25 Chemical Genetics Identifies New Chilling Stress Determinants in *Arabidopsis*

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Introduction

Cold stress limits plant survival and productivity in agriculture and in natural systems worldwide. Although the effects of freezing temperatures have often been of primary interest for plant researchers, chilling temperatures in the range from 0 to 15°C can also be of importance. For example, chilling temperatures during spring can hinder establishment of vegetable seedlings or have damaging effects on already established plants. In Nordic areas especially, it is common that summer growing seasons can have periods of cold weather that cause significant effects on flowering, fruit set and general plant vigour. In the autumn, early chilling episodes can prevent effective winter hardening, resulting in winter damage and reduced crop yields during subsequent growing seasons. Finally, crop losses caused by postharvest chilling, in the field or in storage, can also be significant.

Knowledge gaps in relation to fundamental questions about chilling stress are substantial. The following questions can be asked. How important is chilling stress as a factor limiting plant distribution and success? How do plants sense chilling temperatures? How is the perception of chilling temperatures translated into signal transduction, leading to the activation of genes that enable plants to cope with chilling? When are the critical times of the year in relation to sensitivity to chilling stress: spring, autumn or winter? What is the molecular mechanism(s) of chilling injury? What are the mechanisms of tolerance? Opinions vary with respect to the best experimental approaches to answer these questions. On the one hand, it is often said that research should focus on our most important agricultural and landscape plants, since breakthroughs with these plants would be expected to result in rapid payoffs practically. On the other hand, other researchers emphasize the idea that plant research should be focused on *Arabidopsis* as a model system, keeping in mind the advantages of technologies based on knockout mutants, transformation and gene expression profiling. *Arabidopsis* was introduced as a model system for plant research about 25 years ago and approximately 50% of all plant researchers in the world today study this plant. The extent to which discoveries with *Arabidopsis* will be transferable to other plant species remains to be determined.

The basis for the research reported in the present chapter were investigations pioneered by Professor Norio Murata during the last 10 years, demonstrating that glycine betaine (GB) can confer tolerance to several types of stress at low concentrations, including chilling stress, either after application to plants or in transgenics engineered to overproduce GB. GB is a widely distributed natural product in plants (Rhodes and Hanson, 1993; Ingram and Bartels, 1996) that has been demonstrated to improve

 Plant Cold Hardiness: From the Laboratory to the Field (eds L. Gusta, M. Wisniewski and K. Tanino)

stress tolerance in agriculturally important crops (Allard *et al.,* 1998; Chen *et al.,* 2000; Rahman *et al.,* 2002). Examples of stress-tolerant transgenics engineered to produce GB (Sakamoto and Murata, 2000, 2001; Chen and Murata, 2002; Park *et al.,* 2004) include rice, tobacco, tomato, *Brassica napus*, *Brassica juncos* and *Diospyrus kaki*. GB production has also been shown to improve stress tolerance in bacteria and algae (Nomura *et al.,* 1995; Kempf and Bremer, 1998).

GB levels in transgenic plants are often quite low, and the extreme examples are transgenic tobacco plants with improved salt and chilling tolerance associated with a GB concentration of 0.035 µmol/g fresh weight (Holmström *et al.,* 2000). Given this fact and the demonstration that GB application can affect gene expression (Allard *et al.,* 1998), we hypothesized that GB might confer stress tolerance, at least in part, via effects on gene expression rather than by its known effects on osmotic pressure or protein stability. If this could be shown, then a new approach for identifying chilling stress determinants in plants would be established, using GB in a chemical genetic screen. The rationale of this approach would be that at least some of the genes up- or down-regulated by GB play a role in chilling sensitivity and/or tolerance to chilling stress. In other words, by identifying GB-regulated genes, the potential exists to identify new chilling stress determinants.

Because the best system for conducting these types of experiments was with *Arabidopsis*, we began conducting global gene expression studies with microarrays followed by confirmation on Northern blots to identify GB-regulated genes. Several up-regulated genes were detected in leaves (Einset, 2006) and roots (Einset *et al.,* 2007a). That GB's effect depends on gene expression was first proved by direct functional evidence using a knockout mutant for RabA4c GTPase (At5g47960). Although *Arabidopsis* is usually defined as chilling-resistant because it shows no obvious signs of chilling injury, chilling does have an effect because chilled plants show inhibited root growth upon transfer back to normal temperatures. Remarkably, when wild-type plants were pre-treated with GB, root growth rates after chilling were comparable to non-chilled plants. By contrast, the *RabA4c GTPase* knockout showed no GB response in the chilling test, proving the requirement for a functional *RabA4c GTPase* gene for GB's effect (Einset *et al.,* 2007a). The chemical genetics idea was validated!

