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NO way to treat a cold

Temperature is one of the crucial factors that determines plant survival and distribution on the Earth. Various plant species acquire enhanced freezing tolerance by cold acclimation in which prior exposure to low, but nonfreezing, temperatures boosts the chances of surviving subsequent freezing events. Various physiological and biochemical changes take place during the cold acclimation, for example, an increase in osmolites, such as proline (Zhao *et al.*, 2009), and ice-crystal formation in intercellular spaces (Ashraf & Foolad, 2007). These changes are reflected in a massive reprogramming of both the transcriptome and the metabolome (see e.g. Guy *et al.*, 2008 and Thomashow, 2010 for recent reviews). Now, in this issue of *New Phytologist*, Cantrel *et al.* (pp. 415–427) have clearly established nitric oxide (NO) as a key player in the plant response to cold stress and have demonstrated that it plays a central role in modulating the synthesis of sphingolipid signals. Based on these insights, novel strategies to improve cold tolerance in plants may be expected.

'Crucially, the authors used multiple approaches to determine NO production.'

How cold stress can be sensed by plants

Plants can sense cold stress by physical changes in cell constituents, such as a decrease in membrane fluidity, which will, among other factors, influence the activity of enzymes that act on membrane lipids, such as diacylglycerol kinase (DAGK) and phospholipase D (PLD) (Ruelland *et al.*, 2002). Vaultier *et al.* (2006) used desaturase mutants to show that membrane rigidification is a key early response in cold perception that induces cold responsive (*COR*) genes, for example, in *Brassica napus* (Orvar *et al.*, 2000; Sangwan *et al.*, 2001). Cold-induced membrane rigidification leads to an increase in cytosolic Ca^{2+} via ligand-activated calcium channels (Komatsu *et al.*, 2007), whilst DAGK/PLD-derived phosphatidic acid may trigger an increase in the production of reactive oxygen species (ROS). However, any beneficial effects of ROS in cold acclimation are probably concentration-dependent, as the cold-sensitive Arabidopsis frostbite1 (*fro1*) mutant (mutated at the site of complex 1 of the mitochondrial electron transport chain) produces high concentrations of ROS and was shown to be impaired in *COR* gene induction (Suzuki & Mittler, 2006). Clearly, plants have developed elaborate and efficient ROS scavenging and antioxidant defense mechanisms that suppress toxic concentrations of ROS and allow the use of low concentrations of ROS as signal transduction mediators. In this context, it is relevant to note that NO can act as an antioxidant in various stress conditions (Beligni & Lamattina, 1999) and this function should be considered along with direct signalling roles for NO in cold acclimation.

A specific role of NO during cold stress

During the past decade, NO has emerged as an important signalling molecule in plants that is involved in a multitude of physiological and developmental processes (see Besson-Bard *et al.*, 2008 for a review). Plants contain various pathways for NO production: these are nitrite-dependent and L-arginine (L-Arg)-dependent pathways (Besson-Bard *et al.*, 2008). It is still uncertain whether a nitric oxide synthase (NOS)-like enzyme exists in plants (Moreau *et al.*, 2010). However, several reports show increased NOS activity upon supply of L-Arg and also under various stresses (Corpas *et al.*, 2009). To date, the most intensively studied NO-producing enzyme is nitrate reductase (NR). Nitrate reductase is encoded by two genes (*NIA1* and *NIA2*), which encode proteins that, respectively, contribute *c.* 10% and 90% of the total NR activity (Wilkinson & Crawford, 1993).

Within the context of temperature stress, NO is involved in the plant response to high- and low-temperature stress. Nitric oxide-mediated signal transduction in various abiotic stresses is associated with ABA, Ca^{2+} and H_2O_2, features shared by signalling events linked to cold stress. In addition, the supply of exogenous NO increased cold tolerance in various plant species such as wheat, maize and tomato (Neill *et al.*, 2003). Equally, temperature-responsive roles for NO have been related to the antioxidative property of NO (Beligni *et al.*, 2002) by suppressing peroxidative metabolism (Neill *et al.*, 2002).

Nitric oxide can influence cellular processes through the *S*-nitrosylation of protein thiols to form *S*-nitrosothiols. *S*-nitrosylation can activate or repress protein functions. Abat & Deswal (2009) detected several *S*-nitrosothiols in *Brassica juncia* seedlings exposed to cold for 1–6 h, and cold-induced nitrosylation changes were noted in *c.* 20 proteins, including antioxidant enzymes and both subunits of Rubisco.

