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A novel cold-inducible gene from *Arabidopsis*, *RCI3*, encodes a peroxidase that constitutes a component for stress tolerance

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Summary

A cDNA from *Arabidopsis* corresponding to a new cold-inducible gene, *RCI3* (for *R*are *C*old *I*nducible gene 3), was isolated. Isoelectric focusing electrophoresis and staining of peroxidase activity demonstrated that *RCI3* encodes an active cationic peroxidase. RNA-blot analysis revealed that *RCI3* expression in response to low temperature is negatively regulated by light, as *RCI3* transcripts were exclusively detected in etiolated seedlings and roots of adult plants. *RCI3* expression was also induced in etiolated seedlings, but not in roots, exposed to dehydration, salt stress or ABA, indicating that it is subjected to a complex regulation through different signaling pathways. Analysis of transgenic plants containing *RCI3::GUS* fusions established that this regulation occurs at the transcriptional level during plant development, and that cold-induced *RCI3* expression in roots is mainly restricted to the endodermis. Plants overexpressing *RCI3* showed an increase in dehydration and salt tolerance, while antisense suppression of *RCI3* expression gave dehydration- and salt-sensitive phenotypes. These results indicate that RCI3 is involved in the tolerance to both stresses in *Arabidopsis*, and illustrate that manipulation of *RCI3* has a potential with regard to plant improvement of stress tolerance.

Keywords: peroxidase, abiotic stress, *Arabidopsis*, dehydration tolerance, salt tolerance, freezing tolerance.

Introduction

Low temperatures are among the most important environmental factors influencing plant distribution, development and survival. Many plants from temperate regions have developed natural adaptive mechanisms to tolerate low and freezing temperatures. Central to this adaptation is the process of cold acclimation, by which plants can adjust their metabolism to cold and increase their tolerance to freezing in response to low-non-freezing temperatures (Levitt, 1980). The majority of these changes are regulated by low temperatures through alterations in gene expression and, in recent years, many genes whose expression is induced during cold acclimation have been isolated and characterized in several species (Thomashow, 1999). Some cold-inducible genes have been shown to be involved in freezing tolerance (Artus *et al*., 1996; Jaglo-Ottosen *et al*., 1998; Kasuga *et al*., 1999; Kim *et al*., 2001; Liu *et al*., 1998; Tähtiharju and Palva, 2001), however, the relative importance of these genes in cold acclimation is, in general, not well understood.

Although the expression of some cold-inducible genes seems to be specifically regulated by low temperatures, in most cases it is also responsive to dehydration, salt stress and abscisic acid (ABA) (Thomashow, 1999), indicating that plant responses to all these treatments share common features. In fact, low non-freezing temperatures lead to water stress by imparing water absorption by the roots and water transport to the shoot (Hale and Orcutt, 1987). Freezing damage is mainly a consequence of

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freezing-induced dehydration stress (Levitt, 1980). As for ABA, it mimics the effects of low temperature by increasing freezing tolerance (Chen and Gusta, 1983; Cloutier and Siminowitch, 1982; Mäntylä *et al.*, 1995). Some cold-inducible genes such as *RD29A/COR78/LTI78* and *COR15A* from *Arabidopsis* contain ABRE and DRE/CRT *cis*-regulatory elements in their promoters (Baker *et al.*, 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). Whereas the ABRE element is responsive to ABA, the DRE/CRT element mediates cold- and dehydration-regulated gene expression through an ABA-independent pathway (Shinozaki and Yamaguchi-Shinozaki, 2000). Transcription factors that specifically bind to DRE/CRT have been identified (Liu *et al.*, 1998; Stockinger *et al.*, 1997), and the corresponding genes, named *CBFs* or *DREBs*, shown to be induced by low temperature or dehydration and salt stress (Gilmour *et al.*, 1998; Liu *et al.*, 1998; Medina *et al.*, 1999). The overexpression of some of these genes leads to enhanced cold-regulated gene expression and increased plant tolerance to freezing, drought, and salt stresses (Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999).

The expression of cold-inducible genes is also regulated by intrinsic developmental cues (Baker *et al.*, 1994; Medina *et al.*, 2001; Pearce *et al.*, 1998; Sistrunk *et al.*, 1994; Wang and Cutler, 1995; Xu *et al.*, 1995; Yamaguchi-Shinozaki and Shinozaki, 1993), an expected result considering that freezing tolerance of plant tissues is affected by their developmental stage (Wang and Cutler, 1995) and low temperatures have pleiotropic effects on many developmental processes (Thomashow, 1999). Interestingly, most of these genes are not uniformly regulated by cold along the plant, suggesting that the molecular mechanisms of cold acclimation are differentially modulated in different tissues. Recent results have shown that changes in cytosolic calcium concentration in response to low temperatures are different in different cell types of *Arabidopsis* roots (Kiegle *et al.*, 2000), indicating that, in some cases, the cold response is even cell-type specific. In spite of the high number of cold-inducible genes so far identified none of them has been, however, reported to be specifically regulated by low temperature at cell or tissue level. The isolation and characterization of such genes would provide new clues to understand the molecular mechanisms governing the cold-acclimation response, and would help to reveal how environmental conditions interact with internal developmental programmes.

In an attempt to identify genes showing novel cold-inducible expression patterns, a cDNA library from cold-acclimated etiolated seedlings of *Arabidopsis* was screened with a substracted probe enriched in cold-induced transcripts. Thus, two cold-specific-inducible genes, *RCI1A (*for *R*are *C*old *I*nducible *1 A* gene) and *RCI1B*, encoding multifunctional regulatory 14-3-3 proteins were isolated (Abarca *et al.*, 1999; Jarillo *et al.*, 1994). In addition, *RCI2A* and *RCI2B*, two genes whose expression is transiently induced by cold and in response to drought, salt stress and ABA, and encode putative regulators of the plasma membrane potential, were also identified (Capel *et al.*, 1997; Medina *et al.*, 2001). Here, we report the isolation and molecular characterization of a new *R*are *C*old *I*nducible gene, *RCI3*, which encodes an active cationic peroxidase. Expression analysis showed that *RCI3* is subjected to an intricated regulation in response to environmental and developmental cues. Interestingly, overexpression of *RCI3* increases *Arabidopsis* tolerance to drought and salt stresses while suppression of *RCI3* expression reduces the tolerance of *Arabidopsis* to these adverse conditions. On the basis of these results, a putative role for RCI3 in stress tolerance is discussed.

Results

Isolation and molecular characterization of RCI3

RCI3 was isolated by screening a cDNA library prepared from cold-acclimated etiolated seedlings of *Arabidopsis* with a subtracted cDNA probe enriched in cold-induced transcripts. Northern-blot hybridizations with total RNA extracted from cold-acclimated and non-acclimated etiolated seedlings indicated that *RCI3* specifically hybridized with a cold-induced transcript of approximately 1.2 kb (data not shown). The nucleotide sequence of this clone (deposited in the GenBank database with the accession number U97684) was 1171 bp and contained an ORF of 978 bp that would encode a protein of 326 amino acids with an ATG codon at nucleotide 35 and the poly $(A)^+$ tail starting 159 nucleotides downstream from the stop codon.

