2-2013

The β-cyanoalanine pathway is involved in the response to water deficit in Arabidopsis thaliana.

Marylou Machingura

Aissatou Sidibe

Andrew J Wood

Stephen Ebbs
Southern Illinois University Carbondale, sebbs@plant.siu.edu

Recommended Citation
Machingura, Marylou, Sidibe, Aissatou, Wood, Andrew J and Ebbs, Stephen. "The β-cyanoalanine pathway is involved in the response to water deficit in Arabidopsis thaliana.." *Plant physiology and biochemistry* 63 (Feb 2013): 159-169. doi:10.1016/j.plaphy.2012.11.012.

TITLE: The β-cyanoalanine pathway is involved in the response to water deficit in *Arabidopsis thaliana*

Running head: The β-cyanoalanine pathway and water deficit in Arabidopsis

Marylou Machingura, Aissatou Sidibe Niang, Andrew J. Wood, and Stephen D. Ebbs*

Department of Plant Biology and Center for Ecology, Southern Illinois University Carbondale, 420 Life Science II, 1125 Lincoln Drive, Carbondale, IL 62901-6509 USA

*Corresponding author: Phone +1 618 453-3226; FAX +1 618 453-3441; sebbs@plant.siu.edu

Abstract

The β-cyanoalanine pathway is primarily responsible for detoxification of excess cyanide produced by plants. Recent evidence suggests that cyanide detoxification via this pathway may be involved in the response and tolerance to water deficit in plants. The aim of this study was to explore this role in *Arabidopsis thaliana* in greater detail. The first objective was to establish responsiveness of the pathway to the magnitude and duration of water deficit. The second objective was to examine how interruption of single genes (*AtCysA1, AtCysC1 and AtNIT4*) encoding enzymes of the pathway influenced the ability to metabolize cyanide and withstand water deficit. Arabidopsis plants were exposed to conditions which emulated acute and chronic water deficit, followed by measurement of tissue cyanide concentration, activity of enzymes, and physiological parameters. The results for wild-type Arabidopsis demonstrated a transient increase in cyanide concentration and β-cyanoalanine synthase activity, followed by a decrease in both. The increase in enzyme activity was localized to the tissue in direct proximity to the stress. The knockdown *AtCysA1* mutant did not differ from wild-type while *AtCysC1* mutants were slightly more sensitive to water deficit. The *AtNIT4* mutant was the most sensitive showing decreased growth along with altered chlorophyll content under water deficit as compared to wild-type. Collectively, the results indicated that the pathway is responsive to water deficit although the severity of stress did not alter the nature of the response, implying that the capacity to remove cyanide generated during water deficit may contribute to tolerance to this stress in Arabidopsis.

Author keywords Arabidopsis thaliana; Cyanide; Cyanoalanine synthase; Nitrilase; Water deficit

1. 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 [1] and ethylene biosynthesis [2, 3]. While formation of cyanogenic glycosides and cyanolipids is limited to ~2,500 plant species, ethylene biosynthesis is the 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 (ACO)(EC 1.14.17.4), releasing hydrogen cyanide and carbon dioxide as co-products [2]. The β-cyanoalanine synthase pathway is the principle pathway for cyanide detoxification in plants. In the first step of the pathway catalyzed by β-cyanoalanine synthase (EC 4.4.1.9), cyanide reacts with cysteine to form β-cyanoalanine, releasing bisulphide [4]. The second step is mediated by a dual function nitrile hydratase/nitrilase (EC 3.5.5.1) designated in Arabidopsis as nitrilase 4 (NIT4). The NIT4 enzyme catalyzes reactions that convert cyanoalanine to either asparagine or aspartate in conjunction with ammonia, respectively [5].

