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Activity of the beta-cyanoalanine synthase pathway is associated with the response to abiotic stress by Arabidopsis thaliana.

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ACTIVITY OF THE β-CYANOALANINE SYNTHASE PATHWAY IS ASSOCIATED WITH THE RESPONSE TO ABIOTIC STRESS BY *ARABIDOPSIS THALIANA*

by

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ABSTRACT: Cyanide is produced throughout a plant's life cycle alongside the hormone ethylene by oxidation of 1-aminocyclopropane-1-carboxylic acid. Production increases during certain developmental stages such as seed germination, seedling elongation, fruit ripening and senescence. Abiotic stresses increase ethylene production giving rise to 'stress cyanide'. Cyanide also comes from metabolism of cyanogenic compounds. Cyanide is however, a toxic chemical which readily binds to metallo-enzymes inhibiting primary metabolic processes. Plants have mechanisms to maintain cyanide homeostasis such as the β-cyanoalanine pathway whereby cysteine reacts with cyanide forming β-cyanoalanine, mediated by β-cyanoalanine synthase and cysteine synthase. A dual nitrilase 4 enzyme then converts the β-cyanoalanine into asparagine or aspartate and ammonium. Studies have suggested that the physiological function of the pathway is not restricted to detoxification and assimilation of excess cyanide. The overall research goal was to investigate the role of the pathway in plant tolerance to water deficit and exogenous cyanide exposure in *Arabidopsis thaliana*. The first objective was to investigate responsiveness of the pathway to duration and intensity of water deficit and cyanide exposure. The second was to investigate the contribution of enzymes associated with the pathway to cyanide metabolism. The questions addressed were whether there is enzymatic redundancy in enzymes associated with the first step of cyanide detoxification and whether there is pathway redundancy between

the β-cyanoalanine and an alternative sulfurtransferase pathway. *A. thaliana* Col-0 and three SALK-line mutants with a T-DNA insertion for the genes *AtCysA1*, *AtCysC1* and *AtNIT4* were grown and exposed to water stress. Physiological and biochemical measurements were taken. The results showed a transient increase in cyanide concentration and β-cyanoalanine synthase activity on exposure to stress. The response pattern was similar regardless of intensity or duration of stress. Knocking out *AtCysA1* or *AtCysC1* did not impair the ability of plants to metabolize cyanide and tolerate stress i.e the enzymes were functionally redundant. The *AtNIT4* mutant however, was impaired in cyanide metabolism and exhibited a sensitive phenotype under both stresses, suggesting that the cyanoalanine pathway is the sole pathway in cyanide detoxification. The results show that the pathway may be an important tool in improvement of plant tolerance to abiotic stress.

DEDICATION

I dedicate this dissertation to my mom and dad, with love and appreciation.

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DISTRIBUTION AND FUNCTIONS OF THE CYANOALANINE PATHWAY

1.0 Introduction

Cyanide is a surprisingly ubiquitous compound in nature, arising from both anthropogenic and natural sources. Cyanide is used in or is a by-product of organic chemicals, plastic and rubber synthesis, electroplating and steel production, the extraction of precious metals from ore, and the photographic, aluminum, and manufactured gas industries (ATSDR, 1997). Depending upon the industry and the process involved, cyanide concentrations used or present in the waste streams can vary from <1 to >65,000 mg L^{-1} (Dash et al., 2009). Soil bacteria and fungi are among the most prevalent natural sources of cyanide (Ghosh et al., 2005). Cyanogenic bacteria include species from the genera *Chromobacterium anacystis, Nostoc, Plectonema, Rhizobium* and *Serratia plymuthica* (Antoun et al., 1998; Blom et al., 2011; Castric, 1981; Gallagher and Manoil, 2001). Some species of *Pseudomonas* release cyanide into the rhizosphere, with concentrations in excess of 100 mg cyanide kg^{-1} soil reported in some cases (Barber et al., 2003; Crutzen and Andreae, 1990; Kesler-Arnold and O'Hearn, 1990; Owen and Zdor, 2001; Rudrappa et al., 2008). Fungal cyanogenesis by *Marasmius, Ascomycetes, Basidomycetes* and several other genera has also been demonstrated (Knowles, 1976). Forest fires are a significant source of cyanide in the environment as incomplete burning releases cyanide from pyrolysis of amino acids, N-heterocyles and dicarboxylic acids (Johnson and Kang, 1971). Burning cyanogenic glycosides in organic materials produces 25-31 mg cyanide kg^{-1} burned (Andreae and Merlet, 2001). While the cyanide may be released into the atmosphere,

studies have shown that oceans are the ultimate sink for this cyanide (Li et al., 2000). Once released, atmospheric cyanide has a half life of a 1-3 years (ATSDR, 1995) while the half life in oceans predicted by model simulation is 1-2 months (Li et al., 2000). Up to 49 μg HCN L^{-1} have been detected in stormwater runoff after a wildfire and such concentrations are toxic to aquatic life (Crutzen and Andreae, 1990). Lower plants, for example algae, *Chlorella vulgaris* are known to produce cyanide during oxidation of some amino acids such as histidine (Pistorius et al., 1977).

Higher plants possess several pathways for the biosynthesis and release of cyanide (Figure 1.1). There are >3,000 species of cyanogenic plants, so named because they produce cyanogenic glycosides as storage forms of nitrogen and defensive compounds (Selmar et al., 1988; Vetter, 2000). While the majority of these species are angiosperms, there are some ferns (*Cheilanthes intramarginalis, C. marginata and Notholaena aurea*) that synthesize these compounds (Balick et al., 1978). Cyanide is released from these glycosides by hydrolysis in response to herbivory or other tissue damage (Kadow et al., 2012; Seigler, 1991) or during the decomposition of plant tissues in the soil (Widmer and Abawi, 2002). Other processes which lead to cyanide emanation include the degradation of glucosinolates produced by members of the Cruciferae (Donkin et al., 1995) and camalexin biosynthesis in *Arabidopsis thaliana* (Böttcher et al., 2009). A ubiquitous source of cyanide in higher plants is ethylene biosynthesis. Cyanide is a co-product of the synthesis of this gaseous plant growth regulator from aminocyclopropane-1-carboxylic acid (Peiser et al., 1984). Ethylene synthesis occurs throughout plant growth and development, but increases significantly when plants are subjected to biotic or abiotic stress (Morgan and Drew, 1997; Seo et al., 2011), thus so does cyanide synthesis (Liang, 2003; Woodrow et al., 2002). There are also reports that cyanide may be enzymatically produced using

glyoxylate from photorespiration and hydroxylamine from nitrate assimilation (Hucklesby et al., 1982; Solomonson and Spehar, 1981), but if this occurs in vivo then this may be relevant only to plants carrying out C3 photosynthesis.

2.0 Biological detoxification of cyanide

Because cyanide is a potent inhibitor of electron transport and numerous metallo-enzymes (Echevarria et al., 1984; Solomonson, 1974; Solomonson, 1981), organisms exposed to cyanide rapidly metabolize this poison to reduce or prevent the adverse effects. There are two fundamental strategies for removing cyanide from biological systems, either through degradation (breakdown to simpler inorganic molecules) or assimilation (incorporation of cyanide into primary metabolites). Degradation represents the primary strategy for cyanide detoxification in eubacteria and includes hydrolytic, reductive, or oxidative pathways forming simple nitrogenous compounds such as formamide and ammonium (Gupta et al., 2010; Samiotakis and Ebbs, 2004). Fungi (e.g., *Fusarium sp*.) are also capable of degrading cyanide via hydrolytic pathways (Cluness et al., 1993; Dumestre et al., 1997).

The assimilation of cyanide can occur via two possible pathways (Figure 1.2). One pathway involves sulfurtransferases (e.g., rhodanese) that transfer the sulfur atom from thiosulfate to cyanide to form thiocyanate (Ezzi-Mufaddal and Lynch, 2002; Hatzfeld and Saito, 2000; Papenbrock and Schmidt, 2000a; Saidu, 2004; Sörbo, 1955). The sulfurtransferase pathway has long been considered to be the principal pathway for cyanide detoxification in animals, although the associated enzymes are thought to have more important functions in cellular sulfur metabolism (Saidu, 2004). Sulfurtransferase enzymes provide cyanide detoxification in species of *Thiobacillus* (Gardner and Rawlings, 2000) and species from the

fungal genera *Trichoderma* (Ezzi-Mufaddal and Lynch, 2002). Genes for these enzymes have been found in plants (Hatzfeld and Saito, 2000; Nakamura et al., 2000; Papenbrock et al., 2011), but efforts to demonstrate a significant role in cyanide detoxification have thus far been inconclusive (Meyer et al., 2003; Papenbrock and Schmidt, 2000a; Papenbrock and Schmidt, 2000b).

The second pathway for cyanide assimilation involves incorporation of cyanide into nitrogen metabolism through formation of asparagine and aspartate. Evidence for cyanide assimilatory pathways has been found in a limited number of eubacteria (e.g., *Chromobacterium violaceum, Bacillus megaterium, and Escherichia coli*) and fungal (e.g., *Rhizoctonia solani, Rhizopus oryzae*) species where they often function concomitantly with a degradation pathway and/or with rhodanese activity (Ezzi-Mufaddal and Lynch, 2002; Mundy et al., 1973; Raybuck, 1992). The most prevalent example of this type of cyanide assimilation is the β-cyanoalanine pathway (Miller and Conn, 1980; Yu et al., 2012). The first step is catalyzed by β-cyanoalanine synthase (β-CAS), which mediates a reaction that substitutes the sulfhydryl moiety of cysteine (or another alanyl donor like serine) with cyanide, forming the nitrile cyanoalanine with the concomitant release of hydrogen sulfide (Warrilow and Hawkesford, 1998). The second step is catalyzed by a bi-functional enzyme with both nitrile hydratase and nitrilase activity. The nitrile hydratase activity converts cyanoalanine to asparagine while the nitrilase activity results in the formation of aspartate and ammonium (Piotrowski et al., 2001). The scope of this review is limited to a discussion of the β-cyanoalanine pathway in plants. We summarize here the distribution of the pathway in higher plants. The contribution of the pathway to cyanide detoxification is discussed, along with recent evidence suggesting broader roles for the pathway in plant metabolism.

3.0 Discovery of the β-cyanoalanine pathway

The β-cyanoalanine pathway is known to be the principal pathway responsible for cyanide detoxification in insects (Meyers and Ahmad, 1991; Ogunlabi and Agboola, 2007) and plants. The first evidence for this role in plants came from investigations of cyanogenic glycoside metabolism. Feeding studies using $H^{14}CN$ were conducted to determine the source of the nitrile group associated with plant cyanogenic glycosides (Blumenthal-Goldschmidt et al., 1963). Surprising to the researchers involved was the observation that for many cyanogenic and acyanogenic plant species, the majority of the ^{14}C radiolabel was detected in the amino acid asparagine, not in the cyanogenic glycosides. Subsequent work (Blumenthal et al., 1968; Floss et al., 1965; Fowden and Bell, 1965; Nigam and Ressler, 1964; Ressler et al., 1963; Tschiersch, 1964) demonstrated that cyanoalanine was the principal product formed shortly after exposure to cyanide. Although this pathway has been studied for nearly fifty years, results from the past two decades have provided the most significant advances.

4.0 Distribution of the β-cyanoalanine pathway in plants

4.1 Distribution across species

The β -cyanoalanine pathway appears broadly distributed across the angiosperms as activity of the enzyme that mediates the first step of the pathway, β -cyanoalanine synthase, has been detected in numerous species (Table 1.1). The list of plants that possess this pathway may be considerably longer if indirect evidence of cyanide assimilation is also considered. Some studies that have examined the ability of plants to remove cyanide from solution have used a Michaelis-Menten kinetics to model cyanide disappearance, equating disappearance (and the

lack of accumulation of cyanide in the tissues), with metabolism of cyanide by the β-cyanoalanine pathway (Larsen et al., 2004; Yu et al., 2004). If true, then at least 25 additional plant species, including both cyanogenic and acyanogenic and both woody and herbaceous plants could also be included (Table 1.1). One early contention regarding the β-cyanoalanine pathway was that cyanogenic plants naturally displayed a greater inherent pathway activity than acyanogenic plants. For example, β-CAS activity in leaves of cyanogenic lima bean (*Phaseolus lunatus*) was nearly 50-fold higher than activity in acyanogenic kidney bean (*Phaseolus vulgaris*) seedlings (Miller and Conn, 1980). β-cyanoalanine synthase activity in whole leaves of leek (*Allium porum* L.), pea (*Pisum sativum* L.), maize (*Zea mays* L.), and cyanogenic sorghum (*Sorghum bicolor x Sorghum sudanense*) in the absence of cyanide exposure was 11, 3.8, 44, and 75 mg S^{2-} min^{-1} mg^{-1} protein, respectively (Wurtele et al., 1984). However, because the magnitude of the difference in activity between cyanogenic and acyanogenic species tends more commonly to be <10-fold and depends upon the specific tissue sampled and the stage of development of those tissues, current opinion now indicates that the cyanogenic plants do not necessarily have an inherently enhanced capacity to assimilate cyanide via the β-cyanoalanine pathway (Larsen et al., 2004; Wurtele et al., 1984; Yu et al., 2004). Activity of the pathway does not appear to be restricted to any particular organ. Activity of both β-CAS and the nitrilase/nitrile hydratase have been identified in numerous tissues, including roots, stems, tubers, leaves, buds, cotyledons, fruits, and seeds of numerous plant species (Table 1.1). The level of enzyme activity also tends to be variable in each tissue. For example, β-CAS was isolated from tuber, leaf and rind of cassava (*Manihot esculenta* Crantz), and the β-CAS activity was higher in tuber (150 mg $H_2S.min^{-1}$) than in rind (105 mg $H_2S.min^{-1}$) and leaf (108 mg $H_2S.min^{-1}$) (Merina

et al., 1997). Sorghum and spinach (*Spinacea oleracea*) also show such variability in their tissues (Table 1.1).

4.2 Spatial distribution of the pathway within plant cells and tissues

The spatial distribution of the pathway within specific tissues has been examined in a limited number of studies but only in terms of β-CAS activity. The results have indicated that activity of this enzyme in leaves is greatest in the mesophyll layer. Fractionation studies with sorghum, maize, pea, and leek detected β-CAS activity in the inner and outer of tissues epidermis, the mesophyll, and for the monocots the bundle sheath cells (Wurtele et al., 1984). When β-CAS activity in each of these cell types was expressed as a percentage of activity in the whole leaf, the mesophyll cells showed the greatest activity, due primarily to the fact that there are considerably more cells of this type per leaf than other cell types. For leek, sorghum, and pea, >65% of the activity was localized to the mesophyll, a percentage similar to that reported for the mesophyll layer of barley (*Hordeum vulgare*) leaves (Wurtele et al., 1984). The distribution of activity across the leaf appears to be influenced by developmental factors, at least in some plant species. The activity of β-CAS increased steadily in seedlings of blue lupine (*Lupinus angustifolia*) and asparagus (*Asparagus officinalis*) with seedling age over 10 and 25 d, respectively (Cooney et al., 1980; Hendrickson and Conn, 1969). The spatial distribution of β-CAS activity along the length of individual barley leaves increased from base to tip. Activity in the older apical segments of the leaf represented 70% of the whole leaf enzyme activity and was 3.5-fold greater than activity in the basal segments (Wurtele et al., 1984). Within those apical segments, activity was localized predominantly (~70%) to the mesophyll cells while in the basal

leaf segments, the converse was observed, with the mesophyll cells contributing only about 30% to the total β-CAS activity in that segment of the leaf. The increase in activity along the developmental axis of the leaf was attributed in this case to a specific increase in β-CAS activity in the mesophyll cell layer with cell age. These results contrast with those for maize roots in which greater activity was associated with the younger cells of the tip as compared to the older segments of the root (Stulen et al., 1979). Despite spatial differences in the magnitude across and within plant tissues, activity of β-CAS would be required to some degree in all cells because cyanide produced endogenously or taken up from the rhizosphere would exist at physiological pH as HCN (Dzombak et al., 2005; Ghosh et al., 2005) and would therefore be capable of diffusing throughout plant tissues.

The β-cyanoalanine synthase enzyme is localized to the mitochondria of plant cells (Hatzfeld et al., 2000; Hendrickson and Conn, 1969; Lai et al., 2009; Maruyama et al., 2001; Warrilow and Hawkesford, 1998; Watanabe et al., 2008; Wurtele et al., 1984) There are some reports in the literature of cytosolic β-CAS activity (Hasegawa et al., 1995a; Liang and Li, 2001; Maruyama et al., 1998) and this activity is now known to be mediated by cytosolic cysteine synthases. The primary localization of β-cyanoalanine synthase to the mitochondria is perhaps logical given that cyanide can bind irreversibly to the terminal cytochrome *c* oxidase of the mitochondrial electron transport chain, inhibiting electron flow. Additional protection from cyanide would be provided by the cytosolic cysteine synthase which has been reported to share β-CAS activity (Watanabe et al., 2008). In addition to several versions of cytochrome *c* oxidase (McIntosh, 1994; Siedow, 1982), plants possess an alternative oxidase (AOX) which provides a parallel pathway for mitochondrial electron flow from Complex I and ubiquinone to oxygen by diverting electron flow to oxygen prior to Complex III and IV. The alternative pathway does not

make the same contribution to the proton gradient across the inner mitochondrial membrane as flow through cytochrome *c* oxidase so there is a concomitant reduction in ATP synthesis (Siedow and Day, 2000). The AOX does not contribute to the removal of cyanide, so the impact of ATP synthesis would presumably increase with the duration and/or concentration of cyanide exposure. The localization of β-CAS to the mitochondria would therefore act to protect ATP synthesis through the removal of cyanide. The nitrilase/nitrile hydratase that mediates the conversion of cyanoalanine is likely a cytosolic protein as GFP fusions with the Arabidopsis protein localize to the cytosol (Piotrowski, 2008). This is in agreement with a proposed model in tobacco (*Nicotiana tabacum* L.) where under normal conditions nitrilase-like proteins interact with ethylene response element–binding proteins (EREBPs) in the cytosol. The complex presumably helps to retain EREBPs in the cytoplasm. However, upon signaling the complex disintergrates and EREBPs are released and translocated to the nucleus to activate expression of defense genes such as tomato *Pto* kinase (Xu et al., 1998). Nitrilase expression has also been detected in the nucleus potentially in a regulatory role for expression of ethylene-associated genes (Piotrowski, 2008).

5.0 Biological roles of the β-CAS pathway

5.1 Cyanide detoxification

Since the initial discovery in the 1960's, the primary role attributed to the β-cyanoalanine pathway has been cyanide detoxification. Because cyanide is a well-known enzyme inhibitor and uncoupler, it seems obvious that there should be a detoxification pathway to protect sensitive proteins. Cyanogenic plants clearly require activity of this pathway to detoxify the cyanide

released from cyanogenic glycosides and cyanolipids. Cyanide detoxification would seem most critical in cyanogenic plants to prevent autotoxicity from the cyanide used in herbivory defense. Yet as indicated in Table 1.1, activity of the β-cyanoalanine pathway is found in a wide variety of acyanogenic species. What's more, evidence demonstrates that activity is not inherently higher in cyanogenic species as compared to acyanogenic species (Larsen et al., 2004; Wurtele et al., 1984; Yu et al., 2004), so the functional significance would seem to extend further. The sole function in cyanide detoxification seems inadequate to justify the ubiquitous existence in plants of this pathway. New insights emerging from recent study of this pathway in plants now suggest that the functional contributions of the pathway and/or its individual enzymes are more diverse (Table 1.2). Continued reference to this pathway solely to cyanide detoxification may therefore be an oversimplification of its occurrence and biological roles.

5.2 Detoxification of cyanide from cyanogenic glycosides and cyanolipids

Cyanogenic glycosides are polar, water soluble β-hydroxynitriles (cyanohydrins) or β-glycosides. These compounds have been detected in >3,000 taxa across the families Fabaceae, Rosaceae, Linaceae, Asteraceae, and others. Examples of common cyanogenic glycosides include dhurrin, amygdalin, prunasin, and linamarin. The concentration of these compounds varies widely in plants and is largely a result of complex interaction of several factors. These include genetic factors within species and populations (Ballhorn et al., 2009; Vetter, 2000) and biotic factors such as fungal infection (Ballhorn, 2011). Greater amounts of cyanogenic glycosides have been observed in young than in mature lima bean leaves (Ballhorn 2005). Seasonal, and diurnal changes in cyanogenic content of plants have been reported (Fieldes and

Gerhardt, 2001; Hayden and Parker, 2002) and the effect of nutritional status was demonstrated in young sorghum plants where application of nitrogen increased the content of cyanogenic glycosides (Busk and Moller, 2002). Not all plants are cyanogenic, but for those that are, the release of cyanide from these stored glycosides provides the substrate required for cyanoalanine synthase activity. In highly cyanogenic plant species such as cassava, bamboo (*Bambusa* spp), sorghum (*Sorghum bicolor*), lima bean and almond (*Prunus* spp.) (Table 1.1), the concentration of cyanogenic glycosides in tissues where storage is localized can be as high as 2,000 to 8,000 mg kg^{-1}FW. Other tissues of these same plants that are not principal storage sites of cyanogenic glycosides as well as tissues from moderately cyanogenic species such as *Eucalyptus*, flax (*Linum usitatissimum*) and white clover (*Trifolium repens* L.) may have concentrations ranging from below the limit of detection up to 1,000 mg cyanide kg^{-1} FW (Wong-Chong et al., 2005a). Some species such as wheat (*Triticum aestivum),* sweet almonds (*Prunus amygdalus dulcis*) and corn (*Zea mays* L) are referred to as weakly cyanogenic because they produce only trace amounts of cyanogenic glycosides. Plants whose tissue contain >200 mg cyanide kg^{-1} FW are considered "poisonous" and therefore a danger to human and animal health (Kingsbury, 1964).

A principal role of cyanogenic glycosides is as a deterrent to herbivory (Ballhorn et al., 2009). Cyanogenic glycosides are stored principally in the cell vacuole. The subsequent release of cyanide (as HCN) can occur non-enzymatically but is also facilitated by β-glucosidases and hydroxynitrile lyases (Kadow et al., 2012). The enzymes responsible for the release of cyanide are spatially separated from the cyanogenic glycosides either through storage in adjacent cells, a separate subcellular compartment (e.g., the plastid), or in the apoplastic space (Gruhnert et al., 1994; Morant et al., 2008a; Seigler, 1991; Selmar, 1993). During herbivory, stored cyanogenic glycosides or glycosides synthesized *de novo* come in contact with the hydrolytic enzymes. β-

glucosidases cleave the glycoside to release the sugar and a cyanohydrin molecule. For highly cyanogenic plants, this so called "cyanide bomb" system (Morant et al., 2008a) provides an effective deterrent to those herbivores that cannot detoxify, metabolize, or sequester the dose of cyanide received. The release of such high concentrations of cyanide during the metabolism of cyanogenic glycosides presents a risk of autotoxicity to the plant that must be overcome via cyanide detoxification. While activity of the sulfurtransferase enzyme rhodanese has been detected in cyanogenic tissues (Elias et al., 1997; Nambisan and Sundaresan, 1994), the majority of evidence demonstrates that the β-cyanoalanine pathway is principally responsible for the removal of the cyanide released from cyanogenic glycosides. Studies with numerous cyanogenic plants have shown that the activity of enzymes associated with the β-cyanoalanine pathway tends to be high in tissues with the highest concentrations of cyanogenic glycosides or the highest rate of glucosidase activity (Elias et al., 1997; Forslund and Jonsson, 1997; Kongsawadworakul et al., 2009; Mizutani et al., 1991; Nambisan and Sundaresan, 1994; Selmar et al., 1988). An examination of nine cyanogenic plants found that β-cyanoalanine synthase activity in cyanogenic tissues was comparable to or as much as 10-fold higher than the corresponding cyanide production potential (Miller and Conn, 1980). Similar trends were observed in cassava between the activity of the β-hydroxynitrile lyase involved in the metabolism of the cyanogenic glycoside linamarin and the nitrilase activity from the second step of the β-cyanoalanine pathway (Elias et al., 1997). Contrary to the above, other authors have found no specific relationship between the distribution of enzyme activity for enzymes of the β-cyanoalanine pathway and the distribution of cyanogenic glycosides in plant tissues. Cyanoalanine synthase activity was found across the different cell types of sorghum leaves in a pattern that did not correlate with the distribution of the cyanogenic glycoside dhurrin (Wurtele et al., 1984). High concentrations of the cyanogenic

glycoside linamarin are found in seeds of the rubber tree (*Hevea brasiliensis*) but the cyanoalanine synthase activity is localized to the developing seedling after germination (Selmar et al., 1988). This separation of enzymatic activity is thought to reflect a related role for this pathway in the metabolism of nitrogen stored as cyanogenic glycosides. Several studies (Cutler and Conn, 1982; Selmar et al., 1988) have shown that cyanogenic glycosides are mobilized from sites of storage to sink tissues (e.g., developing leaves or other organs) where the cyanogenic glycosides are broken down to release cyanide. The β-cyanoalanine pathway then acts to detoxify the liberated cyanide, using that molecule to provide those rapidly growing tissues with specific amino acids (e.g., asparagine, aspartate) as well as ammonium for the synthesis of other amino acids. This relationship has been observed for linamarin catabolism in early seedling growth on *Brasiliensis* in which the authors demonstrated that cyanogenic glycosides are mobilized and metabolized to increase seedling growth (Selmar et al., 1988). Linamarin is hydrolysed to linustatin, a mobile form not hydrolyzed by β-glucosidases. While no HCN was detected, the activity of β-CAS was high, suggesting assimilation of HCN into amino acids. The same trend has been reported in almonds (Sánchez-Pérez et al., 2008) and in barley where the activity of β-CAS is proportional to the level of cyanogenic glycosides (Forslund and Jonsson, 1997). In some tissues (e.g. roots), β-CAS activity was ~500 fold higher than the rate of cyanogenic glycoside catabolism.

5.3 Assimilation of ethylene-associated cyanide

The β-CAS pathway primarily metabolizes ethylene-associated cyanide and activity of the pathway increases concomitantly with increases in ethylene synthesis to provide cyanide homeostasis. The production of ethylene-associated cyanide in specific tissues would create a

local demand for cyanide detoxification which may therefore represent the more universal role for this pathway in plants. Cyanide detoxification via the β-cyanoalanine pathway may in some respects be expressed synonymously as cyanide assimilation since detoxification is achieved through assimilation rather than through the degradation, reduction, or oxidation pathways found in bacteria and fungi (Ebbs, 2004; Gupta et al., 2010; Raybuck, 1992). Describing activity more broadly in terms of assimilation also removes the presumption that cyanide need be present in potentially toxic concentrations for the pathway to be functionally relevant. This context would be an appropriate description for the assimilation of ethylene-derived cyanide by the β-cyanoalanine pathway.

The steady state concentration of cyanide in plant tissues is very low (e.g., ~0.2 μM) with the most cyanide-sensitive enzymes inhibited by concentrations exceeding ~10 μM (Yip and Yang, 1988). Tissue cyanide concentrations of up to 350 μM have been reported for some climacteric fruit at the peak of ethylene synthesis during ripening (Mizutani et al., 1988). Other estimates of cyanide production during periods of ethylene synthesis range from 5-50 pmol min^{-1} g^{-1} FW in barley (Wurtele et al., 1985) to values as high as ~150 nmol min^{-1} g^{-1} FW reported in apples (*Malus domestica*) and avocado (*Persea americana*) (Yip and Yang, 1998). In tobacco a 10-fold increase from a baseline of 80 nmol g^{-1} FW was reported when plants were subjected to water deficit stress (Liang, 2003). The increases in cyanide in response to different conditions is however transient, returning to basal levels when the disturbance is removed. While the transient response is attributed to induction of β-CAS activity, it is important to note that ethylene production in response to a disturbance is also a transient signaling event (Takahashi et al., 2006). Thus, because synthesis and removal of cyanide are not perfectly coordinated, the two mechanisms are needed to balance cyanide homeostasis. Both ethylene and cyanide

concentrations vary with species, developmental stage, and both abiotic and biotic conditions the plant is experiencing.

5.4 Programmed cell death

The role of the β-CAS pathway in programmed cell death (PCD) may be considered as indirect via the interaction of ethylene and cyanide. PCD is an active process of cell death which occurs as a developmental stage or in response to stressful environmental conditions (Samuilov et al., 2002). On the one hand, cyanide is known to induce PCD in plants via inhibition of antioxidant enzymes, catalase and peroxidases. Inhibition of the enzymes causes accumulation of H_2O_2 and consequently hydroxyl radicals that destroy DNA, proteins and lipids (Samuilov et al., 2000). On the other hand one study has reported a strong correlation of ethylene evolution and ozone-induced cell death in birch (*Betula pendula*) ethylene insensitive mutants (Vahala et al., 2003). These mutant plants would be impaired in cyanide detoxification since ethylene is also known to regulate the β-CAS gene (Goudey et al., 1989). The same correlation could be said of cyanide since the two gases are produced in equimolar amounts. Indeed, in many studies the effects of cyanide and ethylene are often inseparable. This study also showed that O_3-induced activation of β-CAS was dependent on ethylene biosynthesis. Application of 100-300 μM cyanide induced PCD and ethylene insensitive plants produced lesions on exposure to O_3, which was attributed to defective cyanide metabolism. In tobacco plants infected with tobacco mosaic virus, there was increased cyanide production localized to the affected area, preceded by suppression of β-CAS activity, necrotic lesions, followed by cell death (Siefert et al., 1995). The suppression of β-CAS enzyme activity thus appears to be a deliberate mechanism to induce death by cyanide toxicity. Separate compartmentation of cyanide biosynthesis and degradation has also

been postulated as a possible mechanism for cell death in some species. While cyanide is released in the cytoplasm by ACC oxidase, its degradation is effectively in mitochondria by β-CAS. When 2,4-D was applied to barley and soybean (*Glycine max* L.), the response was an increase in cyanide and ethylene in both species with a greater magnitude of increase in soybean (Tittle et al., 1990). While this increase was accompanied by increased β-CAS enzyme activity in soybean, there was no change in β-CAS activity in barley. The authors suggested that in herbicide sensitive grasses, cell death may be due to accumulation of cyanide coupled to an inefficiency to detoxify the cyanide because of separate compartmentation. The results also suggest that there are species specific differences in mechanisms of induction of the β-CAS enzyme. An increase in ethylene production during pathogen infection would generally, be an expected response but a concomitant down-regulation of β-CAS observed in tobacco (Siefert et al., 1995) would be contrary to tolerance mechanisms. It was thus speculated that when cell death is required during stress and PCD, β-CAS activity is depressed resulting in a build-up of cyanide, and localized cell death.

5.5 Contribution of the pathway to plant growth and development

5.5.1 Seed germination

Several studies have demonstrated that the synthesis of amino acids resulting from cyanide detoxification by the β-CAS pathway also fulfills a fundamental biological role during seed germination (Esashi et al., 1996; Maruyama et al., 1997; Selmar et al., 1988). Seeds of a variety of species store cyanogenic compounds as a source of nitrogen (Sanchez-Perez et al., 2008; Yildirim and Askin, 2010). Immediately prior to and during germination there is increased evolution of endogenous HCN as the cyanogenic nitrogen is liberated (Esashi et al., 1991). Even

for non-cyanogenic species, a burst of ethylene was also observed during germination (Gianinetti et al., 2007). The cyanide is thought to be involved in breaking seed dormancy and promoting germination (Bogatek and Lewak, 1991; Esashi et al., 1996; Maruyama et al., 1996; Oracz et al., 2008). The two isoforms belonging to the beta-substituted alanine synthase (Bsas) family of enzymes, implicated in the detoxification of cyanide were identified in unimbibed seeds of rice (*Oryza sativa* L.) and cocklebur (*Xanthium pennsylvanicum* Wallr.) and some residual β-CAS-like activity was detected (Hasegawa et al., 1995a). The β-CAS activity increased considerably during imbibition after induction following an ethylene burst. Similarly, hydrogen sulfide was evolved along with β-cyanoalanine during germination of *Amaranthus albus* and *Lactuca sativa* seeds (Taylorson and Hendricks, 1973). In this study addition of β-cyanoalanine was the most effective in promoting seed germination as compared to other amino acids. The presumption is that there was increased activity of the β-CAS pathway resulting in more asparagine, aspartate and ammonia, and subsequent incorporation of the amino acids into protein. Although the mechanisms by which the pathway is involved in seed germination are variable and not clear (Vahala et al., 2003), the studies seem to show a strong correlation between β-CAS activity and changes in corresponding amino acid pools in imbibed seeds. What is clear though, is that cyanogenic compounds in seeds provide nitrogen and carbonyl compounds for incorporation into amino acids necessary to initiate seed germination (Maruyama et al., 1998; Maruyama et al., 1997). In these studies there were increases in the concentrations of some amino acids during germination of cocklebur seeds. In addition to providing the embryo with amino acids for protein synthesis, another hypothesis has been proposed that the increase in the size of the amino acid pool decreases water potential of the embryonic axis, which favors water influx and radical elongation (Grossmann, 1996). A second benefit from activity of the β-cyanoalanine pathway

during germination and seedling establishment is an increase in the pool of free thiols (Vahala et al., 2003). Thiols, particularly H_2S, have been shown to promote seed germination in wheat seeds exposed to Cu stress indirectly through accumulation of free amino acids (Zhang et al., 2008).

5.5.2 Root elongation

Hydrogen cyanide has negative effects on root metabolism. There are reports that microorganisms in the rhizosphere produce toxic levels of cyanide which inhibit root growth (Kremer and Souissi, 2001). It is well established that cyanide inhibits cytochrome respiration and several other biochemical processes (Grossmann, 1998). A recent study has reported that cyanide (as low as 100µM) suppresses auxin biosynthesis at the root tip thereby inhibiting primary root growth in *A. thaliana* (Rudrappa et al., 2008). The hormonal crosstalk could be more complicated since gibberellins and cytokinins are involved in root initiation and growth. Similarly, cyanide metabolism is implicated in root hair formation and elongation in *A. thaliana* (García et al., 2010). In this study the authors showed defects in root hair formation and lack of elongation when the β-CAS gene was disrupted. Plants may have low levels of cyanide to prevent inhibition of respiration but the levels seem sufficient to inhibit root hair elongation. When cyanide accumulates, it is presumed to become a repressive signal for genes encoding enzymes involved in cell wall rebuilding, root tip development, and ethylene signaling (García et al., 2010). Therefore β-CAS activity keeps that low level of cyanide to maintain proper root hair development. In agreement, Howden et al. (2009) reported that impaired cyanide metabolism in *A. thaliana NIT4* mutants caused root physiological defects while increased expression of nitrilase activity in wild type plants improved root development significantly.

5.6 Role of β-CAS pathway in primary metabolism

5.6.1 Nitrogen metabolism

Some evidence suggests that cyanide regulates nitrogen assimilation by acting as a signaling molecule (Yip and Yang, 1998) perhaps by regulating nitrate reductase (Solomonson and Spehar, 1979). If this is true, the β-CAS pathway would be involved in the shifts in amino acids pools mentioned above as it involves allocating nitrogen and carbon. In addition cyanogenic glycosides as storage compounds are broken down to supply nitrogen and carbon when they are required. Siegien and Bogatek (2006) have proposed a mechanism in which the presence of cyanide presumably changes the abundance of genes associated with primary nitrogen metabolism at transcriptional level, in favor of the β-CAS pathway. When spinach plants were deprived of nitrogen and nitrogen/sulfur, the mRNA levels of β-CAS increased significantly (Takahashi and Saito, 1996). Nevertheless, it is not apparent whether the mRNA was translated into enzyme activity. Plants generally take up nitrogen in the form of nitrate and ammonia and it is in these two forms that fertilizers are conventionally added to soil. However most of the nitrogen in the soil is in bound organic form and require microbial processes to degrade and make nitrogen available (Schulten and Schnitzer, 1998). Consequently, many rhizobacteria are able to degrade these nitrile compounds in addition to forming associations with plant roots (Howden et al., 2009). Plants may then among other mechanisms, use the β-CAS pathway to acquire nitrogen from the available cyanide in the rhizosphere. Indeed various plant species have been shown to take up cyanide from growth medium and the cyanide is quickly metabolized and assimilated (Larsen et al., 2004; Yu et al., 2007; Yu et al., 2004; Yu et al., 2005b). Although in these studies the authors do not definitively mention the nitrogen flux through the β-CAS pathway and also a crosstalk with the primary nitrogen pathways, it is

apparent that the ability to metabolize and assimilate cyanide could be attributed to this pathway. The role of β-CAS in metabolism of cyanide has recently been shown by an upregulation of β-CAS enzyme activity in rice seedlings exposed to KCN (Yu et al., 2012). When sorghum and wheat plants were deprived of nitrogen and exposed to labeled cyanide, more ^{15}N was observed in these plants than in plants with standard N-supply (Ebbs et al., 2010). An increased activity of the β-CAS and asparaginase enzymes in wheat plants exposed to cyanide was also reported (Machingura and Ebbs, 2010), demonstrating that exogenous cyanide makes contribution to plant nitrogen metabolism (Ebbs et al., 2003; Samiotakis and Ebbs, 2004). The direct assimilation of nitrogen into asparagine and aspartate presents yet another benefit of cyanide detoxification by increasing amino acid pools. In the simplest context, cyanide assimilation could be described as a metabolic effort to recycle cyanogenic carbon and nitrogen to prevent the loss of the macronutrients to volatilization. In addition to recycling, the pathway provides an alternate source of ammoniacal nitrogen derived from endogenous or exogenous cyanide. In addition to amino acids, ammonia has since been established to be an ultimate product of cyanide detoxification and assimilation, and this ammonia is directly incorporated into primary nitrogen metabolism.

