Plant Mol Biol (2007) 64:205–217
DOI 10.1007/s11103-007-9145-z

Altered ABA, proline and hydrogen peroxide in an Arabidopsis glutamate:glyoxylate aminotransferase mutant

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Received: 7 December 2006 / Accepted: 28 January 2007 / Published online: 23 February 2007

Abstract Plant responses to abiotic stress are determined both by the severity of the stress as well as the metabolic status of the plant. Abscisic acid (ABA) is a key component in integrating these various signals and controlling downstream stress responses. By screening for plants with decreased *RD29A:LUC* expression, we isolated two alleles, *glutamate:glyoxylate transferase1-1* (*ggt1-1)* and *ggt1-2*, of a mutant with altered ABA sensitivity. In addition to reduced ABA induction of *RD29A*, *ggt1-1* was altered in ABA and stress regulation of *Δ^1-pyrroline-5-carboxylate synthase*, *proline dehydrogenase* and *9-cis-epoxycarotenoid dioxygenase 3*, which encode enzymes involved in Pro and ABA metabolsim, respectively. *ggt1-1* also had altered ABA and Pro contents after stress or ABA treatments while root growth and leaf water loss were relatively unaffected. A light-dependent increase in H_2O_2 accumulation was observed in *ggt1-1* consistent with the previously characterized role of GGT1 in photorespiration. Treatment with exogenous H_2O_2, as well as analysis of a mutant in *nucleoside diphosphate kinase 2* which also had increased H_2O_2 content but is not involved in photorespiration or amino acid metabolism, demonstrated that the greater ABA stimulation of Pro accumulation in these mutants was caused by altered H_2O_2 content as opposed to other metabolic changes. The results suggest that metabolic changes that alter H_2O_2 levels can affect both ABA accumulation and ABA sensitivity.

Keywords Abiotic stress · Abscisic acid · Glutamate:glyoxylate aminotransferase · Hydrogen peroxide · Nucleoside diphosphate kinase 2 · Proline

Abbreviations

ABA	Abscisic acid
COR15	Cold responsive 15
GGT1	Glutamate:glyoxylate transferase 1
NCED3	9-*cis*-Epoxycarotenoed dioxygenase 3
NDPK2	Nucleoside diphosphate kinase 2
P5CS1	Δ^1-Pyrroline-5-carboxylate synthase 1
PEG	Polyethylene glycol 8000
ProDH	Proline dehydrogenase
QPCR	Quantitative real-time reverse transcriptase PCR
RAB18	Responsive to ABA18
RD29A:LUC	RD29A (responsive to dessication29A) promoter controlling expression of the luciferase coding region
ROS	Reactive oxygen species
SA	Salicylic acid
SHMT1	Serine-hydroxylmethyl transferase

Electronic supplementary material The online version of this article (doi:10.1007/s11103-007-9145-z) contains supplementary material, which is available to authorized users.

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Introduction

Plants must sense and respond to many types of abiotic stresses and integrate these signals with internally generated signals which reflect factors such as metabolic status or development state. The combined output of these signals then modifies gene expression, metabolism and growth and development to adapt to the stress (Zhu et al. 2002). Many of these stress responses are regulated by abscisic acid (ABA). ABA accumulation occurs in response to various stresses that cause a decrease in tissue water content, such as dehydration induced by low water potential and salinity (Zeevaart 1999; Finkelstein et al. 2002; Xiong and Zhu 2003). ABA acts through a complex signaling network and most known ABA signaling components control only a subset of ABA responses (Finkelstein et al. 2002).

A preponderance of evidence supports ABA as a required component of normal stress response in plants (Finkelstein et al. 2002; Zhu 2002). However, the involvement of other signals and how these other signals interact with the ABA signal remain unanswered questions. These questions arise, at least in part, from a number of observations that have shown that ABA applied to unstressed plants often does not elicit the same response as ABA accumulation that occurs under stress conditions (Sharp et al. 1994; Imai et al. 1995; Verslues and Bray 2006). Control of ABA accumulation and sensitivity also appears to involve extensive feedback regulation (Xiong et al. 2002; Verslues and Bray 2006).

These observations highlight the importance of characterizing factors that may modulate responses to ABA under different conditions. One specific hypothesis worthy of investigation is that reactive oxygen species (ROS), generated by a number of metabolic mechanisms, can alter ABA sensitivity. There is specific genetic evidence that H_2O_2 generated by NADPH oxidases acts downstream of ABA in mediating stomatal closure (Zhang et al. 2001; Kwak et al. 2003; Bright et al. 2006). H_2O_2 may also be involved in signaling a variety of other hormone and stress responses (Chang et al. 2004; Mori and Schroeder 2004; Shin and Schachtman 2004; Kwak et al. 2006). Abitotic stress can cause many metabolic changes and is known to increase ROS levels in plant tissue (Foyer and Noctor 2003, 2005). ROS, along with ABA accumulation, has been proposed to be a key component of "cross tolerance" to multiple types of stress (Pastori and Foyer 2002). High levels of ROS lead to cellular damage and are involved in programmed cell death (Pavet et al. 2005; Van Breusegem and Dat 2006). However, increasing evidence suggests that ROS can have effects on signaling and metabolism at levels well below that required to cause general cellular damage. Thus even relatively small changes in the production of ROS, or the antioxidants needed to control ROS levels, can have significant effects on signal transduction. In addition, there is evidence that the specific chemical identity of the ROS or antioxidant molecules involved and the site of ROS production can determine the specificity of the response (op den Camp et al. 2003; Ball et al. 2004; Foyer and Noctor 2005; Mullineaux et al. 2006).

Of the ROS generated in plant tissue, H_2O_2 is the least reactive and has the potential to persist longer in the cell and may move between cellular compartments. H_2O_2 can be generated via several metabolic mechanisms: in the chloroplasts through the Mehler reaction of photosystem I (Asada 2006), in peroxisomes by the photorespiratory glycolate oxidase reaction (del Rio et al. 2006), in mitochondria through electron transport (Rhoads et al. 2006) and on the plasma membrane by NADPH-oxidases (Sagi and Fluhr 2006; Kwak et al. 2006). Under conditions of high light or where photorespiration is inhibited, the chloroplast and peroxisome will be the main source of H_2O_2; however, how an increase in H_2O_2 from these sources will affect other ROS dependent signaling, such as ABA signaling, is not well understood. In addition to these H_2O_2 sources, other genes, whose relation to H_2O_2 production are more obscure, also are involved in H_2O_2 signaling. One regulator which has been identified is nucleoside diphosphate kinase 2 (NDPK2). *ndpk2* mutants have increased ROS accumulation and sensitivity and NDPK2 physically interacts with mitogen-activated protein kinase 3 (MAPK3) and MAPK6 which are also known to be involved in H_2O_2 signaling (Moon et al. 2003).

