

2-2013

# The $\beta$ -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

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## Recommended Citation

Machingura, Marylou, Sidibe, Aissatou, Wood, Andrew J and Ebbs, Stephen. "The  $\beta$ -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.

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1 **TITLE: The  $\beta$ -cyanoalanine pathway is involved in the response to water deficit in**  
2 *Arabidopsis thaliana*

3

4 Running head: The  $\beta$ -cyanoalanine pathway and water deficit in Arabidopsis

5

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

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8 Department of Plant Biology and Center for Ecology, Southern Illinois University Carbondale,

9 420 Life Science II, 1125 Lincoln Drive, Carbondale, IL 62901-6509 USA

10

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

12 **Abstract**

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

32 **Author keywords** *Arabidopsis thaliana*; Cyanide; Cyanoalanine synthase; Nitrilase; Water  
33 deficit

34 **1. Introduction**

35 Cyanide is produced by higher plants via multiple metabolic pathways. The two most  
36 prevalent sources of endogenous cyanide are the turnover of cyanogenic glycosides or  
37 cyanolipids [1] and ethylene biosynthesis [2, 3]. While formation of cyanogenic glycosides and  
38 cyanolipids is limited to ~2,500 plant species, ethylene biosynthesis is the ubiquitous source of  
39 endogenous cyanide in plants. Ethylene in higher plants is synthesized by oxidation of 1-amino-  
40 cyclopropane-1-carboxylic acid (ACC) by ACC oxidase (ACO)(EC 1.14.17.4), releasing  
41 hydrogen cyanide and carbon dioxide as co-products [2]. The  $\beta$ -cyanoalanine synthase pathway  
42 is the principle pathway for cyanide detoxification in plants. In the first step of the pathway  
43 catalyzed by  $\beta$ -cyanoalanine synthase (EC 4.4.1.9), cyanide reacts with cysteine to form  $\beta$ -  
44 cyanoalanine, releasing bisulphide [4]. The second step is mediated by a dual function nitrile  
45 hydratase/nitrilase (EC 3.5.5.1) designated in Arabidopsis as nitrilase 4 (NIT4). The NIT4  
46 enzyme catalyzes reactions that convert cyanoalanine to either asparagine or aspartate in  
47 conjunction with ammonia, respectively [5].

48 Recent evidence suggests a role of the  $\beta$ -CAS pathway in plant response and acclimation  
49 to abiotic stress. For example, when birch plants were exposed to ozone, there was an increase in  
50 ethylene production and increased  $\beta$ -CAS transcript abundance [6]. Tissue cyanide  
51 concentrations and  $\beta$ -CAS activity showed concomitant increases when tobacco (*Nicotiana*  
52 *tabacum* L.) plants were subjected to water deficit [7]. Cyanide concentration decreased after  
53 two days of stress due to the action of  $\beta$ -CAS enzyme, even though ethylene production was still  
54 high. The conclusion offered by the authors was that water deficit induced cyanide production,

55 and that removal of cyanide by the  $\beta$ -CAS enzyme contributed to the response, if not also the  
56 tolerance, to water deficit.

57         The overarching goal of the work here was to further investigate the contribution of the  
58  $\beta$ -cyanoalanine synthase pathway to the response to water deficit. *Arabidopsis thaliana* (Col-0)  
59 and three SALK T-DNA insertion lines for the three genes directly associated with the pathway  
60 [5, 8], namely O-acetylserine(thiol) lyase (OASTL) (EC 2.5.1.47) (*AtCysAI*),  $\beta$ -cyanoalanine  
61 synthase (*AtCysCI*), and nitrilase 4 (*AtNIT4*), were used. There were two main objectives for  
62 this study. The first was to examine the response of the pathway in wild type *Arabidopsis* Col-0  
63 to the magnitude and duration of water deficit. Since ethylene production depends on the severity  
64 and duration of stress [9], the concomitant production of cyanide should also be variable.  
65 Activity of  $\beta$ -CAS may also vary in order to maintain cyanide at a steady state concentration  
66 below that potentially inhibitory for metabolism [3]. The second objective was to examine how  
67 interruption of single genes encoding enzymes of the pathway influenced the ability of  
68 *Arabidopsis* to metabolize cyanide and respond to water deficit. Given that  $\beta$ -CAS (*AtCysCI*,  
69 *Bsas* 3;1) and one OASTL (*AtCysAI*, *Bsas* 1;1) are the only enzymes with  $\beta$ -cyanoalanine  
70 synthase activity under physiological conditions [8], the efforts here sought to determine if both  
71 of these genes were required for the function of the pathway *in vivo* or if one gene alone was  
72 sufficient to maintain cyanide assimilation under water deficit conditions. As a single gene in  
73 *Arabidopsis* [5], the use of the nitrilase 4 SALK line allows for an examination of the impact of  
74 water deficit when the second step of the pathway is specifically interrupted.

