

Membrane-trafficking RabA4c involved in the effect of glycine betaine on recovery from chilling stress in *Arabidopsis*

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Glycine betaine (GB) can confer tolerance to several types of stress at low concentrations, either after application to plants or in transgenics engineered to overproduce GB. Based on earlier studies on levels of GB in plants and evidence for effects on gene expression, we hypothesized that at least part of this effect could be ascribed to the activation of the expression of stress tolerance genes. Using a strategy based on high-throughput gene expression analysis with microarrays followed by confirmation with northern blots, we identified *Arabidopsis* genes upregulated in roots that reinforce intracellular processes protecting cells from oxidative damage and others that appear to be involved in reinforcing a scavenging system for reactive oxygen species (ROS) in cell walls. Upregulated genes in roots include those for the membrane-trafficking RabA4c, the root-specific NADPH-dependent ferric reductase (FRO2) localized to the plasma membrane, mitochondrial catalase 2 and the cell wall peroxidase ATP3a. Comparative studies with wild-type *Arabidopsis* and knockout mutants for the membrane-trafficking RabA4c gene demonstrated that the mutants respond only slightly to GB, if at all, compared with wild-type in relation to root growth recovery after chilling stress, demonstrating the role of RabA4c in relation to the GB effect. The results point toward links between oxidative stress, gene expression, membrane trafficking and scavenging of ROS such as superoxide and hydrogen peroxide in relation to GB effects on chilling tolerance in plants.

Introduction

Glycine betaine (GB) is a widely distributed natural product in plants that accumulates under stress conditions (Ingram and Bartels 1996, Rhodes and Hanson 1993) and has been used commercially to improve stress tolerance in agriculturally important crops (Allard et al. 1998, Chen et al. 2000, Rahman et al. 2002). With the advent of plant transformation methodologies, transgenic plants overproducing GB and having increased stress tolerance have been obtained. Examples of stress-tolerant transgenics engineered to produce GB (Chen and Murata 2002, Park et al. 2004, Sakamoto and Murata 2000, 2001)

Abbreviations – DAB, 3,3′-diaminobenzidine; FW, fresh weight; GB, glycine betaine; NBT, nitroblue tetrazolium; PCR, polymerase chain reaction; ROS, reactive oxygen species; YFP, yellow fluorescent protein.

include *Arabidopsis*, rice, tobacco, tomato, *Brassica napus*, *Brassica juncos* and *Diospyrus kaki*. In addition, GB production has been shown to improve stress tolerance in organisms such as bacteria and algae (Kempf and Bremer 1998, Nomura et al. 1995).

Intracellular accumulation of GB to high levels can enable cells to equilibrate their osmotic pressure with high external osmotic forces under stress conditions such as dehydration caused by high salt or freezing. Alternatively, GB can also have direct osmoprotectant effects on enzymes by preventing protein denaturation under extreme conditions. For example, a study by Diamant et al. (2001) showed that GB could prevent in vitro aggregation of enzymes at 42°C as well as promote chaperone-mediated refolding of enzymes after heat denaturation.

Even though genetic engineering can increase GB levels in transgenic plants, thus improving tolerance to chilling, high light intensity, salt, cold and freezing stresses, the levels of GB obtained are often quite low in comparison to GB levels in plants that normally accumulate this compound to high tissue concentrations or, in fact, in relation to GB concentrations required to cause effects on protein stability in vitro. For example, GB levels in sugar beet (*Beta vulgaris*) can be >40 μmol g^{-1} fresh weight (FW), while GB levels in transgenic plants rarely exceed 1–5 μmol g^{-1} FW. The extreme examples are transgenic tobacco plants with improved salt and chilling tolerance associated with a GB concentration of 0.035 μmol g^{-1} FW (Holmström et al. 2000).

Low levels of GB production in transgenic plants are probably because of limitations in the production of GB's precursor choline and its transport into the chloroplast in these plants (Huang et al. 2000, MacNeil et al. 2000, 2001). At the same time, the fact that such a large discrepancy can exist between GB levels in stress-tolerant transgenics as opposed to levels in natural GB overproducers suggests that GB might have effects over and above its known osmotic and protein-stabilizing effects.