Our laboratory has used a forward genetics approach to study the regulatory mechanisms controlling abiotic stress and ABA responses through identification of factors that affect the expression of Arabidopsis *RD29A* and other stress regulated genes. *RD29A* is known to be induced by cold, salinity, dehydration and exogenous ABA (Yamaguchi-Shinozaki and Shinozaki 1993; Ishitani et al. 1997; Xiong et al. 1999; Zhu 2002). Here we describe two mutant alleles (*ggt1-1* and *ggt1-2*) of glutamate:glyoxylate transferase 1 (GGT1) identified through screening for reduced *RD29A* induction by stress and ABA. GGT1 activity is required for photorespiration and *ggt1-1* also has altered gene expression, ABA content and proline accumulation in response to abiotic stress or ABA treatments. *ggt1* also has increased H_2O_2 content which

is likely to be the principal factor causing the altered ABA response. The relationship of the increased ABA-responsive proline accumulation and other *ggt1* phenotypes to H_2O_2 is further supported by similar changes in *ndpk2*, which also has increased H_2O_2 but is not involved in photorespiration or amino acid metabolism. The results suggest that many types of metabolic changes that increase tissue H_2O_2 levels are likely to alter ABA response.

Results

ggt1-1 has altered ABA- and stress-responsive gene expression

Previously, we have described large scale screening of ethyl methanesulfonate mutagenized plants containing a construct where the promoter of *RD29A* drives expression of a luciferase reporter gene (Ishitani et al. 1997). *ggt1-1* was isolated from this screen as a mutant with reduced ABA-responsive *RD29A* expression. In *ggt1-1*, expression of *RD29A:LUC* was approximately one-third of the wild-type level at 3 h after application of 100 μM ABA (Fig. 1a). Cold treatment, which does not induce a high level of ABA accumulation (Zhu et al. 2005), produced the same induction of *RD29A:-LUC* as in wild-type (Fig. 1b). Transfer of seedlings to 300 mM NaCl produced a similar increase in *RD29A* expression in mutant and wild-type until 5 h after transfer when *ggt1-1* had less expression than wild-type (Fig. 1c). At this time point, it is likely that the expression of *RD29A:LUC* was also affected by reduced ABA accumulation in the mutant (see below).

We used quantitative RT-PCR (QPCR) to quantify expression of the endogenous *RD29A* gene and found that it was also less highly expressed in *ggt1-1* than wild-type at 4 h after transfer of seedlings to -1.2 MPa polyethylene glycol (PEG), 150 mM NaCl or 100 μM ABA (Fig. 2a). The difference between *ggt1-1* and wild-type was particularly pronounced in the PEG-treated seedlings. This is likely because of the high level of induction of *RD29A* in wild-type seedlings and the reduced ABA accumulation in PEG-treated *ggt1-1* seedlings (see below). In the same experiments, two other commonly examined stress- and ABA-regulated genes, *RAB18* and *COR15*, did not have consistently altered expression in *ggt1-1* (Fig. 2b, c). This suggests that *ggt1-1* does not affect all of the mechanisms by which ABA regulates gene expression.

We did, however, observe altered expression of genes involved in Pro and ABA metabolism. Stress- and ABA-induced upregulation of Δ^1-pyrroline-5-carboxylate synthase (*P5CS1*), the rate-limiting enzyme in stress-induced Pro synthesis (Zhang et al. 1995) was reduced in *ggt1-1* (Fig. 2d). Similarly, down-regulation of proline dehydrogenase (*ProDH*), which catalyzes the catabolism of Pro (Kiyosue et al. 1996), was also impaired in *ggt1-1* (Fig. 2e). Likewise, expression of the gene encoding a key enzyme in stress-induced ABA biosynthesis, 9-*cis*-epoxycarotenoed dioxygenase 3 (*NCED3*; Tan et al. 2003) was also induced less strongly in *ggt1-1* than in wild-type (Fig. 2f). The change in ABA-induced *NCED3* expression in particular suggests that feedback regulation of ABA metabolism was affected in *ggt1-1*.

ggt1 is mutated in an aminotransferase required for photorespiration and has increased H_2O_2 content

During the initial characterization of *ggt1-1*, we noticed that *ggt1-1* plants had a pale green color and grew more slowly than wild-type (Supplementary Fig. 1). We also noted that although *ggt1-1* did not affect cold tolerance of plants kept in the dark, *ggt1-1* plants subjected to cold and light treatment exhibited severe leaf chlorosis (data not shown). This light sensitivity of *ggt1-1* could be alleviated either by reduced light intensity (data not shown) or by growth on media containing sucrose (Supplementary Fig. 1). These phenotypes suggested that the altered stress responses in *ggt1-1* may be associated with an impairment in photosynthesis or light sensing.

The light sensitivity of *ggt1-1* was used to isolate the mutated gene by a map based cloning strategy. Genetic analysis of *ggt1-1* showed it to be recessive (data not shown) and mapping narrowed the location of the mutation to BAC F26F24. Twenty-nine candidate genes were sequenced and a C to T change was found at position 46956 in *At1g23310* (Fig. 3a). After the *ggt1-1* mutation was identified, we discovered a T-DNA insertion mutant of the same gene with a similar phenotype (reduced *RD29A:LUC* expression and light sensitivity) in a T-DNA mutagenized population of *RD29A:LUC* [this T-DNA mutagenized population is described in Zhu et al. (2002)]. The T-DNA mutant line was designated as *ggt1-2* and has an insertion at position 46263 in the seventh exon of *At1g23310* (Fig. 3a). F_1 seedlings of a cross between the two mutants exhibited the same light sensitive phenotype as either parental line (Fig. 3b); thus establishing that the phenotype is caused by the same gene in both mutant lines.

ggt1-2 is almost certainly a null allele and this was confirmed by the absence of transcript in *ggt1-2* under conditions where the wild-type transcript is present

