Systemic low temperature signaling in Arabidopsis

Peter A. Gorsuch1, Alexander W. Sargeant1,2, Steven D. Penfield3, W. Paul Quick4 and Owen K. Atkin1,5,*

1Department of Biology, University of York, PO Box 373, York YO10 5YW, UK
2School of Biological and Biomedical Sciences, Durham University, Durham DH1 3LE, UK
3Centre for Novel Agricultural Products, Department of Biology, University of York, PO Box 373, York YO10 5YW, UK
4Department of Animal and Plant Sciences, University of Sheffield, Western Bank, Sheffield S10 2TN, UK
5Plant Science Division, Research School of Biology, Building 46, The Australian National University, Canberra, ACT, 0200, Australia

*Corresponding author: E-mail, Owen.Atkin@anu.edu.au; Fax, +61-2-6125-5095
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Introduction

Plants exhibit a wide range of phenotypes when exposed to different environments. For example, growing entire plants under high light results in leaves whose anatomical and physiological traits differ markedly from those of their low-light grown counterparts (Hanson 1917, Terashima et al. 2006, Athanasiou et al. 2010). Similarly, the phenotype of plants is strongly influenced by the growth temperature directly experienced by tissues during their development (Strand et al. 1999, Loveys et al. 2003, Atkin et al. 2006). Whether such changes in leaf phenotype can also occur in response to conditions experienced by other tissues on the same plant has increasingly been a focus of research, with numerous studies showing that the phenotype of tissues can be altered systemically in response to remote, localized stimuli experienced elsewhere on the same plant. Examples include pathogen attack (Chester 1933) and leaf injury (Green and Ryan 1972), with jasmonic acid a possible systemic signal in leaf defense (Li et al. 2002, Wasternack et al. 2006). Similarly, the treatment of mature, expanded leaves of Arabidopsis with air of known atmospheric CO2 concentration alters stomatal initiation and density in developing leaves on the same plant (Lake et al. 2001, Lake et al. 2002). Systemic effects have also been observed in response to remote, localized excess light treatment (Karpinski et al. 1999, Lake et al. 2001, Yano and Terashima, 2001, Thomas et al. 2003, Rossel et al. 2007). A common conclusion to these investigations is that developing leaves are unable to sense directly and accurately their immediate environment, and that they rely on signals from developed tissues to alter leaf phenotypes. The eventual phenotype of leaves is to some degree determined by exposure to cold for a short period early in development (Gorsuch et al. 2010a). Whether systemic signaling plays a role in determining how plants sense and respond to changes in temperature is, however, not known.

One of the most intensively studied fields of environmental plant science is that of low temperature responses of cold-tolerant plants, and subsequent acclimation. In the cold, enzyme function is constrained by low kinetic activity of the

Abbreviations: ANOVA, analysis of variance; COR, cold-responsive; LSD, least significant difference; LUC, luciferase; ROS, reactive oxygen species; RT–PCR, reverse transcription–PCR; RH, relative humidity; sHSP, small heat-shock protein.

Keywords: Cold acclimation • Cold stress • C-repeat binding factor • KIN2 • Leaf development • Systemic signaling.

When leaves are exposed to low temperature, sugars accumulate and transcription factors in the C-repeat binding factor (CBF) family are expressed, which, together with CBF-independent pathways, are known to contribute to the cold acclimation process and an increase in freezing tolerance. What is not known, however, is whether expression of these cold-regulated genes can be induced systemically in response to a localized cold treatment. To address this, pre-existing, mature leaves of warm-grown Arabidopsis thaliana were exposed to a localized cold treatment (near 10°C) whilst conjoined newly developing leaves continued only to experience warmer temperatures. In initial experiments on wild-type A. thaliana (Col-0) using real-time reverse transcription–PCR (RT–PCR) we observed that some genes— including CBF genes, certain downstream cold-responsive (COR) targets and CBF-independent transcription factors— respond to a direct 9°C treatment of whole plants. In subsequent experiments, we found that the treatment of expanded leaves with temperatures near 10°C can induce cold-associated genes in conjoined warm-maintained tissues. CBF1 showed a particularly strong systemic response, although CBF-independent transcription factors also responded. Moreover, the localized cold treatment of A. thaliana (C24) plants with a luciferase reporter fused to the promoter region of KIN2 indicated that in warm-maintained leaves, KIN2 might respond to a systemic signal from remote, directly cold-treated leaves. Collectively, our study provides strong evidence that the processes involved in cold acclimation are partially mediated by a signal that acts systemically. This has the potential to act as an early-warning system to enable developing leaves to cope better with the cold environment in which they are growing.