The chapter begins by giving an update on studies using GB-based chemical genetics. After demonstrating the overall approach with results from a putative basic leucine zipper (bZIP) transcription factor, evidence is presented further implicating membrane trafficking processes and reactive oxygen species (ROS) in chilling stress. Finally, a model for ROS signalling in chilling stress is presented along with suggestions about important areas for future research.

Materials and Methods

Plant materials, growth conditions, chilling and chemical treatments

Arabidopsis thaliana plants were grown in 8.5 cm diameter plastic Petri plates containing MS medium, 30 g sucrose/l and 8 g agar/l under controlled environment conditions at 24°C, 16 h photoperiod and light intensity of 80 µM/m^2/s. Seed for making homozygous knockouts of the *bZIP* gene (knockout SALK_004683) were obtained through SIGnAL (http://signal.salk.edu/tdna_protocols.html). In experiments testing recovery from chilling, plants at the rosette stage of growth, treated for 24 h either with GB or with water as controls, were transferred to 4°C and 30 µM/m^2/s continuous light for 2 days, after which time they were transferred back to 24°C, 16 h photoperiod and 80 µM/m^2/s 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 mM GB only while control plants were sprayed with water. For brefeldin A (BFA) experiments, plants were treated with a solution containing 10 µM BFA immediately before transfer to chilling conditions.

Detection of superoxide

For detecting superoxide, plant material was vacuum-infiltrated with 0.1 mg nitroblue tetrazolium (NBT)/ml in 25 mM 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 mM $MnCl_2$ and 10 units of superoxide dismutase/ml 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 colour camera. The Leica DC program was used to import images of NBT-stained roots into Adobe Photoshop Elements 2.0. Images were then analysed 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 either GB or BFA (10 μM solution) pre-treatments were analysed 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

Pursuing the goal of identifying genes regulated by GB treatments, we began gene expression profiling using DNA microarrays five years ago. Our first experiments were done with Incyte Genomics *Arabidopsis* GEM1 chip with 6000+ different genes. Next, we began collaboration with Atle Bones' group at the Norwegian University of Science and Technology in Trondheim. Over the years, the Trondheim Group has developed their own unique oligonucleotide sets for genes that have been implicated in stress responses. One array (1.1K chip) comprises eight copies each of 1100 genes, while the other custom array has eight copies each of 2000 genes (2K chip). Lately, we have used arrays printed with the 2K gene set plus Operon Biotechnologies GmbH's 70-mer oligonucleotide set for over 26,000 *Arabidopsis* genes.

In conducting experiments, tissue samples from control plants and 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. After quantification of signal intensities, the data were normalized to correct for differences in probe labelling, background levels, inconsistency in replicates on the same array and non-linearity of intensity distributions. Next, we identified genes showing significant and consistent up-regulation by GB in at least three independent experiments. Finally, to confirm up-regulation using an alternative method, we conducted gene expression analyses using Northern blots. So far, we have obtained Northern blot confirmation for significant up-regulation of the genes encoding the following in roots and leaves: membrane trafficking RabA4c GTPase, a bZIP transcription factor (At3g62420), mitochondrial catalase 2 (At4g35090), cell wall peroxidase ATP3a (At5g64100) and a glutathione *S*-transferase (At1g02930). In roots only, we have confirmed up-regulation of genes encoding tonoplast aquaporin (At5g47450) and the NADPH-dependent ferric reductase FRO2 (At1g01580) localized to the plasma membrane.

The fact that the level of mRNA for a particular gene increases upon GB pre-treatment is not sufficient by itself to show that GB's effect on chilling tolerance requires this up-regulation of mRNA levels, even if the particular gene involved has already been implicated in relation to stress in other systems. What is needed is a direct demonstration of the role of specific genes in relation to stress tolerance and the effect of GB. This has already been accomplished using mutants for the membrane trafficking RabA4c GTPase (Einset *et al.,* 2007a) and FRO2 (Einset *et al*., 2008). Figure 25.1 presents a similar demonstration for the putative bZIP transcription factor, showing the effect of GB pre-treatments on chilling recovery by wild-type plants and knockout mutants for the putative *bZIP* transcription gene. Twenty-four hours after treatment with GB, plants were transferred to