75

76

77        **2. Results**

78        *2.1 Response of the  $\beta$ -cyanoalanine pathway to the magnitude and duration of water deficit*

79            Acute water deficit significantly increased the relative abundance of *AtCysCI* transcript,  
80 but by less than two-fold overall in whole *Arabidopsis* seedlings (Figure 1A). A two-fold  
81 increase in  $\beta$ -CAS-like enzyme activity was also observed in response to acute water deficit  
82 (Figure 1B). While there was a significant increase in expression of *AtCysCI* transcript and  $\beta$ -  
83 CAS-like enzyme activity, there was no significant difference in tissue cyanide content between  
84 control plants and plants exposed to water deficit treatments (data not shown). No significant  
85 difference was observed also in expression of *AtNIT4* transcript in response to water deficit as  
86 compared to control plants (data not shown).

87            The acute water deficit treatment was repeated with plants removed from the growing  
88 medium and allowed to air dry for 20 min intervals up to 60 min. As expected, RWC of the  
89 tissues fell significantly over the course of 1 h, from 96% to ~85% after 20 min and to ~64% by  
90 60 min. When the tissues were analyzed for cyanide concentration and  $\beta$ -CAS-like activity, both  
91 were observed to follow similar, transient changes (Figure 2). Both tissue cyanide and  $\beta$ -CAS-  
92 like activity increased at 20 min by 2.5- and 10-fold higher than the basal levels, respectively,  
93 although these changes were not significant. After 20 min, the values for each decreased so that  
94 at 60 min, both tissue cyanide concentration and  $\beta$ -CAS-like activity were significantly lower  
95 than the peak at 20 min. To confirm that the patterns in the data represented specific changes in  
96 tissue cyanide and  $\beta$ -CAS-like activity and not changes driven by differences in fresh weight  
97 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<sup>-1</sup> (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  $\beta$ -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  $\beta$ -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  $\beta$ -  
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  $\beta$ -CAS pathway to water deficit was generally similar regardless of the magnitude and

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

125

## 126 *2.2 The pathway response is localized to stressed tissue*

127 To demonstrate that the response of the  $\beta$ -cyanoalanine synthase pathway to water deficit  
128 stress was a phenomenon localized to tissues experiencing the water deficit, a split-root  
129 technique was employed. As anticipated, discontinuing watering led to a significant decrease in  
130 the volumetric soil water content, reaching a value of 20% in root compartment 2 as compared to  
131 58% in the watered root compartment 1. The volumetric soil water content of the two root  
132 compartments for control plants were each >80%. These differences in soil water between the  
133 two equally-watered compartments for control plants and the watered side of the pots receiving a  
134 water deficit treatment suggested that water was drawn more heavily from the watered pots  
135 opposite the water deficit pots, allowing the plant to compensate for the deficit in the dry  
136 compartment. Despite these differences in soil hydration, the RWC of the shoots of all plants  
137 was unaffected, with no significant difference between the treated and control plants (88 and  
138 85%, respectively). At harvest, there was no significant difference in the root tissue cyanide  
139 concentration between the dry and wet compartments or in comparison to the two wet  
140 compartments of the control split-root. The tissue cyanide concentration was significantly higher  
141 in shoots of plants that had a dry root compartment as compared to control plants with both root



142 compartments well watered (Figure 5). While there was no significant difference in  $\beta$ -CAS-like  
143 activity in the shoots between treatments, enzyme activity in the roots experiencing water deficit  
144 was significantly greater than well-watered roots (Figure 6). These trends in the data did not  
145 change when the data were recalculated as former in pmol cyanide or as nmol product  $\text{h}^{-1}$  (Figure  
146 S2).

147

### 148 *2.3 Response of the $\beta$ -cyanoalanine synthase pathway mutants to water deficit*

149 Consistent with studies by others [8], the established SALK lines for  $\beta$ -CAS (*AtCysC1*)  
150 and the cytosolic OASTL (*AtCysA1*) were shown to be knockdowns for enzyme activity (Figure  
151 7A). The  $\beta$ -CAS-like activity decreased by 22% for *AtCysA1* and 42% for *AtCysC1* lines as  
152 compared to Col-0 plants. The magnitude of the decrease for each line was similar to the 33%  
153 and 50% decrease, respectively, observed previously for these SALK lines [8]. A surprising  
154 observation made here and elsewhere [15, 16] was that the interruption of *AtNIT4* had a  
155 concomitant influence on  $\beta$ -CAS-like activity, with a decrease in activity of 71% in comparison  
156 to Col-0 plants (Figure 7A). Analysis of *AtNIT4* enzyme activity for the *AtCysA1* and *AtCysC1*  
157 lines was not significantly different from Col-0 (Figure 7B). The *AtNIT4* mutant line did display  
158 nitrile hydratase activity although at a rate significantly lower (~20%) than for Col-0 and the  
159 other two lines (Figure 8A). There was however no significant difference in nitrilase activity  
160 under control conditions (4.1 and 3.6 nKat  $\text{mg}^{-1}\text{protein h}^{-1}$  respectively for Col-0 and *AtNIT4*) or  
161 in response to KCN treatment (3.9 and 4.0 nKat  $\text{mg}^{-1}\text{protein h}^{-1}$ , respectively). Activity of this  
162 enzyme was not cyanide inducible in either line (Figure 8B), indicating that the presence of