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system, and compensatory mechanisms, such as an increase in enzyme activity (particularly those involved in sucrose synthesis) can occur (Guy et al. 1992, Holaday et al. 1992, Strand et al. 1999, Stitt and Hurry, 2002). Exposure to cold results in depolarization of the plasma membrane, along with a rise in cytosolic calcium which leads to further signaling, and changes in gene expression (Knight et al. 1996, Plieth et al. 1999, Knight, 2002, Penfield, 2008). Exposure to cold results in depolarization of the plasma membrane, along with a rise in cytosolic calcium which leads to further signaling, and changes in gene expression (Knight et al. 1996, Plieth et al. 1999, Knight, 2002, Penfield, 2008). Extended exposure to cold also stimulates large-scale changes in the transcriptome (Fowler and Thomashow, 2002, Chinnusamy et al. 2007, Robinson and Parkin, 2008), metabolome (Cook et al. 2004, Gray and Heath, 2005) and proteome (Goulas et al. 2006, Herman et al. 2006).

In the last 15 years, a family of transcription factors highly up-regulated by cold known as C-repeat binding factor genes (CBF1–CBF3) has received particular scrutiny, along with a subset of downstream targets, cold-responsive (COR) genes (e.g. COR6.6/KIN2; Kurkela and Franck 1990, Kurkela and Borg-Franck 1992, Kreps et al. 2002). CBF genes appear to play an important role in configuring the low temperature transcriptome at large (Fowler and Thomashow, 2002, Chinnusamy et al. 2007) and, together with genes on the CBF regulon, have been shown to enhance freezing tolerance (Jaglo-Ottosen et al. 1998, Steponkus et al. 1998, Gilmour et al. 2004). Other, independent pathways also operate (Fowler and Thomashow, 2002, Medina et al. 2005, Vogel et al. 2005, Chinnusamy et al. 2007). Such studies have typically exposed plants to low temperatures (e.g. 4°C). However, increased expression of CBF has also been demonstrated from around 12°C in plants experiencing a gradual decrease in temperature (Zarka et al. 2003). What is not known, however, is whether expression of such genes is increased in response to remote, localized cold stimuli experienced elsewhere on the same plant.

In this study, we addressed the question of whether there is evidence of cold-induced systemic signaling in Arabidopsis thaliana Col-0 and A. thaliana C24; to do this, we exposed pre-existing mature leaves of warm-grown plants to a localized cold treatment, whilst conjoined newly developing leaves continued to experience only warmer temperatures. Using a combination of real-time reverse transcription–PCR (RT–PCR) and luciferase reporter experiments, we found that exposure of warm-grown expanded leaves to low temperature induces the expression of cold-associated genes in conjoined warm-maintained tissues, strongly suggesting the existence of systemic temperature signaling.

Results

Direct temperature treatment

An initial real-time RT–PCR experiment was performed to quantify temporal changes in gene expression when whole plants were directly exposed to moderately low (9°C) temperatures, relative to control plants kept at 20°C (Fig. 1). The resulting relative induction data also enabled us to determine which candidate target genes to analyze at three time points:

![Fig. 1](image-url)
points (2, 6 and 24h) in subsequent localized cold treatment experiments (induction data for genes that were analyzed in the initial experiment but did not respond are not shown). Leaf insertion 18 and the central meristematic ‘plug’ were analyzed from each of four 6-week old A. thaliana (Col-0) at each time point.