Recent evidence 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 increased β-CAS transcript abundance [6]. Tissue cyanide concentrations and β-CAS activity showed concomitant increases when tobacco (*Nicotiana tabacum* L.) plants were subjected to water deficit [7]. Cyanide concentration decreased after two days of stress 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 [5, 8], namely O-acetylserine(thiol) lyase (OASTL) (EC 2.5.1.47) (*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 Arabidopsis Col-0 to the magnitude and duration of water deficit. Since ethylene production depends on the severity and duration of stress [9], 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 [3]. The second objective was to examine how interruption of single genes encoding enzymes of the pathway influenced the ability of Arabidopsis to metabolize cyanide and respond to water deficit. Given that β-CAS (*AtCysC1*, Bsas 3;1) and one OASTL (*AtCysA1*, Bsas 1;1) are the only enzymes with β-cyanoalanine synthase activity under physiological conditions [8], 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. As a single gene in Arabidopsis [5], 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. Results

2.1 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 Arabidopsis seedlings (Figure 1A). A two-fold increase in β-CAS-like enzyme activity was also observed in response to acute water deficit (Figure 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 also 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, 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 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. To confirm that the patterns in the data represented specific changes in tissue cyanide and β-CAS-like activity and not changes driven by differences in fresh weight biomass or total protein content, respectively, the data were recalculated to express the former in

98 pmol cyanide and the latter as nmol product h^{-1} (Figure S1) and the same general trends were
99 obtained although the difference for each parameter as a function of time was not significant.
100 One approach to apply a more environmentally-relevant, chronic water deficit was to
101 simply withhold watering and monitor the water status of the growing medium. Plants were first
102 harvested for measurement four days after watering ceased when the volumetric water content of
103 the medium fell below 50%. Additional harvests were carried out on the consecutive fifth and
104 sixth days after watering ceased. Tissue cyanide concentration was significantly higher in plants
105 that had been deprived of water for four and five days as compared to plants prior to the onset of
106 water deficit treatment (Figure 3). After six days without water, tissue cyanide concentrations
107 were not significantly different from untreated plants. A significant difference in β-CAS-like
108 activity was observed between plants deprived of water and plants prior to onset of treatment.
109 There was however no significant difference in β-CAS activity between sampling dates. To
110 determine the response of the pathway to a longer duration of water deficit, the experiment was
111 essentially repeated except that plants were harvested after 5, 10, and 15 d without watering, at
112 which point there was an obvious loss of turgor in the leaves of all treated plants. The RWC of
113 the treated tissues decreased significantly from 84% to 64% during the 15 d period, while the
114 well watered control plants maintained a RWC of 84-88%. Compared to control plants, tissue
115 cyanide concentration was significantly higher after 5 d without watering but significantly lower
116 than untreated controls after 10 and 15 d (Figure 4). There was no significant difference in β-
117 CAS-like activity between well watered control plants and plants deprived of water for 5 or 10
118 days. However, enzyme activity decreased significantly after 15 d without water. Overall, the
119 activity of β-CAS pathway to water deficit was generally similar regardless of the magnitude and

duration of the stress. As for the acute water deficit exposure, recalculating the results from the two chronic water deficit exposures as pmol cyanide and as nmol product h^{-1} (Figure S1), the same general trends were again obtained. The differences for each parameter as a function of time did show significant differences in this case with the same transient patterns as those observed in Figs. 3-4.

2.2 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. 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 soil hydration, 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 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 6). These trends in the data did not change when the data were recalculated as former in pmol cyanide or as nmol product h^{-1} (Figure S2).

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

Consistent with studies by others [8], the established SALK lines for β-CAS (*AtCysC1*) and the cytosolic OASTL (*AtCysA1*) were shown to be knockdowns for enzyme activity (Figure 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 [8]. A surprising observation made here and elsewhere [15, 16] 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 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 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). Activity of this enzyme was not cyanide inducible in either line (Figure 8B), indicating that the presence of

cyanide produced during water deficit did not produce the results in Figure 8A due to differential induction of nitrile hydratase activity in the two Arabidopsis lines.

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 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 (Figure S3). 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 and fresh weight biomass after15 d of water deficit for each line as compared to corresponding well-watered plants of the same line (Figure 9). The magnitude of the decrease was not significantly different between the *AtCysA1* mutant line and the Col-0 plants (Table 1). The *AtCysA1* mutant had a 35% decrease in leaf area. The *AtCysC1* mutant had 50% reductions in leaf area and significantly different from 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* mutant plants were slightly larger (Table 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 1).

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 10). 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 11). 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.

