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LOS2, a genetic locus required for cold-responsive gene transcription encodes a bi-functional enolase

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The *Arabidopsis* mutation, *los2*, impairs cold-responsive gene transcription, acquired freezing tolerance and plant resistance to chilling under certain conditions. *LOS2* was isolated through positional cloning and shown to encode an enolase in the glycolytic pathway. In animal cells, enolase has also been known to function as a transcription factor that represses the expression of c-*myc* by binding to the c-*myc* gene promoter. LOS2 fused to green fluorescent protein is targeted to the nucleus as well as to the cytoplasm. LOS2/enolase protein can bind to the *cis*-element of the human c-*myc* gene promoter and to the gene promoter of STZ/ZAT10, a zinc finger transcriptional repressor from *Arabidopsis*. *STZ/ZAT10* expression is induced rapidly and transiently by cold in the wild type, and this induction is stronger and more sustained in the *los2* mutant. Furthermore, the expression of a *RD29A-LUC* reporter gene is repressed significantly by STZ/ZAT10 in transient expression assays in *Arabidopsis* leaves. Our results demonstrate that cold-responsive gene transcription in plants is controlled by a bi-functional enolase.

Keywords: cold signaling/enolase/LOS2/transcription factor/ZAT10 (STZ)

Introduction

Low temperature is an important environmental factor influencing plant growth and development. Some plants can cold acclimate, i.e. their freezing tolerance can be increased by being exposed to low but non-freezing temperatures (Levitt, 1980). Cold-regulated gene expression is a critical aspect of cold acclimation. Low temperatures induce the expression of a diverse array of plant genes, many of which are known as *COR* (for cold regulated), *KIN* (for cold induced), *LTI* (for low temperature induced) or *RD* (for responsive to desiccation) genes. Some of the gene products have been shown to contribute functionally to freezing tolerance. For example, the chloroplastic COR15A protein acts as a cryoprotectant by stabilizing membranes against freezing injury (Steponkus *et al*., 1998).

Substantial progress has been made towards understanding the transcriptional regulation of cold-responsive genes. Many of these genes have in their promoters one or several copies of the *DRE/CRT cis*-element, which has the core sequence CCGAC (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger *et al*., 1997). A small family of transcription factors known as CBFs or DREB1s (Stockinger *et al*., 1997) binds to this element and activates transcription of the downstream *COR/RD/KIN/LTI* genes. Interestingly, the *CBF/DREB1* genes are themselves induced by low temperatures, and this induction is transient and precedes that of downstream cold-regulated genes with the *DRE/CRT cis*-element (Medina *et al*., 1999). Therefore, there is a transcriptional cascade leading to the expression of the *DRE/CRT* class of genes under cold stress. Ectopic expression of *CBF/DREB1* genes in plants turns on downstream cold-responsive genes even at warm temperatures (Jagglo-Ottosen *et al*., 1998). In transgenic *Arabidopsis* plants ectopically expressing *CBF3*, higher levels of proline and soluble sugars were found, which correlate with increased freezing tolerance (Gilmour *et al*., 2000).

CBF1 physically interacts with a histone acetyltransferase and a putative transcriptional adaptor protein (Stockinger *et al*., 2001). Recently, a negative regulator of cold-induced *CBF* expression was cloned (Lee *et al*., 2001). This regulator, *HOS1*, was identified in a genetic screen for *Arabidopsis* mutants with de-regulated *RD29A* expression (Ishitani *et al*., 1998). In homozygous recessive *hos1* mutant plants, cold induction of *RD29A* and its upstream regulators, *CBFs*, is enhanced. *HOS1* encodes a variant RING finger that exists in the cytoplasm at warm temperatures but appears in the nucleus when plants are incubated in the cold (Lee *et al*., 2001). Since some RING finger proteins can serve as ubiquitin E3 ligases that help degrade specific target proteins, HOS1 has been proposed to function by targeting the transcriptional activator of CBFs for degradation.

In order to have a better understanding of cold sensing and signaling in plants, more regulatory components need to be identified. We report here the characterization and cloning of a mutation, *los2*, which specifically impairs cold-induced transcript accumulation of stress genes. *LOS2* is also critical for cold acclimation, and for chilling resistance under some conditions. *LOS2* encodes an enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) that converts 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway. In animal cells, part of the enolase protein has been shown to bind to the promoter element of the c-*myc* gene and to repress c-*myc* expression (Feo *et al*., 2000; Subramanian *et al*., 2000). The LOS2 protein can bind to the c-*myc* promoter as well as to the promoter of the zinc finger *STZ/ZAT10* from *Arabidopsis*. *STZ/ZAT10* expression is induced rapidly and transiently by cold in wild-type plants, and the induction is significantly enhanced and prolonged by the *los2* mutation.

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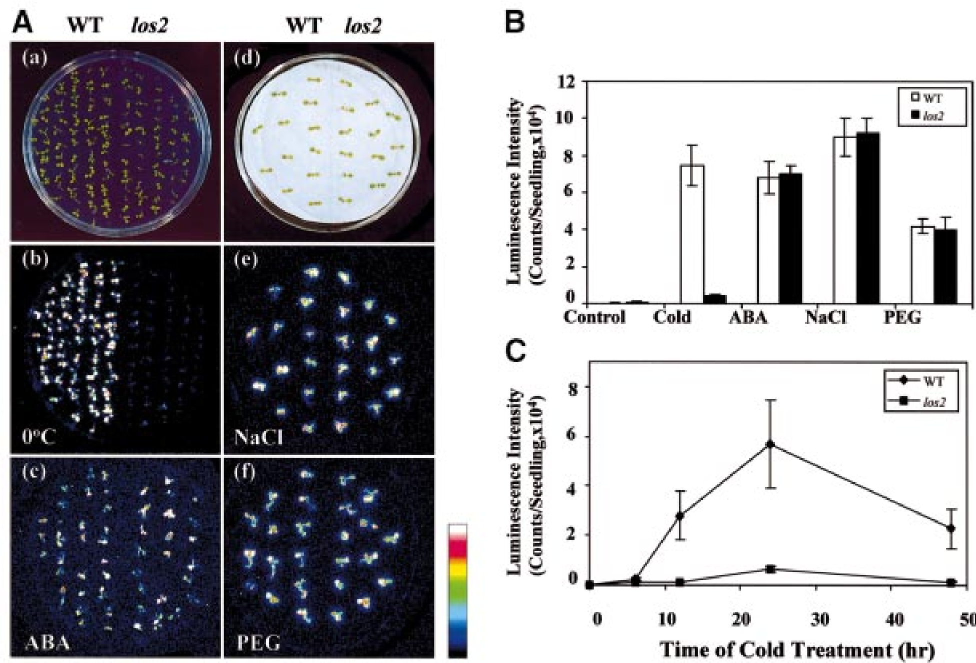

Fig. 1. The *los2* mutation impairs cold-induced luminescence expression. (**A**) Luminescence images of wild-type and *los2* plants. (a) Wild-type and *los2* seedlings used for cold or ABA treatment. (b and c) Luminescence of wild-type and *los2* seedlings after low temperature (0°C, 24 h) or ABA treatment (100 µM ABA, 3 h). (d) Wild-type and *los2* seedlings used for NaCl or PEG treatment. (e and f) Luminescence of wild-type and *los2* seedlings after NaCl (300 mM NaCl, 5 h) or PEG treatment (30% PEG 6000, 5 h). The color scale on the right shows the luminescence intensity from dark blue (lowest) to white (highest). (**B**) Luminescence intensity in wild-type and *los2* plants under different stresses. Shown are the mean values ± SE from 20 individual seedlings. Stress treatments were as described in (A). (**C**) Time course of luminescence expression in wild-type and *los2* plants during cold treatment. The wild-type and *los2* plants on the same agar plate were incubated at 0°C for the indicated time and luminescence images were taken. Shown are the mean values ± SE from 20 individual seedlings.

These results suggest that LOS2 has a direct regulatory function in controlling gene expression under low temperature stress.

