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The effect of short-term low-temperature treatments on gene expression in *Arabidopsis* correlates with changes in intracellular Ca^{2+} levels

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ABSTRACT

The role of changes in intracellular calcium ion concentration ($[Ca^{2+}]_i$) in low-temperature signal transduction in plants has lately been supported by several studies. An analysis to determine whether the low-temperature-induced increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) could be correlated with a downstream response such as gene expression was carried out. The induction of the low-temperature-regulated gene *LTI78* was used as an end point marker of the signal transduction pathway. It was found that this gene is induced by very brief low-temperature exposures and that the induction does not depend on a continuous exposure to low temperature. By altering the cooling rate, different patterns of the Ca^{2+} response were obtained which could be correlated with different patterns of *LTI78* induction. Furthermore, reducing the Ca^{2+} transients by pre-treatment with the Ca^{2+} channel blocker La^{3+} also led to a reduced level of gene induction. The results show that brief exposures to low temperature results in the onset of a signalling pathway that leads to the induction of gene expression. This indicates the involvement of changes in $[Ca^{2+}]_{cyt}$ in low-temperature signalling leading to *LTI78* expression but the presence of multiple signalling pathways is suggested.

Key-words: aequorin; Ca^{2+} inhibition; cold signalling; cooling rate; *LTI78*.

INTRODUCTION

Plants in temperate climates often experience low temperatures and in many plant species an exposure to low temperature is accompanied by changes in gene expression and cold acclimation, namely an increase in the level of freezing tolerance. To obtain these downstream effects, which might be necessary for survival, the plant must perceive the low temperature, or the change in temperature, and be able to convert the signal into the appropriate response.

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The role of calcium ions as a mediator of the signal transduction of low-temperature (LT) responses has become evident during recent years. An increase in intracellular calcium ion concentration ($[Ca^{2+}]_i$) in response to LT treatment has been observed in *Arabidopsis* (Knight, Trewavas & Knight 1996; Polisensky & Braam 1996), tobacco (Knight *et al*. 1991) and tomato (Sebastiani, Lindberg & Vitagliano 1999) as well as in the moss *Physcomitrella* (Russel *et al*. 1996). The increase in $[Ca^{2+}]_i$ that is observed as a response to LT treatment is transient; the $[Ca^{2+}]_i$ has returned to resting levels within a few minutes, even during LT exposures by a slow cooling (Knight *et al*. 1996; Plieth *et al*. 1999).

In studies on the LT signalling process changes in gene expression and development of freezing tolerance have been used as end point markers. LT-induced genes have been identified and characterized in many plant species (Thomashow 1999). Many of the genes encode proteins with a known or putative function but for a large group of genes no function has been determined for the encoded proteins (Thomashow 1999). For one set of genes in *Arabidopsis* the transcription factor(s) responsible for the LT-induced expression has been identified. The CBF/DREB1 proteins have been found to bind to the promoter regions of LT-regulated genes containing an element called DRE or C-repeat (Stockinger, Gilmour & Thomashow 1997; Liu *et al*. 1998; Sakuma *et al*. 2002). The induction of the *DREB1/CBF* genes has been shown to be LT regulated (Gilmour *et al*. 1998; Liu *et al*. 1998; Shinwari *et al*. 1998) but the mechanism by which *DREB1/CBF* regulates transcription has not been determined. Overexpression of the *CBF1* or *DREB1A* genes in transgenic plants was found to lead to the induction of the downstream genes as well as an increased cold acclimation capacity (Jaglo-Ottosen *et al*. 1998; Kasuga *et al*. 1999).

The expression of LT-induced genes has been correlated with LT-induced changes in $[Ca^{2+}]_i$ by analyses of the effect of Ca^{2+} channel blockers and Ca^{2+} chelators (Monroy, Sarhan & Dhindsa 1993; Knight *et al*. 1996; Polisensky & Braam 1996; Tähtiharju *et al*. 1997; Sangwan *et al*. 2001). It has also been shown that treatment with a Ca^{2+} ionophore results in the induction of LT-responsive genes without any

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LT exposure (Monroy & Dhindsa 1995; Sangwan *et al.* 2001). In addition, treatment with these substances was found to affect the capacity of alfalfa plants to cold acclimate (Monroy *et al.* 1993). These results have led to the conclusion that an alteration of $[Ca^{2+}]_i$ is required as a component of the LT signal transduction pathway. Recent work on alfalfa, *Brassica napus* and *Arabidopsis*, has demonstrated that, in addition to changes in Ca^{2+}, membrane rigification, cytoskeleton reorganization and protein phosphorylation/dephosphorylation are part of the LT perception and signal transduction, leading to altered gene expression and cold acclimation (Monroy, Sangwan & Dhindsa 1998; Örvar *et al.* 2000; Sangwan *et al.* 2001; Tähtiharju & Palva 2001)

In the present study we have analysed whether the induction of the LT-regulated gene *LTI78 (*also known as *RD29A* or *COR78*) (Horvath, McLarney & Thomashow 1993; Nordin, Vahala & Palva 1993; Yamaguchi-Shinozaki & Shinozaki 1993) could be correlated with the elevation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in response to LT exposure. *Arabidopsis* seedlings expressing a 35S-aequorin construct were employed in an experimental set-up in which the LT treatment was applied in different ways. This allowed the possibility of LT exposures on whole seedlings, with a slower cooling than the cold shock treatments previously used in analyses of changes in Ca^{2+} (Knight *et al.* 1996; Polisensky & Braam 1996). The LT exposures also resulted in the induction of *LTI78* which made it possible to analyse which effects different LT conditions and Ca^{2+} channel blockers or Ca^{2+} chelators had on both changes in $[Ca^{2+}]_{cyt}$ and *LTI78* expression.

MATERIALS AND METHODS

Plant material and growth conditions

Transgenic *Arabidopsis* plants expressing cytoplasmic apoaequorin (a kind gift from J. Braam and D.H. Polisensky, Rice University, Houston, TX, USA) were employed for $[Ca^{2+}]$ measurements and gene expression analyses by Northern blot. Seeds were germinated and grown on 0.5 mL full strength Murahige and Skoog (MS) medium in the Ellerman tubes that were used as luminometer cuvettes. The seedlings were grown in constant light and used for LT exposures when 11–13 days old. Samples that were used for Ca^{2+} measurement contained two seedlings and samples used for RNA extraction contained 10–15 seedlings. Leaves were used as experimental material since they have a known capacity to sense LT, as indicated by their ability to cold acclimate (Thomashow 1994) and by the fact that the *LTI78* gene is strongly induced by LT in leaves (Horvath *et al.* 1993; Nordin *et al.* 1993).

Transgenic *LTI78::LUC* plants were produced by *Agrobacterium*-mediated root transformation of *Arabidopsis*, ecotype C24. The transformed construct consisted of a 1200 bp fragment of the *LTI78* promoter, including the 5′ untranslated leader, fused at the translation initiation codon with the firefly luciferase gene, a kind gift from Dr Teemu Teeri (University of Helsinki, Finland). One of the obtained lines, containing a single insert, was used for analyses of LT-regulated LUC activity.