Comparison of the predicted polypeptide RCI3 with sequences available in databases revealed high similarity with peroxidases from several plant species. Figure 1 shows the amino acid alignment of the deduced RCI3 sequence with those of TPX1, TPX2 and HRP-C peroxidases from tomato and horseradish. TPX1 and TPX2 are the reported peroxidases (Botella *et al.*, 1993), having highest similarity to RCI3 (76 and 78%, respectively). HRP-C has been extensively characterized and can be considered a model peroxidase (Fujiyama *et al.*, 1988; Welinder, 1992). RCI3 contains eight conserved Cys residues implicated in the formation of four disulfide bridges (Kjaersgard *et al.*, 1997), as well as the distal and proximal catalytic sites characteristic of plant peroxidases (Welinder, 1992). Moreover, as reported for other plant peroxidases, RCI3 includes an endoplasmic reticulum signal peptide but lacks the carboxy-terminal propeptide displayed by the vacuole-targeted peroxidases such as HRP-C (Johansson *et al.*, 1992). The presence of the signal peptide indicates that RCI3 may be processed in a mature protein of 302 amino acids with a calculated molecular mass of 32.4 kDa and a pI of 8.5, indicating that it is a cationic peroxidase. The mature form

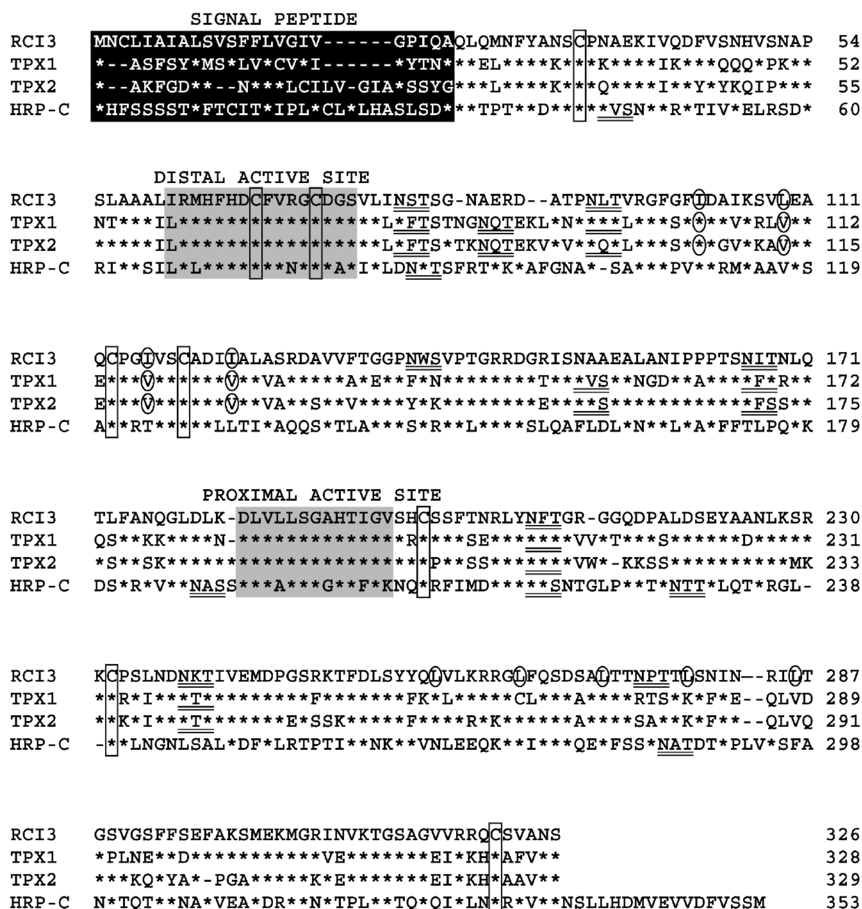

Figure 1. Sequence alignment between RCI3 and other peroxidases.
The amino acid sequence deduced from *Arabidopsis RCI3* cDNA was aligned with TPX1 and TPX2 proteins from tomato (Botella *et al.*, 1993) and HRP-C from horseradish (Fujiyama *et al.*, 1988). Amino acid residues identical to RCI3 protein sequence at a given position are indicated by asterisks. Dashes have been included to optimize alignments. The black box highlights the putative signal peptides. Grey boxes mark the distal and proximal active sites. Vertical rectangles indicate cysteins involved in disulfide bridge formation. Circles mark amino acids that could be involved in leucine zippers. Putative glycosylation sites are double underlined, while the carboxy-terminal peptide in HRP-C is single underlined.

of RCI3 exhibits seven putative glycosylation sites (Asn-Xxx-Thr/Ser) (Gavel and von Heijne, 1990), and two putative leucine zippers that could be involved in protein–protein interactions (O'Shea *et al.*, 1989).

Analysis of the *Arabidopsis* genome sequence revealed that *RCI3* is located at the top of chromosome 1, in a genomic fragment cloned in YAC yUP8H12 (GenBank accession no. AC000098). Comparison of the cDNA with the genomic sequence showed that the *RCI3* gene contains three introns and four exons, the border regions coinciding in all cases with the consensus described for *Arabidopsis* genes (Brown, 1996). Analysis of the 5′ untranslated region of *RCI3* uncovered a putative TATA box sequence (TATATAAA) located at −94 bp from the initiation ATG codon, as well as several sequences sharing similarity to regulatory elements (DRE/CRT, G-box-like, MYC, GT1 and ROOT) involved in stress and organ-specific responses that could account for the complex expression pattern shown by this gene (see next two sections). Southern analysis of genomic DNA confirmed that a 3′ cDNA fragment containing 963 nucleotides of *RCI3* specifically hybridized to the *RCI3* restriction fragments without producing cross-hybridization (data not shown) and could be used as a probe for expression analyses.

The expression of RCI3 in response to low temperature is negatively regulated by light

Since *RCI3* had been identified from a cDNA library prepared from cold-acclimated etiolated seedlings, the expression of *RCI3* in response to low temperature was first characterized in etiolated seedlings of *Arabidopsis*. As shown in Figure 2(a), *RCI3* mRNAs gradually accumulated by cold treatment, attaining its maximal steady state level after 1 day of exposure to 4°C. Expression analyses carried out in adult plants of *Arabidopsis* showed that *RCI3* transcripts only accumulated in response to low-temperature in roots (Figure 2b).