CBF1, 2 and 3 and downstream COR genes COR15a and KIN2 showed similar temporal responses (Fig. 1A–E) to those previously described (Gilmour et al. 1998, Vogel et al. 2005). A gene encoding a small heat-shock protein (sHSP, At2g29500), known to be induced by high light treatment (Rossel et al. 2007), was also induced in leaf insertion 18 after 2h of direct cold treatment (Fig. 1F). The alternative NAD(P)H dehydrogenase gene, NDB2, exhibited a >3-fold increase in expression in the central meristematic plug after 6h of direct cold treatment (Fig. 1G). Past studies have reported NDB2 transcript levels increasing following exposure to a range of abiotic stresses, including sustained cold (Gutierrez et al. 1997, Clifton et al. 2005, Armstrong et al. 2008). RCI2A [a small hydrophobic protein whose expression affects freezing tolerance and which is independent of CBF control (Capel et al. 1997, Novillo et al. 2004, Medina et al. 2005)] and ZAT12 [a transcription factor central to the cold response which is CBF independent (Fowler and Thomashow, 2002, Davletova et al. 2005, Vogel et al. 2005)] were induced at all time points in leaf insertion 18, but not the central meristematic plug (Fig. 1H and J, respectively). The induction of RCI2A and ZAT12 in the expanding leaf is also consistent with previous studies (Capel et al. 1997; Nylander et al. 2001, Fowler and Thomashow, 2002, Davletova et al. 2005, Vogel et al. 2005). The expression pattern of ZAT10 also matched previous data (Vogel et al. 2005), with expression levels in leaf insertion 18 being enhanced 2 and 6h after commencement of cold treatment (Fig. 1I). Taken together, these results show that the moderately low temperature of 9°C is low enough to induce the expression of genes related to cold acclimation in Arabidopsis in the growth conditions used in our study.

Localized low temperature treatment

To test the hypothesis that a localized cold treatment affects gene expression in remote, warm-maintained tissues, we used the apparatus shown in Fig. 2 (see also Supplementary Fig. S1) to expose most fully expanded leaves on the rosette to a mean treatment temperature of 9.8±0.6°C and quantified patterns of gene expression (using the findings of the direct temperature treatments to select target genes that were cold induced) in three tissue types: (i) directly cold-treated mature leaves; (ii) warm-maintained expanding leaves; and (iii) warm-maintained tissues in the central meristematic plug. Fig. 3 shows the patterns of relative gene expression in two tissue types [the warm-maintained expanding leaf (insertion 19) and the central meristematic plug] for all targeted genes (with analysis restricted to the time points where gene expression was increased in the direct cold treatment experiments). For each gene, expression was once again increased in the fully expanded mature leaves that were directly cold treated, across three biological replicates (data not shown). However, in several cases, expression of cold-inducible genes was also found to be increased in warm-maintained, expanding leaves whose conjoined mature leaves were cold treated. For example, CBF1

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**Fig. 2** The cold clamp apparatus. Pre-existing leaves were kept within an airspace between two cold clamps. Developing leaves grew into ambient, warm air, and were also supplied with additional piped ambient air to ensure that these leaves were not inadvertently exposed to a direct cold stimulus. All clamp walls were made from clear Perspex and newly developing leaves were insulated from the cold clamps using compressed foam. Plant roots were in hydroponics solution. (A) Cross-section; (B) photograph; and (C) false color, infrared image of the cold-treated plants of a run whose mean treatment temperature was 7.7°C at the time of the photograph, according to thermistors placed inside the clamps. The mean temperature at the centre of the rosettes was 20.1°C. Note that the condensation in B was an exceptional event and did not occur during the runs described in the text. Further details on the experimental clamp apparatus are shown in Supplementary Fig. S1.
expression was several fold higher after 2 and 24 h in warm-maintained expanding leaves from plants whose mature leaves were cold treated (compared with entirely warm-maintained control plants; Fig. 3A). Similarly, expression levels of CBF2 (Fig. 3B), RC12A (Fig. 3H), ZAT10 (Fig. 3I) and ZAT12 (Fig. 3J) were increased up to 3.0-, 2.0-, 1.7- and 3.0-fold, respectively, in warm-maintained, expanding leaves in plants where the remaining rosette was cold treated. In contrast, there was less evidence of systemic cold-induced gene expression of the other genes (CBF3 (up to 1.5-fold increase; Fig. 3C), COR15A (1.2-fold; Fig. 3D), KIN2 (1.6-fold; Fig. 3E) and NDB2 (1.3-fold; Fig. 3G)), with there being no evidence of induction of sHSP (Fig. 3F). Moreover, compared with expanding leaves, there tended to be less evidence of systemic cold-induced gene expression in the central meristematic plug.