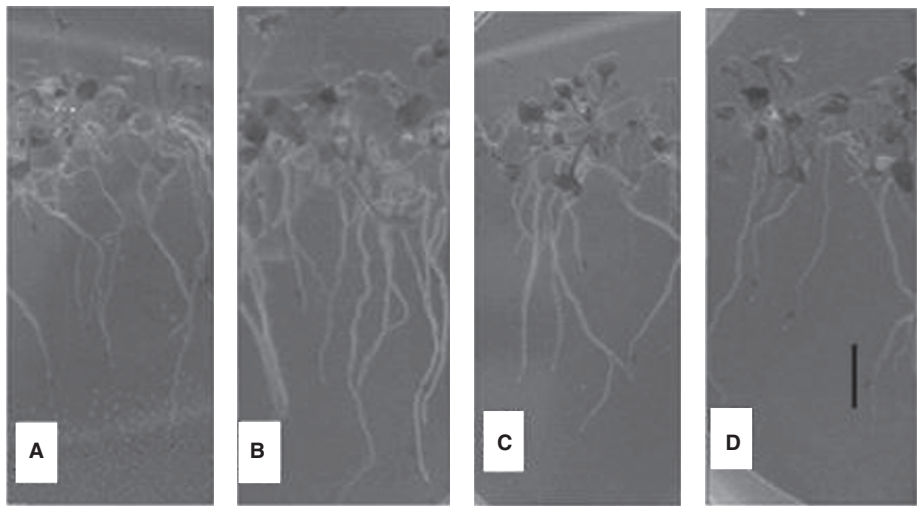

Fig. 25.1. Effect of glycine betaine (GB) on recovery from chilling stress by wild-type and *bZIP*-knockout mutant *Arabidopsis*. Plants approximately 2 weeks old were sprayed with 100 mM GB, then incubated 24 h at 24°C, 16 h photoperiod and 40 μM/m²/s light intensity before being transferred to chilling conditions at 4°C and 30 μM/m²/s light intensity for 48 h. Photographs show plants four days after transfer back to normal growing conditions at 24°C, 16 h photoperiod and 40 μM/m²/s light intensity and incubated vertically. All photographs are presented with the same magnification and the vertical bar in (D) corresponds to 1 cm. Treatments were: (A) Columbia without GB pre-treatment; (B) Columbia with GB pre-treatment; (C) *bZIP* knockout without GB pre-treatment; (D) *bZIP* knockout with GB pre-treatment.

chilling conditions at 4°C and 20 μM/m²/s light intensity. 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. 25.1 shows, wild-type Columbia responded markedly to GB pre-treatments, as demonstrated by the large difference in shoot and root growth plus versus minus GB, while *bZIP* knockouts showed little, if any, response. This proves that bZIP is required for GB's effect on chilling recovery. Figure 25.2

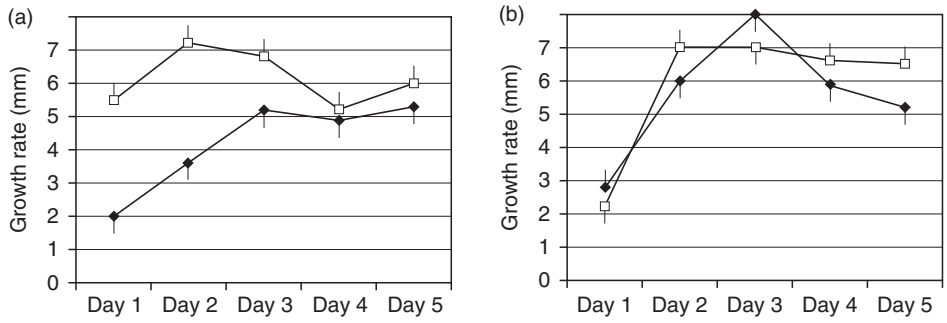

Fig. 25.2. Root growth rates during recovery from chilling by (a) wild-type Columbia and (b) *bZIP*-knockout *Arabidopsis* plants, pre-treated with glycine betaine (GB) (–□–) or with no GB pre-treatment (–■–). Daily growth increments, measured as mm growth per day, were determined for randomly selected 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 40 μM/m²/s light intensity. Each point is mean value based on pooled results from five independent experiments with at least ten independent measurements per data point; standard errors are represented by vertical bars.

presents root growth rates during recovery and also shows that the response to GB by wild-type plants can be seen by measuring root growth as early as one day after plants were transferred back to normal growth conditions after the 2 days of chilling treatment. Five days later, all treatments had similar root growth rates, indicating that the *bZIP* knockouts are not simply less robust in their growth under normal conditions.