The specific accumulation of cold-induced *RCI3* transcripts in etiolated seedlings and roots suggested that light could exert a negative effect on *RCI3* expression. To test this possibility, *RCI3* expression was analyzed in 5-day-old seedlings of *Arabidopsis* that had been grown under dark or light conditions at control temperature (20°C) or exposed for 1 day to 4°C. Whole seedlings, aerial parts (i.e. hypocotyl plus cotyledons) and roots were used for the experiments. The results obtained revealed that under dark conditions all samples analyzed accumulated *RCI3* transcripts in response to low temperature (Figure 2c). In contrast, accumulation

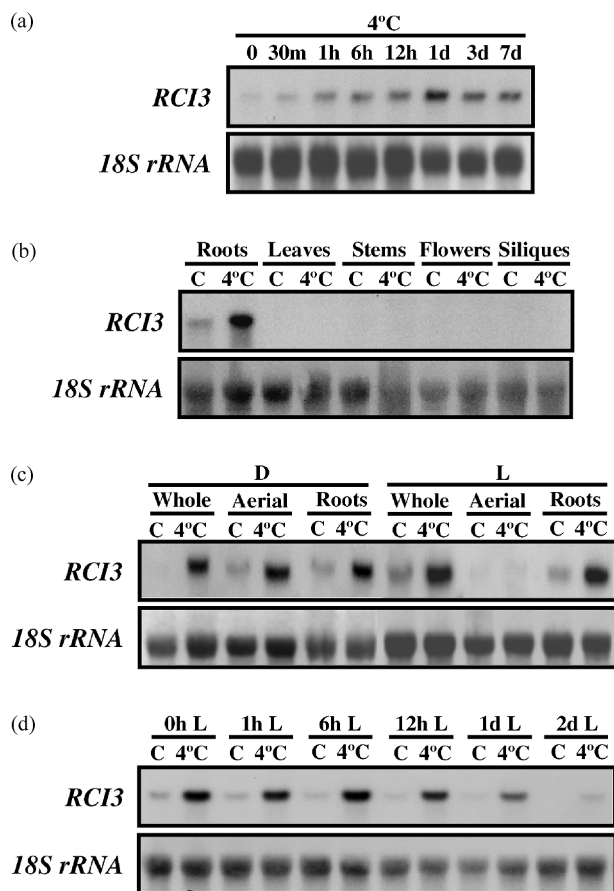

Figure 2. Regulation of *RCI3* expression by low temperature and light.
RNA-blot hybridizations performed with total RNA (10 μg per lane) and the *RCI3*-specific probe (see Experimental procedures). Equal RNA loading was verified using a *18S rRNA* probe (see Experimental procedures).
(a) RNA from 4-day-old whole etiolated seedlings exposed to 4°C for the indicated times.
(b) RNA from different organs of 8-week-old plants grown at control temperature (C) or exposed for 1 day at 4°C (4°C).
(c) RNA from 5-day-old seedlings (whole ones, their aerial parts or their roots) grown in the dark (D) or in the light (L) at control temperature (C) or at 4°C for 1 day (4°C).
(d) RNA from the aerial part of etiolated seedlings that were exposed to light (L) during the indicated times until becoming 4 days old, and then maintained at control temperature (C) or placed at 4°C (4°C) for 1 additional day in the dark.

of *RCI3* transcripts by cold under light conditions was only detected in whole seedlings and roots (Figure 2c). Therefore, cold-induced *RCI3* expression seems to be negatively regulated by light. To get an insight on how light can negatively regulate cold-induced *RCI3* expression, 4-day-old etiolated seedlings that had been exposed to light for different periods of time were subsequently incubated at 4°C or maintained at 20°C in the dark for 1 additional day. RNA-blot hybridizations performed with their aerial parts showed that light exposure gradually reduced *RCI3* transcript accumulation in response to low temperature, being almost abolished after 2 days of light treatment (Figure 2d).

The expression of RCI3 is also regulated by dehydration, NaCl and ABA

Since many cold-inducible genes are also responsive to exogenous ABA and osmotic-related stresses (Thomashow, 1999), the effect of dehydration, high salt and ABA on *RCI3* mRNA levels was analyzed. Taking into consideration that the expression of *RCI3* was differentially regulated by low temperature in etiolated seedlings and adult plants of *Arabidopsis* (see above), this study was carried out in etiolated seedlings, and leaves and roots of adult plants. In etiolated seedlings, *RCI3* mRNAs accumulated in response to all treatments although the attained levels were always lower than those obtained in response to low temperature (Figure 3a). No accumulation of *RCI3* transcripts was detected in leaves of adult plants under any circumstance (data not shown). In the case of roots, only a slight increase of *RCI3* mRNAs levels could be observed after dehydration

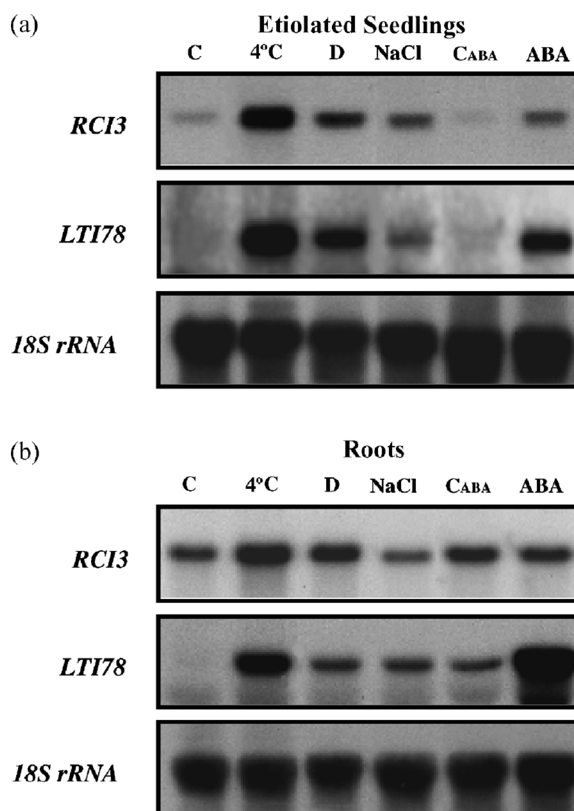

Figure 3. Accumulation of *RCI3* transcripts in response to dehydration, salt stress and ABA.
RNA-blot hybridizations were performed as in Figure 2. The efficacy of treatments was controlled with a *LTI78* probe (see Experimental procedures).
(a) RNA from 5-day-old whole etiolated seedlings grown at control temperature (C), exposed for 1 day at 4°C (4°C), dehydrated until losing 50% of their FW (D), treated with 250 mM NaCl (NaCl), with 100 μM ABA (ABA) or with the ABA solvent (C_{ABA}).
(b) RNA from roots of plants grown in liquid medium exposed to the same treatments as those described in panel (a).