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

165 Growth parameters (e.g., leaf area and biomass) were similar for the Col-0 plants and the  
166 *AtCysA1* mutant line under nominal conditions. Leaf area and fresh weight biomass for *AtCysC1*  
167 were significantly different from Col-0 while the dry weight biomass was not different. There  
168 were no significant differences in these parameters between the *AtCysA1* and *AtCysC1* lines  
169 under well-watered conditions. The *AtNIT4* plants showed significantly less leaf area and  
170 biomass than the Col-0 and *AtCysA1* line under nominal growth conditions but none of the values  
171 were significantly different from the *AtCysC1* line (Figure 9). For the *AtNIT4* line, the plants  
172 also displayed a smaller rosette diameter and leaf size, but not a decrease in leaf number  
173 compared to the other lines (Figure S3). On imposition of water deficit there was a statistically  
174 significant decrease in RWC for all lines and the magnitude of the decrease was the same for  
175 each line. Well-watered controls across all four lines had a RWC of 83% while the plants  
176 deprived of water had a RWC of 71%. Although the magnitude of the decrease in RWC was the  
177 same for all the lines, the effect of this mild water deficit in terms of the subsequent effect on  
178 growth varied for each of the mutants. There was a significant decrease in leaf surface area and  
179 fresh weight biomass after 15 d of water deficit for each line as compared to corresponding well-  
180 watered plants of the same line (Figure 9). The magnitude of the decrease was not significantly  
181 different between the *AtCysA1* mutant line and the Col-0 plants (Table 1). The *AtCysA1* mutant  
182 had a 35% decrease in leaf area. The *AtCysC1* mutant had 50% reductions in leaf area and  
183 significantly different from Col-0. The *AtNIT4* mutant was the most sensitive, with the same  
184 parameter decreasing by 66%. For dry weight biomass, the *AtCysA1* showed a small decrease

185 while the *AtCysCI* mutant plants were slightly larger (Table 1). The *AtNIT4* plants decreased  
186 significantly in dry weight biomass (25.4%) relative to well watered plants of the same line. The  
187 magnitude of decrease in dry weight biomass was however greatest for the Col-0 plants at 42%  
188 (Table 1).

189 The relative chlorophyll content of Col-0, the *AtCysAI* and *AtCysCI* lines all showed a  
190 significant increase with time as compared to well-watered plants of the same line (Figure 10).  
191 The measured values for these lines were higher than the control values, representing relative  
192 chlorophyll values greater than the well watered plants of the same line. The *AtCysAI* and  
193 *AtCysCI* mutants were however not different from Col-0 except at the final time point where the  
194 *AtCysAI* line showed the greatest relative chlorophyll content. Contrary to the above, the  
195 relative chlorophyll content of the *AtNIT4* mutant showed an initial decline in the first 5 d after  
196 initiation of water deficit and stabilized thereafter at 90% of the untreated plants of the same line.  
197 At the end of the water deficit period (day 15), the only plants showing an appreciable increase  
198 in tissue cyanide was the *AtNIT4* mutant under water deficit (Figure 11). There was no difference  
199 in  $\beta$ -CAS-like activity between the four lines or between treatments (data not shown). As  
200 compared to previous experiments with Col-0 only, the magnitude of the values for  $\beta$ -CAS  
201 activity for all lines (Col-0 and the mutants) was lower, in these plants which seemed reasonable  
202 since the cyanide content had stabilized at low levels.

203

### 204 **3. Discussion**

205 Water deficit is a limiting factor for plant growth and development. Plants utilize a  
206 number of mechanisms at the cellular level to stave off the negative effects of water deficit,

207 including osmotic adjustment, synthesis of compatible solutes, increased synthesis of abscisic  
208 acid, and upregulated expression of DREB transcription factors and late embryogenesis proteins  
209 [17]. Plants also need energy under stress and the relative importance of the mitochondria as the  
210 principal organelle for ATP production increases [18]. While other work has shown that under  
211 intense water stress, mitochondria activity decreases [19], some have reported an increase in  
212 mitochondrial function under moderate, slow onset stress [20]. Water stress generates ethylene,  
213 and would also therefore generate cyanide. Since a principle target of cyanide inhibition is the  
214 terminal oxidase of the mitochondria, cyanide detoxification by the  $\beta$ -CAS pathway is likely  
215 necessary to help offset the negative effects of cyanide on the mitochondria [21], and therefore  
216 contribute to the response to water deficit.

