The Role of SNF1-Related Protein Kinase 1 (SnRK1) in Regulating Intermediary Metabolism in Arabidopsis thaliana

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You Wang

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ABSTRACT

The Role of SNF1-Related Protein Kinase 1 (SnRK1) in Regulating Intermediary Metabolism in Arabidopsis thaliana

You Wang

Advisors:
Dr. Michael J. Emes
Dr. Ian J. Tetlow

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The Sucrose Non-fermenting-1 (SNF1)-Related Protein Kinase 1 (SnRK1) is a highly conserved heterotrimeric protein kinase which plays an important role in the control of energy balance in plants. It possesses a catalytic subunit (α) and two regulatory subunits (β and γ). This research project focuses on understanding the role of one of its regulatory subunits, AKINβ1, on metabolism in Arabidopsis.

The effects of altered expression of AKINβ1 on carbohydrate metabolism in plants was investigated in an Arabidopsis T-DNA insertion mutant. The concentrations of key intermediates in the tricarboxylic acid cycle of the mutant leaves were markedly reduced throughout the diurnal cycle, and this was coupled with a decrease in measurable respiration rate. The subcellular localization of AKINα1 (AKIN11), AKINβ1, and AKINγ1 subunits of SnRK1 was investigated in tobacco and Arabidopsis leaves, using fluorescence-tagged proteins. The regulatory function of N-myristoylation on the subcellular localization of AKINβ1 was also investigated, indicating that AKINβ1 binds to the Golgi stack, and that the N-terminal 74-amino acids of AKINβ1 possesses a putative nuclear localization signal. Recombinant fusion proteins (AKINα1, AKINβ1 and AKINγ1) of SnRK1 were expressed in E. coli and used as bait to study their interaction with other proteins in plant leaf cells.
LHCB1.1/LHCB1.2 or/and LHCB1.3 were identified as putative interacting protein(s) of AKINα1. Finally, the effect of altered expression of AKINβ1 on transcriptional regulation was studied. Compared to WT, 2485 genes and 188 genes were expressed differentially in the akinβ1 mutant leaves in response to light and darkness respectively. Very large changes in expression were observed in several genes including glycerol-3-phosphate acyltransferase 5 (-243 fold), NADP-dependent malic enzyme 1 (-70 fold), and nitrate transporter 1.8 (-52 fold), indicating that the AKINβ subunit plays a significant role in modulating carbohydrate, lipid, and nitrogen metabolism. A model is hypothesized to explain the effects of AKINβ1 on metabolism in Arabidopsis. The results in this study provide new insight into the role of SnRK1, especially AKINβ1, in regulating plant metabolism.
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List of Abbreviations

ABA       abscisic acid
ABI       abscisic acid insensitive
ABRC      The Arabidopsis Biological Resource Center
ACS1      acetyl-CoA synthetase
Adi3      AvrPto-dependent Pto-interacting protein 3
ADP       adenosine diphosphate
ADPG      adenosine-5’-diphosphoglucose
AGPase    adenosine-5’-diphosphoglucose pyrophosphorylase
AIS       auto-inhibitory sequence
AMP       adenosine monophosphate
AMPK      AMP-activated protein kinase
APX       ascorbate peroxidase
AREBP     ABA responsive element binding protein
ATP       adenosine triphosphate
BAM       β-amylase
BCIP      5-bromo-4-chloro-3-indolylphosphate
BiFC      bimolecular fluorescence complementation
bZIP      basic region-leucine zipper transcription factor
CBC       the Calvin-Benson Cycle
CBD       chitin binding domain
CBM       carbohydrate-binding module
CBS       cystathionine β-synthase motif
cDNA      complementary DNA
CIPK15    calcineurin B-like-interacting protein kinase 15
CNBr      cyanogen bromide
CoASH     coenzyme A
CRK       cysteine-rich receptor-like kinase
CTD       C-terminal domain
DHAP      dihydroxyacetone phosphate
DTT       1,4-dithiothreitol
EC        Enzyme Commission
E.coli    *Escherichia coli*
EEL/bZIP12 enhanced EM level
EDTA      ethylenediaminetetraacetic acid
FAD       flavin adenine dinucleotide
FADH$_2$  flavin adenine dinucleotide (reduced form)
FBPase    fructose-1,6-bisphosphatase
F2,6BP    fructose-2,6-bisphosphate
FDR       False Discovery Rate
FER4      Ferritin 4
F2KP      6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase
F6P  fructose-6-phosphate
FKPM  fragments per kilobase of transcript per million mapped reads
FRO3  ferric reduction oxidase 3
Fru-1,6-BP  fructose 1,6-bisphosphate
Fru-2,6-BP  fructose-2,6-bisphosphate
FSD1  Fe-superoxide dismutase 1
FUS3  FUSCA3
Ga3P  glyceraldehyde-3-phosphate
Gal1P  galactose-1-phosphate
GAPDH  glyceraldehyde 3-phosphate dehydrogenase
3-GAP  3-phosphoglyceraldehyde
G1,6BP  glucose-1,6-bisphosphate
gDNA  genomic DNA
GER3  GERMIN 3
gin  glucose insensitive
GO  Gene Ontology
G1P  glucose-1-phosphate
G6P  glucose-6-phosphate
GLUT1  glucose transporter type 1
GLUT4  glucose transporter type 4
Gly3P  glycerol-3-phosphate
GPAT  glycerol-3-phosphate sn-2-acyltransferase
GPC  gel permeation chromatography
GRIK  Geminivirus Rep-interacting kinase
GST  glutathione S-transferase
HEPES  4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HMGR  3-hydroxymethyl-3-methylglutaryl coenzyme A reductase
H2O2  hydrogen peroxide
IAA  indole-3-acetic acid
ICL1  isocitrate lyase
IDD  indeterminate domain
Imidazole  1,3-Diaza-2,4-cyclopentadiene
IPTG  isopropyl β-D-1-thiogalactopyranoside
ISA  isoamylase
kDa  kilodalton
KA1  kinase-associated 1 domain
KEGG  Kyoto Encyclopedia of Genes and Genomes
KO  KEGG Orthology
LC-ESI-Q-TOF-MS  liquid chromatography-electrospray ionization-quadrupole-time of flight-mass spectrometry
LC-MS/MS  liquid chromatography coupled to tandem mass spectrometry
LHCB  light harvesting chlorophyll a/b binding protein
LHC  light-harvesting chlorophyll a/b-protein complex
LPA  lysophosphatidic acid
Man6P  mannose-6-phosphate
MBP  maltose-binding protein
MLS1  malate synthase
mMDH  mitochondrial malate dehydrogenase
MMR  multiple mapped reads
MOS  malto-oligosaccharides
mRNA  messenger RNA
Myr  myristoylation
NAD  nicotinamide adenine dinucleotide
NADH  nicotinamide adenine dinucleotide (reduced form)
NBT  nitro-blue tetrazolium
NDH  NAD(P)H dehydrogenase
NGS  next-generation sequencing
nttl  N-myristoyltransferase 1
NP-40  Nonidet P40
np-Ga3PDHase  non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase
NR  nitrate reductase
NRT  nitrate transporter
NusA  N-utilization substance protein A
NVR  N-terminal variable region
OD  optical density
2-OG  2-oxoglutarate
OPPP  The oxidative pentose phosphate pathway
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate buffered saline
PCK1  phosphoenolpyruvate carboxykinase
PCR  polymerase chain reaction
PDF1  protodermal factor 1
PEPC  phosphoenolpyruvate carboxylase
PEP  phosphoenolpyruvate
PFK  ATP-dependent phosphofructokinase
PFP  pyrophosphate-dependent phosphofructokinase
3-PGA  3-phosphoglycerate
PGC1α  peroxisome proliferator-activated receptor-γ co-activator 1α
Pi  phosphate
PK  pyruvate kinase
PP2CA  protein phosphatase 2CA
PP2C2  C-type protein phosphatase
PRK  phosphoribulokinase
PPT  phosphate translocator
PSI  photosystem I
PSII  photosystem II
5PTase13  myoinositol polyphosphate 5-phosphatase
QC  quality control
CHAPTER I
GENERAL INTRODUCTION
Most plants are rooted in one location and do not move during their life cycle. This means that plants have to face varied environmental challenges over a diurnal cycle, involving abiotic and biotic stresses in order to survive and maintain growth, development and reproduction. To adapt to their living environment, plants have evolved many physiological mechanisms which facilitate metabolic regulation and sustain energy balance. Among these mechanisms, regulation of carbohydrate metabolism is very important given the need for sugars to maintain plant growth and development, and also because metabolites are used as signaling molecules to regulate important developmental and physiological processes in plants. As autotrophs, plants produce carbohydrates by photosynthesis, which is affected by changes in environmental factors such as temperature, light intensity and water availability. Such variables have led to the evolution of regulatory mechanisms for carbohydrate metabolism to restore and maintain energy balance in plants. Recently, several lines of evidence show that the orthologues of mammalian AMP-activated protein kinase (AMPK)/yeast sucrose non-fermenting 1 (SNF1) family in plants, known as SNF1-related kinase 1 (SnRK1), play a vital role in the regulation of carbohydrate metabolism and energy balance (Polge and Thomas, 2007; BaenaGonzalez and Sheen, 2008; Rodrigues et al., 2013; Cho et al., 2016). The AMPK/SNF1/SnRK1 protein kinase complexes are a family of highly conserved heterotrimeric serine/threonine kinases that have been identified in all eukaryotes including animals (AMPK), yeast (SNF1), and plants (SnRK1). The principal role of these kinases is the integration of cellular internal and external signals such as nutrient availability, environmental stress signals and
energy expenditure in order to maintain cell survival by triggering the required adaptations to maintain energy homeostasis (Hardie, 2007; Polge and Thomas, 2007; Hedbäcker and Carlson, 2008).

1.1 INTRODUCTION TO AMPK/SNF1/SnRK1 PROTEIN KINASES

The AMPK/SNF1/SnRK1 kinases are conserved throughout all eukaryotes and share a similar αβγ heterotrimeric structure (Carling et al., 2012). AMPK from rat liver was identified in 1973 (Beg et al., 1973; Carlson and Kim, 1973) and SNF1 was identified in yeast in 1981 (Carlson et al., 1981). RKin1 from rye endosperm was identified as the first SnRK1 gene on the basis of its ability to complement the yeast snf1Δ mutation (Alderson et al., 1991). AMPK from rat, SNF1 from yeast, and RKin1 from rye were identified as members of a conserved protein kinase family maintained throughout evolution (Carling et al., 1994). In Arabidopsis, two SnRK1 α-subunits (AKIN10 and 11) are known that they are capable of functionally complementing the yeast snf1Δ mutation (Bhalerao et al., 1999).

The protein kinases in AMPK/SNF1/SnRK1 family comprise generally a catalytic α subunit and two regulatory β and γ subunits responsible for the formation of a stable complex with protein kinase activity. Theoretically, the number of kinase complexes that can be formed diverges significantly between different organisms. For example, one α subunit (Snf1), three alternate β subunits (Sip1, Sip2 and Gal83) and one γ subunit (Snf4) have been identified in yeast, which means three alternate SNF1 complexes exist in yeast (Ghillebert et al., 2011). However, two isoforms of the α subunits (a1 and 2), two β subunits (β1 and 2) and three alternate γ subunits (γ1, 2 and 3) have been
identified in humans and are encoded by different genes, which means that at least 12
different AMPK complexes could be formed in humans (Ghillebert et al., 2011). These
different kinase complexes could be subject to particular tissue and subcellular
distribution, and regulate specific signaling pathways (Steinberg and Kemp, 2009).

As central metabolic sensors and regulators, AMPK/SNF1/SnRK1 protein kinases play
a central role in the regulation of metabolism by responding to cellular energy charge
(Ghillebert et al., 2011). Essentially, the protein kinases are activated by nutrition-
depletion and energy-depletion stress conditions in order to maintain energetic
homeostasis and protect against marked changes in energy (ATP) levels in cells. They
achieve this by activating catabolic processes (e.g. glycolysis, fatty acid oxidation,
sucrose, amino acid, and protein degradation processes) and inhibiting anabolic
metabolism (e.g. protein biosynthesis, fatty acid biosynthesis and ribosome biogenesis)
to produce energy and maintain cell survival. Moreover, these protein kinases play key
roles in regulating normal growth and development at the cellular level (Ghillebert et
al., 2011). The kinases work with other central signaling components such as
metabolites and hormones to control important physiological and biochemical
processes such as carbohydrate uptake and metabolism, fatty acid and lipid biosynthesis,
and the storage of carbon energy reserves (Ghillebert et al., 2011).

1.1.1 AMPK

The mammalian AMPK was the first protein kinase identified in this family and has
been well studied. When AMP is elevated and ATP is depressed, AMPK senses
adenylate energy charge by competitive binding of AMP, ADP, and ATP to three
cystathionine β-synthetase (CBS) motifs (CBS1, CBS3, and CBS4) in its γ-subunit (Hardie and Carling, 1997; Wang et al., 2003). AMP and ADP stimulate phosphorylation of AMPK on Thr172, which is required to activate AMPK (Oakhill et al., 2011). To restore and maintain energy homeostasis, AMPK triggers catabolic pathways to generate ATP and inhibits biosynthetic pathways to conserve ATP in response to energetic stress. One important function of AMPK is that it promotes glucose uptake by stimulating translocation of glucose transporter type 4 (GLUT4) to the plasma membrane and enhancing the transporter function of glucose transporter type 1 (GLUT1) to stimulate glucose catabolism during muscle contraction (Barnes et al., 2002; Jørgensen et al., 2004). AMPK also accelerates fatty acid uptake into cardiac myocytes (Habets et al., 2009). Subsequently, AMPK stimulates glucose catabolism by increasing glycolysis via protein phosphorylation of phosphofructokinase 2 on Ser466, which stimulates the rate of glycolysis in humans. It also stimulates fatty acid catabolism by promoting the transfer of fatty acids into mitochondria and their degradation by the β-oxidation pathway (Barnes et al., 2002; Habets et al., 2009; Wu and Wei, 2011). Moreover, AMPK also regulates mitochondrial biogenesis and mitophagy. AMPK can activate mitochondrial biogenesis by the direct phosphorylation of peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α). This is a co-activator which increases the activity of several transcription factors that regulate the expression of nuclear-encoded mitochondrial genes (Lin et al., 2005; Jäger et al., 2007; Cantó et al., 2010). AMPK is also involved in mitochondrial mitophagy. This is a special form of autophagy, which involves phosphorylating and activating UNC-51-
like kinase 1 (ULK1), the mammalian orthologue of the yeast kinase Atg1 which, in turn, initiates the autophagy cascade (Behrends et al., 2010; Egan et al., 2011; Kim et al., 2011). Furthermore, AMPK inhibits almost all anabolic pathways such as lipid, carbohydrate, protein and ribosomal RNA biosynthesis. This is achieved by inhibiting gene expression of some of the proteins involved, or by post-translational phosphorylation of proteins that are involved in these pathways (Hardie et al., 2012).

1.1.2 SNF1

SNF1 is the orthologue of AMPK found in yeast and plays a key role in yeast sucrose utilization (Carlson et al., 1981). SNF1 is activated in response to the stress of glucose deprivation (Hardie et al., 1998). SNF1 activity requires phosphorylation of the catalytic subunit on Thr210 (McCartney and Schmidt, 2001). ADP binds to Snf4 and protects SNF1 against dephosphorylation of Thr210 (Mayer et al., 2011). SNF1 phosphorylates enzymes in the cytosol to launch short-term adjustments in response to low glucose conditions (Schluepmann et al., 2012). SNF1 also phosphorylates genomic transcriptional components and histones in the nucleus to stimulate longer-term adjustments in response to low glucose conditions (De Virgilio, 2012). SNF1 primarily regulates transcription repressors such as Mig1, Nrg1 and Nrg2 to affect carbon metabolism in yeast. SNF1 regulates the transcription of genes involved in metabolism of alternative carbon sources such as sucrose, galactose, and maltose mainly by inhibiting the activity of the transcription repressor Mig1, which contains two Cys2His2 (C2H2) zinc finger motifs (Lutfiyya and Johnston, 1996; Lutfiyya et al., 1998; Westholm et al., 2008). The other two main SNF1-regulated repressors are Nrg1 and
Nrg2 that also contain the C2H2 zinc finger motif (Santangelo, 2006). Both Nrg1 and Nrg2 have various functions in the regulation of glucose-repressed genes (Berkey et al., 2004). Nrg1 and Nrg2 contribute to repression of many glucose-repressed genes such as suc2, gal1, mal involved in maltose utilization, flo11 involved in filamentous growth, and stal and sta2 for encoding glucoamylase isozymes (Kim et al., 2004). SNF1 also regulates transcriptional activators such as CAT8 and SIP4, ADR1 and MSN2 to affect carbon metabolism in yeast. CAT8 and SIP4 are C6 zinc cluster DNA-binding activators with similar structures, activate the expression of genes encoding enzymes such as isocitrate lyase (ICL1) (isocitrate metabolism), fructose-1,6-bisphosphatase (FBPase) and phosphoenolpyruvate carboxykinase (PCK1) (gluconeogenesis), malate synthase (MLS1) (glyoxylate cycle), and acetyl-CoA synthetase (ACS1) (gluconeogenesis, respiratory metabolism and the glyoxylate cycle) in response to low glucose levels (Randez-Gil et al., 1997; Vincent and Carlson, 1998; Bojunga and Entian, 1999). ADR1 activates more than 100 glucose-repressed genes primarily by binding to their promoters in a SNF1-dependent manner (Young et al., 2009). SNF1 is involved in the regulation of the stress-response transcription factor MSN2 and stimulates a longer-term adaptation to carbon stress (Mayordomo et al., 2002; De Wever et al., 2005).

SNF1 also regulates enzymes and hexose transport to affect carbon metabolism in yeast. SNF1 activates glycogen synthase, GSY2, by inactivating PHO85 kinase and activating the Glc7/Gac1 protein phosphatase in response to low glucose levels (Hardy et al., 1994; Francois and Parrou, 2001). SNF1 directly phosphorylates hexokinase 2 (HKX2) on Ser14 to inhibit the predominant glucose kinase in yeast (Fernández-García et al., 2012).
The protein kinase is also a transcriptional regulator that affects the expression of several genes regulated by Mig1 in the nucleus (Fernández-García et al., 2012). SNF1 directly regulates RNA polymerase II holoenzyme for transcriptional control in response to important signals in yeast (Kuchin et al., 2000), and affects expression of the hexose carrier genes by inhibiting MIG1 activity (Tomas-Cobos and Sanz, 2002; Kaniak et al., 2004).

1.1.3 SnRK1

SnRK1 is the orthologue of SNF1 identified in plants. SnRK1 phosphorylates and inactivates several important metabolic enzymes such as nitrate reductase (NR), sucrose phosphate synthase (SPS) and 3-hydroxymethyl-3-methylglutaryl coenzyme A reductase (HMGR) (Sugden et al., 1999b; Kulma et al., 2004) as well as regulating gene expression of a variety of genes encoding proteins involved in carbohydrate metabolism, protein metabolism and nucleotide metabolism (Baena-Gonzalez et al., 2007). SnRK1 is involved in various stress responses such as sugar deprivation, drought, cold, hypoxia, salt and osmotic stress, and viral infection (Baena-Gonzalez and Sheen, 2008). Moreover, the protein kinase is necessary for plant growth during darkness (Thelander et al., 2004). More detailed information about the structure and functions of SnRK1 in plants will be described in the following parts.

1.2 REGULATION OF THE STRUCTURE AND ACTIVITY OF SnRK1 IN PLANTS

1.2.1 The structure of SnRK1

As members of the AMPK/SNF1 kinase family, SnRK protein kinases have been
divided into three subgroups: SnRK1, SnRK2 and SnRK3 on the basis of amino acid sequence similarity and domain structure (Halford and Hey, 2009). SnRK1 plays a major role in the regulation of carbon metabolism and energy status, while SnRK2 and SnRK3 have potential functions in stress and abscisic acid (ABA)-mediated signaling pathways (Coello et al., 2012). Compared with SnRK1, SnRK2 and SnRK3 share less sequence similarity with SNF1, do not complement yeast snf1Δ mutants, and are unique to plants (Halford et al., 2003), which demonstrates that only SnRK1 belongs to the conserved AMPK/SNF1 kinase family. As the orthologues of the highly conserved AMPK/SNF1 protein kinase family in plants, the SnRK1 kinases share the αβγ heterotrimeric structure (Crozet et al., 2014). The homologues of all subunits of SnRK1 identified in *Arabidopsis thaliana* are named AKIN. Two α subunits (AKIN10 and 11), three β subunits (AKINβ1, AKINβ2 and AKINβ3) and two γ subunits (AKINγ and AKINβγ) have been identified in *Arabidopsis*.

The catalytic α-subunit of SnRK1 is composed of two domains: the kinase catalytic domain and the regulatory domain (Figure 1.1). The kinase catalytic domain has a canonical fold with 11 sub-domains and contains the activation loop called the T-loop (Hanks and Hunter, 1995). The regulatory domain has two main parts: an ubiquitin-associated (UBA) domain and a kinase-associated 1 (KA1) domain. The UBA domain mediates the interaction with ubiquitinated proteins instead of an auto-inhibitory sequence (AIS) that inhibits kinase activity in the regulatory domain of α-subunits of the AMPK/SNF1 kinases in mammals and yeast (Farras et al., 2001; Pang et al., 2007; Chen et al., 2009). The KA1 domain is responsible for the interaction with the
regulatory subunits and upstream phosphatases (Kleinow et al., 2000; Amodeo et al., 2007; Xiao et al., 2011; Rodrigues et al., 2013). Arabidopsis expresses two SnRK1 catalytic subunits, α1 (AKIN11 or SnRK1.2) and α2 (AKIN10 or SnRK1.1), which comprise an amino-terminal kinase domain followed by a UBA domain and a KA1 motif towards the carboxy terminal. Compared with AKIN11, AKIN10 plays the principal role in sugar and ABA signaling and miRNA-mediated energy signaling since AKIN10 is the dominant catalytic subunit in leaves, accounting for 90% of the total SnRK1 complexes (Jossier et al., 2009; Tsai and Gazzarrini, 2012b; Rodrigues et al., 2013; Confraria et al., 2013). In addition, a non-expressed SnRK1α3 (AKIN12) without detectable kinase activity has been also identified as a pseudogene in the Arabidopsis genome (Hrabak et al., 2003; Fragoso et al., 2009; Jossier et al., 2009).

**Figure 1.1** SnRK1 complex and its subunit domain architecture in Arabidopsis. The major regulatory phosphorylation sites (the T–loop threonine residue) within the catalytic domain of α subunit are shown. SnRK1 γ1 is not involved in the formation of the kinase complex (Emanuelle et al., 2015). The color lines arrow the subunits of SnRK1 participate in the formation of SnRK1 complex. A putative phosphorylation site of AvrPto-dependent Pto-interacting protein 3 (Adi3), Ser27, on AKINβ1/2 has been shown. UBA: ubiquitin-associated domain; KA1: kinase-associated 1 domain; NVR: N-terminal variable region; CBD: carbohydrate-binding domain; CBM: carbohydrate-binding module; CTD: C-terminal domain; CBS: cystathionine β-synthase motif; (Modified from Crozet et al., 2014 and Emanuelle et al., 2016).
The regulatory β-subunit of SnRK1 is composed of three domains: an amino-terminal variable region (NVR), a mid-module carbohydrate-binding domain and a carboxyl-terminal domain (CTD) (Figure 1.1; Iseli et al., 2005; Koay et al., 2010). In Arabidopsis, two β isoforms with the classical structure, AKINβ1 and AKINβ2, and a plant-specific β isoform that comprises solely a CTD, AKINβ3, have been identified (Gissot et al., 2004; Polge et al., 2008). The NVR domain can be N-myristoylated, which negatively regulates nuclear SnRK1 activity by sequestering the kinase complex at the plasma membrane (Pierre et al., 2007). Interestingly, the divergent N-terminal extension of the β-subunits can direct the SNF1 kinases to different cellular localizations in response to glucose availability and also facilitates association with downstream targets in yeast (Vincent et al., 2001; Hedbacker et al., 2004a; Hedbacker et al., 2004b; Hedbacker and Carlson, 2008). The β-subunits of SnRK1 likely have similar functions since AKINβ1 and AKINβ2 can change their locations by N-myristoylation-mediated regulation (Pierre et al., 2007) and AKINβ1 and AKINβ3 can interact with nitrate reductase that can be bound and phosphorylated by SnRK1 (Polge et al., 2008). Compared with the CBD domain in rat AMPKβ1 that can bind glycogen (Koay et al., 2010), the CBD in AKINβ2 is a conserved region in terms of overall topology and the majority of binding site residues identified as involved in protein-carbohydrate interactions (Avila-Castañeda et al., 2014). However, the experimental evidence on the carbohydrate-binding capability of these AKINβ subunits is contradictory. Binding experiments using the AKINβ2 recombinant protein indicated that AKINβ2 could associate with starch but exhibited little association with a mixture of amylose and amylopectin (Avila-
Castañeda et al., 2014). However, other binding experiments using the recombinant CBD domains of AKINβ1 and AKINβ2 indicated that neither of the two carbohydrate-binding modules located in the AKINβ1 and AKINβ2 subunits interact with various carbohydrates, including starch (Emanuelle et al., 2015). The CTD domain of the β subunit works as a scaffold to tether the α and the γ subunits. This could be a primary function of the SnRK1 β subunits since the plant-specific AKINβ3 subunit that contains only the CTD region is able to complement the yeast mutant gal83 Asip1 Asip2Δ that lacks all three β-subunits in yeast (Gissot et al., 2004).

The regulatory γ-subunits of SnRK1 typically comprise four tandem cystathionine-β-synthase motifs (a tandem pair of CBS motifs in one Bateman domain) that can bind adenine nucleotides to regulate kinase catalytic activity in AMPK and SNF1 (Figure 1.1; Mayer et al., 2011; Oakhill et al., 2012). Two types of γ-subunits have been identified in plants, including Arabidopsis, which are γ and βγ. The γ subunit of SnRK1 possesses the typical structure of γ-subunits, having four conserved CBS motifs (Ramon et al., 2013). However, the γ subunit of SnRK1 does not participate in SnRK1 heterotrimers based on co-immunoprecipitation experiments in vitro (Ramon et al., 2013; Emanuelle et al., 2015) and does not complement a yeast mutant lacking the γ subunit of SNF1 (snf4Δ) (Bouly et al., 1999; Ramon et al., 2013). In contrast to this, it has been shown that AKINγ can interact with AKINβ1, AKINβ2 and AKIN10 respectively in yeast two-hybrid assays (Bouly et al., 1999). It is possible that AKINγ is not directly involved in SnRK1 signaling and could have some unknown biological functions that may be related to the role of SnRK1 in plants (Bouly et al., 1999; Ramon
et al., 2013). The βγ subunit of SnRK1 is restricted to the plant kingdom (Gissot et al., 2006) and comprises an N-terminal carbohydrate-binding module (CBM) that is homologous to the CBD found in β-subunits, as well as the typical structure of the four CBS motifs found in γ-subunits that are necessary for binding with AKINαs (Gissot et al., 2006). The βγ subunit of SnRK1 is indispensable for the formation of functional SnRK1 heterotrimers, at least in Arabidopsis. The βγ subunit of SnRK1 can also complement the yeast mutant snf4Δ which lacks the γ-subunit of the SNF1 kinase, suggesting the βγ subunit can act as the sole canonical γ-subunit of the SNF1 (Ramon et al., 2013; Emanuelle et al., 2015). In Arabidopsis, the βγ-subunit can bind with one of two α-subunits (AKIN10 and AKIN11) and one of three β-subunits (AKINβ1, AKINβ2 and AKINβ3) to form six possible functional SnRK1 isoenzymes (Emanuelle et al., 2015).

1.2.2 Regulation of SnRK1 activity in plants

The protein kinases which belong to the AMPK/SNF1/SnRK1 family are regulated by a complex range of regulatory controls and feedback loops. The mechanisms by which these occur has been revealed largely from studies of AMPK and, to a lesser extent, SNF1. Many of these mechanisms are common, but there are also some plant-specific regulatory mechanisms (Emanuelle et al., 2015).

The activity of SnRK1 can be regulated by reversible phosphorylation (Figure 1.2). Like AMPK, the conserved threonine residues are located in the T-loop of SnRK1 α-subunits (Thr 175 in AKIN10 and Thr 176 in AKIN11). As the major regulatory phosphorylation sites in the catalytic subunits, phosphorylation of the Thr residues in
the T-loop is required for the activation of the protein kinases, thus “switching on” the protein kinases (Sugden et al., 1999a; Baena-Gonzalez et al., 2007; Shen et al., 2009; Crozet et al., 2010; Emanuelle et al., 2015). However, the T-loop phosphorylation state of SnRK1 does not affect the expression of genes regulated by SnRK1 in either cell-based assays (Baena-Gonzalez et al., 2007) or detached Arabidopsis leaves (Rodrigues et al., 2013). Further, the T-loop phosphorylation state of SnRK1 is unchanged even if the activity of SnRK1 is changed in response to different treatments (Fragoso et al., 2009; Coello et al., 2012). These results suggest that SnRK1 is activated by additional/other mechanisms. In addition, the conserved ATP binding residues (involving K48 in AKIN10 and K49 in AKIN11) are necessary for kinase activity, since mutation of these lysine residues inactivates the kinase (Baena-Gonzalez et al., 2007).

Several putative upstream kinases of SnRK1 have been identified. Two upstream kinases, the Gemini virus Rep-interacting kinases 1 and 2 (GRIK1 and GRIK2), that are also SnRK1-activating kinases, SnAK1 and SnAK2, have been identified (Shen et al., 2009). SnRK1 can reciprocally phosphorylate either GRIKs in vitro, on a specific serine residue (Ser260 in AtSnAK1 and Ser261 in AtSnAK2) to inactivate these two kinases. This represents a negative feedback cycle that may provide an additional level of regulating SnRK1 activation (Crozet et al., 2010). A potential upstream kinase of SnRK1, calcineurin B-like-interacting protein kinase 15 (CIPK15) was reported in rice (Lee et al., 2009). But whether CIPK15 can directly phosphorylate and activate SnRK1 has to be confirmed. In addition, protein kinase AvrPto-dependent Pto-interacting protein3, which is a suppressor of cell death triggered by pathogens in tomato, can
interact with SnRK1α1 and phosphorylate the Gal83 β-subunit at Ser26, which in turn inhibits the activity of the tomato SnRK1 complex (Avila et al., 2012). Several putative upstream phosphatases of SnRK1 have also been revealed. Two upstream phosphatases, abscisic acid insensitive (ABI) 1 and protein phosphatase 2CA (PP2CA), inactivate SnRK1 by dephosphorylation, indicative of a relationship between the SnRK1-related pathway and ABA signaling pathway (Rodrigues et al., 2013). Another putative myristoylated 2C-type protein phosphatase (PP2C), PP2C74, interacts with AKIN10 in vitro and in yeast two-hybrid assays (Tsugama et al., 2012), suggesting the phosphatase is a potential upstream regulator of SnRK1. Although upstream kinases and phosphatases of SnRK1 have been identified, the detailed regulatory mechanism for SnRK1 by reversible phosphorylation is unclear.

The activity of SnRK1 can also be regulated by other post-translational mechanisms, including ubiquitination and the small ubiquitin-like modifier (SUMO) protein modification (SUMOylation) (Figure 1.2). For example, AKIN10 degradation is mediated by the CUL4-based E3 ubiquitin ligase (Lee et al., 2008). In addition, interactions of AKIN10 with E2 SUMO-conjugating enzyme (SCE) and SUMO protease (ESD4) have been identified in a yeast two-hybrid screen (Elrouby and Coupland, 2010). AKIN10 is SUMOylated by both SUMO1 and SUMO3 isoforms in an E. coli-based, high-throughput assay and AKIN10 has putative SUMO attachment sites based on a bioinformatic analysis (Elrouby and Coupland, 2010). Moreover, the activity of SnRK1 also can be regulated by N-myristoylation, which targets AKINβ1 and AKINβ2 to the plasma membrane. When N-myristoylation is prevented, AKINβ1
is relocated to the nucleus and AKINβ2 is relocated to the cytoplasm. SnRK1 activity increases five-fold in the N-myristoyltransferase 1 (nmt1) mutant while AKINβ1 gene expression increases two-fold, in contrast to the gene expression levels of AKINβ2 and AKINβ3 which are decreased (Pierre et al., 2007). These results suggest that N-myristoylation of the AKINβ subunits could be a negative regulator of SnRK1 activity.

**Figure 1.2** Summary of the regulatory mechanisms of the activity of the SnRK1 kinases described in Section 1.2.1. Broken lines and full lines represent indirect links and direct connections respectively. ○: phosphorylation; Ub, ubiquitination; SUMO, small ubiquitin-like modifier; Myr, myristoylation (Modified from Crozet et al., 2014).

The activity of SnRK1 can be regulated by several sugars and sugar-phosphates (Figure 1.2). Unlike AMPK, SnRK1 is not allosterically activated by adenine nucleotides (Sugden et al., 1999a; Emanuelle et al., 2015). SnRK1 is inactivated by several sugars and sugar-phosphates such as trehalose-6-phosphate (T6P), glucose-1-phosphate (G1P), glucose-6-phosphate (G6P), fructose-1,6-bisphosphate, glucose and sucrose (Baena-González et al., 2007; Piattoni et al., 2011; Nunes et al., 2013). T6P has been shown to be a potent inhibitor of SnRK1 extracted from young Arabidopsis leaf tissue (Zhang et al., 2009), wheat grain (Martinez-Barajas et al., 2011) and potato tubers (Debast et al.,
In the presence of an unknown factor, likely a protein due to its heat lability, T6P inhibits the activity of Arabidopsis SnRK1 at physiological concentrations in vitro (Zhang et al., 2009). The unknown protein is only present in young growing tissues such as seedlings and young Arabidopsis (Zhang et al., 2009). G1P and G6P also inhibit SnRK1 from Arabidopsis seedlings, in the presence of the unknown protein (Nunes et al., 2013). In addition, T6P and G1P inhibit the kinase synergistically and ribose 5-phosphate (R5P) and ribulose 5-phosphate (Ru5P) also can inhibit SnRK1 activity, whereas fructose-6-phosphate (F6P) and uridine-5’-diphosphoglucose (UDPG) do not inhibit SnRK1 (Nunes et al., 2013). SnRK1 activity was inhibited by glucose and sucrose (5-50mM) (Baena-Gonzalez et al., 2007). SnRK1 activity is inhibited by sugars and sugar-phosphates, reflecting the energy status (carbon availability) of a plant. When carbon availability decreases, a concomitant drop in the levels of sugars and sugar-phosphates likely releases the inhibition of SnRK1 activity. The activated SnRK1 phosphorylates and inactivates several important metabolic enzymes that control steroid and isoprenoid biosynthesis, nitrogen assimilation for amino acid and nucleotide synthesis, sucrose synthesis, and carbon partitioning (Sugden et al., 1999b; Kulma et al., 2004). It also induces extensive transcriptional changes in the transcriptome to promote catabolic pathways and repress anabolic pathways (Baena-Gonzalez et al., 2007), triggering a complex signaling pathway to restore the energy balance of the plant.

The activity of SnRK1 can be regulated by other mechanisms. Based on in vitro and yeast two-hybrid system studies, SnRK1 can interact with a myoinositol polyphosphate 5-phosphatase (5PTase13) that is involved in the inositol signaling pathway. Under low-
nutrient or low-sugar conditions, 5PTase13 upregulates SnRK1 activity. However, under severe starvation conditions, 5PTase13 downregulates SnRK1 activity (Ananieva et al., 2008). In addition, recombinant SnRK1 and endogenous SnRK1 from 3-week-old Arabidopsis rosettes are both inhibited by a proteinaceous factor (>30kDa) extracted from young Arabidopsis leaves (Emanuelle et al., 2015). The expression of AKINβ regulatory subunits is regulated by sugar and light, which would affect the activity of SnRK1 indirectly. Expression of akinβ1 is repressed by sucrose and is promoted by darkness, though these signals function independently (Polge et al., 2008). Expression of akinβ2 is not regulated by light (Polge et al., 2008). The expression of akinβ3 is only upregulated in darkness and is sugar independent (Polge et al., 2008). These different regulatory patterns of gene expression of the AKINβ subunits are a function of the specific regulatory sequences in their own promoters (Polge et al., 2008).

1.3 FUNCTIONS OF SnRK1

During evolution, plants have developed integrated systems to maintain energy homeostasis. Key components of these systems are “molecular sensors” that can monitor fluctuations in plant cellular energy status continuously, and reprogram metabolism in response to varied energy condition quickly. As one of these energy sensors, SnRK1 phosphorylates enzymes and regulates gene expression of many proteins to effect rapid adjustments and restore energy balance in plants. SnRK1 is a central regulator for metabolic homeostasis in plants and is crucial for normal plant growth and development as well as response to different stresses (Emanuelle et al., 2015).
SnRK1 phosphorylates several enzymes involved in metabolic pathways (Table 1.1). *In vitro*, SnRK1 can phosphorylate and inactivate several metabolic enzymes such as a) 3-hydroxymethyl-3-methylglutaryl coenzyme A reductase which is a key enzyme in steroid and isoprenoid synthesis pathway, b) sucrose phosphate synthase involved in sucrose biosynthesis pathway, c) nitrate reductase, involved in the first step of nitrate assimilation, and d) trehalose-6-phosphate synthase 5 (TPS5) which is involved in trehalose-6-phosphate biosynthesis (Sugden *et al.*, 1999b; Harthill *et al.*, 2006; Polge and Thomas, 2007). In addition, SnRK1 also can phosphorylate the non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (np-Ga3PDHase) in wheat that is involved in catalyzing the irreversible oxidation of glyceraldehyde-3-phosphate (Ga3P) to 3-phosphoglycerate (3-PGA) and 3-PGA is an inhibitor of SnRK1 in glycolysis (Piattoni *et al.*, 2011). Moreover, Arabidopsis 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (F2KP) can be phosphorylated by rat liver mammalian AMPK and AKIN10 (Kulma *et al.*, 2004; Cho *et al.*, 2016). F2KP regulates the content of fructose-2,6-bisphosphate (Fru-2,6-BP), which is an important signaling metabolite involved in regulating photosynthetic carbon partitioning in plants (Kulma *et al.*, 2004). SnRK1 can phosphorylate sucrose synthase on a serine site (S15), which indirectly regulates the activity of the key enzyme in sucrose degradation (Hardin *et al.*, 2003). These results show that SnRK1 is an important integrator that is involved in regulating carbon, nitrogen, and lipid metabolism in plants.
Table 1.1 Summary of enzymes phosphorylated by SnRK1 or interacting with SnRK1.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Metabolic pathway(s)</th>
<th>Effect of SnRK1 on activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-hydroxymethyl-3-methylglutaryl coenzyme A reductase</td>
<td>Steroid and isoprenoid synthesis pathway</td>
<td>Inactivated</td>
<td>Sugden et al., 1999b</td>
</tr>
<tr>
<td>Sucrose phosphate synthase</td>
<td>Sucrose biosynthesis pathway</td>
<td>Inactivated</td>
<td>Sugden et al., 1999b</td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>Nitrate assimilation</td>
<td>Inactivated</td>
<td>Sugden et al., 1999b</td>
</tr>
<tr>
<td>Trehalose-6-phosphate synthase 5</td>
<td>Trehalose-6-phosphate biosynthesis</td>
<td>Inactivated</td>
<td>Harthill et al., 2006</td>
</tr>
<tr>
<td>Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Glycolysis</td>
<td>Unknown</td>
<td>Piattoni et al., 2011</td>
</tr>
<tr>
<td>6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase</td>
<td>Fructose-2,6-bisphosphate metabolism</td>
<td>Unknown</td>
<td>Cho et al., 2016</td>
</tr>
<tr>
<td>Sucrose synthase</td>
<td>Sucrose degradation</td>
<td>Unknown</td>
<td>Hardin et al., 2003</td>
</tr>
</tbody>
</table>
SnRK1 also phosphorylates some regulatory enzymes involved in metabolic pathways. For example, AKIN10 phosphorylates TPS 7 and TPS 8, by which SnRK1 decreased the level of T6P to maintain its activity. AKIN10 can also phosphorylate the translation initiation factor eIFiso4G1, and protein tyrosine phosphatase 1 that is a primary target of reactive oxygen species (ROS) \textit{in vitro} (Cho \textit{et al.}, 2016).

SnRK1 can launch extensive transcriptional reprogramming to regulate the vital metabolic pathways in plants. SnRK1 can interact and phosphorylate several transcription factors in Arabidopsis such as the B3-domain transcription factor FUSCA3 (FUS3), transcription factor bZIP63, the indeterminate domain (IDD)-containing transcription factor IDD8 and a trihelix transcription factor PETAL LOSS (PTL) (Tsai and Gazzarrini, 2012a; Mair \textit{et al.}, 2015; Jeong \textit{et al.}, 2015; Brien \textit{et al.}, 2015). In addition, the extent of AKIN10 transcriptional regulation has been studied and 278 genes were identified on the basis of being activated by AKIN10 transfected into Arabidopsis protoplasts and 322 genes were also identified as being repressed (Baena-González \textit{et al.}, 2007). These AKIN10-activated genes are mainly involved in various catabolic pathways such as cell wall, starch, sucrose, amino acid, lipid, and protein degradation processes that produce alternative sources of energy and metabolites to maintain cellular energy balance. However, numerous genes co-repressed by AKIN10 are involved in energy-consuming ribosome biogenesis and anabolism (Baena-González \textit{et al.}, 2007). In addition, AKIN10 also up-regulates gene expression of trehalose-6-phosphate synthase 8-11 involved in the biosynthesis of trehalose-6-phosphate that is an indispensable signal metabolite involved in the
regulation of plant growth and development in response to carbon availability (Conte

 Antisense repression of the wheat SnRK1 represses transient activity of the α-amy

 Amylase 2 gene promoter in cultured wheat embryos (Laurie et al., 2003). Antisense suppression of potato SnRK1 (PKIN1) downregulated gene expression and activity of sucrose synthase but did not affect expression

 activity of neutral or acid invertases in potato tubers (Purcell et al., 1998). Moreover, AKIN10 also positively activates autophagy by transcriptional regulation in plants (Baena-González et al., 2007). These results reinforce the proposal that SnRK1 plays a significant role in maintaining plant energy balance by regulating gene expression of genes encoding proteins related to plant metabolism.

 SnRK1 also plays an important regulatory role in plant growth and development. SnRK1 promotes seed maturation and inhibits seed germination by positively regulating ABA signaling (Radchuk et al., 2006; Radchuk et al., 2010). SnRK1 can regulate some transcription factors involved in ABA signaling, such as the basic region-leucine zipper transcription factor (bZIP)-type transcription factors ABA insensitive 5 and enhanced EM level (EEL/bZIP12) that are regulators in seed maturation and germination (Bensmihen et al., 2002; Lopez-Molina et al., 2002; Bitrián et al., 2011). Pea seeds with antisense-repressed SnRK1 activity exhibit decreased ABA content and decreased gene expression of B3-domain transcription factor ABA insensitive 3 that is a necessary component of the regulatory network for seed development and maturation in Arabidopsis (Radchuk et al., 2006; Radchuk et al., 2010). Moreover, SnRK1 positively regulates the B3-domain transcription factor FUSCA3 which is a key
regulator of seed development and an important component in hormone interaction networks in Arabidopsis by phosphorylation (Tsai and Gazzarrini, 2012a; Tsai and Gazzarrini, 2012b). ABA positively regulates gene transcription of SnRK1, and also post-translationally regulates it by inactivating Group A protein phosphatases type 2C (PP2Cs) that can dephosphorylate and inactivate SnRK1 (Sugden et al., 1999a; Radchuk et al., 2010; Rodrigues et al., 2013). These results show that SnRK1 is necessary for ABA-mediated seed maturation, and incorporates a feedback mechanism involving ABA regulation of the SnRK1 complex at multiple levels. The antagonistic function between gibberellins and ABA plays a vital role in regulating the developmental transition from formation and development of embryo to seed germination (Gómez-Cadenas et al., 2001). Gibberellic acid that stimulates plant growth and development inhibits the expression of different SnRK1 subunits, which proves that SnRK1 is involved in ABA signaling pathway (Bradford et al., 2003).

Moreover, several transcription factors in Group-S basic region/leucine zipper motif transcription factors in Arabidopsis such as bZIP11 mediate SnRK1 signaling (Baena-González et al., 2007; Rodrigues et al., 2013). Overexpression of bZIP11 inhibits plant growth by reprogramming metabolism such as reducing trehalose-6-phosphate level (Hanson et al., 2008; Ma et al., 2011). Consequently, overexpression of bZIP11 or SnRK1 rescues growth inhibition caused by an increased level of T6P, which suggests cross regulation between SnRK1-bZIP and T6P (Schluepmann et al., 2004; Delatte et al., 2011). In addition, AKIN10 phosphorylates Group-A bZIP transcription factors such as ABI5 and EEL/bZIP12 that mediate ABA signaling as well as ABA responsive
element binding proteins (AREBPs) that mediates the gene expression induced by ABA (Zhang et al., 2008; Bitrián et al., 2011). SnRK1 is activated by ABA which inhibits PP2C, promoting SnRK1 signaling during stress, and the elimination of energy deficit conditions in Arabidopsis leaf discs (Rodrigues et al., 2013). SnRK1 and PPC2 are considered the important components of the ABA signaling pathway (Jossier et al., 2009; Rodrigues et al., 2013). Therefore, PP2Cs and bZIPs would be convergence points between SnRK1, ABA and T6P signaling pathways and may integrate different signaling pathways to maintain normal plant growth and development (Figure 1.3).

Figure 1.3 Model of SnRK1-mediated response to changes in sugars. Elevated sugar content inhibits the activity of SnRK1 by causing increased T6P, leading to repression of genes regulated by bZIP transcription factors. Where a decrease in sugar level occurs, the activity of SnRK1 is increased as a result of decreased T6P level, leading to bZIP-mediated gene expression, promoting catabolic processes to achieve a balanced energy condition in plants. Glucose positively regulates the expression of both ABA biosynthesis and ABA signaling genes (León and Sheen, 2003). The dephosphorylation of SnRK1 is decreased due to inactivation of PP2C phosphatases caused by ABA in response to high sugar level, which lessens the effect of sugar on SnRK1 repression (Modified from Rodrigues et al., 2013; Tsai and Gazzarrini, 2014; Hulsmans et al., 2016).

SnRK1 also plays an important regulatory role in vegetative development, senescence and flowering. Arabidopsis akin10/akin11 double-mutants produce flowers and senesce early, while overexpression of SnRK1 delays vegetative growth, senescence, and flowering (Baena-González et al., 2007). AKIN10 and AKIN11 are expressed throughout the entire Arabidopsis plant life cycle, including vegetative development
and flowering, and the expression of the catalytic isoforms is invariable in plants under normal growth conditions (Fragoso et al., 2009). Moreover, SnRK1 kinase activity shows an inverse relation with plant age, i.e. in younger leaves, the activity of the kinase is higher compared to older leaves (Tsai and Gazzarrini, 2014).

1.4 THE EFFECTS OF SnRK1 ON CARBOHYDRATE METABOLISM AND SUGAR SIGNALING IN PLANT LEAVES

Carbohydrate metabolism is a complex process that includes various biochemical pathways and is responsible for the biosynthesis, degradation and interconversion of carbohydrates in living organisms. In plant source organs such as leaves, sucrose and starch are synthesized as a result of photosynthesis in cytosolic and plastidic compartments respectively. Sucrose is exported to sink organs such as roots, tubers and developing seeds via the phloem, or else is degraded to hexoses (glucose and fructose) or hexose phosphate to feed the glycolytic pathway and maintain respiration. At night, leaf starch is degraded to maltose (Zeeman, 2015). Moreover, carbohydrate metabolites such as sucrose, glucose and trehalose-6-phosphate also serve as important signaling molecules in response to both cellular energy status, as well as biotic and abiotic stress (Smeekens and Hellmann, 2014). As mentioned above, SnRK1 directly regulates several metabolic enzymes involved in carbohydrate metabolism by posttranslational modification. Moreover, gene expression analysis shows that AKIN10 also regulates expression of various genes involved in photosynthesis, starch/sucrose metabolism, glycolysis/gluconeogenesis and the oxidative pentose pathway (Baena-Gonzalez et al., 2007).
1.4.1 The effects of SnRK1 on photosynthesis and photorespiration

Photosynthesis is a physico-chemical process in which plants, algae and some prokaryotes directly use light energy to synthesize organic compounds from CO₂. SnRK1 has been shown to promote the gene expression of proteins involved in photosynthesis (Zhang et al., 2009). AKIN10 activates the expression of several genes involved in photosynthesis and photorespiration in Arabidopsis (Table 1.2; Baena-Gonzalez et al., 2007). For example, AKIN10 upregulates the expression of the chloroplastic calvin cycle protein CP12-2, which binds with two dimers of phosphoribulokinase (PRK) and two tetramers of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to form a stable and reversible GAPDH-CP12-PRK complex resulting in inactivation of both enzymes (Graciet et al., 2003; Baena-Gonzalez et al., 2007; Avilan et al., 2012). PRK and GAPDH are essential catalytic enzymes in the regeneration phase and in the reduction phase of Calvin-Benson cycle respectively. Moreover, AKIN10 upregulates the gene expression of glyceraldehyde 3-phosphate dehydrogenase GAPA2 which is a subunit for the formation of GAPDH tetramer complex (Baena-Gonzalez et al., 2007; Niyogi et al., 2015). Thus, AKIN10 would play a sophisticated role in the regulation of Calvin-Benson cycle in plants involving activation and inhibition. Moreover, AKIN10 also upregulates the expression of D-glycerate 3-kinase that catalyzes formation of 3-PGA from glycerate in photorespiration, and links the latter to the Calvin-Benson cycle facilitating reuse of glycolate-derived carbon (Kleczkowski and Randall, 1988; Boldt et al., 2005). In addition, the photosynthetic intermediates R5P and Ru5P can inhibit SnRK1 activity, which would...
Table 1.2 Proteins whose expression is regulated by AKIN10, and their functions in photosynthesis and photorespiration.