Given that GB can confer stress tolerance in transgenics at levels corresponding to hormonal concentrations and based on earlier demonstrations that GB affects gene expression (Allard et al. 1998), we hypothesized that at least part of GB's effect could be ascribed to factors other than its known effects on osmotic pressure or on protein stability; e.g. GB may activate the expression of stress tolerance genes. These considerations led us to use a strategy based on high-throughput gene expression analysis with microarrays to identify genes activated by GB followed by confirmation of effects on gene expression with northern blots. This report describes the identification of genes activated by GB in roots that can be seen as determinants that reinforce intracellular processes protecting cells from oxidative damage and that also set up a scavenging system for reactive oxygen species (ROS) in walls via membrane trafficking. Focusing directly on a gene for the membrane-trafficking RabA4c, we have then used knockout mutants to demonstrate the direct role of this gene in the GB response of plants in relation to recovery from chilling stress. Experiments with transgenics containing constructs composed of the RabA4c promoter driving expression of yellow fluorescent protein (YFP) indicate that RabA4c expression patterns in roots correspond to the regions of high ROS accumulation under chilling conditions.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana plants were grown in 8.5-cm-diameter plastic petri plates containing MS medium, 30 g l^{-1} sucrose and 8 g l^{-1} agar under controlled environment conditions at 24°C, 16-h photoperiod and a 40 μM m^{-2} s^{-1} light intensity. Seed for making homozygous knockouts of the RabA4c gene (knockout SALK_005306) were obtained through SIGnAL (http://signal.salk.edu/tdna_protocols.html). In experiments testing 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 μM m^{-1} s^{-1} continuous light for 2 days after which time they were transferred back to 24°C, 16-h photoperiod and a 40 μM m^{-2} s^{-1} light intensity and incubated vertically. Daily root growth increments were measured with a ruler.

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, as described earlier in studies of uptake and translocation of GB in crop plants (Makela et al. 1996). Control plants were sprayed with water.

RNA analysis

Total RNA was extracted directly from roots, excised from plants and analyzed by northern blots as described previously (Liu and Zhu 1997). Hybridization probes were obtained by amplifying gene-specific regions of genomic DNA with the following primers: RabA4c left upper (5′-TGTTGCTAATGAGGAAGGAGA-3′) and RabA4c right lower (5′-TTAGTATGCAGTGTTCAACTC-3′); FRO2 left upper (5′-AAAGCAATAACGGTGGTTCG-3′) and FRO2 right lower (5′-ATACGCAGATGTGGCAA′C-3′);

FRO6/FRO7 left upper (5′-AAGTGGAAGCAAATGG-CAA-3′) and FRO6/FRO7 right lower (5′-CCCATTTCC-CATTCAATGAC-3′); ATP3a left upper (5′-CTCCGTTGC-TAAGCAGAAGC-3′) and ATP3a right lower (5′-CCC-TCGTCTAACTCCACTCG-3′) and catalase 2 left upper (5′-CGTCGAGGTATGACCAGGTT-3′) and catalase 2 right lower (5′- AGGGCATCAATCCATCTCTG-3′). Details of the composition of all four microarrays (1.1-K chip comprising eight copies each of 70-mers for 1100 genes, 2-K chip with eight copies each of 70-mers for 2000 genes, Incyte Genomics (St. Louis, MO) *Arabidopsis* GEM1 chip with 6000+ different genes and a chip based on 70-mer probes from Operon's (Cologne, Germany) *Arabidopsis* genome oligo set 1.0 representing 26 000+ genes) can be obtained at http://arken.umb.no/~johnei/. RNA for microarray analysis with the 1.1-K and 2-K chips and the Operon genomic chip was isolated using RNeasy (Qiagen, Oslo, Norway) then processed using Genisphere (Hatfield, PA) 3DNA Array 350 technology. After hybridization in a Tecan (Zurich, Switzerland) HS 4800 hybridization station and dendrimer labeling, each slide was scanned twice at different laser intensities using a Packard BioScience (Meriden, CT) Scan Array Express HT scanner, after which the individual scans were analyzed by a GenePix (Sunnyvale, CA) Pro 5.1 imaging program. Signal intensities were background corrected and normalized (Quackenbush 2002). In addition to running several independent experiments with different microarrays, we conducted dye-swap tests to demonstrate that observed experimental artifacts did not cause differences.

