Biochemical Characterization of Sinapate Ester Metabolism in *Arabidopsis thaliana* in Response to Transient Abiotic Stress

by

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ABSTRACT

BIOCHEMICAL CHARACTERIZATION OF SINAPATE ESTER METABOLISM IN *ARABIDOPSIS THALIANA* IN RESPONSE TO TRANSIENT ABIOTIC STRESS

Kevin Neil **Advisor:**

University of Guelph, 2015 **Gale G. Bozzo**

Flavonol bisglycosides and sinapate esters are metabolites that accumulate in plants with abiotic stress. In *Arabidopsis thaliana*, flavonol bisglycoside levels increase during nitrogen deficiency and low temperature (NDLT), and are lost during recovery from NDLT. Flavonol 3-*O*-β-glucoside-7-*O*-α-rhamnoside catabolism is dependent upon β-glucosidase (i.e., BGLU15) activity. Here, 1-*O*-sinapoyl-β-D-glucose and 1,2-bis-*O*-sinapoyl-β-glucoside levels increased in *Arabidopsis* after 7 d of NDLT, and gradually disappeared within 5 d of recovery. BGLU16 is phylogenetically related to BGLU15, and their transcript levels increased during recovery from NDLT. Following expression in bacteria and purification to homogeneity, biochemical assays revealed that the recombinant BGLU16 utilizes the artificial substrate *p*-nitrophenyl-β-D-glucoside and quercetin 3-*O*-β-glucoside; other flavonol glycosides were not hydrolyzed. BGLU16 incubated with an *Arabidopsis* extract containing phenylpropanoids and flavonols yielded sinapate as the product, implying that BGLU16 hydrolyzes sinapate esters. This information is important for designing biochemical studies aimed at elucidating the relevance of sinapate ester catabolism in plants.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	absorbance
At	*Arabidopsis thaliana*
BGLU	β-glucosidase
brt1	*bright trichomes 1*
°C	degrees Celsius
C	carbon
d	day
DAD	diode array detector
EDTA	ethylenediaminetetraacetic acid
FAH	*FERULIC ACID 5-HYDROXYLASE*
FM	fresh matter
FPLC	fast protein liquid chromatography
g	gravity
g	gram(s)
GC-MS	gas chromatography-mass spectroscopy
GH1	glycoside hydrolase family 1

h	hour(s)
Heynh.	Heynhold
IMAC	immobilized metal ion affinity chromatography
IPTG	isopropyl β-D-1-thiogalactopyranoside
K3G7R	kaempferol 3-O-β-glucoside-7-O-α-rhamnoside
K3R7R	kaempferol 3-O-α-rhamnoside-7-O-α-rhamnoside
K3Ru7R	kaempferol quercetin 3-O-β-rutinoside-7-O-α-rhamnoside
kDa	kiloDalton
L.	Linnaeus
m/z	mass to charge ratio
MES	2-morpholinoethanesulfonic acid
µg	microgram(s)
µL	microlitre
µmol	micromole(s)
µM	micromolar
mAU	milliabsorbance units
mg	milligram(s)

mM	millimolar
min	minute(s)
MS	mass spectroscopy
MYB	transcription factor: myeloblastoma
NDLT	nitrogen deficiency and low temperature
nmol	nanomole(s)
NSHT	nitrogen sufficiency and high temperature
Os	*Oryza sativa*
PAP	production of anthocyanin pigment
pmol	picomole(s)
*p*NP	*p*-nitrophenyl
*p*NP-glucoside	*p*-nitrophenyl-β-D-glucoside
Q3G	quercetin 3-O-β-glucoside
Q3G7R	quercetin 3-O-β-glucoside-7-O-α-rhamnoside
Q3R7R	quercetin 3-O-α-rhamnoside-7-O-α-rhamnoside
Q3Ru7R	quercetin 3-O-β-rutinoside-7-O-α-rhamnoside
REF	*REDUCED EPIDERMAL FLUORESCENCE*

s	second(s)
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
SD	standard deviation
SE	standard error
T-DNA	transfer deoxyribonucleic acid
TT	*TRANSPARENT TESTA*
UDP	uridine diphosphate
UGT	uridine diphosphate-glycosyltransferase
UHPLC	ultra high performance liquid chromatography
UV	ultraviolet
V	volts

CHAPTER ONE – LITERATURE REVIEW

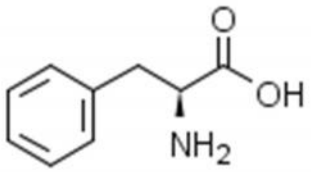
1.1 INTRODUCTION

This literature review discusses the biological role of phenylpropanoids and their derived metabolites in plants. Emphasis will be placed on the structurally-related natural products, flavonols and sinapate esters, including accumulation of their glycosylated end products with abiotic stress. Current literature on the biochemistry governing the disappearance of these small molecule glycosides during the recovery from abiotic stress *in planta* will also be considered. Here, details on the biochemical properties of plant β-glucosidases (BGLUs) will be provided.

1.2. PHENYLPROPANOID CHEMISTRY

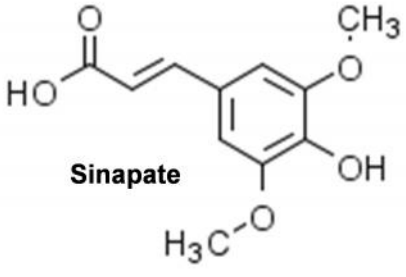
Phenylpropanoids are a large family of secondary metabolites produced exclusively in plants, which includes hydroxycinnamic acids; derived compounds include coumarins, lignins, lignans, and flavonoids (Fig. 1.1). All aforementioned metabolites are derived from *p*-coumaroyl CoA, a metabolic intermediate formed from the aromatic ring of phenylalanine via the concerted action of phenylalanine ammonia lyase, cinnamate-4-hydroxylase and *p*-coumaroyl-CoA-ligase (Dixon and Paiva, 1995; Fraser and Chapple, 2011). Diversity of phenylpropanoids and their derived flavonols is afforded by various hydroxylation, cyclization, methoxylation, methylation, prenylation, acylation and glycosylation events (Vogt, 2010). Glycosylation of these molecules renders them more soluble in the vacuole, although their bioactivities could be compromised *in planta* (Lim and Bowles, 2004; Vaistij et al., 2009).

A.

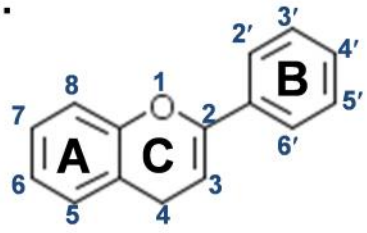


Phenylalanine

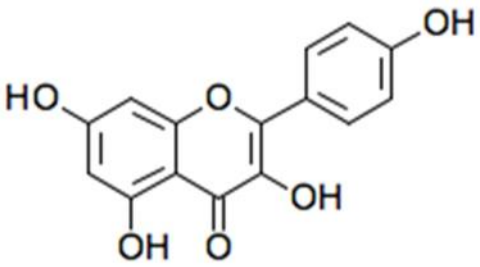
B.



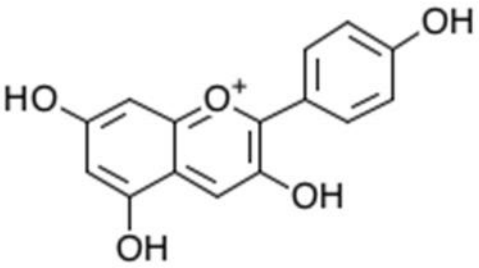
C.



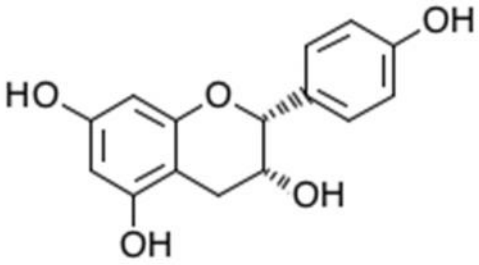
Flavonoid



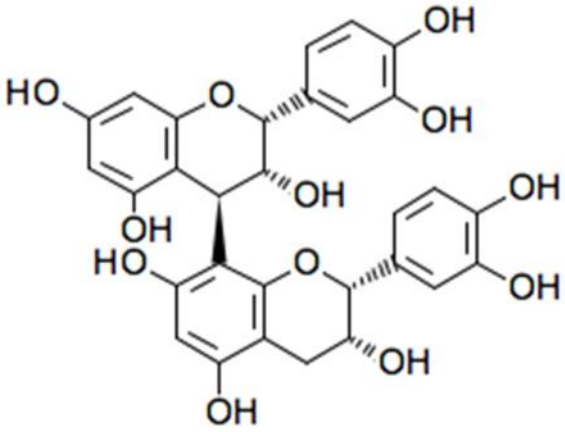
Flavonol



Anthocyanidin



Flavan-3-ol



Proanthocyanidin

Figure 1.1. Structural diversity of phenylpropanoids and their derived metabolites. (A) Phenylalanine is the common precursor of all phenylpropanoids. (B) Sinapate is an example of hydroxycinnamate esters formed from the phenylpropanoid derivative *p*-coumarate. (C) The flavonoid backbone is generated from *p*-coumarate and 3 malonyl-CoA molecules; it consists of aromatic A and B rings fused together by a heterocyclic C ring. The level of C ring oxidation is the distinguishing feature between flavonoid subclasses, including flavonols, flavan-3-ols, anthocyanidins and proanthocyanidins.

1.3. FLAVONOL ANABOLISM

Flavonoids represent the largest sub-class of phenylpropanoid-derived compounds occurring in higher plants, non-flowering liverworts, and horsetails (Shaw et al., 2006; Pereira et al., 2009; Martens et al., 2010; Cartea et al., 2011). Flavonoids tend to reside within the epidermis of plant leaves during exposure to UV light, possibly providing photo-protection to underlying photosynthetic mesophyll cells, although more recent evidence suggests their distribution within both tissues (Sheahan, 1996; Agati and Tattini, 2010). To date, approximately 10,000 flavonoids have been described, and thus are the third largest family of plant secondary metabolites, after alkaloids and terpenoids (Martens et al., 2010). Structurally, flavonoids consist of two aromatic rings (A and B rings) fused together by a three carbon bridge (C ring) (Fig. 1.1). Flavonoids are sub-classified into nine groups based on the degree of oxidation of the C ring. These sub-classes include flavanones (dihydroflavones), dihydroflavonols, flavan-3,4-diols, anthocyanidins, flavones, flavonols, flavan-3-ols, proanthocyanidins, and isoflavonoids, with the latter group being prominent only in legumes (Fig.1.1; Winkel-Shirley, 2002; Martens et al., 2010). In addition, these compounds can be modified by prenylation, methylation, acylation, and glycosylation (Laflamme et al., 1993; Hatano et al., 1997; Schröder et al., 2004; Cartea et al., 2011). In the model plant, *Arabidopsis thaliana* (L.) Heynh., flavonols and anthocyanins co-occur in vegetative tissues, whereas proanthocyanidins and flavonol predominate in seeds (Lepiniec et al., 2006; Routaboul et al., 2006; Saito et al., 2013). In this species, flavonols exist as the glycosides of isorhamnetin, kaempferol and quercetin (Saito et al., 2013). The flavonol aglycone backbone is derived from phenylalanine by the concerted action of phenylalanine

ammonia lyase, cinnamate 4-hydroxylase, *p*-coumaroyl-CoA ligase, chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, flavonoid 3'-hydroxylase, and flavonol synthase (Fig. 1.2; Fraser and Chapple, 2011). Notably, the intermediate *p*-coumaroyl-CoA formed via *p*-coumaroyl-CoA ligase is a precursor for hydroxycinnamic acids, as well as flavonoids (Fraser and Chapple, 2011). Production of the flavonol, kaempferol, depends upon a fully functional biosynthetic pathway, including flavanone 3-hydroxylase (an enzyme forming dihydrokaempferol from naringenin) and flavonol synthase, both of which are induced in *Arabidopsis* following UV-B exposure (Pelletier and Shirley, 1996; Pelletier et al., 1997). Dihydroquercetin and quercetin are produced from dihydrokaempferol and kaempferol, respectively, via a flavonoid 3'-hydroxylase (Schoenbohm et al., 2000). The known functional importance of structural and regulatory proteins for flavonol biosynthesis is the result of numerous studies with *Arabidopsis transparent testa* mutants; these mutants have pale or yellow coloured seeds, which is consistent with lower flavonol levels than the brown wild type seeds (Lepiniec et al., 2006). An *Arabidopsis* flavonoid 3'-hydroxylase mutant (*tt7*) is deficient in quercetin glycosides and is UV-B sensitive; however, the accumulation of kaempferol glycosides and hydroxycinnamic esters is unaffected (Ryan et al., 2001). In *Arabidopsis*, most enzymes of the flavonol biosynthesis pathway are encoded by a single gene, whereas flavonol synthase is encoded by multiple loci (Pelletier et al., 1997; Wisman et al., 1998). Flavonol synthase produces the flavonols kaempferol and quercetin from dihydrokaempferol and dihydroquercetin, respectively (Wisman et al., 1998).