Results

Identification of the LOS2 locus

To facilitate genetic dissection of cold signaling, we previously constructed *Arabidopsis* plants that emit bioluminescence in response to cold temperatures. The plants express the firefly luciferase reporter gene (*LUC*) under the control of the *RD29A* promoter, which contains the cold- and osmotic stress-responsive *DRE/CRT* element and the abscisic acid (ABA)-responsive *ABRE* element (Ishitani *et al*., 1997). The *RD29A-LUC* plants (referred to herein as wild type) were mutagenized by ethylmethane sulfonate, and mutants with aberrant luminescence responses were screened from the resulting M_2 population by luminescence imaging using a cooled CCD camera (Ishitani *et al*., 1997).

One mutant, designated as *los2*, shows a substantially reduced luminescence response specifically under cold stress, and was chosen for detailed characterization. Figure 1A shows the *RD29A-LUC* transgene expression phenotype of *los2* and wild-type plants when treated with cold, ABA, NaCl or polyethylene glycol (PEG). Wild-type and mutant plants were grown on the same agar plate and bioluminescence images were taken after the respective stress treatments. Without stress treatment, no *RD29A-LUC* expression was detected in either the wild-type or *los2* plants (data not shown). In response to cold treatment for 24 h, wild-type plants showed strong luminescence emission. In contrast, luminescence emission from cold-treated *los2* plants was very weak (Figure 1Ab). ABA, NaCl or PEG treatment induced similar levels of luminescence in wild-type and *los2* plants (Figures 1Ac, e and f). These luminescence images indicate that the *los2* defect in *RD29A-LUC* expression is specific to cold stress. A quantification of the luminescence shows clearly that *RD29A-LUC* expression in *los2* is virtually non-responsive to cold, but is still fully responsive to ABA, NaCl or PEG. The cold-induced luminescence was 9.2 times lower in *los2* compared with the wild type (Figure 1B).

To test whether *los2* plants display similarly reduced expression of the *RD29A-LUC* transgene at different time points of cold treatment, 10-day-old seedlings on agar plates were cold treated for 0, 6, 12, 24 or 48 h and then used for luminescence imaging. As shown in Figure 1C, the luminescence intensity of wild-type plants increased quickly in response to cold treatment, and reached a peak level after 24 h of cold treatment. In *los2* plants, the luminescence intensity was very low throughout the time course of cold treatment.

To test the dominance of the *los2* mutation, F_1 plants were obtained by backcrossing *los2* plants with the wild type. All F_1 plants exhibited the wild-type phenotype of luminescence expression in response to cold treatment (data not shown). The F_2 progeny of the selfed F_1 showed an ~3:1 segregation for wild type/mutant, when imaged for luminescence after cold treatment (data not shown). These results indicate that *los2* is a recessive mutation in a single nuclear gene.

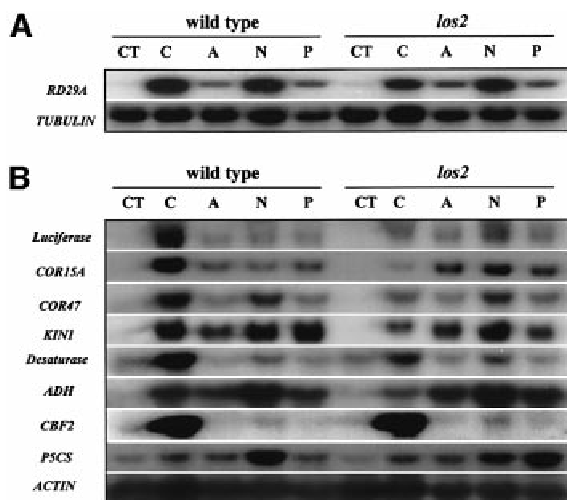

Fig. 2. *los2* blocks cold induction of *COR/KIN/RD/LTI* but not *CBF* genes. (**A**) Endogenous *RD29A* expression in wild-type and *los2* plants under different stress treatments. (**B**) The expression of other stress-responsive genes in wild-type and *los2* plants. Wild-type and *los2* plants were grown in the same agar plates for 3 weeks under continuous light at 22 ± 2°C. The seedlings were then treated with cold (0 ± 0.1°C for 24 h), ABA (100 μM for 3 h), NaCl (300 mM for 3 h) or PEG (30% for 5 h). Genes for β-tubulin or actin were used as loading controls. CT, control; C, cold; A, ABA; N, NaCl; P, PEG.

los2 blocks cold induction of *COR/KIN/RD/LTI* but not *CBF* genes

RNA blot analysis was carried out to determine the impact of *los2* mutation on the transcript levels of stress-responsive genes. The endogenous *RD29A* transcript level was greatly induced in the wild-type plants but this induction was lower in *los2* mutant plants (Figure 2A). Induction of the *RD29A* transcript by ABA, NaCl or PEG treatment was not affected by the *los2* mutation (Figure 2A). The effect of *los2* on the cold induction of endogenous *RD29A* is less dramatic compared with the effect on *RD29A-LUC*. This is probably because the *RD29A-LUC* transgene contains only part of the *RD29A* promoter (a fragment containing –646 to –1 upstream of the transcription start site), and there may be additional unknown regulatory elements in the endogenous *RD29A* promoter or even in the untranslated regions or introns of the endogenous *RD29A* gene. The difference between *RD29A-LUC* and endogenous *RD29A* expression levels has also been noticed in other mutants such as *hos1* (Ishitani *et al.*, 1998).

In contrast to the reduction of cold activation of *RD29A* expression, the *los2* mutation virtually blocked the cold induction of *LUC*, *COR15A*, *COR47*, *KIN* and *ADH* (alcohol dehydrogenase) genes (Figure 2B). As with the induction of *RD29A*, induction of *LUC*, *COR15A*, *COR47*, *KIN* and *ADH* by ABA, NaCl or PEG was not significantly affected or not at all affected by the *los2* mutation.

The *CBF/DREB1* transcription factor genes are induced specifically by cold treatment. We compared the expression of one of them, *CBF2*, in wild-type and *los2* plants (Figure 2B). The *los2* mutation had no effect on the cold regulation of *CBF2*, implying that *LOS2* does not function upstream of the *CBF* genes.

Membrane fluidity is an important determining factor for cell adaptation to both high and low temperature stresses (Levitt, 1980). Plants are known to increase in polyunsaturated phospholipids to maintain proper membrane fluidity under cold stress (Miquel *et al.*, 1993). We determined the expression of one of the desaturase genes, Δ9-desaturase. The result shows that expression of this desaturase gene was highly induced by cold stress in the wild type (Figure 2B). The induction was much diminished in *los2* mutant plants, indicating that polyunsaturation of membrane phospholipids may not occur in *los2* in response to cold stress.

The compatible osmolyte, proline, plays an important role in freezing tolerance (Delauney *et al.*, 1993). The *P5CS* gene encodes the rate-limiting enzyme, Δ^1-pyrroline-5-carboxylate synthetase, in proline biosynthesis. *P5CS* expression was induced slightly by cold treatment, and this induction was not affected by the *los2* mutation. Curiously, *P5CS* induction by NaCl appeared to be reduced whereas its induction by PEG was enhanced in the *los2* mutant (Figure 2B).

los2 mutant plants are chilling sensitive in the light but not in the dark

Unless indicated otherwise, all our cold treatments were carried out in the dark. Under the dark cold treatments as well as under normal growth temperatures, *los2* plants behaved just like the wild-type plants, and no visible phenotypic changes could be detected. However, when *los2* mutant plants were incubated in the cold under light, a striking phenotype was observed: the mutant plants wilted. The wilting of *los2* plants first became visible at the leaf tips after 2 days at 4°C and under light. After 1 week, the mutant leaves were entirely desiccated but maintained their green color (Figure 3Ab). With prolonged cold and light exposure, *los2* plants became bleached and dead (Figure 3Ad). In comparison, the vigor and survival of wild-type plants were not visibly affected by even prolonged cold and light treatment.