(Figure 3b). Taken together, these results show that the accumulation of *RCI3* transcripts in response to dehydration, NaCl and ABA is also differentially regulated in etiolated seedlings and in different organs of adult plants. A probe that recognizes the *LTI78* gene from *Arabidopsis*, the expression of which is induced by low temperature, ABA, dehydration and NaCl treatments (Yamaguchi-Shinozaki and Shinozaki, 1993), was used as a positive control for treatments.

The expression of RCI3 is mainly restricted to the root endodermis

To investigate *RCI3* expression at tissue level during plant development and in response to different treatments, a 2.4-kb fragment of *RCI3* promoter (−2459 to −24 from ATG) was isolated, fused to the *uidA* (*GUS*) reporter gene, and the resulting construct introduced into *Arabidopsis* plants. Stable transformant lines were obtained and five independent representative ones analyzed. Results obtained with individual plants from these lines were, in all cases, very similar. RNA-blot experiments with etiolated transgenic seedlings that had been exposed to 4°C, dehydrated, or treated with NaCl or ABA revealed that *GUS* mRNAs accumulated in response to all treatments (Figure 4a). Experiments with roots from adult transgenic plants exposed to the same treatments showed that *GUS* messengers only accumulated significantly upon exposure to 4°C (Figure 4b). These expression patterns resembled very much those

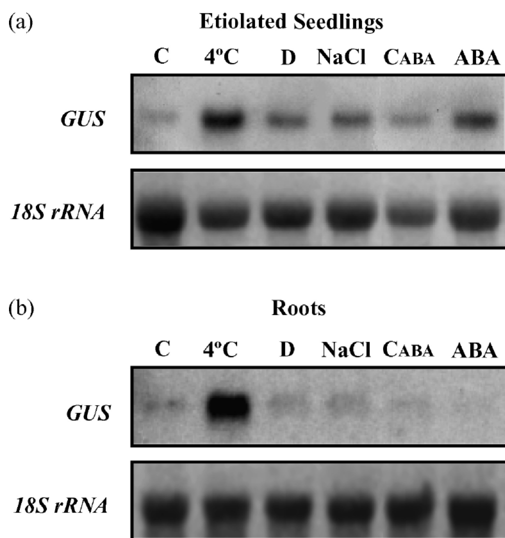

Figure 4. *GUS* expression in transgenic *Arabidopsis* plants containing *RCI3-GUS* fusions.
RNA-blot hybridizations using a specific probe for the *uidA* (*GUS*) gene (see Experimental procedures). Total RNA (10 μg per lane) was extracted from 5-day-old whole etiolated seedlings (a) and roots of plants grown in liquid medium (b) that had been exposed to the same treatments as in Figure 5 (a). Equal RNA loading was verified with a *18S rRNA* probe (see Experimental procedures).

observed for endogenous *RCI3* transcripts (Figure 3a,b), suggesting that the isolated promoter region contained the *cis*-elements involved in the regulation of *RCI3* in response to low temperature, dehydration, salt stress and ABA. In addition, these results established that the expression of *RCI3* in response to these treatments is regulated at the transcriptional level.

Once proved that the isolated *RCI3* promoter fragment was able to drive the transcription of the *GUS* gene in a correct way, the reporter-gene assay was used to follow the expression of *RCI3 in situ* during *Arabidopsis* development. Histochemical analysis of GUS activity did not show staining in *RCI3::GUS* mature seeds (Figure 5a). After 1 day of germination, *RCI3::GUS* transgenic seedlings showed GUS staining in the radical, including the radical hairs, although not in the root tip or the hypocotyl (Figure 5b). This pattern of GUS activity was maintained during germination, and cotyledons did not display any GUS staining either (Figure 5c–f). After germination, GUS activity was analyzed in 7-day-old etiolated *RCI3::GUS* seedlings. Under unstressed conditions only roots from these seedlings showed some GUS staining (Figure 5g). When 7-day-old etiolated transgenic seedlings were exposed to low temperature, dehydration, salt stress or ABA, the patterns of GUS staining were in all cases very similar. The results obtained in cold-treated seedlings are shown as a representative example (Figure 5h). High GUS activity was detected in roots and vascular tissues of hypocotyls but not in cotyledons or root tips.

GUS staining in fully-developed *RCI3::GUS* transgenic plants was also studied. The patterns of GUS expression under unstressed conditions and after exposition to low temperature, dehydration, salt stress or ABA were identical with just one difference. This difference was detected when measuring GUS activity, cold-treated roots showing higher values than control ones (data not shown). The results obtained in cold-treated plants are presented as a representative example (Figure 5i–q). High GUS staining was detected in both main and lateral roots, markedly in the root vascular cylinder, but not in root tips (Figure 5i–j). Analyses of cross-sections from roots revealed GUS activity in the cortex, the endodermis and the stele cell layers, the endodermis having especially high levels (Figure 5k). Stems, leaves and flowers did not show GUS staining (Figure 5l–p), which was only detected in the abscission zones of mature siliques (Figure 5q). Taken together, these data demonstrate that the expression of *RCI3* is tightly regulated during *Arabidopsis* development and is mainly confined to the root endodermis.

RCI3 encodes an active cationic peroxidase

Isoelectric focusing electrophoresis and staining of peroxidase activity was used to ascertain whether *RCI3* encoded

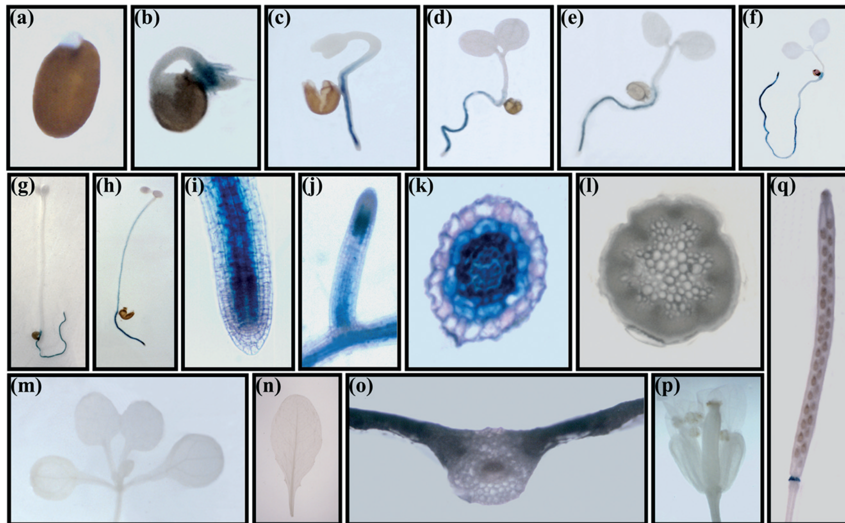

Figure 5. Histochemical localization of GUS activity in transgenic *Arabidopsis* plants containing *RCI3-GUS* fusions.(a) Mature seed. (b–f) Light-grown seedlings 1, 2, 3, 4 and 7 days after germination. (g) Seven-day-old etiolated seedling grown under control conditions. (h) Seven-day-old etiolated seedling exposed 1 day at 4°C. (i–q) Adult plants exposed to 4°C for 1 day. (i) Main root. (j) Secondary root. (k) Cross-section of a root. (l) Cross-section of a stem. (m) Three-week-old rosette. (n) Leaf. (o) Cross-section of a leaf. (p) Flower. (q) Mature silique.

