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RESEARCH PAPER

Phylogenetic and functional analysis of *Arabidopsis RCI2* genes

Joaquín Medina[1], María Luisa Ballesteros[1] and Julio Salinas[1,2,*]

[1] *Departamento de Biotecnología, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Carretera de la Coruña, Km. 7, 28040 Madrid, Spain*

[2] *Departamento de Biología de Plantas, Centro de Investigaciones Biológicas (CIB-CSIC), Ramiro de Maeztu 9, 28040 Madrid, Spain*

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Abstract

Six new *Arabidopsis thaliana* genes (*AtRCI2C-H*) have been identified that show high homology to *AtRCI2A* and *AtRCI2B*. Sequence comparisons revealed that *AtRCI2*-related genes are widely spread among very different organisms, including other plant species, prokaryotes, fungi, and simply organized animals, and are also organized in gene families. Most *RCI2* genes show a similar exon–intron organization, which indicates that they have been structurally conserved during evolution, and encode small, highly hydrophobic proteins containing two putative transmembrane domains. Consistently, the majority of AtRCI2 proteins localize in the plasma membrane. RCI2 proteins exhibit an elevated level of sequence similarity and seem to have evolved from a common ancestor. In spite of their high similarity, conserved subcellular localization, and common origin, experimental evidence is presented suggesting that different RCI2 proteins may have distinct functional roles. Thus, as previously demonstrated for *AtRCI2A* and *AtRCI2B*, the newly identified *AtRCI2* genes (*AtRCI2C–H*) are differentially regulated in *Arabidopsis* organs and in response to abiotic stresses and ABA treatment. Furthermore, only the AtRCI2 proteins that do not contain the C-terminal hydrophilic tail (i.e. AtRCI2A–C and AtRCI2H) are able to complement for the loss of the yeast *AtRCI2*-related gene *PMP3*. On the basis of these results, different aspects on the evolution and roles of *RCI2* genes are discussed.

Key words: ABA, abiotic stress responses, *Arabidopsis*, cold acclimation, dehydration, gene family, *RCI2* genes.

Introduction

During recent years, a large effort has been made to understand the molecular mechanisms that plants have evolved to survive adverse environmental conditions. *Arabidopsis thaliana* transcriptome analyses have revealed that hundred of genes are regulated by low temperature, dehydration or high salt, and that different signalling pathways are involved in this regulation (Fowler and Thomashow, 2002; Jiang and Deyholos, 2006; Oono *et al*., 2006). Some of these genes are also regulated by developmental cues, which is not unexpected considering that environmental stresses have pleiotropic effects on developmental processes (Salinas, 2002; Shinozaki and Yamaguchi-Shinozaki, 2007). Interestingly, several genes are not induced in response to low temperature, dehydration or high salt in all plant organs (Medina *et al*., 2001; Chen *et al*., 2002; Fukuda *et al*., 2004), suggesting that plant responses to abiotic stresses are differentially modulated in different tissues.

Genes that respond to abiotic stresses often belong to gene families (Yokoi *et al*., 2002; Tran *et al*., 2004; Shigaki *et al*., 2006). Why these genes have evolved as gene families, and whether they have redundant or complementary functions are important questions that, in most cases, still remain to be answered. Gene families arise from duplication of ancestral genes and subsequent sequence divergence, which can lead to functional specialization allowing plants to adapt better to unfavourable environments (Paterson *et al*., 2006). The sequence of the *Arabidopsis* genome revealed that 60% is segmentally duplicated, significantly more than any other sequenced eukaryotic genome (Blanc *et al*., 2000; Simillion *et al*., 2002), and 17% organized in tandemly repeated

* To whom correspondence should be addressed. E-mail: salinas@cib.csic.es

segments (Bancroft, 2001). The analysis of the deduced genes unveiled the importance of gene families, in terms of both number and size, when considering the organization and evolution of the *Arabidopsis* genome (Arabidopsis Genome Initiative, 2000).

In an attempt to identify new genes from *Arabidopsis* involved in the low-temperature response, *RCI2A* and *RCI2B* (hereafter referred as *AtRCI2A* and *AtRCI2B*, respectively), two homologous genes that are tandemly organized were isolated (Capel *et al*., 1997). The characterization of *AtRCI2A* and *AtRCI2B* expression uncovered that they are induced not only by low temperature but also by ABA, dehydration, and salt treatments. Analysis of *Arabidopsis* transgenic plants harbouring *AtRCI2* promoters fused to the *uidA* (*GUS*) reporter gene revealed that the expression of *AtRCI2A* and *AtRCI2B* is differentially regulated at the tissue level during plant development and in response to abiotic stresses (Medina *et al*., 2001). Both genes encode small, highly hydrophobic proteins with two putative transmembrane domains (Capel *et al*., 1997). The absence of any signal for organelle targeting allowed it to be proposed that AtRCI2A and AtRCI2B proteins might localize to the plasma membrane (Capel *et al*., 1997), which is considered a primary site of injury by freezing (Lyons, 1973). A possible role for AtRCI2 proteins in maintaining membrane function and/or integrity when plants have to adapt to any environmental condition that reduces water availability was suggested. Alternatively, it was proposed that they could interact with other membrane proteins to control the hydric balance of the cells (Capel *et al*., 1997). A yeast plasma membrane protein, PMP3, was identified that showed high sequence similarity to AtRCI2A and AtRCI2B (Navarre and Goffeau, 2000). Deletion of the *PMP3* gene results in an increase in Na^+ and K^+ uptake due to a hyperpolarization of the plasma membrane, which suggests a role for PMP3 in regulating plasma membrane potential. Interestingly, AtRCI2A can substitute for the loss of PMP3 (Navarre and Goffeau, 2000; Nylander *et al*., 2001), indicating that both proteins have a similar function. However, little is known about the precise role of AtRCI2A and AtRCI2B in stress response. Recently, the isolation of *Arabidopsis* transgenic plants containing disrupted or overexpressed *AtRCI2A* have been described (Mitsuya *et al*., 2005, 2006). The characterization of these plants suggested that AtRCI2A may be involved in the salt response by avoiding over-accumulation of Na^+ and K^+ ions (Mitsuya *et al*., 2005, 2006).

In this paper, the identification and characterization of six new *Arabidopsis* genes (*AtRCI2C-H*) that show high homology to *AtRCI2A* and *AtRCI2B* are reported. In addition, it is shown that *AtRCI2*-related genes are widespread among very different organisms, including other plant species, prokaryotes, fungi, and animals, where they are also organized as gene families. The analysis of the *AtRCI2*-related genes from rice and *Caenorhabditis elegans*, whose genomes have been completely sequenced, and their comparison with the corresponding genes from *Arabidopsis* provided valuable information on the structure and organization of *RCI2* genes. Remarkably, these genes and the corresponding predicted proteins are highly conserved at both sequence and structural levels, and seem to have a common evolutionary origin. Subcellular localization of AtRCI2 proteins indicates that, excluding AtRCI2D, all of them localize to the plasma membrane. Expression and functional analysis suggest that, in spite of their similarity, conserved subcellular localization, and common origin, different AtRCI2 proteins may have distinct roles in response to abiotic stresses. On the basis of these results, some aspects on the evolution and functions of *RCI2* genes are discussed.

Materials and methods

Screening of databases, sequence analyses, and chromosome localizations

Novel *Arabidopsis RCI2* genes (*AtRCI2C-H*) were identified by comparing AtRCI2A and AtRCI2B proteins (Capel *et al*., 1997) with entries in GenBank (http://www.ncbi.nlm.nih.gov) and *Arabidopsis* MIPS (http://mips.gsf.de/proj/thal/db/index.html) databases, using BLASTP and TBLASTN algorithms (Altschul *et al*., 1997). Subsequent pair-wise DNA sequence comparisons were applied to identify and exclude double annotations. The AtRCI2 proteins and the same algorithms were used to uncover *AtRCI2*-related genes in rice (*Oryza sativa*) and *C. elegans*. Rice *AtRCI2*-related genes (*OsRCI2-1–12*) were searched in the GenBank at the NCBI (http://www.ncbi.nlm.nih.gov/) and TIGR (http://www.tigr.org/tdb/e2k1/osa1/) databases derived from *O. sativa* ssp *japonica*, and in GenBank at the NCBI database derived from *O. sativa* ssp *indica*. In the case of *C. elegans*, the complete set of *AtRCI2*-related genes (*CeRCI2-1–13*) was obtained by searching in the WormBase (http://www.wormbase.org; Stein *et al*., 2001). The search for *AtRCI2*-related genes in other organisms was carried out in the different EST collections available by April 2007 in GenBank, using the TBLASTN algorithm and all AtRCI2 proteins as queries. Redundant sequences were identified by comparing BLAST results, and only the longest ones were considered for further analyses. The accession numbers of the selected ESTs are listed in Supplementary Table 1 at *JXB* online.