Detection of superoxide and hydrogen peroxide

For detecting superoxide, plant material was vacuum infiltrated with 0.1 mg/ml 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 superoxide dismutase were added to the buffer, in addition to NBT. These controls gave no formazan product. For visualization of hydrogen peroxide, plant material was vacuum infiltrated with 0.1 mg/ml 3,3′-diaminobenzidine (DAB) in 50 m*M* Tris–acetate buffer (pH 5.0) at room temperature in the dark for 48 h. In control treatments to test the validity of the method, 10 m*M* ascorbic acid was added to the buffer, in addition to DAB. Method controls gave no gold-brown product.

Construction of plasmids and transformed plants

The A4c promoter was polymerase chain reaction (PCR) amplified from isolated genomic DNA using the primers ERIK177 (5′-GGAATTCGACGGTTATCTG-3′) and ERIK178 (5′-CATGCCATGGTTCACACCAAAATCACAAG-3′). These primers were designed to include EcoRI and NcoI restriction sites at the 5′ and 3′ ends, respectively. The PCR product was digested with *Eco*RI and *Nco*I. The amplified promoter fragment was then inserted into pCAMBIA-EYFP-C1 digested with *Eco*RI and *Nco*I (Preuss et al. 2004). This created the pCAMBIA-A4cPromoter-EYFP vector. Plants were transformed using *Agrobacterium tumefaciens* by the floral dip method (Clough and Bent 1998).

Results

Analysis of gene expression profiles of GB-treated plants

Gene expression analysis using DNA microarrays has become a powerful tool for high-throughput genetic analysis; e.g. to identity genes associated with cold acclimation (Fowler and Thomashow 2002). To focus on the possible effects of GB on stress-related genes, we constructed two different oligonucleotide arrays that are enriched for genes that have been implicated in stress responses. One array (1.1-k chip) is composed of eight copies each of 70-mers for 1100 genes, while the other custom array has eight copies each of 70-mers for 2000 genes. These arrays plus the Incyte Genomics Arabidopsis GEM1 chip with 6000+ different genes and a chip based on 70-mer probes from Operon's *Arabidopsis* genome oligo set 1.0 representing 26 000+ genes were used to analyze gene expression profiles in whole plants, excised roots and leaves.

Tissue samples from control plants and from GB-treated plants were used for RNA isolation and then this RNA was used as template to make cDNA that was hybridized to the microarrays. Twelve independent biological experiments (RNA isolated from GB-treated and control-treated plants) were used in 22 separate hybridizations. After quantitation of signal intensities, the data were normalized to correct for differences in probe labeling, background levels, inconsistency in replicates on the same array and non-linearity of intensity distributions. Next, we identified genes showing significant and consistent upregulation by GB in at least four independent experiments. From the more than 26 000 genes that were analyzed in repeated experiments and using different microarrays, we could identify upregulation of genes for transcription factors, membrane-trafficking components, ROS-scavenging enzymes and for plasma membrane NADPH-dependent ferric reductases.

Focusing on genes activated in roots only, we isolated RNA directly from roots, then used northern blot

hybridization as an alternative gene expression technology to confirm our earlier results using root RNA and microarrays. Fig. 1 shows that the expression of genes in roots for the membrane-trafficking RabA4c (At5g47960), NADPH-dependent ferric reductase FRO2 (At1g01580), mitochondrial catalase 2 (At4g35090) and the cell wall peroxidase ATP3a (At5g64100) was significantly upregulated 24 h after GB treatment (Fig. 1).

Effect of GB on recovery from chilling stress

Demonstration of the activation of gene expression by GB is not sufficient by itself to show that GB causes its effect by turning on genes for stress tolerance, even if several of the target genes have functions that have already been implicated in relation to stress. What is needed is a direct demonstration of the role of specific genes in relation to stress tolerance and the effect of GB. Fig. 2 shows the effect GB pretreatments on chilling recovery by wild-type plants and knockout mutants for the membrane-trafficking RabA4c gene. Twenty-four hours after treatment with GB, plants were transferred to chilling conditions at 4°C and approximately 20 μM 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. 3 shows, wild-type Columbia responded markedly to GB pretreatments, as shown by the large difference in shoot and root growth plus or minus GB, while RabA4c