Both NR (Zhao *et al.*, 2009) and a NOS-like enzyme have been suggested as sources of NO in response to cold (Corpas *et al.*, 2008). This controversy has now been resolved by careful experimentation carried out by Cantrel *et al.* The authors demonstrated rapid production of NO when Arabidopsis plants were exposed to cold. Crucially,

the authors used multiple approaches to determine NO production. Nitric oxide emission measurements were made using the widely employed diamino fluorescein diacetate (DAF-2DA) dye together with a chemiluminescence method. This type of dual check is necessary in NO research because no single method yields unambiguous results. Each method has its advantages and disadvantages. The advantage of DAF-2DA is that it can penetrate into the cells, close to NO-producing sites and gives fluorescence, but it suffers from a lack of specificity for NO because it can also react with other oxidized products (Planchet & Kaiser, 2006). Chemiluminescence is a widely accepted method but measures NO only in the gas phase and thus only represents a fraction of *in planta* NO production. In addition, the half life of NO under normoxic conditions needs to be considered.

Using both assays, Cantrel *et al.* showed that NR was the source of NO during cold exposure. Initially, tungstate treatment (at the recommended concentration) was shown to suppress NO production. However, because of the dubious specificity of tungstate for NR, as it can also inhibit other haem-containing enzymes, the authors verified this

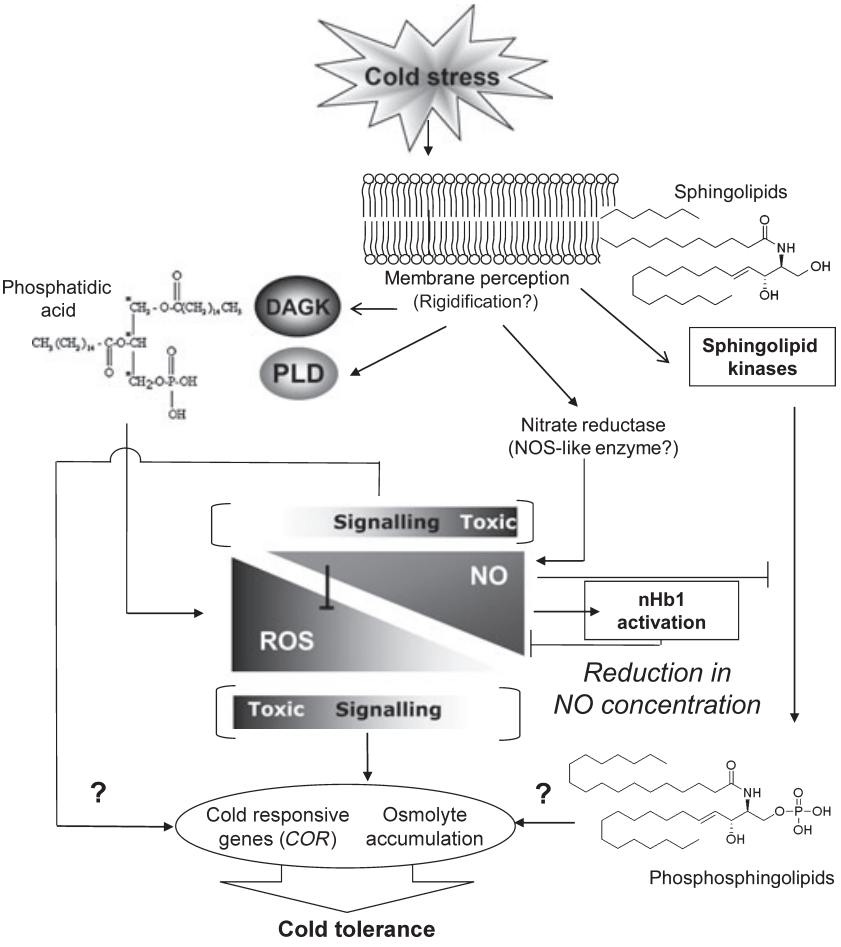

Fig. 1 Contributions of nitric oxide (NO) and sphingolipids to cold tolerance in plants. A model is presented highlighting two novel contributors to cold stress tolerance: an interactive reactive oxygen species (ROS)–NO concentration-dependent signalling mechanism; and phosphosphingolipids. Cold stress is perceived, probably as a result of reduced phospholipid mobility (rigidification), which results in the production of phosphatidic acid through the activation of diacylglycerol kinase (DAGK) and/or phospholipase D (PLD). Phosphatidic acid activates the production of ROS. Simultaneously, NO is generated by nitrate reductase, with a possible contribution from a nitric oxide synthase (NOS)-like enzyme. Both NO and ROS are toxic at higher concentrations so they require modulation to concentrations that allow ROS- and NO-mediated signalling mechanisms to occur. These are likely to include initiating cold responsive gene (*COR*) expression and osmolyte accumulation. The ROS concentrations are likely to be directly suppressed by NO. However, the content of NO is reduced to a subtoxic concentration by haemoglobins (Hb). Haemoglobin is boxed because increased effectiveness of this enzyme could prevent toxic concentrations of NO accumulating during cold stress and may encourage signalling, leading to the development of plant tolerance. Therefore, Hb could be targeted in strategies aiming to increase cold tolerance in crops. Another signalling mechanism is the cold activation of sphingolipid kinases to phosphophorylate sphingolipids (a ceramide is illustrated). An association between phosphosphingolipids and *COR* and osmolyte accumulation is hypothesized, and could equally contribute to the ROS–NO regulation mechanism or act via an independent route. The sphingokinase is boxed as another potential target through which cold tolerance could be manipulated. nHb, nonsymbiotic haemoglobins.