Three tests were considered valid to analyze systemic cold induction for each gene shown in Fig. 3: least significant difference (LSD) post-hoc tests from one-way analyses of variance (ANOVA) combining all gene–time point combinations; independent t-tests for each time point; and two-way ANOVAs (with ‘temperature treatment’ and ‘time’ as factors). LSD tests from one-way ANOVAs showed significant induction of RC12A at 24 h in the warm-maintained expanding leaf whose mature leaves experienced a localized cold treatment (P < 0.05), and an increase in CBF1 (P = 0.090) in the same insertion at 2 h. Independent samples t-tests for each time point showed differences of marginal significance between warm control and expanding leaves for CBF1 at 2 h (P = 0.051), and in the central meristematic plug at 6 h for both RC12A (P = 0.053) and ZAT12 (P = 0.078). Two-way ANOVAs showed overall induction (across all time points) to be marginal for CBF1 (P = 0.079), CBF2 (P = 0.060) and RC12A (P = 0.055) in expanding leaves (Supplementary Table S4). Thus, while the statistical significance of induction varied among the genes (and was dependent on the tests used), collectively the results suggest that expression of several genes [in particular CBF1 (Fig. 3A) and RC12A (Fig. 3H)] was increased in warm-maintained expanding leaves receiving a remote, localized cold stimulus. The most responsive gene was CBF1, which showed induction in the cold treatment to at least a 10% significance level in all tests, apparently due to strong induction following 2 h of localized cold treatment.

Long-term exposure to localized cold did not significantly (one-way ANOVA) increase concentrations of the compatible solute sucrose in clamped mature leaves directly experiencing the cold (relative to warm-maintained, clamped leaves) (Fig. 4). There was no difference in sucrose concentrations exhibited by unclamped leaves, irrespective of treatment. (Fig. 4).

Data were analyzed using real-time RT–PCR; induction values are relative to warm control values for each time point, according to the 2−ΔΔCt method. Values are the mean of three biological replicate 6-week-old Arabidopsis thaliana (Col-0) plants at each time point (bars represent RQmin and RQmax, as described in Livak 2001).

Fig. 3 Induction of gene expression in warm-maintained tissues of wild-type Arabidopsis plants on which the mature leaves were subjected to a localized cold (9.8°C) treatment. (A) CBF1, (B) CBF2, (C) CBF3, (D) COR15a, (E) KIN2, (F) sHSP, (G) NDB2, (H) RC12A, (I) ZAT10, (J) ZAT12. Filled circles, leaf insertion 18; open circles, central meristematic ‘plug’. PDF2 was used as an endogenous control.
KIN2::LUC experiment: localized temperature treatment

In a parallel experiment to the real-time RT–PCR work, we analyzed the effect of localized cold treatment on transgenic Arabidopsis with a luciferase reporter fused to the promoter region of KIN2 (Fig. 5A, B). Across seven experimental runs, the unclamped leaves experienced a similar temperature to that of the warm control leaves (19.9±0.1°C and 19.3±0.1°C, respectively). Statistical analysis of control–treatment pairs across all runs (Wilcoxon signed ranks test, n = 42) showed a small but significant induction of KIN2 both in the directly treated region (P<0.005) and in the unclamped region of the plant which did not directly receive a cold stimulus (P<0.05; Fig. 5C).

Discussion

Building on previous studies describing the systemic nature of plant responses to the irradiance and atmospheric CO₂ concentration environment of mature leaves (Karpinski et al. 1999, Lake et al. 2001, Lake et al. 2002, Coupe et al. 2006, Rossel et al. 2007), our data demonstrate the existence of cold-induced systemic induction of gene expression in Arabidopsis. Having the ability to respond to a systemic temperature signal could be an advantage to developing leaves, as the shoot apex is often enclosed and thus not subject to the same aerial environment occupied by mature leaves; moreover, transpiration reduces the daytime temperature of mature leaves. As a result, the temperature of mature leaves can be substantially different from that at the shoot apex, particularly when the apex is close to the soil (Larcher 2003). This may make direct sensing of air temperature difficult and necessitate long-distance sensing/signaling.

Fig. 4 Comparison of sucrose concentrations in clamped mature leaves and unclamped, developing leaves of wild-type Arabidopsis thaliana following long-term (23 d) growth of plants in the experimental set up shown in Fig. 2. Open bars are for control plants where the clamped and unclamped leaves were maintained in the warm (20°C). Filled bars are for plants where the clamped mature leaves were at 9.5°C, and where unclamped leaves were warm-maintained [n = 3 (±SE)].