3. 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 [17]. Plants also need energy under stress and the relative importance of the mitochondria as the principal organelle for ATP production increases [18]. While other work has shown that under intense water stress, mitochondria activity decreases [19], some have reported an increase in mitochondrial function under moderate, slow onset stress [20]. Water stress generates ethylene, and would also therefore generate cyanide. Since a principle target of cyanide inhibition is the terminal oxidase of the mitochondria, cyanide detoxification by the β-CAS pathway is likely necessary to help offset the negative effects of cyanide on the mitochondria [21], and therefore 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 Arabidopsis the *AtCysC1* gene shows high, constitutive expression that is not responsive to cyanide exposure or stress [22], hence the significant yet minor difference in transcript abundance even in response to an acute exposure to water deficit was not wholly unexpected (Figure 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 [23]. The generally parallel changes in tissue cyanide and β-CAS-like enzyme activity observed here in response to water deficit (Figures 2-4) are consistent with reports from a similar water deficit study with tobacco [7]. Both studies showed a transient increase in β-CAS-like enzyme activity coinciding with a water deficit-induced spike in tissue cyanide. 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 Arabidopsis seedlings (Figures 2-4) suggestive of a coordinated response that includes metabolic components beyond the β-CAS pathway. With little evidence for an induction of *AtCysC1*gene, the results suggests that innate activity of the existing pool of enzymes in Arabidopsis 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.

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 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. The tissue cyanide concentration from the split root experiment was ~two-fold higher as compared to the other drying techniques (Figure 5). Increased ethylene production was also reported in tomato plants treated as split-roots [24] 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 an increase in β-CAS activity (Figure 6). As reported in other split-root studies [e.g., 25] 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 maintained 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. However, considering the volatile nature of the HCN molecule and the widespread detoxification system in all living tissues [26], 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 [e.g. 24]. In that study increased abscisic acid (ABA) and ethylene were reported in the xylem sap of split-root treated plants. 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 [27]. 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. Simultaneous analysis of ethylene and cyanide would be necessary in future studies to provide confirmation of this supposition.

The efforts to evaluate how the β-CAS mutants responded to the imposition of water deficit were complicated by the inherent differences in growth characteristics for the lines (Figure 9) and the specific response of each to the mild water deficit imposed (Table 1, Figures 9-11). Collectively the decrease in RWC, dry weight biomass, and leaf area (Figure 9) all indicate that withholding water did affect the water status of all four lines. The progressively greater decrease in these values from the *AtCysA1* to *AtCysC1* and *AtNIT4* lines (Table 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 11). 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. 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 [28], although for this study the growth enhancement was reflected through to maturity at 10 weeks. No statistically significant evidence of this was observed here.

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 [29]. The divergence at Day 15 (Figure 10) with a sustained increased 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.

In the absence of additional data, one speculative explanation for the differences in response of the *AtCysA1* and *AtCysC1* mutant 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 [3, 30], with cyanide enhancing expression of genes such as *ACS6* associated with ethylene synthesis [31] and displaying crosstalk with elements of the ethylene signaling pathway during germination of sunflower seed [32]. There is a growing body of evidence indicating interactions between ethylene and ABA in the control of growth under water stress [33]. Increased production of reactive oxygen species (ROS) under water deficit has been demonstrated [34] 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 and may have direct roles in modulating production of ROS. There is emerging evidence of signaling roles of hydrogen sulfide in plant cells. As enzymes with dual function in cysteine synthesis and β-CAS enzyme activity, 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 of 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

316 require extensive additional work to verify, but these two possibilities offer some explanation for
317 the results obtained for these two lines.

318 The results of this work demonstrated that the insertional mutation of *AtNIT4* has the
319 most drastic effect on growth under both nominal and water limiting conditions as compared to
320 *AtCysA1* or *AtCysC1*. The results from Figure 8B illustrate that the latter is not due to a lack of
321 enzyme inducibility resulting from the t-DNA insertion as neither the Col-0 nor *AtNIT4* line
322 showed increased activity in response to cyanide exposure. The phenotypic difference in nitrile
323 hydratase activity would be expected then to remain the same between the Col-0 and *AtNIT4*
324 lines in the presence of water deficit-elicited cyanide. While sulfurtransferases in plants have
325 been identified and do show metabolism of cyanide to thiocyanate *in vitro* [35], the data here on
326 hypersensitivity of the *AtNIT4* mutant indirectly supports prior studies which demonstrated that
327 sulfurtransferases show no specific role in cyanide homeostasis *in planta*. The results offer
328 additional evidence that the β-CAS pathway is the primary pathway for cyanide detoxification in
329 Arabidopsis. The *AtNIT4* mutant plants are not only knockdowns for nitrile hydratase activity but
330 also knockdowns for β-CAS activity (Figure 7 and 8). The knockdown in β-CAS activity may be
331 indicative of feedback control of the pathway. With decreased activity of both enzymes in the
332 pathway, there would be an accumulation of β-cyanoalanine and decreased assimilation of
333 cyanide. Accumulation of both cyanide and β-cyanoalanine would be potentially detrimental to
334 the plants [8], especially in the absence of any activity from the sulfurtransferase pathway for
335 cyanide assimilation. Although ostensibly a t-DNA insertional mutant, the modest decrease in
336 nitrile hydratase and lack of a difference in nitrilase activity suggest that this line may be leaky.
337 While *AtNIT4* is a single gene in Arabidopsis which shows high specificity *in vitro* for β-