5.6.2 Sulfur metabolism

The β-cyanoalanine synthase enzyme may itself have an accessory function in sulfur metabolism stemming from its capacity to mediate cysteine synthesis. β-cyanoalanine synthase is a bifunctional enzyme by virtue of a pyridoxal phosphate cofactor found in all members of the Bsas family of enzymes. In addition to cyanide removal, the enzyme has been shown to catalyze cysteine biosynthesis using o-acetyl-serine (OAS) and sulfide as substrates (Hatzfeld et al., 2000;

Yamaguchi et al., 2000), albeit with lower efficiency (e.g., higher K_M value). Cysteine biosynthesis is a key step in S-assimilation which incorporates inorganic sulfur into organic form. While *A. thaliana* is, to our knowledge, the only species with two Bsas enzymes in the mitochondria, Bsas2.2 and Bsas3.1 (Hesse et al., 1999; Hesse et al., 2004), a high level of mitochondrial cysteine biosynthesis for the Bsas2.2 mutant has been demonstrated (Watanabe et al., 2008). Although we cannot conclude that β-CAS is involved in cysteine biosynthesis *in vivo*, it is reasonable to assume that β-CAS is responsible for the mitochondrial cysteine synthase activity observed in the Bsas2.2 mutant. Consequently, it has also been demonstrated that β-CAS enzyme activity in spinach is increased in response to sulfur-deprivation for 7 and 15 d, and yet no change was reported in cysteine synthase activity (Warrilow and Hawkesford, 1998). The authors suggested that a disturbance in N and S metabolism may have led to increased cyanide production, hence the increase in β-CAS activity. However, this increase may also be explained by a direct involvement of β-CAS in cysteine biosynthesis. By virtue of lower efficiency of β-CAS for the cysteine synthase reaction, more of this protein would be synthesized to compensate for the low efficiency. Thus the increase was perhaps not cyanide or stress related.

Hydrogen sulfide is one of the products released alongside with β-cyanoalanine. A recent study implicates the role of H_2S in enhancing expression of enzymes involved in thiol redox modification in spinach and promoting chloroplast biogenesis (Juan et al., 2011). Through these mechanisms elevated levels of H_2S led to increased photosynthesis. In *A. thaliana*, H_2S released from metabolic processes induced expression of drought tolerance genes (Jin et al., 2011). Lisjak et al., (2010) used a compound GYY4137 to show that slow and sustained production of H_2S is involved in signaling and closure of stomata. Evidence suggests that the function of H_2S will depend on the form in which it exists in the cell as governed by pH. At low pH up to about

6.0, H_2S is the predominant species which would be involved in signaling or would also be lost through volatilization. However in cell compartments where pH is 7.0 or greater, the hydrogen sulfide will readily ionize, and gets incorporated into sulfur compounds. One study showed that 40% of H_2S ionizes and is converted to glutathione and other reduced S-compounds involved in stress tolerance (Kok et al., 1985). The fate of H_2S produced in the β-CAS pathway however, requires more research.

5.7 Response to biotic and abiotic stress

Cyanide metabolism is implicated in the tolerance of plants to biotic and abiotic stresses and in the mechanism of herbicide action. Increased ethylene production is a pronounced response to stress and is known to induce β-CAS activity. One study has shown increased cyanide and activity of this enzyme when tobacco plants were subjected to water deficit for one day (Liang, 2003). Additionally a strong correlation between enzyme activity and ethylene production was demonstrated (Miller and Conn, 1980). The trend was the same regardless of whether the species was cyanogenic or not. Other examples that demonstrate the elevated ethylene production levels during abiotic stress include wheat subjected to heat stress (38^{o}C) (Hays et al., 2007)**,** application of 2,4-D in barley (Tittle et al., 1990) and in mungbean (*Vigna radiata*) seedlings exposed to osmotic stress (Ke and Sun, 2004). In all cases, the authors concluded that ethylene was responsible for regulation of the particular stress. It is presumed that this transient response will elicit downstream pathways that impart stress tolerance.

Many bacteria in the rhizosphere produce HCN and studies demonstrate that cyanide has functional roles in biological control of soil-borne pathogens (Bagnasco et al., 1998; Goel et al.,

2002). In one study tomato (*Lycopersicum esculentum*) roots were treated with a glycoprotein CWP produced by *Pythium oligandrum.* The *LeCAS* gene was among the highly induced genes and the authors suggested association of the cyanide detoxification mechanism with signaling pathway for induced resistance (Hase et al., 2006; Takahashi et al., 2006). Increased expression of *LeCAS* coincided with ethylene production. The same induction of ethylene production was observed in cell cultures of tomato and potato (*Solanum tuberosum*) treated with elicitors from *Phytopthora spp* (Basse and Boller, 1992). These studies suggest that the β-CAS pathway is involved in induced resistance to biotic stress by fungal glycoprotein elicitor. Other examples in which cyanide is involved in biotic stresses include tobacco black rot and tomato crown and root rot (Duffy et al., 2004; Voisard et al., 1989). The increase in plant resistance to pathogenic attack (Grossmann, 1996) is effected through localized CN-mediated cell death (Siefert et al., 1995). Further indication of a role of this pathway in the detoxification of cyanide was found in the rubber tree where an imbalance between the rate of cyanide release from cyanogenic glycosides and the activity of β-cyanoalanine synthase resulted in increased cyanide concentration in plant tissues, which the authors suggested might be a causal agent for the susceptibility to bark necrosis syndrome in that plant species (Chrestin et al., 2004).

The mediation of the β-CAS pathway as in biotic stress response could further be extended to abiotic stress response as in the phytotoxic action for herbicides on noxious weeds. Quinclorac is a selective, auxinic herbicide for control of dicot, but it also controls some monocot weeds. The mode of action of quinclorac in sensitive plants such as tomato and barnyard grass (*Echinocloa crus-gali* L.) involves stimulation of excess cyanide production coupled to low activity of the β-CAS enzyme (Abdallah et al., 2006; Grossmann and Kwiatkowski, 2000). In a study with resistant and susceptible crabgrass (*Digitaria ischaemum*)

quinclorac induces ACC synthase activity in the susceptible biotype leading to ethylene and also cyanide accumulation, with insignificant change in the resistant biotype (Abdallah et al., 2006). The selective induction of ACC synthase has been suggested as basis of resistance or susceptibility in different species (Grossmann and Kwiatkowski, 1995). Similarly, tolerant rice plants did not show differences in cyanide and ethylene production or β-CAS activity (Grossmann and Kwiatkowski, 1995; Grossmann and Scheltrup, 1997). Another auxinic herbicide, 2,4-D had the same effect on susceptible soybean and less effect in barley (Tittle et al., 1990), so did other herbicides in the same class of auxins in susceptible dicots (Grossmann, 1996). It is probable that ethylene is only responsible for the stress responses preceding death, while cyanide is the actual cause of death (Grossmann, 1996).

5.8 Assimilation of exogenous cyanide

Plants are constantly exposed to cyanide from a wide range of nitrile compounds in the environment and through associations with cyanide producing microorganisms. Since cyanogenic bacteria and fungi are ubiquitous in the plant environment (Ramette et al., 2003), it is reasonable that these microbes in the plant-soil environment must be able to metabolize cyanide. Studies have reported that cyanide is a major source of nitrogen for these organisms. PinA, a nitrilase 4 ortholog found in bacteria that associate with plants showed 44% sequence similarity with the plant nitrilase 4 (Howden et al., 2009). Expression of PinA was induced by *A. thaliana* and sugarbeet (*Beta vulgaris*) seedlings. The authors concluded that expression of PinA required a plant-derived signal and that it confers tolerance to high levels of cyanide in the environment. Reports of growth producing rhizobateria (PGPR) in the rhizosphere are abundant (Bertrand et

al., 2000; Bloemberg and Lugtenberg, 2001). Among several mechanisms of action, production of cyanide is often referred to as an anti-fungal metabolite, indirectly enhancing plant growth (Nelson, 2004). In view of the β-CAS pathway, it may be appropriate to consider this mechanism as directly providing cyanide as an alternative form of nitrogen to the plant.

The functional role of the β-CAS pathway seems to be of a greater magnitude than the ecologically expressed levels and may extend to utilization of high concentrations of exogenous cyanide released from industrial wastes. Studies have reported willow (*Salix spp*) plants thriving in cyanide contaminated environments (Trapp et al., 2001). Diamond willow (*Salix eriocephala*) did not show signs of stress in 2 mg CN L^{-1} (Ebbs et al., 2003) while grass crops performed well in 50 mg CN L^{-1} (Samiotakis and Ebbs, 2004). It is clear from these examples that the degree of tolerance is species specific. Because the magnitude of β-CAS activity in plants is much greater than the basal rate of ethylene production (Yip and Yang, 1998) we may speculate that the ability to metabolize cyanide and prevent toxicity is fairly elastic and is still applicable under non-physiological conditions. Activity of this pathway does contribute to detoxification of cyanide obtained from exogenous sources (Ebbs et al., 2003; Larsen et al., 2004; Yu et al., 2005a; Yu et al., 2004), but the cyanide generated by natural sources including bacterial and fungal cyanogenesis (Castric, 1981; Estes et al., 1988; Knowles, 1976), release of cyanide from decomposing cyanogenic plants (Widmer and Abawi, 2002) and forest fires (Li et al., 2000) rarely reaches concentrations phytotoxic to plants. Cyanide concentrations in excess of 100 mg kg^{-1} DW soil have been reported in the rhizosphere of some plants colonized by cyanogenic bacteria (Kesler-Arnold and O'Hearn, 1990; Owen and Zdor, 2001) but there is differential sensitivity of plants to such concentrations. Corn for example, growing in the presence of these cyanogenic bacteria showed no significant reduction in growth whereas velvetleaf (*Abutilon*

theophrasti) showed growth reductions of up to 80% (Owen and Zdor, 2001). Even so, soils with naturally occurring cyanide concentrations high enough to be phytotoxic would be highly localized and intermittent. The increasing use and release of cyanide by industry represents a significant anthropogenic source of cyanide in terrestrial and aquatic environments, with concentrations exceeding 1,000 mg kg^{-1} and 10,000 mg L^{-1} respectively in some instances (Grosse, 1990; Henny et al., 1994), but this is a contemporary phenomenon.

5.9 Cyanide microcycles

Aside from these innate biological roles of the pathway and its enzymes, the β-CAS pathway in plants also serves an ecological role in the cycling of cyanogenic nitrogen within some communities as well as a more contemporary function in protecting plants from the cyanide exposures that result from anthropogenic activities. Some reports have proposed natural microcycles between those organisms that produce and those that degrade and assimilate cyanide (Allen and Strobel, 1966; Thatcher and Weaver, 1976). Cyanide is produced by plants as glycosides and also by bacteria and fungi during metabolic processes. The cyanide is converted by microbes to forms of carbon and nitrogen which are assimilated by plants. Many species for example, sorghum, have been shown to have pathogenic fungi which convert cyanide to formamide, then to CO_2 and NH_3 (Thatcher and Weaver, 1976). In view of the many biological transformations, and natural sinks, it is not surprising that there are currently no reports of bioaccumulation of free cyanide in the environment.

Table 1.1 Distribution of the β-CAS pathway in plants and localization within tissues. The number of plus signs indicates the level of enzyme activity relative to the basal level: <35 nmol mg^{-1}Protein hr^{-1}).

Species Common name	Latin name	Whole seedling	Shoots	Leaves/ cotyledon	Fruit/Seed	Roots/tuber	Reference
Alfalfa	*Medicago sativa*	+					(Miller and Conn, 1980)
Arabidopsis	*Arabidopsis thaliana*		+ +	+		+	(Hatzfeld et al., 2000; Yamaguchi et al., 2000)
Apple	*Pyrus malus*				+		(Han et al., 2007)
Barley	*Hordeum vulgare*	+++		+	+		(Wurtele, Nikolau, and Conn, 1985; Goudey, Tittle, and Spencer, 1989)
Blue lupine	*Lupinus angustifolia*	+	+	+			(Hendrickson and Conn, 1969; Akopyan, Braunstein, and Goryachenkova, 1975; Miller and Conn, 1980),
Cabbage	*Brassica oleracea*	++		+++			(Miller and Conn, 1980)
California poppy	*Eschscholzia californica*	+					(Miller and Conn, 1980)
Cassava	*Manihot esculenta*		+	+++		+++++	(Elias, Sudhakaran, and Nambisan, 1997)
Common vetch	*Vicia sativa*	+					(Miller and Conn, 1980) Ressler
Common flax	*Linum usitatissimum*		+			+	(Miller and Conn, 1980)
Clover	*Trifolium repens*	+					(Miller and Conn, 1980)
Cocklebur	*Xanthium pennysylvanicum*	+			+		(Hasegawa et al., 1994; Maruyama et al., 1996)
Cucumber	*Cucumis satiyus*				+		(Hasegawa et al., 1995)
Leek	*Allium porrum*			+			(Wurtele, Nikolau, and Conn, 1984)
Lettuce	*Lactuca sativa*	+					(Goudey, Tittle, and Spencer, 1989)
Lima bean	*Phaseolus lunatus*			++++			(Miller and Conn, 1980)
Leaf mustard	*Brassica juncea*	+					(Miller and Conn, 1980)
Loquat	*Eryobotrya japonica*	+					(Miller and Conn, 1980)
Lotus	*Lotus tenuis*	+					(Miller and Conn, 1980)
Maize/corn	*Zea mays*			++			(Wurtele, Nikolau, and Conn, 1984)
Mungbean	*Phaseolus aureus*	+					(Miller and Conn, 1980; Goudey, Tittle, and Spencer, 1989)
Potato	*Solanum tuberosum*					++	(Wen et al., 1997; Maruyama, Saito, and Ishizawa, 2001)
Pea	*Pisum sativum*	+		+		+	(Wurtele, Nikolau, and Conn, 1984; Goudey, Tittle, and Spencer, 1989)
Rice	*Oryza sativa*	+		+	+		(Lai et al., 2009)
Soybean	*Glycine max*	+					(Tittle, Goudey, and Spencer, 1990)
Spinach	*Spinacea*	+		+++++		+	(Warrilow and Hawkesford, 1998; Hatzfeld et al., 2000)
Sorghum	*Sorghum bicolor*		++++	++		++	(Miller and Conn, 1980; Wurtele, Nikolau, and Conn, 1984)
Tobacco	*Nicotiana tabacum*			+++		+	(Liang, 2003)
Wheat	*Triticum aestivum*	+++					(Goudey, Tittle, and Spencer, 1989)
Wild mustard	*Sinapis arvensis*	++					(Goudey, Tittle, and Spencer, 1989)
Avocado	*Persea gratissima*				+++++		(Yip and Yang, 1988)

Table 1.2 Summary of the physiological and ecological functions of the β-CAS pathway

Function	**Species**	**Mechanism of action**	**References**
Detoxification and assimilation of ethylene-associated cyanide	Arabidopsis, tobacco, potato	Direct reaction of cyanide and cysteine,	(Smith and Arteca, 2000) (Liang, 2003), (Wen et al., 1997)
Assimilation of exogenous cyanide from anthropogenic sources	Willow	Cross talk with primary N metabolism, suppression of nitrate reductase	(Ebbs et al., 2003), (Larsen et al., 2004)
Detoxification and assimilation of cyanide from degradation of cyanogenic glycosides	Barley, almond, sorghum, cassava	Direct reaction of cyanide and cysteine	(Forslund and Jonsson,1997),(Sánchez-Pérez et al., 2008), (Elias et al., 1997)
Metabolism of nitrogen stored in cyanogenic glycosides, N-recycling back into primary metabolism	Brasiliensis	Incorporation on C and N from cyanide into primary amino acids Signaling molecule, represses nitrate reductase	Selmar et al., 1988 (Siegien and Bogatek, 2006)
Breaking seed dormancy Seed germination	Sunflower Cocklebur, lactuca amaranthus Wheat	Accumulation of ROS increases carbonylation of proteins Direct assimilation into asparagine and aspartate increases amino acid pools, decreases water potential, promoting water uptake , Release of H_2S	(Oracz et al., 2007; Oracz et al., 2008) (Esashi et al., 1996), (Maruyama et al., 1997) (Hasegawa et al., 1995b) (Zhang et al., 2008)
Sulfur metabolism	Arabidopsis	Cysteine biosynthesis in mitochondria Release of H_2S, increase in supply of thiol group promotes chloroplast biogenesis, confers drought tolerance	(Hatzfeld et al., 2000), (Juan et al., 2011) (Jin et al., 2011)

Table 1.2 (continued)

Root elongation, root hair formation	Arabidopsis	Maintains cyanide homeostasis	(García et al., 2010) (Howden et al., 2009)
Programmed cell death	Tobacco	Separate compartments of cyanide production and removal causes inefficient cyanide removal	(Samuilov et al., 2000) (Siefert et al., 1995)
Confers tolerance to abiotic stress	Wheat, tobacco, birch, mungbean	Transient ethylene/cyanide signals tolerance mechanisms	(Hays et al., 2007) (Vahala et al., 2003)
Defense against herbivory	Cassava, sorghum, lotus	The 'cyanide bomb' Products and intermediates of cyanide metabolism are toxic	(Morant et al., 2008a; Zagrobelny et al., 2004)
Defense against pathogens, disease	Rubber tree, tobacco, rice	Cyanide as an anti-fungal/viral metabolite, Induction of alternative oxidase	(Chrestin et al., 2004) (Chivasa and Carr, 1998)
Mode of action in herbicides quinclorac & 2.4-D	Tomato, barley, soybean, barnyard grass	Ethylene and cyanide burst coupled to down-regulated β-CAS activity	(Grossmann and Kwiatkowski, 2000; Tittle et al., 1990),
Ecological N cycling	Plants that form associations with PGPR e.g sorghum, Arabidopsis, sugarbeet	Rhizosphere microbes release HCN during breakdown of carbonyl compounds Cyanide is an alternative nitrogen source	(Thatcher and Weaver, 1976), (Howden et al., 2009)

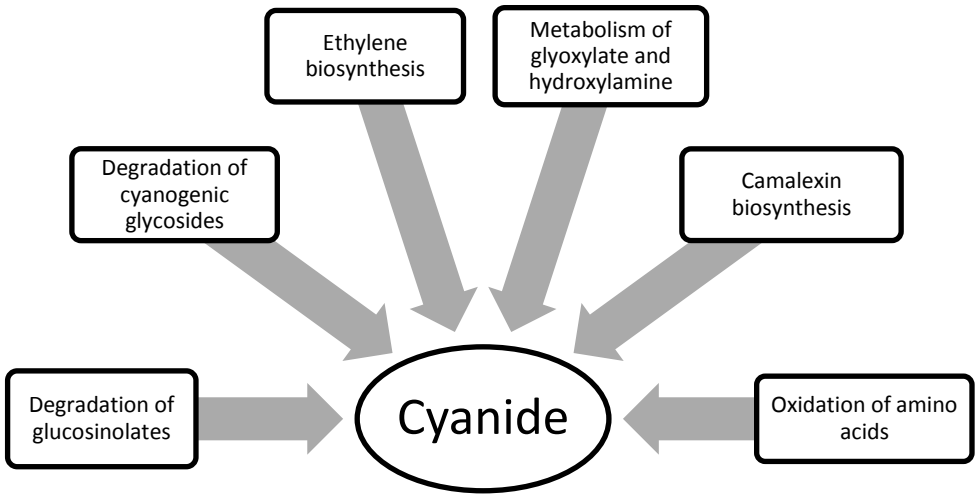

Figure 1.1 Pathways for cyanide biosynthesis in higher plants.

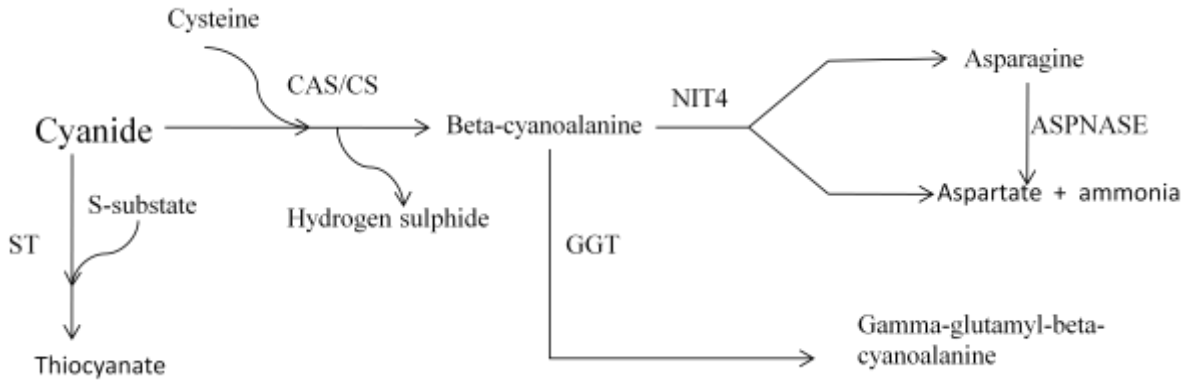

Figure 1.2 Two pathways for cyanide detoxification and assimilation in higher plants. In capital letters: names of enzymes mediating the reactions. CAS=β-cyanoalanine synthase, CS=cysteine synthase, NIT4=nitrilase 4, ASPNASE=asparaginase, ST=sulfurtransferase, GGT=γ-glutamyl-transferase.

CHAPTER 2

PHYLOGENY AND ENZYMOLOGY OF β-CYANOALANINE PATHWAY ENZYMES

1.0 Introduction

Cyanide is produced in higher plants during breakdown of cyanogenic glycosides and cyanolipids for those species that have the secondary compounds (Jones, 1998; Sánchez-Pérez et al., 2008) and during ethylene biosynthesis in all higher plants (Yip and Yang, 1988). The concentrations of endogenous cyanide generated by these pathways are typically quite low (<10 μM) but vary in magnitude depending on the species, growing conditions and developmental stage of the plant. For example, higher concentrations of tissue cyanide coincident with increased ethylene synthesis have been reported during periods of abiotic stress (Liang, 2003; Vahala et al., 2003), in ripening fruit (Yip and Yang, 1998) and senescing tissues (Manning, 1986). Cyanide is a metabolic inhibitor of metalloenzymes, particularly cytochrome *c* oxidase, inhibiting respiration and other vital metabolic processes (Solomonson, 1981). Plants detoxify cyanide to maintain cyanide homeostasis in the tissues and this detoxification is achieved by assimilating the carbon and nitrogen into primary metabolism. There are two possible metabolic pathways that could provide cyanide detoxification. One pathway is mediated by sulfurtransferases (e.g rhodanese and 3-mercaptopyruvate sulfurtransferase), multifunctional enzymes in bacteria, animals and plants encoded by a small gene family. The primary function of sulfurtransferases is clearly associated with sulfur metabolism. As the name implies, these enzymes transfer the thiol group from a donor to a recipient molecule. In the case of cyanide detoxification, the thiol is transferred to cyanide to form thiocyanate (Meyer et al., 2003). In animals, thiocyanate is excreted in urine (Papenbrock and Schmidt, 2000a; Ressler and Tatake, 2001; Saidu, 2004) and in plants (e.g. cabbage) thiocyanate is methylated by thiol

methyltransferase (Attieh et al., 2000). Protein sequence alignment of several thiol methyltransferases reveal high homology (Attieh et al., 2002), suggesting a common mechanism of thiocyanate detoxification. There are sulfurtransferase genes in plants (Meyer et al., 2003) but there is limited evidence of thiocyanate synthesis via this pathway in plants (Kakes and Hakvoort, 1992; Miller and Conn, 1980; Nakamura et al., 2000). Efforts to provide convincing evidence for the role of sulfurtransferases in cyanide detoxification in plants have not been successful (Papenbrock and Schmidt, 2000a; Papenbrock and Schmidt, 2000b).

The primary biochemical pathway for cyanide detoxification in plants is the β-cyanoalanine synthase (β-CAS) pathway. While the release of HCN in plants was first detected in 1802 in bitter almonds (*Prunus amygdalus* var. *amara*), the β-CAS pathway was not discovered in plants until the 1960s. Since then, considerable work has been conducted to characterize the enzymes associated with two sequential steps and the corresponding genes. Some of the most significant work on this pathway has come in the past 10-15 years. This pathway has two enzymatic steps (Equation 1 and 2). The first is mediated by two isoenzymes of the beta-substituted alanine synthase (Bsas) family while a nitrilase/nitrile hydratase mediates the second step.

$$\text{Cysteine} + \text{HCN} \longrightarrow \beta - \text{cyanoalanine} + H_2S \qquad \text{eq. 1}$$

$$\beta - \text{cyanoalanine} + H_2O \longrightarrow \text{asparagine} + \text{aspartate} + NH_4^+ \qquad \text{eq. 2}$$

The principal enzyme carrying out the first step of the pathway is β-cyanoalanine synthase [EC 4.4.1.9]. This enzyme transfers the sulfhydryl moiety from a cysteine residue to cyanide to form the non-protein amino acid β-cyanoalanine (Blumenthal et al., 1968; Hendrickson and Conn, 1969). β-Cyanoalanine was initially thought to be hydrolyzed by cyanoalanine hydrolase [EC

4.2.1.65] or cyanoalanine hydratase [4.2.1.66] into asparagine and/or aspartate and ammonia (Castric et al., 1972). Studies with *A. thaliana* and tobacco later showed that the enzyme responsible for this step, designated as a nitrilase 4 (NIT4) [3.5.5.1], is bifunctional and capable of carrying out both nitrilase and nitrile hydratase activities simultaneously, using β-cyanoalanine as the substrate to form the indicated products (Piotrowski et al., 2001; Piotrowski and Volmer, 2006).

Although β-cyanoalanine synthase and nitrilase 4 activities make major contributions to cyanide detoxification and assimilation, emerging information suggests that there are other enzymes that are associated with the pathway. The cytosolic cysteine synthase [EC 2.5.1.47] also belonging to the Bsas family, possesses β-cyanoalanine synthase-like activity (Maruyama et al., 2000; Warrilow and Hawkesford, 1998; Watanabe et al., 2008) although the primary function of this enzyme is in cysteine synthesis (Lopez-Martin et al., 2008). Asparaginase [EC 3.5.1.1] could be considered an extension of this pathway as this enzyme is involved in a deamination reaction that converts asparagine to aspartate and ammonia. Gamma-glutamyl transferase [EC 2.3.2.2] has been shown in *Vicia* and *A. thaliana* to utilize β-cyanoalanine to form γ-glutamyl-β-cyanoalanine (Ressler et al., 1969b; Watanabe et al., 2008). This review provides a discussion on current knowledge on the enzymology and phylogeny of the principal enzymes and genes in angiosperms associated with the cyanoalanine synthase pathway. Discussion of the possible evolutionary pressures that gave rise to these genes and their recruitment into the pathway is also included as is growing evidence of crosstalk between this cyanide assimilation pathway and other pathways associated with growth, development, and the response to biotic and abiotic stresses.

2.0 Phylogeny of β-cyanoalanine synthase

β-Cyanoalanine synthase belongs to the Bsas superfamily of genes. The family has been resolved into six subfamilies representing three primary functional groups of enzymes (Hatzfeld et al., 2000). Three subfamilies (*Bsas1*, *Bsas2*, and *Bsas4*) are *O*-acetylserine(thiol)lyases (OAS-TL), although the term cysteine synthase is perhaps more commonly used (Blumenthal et al., 1968; Elias et al., 1997; Hasegawa et al., 1995b). The genes *Bsas1* and *Bsas4* encode cytosol enzymes while *Bsas2* encodes a plastid-localized protein (Hatzfeld et al., 2000; Lunn et al., 1990; Rolland et al., 1992; Watanabe et al., 2008). The *Bsas5* and *Bsas6* subfamilies are largely uncharacterized and the localization of the gene products has not been conclusively established. The Bsas5.1 gene has been shown to encode the enzyme S-sulfocysteine synthase that mediates cysteine synthesis by accepting thiosulfate instead of sulfide (Bermúdez et al., 2010), although there is speculation that this may not be its primary function (Hatzfeld et al., 2000). Enzymes involved in cysteine synthesis must have an obligate interaction with serine-acetyl transferase (SAT) (Droux et al., 1998; Wirtz and Hell, 2006) and yet this Bsas5.1 encoded enzyme does not show this property (Bonner et al., 2005). Recently Wirtz et al. (2010) provided evidence for its role in chloroplast function and redox control in agreement with localization to the plastid. β-cyanoalanine synthase is the only representative of the *Bsas3* subfamily (Hatzfeld et al., 2000; Lai et al., 2009) . The gene encoding this enzyme is widely distributed across species having been detected in all higher plants tested. Table 2.1 gives examples of the β-CAS-like enzymes as characterized in some selected species and, the kinetic parameters are quite variable across the species. This is in agreement with comparison of sequence alignments of rice-CAS and β-CAS-like proteins from other plant species which reports 69-71% homology (Lai et al., 2009).

The *Bsas* genes show homology to both bacterial cysteine synthases and to fungal cystathionine β-synthases (CBS). There is speculation that the *Bsas5* and *Bsas6* subfamilies may represent plant CBS genes (Hatzfeld et al., 2000), with the remaining four subfamilies representing homologous cysteine synthases derived from a common ancestral gene (Janowitz et al., 2009; Piotrowski, 2008). Presumably, the *Bsas3* subfamily diverged such that the genes now encode a mitochondrial enzyme specialized for cyanide detoxification but with a secondary vestigial role in cysteine synthesis in that organelle. A phylogenetic analysis using 18 mitochondrial β-CAS protein sequences from different species revealed high homology (87-100%) among the proteins (Figure 2.1). The analysis showed β-CAS proteins as one cluster, in agreement with earlier observations (Jost et al., 2000; Marrero-Degro, 2010), with a clear separation between the monocots and eudicots.

The distribution of the *Bsas* family of genes and the catalytic capacities of the encoded enzymes within each subfamily has been attributed to selection pressures that altered the substrate specificity of the various enzymes, narrowing that specificity in most cases (Hatzfeld et al., 2000). One pressure that may have prompted the divergence to produce the *Bsas3* subfamily may have been the evolutionary change in ethylene synthesis to the current pathway involving 1-aminocyclopropane-1-carboxylic acid (ACC) as the immediate precursor (Percudani and Peracchi, 2003). In non-seed plants, ethylene synthesis does not involve the intermediate ACC (Osborne et al., 1996). Presumably therefore there would be no cyanide production via this pathway as there is for ACC-mediated ethylene synthesis. The enzyme catalyzing the last step in ACC-mediated ethylene synthesis, ACC oxidase, first appeared in higher vascular plants at the end of the Devonian and Carboniferous periods (Osborne et al., 1996) which would date back to about 400 MYA. The shift to ACC-mediated ethylene synthesis represents a single evolutionary

change prior to divergence of monocotyledonous and eudicotyledonous lineages (Osborne et al., 1996), and is believed to be associated with the alteration of substrate specificity of a 2-oxoacid-dependent dioxygenase (John, 1997). The first plants to show such direct conversion belong to the Gnetaceae and Ephedraceae, after which all gymnosperms and angiosperms exhibit ACC oxidase activity (Reynolds and John, 2004). The catalytic activity of ACC oxidase is responsible for the appearance of cyanide as a co-product of ethylene synthesis. This perhaps may have created the selection pressure that caused the divergence of β-cyanoalanine synthase from the large cysteine synthase family. Localization of ACC oxidase and β-CAS to the mitochondria (Hatzfeld et al., 2000; Vinkler and Apelbaum, 1985) has further provided evidence for the relationship of β-CAS and ethylene biosynthesis. One could speculate that this same evolutionary change may have also contributed to the divergence of the nitrilase/nitrile hydratase that mediates the second step of the β-cyanoalanine pathway from the other closely related nitrilases.

3.0 Enzymology of β-Cyanoalanine synthase

Structurally, there are two types of β-CAS found in plants (Hasegawa et al., 1995a; Ikegami et al., 1988a). The first is a monomeric enzyme (52 kD) isolated from blue lupine and contains one pyridoxal-5-phosphate (PLP) cofactor (Akopyan et al., 1975). The second type, isolated from spinach, cocklebur, rice, and *Lathyrus latifolius*, is a homodimer (Ikegami et al., 1988a; Ikegami et al., 1988b; Lai et al., 2009; Maruyama et al., 1998) with monomers (28-30 kD) having α/β structural domains (Bonner et al., 2005). At the amino acid level, β-CAS shows a 55-61% homology to the cysteine synthases (Hatzfeld et al., 2000; Maruyama et al., 2001). Evidence from potato demonstrates that β-CAS is immunologically distinct from the cysteine

synthases as antibodies raised against purified cysteine synthase enzymes failed to recognize β-CAS, while antibodies raised to purified β-CAS cross-reacted only with β-CAS (Maruyama et al., 2001). As β-substituted alanine synthase proteins, all *Bsas* enzymes including β-cyanoalanine synthase utilize *O*-acetylserine (OAS), L-serine, or L-cysteine as the donor of the alanyl moiety (Akopyan et al., 1975) and are characterized by a PLP cofactor that contributes the catalytic promiscuity of the enzymes (Alexander et al., 1994; Ikegami and Murakoshi, 1994; Percudani and Peracchi, 2003). However β-CAS shows a substrate preference that provides further justification for the establishment of β-CAS as a distinct subfamily relative to the cysteine synthases. There are notable substitutions in the PLP binding domain of β-CAS (Lai et al., 2009) which perhaps may account for the differences in specificity.

In vitro studies with purified enzymes have shown that for β-CAS and the synthesis of cyanoalanine, K_M values of 0.4 mM cysteine and 0.5 mM KCN were obtained (Castric et al., 1972). These values may not accurately reflect *in vivo* activity as the K_M value for KCN is higher than the cyanide concentration found in many plant tissues (Warrilow and Hawkesford, 1998; Yip and Yang, 1998). Calculations of steady state cyanide concentrations in plants producing cyanide indicate that enzymatic activity is 500-times greater than the rate of cyanide production (Yip and Yang, 1988; Yip and Yang, 1998), suggesting that the *in vivo* K_M is perhaps substantially lower. Recent reports supported this contention, indicating K_M values of 0.24 to 1.0 μM KCN for the formation of cyanoalanine (Hatzfeld et al., 2000; Warrilow and Hawkesford, 1998). The initial studies of cyanoalanine synthesis demonstrated that the purified β-cyanoalanine synthase could use methanethiol, ethanethiol, 2-mercaptoethanol as substrates *in vitro*, but at reaction rates that were 75-80% slower than the reaction forming cyanoalanine (Akopyan et al., 1975; Hendrickson and Conn, 1969). The β-cyanoalanine synthase enzyme did

not utilize other sulfur-containing compounds, such as homocysteine, reduced glutathione, dithiothreitol, sulfate or thiocyanate as substrates (Akopyan et al., 1975).

These *in vitro* studies further demonstrated that β-CAS can mediate the same reaction as cysteine synthase (CS), resulting in the synthesis of cysteine. Similarly, cysteine synthase can mediate the synthesis of cyanoalanine. Nevertheless, kinetic studies of the enzymatic activity of specific isoforms of β-CAS and CS from plants revealed a marked specificity of β-CAS for cyanoalanine synthesis and CS for cysteine synthesis (Hatzfeld et al., 2000; Maruyama et al., 2001; Warrilow and Hawkesford, 1998). For example, the K_M value for cyanoalanine synthesis was 30- to 130-fold lower when β-CAS catalyzed the reaction rather than CS. Conversely, the K_M value for cysteine synthesis was 30-fold lower for CS as compared to β-CAS (Hatzfeld et al., 2000). This fundamental difference in substrate specificity is another reason for the classification into specific *Bsas* subfamilies based upon whether they are true cysteine synthase or o-acetyl-serine-thiol lyases (i.e., OAS-TL) enzymes or whether, like β-CAS, the enzymes are OAS-TL-like proteins (Lunn et al., 1990; Rolland et al., 1992). The "true" OAS-TLs are defined by ability to form a complex with serine acetyl transferase (SAT), thereby regulating activity of SAT. Three key amino acid residues required for this association with SAT are not conserved in β-CAS (Lai et al., 2009), presumably precluding formation of the enzyme complex and reducing the overall activity of β-CAS for cysteine. Based on the amino acid sequences (Maruyama et al., 1997; Saito et al., 1994), another difference between the β-CAS and CS isoforms is in a signal peptide within their sequence for mitochondrial or chloroplast targeting, respectively.

