Characterization of Glyoxylate/Succinic Semialdehyde Reductases in Plants and Impact of Elevated CO₂ on γ-Aminobutyrate Metabolism in ‘Empire’ Apple Fruit Stored Under Controlled Atmosphere Conditions

by

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ABSTRACT

Characterization of Glyoxylate/Succinic Semialdehyde Reductases in Plants and Impact of Elevated CO$_2$ on $\gamma$-Aminobutyrate Metabolism in ‘Empire’ Apple Fruit Stored Under Controlled Atmosphere Conditions

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Plant NADPH-dependent glyoxylate/succinic semialdehyde reductases 1 and 2 (GLYR1 and GLYR2) are hypothesized to detoxify the reactive aldehydes, photorespiratory-derived glyoxylate and $\gamma$-aminobutyrate (GABA)-derived succinic semialdehyde (SSA), into glycolate and $\gamma$-hydroxybutyrate (GHB), respectively. Here, recombinant GLYR1 and/or GLYR2 proteins from apple, rice and Arabidopsis thaliana were produced and used for kinetic analyses of enzyme activity. Plant GLYRs used NADPH more efficiently than NADH, preferred glyoxylate to SSA, and were feedback inhibited by a high NADP$^+$/NADPH ratio, suggesting that GLYRs are redox regulated in planta. Furthermore, green fluorescent protein-GLYR fusions were generated to investigate their subcellular localizations using various transient and stable expression systems. GLYR1 was cytosolic, whereas GLYR2 was dual localized to mitochondria and plastids, which was corroborated by the isolation and recovery of glyoxylate reductase activity in Percoll gradient-purified mitochondria from an atglyr1 mutant. Plant GLYRs are proposed to have specialized functions related to GABA- and photorespiratory stress-derived aldehydes within their respective cellular compartments.
Abiotic stress causes GABA accumulation in plants and plant organs such as apple fruit, notably under controlled atmosphere (CA) storage used to preserve apple quality. CA is comprised of multiple abiotic stresses (i.e., elevated CO₂, low O₂ and chilling) and can cause physiological disorders, which may be associated with GABA. GABA can originate from the decarboxylation of glutamate or the terminal oxidation of the polyamines putrescine and spermidine. Here, we determined the incidence of CO₂ injury and profiled the metabolites and transcripts associated with GABA metabolism in ‘Empire’ apples stored with 0.03 or 5 kPa CO₂ and 2.5 kPa O₂ at 0 or 3°C, and sampled over a 16-week period. Fruit stored at elevated CO₂, particularly at 0°C, developed a higher incidence of external CO₂ injury, which was more associated with polyamines than GABA. Discrepancy between metabolite and gene expression patterns suggests that GABA is primarily derived from glutamate and that it is catabolized to GHB, rather than succinate, due to a highly reduced redox environment within the fruit. These findings are interpreted as evidence for the involvement of both gene-dependent and -independent mechanisms for the metabolism of GABA in apples stored under CA conditions.
ACKNOWLEDGEMENTS

As my graduate career comes to a close, I feel privileged to have learned so much in the company of such extraordinary people. The undertaking and completion of this thesis was made possible with the constant support and guidance of my advisors Dr. Barry Shelp and Dr. Gale Bozzo. I deeply appreciate your generosity, patience and counselling, as well as your availability for any questions I may have had. Thank you to Dr. Robert Mullen and Dr. Satinder Gidda for use of their lab and equipment as well as their valuable advice and expertise, and to Dr. Jennifer DeEll for her insight and assistance with the apple project as well as this thesis. I would not have grown into the researcher I am today without the support of Dr. Adel Zarei, Dr. Chris Trobacher, Dr. Vikram Bajwa and Dr. Gord Hoover who significantly contributed to this thesis and helped me with many protocols and methods; thank you for your availability to discuss results, questions and concerns.

I am incredibly grateful to my wonderful parents, close friends and partner, Angelo, for grounding me by reminding me of the important things in life, inspiring me with your courage and compassion and encouraging me to push myself in accomplishing this milestone. To my lab mates and friends, thank you for the camaraderie; I feel very fortunate to have been part of this community where we shared many ideas, laughs and beers. Lastly, this research would not have been possible without the generosity of funding agencies such as Apple Growers of Ontario, MITACS Inc., Rohm and Haas, the Natural Sciences and Engineering Research Council of Canada, and the Ontario Ministry of Agriculture & Food. I am also honoured to have received various University of Guelph and Ontario Agriculture College awards.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>OH</td>
<td>hydroxyl radical</td>
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<tr>
<td>µg</td>
<td>microgram</td>
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<td>µL</td>
<td>microliter</td>
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<td>micromolar</td>
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<tr>
<td>1-MCP</td>
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<td>alanine transaminase</td>
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<td>β-hydroxyacid dehydrogenase</td>
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<td>tobacco bright yellow-2</td>
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<td>CA</td>
<td>controlled atmosphere</td>
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<td>Ca&lt;sup&gt;2+&lt;/sup&gt;/CaM</td>
<td>calcium/calmodulin</td>
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<td>CP-PLS</td>
<td>canonical-powered partial least squares</td>
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<td>cetyltrimethylammonium bromide</td>
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<td>Cu</td>
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<td>d</td>
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<td>ethylenediaminetetraacetic acid</td>
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<td>glutamate decarboxylase</td>
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CHAPTER ONE – GENERAL INTRODUCTION

γ-Aminobutyrate (GABA) is a non-protein amino acid first discovered in potato tubers, and is now mostly recognized as a neurotransmitter in the mammalian central nervous system (see Kinnersley and Turano, 2000). In plants, the precise role of GABA remains highly speculative, but evidence suggests that it is involved in various processes such as biochemical pH-stat, tricarboxylic acid cycle (TCA) bypass, nitrogen storage, plant development and plant defense (Shelp et al., 1999; Kinnersley and Turano, 2000). Furthermore, it is clear that GABA is associated with the stress response, since it accumulates many-fold in response to a variety of both biotic and abiotic stresses, including cellular acidosis, mechanical damage, cold, O$_2$ deficiency, heat, drought and salinity in virtually every plant organ or tissue examined to date (Kinnersley and Turano, 2000; Shelp et al., 2009).

GABA is produced in the cytosol from the amino acid glutamate via glutamate decarboxylase (GAD) activity, which is activated by stress-induced calcium/calmodulin (Ca$^{2+}$/CaM) or stimulated by acidic pH (Shelp et al., 1999). Theoretically, GABA can also originate from the terminal catabolism of the polyamines putrescine and spermidine via the action of O$_2$-dependent Cu-containing amine oxidases (AO) producing 4-aminobutanal, which in turn is converted into GABA by NAD$^+$-dependent aminoaldehyde dehydrogenase (AMADH) (Shelp et al., 2012b; Tiburcio et al., 2014; Zarei et al., 2015). GABA enters the mitochondrion through a GABA permease and is converted to succinic semialdehyde (SSA) by glyoxylate or pyruvate-dependent GABA transaminase (GABA-T). SSA is oxidized to the TCA cycle intermediate, succinate, by NAD$^+$-dependent succinic semialdehyde dehydrogenase (SSADH), or reduced to γ-
hydroxybutyrate (GHB) by cytosolic and plastidial NADPH-dependent glyoxylate reductases (GLYR) 1 and 2, respectively.

GLYRs are so named because they have the dual function of reducing both GABA-derived SSA with milimolar affinity and photorespiratory-derived glyoxylate with micromolar affinity (see Allan et al., 2009). During photorespiration, glyoxylate is produced from glycolate in the peroxisome by the O$_2$-dependent enzyme glycolate oxidase. Glyoxylate and SSA are reactive aldehydes that are detrimental to plant health and photosynthesis, and may accumulate as a result of abiotic and photorespiratory stresses. Plant GLYRs are therefore thought to play an important role in maintaining plant health by reducing glyoxylate and SSA, which may have leaked from their respective cellular compartments, to their less reactive corresponding alcohols, glycolate and GHB, respectively (Allan et al., 2009). To date, knowledge of plant GLYRs originates mostly from analysis of structural, kinetic and subcellular localization of Arabidopsis thaliana GLYRs (Breitkreuz et al., 2003; Hoover et al., 2007a, b; Simpson et al., 2008; Ching et al., 2012; Hoover et al., 2013). AtGLYR1 (cytosolic) and AtGLYR2 (plastidial) isoforms are similar in their amino acid sequence, except for an N-terminal signal peptide present in GLYR2 (Simpson et al., 2008, Ching et al., 2012). Both AtGLYRs prefer glyoxylate over SSA in vitro, are more active with NADPH than NADH as a co-factor, and are product inhibited, especially by NADP$^+$, indicating that they could be redox regulated in vivo (Hoover et al., 2007a, b, Simpson et al., 2008). Despite an in vitro preference for glyoxylate, the use of glyr1 and glyr2 knockout mutants of Arabidopsis suggests that GHB can be derived from SSA via both GLYR isoforms in vivo (Allan et al., 2012).
Abiotic stress is known to cause the accumulation of GABA and to enhance the NAD(P)H/NAD(P)+ ratio such that GABA-derived SSA is diverted from succinate production via NAD+-dependent SSADH to GHB production via NADPH-dependent GLYRs (Allan et al., 2003, 2008, 2012); this provides strong support for the redox regulation of GLYRs and their involvement in aldehyde detoxification during stress. In Chapter Three of this thesis, we further tested the hypothesis that plant GLYRs function in the NADPH-dependent reduction of glyoxylate and SSA to glycolate and GHB, respectively. GLYRs were cloned from apple, Arabidopsis and rice cDNAs, and the corresponding recombinant proteins were individually produced in, and purified from *Escherichia coli* using affinity chromatography, and used to determine kinetic parameters in order to assess substrate and co-factor preference. Furthermore, Arabidopsis, apple and rice GLYRs were fused to green fluorescent protein (GFP) and used to assess GLYR subcellular localization in tobacco bright yellow-2 (BY-2) cells, Arabidopsis protoplasts and stably-transformed Arabidopsis seedlings expressing inducible-Ar*GLYR2*-GFP. In addition, GLYR activity was measured in a Percoll-purified isolated mitochondrial fraction from an *atglyrl* mutant. Rice (a monocotyledonous plant) and apple as well as Arabidopsis (dicotyledonous plants) represent a diverse array of plant species, and studies of their GLYR enzyme kinetics and subcellular localization should provide a broader view of GLYR function in aldehyde detoxification throughout the plant kingdom.

The GABA pathway has been extensively studied in photosynthetic plant organs. Much less information is available on GABA metabolism in non-photosynthetic organs such as apple fruit, which were recently shown to accumulate GABA as a consequence of controlled atmosphere (CA) storage and associated storage disorders (Deewatthanawong
and Watkins, 2010; Lee et al., 2012; Trobacher et al., 2013a; Deyman et al., 2014b; Lum, 2014; Chiu et al., 2015). CA storage employs multiple abiotic stresses (i.e., low O₂, low temperature and high CO₂) to delay ripening and senescence processes, and can lead to storage disorders such as external CO₂ injury and flesh browning of ‘Empire’ apples (Watkins and Liu, 2010). The biochemical mechanisms underlying physiological injury in apple fruits during CA storage are uncertain. Moreover, apple fruit stored under CA conditions provide an interesting model to assess metabolic processes under high CO₂ combined with O₂ deficiency, especially since O₂ availability is known to be restricted in bulky fruit such as apple (Ho et al., 2011). For example, the activities of O₂-dependent enzymes such as polyamine oxidase (PAO) and AO, as well as NAD(P)⁺-dependent enzymes such as AMADH, are predicted to be limited, whereas the activities of enzymes such as GAD and GLYR are predicted to be stimulated under low O₂ stress and elevated NAD(P)H/NAD(P)⁺ ratios (Shelp et al. 2012b).

In Chapter Four of this thesis, we tested the following hypotheses: i) GABA production in CA-stored apple fruit is supported primarily by glutamate decarboxylation, rather than polyamine catabolism; ii) GABA catabolism is diverted to GHB production rather than succinate; and iii) GABA levels are associated with CO₂-induced external injury. This study was based on a 16-week experiment conducted in 2009 with ‘Empire’ apples stored under high (5 kPa) or low (0.03 kPa) CO₂, at low O₂ (2.5 kPa), and either high (3°C) or low (0°C) temperature (Deyman et al. 2014b). The apples were collected over the time course and GABA pathway- as well as polyamine catabolism-related transcripts were analyzed using quantitative real-time polymerase chain reaction (qPCR). Amino acids and other metabolites associated with these pathways were extracted and
analyzed via multiple methods including gas chromatography coupled to tandem mass spectroscopy (GC-MS/MS), high performance liquid chromatography (HPLC) and enzyme-linked assays. Canonical powered partial least squares (CP-PLS) correlation analyses were performed to assess metabolite-metabolite, metabolite-transcript and metabolite-disorder relationships to further elucidate mechanisms involved.
CHAPTER TWO – LITERATURE REVIEW

The following literature review provides an overview of GABA in various plants species, its production via glutamate decarboxylation or polyamine catabolism, and metabolism via the GABA shunt and GLYRs in response to various abiotic stresses.

2.1. GLUTAMATE-DERIVED GABA: MULTIPLE ROLES IN PLANTS, INCLUDING STRESS METABOLITE

GABA is a ubiquitous zwitterionic, non-proteinaceous amino acid (Fig. 2.1); and its role in plants remains uncertain (Bown and Shelp, 1997; Bown et al., 2006; Shelp et al., 1999, 2006, 2009; Kinnersley and Turano, 2000; Bouché et al., 2003b, Bouché and Fromm, 2004). GABA is primarily derived from the amino acid glutamate by the enzyme GAD, is a major component of the free amino acid pool, and typically accumulates in response to stress (Satya Naryan and Nair, 1990; Bown and Shelp 1997; Shelp et al., 1999, 2003, 2009; Kinnersley and Turano, 2000).

![GABA molecule](image)

**Figure 2.1.** GABA, the ubiquitous zwitterionic non-protein four carbon amino acid.

Radiolabelling studies demonstrate that GABA can be derived from the decarboxylation of glutamate (Jordan and Givan, 1979; Wallace et al., 1984). [U-\(^{14}\)C]glutamate supplied to soybean cotyledons is converted to [\(^{14}\)C]GABA, as well as \(^{14}\)CO\(_2\), and is incorporated into the free amino acid pool, whereas [1-\(^{14}\)C]glutamate results
in the production of $^{14}$CO$_2$, but not $[^{14}C]$GABA (Micallef and Shelp, 1989; Chung et al., 1992; Tuin and Shelp, 1994, 1996).

In plants, GABA concentrations range from 0.03 to 2.0 µmol g$^{-1}$ fresh mass (FM), but accumulate to concentrations exceeding those of amino acids involved in protein synthesis, following exposure to various stresses (Shelp et al., 1999; Kinnersley and Turano, 2000). For example, GABA accumulation (as % of control) is stimulated within a few seconds to several days by: mechanical damage in soybean (1800-2700%) and tobacco leaves (558%); cold stress in soybean (2000%) and Arabidopsis (225%); O$_2$ deficiency in rice roots (750%), tea leaves (4000%), soybean sprouts (180%), tobacco (800) and Arabidopsis leaves (600%); cytosolic acidification in asparagus mesophyll cells (300%) and carrot suspension cells (235%); and, UV stress in Arabidopsis plants (500%) (Kinnersley and Turano, 2000; Shelp et al., 2009). Despite being thoroughly studied, the precise role of GABA accumulation during abiotic stress remains unclear.

In animals, GABA functions in neural transmission and may also serve as a signalling molecule (Shelp et al., 2009). Bioinformatic analysis suggests high sequence and structural similarity between mammalian ligand-gated ionotropic GABA receptors and plant glutamate receptors, which regulate calcium entry into the cell (Kang and Turano, 2003). Moreover, efficient growth of Arabidopsis seedlings on GABA as the sole nitrogen source offers evidence for GABA transporters (Breitkreuz et al., 1999). The known amino acid transporters amino acid permease 2 (AAP2) and proline transporter 2 (ProT2) were identified as GABA transporters by heterologous complementation of a GABA uptake-deficient yeast mutant with an Arabidopsis cDNA library (Breitkreuz et al., 1999). In 2006, Meyer et al. identified an Arabidopsis transporter AtGAT1 based on
sequence homology to the ProT family. *Ar*GAT1 possesses a predicted transmembrane domain, which transports GABA with high affinity ($K_m = 43 \mu M$) at an acidic optimal pH (pH 4.5-5.5) upon functional characterization in *Saccharomyces cerevisiae* and *Xenopus laevis* oocytes. Moreover, this transporter displays elevated gene expression during wounding stress. When fused to GFP, *At*GAT1 localizes to the plasma membrane in tobacco protoplasts, providing evidence of GABA transport into the plant cell. In 2011, Michaeli et al. identified an Arabidopsis GABA permease (GABP) in the acid/polyamine/organocation (APC) transporter family, which is homologous to bacteria and fungi GABA transporters. GABP complements a yeast mutant deficient in GABA transport and is located in mitochondria, based on transient expression of the GFP fusion protein in tobacco leaves and protoplasts, as well as subcellular fractionation, although mitochondrial membrane boundedness was not investigated. $[^{3}]$H[GABA uptake was impaired in isolated mitochondria of *gapb* mutants, albeit not completely abolished (Michaeli et al., 2011), suggesting the existence of multiple mitochondrial GABA transporters (Shelp et al., 2012d).

Extracellular GABA may function in signalling and communication within and among plants, fungi, bacteria and insects. In the Arabidopsis gametophyte, an increasing gradient of GABA concentration is associated with pollen tube growth (Palanivelu et al., 2003; Bouché and Fromm, 2004). Indeed, application of exogenous GABA onto tobacco pollen grain and pollen tube plasma membranes was found to modulate putative Ca$^{2+}$ channels by increasing Ca$^{2+}$ influx, indicating that GABA triggers a signalling mechanism for pollen tube growth (Yu et al., 2014). When the fungal hyphae *Cladosporium fulvum* infect tomato roots, plant GABA concentrations increase by 375%,
after which GABA is metabolized as a nutrient source (Solomon and Oliver, 2001, 2002; Oliver and Solomon, 2004; Shelp et al., 2006). Infection of wounded tomato stems by the tumour-inducing bacterium Agrobacterium tumefaciens increases the extracellular concentration of GABA, which in turn enters the bacterium via the GABA transporter Bra and reduces Agrobacterium virulence compared with control plants (Chevrot et al., 2006; Shelp et al., 2006). Notably, ingestion of GABA appears to act on GABA-activated Cl⁻ channels at the neuromuscular junction of insects, thereby inhibiting action potentials leading to reversible paralysis (Bown et al., 2006). Mechanically-simulated herbivory and leaf damage in soybean results in 10- to 25-fold increases in GABA levels, which may function to deter further damage (Ramputh and Bown, 1996). Indeed, oblique-banded leafroller larvae raised on high GABA diets display reduced growth rates and survivability. Furthermore, transgenic tobacco plant roots possessing constitutively expressed GAD show elevated GABA levels and less nematode egg mass than the wild-type (WT) (McLean et al., 2003). In addition, tobacco budworm larvae consume 6-12 times more WT tobacco leaves than transgenic leaves overexpressing GAD (MacGregor et al., 2003). These findings indicate that GABA accumulation as a result of herbivory may be a natural defence mechanism against invertebrate pests.

Evidence suggests that GABA is involved in plant development. For example, transgenic plants lacking the GAD autoinhibitory CaM-binding domain possess higher GABA and lower glutamate concentrations than WT plants, and exhibit growth abnormalities such as short stems with atypical cortex parenchyma (Baum et al., 1996). Immuno-staining of GABA revealed its localization in the root and hypocotyl of pine seedling, specifically in the differentiating xylem, ray parenchyma and epithelial resin
duct cells (Molina-Rueda et al., 2015). GABA accumulation is correlated with sterility in the flowers of Arabidopsis gaba-1 mutants (Palanivelu et al., 2003) and infertility, as well as dwarfism in tomato SlGABA-T1RNAi lines (Koike et al., 2013). The similar mutant atgaba-t/pop2-1 shows root, hypocotyl and cell wall defects (Renault et al., 2013). Arabidopsis pop-2 and enf1 mutants of GABA catabolism show defects in pollen tube growth (Renault et al., 2011) and abaxial-adaxial leaf development (Toyokura et al., 2011), respectively.

GABA plays a role in nitrogen and carbon metabolism. It is thought to function in nitrogen storage as it is the fourth prominent amino acid in the free amino acid pool of soybean cotyledons, as determined by [14C]glutamate metabolism (Micallef and Shelp, 1989). Glutamate conversion to GABA is elevated under conditions that reduce glutamine and protein synthesis and promote protein degradation (Satya Narayan and Nair, 1990). Diurnal fluctuations of glutamate and GABA are observed in tobacco and Arabidopsis leaves, with a shift between source and sink leaves (Masclaux-Daubresse et al., 2002; Allan and Shelp, 2006). Moreover, during continuous nitrogen supply, a close relationship between glutamate and GABA is observed; upon nitrogen interruption, GABA levels decline following a sharp increase in glutamate levels in older Arabidopsis leaves whereas GABA levels remain steady in younger leaves, suggesting that GABA is involved in source-sink nitrogen requirements (Allan and Shelp, 2006). In roots of atgaba-t/pop2-1 mutants of GABA catabolism exposed to salt stress, glutamate and GABA levels increase whereas succinate, starch, glucose, fructose and sucrose levels decrease along with increased expression of genes responsible for sucrose and starch catabolism (Renault et al., 2013). Depriving the TCA cycle of succinate in atgabat/pop2-
mutants could trigger sucrose and starch catabolism to re-supply TCA cycle intermediates (Renault et al., 2013). Upon antisense suppression of TCA cycle enzyme 2-oxoglutarate dehydrogenase in tomato, feeding radiolabelled glucose or glutamate results in significantly higher radiolabelled GABA and succinate, compared with WT, meaning that GABA catabolism is compensating for TCA cycle intermediates (Araújo et al., 2012). These results link GABA metabolism to nitrogen and carbon metabolism.

2.2. GABA ACCUMULATION IN DISORDERED FRUIT FROM CONTROLLED ATMOSPHERE STORAGE

In Canada, apples are an economically important food commodity, representing approximately 158.9 million dollars in revenue in 2010 (http://www.statcan.gc.ca/pub/21-011-x/2011002/t031-eng.htm). In Ontario alone, the estimated farm gate value of apples sold in 2010 was 68.6 million dollars. To preserve apples for year-round supply, these fruit are stored under CA conditions, and approximately 42% of Canada’s stored apples reside in Ontario (http://www4.agr.gc.ca/IH5_Reports/faces/cognosSubmitter.jsp).

Standard CA conditions, defined as low O₂, high CO₂ and low temperature (2.5-3 kPa O₂, 2.0-4.5 kPa CO₂, 0-3 °C) are used to delay ripening and can vary with apple cultivars (http://www.omafra.gov.on.ca/english/crops/hort/news/orchnews/2012/on-0912a12.htm). CA storage at high CO₂ and/or low O₂ inhibits the production of the ripening/senescence hormone ethylene by suppressing the expression of 1-aminocyclopropane-1-carboxylate (ACC) synthase, as well as the expression and activity of ACC oxidase, the enzymes responsible for ethylene synthesis (Gorny and Kader, 1997). Moreover, the rate of respiration is reduced in CA-stored apples due to inhibition of the TCA cycle enzyme
succinate dehydrogenase and depletion of TCA cycle intermediates (Frenkel and Patterson, 1973; Knee, 1973). Therefore, CA conditions prolong apple storage life by preventing ripening and reducing respiration.

CA storage conditions are not always optimal and may cause storage-related physiological disorders, resulting in a net economic loss of stored apples. Postharvest browning disorders are generally associated with the formation of reactive oxygen species (ROS) leading to oxidative stress and cellular breakdown (Veltman et al., 2003; Franck et al., 2007). These symptoms can be cultivar specific and aggravated by certain CA conditions. For example, low-temperature storage at 0°C promotes vascular breakdown, as well as core browning (well defined necrotic cortex or core tissue) of ‘Cortland apples’ (Blanpied et al., 1990; DeEll et al., 1998) and flesh browning (firm and moist browning tissue) of ‘Empire’ apples (Watkins and Liu, 2010). Conversely, a warmer storage temperature of 3°C stimulates the incidence of external CO₂ injury (irregular, well defined, roughened brown peel lesions) and internal senescent breakdown in ‘Empire’ apples (Blanpied et al., 1990; Watkins and Liu, 2010). A storage temperature of 2°C reduce flesh browning of ‘Fuji’ apples, as well as firmness and titratable acidity (Kweon et al., 2013). Other low-temperature storage disorders include bitter pit, which is characterized by brown necrotic regions in the calyx, and superficial scald, which is depicted as discoloured peel (Meheriuk et al., 1994). Superficial scald of ‘Granny Smith’ and bitter pit of ‘Golden Reinder’ apples can be significantly reduced by pre-treatment with low O₂ (1-2%) at 20°C for 7-10 d prior to cold storage (Pesis et al., 2007; Val et al., 2009). Pre-treatment with low O₂ stress can trigger the formation of CO₂, acetaldehyde
and ethanol within the fruit, which at high concentrations can reduce ethylene production, delay ripening and prevent storage disorders (Pesis, 2005; Pesis et al., 2007).

Certain CO₂ and O₂ partial pressures can exacerbate the incidence of storage disorders at cultivar-specific CA conditions. High CO₂ and low O₂ promote internal browning in ‘Braeburn’ apples (2-5 kPa CO₂, 1 kPa O₂) (Elgar et al., 1998) and external CO₂ injury in ‘Empire’ apples (5 kPa CO₂, 2 kPa O₂) (Watkins et al., 1997). Alternatively, high CO₂ (2.5 kPa) aggravates flesh browning, a chilling-related disorder, in mature ‘Fuji’ apples, regardless of O₂ concentration (Kweon et al., 2013). Similarly, a high CO₂ concentration (up to 5 kPa) promotes external CO₂ injury and flesh browning in ‘Empire’ apples (Watkins and Liu, 2010). Alternatively, Deyman et al. (2014b) found marked higher external injury, but only slightly higher flesh browning, if at all, in ‘Empire’ apples stored at high CO₂ (5 kPa) compared with low CO₂ (0.03 kPa), at both 3°C and 0°C. These results highlight differences in susceptibility of apple cultivars to various storage disorders, and uncertainty about biochemical mechanisms underlying these physiological injuries in CA-stored apples.

Notably, certain storage disorders have been associated with specific metabolites, enzymes or transcripts in some apple cultivars. For example, the accumulation of α-farnesene oxidation products and triterpenoids is associated with superficial scald in ‘Granny Smith’ apples stored at 0.5-1°C in air (Rudell et al., 2009; Leisso et al., 2013). Also, the elevated activity of ascorbate peroxidase, which is involved in antioxidant metabolism, is associated with flesh browning development in ‘Empire’ apples stored at 3°C, 2 kPa CO₂, 2 kPa O₂ (Lee et al., 2012). Differentially expressed internal browning-specific transcripts have been identified in CA-stored ‘Braeburn’ apples (0.7 kPa CO₂, 3
kPa O₂ and 1°C): i) pyruvate dehydrogenase, which is associated with the TCA cycle, is up-regulated; and ii) 1-aminocyclopropane-1-carboxylase, which is associated with ethylene biosynthesis, is down-regulated in browning tissue (Mellidou et al., 2014).