The exon–intron boundaries of the *RCI2* genes were determined with the GenScan program (http://genes.mit.edu/GENSCAN.html; Burge and Karlin, 1997), and verified, when possible, with available EST and full-length cDNA sequences. The chromosome localization of the *AtRCI2* genes was established with the Map Viewer Tool from The Arabidopsis Information Resource (http://www.arabidopsis.org/servlets/mapper). The chromosome localization of the *OsRCI2* and *CeRCI2* genes was determined according to the rice TIGR database and WormBase, respectively. Duplications of *AtRCI2* genes and the presence of *AtRCI2* genes on duplicated segments were investigated by using the MIPS redundancy viewer (http://mips.gsf.de/proj/thal/db/gv/rv/) and the Simillion database (http://www.psb.rug.ac.be/bioinformatics/simillion_pnas02; Simillion *et al*., 2002).

Protein alignments and similarity values were generated with the Clustal X program (Thompson *et al*., 1997). Specific targeting

sequences were predicted with the PSORT programs (http://psort.nibb.ac.jp/; Nakai and Kanehisa, 1992). Hydropathy analyses were performed using the ProtScale program (http://ca.expasy.org/cgi-bin/protscale.pl) with the Kyte and Doolittle option and a window of nine amino acids (Kyte and Doolittle, 1982). The superposition of RCI2 hydropathy plots was generated after transferring the raw data with the Microsoft Excel application (Microsoft, Redmond, WA) and aligning the sequences to match the two proline residues conserved in the first transmembrane domains. The prediction of transmembrane domains was realized with the TMHMM 2.0 program (http://www.cbs.dtu.dk; Krogh *et al*., 2001). The compilation of RCI2 transmembrane domains was generated, as in the case of the hydropathy plots described above. The phylogenetic tree was constructed using the Neighbor–Joining method (Saitou and Nei, 1987), and the bootstrap test carried out with 1000 iterations.

Plant material, growth conditions, and treatments

Seeds from *A. thaliana* plants, ecotype Columbia (Col), were obtained from Lehle Seeds (Round Rock, TX, USA). Plant growth conditions and treatments were as described (Medina *et al*., 2001). The onion (*Allium cepa*) bulbs used for particle bombardment experiments were purchased from local markets.

Molecular biology methods

Total RNA was extracted from different *Arabidopsis* organs and seeds according to Logeman *et al.* (1987) and Lara *et al.* (2003), respectively. DNA digestions and cloning were performed following standard protocols (Sambrook *et al*., 1989). RNA-blot hybridizations were performed as previously described by Medina *et al.* (2001). The specific probes for *AtRCI2A* and *AtRCI2B* were those described by Capel *et al.* (1997). DNA fragments partially encompassing the corresponding 3′-UTRs were used as specific probes for the rest of *AtRCI2* genes. These fragments were generated by PCR with primers shown in Supplementary Table 2 at *JXB* online. RNA samples from each experiment were analysed in at least two independent blots, and each experiment was repeated at least twice.

Expression analysis in yeast was performed by RT-PCR using 2 μg of total RNA from WT303 and transformed YR93-31 yeast strains and M-MuLV Reverse transcriptase (USB, Cleveland, USA). RNAs were isolated according to Schmitt *et al.* (1990). *AtRCI2* transcripts were detected using gene-specific primers indicated in Supplementary Table 3 at *JXB* online. The yeast *ACT1* transcript was used as the endogenous control and detected using specific primers 5′-GATCTTGAGTGAACGTGGT-3’ and 5′-TTCTGGGGCTCTGAATCTT-3′. PCR products were separated on 1.5% (w/v) agarose gel containing ethidium bromide and visualized on UV light.

Isolation of AtRCI2 *cDNA clones and transformation of yeast strains*

Full-length cDNAs corresponding to *AtRCI2A–F* genes were obtained by RT-PCR using 2 μg of total RNA extracted from 4-week-old plants exposed to low temperature. The full-length *AtRCI2H* cDNA was synthesized by PCR using the EST no. AV824849 (Asamizu *et al*., 2000) as a template. The specific primers employed to obtain the cDNAs are shown in Supplementary Table 3 at *JXB* online. The truncated *AtRCI2D-Δ1* cDNA, which contains a stop codon that eliminates the last 18 amino acids from the AtRCI2D protein, was generated by PCR, using the full-length *AtRCI2D* cDNA as a template and the oligonucleotides *AtRCI2D* (F) and *AtRCI2D-Δ1* (see Supplementary Table 3 at *JXB* online) as specific primers. All primers contained *Bam*HI restriction sites that were used for directly cloning into the pRS416 plasmid (Navarre and Goffeau, 2000) between the promoter and terminator of *PMP3*. Constructs were sequenced and used to transform *Saccharomyces cerevisae* mutant strain YR93-31 that has the *PMP3* gene deleted (Navarre and Goffeau, 2000). Transformants were selected on 0.67% (w/v) yeast nitrogen base medium, containing 2% (w/v) agar, 4% (w/v) glucose and all micronutrients except tryptophan and uracil.

Yeast sensitivity assays to NaCl

Transformed YR93-31 strains as well as 303 wild-type cells were grown in YPD medium to saturation, and their OD at 600 nm determined. Cultures were normalized to the same value (OD_{600}=0.5), and serial 10-fold dilutions plated onto agar–YPD plates supplemented with different NaCl concentrations (0, 0.25, 0.5, and 1.0 M). Yeasts were grown for 3 d at 30 °C, and the highest NaCl concentration where they were able to grow was determined.

Subcellular localization of GFP-AtRCI2 fusion proteins

To construct translational fusions between cDNAs corresponding to *GFP* and *AtRCI2* genes, *AtRCI2* cDNAs were amplified by PCR with the oligonucleotides shown in Supplementary Table 3 at *JXB* online. cDNAs were subsequently cloned into the *Bgl*II site of the expression vector pAVA393 (Von Arnin *et al*., 1998) to generate *GFP-AtRCI2* fusions under the control of the CaMV 35S promoter. The *GFP-AtRCI2* fusions were transiently expressed in onion epidermal cells by particle bombardment, or in *Arabidopsis* protoplasts by PEG-mediated transformation, essentially as described by Yokoi *et al.* (2002) and Milla *et al.* (2006), respectively.

Fluorescence was observed by confocal microscopy (Leica TCSSP2, Leica Microsystem, Wetzlar, Germany). For imaging GFP fusions alone or in combination with YFP fusions, the excitation lines were 488 nm for GFP and 515 nm for YFP. Fluorescence was detected in a 500–538 nm band for GFP, and a 560–615 nm band for YFP. Filter sets were used to visualize and to discriminate between fluorophores.