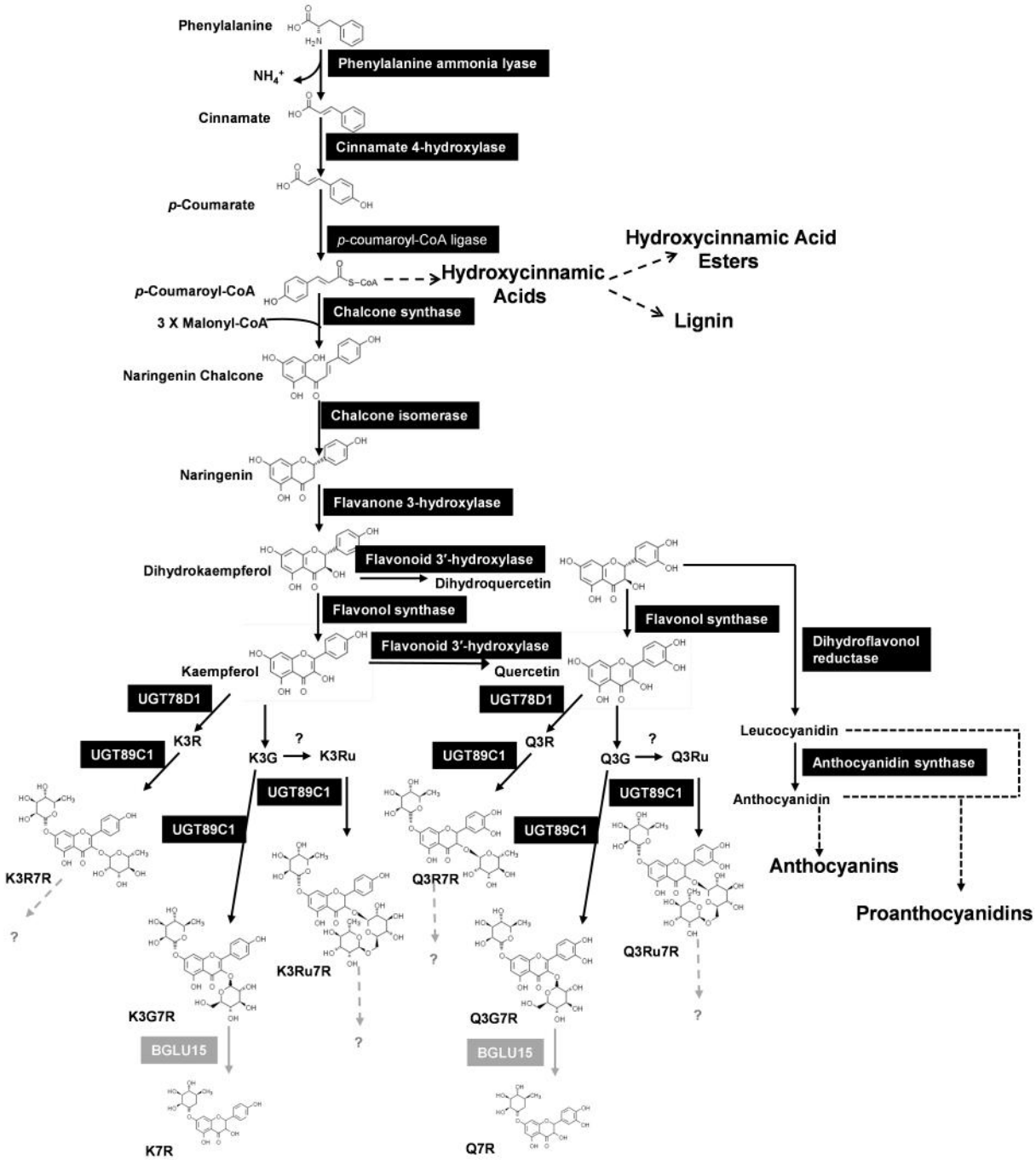

Phenylalanine
Phenylalanine ammonia lyase
NH_4^+
Cinnamate
Cinnamate 4-hydroxylase
p-Coumarate
p-coumaroyl-CoA ligase
Hydroxycinnamic Acid Esters
p-Coumaroyl-CoA
Hydroxycinnamic Acids
Lignin
Chalcone synthase
3 X Malonyl-CoA
Naringenin Chalcone
Chalcone isomerase
Naringenin
Flavanone 3-hydroxylase
Dihydrokaempferol
Flavonoid 3′-hydroxylase
Dihydroquercetin
Flavonol synthase
Flavonol synthase
Dihydroflavonol reductase
Kaempferol
Flavonoid 3′-hydroxylase
Quercetin
UGT78D1
UGT78D1
Leucocyanidin
K3R
K3G
K3Ru
Q3R
Q3G
Q3Ru
UGT89C1
UGT89C1
UGT89C1
UGT89C1
UGT89C1
UGT89C1
Anthocyanidin synthase
Anthocyanidin
K3R7R
Q3R7R
Anthocyanins
Proanthocyanidins
K3Ru7R
Q3Ru7R
K3G7R
Q3G7R
BGLU15
BGLU15
K7R
Q7R

Figure 1.2. Schematic representation of flavonol bisglycoside metabolism in the model plant *Arabidopsis*. The biosynthetic enzymes are indicated by white text within black- boxes, whereas catabolic enzymes are indicated by white text within dark-grey boxes. Dashed arrows indicate processes involving more than one enzyme. Black question mark symbols represent uridine diphosphate-glycosyltransferase (UGT) reactions with no known gene; grey question mark symbols represent flavonol bisglycoside catabolism reactions yet to be characterized. For simplicity, only stress-related flavonol metabolism is shown. Abbreviations include: BGLU15, flavonol 3-O-β-glucoside-7-O-α-rhamnoside glucosidase; K3G, kaempferol 3-*O*-β-glucoside; K3R, kaempferol 3-*O*-α-rhamnoside; K3Ru, kaempferol 3-*O*-β-rutinoside; K3G7R, kaempferol 3-*O*-β-glucoside-7-*O*-α-rhamnoside; K3R7R, kaempferol 3-*O*-α-rhamnoside-7-*O*-α-rhamnoside; K3Ru7R, kaempferol 3-*O*-β-rutinoside-7-*O*-α-rhamnoside; Q3G, quercetin 3-*O*-β-glucoside; Q3R, quercetin 3-*O*-α-rhamnoside; Q3Ru, quercetin 3-*O*-β-rutinoside; Q3G7R, quercetin 3-*O*-β-glucoside-7-*O*-α-rhamnoside; Q3R7R, quercetin 3-*O*-α-rhamnoside-7-*O*-α-rhamnoside; Q3Ru7R, quercetin 3-*O*-β-rutinoside-7-*O*-α-rhamnoside UGTs, uridine diphosphate glycosyltransferases; UGT78D1, UDP-rhamnose: flavonol-3-*O*-rhamnosyltransferase; UGT78D2, UDP-glucose: flavonol 3-*O*-glucosyltransferase; and UGT89C1, UDP-rhamnose: flavonol 7-*O*-rhamnosyltansferase.

Flavonol aglycones are found in the nucleus and plasma membrane (Buttery and Buzzell, 1973; Peer et al., 2001; Debeaujon et al., 2003; Martens et al., 2010; Rodas et al., 2014), but mainly exist as glycosides within the vacuole and cell wall (Strack et al., 1988; Murphy et al., 2000; Zhao et al., 2011). Approximately 35 flavonols exist in *Arabidopsis*, including kaempferol, quercetin, and isorhamnetin with glucose, rhamnose, or arabinose attached to the 3- and/or 7-hydroxy positions (Corradini et al., 2011; Saito et al., 2013). Flavonol bisglycosides occurring in *Arabidopsis* vegetative tissues include kaempferol-3-*O*-β-glucoside-7-*O*-α-rhamnoside, kaempferol-3-*O*-β-glucoside-7-*O*-α-rhamnoside (K3G7R), kaempferol 3-*O*-β-rutinoside-7-*O*-α-rhamnoside and their quercetin counterparts (Olsen et al., 2009; Saito et al., 2013; Roepke and Bozzo, 2015). Flavonol glycosides are produced by UDP-sugar dependent glycosyltransferases (UGTs; Bowles et al., 2006).

The *Arabidopsis* genome consists of 120 putative UGTs (Caputi et al., 2012). Included in this family of carbohydrate-active enzymes are flavonol-utilizing UGTs, some of which are co-expressed in developing plants tissues (Yonekura-Sakakibara et al., 2008). Modification of flavonol aglycones to monoglycosides and finally bisglycosides requires flavonol-specific UGTs in this organism (Fig. 2.2; Lim, et al., 2004; Yonekura-Sakakibara et al., 2007; Yonekura-Sakakibara et al., 2014). *Arabidopsis* UGT73C6 catalyzes the 3-O-rhamnosylation of flavonols yielding flavonol 3-*O*-rhamnosides (Jones et al., 2003). Flavonol 3-*O*-β-glucosides are formed from their aglycone precursors by UGT78D2, a UDP-glucose glucosyltransferase; consequently, no flavonol bisglycoside is formed in the *ugt78d2* knockout of *Arabidopsis* (Tohge et al., 2005). Flavonol monoglycosides can be modified further at the 7-hydroxy position, resulting in flavonol

bisglycosides. Yonekura-Sakakibara et al. (2007) demonstrated that UGT89C1 is required for the biosynthesis of K3G7R from kaempferol 3-*O*-glucoside and UDP-rhamnose. Another glucosyltransferase, UGT79B6, is responsible for the biosynthesis of kaempferol and quercetin 3-*O*-glucosyl-(1→2)-glucosides, producing novel flavonol glycosides specific to pollen (Yonekura-Sakakibara et al., 2014). *Arabidopsis* UGT78D3 is an arabinosyltransferase, responsible for the biosynthesis of quercetin 3-*O*-arabinoside from quercetin (Yonekura-Sakakibara et al., 2008).

1.4. RESPONSE OF FLAVONOL BISGLYCOSIDES TO ABIOTIC STRESS

The biological functions of flavonol bisglycosides include plant defense, modification of polar auxin transport, and stress response (Jacobs and Rubery, 1988; Li et al., 1993; Brown et al., 2001; Buer and Muday, 2004; Martens et al., 2010; Fraser and Chapple, 2011; Misyura et al., 2012; Yin et al., 2014). In *Arabidopsis*, flavonols and their bisglycosides, such as quercetin 3-*O*-β-glucoside-7-*O*-α-rhamnoside (Q3G7R), accumulate in response to biotic and abiotic factors, including UV radiation, pathogen attack, cold stress, and nitrogen deficiency (Dixon and Paiva, 1995; Landry et al., 1995; Winkel-Shirley, 2002; Peng et al., 2008; Olsen et al., 2009; Falcone Ferreyra et al., 2010). Exposure to UV and sunlight enhances levels of flavonol glycosides in leaves of *Arabidopsis* (Sheahan, 1996), privet (Agati et al., 2009), large-leaved lime trees (Majer et al., 2014), and grape berries (Kolb et al., 2003). In contrast, Siipola et al. (2015) determined that quercetin bisglycoside levels are higher in pea leaves exposed to solar blue light than UV radiation (Siipola et al., 2015). These aforementioned changes are associated with induction of the biosynthetic pathway. Expression of genes for early

biosynthesis enzymes, including chalcone synthase and chalcone isomerase, is both induced by UV and downregulated by its absence (Kubasek et al., 1992; Christie and Jenkins, 1996). Moreover, *Arabidopsis tt4* and *tt5* mutants, which are defective in chalcone synthase and chalcone isomerase, respectively, contain lower levels of kaempferol glycosides and sinapate ester levels than wild type plants (Li et al., 1993). Kaempferol glycosides are the predominant flavonols in *Arabidopsis* plants grown in the absence of UV light; biosynthesis of quercetin glycosides is detectable following exposure to UV light (Veit and Pauli, 1999). This is also evident for other abiotic stresses, such as nutrient deficiency (Lillo et al., 2008). Flavonol accumulation in vegetative tissues is well correlated with growth under nitrogen deficiency (Bongue-Bartelsman and Phillips, 1995; Stewart et al., 2001; Olsen et al., 2009; Larbat et al., 2012). These metabolic shifts are dependent upon transcriptional regulation of the biosynthetic pathway. For example, tobacco plants cultivated under low nitrate (0.2 mM) accumulate quercetin 3-*O*-β-rutinoside, a phenomenon that is consistent with increased expression of *PHENYLALANINE AMMONIA LYASE*, *CINNAMATE 4-HYDROXYLASE* and *4-COUMAROYL COA LIGASE* (Fritz et al., 2006). A dramatic increase in transcript levels for *CHALCONE SYNTHASE*, *CHALCONE ISOMERASE*, *FLAVONONE 3-HYDROXYLASE* and *FLAVONOID 3'HYDROXYLASE* occurs within 7 d of the transfer of *Arabidopsis* plants from 14 mM to 0.3 mM nitrate (Peng et al., 2008). Nitrogen limitation results in higher transcript levels for chalcone synthase and dihydroflavonol reductase, as well as higher flavonol concentrations in leaves of tomato (Stewart et al., 2001). Tomato plants subjected to low nitrogen (0.15 mM NO_3^-, 0.01 mM NH_4^+) growth conditions for 10 d after a period of adequate supply (15 mM NO_3^-, 1.2 mM NH_4^+) have

elevated flavonol glycoside and anthocyanin levels; by contrast, changes are minimal under full nitrogen (Larbat et al., 2012). In *Arabidopsis*, an increased abundance of transcripts for positive regulators of the flavonol pathway, such as *PRODUCTION OF ANTHOCYANIN PIGMENT1* (*PAP1*), *PAP2* and *MYELOBLASTOMA12* (*MYB12*), is evident during nitrogen-deficient growth (Lea et al., 2007; Lillo et al., 2008; Olsen et al., 2009). In senescing *Arabidopsis* leaves, *PAP1* transcripts accumulate in response to the application of exogenous sucrose (Pourtau et al., 2006). It is interesting to note that the accumulation of flavonol glycosides in tomato following re-exposure to abiotic stress is greater than levels present during the initial exposure (Larbat et al., 2012).

Simultaneous application of abiotic stresses, such as nitrogen deficiency (0 mM nitrate) and low temperature (e.g., 10 °C) (NDLT) dramatically increases kaempferol- and quercetin bisglycoside levels in *Arabidopsis* (Olsen et al., 2009; Roepke and Bozzo, 2015). K3G7R and Q3G7R concentrations are enhanced 2.4- and 29.3-fold, respectively, during a 7-d exposure to NDLT (Roepke and Bozzo, 2015). Flavonol glycoside concentrations increase synergistically in tomato leaves following simultaneous exposure to high light intensity and nitrogen deficiency (Løvdal et al., 2010). Upon transfer of tomato plants stressed with short-term nitrogen limitations (10 d at 0.15 mM NO_3^- and 0.01 mM NH_4^+) to optimal growth conditions (15 mM NO_3^- and 1.2 mM NH_4^+), flavonol bisglycoside concentrations decrease to pre-stress levels (Larbat et al., 2004). Similarly, a 50-100% loss in flavonol bisglycosides occurs within 5 d of the transfer of NDLT-stressed *Arabidopsis* plants to nitrogen sufficiency and high temperature (21°C; NSHT) conditions (Olsen et al., 2009; Ropeke and Bozzo, 2015). It appears these losses coincide

with a sharp decline in the expression of genes corresponding to the flavonol biosynthesis pathway (Olsen et al., 2015).

1.5. FLAVONOL BISGLYCOSIDE CATABOLISM

Early plant flavonol research demonstrated that plant cell cultures degrade flavonol glycosides, as supplied [^{14}C]-kaempferol-3-*O*-β-glucoside is converted to [^{14}C]-glucose (Muhle et al., 1976), implying that hydrolysis of the β-*O*-linked sugar is possible. In fact, losses of flavonols and structurally-related flavonoids in soybean (Graham, 1991), onion (Takahama and Hirota, 2000), tartary buckwheat (Suzuki et al., 2007) and *Arabidopsis* (Ropeke and Bozzo, 2015) are correlated with the induction of β-glucosidase (BGLU) activity. In onion bulb, high levels of quercetin 4′-*O*-β-glucoside, the product of quercetin-3,4′-bisglucoside hydrolysis, are present in senescing tissues; this phenomenon that is associated with higher quercetin-3,4′-bisglucoside BGLU activity relative to fleshy tissues (Takahama and Hirota, 2000) . Suzuki et al. (2005) also demonstrated co-localization of rutin BGLU activity and rutin in young developing leaves of tartary buckwheat.