Comparative metabolite profiling of injured and uninjured tissues from storage-disordered pear fruit (3 kPa O₂, 1 kPa CO₂, 1°C) reveals the accumulation of TCA cycle components and GABA in brown tissue (Pedreschi et al., 2009). After 4 months of storage, malate, succinate and GABA concentrations are approximately 25%, 30% and 33% higher, respectively, in brown disordered pear tissue than in sound tissue (Pedreschi et al., 2009). Tomato fruit stored at 30°C under CA (11 kPa O₂, 9 kPa CO₂) accumulate approximately 48% more GABA than fruit stored in air at 15°C (Makino et al., 2008). Moreover, at 13°C tomato fruit stored in 10 kPa CO₂ accumulate an estimated 30% more GABA than fruit stored in air (Deewatthanawong et al., 2010b). GABA also returns to control baseline levels when transferred back to air. Similarly, GABA accumulates in cherimoya fruit exposed to 20 kPa CO₂, and then decreases upon transfer of the fruit to air (Merodio and Cura, 1998). GABA accumulation in strawberries stored at 2°C and 20 kPa CO₂ is cultivar dependent (Deewatthanawong et al., 2010b). Notably, the GABA level is approximately 100% higher in skins of ‘Empire’ apples stored at 2.5 or 5 kPa CO₂ than in skins of apples stored at 1 kPa CO₂ (Deewatthanawong and Watkins, 2010), and transfer from CA storage (3 ºC, 2.5 kPa O₂, 2.5 kPa CO₂) to ambient conditions (0.038 kPa CO₂, 21 kPa O₂, 25 ºC) decreases the GABA concentration of intact fruit by 60% (Trobacher et al., 2013a). Moreover, GABA levels in CA-stored ‘Empire’ apples (2 kPa O₂, 2 kPa CO₂, 3.3 ºC) increase with storage time and are higher under treatment with the ethylene inhibitor 1-methylcyclopropene (1-MCP), which is associated with
enhanced flesh browning (Lee et al., 2012). Similarly, elevated GABA levels are associated with CA-related injury in ‘Honeycrisp’ apples (Lum, 2014; Chiu et al., 2015; Leisso et al., 2015). Recently, Deyman et al. (2014a) described a linear accumulation of GABA in both 1-MCP treated and untreated ‘Empire’ apples during CA storage (2.5 kPa O₂, 0.03 kPa and 2.5 kPa CO₂, 3 °C), even in cases where the incidences of external CO₂ injury and internal flesh browning are low. Together, these results suggest that CA storage (i.e., chilling, elevated CO₂, low O₂) is analogous to abiotic stress. However, it is unclear whether a positive correlation exists between GABA accumulation and CO₂-induced external injury. In particular, some of these findings must be viewed with caution because the fruits or fruit parts were handled or subjected to mechanical injury before being frozen, which can result in the stimulation of GAD activity and elevated GABA concentration (Allan and Shelp, 2006; Shelp et al., 2012a).

2.3. THE GABA SHUNT

GABA is produced from glutamate and then converted to succinate via a series of enzymes known as the GABA shunt: GAD, GABA-T, and SSADH (Fig. 2.2). First, glutamate is irreversibly α-decarboxylated to GABA via cytosolic GAD (Breitkreuz and Shelp, 1995). Multiple GAD genes are present in various plant species such as Petunia, tomato, tobacco, Arabidopsis, rice and apple (Shelp et al., 1999; Akama et al., 2001; Yevtushenko et al., 2003; Akama and Takaiwa, 2007; Akihiro et al., 2008, Trobacher et al., 2013b). Unlike the enzyme from bacteria and animals, plant GADs are typically activated by Ca²⁺/CaM binding to a C-terminal domain (Baum et al., 1993; Ling et al.,
**Figure 2.2.** The GABA shunt pathway. GAD: glutamate decarboxylase; GABA: γ-aminobutyric acid; GABA-T: GABA-transaminase, SSA: succinic semialdehyde; SSADH: succinic semialdehyde dehydrogenase.

1994; Arazi et al., 1995; Snedden et al., 1995, 1996; Cholewa et al., 1997; Turano and Fang, 1998; Zik et al., 1998; Yevtushenko et al., 2003, Gut et al., 2009). In general, GAD is autoinhibited at neutral pH, where the CaM binding domain physically covers the six active sites. With the presence of Ca$^{2+}$, CaM undergoes a conformational change, allowing it to bind to the GAD CaM-binding domains, thereby relieving autoinhibition and leading to activation of GADs (Shelp et al., 2012a). Autoinhibition of the CaM-binding domain can also be released at acidic pH, leading to stimulation of GAD activity (Shelp et al., 2012a). Apple fruit *MdGAD1* and *MdGAD2* display common biochemical properties of plant GADs; however, the activity of *MdGAD3* is not activated by Ca$^{2+}$/CaM at near physiological pH, and much less stimulated by acidic pH, probably because it does not possess a C-terminal autoinhibitory domain (Trobacher et al., 2013b). Notably, *OsGAD2* does not bind CaM, but the C-terminal domain remains autoinhibitory (Akama et al., 2001), and truncation of this domain results in constitutive activation, leading to 100-fold higher GABA concentration compared with WT (Akama and Takaiwa, 2007).
An *in vivo* role for Ca\(^{2+}/\)CaM-mediated activation of GADs is implied from evidence for the increase in cytosolic Ca\(^{2+}\) during cold shock in tobacco (Knight et al., 1991) and asparagus (Cholewa et al., 1997), mechanical stress in *Arabidopsis* (Braam and Davis 1990), and heat shock in *Arabidopsis* (Braam, 1992). Kinetic analyses reveal that a recombinant petunia GAD exhibits hyperbolic activity (*V_\text{max}*= 30 µmol min\(^{-1}\) mg\(^{-1}\) protein) and possesses a *K_m* (glutamate) = 8.2 mM when 1 mM Ca\(^{2+}\) and 50 nM CaM are added (Snedden et al., 1996). Moreover, GAD is known to possess an acidic pH optimum (Tsushida and Murai, 1987; Snedden et al., 1992, 1995, 1996), which is consistent with observations that GABA accumulates as a response to cellular acidosis from hypoxia, which would stimulate *in planta* GAD activity (Carroll et al., 1994; Crawford et al., 1994; Shelp et al., 1999). Moreover, GABA decarboxylation via GAD requires H\(^+\) for catalysis; therefore, GABA accumulation could contribute to pH regulation during cytosolic acidification (Shelp et al., 1999).

Microarray analysis shows that *AtGAD* gene homologs in *Arabidopsis* are differentially expressed throughout the plant: *AtGAD1* is expressed mostly in roots; *AtGAD2* is abundant in root, shoot, flower and immature siliques; *AtGAD3* and *AtGAD4* are present in young leaves, sepals, carpels and immature siliques; and, *AtGAD5* is expressed in stamens and mature pollen (Shelp et al., 2012c). In response to various abiotic stresses, *AtGADs* are the most responsive genes in the *Arabidopsis* GABA shunt pathway, where *AtGAD1* is the most inducible. *AtGAD3* and *AtGAD4* expression is low throughout the plant, but is highly induced in roots and shoots as a response to low O\(_2\) stress, whereas *AtGAD2* is the most abundant isoform in the plant, yet not induced under this stress. Microarray analysis also revealed the *AtGAD* genes are differentially
responsive to various stresses (Kilian et al., 2007; Shelp et al., 2012c). In shoots, most stresses result in slight up-regulation of \textit{AtGAD1} and \textit{AtGAD5}. Stresses such as salt, UV-B, osmotic, drought and wounding up-regulate \textit{AtGAD3} and \textit{AtGAD4}, whereas heat stress has no effect on \textit{AtGAD} expression. In roots, \textit{AtGAD1} and \textit{AtGAD2} are not responsive to any stress, whereas salt, cold, osmotic and UV-B stresses upregulate \textit{AtGAD3} and \textit{AtGAD4}, and salt as well as heat stress slightly up-regulate \textit{AtGAD5}. In addition, the expression of a \textit{PgGAD} gene in \textit{Panax ginseng} is enhanced in response to various abiotic stresses such as cold, heat, salt, anoxia and wounding, although the time patterns differ among the stresses (Lee et al., 2010); these results are consistent with increasing cytosolic \textit{Ca}^{2+} concentrations under similar conditions (Braam and Davis, 1990; Knight et al., 1991). In addition, \textit{AtGAD4} expression is elevated under drought (Urano et al., 2009), \textit{O$_2$} deficiency (Miyashita and Good, 2008) and cold (Kaplan et al., 2007).

\textit{GAD} expression can be associated with GABA accumulation. In germinating rice (\textit{Oryza sativa}) subjected to anoxia, GABA levels increase, coinciding with peak expression of two of five \textit{OsGADs} after 12 h of stress (Narsai et al., 2009). In cold-acclimated Arabidopsis, \textit{AtGAD4} expression is induced and precedes the GABA accumulation peak (Kaplan et al., 2004, 2007). However, in frost-resistant barley (\textit{Hordeum vulgare}) seedlings exposed to freezing stress the GABA level increases 15-fold without any change in \textit{HvGAD} expression, although \textit{HvGAD} expression is induced upon longer-term cold acclimation, which also results in GABA accumulation (Mazzucotelli et al., 2006). Salt stress increases GABA levels in Arabidopsis plants by 50-280\% over the course of 4 d, corresponding with an induction in \textit{AtGAD2} and \textit{AtGAD4} expression, whereas GAD activity is shown to decrease 24 h after salt stress.
(Renault et al., 2010). These expression profiles for various GAD orthologs highlight the gene-dependent and -independent mechanisms involved in GABA accumulation, particularly under abiotic stress.

In the second step of the pathway, GABA is reversibly metabolized into SSA via GABA-T, which catalyzes the conversion of pyruvate and glyoxylate to alanine and glycine, respectively (Clark et al., 2009a, b). In Arabidopsis, there exists only one GABA-T isoform, which is localized in the mitochondrion (Clark et al., 2009b), whereas there are three tomato GABA-T isoforms that are localized in the mitochondrion (SlGABA-T1), cytosol (SlGABA-T3) or plastid (SlGABA-T2) (Clark et al., 2009b; Koike et al., 2013). Notably, both apple MdGABA-Ts reside exclusively in the mitochondrion (Trobacher et al., 2013a). AtGABA-T has an optimum pH range between pH 8 and 10, and a $K_m$ for GABA (fixed pyruvate), GABA (fixed glyoxylate), pyruvate and glyoxylate (fixed GABA) of 340, 180, 140 and 110 µM, respectively (Clark et al., 2009a). Moreover, this enzyme catalyzes irreversible glyoxylate-dependent and reversible pyruvate-dependent reactions, but has no activity with 2-oxoglutarate, unlike mammalian GABA-T (Clark et al., 2009a). Recombinant apple MdGABA-T1 and MdGABA-T2 display maximal activity between a pH of 8.5 and 9.5, and a $K_m$ for GABA (fixed pyruvate), GABA (fixed glyoxylate), pyruvate and glyoxylate (fixed GABA) of 165, 135, 23 and 39 µM, respectively for MdGABAT-1, and 77, 100, 19 and 16 µM, respectively, for MdGABAT-2, and the catalytic efficiency for MdGABA-T1 is two times that of MdGABA-T2 (Trobacher et al., 2013a). Notably, MdGABA-Ts are effectively inhibited to less than 5% of the control by the pyridoxal phosphate inhibitor aminoxyacetate and the GABA analogs gabaculine and vigabatrin, much like their
Arabidopsis and tobacco counterparts (Van Cauwenberghe and Shelp, 1999; Clark et al., 2009a). Therefore, GABA-Ts appear to have multiple subcellular locations across plant species, and display kinetic properties within the same order of magnitude, at least in Arabidopsis and apple.

*GABA-T* transcripts in Arabidopsis are found throughout the plant according to microarray analysis, and are slightly induced in shoots during osmotic and salt stress (Kilian et al., 2007). Similarly, *AtGABA-T* expression is slightly induced in whole plants during salt stress (Renault et al., 2010), as well as in germinating rice in response to anoxia (Narsai et al., 2009). In contrast, *GABA-T* expression is not affected by hypoxia in Arabidopsis roots (Miyashita and Good, 2008), or cold and freezing stresses in a frost-resistant barley (Mazzucotelli et al., 2006). Tomato *SlGABA-T1* and *SlGABA-T3* expression is strong during the ripening stages of tomato fruit and their activity is highest when GABA levels decrease after the tomato breaker stage, implying that GABA is being catabolised by GABA-T (Akihiro et al., 2008). During cold shock (1 h exposure to 4°C) an Arabidopsis mitochondrial *GABA-T* overexpression mutant accumulates 30% less GABA than WT plants, whereas no effect on GABA levels is apparent in non-stressed plants, suggesting that GABA-T catabolizes stress-produced GABA (Simpson et al., 2010). Moreover, the Arabidopsis *GABA-T* knockout mutants *gaba-t1* and *pop2-3* exhibit minimal growth compared with WT when grown on GABA as the sole source of nitrogen (Clark et al., 2009a), suggesting that GABA-T is essential for the catabolism of GABA accumulated during abiotic stress.

In the third step of the pathway, SSA is oxidized irreversibly to succinate via SSADH, which is localized in the mitochondrial matrix (Busch and Fromm, 1999). This
enzyme has an optimum pH of approximately 9 and utilizes NAD$^+$ as a cofactor. In Arabidopsis, AtSSADH is expressed throughout the plant and is up-regulated by cold (Shelp et al., 2012c), as well as osmotic and salt stresses (Kilian et al., 2007; Renault et al., 2010). Exposure of germinating rice to anoxia up-regulated OsSSADH expression (Narsai et al., 2009), whereas no change in expression is observed in frost-resistant barley submitted to cold or freezing stress, although a slight induction occurs during longer term cold acclimation (Mazzucotelli et al., 2006). In cold-acclimated Arabidopsis plants, the peak of AtSSADH expression coincides with a decline in GABA (Kaplan et al., 2007). In general, there appears to be an association between abiotic stress-triggered GABA accumulation and AtSSADH expression; however, the activity of this enzyme could be under biochemical control.

Kinetic analysis of a recombinant Arabidopsis enzyme revealed that SSADH follows Michaelis-Menten kinetics for NAD$^+$ ($K_m = 130$ µM) but not for SSA ($K_{0.5} = 15$ µM) (Busch and Fromm, 1999). Furthermore, the enzyme is feedback inhibited by AMP (competitive), ADP (mixed competitive) and ATP (non-competitive), indicating that in planta ATP/ADP ratios may be important in regulating the activity of this enzyme. Studies using fluorescence spectroscopy to monitor cofactor displacement showed that AMP displaces all bound NADH, whereas ATP has no effect (Busch et al., 2000). Notably, the activity of Arabidopsis recombinant SSADH is completely inhibited by an NADH/ NAD$^+$ ratio of 2, but increases by 2-fold as this ratio decreases to 0.1 (Busch and Fromm, 1999), suggesting that a high NADH/ NAD$^+$ ratio limits or inhibits the function of NAD$^+$-dependent SSADH and contributes to the regulation of GABA accumulation (Busch and Fromm, 1999; Shelp et al., 1999). Other research suggests that SSADH is
linked to redox regulation since Arabidopsis ssadh plants exposed to high light exhibit reduced growth and greater incidence of necrosis due to the accumulation of reactive oxygen species (ROS) than WT plants (Bouché et al., 2003a). These data highlight the importance of SSADH, as well as the GABA shunt, in regulating ROS accumulation (Bouché et al., 2003a). However, Arabidopsis gaba-t/ssadh double mutants have a suppressed ssadh phenotype and do not accumulate toxic peroxides, indicating that SSA accumulation can probably explain the toxicity phenotype observed in Bouché et al. (2003a) (Ludewig et al., 2008).

ROS production and consumption is mediated by the pyridine dinucleotides NAD(P)(H) (Bouché et al., 2003; Allan et al., 2009). Notably, NAD(P)H oxidases generate ROS at the plasma membrane, which then serve as signalling molecules to activate downstream genes implicated in abiotic stress tolerance (Hunt et al., 2004). Moreover, certain CaM-dependent NAD kinases are activated as a result of intracellular Ca\(^{2+}\) influx during abiotic stress, resulting in the phosphorylation of NAD(H) (Allan et al., 2012). The ratio of NAD(P)H/NAD(P)\(^+\) is important in plant redox homeostasis, and can increase as a result of abiotic stresses, notably oxidative stress (Allan et al., 2008, 2009, 2012). During exposure to these stresses, reducing power (NADH and NADPH) would accumulate, thereby diminishing the availability of NAD\(^+\) and NADP\(^+\) as electron acceptors in mitochondria and chloroplasts, and resulting in cellular damage (Scheibe et al., 2005). Under oxidative stress in particular, the mitochondrial electron transport chain would be restricted due to lack of O\(_2\) as the terminal electron acceptor, thereby limiting NADH and ATP recycling (Breitkreuz et al., 2003; Allan et al., 2008). Indeed, studies have shown that low O\(_2\) stress increases NAD(P)H/NAD(P)\(^+\) ratios. Tobacco leaf
NADPH/NADP$^+$ increases from 2 to 3 (Allan et al., 2008) and Arabidopsis leaf
NADH/NAD$^+$ ratio increases from 1 to 8, whereas NADPH/NADP$^+$ ratio increases from
1 to 6 (Allan et al., 2012) as a response to $O_2$ deprivation via submergence. Under anoxic
conditions, fermentation processes would be initiated, resulting in elevated lactate
dehydrogenase activity, which oxidizes NADH to NAD$^+$ for use in ATP production via
glycolysis (Sachs et al., 1980).

The GABA shunt is found in virtually all organisms, highlighting its metabolic
importance (Shelp et al., 1999). Radiolabelling studies have demonstrated that GABA-
derived succinate generated by a mitochondrial SSADH enters the TCA cycle and acts as
a carbon source (Tuin and Shelp, 1994, 1996). Succinate catabolized by the GABA shunt
may function as a TCA cycle bypass, by circumventing the enzymes NAD-dependent 2-
oxoglutarate dehydrogenase and the ADP-dependent succinyl-CoA ligase (Shelp et al.,
1999; Studart-Guimarães et al., 2007; Araújo et al., 2012). This bypass could provide
immediate substrate upon recovery from abiotic stresses such as hypoxia, which impair
respiration and increase the NAD(P)H/NAD(P)$^+$ ratio (Shelp et al., 1999; Ludewig et al.,
2008). Interestingly, succinic Co-A ligase-deficient mutants of Arabidopsis, which are
unable to convert succinyl-CoA to succinate, have elevated levels of GAD activity and
GABA (Studart-Guimarães et al., 2007), although biochemical regulatory mechanisms
for GAD, such as cytosolic calcium or cellular acidosis, were not investigated. Moreover,
knockouts of the TCA cycle enzyme isocitrate dehydrogenase in Arabidopsis are not
phenotypically disadvantaged and accumulate 50-250% more GABA than WT (Lemaitre
et al., 2007), indicating that the GABA shunt may compensate for reduced TCA cycle
activity. Although the GABA shunt is theoretically less energetically favourable (1 mol
NADH) than the TCA cycle (1 mol NADH plus 1 mol ATP), it offers metabolic
flexibility (Allan et al., 2009). Together, these findings indicate that the GABA shunt
may play an important role in TCA cycle-compromised plants, providing an alternative
source of succinate upon recovery from stress.

2.4. POLYAMINE-DERIVED GABA

Theoretically, GABA can be formed indirectly via polyamine catabolism
(reviewed in Shelp et al., 2012b; Zarei et al., 2014). In plants, polyamines accumulate in
response to a variety of abiotic stresses, notably drought, oxidative stress, cold and
salinity (Alcázar et al., 2010; Tiburcio et al., 2014). They function to stabilize
membranes, scavenge free radicals, interact with phytochrome and hormones, as well as
stimulate DNA replication and gene expression, thereby affecting plant growth,
development and stress tolerance. Polyamines are comprised mainly of putrescine,
spermidine and spermine and exist either as free form or conjugated to various molecules
such as proteins, nucleic acids and phenolic acids. Production of putrescine is mediated
by a multistep pathway involving arginine or ornithine decarboxylation (Shelp et al.,
2012b; Majumdar et al., 2013). Putrescine can then be converted to spermidine and
spermine via spermidine and spermine synthases (Shelp et al., 2012b). Polyamines can be
converted and back-converted into each other by synthases and O$_2$-dependent back-
conversion PAOs. To form GABA, putrescine and spermidine are catabolized via O$_2$-
dependent copper-containing AO resulting in 4-aminobutanal (Planas-Portell et al., 2013,
Zarei et al., 2014) (Fig. 2.3). In turn, 4-aminobutanal can then be converted into GABA
via NAD$^+$-dependent AMADH activity (Zarei et al., 2015).
Exogenously-supplied radiolabeled putrescine results in the appearance of radiolabeled GABA in various plant parts and tissues such as roots of intact maize (DiTomaso et al., 1992) and Limonium tartaricum (Duhaze et al., 2002), discs of tomato pericarp (Rastogi and Davies, 1989), and excised cultured cotyledons of Pinus ratiata (Kumar and Thorpe, 1989, Shelp et al., 2012b). The production of radiolabelled GABA is inhibited by addition of the amine oxidase inhibitor aminoguanidine and elevated by the GABA-T inhibitor gabaculine, thereby linking polyamine, notably putrescine, catabolism to GABA production. Various mutant studies indicate that putrescine catabolism would be the likely source of GABA, but it has been argued that GABA production in WT plants exposed to abiotic stress may be limited by changes in cellular redox balance, rather than putrescine availability, and that the resulting GABA is derived from glutamate (Shelp et al., 2012b).

Regarding FAD-containing PAOs, the Arabidopsis proteins are best characterized wherein AtPAO2-4 isoforms are peroxisomal and AtPAO1 and AtPAO5 are cytosolic (Tiburcio et al., 2014). The recombinant AtPAO1-4 isoforms have been kinetically characterized, demonstrating that AtPAO3 catalyzes the conversion of spermine to spermidine and spermidine to putrescine (Moschou et al. 2008), and that all isoforms have $K_m$ values for spermine and spermidine in the micromolar range, except for AtPAO1 which does not use spermidine as a substrate (Fincato et al., 2011). To date, plant copper-containing AOs have been identified as having either an N-terminal extracellular signal peptide (e.g., AtCuAO1, AtAO1, Nicotiana tabacum NtAO1, NtDAO1 and MdAO2) or a
Figure 2.3. Polyamine catabolism and GABA shunt pathways. Abbreviations: AO, amine oxidase; PAO, polyamine oxidase; PDH, Δ₁-pyrroline dehydrogenase; AMADH, aminoaldehyde dehydrogenase; GAD, glutamate decarboxylase; GABA, γ-aminobutyric acid; GABA-T, γ-aminobutyric acid transaminase; GLYR/SSAR, glyoxylate/succinic semialdehyde reductase; GHB, γ-hydroxybutyrate (adapted from Shelp et al., 2012b).
C-terminal peroxisomal targeting signal (e.g., *Pisium sativum* *PsCuAO*, *AtCuAO2*, *AtCuAO3* and *MdAO1*) (Planas-Portell et al., 2013; Zarei et al., 2014). Arabidopsis kinetic analyses have revealed that *AtCuAOs* use putrescine and spermidine as their substrates (Planas-Portell et al., 2013), whereas apple *MdAO1* has high catalytic efficiency for 1,3-diaminopropane, putrescine and cadaverine, and *MdAO2* has high catalytic efficiency for aliphatic and aromatic monoamines. Therefore, *MdAO1* could contribute to GABA production through the catabolism of putrescine (Zarei et al., 2014). Plant AOs are thought to be involved in responses to biotic and abiotic stresses, as well as wound healing, detoxification and fruit set and ripening (Walters, 2003; Cona et al., 2006; Zarei et al., 2014). Several putative *AtAOs* are subject to transcriptional regulation under abiotic stress (i.e., chilling, salt and osmotic stress) (Shelp et al., 2012b). Furthermore, AO activity could be limited by the availability of molecular O$_2$ for catalysis. In bulky plant organs such as tubers and fruit, it is well known that a decreasing O$_2$ gradient exists from the outer to the inner part of the fruit. In melon fruit, this declining O$_2$ gradient influences the surrounding metabolic processes such that GABA, alanine and ethanol accumulate and the adenylate energy charge as well as ATP/ADP ratio decrease as a result of O$_2$ deficiency and fermentation (Biais et al., 2010; Shelp et al., 2012b).

Once putrescine and spermidine are oxidized into 4-aminobutanal, this metabolite can be directly converted into GABA via NAD$^+$-dependent AMADH (Shelp et al., 2012b; Zarei et al., 2015). Alternatively, 4-aminobutanal is in rapid non-enzymatic equilibrium with Δ$^1$-pyrroline which can then be dehydrogenated to GABA by either AMADH or NAD$^+$-dependent Δ$^1$-pyrroline dehydrogenase (PDH). In Arabidopsis, there
are two candidate genes for AMADH, which are only marginally responsive to abiotic stresses such as salinity and cold (Missihoun et al., 2011; Shelp et al., 2012b). In apple there are also two AMADH isoforms, cytosolic \( MdAMADH1 \) and peroxisomal \( MdAMADH2 \), that catalyze NAD\(^+\)-dependent reactions for the conversion of 3-aminopropanal to \( \beta \)-alanine with high catalytic efficiency, and 4-aminobutanal to GABA with lower catalytic efficiency, at a pH optimum of 9.8 (Zarei et al., 2015). AMADH and PDH activity could be biochemically controlled by the high \( \text{NAD(P)H}/\text{NAD(P)}^+ \) ratio observed in plants subjected to abiotic stress, either by the limited availability of \( \text{NAD}^+ \) as substrate or feedback inhibition by NADH. For example, in ‘Empire’ apple fruit stored under chilling, low \( \text{O}_2 \) and elevated high \( \text{CO}_2 \) (2.5 kPa \( \text{CO}_2 \)) conditions, GABA production seems to be more correlated to glutamate decarboxylation than to polyamine oxidation (Deyman et al., 2014a). Recent findings suggest that GABA can also be non-enzymatically derived from the amino acid proline (Fig. 2.3) (Signorelli et al., 2015). Proline accumulates in plants exposed to abiotic stress, serving various protective roles such as non-enzymatic antioxidant defense via scavenging of reactive hydroxyl (OH) radicals. Reaction with \( \cdot \text{OH} \) results in spontaneous decarboxylation of proline, forming pyrrolidin-1-yl, which can be converted non-enzymatically to \( \Delta^1 \)-pyrroline, the common product of polyamine catabolism. \( \Delta^1 \)-Pyrroline can then be converted to GABA by PDH or AMADH, providing an alternative route for GABA production under abiotic stress. However, as previously mentioned, PDH and AMADH activities could be biochemically regulated by the \textit{in vivo} redox status.
2.5. SUCCINIC SEMIALDEHYDE AND GLYOXYLATE REDUCTION IN PLANTS

Complementation of a SSADH-deficient yeast mutant with an Arabidopsis cDNA library identified a novel cDNA (now designated as glyoxylate/succinic semialdehyde reductase or GLYR1, rather than SSA reductase or GHB dehydrogenase) (Breitkreuz et al., 2003). When AtGLYR1 is reinserted into the mutant, GHB accumulates when grown on GABA as the sole N source. These preliminary findings suggested that GLYR1 exhibits SSA reductase activity, which could divert GABA shunt from the production of succinate to production of GHB. GHB is a short chain fatty acid and was first demonstrated to accumulate in Arabidopsis and tea leaves (273 to 739 nmol g\textsuperscript{-1} FM), as well as soybean sprouts (10 to 155 nmol g\textsuperscript{-1} FM) subjected to O\textsubscript{2} deficiency, either by submergence or the use of low O\textsubscript{2} (Allan et al., 2003; Breitkreuz et al., 2003). AtGLYR1 transcript abundance, as measured by relative reverse transcriptase polymerase chain reaction (RT-PCR), was found to decrease in Arabidopsis within 2 h of submergence even though GABA and GHB concentrations appear higher than in non-submerged control plants (Breitkreuz et al., 2003). Subsequently, transcript analyses performed via the more precise technique of qPCR demonstrated that AtGLYR1 transcript abundance is elevated, and GHB as well as GABA accumulate in Arabidopsis plants subjected to salinity, drought, submergence, cold or heat (Allan et al., 2008).

Arabidopsis ssadh mutants accumulate more GHB and ROS, which is coincident with a higher incidence of cell death under high light, than in WT plants (Fait et al., 2005). The use of $\gamma$-vinyl-$\gamma$-aminobutyrate, a GABA-T inhibitor, and an atgaba-t/ssadh double mutant partially alleviates these detrimental symptoms and suggests that SSA or
GHB accumulation, rather than ROS accumulation, is more likely to explain the toxicity (Fait et al., 2005; Ludewig et al., 2008). In vitro assays of crude cell-free extracts from Arabidopsis reveal SSA reduction throughout the plant, notably in leaves at all developmental stages, as well as in reproductive tissues (Hoover et al., 2007b). Therefore, the aforementioned evidence suggests SSA reduction into GHB is mediated via GLYR in plant tissues.