Results

AtRCI2A *and* AtRCI2B *belong to a gene family*

To investigate the presence of *AtRCI2A* and *AtRCI2B* homologues in the *Arabidopsis* genome, BLAST searches (Altschul *et al*., 1997) were conducted with the predicted AtRCI2A and AtRCI2B proteins (Capel *et al*., 1997). Six putative genes, *AtRCI2C–H*, were identified that would encode proteins showing 53–94% sequence similarity to AtRCI2A and AtRCI2B, and together with *AtRCI2A* and *AtRCI2B* constitute the *AtRCI2* gene family (Table 1; Fig. 1). The sequence of *AtRCI2G*, which was not recognized as a putative gene during the *Arabidopsis* automated annotation process, has been submitted to GenBank and registered with the accession number AT4G28088 (Table 1). The chromosome localization of the eight *AtRCI2* genes is indicated in Table 1 and displayed in Fig. 2A. In summary, *AtRCI2E*, *AtRCI2F*, and *AtRCI2G* localize to chromosome 4, *AtRCI2E* and *AtRCI2F* being arranged as a tandem. In a similar way, *AtRCI2A* and *AtRCI2B* are

Table 1. *Identified* AtRCI2 *genes*

Name[a]	Arabidopsis (AGI) ID[b]	Other names[c]	Chromosome[d]	Start–Stop[e]	Length[f] (aa)	Mr[g]	pI[h]
AtRCI2A	AT3G05880	*Lti6a*[1]	3	1755503–1756546	54	5961	6.18
AtRCI2B	AT3G05890	*Lti6b*[1]	3	1757637–1758498	54	6080	7.98
AtRCI2C	AT1G57550	–	1	21378068–21378336	52	5841	6.26
AtRCI2D	AT2G24040	–	2	10231057–10231686	75	8624	5.02
AtRCI2E	AT4G30650	–	4	14954333–14954920	73	7904	4.59
AtRCI2F	AT4G30660	–	4	14955461–14956068	74	8518	5.10
AtRCI2G	AT4G28088	–	4	13961250–13961566	77	8771	4.59
AtRCI2H	AT2G38905	–	2	16251177–16251562	54	5948	3.97

[a] Names of the identified *AtRCI2* genes.
[b] Accession numbers of the identified *AtRCI2* genes.
[c] Other names [Nylander *et al.* (2001)[1]] of the identified *AtRCI2* genes.
[d] Chromosome localizations of the identified *AtRCI2* genes.
[e] Chromosome co-ordinates of the identified *AtRCI2* genes.
[f] Number of amino acids of the corresponding proteins.
[g] Predicted molecular weights of the corresponding proteins.
[h] Predicted isoelectric points of the corresponding proteins.

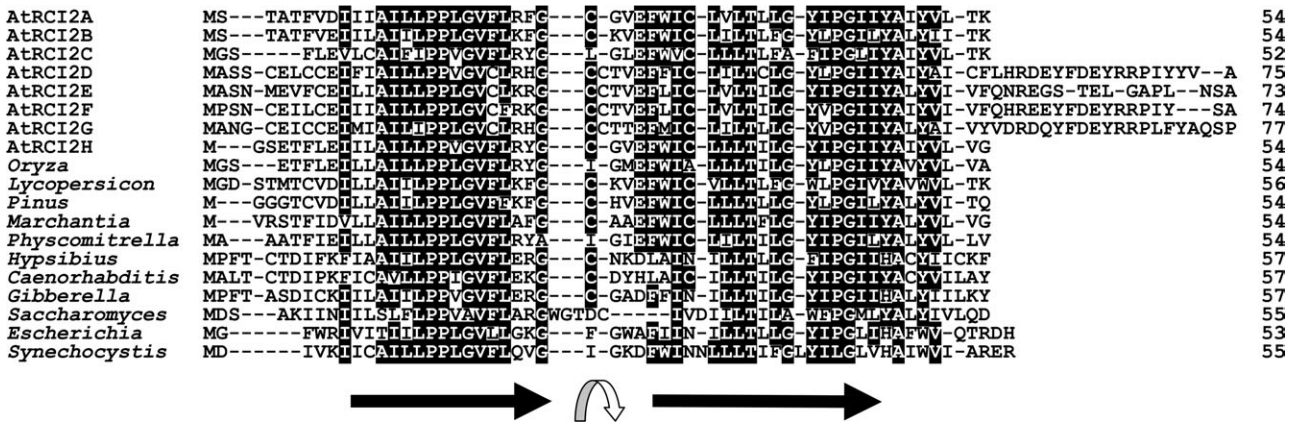

Fig. 1. Sequence comparison of AtRCI2 and AtRCI2-related proteins. Alignment of amino acid sequences of AtRCI2 and representative AtRCI2-related proteins from different organisms, including *Oryza sativa* (OsRCI2-7; accession no. AU088623), *Lycopersicon esculentum* (AW093948), *Pinus taeda* (AW754907), *Marchantia polymorpha* (BJ866789), *Physcomitrella patens* (BJ590913), *Hypsibius dujardini* (CF544790.1), *Caenorhabditis elegans* (CeRCI2-11; BJ113898), *Gibberella zeae* (CD458606), *Saccharomyces cerevisiae* (PMP3; DB661484), *Escherichia coli* (NP-289216), and *Synechocystis* sp. PCC 6803 (NP-442657). Amino acids shown in black boxes correspond to residues conserved in at least 11 of the 19 sequences analysed. The number of residues in each sequence is indicated on the right side. Dashes represent gaps introduced to improve alignments. Straight arrows mark predicted transmembrane domains. The curved arrow indicates a putative loop structure.

organized as a tandem in chromosome 3, while *AtRCI2D* and *AtRCI2H* localize to chromosome 2 and *AtRCI2C* in chromosome 1. Chromosome 5 does not contain any *AtRCI2* gene. Comparison among genomic and deduced coding sequences revealed that, as in the case of *AtRCI2A* and *AtRCI2B* (Medina *et al*., 2001), *AtRCI2C–H* genes harbour two exons separated by an intron of variable size (82–275 bp). Nevertheless, they do not seem to have the additional intron that *AtRCI2A* and *AtRCI2B* contain into their 3′-UTRs (Medina *et al*., 2001) (Fig. 2B).

Table 1 shows some features of deduced AtRCI2 proteins, namely number of amino acids, molecular weights, and isoelectric points. According to their lengths, AtRCI2 proteins can be divided into two different groups. The first one includes AtRCI2A, AtRCI2B, AtRCI2H (54 amino acids each) and AtRCI2C (52 amino acids) (Table 1; Fig. 1). The second group comprises AtRCI2D, AtRCI2E, AtRCI2F, and AtRCI2G (75, 73, 74 and 77 amino acids, respectively), all of them having an extra C-terminal tail of about 20 amino acids rich in charged residues (Table 1; Fig. 1). Hydropathy analyses (Kyte and Doolittle, 1982) indicated that all AtRCI2 proteins are highly hydrophobic with two clear hydrophobic stretches and a region of lower hydrophobicity between them (Fig. 3A). Proteins belonging to the second group (AtRCI2D–G) present an additional hydrophilic tract corresponding to their C-terminal tails (Fig. 3A). The membrane topology prediction program TMHMM (Krogh *et al*., 2001) anticipates that AtRCI2 proteins contain two putative transmembrane domains corresponding to the hydrophobic stretches mentioned above (Fig. 3B). The sequence connecting the transmembrane domains would form

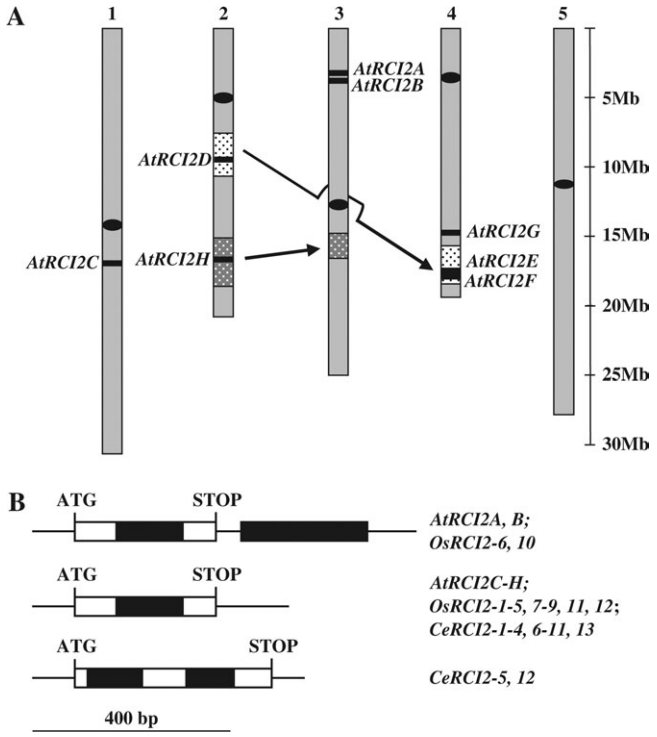

Fig. 2. Chromosome localization and structure of *AtRCI2* genes. (A) *AtRCI2* genes are localized in the chromosomes according to the BAC and YAC contigs from the *Arabidopsis* database. The representation is drawn to scale. Highlighted blocks and arrows indicate large-scale duplications involving *AtRCI2* genes. Centromeres are indicated by ellipses. (B) Structure of *AtRCI2* and *AtRCI2*-related genes. A scheme representing the different *RCI2* genes from *Arabidopsis*, rice, and *C. elegans* is drawn to scale. Exons and introns are indicated by white and black boxes, respectively.

a putative turn (Fig. 3B). These results are consistent with those obtained from the PSORT program (Nakai and Kanehisa, 1992), which unveiled that deduced AtRCI2 sequences do not contain any obvious consensus organelle targeting or retention signals (data not shown), suggesting, therefore, plasma membrane localization.