BGLUs hydrolyze the β-*O*-linked sugars conjugated to small molecules and oligosaccharides (Davies and Henrissat, 1995; Morant et al., 2008). BGLUs are ubiquitous in all living organisms and are involved in a variety of metabolic processes, including plant defense and stress response (Hösel et al., 1978; Thorlby et al., 2004; Morant et al., 2008; Yu et al., 2009). As β-linked sugar conjugates are abundant in nature, a number of substrate-specific BGLUs exist in plants; this group of enzymes can hydrolyze sugars other than glucose, albeit with lower specificity, and therefore display

lower β-fucosidase, β-galactosidase, or β-mannosidase activities (Minic, 2008). BGLUs share common tertiary structure characteristics, including a β_8- or α_8-barrel fold, consisting of two conserved motifs, TFNEP and (I/V)TENG, each harbouring a catalytic glutamate residue (Henrissat and Davies, 1997; Czjzek et al, 2000; Xu et al,. 2004). The active site of members of the glycoside hydrolase 1 (GH1) family contains two glutamates, one of which promotes the nucleophilic attack of the sugar and the other donates a proton to the aglycone (Czjzek et al., 2000).

In *Arabidopsis*, BGLUs belong to the GH1 gene family, which consists of 47 members sorted into ten subfamilies based on phylogeny (Xu et al., 2004). *BGLU1-47* are highly related and share evolutionary lineage; a pseudo BGLU, *SENSITIVE TO FREEZING2*, is distinct and more phylogenetically related to bacterial than plant BGLUs (Xu et al., 2004). Moreover, ten GH1 subfamilies are distinguished on the basis of their metabolic roles within the cell and substrate specificities (Xu et al., 2004). GH1 family BGLUs from maize, sorghum, and wheat tend to be phylogenetically distinct from those occurring in *Arabidopsis* and rice (Cairns and Esen, 2010). GH1 enzymes are known to display specificity for the aglycone core, as well as the sugar groups attached to the aglycone (Cairns and Esen, 2010). With respect to subcellular localization, evidence exists for GH1 family members residing in the cell wall, vacuole, peroxisome and endoplasmic reticulum (Chivasa et al., 2002; Matsushima et al., 2003; Xu et al., 2004; Minic, 2008). An apoplastic BGLU isolated from soybean roots catabolizes isoflavone 7-*O*-(6′′O-malonyl–β-D-glucoside) compounds, a group of phenolic metabolites with a structural similarity to flavonols (Suzuki et al., 2006). More recent evidence indicates that transcript levels for the soybean isoflavone 7-*O*-(6′′O-malonyl–β-D-glucoside) hydrolase

are elevated in response to infection by *Phytophthora sojae*, as well as flooding, drought, and plant hormones (Yoo et al., 2013). Moreover, accumulation of the phytoalexin medicarpin and is associated with increased *BGLU* expression in barrel clover treated with yeast elicitors or methyl jasmonate (Naoumkina et al., 2007).

Roepke and Bozzo (2015) demonstrated that a 250% increase in BGLU activity coincides with the loss of flavonol bisglycosides, including K3G7R and Q3G7R, during recovery from NDLT. K3G7R and Q3G7R concentrations are reduced by 33% and 56%, respectively, within 2 d after NDLT-stressed plants are resupplied with nitrate and transfer to 21 °C. In the *Arabidopsis* genome, a *GH1* family clade consisting of *BGLU12* to *17*, inclusive, is phylogenetically similar to legume BGLUs which utilize isoflavone conjugates; in addition, this clade is phylogenetically distinct from BGLUs specific for monolignol glucosides, abscisic acid conjugates, and glucosinolates (Xu et al., 2004; Suzuki et al., 2006; Naoumkina et al., 2007; Roepke and Bozzo, 2015). Interestingly, transcript levels for *BGLU12*, *15* and *16* are 68-300% higher in vegetative *Arabidopsis* tissues after only 1 d of recovery from NDLT (Roepke and Bozzo, 2015).

In vitro analysis of the recombinant thiroedoxin-His_6 tagged mature BGLU15 revealed a preference for K3G7R and Q3G7R (Fig. 1.2); kaempferol 3-*O*-β-glucoside and quercetin 3-*O*-β-glucoside (Q3G) also serve as substrates, although these are catalyzed with lower efficiencies. This, together with the inability of BGLU15 to use other quercetin conjugates such as quercetin 3-*O*-β-galactoside, rutin, and quercetin 3-*O*-α-rhamnoside, indicates that this enzyme is a BGLU with specificity for glucose attached to the 3-hydroxy position of flavonols. Functional studies using T-DNA insertional-inactivation mutants of the *BGLU15* gene (*At2g44450*) have revealed that flavonol 3-*O*-

β-glucoside-7-*O*-α-rhamnosides are not catabolized in the absence of this enzyme, whereas other flavonol bisglycosides are degraded (Roepke, 2015). This suggests that BGLU12 and BGLU16 are not involved in the catabolism of flavonol 3-*O*-β-glucoside-7-*O*-α-rhamnosides, but may be involved in the loss of the related compounds flavonol 3-*O*-β-rutinoside-7-*O*-α-rhamnosides. In addition, the levels of sinapate esters (i.e., 1-*O*-sinapoyl-β-glucoside) decline in *Arabidopsis* leaves during the recovery from NDLT, regardless of whether BGLU15 is present. Thus, the possibility remains that sinapate esters, such as 1-*O*-sinapoyl-β-glucoside, are hydrolyzed by BGLU12 and/or BGLU16. Flavonol conjugates accumulate together with higher levels of sinapate esters in response to UV-B (Sheahan, 1996), but it is not known whether these compounds accumulate with other abiotic stresses, such as NDLT.

1.6. SINAPATE ESTER METABOLISM IN PLANTS

Hydroxycinnamates such as sinapate esters are somewhat structurally similar to flavonols, as they have an aromatic ring and three carbon structure (C_6-C_3 skeleton), and they are both derived from *p*-coumarate (Fig.1D; Cartea et al., 2011). Hydroxycinnamates exist as ester or amide conjugates (Strack, 2001; Cartea et al., 2011), and tend to accumulate in organ-specific patterns (Fraser and Chapple, 2011). Most of these phenolic compounds are widely distributed throughout the plant kingdom; sinapate and its derived esters are hallmark compounds in Brassicaceae species and their edible parts (Milkowki and Strack, 2010), and these compounds are also present in strawberries, raspberries, and herbs such as dill, rosemary, sage and anise (Nićiforović and Abramović, 2014). In broccoli florets, sinapate can approximate 100 $\mu g\ g^{-1}$fresh matter (FM), which

is in excess of their flavonol levels (Bhagwat et al., 2014; Nićiforović and Abramović, 2014). The sinapate ester sinapoylcholine (also known as sinapine) is present at high levels in canola seeds, and are problematic as they can render their oils indigestible and astringent; thus, current *Brassica napus* L. breeding efforts are now looking at developing germplasm with low seed sinapoylcholine levels (Milkowski and Strack, 2010). Similarly, sinapoylcholine predominates in seeds of *Arabidopsis* (Landry et al., 1995), whereas sinapoylmalate is the major esterified form in seedlings (Ruegger and Chapple, 2001). In fact, sinapoylmalate occurs in leaf epidermal cells, serving a role in UV light protection (Lorenzen et al., 1996; Li et al., 2010). Sinapate esters absorb UV-B light and emit a blue fluorescence, which permits their quantification *in vitro* and visualization *in situ* (Chapple et al., 1992; Li et al., 2010). Consequently, many of the steps pertaining to sinapate biosynthesis were discovered via the genetic characterization of *Arabidopsis reduced epidermal fluorescence* (*ref*) mutants, derived from ethyl methanesulfonate-mutagenized plants (Ruegger and Chapple, 2001; Franke et al., 2002; Fraser and Chapple, 2011; Kim et al., 2014).

The biosynthetic pathways yielding flavonols and sinapate share metabolic intermediates up to *p*-coumaroyl-CoA (Fig. 1.3; Fraser and Chapple, 2011). The main biosynthetic pathway for sinapate production involves conversion of *p*-coumaroyl-CoA to *p*-coumaroylshikimate by hydroxycinnamoyl-coenzyme shikimate:quinate hydroxycinnamoyl-transferase, and hydroxylation by *p*-coumaroyl shikimate 3'-hydroxylase, CoA-mediated conversion to caffeoyl-CoA, followed by subsequent 3-O-methylation, reduction, 5-hydroxylation, methylation and oxidation of the formed

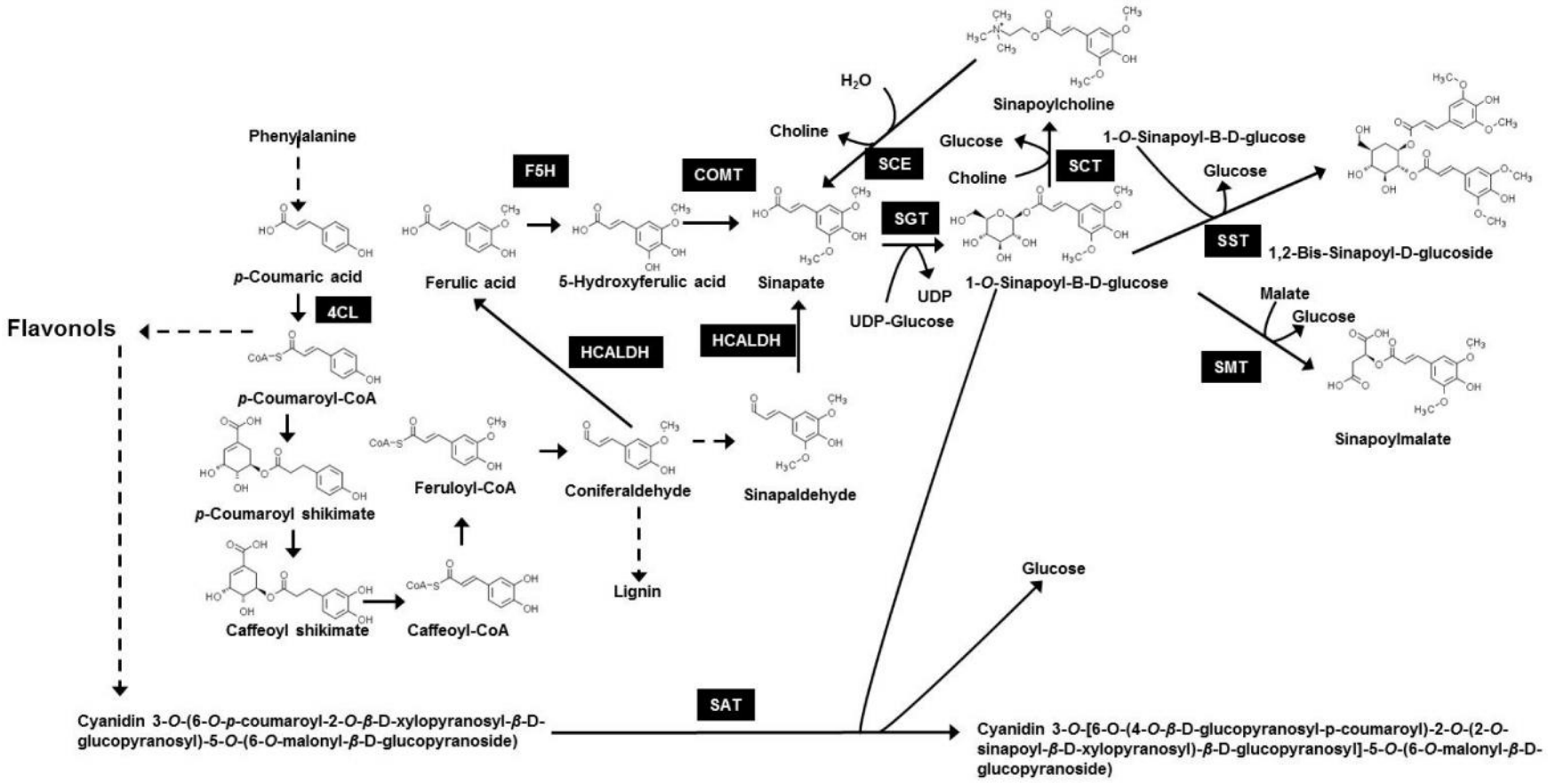

Phenylalanine
p-Coumaric acid
4CL
Flavonols
p-Coumaroyl-CoA
p-Coumaroyl shikimate
Caffeoyl shikimate
Caffeoyl-CoA
Feruloyl-CoA
Ferulic acid
F5H
5-Hydroxyferulic acid
COMT
Coniferaldehyde
Sinapaldehyde
HCALDH
HCALDH
Lignin
Sinapate
SGT
UDP
UDP-Glucose
1-O-Sinapoyl-B-D-glucose
H2O
Choline
SCE
Sinapoylcholine
Choline
SCT
Glucose
1-O-Sinapoyl-B-D-glucose
Glucose
SST
1,2-Bis-Sinapoyl-D-glucoside
Malate
Glucose
SMT
Sinapoylmalate
Glucose
SAT
Cyanidin 3-O-(6-O-p-coumaroyl-2-O-β-D-xylopyranosyl-β-D-glucopyranosyl)-5-O-(6-O-malonyl-β-D-glucopyranoside)
Cyanidin 3-O-[6-O-(4-O-β-D-glucopyranosyl-p-coumaroyl)-2-O-(2-O-sinapoyl-β-D-xylopyranosyl)-β-D-glucopyranosyl]-5-O-(6-O-malonyl-β-D-glucopyranoside)

Figure 1.3. Sinapate ester metabolism in *Arabidopsis*. Enzymes are indicated by white text within black boxes. Dashed arrows indicate processes involving more than one enzyme. The pathway from *p*-coumaroyl CoA to coniferaldehyde and sinapaldehyde is not described in detail here. Abbreviations include: 4CL, *p*-coumaroyl CoA-ligase; COMT, caffeic acid/5-hydroxyferulic acid O-methyltransferase; F5H, ferulate 5-hydroxylase; HCALDH, hydroxycinnamaldehyde dehydrogenase; SAT, sinapoylglucose:anthocyanin sinapoyltransferase; SCE, sinapoylcholinesterase; SCT, sinapoylglucose:choline sinapoyltransferase; SGT, sinapate:UDP-glucose glucosyltransferase; SMT, sinapoylglucose:malate sinapoyltransferase; SST, sinapoylglucose:sinapoylglucose sinapoyltransferase. The illustration was adapted from Milkowski and Strack (2010) and Fraser and Chapple (2011).

sinapaldehyde via hydroxycinnamaldehyde dehydrogenase (Fig. 1.3). In *Arabidopsis*, the enzyme is encoded by *REF1*; *ref1* mutants contain 90% lower sinapoylmalate levels than wild type plants, suggesting that a hitherto unknown redundant enzyme(s) exists (Nair et al., 2004; Fraser and Chapple, 2011).