ArGLYR1 has an approximate size of 33 kDa and displays an N terminal dinucleotide cofactor-binding domain known as the Rossmann fold, as well as an arginine residue believed to be critical for NADPH binding (Breitkreuz et al., 2003). Both of these regions are conserved in known mammalian and bacterial SSA reductases and GHB dehydrogenases, despite low amino acid similarity. Based on biochemical function, it seemed likely that GLYR1 belongs to one of two large enzyme superfamilies known as NADPH-dependent aldo-keto reductases or the short-chain dehydrogenases, but conservation of likely active site amino acids suggested that GLYR1 belongs to a smaller family known as the β-hydroxyacid dehydrogenase (β-HAD) superfamily, which includes 6-phosphogluconate dehydrogenase, tartronic semialdehyde dehydrogenase and 3-hydroxyisobutyrate dehydrogenase (Hoover et al., 2007a; Simpson et al., 2008). Subsequently, 3D modelling and site-directed mutagenesis of AtGLYR1 provided evidence for: i) two main domains where domain I contains the dinucleotide binding region with a typical Rossmann fold characterized by two α/β units, and domain II contains α helices; ii) an overall tetrameric structure; iii) an active site consisting S121, K170, N174, F231 and D239; and, iv) the involvement of a critical lysine residue (K170) in catalysis (Hoover et al., 2013).
When an NADPH-dependent GLYR activity was first isolated from spinach leaves, both cytosolic and chloroplastic activities were observed via subcellular fractionation (Givan and Kleczkowski, 1992). The cytosolic and chloroplast activities, respectively, can likely be attributed to GLYR1 and a second putative GLYR isoform, GLYR2, which was identified in Arabidopsis using a bioinformatics approach (Simpson et al., 2008). AtGLYR2 has 57% amino acid sequence identity to AtGLYR1, with a molecular mass of 36.2 kDa and a 51 amino acid N-terminal extension containing a plastid-targeting sequence characterized by an enrichment of serine, threonine and small hydrophobic amino acid residues. Transient GFP tagged-AtGLYR1 and -AtGLYR2 expression in tobacco BY-2 cell cultures suggest that these proteins are localized to the cytosol and plastid stroma, respectively (Simpson et al., 2008). Later, AtGLYR1 was predicted to reside within the peroxisome based on proteome analysis of Arabidopsis leaf peroxisomes and a C-terminal tripeptide consisting of serine-arginine-glutamate, similar to the type 1 peroxisomal targeting signal which includes serine-lysine-leucine (Ching et al., 2012). Moreover, the C-terminal GLYR1-GFP fusion protein cloned in Simpson et al. (2008) may have masked the peroxisomal-like targeting signal, rendering the protein cytosolic. However, in transiently transformed Arabidopsis suspension cells and stably transformed Arabidopsis plants, AtGLYR1 was exclusively localized to the cytosol and not the peroxisome, potentially due to the acidic glutamate residue of the C-terminal tripeptide replacing the large hydrophobic residue which is typical of the peroxisomal targeting signal (Ching et al., 2012).

Although there is evidence for in vivo conversion of SSA to GHB as discussed above, GLYR1 and 2 prefer glyoxylate as a substrate in vitro (Hoover et al., 2007a, b;
Recombinant AtGYR1 and AtGLYR2 proteins display Michaelis-Menten kinetics for SSA and glyoxylate, and optimal enzymatic activities at pH 7.8. Under saturating NADPH levels, GLYR2 exhibits a 350-fold higher affinity for glyoxylate ($K_m = 34 \, \mu M$) than for SSA ($K_m = 9 \, \text{mM}$) (Simpson et al., 2008), whereas GLYR1 exhibits a 250-fold higher affinity for glyoxylate ($K_m = 4.5 \, \mu M$) than for SSA ($K_m = 870 \, \mu M$) (Hoover et al., 2007b). In either case, GLYR affinity for glyoxylate may be due to the substrate’s strong electron-withdrawing carboxyl group attached to a much weaker electron-withdrawing aldehyde group, which are optimal characteristics for aldehyde reduction to an alcohol (Hoover et al., 2007b). Moreover, the rate of the GLYR1 forward reaction is dramatically faster than the reverse reaction and much greater in the presence of NADPH than NADH (Hoover et al., 2007a, b; Simpson et al., 2008).

Interestingly, consumption of subsaturating levels of glyoxylate and SSA can occur simultaneously at saturating NADPH in a preparation of recombinant AtGLYR1 (Allan et al., 2012). These results suggest that the essentially irreversible reduction of SSA/glyoxylate to GHB/glycolate is more likely than their corresponding oxidation, and that there is a potential for competition between SSA and glyoxylate.

Mechanistic analyses were performed to further characterize the GLYR1 reactions. The kinetic mechanism is described as ordered Bi-Bi, in which NADPH binds to the enzyme before SSA or glyoxylate, and NADP$^+$ is released before the corresponding alcohol (Hoover et al., 2007a). NADP$^+$ is a more effective inhibitor ($K_i = 3.1 \, \mu M$) than GHB ($K_i = 79.9-93.5 \, \mu M$) or glycolate ($K_i = 22.1-23.7 \, \mu M$) (Hoover et al., 2007a). Inhibition by NADP$^+$, as well as high affinity for NADPH, suggests that GLYR1 and
GLYR2 are influenced by the NADPH/NADP\(^+\) ratio, and could be redox regulated in planta.

As previously mentioned, SSA is an intermediate in GABA metabolism; however, glyoxylate, GLYR’s preferred substrate in vitro, is a photorespiratory intermediate. Abiotic stresses such as drought, heat and high light are known to elicit stomatal closure resulting in declining CO\(_2\) concentrations within the leaf, thereby enhancing photorespiration (Allan et al., 2009). Photorespiration and the associated peroxisomal glycolate pathway are essential in dissipating heat during leaf exposure to high light and temperature (Wingler et al., 2000). Glyoxylate is formed from glycolate by glycolate oxidase in the peroxisome (Campbell and Ogren, 1990) and could be formed in excess during periods of photorespiratory stress (Allan et al., 2009).

SSA and glyoxylate are aldehydes containing the chemical grouping H-C=O, and can be harmful to plant health if they accumulate (Weber et al., 2004; Kotchoni et al., 2006). In general, aldehydes are intermediates involved in many metabolic processes such as carbohydrate, protein, amino acid, steroid or lipid metabolism (Yoshida et al., 1998). Upon accumulation, aldehydes can peroxidize lipids and reduce membrane integrity, create chromosomal anomalies and DNA adducts, trigger the transcription of stress-related genes, and modify proteins, thereby compromising cellular and developmental processes (Kotchoni et al., 2006). Therefore, aldehyde accumulation can cause toxic effects if they are not reduced to their corresponding less toxic alcohols by reductases such as GLYRs (Allan et al., 2008, 2009, 2012). For example, it has been reported that ribulose bisphosphate carboxylase oxygenase (Rubisco), the enzyme responsible for photosynthetic CO\(_2\) fixation, is inhibited by glyoxylate (Campbell and...
Ogren, 1990), and SSA accumulation is linked to a necrotic phenotype, as was previously mentioned (Ludewig et al., 2008). During photorespiratory stress, cytosolic GLYR1 and plastidial GLYR2 are hypothesized to partly function as a high affinity scavenging enzyme, reducing glyoxylate which may have leaked from the peroxisome and into the cytosol or chloroplast, thereby preventing inhibition of Rubisco (Allan et al., 2009).

However, the photorespiratory enzyme hydroxypyruvate reductase (HPR) is also involved in glyoxylate, as well as hydroxypyruvate reduction (Givan and Kleczkowski, 1992; Hoover et al., 2013). HPR2 and HPR3 are localized in the cytosol and plastid, respectively, and both have higher preference for NADPH than NADH and similar preference for glyoxylate and hydroxypyruvate. HPR1 is peroxisomal, and has higher preference for NADH and hydroxypyruvate over NADPH and glyoxylate (Timm et al., 2008, 2011, Hoover et al., 2013), although early enzyme kinetics have shown that $K_m$ values are in the milimolar range (Givan and Kleczkowski, 1992). Therefore, GLYRs, like HPRs, appear to be associated with photorespiratory glyoxylate detoxification.

Exposure to other stresses triggers the GABA shunt pathway and could involve GLYRs. Microarray analysis of Arabidopsis shows that $AtGLYR$s are expressed throughout the plant, and $AtGLYR2$ is more highly expressed than $AtGLYR1$ in rosette leaves, which is consistent with its localization within the chloroplast (Shelp et al., 2012c). Similarly, in cold-acclimated Arabidopsis plants, $AtGLYR$ transcript levels are unchanged whereas GHB levels accumulate within 48 h of stress (Kaplan et al., 2004, 2007). Alternatively, in response to various abiotic stresses such as cold, drought, osmotic and salt, $AtGLYR$s appear unaffected or slightly down-regulated in Arabidopsis seedlings (Kilian et al., 2007), whereas in germinating rice exposed to anoxia, abundance
of OsGLYR transcripts is correlated to peak GHB accumulation within 4 h of stress (Narsai et al., 2009). Arabidopsis leaves exposed to water, temperature and oxidative stress, the latter of which is simulated by submergence leading to O$_2$ restriction, exhibit increased AtGLYR1 and/or AtGLYR2 transcript levels, as well as elevated GABA, GHB and NADPH/NADP$^+$ levels over time (Allan et al., 2008). Similarly, tobacco leaves exposed to submergence also display increased GABA, GHB and NADPH/NADP$^+$ levels over time. Upon recovery from 3 h of submergence, tobacco leaf GABA, GHB, and phosphorylated pyridine dinucleotides return to control levels within 9 h; however, after 6 h or 9 h of submergence, these metabolites fail to recover. In a similar study, the levels of AtGLYR1 and AtGLYR2 transcripts are higher in leaves of WT Arabidopsis (Columbia and Wassilewskija ecotypes) subjected to 6 h of submergence stress, than in the non-submerged control (Allan et al., 2012). Both WTs display higher levels of GABA, GHB and NAD(P)H/NAD(P)$^+$ ratios in submerged versus non-submerged plants, whereas atglyr1 and atglyr2 mutants possess lower levels of these metabolites, possibly due to SSA accumulation and feedback inhibition of GABA-T. Moreover, GLYR1 appears to be more important in GHB production than GLYR2. In these studies, GLYR transcript abundance is sometimes correlated with GABA and GHB accumulation, as well as elevated NAD(P)H/NAD(P)$^+$ ratios during abiotic stress, notably O$_2$ deficiency from submergence. However, GLYR activity is likely regulated at the post-transcriptional level via in vivo redox status.

Interestingly, there is no effect on GHB levels in the Arabidopsis NADK1 suppression mutant in absence of stress compared with WT, even though the NADPH/NADP$^+$ ratio decreases, probably due to compensatory NADK activity from
other isoforms NADK2 and NADK3 (Allan et al., 2012). In this same study, submerged leaves of an NADK1 overexpression line of Arabidopsis have elevated levels of GHB compared with WT; however, the NADPH/NADP⁺ ratio remains unchanged, potentially due to limiting SSA. Moreover, the NADK1 suppression mutant under submergence stress displays marginally elevated GHB, potentially due to limited NAD(H) phosphorylation. Therefore, GLYR activity appears to be redox regulated, possibly supported by NADK1. These results provide evidence for transcriptional- and redox-regulated SSA reduction via GLYRs in vivo, despite the higher affinity for glyoxylate in vitro. Based on these findings, GLYRs are hypothesized to be involved in the detoxification of SSA and glyoxylate, which may accumulate in response to various abiotic stresses, by reduction to their corresponding alcohols (Allan et al., 2003; Breitkreuz et al., 2003; Hoover et al., 2007b).

2.6. CONCLUDING REMARKS

In plants, GABA is associated with biotic stress, development, carbon:nitrogen balance and nitrogen storage, and most of all abiotic stress. The GABA catabolite, GHB, is also associated with abiotic stress, although much less is known about its metabolic role(s) and fate(s). With CA-stored apple fruit, conditions that reduce ripening and respiration rates should be considered as abiotic stresses, which could lead to GABA accumulation and possibly physiological injury. GABA can theoretically originate from both glutamate decarboxylation and polyamine catabolism. Genes associated with GABA production and metabolism are not always expressed in accordance with abiotic stress, and the proteins encoded by these genes could be biochemically regulated by O₂
limitation, altered redox balance, \( \text{Ca}^{2+}/\text{CaM} \) activation or cellular acidosis, depending on the enzymatic activity under consideration. The function of GLYRs seems to straddle GABA metabolism and photorespiration, thereby providing various strategies for detoxifying two different aldehydes: glyoxylate and SSA. Most of these enzymes and their corresponding isoforms are localized in various cellular compartments, highlighting the wide scope of GABA production and catabolism in the plant cell, and potential interaction among multiple pathways involved in GABA metabolism.
CHAPTER THREE – PLANT GLYOXYLATE/SUCCINIC SEMIALDEHYDE REDUCTASES ARE LOCATED IN THE PLASTID, MITOCHONDRION AND CYTOSOL

3.1. CONTRIBUTIONS

This chapter, which is in preparation for submission to *Plant Physiology*, derives from my primary research project and I had an integral role in planning and deciding the direction of the research. Adel Zarei assisted with transcript analysis of the *atglyr1* mutant, prepared the Arabidopsis protoplasts for transient expression, and assisted with the purification of *atglyr1* mitochondria using Percoll. Vikramjit Bajwa cloned the rice and Arabidopsis GLYR genes and conducted kinetic analysis of the *OsGLYR* proteins. Christopher Trobacher cloned the apple GLYR genes. I performed the remaining experiments and data analysis, including kinetic analyses of the apple and Arabidopsis GLYR proteins, cloning of the *MdGLYR*-GFP fusion proteins and the methoxyfenozide-inducible *ArGLYR2*-GFP, producing and characterizing the stable Arabidopsis line expressing the methoxyfenozide-inducible *ArGLYR2*-GFP, all transient and stable expression experiments and associated imaging, and assay of marker proteins in Percoll-purification experiments. I also prepared most of the manuscript in conjunction with Barry Shelp and Gale Bozzo. Jennifer DeEll, Kazuhito Akama and Robert Mullen provided many helpful comments and molecular reagents during the research and editing of the manuscript. The apple GLYR gene sequences have been submitted to GenBank as *MdGLYR1* and *MdGLYR2* (GenBank Acc. Nos. KT202799 and KT202800, respectively).
3.2. ACKNOWLEDGEMENTS

This research was supported by funding from the Natural Sciences and Engineering Research Council (NSERC) of Canada as a Strategic Project Grant and Individual Discovery Grants, and from the Ministry of Agriculture, Food and Rural Affairs, AgroFresh Inc., MITACS Inc., and the Ontario Apple Growers. C.J.B and C.P.T. acknowledge receipt of MITACS Inc. fellowships. Thanks to Dr. Michael Shaw (Dow Agrosciences, Indianapolis, Indiana, for providing a sample of Intrepid 2F insecticide, Drs. William Plaxton and Joonho Park (Queen’s University, Kingston, ON) for providing the pSAT4-(PDCpl-E2)-mCherry plasmid and Dr. Jaideep Mathur for the use of his epifluorescence microscope.
3.3. ABSTRACT

Plant NADPH-dependent GLYR1 and GLYR2 are hypothesized to detoxify photorespiratory glyoxylate and GABA-derived SSA by conversion into their corresponding less toxic alcohols glycolate and GHB, respectively. Photorespiration and the GABA pathway, as well as the ratio of NADPH/NADP⁺, are known to be stimulated by abiotic stress conditions. Here, we cloned and heterologously expressed Arabidopsis thaliana, apple (Malus × domestica Borkh.) and rice (Oryza sativa) GLYR1 and GLYR2 in Escherichia coli. The purified GLYRs had an affinity for glyoxylate and SSA, respectively, in the low micromolar and millimolar ranges and were feedback inhibited by NADP⁺, supporting a potential role for GLYRs in redox homeostasis during abiotic stress. Transient expression of GLYR-GFP fusions in tobacco suspension cells or Arabidopsis protoplasts confirmed that MdGLYR1 is localized in the cytosol. In contrast to previous evidence for a plastidial AtGLYR2, we demonstrated that GLYR2s from apple, rice and Arabidopsis were dual localized in the plastid and mitochondrion. Furthermore, methoxyfenozide-inducible AtGLYR2-GFP in stably-transformed Arabidopsis plants was also dual localized in mitochondria and plastids, and GLYR activity was detected in a Percoll-purified mitochondrial fraction from an atglyr1 knockout mutant, providing novel evidence for a mitochondrial-localized GLYR in higher plants. Cytosolic GLYR1 and plastidial GLYR2 are hypothesized to scavenge excess glyoxylate during photorespiratory stress, whereas mitochondrial GLYR2 may be responsible for the metabolism of GABA-derived SSA produced by mitochondrial GABA transaminase. This study contributes to our understanding of the subcellular localization of plant GLYRs, and their potential biochemical roles during abiotic stress.
3.4. INTRODUCTION

Plant NADPH-dependent GLYRs are hypothesized to detoxify harmful reactive aldehydes into their corresponding less toxic alcohols, thereby preserving plant health during various abiotic stresses (Allan et al., 2009). Glyoxylate, a photorespiratory intermediate, is produced in the peroxisome via oxidation of glycolate and can accumulate under conditions such as drought or salt stress, which elicit stomatal closure (Wingler et al., 2000). Micromolar levels of glyoxylate inhibit the activation of Rubisco in intact, lysed and reconstituted chloroplasts (Campbell and Ogren, 1990), and glyoxylate can react with DNA strands forming glyoxylated adducts or polypeptide side chains, modifying arginine and lysine residues (Schmitt et al., 2005; Maekawa et al., 2006). GLYRs may reduce glyoxylate into less harmful glycolate (Givan and Kleczkowski, 1992; Allan et al., 2009). In contrast, SSA is a toxic intermediate in GABA metabolism, and is produced in the mitochondrion via transamination of GABA (Shelp et al., 2012d). GABA is well known to accumulate under various stresses such as cold, drought and hypoxia (Shelp et al., 1999; Kinnersley and Turano, 2000; Allan et al., 2008), which have been linked with elevated NAD(P)H/NAD(P)⁺ ratios, reduction of SSA into GHB via NADPH-dependent GLYRs, and inhibition of SSA conversion to succinate via NAD⁺-dependent SSADH (Allan et al., 2009, 2012).

GLYRs were first isolated from spinach and pea leaves, and found in both cytosolic and purified plastid fractions, with 10-20% of the total leaf GLYR activity being present in isolated chloroplasts (Zelitch, 1953; Givan and Kleczkowski, 1992). To date, the best characterized plant GLYRs in terms of biochemical, kinetic and structural
properties are from Arabidopsis (Breitkreuz et al., 2003; Hoover et al., 2007a, b; Simpson et al., 2008; Hoover et al., 2013). ArGLYR1 (GenBank accession no. AY044183) is localized in the cytosol, whereas ArGLYR2 (GenBank accession no. AAP42747) is localized in the plastid (Simpson et al., 2008; Ching et al., 2012). Both enzymes prefer NADPH over NADH as a cofactor and display a higher affinity for glyoxylate ($K_m$ in the low micromolar range) than for SSA ($K_m$ in the low millimolar range) (Hoover et al., 2007b; Simpson et al., 2008). NADP$^+$ competitively inhibits ArGLYR1, indicating that NADPH/NADP$^+$ ratios may regulate GLYR activity in planta. Moreover, ArGLYR1 is a member of the β-HAD superfamily, all of which possess a conserved glycine-rich fingerprint motif at their N-terminus known as the Rossmann fold, where NADPH typically binds (Hoover et al., 2013).

In this paper, we assessed the potential involvement in aldehyde detoxification of GLYRs from apple (Malus × domestica Borkh.), a dicotyledonous species, and rice (Oryza sativa L.), a monocotyledonous species. These results caused us to revisit the previously characterized ArGLYRs. The GLYRs from all three species possessed the conserved glycines belonging to the β-HAD superfamily, and a similar sequence for the glycine-rich fingerprint motif. In general, GLYRs displayed higher affinity and catalytic efficiency for glyoxylate than for SSA, and were inhibited by the reaction product NADP$^+$. GLYR1 was cytosolic whereas all GLYR2s were localized in both plastids and mitochondria with all subcellular localization systems tested (i.e., transient expression in tobacco BY-cells or protoplasts from Arabidopsis suspension cells, and methoxyfenozide-induced expression of ArGLYR2-GFP in stably-transformed Arabidopsis). The mitochondrial localization for GLYR2 was supported via the
preparation and analysis of glyoxylate reductase activity in Percoll-gradient-purified fractions enriched in mitochondria from a glyr1 knockout mutant. These results highlight the potential regulation of plant GLYRs by NADPH/NADP⁺ ratios in planta, and their roles in toxic aldehyde reduction throughout the plant cell during abiotic stress.

3.5. MATERIALS AND METHODS

3.5.1. Plant Material, RNA and DNA Extraction

Apple total RNA was extracted from 1 g of liquid N₂-ground powder from mature apple (Malus × domestica Borkh. cv. ‘Empire’) fruit that had been stored at 0°C under 5 kPa CO₂ for 8 weeks postharvest and used to prepare cDNA as described previously (Trobacher et al., 2013b). Rice total RNA was extracted using a Qiagen RNAeasy kit (Qiagen, Valencia, CA) from 50 mg of liquid N₂-ground powder from leaves of 20-d old rice plants (Oryza sativa L. cv. Kaybonnet) grown in a controlled environment chamber at 28°C temperature and 16 h photoperiod (Brauer et al., 2011). Total Arabidopsis thaliana [L.] Heynh ecotype Columbia RNA was extracted using 100 mg of liquid N₂-frozen rosette leaf tissue from a methoxyfenozide-inducible AtGLYR2-GFP stable line and a glyr1 knockout mutant. This RNA was used for synthesis of cDNA and qPCR analyses as described previously (Zarei et al., 2011, 2014). The housekeeping gene elongation factor 1 (EF-1) was chosen based on the research of Poupard et al. (2003) and Czechowski et al. (2005). Arabidopsis genomic DNA was extracted from 100 mg of frozen leaf tissue as described by Montiel et al. (2011). For characterization of the Arabidopsis transgenic line expressing inducible AtGLYR2-GFP, 100 mg of rosette
tissue from two plants was pooled for analysis. For characterization of the *atglyr1* mutant, data are means of three separately extracted biological replicates.

3.5.2. Identification and Cloning of cDNAs Encoding Apple and Rice GLYRs and Arabidopsis GLYR2

The predicted amino acid sequence of Arabidopsis GLYR1 (AT3G25530) was used as a query for a BLAST search of GenBank for similar predicted peptides. Among the best matched hits were a barley cDNA (AK25268), a tomato succinic semialdehyde reductase 1 (AB359919), a wheat cDNA (AK333249), a *Vitis vinefera* mRNA (XM_002266216), a poplar mRNA (XM_002320512), and a *Lotus japonicus* cDNA (AK337476). The peptide sequences were aligned and used to identify two highly conserved regions including VGFLGLGIMG, just inside the start codon, and QQKDMRLALAL, approximately 43 amino acids upstream of the C-terminus of *AtGLYR1*. Degenerate primers (CT-F4 and CT-R4, see Appendix A, Supplementary Table S3.1 for a list of all primers used in this study) corresponding to these sequences amplified two products from the apple fruit cDNA. The first product, when translated, encodes a 213 amino acid peptide that is 76% identical to *AtGLYR1*. The second product encodes a 223 amino acid peptide that is 82% identical to *AtGLYR2* (AT1G17650). The two predicted peptides are 53% identical to each other. These sequences were used to design primers for 5'- and 3'-Rapid Amplification of cDNA Ends (RACE). Primers CT-R7 and CT-R8 were used for 5'-RACE and nested PCR, respectively, on the *GLYR1*-like gene. Primers CT-F7 and CT-F8 were used for 3'-RACE and nested PCR, respectively. Primers CT-R9 and CT-R10 were used for 5'-RACE and nested PCR, respectively, on the
GLYR2-like gene. Primers CT-F9 and CT-F10 were used for 3'-RACE and nested PCR, respectively. Sequences obtained from the RACE reactions were used to design primers matching the 5' and 3' untranslated regions (CT-F12 and CT-R12 for the GLYRI-like gene, and CT-F13 and CT-R13 for the GLYR2-like gene). A 958 bp product and an 1166 bp product, designated as *MdGLYR1* (GenBank Acc No. KT202799) and *MdGLYR2* (GenBank Acc No. KT202800) respectively, were amplified and sub-cloned into the cloning vector pCR2.1-TOPO (Invitrogen). The predicted translation products are 58% identical to each other. *MdGLYR1* is 80% identical to *AtGLYR1*, and *MdGLYR2* is 78% identical to *ArGLYR2*. Identical sequences have now been identified from the apple genome database (www.rosaceae.org): *MdGLYR1*, MDP0000149834; *MdGLYR2*, MDP0000158245.

The primers CT-F17 and CT-R17 were used to amplify *MdGLYR1* with 5' *NdeI* and 3' *BamHI* restriction sites. TargetP (v1.1; http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson et al., 2000) predicted that *MdGLYR2* has an N-terminal 52 amino acid plastid targeting sequence. The primers CT-F17 and CT-R17 were used to amplify *MdGLYR2* minus the sequence encoding the putative plastid transit peptide with 5' *NdeI* and 3' *BamHI* restriction sites. Both PCR products were digested with *NdeI* and *BamHI*, gel purified, and ligated into pET15b expression vector (Novagen, Madison, WI) digested with the same enzymes to produce pET15b-*MdGLYR1* and pET15b-*MdGLYR2Δ54*. When expressed, the truncated GLYR2 possesses an N-terminal 6xHis tag.

The *AtGLYR1* and *AtGLYR2* cDNAs (AY044183 and AAP42747, respectively) were blasted against the rice (cv. Nipponbare) genome annotation project (http://rice.plantbiology.msu.edu/) to reveal two full length cDNA sequences,
LOC_Os02g35500 and LOC_Os01g39270, which are 81% and 65% identical to
AtGLYR1 and AtGLYR2, respectively. These sequences were designated as putative
OsGLYR1 and OsGLYR2, respectively. Putative full length OsGLYR1 and OsGLYR2 were amplified from the rice cDNA using forward and reverse primers, VB-F1 and VB-
R1 for OsGLYR1, and VB-F2 and VB-R2 for OsGLYR2. The primers VB-F1 and VB-R1 were used to amplify OsGLYR1 with 5’ NdeI and 3’ BamHI restriction sites. TargetP
(v1.1; http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson et al., 2000) predicted that
OsGLYR2 has an N-terminal 35 amino acid plastid targeting sequence. The primers VB-
F2 and VB-R2 were used to amplify OsGLYR2 minus the sequence encoding the putative
plastid transit peptide with 5’ NdeI and 3’ BamHI restriction sites. Both PCR products
were digested with NdeI and BamHI, gel purified, and ligated into pET15b digested with
the same enzymes to produce pET15b-OsGLYR1 and pET15b-OsGLYR2Δ35. When
expressed the truncated GLYR possesses an N-terminal 6xHis tag for purification.

Notably, the OsGLYR1 and truncated OsGLYR2 amino acid sequences from the
Kaybonnet cultivar are 100% identical to the Nipponbare cultivar. Nucleotide sequences
are also identical between the cultivars, except for one single nucleotide polymorphism in
the OsGLYR2 sequence.

A truncated Arabidopsis AtGLYR2 lacking 58 amino acids of its N-terminal
plastid targeting sequence (AtGLYR2 Δ58) was amplified from the Arabidopsis cDNA
using primers VB-F3 and VB-R3 and cloned into pET15b expression vector using 5’
NdeI and 3’ BamHI restriction sites. The PCR product was digested with NdeI and
BamHI, gel purified, and ligated into pET15b digested with the same enzymes to produce
pET15b-AtGLYR2Δ58 construct. When expressed, the truncated AtGLYR possesses an N-terminal 6xHis tag for purification.