So far, three genes have been identified with this approach: those encoding a membrane trafficking RabA4c GTPase, a NADPH-dependent ferric reductase (FRO2) and a bZIP transcription factor. The fact that both the RabA4c GTPase and the plasma membrane NADPH-dependent ferric reductase FRO2 are both membrane proteins in epidermal cells of *Arabidopsis* roots has focused our attention on the possibility that membrane trafficking processes might be central to chilling sensitivity. For example, does the increase in ROS associated with chilling require membrane trafficking? An experiment to answer this question is shown in Fig. 25.3. In this case, roots of wild-type Columbia were pre-treated with a solution containing 10 μM BFA, an inhibitor of Golgi to plasma membrane vesicle trafficking, or with 100 mM GB prior to placing plants in the cold. Two days later, plants were removed from chilling and superoxide was visualized in roots using NBT staining and ROS staining was analysed in photographs using ImageJ. As Fig. 25.3 shows, ROS accumulation was markedly decreased in both GB- and BFA-treated roots compared with control roots, indicating that inhibition of membrane trafficking can cause an inhibition of ROS accumulation during chilling. ROS staining of GB- versus BFA-treated roots was not significantly different.

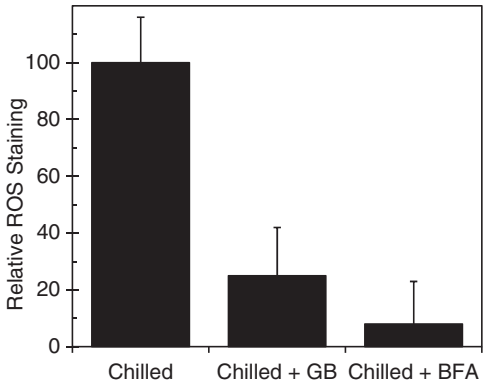

Fig. 25.3. Levels of reactive oxygen species (ROS), measured as blue pixel intensities using ImageJ with nitroblue tetrazolium-stained roots, in chilled *Arabidopsis* plants of wild-type Columbia not pre-treated with glycine betaine (GB), pre-treated with GB or pre-treated with brefeldin A (BFA). Data are means based on pooled results from three independent experiments with standard errors represented by vertical bars.

Discussion

Before the introduction of *Arabidopsis* as a model system, experiments to identify processes and genes involved in chilling tolerance were based on hypotheses generated via understanding of fundamental processes. In the 1970s, for example, Lyons and Raison proposed an explanation for the molecular mechanism determining chilling sensitivity based on membrane phase transitions occurring at low temperatures and resulting in destructive events such as membrane damage, ion leakage, impaired photosynthesis and respiration, as well as the production of toxic compounds (Lyons and Raison, 1970; Lyons, 1973). This focus on membrane phase transitions led to several studies examining the role of unsaturated membrane lipids on chilling sensitivity (Murata, 1983). Among other findings, a correlation was reported between the unsaturation level of membrane lipids and chilling sensitivity in tests of several herbaceous species (Nishida and Murata, 1996). In addition, it was shown that manipulation of the unsaturation in chloroplast membrane lipids in transgenic plants and blue-green algae had effects on chilling sensitivity (Murata *et al*., 1992). On the other hand, it has also been shown that many chilling-resistant plants do not have high levels of unsaturated membrane lipids. In addition, examples are found in *Arabidopsis*, for example, where mutants having large changes in chloroplast membrane lipids had no change in chilling sensitiv-

ity (Wu and Browse, 1995). The conclusion, therefore, has been that membrane lipid composition and membrane phase transitions are not the only factors determining chilling sensitivity.

With the beginning of the *Arabidopsis* era during the 1980s, new hypothesis-generating approaches became possible based on: (i) rapid screening for mutants that are less tolerant (Warren *et al*., 1996; Thorlby *et al*., 2004) or more tolerant (Xin and Browse, 1998) to cold temperatures; (ii) gene expression studies to identify genes affected by cold temperatures (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger *et al*., 1997; Thomashow, 2001; Viswanathan and Zhu, 2002); and (iii) chemical genetics approaches such as the one described in this chapter.

At the same time that we were conducting experiments to identify genes required for GB's effect on chilling tolerance, a close relationship was found between ROS accumulation and chilling stress. To summarize several independent lines of evidence in this regard, we found that: (i) chilling results in increases in ROS levels (superoxide and hydrogen peroxide) in both roots and leaves; (ii) during recovery from chilling under normal growth conditions, ROS disappears in parallel with growth recovery; (iii) GB pre-treatments prevent ROS accumulation during chilling, as well as the root growth inhibition seen after transfer of plants to normal growth temperatures; (iv) GB pre-treatments up-regulate several genes known to be involved in ROS scavenging; (v) mutants that are unresponsive to GB accumulate ROS with or without GB pre-treatments; (vi) GB does not prevent chilling effects in these mutants; and, finally, (vii) treatment of plants with hydrogen peroxide (0.05%) alone without chilling results in the same kinds of inhibition of root growth and elevated ROS levels as seen in chilled plants.