AtRCI2-*related genes are widespread in plants*

To explore the occurrence of *AtRCI2*-related genes in plant species other than *Arabidopsis*, the EST sequences available in plant databases were analysed. A BLAST search using all the deduced AtRCI2 proteins as queries identified more than 4200 ESTs coding for putative proteins with significant sequence similarity (>35%) to them. Redundant sequences were identified, and only the longest one in each case was considered for further analyses. The accession numbers of the selected ESTs (1373) are displayed in Supplementary Table 1 at *JXB* online. These sequences belong to more than 150 different species, including eudicots, monocots, gymnosperms, and bryophytes. The comparison of representative examples of deduced AtRCI2-related proteins from these phyla with AtRCI2 proteins revealed a high level of sequence similarity (49–92%) (Fig. 1). The hydropathy profiles of the representative AtRCI2-related proteins displayed in Fig. 1 were very similar to those of AtRCI2 proteins (see Supplementary Fig. 1A at *JXB* online), which suggests they are also located in the plasma membrane. In fact, the prediction program TMHMM showed that they also contain two putative transmembrane domains corresponding to hydrophobic regions, even if the first domain is not perfectly conserved in some cases (see Supplementary Fig. 1B at *JXB* online).

A detailed analysis of the data obtained from the BLAST search indicated that, as in *Arabidopsis*, *AtRCI2*-related genes may be organized in gene families as well. For instance, ESTs corresponding to at least seven different *AtRCI2*-related genes were identified from wheat (*Triticum aestivum*), five from maize (*Zea mays*), and four from soybean (*Glycine max*). The genome of rice (*Oryza sativa*), which has been sequenced (Yu *et al*., 2002; Goff *et al*., 2002), allowed the study of the structure and organization of *RCI2* genes in a plant species other than *Arabidopsis*. The available genome sequence for the *japonica* and *indica* subspecies was analysed separately. A total of 11 genes were identified in *japonica* and another 11 in *indica*, allowing the compilation of 12 non-redundant *AtRCI2*-related genes in rice (*OsRCI2-1-12*) (Table 2). Comparison among genomic and coding sequences revealed that the structure of *OsRCI2* genes is similar as that of *AtRCI2* genes (Fig. 2). *OsRCI2* genes are distributed in at least six out of the 12 rice chromosomes, *OsRCI2-3* and *OsRCI2-4* being arranged as a tandem in the *indica* genome (Table 2).

Predicted OsRCI2 proteins exhibit 41–92% of sequence similarity to AtRCI2s and can also be classified in two groups depending on their predicted sizes (Table 2; see Supplementary Fig. 2A at *JXB* online). Moreover, OsRCI2 proteins are also anticipated to have two transmembrane domains that would correspond to their hydrophobic stretches (see Supplementary Fig. 2B at *JXB* online). Other characteristics of OsRCI2s, including their molecular weights and isoelectric points, are similar to those of AtRCI2 proteins too (Table 2).

AtRCI2-*related genes are present in organisms other than plants*

To extend the study on *AtRCI2*-related genes further, the available EST databases were searched for similar sequences in other organisms than plants using all the AtRCI2 proteins as queries. Results revealed that *AtRCI2*-related genes are present in different species, including metazoans (nematodes and protostomes), fungi (ascomycota and basidiomycota), and prokaryotes (cyanobacteria, proteobacteria, planctomycetes, and firmicutes). Interestingly, *AtRCI2*-related sequences were not found in

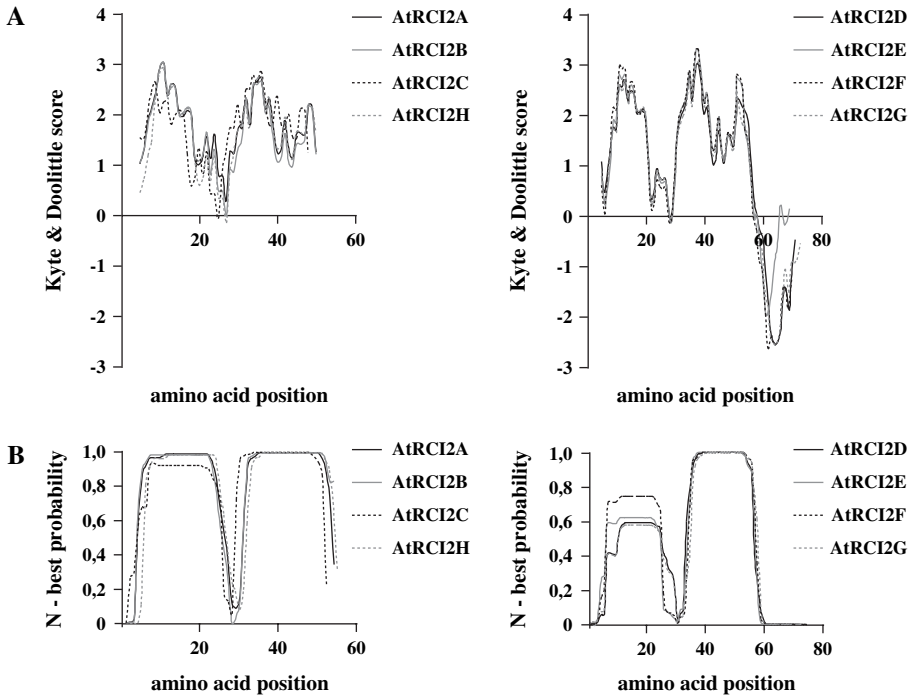

Fig. 3. Hydropathy profiles and transmembrane predictions for AtRCI2 proteins. (A) Compilation of Kyte and Doolittle profiles for AtRCI2A–C and AtRCI2H (left panel), and AtRCI2D–G (right panel). (B) Compilation of profiles generated by the TMHMM 2.0 program for AtRCI2A–C and AtRCI2H (left panel), and AtRCI2D–G (right panel).