Low-temperature treatments

The seedlings were exposed to different LT conditions by submerging the cuvettes containing the seedlings in ice, in an ice/water bath or in a water bath maintained at 8.5 °C. The temperature of the air inside the cuvette was followed using a thermocouple placed inside the cuvette. The final temperature experienced by the seedling was around 1 °C both for cuvettes submerged in ice or in ice/water. In cuvettes submerged in 8.5 °C water the final temperature of the air inside the cuvette was 10 °C. The different types of LT exposures also resulted in different cooling rates, with the fastest cooling obtained during immersion in ice/water. After different time periods of immersion, the cuvettes were withdrawn and immediately placed in the luminometer for luminescence measurements. Samples that were to be used for subsequent RNA extraction were withdrawn from the LT exposures and were either collected and frozen in liquid nitrogen immediately or left at ambient room temperature for different time periods before freezing.

In order to obtain a slower temperature decrease the cuvettes were immersed in a water bath tempered at 19.5 °C, after which the water bath was set at 6.8 °C. Cooling of the water bath resulted in a cooling of the air inside the cuvette from 20 to 8.5 °C in 11 min. Samples were withdrawn for luminescence measurements at 20, 18, 16, 14, 12, 10 and 8.5 °C. Samples to be used for RNA extraction were withdrawn at 10 °C and then harvested as described above.

Aequorin reconstitution and $[Ca^{2+}]_{cyt}$ measurements

Cytosolic $[Ca^{2+}]$ was measured in seedlings producing apoaequorin after reconstitution of aequorin *in vivo* by adding two 2 μL droplets of 2 μM coelenterazine (Molecular Probes Europe BV, Leiden, The Netherlands) onto the newly emerging leaves of the seedlings. The seedlings were left overnight and LT exposures and luminescence measurements were performed the following day. Luminescence was measured using a chemiluminometer consisting of an EMI photomultiplier tube (Model 9829 A) and a FACT50 cooling system (Electron Tubes Ltd, Ruislip, UK) as described previously (van der Luit *et al.* 1999). Following the LT exposures the tubes containing the seedlings were immediately transferred to the luminometer and the emitted light was recorded in 0.2 s intervals. The remaining reconstituted aequorin was extracted and discharged by adding an excess of $CaCl_2$. The luminescence values were converted into Ca^{2+} concentrations according to the equation described by van der Luit *et al.* (1999) using K_R and K_{TR} values for native coelenterazine. The first $[Ca^{2+}]$ value obtained from each trace was considered to be the $[Ca^{2+}]_{cyt}$ for that sample.

RNA extraction and Northern blot analysis

Seedlings exposed to LT were left at ambient room temperature for different periods of time before the aerial parts of the seedlings were collected and frozen. For each time point the plant material from three or four tubes, each containing 10–15 seedlings, was pooled. Total RNA was extracted according to Verwoerd, Dekker & Hoekema (1989). Ten micrograms of RNA was separated on formaldehyde containing denaturing gels and blotted onto positively charged nylon membranes (Roche Molecular Biochemicals, Roche Diagnostics Scandinavia AB, Bromma, Sweden). The membranes were hybridized with digoxigenin (DIG)-labelled (Roche Molecular Biochemicals) DNA fragments corresponding to the genes *LTI78*, *LTI30* or *DREB1A*. The *LTI78* probe consisted of a 1.2 kb fragment of the 3′-end of the *LTI78* gene (Nordin *et al*. 1993). The *LTI30* probe corresponded to the *LTI30* cDNA (Welin *et al*. 1994) and was obtained from the plasmid pLTI30R (Svensson, Palva & Welin 2000), a kind gift from Jan Svensson. The *DREB1A* probe was amplified using the primer pair 5′-TTGGCTCCGATTACGAGTCCTC-3′ and 5′-GGCCAAAAGCATCCCTTCTGC-3′, resulting in a 544 bp DNA fragment, that was confirmed to correspond to *DREB1A* by sequencing. After hybridization and stringency washes, the DIG-labelled probes were immunodetected and visualized using chemiluminescence as described by the manufacturer (Roche Molecular Biochemicals) and exposed to chemiluminescent detection film (Roche Molecular Biochemicals).

Luciferase activity measurements

Arabidopsis seedlings expressing a *LTI78* promoter::*LUC* fusion was exposed to LT by immersion in ice/water for different time periods. The luciferase substrate was added to the seedlings immediately after the LT exposure was interrupted. The seedlings were left at ambient room temperature and the *in vivo* luminescence from the seedlings was measured at intervals during the post-treatment period using a luminometer.

Inhibitor pre-treatments

Seedlings were pre-treated with either 10 mm ethyleneglycoltetraacetic acid (EGTA) or 2 mm $LaCl_3$ on the day before the LT exposures. The inhibitor solutions were pipetted into the tubes, covering the whole seedlings and left for 3 h after which as much as possible of the solutions was removed. Control seedlings were treated the same way but with water. Coelenterazine was added to samples that were to be used for Ca^{2+} measurements as described above, after the inhibitor pre-treatment. The seedlings were left overnight before LT exposure and subsequent Ca^{2+} measurements or RNA extractions.

RESULTS

Changes in $[Ca^{2+}]_{cyt}$ in response to low-temperature treatment

To correlate the gene induction response to low temperature with the response regarding changes in $[Ca^{2+}]_{cyt}$ we used different ways of performing the LT treatments. Seedlings were grown in luminometer cuvettes and exposed to LT by submerging the cuvettes in ice, ice/water or water maintained at 8.5 °C. This way the seedling experienced the temperature of the air inside the cuvette without being in direct contact with the ice or the cold water. The final temperatures for these different treatments were around 1 °C for the ice/water and ice treatments and 10 °C for seedlings submerged in 8.5 °C water. The different LT exposures also resulted in different cooling rates.

We found that the strength and kinetics of the Ca^{2+} response depended on how the treatment was performed. Placing the cuvette in ice/water resulted in a rapid elevation of $[Ca^{2+}]_{cyt}$ to approximately 1.2 μM (Fig. 1a). If the cuvettes containing the seedlings were placed on ice the response was not as strong and the Ca^{2+} peak occurred later and was broader (Fig. 1a). Placing the cuvettes in 8.5 °C water resulted in a third type of response with a rather sharp peak but not as strong as after the ice/water exposure (Fig. 1a). During the exposures the $[Ca^{2+}]_{cyt}$ increase was transient and decreased again rather rapidly. However, except for the exposure by immersion in 8.5 °C water, the $[Ca^{2+}]_{cyt}$ remained slightly elevated throughout the exposure periods used (Fig. 1a).