The wilting of *los2* plants in response to cold and light treatment appeared to be a result of a water deficit because no wilting occurred when the plants were covered with a plastic film (data not shown). However, the mutant plants did not lose more water than the wild-type plants in the cold (in the light) or at normal growth temperatures (data not shown). Furthermore, the wilting occurred regardless of the amount of water supplied to the roots. We hypothesized that the cold-induced wilty phenotype of *los2* plants may be caused by impaired water absorption or conduction by the roots. It has been well documented that some chilling-sensitive plants such as mung bean and tomato experience a sharp decrease in root hydraulic conductance and subsequent wilting when they are exposed to chilling temperatures (Markhart *et al.*, 1979; Bagnall *et al.*, 1983).

We performed a series of grafting experiments to test the 'water absorption/conduction' hypothesis. The results show that wilting occurred when the grafted plants had *los2* shoots, but no wilting occurred when they had wild-type shoots, regardless of the root genotypes (Figure 3Ag–j). Because the wilting phenotype has nothing to do with the root genotype, the results clearly reject the 'water absorption/conduction' hypothesis.

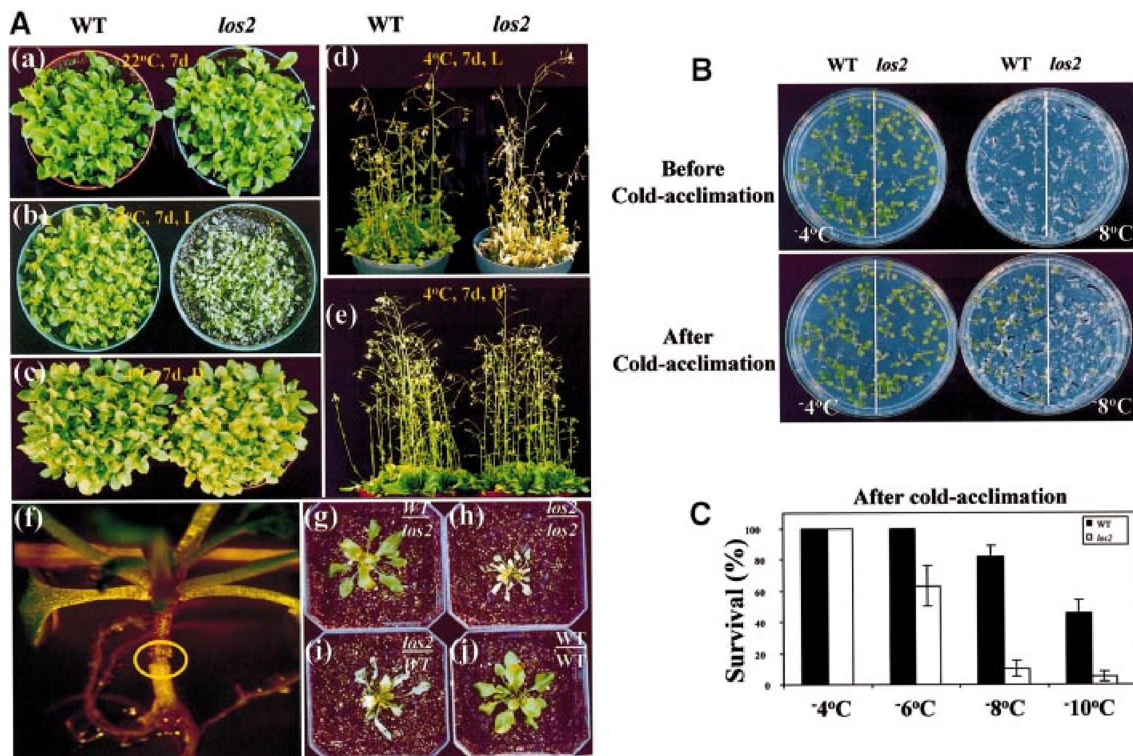

Fig. 3. Chilling and freezing sensitivity in *los2* plants. (**A**) Morphology of wild-type and *los2* plants with or without cold treatment and grafting experiment. (a) Wild-type and *los2* plants are normal when grown at 22°C. (b and d) *los2* but not wild-type plants wilt when incubated under light (20 μmol/m^2/s) at 4°C for 7 days. (c and e) Neither *los2* nor wild-type plants wilt when incubated in the dark at 4°C for 7 days. (f–j) Grafting experiment showing that wilting is not caused by the *los2* root. (f) A successfully grafted plant. The circle highlights the graft junction. (g–j) Chilling sensitivity of grafted plants at 4°C. Grafted plants in soil were incubated in the cold room for 10 days under continuous light (20 μmol/m^2/s) before pictures were taken. (g) Seedling with a wild-type shoot and *los2* root. (h) Seedling with a *los2* shoot and root. (i) Seedling with a *los2* shoot and wild-type root. (j) Seedling with a wild-type shoot and root. (**B**) Morphology of wild-type and *los2* plants after freezing stress, with or without cold acclimation treatment. (**C**) Quantification of the freezing survival rate for wild-type and *los2* plants with cold acclimation treatment.

Since *los2* mutant plants show light-dependent chilling sensitivity, we performed experiments to determine whether the effect of the mutation on cold-responsive gene expression is also light dependent. Luminescence imaging showed that reduced cold induction of *RD29A-LUC* expression in *los2* seedlings occurred in either light or dark conditions (data not shown). RNA blot analysis revealed that cold-induced expression of *RD29A*, *COR15A* and *COR47* was diminished by the *los2* mutation in either light or dark conditions (data not shown). In contrast, cold-induced *CBF2* expression in either light or dark conditions was not reduced by the *los2* mutation (data not shown). These results demonstrate that although *los2* plants show light-dependent chilling sensitivity, the effect of the mutation on cold-responsive gene expression is not dependent on light.

Chilling-induced cell death and freezing sensitivity in los2 mutant plants

We attempted to examine the effect of cold acclimation on the freezing tolerance of *los2* plants. Wild-type and *los2* plants were treated at 4°C under light for 2 days, and covered with plastic film to avoid the wilting of the mutant. The leaves were then excised and subjected to a standard electrolyte leakage assay (Ishitani *et al*., 1998). As expected, the wild-type plants showed an increase in freezing tolerance and a decrease in electrolyte leakage, in response to the cold acclimation treatment (Figure 4A). Surprisingly, *los2* leaves showed a dramatic increase in electrolyte leakage after the cold acclimation treatment (Figure 4A). The results suggest that *los2* cell membranes became leaky when the mutant plants were exposed to cold and light, consistent with the chilling-sensitive phenotype.

The *los2* leaves that were treated for 2 days at 4°C under light (under a plastic film cover to prevent wilting) were examined under a transmission electron microscope. Under the electron microscope, *los2* mesophyll cells were found to have disintegrated cell membranes (Figure 4B). Membrane blebbing was prevalent in *los2* cells. These cytological changes indicate that the cold and light treatment induced cell death in *los2* leaves.

In order to avoid chilling sensitivity during cold acclimation treatment, wild-type and *los2* plants were incubated for 2 days at 4°C in the dark. The method of Xin and Browse (1998) was then used to determine cold-induced freezing tolerance. Without cold acclimation treatment, wild-type and *los2* plants had the same level of freezing tolerance (Figure 3B). For example, both the wild type and *los2* mutant survived –4°C freezing but neither survived –8°C freezing (Figure 3B). However, after the 2 day cold acclimation treatment in the dark, wild-type plants became substantially more tolerant to freezing than *los2* plants (Figure 3B). Most of the *los2*