an active peroxidase. The profiles of peroxidases extracted from 7-day-old whole seedlings, or their corresponding aerial parts, grown under light or dark conditions at 20°C and 4°C are shown in Figure 6(a). A band with a pI value of 8.6, which is very close to the theoretical pI value of the mature RCI3 protein (8.5), was detected in both dark- and light-grown whole seedlings developed at 20°C or subjected to 4°C. The intensity of this band was always higher in cold-treated seedlings than in control ones. The same isozyme was also detected in the aerial part of etiolated seedlings exposed to low temperature. The aerial part of light-grown seedlings did not reveal the band of pI at 8.6 band under any temperature condition. Extracts from leaves of 3-week-old *Arabidopsis* maintained at control

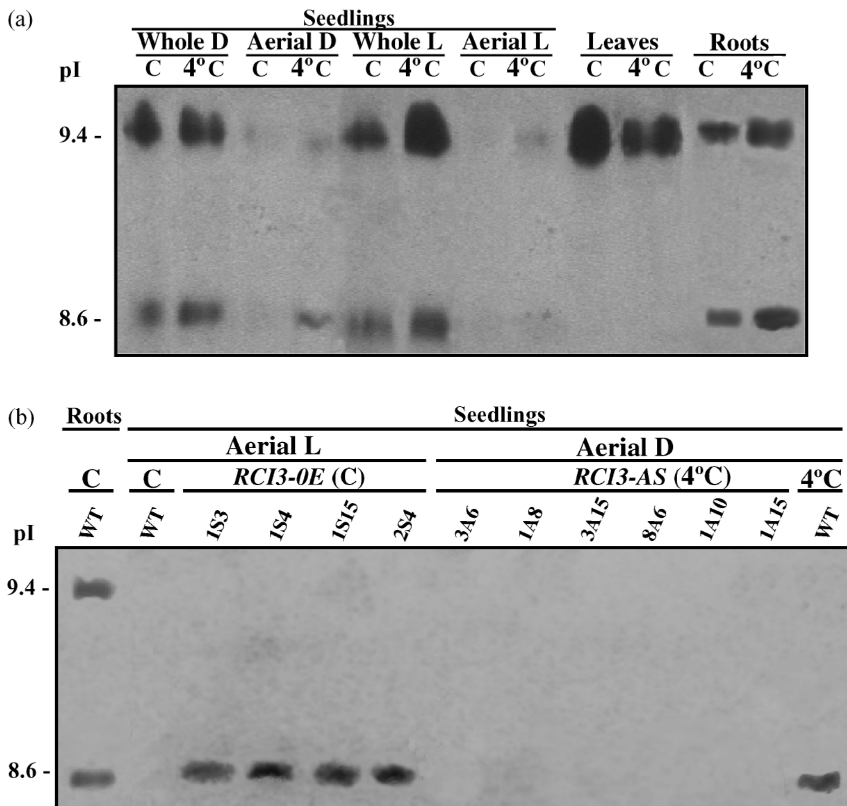

Figure 6. Peroxidase activity of RCI3.
Isoelectric focusing electrophoresis in polyacrylamide gels of crude plant extracts stained for peroxidase activity. The pI of the isozymes is indicated.
(a) Extracts from 7-day-old whole seedlings, or their corresponding aerial parts, grown under dark (D) or light (L) conditions at control temperature (C) or exposed for 3 days at 4°C (4°C). Extracts were also obtained from leaves of 3-week-old plants and roots of plants grown in liquid medium maintained at control temperature (C) or subjected to 4°C for 3 days (4°C).
(b) Extracts from the aerial part of 7-day-old seedlings grown under light (L) or dark (D) conditions at control temperature (C) or exposed 3d at 4°C (4°C). Extracts from roots of plants grown in liquid medium at control temperature (C) were also obtained. WT, *RCI3-OE* and *RCI3-AS* indicate Columbia wild-type, *RCI3* overexpressing transgenic lines and *RCI3* antisense transgenic lines, respectively.

temperature or placed at 4°C did not show this band either. In the case of roots, the isozyme of pI at 8.6 was detected in both control and cold-treated extracts. Nevertheless, its intensity was always higher in roots exposed to 4°C (Figure 6a). In addition to the pI 8.6 band, another unrelated band having a pI value of 9.4 was revealed in some extracts. The accumulation patterns of the 8.6 pI isozyme correlated to the accumulation patterns of *RCI3* transcripts described above (Figure 2), suggesting that this peroxidase activity could be the product of the *RCI3* gene. To confirm this possibility, transgenic *Arabidopsis* plants containing the *RCI3* cDNA in sense and antisense orientation under the control of the constitutive cauliflower mosaic virus 35S (CaMv35S) promoter were obtained. Four independent overexpressing lines homozygous for a single copy of the transgene and showing high levels of *RCI3* expression (data not shown) were selected to analyze their peroxidase profiles. Extracts obtained from the aerial part of 7-day-old overexpressing seedlings grown under light conditions at control temperature revealed, in all cases, one band of pI at 8.6 as that detected in extracts from wild-type roots maintained at 20°C (Figure 6b). This band, however, was not detected in 7-day-old wild-type seedlings grown under light conditions at control temperature (Figure 6b). On the other hand, six independent antisense transgenic lines homozygous for a single copy of the fusion were selected after verifying, by RNA-blot hybridization, that they showed null levels of *RCI3* mRNA in etiolated seedlings exposed to low temperature (data not shown). Contrary to the extracts from the aerial part of dark-grown wild-type seedlings exposed to 4°C that showed the 8.6 pI band, extracts from the aerial part of 7-day-old antisense seedlings grown under the same conditions did not reveal any band in any case (Figure 6b). All these results substantiate that the isozyme of pI at 8.6 corresponds to the RCI3 protein, and indicate that the *RCI3* gene encodes an active cationic peroxidase.

The expression levels of RCI3 correlate to stress tolerance in Arabidopsis

To explore a possible role of *RCI3* in stress tolerance, the transgenic plants of *Arabidopsis* that showed altered levels of RCI3 activity (see above) were analyzed for freezing, dehydration and salt tolerance. None of the transgenic plants analyzed exhibited any obvious morphological or developmental abnormality.

Tolerance to freezing was examined in both *RCI3* transgenic and wild-type plants, before and after cold acclimation. In the case of non-acclimated plants, freezing tolerance was determined after exposition to −7°C for 6 h. For cold-acclimated plants, tolerance was established after incubation to −11°C for 6 h. Cold acclimation was previously triggered by placing plants for 7 days at 4°C. No significant differences between transgenic and wild-type plants were found in any case, all being unable to resume growth after −7°C or −11°C treatment (data not shown).