The $[Ca^{2+}]_{cyt}$ peak was obtained very shortly after the cuvette was submerged. For ice/water and 8.5 °C water exposures a substantial increase in $[Ca^{2+}]_{cyt}$ could be detected already after 10 seconds (Fig. 1a). At this point the temperature had not decreased by more than a few degrees. Therefore the seedlings might be responding to the *change* in temperature and it appears as if the rate of the temperature decrease is important. The rate of temperature decrease was different depending on how the LT treatments were performed and for each LT exposure a correlation was found between the cooling rate and the timing of the Ca^{2+} response (Fig. 2). During treatments by immersion in ice/water or in 8.5 °C water, where the more rapid decreases in temperature were obtained, the timing of the $[Ca^{2+}]_{cyt}$ peaks coincided well with the time points at which the highest cooling rate was observed. After immersion in ice the cooling rate was slower and almost linear during a large part of the cooling, and a broader peak in the $[Ca^{2+}]_{cyt}$ transient was observed (Fig. 2).

Submerging the cuvettes in different temperatures still resulted in a rather rapid cooling of the air inside the tube and of the seedling. We therefore performed a slower cooling by placing the cuvettes in a water bath tempered at 20 °C and then setting the temperature at 6.8 °C. This resulted in a final temperature of around 8.5 °C inside the cuvette and the cooling took 11 min. Samples were withdrawn at 20, 18, 16, 14, 12, 10 and 8.5 °C and placed in the luminometer for luminescence measurement and subse-

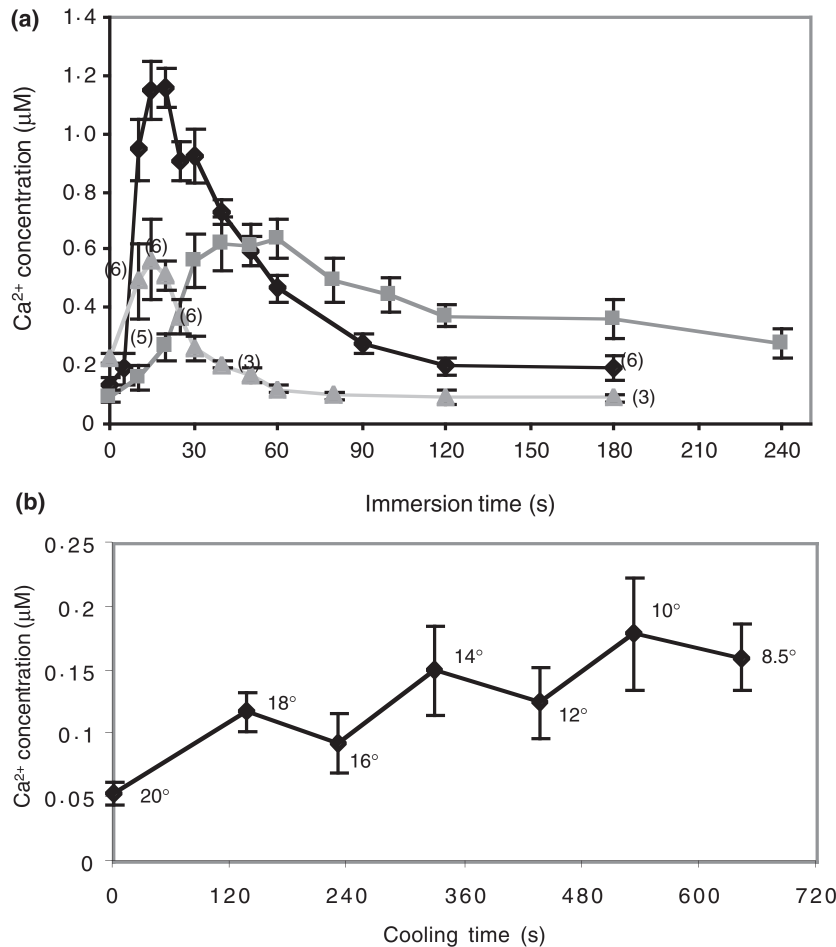

Figure 1. The Ca^{2+} response depends on how the LT exposures are performed. (a) Seedlings in tubes were immersed in an ice/water bath (◆), in ice (■) or in a water bath maintained at 8.5 °C (▲) for different time periods. After immersion the tubes were immediately transferred to the luminometer and for each immersion time a trace was obtained that was converted into $[Ca^{2+}]_{cyt}$. The very first $[Ca^{2+}]_{cyt}$ value from each trace was used as the $[Ca^{2+}]_{cyt}$ in the seedlings after that particular immersion time. The graph shows the average of the $[Ca^{2+}]_{cyt}$ values obtained from three experiments in which two or three replicates were used for most immersion times. Error bars indicate standard error, $n = 8–9$, except when indicated within brackets in the graph. (b) Tubes with seedlings were placed in a water bath tempered at 19.5 °C after which the water bath was set at 6.8 °C resulting in a cooling of the temperature inside the tubes from 20 to 8.5 °C. Samples were withdrawn at the temperatures indicated in the figure and luminscence was recorded and converted into $[Ca^{2+}]$ as described above. Each point represents the mean value of 14 samples taken during four separate experiments except for the 8.5 °C value for which $n = 10$. Error bars indicate standard error.

quent $[Ca^{2+}]_{cyt}$ determination. The $[Ca^{2+}]_{cyt}$ observed during this experiment demonstrated a slight and consistent increase (Fig. 1b). The rate of the temperature decrease during this slower cooling was constant throughout the cooling. The samples which showed the least variation and the most statistically significant increase in $[Ca^{2+}]_{cyt}$, were those withdrawn already at 18 °C (Fig. 1b).

Gene expression in response to low-temperature exposure

To correlate the changes in $[Ca^{2+}]_{cyt}$ with a downstream response we used the expression of the LT-induced gene *LTI78* as an end point marker. Since the changes in $[Ca^{2+}]_{cyt}$ that we observed in our different treatments all occurred very rapidly and $[Ca^{2+}]_{cyt}$ levels had decreased within a few minutes, we were interested to determine whether gene expression could be affected by short LT treatments. In previous studies of gene induction by Northern analysis a LT treatment of a few hours was usually required to result in the accumulation of detectable levels of *LTI78* mRNA (Nordin, Heino & Palva 1991; Yamaguchi-Shinozaki & Shinozaki 1993). To separate the signal occurring early during LT exposure from more long-term effects of the low temperature, short-term LT treatments were applied after which the plants were left at ambient room temperature for a period of time, before samples were taken for RNA extraction. During this post-treatment period *LTI78* mRNA was found to accumulate to significant levels. If the seedlings were immersed in ice/water for different time periods, *LTI78* mRNA could be detected 30 min after the LT exposure was interrupted and at 60–90 min after treatment, high levels of *LTI78* mRNA were observed (Fig. 3a). An immersion period as brief as 30 s was found to be sufficient to result in the accumulation of *LTI78* mRNA when RNA was extracted 30 or 60 min after treatment. (Fig. 3a).