<table>
<thead>
<tr>
<th>Gene expression activated by AKIN10</th>
<th>Functions in photosynthesis/photorespiration</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplastic Calvin cycle protein CP12-2</td>
<td>Regulation of the activity of two Calvin cycle enzymes, phosphoribulokinase and glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Graciet et al., 2003; Baena-Gonzalez et al., 2007; Avilan et al., 2012</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate dehydrogenase GAPA2</td>
<td>The formation of GAPDH tetramer complex that catalyzes the form of glyceraldehyde-3-phosphate in Calvin-Benson cycle</td>
<td>Baena-Gonzalez et al., 2007; Niyogi et al., 2015</td>
</tr>
<tr>
<td>NDH-dependent cyclic electron flow 1 (NDF4)</td>
<td>A subunit of the chloroplastic NAD(P)H dehydrogenase (NDH) complex involved in photosystem I (PSI) cyclic and chlororespiratory electron transport</td>
<td>Baena-Gonzalez et al., 2007; Takabayashi et al., 2008</td>
</tr>
<tr>
<td>PSI reaction center subunit D-2 (PSAD-2)</td>
<td>A ferredoxin-docking protein which forms complexes with ferredoxin and ferredoxin-oxidoreductase in PSI reaction center</td>
<td>Baena-Gonzalez et al., 2007; Yu et al., 2008</td>
</tr>
<tr>
<td>Photosynthetic NDH subcomplex L2 (PQL2)</td>
<td>A subunit of the chloroplastic NAD(P)H dehydrogenase (NDH) complex involved in PSI cyclic and chlororespiratory electron transport</td>
<td>Baena-Gonzalez et al., 2007; Suorsa et al., 2010</td>
</tr>
<tr>
<td>Oxygen-evolving enhancer protein 1-2 (PSBO2)</td>
<td>An extrinsic subunit of photosystem II (PSII)</td>
<td>Baena-Gonzalez et al., 2007; Dwyer et al., 2012</td>
</tr>
<tr>
<td>Plastocyanin (PETE1)</td>
<td>A mobile electron carrier between the cytochrome b6f complex and the reaction center of PSI P-700 in photosynthesis</td>
<td>Baena-Gonzalez et al., 2007; Abdel-Ghany, 2009</td>
</tr>
<tr>
<td>Peroxisomal alanine-glyoxylate aminotransferase (AGT)</td>
<td>Catalysis of transamination reactions with multiple substrates in photorespiration</td>
<td>Baena-Gonzalez et al., 2007; Zhang et al., 2013</td>
</tr>
<tr>
<td>D-glycerate 3-kinase</td>
<td>Conversion of glycerate to form 3-phosphoglycerate in photorespiration</td>
<td>Baena-Gonzalez et al., 2007; Boldt et al., 2005</td>
</tr>
</tbody>
</table>
provide feedback regulation of the activity of SnRK1 (Nunes et al., 2013). These results show that SnRK1 plays multiple regulatory roles in photosynthesis and photorespiration.

1.4.2 The effects of SnRK1 on partitioning of photoassimilates between sucrose and starch in plant leaves

During the day, triose phosphates (dihydroxyacetone phosphate (DHAP) and 3-phosphoglyceraldehyde (3-GAP)) synthesized by the Calvin-Benson cycle (photosynthesis) are exported from the chloroplast to the cytosol, and converted to hexose phosphates which are used to synthesize sucrose in the cytosol. The interconversion of fructose 1,6-bisphosphate (Fru-1,6-BP) and F6P in the cytosol is a key step in sucrose biosynthesis, which is regulated by a signal metabolite, Fru-2,6-BP, that integrates photosynthesis, sucrose synthesis, starch synthesis, and glycolysis (Figure 1.4; Nielsen et al., 2004). F2KP is a cytosolic bifunctional enzyme that generates and hydrolyses Fru-2,6-BP to regulate partitioning of photoassimilates between sucrose and starch in plant leaves. Arabidopsis AKIN10 not only phosphorylates F2KP but also activates its gene expression. This has been postulated to switch enzyme activity towards production of Fru-2,6-BP, further promoting carbon flow to glycolysis pathway (Figure 1.4; Cho et al., 2016). During the night, hexose phosphates for sucrose biosynthesis are derived from other source such as glucose, G1P and maltose from transient starch degradation in chloroplast (Zeeman, 2015). SPS is a key enzyme involved in sucrose biosynthesis and SnRK1 can inactivate spinach SPS by phosphorylation on Ser158 (Sugden et al., 1999). Further, AKIN10 upregulates the
expression of a sucrose phosphate synthase isozyme, SPS4F, and downregulates the expression of sucrose-phosphatase 2 (SPP2). The latter catalyzes the formation of sucrose from sucrose-6-phosphate produced by SPS (Lunn et al., 2000; Baena-Gonzalez et al., 2007).

**Figure 1.4** Model of SnRK1-mediated regulation of fructose-2,6-bisphosphate metabolism and regulatory function of the metabolite in the interconversion of fructose-6-phosphate and fructose-1,6-phosphate. Fructose-2,6-bisphosphate is synthesized and degraded by fructose-6-phosphate 2-kinase and fructose-2,6-bisphosphatase respectively. SnRK1 has been postulated to switch the activity of fructose-6-phosphate
2-kinase/fructose-2,6-bisphosphatase towards production of Fru-2,6-BP, further promoting carbon flow to glycolysis (Cho *et al*., 2016). Interconversion of fructose-6-phosphate and fructose-1,6-phosphate is catalyzed by ATP-dependent phosphofructokinase (PFK), pyrophosphate-dependent phosphofructokinase (PFP) and fructose-1,6-bisphosphatase. PEP: phosphoenolpyruvate; F2,6BP: fructose-2,6-bisphosphate. The question marks represent the supposed regulatory target(s).

### 1.4.3 Sucrose degradation

Sucrose can be cleaved by two enzymes: invertases and sucrose synthases that exist as multiple isoenzymes. Sucrose is degraded to form glucose and fructose by invertases or UDP-glucose and fructose by sucrose synthases. UDP-glucose is the substrate for the biosynthesis of cellulose and callose and it also can be converted to G6P by the combined actions of UDP-glucose pyrophosphorylase and phosphoglucomutase to enter glycolysis in the cytosol (Zeeman, 2015). As sucrose synthase can direct carbon flow into polysaccharide biosynthesis, as well as into the pathways of glycolysis and respiration, there is evidence that sucrose synthase plays an important role in cell wall formation (Koch, 2004). SnRK1 from maize leaves can phosphorylate maize sucrose synthase on a serine site (S15) (Hardin *et al*., 2003). Phosphorylation on S15 can change the amino-terminal conformation of the enzyme, which may affect the catalytic activity of sucrose synthase and membrane association (Hardin *et al*., 2004). In addition, phosphorylation of S15 also plays a key role in sucrose synthase degradation, since it is a prerequisite for a second phosphorylation on S170 of maize sucrose synthase that targets it for ubiquitin-mediated degradation by the proteasome (Hardin *et al*., 2003; Koch, 2004). In potato, SnRK1 kinase (PKIN1) positively regulates expression and activity of sucrose synthase, but did not affect activity of neutral and acid invertases in potato tubers (Purcell *et al*., 1998). By contrast, overexpression of AKIN10 in
Arabidopsis protoplasts upregulated the expression of cytosolic alkaline/neutral invertase and vacuolar invertase 2 that is involved in sucrose degradation (Baena-Gonzalez et al., 2007).

1.4.4 The effects of SnRK1 on starch metabolism

Triose phosphates synthesized by the Calvin-Benson cycle can also enter the plastid hexose phosphate pool to support starch biosynthesis. ADP-glucose pyrophosphorylase (AGPase), starch synthases, starch branching enzymes and starch debranching enzymes work together to synthesize starch in plant leaves (Preiss, 1984; Ball et al., 1996; Zeeman et al., 1998; Myers et al., 2000; Zeeman, 2015). SnRK1 has been shown to positively regulate post-translational redox activation of AGPase in potato tubers in response to high sucrose levels (Tiessen et al., 2003), suggesting that SnRK1 might be involved in regulation of the starch biosynthetic pathway. Further, it has been found that both the activity of AGPase and starch content in leaves are increased in tomato overexpressing MhSnRK1 from apple (Wang et al., 2012). Interestingly, overexpression of SnRK1 in transgenic potato tubers and tomato caused increased starch accumulation (McKibbin et al., 2006; Wang et al., 2012), whereas overexpression of AKIN10 in Arabidopsis led to a decrease in the starch content (Jossier et al., 2009).

At night, starch granules in leaves are degraded in chloroplasts and the major products are maltose and glucose which are transported to the cytosol and metabolized to hexose phosphates and sucrose (Smith, 2012). In Arabidopsis leaves, the degradation of starch granules also leads to formation of a smaller amount of maltotriose and longer malto-
oligosaccharides (MOS), as a result of the actions of α-amylase, the debranching enzyme isoamylase 3 (ISA3) and β-amylases (especially β-amylase 3 (BAM3)) (Wattebled et al., 2005; Delatte et al., 2006; Fulton et al., 2008). SnRK1 can regulate the transcription of α-amylase that can contribute to starch degradation (Laurie et al., 2003; Delatte et al., 2006) and AKIN10 can upregulate expression of β-amylase 3 (Baena-Gonzalez et al., 2007). Moreover, starch degradation is significantly decreased in an Arabidopsis akin10/akin11 double mutant, leading to increased starch content during a diurnal cycle (Baena-Gonzalez et al., 2007).

1.4.5 The effects of SnRK1 on the oxidative pentose phosphate pathway

The oxidative pentose phosphate pathway (OPPP) is a major source of reducing power (NADPH) and metabolic intermediates for biosynthetic processes such as the formation of flavonoids, polyphenols, lignin and aromatic amino acids (the shikimate pathway), and nucleic acid synthesis (Kopriva et al., 2000; Kruger and von Schaewen, 2003; Zeeman, 2015). The plastidic pathway contains two phases: the oxidative phase and the non-oxidative phase (Kruger and von Schaewen, 2003). In phototrophic tissues, NADPH produced in the OPPP contributes to redox activation of AGPase (Michalska et al., 2009; Geigenberger, 2011). Ribulose-5-phosphate and ribose-5-phosphate are metabolic intermediates in the OPPP (Kruger and von Schaewen, 2003; Zeeman, 2015) and also inhibit the activity of SnRK1 from Arabidopsis seedlings leaves and wheat grain (Piattoni et al., 2011; Nunes et al., 2013). Moreover, AKIN10 upregulates the expression of two isoforms of glucose-6-phosphate dehydrogenase (1 and 4) that catalyze the first irreversible reaction of the OPPP, but downregulates the expression of
6-phosphogluconate dehydrogenase that catalyzes the production of NADPH by converting 6-phosphogluconate to ribulose 5-phosphate (Baena-Gonzalez et al., 2007; Schluepmann et al., 2012). These results imply that SnRK1 could inhibit NADPH generation for fatty acid and amino acid synthesis, leading to the accumulation of 6-phosphogluconate, in the oxidative phase of the OPPP, and that SnRK1 activity could be regulated by the availability of pentose phosphates.

1.4.6 The effects of SnRK1 on glycolysis and respiration

In plants, glycolysis is the main pathway to fuel respiration since plant mitochondria rarely respire fatty acids (Plaxton, 1996). Moreover, the carbon that flows into glycolysis and the tricarboxylic acid (TCA) cycle is used to synthesize various compounds such as amino acids, nucleic acids, fatty acids and secondary metabolites (Plaxton, 1996). Glycolysis uses sucrose and starch as the principal substrates in higher plants and converts hexose or hexose-6-phosphate, derived from sucrose and starch degradation, to pyruvate to feed the TCA cycle in plants (Plaxton, 1996). Glycolysis occurs independently in two subcellular compartments, the cytosol and plastid. These two glycolytic pathways can communicate via metabolite transporters such as the phosphoenolpyruvate (PEP)/phosphate translocator (PPT) and triose phosphate/phosphate translocator (TPT), located in the inner envelopes of chloroplasts (Plaxton, 1996). Pyruvate kinase (PK) catalyzes the transfer of a phosphate group from PEP to ADP to form pyruvate and ATP in glycolysis. Two potato SnRK1 proteins, P Kin1 and StubSNF1, have been shown to interact with a potato cytosolic PK in vitro and both SnRK1s influence cytosolic PK activity in the SnRK1-repressed potato lines.
in vivo (Beczner et al., 2010). In addition, Arabidopsis AKIN10 upregulates the expression of cytosolic PK (Figure 1.5; Baena-González et al., 2007). AKIN10 downregulates the expression of phosphoenolpyruvate carboxylase (PEPC) 1 which catalyzes the anaplerotic formation of oxaloacetate from PEP, and pyrophosphate-dependent 6-phosphofructose-1-kinase (PFP) that catalyzes the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate (Figure 1.5; Baena-González et al., 2007).

Respiration oxidizes acetyl-CoA produced through the consecutive processes of glycolysis and mitochondrial TCA cycle, producing CO$_2$ and reducing compounds (NAD(P)H and FADH$_2$). Respiration is essential for plant growth, development, and carbon balance (Plaxton and Podesta, 2006). Antisense suppression of several individual reactions of the TCA cycle in tomato produced very little effect on leaf respiration, with the exception of 2-oxoglutarate dehydrogenase (Carrari et al., 2003; Nunes-Nesi et al., 2005; Nunes-Nesi et al., 2007; Studart-Guimarães et al., 2007; Sienkiewicz-Porzucek et al., 2008; Araújo et al., 2008; Sienkiewicz-Porzucek et al., 2010; Araújo et al., 2011). AKIN10 downregulates expression of the enzyme mitochondrial malate dehydrogenase (mMDH) 2 that catalyzes the reversible oxidation of malate to oxaloacetate (Baena-González et al., 2007), which would lead to very little effect on the TCA cycle. Arabidopsis AKIN10 regulates the expression of a mitochondrial S-adenosylmethionine transporter-like protein. S-adenosylmethionine participates in many essential metabolic pathways as the donor of methyl group, methylene groups, amino groups, ribosyl groups and aminopropyl groups in plants (Figure 1.5; Fontecave et al., 2004).
Figure 1.5 The effects of AKIN10 on glycolysis and respiration. AKIN10 regulates activity of pyruvate kinase and expression of pyruvate kinase, phosphoenolpyruvate carboxylase 1, pyrophosphate-dependent 6-phosphofructose-1-kinase, malate dehydrogenase 2, and a mitochondrial S-adenosylmethionine transporter-like protein. Blue box indicates the activity and expression of enzyme are regulated by AKIN10; green box indicates the expression of enzyme is downregulated by AKIN10. SAMT: S-adenosylmethionine transporter.

1.4.7 The effects of SnRK1 on sugar signaling in plants

Sugars are the primary metabolites in plants and provide energy and carbon skeletons for many physiological processes. Sugars also serve as signaling molecules that control and regulate plant growth and development. Several sugar compounds such as hexoses (glucose and fructose), sucrose and trehalose-6-phosphate have been identified as signaling molecules (Rolland et al., 2006; Lastdrager et al., 2014).

1.4.7.1 The effects of SnRK1 on hexose signaling pathway

Both glucose and fructose serve as signaling metabolites in hexose signaling pathways. Glucose, as a signaling metabolite, controls many important physiological processes such as gene transcription and protein expression, primary and secondary metabolism,
cell-cycle progression and growth and developmental programs in plants (early organ
growth and senescence processes) (Jang et al., 1997; Riou-Khamlichi et al., 2000; Koch,
2004; Price et al., 2004; Baena-González et al., 2007; Wingler et al., 2009). Glucose
signaling is sensed and transduced by two main signaling pathways: 1) glucose
signaling sensed directly by glucose sensors such as hexokinase 1 and hexokinase-like
proteins; and 2) glucose signaling sensed indirectly by a variety of energy and
metabolite sensors such as SnRK1 and the glucose-activated target of rapamycin (TOR),
a protein kinase that senses plant nutrient and energy status to stimulate plant cell
proliferation and growth (Xiong and Sheen, 2012). Interestingly, the indirect glucose-
signaling sensor, SnRK1 is inhibited by glucose, whereas TOR kinase is stimulated by
glucose (Baena-González et al., 2007; Xiong and Sheen, 2012). SnRK1 and TOR
control partially overlapping transcriptional networks in plants, which responds to
glucose-derived energy and metabolite signaling associated with glycolysis and
mitochondrial bioenergetics (Baena-González et al., 2007; Xiong et al., 2013).

Fructose has been identified as a signaling metabolite in the hexose signaling pathway
based on the evidence that the Arabidopsis glucose insensitive 2 (gin2) mutant, which
lacks the activity of hexokinase 1, is insensitive to glucose but is sensitive to fructose
(Cho and Yoo, 2011; Li et al., 2011). Fructose signaling results in seedling
developmental arrest, and there is cross-talk with the ABA and ethylene signaling
pathways (Cho and Yoo, 2011). Although there is no evidence that SnRK1 plays a
regulatory role in fructose signaling directly, SnRK1 could indirectly regulate the
interconversion of Fru-1,6-BP and F6P in the cytosol by phosphorylation of F2KP
(Section 1.3 and 1.4.2; Nielsen et al., 2004; Cho et al., 2016) and so be involved in the fructose signaling pathway in plants.

1.4.7.2 The effects of SnRK1 on sucrose-specific signaling pathway

Sucrose, as a significant metabolite, also plays a role in sugar signaling. Sucrose signaling mainly plays a regulatory role in mature and fully functional organs in plants (Koch, 2004). Although a sucrose signaling sensor has not been revealed, Arabidopsis S1 class bZIPs are considered putative sensor candidates for a sucrose-specific signaling pathway (Weltmeier et al., 2009). The Arabidopsis S1 class bZIPs (bZIP1, bZIP2/GBF5, bZIP11, bZIP44, and bZIP53) are positive downstream effectors that regulate many AKIN10 target genes (Baena-Gonzalez et al., 2007; Hanson et al., 2008). Thus sucrose regulates SnRK1 signaling by repressing gene translation of S1 class bZIPs. SnRK1 and sucrose could therefore work together to regulate a complex signaling network to maintain the energy balance in plants. In addition, AKIN10 overexpression repressed the expression of the transcription factor MYB75/PAP1 that induces anthocyanin production, which also is regulated by the sucrose signaling pathway (Teng et al., 2005; Baena-González et al., 2007). As mentioned above, SnRK1 can control sucrose biosynthesis by regulating the activities of sucrose phosphate synthase and sucrose synthase and also be inactivated by sucrose, which shows a tight regulation between SnRK1 and the sucrose signaling pathway (Figure 1.6; Purcell et al., 1998; Sugden et al., 1999b; Hardin et al., 2003; Baena-Gonzalez et al., 2007).
Figure 1.6 Model of SnRK1-mediated regulation of sucrose signaling pathway. SnRK1 inactivates sucrose phosphate synthase by protein phosphorylation and regulates activity of sucrose synthase positively. Meanwhile, sucrose also can inhibit the activity of SnRK1. SnRK1 positively regulates the Arabidopsis S1 class bZIPs that are involved in seed development and metabolic reprogramming in response to stress (Peviani et al., 2016) and inhibits the expression of the transcription factor MYB75/PAP1 involved in anthocyanin production.

1.4.7.3 The effects of SnRK1 on trehalose-6-phosphate signaling

Trehalose-6-phosphate is a metabolite found at trace levels in most plants but plays a central role in the integration of plant metabolism with plant growth and development (Paul et al., 2008). The metabolite is synthesized (Figure 1.7) by the condensation reaction of UDP-glucose and G6P, which is catalyzed by trehalose-6-phosphate synthase (Cabib and Leloir, 1958). T6P is dephosphorylated to form trehalose by trehalose-6-phosphate phosphatase (TPP). Trehalose is eventually hydrolyzed by trehalase into two molecules of glucose (Elbein et al., 2003). T6P is an indispensable signal metabolite involved in the regulation of plant growth and development ranging from control of embryo development to leaf senescence, as well as photosynthesis, carbohydrate utilization and starch biosynthesis in response to carbon availability (Eastmond et al., 2002; Schluepmann et al., 2003; Pellny et al., 2004; Acevedo-Hernández et al., 2005; Lunn et al., 2006; Schluepmann et al., 2012). Moreover, elevated concentrations of T6P are an indicator of high carbon availability, and related
to the concentrations of some important carbohydrates such sucrose, G6P and F6P in vivo (Schluepmann et al., 2003; Paul et al., 2008).

**Figure 1.7** Metabolism of T6P and regulation of SnRK1-bZIP11 by T6P. UDP-glucose and G6P from sucrose degradation are converted by trehalose-6-phosphate synthase to form T6P. T6P is dephosphorylated to form trehalose by trehalose-6-phosphate phosphatase. Trehalose is eventually hydrolyzed by trehalase into two molecules of glucose. When sucrose is abundant, an increase in T6P inhibits SnRK1 and therefore bZIP11-dependent gene expression; whereas when sucrose or carbon level drops, a decrease in T6P activates SnRK1 which drives bZIP11-dependent gene expression (Modified from Ponnu et al., 2011).

SnRK1 can inactivate trehalose-6-phosphate synthase 5 by phosphorylation and AKIN10 upregulates the gene expression of TPS 8-11 (Polge and Thomas, 2007; Baena-Gonzalez et al., 2007). Meanwhile, T6P inhibits the activity of SnRK1 (Zhang et al., 2009). The transcription factor bZIP11 could be the link point between T6P and SnRK1 (Delatte et al., 2011). bZIP11 is involved in regulating gene expression to inhibit plant growth in response to sugar starvation (Hanson et al., 2008) and has a synergistic effect with SnRK1 on regulating gene expression (Baena-González et al., 2007).
2007). Arabidopsis plants overexpressing bZIP11 accumulate T6P and have reduced SnRK1 activity (Delatte et al., 2011). bZIP11 is translationally repressed by sucrose in Arabidopsis (Rook et al., 1998), and transgenic Arabidopsis plants with dexamethasone-induced bZIP11 possess reduced T6P levels following induction (Hanson et al., 2008; Ma et al., 2011). The link between T6P and SnRK1 is explained by a proposed mechanism: when sucrose is abundant, an increase in T6P inhibits SnRK1 and therefore bZIP11-dependent gene expression; whereas when sucrose or carbon level drops, a decrease in T6P activates SnRK1 and then bZIP11-dependent gene expression (Delatte et al., 2011). These evidences shows that SnRK1 and T6P regulate corporately a complex signaling network in response to different carbon levels in plants.

1.5 SUBCELLULAR LOCALIZATION OF SnRK1

Plant cells are organized into structurally and functionally distinct compartments that contain specific sets of proteins and biochemically distinct physiological processes. The appropriate subcellular localization of proteins is crucial and necessary since this offers the proper physiological environment and substrates for their activity and function. Identification of protein subcellular localization can supply useful biological information on their functional characteristics such as their putative interacting partners and post-translational modification machinery and also integrate the proteins into known functional biological networks (Hung and Link, 2011). As a central regulator, SnRK1 plays complex roles in plant energy regulation and some of these roles involve cross-talk with other sugar or hormone signaling pathways in plants, which makes the study on SnRK1 function more complex.
Some studies on subcellular localization of AKIN subunits have been done. AKIN10 and AKIN11 have been shown to localize to the nucleus and cytosol in tobacco leaves based on transient expression assays using a fluorescent-tagged protein (Nietzsche et al., 2014; Williams et al., 2014). AKIN10 did not co-localize with chloroplast, peroxisomes, mitochondria, or Golgi in tobacco leaves (Williams et al., 2014). However, in transgenic Arabidopsis plants overexpressing AKIN10-GFP and/or AKIN11-GFP fusion proteins, AKIN10 and AKIN11 appeared to localize to the chloroplast and cytosol (Fragoso et al., 2009; Bitrián et al., 2011; Williams et al., 2014).

With response to the β subunits of SnRK1, AKINβ2 were detected in close proximity to chloroplasts by employing the rabbit anti-AKINβ2 antibody. Further, chloroplasts were isolated and treated with or without thermolysin (a thermostable neutral metalloproteinase to degrade all proteins external to intact chloroplasts) and analyzed by western blot. AKINβ2 was detected in the untreated sample but not in the sample treated by the protease, suggesting that AKINβ2 binds with the outer membrane of the chloroplasts (Avila-Castañeda et al., 2014). Moreover, AKINβ2 and AKINβ3 were located in both the cytosol and the nucleus by bimolecular fluorescence complementation (BiFC) experiments accomplished by observing interactions between the two AKINβ-subunits and AKINβγ in tobacco leaves (Gissot et al., 2006). In addition, as mentioned previously (Section 1.2.1), inhibition of N-myristoylation results in relocalization of AKINβ1 and AKINβ2 from the plasma membrane to the nucleus and the cytosol respectively (Pierre et al., 2007). With response to the γ subunits of SnRK1, AKINβγ from maize was detected in both nucleus and cytosol in transfected
onion epidermal cells (López-Paz et al., 2009). AKINβγ is also apparently localized within chloroplasts as detected by an anti-AKINβγ antibody. Further, western blots of the proteins from isolated chloroplasts, treated and untreated with thermolysin, indicated that AKINβγ is located inside and outside the organelle (Avila-Castañeda et al., 2014). No information about the subcellular localization of AKINγ is available. Thus there remains considerable ambiguity about the location of different SnRK1 subunits.

1.6 HYPOTHESIS AND OBJECTIVES OF THE PROJECT

The SnRK1 family, of evolutionarily conserved protein kinases, are hypothesized to perceive cellular metabolic state and energy levels and are activated in response to decreased energy levels. Upon activation, SnRK1 kinases initiate complex transcriptional and metabolic reprogramming events to restore and maintain energy balance. They stimulate tolerance to stress conditions, achieved partly by promoting catabolism and repressing anabolism. The evidence suggests that SnRK1, as a global regulator, regulates important carbohydrate and related metabolic pathways such as photosynthesis, starch and sucrose metabolism, and glycolysis and respiration. This is achieved by phosphorylation of proteins within a pathway and by transcriptional reprogramming. The present investigation is based on the hypothesis that SnRK1 plays a key role in regulating carbohydrate metabolism and carbohydrate partitioning in plants to maintain energy balance in plant cells.

This research project focused on the following objectives,
To investigate the role of the regulatory subunit AKINβ1 (AT5G21170) on carbohydrate metabolism and carbon partitioning in *Arabidopsis thaliana*.

An Arabidopsis T-DNA mutant with significantly decreased expression of *AKINβ1* (SALK_008325) has been employed to determine the effects of altered *AKINβ1* on carbohydrate metabolism and carbon partitioning in plants. Although β subunits are important components of the kinase complexes, no study on the effect of any regulatory subunit of SnRK1 on carbohydrate metabolism and carbon partitioning in Arabidopsis has been carried out to date; further, bioinformatic analysis suggests that at least one splice variant of the *AKINβ1* subunit sequence includes a putative transit peptide which could direct the protein to chloroplasts. Moreover, unlike *AKINβ2* and *AKINβ3*, the expression of *AKINβ1* is upregulated by AKIN10, indicative of a close relationship between AKIN10 and AKINβ1. To reveal changes in carbohydrate metabolism and carbon partitioning in the *akinβ1* mutant, starch and soluble sugars were determined by enzymatic analysis. Carbohydrate and organic acid metabolic profiles were analyzed by high-performance anion-exchange liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and respiration and photosynthesis rates were measured by gas exchange (see Chapter II).

To determine the subcellular localization of three subunits of SnRK1 *in vivo*. AKIN11, AKINβ1 and AKINγ were fused with different fluorescent proteins and localization determined by confocal laser microscopy. This was undertaken using both transient expression systems in *N. benthamiana* and also following stable
transformation in *Arabidopsis thaliana*. To identify accurate subcellular localization of SnRK1 subunits in *N. benthamiana*, a suite of fluorescent compartment-specific markers was also employed. The literature is unclear and there is a need to provide definition of subcellular compartmentation if the roles of SnRK1 subunits are to be properly understood (see Chapter III).

**III** To express recombinant proteins of SnRK1 subunits in *E. coli* and study the interactions of SnRK1 with other proteins in plant leaf cells.

AKIN11, AKINβ1 and AKINγ were cloned in a modified pET32a plasmid and expressed in *E. coli*. Each subunit was immobilized to an inert support and used as bait to identify putative interacting proteins in plant leaf extracts (see Chapter IV).

**IV** To study the role of the regulatory subunit AKINβ1 on transcriptional regulation in *Arabidopsis*.

RNA extracts from the *Arabidopsis* *akinβ1* mutant leaves and *Arabidopsis* Columbia (Col-0) leaves were made during darkness and light. High-throughput RNA-sequencing (RNA-Seq) was used to determine transcriptome expression in wild-type and mutant leaves of *Arabidopsis* (see Chapter V).
CHAPTER II
EFFECTS OF DOWN REGULATION OF AKINβ1 ON LEAF METABOLISM AND PHYSIOLOGY IN *ARABIDOPSIS THALIANA*
2.1 INTRODUCTION

The previous chapter identified SnRK1 as a global metabolic sensor and regulator of energy homeostasis in plants. In metabolic terms, the protein kinase is thought to activate catabolic pathways to produce energy and to inhibit anabolic pathways in order to conserve energy. Considerable evidence points to a role for SnRK1 in the regulation of several metabolic pathways including photosynthesis/photorespiration, sucrose metabolism, starch metabolism, OPPP, glycolysis and respiration. This is achieved by regulating gene expression and by post-translational protein phosphorylation (Baena-González et al., 2007; Jossier et al., 2009; Cho et al., 2016).

Carbon partitioning (Figure 2.1) in plants is a complex regulatory process related to plant survival, growth and development and the effects of SnRK1 (α subunit) on carbon partitioning has been revealed in pea embryos (Radchuk et al., 2010). At the whole-plant level, carbon partitioning is the process whereby photoassimilates are distributed throughout the whole plant from source tissues to sink tissues. For most plants, this involves partitioning of carbon to sucrose synthesis in leaves, and transport to sink tissues via the phloem (Turgeon, 1989; van Bel, 2003). Within photosynthetic cells, carbon partitioning represents the distribution of photosynthate between export, respiration and other metabolic pathways, and has a significant influence on the rate of plant growth and development. Carbon partitioning between starch and sucrose biosynthesis fluctuates with photosynthetic rate. Low carbon partitioning to starch is a response to low rates of photosynthesis, whereas much higher proportions of fixed carbon are diverted to starch at higher rates of photosynthesis (Sharkey et al. 1985).
During photosynthetic CO$_2$ fixation, triose phosphates are produced and used in the Calvin-Benson cycle to regenerate ribulose-1,5-bisphosphate in the chloroplast with net gain of carbon in the form of triose phosphate. As a result, the levels of triose phosphates and 3-phosphoglycerate (3-PGA) are increased, and the level of phosphate (Pi) in the chloroplast is decreased, the latter a result of ATP synthesis by photophosphorylation in chloroplast. 3-PGA is an activator and Pi is an inhibitor of ADP-glucose pyrophosphorylase and a change in the ratio of these two, therefore regulates AGPase activity (Gosh and Preiss, 1966). Alternatively, triose phosphates are translocated to the cytosol in exchange for Pi, thereby restoring the Pi balance of the chloroplast. In the cytosol, triose phosphates are converted to Fru-1,6-BP and then to hexose phosphates and UDP-glucose, providing the substrates (F6P and UDP-glucose) and activator (G6P) for SPS in the pathway of sucrose synthesis. The exchange of cytosolic Pi with plastidic triose phosphate leads to decreased Pi levels in the cytosol, which also relieves the inhibition of SPS activity (Preiss, 1984; Nielsen et al., 2004). Moreover, dihydroxyacetone phosphate (triose phosphate) is directly used for glycolysis in the cytosol. Carbon from sucrose and starch degradation can also be converted to Fru-1,6-BP and then to DHAP and pyruvate in the cytosol to feed into the TCA cycle in the mitochondria. So the cytosolic interconversion between F6P and Fru-1,6-BP is a vital point for control of carbon partitioning between sucrose metabolism, starch metabolism and respiration. The conversion of F6P to Fru-1,6-BP is catalyzed by ATP-dependent phosphofructokinase (PFK) and pyrophosphate-dependent phosphofructokinase (PFP) and the conversion of Fru-1,6-BP to F6P is catalyzed by
fructose-1,6-bisphosphatase (FBPase) or a reversal of the PFP reaction. Although ATP-dependent PFK is not regulated by Fru-2,6-BP, Fru-2,6-BP could regulate the interconversion between F6P and Fru-1,6-BP by its action as an activator of PFP and an inhibitor of FBPase in the cytosol (Stitt, 1990; Nielsen, 1995; Nielsen et al., 2004). Arabidopsis AKIN10 not only phosphorylates 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (F2KP), which is a cytosolic bifunctional enzyme that generates and hydrolyses Fru-2,6-BP, but also activates its gene expression. It has been proposed that phosphorylated F2KP activity favors production of Fru-2,6-BP, promoting carbon flux through glycolysis (Cho et al., 2016).

![Diagram](image)

**Figure 2.1** Schematic representation of carbon partitioning within source tissues. Abbreviated names of the key enzymes that regulate flux are shown adjacent to red and green arrows. Abbreviated names of metabolites are shown in circles. Solid lines show reactions and dashed lines indicated allosteric regulations. Metabolic reactions occurring only at daytime are shown in red, metabolic reactions occurring only at night are shown in black and reactions occurring during day and night are shown in green. Carbon dioxide fixed in the Calvin-Benson Cycle (CBC) during the day is reduced to form triose-phosphates (TP). TP is partitioned between starch synthesis in the
chloroplast and sucrose synthesis in the cytosol. Starch synthesis is regulated by two irreversible enzymes: stromal fructose-1,6-bisphosphatase (sFBPase) and ADP-glucose pyrophosphorylase (AGPase). Sucrose synthesis is regulated by two irreversible enzymes: cytosolic FBPase (cFBPase) and sucrose-phosphate synthase (SPS). The activity and transcript level of cFBPase increase in the light (Khayat et al., 1993). cFBPase is involved in sucrose synthesis predominantly during the day since TP is derived from the CBC. FBPase can be allosterically inhibited by fructose-2,6-bisphosphate, which is synthesised from F6P by the bifunctional enzyme F2KP. At night, the substrates for sucrose synthesis are derived from maltose (M), glucose (G) and glucose-1-phosphate (G1P) arising from starch degradation. β-amylase (BAM), one of the enzymes of starch degradation, is shown for clarity. Sucrose is synthesized by using hexose phosphates in the cytosol hexose phosphate pool (G1P, G6P and F6P) during day and night and is exported to sink tissues (Taken from Pokhilko et al., 2014; Pokhilko and Ebenhöh, 2015).

As mentioned in the previous chapter, SnRK1 directly regulates several metabolic enzymes involved in carbohydrate metabolism by posttranslational modification and also regulates expression of various genes involved in photosynthesis, starch/sucrose metabolism, glycolysis/gluconeogenesis and the oxidative pentose pathway (Baena-Gonzalez et al., 2007), indicating that SnRK1 plays various roles in carbohydrate metabolism.

Metabolomics (metabolic profiling) is a powerful tool which provides quantitative analysis and qualitative profiling of large numbers of cellular metabolites in biological processes and metabolic pathways in cells or organ (Gika et al., 2014; Peng et al., 2015). By studying global metabolite profiles, measurement of the changes in the concentrations of cellular metabolites, as a result of some form of perturbation, provide biochemical insight into metabolic processes in cells (Theodoridis et al., 2008). In addition, metabolic profiling supplies a large amount of information on metabolite concentrations and the real-time metabolic status which can enrich and help explain
genomic, transcriptomic and proteomic data (Gika et al., 2014). Metabolic profiling has been used to understand mechanisms involved in metabolic regulation. For example, metabolic profiling has been used to demonstrate a light-dependent accumulation of many compounds including many sugars and sugar derivatives, TCA cycle intermediates, and amino acids in Arabidopsis plants grown under different light conditions (Jänkänpää et al., 2012). Pertinent to the present study, metabolic profiling of embryos from a homozygous pea line, with repressed SnRK1 activity, revealed altered levels of several organic and amino acids, sugars and glycolytic intermediates, indicating decreased carbon partitioning into subsequent pathways such as the TCA cycle and amino acid biosynthesis (Radchuk et al., 2010).

So far, many studies on SnRK1 have focused on the catalytic subunits, especially AKIN10 in relation to sugar and ABA signaling (Jossier et al., 2009; Rodrigues et al., 2013). However, a few studies on effects of the regulatory β and γ subunits on carbohydrate metabolism in plants were performed. The regulatory β subunits appear to play an important role in determining the subcellular localization of the kinase complexes, and in associating with downstream targets due to their unique NVR domain. As mentioned in previous chapter, AKINβ1 and AKINβ2 can change their locations by N-myristoylation-mediated regulation (Pierre et al., 2007) and AKINβ1 and AKINβ3 can interact with nitrate reductase that can be bound and phosphorylated by SnRK1 (Polge et al., 2008). Further, expression of AKINβ1 is regulated tightly by AKIN10 (Pierre et al., 2007; Baena-González et al., 2007). Further, the expression of AKINβ1 is apparently upregulated by darkness and repressed by sucrose (Polge et al.,
2008), consistent with the hypothesis that SnRK1 is activated or deactivated in response to changes in energy status.

In this study, the role of the regulatory subunit, AKINβ1 (AT5G21170), was studied by comparing metabolism in the wild-type (WT) with a T-DNA insertion mutant (Salk-008325). Two splice variants (AT5G21170.1 and AT5G21170.2) giving rise to two mRNAs from the same gene have been identified. Insertion of T-DNA into the Arabidopsis Columbia (Col-0) mutant line selected was investigated by polymerase chain reaction (PCR). Effects of the T-DNA insert on expression of akinβ1 were detected by reverse transcription (RT)-PCR and quantitative reverse transcription (qRT)-PCR respectively. Using both wild-type and the T-DNA insertion line, starch and soluble sugars were determined by enzymatic analysis, and carbohydrate and organic acid metabolic profiles were analyzed by LC-MS/MS. Effects on respiration and photosynthesis rates were measured by gas exchange. Thus changes in carbohydrate metabolism and carbon partitioning in the akinβ1 mutant were identified in comparison to the wild-type in order to gain further understanding of the role played by this kinase in plant carbon metabolism.

2.2 MATERIAL AND METHODS

2.2.1 Arabidopsis lines and plant growth

Arabidopsis Columbia (Col-0) ecotype was used as wild-type in this study. Seeds of Arabidopsis Columbia (Col-0) mutant containing a T-DNA insert in akinβ1 (SALK_008325) were obtained from The Arabidopsis Biological Resource Center (ABRC) at Ohio State University, USA. Before sowing, seeds were placed in a 1.5-ml
Eppendorf tube at 4°C for 2 days. About 200 Arabidopsis seeds were transferred to a fresh 1.5-ml Eppendorf tube and 0.1% agar solution was added and mixed. About 3 seeds per well were dropped into a 24-cell plastic tray containing wet soil, held by a plastic half-flat tray. After sowing seeds, the tray was placed in Conviron E8 growth chamber (Conviron, Winnipeg, Canada) and covered with a clear plastic cover for one week. Arabidopsis plants were grown in long-day conditions (16-h light/8-h darkness), at a light intensity of 150µmol photon/m²/sec, 23°C (daytime) and 18°C (darkness), and 60% humidity. Arabidopsis plants were watered once per week.

### 2.2.2 Arabidopsis plant genomic DNA (gDNA) extraction

To identify exogenous gene inserts in the Arabidopsis genome, a whole Arabidopsis leaf was harvested in a 1.5-ml Eppendorf tube to extract genomic DNA. 200µl of diluted Edwards Solution (final concentrations, 29mM Tris-HCl (pH7.5), 25mM NaCl, 3.4mM EDTA, and 0.05% SDS) was added to the tube. The leaf was crushed with a plastic rod against the tube wall until the solution turned transparent green and visible tissue residue was left in the solution. The solution was centrifuged at 14,000 rpm for 5 min and the supernatant transferred to a fresh 1.5-ml Eppendorf tube. The supernatant was stored at -20°C for future use.

### 2.2.3 Polymerase chain reaction

The KOD Hot Start DNA polymerase (Novagen, Billerica, USA) was employed for PCR reactions unless otherwise mentioned. The components of PCR reaction mixture (50μl) were 5μl 10×KOD Hot Start DNA Polymerase Buffer, 3μl 25mM MgSO₄, 5μl dNTPs (2mM each), 32.5μl distilled water (dH₂O), 1.5μl Sense (5’) primer, 1.5μl Anti-
Sense (5’) primer, 1μl template DNA and 0.5μl polymerase. Cycling protocol used:

Initial denaturation: 95ºC for 5min;

Denaturation: 95ºC for 30s;

Annealing: lowest melting temperature (Tm) of primer for 30s;

Extension: 72ºC for 20s/kb, 35 cycles;

Final extension: 72ºC for 10min.

The PCR product was stored at -20ºC for future use.

2.2.4 Identification of T-DNA insertion in AKINβ1 genes of SnRK1 in Arabidopsis akinβ1 mutant line

Seeds of the akinβ1 mutant were sown as described above (Section 2.2.1). A rosette leaf from each of 10 independent plants was harvested and Arabidopsis genomic DNA extracted. Homozygous, single mutant lines were detected by standard PCR amplification of the mutant allele from genomic DNA, using specific primers. PCR primers used for mutant allele identification hybridize to the T-DNA left border (LBB1.3, 5’-ATTTTGCCGATTTCGGAAC-3’), to the left genomic sequence of akinβ1 (Beta F, 5’-TTTTTGTGTATCTTGGTTCAGG-3’), and to the right genomic sequence of akinβ1 (Beta R, 5’-GTGACGCATAGGTGAGCAA-3’).

2.2.5 Agarose gel electrophoresis

0.8%-1% (w/v) agarose gel was employed for DNA or RNA separation. Agarose powder was dissolved completely in Tris-Acetate-EDTA (TAE) buffer (40mM Tris-acetate (pH8.0) and 1mM EDTA) by heating in a microwave oven. The boiled solution was cooled to 50-60ºC and ethidium bromide added to a final concentration of 0.2μg/ml.
The gel was poured into a gel tray and a “comb” was placed at one end of the tray to make sample wells. TAE buffer was overlaid on the gel. DNA or RNA samples mixed with 6×DNA loading dye or 2×RNA loading dye (Thermo Scientific, Waltham, USA) were loaded onto the gel. Electrophoresis was carried out at 90V for 40-50 min. DNA or RNA bands in gels were visualized on a ChemiDoc™ XRS gel imaging and documentation system (BioRad, Berkeley, USA).

2.2.6 Total RNA extraction, RT-PCR and qRT-PCR

To test the expression level of AKINβ1 gene in the akinβ1 mutant and WT, RT-PCR and qRT-PCR were used respectively. About 0.5g leaves of 4-week-old plants were harvested into 15-ml Falcon tubes every 4 hours over a 24-hour cycle or 1 hour into the dark period and were frozen in liquid nitrogen immediately. About 100 mg frozen leaf sample was ground to a fine powder, in the tube, at liquid nitrogen temperature using a pre-cooled plastic rod. Total RNA from Arabidopsis leaf cells was isolated and purified by employing Plant/Fungi Total RNA Purification Kit (Norgen BIOTEK CORP, Thorold, Canada).

Reverse transcription of total RNA to cDNA was done by employing qScript cDNA SuperMix kit (Quanta Biosciences, Gaithersburg, USA). 2μl RNA samples containing 1μg of the total RNA, 4μl of 5×qScript cDNA SuperMix, 14μl RNase/DNase-free water were added together in a PCR tube and mixed thoroughly. cDNA first-strand synthesis was performed: 25ºC for 5 min, 42ºC for 30 min and 85ºC for 5 min. Two more cDNA first-strand samples, taken at 8 hours into darkness, were prepared to create standard curves for the two sets of qRT-PCR primers of akinβ1 and ACTIN2 (ACT2). The primers
were designed based on the cDNA sequence of *akinβ1* after its first intron and the cDNA sequence of *ACT2* respectively using Primer3 software (*akinβ1* qRT-PCR1 F: AGCAA GGATGGACTCTCTCAA, *akinβ1* qRT-PCR1 R: TACCGTGTGAGCGGTTTGT; *akinβ1* qRT-PCR2 F: GCAAGGAATCCCTACCATCA, *akinβ1* qRT-PCR2 R: TTCCCC TGATTTCCTGCAGCTT; *ACT2* qRT-PCR1 F: GCAGGAGATGGAAACCTCAA, *ACT2* qRT-PCR1 R: GGGCATCTGAATCTCTCAGC; *ACT2* qRT-PCR2 F: GGTAA CATTGTGCTCAGTGGTG, *ACT2* qRT-PCR2 R: ACGACCTTAATCTTCATGC TGC).

For RT-PCR, 2μl of the cDNA first-strand reaction sample, from the RNA sample made 1 hour into darkness, was used to amplify the full-length cDNA coding AKINβ1 by PCR reactions with the primers (*AKINβ1* Exon1 F: ATGGGAAATGCG AACGGCAA, *AKINβ1* Exon4 R: TTACCGTGTGAGCGGTTTGT).

For qRT-PCR, 20μl of the cDNA samples from leaf extracts, harvested every 4 hours over a 24-hour cycle, were diluted 5 times with dH2O into 100μl. For each qRT-PCR reaction, 11μl of the diluted cDNA samples, 12.5μl of 2×QuantiTect SYBR Green PCR Master Mix (QIAGEN, Venlo, Netherlands), 1.5μl of each specific primer (*akinβ1* qRT-PCR1 F/R; *ACT2* qRT-PCR2 F/R; *AKINβ1* Exon1 F/R) (10μM) were added in a 96-well PCR plate and mixed thoroughly. Thermal cycling: 50°C for 2 min, 95°C for 2 min, and 95°C for 15s and 60°C for 1 min for 40 cycles, employing the Applied Biosystems 7300 Real-Time PCR System (Thermo Scientific, Waltham, USA).
2.2.7 Measurement of starch content

To measure leaf starch content, approximate 0.5g leaves of 4-week-old plants were harvested into 15-ml Falcon tubes respectively, and frozen in liquid nitrogen immediately after weighing. Leaves were harvested from three biological replicates.

10ml 80% (v/v) ethanol was added into each sample and incubated in a boiling water bath for 30 min. The samples were centrifuged at 3000g for 10 min at room temperature and the supernatant discarded. This ethanol extraction was repeated twice, discarding the supernatant. Each pellet was transferred to a mortar, dried and homogenized thoroughly at room temperature. The homogenized pellets were transferred back to their respective tubes. 5ml sterilized water was used to wash each mortar and added to the sample. 0.5 ml of each homogenate was added to each of four 1.5-ml Eppendorf tubes. The samples were incubated at 100 °C for 10 min to gelatinize starch granules. Samples were cooled to room temperature and 0.5ml 200mM sodium acetate (pH5.5) added to each tube. About 6 units of α-amylglucosidase and 1 unit of α-amylase were added to three tubes of each sample, and an equivalent volume of water was added to one tube of each sample as a control. All samples were incubated at 37°C overnight with rotating. After overnight incubation, the samples were centrifuged at 10000g for 5 min at room temperature. 20μl of each supernatant was used to measure glucose content (Section 2.2.9) and the content of starch was calculated in each sample according to Smith and Zeeman (2006).

2.2.8 Soluble sugar extraction from leaves

About 0.15g leaves, from each of three biological replicates, were harvested into 1.5-
ml Eppendorf tubes respectively, frozen in liquid nitrogen immediately after weighing and stored at -80 °C for future use.

Each sample was ground into powder in tubes standing in a container with liquid nitrogen. 1ml 80% (v/v) ethanol was added into each tube and incubated in a boiling water bath for 1 hour. Samples were centrifuged at 12000g for 10 min at room temperature and each supernatant transferred to a fresh 1.5-ml Eppendorf tube. This ethanol extraction was repeated twice more. 3ml of supernatant for each sample was divided into three Eppendorf tubes. The supernatant from each sample was freeze-dried and the pellets dissolved in 100μl dH2O. Solutions were transferred into one of the three tubes and 150μl dH2O was used to wash each of the other two tubes, transferring the 300μl dH2O into the sample. The total volume obtained for each sample was 600μl. Samples were stored at -20°C for future use.

2.2.9 Measurement of soluble sugars

Sugars were measured by enzymatic determination against appropriate standards of known concentrations.

For glucose determination, 40μl of soluble sugar extract was mixed with 1ml triethanolamine buffer (0.75M triethanolamine hydrochloride, 10mM MgCl2 (pH7.6)), 1.88ml dH2O, 100μl nicotinamide-adenine dinucleotide phosphate solution (11.5mM NADP-Na) and 100μl adenosine-5’-triphosphate solution (81mM ATP-Na2H2, 0.36mM NaHCO3) in sequence and absorbance at 340nm measured. 20μl Hexokinase/glucose-6-phosphate dehydrogenase mixture (2mg/ml HK, 1mg/ml G6P DH in 3.2M
(NH$_4$)$_2$SO$_4$ dissolved in 50mM Tris-HCl (pH7.5) and 1mM MgCl$_2$) (Sigma-Aldrich, St. Louis, USA) was added to each assay and mixed thoroughly. Absorbance at 340 nm of each solution was monitored until no further increase was detectable.

For fructose determination, 40μl of each soluble sugar extract was mixed with the same reaction mix as for glucose prior to addition of 20μl of phosphoglucose isomerase mixture (0.1U/μl phosphoglucose isomerase in 50mM Tris-HCl (pH7.5), 1mM MgCl$_2$) (Sigma-Aldrich, St. Louis, USA) and the solution was incubated at 25°C for 30 min. In this assay, fructose in each sample was converted to F6P and then G6P by hexokinase and phosphoglucose isomerase in sequence. Glucose in the same sample was also converted to G6P by hexokinase. Thus the amount of fructose in each sample is equal to the difference in values before and after addition of phosphoglucose isomerase.

For sucrose determination, 30μl of each soluble sugar extract was mixed with 20μl of invertase (1.67mg/ml invertase in citrate buffer (164mM citrate acid and 155mM trisodium citrate (pH4.6)) (Sigma-Aldrich, St. Louis, USA) and was incubated at 55°C for 30 min. Each sample was then mixed with the same reaction mix as for glucose determination. Meanwhile, 30μl of each soluble sugar extract without the addition of invertase was mixed with the same reaction mix as for glucose determination. In this assay, sucrose was hydrolyzed by invertase to form glucose and fructose. Sucrose content was calculated as the difference in the amount of glucose measured with and without the addition of invertase.

For maltose determination, 30μl of soluble sugar extract was mixed with 20μl of α-
glucosidase (0.81mg/ml α-glucosidase in citrate buffer (164mM citrate acid and 155mM trisodium citrate (pH6.6)) (Sigma-Aldrich, St. Louis, USA) and was incubated at 25°C for 2 hours. Each sample was mixed with the same reaction mix as for glucose determination. In this assay, maltose is hydrolyzed by α-glucosidase to form glucose, and sucrose is also hydrolyzed by α-glucosidase to form glucose and fructose. Thus glucose from maltose, glucose from sucrose, as well as the endogenous amount of glucose in each sample were determined. Moreover, one molecule of maltose is hydrolyzed to form two molecules of glucose. Thus calculation of the amount of maltose is deduced from separate measurements taking account of the amounts of sucrose and glucose in each sample.

For malto-oligosaccharide (MOS) determination, 30μl of each soluble sugars extract was mixed with 20μl of amyloglucosidase (0.825U/μl amyloglucosidase in 3.2M (NH₄)₂SO₄ solution (pH 4.5)) (Sigma-Aldrich, St. Louis, USA) and was incubated at 37°C for 2 hours. Each sample was then mixed with the same reaction mix as for glucose determination. In this assay, MOS and maltose were hydrolyzed by amyloglucosidase to form glucose. Thus the amount of glucose from MOS is deduced by taking account of the amounts of glucose derived from maltose, and the amount of glucose in each sample. One molecule of MOS is hydrolyzed to form n molecules of glucose (n=3-10; Weill and Hanke, 1962). Because the molecular weights of MOS are not a single value, the molar mass is presented as maltose equivalents.