Fig. 5 (A and B) Example of KIN2::LUC induction following addition of luciferin. (A) A bright field images of two selected plants (left, plants subjected to a localized cold treatment in the clamped region; right, control plants whose clamped leaves were kept in the warm. In B, the white line shows the approximate outline of each plant and the demarcation between unclamped (UC) and clamped (C) regions. (C) Treatment fluorescence (per unit fresh mass) induction, expressed relative to the warm control value for each treatment–control pair. Both clamped (P<0.005) and unclamped (P<0.05) leaves of cold-treated plants had higher fluorescence than their control counterparts (Wilcoxon signed ranks test, n = 42).
A transcription factor of central importance to the cold response, CBF1, was amongst the genes showing the strongest systemic induction (Fig. 3). Moreover, its induction followed similar kinetics to the induction by direct cold treatment reported previously (Gilmour et al., 1998), suggesting that the observations of direct treatment studies may even reflect the consequences of rapid systemic signaling rather than the direct effects of cold per se. One other transcription factor, ZAT12, which also showed evidence of systemic induction (Fig. 3), was found by Rossel et al. (2007) to be systemically induced following excess irradiance treatment. Similarities between the systemic responses to light and cold are consistent with the observation that direct application of each stimulus can result in increased reduction of the photosynthetic electron transport chain and subsequent generation of reactive oxygen species (ROS), particularly in combination (Apel and Hirt 2004, Asada 2006, Armstrong et al. 2007), as in the treatment conditions for real-time RT-PCR experiments. However, COR15a and sHSP, which were directly induced by cold (Fig. 1) and systemically by light (Rossel et al., 2007), showed no evidence of systemic response to low temperature in the current study, indicating divergence between the stimuli. Systemic responses of ZAT12 and RCi2A may be of particular importance in that these genes have previously been shown to display independence to CBF activity (Fowler and Thomashow 2002, Novillo et al. 2004, Medina et al. 2005, Vogel et al. 2005), suggesting that, just as in directly cold-treated tissues, multiple cold signaling pathways may be systemically induced.

Use of a luciferase construct showed that the expression of KIN2 is significantly higher in warm-maintained leaves responding to a systemic signal from remote, directly cold-exposed leaves (Fig. 5C), but that the magnitude of this induction was slight compared with that seen in directly treated leaves (Fig. 1E). No significant difference was seen in the real-time RT-PCR experiments (Fig. 3E). The observed significance of the systemic induction of KIN2 using luciferase fluorescence (Fig. 5C) may therefore reflect a sample size effect (i.e., n = 42 in Fig. 5C, vs. n = 3 in Fig. 3E). A further discrepancy in KIN2 expression is seen in the response of directly treated tissues between experiments. In whole plants directly exposed to 9°C for 24 h, a developing leaf showed 27-fold induction of KIN2 (Fig. 1E). In contrast, transgenic luciferase reporter fluorescence data of similarly treated plants (at 9.5°C) implied only a 2-fold induction. Moreover, two adjoining, directly cold-treated (9.1°C) leaves on plants whose other leaves were kept warm showed much smaller increases in KIN2 (2.5- and 5-fold, respectively; data not shown). Similar, but slightly less pronounced, differences between the whole-plant and localized cold treatment real-time RT-PCR experiments were observed for COR15a, and the trend of moderated expression in the directly treated parts of plants treated with only localized cold was widespread. Further investigation is required to determine whether these differences indicate a temperature response threshold or the systemic moderation of expression by the parts of the plant that were not chilled.

Most previous studies of gene induction under a uniform cold stimulus have used temperatures of ≤5°C (Gilmour et al., 1998, Lee et al. 1999, Wanner and Junttila 1999, Fowler and Thomashow 2002, Kreps et al. 2002, Chinnusamy et al. 2003, Takagi et al. 2003, Gilmour et al. 2004, Knight et al. 2004, Clifton et al. 2005, Hannah et al. 2005, Lee et al. 2005, Oono et al. 2006, Swindell, 2006, Kaplan et al. 2007, Armstrong et al. 2008). In contrast, our results show that induction of classic cold-regulated genes can occur by a direct treatment of 9°C (Fig. 1). Our finding supports the results of Zarka et al. (2003), who found that increased expression of CBF transcripts commences when A. thaliana experiences direct exposure to 12°C (Zarka et al. 2003). The present study shows, therefore, that cold-regulated genes in this species are strongly induced by a temperature treatment of 9°C, and that induction of some of these genes is systemic.