The post-treatment induction of *LTI78* seemed to be regulated at the transcriptional level. Transgenic seedlings harbouring a *LTI78* promoter::*LUC* fusion construct were subjected to LT by immersion in ice/water for 1 or 3 h. After interrupting the LT exposure the luciferase substrate was added and LUC activity was measured at intervals during 90 min. An increase in *in vivo* LUC activity was found during the post-treatment period (Fig. 4). Similar results were obtained when the LUC activity was analysed in protein extracts of seedlings treated the same way (not shown).

We then analysed whether the post-treatment induction was specific to *LTI78* by studying how other LT-induced genes responded during the post-treatment period. A 5 min immersion in ice/water was found to be sufficient to result

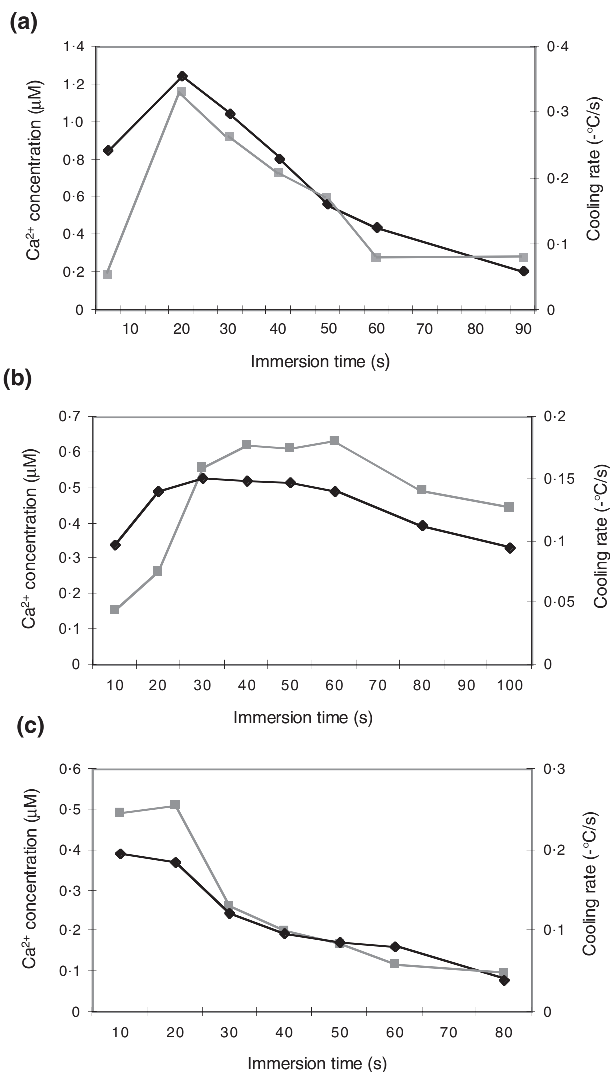

Figure 2. Changes in $[Ca^{2+}]_{cyt}$ and cooling rate during different types of LT exposure. $[Ca^{2+}]_{cyt}$ (■) and cooling rate (◆) during immersion in ice/water (a), in ice (b) or in water maintained at 8.5 °C (c) was plotted against the immersion time. The temperature was followed using a thermocouple inside one of the tubes.

in the accumulation of mRNA corresponding to the LT-regulated dehydrin gene *LTI30*. The kinetics of *LTI30* accumulation was similar as for *LTI78* but *LTI30* RNA did not accumulate to the same high level (Fig. 3b).

In contrast, the same kind of treatment did not result in a detectable accumulation of *DREB1* mRNA. In Fig. 3c Northern blots hybridized simultaneously with probes for *LTI78* and *DREB1A* are shown. Since the *DREB1A* probe was not gene specific, the hybridizations using this probe probably showed the accumulation of all three *DREB1* genes. After 5 or 20 min of LT exposure by immersion in ice/water no detectable amount of *DREB1* mRNA had accumulated. After a 3 h LT exposure an accumulation of *DREB1* transcripts could be detected immediately after the LT treatment was interrupted. During the post-treatment period the *DREB1* mRNA disappeared and *LTI78* mRNA started to accumulate (Fig. 3c). This indicates that the post-treatment accumulation of *LTI78* does not require an induction of the *DREB1* genes, but instead might be regulated by DREB1 protein already present in the seedlings.

Taken together, these results show that a very brief exposure to LT gives rise to a signal that is sufficient to result in subsequent induction of LT-regulated gene expression. Furthermore, a continuous exposure to LT is not necessary for the gene induction.

Correlation between low-temperature-induced changes in $[Ca^{2+}]$ and *LTI78* expression

Since the different LT exposures gave rise to different patterns of $[Ca^{2+}]_{cyt}$ increase, we analysed whether the induction of *LTI78* showed a difference depending on how the LT exposures were performed. After brief LT exposures a correlation between the way the LT treatment was performed and *LTI78* induction was observed. An immersion in ice/water for 30 s or 1 min resulted in a clear induction of *LTI78* whereas after such short immersions in ice hardly any *LTI78* mRNA accumulation could be detected (Fig. 5a). After 20 min of immersion the difference in the induction was less clear and after an even longer exposure no difference was observed between the LT treatment and *LTI78* mRNA accumulation in seedlings exposed to the same temperature. In seedlings exposed to 10 °C (submerging in 8.5 °C), a reduced accumulation of *LTI78* RNA was observed (Fig. 5a). This lower level of induction was detected at all immersion periods tested.

The induction of *LTI78* after a direct immersion in 8.5 °C (resulting in 10 °C inside the cuvette) was also compared with the induction after an exposure to a slow cooling to 10 °C. After the slow cooling, from 20 to 10 °C in 9 min, very low levels of *LTI78* mRNA could be detected (Fig. 6b). In contrast, a 9 min as well as a 5 min immersion directly in 8.5 °C water resulted in a clear induction of *LTI78* (Fig. 5b).

During the brief exposures to different LT conditions the temperature only decreased a few degrees. However, at the time points when the samples were taken the temperature that the seedlings had experienced was different in the different LT treatments, which might explain the difference in gene expression. Since a Ca^{2+} transient, that was dependent on how the LT exposure was performed, did occur during the very brief exposures we wanted to investigate if a more direct correlation between the Ca^{2+} response and gene induction could be found. We therefore carried out tests to determine what effect an inhibition/reduction of the Ca^{2+} transient during immersion in ice/water would have on *LTI78* expression. Pre-treating the seedlings with EGTA, which chelates extracellular Ca^{2+} ions, or La^{3+}, a putative inhibitor of plasma membrane Ca^{2+} channels, resulted in reduced responses regarding changes in $[Ca^{2+}]_{cyt}$ during LT exposure (Fig. 6a). The immediate strong peak in Ca^{2+} obtained in seedlings immersed in ice/water was reduced but the 'tail' of the peak, i.e. $[Ca^{2+}]_{cyt}$ in the seedlings after an immersion time of more than 1 min, showed the same