For data calculations, the general formula used to calculate the concentration of glucose
is:

\[ C = \frac{(V \times MW)}{(\varepsilon \times d \times v \times 1000)} \times (A_{\text{sample before reaction}} - A_{\text{sample after reaction}}) \text{ (mg/ml)} \]

- \( V \) is final volume (ml)
- \( v \) is sample volume (ml)
- \( MW \) is molecular weight of the substance to be assayed
- \( d \) is light path (cm)
- \( \varepsilon \) is absorption coefficient of NADPH at 340 nm which is 6.22 (L/mM×cm)
- \( A \) is measured absorbance of NADPH at 340nm

### 2.2.10 Metabolite determination

Four-week-old leaf material was harvested directly in the growth chamber in ambient light, and immediately placed in labelled tubes sitting in liquid nitrogen. Leaves were harvested from five biological replicates. Frozen leaf samples were ground to a fine powder, in the tubes, at liquid nitrogen temperature using pre-cooled plastic rods. 15-20mg of the frozen leaf powder was transferred into a pre-cooled, 2-ml Safe-lock microcentrifuge tubes (Eppendorf, Wesseling-Berzdorf, Germany). 175\( \mu \)l of ice-cooled 30% (v/v) CHCl\(_3\) in CH\(_3\)OH was added to each sample while the tubes were sitting in liquid nitrogen. After thawing on ice, each sample was mixed thoroughly with the CHCl\(_3\)/CH\(_3\)OH solvent by agitation of each tube. 175\( \mu \)l of ice-cooled 30% (v/v) CHCl\(_3\) in CH\(_3\)OH was used to wash tiny leaf debris from the wall of each tube into the initial extract in each tube. After the samples were placed at -20°C for 3 hours with occasional mixing, 350\( \mu \)l ice-cooled dH\(_2\)O was added to each sample. Samples were warmed to 4°C with repeated shaking and centrifuged at 13000g at 4°C for 10 min. The upper, aqueous-CH\(_3\)OH phase in each tube was transferred into a fresh 1.5-ml tube and kept at 4°C. 300\( \mu \)l ice-cooled dH\(_2\)O was used to re-extract the CHCl\(_3\) phase in each tube by
vortexing and centrifuging as described above. The second aqueous-CH₃OH phase was added to the first aqueous-CH₃OH extract. Each aqueous-CH₃OH extract was evaporated to dryness at 35°C. Samples were sent to Dr. John Lunn (Max Planck Institute of Molecular Plant Physiology, Germany) for high-performance anion-exchange liquid chromatography coupled to tandem mass spectrometry analysis of metabolites. Metabolites were measured as described by Lunn et al. (2006) and Figueroa et al. (2016). The LC-MS/MS machine was calibrated by using known amounts of the pure compound for each of the metabolites measured and then plotting the integrated peak area against the amount used set up the calibration curves. In addition, compounds labelled with stable isotopes as internal standards were also used to correct for ion suppression or other matrix effects. The integration and calculation of chromatograms was performed using Analyst software (SCIEX).

2.2.11 Measurement of photosynthesis and respiration

Photosynthesis and respiration were measured using the LI-6400 Portable Photosynthesis System (LI-COR Biosciences, Lincoln, USA). Two trays of each set of plants were grown at 23°C for 16-h light and at 18°C for 8-h darkness for 4 weeks. Two days before measurement, one tray of each set of plants was transferred to another incubator with the same diurnal setting, except the temperature at night was 23°C. 8 or 10 rosette leaves with similar size from each set of plants were used to measure photosynthesis or respiration. Measurements were started 1 hour after the beginning of the light or dark period. The CO₂ concentration used for photosynthesis measurements was 438µmol/mol and the CO₂ concentration used for respiration measurements were
and 433µmol/mol (23°C) and 448µmol/mol (18°C) respectively.

2.3 RESULTS

2.3.1 Identification of T-DNA insertion in AKINβ1 gene of mutant, and effects on gene expression

Arabidopsis AKINβ1 (AT5G21170) gene is located on chromosome 5 and has two protein-coding gene splice variants (AT5G21170.1 and AT5G2110.2). For AT5G21170.1, its genomic DNA sequence contains 4 exons and 3 introns and is 2935 base pairs (bp) in length. For AT5G21170.2, its genomic DNA sequence contains 5 exons and 4 introns and is 2830bp in length (Figure 2.2). SALK_008325 is an Arabidopsis (Columbia-0) akinβ1 mutant line generated by T-DNA insertion. The T-DNA was inserted in the first intron of the genomic DNA of AKINβ1 gene common to both splice variants.

![Figure 2.2 Structure of AKINβ1 gene. Genomic DNA schematics are presented for two gene splice variants of AKINβ1 gene (AT5G21170) of SnRK1 in Arabidopsis, showing the T-DNA insertion site. Deep blue boxes represent exons and black lines represent introns. Pale blue boxes represent 5’ untranslated region (5’ UTR) and 3’ untranslated region (3’ UTR) respectively. Arrows represent the primers used in PCR-based genotyping.](image)

The T-DNA insertion lines were confirmed by PCR amplification (Figure 2.3 A) of
plant genomic DNA using a left-border primer (LBb1.3) and the specific \textit{akin\textbeta{}1} gene primer (5’-TTTTTGTGTTATCTTTGGTTCAGG-3’), showing that only the mutant line produced PCR products of the expected size (1146bp). Homozygous mutant plants were identified by PCR amplification using a pair of specific \textit{akin\textbeta{}1} gene primers, 5’-TTTTTGTGTTATCTTTGGTTCAGG-3’ and 5’-GTGACGCGATAGGTGAGCAAA-3’, by which one PCR product (1000bp) can be amplified from genomic DNA of WT or heterozygous lines, whereas no PCR product can be obtained from homozygous mutant plants (Figure 2.3 A).

Given that the T-DNA insert is in a non-coding region of the gene, the expression level of \textit{AKIN\textbeta{}1} in the \textit{Arabidopsis} mutant line was investigated by RT-PCR and qRT-PCR respectively. \textit{ACT2} was used as a reference (Kim \textit{et al}., 2008; Kim \textit{et al}., 2010; Hemmes \textit{et al}., 2012). The results show that expression of the \textit{AKIN\textbeta{}1} was reduced significantly in the \textit{akin\textbeta{}1} mutant leaves harvested 1 hour into the dark period (Figure 2.3 B). This is consistent with previous RT-PCR results showing reduced expression of \textit{AKIN\textbeta{}1} in the \textit{akin\textbeta{}1} mutant (Li \textit{et al}., 2009).

qRT-PCR data show that expression of \textit{AKIN\textbeta{}1} was higher in WT than in the \textit{akin\textbeta{}1} mutant over a 24-hour time course. The expression pattern of \textit{AKIN\textbeta{}1} gene in leaves of both WT and the \textit{akin\textbeta{}1} mutant was similar but “dampened” in the latter. During the light period, expression of \textit{AKIN\textbeta{}1} increased from the beginning of light, peaking at 8 hours into the light period, before decreasing until the end of the light period in both lines. Expression of \textit{AKIN\textbeta{}1} was significantly higher in WT than in the mutant at two
time points, at the beginning of illumination (end of darkness) and 4 hours into the light period. During darkness, expression of AKINβ1 increased from the beginning of darkness, peaked at 4 hours into the dark period and decreased from the time point to the end of the darkness in both lines. Expression of AKINβ1 was significantly higher in WT than in the mutant at two time points, 4 hours into the dark period and 8 hours into the dark period (Figure 2.3 C). These results indicate that the akinβ1 mutation does not lead to complete loss of expression but produces a knockdown of AKINβ1 expression.

Figure 2.3 Identification of T-DNA insertion in AKINβ1 gene in the akinβ1 mutant and expression of AKINβ1 in the wild-type and mutant. (A) Detection of T-DNA insert in AKINβ1 gene in genomic DNA of WT and the mutant line. PCR was performed with gene-specific primers. Genomic DNA was prepared from leaves of WT and the mutant. Only the mutant line produced a PCR product of the expected size (1146bp). Homozygous mutant plants were confirmed by PCR amplification using a pair of specific AKINβ1 gene primers by which one PCR product (1000bp) can be amplified from genomic DNA of WT or heterozygous lines, whereas no PCR product can be obtained from homozygous mutant plants. (B) Expression of AKINβ1 determined by RT-PCR. ACT2 was used as a reference gene. (C) Detection of the expression of
AKINβ1 in the Arabidopsis mutant and wild-type by qRT-PCR. ACT2 was used as a reference gene. Asterisks represent significant differences at P<0.05. Error bars represent Standard deviation (n=3).

2.3.2 Effect of altered AKINβ1 expression on leaf starch, sugars and intermediary metabolites over a diurnal cycle

2.3.2.1 Starch

Leaf starch content was measured in Arabidopsis WT and the akinβ1 mutant at 6 time points over a diurnal cycle (16-hour day/8-hour night cycle) (Figure 2.4 A). The akinβ1 mutant accumulated starch during the day time and degraded starch during the night in a pattern similar to WT. However, a higher starch content was consistently observed in the mutant throughout the diurnal cycle. The starch contents in the akinβ1 mutant were significantly different from WT at two time points, the beginning of light (end of darkness) and 8 hours into the light period. Nonetheless, net turnover (the difference between average values at end of light and end of the dark period) was nearly identical (41.7µmol/g in WT and 40.5umol/g in the mutant). Under the long day cycle, starch was not completely degraded by end of night in either the mutant or WT lines. When measured over a short-day diurnal cycle (8-hour day/16-hour night cycle) (Figure 2.4 B), the starch contents in the akinβ1 mutant were significantly different from WT at three time points (4 hours into the light period, at the end of light, and 4 hours into the dark period) and starch was almost completely degraded in both lines at the end of darkness. This indicates that the akinβ1 mutant accumulated less starch during the day time (39.09±1.76µmol/g in WT and 34.68±2.3µmol/g in the mutant; P=0.003).
Figure 2.4 Starch content in Arabidopsis leaves over different diurnal cycles. Leaves were harvested every 4 hours during a 16-hour light (white)/8-hour dark (gray) cycle (A) and under an 8-hour light (white)/16-hour dark (gray) cycle (B). Starch quantification is expressed in units of µmol glucose equivalents/g fresh weight. Starch quantification is also expressed in units of mg starch/g fresh weight for the long day cycle (C) and the short day cycle (D). Each data point is the average of three independent plants. Asterisks represent significant differences at P<0.05. Error bars represent standard deviation (n=3).

2.3.2.2 Soluble sugars

Glucose, fructose, sucrose, maltose and MOS are key metabolites arising from starch degradation and sucrose metabolism in plant leaves.

In WT, sucrose accumulated gradually during the light period and decreased in the darkness (Figure 2.5 A). The data are consistent with the overall pattern of a long day cycle (16-h day/8-h dark) for sucrose observed in other studies (Rojas-González et al., 2015). In the akinβ1 mutant, more sucrose accumulated during the light period, declining only after 4 hours of darkness. The differences in sucrose contents were significantly higher in the akinβ1 mutant compared to WT at four time points (8h, 12h, 16h and 20h).
Maltose and MOS contents decreased gradually during the light period and increased gradually later in the dark period but dropped markedly at the end of darkness, beginning of light (Figure 2.5 B and C), in WT, again consistent with the overall pattern of a long day cycle observed previously (Lu et al., 2005; Lu et al., 2006; Critchley et al., 2001; Dumez et al., 2006). While the patterns of maltose and MOS turnover in the mutant were broadly similar to those of WT, the maltose and MOS contents in the akinβ1 mutant were significantly higher, most prominently at night.

In WT, glucose and fructose accumulated during the middle of the light period and increased as darkness approached, and both showed some accumulation and turnover during the dark period (Figure 2.5 D and E). This is consistent with the overall pattern of a long day cycle (16-h day/8-h dark) for glucose and fructose previously observed in the Columbia ecotype (Rojas-González et al., 2015). Except for the time point at the end of darkness, the akinβ1 mutant exhibits a similar overall pattern of glucose and fructose turnover as WT. Compared with WT, more glucose accumulated during the middle of the light period in the akinβ1 mutant, but was significantly lower later during the light period and early period of darkness (as was fructose). Unlike in WT, glucose and fructose accumulation continued to increase in the akinβ1 mutant until the end of darkness (Figure 2.5 D and E).
**Figure 2.5** Soluble sugar turnover in Arabidopsis leaves during a diurnal cycle. A: Sucrose; B: Maltose; C: MOS; D: Glucose; E: Fructose. Leaves were harvested every 4 hours during a 16-hour light (white)/8-hour dark (gray). Sugar quantification is in units of μmol sugar indicated in each figure/g fresh weight. Because MOS are oligosaccharides and their molecular weights are not a single value, MOS quantification is in units of μmol maltose/g fresh weight. Each data point is the average of three independent plants. Asterisks represent significant differences at P<0.05. Error bars represent standard deviation (n=3).

### 2.3.2.3 Sugar phosphates and organic acids

#### 2.3.2.3.1 Sugar phosphates

LC-MS/MS analysis was used to determine profiles for 24 metabolites involved in glycolysis gluconeogenesis, starch biosynthesis, the TCA cycle and shikimate pathway (Figure 2.6). Among them, 15 metabolites were intermediary metabolites including 1
signaling metabolite (T6P), 2 nucleotide sugars (ADP-glucose (ADPG) and UDP-glucose (UDPG)), and 7 organic acids in the TCA cycle.

ADPG is the precursor for starch biosynthesis, and highly susceptible to variation if metabolism is not quenched on extraction (Kusano et al., 2011; Tohge et al., 2011). ADPG measurement therefore also served as a useful reference point and quality control in terms of reliable quantification of all metabolite profile data. In both WT and mutant, the content of ADPG increased sharply from the beginning of the light until 8 hours into the light period, gradually declining thereafter, and decreased to very low levels during darkness. The content of ADPG in the akinβ1 mutant was lower than in WT during the light period, and both were similarly low during the dark period (Figure 2.6 A). By contrast, the content of UDPG did not fluctuate greatly, although it was higher in the akinβ1 mutant during the first 8 hours of light (Figure 2.6 B). Examination of the ratio of ADPG/UDPG (Figure 2.7 A) shows a clear increase in the ratio from the onset of illumination up until 8 hours into the light period. Moreover, the ADPG/UDPG ratios in the akinβ1 mutant were statistically lower than in WT throughout most of the light period. ADPG and UDPG are key metabolites for starch biosynthesis and sucrose metabolism respectively and the ratio of starch/sucrose (Figure 2.7 B) shows that the starch/sucrose ratios increased during the light period and decreased during the darkness in WT. By contrast, the starch/sucrose ratios exhibit no great fluctuation in the akinβ1 mutant during the light and the ratio decreased during the darkness. There was a statistically significant difference between the starch/sucrose ratios in WT and the mutant at the end of darkness.
T6P is a signaling metabolite. The content of T6P peaked after 4 hours in the light, then decreased sharply by 8 hours into the light period. T6P content increased again until the end of illumination and then decreased during the darkness in both WT and the mutant. The content of T6P in the akinβ1 mutant was similar to WT during the light period except at 4 hours into the light period at which point the content of T6P in the akinβ1 mutant was 14.52% higher in the mutant. During darkness, the content of T6P declined in both mutant and WT and was significantly lower than in WT after 4 hours into darkness (Figure 2.6 C). T6P/Sucrose ratio (Figure 2.7 C) has been proposed as a critical parameter for Arabidopsis, which forms part of a homeostatic mechanism to maintain sucrose levels within a range in Arabidopsis (Yadav et al., 2014). The pattern of the T6P/Sucrose ratio was similar in both WT and the mutant during the diurnal cycle, and reasonably constant except for single set of measurements made after 4-hour illumination, in which the ratio rose.

The contents of seven intermediate metabolites including G1P, galactose-1-phosphate (Gal1P), glucose-1,6-bisphosphate (G1,6BP) (also an intermediate in the interconversion of G1P and G6P), G6P, F6P, mannose-6-phosphate (Man6P), Fru-1,6-BP (Figure 2.6 E-K) were measured. Each was higher in the mutant than in WT during the light period, but this situation was reversed during the dark period. The content of sucrose-6-phosphate (Suc6P) in the akinβ1 mutant (Figure 2.6 D) was similar to WT throughout the diurnal cycle. But compared with WT, the contents of Suc6P in the mutant increased after 4 hours in the light, and decreased to greater extent until the end of the darkness. Fru-1,6-BP showed a marked diurnal turnover, increasing from the
beginning of the light period, gradually decreasing during the remainder of the day, and falling markedly during darkness. In WT, the contents of both G1P and G6P decreased up until 8 hours into the light and then increased until the end of darkness. In the akinβ1 mutant, fluctuations in these hexose-phosphates were less pronounced. The content of F6P did not change markedly over the diurnal cycle. Though, similar to G1P and G6P, the contents of F6P in the akinβ1 mutant were higher than in WT during the light period and lower than in WT during darkness. The ratio of F6P/Fru-1,6-BP is a function of carbon flux between sucrose metabolism and glycolysis, and carbon flux in the Calvin-Benson cycle. The ratio of F6P/Fru-1,6-BP (Figure 2.7 D) shows that there were no differences between the lines, but substantial changes over the diurnal cycle.

3-PGA, PEP, pyruvate, glycerate, glycerol-3-phosphate (Gly3P) and shikimate are involved in cytosolic glycolysis, Calvin-Benson cycle, photorespiration, and the plastidic shikimate pathway. Generally, their contents in the akinβ1 mutant (Figure 2.6 L-Q) were lower than in WT during the light period, but higher than, or similar to, WT during the dark period. The ratio of PEP/pyruvate (Figure 2.7 E) which could be influenced by carbon flux from glycolysis to the TCA cycle (Zabalza et al., 2009) shows that there were no significant differences between the lines over the diurnal cycle.
Figure 2.6 Metabolite profiling in leaves of Arabidopsis WT and the akinβ1 mutant during a diurnal cycle. 17 metabolites were measured by LC-MS/MS analysis and five replicates for each sample at each time point were measured. Asterisks represent significant differences at P<0.05. Error bars represent standard deviation (n=5).
Figure 2.7 Ratios of sugars and their intermediates. Five replicates for each sample at each time point were measured for ADPG/UDPG, F6P/Fru1,6BP and PEP/Pyruvate. Three replicates (values presented as glucose equivalents) for each sample at each time point were measured for starch/sucrose. For ratios of T6P/sucrose, the average values for T6P and sucrose at each time point were used to calculate the ratios since they had different number of replicates, so no error bars were shown. Starch or sucrose quantification is in units of nmol starch or sucrose/g fresh weight. Asterisks represent significant differences at P<0.05. Error bars represent standard deviation (n=5 or n=3).

2.3.2.3.2 Organic acids

Diurnal changes in the contents of five organic acids (citrate, 2-oxoglutarate (2-OG), succinate, fumarate and malate) in WT are consistent with previous observations in Arabidopsis (Fahnenstich et al., 2007), although comparative data on diurnal changes in the contents of aconitate and isocitrate in WT over a long-day diurnal cycle are not
available. Although there are several pathways for citrate metabolism in plant cells, the most significant involves citrate metabolism in the TCA cycle (Popova and Pinheiro de Carvalho, 1998). Interestingly, the contents of the seven organic acids in the akinβ1 mutant were much lower than those in WT over the diurnal cycle (Figure 2.8 A-G). The contents of citrate, aconitate, isocitrate, and succinate in both mutant and WT lines decreased during the light and increased during the darkness, whereas the contents of 2-OG, fumarate and malate in both lines increased from the beginning of illumination up until 12 hours into the light period, and decreased during the remaining diurnal cycle.

The product/reactant ratios of these organic acids in the TCA cycle and pyruvate measured were calculated. Ratios of citrate/malate and succinate/2-OG were calculated since the contents of oxaloacetate and succinyl-CoA were not determined. Since acetyl-CoA content was also not measured and acetyl-CoA and oxaloacetate combine to form citrate in the TCA cycle, the ratio of citrate to pyruvate was calculated as a “proxy” for the citrate synthase reaction. The ratios of citrate/malate (Figure 2.9 A) in the akinβ1 mutant were lower than in WT during the light period (significant at all time points) and similar to WT during the darkness. Similarly, the ratios of citrate/pyruvate (Figure 2.9 B) in the akinβ1 mutant were significantly lower than in WT at two time points (8 hours and 12 hours) during the light, and similar to WT during the darkness. The ratios of isocitrate/aconitate (Figure 2.9 D) in the akinβ1 mutant were markedly higher than in WT throughout the light period. Moreover, the value of the ratio increased in the mutant throughout the light period, declining in darkness, whereas there was little fluctuation in this ratio in the WT. The ratio of succinate/2-OG (Figure 2.9 F) was stable
for most of the light period in the mutant and WT, and rose during darkness where the ratio was higher in the WT at the end of darkness (beginning of light). The ratio of fumarate/succinate (Figure 2.9 G) increased in light and fell during darkness in both WT and mutant. This ratio was lower in the akinβ1 mutant than in WT during the light period. The ratios of aconitate/citrate (Figure 2.9 C) and 2-OG/isocitrate (Figure 2.9 E) and malate/fumarate (Figure 2.9 H) were not significantly different between the akinβ1 mutant and WT over the diurnal cycle.
Figure 2.8 Metabolite profiling of Arabidopsis WT and the akinβ1 mutant leaves during a diurnal cycle. Seven organic acids in the TCA cycle were measured by LC-MS/MS analysis and five replicates for each sample at each time point were measured. Asterisks represent significant differences at P<0.05. Error bars represent standard deviation (n=5).
Figure 2.9 Ratios of the organic acids measured. Five replicates for each sample at each time point were measured for each intermediate ratio. Asterisks represent significant differences at P<0.05. Error bars represent standard deviation (n=5).
2.3.3 Photosynthesis and respiration rates

Photosynthesis and respiration rates in rosette leaves of the \textit{akinβ1} mutant and WT were measured. The results (Figure 2.10) are consistent with photosynthesis and respiration rates observed previously (Ribeiro \textit{et al.}, 2012; Boex-Fontvieille \textit{et al.}, 2014) and show there is no significant difference between photosynthesis in the WT and the \textit{akinβ1} mutant at 23°C. However, there is a significant difference in respiration rates when measured in plants maintained at either 18°C or 23°C in darkness. The respiratory rate in the \textit{akinβ1} mutant was significantly lower (40%) than that observed in WT at 18°C in darkness. The respiration rate in both lines was higher in plants held at 23°C in comparison to those maintained at 18°C, and the respiratory rate in the \textit{akinβ1} mutant was, again, significantly lower (18%) than in WT.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2_10.png}
\caption{Photosynthesis and respiration of the \textit{akinβ1} mutant and WT. Values were measured in situ using a LI-6400 Portable Photosynthesis System. In order to facilitate comparison of photosynthesis with respiration at 23°C, respiration was measured in 23°C.}
\end{figure}
plants maintained at either 18°C or 23°C in darkness. Asterisks represent significant differences at P<0.05. Error bars represent standard deviation (for net photosynthesis rates, n=10; for respiration rates, n=8).

2.4 DISCUSSION

In this study, an Arabidopsis T-DNA insertion line (Salk_008325) for the regulatory subunit, AKINβ1, was used to study the effect of SnRK1 on metabolism in Arabidopsis. However, all publically available Arabidopsis mutant lines have an identified T-DNA insert in either the promoter region or different introns of the AKINβ1 gene, not in open reading frames. However, several publically available Arabidopsis mutant lines (PERL0913060, PERL0913061, PERL0913062, PERL0913064 and ossowski_885203) have an identified single nucleotidic substitution in exons of AKINβ1 gene, but these substitutions don’t form new stop codons in these modified gene sequences and lead to production of truncated protein sequences. An adapter ligation-mediated PCR strategy was employed to identify the location of the T-DNA insert in the Arabidopsis genome and select putative Arabidopsis mutant lines possessing the T-DNA insertion, by which the akinβ1 mutant line was identified. In this selection process, only the target T-DNA-gDNA junction can be exclusively amplified with the T-DNA and adapter primers by PCR (O'Malley et al., 2007), which would ensure that the T-DNA segment was only inserted in the AKINβ1 gene in the mutant line. In the current study, expression of AKINβ1 was detected by RT-PCR and qRT-PCR (Figure 2.3 B and C). Both methods showed that expression of AKINβ1 is decreased in the mutant relative to the wild-type. The expression pattern of AKINβ1 during a diurnal cycle was also investigated (Figure 2.3 C). Expression of AKINβ1 increased gradually during day light, falling after 8 hours
and then increasing again during darkness before falling again. The expression of *AKINβ1* is repressed by sucrose and is promoted by darkness independently (Polge *et al.*, 2008). Consistent with this, the expression pattern of *AKINβ1* was inversely related to the changes in sucrose content during daylight (Figure 2.5 A). There was an observed decrease in sucrose content from the beginning of light up until 4 hours, during which expression of *AKINβ1* peaked at 8 hours into the light period. As leaf sucrose content continued to increase during the light period, expression of *AKINβ1* decreased. The second phase of increased expression of *AKINβ1* during the dark period peaked at 4 hours (measurable increase in expression of *AKINβ1* was detectable after 1 hour in darkness (see Chapter V)), when sucrose was still high (notably in the mutant), implying that the effect of darkness on expression is decoupled from sucrose levels.

The same Arabidopsis mutant line with decreased expression of *AKINβ1* has previously been used to study the regulatory function of *AKINβ1* in nitrogen metabolism and sugar signaling (Li *et al.*, 2009). Li *et al.* (2009) reported that in the mutant with lower *AKINβ1* expression, the activity of nitrate reductase increased about three-fold compared to the wild-type, whereas in lines overexpressing *AKINβ1*, NR activity was 10-180 times lower than that observed in the wild-type. Thus altered *AKINβ1* expression can clearly have an effect on assimilatory pathways, in this case on nitrate assimilation.

The effects of altered expression of *AKINβ1* on metabolite profiles, and diurnal sugar turnover, are summarized in Figure 2.11. The results indicate significantly decreased
contents of citrate, aconitate, isocitrate, 2-OG, succinate, fumarate and malate (in the TCA cycle) in the akinβ1 mutant. Differences from the WT were most prominent during illumination. Interestingly, metabolic profiling of SnRK1-repressed pea embryos (Radchuk et al., 2006) harvested in the middle of the light phase also showed decreased contents of the same organic acids (citrate, aconitate, isocitrate, 2-OG, succinate, fumarate and malate) from the TCA cycle, whereas contents of glycolytic intermediates (G1P, G6P, F6P, UDPG and 3-PGA) were increased or unchanged (Radchuk et al., 2010). Moreover, sucrose and maltose contents were increased, while starch and free hexose (glucose and fructose) contents were decreased in SnRK1-repressed pea embryos. These results, from pea, are also indicative of decreased carbon partitioning and/or flux from the glycolytic pathway into subsequent pathways such as the TCA cycle and amino acid biosynthesis (Radchuk et al., 2006; Radchuk et al., 2010). There is a notable parallel between the study of SnRK1-repressed pea embryos and the akinβ1 mutant studied here. In addition to the decrease in TCA cycle intermediates, the contents of sugars and their intermediates such as sucrose, maltose, G1P, G6P, F6P, and UDPG were a little higher in the akinβ1 mutant during the day time compared to wild-type. The increased contents of sucrose in the akinβ1 mutant could be due to many factors, including the possibility of higher activity of sucrose phosphate synthase, which might arise in the mutant, since SnRK1 inactivates sucrose phosphate synthase by phosphorylation (Sugden et al., 1999). In addition, the increased content of sucrose in the akinβ1 mutant during the light is consistent with the decrease in glucose and fructose observed after 8 hours in the light period, since glucose and fructose can be
derived from sucrose degradation. Starch content was decreased in SnRK1-repressed embryos (Radchuk et al., 2010), whereas in the Arabidopsis akinβ1 mutant, the starch content was not significantly altered, and indeed showed a small increase. This may be a reflection of the fact that metabolic pathways involved in starch biosynthesis are different between source and sink tissues. Carbon for starch biosynthesis is derived from the Calvin-Benson cycle in photosynthetic tissues, whereas in sink tissues (e.g. pea embryos) it is derived from sucrose translocated through the phloem (Ohdan et al., 2005). Consistent with this hypothesis are the observations that overexpression of SnRK1 in transgenic potato tubers caused increased starch accumulation (McKibbin et al., 2006), whereas overexpression of AKIN10 in Arabidopsis led to a decrease in leaf starch content and an increase in the starch level in leaves of Arabidopsis akin10/akin11 double-mutant plants (Baena-Gonzalez et al., 2007; Jossier et al., 2009).

As mentioned above, it has been suggested that AKIN10/11 play an unknown regulatory role in starch mobilization at night (Baena-Gonzalez et al., 2007). The effects on starch accumulation in akin10 plants was more apparent during phosphate starvation, which may reflect less starch mobilization at night during phosphate starvation (Fragoso et al., 2009). Further, SnRK1 can upregulate expression of BAM3 (Baena-Gonzalez et al., 2007), a key enzyme of leaf starch degradation (Fulton et al., 2008). SnRK1 has also been shown to positively regulate post-translational redox
Summary of carbohydrate contents and metabolite profiling in the *akinβ1* mutant and WT leaves. The putative regulatory mechanisms of AKINβ1 and its predicted subcellular localizations have been shown and will be considered in more detail in the next chapter. The six rectangles for each intermediate represent the six time points at which leaf samples were collected. Orange rectangles indicate significantly higher content in mutant leaves; green rectangles indicate significantly lower content in mutant leaves; gray rectangles indicate no significant difference. Student’s t-test, $P<0.05$, for starch and soluble sugars (glucose, fructose, sucrose, maltose and MOS), $n=3$, for intermediates in metabolite profiling, $n=5$.

**Figure 2.11** Summary of carbohydrate contents and metabolite profiling in the *akinβ1* mutant and WT leaves. The putative regulatory mechanisms of AKINβ1 and its predicted subcellular localizations have been shown and will be considered in more detail in the next chapter. The six rectangles for each intermediate represent the six time points at which leaf samples were collected. Orange rectangles indicate significantly higher content in mutant leaves; green rectangles indicate significantly lower content in mutant leaves; gray rectangles indicate no significant difference. Student’s t-test, $P<0.05$, for starch and soluble sugars (glucose, fructose, sucrose, maltose and MOS), $n=3$, for intermediates in metabolite profiling, $n=5$.
starch degradation at different times of day and in different tissues. The mechanism by which starch degradation is decreased in the *akin10* mutant during phosphate starvation was hypothesized to involve binding of SnRK1 directly to starch through the CBD domain of AKINβ subunits, which could either be regulated directly by SEX4 or in an independent manner (Niittylä *et al*., 2006; Fragoso *et al*., 2009). This hypothesis is dependent on understanding the localization of SnRK1 subunits.

The concentrations of intermediates in the TCA cycle of mutant leaves were significantly reduced compared to the WT, consistent with the observed decrease in respiration rates (Figure 2.10). The decrease in dark respiration in the mutant leaves may contribute to elevated sucrose and starch contents as a result of decreased carbon catabolism, reflecting reduced carbon flux entering into the TCA cycle from glycolysis in the mutant during darkness. Curiously, in tomato lines expressing tomato mitochondrial malate dehydrogenase (mMDH) gene in the antisense orientation, the *mMDH*-repressed lines exhibited higher contents of malate and lower levels of transitory starch and sucrose, whereas in tomato lines expressing tomato *fumarase* in the antisense orientation, the *fumarase*-repressed lines possessed lower contents of malate and higher levels of these carbohydrates (Centeno *et al*., 2011). This is consistent with the observation that the *akinβ1* mutant contained lower contents of malate and increased starch and sucrose during both darkness and the light period. The observation of higher accumulation of starch and sucrose and lower contents of TCA cycle intermediates in the mutant, during illumination, would support the hypothesis that starch synthesis is stimulated when less carbon is stored in organic acids (Fahnenstich
et al., 2008). Although starch was degraded in the mutant, decreased carbon flux entering glycolysis and then feeding the TCA cycle may account for the accumulation of maltose and MOS observed during the dark period. These results show the interplay between carbohydrate metabolism in chloroplast and cytosol, and the metabolism of organic acids in the TCA cycle in mitochondria.

The decrease in contents of citrate, aconitate, and isocitrate during the light period and their subsequent increase during the darkness (Figure 2.8) in both the mutant and WT are consistent with the observation that mitochondrial pyruvate dehydrogenase complex, citrate synthase, aconitase, and isocitrate dehydrogenase activities decrease in the light and increase in darkness in Arabidopsis (Lee et al., 2010). It might be expected that the decrease in isocitrate dehydrogenase activity in the light and increase in darkness would also cause the content of 2-OG to decrease during the light and increase in darkness. However, citrate and isocitrate are exported to the cytosol to produce 2-OG in the cytosol in illuminated leaves (Hanning and Heldt, 1993; Lee et al., 2010). Glutamate dehydrogenase is a mitochondrial enzyme that catalyzes the reversible amination/deamination reaction between 2-OG and glutamate (Marchi et al., 2014). The amination activity of glutamate dehydrogenase (generating glutamate) showed no significant changes in both darkness and the light, but the deamination activity of glutamate dehydrogenase (generating 2-oxoglutarate) decreased in darkness and increased rapidly in the 2 hours after illumination (Lee et al., 2010), which could lead to decrease in the content of 2-OG in darkness and increase in its content in the light. Moreover, 2-OG dehydrogenase activity increases in darkness (Lee et al., 2010),
which could also decrease the content of 2-OG in darkness. These metabolic changes are therefore consistent with the observed increase in 2-OG content in the light and decrease in darkness in both WT and mutant.

Succinate content decreases during the light period and increases during darkness (Figure 2.8 E). Succinyl-CoA synthase has been shown to be activated in response to light (Steer and Gibbs, 1969; Stobart and Pinfield, 1970), whereas the activity of succinate dehydrogenase decreases in light and increases in darkness (Popov et al., 2010). This might be expected to lead to increased succinate during the light period and a decrease during darkness, which is the converse of what is observed. However, although organic acid metabolism follows the conventional cyclic flux mode of the TCA cycle in the dark, during the light, the activity of the mitochondrial pyruvate dehydrogenase complex is partially repressed and there is a very low carbon flux through succinyl-CoA synthase and succinate dehydrogenase (Tcherkez et al. 2009). This shows that there is a non-cyclic flux mode of the TCA cycle which bypasses succinate (Sweetlove et al., 2010; Araújo et al., 2012) and thus would lead to the observed change in turnover of succinate during the diurnal cycle. That malate and fumarate contents increase during the light and decrease during darkness in both lines could be related to the observed increase in fumarase activity and mMDH 2 protein abundance during the light period (Lee et al., 2010). The diurnal turnover of both organic acids are similar to those of starch and sucrose, and it has been suggested that these organic acids may function as transient carbon storage molecules (Fahnenstich et al., 2007).
Lack of data on the concentrations of products and reactants involved in each reaction of the TCA cycle such as NADH, NAD\(^+\), ADP, ATP, coenzyme A (CoASH), FAD, FADH\(_2\) and H\(_2\)O, renders it impossible to calculate the mass-action ratio for each reaction in the TCA cycle. However, the product/reactant ratios of TCA cycle intermediates and pyruvate (Figure 2.9) show a clear difference between the mutant and WT. The citrate/malate ratio is significantly lower in the mutant than WT throughout the light period. The citrate/pyruvate ratio exhibits a similar pattern and is significantly lower in the mutant than WT at two time points during the light, whereas the ratio of isocitrate/aconitate is markedly higher in the mutant during the light. These changes indicate that regulation of carbon flux in the TCA cycle is altered in the \textit{akinβ1} mutant.

One possible reason is that the regulation of these enzymes is altered in the mutant.

It has been reported that two potato SnRK1 proteins, PGIN1 and StubSNF1, interact with a potato cytosolic pyruvate kinase \textit{in vitro} and influence activity of cytosolic pyruvate kinase in the SnRK1-repressed potato lines \textit{in vivo} (Beczner \textit{et al.}, 2010). In addition, Arabidopsis AKIN10 upregulates the expression of cytosolic pyruvate kinase and downregulates the expression of phosphoenolpyruvate carboxylase 1, which catalyzes the anaplerotic formation of oxaloacetate from PEP (Baena-González \textit{et al.}, 2007). Thus altered activity of SnRK1 in the mutant would decrease pyruvate content directly or indirectly, contributing to the change in the citrate/pyruvate ratio. Moreover, activities of mitochondrial citrate synthase, mitochondrial fumarase, cytosol ATP-citrate lyase (converts citrate to acetyl-CoA for lipid biosynthesis) and mitochondrial succinate dehydrogenase are regulated by thioredoxin-mediated redox \textit{in vivo}.
(Schmidtmann et al., 2014; Daloso et al., 2015). AKIN10 regulates expression of genes encoding several proteins including two mitochondrial proteins involved in redox regulation (Baena-Gonzalez et al., 2007) and Arabidopsis AKINβγ (γ subunit of SnRK1) is thought to be critical for the regulation of reactive oxygen species levels, by regulating the biogenesis of mitochondria and peroxisomes in pollen (Gao et al., 2016). This shows that SnRK1 could affect activities of the enzymes in the TCA cycle by regulating redox conditions. In addition, a metabolic control analysis showed that much of the control of flux through the TCA cycle is resident in aconitase, fumarase, malate dehydrogenase and 2-oxoglutarate dehydrogenase, suggesting that these enzymes would be sensitive targets for flux regulation (Nunes-Nesi et al., 2008; Araújo et al., 2012). Three of them, aconitase, fumarase, and malate dehydrogenase, are involved in the reactions in which product/reactant ratios are seen to alter in the akinβl mutant.

However, although organic acids are mainly synthesized in the TCA cycle in mitochondria (López-Bucio et al., 2000), they can be exported and contribute to other aspects of metabolism. For example, 2-OG and oxaloacetate provide carbon skeletons for nitrogen assimilation and amino acid biosynthesis/degradation. In turn, other anaplerotic reactions also feed into the TCA cycle such as oxaloacetate and malate from PEP and glyoxylate, succinate from γ-aminobutyric acid degradation, and fumarate from arginine and purine nucleotide biosynthesis (Sweetlove et al., 2010; Araújo et al., 2012). Thus it is difficult to pin-point putative SnRK1-regulated enzyme(s) or transporter(s) in the TCA cycle only on the basis of the ratios of product/reactant. Measurement of the activities of mitochondrial enzymes in the TCA cycle would be a
useful source of further information, as would determination of amino acid contents.

Considering the results in this study and others, it can be postulated that SnRK1 positively regulates respiration in source organs, thus promoting catabolism to generate ATP, and inhibiting biosynthetic pathways in order to conserve ATP. Thus SnRK1 plays a regulatory role in partitioning carbon between starch metabolism, sucrose metabolism and the TCA cycle.

An aspect that requires further consideration is the role of T6P. T6P is an important signaling metabolite which plays a significant role in plant metabolism, growth, and development. It has been observed that T6P levels can be induced by sucrose and that high T6P levels can lower sucrose levels in plants, leading to the proposal of T6P is a negative feedback regulator of sucrose levels and there is a bidirectional regulation between T6P and sucrose (Yadav et al., 2014; Figueroa et al., 2016). A T6P-sucrose nexus has been proposed in which high sucrose levels lead to an increase in T6P content which, in turn, leads to a decrease in sucrose. Thus photoassimilates can be partitioned to generate carbon skeletons for amino acid synthesis instead of sucrose biosynthesis (Yadav et al., 2014; Figueroa et al., 2016). In this study, the changes in T6P content in WT (Figure 2.6 C) during the long-day diurnal cycle are consistent with those observed previously (Wahl et al., 2013). During the light period, sucrose content increased gradually (Figure 2.5 A), leading to increase T6P contents in WT. During the dark period, decreased sucrose content was accompanied by a decrease in T6P, consistent with the notion that T6P levels in plant tissues change in parallel with either endogenous
or imposed fluctuations in sucrose content (Figueroa et al., 2016). Similar changes in contents of sucrose and T6P were also observed in the akinβ1 mutant. As mentioned in Chapter I, there is a bidirectional regulation between SnRK1 and T6P. T6P inhibits the activity of SnRK1 (Zhang et al., 2009). In turn, SnRK1 can inactivate trehalose-6-phosphate synthase 5 by phosphorylation, and AKIN10 upregulates the gene expression of TPS8-11 (Polge and Thomas, 2007; Baena-Gonzalez et al., 2007), showing that SnRK1 would play role in regulation of T6P content in Arabidopsis. However, there is no significant change in T6P content between the mutant and WT over the diurnal cycle in this study, which would show that loss of AKINβ1 has no effect on T6P content in four-week old Arabidopsis leaves. This is supported by the observation that there was no relationship between changes in SnRK1 activity and changes in T6P content in whole grains during wheat grain development (Martínez-Barajas et al., 2011).

As mentioned in Chapter I, there is evidence that SnRK1 plays various regulatory roles in carbohydrate metabolism including starch metabolism in chloroplasts, sucrose metabolism and glycolysis in the cytosol, and respiration in mitochondria. Thus many possibilities underlie the changes observed in sugar contents and organic acids in this study. Among these possible mechanisms, the conversion between F6P and Fru-1,6-BP could be a possible regulatory point which leads to changes in carbon partitioning in the akinβ1 mutant since it has been proposed that SnRK1 phosphorylates F2KP to switch enzyme activity towards production of Fru-2,6-BP, further promoting carbon flow to glycolysis (Cho et al., 2016). Changes in Fru-2,6-BP contents in the akinβ1 mutant should be measured to test this hypothesis.
It has been shown that SnRK1 regulates different enzymes by direct protein phosphorylation and altered gene expression of proteins involved in pathways of carbohydrate metabolism (see Chapter I). The subcellular localization of AKINβ1 proteins encoded by the two splice variants of akinβ1 gene (AT5G21170.1 and AT5G21170.2) were predicted using SUBA3 (http://suba3.plantenergy.uwa.edu.au/), a subcellular location database for Arabidopsis proteins which combines experimental results on subcellular proteomics, fluorescent protein visualization, and protein–protein interaction datasets as well as subcellular targeting prediction of Arabidopsis proteins form 22 prediction programs (Tanz et al., 2013). The bioinformatics approach suggests that both splice variants could be localized in one or more of the cytosol, nucleus, chloroplast and mitochondria. Thus studies on the localization of AKINβ1 would be an important step in understanding the basis for the effects of the mutant.

In conclusion, changes in the gene expression of the AKINβ1 subunit of SnRK1 affects carbon partitioning in Arabidopsis. Decreased expression of akinβ1 likely causes a decrease in the level of AKINβ1 subunit (although not directly shown), resulting in decreased activity of SnRK1 in the akinβ1 mutant. Net photosynthesis rates in both the mutant leaves and the WT leaves exhibited no significant change. The increased contents of starch and sucrose and the decreased respiration in the mutant leaves may reflect changes in carbon partitioning between sucrose biosynthesis, starch metabolism and the TCA cycle in Arabidopsis. These results show that SnRK1 plays a regulatory role in carbon partitioning possibly by promoting the TCA cycle at the expense of sucrose biosynthesis and starch metabolism in plants. This possibility is consistent with
the hypothesis that SnRK1 activates catabolic processes to produce energy and inhibits anabolic metabolism to conserve energy, to restore and maintain energy balance in plant cells.

In the next chapter, the in vivo subcellular localization of three subunits of SnRK1 (AKIN11, AKINβ1 and AKINγ) are investigated by using both transient expression systems in N. benthamiana, and stable transformation in A. thaliana. Understanding the localization of SnRK1 subunits, especially AKINβ1, is of prime importance in understanding the phenomena observed in this chapter.
CHAPTER III
CELLULAR LOCALIZATION OF AKINα1, AKINβ1 AND AKINγ1 SUBUNITS OF SnRK1 IN PLANT LEAVES
3.1 INTRODUCTION

The previous chapter showed that SnRK1 may play a novel role in regulating carbon partitioning between starch metabolism, sucrose metabolism and the TCA cycle in Arabidopsis leaves. Plant cells are organized into a complex and elaborate network of subcellular compartments, which leads to most biological processes such as carbohydrate metabolism and organic acid metabolism being spatially restricted to well-defined locations (Rost et al., 2003; Koroleva et al., 2005; Li et al., 2006). To fully understand the physiological functions and metabolism that subunits of SnRK1 are involved in, it is important to understand their subcellular localization.

Fluorescent protein-based methods are widely used approaches to identify protein subcellular localization in plants (Bhat et al., 2006). The variety of fluorescent proteins available are derived from two fluorescent proteins, green-fluorescent protein (GFP) from the jellyfish Aequoria victoria and a red fluorescent protein (RFP), DsRed, from the soft coral Dictyosoma. Numerous different point mutations in the original GFP and RFP have been exploited (Chudakov et al., 2005; Chudakov et al., 2010; Matz et al., 1999). For example, yellow fluorescent proteins (YFP) are derived from genetic mutation of GFP (Nagai et al., 2002). Fluorescent proteins covering the spectral range from blue to far-red have been now available (Piatkevich et al., 2010). When selecting fluorescent proteins for determination of subcellular localization, several general requirements should be considered: Fluorescent proteins should: i) be expressed efficiently in the experimental system, such that the fluorescent signal is sufficiently stronger than autofluorescence and can be reliably imaged. ii) possess enough
photostability to avoid photobleaching during experimental observation. iii) be a monomer with no cellular toxicity. iv) be insensitive to environmental factors such as acidic pH. v) have minimal crosstalk in their excitation and emission channels in multiple-labeling experiments (Shaner et al., 2005).

Agrobacterium-mediated DNA transfer is a simple, efficient and popular method to transfer exogenous genes into the nucleus for gene function analysis in various plant species such as tobacco, tomato, Arabidopsis, grapevine, radish and pea (van der Hoorn et al. 2000; Wroblewski et al. 2005; Tzfira and Citovsky, 2006; Sheludko et al. 2007; Santos-Rosa et al. 2008). Agrobacterium tumefaciens, which is a gram-negative soil bacterium, naturally infects the wound sites in dicotyledonous plants and causes the formation of the crown gall tumors. A small DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid from bacterial cells is transferred into host plant cells, and then stably integrated into the host genome and transcribed through a bacterial type IV secretion system (T4SS) (Nester et al., 1984; Binns and Thomashow, 1988; Fronzes et al., 2009). Agrobacterium can also mediate the transfer of exogenous DNA into some monocotyledonous plants such as rice and maize (Hiei et al., 1997; Reyes et al., 2010). Agrobacterium-mediated transient gene expression in different plant tissues is used widely to identify and analyze gene functions and subcellular localization, and has previously been used to study the location of AKIN10 and AKIN11 (Nietzsche et al., 2014; Williams et al., 2014). Currently, two platforms for transient gene expression have been well-established: Agrobacterium-mediated tobacco leaf infiltration and Arabidopsis mesophyll protoplast transfection (Yoo et al., 2007; Vaghchhipawala et al., 2007;
Transient expression methods by *Agrobacterium*-mediated leaf infiltration have also been established for other plants such as lettuce, tomato and Arabidopsis (Sheludko *et al.* 2007). *Agrobacterium*-mediated leaf infiltration is a simple, effective and convenient technique, involving the injection of *Agrobacterium tumefaciens* containing the cDNA of interest, in a binary vector, into plant leaf cells with intact cell walls followed by observation of gene expression within the infiltrated leaves after a few days (Sparkes *et al.* 2006). Before establishment of a stable transgene expression line, initial evaluation of the expression efficiency of one or more transgene vectors *in vivo*, or observation of a phenotype that should display in stably transgenic plants, can be completed by an *Agrobacterium*-mediated tobacco leaf infiltration assay. However, for subcellular localization studies, some Arabidopsis proteins fused with GFP were mislocalized when expressed in tobacco epidermal cells (Marion *et al.*, 2008). In addition, although development of stable transgenic plants is time-consuming and labour-intensive, they offer a sustainable supply of plant material with expression of target protein for studying the physiological activity, regulation, subcellular localization and functions of a target gene throughout all tissues and cell types, as well as a steady source of transgenic plant seeds. The “floral-dip” method of *Agrobacterium*-mediated transformation of Arabidopsis is an established method for stable gene expression.

In this study, plant leaves were used to determine the subcellular location of AKIN subunits. A plant leaf generally consists of four layers of cells: upper epidermis which produces cuticle and has few chloroplasts, palisade mesophyll which have many chloroplasts in the cytoplasm, spongy mesophyll which also contains chloroplasts and
have xylem and phloem in veins, and the lower epidermis containing guard cells which form stomata (Figure 3.1 A). The main organelles in the cytoplasm of leaf cells includes nucleus, chloroplasts, mitochondria, peroxisomes, Golgi apparatus, endoplasmic reticulum, and vacuole (Figure 3.1 B). Based on their appearance under the microscope, organelles in leaf cells can be divided into two groups: organelles such as plastids, mitochondria and peroxisomes that are predominantly small and round, and typically present in many copies per cell that leads to a punctate pattern when co-located with fluorescent proteins or markers. Other organelles such as the Golgi apparatus and endoplasmic reticulum, that have a long, extended morphology, are usually observed as a single interconnected structure that stretches throughout the cytoplasm, leading to a tubular or sheet-like morphology (Nelson et al., 2007). Plastids are often the largest of the punctate organelles, ranging in size from 2-3µm for proplastids to roughly 5µm for fully developed chloroplasts. Chloroplasts can be easily identified by their green color in bright-field microscopy and by their typical red chlorophyll fluorescence (Nelson et al., 2007). Mitochondria are typically small (approximately 0.5µm in diameter) and spheroidal organelles that are present in high copy numbers in every cell. Moreover, their morphology can vary dramatically from one cell to the next (e.g. worm-like or a beads-on-a-string shape) due to the fusion and fission of these organelles (Logan, 2006), but within a given cell all mitochondria display similar morphology. Peroxisomes also are small, typically round organelles; their sizes vary from less than 0.5 to 2µm within a single cell, and the number of peroxisomes varies from cell to cell (Nelson et al., 2007). The Golgi apparatus comprises many small individual membrane
stacks, which are less than 1µm in size. These stacks appear as round, flattened discs, small rings or short lines, depending on orientation and status (Nebenführ et al., 1999). Lipid bodies are tiny, round membrane-bound organelles whose sizes range from about 0.5 to 2.5µm and are formed by pinching off from the endoplasmic reticulum (Van der Schoot et al., 2011; Fujimoto and Parton, 2011). The plasma membrane surrounds the cytoplasm on all sides as a single layer. Hence, proteins that localize to this membrane display uniform labeling along the surface periphery of the cell (Nelson et al., 2007). Nuclei within plants differed considerably in size and shape. The shape ranged from spherical (the most usually observed morphology) to a highly elongated, almost rod-like shape. Variation in nuclear size was observed in Arabidopsis plants (Chytílova et al., 1999).

Figure 3.1 Cartoons of plant leaf structure (A) and organelles (B) in a plant leaf cell.

The previous chapter showed that changes in the expression of the AKINβ1 subunit of SnRK1 affects carbon metabolism in Arabidopsis. In this study, the subunits of SnRK1 in Arabidopsis: AKINα1 (AKIN11; At3g29160.1), AKINβ1 (At5g21170.1) and AKINγ1 (At3g48530.1) were chosen to study their subcellular localization in vivo.
Although the subcellular localization of AKIN10 and 11 have previously been reported as located in the nucleus, chloroplast and cytoplasm (Fragoso et al., 2009), whether these catalytic subunits, especially AKIN11, are also localized in other organelles such as peroxisomes and mitochondria is unknown. Understanding the subcellular localization of AKINβ1 would provide more information about its role in regulating carbohydrate metabolism. Because a putative chloroplast transit peptide that covers amino acids 1-74 at the N-terminus of AKINβ1 is predicted using ChloroP 1.1 (cleavage site score=0.927, http://www.cbs.dtu.dk/services/ChloroP/), a truncated AKINβ1 lacking this peptide was used to study the role of this peptide region in subcellular localization of AKINβ1. Although AKINγ1 does not participate in the formation of a functional protein kinase complex (Ramon et al., 2013; Emanuelle et al., 2015) and does not complement a yeast mutant lacking the γ subunit of SNF1 (snf4) (Bouly et al., 1999; Ramon et al., 2013), it has been shown that AKINγ1 can interact with AKINβ1, AKINβ2 and AKIN10 respectively in yeast two-hybrid assays (Bouly et al., 1999). Thus AKINγ1 may have some unknown biological functions that may be related to SnRK1 in plants (Bouly et al., 1999; Ramon et al., 2013). The subcellular localization of AKINγ1 is presently unknown. The experiments described in this chapter indicate how subunits were fused with enhanced yellow fluorescent protein (EYFP), monomeric red fluorescent protein (mRFP) and green fluorescent protein (GFP) respectively for studying their co-localization in vivo. All fluorescent proteins were fused to the C-terminus of each subunit. AKIN11 fused with a fluorescent protein at its C-terminus has previously been used for subcellular localization studies (Fragoso
et al., 2009; Bitrián et al., 2011; Williams et al., 2014) and AKINβ1 has a N-terminal variable region (NVR) domain at its N-terminus that plays an important role in subcellular localization (Pierre et al., 2007) and possesses a predicted chloroplast transit peptidase at its N-terminus (74 amino acids) that would direct AKINβ1 to chloroplasts that is an important organelle for carbon metabolism. Since no subcellular localization of AKINγ1 has been reported at present, AKINγ1 fused with green fluorescent protein at the C-terminus of AKINγ1 was used to detect its localization in vivo.