4.0 Phylogeny of Nitrilase/nitrile hydratase

The nitrilase/nitrile hydratase that mediates the second step of the β-cyanoalanine pathway is encoded by a gene designated nitrilase 4. This gene belongs to, but has diverged from, a larger superfamily of nitrilase enzymes. The nitrilases are believed to be an ancient gene family and because of their wide distribution presumably appeared early in the diversification of eukaryotes (Pace and Brenner, 2001). The superfamily has 13 families, one of which is a small group of cyanide-hydrolyzing enzymes subdivided into nitrilases, cyanide hydratases and cyanide dihydratase based on their enzymatic activities (O'Reilly and Turner, 2003). Discovery of the first nitrilases in barley and some fungi dates back to the 1960s and was associated with auxin biosynthesis (Thimann and Mahadevan, 1964). In agreement with the ubiquity of auxins, recent phylogenetic analyses show wide distribution of plant nitrilases in both non-vascular and vascular plants (Janowitz et al., 2009). Generally nitrilases catalyze the hydrolysis of nitriles to a carboxylic acid and ammonia. The specific biological role of a nitrilase is dictated by their substrate specificity (Howden and Preston, 2009). Although nitrile hydratases (EC 4.2.1.84) convert nitriles to amides, they have also been referred to as nitrilases in older literature but their phylogenetic origins are not the same.

Four well-conserved nitrilase genes have been identified in *A. thaliana*, designated as NIT1, 2, 3 and 4 (Bartel and Fink, 1994). The first three, NIT1, 2 and 3, belong to one group (referred to as the NIT1 group). These three genes are clustered on chromosome 3 while the NIT4 gene is found on chromosome 5 (Hillebrand et al., 1996; Hillebrand et al., 1998). Phylogenetic analysis suggests the genes originated from a common ancestor. It is believed that the NIT4 gene is the oldest having been traced as far back in non-vascular plants, and that a gene

duplication event 70 MYA gave rise to NIT1 (Paterson et al., 2004; Piotrowski, 2008). Figure 2.2 shows occurrence of the *NIT4* gene in monocots and eudicots. Subsequent duplications of NIT1 resulted in NIT2 and NIT3. Further duplication likely occurred after divergence of the Poaceae to result in two/three NIT4 homologs only in this family designated NIT4A/B (Jenrich et al., 2007). Duplications of gene families are generally attributed to selection pressure and the need to widen substrate specificity (Lan et al., 2009). Distribution of the NIT1 genes has been detected only in Brassicaceae where they are involved in glucosinolate metabolism (Janowitz et al., 2009) while NIT4 are wide spread in almost all higher plants tested. Studies suggest that the presence of glucosinolates in Brassicaceae would perhaps call for wider substrate specificity. Consequently the substrate specificities of the NIT1 group are different from that of the NIT4. Both NIT1 and NIT4 genes consist of 4 introns and 5 exons with conserved positions at the intron-exon junctions (Dohmoto et al., 2000; Hillebrand et al., 1996). While the internal exons (2, 3, and 4) are highly conserved, exons 1 and 5 show less conservation between genes. The sizes of the introns are variable (Dohmoto et al., 1999), hence the genes tend to vary in length.

5.0 Enzymology of Nitrilase/nitrile hydratase

5.1 Structure of nitrilases

The nitrilase enzyme is a polypeptide made up of α-helices and β-sheets with a molecular mass between 32-45 kDa (Bartel and Fink, 1994; O'Reilly and Turner, 2003). A comparison of the amino acid sequences of cyanide hydrolysing enzymes, a sub-branch of the nitrilase superfamily showed little divergence (O'Reilly and Turner, 2003). Protein sequences of NIT1group are 82% identical to each other but NIT4 has only 65% amino acid identity to NIT1

(Piotrowski et al., 2001). Nitrilases form aggregates of 4-26 individual enzymes to achieve activation. For example, a decameric structure was proposed for a nitrilase from *Rhodococcus rhodhochrous* basing on the molecular mass of the resulting quaternary protein (Nagasawa et al., 2000). In sorghum (*Sorghum bicolor*) Jenrich *et al*., (2007) found dimers of 40 and 42 kDa which the authors proposed would associate to form larger units. The mechanism of catalysis involves a novel catalytic triad (Glu-Lys-Cys) present at the active site of all nitrilases (Pace and Brenner, 2001). In addition X-ray diffraction has shown that a Mg cofactor is also required for activation.

The structure of some nitrilases from the Poaceae is unique in that there is a dimerization of NIT4 homologs (Jenrich et al., 2007). Analyses have shown two NIT4 homologs, NIT4A and B, in rice (Lai et al., 2009) and in sorghum three isoforms have been identified as NIT4A, NIT4B1 and B2 (Jenrich et al., 2007). The individual NIT4 homologs in *A. thaliana* and tobacco could metabolize β-cyanoalanine, and yet the individual NIT4A/B1/B2 in Poaceae must form a heteromeric complexes in order to achieve high catalytic activity for β-cyanoalanine (Jenrich et al., 2007). Site-directed mutagenesis of the essential catalytic cysteine residue showed that NIT4A has the active site for cyanoalanine hydrolysis while NIT4B has active site for other substrates (Jenrich et al., 2007). The composition of the complex then becomes an important factor in defining substrate specificity for cyanide metabolism as each subunit has a binding site specific to a substrate.

5.2 Substrate specificity

In Brassicaceae the NIT1 group are involved in hydrolysis of indole-3-acetonitrile (IAN) to indole-3-acetic acid and indole-acetamide (Pollmann et al., 2006). The hydrolysis of IAN by

nitrilases however, is a very slow reaction, and the same nitrilases have shown higher activities with other nitriles as substrates (Vorwerk et al., 2001). In addition, maize and sorghum NIT4A homologs had no detectable activity with IAN. It is now generally agreed that the primary role of nitrilases may not be in auxin biosynthesis but for degradation of cyanogenic glycosides and glucosinolates (Piotrowski, 2008) and general aspects of nitrile metabolism (O'Reilly and Turner, 2003). A range of glucosinolate-derived nitriles has been identified as potential substrates of NIT1 homologs (Janowitz et al., 2009; Piotrowski, 2008; Vorwerk et al., 2001). The NIT4 homolog however, exhibits different substrate specificity and does not accept the aliphatic and aromatic nitriles (e.g 3-phenylpropionitrile) that are the major substrates for the NIT1 group. Instead the enzyme has high specificity for β-cyanoalanine (Piotrowski et al., 2001). In maize NIT4A/B (formerly mistaken for NIT1 and 2) displayed dual activity for auxin biosynthesis and also β-cyanoalanine hydrolysis (Kriechbaumer et al., 2007).

NIT4 proteins are bifunctional, possessing both nitrilase and nitrile hydratase activity. The enzymes have shown the capability of hydrolyzing β-cyanoalanine to asparagine or aspartate and ammonia (Piotrowski et al., 2001). The ratio of asparagine:aspartate product depends on the species. For example, in Arabidopsis and tobacco a 1:1 ratio of asparagine to aspartate was reported (Piotrowski et al., 2001) and in blue lupine and sorghum the ratio was 3:1 (Piotrowski and Volmer, 2006). Additionally, ^{14}C-labelling studies traced carbon from cyanide and found it to be predominantly in asparagine and not aspartate (Castric and Conn, 1971). The disparity in the ratio between species prompted further studies with respect to this characteristic. Studies on the catalytic center for both nitrilase and hydratase activities revealed a cysteine residue (cys-163) that seems to be an important regulatory point for activity (Kobayashi et al., 1993; Kobayashi et al., 1992). It is likely that this amino acid changes states and influences the ratio to

regulate the amount of ammonia being directly released (Piotrowski, 2008). The switch is an inherent property of the different NIT4 homologs and not a result of mutation (Piotrowski and Volmer, 2006). In another study, Kiziak and Stolz (2009) demonstrated that a single amino acid change in the catalytic region of *Pseudomonas fluorescens* could convert a nitrilase from forming an acid to an amide. It remains unclear what causes the shift but it is likely that the mechanism might be species specific.

6.0 Regulation of the β-cyanoalanine pathway

6.1 Regulation of β-CAS gene expression

The assimilation of cyanide by the β-cyanoalanine pathway is highly regulated by a range of regulatory mechanisms. OAS-TL genes exhibit semi-constitutive expression inducible by abiotic stresses (Hell et al., 1994; Hesse et al., 1999). In this type of expression pattern, a protein is widely expressed in the tissues but the promoter region is not always active (Carpenter et al., 1993). It is therefore likely that the different putative promoter areas in OAS-TLs likely give rise to specific regulatory patterns (Jost et al., 2000). Several abiotic stresses have been shown to up-regulate OAS-TLs at the transcriptional level. In *A. thaliana*, salt and heavy metals (cadmium) induced mRNA of the cytosolic OAS-TL 7-fold with evidence that response is mediated by ABA (Barroso et al., 1999). Several studies have shown that expression of OAS-TL genes is upregulated by sulfur and nitrogen limitation with mRNA increases of 2-3 fold (Hell et al., 1994; Hesse et al., 1999; Takahashi and Saito, 1996). While two OAS-TLs in *A. thaliana* showed a 2-fold transient increase induced by sulfur, in the long term they did not differ significantly from the control (Takahashi and Saito, 1996). The studies show a clear pattern that

abiotic stresses upregulate OAS-TL gene expression. In *A. thaliana* the *AtCysC1* gene encoding the β-CAS enzyme shows high, constitutive expression that is not responsive to cyanide exposure or stress (Kaplan et al., 2007; Kreps et al., 2002; Matsui et al., 2008; Yamaguchi et al., 2000). In birch plants exposed to ozone, there was accumulation of β-CAS transcript (Vahala et al., 2003). In spinach expression of the CysC1 gene was up-regulated 5-fold by nitrogen deficiency, and this accumulation was presumably needed to remove excess sulfide coming from degradation of S-containing compounds to release N (Takahashi and Saito, 1996).

Hormones also tend to regulate OAS-TL gene expression and ethylene has probably the greatest and most direct effect on β-CAS gene expression. Ethylene seems to have both promotional and inhibitory effects on the transcription of this gene. The inhibitory effects of ethylene were demonstrated in a comparison of two cDNAs encoding PCAS1 and PCAS2 in potato (*Solanum tuberosum* L.) in which there was accumulation of β-CAS protein with no effect on β-CAS transcript when endogenous ethylene dropped to non-detectable levels (Hasegawa et al., 1995a; Meyer et al., 2003). It is not surprising that auxin also has profound effects on β-CAS expression because of its close association with ethylene. When 2,4-D, a synthetic auxin was used to induce ethylene in soybean, β-CAS transcription was induced although there was no change in barley (Tittle et al., 1990). In rice, auxin induced the accumulation of β-CAS transcript (Lai et al., 2009). The contradicting results suggest that the effect may be species specific or perhaps require additional factors not necessarily considered in those experiments. The proposed mechanism of action is that auxin induces ethylene biosynthesis by promoting ACC synthase and ACC oxidase transcription (Kim et al., 1992). While auxin and ethylene effects on β-CAS transcription are antagonistic, the promotional effects of auxin tend to be stronger than the suppressive effect of ethylene (Maruyama et al., 2001) and perhaps this could

also be species specific. Methyl jasmonate, a signal for wounding, inhibits β-CAS expression at transcriptional level but has no effect on the cytosolic OAS-TL. Other phytohormones (ABA, GA) had no effect on β-CAS expression (Maruyama et al., 2001).

6.2 Regulation of β-CAS at protein level

One recent study found three subspecies of OAS-TLC enzyme and speculated that there may be post-translational modifications regulating β-CAS activity as has been demonstrated in other OAS-TLs (Wirtz et al., 2010). Activity of the β-CAS protein is regulated by ethylene, auxin, environmental conditions (including biotic and abiotic stresses) as well as developmental stage of the plant. There however tends to be in some cases only little variation in response to those factors. In eudicot species such as bean, soybean, lettuce, wild mustard and in barley, external ethylene increased β-CAS activity at a rate proportional to ethylene concentration (Goudey et al., 1989). Ethylene increases during certain developmental stages (e.g., seed germination, fruit ripening) and in response to wounding and this increase is followed by increased β-CAS activity (Manning, 1988). For example in *Petunia* flowers an increase in ACC oxidase activity was followed by increased β-CAS activity (Manning, 1986). In other studies a burst of stress ethylene induced enzyme activity (Liang, 2003; Wen et al., 1997) and addition of the ethylene precursor ACC also increased ethylene production in *A.thaliana* (Meyer et al., 2003). Alongside with ethylene, cyanide is also produced, hence β-CAS is presumably induced to detoxify the excess cyanide. Exogenous application of ethylene does not however, involve cyanide emanation and a decrease was in fact reported in *A. thaliana* seedlings fumigated with ethylene (Meyer et al., 2003). The increase in β-CAS enzyme activity in this study is not likely

due to the direct induction by this ethylene-associated cyanide because studies with *A. thaliana*, wheat (*Triticum aestivum* L.), and potato have shown no response of enzyme activity to cyanide treatment (Kosma, 2005; Maruyama et al., 2001; Meyer et al., 2003; Sidibe, 2008).

The pH is an important regulator of OAS-TL activity, with activity favored under alkaline conditions (Yamaguchi et al., 2000). The reaction mediated by β-CAS is also more rapid at higher pH (Burandt et al., 2002). Since cyanide mostly exists in ionic form (i.e., CN^-) at higher pH, it is in this form that cyanide easily binds to the enzyme and so it is reasonable to assume that substrate availability becomes relatively higher as pH increases.

In *A. thaliana* and spinach there was increased activity of the β-CAS protein in response to nitrogen deficiency (Machingura and Ebbs, 2010; Takahashi and Saito, 1996). In tobacco, water deficit led to increased β-CAS activity during the period of stress and decline in activity on re-watering (Liang, 2003). β-CAS was also induced in tomato roots treated with a fungal glycoprotein elicitor (Takahashi et al., 2006) and during ripening in response to ethylene (Han et al., 2007). In this study, the use of inhibitors of ethylene receptors verified that functional ethylene signaling is required to activate β-CAS, otherwise cyanide detoxification would be compromised. It is therefore clear that stress induces cyanide release, but it is not the cyanide which stimulates β-CAS activity. Taken together these studies suggest β-CAS expression is highly regulated by various other pathways. Other environmental factors such as light and dark, and the developmental stage of the plant all regulate β-CAS activity (Burandt et al., 2001; Wurtele et al., 1984).

6.3 Regulation of nitrilases at gene level

Nitrilase genes exhibit differential expression in a tissue specific manner, a characteristic reflected in their promoter regions. In *A. thaliana* the NIT1 gene is expressed in rosette leaves, NIT2 in siliques, NIT3 in the roots, and NIT4 in root and shoot tips of 12 day-old seedlings (Bartel and Fink, 1994). The expression seems to be developmentally and environmentally regulated as expression of NIT4 spread to all vegetative parts (leaf, stipule, sepal and siliques) in mature 5 week-old plants (Bartel and Fink, 1994; Bartling et al., 1994). Similarly, NIT4 mRNA was abundant in mature green tissues increasing during senescence (Oracz et al., 2008). The sites of nitrilase expression coincide with sites of auxin biosynthesis, which are also sites of increased ethylene biosynthesis (Bartel and Fink, 1994).

Generally nitrilase genes tend to have low level of expression as suggested by an A-T rich (71%) upstream sequence and absence of CAAT box upstream of the TATA box (Dohmoto et al., 1999). Consequently, studies on expression of NIT4 gene revealed low levels of expression in various organs of tobacco (Dohmoto et al., 1999) and in blue lupine NIT4A/B expression in leaves and cotyledons increased over a period 5-13 days (Piotrowski and Volmer, 2006). NIT2 gene showed low levels of expression, with a steady increase in mRNA in leaves during senescence induced by salicylic acid (Quirino et al., 1999). The genes were however not induced by ABA and ACC (Dohmoto et al., 1999). It is therefore not surprising that increased expression of NIT4 transcript was observed in *A. thaliana* under abiotic stress coinciding with increased β-CAS activity (Kilian et al., 2007). NIT4 expression was however not induced by pathogen invasion (Bartel and Fink, 1994). Additionally, the ethylene-responsive element and ABA responsive element found in the upstream region of the NIT4 gene (Dohmoto et al., 1999) suggest that ethylene and ABA may be involved in its regulation.

6.4 Regulation of nitrilases at protein level

Nitrilases enzymes are induced by light (Park et al., 2003), indole-3-acetonitrile (IAN), and in the case of nitrilase 4 by β-cyanoalanine (Kriechbaumer et al., 2007). In maize, nitrilase activity was detected in kernel tissues, young roots, and on tips of coleoptiles (Park et al., 2003). Protein expression was highest during germination with a steady decline thereafter. Kriechbaumer et al. (2007) used western analysis to investigate how nitrilase concentration is modulated in maize. Jasmonic acid was shown to repress protein abundance, opposing auxin effect. This is in agreement with another study which reported in a decrease in protein concentration and transcription on wounding (Cheong et al., 2002) because jasmonic acid is a signal for wounding. Also in response to wounding there was an increase in protein aggregation (Cutler and Somerville, 2005), a characteristic which leads to increased enzyme activity (Piotrowski, 2008). Other substrates, IAN, OAS and β-cyanoalanine induced nitrilase (ZmNIT1/ZmNIT2) in maize activity (Kriechbaumer et al., 2007). Later, it was discovered that these two homomers are in fact ZmNIT4 complexes (Piotrowski, 2008). NIT3 protein activity was induced 35-fold when plants were exposed to sulfur deficiency and this induction has been associated with degradation of glucosinolates (Kutz et al., 2002). It is likely that regulation of protein activity may also be species specific. Other hormones (GA, ABA and brassinolide) and kinetin had no detectable effect on protein abundance. Thimann and Mahadevan (1964) found no cyanide inhibition of barley nitrilase.

7.0 Accessory enzymes in the β-CAS pathway

7.1 γ-Glutamyl transpeptidases (EC.2.3.2.2)

The conversion of β-cyanoalanine to asparagine occurs very quickly such that the intermediate is minimally detectable in most plants. There is evidence in a limited number of plant species (e.g., *A. thaliana*, *Vicia* spp., *Lathyrus* spp.) that there may be an alternate fate for the cyanoalanine formed by β-cyanoalanine synthase activity. Past studies (Blumenthal et al., 1968; Fowden and Bell, 1965) as well as one recent study (Watanabe et al., 2008) have suggested that β-cyanoalanine may also be converted to γ-glutamyl-β-cyanoalanine by transpeptidases. In *A. thaliana*, γ-glutamyl transpeptidases such as AtGGT1 (At4g39640), AtGGT2 (At4g39650), and/or AtGGT3 (At4g29210) were suggested as possible candidates. Bsas3;1 knockdown mutants in *A. thaliana* accumulated significantly less γ-glutamyl-β-cyanoalanine than wild type or other CS knockouts. In a separate experiment, when agar-grown wild type and Bsas3;1 mutants were exposed to 100 μM β-cyanoalanine, the wild type displayed significantly higher concentrations of γ-glutamyl-β-cyanoalanine than the Bsas3;1 mutant, while the converse was observed when the β-cyanoalanine concentrations was increased to 1 mM (Watanabe et al., 2008). The authors suggested that synthesis of γ-glutamyl-β-cyanoalanine could be mediated by apoplastic enzymes encoded by *AtGGT1* and *AtGGT2* (Ohkama-Ohtsu et al., 2007a) or by the vacuolar enzyme encoded by *AtGGT3* (Ohkama-Ohtsu et al., 2007b). However, they acknowledged that this would require specific subcellular trafficking to direct cyanoalanine to one or both of these compartments. Studies with seeds of *Vicia* spp. and *Lathyrus* spp. have suggested that γ-glutamyl-β-cyanoalanine is a possible but not obligatory intermediate in the β-cyanoalanine pathway between β-cyanoalanine and asparagine (Ressler et al., 1969a). Since asparagine can also be synthesized from γ-glutamyl-β-cyanoalanine (Ressler et al., 1969a; Ressler et al., 1969b) the formation of these two compounds may in fact occur in parallel as suggested by the recent work in *A. thaliana* (Watanabe et al., 2008). The ubiquity of

γ-glutamyl-β-cyanoalanine formation in plants has not been established, nor has the role for GGTs in its synthesis. Expression patterns of the four genes have done very little to shed light on the functional roles (Destro et al., 2011).

7.2 Asparaginase (EC.3.5.1.1)

The enzyme asparaginase belongs to a superfamily of N-terminal nucleophile amidohydrolases that act on amide bonds. Small subunit polypeptides aggregate to make protein of approximately 75 kDa with optimum enzyme activity at pH 8.2. To our knowledge four genes in *A. thaliana* have been identified (Bruneau et al., 2006), and in soybean there are two copies. Results from phylogenetic analysis suggest divergence of the genes into distinct subfamilies of K^+-dependent and K^+-independent respectively (Bruneau et al., 2006; Sodek et al., 1980). Comparison of amino acid sequences revealed only 55% identity and their substrate specificities also reflect the divergence. The K^+-dependent group has a higher affinity for asparagine (K_M 0.14) and did not accept β-aspartyl dipeptides (Hejazi et al., 2002) in contrast to the K^+-independent group (10mM) (Bruneau et al., 2006). The distribution of asparaginase is ubiquitous in plants having been detected in most species. Expression however seems to be confined to sink tissues in different plant species. In *Lupinus albus* the enzyme was detected in cotyledons, in legume seeds and in *A. thaliana,* stamens and mature pollen. At the subcellular level asparaginase is localized to the cytosol (Ireland and Joy, 1983), the same location as NIT4 and hence where asparagine is produced. Since there are several routes for synthesis and degradation of asparagine (Sieciechowicz et al., 1988), additional studies are required to establish a specific relationship between the activity of cyanoalanine synthase and asparaginase. The significance of asparaginases in plants is to supply/recycle N and C to sink tissues and

prevent loss. Given the several biosynthesis and degradation pathways for asparagine in plants, it would be interesting to determine the level of importance of the β-CAS pathway to this metabolic network.

While many studies have established that the final step to assimilate carbon and nitrogen from cyanide back into primary metabolism is conversion of asparagine into aspartate and ammonium (Cho et al., 2007), asparagine is the main product of cyanide metabolism in most plant species (Blumenthal et al., 1968; Peiser et al., 1984). This step is mediated by a NIT4 homolog with both nitrilase and nitrile hydratase activities. Asparagine has been shown to accumulate in plant tissues during stress and senescence (Lea et al., 2007). To trace the path of C and N from cyanide, studies with labeled ^{15}N confirmed greatest enrichment of labeled ^{15}N in asparagine (Ebbs et al., 2003; Siritunga and Sayre, 2004). The asparagine can also be converted through a deamination reaction mediated by asparaginase to aspartate and ammonium (Elias et al., 1997; Ressler et al., 1969a; Ressler et al., 1969b). One study on response of asparaginase to cyanide showed significant up-regulation of activity by wheat plants when cyanide was the sole source of nitrogen (Machingura and Ebbs, 2010). These results suggest that there may be shared regulatory elements of the β-CAS pathway and asparaginase.

8.0 Conclusion

The phenomenon of cyanogenesis is not only found in higher plants but also present in lower plants for example in the alga *Chlorella vulguris*, the cyanide emanating from amino acid oxidation and cyanogenic glycosides in ferns. Consequently cyanide would have been present in lower plants in minute levels. Study on cyanide in lower plants has been marginalized and so the physiological implications of cyanide remained unclear. Acquisition of ACC oxidase by land

plants ~400 MYA would have resulted in increased cyanide emanation and created the need for a more specific homeostatic mechanism. The β-CAS pathway may thus be traced back to early land plants. Because the pathway involves two independent enzyme families, the lyase and hydrolases, it is challenging to reconcile events around the *Bsas* and nitrilase protein families leading to recruitment into the β-CAS pathway. The information may not be as complete but seems sufficient to presume a recruitment of pre-existing enzymes into cyanide metabolism. According to Jensen (1976), herein the evolution of β-CAS initiated a multistep pathway. The assumption of presence of intermediates was met since ethylene and ACC were already present. Additional information allows speculation on the order of integration of the steps into one pathway. The β-CAS pathway is not confined to a few plant species but is ubiquitous in all higher plants. Additionally, *Bsas* genes would have been present in lower plants for their primary function in cysteine synthesis. Phylogenetic analysis reveals that OAS-TL and SAT are found in lower plants (*Selaginella moellendorffii*), though with fewer isoforms than in higher plants (Kopriva, 2007). In this phylogenetic analysis one lower OAS-TL isoform groups quite closely with *A. thaliana* β-CAS. By speculation, divergence of β-CAS gene specifically for cyanide removal may have occurred after acquisition of ACC oxidase. There is evidence of a mutation in the β-CAS gene which would have given way to specialization in cyanide detoxification (Lai, 2007). Nitrilase 4 activity also exists in almost all land plants. Piotrowski (2008) provides a phylogenetic analysis of the nitrilases and traces β-cyanoalanine activity as far back as moss, for example *Physcomitrella patens*, a non-vascular group of plants. The occurrence of NIT4 activity in non-vascular plants may have served a primary function considering the nitrilases are characterized by broad substrate specificity. Alternatively, some regulatory controls may have kept low level of expression of nitrilases as suggested by Dohmoto

et al. (1999) who reported some NIT4 sequence characteristics typical of low expression. The NIT4 gene was perhaps adopted into the pathway second, after divergence of β-CAS to prevent accumulation of another toxic compound, β-cyanoalanine.

Table 2.1 Functional and kinetic characteristics of β-CAS-like proteins in different plant species.

Species	*Accession number	Molecular mass (kD)	β-CAS activity (μmol H_2S $min^{-1}mg^{-1}$)	CS activity (μmol Cysteine $min^{-1}mg^{-1}$)	K_M for cysteine	K_M for cyanide	Reference
Lupinus angustifolia L.		53	33.8	1.27	2.5	0.55	(Hendrickson and Conn, 1969)
L. angustifolia		52	43.5				(Akopyan et al., 1975)
Lathyrus latifolius		56	132		16	0.51	(Ikegami et al., 1988a)
Spinacia oleracea		60	212		2.3	0.73	(Ikegami et al., 1988b)
A.thaliana	AJ010505	39.9	62.1	0.4	2.54	0.06	(Hatzfeld et al., 2000)
Spinach	D37963	58	157	0.4	2.14	0.1	(Hatzfeld et al., 2000)
Potato	AB027000	38.2	66.2	49.5	2.76	0.134	(Maruyama et al., 2001)
Apple	DQ471308	40.9	12				(Han, 2007)
Apple	DQ471309	40.5	12				
Rice	XP_474584	36.5	13.3	-	0.84	0.27	(Lai, 2007)
Cassava:leaf		51	108			7	(Elias et al., 1997)
r ind			105		2.5	8	
tuber			150			5	

*Accession numbers: Lai, 2007, Han, 2007.

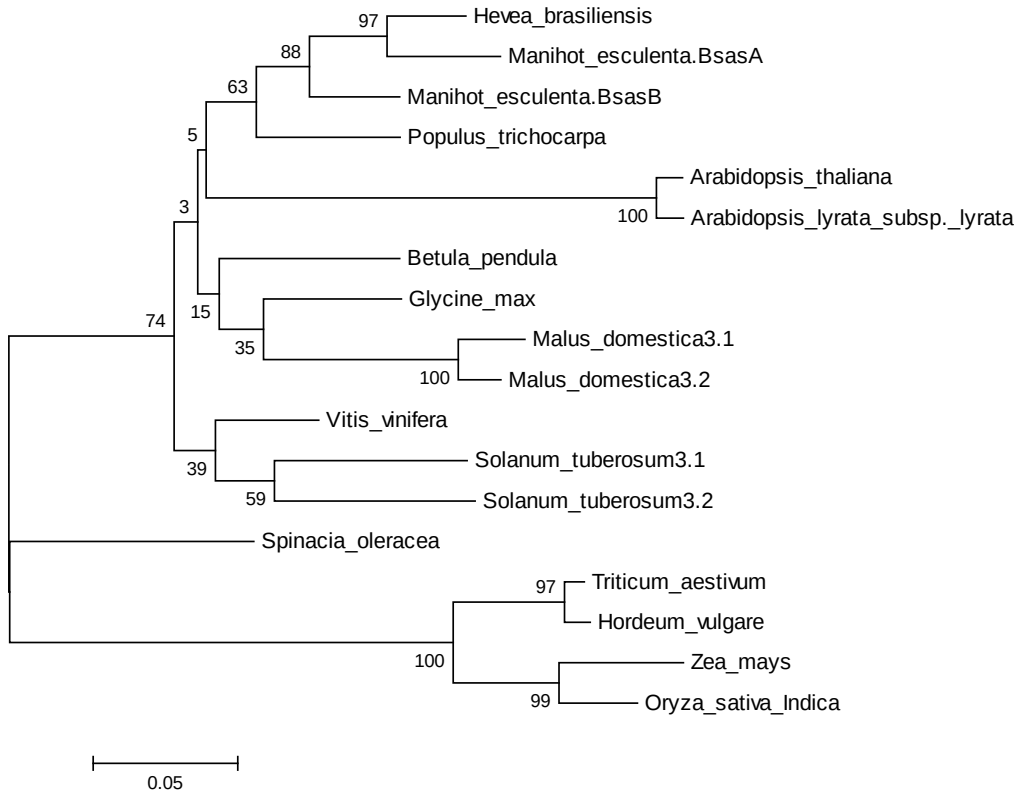

Figure 2.1 Phylogenetic analysis of mitochondrial β-cyanoalanine synthase enzymes in higher plants. Multiple sequence alignment was done in ClustalW2 using default parameters. The tree was constructed using the Maximum Likelihood program.

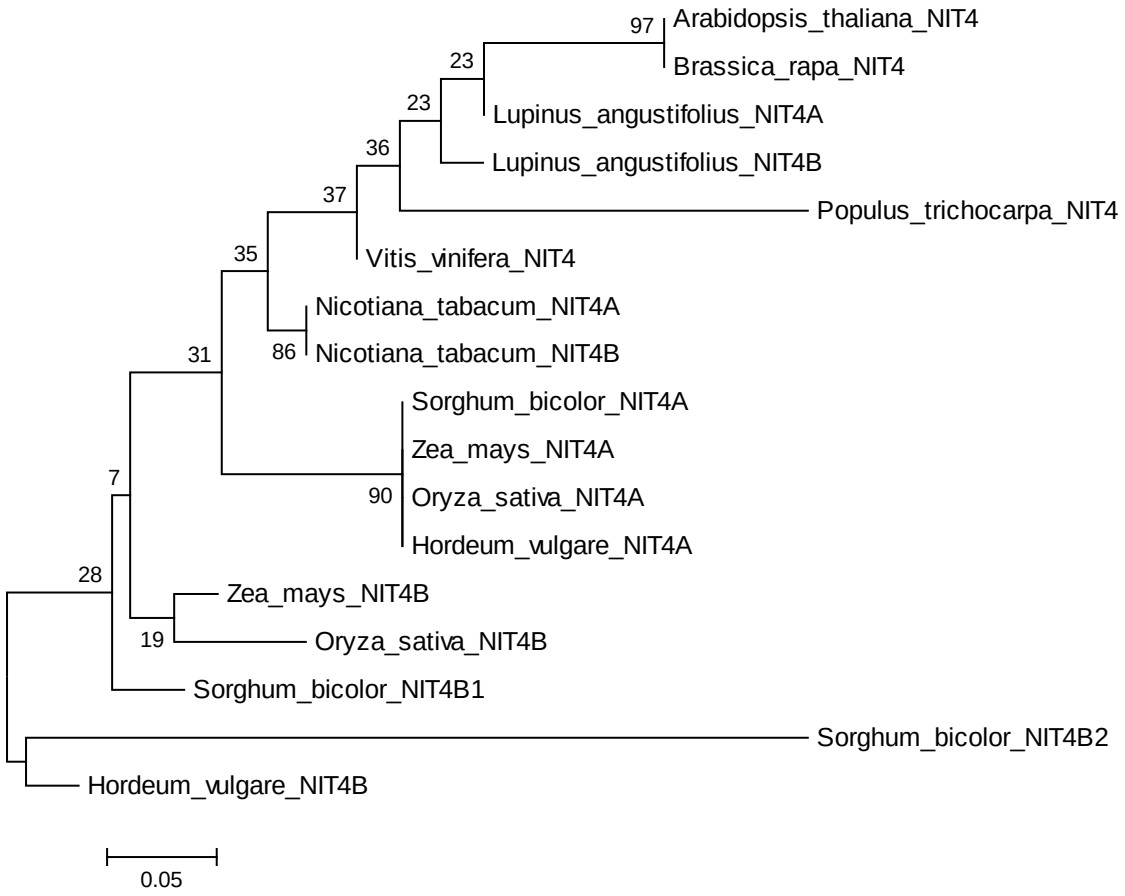

Figure 2.2 Phylogenetic analysis of the sub group of nitrilase 4 in higher plants. Multiple sequence alignment was done using ClustalW2 using default parameters. The tree was constructed using Maximum Likelihood program.

CHAPTER 3

THE β-CYANOALANINE PATHWAY IS INVOLVED IN THE RESPONSE TO WATER DEFICIT IN ARABIDOPSIS THALIANA

1.0 Introduction

Cyanide is produced by higher plants via multiple metabolic pathways. The two most prevalent sources of endogenous cyanide are the turnover of cyanogenic glycosides or cyanolipids (Jones, 1998; Poulton, 1990) and ethylene biosynthesis (Peiser et al., 1984; Yip and Yang, 1988). While formation of cyanogenic glycosides and cyanolipids is limited to ~2,500 plant species, ethylene biosynthesis is ubiquitous source of endogenous cyanide in plants. Ethylene in higher plants is synthesized by oxidation of 1-amino-cyclopropane-1-carboxylic acid (ACC) by ACC oxidase, releasing hydrogen cyanide and carbon dioxide as co-products. Ethylene production (and hence cyanide production) increases when plants experience biotic and abiotic stresses such as viral infection, water deficit, or ozone (Liang, 2003; Peiser et al., 1984; Siefert et al., 1995; Vahala et al., 2003). Plants have evolved mechanisms to remove cyanide from such sources to prevent autotoxicity. The β-cyanoalanine synthase pathway is the principal pathway for cyanide detoxification in plants. In the first step of the pathway, cyanide reacts with cysteine to form β-cyanoalanine, releasing bisulfide in a reaction catalyzed by β-cyanoalanine synthase or cysteine synthase (Blumenthal et al., 1968; Hendrickson and Conn, 1969; Warrilow and Hawkesford, 1998). The second step of the β-CAS pathway is mediated by a dual function nitrile hydratase/nitrilase designated in *A. thaliana* as nitrilase 4 (NIT4). The NIT4 enzyme catalyzes reactions that convert cyanoalanine to either asparagine or aspartate in conjunction with ammonia, respectively (Piotrowski et al., 2001).

Recent evidence also suggests a role of the β-CAS pathway in plant response and acclimation to abiotic stress. For example, when birch plants were exposed to ozone, there was an increase in ethylene production (and also cyanide) and increased β-CAS transcript abundance (Vahala et al., 2003). In another study, tissue cyanide concentrations and β-CAS activity showed concomitant increases when tobacco (*Nicotiana tabacum* L.) plants were subjected to water deficit (Liang, 2003). Cyanide concentration decreased after two days of stress, presumably due to the action of β-CAS enzyme, even though ethylene production was still high. The conclusion offered by the authors was that water deficit induced cyanide production, and that removal of cyanide by the β-CAS enzyme contributed to the response, if not also the tolerance, to water deficit.