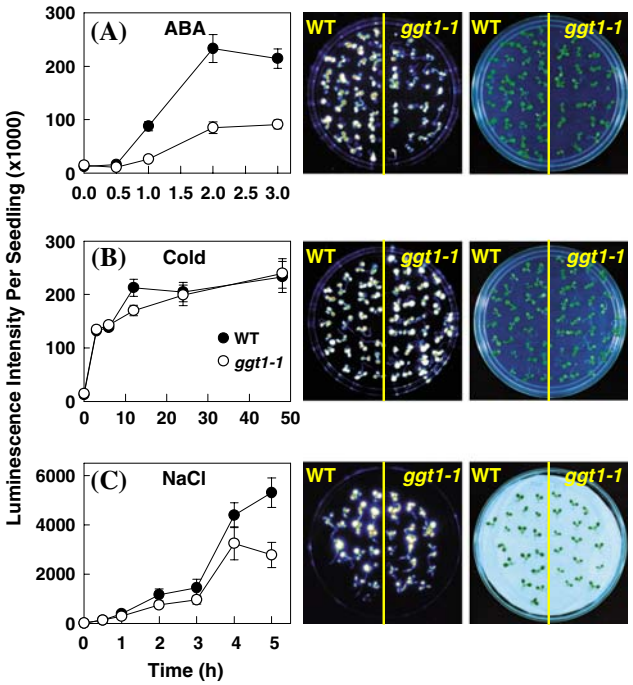


Fig. 1 RD29A:LUC expression in wild-type and *ggt1-1* seedlings. *Left panels* are luminescence intensities quantified from individual seedlings. Data are means ± SE ($n = 10$). *Middle panels* are luminescence images at the final time point for each treatment. *Right panels* are pictures of the same plate of seedlings used for the luminescence image shown in the *middle panel*. (**A**) Luminescence intensities after spraying seedlings with 100 μM (±)ABA. (**B**) Luminescence intensities after transfer of seedlings to 0°C. (**C**) Luminescence intensities after transfer of seedlings to 300 mM NaCl

(Fig. 3c). *ggt1-1* still produces full-length transcript (Fig. 3c) but the mutation changes the highly conserved Leu^{109} to Phe. To confirm that *ggt1-1* is also a null mutation, *GGT1* cDNAs were isolated from both the wild-type and *ggt1-1*; expressed as glutathione S-transferase fusion proteins in *Escherichia coli*; and assayed to show that the *ggt1-1* protein lacked the alanine aminotransferase activity of the wild-type protein (Supplementary Fig. 2). This analysis confirmed that the loss of aminotransferase activity was responsible for both the *ggt1-1* and *ggt1-2* phenotypes.

GGT1 [also referred to as GGAT1 by Igarashi et al. (2003, 2006)] has been shown to catalyze four amino transferase reactions (Liepman and Olsen 2001, 2003; Igarashi et al. 2003): Glu-glyoxylate aminotransfer, Ala-glyoxylate aminotransfer, Glu-pyruvate aminotransfer and Ala-2-ketoglutarate aminotransfer. GGT1 contains a type I peroxisomal targeting signal and has been shown to be localized to peroxisomes (Liepman and Olsen 2003). Strong evidence supports a role for GGT1 in photorespiratory metabolism. First, Glu-glyoxylate and/or Ala-glyoxoylate aminotransferases are required for photorespiration because these reactions generate glycine in the peroxisome (Wingler et al. 2000; Liepman and Olsen 2003). A light sensitive phenotype which can be alleviated by addition of sucrose, as seen in *ggt1*, is consistent with impaired photorespiration and Igarashi et al. (2003) have also shown that the impaired growth of a *ggt1* [referred to as *aoat1* in Igarashi et al. (2003)] knockout mutant can be rescued by high CO_2. Also consistent with a photorespiratory role of GGT1 is the observation that *GGT1* expression is down regulated in the dark [Fig. 3c; Liepman and Olsen (2004)]. Overexpression of GGT1 increases Ser, Gly and citrulline levels, demonstrating that it is also important in amino acid metabolism (Igarashi et al. 2006).

Identification of the mutated gene in *ggt1-1* and *ggt1-2* offered a ready explanation for the light sensitivity of these plants. It did not, however, offer as ready an explanation for the altered ABA and abiotic stress responses. One possibility is that the impaired photorespiration in *ggt1* could lead to increased ROS levels which could in turn influence ABA and stress responses. Increased ROS has been observed in a serine-hydroxylmethyl transferase (SHMT1) mutant (Moreno et al. 2005) and photorespiratory metabolism in the chloroplast and peroxisome is known to be a source of H_2O_2 (Foyer and Noctor 2005). We first investigated the ROS content of *ggt1-1* by staining wild-type and *ggt1-1* leaves with NBT or DAB to detect O_2^- or H_2O_2, respectively (Supplementary Fig. 3). The results indicated an increased level of H_2O_2 but little difference in O_2^-. This difference in H_2O_2 was further investigated by quantitative determination of seedling H_2O_2 using an Amplex Red assay (Shin and Schachtman 2004). Light grown *ggt1-1* seedlings, but not dark grown seedlings, had increased levels of H_2O_2 relative to wild-type (Fig. 3d), consistent with a photorespiratory origin of the increased H_2O_2 in *ggt1-1*.

Altered ABA and stress responses in *ggt1-1* are associated with increased H_2O_2

The increased H_2O_2 content of unstressed *ggt1-1* suggests a possibility as to how a mutation affecting photorespiration could also alter ABA responses. We performed further experiments to characterize the extent to which ABA responses are altered in *ggt1-1* and to test whether these alterations are indeed linked to the increased H_2O_2 content of *ggt1-1*. Because H_2O_2 has already been implicated in guard cell ABA signaling and we observed changes in gene expression in *ggt1-1* related to ABA and Pro metabolism (Fig. 2), we focused on experiments testing the ABA and stress