Table 2. *Identified* OsRCI2 *genes*

Name[a]	Rice (TIGR) ID[b]	Other names[c]	*Indica* Contig name[d]	*Japonica* BAC/PAC name[e]	Chromosome[f]	Start–Stop[g]	Length[h] (aa)	Mr[i]	pI[j]
OsRCI2-1	LOC_Os01g18390	–	AAAA01003450.1	P0511C01	1	38352–38672	56	6199	6.16
OsRCI2-2	–	–	AAAA01004913.1	P0511C01	1	24254–24679	74	8284	6.70
OsRCI2-3	LOC_Os03g25460	–	AAAA01010593.1	OSJNBa0013M12.23	3	136937–136612	64	7163	4.70
OsRCI2-4	–	–	AAAA01010593.1	Not found	3	136011–135776	57	6409	7.03
OsRCI2-5	LOC_Os03g17790	–	AAAA01007293.1	OSJNBb0022M22	3	34666–34381	54	5958	3.96
OsRCI2-6	LOC_Os05g04700	*OsLTI6B*[1]	AAAA01002393.1	OSJNBa0069I13	5	29293–29013	55	6015	6.98
OsRCI2-7	LOC_Os05g03130	–	AAAA01006035.1	OJ1729E02	5	36761–36528	54	5970	4.15
OsRCI2-8	LOC_Os06g08564	–	AAAA01000911.1	P0554A06	6	110717–110375	75	8481	5.00
OsRCI2-9	LOC_Os06g44220	–	AAAA01001095.1	P0453H04	6	157741–158080	78	8504	4.49
OsRCI2-10	LOC_Os07g44180	*OsLTI6A*[1]	AAAA01006348.1	P0487A05	7	138279–137997	56	6226	4.36
OsRCI2-11	LOC_Os09g38560	*OSR8*[2]	AAAA01000379.1	OJ1065_E04	9	11088–12179	72	8037	4.81
OsRCI2-12	–	–	Not found	AACV01019593.1	9	5757–6057	65	7648	4.84

[a] Names of the identified *OsRCI2* genes in the corresponding BAC/PACs.
[b] Accession numbers of the identified *OsRCI2* genes in the corresponding BAC/PACs.
[c] Other names [Morsy *et al*. (2005)[1]; Koike and Imai (GenBank accession number Q9LRI7)[2]] of the identified *OsRCI2* genes in the corresponding BAC/PACs.
[d] Contig names at the NCBI *O*. *sativa* BLAST page.
[e] BAC/PAC clone names at the rice TIGR database.
[f] Chromosome localizations of the identified *OsRCI2* genes in the corresponding BAC/PACs.
[g] Coordinates of the identified *OsRCI2* genes in the corresponding BAC/PACs.
[h] Number of amino acids of the corresponding proteins.
[i] Predicted molecular weights of the corresponding proteins.
[j] Predicted isoelectric points of the corresponding proteins.

animals having more complex organization such as arthropods and vertebrates. The accession numbers of the longest non-redundant ESTs identified (340) are listed in Supplementary Table 1 at *JXB* online. Figure 1 displays the comparison among some representative examples of deduced AtRCI2-related proteins from nematodes, protostomes, fungi, and prokaryotes, and RCI2 proteins from *Arabidopsis* and other plant species. Sequence similarity ranges between 41% to 78%, indicating that RCI2 proteins are conserved during evolution. The hydropathy

profiles of these representative AtRCI2-related proteins resemble those of AtRCI2 proteins (see Supplementary Fig. 1A at *JXB* online), and also suggest a plasma membrane localization. Furthermore, the TMHMM program predicts two putative transmembrane domains corresponding to their hydrophobic regions (see Supplementary Fig. 1B at *JXB* online).

The analysis of non-redundant identified ESTs (see Supplementary Table 1 at *JXB* online) indicated that *AtRCI2*-related genes from organisms other than plants can also be organized into gene families. Additional information on the structure and genome organization of these genes was obtained from the sequenced genome of *Caenorhabditis elegans* (*C. elegans* sequencing consortium, 1998). BLAST searches performed in the WormBase (Stein *et al.*, 2001) identified 13 *AtRCI2*-related genes (*CeRCI2-1–13*) (Table 3). The predicted exon–intron organization of these genes resembles that of *Arabidopsis* and rice *RCI2* genes, except *CeRCI2-5* and *CeRCI2-12* that contain two introns into their coding regions (Fig. 2). *CeRCI2* genes are distributed on six chromosomes of *C. elegans*, *CeRCI2-7-8* and *CeRCI2-11-13* genes being organized in tandem on chromosomes 5 and X, respectively (Table 3).

The deduced CeRCI2 proteins can be arranged, as their *Arabidopsis* and rice RCI2 counterparts, into two groups with different predicted lengths (Table 3; see Supplementary Fig. 3A at *JXB* online). In addition, they are also anticipated to have two putative transmembrane domains, although the first domain in CeRCI2-6 and CeRCI2-11-13 is not fully conserved (see Supplementary Fig. 3B at *JXB* online). CeRCI2 proteins have similar molecular weights and isoelectric points as those of RCI2 proteins from *Arabidopsis* and rice (Table 3).

Most AtRCI2 proteins localize to the plasma membrane

Results described above indicate that *RCI2* genes encode small, highly hydrophobic proteins, containing two conserved putative transmembrane domains. Furthermore, in some cases, RCI2 proteins hold additional hydrophilic tails with charged residues. Consistent with these data, our *in silico* analyses suggest that most RCI2 proteins should localize in the plasma membrane. In an attempt to confirm this prediction experimentally, the subcellular localization of AtRCI2 isoforms was investigated. Translational fusions between full-length cDNAs corresponding to *GFP* and *AtRCI2* genes were generated and placed under the control of the CaMV 35S promoter. In the case of *AtRCI2G*, we repeatedly failed to obtain the corresponding cDNA by RT-PCR (data not shown) and the GFP-AtRCI2G fusion could not be generated. This result is consistent with the absence of ESTs for *AtRCI2G* in databases and suggests that this gene must be expressed at very low levels if at all. The constructs encoding GFP–AtRCI2 fusion proteins were transiently expressed in onion epidermal cells by particle bombardment. The patterns of fluorescence observed by confocal microscopy revealed that AtRCI2A–C and AtRCI2E–H fusion proteins are localized in the plasma membrane, confirming the *in silico* analyses described above (Fig. 4A). In addition, in several cells transformed with AtRCI2C, AtRCI2E, and AtRCI2F fusion proteins fluorescence was also detected in punctate structures and dots of different sizes (Fig. 4A). Interestingly, the GFP–AtRCI2D fusion protein did not localize in the plasma membrane but mostly in intracellular membranes and numerous small dots (Fig. 4A), suggesting it can be associated to membranes of endoplasmic reticulum (ER) and Golgi

Table 3. *Identified* CeRCI2 *genes*

Name[a]	*C. elegans* ID[b]	Chromosome[c]	Start–Stop[d]	Length[e] (aa)	Mr[f]	pI[g]
CeRCI2-1	W10C8.6	1	2858618–2859839	84	9800	8.3
CeRCI2-2	T23B3.2	1	6715997–6716795	92	10588	5.90
CeRCI2-3	F25H5.8	1	91177738–91178688	62	7157	6.80
CeRCI2-4	CO4G6.5	2	5085803–5086041	59	6945	4.34
CeRCI2-5	ZK632.10	3	9826543–9827046	80	8707	5.33
CeRCI2-6	WO2A2.9	4	13347656–13348544	57	6200	5.51
CeRCI2-7	R10D12.6	5	13949126–13949447	91	10646	5.70
CeRCI2-8	R10D12.7	5	13949873–13950201	91	10700	6.50
CeRCI2-9	T06C12.9	5	15885465–15885862	91	10538	7.00
CeRCI2-10	F47B7.1	X	3788003–3788515	59	6839	6.02
CeRCI2-11	T23F2.3	X	5512046–5512265	57	6249	5.45
CeRCI2-12	T23F2.4	X	5514781–5515009	77	6300	7.29
CeRCI2-13	T23F2.5	X	5516878–5517101	57	6273	7.10

[a] Names of the identified *CeRCI2* genes.
[b] Gene identification numbers at the Wormbase of the identified *CeRCI2* genes.
[c] Chromosome localizations of the identified *CeRCI2* genes.
[d] Chromosome co-ordinates of the identified *CeRCI2* genes.
[e] Number of amino acids of the corresponding proteins.
[f] Predicted molecular weights of the corresponding proteins.
[g] Predicted isoelectric points of the corresponding proteins.