The overarching goal of the work here was to further investigate the contribution of the β-cyanoalanine synthase pathway to the response to water deficit. *Arabidopsis thaliana* (Col-0) and three SALK t-DNA insertion lines for the three genes directly associated with the pathway (Piotrowski et al., 2001; Piotrowski and Volmer, 2006; Watanabe et al., 2008), namely cysteine synthase (*AtCysA1*), β-cyanoalanine synthase (*AtCysC1*), and nitrilase 4 (*AtNIT4*), were used. There were two main objectives for this study. The first was to examine the response of the pathway in wild type *A. thaliana* Col-0 to the magnitude and duration of water deficit. Since ethylene production depends on the severity and duration of stress (Morgan and Drew, 1997), the concomitant production of cyanide should also be variable. Activity of β-CAS may also vary in order to maintain cyanide at a steady state concentration below that potentially inhibitory for metabolism (Yip and Yang, 1988) as was observed in the prior study with tobacco and water deficit (Liang, 2003). The second objective was to examine how interruption of single genes encoding enzymes of the pathway influenced the ability of *A. thaliana* to metabolize cyanide and

respond to water deficit. Given that β-CAS (*AtCysC1*, Bsas 3;1) and one cysteine synthase (*AtCysA1*, Bsas 1;1) are the only enzymes with β-cyanoalanine synthase activity (Watanabe et al., 2008), the efforts here sought to determine if both of these genes were required for the function of the pathway *in vivo* or if one gene alone was sufficient to maintain cyanide assimilation under water deficit conditions (i.e. redundant compensation). As a single gene in *A. thaliana* (Bartel and Fink, 1994), the use of the nitrilase 4 SALK line allows for an examination of the impact of water deficit when the second step of the pathway is specifically interrupted.

2.0 Materials and Methods

2.1 Plant culture

Seeds of wild type *Arabidopsis thaliana* (Col-0) and the SALK t-DNA insertional mutants for cysteine synthase (*AtCysA1*, At4g14880, SALK_72213), β-cyanoalanine synthase (*AtCysC1*, At3g61440, SALK_22479) and nitrilase 4 (*AtNIT4*, At5g22300, SALK_016289C) were obtained from TAIR (www.Arabidopsis.org). For some experiments, seeds were surface sterilized by fumigation according to Clough and Bent (1998) and germinated on plates containing half-strength MS medium with 1% agar and 0.5% sucrose. After a three day vernalization period at 4ºC in the dark the plates were placed at a 30° angle in a Percival growth chamber (Model E-36 L, Des Moines, IA, USA) at ambient humidity with an 8 h photoperiod at a light intensity of ~150 μM m^{-2} s^{-1} provided by a combination of fluorescent and incandescent bulbs. The day/night temperatures were 22° and 18°C respectively, and plants were left to grow for three weeks. Thereafter plants were removed from plates and transferred to sterile perlite and vermiculite (1:1) and left to grow for an additional three weeks to establish biomass. For one

particular experiment, plants were transferred from plates to a hydroponic solution with the following composition: 6 mM KNO_3, 4 mM $Ca(NO_3)_2$, 0.1 mM $NH_4H_2PO_4$, 1 mM $MgSO_4$, 50 μM KCl, 12.5 μM H_3BO_3, 1 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.1 μM $NiSO_4$, and 0.016 μM $(NH_4)_6Mo_7O_{24}$ (Ebbs et al., 2003). The solution was buffered with 1 mM *n*-morpholinoethanesulfonic acid (MES) titrated to pH 6.0 with MES-TRIS. Iron was provided as 10 μM Fe-EDTA, the solution was continuously aerated, and the growth conditions were as indicated above. After three weeks of growth plants were transferred from the hydroponic culture to sterile sand for the split-root experiment described below. Surface sterilized seeds of wild type (Col-0) for one additional experiment were also germinated directly in sterile potting mix using the same vernalization and growth conditions as above. These plants were watered with 50 mL of the same nutrient solution as above and 100 mL water per week. The 100 mL of water was split into two applications of 50 mL. The plants were grown for six weeks under the same growth conditions and photoperiod as above to establish biomass and were then subjected to the desired treatments.

2.2 Response of the β-cyanoalanine synthase pathway to magnitude and duration of water deficit

Two different strategies were used to impose water deficit on *A. thaliana* Col-0 plants to observe the response of the β-cyanoalanine synthase pathway. For the first, the goal was to examine the response to a severe, short-term (i.e., acute) water deficit. Three week old plants grown on MS plates were subjected to this acute water deficit by removing the plants from the plate and exposing the whole seedlings to air for 20 min. This method of creating water deficit was used previously in a transcriptome study that examined global changes in gene expression in

A. thaliana in response to various abiotic stresses (Kilian et al., 2007). There were three replicates each for the acute water deficit treatment and the control (i.e., plants remaining on the MS plates). Whole plants were harvested, snap-frozen in liquid nitrogen, and stored at -80°C so that subsequent analyses could quantify whole plant transcript abundance via quantitative RT-PCR (qRT-PCR). Additional plants, grown three weeks in MS plates and then three weeks in potting mix were left unwatered for seven days prior. An acute water deficit treatment was imposed by gently removing the seedlings from the potting mix, removing adhering soil particles, and exposing the roots to air for 45 min. Following the treatments, whole plants (three replicates each of control or treated plants) were snap-frozen in liquid nitrogen for later analysis of whole plant tissue cyanide concentration and β-CAS-like activity. To provide information on the time-dependence of the response, additional plants were germinated and grown in perlite and vermiculite up to the 10-12 leaf stage. These plants were simply pulled out of the media and air dried for 60 min to impose acute water deficit. Plants were sacrificed at 20 min intervals for 60 min and snap-frozen in liquid nitrogen. Replicate samples of treated and control plants (n=3) were analyzed for relative water content, tissue cyanide and β-CAS-like activity in the whole plant.

The second strategy for imposing water deficit was intended to be more physiologically and environmentally relevant (i.e. chronic water deficit). For one experiment, watering of Col-0 plants grown in perlite and vermiculite was withdrawn and the volumetric water content of the medium was monitored using an Echo EC-5 soil moisture sensor (Decagon Devices Inc, Pullman WA USA). After 4 days, when the volumetric water content of the medium decreased to 50%, plants were harvested at one day intervals for three days. These samples, replicated three times per treatment and time point, were analyzed for relative water content, tissue cyanide and β-

CAS-like activity. In a second iteration of this type of experiment, watering was discontinued for 15 days. Subsequently, plants were harvested every five days for 15 days, at which point all treated plants were showing a significant loss of turgor. As above, relative water content of the shoots, tissue cyanide and β-CAS-like activity were determined, with four replicates per treatment and time point.

To examine whether the response was localized to tissues in contact with dry soil, a split-root experiment was performed (Davies, 2000). Six-week-old Col-0 plants grown for three weeks in hydroponics were used. The plants were grown hydroponically to reduce the damage associated with their transfer and to allow for an easier separation of the root system into two roughly comparable components. The two components of the root systems were each established in separate pots containing sterile sand. The plants were provided one week to acclimate to the new growing medium. During this recovery period, there were no signs of stress and new growth was evident. After the recovery period, the split-root systems were assigned to one of two treatment groups, control and treated. For the control plants, the two pots containing the split-root systems were each watered with 50 mL nutrient solution once per week and two applications of 50 mL of water twice per week. For the treated plants, water was completely withheld from one compartment, while the other compartment received the same watering pattern as used for the control plants. Plants were harvested on day 7 and separated into shoots and the roots from each pot. The relative water content of shoots, tissue cyanide concentration in roots and shoots, and β-CAS-like activity in roots and shoots were determined for these samples. There were four replicates of each treatment in this experiment with a replicate represented by a single split-root plant.

2.3 Response of the β-cyanoalanine synthase pathway mutants to water deficit

The decrease in β-cyanoalanine synthase-like activity and/or nitrilase/nitrile hydratase activity in the insertional mutants was demonstrated using the procedures described below. Plants were germinated and grown in plates under the same growth conditions as above. After three weeks, seedlings were transferred from MS plates to hydroponic culture and allowed to grow for two additional weeks. Whole plants were harvested, rinsed, snap-frozen in liquid nitrogen and stored at -80°C prior to analysis. The assay for β-cyanoalanine synthase-like activity was conducted on whole seedlings and each line was replicated four times. Similarly, nitrilase/nitrile hydratase activity was determined in Col-0 and the *AtNIT4* mutants. Nitrilase/nitrile hydratase activity was determined in untreated plants of each line and in plants treated for 36 h with 1 mM cyanide (as KCN). Three week old seedlings were transferred from MS plates to hydroponic culture as above, and grown for three weeks. The 36 h cyanide treatment was then imposed on half the Col-0 and *AtNIT4* plants. The composition of the hydroponic cyanide treatment solution was similar to the basal nutrient solution except that the phosphate concentration was lowered to 0.02 mM, $MnSO_4$ concentration was lowered to 1.6 μM, and iron was omitted to preclude formation of metal cyanide solids (Ebbs et al., 2003). Aeration of the nutrient solution was discontinued to reduce cyanide volatization (Ebbs et al., 2003; Ebbs et al., 2008; Samiotakis and Ebbs, 2004). After 36 h of exposure, whole seedlings were harvested, rinsed, snap-frozen in liquid nitrogen, and stored at -80°C until the nitrile hydratase/nitrilase assays were performed. Each line and treatment had four replicates.

To examine how the putative lines for β-cyanoalanine synthase (*AtCysC1*), the cytosolic cysteine synthase (*AtCysA1*), and the nitrile hydratase/nitrilase (*AtNIT4*) responded to water deficit, seeds of each line were germination and grown for three weeks on MS plates and then

three weeks in 1:1 sterile perlite and vermiculite. A subset of plants of each line was harvested and the whole plants were snap-frozen in liquid nitrogen for determination of basal β-CAS-like activity. For the remaining plants, watering was withheld for 15 days and the physiological status of the plants was monitored by measuring relative chlorophyll content and chlorophyll fluorescence (F_V/F_M ratio) every five days as well as relative water content at harvest. The plants were harvested and leaf area was measured (Li-COR leaf area meter, model LI-3000 A, Lincoln NE USA). The tissues were then subdivided into two subsamples. The first was used for the determination of relative water content. Using the dry weight and the relative water content of this subsample, the total dry weight of the tissue at harvest was calculated. The second tissue subsample was rinsed with deionized water to remove adhering soil particles, and snap-frozen for storage as described above for determination of tissue cyanide concentration and β-CAS activity.

2.4 Biochemical and physiological measurements

2.4.1 Quantitative RT-PCR

To assess expression of genes of interest, total RNA was extracted from whole seedlings using the E.Z.N.A Plant RNA Kit (OMEGA Bio-tek, Norcross, GA, USA) according to the manufacturer's instructions. The RNA concentration was determined using a Nanodrop spectrophotometer, (NanoDrop ND-1000, Wilmington, DE, USA). First strand cDNA was synthesized from 1 μg of RNA using the SuperScript^{TM} III First Strand Synthesis System for RT-PCR (Invitrogen, CA, USA) according to the manufacturer's instructions. Real-time PCR was conducted using gene specific primers (Han and Kim, 2006) and the Chromo4 real-time PCR

system (Bio-Rad Laboratories, Hercules, CA, USA). Quantification of transcript abundance was achieved using a standard curve which related DNA concentration to the Ct value (Livak and Schmittgen, 2001). Expression of genes was normalized to the ubiquitin 10 gene (At4g05320) and melting curves were used to confirm that only a single transcript was being amplified.

2.4.2 Measurement of plant tissue cyanide

Tissue cyanide content was determined using a microdiffusion technique (ASTM, 1998; Brinkler and Seigler, 1992) as previously modified (Ebbs et al., 2008) to consist of a 50 mL conical centrifuge tube and a 5 mL glass tube as a gas trap. Plants were ground under liquid nitrogen and then ground again in an extraction buffer (50 mM of K_2HPO_4, pH 8.9) using 10 mL of buffer for each gram of fresh tissue. The slurry was transferred to a 15 mL centrifuge tube and centrifuged for 10 min at 3,000 *g* at 4^{o}C. The supernatant was transferred to the 50 mL Falcon tube. The smaller inner tube, which contained 1.5 mL of 5 mM K_2HPO_4 and 0.5 mL of phenolphthalin reagent, was inserted into the 50 mL centrifuge tube. To force the cyanide into the volatile phase, 18 M H_2SO_4 was added to the supernatant in the 50 mL tube to a final concentration of 0.41 M and swirled gently to mix. The Falcon tube was sealed and incubated at 25^{o} C for 1 h. After the incubation, the smaller gas trap was removed and 0.5 mL of 0.1% KOH was added. After a period of color development, the absorption of the trap solution was then read at 550 nm wavelength with a Cary 50 UV-Visible spectrophotometer (Varian Inc., Mulgrave, Australia), and compared to a standard curve constructed from known concentrations of KCN.

2.4.3 DMPDA assay for β-cyanoalanine synthase activity

To determine activity of β-CAS in the seedlings, whole plants or individual tissues were ground under liquid nitrogen and then under a buffer consisting of 2 mM EDTA-Na_2, 10 mM cysteine, 0.1 M Tris-HCl, pH 9.5. The homogenate was centrifuged at 4,000 *g* for 10 min at 4°C and then split into three subsamples. One subsample was used for determination of the total protein content using the Pierce BCA assay Kit (Thermo Scientific) according to the manufacturer's instructions. The other two samples were used for the determination of β-CAS activity. One of these two tubes was boiled for 10 min to provide a heat-killed control to correct for background absorbance. The activity of β-CAS was assayed using the DMPDA assay (Liang, 2003; Warrilow and Hawkesford, 1998). The crude protein supernatant was mixed 1:1 (v/v) with the substrate (10 mM cysteine, 3 mM KCN, 160 mM 2-amino-2-methyl-1-propanol, pH 9.8). The reaction mixture was incubated in sealed vials at 26º C for 20 min. The reaction was terminated with the addition of 0.5 mL of acidic dye precursor reagent (15 mM N,N-dimethyl-1,4-phenylenediamine dihydrochloride, 3 mM ferric chloride, 4.2 mM HCl). After a 20 min period of color development, methylene blue formation was measured spectrophotometrically at 745 nm. Enzyme activity data was normalized to the protein concentration.

2.4.4 Colorimetric determination of nitrilase and nitrile hydratase activity

Nitrilase and nitrile hydratase activity was assayed via determination of ammonia according to Piotrowski et al. (2001). Briefly, 1 g (FW) of tissue was ground in liquid nitrogen and then in 3 ml of 100 mM KH_2PO_4 buffer, pH 8.9. The homogenate was centrifuged at 4000 *g*

for 15 min at 4°C. The supernatant was decanted and centrifuged again for 20 min to obtain a crude extract. Total protein in the extract was determined as indicated above. For determination of background ammonia in the extract, an aliquot containing 100 μg of total protein was heat denatured in boiling water for 10 min and then incubated with substrate (3 mM β-cyanoalanine in 0.05 M potassium phosphate buffer, pH 8.9) in a total volume of 1 mL. Nessler's reagent (1.33 mL) was added and after 10 min of color development the absorption was read at 480 nm. A second aliquot of the crude extract containing 100 μg of total protein was incubated with the same substrate in a total volume of 1 mL for 30 min at 30°C after which the sample was boiled at 100°C for 10 min to stop the reaction. For parallel determination of nitrilase and nitrile hydratase activity, two aliquots, each 0.1 mL were taken from the reaction mixture and each was diluted to 1 mL with deionized water. To one sample, 1.33 mL Nessler's reagent was added for determination of ammonia resulting from the nitrilase activity. The second sample was incubated with 0.25 units of asparaginase at 37°C for 30 min, allowing for determination of ammonia resulting from both nitrilase and nitrile hydratase activity. After incubation, 1.33 mL Nessler's reagent was added and both samples were read at 480 nm. Nitrile hydratase activity expressed as the difference between ammonia produced from the combined activity of the two enzymes and the activity of nitrilase (Piotrowski et al., 2001).

2.4.5 Relative water content

Samples were immediately weighed after harvest to determine fresh weight (FW). The samples were placed in the dark overnight in vials containing DI water at 4°C and then weighed to determine turgid weight (TW). The samples were oven dried to constant mass at 55°C and the

dry weight (DW) was obtained. Relative water content (RWC) was determined according to Barr and Weatherley (1962):

$$RWC\ (\%) = \frac{FW - DW}{TW - DW} * 100$$

2.4.6 Relative chlorophyll content and chlorophyll fluorescence

Relative chlorophyll content of leaves was determined using a SPAD 502+ Chlorophyll Meter (Konica Minolta Sensing Inc., Osaka, Japan). Chlorophyll fluorescence was measured using an OS1-FL chlorophyll fluorometer (Opti-Sciences; Hanover, NH, USA) following the manufacturer's instructions. Both readings were taken on fully expanded leaves between 11 am and 12 pm. Leaves were dark-adapted for 20 minutes using the manufacturer's clips prior to determination of chlorophyll fluorescence as the F_V/F_M ratio.

2.5 Statistical analyses

Data from the experiments using only Col-0 plants were analyzed using SAS package Version 9.1 as one-way ANOVA with Tukey's test used for post hoc analysis. Data from experiments with Col-0 and the three mutant lines was first analyzed as a two-way ANOVA. Where no significant interactions between the main effects for a parameter were observed, the main effects were analyzed separately by one-way ANOVA with Tukey's test used for post hoc analysis. When a significant interaction between main effects did occur, the data were reanalyzed by one-way ANOVA with each interaction mean (line x treatment) representing an individual treatment. Tukey's test was again used for the post hoc analysis.

3.0 Results

3.1 Response of the β-cyanoalanine pathway to the magnitude and duration of water deficit

Acute water deficit significantly increased the relative abundance of *AtCysC1* transcript, but by less than two-fold overall in whole *A. thaliana* seedlings (Figure 3.1A). A two-fold increase in β-CAS-like enzyme activity was also observed in response to acute water deficit (Figure 3.1B). While there was a significant increase in expression of *AtCysC1* transcript and β-CAS-like enzyme activity, there was no significant difference in tissue cyanide content between control plants and plants exposed to water deficit treatments (data not shown). No significant difference was observed in expression of *AtNIT4* transcript in response to water deficit as compared to control plants (data not shown).

The acute water deficit treatment was repeated with plants removed from the growing medium and allowed to air dry for 20 min. intervals up to 60 min. As expected, relative water content (RWC) of the tissues fell significantly over the course of 1 h, from 96% to ~85% after 20 min and to ~64% by 60 min. When the tissues were analyzed for cyanide concentration and β-CAS-like activity, both were observed to follow similar, transient changes (Figure 3.2). Both tissue cyanide and β-CAS-like activity increased at 20 min by 2.5- and 10-fold higher than the basal levels, respectively, although these changes were not significant. After 20 min., the values for each decreased so that at 60 min, both tissue cyanide concentration and β-CAS-like activity were significantly lower than the peak at 20 min.

One approach to apply a more environmentally-relevant, chronic water deficit was to simply withhold watering and monitor the water status of the growing media. Plants were first harvested for measurement four days after watering ceased when the volumetric water content of the medium fell below 50%. Additional harvests were carried out on the consecutive fifth and sixth days after watering ceased. Tissue cyanide concentration was significantly higher in plants

that had been deprived of water for four and five days as compared to plants prior to the onset of water deficit treatment (Figure 3.3). After six days without water, tissue cyanide concentrations were not significantly different from untreated plants. A significant difference in β-CAS-like activity was observed between plants deprived of water and plants prior to onset of treatment. There was however no significant difference in β-CAS activity between sampling dates. To determine the response of the pathway to a longer duration of water deficit, the experiment was essentially repeated except that plants were harvested after 5, 10, and 15 d without watering, at which point there was an obvious loss of turgor in the leaves of all treated plants. The RWC of the treated tissues decreased significantly from 84% to 64% during the 15 d period, while the well watered control plants maintained a RWC of 84-88%. Compared to control plants, tissue cyanide concentration was significantly higher after 5 d without watering but significantly lower than untreated controls after 10 and 15 d (Figure 3.4). There was no significant difference in β-CAS-like activity between well watered control plants and plants deprived of water for 5 or 10 days (Figure 3.4). However, enzyme activity decreased significantly after 15 d without water. Overall, the activity of β-CAS pathway to water deficit was generally similar regardless of the magnitude and duration of the stress.

3.2 The pathway response is localized to stressed tissue

To demonstrate that the response of the β-cyanoalanine synthase pathway to water deficit stress was a phenomenon localized to tissues experiencing the water deficit, a split-root technique was employed in which one half of the root system of an *A. thaliana* plant was watered normally (root compartment 1) but the other side was left unwatered for seven days (root

compartment 2). Control plants for this experiment consisted of split-root plants with both root compartments watered normally. As anticipated, discontinuing watering led to a significant decrease in the volumetric soil water content, reaching a value of 20% in root compartment 2 as compared to 58% in the watered root compartment 1. The volumetric soil water content of the two root compartments for control plants were each >80%. These differences in soil water between the two equally-watered compartments for control plants and the watered side of the pots receiving a water deficit treatment suggested that water was drawn more heavily from the watered pots opposite the water deficit pots, allowing the plant to compensate for the deficit in the dry compartment. Despite these differences in moisture content, the RWC of the shoots of all plants was unaffected, with no significant difference between the treated and control plants (88 and 85%, respectively). At harvest, there was no significant difference in the root tissue cyanide concentration between the dry and wet compartments or in comparison to the two wet compartments of the control split-root. The tissue cyanide concentration was significantly higher in shoots of plants that had a dry root compartment as compared to control plants with both root compartments well watered (Figure 3.5). While there was no significant difference in β-CAS-like activity in the shoots between treatments, enzyme activity in the roots experiencing water deficit was significantly greater than well-watered roots (Figure 3.6).

3.3 Response of the β-cyanoalanine synthase pathway mutants to water deficit

Consistent with studies by others (Watanabe et al., 2008), the established SALK lines for β-CAS (*AtCysC1*) and the cytosolic cysteine synthase (*AtCysA1*) were shown to be knockdowns for enzyme activity (Figure 3.7A). The β-CAS-like activity decreased by 22% for *AtCysA1* and

42% for *AtCysC1* lines as compared to Col-0 plants. The magnitude of the decrease for each line was similar to the 33% and 50% decrease, respectively, observed previously for these SALK lines (Watanabe et al., 2008). A surprising observation made here and elsewhere (Piotrowski, 2008; Sidibe, 2008) was that the interruption of *AtNIT4* had a concomitant influence on β-CAS-like activity, with a decrease in activity of 71% in comparison to Col-0 plants (Figure 7A). Analysis of AtNIT4 enzyme activity for the *AtCysA1* and *AtCysC1* lines was not significantly different from Col-0 (Figure 3.7B). The *AtNIT4* mutant line did display nitrile hydratase activity although at a rate significantly lower (~20%) than for Col-0 and the other two lines (Figure 3.8A). There was however no significant difference in nitrilase activity under control conditions (4.1 and 3.6 nKat mg^{-1}protein h^{-1} respectively for Col-0 and *AtNIT4*) or in response to KCN treatment (3.9 and 4.0 nKat mg^{-1}protein h^{-1}, respectively).

Growth parameters (e.g., leaf area and biomass) were similar for the Col-0 plants and the *AtCysA1* mutant line under nominal conditions. Leaf area and fresh weight biomass for *AtCysC1* were significantly different from Col-0 while the dry weight biomass was not different. There were no significant differences in these parameters between the *AtCysA1* and *AtCysC1* lines under well-watered conditions. The *AtNIT4* plants showed significantly less leaf area and biomass than the Col-0 and *AtCysA1*line under nominal growth conditions but none of the values were significantly different from the *AtCysC1* line (Figure 3.9). For the *AtNIT4* line, the plants also displayed a smaller rosette diameter and leaf size, but not a decrease in leaf number compared to the other lines (Appendix A). On imposition of water deficit there was a statistically significant decrease in RWC for all lines and the magnitude of the decrease was the same for each line. Well-watered controls across all four lines had a RWC of 83% while the plants deprived of water had a RWC of 71%. Although the magnitude of the decrease in RWC

was the same for all the lines, the effect of this mild water deficit in terms of the subsequent effect on growth varied for each of the mutants. There was a significant decrease in leaf surface area after15 d of water deficit for each line as compared to corresponding well-watered plants of the same line (Figure 3.9). The magnitude of the decrease was not significantly different between the *AtCysA1*mutant line and the Col-0 plants (Table 3.1). The *AtCysA1* mutant had a 35% decrease in leaf area. The *AtCysC1* mutant had 50% reduction in leaf area and significantly different from *AtCysA1* and Col-0. The *AtNIT4* mutant was the most sensitive, with the same parameter decreasing by 66%. For dry weight biomass, the *AtCysA1* showed a small decrease while the *AtCysC1*mutants were slightly larger (Table 3.1). The *AtNIT4* plants decreased significantly in dry weight biomass (25.4%) relative to well watered plants of the same line. The magnitude of decrease in dry weight biomass was however greatest for the Col-0 plants at 42% (Table 3.1).

Chlorophyll fluorescence (F_V/F_M ratio) was also measured every third day after imposition of the treatment for 15 days as an indicator of stress. The Col-0 plants showed no change in the F_V/F_M ratio over time or between treatments (Figure 3.10A). Plants of the *AtCysA1* mutant line showed significantly higher ratios under the water deficit conditions as compared to well-watered plants of the same line. The values for the *AtCysA1* plants under water deficit increased with time and were significantly greater that Col-0 for the latter two time points (Figure 3.10B). The F_V/F_M ratio for well watered *AtCysA1* plants was significantly lower than the treated plants of the same line and control Col-0 plants on Day 5 and 15 but not on Day 10. Under mild water deficit, *AtCysC1* and *AtNIT4* mutants had significantly lower F_V/F_M ratio than the corresponding well-watered plants of the same lines (Figure 3.10C,D). The ratios for both lines on the latter two sampling days were generally significantly lower than comparable

values for Col-0. The relative chlorophyll content of Col-0, the *AtCysA1* and *AtCysC1* lines all showed a significant increase with time as compared to well-watered plants of the same line (Figure 3.11). The measured values for these lines were higher than the control values, representing relative chlorophyll values greater than the well watered plants of the same line. The *AtCysA1* and *AtCysC1*mutants were however not different from Col-0 except at the final time point where the *AtCysA1* line showed the greatest relative chlorophyll content. Contrary to the above, the relative chlorophyll content of the *AtNIT4* mutant showed an initial decline in the first 5 d after initiation of water deficit and stabilized thereafter at 90% of the untreated plants of the same line. At the end of the water deficit period (day 15), the only plants showing an appreciable increase in tissue cyanide was the *AtNIT4* mutant under water deficit (Figure 3.12). There was no difference in β-CAS-like activity between the four lines or between treatments (data not shown). As compared to previous experiments with Col-0 only, the magnitude of the values for β-CAS activity for all lines (Col-0 and the mutants) was lower, in these plants which seemed reasonable since the cyanide content had stabilized at low levels.

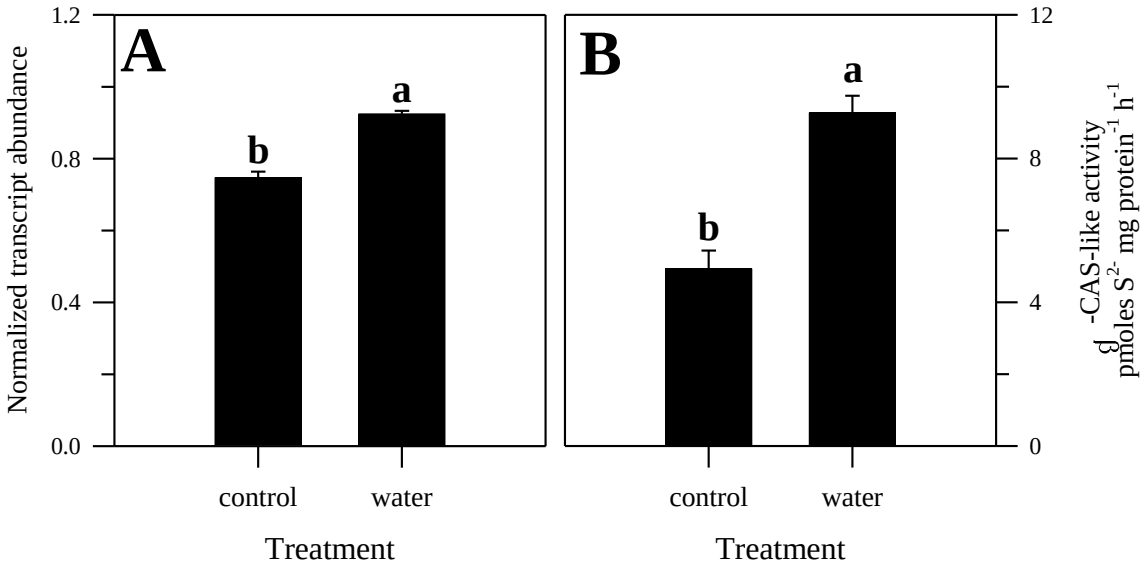

Figure 3.1 Transcript abundance for *AtCysC1* (**A**) and β-CAS-like enzymatic activity in crude protein extracts (**B**) in 3-week-old whole seedlings *A. thaliana* (Col-0) plants grown in agar plates under normal conditions (control) and after exposure to water deficit. Transcript abundance was determined by qRT-PCR relative to ubiquitin 10 in plants subjected to a 20 min air exposure of whole seedling after removal from plates. Enzyme activity was determined for whole plants left unwatered for 7 d followed by a 45 min air exposure of whole seedling after removal from the pot. Each bar represents the mean and standard error (n=3). Within a panel, bars with different letters are significantly different from one another (α=0.05).

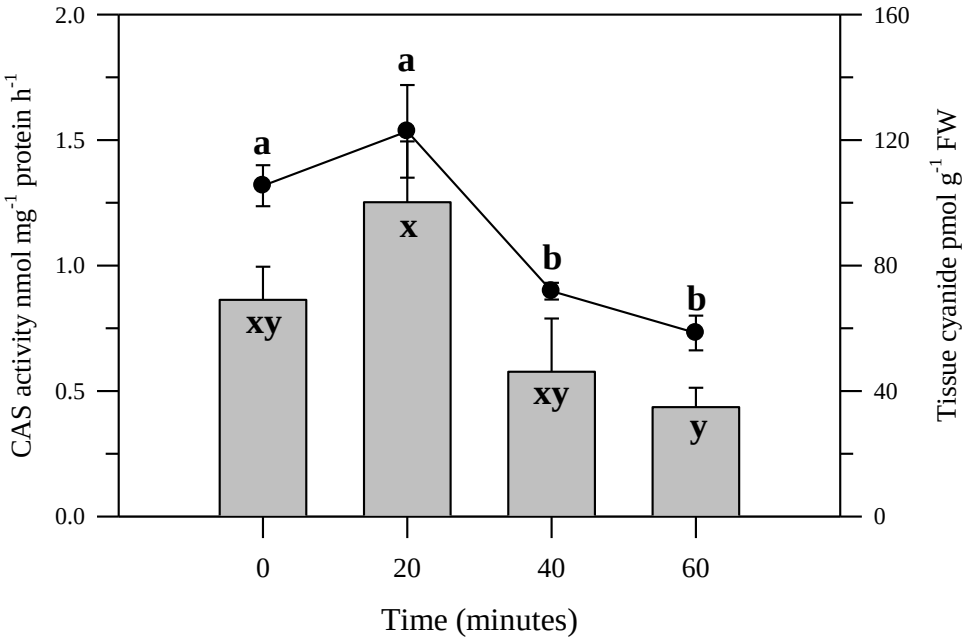

Figure 3.2 Cyanide concentration and CAS-like enzymatic activity in crude protein extracts from shoots of 10 week old *A. thaliana* Col-0 grown in perlite and vermiculite and subjected to acute water deficit by uprooting and exposing to dry air for up to 60 min. The grey bars represent β-CAS activity and the line graph represents tissue cyanide concentration. Each point represents the mean and standard error (n=3). Letters (a-b for tissue cyanide concentrations, x-y for β-CAS-like activity) are used to indicate values that are significantly different from one another (α=0.05).

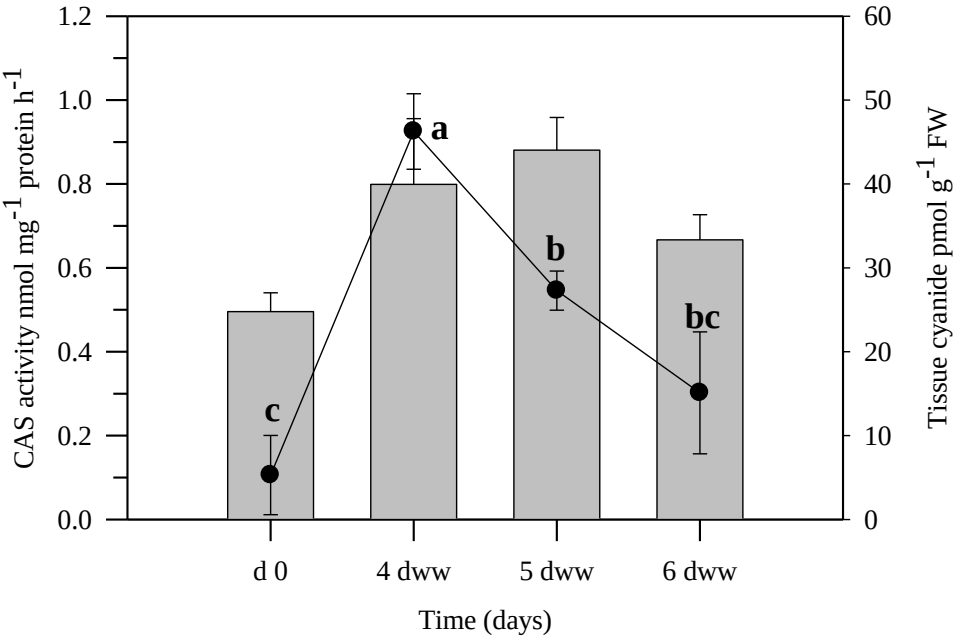

Figure 3.3 Cyanide concentration and β-CAS-like enzymatic activity in crude protein extracts from shoots of 10 weeks old *A. thaliana* Col-0 grown in perlite and vermiculite. Watering was withdrawn for 6 days, dww stands for days after withdrawal of watering. The grey bars represent β-CAS activity and the line graph represents tissue cyanide concentration. Each point represents the mean and standard error (n=4). Letters (a-c for tissue cyanide concentrations) are used to indicate values that are significantly different from one another (α=0.05).

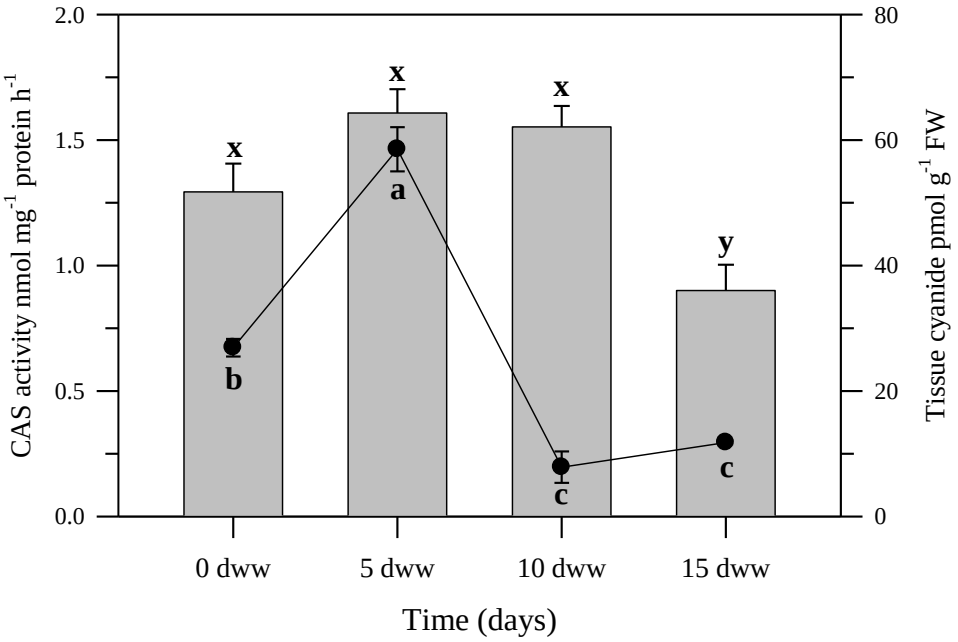

Figure 3.4 Cyanide concentration and CAS-like enzymatic activity of *A. thaliana* Col-0 plants. Plants were grown in perlite:vermiculite medium and subjected to continuous water deficit for 15 days. Grey bars represent CAS activity and the line graph represents cyanide concentration in tissues. Each point represents the mean and standard error (*n*=4). Letters (a-c for tissue cyanide concentrations, x-y for β-CAS-like activity) are used to indicate values that are significantly different from one another (α=0.05).