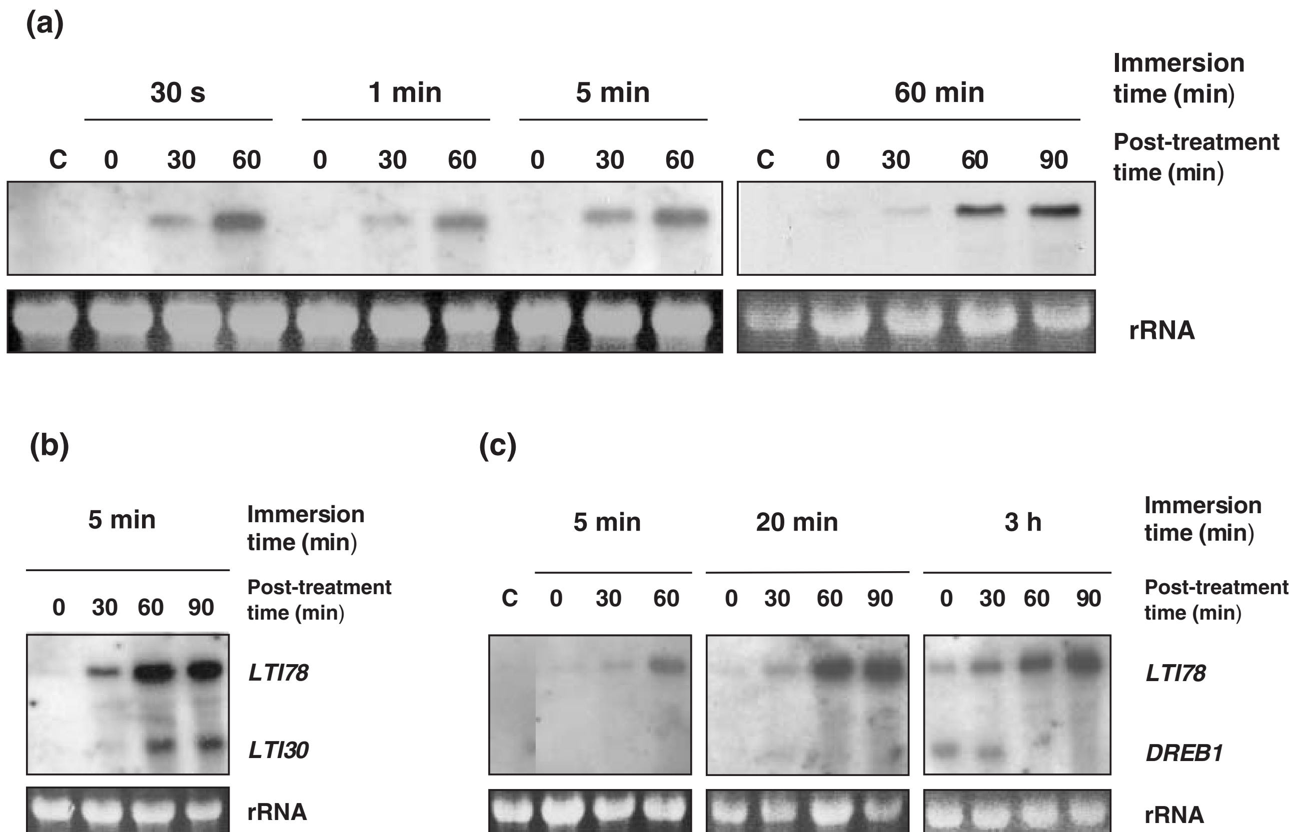

Figure 3. Induction of LT-regulated genes during a period after LT exposures. Seedlings were exposed to LT by immersion in ice/water for the time periods indicated in the figure. C indicates non-treated samples. The post-treatment time indicates the time period that the seedlings were left at ambient room temperature before being collected and frozen. RNA was transferred to nylon membranes and hybridized with *LTI78* (a), *LTI78* and *LTI30* simultaneously (b) or *LTI78* and *DREB1* simultaneously (c). Ten micrograms of total RNA was run in each lane. The ethidium bromide-stained rRNA is shown as a loading comparison.

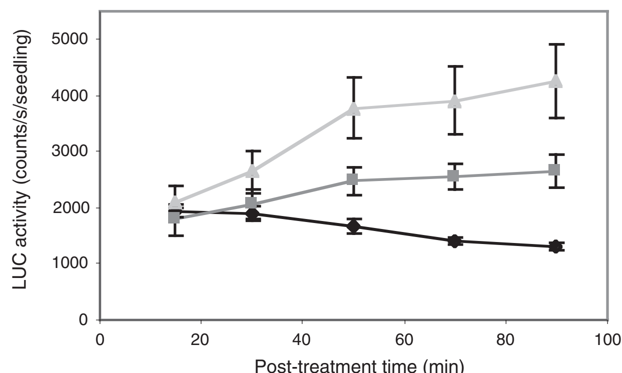

Figure 4. *In vivo* LUC activity in *LTI78*::*LUC* transgenic plants during the post-treatment period. Tubes with seedlings were placed on ice for 1 h (■) or 3 h (▲). The LT exposure was interrupted (time 0) and the luciferase substrate was added immediately. The seedlings were then left at ambient room temperature and luminescence measurements were perfomed after 15, 30, 50, 70 and 90 min. Control seedlings (◆) were left at room temperature throughout the experiment and LUC activity was measured at the same time points after the addition of the luciferin substrate as for the LT-treated seedlings. Mean values are shown and error bars indicate standard error ($n = 7$ for ice-treated samples, $n = 8$ for control samples).

level in inhibitor treated seedling as in control seedlings. The Ca^{2+} peak was reduced to a greater extent in EGTA-treated seedlings than in La^{3+}-treated plants (Fig. 6a). Since the $[Ca^{2+}]_{cyt}$ peak was not completely inhibited by the these inhibitor pre-treatments, part of the $[Ca^{2+}]_{cyt}$ increase might result from the release of Ca^{2+} from intracellular sources. There is also a possibility that the inhibitors do not penetrate completely into the leaf tissue and therefore not all cells are inhibited.

Pre-treating the seedlings with La^{3+} resulted in a clear inhibition of the subsequent *LTI78* induction. After an immersion in ice/water for 1 min or 20 min no accumulation of *LTI78* mRNA was detected in La^{3+}-pre-treated seedlings when compared with water-pre-treated seedlings (Fig. 6b). EGTA pretreatment on the other hand did not show any clear reduction in *LTI78* induction (Fig. 6b).

These results indicate that the increase in $[Ca^{2+}]_{cyt}$ that is obtained in response to the type of LT exposure used in this study is at least partly dependent on the influx of extracellular Ca^{2+} into the cells. In addition, the induction of *LTI78* after brief LT exposures correlates with the increase in $[Ca^{2+}]_{cyt}$ that is obtained early during the LT treatment.