3.5.3. Production, Purification and Kinetic Analysis of Recombinant Apple, Rice and Arabidopsis GLYRs

The constructs pET15b-MdGLYR1 and pET15b-MdGLYR2Δ54 were individually expressed in E. coli BL-21(DE3) Rosetta (pLysS) cells (Novagen) co-expressing the GroES/GroEL chaperone complex, and the resulting soluble recombinant proteins were passed through a Ni²⁺ affinity column essentially as described previously (Clark et al., 2009a). Also, the pET15b-AtGLYR1, pET15b-AtGLYR2Δ58, pET15b-OsGLYR1 and pET15b-OsGLYR2Δ35 constructs were expressed in E. coli BL-21(DE3) cells and the recombinant proteins purified as described in Hoover et al. (2007b). Each eluted protein was precipitated in 80% ground ammonium sulfate and stored at -80°C. As necessary, the pellet was resuspended in 100 µL of reaction buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid (HEPES), 10% sorbitol, pH 7.8 for AtGLYR1 and AtGLYR2, pH 7.5 and 7.3 for MdGLYR2 and MdGLYR2, and pH 6.5 and 7.1 for OsGLYR1 and OsGLYR2 Δ35, respectively)

Enzymatic activity was monitored as the oxidation of NADPH in nmol/min at A₃₄₀ at room temperature using a Spectramax Plus384 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, California). All assays were conducted using a 96-well flat bottom polystyrene plate (Corning Incorporated, Corning, NY) at a final reaction volume of 250 µL and an enzyme concentration within the linear range (2-50 nM). The reaction was initiated with a pre-made master mix of reaction buffer, co-factor and
substrate brought to volume with double distilled H\textsubscript{2}O. The reaction was briefly mixed before reading the initial enzymatic rate. The optimal pH for enzymatic activity at saturating co-factor and glyoxylate was determined using buffers (50 mM) with overlapping pH range (2-morpholino-ethanesulfonic acid (MES) for pH 5.5-6.8; HEPES for pH 6.8-8.2; and N-tris(hydroxymethyl)methyl-4-aminobutanesulfonic acid (TABS) for pH 8.2-9.6). The three buffers were used separately for the apple isoforms, whereas they were combined for rice. The kinetic parameters ($V_{\text{max}}$, $K_m$, $k_{\text{cat}}$) were determined using saturating co-factor and varying substrate or varying co-factor and saturating substrate. All measurements were done in quadruplicate and the kinetic data were fit to the Michaelis-Menten equation ($V=V_{\text{max}}[S]/K_m + [S]$) using non-linear least square analysis (SigmaPlot2000, version 6.1; Enzyme Kinetics Module, version 1.0). From the kinetic parameters, a performance constant ($k_{\text{cat}}/K_m$) was calculated for each substrate.

The influence of redox ratio on the activities of \textit{MdGLYR1} and \textit{MdGLYR2} was investigated using saturating glyoxylate, saturating NADPH and varying \textit{NADP}$. Data typically represent the means ± SE of three independent enzyme preparations. Protein concentration was determined via the Bradford method (Bradford, 1976) using the assay kit provided by Bio-Rad Laboratories Ltd.; bovine serum albumin (fraction V, Pierce) was used as the standard.

3.5.4. Transient Expression and Subcellular Localization of Apple, Rice and \textit{Arabidopsis} GLYRs in Tobacco BY-2 Cells and \textit{Arabidopsis} Protoplasts

The full-length open reading frame of \textit{MdGLYR1} was amplified with \textit{NcoI} restriction sites using the primers CB-F1 and CB-R1, whereas the \textit{MdGLYR2} open
reading frame was amplified with NheI restriction sites using the primers CB-F2 and CB-R2. The resulting PCR products were digested and sub-cloned into pUC18/NcoI-mGFP, or pUC18/NheI-mGFP (as described by Simpson et al., 2008) to produce GLYRs with monomeric GFP fused to their C-terminus. The full-length open reading frame of AtGLYR2 was amplified with NheI restriction sites using the primers NheI_AtGLYR2_F and NheI_AtGLYR2_R from WT Arabidopsis cDNA. The resulting PCR products were digested and sub-cloned into pUC18/NheI-mGFP to produce AtGLYR2-GFP fusion protein constructs, and screened based on ampicillin resistance. The open reading frame of the OsGLYR2 cDNA clone from the cultivar Nipponbare (AK064876 from National Institute Agrobiological Sciences, Tsukuba, Japan) was amplified with the NotI and SalI restriction sites. The resulting PCR products were digested and subcloned into the pTH-2 vector (including GFP) used in Englert et al. (2007).

A total of 5 µg of GFP plasmid DNA with or without 1 µg of the cytosolic marker pSAT4A-mCherry-N1 plasmid DNA (Arabidopsis Biological Resource Center) or plastidial pyruvate decarboxylase E2-mCherry (pSAT4-(PDCpl-E2))-mCherry plasmid DNA (Park et al., 2012) were transiently expressed in tobacco BY-2 suspension cells via tungsten particle bombardment using a Biolistics PDS-1000/HE particle delivery system (Bio-Rad). After incubation for 8 h, cells were fixed in 4% w/v formaldehyde then imaged as previously described (Trobacher et al., 2013b). Mitochondria were immunolabelled using rabbit anti-cytochrome c oxidase II (COXII) primary antibody and goat anti-rabbit rhodamine red X secondary antibody (Jackson Immunoresearch Laboratories) as described by Frelin et al. (2012). Transformations were replicated at least three times, and a minimum of 10 cells were imaged per transformation using
confocal laser scanning microscopy (CLSM) as previously described (Trobacher et al., 2013b). Aperture was manually controlled by closing the diaphragm by 1/3, and regulating pinhole size (1.0 airy unit). Fluorophore emissions were imaged sequentially, and no detectable bleedthrough was observed with the same acquisition settings used in data collection.

Arabidopsis protoplasts cells were prepared by enzyme digestion of 1-d-old subcultured Arabidopsis suspension cells, as described by Zarei et al. (2011). A total of 10 µg of GFP fusion proteins with or without 5 µg of pSAT4-(PDCpl-E2)-mCherry plastid marker was mixed with 125 µL of concentrated protoplast solution. Arabidopsis protoplast mitochondria were stained with 100 nM Mitotracker Red CMXRos (Invitrogen Life Technologies), incubated for 25 min at 32°C in the dark, followed by 3 x 5 min washing in protomedium. Cells were imaged immediately after staining as previously described (Trobacher et al., 2013b) using CLSM. All fluorescent images are representative of at least three independent transformation replications with a minimum of 10 transformed cells imaged per transformation.

3.5.5. Stable Expression and Subcellular Localization of AtGLYR2 in Arabidopsis Seedlings

A stable Arabidopsis line expressing methoxyfenozide-inducible AtGLYR2-GFP was cloned following the methoxyfenozide-inducible promoter system outlined in Dietrich et al. (2008). The full-length open reading frame of AtGLYR2-GFP was amplified from pUC18-AtGLYR2-GFP with 5' PacI and 3' SpeI restriction sites using the primers F-PacI-LAtGR2-GFP and R-SpeI-LAtGR2-GFP, respectively. The resulting
PCR product was digested and subcloned into the plasmid CD1660-1-5XG-M35S, resulting in the construct CD-1660-1-5XG-M35S::AtGLYR2-GFP; positive clones were screened based on ampicillin resistance and sequenced. From this construct, the 5XG-M35S-AtGLYR2-GFP cassette was digested with NotI and ApaI and subcloned into the plasmid CD1468-1 possessing a promoter-binding VGE element, resulting in the construct CD1468-1-VGE-5XG-M35S::AtGLYR2-GFP; positive clones were screened based on ampicillin resistance and sequenced. This cassette was then digested with AscI and subcloned into the binary vector pEC291, resulting in the construct methoxyfenozide inducible pEC291-AtGLYR2-GFP; positive clones were screened based on kanamycin resistance and sequenced. The empty vectors CD1660-1-5XG-M35S, CD1468-1-VGE-5XG-M35S and pEC291 were kindly provided by Dr. Robert Mullen.

The pEC291-AtGLYR2-GFP plasmid was transformed into EHA105 Agrobacterium cells and WT Arabidopsis plants were stably transformed with pEC291-AtGLYR2-GFP via the floral dip method (Clough and Bent, 1998). Plants were allowed to self-pollinate and grow to maturity in a controlled environment growth chamber (Enconair Ecological Chambers, Model GC8-2H) set at 70 μmol s⁻¹ m⁻² photosynthetic photon flux density for 16 h at 23°C and in the dark for 8 h at 21°C). Seeds were first treated with 95% ethanol for 30–60 s, then with 30% bleach (1.57% sodium hypochlorite, final volume) containing 0.05% Tween 20 for 5 min, followed by three rinses with sterile water. Sterilized seeds were suspended in 0.1% sterile agarose and screened on solid half-strength MS medium containing 30 g L⁻¹ sucrose, as well as 100 mg L⁻¹ timentin and 16 μg mL⁻¹ hygromycin. Fourteen-d-old transformants were transferred to Sunshine LC1 potting mix (Sun Gro Horticulture, Canada), allowed to self-pollinate and grown to
maturity. T2 seeds were sterilized and screened as described above, and highly expressing GFP lines were selected by viewing 14-d-old seedlings under an epifluorescent microscope (Leica DM-6000CS). The selected lines were grown to maturity and the T3 progeny was harvested.

Localization analysis of Arabidopsis stably expressing methoxyfenozide-inducible-AtGLYR2-GFP was performed using 14-d-old T3 seedlings. Expression of AtGLYR2-GFP was induced by foliar application of a 1:10,000 dilution in autoclaved double distilled water of Intrepid 2F pesticide containing the active ingredient methoxyfenozide. Seedlings were incubated for 48 h before imaging. Mitochondria were stained by immersing seedlings in a solution of 500 nM Mitotracker Red CMXRos diluted in water, gently shaken for 25 min at 37ºC, then washed 3 x 5 min with water.

Microscopy was performed as previously described by Trobacher et al. (2013a) using CLSM. GFP and chlorophyll were excited with a 488 nm argon ion laser at 25% power, and the emitted light was detected at 500-530 nm for GFP and 685-750 for chlorophyll. Mcherry and Mitotracker were excited with a 543 nm Ar/HeNe laser at 85% power, and emitted light was detected at 590-650 nm for mCherry and 579-599 nm for Mitotracker.

3.5.6. Mitochondrial Localization of AtGLYR2 in WT Arabidopsis and glyr1 Knockout Mutant Using Percoll Gradient Centrifugation

An Arabidopsis glyr1 knockout mutant (Salk_057410) was twice backcrossed to the wild type. Plants homozygous for glyr1 T-DNA were identified using the primers glyr1-WT-F and glyr1-WT-R for the wild type allele, and LBb1.3-F and LBb1.3-R for
the T-DNA, as recommended by the Salk Institute (Appendix A, Supplementary Table S3.1). The primers were designed using the Salk iSECT tool online software.

Initially, the total GLYR activity in rosette leaf tissue from 4-week-old Arabidopsis WT or glyr1 mutant, grown as described above, was extracted and assayed at saturating substrate and co-factor levels following Hoover et al. (2007b). Subsequently, mitochondria were isolated from 55-60 g of polytron-homogenized rosette tissue from either of the lines using linear Percoll density gradient centrifugation (Taylor et al., 2014). The WT was grown in a naturally-lighted greenhouse set at 23/19°C under a 16-h light/8-h night regime, whereas the atglyr1 mutant was grown in an Enconair controlled environment growth chamber (Model GC8-2H) set at 70 μmol m⁻² s⁻¹ photosynthetic photon flux density under a 23/19°C under a 11-h light/8-h night regime. Plants were watered as required with tap water. Aliquots of the crude homogenate, enriched total organelle pellet and Percoll-purified mitochondrial fractions were sonicated for 10 s in order to burst organelles (Fischer Scientific sonic dismembrator Model 120). Fractions were loaded onto Econo-Pac 10DG desalting columns (Biorad) and eluted with reaction buffer prior to enzymatic assays. GLYR activity was assayed at saturating substrates and mitochondrial fumarase and plastid phosphoribulokinase were assayed as described by Taylor et al. (2014), with the exception that the reaction volume was 250 μL and the wavelength for the fumarase assay was 240 nm (Puchegger et al., 1990), rather than 340 nm as given. Blank measurements consisting of non-substrate initiated assays were subtracted from the substrate-initiated assays. Data represent means of six technical replicates. The experiment was repeated twice.
3.6. RESULTS

3.6.1. Production and Characterization of Recombinant Apple, Rice and Arabidopsis GLYRs

The N-terminal targeting presequences were predicted using TargetP (http://www.cbs.dtu.dk/services/TargetP/), WoLF PSORT (http://www.genscript.com/psort/wolf_psort.html), SherLoc (http://abi.inf.uni-tuebingen.de/Services/SherLoc2/) and YLoc (http://abi.inf.uni-tuebingen.de/Services/YLoc/webloc.cgi). These sequences were removed from the full-length GLYR2s from apple (Δ54), rice (Δ35) and Arabidopsis (Δ58) and the truncated sequences, together with the full-length GLYR1s were individually expressed in Escherichia coli (Appendix A, Supplementary Fig. S3.1). This allowed for the recovery of a significant portion of the recombinant proteins in the corresponding soluble fraction (Appendix A, Supplementary Fig. S3.2). In all fractions eluted from Ni\(^{2+}\)-affinity columns, the recombinant protein was purified to near homogeneity. Predicted protein masses for MdGLYR1, MdGLYR2Δ54, OsGLYR1, OsGLYR2Δ35, AtGLYR1 and AtGLYR2Δ58 were 31.8, 33.1, 30.5, 31.4, 30.7 and 33.2 kDa, respectively. The mass of recombinant AtGLYR2Δ58 appeared slightly larger than predicted, despite repeated cloning of the gene and preparation of the recombinant protein; this phenomenon has been reported previously (Shi et al., 2012). The activities of MdGLYR1, MdGLYR2 and OsGLYR1 were maximal over a pH range of approximately 6.8-7.5, whereas the maximum for OsGLYR2 was found at a slightly more acidic pH of 6-6.5 (Appendix A, Supplementary Fig. S3.3). These results are similar to the previously reported pH optima for recombinant AtGLYR1 and AtGLYR2 between 6.8-7.8 (Hoover et al., 2007b;
Simpson et al., 2008).

Enzyme kinetics were determined with the various recombinant proteins to assess their substrate specificity. Overall, the catalytic efficiencies ($k_{\text{cat}}/K_m$) of all GLYRs across species were within the same order of magnitude for each separate substrate, and the $K_m$ values for glyoxylate and NADPH were in the micromolar range, whereas the $K_m$ value for SSA was in the millimolar range (Table 3.1 and Appendix A, Supplementary Figs. S3.4-S3.6). Glyoxylate and SSA-dependent kinetics for the proteins encoded by newly cloned AtGLYRs were similar to previously published data (Hoover et al., 2007b; Simpson et al., 2008); therefore, NADPH kinetics were not re-investigated. Notably, the kinetic analyses conducted here used microtitre plates, rather than cuvettes as in Hoover et al. (2007b) and Simpson et al. (2008), but similar results were obtained. Across species and isoforms, the highest catalytic efficiencies were observed for NADPH, mostly at fixed glyoxylate, followed by glyoxylate at fixed NADPH, whereas catalytic efficiencies for SSA were the lowest in all isoforms (Table 3.1; Hoover et al., 2007b, Simpson et al., 2008). There were no clear or uniform catalytic differences between GLYR1 and GLYR2 isoforms, indicating that across all species, GLYRs prefer glyoxylate over SSA, and have a high affinity for their co-substrate NADPH. The activities of $MdGLYR1$ and $MdGLYR2$ were inhibited by increasing NADP$^+$/NADPH ratio (Appendix A, Supplementary Fig. S3.7), and their reaction rates were reduced by half with an NADP$^+$/NADPH ratio of 5, indicating that GLYRs could be feedback inhibited in vivo.
Table 3.1. Kinetic parameters for purified recombinant GLYRs from apple, rice and Arabidopsis. Data represent the mean ± SE of two (Arabidopsis) or three (apple and rice) typical enzyme preparations.

<table>
<thead>
<tr>
<th>Varied Substrate</th>
<th>Fixed Substrate</th>
<th>$K_m$ $\mu M$</th>
<th>$V_{max}$ $\mu mol$ mg$^{-1}$ protein min$^{-1}$</th>
<th>$k_{cat}$ s$^{-1}$</th>
<th>$k_{cat}/K_m$ s$^{-1}$ mM$^{-1}$</th>
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<tr>
<td><strong>MdGLYR1</strong></td>
<td>Glyoxylate</td>
<td>14.1 ± 2.1</td>
<td>54.7 ± 6.7</td>
<td>30.9 ± 3.8</td>
<td>2230 ± 153</td>
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<td>NADPH</td>
<td>Glyoxylate</td>
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<td>39.4 ± 6.1</td>
<td>22.3 ± 3.4</td>
<td>6803 ± 278</td>
</tr>
<tr>
<td>SSA</td>
<td>NADPH</td>
<td>1133 ± 273</td>
<td>8.1 ± 2.9</td>
<td>4.6 ± 1.6</td>
<td>5.1 ± 2.3</td>
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<td>NADPH</td>
<td>SSA</td>
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<td>9.8 ± 1.0</td>
<td>5.5 ± 0.6</td>
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<td>NADPH</td>
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<td>28.2 ± 2.7</td>
<td>15.7 ± 1.5</td>
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<td>21.9 ± 4.2</td>
<td>5.4 ± 0.9</td>
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<td>21.4 ± 5.0</td>
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<td>1040 ± 73</td>
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<td>28.4 ± 2.8</td>
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<tr>
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<td>19.3 ± 4.0</td>
<td>36.8 ± 19.9</td>
<td>18.4 ± 10.0</td>
<td>906 ± 353</td>
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3.6.2. Subcellular Localization of Apple, Rice and Arabidopsis GLYRs in Various Transient and Stable Expression Systems

*In silico* comparison of the GLYR sequences from apple, rice and Arabidopsis revealed that GLYR1s and GLYR2s are 78-80% and 67-77% identical, respectively, and GLYR1s are 54-57% identical to GLYR2s among species (Appendix A, Supplementary Fig. S3.1). All GLYRs possess the glycine-rich fingerprint motif near their N terminus, which is important in NADPH binding within the Rossmann fold (Hoover et al., 2013; Appendix A, Supplementary Fig. S3.1). Recently, AtGLYR1 was thought to contain a C-terminus peroxisomal-like targeting tripeptide (SRE), similar to the type 1 peroxisomal target signal (SKL); however, multiple lines of evidence showed exclusive localization to throughout the cytosol and not the peroxisome (Ching et al., 2012). Notably, *Md* and *Os*GLYR1s appear to lack C-terminal peroxisomal-like targeting signals, as well as N-terminal targeting sequences (Appendix A, Supplementary Fig. S3.1); therefore, GLYR1s are hypothesized to be localized in the cytosol, as was reported for AtGLYR1 in tobacco BY-2 cells, Arabidopsis suspension cells and stably transformed Arabidopsis plants (Simpson et al., 2008; Ching et al., 2012).

All GLYR2s possess an N-terminal targeting sequence of varying lengths, and are generally more highly predicted to be localized in the chloroplast than in the mitochondria by various subcellular localization prediction programs (Appendix A, Supplementary Fig. S3.1). However, mitochondrial specific programs such as MitoProt predicted a high probability of mitochondrial localization for all three GLYR2s, and DualPred specific for dual targeting predicted mitochondrial localization for *Md* and *Os*GLYR2 (Appendix A, Supplementary Fig. S3.1). The subcellular localization database
for Arabidopsis proteins (SUBA3 http://suba3.plantenergy.uwa.edu.au/) suggested that
AtGLYR2 is localized in the plastid based on MS/MS analyses; however, these
conclusions are based solely on chloroplast, and not mitochondrial isolation experiments.
To assess subcellular localization, MdGLYR-GFP fusion proteins were transiently
expressed in tobacco BY2 cells using biolistic bombardment. As predicted based on
previous results for AtGLYR1 (Ching et al., 2012), MdGLYR1-GFP displayed diffuse
localization throughout the cell (Fig. 3.1A) and co-localized with the cytosolic marker
protein mCherry (Fig. 3.1C). The subcellular localization of OsGLYR1 was not assessed
as it, like At and MdGLYR1, lacks an N-terminal targeting signal (Appendix A,
Supplementary Fig. S3.1), and therefore was also presumed to be cytosolic. MdGLYR2-
GFP expression was concentrated in large globular structures (Fig. 3.1E) and co-localized
with the plastid marker PDCpl-E2-mCherry (Fig. 3.1G), and therefore appears to be
localized in the plastid. Notably, in the majority (approximately 70%) of tobacco BY-2
cells transiently expressing MdGLYR2-GFP, smaller puncta were also observed (Fig.
3.2A), which did not co-localize with the plastid marker (Fig. 3.2, C and D). The smaller
MdGLYR2-GFP-containing puncta were not previously noticed by Simpson et al. (2008),
who suggested that AtGLYR2-GFP was exclusively localized in the plastid. When cells
expressing MdGLYR2-GFP were immunostained with mitochondrial anti-COXII-IgG
(Fig. 3.2G), the smaller puncta co-localized with the mitochondrial antibody (Fig. 3.2, H
and I). Therefore, MdGLYR2-GFP appears to be targeted to both plastids and with
mitochondrial anti-COXII-IgG (Fig. 3.3, C and D). Furthermore, we tested the localization
of OsGLYR2-GFP (Fig. 3.3F) and similar findings were observed (Fig. 3.3, H and I).
GLYRs from multiple plant species including both monocotyledonous (rice) and
**Figure 3.1.** Subcellular localization of *MdGLYRs* in tobacco BY-2 cells. Cells were co-bombarded with *MdGLYR1*-mGFP (A) and the cytosolic marker mCherry (B) or *MdGLYR2*-mGFP (E) and the plastid marker PDC_{pt}-E2-mCherry (F), and allowed to express for 8 h before visualization using CLSM. Co-localization between GFP and mCherry is observed by the yellow colour in the merged image (C and G). D and H are the corresponding transmitted light images and scale bars represent 10 µm. mitochondria in tobacco BY-2 cells. The finding that *MdGLYR2*-GFP appears to be dual localized prompted us to revisit AtGLYR2-GFP localization. Tobacco BY-2 cells expressing AtGLYR2-GFP also exhibited smaller puncta (Fig. 3.3A), which co-localized
Figure 3.2. Dual Localization of *MdGLYR2* in tobacco BY-2 cells. Cells were bombardered with *MdGLYR2-GFP* (A and F) with PDCpl-E2-mCherry (B) or alone (F) and allowed to express for 8 h. Cells in F were immunostained with mitochondrial specific anti-COXII-IgG (G) before visualization using CLSM. Co-localization between GFP and mCherry (C) or anti-COXII-IgG (H) is observed by the yellow colour in the merged images. In the higher magnification images (D, I), filled arrows indicate co-localization whereas open arrows indicate lack of co-localization. E and J are the corresponding transmitted light images. Line and dashed scale bars represent 10 and 3 µm, respectively.
Figure 3.3. Dual Localization of \( \text{AtGLYR2} \) and \( \text{OsGLYR2} \) in tobacco BY-2 cells. Cells were bombarded with \( \text{AtGLYR2-GFP} \) (A) or \( \text{OsGLYR2-GFP} \) (F), left to express for 8 h then immunostained with mitochondrial anti-COXII-IgG (B and G) before visualization using CLSM. Co-localization between GFP and anti-COX-II is observed by the yellow colour in the merged images (C and H). In the higher magnification images (D and I), filled arrows indicate co-localization whereas open arrows indicate lack of co-localization. E and J are the corresponding transmitted light images. Line and dashed scale bars represent 10 and 3 \( \mu \text{m} \), respectively.
dicotyledonous (apple and Arabidopsis) plants appear to be dual localized to plastids and mitochondria in tobacco BY-2 cells. In order to exclude the possibility of artifacts derived from expressing non-native proteins in tobacco BY-2 cells, the localization of AtGLYR2-GFP was investigated using Arabidopsis protoplasts (Fig. 3.4). In all Arabidopsis protoplast cells, AtGLYR2-GFP localized to plastids and mitochondria (Fig. 3.4, C and D). Dual localization to mitochondria and plastids was also observed in all Arabidopsis protoplast cells expressing either MdGLYR2-GFP (Fig. 3.4, H and I) or OsGLYR2-GFP (Fig. 3.4, C and D).

To confirm the above results and to eliminate the possibility of localization artifacts from ectopic transient expression in a non-native system, we generated stably transformed Arabidopsis plants expressing methoxyfenozide-inducible AtGLYR2-GFP. Presence of the AtGLYR2-GFP transgene was confirmed via gene-specific PCR amplification, and qPCR-assessed expression of AtGLYR2 was observed to be four times higher in transgenic plants induced with methoxfenozone after 48 h and 72 h, than in WT and non-induced controls, which displayed similar AtGLYR2 expression (Appendix A, Supplementary Fig. S3.8). In the presence or absence of methoxyfenozide, non-specific GFP expression or Mitotracker staining was not observed in WT controls (Appendix A, Supplementary Fig. 3.9A, D and G). Moreover, no bleed-through between the blue, green and red channels using CLSM could be distinguished. In seedlings stably expressing AtGLYR2-GFP, GFP expression did not occur in the absence of methoxyfenozide induction (Appendix A, Supplementary Fig. S3.9, I). Upon induction, AtGLYR2-GFP displayed consistent dual localization to the plastid (Fig. 3.5, C and D) and mitochondrion (Fig. 3.5, F and G), corroborating the results found with transient
Figure 3.4. Dual Localization of AtGLYR2, MdGLYR2 and OsGLYR2 in Arabidopsis protoplasts. Protoplast cells were transfected with At (A), Md (F) or OsGLYR2-GFP (K), allowed to express overnight, and stained with mitochondrial specific Mitotracker (B, G, L) before visualization using CLSM. Co-localization between GFP and Mitotracker is observed by the yellow colour in the merged images (C, H, M). In the higher magnification images (D, I, N), filled arrows indicate co-localization whereas open arrows indicate lack of co-localization. E, J, O are the corresponding transmitted light images. Line and dashed scale bars represent 10 and 3 µm, respectively.
Figure 3.5. Dual localization of methoxyfenozide-inducible AtGLYR2-GFP in stably transformed Arabidopsis. Leaves of 14 d old Arabidopsis seedlings stably expressing AtGLYR2-GFP were imaged via CLSM after induction with methoxyfenozide (A). Panels B and E represent the corresponding chlorophyll autofluorescence and Mitotracker stain, respectively. Co-localization of GFP and chlorophyll is observed in cyan (C) and co-localization of GFP and Mitotracker is observed in yellow (F). In the higher magnification images (D and G), co-localization is indicated by a filled arrow, whereas lack of co-localization is indicated by an open arrow. H is the corresponding transmitted light image. Line and dashed scale bars represent 30 and 3 µm, respectively.
expression of AtGLYR2-GFP in tobacco BY-2 cells and Arabidopsis protoplasts.

3.6.3. Mitochondrial Localization of AtGLYR2 Using Percoll Gradient Centrifugation

To minimize the possibility of cytosolic AtGLYR1 contamination, we investigated the localization of AtGLYR2 in mitochondria purified from an atglyr1 mutant using Percoll gradient centrifugation. In crude Arabidopsis extracts, GLYR activity was approximately seven times higher in WT than in atglyr1 (Fig. 3.6A), indicating that AtGLYR1 is responsible for the majority of the cellular glyoxylate reductase activity. Moreover, qPCR expression analysis confirmed the virtual absence of AtGLYR1 transcript in the atglyr1 mutant compared with WT (Fig. 3.6B). These results indicate that the AtGLYR1 gene was successfully knocked down in the mutant and that the remaining GLYR activity (i.e., ~15% of the total) can be mostly attributed to AtGLYR2. During the process of mitochondria isolation, tissue homogenate, enriched total organelle pellet, and Percoll gradient-purified mitochondria from the atglyr1 mutant were sampled for enzymatic assays (Fig. 3.6C). Plastidial phosphoribulokinase had the highest activity in tissue homogenate, followed by mitochondrial fumarase and then GLYR. In the enriched total organelle pellet, only 1.7% of the phosphoribulokinase activity was recovered, whereas 18% and 24% of fumarase and GLYR activities, respectively, were recovered. In the Percoll-purified mitochondrial fraction, phosphoribulokinase activity was undetectable, confirming that the mitochondrial fraction was essentially free of plastid contamination and hence, plastidial GLYR2. In
**Figure 3.6.** Distribution of glyoxylate/succinic semialdehyde (GLYR) activity in rosette leaf tissue from Arabidopsis. Total GLYR activity (A) and GLYRI expression (B) in WT and atglyr1 knockout mutant. Data represent the mean ± SE of three biological replicates.