The glucose ester of sinapate, 1-*O*-sinapoyl-β-D-glucose, is formed from sinapate by a sinapate:UDP-glucose sinapoyltransferase in *Arabidopsis* and *B. napus* (Strack, 1980; Milkowski et al., 2004; Li et al., 2010), and it is the immediate precursor of sinapoylmalate and sinapoylcholine in vegetative tissues and seeds, respectively. *SINAPATE:UDP-GLUCOSE SINAPOYLTRANSFERASE* transcript and 1-*O*-sinapoyl-β-D-glucose levels increase during the early stages of seed embryogenesis (Milkowski et al., 2004). In *Arabidopsis*, four UGTs, including UGT84A2, which are proximal to one another in the genome, catalyze the glucosylation of sinapate (Milkowski et al., 2000). The *Arabidopsis bright trichomes 1* (*brt1*) mutant is genetically complemented by *UGT84A2* (Sinlapadech et al., 2007). A reduction in the levels of 1-*O*-sinapoyl-β-D-glucose and its derived sinapoylmalate and sinapoylcholine is apparent in *Arabidopsis brt1* seedlings and seed, respectively; the accumulation of a sinapate polyketide culminates in hyper-fluorescence of *brt1* trichomes relative to their wild type counterparts (Sinlapadech et al., 2007). Residual levels of sinapoylmalate and sinapate:UDP-glucose sinapoyltransferase activity in *brt1* seedlings suggests that functional redundancy exists in *Arabidopsis*. In fact, *in vitro* studies have shown that UGT84A1, UGT84A3 and UGT84A4 utilize sinapate to some extent (Milkowski et al., 2000). UV-B irradiation of *Arabidopsis* is associated with elevated levels of *UGT84A1*, *UGT84A2*, *UGT84A3* and *UGT84A4* transcripts and sinapate ester (Meißner et al., 2008). Apart from sinapate modification, it is known that the major anthocyanin in *Arabidopsis* is sinapoylated (i.e., cyanidin 3-*O*-[6-O-(4-*O*-β-D-

glucopyranosyl-p-coumaroyl)-2-*O*-(2-*O*-sinapoyl-β-D-xylopyranosyl)-β-D-glucopyranosyl]-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside) (Bloor and Abrahams, 2002). A query of publically-available microarray databases revealed *UGT84A2* clusters with anthocyanin biosynthetic genes, including an *ANTHOCYANIN 5-O-GLUCOSYLTRANSFERASE*, *ANTHOCYANIN 3-O-GLUCOSIDE: 6àà-O-P-COUMAROYLTRANSFERASE*, and *ANTHOCYANIN 5-O-GLUCOSIDE: 6àà-O-MALONYLTRANSFERASE* (Yonekura-Sakakibara et al., 2012). Moreover, the level of the major anthocyanin, cyanidin 3-*O*-[6-O-(4-*O*-β-D-glucopyranosyl-p-coumaroyl)-2-*O*-(2-*O*-sinapoyl-β-D-xylopyranosyl)-β-D-glucopyranosyl]-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside), is reduced in *Arabidopsis ugt84a2* (*brt1*) mutants (Yonekura-Sakakibara et al., 2012; Saito et al., 2013). These findings demonstrate that UGT84A2 participates in anthocyanin modification by conjugating 1-*O*-sinapoyl-β-D-glucose to cyanidin 3-*O*-(6-*O*-*p*-coumaroyl-2-*O*-β-D-xylopyranosyl-β-D-glucopyranosyl)-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside) (Fig. 1.3), suggesting that it acts in concert with other anthocyanin-modifying enzymes in the production of these pigments in *Arabidopsis*.

Sinapoylcholine is a major sinapate ester in developing seeds and is formed from 1-*O*-sinapoyl-β-D-glucose and choline in the presence of sinapoylglucose:choline sinapoyltransferase (Bopp and Lüdicke, 1980; Strack et al., 1982); this compound serves as a storage form of choline in many Brassicaceae species, including *Arabidopsis* (Strack et al., 1980; Milkowski et al., 2004; Fraser and Chapple, 2011). A widespread expression analysis of genes involved in sinapate metabolism in *B. napus* organs indicates that *SINAPOYLGLUCOSE:CHOLINE SINAPOYLTRANSFERASE* transcripts are detected solely within developing seeds (Milkowski et al., 2004). *Sinapis alba* L. seeds labelled with [^{14}C]-phenylalanine accumulate [^{14}C]-sinapoylcholine within their cotyledons during seed maturation, but it is degraded to 1-*O*-

sinapoyl-β-D-glucose in germinating seeds (Bopp and Lüdicke, 1980). Furthermore, pulse chase labelling experiments demonstrate that [^{14}C]-choline fed to developing *Raphanus sativus* L. seeds is incorporated into sinapoylcholine; choline released from this ester is used to produce phosphatidylcholine in developing seedlings (Strack, 1981). Sinapoylcholine esterase is the enzyme responsible for releasing choline from sinapoylcholine during seed germination and early seedling development; the free choline is used in phosphatidylcholine biosynthesis, a component of cell membranes (Strack et al., 1980; Fraser and Chapple, 2011).

The high free energy of hydrolysis of 1-*O*-sinapoyl-β-D-glucose allows for the sinapoyl moiety to be conjugated to malate by a vacuolar 1-*O*-sinapoyl-β-D-glucose:malate sinapoyltransferase, yielding sinapoylmalate (Strack, 1982; Sharma and Strack, 1985; Mock and Strack, 1993; Lehfeldt et al., 2000). Sinapoylmalate is the most abundant sinapate ester in *Arabidopsis*, and it accumulates in the upper (adaxial) leaf surface (Lorenzen et al., 1996; Li et al., 2010; Santos-Filho et al., 2012). An increase in this ester occurs with differentiation of cotyledons (Milkowski et al., 2004). Sinapoylmalate is a major UV-B-absorbing compound in *Arabidopsis*, and is generally present at high levels relative to all other UV-B absorbing compounds (e.g., kaempferol; Li et al., 1993). The 1-*O*-sinapoyl-β-D-glucose:malate sinapoyltransferase step is encoded by *SINAPOYLGLUCOSE ACCUMULATOR1* (*SNG1*) in *Arabidopsis* (Lorenzen et al., 1996). The main sinapate ester in the leaves of *Arabidopsis sng1* mutant plants is 1-*O*-sinapoyl-β-D-glucose, approximating levels of sinapoylmalate in wild type plants (Lorenzen et al., 1996). The *sng1* phenotype occurs in approximately 4% of the natural population of *Arabidopsis* (Li et al., 2010). Sinapoylmalate production appears to be indirectly dependent on nitrate reductase activity, the enzyme required for nitric oxide production (Santos-Filho et al., 2012). Nitric oxide is a known signalling molecule in plants, and it is involved in

plant development, as well as biotic and abiotic stress resistance (Wang and Wu, 2005; Besson-Bard et al., 2008; Wilson et al., 2008). *Arabidopsis* nitrate reductase double mutants (*nia1 nia2*) contain less sinapoylmalate and more 1-*O*-sinapoyl-β-D-glucose than wild type plants; this metabolic phenotype is reversible upon addition of malate and nitric oxide (Santos-Filho et al., 2012).

Apart from esterification, sinapate can be metabolized to sinapyl alcohol via cinnamoyl alcohol dehydrogenase; sinapoyl alcohol is a precursor of syringyl monolignol subunits of lignin (Boerjan et al., 2003). Lignin is a polymer of monolignols and is critical for plant structural support and protection (Boerjan et al., 2003). In the absence of sinapate biosynthesis, the *Arabidopsis ref8* mutant, which encodes for *p*-coumaroyl shikimate 3'-hydroxylase, accumulates *p*-coumaroyl based lignin polymers (Franke et al., 2002). Moreover, this mutant is associated with a stunted growth phenotype and infertile plants (Kim et al., 2014). This could be due to the importance of lignin in the composition of vascular tissues required for water movement in the plant (Boerjan et al., 2003).

The biosynthesis of sinapate esters is induced in leaf epidermal cells following exposure to UV-B, a mechanism that affords photosynthetic tissues protection against damage to DNA, RNA, and proteins (Caldwell et al., 1983; Li et al., 1993; Landry et al., 1995). The production of sinapate esters is blocked in *Arabidopsis ferulic acid 5-hydroxylase* (*fah1*) mutants, which are defective in ferulate-5-hydroxylase activity, an enzyme forming the sinapate precursor 5-OH coniferaldehyde (Chapple et al., 1992). The *Arabidopsis fah1* mutant is relatively more sensitive to UV-B irradiation than a kaempferol-deficient *transparent testa5* (*tt5; chalcone isomerase*) mutant, suggesting that sinapate esters are more important for UV-B protection than flavonols (Li et al., 1993). Apart from UV-B stress, evidence exists for the accumulation of sinapate esters

and their related metabolites with other abiotic stresses. The levels of hydroxycinnamate-glucose esters are greater in leaves of *Populus* plants cultivated hydroponically under low nitrate (0.25 mM) than in plants cultivated under 2.5 mM nitrate; there was no evidence for accumulation of sinapate glucose esters (Babst et al., 2014). Cold stress causes an increase in the concentration of aromatic amino acids (i.e., tryptophan, tyrosine, and phenylalanine) and intermediates of the phenylpropanoid pathway, including sinapate (Kaplan et al., 2004). The aforementioned study used a GC-MS approach, whereby only non-conjugated forms of metabolites are derivatized, thus excluding the possibility of accounting for predominant glycosylated or esterified forms of sinapate. Sinapate esters levels are enhanced nearly 50% in *Arabidopsis* rosette stage plants grown hydroponically for 5 d in nitrogen-free medium (Feyissa et al., 2009). Simultaneous increases in sinapate esters (e.g., 1-*O*-sinapoyl-β-D-glucose) and flavonol bisglycosides (e.g., Q3G7R) are apparent in sulfur-deficient *Arabidopsis* (Zhang et al., 2011). The possibility remains that levels of sinapate esters are transiently enhanced by the application of simultaneous abiotic stresses, such as NDLT, which is known to induce flavonol bisglycoside production in *Arabidopsis*. More importantly, recovery from NDLT is associated with catabolism of these natural products (Olsen et al., 2009; Roepke and Bozzo, 2015).

1.7. CONCLUDING REMARKS

In summary, flavonol bisglycosides and sinapate esters in *Arabidopsis* accumulate in response to abiotic stresses, including UV-B irradiation, nutrient deficiencies, and low temperature (Jacobs and Rubery, 1988; Chapple et al,. 1992; Li et al., 1993; Misyura et al., 2012). Recent biochemical and molecular evidence indicate that flavonol bisglycosides (i.e., flavonol 3-*O*-β-glucoside-7-*O*-α-rhamnosides) are degraded in *Arabidopsis* during recovery from NDLT stress, a process requiring an initial hydrolysis by BGLU15. *Arabidopsis* BGLU16 is phylogenetically related to BGLU15, as well as other flavonoid glycoside-utilizing BGLUs from other species (Roepke and Bozzo, 2015). Similarly, *BGLU16* transcripts are more abundant during the recovery from NDLT than with NDLT or NSHT conditions alone.

This thesis focuses on the relationship between phenylpropanoid metabolism, specifically sinapate esters, and abiotic stresses in plants. I tested the following hypothesis: sinapate esters are catabolized in *Arabidopsis* by BGLU activity during recovery from abiotic stress. My first objective was to determine the response of sinapate ester levels in rosette leaves of *Arabidopsis* subjected to NDLT for 7 d, followed by a 5 d recovery period. My second objective was to investigate the biochemical properties of a recombinant BGLU16 protein.

CHAPTER TWO – MATERIALS AND METHODS

2.1. BIOCHEMICAL REAGENTS

Biocatalytic synthesis of Q3G7R from Q3G was performed essentially as described previously (Roepke and Bozzo, 2013). Esculetin, esculin, isorhamnetin, isorhamnetin 3-O-β-glucoside, kaempferol 3-O-β-glucoside, naringenin 7-O-β-glucoside, phloretin, phloridizin, quercetin 3-O-β-galactoside, Q3G, and quercetin 3-O-α-rhamnoside were purchased from Extrasynthese (Genay, France). Kaempferol, quercetin and quercetin 3-O-β-rutinoside were purchased from Indofine Chemical Company (Hillsborough, NJ, USA). Unless mentioned elsewhere, all other materials were from Sigma–Aldrich (Oakville, ON, Canada).

2.2. PLANT MATERIALS, ABIOTIC STRESS AND RECOVERY TREATMENTS

Changes in sinapate ester concentrations as a function of transient abiotic stress in *Arabidopsis* plants were calculated from UHPLC-MS^n-DAD data collected from a previous time course experiment (Roepke and Bozzo, 2015). The details of the method used by Roepke and Bozzo (2015) are provided here. Briefly, *Arabidopsis* Heynh. (Columbia-0 ecotype) (Round Rock, TX) was grown on nutrient-free Sunshine Mix #2/LB2 soil (Sun Gro Horticulture Canada, Ltd.) fertilized with a modified Hoagland's nutrient solution (14 mM nitrate [6 mM KNO_3, 4 mM $Ca(NO_3)_2$], 2 mM $MgSO_4$, 10 mM KH_2PO_4, 1 mM FeNaEDTA, 20 µM H_3BO_3, 4 µM $MnCl_2 \cdot 4H_2O$, 0.4 µM $ZnCl_2$, 0.2 µM MoO_3, and 0.5 mM MES, to a final pH of 5.5). No copper or ammonia was added to this modified Hoagland's nutrient solution. Nutrient solution was supplied on day 7, 14, 21,

and 29 after germination. Plants were cultivated in an environmentally-controlled growth chamber at 21°C with 16 h light / 8 h dark photoperiod and a photosynthetic photon flux density of 180 µmol m^{-1} s^{-1}; this treatment will be referred to as NSHT from herein. Thereafter, three trays consisting of 48 pots each (one rosette-stage plant per pot) were transferred to a second environmentally-controlled growth chamber, fertilized with modified Hoagland's medium (0 mM nitrate) and maintained at 10°C for 7 d thereafter (from herein, this treatment will be referred to as NDLT). At the end of the NDLT stress period, all plants were transferred to NSHT conditions for an additional 5 d. As a control, an equivalent number of plants were maintained under NSHT conditions for the same time period. Control and treated plants were harvested periodically during and after the NDLT stress period. Harvested plants were immediately pulverized under liquid N_2, and the powder was stored at -80°C prior to metabolite extraction.

2.2.1. UHPLC-MS^n-DAD Analysis of *Arabidopsis*

For metabolite extraction, 100 mg of the frozen powder from *Arabidopsis* tissue was extracted with five volumes of methanol : acetic acid : Milli-Q H_2O (9:1:10 v/v/v) in an ultrasonication bath for 1 min, rotated on an orbital shaker at ambient temperature for 20 min, then centrifuged at 10000 *g* for 10 min. The pellet was re-extracted twice as described above, and the supernatants from each successive extraction were pooled and dried under vacuum. The dried residue was stored at -80 °C until used for metabolite analysis.