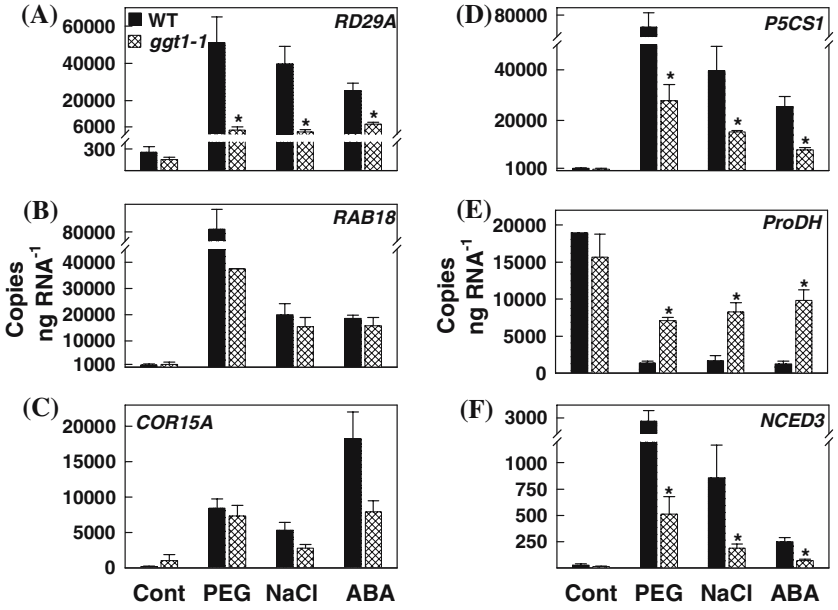


Fig. 2 Quantitative PCR analysis of *RD29A* (**A**), *RAB18* (**B**), *COR15* (**C**), Δ^1*-pyrroline-5-carboxylate synthase1* (**D**), *Pro dehydrogenase* (**E**) and *9*-cis-*epoxycarotenoid dioxygenase3* (**F**) expression in wild-type (*WT*) and *ggt1-1* seedlings. Seven-day-old seedlings were transferred from control plates to either fresh control plates or plates containing PEG (-1.2 MPa), NaCl (150 mM) or ABA (100 μM). Samples were collected at 4 h after transfer and used for RNA extraction and QPCR as described in Sect. ''Methods.'' Data are means ± SE (n = 3–5). *Asterisks* indicate a significant difference ($P \leq 0.05$) between the mutant and *WT* exposed to the same treatment

sensitivity and stress-induced ABA and Pro accumulation of *ggt1-1*.

To quantify the effect of *ggt1-1* on ABA sensitivity, we measured three ABA-regulated traits: seed germination, Pro accumulation and root elongation. Seed germination is the most commonly studied ABA-regulated trait and has been subjected to extensive genetic analysis (Finkelstein et al. 2002). *ggt1-1* was unaffected in ABA sensitivity of seed germination under these experimental conditions (Fig. 4a). This indicated either that *ggt1-1* did not have altered H_2O_2 levels in non-photosynthetic tissues such as germinating seeds or that H_2O_2 status does not have a major role in germination. To test whether altered H_2O_2 metabolism could affect seed germination, we quantified the response of wild-type seed germination to ABA in the presence of exogenous H_2O_2 (2 mM) or the H_2O_2 scavenger ascorbate (Asc, 2 mM). Exogenous H_2O_2 had little effect on germination; but, Asc blocked the inhibition of seed germination by high levels of ABA (Fig. 4b). These results were consistent with the lack of altered germination in *ggt1-1*. The Asc treatment indicated that H_2O_2 is required for the inhibition of seed germination by ABA. Thus, when Asc is present to remove H_2O_2 from the seed, the ability of ABA to inhibit germination is decreased. However, adding more H_2O_2 does not enhance the ABA response, either because some other signaling components are required to get more response to ABA or because the exogenous H_2O_2 does not have the same effect or does not reach the same cellular compartments within the seed, as endogenously produced H_2O_2.

Pro accumulation is also of interest because the response of Pro accumulation to ABA is clearly dependent not only on the amount of ABA but also on the plant's sensitivity, or competency, to respond to the ABA (Verslues and Bray 2006). Pro accumulation was higher in untreated *ggt1-1* seedlings and also increased to a higher than wild-type level upon ABA treatment (Fig. 4c). This is consistent with the fact that the Pro measurements were conducted in growing seedlings where *ggt1-1* had a significantly increased H_2O_2 content. H_2O_2 treatment (500 μM) of wild-type seedlings increased Pro content of untreated and ABA treated seedlings in a manner similar to that seen in *ggt1-1* (Fig. 4d), suggesting that this phenotype of *ggt1-1* may be caused by its increased H_2O_2 content. Conversely, treatment with the H_2O_2 scavenger Asc blocked ABA-induced Pro accumulation (Fig. 4d). The increased Pro accumulation after H_2O_2 treatment was different than what might have been predicted based on the decreased ABA induction of *P5CS1* and decreased repression of *ProDH* (Fig. 2d, e). This suggests that H_2O_2 may affect Pro accumulation by other mechanisms in addition to

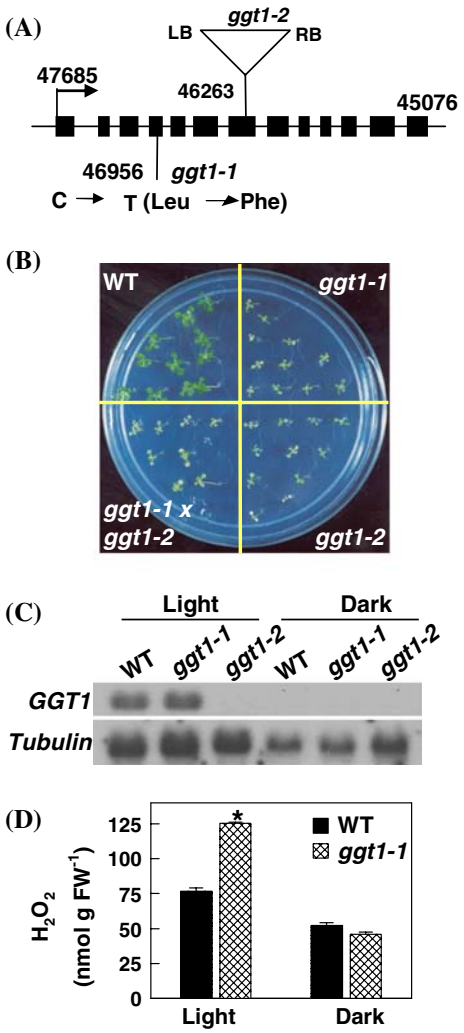


Fig. 3 *ggt1-1* and *ggt1-2* are mutated in a peroxisomal aminotransferase and have increased H_2O_2 content. (**A**) Diagram showing the gene structure of *GGT1* and the positions of the mutation in *ggt1-1* and the T-DNA insertion in *ggt1-2*. *Numbers* in the diagram refer to position within BAC F26F24. (**B**) Allelism test of *ggt1-1* and *ggt1-2*. Wild-type (*WT*), *ggt1-1*, *ggt1-2* and F1 seedlings of *ggt1-1* × *ggt1-2* were plated on MS media and kept under normal light for 1 week before being photographed. (**C**) RNA gel blot analysis of light regulated expression of *GGT1*. RNA was isolated from light or dark grown seedlings and 10 μg of total RNA loaded in each *lane*. *GGT1* transcript was not detected in the *ggt1-2* mutant. The blot was probed with tubulin as a loading control. (**D**) H_2O_2 content of light and dark grown seedlings of *WT* (C24) and *ggt1-1*. Seedlings were plated on half-strength MS with 0.5% sucrose and after stratification either grown under normal light conditions (70 μmol m^{-2} s^{-1}) or kept in darkness. Seedlings were harvested after 7 days and H_2O_2 quantified. Data are means ± SE, $n = 4$. *Asterisk* indicates that the H_2O_2 content of *ggt1-1* was significantly different than that of *WT* ($P \leq 0.05$)

transcriptional regulation, such as stimulation of P5CS1 enzyme activity or repression of ProDH activity. It should be noted that because MES buffer was used in all experiments, addition of Asc did not affect the pH of the growth media.