Distribution of phosphoribulokinase, fumarase and glyoxylate/succinic semialdehyde (GLYR) activity in enriched and Percoll-purified mitochondrial fractions from an atglyr1 knockout mutant (C). Data are derived from a typical preparation of 50-60 g of rosette leaf tissue. ND indicates not detected.
addition, 21% of the fumarase activity loaded onto the gradient was recovered, indicating that the fraction was indeed mitochondrial. Lastly, 1.1% of the GLYR activity loaded was recovered, indicating that approximately 5% of the GLYR2 activity loaded onto the gradient is mitochondrial, whereas the remaining activity is plastidial. Notably, the specific activities of both fumarase and GLYR2 were enriched in the purified mitochondrial fraction (i.e., approximately three to six times compared with total organelle pellet), indicating that AtGLYR2 was localized, at least in part, in mitochondria.

3.7. DISCUSSION

GLYRs straddle two different branches of plant metabolism: the GABA pathway and the photorespiratory pathway. We hypothesized that *in planta*, GLYRs are involved in reducing GABA-derived SSA and photorespiratory-derived glyoxylate into their corresponding less toxic alcohols, GHB and glycolate, respectively. Our kinetic analyses (Table 3.1) showed that GLYRs from a wide variety of plant species, including monocotyledonous rice as well as dicotyledonous apple and Arabidopsis, behaved very similarly with respect to their catalytic efficiencies, probably because of their conserved active site residues and NADPH-binding sequence (Appendix A, Supplementary Fig. S3.1). The affinities of GLYRs for glyoxylate and NADPH were in the micromolar range, whereas the affinity for SSA was in the millimolar range (Table 3.1); the values are similar to those reported previously for glyoxylate reductase activity purified from spinach leaves (Kleczkowski et al., 1995) and for recombinant AtGLYR activities (Hoover et al., 2007b; Simpson et al., 2008). Notably, evidence exists for glyoxylate and
SSA reduction in planta. For example, NADPH-dependent glyoxylate reductase activity has been reported for leaves of lettuce, millet, pea, red beet, sorghum, spinach and tomato (Kleczkowski et al., 1988); however, this activity could also be attributed, at least in part, to the three isoforms of the photorespiratory enzyme HPR, which can use both NADH and NADPH, as well as glyoxylate and hydroxypyruvate (Timm et al., 2008, 2011). In addition, SSA reductase activity has been reported throughout Arabidopsis plants, particularly in vegetative and reproductive organs (Hoover et al., 2007b). Together, these findings highlight the potential importance of both the glyoxylate and SSA reductase reactions in vivo. Moreover, our results suggested that recombinant OsGLYRs, like the AtGLYRs, use NADPH more efficiently than NADH, and MdGLYRs are inhibited by NADP+, as are the purified spinach GLYR (Kleczkowski et al., 1986, Kleczkowski 1995) and the recombinant AtGLYRs (Hoover et al., 2007b), suggesting that plant GLYR activity can be regulated in vivo by the NADPH/NADP+ ratio.

GLYRs appear to be located throughout the plant cell. Here, we used several in vivo transient ectopic expression systems including tobacco BY-2 cells and Arabidopsis protoplasts, as well as a stable and inducible in planta Arabidopsis expression system. In tobacco BY-2 cells, MdGLYR1 was cytosolic (Fig. 3.1), as was previously reported for AtGLYR1 in BY-2 cells (Simpson et al., 2008), Arabidopsis suspension cells and stably transformed Arabidopsis plants, despite the type 1 peroxisomal target signal-like tripeptide on the C-terminus of AtGLYR1 (Ching et al., 2012). In the case of GLYR2, a variety of in silico programs predicted non-unanimous combinations of chloroplast and/or mitochondrion localizations for At, Md and OsGLYR2s (Appendix A, Supplementary Fig. S3.1), highlighting the need for using multiple expression systems and different lines
of evidence in order to assess protein localization (Denecke et al., 2012; Millar et al., 2009). Empirical evidence clearly established that all the GLYR2-GFP fusions studied were dual targeted to the mitochondrion and chloroplast (Figs. 3.3-3.5), contrary to a previous report wherein AtGLYR2-GFP was exclusively plastidial (Simpson et al., 2008). Dual targeting of AtGLYR2-GFP in BY-2 cells could have been missed previously due to the use of fewer transformation events and expression systems (Simpson et al., 2008).

Recovery of AtGLYR2 activity in an enriched mitochondrial fraction of an atglyr1 mutant, free from cytosolic AtGLYR1 or plastidial AtGLYR2 contamination, provides in planta support for the dual targeting of AtGLYR2-GFP (Fig. 3.6). As previously mentioned, HPR can also reduce glyoxylate in vivo. AtHPR1, AtHPR2 and AtHPR3 are localized in the peroxisome, cytosol and chloroplast, respectively, where AtHPR1 prefers NADH and hydroxypyruvate over NADPH and glyoxylate, AtHPR2 prefers NADH and utilizes hydroxypyruvate and glyoxylate similarly, and AtHPR3 prefers NADPH and glyoxylate over NADH and hydroxypyruvate (Timm et al., 2008, 2011). Formal kinetic analyses have yet to be performed on recombinant HPR proteins; however, we can propose that plastidial HPR3 together with plastidial GLYR2 could support the optimum NADPH-based reduction of photorespiratory glyoxylate within the chloroplast (Hoover et al., 2013), whereas GLYR1 could be mostly responsible for NADPH-based glyoxylate reduction within the cytosol. To date, no evidence for a HPR isoform in the mitochondrion exists, suggesting that the observed glyoxylate reductase activity in Percoll-purified enriched mitochondria is attributable solely to mitochondrial AtGLYR2 (Fig. 3.6).
Earlier studies, using the photosynthesizing protist *Euglena gracilis*, reported the presence of mitochondrial NADPH-dependent GLYR activity in purified mitochondria, specifically localized in the intermembrane space, constituting approximately 2% of total mitochondrial protein (Yokota and Kitakoa, 1979; Yokota et al., 1985). The $K_m$ values for NADPH and glyoxylate are in the low micromolar range and the pH optimum is 6.45 (Yokota et al. 1985), which are similar to our findings with *At*, *Md* and *Os*GLYRs. The novel mitochondrial GLYR2 present in higher plants could therefore descend from early photosynthesizing unicellular organisms such as *E. gracilis*. Dual targeting of proteins to mitochondria and chloroplasts could be a consequence of alternative gene splicing, alternative transcription and translation initiation sites, as well as an ambiguous targeting signal that is recognized by the import machinery in both organelles (Carrie and Small, 2013). In the case of GLYR2, The Arabidopsis Information Resource (TAIR https://www.arabidopsis.org/) provides only one version of the *AtGLYR2* gene and no splice variants. Moreover, cloning of *At*, *Md* or *OsGLYRs* from total cDNA resulted in only two clear sequences corresponding to GLYR1 and GLYR2, indicating that GLYR2s are not alternatively spliced or transcribed. A careful look at the *At* and *MdGLYR2* sequences (Appendix A, Supplementary Fig. S3.1) revealed that the N-terminal starting methionine residue is closely followed by a conserved second methionine 16-18 amino acids downstream, which could be interpreted as two translation initiation sites resulting in two separate proteins. However, the second conserved methionine in the *OsGLYR2* sequence is only four amino acids downstream, which would likely not translate into two different proteins destined for separate organelles, but did result in dual targeting, like *At* and *OsGLYR2* (Figs. 3.3-3.5). With respect to an ambiguous dual targeting signal
peptide, no precise consensus yet exists as to the important amino acids involved (Carrie et al., 2009). For example, these sequences have been reported to be enriched in positively charged residues such as arginine, histidine and lysine and deficient in glycine and acidic residues such as aspartate and glutamate (Carrie et al., 2009), or enriched in phenylalanine, leucine and serine and deficient in glycine (Berglund et al., 2009). In general, GLYR2 sequences do appear to follow these guidelines (Appendix A, Supplementary Fig. S3.1) and therefore likely possess ambiguous targeting signals, resulting in their dual targeting to plastids and mitochondria.

Plant cytosolic GLYR1, as well as mitochondrial and plastidial GLYR2, could serve physiological roles in both GABA metabolism and photorespiration (Fig. 3.7). Under certain conditions of abiotic stress such as drought, heat and salinity, plant stomata close to avoid water loss, leading to reduced intracellular CO₂/O₂ ratio and resulting in elevated rates of Rubisco oxygenation and photorespiration, theoretically leading to an accretion of glyoxylate (Allan et al., 2009). A barley mutant deficient in glycine decarboxylase, the photorespiratory enzyme responsible for converting glyoxylate-derived glycine into serine, displays elevated GLYR activity relative to WT (Igamberdiev et al., 2001). Moreover, barley mutants in the nitrogen assimilation enzymes glutamine synthetase and ferredoxin-dependent glutamate synthase showed a negative correlation between glyoxylate levels and Rubisco activation (Hausler et al., 1996), indicating that photorespiratory-derived glyoxylate accumulation can be detrimental to photosynthesis. Arabidopsis plants exposed to photorespiratory stresses such as drought, heat and salinity, have elevated levels of GLYR expression, as well as NADPH/NADP⁺ ratio (Allan et al., 2008), suggesting that NADPH-dependent GLYR activity would be
Figure 3.7. Proposed roles for GLYRs in the stress response. GLYRs can reduce
glyoxylate and SSA in the cytosol, chloroplast and mitochondrion. SSA is an
intermediate in γ-aminobutyric acid (GABA) metabolism. GABA is formed in the cytosol
by glutamate decarboxylase (GAD), which is in turn converted to succinic semialdehyde
(SSA) by mitochondrial GABA-transaminase (GABA-T). SSA can either be converted to
succinate, to enter the tri-carboxylic acid cycle (TCA), by mitochondrial succinic
semialdehyde dehydrogenase (SSADH), or it can be converted to γ-hydroxybutyrate
(GHB) by mitochondrial, plastidial or cytosolic glyoxylate reductase 1 and/or 2 (GLYR1,
GLYR2). Glyoxylate is an intermediate in the photorespiratory cycle, and formed in the
peroxisome by glycolate oxidase (GOX). Glyoxylate can thereafter be reduced to
glycolate by GLYRs (modified from Shelp et al., 2012d).
stimulated. Also, the NADH/NAD$^+$ ratio increases, possibly due to elevated glycine decarboxylase activity (Allan et al., 2009). Under these conditions, cytosolic GLYR1 and newly identified mitochondrial GLYR2 are hypothesized to scavenge with high affinity the surplus of glyoxylate produced from photorespiratory stress that may escape from peroxisomes, with plastidial GLYR2 and HPR3 providing extra assurance before photosynthesis can be inhibited.

Under O$_2$ deficiency stresses such as hypoxia and submergence, the activity of photorespiratory O$_2$-dependent glycolate oxidase would likely be inhibited and thereby unable to generate glyoxylate (Narsai et al., 2009). With submergence, GABA, GHB and GLYR transcripts accumulate in Arabidopsis and tobacco leaves, together with an increasing NAD(P)H/NAD(P)$^+$ ratio, probably due to restriction of mitochondrial electron transport chain (Allan et al., 2008, 2012). Hence, under low O$_2$ stress GABA-derived SSA may be diverted from the production of the TCA cycle intermediate succinate via NAD$^+$-dependent SSADH, and instead be detoxified into GHB via the GLYRs (Fig. 3.7). SSA is produced following the transamination of GABA via mitochondrial GABA-T, and was originally thought to be exported into the cytosol for detoxification into GHB via GLYR1 (Allan et al., 2009). However, we can now hypothesize that the novel mitochondrial GLYR2 would reduce SSA directly following its synthesis within the organelle, thereby minimizing the escape of this toxic aldehyde from the mitochondrion. Thereafter, cytosolic GLYR1 and plastidial GLYR2 could provide additional capacity for reducing aldehydes throughout the rest of the plant cell, and producing GHB for a yet unknown purpose (Allan et al., 2009).
GHB production has also been observed with many other abiotic stresses such as salinity, drought, cold and heat, which would theoretically result in the accumulation of both glyoxylate and SSA (Allan et al., 2008). Despite differences in substrate affinities of GLYRs, recombinant AtGLYR1 supplied with glyoxylate and SSA levels near their respective $K_m$ values can produce both glycolate and GHB simultaneously (Allan et al., 2012), suggesting that there is potential for both substrates to compete for GLYR activity in vivo. GLYRs appear to be biochemically redundant, and could perform specialized cellular functions depending on the metabolic status of the cellular compartment in which they reside.
CHAPTER FOUR – DECIPHERING PATHWAYS FOR γ-AMINOBUTYRATE (GABA) METABOLISM IN ‘EMPIRE’ APPLE FRUIT STORED UNDER ELEVATED CO2 CONDITIONS

4.1. CONTRIBUTIONS

This chapter, which is in preparation for submission to *Plant, Cell and Environment*, derives from my primary research project and I had an integral role in planning and deciding the direction of the research. Adel Zarei conducted the AMADH, GAD1, GAD2 and GABAT1,2 transcript analyses, whereas Greta Chiu, Kristen Deyman and Jingyun Liu, respectively, conducted the GHB, polyamine and amino acid analyses in their thesis works. Christopher Trobacher cloned several of the previously unknown genes from apple, and Geoffrey Lum and Sanjeena Subedi provided guidance for the CP-PLS analysis. I performed the remaining metabolite and transcript analysis, and all statistical analysis presented here; this includes the levels of pyridine dinucleotides, succinate, and GLYR1, GLYR2, GAD3, SSADH1 and SSADH2 transcripts, as well as the CP-PLS statistical analysis. I also prepared most of the manuscript in conjunction with Barry Shelp and Gale Bozzo. Jennifer DeEll provided many helpful comments and materials during the research and editing of the manuscript. The apple PAO gene sequences have been submitted to GenBank as *MdPAO1, MdPAO2, MdPAO3, MdPAO4, MdPAO5* and *MdPAO6* (GenBank Acc. Nos. KT184496-KT184501).
4.2. ACKNOWLEDGEMENTS

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4.3. ABSTRACT

Apple (Malus × domestica Borkh.) quality deteriorates quickly under ambient environmental conditions. CA storage is commonly used to delay fruit ripening and
decay, but can result in physiological injury and the accumulation of GABA, a well-known marker of abiotic stress. In the present study, profiling of metabolites and transcripts associated with the production of GABA from glutamate and polyamines and with its subsequent catabolism was conducted to determine their relationship with CO₂-induced external injury. 'Empire' apples were stored with 0.03 or 5 kPa CO₂ and 2.5 kPa O₂ at two chilling temperatures (0 and 3°C) and sampled over a 16-week period. Fruit stored under elevated CO₂, particularly at 0°C, were more prone to external CO₂ injury, and the polyamines were more associated with this disorder than GABA. A CO₂ effect was apparent for metabolites, whereas their associated gene transcripts displayed both short-and long-term responses. A highly reduced redox status within intact apple fruit is proposed to favour GABA production from glutamate and its catabolism to GHB. These findings provide a relatively complete picture of gene-dependent and -independent processes involved in GABA accumulation and their association with physiological disorders during CA apple storage.

4.4. INTRODUCTION

Apple (Malus x domestica Borkh.) is the most important rosaceous fruit worldwide [Food and Agriculture Organization of the United Nations: Statistics (FAOSTAT 2012)]. In Canada, the farm gate value for the apple industry was approximately $158.9 million in 2010 (http://www.statcan.gc.ca/pub/21-011-x/2011002/t031-eng.htm) and about 40% of this was produced in the province of Ontario (http://www4.agr.gc.ca/IH5_Reports/faces/cognosSubmitter.jsp). CA storage (i.e., low temperature, low O₂ and elevated CO₂ partial pressures) is used to delay fruit ripening
and prolong the period in which locally-grown apples are available (http://www.omafra.gov.on.ca/english/crops/hort/news/orchnews/2012/on-0912a12.htm). However, CA storage may lead to the development of physiological disorders in a cultivar-dependent manner. For example, external CO\(_2\) injury, characterized by roughening of the skin, often partly sunken, with sharply defined edges (Blanpied et al., 1990), is linked with CO\(_2\) partial pressures above 2 kPa; in ‘Empire’ apples, it is more pronounced at 1°C than 3°C and exacerbated by O\(_2\) partial pressures below 2 kPa (Burmeister and Dilley, 1995). High CO\(_2\) and low O\(_2\) levels also promote CO\(_2\) injury in ‘Empire’ apple fruit stored at 5 kPa CO\(_2\), 2 kPa O\(_2\) and 2°C, compared to apples stored at 2 kPa CO\(_2\) (Watkins et al., 1997). Another study showed that CO\(_2\) injury is correlated with CO\(_2\) partial pressures from 0 to 5 kPa CO\(_2\), and either unaffected or worsened with increasing storage temperatures (0.5-3°C) in an orchard-dependent manner (Watkins and Liu, 2010). Flesh browning, an internal chilling injury, characterized by firm and moist necrotic regions, is also exacerbated at higher CO\(_2\) conditions (5 kPa vs 2 kPa) and more apparent at 0.5°C than at 3°C, although results appear to be orchard-dependent (Watkins and Liu, 2010). Other research indicates that external CO\(_2\) injury is markedly higher in ‘Empire’ apple fruit stored with 5 kPa CO\(_2\) than with 0.03 kPa CO\(_2\) at both 3°C and 0°C; however, there appears to be little effect on internal flesh browning (Deyman et al., 2014b).

Mechanisms underlying physiological injury in apple fruits during CA storage are uncertain. However, TCA cycle constituents (i.e., malate, succinate) and the non-protein amino acid GABA have been shown to accumulate in the browning flesh of CA-stored pear fruit (3 kPa O\(_2\), 1 kPa CO\(_2\), 1°C) (Pedreschi et al., 2009). Moreover, the GABA level
in tomato fruit is greater at 30°C under CA conditions (11 kPa O₂, 9 kPa CO₂) than at 15°C in air (Makino et al., 2008), and at 13°C under 10 kPa CO₂ than in air, before returning to baseline levels upon transfer to air (Deewatthanawong et al., 2010b). GABA accumulates in cherimoya, strawberry and apple fruits exposed to elevated CO₂, and decreases upon transfer of the fruit to air (Merodio et al., 1998; Deewatthanawong and Watkins 2010; Deewatthanawong et al., 2010a, b; Lee et al., 2012; Trobacher et al., 2013a; Lum 2014). Multiple metabolites, including GABA, have been associated with the development of flesh browning symptoms in ‘Empire’ and ‘Honeycrisp’ apples (Lee et al., 2012; Lum, 2014; Chiu et al., 2015), and CA-related injury in ‘Honeycrisp’ apples (Chiu et al., 2015). However, there has been little research on mechanisms responsible for external CO₂ injury.

It is well known that GABA may be derived from glutamate in plants exposed to various abiotic stresses via activity of the enzyme GAD, which can be activated by Ca²⁺/CaM or stimulated by cytosolic acidification (Shelp et al., 2012a, c). In turn, GABA is converted to SSA via GABA-T and then to succinate via NAD⁺-dependent SSADH or to GHB via NADPH-dependent GLYR. Much less attention has been paid to the derivation of GABA from polyamines (Shelp et al., 2012b; Zarei et al., 2014, 2015). This can occur by the terminal catabolism of putrescine or spermidine to 4-aminobutanal via O₂-dependent Cu-containing AOs (Zarei et al., 2014) or the non-enzymatic decarboxylation of proline to pyrrolidin-1-yl, which is easily converted to Δ¹-pyrole/4-aminobutanal (Signorelli et al., 2015). In turn, 4-aminobutanal can be converted to GABA via NAD⁺-dependent AMADH (Zarei et al., 2015). There is considerable evidence to suggest that O₂ availability is limited in bulky fruit, which may in turn
influence redox balance and the activities of AO, AMADH, SSADH and GLYR (Allan et al., 2008, 2012; Ho et al., 2011, 2013; Shelp et al., 2012b). If so, CA storage of fruit with elevated CO₂ could further limit the contribution of polyamines to GABA production and divert GABA carbon to GHB, rather than succinate (Shelp et al., 2012b).

Mutants of the routes for GABA metabolism are not readily available for apple, and radiolabelled precursors cannot be supplied to intact apple fruit without perturbing the internal gaseous environment. Recently, Gapper et al. (2013) used RNAseq to explore the transcriptome of the ‘Empire’ apple peel during the development of external CO₂ injury, but there was little discussion of the potential mechanisms involved. In the present study, we utilized frozen powders and cDNA previously prepared from apples collected in a 2009 CA study to monitor GABA-related metabolites and gene transcripts during the 16-week storage period (Deyman et al., 2014b). The apples had been stored at 0.03 kPa CO₂ (ambient) or 5 kPa CO₂ (elevated), which is greater than is typically used for standard CA storage (2.0 kPa CO₂) of Ontario-grown ‘Empire’ apple fruit, and there was evidence of considerable external CO₂ injury, but little internal injury. Intact fruit were analyzed to circumvent the potential influence of mechanical stress during peel removal (Chehab, 2009; Benikhlef et al., 2013; Trobacher et al., 2013a). Real-time polymerase chain reaction (i.e., quantitative PCR) enabled us to focus on GABA-related transcripts only. The objectives of this study were to investigate: (i) the impact of elevated CO₂ on the relative importance of glutamate and polyamines in the biosynthesis of GABA; (ii) the impact of elevated CO₂ on the production of succinate and GHB; and (iii) the relationship between CO₂-induced external injury and GABA metabolism.
4.5. MATERIALS AND METHODS

4.5.1. Controlled Atmosphere Storage of Apple Fruit

The harvest and CA storage in 2009 of apple (*Malus × domestica* Borkh cv. Empire) fruit have been described elsewhere (Deyman et al., 2014b). Briefly, the storage facility consisted of four separate temperature-controlled rooms, each containing four portable chambers supplied with unique atmospheric gas concentrations. A split plot experimental design consisting of four CA treatment replicates was employed to minimize the possibility of chamber effects. Briefly, two random rooms were set at 3 or 0°C; within each room, two random chambers were supplied with 2.5 kPa O\(_2\) and either 5 or 0.03 kPa CO\(_2\). Four apples were randomly collected from each treatment replicate after 2, 4, 8 and 16 weeks, frozen as quickly as is practicable in liquid N\(_2\), and then individually pulverized and treated as subsamples. Eight apples were also collected for assessment of storage-related disorders as described previously (Deyman et al., 2014b).

4.5.2. Extraction and Analysis of Metabolites

Extraction of whole frozen fruit and determination of levels of various amino acids, including GABA, and the free forms of putrescine, spermidine and spermine in each treatment replicate were determined by reverse-phase high performance liquid chromatography as described elsewhere (Allan and Shelp, 2006; Liu, 2011; Deyman et al., 2014a). Also, methods for extraction and determination of GHB in each treatment replicate by gas chromatography coupled to tandem mass spectrometry were described previously (Chiu, 2013).
Succinate was extracted from each treatment replicate by homogenizing 100 mg of apple tissue, comprised of 25 mg from each of the replicate subsamples, in 500 µL of ice cold 1 M perchloric acid. The extract was adjusted to pH 8 with 4 M KOH, and the final volume was made up to 1 mL. Then, 0.03 g mL\(^{-1}\) of polyvinylpyrrolidone (PVPP) (Sigma) was added and the solution incubated for 20 min, shaking on ice. Samples were stored on ice for an additional 20 min to allow precipitation of KClO\(_4\), then centrifuged at 15,000 x g for 15 min at 4°C. The clarified supernatant was used in an endpoint enzyme-linked cuvette assay (K-SUCC, Megazyme International Ireland, Wicklow, Ireland) that was adapted for a microplate reader (SpectraMax Plus384, Sunnyvale, CA). A linear standard curve ranging from 0 to 115 nmol succinate was constructed using internal standards submitted to the extraction process in apple tissue background. In order of addition into a 96-well plate, each reaction included 100 µL of clarified extract or standard, 86 µL ddH\(_2\)O, 20 µL of solution one (buffer), 20 µL of solution two (NADH), 20 µL of solution three (ATP, phosphoenolpyruvate, coenzyme A), and 2 µL of suspension four (pyruvate kinase, L-lactate dehydrogenase). This mixture was mixed and the initial absorbance determined at 340 nm. The reaction was initiated by mixing in 2 µL of suspension five (succinyl-coA synthetase), giving a final volume of 250 µL. The final absorbance was determined 30 min after initiation. A blank obtained with a non-initiated assay was subtracted from the difference between initial and final absorbance, and the result was compared with the internal standard curve to quantify the amount of succinate.

Pyridine dinucleotides were extracted and assayed essentially as reported by Queval and Noctor (2007), with minor modifications. Samples consisted of 100 mg of frozen pulverized apple tissue comprised of 25 mg from four fruit of one treatment.
replicate, where data represent the average of four treatment replicates ± SE. NAD(P)^+ and NAD(P)H were extracted in 1 mL ice cold 0.2 N HCl or NaOH, respectively, containing 0.03 mg mL\(^{-1}\) PVPP, then shaken on ice for 20 min before being processed. Acidic and basic samples were neutralized with 1 M NaOH and 2 N HCl, respectively, to a final pH of 7, and the final extract volume was made up to 1 mL. NAD(P)^+ assays included 100 µL of extract and were initiated with a master mix containing 45 µL of 0.1 M HEPES + 2 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.5), whereas NAD(P)H assays containing 10 µL of extract were initiated with a master mix containing 130 µL of 0.1 M HEPES + 2 mM EDTA (pH 7.5), as well as the other assay reagents. The linear rate was recorded as the decrease in A\(_{600}\) within the first minute. Pyridine dinucleotides were quantified with an internal standard curve, and values were corrected for the dilution factor, as well as the recovery of a spiked standard, and subtracted from a blank consisting of buffer in place of extract.

4.5.3. RNA Extraction and cDNA Synthesis

RNA was isolated from the frozen apple fruit powder, essentially according to Gasic et al. (2004), with some modifications. Briefly, 15 mL of pre-warmed extraction buffer (60°C, 2% cetyltrimethylammonium bromide (CTAB) 2% polyvinylpyrrolidone K-30, 100 mM Tris HCl (pH 8), 25 mM EDTA, 2.0 M NaCl, 0.5 g L\(^{-1}\) spermidine (free acid), 2% 2-mercaptoethanol (added just before use) was added to 1.5 g of apple powder and incubated for 15 min in a 60°C water bath, with intermittent vortexing. An equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added and the mixture was agitated for 5 min and then centrifuged at 10,000 x g for 15 min at 4°C. The upper aqueous phase
was transferred to a clean tube and mixed with another volume of chloroform:isoamyl alcohol and then agitated and centrifuged. The RNA in the aqueous phase was precipitated by adding 8 M LiCl to a final concentration of 2 M and stored overnight at 4°C. Tubes containing RNA were centrifuged at 12,000 x g for 40 min at 4°C, then the supernatant was discarded and the pellet washed with 70% ethanol and dried by speedvac (SVC 100H, Savant). The RNA was dissolved in 30 µL of autoclaved double distilled water. RNA integrity was verified using formaldehyde RNA gel electrophoresis. RNA (10 µg) was treated with DNAase I using the Turbo DNA-free kit (Applied Biosystems) according to the manufacturer’s protocol. For first strand cDNA synthesis, 10-100 ng total RNA was incubated with oligo(dT)20 and Superscript III RT (Invitrogen) at 50°C, followed by 55°C for 30 min.