cyanoalanine as a substrate [5], there seem to be no published studies with *AtNIT4* mutants in Arabidopsis 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 [36]. 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 [36]. Nonetheless, the sensitivity of *AtNIT4* plants is reflected in the magnitude of the reduction in leaf area and fresh weight biomass (Figure 9), and relative chlorophyll content (Figure 10). The small phenotype is comparable to what might be expected for an auxin-deficient mutant, especially since nitrilases are also involved in auxin biosynthesis [37]. However, given the purported substrate specificity of the AtNIT4 protein for β-cyanoalanine [5], this argues against *AtNIT4* as an auxin-deficient mutant. If the *AtNIT4* is indeed a single gene in Arabidopsis responsible for hydrolysis of β-cyanoalanine , knocking down this gene should cause a build-up of cyanide (Figure 11) as well as β-cyanoalanine. β-cyanoalanine can be toxic to plants [8] via inhibition of Asparagine:tRNA synthatase [38] and inhibition of root growth [37, 39]. 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.

In conclusion, the 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 [8] 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). 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 [40]. Tolerance to such herbicides has been linked to CAS activity suggesting both stress and herbicide tolerance may be provided in plants in part by the β-CAS pathway. An intriguing question for future study would be to examine how plants respond to simultaneous exposure to water deficit in cyanide contaminated soils. As tolerance to both stresses may require action of the β-CAS pathway, such studies could provide information on the capacity of the pathway to respond under more extreme conditions in the presence of multiple stresses. The complexity of the results obtained here and the interaction of the β-CAS pathway with

382 mitochondrial function and several important cellular signaling molecules (e.g., ethylene, ROS,
383 hydrogen sulfide) also imply a broader contribution to cellular function than simple cyanide
384 detoxification.
385
386 **4. Materials and Methods**
387 *4.1 Plant culture*
388 Seeds of wild type *Arabidopsis thaliana* (Col-0) and the SALK t-DNA insertional
389 mutants for cysteine synthase (*AtCysA1*, At4g14880, SALK_72213), β-cyanoalanine synthase
390 (*AtCysC1*, At3g61440, SALK_22479) and nitrilase 4 (*AtNIT4*, At5g22300, SALK_016289C)
391 were obtained from TAIR (www.Arabidopsis.org). For some experiments, seeds were surface
392 sterilized by fumigation and germinated on plates containing half-strength MS medium with 1%
393 agar and 0.5% sucrose. After a three day vernalization period at 4ºC in the dark the plates were
394 placed at a 30° angle in a Percival growth chamber (Model E-36 L, Des Moines, IA, USA) at
395 ambient humidity with an 8 h photoperiod at a light intensity of ~150 μM m^{-2} s^{-1}. The day/night
396 temperatures were 22° and 18°C respectively, and plants were left to grow for three weeks.
397 Plants were removed from plates and transferred to sterile perlite and vermiculite (1:1) and
398 grown for an additional three weeks. For one experiment, plants were transferred from plates
399 directly to hydroponic solution with the following composition: 6 mM KNO_3, 4 mM $Ca(NO_3)_2$,
400 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 $ZnSO_4$,
401 0.5 μM $CuSO_4$, 0.1 μM $NiSO_4$, and 0.016 μM $(NH_4)_6Mo_7O_{24}$ [10]. The solution was buffered
402 with 1 mM *n*-morpholinoethanesulfonic acid (MES) titrated to pH 6.0 with MES-TRIS. Iron
403 was provided as 10 μM Fe-EDTA, the solution was continuously aerated, and the growth