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result using the *nia1nia2* NR double mutant that also showed no cold-induced increase in NO production. Using the mammalian NOS inhibitor N^G-nitro-L-arginine-methyl ester (L-NAME), the authors established that NOS-like activity was not responsible for the cold-induced NO production. This is in contrast to the report of Corpas *et al.* (2009), emphasizing, in our opinion, the importance of using multiple methods to measure NO.

A role for nonsymbiotic haemoglobins under cold stress

Plant nonsymbiotic haemoglobins (nHb) are known to scavenge NO (Dordas *et al.*, 2003). There are two classes of nonsymbiotic haemoglobins within plants: one forms a 3-on-3 α-helical sandwich embracing a haem and the other, a truncated version, forms a 2-on-2 α-helical sandwich (Wittenberg *et al.*, 2002). In *Arabidopsis* there are two differing 3-on-3 α-3 forms (these are encoded by Hb1 (At2g16060) and Hb2 (At3g10520)), and a 2-on-2-fold form denoted Glb3 (At4g32690) has also been identified in plants (Watts *et al.*, 2001). One very interesting observation made by Cantrel *et al.* was Hb up-regulation in response to cold (Fig. 1). This suggests that the Hb could be a modifier of the cold response through the modulation of NO and sphingolipid production. This is substantiated by the observation that Hb over-expressing lines showed reduced expression of cold-induced genes. Interestingly, this effect was highly gene specific, as only the expressions of *CBF1* and *CBF3* were reduced, not that of *CBF2*. This differential regulation of the CBF transcription factors is in agreement with the results of earlier investigations (Novillo *et al.*, 2004, 2007; Gery *et al.*, 2011). Clearly, these observations need to be developed further to establish the roles of specific Hb genes and, most importantly, their effects on cold acclimation and freezing tolerance. This could establish whether modulating the expression of Hb in crop species, either through selection of suitable germplasm from breeding populations or via genetic modification (GM) approaches, could be an appropriate strategy to increase freezing tolerance in agriculturally relevant species.

The involvement of NO in lipid-based cold signalling

Phosphatidic acid has been shown to be involved in the early steps of plant cold signalling (Ruelland *et al.*, 2002). However, Cantrel *et al.* demonstrate, with the help of inhibitors, that the increase in phosphatidic acid is not mediated by the cold-triggered NO production. Instead, they provide evidence for an involvement of NO in phosphosphingolipid signalling. It is shown that sphingolipids are transiently phosphorylated in response to cold exposure and that NO serves as a negative regulator of this phosphorylation. This is the first time that either sphingolipid phosphorylation or its regulation by NO have been implicated in plant cold signal transduction and this finding opens the way for a wide array of future studies that will significantly increase our understanding of these complex and inter-related processes.

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Key words: chemiluminescence, cold acclimation, nitrate reductase, nitric oxide, nitric oxide synthase.

A new tool for functional genomics in maize

Maize provides one of the fundamental sources of nutrition for the world's population. Continued improvement in the performance of maize is essential to meet the needs as the global population increases. In many ways maize research is poised for a wave of progress as its rich genetic diversity can now be more effectively exploited through resources such as the recently completed maize B73 genome sequence (Schnable *et al.*, 2009), other genome sequences that are nearing completion (e.g. Mo17 at http://www.phytozome.net/maize and Palomero Toluqueno http://www.palomerotoluqueno.org/) and the 5000-line nested association mapping population (McMullen *et al.*, 2009). However, utilization of genome sequence and the linking of DNA sequence to function often require research tools that have been lacking in maize, in which it is particularly laborious to generate transgenic plants. In this issue of *New Phytologist*, van der Linde *et al.* (pp. 471–483) report significant progress that should facilitate the process of establishing the function of maize genes.

'The development of efficient VIGS tools for maize is a very significant advance for maize research.'

In van der Linde *et al.*'s report, a virus-induced gene silencing (VIGS) system is utilized to assess whether candidate genes have essential functions in determining the outcome of interactions between maize and the biotrophic fungal pathogen *Ustilago maydis*, the causal agent of corn smut. In addition to being a major pathogen of maize, the *U. maydis*–maize interaction is one of the best-developed model fungal pathosystems of maize. During the initial 12 h of infection, defense genes are highly expressed in both compatible and incompatible *U. maydis*–maize interactions (Doehlemann *et al.*, 2008, 2009). At the 24 h time-point during the compatible interaction, when the biotrophic interface has been established, expression of defense genes is suppressed and expression of the well-known suppressor of cell death, Bax-inhibitor 1 (*BI1*), is strongly induced (Doehlemann *et al.*, 2008). By contrast, in the incompatible interaction with a mutant strain of *U. maydis* lacking the effector protein, Pep1, suppression of the defense genes does