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The FRO2 ferric reductase is required for glycine betaine's effect on chilling tolerance in *Arabidopsis* roots

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FRO2 (At1g01580) codes for an NADPH-dependent ferric reductase in plasma membranes of root epidermal cells with a demonstrated role in iron uptake by plants. Ferric reductase activity has been shown to be the rate-limiting step for iron uptake in strategy I plants like *Arabidopsis* and in rice, but it has been unclear whether *FRO* genes have other physiological functions. We hypothesized that *FRO2* was involved in chilling stress tolerance because its expression was upregulated by treatment of plants with glycine betaine (GB), a chemical that prevents reactive oxygen species (ROS) signaling in chilling stress. This idea was confirmed by showing that the *FRO2* null mutant *frd1-1* failed to respond to GB in chilling assays either in relation to root growth recovery or inhibition of ROS accumulation. Measurements of ferric reductase activity in wild-type plants treated with GB before chilling showed no significant GB effect compared with controls. In addition, 35S-*FRO2* transgenics with elevated mRNA levels did not have improved chilling tolerance. However, ferric reductase activity in wild-type plants or 35S-*FRO2* transgenics pretreated with GB was several-fold higher after chilling compared with non-pretreated controls. These experiments identify a new physiological function for *FRO2,* i.e. blocking ROS accumulation during chilling. They also suggest that GB has a major effect on *FRO2* activity posttranscriptionally in the cold.

Introduction

Iron performs essential functions in plant biochemistry, mediating oxidation–reduction reactions in over 100 enzymes involved in photosynthesis, respiration and nitrogen fixation and in the synthesis of DNA nucleotides and ethylene. In addition, iron participates in several reactions (Apel and Hirt 2004, Suzuki and Mittler 2006) producing or scavenging reactive oxygen species (ROS). Excess iron concentrations can occur in plants under anaerobic conditions or at low pH when the concentrations of Fe(II) in the growth media become elevated. Inasmuch as the major iron transporter in roots (IRT1) transports the Fe(II) ion (Eide et al. 1996, Korshunova et al. 1999, Vert et al. 2002), these conditions can lead to elevated iron levels, resulting in symptoms such as leaf browning in rice as well as damage caused by production of the highly toxic hydroxyl radical from H_2O_2 via the Fenton reaction (Halliwell and Gutteridge 1992). Deficiency of iron is also common, especially on alkaline soils. Low iron causes a different set of morphological symptoms in growing roots as well as leaf yellowing resulting from defective Chl production. It also leads to increases in the concentrations of other heavy metals, such as Cu, Zn and Mn in both roots and shoots.

In *Arabidopsis,* low iron conditions also cause an adaptive upregulation of genes for a H^+-ATPase,

Abbreviations – GB, glycine betaine; NOX, NADPH oxidase; NBT, nitroblue tetrazolium; ROS, reactive oxygen species.

NADPH-dependent ferric reductases (*FRO* genes) and IRT1 involved in uptake of the Fe(II) ferrous ion (Guerinot and Yi 1994, Kim and Guerinot 2007). Acidification followed by iron reduction and uptake is known as strategy I iron uptake as opposed to strategy II iron uptake, which is characterized by chelation of external Fe(III) by phytosiderochromes followed by uptake of the Fe(III)-phytosiderochrome complex (Guerinot 2007). The genome of *Arabidopsis* contains eight *FRO* genes coding for membrane-associated NADPH-dependent ferric reductases (Mukherjee et al. 2006, Wu et al. 2005). In most cases, these enzymes are localized to the plasma membrane of cells, where they shuttle reductant from cytoplasmic NADPH to external Fe(III) forming Fe(II). Proof of function in iron uptake for the major *FRO* gene in roots (*FRO2*) was provided by the phenotype of its mutant *frd1-1*, which has an elevated iron requirement because it lacks the ability to reduce external Fe(III) (Robinson et al. 1999, Yi and Guerinot 1996). It has also been shown that overexpression of *FRO* in transgenic rice has been shown to improve yields in alkaline soils (Ishimaru et al. 2007). Whether *FRO* genes have other functions in addition to a role in iron uptake has been unclear.