The dried residue was resuspended in 200 µL of Solvent A (Milli-Q H_2O : acetonitrile : formic acid; 90 : 10 : 0.1; v/v/v), and the solution was passed through a 0.45

µm syringe filter (Mandel Scientific Company Inc., Guelph, ON) and analyzed on a Dionex UltiMate 3000 UHPLC (Thermo Scientific) coupled to a Bruker AmaZon SL MS^n and separated on a Poroshell 120 C-18 column (150 x 4.66 mm; 2.7 µm; Agilent Technologies, Mississauga, ON) using a 20 µL injection volume. Metabolites were eluted with a linear 5-100% solvent B (acetonitrile : formic acid, 100 : 0.1) at 0.4 mL min^{-1}. The gradient profile was as follows: 5% B, 0-5 min; 5-100% B, 5-35 min; 100% B, 35-40 min. Detection of the phenylpropanoid metabolites was performed at 360 nm with a DAD whereas sinapate ester were identified with an AmaZon SL ion trap mass spectrometer interfaced with electrospray ionization and analyzed in the negative ion mode using the conditions described by Roepke and Bozzo (2015).

2.3. CLONING OF RECOMBINANT *ARABIDOPSIS* BGLU16

BGLU16 cDNA (Genbank accession number AY045953) Lambda Zap III construct was from the RIKEN Plant Science Center. Bioinformatic analysis of the nucleotide sequence with web-based tools (i.e., SignalP;www.cbs.dtu.dk/services/ signalP/) predicted a 23 amino acid signal sequence, and a cloning strategy was selected to yield the mature protein (Δ23BGLU16). Briefly, forward (5' - CAAGCATTCCACAAGG CCTAGA) and reverse (3' - TTAAATAACAGCCACCTGCTC) primers were used to PCR amplify the mature BGLU16 with 5' *NcoI* and 3' *SacI* restriction enzymes and cloned into pGEM®-T (Promega Corporation, Madison, WI) using standard molecular biology techniques (Sambrook et al. 1989). The pGEM®-T-Δ23BGLU16 construct was digested with *Nco*I and *Sac*I and ligated into the corresponding sites of pET32b (EMD Millipore, Etobicoke,

ON). This strategy was chosen to generate recombinant Δ23BGLU16 with an N-terminal His_6 tag. All constructs were confirmed by sequencing.

2.4. RECOMBINANT BGLU16 BACTERIAL EXPRESSION AND PURIFICATION

The pGEM®-T-Δ23BGLU16 construct was transformed into Origami™ 2(DE3) pLysS thermocompetent cells (EMD Millipore, Etobicoke, ON) and cultured in lysogeny broth (6 L) containing 50 µg mL^{-1} kanamycin, 50 µg mL^{-1} ampicillin, and 10 µg mL^{-1} tetracycline at 37°C and shaken at 200 rpm until the A_{600} reached mid-logarithmic growth phase (0.4-0.6). Thereafter, isopropyl β-D-1-thiogalactopyranoside was added at a final concentration of 0.5 mM, and cultures were incubated at 20°C for 4 h and shaken at 200 rpm. Cells were pelleted by centrifugation at 3500 *g*, flash frozen in liquid N_2, and stored at -80°C for up to one week prior to purification.

Extraction of Δ23BGLU16 transformants was performed at 0-4°C. The frozen bacterial cell pellets were resuspended in 150 mL of lysis buffer, containing 50 mM NaH_2PO_4 (pH 8.0), 30 mM imidazole, 500 mM NaCl, 1 mM phenylmethanesulfonyl-fluoride, 1 mM ethylenediaminetetraacetic acid, 12 mM β-mercaptoethanol, 1% (v/v) Triton X-100, 1x Sigma Protease Cocktail, 0.01 mg mL^{-1} soybean trypsin inhibitor, and 10% (v/v) glycerol. The resuspended cell material was lysed with a Fisher Scientific™ Model 120 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) using ten 30 s pulses at 30% amplitude; a 30-s incubation on ice was used between successive pulses. The bacterial lysate was centrifuged at 18, 300 *g* for 30 min. The supernatant was collected

and re-centrifuged prior to passage through a 0.45 µm polyvinylidene fluoride filter (Millex-HV; EMD Millipore, Etobicoke, ON).

The filtered lysate was absorbed at 1 mL min^{-1} onto an immobilized metal ion affinity (IMAC) column (1 mL HisTrap^{TM} HP, GE Healthcare, Mississauga, ON) coupled to an ÄKTA FPLC. The IMAC column was pre-equilibrated in 50 mL of Buffer A (50 mM NaH_2PO_4 [pH 8.0], 30 mM imidazole, and 500 mM NaCl). Following absorption of the cell lysate, the column was washed until the A_{280} decreased to baseline. The recombinant thioredoxin-His_6-BGLU16 was eluted from the IMAC column with a linear gradient of 0-50 mM imidazole (10 mL), followed by a second linear gradient of 50-500 mM imidazole (20 min). The fraction containing the homogenous BGLU16 preparation was brought to 20% (v/v) glycerol, divided into 100 µL aliquots, flash frozen in liquid N_2, and stored at -80 °C until used for activity assays. Protein was estimated by dye-binding (Bradford, 1976) using bovine serum albumin as the standard.

2.5. SDS-PAGE ELECTROPHORESIS AND IMMUNOBLOTTING

The recombinant Δ23BGLU16 was analyzed by SDS-PAGE (10 % w/v acrylamide) and polyvinylidene fluoride membrane immunoblotting was performed using standard protocols (Bozzo et al., 2006; Laemmli, 1970). For immunoblotting, the polyvinylidene fluoride membrane was probed with a His-probe mouse monoclonal IgG (Santa Cruz Biotechnology; Dallas, TX), followed by alkaline phosphatase-tagged anti-mouse IgG (Sigma-Aldrich). An alkaline conjugate substrate kit (Bio-Rad) was used to visualise the probed protein according to the manufacturer's protocols.

2.6. BGLU ACTIVITY ASSAYS

For all biochemical assays, homogenous preparations of BGLU16 were desalted on a PD SpinTrap^{TM} G-25 column (GE Healthcare) pre-equilibrated with assay buffer and collected by centrifugation at 800 *g* for 2 min. Unless otherwise mentioned, assays were prepared to a final volume of 200 µL consisting of 50 mM MES-NaOH (pH 5.5), 500 µM substrate, and 1.4 µg of the recombinant mature BGLU16 protein. The impact of pH on BGLU16 activity was investigated with assays containing 500 µM *p*-nitrophenyl-β-D-glucoside (*p*NP-glucoside). The biological buffers were tested at a final concentration of 50 mM in the range of 3 to 8, and included citric acid - Na-citrate (pH 3-6), acetic acid-Na-acetate (pH 4.5-5.5), MES-NaOH (pH 5.5-6.5), and Na_2HPO_4 - NaH_2PO_4(pH 6-8). All single substrate assays were incubated at 30°C for 30 min.

Parallel assays were performed in the presence of an *Arabidopsis* extract containing phenylpropanoid and flavonols. For this, an acidified methanolic extract (using *Arabidopsis* plants samples after 7 d of NDLT stress, as described in section 2.2.1) previously stored under cryogenic conditions was concentrated under vacuum to 40% of the original volume. The concentrated extract (100 µL) was combined with all other assays components as described above and incubated at 30 °C for 40 min. The initial concentration of flavonol bisglycosides and sinapate esters in the assay (expressed as Q3G equivalents) were determined by UHPLC-DAD-MS^n analysis as described below. The initial substrate concentrations in solution were as follows: 90 µM Q3Ru7R, 336 µM K3Ru7R, 184 µM Q3G7R, 410 µM K3G7R, 273 µM Q3R7R, 383 µM K3R7R, 991 µM sinapoylmalate, 39 µM 1,2-bis-*O*-sinapoyl-β-glucoside, and 264 µM 1-*O*-sinapoyl-β-glucoside. All assays were initiated by the addition of the protein, and stopped by flash

freezing in liquid N_2. To account for spontaneous hydrolysis of each substrate, all assays were also performed in the absence of the BGLU16 enzyme.

An equal volume of water-saturated butanol was added to the frozen assays and mixed by vortexing for 30 s. Partitioning of the butanol extracts was performed by centrifuging at 21, 000 *g* for 10 min. The butanol layer was collected and the aforementioned procedure was repeated twice. The pooled butanol layers were dried under vacuum and the dried residue was resuspended in 200 µL H_2O:CH_3CN:HCO_2H (90:10:0.1). For the single substrate assays, a 10 µL aliquot of the BGLU16 reaction products was analysed on an Agilent 1200 HPLC coupled to a DAD and separated on a Kinetex pentafluorophenyl column (10 x 4.6 mm, 2 µm Phenomenex; Torrence, CA) at 0.8 mL min^{-1}, as described by Roepke and Bozzo (2013). Detection of the flavonol aglycones was performed at 360 nm, *p*-nitrophenol (*p*NP) at 320 nm, and phloretin at 280 nm, and all were compared to known amounts of authentic standards. For Q3G7R, product peak areas were compared to known amounts of a quercetin standard, as the molar extinction coefficient of quercetin 7-*O*-α-rhamnoside is unknown.

Assays performed in the presence of an *Arabidopsis* extract containing phenylpropanoids and flavonols were analysed by UHPLC-DAD-MS^n. A 20 µL aliquot of the BGLU16 reaction products was analyzed on a Dionex UltiMate 3000 UHPLC (Thermo Scientific) coupled to a Bruker AmaZon SL MS^n and separated on a Poroshell 120 C-18 column (50 x 4.6 mm; 2.7 µm; Agilent Technologies, Mississauga, ON). Metabolites were eluted with a linear 5-100% solvent B (acetonitrile : formic acid, 100:0.1) gradient at a flow rate of 0.4 mL min^{-1} for 40 min. Detection of flavonol products was done at 360 nm. The masses of the eluted compounds were detected with an

AmaZon SL ion trap mass spectrometer interfaced with electro-spray ionization and analyzed in the negative ion mode with the gas flow at 1 mL min^{-1} and the nebulizer pressure at 35 psi. The drying gas temperature was 300°C with capillary voltages of 4 kV. The capillary exit of the source was -140 V and the end of the plate was offset by -500 V. Eluted masses were scanned at 70-1000 *m/z*. The ion trap was set to auto MS/MS, which fragmented the two most abundant precursors. An authentic standard of quercetin 3-*O*-glucoside was used for comparison.

CHAPTER THREE – RESULTS

3.1. EVIDENCE FOR THE TRANSIENT ACCUMULATION OF SINAPATE ESTER CONJUGATES IN *ARABIDOPSIS*

The analysis of UHPLC-MS^n data revealed that the concentration of the sinapate ester**,** 1-*O*-sinapoyl-β-glucoside, in *Arabidopsis* plants was 1.5-fold higher after 7 d exposure NDLT than the level at day 0 (Fig. 3.1A). The levels of this conjugate were relatively unchanged in plants cultivated under NSHT over the same time period (Fig. 3.1B).The transfer of NDLT-acclimated plants to NSHT was associated with a near-complete loss of this stress-inducible 1-*O*-sinapoyl-β-glucoside by day 1 of the recovery period, and by day 5 of the recovery period the level was similar to that prior to NDLT. No further change was apparent in plants left under continual NSHT. Similarly, UHPLC-MS^n analysis identified a peak eluting at 20.5 min as 1,2-bis-*O*-sinapoyl-β-glucoside. The level of this sinapate conjugate increased steadily with NDLT exposure, and by day 7 it was 2.7-fold higher than in plants sampled at the start of the stress period (Fig. 3.2). A 50% decline in 1,2-bis-*O*-sinapoyl-β-glucoside occurred within 1 d of the transfer of NDLT-stressed plants to NSHT. Overall, a 70% loss in this metabolite was apparent by day 5 of the recovery period. When plants were left under continual NSHT, 1,2-bis-*O*-sinapoyl-β-glucoside levels were relatively unchanged (Fig. 3.2B). A third sinapate derivative eluting at 19.1 min demonstrated a MS^n fragmentation pattern typical of sinapoylmalate. Interestingly, levels of sinapoylmalate were high and unchanged, regardless of treatment (Fig. 3.3).

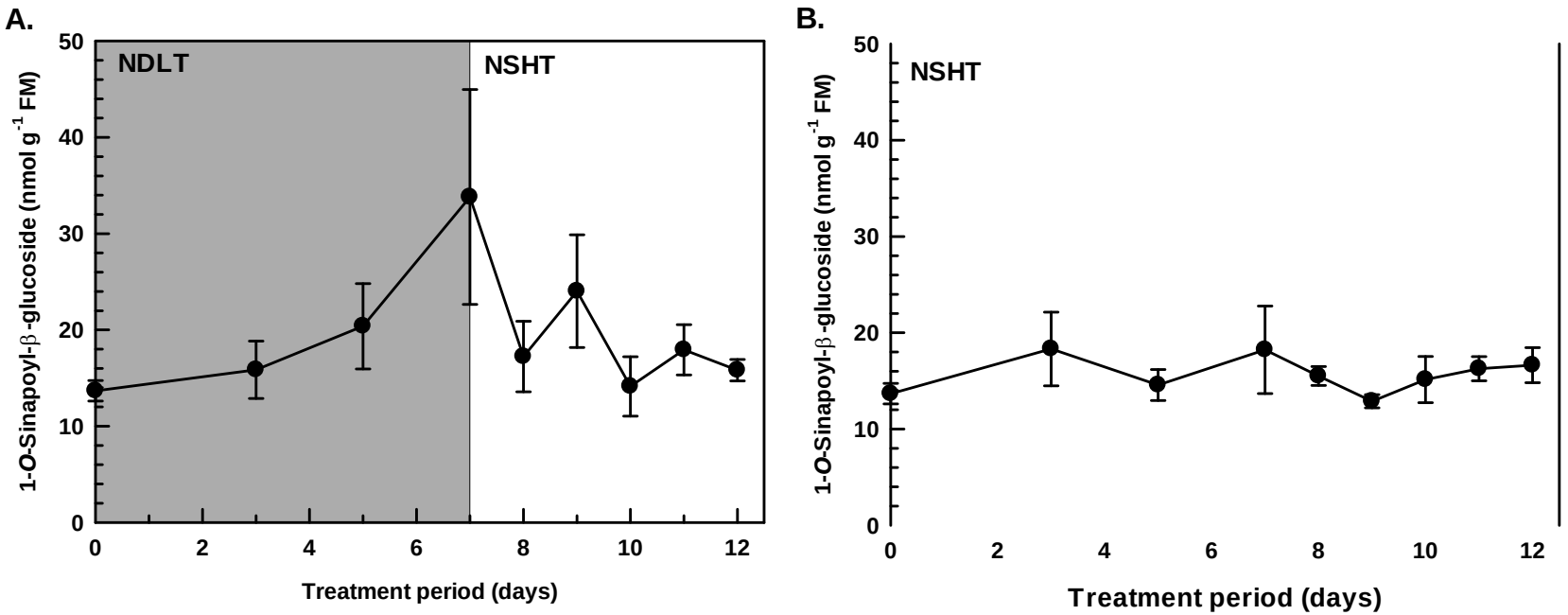

A.
NDLT
NSHT
1-O-Sinapoyl-β-glucoside (nmol g-1 FM)
Treatment period (days)
B.
NSHT
1-O-Sinapoyl-β-glucoside (nmol g-1 FM)
Treatment period (days)

Figure 3.1. Evidence for changes in 1-O-sinapoyl-β-glucoside levels in *Arabidopsis* (A) during NDLT and recovery therefrom, and (B) continual NSHT. (A) Plants were transferred to 0 mM nitrate at 10 °C (NDLT; represented as the grey-shaded portion of the time course) for 7 d. Thereafter, plants were re-supplied with 14 mM nitrate and maintained at 21 °C (NSHT; represented as the non-shaded portion of the time course) for 5 d. (B) As a control, plants of similar age were left under continual NSHT. 1-*O*-Sinapoyl-β-glucoside concentrations were determined by UHPLC-DAD-MS^n analysis of acidified methanolic extracts. A_{360} peak areas at retention time = 16.3 min were compared to known amounts of Q3G and corrected for amount of fresh matter used for the extraction. MS^n analysis confirmed the identity of 1-*O*-sinapoyl-β-glucoside with an [M-H]-parent ion of 385 and fragment ions of 247 and 223 (Horai et al., 2010). For all plots, each datum represents the mean ± SE of three separate experiments.