217         The first objective for this study was to establish how responsive the  $\beta$ -CAS pathway is  
218 to the magnitude and duration of water deficit. In *Arabidopsis* the *AtCysC1* gene shows high,  
219 constitutive expression that is not responsive to cyanide exposure or stress [22], hence the  
220 significant yet minor difference in transcript abundance even in response to an acute exposure to  
221 water deficit was not wholly unexpected (Figure 1). The high protein expression is perhaps  
222 necessary to compensate for the fact that the cysteine synthesis activity displayed by  $\beta$ -CAS  
223 represents a competing reaction for cyanide assimilation [23]. The generally parallel changes in  
224 tissue cyanide and  $\beta$ -CAS-like enzyme activity observed here in response to water deficit  
225 (Figures 2-4) are consistent with reports from a similar water deficit study with tobacco [7].  
226 Both studies showed a transient increase in  $\beta$ -CAS-like enzyme activity coinciding with a water  
227 deficit-induced spike in tissue cyanide. It was interesting to note that the pattern displayed for  
228 cyanide concentration and  $\beta$ -CAS-like activity was similar for each combination of water deficit

229 intensity and duration imposed on the Arabidopsis seedlings (Figures 2-4) suggestive of a  
230 coordinated response that includes metabolic components beyond the  $\beta$ -CAS pathway. With  
231 little evidence for an induction of *AtCysC1* gene, the results suggests that innate activity of the  
232 existing pool of enzymes in Arabidopsis may be sufficient to assimilate pulses of cyanide elicited  
233 during stress-induced cyanide synthesis. The same may be true for the NIT4 enzyme and may  
234 therefore explain the lack of a response at the level of transcription.

235         The split-root experiment further demonstrated that the response of the  $\beta$ -CAS pathway  
236 to water deficit was localized predominantly to the tissue directly subjected to the stress as  
237 illustrated by the significant increase in  $\beta$ -CAS-like activity in roots from the dry compartment as  
238 compared to the well watered compartment (Figure 6). Within the dry compartment, the  
239 imposition of water deficit would presumably promote a localized increase in stress ethylene  
240 which would in turn be expected to increase local activity of  $\beta$ -CAS. The tissue cyanide  
241 concentration from the split root experiment was ~two-fold higher as compared to the other  
242 drying techniques (Figure 5). Increased ethylene production was also reported in tomato plants  
243 treated as split-roots [24] suggesting that the splitting of the roots imposed an additional stress,  
244 perhaps mechanical. It was however, unexpected that the shoots of treated plants had higher  
245 cyanide concentrations as compared to the controls and this accumulation of cyanide was not  
246 accompanied by an increase in  $\beta$ -CAS activity (Figure 6). As reported in other split-root studies  
247 [e.g., 25] the RWC result here did not indicate water deficit stress in the shoots. The cyanide  
248 detected in that tissue would not immediately seem to arise as a collateral result of the root-level  
249 treatment since the shoots of the treated plants maintained their hydration by drawing the  
250 necessary water from the well-watered compartment. Two hypotheses may explain the source of

251 the cyanide detected in the shoots. The first is that cyanide was produced in the drying roots and  
252 moved by diffusion into the dead xylem cells resulting in its translocation to shoots *via* the  
253 transpiration stream. However, considering the volatile nature of the HCN molecule and the  
254 widespread detoxification system in all living tissues [26], an alternate explanation is that  
255 cyanide was synthesized in the shoots in response to chemical signals from roots. Studies have  
256 demonstrated chemical signaling between tissues upon perception of stress [e.g. 24]. In that  
257 study increased abscisic acid (ABA) and ethylene were reported in the xylem sap of split-root  
258 treated plants. It is also well established that production of ACC, the ethylene precursor is  
259 increased in response to stress and may be translocated in the xylem [27]. An increase in ACC  
260 production in the roots from the dry root compartment followed by translocation to the shoots  
261 may have thus resulted in increased ethylene production in shoots, and therefore cyanide  
262 production. Simultaneous analysis of ethylene and cyanide would be necessary in future studies  
263 to provide confirmation of this supposition.