That CBF1 (and perhaps CBF2) was systemically cold induced, but induction of CBF3 and COR target genes was slight at most in indirectly treated tissues, is intriguing. It may be that the patterns of gene expression seen in these developing leaves effectively provide a model for the separation of the action of these factors from other cold-induced genes, without recourse to transgenic plants. If so, the data available would suggest that induction of CBF1 and CBF2 on the scale shown in Fig. 3, and in the absence of CBF3, is insufficient eventually to result in COR induction. Added complexity arises from the differential functions of CBF proteins as previously described—CBF1 and CBF3 appear to have similar functions, but are negatively regulated by CBF2 (Novillo et al. 2004, Novillo et al. 2007). Systemic regulation of CBF1, and not CBF3, indicates a possible divergence in the function of the two genes. It may be that the relatively small systemic induction of KIN2, and the lack of evidence for such a response of COR15a, is indicative of the negative regulation of CBF1 in indirectly treated tissues, either by CBF2 or by ZAT12, which both showed some evidence of systemic induction and have previously been suggested as negative regulators of the effects of CBF1 and CBF3 (Vogel et al. 2005, Novillo et al. 2007). Further work incorporating protein quantification is required for the confirmation of such hypotheses.

What is the systemic agent responsible for the induction of cold gene expression in warm-maintained tissues? Similar questions for other stimuli remain unanswered, although ABA is a front-runner due to its induction by many environmental stimuli including low temperature (Leung and Giraudat 1998) and its systemic distribution (Jackson 1997). ABA induces the expression of CBF genes, RCi2A and ZAT12 (Medina et al. 2001, Knight et al. 2004, Sakamoto et al. 2004, Davletova et al. 2005), which all showed evidence of systemic induction in the current study. However, Rossel et al. (2007) demonstrated that systemic induction of high irradiance responses, which otherwise showed similarities to the data presented here, was not greatly attenuated in mutants with impaired ABA signaling. Sugar levels would also be expected to increase in the cold (Campbell et al. 2007). One might expect that changes in sugar status resulting from localized cold treatment might be
detected by distal warm-maintained organs—if so, this would make sugars potential mediators of a systemic signal, as suggested by Coupe et al. (2006). However, in our study, we found that sucrose levels did not change systemically in response to localized cold (Fig. 4). ROS may also be involved, as in the systemic response to excess irradiance (Karpinski et al. 1999, Rossel et al. 2007), but APX2 plays no part as it was not cold induced in our initial real-time RT–PCR experiment (data not shown). ROS themselves are unable to travel throughout the plant due to their limited diffusive range (Møller et al. 2007, Rossel et al. 2007). A further candidate may be found among microRNAs, as posited by Coupe et al. (2006). MicroRNAs are capable of conveying genetic code sequences between adjacent leaves (Haywood et al. 2005) and over longer distances such as between shoot and root (Pant et al. 2008). Such a signal would be consistent with Rossel et al. (2007), who concluded that the irradiance signal is probably not a phytohormone, but a novel signaling molecule.

In summary, our study provides the first evidence of systemic cold signaling in plants, and evidence that a suite of cold-induced genes are increased in expression when leaves are treated with moderately low temperature near 10°C. While it is clear that further work is needed to assess the downstream consequences of the observed changes in gene expression, the results presented here suggest that induction of cold-induced genes in developing tissues is not solely dependent on the direct perception of cold—some changes can occur in response to signals from remote parts of the plant that have been directly cold treated. Systemic cold induction of gene expression might enable developing tissues receiving the systemic signal to develop a cold phenotype more rapidly during their later development, potentially acting as an early-warning system to enable developing leaves to better cope with the cold environment in which they are growing.

Materials and Methods

Plant material and growth conditions

For all experiments described except those involving KIN2::LUC, wild-type A. thaliana (Col-0) plants were sown in peat-based compost, in a controlled-environment growth chamber (Microclima 1750, Snijders Scientific, or AR75L Percival Scientific Inc.). For reporter gene experiments, A. thaliana (C24) seeds containing the transgene KIN2::LUC [as described by Foster and Chua (1999)] were obtained from Professor Marc Knight (University of Durham, UK) and grown as for Col-0. An 8/16 h day/night temperature regime of 25/20°C was imposed [with 70% relative humidity (RH)], with 150 μmol photons m⁻¹ s⁻¹ daytime irradiance being provided during seedling establishment. When 25 insertions had visibly emerged from the meristem (~5 weeks from sowing), plants were transferred to aerated, modified Hoagland's hydroponics solution (Poorter and Remkes 1990), maintained at pH 5.8. Plants were held singly on foam discs until about 10 further insertions had emerged. They were then transferred to the treatment growth cabinets (see below).