Dehydration was induced by maintaining plants on a dry filter paper for 1 day without watering. The rate of dehydration was determined as the percentage of initial FW remaining after treatment. Wild-type and *RCI3* transgenic plants did not show significant differences in their initial FW values (data not shown). However, after dehydration, wild-type plants maintained an average of 15% of their initial FW, while plants overexpressing the *RCI3* cDNA in sense or antisense orientation maintained 27% and 5%, respectively (Figure 7a). As expected, these different rates of water loss correlated with the capacity of plants to survive after drought stress. In fact, whereas *RCI3* antisense plants were severely affected after 1 day without watering, plants overexpressing *RCI3* did not show apparent alterations in their phenotypes. Wild-type plants presented intermediate phenotypes (Figure 7b).

The influence of *RCI3* expression in salt stress tolerance was estimated by determining the number of green leaves in transgenic and wild-type plants after growing for 7 days in a medium containing 150 mM NaCl. The FW of the plants after the treatment was also considered to be an estimate of their salt tolerance. Wild-type and *RCI3* transgenic plants not subjected to the stress showed very similar values of both green leaves and FW (data not shown). On the contrary, marked differences in the number of green leaves and FW were observed among wild-type and *RCI3* transgenic plants after being exposed to NaCl. In fact, transgenic plants overexpressing *RCI3* showed similar values of green leaf number and FW to unstressed wild-type plants (Figure 7c), indicating that the overexpression of *RCI3* is enough to overcome the stress conditions imposed by this NaCl treatment. Wild-type plants subjected to salt stress displayed 80% and 70% of the green leaf number and FW they displayed under control conditions. In turn, *RCI3* antisense plants only showed 54% and 48% of the green leaf number and FW shown by unstressed wild-type plants (Figure 7c). As could be anticipated from these results, the capacity of *Arabidopsis* plants to develop and survive under salt stress conditions correlated to the levels of *RCI3* transcripts (Figure 7d). Taken together, all these results provide clear evidence for a positive relationship between the expression of *RCI3* and *Arabidopsis* tolerance to dehydration and salt stress.

Discussion

This work describes the identification and molecular characterization of a new low-temperature regulated gene from *Arabidopsis*, *RCI3*, whose expression is restricted to specific root cell types. *RCI3* encodes a functional cationic peroxidase which contains all the typical motifs of these

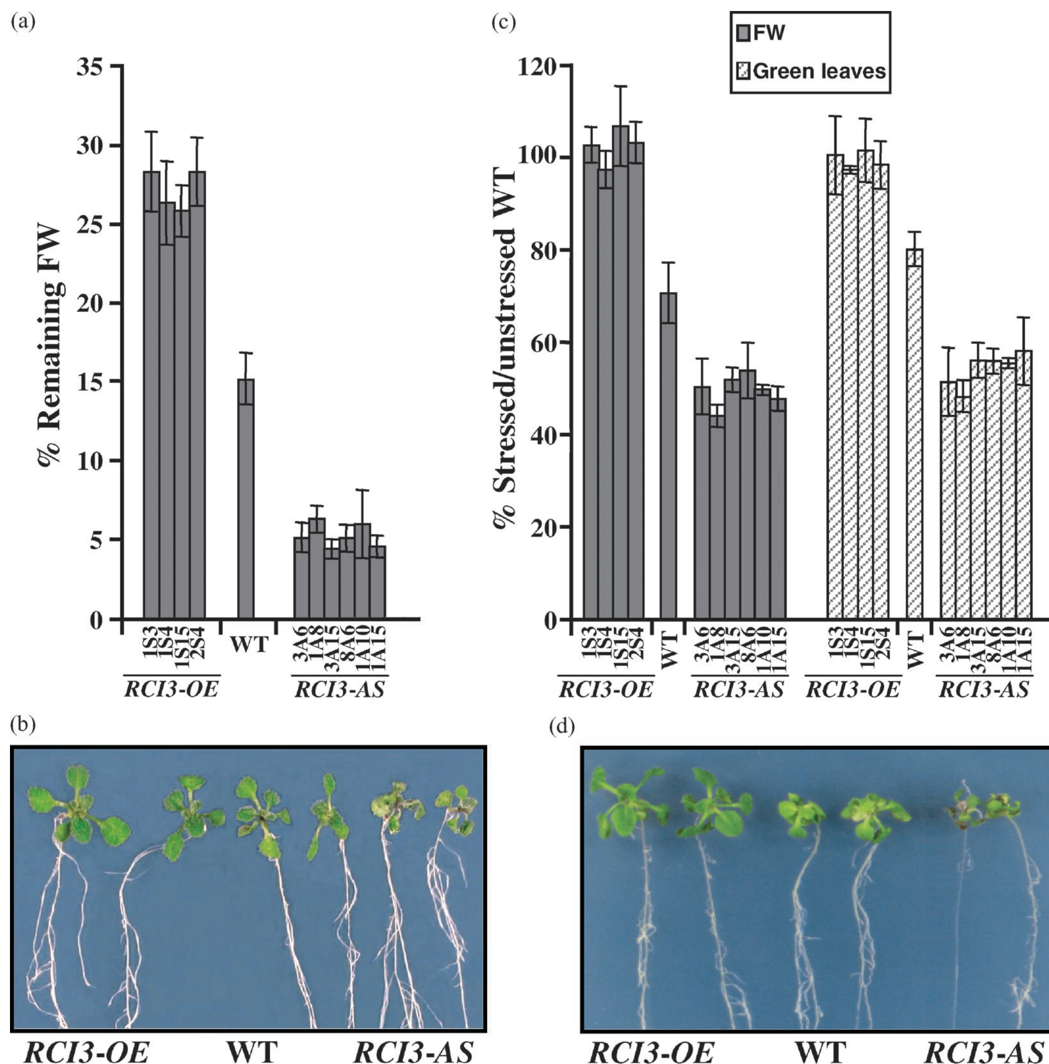

Figure 7. Stress tolerance of transgenic *Arabidopsis* plants overexpressing *RCI3* in sense and antisense orientation.
(a) Dehydration tolerance of 3-week-old wild-type (WT) and transgenic plants overexpressing *RCI3* in sense (*RCI3-OE*) or antisense orientation (*RCI3-AS*). Tolerance was estimated as the percentage of initial FW that remained after transferring plants to a dry filter paper and allowing them to develop for 1 day without watering. Data are expressed as means ($n = 20$). Bars indicate SE. In all cases, values obtained from overexpressing, wild-type and antisense plants were significantly different ($P < 0.05$) as determined by Student's *t*-test.
(b) Representative wild-type and *RCI3* transgenic plants after dehydration treatment.
(c) Salt tolerance of 3-week-old wild-type (WT) and transgenic plants (*RCI3-OE*, *RCI3-AS*). Tolerance was estimated by determining the FW and number of green leaves of plants transferred to a medium containing 150 mM NaCl for 7 days. These values are represented as a percentage of FW and number of green leaves of wild-type unstressed plants. Data are expressed as means ($n = 20$). Bars indicate SE. In all cases, values obtained from overexpressing, wild-type and antisense plants were significantly different ($P < 0.05$) as determined by Student's *t*-test.
(d) Representative wild-type and *RCI3* transgenic plants after salt treatment.