264         The efforts to evaluate how the  $\beta$ -CAS mutants responded to the imposition of water  
265 deficit were complicated by the inherent differences in growth characteristics for the lines  
266 (Figure 9) and the specific response of each to the mild water deficit imposed (Table 1, Figures  
267 9-11). Collectively the decrease in RWC, dry weight biomass, and leaf area (Figure 9) all  
268 indicate that withholding water did affect the water status of all four lines. The progressively  
269 greater decrease in these values from the *AtCysA1* to *AtCysC1* and *AtNIT4* lines (Table 1)  
270 appears inversely related to the apparent importance of these genes and their encoded enzymes  
271 for the  $\beta$ -CAS pathway in that *AtCysC1* is responsible for a larger proportion of  $\beta$ -CAS enzyme  
272 activity than *AtCysA1* while *AtNIT4* acts at a critical bottleneck on the pathway. This

273 relationship is not fully supported though by the data for tissue cyanide and  $\beta$ -CAS enzyme  
274 activity. With the exception of the *AtNIT4* line, this mild water deficit did not produce an  
275 increase in  $\beta$ -CAS enzyme activity or an increase in tissue cyanide for the *AtCysAI* and *AtCysCI*  
276 lines (Figure 11). Even in the absence of an increase in cyanide in those compartments, the  
277 imposition of mild water deficit must have interacted in a different way with these two mutant  
278 lines to influence the change in leaf area and fresh weight biomass. The decrease in dry weight  
279 biomass for the Col-0 line further indicates that the change in water status did have a  
280 concomitant effect on growth of that line. The *AtCysAI* and *AtCysCI* lines did not show a  
281 comparable decrease in dry weight biomass, suggesting that each line was more robust than Col-  
282 0 in growth following the imposition of the mild water deficit. An increase in rosette size at the  
283 seedling stage was reported previously for the *AtCysAI* mutant [28], although for this study the  
284 growth enhancement was reflected through to maturity at 10 weeks. No statistically significant  
285 evidence of this was observed here.

286         While these two lines and Col-0 showed an increase in relative chlorophyll content under  
287 water deficit as compared to well-watered plants of the same line, these results may not  
288 specifically indicate a change in chlorophyll synthesis. The apparent change in chlorophyll  
289 could be due to the reduced leaf area since changes in chloroplast size, number, and/or  
290 chlorophyll content may be independent of changes in leaf area [29]. The divergence at Day 15  
291 (Figure 10) with a sustained increase demonstrated by the *AtCysAI* line as compared to a sharp  
292 decrease for Col-0 and *AtCysCI* suggest the possible onset of more distinct differences between  
293 the two mutant lines in response to the mild water stress imposed.

294 In the absence of additional data, one speculative explanation for the differences in  
295 response of the *AtCysA1* and *AtCysC1* mutant may involve the interaction of the  $\beta$ -CAS pathway  
296 with signaling molecules such as ethylene, ABA, reactive oxygen species, and hydrogen sulfide.  
297 The interaction between cyanide and ethylene synthesis is well documented [3, 30], with cyanide  
298 enhancing expression of genes such as *ACS6* associated with ethylene synthesis [31] and  
299 displaying crosstalk with elements of the ethylene signaling pathway during germination of  
300 sunflower seed [32]. There is a growing body of evidence indicating interactions between  
301 ethylene and ABA in the control of growth under water stress [33]. Increased production of  
302 reactive oxygen species (ROS) under water deficit has been demonstrated [34] and these  
303 molecules are both sources of cellular damage and cellular signals. The ROS are thought to be  
304 important for regulating activity of the cytochrome and alternative oxidase pathways of  
305 mitochondrial electron transport. Cyanide also influences these two pathways via its inhibition  
306 of the terminal oxidase of the cytochrome pathway and may have direct roles in modulating  
307 production of ROS. There is emerging evidence of signaling roles of hydrogen sulfide in plant  
308 cells. As enzymes with dual function in cysteine synthesis and  $\beta$ -CAS enzyme activity,  
309 insertional mutations of either *AtCysA1* or *AtCysC1* could alter the regulation of both cyanide  
310 and hydrogen sulfide concentration. Given the numerous potential interactions of these two  $\beta$ -  
311 CAS pathway enzymes in this landscape of signaling molecules, a complex network of  
312 interactions could emerge that could influence growth in the presence of absence of mild water  
313 stress, stomatal closure, and by extension photosynthetic activity and photochemical quenching  
314 for PS II protection. Obviously a possible disruption to this signaling network and/or a shift in  
315 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  $\beta$ -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  $\beta$ -CAS activity (Figure 7 and 8). The knockdown in  $\beta$ -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  $\beta$ -cyanoalanine and decreased assimilation of  
333 cyanide. Accumulation of both cyanide and  $\beta$ -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  $\beta$ -