Our focus on ferric reductase in relation to chilling stress resulted from investigations using a chemical genetic screen based on glycine betaine (GB) to identify new determinants of stress tolerance in *Arabidopsis* (Einset 2006). It was known that application of GB to plants could improve tolerance to stress caused by chilling (Park et al. 2004), frost, salt, drought and high light intensities, and that this effect was accompanied by gene expression changes, but whether the gene expression changes were implicated in the effect of GB and the genes that were involved has been unclear. By conducting global gene expression studies with microarrays followed by confirmation on Northern blot[s], we detected upregulation of genes in leaves and roots for transcription factors, membrane-trafficking components such as Rab GTPases (Preuss et al. 2004), ROS-scavenging enzymes and for FRO ferric reductases (Einset et al. 2007a, 2007b). That GB's effect depends on gene expression was proven by direct functional evidence using a knockout mutant for *RabA4c*. Although *Arabidopsis* is usually defined as chilling resistant as it shows no obvious signs of chilling injury, chilling does have a negative effect because chilled plants show inhibited root growth upon transfer back to normal temperatures. Remarkably, when wild-type plants were pretreated with GB, root growth rates after chilling were comparable to non-chilled plants. By contrast, the *RabA4c* knockout showed no GB response in the chilling test, proving the requirement for a functional *RabA4c* gene for GB's effect (Einset et al. 2007a, 2007b).

In this report, we present similar functional experiments proving the role of *FRO2* (At1g01580) in GB's effect by using a null mutant *frd1-1*, thereby demonstrating a new physiological function for FRO2 in relation to the effect of GB on chilling tolerance in *Arabidopsis* roots. Two types of experiments using wild-type and mutant plants are presented: (1) recovery of root growth after chilling and (2) ROS accumulation during chilling. As far as the first type of experiment is concerned, it can be shown that root growth of plants receiving a chilling treatment is significantly inhibited during the first few days after plants are returned to normal growing conditions. GB pretreatment alleviates this inhibition in wild-type but not the *frd1-1* mutant. The second type of experiment involves measurements of ROS accumulation during chilling. It is shown that chilling causes accumulation of superoxide in roots of *Arabidopsis* and that GB pretreatment of plants blocks this accumulation in wild-type but not in *frd1-1* mutant. In addition to demonstrating that a functional *FRO2* gene is required for GB's effect in roots, this paper also reports experiments using 35S-*FRO2* transgenics with elevated *FRO2* mRNA levels to test for the possibility that they might have improved chilling tolerance. The observation that these transgenics do not have altered chilling tolerance compared with controls led to further experiments directly showing that ferric reductase activity was increased significantly in GB-pretreated plants that had been subjected to chilling but not in GB-treated plants before the chilling treatment. This suggests that GB exerts a major effect during chilling in relation to ferric reductase activity by affecting *FRO2* expression posttranscriptionally.

Materials and methods

Plant materials, growth conditions, chilling and GB treatments

The *frd1-1* mutant was isolated in a screen for ferric reductase-defective mutants of *Arabidopsis thaliana* from an EMS-mutagenized population, i.e. mutants that failed to show iron-deficiency-inducible root ferric reductase activity (Yi and Guerinot 1996). Production of the 35S-*FRO2* lines is described by Connolly et al. (2003). Each of the 35S-*FRO2* lines has a single copy of the transgene based on 3:1 ratio of Kan resistance :Kan sensitivity in the T2 generation and 100% Kan resistance in the T3. Plants were grown in 8.5 cm diameter plastic Petri plates. In experiments, plants were grown in Petri plates containing Murashige and Skoog (MS) medium, 30 g l^{-1} sucrose and 8 g l^{-1} agar under controlled environmental conditions at 24°C, 16-h photoperiod and a 80 μmol m^{-2} s^{-1} light intensity. To test for recovery from chilling, plants at the

rosette stage of growth, either treated for 24 h with GB or with water as controls, were transferred to 4°C and 30 μmol m^{-2} s^{-1} continuous light for 2 days after which time they were transferred back to 24°C, 16-h photoperiod and a 80 μmol m^{-2} s^{-1} light intensity and incubated vertically. Daily root growth increments were measured with a ruler. All data were subjected to ANOVA in groups. Significant differences between treatments were evaluated at the 95% confidence interval. For GB treatments, approximately 3-week-old plants at the rosette stage were sprayed on both leaves and roots with an aqueous solution containing 100 m*M* GB only, while control plants were sprayed with water. GB had no effect on growth of plants during the 24 h prior to transfer of plants to chilling conditions (data not shown).

Detection of superoxide

For detecting superoxide, plant material was vacuum infiltrated with 0.1 mg ml^{-1} nitroblue tetrazolium (NBT) in 25 m*M* HEPES buffer, pH 7.6. Samples were incubated at room temperature in the dark for 0.5 h. In control treatments to test the validity of the method, 10 m*M* $MnCl_2$ and 10 units ml^{-1} superoxide dismutase were added to the buffer, in addition to NBT. These controls gave no formazan product.