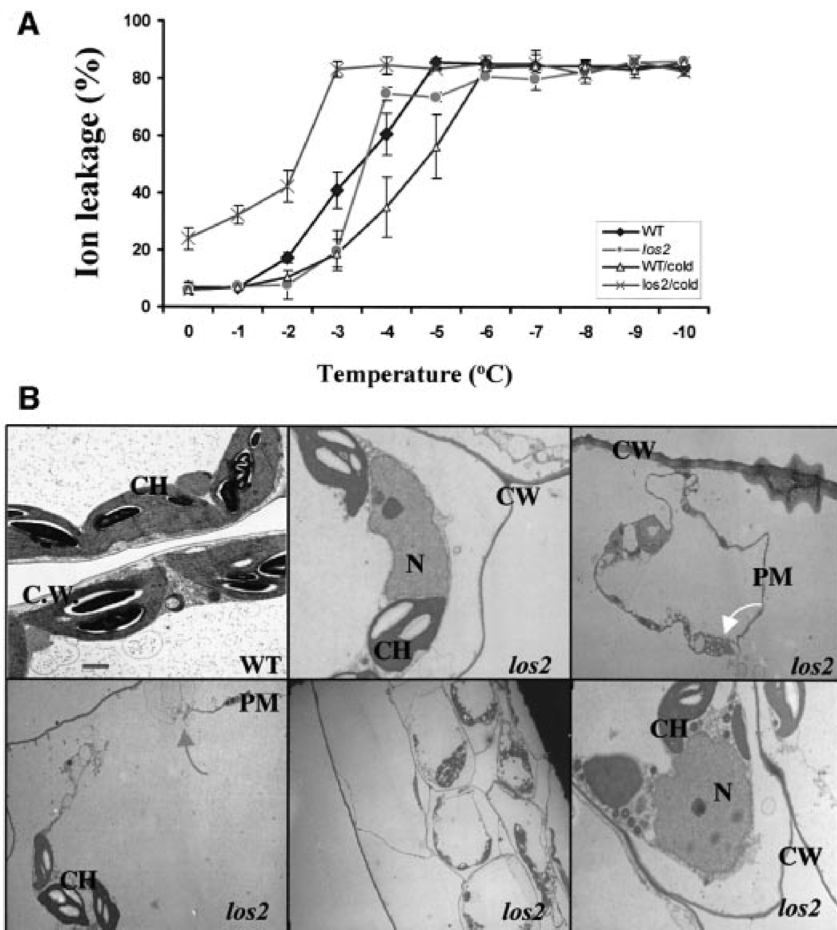

Fig. 4. *los2* plants are defective in membrane integrity under light and cold treatment. (**A**) Ion leakage assay. Wild-type and *los2* mutant leaves from 3-week-old plants grown in soil were used for the assay. For cold acclimation (CA), plants were incubated at 4°C for 2 days under light (20 μmol/m^2/s) before the ion leakage assay. Shown are the mean values and standard errors from three replicates. (**B**) Protoplast shrinkage and plasma membrane damage revealed by transmission electron microscopy. Leaves from wild-type and *los2* plants incubated at 4°C for 2 days under light (20 μmol/m^2/s) are shown. Bar = 1 μm. CW, cell wall; PM, plasma membrane; N, nucleus; CH, chloroplast. The gray arrow indicates broken plasma membrane in *los2* leaf cells. The white arrow indicates membrane blebbing in *los2* leaf cells.

plants did not survive freezing at –8°C. On the contrary, ~81% of the wild-type plants survived the –8°C freezing. Even after freezing at –10°C, ~43.5% of the wild-type plants survived (Figure 3C). The data indicate that *los2* mutant plants are defective in cold acclimation.

Positional cloning reveals that LOS2 encodes an enolase

To map the *LOS2* gene genetically, *los2* plants in the C24 ecotype were crossed with wild-type plants of the Columbia ecotype. The resulting F_1 plants were selfed and the F_2 progeny were planted and grown on MS agar plates for 10 days. Luminescence images of plants after 24 h cold treatment were obtained to select homozygous *los2* plants for genetic mapping. Representative molecular markers that cover five *Arabidopsis* chromosomes were surveyed, and the *LOS2* gene was located to chromosome II. Further mapping positioned *LOS2* to the lower arm of chromosome 2, north of nga168. More refined mapping delimited *LOS2* to a region covered by 10 bacterial artificial chromosome (BAC) clones T20F21, F11F19, F9C22, F2H17, F1O11, F13K3, TIJ8, T2N18, F3G5 and F13M22 (Figure 5A). For the fine mapping, simple sequence repeats were identified from the BAC sequences, and PCR primer pairs were designed to amplify selected repeats to identify simple sequence length polymorphism (SSLP) markers. Also, sequence polymorphisms between Col and C24 ecotypes identified during candidate gene sequencing to find a *los2* mutation were utilized to develop molecular markers (see Materials and methods for details). In summary, with a total of 4563 *los2* plants, the *los2* mutation was delimited to an ~28 kb region between the F1O11.12 and F1O11.20 markers (Figure 5A). There are seven predicted open reading frames (ORFs) in this region. All genomic DNA in this region including the seven ORFs was amplified from the wild-type C24 and *los2* mutant plants and sequenced. This sequence analysis revealed a single G to A mutation in the F1O11.16 gene that encodes an enolase (Van Der Straeten *et al.*, 1991). The mutation would result in the substitution of Gly325 by serine in the *los2* mutant.

To confirm that F1O11.16 is the *LOS2* gene, the F1O11 BAC clone was digested with restriction enzymes *Eco*RI and *Sac*I, which yielded a 5157 bp fragment containing the enolase ORF plus 886 bp upstream of ATG and 1386 bp downstream of the stop codon. The enolase fragment was

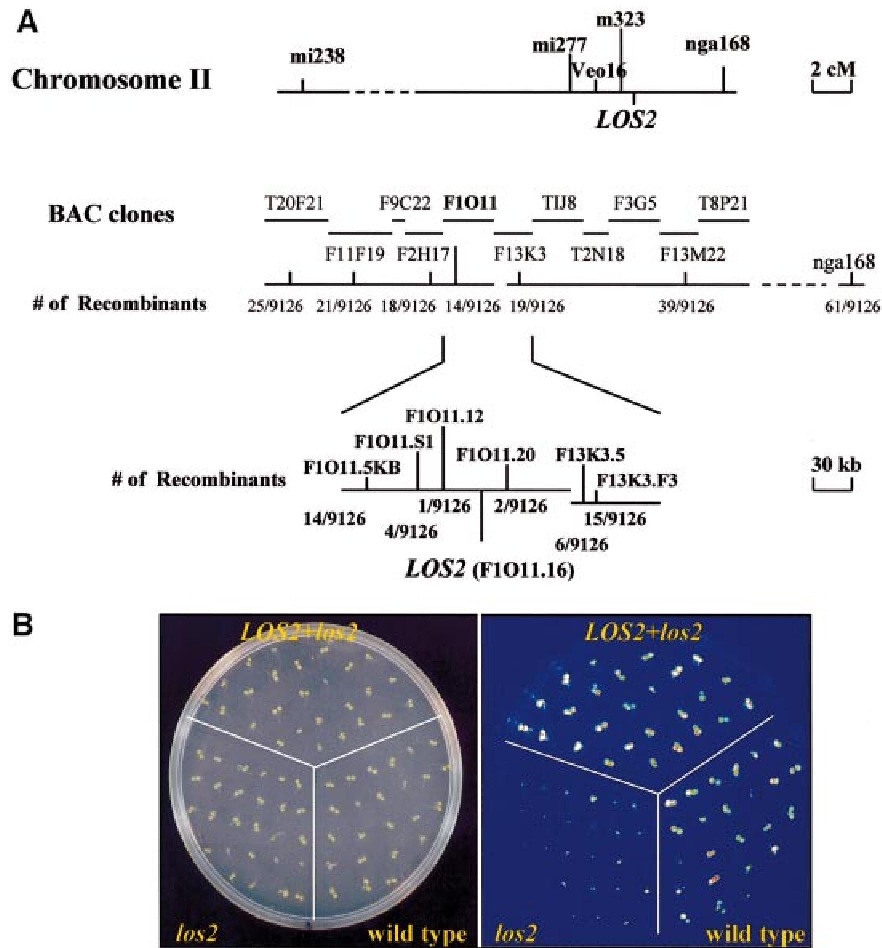

Fig. 5. Positional cloning of *LOS2* and mutant complementation assay. (**A**) Positional cloning. Genetic mapping with SSLP markers positioned *LOS2* to the BAC clone F1O11. Sequence analysis identified a single nucleotide mutation that would result in an amino acid sequence change (Gly325 to serine) in the ORF F1O11.16. (**B**) Complementation assay. *RD29A-LUC* luminescence expression (after 2 days treatment at 0°C) is shown for one representative homozygous T_3 line (*LOS2*+*los2*) transformed with a construct containing a wild-type F1O11.16 gene. Also included in the agar plate are wild-type and *los2* plants as controls.

cloned into the binary vector pCAMBIA 1200 and introduced into *los2* mutant plants via *Agrobacterium*-mediated transformation. Forty-five hygromycin-resistant individuals were selected, and their T_2 and T_3 progeny were subjected to complementation analysis with luminescence imaging. One representative line in the T_3 generation was chosen to illustrate its luminescence response to cold treatment. The result is presented in Figure 5B, which shows that the F1O11.16 gene complemented the luminescence phenotype of *los2* mutant plants. In addition, all of the 45 transgenic *los2* plants expressing the wild-type F1O11.16 transgene were exposed to 4°C under light for a week. None of the transgenic plants wilted, proving that the F1O11.16 gene also complemented the chilling-sensitive phenotype of *los2* mutant.