338 cyanoalanine as a substrate [5], there seem to be no published studies with *AtNIT4* mutants in  
339 Arabidopsis to demonstrate that a t-DNA insertion in this gene abolishes enzymatic activity. The  
340 nitrilase/nitrile hydratase from maize (*Zea mays*), sorghum (*Sorghum bicolor*) and tobacco are  
341 heterodimers [36]. Loss of one protein of the heterodimer may not completely abolish the  
342 enzyme activity but may decrease the overall rate and/or efficiency of enzymatic activity [36].  
343 Nonetheless, the sensitivity of *AtNIT4* plants is reflected in the magnitude of the reduction in leaf  
344 area and fresh weight biomass (Figure 9), and relative chlorophyll content (Figure 10). The small  
345 phenotype is comparable to what might be expected for an auxin-deficient mutant, especially  
346 since nitrilases are also involved in auxin biosynthesis [37]. However, given the purported  
347 substrate specificity of the *AtNIT4* protein for  $\beta$ -cyanoalanine [5], this argues against *AtNIT4* as  
348 an auxin-deficient mutant. If the *AtNIT4* is indeed a single gene in Arabidopsis responsible for  
349 hydrolysis of  $\beta$ -cyanoalanine, knocking down this gene should cause a build-up of cyanide  
350 (Figure 11) as well as  $\beta$ -cyanoalanine.  $\beta$ -cyanoalanine can be toxic to plants [8] via inhibition of  
351 Asparagine:tRNA synthetase [38] and inhibition of root growth [37, 39]. These effects could  
352 then be additive to or synergistic with the hypothesized cellular changes resulting from the  
353 decreased  $\beta$ -CAS activity in the *AtCysA1* or *AtCysC1* lines.

354 In conclusion, the study has shown that the  $\beta$ -CAS pathway in Arabidopsis Col-0 shows a  
355 transient response to water deficit stress. The pattern of response is similar even with differences  
356 in the magnitude and duration of stress. The results have shown that the response of the  $\beta$ -CAS  
357 enzyme is specific to the tissue directly experiencing the stress. This study has also shown  
358 enzymatic functional redundancy between the *AtCysA1* and *AtCysC1* proteins and supports prior  
359 results [8] demonstrating that *AtCysC1* makes a greater contribution to  $\beta$ -CAS activity than

360 *AtCysA1*. Insertional mutation of one of the corresponding genes does not appear to affect  
361 cyanide homeostasis under water deficit but does give rise to distinct differences in growth  
362 parameters associated with water status (i.e., leaf surface area and fresh weight biomass). Such  
363 results suggest a complex metabolic network involving the proteins encoded by *AtCysA1* and  
364 *AtCysC1*. The results from the *AtNIT4* line demonstrate the innate differences in growth of this  
365 line under both normal and water limited conditions. A functional sulfurtransferase pathway,  
366 which should theoretically be able to remove excess cyanide during water deficit, did not  
367 function as such in the *AtNIT4* insertional mutants. Given the modest reduction in nitrile  
368 hydratase activity in this line, further investigation will be needed to examine the contribution of  
369 the encoded enzyme to nitrilase and nitrile hydratase activity and potential *in vivo* redundancies  
370 that may exist. The degree to which  $\beta$ -CAS activity contributes to abiotic stress tolerance will  
371 also require further examination to establish the extent of cyanide production under other water  
372 deficit scenarios and in response to other abiotic stresses. The results obtained are relevant to  
373 both the fundamental study of abiotic stress in plants and to the herbicide industry in that the  
374 mode of action of auxinic herbicides is induction of ethylene biosynthesis and cyanide  
375 autotoxicity [40]. Tolerance to such herbicides has been linked to CAS activity suggesting both  
376 stress and herbicide tolerance may be provided in plants in part by the  $\beta$ -CAS pathway. An  
377 intriguing question for future study would be to examine how plants respond to simultaneous  
378 exposure to water deficit in cyanide contaminated soils. As tolerance to both stresses may  
379 require action of the  $\beta$ -CAS pathway, such studies could provide information on the capacity of  
380 the pathway to respond under more extreme conditions in the presence of multiple stresses. The  
381 complexity of the results obtained here and the interaction of the  $\beta$ -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 (*AtCysAI*, At4g14880, SALK\_72213),  $\beta$ -cyanoalanine synthase  
390 (*AtCysCI*, At3g61440, SALK\_22479) and nitrilase 4 (*AtNIT4*, At5g22300, SALK\_016289C)  
391 were obtained from TAIR ([www.Arabidopsis.org](http://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 4°C 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  $\sim 150 \mu\text{M m}^{-2} \text{s}^{-1}$ . 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<sub>3</sub>, 4 mM Ca(NO<sub>3</sub>)<sub>2</sub>,  
400 0.1 mM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, 50  $\mu\text{M}$  KCl, 12.5  $\mu\text{M}$  H<sub>3</sub>BO<sub>3</sub>, 1  $\mu\text{M}$  MnSO<sub>4</sub>, 1  $\mu\text{M}$  ZnSO<sub>4</sub>,  
401 0.5  $\mu\text{M}$  CuSO<sub>4</sub>, 0.1  $\mu\text{M}$  NiSO<sub>4</sub>, and 0.016  $\mu\text{M}$  (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub> [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  $\mu\text{M}$  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 $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -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