Microscopy

Microscopy was performed using a Leitz Laborlux K microscope with a Leica DC300F color camera (Wetzlar, Germany). The Leica DC program was used to import images of NBT-stained roots into Adobe Photoshop Elements 2.0 (San Jose, CA). Images were then analyzed for blue pixel intensities, representing formazan staining, using public-domain ImageJ (http://rsb.info.nih.gov/ij/). At least eight roots from each chilled treatment with or without GB pretreatments were analyzed relative to controls with at least three independent experiments giving similar results. Data were normalized for chilled wild-type Columbia roots and evaluated using ANOVA between groups. Mean differences at the 95% confidence level were considered as significant.

Results

A functional FRO2 gene is required for GB's effect

GB is being used commercially to improve stress tolerance in agriculturally important crops. Based on this fact and given that GB can confer stress tolerance in transgenics at levels corresponding to hormonal concentrations (Holmström et al. 2000), we hypothesized that at least part of GB's effect might be caused by activation of the expression of stress tolerance genes. To pursue this idea, we conducted gene expression profiling experiments using microarrays followed by confirming analysis using Northern blot[s]. Among the genes that were shown to be strongly upregulated by GB treatments were *FRO2* (At1g01580) in roots and *FRO6*/*FRO7* (At5g49730/At5g49740) in leaves.

Demonstration of gene upregulation by GB is not sufficient by itself to show that GB causes its effect via *FRO* genes. What is needed is a *FRO* gene null mutant to conduct functional studies, testing whether specific genes play a role in relation to stress tolerance, ROS signaling and the effect of GB. Fig. 1 shows the effect of GB pretreatments on chilling recovery by wild-type plants, the *FRO2* null mutant *frd1-1* and a 35S-*FRO2* line. Twenty-four hours after treatment with GB, plants were transferred to chilling conditions at 4°C and approximately 30 μmol m^{-2} s^{-1}. Two days later, plants were transferred back to normal growing conditions and recovery was monitored as root growth on Petri plates incubated vertically. As Fig. 1 shows, wild-type Columbia and the 35S-*FRO2* line clearly responded to GB pretreatments, as shown by the large differences in shoot and root growth plus vs minus GB, while *frd1-1* showed little, if any, response. This proves that a functional *FRO2* gene is required for GB's effect on chilling recovery. Fig. 2 shows that the response to GB by wild-type plants can be seen by measuring root growth as early as 1 day after plants were transferred back to normal growth conditions after the 2 days chilling treatment. The *frd1-1* mutant shows no response to GB on days 1 and 2, then a small effect on day 3. Four days after transfer of plants back to normal growing conditions, all treatments had similar root growth rates, indicating that the non-responsiveness of *frd1-1* is not simply a result of less robust growth under these conditions.

As previous studies had shown a close linkage between chilling stress and ROS accumulation (Suzuki and Mittler 2006), we were interested in investigating ROS in relation to GB treatments and chilling sensitivity in wild-type and *frd1-1* plants. As indicated in Fig. 3, both wild-type and *frd1-1* plants accumulated superoxide in roots in response to chilling conditions, whereas pretreatment with GB significantly reduced superoxide in wild-type plants, while *frd1-1* mutants still showed significant staining. This proves that a functional *FRO2* gene is required for GB's effect on ROS accumulation during chilling. To quantify GB's effect, we used the public-domain ImageJ program to analyze digital images produced with our microscope setup equipped with a color digital camera coupled to a computer. Fig. 4 summarizes the results from three experiments and shows that ROS accumulation by non-pretreated Columbia, non-pretreated *frd1-1* as well as GB-pretreated *frd1-1* were