3.2 MATERIAL AND METHODS

3.2.1 Plant growth

Tobacco (Nicotiana benthamiana) plants and Arabidopsis Columbia (Col-0) plants were cultured and grown as described in Section 2.2.1, but tobacco plants were grown at 24°C under long-day conditions (16-h light/8-h darkness).

3.2.2 Isolation and purification of total RNA from WT Arabidopsis leaf cells

About 100mg Arabidopsis Columbia (Col-0) WT leaf was harvested to prepare total RNA as described in Section 2.2.6.

3.2.3 cDNA synthesis of AKINα1, AKINβ1 and AKINγ1 genes

2μl of the total RNA was used to make the first-strand cDNA as described in Section 2.2.6. 2μl of the first-strand cDNA was used to amplify each full-length cDNA coding sequence of AKINα1, AKINβ1 and AKINγ1 cDNA by PCR reactions.

For PCR amplification of AKINα1 cDNA (AT3G29160), the primers (AKINα1 F: 5’ A TGGATCATTCATCAAAT 3’ and AKINα1 R: 5’ TCAGATCACACGAAGCTCTG
T 3’) were used. For PCR amplification of \textit{AKIN}\textbeta\textit{1} cDNA (AT5g21170), the primers (AKIN\textbeta\textit{1} F: 5’ ATGGGAAATGCGAACGGC 3’ and AKIN\textbeta\textit{1} R: 5’ TTACCGTGTG AGCGGTTTTGTA 3’) were used. For PCR amplification of \textit{AKIN}\textgamma\textit{1} cDNA (AT3G48530), the primers (AKIN\textgamma\textit{1} F: 5’ ATGGCGACTGTTCCGGAG 3’ and AKIN\textgamma\textit{1} R: 5’ TCAGACTCGGTAGTTTTCGGG 3’) were used.

\textbf{3.2.4 Cloning \textit{AKIN}\textalpha\textit{1}, \textit{AKIN}\textbeta\textit{1} and \textit{AKIN}\textgamma\textit{1} cDNA into binary plasmid 943}

Plasmids 943, and 83 and JM11 were kindly provided by Dr. Jaideep Mathur (Department of Molecular and Cellular Biology, University of Guelph, Canada). Plasmid 943 was used as a binary plasmid to clone the SnRK1 subunits, and plasmids 83 and JM11 were used as templates to amplify EYFP and GFP respectively. A red fluorescent protein gene has been inserted in the binary plasmid 943 between \textit{BamHI} and \textit{SacI} sites.

Using overlap extension PCR, the \textit{AKIN}\textalpha\textit{1}-\textit{YFP} and \textit{AKIN}\textgamma\textit{1}-\textit{GFP} fusion genes were cloned and inserted first in plasmid pUC19 (New England BioLabs, Ipswich, USA) before the fusion genes were cloned into plasmid 943, which simplified further cloning experiments. For the \textit{AKIN}\textalpha\textit{1}-\textit{YFP} or \textit{AKIN}\textgamma\textit{1}-\textit{GFP} fusion gene, the cDNA segments of \textit{AKIN}\textalpha\textit{1}, \textit{AKIN}\textgamma\textit{1}, \textit{YFP}, and \textit{GFP} were amplified by using the corresponding primers (Table 3.1). The cDNAs were purified by gel extraction and fused together by overlap extension PCR. For the latter, 1\mu l the \textit{AKIN}\textalpha\textit{1} or \textit{AKIN}\textgamma\textit{1} cDNA segment (198.3ng/\mu l or 203.3ng/\mu l) and 1\mu l the \textit{YFP} or \textit{GFP} cDNA segment (102ng/\mu l or 69ng/\mu l) were mixed together respectively as DNA templates for PCR.
By employing CloneEZ® PCR Cloning Kit (GenScript, Piscataway, USA), the AKINα1-YFP and AKINγ1-GFP fusion genes were cloned in plasmid pUC19 linearized by BamHI (Thermo Scientific, Waltham, USA) respectively. Prior to sequencing, the pUC19 plasmids were digested by restriction enzymes to confirm the presence of inserts of the expected size.

The cDNAs of AKINα1-YFP in pUC19, AKINβ1 from total RNA of Arabidopsis rosette leaves, AKINβ1T-RFP from AKINβ1-RFP in plasmid 943, and AKINγ1-GFP in pUC19 were amplified by the corresponding primers respectively and cloned in linearized plasmid 943 by using the corresponding ligases as shown (Table 3.2). Putative positive plasmids were verified by digestion with the corresponding restriction enzymes (Table 3.2).

1μl of each plasmid 943 containing either AKINα1-YFP, AKINβ1-RFP, AKINβ1T-RFP or AKINγ1-GFP was transformed into 50μl agrobacterium GV3101 competent cells, respectively, by electroporation transformation using Gene Pulser II electroporation
Table 3.1 Primers used to amplify the cDNA segments of *AKIn*α1, YFP, *AKIn*γ1, and GFP for U19 cloning and *AKIn*α1-YFP and *AKIn*γ1-GFP for overlap extension PCR.

<table>
<thead>
<tr>
<th>Fusion gene</th>
<th>Cloned cDNA</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AKInα1-YFP</strong></td>
<td>AKInα1</td>
<td>AKInα-pUC19 F: 5’ GAGCTCGGTACCCGGACTAGTATGGATCATTCATCA 3’</td>
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<tr>
<td></td>
<td></td>
<td>AKInα-pUC19 R: 5’ GGATCCGATCACACGAAGCTC 3’</td>
</tr>
<tr>
<td></td>
<td>YFP</td>
<td>EYFP-pUC19 F: 5’ GAGCTTCGTGTGATCGGATCCATGGTGAGCAAGGGC 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EYFP-pUC19 R: 5’ CTTGCATGCCTGAGGAGCTCTCTACTTGTACAGCTCGTC 3’</td>
</tr>
<tr>
<td><strong>AKInγ1-GFP</strong></td>
<td>AKInγ1</td>
<td>AKInγ-pUC19 F: 5’ GAGCTCGGTACCCGGTCTAGAATGGCGACTGTTCCG 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AKInγ-pUC19 R: 5’ GGATCCGACTCGGTAGTTTTCC 3’</td>
</tr>
<tr>
<td></td>
<td>GFP</td>
<td>GFP-pUC19 F: 5’ GAAAACTACCGAGTCGGATCCATGGGTAAGGGAGAA 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GFP-pUC19 R: 5’ CTTGCATGCTCAGGAGCTCTCTACTTGTACAGCTCGTC 3’</td>
</tr>
<tr>
<td><strong>AKInγ1-GFP</strong></td>
<td>AKInγ1</td>
<td>AKInγ-pUC19 F: 5’ GAGCTCGGTACCCGGTCTAGAATGGCGACTGTTCCG 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AKInγ-pUC19 R: 5’ GGATCCGACTCGGTAGTTTTCC 3’</td>
</tr>
<tr>
<td></td>
<td>GFP</td>
<td>GFP-pUC19 F: 5’ GAAAACTACCGAGTCGGATCCATGGGTAAGGGAGAA 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GFP-pUC19 R: 5’ CTTGCATGCTCAGGAGCTCTCTACTTGTACAGCTCGTC 3’</td>
</tr>
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</table>
Table 3.2 Primers and enzymes used to clone cDNA sequences of *AKINα1-YFP*, *AKINβ1*, *AKINβ1T-RFP*, and *AKINγ1-GFP* into binary vector 943.

<table>
<thead>
<tr>
<th>Cloned gene</th>
<th>Primers used to amplify the cDNA sequence</th>
<th>Restriction enzymes for cDNA digestion</th>
<th>Restriction enzymes for 943 digestion</th>
<th>Ligase</th>
<th>Restriction enzymes for verification of resulting plasmids</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>AKINα1-YFP</em></td>
<td>F: 5' GGAGAGAACACGGGGGACACCTAGTATGGGATCCATCA 3'</td>
<td>XbaI/SacI</td>
<td>CloneEZ enzyme</td>
<td>XbaI/BamHI</td>
<td>BamHI/SacI, BamHI/EcoRI, BamHI/HindIII HindIII/EcoRI</td>
</tr>
<tr>
<td></td>
<td>R: 5' GAACGATCGGGGAAATTCGAGCTCTCACTTGATACAGCTCGTC 3'</td>
<td>XbaI/SacI</td>
<td>CloneEZ enzyme</td>
<td>XbaI/BamHI</td>
<td>BamHI/SacI</td>
</tr>
<tr>
<td><em>AKINβ1</em></td>
<td>F: 5' GCTCTAGAATGGGAAATGCGAACGGC 3'</td>
<td>XbaI/BamHI</td>
<td>T4</td>
<td>XbaI/EcoRI</td>
<td>HindIII/BamHI, BamHI/SacI</td>
</tr>
<tr>
<td></td>
<td>R: 5' CGCGGATCCCCGTGTGAGCGCTTTGTA 3'</td>
<td>XbaI/SacI</td>
<td>CloneEZ enzyme</td>
<td>XbaI/BamHI</td>
<td>BamHI/EcoRI</td>
</tr>
<tr>
<td><em>AKINβ1T-RFP</em></td>
<td>F: 5' GGAGAGAACACGGGGGACTCTAGAATGGGCAAACGCCCCTCCT 3'</td>
<td>XbaI/SacI</td>
<td>CloneEZ enzyme</td>
<td>XbaI/BamHI</td>
<td>BamHI/SacI, BamHI/EcoRI, BamHI/HindIII HindIII/EcoRI</td>
</tr>
<tr>
<td></td>
<td>R: 5' GAACGATCGGGGAAATTCGAGCTCTCAGGGCGCCGCTTGAGTG 3'</td>
<td>XbaI/SacI</td>
<td>CloneEZ enzyme</td>
<td>XbaI/BamHI</td>
<td>BamHI/SacI, BamHI/EcoRI, BamHI/HindIII HindIII/EcoRI</td>
</tr>
<tr>
<td><em>AKINγ1-GFP</em></td>
<td></td>
<td>XbaI/SacI</td>
<td>T4</td>
<td>XbaI/BamHI</td>
<td>BamHI/SacI, BamHI/EcoRI, BamHI/HindIII HindIII/EcoRI</td>
</tr>
</tbody>
</table>
system (Bio-Rad, Berkeley, USA) at a setting of 1.8KV (voltage), 200Ω (resistance), 250μFD (capacitance extender), and 25μFD (capacitance).

3.2.5 Cloning YFP, RFP, GFP and AKINβ1(G2A)-RFP cDNA into binary plasmid 943

The cDNAs of YFP from plasmid 83, RFP from original plasmid 943, and GFP from plasmid JM11 were amplified respectively using the corresponding primers (Table 3.3).

An AKINβ1 mutant (Gly to Ala (G2A) codon substitution at position 2) fused with RFP was generated to study the effect of N-myristoylation on the subcellular location of AKINβ1. The cDNA of AKINβ1 containing the GA to CC mutation at 5 and 6bp (Gly to Ala) was amplified using the corresponding primers (Table 3.3) and AKINβ1-RFP in 943 used as DNA template.

Except that the plasmid backbone released from the XbaI/SacI-digested mixture of both AKINβ1T-RFP in 943 and AKINγ1-GFP in 943 was used, the homologous recombination-based cDNA cloning process and agrobacterium electroporation transformation were performed as described in Section 3.2.4.
<table>
<thead>
<tr>
<th>Cloned gene</th>
<th>Primers used to amplify the cDNA sequence</th>
<th>Restriction enzymes for verification of resulting plasmids</th>
</tr>
</thead>
</table>
| **YFP**    | F: 5’ GGAGAGAACACGGGGGACGGATCCATGGTGAGCAAGGGCGAG 3’  
             R: 5’ GAACGATCGGGGAAATTCGAGCTCTCACTTTGTACAGCTCGTC 3’ | **BamHI/SacI, HindIII/SacI, BamHI/EcoRI**            |
| **RFP**    | F: 5’ GGAGAGAACACGGGGGACGGATCCATGGCCTCCTCCGAGGAC 3’  
             R: 5’ GAACGATCGGGGAAATTCGAGCTCTCAGGGCGCGGTGGAGTG 3’ | **HindIII/SacI, BamHI/SacI, BamHI/EcoRI**         |
| **GFP**    | F: 5’ GGAGAGAACACGGGGGACGGATCCATGGGTAAGGGAGAAGA 3’  
             R: 5’ GAACGATCGGGGAAATTCGAGCTCTCATTTGTATAGTTCATC 3’ | **BamHI/SacI, HindIII, BamHI/EcoRI**            |
| **AKINβ1(G2A)-RFP** | F: 5’ GGAGAGAACACGGGGGACCTCTAGAATGGCCAATGCGAACGGCAAAGAC 3’  
                            R: 5’ GAACGATCGGGGAAATTCGAGCTCTCAGGGCGCC 3’ | **XbaI/BamHI/SacI, MlsI**                 |
3.2.6 Agrobacterium-mediated tobacco leaf infiltration

Agrobacterium cells transformed with the relevant plasmid containing a particular AKIN construct and fluorescent tag were grown on YEB plates containing 10μg/ml rifampicin, 30μg/ml gentamycin and 75μg/ml kanamycin. Colonies were picked by a 1-ml blue tip and dissolved in 1ml of Agrobacterium infiltration medium (500μl 0.5M 2-(N-morpholino) ethanesulfonic acid (MES) buffer, 500μl 1M MgCl₂ and 7.5μl 1M acetasyringone in 50ml dH₂O) and mixed thoroughly. The cells were incubated at room temperature for 2 hours and diluted to OD₆₀₀=0.8 with Agrobacterium infiltration medium. A 1-ml, needleless, plastic syringe was used to infiltrate the bacterial suspension into the underside of two four-week tobacco leaves respectively. The areas infiltrated on the leaves were labelled by a marker pen. The tobacco plant was placed in room temperature under continuous light (400μmol photon/m²/sec) for 48 hours.

3.2.7 Arabidopsis floral dip transformation and positive transformant selection

The first bolts of healthy Arabidopsis plants were clipped to encourage proliferation of secondary bolts. About one week after clipping, plants with many immature flower clusters and not many fertilized siliques were selected for transformation. Three days before floral dip transformation, a colony of the agrobacterium strain GV3101 containing each cDNA of interest (AKINα₁, AKINβ₁, AKINβ₁Τ and AKINγ₁) in the binary vector 943, was inoculated with 5ml lysogeny broth (LB) containing 10μg/ml rifampicin, 30μg/ml gentamycin and 75μg/ml kanamycin and was incubated at 28°C with shaking (250rpm) for 48 hours. On the day when floral dipping was performed, 2-mL of each culture was inoculated with 200ml of LB broth containing 10μg/ml
rifampicin, 30μg/ml gentamycin and 75μg/ml kanamycin, and was incubated at 28°C with shaking (250rpm) until its OD$_{600}$ reached 1.5. The *agrobacterium* cultures were centrifuged and cells resuspended to OD$_{600}=0.8$ in 5% (w/v) sucrose solution with 0.05% (w/v) Silwet L-77. The above-ground parts of 12 Arabidopsis plants were dipped in each *agrobacterium* solution for 30 seconds, with gentle agitation. These dipped plants were watered and placed in a plastic bag and transferred to a growth chamber. The bags were sealed overnight to maintain high humidity under weak light. Next day, the plants were moved from the bags and watered normally until seeds became mature. The plants were dipped one more time after a seven-day interval for higher rates of transformation.

The seeds were selected for positive transformants by placing 500 seeds (about 10mg) sterilized in 20% (v/v) bleach solution for 15min on one Murashige and Skoog (MS) medium plate containing 2% (w/v) sucrose, 0.8% (w/v) agar and 25μg/ml hygromycin B. In total, 5000 seeds collected from 12 plants transformed with each AKIN cDNA of interest were selected. The seeds were placed at 4°C under darkness for 2 days and then grown under continuous light for 7-10 days. Seedlings that displayed normal root growth, green true leaves and long hypocotyls were selected as putative positive transformants. These selected seedlings were confirmed using an ECLIPSE 80i fluorescent microscope (Nikon, Tokyo, Japan) to observe the corresponding fluorescent signal. Seedlings possessing fluorescent signals were transferred to soil and transformation confirmed by PCR. These confirmed transformants were grown and T$_1$ seeds harvested. Seedlings from the T$_2$ generation of each heterozygous transformed
Arabidopsis line were used for determination of subcellular localization of SnRK1 subunit proteins in Arabidopsis leaf cells.

3.2.8 Determination of subcellular localization of the AKINα1, AKINβ1 and AKINγ1, AKINβ1T and AKINβ1(G2A) in infiltrated tobacco leaves

To determinate the subcellular localizations of the AKINα1, AKINβ1 and AKINγ1 subunits, AKINβ1T and AKINβ1(G2A) in infiltrated tobacco leaves, a 1-cm² leaf piece was cut and placed in water in an 1.5-ml Eppendorf tube and degassed with a needleless plastic syringe. The leaf samples were placed in a depression slide in distilled H₂O under a glass coverslip. In total, four leaf pieces for each infiltrated tobacco plant were prepared.

The leaf samples were observed by using an upright Leica DM 6000B microscope connected to a Leica TCS SP5 system with eight visible laser excitations and, additionally, a Radius 405nm laser and a Chameleon Ultra Infrared Laser (Leica MICROSYSTEMS, Wetzlar, Germany). Images were captured and processed using the Leica LAS AF Imaging software (Leica MICROSYSTEMS, Wetzlar, Germany). Fluorescent excitation and emission collection was at 488nm/500–530nm for GFP, 514nm/525–560nm for YFP, 543nm/565–585nm for RFP. The emission collection for chlorophyll autofluorescence was 670-700nm. WT tobacco leaves were used to set the smart gain to remove any background fluorescent signal. Leaf samples infiltrated with GV3101 transformed with plasmid 943 containing either YFP, RFP or GFP only, were used as controls when investigating localization of the fusion proteins.
3.2.9 Immunodetection of expressed protein

Proteins from tobacco leaves observed were extracted in 50mM Tris-HCl buffer (pH7.9) containing 1mM EDTA and 150mM NaCl. The extracts were centrifuged at 14000g for 10min at 4°C. The supernatants were transferred to fresh 1.5ml Eppendorf tubes respectively and total protein concentration for each sample was measured using Protein Assay Dye Reagent (Bio-Rad Laboratories (Canada), Mississauga, Canada) and an UV-Visible Spectrophotometer (UV-1601) (SHIMADZU, Kyoto, Japan) at a wavelength of 595 nm. 50μg protein from each leaf sample was used to perform western blot analysis. For immunoblotting, gels were transblotted onto nitrocellulose membranes (BioRad, Berkeley, USA) using transfer buffer (20% (v/v) methanol in 1×tris-glycine running buffer) in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Berkeley, USA) at 30V for 90 min. Transblotted membranes were incubated in blocking solution (1.5% BSA in 1xTBS (50mM Tris-Cl (pH 7.5), 150mM NaCl) for 15 min at room temperature with shaking. Rabbit anti-GFP antibody (A-11122; Thermo Fisher Scientific, Waltham, USA) or mouse anti-RFP antibody (MA5-15257; Thermo Fisher Scientific, Waltham, USA) was used at 1:1500 dilution blocking solution (1.5% BSA in 1xTBS). Alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse IgG (Sigma-Aldrich, Billerica, USA) was used as a secondary antibody. Blots were developed in 5-bromo-4-chloro-3-indolylphosphate (BCIP)/Nitro-Blue Tetrazolium (NBT) liquid substrate system (Sigma-Aldrich, Billerica, USA) until protein bands become visible.
3.2.10 Identification of organelles in tobacco leaves

Six different fluorescent organelle markers in binary plasmids were obtained from Dr. Robert Mullen (Department of Molecular and Cellular Biology, University of Guelph, Canada) as shown in Table 3.4. Tobacco leaf infiltration was performed as described in Section 3.2.6 except that 600μl of GV3101 strain containing each SnRK1 subunit fusion protein, and 400μl of GV3101 strain containing a known organelle marker, were incubated individually at room temperature, mixed and co-infiltrated into the tobacco leaves (Table 3.4). Fluorescent excitation and emission collection was at 543nm/550–630nm for mcherry, 488nm/500–530nm for GFP, 514nm/525–560nm for YFP, 543nm/565–585nm for RFP. Emission collection for chlorophyll autofluorescence was between 670-700nm. WT tobacco leaves were used to set the smart gain to remove any background fluorescent signal. Western blots were performed as described in Section 3.2.9.

Table 3.4 Mixtures used to perform tobacco leaf co-infiltration.

<table>
<thead>
<tr>
<th>Location</th>
<th>SnRK1 subunit fusion protein</th>
<th>Organelle marker (fluorescent protein fused)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td>AKINα1-YFP</td>
<td>CD3-991 (GFP)</td>
</tr>
<tr>
<td></td>
<td>AKINβ1-RFP/AKINβ1T-RFP</td>
<td>CD3-987 (GFP)</td>
</tr>
<tr>
<td></td>
<td>AKINγ1-GFP</td>
<td>CD3-991 (RFP)</td>
</tr>
<tr>
<td>Peroxisome</td>
<td>AKINα1-YFP</td>
<td>pRCS2/mCherry-PTS1 (RFP)</td>
</tr>
<tr>
<td></td>
<td>AKINβ1-RFP/AKINβ1T-RFP</td>
<td>CD3-979 (GFP)</td>
</tr>
<tr>
<td></td>
<td>AKINγ1-GFP</td>
<td>pRCS2/mCherry-PTS1 (RFP)</td>
</tr>
<tr>
<td>Golgi</td>
<td>AKINβ1-RFP/AKINβ1T-RFP/</td>
<td>CD3-963 (GFP)</td>
</tr>
<tr>
<td></td>
<td>AKINβ1(G2A)-RFP</td>
<td></td>
</tr>
<tr>
<td>Lipid body</td>
<td>AKINβ1-RFP/AKINβ1T-RFP</td>
<td>Oleosin-GFP</td>
</tr>
</tbody>
</table>
3.2.11 Determination of subcellular localization of the AKINα1, AKINβ1, AKINβ1T and AKINγ1 in leaves of transformed Arabidopsis lines

Three leaves from each of three, four-week old T2-generation Arabidopsis lines overexpressing either AKINα1-YFP, AKINβ1-RFP, or AKINβ1T-RFP, and from two, four-week T2-generation Arabidopsis lines expressing AKINγ1-GFP, were harvested and prepared for microscopy as described in Section 3.2.8. WT Arabidopsis leaves were used to set the smart gain to remove any background fluorescent signal.

3. 3 RESULTS

3.3.1 Isolation and purification of total RNA from Arabidopsis leaf cells

To clone AKINα1, AKINβ1 and AKINγ1 cDNA, total RNA from wild-type Arabidopsis (Col-0) leaf cells was extracted. About 100 mg Arabidopsis leaf was used to extract total RNA and finally the RNA sample was dissolved in 50μl DNase/RNase-free distilled water. 2μl of the RNA solution was run on a 0.8% (w/v) agarose gel to check the quality of the RNA sample (Figure 3.2). There were three ribosomal RNA (rRNA) bands (28s, 18s and 5s) on the gel and no RNA degradation was visible.

![Figure 3.2 Total RNA sample on a 0.8% (w/v) agarose gel containing ethidium bromide.](image-url)
3.3.2 Synthesis of cDNAs for AKINα1, AKINβ1 and AKINγ1

The full-length cDNA sequences of AKINα1, AKINβ1 and AKINγ1 in Arabidopsis were obtained from the website of The Arabidopsis Information Resource (TAIR) and are 1539bp, 851bp and 1275bp respectively. The PCR products of the full-length coding sequences of the three subunits were visualized on agarose gels (Figure 3.3).

![PCR products of the full-length cDNA coding AKINα1, AKINβ1, and AKINγ1 genes](image)

**Figure 3.3** PCR products of the full-length cDNA coding AKINα1 (1539bp), AKINβ1 (851bp) and AKINγ1 (1275bp) genes of SnRK1 in Arabidopsis on a 1% (w/v) agarose gel containing ethidium bromide.

3.3.3 Cloning the AKINα1-YFP, AKINβ1, AKINβIT and AKINγ1-GFP cDNAs into binary plasmid 943

3.3.3.1 Cloning the AKINβ1 cDNA into binary plasmid 943

The binary plasmid 943 (11500bp) contained a full-length coding sequence of Arabidopsis calnexin 1 (252bp), inserted between the restriction sites for XbaI and BamHI, fused with a RFP gene (678bp) inserted between the restriction sites for BamHI and SacI. The plasmid was digested by XbaI and BamHI to remove the Arabidopsis chaperone cDNA insert. Following PCR amplification, the AKINβ1 cDNA (without a stop codon) had new XbaI and BamHI restriction sites generated at its 5’-terminal and...
3’-terminal ends and the PCR product was digested by *XbaI* and *BamHI* (Figure 3.4 A). After DNA ligation using T4 ligase and *E. coli* (DH5-α) transformation, 12 colonies were selected to identify positive plasmids by digestion with *XbaI* and *BamHI*. Positive plasmids (12097bp) should release two DNA bands, one vector band (11242bp) and one *AKINβ1* insert band (855bp) (Figure 3.4 B). A positive plasmid was double checked by digestion with *HindIII/BamHI*, *XbaI/EcoRI* and *BamHI/SacI* respectively (Figure 3.4 C), releasing DNA bands of the expected sizes (1697bp/10400bp (Lane 1), 1792bp/10305bp (Lane 2) and 685bp/11412bp (Lane 3) respectively.

![Figure 3.4 Cloning of *AKINβ1* cDNA into binary plasmid 943. (A) The *XbaI-BamHI* digested DNA bands of plasmid 943 (11242bp) and the PCR product *AKINβ1* (866bp). (B) The *XbaI-BamHI* digested plasmids from 12 transformed colonies chosen randomly to identify positive clones of plasmid 943 containing the *AKINβ1* cDNA insert. (C) The positive plasmid (No. 9) was confirmed by digestions with *HindIII/BamHI* (Lane 1), *XbaI/EcoRI* (Lane 2) and *BamHI/SacI* (Lane 3) respectively, releasing the DNA bands of the expected sizes.](image)

### 3.3.3.2 Cloning the *AKINα1-YFP* and *AKINγ1-GFP* cDNAs into plasmid pUC19

The cDNA sequences of *YFP* and *GFP* amplified from plasmids 83 and JM11 were fused to the 3’-terminus of *AKINα1* and *AKINγ1* respectively by employing an overlap extension PCR (Figure 3.5). After amplification of *AKINα1* and *AKINγ1*, new *SpeI* and
BamHI restriction sites were generated at the 5’-terminal and 3’-terminal ends of AKINα1 respectively. New XbaI and BamHI restriction sites were generated at the 5’-terminal and 3’-terminal ends of AKINγ1 respectively to facilitate further binary plasmid cloning. After amplification of YFP and GFP, new BamHI and SacI restriction sites were generated at 5’-terminal and 3’-terminal ends of both YFP and GFP respectively. Moreover, a 21-bp overhang sequence for the 3’-terminal end of AKINα1 was generated at the 5’-terminal end of the YFP segment, and a 21-bp overhang sequence for the 3’-terminal end of AKINγ1 was generated at the 5’-terminal end of the GFP segment, which facilitates overlap extension PCR. The new fusion genes had two different 15-bp overhang sequences from the vector pUC19 on both their 5’ and 3’ termini to facilitate homologous recombination. The unpurified PCR products were digested by BamHI and NcoI respectively to verify that they were of the expected sizes. Because there was a non-specific PCR band (about 560bp) in both PCR reactions respectively (Figure 3.5 B), three bands were apparent on the gels after restriction enzyme digestion (Figure 3.5 C). After digestion with BamHI and NcoI respectively, AKINα1-YFP and AKINγ1-GFP both released two DNA bands of the expected sizes (Figure 3.5 C), demonstrating that the fusions were successful.
Figure 3.5 cDNA fusions of AKINα1-YFP and AKINγ1-GFP by overlap extension PCR. (A) PCR products of YFP, AKINα1, GFP, and AKINγ1 following overlap extension PCR. (B) The overlap extension PCR products of AKINα1-YFP and AKINγ1-GFP. (C) Verification of the fusion cDNAs (AKINα1-YFP and AKINγ1-GFP) by digestion with BamHI and NcoI. AKINα1-YFP digestion released two DNA bands, 1558bp/746bp and 1562bp/742bp, respectively. AKINγ1-GFP digestion released two DNA bands, 1294bp/743bp and 1298bp/739bp, respectively. Because unpurified PCR products were used to perform the digestion, a non-specific PCR band (about 560bp, arrow) was also detected.

AKINα1-YFP and AKINγ1-GFP were inserted into pUC19 digested by BamHI respectively by employing recombination-based cloning. After digestion with SacI, the plasmid pUC19 containing AKINα1-YFP (4948bp) should release three DNA bands (2664bp, 1954bp and 330bp) and the plasmid pUC19 containing AKINγ1-GFP (4681bp) should release two DNA bands (2665bp and 2016bp) (Figure 3.6 A and C). Thus pUC19 plasmids No. 1 and No. 8 contain AKINα1-YFP and AKINγ1-GFP respectively. The pUC19 plasmid containing AKINα1-YFP was digested by EcoRI/BamHI/SalI, EcoRI/NcoI/SalI and EcoRI/PshAI/SalI respectively to check the plasmid, all of which released three DNA bands (2659bp/1557bp/732bp, 2659bp/1561bp/728bp and
2659bp/1217bp/1072bp respectively), confirming the presence of $AKIN\alpha 1$-$YFP$ (Figure 3.6 B). The pUC19 plasmid containing $AKIN\gamma 1$-$GFP$ was digested by $XbaI/BamHI/SacI$, releasing three DNA bands (2676bp/1278bp/727bp) as predicted (Figure 3.6 D).

**Figure 3.6** Cloning of $AKIN\alpha 1$-$YFP$ and $AKIN\gamma 1$-$GFP$ fusion cDNA into pUC19 digested by $BamHI$. (A) Digestion of recombinant plasmids from 10 colonies of DH5-$\alpha$ by $SacI$. The results show that plasmid No. 1 released three DNA bands of the expected sizes (2664bp, 1954bp and 330bp) for $AKIN\alpha 1$-$YFP$. (B) Verification of plasmid pUC19 containing $AKIN\alpha 1$-$YFP$ by digestion with $EcoRI/BamHI/Sall$ (Lane 1), $EcoRI/NcoI/Sall$ (Lane 2) and $EcoRI/PshAI/Sall$ (Lane 3) respectively. The plasmid released three DNA bands of the expected sizes (2659bp/1557bp/732bp, 2659bp/1561bp/728bp, and 2659bp/1217bp/1072bp). (C) Digestion of recombinant plasmids from 9 colonies of DH5-$\alpha$ by $SacI$. The results show that plasmid No. 8 released two DNA bands having the expected sizes (2665bp and 2016bp) for $AKIN\gamma 1$-$GFP$. (D) Verification of plasmid pUC19 containing $AKIN\gamma 1$-$GFP$ by digestion with $XbaI/BamHI/SacI$. Three DNA bands of the expected sizes (2676bp/1278bp/727bp) were detected.
3.3.3 Cloning the AKINα1-YFP, AKINβ1T-RFP and AKINγ1-GFP cDNAs into binary plasmid 943

AKINα1-YFP cDNA (2310bp) and truncated AKINβ1-RFP cDNA (1359bp) were cloned into the binary plasmid 943 (10554bp) linearized by XbaI and SacI respectively (Figure 3.7 A, B, C). For the AKINα1-YFP cDNA segment cloning, the plasmids from 18 transformed DH5-α colonies were verified by digestion with SacI and two positive clones (No. 4 and 5, 12826bp) were identified, each of which released two DNA bands of the expected sizes (10873bp and 1953bp) following restriction enzyme digestion. Both plasmids were double checked by digestion with BamHI/SacI, BamHI/EcoRI, BamHI/HindIII and HindIII/EcoRI respectively, releasing DNA bands of the expected sizes (Figure 3.7 B). For cloning of the truncated AKINβ1-RFP cDNA, the plasmids from 12 transformed DH5-α colonies were verified by digestion with XbaI and SacI and one positive clone (No. 2, 11875bp) was identified, which released two DNA bands of the expected sizes (10554bp and 1324bp). The plasmid was confirmed by digestion with XbaI/BamHI/SacI, BamHI/EcoRI, BamHI/HindIII and HindIII/EcoRI respectively and released DNA bands of the expected sizes (Figure 3.7 C).

By employing T4 ligase, the AKINγ1-GFP cDNA segment digested by XbaI and SacI (2005bp) was cloned into plasmid 943 (10554bp), linearized by XbaI and SacI respectively (Figure 3.7 A, D). The plasmids from 18 transformed DH5-α colonies were checked by digestion with XbaI and SacI and six positive clones (No. 2, 7, 12, 14, 17, 18, 12559bp) were identified, each releasing two DNA bands with the expected sizes (10554bp and 2005bp). All plasmids were confirmed by digestion with
**XbaI/BamHI/SacI, BamHI/EcoRI, BamHI/HindIII and HindIII/EcoRI** respectively (Figure 3.7 D).

**Figure 3.7** Cloning of AKInα1-YFP, truncated AKInβ1-RFP and AKInγ1-GFP fusion cDNA into binary plasmid 943 linearized by XbaI and SacI. (A) The four DNA segments of linearized binary plasmid 943 (10554bp), AKInα1-YFP (2310bp), truncated AKInβ1-RFP (1359bp) and AKInγ1-GFP (2005bp) were used. (B) Selection of plasmid 943 containing AKInα1-YFP and confirmation of the positive clones (numbers in red). Lane 1, 2, 3 and 4 show the plasmids digested by BamHI/SacI, BamHI/EcoRI, BamHI/HindIII and HindIII/EcoRI respectively. (C) Selection of plasmid 943 containing truncated AKInβ1-RFP and confirmation of the positive clone.
Lane 1, 2, 3 and 4 show the plasmid digested by XbaI/BamHI/SacI, BamHI/EcoRI, BamHI/HindIII and HindIII/EcoRI respectively. (D) Selection of plasmid 943 containing AKINγ1-GFP and confirmation of the positive clones (numbers in red or yellow). Lane 1, 2, 3 and 4 show the plasmids digested by XbaI/BamHI/SacI, BamHI/EcoRI, BamHI/HindIII and HindIII/EcoRI respectively.

3.3.4 Cloning of YFP, RFP and GFP cDNAs into binary plasmid 943

The cDNA segments of YFP (768bp), RFP (726bp), and GFP (765bp) were cloned into binary plasmid 943 digested by XbaI/SacI (Figure 3.8 A). For YFP cDNA cloning, the plasmids from 12 transformed DH5-α colonies were verified by digestion with BamHI/SacI and eight clones released a DNA band of the expected size (about 730bp). These plasmids were double checked by digestion with HindIII/SacI and BamHI/EcoRI respectively and one positive plasmid (No. 6, 11276bp) was identified (Figure 3.8 B).

For RFP cDNA cloning, the plasmids from 10 transformed DH5-α colonies were verified by digestion with HindIII/SacI and two positive clones (No. 8 and 9, 11234bp) identified. The plasmid identity was confirmed by digestion with BamHI/SacI and BamHI/EcoRI (Figure 3.8 C). For GFP cDNA cloning, the plasmids from 12 transformed DH5-α colonies were checked by digestion with BamHI/SacI and ten putative positive clones identified. The plasmids were verified by digestion with HindIII and BamHI/EcoRI respectively and two positive clones (No 1 and 9) were identified (Figure 3.8 D).
3.3.5 Cloning of AKINβ1(G2A)-RFP cDNA into binary plasmid 943

The cDNA segment of AKINβ1(G2A)-RFP (1581bp) was cloned into binary plasmid 943 digested by XbaI/SacI (Figure 3.9 A). The plasmids from 20 transformed DH5-α colonies were verified by digestion with XbaI/BamH1/SacI and four clones released two DNA bands of the expected sizes (855bp and 688bp) (Figure 3.9 B). When the Gly-to-
Ala (G2A) codon substitutions at position 2 of the AKINβ1 subunit was generated, the wild-type cDNA sequence of AKINβ1 (5’ ATGGGAAAT 3’) was mutated to the gene sequence (5’ ATGGCCAAT 3’), which is a new MlsI digestion site (5’ TGG↓CCA 3’).

Compared with the wild-type AKINβ1-RFP, one more DNA band should appear following digestion of AKINβ1(G2A)-RFP with MlsI (Figure 3.9 C). DNA sequencing confirmed that the base-pair substitutions were successful (Figure 3.9 D).

![Figure 3.9](image)

**Figure 3.9** Cloning of AKINβ1(G2A)-RFP cDNA into binary plasmid 943 digested by Xbal/Sacl. (A) The two cDNA segments, plasmid backbone released from plasmid mixture of both 943 containing AKINβ1T-RFP and 943 containing AKINγ1-GFP (10554bp) and AKINβ1(G2A)-RFP (1581bp) were used for cloning. (B) Selection of
putative positive plasmid containing AKINβ1(G2A)-RFP using XbaI/BamHI/SacI digestion. Positive clones are labelled in red. (C) Verification of the putative positive plasmids containing AKINβ1(G2A)-RFP using MlsI digestion. A new MlsI digestion site is generated when the base-pair substitutions are successful. Compared with the wild-type AKINβ1-RFP (WT), one more DNA band appeared in all putative positive plasmids after digestion with MlsI. The original DNA band in WT and the two new-generated DNA bands in the mutant are labelled by black arrow and red arrows. (D) DNA sequencing data confirmed that the base-pair substitutions (GA in wild-type AKINβ1 sequence to CC in the AKINβ1 mutant) were successful. The base pairs for the second amino acid on both protein sequences are labelled.

3.3.6 Arabidopsis floral dip transformation and positive transformant selection

Although transient expression provides a valuable method in terms of testing new cloned plasmids and generating data that may not necessarily be feasible through the more labor-intensive generation of stable lines (Sparkes et al., 2006), one potential problem of tobacco leaf transient expression system is that overexpression and saturation can alter the subcellular distribution of proteins of interest through any given pathway (Sparkes et al., 2006). Therefore, in order to study the subcellular localizations of the three SnRK1 subunits, as well as the function of the predicted chloroplast transit peptide in AKINβ1 subunit of SnRK1, binary plasmids containing either AKINα1-YFP, AKINβ1-RFP, truncated AKINβ1-RFP, or AKINγ1-GFP were transformed into wild-type Arabidopsis (Col-0) lines. Seeds from the T0 generation were selected on MS plates using 12.5μg/ml Hygromycin B. After 10 days of culture under continuous light, most seeds germinated but many had very short roots, bent cotyledons and did not sprout true leaves. About 1% of seedlings grew long root as normal, and sprouted green true leaves (Figure 3.10) and were transferred to soil for further testes by microscopic observation and PCR. After microscopic observation and PCR using genomic DNA of transformed seedlings and corresponding primers (Figure 3.11), six Arabidopsis lines
expressing AKINα1-YFP, five Arabidopsis lines expressing AKINβ1-RFP, two Arabidopsis lines expressing AKINγ1-GFP and four Arabidopsis lines expressing the truncated AKINβ1-RFP were identified.

**Figure 3.10** Photographs of transformed Arabidopsis seedlings (T₀ generation) grown on MS plates with 12.5µg/ml Hygromycin B. Putative positive seedlings were marked by red arrows.
Figure 3.11 PCR of Arabidopsis lines (T0 generation) expressing either AKINα1-YFP, AKINβ1-RFP, AKINγ1-GFP or the truncated AKINβ1-RFP. PCR products of AKINα1-YFP (2262bp), AKINβ1-RFP (1533bp), AKINγ1-GFP (1995bp) and AKINβ1T-RFP (1600bp) were produced using genomic DNA extracts from positive seedlings expressing AKINα1-YFP (A), AKINβ1-RFP (B), AKINγ1-GFP (C) and AKINβ1T-RFP (D) respectively. Lanes P and G are PCR products using plasmid 943 containing either AKINα1-YFP, AKINβ1-RFP, AKINγ1-GFP or the truncated AKINβ1-RFP and WT Arabidopsis genomic DNA respectively as positive and negative controls. Positive lines are labelled in red.

3.3.7 Fluorescence-based localization studies

3.3.7.1 Determination of subcellular localizations of YFP, RFP and GFP infiltrated in tobacco leaves

Transient transformation of tobacco leaf cells is a relatively fast technique with a very high frequency of transformation to assess expression of genes of interest. Following the successful cloning of AKIN fusion cDNAs or fluorescent protein cDNAs into the binary plasmid respectively, these resulting binary plasmids were used to investigate
the subcellular localizations of AKIN proteins in tobacco leaves. Each cDNA of three fluorescent proteins, *YFP*, *RFP* and *GFP*, in plasmid 943 were transformed into tobacco leaf cells by *Agrobacterium*-mediated infiltration. Following infiltration, tobacco plants were grown under 48-h continuous light and leaf samples were observed by confocal fluorescent microscopy. The confocal microscope was set to remove background fluorescent signals using non-infiltrated WT tobacco leaves before observing the subcellular localization of each fluorescent protein (Figure 3.12 A). In tobacco leaf cells (epidermal cells and mesophyll cells), the fluorescent proteins (YFP, RFP and GFP) were localized in the cytosol (Figure 3.12 B, C, D). Western blots show that clear protein bands of YFP (27.2kDa), RFP (26.8kDa) and GFP (25.4kDa) could be detected in the leaf samples (Figure 3.13).
Figure 3.12 Subcellular localizations of YFP, RFP and GFP in tobacco leaf cells. The confocal microscope was set to remove background fluorescent signals using non-infiltrated WT tobacco leaves before observation of the subcellular localization of each fluorescent protein (A). YFP (B), RFP (C), and GFP (D) were co-localized in the cytosol in both epidermal cells (E) and mesophyll cells (M). Chlorophyll autofluorescence was defined as blue to facilitate the observation of signal from the red fluorescent protein fused with AKINβ1. During observation, different magnifications (as shown in scale bars) were used to obtain clear images using 10×eyepiece lens and 25×objective lens, and different values of zoom factor from 18 to 32 were used.
Figure 3.13 Western blots of YFP, RFP and GFP expressed in infiltrated tobacco leaves. 50μg protein from each leaf extract was used. YFP (27.2kDa), RFP (26.8kDa) and GFP (25.4kDa) were detected (red arrows) using anti-GFP or anti-RFP antibodies. Neither was detected in WT.

3.3.7.2 Subcellular localization of AKINα1, AKINβ1, and AKINγ1 in tobacco leaves

The four fusion proteins, AKINα1-YFP, AKINβ1-RFP, AKINβ1T-RFP and AKINγ1-GFP, were transformed in tobacco leaf cells to test their expression and determine their subcellular localization in vivo. The confocal microscope was set to remove background fluorescent signals as described previously (Figure 3.14 A). AKINα1 co-localized with the nucleus and cytosol in epidermal cells, but appeared to be co-localized with chloroplasts, as well as the nucleus and cytosol, of mesophyll cells (Figure 3.14 B). AKINβ1 co-localized with the nucleus and cytosol in epidermal cells (Figure 3.14 C, the top line) and in areas in between the chloroplasts (Figure 3.14 C, the middle line), appearing as puncta in the cytosol (Figure 3.14 C, the bottom line). AKINγ1 co-localized with the nucleus and cytosol of epidermal cells (Figure 3.14 D, the top line) and the cytosol of mesophyll cells (Figure 3.14 D, the bottom line). The truncated form
of AKINβ1 co-localized with the nucleus and cytosol in epidermal cells and the cytosol in mesophyll cells (Figure 3.14 E). Western blots show that AKINα1-YFP, AKINβ1-RFP, AKINβ1T-RFP and AKINγ1-GFP fusion proteins were all present in the leaf samples (Figure 3.15). AKINβ1-RFP and AKINβ1T-RFP migrated to higher molecular weights than their predicted sizes of 56.3kDa and 49kDa, respectively, a point which will be considered in Chapter IV.

(A) Non-infiltrated tobacco leaves  

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| RFP | ![RFP Image](image4) | ![Autofluorescence Image](image5) | ![Merge Image](image6) |

| GFP | ![GFP Image](image7) | ![Autofluorescence Image](image8) | ![Merge Image](image9) |
Subcellular localization of the AKINα1, AKINβ1, AKINβ1T, and AKINγ1 subunits following transient expression in tobacco leaf cells. The confocal microscope was set to remove background fluorescent signals using non-infiltrated WT tobacco leaves before observation of the subcellular localization of each subunit protein (A). AKINα1 (B), AKINβ1 (C), AKINγ1 (D), and AKINβ1T (E) co-localized with the nucleus and cytosol in epidermal cells (E). In mesophyll cells (M), AKINα1 (B) was localized in cytosol, the chloroplasts and the nucleus; AKINβ1 (C) was localized in areas in between the chloroplasts and signals appeared punctate; AKINγ1 (D) was localized in cytosol as was the AKINβ1T (E). Chlorophyll autofluorescence was defined as blue to facilitate the observation of signal from the red fluorescent protein fused with AKINβ1. During observation, different magnifications (as shown in scale bars) were used to obtain clear images using 10×eyepiece lens and 25×objective lens and different values of zoom factor varied from 18 to 32.
Figure 3.15 Western blots of AKINα1-YFP, AKINβ1-RFP, AKINβ1T-RFP and AKINγ1-GFP expressed in infiltrated tobacco leaves. 50μg protein from each leaf extract was loaded into each lane. Proteins were detected using anti-GFP or anti-RFP antibodies. No protein bands representing fusion proteins were detected in WT.

3.3.7.3 Localization of AKINα1, AKINβ1, and AKINγ1 in plants co-infiltrated with organelle-specific fluorescent markers

Six different fluorescent-tagged organelle markers in binary plasmids (Table 3.4) were used to investigate the subcellular localization of each SnRK1 subunit in detail. The subunit proteins with their fluorescent tags and the organelle marker proteins were readily detected in the confocal microscope. The results (Figure 3.16 A, B, C, D) show AKINα1-YFP, AKINβ1-RFP, AKINβ1T-RFP, and AKINγ1-GFP did not co-localize with mitochondria or peroxisomes in tobacco leaves. AKINα1-YFP was localized within the chloroplasts in mesophyll cells (Figure 3.16 A), consistent with the observation made previously (Figure 3.14 B). Moreover, puncta that were associated with AKINβ1 in mesophyll cells (Figure 3.14 C) were identified as Golgi stacks, based on co-localisation using a Golgi-stack fluorescent marker (Figure 3.16 E). The NVR
domain (amino acid 1 to 100) on the AKINβ1 protein sequence (López-Paz et al., 2009) has previously been shown to be involved in regulating subcellular localization of AKINβ1 (re-localization from plasma membrane to the nucleus) due to N-myristoylation (Pierre et al., 2007). Moreover, a transit peptide (the first 74 amino acids) is predicted on AKINβ1 protein sequence, which would be presumed to direct the subunit to the chloroplasts (Figure 3.14 C). Thus, the truncated AKINβ1, lacking the predicted chloroplast transit peptide (74 amino acids), fused with RFP was used to test the regulatory function of this region. The truncated AKINβ1 was localized within the cytosol, in contrast to the localization of the wild-type AKINβ1 that was associated with punctate bodies in areas in between the chloroplasts (Figure 3.14 C, Figure 3.16 F). These observations suggest that the predicted chloroplast transit peptide on AKINβ1 is not involved in directing the protein to chloroplasts, but that this region does direct the subunit to Golgi stacks. Neither AKINβ1-RFP or AKINβ1T-RFP co-localized with the marker for lipid bodies (Figure 3.16 E, F).

Extracts from tobacco leaves co-infiltrated with AKIN proteins fused with different fluorescent tags and the organelle markers fused with GFP or RFP (Table 3.4) were assayed using anti-GFP or anti-RFP antibodies. Western blots show that AKIN-fluorescent fusion proteins were all present in the leaf samples (Figure 3.17).
Figure 3.1 Co-localization of SnRK1 subunit fusion proteins with organelle markers. Mitochondrial and peroxisome markers were used to study the subcellular localization of AKINα1-YFP (A), AKINβ1-RFP (B), AKINβ1T-RFP (C), and AKINγ1-GFP (D) in tobacco mesophyll cells. Golgi stack and lipid body markers were used to detect the subcellular localization of AKINβ1-RFP (E) and AKINβ1T-RFP (F) in tobacco.
mesophyll cells. Chlorophyll autofluorescence was defined as blue to facilitate the observation of signal from the AKIN subunit fusion proteins fused with red fluorescent protein. During observation, different magnifications (as shown in scale bars) were used to obtain clear images using 10× eyepiece lens and 25× objective lens and different values of zoom factor from 18 to 32.

Figure 3.17 Western blots of extracts from tobacco leaves co-infiltrated with AKINα1-YFP, AKINβ1-RFP, AKINβ1T-RFP, AKINγ1-GFP and corresponding organelle markers fused with RFP or GFP. Organelle markers for mitochondria (CD3-991(RFP) and CD3-987 (GFP)) and peroxisomes (PTS1 (RFP) and CD3-979 (GFP)) were used with AKIN proteins fused with YFP/GFP or RFP respectively (A). Organelle markers for Golgi stacks (CD3-963 (GFP)) and lipid bodies (oleosin-GFP) were used with AKINβ1 fusion proteins respectively (B). 50μg protein from each extract of co-infiltrated tobacco leaves was loaded into each lane. The names of AKIN fusion protein (top line) and its organelle marker (bottom line) in combination are listed. Proteins (name in red) were detected (red arrows) by anti-GFP or anti-RFP antibodies (under each figure). No protein bands representing fusion proteins and organelle markers were detected in WT. O: oleosin-GFP.
3.3.7.4 Investigation of N-myristoylation of AKINβ1

It has been shown in the previous sections that AKINβ1 co-localized with punctate bodies in mesophyll cells (Figure 3.14 C) and these puncta were identified as Golgi (Figure 3.16 E). It is known that N-myristoylation, which is an important irreversible lipid modification in plants, allows a key subset of proteins to be targeted at the periphery of specific membrane compartments such as Golgi (Pierre et al., 2007; Renna et al., 2013). Whether Golgi localization of AKINβ1 is related to N-myristoylation was investigated by studying the subcellular localization of the mutated subunit in which a putative myristoylation site, glycine at position 2, was mutated to alanine (G2A). The results show that the AKINβ1(G2A)-RFP was localized in nuclei and cytosol in epidermal cells and in mesophyll cells (Figure 3.18 A). Moreover, in mesophyll cells, the mutated AKINβ1 co-localized with areas in between the chloroplasts (cytosol) (Figure 3.18 A) as observed for AKINβ1T (Figure 3.14 E), and in the nucleus (Figure 3.18 A). The AKINβ1(G2A) re-localized to nucleus and cytosol (areas in between the chloroplasts) compared to WT AKINβ1 that co-localized with the Golgi marker (Figure 3.18 B).