The transcript of the *LOS2* gene was constitutively present in *Arabidopsis* seedlings (Figure 7A). The transcript level appeared to be slightly up-regulated in response to cold treatment. The *los2* mutation did not alter this pattern of *LOS2* transcript expression (Figure 7A). We fused the *LOS2* promoter region (–1055 to –1 from ATG) to the α-glucuronidase reporter gene (*GUS*), and the resulting construct was introduced into wild-type *Arabidopsis* plants. *GUS* expression was detected in all plant tissues examined (Figure 7B). The LOS2 protein level was also up-regulated slightly by cold treatments (Figure 7C). LOS2 protein was present in *los2* mutant seedlings, but the level appeared to decline after a 2 day cold treatment (Figure 7C).

The los2 mutation reduces enolase activity at both cold and warm temperatures

Both the wild-type and mutant *LOS2* ORFs were expressed in bacteria to produce recombinant enolase proteins. *In vitro* assays carried out at 22°C showed that the wild-type LOS2 protein had a high enolase activity. In comparison, the *los2* mutant protein had a significantly reduced activity at 22°C (Figure 7D). When the assays were carried out at 0°C, both the wild-type and mutant proteins displayed much lower activities.

At 22°C, tissue extracts from *los2* mutant also had a significantly lower activity compared with that from wild-type plants (Figure 7D). At 0°C, tissue extracts from both wild-type and *los2* mutant plants had low activities, and the difference between the wild-type and *los2* plants was reduced (Figure 7D). These results show that LOS2 is

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LOS2              1:MATITVVKARQIFDSRGNPTVEVDIHTSNGIKVTAAVPSGASTGIYEALELRDGG-SDYLGKGVSKAVGNVNNIIGPALI 79
                    *       ** *************   ** *     *******************      * ********    *  * ***
Human α-enolase   1:MSILK-IHAREIFDSRGNPTVEVDLFTSKGL-FRAAVPSGASTGIYEALELRDNDKTRYMGKGVSKAVEHINKTIAPALV 78

LOS2             80:G-K-DPTQQTAIDNFMVHELDGTQNEWGWCKQKLGANAILAVSLAVCKAGAVVSGIPLYKHIANLAGNPKIVLPVPAFNV 157
                     *    * *   **   *   *  ***   *     * * ****** ***********   * *** *** ****     ********
Human α-enolase  79:SKKLNVTEQEKIDKLMI-EMDGT--E-N--KSKFGANAILGVSLAVCKAGAVEKGVPLYRHIADLAGNSEVILPVPAFNV 152
                                   └──► MBP-1                          DNA binding domain

LOS2            158:INGGSHAGNKLAMQEFMILPVGAASFKEAMKMGVEVYHHLKSVIKKKYGQDATNVGDEGGFAPNIQENKEGLELLKTAIE 237
                    ************************ * ***  * **** ** *** *** ************** *************
Human α-enolase 153:INGGSHAGNKLAMQEFMILPVGAANFREAMRIGAEVYHNLKNVIKEKYGKDATNVGDEGGFAPNILENKEGLELLKTAIG 232

LOS2            238:KAGYTGKVVIGMDVAASEFYSEDKTYDLNFKEENNNGSQKISGDALKDLYKSFVAEYPIVSIEDPFDQDDWEHYAKMTTE 317
                    ***** *************     * *** **        *  ** * * ******    ** ************    * *
Human α-enolase 233:KAGYTDKVVIGMDVAASEFFRSGK-YDLDFK-SPDDPSRYISPDQLADLYKSFIKDYPVVSIEDPFDQDDWGAWQKFTAS 310

LOS2            318:CGTEVQIVGDDLLVTNPKRVAKAIAEKSCNALLLKVNQIGSVTESIEAVKMSKKAGWGVMTSHRSGETEDTFIADLAVGL 397
                      *   * ***** ****** ***   ***** **************  * *       ***** *************** ***
Human α-enolase 311:AG--IQVVGDDLTVTNPKRIAKAVNEKSCNCLLLKVNQIGSVTESLQACKLAQANGWGVMVSHRSGETEDTFIADLVVGL 388

                                        Repression domain

LOS2            398:STGQIKTGAPCRSERLAKYNQLLRIEEELGSEAIYAGVNFRKPVEPY                                  444
                     ****************************** *  ** *** *
Human α-enolase 389:CTGQIKTGAPCRSERLAKYNQLLRIEEELGSKAKFAGRNFRNPLAK-                                  434
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Fig. 6. Amino acid sequence comparison between LOS2 protein and human α-enolase. In human α-enolase, the DNA-binding domain is underlined, and the transcriptional repression domain is double-underlined. The arrow indicates the start site of alternative translation for the c-*myc* promoter-binding protein (MBP-1).

indeed an enolase, and the *los2* mutation disrupts the enolase activity at both warm and cold temperatures. Therefore, the *los2* mutation is not a cold-sensitive allele of *LOS2*. The fact that *los2* tissue extracts had a much lower activity compared with the wild-type extracts suggests LOS2 as the major contributor to enolase activity in *Arabidopsis*.

LOS2–GFP fusion protein is targeted to the nucleus as well as cytoplasm

Enolase has been implicated as a multifunctional protein in animals and fungi. For example, in human cells, part of the enolase has been identified as MBP1 that binds to the c-*myc* promoter and represses c-*myc* gene expression (Ray and Miller, 1991; Ghosh *et al*., 1999a; Feo *et al*., 2000). MBP1 is an alternatively translated form of enolase that simply lacks the first 93 amino acid residues (Feo *et al*., 2000; Subramanian *et al*., 2000; Figure 6). As a glycolytic enzyme, enolase is localized in the cytoplasm. As a transcription factor, MBP1 is localized in the nucleus (Feo *et al*., 2000).

To examine the localization of LOS2 protein, green fluorescent protein (GFP) was fused in-frame to the C-terminus of LOS2, and the fusion protein expressed in *Arabidopsis* plants under the cauliflower mosaic virus (CaMV) 35S promoter. Fifteen independent T_1 lines were selected and examined for GFP expression. Undoubtedly, GFP expression was detected in the cytoplasm (Figure 8A). However, GFP was also clearly detected in the nucleus (Figure 8A). The nuclear GFP expression was detected at both cold and normal growth temperatures (Figure 8A). GFP fusion with several truncated versions of LOS2 (e.g. starting with Met93 or Met187) gave similar localization patterns (data not shown).

LOS2 binds to the promoter and down-regulates the expression of ZAT10, an early cold-responsive gene encoding a transcriptional repressor

Human α-enolase (ENO1) has been shown to bind to the c-*myc* promoter and acts as a negative regulator of the c-*myc* gene (Ray and Miller, 1991; Ghosh *et al*., 1999a). Comparison of LOS2 and human ENO1 sequences revealed that the putative DNA-binding and repression domains are conserved in LOS2 (Figure 6), suggesting that LOS2 may also be capable of binding to certain DNA sequences, perhaps sequences similar to the animal c-*myc* promoter element. To address this possibility, we performed an electrophoretic mobility shift assay (EMSA) using His-LOS2 fusion protein. As shown in Figure 8B, several DNA-shifted bands were observed with a DNA probe containing the human enolase-binding site in the c-*myc* promoter. The binding of LOS2 to the labeled human c-*myc* probe was competed off by unlabeled cold c-*myc* probe but not by a 1000-fold excess of poly(dI–dC), indicating the specificity of the LOS2–DNA interaction (Figure 8B).