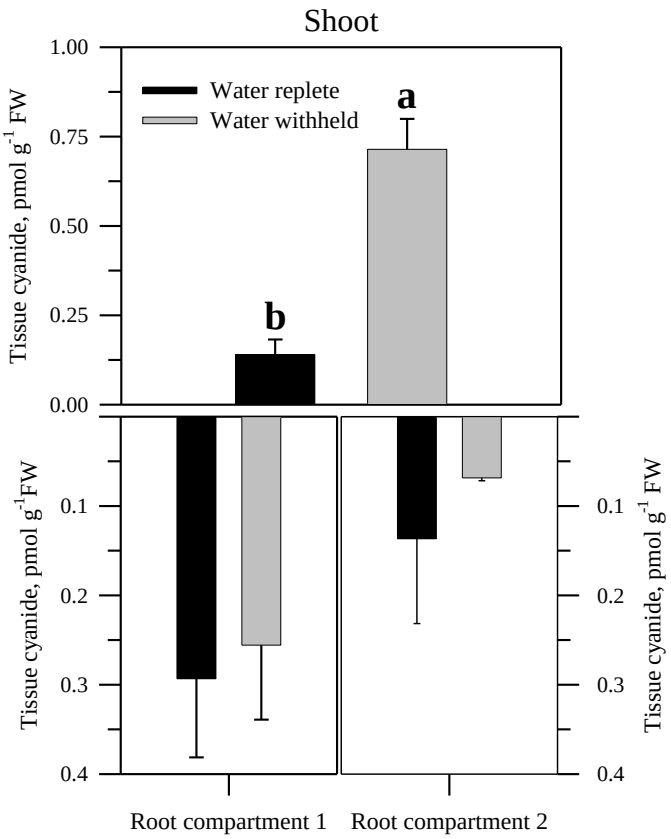

Figure 3.5 Cyanide concentration in crude protein extracts from A. *thaliana* Col-0 plants grown in perlite and vermiculite and subjected to water deficit by splitting the roots into two compartments. Control plants (black bars) were well watered. For the treated plants (grey bars), compartment 1 was watered as the controls, watering was withheld from compartment 2 for 7 days. Each bar represents the mean and standard error (n=4). Bars with different letters are significantly different from each other (α=0.05).

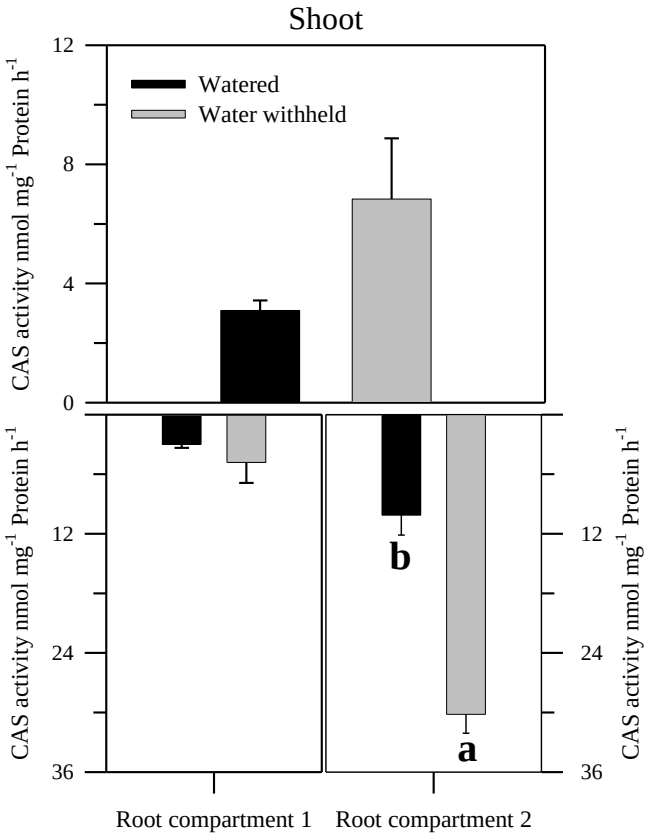

Figure 3.6 β-CAS-like enzymatic activity in crude protein extracts from A. *thaliana* Col-0 plants grown in perlite and vermiculite and subjected to water deficit by splitting the roots into two compartments. Control plants (black bars) were well watered. For the treated plants (grey bars), compartment 1 was watered as the controls, watering was withheld from compartment 2 for 7 days. Each bar represents the mean and standard error (n=4). Bars with different letters are significantly different from each other (α=0.05).

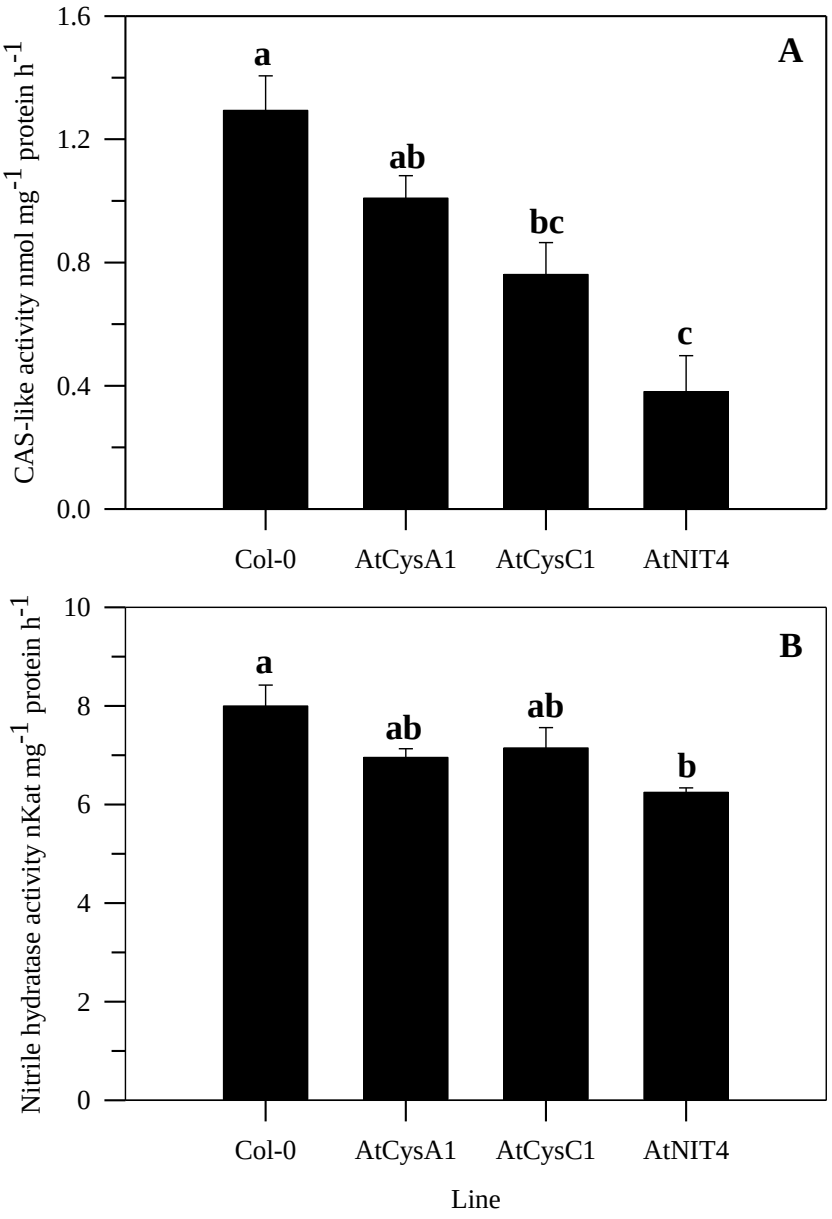

Figure 3.7 Basal β-CAS-like activity (**A**) and nitrile hydratase activity (**B**) in crude protein extracts from 5 week whole plants of *A. thaliana* Col-0 and three mutants *AtCysA1, AtCysC1* and *AtNIT4* grown in hydroponics. Each bar represents the mean and standard error (n=4). Bars with different letters are significantly different from each other (α=0.05). (1 Katal = 1 mol s^{-1})

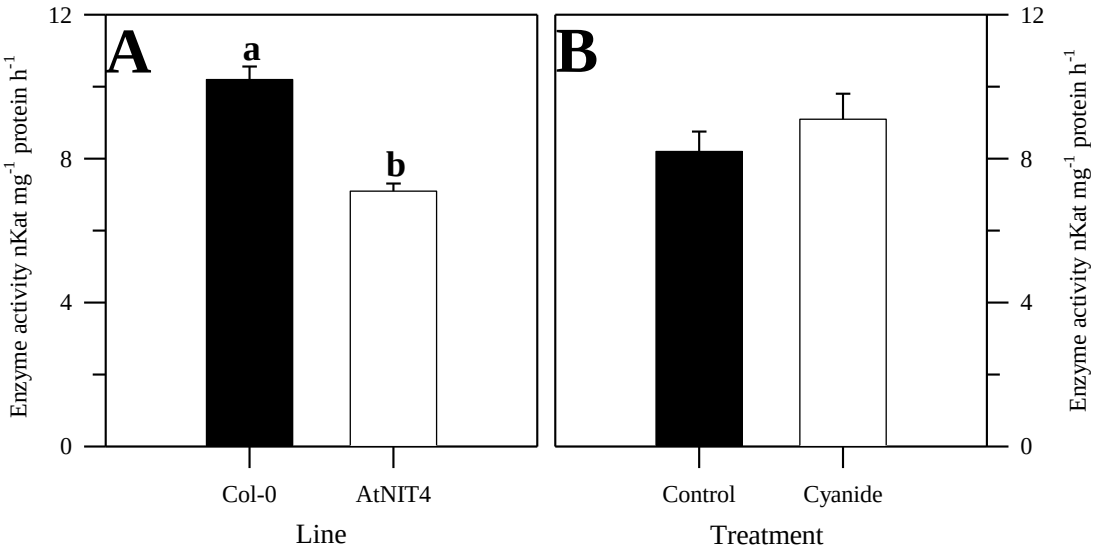

Figure 3.8 Nitrile hydratase activity in crude protein extracts from 5 week whole plants of *A. thaliana* Col-0 and the *AtNIT4* mutant grown in hydroponics and exposed to 1mM cyanide. Results indicate the nitrile hydratase activity between lines (**A**) and within both lines in response to cyanide treatment (**B**). Each bar represents the mean and standard error (n=4). Within a panel, bars with different letters are significantly different from one another (α=0.05). (1 Katal = 1 mol s^{-1})

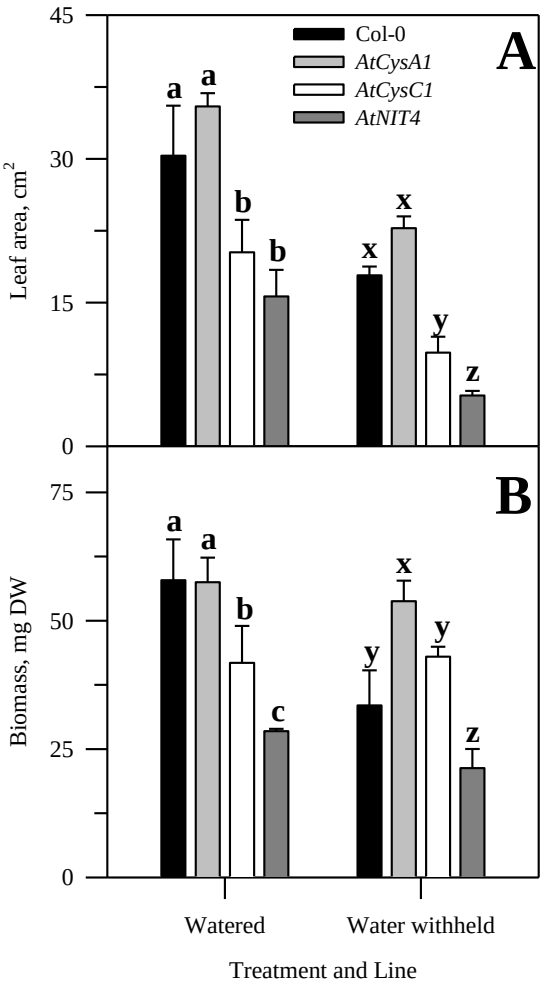

Figure 3.9 Leaf area (**A**) and DW biomass (**B**) of *A.thaliana* Col-0 and three mutants *AtCysA1, AtCysC1* and *AtNIT4* under well watered conditions and following 15 days without water. Plants were geminated and grown for three weeks on MS medium, transferred to perlite and vermiculite and grown for 3 weeks. Plants were then subjected to water deficit for 15 days and harvested. Each bar represents the mean and standard error (n=4). Within a panel and for each treatment, bars with different letters are significantly different from one another (α=0.05).

Table 3.1 Percent change in leaf area, fresh weight (FW) and dry weight (DW) biomass in response to a 15 d period of water deficit by plants of *A. thaliana* Col-0 and three mutants for genes associated with the β-cyanoalanine synthase pathway.

	Percent increase (+) or decrease (-) in measured parameter		
Line	Leaf area	FW biomass	DW biomass
Col-0	-37.2 c	-40.3 b	-42.2 a
AtCysA1	-35.2 c	-41.3 b	-6.6 c
AtCysC1	-50.2 b	-58.1 a	+2.3 c
AtNIT4	-68.0 a	-69.3 a	-25.4 b

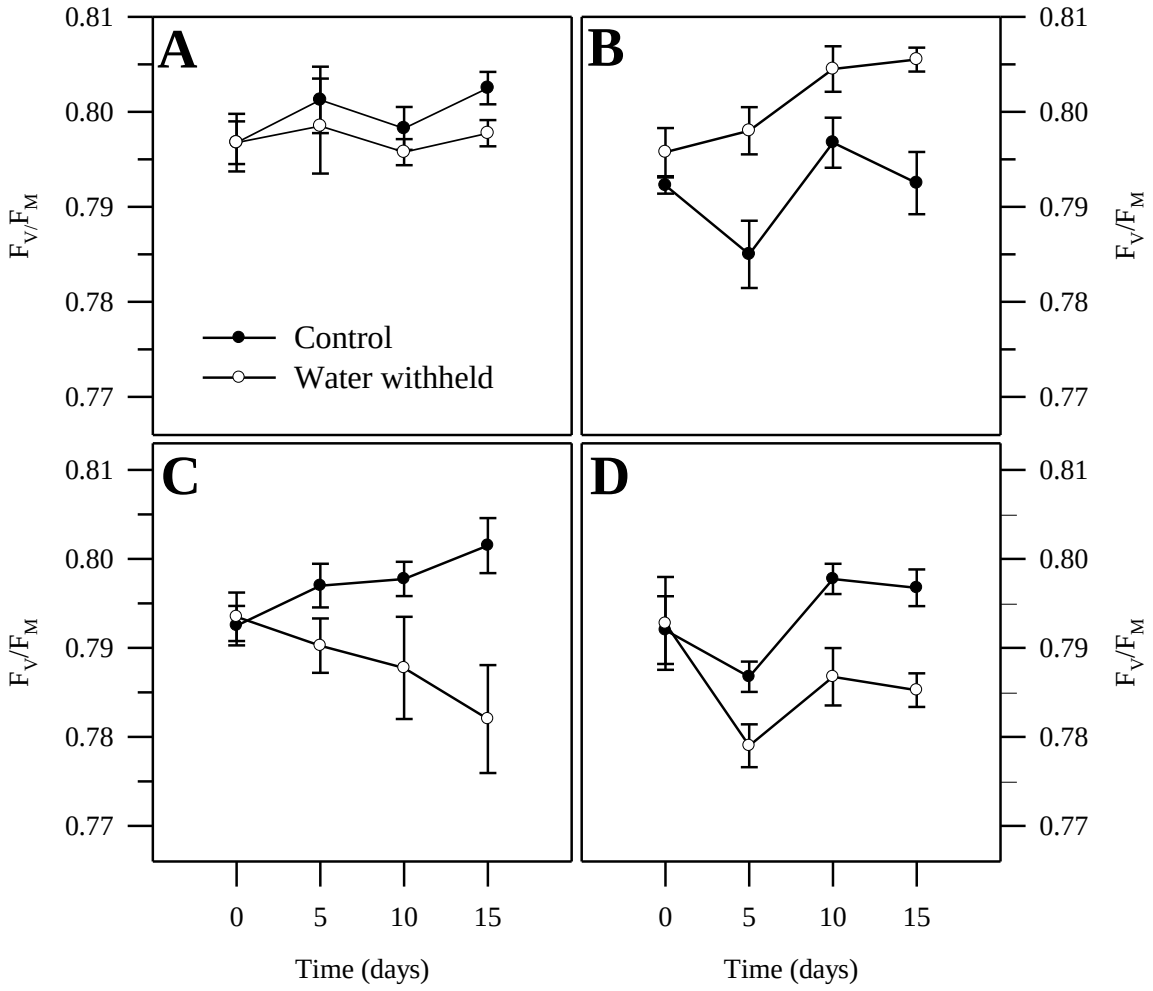

Figure 3.10 Chlorophyll fluorescence as measured by F_V/F_M ratio of *A. thaliana* Col-0 (**A**) and three mutants *AtCysA1* (**B**), *AtCysC1* (**C**) and *AtNIT4* (**D**). Plants were geminated and grown for three weeks on MS medium, transferred to perlite and vermiculite and grown for 3 weeks. Plants were subjected to water deficit for 15 days and measurements taken. The closed circles represent control and the open circles represent the drying treatment. Each point on the line represents the mean and standard error (*n*=4).

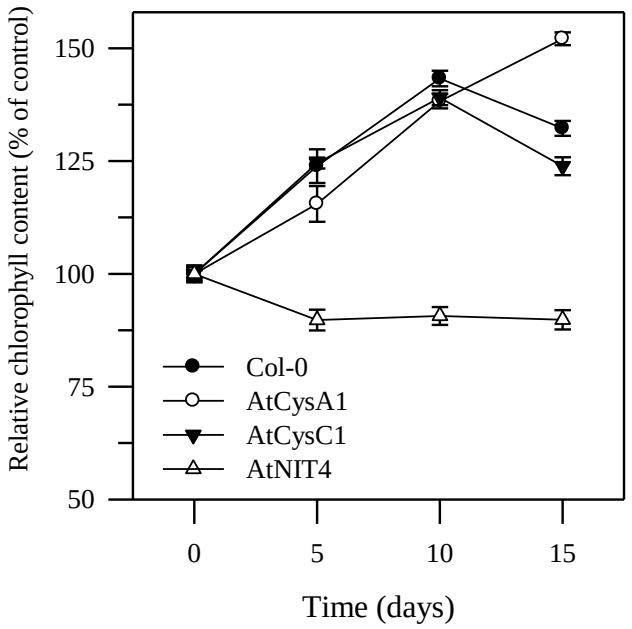

Figure 3.11 Relative chlorophyll content of *A. thaliana* Col-0 and three mutants *AtCysA1*, *AtCysC1* and *AtNIT4*. Plants were geminated and grown for three weeks on MS medium, transferred to (1:1) perlite and vermiculite and grown for 3 weeks. Plants were subjected to water deficit for 15 days and measurements taken during the period. Each point on the line represents the mean and standard error (n=4).

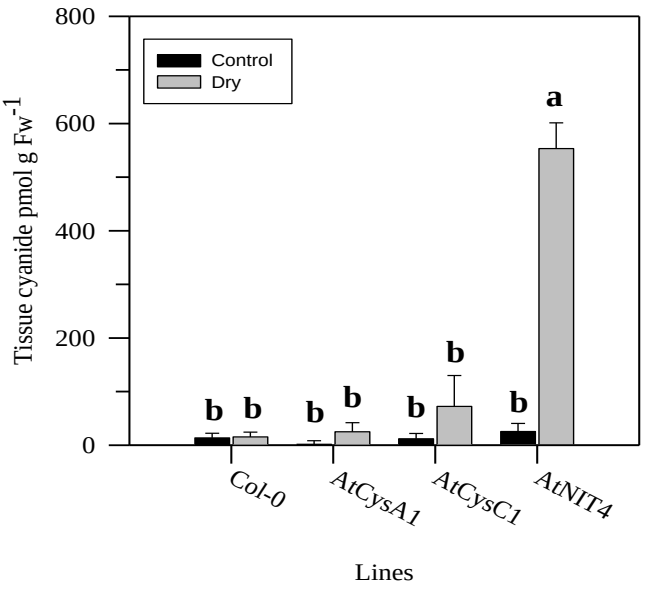

Figure 3.12 Cyanide concentration in crude protein extracts from *A. thaliana* Col-0 and three mutants *AtCysA1*, *AtCysC1* and *AtNIT4*. Plants were geminated and grown for three weeks on MS medium, transferred to perlite and vermiculite and grown for three weeks. Plants were subjected to water deficit for 15 days. Each bar represents the mean and standard error (*n*=4). Bars with different letters are significantly different from one another (α=0.05).

4.0 Discussion

Water deficit is a limiting factor for plant growth and development. Plants utilize a number of mechanisms at the cellular level to stave off the negative effects of water deficit, including osmotic adjustment, synthesis of compatible solutes, increased synthesis of abscisic acid, and upregulated expression of DREB transcription factors and late embryogenesis proteins (e.g., Agarwal et al., 2006; Farooq et al., 2009). Plants also need energy under stress and the relative importance of the mitochondria as the principal organelle for ATP production increases under water stress (Bartoli et al., 2005). While most work has shown that under intense water stress, mitochondria activity decreases (Generozova et al., 2009; Ribas-Carbo et al., 2005), others have reported an increase in mitochondrial function under moderate, slow onset stress (Atkin and Macherel, 2009). Water stress generates ethylene (Gu et al., 2007; Yang et al., 2004), and would also therefore generate cyanide. Since a principle target of cyanide inhibition is the terminal oxidase of the mitochondria, a reasonable hypothesis is that cyanide detoxification by the β-CAS pathway is necessary to help offset the negative effects of cyanide on the mitochondria, and therefore by extension contribute to the response to water deficit.

The first objective for this study was to establish how responsive the β-CAS pathway is to the magnitude and duration of water deficit. In *A. thaliana* the *AtCysC1* gene shows high, constitutive expression that is not responsive to cyanide exposure or stress (Kaplan et al., 2007; Kreps et al., 2002; Matsui et al., 2008; Yamaguchi et al., 2000), hence the significant yet minor difference in transcript abundance even in response to an acute exposure to water deficit was not wholly unexpected (Figure 3.1). The high protein expression is perhaps necessary to compensate for the fact that the cysteine synthesis activity displayed by β-CAS represents a competing

reaction for cyanide assimilation (Hatzfeld et al., 2000). The generally parallel changes in tissue cyanide and β-CAS-like enzyme activity observed here in response to water deficit (Figures 3.2-3.4) are consistent with reports from a similar water deficit study with tobacco (Liang, 2003). Both studies showed a transient increase in β-CAS-like enzyme activity coinciding with a water deficit-induced spike in tissue cyanide. The magnitude of the increase for both cyanide and β-CAS-like enzyme activity was greater for tobacco than was generally observed here for *A. thaliana,* which may indicate that regulation of the *AtCysC1* gene is perhaps different in these two species. The difference may also be related to the examination of individual tissues in the tobacco study compared to whole seedlings here or perhaps the developmental age of the plants used. One prior study illustrated that activity of β-CAS increased with the developmental age of barley (*Hordeum vulgare* L.) leaves (Wurtele et al., 1985). The *A. thaliana* seedlings used here may not have been developmentally equivalent to the tobacco plants used in that prior study (10-leaf stage tobacco seedlings).

While changes in gene expression might account for part of the increased activity of β-CAS in response to water deficit, enzyme activity was more likely directly influenced by ethylene elicited by the abiotic stress as suggested by several studies. Increases in ethylene production have frequently been observed in plants subjected to abiotic stress (e.g., Poor and Tari, 2011; Yang and Hoffman, 1984). An overproduction of ethylene was reported in rice (*Oryza sativa* L.) plants subjected to severe water deficit, while production was lower when the magnitude of stress was moderate (Yang et al., 2004). Since ethylene regulates β-CAS activity (Goudey et al., 1989; Hasegawa et al., 1995a; Hasegawa et al., 1994; Maruyama et al., 1996; Maruyama et al., 1997; Matilla, 2000; Wen et al., 1997) post-transcriptionally (Maruyama et al.,

2001) and through crosstalk with the ethylene signaling pathway (Oracz et al., 2008; Vahala et al., 2003), a concomitant increase in cyanide production in response to water deficit followed by increased activity of β-CAS would be reasonably anticipated. The increase in β-CAS enzyme activity in this study is not likely due to the direct induction by this ethylene-associated cyanide because studies with *A. thaliana*, wheat (*Triticum aestivum* L.), and potato (*Solanum tuberosum* L.) have shown no response of enzyme activity to cyanide treatment (Kosma, 2005; Maruyama et al., 2001; Meyer et al., 2003; Sidibe, 2008). The decreases in β-CAS-like activity in this study were only observed after extended periods of exposure to water deficit. Such decreases may simply reflect overcompensation in terms of cyanide removal, drawing down the cyanide concentration below that typically observed in the plant tissue thereby establishing a new level of cyanide homeostasis during the exposure to the stress. Prior studies have suggested that a cyanide homeostasis in plant tissues maintains concentrations below those that are potentially inhibitory but also within a range consistent with some possible degree of physiological relevance (Yip and Yang, 1998). It was interesting to note that the pattern displayed for cyanide concentration and β-CAS-like activity was similar for each combination of water deficit intensity and duration imposed on the *A. thaliana* seedlings (Figures 3.2-3.4) suggestive of a coordinated response that includes metabolic components beyond the β-cyanoalanine synthase pathway. With little evidence for an induction of *AtCysC1*gene, the results suggests that innate activity of the existing pool of enzymes in *A. thaliana* may be sufficient to assimilate pulses of cyanide elicited during stress-induced cyanide synthesis. The same may be true for the NIT4 enzyme and may therefore explain the lack of a response at the level of transcription to imposition of water deficit.

The split-root experiment further demonstrated that the response of the β-CAS pathway to water deficit was localized predominantly to the tissue directly subjected to the stress as illustrated by the significant increase in β-CAS-like activity in roots from the dry compartment as compared to the well watered compartment (Figure 3.6). Within the dry compartment, the imposition of water deficit would presumably promote a localized increase in stress ethylene which would in turn be expected to increase local activity of β-CAS. Localized ethylene production was also reported in banana (*Musa acuminata*) in response to wounding and in bean (*Phaseolus vulgaris* L) in response to viral infection (Delaat and Vanloon, 1983; Donminguez and Vendrell, 1993). The tissue cyanide concentration from the split root experiment was ~two-fold higher as compared to the other two drying techniques (Figure 3.5). Increased ethylene production was also reported in tomato plants treated as split-roots (Hussain et al., 2000) suggesting that the splitting of the roots imposed an additional stress, perhaps mechanical. It was however, unexpected that the shoots of treated plants had higher cyanide concentrations as compared to the controls and this accumulation of cyanide was not accompanied by a significant increase in β-CAS activity, although the results did perhaps trend in that direction (Figure 3.6). As reported in other split-root studies (e.g., Gowing et al., 1990) the RWC result here did not indicate water deficit stress in the shoots. The cyanide detected in that tissue would not immediately seem to arise as a collateral result of the root-level treatment since the shoots of the treated plants maintain their hydration by drawing the necessary water from the well-watered compartment. Two hypotheses may explain the source of the cyanide detected in the shoots. The first is that cyanide was produced in the drying roots and moved by diffusion into the dead xylem cells resulting in its translocation to shoots *via* the transpiration stream. This proposition however

seems unlikely considering the volatile nature of the HCN molecule and the widespread detoxification system in all living tissues (Wurtele et al., 1984). An alternate explanation is that cyanide was synthesized in the shoots in response to chemical signals from roots. Studies have demonstrated chemical signaling between tissues upon perception of stress (Davies, 2000; Hussain et al., 2000; Schachtman and Goodger, 2008). In these studies increased abscisic acid (ABA) and ethylene were reported in the xylem sap of split-root treated plants. In another partial root drying experiment the authors reported increased ethylene evolution in tomato (*Lycopersicum esculentum*) leaves of treated plants while there was no difference in leaf water potential (Sobeih et al., 2004). It is also well established that production of ACC, the ethylene precursor is increased in response to stress and may be translocated in the xylem (Else and Jackson, 1998). An increase in ACC production in the roots from the dry root compartment followed by translocation to the shoots may have thus resulted in increased ethylene production in shoots, and therefore cyanide production.

The efforts to evaluate how the β-CAS mutants responded to the imposition of water deficit was complicated by the inherent differences in growth characteristics for the lines (Figure 3.9) and the specific response of each to the mild water deficit imposed (Table 3.1, Figures 3.10-3.12). Collectively the decrease in RWC, decrease in fresh weight biomass, and apparent reduction of leaf expansion indicated by the decrease in leaf area (Figure 3.9) all indicate that withholding water did affect the water status of plants from all four lines. The progressively greater decrease in these two values from the *AtCysA1* to *AtCysC1* and *AtNIT4* lines (Table 3.1) appears inversely related to the apparent importance of these genes and their encoded enzymes for the β-CAS pathway in that *AtCysC1* is responsible for a larger proportion of β-CAS enzyme

activity than *AtCysA1* while *AtNIT4* acts at a critical bottleneck on the pathway. This relationship is not fully supported though by the data for tissue cyanide and β-CAS enzyme activity. With the exception of the *AtNIT4* line, this mild water deficit did not produce an increase in β-CAS enzyme activity or an increase in tissue cyanide for the *AtCysA1* and *AtCysC1* lines (Figure 3.12). For those two lines, the redundant β-CAS activity provided by the functional enzyme may have been sufficient to metabolize any cyanide produced preventing any detectable rise in tissue cyanide. Alternately, this mild water stress may not have been significant enough to elicit a strong ethylene response in most lines, which would limit formation of ethylene-associated cyanide. While the overall concentrations may not have been elevated in tissues of these two mutant lines, the loss of the cytosolic β-CAS enzyme activity of AtCysA1 or the mitochondrial β-CAS enzyme activity AtCysC1 in that line may not have left cyanide-sensitive biochemical processes in that compartment sensitive to local increases in cyanide. Even in the absence of an increase in cyanide in those compartments, the imposition of mild water deficit must have interacted in a different way with these two mutant lines to influence the change in leaf area and fresh weight biomass. Curiously, the decrease in dry weight biomass for the Col-0 line further indicates that the change in water status did have a concomitant effect on growth of that line. The *AtCysA1* and *AtCysC1* lines did not show a comparable decrease in dry weight biomass, suggesting that each line was more robust than Col-0 in growth following the imposition of the mild water deficit. An increase in rosette size at the seedling stage was reported previously for the *AtCysA1* mutant (Lopez-Martin et al., 2008), although for this study the growth enhancement was reflected through to maturity at 10 weeks. No statistically

significant evidence of this was observed here (Appendix A) but the plants used in the experiments performed had not reached this developmental stage.

While these two lines and Col-0 showed an increase in relative chlorophyll content under water deficit as compared to well-watered plants of the same line, these results may not specifically indicate a change in chlorophyll synthesis. The apparent change in chlorophyll could be due to the reduced leaf area since changes in chloroplast size, number, and/or chlorophyll content may be independent of changes in leaf area (Boardman, 1977). The divergence at Day 15 (Figure 3.11) with a sustained increase demonstrated by the *AtCysA1* line as compared to a sharp decrease for Col-0 and *AtCysC1* suggest the possible onset of more distinct differences between the two mutant lines in response to the mild water stress imposed. The disparate pattern of change in chlorophyll fluorescence displayed by the *AtCysA1* and *AtCysC1* lines indicates a potential increase in PS II efficiency for the former and a decrease in efficiency for the latter (Baker, 2008; Yordanov et al., 2000).

In the absence of additional data, only speculative explanations for the differences in response of the *AtCysA1* and *AtCysC1* mutant can be offered. Given the differences in chlorophyll fluorescence under water deficit, one possibility may relate to the coordination between the mitochondria and the chloroplast necessary during periods of water deficit. In C3 plants under water deficit, photosynthesis, photorespiration, and mitochondrial respiration are tightly coordinated to maintain cellular energy and carbon status. This coordination involves an exchange of carbon skeletons, reducing equivalents, and ATP to insure carbon balance between these two organelles (Atkin and Macherel, 2009), and under the gradual onset of water deficit stable or increased carbon metabolism (Bogeat-Triboulot et al., 2007; Hummel et al., 2010; Yu

and Setter, 2003). In sunflower (*Helianthus annuus* L.) under water stress, the mitochondria, which are less susceptible to moderate water stress than the chloroplast (Tezara et al., 2008), export ATP to the chloroplast to support photosynthetic activity (Atkin and Macherel, 2009; Raghavendra and Padmasree, 2003). Results from barley indicate that the PSII D1 protein in barley leaves is highly vulnerable to damage under water deficit stress (Yuan et al., 2005). Repair of the D1 complex would require sufficient ATP and carbon from the mitochondria and additional nitrogen allocation for reassembly of the PS II complex. The metabolic network that supports these metabolic activities under water deficit could have been differentially affected by the insertional mutation of *AtCysA1* or *AtCysC1* leading to contrasting effects on the efficiency of photochemical quenching and hence chlorophyll fluorescence (Figure 3.10). If this differential effect in the two mutant lines extended to effects on stomatal closure and evapotranspiration under the mild water stress here, then this might provide some explanation for the significant difference in decrease in leaf area and fresh weight biomass between the lines.

An alternate explanation may involve the interaction of the β-CAS pathway with signaling molecules such as ethylene, ABA, reactive oxygen species, and hydrogen sulfide. The interaction between cyanide and ethylene synthesis is well documented (Yip and Yang, 1988; Yip and Yang, 1998), with cyanide enhancing expression of genes such as *ACS6* associated with ethylene synthesis (Smith and Arteca, 2000) and displaying crosstalk with elements of the ethylene signaling pathway during germination of sunflower seed (Oracz et al., 2008). There is a growing body of evidence indicating interactions between ethylene and ABA in the control of growth under water stress (Wilkinson and Davies, 2010). Increased production of reactive oxygen species (ROS) under water deficit has been demonstrated (Hu et al., 2006) and these

molecules are both sources of cellular damage and cellular signals. The ROS are thought to be important for regulating activity of the cytochrome and alternative oxidase pathways of mitochondrial electron transport. Cyanide also influences these two pathways via its inhibition of the terminal oxidase of the cytochrome pathway (Nuskova et al., 2010) and may have direct roles in modulating production of ROS (Chun et al., 2004; Prabhakaran et al., 2004). There is emerging evidence of signaling roles of hydrogen sulfide in plant cells. As enzymes with dual function in cysteine synthesis (which consumes hydrogen sulfide) and β-CAS enzyme activity (releasing hydrogen sulfide), insertional mutations of either *AtCysA1* or *AtCysC1* could alter the regulation of both cyanide and hydrogen sulfide concentration. Given the numerous potential interactions of these two β-CAS pathway enzymes in this landscape of signaling molecules, a complex network of interactions could emerge that could influence growth in the presence or absence of mild water stress, stomatal closure, and by extension photosynthetic activity and photochemical quenching for PS II protection. Obviously a possible disruption to this signaling network and/or a shift in cellular carbon balance resulting from one of the two specific insertional mutations would require extensive additional work to verify, but these two possibilities offer some explanation for the results obtained for these two lines.