Western blots show that AKINβ1(G2A)-RFP fusion protein was expressed successfully in the infiltrated tobacco leaves (Figure 3.19 A) and the AKINβ1 fusion proteins were all present in the leaf samples (Figure 3.19 B).
Figure 3.18 Subcellular localization of AKINβ1(G2A) in tobacco leaf cells. (A) Non-infiltrated WT tobacco leaves were used to remove background fluorescent signals. Subcellular localization of AKINβ1(G2A) was detected in epidermal cells and mesophyll cells. (B) Golgi stack marker (CD3-963) was used to study subcellular localization of AKINβ1(G2A) in tobacco leaves. Chlorophyll autofluorescence was defined as blue to facilitate the observation of signal from the red fluorescent protein fused with the AKINβ1 proteins. E=epidermal cells, M=mesophyll cells. During observation, different magnifications (as shown in scale bars) were used to obtain clear images using 10×eyepiece lens and 25×objective lens and different values of zoom factor from 18 to 32.
Figure 3.19 Western blots of AKINβ1(G2A)-RFP, AKINβ1-RFP and Golgi marker expressed or co-expressed in tobacco leaves. 50μg protein per lane from each leaf extract was used. (A) Western blot of AKINβ1(G2A)-RFP in the infiltrated tobacco leaves. (B) Western blots of organelle marker for Golgi stacks (CD3-963 (GFP)) and the AKINβ1 fusion proteins. Proteins were detected using anti-GFP or anti-RFP antibodies. No protein bands representing fusion proteins and organelle markers were detected in WT. The identified protein bands are indicated by arrows. 963=CD3-963.

3.3.7.5 Subcellular localization of AKINα1, AKINβ1, AKINβ1T and AKINγ1 in leaves of Arabidopsis following stable transformation

The Arabidopsis lines expressing AKINα1-YFP, AKINβ1-RFP, truncated AKINβ1-RFP and AKINγ1-GFP were identified and their leaves from the T2 generation were used to observe their subcellular localization. WT Arabidopsis leaves were used to set the microscope to remove any background signal (Figure 3.20 A). AKINα1 co-localized with the nucleus and cytosol in epidermal cells and the chloroplasts in mesophyll cells (Figure 3.20 B), consistent with the observations made during transient expression in tobacco leaves (Figure 3.14 B). AKINβ1 co-localized with puncta and areas surrounding the chloroplasts, non-evenly, in mesophyll cells (Figure 3.20 C), but no fluorescent signal was observed in Arabidopsis epidermal cells in contrast to the situation during transient expression in tobacco leaves (Figure 3.14 C). AKINγ1 co-
localized with the nucleus and cytosol in epidermal cells and cytosol in mesophyll cells (Figure 3.20 D), again consistent with the observation in tobacco leaves (Figure 3.14 D). Compared to AKINβ1, AKINβ1T was localized evenly within areas surrounding the chloroplasts in mesophyll cells (Figure 3.20 E).

(A) Non-infiltrated Arabidopsis leaves

![Images](image_url)
**Figure 3.20** Subcellular localization of the AKINα1, AKINβ1, AKINβ1T, and AKINγ1 subunits following stable expression in Arabidopsis leaf cells. The confocal microscope was set to remove background fluorescent signals using non-infiltrated WT tobacco leaves before observation of the subcellular localization of each subunit protein (A). The subcellular localization of AKINα1 (B), AKINβ1 (C), AKINγ1 (D), and AKINβ1T (E) in Arabidopsis leaves are shown. Chlorophyll autofluorescence was defined as blue.
to facilitate the observation of signal from the red fluorescent protein fused with AKINβ1. E=epidermal cells, M=mesophyll cells. During observation, different magnifications (as shown in scale bars) were used to obtain clear images using 10×eyepiece lens and 25×objective lens and different values of zoom factor from 18 to 32.

The subcellular localizations of AKINα1, AKINβ1 and AKINγ1 observed following transient expression in tobacco leaves or stable expression in Arabidopsis leaves are summarized in Table 3.5.
Table 3.5 Summary of subcellular localization of AKINα1, AKINβ1 and AKINγ1 following transient expression (T) in tobacco leaves and stable expression (S) in Arabidopsis leaves. E, epidermal cells, M, mesophyll cells.

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3.4 DISCUSSION

The study of a protein’s subcellular localization is critical in understanding its cellular functions. Transient expression and stable transformation of fluorescence-tagged proteins are methods which have been widely used to study protein localization in vivo in different systems (Cui et al., 2016). In this study, YFP, RFP and GFP were used as fusion tags to detect the subcellular localization of AKINα1, AKINβ1 and AKINγ1 in vivo using a tobacco-based, transient expression system and Arabidopsis-based, stable expression system respectively. Known protein-markers, fused with RFP (mcherry) or GFP, were also used to identify particular organelles in vivo. During the identification of localization of AKIN proteins, autofluorescence, which is the natural emission of cellular components such as chlorophyll, NAD(P)H, and flavins, should be prevented from affecting the spectral detection of some fluorophores such as RFP (Mylle et al., 2013). Moreover, fluorescent “bleed-through” (crosstalk) is a fundamental problem that must be considered in this study. Many fluorophores like fluorescent proteins have broad bandwidths and asymmetrical spectra. When more than one fluorophore is excited and imaged at a time, if overlap of their spectral emissions occurs, bleed-through occurs. Bleed-through can give rise to artefacts suggesting co-localization when there is none. In the present work, the RFP (AKINβ1)/GFP (organelle markers) and GFP (AKINγ1) or YFP(AKINα1)/mcherry (organelle markers) were used in combination. When RFP or mcherry are excited at 561nm or 543nm, neither GFP or YFP respond. Conversely, however, when GFP or YFP are excited at 488nm or 514nm, then RFP or mcherry can be partially excited due to the significant overlap of the two
excitation spectra. This can lead to misinterpretation of co-localization data in vivo due to the emissions of the RFPs contributing to the imaged GFP/YFP signal. Thus, wild-type tobacco leaves were used as background control, with each laser and detection channel, to set the limits of signal gain and offset to be used for the final imaging series, in order to minimize such artefacts.

AKINα1 (AKIN11) was identified within chloroplasts, as well as the nucleus and cytosol in tobacco leaves and Arabidopsis leaves (Figure 3.14 B and Figure 3.20 B), consistent with previous studies on the subcellular localization of AKIN10 and AKIN11 (Fragoso et al., 2009; Bayer et al., 2012; Tsai and Gazzarrini, 2012; Nietzsche et al., 2014; Williams et al., 2014). The apparent multiple localization of AKINα1 is in line with its proposed role in regulating gene expression in the nucleus, phosphorylation of SPS and F2KP in cytoplasm, and phosphorylation of plastidic acetyl-CoA synthetase in chloroplasts (Kulma et al., 2004; Baena-González et al., 2007; Cho et al., 2016).

Moreover, ribulose-5-phosphate and ribose-5-phosphate exist in chloroplasts and cytoplasm in vivo in plants (Schnarrenberger et al., 1995; Kopriva et al., 2000) and ribose-5-phosphate is in the nucleus as a precursor for the synthesis of nucleotides (Zeeman, 2015). Both compounds inhibit the activity of SnRK1 from wheat endosperm and Arabidopsis (Piattoni et al., 2011; Nunes et al., 2013), which implies that SnRK1 would be located within chloroplasts, cytoplasm, and the nucleus in vivo. The observation here and in the literature that they are co-located suggest that AKIN11 an AKIN10 play complementary roles in the SnRK1 signaling pathway.
AKINγ1 co-localizes with the nucleus and cytosol in tobacco leaves and Arabidopsis leaves (Figure 3.14 D, Figure 3.20 D), consistent with bioinformatic prediction by the SUBA3 (http://suba.plantenergy.uwa.edu.au). Interestingly, although interactions of AKINγ1 with AKIN10, AKINβ1 and AKINβ2 have been identified in the yeast two-hybrid system (Bouly, 1999), there is no interaction with AKINβ3 in the yeast two-hybrid system (Gissot et al., 2004). Further evidence suggests that AKINγ1 does not bind to the AKINβ subunits based on co-immunoprecipitation (Ramon et al., 2013; Emanuelle et al., 2015), and does not complement a yeast mutant lacking the γ subunit of SNF1 (Bouly et al., 1999). Thus, it is difficult to determine whether AKINγ1 is involved in the formation of SnRK1 or not. However, the subcellular localization of AKINγ1 with AKINα1 in the nucleus and cytosol nonetheless are consistent with a putative role in regulating the activity of the kinase.

AKINβ1 was found to be localized within the nucleus and the cytosol in tobacco leaves and areas surrounding the chloroplasts in tobacco leaves and Arabidopsis leaves. Interestingly, AKINβ1 also co-localizes within puncta in tobacco leaves and Arabidopsis leaves, which were identified as Golgi stacks during transient expression in tobacco leaves. The predicted subcellular localization of AKINβ1 using the subcellular location database for Arabidopsis proteins (SUBA3), indicates that it would predicted to be localized within the cytosol, nucleus and chloroplasts.

The regulatory β-subunits of SnRK1 are composed of three domains: an N-terminal variable region (NVR), a carbohydrate-binding domain and a C-terminal domain (CTD)
The NVR domain in AKINβ1 covers amino acids 1-100 of the N-terminus (López-Paz et al., 2009). AKINβ1 co-localized with Golgi (Figure 3.16 E) and areas surrounding the chloroplasts (Figure 3.14 C). Based on the bioinformatic analysis, a truncated AKINβ1 lacking a predicted chloroplast transit peptide (74 amino acids) was used to investigate its location further, and was found distributed in the cytosol (Figure 3.14 E), suggesting that this region plays a role in the subcellular localization of AKINβ1.

Protein N-myristoylation is a lipid modification that occurs during both co-translational protein synthesis and post-translational protein modification, in which a 14-carbon saturated fatty acid (myristic acid) is added irreversibly to an N-terminal glycine of some proteins, after removal of the N-terminal methionine, in a reaction catalyzed by N-myristoyltransferases (NMTs) (Renna et al., 2013; Varland et al., 2015). Protein N-myristoylation is necessary to target proteins to the periphery of specific membrane compartments such as Golgi by the insertion of their N-myristoyl group into Golgi membranes (Banfield, 2011; Renna et al., 2013). It has previously been observed that the glycine at position 2 of both AKINβ1 and AKINβ2 subunits can be N-myristoylated (Pierre et al., 2007). Thus, the regulatory role of this putative transit peptide may reside more in the subcellular localization of AKINβ1 to the Golgi. The importance of N-myristoylation was studied by investigation of the location of an AKINβ1(G2A) mutant. Mutation resulted in loss of the ability to be N-myristoylated and thus the protein localised within the cytosol and also the nucleus, but was no longer observed in Golgi (Figure 3.18 B). These results support the hypothesis that N-myristoylation on glycine
at position 2 of the AKINβ1 subunit is responsible for its localisation with the Golgi stacks. Thus, N-myristoylation is involved in regulation of localization of AKINβ subunits and N-myristoylation of the AKINβ subunits negatively regulates SnRK1 activity (Pierre et al., 2007).

In support of this possibility, activity of SnRK1 increased by five fold in the NMT1-knockout Arabidopsis line compared to WT, which was measured using a peptide kinase (AMARA and SAMS) assay (Radchuk et al., 2006; Pierre et al., 2007). Further, activities of SnRK1 were decreased in Arabidopsis nmt1 lines which were complemented with human NMT1 or S. cerevisiae NMT (both under the control of Arabidopsis NMT1 promoter), when compared to the Arabidopsis nmt1 line (Pierre et al., 2007). AKINβ1 and AKINβ2 containing the G2A mutation were re-localized from the plasma membrane, in wild-type, to the nucleus for the AKINβ1 mutant and to the cytosol for the AKINβ2 mutant, respectively, in onion epidermal cells (Pierre et al., 2007). The results suggest that N-myristoylation of the AKINβ subunits negatively regulates SnRK1 activity, which would be caused by sequestering the complex at the plasma membrane (Pierre et al., 2007). Although N-myristoylation is irreversible, myristate is not sufficiently hydrophobic to allow stable anchorage to cellular membranes (Sonnenburg and Gordon, 2013). So most N-myristoylated proteins employ an additional attachment mode. For example, S-palmitoylation involves a reversible covalent attachment of palmitate (a 16-carbon saturated fatty acid) to cysteine and can be used as a supplementary membrane tether (Sonnenburg and Gordon, 2013). Positively charged, exposed, amino acid residues can interact electrostatically with
negatively charged groups in membrane lipids. The myristoyl moiety on some N-
myristoylated proteins can be reversibly exposed to regulate their association with
membranes (Sonnenburg and Gordon, 2013). However, no putative palmitoylation site
on AKINβ1 is predicted using CSS-Palm (http://csspalm.biocuckoo.org/online.php).
Although the structure of AKINβ1 is not available at present, it may be that tertiary
folding facilitates N-myristoylation-based membrane association. Thus, it is possible
that membrane association of N-myristoylated AKINβ1 could be regulated reversibly
by conformational change of the subunit protein \textit{in vivo}, although this is speculative.
Moreover, AKIN10 and AKIN11 co-localized within chloroplasts, the nucleus and
cytosol (Fragoso et al., 2009; Bayer et al., 2012; Tsai and Gazzarrini, 2012; Nietzsche
et al., 2014; Williams et al., 2014 and present study) but do not localize within Golgi
(AKIN10, Williams et al., 2014, AKIN11, present study). AKINβ1, as an important
regulatory subunit of SnRK1, was found in areas surrounding the chloroplasts as well
as Golgi (Figure 3.14 C, Figure 3.20 C).

These results indicate that AKIN10/11 and AKINβ1 do not share the same localization
in leaf cells, which raises a conundrum in that AKINα subunits and AKINβ1 would not
have chance to form a functional SnRK1 complex \textit{in vivo}. However, the localization of
AKINα subunits within the nucleus and cytosol (Fragoso et al., 2009; Tsai and
Gazzarrini, 2012; Nietzsche et al., 2014; Williams et al., 2014) is consistent with the
location of the AKINβ1(G2A) mutant (Figure 3.18 A). AKINβγ has been shown to be
indispensable for the formation of functional SnRK1 heterotrimers in Arabidopsis
(Gissot et al., 2006) but lacks a putative N-myristoylation site based on NMT-The
Predictor (http://mendel.imp.ac.at/myristate/SUPLpredictor.htm). AKINβγ from maize was detected in both the nucleus and cytosol in transfected onion epidermal cells (López-Paz et al., 2009) and AKINβγ is also apparently located inside and outside chloroplasts in Arabidopsis (Avila-Castañeda et al., 2014). Based on these results, non-N-myristoylated AKINβ1 could localize with the nucleus and cytosol and bind to AKIN10/11 and AKINβγ to form a functional SnRK1, whereas N-myristoylated AKINβ1 would re-localize to the membranes of Golgi stacks, thus preventing formation of SnRK1.

The tobacco plants studied here were grown under light after the infiltration, which shows that N-myristoylation on AKINβ1 occurs under illumination at least. Moreover, gene expression of N-myristoyltransferase 1 was down-regulated in response to light (Cheng et al., 2013). A hypothesis could be raised that in the light, AKINβ1 is N-myristoylated and binds to Golgi stacks, which decreases the formation of functional SnRK1 and SnRK1 activity. In darkness, N-myristoylated AKINβ1 would re-localize to cytosol due to putative change in its conformation. This would facilitate binding of AKINβ1 to AKIN10/11 and AKINβγ to form functional SnRK1 and then direct the kinase to the nucleus or stay in cytosol, and regulate gene expression and protein activity in the nucleus and cytosol. This hypothesis is consistent with the observation that lack of light (darkness) stimulates leaf SnRK1 activity (Baena-Gonzalez et al., 2007; Pokhilko and Ebenhöh, 2015) although a detailed diurnal analysis of SnRK1 activity is lacking for leaves. The relationship between illumination and N-
myristoylation of AKINβ subunits would therefore be an area for further investigation to test this hypothesis.

The observation that AKINβ1 was localized within areas surrounding the chloroplasts in tobacco leaves and Arabidopsis leaves (Figure 3.14 C, Figure 3.20 C) indicates that not all AKINβ1 was retained in the Golgi. Although N-myristoylation is mainly a co-translational modification (Varland et al., 2015), similar effects have been observed for Arabidopsis subgroup 2 h-type thioredoxins (Trxh2, Trxh7 and Trxh8). These can be N-myristoylated to localize them mainly with ER/Golgi, or else are localized in the cytosol (using the onion epidermal cell system). Their partitioning between the membrane compartment and the cytosol has been shown to be correlated with the catalytic efficiency of the N-myristoyltransferase 1 acting at the N terminus of these subgroup 2 h-type thioredoxins (Traverso et al., 2013), which suggests that similar NMT-dependent cellular compartment partitioning of AKINβ1 could exist (Figure 3.21). Thus, it is possible that AKINβ1 could be located in areas surrounding the chloroplasts and could bind to the other AKIN subunits to form functional SnRK1 in the light. The subcellular compartment of AKINβ1 therefore provides a possible mechanism to understand the effects of down-regulation of akinβ1 during the light and in darkness. Based on expression analysis (Figure 2.3 C), it might be expected that SnRK1 activity would be higher in the light, whereas measurement of SnRK1 activity indicates that opposite is true i.e. it is more active in darkness (Baena-Gonzalez et al., 2007; Pokhilko and Ebenhöh, 2015). A change in localization of AKINβ1 protein between the Golgi and cytosol may be affected differentially between light and
darkness *i.e.* if a lower proportion of AKINβ1 is sequestered to the Golgi in darkness, the ability to form an active SnRK1 complex would be increased. It is not clear whether the catalytic efficiency of NMT 1 acting at the N terminus of AKINβ1 in both WT and the *akinβ1* mutant would be the same in light and darkness, although gene expression profiles of transient *AKIN10* expression in protoplasts showed that AKIN10 had no effect on gene expression of either NMT 1 (AT5G57020) or 2 (AT2G44170) in Arabidopsis (Baena-González et al., 2007). As indicated earlier, it is not clear what other mechanisms may influence localization of AKINβ1 into Golgi.

SnRK1 positively regulates contents of TCA cycle intermediates (see Chapter II; Radchuk *et al*., 2010). Decrease in the contents of these organic acids in the TCA cycle and respiration in the *akinβ1* mutant (Figure 2.8 and Figure 2.10) could be caused by a decrease in SnRK1 activity linked to the relative decrease in cytosol-localized AKINβ1 subunit in the mutant. The relative impact of the mutation on TCA cycle intermediates is, in most cases, more pronounced in light compared to darkness (Figure 2.8). This may mean that the proportional change in SnRK1 activity in the mutant compared to the WT is greater during the period of illumination compared to darkness. In other words, there is a proportionally greater reduction in cytosol-localized AKINβ1 in the mutant in the light than in darkness. While this is speculative, the model provides scope for further investigation.
Figure 3.2 Proposed model of NMT-dependent subcellular compartmentation of AKINβ1 in Arabidopsis leaves. In the light (A), AKINβ1 can be N-myristoylated and is sequestered to the Golgi, which prevents the formation of functional SnRK1. A portion of AKINβ1 (N-myristoylation status unclear) is localized in the cytosol, which facilitates the formation of functional SnRK1. The hypothesis is that AKINβ1 localization regulates SnRK1 activity. In the light (A), N-myristoylated AKINβ1 is partially localized in Golgi. In darkness (B), a greater proportion of N-myristoylated AKINβ1 would re-localize to cytosol by some unknown mechanism (putative change in AKINβ1 conformation). AKINβ1 subunits in the cytosol can facilitate the formation of functional SnRK1.
PP2C74, which is a 2C-type protein phosphatase (PP2C) in Arabidopsis, has been shown to interact with AKIN10 using yeast two-hybrid analysis and GST pull-down analysis (Tsugama et al., 2012), suggesting the phosphatase is a potential upstream regulator of SnRK1. This phosphatase has glycine at position 2, suggesting a putative N-myristoylation site, and thus its subcellular localization could also be regulated by N-myristoylation (Pierre et al., 2007; Tsugama et al., 2012). These results show that N-myristoylation could play an important role in regulating SnRK1 activity.

AKINα and AKINβγ subunits play vital roles in plant growth and development (Baena-Gonzalez et al., 2007; Gissot et al., 2006; Gao et al., 2016). The akin10/akin11 double mutation is lethal and the isolation of homozygous AKINβγ knockout plants has also been unsuccessful (Baena-Gonzalez et al., 2007; Ramon et al., 2013; Gao et al., 2016). Based on the proposed role of AKINβ1 in regulating the subcellular localization and activity of the SnRK1 complex, and its identified role in the regulation of nitrogen metabolism and sugar signaling in Arabidopsis (Li et al., 2009), AKINβ1 would also play an essential role in plant growth and development. This may explain why only Arabidopsis knock-down AKINβ1 mutants have been identified, since knock-out mutants may also prove lethal.

In this study, the subcellular locations of AKINα1, AKINβ1 and AKINγ1 have been identified by transient expression in tobacco leaves and stable expression in Arabidopsis leaves. Further, a possible regulatory role of N-myristoylation in Golgi-localization of AKINβ1 has been identified. Identification of putative interacting-
proteins of SnRK1 subunits has the potential to facilitate an understanding of their role(s) in the regulation of metabolism in plant leaves. In the next chapter, recombinant proteins (AKINα1 and AKINβ1) were used in an attempt to investigate such interactions biochemically.
CHAPTER IV

EXPRESSION OF RECOMBINANT AKINα1, AKINβ1 AND AKINγ1 SUBUNITS OF SnRK1 IN E.COLI
4.1 INTRODUCTION

Work described in the previous chapters suggested that SnRK1 may play a novel role in regulating carbon partitioning between starch metabolism, sucrose metabolism and the TCA cycle in Arabidopsis leaves. The subcellular localization of the SnRK1 subunits such as the plastid (AKINα1) and Golgi (AKINβ1) suggest their potential to interact with proteins in different compartments. Identification of putative binding-proteins of SnRK1 subunits should be an important step to understand their role in the regulation of metabolism in plant leaves.

Structural and functional studies of eukaryotic protein kinases in vitro are often restricted by the availability of the pure, catalytically active protein (Sonkoly et al., 2011). Recombinant protein expression is a very powerful and useful tool that overcomes this problem. Two kinds of recombinant protein expression systems have been established: in vitro protein expression (cell-free protein expression) and in vivo protein expression. Cell-free protein expression is a powerful technique that can rapidly express small amounts of recombinant proteins by employing the translation machinery in cell extracts from E.coli, wheat germ, insects and mammalian cells with supplemented amino acids, RNA polymerase, salts and nucleoside triphosphates (Brödel and Kubick, 2014). The in vivo protein expression systems are classified into two groups: prokaryotic protein expression systems and eukaryotic protein expression systems. In the former, eukaryotic cells from yeast, insect and mammalian cells are used to express the recombinant proteins of interest. In the prokaryotic protein expression system, E. coli is most often used as a host for recombinant protein
expression. Although many advantages of cell-free protein expression - such as it being simple, fast and convenient - have been shown, this technique is expensive, is not always reproducible, and unsuitable for large-scale recombinant protein expression (Brödel and Kubick, 2014). The eukaryotic protein expression systems have some advantages including availability of most protein post-translational modifications and protein folding machinery and increased expression level of soluble proteins. However, these systems also suffer some disadvantages such as being expensive, time-consuming, sometimes providing relative low yields of protein and non-typical characteristic N-linked glycan structures of expressed proteins. Therefore, in the present study, the widely used *E. coli*-based prokaryotic protein expression system was employed to express recombinant proteins. This system is well-established, relatively inexpensive, easy to scale up and easy to achieve with simple equipment. Moreover, the expression system offers speed and high levels of protein expression due to the simple and well-characterized genetics of *E. coli*. In addition, some strategies have been developed to overcome the disadvantages of bacterial systems for expressing active eukaryotic proteins such as *E. coli* host strain modification, lower-temperature protein expression and co-expression of molecular chaperones (Rosano and Ceccarelli, 2014).

Before setting up an *E. coli*-based prokaryotic protein expression system, several factors should be considered: *E. coli* host strain, a protein expression plasmid with tags that would enhance the solubility of the recombinant protein and facilitate its purification, and strategies for tag removal. There are many modified *E. coli* host strains available for protein expression and all of these hosts have their own advantages and
disadvantages. Generally, BL21 (DE3) from the B lines or derivatives of K-12 lineage strains are used for initial expression screening (Studier and Moffatt, 1986; Joseph et al., 2015). In this study, an E. coli strain from the B lines, the ArcticExpress (DE3) strain (Agilent Technologies) which holds a chromosomally integrated prophage (λDE3) encoding for the T7 RNA polymerase under the transcriptional control of the lacUV5 promoter, was used (Studier and Moffatt, 1986). IPTG-inducible expression of T7 RNA polymerase leads to expression of the T7 promoter-driven protein. The strain has been engineered to express the cold-adapted chaperonin Cpn60 and co-chaperonin Cpn10 from the psychrophilic bacterium, Oleispira antarctica (Ferrer et al., 2004), which assist correct protein folding by binding to unfolded or partially folded proteins, thus preventing or reducing protein mis-folding and aggregation. Both chaperonins display high protein refolding activities at temperatures of 4–12°C and also enhance the ability for the cells to grow at lower temperatures (Ferrer et al., 2003), which results in decreased rates of protein production and decreased cellular protein concentration, thus facilitating correct recombinant protein folding and increasing the amount of available soluble protein (Rosano and Ceccarelli, 2014).

Various peptides and proteins used as tags in expression plasmids have been developed to facilitate expression and purification of recombinant proteins. Several peptide tags and/or protein tags can be expressed in tandem with the protein of interest to form a fusion protein, facilitating improved solubility and detection of the target protein during the process of protein expression and purification (Nilsson et al., 1997). Small peptide tags such as Arg-tag (5 or 6 arginines), His-tag (6 histidines), S-tag (an N-terminal 15-
amino-acid peptide of bovine ribonuclease A), FLAG-tag (a hydrophilic 8-amino-acid peptide, DYKDDDDK), HA-tag (YPYDVPDYA) and Strep-tag (a synthetic 8-amino-acid peptide, WSHPQFEK) are commonly used for recombinant protein detection and purification since commercial antibodies and specific resins/gels/column are available for purification of all of these small tags. Protein tags such as the maltose-binding protein (MBP), N-utilization substance protein A (NusA), glutathione S-transferase (GST) and thioredoxin A (TrxA) are utilized to enhance solubility of recombinant proteins but the mechanisms by which they do so remain unknown (Raran-Kurussi and Waugh, 2012; Rosano and Ceccarelli, 2014). By comparison, NusA, MBP and TrxA show stronger ability to increase the solubility of fusion proteins, whereas GST displays the weakest (Hammarstrom et al., 2006; Bird, 2011; Costa et al., 2013). These tags should be fused with the solvent-accessible end of a protein of interest to prevent misfolding of the target protein. The plasmid, pET32a, with the TrxA tag under control of the strong bacteriophage T7 transcription and translation signals was employed in this study after attempting to use several pET plasmids for expression of the soluble subunit proteins unsuccessfully. After the protein of interest is expressed successfully, protein purification is the next step to be considered. Protein purification is a set of physical and chemical processes isolating proteins of interest from a complex mixture of cells, tissues or whole organisms. Protein purification methods which include: liquid chromatography methods, antibody protein precipitation, gel electrophoresis, and centrifugation, filtration and other fractionation techniques. In this study, common affinity chromatography methods were used to purify the target recombinant proteins.
by utilizing affinity tags (Hexa-His tag and chitin-binding domain). Before proceeding
to protein structural or biochemical studies, ideally these tags should be removed since
they could affect the catalytic activity and structure of proteins of interest. They can be
removed by two common methods: enzymatic cleavage or chemical cleavage (Rosano
and Ceccarelli, 2014). Enterokinase, thrombin, factor Xa and the tobacco etch virus
(TEV) protease are common proteases used for tag removal by cleaving protease
cleavage sites within the expression plasmids. Cyanogen bromide (CNBr) is the most
common chemical cleavage reagent that hydrolyzes peptide bonds at the C-terminal of
methionine residues, which requires there is no methionine residue in proteins of
interest except for the methionine residue encoded by start codon (ATG) (Rais-
Beghdadi et al., 1998). In this study, the novel intein-tag fused with a chitin binding
domain (CBD) from pTYB21, which is an E. coli cloning vector designed for
recombinant protein expression and purification (NEB, Ipswich, USA), was used for
tag removal. The intein protein encoded within the yeast VMA1 gene has an inducible
self-splicing capability as a result of the thioester bond located at its N-terminal peptide.
Cleavage can be induced by thiol reagents such as 1,4-dithiothreitol (DTT), β-
mercaptoethanol or cysteine (Chong et al., 1997), which are convenient, easy and have
high specificity.

A variety of protein-protein interaction assays have been developed and used to identify
and characterize interactions between two or more proteins. An in vitro protein pull-
down assay in combination with protein sequencing techniques is, in principle, a simple
way to identify putative binding protein(s) for target proteins. Using immobilized
protein pull-down assays, AKIN10/AKIN11-binding proteins such as IDD8, PP2C74 and FUS3 have been identified (Tsai and Gazzarrini, 2012b; Tsugama et al., 2012; Jeong et al., 2015). Moreover, Adi3 has been identified as a tomato SlSnRK1-binding protein by co-immunoprecipitation (Avila et al., 2012).

In this study, an E. coli-based protein expression system was established to express soluble fusion proteins of AKINα1, AKINβ1, a truncated AKINβ1 (lacking the putative plastid transit peptide) and AKINγ1 and attempts made to detect their activities. A chitin-binding domain-based protein pull-down assay was utilized to identify putative interacting protein(s) of AKINβ1 as well as AKINα1 in order to reveal more information about their function in regulating metabolism in Arabidopsis leaves.

4.2 MATERIAL AND METHODS

4.2.1 Cloning AKINβ1 cDNA of SnRK1 into pET32a

The full-length cDNA sequence of AKINβ1 was amplified by using the primers (AKINβ1-pET32a F: 5’AAGGCCATGGCTGATATGGGAAATGCAGGACGGC 3’ and AKINβ1-pET32a R: 5’GAATTCGGATCCGATCCGTGTGAGCGGTTTGTA 3’) from the plasmid pUC19 with AKINβ1-RFP and cloned in protein expression plasmid pET32a (Novagen, Billerica, USA) digested by EcoRV (Thermo Scientific, Waltham, USA) with CloneEZ® PCR Cloning Kit (GenScript, Piscataway, USA). Before the plasmids were sequenced, the plasmids were confirmed by digestion with NcoI/EcoRI restriction enzymes (Thermo Scientific, Waltham, USA).
4.2.2 Expression of AKINβ1 in *E. coli* ArcticExpress (DE3) cells

1μl of the expression plasmid pET32a with AKINβ1 cDNA was transformed into 50μl of *E. coli* ArcticExpress (DE3) competent cells (Agilent Technologies, Santa Clara, USA) by heat shock. One colony of ArcticExpress (DE3) cells was used to inoculate 5ml LB broth containing 20μg/ml of gentamycin and 100μg/ml ampicillin. The culture was incubated at 37°C with shaking at 250rpm overnight. Next morning, 2ml of the culture were sub-cultured in 200-ml LB broth without antibiotic selection in a 500-ml flask and incubated at 30°C with shaking at 250rpm for about 3.5 hours (OD<sub>600</sub>=0.6). 5ml of the culture was transferred into a clean 15-ml cell culture tube as the non-induced control samples. The two cultures were transferred to 10°C and incubated with shaking at 250rpm for about 10 min to cool the cultures to 10°C. 1M isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the 195-ml culture to a final concentration of 1mM and the two cultures were incubated at 10°C with shaking at 250rpm for 24 hours. Following incubation, the cultures were pelleted by centrifugation at 3000g for 10 min at 4°C, frozen rapidly in liquid nitrogen and stored at -80°C for further use.

The *E. coli* cell pellets were allowed to thaw on ice and resuspended in room-temperature BugBuster Protein Extraction Reagent (Novagen, Billerica, USA) by using 5ml of the reagent for each 1g of wet cell paste, with addition of 50μl protease inhibitor cocktail (Sigma-Aldrich, St. Louis, USA), 5μl Benzonase Nuclease (Novagen, Billerica, USA), and 5KU rLysozyme solutions (Novagen, Billerica, USA). The mixtures were then incubated on a rotating mixer for 30 min at room temperature. The soluble and
insoluble fractions of the cell lysates were separated by centrifugation at 16000g for 20 min at 4°C, and frozen in liquid nitrogen and stored at -80°C until used (Figure 4.1 A).

**Figure 4.1** Schematic representation of the structures of the recombinant proteins expressed in this study. (A) The recombinant AKINβ1 protein expressed using pET32a. There was an enterokinase cleavage site between the tags and AKINβ1 proteins. (B) The recombinant AKINβ1 protein expressed using pET32a with intein-CBD domain. An intein cleavage site was generated in front of the enterokinase cleavage site between the tags and AKINβ1 proteins. (C) The recombinant subunit proteins expressed using pET32a with intein-CBD domain. The enterokinase cleavage site was removed. The subunit proteins are fused with three tags from the vector including a TrxA protein tag (109 amino acids, 11.99kDa), a histidine tag (6 amino acids, 0.84kDa), and an S tag (15 amino acids, 1.88kDa), and two Ala residues at the N-terminus of the target proteins.

### 4.2.3 Recombinant protein purification

1ml of a 50% (v/v) slurry of Ni-NTA resin was loaded onto a 10-ml Poly-Prep chromatography column (BioRad, Berkeley, USA) and washed with 5ml of Equilibration Buffer (50mM NaH₂PO₄ (pH8.0), 300mM NaCl). After the buffer was eluted, about 4mg of the recombinant AKINβ1 in 3mL the BugBuster Protein Extraction Reagent mixture (Section 4.2.2) was added onto the column and sealed with a yellow cap. After 40-min incubation with rotation at 4°C, the crude protein sample solution was decanted and stored at -80°C for further use. The wet resin with bound recombinant AKINβ1 protein was allowed to settle and washed immediately with 15ml
Washing Buffer (50mM NaH$_2$PO$_4$ (pH8.0), 300mM NaCl, 20mM imidazole). The recombinant protein was eluted with 1.5 ml of Elution Buffer (50mM NaH$_2$PO$_4$(pH8.0), 300mM NaCl, 250mM imidazole) and the eluate collected. The elution process was repeated twice more and eluate from each kept separately. Eluates were stored at -80°C for future use.

The recombinant AKINβ1 fusion protein was subjected to gel permeation chromatography (GPC), using a Superdex 200 10/300GL column (Amersham Pharmacia Biotech, Little Chalfont, UK), in order to determine the oligomeric/monomeric state of the protein.

The column, connected to an AKTA FPLC (Amersham Biosciences, Little Chalfont, UK), was calibrated using low molecular weight and high molecular gel filtration kits (Amersham Biosciences, Little Chalfont, UK). Standard proteins used were aprotinin (6.5 kDa), ribonuclease A (13.7 kDa), ovalbumin (44kDa), conalbumin (75kDa) from the low molecular weight kit and aldolase (158 kDa) and ferritin (440 kDa) from the high molecular weight kit. The calibration proteins were run in two separate groups to ensure clear resolution of their peaks for accurate elution volume measurements. Each group of proteins was loaded onto the column pre-equilibrated in 50mM Tris-HCl (pH7.9) with 1mM EDTA and 150mM NaCl, at a flow rate of 0.25ml/min. Proteins were eluted and detected by UV absorption at 280nm. The peak elution volume of each standard protein verses the common logarithm of its molecular weight was plotted and
a standard curve produced (Figure 4.2). The void volume of the column (7.55ml) was
determined using Blue Dextran 2000.

For isolation of the recombinant AKINβ1 fusion protein from *E. coli* lysates, the
extracts were thawed on ice and then centrifuged at 14000g for 20 min. 4ml of
supernatant was concentrated by centrifugation with an Amicon Ultra-4 centrifugal
filter unit with a membrane nominal molecular weight limit (NMWL) of 10kDa (EMD
Millipore, Billerica, USA) at 3000g for 30 min at 4°C. 0.5ml of the concentrated
supernatant (about 9.2mg/ml proteins) was loaded onto the Superdex 200 10/300GL
column pre-equilibrated with 50mM Tris-HCl (pH7.9), 1mM EDTA, 150mM NaCl and
the proteins eluted in the same buffer at a flow rate of 0.25ml/min, collecting
0.5ml/fraction.

![Figure 4.2](image)

**Figure 4.2** Calibration of Superdex 200 10/300GL GPC column. Log₁₀ molecular
weights of known proteins were plotted against their elution volume from the gel
permeation column.
4.2.4 SDS-PAGE, purification of polyclonal AKIN subunit antibodies and immunoblotting

Proteins were separated on 10% (v/v) SDS-PAGE gels (7.5ml resolving gel (3.8ml H$_2$O, 3.4ml 30% (v/v) acrylamide solution, 2.6ml 1.5M Tris pH8.8, 0.1ml 10% (w/v) SDS, 0.1ml 10% (w/v) APS and 0.01ml TEMED per 10ml gel solution) and 2.5ml stacking gel (7ml H$_2$O, 1.68ml 30% (v/v) acrylamide solution, 1.28ml 1.5M Tris pH8.8, 0.1ml 10% (w/v) SDS, 0.112ml 10% (w/v) APS and 0.008ml TEMED per 10ml gel solution); 7cm wide×8cm long×1.5mm thick) in 1×SDS Tris-glycine running buffer (25mM Tris-HCl (pH 8.0), 10% (w/v) SDS, 20mM EDTA, 5% (v/v) β-mercaptoethanol, 0.1% (w/v) bromophenol blue, 50% (v/v) glycerol) and boiled for 5 min. Gels were run at 120V and stopped when the loading dye reached the end of the resolving gel.

Arabidopsis polyclonal anti-AKINα1 and anti-AKINβ1 antibodies (the peptide of AKINα1 (310-326): NQVLESLRNRTQNDATV and the peptide of AKINβ1 (118-133): SWDNWRSRKKLQKSGK were used as antigens respectively; ANASPEC, Fremont, USA) were purified from crude rabbit antisera using prepared peptide affinity columns. To make a 1-ml peptide affinity column, 2mg of each respective synthetic peptide of AKINα1 or AKINβ1 shown above was dissolved in 1ml of 50mM Tris-HCl (pH 8.5) with 5mM EDTA. 2ml Pierce sulpho-link resin slurry (Thermo Fisher Scientific, Waltham, USA) was washed with 1ml Tris-HCl (pH 8.5) 6 times. The dissolved peptide was then added to the washed resin in a 1.5-ml tube and incubated on a rotator for 15 min at room temperature and for additional 30 min without rotating.
The peptide-resin mixture was then added to a Bio-Rad Poly-Prep chromatography column (Bio-Rad, Berkeley, USA) and washed with 3ml Tris-HCl (pH 8.5) and then blocked with 1ml of 50mM cysteine in the same washing buffer by incubating on a rotator for 15 min and for additional 30 min without rotating. The peptide-resin was washed with 16ml Tris-HCl (pH 8.5) with 1M NaCl and 16ml Tris-HCl (pH 8.5) with 0.05% Na Azide consecutively. 5ml antisera containing the polyclonal antibodies mixed with 3ml of PBS and 0.01% (w/v) sodium azide were applied to the column and mixed on a rotator at 4°C overnight. The next day, the column was washed with 10ml RIPA (50mM Tris-HCl (pH 7.5), 150mM NaCl, 1% (w/v) nonyl phenoxypolyethoxyl ethanol (NP-40), 0.5% (w/v) Na-deoxycholate, and 0.1% (w/v) SDS). The column was further washed with 10ml NETN-salt buffer (20mM Tris-HCl (pH 8.0), 1M NaCl, 1mM Na2-EDTA, and 0.5% (w/v) NP-40), followed by washing again with 10ml of 10mM Tris-HCl (pH 7.8). The antibody bound to the column was eluted with 0.5ml of 100mM glycine (pH 2.5) into a tube containing 0.5ml 1M Tris-HCl (pH 7.8) and the protein content measured. The column was neutralized by adding 10ml of 10mM Tris-HCl (pH 7.8) with 0.05% (w/v) sodium azide and stored at 4°C.

For immunoblotting, gels were transblotted onto nitrocellulose membranes (BioRad, Berkeley, USA) using transfer buffer (20% (v/v) methanol in 1×tris-glycine running buffer) in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Berkeley, USA) at 30V for 90 min. Transblotted membranes were incubated in blocking solution (1.5% BSA in 1×TBS (50mM Tris-Cl (pH 7.5), 150mM NaCl) for 15 min at room temperature with shaking. Purified antibody (rabbit anti-AKINα1 antibody or anti-AKINβ1
antibody or mouse anti-S tag antibody (Sigma-Aldrich, St. Louis, USA)) was used at 1:1500 dilution in blocking solution (1.5% BSA in 1xTBS). Alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse IgG (Sigma-Aldrich, Billerica, USA) was used as a secondary antibody. Blots were developed in 5-bromo-4-chloro-3-indolylphosphate (BCIP)/Nitro-Blue Tetrazolium (NBT) liquid substrate system (Sigma-Aldrich, Billerica, USA) until protein bands become visible.

4.2.5 Cleavage of recombinant protein fusion tags by enterokinase

To remove the tags on the recombinant fusion protein of AKINβ1, 2μl (1unit/μl) of recombinant enterokinase (Invitrogen, Waltham, USA) was used to remove the tags that were expressed by pET32a by cleaving the enterokinase recognition site in the recombinant protein that was purified by GPC (Fraction B15) at 20ºC for 16 hours and 24 hours respectively. The effectiveness of the cleavage was determined by western blotting as described in Section 4.2.4 using the rabbit anti-AKINβ1 antibody.

4.2.6 Cloning intein-CBD cDNA into pET32a with AKINβ1 cDNA

The intein-CBD cDNA was amplified from pTYB21 (New England BioLabs, Ipswich, USA) using the primers (intein-CBD pET32a F: 5’ACAGCCCAGATCTGGGTATGAAAATCGAAGAAGGTA3’ and intein-CBD pET32a R: 5’TTGTCGTCGTCGTCGGCGTTCTGTACAACCTG3’). The intein-CBD domain cDNA was cloned into the pET32a with AKINβ1 cDNA digested by KpnI (Thermo Scientific, Waltham, USA) by employing CloneEZ® PCR Cloning Kit (GenScript, Piscataway, USA). Positive plasmids were identified by digestion with XbaI/EcoRI (Thermo Scientific, Waltham, USA) and were sequenced.
4.2.7 Expression of AKINβ1 fusion protein in the modified pET32a in *E. coli* ArcticExpress (DE3) strain

The process of fusion protein expression of AKINβ1 possessing intein-CBD in the modified pET32a plasmid in *E. coli* ArcticExpress (DE3) cells was followed as described in Section 4.2.2 (Figure 4.1 B).

4.2.8 Purification of AKINβ1 fusion protein and removal of cleavage tags by DTT

His-tagged AKINβ1 fusion protein was purified on Ni-NTA beads as described in Section 4.2.3. 75μl of 1M DTT solution was added into 1.5ml of the second and third imidazole-eluted fraction respectively to a final concentration of 50mM. The samples were incubated with rotation at 4°C for 40 hours. 30μl of the DTT-treated samples were analysed on western blots to determine whether cleavage of the intein domain was successful.

4.2.9 Cloning *AKINα1, AKINβ1, AKINβ1* lacking the predicted transit peptide and *AKINγ1* genes of SnRK1 into modified pET32a

The full-length cDNA sequence of *AKINα1* cDNA was amplified using the primers (AKINα1 for M32a F: 5’GTTGTACAGAACGCCGCCATGGATCATTCATCAAAT 3’ and AKINα1 for M32a R: 5’ACGGGAGCTCGAATTCGGATCCTCAGATCAGCGAAGCTC 3’) from the plasmid pUC19 containing *AKINα1-YFP* (Section 3.2.4). It was cloned in the modified plasmid pET32a (Section 4.2.6) digested by Ncol/BamHI (Thermo Scientific, Waltham, USA), which released the AKINβ1 cDNA, by employing CloneEZ® PCR Cloning Kit (GenScript, Piscataway, USA). Before the plasmids were sequenced, putative positive plasmids (8987bp) were identified by digestion with
BglII/BamHI (Thermo Scientific, Waltham, USA) and verified by digestion with SacI (Thermo Scientific, Waltham, USA).

A 9-amino acid peptide from the backbone of pET32a, lying between the intein-CBD and AKINβ1 subunit in the modified plasmid pET32a (Section 4.2.6), was removed to obtain an AKINβ1 protein lacking these extra amino acids following DTT-cleavage.

The AKINβ1 cDNA was re-cloned in the modified plasmid. The full-length cDNA sequence of AKINβ1 was amplified by using the primers (AKINβ1 for M32a F: 5’ GT TGTACAGAAGCGCCCATGGGAAATGCGAACGGC 3’ and AKINβ1 for M32a R: 5’ ACGGAGCTCGAATTCGGATCCTCACCGTGTGAGCGGTTTGTA3’) from the plasmid pUC19 containing AKINβ1-RFP as template (Section 3.2.4), and cloned in the modified plasmid pET32a digested by NcoI/BamHI (Thermo Scientific, Waltham, USA) by employing CloneEZ® PCR Cloning Kit (GenScript, Piscataway, USA).

Before the plasmids were sequenced, putative positive plasmids (8300bp) were identified by digestion with XbaI/BamHI (Thermo Scientific, Waltham, USA) and double-checked by digestion with BglII (Thermo Scientific, Waltham, USA).

The sequence of AKINβ1 cDNA, without the predicted chloroplast transit peptide, was amplified by using the primers (AKINβ1T for M32a F: 5’ GTTGTACAGAACGCAGCCG CCGACACCGCCTCCTCCTCT 3’ and AKINβ1 for M32a R: 5’ ACGGAGCTCGA ATTCGGATCCTCACCGTGTGAGCGGTTTGTA 3’) from the plasmid pUC19 containing AKINβ1-RFP as template and cloned in the modified plasmid pET32a digested by NcoI/BamHI (Thermo Scientific, Waltham, USA) by employing
CloneEZ® PCR Cloning Kit (GenScript, Piscataway, USA). Before the plasmids were sequenced, putative positive plasmids (8081bp) were identified by digestion with XbaI/BamHI (Thermo Scientific, Waltham, USA) and verified by digestion with BglII/BamHI (Thermo Scientific, Waltham, USA).

The full-length cDNA sequence of AKINγ1 was amplified by using the primers (AKINγ1 for M32a F: 5’ GTTGTACAGAACGCCGCCATGGCGACTGTTCCGGAG 3’ and AKINγ1 for M32a R: 5’ACGGAGCTCGAATTCGGATCCTCAGACTCGGTAGTTTTC 3’) from the plasmid pUC19 containing AKINγ1-GFP as template (Section 3.2.4) and cloned in the modified plasmid pET32a digested by NcoI/BamHI (Thermo Scientific, Waltham, USA) by using CloneEZ® PCR Cloning Kit (GenScript, Piscataway, USA). Before the plasmids were sequenced, putative positive plasmids (8723bp) were identified by digestion with XbaI/BamHI (Thermo Scientific, Waltham, USA).

4.2.10 Protein expression and purification of subunit fusion proteins

Expression of these subunit fusion proteins (Section 4.2.9) in E. coli ArcticExpress (DE3) cells was followed as described in Section 4.2.2 (Figure 4.1 C). Protein purification by Ni-NTA beads was as described in Section 4.2.3.

4.2.11 Removal of tags from the recombinant subunit proteins by DTT

75μl of 1M DTT solution was added into the third imidazole-eluted fraction of AKINβ1 (Section 4.2.10) to a final concentration of 50mM. The samples were incubated with rotation at 4°C for 24 hours and 72 hours respectively. For the recombinant proteins
AKINα1 and the truncated AKINβ1 (lacking putative plastid transit peptide), DTT was used to remove the tags at a final concentration of 50mM and the samples were incubated with rotation at 4°C for 72 hours. 40μl of the DTT-treated samples were used for western blot analysis to determine whether cleavage was successful based on molecular mass before and after treatment with DTT. Due to the lack of a peptide-specific antibody for AKINγ1, the fusion protein was not treated with DTT for tag cleavage. All samples were stored at -80°C for future use.

To confirm that the proteins released from their fusion proteins after the DTT incubation were AKINα1, AKINβ1 and AKINβ1T respectively, the above DTT-treated protein samples stored at -80°C were loaded on an SDS-PAGE gel. After electrophoresis, the gel was divided into halves (each of three DTT-treated protein samples and protein marker on each half gel). One half of the gel was used to perform western blotting using anti-AKINα1 antibody or anti-AKINβ1 antibody respectively, and the other half gel was subjected to silver staining. Following western-blotting, the corresponding bands were identified on the silver-stained gel, cut from the gel and sent to the Mass Spectrometry Facility (Advanced Analysis Centre, University of Guelph) for trypsin digestion and mass spectrometric (MS) analysis.

4.2.12 Arabidopsis chloroplast isolation

Arabidopsis chloroplasts were isolated using the method of Schulz et al., (2004) with modification. Approximately 40g of four-week-old fresh Arabidopsis rosette leaves were harvested and homogenized with a KINEMATICA Polytron in 400ml ice-cold homogenization buffer (50mM HEPES-KOH (pH 7.5), 2mM Na$_2$-EDTA, 1mM MnCl$_2$, 176
1mM MgCl₂, 330mM sorbitol, 1mM DTT, 0.25% (w/v) bovine serum albumin (BSA)) at 10-second intervals. The cell homogenates were filtered through two layers of miracloth (CalBiochem, San Diego, USA) into a 500ml ice-cold glass beaker and transferred into four 50-ml centrifuge tubes and centrifuged for 8 min at 1000g at 4°C in the SX4250 rotor using a Beckman Coulter Allegra X-22R Centrifuge. The pellet was resuspended using an oil-painting brush in 8ml of fresh homogenization buffer, and 2ml layered on to each of four 15-ml Percoll step-gradients (8ml 40% (v/v) Percoll in homogenization buffer layered on the top of 7ml 85% (v/v) Percoll in homogenization buffer) in 50-ml centrifuge tubes. Samples were centrifuged at 3000g for 15 min at 4°C using a Beckman Coulter Allegra X-22R Centrifuge with slow deceleration. Intact chloroplasts were separated from broken chloroplasts and cell debris; intact chloroplasts were found at the interface between the layers of 40% Percoll and 85% Percoll, while the broken chloroplasts and the cell debris were suspended on top of the 40% Percoll solution. The intact chloroplasts were transferred to a chilled 50-ml tube containing 40ml ice-cold HEPES-sorbitol buffer (50mM HEPES-KOH (pH 7.5), 330mM sorbitol) using a Pasteur pipette. Chloroplasts were then centrifuged at 1000g for 8 min at 4°C. The supernatant was carefully discarded and the pellet containing intact chloroplasts was then lysed by resuspension in 3ml ice-cold rupturing buffer (RB) containing 100mM Tricine/KOH (pH 7.8), 1mM DTT, 5mM MgCl₂, and 10µl/ml protease inhibitor cocktail (GBioScience, St. Louis, USA). Samples were aliquoted and stored at -80°C for future use. Before further use, chloroplast lysates were
centrifuged at 13000g for 10 min to remove thylakoid and other membranes, and other insoluble material such as starch.