404 conditions were as indicated above. After three weeks of growth plants were transferred from
405 the hydroponic culture to sterile sand for the split-root experiment described below. Surface
406 sterilized seeds of wild type (Col-0) for one additional experiment were also germinated directly
407 in sterile potting mix using the same vernalization and growth conditions as above. These plants
408 were watered with 50 mL of the same nutrient solution as above and 100 mL water per week.
409 The 100 mL of water was split into two applications of 50 mL. The plants were grown for six
410 weeks under the same growth conditions and photoperiod as above to establish biomass and were
411 then subjected to the desired treatments.
412
413 *4.2 Response of the β-cyanoalanine synthase pathway to magnitude and duration of water deficit*
414 Two different strategies were used to impose water deficit on Arabidopsis Col-0 plants to
415 observe the response of the β-cyanoalanine synthase pathway. For the first, the goal was to
416 examine the response to a severe, short-term (i.e., acute) water deficit. Three week old plants
417 grown on MS plates were subjected to this acute water deficit by removing the plants from the
418 plate and exposing the whole seedlings to air for 20 min. This method of creating water deficit
419 was used previously in a study that examined global changes in gene expression in Arabidopsis
420 in response to abiotic stresses [11]. There were three replicates each for the acute water deficit
421 treatment and the control. Whole plants were harvested, snap-frozen, and stored at -80°C.
422 Additional plants, grown three weeks in MS plates and then three weeks in potting mix were left
423 unwatered for seven days prior. An acute water deficit treatment was imposed by gently
424 removing the seedlings from the potting mix, removing adhering soil particles, and exposing the
425 roots to air for 45 min. Following the treatments, whole plants (three replicates each of control or

426 treated plants) were snap-frozen in liquid nitrogen for later analysis of whole plant tissue cyanide
427 concentration and β-CAS-like activity. Additional plants were germinated and grown in perlite
428 and vermiculite up to the 10-12 leaf stage. These plants were pulled out of the medium and air
429 dried for 60 min to impose acute water deficit. Plants were sacrificed at 20 min intervals for 60
430 min and snap-frozen in liquid nitrogen. Replicate samples of treated and control plants (n=3)
431 were analyzed for relative water content, tissue cyanide and β-CAS-like activity in the whole
432 plant.

433 The second strategy for imposing water deficit was more physiologically and
434 environmentally relevant (i.e. chronic water deficit). For one experiment, watering of Col-0
435 plants grown in perlite and vermiculite was withdrawn and the volumetric water content of the
436 medium was monitored using an Echo EC-5 soil moisture sensor (Decagon Devices Inc, Pullman
437 WA USA). After 4 days, when the volumetric water content of the medium decreased to 50%,
438 plants were harvested at one day intervals for three days. These samples (n=3) were analyzed for
439 relative water content, tissue cyanide and β-CAS-like activity. In a second experiment, watering
440 was discontinued for 15 days. Plants (n=4) were harvested every five days for 15 days, at which
441 point all treated plants were showing a significant loss of turgor. Relative water content of the
442 shoots, tissue cyanide and β-CAS-like activity were determined.

443 To examine whether the response was localized to tissues in contact with dry soil, a split-
444 root experiment was performed using six-week-old Col-0 plants grown for three weeks in
445 hydroponics. The plants were grown hydroponically to reduce the damage associated with their
446 transfer and to allow for an easier separation of the root system into two roughly comparable
447 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.

4.3 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 [10]. Aeration of the nutrient solution was discontinued to reduce cyanide volatilization [10]. 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 OASTL (*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. 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.

4.4 Biochemical and physiological measurements

4.4.1 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™ 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 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 [12]. 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.