enzymes, including proximal and distal active domains (Welinder, 1992), cystein residues (Kjaersgard *et al.*, 1997) and glycosilation sites (Carpin *et al.*, 1999). RCI3 also contains an endoplasmic reticulum signal peptide (Johansson *et al.*, 1992), suggesting that it is secreted to the cell wall from where it can diffuse out of the cell (Bednarek and Raikhel, 1992). In addition, RCI3 presents two repetitions of four hydrophobic amino acids (i.e. leucine, isoleucine or valine) in a similar way as those described to form leucine zippers domains involved in protein–protein interactions (O'Shea *et al.*, 1989). RCI3 shows high similarity to TPX1 and TPX2, two homologous peroxidases from tomato (Botella *et al.*, 1993).

Compared with other cold-inducible genes, *RCI3* is subjected to a complex regulation. In response to low temperature, *RCI3* transcripts accumulate in the aerial part and in roots of etiolated seedlings but only in roots of light-grown seedlings, suggesting that *RCI3* is negatively regu-

lated by light. This is confirmed by the fact that *RCI3* mRNA levels gradually decrease in the aerial part of cold-treated etiolated seedlings when subjected to light, almost disappearing after 2 days of exposition. Consistent with this regulation, the accumulation of *RCI3* transcripts in adult plants of *Arabidopsis* exposed to low temperature is root specific. From these results, it is tempting to speculate that a chloroplast signal could be involved in the negative regulation of *RCI3*. Some cold-regulated genes have been described to be induced by low temperature only in the presence of light (Chauvin *et al*., 1993; Leyva *et al*., 1995), however, to our knowledge, *RCI3* is the first gene thus far whose expression in response to low temperature is negatively regulated by light. Still, confirming its complex regulation, the results presented show that *RCI3* expression is not completely under light control. Thus, *RCI3* is induced in etiolated seedlings but not in roots in response to different cold-related treatments such as dehydration, salt stress and exogenous ABA. All these data indicate that, during *Arabidopsis* development and in response to different stress situations, the regulation of *RCI3* must be accomplished through multiple signal transduction pathways.

Histochemical determination of GUS activity in transgenic *Arabidopsis* plants containing a *RCI3* promoter::*GUS* chimeric fusion allowed to detect specific staining in the radicles of 1-day-old transgenic seedlings excluding the root tips, indicating that *RCI3* expression is tightly regulated since very early during germination. This pattern of root-restricted GUS activity was maintained during all the *Arabidopsis* development. A minute study of GUS staining distribution in the main and lateral roots showed that *RCI3* expression is confined to the cortex, the endodermis and the stele, with the endodermis, which is the main lignification zone (Cruz *et al*., 1992; Steudle and Peterson, 1998), displaying the highest levels of GUS activity. In addition to this, the *RCI3* promoter was also active in the abscission zones of mature siliques. When transgenic plants were exposed to low temperature, dehydration, salt stress or ABA, the pattern of GUS staining was, in all cases, very similar to that observed under control conditions with the exception of cold-treated plants that showed an increase of GUS activity in their roots. The tissular specificity of *RCI3* induction in response to low temperature indicates that, in some cases, different cell types have different competences to sense and/or respond to the cold stress. This has also been shown for different root cell types of *Arabidopsis* that experience different changes in cytosolic calcium concentration after low temperature treatment (Kiegle *et al*., 2000). *TPX1* and *TPX2*, the peroxidase-encoding genes having highest similarity to *RCI3*, are also subjected to developmental and environmental regulation, although show different expression patterns from *RCI3*. *TPX1* transcripts constitutively accumulate in root tissues, preferentially in epidermal and sub-epidermal cells, this accumulation increasing by NaCl treatment (Botella *et al*., 1994a). After wounding, however, *TPX1* mRNA accumulates in the stem and main vein of leaves (Botella *et al*., 1994b). *TPX2* expression is constitutive only in roots from seedlings, and is not induced by salt stress or wounding (Medina *et al*., 1997). The response of these genes from tomato to low temperature has not been described.

A functional analysis of RCI3 was carried out in *Arabidopsis* transgenic plants with altered levels of RCI3 activity. Under control conditions, transgenic plants showed no obvious differences in plant growth and development with wild-type plants. When transgenic and wild-type plants were tested for freezing tolerance, no significant differences were observed either. However, striking differences were found after dehydration and salt stress treatments. In fact, overexpression of *RCI3* increased dehydration and salt tolerance on *Arabidopsis* plants, while supression of *RCI3* expression decreased plant tolerance to these stresses. Since *RCI3* expression is not induced in adult plants by dehydration or high salt, these results demonstrate that the low constitutive expression of *RCI3* ensures part of the *Arabidopsis* intrinsic tolerance to these stresses, and substantiate that RCI3 plays a role in dehydration and salt tolerance. In contrast, *RCI3* alone, in spite of its cold inducibility, does not seem to have a relevant role in *Arabidopsis* freezing tolerance. Peroxidase isozymes are involved in different plant responses to external signals by modifying cell walls (Bowles, 1990). In this way, peroxidases have been reported to be implicated in lignin and suberin deposition in root tissues under osmotic-related stresses (Botella et al., 1994a). We propose that RCI3 could be involved in promoting the lignification and suberization of root tissues, which would result in avoiding water loss. The present work illustrates that manipulation of genes not involved in signal transduction can also be used to understand the molecular mechanisms gathering plant responses to environmental stresses and to increase stress tolerance, in this case specifically to dehydration and high salt.

Experimental procedures

Plant materials, growth conditions and treatments

Seeds from *Arabidopsis thaliana* (L) Heynh, ecotype Columbia (Col) were purchased from Lehle Seeds (Round Rock, TX, USA). Seeds, seedlings and plants were used for the experiments. To obtain seedlings, seeds were sown under sterile conditions in Petri dishes containing mineral nutrient solution (ATM; Haughn and Sommerville, 1986), solidified with 0.8% (w/v) agar. To obtain soil-grown plants, seeds were sown in pots containing a mixture of organic substrate and vermiculite (3 : 1 v/v) and irrigated with water and ATM once a week. Liquid-grown plants were obtained by sowing seeds under sterile conditions in flasks containing GM

medium (MS medium, Murashige and Skoog, 1962; supplemented with 1% sucrose) that were cultured with shaking for 3 weeks. Plants for dehydration- and salt-tolerance assays were obtained from seeds sown under sterile conditions in Petri dishes containing GM medium solidified with 0.8% (w/v) agar. Both, seedlings and plants were grown at 20°C under long-day photoperiod (16 h of cool-white fluorescent light, photon flux of 70 μM $m^{-2} sec^{-1}$). Etiolation was achieved by covering seedlings and plants with aluminium foil.