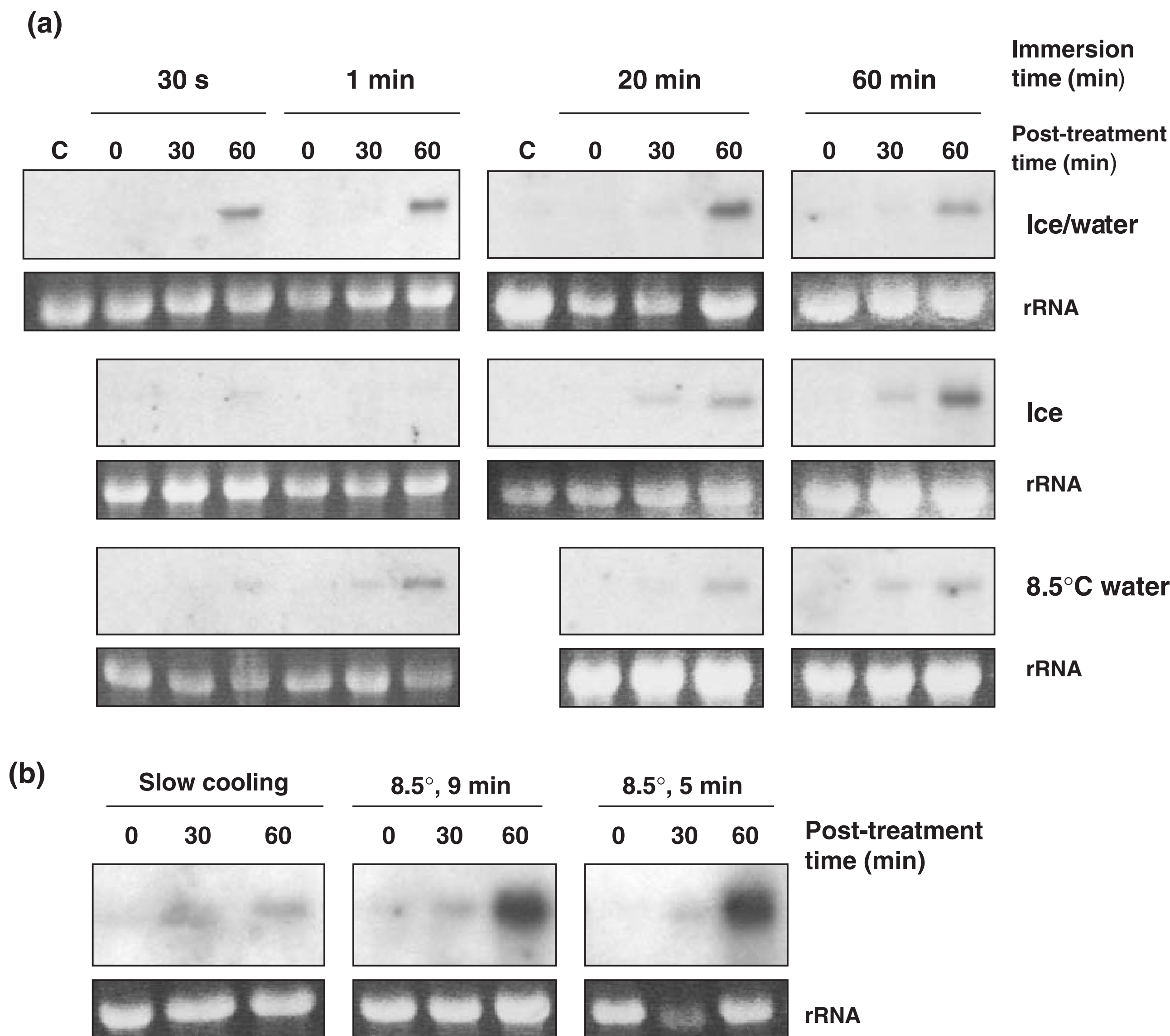

Figure 5. Accumulation of *LTI78* mRNA after exposures to different LT conditions. Total RNA (10 μg in each lane) from seedlings exposed to different LT conditions was hybridized with a *LTI78* probe. (a) Seedlings were exposed to LT by immersion in ice/water, ice or 8.5 °C water for the indicated time periods. C indicates non-treated samples. The samples from 30 s and 1 min treatments and from 20 min and 60 min treatments, respectively, were run in the same gel and hybridized and exposed together. (b) Total RNA was isolated from seedlings exposed to a slow cooling (from 20 to 10 °C in 9 min) or immersed in water maintained at 8.5 °C for 9 or 5 min. The RNA was blotted onto a nylon membrane and hybridized and exposed together. The post-treatment time indicates the time period that the seedlings were left at ambient room temperature after the LT exposure before being collected and frozen. Ethidium bromide-stained rRNA is shown as a loading comparison.

DISCUSSION

Effect of cooling rate on $[Ca^{2+}]_{cyt}$ transients

Previous studies using cold treatment to induce changes in intracellular $[Ca^{2+}]$ have to a large extent used cold shock as the stimulus. We wanted to use LT treatments that would result in different kinds of $[Ca^{2+}]_{cyt}$ transients as well as a subsequent induction of LT-regulated gene expression that could be detected using Northern blot analysis. In addition, the analyses were performed on leaves, for which the effect of different types of LT exposure on $[Ca^{2+}]_{cyt}$ had not been examined previously. By using the method of submerging tubes containing the seedlings in ice or water of different temperatures, the aerial parts of the seedling were only exposed to the temperature of the air inside the tube. The rate of cooling of the air was dependent on how the treatment was performed. It was found that these types of LT exposures also induced transient increases in $[Ca^{2+}]_{cyt}$. The responses were different depending on how the treatment was performed. A maximum response was obtained during