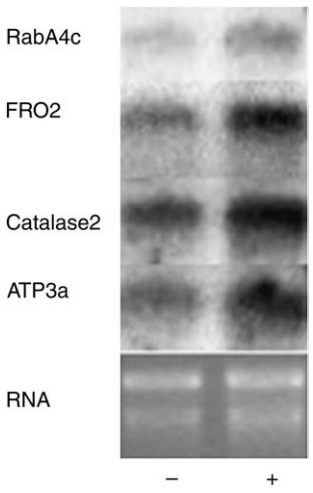

Fig. 1. GB upregulation of selected genes expressed in roots. Three-week-old rosette -stage plants of Columbia grown on MS medium plus 30 g/l sucrose and 8 g/l agar were sprayed with a 100 mM GB solution and then RNA was isolated from excised roots after 24 h. The RNA samples were zero time control (left, −) and 24 h after GB treatment (right, +). Gene probes used for hybridization to gel blots of RNA (approximately 10 μg per sample) are indicated at left.

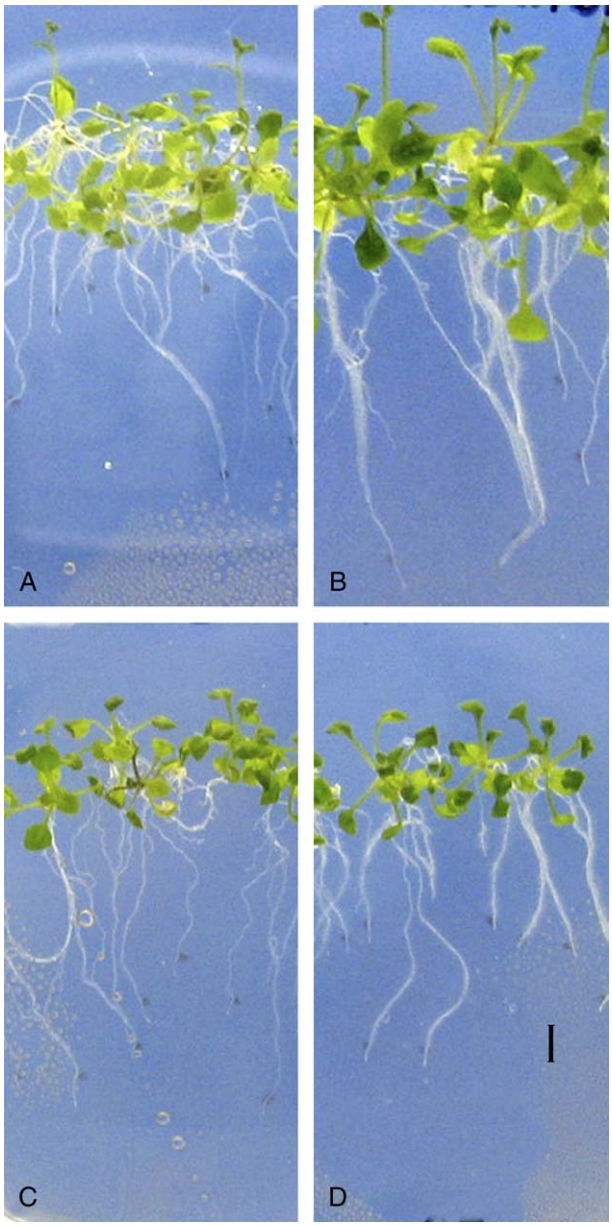

Fig. 2. Effect of GB on recovery from chilling stress by wild-type and RabA4c knockout mutant *Arabidopsis*. Approximately 3-week-old plants were sprayed with 100 mM GB, then incubated for 24 h at 24°C, 16-h photoperiod and a 40 μM m^{-2} s^{-1} light intensity before being transferred to chilling conditions at 4°C and 30 μM m^{-2} s^{-1} light intensity for 48 h. Photos show plants 4 days after transfer back to normal growing conditions at 24°C, 16-h photoperiod and a 40 μM m^{-2} s^{-1} light intensity and incubated vertically. All photos are presented with the same magnification and the vertical bar in D corresponds to 1 cm. Treatments were (A) Columbia without GB pretreatment, (B) Columbia with GB pretreatment, (C) RabA4c knockout without GB pretreatment and (D) RabA4c knockout with GB pretreatment.