The results of this work clearly demonstrate that the insertional mutation of *AtNIT4* has the most drastic effect on growth under both nominal and water limiting conditions as compared to *AtCysA1* or *AtCysC1*. While sulfurtransferases in plants have been identified and do show metabolism of cyanide to thiocyanate *in vitro* (Meyer et al., 2003; Papenbrock and Schmidt, 2000a), the data here on hypersensitivity of the *AtNIT4* mutant indirectly supports prior studies which demonstrated that sulfurtransferases show no specific role in cyanide homeostasis *in*

planta (Meyer et al., 2003). The results offer additional evidence that the β-CAS pathway is the primary pathway for cyanide detoxification in Arabidopsis. The *AtNIT4* mutant plants are not only knockdowns for nitrile hydratase activity but also knockdowns for β-CAS activity (Figure 3.7 and 3.8). The knockdown in β-CAS activity may be indicative of feedback control of the pathway. With decreased activity of both enzymes in the pathway, there would be an accumulation of β-cyanoalanine and decreased assimilation of cyanide. Accumulation of both compounds (cyanide, β-cyanoalanine) would be potentially detrimental to the plants (Piotrowski and Volmer, 2006; Watanabe et al., 2008), especially in the absence of any activity from the sulfurtransferase pathway for cyanide assimilation. Although ostensibly a t-DNA insertional mutant, the modest decrease in nitrile hydratase and lack of a difference in nitrilase activity suggest that this line may be leaky or may not in fact be homozygous for the insertion. While *AtNIT4* is a single gene in *A. thaliana* which shows high specificity *in vitro* for β-cyanoalanine as a substrate (Piotrowski et al., 2001), there seem to be no published studies with *AtNIT4* mutants in *A. thaliana* to demonstrate that a t-DNA insertion in this gene abolishes enzymatic activity. The nitrilase/nitrile hydratase from maize (*Zea mays*), sorghum (*Sorghum bicolor*) and tobacco are heterodimers (Howden et al., 2009; Jenrich et al., 2007). Loss of one protein of the heterodimer may not completely abolish the enzyme activity but may decrease the overall rate and/or efficiency of enzymatic activity (Jenrich et al., 2007). Nonetheless, the sensitivity of *AtNIT4* plants is reflected in the magnitude of the reduction in leaf area and fresh weight biomass (Figure 3.9), the decrease in chlorophyll fluorescence (Figure 3.10D) and relative chlorophyll content (Figure 3.11). The small phenotype is comparable to what might be expected for an auxin-deficient mutant (Ya, 2003), especially since nitrilases are also involved in auxin

biosynthesis (Kriechbaumer et al., 2007; Park et al., 2003). However, given the purported substrate specificity of the AtNIT4 protein for β-cyanoalanine (Piotrowski et al., 2001), this argues against *AtNIT4* as an auxin-deficient mutant. If the *AtNIT4* is indeed a single gene in *A. thaliana* responsible for hydrolysis of β-cyanoalanine (Piotrowski et al., 2001; Piotrowski and Volmer, 2006), knocking down this gene should cause a build-up of cyanide (Figure 3.12) as well as β-cyanoalanine. β-cyanoalanine can be toxic to plants (Watanabe et al., 2008) via inhibition of Asparagine:tRNA synthatase (Lea and Fowden, 1973) and inhibition of root growth (Howden et al., 2009; Kriechbaumer et al., 2007). These effects could then be additive to or synergistic with the hypothesized cellular changes resulting from the decreased β-CAS activity in the *AtCysA1* or *AtCysC1* lines.

5.0 Conclusion

In conclusion, this study has shown that the β-CAS pathway in Arabidopsis Col-0 shows a transient response to water deficit stress. The pattern of response is similar even with differences in the magnitude and duration of stress. The results have shown that the response of the β-CAS enzyme is specific to the tissue directly experiencing the stress. This study has also shown enzymatic functional redundancy between the AtCysA1and AtCysC1 proteins and supports prior results (Watanabe et al., 2008) demonstrating that AtCysC1 makes a greater contribution to β-CAS activity than AtCysA1. Insertional mutation of one of the corresponding genes does not appear to affect cyanide homeostasis under water deficit but does give rise to distinct differences in growth parameters associated with water status (i.e., leaf surface area and fresh weight biomass) and chlorophyll fluorescence. Such results suggest a complex metabolic

network involving the proteins encoded by *AtCysA1*and *AtCysC1*. The results from the *AtNIT4* line demonstrate the innate differences in growth of this line under both normal and water limited conditions. A functional sulfurtransferase pathway, which should theoretically be able to remove excess cyanide during water deficit, did not function as such in the *AtNIT4* insertional mutants. Given the modest reduction in nitrile hydratase activity in this line, further investigation will be needed to examine the contribution of the encoded enzyme to nitrilase and nitrile hydratase activity and potential *in vivo* redundancies that may exist. The degree to which β-CAS activity contributes to abiotic stress tolerance will also require further examination to establish the extent of cyanide production under other water deficit scenarios and in response to other abiotic stresses. The results obtained are relevant to both the fundamental study of abiotic stress in plants and to the herbicide industry in that the mode of action of auxinic herbicides is induction of ethylene biosynthesis and cyanide autotoxicity (Bartel and Fink, 1994; Grossmann, 1996; Grossmann, 1998; Grossmann and Kwiatkowski, 1995). Tolerance to such herbicides has been linked to β-CAS activity (Grossmann and Kwiatkowski, 2000) suggesting both stress and herbicide tolerance may be provided in plants in part by the β-CAS pathway. The complexity of the results obtained here and the interaction of the β-CAS pathway with mitochondrial function and several important cellular signaling molecules (e.g., ethylene, ROS, hydrogen sulfide) imply a broader contribution to cellular function than simple cyanide detoxification.

CHAPTER 4

THE β-CYANOALANINE PATHWAY IS INVOLVED IN TOLERANCE TO CYANIDE IN ARABIDOPSIS THALIANA

1.0 Introduction

Plants can be exposed to cyanide from both endogenous and exogenous sources. Natural exogenous sources include cyanogenesis by bacteria, fungi, algae, and plants. Rhizosphere concentrations of 100 mg cyanide kg^{-1} DW soil have been reported in soils with cyanogenic bacteria (Owen and Zdor, 2001). Release of cyanide from dead and decomposing plant residues depends on the cyanogenic content of the tissues with reports of <2 μg g^{-1} tissue in sudangrass (*Sorghum sudanense*) leaf tissue (Widmer and Abawi, 2002), but may be >100 mg kg^{-1} cassava (*Manihot esculenta* Crantz) roots and leaves and sorghum (*Sorghum bicolor* L.) leaves (Busk and Moller, 2002; Kobawila et al., 2005). The greater amount of exogenous cyanide in the environment comes from anthropogenic sources, mostly industrial waste (Wong-Chong et al., 2005b). Soils contaminated with industrial wastes from various industries may contain >1,000 mg kg^{-1} DW soil (Henny et al., 1994). Higher plants produce cyanide endogenously during the oxidation of 1-amino-cyclopropane-1-carboxylic acid to ethylene (Bleecker and Kende, 2000; Tsuchisaka et al., 2009). Under nominal conditions, concentrations ~4 μg g^{-1} have been reported in apple (*Malus domestica*), which may increase during fruit ripening and senescence to >8 μg g^{-1} (Mizutani et al., 1987; Yip and Yang, 1988). Some plants that produce cyanogenic glycosides and cyanolipids may release some of that cyanide upon degradation of these compounds (Morant et al., 2008b). The amount of cyanide produced by such plant species is variable but the cyanide

potential may be as high as 2.5 g kg^{-1} for sorghum and almond (*Prunus dulcis* L.) and >3 g kg^{-1} for lima bean (*Phaseolus lunatus* L.) (Fokunang et al., 2001).

Recent studies have shown that plants will take up free cyanide and other metallo-cyanide compounds when present in the root zone (e.g.Ebbs et al., 2003; Ebbs et al., 2010; Larsen and Trapp, 2006; Yu et al., 2005b). While free cyanide will readily diffuse across membranes, the mechanisms for the uptake of metallo-cyanide compounds are not yet clear (Ebbs et al., 2008; Yu et al., 2012). Within plant tissues, cyanide is known to inhibit biological processes including respiration, carbon and nitrate assimilation by binding to metallo-enzymes (Leavesley et al., 2008; Solomonson, 1981). Cyanide also promotes the production of reactive oxygen species (ROS) and has been shown to increase hydrogen peroxide (H_2O_2) in embryonic axes of sunflower (*Helianthus annuus* L.) by stimulating NADPH oxidase while also inhibiting antioxidant enzymes such as catalase (e.g.Gunasekar et al., 1998; Oracz et al., 2009; Siegien and Bogatek, 2006). There are reports of growth suppression of lettuce (*Lactuca sativa* L.), barnyard grass (*Echinochloa crus-galli*) and green foxtail (*Setaria viridis* L.) by cyanogenic rhizobacteria and exogenous cyanide concentrations of 12.5 to 100 µM in the external solution (Grossmann and Kwiatkowski, 1995; Kremer and Souissi, 2001). Significant reduction in root growth was observed for *A. thaliana* plants exposed to 50 µM KCN (Smith and Arteca, 2000) and nuclei degradation in pea (*Pisum sativum* L.) leaves exposed to 2.5 mM KCN (Samuilov et al., 2006).

Plants have biochemical mechanisms to detoxify excess cyanide to evade the toxic effects while also maintaining cyanide at concentrations necessary for the purported signaling function of this molecule (Siegien and Bogatek, 2006). Low concentrations of cyanide (<10 µM) can be

detected in plant tissues but a tight homeostasis is typically maintained to protect sensitive enzymes and processes within the cells (Yip and Yang, 1988). During periods of increased ethylene synthesis, such as ripening and senescence (Mizutani et al., 1987), or during periods of biotic or abiotic stress (Djanaguiraman and Prasad, 2010; Hase et al., 2006) there may be transient increases in tissue cyanide that must be compensated for. Yu *et al*., (2011) reported an increasing cyanide assimilation rate with increasing cyanide concentration in rice (*Oryza sativa* L.) seedlings. In basket willow (*Salix viminalis* L.), exogenous cyanide was quickly metabolized to below inhibitory levels in the plant tissues (Larsen et al., 2005).

Two metabolic pathways are implicated in the detoxification and assimilation of excess cyanide in higher plants. One pathway is mediated by sulfurtransferases such as rhodanese (Blumenthal et al., 1968; Meyer et al., 2003). Sulfurtransferases transfer the cyanide moiety to thiosulfate to produce the less toxic compound thiocyanate. In animals sulfurtransferases are the principle means of detoxifying cyanide. In plants available evidence suggests that the role of rhodanese in cyanide detoxification may be incidental (Meyer et al., 2003; Papenbrock et al., 2011). Reports of low rhodanese activity in some plants (Kakes and Hakvoort, 1992; Miller and Conn, 1980) provide little evidence that this activity contributes to cyanide detoxification.

The principal mechanism for maintaining cyanide homeostasis in higher plants is the β-cyanoalanine (β-CAS) pathway. In this two-step pathway, cyanide is substituted for the sulfhydryl group of cysteine to form β-cyanoalanine with the release of hydrogen sulfide (Blumenthal et al., 1968). The β-cyanoalanine is further hydrolyzed in the second step by a dual enzyme with both nitrilase and nitrile hydratase activity, forming asparagine or aspartate and

ammonia, respectively (Piotrowski et al., 2001). In plants such as *A. thaliana*, the genes that encode enzymes capable of forming β-cyanoalanine belong to the β-substituted alanine synthase (Bsas) family. Work by Watanabe et al., (2008) has shown that only two proteins, encoded by *AtCysA1* and *AtCysC1*, possess β-CAS activity, with the latter displaying greater activity than the former. In *A. thaliana* the nitrilase/nitrile hydratase designated as nitrilase 4 (*AtNIT4)* is reportedly encoded by a single gene (Piotrowski et al., 2001).

The overall goal of this study was to further explore the contribution of each gene in the β-CAS pathway to cyanide tolerance. The study used *Arabidopsis thaliana* (Col-0) and SALK t-DNA insertion lines in the Col-0 background for the genes associated with the pathway, namely cysteine synthase (*AtCysA1*), β-cyanoalanine synthase (*AtCysC1*), and nitrilase 4 (*AtNIT4*). The specific objectives were two-fold. The first objective was to determine the extent to which disruption of a single gene (*AtCysA1* or *AtCysC1)* impairs the ability to metabolize cyanide. The intent was to address whether the two enzymes are fully redundant in their role for β-cyanoalanine formation such that even with the loss of one, the other maintains sufficient activity to maintain cyanide homeostasis and detoxification. The second objective was also a question associated with pathway redundancy. The second step of the pathway mediated by nitrilase 4 is a potential bottleneck in the β-CAS pathway since the nitrilase 4 is a single gene. Studies have attempted to demonstrate a role of the sulfurtransferase pathway in plants (Meyer et al., 2003; Papenbrock and Schmidt, 2000a; Papenbrock and Schmidt, 2000b) but have done so in the presence of a fully functional β-CAS pathway. The contribution of the sulfurtransferase pathway to cyanide detoxification may therefore be masked by the more prominent β-CAS pathway. Disrupting *AtNIT4* to decrease cyanide detoxification via the β-CAS pathway presents an

opportunity to indirectly investigate whether activity of the sulfurtransferase pathway can compensate to provide cyanide homeostasis and detoxification if necessary.

2.0 Materials and methods

2.1 Plant culture

Seeds of wild type *Arabidopsis thaliana* (Col-0) and SALK line mutants for cysteine synthase (*AtCysA1*, SALK_72213), β-cyanoalanine synthase *(AtCysC1*, SALK_22479) and nitrilase 4 (*AtNIT4* SALK_016289C*)* were obtained from the Arabidopsis Information Resource, TAIR (www.Arabidopsis.org). The first two lines are the same as those used in prior research (Watanabe et al., 2008). The *AtNIT4* mutant was used in one study (Howden et al., 2009) and showed impaired metabolism of β-cyanoalanine. Earlier work using qRT-PCR and/or enzyme activity established the status of each line as knockdowns in the activity of the respective enzymes (Chapter 3). The seeds used here were surface sterilized by the vapor-phase sterilization method (Clough and Bent, 1998). Briefly, seeds were placed in a 1.5 mL micro centrifuge tubes in a desiccator jar with the tubes left open. A 250 mL beaker containing 100 mL bleach was also placed in the middle of the desiccator. The desiccator was placed in a fume hood. Concentrated HCl (3 mL) was added to the bleach and the jar was immediately sealed. The desiccator was left undisturbed for 4 h to allow the generated chlorine gas to surface sterilize the seeds. After 4 h, the seal on jar was released and a period of 1 h allowed for evacuation of the chlorine. Thereafter the seeds were removed and stored at 4^{o}C in the closed vials. The sterilized seeds were mounted

on plates containing half-strength MS media with 1% agar and 0.5% sucrose and placed in the dark for 2 d at 4ºC for vernalization. Thereafter the plates were placed at a 30° angle in a Percival growth chamber for germination (Model E-36 L, Des Moines, IA, USA) at ambient humidity with an 8 h photoperiod at a light intensity of ~150 μM m^{-2} s^{-1} provided by a combination of fluorescent and incandescent bulbs. The day/night temperatures were 22° and 18°C respectively. After three weeks of growth on MS plates, seedlings were transferred to black 2-L pots and cultured in hydroponics with nutrient solution under the same growth chamber conditions. The nutrient solution had the following composition: 6 mM KNO_3, 4 mM $Ca(NO_3)_2$, 0.1 mM $NH_4H_2PO_4$, 1 mM $MgSO_4$, 50 μM KCl, 12.5 μM H_3BO_3, 1 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.1 μM $NiSO_4$, and 0.016 μM $(NH_4)_6Mo_7O_{24}$. The solution was buffered with 1 mM *n*-morpholinoethanesulfonic acid (MES) titrated to pH 6.0 with MES-TRIS. Iron was provided as 10 μM Fe-EDTA (Ebbs et al., 2003). The solution was continuously aerated and changed every week. Plants were left to grow for three weeks in the hydroponic culture to accumulate sufficient biomass. Thereafter cyanide treatments for the experiments below were imposed.

2.2 Dose-response of the β-CAS pathway mutants to cyanide exposure

The experiment was carried out under the same growth chamber conditions described above. The composition of the cyanide treatment solution was similar to the basal nutrient solution except that the phosphate concentration was decreased to 0.02 mM, the $MnSO_4$ concentration was 1.6 μM, iron was omitted from the solution to preclude formation of iron

cyanide solids, and aeration was discontinued to minimize volatilization (Ebbs et al., 2003; Ebbs et al., 2008; Samiotakis and Ebbs, 2004). The nutrient solution was autoclaved to prevent microbial degradation of added cyanide. To characterize the *A. thaliana* lines and provide a basis for selecting physiologically relevant cyanide concentrations for future experiments, six week old plants were exposed to nine cyanide concentrations and a control (0.01, 0.1, 0.5, 1, 2, 5, 10, 20, 30 mg cyanide L^{-1}) for seven days. During the treatment period, transpiration was measured by weighing the 2 L pots every day, correcting for simple evaporation using pots with no plants. Relative chlorophyll content of leaves was determined using a SPAD 502+ Chlorophyll Meter (Konica Minolta Sensing Inc., Osaka, Japan). The readings were taken on fully expanded leaves between 11 am and 12 pm on the days of measurement. Plants were harvested after 7 d, rinsed in deionized water, and fresh weight was recorded.

2.3 Evaluation of cyanide toxicity and tolerance in β-cyanoalanine pathway mutants

To investigate how these knockdown mutant lines for enzymes of the β-cyanoalanine pathway would influence cyanide metabolism, three concentrations from the dose-response experiment were used. A concentration of 2.5 mg cyanide L^{-1} was chosen for an experiment to examine in greater detail the effect of low cyanide treatment. Plants were grown as above and subjected to 2.5 mg cyanide L^{-1} treatment for 10 d. Relative chlorophyll content was measured on day 10, thereafter plants were harvested and biomass (g FW) was recorded. In a related experiment, plants of the four lines were exposed to 5 mg cyanide L^{-1} for 10 d and total biomass was measured at harvest.

A more detailed series of experiments were carried out using 30 mg cyanide L^{-1} since this concentration resulted in the greatest separation between the lines in terms of final biomass during the dose-response experiment. Plants were grown as above and then exposed to 30 mg cyanide L^{-1} in 2 L hydroponic pots. Relative chlorophyll content and chlorophyll fluorescence were measured after 0, 4, 7 and 10 d of exposure. Chlorophyll fluorescence was measured using an OS1-FL chlorophyll fluorometer (Opti-Sciences; Hanover, NH, USA) following the manufacturer's instructions. For chlorophyll fluorescence leaves were first dark-adapted for 20 min. using the manufacturer's clips. Data were expressed as the F_V/F_M ratio. The plants of the Col-0, *AtCysA1*and *AtCysC1* lines were harvested after 10 d of treatment and total fresh weight was measured. The *AtNIT4* plants showed severe chlorosis and wilting in response to treatment, requiring these plants to be measured and harvested after 7 d of treatment.

As transpiration is a parameter used frequently to assess the effect of cyanide exposure on plants (Larsen and Trapp, 2006; Trapp et al., 2000; Yu et al., 2012), a separate short term experiment was performed to determine if the lines showed a differential decrease in transpiration in response to cyanide exposure. A technique used previously to measure transpiration was again used here (Ebbs et al., 2010). Plants grown as above were transferred to 150 mL Erlenmeyer flasks containing 125 mL of the cyanide treatment solution containing 30 mg cyanide L^{-1}. The mouths of the flasks were sealed tightly around the plant stem with parafilm to prevent water loss by evaporation. The mass of the flask, plug, nutrient solution and plant were determined at the onset of treatment. Thereafter the flasks were weighed every day for 7 d. The daily water loss was attributed to transpiration. Relative water content of the tissues was also determined at harvest. Plant samples were immediately weighed after harvest to

determine fresh weight (FW). The samples were placed in vials containing deionized water at 4^{o}C and left in the dark overnight. The next morning samples were re-weighed to determine turgid weight (TW). Thereafter the samples were oven dried to constant mass at 55^{o}C and the dry weight (DW) was obtained. Relative water content (RWC) was determined according to Barr and Weatherley (1962):

$$RWC\ (\%) = \frac{FW - DW}{TW - DW} * 100$$

In a third experiment, a set of plants grown as above was exposed to 30 mg cyanide L^{-1} in 2-L pots, harvested at three time points: 0, 24 and 36 h, rinsed in deionized water and snap frozen for determination of β-CAS activity. To determine β-CAS activity in the samples 1g of whole plant tissue was homogenized under liquid nitrogen and then in 1 mL buffer (2 mM EDTA-Na_2, 10 mM cysteine, 0.1 M Tris-HCl, pH 9.5). The homogenate was centrifuged at 4,000 *g* for 10 min at 4°C and the supernatant split into three subsamples. The first subsample was used for determination of the total protein content using the Pierce BCA assay Kit (Thermo Scientific) according to the manufacturer's instructions. The other two subsamples were used for the determination of β-CAS activity. One subsample was boiled for 10 min. to provide a heat-killed control to allow for determination of background absorbance. The activity of β-CAS was assayed using the DMPDA assay (Liang, 2003; Warrilow and Hawkesford, 1998). The crude protein extract was mixed 1:1 (v/v) with the substrate (10 mM cysteine, 3 mM KCN, 160 mM 2-amino-2-methyl-1-propanol, pH 9.8). The reaction mixture was incubated in sealed vials at 26º C for 20 min. The reaction was terminated with the addition of 0.5 mL of acidic dye precursor reagent (15 mM N,N-dimethyl-1,4-phenylenediamine dihydrochloride, 3 mM ferric chloride, 4.2

mM HCl). After a 20 min period of color development, methylene blue formation was measured spectrophotometrically at 745 nm. Enzyme activity data was normalized to the protein concentration.

To gather information on onset of oxidative stress in these lines in response to cyanide exposure, plants were again grown as above, exposed to 30 mg cyanide L^{-1}, and harvested after 5 d (prior to the onset of severe toxicity symptoms in the *AtNIT4* line). Oxidative stress was assessed by examining production of H_2O_2 and activity of catalase. To visualize the distribution of H_2O_2 across the leaves of treated plants, leaves were detached and infiltrated with 1 mg mL^{-1} 3,3'-diamino-benzidine (DAB) solution (pH 3.8) for 8 h according to Thordal-Christensen *et al*. (1997). The leaves were subsequently immersed in boiling alcohol (96%) for <10 minutes to remove chlorophyll. The leaves were dipped in glycerol to maintain the pliability of the leaves, and placed on a glass slide for examination. Images were captured on the Olympus SZX12 Stereo Microscope (Hitschfel Instruments Inc, Missouri, USA) equipped with Optronics Microfire Camera (California, USA). Hydrogen peroxide in the tissues was also quantified according to Velikova *et al*. (2000). To maintain a low temperature, all steps were performed in a cold room at 4^oC. Whole plants (~0.5 g fresh weight) were homogenized in 5 mL 0.1% (w/v) TCA. The crude extract was centrifuged at 12,000 *g* for 15 min at 4^oC. From the supernatant, 0.5 mL was taken and 0.5 mL of 10 mM KH_2PO_4 buffer (pH 7.0) and 1 mL of 1 M potassium iodide were added. Absorbance was read at 390 nm immediately after the reagents were mixed. Content of H_2O_2 was determined by comparison to a standard curve developed using known concentrations of H_2O_2. Catalase activity was determined according to Gomes-Junior et al. (2006). Whole plant tissues were homogenized in liquid nitrogen and then in 100 mM KH_2PO_4

buffer (pH 7.5) containing 1 mM ethylene diaminetetraacetic acid (EDTA), 3 mM dithiothreitol and 5% (w/v) insoluble polyvinylpolypyrrolidone (PVPP) at a ratio of 0.5 g FW to 1 mL buffer. The extract was centrifuged at 10,000 g for 30 min. and the supernatant immediately used for enzyme analysis. Catalase activity was determined spectophotometrically in a reaction mixture with 1 mL 100 mM KH_2PO_4 (pH 7.5) and 2.5 µL H_2O_2 (30% solution). The H_2O_2 was added immediately before the assay was initiated. The reaction was initiated by adding 15 µL of enzyme extract. Catalase activity was determined by tracking the removal of H_2O_2 at 240 nm at 10 second intervals over 1 min. using a Cary 50 UV-Visible spectrophotometer (Varian Inc., Mulgrave, Australia) against a plant extract-free blank. Catalase activity was expressed as µmol mg^{-1} protein min^{-1}.

2.4 Toxicity of β-cyanoalanine to β-cyanoalanine pathway mutants

Three week old plants were transferred from MS plates to 30 mL shell vials. The plants were exposed to four concentrations of β-cyanoalanine (0.05, 0.1, 0.5 and 1 mM), corresponding to (1.3, 2.6, 13, and 26 mg L^{-1}) added to the standard nutrient solution. This range of concentrations is comparable to that used by Watanabe et al. (2008) in a similar experiment. The plants wilted and died after 4 d and % mortality was recorded.

2.5 Statistical analyses

Data were analyzed using SAS package Version 9.1 as two-way ANOVA with Tukey's test used for post hoc analysis at the 0.05 level of significance. Where no significant interactions between the main effects for a parameter were observed, the main effects were analyzed separately by one-way ANOVA with Tukey's test used for post hoc analysis. When a significant interaction between main effects did occur, the data were reanalyzed by one-way ANOVA with each interaction mean (line x treatment) representing an individual treatment.

3.0 Results

3.1 Dose-response of the β-CAS pathway mutants to cyanide exposure

The dose-response curve showing the final fresh weight biomass in response to cyanide treatment over 7 d is shown in Figure 4.1A. Cyanide concentrations of <10 mg L^{-1} had a stimulatory effect for the Col-0 and *AtCysA1* lines. Plants from the *AtCysC1* line showed a slight stimulation at some of the low cyanide concentrations and a slow but consistent decline in biomass over most cyanide concentrations. These hormetic increases in biomass were greatest at a concentration of 1 mg cyanide L^{-1} for *AtCysC1*, up to 5 mg cyanide L^{-1} for *AtCysA1* and 10 mg cyanide L^{-1} for Col-0 (Figure 4.1A). A modest but steady decline in biomass was observed for these lines after the hormetic increase in biomass. The *AtNIT4* mutant showed the greatest sensitivity, decreasing consistently as cyanide concentration increased. After 7 d of treatment the final biomass of the *AtCysA1*, *AtCysC1*, and Col-0 plants did not differ significantly and was 10-15% lower than the untreated control plants of the same line. Final biomass of the *AtNIT4* plants was significantly lower than the other three lines and was 60% lower than the untreated

plants of the same line. There was also a general decline in the chlorophyll content of all lines relative to their untreated controls with no significant difference between the lines at lower cyanide concentrations. At 20 mg cyanide L^{-1} the *AtNIT4* had lower chlorophyll compared to the other lines (Figure 4.1B) and at 30 mg cyanide L^{-1} *AtNIT4* plants were severely chlorotic, wilted and difficult to measure. Absolute and cumulative transpiration showed a declining trend as concentration increased, although there was no apparent difference between the lines (Appendices B and C).

3.2 Evaluation of cyanide toxicity and tolerance in β-cyanoalanine pathway mutants

For final fresh weight biomass, there was no significant interaction between the two primary effects (line and treatment) in response to exposure to a chronic treatment of 2.5 mg cyanide L^{-1} for 10 d. There was no difference between lines in fresh weight biomass (Figure 4.2A). Across all lines, exposure to cyanide resulted in a significant overall decrease in fresh weight biomass of ~48% (Figure 4.2B). There was a significant interaction between line and treatment for relative chlorophyll content. There was generally no difference in relative chlorophyll content of leaves of the untreated or treated Col-0, *AtCysA1*, or *AtCysC1* mutants. The only exception was the relative chlorophyll content for the two mutant lines relative to each other in response to treatment. There was a significant decrease in relative chlorophyll content for the *AtNIT4* line relative to all lines for the cyanide treatment (Figure 4.3).

Plants from each of the lines were also subjected to a chronic treatment of 5 mg cyanide L^{-1}. This cyanide concentration produced the growth stimulation observed for some lines in the dose-response experiment above (Figure 4.1). After 10 d of exposure to this treatment regime, the *AtNIT4* plants displayed obvious signs of stress (i.e., chlorosis, loss of turgor). The Col-0, *AtCysA1* and *AtCysC1* lines developed a purplish color indicative of increase in anthocyanin pigmentation (Figure 4.4). The fresh weight biomass of *AtCysA1, AtCysC1* and *AtNIT4* mutants were not significantly different from Col-0 plants under nominal conditions (Figure 4.5A). However after 10 d of cyanide exposure there was a significant overall decrease in biomass for all the lines (Figure 4.5B). The magnitude of decrease was ~69% and not significantly different across the lines (α=0.05) (Table 4.1).

To quantify the relative importance of each gene in cyanide metabolism, the highest concentration from the dose response curve (i.e. 30 mg cyanide L^{-1}) was chosen to represent a more overtly toxic cyanide exposure. The biomass of *AtCysA1* and *AtCysC1* was not significantly different from Col-0 plants on day 10, however the *AtNIT4* plants had significantly lower biomass (Figure 4.6A). Across all lines, plants treated with cyanide had significantly lower biomass that control plants (Figure 4.6B). After treatment with cyanide, only the *AtNIT4* mutant had significantly lower fresh weight biomass compared to Col-0 and showed the same signs of toxicity (i.e chlorosis, and loss of turgor followed by wilting by day 7) as observed previously (Figure 4.4).

Additional measurements were taken to assess the physiological status of the *A. thalina* lines. While there was no significant change in the chlorophyll content within the first 7 d of

cyanide treatment for the *AtCysA1* and *AtCysC1* lines as compared to Col-0, there was a notable drop after day 7 to between 70 and 85% of the untreated plants of the same line (Figure 4.7). A declining trend was observed for the chlorophyll content of *AtNIT4* plants, reaching a significant reduction in chlorophyll content of 65% by day 7. The same response pattern was observed in chlorophyll fluorescence as measured by the F_V/F_M ratio. The ratio for Col-0, the *AtCysA1* and *AtCysC1* mutants was not significantly different from the untreated control plants of the same line. Within a line there were similar fluctuations over the 10 day period (Figure 4.8A-C). The *AtNIT4* line showed a steady decline in the F_V/F_M ratio over 7 d after which the plants started wilting and were harvested (Figure 4.8D). Taken together, these results show that only the *AtNIT4* mutant was consistently sensitive to cyanide exposure with significantly lower biomass, chlorophyll content and chlorophyll fluorescence.

To evaluate the inhibitory effect of cyanide on transpiration, additional plants were subjected to 30 mg cyanide L^{-1}. There was an overall decrease in cumulative transpiration of cyanide treated plants as compared to untreated plants of the same line (Figure 4.9). The magnitude of decrease was however not significantly different across the *A. thaliana* lines as shown in Table 4.2. The amount of water lost per day (absolute transpiration g d^{-1}) decreased uniformly across the lines over the treatment period with normal fluctuations in the control plants (Appendix D). After 7 d the relative water content of the shoots in the mutants was not significantly different from Col-0 (Appendix E).

There was however differential response in the CAS-like enzyme activity of the lines when exposed to 30 mg cyanide L^{-1}. The level of activity at 5 mg cyanide L^{-1} (data not shown)

was not significantly different from that at 30 mg cyanide L^{-1}. As expected the Col-0 plants had the highest enzyme activity which however did not vary significantly after 24h or 36 h (Figure 4.10), in agreement with previous studies that have reported no induction of the CAS enzyme by cyanide (Maruyama et al., 2001; Meyer et al., 2003). β-CAS activity in the mutant lines was significantly lower than the Col-0 plants. The magnitude of decrease was ~30% for the *AtCysA1* mutant and ~50% for the *AtCysC1* and *AtNIT4* lines, also in agreement with prior studies (Watanabe et al., 2008) and results obtained during this research (Chapter 3).

To examine the production of ROS in response to the cyanide treatment, plants of the four lines were exposed to 30 mg cyanide L^{-1} for 5 d. On day 5 there were visible signs of toxicity (Figure 4.4) and the leaves were stained with DAB reagent. The DAB staining was most evident in the vascular bundles and in trichomes for all lines (Figure 4.11A-H). However staining was more intense in *AtNIT4* plants as compared to other lines. The concentration of H_2O_2 in the whole plant tissues was determined and results showed a significant increase in H_2O_2 content of the three mutant lines compared to Col-0 plants (Figure 4.12). The magnitude of increase in H_2O_2 content was ~2-fold in the *AtNIT4* and *AtCysC1* mutants and lowest in Col-0 with 55% (Table 4.3). The results are in agreement with the visual DAB staining in which the *AtNIT4* shows the greatest intensity of staining. To quantify the antioxidant activity in plant tissues, catalase activity was also determined. The activity of catalase in the three mutant lines *AtCysA1, AtCysC1* and *AtNIT4* were all not significantly different from Col-0 plants (Figure 4.13A). However *AtCysA1* had the lowest activity of this enzyme, significantly different from *AtCysC1* and *AtNIT4*. The overall effect of cyanide was a significant increase in catalase activity (Figure 4.13B). Since catalase activity was not impaired in the mutants, the results together

suggest enhanced rate of H_2O_2 production but may not have had a prominent effect on potential removal by catalase.

3.3 Toxicity of β-cyanoalanine to β-cyanoalanine pathway mutants

To examine whether β-cyanoalanine could be an element of the toxicity displayed by *AtNIT4*, Col-0 and *AtNIT4* plants were subjected to four concentrations of β-cyanoalanine (0, 1.3, 2.6, 13, and 26 mg L^{-1}). At 26 mg L^{-1} β-cyanoalanine was toxic to both Col-0 and *AtNIT4* plants with 75% mortality. There was no mortality in the Col-0 plants at 1.3 and 2.6 mg L^{-1} β-cyanoalanine while the *AtNIT4* plants showed 50% mortality at both concentrations (Table 4.4). Control plants grew well with no signs of stress or mortality. These results are consistent with the interruption of the activity of the enzyme encoded by *AtNIT4* and are consistent with other studies (Howden et al., 2009; Watanabe et al., 2008) that have demonstrated β-cyanoalanine toxicity to plants.

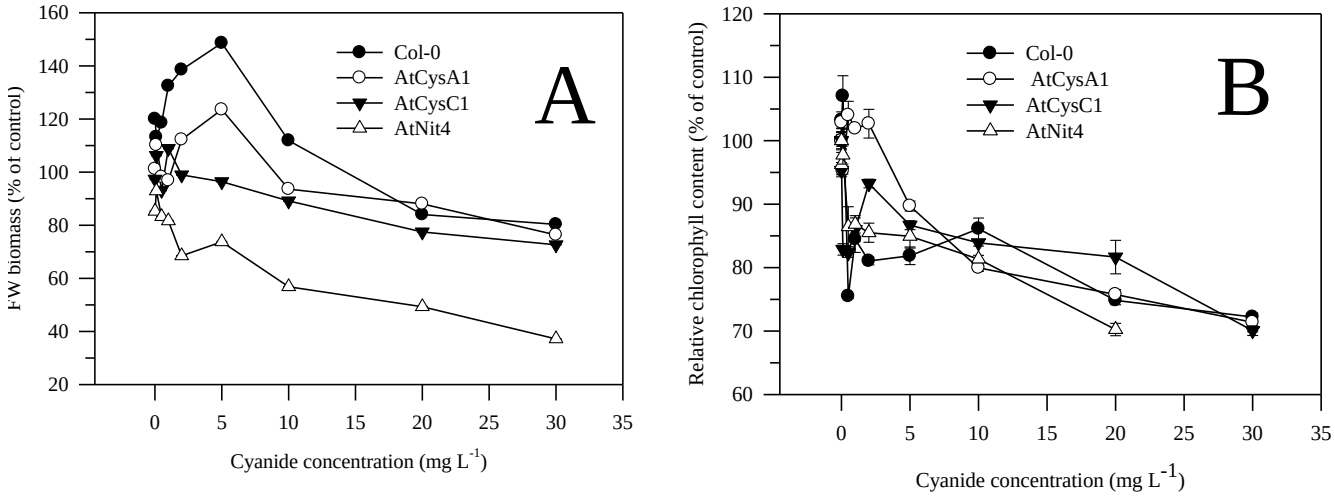

Figure 4.1 Plant fresh weight (FW) (**A**) and relative chlorophyll content (**B**) of 6-week old *A. thaliana* wild type (Col-0), *AtCysA1*, *AtCysC1* and *AtNIT4* mutants after 7 d exposure to nine concentrations of cyanide. Relative chlorophyll content of *AtNIT4* at 30 mg cyanide was not measurable, plants were chlorotic and wilted. Data represents mean values of 3 replicates.

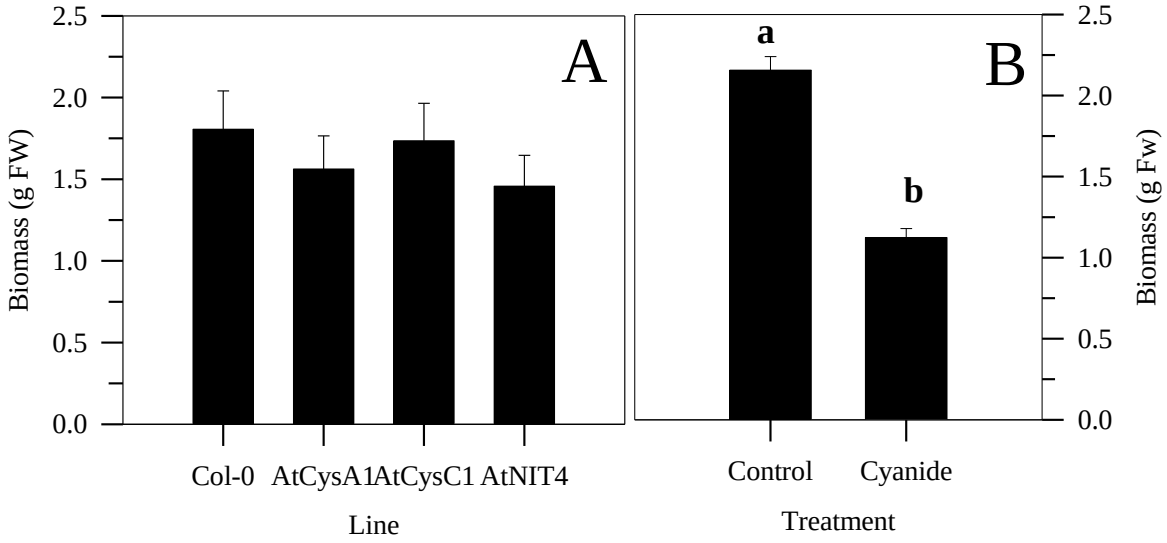

Figure 4.2 Biomass of 5-week old *A. thaliana* (Col-0), *AtCysA1*, *AtCysC1* and *AtNIT4* mutants exposed to 2.5 mg cyanide L^{-1} for 10 days. Panels represent (**A**) Line effects and (**B**) cyanide treatment effects. Each bar represents the mean and standard error (n=4). Bars with different letters are significantly different from each other (α=0.05).