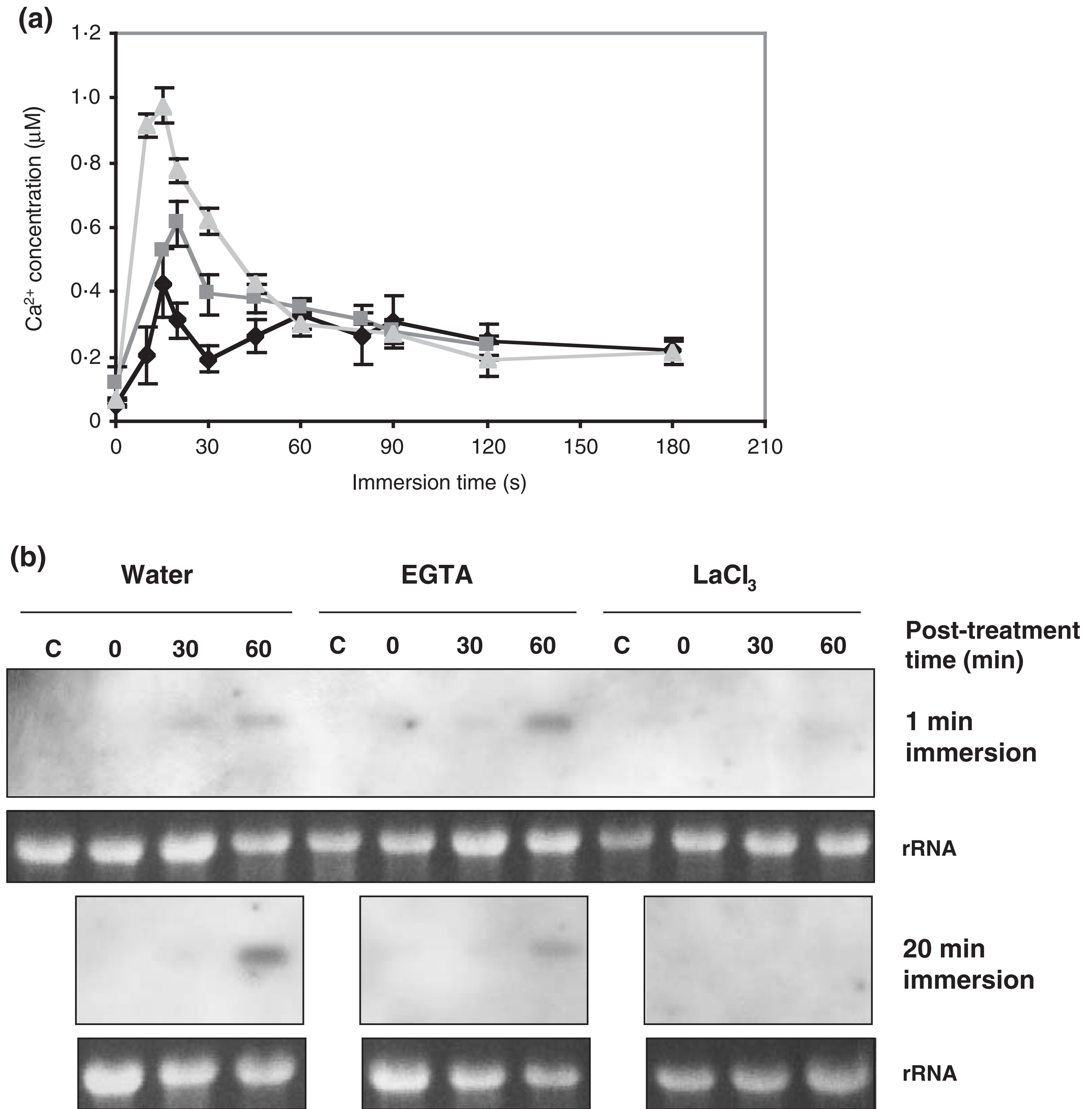

Figure 6. Pre-treatment with EGTA or $LaCl_3$ reduces the low-temperature-induced increase in $[Ca^{2+}]_{cyt}$ and affects *LTI78* expression. (a) Seedlings in tubes were pre-treated with EGTA (◆), $LaCl_3$ (■) or water (▲) and subsequently immersed in an ice/water bath for different time periods. The $[Ca^{2+}]_{cyt}$ for each immersion time was calculated as described in Fig. 1. The experiment was performed several times with at least two replicates for each immersion time in each experiment. The graph shows the mean values of the $[Ca^{2+}]_{cyt}$ obtained from the different immersion times and pre-treatments. Error bars indicate standard error, n = 8–12 for EGTA pre-treated samples, n = 8–9 for $LaCl_3$ pre-treated samples and n = 12–16 for water pre-treated samples.(b) Total RNA (10 µg in each lane) from seedlings pre-treated with water, EGTA or $LaCl_3$ and subsequently exposed to LT by immersion in ice/water for the time periods indicated, was hybridized with a *LTI78* probe. The post-treatment time indicates the time period that the seedlings were left at ambient room temperature before being harvested and frozen. The samples shown were run in the same gel and hybridized and exposed together. The ethidium bromide-stained rRNA is shown as a loading comparison.

the cooling by immersion in ice/water when the cooling rate was the largest. Furthermore, the increase in $[Ca^{2+}]_{cyt}$ had already started when the temperature had only decreased by a few degrees. Therefore it appears as if the Ca^{2+} response is dependent on the rate of cooling. These results are in agreement with the findings by Plieth *et al*. (1999). In their experiments using *Arabidopsis* roots the kinetics of the Ca^{2+} response was different depending on the initial cooling rate and as the cooling rate was lowered a biphasic response was observed (Plieth *et al*. 1999). A biphasic response was also observed during a gradual cooling of *Arabidopsis* seedlings from 20 to 0 °C (Knight & Knight 2000). No clear biphasic response was obtained in our experiments although during the ice/water exposure (Fig. 1) the small second peak that followed the main peak could indicate the presence of a similar type of response. Similarly, during the ice treatment the peak flattened out and a slight second peak might be present although insufficient numbers of measurements at these time points prevent confirmation of this.

Post-treatment induction of gene expression

Our results show that a very brief LT exposure is sufficient for the onset of a signalling pathway leading to an induction of gene expression. This is not only true for the *LTI78* gene, since another LT-regulated gene, *LTI30*, was also found to be induced in a similar way. Furthermore, a continuous exposure to LT does not seem to be necessary to result in gene induction. These results confirm previous findings by Knight *et al*. (1996) who found that a cold shock followed by ambient temperature resulted in the induced expression of the *kin1* gene. The capacity to regulate gene expression in response to transient temperature changes might be of importance for preparing the plant for subsequent, more severe, exposures to low temperatures.

Induction of *LTI78* expression has been shown to be regulated by the *DREB1*/*CBF* transcription factors and the induction of the *DREB1*/*CBF* genes has in turn been shown to be LT regulated (Gilmour *et al*. 1998; Liu *et al*. 1998; Shinwari *et al*. 1998). We found that the induction of *LTI78* after a brief LT exposure and post-treatment period seemed to be on the transcriptional level but did not seem to require the transcriptional activation of the genes encoding the DREB1/CBF transcription factors. It is possible that the *DREB1*/*CBF* genes are induced during the brief LT exposures but that too low levels of mRNA are accumulating to be detected in our experiments. However, if the *DREB1*/*CBF* genes are not induced during the treatments that did result in the induction of *LTI78*, the expression of *LTI78* might be regulated by DREB1/CBF proteins already present in the cells. It has been suggested that CBF1 (DREB1B) binds to the CRT/DRE element of the promoter of a target gene already at normal temperature and that a cold denaturation of the CBF1 protein leads to the transformation from a repressor to an activator (Kanaya *et al*. 1999). It could be that the changes in temperature that result from the brief LT exposures that were employed in this study were sufficient to lead to alterations of the activation capacity of the CBF/DREB1 proteins. Another likely possibility is that other post-translational modifications such as phosphorylation/dephosphorylation of the transcription factors are involved. The involvement of phosphatases or kinases in responses to LT has been observed in alfalfa (Jonak *et al*. 1996; Monroy *et al*. 1998), *Arabidopsis* (Tähtiharju & Palva 2001; Matsuoka *et al*. 2002) and rice (Saijo *et al*. 2000). In the case of the rapid induction of gene expression that we have observed, if phosphatases or kinases are involved, it is possible that their corresponding genes are not regulated at the transcriptional level but instead their activities might be modulated by the change in temperature. This has been observed for the *Arabidopsis* MAPK kinase AtMEK1 (Matsuoka *et al*. 2002). Their activation might be caused directly by the brief LT exposure or by another signalling component that is activated during the LT exposure, for example, the increase in $[Ca^{2+}]_{cyt}$.