4.4.2 Measurement of plant tissue cyanide

Tissue cyanide content was determined using a microdiffusion technique [13] 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

513 tube and centrifuged for 10 min at 3,000 *g* at 4°C. The supernatant was transferred to the 50 mL
514 Falcon tube. The smaller inner tube, which contained 1.5 mL of 5 mM K_2HPO_4 and 0.5 mL of
515 phenolphthalin reagent, was inserted into the 50 mL centrifuge tube. To force the cyanide into
516 the volatile phase, 18 M H_2SO_4 was added to the supernatant in the 50 mL tube to a final
517 concentration of 0.41 M and swirled gently to mix. The Falcon tube was sealed and incubated at
518 25° C for 1 h. After the incubation, the smaller gas trap was removed and 0.5 mL of 0.1% KOH
519 was added. After a period of color development, the absorption of the trap solution was then
520 read at 550 nm wavelength with a Cary 50 UV-Visible spectrophotometer (Varian Inc.,
521 Mulgrave, Australia), and compared to a standard curve constructed from known concentrations
522 of KCN.
523
524 4.4.3 DMPDA assay for β-cyanoalanine synthase activity
525 To determine activity of β-CAS in the seedlings, whole plants or individual tissues were
526 ground under liquid nitrogen and then under a buffer consisting of 2 mM EDTA-Na_2, 10 mM
527 cysteine, 0.1 M Tris-HCl, pH 9.5. The homogenate was centrifuged at 4,000 *g* for 10 min at 4°C
528 and then split into three subsamples. One subsample was used for determination of the total
529 protein content using the Pierce BCA assay Kit (Thermo Scientific) according to the
530 manufacturer's instructions. The other two samples were used for the determination of β-CAS
531 activity. One of these two tubes was boiled for 10 min to provide a heat-killed control to correct
532 for background absorbance. The activity of β-CAS was assayed using the DMPDA assay [7].
533 The crude protein supernatant was mixed 1:1 (v/v) with the substrate (10 mM cysteine, 3 mM
534 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.

4.4.4 Colorimetric determination of nitrilase and nitrile hydratase activity

Nitrilase and nitrile hydratase activity was assayed via determination of ammonia according to Piotrowski et al. [5]. 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 4ºC. 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 30ºC after which the sample was boiled at 100ºC for 10 min to stop the reaction. For parallel determination of nitrilase and 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 37ºC 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 [5].

4.4.5 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 4ºC and then weighed to determine turgid weight (TW). The samples were oven dried to constant mass at 55ºC and the dry weight (DW) was obtained. Relative water content (RWC) was determined according to Barr and Weatherley [14]:

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

4.4.6 Relative chlorophyll content

Relative chlorophyll content of leaves was determined using a SPAD 502+ Chlorophyll Meter (Konica Minolta Sensing Inc., Osaka, Japan).

4.5 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

579 significant interactions between the main effects for a parameter were observed, the main effects
580 were analyzed separately by one-way ANOVA with Tukey's test used for post hoc analysis.
581 When a significant interaction between main effects did occur, the data were reanalyzed by one-
582 way ANOVA with each interaction mean (line x treatment) representing an individual treatment.
583 Tukey's test was again used for the post hoc analysis.
584

585 5. **Acknowledgments**

586 The authors would like to thank the Fulbright Scholars Program for supporting the graduate
587 study of Aissatou Sidibe Niang and to the Department of Plant Biology at Southern Illinois
588 University for supporting the graduate study of Marylou Machingura. Additional Financial
589 support for this research was provided by a Faculty SEED Grant from Southern Illinois
590 University to Stephen Ebbs.

591 REFERENCES
592
593 [1] J.E. Poulton, Cyanogenesis in Plants, Plant Physiology, 94 (1990) 401-405.
594 [2] G.D. Peiser, T.-T. Wang, N.E. Hoffman, S.F. Yang, H.-W. Liu, C.T. Walsh, Formation of
595 cyanide from carbon 1 of 1-aminocyclopropane-1-carboxylic acid during its conversion to
596 ethylene, Proceedings of the National Academy of Science of the United States of America, 81
597 (1984) 3059-3063.
598 [3] W.-K. Yip, S.F. Yang, Cyanide metabolism in relation to ethylene production in plant tissues,
599 Plant Physiology, 88 (1988) 473-476.
600 [4] H.R. Hendrickson, E.E. Conn, Cyanide metabolism in higher plants. IV. Purification and
601 properties of the beta-cyanoalanine synthase of blue lupine, Journal of Biological Chemistry, 244
602 (1969) 2632-2640.
603 [5] M. Piotrowski, S. Schonfelder, E.W. Weiler, The *Arabidopsis thaliana i*sogene NIT4 and its
604 orthologs in tobacco encode beta-cyano-L-alanine hydratase/nitrilase, Journal of Biological
605 Chemistry, 276 (2001) 2616-2621.
606 [6] J. Vahala, R. Ruonala, M. Keinanen, H. Tuominen, J. Kangasjarvi, Ethylene insensitivity
607 modulates ozone-induced cell death in birch, Plant Physiology, 132 (2003) 185-195.
608 [7] W.S. Liang, Drought stress increases both cyanogenesis and beta-cyanoalanine synthase
609 activity in tobacco, Plant Sci, 165 (2003) 1109-1115.
610 [8] M. Watanabe, M. Kusano, A. Oikawa, A. Fukushima, M. Noji, K. Saito, Physiological roles
611 of the β-substituted alanine synthase gene family in Arabidopsis, Plant Physiology, 146 (2008)
612 310-320.