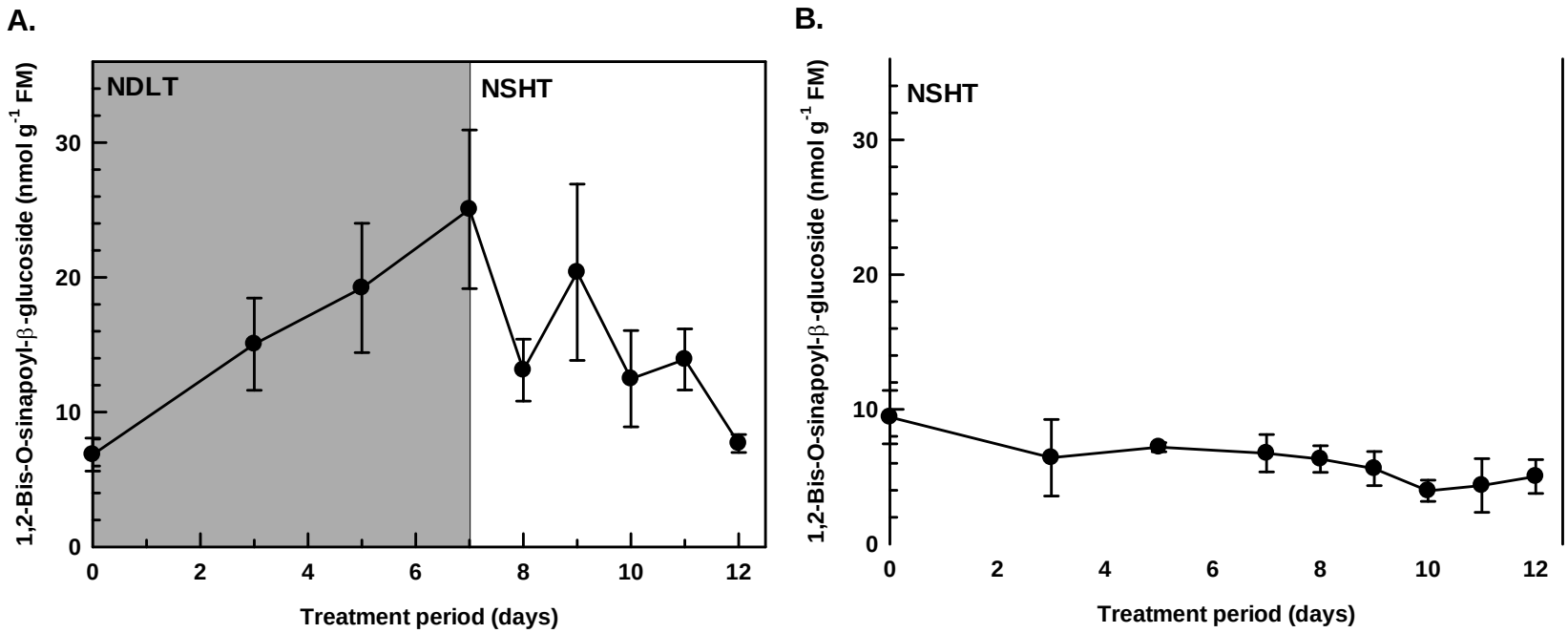

A.
NDLT
NSHT
1,2-Bis-O-sinapoyl-β-glucoside (nmol g-1 FM)
0
10
20
30
0
2
4
6
8
10
12
Treatment period (days)
B.
NSHT
1,2-Bis-O-sinapoyl-β-glucoside (nmol g-1 FM)
0
10
20
30
0
2
4
6
8
10
12
Treatment period (days)

Figure 3.2. Evidence for changes in 1,2-bis-*O*-sinapoyl-β-glucoside levels in *Arabidopsis* (A) during NDLT and recovery therefrom, and (B) continual NSHT. (A) Plants were maintained under 0 mM nitrate at 10 °C (NDLT; represented as the grey-shaded portion of the time course) for 7 d. Thereafter plants were supplied with 14 mM nitrate and maintained at 21 °C (NSHT; represented as the non-shaded portion of the time course) for 5 d. (B) As a control, plants of similar age were left under continual NSHT. 1,2-Bis-*O*-sinapoyl-β-glucoside concentrations were determined by UHPLC-DAD-MS^n analysis of acidified methanolic extracts. A_{360} peak areas at a retention time = 20.5 min were compared to known amounts of Q3G and corrected for amount of fresh matter used for the extraction. MS^n analysis confirmed the identity of 1,2-bis-*O*-sinapoyl-β-glucoside with an [M-H]- parent ion of 591 and fragment ions of 367 and 223 (Schymanshi and Neumann, 2013). For all plots, each datum represents the mean ± SE of three separate experiments.

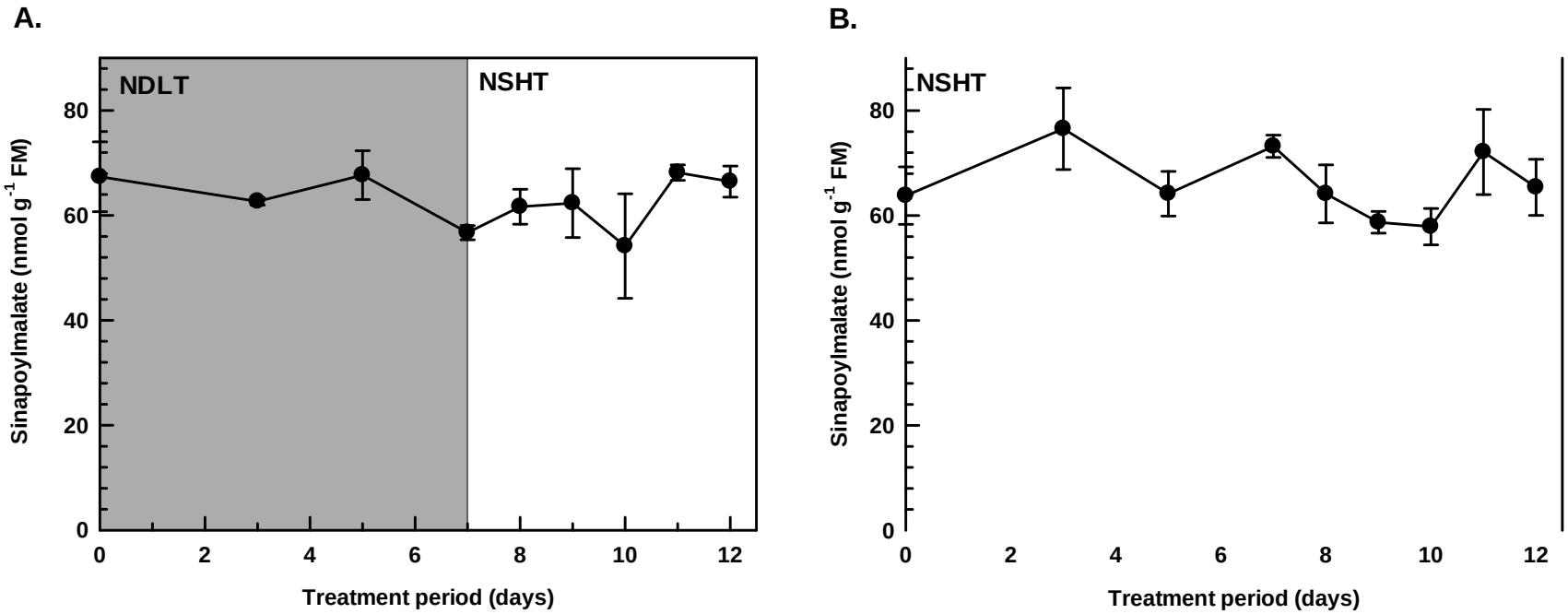

A.
NDLT
NSHT
Sinapoylmalate (nmol g-1 FM)
Treatment period (days)
0
20
40
60
80
0
2
4
6
8
10
12
B.
NSHT
Sinapoylmalate (nmol g-1 FM)
Treatment period (days)

Figure 3.3. Evidence for changes in sinapoylmalate levels in *Arabidopsis* (A) during NDLT and recovery therefrom; and (B) continual NSHT. (A) Plants were maintained under 0 mM nitrate at 10 °C (NDLT; represented as the grey-shaded portion of the time course) for 7 d. Thereafter plants were supplied with 14 mM nitrate and maintained at 21 °C (NSHT; represented as the non-shaded portion of the time course) for 5 d. (B) As a control, plants of similar age were left under continual NSHT. Sinapoylmalate concentrations were determined by UHPLC-DAD-MS^n analysis of acidified methanolic extracts. A_{360} peak areas at a retention time = 19.1 min were compared to known amounts of Q3G and corrected for amount of fresh matter used for the extraction. MS^n analysis confirmed the identity of sinapoylmalate with an [M-H]- parent ion of 339 and fragment ions of 223, 164, 133 (Horai et al., 2010). For all plots, each point represents the mean ± SE of three separate experiments.

3.2. BACTERIAL EXPRESSION AND PURIFICATION OF RECOMBINANT BGLU16

The recombinant thioredoxin-His_6 tagged mature BGLU16 was expressed in *E. coli* Origami^{TM} 2 (DE3) cells and purified using an IMAC column. Protein was eluted from the IMAC as two major peaks, although not well resolved from each other (Fig. 3.4). Separate fractions from the eluate were analyzed by SDS-PAGE and the corresponding immunoblots were probed with a His_6-antibody. Only fraction 7 revealed a homogenous preparation containing a single band of 73.9 kDa (Fig. 3.4A), which was free of immunoreactive degradation products (Fig. 3.5B). The purification strategy yielded 4 ± 1.7 mg (mean ± SE of three separate enzyme preparations) of the recombinant thioredoxin-His_6-mature BGLU16 from 6 L of bacterial culture.

3.3. EFFECT OF ASSAY pH ON RECOMBINANT BGLU16 ACTIVITY

A preliminary experiment revealed that BGLU16 hydrolysis activity was linear with assay time (5 to 40 min) and protein (up to 1.7 µg; Fig. 3.6) in the presence of 500 µM *p*NP-glucoside, an artificial substrate known to be used by many BGLUs (Ahn et al., 2010; Roepke and Bozzo, 2015). Thereafter, the effect of assay pH on the activity of the recombinant mature BGLU16 was assessed as the hydrolysis of *p*NP-glucoside across a broad pH range. The optimal pH was determined to be 5.5 (Fig. 3.7). All other biochemical assays were performed at pH 5.5.

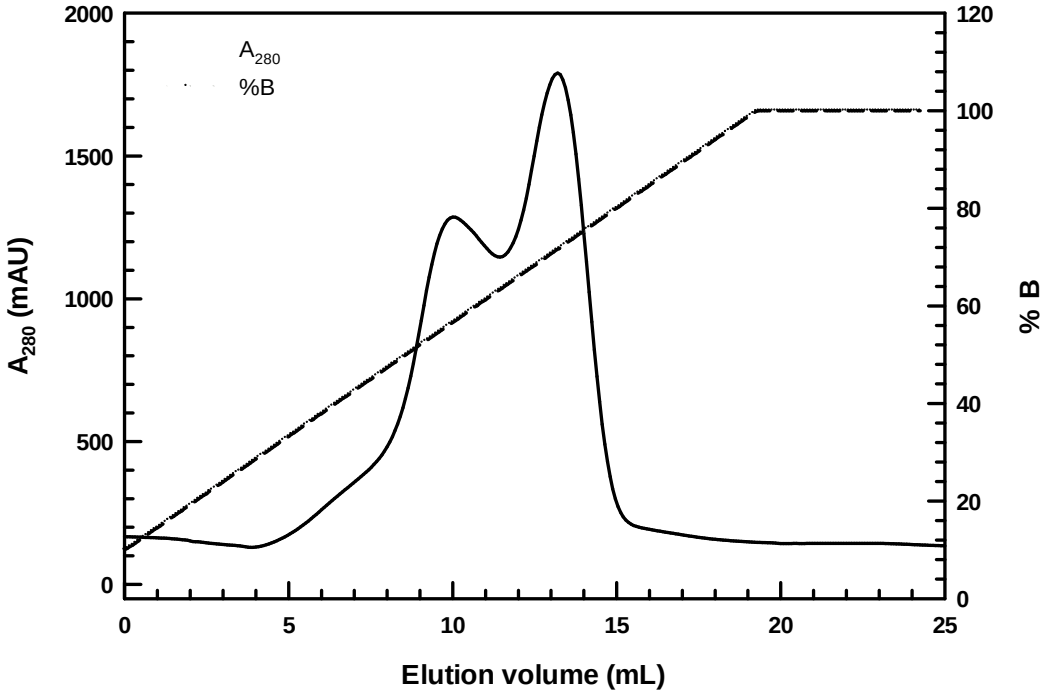
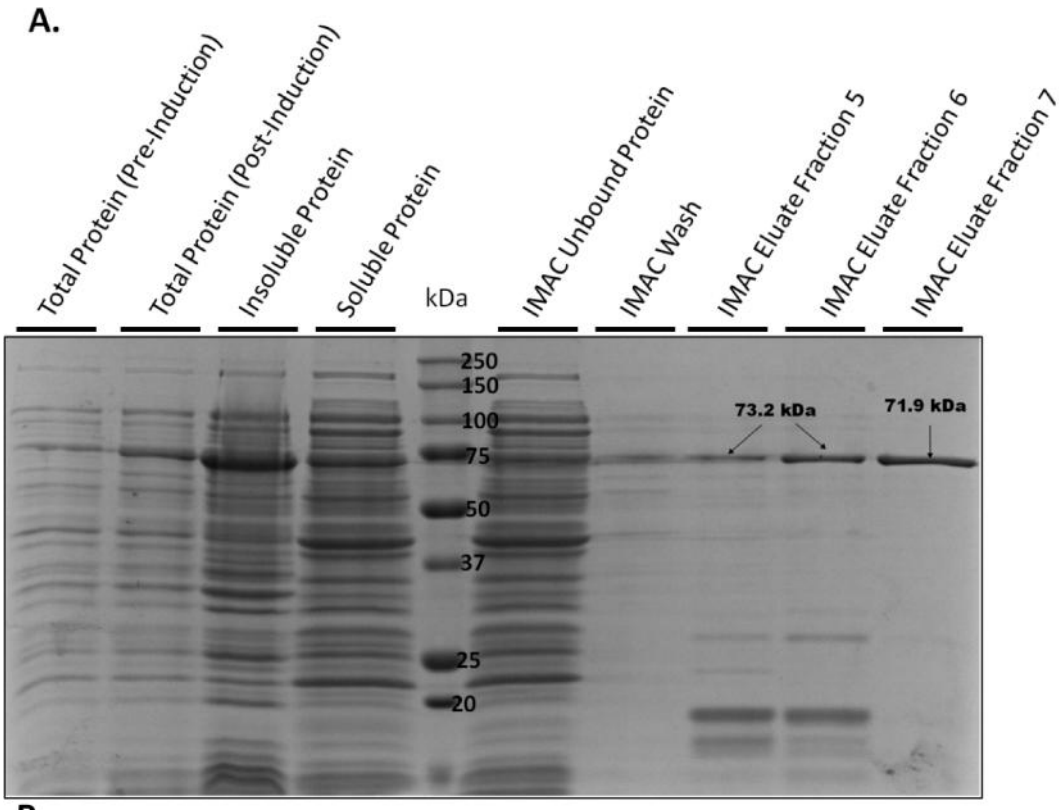


Figure 3.4. Immobilized metal affinity chromatography of the recombinant mature BGLU16. The column was developed with a linear imidazole gradient (10-100% buffer B; 50-500 mM imidazole) and fractions of 2 mL were collected. The protein eluting between 341 and 390 mM imidazole contained BGLU16 and no other protein contaminants as determined by SDS-PAGE analysis.