The response of root elongation to exogenous ABA was also quantified. We focused on treatments with low levels of exogenous ABA because these treatments are more consistent with both the level of ABA found in stressed plants (Verslues and Bray 2006) and the physiological role of ABA in maintaining or promoting, rather than inhibiting, root growth under stress (Sharp et al. 1994). Treatment with 0.5 μM ABA stimulated root elongation in each genotype relative to its control root elongation but this effect was the same in both wild-type and *ggt1-1* (Fig. 4e). In the absence of ABA treatment, root elongation of *ggt1-1* was reduced nearly 50% compared to wild-type (33.1 ± 0.9 mm for wild-type versus 17.8 ± 0.6 mm for *ggt1-1*; Supplementary Fig. 4). These data suggest that while *ggt1-1* does affect root elongation, it does not affect the response of root elongation to ABA.

ggt1-1 differs from wild-type in stress-induced ABA accumulation

Previous measurements in this experimental system (Verslues et al. 2006; Verslues and Bray 2006) have shown that ABA content increases rapidly after transfer of seedlings to PEG-infused plates and reaches a maximal level by 8 h after transfer (Verslues et al. 2006; Verslues and Bray 2006). Thus, ABA content of wild-type and *ggt1-1* was measured at 8 h after transfer to low water potential (PEG) or NaCl stress. *ggt1-1* had decreased ABA accumulation after transfer to either PEG or NaCl (Fig. 5a). This is consistent with the decreased induction of *NCED3* in *ggt1-1* (Fig. 2f) and again suggests that *ggt1-1* may affect feedback regulation of ABA synthesis.

As a broader measure of the stress resistance of *ggt1-1*, root elongation of low water potential- or NaCl-treated seedlings was quantified. This analysis showed that the relative root growth of *ggt1-1* was greater than wild-type at all severities of low water potential tested (Fig. 5b). The greater relative root growth of *ggt1-1* likely reflects in large part the reduced root elongation of unstressed *ggt1-1* seedlings. However, the lesser inhibition of *ggt1-1*, as well as the lack of apparent bleaching or other damage to the *ggt1-1* seedlings (Supplementary Fig. 4) also indicates that under these growth conditions *ggt1-1* is not inherently more sensitive to low water potential. A similar result is observed for NaCl-stressed seedlings where relative root elongation of *ggt1-1* is greater than that of wild-type at 50 mM NaCl and only slightly more affected or the same as wild-type at 100 and 150 mM (Fig. 5c).

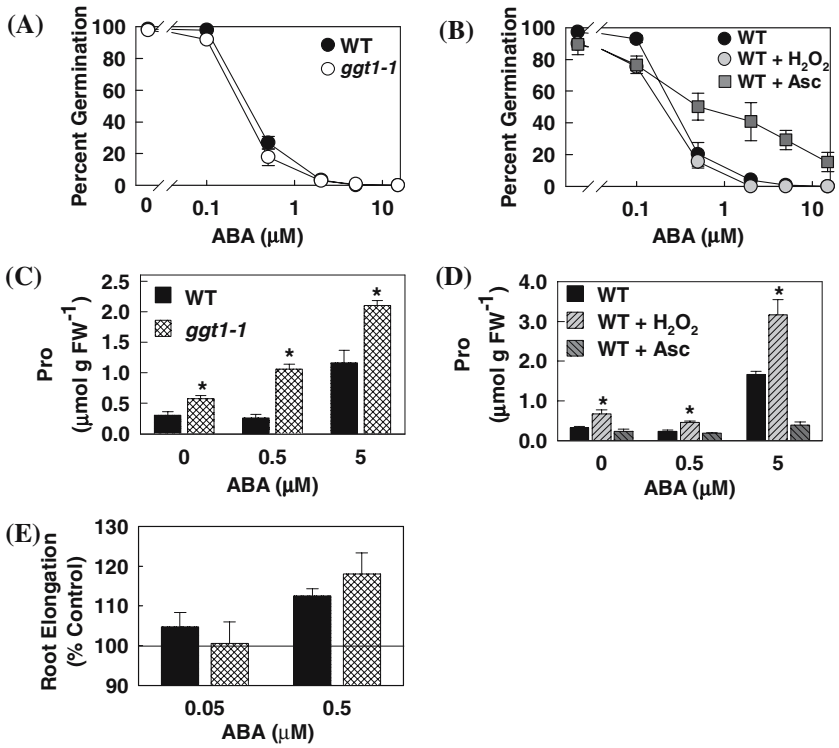
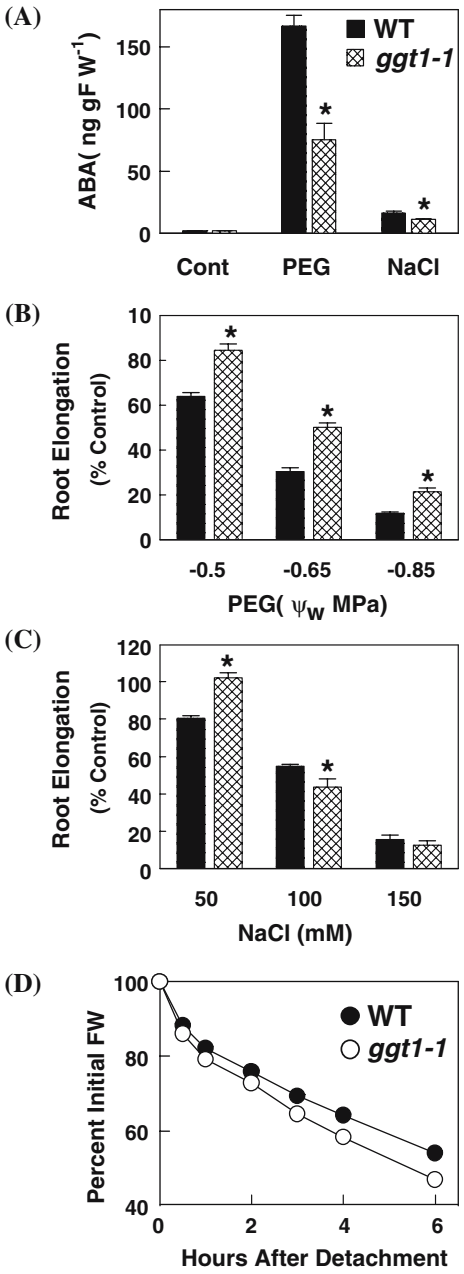