4.5.4. Quantitative Real Time-PCR

Primers used for qPCR were designed using Primer Express 3 software (Applied Biosystems) with the following default conditions: 60°C primer melting temperature; 50-80 bp amplicon length; and, 40-60% primer GC content. The list of primers used here is provided in Appendix B, Supplementary Table S4.1. We were not able to design primers that enabled separate monitoring of the distinct apple GABA-Ts (Trobacher et al., 2013a). Quantitative PCR was performed in a 96 well plate iQ5 Multicolor Real-Time PCR Detection System (BioRad) as previously described (Zarei et al., 2015). Dissociation curve analysis was performed after 40 cycles of qPCR. Efficiency of the primer pairs ranged from 90 to 105%. Data were analyzed and relative expression calculated using the 2^{-ΔCT} method (Livak and Schmittigen, 2001). The expression of each
target gene was normalized to the housekeeping apple \textit{EF-1}\textalpha\ gene (MD0000294265) (Poupard et al., 2003; Czechowski et al., 2005). Each treatment replicate was analyzed in duplicate.

4.5.5. Identification of Apple Genes

The apple genes for three \textit{GAD}s, two \textit{GABA-T}s, two \textit{GLYR}s, five Cu-containing \textit{AO}s and two \textit{AMADH}s have been described elsewhere (Trobacher et al., 2013a, 2013b; Zarei et al., 2014, 2015; see Chapter Three). Putative apple \textit{SSADH1} and \textit{SSADH2} genes were identified using a BLAST search of the Arabidopsis \textit{SSADH} sequence (GenBank Acc No. NM_106592) against the apple genome (www.rosaceae.org). Two sequences were highly similar to the Arabidopsis sequence: MDP0000147030 and MDP0000229588, designated as \textit{MdSSADH1} and \textit{MdSSADH2}, and encode for 538 and 613 amino acid protein, respectively. The \textit{SSADH1} proteins from Arabidopsis and apple are 87% identical, whereas the \textit{SSADH2} proteins are 79% identical; \textit{MdSSADH1} and \textit{MdSSADH2} are 92% identical to each other.

The 543 amino acid sequence of \textit{Arabidopsis thaliana} alanine aminotransferase 1 (\textit{AtAlaAT1}; GenBank Acc. No. AF275372) was used as a query for a BLAST search of GenBank for similar predicted peptides. Similar proteins including \textit{AtAlaAT2}, alanine aminotransferases from barley (GenBank Acc. No. CAA81231.1) and corn (GenBank Acc. No. AAC62456.1), and predicted peptides from grape (GenBank Acc. No.XP_002265294.1) and poplar (GenBank Acc. No. XP_002304255.1) were used to design degenerate primers CT-F27 and CT-R27 that amplified a 485 bp fragment. A translation of the fragment revealed that it was 79 to 89% identical to the sequences used
to design the degenerate primers. The sequence was used to design primers for 5’ and 3’ for RACE. The primers CT-F30 and CT-F31 were used for 5’ RACE and nested PCR, respectively; CT-R30 and CT-R31 were used for 3’ RACE and nested PCR, respectively. Sequences obtained from the RACE reactions were combined to assemble a contig representing an apple alanine aminotransferase (MdAlaAT). The translated MdAlaAT is 490 amino acids in length and 77 to 90% identical to the sequences used to design the degenerate primers. MdAlaAT is 97% identical to the last 507 amino acids of a 560 amino acid predicted peptide from the apple genome, MDP0000168683. The MdAlaAT sequence was used to design primers for qPCR.

4.5.6. Statistical Analysis

Time course data for physiological injury and levels of metabolites or transcripts are represented as means of three to four storage replicates ± standard error. Assumptions of randomness, homogeneity, normality, and independence of errors were confirmed with plots of residuals using the Proc Mixed method in SAS 9.3 (SAS Institute Inc., Cary, NC).

The relationship between physiological injury and metabolites or transcripts was analyzed using CP-PLS. All CP-PLS analyses were performed using R (R Core Team 2013) with the pls package (Mevik et al., 2012). PLS regression analysis relates the variation in the response variables (Y-variables) to the variation in the predictor variables (X-variables) (Pérez-Enciso and Tenenhaus, 2003). This is achieved by extracting latent variables (LVs) from the predictor variables, in order to explain as much possible covariance between the X- and Y-variables. The first and second LVs are most relevant
for the prediction of the responses. CP-PLS is an extension of PLS and canonical correlation analysis (Indahl et al., 2009). The advantage of CP-PLS for interpretation of results is the model incorporates experimental treatments and factors, in addition to predictor and response variables. Moreover, CP-PLS yields optimal components comprised of both continuous and categorical variables (Indahl et al., 2009). For these studies, metabolites or transcripts were considered as the predictor variables, whereas the experimental factors, storage duration and physiological disorders were considered response variables. The experimental factors included CO₂ (0.03 and 5 kPa), O₂ (2.5 kPa) and temperature (0 and 3°C) treatments. These experimental factors were incorporated as categorical variables, whereas metabolites or transcripts, storage duration and physiological disorders were incorporated as continuous variables. Scaling of all metabolites or transcripts, storage duration and physiological disorder data to unit was conducted to enable equal chance for all variables to influence the model. The percentage data generated from assessment of physiological injury were log-transformed prior to analysis.

4.6. RESULTS

4.6.1. Physiological Injury in CA-stored Apple Fruit

Previously, we reported information on the quality traits of ‘Empire’ apple fruit stored at two CO₂ levels (5 or 0.03 kPa CO₂), 2.5 kPa O₂, and two chilling temperatures (3 or 0 °C) using apples harvested from two separate orchards in 2009 (Deyman et al., 2014b). Since differences between the two orchards were small, we chose the apple fruit from orchard one only for determination of the levels of metabolites and gene transcripts
associated with GABA metabolism. Here, the data for the incidence of physiological injury are reproduced. The percent incidence of internal flesh browning was minor and did not exceed 22% over all treatments (Fig. 4.1A). The percent incidence of external CO₂ injury was stimulated by 5 kPa CO₂ and increased steadily with storage time regardless of temperature, from 30-40% at week 2 to 55-80% at week 8 (Fig. 4.1B), compared with 0-30% maximum incidence in the low CO₂ treatment. The early appearance and high incidence of external CO₂ injury are consistent with results reported previously (Fawbush et al., 2008).

4.6.2. Levels and Ratios of Pyridine Dinucleotides in CA-stored Apple Fruit and Rosette Leaves of Arabidopsis

The major non-phosphorylated and phosphorylated pyridine dinucleotides in both freshly harvested and CA-stored ‘Empire’ fruit were the reduced forms (Fig. 4.2). The concentrations of NAD⁺, NADH and NADP⁺ in CA-stored apple fruit generally decreased with storage time, whereas NADPH increased, resulting in lower levels of NAD(H) and higher levels of NADP(H). Overall, there was an approximately 60% decrease in the concentrations of total pyridine dinucleotides, a 50% decrease in the NADH/NAD⁺ ratio, and a 2-fold increase in NADPH/NADP⁺ ratio. Notably, the pyridine dinucleotide ratios ranged from 20-70 for NADH/NAD⁺ and 60-150 for NADPH/NADP⁺, and CO₂-specific effects were not evident. To validate the method, we determined the concentrations of pyridine dinucleotides in photosynthesizing leaves from Arabidopsis plants grown under non-stress conditions (Appendix B, Supplementary Fig.)
Figure 4.1. Impact of elevated CO$_2$ at two chilling temperatures on the incidence of internal flesh browning (A) and external CO$_2$ injury (B) in ‘Empire’ apple fruit during CA storage. Storage conditions: 0°C, 5 kPa CO$_2$ (●); 0°C, 0.03 kPa CO$_2$ (○); 3°C, 5 kPa CO$_2$ (▼); 3°C, 0.03 kPa CO$_2$ (Δ). All data represent the mean of four treatment replicates. (Raw data were taken from Deyman et al., 2014b.)
Figure 4.2. Impact of elevated CO₂ at two chilling temperatures on the pyridine
dinucleotide status in ‘Empire’ apple fruit during CA storage. A-G, Concentrations of
various components. H-I, Ratios of key components. Storage conditions: 0°C, 5 kPa CO₂
(●); 0°C, 0.03 kPa CO₂ (○); 3°C, 5 kPa CO₂ (▼); 3°C, 0.03 kPa CO₂ (Δ). All data
represent the mean of four treatment replicates.
S4.1). These data are in close agreement with values reported by Queval and Noctor (2007). Thus, it can be concluded that the internal environment of the apple fruit is already reduced at the beginning of the storage experiment and it becomes increasingly so with storage time; however, there does not appear to be a specific response to elevated CO₂.

4.6.3. Levels of GABA and Closely Related Metabolites and Gene Transcripts in CA-Stored Apple Fruit

The major amino acids in both freshly harvested and stored ‘Empire’ apple fruit were aspartate, asparagine and glutamate (Appendix B, Supplementary Table S4.3). However, the concentrations of total amino acids (TAA), GABA and GABA-related amino acids (i.e., glutamate and alanine) were initially increased by CA storage, and 5 kPa CO₂ at 0°C tended to generate higher GABA and alanine levels than the corresponding value at 0.03 kPa CO₂ (Fig. 4.3, A-D). Thereafter, the concentrations of these metabolites generally declined to their original levels, although GABA and to a lesser extent glutamate increased once again with 5 kPa CO₂ at 0°C. There was a distinct increase in the initial response of succinate with 5 kPa only at both 0 and 3°C, and this was followed by a decline to original levels (Fig. 4.3F). Likewise, the concentrations of GHB showed a rapid increase and tended to be higher with elevated CO₂ (Fig. 4.3E). Notably, this increase was even more dramatic and rapid than that for GABA. The concentration of GHB at week 16 under 5 kPa CO₂ and 0 °C was eight times the initial GHB concentration and markedly higher than the corresponding value at 3°C. These findings reflect the general nature of the GABA and GHB responses to CA storage, as well as the
Figure 4.3. Impact of elevated CO$_2$ at two chilling temperatures on the levels of GABA and related metabolites in ‘Empire’ apple fruit during CA storage. Storage conditions: 0 °C, 5 kPa CO$_2$ (●); 0°C, 0.03 kPa CO$_2$ (○); 3°C, 5 kPa CO$_2$ (▼); 3°C, 0.03 kPa CO$_2$ (Δ). All data represent the mean of four treatment replicates. TAA represents total amino acids. (Raw data for amino acids and GHB were taken from Liu (2011) and Chiu (2013), respectively).
existence of distinct CO$_2$ responses early and late in the storage period. It was predicted that closely related polyamines and gene transcripts would show similar patterns over time, thereby revealing metabolite/metabolite or metabolite/transcript relationships that would account for these CO$_2$-induced responses.

The expression of alanine transaminase (Ala-T), which is generally considered to be an indicator of O$_2$ deficiency (Good and Crosby, 1989; Muench and Good, 1994), was induced within 2 weeks of storage, and there was little difference among treatments (Fig. 4.4I). Thereafter, there was a general decline, but this was slowest with elevated CO$_2$ at 0°C. The MdGAD1 transcript was highly abundant, tended to respond more slowly to 5 kPa CO$_2$ than 0.03 kPa CO$_2$, and peaked at about 8 weeks (Fig. 4.4A). The MdGAD2 transcript was moderately abundant, responded less quickly to 5 kPa CO$_2$ at 0°C than at 3°C, and peaked at 2-8 weeks depending upon the CO$_2$ response (Fig. 4.4B). The level of the low abundance MdGAD3 transcript was essentially unresponsive to CO$_2$ and declined with storage time (Fig.4.4C). MdGABA-T1,2 (Fig. 4.4D) and MdSSADH1 (Fig 4.4G) transcripts were moderately abundant and displayed similar CO$_2$ responses and patterns as GAD1, whereas the MdSSADH 2 (Fig. 4.4H) transcript was much less abundant and rapidly declined in a CO$_2$-independent manner with storage time. The transcripts for MdGLYR1 and MdGLYR2 were moderately abundant and spiked transiently, albeit only to a slight degree, immediately following the spike in MdGAD2 transcript abundance (Fig. 4.4, E and F). Similarities and differences in expression patterns could suggest that: (i) MdGAD2 and MdGAD1, respectively, are associated, at least in part, with the short- and long-term accumulation of GABA that occurs in response to CA storage; (ii) MdGABA-T1,2 and MdSSADH1 are linked to the long-term accumulation; and (iii)
Figure 4.4. Impact of elevated CO$_2$ at two chilling temperatures on the expression of genes associated with the biosynthesis and catabolism of GABA from glutamate in ‘Empire’ apple fruit during CA storage. Storage conditions: 0°C, 5 kPa CO$_2$ (●); 0°C, 0.03 kPa CO$_2$ (○); 3°C, 5 kPa CO$_2$ (▼); 3°C, 0.03 kPa CO$_2$ (Δ). All data represent the mean of three to four treatment replicates. Abbreviations: Ala-T, alanine transaminase; GABA-T, GABA transaminase; GAD, glutamate decarboxylase; GLYR, glyoxylate/succinic semialdehyde reductase; SSADH, succinic semialdehyde dehydrogenase.
MdSSADH2, MdGLYR1 and MdGLYR2 are linked to short-term GABA accumulation. However, none of the gene transcripts monitored here displayed a CO2-induced response that could be linked directly to the distinct peaks in GABA levels.

4.6.4. Levels of Polyamines and Expression of Genes Associated with their Catabolism to GABA in CA-stored Apple Fruit

The major polyamines in both freshly harvested and stored ‘Empire’ apple fruit were putrescine and spermidine, with minor concentrations of spermine (Fig. 4.5). The concentrations of putrescine were transiently increased after 2-4 weeks with 5 kPa CO2 at both 0 and 3°C (Fig. 4.5A), whereas the concentrations of both spermidine and spermine were transiently increased by 5 kPa CO2 at 0 °C only (Fig. 4.5, B and C). In general, there was little, if any evidence for increasing polyamines with 0.03 kPa over the time course, although a temperature effect was occasionally evident and spermine did decrease during late storage. Overall, the total polyamines accumulated significantly only during early storage with 5 kPa CO2 at 0°C, then decreased to control levels by week 8 of storage (Fig. 4.5D). These findings indicate that specific polyamines increased only during the early part of the storage period and this was a CO2-specific response that might be associated with short-term GABA and polyamine accumulation.

The MdAO2 transcript was the most abundant of the five MdAO genes; it responded similarly to 5 and 0.03 kPa CO2, and peaked late in the storage period (Fig. 4.6B). The MdAO1 transcript was moderately abundant, responded slightly more strongly to 5 kPa CO2 at 0°C than at 3°C, and peaked at 2-4 weeks (Fig. 4.6A). The MdAO3-5
Figure 4.5. Impact of elevated CO₂ at two chilling temperatures on the polyamine levels in ‘Empire’ apple fruit during CA storage. Storage conditions: 0°C, 5 kPa CO₂ (●); 0°C, 0.03 kPa CO₂ (○); 3°C, 5 kPa CO₂ (▼); 3°C, 0.03 kPa CO₂ (Δ). All data represent the mean of four treatment replicates. (Raw data were taken from Deyman 2014.)
Figure 4.6. Impact of elevated CO$_2$ at two chilling temperatures on the expression of genes associated with the catabolism of polyamines to GABA in ‘Empire’ apple fruit during CA storage. Storage conditions: 0°C, 5 kPa CO$_2$ (●); 0°C, 0.03 kPa CO$_2$ (○); 3°C, 5 kPa CO$_2$ (▼); 3°C, 0.03 kPa CO$_2$ (Δ). All data represent the mean of three to four treatment replicates. Abbreviations: AO, amine oxidases; AMADH, aminoaldehyde dehydrogenase.
transcripts were present in low abundance and peaked transiently at 4-8 weeks depending upon the CO$_2$ and temperature response; *MdAO4* and *MdAO5* transcripts showed CO$_2$-dependent stimulation at both temperatures during late storage (Fig. 4.6, C-E). The *MdAMADH* transcripts were moderately abundant and tended to peak by 2-4 weeks, although a response to elevated CO$_2$ at 0$^\circ$C tended to be delayed (Fig. 4.6, F and G). The expression patterns alone suggest that: (i) *MdAO1* and *MdAO2*, respectively, are associated, at least in part, with the short- and long-term accumulation of GABA that occurs in response to CA storage; and, (ii) *MdAMADH1* and *MdAMADH2* are linked to the long-term accumulation. None of the gene transcripts monitored here displayed a CO$_2$-induced response which could be linked directly to the overall pattern in GABA levels. Subsequently, a correlation analysis was utilized to more closely examine metabolite- transcript, metabolite-metabolite, and physiological injury-metabolite relationships during CA storage with elevated CO$_2$.

4.6.5. Correlation Analyses of Physiological Injury and Metabolite or Gene Transcript Levels

The data set was analyzed by CP-PLS to simultaneously fit a single model that represents the variation for all responses to the variation of the factors (Fig. 4.7). CP-PLS scores demonstrate the similarities or dissimilarities of each treatment with respect to fruit metabolites or transcripts; scores that are clustered closer together are considered to be more metabolically similar than those that are more spatially distributed. Score plots revealed a shift in GABA-related metabolite or transcripts levels of the whole apple fruit with storage time, away from the fresh harvest control and from each other (Fig. 4.7A).
Figure 4.7. CP-PLS score and loading plots of GABA pathway metabolites and transcripts in ‘Empire’ apple fruit stored under two CO$_2$ levels at two chilling temperatures for up to 16 weeks. Score plots A-C: fresh harvest control (*) and all variables at 2 (red), 4 (green), 8 (blue) and 16 (pink) weeks of storage. A: all variables under all treatments. B: all variables at 0°C, comparing 0.03 kPa CO$_2$ (open squares) and 5 kPa (filled squares) CO$_2$. C: all variables at 3°C, comparing 0.03 (open triangles) and 5 kPa (filled triangles) CO$_2$. D represents a correlation loading plot of all response (experimental factors: grey; disorders and storage: red) and predictor (metabolites: black; transcripts: blue) variables.
The divergence of metabolites and transcripts was already evident within 2 weeks at 5 kPa CO$_2$. Moreover, a CO$_2$ effect was observed at both 0 and 3°C for most variables, where scores for 5 and 0.03 kPa CO$_2$ tended to cluster separately (Fig. 4.7, B and C). On the other hand, loading plots reveal the % variance explained by one or more components of the model, where points or orthogonal intersection points further from the origin represent a higher percentage of explained variance. The first two components of the model indicate that the internal disorder response variable was not well explained (11%), whereas the external disorder (51%) and storage time (77%) response variables appeared to be much better explained (Appendix B, Supplementary Table S4.4).

In order to correlate response variables to predictors, an imaginary line is drawn from the response through the origin. Predictors are perpendicularly projected onto this line, and intersecting points that are on the same side of the origin as the response are considered to be positively correlated, whereas intersecting points that are on the opposite side of the origin are considered to be negatively correlated. Variables that are clustered together are considered to be positively correlated. Based on metabolites, 5 kPa CO$_2$ was highly positively correlated to external disorder and putrescine, weakly positively correlated to spermine, succinate, GHB, internal disorder and GABA, and weakly negatively correlated to TAA and NADPH/NADP$^+$ (Fig. 4.7D). Based on transcripts, 5 kPa CO$_2$ was most highly correlated to $MdGAD2$, followed by $MdAla-T$ and $MdAO1$, and negatively correlated to $MdGAD1$, $MdGABA-T$, $MdGLYR2$, $MdGLYR1$ and $MdSSADH2$. GABA itself was weakly correlated to putrescine, spermine, succinate, alanine and total polyamines, $MdGAD2$ and $MdAO1$, and negatively correlated to NADPH/NADP$^+$, $MdGAD1$, $MdGABA-T$, $MdGLYR2$, $MdAMADH2$ and $MdAMADH1$. GHB was positively
correlated to putrescine, \( M_d GAD2 \), spermine, \( M_d AO2 \) and succinate, and weakly negatively correlated to \( M_d GLYR2 \), \( M_d GLYR1 \) and \( M_d SSADH2 \).

### 4.7. DISCUSSION

#### 4.7.1. GABA and CO\(_2\)-induced Physiological Injury During CA Storage

GABA is a ubiquitous, non-proteinaceous four-carbon amino acid that functions in metabolism and cell signaling. In higher plants, it has been linked to central carbon and nitrogen metabolism (Bown and Shelp, 1997; Shelp et al., 1999; Zhang et al., 2011; Araújo et al., 2012; Renault et al., 2013), osmoregulation (Shelp et al., 1999), development (Renault et al., 2011; Toyokura et al., 2011; Koike et al., 2013; Molina-Rueda et al., 2015), defence (Bown et al., 2006) and communication within and among plants and other organisms (Bouché et al., 2003b; Shelp et al., 2006). GABA is known to accumulate in plants and plant parts, including fruits, subjected to various biotic and abiotic stresses such as mechanical damage, hypoxia, chilling and elevated CO\(_2\) (Shelp et al., 1999, 2009, 2012c), and may serve as a biomarker for various physiological injuries in pome fruit, including CO\(_2\)-induced flesh disorders in apple (Pedreschi et al., 2009; Lee et al., 2012; Lum, 2014; Chiu et al., 2015; Leisso et al., 2015). Recently, Gapper et al. (2013) selected 35 gene candidates in ‘Empire’ apple peel based on up-regulation with the ethylene binding inhibitor 1-MCP, and either repression or no change with the antioxidant diphenylamine. Unfortunately, few predictive biomarkers were identified for the development of external CO\(_2\) injury, given the greater influence of storage duration on the transcriptome than either 1-MCP or diphenylamine, and no hypotheses were proposed for how candidates might be involved.
In the present study, we presented profiles for GABA-related metabolites and transcripts from intact ‘Empire’ apple fruit stored under hypoxic CA conditions that allowed the development of external CO₂ injury at two chilling temperatures, while limiting internal injury (Fig. 4.1; Deyman et al., 2014b). The low O₂ status of freshly harvested or stored fruit was established from the much higher ratio of reduced to oxidized pyridine dinucleotides (Fig. 4.2) than in photosynthesizing unstressed Arabidopsis leaves (Appendix B, Supplementary Table Fig. S4.1), and the induction and maintenance of MdAla-T expression during storage (Fig. 4.4). Notably, NADH/NAD⁺ slightly declined over the storage period, whereas NADPH/NADP⁺ increased; however, clear trends could not be discerned among the CA conditions. Hypoxia/anoxia and CA storage in particular have been shown to influence redox and energy status with the transition from aerobic to anaerobic (i.e., respiration to fermentation) metabolism within bulky fruit and tubers (Sachs et al., 1980; Geigenberger, 2003; Ho et al., 2011, 2013).

Previous studies have shown that submergence-induced low O₂ stress alters the NAD(P)H/NAD(P)⁺ ratios: tobacco leaf NADPH/NADP⁺ increases from 2 to 3 (Allan et al., 2008); Arabidopsis leaf NADH/NAD⁺ increases from 1 to 8; and Arabidopsis leaf NADPH/NADP⁺ increases from 1 to 6 (Allan et al., 2012).

Non-photosynthesizing or non-illuminated tissue such as CA-stored apple fruit cannot produce NADPH via terminal electron acceptance of photosystem I. A major source of NADPH production would be the oxidative pentose phosphate pathway, wherein glucose-6-phosphate is converted to ribulose-5-phosphate via NADP⁺-dependent glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase activities (Kruger and von Schaewen, 2003). Interestingly, G6PDH activity in ‘Empire’
apple at harvest is 20-fold higher than that in fruit stored for up to 3 months at 5 °C (Adyanthaya et al., 2009). Moreover, two classes of plastidial G6PDH exist in plants; expression of potato G6PDH P1 is abundant in green tissues, whereas expression of potato G6PDH P2 is abundant throughout the plant, especially stems and roots, and the corresponding activity of G6PDH P2 is less sensitive than that of P1 to feedback inhibition by an increased NADPH/NADP⁺ ratio. Another source of NADPH production during abiotic stress could originate from a stress-induced calcium/CaM-regulated NAD kinase, which phosphorylates NADH and NAD⁺ creating NADPH and NADP⁺, respectively (Allan et al., 2008, 2012). Our data demonstrated decreasing NAD(H) and increasing NADPH (Fig. 4.2), suggesting that NAD(H) could be increasingly phosphorylated throughout the storage period. Thus, the high NADPH/NADP⁺ ratio at the beginning of storage may be attributed to high G6PDH P2 activity, and the increasing ratio may be the result of stimulated NADK activity. Overall, it can be concluded that both fresh and CA-stored ‘Empire’ apples are in a highly reduced state, indicative of O₂ limitation, which would influence GABA-related metabolism (Shelp et al., 2012b).

The major amino acids in both freshly harvested and stored ‘Empire’ fruit were aspartate, asparagine and glutamate, whereas polyamines consisted of similar levels of putrescine and spermidine, together with minor levels of spermine (Figs. 4.3 and 4.5; Appendix B, Supplementary Table S4.3). There was an early storage phase in which TAA, glutamate, GABA and alanine transiently accumulated, regardless of CO₂ treatment or chilling temperature, although GABA and alanine accumulation appeared to be greatest with elevated CO₂ at 0°C. This was accompanied by relatively stable levels of polyamines, succinate and GHB with ambient CO₂, but transient accumulation of these
same metabolites with elevated CO₂, especially at 0°C. Notably, the succinate level further declined thereafter, whereas GABA and GHB tended to increase over the longer term. Previously, Magné et al. (1997) described similar findings for the major amino acids and polyamines with ‘Granny Smith’ apple fruit stored at 2°C, although putrescine tended to accumulate with storage duration. Furthermore, they demonstrated an early transient increase, followed by a steady decline, in proteolytic activity. Together, these data suggest that: (i) cold-induced protein hydrolysis was primarily responsible for the enhanced availability of amino acids in ‘Empire’ apple fruit during the early storage phase, and the higher glutamate level had more impact on the levels of GABA than polyamines (Shelp et al., 1999; Scott-Taggart et al., 1999; Majumdar et al., 2013); and (ii) elevated CO₂ had distinct effects on the production of GABA and polyamines, as well as succinate and GHB, during the initial phase, and on GABA and GHB production over the longer term. Thus, a complex pattern of GABA-related metabolites can be recognized in ‘Empire’ apple exposed to a combination of O₂ deficiency, chilling and elevated CO₂.

The incidence of internal physiological injury was relatively low regardless of CO₂ level, but the onset of CO₂-induced external injury was particularly rapid, regardless of chilling temperature, reaching a maximum after only 8 weeks (Fig. 4.1), and highly correlated to putrescine, followed by spermine, GHB, succinate, total polyamines and GABA (Fig. 4.7). Changes in the metabolism of polyamines, like GABA and GHB, are well known to occur in fruit and other tissues in response to low temperature or O₂ deficiency, but the physiological importance of these processes is still unclear (McDonald and Kushad, 1986; Kramer et al., 1989, Kaplan et al., 2004; Narsai et al., 2009; Pedreschi et al., 2009, Alcázar et al., 2010; Shelp et al., 2012b; Tiburcio et al., 2014). Our findings...
suggest that the accumulation of polyamines, especially putrescine and spermine, could serve as a potential biomarker for CO$_2$-induced external injury, although this interpretation is complicated by the presence of multiple stress conditions during CA storage (Ahuja et al., 2010; Rasmussen et al., 2013; Rejeb et al., 2014; Sewelam et al., 2014).

### 4.7.2. Relative Importance of Polyamines and Glutamate in CO$_2$-induced Synthesis of GABA in CA-stored Apple Fruit

In the present study, we tested the hypothesis that glutamate decarboxylation, rather than polyamine catabolism, is primarily responsible for CO$_2$-induced GABA production in apple fruit. Our approach was to compare metabolite-transcript relationships associated with the alternative routes for GABA biosynthesis. Figure 4.8 summarizes the most important relationships and places them in context of our current knowledge about the biochemical role(s) of the proteins and their subcellular compartmentation. The most studied route is from glutamate, a reaction that can be catalyzed by three different cytosolic GADs in apple fruit; the expression of both $MdGAD1$ and $MdGAD2$ is relatively abundant and the encoded proteins possess a C-terminal calmodulin-binding domain, whereas $MdGAD3$ is much less abundant and the corresponding protein does not bind calmodulin (Trobacher et al., 2013b). Putrescine and spermidine also represent potential sources of GABA via the metabolite 4-aminobutanal.