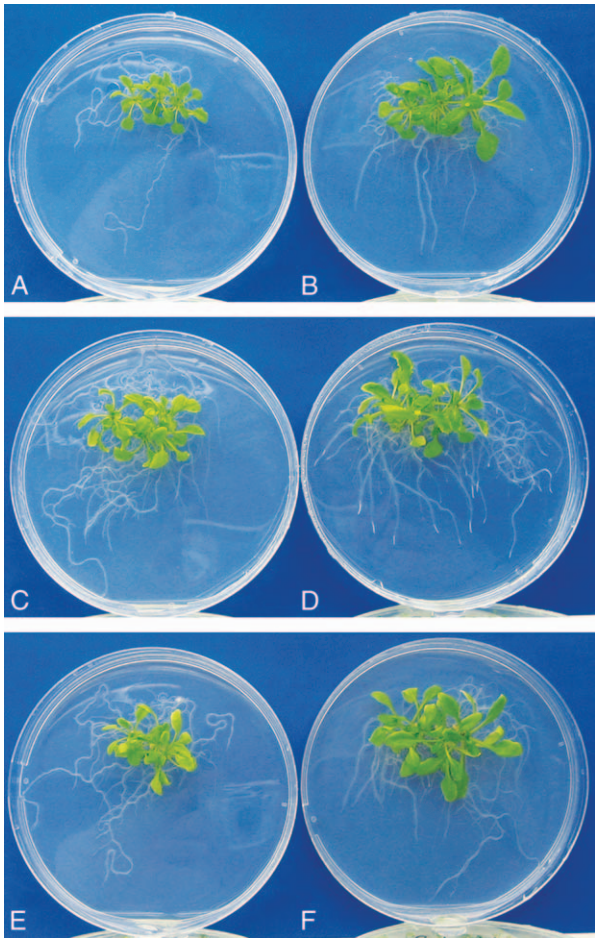

Fig. 1. Effect of GB pretreatments on recovery from chilling stress by wild-type, *frd1-1* mutant and 35S-FRO2 transgenic *Arabidopsis*. Twelve-day-old plants were sprayed with 100 m*M* GB or water then incubated 24 h at 24°C, 16-h photoperiod and a 80 μmol m^{-2} s^{-1} light intensity before being transferred to chilling conditions at 4°C and 30 μmol m^{-2} s^{-1} continuous light intensity for 48 h. Photos show plants 5 days after transfer back to normal growing conditions at 24°C, 16-h photoperiod and a 80 μmol m^{-2} s^{-1} light intensity and incubated vertically. All photos are presented with the same magnification; Petri plates are 8.5 cm in diameter. Treatments were (A) Columbia without GB pretreatment, (B) Columbia with GB pretreatment, (C) *frd1-1* without GB pretreatment, (D) *frd1-1* with GB pretreatment, (E) 15G 35S-FRO2 *frd1-1* without GB pretreatment and (F) line 15G 35S-FRO2 with GB pretreatment.

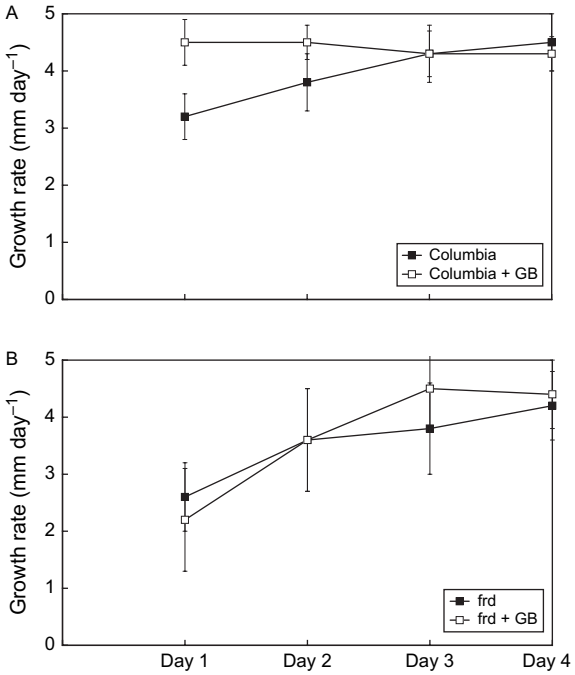

Fig. 2. Root growth rates during recovery from chilling by wild-type Columbia and *frd1-1* plants, pretreated with GB (+) or with no pretreatment (−). Daily growth increments, measured as millimeters of growth per day, were determined for randomly selected roots of plants grown vertically on Petri plates after transfer from chilling to normal growth conditions at 24°C, 16-h photoperiod and a 80 μmol m^{-2} s^{-1} light intensity. Each point is based on pooled results from three independent experiments with at least 10 independent measurements per data point and with standard errors indicated.

indistinguishable, while GB-pretreated Columbia had markedly lower ROS staining.

35S-FRO2 transgenics do not have improved chilling tolerance

Based on evidence for *FRO2* upregulation by GB and the demonstration that a functional *FRO2* is required for GB's effect, it was of interest to determine whether transgenics expressing a constitutive *FRO2* gene driven by the strong 35S promoter might have improved chilling tolerance. Fig. 5 compares superoxide staining for two transgenic lines, characterized by Connolly et al. (2003) that express 35S-*FRO2* constructs. Although both transgenic lines have strongly upregulated *FRO2* mRNA levels, they showed similar superoxide accumulation during chilling compared with wild-type Columbia. They also responded similarly to GB pretreatments. Tests of root growth rates after chilling with or without GB pretreatments also indicated that the 35S-*FRO2* transgenics had chilling tolerances and chilling recovery abilities similar to wild-type Columbia (data not shown).