Table 4.1 Magnitude of decrease (%) in fresh weight biomass of 5-week old *A. thaliana* Col-0, *AtCysA1*, *AtCysC1* and *AtNIT4* mutants after exposure to 2.5 and 5 mg cyanide L^{-1} for 10 d.

Cyanide concentration (mg L^{-1})	Magnitude of decrease (%)			
	Col-0	*AtCysA1*	*AtCysC1*	*AtNIT4*
2.5	49.4	45.6	45.7	50.5
5	66.8	73.9	65.9	70.4

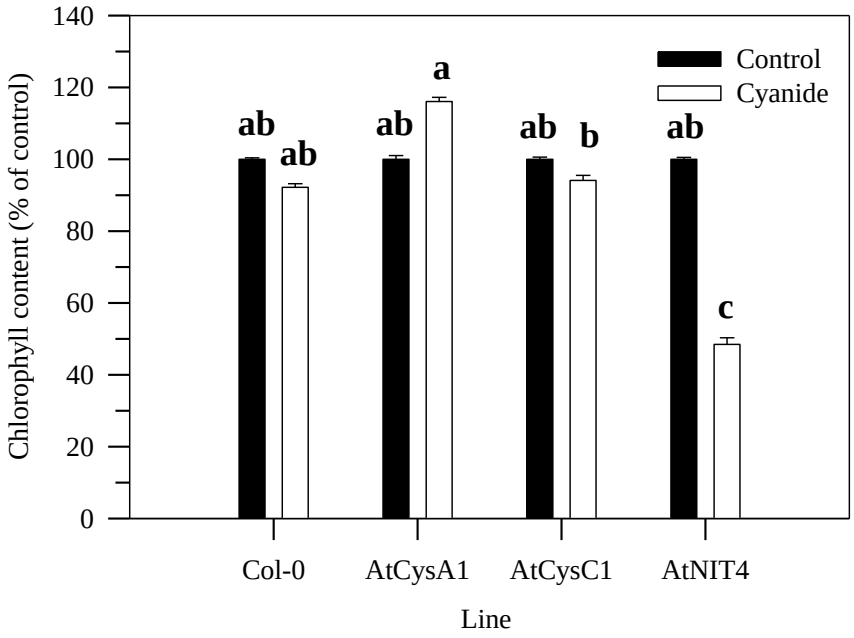

Figure 4.3 Chlorophyll content of 5-week old *A. thaliana* wildtype (Col-0), *AtCysA1*, *AtCysC1* and *AtNIT4* mutants exposed to 2.5 mg cyanide L^{-1} for 10 days. Black bars represent the mean and standard error (n=4) of control plants, open bars represent plants treated with cyanide. Bars with different letters are significantly different from each other (α=0.05).

Scale bar = 4.5 cm

Figure 4. 4 Images of 6-week old plants of *A. thaliana* (Col-0) (**A)**, *AtCysA1***(B)**, *AtCysC1***(C)** and *AtNIT4***(D)** mutants grown in hydroponics, and exposed to 30 mg cyanide L^{-1} cyanide for 5 days.

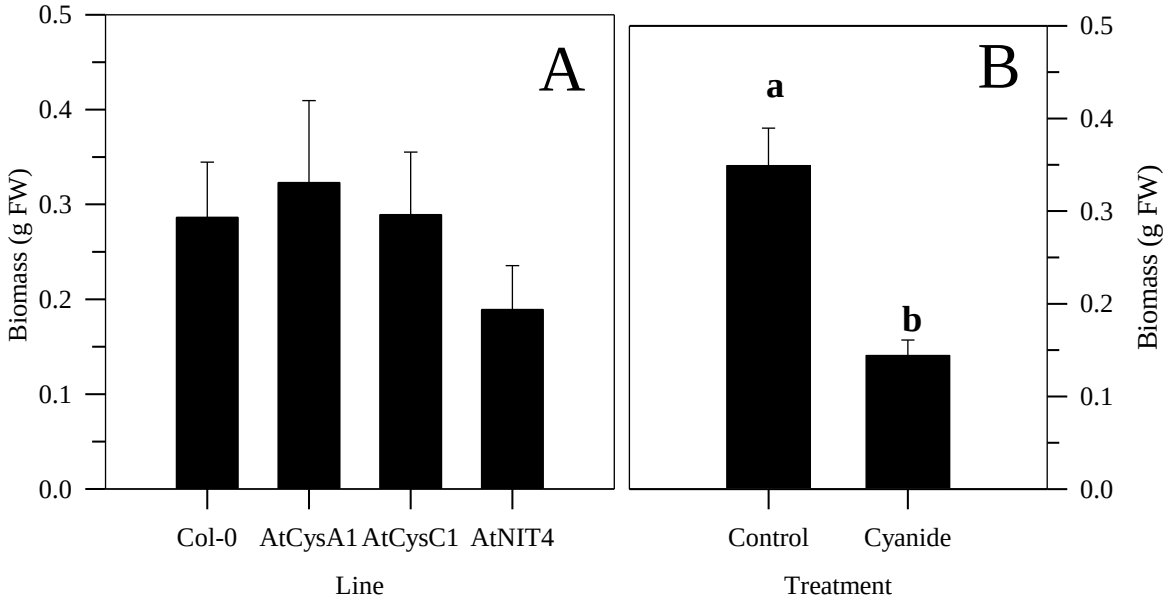

Figure 4.5 Biomass of 5-week old *A. thaliana* wild type (Col-0), *AtCysA1*, *AtCysC1* and *AtNIT4* mutants exposed to 5 mg cyanide L^{-1} for 10 days. Panels represent (**A**) Line effects and (**B**) cyanide treatment effects. Each bar represents the mean and standard error (n=4). Bars with different letters are significantly different from each other (α=0.05).

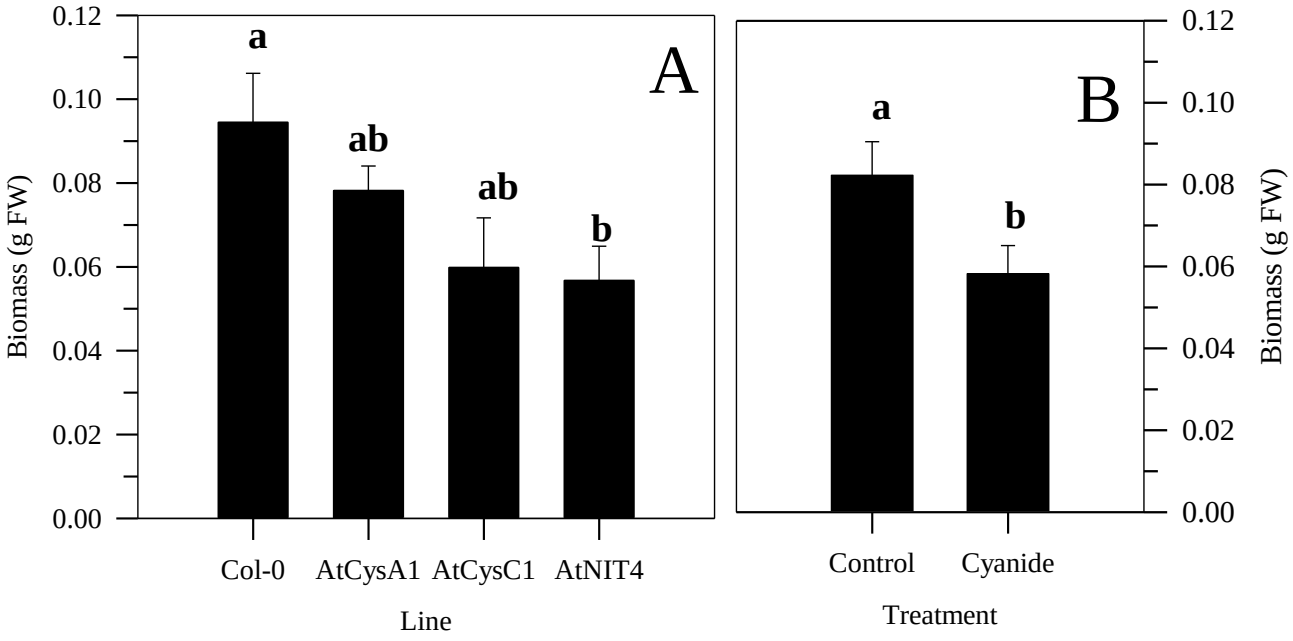

Figure 4.6 Biomass of 6-week old *A. thaliana* wild type (Col-0), *AtCysA1*, *AtCysC1* and *AtNIT4* mutants exposed to 30 mg cyanide L^{-1} for 10 days. Panels represent (**A**) Line effects and (**B**) cyanide treatment effects. Each bar represents the mean and standard error (n=4). Bars with different letters are significantly different from each other (α=0.05).

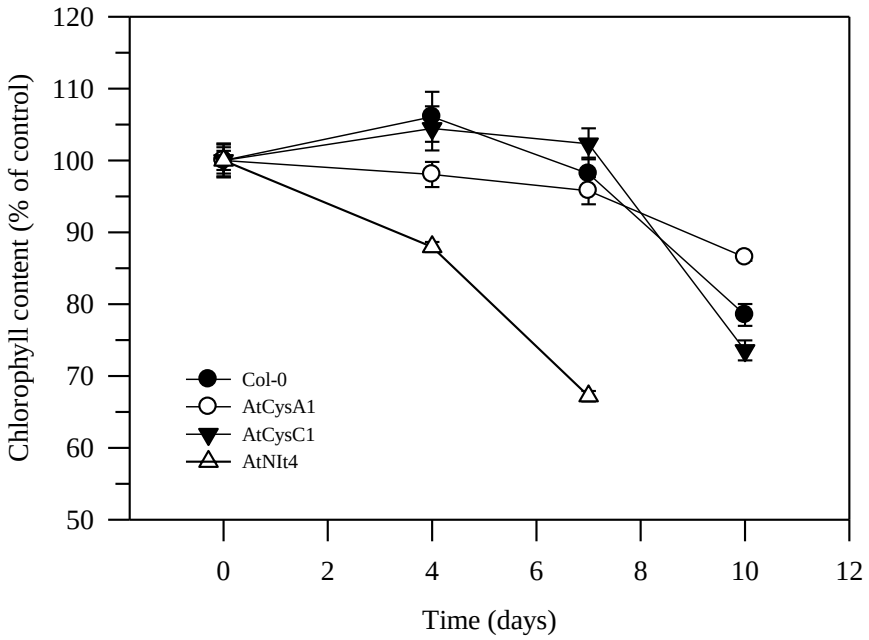

Figure 4.7 Relative chlorophyll content of 6-week old *A. thaliana* wild type (Col-0) and three mutants *AtCysA1*, *AtCysC1* and *AtNIT4* treated with 30 mg cyanide L^{-1} for 10 days. Chlorophyll content was read using a Spad meter and each point represents the mean and standard error (n=4).

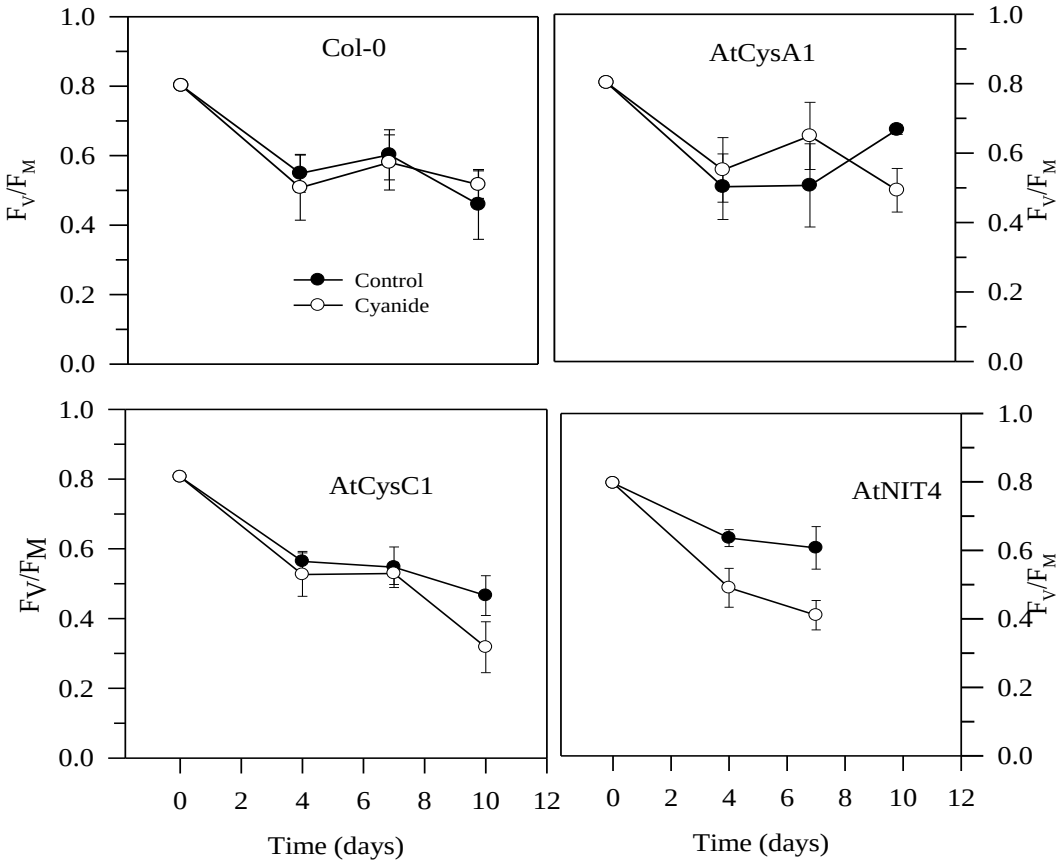

Figure 4.8 Chlorophyll fluorescence as measured by F_V/F_Mratio of 6-week *A. thaliana* wild type (Col-0) and three mutants *AtCysA1*, *AtCysC1* and *AtNIT4* treated with 30 mg CN L^{-1} for 10 d. Each point represents the mean and standard error (n=4).

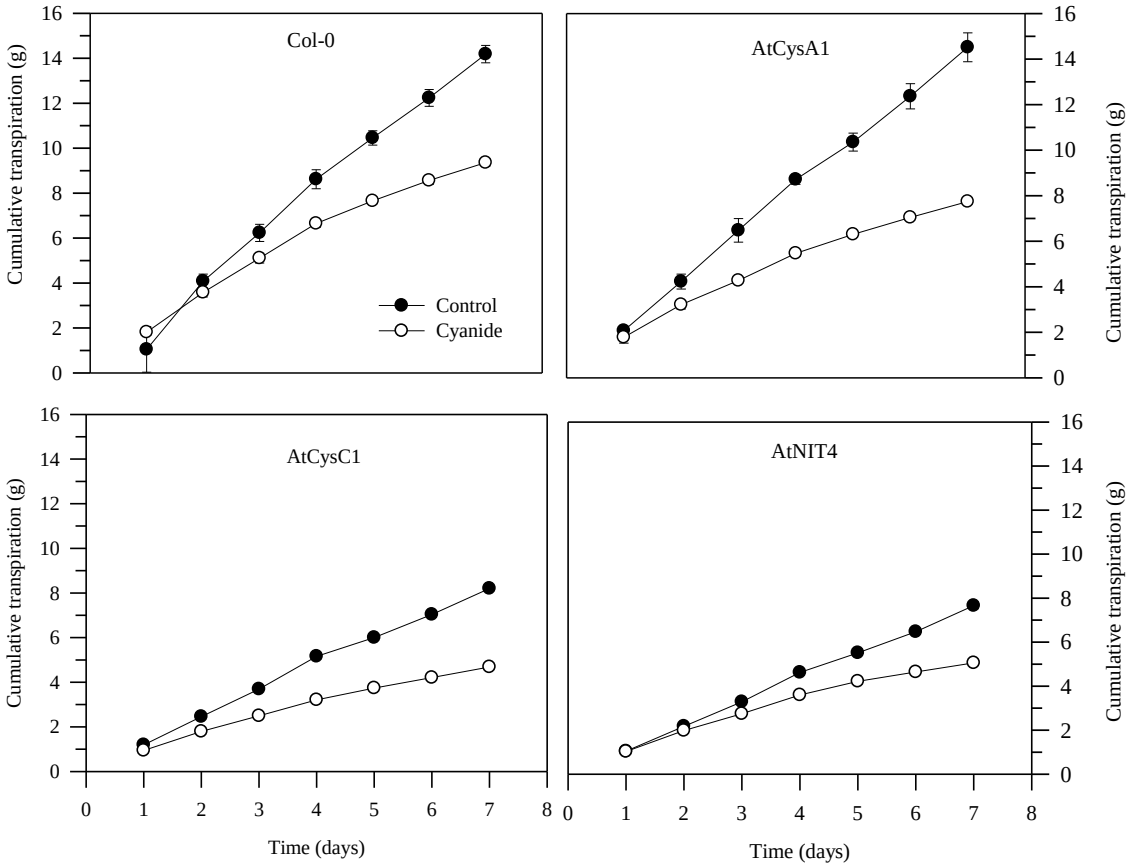

Figure 4.9 Cumulative transpiration of 6-week old *A. thaliana* wild type (Col-0) and three mutants *AtCysA1*, *AtCysC1* and *AtNIT4* treated with 30 mg cyanide L^{-1}. Transpiration was measured by weighing Erlenmeyer flasks every 24 h for 7 d.

Table 4.2 Cumulative transpiration (g) over 7 d and Overall decrease in transpiration of 6-week old *A. thaliana* Col-0, *AtCysA1, AtCysC1* and *AtNIT4* mutants subjected to 30 mg cyanide L^{-1}. Transpiration was measured by weighing Erlenmeyer flasks every 24 h for 7 d.

Line	Control (g)	Cyanide (g)	Overall decrease (%)
Wild type (Col-0)	14.18	9.36	33.9
AtCysA1	13.79	7.84	43.1
AtCysC1	8.20	4.69	42.8
AtNIT4	7.66	5.06	33.9

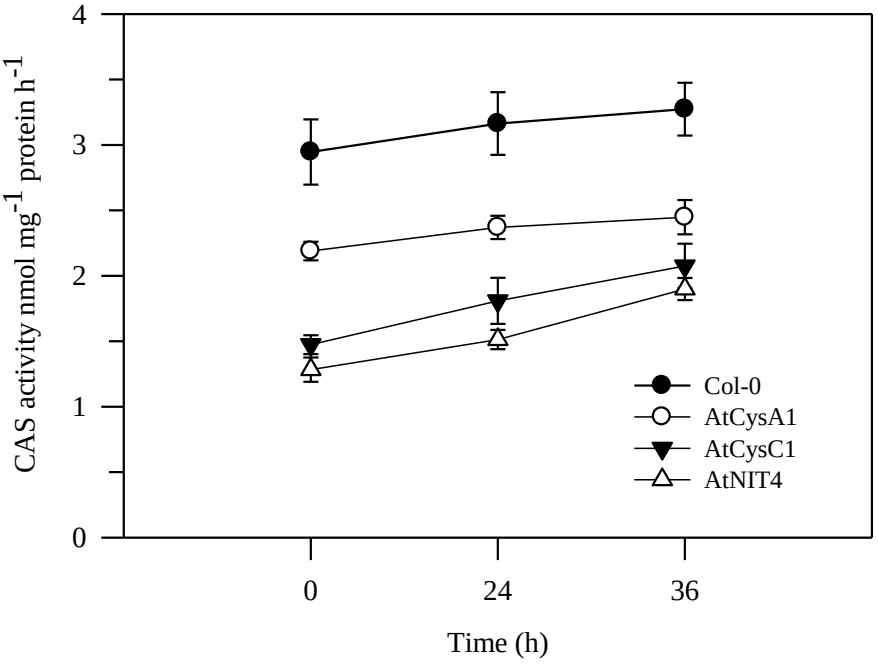

Figure 4.10 CAS-like activity in crude protein extracts from 6-week old plants of *A. thaliana* (Col-0), *AtCysA1, AtCysC1* and *AtNIT4* mutants grown in hydroponics and exposed to 30 mg L^{-1} cyanide for 36 h. Each bar represents the mean and standard error (n=4). Differences between the wild type and mutants analyzed by one-way Anova were statistically significant $p<0.01$. Bars with different letters are significantly different from each other ($\alpha=0.05$).

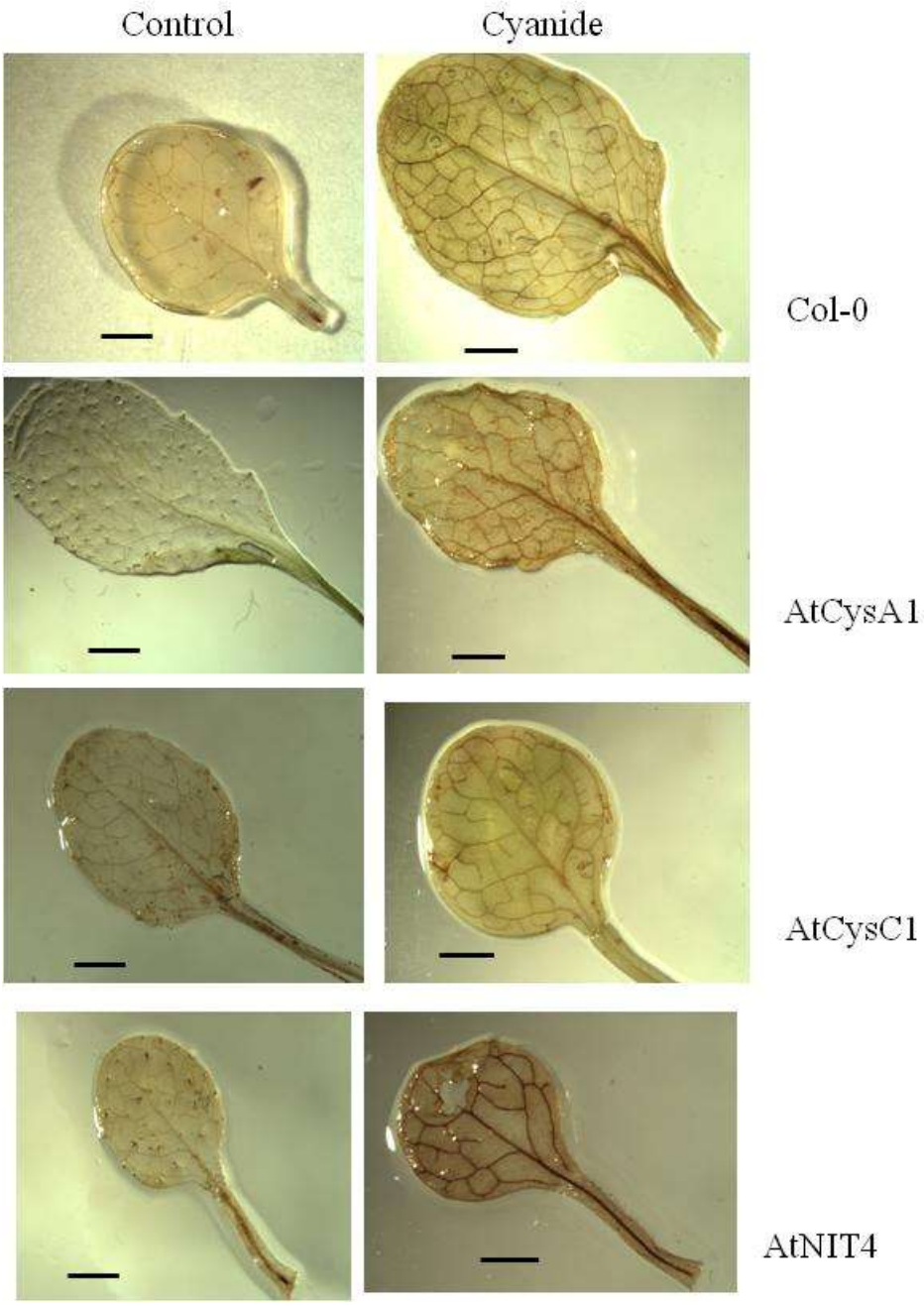

Figure 4.11 DAB stained leaves from 6-week old plants of *A. thaliana* (Col-0), *AtCysA1, AtCysC1* and *AtNIT4* mutants grown in hydroponics, and exposed to 30 mg cyanide L^{-1} cyanide for 5 d. Scale bar = 2mm.

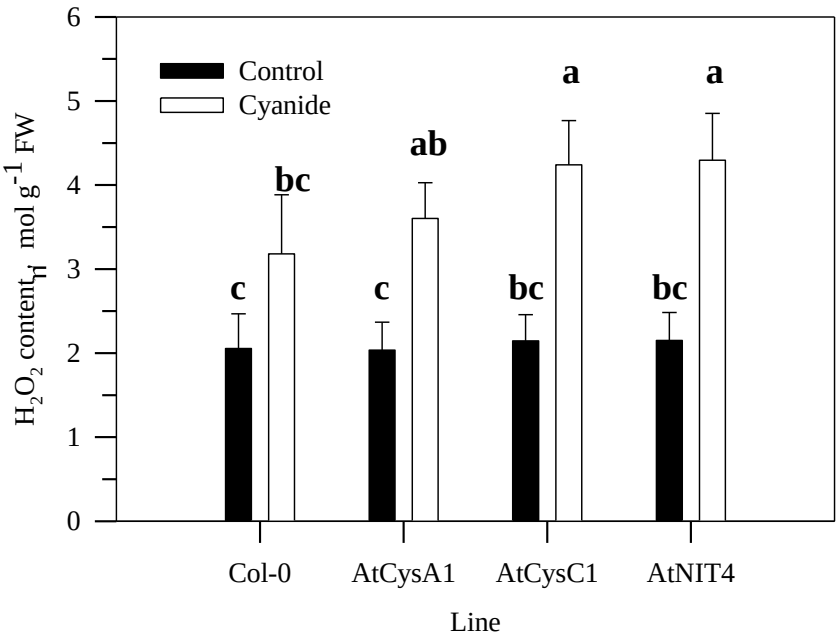

Figure 4.12 Hydrogen peroxide content of 6-week old plants of *A. thaliana* (Col-0), *AtCysA1*, *AtCysC1* and *AtNIT4* mutants grown in hydroponics, and exposed to 30 mg cyanide L^{-1} for 5 d. Each bar represents the mean and standard error (n=4). Bars with different letters are significantly different from each other (α=0.05).

Table 4.3 H_2O_2 content (μmol g^{-1} FW) and overall increase (%) after 5 d exposure of 6-week old *A. thaliana* Col-0, *AtCysA1*, *AtCysC1* and *AtNIT4* mutants to 30 mg cyanide L^{-1}. Each data value represents the mean (*n*=4).

Line	Control	Cyanide	Increase in H_2O_2 (%)
Col-0	2.05	3.18	55.1
AtCysA1	2.03	3.60	77.3
AtCysC1	2.14	4.24	98.1
AtNIT4	2.15	4.29	99.5

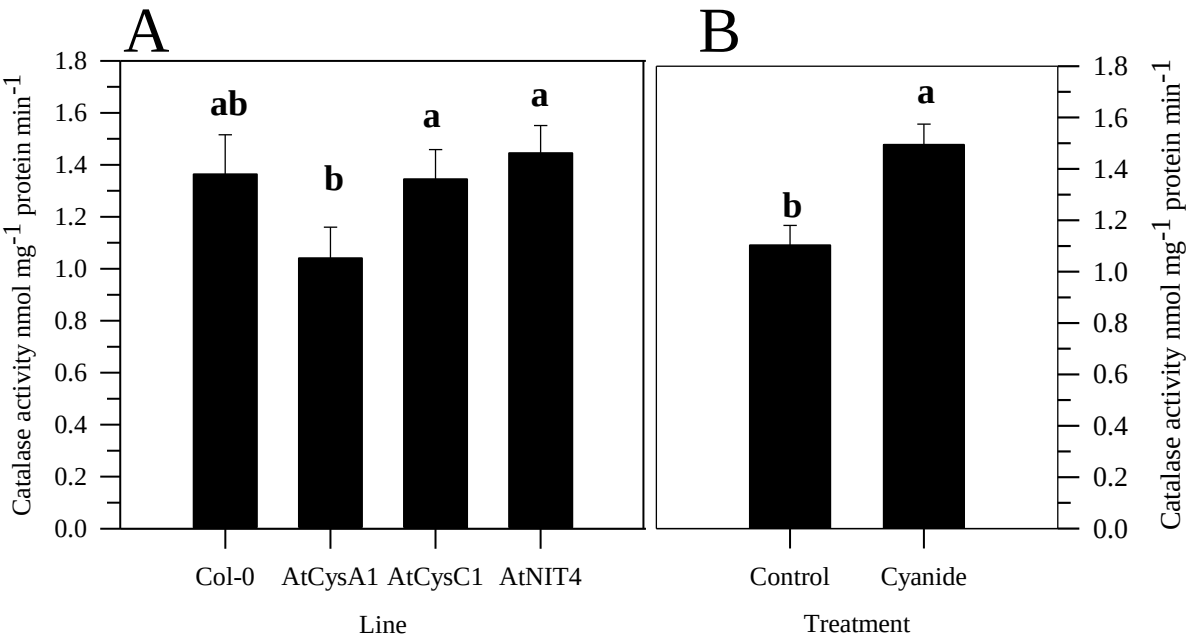

Figure 4.13 Catalase activity of 6-week old plants of *A. thaliana* (Col-0), *AtCysA1*, *AtCysC1* and *AtNIT4* mutants grown in hydroponics, and exposed to 30 mg cyanide L^{-1} for 5 d. Panels represent (**A**) Line effects and (**B**) cyanide treatment effects. Each bar represents the mean and standard error (n=4). Bars with different letters are significantly different from each other (α=0.05).

Table 4.4 Percent survival of three week old *A. thaliana* Col-0 and *AtNIT4* plants exposed to four concentrations of β-cyanoalanine. Plants were grown in plates and transferred to hydroponic vials for treatment. Each treatment comprised 4 replicates.

β-cyanoalanine concentration (mg L^{-1})	Mortality (%)	
	Col-0	*AtNIT4*
0	0	0
1.3	0	50
2.6	0	50
13	0	50
26	75	75

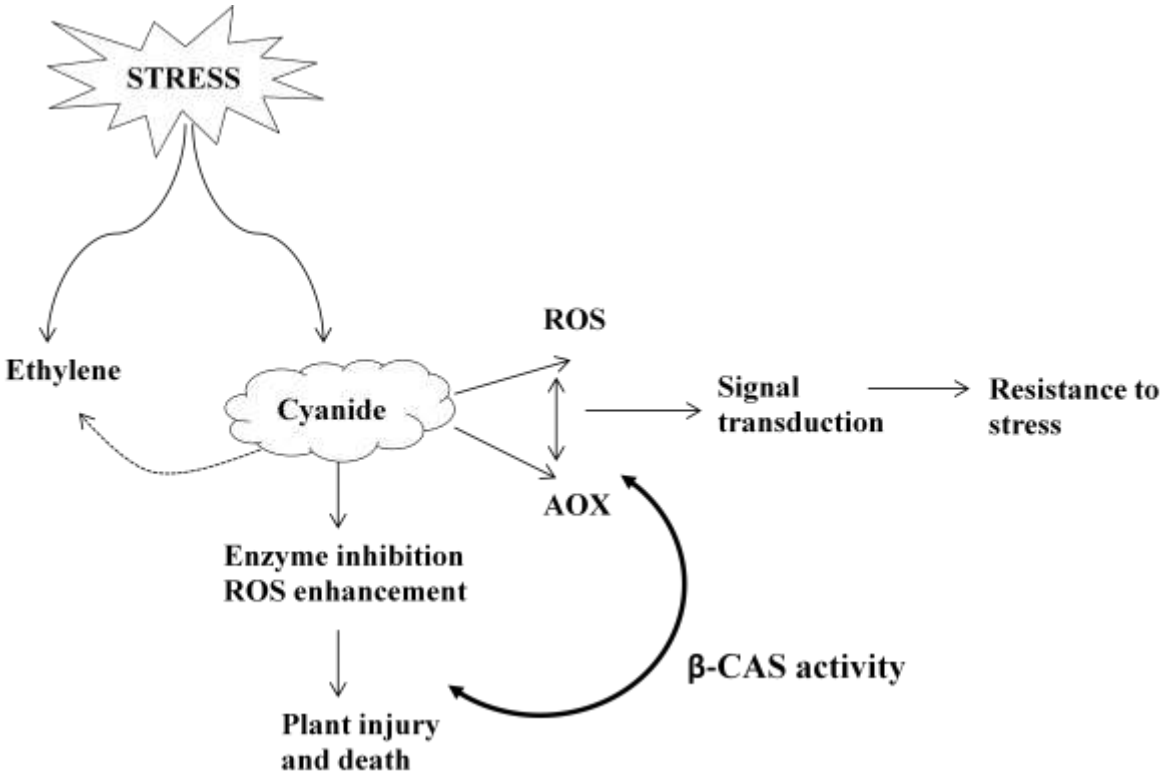

Figure 4.14 Role of the β-cyanoalanine pathway in tolerance to abiotic stress conditions – modified from Siegien and Bogatek, (2006). The dotted arrow indicates stimulation of ethylene by cyanide. The curved arrow indicates regulation of cyanide concentration by the β-CAS pathway. CAS=cyanoalanine synthase, CS=cysteine synthase, NIT4=nitrilase 4, ROS=reactive oxygen species, AOX=alternative oxidase

4.0 Discussion

The β-CAS pathway has been the focus of study in recent years and considerable evidence has emerged to demonstrate the specific genes and enzymes involved in the pathway. The enzymes AtCysA1 and AtCysC1 cooperatively mediate the first step forming β-cyanoalanine (Watanabe et al., 2008) and AtNIT4 mediates the second step by hydrolyzing β-cyanoalanine to amino acids (Piotrowski et al., 2001). Studies on cyanide uptake have already shown that on exposure to cyanide, plants readily take up, transport and metabolize the cyanide (Ebbs et al., 2010; Larsen et al., 2005; Yu et al., 2007). Other studies have reported up-regulation of the enzymes associated with the pathway when plants are exposed to cyanide (Yu et al., 2012). More information on the molecular genetics of *AtCysA1, AtCysC1* and *AtNIT4* has become available and the relative contribution of each gene is known as is the specific cell compartments where each enzyme occurs (Hatzfeld et al., 2000; Jost et al., 2000). The overall objective of this study was to determine the extent to which the interruption of the genes encoding enzymes of the β-CAS pathway altered the ability of *A. thaliana* mutants to tolerate cyanide. The approaches used sought to confirm potential redundancies between the two enzymes with demonstrated β-CAS activity (AtCysA1 and AtCysC1) and between the β-CAS pathway and the sulfurtransferase pathway.

In terms of the redundancy between AtCysA1 and AtCysC1, the consistently similar results obtained across the experiments for Col-0 and both the *AtCysA1* and *AtCysC1* mutants demonstrate that even in the absence of one of the two enzymes capable of β-CAS activity, the remaining functional enzyme appears capable of metabolizing the accumulated cyanide to maintain a phenotype similar to that of Col-0. Cyanide concentrations > 5 mg L^{-1} have been

reported toxic in *A. thalina* and willow (Larsen et al., 2005; Smith and Arteca, 2000) while the expected deleterious effects of cyanide were observed at 30 mg L^{-1}. The small decrease in the fresh weight biomass of the two mutants, *AtCysA1* and *AtCysC1* was not statistically significant (Figure 4.5A). Although the significant decrease in transpiration was an expected result (Figure 4.9 and Appendix D), it was surprising that the magnitude of decrease did not vary across the lines (including *AtNIT4,* Table 4.2), suggesting that the activity of the β-CAS pathway has no direct association with the effect of cyanide on transpiration. The decreases in chlorophyll content particularly after 7 d for Col-0, *AtCysA1* and *AtCysC1* (Figure 4.7) and in F_V/F_M ratio for *AtCysC1* show comparable responses to the biochemical effects of cyanide exposure. The decreases may be attributed to cyanide inhibition of enzymes involved in several metabolic processes (Samuilov et al., 2006; Solomonson, 1981). Nevertheless, *AtCysA1* and *AtCysC1* mutants were still phenotypically similar to Col-0.