Spermidine and spermine, respectively, are known to be back-converted to putrescine and/or spermidine in dicotyledonous plants (Fincato et al., 2011; Shelp et al., 2012b; Tiburcio et al., 2014), and preliminary assessment from our laboratory, based on
in silico analysis, suggests that specific apple FAD-polyamine oxidases (MdPAO2-4) are peroxisomal (Appendix B, Supplementary Methods S4.1). Five Cu-MdAOs are present in ‘Empire’ apple fruit and the expression of two of these is relatively abundant; MdAO1 is peroxisomal and has high affinity for diamines such as putrescine and 1,3-diaminopropane, whereas MdAO2 is extracellular and exclusively uses monoamines (Zarei et al., 2014). There is evidence, albeit preliminary, for the terminal oxidation of spermidine to 4-aminobutanal in Arabidopsis via the action of AtCuAO2 and AtCuAO3 and in monocots via FAD-PAOs, but there is no support for the involvement of FAD-PAOs in these reactions in dicots such as apple (Moschou et al., 2008; Fincato et al., 2011; Planas-Portell et al., 2013). Furthermore, two NAD⁺-dependent MdAMADHs have been recently shown to convert 4-aminobutanal to GABA, as well as 3-aminopropanal to β-alanine (Zarei et al., 2015). The available evidence suggests that MdAMADH2 is peroxisomal and MdAMADH1 is cytosolic even though both enzymes contain a C-terminal peroxisomal targeting sequence, causing the authors to propose that MdAMADH1 could be targeted to the peroxisome in a stress-specific manner. Therefore, MdAO1, in combination with one or both of the MdAMADHs, would represent a likely path for putrescine oxidation to GABA in apple fruit (Fig. 4.8).

The short-term response of GABA to elevated CO₂ under hypoxic CA conditions was correlated with the up-regulation of MdGAD2, MdAO1 and MdAla-T, as well as elevated levels of putrescine and spermine, whereas the long-term response was
Figure 4.8. Model for the oxidation of polyamines to GABA and GHB in CA-stored apple fruit. The expression of genes encoding the enzymes highlighted in yellow was monitored in this study. Abbreviations: 4-ABAL, 4-aminobutanal; AO, amino oxidase; AMALDH, aminoaldehyde dehydrogenase; 3-APAL, 3-aminopropanal; 1,3-DAP, 1,3-diaminopropane; GAD, glutamate decarboxylase; GABA, 4-aminobutyrate; GABA-T, 4-aminobutyrate transaminase; GHB, 4-hydroxybutyrate; GLYR, glyoxylate/succinic semialdehyde reductase; PDH, pyrroline dehydrogenase; SSA, succinic semialdehyde; SSADH succinic semialdehyde dehydrogenase; TCA, tricarboxylic acid. (Adapted from Shelp et al., 2012b).
correlated with up-regulation of a different suite of genes (i.e., \textit{MdGAD1}, \textit{MdAO2}, \textit{MdAMADH1} and \textit{MdAMADH2}) (Figs. 4.3-4.7). There has been little research on the impact of elevated CO\textsubscript{2} on metabolite-transcript relationships, let alone on GABA pathways in CA-stored apple fruit, which represent a developmental stage that is independent of cell division, growth and cell expansion, and is slated for ripening and senescence. However, research on O\textsubscript{2} or chilling stress with other developmental stages can provide useful information. For example, the oxidative pentose phosphate pathway is up-regulated in soybean leaves in response to elevated CO\textsubscript{2}, suggesting that the supply of NADPH would be improved (Ainsworth et al., 2006). Furthermore, two GABA peaks are found in rice being germinated under anoxia and the first peak is associated with slight up-regulation of at least one of five \textit{OsGAD}s and down-regulation of both \textit{OsGABA-T} and \textit{OsSSADH} (Narsai et al., 2009), whereas a single transient GABA peak is found in shoots of chilled Arabidopsis and it is associated with up-regulation of both \textit{AtGAD4} and \textit{AtSSADH} (Kaplan et al., 2007). From this information, it can be suggested that the pathways supplying GABA in apple fruit are being reprogrammed during elevated CO\textsubscript{2} storage under CA conditions; however, it is difficult to posit mechanisms to explain the metabolite-transcript relationships observed. While distinct transcriptional (gene-dependent) events appear to be linked to the \textit{GAD}s, biochemical mechanisms (i.e. gene-independent or post-transcriptional) involving Ca\textsuperscript{2+}/CaM or cytosolic acidification could be responsible for a stress-induced increase in GAD activity (Shelp et al., 1999, 2012a, 2012b; Kinnersley and Turano, 2000; Trobacher et al., 2013b). The elevated polyamine levels could be linked with the up-regulation of \textit{MdAO1} by an unknown mechanism, but given that both AO and PAO activities rely on molecular O\textsubscript{2} for catalysis (Juda et al.,
2006; Mukherjee et al., 2008; Zarei et al., 2014), AMADHs are NAD\(^+\)-dependent (Zarei et al., 2015), and CA-stored apple fruit are O\(_2\) limited with an elevated NAD(P)H/NAD(P)\(^+\) ratio (see discussion above; Ho et al., 2011, 2013), it seems more likely that restricted AO, PAO, and AMADH activities caused the accumulation of putrescine and possibly spermine. Therefore, we propose that GABA generated in apple fruit during CA storage with elevated CO\(_2\) is derived from glutamate, rather than polyamines, but research is required to investigate mechanisms for the transcriptional regulation of GAD, AO and AMADH.

**4.7.3. GHB Metabolism in CA-stored Apple Fruit**

In the present study, we tested the hypothesis that GABA carbon is diverted from succinate production to GHB during the CO\(_2\)-induced stress response. The most common routes for succinate production involve ADP-dependent succinyl-coenzyme A ligase, an important component of the TCA cycle, and NAD\(^+\)-dependent SSADH, a component of the GABA shunt (Busch and Fromm, 1999; Studart-Guimarães et al., 2007). It is noteworthy that the GABA shunt can be up-regulated to compensate for the down-regulation of succinyl-coenzyme A ligase in illuminated tomato leaves (Studart-Guimarães et al., 2007), and succinic semialdehyde can be converted to GHB via NADPH-dependent GLYR activity (Bouché et al., 2003a; Breitkreuz et al., 2003; Fait et al., 2005; Allan et al., 2008, 2012; Simpson et al., 2008; Bao et al., 2015) (see Chapter 3).

Here, we determined the expression of two putative mitochondrial SSADHs in apple fruit, as well as cytosolic GLYR1 and plastid/mitochondrial GLYR2 (Figs. 4.4 and 4.8; see Chapter 3). The initial accumulation of succinate and GHB might be simply
explained, at least in part, by elevated *MdGAD2* levels; however, the accumulation of GHB preceded that of GABA and there was no positive relationship between succinate or GHB accumulation and *MdSSADH* or *MdGLYR* expression (Figs. 4.3 and 4.4). Previous studies have noted that succinate accumulates continuously for up to 50 h in rice germinating under anoxia, with GHB accumulating prior to GABA (Narsai et al., 2009), and a lack of correlation between *GLYR* or *SSADH* expression and GHB accumulation in submerged *Arabidopsis* (Allan et al., 2008, 2012) and rice germinated under anoxia (Narsai et al., 2009; Shelp et al., 2012c). Therefore, under the reducing conditions evident in CA-stored fruit (Fig. 4.2) it seems more likely that the activities of the catabolic enzyme for succinate, NAD$^+$-succinate dehydrogenase, and of the TCA cycle was restricted by the elevated CO$_2$/O$_2$ and reducing environment within the apple (Frenkel and Patterson, 1973; Ke et al., 1993; Tcherkez et al., 2008; Narsai et al., 2009). These same conditions would restrict SSADH activity and increase NADPH/NADP$^+$ ratios, resulting in the diversion of SSA to the production and accumulation of GHB (Allan et al., 2003, 2008, 2012; Breitkreuz et al., 2003).

The second CO$_2$-induced peak in GHB is highly correlated to GABA, but not succinate (Fig. 4.3). Previously, Kaplan et al. (2007) demonstrated that a transient increase in GHB level in shoots of chilled Arabidopsis plants follows a transient increase in GABA and it is independent of *AtGLYR* expression (see Shelp et al., 2012c). However, there was a concomitant and sustained increase in succinate level, which could be interpreted as support for the operation of a reductive TCA cycle (Vanlerberghe et al., 1989; Shelp et al., 2012c). Our findings might suggest that the carbon/nitrogen balance in CA-stored apple fruit was altered with the extended period of stress, resulting in carbon
limitation of the TCA cycle and diversion of GABA carbon to GHB, rather than succinate, because of the unfavorable redox conditions (Fait et al., 2011; Renault et al., 2013; Mustroph et al., 2014). Exploration of the function of GHB in plants during exposure to elevated CO$_2$, low O$_2$ and/or chilling, using single and double overexpression or knockout mutants of GABA-T and GLYR, is warranted.

4.8. CONCLUDING REMARKS

Apple fruit face chilling, low O$_2$ and elevated CO$_2$ during CA storage. These stresses can inhibit respiration, delay ripening, and result in physiological injury. In this study, we focused on the impact of elevated CO$_2$ during CA storage on the incidence of external CO$_2$ injury and metabolites and gene transcripts associated with pathways for GABA and GHB production: glutamate and polyamines. Our findings indicated that both gene-independent and gene-dependent processes were involved in the onset on external CO$_2$ injury, and that putrescine would best serve as a biomarker for the early detection of this external disorder. CA storage in general increased levels of amino acids potentially due to proteolysis, thereby enhancing glutamate substrate availability for GABA production. Elevated CO$_2$ appeared to have more of an influence on polyamine and GABA-related metabolites than their associated transcripts, which showed two distinct expression patterns associated with short- and long-term GABA accumulation. Given this transcript/metabolite discrepancy, biochemical or post-transcriptional mechanisms are proposed to be involved in the regulation and/or stimulation of enzymes associated with GABA and polyamine metabolism. Apple fruit were found to be in a highly reduced state, potentially due to the high CO$_2$/O$_2$ environment, which would favour enzymatic
activity towards GABA production from glutamate rather than polyamines, and divert
GABA carbon to GHB rather than succinate.
CHAPTER FIVE – SUMMARY AND FUTURE PROSPECTS

Plant NADPH-dependent GLYR activity was first identified in partially purified spinach leaves, and was later found to be composed of two separate isoforms: cytosolic GLYR1 and plastidial GLYR2 in Arabidopsis. GLYRs are so named because of their micromolar affinity for the photorespiratory intermediate glyoxylate. HPRs are also able to reduce glyoxylate in vivo, where HPR1, HPR2 and HPR3 isoforms exist in the peroxisome, cytosol and plastid, respectively. GLYRs can also reduce SSA, a catabolite of GABA, into GHB with millimolar affinity. This catalytic function was first demonstrated by complementation of an ssadh-deficient yeast mutant with an Arabidopsis cDNA library, followed by growth on GABA as the sole source of nitrogen. Glyoxylate and SSA are reactive aldehydes, and would be detrimental if they accumulate as a result of stress; therefore, GLYRs are important in reducing these aldehydes into their corresponding, less toxic alcohols glycolate and GHB, and could be key to metabolically engineering abiotic stress-tolerant plants. To date, most information about the GLYRs has been derived from research on Arabidopsis; therefore, a more thorough investigation of plant GLYRs was warranted.

In Chapter Three of this thesis, I further tested the hypothesis that plant GLYRs function in NADPH-dependent glyoxylate and SSA reduction into glycolate and GHB, respectively. GLYR1 and/or GLYR2 were cloned from apple, rice and Arabidopsis cDNA, and the recombinant proteins were separately expressed in E. coli and purified to near homogeneity using affinity chromatography. These purified recombinant proteins were used to determine the specificity for the substrates glyoxylate and SSA and the cofactor NADPH. Across species, catalytic efficiencies were highest for NADPH, followed
by glyoxylate (in the micromolar range) and then SSA (in the millimolar range), with no clear kinetic differences between GLYR1 and GLYR2 isoforms, findings that are similar to those previously published for AtGLYRs. Also, MdGLYRs were product inhibited by an increasing ratio of NADP+/NADPH, and OsGLYRs preferred NADH over NADPH, supporting the notion that plant GLYRs could be regulated by the NADPH/NADP+ ratio in vivo.

GLYR sequences examined here were similar between isoforms and among species, except for an N-terminal targeting sequence present in GLYR2s. In this regard, online targeting programs predicted various combinations of chloroplastic and mitochondrial localization, whereas empirical research had reported that AtGLYR2 is exclusively localized to the plastid, justifying the need for multiple experimental systems in which to confirm/establish the subcellular localization of GLYR2. MdGLYR1, MdGLYR2, AtGLYR2 and OsGLYR2 sequences were fused to GFP, and their subcellular localizations were assessed in tobacco BY-2 cells, as well as Arabidopsis protoplasts. In addition, an Arabidopsis line with stably expressed methoxyfenozide-inducible AtGLYR2-GFP was created. In tobacco BY-2 cells, MdGLYR1 was determined to be cytosolic, like previously published AtGLYR1. In all expression systems tested, apple, rice and Arabidopsis GLYR2s localized to both chloroplast and mitochondrion, which was confirmed with the detection of glyoxylate reductase activity in planta in the purified mitochondrial fraction of an Arabidopsis glyr1 mutant free from chloroplastic GLYR2 and cytosolic GLYR1 contamination. The only other mention of a mitochondrial NADPH-dependent GLYR is in early
literature referring to the photosynthesizing protist *E. gracilis*. It was suggested that dual localization of plant GLYR2s is probably the result of an ambiguous targeting signal at the N-terminus of the amino acid sequence.

This study led us to conclude that plant GLYRs are responsible for the detoxification of both GABA-derived SSA and photorespiratory-derived glyoxylate. Abiotic stresses such as drought, heat, salinity and hypoxia, which may result in the differential accumulation of either SSA or glyoxylate, have been shown to elevate expression of *AtGLYR1* and *AtGLYR2*, NAD(P)H/NAD(P)+ ratio, and levels of GABA and GHB, suggesting that glyoxylate and SSA could be simultaneously detoxified in plant cells, as shown to occur with recombinant *AtGLYR1* *in vitro*.

Under abiotic stress, we proposed that: i) cytosolic GLYR1 scavenges the surplus of glyoxylate escaping from the peroxisome and/or SSA leaking from the mitochondrion; ii) plastidial GLYR2 would reduce glyoxylate that has entered the chloroplast thereby preventing the inhibition of photosynthesis; and, iii) mitochondrial GLYR2 reduces excess glyoxylate and SSA, directly following its synthesis within the organelle, thereby preventing these toxic aldehydes from escaping the mitochondrion and causing further damage throughout the plant cell. Furthermore, plastidial GLYR2 could assist in preventing the over-reduction of photosystem I on the chloroplastic electron transport chain under stresses such as high light, which would result in lower levels of NADP+ (Allan et al., 2009). The catalytic activity of GLYR2 could cycle excess NADPH, thereby preventing the diversion of electrons to oxygen and the creation of ROS. Therefore, plant GLYRs appear biochemically redundant but could serve specialized roles depending on the metabolic status of their respective compartments.
Preliminary work with Arabidopsis has demonstrated that *GLYR1* over-expression and *glyr1/glyr2* double knockout mutants are severely and mildly affected, respectively, by 150 mM salt stress (Chiu, 2013). This phenotype could potentially be due to a four-fold higher accumulation of GHB in the over-expression mutant, compared with the WT; however, the fate and function of GHB is still unclear. Results in Chapter Three indicated that GLYR1 is responsible for approximately 85% of the glyoxylate reductase activity *in planta*, barring the potential overlapping activities of the HPRs, making our understanding of the physiological relevance of GLYR2 somewhat elusive. However, the dual localization of GLYR2 in the plant cell, especially in the mitochondrion where HPR isoforms are not present, illustrates the potential importance of this isoform in targeted aldehyde detoxification within mitochondria and chloroplasts. Future directions could involve phylogenetic analyses to investigate the evolution of plastidial/mitochondrial GLYRs from photosynthesizing unicellular organisms such as *E. gracilis* to higher plants. The relative physiological importance and relevance of mitochondrial versus plastidial GLYR2s could be investigated by mutating the N-terminal signal sequence in order to change their respective localizations, and assessing potential phenotypic differences in these mutants under various stresses, compared with ambient conditions. In addition, the relative importance of GLYRs vs HPRs could be investigated *in vitro* by assessing the kinetic characteristics of HPRs, as well as *in vivo* by crossing an *atglyr1* knock-out mutant with an *athpr2* over-expressing mutant, given that most of the glyoxylate reductase activity is attributed to
GLYR1, to assess if a phenotype is rescued under abiotic stress. Finally, the function of GHB accumulation and its catabolism could be investigated with an SSA or GHB feeding experiment using WT and atg lyr1 and atg lyr2 single and double knock-out mutants. Up- and down-regulated genes could be compared between genotypes in order to determine candidate genes involved in GHB metabolism and catabolism, as well as their putative functions.

During abiotic stress, GABA is known to accumulate several-fold in most plant tissues studied to date, notably in apple fruit exposed to CA storage. Apples are an important fruit crop both worldwide and within Canada, where Ontario is responsible for 40% of production. Notably, ‘Empire’ is a major apple cultivar grown in Ontario, New York and Michigan, and are highly marketable because of their ability to be stored for extended periods. CA storage (i.e., low O₂, high CO₂, low temperature) is used to delay ripening and senescence but can also cause physiological disorders such as CO₂–induced injuries characterized by sharply defined rough and sunken regions of the peel. GABA has been linked to flesh browning disorders in ‘Empire’ and ‘Honeycrisp’ apples; however, little is known regarding the mechanisms involved in the onset of external CO₂ injury, as well as the metabolism of GABA in non-photosynthesizing plant organs such as fruit. GABA can be derived by either direct decarboxylation of glutamate via GADs or indirectly as the oxidation of the polyamines putrescine and spermidine to 4-aminobutanal via O₂-dependent AOs, which can then be converted to GABA by NAD⁺-dependent AMADH. Therefore, investigations of both GABA and polyamine metabolism were required to understand their potential involvement in the development of storage disorders.
In Chapter Four of this thesis, I tested the hypotheses: i) GABA production in CA-stored apple fruit is supported primarily by glutamate decarboxylation, rather than polyamine catabolism; ii) GABA catabolism is diverted to GHB production rather than succinate; and iii) GABA levels are associated with CO₂-induced external injury. ‘Empire’ apple fruit were stored under specific CA conditions promoting external CO₂ injury (5 vs. 0.03 kPa CO₂ and 2.5 kPa O₂) in combination with two chilling temperatures (0 and 3°C) for a period of 16 weeks. Apples representative of four storage replicates were sampled periodically, assessed for physiological disorders, and intact apples were flash frozen and pulverized. Total apple RNA was extracted from which cDNA was synthesized, and the abundance of gene transcripts associated with polyamine catabolism and GABA metabolism were determined via qPCR. Metabolites involved in these two pathways were quantified using a combination of HPLC, GC-MS/MS and enzyme-linked spectrophotometric assays. Correlations between physiological disorders, transcripts and metabolites were performed using CP-PLS.

Apples stored under elevated CO₂ in the hypoxic environment with chilling exhibited minor internal disorders in combination with a rapid and high incidence of external CO₂ injury. Intact apple fruit were in a highly reduced state prior to, and during CA storage, as demonstrated by a high NAD(P)H/NAD(P)⁺ ratio and induced expression of Ala-T, which are probably due to limiting O₂ diffusion within the bulky fruit. With storage, there was initially a transient increase in TAA, including glutamate and alanine, presumably due to enhanced
proteolysis, which likely contributed to the transient accumulation of GABA and GHB, particularly with elevated CO$_2$ at 0°C. Thereafter, both GABA and GHB displayed a second increase with elevated CO$_2$ at 0°C. Succinate also increased upon CA storage at high CO$_2$, but decreased to control levels in the later stage of storage, findings attributed to restricted succinate dehydrogenase and TCA cycle activities. GABA-related transcripts displayed short- and long-term expression patterns; however, no CO$_2$-specific effects were observed that could be directly linked to the two peaks in GABA accumulation. Polyamines transiently accumulated and could be associated with short-term GABA accumulation. Polyamine catabolism genes, like GABA-related transcripts, showed both short- and long-term accumulation patterns; however, again no CO$_2$-specific effects were observed. CP-PLS analyses revealed that a time-dependent CO$_2$ effect on metabolites and transcripts variables was evident, and that the elevated CO$_2$ treatment was highly positively correlated to external CO$_2$ injury, putrescine and GAD2, and weakly positively correlated to spermine, succinate, GHB, GABA and AO1.

The inconsistency between metabolite and transcript patterns suggests the importance of post-transcriptional or biochemical regulation of GABA- and polyamine-related enzymes. Within the highly reduced and O$_2$-deprived environment in the apple, the abiotic stress-stimulated and Ca$^{2+}$/CaM activated GAD activity would be enhanced, as would the NADPH-dependent GLYR activity. In contrast, O$_2$-dependent AO and NAD$^+$-dependent AMADH, SSADH and succinate dehydrogenase activities would be inhibited, resulting in the initial accumulation of succinate and polyamines under elevated CO$_2$. Therefore, GABA accumulation in CA-stored ‘Empire’ apples would be the result of glutamate decarboxylation, and GABA carbon would be diverted from succinate to
GHB production. Nevertheless, putrescine and spermine were more highly correlated with external CO$_2$ injury than GABA, and may therefore be involved with the onset of this physiological injury. Overall, our findings are in agreement with first and second hypotheses, but the third hypothesis must be rejected.

Under CA storage with elevated CO$_2$, the contribution of polyamines to GABA production is proposed to be restricted, and this theory could be further explored using radiolabelled polyamines with a model plant system such as Arabidopsis. For example, the metabolism [$^{14}$C] putrescine applied to the roots of Arabidopsis could be compared between plants temporarily shifted to CA conditions, and plants under ambient conditions. Relevant metabolites such as GABA, succinate and GHB could be extracted, and the potential differences in the proportion of labelled versus non-labelled metabolites in CA-stressed and control plants could be determined. In light of the diversion of GABA carbon from succinate to GHB production under CA conditions, the function of GHB accumulation and its metabolism under high CO$_2$/O$_2$ and chilling should be further investigated. These studies could compare the response of Arabidopsis single and double overexpression or knockout mutants of GLYR1 and GLYR2, which are temporarily submitted to CA stress or ambient conditions, by assessing phenotypic, transcript and metabolite differences with respect to the GABA pathway. These future directions would benefit from the readily available of Arabidopsis mutants to elucidate a more complete picture of gene-dependent and independent processes associated with GABA metabolism under CA stress.
CHAPTER SIX - LITERATURE CITED


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APPENDICES

APPENDIX A: CHAPTER THREE – PLANT GLYOXYLATE/SUCCINIC SEMIALDEHYDE REDUCTASES ARE LOCATED IN THE PLASTID, MITOCHONDRION AND CYTOSOL

Supplementary Table S3.1. Synthetic oligonucleotides

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A. Multiple sequence alignment for GLYRs

AtGLYR1

MdGLYR1

OsGLYR1

AtGLYR2

MdGLYR2

OsGLYR2

AtGLYR1

MdGLYR1

OsGLYR1

AtGLYR2

MdGLYR2

OsGLYR2

AtGLYR1

MdGLYR1

OsGLYR1

AtGLYR2

MdGLYR2

OsGLYR2

AtGLYR1

MdGLYR1

OsGLYR1

AtGLYR2

MdGLYR2

OsGLYR2

AtGLYR1

MdGLYR1

OsGLYR1

AtGLYR2

MdGLYR2

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AtGLYR2

MdGLYR2

OsGLYR2

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OsGLYR1

AtGLYR2

MdGLYR2

OsGLYR2

AtGLYR1

MdGLYR1

OsGLYR1

AtGLYR2

MdGLYR2

OsGLYR2
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B. Predicted subcellular localization of GLYR2s

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<td>Mit(^b)</td>
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\(^a\)Chl, chloroplast;

\(^b\)Mit, mitochondria
**Supplementary Figure S3.1.** Multiple sequence alignment, percent identity matrix and predicted subcellular localization of apple (*Malus x domestica*), rice (*Oryza sativa*) and Arabidopsis (*Arabidopsis thaliana*) (A). The alignment was created with ClustalW2 (EMBLE-EBI). Identical residues are marked with an asterisk, and similar and non-similar residues are marked with a colon and period, respectively. Active site residues are highlighted in grey and predicted N-terminal targeting sequences are underlined. The blue bar denotes the glycine-rich fingerprint motif and conserved glycines are highlighted in yellow. *In silico* analysis of subcellular localization was conducted using various websites ([http://www.cbs.dtu.dk/services/TargetP/](http://www.cbs.dtu.dk/services/TargetP/); [http://www.genscript.com/psort/wolf_psort.html](http://www.genscript.com/psort/wolf_psort.html); [http://www.csbio.sjtu.edu.cn/bioinf/plant/](http://www.csbio.sjtu.edu.cn/bioinf/plant/); [http://bioinfo3.noble.org/AtSubP/?dowhat=About](http://bioinfo3.noble.org/AtSubP/?dowhat=About); [http://ihg.gsf.de/ihg/mitoprot.html](http://ihg.gsf.de/ihg/mitoprot.html); [http://www.cbs.dtu.dk/services/ChloroP/](http://www.cbs.dtu.dk/services/ChloroP/); [http://abi.inf.uni-tuebingen.de/Services/MultiLoc/](http://abi.inf.uni-tuebingen.de/Services/MultiLoc/); [http://suba3.plantenergy.uwa.edu.au/](http://suba3.plantenergy.uwa.edu.au/); [http://pcmpred.bicpu.edu.in/pcmpred.php](http://pcmpred.bicpu.edu.in/pcmpred.php)) (B). A higher or more positive number in each row of the subcellular localization table indicates higher probability of the predicted localization. Dashed lines indicate lack of predictions.
At GLYR1

At GLYR2

Δ 58

26 kDa-
34 kDa-
72 kDa-
72 kDa-
34 kDa-
26 kDa-
55 kDa-
43 kDa-
55 kDa-
43 kDa-

Eluate

Eluate

MdGLYR1

MdGLYR2 Δ54

A

B

A GLYR1

A GLYR2 Δ58

C

QsGLYR1

QsGLYR2 Δ55

M

Eluate

Eluate

M

Eluate

M

Eluate

M

Eluate
**Supplementary Figure S3.2.** Purification of recombinant apple (A), Arabidopsis (B) and rice (C) GLYRs. In A and B, top and bottom panels represent coomassie brilliant blue-stained SDS-PAGE gels and the corresponding immunoblots probed with an anti-His-tag antibody of the various fractions obtained during purification of \(Md\)GLYR1 and \(Md\)GLYR2\(\Delta\)54 and \(At\)GLYR1 and \(At\)GLYR2\(\Delta\)58 from \(E.\ coli\). In C, an immunoblot probed with an anti-His-tag antibody of the eluate fraction obtained after purification of \(Os\)GLYR1 and \(Os\)GLYR2\(\Delta\)35. Molecular weight markers are shown in kDa.
**Supplementary Figure S3.3.** Dependence of GLYR1 (A, C) and GLYR2 (B, D) activities from apple (A, B) and rice (C, D) on pH. Activity was determined using saturating glyoxylate and NADPH as co-substrates in a combination of MES, HEPES and TABS buffers either separately, or together. Data represent the mean (± SD) of three technical replicates from a typical enzyme preparation.
V\text{max} = 41.6 \text{ µmol/min/mg} \\
K_m = 10.2 \text{ µM}

V\text{max} = 14.6 \text{ µmol/min/mg} \\
K_m = 6.8 \text{ µM}

V\text{max} = 17.2 \text{ µmol/min/mg} \\
K_m = 6.8 \text{ mM}

V\text{max} = 40.6 \text{ µmol/min/mg} \\
K_m = 2.4 \text{ µM}

V\text{max} = 29.1 \text{ µmol/min/mg} \\
K_m = 2.4 \text{ µM}

V\text{max} = 11.6 \text{ µmol/min/mg} \\
K_m = 10 \text{ µM}

V\text{max} = 23.7 \text{ µmol/min/mg} \\
K_m = 1.8 \text{ µM}

V\text{max} = 17.2 \text{ µmol/min/mg} \\
K_m = 6.8 \text{ mM}

V\text{max} = 2.6 \text{ µmol/min/mg} \\
K_m = 1.5 \text{ mM}
Supplementary Figure S3.4. Dependence of recombinant GLYR1 (A-D) and GLYR2 (E-H) activities from apple on glyoxylate in the presence of NADPH (A, E), NADPH in the presence of glyoxylate (B, F), SSA in the presence of NADPH (C, G), and NADPH in the presence of SSA (D, H). Data represent the mean ± SD of four technical replicates from a typical enzyme preparation.
V_{max} = 78.6 \mu\text{mol/min/mg} \quad K_m = 47.4 \mu\text{M}

V_{max} = 36.6 \mu\text{mol/min/mg} \quad K_m = 12.6 \mu\text{M}

V_{max} = 43.6 \mu\text{mol/min/mg} \quad K_m = 4.3 \text{mM}

V_{max} = 18.5 \mu\text{mol/min/mg} \quad K_m = 35.5 \mu\text{M}

V_{max} = 14.1 \mu\text{mol/min/mg} \quad K_m = 21.4 \mu\text{M}

V_{max} = 12.6 \mu\text{mol/min/mg} \quad K_m = 6.7 \mu\text{M}

V_{max} = 19.0 \mu\text{mol/min/mg} \quad K_m = 1.7 \text{mM}

V_{max} = 10.9 \mu\text{mol/min/mg} \quad K_m = 13.1 \mu\text{M}
Supplementary Figure S3.5. Dependence of recombinant GLYR1 (A-D) and GLYR2 (E-H) activities from rice on glyoxylate in the presence of NADPH (A, E), NADPH in the presence of glyoxylate (B, F), SSA in the presence of NADPH (C, G), and NADPH in the presence of SSA (D, H). Data represent the mean ± SD of four technical replicates from a typical enzyme preparation.
Supplementary Figure S3.6. Dependence of recombinant GLYR1 (A) and GLYR2 (B) activities from Arabidopsis on glyoxylate in the presence of NADPH. Data represent the mean ± SD of four technical replicates from a typical enzyme preparation.
Supplementary Figure S3.7. Dependence of MdGLYR1 (A) and MdGLYR2Δ54 (B) activities on NADP⁺/NADPH ratio. Activity was determined using saturating glyoxylate and NADPH, and increasing concentrations of NADP⁺. Data represent the mean ± SD of four technical replicates from a typical enzyme preparation.
Supplementary Figure S3.8. Characterization of a stable AtGLYR2-GFP transgenic line.