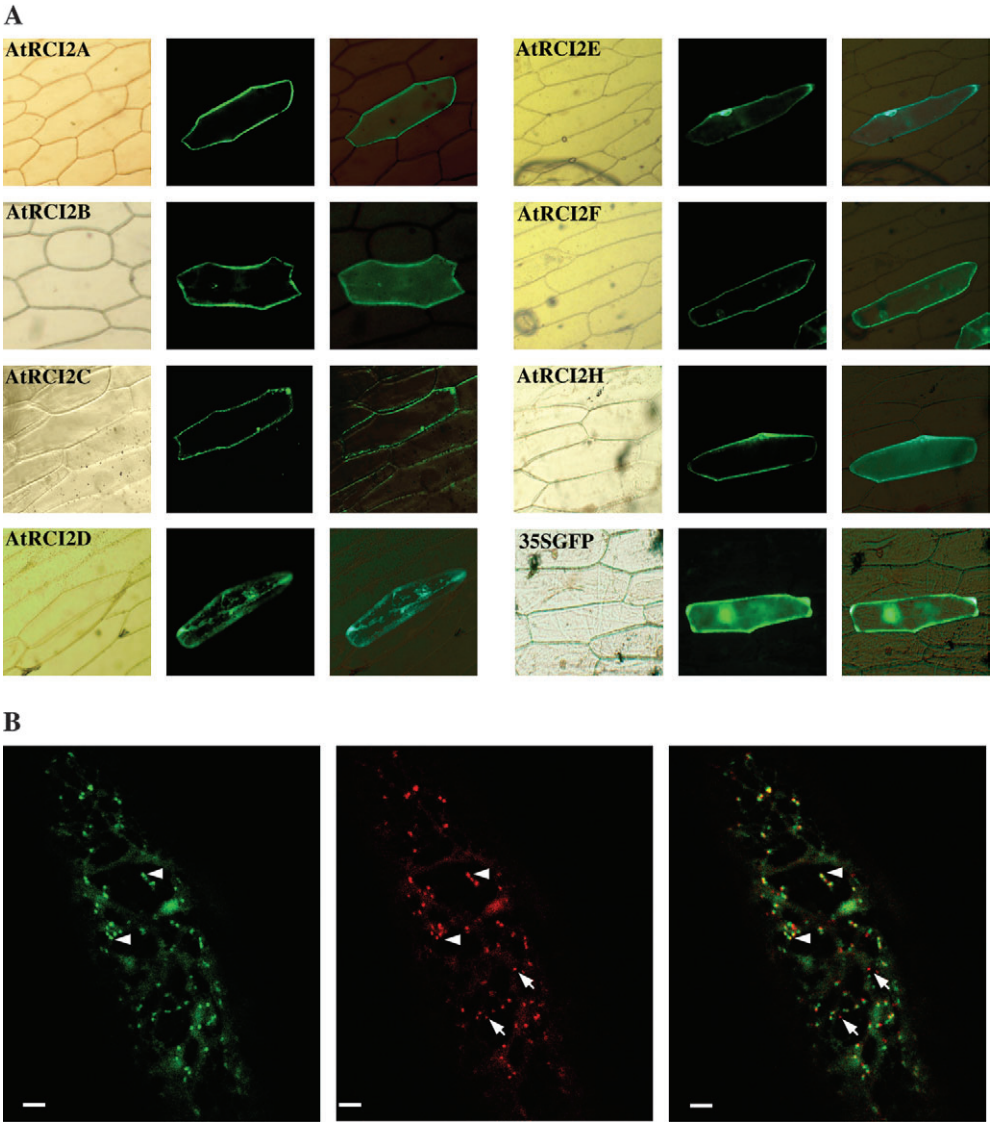

Fig. 4. Subcellular localization of GFP–AtRCI2s fusion proteins. (A) Confocal cross-sections of onion epidermal cells bombarded with different GFP–AtRCI2 fusion proteins or a GFP vector alone. Left panels: bright-field images of onion cells bombarded with the fusions indicated in the top left; middle panels: fluorescence images of the cells from left panels; right panels: overlays of left and middle panel images. (B) Confocal cross-sections of onion epidermal cells co-bombarded with plasmids encoding the GFP–AtRCI2D fusion protein and the Erd2-YFP ER/Golgi marker. The GFP and YFP fusions are pseudo-coloured in green (left panel) and red (central panel), respectively. Co-localization is pseudo-coloured in yellow (right panel). Bars represent 10 μm. Arrowheads indicate small structures where GFP-AtRCI2D co-localizes with the Erd2-YFP marker. Arrows indicate punctate structures only labelled by Erd2-YFP.

apparatus. Similar results were obtained when the subcellular localization of GFP–AtRCI2 fusion proteins was analysed in *Arabidopsis* protoplasts transiently transformed with *GFP–AtRCI2* constructs (see Supplementary Fig. 4 at *JXB* online). To evaluate the hypothesis that AtRCI2D associates to membranes of the ER and the Golgi apparatus, the *GFP–AtRCI2D* construct was cotransformed together with the *Erd2–YFP* translational fusion into onion epidermal cells. *Erd2* encodes the *Arabidopsis* HDEL receptor, an ER and Golgi marker (Brandizzi *et al*., 2002). Figure 4B shows that the GFP-AtRCI2D fusion protein mainly colocalized with the Erd2 marker (Fig. 4B, arrowheads), although some fluorescence that localized independently from Erd2 could also be discerned in some cells (Fig. 4B, arrows). From all these results, it is concluded that AtRCI2A–C and AtRCI2E–H proteins are localized in the plasma membrane while AtRCI2D is mainly associated to ER and Golgi organelles.

AtRCI2 *genes are differentially regulated in Arabidopsis* organs and in response to abiotic stresses

The presence of multiple isoforms of RCI2 proteins in different organisms, most of them showing a relevant

level of similarity and a predicted common subcellular localization, raises the question as to whether or not they have redundant functions. Previously, it was demonstrated that *AtRCI2A* and *AtRCI2B* are differentially regulated during *Arabidopsis* development and in response to abiotic stresses such as low temperature, dehydration, and high salt (Capel *et al*., 1997; Medina *et al*., 2001), which suggested that all AtRCI2 isoforms do not play exactly the same role. To investigate this possibility further, the expression of *AtRCI2C–F* in seeds and different organs of *Arabidopsis* plants grown under control conditions was analysed first by RNA-blot hybridizations with specific probes. In contrast to *AtRCI2A* and *AtRCI2B*, whose transcripts mainly accumulate in stems and roots, Fig. 5A shows that *AtRCI2C* transcripts were found at low levels in all the organs analysed except seeds. *AtRCI2D* expression was mainly detected in roots and flowers, and moderately in leaves and stems. As in the case of *AtRCI2A*, *AtRCI2B*, and *AtRCI2C*, the expression of *AtRCI2D* was not detected in seeds (Fig. 5A). *AtRCI2E* was expressed at low levels in all organs, including seeds, while *AtRCI2F* expression was principally disclosed in stems, roots, and seeds (Fig. 5A). It was not possible to detect *AtRCI2G* transcripts by RNA-blot hybridizations (data not shown), which is consistent with the absence of corresponding ESTs in databases. Regarding *AtRCI2H*, its expression is restricted to seeds (Fig. 5A).

The expression of *AtRCI2* genes in response to different treatments (Fig. 5B) was also examined. As previously reported, *AtRCI2A* and *AtRCI2B* were induced by low temperature, dehydration, high salt, and exogenous ABA (Capel *et al*., 1997; Medina *et al*., 2001; Nylander *et al*., 2001). On the other hand, while *AtRCI2C* expression was unaffected by these treatments, *AtRCI2D–F* messengers accumulated to different extents in response to them (Fig. 5B). Transcripts corresponding to *AtRCI2G* and *AtRCI2H* could not be detected (data not shown). Taken together, all these data demonstrate that *AtRCI2* genes are differentially regulated in *Arabidopsis* organs and in response to abiotic stresses, indicating that the activity of the different AtRCI2 isoforms is confined to particular tissues under control and stressed conditions, and, therefore, that most likely they do not have redundant functions.