Fig. 4 Effect of *ggt1-1* and H_2O_2 or Asc treatments on seed germination, Pro accumulation and root elongation responses to exogenous ABA. For all experiments, the control media was half-strength MS with MES buffer without sugar with other growth conditions as indicated in Sect. ''Methods.'' (**A**) Response of wild-type (*WT*, ecotype C24) and *ggt1-1* seed germination to ABA. Germination was scored as radicle emergence 96 h after the end of stratification. Data are means ± SE, $n = 4$. (**B**) Effect of exogenous H_2O_2 (2 mM) or Asc (2 mM) added to the media before stratification on ABA inhibition of seed germination. Data are means ± SE, $n = 4$. (**C**) ABA-induced Pro accumulation in *WT* and *ggt1-1*. Seven-day-old seedlings were transferred from control media to the indicated treatments and seedling Pro content measured 96 h later. Data are means ± SE, $n = 6$–8. *Asterisks* indicate treatments where the Pro content of *ggt1-1* was significantly different than that of *WT* ($P \leq 0.05$). (**D**) Effect of exogenous H_2O_2 (500 μM) or Asc (2 mM) on ABA-induced Pro accumulation. Experimental procedures and data are as in (**C**). (**E**) Effect of exogenous ABA on root elongation in *WT* and *ggt1-1*. Root elongation is presented as a percent of the elongation on control media for each genotype. Data are means ± SE, $n = 14$–16. Root elongation of *ggt1-1* was not significantly different than that of *WT* in either treatment. Pictures of representative seedlings and mean root length increases for each treatment are given in Supplementary Fig. 4

It was also of interest to determine whether *ggt1-1* was affected in its control of leaf water loss because of the extensive evidence suggesting a role of H_2O_2 as an ABA signaling intermediate in stomatal movement. *ggt1-1* had a slightly greater rate of leaf water loss than wild-type (Fig. 5d). This suggests that despite the evidence that H_2O_2 acts downstream of ABA in stomatal closure, the increased leaf H_2O_2 content of *ggt1-1* was insufficient to promote stomatal closure.

ndpk2 also has increased H_2O_2 and altered ABA response

To further determine that it was the increased H_2O_2 and not other metabolic changes that caused the altered ABA response of *ggt1-1*, we sought to examine the ABA response of another, metabolicly unrelated, mutant which also accumulates more H_2O_2 than wild-type. Besides providing confirmation of the effects of elevated H_2O_2, examination of such a mutant is advantageous because many other photorespiratory mutants, which are likely to have increased H_2O_2, also have severely retarded growth and development making them difficult to examine under the experiment conditions used for *ggt1*. A knockout of *ndpk2* has been reported to have increased ROS (Moon et al. 2003); however, it does not have any known function in photorespiration or amino acid metabolism. We found that both unstressed and PEG-treated *ndpk2* seedlings had increased H_2O_2 content (Fig. 6a). We also found that *ndpk2* seedlings germinated poorly (approximately half of the *ndpk2* seed was inviable or germinated more slowly than wild-type under our standard growth conditions) and grew more slowly than wild-



◀ **Fig. 5** Stress responses of wild-type (*WT*, ecotype C24) and *ggt1-1*. Control media for all experiments was half-strength MS with 6 mM MES but without added sugar. (**A**) ABA contents 8 h after transfer of 7-day-old seedlings to fresh control media or plates containing PEG (–1.2 MPa) or NaCl (150 mM). Data are means ± SE, n = 4–8. *Asterisks* indicate treatments where the ABA content of *ggt1-1* was significantly different than that of *WT* ($P \leq 0.05$). (**B**) and (**C**) Root elongation of *WT* and *ggt1-1* seedlings transferred to the indicated PEG (**B**) or NaCl (**C**) treatments. Seedlings were 4 days old at the time of transfer and root elongation over the 6 days following transfer was quantified. Data presentation is as in Fig. 4e. Data are means ± SE, n = 14–16. *Asterisks* indicate treatments where root elongation of *ggt1-1* was significantly different ($P \leq 0.05$) than that of *WT*. Pictures of representative seedlings and mean root length increases for each treatment are given in Supplementary Fig. 4. (**D**) Leaf water loss of *WT* and *ggt1-1*. Data are means ± SE, n = 6. Water content of *ggt1-1* leaves was significantly less ($P \leq 0.02$) than that of *WT* at all time points from 0.5 to 6 h after detachment

type under control conditions (Fig. 6a). Root length increase of wild-type (ecotype Columbia) seedlings over 6 days was 45.4 ± 1.1 mm versus 26.4 ± 1.8 mm for *ndpk2*.

Like *ggt1-1*, *ndpk2* had increased Pro in both control and ABA treated seedlings (Fig. 6b). This result further suggests that it was the increased H_2O_2 content, and not altered amino acid metabolism, that caused the increased Pro levels in these mutants. Also consistent with an altered ABA response in *ndpk2* was the dramatic stimulation of *ndpk2* root growth by ABA (Fig. 6c; representative seedlings are shown in Fig. 5). Such an ABA stimulation of root growth would not be expected in *ggt1-1* because the increased H_2O_2 production occurs in photosynthetic tissue.

Discussion

ggt1-1 affects stress and ABA responses indirectly by altering H_2O_2 status

Determining the molecular mechanisms of ROS and metabolic signaling offers a particular challenge because it must be determined whether the effects attributed to a particular gene or protein represent a direct involvement in signaling or an indirect effect caused by more general changes in metabolic status. In many cases, the basic metabolic functions, but not the specific signaling functions, of the proteins involved may already be known. Perhaps the best studied example of this is Arabidopsis *HEXOKINASE1* (*HXK1*) which has distinct roles in both sugar metabolism and signal transduction. *hxk1* mutants that lack catalytic activity can still interact with other proteins to regulate glucose-responsive transcriptional changes in the nucleus (Moore et al. 2003; Cho et al. 2006). In other cases, a metabolic enzyme may still have a substantial effect on the output of a particular signaling pathway, but the mechanism may be more indirect through changes in levels of key metabolites or generation of ROS. Our data indicate that *ggt1-1* likely has such an indirect effect on stress and ABA response through inhibited photorespiration which leads to increased H_2O_2 levels.