Ferric reductase assays indicate posttranscriptional regulation

To obtain direct biochemical evidence that ferric reductase activity is affected by GB pretreatments, we measured enzyme activity in wild-type plants before and after chilling, with or without GB pretreatments. Fig. 6 shows the results of a representative experiment, demonstrating

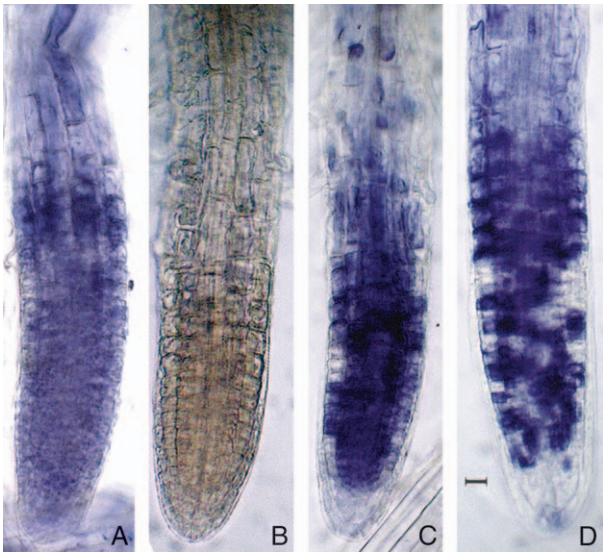

Fig. 3. Comparison of superoxide staining using NBT for roots from chilled plants of wild-type Columbia and *frd1-1* either not pretreated with GB or pretreated with GB. Plants were stained with NBT immediately after being removed from chilling. All photos are presented with the same magnification and the bar indicates 25 μm. Treatments were (A) Columbia without GB pretreatment, (B) Columbia with GB pretreatment, (C) *frd1-1* without GB pretreatment and (D) *frd1-1* with GB pretreatment.

that ferric reductase levels prior to chilling were unaffected by GB pretreatments, while enzyme levels after chilling were significantly increased in GB-pretreated plants.

Fig. 7 compares GB effects on post-chilling ferric reductase levels in wild-type and the 5A line of 35S-*FRO2* transgenics. Similar results were obtained with the 15G 35S-*FRO2* transgenic (data not shown). In all cases, significant increases in ferric reductase activity in GB-pretreated plants, ranging from two- to six-fold in repeat experiments, were observed after chilling even though these increases were similar in wild-type Columbia and 35S-*FRO2* transgenic plants.

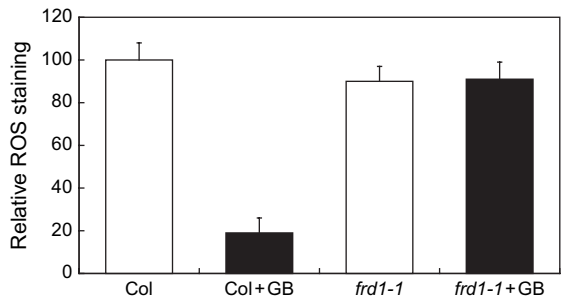

Fig. 4. ROS levels, measured as blue pixel intensities using ImageJ with NBT-stained roots, in chilled plants of wild-type Columbia and *frd1-1* either not pretreated with GB or pretreated with GB. Each value is based on pooled results from three independent experiments with standard errors indicated. The total number of stained roots analyzed were 19, 21, 25 and 25 for Columbia (Col) without GB, Columbia with GB, *frd1-1* no GB and *frd1-1* GB, respectively.

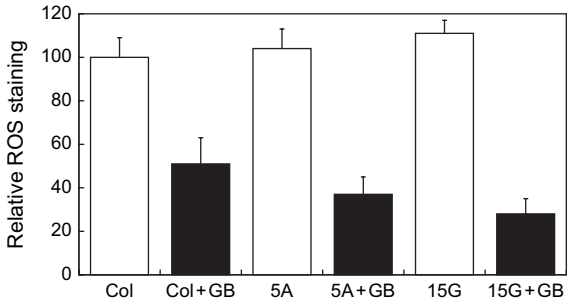

Fig. 5. Effect of GB pretreatments on ROS levels in chilled roots of Columbia and 35S-*FRO2* lines 5A, 11E and 15G either not pretreated with GB or pretreated with GB. Each value is based on analysis of randomly selected roots from at least eight randomly selected roots in three independent experiments giving similar results with standard errors indicated. Relative ROS staining for GB-non-pretreated Columbia (Col), 5A and 15G were not significantly different from one another, while the values for GB-pretreated Columbia, 5A and 15G were also not significantly different from one another.