[9] P.W. Morgan, M.C. Drew, Ethylene and plant responses to stress, Physiologia Plantarum, 100 (1997).

[10] S. Ebbs, J. Bushey, S. Poston, D. Kosma, M. Samiotakis, D. Dzombak, Transport and metabolism of free cyanide and iron cyanide complexes by willow, Plant Cell and Environment, 26 (2003) 1467-1478.

[11] J. Kilian, D. Whitehead, J. Horak, D. Wanke, S. Weinl, O. Batistic, C. D'Angelo, E. Bornberg-Bauer, J. Kudla, K. Harter, The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses, Plant Journal, 50 (2007) 347-363.

[12] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method, Methods, 25 (2001) 402-408.

[13] S.D. Ebbs, R.C.R. Piccinin, J.Q.D. Goodger, S.D. Kolev, I.E. Woodrow, A.J.M. Baker, Transport of ferrocyanide by two eucalypt species and sorghum, Int J Phytoremediat, 10 (2008) 343-357.

[14] H.D. Barr, P.E. Weatherley, A re-examination of the relative turgidity technique for estimating water deficit in leaves, Aust. J. Biol. Sci., 15 (1962) 413-428.

[15] A. Sidibe, Effect of Abiotic stresses and cyanide treatment on the cyanide assimilatory pathway in *Arabidopsis thaliana*, in: Plant Biology, Southern Illinois University, Carbondale, Carbondale, 2008, pp. 176.

[16] M. Piotrowski, Primary or secondary? Versatile nitrilases in plant metabolism, Phytochemistry, 69 (2008) 2655-2667.

634 [17] P.K. Agarwal, P. Agarwal, M.K. Reddy, S.K. Sopory, Role of DREB transcription factors in
635 abiotic and biotic stress tolerance in plants, Plant Cell Reports, 25 (2006) 1263-1274.
636 [18] C.G. Bartoli, F. Gomez, G. Gergoff, J.J. Guiamet, S. Puntarulo, Up-regulation of the
637 mitochondrial alternative oxidase pathway enhances photosynthetic electron transport under
638 drought conditions, Journal of Experimental Botany, 56 (2005) 1269-1276.
639 [19] I.P. Generozova, S.N. Maevskaya, A.G. Shugaev, The inhibition of mitochondrial metabolic
640 activity in etiolated pea seedlings under water stress, Russian Journal of Plant Physiology, 56
641 (2009) 38-44.
642 [20] O.K. Atkin, D. Macherel, The crucial role of plant mitochondria in orchestrating drought
643 tolerance, Annals of Botany, 103 (2009) 581-597.
644 [21] I. García, J.M. Castellano, B. Vioque, R. Solano, C. Gotor, L.C. Romero, Mitochondrial β-
645 cyanoalanine synthase is essential for root hair formation in *Arabidopsis thaliana*, Plant Cell, 22
646 (2010) 3268-3279.
647 [22] A. Matsui, J. Ishida, T. Morosawa, Y. Mochizuki, E. Kaminuma, T.A. Endo, M. Okamoto,
648 E. Nambara, M. Nakajima, M. Kawashima, M. Satou, J.M. Kim, N. Kobayashi, T. Toyoda, K.
649 Shinozaki, M. Seki, Arabidopsis transcriptome analysis under drought, cold, high-salinity and
650 ABA treatment conditions using a tiling array, Plant and Cell Physiology, 49 (2008) 1135-1149.
651 [23] Y. Hatzfeld, A. Maruyama, A. Schmidt, M. Noji, K. Ishizawa, K. Saito, β-Cyanoalanine
652 synthase is a mitochondrial cysteine synthase-like protein in spinach and Arabidopsis, Plant
653 Physiology, 123 (2000) 1163-1171.