Possibly the most remarkable finding cited above was that plants pre-treated with GB do not show reduced growth rates after chilling. This observation makes it seem as though chilled plants can avoid the effects of chilling stress as long as they can avoid ROS accumulation. To stimulate further mechanistic studies in relation to the role of ROS in chilling stress, we present a model involving ROS signalling as the molecular mechanism determining chilling sensitivity in Fig. 25.4. According to this model, chilling stress begins with perception of low temperature by an unknown receptor, followed by activation of membrane NADPH oxidase (NOX), causing an increase in ROS levels which signals altered gene expression, resulting in inhibited growth under normal growing conditions in *Arabidopsis* and in more severe effects in chilling-sensitive plants.

Although the actual mechanism of ROS production in the cold is not known, NOX activity is an interesting possibility inasmuch as NOX has been demonstrated in *Arabidopsis* 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). Considerable knowledge gaps exist with regard to plant NOX enzymes. In neutrophils activation of NOX involves membrane trafficking as well as other proteins such as membrane trafficking Rop GTPases (Bedar and Krause, 2007). If similar processes are occurring in *Arabidopsis* roots, this could

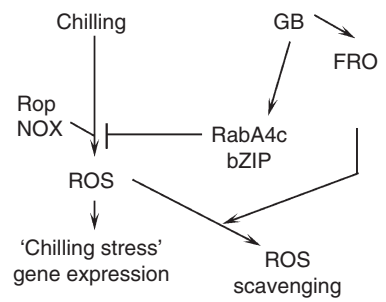

Fig. 25.4. Schematic representation of a model for chilling stress as reactive oxygen species (ROS) signalling and gene expression. A competing pathway based on glycine betaine (GB) action via gene expression, membrane trafficking and extracellular ferric ion reduction blocks ROS accumulation and alleviates chilling stress. bZIP, basic leucine zipper transcription factor; FRO, NADPH-dependent ferric reductase; NOX, NADPH oxidase; RabA4c, RabA4c GTPases; Rop, Rop GTPases.

explain our observation that BFA blocks ROS accumulation during chilling. In fact, a recent paper by Jones *et al*. (2007) suggests that enhanced Rop GTPase activity can heighten ROS production regulating root hair development in *Arabidopsis*. Based on all of these lines of evidence, we suggest that Rop GTPases may affect ROS production during chilling via NOX activation. As the model suggests, the working hypothesis is that GB upregulates membrane trafficking associated with RabA4c GTPases and that this process competes with Rop GTPase membrane trafficking associated with NOX activation.

Another feature of the model is the involvement of Fe via markedly elevated levels of NADPH-dependent ferric reductase occurring during chilling in plants pre-treated with GB (Einset *et al*., 2007b). If one makes the assumption that NOX in plants is similar to neutrophil NOX (Bedar and Krause, 2007), then superoxide would initially be produced outside the plasma membrane. By increasing plasma membrane-localized ferric reductase activities, then GB increases transfer of reductant potential from cytoplasmic NADPH to the cell wall of root epidermal cells, effectively stopping ROS build-up in roots, both extra- and intracellularly, along with attendant ROS accumulation associated with chilling stress.

Conclusion

In conclusion, results from chemical genetic studies on chilling stress seem to be focusing attention on ROS as well as associated processes such as membrane trafficking and Fe metabolism. Major unanswered questions involve the mechanism of ROS production and the factors that regulate it. Further questions revolve around downstream components of the ROS signalling system. In animal systems, ROS levels comparable to those we see in chilled *Arabidopsis* roots activate protein kinases (Burgoyne *et al*., 2007), initiating signalling cascades. The possibility exists that ROS might be regulatory in *Arabidopsis* in relation to mitogen-activated protein (MAP) kinase signalling, for example. If so, then one would have to ask whether it is ROS that is dominant in determining the system's dynamics or other components such as the MAP kinases. Finally, by analogy to ROS signalling during root hair formation, the involvement of Ca^{2+} channels is likely.

All in all, only the results of future experiments will be able to resolve the issue of whether chemical genetic studies with *Arabidopsis* can give us a fundamentally better understanding of cold tolerance mechanisms with the view to producing improved plant types for cold stress.

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