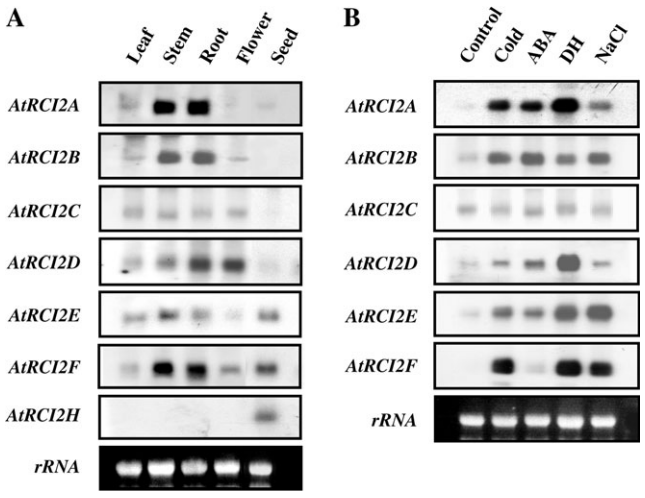

Fig. 5. Expression of *AtRCI2* genes in different organs of *Arabidopsis* and in response to different treatments. RNA-blot hybridizations were performed with total RNA (40 μg per line) extracted from (A) seeds and different organs of 8-week-old *Arabidopsis* plants or (B) leaves of 4-week-old *Arabidopsis* plants grown under control conditions (Control), exposed to 4 °C (Cold) for 24 h, sprayed with 100 μM ABA (ABA) for 6 h, dehydrated until losing 50% of their fresh weight (DH), or treated with 250 mM NaCl (NaCl) for 12 h. Specific probes were used for each *AtRCI2* gene. *rRNA* stained with ethidium bromide is shown at the bottom as a control of equal RNA loading.

A subset of AtRCI2 *genes complement the PMP3* deletion in yeast

The functional analysis of RCI2 proteins was more directly approached by investigating the capacity of *AtRCI2* genes to complement the deletion of the yeast *AtRCI2*-related gene *PMP3*. cDNAs corresponding to *AtRCI2A–F* and *AtRCI2H* were cloned into a yeast expression vector containing the promoter and terminator of *PMP3*, and used to transform *PMP3*-defficient yeast cells which exhibit high sensitivity to Na^+ (Navarre and Goffeau, 2000). Adequate expression of all transformed cDNAs in yeast cells was assessed by RT-PCR (data not shown). The sensitivity of transformed cells to increasing Na^+ concentrations was subsequently estimated. Figure 6 shows that, as reported by Navarre and Goffeau (2000), the cDNA corresponding to *AtRCI2A* was able to complement the deletion of *PMP3*. In spite of the high similarity existing among *AtRCI2* genes, only *AtRCI2B*, *AtRCI2C*, and *AtRCI2H* were also able to complement the *PMP3* deletion though to a lesser extent than *AtRCI2A* (Fig. 6).

Because the *AtRCI2* genes that encode AtRCI2 long proteins (AtRCI2D–F) were not able to complement the *PMP3* deletion, the hypothesis that the extra C-terminal hydrophilic tail characteristic of these proteins could play an important role in their complementation capacities was evaluated. With this aim, the C-terminal tail of AtRCI2D, comprising leucine 58 to alanine 75 (Fig. 1), was deleted to generate the AtRCI2D-Δ1 truncated protein. The corresponding *AtRCI2D-Δ1* cDNA was cloned into the yeast expression vector described above, which was used to transform *PMP3*-deficient yeast cells. Interestingly, AtRCI2D-Δ1 complemented the deletion of *PMP3* (Fig. 6), indicating that the extra C-terminal tails of the long AtRCI2 proteins may be critical for their complementation capacities and, therefore, may determine their function(s). All these results confirm that all AtRCI2 isoforms are not functionally redundant.

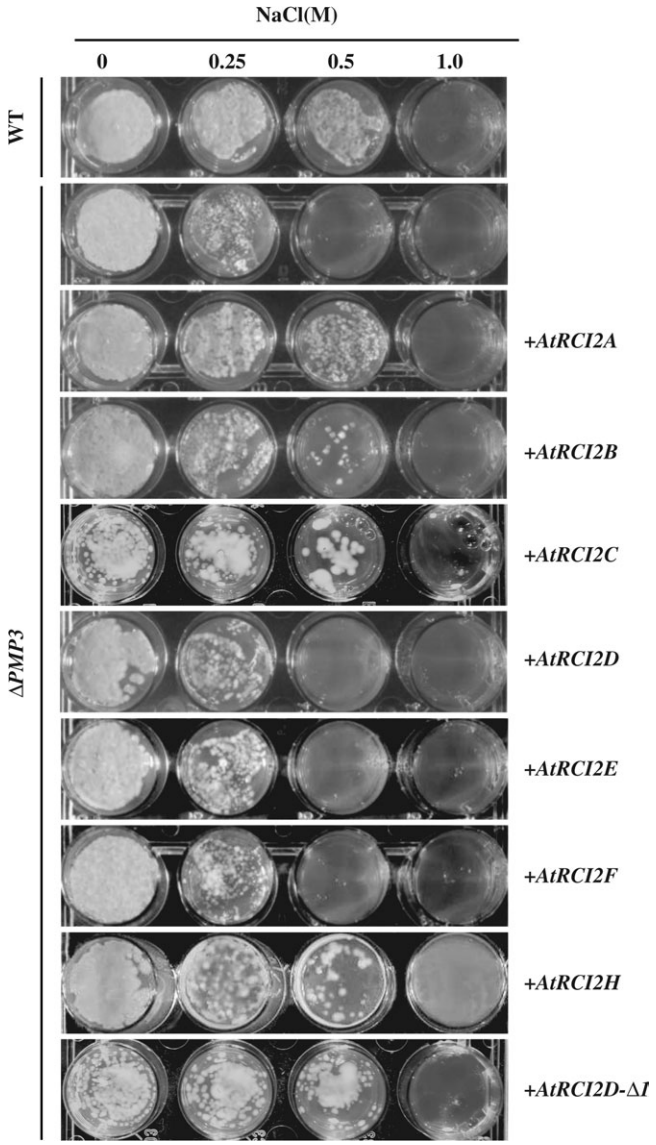

Fig. 6. Complementation of the Δ*PMP3* yeast mutant with *AtRCI2* cDNAs. Yeast cells having deleted the *PMP3* gene (Δ*PMP3*) were transformed with *AtRCI2* cDNAs (+*AtRCI2A–F* and +*AtRCI2H*) or with a truncated *AtRCI2D* cDNA mutant lacking the C-terminal domain (+*AtRCI2D-Δ1*). Wild type (WT), Δ*PMP3*, and transformed strains were grown in YD medium to saturation and normalized to a 0.5 OD_{600} value. Hundred-fold dilutions of the saturated cultures were plated onto agar–YPD plates supplemented with different NaCl concentrations (0, 0.25, 0.5, and 1.0 M). Plates were grown for 3 d at 30 °C.

Discussion

Six new *Arabidopsis* genes homologous to *AtRCI2A* and *AtRCI2B* have been identified and characterized. The eight *AtRCI2* genes (*AtRCI2A–H*) are distributed among four out of the five *Arabidopsis* chromosomes, and constitute a novel gene family. Large-scale duplications have been claimed to contribute greatly to the current shape of the *Arabidopsis* genome (Blanc *et al*., 2000; Bowers *et al*., 2003), with deletions and frequent tandem duplications of individual genes changing the microstructure of the duplicated segments (Bancroft, 2001). Our data suggest that *AtRCI2* genes have been implicated in some of these chromosome rearrangements. Thus, *AtRCI2D* is localized in a region of chromosome 2 that is duplicated in chromosome 4 (Blanc *et al*., 2000). The duplicated region contains *AtRCI2E* and *AtRCI2F* organized as a tandem. The organization of *AtRCI2A* and *AtRCI2B* in chromosome 3 indicates that, in all likelihood, they have also been originated by tandem duplication as *AtRCI2E* and *AtRCI2F*. On the other hand, *AtRCI2H* is localized in a region of chromosome 2 that is duplicated in chromosome 3 (Blanc *et al*., 2000). This duplicated region, however, does not contain any *AtRCI2* gene, probably because of a subsequent partial deletion.