A.
Total Protein (Pre-Induction)
Total Protein (Post-Induction)
Insoluble Protein
Soluble Protein
kDa
IMAC Unbound Protein
IMAC Wash
IMAC Eluate Fraction 5
IMAC Eluate Fraction 6
IMAC Eluate Fraction 7
250
150
100
75
50
37
25
20
73.2 kDa
71.9 kDa

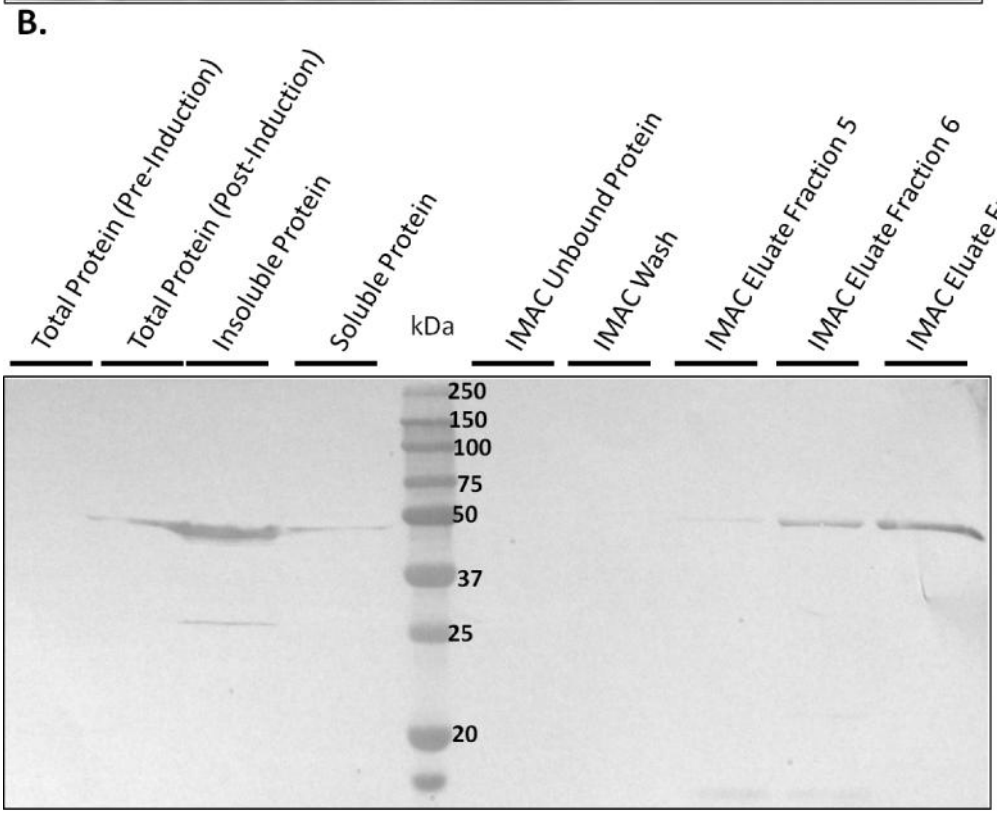
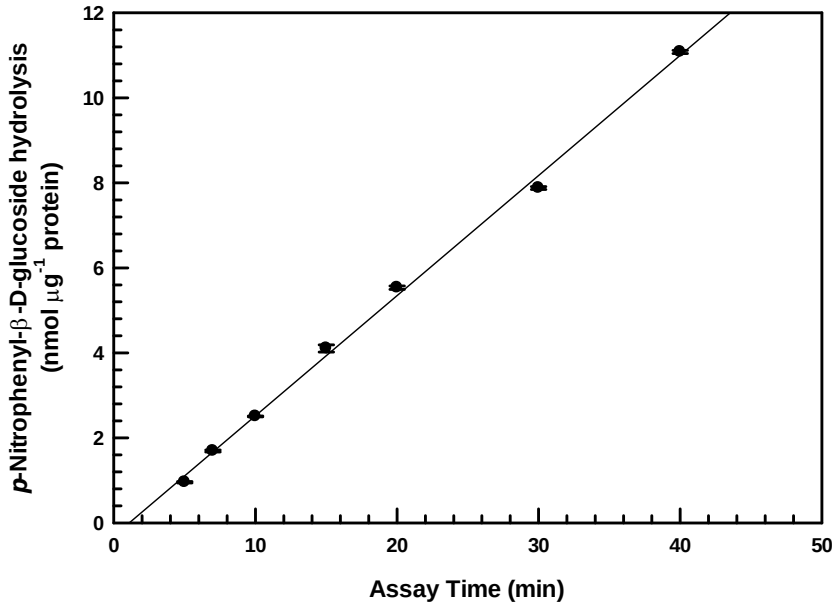

B.
Total Protein (Pre-Induction)
Total Protein (Post-Induction)
Insoluble Protein
Soluble Protein
kDa
IMAC Unbound Protein
IMAC Wash
IMAC Eluate Fraction 5
IMAC Eluate Fraction 6
IMAC Eluate Fraction 7
250
150
100
75
50
37
25
20

Figure 3.5. Coomassie Brilliant Blue SDS-PAGE (A) and immunoblot (B) analysis of the expression and purification of the thioredoxin-His_6 tagged mature BGLU16 from *E. coli* Origami™ 2 (DE3) cells. For each panel, the central lane corresponds to the size in kDa of the molecular mass standards. In cases where the protein amount is not given, each lane was loaded with 8 µL of the sample mixed with 8 µL of 2x SDS sample buffer. The immunoblot was probed with an anti-His_6 antibody.

A.


p-Nitrophenyl-β-D-glucoside hydrolysis
(nmol μg-1 protein)
0
2
4
6
8
10
12
0
10
20
30
40
50
Assay Time (min)

B.

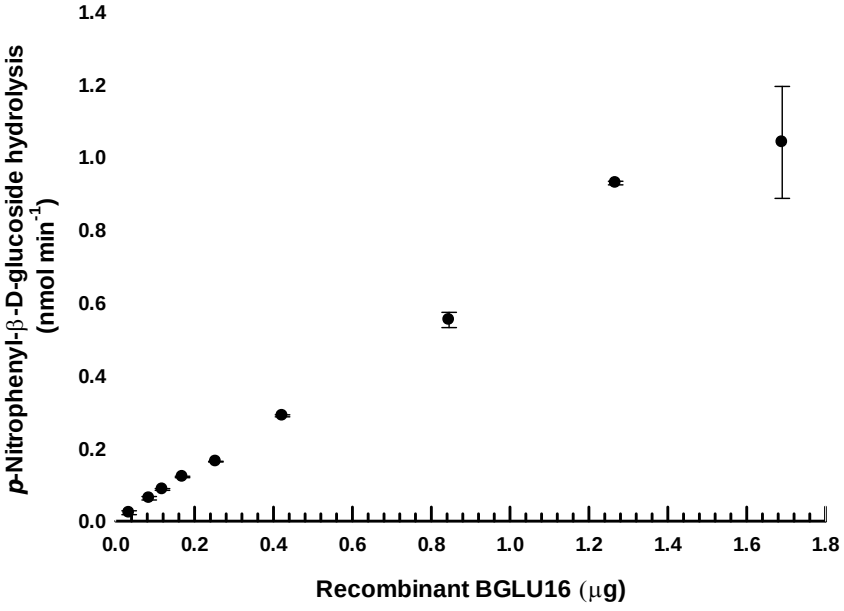

p-Nitrophenyl-β-D-glucoside hydrolysis
(nmol min-1)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Recombinant BGLU16 (μg)

Figure 3.6. Linearity of the BGLU16 assay with time and protein. (A) To assess whether the BGLU16 hydrolysis assay was linear with assay time, 500 µM *p*-nitrophenyl-β-D-glucoside was incubated with 230 ng of a representative homogenous BGLU16 preparation for various times in citrate acid : Na-citrate buffer at pH 5. **(B)** To assess the range of protein amount corresponding to a linear BGLU hydrolysis assay, reactions containing 0.03-1.69 µg of recombinant mature BGLU16 were incubated for 20 min. Data represents the mean ± SD of three separate determinations of a representative enzyme preparation. Assays were performed at 30°C.

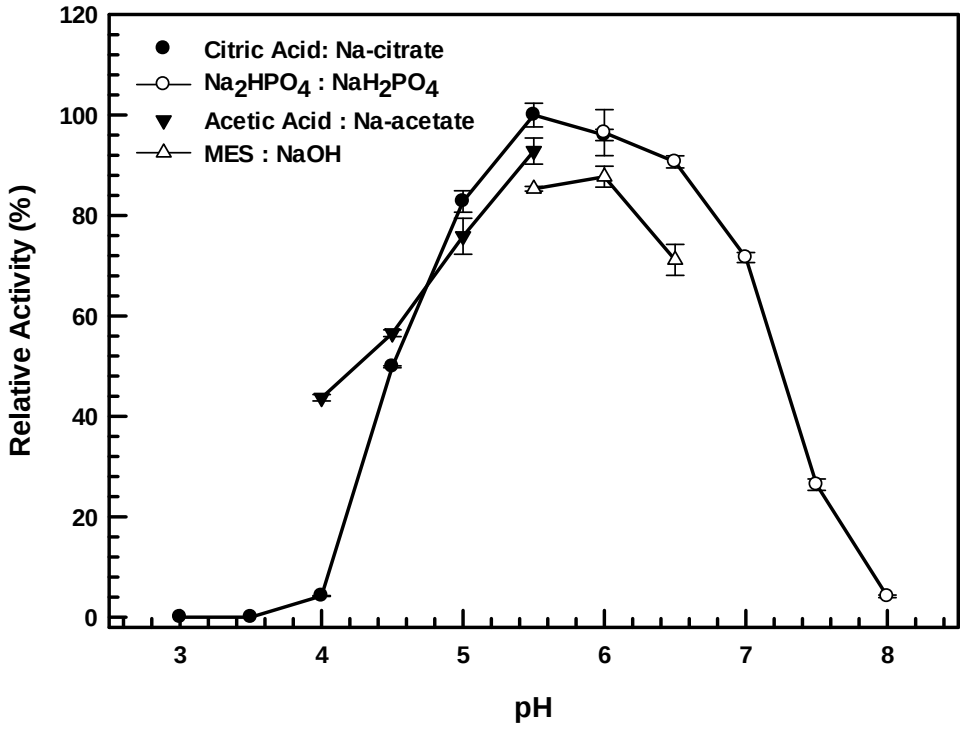


Figure 3.7. Dependence of recombinant BGLU16 activity on assay pH.

Activity was measured using 0.8 µg BGLU16 in the presence of 500 µM *p*NP-glucoside and a 30 min incubation period. For the pH profile, assays were buffered in 50 mM solutions of different biological buffers, including citric acid : Na-citrate (closed circles), MES : NaOH (open triangles), acetic acid : Na-acetate (solid inverted triangles) and Na_2HPO_4 : NaH_2PO_4 (open circles). In all cases, assay products were quantified by HPLC-DAD and peak areas of the product compared against known amounts of *p*-nitrophenol. All values represent the mean ± SE of three separate recombinant BGLU16 preparations. The relative activity corresponding to 100% is equivalent to a maximal activity 0.73 ± 0.20 µmol mg protein^{-1} min^{-1} (mean ± SE of three enzyme preparations) in citric acid : Na-citrate (pH 5.5).

3.4. RECOMBINANT BGLU16 UTILIZES QUERCETIN 3-O-β-GLUCOSIDE AND SINAPATE ESTERS

To assess substrate utilization by BGLU16, hydrolysis activity was monitored at a fixed substrate concentration (500 µM) as demonstrated in Fig. 3.8. BGLU16 hydrolyzed the artificial substrate pNP-glucoside. However, activity was also shown for Q3G, albeit this was much lower. Hydrolytic activity was absent in the presence of all other quercetin monoglucosides and the bisglycoside, Q3G7R. Esculin and kaempferol 3-*O*-β-glucoside were also used by the enzyme, albeit at dramatically lower rates relative to *p*NP-glucoside and Q3G (Fig 3.8).

BGLU16 assays with an *Arabidopsis* phenylpropanoid/flavonol extract mixture was analyzed by UHPLC-DAD-MS^n (Fig. 3.9). This revealed that the levels of flavonol bisglycosides in the assay were unchanged following incubation with the mature recombinant BGLU16. Although the abundance of sinapate esters was not significantly affected, there was evidence for the production of sinapate following 40 min incubation with BGLU16. Since the concentrations of both 1-*O*-sinapoyl-β-glucoside and 1,2-bis-*O*-sinapoyl-β-glucoside were not significantly affected by the protein, it is not clear which metabolite was being catabolised by BGLU16.