Since no c-*myc* homolog is present in *Arabidopsis*, other genes might be targets for LOS2. The C-terminal region of LOS2 is very similar to that of human enolase (Figure 6), which serves as a repression domain (Ghosh *et al*., 1999a). To identify possible target genes of LOS2, we surveyed the promoter sequences of *Arabidopsis* genes encoding putative transcriptional repressors. We found that the region from –774 to –748 upstream of the translation initiation site of *STZ/ZAT10*, which encodes a putative transcriptional repressor (Ohta *et al*., 2001), has similarity to the enolase/MBP1-binding sequence in the human c-*myc* promoter (Figure 8D). As shown in Figure 8C, LOS2 was also capable of binding to this *STZ/ZAT10* promoter fragment (–775 to –747). Competition

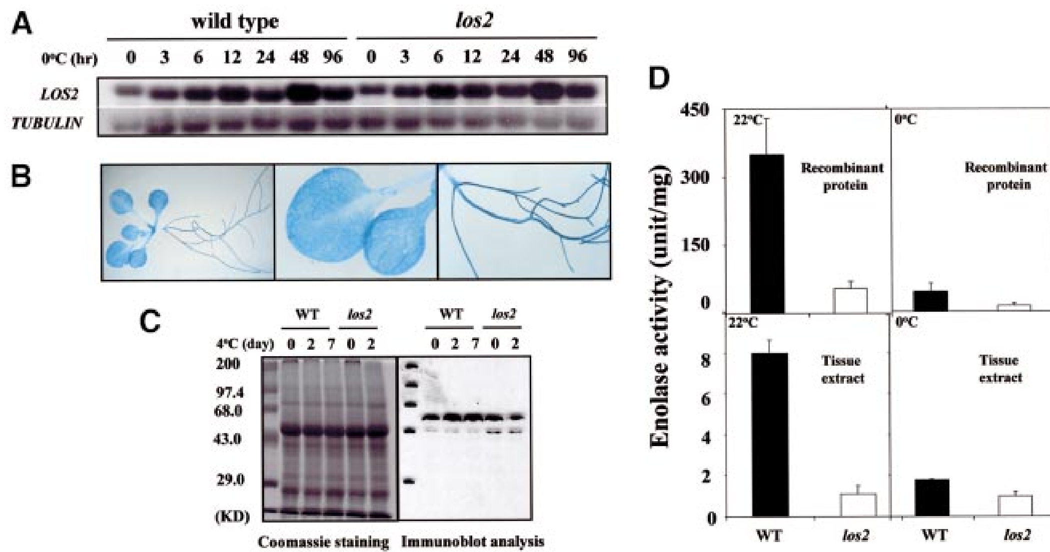

Fig. 7. Gene expression pattern and enolase activity of LOS2. (**A**) Expression of the *LOS2* gene transcript. Full-length LOS2 cDNA was used as a probe. The β-tubulin gene was used as a loading control. (**B**) *LOS2 promoter::GUS* expression. Wild-type plants containing *LOS2 promoter::GUS* were stained with X-Gluc for 15 h and visualized under a microscope. (**C**) Immunoblot analysis. A 20 μg aliquot of total protein from wild-type and *los2* leaves was extracted and fractionated in a 12.5% SDS–polyacrylamide gel. Shown are the gel picture of Coomassie staining and immunodetection with polyclonal anti-enolase. Molecular size markers are shown on the left. (**D**) Wild-type and mutant enolase activity. Shown are the mean values ± SE from three replicates.

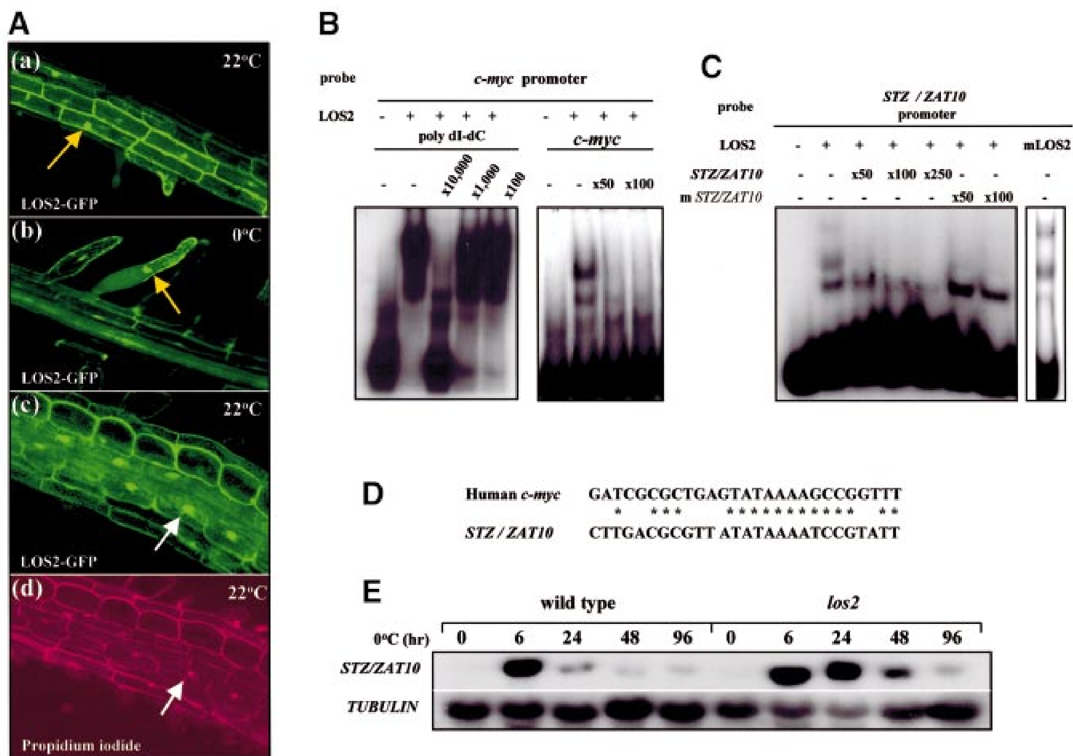

Fig. 8. LOS2 subcellular localization and binding to promoter elements. LOS2–GFP localizes in the nucleus as well as in the cytoplasm and binds specifically to the promoter of c-*myc* and *STZ/ZAT10*. (**A**) LOS2–GFP fusion protein is localized in the nucleus as well as in the cytoplasm. (a–c) Confocal images of root tissues expressing LOS2–GFP fusion protein at 22 or 0°C. Arrows point to nuclei. (d) The same root tissue shown in (c) was stained with propidium iodide for nuclei. (**B**) EMSA with the human c-*myc* promoter DNA probe. Cold competitors [poly(dI–dC) in the left panel and the c-*myc* promoter DNA probe in the right panel] were added to determine the binding specificity. (**C**) EMSA was carried out with the *STZ/ZAT10* promoter DNA probe in the presence or absence of wild-type (*STZ/ZAT10*) or mutated (m*STZ/ZAT10*) cold probes as competitors. (**D**) Sequence alignment between human c-*myc* and *Arabidopsis STZ/ZAT10* promoter elements included in the probes used in the EMSA. (**E**) Expression of *STZ/ZAT10* in response to cold stress in wild-type and *los2* plants.