4.2.13 Leaf protein extraction

Approximately 30g of rosette leaves from four-week-old Arabidopsis plants were harvested, frozen in liquid nitrogen, and ground into fine powder using a pre-chilled mortar and pestle under frozen condition. The frozen powder was mixed with 100ml ice-cold buffer containing 50mM Tris-HCl (pH7.9) with 1mM EDTA, 150mM NaCl, and 10µl/ml protease inhibitor cocktail (GBioScience, St. Louis, USA) and further incubated on ice for 20 min followed by centrifugation at 14000g for 10 min at 4°C using a Beckman Coulter Microfuge 22R Centrifuge and the F241:5P rotor. The supernatant was transferred to 50-ml tubes and stored at -80°C for future use.

4.2.14 Detection of recombinant AKINα1 and AKINβ1 interactions with Arabidopsis leaf and chloroplast proteins respectively

The steps involved in the “pull-down” assay are summarized in Figure 4.3. 2mg protein from E. coli expressing AKINα1 or AKINβ1 (both with Trx-His-S-Intein CBD tags) and E. coli expressing only the tag (Trx-His-S-Intein CBD) were purified on 110µl Ni-NTA beads as described in Section 4.2.3. 10µl Ni-NTA beads bound with recombinant proteins AKINα1, AKINβ1 and the tag-only protein respectively were retained to detect whether each protein was bound to beads. The proteins on remaining 100µl Ni-NTA beads were eluted respectively for the following steps.

430ul chitin beads were washed with 10ml column buffer (20mM Na-HEPES (pH8.5), 500mM NaCl, 0.1% (v/v) Tween-20, and 1mM EDTA). Each of the three purified
proteins from the second and third imidazole-eluted fractions of the above Ni-NTA purification were incubated with 110µl chitin affinity beads (2mg of recombinant protein per ml of chitin resin (New England BioLabs, Ipswich, USA)) at 4ºC with rotating overnight respectively. 100µl chitin beads were incubated with the Ni-NTA elution buffer as control (empty beads). The chitin beads bound with purified AKINα1, AKINβ1 and the tag-only proteins as well as the above empty beads as control were washed with 20ml column buffer respectively. After incubation and washing, 10µl of the 110µl chitin beads bound with each fusion protein respectively were retained to detect whether each protein was bound to chitin beads.

Each 100µl of chitin beads were aliquoted into three fractions: 30µl beads as control; 35µl beads were incubated with 10ml Arabidopsis leaf proteins (2.7mg/ml) in buffer (50mM Tris-HCl (pH7.9), 150mM NaCl, 1mM EDTA, 5mM MgCl₂, 1mM ATP, and 10µl/ml protease inhibitor cocktail); 35µl beads were incubated with 3ml chloroplast stroma proteins (1.5mg/ml) in buffer (100mM Tricine/KOH (pH 7.8), 1mM EDTA, 5mM MgCl₂, 1mM ATP, and 10µl/ml protease inhibitor cocktail). Samples were incubated at 4ºC for 1 hour with rotating. Each sample of beads was washed with 10ml buffer containing 20mM Tris-HCl (pH8.0) and 0.1% (v/v) Tween-20, followed by 10ml of the same buffer containing 500mM NaCl. After washing, the slurries of beads were aliquoted into two equal-volume fractions respectively. One aliquot was dissolved in 70µl 2×loading sample dye for SDS gel electrophoresis and the other was incubated in 200µl “cleavage” buffer (20mM Na-HEPES (pH 8.5), 500mM NaCl, 0.1% (v/v) Tween-20, 1mM EDTA and 50mM DTT) at 4ºC for 48 hours with rotating to release
recombinant bait protein from the beads. The 100µl chitin beads (empty) used as a control were treated identically. The supernatants from the DTT-treated samples were transferred to 1.5-ml tubes respectively. All samples were stored at -80ºC for future use.

4.2.15 SDS-PAGE protein staining

10µL solution of beads in 70µl 2×loading sample dye and 20µl solution of beads treated with 50mM DTT were used to run SDS-PAGE gels. Each of the gels was fixed in 100ml 50% (v/v) methanol/5% (v/v) acetic acid solution for 20 min and washed in 100ml 50% (v/v) methanol solution for 10 min and 100ml ultrapure H₂O overnight with shaking. The gels were sensitized in 100ml 0.02% (w/v) sodium thiosulfate solution for 1 min and washed twice with 100ml ultrapure H₂O for 1 min consecutively. The gels were incubated with pre-cooled 100ml 0.1% (w/v) silver nitrate solution at 4ºC for 20 min and washed twice with 100ml ultrapure H₂O for 1 min, consecutively. The gels were developed with 0.04% (v/v) formalin in 2% (w/v) sodium carbonate solution until protein bands appeared, and the development process was stopped by incubating the gels with 5% (v/v) acetic acid for 5 min. Finally, the gels were washed twice in 100ml ultrapure H₂O for 30 min with shaking.

For Coomassie Blue staining, after electrophoresis, SDS-PAGE gels were stained in 100ml Coomassie Staining Solution (0.1% (w/v) Coomassie Brilliant Blue, 10% (v/v) acetic acid, 40% (v/v) methanol) overnight. Excess stain was removed from the gels by washing once with 100ml Destaining Solution (20% (v/v) methanol, 10% (v/v) acetic acid) overnight.
Protein bands identified as potentially interacting with recombinant bait proteins were excised and sent to Mass Spectrometry Facility (Advanced Analysis Centre, University of Guelph) for MS analysis.
**Figure 4.3** Flow chart of the pull-down assay used to detect interaction of AKINα1 and AKINβ1 with Arabidopsis leaf proteins and chloroplast proteins.
4.3 RESULTS

4.3.1 Cloning and protein expression of AKINβ1 cDNA

Due to the low protein content of regulatory enzymes, such as SnRK1 in Arabidopsis, it is unrealistic to attempt to isolate each endogenous subunit of this kinase from leaf material. Attempts were therefore made to express recombinant proteins of AKINα1, AKINβ1, the truncated AKINβ1 and AKINγ1 in *E. coli* strain ArcticExpress (DE3). Before proteins were expressed in plasmid pET32a, several protein expression pET vectors including pET29, pET41 and pET43.1 were used to try and express the subunits in the *E. coli* strain ArcticExpress (DE3), but these experiments were unsuccessful since only very low levels of soluble subunit proteins were detected (data not shown). By employing recombination-based cloning technology, the PCR product of *AKINβ1* gene (879bp) without a stop codon was cloned into plasmid pET32a (5900bp) linearized by *EcoRV* and a positive clone (6749bp) was confirmed by digestion with *NcoI* and *EcoRI*, which released two DNA bands of the expected size (5880bp and 869bp) (Figure 4.4 A).

Expressed soluble recombinant AKINβ1 from ArcticExpress (DE3) cell lysates was separated by 10% SDS-PAGE, followed by immunodetection using a rabbit polyclonal anti-AKINβ1 antibody. Western blot shows that there was a clear protein band on the gel, indicating the successful expression of recombinant AKINβ1 in ArcticExpress (DE3) cells. The position of the recombinant AKINβ1 on the gel was larger than its expected size (51.5kDa) (Figure 4.4 B). Purification of the recombinant AKINβ1 fusion
protein using Ni-NTA beads was performed. Fractions from each step of the purification process were separated by 10% SDS-PAGE, followed by Coomassie Blue staining (Figure 4.4C). The result of recombinant AKINβ1 purification shows that a significant level of the recombinant protein bound to the affinity resin was eluted, but also that a high amount of the recombinant protein was not bound by the beads (Figure 4.4 C Lane 2). Soluble extract from ArcticExpress (DE3) cells containing recombinant AKINβ1 protein was separated through a Superdex 200 10/300GL gel permeation column and the collected fractions analyzed by SDS-PAGE and immunoblotting, using the peptide-specific anti-AKINβ1 antibody. The elution peak was identified at Fraction B10 using Molecular Imager ChemiDoc XRS+ system (Bio-Rad, Berkeley, USA) corresponding to a molecular mass of approximately 160kDa (Figure 4.4 D, E). Simplistically, this could represent a trimer of recombinant AKINβ1 protein, although interpretation of GPC data is to be treated with caution. Protein having a monomeric mass (51.5kDa) would be expected in Fraction B15 and the recombinant protein in Fraction B15 was used to test the efficacy of enterokinase-based cleavage, but immunodetection indicated that after either 16-hour or 24-hour incubation, there was no significant cleavage product of the expected size (34.54kDa) (Figure 4.4 F).
Figure 4.4 cDNA cloning, expression and purification of the AKINβ1 subunit of SnRK1. (A) The two DNA segments, AKINβ1 PCR product (879bp, Lane 1) and the linearized pET32a (5900bp, Lane 2), were ligated by employing recombination-based cloning technology. The positive clone was digested by NcoI and EcoRI (Lane 3) and released two expected DNA bands (5880bp and 869bp). (B) Immunodetection using rabbit polyclonal anti-AKINβ1 antibody of the soluble AKINβ1 recombinant protein from ArcticExpress (DE3) cell lysates shows a clear protein band indicated by a red arrow. (C) AKINβ1 recombinant protein purification by metal affinity chromatography. Lanes 1-3 are, respectively, crude cell lysate, the flow-through of the cell lysate after incubation with beads (recombinant proteins unbound to the affinity beads), Ni-NTA beads after incubation (recombinant proteins bound to the beads). Lanes 4-5 are the wash fractions, Lanes 6-9 are the imidazole-eluted fractions. 30µl of each fraction was loaded on an SDS-PAGE gel. (D) Immunodetection of the recombinant protein fractions separated through a Superdex 200 10/300GL gel permeation column. (E) Densitometric scan of samples from “D” using Molecular Imager ChemiDoc XRS+ system. Elution peak indicated by the arrow. (F) Immunodetection following incubation of the enterokinase-treated recombinant protein at 20°C for 16 hours (Lane 1) and 24 hours (Lane 2). The bar indicates the expected size (34.5kDa) of AKINβ1 following enterokinase-based cleavage.
4.3.2 Cloning of intein-CBD cDNA into pET32a with AKINβ1 cDNA

Because cleavage of the tag from the recombinant AKINβ1 protein using enterokinase appeared to be unsuccessful, an intein-CBD tag that can be cleaved by DTT was cloned into the plasmid pET32a with the AKINβ1 gene digested by KpnI. The PCR product of the intein-CBD domain (1575bp, Figure 4.5 A) containing a 17bp overhang sequence on the N-terminal end and a 14bp overhang sequence on the C-terminal end of the plasmid pET32a was cloned into plasmid pET32a with the AKINβ1 cDNA (6749bp, Figure 4.5 A), which also generated two additional Ala residues (6bp) (to facilitate DTT-induced cleavage at the C-terminus of the intein-CBD domain). The plasmids in 10 transformed E. coli colonies were checked by digestion with XbaI and EcoRI to identify potential positive clones (8252bp). After digestion with XbaI and EcoRI, positive clones should release two DNA bands, 5288bp and 2964bp respectively, as shown for clones 7 and 10 (Figure 4.5 B).

Figure 4.5 Cloning of intein-CBD domain gene into plasmid pET32a with AKINβ1 subunit cDNA. (A) The PCR product of the intein-CBD domain gene (1575bp) contained a 17bp overhang sequence on the N-terminus and a 14bp overhang on the C-terminus of the plasmid for DNA ligation and incorporation of two extra Ala residues (6bp). The DNA band of the plasmid pET32a with AKINβ1 subunit digested by KpnI. (B) Identification of positive clones by digestion with XbaI and EcoRI. Clones 7 and 10 released two DNA bands of the expected size (5288bp and 2964bp).
4.3.3 Protein expression and purification of AKINβ1 in a modified pET32a plasmid and cleavage of tag by DTT

Soluble recombinant AKINβ1 protein fused with an intein-CBD tag (1575bp, 58.5kDa) from ArcticExpress (DE3) cell lysates was separated by 10% SDS-PAGE, followed by Coomassie Blue staining and immunodetection using the rabbit polyclonal anti-AKINβ1 antibody respectively. Both the Coomassie Blue staining and immunodetection results show that there was a clear protein band of the expected size (109kDa; Figure 4.1 B) on the gel, indicating successful expression (Figure 4.6 A).

Purification of recombinant AKINβ1 fusion protein using Ni-NTA beads was performed. Protein fractions from each step of the purification were subjected to 10% SDS-PAGE, followed by Coomassie Blue staining (Figure 4.6 B). The results show that a significant proportion of the recombinant protein that bound to the affinity resin was eluted from the beads (Figure 4.6 B). The following DTT-induced cleavage process requires long-incubation times (40h) (Chong et al., 1997) and there were non-specific proteins that would contain *E. coli* proteases in all imidazole-eluted fractions (Figure 4.6 B Lanes 3-5). In order to decrease the possibility of target protein degradation and obtain detectable cleaved protein bands, later imidazole-eluted fractions (Figure 4.6 B Lanes 4 and 5), containing fewer non-specific proteins and sufficient AKINβ1 fusion protein, were used for DTT-induced cleavage. 50mM DDT was used to cleave the recombinant protein in the imidazole-eluted fractions (Figure 4.6 B Lanes 4 and 5) from Ni-NTA bead purification. Western blotting indicated that there was a clear protein band on the gel after 40-hour cleavage with DTT, though a high proportion of
recombinant protein remained uncleaved (Figure 4.6 C). The position of the recombinant AKINβ1 on the gel was larger than its expected size. The calculated molecular weight of AKINβ1 plus 38 amino acids from the vector (2 Ala residues and 9 amino acids between the C-terminus of the intein-CBD and the N-terminus of AKINβ1, plus 27 amino acids between the C-terminus of AKINβ1 and the stop codon of multiple cloning site region of pET32a) is 35kDa, whereas the estimated size from western blot (Figure 4.6 C) was 43kDa.

**Figure 4.6** Protein expression and purification of AKINβ1 fused with intein-CBD, and tag removal by 50mM DTT. (A) 10% SDS-PAGE gel of protein extracted from ArcticExpress (DE3) cells. The results of Coomassie Blue staining (Lane 1) and immunodetection (Lane 2) indicate the recombinant protein (109kDa). The subunit proteins (35kDa) are fused with three tags from the vector including a TrxA protein tag (109 amino acids, 11.99kDa), two histidine tags (6 amino acids, 0.84kDa), and an S tag (15 amino acids, 1.88kDa), and the intein-CBD fragment (525 amino acids, 58.5kDa). (B) Coomassie Blue staining of fractions eluted from Ni-NTA beads. Lane 1, crude cell lysate; Lane 2, wash fraction; Lanes 3-5, three imidazole-eluted fractions. 30µl of each fraction was loaded on the SDS-PAGE gel. (C) Cleavage of purified recombinant protein from Lanes 4 and 5 in (B) respectively, by incubation in 50mM DTT at 4°C for 40 hours. A clear cleaved protein band (red arrow) was observed but a high proportion of recombinant protein was not cleaved during the process.

**4.3.4 Cloning AKINα1, AKINβ1, the truncated AKINβ1 and AKINγ1 genes into the modified pET32a vector**

To express soluble AKINα1, AKINβ1, AKINβ1 without the predicted chloroplast transit peptide and AKINγ1 protein for future use, the three full-length *AKINα1*,
AKINβ1 and AKINγ1 cDNAs and the truncated AKINβ1 cDNA were cloned into the modified pET32a vector. The PCR products of the full-length cDNAs of AKINα1 (1578bp), AKINβ1 (891bp), and AKINγ1 (1314bp) and the truncated AKINβ1 (669bp) (with overhang sequences from the modified pET32a) (Figure 4.7 A) were ligated with the modified pET32a linearized by NcoI and BamHI (7389bp, Figure 4.7 A). For the modified pET32a containing the AKINα1 sequence, six plasmid samples were used to identify positive clones which should release three DNA bands (5857bp, 2590bp and 540bp) following the digestion with BglII and BamHI. One positive plasmid was identified and further checked by digesting with SacI, showing the plasmid released products of the expected sizes (7751bp and 1236bp) (Figure 4.7 B and C). For AKINβ1, six plasmid samples were used to identify positive clones which should release two DNA bands (5381bp and 2919bp) following digestion with XbaI and BamHI. Three positive plasmids were identified and further checked with BglII, and the plasmids released DNA bands with the expected sizes (6643bp, 1126bp and 540bp) (Figure 4.7 B and C). For AKINβ1T (lacking the predicted chloroplast transit peptide), six plasmid samples were used to identify positive clones which should release two DNA bands (5381bp and 2700bp) following digestion with XbaI and BamHI. Three positive plasmids were identified and checked with BglII and BamHI, showing DNA bands of the expected sizes (5857bp, 1684bp and 540bp) (Figure 4.7 B and C). Four plasmid samples were checked to identify positive clones of AKINγ1 which should release two
DNA bands (7990bp and 733bp) following digestion with EcoRV and BamHI. One plasmid releasing the DNA bands of the expected sizes was identified (Figure 4.7 B).

Figure 4.7 cDNA cloning of AKInα1, AKInβ1, AKInβ1T (lacking the predicted chloroplast transit peptide) and AKInγ1 subunit into pET32a with intein-CBD for protein expression. (A) The PCR products of AKInα1, AKInβ1, AKInβ1T and AKInγ1 subunits and the digested vector pET32a. (B) Identification of positive clones from the four ligation reactions. Lane 1 was a positive clone for AKInα1. Lanes 7, 8 and 11 were positive clones for AKInβ1. Lanes 13, 15 and 17 were positive clones for AKInβ1T. Lane 20 was a positive clone for AKInγ1. (C) Confirmation of positive clones by restriction enzyme digestion. The seven positive clones were digested with SacI, BglII and BamHI/BglII respectively to confirm they possessed inserts of the expected size.

4.3.5 Protein expression and purification of the subunits of SnRK1 in E. coli ArcticExpress (DE3) cells

The recombinant proteins were fused with four tags from the vector pET32a including a TrxA protein tag, a His-tag, an S-tag and the intein-CBD fragment and two Ala residues in sequence on the N-terminal end of the target proteins (Figure 4.8 A). The two Ala residues between the intein-CBD domain and the proteins of interest facilitate the efficiency of cleavage. Soluble recombinant proteins fused with these tags from
ArcticExpress (DE3) cell lysates were separated by 10% SDS-PAGE, followed by immunodetection using rabbit polyclonal anti-AKINα1 and anti-AKINβ1 antibodies and mouse monoclonal S-tag antibody for AKINγ1 respectively. Four clear protein bands of the expected sizes, 131kDa, 106kDa, 98kDa and 121kDa, indicated the successful expression of recombinant AKINα1, AKINβ1, AKINβ1T and AKINγ1 respectively (Figure 4. 8B). Purification of the recombinant proteins using Ni-NTA beads was performed, and bound proteins eluted as described in Section 4.2.3. Protein fractions from each step of the purification process were separated by 10% SDS-PAGE, followed by Coomassie Blue staining (Figure 4.8C). The results show a significant level of the recombinant proteins bound to the affinity resin, which could be eluted from Ni-NTA beads by imidazole as described in Section 4.2.3 (Figure 4.8C).
Figure 4.8 Protein expression and purification of AKINα1, AKINβ1, AKINβ1T and AKINγ1 fused with the tags. (A) Schematic representation of the structures of the recombinant proteins. The recombinant proteins are fused with three tags from the vector including a TrxA protein tag (109 amino acids, 11.99kDa), a histidine tag (6 amino acids, 0.84kDa), and an S tag (15 amino acids, 1.88kDa), and the intein-CBD fragment (525 amino acids, 58.5kDa) and two Ala residues (0.22kDa) at the N-terminus of the target proteins. The intein cleavage site is located at the C-terminal end of the intein-CBD fragment. (B) Immunodetection of soluble recombinant proteins in cell lysates. Lane 1 to 4 represent recombinant AKINα1 (131kDa), AKINβ1 (106kDa),
AKINβ1T (98kDa) and AKINγ1 (121kDa) fusion proteins respectively, using antibodies to AKINα1, AKINβ1 or the S-tag as appropriate. (C) Coomassie Blue staining following protein purification of the soluble AKIN recombinant proteins using Ni-NTA beads. Lane 1 to 5 represent cell lysate, column flow through after incubation of cell lysate with the beads, and imidazole-eluted fractions. 30µl of each fraction was loaded on the SDS-PAGE gel. The triangle markers show beginning of protein elution with imidazole.

**4.3.6 Removal of tags from recombinant AKINα1, AKINβ1, and AKINβ1T by DTT and protein sequencing**

The DTT-induced cleavage process requires long-incubation times (24 or 72h) (Chong *et al.*, 1997) and there were non-specific proteins that would contain *E. coli* proteases in all imidazole-eluted fractions of each recombinant AKIN subunit protein (Figure 4.8 C Lanes 3-5). Thus, to test the ability of the intein cleavage site to be cleaved, the third imidazole-eluted fraction for each recombinant protein that contains fewer non-specific proteins and sufficient target protein were used for DTT-induced cleavage to decrease the possibility of target protein degradation and obtain detectable cleaved protein bands using western blot. 50mM DTT was incubated with the recombinant AKINβ1 protein (Figure 4.8 C). Following 24-hour incubation with DTT, a clear protein band of 39kDa was detected, although a large proportion of recombinant protein was clearly not cleaved during the process. Following 72-hour incubation with DTT, the amount of cleaved AKINβ1 protein increased, but cleavage was still incomplete (Figure 4.9 A). The recombinant proteins of AKINα1 and the truncated AKINβ1 were also cleaved by DTT (Figure 4.9 B and C) following 72-hour incubation in DTT. Cleaved protein bands were detected, but a high proportion of each recombinant protein remained uncleaved. The expected MWs of AKINβ1 and the truncated AKINβ1 proteins (including the two
additional Ala residues) are approximately 31kDa and 23.5kDa, but SDS-PAGE-calculated MWs of the proteins (about 39kDa and 31kDa) were larger than expected. The predicted MW of AKINα1 is approximately 59kDa, but based on SDS-PAGE, the recombinant protein (about 50kDa) seems smaller.

**Figure 4.9** Immunodetection of recombinant tagged proteins of AKINα1, AKINβ1, and AKINβ1T before and after cleavage with DTT using anti-AKINα1 antibody or anti-AKINβ1 antibody respectively. The AKINα1, AKINβ1, and AKINβ1T proteins cleaved from their fusion proteins are indicated by red arrows.

To confirm the identity of the fusion proteins, post-DTT incubation, the corresponding bands on a silver-stained gel were used to perform MS analysis. The released protein bands shown by western blotting using anti-AKINα1 antibody or anti-AKINβ1 antibody respectively (Figure 4.10 A) were identified and cut from the silver-stained gel (Figure 4.10 B). The gel slices were destained, followed by an overnight in-gel trypsin digestion. Following extraction from the gel slices, the resulting peptides from each sample were analyzed by liquid chromatography-electrospray ionization-quadrupole-time of flight-mass spectrometry (LC-ESI-Q-TOF-MS). MS results were analyzed by Peaks 8.0 software with a cut-off at 5% False Discovery Rate (FDR). 34, 14 and 4 peptide sequences were identified from each band of protein released from the
recombinant AKINα1, AKINβ1 and AKINβ1T fusion proteins respectively. By matching the peptide sequences with NCBI non-redundant protein database limited to viridiplantae (green plants), 32, 13 and 4 peptides were matched respectively (this includes peptide sequences that were sequenced twice plus the same peptide sequences with posttranslational modification). Finally, 22, 10 and 4 non-repetitive peptide sequences were identified and matched to AKINα1, AKINβ1 and AKINβ1T protein respectively (Table 4.1, Figure 4.11), confirming that the DTT-released proteins were AKINα1, AKINβ1 and AKINβ1T respectively. Thus, full-length, soluble AKINα1, AKINβ1 and the truncated AKINβ1 were obtained successfully, but appear to run anomalously on SDS-gels.

**Figure 4.10** The bands of proteins released from the AKINα1, AKINβ1 and AKINβ1T fusion proteins after DTT incubation for MS analysis. Compared with these released protein bands shown by western blotting using anti-AKINα1 antibody or anti-AKINβ1 antibody respectively (A), their corresponding protein bands on the silver-stained gel were identified and cut from the gel for further MS analysis (B). These protein bands were labelled using red arrows.
Table 4.1 Identification of the proteins released from the AKINα1 fusion protein, AKINβ1 fusion protein and AKINβ1T fusion protein after DTT incubation. OS stands for Organism Name, GN is Gene Name, PE stands for Protein Existence, and SV is Sequence Version. The peptides from AKINβ1T sequencing result was matched as a part of AKINβ1, thus, the identification result in Column 3 are shown as AKINβ1.

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Figure 4.11 Mass spectrometric confirmation of the identity of recombinant proteins. The identified proteins were released from the AKINo1 fusion protein, AKINβ1 fusion protein and AKINβ1T fusion protein following DTT incubation of intein-tagged polypeptides using Peaks 8.0 software. 22, 10 and 4 non-repetitive peptides were matched on each of the three protein sequences respectively. The black arrow shows the first amino acid in the truncated AKINβ1. Oxidation, carbamidomethylation and Deamidation.
4.3.7 Use of recombinant AKINα1 and AKINβ1 as bait to detect interacting Arabidopsis leaf and chloroplast proteins

The unprocessed recombinant proteins AKINα1 and AKINβ1 (i.e. with tags) and the expressed tag protein (not fused to any subunit), were purified by Ni-NTA affinity chromatography. The products were then bound to chitin affinity beads as bait in order to detect interacting proteins from Arabidopsis leaf and chloroplast extracts.

Purification of the recombinant proteins using Ni-NTA beads was performed and bound proteins eluted as described in Section 4.2.3. Protein fractions from each step of the purification process were separated by 10% SDS-PAGE, followed by Coomassie Blue staining and western blot using antibodies to AKINα1, AKINβ1 or the S-tag as appropriate (Figure 4.12 A). The results show a significant amount of the recombinant proteins bound to the affinity resin, which was eluted from Ni-NTA beads by imidazole (Figure 4.12 A). Comparing the first imidazole-eluted fraction (Figure 4.12 A Lane 8), there were fewer non-specific protein bands in subsequent fractions (Figure 4.12 A Lane 9 and 10), while the bait protein remained abundant. Thus the samples in Lane 9 and 10 were combined and used for further immobilization on chitin beads. Following chitin-based protein immobilization, fractions eluted from each step of the purification process were separated by 10% SDS-PAGE, and stained for protein (Coomassie) and subjected to western blotting as before (Figure 4.12 B). The results show a significant level of the recombinant proteins bound to the chitin affinity resin (Figure 4.12 B). Although multiple protein bands were detected after purification (Figure 4.12 B Lane
Figure 4.12 Recombinant AKINα1, AKINβ1 and the tag-only proteins purified by Ni-
NTA and chitin affinity chromatography. (A) Recombinant AKINα1, AKINβ1 and the tag-only proteins purified from Ni-NTA beads. Lane M: protein marker, Lane 1: bacterial cell lysate, Lane 2: column flow through after cell lysate incubated with Ni-NTA beads, Lanes 3-7: wash fractions, Lanes 8-11: imidazole-eluted fractions. (B) Imidazole-eluted fractions of AKINα1, AKINβ1 and the tag-only proteins were bound to chitin beads. Lane M: protein marker, Lane 1: purified proteins from the previous Ni-NTA purification process, Lane 2: column flow through after the purified proteins incubated with chitin beads, Lanes 3-6: wash fractions, Lane 7: proteins on chitin beads. The arrows and numbers show, respectively, the protein band and molecular weight for each recombinant protein. The triangle markers show beginning of column washing (hollow) or imidazole elution/protein immobilization (solid).

7), some of these are likely to be proteolytically cleaved, truncated recombinant proteins which contained the chitin-CBD tag since they could also bind to chitin resin.

Recombinant AKINα1 and AKINβ1 proteins bound to the chitin beads were used as bait to detect interacting proteins from Arabidopsis leaf and chloroplast, taking account of contaminating polypeptides observed in the controls (tag only).

Several controls were included: a) “empty” chitin beads incubated with leaf proteins or chloroplast proteins only; b) “empty” chitin beads incubated with recombinant AKINα1 or AKINβ1 or the tag-only protein only; c) “empty” chitin beads incubated with the recombinant tag-only protein followed by interaction with leaf proteins or chloroplast proteins. These controls were included to take account of the large amount of non-specific protein binding.

Using the recombinant subunits as bait, three protein bands putatively interacting with recombinant AKINα1, and one putative interacting protein with recombinant AKINβ1, were identified when whole leaf extracts were incubated with each respective recombinant protein (Figure 4.13). The four protein bands were cut from gels and
destained, followed by an overnight in-gel trypsin digestion. The resulting peptides from each protein band sample were extracted from the gel slices, dried and analyzed by LC-ESI-Q-TOF-MS. The MS analysis results were analyzed by Peaks 8.0 software with a cut-off at 5% FDR. After MS analysis, 15 peptide sequences were identified from the protein band labelled as ‘2’ (Figure 4.13 A). By matching the peptide sequences with NCBI non-redundant protein database limited to viridiplantae (green plants), 6 peptides ranging from 8 to 36 amino acids were matched (Table 4.2) and two protein sequences were identified (Table 4.3). The first peptide sequence (chlorophyll a/b binding protein (LHCP AB 180)) is also part of the second peptide (chlorophyll A/B binding protein-3/chlorophyll A/B-binding protein-2). This protein band could be chlorophyll A/B binding protein-3/chlorophyll A/B-binding protein-2 and/or chlorophyll A/B binding protein-1 since all these proteins contain the peptide sequence (chlorophyll a/b binding protein (LHCP AB 180)) (Figure 4.13 and 4.14). No peptide was identified from the other three protein bands labelled as ‘1’, ‘3’ and ‘4’, which could be caused by various factors such as insufficient protein in the sample, poor protease-based digestion, or poor fragmentation (peptides are too small or too large) for MS analysis. Compared with about 27mg Arabidopsis leaf proteins used, far less stromal protein (about 4.5mg) was used to perform the “pull-down” assay, which could have contributed to the failure to detect interacting polypeptides from chloroplasts.
Figure 4.13 Identification of proteins which interact with immobilized, recombinant AKINα1 or AKINβ1. Soluble extracts from leaves (A, C) or chloroplasts (B, D) were incubated with either AKINα1 (α), AKINβ1 (β) or the tag-only polypeptide (T) covalently bound to chitin beads. EB=samples incubated with “empty beads” lacking covalently bound polypeptide. Gels were silver-stained for proteins bound to the chitin-resin prior to elution with DTT (A, B) or released following elution with 50mM DTT for 48h (C, D). Red arrows indicate proteins used for MS analysis.
Table 4.2 MS analysis of putative interacting polypeptides following incubation of leaf cell lysates with recombinant AKINα1. After in-gel trypsin digestion and MS analysis, 6 peptides were identified from NCBI non-redundant protein database limited to viridiplantae (green plants).

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Table 4.3 Identification of leaf proteins that interact with AKINα1.

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Figure 4.14 The identified AKINα1-interacting protein candidate(s) identified by Peaks 8.0 software. 6 peptides were matched on each of the two protein sequences. The numbers under blue lines represents the number assigned to each of the 6 peptides. carbamidomethylation and Deamidation.

Chlorophyll a/b binding protein (LHCP AB 180)

1 MRKTVAKPKG PSGSPWyGSD RVKYLGFPG ESPSYLTHEF PGDYGWDTAG LSADPETFAR NNELEVIHSS WAMLGALGCV
2 4 3
81 FPELLARNGV KFGEAVWFKK GSIIFSDDSGL DYLGNPSLVH AQSLAIAWAT QVIIMGAVEX GRY VAGNPGPLG EAEDILLYPGE
1 6
161 SFDPDLGATD PEFAELKVK ELKNGLAMF SMFGFFVQAIR TGKGPIENL ADHLADPVNN NAWAFATNFV PGK

Chlorophyll A/B binding protein 3/chlorophyll A/B binding protein 2

1 MAASTMALS GAFAGKAVNL SPAASEVLGSS GRTVMRKTVK KPKGPGSGWP YGSDRVRKYLGF PSGEPSYLT GEFPGDYGKW
2 4 3
81 DTAGLSADPE TFARNRELEV IHSRWAMLG ALGVPPPELLA RNIGVR FGIAV WFKKAGSGIFS DGGLDYLGNP SLVHAQSLA
1 6
161 IWATQVILMG AVEGYR VAGN GPLGAEEDLL YPGGSFDPLG LATDPEFAELKVKELKNGR LAMFSMGFF VQAIVTCKGP
2 6

241 LENLADHLAD PVNNNAWAFATNFVPGK
4.4 DISCUSSION

In vitro biochemical studies on structure, function and protein interactions of eukaryotic protein kinases are often constrained by the availability of the catalytically active protein kinase, as they are often in low abundance in the cell and hence difficult to purify from tissue extracts by traditional biochemical methods. Recombinant protein expression technology offers a convenient way to resolve this problem. In this study, a protein expression system in E. coli has been established for the successful expression of soluble AKIN subunits. The main problem of protein expression of AKIN subunits was that a large number of AKIN subunits, especially AKINα1, were in the form of inclusion bodies (data not shown) in this study. Several useful strategies, which ameliorate the factors that can lead to inclusion body formation such as improper pH, redox potential, and folding mechanisms in E. coli cells, have been developed. They include facilitating disulfide bond formation, co-expressing chaperone/supplementing chemical chaperones and cofactor, and slowing down recombinant protein production rate (Rosano and Ceccarelli, 2014). Disulfide bond formation is sometimes necessary for formation of stable secondary and tertiary structures of recombinant proteins (Wong, 2005). For the E. coli-based expression system used in this study, the tag protein, TrxA (thioredoxin-1), was employed due to its ability to increase fusion protein solubility. It is postulated that TrxA facilitates disulfide bond formation in the cytosol and helps reduce formation of protein aggregates (Wong, 2005; Hammarstrom et al., 2006; Bird, 2011; Costa et al., 2013). AKINα1 and AKINγ1 have 3 and 1 putative disulfide bonds respectively, as predicted by DiANNA (http://clavius.bc.edu/~clotelab/DiANNA/).
Although the elution peak of the recombinant AKINβ1 protein fused with TrxA was identified as a form of trimer (160kDa) (Figure 4.4 D), fractions likely to contain monomers of the fusion protein were identified by GPC analysis. Soluble recombinant AKINα1 protein fused with TrxA was obtained (Figure 4.8). Soluble recombinant AKINγ1 protein fused with TrxA was also obtained but not used for DTT-induced tag cleavage and pull-down assays due to lack of a peptide-specific antibody (Figure 4.8). Thus TrxA may facilitate disulfide bond formation in the cytosol, leading to the successful expression of recombinant fusion proteins of AKIN subunits.

Tag-based affinity chromatography is a powerful tool for protein purification. Using a high affinity interaction of His-tag with immobilized ion (Ni$^{2+}$, Co$^{2+}$, Cu$^{2+}$ and Zn$^{2+}$) on a resin matrix, recombinant proteins fused with His-tag can be bound to the metal-charged resin by coordinate covalent bonds. Imidazole can elute recombinant proteins from the resin since this compound competes with the His-tag (an imidazole side chain on His residue) for binding to the metal-charged resin (Porath et al., 1975). Most other proteins in crude lysate can not be bound to the resin or bound weakly. Thus, His-tag-based affinity chromatography can provide relatively pure recombinant protein from a crude lysate (Terpe, 2003). In this study, a significant amount of non-specific binding proteins were removed from the target AKIN recombinant proteins using His tag-based affinity chromatography (Figure 4.8 and 4.12). Tag removal is a key step for the successful expression of recombinant proteins. Enzymatic cleavage is a technically practical way to remove tags from the recombinant proteins and enterokinase, a serine protease, is used widely in this enzymatic cleavage process. In this study, enterokinase
was used in an attempt to remove the tags (Trx-His-S) from the recombinant AKINβ1 protein, but no significant cleavage product of the expected size (34.5kDa) was detected (Figure 4.4 F). Although there is no other enterokinase cleavage site in the AKINβ1 fusion protein, it is known that enterokinase does not exhibit high stringency in its specificity for this sequence, which could lead to the fusion protein being cleaved by the protease at other non-specific sites, rather than the designated site (Waugh, 2011). In addition, enterokinase requires optimal reaction conditions e.g. pH, temperature, and salt concentration, and further work is needed to optimize reaction conditions.

A modified protein splicing element (intein) from *Saccharomyces cerevisiae* can be induced to undertake a self-cleavage reaction at its N-terminal peptide linkage by DTT, β-mercaptoethanol or cysteine at low temperatures such as 4ºC, and over a broad pH range (pH5.5-9) (Chong et al., 1997). The inducible self-cleavage activity of intein in protein splicing has been employed to separate a target protein from an affinity tag. In this study, the intein combined with a chitin-binding domain (used to bind the fusion protein to chitin beads) was used to facilitate separation of the tags from the recombinant AKIN subunit proteins of interest in less harsh conditions. The recombinant proteins, AKINα1 and AKINβ1, were released from the fusion tag after 72-hour DTT treatment, but the cleavage of the target proteins was incomplete (Figure 4.9). This suggests that efficiency of the DTT-based cleavage was low and some attempts such as varying the incubation temperature and pH should be undertaken to optimise conditions for this cleavage reaction in the future.
Determination of protein molecular weight using SDS-PAGE gel electrophoresis is a commonly used method in biochemical research. However, occasionally, protein migration following SDS-PAGE does not correlate with predicted molecular weights of proteins (Rath et al., 2009; Guan et al., 2015). Such abnormal electrophoretic migration of proteins can be caused by many different factors such as the primary amino acid sequence of target protein, chemical modification of target proteins or amino acid substitutions (Shi et al., 2012; Guan et al., 2015). The SnRK1 subunits studied (AKINα1, AKINβ1 and AKINβ1T) all show abnormal SDS-PAGE migration (Figure 4.9), following cleavage by DTT. The primary amino acid sequence of each target protein affects the net charge of its protein sequence, all these target proteins have more negatively charged residues than positively charged residues on the basis of analysis using the ProtParam tool (http://web.expasy.org/protparam/). This feature could result in apparently lower MWs of these proteins when observed on SDS gels than expected. This is the case for AKINα1, but not for the other two proteins (AKINβ1 and AKINβ1T) which appear larger than expected. Moreover, posttranslational modification of these three proteins in the E. coli strain used could also contribute to their abnormal electrophoretic migration. Posttranslational modification(s) of these three proteins in the E. coli strain used, which have been identified by MS analyzed such as oxidation, carbamidomethylation, deamidation (Figure 4.11) and would occur such as phosphorylation and glycosylation, may have contributed to altered protein mobility on SDS gels. Although all these target proteins (AKINα1, AKINβ1 and AKINβ1T) show abnormal SDS-PAGE migration, the MS analysis result of these proteins released from
the recombinant AKINα1, AKINβ1 and AKINβ1T fusion proteins respectively (Figure 4.11) strongly confirmed that these released proteins were AKINα1, AKINβ1 and AKINβ1T respectively, proving that AKINα1, AKINβ1 and AKINβ1T fused with tags respectively have been expressed successfully.

Earlier papers have reported that AKINα subunits fused with GST could be expressed in E. coli, and these affinity-purified AKINα fusion proteins were used directly for pull-down assays in vitro (Tsugama et al., 2012; Jeong et al., 2015). These results show that E. coli-based expression of soluble AKINα subunits have the correct conformation for protein-protein interaction. In the present study, AKINα1 fusion-protein and AKINβ1 fusion-protein, purified by two-step affinity purification, were used in protein-protein interaction studies to identify potential interacting proteins from Arabidopsis leaf lysates and chloroplast lysates. Two protein sequences from the putative binding protein(s) of AKINα1 were identified as belonging to the chlorophyll a/b binding protein family (Table 4.3). The argument that chlorophyll a/b binding protein(s) interacts with AKINα1 is strengthened by the observation that it was not detected when using the immobilised tags (Trx-His-S-Intein) alone (Figure 4.13 A). In fact, chlorophyll a/b binding protein 3 (light harvesting chlorophyll a/b binding protein (LHCB) 1.2; 267 amino acids, 28kDa) is the precursor of LHCP AB 180 (233 amino acids, 25kDa) (Leutwiler et al., 1986). The protein band observed was around 25kDa (Figure 4.13 A 2), suggesting it could be LHCP AB 180. Moreover, the mature proteins encoded by Lhcb1.1 (AT1G29920, encoding chlorophyll a/b binding protein 2), Lhcb1.2 (AT1G29910) and Lhcb1.3 (AT1G29930, encoding chlorophyll a/b binding
protein 1) are identical (Jansson, 1999; Galka et al., 2012). Thus either or both LHCB1.1/1.2, which are encoded by two different genes, Lhcb1.1 and Lhcb1.2, could interact with AKINα1 as they share a common amino acid sequence. The other possibility is that the interacting protein is chlorophyll a/b binding protein 1 (LHCB1.3) since LHCB1.3 also contains the same peptide sequence. Thus, these detected peptides could belong to LHCB1.1/LHCB1.2 and/or LHCB1.3.

The chlorophyll binding proteins including LHCB1.1, LHCB1.2 and LHCB1.3 of the light-harvesting chlorophyll a/b-protein complex II (LHCII) are encoded by nuclear genes and then translated as precursor polypeptides in the cytoplasm. After import into the chloroplast throughout the outer and inner envelope membranes of chloroplasts, these precursor polypeptides are cleaved at their N-terminus releasing the chloroplast transit peptide to form their mature proteins. These mature proteins with thylakoid-targeting signal sequence are inserted into thylakoid membranes in which these proteins noncovalently bind chlorophyll a, chlorophyll b, and carotenoid molecules to form pigment-protein complexes that harvest light energy for photosynthesis (Leutwiler et al., 1986; Robinson and Mant, 2005; Nick et al., 2013). AKINα1 would have to bind with each mature protein in the chloroplast stroma and/or in thylakoid membranes, which is supported by the observation that AKINα1 is also localized in chloroplast (Fragoso et al., 2009). It was therefore surprising that when chloroplast lysates were used to perform the pull-down assay, no putative binding peptide was identified. However, after chloroplast isolation, intact chloroplasts were lysed by resuspension in rupturing buffer and centrifuged to remove membranes and other particulate matter
Thus membrane-bound LHCB1 may have been removed during centrifugation, whereas it is possible that some remained in the supernatant of the whole leaf extract. Further, about 27 mg Arabidopsis leaf soluble protein was used, compared to only 4.5 mg chloroplast soluble protein were used to perform the pull-down assay. Thus it is possible that insufficient precursor and mature LHCB1 proteins limited the possibility of detection.

Trimeric LHCII is regulated by reversible phosphorylation to balance the relative excitation of photosystem I (PSI) and photosystem II (PSII), dependent on changes in light quantity or quality (Mekala et al., 2015). The light quantity-dependent control of thylakoid protein phosphorylation drastically differs from the light quality-dependent protein phosphorylation (Tikkanen et al., 2010). Under natural conditions, large and sudden changes in light quantity are more common than changes in light quality. Thus changes in light intensity would be the most important drivers of LHCII phosphorylation and dephosphorylation to balance PSI and PSII excitation (Rintamäki et al., 2000). When light intensity increases, the phosphorylation level of LHCII decreases. Whereas, when light intensity decreases, the phosphorylation of the LHCII proteins strongly increases (Rintamäki et al., 1997; Rintamäki et al., 2000). LHCII phosphorylation is a prerequisite to form a PSI-LHCII complex (Pesaresi et al., 2009; Järvi et al., 2011), which increases the absorption capacity of PSI. Moreover, LHCII trimers are composed of LHCB1, LHCB2, and LHCB3 proteins and the LHCB1 proteins including LHCB1.1, 1.2 and 1.3 proteins have been shown to be phosphorylated (Bellafiore et al., 2005; Pietrzykowska et al., 2014). This suggests that
SnRK1 could affect photosynthesis by regulating LHCII phosphorylation under decreased light intensity. Interestingly, a minimal recognition motif for SnRK1 in animals and plants was proposed using variant peptide substrates of SnRK1, which is M/V/L/I-(R/K/H, X, X)-X-S/T-X-X-M/V/L/I (oblique indicates alternatives, commas indicate that the order of residues can be changed, and the X residues represent any amino acid) (Weekes et al., 1993). All LHCB1.1, 1.2 and 1.3 proteins have this recognition motif for SnRK1 (Figure 4.15). Moreover, the crystal structure of spinach LHCB1.3 (PDB entry 4LCZ) showed that this recognition motif is on the stromal surface of LHCII (Wan et al., 2014) and the protein sequence of the spinach LHCB1.3 is highly similar (93% identity) to the protein sequence of Arabidopsis LHCB1.3 based on Protein BLAST analysis. This suggests that SnRK1 could phosphorylate LHCB proteins, consistent with the in vitro interaction observed in this study. Moreover, it has been shown that the leaf photosynthetic rate in transgenic tomatoes overexpressing the MhSnRK1 gene (an α subunit encoding gene from Malus hupehensis Rehd) was increased (Wang et al., 2012). This is consistent with the hypothesis that SnRK1 activates catabolic processes to produce energy in plant cells and shows that SnRK1 could play a role in regulation of photosynthesis.

Figure 4.15 A recognition motif for SnRK1 in Arabidopsis LHCB1 proteins. (A) A recognition motif for SnRK1 in animals and plants was proposed (Weekes et al., 1993). The residues are required for recognition as follows: phosphorylated serine (S) or threonine (T), hydrophobic residues at P-5 and P+4 and basic residues at either P-2, P-3 or P-4. The amino acids in the same vertical line are alternative. The order of these
amino acids (in red) in the same row can be changed. X residues represent any amino acid. (B) The recognition motif for SnRK1 is also identified in LHCBI.1, 1.2 and 1.3 proteins respectively.

So far, a protein kinase, STATE TRANSITION (STN) 7, has been shown to be essential for the rapid phosphorylation of the light-harvesting antenna LHCII that regulates its displacement from PSII to PSI (Bellafiore et al., 2005). A homologous kinase of STN7, STN8, can also phosphorylate LHCII. However, its phosphorylation activity toward the LHCII complex is much lower than that of STN7 (Bonardi et al., 2005; Leoni et al., 2013). Moreover, LHCBI and LHCIB2 are also phosphorylated at other sites and these phosphorylations are unlikely to be catalyzed by either STN7 or STN8 (Ingelsson and Vener, 2012), showing that other protein kinase(s) are likely to be involved in LHCBI protein phosphorylation.

Proteins which could interact with recombinant AKINβ1, whose size is between 75kDa-100kDa, were detected (Figure 4.13 A Lane 5) but the concentration of protein(s) in the band was too low to analyze using MS. Given the subcellular localization of AKINβ1 (Golgi in tobacco leaf cells, see Chapter III) and the fact that soluble leaf proteins were used to perform the protein-protein interaction study, it is possible that AKINβ1-interacting proteins bound to Golgi were pelleted with membrane debris, thus compromising their detection. Moreover, as AKINα1 requires ATP to interact with its substrate proteins, it is possible that AKINβ1 maybe require some specific reaction conditions to interact with its interacting proteins.

Although no putative interacting protein was identified for the Golgi-localised AKINβ1 subunit, it appears to play a novel role in regulating carbon partitioning between starch
metabolism, sucrose metabolism and the TCA cycle in Arabidopsis leaves (see Chapter II). It has been shown previously that SnRK1 can directly regulate several metabolic enzymes involved in carbohydrate metabolism by posttranslational modification. Most importantly, SnRK1 can also regulate expression of various genes involved in photosynthesis, starch/sucrose metabolism, glycolysis/gluconeogenesis and the oxidative pentose pathway (Baena-Gonzalez et al., 2007). In the next chapter, high-throughput RNA-sequencing (RNA-Seq) was used to determine putative transcriptome expression changes in the akinβ1 mutant leaves of Arabidopsis, in order to investigate the putative role of SnRK1, especially AKINβ1, in gene expression regulation.
CHAPTER V
EFFECTS OF MUTATION OF AKINβ1 ON TRANSCRIPTION AND IN RESPONSE TO ILLUMINATION
5.1 INTRODUCTION

The previous chapter showed that LHCBI.1/LHCBI.2 and/or LHCBI.3 interact with AKINα1 using a chitin bead-based pull-down assay. SnRK1 can not only directly regulate several metabolic enzymes and proteins, but also regulate expression of various genes involved in photosynthesis, starch/sucrose metabolism, glycolysis/gluconeogenesis and the oxidative pentose pathway (Baena-Gonzalez et al., 2007). Given the significant effect that loss of AKINβ1 has on metabolism (see Chapter II), the question is raised as to whether transcriptome expression also changes in the akinβ1 mutant leaves of Arabidopsis. The transcriptome represents the whole set of all messenger RNA (mRNA) molecules expressed by a cell, tissue or organism (Adams, 2008). Transcriptome studies offer

• insights into spatio-temporal expression pattern of genes,

• quantitation of gene expression,

• identification and isolation of genes of interest and their variants (e.g. alternative splicing (RNA editing) or alternative transcription initiation and termination sites),

• identification and development of functional molecular markers,

• large scale comparative genomic studies (Garg and Jain, 2013).

The extent of AKIN10 transcriptional regulation has been studied, and over a thousand AKIN10-target genes involved in different metabolic pathways, such as such as cell wall, starch, sucrose, amino acid, lipid, and protein degradation processes, trehalose-6-
phosphate biosynthesis and ribosome biogenesis have been identified (Baena-González et al., 2007; Contento et al., 2004; Ramon et al., 2009). Although studies on transcriptional regulation of the catalytic subunit (AKIN10) of SnRK1 have been performed, little is known about the importance of the regulatory subunits (AKINβ and AKINγ) in regulating gene expression are available. This chapter is concerned with understanding the role of AKINβ in regulating gene expression.

With the great advance in methods to deduce and quantify gene expression at the whole genome level (Wang et al., 2009), two popular methods, DNA microarray and RNA-sequencing, have been developed and employed for genome-wide transcriptome profiling. DNA microarray technology was developed in the 1990s and is used as a tool for studying the transcriptome and global gene expression patterns of plants. The high-throughput technology offers parallel gene expression analysis for thousands of genes with known and unknown function, or DNA homology analysis to identify gene polymorphisms and mutations in genomic DNA (Jaluria et al., 2007). A recently developed alternative technology for transcriptome analysis, RNA sequencing (RNA-Seq, also known as transcriptome shotgun sequencing), is replacing microarray technology in gene expression studies due to the advantages of RNA-Seq over microarrays. RNA-Seq: a) has a greatly increased specificity, offering superior resolution at base-pair level; b) has a high sensitivity, offering a broader dynamic range for detecting rare and low-abundance transcripts. In principle, RNA-Seq has an unlimited dynamic range that only depends on the sequencing depth (the number of times a nucleotide is read during the sequencing process); c) does not require any
information on the sequences to be investigated for revealing new sequences such as paralogous sequences and unknown sequences; d) can reliably analyze known sequences, and existing data of RNA-Seq can be easily reanalyzed in relation to any new available genome-build datasets (Shendure, 2008; Oshlack et al., 2010; Kangaspeska et al., 2012). Thus, in this study, RNA-Seq technique was employed to study transcriptome changes in the *akinβ1* mutant in response to light and darkness.