Direct temperature treatment

To explore the effects of direct exposure to a moderately low temperature on gene expression, plants established above were shifted to another Microclima 1750 growth cabinet (constant 20°C, 60% RH), at high growth irradiance (350 μmol photons m⁻¹ s⁻¹). After a short period of establishment in the new cabinet, a subset of 12 plants was then transferred to a cold, but otherwise matched, Microclima 1750 cabinet, with whole plants maintained at 9°C whilst the others were kept at 20°C. After 2, 6 or 24 h, samples were taken of the leaf material from both temperature treatments (four plants were harvested per treatment at each time period). Two tissue types were taken from each plant: (i) a near fully expanded leaf (leaf insertion 18); and (ii) the central meristematic ‘plug’, consisting of the seven smallest visible insertions (the largest of which was about 1 mm²), the apical meristem and the underlying green stem. Tissues were immediately weighed and frozen in liquid N₂. Samples were stored at −80°C awaiting analysis.

Localized low temperature treatment for real-time RT–PCR

To investigate the effects of 2, 6 or 24 h remote-localized cold treatments on gene expression, we used a set of custom-made Perspex water jackets [York Plastics (Engineering) Ltd.) (Fig. 2; see also Supplementary Fig. S1 for further detail). The upper and lower water jackets were identical, each with cold water circulating through a central space with a thermocirculator (Ecoline RE-112, Lauda). Between the two water jackets was a Perspex spacer, to provide airspace for the clamped pre-existing leaves. Two clamps were used per plant (with the exception of the KIN2::LUC experiments, which used only one). Clamped leaves were held away from the internal water jacket surfaces with thin wire mesh to prevent condensation, and insertions 3–21 (apart from 19) were directly treated on each plant. The 19th insertion, and all those younger than the 22nd, were not clamped, developing in ambient (warm) air. Ambient air was circulated around the unclamped parts of all plants, to ensure that the low temperature treatment only affected the clamped leaves in the cold treatment. Pressure exerted on the petioles by the clamp’s foam seal was insufficient to result in damage. Three plants were transferred to the cold treatment clamps, and three to identical control clamps alongside at ambient temperature (20°C). The apparatus was in a plant growth cabinet (Microclima 1750) under the conditions described above for the warm cabinet direct effects treatment (i.e. constant 20°C). Cabinet air was dehumidified to minimize condensation on the cold plastic surface, using a dehumidifier (WDH-920-12, New Widetech Industries) whose outflow was directed at the apparatus, where ambient RH was 25–35%. One experimental run was performed for each time point (i.e. n = 3 biological replicates for real-time RT–PCR experiments).
Temperatures were monitored at 5 min intervals with a data-logger (DL2E, Delta-T Devices). One leaf thermistor was held in the centre of each clamp airspace, and one was held adjacent to the center of each rosette, at the estimated coldest point. Across all plants and runs, the mean temperature of directly treated, expanded leaves was 9.8°C. At the centre of the treatment rosettes the mean was 18.5°C (i.e. 13°C below control levels). Infrared images (B2, ThermoCAM) taken during later experiments in the same apparatus confirmed that the thermistor temperatures were consistently representative of the growing environment, and that temperatures outside the cold treatment were near ambient levels (Fig. 2C).

Clamp RH was controlled to prevent condensation and minimize humidity differences between control and treatment clamps. Air within the clamps was circulated at 1 liter min⁻¹ throughout, with cold treatment RH regulated by passing cold treatment air through a copper coil in a water trap at 2°C. Control clamp air was humidified by bubbling through cooled (11–14°C) water, fine-tuned so that control clamp RH would match that of the treatment clamp. RHs were measured regularly using a Li-6400 gas analyzer (Li-Cor). Actual clamp RH levels were 50–70%, calculated from Li-6400 readings as outlined in Buck (1981).

Sampling of the of leaf material from the clamp experiments took place once the appropriate period had elapsed (i.e. duration of cold treatment; see Results section). Plants were harvested as quickly as possible, in the same order in which they were installed. For each plant, insertions 17 (clamped), 18 (clamped in the other clamp to 17) and 19 (unclamped), and the central plug were excised, weighed and immediately frozen in liquid N₂. Approximately 8 min elapsed between opening the clamp and freezing the central plug.