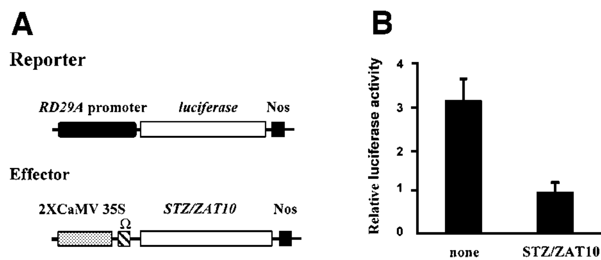

Fig. 9. STZ/ZAT10 can repress the expression of *RD29A* in *Arabidopsis* leaves. (**A**) Schematic representation of the reporter and effector plasmids used in transient expression assays. A portion of the *RD29A* promoter (black oval) was fused to firefly luciferase. Nos denotes the terminator signal of the gene for nopaline synthase. Ω indicates the translational enhancer of tobacco mosaic virus. (**B**) Relative luciferase activities after transfection with *RD29A-LUC* and 2× *35S-STZ/ZAT10*. To normalize values obtained after each transfection, pPTRL DNA that contained the CaMV 35S promoter and a gene for luciferase from *Renilla* was used as internal control. Luciferase activity was expressed in arbitrary units relative to the activity of *Renilla* luciferase (as described in Ohta *et al*., 2001). The values are averages of three bombardments, and error bars indicate standard deviations.

experiments with mutated *STZ/ZAT10* promoter fragment confirmed specific binding of LOS2 to this sequence. Mutant LOS2 protein could also bind to the fragment (–775 to –747) (Figure 8C).

RNA blot analysis showed that *STZ/ZAT10* expression was induced rapidly and transiently by cold stress in the wild type whereas, in the *los2* mutant, the cold induction was stronger and more sustained (Figure 8E). These results suggest that LOS2 negatively regulates the expression of *STZ/ZAT10* by binding to its promoter.

To examine whether *STZ/ZAT10* represses the expression of the *RD29A* gene, transient expression assays were performed in *Arabidopsis* leaves. The *RD29A-LUC* transgene, containing an upstream sequence (–646 to –1 from the transcription start site) of the *RD29A* gene fused to the *LUC* reporter gene was delivered into *Arabidopsis* leaves by particle bombardment. When the effector plasmid containing *STZ/ZAT10* under the control of the CaMV 35S promoter (Figure 9A) was co-transfected with *RD29A-LUC*, the LUC activity was reduced to ~30% (Figure 9B). This observation suggests that *STZ/ZAT10* represses the expression of the *RD29A* gene.

Discussion

In the present study, we identified and cloned *LOS2*, a positive regulator of cold-responsive gene transcription. We identified the *los2* mutant by its reduced expression of the *RD29A-LUC* transgene under cold treatment. Normal responses of *los2* plants to ABA, salt and PEG indicate that *los2* has a specific defect in cold signaling. Besides its effect on the *RD29A-LUC* transgene, *los2* also specifically impairs cold-regulated expression of the endogenous *COR/KIN/RD/LTI* genes. The *COR/KIN/RD/LTI* genes are regulated by cold through the *CRT/DRE* element in their promoters (Thomashow, 1999). CBF transcription factors bind to the *CRT/DRE* sequence and activate *COR/KIN/RD/LTI* expression. However, no difference was detected in the expression of *CBF* genes between wild-type and *los2* plants (Figure 2B). These results suggest that *LOS2* has a function downstream of the CBF transcription factors or is required directly or indirectly for CBF activation of the *COR/KIN/RD/LTI* genes. The effect of *los2* on *COR/KIN/RD/LTI* but not *CBF* genes is similar to that of *sfr6*, the only other *Arabidopsis* mutation known to reduce cold induction of genes (Knight *et al*., 1999). However, the effect of *sfr6* is not cold specific because the mutation also blocks osmotic stress or ABA induction of the *COR/KIN/RD/LTI* genes (Knight *et al*., 1999).

LOS2 is not only critical for freezing tolerance, it is also important for chilling resistance. In the light, *los2* mutant plants are chilling sensitive. The chilling sensitivity is a consequence of cold- and light-induced cell death. The molecular basis of the induced cell death is unclear. It may be caused by metabolic imbalance because the *los2* mutation disrupts enolase activity. It may also have to do with the defective gene regulation in the *los2* mutant. As shown in Figure 2B, the transcript level of a desaturase gene is induced by cold in the wild type but this induction is greatly decreased in *los2*. Membrane fluidity is an important factor for plant survival under cold temperatures (Levitt, 1980). The defect in the desaturase gene induction may contribute to the chilling sensitivity of *los2* plants. Interestingly, *los2* plants are not chilling sensitive in the dark, although *los2* plants are impaired in cold-responsive gene expression in both light and dark. These observations suggest that the chilling sensitivity of *los2* plants in the light may also be related to metabolic imbalance caused by photosynthesis. Alternatively, the chilling sensitivity may be a manifestation of programmed cell death. A connection between human enolase and apoptosis has been demonstrated before. Part of human enolase, MBP1, when transfected into fibroblast cells, caused rapid cell death (Ray, 1995). The cold- and light-induced cell death in *los2* mutant plants has some hallmarks of programmed cell death, e.g. cell shrinkage, membrane blebbing (Figure 4B), nuclear condensation and DNA fragmentation (data not shown). Therefore, it is possible that the chilling-sensitive phenotype of *los2* is a result of programmed cell death caused by the interplay between defective gene regulation, and cold and light signals.

The *los2* mutation was found in an enolase gene that converts 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway. This enolase gene was able to complement the *los2* mutant phenotypes (Figure 5B). *LOS2* transcript can be detected at normal growth temperatures, and cold treatment enhanced transcript accumulation in both wild-type and *los2* plants. Since a CCGAC core sequence of *CRT/DRE* is found in the enolase gene promoter, it is possible that CBF1/DREB1 may regulate the expression of enolase under cold stress.

Interestingly, MBP1, part of human α-enolase, was shown to be targeted to the nucleus and binds to the c-*myc* promoter (Feo *et al*., 2000). MBP1 binds just 5′ to the P2 TATA motif and negatively regulates the expression of c-*myc* by preventing TATA-binding protein (TBP) from binding to the TATA motif (Ray and Miller, 1991). In addition to the inhibition of TBP binding, MBP1 has active repression domains in both its N- and C-terminal regions (Ghosh *et al*., 1999a; Figure 6). The active repression may be mediated partly through interaction with histone deacetylase (HDAC) (Ghosh *et al*., 1999b). Consistent with these results on c-*myc* repression, transfection of

human breast carcinoma cells with the *MBP1* cDNA inhibits tumor formation (Ray *et al*., 1995).

We suspected that LOS2/enolase might have a similar function as a transcriptional repressor that regulates cold-responsive gene expression. We found that the LOS2–GFP fusion protein is targeted to the nucleus (Figure 8A). In addition, LOS2 protein can bind to the TATA box region of the c-*myc* promoter. There is no bona fide c-*myc* homolog in *Arabidopsis*. However, we have identified an *Arabidopsis* zinc finger gene, *STZ/ZAT10*, which may be a direct target for LOS2/enolase regulation. The *STZ/ZAT10* gene promoter contains a similar region to the *cis*-element in c-*myc* where MBP1 binds. EMSAs show that LOS2 protein can bind to this *STZ/ZAT10* promoter element. The sequence of LOS2 is highly conserved with that of human α-enolase, especially in their C-terminal region that is necessary for active repression (Ghosh *et al*., 1999a; Figure 6). We showed that both the wild-type and mutant LOS2 proteins could bind to the sequence of the *STZ/ZAT10* promoter, indicating that mutation does not affect the binding activity of LOS2. Since the mutation in the *los2* allele substitutes Gly325 to serine that is located near the putative repression domain (Ghosh *et al*., 1999a), the mutation probably causes a defect in repression activity. We hypothesize that LOS2 represses *STZ/ZAT10* expression, which in turn may repress the transcription of *COR/KIN/RD/LTI* genes. *STZ/ZAT10* is induced rapidly and transiently by cold treatment, suggesting an important role for *STZ/ZAT10* in controlling genes that are induced more slowly by cold (e.g. the *COR/KIN/RD/LTI* genes). Our finding that *STZ/ZAT10* expression is increased in the *los2* mutant plants is consistent with LOS2 having a role in repressing *STZ/ZAT10* transcription.