Involvement of Ca^{2+} in gene regulation

The role of Ca^{2+} as a mediator of the response to changes in temperature has been implied by several previous studies. An inhibition of a downstream response, for example, the induction of cold-regulated gene expression by pretreatment with Ca^{2+} channel blockers or Ca^{2+} chelators has been observed in alfalfa (Monroy *et al*. 1993; Monroy & Dhindsa 1995), *Brassica napus* (Sangwan *et al*. 2001) and *Arabidopsis* (Knight *et al*. 1996; Polisensky & Braam 1996; Tähtiharju *et al*. 1997). In most of these studies cell cultures were used or the effect of the inhibitor pre-treatment on the cold-induced $[Ca^{2+}]_i$ increase was not analysed. This is, to our knowledge, the first report on the involvement of Ca^{2+} in the LT-induced expression of *LTI78* and on the correlation between different Ca^{2+} response patterns with an end response using whole plants. The results indicate that the induction of *LTI78* expression after a brief LT exposure correlates with the increase in $[Ca^{2+}]_i$ that occurs as a result of the cooling rate. The results also suggest an additional pathway leading to *LTI78* expression, depending more on the temperature to which the seedlings are exposed. After longer LT exposure times, during which the seedlings were exposed to 1 °C for most of the treatment time, no obvious difference in the level of gene expression could be detected even though the Ca^{2+} response was different during the beginning of the treatments. Treatments at a higher temperature also resulted in a weaker induction of *LTI78* expression. Although these results show a correlation between the gene expression and Ca^{2+} response it is only indirect. It might still be possible that the gene expression is dependent on the exposure temperatures also after the very brief treatments. After 30 s on ice, when no gene induction could be detected, the temperature had only decreased to around 19 °C inside the cuvette. In contrast, in cuvettes immersed in ice/water the temperature had decreased to approximately 13 °C in the same period of time and this treatment resulted in a clear *LTI78* induction.

However, the results from the slow cooling indicate that it is not only the temperature that is important. The slow cooling gave rise to a much weaker induction than a transfer directly to 8.5 °C water, even though the final temperature was the same (10 °C). It could be argued that the length of the exposure to a specific temperature also had an effect, since after the slow cooling the treatment was interrupted as soon as the temperature inside the cuvettes had reached 10 °C. This meant that the seedlings had only experienced a temperature below 12 °C for less than 2 min. In cuvettes that were placed directly in an 8.5 °C water bath and left for 5 min, the temperature was below 12 °C for approximately 3.5 min. This difference in exposure time is very short but we cannot rule out that it has an effect on the level of gene induction.

A more direct correlation between the Ca^{2+} response and *LTI78* expression came from the results using the Ca^{2+} channel inhibitor La^{3+}. Pre-treating the seedlings with La^{3+} resulted in a reduced Ca^{2+} response and a complete inhibition of the induction of *LTI78* after brief LT exposures. This indicates that the release of Ca^{2+} from external sources, which is inhibited by the Ca^{2+} channel blocker La^{3+}, is necessary for the induction of *LTI78* after brief LT exposures. Since the 'tail' of the transient was similar to the one obtained in control seedlings the main component in regulating the induction of gene expression appears to be the large initial $[Ca^{2+}]$ peak. The failure to get any inhibition of gene expression by treatment with 10 mM EGTA was somewhat unexpected since EGTA pre-treatment did result in a reduction of the $[Ca^{2+}]$ peak. It might be explained by the likely difference between the way La^{3+} and EGTA function in calcium signalling inhibition. It has been show that a long-term deprivation of external Ca^{2+} by EGTA treatment leads to a gradual restoration of the sensitivity to stimuli that involve extracellular Ca^{2+} influx, even though the extracellular Ca^{2+} is chelated (Cessna & Low 2001). If a similar restoration occurs in the plants in our experiments the *LTI78* gene might be induced by LT also in the EGTA-treated plants. In contrasts, in the La^{3+}-treated plants in which the Ca^{2+} channels are blocked, Ca^{2+} influx and gene induction would be inhibited.

The mechanisms by which plants perceive a change in temperature and relay the signal to a downstream response are still unclear. It has been suggested that membrane rigification, caused by the lowered temperature, leads to actin filament reorganization and influx of Ca^{2+}, which in turn results in induction of gene expression (Örvar *et al*. 2000). In our experiments we found that the Ca^{2+} response occurred very early during the lowering of the temperature, at a time point when the temperature had decreased by only a few degrees. It seems unlikely that membrane rigification and actin reorganization could occur during this very brief time period and be induced by the small temperature decrease that precedes the increase in $[Ca^{2+}]_{cyt}$. A small downward shift in temperature is not likely to result in a change in the molecular motion of membrane lipids that is large enough to be detected by a possible sensor (Murata & Los 1997). In the study by Monroy & Dhindsa (1995), no influx of Ca^{2+} into alfalfa protoplasts was detected at temperatures above 15 °C and the Ca^{2+} influx was measured at time points much later during the LT exposure than the ones we have used. It is therefore difficult to compare how the alfalfa cells responded regarding changes in Ca^{2+} with the timing of the Ca^{2+} peak that we obtained in our experiments. We have not analysed the involvement of any other components in the signal transduction but it could be that the regulation by temperature that we observed after longer LT exposures might involve the components suggested by Örvar *et al*. 2000). These responses would then only occur if the LT exposures were present for longer time periods. This indicates the presence of two signalling pathways in response to changes in temperature. One involves a rapid change in temperature and an accompanying immediate increase in $[Ca^{2+}]_{cyt}$ that leads to changes in gene expression which are not dependent on a continuous presence of a low temperature. The other pathway, which might involve membrane rigification and a subsequent change in $[Ca^{2+}]_{cyt}$, would lead to a temperature-dependent induction of gene expression. The presence of multiple signalling pathways in response to abiotic stress has lately become evident (reviewed by Xiong, Schumaker & Zhu 2002). The different pathways include signalling components such as Ca^{2+}, reactive oxygen species, phospholipids and phosphorylation cascades. Furthermore, different sensors that perceive the initial signal might also exist (Xiong *et al*. 2002). A putative sensor protein has been proposed that might detect physical phase transitions in microdomains of the plasma membrane as a result of temperature shifts (Murata & Los 1997). It could be speculated that such a sensor could detect a rapid cooling, with a temperature drop of only a few degrees, and in turn activate the Ca^{2+} channels responsible for the early $[Ca^{2+}]_{cyt}$ increase observed in this study.

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