The decrease in β-CAS enzyme activity observed in each line was of the expected magnitude (Watanabe et al., 2008). The lack of induction in β-CAS activity by cyanide at 5 and 30 mg cyanide L^{-1} was also expected since the *AtCysC1* gene is highly and constitutively expressed (Alvarez et al., 2010) and not responsive to abiotic stress (Kilian et al., 2007) and cyanide exposure (Maruyama et al., 2001). This latter point is evident from the lack of a change in enzyme activity after exposure to cyanide 24 and 36 h (Figure 4.10). Since the remaining activity of the functional enzyme did not change when the parallel enzyme in the pathway was disabled, the level of activity of either AtCysA1 or AtCysC1 was apparently sufficient to maintain cyanide homeostasis. The results provide further evidence for functional redundancy between these two proteins even though AtCysC1 makes a greater contribution to β-CAS-like

activity than AtCysA1. The results on oxidative stress (Figure 4.11 A-H) show tissue distribution and localization of H_2O_2 in vascular bundles and trichomes in agreement with other studies that have reported increase in H_2O_2 on exposure to abiotic stresses (Mittler, 2002). H_2O_2 is required for the process of lignification in metaxylem cells (Barcelo, 2005; Ros Barcelo, 1998) and is known to readily diffuse across membranes of xylem parenchyma cells via specific aquaporins (Bienert et al., 2007; Henzler and Steudle, 2000). The level of oxidative stress for Col-0 and the *AtCysA1* mutant was not significantly different, while the two mutants (*AtCysA1* and *AtCysC1*) were also phenotypically similar (Figure 4.12). With a uniform magnitude of increase in catalase activity in all the lines (Figure 4.13 B) the results are in agreement with functional redundancy between AtCysA1 and AtCysC1in cyanide detoxification and the greater contribution of AtCysC1. This redundancy was apparent under the conditions used in these experiments and additional study will be required to determine whether that redundancy persists over longer periods of exposure to cyanide and/or to higher cyanide concentrations.

With regard to possible redundancies between these two pathways, the results support the contention that the β-CAS pathway is the principle mechanism for cyanide detoxification in *A. thaliana.* Given the degree of sensitivity to cyanide demonstrated by the *AtNIT4* mutants, this could be an indication that the sulfurtransferase pathway was unable to rescue the plants from cyanide toxicity. The decrease in transpiration did not vary across the lines implying that cyanide did not affect water relations and guard cell function. Image analysis of the stoma did not provide conclusive evidence for closure of stomata (data not shown). The effect of cyanide on *AtNIT4* leading to chlorosis and wilting may not be water related but biochemical, as indicated by the decrease in chlorophyll content and fluorescence (Figure 4.7 and 4.8D). The same

inhibition effects of cyanide on metallo-enzymes (Berg and Krogmann, 1975; Samuilov et al., 2006) collectively led to reduced growth. The hypersensitivity of *AtNIT4* mutant was also reflected in accumulation of H_2O_2. With catalase activity not compromised in the mutant, the result suggests enhanced capacity to generate H_2O_2 in the mutant.

The *AtNIT4* plants are knockdowns in both AtNIT4 and β-CAS enzyme activity (Chapter 3) in that the mutant plants are impaired not only in hydrolysis of β-cyanoalanine, but also in the initial detoxification of cyanide to β-cyanoalanine. This would potentially result in accumulation of cyanide and/or β-cyanoalanine. Results here show that β-cyanoalanine is toxic to *A. thaliana*. Another study with the *AtNIT4* mutant reported impaired metabolism of β-cyanoalanine which resulted in root growth inhibition (Howden et al., 2009). With the β-CAS pathway impaired, one would expect the alternative sulfurtransferase pathway to compensate in cyanide removal and rescue the plants, but this was not apparent in the *AtNIT4* phenotype. Hence, there is no pathway redundancy in cyanide detoxification in *A. thaliana*. The inadequate removal of excess cyanide by sulfurtransferases, the possible accumulation of β-cyanoalanine, and perhaps some combination of effects of inhibition of enzymes by cyanide all contribute to the sensitive phenotype of this mutant.

One notable observation in the dose response curve was the hormetic effect of cyanide at lower concentrations for Col-0 plants and the mutants *AtCysA1* and *AtCysC1*. The magnitude of stimulation was variable across the lines with 150%, 125% and 110% respectively. Low cyanide doses (<5 mg cyanide L^{-1}) have also been reported to stimulate growth in willows (Yu et al., 2007) and may impose a hormetic effect. The concept of hormesis is still controversial and even

though several mechanisms have been postulated (Cedergreen et al., 2007), changes in respiration, photosynthesis, protein content, free amino acids or soluble carbohydrates in response to several herbicides were reportedly not sufficient to explain hormesis (Wiedman and Appleby, 1972). One general explanation for hormetic effects is overcompensation to an early disruption in homeostasis (Calabrese, 1999). In terms of the stimulation of growth observed here, the results may be related to the role of cyanide as an inhibitor/regulator of metallo-enzymes such as nitrate reductase (Solomonson and Spehar, 1979). There have been some studies demonstrating that plants are capable of utilizing cyanide as a supplemental source of nitrogen (Ebbs et al., 2010) perhaps due to opportunistic or even preferential assimilation of cyanide via the β-CAS pathway as a source of nitrogen (Siegien and Bogatek, 2006). There was perhaps overcompensation after disruption in nitrogen metabolism as the plants switched from ammoniacal nitrogen to cyanogenic nitrogen. The strong stimulatory response to low cyanide concentrations displayed by Col-0 and *AtCysA1* as compared to the small response shown by *AtCysC1* suggests that *AtCysC1* is specifically required for this beneficial response. Altogether the results provide evidence for a role of the β-CAS pathway to tolerance to stress summarized in the model (Figure 4.14). On exposure to stress, elevated levels of ethylene are produced concomitantly with cyanide. In the presence of this pathway however, cyanide homeostasis is maintained. The increase in cyanide concentration, coupled to low activity of the β-CAS pathway results in enzyme inhibition and consequently, cell death (Grossmann, 1996). Results with the *AtNIT4* mutant have shown that impairing the β-CAS pathway renders the plants susceptible to damage. The transient cyanide peak on exposure to stress becomes a signaling

event, turning on a cascade of events leading to tolerance mechanisms (Siegien and Bogatek, 2006).

In conclusion the study has shown that the *AtCysA1* and *AtCysC1* mutants are not impaired in cyanide metabolism confirming functional redundancy between these proteins. The hypersensitivity of the *AtNIT4* mutant means that the mutant is impaired in cyanide metabolism. With the CAS pathway impaired the study confirmed that there is no pathway redundancy cyanide metabolism i.e the sulfurtransferase pathway is not involved in stress cyanide removal and so the β-cyanoalanine pathway is the sole pathway for cyanide assimilation in *A. thaliana*.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The β-cyanoalanine pathway is the primary pathway for cyanide detoxification and assimilation in higher plants. Available evidence now suggests that the pathway may have other physiological functions. The overall goal of this study was to investigate the contribution of the pathway to tolerance to water deficit and cyanide exposure. To achieve this goal the study used *Arabidopsis thaliana* (Col-0) plants and three single knockout mutants cysteine synthase (*AtCysA1*), β-cyanoalanine synthase (*AtCysC1*) and nitrilase 4 (*AtNIT4*) to establish the relative importance of each of the genes encoding enzymes associated with the pathway. Specific objectives were developed to address the main goal.

Objective 1: To establish responsiveness of the β-cyanoalanine pathway in Col-0 plants to the magnitude and duration of water deficit stress.

Objective 2: To establish responsiveness of the β-cyanoalanine pathway in Col-0, and three mutants plants *AtCysA1, AtCysC1 and AtNIT4* to cyanide application.

Objective 3: To examine how loss of single genes (*AtCysA1, AtCysC1 and AtNIT4*) encoding enzymes of the β-cyanoalanine pathway influence the ability of *A. thaliana* Col-0 and three mutants (*AtCysA1, AtCysC1 and AtNIT4)* to tolerate water deficit stress.

Objective 4: To determine whether disruption of single genes encoding enzymes of the pathway altered the ability of the mutants to metabolize cyanide. One research question associated with objectives 2 and 3 was whether there is functional redundancy between the enzymes AtCysA1

and AtCysC1 in mediating the first step of the β-CAS pathway. The second question was whether activity of the alternative sulfurtransferase pathway can fully compensate to provide cyanide detoxification and homeostasis using the *AtNIT4* mutant.

The first finding was that the pathway in Col-0 plants was not wholly responsive to mild water deficit stress with little evidence for induction of the *AtCysC1* gene and no change in β-CAS-like enzyme activity. The transient increase in cyanide concentration in the short term was followed by an insignificant increase in β-CAS-like activity. The response pattern was the same regardless of intensity and duration of the stress with a general tendency to return to a new and lower level of cyanide homeostasis. While the result is consistent with other conclusions that the β-CAS-like activity is constitutive and highly expressed in *A. thaliana*, it is contrary to other reports that have reported a significant up-regulation of transcript abundance and β-cyanoalanine synthase enzyme activity in tobacco and birch. Together the results provide evidence that the β-CAS-like enzymes are regulated at different levels (transcriptional and post-transcriptional) (Maruyama et al., 2001) and that regulation is species specific. Another finding from the study was that pathway response is not global, but rather is localised to the tissue in direct proximity to the stress. This result provides support for hypotheses on hormonal signaling between tissues, that biochemical signals are produced and translocated to other tissues for stress response. Responsiveness of the β-CAS pathway therefore involves complex interaction with other pathways such as ethylene and ROS leading to stress tolerance.

Loss of single genes (*AtCysA1 and AtCysC1*) encoding enzymes for cyanide detoxification did not impair the ability of the mutants to metabolize stress cyanide and tolerate water deficit. The findings suggest that these two enzymes (and perhaps other members of the

Bsas family) are functionally redundant. It is most probable that the enzymes are not fully redundant because of the minor deviations in phenotypes of the mutants. This phenomenon of functional redundancy thus complicated the efforts to quantify the relative contribution of each gene to cyanide removal, but it was apparent that AtCysC1 makes greater contribution to β-CAS-like activity than AtCysA1 as reported by (Watanabe et al., 2008). Having established the redundancy of these two, it is not clear whether the remaining eight *Bsas* enzymes may compensate for reduced activity. It would be interesting to utilize a double knockout mutant for AtCysA1 and AtCysC1 to determine whether other members of the Bsas enzymes compensate for the loss of these two.

The key role of the *AtNIT4* gene in the pathway was illustrated by the *AtNIT4* mutant impaired in hydrolysis of β-cyanoalanine. The findings on sensitivity of the *AtNIT4* mutant to water deficit and cyanide exposure provide more evidence that the *AtNIT4* gene is indeed a sole member of the subfamily mediating the second step of the pathway (Piotrowski et al., 2001), and that it is critical to functionality of the pathway. Disrupting the function of this gene would create a bottleneck at β-cyanoalanine removal and perhaps a negative feedback on cyanide detoxification causing a build-up of these metabolites. The susceptibility of *AtNIT4* mutants to water deficit and cyanide exposure stress is evidence that the alternative sulfurtransferase pathway is not involved in excess cyanide removal, at least in *A. thaliana* . Thus the β-CAS pathway is the sole pathway for cyanide detoxification and assimilation and maintenance of cyanide homeostasis. One question that arose from this is the exact cause of *AtNIT4* sensitivity. Additional studies could be done with the *AtNIT4* mutant to investigate whether there is accumulation of cyanide or β-cyanoalanine or both resulting in toxicity. While our findings show

that β-cyanoalanine is also toxic to *A. thaliana*, it is not clear whether there is accumulation of this intermediate in the *AtNIT4* mutant. It was however evident that there is enhanced production of reactive oxygen species in this mutant in agreement with one report that cyanide stimulates NADPH oxidase (Oracz et al., 2009). Another finding with the dual AtNIT4 enzyme was that nitrile hydratase activity is more important than nitrilase activity in β-cyanoalanine metabolism. The ratio of the two activities in *A. thaliana* is1:1 but varies with species. Results in this study show that there is a tendency to shift towards nitrile hydratase activity on exposure to stress. The cause of this shift is not clear, though one study reported that a single amino acid change in the catalytic region of *Pseudomonas* resulted in a similar shift (Kiziak and Stolz, 2009). It would be interesting to establish the physiological implications of the changes in this ratio.

The phenotype of the *AtNIT4* mutant could also be likened to auxin mutants. The plant hormone auxin is known to control primary root elongation. Two pieces of evidence suggest that the *AtNIT4* phenotype may be associated with auxin. The first is that nitrilases are generally implicated in auxin biosynthesis (Kriechbaumer et al., 2007). Although the specificity of *AtNIT4* is β-cyanoalanine, it is reasonable to speculate that there may be residual activity in auxin metabolism. Second, cyanide has been shown to suppress auxin biosynthesis/perception at the root tip (Rudrappa et al., 2008), or rather suppression at root tip imposes negative effect on shoot growth. It would be interesting to further study the metabolism of auxin in this mutant.

One unexpected result was increased dry weight biomass accumulation in the mutants relative to Col-0 plants after exposure to 15 d of water deficit. It is not apparent from the study why the mutants would have increased growth and it raises questions on how the pathway is connected to key metabolic processes of carbohydrate accumulation i.e the intricate relationship

between the chloroplasts and mitochondria. Mitochondria have been shown to play a key role in plant tolerance to stress (Atkin and Macherel, 2009), and the β-CAS pathway is one mechanism to protect the mitochondria. Further studies could be done on how the β-CAS pathway and cyanide interact with carbohydrate metabolism. For example, one product of the pathway, hydrogen sulfide not only has direct effects on photosynthetic activity (Chen et al., 2011) but also protects soybean seedling against drought (Zhang et al., 2010). The phenomenon of hormesis at low cyanide doses was one result worth noting. The mechanisms for this response are not clear and could be a subject of further study. It was however interesting to note that the *AtCysC1*gene is required for this response, suggesting for a role of the β-CAS pathway in primary metabolic processes.

While the primary role of the β-CAS pathway has been continuously alluded to as stress cyanide detoxification, some reports suggest that there are more physiological functions in regards to lateral root development, regulating nitrate assimilation, signaling, and resistance to pathogen infection. These functions of the β-CAS pathway may be direct, such as removal of cyanide and prevention of toxicity, or indirect via maintenance of cyanide homeostasis. This study suggests that the pathway has a role in tolerance to water deficit stress and also cyanide exposure. Taken together the results provide a better understanding of the β-CAS pathway. The pathway is indeed a potential tool for use in improving plants for tolerance to abiotic stress.

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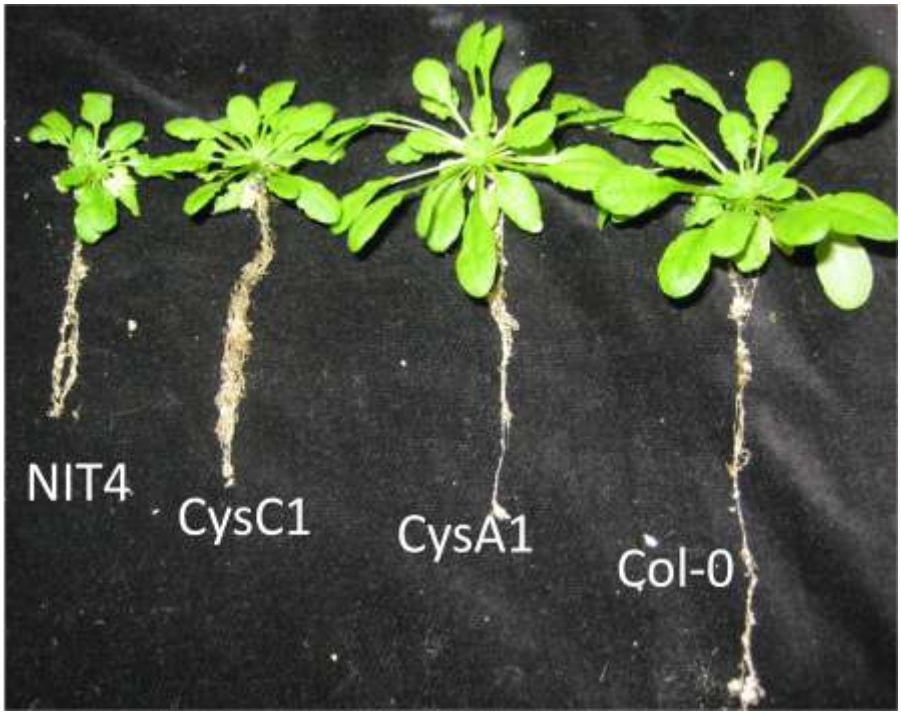
APPENDICES

APPENDIX A

Phenotype of *A. thaliana* Col-0, three mutants *AtCysA1, AtCyC1* and *AtNIT4*.



Appendix A. Phenotype of *A. thaliana* (Col-0), three single mutants *AtCysA1, AtCyC1* and *AtNIT4* after 15 d of water deficit. Plants were geminated and grown for three weeks on MS media, transferred to perlite and vermiculite and grown for three additional weeks. Plants were then subjected to water deficit for 15 days.

APPENDIX B

Cumulative transpiration of *A. thaliana* Col-0, *AtCysA1*, *AtCysC1* and *AtNIT4* mutants

Plants germinated in MS plates were cultured in hydroponics until six weeks of growth. Plants were exposed to cyanide treatments 30 mg L^{-1} for 7 days. Each data point represents the mean and standard error (*n*=3).

Line	Cyanide concentration (mg L^{-1})	24 h	48 h	72 h	96 h	120 h	144 h
Col-0	0	2.07 (0.25)	1.53 (0.12)	2.60 (0.32)	1.90 (0.03)	2.00 (0.15)	1.77 (0.22)
	0.01	1.57 (0.29)	1.43 (0.20)	2.37 (0.10)	1.77 (0.19)	1.30 (0.43)	1.57 (0.15)
	0.1	0.83 (0.24)	1.60 (0.18)	2.10 (0.36)	1.37 (0.21)	0.93 (0.17)	1.30 (0.52)
	0.5	1.23 (0.12)	0.97 (0.19)	1.57 (0.18)	0.57 (0.12)	1.50 (0.06)	1.20 (0.23)
	1	1.03 (0.27)	1.60 (0.34)	1.67 (0.45)	0.73 (0.18)	0.80 (0.17)	0.47 (0.31)
	2	1.87 (0.09)	1.43 (0.03)	0.90 (0.09)	0.70 (0.12)	0.57 (0.09)	0.57 (0.06)
	5	1.93 (0.24)	1.37 (0.39)	1.43 (0.28)	0.97 (0.12)	0.73 (0.09)	0.87 (0.27)
	10	1.53 (0.15)	1.63 (0.15)	1.37 (0.17)	0.80 (0.09)	0.40 (0.47)	0.33 (0.20)
	20	2.10 (0.06)	0.97 (0.12)	1.07 (0.19)	0.80 (0.36)	0.93 (0.25)	0.30 (0.21)
	30	1.70 (0.32)	1.77 (0.18)	1.10 (0.47)	0.50 (0.01)	0.63 (0.12)	0.37 (0.15)
AtCysA1	0	3.53 (0.44)	1.93 (0.39)	1.83 (0.57)	1.27 (0.35)	1.97 (0.56)	1.73 (0.08)
	0.01	3.00 (0.55)	2.03 (0.49)	1.87 (0.58)	1.37 (0.19)	1.20 (0.25)	1.33 (0.20)
	0.1	2.03 (0.51)	2.10 (0.57)	1.83 (0.30)	1.43 (0.27)	1.00 (0.21)	1.57 (0.52)
	0.5	3.10 (0.38)	1.73 (0.48)	2.27 (0.50)	2.17 (0.55)	1.80 (0.50)	1.37 (0.47)
	1	2.60 (0.31)	2.67 (0.32)	0.33 (0.19)	0.70 (0.12)	1.17 (0.35)	1.00 (0.36)
	2	2.70 (0.45)	0.87 (0.13)	0.83 (0.22)	0.67 (0.07)	0.47 (0.09)	0.37 (0.07)
	5	2.33 (0.41)	1.10 (0.35)	0.87 (0.38)	0.70 (0.26)	0.80 (0.20)	0.60 (0.31)
	10	1.67 (0.22)	1.20 (0.12)	1.67 (0.52)	0.93 (0.07)	0.73 (0.15)	0.80 (0.15)
	20	2.13 (0.19)	1.23 (0.19	1.63 (0.19)	1.97 (0.12)	0.63 (0.09)	0.70 (0.06)
	30	2.60 (0.22)	1.17 (0.13)	0.53 (0.19)	0.93 (0.09)	0.70 (0.12)	0.57 (0.07)
AtCysC1	0	1.53 (0.19)	2.17 (0.24)	1.80 (0.32)	2.00 (0.35)	1.50 (0.17)	1.13 (0.19)
	0.01	2.10 (0.23)	2.17 (0.20)	1.73 (0.23)	1.20 (0.21)	1.53 (0.24)	1.30 (0.10)
	0.1	2.53 (0.12)	2.57 (0.18)	2.30 (0.31)	1.17 (0.23)	0.83 (0.12)	0.57 (0.13)
	0.5	2.00 (0.29)	1.90 (0.25)	1.50 (0.40)	1.33 (0.27)	1.20 (0.25)	1.40 (0.55)
	1	2.23 (0.35)	2.20 (0.55)	1.80 (0.49)	1.50 (0.40)	1.50 (0.50)	1.23 (0.50)
	2	2.00 (0.50)	1.97 (0.53)	1.77 (0.48)	0.87 (0.20)	1.30 (0.10)	0.90 (0.21)
	5	1.80 (0.36)	1.97 (0.22)	1.23 (0.12)	0.83 (0.20)	0.80 (0.12)	0.60 (0.06)
	10	2.17 (0.45)	1.70 (0.46)	1.60 (0.25)	1.30 (0.12)	0.77 (0.13)	0.63 (0.15)
	20	1.10 (0.06)	1.23 (0.13)	2.07 (0.32)	1.77 (0.33)	0.87 (0.17)	0.37 (0.13)
	30	2.13 (0.52)	0.87 (0.22)	1.37 (0.47)	1.17 (0.30)	0.90 (0.17)	0.43 (0.13)
AtNIT4	0	1.93 (0.53)	1.77 (0.24)	1.93 (0.27)	1.53 (0.29)	1.67 (0.52)	1.63 (0.37)
	0.01	1.93 (0.47)	1.60 (0.20)	1.97 (0.43)	2.23 (0.34)	1.33 (0.18)	1.23 (0.18)
	0.1	2.10 (0.20)	2.07 0.23)	2.00 (0.40)	1.67 (0.09)	1.33 (0.26)	0.93 (0.33)
	0.5	2.00 (0.40)	1.40 (0.26)	1.93 (0.09)	2.23 (0.20)	1.47 (0.13)	0.93 (0.26)
	1	1.60 (0.26)	1.80 (0.35)	1.43 (0.22)	1.30 (0.06)	0.93 (0.18)	0.47 (0.09)
	2	1.87 (0.33)	1.20 (0.42)	1.00 (0.35)	1.67 (0.54)	1.40 (0.49)	0.83 (0.29)
	5	2.63 (0.14)	1.47 (0.23)	1.40 (0.12)	1.80 (0.35)	0.80 (0.12)	0.37 (0.07)
	10	2.30 (0.10)	1.50 (0.16)	1.47 (0.28)	1.20 (0.35)	1.10 (0.10)	0.63 (0.17)
	20	1.90 (0.15)	1.23 (0.24)	1.53 (0.13)	1.40 (0.10)	1.07 (0.23)	0.47 (0.13)
	30	1.80 (0.21)	1.27 (0.07)	1.50 (0.25)	0.80 (0.06)	0.80 (0.35)	0.43 (0.19)

APPENDIX C

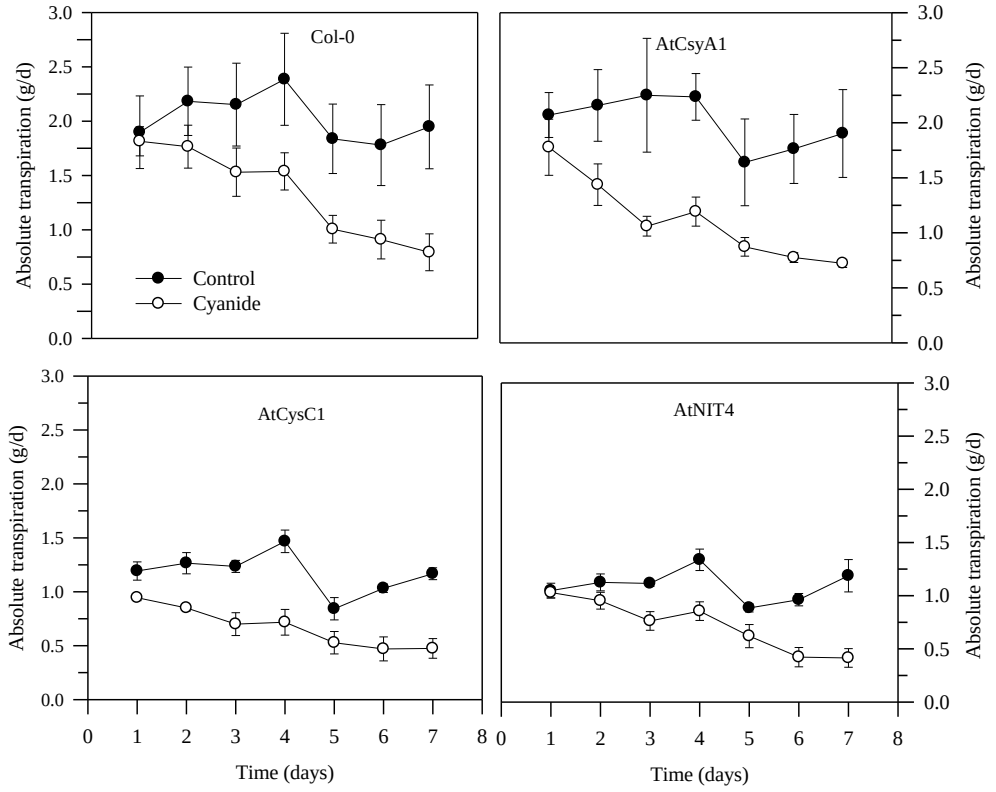
Absolute transpiration of *A. thaliana* Col-0, *AtCysA1*, *AtCysC1* and *AtNIT4* mutants

Plants germinated in MS plates were cultured in hydroponics until six weeks of growth. Plants were exposed to cyanide treatments 30 mg L^{-1} for 7 days. Each data point represents the mean and standard error (*n*=3).

Line	Cyanide concentration (mg L^{-1})	24 h	48 h	72 h	96 h	120 h	144 h
Col-0	0	2.07 (0.25)	3.60 (0.12)	6.20 (0.32)	8.10 (0.03)	10.1 (0.15)	11.87 (0.22)
	0.01	1.57 (0.29)	3.00 (0.20)	5.37 (0.10)	7.13 (0.19)	8.43 (0.43)	10.00 (0.15)
	0.1	0.83 (0.24)	2.43 (0.18)	4.53 (0.36)	5.90 (0.21)	6.83 (0.17)	8.13 (0.52)
	0.5	1.23 (0.12)	2.20 (0.19)	3.77 (0.18)	4.33 (0.12)	5.83 (0.06)	7.03 (0.23)
	1	1.03 (0.27)	2.63 (0.34)	4.30 (0.45)	5.03 (0.18)	5.83 (0.17)	6.30 (0.31)
	2	1.87 (0.09)	3.30 (0.03)	4.20 (0.09)	4.90 (0.12)	5.47 (0.09)	6.03 (0.06)
	5	1.93 (0.24)	3.30 (0.39)	4.73 (0.28)	5.70 (0.12)	6.43 (0.09)	7.30 (0.27)
	10	1.53 (0.15)	3.17 (0.15)	4.53 (0.17)	5.33 (0.09)	5.73 (0.57)	6.07 (0.20)
	20	2.10 (0.06)	3.7 (0.12)	4.13 (0.19)	4.93 (0.36)	5.87 (0.25)	6.17 (0.21)
	30	1.70 (0.32)	3.47 (0.18)	4.57 (0.47)	5.07 (0.01)	5.70 (0.12)	6.07 (0.15)
AtCysA1	0	3.53 (0.44)	5.47 (0.39)	7.30 (0.57)	8.57 (0.35)	10.5 (0.56)	12.27 (0.38)
	0.01	3.00 (0.55)	5.03 (0.49)	6.90 (0.58)	8.27 (0.19)	9.47 (0.25)	10.80 (0.20)
	0.1	2.03 (0.51)	4.13 (0.57)	5.97 (0.30)	7.40 (0.27)	8.40 (0.21)	9.97 (0.52)
	0.5	3.10 (0.38)	4.83 (0.48)	7.10 (0.50)	9.27 (0.55)	11.07 (0.6)	12.43 (0.47)
	1	2.60 (0.31)	5.27 (0.42)	5.60 (0.19)	6.30 (0.12)	7.47 (0.35)	8.47 (0.36)
	2	2.70 (0.45)	3.57 (0.13)	4.40 (0.22)	5.07 (0.17)	5.53 (0.19)	5.90 (0.17)
	5	2.33 (0.41)	3.43 (0.35)	4.30 (0.38)	5.00 (0.26)	5.80 (0.20)	6.40 (0.31)
	10	1.67 (0.22)	2.87 (0.12)	4.53 (0.52)	5.47 (0.17)	6.20 (0.15)	7.00 (0.15)
	20	2.13 (0.19)	3.37 (0.19)	5.00 (0.19)	6.97 (0.12)	7.60 (0.19)	8.30 (0.16)
	30	2.60 (0.22)	3.77 (0.13)	4.30 (0.19)	5.23 (0.19)	5.93 (0.12)	6.50 (0.17)
AtCysC1	0	1.53 (0.19)	3.70 (0.24)	5.50 (0.32)	7.50 (0.35)	9.00 (0.17)	10.13 (0.19)
	0.01	2.10 (0.23)	4.27 (0.20)	6.00 (0.23)	7.20 (0.21)	8.73 (0.24)	10.03 (0.10)
	0.1	2.53 (0.12)	5.10 (0.18)	7.40 (0.31)	8.57 (0.23)	9.40 (0.12)	9.97 (0.13)
	0.5	2.00 (0.29)	3.90 (0.25)	5.40 (0.40)	6.73 (0.27)	7.93 (0.25)	9.33 (0.55)
	1	2.23 (0.35)	4.43 (0.55)	6.23 (0.49)	7.73 (0.40)	9.23 (0.50)	10.47 (0.50)
	2	2.00 (0.50)	3.97 (0.53)	5.73 (0.48)	6.60 (0.20)	7.90 (0.10)	8.80 (0.21)
	5	1.80 (0.36)	3.77 (0.22)	5.00 (0.12)	5.83 (0.20)	6.63 (0.12)	7.23 (0.16)
	10	2.17 (0.45)	3.87 (0.46)	5.47 (0.25)	6.77 (0.12)	7.53 (0.13)	8.17 (0.15)
	20	1.10 (0.06)	2.33 (0.13)	4.40 (0.32)	6.17 (0.33)	7.03 (0.17)	7.40 (0.13)
	30	2.13 (0.52)	3.00 (0.22)	4.37 (0.47)	5.53 (0.30)	6.43 (0.17)	6.87 (0.13)
AtNIT4	0	1.93 (0.53)	3.70 (0.24)	5.63 (0.27)	7.17 (0.29)	8.83 (0.52)	10.47 (0.37)
	0.01	1.93 (0.47)	3.53 (0.20)	5.50 (0.43)	7.73 (0.34)	9.07 (0.18)	10.30 (0.18)
	0.1	2.10 (0.20)	4.17 (0.23)	6.17 (0.40)	7.83 (0.09)	9.17 (0.26)	10.10 (0.33)
	0.5	2.00 (0.40)	3.40 (0.26)	5.33 (0.09)	7.57 (0.20)	9.03 (0.13)	9.97 (0.26)
	1	1.60 (0.26)	3.40 (0.35)	4.83 (0.22)	6.13 (0.06)	7.07 (0.18)	7.53 (0.09)
	2	1.87 (0.33)	3.07 (0.42)	4.07 (0.35)	5.73 (0.54)	7.13 (0.49)	7.97 (0.29)
	5	2.63 (0.14)	4.10 (0.23)	5.50 (0.12)	7.30 (0.35)	8.10 (0.12)	8.47 (0.07)
	10	2.30 (0.10)	3.80 (0.06)	5.27 (0.28)	6.47 (0.35)	7.57 (0.10)	8.20 (0.17)
	20	1.90 (0.15)	3.13 (0.24)	4.67 (0.13)	6.07 (0.10)	7.13 (0.23)	7.60 (0.13)
	30	1.80 (0.21)	3.07 (0.07)	4.57 (0.25)	5.37 (0.06)	6.17 (0.35)	6.60 (0.09)

APPENDIX D

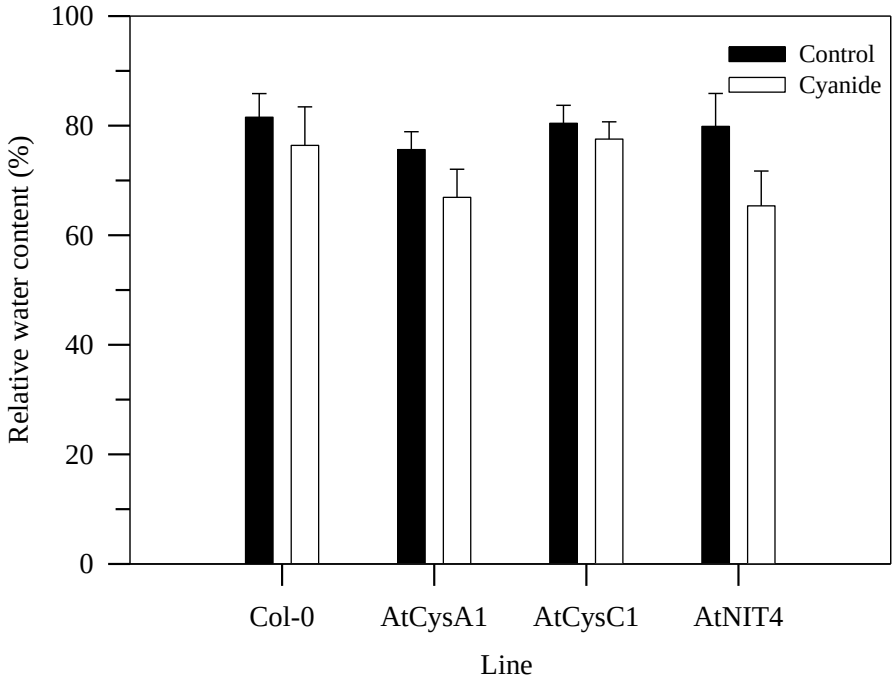
Absolute transpiration of *A. thaliana* (Col-0), *AtCysA1*, *AtCysC1* and *AtNIT4* mutants.



Appendix D. Absolute transpiration of *A. thaliana* (Col-0), *AtCysA1*, *AtCysC1* and *AtNIT4* mutants. Plants germinated in MS plates were cultured in hydroponics for 6 weeks. Plants were transferred to Erlenmeyer flasks for cyanide treatments 30 mg L^{-1} for 7 d. The closed circles represent control and the open circles represent the cyanide treatment. Each point on the line represents the mean and standard error (n=4).

APPENDIX E

Relative water content of *A. thaliana* Col-0, *AtCysA1*, *AtCysC1* and *AtNIT4* mutants.



Appendix E. Relative water content of *A. thaliana* Col-0, *AtCysA1*, *AtCysC1* and *AtNIT4* mutants. Six week old plants cultured in hydroponics were subjected to 30 mg cyanide L^{-1} for 7d. The black bars represent control and the open bars represent the cyanide treatment. Each bar is the mean and standard error (n=4).

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Ebbs, S. D., Kosma, D. K., Nielson, E. H., Machingura, M., Baker, A. J. M. and Woodrow, I. E. (2010), Nitrogen supply and cyanide concentration influence the enrichment of nitrogen from cyanide in wheat (*Triticum aestivum* L.) and sorghum (*Sorghum bicolor* L.). Plant, Cell & Environment, 33: 1152–1160

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