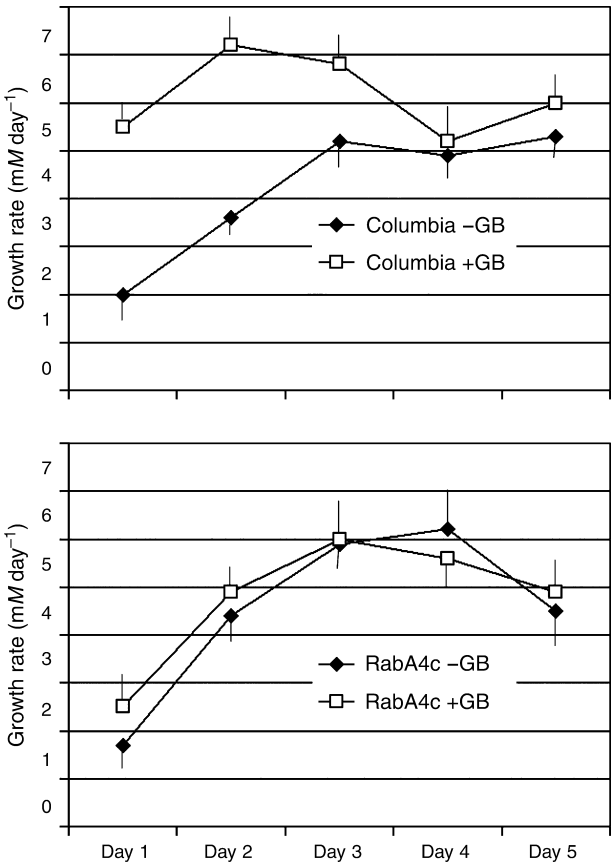

Fig. 3. Root growth rates during recovery from chilling by wild-type Columbia and RabA4c knockout plants, pretreated with GB (+) or with no pretreatment (−). Daily growth increments, measured as millimeter growth per day, were determined for randomly selected roots of plants grown on petri plates incubated vertically after transfer from chilling conditions to normal growth conditions at 24°C, 16-h photoperiod and a 40 μM m^{-2} s^{-1} light intensity. Each point is based on pooled results from five independent experiments with at least 10 independent measurements per data point and with standard errors indicated.

Fig. 4. Comparison of superoxide staining using NBT for roots from chilled plants (left) and from plants grown under normal growth conditions at 24°C, 16-h photoperiod and a 40 μM m^{-2} s^{-1} light intensity (right). Both photos are presented with the same magnification. The bar indicates 20 μm.

knockouts showed little, if any, response. This shows that RabA4c is required for GB's effect on chilling recovery. Fig. 4 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-day chilling treatment. Five days later, all treatments had similar root growth rates, indicating that the RabA4c knockouts are not simply less robust in their growth under normal conditions, generally.

Oxidative stress associated with chilling in leaves

On the basis of the types of genes affected by GB, we hypothesized that chilling leads to ROS buildup in roots and that GB prevents accumulation of ROS to growth-inhibiting levels. As expected, chilled roots stained intensely with NBT for superoxide compared with roots of non-chilled plants (Fig. 4). Fig. 5 shows superoxide staining of representative roots immediately after chilling (Day 1) compared with staining during recovery (Day 2 and Day 4). Heavy formazon staining of plants not GB pretreated on Day 1 rapidly diminished by Day 2 and then reached nearly negligible levels by Day 4. This time course for superoxide disappearance corresponds to the time course for recovery of optimal root growth (Fig. 3). As Fig. 5 also shows, pretreatment of plants with GB significantly reduced superoxide staining after chilling, an observation that corresponds to the observation that root growth after chilling was not inhibited when plants were pretreated with GB (Fig. 3).

To confirm that the effects of chilling and GB are related to ROS, Fig. 6 shows DAB staining for hydrogen

Fig. 5. GB inhibits ROS accumulation in roots caused by chilling. Photographs show duplicate NBT-stained roots on successive days after chilling treatment. Plants were either pretreated with GB (+) or not pretreated (−) prior to chilling.

peroxide with roots from chilled plants, either pretreated with GB or not pretreated. Hydrogen peroxide staining was clearly demonstrated in roots after chilling, while GB pretreatments prevented this hydrogen peroxide accumulation.