Discussion

The results reported here show the requirement for a functional *FRO2* gene to obtain GB's beneficial effect on chilling tolerance. The results also show that GB causes significant increases in the activity of ferric reductase in chilled plants, providing direct biochemical

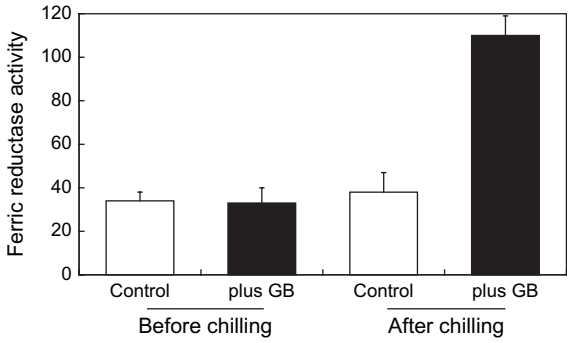

Fig. 6. Ferric reductase levels in wild-type Columbia based on six independent experiments giving similar results. Results of a representative experiment are shown involving six replicate plants per treatment with standard deviations indicated. The first two bars on the left show ferric reductase activities 24 h after GB treatment before plants are transferred to chilling, while the next two bars show ferric reductase activities after two days of chilling. A ferric reductase value of 100 in the figure corresponds to 0.01 μmol Fe^{2+} produced per gram FW of root per hour.

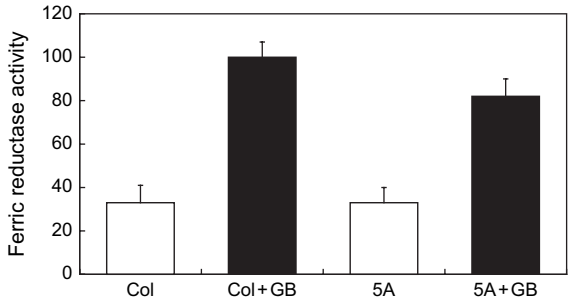

Fig. 7. Comparison of wild-type Columbia (Col) vs 35S-FRO2 transgenic line 5A with respect to GB-induced increases in ferric reductase during chilling based on four independent experiments giving similar results. Results of a representative experiment are shown involving six replicate plants per treatment with standard deviations indicated. A ferric reductase value of 100 in the figure corresponds to 0.01 μmol Fe^{2+} produced per gram FW of root per hour.

evidence that a mechanism for transferring reductant potential from cytoplasmic NADPH to the cell wall is activated by GB pretreatments. A plausible interpretation of these findings is that GB action increases reductant concentrations in the wall of root epidermal cells via *FRO2*-coded ferric reductase, effectively stopping ROS buildup in roots, both extra- and intracellularly, along with attendant ROS signaling associated with chilling stress (Einset et al. 2007b). In addition to increasing the potential for ROS scavenging outside of cells, increased ferric reductase activity could also increase iron availability for cells by increasing ferrous ion levels for uptake by IRT1. A consequence of increased iron levels in cells might also be to stabilize cytosolic aconitase (Dupuy et al. 2006), enhancing its ability to produce isocitrate substrate to NADP-dependent isocitrate dehydrogenase, one of the major producers of NADPH in the cytosol of animal cells (Lee et al. 2002).

To determine whether increased *FRO2* mRNA levels alone are responsible for GB's effect, we tested 35S-*FRO2* transgenics overexpressing FRO2 mRNA for recovery from chilling, response to GB and ROS accumulation compared with wild-type Columbia. All tests showed that the 35S-*FRO2* transgenics had chilling sensitivities and GB responses similar to wild-type plants. This indicates that GB's effect on chilling tolerance is primarily because of processes other than upregulation of *FRO2* mRNA levels, namely that GB exerts effects on posttranscriptional regulation of *FRO2* during chilling.

Connolly et al. (2003) used the same 35S-*FRO2* transgenics in studies of ferric reductase induction under low iron conditions. Consistent with our results, they also found no correlation between mRNA levels and measurable ferric reductase, suggesting that posttranscriptional regulation is involved in relation to low iron effects too. The FRO2 protein has five lysine residues for ubiquitination in the cytoplasmic loop that also contains binding sites for NADPH and FAD (Robinson et al. 1999), so post-transcriptional regulation via ubiquitination followed by protein degradation is a plausible level of control for the FRO2 protein. Connolly et al. (2002) have presented evidence suggesting that the levels of IRT1 protein are also regulated posttranscriptionally. In a recent report by Kerkeb et al. (2008), the hypothesis was tested that levels of the IRT protein are posttranslationally regulated via ubiquitination. They mutated one or both lysine residues (lys-146 and lys-171) to arginine in the major cytoplasmic loop of the IRT protein presumed to be ubiquitin attachment sites. Either one or both of these changes was found to abolish iron-induced control of posttranscriptional turnover of the IRT protein, providing support for the ubiquitination hypothesis with IRT. With regard to cold-regulated protein degradation, a precedent in *Arabidopsis* involves the *HOS1* gene/*ICE1* system in which the HOS1 RING E3 ligase ubiquitinates ICE1, causing its degradation in the cold but not at 22°C (Dong et al. 2006, Zhu et al. 2007).