STZ/ZAT10 is a TFIIIA/Krüppel-like zinc finger protein that has been shown to repress the *trans*-activation of genes through an essential repression motif (Ohta *et al*., 2001). *STZ/ZAT10* is similar to other zinc finger proteins such as petunia *ZPT2-2* (*EPF2-5*) (Takatsuji and Matsumoto, 1996). Binding experiments have demonstrated that ZPT2-2 recognizes similar sequences containing the core sequence, AGT (or ACT in a reverse orientation) (Takatsuji and Matsumoto, 1996). In the case of ZPT2-2, the target sequence consists of a tandem repeat of two AGTs, and the spacing between the two AGT sequences is important for ZPT2-2 to distinguish target sequences (Takatsuji and Matsumoto, 1996). Interestingly, we found two tandem AGT sequences with appropriate spacing between them, from –554 to –522 upstream of the transcription initiation site in the *RD29A* gene promoter (ACTAGTGTA-N_{13}-TCTAGTAAG). This suggests that *RD29A* may be one of the direct target genes for *STZ/ZAT10*. All the other genes such as *COR15A*, *COR47*, *KIN1* and the desaturase that are blocked by the *los2* mutation also have in their promoters similar tandem AGT repeats. Moreover, our transient expression assays revealed that indeed STZ/ZAT10 represses *RD29A-LUC* expression, suggesting that STZ/ZAT10 may interact directly with the sequences in the *RD29A* promoter. In conclusion, our data provide the first genetic evidence that LOS2/enolase is a positive regulator of the *RD29A* gene, by controlling the expression of STZ/ZAT10 that represses the *RD29A* gene. Thus, LOS2/enolase and STZ/ZAT10 along with other transcription factors such as CBFs/DREB1s constitute a fine-tuned transcriptional regulatory circuitry for optimal cellular responses to cold environments.

Materials and methods

Mutant isolation, luminescence imaging and plant growth

Mutant screening, procedures for stress treatment and bioluminescence imaging, and conditions for growing plants in soil were described previously (Ishitani *et al*., 1997; Lee *et al*., 2001).

RNA analysis

Two-week-old wild-type and mutant plants grown on standard 0.6% agar medium containing Murashige and Skoog (MS) salt supplemented with 3% sucrose were treated with either low temperature, ABA, NaCl or PEG. Whole seedlings (~0.3 g) were homogenized and total RNA was extracted as described (Lee *et al*., 2001). Gene-specific DNA probes were as described (Ishitani *et al*., 1998; Lee *et al*., 2001). For the Δ9-desaturase gene, a full-length cDNA was used as the probe.

Grafting experiment

The hypocotyls of 1-week-old seedlings grown vertically on MS agar plates were excised with micro-dissecting scissors (Fisher Scientific) under a dissecting microscope, and wild-type or mutant shoots were placed carefully on top of mutant or wild-type roots, respectively. The re-connected seedlings were incubated in sealed agar plates in order to heal under high humidity. Since the rate of success grafting was very low (~5%), a large number of seedlings was used. Successfully grafted seedlings were transferred to soil and grown to the rosette stage for the examination of chilling sensitivity.

Electrolyte leakage and freezing tolerance assays

The electrolyte leakage assay was carried out as described previously (Ishitani *et al*., 1998).

The freezing tolerance assay was carried out as described by Xin *et al*. (1998). Briefly, seedlings were grown in a standard 0.6% agar medium for 2 weeks under continuous light. Plates with seedlings were placed in a freezing chamber set to –1°C and treated for 16 h prior to decreasing the temperature at 1°C/h. Freezing was initiated by the addition of ice chips to the plates. Plates were removed at the desired temperatures and placed at 4°C overnight for thawing. After recovery for 2 days, the number of surviving plants was scored. For cold acclimation, plates were incubated at 4°C in the dark for 2 days before the freezing treatment.

Genetic mapping and cloning of LOS2

For genetic mapping of the *los2* mutation, the *los2* mutant of the *Arabidopsis* C24 ecotype was crossed with wild-type plants of the Columbia ecotype. A total of 4563 homozygous *los2* mutants were chosen from the segregating F_2 population with the phenotype of low expression of *RD29A-LUC* after cold treatment. Genetic mapping was carried out as described (Lee *et al*., 2001).

For *los2* complementation analysis, a genomic fragment containing the enolase ORF plus 886 bp of upstream sequence and 1386 bp of downstream sequence was digested with *Eco*RI and *Sac*I and inserted into the binary vector pCAMBIA 1200. The resulting plasmid was transferred to the *los2* mutant via *Agrobacterium*-mediated transformation. Forty-five hygromycin-resistant T_1 transformants were selected, seedlings of T_2 and T_3 progeny were subjected to cold treatment and luminescence images were taken. In addition, all of the 45 transgenic *los2* plants expressing the wild-type F1O11.16 transgene were exposed to 4°C under light for a week to test the complementation of the chilling-sensitive phenotype of *los2* plants.

For the *LOS2-GUS* construct, a *LOS2* promoter region from –1055 to –1 (relative to the ATG start codon) was amplified and inserted into *Sal*I–*Sma*I sites of the binary vector pCAMBIA1391Z. The plasmid was transferred to wild-type C24 plants. Twenty-five hygromycin-resistant T_1 transformants were selected, and seedlings of T_2 progeny were stained with X-Gluc for 15 h as described (Lee *et al*., 2001).

Expression of LOS2–GFP fusion protein

Wild-type *LOS2* cDNA was amplified by RT–PCR and inserted into the *Nco*I site of the binary vector pCAMBIA1200/35SP/GFP. Forty lines of hygromycin-resistant T_1 transformants were selected and their segregating T_2 progeny were examined for GFP expression (Lee *et al*., 2001).

Enolase activity assay and immunoblot analysis

The wild-type and mutant *LOS2* cDNAs were amplified by RT–PCR and inserted into the *Nco*I site of the expression vector pET14b. The plasmids were transferred to *Escherichia coli* cells (BL21 DE3, Codon Plus). Expression of His-LOS2 fusion protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1.5 mM. Purification of wild-type and mutant His-LOS2 protein was performed according to the manufacturer's instructions (His-Bind Buffer Kit, Novagen, WI). The enolase activity assay and immunoblot analysis were as described (Van Der Straeten *et al*., 1991).

DNA binding assay

EMSAs were carried out as described (Hao *et al*., 1998). The following double-stranded oligonucleotides were used for probes and competitors in EMSAs. Human c-myc, 5′-GGAGGGATCGCGCTGAGTATAAAAG-CCGGTTTCGG-3′ (Ray and Miller, 1991); ZAT10, 5′-GGTCTTGAC-GCGTTATATAAAATCCGTATTA-3′; and m-ZAT10, 5′-GGAGGT-CATATGGCGCGCCCCGAATGCGG-3′. These probes were end-labeled with [α-^{32}P]dCTP by Klenow fragment and purified through a Sephadex G-50 column. The labeled probes were incubated with 5 μg of purified His-LOS2 fusion protein in 1× binding buffer (Hao *et al*., 1998) supplemented with 20 pmol poly(dI–dC) for 20 min at room temperature. The resulting DNA–protein complexes were resolved by electrophoresis on a 6% polyacrylamide gel in 0.5× TBE buffer and visualized by autoradiography. For competition experiments, unlabeled competitors were incubated with the His-LOS2 fusion protein on ice for 30 min prior to adding labeled probes.

Transient expression assay

For the reporter gene construct, a fragment containing –646 to –1 upstream of transcription start site of the *RD29A* gene was fused transcriptionally to firefly *LUC* (Promega). A plasmid for the expression of *STZ/ZAT10* was constructed as follows. The CaMV 35S promoter and the GUS reporter gene in pBI221was replaced by the 2× CaMV 35S promoter to generate 2× 35S-Nos. The cDNA for *STZ/ZAT10* was amplified by PCR, digested with *Sal*I and inserted into *Sma*I and *Sal*I sites of 2× 35S-Nos. Plasmid DNA was delivered to *Arabidopsis* leaves using particle bombardment (Ohta *et al*., 2001).

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