Fig. 7 compares RabA4c expression in roots, visualized with a RabA4c-YFP construct in transgenics, with the pattern of superoxide accumulation in RabA4c knockouts in response to chilling stress. Both RabA4c expression and superoxide accumulation in the knockout appeared to be most pronounced in the region of active cell division in the root tip behind the root cap.

Discussion

The hypothesis that GB treatments can confer stress tolerance, at least in part, via gene activation provided the opportunity to use a chemical genetics approach to identify genes that might be involved in this response. As with so-called mutational genetics, there was no a priori bias about the kinds of genes that would be identified, e.g. in relation to cellular localization or specific enzyme

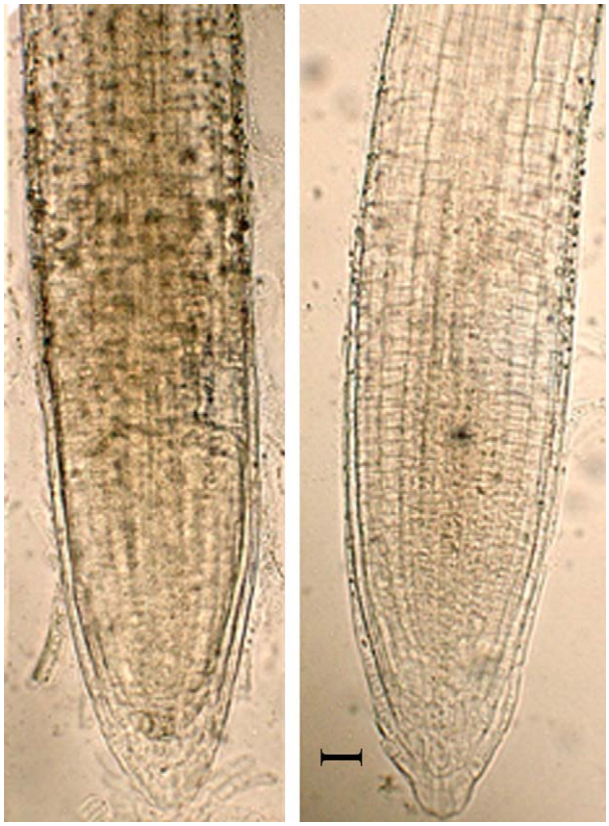

Fig. 6. Comparison of hydrogen peroxide staining using DAB for roots from chilled plants either not pretreated with GB (left) or pretreated with GB (right). Plants were treated with DAB immediately after being removed from chilling. Both photos are presented with the same magnification. The bar indicates 20 μm.

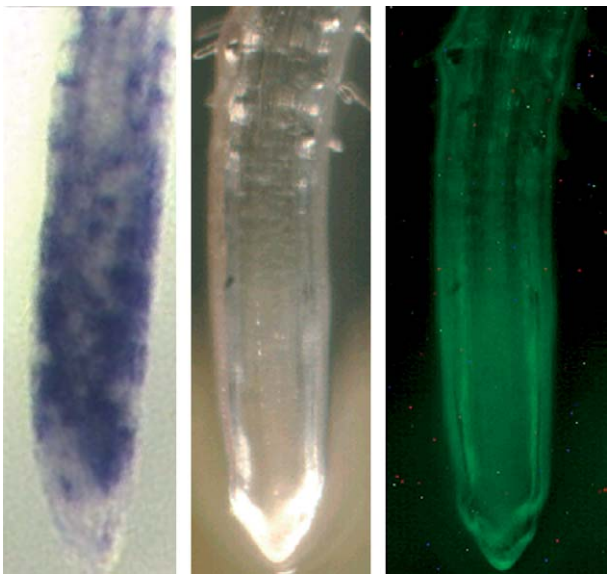

Fig. 7. Distribution of superoxide accumulation in roots after chilling (left) compared with YFP expression in a transgenic producing YFP under control of the RabA4c promoter (right). White-light photograph of the transgenic is the center picture. Superoxide levels were visualized using NBT staining as in Figs 4 and 5.

systems. Experiments with microarrays and northern blots led to the identification of GB target genes in roots such as the membrane-trafficking RabA4c, the root-specific NADPH-dependent ferric reductase (FRO2) localized to the plasma membrane, mitochondrial catalase 2 and the cell wall peroxidase ATP3a.