RNA-Seq reveals the presence and quantity of gene transcripts in a biological sample by employing next-generation sequencing (NGS, also known as high-throughput sequencing) and exploiting different sequencing technologies including Illumina (Solexa) sequencing, Roche 454 sequencing, the ThermoFisher Ion torrent (ionPGM/ ion Proton) sequencing and the Applied Biosystems SOLiD sequencing (Reuter et al., 2015). Illumina sequencing technology (also called sequencing by synthesis) employs clonal array formation and fluorescently-labeled, reversible terminator technology for fast and precise sequencing on a large scale. A typical RNA-Seq analysis involves the following steps: (1) total RNA isolation from cells or tissues of interest and conversion of the total RNA or fractionated RNA into complementary DNA (cDNA); (2) ligation of platform-specific adapters to each cDNA fragment at one or both ends to prepare the sequencing library, and immobilization of the DNA fragments on a solid support; (3) sequencing cDNA fragments with or without amplification by a massively parallel, deep sequencing-based, approach to obtain short sequences from one end (single-end sequencing) or both ends (pair-end sequencing) of the cDNA fragments. Either by aligning these resulting short sequencing reads onto the reference genome or the
reference transcripts, or by assembling these reads de novo, a genome-scale transcription map is established to reveal the transcriptional structure and expression level of genes related to the experimental condition of interest (Mortazavi et al., 2008; Wang et al., 2009; Hoeijmakers et al., 2013).

Transcriptome analysis has previously been used to study the change of gene expression and the identification of early target genes of AKIN10 (Baena-González et al., 2007). The extent of AKIN10 transcriptional regulation has been studied by employing a protoplast transient-expression assay in Arabidopsis and gene chip-based method (DNA microarrays). Following stringent and multi-step filtering processes, 278 genes were identified as being activated by AKIN10 and 322 genes as being repressed (Baena-González et al., 2007). Many of the genes exhibiting altered expression encoded proteins involved in carbohydrate metabolism such as starch and sucrose degradation (see Chapter I). In the present study, RNA-Seq was used to analyze changes in gene expression pattern in wild-type and the akinβ1 mutant in response to light and darkness. The hypothesis is that identifying putative genes which are regulated by the AKINβ1 subunit, may provide insight into the changes in metabolism observed (see Chapter II).

5.2 MATERIAL AND METHODS

5.2.1 Total RNA extraction from the rosette leaves of the akinβ1 mutant and WT

To prepare total RNA samples for RNA-Seq, 4-week-old Arabidopsis leaves from two individual akinβ1 mutant plants or two WT plants grown under long day conditions (16-hour light/8-hour darkness) were harvested into 15-ml Falcon tubes. Single leaf samples were taken after 1-h light or 1-h darkness and frozen in liquid nitrogen. For
each time point, six leaf samples from each of the *akinβ1* mutant or WT were collected and frozen in liquid nitrogen. The frozen sample was ground to powder in a pre-cooled mortar. 80mg leaf powder from each sample was used to extract total RNA by employing mirVana™ miRNA Isolation Kit (Thermo Fisher Scientific, Waltham, USA). Total RNA samples were dissolved in RNase-free H₂O and optical density at 280nm measured on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA), to meet the required purity for RNA-Seq (OD260/280 = 1.8-2.2; OD260/230 ≥ 2.0). 5μg of each sample was frozen in dry ice and sent to Novogene Company (Beijing, China) for RNA-Seq analysis.

The whole process of RNA-Seq analysis has been summarized in Figure 5.1. From the RNA sample preparation to the final sequencing data, each step including sample test, library preparation and sequencing influences the RNA-Seq data quality and further impacts the final analysis results. To guarantee the reliability of the data, quality control (QC) is performed at each step of the procedure.
5.2.2 Library preparation and sequencing

5.2.2.1 Total RNA sample QC

Three samples with the highest quality from each of the four groups of RNA samples were chosen. All samples were tested through the following four steps before library construction: (1) RNA degradation and potential contamination was tested by agarose gel electrophoresis; (2) RNA purity (OD260/OD280) was tested by Nanodrop spectrophotometry; (3) RNA concentration was quantified on a Qubit fluorometer; (4) RNA integrity was determined by Agilent 2100 Bioanalyzer.

5.2.2.2 Library construction

To construct cDNA libraries, mRNA from the chosen samples was enriched by using oligo (dT) beads. The enriched mRNA samples were fragmented randomly in fragmentation buffer, followed by cDNA synthesis using random hexamers. After first-
strand cDNA synthesis, a custom second-strand synthesis buffer (Illumina) was added with dNTPs, RNase H and *E. coli* polymerase I to generate the second strand by nick-translation. The final cDNA libraries were ready after one round of purification, terminal repair, A-tailing (addition of 3'-A overhangs), ligation of sequencing adapters, size selection and PCR enrichment (Figure 5.2).

![Diagram of library construction](image)

**Figure 5.2** The workflow chart of library construction for RNA-Seq.

**5.2.2.3 Library QC**

To test the quantity of these cDNA libraries, the concentration of each library was quantified using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, USA), and then was diluted to 1ng/μl before checking insert size on an Agilent 2100
Bioanalyzer (Agilent Technologies, Santa Clara, USA) and quantifying to greater accuracy by quantitative PCR (library activity>2nM).

5.2.2.4 Sequencing

To sequence the libraries, libraries were fed into Illumina HiSeq™ Sequencing machines according to library activity and expected data volume.

5.2.2.5 Data analysis workflow

To process and analyze the RNA-Seq data by employing the different software packages utilized, the raw data analysis was done based on the Arabidopsis TAIR10 reference genome (Figure 5.3).

![Figure 5.3 The workflow chart of the raw data analysis.](image)

5.2.3 qRT-PCR of the leaf RNA samples from the akinβ1 mutant and WT

To confirm the expression level of AKINβ1 gene in the akinβ1 mutant and WT, the three RNA samples used for RNA-Seq analysis were used for qRT-PCR reactions. The whole process from cDNA first-strand synthesis to qRT-PCR was followed as described in Section 2.2.6.
5.3 RESULTS

5.3.1 RNA-Seq correlation

The total RNA samples were tested by detecting RNA degradation and potential contamination, RNA purity, RNA concentration and RNA integrity and three samples with the highest quality from each sample groups were chosen for RNA-Seq analysis.

Biological replication is necessary for any biological experiment including RNA-Seq experiments (Hansen et al., 2011). In RNA-Seq experiments, biological replicates have two main purposes. Firstly, they demonstrate whether the experiment is repeatable; secondly, replicates can reveal differences in gene expression between samples. Correlations are usually calculated in RNA-Seq data to check whether there is any dependence between two sets of data (i.e. technical or biological replicates are expected to be closely correlated). The correlation between samples is an important indicator for testing the reliability of the experiment. The closer the correlation coefficient is to 1, the greater the similarity of the samples. For a straightforward linear regression, goodness of fit equals the square of the Pearson correlation coefficient (R) (ENCODE, 2011). The $R^2$ should be larger than 0.92 under ideal experimental conditions (ENCODE, 2011). In this project, the $R^2$ is required to be larger than 0.8. The results (Figure 5.4) show the Pearson correlation between the samples in the same group are from 0.98 to 0.99 and the Pearson correlation between the samples in the different groups are from 0.977 to 0.988, showing that the biological replicates have high
similarity.

**Figure 5.4** The Pearson correlation between RNA samples from the four groups of RNA samples. L1-3 represent three RNA samples from Arabidopsis plants taken in the light and D1-3 represent three RNA samples from Arabidopsis plants taken in darkness.

### 5.3.2 Raw data quality control summary

The quality of the raw data was tested by three aspects: error rate, A/T/G/C content distribution and data filtering. The error rate for each base can be transformed to the Phred score (Q score) by using this equation: Phred score (Q score) = -10\log_{10}e, e= base calling error. So, Q20 represents base call accuracy is 99% and Q30 represents a base call accuracy of 99.9%. For A/T/G/C content distribution, GC content distribution is evaluated to detect potential GC-content bias, which affects subsequent gene expression quantification. Theoretically, G should be equal to C, and A should be equal to T throughout the whole sequencing process for non-stranded libraries, whereas GC-content bias is normally observed in stranded libraries in which only the first strand
cDNAs are amplified to generate a cDNA library (Zhao et al., 2015). Digital Gene Expression is a sequence-based RNA-sequencing method for gene expression analyses, which directly quantifies transcript abundance counts (a digital output) at an unparalleled level of sensitivity (Hong et al., 2012; Jiang et al., 2015). For Digital Gene Expression libraries, a large variation of sequencing error in the first 6-7 bases are allowed due to the use of random primers in library construction. For data filtering, raw reads are filtered to remove reads containing adapters or reads of low quality (e.g. 1) reads with adaptor contamination; 2) reads in which uncertain nucleotides constitute more than 10% of either read; 3) reads in which low quality nucleotides (base quality < 20) constitute more than 50% in a read), by which clean reads were obtained and used to perform downstream analyses. The results (Table 5.1) show that the error rate for each base in each sample was 0.03%. Moreover, Q20(%) and Q30 (%) were at least 93.19% and 87.12% respectively, and the GC content was about 45%, which shows that the raw sequencing data was of good quality.

Table 5.1 Raw data quality control summary.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Raw reads</th>
<th>Clean reads</th>
<th>Clean bases</th>
<th>Error rate(%)</th>
<th>Q20(%)</th>
<th>Q30(%)</th>
<th>GC content(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT_D1</td>
<td>25729170</td>
<td>23650532</td>
<td>2.96G</td>
<td>0.03</td>
<td>94.01</td>
<td>88.01</td>
<td>45.32</td>
</tr>
<tr>
<td>WT_D2</td>
<td>22611122</td>
<td>20850826</td>
<td>2.61G</td>
<td>0.03</td>
<td>94.14</td>
<td>88.22</td>
<td>45.60</td>
</tr>
<tr>
<td>WT_D3</td>
<td>27470164</td>
<td>25175134</td>
<td>3.15G</td>
<td>0.03</td>
<td>94.08</td>
<td>88.13</td>
<td>45.82</td>
</tr>
<tr>
<td>Mut_D1</td>
<td>26048948</td>
<td>23869894</td>
<td>2.98G</td>
<td>0.03</td>
<td>93.46</td>
<td>87.12</td>
<td>45.51</td>
</tr>
<tr>
<td>Mut_D2</td>
<td>24747612</td>
<td>22609822</td>
<td>2.83G</td>
<td>0.03</td>
<td>93.19</td>
<td>86.63</td>
<td>45.42</td>
</tr>
<tr>
<td>Mut_D3</td>
<td>27639096</td>
<td>25342152</td>
<td>3.17G</td>
<td>0.03</td>
<td>93.42</td>
<td>87.06</td>
<td>45.61</td>
</tr>
<tr>
<td>WT_L1</td>
<td>27187800</td>
<td>25013234</td>
<td>3.13G</td>
<td>0.03</td>
<td>94.03</td>
<td>88.02</td>
<td>46.06</td>
</tr>
<tr>
<td>WT_L2</td>
<td>26849476</td>
<td>24306024</td>
<td>3.04G</td>
<td>0.03</td>
<td>93.90</td>
<td>87.79</td>
<td>45.93</td>
</tr>
<tr>
<td>WT_L3</td>
<td>25261562</td>
<td>23195902</td>
<td>2.9G</td>
<td>0.03</td>
<td>93.89</td>
<td>87.76</td>
<td>45.97</td>
</tr>
<tr>
<td>Mut_L1</td>
<td>26892554</td>
<td>26259284</td>
<td>3.28G</td>
<td>0.03</td>
<td>96.39</td>
<td>92.69</td>
<td>46.07</td>
</tr>
<tr>
<td>Mut_L2</td>
<td>29803520</td>
<td>29057882</td>
<td>3.63G</td>
<td>0.03</td>
<td>96.39</td>
<td>92.70</td>
<td>46.06</td>
</tr>
<tr>
<td>Mut_L3</td>
<td>30963010</td>
<td>30282320</td>
<td>3.79G</td>
<td>0.03</td>
<td>96.54</td>
<td>92.98</td>
<td>45.75</td>
</tr>
</tbody>
</table>

Note: (1) Raw Reads: the original sequencing read counts; (2) Clean Reads: counts of reads after data filtering; (3) Clean Bases: clean read numbers multiply read length in G unit; (4) Error Rate: average sequencing error rate, which is calculated by Phred
score (Q score) = -10\log_{10} e; (5) Q20: percentages of bases whose correct base recognition rates are greater than 99% in total bases; (6) Q30: percentages of bases whose correct base recognition rates are greater than 99.9% in total bases; (7) GC content: percentages of G and C in total bases.

5.3.3 Overview of mapping status

TopHat (v2.0.12), which is an efficient read-mapping algorithm, was used for mapping sequences to the reference Arabidopsis genome (TAIR10 genome release). The TopHat2 algorithm can align reads to a reference transcriptome and map reads to the exons as well as map reads that are segmented to the adjacent exons. The mismatch parameter was set to two by default, and other parameters were set to default. Only clean reads were used to analyze the mapping status of RNA-Seq data against the reference genome. When the reference genome is appropriate and the experiment is contamination-free, the total mapped reads (TMR) should be larger than 70% and multiple mapped reads (MMR) should be no more than 10%. The results of clean reads mapping to the reference genome (Table 5.2) show that the total mapped reads for all samples were larger than 89% and the multiple mapped reads for all samples were smaller than 1.4%. This shows the chosen reference genome is appropriate and that the experiment is contamination-free. At least 88% of reads were uniquely mapped to the reference genome in all samples sequenced, and about half of these uniquely mapped reads for each sample were mapped to the positive strand (+) and half to the negative strand (-). About two thirds of these uniquely mapped reads for each sample were mapped entirely to a single exon, and about one third of these uniquely mapped reads for each sample were mapped to two exons (also named “junction reads”).
Table 5.2 Overview of mapping clean reads to the reference genome.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>WT_D1</th>
<th>WT_D2</th>
<th>WT_D3</th>
<th>Mut_D1</th>
<th>Mut_D2</th>
<th>Mut_D3</th>
<th>WT_L1</th>
<th>WT_L2</th>
<th>WT_L3</th>
<th>Mut_L1</th>
<th>Mut_L2</th>
<th>Mut_L3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Total reads</td>
<td>23650532</td>
<td>20850826</td>
<td>25175134</td>
<td>23869894</td>
<td>22609822</td>
<td>25342152</td>
<td>25013234</td>
<td>24306024</td>
<td>23195902</td>
<td>26259284</td>
<td>29057882</td>
<td>30282320</td>
</tr>
<tr>
<td>(2) Total mapped</td>
<td>(89.69%)</td>
<td>(90%)</td>
<td>(89.85%)</td>
<td>(89.47%)</td>
<td>(89.02%)</td>
<td>(89.39%)</td>
<td>(89.88%)</td>
<td>(89.49%)</td>
<td>(89.72%)</td>
<td>(94.33%)</td>
<td>(94.47%)</td>
<td>(94.63%)</td>
</tr>
<tr>
<td>(3) Multiple mapped</td>
<td>(0.78%)</td>
<td>(0.74%)</td>
<td>(0.73%)</td>
<td>(0.75%)</td>
<td>(1.05%)</td>
<td>(1.37%)</td>
<td>(1.13%)</td>
<td>(0.77%)</td>
<td>(0.75%)</td>
<td>(1.4%)</td>
<td>(0.73%)</td>
<td>(0.8%)</td>
</tr>
<tr>
<td>(4) Uniquely mapped</td>
<td>(88.91%)</td>
<td>(89.26%)</td>
<td>(88.93%)</td>
<td>(88.71%)</td>
<td>(87.98%)</td>
<td>(88.02%)</td>
<td>(88.75%)</td>
<td>(88.72%)</td>
<td>(88.97%)</td>
<td>(92.93%)</td>
<td>(93.73%)</td>
<td>(93.84%)</td>
</tr>
<tr>
<td>(5) Reads map to '+'</td>
<td>10514570</td>
<td>9308644</td>
<td>11196619</td>
<td>10589877</td>
<td>9945787</td>
<td>11154173</td>
<td>11104725</td>
<td>10784211</td>
<td>10321614</td>
<td>12204100</td>
<td>13621452</td>
<td>14213081</td>
</tr>
<tr>
<td>Reads map to '-'</td>
<td>10512385</td>
<td>9303202</td>
<td>11109085</td>
<td>10586076</td>
<td>9945304</td>
<td>11105826</td>
<td>11095197</td>
<td>10780220</td>
<td>10316484</td>
<td>12191153</td>
<td>13615847</td>
<td>14209240</td>
</tr>
<tr>
<td>(6) Splice reads</td>
<td>7768352</td>
<td>6927357</td>
<td>8406344</td>
<td>7852645</td>
<td>7443396</td>
<td>8304046</td>
<td>8052032</td>
<td>7776888</td>
<td>7458042</td>
<td>8851160</td>
<td>9848203</td>
<td>10346658</td>
</tr>
<tr>
<td>Non-splice reads</td>
<td>13258803</td>
<td>11684489</td>
<td>13981260</td>
<td>13323310</td>
<td>12447695</td>
<td>14000953</td>
<td>14147890</td>
<td>13787543</td>
<td>13180056</td>
<td>15552093</td>
<td>17389096</td>
<td>18069383</td>
</tr>
</tbody>
</table>

Note: (1) Total number of clean data; (2) Total number of reads that can be mapped to the reference genome. In general, this number should be larger than 70% when there is no contamination and the correct reference genome is chosen; (3) Number of reads that can be mapped to multiple sites in the reference genome, which is usually less than 10% of the total; (4) Number of reads that can be uniquely mapped to the reference genome; (5) Number of reads that map to the positive strand (+) or the negative strand (-); (6) Splice reads can be segmented and mapped to two exons (also named “junction reads”), whereas non-splice reads (7) can be mapped entirely to a single exon. The ratio of splice reads depends on the insert size used in the RNA-Seq experiments.
5.3.4 Expression quantification

Gene expression level is measured by transcript abundance. In current RNA-Seq analysis, gene expression was estimated by counting the reads that map to genes or exons. Read count is proportional to not only the actual gene expression level but also the gene length and the sequencing depth. In order for the gene expression levels estimated from different genes and experiments to be comparable, the FPKM (Fragments Per Kilobase of transcript per Million mapped reads) is used. In RNA-Seq analysis, FPKM is the most common method of estimating gene expression levels, which takes into account the effects of both sequencing depth and gene length on counting of fragments (Trapnell et al., 2009). HTSeq (v0.6.1) software was used to analyze the gene expression levels in this experiment, using the union mode (Anders et al., 2015). The Result Files present the number of genes that were expressed at different levels and the gene expression level of each single gene. In general, an FPKM value that is smaller than 1 indicates the gene is not expressed. The results of gene expression in the akinβ1 mutant in comparison to WT in response to light or darkness were shown in Appendix A. The results (Table 5.3) show that in the four FPKM ranges, there is little difference in gene expression between the akinβ1 mutant and WT in response to either light or dark condition. However, there are some differences in overall gene expression between the akinβ1 mutant and WT when comparing samples between darkness and light. As an example, around 19% of genes were expressed at an FPKM level (15-60) in the dark, while around 16% of genes were expressed at this level in the light.
Table 5.3 Comparison of overall gene expression levels between WT and the *akinβ1* mutant in darkness and light.

<table>
<thead>
<tr>
<th>FPKM Interval</th>
<th>WT_D1</th>
<th>WT_D2</th>
<th>WT_D3</th>
<th>Mut_D1</th>
<th>Mut_D2</th>
<th>Mut_D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>10578(38.58%)</td>
<td>10508(38.33%)</td>
<td>10540(38.44%)</td>
<td>10723(39.11%)</td>
<td>10320(37.64%)</td>
<td>10158(37.05%)</td>
</tr>
<tr>
<td>1-3</td>
<td>2585(9.43%)</td>
<td>2642(9.64%)</td>
<td>2641(9.63%)</td>
<td>2514(9.17%)</td>
<td>2641(9.63%)</td>
<td>2660(9.70%)</td>
</tr>
<tr>
<td>3-15</td>
<td>7005(25.55%)</td>
<td>7020(25.61%)</td>
<td>7014(25.58%)</td>
<td>6838(24.94%)</td>
<td>6942(25.32%)</td>
<td>7119(25.97%)</td>
</tr>
<tr>
<td>15-60</td>
<td>5108(18.63%)</td>
<td>5085(18.55%)</td>
<td>5017(18.30%)</td>
<td>5175(18.88%)</td>
<td>5268(19.22%)</td>
<td>5286(19.28%)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>2140(7.81%)</td>
<td>2161(7.88%)</td>
<td>2204(8.04%)</td>
<td>2166(7.90%)</td>
<td>2245(8.19%)</td>
<td>2193(8.00%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>FPKM Interval</th>
<th>WT_L1</th>
<th>WT_L2</th>
<th>WT_L3</th>
<th>Mut_L1</th>
<th>Mut_L2</th>
<th>Mut_L3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>10927(39.86%)</td>
<td>10925(39.85%)</td>
<td>11049(40.30%)</td>
<td>11311(41.26%)</td>
<td>11236(40.98%)</td>
<td>10929(39.86%)</td>
</tr>
<tr>
<td>1-3</td>
<td>2998(10.94%)</td>
<td>2961(10.80%)</td>
<td>2916(10.64%)</td>
<td>2804(10.23%)</td>
<td>2785(10.16%)</td>
<td>2767(10.09%)</td>
</tr>
<tr>
<td>3-15</td>
<td>7294(26.60%)</td>
<td>7332(26.74%)</td>
<td>7204(26.28%)</td>
<td>7054(25.73%)</td>
<td>7038(25.67%)</td>
<td>7144(26.06%)</td>
</tr>
<tr>
<td>15-60</td>
<td>4249(15.50%)</td>
<td>4252(15.51%)</td>
<td>4304(15.70%)</td>
<td>4303(15.70%)</td>
<td>4369(15.94%)</td>
<td>4573(16.68%)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>1948(7.11%)</td>
<td>1946(7.10%)</td>
<td>1943(7.09%)</td>
<td>1944(7.09%)</td>
<td>1988(7.25%)</td>
<td>2003(7.31%)</td>
</tr>
</tbody>
</table>
5.3.5 Differential gene expression analysis

Differential gene expression is analyzed by DESeq (v1.10.1) based on the read counts of genes from expression analysis (Anders and Huber, 2010). Four comparisons were performed to identify differentially expressed genes (DEGs): 1) WT in dark vs WT in light; 2) mutant in dark vs mutant in light; 3) mutant vs WT in dark; and 4) mutant vs WT in light. Volcano plots (Figure 5.5) are used to reveal the overall distribution of DEGs (the threshold was set as p-value adjusted (padj)<0.05).

Figure 5.5 Volcano plots for DEGs in the akinβ1 mutant and WT. The x-axis shows the

- Up-regulated genes
- Down-regulated genes
fold change in gene expression between different samples, and the y-axis shows the statistical significance of the differences (the threshold was set as padj<0.05; -log_{10}(padj)>1.3). Significantly up and down regulated genes are highlighted in red and green respectively. Genes which did not express differently between treatment group and control group are in blue.

Cluster analysis (Figure 5.6) is used to identify genes with similar expression patterns in response to light or darkness, and identify the groups of genes with similar functions that exhibit different expression patterns in the akinβ1 mutant and WT in response to light or darkness. By clustering genes with similar expression patterns, it is possible to reveal unknown functions of known genes or putative functions of unknown genes. In the hierarchical clustering, areas of different colors denoted different groups of genes and genes within each cluster may have similar functions or take part in the same biological process. The outcome of this cluster analysis shows that in response to illumination change (i.e. darkness vs light), gene expression of most genes in both the akinβ1 mutant and WT has been changed conversely. Compared with gene expression in WT, further, expression patterns of some genes in the akinβ1 mutant were clearly different from WT in responding to light or dark condition either conversely or with a significantly different expression levels (Figure 5.6).
Figure 5.6 The FPKM cluster analysis of the DEGs in the akinβ1 mutant and WT in response to light and darkness. The results were clustered using the log_{10}(FPKM+1) value. Red denotes genes with high expression levels, and blue denotes genes with low expression levels. The color range from red to blue represents the log_{10}(FPKM+1) value from large to small.

In this study, the comparison of differential gene expression in the akinβ1 mutant and WT in response to light and darkness has been focused on. The comparative results for differential gene expression in the akinβ1 mutant and WT are summarized in Table 5.4.

Table 5.4 Summary of the DEGs identified in the akinβ1 mutant in response to light and darkness.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>The number of DEGs (↑ up-regulated, ↓ down-regulated)</th>
<th>The number of DEGs with a two-fold change (↑ up-regulated or ↓ down-regulated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mut_D vs WT_D</td>
<td>188 (90 ↑, 98 ↓)</td>
<td>17 ↑, 21 ↓</td>
</tr>
<tr>
<td>Mut_L vs WT_L</td>
<td>2485 (1103 ↑, 1382 ↓)</td>
<td>130 ↑, 252 ↓</td>
</tr>
</tbody>
</table>
The Venn diagram (Figure 5.7) shows that 2485 DEGs were identified in the comparison of the akinβ1 mutant with WT in response to light and 188 DEGs were identified in the comparison of the akinβ1 mutant with WT in response to darkness.

![Venn diagram](image)

**Figure 5.7** Venn diagram of DEGs in the akinβ1 mutant and WT in response to light and darkness. 2485 DEGs were identified in the comparison of the akinβ1 mutant with WT in response to light and 188 genes were differentially expressed in response to darkness. 117 genes were expressed differentially in response to both light and darkness.

### 5.3.6 Expression of AKINβ1 in the akinβ1 mutant and WT

As demonstrated in Chapter II, the expression of AKINβ1 is decreased significantly in the akinβ1 mutant compared to WT. The RNA-Seq results show that the expression of AKINβ1 in the mutant was 20% lower than in WT at 1h into the dark period. Whereas the expression of AKINβ1 in the mutant was 43% higher than in WT at 1h into the light period (Table 5.5). qRT-PCR results (Figure 5.8) show that the expression of AKINβ1 in the mutant was about 44% lower than in WT at 1h into the dark period. AKINβ1 expression in the akinβ1 mutant was about 29% higher than in WT at 1h into the light period, consistent with the RNA-Seq results.
Table 5.5 RNA-Seq results for expression of AKINβ1 in the mutant and WT in response to light and darkness.

<table>
<thead>
<tr>
<th></th>
<th>Read count in the light</th>
<th>Read count in darkness</th>
</tr>
</thead>
<tbody>
<tr>
<td>The akinβ1 mutant</td>
<td>220.3</td>
<td>561.5</td>
</tr>
<tr>
<td>WT</td>
<td>153.6</td>
<td>675.8</td>
</tr>
</tbody>
</table>

Figure 5.8 The expression of AKINβ1 gene in the mutant leaves and WT leaves detected by RT-qPCR. The expression of AKINβ1 in the akinβ1 mutant was significantly lower than WT at 1h into the dark period. Expression of AKINβ1 in the akinβ1 mutant was higher significantly than in WT at 1h into the light period. Asterisks represent significant differences at P<0.05. Error bars represent Standard deviation (n=3).

5.3.7 Differentially expressed genes (DEGs)

117 DEGs were identified in the akinβ1 mutant in response to both light and darkness, including 14 DEGs with at least 2-fold change (Table 5.6). The changes in the remaining 103 DEGs were less than 2 fold, including genes encoding chlorophyll a/b binding proteins 2 (AT1G29920; log₂foldchange=-0.7397 (darkness) and -0.35758 (light)) and 3 (AT1G29910; log₂foldchange=-0.77528 (darkness) and -0.55848 (light)) discussed in the previous chapter.

Other relatively small changes in associated gene expression were also observed, such
as glutamyl-tRNA reductase (AT1G58290; \( \log_2 \text{foldchange} = -0.91268 \) (darkness) and -0.31846 (light)) involved in tetrapyrrole biosynthesis (tetrapyrrole such as chlorophyll, heme, siroheme, and phytochromobilin plays vital roles in photosynthesis and respiration (Zhao et al., 2014)), protochlorophyllide oxidoreductase B (AT4G27440; \( \log_2 \text{foldchange} = -0.49023 \) (darkness) and \(-1.6773 \) (light)) in chlorophyll synthesis (Garrone et al., 2015), and glycosylphosphatidylinositol-anchored lipid protein transfer 6 (AT1G55260; \( \log_2 \text{foldchange} = -0.70703 \) (darkness) and \(-0.6226 \) (light)) in lipid transport (Deng et al., 2016).

DEGs exhibiting very large changes in the akinβ1 mutant in comparison to WT (at least 8-fold in the light and at least 4-fold in the darkness) are summarized in Table 5.7. These included genes encoding enzymes of lipid metabolism such as: glycerol-3-phosphate acyltransferase 5 (GPAT5, AT3G11430) which catalyzes the acylation at \( sn-1 \) position of glycerol-3-phosphate to produce lysophosphatidic acid (LPA) that is a substrate for the production of several important glycerolipid intermediates such as extracellular lipid polyesters, storage lipids, and membrane lipids (Chen et al., 2011); 3-ketoacyl-CoA synthase 16 (AT4G34250) which is responsible for fatty acid elongation (biosynthesis of very long chain fatty acids) (Lokesh et al., 2013); and NADP-dependent malic enzyme (ME) 1 (AT2G19900) that catalyzes the oxidative decarboxylation of malate to pyruvate in cytosol and also produces NADPH (Gerrard Wheeler et al., 2005).
Table 5.6 DEGs with minimal 2-fold change from the 117 genes that were expressed differentially in the akinβ1 mutant in comparison to WT in response to both light and darkness.

<table>
<thead>
<tr>
<th>Gene identity</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Down-regulated</strong></td>
<td></td>
</tr>
<tr>
<td>AT4G36850</td>
<td>PQ-loop repeat family protein/transmembrane family protein</td>
</tr>
<tr>
<td>AT5G48490</td>
<td>DIR1-LIKE (a protein with similarity to a lipid transfer protein)</td>
</tr>
<tr>
<td>AT1G22550</td>
<td>Major facilitator superfamily protein</td>
</tr>
<tr>
<td>AT1G66760</td>
<td>Multidrug and toxic compound extrusion protein</td>
</tr>
<tr>
<td>AT4G01870</td>
<td>TolB protein-like protein</td>
</tr>
<tr>
<td>AT2G19640</td>
<td>Histone-lysine N-methyltransferase (ASHR2)</td>
</tr>
<tr>
<td>AT1G11850</td>
<td>Transmembrane protein (F12F1.31)</td>
</tr>
<tr>
<td>AT5G20630</td>
<td>Germin-like protein subfamily 3 member 3</td>
</tr>
<tr>
<td>AT1G23205</td>
<td>Plant invertase/pectin methylesterase inhibitor superfamily protein (T26J12.3)</td>
</tr>
<tr>
<td>AT4G28780</td>
<td>GDSL esterase/lipase</td>
</tr>
<tr>
<td>AT4G30610</td>
<td>Serine carboxypeptidase 24</td>
</tr>
<tr>
<td><strong>Up-regulated</strong></td>
<td></td>
</tr>
<tr>
<td>AT5G62165</td>
<td>MADS-box protein (AGL42)</td>
</tr>
<tr>
<td>AT4G38340</td>
<td>NLP3</td>
</tr>
<tr>
<td>AT1G70260</td>
<td>WAT1-related protein</td>
</tr>
</tbody>
</table>
Table 5.7 DEGs showing larger expression changes (minimal 8 fold in the light and 4 fold in darkness) identified in the akinβ1 mutant, in the comparison to WT.

<table>
<thead>
<tr>
<th>Gene identity</th>
<th>Gene name</th>
<th>log(_2) FoldChange</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Decreased expression in the mutant in the light</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT3G11430</td>
<td>Glycerol-3-phosphate acyltransferase 5</td>
<td>-7.9255</td>
</tr>
<tr>
<td>AT1G03790</td>
<td>Zinc finger CCCH domain-containing protein 2</td>
<td>-6.4217</td>
</tr>
<tr>
<td>AT2G19900</td>
<td>NADP-dependent malic enzyme 1</td>
<td>-6.134</td>
</tr>
<tr>
<td>AT4G21680</td>
<td>Nitrate transporter 1.8</td>
<td>-5.692</td>
</tr>
<tr>
<td>AT3G17520</td>
<td>Late embryogenesis abundant protein (LEA) family protein</td>
<td>-5.34</td>
</tr>
<tr>
<td>AT4G37390</td>
<td>Auxin-responsive GH3 family protein</td>
<td>-5.1405</td>
</tr>
<tr>
<td>AT3G01420</td>
<td>α-dioxygenase 1 (DOX1)</td>
<td>-5.0227</td>
</tr>
<tr>
<td>AT3G60140</td>
<td>β glucosidase 30</td>
<td>-4.8552</td>
</tr>
<tr>
<td>AT2G37390</td>
<td>Sodium potassium root defective 2</td>
<td>-4.4106</td>
</tr>
<tr>
<td>AT2G32660</td>
<td>Receptor like protein 22</td>
<td>-4.3193</td>
</tr>
<tr>
<td>AT3G09220</td>
<td>Putative laccase</td>
<td>-4.1494</td>
</tr>
<tr>
<td>AT1G73260</td>
<td>Arabidopsis Kunitz trypsin inhibitor 1</td>
<td>-4.1063</td>
</tr>
<tr>
<td>AT1G73620</td>
<td>Pathogenesis-related thaumatin superfamily protein</td>
<td>-4.0153</td>
</tr>
<tr>
<td>AT2G42840</td>
<td>Protodermal factor 1</td>
<td>-4.0117</td>
</tr>
<tr>
<td>AT2G35730</td>
<td>Heavy metal transport/detoxification superfamily protein</td>
<td>-3.6469</td>
</tr>
<tr>
<td>AT5G66390</td>
<td>Peroxidase 72</td>
<td>-3.5338</td>
</tr>
<tr>
<td>AT1G14250</td>
<td>GDA1/CD39 nucleoside phosphatase family protein</td>
<td>-3.259</td>
</tr>
<tr>
<td>AT5G50760</td>
<td>Small auxin up-regulated RNA 55</td>
<td>-3.2328</td>
</tr>
<tr>
<td>Gene ID</td>
<td>Description</td>
<td>Expression Ratio</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>AT4G34250</td>
<td>3-ketoacyl-CoA synthase 16</td>
<td>-3.2244</td>
</tr>
<tr>
<td>AT5G42180</td>
<td>Peroxidase 64</td>
<td>3.8246</td>
</tr>
<tr>
<td>AT5G22570</td>
<td>WRKY38</td>
<td>3.6714</td>
</tr>
<tr>
<td>AT2G26020</td>
<td>Plant defensin 1.2b</td>
<td>3.5026</td>
</tr>
<tr>
<td>AT2G40300</td>
<td>Ferritin 4</td>
<td>-2.0892</td>
</tr>
<tr>
<td>AT3G18550</td>
<td>TCP family transcription factor (Branched 1)</td>
<td>2.9429</td>
</tr>
<tr>
<td>AT1G70260</td>
<td>UMAMIT36</td>
<td>2.9288</td>
</tr>
<tr>
<td>AT2G30766</td>
<td>Unknown</td>
<td>2.3663</td>
</tr>
<tr>
<td>AT5G05250</td>
<td>Unknown</td>
<td>2.3394</td>
</tr>
<tr>
<td>AT1G47400</td>
<td>Unknown</td>
<td>2.1695</td>
</tr>
</tbody>
</table>

Increased expression in the mutant in the light

Decreased expression in the mutant in darkness

Increased expression in the mutant in darkness
5.3.8 Gene Ontology enrichment analysis of the DEGs

Gene Ontology (GO, http://www.geneontology.org/) is a major bioinformatics initiative to standardize the presentation of gene and gene product attributes across all species. GO is widely used to describe cellular components, molecular function and biological processes in which genes are involved. The 117 DEGs which overlapped in both dark and light conditions were analyzed by agriGO (http://bioinfo.cau.edu.cn/agriGO/analysis.php). The Significance Level was set at 0.05 and 49 GO terms were enriched significantly such as photosynthesis (GO:0015979), response to stimulus (GO:0050896), macromolecule localization (GO:0033036), cellular nitrogen compound metabolic process (GO:0034641), lipid localization (GO:0010876)/transport (GO:0006869), transport (GO:0006810), response to stress (GO:0006950)/stimulus (GO:0050896) and protein folding (GO:0006457). Six out of the 49 GO terms were related to molecule localization and transport. The selected GO terms are listed in Table 5.8.

Compared with WT, the DEGs with at least 2-fold change in the *akinf1* mutant in response to light and dark conditions respectively were analyzed by agriGO (Significant Level was 0.05). Selected GO terms with DEG of at least 2 fold are listed in Table 5.9.
Table 5.8 Selected GO terms with the 117 DEGs overlapped in both dark and light conditions.

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Ontology</th>
<th>Description</th>
<th>p-value</th>
<th>False discovery rate (FDR)</th>
<th>Differentially expressed genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0015979</td>
<td>P</td>
<td>Photosynthesis</td>
<td>5.9e-08</td>
<td>9.4e-06</td>
<td>AT2G05070, AT1G29920, AT1G29910, AT5G54270, AT2G40300</td>
</tr>
<tr>
<td>GO:0010876</td>
<td>P</td>
<td>Lipid localization</td>
<td>4e-10</td>
<td>1.3e-07</td>
<td>AT1G27950, AT1G12090, AT1G55260</td>
</tr>
<tr>
<td>GO:0051179</td>
<td>P</td>
<td>Localization</td>
<td>0.00028</td>
<td>0.011</td>
<td>AT1G07000, AT1G27950, AT1G22550, AT3G16240</td>
</tr>
<tr>
<td>GO:0034641</td>
<td>P</td>
<td>Cellular nitrogen compound metabolic process</td>
<td>0.0011</td>
<td>0.031</td>
<td>AT1G58290, AT4G27440, AT2G27820, AT3G45640</td>
</tr>
<tr>
<td>GO:0050896</td>
<td>P</td>
<td>Response to stimulus</td>
<td>1.2e-05</td>
<td>0.0011</td>
<td>AT3G27060, AT1G3308, AT1G69850</td>
</tr>
<tr>
<td>GO:0051704</td>
<td>P</td>
<td>Multi-organism process</td>
<td>0.00071</td>
<td>0.023</td>
<td>AT2G37130, AT4G27300, AT1G65790, AT4G27300</td>
</tr>
<tr>
<td>GO:0009628</td>
<td>P</td>
<td>Response to abiotic stimulus</td>
<td>0.002</td>
<td>0.046</td>
<td>AT3G50060, AT5G20630, AT4G02520</td>
</tr>
<tr>
<td>GO:0009507</td>
<td>C</td>
<td>Chloroplast</td>
<td>3.3e-09</td>
<td>1.3e-07</td>
<td>AT2G27820, AT4G19170, AT4G08390, AT4G25100</td>
</tr>
</tbody>
</table>


Table 5.9 Selected GO terms and DEGs with minimal 2-fold change in the akinβ1 mutant in response to light and darkness, compared to WT.

<table>
<thead>
<tr>
<th>Gene identity</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2-fold down-regulated in dark condition (1h into the dark period)</strong></td>
<td></td>
</tr>
<tr>
<td>GO:0006950 (response to stress)</td>
<td></td>
</tr>
<tr>
<td>AT4G25100</td>
<td>Fe-superoxide dismutase 1 (FSD1)</td>
</tr>
<tr>
<td>AT1G07890</td>
<td>Ascorbate peroxidase 1 (APX1)</td>
</tr>
<tr>
<td>AT2G40300</td>
<td>Ferritin 4 (FER4)</td>
</tr>
<tr>
<td>GO:0042221 (response to chemical stimulus)</td>
<td></td>
</tr>
<tr>
<td>AT4G30610</td>
<td>Serine carboxypeptidase 24 precursor</td>
</tr>
<tr>
<td>GO:0050896 (response to stimulus)</td>
<td></td>
</tr>
<tr>
<td>AT5G20630</td>
<td>GERMIN 3 (ATGER3)</td>
</tr>
<tr>
<td>AT1G66760</td>
<td>MATE efflux family protein</td>
</tr>
<tr>
<td><strong>2-fold up-regulated in dark condition (1h into the dark period)</strong></td>
<td></td>
</tr>
<tr>
<td>No significantly enriched GO term</td>
<td></td>
</tr>
<tr>
<td>AT3G18550</td>
<td>TCP transcription factor (AtBRC1)</td>
</tr>
<tr>
<td>AT5G62165</td>
<td>MADS-box transcription factor (AGAMOUS-like 42)</td>
</tr>
<tr>
<td>AT4G38340</td>
<td>NIN-like transcription factor (NIN-like protein 3)</td>
</tr>
<tr>
<td>Gene ID</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>AT5G53450</td>
<td>Transcription factor OBP3-RESPONSIVE GENE 1</td>
</tr>
<tr>
<td>AT5G13740</td>
<td>Zinc induced facilitator 1</td>
</tr>
<tr>
<td>AT1G70260</td>
<td>Usually multiple acids move in and out transporters 36 (UMAMIT36)</td>
</tr>
<tr>
<td>AT1G23020</td>
<td>Ferric reduction oxidase 3 (FRO3)</td>
</tr>
<tr>
<td>AT1G48300</td>
<td>Diacylglycerol acyltransferase 3</td>
</tr>
<tr>
<td></td>
<td><strong>2-fold down-regulated in light condition (1h into the light period)</strong></td>
</tr>
<tr>
<td></td>
<td>GO:0009699 (lipid localization)</td>
</tr>
<tr>
<td>AT1G62510</td>
<td>Lipid-transfer protein</td>
</tr>
<tr>
<td>AT5G48490</td>
<td>Lipid-transfer protein</td>
</tr>
<tr>
<td>AT2G13820</td>
<td>Lipid-transfer protein (Xylogen protein 2)</td>
</tr>
<tr>
<td>AT4G22470</td>
<td>Lipid-transfer protein</td>
</tr>
<tr>
<td></td>
<td>GO:0019748 (secondary metabolic process)</td>
</tr>
<tr>
<td>AT3G11430</td>
<td>Glycerol-3-phosphate sn-2-acyltransferase 5 (GPAT5)</td>
</tr>
<tr>
<td>AT5G25980</td>
<td>Glucoside glucohydrolase 2</td>
</tr>
<tr>
<td>AT5G26000</td>
<td>Thioglucoside glucohydrolase 1</td>
</tr>
<tr>
<td>AT5G17220</td>
<td>Glutathione s-transferase 26</td>
</tr>
<tr>
<td>AT2G23910</td>
<td>NAD(P)-binding Rossmann-fold superfamily protein</td>
</tr>
<tr>
<td></td>
<td>GO:0051726 (regulation of cell cycle)</td>
</tr>
<tr>
<td>AT1G15570</td>
<td>CYCLIN A2;3</td>
</tr>
<tr>
<td>AT1G44110</td>
<td>CYCLIN A1;1</td>
</tr>
<tr>
<td>AT4G34160</td>
<td>CYCLIN D3;1</td>
</tr>
<tr>
<td>AT2G26760</td>
<td>CYCLIN B1;4</td>
</tr>
<tr>
<td></td>
<td>GO:0009699 (phenylpropanoid biosynthetic process)</td>
</tr>
<tr>
<td>AT3G11430</td>
<td>Glycerol-3-phosphate sn-2-acyltransferase 5</td>
</tr>
<tr>
<td>Gene ID</td>
<td>Protein Name</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>AT3G29590</td>
<td>Malonyl-CoA:anthocyanidin 5-O-glucoside-6&quot;-O-malonyltransferase</td>
</tr>
<tr>
<td>AT5G42800</td>
<td>Dihydroflavonol 4-reductase</td>
</tr>
<tr>
<td></td>
<td><strong>GO:0009813 (flavonoid biosynthetic process)</strong></td>
</tr>
<tr>
<td>AT4G22880</td>
<td>Anthocyanidin synthase</td>
</tr>
<tr>
<td>AT1G53520</td>
<td>Fatty-acid-binding protein 3</td>
</tr>
<tr>
<td>AT3G29590</td>
<td>Malonyl-CoA:anthocyanidin 5-O-glucoside-6&quot;-O-malonyltransferase</td>
</tr>
<tr>
<td></td>
<td><strong>GO:0042398 (cellular amino acid derivative biosynthetic process)</strong></td>
</tr>
<tr>
<td>AT1G01480</td>
<td>1-amino-cyclopropane-1-carboxylate synthase 2</td>
</tr>
<tr>
<td>AT2G23910</td>
<td>NAD(P)-binding Rossmann-fold superfamily protein</td>
</tr>
<tr>
<td></td>
<td><strong>GO:0005975 (carbohydrate metabolic process)</strong></td>
</tr>
<tr>
<td>AT4G29360</td>
<td>O-Glycosyl hydrolases family 17 protein</td>
</tr>
<tr>
<td>AT2G22620</td>
<td>Rhamnogalacturonate lyase family protein</td>
</tr>
<tr>
<td>AT1G35910</td>
<td>Trehalose-6-phosphate phosphatase D</td>
</tr>
<tr>
<td>AT1G10640</td>
<td>Pectin lyase-like superfamily protein</td>
</tr>
<tr>
<td>AT2G14620</td>
<td>Xyloglucan endotransglucosylase/hydrolase 10</td>
</tr>
<tr>
<td>AT4G15210</td>
<td>β-Amylase 5</td>
</tr>
<tr>
<td>AT5G40610</td>
<td>Plastidic glycerol-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td></td>
<td><strong>GO:0016798 (hydrolase activity, acting on glycosyl bonds)</strong></td>
</tr>
<tr>
<td>AT1G65610</td>
<td>Glycosyl hydrolase 9A2</td>
</tr>
<tr>
<td>AT4G29360</td>
<td>O-Glycosyl hydrolases family 17 protein</td>
</tr>
<tr>
<td>AT4G25810</td>
<td>Xyloglucan endotransglucosylase/hydrolase 23</td>
</tr>
<tr>
<td>AT1G65310</td>
<td>Xyloglucan endotransglucosylase/hydrolase 17</td>
</tr>
<tr>
<td>AT1G52400</td>
<td>β-glucosidase 1</td>
</tr>
<tr>
<td>AT5G57560</td>
<td>Xyloglucan endotransglucosylase/hydrolase 22</td>
</tr>
</tbody>
</table>

245
| GO:0030234 (enzyme regulator activity) | AT1G08340 | Rho GTPase activating protein with PAK-box/P21-Rho-binding domain |
| AT5G06150 | CYCLIN B 1;2 |
| AT5G20740 | Plant invertase/pectin methylesterase inhibitor superfamily protein |
| GO:0019207 (kinase regulator activity) | AT3G11520 | CYCLIN B1;3 |
| AT5G06150 | CYCLIN B 1;2 |
| AT2G26760 | CYCLIN B1;4 |
| GO:0016491 (oxidoreductase activity) | AT5G39320 | UDP-glucose dehydrogenase 4 |
| AT5G05340 | Peroxidase 52 |
| AT3G49120 | Peroxidase 34 |
| GO:0004553 (hydrolase activity, hydrolyzing O-glycosyl compounds) | AT1G65610 | Glycosyl hydrolase 9A2 |
| AT4G29360 | O-Glycosyl hydrolases family 17 protein |
| AT3G60140 | β-glucosidase 30 |
| AT2G14620 | Xyloglucan endotransglucosylase/hydrolase 10 |
| GO:0003824 (catalytic activity) | AT2G19900 | NADP-dependent malic enzyme 1 |
| AT4G37390 | Auxin-responsive GH3 family protein |
| AT1G06360 | Delta-9 desaturase-like 5 protein |

**2-fold up-regulated in light condition (1h into the light period)**

<p>| GO:0009743 (response to carbohydrate stimulus) | AT4G23810 | WRKY53 transcription factor |</p>
<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT2G46400</td>
<td>WRKY46 transcription factor</td>
</tr>
<tr>
<td>AT3G44350</td>
<td>NAC domain containing protein 61</td>
</tr>
<tr>
<td>AT2G17040</td>
<td>NAC domain containing protein 36</td>
</tr>
<tr>
<td></td>
<td>GO:0006468 (protein amino acid phosphorylation)</td>
</tr>
<tr>
<td>AT5G59680</td>
<td>Leucine-rich repeat protein kinase family protein</td>
</tr>
<tr>
<td>AT1G34420</td>
<td>Leucine-rich repeat transmembrane protein kinase family protein</td>
</tr>
<tr>
<td>AT1G35710</td>
<td>Protein kinase family protein with leucine-rich repeat domain</td>
</tr>
<tr>
<td>AT4G23230</td>
<td>Cysteine-rich receptor-like protein kinase 15</td>
</tr>
<tr>
<td>AT5G39020</td>
<td>Malecin/receptor-like protein kinase family protein</td>
</tr>
<tr>
<td>AT1G65790</td>
<td>Receptor kinase 1</td>
</tr>
<tr>
<td>AT4G18250</td>
<td>Putative receptor serine/threonine kinase</td>
</tr>
<tr>
<td>AT1G51790</td>
<td>Leucine-rich repeat protein kinase family protein</td>
</tr>
<tr>
<td>AT1G56120</td>
<td>Leucine-rich repeat transmembrane protein kinase</td>
</tr>
<tr>
<td></td>
<td>GO:0042221 (response to chemical stimulus)</td>
</tr>
<tr>
<td>AT3G28580</td>
<td>P-loop containing nucleoside triphosphate hydrolases superfamily protein</td>
</tr>
<tr>
<td>AT5G26920</td>
<td>Calmodulin-binding protein 60-like G</td>
</tr>
<tr>
<td>AT3G09940</td>
<td>Monodehydroascorbate reductase 3</td>
</tr>
<tr>
<td>AT2G14560</td>
<td>Late up-regulated in response to <em>hyaloperonospora parasitica</em></td>
</tr>
<tr>
<td>AT1G16420</td>
<td>Metacaspase 8</td>
</tr>
<tr>
<td></td>
<td>GO:0051704 (multi-organism process)</td>
</tr>
<tr>
<td>AT3G57240</td>
<td>β-1,3-glucanase 3</td>
</tr>
<tr>
<td>AT1G33960</td>
<td>AvrRpt2-induced gene 1</td>
</tr>
<tr>
<td>AT3G09940</td>
<td>Monodehydroascorbate reductase 3</td>
</tr>
</tbody>
</table>
5.3.9 KEGG Pathway enrichment analysis of the DEGs

KEGG (Kyoto Encyclopedia of Genes and Genomes) is a collection of manually curated databases dealing with genomes, biological pathways, diseases, drugs, and chemical substances. The Enzyme Commission (EC) number was used as a primary identifier for matching genes in the genome and their encoded enzymes in the metabolic pathway to reconstruct the metabolic pathway from the genome (Kanehisa et al., 2004). About 90 graphical diagrams for the reference metabolic pathways have been established and each reference pathway can be regarded as a network of enzymes (EC numbers) (Kanehisa and Goto, 2000). Organism-specific pathways could be generated automatically by matching enzyme-encoding genes with the EC numbers against the enzyme (EC numbers) networks of the KEGG reference metabolic pathways (Kanehisa, 1996; Masoudi-nejad et al., 2007). The ortholog identifiers were introduced to identify nodes (proteins) in the regulatory pathways, linking with the genomic information (Kanehisa and Goto, 2000). Moreover, the ortholog identifiers are used to replace the EC numbers in the metabolic pathways in order to overcome various problems caused by the EC number-based scheme (e.g. it is unable to distinguish multiple genes that match one EC number) (Kanehisa and Goto, 2000). The KEGG Orthology (KO) is a further extension of the ortholog identifiers, which is a database in which information of molecular functions of genes and proteins is associated with the concept of functional orthologs (Mao et al., 2005). Pathway enrichment analysis identifies significantly enriched metabolic pathways or signal transduction pathways associated with DEGs compared with the whole genome background. The KEGG Pathway enrichment
analysis of the DEGs was performed by employing KOBAS (v2.0; Corrected P-Value<0.05; Mao et al., 2005; Xie et al., 2011) and the result (Table 5.10) shows that in the akinβ1 mutant in darkness, the down-regulated DEGs were significantly enriched in pathways such as photosynthesis (ath00195), photosynthesis-antenna proteins (ath00196), porphyrin and chlorophyll metabolism (ath00860), whereas the up-regulated DEGs were not enriched significantly in any pathway. In the akinβ1 mutant in response to light, the down-regulated DEGs were significantly enriched in KEGG pathway of ribosome (ath03010). The up-regulated DEGs were enriched in pathways involved in plant-pathogen interactions (ath04626), control of the plant circadian rhythm (ath04712), and inositol phosphate metabolism (ath00562).