448 containing sterile sand. The plants were provided one week to acclimate to the new growing  
449 medium. During this recovery period, there were no signs of stress and new growth was evident.  
450 After the recovery period, the split-root systems were assigned to one of two treatment groups,  
451 control and treated. For the control plants, the two pots containing the split-root systems were  
452 each watered with 50 mL nutrient solution once per week and two applications of 50 mL of  
453 water twice per week. For the treated plants, water was completely withheld from one  
454 compartment, while the other compartment received the same watering pattern as used for the  
455 control plants. Plants were harvested on day 7 and separated into shoots and the roots from each  
456 pot. The relative water content of shoots, tissue cyanide concentration in roots and shoots, and  $\beta$ -  
457 CAS-like activity in roots and shoots were determined for these samples. There were four  
458 replicates of each treatment in this experiment with a replicate represented by a single split-root  
459 plant.

460

#### 461 *4.3 Response of the $\beta$ -cyanoalanine synthase pathway mutants to water deficit*

462 The decrease in  $\beta$ -cyanoalanine synthase-like activity and/or nitrilase/nitrile hydratase  
463 activity in the insertional mutants was demonstrated using the procedures described below.  
464 Plants were germinated and grown in plates under the same growth conditions as above. After  
465 three weeks, seedlings were transferred from MS plates to hydroponic culture and allowed to  
466 grow for two additional weeks. Whole plants were harvested, rinsed, snap-frozen in liquid  
467 nitrogen and stored at  $-80^{\circ}\text{C}$  prior to analysis. The assay for  $\beta$ -cyanoalanine synthase-like  
468 activity was conducted on whole seedlings and each line was replicated four times. Similarly,  
469 nitrilase/nitrile hydratase activity was determined in Col-0 and the *AtNIT4* mutants.

470 Nitrilase/nitrile hydratase activity was determined in untreated plants of each line and in plants  
471 treated for 36 h with 1 mM cyanide (as KCN). Three week old seedlings were transferred from  
472 MS plates to hydroponic culture as above, and grown for three weeks. The 36 h cyanide  
473 treatment was then imposed on half the Col-0 and *AtNIT4* plants. The composition of the  
474 hydroponic cyanide treatment solution was similar to the basal nutrient solution except that the  
475 phosphate concentration was lowered to 0.02 mM, MnSO<sub>4</sub> concentration was lowered to 1.6 μM,  
476 and iron was omitted to preclude formation of metal cyanide solids [10]. Aeration of the nutrient  
477 solution was discontinued to reduce cyanide volatilization [10]. After 36 h of exposure, whole  
478 seedlings were harvested, rinsed, snap-frozen in liquid nitrogen, and stored at -80°C until the  
479 nitrile hydratase/nitrilase assays were performed. Each line and treatment had four replicates.

480 To examine how the putative lines for β-cyanoalanine synthase (*AtCysCI*), the cytosolic  
481 OASTL (*AtCysAI*), and the nitrile hydratase/nitrilase (*AtNIT4*) responded to water deficit, seeds  
482 of each line were germinated and grown for three weeks on MS plates and then three weeks in  
483 1:1 sterile perlite and vermiculite. A subset of plants of each line was harvested and the whole  
484 plants were snap-frozen in liquid nitrogen for determination of basal β-CAS-like activity. For  
485 the remaining plants, watering was withheld for 15 days and the physiological status of the plants  
486 was monitored by measuring relative chlorophyll content. The plants were harvested and leaf  
487 area was measured (Li-COR leaf area meter, model LI-3000 A, Lincoln NE USA). The tissues  
488 were then subdivided into two subsamples. The first was used for the determination of relative  
489 water content. Using the dry weight and the relative water content of this subsample, the total  
490 dry weight of the tissue at harvest was calculated. The second tissue subsample was rinsed with



491 deionized water to remove adhering soil particles, and snap-frozen for storage as described above  
492 for determination of tissue cyanide concentration and  $\beta$ -CAS activity.