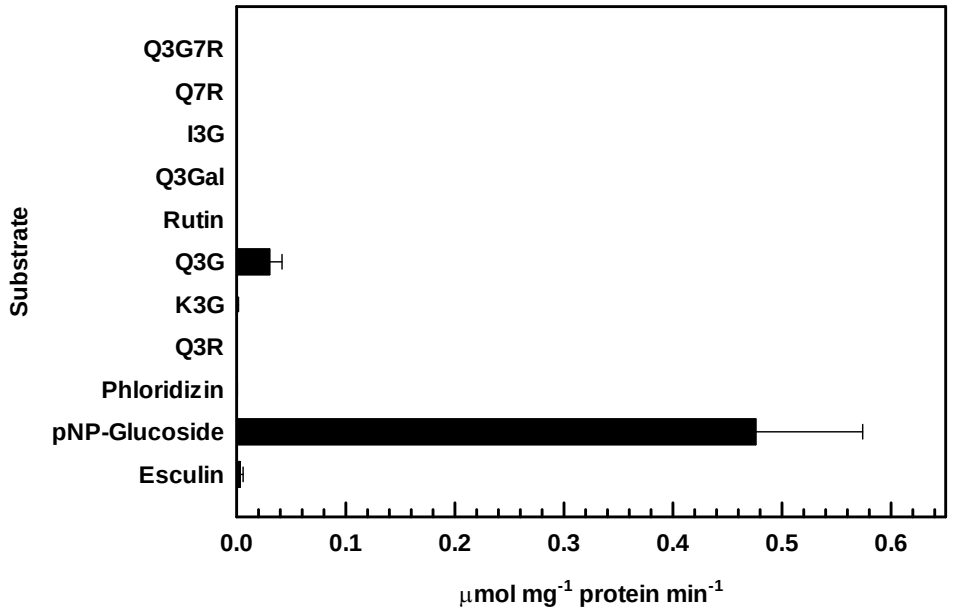


Figure 3.8. Substrate preference of recombinant thioredoxin-His$_6$ tagged mature BGLU16.

Individual *in vitro* hydrolysis activities were measured at 500 μM substrate with 0.8 μg BGLU16 at pH 5.5; assays were incubated for 30 min and products detected by HPLC-DAD. Data represent the mean ± SE of three separate recombinant protein preparations. Abbreviations include: I3G, isorhamnetin 3-O-β-glucoside; K3G, kaempferol 3-O-β-glucoside; *p*NP-glucoside, *p*-nitrophenyl-β-D-glucoside; Q3Gal, quercetin 3-O-β-galactoside; Q3G7R, quercetin 3-O-β-glucoside-7-O-α-rhamnoside; Q3R, quercetin 3-O-α-rhamnoside; Q7R, quercetin 7-O-α-rhamnoside.

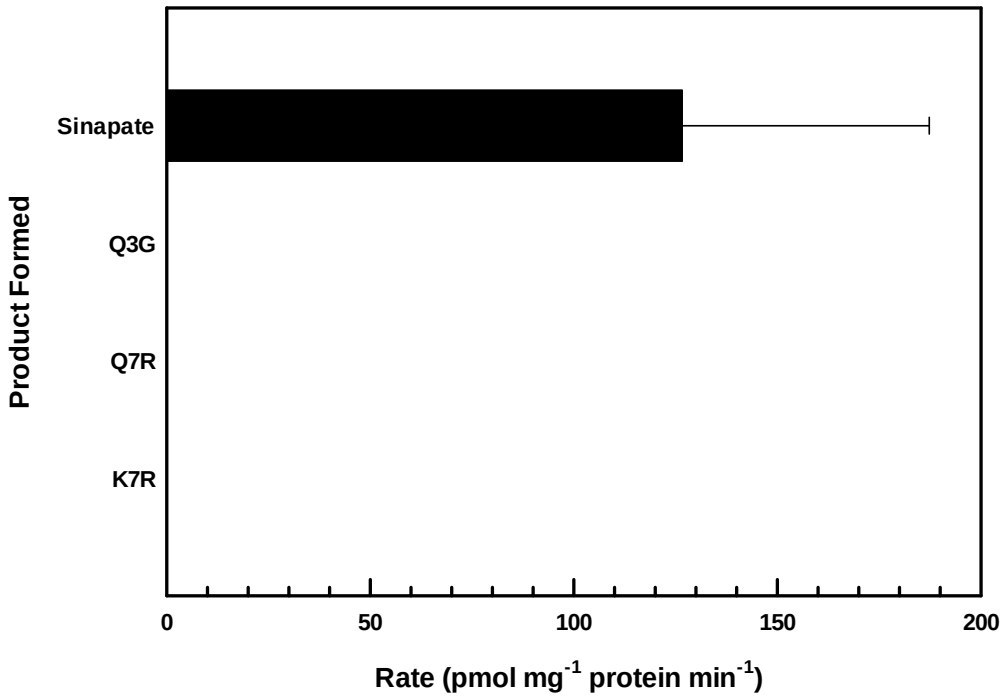


Figure 3.9. Product formation from an *Arabidopsis*-derived phenylpropanoid / flavonol mixture following incubation with mature recombinant BGLU16.

Hydrolysis rates were measured at the final substrate concentrations for flavonol bisglycosides and sinapate esters as described under section 2.6., in assays containing 1.4 µg BGLU16 at pH 5.5; assays were incubated for 40 min and products detected by UHPLC-DAD-MS^n. Reaction rates for sinapate represent the mean of two separate recombinant protein preparations (assays for each preparation included three technical replicates), which ranged from 66 to 187 nmol mg^{-1} protein min^{-1}. Abbreviations include: Q3G, quercetin 3-*O*-β-glucoside; K7R, kaempferol 7-O-α-rhamnoside; Q7R, quercetin 7-O-α-rhamnoside;

CHAPTER FOUR – DISCUSSION

4.1. IMPACT OF TRANSIENT ABIOTIC STRESS ON LEVELS OF SINAPATE ESTERSIN *ARABIDOPSIS*

Sinapate and its esters accumulate in epidermal tissues of leaves in response to UV-B (Dixon and Paiva, 1995). In addition, total sinapate esters are 50% higher in rosette stage *Arabidopsis* leaves after 5 d of nitrogen-deficient growth at 20 °C, relative to nitrogen-sufficient plants (Feyissa et al., 2009). Here, UHPLC-MS^n-DAD analysis revealed a similar relative change for 1-*O*-sinapoyl-β-D-glucose levels in *Arabidopsis* plants that had been subjected to NDLT for 5 d (Fig. 3.1), although a further increase was detected following an additional 2 d of stress. More importantly, larger changes in 1,2-bis-*O*-sinapoyl-β-glucoside were apparent during this period (Fig. 3.2). Plants were subjected to nitrogen deficiency at 10 °C , which appeared to synergistically enhance the levels of sinapate glycoside conjugates relative to an earlier study wherein nitrogen-stressed plants were cultivated at 20 °C (Fessiya et al., 2009). In fact, sinapate esterification is increased in the mesophyll tissue of winter oilseed rape leaves when plants are left at 2 °C for 3 weeks, followed by a brief freezing period (-5 °C for 18 h) (Solecka et al., 1999). The levels of sinapate esters detected in *Arabidopsis* plants after 7 d of NDLT (10-60 nmol g FM^{-1}) are an order of magnitude lower than the flavonol bisglycosides concentrations (200-300 nmol g FM^{-1}) (Olsen et al., 2009; Roepke and Bozzo, 2015). The comparatively lower level of sinapate esters could be due in part to the relative quantification protocol used here as UHPLC-DAD-MS^n peak areas

corresponding to sinapate esters were expressed as Q3G equivalents, a molecule with a greater mass than any sinapate compound. The flavonol glycoside levels described by Bozzo and Roepke (2015) were also expressed in Q3G equivalents. Alternatively, the lower levels could be due to the role of 1-*O*-sinapoyl-β-D-glucose as a metabolic precursor of sinapolylated anthocyanins (Yonekura-Sakakibara et al., 2012). In *Arabidopsis*, glucosylation of sinapate occurs via UGT84A2 (Lim et al., 2001; Sinlapadech et al., 2007). In fact, *UGT84A2* gene expression is upregulated in *Arabidopsis* seedlings in response to nutrient deficiency (Lillo et al., 2008), and is co-expressed with anthocyanin biosynthesis genes (Yonekura-Sakakibara et al., 2012). Anthocyanins levels are markedly increased with NDLT (Olsen et al., 2009).

Interestingly, the levels of the major sinapate ester, sinapoylmalate, were unaffected by NDLT, and they were equivalent to plants cultivated under NSHT (Fig. 3.3), suggesting that this metabolite is not induced in *Arabidopsis* in response to low temperature or nitrogen deficiency. Sinapoylmalate is the most abundant sinapate ester in *Arabidopsis*, and it resides within the epidermis of the adaxial leaf surface (Lorenzen et al., 1996; Li et al., 2010). Sinapoylmalate is a major UV-B absorbing metabolite, which is possibly involved in constitutive photoprotection. Sinapoylmalate biosynthesis is partly dependent on the availability of nitric oxide, a signalling molecule known to function in biotic and abiotic stress resistance, and likely present at elevated levels under NDLT (Wang and Wu, 2005; Besson-Bard et al., 2008; Wilson et al., 2008; Santos-Filho et al., 2012). Nitric oxide availability is associated with higher phenylpropanoid gene expression (Delledonne et al., 1998). The lack of any change in sinapoylmalate

concentration in NDLT-stressed *Arabidopsis* tissues could be indicative of low nitric oxide levels, although this possibility requires further study.

4.2. BGLU16 COULD BE INVOLVED IN CATABOLISM OF SINAPOYL GLUCOSIDES

The formation of flavonol bisglycosides occurs in *Arabidopsis* with NDLT (Olsen et al., 2009; Roepke and Bozzo, 2015). The loss of flavonol 3-*O*-β-glucoside-7-*O*-α-rhamnosides is evident during the recovery from NDLT stress, and is dependent upon BGLU15 activity (Roepke, 2015). BGLU16 is phylogenetically similar to BGLU15 and its transcript levels are elevated by 68% during the recovery from NDLT (Roepke and Bozzo, 2015). The recent metabolic characterization of *bglu15* mutants revealed that K3G7R, Q3G7R and flavonol 3-*O*-β-glucosides are not catabolized *in planta* during recovery from abiotic stress, while other flavonol bisglycosides were lost (Roepke, 2015), suggesting that BGLU16 is involved in hydrolysis of other flavonol bisglycosides and/or their related sinapate esters.

Hydrolysis activity for the of *p*NP-glucoside by BGLU16 at pH 5.5 was approximately 0.73 μmol mg $^{-1}$ protein min^{-1}, approximating that determined previously for BGLU 15 (Fig. 3.8; Roepke and Bozzo, 2015); only Q3G was hydrolyzed at a slightly lower rate by BGLU16 (Fig. 3.8). The rate of hydrolysis of the recombinant *Arabidopsis* BGLU16 was optimal at acidic pH (Fig. 3.7). This is similar to the pH optimum for BGLU15 (Roepke and Bozzo, 2015), an *Arabidopsis* scopolin hydrolase (Ahn et al., 2010), and a soybean isoflavone conjugate hydrolase (Suzuki et al., 2006). The acidic pH optimum for BGLU16 could be physiologically relevant, as this is the pH of the

apoplastic milieu (Husted and Schjoerring, 1995), and the subcellular localization site of phylogenetically-related proteins, such as *Arabidopsis*BGLU15 and soybean isoflavone conjugate hydrolase (Suzuki et al., 2006; Ropeke and Bozzo, 2015). Interestingly, a number of phenolic compounds, including flavonol glycosides, are confined to the cell wall (Strack et al., 1988; Agati and Tattini, 2010) and could be available to co-localized enzymes. The absence of activities with other quercetin conjugates, including Q3G7R, suggests that BGLU16 prefers other physiological substrates.

The recombinant BGLU16 produced sinapate at a rate of 130 nmol min^{-1} mg $protein^{-1}$. The reaction rates detected for BGLU16 appear to be similar to the maximum activities known for other plant BGLUs, including those specific for scopolin glucosides, isoflavone glucosides, monolignol glucosides and flavonol 3-*O*-β-glucoside-7-*O*-α-rhamnosides (Escamilla-Treviño et al., 2006; Naoumkina et al., 2007; Ahn et al., 2010; Roepke and Bozzo, 2015). The substrate range used in these studies was 250 µM to 10 mM. It is difficult to determine whether one or more of the sinapate ester conjugates was hydrolyzed, as the assay was performed under conditions where very little of the substrates were present in the original metabolite mixture, and none were used to a significant degree. The assays used to assess substrate utilization by BGLU15 were conducted at a substrate concentration of 500 µM, and 1-*O*-β-sinapoyl-glucoside and 1,2-bis-*O*-sinapoyl-β-glucoside were present at concentrations of 264 µM and 39 µM, respectively, which may be sub-saturating concentrations. A complete kinetic analysis of BGLU16 in the presence of sinapate esters was not attempted due to the commercial unavailability of these compounds. Analysis of the catalytic efficiency of the enzyme could only be achieved following the biocatalysis of these compounds from sinapate

supplied to cells engineered with UGT84A2, an *Arabidopsis* sinapate:UDP-glucose sinapoyltransferase, using previously published methods (Roepke and Bozzo, 2013). Since BGLU16 is phylogenetically related to hydrolases known to use *O*-β-linked glucosides, it seems plausible that 1-*O*-β-sinapoyl-glucoside and/or 1,2-bis-*O*-sinapoyl-β-glucoside are likely hydrolyzed by this enzyme. BGLU16 has a low activity with the substrates tested here; however, its physiological substrate could be another sinapoylated metabolite, such as cyanidin 3-*O*-[6-O-(4-*O*-β-D-glucopyranosyl-p-coumaroyl)-2-*O*-(2-*O*-sinapoyl-β-D-xylopyranosyl)-β-D-glucopyranosyl]-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside) (Bloor and Abrahams, 2002). Sinapoylated anthocyanins accumulate during NDLT and photooxidative stresses, and are degraded during recovery from abiotic stress conditions (Olsen et al., 2009; Maruta et al., 2014). Unfortunately, the high degree of modification of this major anthocyanin suggests that biocatalysis is infeasible; thus, isolation from *Arabidopsis* tissues would be required to test this possibility. Alternatively, the enzymatic mechanism for BGLU16 may involve a transglycosidase activity. In fact, Os9BGLU31 is a rice transglycosidase that transfers glucose moieties between phenolic acids, flavonoids, and phytohormones (Luang et al., 2013).

It is tempting to speculate that sinapate formed from a BGLU-mediated degradation of 1-*O*-sinapoyl-β-glucoside could be a precursor for lignin biosynthesis. Lignin is a polymer of phenylpropanoids, including *p*-coumaryl, coniferyl and sinapyl alcohol monomers, which are derived from *p*-coumaric acid, caffeic acid and sinapate, respectively (Boerjan et al., 2003). The hydroxycinnamic acids are converted to monolignols by *p*-coumaroyl CoA-ligase, cinnamoyl CoA reductase and cinnamyl alcohol dehydrogenase (Humphreys and Chapple, 2002). The relative proportion of these

monolignols depends on the plant species; angiosperm lignin consists primarily of guaiacyl and syringyl monolignols, and gymnosperm lignins primarily of guaiacyl monolignols (Baucher et al., 1998). The lignin structure depends on the availability of monolignols, the rate of production of reactive oxygen species, and polysaccharides (Terashima et al., 1995). To confirm this metabolic fate, radiolabelled sinapate could be supplied to 1-*O*-β-sinapoyl-glucoside-deficient *brt1* mutants of *Arabidopsis* during the recovery from NDLT.

CHAPTER FIVE - LITERATURE CITED

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