The mechanism of ROS production in the cold is not known, although NADPH oxidase (NOX) activity is an interesting possibility inasmuch as NOX has been demonstrated to be involved in ROS production during root hair development by root epidermal cells (Gapper and Dolan 2006) as well as being implicated in pathogen responses (Torres and Dangl 2005) and stomatal control (Kwak et al. 2006). By analogy to animal systems, the activation of NOX in phagocytes has been studied extensively, revealing several cofactors in addition to phosphorylation events and membrane trafficking (Bedar and Krause 2007). It is also known that phagocyte NOX produces superoxide on the outside of the plasma membrane, which would correspond to the wall region in plant cells. As far as downstream signaling in relation to elevated ROS, processes similar to ROS effects on the activity of protein kinases (Burgoyne et al. 2007) could also be occurring in *Arabidopsis*.

FRO2 was identified as a major ferric reductase in root epidermal cells, where it is upregulated together with *IRT* under conditions of low soil iron (Robinson et al. 1999). Expression of *FRO2* and the other seven *FRO* genes in *Arabidopsis* has been studied by various methods. Bauer et al. (2004), e.g. used RT-PCR followed by gels and hybridization of blots with gene-specific probes. Although their analysis did not measure expression of *FRO1*, *FRO6*, *FRO7* or *FRO8*, they did detect *FRO2*, *FRO3* and *FRO5* expression in roots as well as expression of *FRO3* and *FRO4* in shoots. Wu et al. (2005), on the other hand, used RT-PCR followed by gels as well as

promoter *FRO–GUS* fusions in transgenics. They detected *FRO2* and *FRO3* expression in roots and *FRO6*, *FRO7* and *FRO8* expression in shoots with weaker signals for *FRO3* and *FRO5*. Mukherjee et al. (2006) used RT-PCR and found that the major genes expressed in roots were *FRO2*, *FRO3* and *FRO5*, while in shoots the major genes expressed were *FRO3*, *FRO6* and *FRO7*. Studies of *FRO* gene expression have been hindered by the fact that only two of the eight *FRO* genes are represented on the commonly used Affymetrix chip (Santa Clara, CA), so there exists limited information on the regulation of this gene family in relation to relative expression levels, tissue specificity as well as developmental fluctuations and responses to stress. We have used Operon's oligomers plus our own custom-designed oligos (Einset et al. 2007a) to monitor *FRO* gene expression under various growth conditions. We have also repeated RT-PCR analysis using methods of Mukherjee et al. (2006) to look at *FRO* gene expression and to study GB effects before and after chilling for all eight *FRO* genes. Although the major *FRO* genes expressed in roots are *FRO2*, *FRO3*, *FRO4* and *FRO5* (data not shown), only upregulation of *FRO2* by GB could be shown in microarray, Northern and RT-PCR experiments.

In conclusion, our experiments show that GB's effect on chilling stress in *Arabidopsis* roots involves post-transcriptional processes occurring in the cold and affecting ferric reductase activities coded by *FRO2*. The demonstration that *FRO2* plays a role in GB-mediated chilling tolerance and ROS accumulation represents a new physiological function for *FRO* genes, in addition to their demonstrated role in iron uptake. Earlier gene expression experiments together with functional genomic analysis based on mutants (Einset et al. 2007a, 2007b) have identified two other genes required for GB signaling in chilling stress; namely, *RabA4c* (At5g47960) and a putative bZIP transcription factor (At3g62420). In all cases, the experiments show a tight association between ROS accumulation and chilling stress.

References

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55: 373–399

Bauer P, Thiel T, Klatte M, Bereczky Z, Brumbarova T, Hell R, Grosse I (2004) Analysis of sequence, map position, and gene expression reveals conserved essential genes for iron uptake in Arabidopsis and tomato. Plant Physiol 136: 4169–4183

Bedar K, Krause K-H (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 87: 245–313

Burgoyne JR, Madhani M, Cuello F, Charles RL, Brennan JP, Schröder E, Browning DD, Eaton P (2007) Cysteine redox sensor in PKGIa enables oxidant-induced activation. Science 317: 1393–1397

Connolly EL, Fett JP, Guerinot ML (2002) Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. Plant Cell 14: 1347–1357

Connolly EL, Campbell NH, Grotz N, Prichard CL, Guerinot ML (2003) Overexpression of the FRO2 ferric chelate reductase confers tolerance to growth on low iron and uncovers posttranscriptional control. Plant Physiol 133: 1102–1110

Dong C-H, Agarwal M, Zhang Y, Xie Q, Zhu JK (2006) The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. Proc Natl Acad Sci USA 103: 8281–8286

Dupuy J, Volbeda A, Carpentier P, Darnault C, Moulis JM, Fontella-Camps JC (2006) Crystal structure of human iron regulatory protein 1 as cytosolic aconitase. Structure 14: 129–139