Low temperature treatments were performed by transferring seedlings and plants to a growth chamber set to 4°C for different periods of time in the dark or under the light and photoperiodic conditions described above. Unless otherwise mentioned, illumination conditions were identical to those utilized before cold exposition. Dehydration was induced by removing seedlings and plants from their corresponding media and substrates and allowing them to lose 50% of their initial FW. Salt stress was accomplished by transferring seedlings to new Petri dishes containing the agar medium plus 250 mM NaCl, watering plants with 250 mM NaCl or adding NaCl to the liquid GM medium containing plants to attain a final concentration of 250 mM. In all cases, the NaCl treatment was maintained for 24 h. For ABA treatments, seedlings and soil-grown plants were sprayed with 100 μM ABA. Liquid-grown plants were supplemented with ABA to a final concentration of 100 μM. The ABA stock solution (100 mM) was prepared in DMSO, and control treatments were given with water containing the same final concentration of the ABA solvent. Plant material was always collected after 6 h of ABA treatment. To ensure complete darkness, all manipulations with etiolated material were conducted under green safety light. After treatments, seedlings and plants used for Northern-blot hybridizations and peroxidase activity experiments were immediately frozen in liquid N_2 and stored at −80°C until their use. For analysis of GUS activity, the material was used immediately after treatments.

Freezing tolerance was analyzed by exposing 3-week-old soil-growing plants directly to −7°C for 6 h, or to −11°C for 6 h after being acclimated for 7 days at 4°C. Tolerance to freezing was determined as the capacity of plants to resume growth after returning to control conditions. Dehydration tolerance was investigated by removing GM-growing plants from the medium, placing them on a dry filter paper, and allowing them to develop for 1 day without watering. The rate of dehydration was estimated as the percentage of initial FW that remained after treatment. The phenotype of the plants after the treatment was also studied. NaCl tolerance was checked by transferring GM-growing plants to a new GM medium containing 150 mM NaCl. Tolerance was estimated by determining the number of green leaves and the fresh weight of the plants after 7 days of treatment. The phenotype of the plants after the stress was also analyzed.

Molecular biology methods

The Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA) was used to obtain a full-length cDNA clone of *RCI3*. DNA sequencing was carried out by using specific oligonucleotide primers and a semiautomatic DNA sequencer (model 373 A, Perkin Elmer, San Jose, CA, USA). Nucleotide sequences were always determined in both strands. Databases were searched for sequence similarities using the Basic Local Alignment Search Tool (BLAST) program of the National Center for Biotechnology Information (Altschul *et al.*, 1997). Comparison of the nucleotide and amino acid sequences were performed with the software package Lasergene DNASTAR.

Genomic DNA extractions were carried out according to the method described by Dellaporta *et al.* (1983). Total RNA was isolated following the method of Logeman *et al.*, 1987). Restriction digestions, cloning, and DNA- and RNA-blot hybridizations were performed following standard protocols (Sambrook *et al.*, 1989). The *RCI3*-specific probe was the cDNA from EST no. p175C22T7. This cDNA corresponds to a 3′ fragment of *RCI3* containing 963 nucleotides. The *LTI78* probe (Nordin *et al.*, 1993) consisted of a 1.0-kb fragment obtained by PCR from genomic DNA of L*er* ecotype by using the primers 5′-CGGGATTTGACGGAGAACCA-3′ and 5′-ACCATAATACATCAAAGACG-3′. The *GUS* probe was the 446 bp-fragment obtained by PCR from the pBI101 plasmid (Clontech, Palo Alto, CA, USA) by using the primers 5′-TTACGTCCTG-TAGAAACCCCAAC-3′ and 5′-GATGGATCCCGGCATAGTTAAAG-3′. Equal RNA loading was monitored by using a 0.3-kb *Eco*RI fragment from the *18S rDNA* as a probe (Tremousaygue *et al.*, 1992). The hybridization signals were quantified as described by Capel *et al.* (1997). RNA samples from each experiment were analyzed in at least two independent blots, and each experiment was repeated at least twice.

Transgenic plants

A PCR-based cloning procedure was used to obtain *RCI3* promoter::*GUS* transcriptional fusions. By using the primers 5′-TACAA-GAGGAGCTCGAGG-3′ and 5′-TGAGACTCTCTCTGT-3′, a 2.4-kb fragment 24 bp upstream of the *RCI3* coding region was amplified. This *RCI3* promoter fragment was cloned into the *Sma*I site of *pBluescript SK(+)* (Stratagene Cloning Systems, La Jolla, CA, USA). The plasmid was digested with *Sal*I and *Bam*HI, and ligated into the corresponding restriction sites of the *pBI101.1* vector (Clontech) to yield the *RCI3::GUS* construct. The full-length *RCI3* cDNA was cloned into the *Sma*I site of *pBluescript SK(+)*. This plasmid was digested with *Sac*I and *Eco*RI, filled with Klenow and ligated into the *Sma*I site, downstream of the CaMv35S promoter in the *pROK2* plasmid vector (Baulcombe *et al.*, 1986), in sense (*RCI3-OE*) and antisense (*RCI3-AS*) orientation. The recombinant plasmids, once verified the constructs by DNA sequencing, were introduced into *Agrobacterium tumefaciens* strain C58C1 (Deblaere *et al.*, 1985). Transformation of *Arabidopsis* Col ecotype was performed by vacuum infiltration (Clough and Bent, 1998).

Assays of GUS activity and histochemical staining

Histochemical localization of GUS activity and GUS activity assays were performed as described in Medina *et al.* (2001).

Isoelectric focusing and visualization of peroxidase activity

Frozen tissues were homogenized with pestle and mortar in one volume of 20% sucrose (w/v). Extracts were centrifuged at 13000 g for 10 min at 4°C before application to the gel. Isoelectric focusing was carried out using a Multiphor II electrophoresis unit (Amersham Biosciences, Amersham, UK). The polyacrilamide gels, containing 10% glycerol and 2% w/v ampholytes (Pharmalyte, pH range of 3–10), were 0.25 mm thick and 120 mm wide. 1 M NaOH and 0.33 M citric acid were used as catholyte and anolyte, respectively. Ten micrograms of proteins were loaded at about 1 cm from the anode after a 500-V h^{-1} prefocus, and the run was terminated after 4 kV h^{-1}. Peroxidase activity was visualized following the method described by Liu *et al.* (1990).

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