Our results demonstrate that *ggt1-1* caused a light-dependent increase in H_2O_2 content, consistent with the established role of GGT1 in photorespiratory

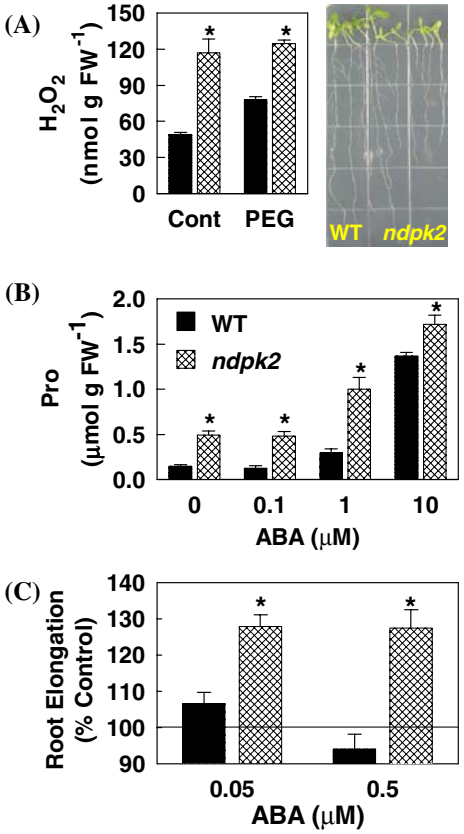


Fig. 6 ABA responses and H_2O_2 content of *ndpk2*. (**A**) H_2O_2 contents (*left panel*) of wild-type (*WT*, ecotype Columbia) and *ndpk2* seedlings under control conditions or 5 h after transfer to –1.2 MPa PEG. Data are means ± SE (n = 4). *Right panel* shows 10-day-old seedlings of *WT* and *ndpk2* grown under control conditions. (**B**) ABA-induced Pro accumulation in *WT* and *ndpk2*. Seven-day-old seedlings were transferred from control media to the indicated treatments and seedling Pro content measured 96 h later. Data are means ± SE, n = 6–8. *Asterisks* indicate treatments where the Pro content of *ndpk2* was significantly different than that of *WT* ($P \leq 0.05$). (**C**) Effect of exogenous ABA on root elongation in *WT* and *ndpk2*. Root elongation is presented as a percent of the elongation on control media for each genotype. Data are means ± SE, n = 8–10. *Asterisks* indicate treatments where the root elongation of *ndpk2* was significantly different than that of *WT* ($P \leq 0.01$)

metabolism in the peroxisome (Liepman and Olsen 2001; Igarashi et al. 2003). This caused a change in the basal level of H_2O_2 in unstressed plants which causes an increase in ABA-induced Pro accumulation. The increased H_2O_2 was also correlated with altered ABA-induced gene expression and reduced ABA accumulation. The reduced ABA accumulation may be caused by a greater sensitivity to ABA feedback in regulating ABA accumulation. These effects likely involve several different mechanisms. ABA and stress induction or repression of gene expression were inhibited because the higher basal H_2O_2 content of *ggt1-1* desensitized the initial responses to stress or ABA. In contrast, some responses, such as proline accumulation which was increased in *ggt1-1* and *ndpk2* even in the absence of ABA treatment, were more directly stimulated by the increased basal level of H_2O_2 and are likely to involve mechanisms other than altered transcription of ABA-responsive genes.

Increased ROS levels stimulate ABA-induced Pro accumulation

The effect of *ggt1-1* and *ndpk2* on Pro content was particularly intriguing. Since increased Pro content could be seen in both *ggt1-1* and *ndpk2* and exogenous H_2O_2 could also elicit an increase in Pro content, it is unlikely that this phenotype is caused by altered amino acid metabolism in *ggt1-1*. Instead, it is consistent with the data of Fabro et al. (2004) who observed that leaves infiltrated with xanthine and xanthine oxidase to generate ROS also had an up to fourfold increase in Pro content. The increase in Pro content was linearly related to the amount of xanthine oxidase infiltrated into the leaves indicating a dependence of Pro content on ROS production. The increased Pro content of *ggt1-1* is also consistent with other proposals that Pro metabolism is connected to redox regulation and that Pro may itself serve as an antioxidant (Hare et al. 1998).

The increased Pro content of *ggt1-1* is also interesting in relation to previous observations that although stress-induced Pro accumulation is dependent on ABA, application of ABA to unstressed plants does not elicit the same level of Pro accumulation seen in stressed plants (Verslues and Bray 2006). Although the levels of Pro seen in *ggt1-1* and *ndpk2* were not increased to the level seen in stressed plants, the results do suggest that H_2O_2 can stimulate ABA-induced Pro accumulation and may be one factor in inducing the high levels of Pro accumulation that can occur under low water potential stress. This effect on Pro accumulation is likely to be via mechanisms other than transcriptional regulation of *P5CS1* and *ProDH* as the changes in *P5CS1* and *ProDH* transcript levels that occurred in the initial stress and ABA response of *ggt1-1* would suggest a decrease in Pro accumulation. One possibility is that the increased Pro levels of *ggt1-1* and *ndpk2* were caused by altered expression of *P5CS2*. Fabro et al. (2004) have shown that *P5CS2* was upregulated by incompatible plant–pathogen interactions that are known to also increase ROS levels. Alternatively, the increased Pro observed in *ggt1-1* and *ndpk2* could be caused by up regulation of P5CS1 enzyme activity, down regulation of ProDH activity,

altered abundance of the P5CS1 or ProDH proteins, altered levels of substrate for Pro synthesis or altered feedback regulation of Pro metabolism.

Moreno et al. (2005) reported that another photorespiratory mutant *shmt1-1*, which is affected in SHMT1 activity, is more susceptible to pathogen infection than wild-type. They concluded that although ROS production is essential for pathogen resistance, constitutive overaccumulation of ROS actually impaired defense responses. This is similar to our conclusion that constitutive overproduction of H_2O_2 in *ggt1-1* impaired ABA regulation of gene expression. Moreno et al. (2005) also suggested the growth of *shmt1-1* seedlings was more sensitive to salt stress than wild-type. This differs from our results; however, their plants were also exposed to a higher light intensity than that used in our experiments. Thus, their results may indicate the interaction of salt stress and light stress. It should also be noted that the plant defense associated hormone salicylic acid (SA) can regulate H_2O_2 levels and may interact with ABA (Guan and Scandalios 1995; Rai et al. 1986). Thus, altered SA levels or signaling could also contribute to the phenotypes of *shmt1-1* as well as *ggt1-1* and *ndpk2*.