Eide D, Broderius M, Fett J, Guerinot ML (1996) A novel iron-regulated metal transporter from plants identified by functional expression in yeast. Proc Natl Acad Sci USA 93: 5624–5628

Einset J (2006) An extracellular mechanism of light protection in plants identified using a chemical genetic screen. Acta Hort (ISHS) 711: 339–344

Einset J, Nielsen E, Connolly EL, Bones A, Sparstad T, Winge P, Zhu JK (2007a) Membrane trafficking RabA4c involved in the effect of glycine betaine on recovery from chilling stress in *Arabidopsis*. Physiol Plant 130: 511–518

Einset J, Winge P, Bones A (2007b) ROS signaling pathways in chilling stress. Plant Signal Behav 2: 365–367

Gapper C, Dolan L (2006) Control of plant development by reactive oxygen species. Plant Physiol 141: 341–343

Guerinot ML (2007) It's elementary: enhancing Fe3+ reduction improves rice yields. Proc Natl Acad Sci USA 104: 7311–7312

Guerinot ML, Yi Y (1994) Iron: nutritious, noxious, and not readily available. Plant Physiol 104: 815–820

Halliwell B, Gutteridge JMC (1992) Biologically relevant metal ion-dependent hydroxyl radical generation. FEBS Lett 307: 108–112

Holmström K, Somersalo S, Mandal A, Palva TE, Welin B (2000) Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine. J Exp Bot 51: 177–185

Ishimaru Y, Kim S, Tsukamoto T, Oki H, Kobayashi T, Watanabe S, Matsuhashi S, Takahashi M, Nakanishi H, Mori S, Nishizawa NK (2007) Mutational reconstructed ferric chelate reductase confers enhanced tolerance in rice to iron deficiency in calcareous soil. Proc Natl Acad Sci USA 104: 7373–7378

Kerkeb L, Mukherjee I, Chatterjee I, Lahner B, Salt DE, Connolly EL (2008) Iron-induced turnover of the *Arabidopsis* IRT1 metal transporter requires lysine residues. Plant Physiol 146: 1964–1973

Kim SA, Guerinot ML (2007) Mining iron: iron uptake and transport in plants. FEBS Lett 581: 2273–2280

Korshunova YO, Eide D, Clark WG, Guerinot ML, Pakrasi HB (1999) The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with broad specificity. Plant Mol Biol 40: 37–44

Kwak JM, Nguyen V, Schroeder JI (2006) The role of reactive oxygen species in hormonal responses. Plant Physiol 141: 323–329

Lee SM, Koh H-J, Park D-C, Song BJ, Huh T-L, Park J-W (2002) Cytosolic $NADP^+$-dependent isocitrate dehydrogenase status modulates oxidative damage to cells. Free Radic Biol Med 32: 1185–1196

Mukherjee I, Campbell NH, Ash JS, Connolly EL (2006) Expression profiling of the *Arabidopsis* ferric chelate reductase (*FRO*) gene family reveals differential regulation by iron and copper. Planta 223: 1178–1190

Park E-J, Jeknic Z, Sakamoto A, DeNoma J, Yuwansiri R, Murata N, Chen THH (2004) Genetic engineering of glycinebetaine synthesis in tomato protects seeds, plants, and flowers from chilling damage. Plant J 40: 474–487

Preuss ML, Serna J, Falbel TG, Bednarek SY, Nielsen E (2004) The *Arabidopsis* Rab GTPase RabA4b localizes to the tips of growing root hair cells. Plant Cell 16: 1589–1603

Robinson NJ, Procter CM, Connolly EL, Guerinot ML (1999) A ferric-chelate reductase for iron uptake from soils. Nature 397: 694–697

Suzuki N, Mittler R (2006) Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. Physiol Plant 126: 45–51

Torres MA, Dangl JL (2005) Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr Opin Plant Biol 8: 397–403

Vert G, Grotz N, Dedaldechamp F, Gaymard F, Guerinot ML, Briat J-F, Curie C (2002) IRT, an *Arabidopsis* transporter essential for iron uptake from the soil and plant growth. Plant Cell 14: 1223–1233

Wu H, Li L, Du J, Youxi Y, Cheng X, Ling H-Q (2005) Molecular and biochemical characterization of the Fe(III) chelate reductase gene family in *Arabidopsis thaliana*. Plant Cell Physiol 46: 1505–1514

Yi Y, Guerinot ML (1996) Genetic evidence that induction of root Fe(III) chelate reductase activity is necessary for iron uptake under iron deficiency. Plant J 10: 835–844

Zhu J, Dong C-H, Zhu J-K (2007) Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. Curr Opin Plant Biol 10: 290–295

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