A. Amplification of the AtGLYR2-GFP transgene from Arabidopsis genomic DNA. Lanes 1 and 2: amplification of GABA-T from WT and AtGLYR2-GFP transgenic mutant, respectively, resulting in two 161 bp amplicons. Lane 3: amplification of AtGLYR2-GFP from WT with primers flanking the AtGLYR2-GFP fusion, resulting in no amplicon. Lane 4: amplification of AtGLYR2-GFP from transgenic AtGLYR2-GFP, resulting in a 384 bp amplicon. PCR product form lane 4 was sequenced, and matched the predicted transgene sequence. B. Expression of AtGLYR2 from cDNA of WT and AtGLYR2-GFP at 0 h, 48 h and 72 h after induction with methoxyfenozide, as measured by qPCR using the house-keeping gene elongation factor 1 (EF-1). Control: RNA treated by DNaseI without reverse transcriptase in cDNA reaction.
**Supplementary Figure S3.9.** Controls for inducible AtGLYR2-GFP expression in stably transformed Arabidopsis. WT seedlings were imaged without (A) or with (D) application of methoxyfenozide. Chlorophyll autofluorescence was imaged in B, E and J. Seedlings were stained with Mitotracker (F) and did not show bleed-through (D) or co-localization with chlorophyll (G). Arabidopsis plants stably expressing AtGLYR2-GFP did not show leaky expression in non-induced seedlings (I). C, H and K are the corresponding transmitted light images. Scale bars represent 10 µm.
### Supplementary Table S4.1. Synthetic oligonucleotides utilized for qRT-PCR in apple fruit.

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<td>CGGTGGGACAGACACAGAGA</td>
</tr>
<tr>
<td><em>MdGAD3</em> reverse</td>
<td>CACTCCGACTAGTAGCATTGGCA</td>
</tr>
<tr>
<td><em>MdGABA-T</em> forward</td>
<td>GAGCATTGCCCCCAAGATTTT</td>
</tr>
<tr>
<td><em>MdGABA-T</em> reverse</td>
<td>TCCCCATATGAGATGGACTGTCACA</td>
</tr>
<tr>
<td><em>MdGLYR1</em> forward</td>
<td>GGTCATACACATCGAACAGTACCTTT</td>
</tr>
<tr>
<td><em>MdGLYR1</em> reverse</td>
<td>TGATGATACAAATGAGTGCCTGTTT</td>
</tr>
<tr>
<td><em>MdGLYR2</em> forward</td>
<td>AAAATAGGACTGGACCCGAAAGT</td>
</tr>
<tr>
<td><em>MdGLYR2</em> reverse</td>
<td>TGGGCCTTTCATTGAGTACATTG</td>
</tr>
<tr>
<td><em>MdSSADH1</em> forward</td>
<td>CAGTGGCACCCCTTTTGC</td>
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<tr>
<td><em>MdSSADH1</em> reverse</td>
<td>GCAGCTAACCCTGCAATTG</td>
</tr>
<tr>
<td><em>MdSSADH2</em> forward</td>
<td>TCATACTTTGATACCTCATTCCAT</td>
</tr>
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<td><em>MdSSADH2</em> reverse</td>
<td>GAGCAGCGATAGAAATTTGAATG</td>
</tr>
<tr>
<td><em>MdEF-1α</em> forward</td>
<td>CTCCACATTGCCGCTCAAG</td>
</tr>
<tr>
<td><em>MdEF-1α</em> reverse</td>
<td>GCCAGATCGCCTGTCGAT</td>
</tr>
<tr>
<td><strong>MdAlaAT forward</strong></td>
<td>CTGCAAAGACAGCCCCCAGAT</td>
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<tr>
<td>---------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td><strong>MdAlaAT reverse</strong></td>
<td>ACAACAACTCCTGTGCGATTGA</td>
</tr>
<tr>
<td><strong>MdAMADH1 forward</strong></td>
<td>AAAGGTGTGACCCTTCTCTAAGG</td>
</tr>
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<td><strong>MdAMADH1 reverse</strong></td>
<td>AGCATGGTTGTGAGCAGTTGAT</td>
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<td><strong>MdAMADH2 forward</strong></td>
<td>GCTGCAGTCATATCGAAAGATTTAGA</td>
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<td>CCCGCTTGAAGGGCCTTA</td>
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<tr>
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<td>GACGTCCCTCCGAGCACAT</td>
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<tr>
<td><strong>MdAO1 reverse</strong></td>
<td>TGCAGCCATCCCGTTGT</td>
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<td><strong>MdAO2 forward</strong></td>
<td>ACCAAAGCACAGGCGAAGAT</td>
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</tr>
<tr>
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<td>CCGAGGTGCATTACCAATTACA</td>
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<tr>
<td><strong>MdAO3 reverse</strong></td>
<td>CCAGCCCATTTTCAGACTTG</td>
</tr>
<tr>
<td><strong>MdAO4 forward</strong></td>
<td>AGTAGCCAAGCCGATTCAGAAC</td>
</tr>
<tr>
<td><strong>MdAO4 reverse</strong></td>
<td>GCTCGATCATACTAAARATGATGC</td>
</tr>
<tr>
<td><strong>MdAO5 forward</strong></td>
<td>AGTAGCCAAGCCGATTCAGAAC</td>
</tr>
<tr>
<td>** MdAO5 reverse**</td>
<td>CAAGGTAAGTCAAAGAAAGCATGTTTT</td>
</tr>
</tbody>
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**Supplementary Table S4.2.** Synthetic oligonucleotides utilized for identification and cloning of apple alanine aminotransferase.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT-F27</td>
<td>GCTTCCCCAGGTGTGCAYHTNATGATGC</td>
</tr>
<tr>
<td>CT-R27</td>
<td>CCTCTCTTTCCGCACCTCTCCRTARTANCC</td>
</tr>
<tr>
<td>CT-F30</td>
<td>GCTGATAAGTTTCAGAGAAGGATGGGATTTCCTTGTCCC</td>
</tr>
<tr>
<td>CT-F31</td>
<td>CCTCAATACCCCTTTGTACTCTGCGTCAATAGCCC</td>
</tr>
<tr>
<td>CT-R30</td>
<td>CCTGCTCGCCATATCCCATAGACCAGGGATACC</td>
</tr>
<tr>
<td>CT-R31</td>
<td>CTGGTTGTCTCGGAAGAACCTGTCTGTGGG</td>
</tr>
</tbody>
</table>

Supplementary Table S4.3. Impact of elevated CO\textsubscript{2} at two chilling temperatures on the amino acid composition of ‘Empire’ apple fruit during 16 weeks of postharvest storage in 2009. Data represent the mean ± SE of four storage replicates. Data taken from Liu (2011).
### Amino Acid Concentration (nmol g⁻¹ FM)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Pre-Storage</th>
<th>0 °C, 0.03 kPa CO₂</th>
<th>0 °C, 5 kPa CO₂</th>
<th>3 °C, 0.03 kPa CO₂</th>
<th>3 °C, 5 kPa CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate</td>
<td>563.14± 65.37</td>
<td>1206.01±55.30</td>
<td>676.68±96.00</td>
<td>839.36±137.02</td>
<td>552.98±75.02</td>
</tr>
<tr>
<td>Glutamate</td>
<td>314.70 ±23.04</td>
<td>854.06±41.76</td>
<td>660.33±41.26</td>
<td>812.97±98.37</td>
<td>663.82±76.61</td>
</tr>
<tr>
<td>Aspargine</td>
<td>895.49±358.31</td>
<td>1006.13±36.35</td>
<td>582.33±175.46</td>
<td>811.24±103.10</td>
<td>590.66±198.54</td>
</tr>
<tr>
<td>Serine</td>
<td>197.79±11.72</td>
<td>340.11±21.33</td>
<td>339.32±25.28</td>
<td>295.66±17.88</td>
<td>250.45±23.91</td>
</tr>
<tr>
<td>Glutamine</td>
<td>176.46±33.93</td>
<td>154.51±19.98</td>
<td>240.04±30.07</td>
<td>107.12±7.17</td>
<td>114.02±21.16</td>
</tr>
<tr>
<td>Histidine</td>
<td>16.06±0.37</td>
<td>27.37±4.46</td>
<td>29.05±1.45</td>
<td>17.99±3.54</td>
<td>17.28±0.69</td>
</tr>
<tr>
<td>Glycine</td>
<td>82.95±3.12</td>
<td>94.72±6.14</td>
<td>99.55±8.67</td>
<td>92.40±6.38</td>
<td>80.76±8.16</td>
</tr>
<tr>
<td>Threonine</td>
<td>46.76±3.31</td>
<td>57.79±5.69</td>
<td>53.65±4.36</td>
<td>39.17±11.84</td>
<td>35.51±3.92</td>
</tr>
<tr>
<td>Arginine</td>
<td>26.74±2.23</td>
<td>78.70±9.27</td>
<td>68.60±6.70</td>
<td>77.94±9.33</td>
<td>56.76±7.59</td>
</tr>
<tr>
<td>Alanine</td>
<td>104.87±13.77</td>
<td>292.13±24.83</td>
<td>430.08±72.83</td>
<td>264.93±34.62</td>
<td>275.84±37.54</td>
</tr>
<tr>
<td>GABA</td>
<td>71.49±10.55</td>
<td>176.19±14.64</td>
<td>162.27±24.67</td>
<td>173.23±14.98</td>
<td>143.18±16.34</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>8.83±0.61</td>
<td>9.81±0.94</td>
<td>13.64±1.24</td>
<td>7.09±0.86</td>
<td>8.08±0.56</td>
</tr>
<tr>
<td>Cysteine</td>
<td>27.48±2.71</td>
<td>30.36±6.68</td>
<td>55.48±21.94</td>
<td>24.12±1.93</td>
<td>34.36±14.54</td>
</tr>
<tr>
<td>Valine</td>
<td>7.91±0.18</td>
<td>45.55±2.88</td>
<td>35.85±1.96</td>
<td>38.45±4.32</td>
<td>28.09±1.88</td>
</tr>
<tr>
<td>Methionine</td>
<td>24.88±1.26</td>
<td>15.46±1.89</td>
<td>13.56±1.38</td>
<td>11.11±1.40</td>
<td>9.53±1.02</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>10.18±0.87</td>
<td>15.02±1.73</td>
<td>25.33±2.96</td>
<td>11.50±1.96</td>
<td>14.70±3.57</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>11.17±0.64</td>
<td>17.31±1.76</td>
<td>14.00±0.97</td>
<td>14.70±2.27</td>
<td>9.67±0.51</td>
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<tr>
<td>Leucine</td>
<td>13.82±0.80</td>
<td>22.60±2.37</td>
<td>16.63±1.92</td>
<td>20.45±1.28</td>
<td>12.41±0.99</td>
</tr>
<tr>
<td>Lysine</td>
<td>9.53±1.15</td>
<td>21.44±8.71</td>
<td>19.41±4.77</td>
<td>9.31±1.73</td>
<td>5.40±0.73</td>
</tr>
<tr>
<td>Total amino acids</td>
<td>2610.27±505.44</td>
<td>4465.26±230.12</td>
<td>3535.81±452.11</td>
<td>3668.74±361.88</td>
<td>2903.50±447.74</td>
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</table>
### Amino Acid Concentration (nmol g$^{-1}$ FM)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>0 °C, 0.03 kPa CO$_2$</th>
<th>0 °C, 5 kPa CO$_2$</th>
<th>3 °C, 0.03 kPa CO$_2$</th>
<th>3 °C, 5 kPa CO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate</td>
<td>1264.49±61.21</td>
<td>763.39±101.67</td>
<td>1218.08±114.18</td>
<td>778.31±72.95</td>
</tr>
<tr>
<td>Glutamate</td>
<td>803.57±54.24</td>
<td>756.73±77.07</td>
<td>862.89±12.78</td>
<td>679.44±47.57</td>
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<tr>
<td>Asparagine</td>
<td>798.81±121.66</td>
<td>716.78±94.96</td>
<td>876.68±139.01</td>
<td>625.16±139.47</td>
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<tr>
<td>Serine</td>
<td>284.08±20.65</td>
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<td>246.50±20.42</td>
<td>205.53±19.95</td>
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<tr>
<td>Glutamine</td>
<td>87.09±3.23</td>
<td>130.37±10.95</td>
<td>108.03±16.74</td>
<td>82.18±7.51</td>
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<tr>
<td>Histidine</td>
<td>26.02±0.79</td>
<td>24.44±0.41</td>
<td>26.56±1.93</td>
<td>24.70±1.24</td>
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<tr>
<td>Glycine</td>
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<td>87.07±4.35</td>
<td>85.1±6.14</td>
<td>83.79±7.02</td>
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<tr>
<td>Threonine</td>
<td>58.64±6.37</td>
<td>59.50±4.16</td>
<td>54.75±8.86</td>
<td>55.47±6.96</td>
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<tr>
<td>Arginine</td>
<td>63.70±4.46</td>
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<td>70.2±4.76</td>
<td>57.2±4.05</td>
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<tr>
<td>Alanine</td>
<td>231.00±24.64</td>
<td>341.73±54.26</td>
<td>233.87±35.31</td>
<td>202.05±21.21</td>
</tr>
<tr>
<td>GABA</td>
<td>141.06±6.18</td>
<td>198.97±26.76</td>
<td>156.50±15.67</td>
<td>143.57±7.13</td>
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<tr>
<td>Tyrosine</td>
<td>9.99±0.63</td>
<td>11.53±0.85</td>
<td>9.43±0.57</td>
<td>9.56±0.48</td>
</tr>
<tr>
<td>Cysteine</td>
<td>31.51±4.21</td>
<td>29.43±3.56</td>
<td>30.32±5.39</td>
<td>30.59±7.77</td>
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<tr>
<td>Valine</td>
<td>43.62±2.35</td>
<td>35.61±1.57</td>
<td>39.86±3.96</td>
<td>30.50±1.72</td>
</tr>
<tr>
<td>Methionine</td>
<td>11.45±0.66</td>
<td>10.37±0.69</td>
<td>10.88±0.99</td>
<td>9.96±0.56</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>14.98±0.77</td>
<td>21.54±1.86</td>
<td>13.26±0.85</td>
<td>12.70±1.21</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>17.99±1.06</td>
<td>12.73±0.75</td>
<td>16.55±1.54</td>
<td>13.38±1.00</td>
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<td>Leucine</td>
<td>20.57±1.04</td>
<td>13.45±1.25</td>
<td>19.84±0.95</td>
<td>15.25±0.99</td>
</tr>
<tr>
<td>Lysine</td>
<td>11.64±3.20</td>
<td>9.16±2.16</td>
<td>16.00±1.68</td>
<td>13.26±1.34</td>
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<tr>
<td>Total amino acids</td>
<td>4005.4±179.8</td>
<td>3590.5±315.1</td>
<td>4095.7±375.5</td>
<td>3072.7±290.4</td>
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</table>
## Amino Acid Concentration (nmol g⁻¹ FM)

<table>
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<th></th>
<th>Week 8</th>
<th>Week 16</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0 °C, 0.03 kPa CO₂</td>
<td>0 °C, 5 kPa CO₂</td>
</tr>
<tr>
<td>Aspartate</td>
<td>799.92±95.55</td>
<td>426.43±24.20</td>
</tr>
<tr>
<td>Glutamate</td>
<td>437.97±11.30</td>
<td>361.12±28.85</td>
</tr>
<tr>
<td>Asparagine</td>
<td>772.61±313.42</td>
<td>554.79±252.32</td>
</tr>
<tr>
<td>Serine</td>
<td>191.35±12.35</td>
<td>160.75±15.26</td>
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<tr>
<td>Glutamine</td>
<td>50.94±10.43</td>
<td>54.41±21.80</td>
</tr>
<tr>
<td>Histidine</td>
<td>18.93±0.52</td>
<td>14.73±1.61</td>
</tr>
<tr>
<td>Glycine</td>
<td>100.54±9.27</td>
<td>75.79±2.52</td>
</tr>
<tr>
<td>Threonine</td>
<td>47.97±2.51</td>
<td>58.85±3.08</td>
</tr>
<tr>
<td>Arginine</td>
<td>35.29±4.58</td>
<td>39.07±2.66</td>
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<tr>
<td>Alanine</td>
<td>119.22±15.24</td>
<td>147.17±47.07</td>
</tr>
<tr>
<td>GABA</td>
<td>70.18±9.51</td>
<td>130.35±43.20</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>9.92±0.85</td>
<td>9.16±2.18</td>
</tr>
<tr>
<td>Cysteine</td>
<td>30.18±4.29</td>
<td>24.33±2.99</td>
</tr>
<tr>
<td>Valine</td>
<td>34.35±3.09</td>
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<tr>
<td>Methionine</td>
<td>3.60±0.54</td>
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</tr>
<tr>
<td>Phenylalanine</td>
<td>10.00±0.63</td>
<td>10.37±3.26</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>23.92±6.50</td>
<td>10.80±0.65</td>
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<tr>
<td>Leucine</td>
<td>21.33±1.74</td>
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<tr>
<td>Lysine</td>
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<td>1.36±0.05</td>
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<tr>
<td>Total amino acids</td>
<td>2779.6±411.5</td>
<td>2116.6±410.8</td>
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</tbody>
</table>
**Supplementary Table S4.4.** CP-PLS total explained variance for the first four latent variables (LVs) relating metabolites or transcripts (X variables) with response variables (Y variables: storage time, physiological disorders) ‘Empire’ apples stored under two CO₂ levels (5 and 0.03 kPa) at two chilling temperatures (3 and 0°C) for up to 16 weeks.

<table>
<thead>
<tr>
<th>Variance Explained (%)</th>
<th>Variable</th>
<th>X</th>
<th>Week</th>
<th>Internal</th>
<th>External</th>
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</thead>
<tbody>
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<td>LV1</td>
<td></td>
<td>21.33</td>
<td>75.46</td>
<td>0.7473</td>
<td>0.0026</td>
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<tr>
<td>LV1 + LV2</td>
<td></td>
<td>27.60</td>
<td>76.70</td>
<td>10.68</td>
<td>51.38</td>
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<tr>
<td>LV1 + LV2 + LV3</td>
<td></td>
<td>36.45</td>
<td>84.79</td>
<td>28.83</td>
<td>53.48</td>
</tr>
<tr>
<td>LV1 + LV2 + LV3 + LV4</td>
<td></td>
<td>42.79</td>
<td>84.82</td>
<td>30.38</td>
<td>53.75</td>
</tr>
</tbody>
</table>
Supplementary Figure S4.1. Pyridine dinucleotide status in rosette leaves of three-week-old Arabidopsis plants. (A) Concentrations of oxidized and reduced forms of NAD(P)H. (B) Ratios of reduced to oxidized forms of NAD(H) and NADP(H). Plants were grown under 21 °C, light intensity of 180 µmol m\(^{-2}\) s\(^{-1}\), 16 h/8 h light/dark period (B). Data represent the mean of four technical replicates.
Supplementary Methods S4.1. Identification and *in silico* subcellular localization of apple PAOs.

Adel Zarei, Christopher P. Trobacher and Barry J. Shelp

Dept. of Plant Agriculture, University of Guelph, Guelph, ON N1G 2W1, Canada

The apple genome database (www.rosaceae.org) was searched utilizing known Arabidopsis *PAOs* (*AtPAO1*, *At5g13700*; *AtPAO2*, *At2g43020*; *AtPAO3*, *At3g59050*; *AtPAO4*, *At1g65840*; *AtPAO5*, *At4g29720*) sequences as queries at the nucleotide and amino acid levels. Six putative apple *PAO* genes were identified with following accession numbers: MDP0000261625; MDP0000321972; MDP0000188855; MDP0000702799; and, MDP0000941459. Total RNA from apple leaves cv. ‘Empire’ was prepared as described elsewhere (Gasic et al., 2004) and treated with DNase I (TURBO DNA-free™ Kit, Ambion). Single-stranded cDNA was generated according to Zarei et al. (2015). cDNA containing open reading frames of the apple *PAO* homologs were amplified using gene specific primers (See Table 1 below). All six putative apple *PAO* genes appeared to be expressed in leaves and were cloned into pCR2.1-TOPO (Invitrogen) vector using the manufacturer’s protocol. At least three clones from independent PCR origin were sequenced for each *PAO* gene. The apple *PAO* gene sequences have been designated as *MdPAO1*, *MdPAO2*, *MdPAO3*, *MdPAO4*, *MdPAO5* and *MdPAO6* (GenBank Acc. Nos. KT184496-KT184501)

*In silico* analysis reveals that the six putative *PAO* genes encode proteins ranging from 488 to 533 amino acids (see Table 2 below). Amino acid sequence identity varies from 24% (*MdPAO1* and *MdPAO6*) to 91% (*MdPAO3* and *MdPAO4*) among the
MdPAOs. MdPAO1 is 72% identical to AtPAO1, MdPAO2 in 79% identical to AtPAO2, and MdPAO3 is 60% identical to AtPAO3. In particular, MdPAO5 and MdPAO6 show a high degree of identity (90%) to each other, as well as AtPAO5.

Sequence comparison and phylogenetic analysis of the six putative MdPAO genes accompanied by Arabidopsis PAO reveals that they can be divided into three distinct groups as described for Arabidopsis (Moschou et al., 2008). MdPAO2, MdPAO3 and MdPAO4, together with AtPAO2, AtPAO3 and AtPAO4 form a cluster possessing a peroxisome targeting signal 1 (see Fig. 1, below). Arabidopsis members of this group are localized in peroxisome (Tiburcio et al., 2014), and similar subcellular localization is predicted for apple members of this group. Moreover, MdPAO1 clustered with AtPAO1, whereas MdPAO5, MdPAO6 and AtPAO5 clustered separately. AtPAO1 and AtPAO5 appear to encode cytosolic proteins (Tiburcio et al. 2014); therefore, MdPAO5 and MdPAO6 are predicted to be cytosolic.
Table 1. List of synthetic oligonucleotide utilized for cloning of apple PAOs.

<table>
<thead>
<tr>
<th>Oligo name</th>
<th>Sequence</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>CT-F50</td>
<td>CTAGCCAACCGATATACACAGCGCCC</td>
<td>Forward primer for MdPAO1</td>
</tr>
<tr>
<td>CT-R50</td>
<td>CGGTCAACAAATCATGGTGTGGCTACTAACCC</td>
<td>Reverse primer for MdPAO1</td>
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<tr>
<td>CT-F51</td>
<td>CAATTTAATTCTTTTCGTTTCCCAATTTTCTTTTTCACCATTCC</td>
<td>Forward primer for MdPAO4</td>
</tr>
<tr>
<td>CT-R51</td>
<td>CCCATCTTTCATGTCTGAAGTATCGGAAAATG</td>
<td>Reverse primer for MdPAO4</td>
</tr>
<tr>
<td>CT-F52</td>
<td>AATTCCAATACCAATTTTCGATTGAATCACC</td>
<td>Forward primer for MdPAO2</td>
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<tr>
<td>CT-R52</td>
<td>GGAGGATGCGGCGGCGG</td>
<td>Reverse primer for MdPAO2</td>
</tr>
<tr>
<td>CT-F53</td>
<td>CAATTTTTTCAAATCCCATTTTCTTTGGAAATTCC</td>
<td>Forward primer for MdPAO3</td>
</tr>
<tr>
<td>CT-R53</td>
<td>GAAATGTAATCTTATCATTCTCATGTACCAAGATG</td>
<td>Reverse primer for MdPAO3</td>
</tr>
<tr>
<td>CT-F54</td>
<td>CCAAAAACCCATTACTTTAATTTCCAGCTGAAAC</td>
<td>Forward primer for MdPAO5</td>
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<tr>
<td>CT-R54</td>
<td>GAAATTAATGTTCAAAAATAGATAAGAATCTCTAAATATTG</td>
<td>Reverse primer for MdPAO5</td>
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<tr>
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<tr>
<td>CT-R55</td>
<td>GTACTTAAAGAGAGAGAAAGCAGAAGATGTTCACAATG</td>
<td>Reverse primer for MdPAO6</td>
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Table 2. Percent global identity of apple and Arabidopsis PAOs created by Clustal 2.1

<table>
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<tr>
<th></th>
<th>AtPAO5</th>
<th>MdPAO5</th>
<th>MdPAO6</th>
<th>AtPAO1</th>
<th>MdPAO1</th>
<th>MdPAO2</th>
<th>AtPAO2</th>
<th>AtPAO3</th>
<th>AtPAO4</th>
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<td>AtPAO5</td>
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<tr>
<td>MdPAO5</td>
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<td>MdPAO6</td>
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<td>AtPAO1</td>
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<td>28.51</td>
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<td>MdPAO3</td>
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<td>61.93</td>
<td>60.33</td>
<td>67.28</td>
<td>91.67</td>
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**Figure 1.** Multiple amino acid sequence alignment of the N- and C-termini of apple and Arabidopsis PAOs. Sequence alignment was performed with the CLUSTALW2 software (Larkin et al., 2007) and edited manually. Number of total amino acids is shown on the right. The presence of C-terminal peroxisomal targeting signals are enclosed by the blue box. Identical residues are shown with a black background, and similar residues are shown with a grey background.
Figure 2. Phylogenetic relationship of PAO members in apple and Arabidopsis. The unrooted neighbor-joining method was utilized to perform the phylogenetic analysis. The phylogram was created by ClustalW2 multiple sequence alignment and MEGA 6.0 software (Tamura et al., 2013). Proteins carrying putative peroxisome targeting signals are circled by a dashed line. Internal numbers give bootstrap frequencies for each clade.