In long-term experiments to assess the effect of localized cold treatment on sucrose concentrations, plants were kept in the experimental apparatus until the first leaf to emerge from the apical meristem following the start of the treatment, the reference leaf, was mature (its growth rate was decreasing, after 23 d; see Gorsuch et al. 2010b for details). The reference leaf, and leaves a few insertions younger than it, were harvested (all were newly developed since start of treatment) along with certain clamped, pre-existing leaves. Analysis of sucrose concentrations was carried out as described in Locyes et al. (2003). Briefly, soluble sugars were extracted by hot ethanol extraction, after which sucrose concentrations were estimated using a micro-titer plate-based assay. The concentration of sucrose plus glucose was estimated via incubation of the extract with invertase (Sigma), followed by measurement of the total glucose concentration. For glucose alone, extracts were incubated in the presence of hexokinase and glucose-6-phosphate dehydrogenase. The reduction of NADP to NADPH was then followed spectrophotometrically at 340 nm (Dyatech Laboratories MRX). Sucrose concentrations were calculated as the difference between sucrose plus glucose minus glucose alone.

cDNA production and subsequent real-time RT–PCR

Total RNA was extracted from three biological replicates (i.e. the desired tissues from three individual plants) for each assay using the RNeasy Plant Mini Kit (Qiagen Ltd.). A 1.8 µg aliquot of RNA was treated with DNase (RQ1, Promega), and cDNA was synthesized from 655 ng of RNA (Impron-II Reverse Transcriptase system, Promega UK Ltd.). Random primers were used for reverse transcription, and RNasin (Promega) was included. cDNA samples were diluted 30-fold following synthesis.

Real-time RT–PCR primer sequences (Eurofins MWG Operon) and their origins are shown in Supplementary Table S1. PDF2 was used as an endogenous control (Czechowski et al. 2005, Armstrong et al. 2008). Each 25 µl real-time RT–PCR contained 0.5 µM primer, 1× SYBR Green Power Mix (Applied Biosystems) and 5 µl of cDNA solution. All plates were run for 45 cycles on an ABI Prism 7300 Sequence Detection System (Applied Biosystems), collated using 7000 System Sequence Detection Software v.1.2.3 (Applied Biosystems), and analyzed using Microsoft Excel. Transcript levels are expressed relative to PDF2, and to the warm control at each respective time point, using the 2⁻ΔΔCt method (Livak and Schmittgen 2001).

Raw cycle number (Ct) data from real-time RT–PCR analysis of whole plants directly subjected to 9°C, and warm controls are shown in Supplementary Table S2. For the localized cold experiment, raw Ct values for each sample are shown in Supplementary Table S3. For the following genes where direct comparative data were available for leaf insertion 18 of warm-grown plants (CBF1, CBF2, CBF3, PDF2 and RC12A), no significant differences (using a one-way ANOVA of pooled values at 2, 6 and 24 h) were found in average Ct values between the two experiments (i.e. unclamped/whole-plant and clamped/localized experiments; see Supplementary Fig. S2A). This indicates that clamping had no significant effect on expression of these genes. Similarly, a one-way ANOVA revealed that there were no significant differences in expression of CBF1, CBF2, CBF3, PDF2 and RC12A in leaf insertion 18 of cold-treated unclamped and cold-treated clamped plants (Supplementary Fig. S2B). Clamping of cold-treated plants was, however, associated with slightly lower (but statistically significant) average Ct values of ZAT12 (Supplementary Fig. S2B).

KIN2::LUC experiment

To explore the spatial patterns of gene expression in plants receiving a localized low temperature treatment, an additional study was performed on transgenic Arabidopsis with a luciferase reporter fused to the promoter region of KIN2. Expanded leaves comprising 25–35% of the above-ground fresh mass were directly cold-treated (at a mean temperature of 9.5°C) in one clamp of the apparatus shown in Fig. 2 and Supplementary Fig. S1. Following the 24 h cold clamp treatment, each whole cold-treated plant was analysed alongside a warm-only control. Luciferin was then applied using an atomizer and
the plants were incubated in the dark for 15 min to allow for permeation of the luciferin and subsidence of Chl fluorescence. Kin2 induction was measured by quantifying fluorescence during 150 s exposure on a photon counting system (HRPCS-2, Photek Ltd.). Seven independent runs were carried out.

**Supplementary data**

Supplementary data are available at PCP online.

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