Altered H_2O_2 can affect many aspects of ABA metabolism and ABA signaling

The effect of *ggt1-1* on ABA accumulation, along with observations that alterations in ROS metabolism can influence ABA metabolism (Pastori et al. 2003; Rossel et al. 2006); that ABA can regulate the expression of genes related to ROS metabolism (Jiang and Zhang 2002; Fryer et al. 2003; Milla et al. 2003); and that ROS status can influence stress-induced ABA accumulation (Hu et al. 2005) indicate that there are likely to be numerous mechanisms by which ABA and ROS metabolism interact and cross regulate one another. At the cellular level, the recent report that the chloroplast-localized Mg-chelatase H subunit specifically binds ABA and is likely to be an ABA receptor (Shen et al. 2006) supports the idea that H_2O_2 produced in the chloroplast, one likely source of the increased H_2O_2 production in *ggt1-1*, could affect ABA signaling. At the tissue level, the stimulation of root elongation by ABA in *ndpk2* but not in *ggt1-1* is consistent with the increased H_2O_2 production in *ggt1-1* being confined to photosynthetic tissues while *ndpk2* overaccumulates H_2O_2 by a different mechanism not dependent on photosynthesis. Interestingly, Mullineaux et al. (2006) have discussed the importance of the tissue and subcellular localization of H_2O_2 production as well as a possible link between ABA metabolism and H_2O_2 via G-proteins which are involved in regulating both H_2O_2 production and ABA signaling. All of these observations strengthen the link between the increased H_2O_2 in *ggt1-1* and its altered ABA response. Overall, our results support the idea that H_2O_2 is part of ABA signaling not just in guard cell responses, as has been well described at the molecular level (Kwak et al. 2006), but also in many other ABA-regulated responses.

Materials and methods

Plant material and growth conditions

Soil grown plants were grown under continuous light (70 μmol m^{-2} s^{-1}) using a standard potting mix (Metro Mix). For seedling analysis, seedlings of *ggt1-1* and its wild-type (ecotype C24) or *ndpk2* and its wild-type (ecotype Columbia) were routinely grown by surface sterilizing seed and plating onto half-strength MS with 6 mM MES (pH 5.7) without the addition of sucrose or other sugars (unless otherwise noted) following the protocols of Verslues et al. (2006). Seeds were stratified for 4 days at 4°C and then transferred to a growth chamber maintained at 23°C and continuous light (70 μmol m^{-2} s^{-1}) and ambient CO_2. For low water potential treatments, seedlings were grown on top of a nylon mesh overlaid on the agar surface and 6-day-old seedlings were transferred using the mesh to PEG-infused plates prepared according to Verslues et al. (2006). Salt, ABA, H_2O_2 and Asc treatments were performed in the same manner by transferring 6-day-old seedlings to half-strength MS plates without sugar but with the indicated chemicals added to the media. For root elongation measurements, 4-day-old seedlings were transferred individually to the stress or ABA treatments, the initial position of the root apex marked and the increase in root length measured 6 days later. For germination assay, seeds were sown on half-strength MS media without sugar and with the indicated concentration of (+)-ABA and stratified for 4 days at 4°C. Germination was scored based on emergence of the radicle 4 days after the end of the stratification treatment.

For leaf water loss measurements, fully expanded leaves were removed from 4- to 5-week-old plants, placed in tared plastic weighing tissues and incubated under the same conditions used for seedling growth with each sample weighed at the indicated times.

For luminescence analysis, seedlings were grown on MS media with 3% sucrose. Luminescence analysis was performed as previously described (Ishitani et al. 1997)

using 7-day-old seedlings. Cold stress was performed by transferring seedlings to 0°C for the indicated amount of time before luminescence measurement. Salt stress was imposed by transferring seedlings to filter paper soaked with MS media containing 300 mM NaCl. For ABA treatment, seedlings were sprayed with 100 μM (±)ABA (Sigma, St Louis, MO, USA) before imaging. Luminescence intensity was quantified for individual seedlings after subtraction of background luminescence.

RT-PCR and RNA blot analysis

For QPCR analysis, total RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). First strand cDNA was synthesized with 1 or 2 μg of total RNA. Real-time PCR quantification of *RD29A, RAB18*, *COR15* and *P5CS1, Pro Ox* and *NCED3* expression was performed essentially as described by Tan et al. (2003) using a Sequence Detection System 7700 instrument (Applied Biosystems, Foster City, CA, USA). Quantification of copy number for each gene was done by amplifying a 400–600-bp portion of each gene containing the binding sites for the real-time PCR primers and TaqMan probe. The amplified DNA fragment was purified, quantified spectrophotometrically and diluted appropriately to generate a standard curve for calculation of the copy number of each transcript. Sequences of the oligonucleotides used for QPCR analysis are given in Supplementary Table 1.

For RNA blot analysis of GGT1 expression RNA from control, stressed or ABA treated seedlings was isolated by phenol:chloroform extraction. Ten micrograms of total RNA was separated by denaturing gel electrophoresis and blotted onto nitrocellulose membrane. ^{32}P-labeled probes were prepared using Prime-It II random primer labeling kit (Stratagene) and hybridization and washing performed using standard techniques. A *GGT1* specific probe was prepared by restriction digestion of the cloned cDNA and gel purification of a fragment from a *GGT1* specific region.

Quantification of ABA, Pro and H_2O_2

ABA was assayed by radioimmunoassay as previously described (Bray and Beachy 1985; Verslues and Bray 2006). Pro was assayed using the ninhydrin-based colorimetric assay of Bates et al. (1973). H_2O_2 was assayed using an Amplex Red H_2O_2 assay kit (Molecular Probes/Invitrogen, Carlsbad, CA, USA) following the protocols of Shin and Schachtman (2004). Briefly, seedling or leaf samples were ground in liquid nitrogen, placed into chilled, preweighed 2 ml microcentrifuge tubes and weighed while frozen to obtain the fresh weight of the sample. At the time of analysis, samples were suspended in 20 mM sodium phosphate buffer and assayed according to the manufacturer's instructions.

Data were statistically analyzed using standard two-tailed *t*-test and significant differences are reported in the figures or figure legends.

Acknowledgements This work was supported by NIH grant 5R01GM059138 to J.-K.Z., by NSF grant IBN-0420152 to J.-K.Z and by an NIH postdoctoral fellowship (1F32GM074445) to P.E.V. We thank Giltsu Choi and Pill-Soon Song (Kumho Life and Environmental Science Laboratory, Korea) for the gift of *ndpk2* seed. We also thank the laboratory of Dr Robert Heath for use of equipment for the Pro measurements and Rebecca Stevenson for technical assistance.

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