Identifying genes activated by GB focused attention on processes such as gene expression, oxidative stress and membrane trafficking which, in turn, led us to experiments with RabA4c knockout mutants to test the idea directly that GB effects were mediated via this process. Using a recovery-from-chilling assay, it was possible to show that GB pretreatments improved the tolerance of wild-type plants to chilling stress but that the knockouts for RabA4c showed little, if any, response. It was also shown that the localization of RabA4c expression in roots coincided with the region of maximum superoxide and hydrogen peroxide accumulation during chilling.

Given the genes for ROS scavenging identified in this study, a question arises as to the source of reductant for ROS scavenging in cell walls inasmuch as cell walls are not autonomous with respect to energy supply. In this respect, it is interesting that GB activates the expression of genes for two NADPH-dependent ferric reductases in leaves (FRO6 and FRO7, data not shown) and the corresponding ferric reductase in roots (FRO2). Mutants null for FRO2 have an elevated iron requirement because they lack the ability to reduce external Fe^{+3} (Robinson et al. 1999). All three enzymes (FRO2, FRO6 and FRO7) could provide a mechanism to shuttle reductant from cytoplasmic NADPH to cell wall Fe^{+3}, producing Fe^{+2} as a reductant source. FRO6 and FRO7 are closely related to FRO2 which is expressed in roots and which reduces external Fe^{+3} to Fe^{+2} that can then be taken up by a Fe^{+2} transporter.

Another question relates to whether RabA4c is normally limiting in *Arabidopsis* during chilling stress. Fig. 3 shows, e.g. that in the absence of a GB pretreatment wild-type shows a similar time course of recovery from chilling as the RabA4c knockouts. Similarly, the levels of superoxide accumulation after chilling are comparable for wild-type and the knockouts in the absence of GB pretreatments. These results suggest that expression of the gene for RabA4c in *Arabidopsis* may only be limiting in relation to the GB effect.

As pointed out by Park et al. (2004), both choline monooxygenase and betaine aldehyde dehydrogenase are targeted to the chloroplast, making it likely that most GB synthesized normally is localized to the chloroplast (Sakamoto and Murata 2000). However, Park et al. (2004) estimated that 60–86% of total GB in transgenic tomato plants is chloroplast localized, so significant amounts of GB might be released from chloroplasts to be available for influencing nuclear gene expression for improved stress tolerance. Likewise, Sakamoto et al. (2000) measured only 60% of the GB as chloroplast localized in their *Arabidopsis* transgenics. Given the positive effects of cytoplasmic GB demonstrated here, it seems likely that release of GB from chloroplasts to the cytoplasm might be selected for evolutionarily in several instances inasmuch as this process would lead to improved stress tolerance. Thus, levels of GB leakage from chloroplasts indicated by Park et al. (2004) and Sakamoto et al. (2000) might be even more pronounced in plants that are naturally stress tolerant. Although GB is produced by *Arabidopsis*, there is no evidence to date that fluctuations in GB levels are involved in regulating stress responses. On the other hand, it is clear that at least some genes activated by GB in *Arabidopsis* have counterparts in other plants. We were interested in the possibility that GB was causing its effect by activating the cold-induced set of genes represented by CBF transcription factors plus downstream products from genes such as COR6.6, COR15a, COR47 and COR78 (Fowler and Thomashow 2002), but we could not detect the activation of any of these genes in microarray experiments (data not shown). Similarly, there was no evidence for activation of SOS genes for salt tolerance such as SOS1, SOS2, SOS3 and SOS4 (Alia et al. 1998). Rather, it appears as though GB is upregulating a different set of genes, involved particularly in oxidative stress tolerance, that conceivably could explain GB effects on improving tolerance to several different kinds of stress affected by GB treatments, including stresses caused by chilling (Sakamoto et al. 2000), frost (Chinnusamy et al. 2005), salt (Alia et al. 1997) and high light intensities (Alia et al. 1999).

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