[24] A. Hussain, C.R. Black, I.B. Taylor, J.A. Roberts, Does an antagonistic relationship between ABA and ethylene mediate shoot growth when tomato (Lycopersicon esculentum Mill.) plants encounter compacted soil?, Plant Cell and Environment, 23 (2000) 1217-1226.

[25] D.J.G. Gowing, W.J. Davies, H.G. Jones, A positive root-sourced signal as an indicator of soil drying in apple *Malus* X *domestica*-Borkh., Journal of Experimental Botany, 41 (1990) 1535-1540.

[26] E.S. Wurtele, B.J. Nikolau, E.E. Conn, Tissue distribution of beta-cyanoalanine synthase in leaves, Plant Physiology, 75 (1984) 979-982.

[27] M.A. Else, M.B. Jackson, Transport of 1-aminocyclopropane-1-carboxylic acid (ACC) in the transpiration stream of tomato (Lycopersicon esculentum) in relation to foliar ethylene production and petiole epinasty, Aust J Plant Physiol, 25 (1998) 453-458.

[28] M.C. López-Martín, M. Becana, L.C. Romero, C. Gotor, Knocking out cytosolic cysteine synthesis compromises the antioxidant capacity of the cytosol to maintain discrete concentrations of hydrogen peroxide in Arabidopsis, Plant Physiology, 147 (2008) 562-572.

[29] N.K. Boardman, Comparative photosynthesis of sun and shade plants, Annual Review of Plant Physiology, 28 (1977) 355-377.

[30] W.K. Yip, S.F. Yang, Ethylene biosynthesis in relation to cyanide metabolism, Botanical Bulletin of Academia Sinica, 39 (1998) 1-7.

[31] J.M. Smith, R.N. Arteca, Molecular control of ethylene production by cyanide in *Arabidopsis thaliana*, Physiologia Plantarum, 109 (2000) 180-187.

[32] K. Oracz, H. El-Maarouf-Bouteau, R. Bogatek, F. Corbineau, C. Bailly, Release of sunflower seed dormancy by cyanide: cross-talk with ethylene signalling pathway, Journal of Experimental Botany, 59 (2008) 2241-2251.

[33] S. Wilkinson, W.J. Davies, Drought, ozone, ABA and ethylene: new insights from cell to plant to community, Plant Cell and Environment, 33 (2010) 510-525.

[34] X. Hu, A. Zhang, J. Zhang, M. Jiang, Abscisic acid is a key inducer of hydrogen peroxide production in leaves of maize plants exposed to water stress, Plant and Cell Physiology, 47 (2006) 1484-1495.

[35] T. Meyer, M. Burow, M. Bauer, J. Papenbrock, Arabidopsis sulfurtransferases: investigation of their function during senescence and in cyanide detoxification, Planta, 217 (2003) 1-10.

[36] R. Jenrich, I. Trompetter, S. Bak, C.E. Olsen, B.L. Moller, M. Piotrowski, Evolution of heteromeric nitrilase complexes in Poaceae with new functions in nitrile metabolism, Proceedings of the National Academy of Sciences of the United States of America, 104 (2007) 18848-18853.

[37] V. Kriechbaumer, W.J. Park, M. Piotrowski, R.B. Meeley, A. Gierl, E. Glawischnig, Maize nitrilases have a dual role in auxin homeostasis and beta-cyanoalanine hydrolysis, Journal of Experimental Botany, 58 (2007) 4225-4233.

[38] P.J. Lea, L. Fowden, Amino acid substrate specificity of asparaginyl-, aspartyl- and glutaminyl-tRNA synthetase isolated from higher plants, in, 1973, pp. 1903-1916.

[39] A.J.M. Howden, C. Jill Harrison, G.M. Preston, A conserved mechanism for nitrile metabolism in bacteria and plants, Plant Journal, 57 (2009) 243-253.

[40] K. Grossmann, A role for cyanide, derived from ethylene biosynthesis, in the development of stress symptoms, Physiologia Plantarum, 97 (1996) 772-775.