As examples, the DEGs enriched significantly in KEGG pathway of photosynthesis-antenna proteins (Figure 5.9) included lhcb1.1 (AT1G29920; -1.7 fold), lhcb1.2 (AT1G29910; -1.7 fold), lhcb2.2 (AT2G05070; -1.4 fold), lhcb2.3/4 (AT3G27690; -1.7 fold), lhcb2.1 (AT2G05100; -1.4 fold), and lhcb3*I (AT5G54270; -1.4 fold). The DEGs enriched in KEGG pathway of TCA (ath00020) are showed in Figure 5.10 and Table 5.11 since respiration rate in the akinβ1 mutant decreased (Section 2.3.5).
Table 5.10 Summary of KEGG Pathway enrichment analysis of DEGs in response to light and darkness.

<table>
<thead>
<tr>
<th></th>
<th>Up-regulation or down-regulation of DEGs</th>
<th>Numbers of KEGG pathways enriched significantly</th>
<th>KEGG pathways enriched significantly (Corrected P-Value&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mut_D vs WT_D</td>
<td>Down-regulation</td>
<td>3</td>
<td>Photosynthesis-antenna proteins (ath00196), Photosynthesis (ath00195), Porphyrin and chlorophyll metabolism (ath00860)</td>
</tr>
<tr>
<td></td>
<td>Up-regulation</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Mut_L vs WT_L</td>
<td>Down-regulation</td>
<td>1</td>
<td>Ribosome (ath03010)</td>
</tr>
<tr>
<td></td>
<td>Up-regulation</td>
<td>8</td>
<td>Plant-pathogen interaction (ath04626), plant circadian rhythm (ath04712), inositol phosphate metabolism (ath00562), regulation of autophagy (ath04140), limonene and pinene degradation (ath00903), stilbenoid, diarylheptanoid and gingerol biosynthesis (ath00945), phosphatidylinositol signaling system (ath04070), plant hormone signal transduction (ath04075)</td>
</tr>
</tbody>
</table>
Figure 5.9 DEGs enriched significantly in KEGG pathway of Photosynthesis-antenna proteins (ath00196) in the akinβ1 mutant in response to darkness. Green boxes indicate genes encoding proteins which are down-regulated in the mutant in response to darkness.
Figure 5.10 DEGs enriched in citrate cycle (ath00020) in the *akinβ1* mutant in response to light compared to WT. The numbers in boxes are enzyme commissions. Green boxes indicate the gene encoding the enzyme is down-regulated in the mutant in response to light. Red boxes indicate the gene encoding the enzyme is up-regulated in the mutant in response to light. The DEGs in the labelled boxes are listed in Table 5.10.
Table 5.11 The DEGs enriched in KEGG pathway of citrate cycle (TCA cycle) (ath00020). EC=Enzyme Commission.

<table>
<thead>
<tr>
<th>Gene identity</th>
<th>Gene name</th>
<th>log2FoldChange</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC 2.3.1.12</td>
<td>AT1G34430 Embryo defective 3003</td>
<td>-0.45624</td>
</tr>
<tr>
<td></td>
<td>AT2G34590 Transketolase family protein</td>
<td>-0.44829</td>
</tr>
<tr>
<td>EC 1.2.4.1</td>
<td>AT4G16155 Dihydrolipoamide dehydrogenase</td>
<td>-0.47899</td>
</tr>
<tr>
<td></td>
<td>AT3G16950 Lipoamide dehydrogenase 1</td>
<td>-0.47225</td>
</tr>
<tr>
<td>EC 1.8.1.4</td>
<td>AT4G16155 Dihydrolipoamide dehydrogenase</td>
<td>-0.47899</td>
</tr>
<tr>
<td></td>
<td>AT3G16950 Lipoamide dehydrogenase 1</td>
<td>-0.47225</td>
</tr>
<tr>
<td>EC 2.3.3.8</td>
<td>AT3G06650 ATP-citrate lyase B-1</td>
<td>-0.29536</td>
</tr>
<tr>
<td></td>
<td>AT5G49460 ATP citrate lyase subunit B 2</td>
<td>-0.4003</td>
</tr>
<tr>
<td></td>
<td>AT1G60810 ATP-citrate lyase A-2</td>
<td>-0.86362</td>
</tr>
<tr>
<td>EC 1.1.1.37</td>
<td>AT1G04410 Cytosolic NAD-dependent malate</td>
<td>-0.25289</td>
</tr>
<tr>
<td></td>
<td>dehydrogenase 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AT3G47520 Plastidic NAD-dependent malate</td>
<td>-0.51058</td>
</tr>
<tr>
<td></td>
<td>dehydrogenase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AT5G09660 Peroxisomal NAD-malate dehydrogenase 2</td>
<td>0.26924</td>
</tr>
<tr>
<td>EC 1.2.4.2</td>
<td>AT5G65750 2-oxoglutarate dehydrogenase, E1</td>
<td>0.33375</td>
</tr>
<tr>
<td></td>
<td>component</td>
<td></td>
</tr>
</tbody>
</table>

5.3.10 The expression pattern of AKIN subunit genes and genes related to starch biosynthesis in WT in response to illumination

Based on RNA-Seq data (Table 5.12), in WT, the expression of $AKIN\beta2$ (AT4G16360) decreased in darkness compared to light, whereas the expression of $AKIN\beta1$ and $AKIN\gamma1$ (AT3G48530) significantly increased in darkness. The expression of $AKIN10$ (AT3G01090), $AKIN11$ (AT3G29160), $AKIN\beta3$ (AT2G28060) and $AKIN\beta\gamma$ (AT1G09020) shows no significant change in response to illumination (Table 5.12). Thus, the expression of the three subunits of SnRK1 is regulated by illumination but
not all subunits show the same direction of changes.

Table 5.12 Expression of SnRK1 subunits in Arabidopsis Columbia (Col-0) in response to light and darkness.

<table>
<thead>
<tr>
<th>Gene identity</th>
<th>Gene name</th>
<th>Read count in the light</th>
<th>Read count in darkness</th>
<th>Significant difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT3G01090</td>
<td>AKIN10</td>
<td>361.0</td>
<td>412.1</td>
<td>No</td>
</tr>
<tr>
<td>AT3G29160</td>
<td>AKIN11</td>
<td>333.7</td>
<td>281.5</td>
<td>No</td>
</tr>
<tr>
<td>AT5G21170</td>
<td>AKINβ1</td>
<td>148.8</td>
<td>610.3</td>
<td>Yes</td>
</tr>
<tr>
<td>AT4G16360</td>
<td>AKINβ2</td>
<td>540.8</td>
<td>372.9</td>
<td>Yes</td>
</tr>
<tr>
<td>AT2G28060</td>
<td>AKINβ3</td>
<td>118.9</td>
<td>111.8</td>
<td>No</td>
</tr>
<tr>
<td>AT3G48530</td>
<td>AKINγ1</td>
<td>196.3</td>
<td>596.0</td>
<td>Yes</td>
</tr>
<tr>
<td>AT1G09020</td>
<td>AKINβγ</td>
<td>567.4</td>
<td>489.3</td>
<td>No</td>
</tr>
</tbody>
</table>

5.4 DISCUSSION

Based on both RNA-Seq and qRT-PCR analysis, the expression of AKINβ1 in the mutant has been affected. The expression of AKINβ1 in the mutant was higher than in WT at 1h into the light period, whereas its expression in the mutant was lower than in WT at 1h into the dark period (Table 5.5 and Figure 5.8), showing the T-DNA insertion in the AKINβ1 gene alters the expression pattern of AKINβ1 gene in the mutant. Moreover, the effect of the akinβ1 mutation on TCA cycle intermediates is more significant in the light than the dark as has been observed in Chapter II, which could be associated with a greater reduction in the cytosol-localized AKINβ1 subunit in the akinβ1 mutant in the light than in darkness (see Chapter III). The expression of N-myristoyltransferase 1 (AT5G57020) is the same in both WT and the akinβ1 mutant and does not change in response to changes in illumination. The observation that the effect of the mutation on the expression of AKINβ1 is more marked in the light than in
darkness is consistent with the observation that global effects on gene expression are greater in the light than the dark (Table 5.4).

In this study, changes in gene expression in the \textit{akinβ1} mutant relative to WT are substantial (Figure 5.6). In the light, 2485 DEGs were identified in the \textit{akinβ1} mutant in comparison to WT, including 382 DEGs with 2-fold changes (Table 5.4). 130 DEGs with 2-fold up-regulation are enriched in 16 GO terms that include post-translational protein modification, response to biotic and chemical stimulus and kinase activity. 252 DEGs with 2-fold down-regulation are enriched in 42 GO terms that include some metabolic processes (carbohydrate metabolic process, secondary metabolic process, amino acid derivative biosynthetic process and lipid metabolic process) but also lipid transport and localization. These results reinforce the hypothesis that SnRK1 is a central regulator of carbon, nitrogen, and lipid metabolism (Emanuelle \textit{et al.}, 2016). In darkness, 188 DEGs were identified in the \textit{akinβ1} mutant compared to WT, including 38 DEGs exhibiting \(\geq 2\)-fold change (Table 5.4). Although 17 DEGs were up-regulated by \(\geq 2\) fold, they were not enriched in any GO term. 21 DEGs showed \(\geq 2\)-fold down-regulation and are enriched in 4 GO terms that include response to biotic and chemical stimulus and oxidoreductase activity. Moreover, 117 DEGs that overlapped in response to both light and darkness in the \textit{akinβ1} mutant (Figure 5.7) were enriched in 49 GO terms that include photosynthesis (chlorophyll A/B-binding proteins), lipid localization/transport, localization, macromolecule localization, transport, nitrogen compound metabolic process, response to stress/stimulus and protein folding, showing SnRK1 may also regulate the localization and transport of molecules in cells to maintain
energy balance. The substantial changes in gene expression in the *akinβ1* mutant relative to WT show that SnRK1 is a global regulator in plant biological processes (Figure 5.11).

![Figure 5.11 Biological processes regulated by SnRK1 (AKINβ1) based on data in this study.](image)

The TCA cycle plays an important role in connecting carbohydrate metabolism, nitrogen metabolism, and lipid metabolism in plants. Amino acid biosynthesis (nitrogen metabolism) uses compounds from the TCA cycle (*e.g.* oxaloacetate and 2-oxoglutarate) and glycolysis (*e.g.* phosphoenolpyruvate, pyruvate and 3-phosphoglycerate), and amino acid degradation leads to several metabolites (*e.g.* pyruvate and acetyl-CoA) that are used by the TCA cycle as an energy source (Pratelli and Pilot, 2014). Moreover, 2-oxoglutarate from the TCA cycle is the substrate for nitrogen assimilation *via* the glutamate synthase reaction (Forde and Lea, 2007; Bernard and Habash, 2009). Acetyl-CoA is an important intermediate between lipid metabolism and carbohydrate metabolism. In fatty acid synthesis, the first committed step is considered to be
conversion of acetyl-CoA to malonyl-CoA in plastids (Sasaki and Nagano, 2004). Since acetyl-CoA can not be imported into plastids, it must be generated within the plastid (Rawsthorne, 2002). Plastidic free acetate, pyruvate produced in plastidic glycolysis, as well as pyruvate and malate imported from the cytosol, can serve as substrates for acetyl-CoA production in plastids (Plaxton, 1996; Rawsthorne, 2002). In fatty acid β-oxidation, a long-chain fatty acid is oxidized to yield acetyl-CoA in mitochondria or microbodies (peroxisomes or glyoxysomes) and acetyl-CoA is oxidized to CO₂ via the TCA cycle in mitochondria (Allenbach and Poirier, 2000).

NADP-dependent malic enzymes catalyze the oxidative decarboxylation of malate to form pyruvate (Chang and Tong, 2003) and four isoforms are present in Arabidopsis (Gerrard Wheeler et al., 2005). Gene expression of NADP-dependent malic enzyme 1 (AT2G19900) which catalyzes the oxidative decarboxylation of malate to pyruvate in the cytosol (Gerrard Wheeler et al., 2005) was significantly down-regulated (70 fold) in the akinβ1 mutant in response to light. Expression of NADP-ME 2 (AT5G11670), which is the predominately expressed isoform of NADP-MEs in the cytosol of Arabidopsis leaves (Gerrard Wheeler et al., 2005), was only 1.28-fold up-regulated in the akinβ1 mutant in response to light, and there were no changes in expression of cytosolic NADP-ME 3 (AT5G25880) and plastidic NADP-ME 4 (AT1G79750) in the mutant both in the light and in darkness. Although NADP-dependent ME 1 appears to be expressed preferentially in Arabidopsis roots, and only a very weak cDNA band of the enzyme was detected previously in Arabidopsis leaves using semi-quantitative RT-PCR (Gerrard Wheeler et al., 2005), RNA-Seq can detect rare and low-abundance
transcripts more easily than RT-qPCR by increasing its sequencing depth (the number of sequenced reads for a given sample) (Conesa et al., 2016). Thus, RNA-Seq results show a 70-fold down-regulation in expression of NADP-dependent ME 1 in the mutant leaves compared to WT in the light. Decarboxylation of malate in the cytosol leads to production of pyruvate (that can enter the mitochondrial TCA cycle or plastidic fatty acid biosynthesis) and NADPH (a vital component in both redox homeostasis and assimilatory metabolism). Decreased expression of cytosolic NADP-dependent ME 1 could affect the production of pyruvate in the cytosol with concomitant effects on mitochondrial TCA cycle metabolism. Thus, SnRK1, especially AKINβ1, may regulate the TCA cycle by affecting the contents of cytosolic and mitochondrial malate in Arabidopsis. The content of malate decreased in the mutant leaves (see Chapter II) possibly as a result of significantly decreased expression of cytosolic NADP-dependent malic enzyme 1, leading to a decrease in the content of malate in the mitochondria. In turn, this could lead to a decrease in TCA cycle activity as observed in the akinβ1 mutant (see Chapter II). Moreover, the observation that a significant decrease in the contents of TCA cycle intermediates in the akinβ1 mutant leaves were most marked in the light (see Chapter II) could be related to changes in gene expression in the akinβ1 mutant. Not only was NADP-dependent ME 1 down-regulated 70-fold, GPAT 5 (AT3G11430) was 243-fold down-regulated in the mutant leaves in the light. The latter could affect both carbon flux into glycerol-3-phosphate to form lysophosphatidic acid, and carbon flux into pyruvate production via glycolysis. In turn, this would affect TCA cycle metabolism in the mutant in the light (discussed in more detail later). Given the
significant change in expression of NADP-ME 1 in the leaves of mutants in this study, and that the gene appears to be expressed preferentially in Arabidopsis roots (Gerrard Wheeler et al., 2005), the effects of the akinβ1 mutation on its expression in roots, root respiration and metabolism are worthy of further investigation.

Expression of β-amylase 5 (AT4G15210) was down-regulated 2.8-fold in the mutant in the light compared to WT. This could contribute to increased contents of sucrose and maltose observed in the mutant in the light (see Chapter II) as a result of decreased β-amylase 5-mediated polyglucan degradation. β-amylase hydrolyzes α-1,4 glucosidic bonds from the nonreducing end of polyglucan chains to produce maltose (Kossmann and Lloyd, 2000). β-amylase 5 is a cytosolic enzyme that accounts for up to 80% of total β-amylase activity in leaves (Lin et al., 1988; Monroe et al., 1991; Monroe et al., 2014) but its function is unknown (Monroe et al., 2014). Maltose is exported from the chloroplast to the cytosol predominantly during starch degradation at night and it is further metabolized to polyglucan by cytosolic amylomaltase (disproportioning enzyme 2) and then to glucose and/or sucrose (Sharkey et al., 2004).

Expression of the nitrate transporter (NRT) 1.8 (AT4G21680), an inducible low-affinity transporter in the nitrate assimilatory pathway (Li et al., 2010), was also down-regulated 52-fold in the akinβ1 mutant in response to light. NRT1.8 unloads nitrate from the xylem sap and transports nitrate across the xylem parenchyma cell membrane (Li et al., 2010). Decreased expression of NRT1.8 would decrease the content of nitrate transported, affecting the content of ammonium for nitrate assimilation. Thus this
appears to be coordinated with the decreased content of organic acids, especially 2-oxoglutarate, in the TCA cycle. These results indicates that SnRK1 may regulate nitrate assimilation by affecting nitrate transport in Arabidopsis as well as via organic acid metabolism.

Glycerol-3-phosphate acyltransferase is the first enzyme in the pathway required for the de novo synthesis of membrane and storage lipids, and catalyzes the transfer of an acyl group from acyl-CoA or acyl-ACP to the sn-1 position of glycerol-3-phosphate to form lysophosphatidic acid (Yang et al., 2012) with nine isoforms found in Arabidopsis (Chen et al., 2011). Expression of GPAT5 (AT3G11430), which is bound to the endoplasmic reticulum membrane and can control the accumulation of C22:0- and C24:0-fatty acid, ω-hydroxy fatty acid, and α, ω-dicarboxylic acid monomers in the suberin of roots and seed coats (Beisson et al., 2007; Chen et al., 2011), is down-regulated 243-fold in the mutant compared to WT in the light. Expression of GPAT3 (AT4G01950) was 1.99-fold down-regulated in the akinβ1 mutant in response to light, and there were no changes in expression of other GPAT genes in the mutant both in the light and in darkness. AtGPAT5 can acylate glycerol-3-phosphate at the sn-2 position with α, ω-dicarboxylic acid-CoA to form sn-2 lysophosphatidic acids (Yang et al., 2010; Chen et al., 2011). In plant cells, glycerol-3-phosphate is synthesized either via glycerol kinase-mediated glycerol phosphorylation or via glycerol-3-phosphate dehydrogenase-mediated reduction of dihydroxyacetone phosphate (DHAP) in the cytosol (Venugopal et al., 2009). Moreover, free fatty acids, which are derived from an acyl-ACP-thioesterase-catalyzed fatty acid degradation in plastids, are exported into the cytosol
and are esterified to form acyl-CoA. Acyl-CoA in the cytosol can be used by endoplasmic reticulum membrane-bound GPATs such as GPAT5 to form lysophosphatidic acids in plants (Lee et al., 2016). Significantly down-regulated expression of GPAT5 in the mutant could lead to increased content of glycerol-3-phosphate (slightly increased content of glycerol-3-phosphate was observed in the akinβ1 mutant in Chapter II), affecting carbon flux into glycolytic pyruvate production. Moreover, significantly reduced expression of GPAT5 would affect the content of acyl-CoA in the cytosolic acyl-CoA pool, affecting fatty acid degradation in plastids. Recombinant AMPKα1 and AMPKα2 have been shown to inhibit hepatic mitochondrial GPAT in a time and ATP-dependent manner. Incubation of AMPK heterotrimers that contain either AMPKα1 or α2, and the non-catalytic subunits AMPKβ1 and AMPKγ1, with rat liver mitochondria affected the activity of mitochondrial GPAT (Muoio et al., 1999). Taken together, these results show that SnRK1/AMPK regulates acyl-CoA channeling away from glycerolipid biosynthesis by changing gene expression of GPAT and/or inhibiting activity of GPAT (Muoio et al., 1999). Moreover, α-dioxygenase 1 (AT3G01420, -32 fold), 3-ketoacyl-CoA synthase 16 (AT4G34250, -9 fold), Δ9 desaturase 1 (AT1G06080, -7 fold) that are also involved in lipid metabolism were significantly down-regulated in the akinβ1 mutant in response to light. Interestingly, expression of diacylglycerol acyltransferase 3 (AT1G48300), which is involved in the synthesis of triacylglycerides (Turchetto-Zolet et al., 2011), was up-regulated 3-fold in the mutant in response to darkness.

Plastidic glycerol-3-phosphate dehydrogenase (AT5G40610) was down-regulated 2-
fold in the *akinβ1* mutant in the light. Plastidic glycerol-3-phosphate dehydrogenase catalyzes the reversible redox conversion of glycerol-3-phosphate and DHAP in plastids. Plastidic and cytosolic glycolysis pathways can be connected by exchange of intermediates (PEP, 3-PGA and DHAP) using highly selective transporters located in the inner plastid envelope (Plaxton, 1996). Changes in expression of the glycerol-3-phosphate dehydrogenase gene may affect contents of DHAP in the cytosol and plastids, further affecting carbon flux into glycolysis and the TCA cycle. These results suggest that SnRK1, via AKINβ1, also affects the TCA cycle by regulating expression of genes encoding proteins involved in carbon metabolism, nitrogen assimilation and lipid metabolism in Arabidopsis leaves.

SnRK1 can communicate hormonal signals such as auxins and ABA with metabolism and development (Radchuk *et al*., 2010). Auxin is a plant growth hormone that is able to stimulate differential development in response to gravity or light stimuli (Simmons *et al*., 1995; Briggs, 1963). Indole-3-acetic acid (IAA), the main auxin in higher plants, has profound effects on plant growth and development (Bonner and Wildman, 1946; Bonner and Bandurski, 1952; Zhao, 2010). Auxin responses are concentration-dependent in most tissues, and different tissues respond in a distinct manner to variable amounts of exogenous auxins (Ludwig-Müller, 2011). IAA-amido synthetases synthesize IAA-amino acid conjugates (inactive IAA) by conjugating amino acid such as Asp, Phe, and Trp to auxin *in vitro* (Takase *et al*., 2004; Staswick *et al*., 2005), which is an important regulatory mechanism (auxin conjugates) for auxin homeostasis. In the *akinβ1* mutant, gene expression of IAA-amido synthetases such as GH3.2
(AT4G37390), GH3.9 (AT2G47750), and GH3.6 (AT5G54510) were 35-fold, 3-fold, and 2-fold down-regulated respectively in the light. Moreover, the distribution and homeostasis of auxin is maintained by auxin transporter family proteins that are known as auxin efflux carriers (PINs) (Mohanta et al., 2015). In the akinβ1 mutant, expression of PIN-LIKES 4 (AT1G76530) was up-regulated 2.4-fold in the light. Moreover, small auxin up-regulated RNA (SAUR) belongs to a gene family that is rapidly and transiently induced in response to auxin, but their functions have remained unknown (Markakis et al., 2013). Expression of SAUR55 (AT5G5760) was down-regulated (9 fold) but expression of SAUR33 (AT3G61900) was up-regulated (2 fold) in the akinβ1 mutant in response to light. These results show that SnRK1 may also be involved in auxin signaling by regulating auxin distribution and homeostasis.

*Arabidopsis thaliana* tandem CCCH zinc finger proteins (AtTZFs) are potent regulators of plant growth and stress responses (Bogamuwa and Jang, 2016). Expression of AtTZF4 (AT1G03790) was down-regulated 86-fold in the mutant compared to WT in the light. AtTZF4 is involved in ABA, gibberellin (GA) and phytochrome mediated seed germination responses (Bogamuwa and Jang, 2016). Gibberellin is an important hormone that controls various plant development processes such as seed germination, leaf expansion, stem elongation, flowering, and seed development (Ogawa et al., 2003). GA₄, a biologically active form of gibberellin, induced transcript accumulation of the protodermal factor 1 (PDF1) gene (AT2G42840) mainly in the epidermis of the embryonic axis (Ogawa et al., 2003). Expression of PDF1 was down-regulated 16-fold in the mutant compared to WT in the light, suggesting that SnRK1 may be involved in
the gibberellin signaling pathway by regulating PDF1 gene expression.

WRKY38 (AT5G22570), a salicylic acid-responsive transcription factor, was 13-fold up-regulated in the mutant in the light. WRKY38 is involved in the salicylic acid response such as exogenous SA inhibiting root cell elongation, abolishing certain root ROS maxima and altering root auxin signaling (Bakshi and Oelmüller, 2014). Taken together, these results suggest that SnRK1 is involved in various signaling pathways of phytohormones such as auxin, ABA, gibberellin and salicylic acid by regulating gene expression.

Three genes, FeSOD (AT4G25100), APX1 (AT1G07890) and stromal-APX (AT4G08390), were 3-fold, 2.5-fold, and 2.5-fold down-regulated respectively in the mutant in response to darkness and are involved in the reactive oxygen species (ROS) scavenging network of Arabidopsis. In the ROS scavenging network, major ROS-scavenging enzymes include superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase, glutathione peroxidase and peroxiredoxin, which provides highly efficient machinery to detoxify \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) in cells (Mittler et al., 2004). Hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) is produced predominantly in plant cells during photosynthesis and photorespiration, and to a lesser extent, in respiration processes (Slesak et al., 2007) and \( \text{O}_2^- \) is mainly produced in mitochondria when over-reduction of the electron transport chain occurs under specific stress conditions (Mittler et al., 2004). The ROS scavenging network maintains ROS at a basal non-toxic level, and any deviation from this balance could be used for ROS signaling reactions (Mittler et al., 2004). AKIN10
has previously been shown to regulate gene expression of monodehydroascorbate reductase 6 (AT1G63940), glutaredoxin 13 (AT1G03850), peroxiredoxin-2D (AT1G60740), and thioredoxin H5 (AT1G45145) that are involved in ROS scavenging network (Baena-Gonzalez et al., 2007; Mittler et al., 2004). Thus, SnRK1 may regulate expression of genes involved in the ROS scavenging network, affecting the level of ROS which are important signal molecules that regulate development, differentiation, redox levels, stress signaling, interactions with other organisms, systemic responses, and cell death in higher plants (Mittler et al., 2016). Further, a cysteine-rich receptor-like kinase (CRK) 6 gene (AT4G23140) was 2.6-fold up-regulated in the mutant in the light. The kinase is involved in the response to extracellular O₃-dependent ROS in Arabidopsis (Idänheimo et al., 2014).

Gene expression of trehalose-6-phosphate phosphatase D (AT1G35910) was 2.6-fold down-regulated in the akinβ1 mutant in the light. Modification of the T6P signaling pathway could affect plant growth and development that range from embryo development to leaf senescence (Tsai and Gazzarrini, 2014). The significant decrease in gene expression of trehalose-6-phosphate phosphatase D in the light could lead to increased content of T6P, which would be related to the statistically significant increases in content of T6P in the mutant at 4h into the light and 4h into the dark periods (see Chapter II), although differences in T6P content were not evident at other time points in the light.

SnRK1-induced genes involved in stress signaling have been identified using a large
gene expression compendium of microarray profiles associated with various types of stress (Baena-González and Sheen, 2008). Leucine-rich repeat family proteins (AT1G34420, AT1G35710 and AT5G59680) that are involved in host responses to biotic and abiotic stresses and many developmental processes (Dievart and Clark, 2004; Chinchilla et al., 2009) were up-regulated in the akinβ1 mutant in the light. Moreover, genes encoding receptor-like proteins 23 (AT2G32680), 34 (AT3G11010), 38 (AT3G23120), 41 (AT3G25010) and 43 (AT3G28890) that are involved in various biological processes such as immunity to microbial infection and plant development (Wu et al., 2016) were also up-regulated in the mutant in the light. These results show that SnRK1 plays a role in response to biotic and abiotic stresses.

The gene expression pattern of AKINs in WT in response to illumination (Table 5.12) shows increased expression of AKINβ2 (AT4G16360) in the light, and increased transcription of AKINβ1 in darkness, which is consistent with previous observations (Polge et al., 2008). These results suggest that differential expression of the two AKINβ subunits in response to illumination could impact regulation of the SnRK1 complex. Moreover, both AKINβ1 and AKINβ2 have similar protein structure (Polge et al., 2008) and show 59% amino acid similarity based on BLAST analysis. AKINβ1 localized in the nucleus, the cytosol and the area in between the chloroplasts in tobacco leaves (see Chapter III). AKINβ2 is located in both the cytosol and the nucleus in tobacco leaves as shown by bimolecular fluorescence complementation (BiFC) experiments (Gissot et al., 2006) and AKINβ2 has been shown to interact with the outer membrane of chloroplasts in Arabidopsis leaves (Avila-Castañeda et al., 2014). Perhaps significantly,
both regulatory subunits of SnRK1 exhibit N-myristoylation-mediated subcellular localization (see Chapter III; Pierre et al., 2007). Inhibition of N-myristoylation results in relocalization of AKINβ1 and AKINβ2 from the plasma membrane to the nucleus and the cytosol respectively (Pierre et al., 2007). These results show that AKINβ1 and AKINβ2 have similar subcellular localization in plant leaves. Taken together, the results of expression patterns of AKINβ1 and AKINβ2 in response to illumination change and their subcellular localization suggest that the two subunits regulate the subcellular localization of SnRK1 complex at different times; AKINβ1 in darkness and AKINβ2 in the light respectively. There was no significant change in the expression of AKIN10 (AT3G01090), AKIN11 (AT3G29160), AKINβ3 (AT2G28060) and AKINβγ (AT1G09020) in response to illumination, whereas expression of AKINγ1 (AT3G48530) increased significantly in darkness, suggesting that it may also play a role in regulating SnRK1, alongside regulation of the expression of the AKINβ1 and AKINβ2 subunits.

SnRK1-related regulation of photosynthesis has been shown in transgenic tomatoes overexpressing the MhSnRK1 gene (an α subunit encoding gene from Malus hupehensis Rehd) which showed increased rates of photosynthesis (Wang et al., 2012). In the current study, gene expression of lhcb1.1 (AT1G29920), lhcb1.2 (AT1G29910), and lhcb1.3 (AT1G29930) were up-regulated significantly in WT in the light compared to darkness, showing that these genes are induced in response to light, and consistent with their function as components of the light-harvesting complex II in photosynthesis (Kouril et al., 2005; Galetskiy et al., 2008). Arabidopsis lines that have decreased LHCB1 protein level, achieved by silencing the five lhcb1 genes (lhcb1.1-1.5), possess
decreased chlorophyll content (Pietrzykowska et al., 2014). LHCB1 is necessary for phosphorylation-driven state transitions that regulates photosynthetic light harvesting in plants (Pietrzykowska et al., 2014). Expression of lhcb1.1 (AT1G29920), lhcb1.2 (AT1G29910), and lhcb1.3 (AT1G29930) was down-regulated slightly (1.28 fold, 1.47 fold, and 1.39 fold) in the akinβ1 mutant in comparison to WT in response to light and no significant change in photosynthetic rate was observed in the mutant leaves, although photosynthesis was not investigated in detail (see Chapter II). Putative AKIN11-interacting protein(s), LHCBI.1/LHCBI.2 and/or LHCBI.3, was identified using a chitin-binding domain-based protein pull-down assay (see Chapter IV), which may suggest that a more detailed analysis of photosynthesis could be warranted.

Transcriptome analysis of the akinβ1 mutant leaves shows that the expression pattern of akinβ1 was altered, leading to a significant change in expression of genes encoding proteins involved in various biological pathways. This included metabolic pathways (carbohydrate metabolic process, amino acid derivative biosynthetic process, lipid metabolic process, and secondary metabolic process), several signaling pathways (plant hormones and signaling molecules (T6P and ROS)), and response pathways to biotic and abiotic stress, reinforcing the view that SnRK1 is a global regulator of gene expression in Arabidopsis, including in response to illumination. Moreover, significant changes in expression of genes encoding proteins such as NADP-dependent ME 1, GPAT 5, and β-amylase 5 could lead to decreased TCA activity and changed carbon partitioning in the akinβ1 mutant (see Chapter II). Taken together, alterations in metabolism (Chapter II), the subcellular localization of AKINβ1 (Chapter III), and
transcriptome analysis (this chapter) lead to a hypothesis for AKINβ1-mediated regulation which will be explored in the final chapter.
CHAPTER VI
SUMMARY AND GENERAL DISCUSSION
SnRK1 is an evolutionarily conserved energy-sensing protein kinase and a central integrator of the plant stress and energy starvation signaling pathways (Cho et al., 2016). SnRK1 has been shown to trigger various metabolic and transcriptional reprogramming to maintain energy homeostasis for plant growth and survival in response to decreasing energy levels (Crozet et al., 2014; Hulsmans et al., 2016). Although the importance of SnRK1 has been studied in different species, understanding of the role of its regulatory subunits such as AKINβ1 is limited. The research presented in this thesis investigated the role of the regulatory subunit AKINβ1 on metabolism and transcriptional regulation in Arabidopsis using an Arabidopsis T-DNA insertion mutant with altered expression of akinβ1 (SALK_008325). Subcellular localization of three subunits of SnRK1 (AKINα1, AKINβ1 and AKINγ1) effects on metabolism, interactions with other proteins, and effects on gene expression were investigated to understand detailed functions of SnRK1 in regulating mechanism in Arabidopsis leaf cells.

6.1 Changes in AKINβ1 expression affects the TCA cycle and respiration in Arabidopsis leaf cells over a diurnal cycle

The TCA cycle is an important aerobic pathway for the final steps of the oxidation of carbohydrates, fatty acids and proteins, releasing carbon dioxide and leading to production of chemical energy in the form of ATP. In this thesis, the concentrations of intermediates (citrate, aconitate, isocitrate, 2-OG, succinate, fumarate and malate) in the TCA cycle of akinβ1 mutant leaves were found to be significantly reduced compared to the WT, and this was accompanied by a decreased in respiration rate (see Chapter II). Analysis of the product/reactant ratios of TCA cycle intermediates and
pyruvate also shows that regulation of carbon flux in the TCA cycle is altered in the akinβ1 mutant. In addition, the contents of sugars and their intermediates such as sucrose, maltose, G1P, G6P, F6P, and UDPG were slightly higher in the akinβ1 mutant during the light period compared to WT. Similar results were also observed in SnRK1-repressed pea embryos (Radchuk et al., 2010). Thus, it can be postulated that SnRK1 plays a regulatory role in TCA cycle metabolism and positively regulates respiration in source organs by regulating carbon partitioning between primary metabolism and the TCA cycle.

Changes in metabolism in the akinβ1 mutant were also revealed using RNA-Seq (Chapter V). For example, expression of glycerol-3-phosphate acyltransferase 5 (AT3G11430) and NADP-dependent malic enzyme 1 (AT2G19900) were markedly down-regulated (234 fold and 70 fold). As discussed in Chapter V, changes in expression of both genes could affect carbon flux into pyruvate production via glycolysis as well as the contents of cytosolic and mitochondrial malate in Arabidopsis leaves, contributing to alterations observed in the TCA cycle in the mutant (see Chapter II). Thus, the decrease in the contents of intermediates in the TCA cycle in the akinβ1 mutant leaves (Chapter II) during the diurnal cycle, especially in the light, can be understood, at least partially, by changes in gene expression regulated by SnRK1.

It is possible that SnRK1 can also affect carbon flux into the TCA cycle by regulating activity of its substrate proteins that locate in different cellular compartments (e.g. cytosol, chloroplasts and nucleus). These cellular compartments are the cellular
location of the catalytic subunits of SnRK1 (AKIN10 and AKIN11) (see Chapter III; Fragoso et al., 2009; Bayer et al., 2012; Tsai and Gazzarrini, 2012; Nietzsche et al., 2014; Williams et al., 2014). For example, two potato SnRK1 proteins, PKN1 and StubSNF1, have been shown to interact with a potato cytosolic pyruvate kinase (glycolysis) in vitro, and both SnRK1s influence cytosolic PK activity in the SnRK1-repressed potato lines in vivo (Beczner et al., 2010). This could also have effects on the concentration of pyruvate available to enter the TCA cycle in the mitochondria. Moreover, AKINα1 (AKIN11) can interact with LHCBS in chloroplasts (see Chapter III), which could affect the formation of PSI-LHCII complexes and the absorption capacity of PSI (Pesaresi et al., 2009; Järvi et al., 2011). This could affect photosynthesis in Arabidopsis leaves, leading to changes in carbon flux in the akinβ1 mutant leaves.

SnRK1 has also been shown to play a regulatory role in nitrogen and lipid metabolism (Radchuk et al., 2010; Zhai et al., 2017). 2-oxoglutarate, an intermediate in the TCA cycle, is also a substrate for amino acid, glucosinolate, flavonoid, alkaloid, and gibberellin biosynthesis (Araújo et al., 2014) and change in 2-oxoglutarate in the TCA cycle were observed (see Chapter II). The contents of amino acids (e.g. glutamine, proline and 4-aminobutyrate) were decreased in SnRK1-repressed pea embryos (Radchuk et al., 2010). Moreover, AKIN10 positively regulates WRII degradation via AKIN10-dependent phosphorylation of WRII (Zhai et al., 2017). WRII is a transcription factor and can induce expression of genes (e.g. plastidial pyruvate kinases and plastidial acetyl-CoA carboxylase BCCP2 subunit) involved in plastidial glycolysis.
and fatty acid biosynthesis (Baud et al., 2007; Baud et al., 2009; Zhai et al., 2017). Down-regulated expression of these WRI1-induced genes (pyruvate kinases (AT5G52920 and AT3G22960, -1.49 fold and -1.27 fold)), acetyl-CoA carboxylase BCCP2 subunit (AT5G15530, -2.25 fold), and phosphoenolpyruvate enolase (AT1G74030, -1.36 fold)) were observed in the akinβ1 mutant, in comparison to WT, in the light (see Chapter V). Although not as dramatic as some of the changes in other genes, this suggests that expression of these WRI1-induced genes has been affected in the mutant. Although the TCA cycle and fatty acid biosynthesis are organized in distinct compartments (mitochondria and plastids) in plants (Rawsthorne, 2002), plastidic and cytosolic glycolysis can interact via transporters present in the inner plastid envelope, by which intermediates such as G1P, G6P, DHAP, 3-PGA and PEP can be transported between the two glycolytic pathways (Plaxton, 1996). Moreover, cytosolic malate can be transported from cytosol to plastids and mitochondria, and can also be decarboxylated to form pyruvate by NAD-dependent malic enzymes in cytosol and plastids (Plaxton, 1996; Gerrard Wheeler et al., 2005). The sharing of intermediates between cytosol, plastids and mitochondria facilitates a relationship between plastidial fatty acid biosynthesis and the mitochondrial TCA cycle, affecting the content of acetyl-CoA that is a substrate in the first step of the TCA cycle and in fatty acid biosynthesis. Thus, SnRK1 could also play a complex regulatory role in the interaction between nitrogen and lipid metabolism, which would be related to changes in carbon flux into the TCA cycle.
6.2 N-myristoylation affects the subcellular localization of AKINβ1

In this thesis, N-myristoylation-dependent Golgi-localization of AKINβ1 was revealed in transient expression assays using tobacco leaves (see Chapter III). Wild-type AKINβ1 co-localizes with Golgi and in the area in between the chloroplasts. However, truncated AKINβ1 (lacking N-terminal 74 amino acids) was found only in the cytosol, whereas the AKINβ1(G2A) mutant protein, preventing N-myristoylation of the subunit, co-localized within cytosol and the nuclei (see Chapter III). These results therefore also suggest that the 74-amino acid N-terminal peptide possesses a putative nuclear localization signal, as well as N-myristoylation site on Gly 2 leading to AKINβ1 binding to Golgi. This hypothesis is also supported by the predicted subcellular localization of the 74 amino-acid peptide when using PSORT II software (https://psort.hgc.jp/form2.html), indicating a nuclear localization. The latter is consistent with the observation that AKINβ1(G2A) re-localizes to the nucleus in onion epidermal cells (Pierre et al., 2007). In fact, the 74 amino-acid N-terminal peptide of AKINβ1 is a predicted chloroplast transit peptide using an online bioinformatics tool (ChloroP), which was one of the original important rationales for this project. However, the subcellular localization of wild-type AKINβ1 in both tobacco leaf transient expression and Arabidopsis stable expression showed that it was found within areas surrounding the chloroplasts not within the organelle (see Chapter III), indicating that some caution is needed when interpreting bioinformatic evidence.

Based on the localizations of wild-type AKINβ1 and AKINβ1 (G2A) mutant, a hypothesis is proposed that N-myristoylated AKINβ1 localizes to Golgi stacks during
illumination, but re-localizes to the nucleus and cytosol in darkness. The partitioning of N-myristoylated AKINβ1 between the Golgi and cytosol/nucleus is N-myristoyltransferase-dependent alteration in response to illumination change (see Chapter III). This model is consistent with a proposed role for SnRK1 in regulating gene expression in the nucleus and phosphorylation of substrate proteins such as PK, SPS and F2KP in the cytosol (Kulma et al., 2004; Baena-González et al., 2007; Cho et al., 2016).

6.3 SnRK1 could interact with LHCB1 protein(s)

In this thesis, MS analysis was used to confirm that full-length, soluble AKINα1, AKINβ1 and the truncated AKINβ1 were successfully obtained as recombinant fusion proteins (see Chapter IV). AKINα1 and AKINβ1 were used in protein-protein interaction studies in an attempt to identify potential interacting proteins from lysates of either Arabidopsis leaves or purified chloroplasts. LHCB1.1/LHCB1.2 and/or LHCB1.3 were identified as putative interacting protein(s) with AKINα1 (see Chapter IV). This is certainly plausible given the plastidic localization of AKINα1 subunits (see Chapter III; Fragoso et al., 2009). Moreover, all LHCB1.1, 1.2 and 1.3 proteins possess the minimal recognition motif for SnRK1 (Figure 4.15) that is L-X-H-X-S-X-X-I (Weekes et al., 1993). The crystal structure of spinach LHCB1.3 (PDB entry 4LCZ) indicates that this recognition motif is on the stromal surface of LHCII (Wan et al., 2014), and its amino acid sequence is 93% identical to that of Arabidopsis LHCB1.3. LHCB1.1, 1.2 and 1.3 proteins have been shown to be phosphorylated (Bellafiore et al., 2005; Pietrzykowska et al., 2014; Mekala et al., 2015) by the protein kinases, STN7.
and 8, responsible for LHCII phosphorylation (Bellaiole et al., 2005; Bonardi et al., 2005; Leoni et al., 2013). However, LHCBI and LHCB2 are also phosphorylated at other sites and these are unlikely to be catalyzed by either STN7 or STN8 (Ingelsson and Vener, 2012), implying that other protein kinase(s) are likely to be involved in LHCBI protein phosphorylation. These results suggest that SnRK1 could phosphorylate LHCBI proteins in chloroplasts.

It has been proposed that SnRK1 activity could increase leaf photosynthetic rate (Wang et al., 2012). In plants, when PSII is preferentially excited, LHCII is phosphorylated and attaches to PSI (Allen, 1992), which forms a PSI-LHCII complex and increases the absorption capacity of PSI (Pesaresi et al., 2009; Järvi et al., 2011). When light intensity increases, the phosphorylation level of LHCII decreases, whereas, when light intensity decreases, the phosphorylation of the LHCII proteins strongly increases (Rintamäki et al., 1997; Rintamäki et al., 2000). Moreover, expression of Lhcb1.1 (AT1G29920), Lhcb1.2 (AT1G29910) and Lhcb1.3 (AT1G29930) were down-regulated in the akinβ1 mutant relative to WT in both light and darkness (see Chapter V). These results show that SnRK1 could regulate photosynthesis by phosphorylating LHCBI proteins as well as by regulating their expression to increase carbon assimilation and maintain energy balance in plants.

6.4 SnRK1, especially AKINβ1, is a global effector of gene expression in plants

SnRK1 regulates expression of a variety of genes encoding proteins involved in carbohydrate metabolism, protein metabolism and nucleotide metabolism (Baena-Gonzalez et al., 2007). AKIN10 and AKIN11 (AKINα1) are localized within the
nucleus, as well as the chloroplasts (see Chapter III, Fragoso et al., 2009, Tsai and Gazzarrini, 2012, Williams et al., 2014, Nietzsche et al., 2014), consistent with regulation of gene expression. In this thesis, a clear and significant change in gene expression in the akinβ1 mutant, in comparison to WT, in response to illumination change has been shown (see Chapter V), which is related to altered expression of AKINβ1 in the mutant. 2673 DEGs were identified in the akinβ1 mutant, in comparison to WT, in response to light or darkness, including 420 DEGs with 2-fold changes. These DEGs were involved in various biological pathways including carbohydrate, lipid, amino acid, and nitrogen metabolism, as well as secondary metabolism, signal transduction and genes which respond to biotic and chemical stimuli, as well as the cell cycle. These results show that SnRK1 is a central regulator integrating carbon, nitrogen, and lipid metabolism (Emanuelle et al., 2016) by regulating gene expression.

In summary, the results presented in this thesis have demonstrated that changes in expression of AKINβ1 in the akinβ1 mutant leads to significantly decreased TCA cycle and significant changes in gene expression in the akinβ1 mutant compared to WT. Moreover, N-myristoylation on Gly 2 of AKINβ1 subunit and N-terminal 74 amino acids of AKINβ1 may play an important role in regulating the subcellular localization of AKINβ1 in vivo. Significantly decreased TCA cycle activity and significant changes in gene expression were observed in the akinβ1 mutant compared to WT, notably in the light.
Based on the results obtained in this thesis, a model of the regulatory role of AKINβ1 in carbohydrate metabolism (the TCA cycle) and gene expression in Arabidopsis leaves is hypothesized (Figure 6.1). In the light, AKINβ1 can be N-myristoylated and bind to Golgi stacks. This would decrease the formation of functional SnRK1 in the cytosol, decreasing SnRK1 activity in leaf cells. In darkness, N-myristoylated AKINβ1 would re-localize to the cytosol, possibly involving changes in its conformation brought about by some other mechanism. This would facilitate binding of AKINβ1 to AKIN10/11 and AKINβγ to form functional SnRK1 in the cytosol, directing the kinase to the nucleus or remaining in the cytosol. Functional SnRK1 could then regulate gene expression in the nucleus and/or directly interact with proteins in the cytosol, leading to changes in carbon partitioning and TCA cycle activity.

**Figure 6.1** Proposed model of regulation of AKINβ1 and its effects on the TCA cycle and gene expression.
Future investigations should be focused on identifying the putative substrate proteins of SnRK1 and the specific regulatory mechanism affecting the TCA cycle. Activities of enzymes involved in the TCA cycle and glycolysis could be determined along with measurements of the content of individual enzymes (e.g. using antibodies) to determine whether their activities have been modified post-translationally. Secondly, additional putative interacting proteins of AKINα1 and AKINβ1 should be identified. As well as more detailed co-immunoprecipitation studies, this could include using yeast two-hybrid system with cDNA libraries prepared from Arabidopsis rosette leaves cultured under stress conditions such as extended darkness. Thirdly, the relationship between the activity of SnRK1 and N-myristoylation of AKINβ subunits needs to be established. The subcellular localizations of AKINβ1 or AKINβ2 should also be investigated during light and darkness, using GFP-fusion proteins, and post-translational modifications of both analyzed by using mass spectrometry. Wild-type Arabidopsis could be used to measure activity of SnRK1 during a diurnal cycle using a peptide kinase assay (AMARA and SAMS). Coupled with analysis of post-translational modifications of both AKINβ1 and AKINβ2 (e.g. using mass spectrometry), investigation of whether N-myristoylation of AKINβ subunits affect SnRK1 activity in vivo could be determined. Finally, the subcellular localizations of all subunits of SnRK1 (AKINα1, AKINα2, AKINβ1, AKINβ2, AKINβ3, AKINγ and AKINβγ) under the control of their native promoters should be determined, using agrobacterium-based tobacco transient expression and Arabidopsis stable expression. This would help to fully understand the physiological functions and metabolism in which each subunit of SnRK1 is involved.
It has been shown that SnRK1 plays a significant role in regulating carbon metabolism, as well as affecting expression of genes encoding proteins involved in carbon, lipid and nitrogen metabolism. This has significant applications for understanding SnRK1-dependent regulation mechanism in global plant metabolism and plant growth and development, which could facilitate crop improvement.
REFERENCES


Bojunga N and Entian KD (1999). Cat8p, the activator of gluconeogenic genes in Saccharomyces cerevisiae, regulated carbon source-dependent expression of NADP-dependent cytosolic isocitrate dehydrogenase (Idp2p) and lactate permease (Jen1p). Molecular Genetics and Genomics, 262: 869-875.


LOSS, a trihelix transcription factor that represses growth in *Arabidopsis thaliana*, binds the energy-sensing SnRK1 kinase AKIN10. The Journal of Experimental Botany, 66(9): 2475-2485.


Eastmond PJ, van Dijken AJ, Spielman M, Kerr A, Tissier AF, Dickinson HG, Jones JD, Smeekens SC and Graham IA (2002). Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for Arabidopsis embryo


Gao XQ, Liu CZ, Li DD, Zhao TT, Li F, Jia XN, Zhao XY and Zhang XS (2016). The Arabidopsis KINβγ subunit of the SnRK1 complex regulates pollen hydration on
the stigma by mediating the level of reactive oxygen species in pollen. PLOS Genetics, 12(7): e1006228.


Halford NG and Hey SJ (2009). Snf1-related protein kinases (SnRKs) act within an intricate network that links metabolic and stress signalling in plants. The Biochemical Journal, 419: 247-259.


Nunes C, Primavesi LF, Patel MK, Martinez-Barajas E, Powers SJ, Sagar R, Fevereiro PS, Davis BG and Paul MJ (2013). Inhibition of SnRK1 by metabolites: tissue-dependent effects and cooperative inhibition by glucose 1-phosphate in
combination with trehalose 6-phosphate. Plant Physiology and Biochemistry, 63: 89-98.


Oshlack A, Robinson MD and Young MD (2010). From RNA-Seq reads to differential expression results. Genome Biology, 11: 220.


coordinates metabolic and hormonal signals during pea cotyledon growth and differentiation. The Plant Journal, 61: 324-338.


Raran-Kurus S and Waugh DS (2012). The ability to enhance the solubility of its fusion partners is an intrinsic property of maltose-binding protein but their folding is either spontaneous or chaperone-mediated. PLoS ONE, 7: e49589.


ferredoxin-thioredoxin system in chloroplasts. PNAS USA, 97: 11644-11649.


Sugden C, Crawford RM, Halford NG and Hardie DG (1999a). Regulation of spinach SNF1-related (SnRK1) kinases by protein kinases and phosphatases is associated with phosphorylation of the T loop and is regulated by 5’-AMP. Plant Journal, 19: 433-439.


Wu SB and Wei YH (2011). AMPK-mediated increase of glycolysis as an adaptive response to oxidative stress in human cells: implication of the cell survival in


APPENDIX
Appendix A: The differentially expressed genes in the akinβ1 mutant in response to light or darkness, compared to WT. These DEGs are listed in a excel file. Mut_LvsWT_L.DEG_up or Mut_LvsWT_L.DEG_down indicates the up-regulated or down-regulated DEGs in the akinβ1 mutant in response to light, compared to WT. Mut_DvsWT_D.DEG_up or Mut_DvsWT_D.DEG_down indicates the up-regulated or down-regulated DEGs in the akinβ1 mutant in response to darkness, compared to WT.