493

#### 494 *4.4 Biochemical and physiological measurements*

##### 495 4.4.1 Quantitative RT-PCR

496 To assess expression of genes of interest, total RNA was extracted from whole seedlings using  
497 the E.Z.N.A Plant RNA Kit (OMEGA Bio-tek, Norcross, GA, USA) according to the  
498 manufacturer's instructions. The RNA concentration was determined using a Nanodrop  
499 spectrophotometer, (NanoDrop ND-1000, Wilmington, DE, USA). First strand cDNA was  
500 synthesized from 1  $\mu$ g of RNA using the SuperScript<sup>TM</sup> III First Strand Synthesis System for RT-  
501 PCR (Invitrogen, CA, USA) according to the manufacturer's instructions. Real-time PCR was  
502 conducted using gene specific primers and the Chromo4 real-time PCR system (Bio-Rad  
503 Laboratories, Hercules, CA, USA). Quantification of transcript abundance was achieved using a  
504 standard curve which related DNA concentration to the Ct value [12]. Expression of genes was  
505 normalized to the ubiquitin 10 gene (At4g05320) and melting curves were used to confirm that  
506 only a single transcript was being amplified.

507

##### 508 4.4.2 Measurement of plant tissue cyanide

509 Tissue cyanide content was determined using a microdiffusion technique [13] to consist  
510 of a 50 mL conical centrifuge tube and a 5 mL glass tube as a gas trap. Plants were ground under  
511 liquid nitrogen and then ground again in an extraction buffer (50 mM of  $K_2HPO_4$ , pH 8.9) using  
512 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<sub>2</sub>HPO<sub>4</sub> 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<sub>2</sub>SO<sub>4</sub> 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<sub>2</sub>, 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

535 sealed vials at 26° C for 20 min. The reaction was terminated with the addition of 0.5 mL of  
536 acidic dye precursor reagent (15 mM N,N-dimethyl-1,4-phenylenediamine dihydrochloride, 3  
537 mM ferric chloride, 4.2 mM HCl). After a 20 min period of color development, methylene blue  
538 formation was measured spectrophotometrically at 745 nm. Enzyme activity data was  
539 normalized to the protein concentration.

540

#### 541 4.4.4 Colorimetric determination of nitrilase and nitrile hydratase activity

542 Nitrilase and nitrile hydratase activity was assayed via determination of ammonia  
543 according to Piotrowski et al. [5]. Briefly, 1 g (FW) of tissue was ground in liquid nitrogen and  
544 then in 3 ml of 100 mM KH<sub>2</sub>PO<sub>4</sub> buffer, pH 8.9. The homogenate was centrifuged at 4000 g for  
545 15 min at 4°C. The supernatant was decanted and centrifuged again for 20 min to obtain a crude  
546 extract. Total protein in the extract was determined as indicated above. For determination of  
547 background ammonia in the extract, an aliquot containing 100 µg of total protein was heat  
548 denatured in boiling water for 10 min and then incubated with substrate (3 mM β-cyanoalanine  
549 in 0.05 M potassium phosphate buffer, pH 8.9) in a total volume of 1 mL. Nessler's reagent  
550 (1.33 mL) was added and after 10 min of color development the absorption was read at 480 nm.  
551 A second aliquot of the crude extract containing 100 µg of total protein was incubated with the  
552 same substrate in a total volume of 1 mL for 30 min at 30°C after which the sample was boiled at  
553 100°C for 10 min to stop the reaction. For parallel determination of nitrilase and hydratase  
554 activity, two aliquots, each 0.1 mL were taken from the reaction mixture and each was diluted to  
555 1 mL with deionized water. To one sample, 1.33 mL Nessler's reagent was added for  
556 determination of ammonia resulting from the nitrilase activity. The second sample was incubated

557 with 0.25 units of asparaginase at 37°C for 30 min, allowing for determination of ammonia  
558 resulting from both nitrilase and nitrile hydratase activity. After incubation, 1.33 mL Nessler's  
559 reagent was added and both samples were read at 480 nm. Nitrile hydratase activity expressed as  
560 the difference between ammonia produced from the combined activity of the two enzymes and  
561 the activity of nitrilase [5].

562

#### 563 4.4.5 Relative water content

564 Samples were immediately weighed after harvest to determine fresh weight (FW). The samples  
565 were placed in the dark overnight in vials containing DI water at 4°C and then weighed to  
566 determine turgid weight (TW). The samples were oven dried to constant mass at 55°C and the  
567 dry weight (DW) was obtained. Relative water content (RWC) was determined according to Barr  
568 and Weatherley [14]:

$$569 \quad RWC (\%) = \frac{FW - DW}{TW - DW} * 100$$

570

#### 571 4.4.6 Relative chlorophyll content

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

574

#### 575 *4.5 Statistical analyses*

576 Data from the experiments using only Col-0 plants were analyzed using SAS package Version  
577 9.1 as one-way ANOVA with Tukey's test used for post hoc analysis. Data from experiments  
578 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.

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