RCI2-related genes have been described in several plant species, including barley, rice, sheep grass, wheat, and wheat grass (Goddard *et al*., 1993; Gulick *et al*., 1994; Hughes and Dunn, 1996; Imai *et al*., 2005; Inada *et al*., 2005; Morsy *et al*., 2005), as well as in the moss *Physcomitrella patens* (Kroemer *et al*., 2004). Here, searching through the available databases, it is shown that *AtRCI2*-related genes are widespread among different organisms, including most plant species, simple metazoans, fungi, and prokaryotes. It is noteworthy that no *AtRCI2*-related genes were noticed in the genomes of organisms like protostomes (arthropods, molluscs) and deuterostomes (echinoderms, chordates), suggesting that the functions of the corresponding proteins are not necessary in these organisms, or have been undertaken by other proteins. At a structural level, *RCI2* genes are remarkably well conserved during evolution, most of them being constituted by two exons separated by a single intron. A detailed analysis of the ESTs identified revealed the presence of several AtRCI2-related isoforms within the same species, indicating that, as *AtRCI2*s, *AtRCI2*-related genes are also organized into gene families. In rice, and *C. elegans*, for instance, 12 and 13 different *AtRCI2*-related genes were found, respectively. Some of these genes are arranged in tandem, as in *Arabidopsis*, which allows speculation that tandem duplications of individual genes have contributed to the evolution of *RCI2* gene families.

Our data clearly show that both sequence and topology have been conserved in AtRCI2 and AtRCI2-related proteins, suggesting that they have a common evolutionary origin. To test this hypothesis, a combined phylogenetic analysis was performed with the eight AtRCI2 proteins and the representative AtRCI2-related proteins shown in Fig. 1. An unrooted tree inferred from a Neighbor–Joining analysis (Saitou and Nei, 1987) is displayed in Fig. 7. The tree indicates that, in fact, RCI2 proteins have evolved from a common ancestor to three evolutionary groups. Group I is constituted by all plant proteins, while group II is composed by the proteins from

prokaryotes, and group III by a cluster of proteins belonging to different organisms including nematodes, protostomes, and fungi. Interestingly, the group of plant proteins can be subdivided in four (A–D) evolutionary branches, AtRCI2s being distributed in branches A, B, and C. Branch A is constituted by AtRCI2C and AtRCI2H isoforms, together with the representative AtRCI2-related protein from *O. sativa*. AtRCI2 proteins containing the extra C-terminal tail (AtRCI2D-G) compose branch B, confirming the common evolutionary pathway predicted for their corresponding genes on the basis of their structure and chromosome distribution. In a similar way, AtRCI2A and AtRCI2B, whose corresponding genes are organized in tandem, are localized in the same evolutionary branch (C), together with representative proteins from other different species. RCI2-related proteins from bryophytes constitute branch D, indicating a relatively early diversification of RCI2 families during plant evolution.

Our *in silico* analysis suggested that AtRCI2 and AtRCI2-related proteins contain two conserved transmembrane domains and localize to the plasma membrane. Consistent with these data, subcellular localization of AtRCI2 proteins, as revealed by transient expression of *GFP-AtRCI2* fusions in onion epidermal cells and *Arabidopsis* protoplasts, indicates that only AtRCI2D does not localize to the plasma membrane, being associated to the ER and Golgi compartment. In agreement with a plasma membrane localization for the majority of AtRCI2-related proteins, the yeast AtRCI2-related protein PMP3 was purified from this cellular component (Navarre and Goffeau, 2000). Moreover, a plasma membrane localization has also been recently reported for the wheat RCI2-related protein WPI6 (Imai *et al*., 2005), and the RCI2-related protein LTI6a was detected in the membrane protein fraction of rice cold-stressed seedlings (Morsy *et al*., 2005), further supporting that most RCI2 proteins are localized in the plasma membrane.

Little is known about the function of RCI2 proteins. The high sequence similarity of AtRCI2 and AtRCI2-related proteins, their predicted common evolutionary origin, and their conserved subcellular localization, suggest that RCI2 proteins may retain a conserved role. In this regard, the yeast AtRCI2-related protein PMP3 has been involved in controlling the plasma membrane potential, which, in turn, would contribute to the regulation of Na^+ uptake in yeast cells (Navarre and Goffeau, 2000), and AtRCI2A has been reported to play a role in salt tolerance by avoiding over-accumulation of Na^+ and K^+ ions in *Arabidopsis* (Mitsuya *et al*., 2005, 2006). Moreover, complementation analysis revealed that AtRCI2A (Navarre and Goffeau, 2000; Nylander *et al*., 2001) and

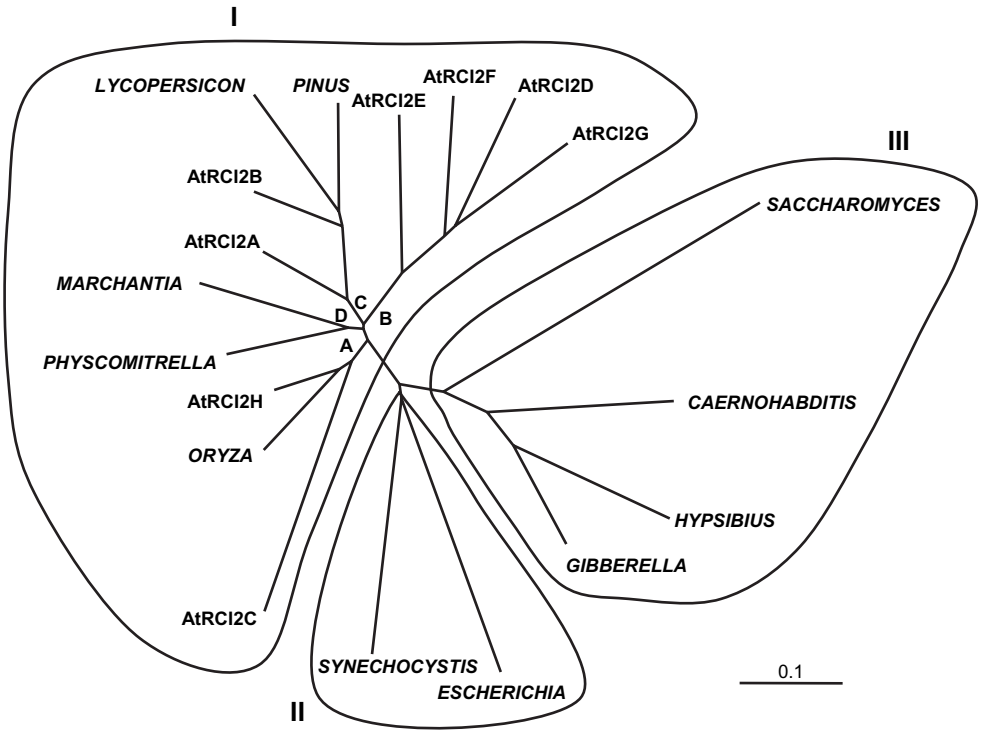

Fig. 7. Phylogenetic tree of AtRCI2 and representative AtRCI2-related proteins from different organisms. The amino acid sequences of proteins displayed in Fig. 1 were aligned with the Clustal X program, and an unrooted tree was constructed by using the Neighbor–Joining method. The branching pattern of the tree shows a distribution into different groups. RCI2 proteins from plants (I), prokaryotes (II), and other organisms, including nematodes, protostomes, and fungi (III), constitute the major groups. Branches into the plant group are indicated (A–D).

RCI2-related proteins WP16 from wheat (Imai *et al*., 2005) and AcPMP3 from sheep grass (Inada *et al*., 2005) restore the plasma membrane potential and the salt-sensitive phenotype of yeast cells lacking PMP3. These data indicate that, besides PMP3 and AtRCI2A, other RCI2 proteins can be involved in regulating plasma membrane potential and, therefore, abiotic stress responses. In this study, however, it is shown that, similarly to what has previously been demonstrated for *AtRCI2A* and *AtRCI2B* (Capel *et al*., 1997; Medina *et al*., 2001) and described for several *AtRCI2*-related genes from rice and sheep grass (Inada *et al*., 2005; Morsy *et al*., 2005), *AtRCI2C*, *AtRCI2D*, *AtRCI2E*, and *AtRCI2F* genes are differentially regulated in *Arabidopsis* organs and in response to abiotic stresses and ABA treatment. These results suggest that, in spite of their high similarity, shared evolutionary origin, and conserved subcellular localization, all RCI2 proteins do not have exactly the same function.

Additional support to the hypothesis that different RCI2 proteins may have different roles, comes from results showing that, in addition to *AtRCI2A*, only *AtRCI2B*, *AtRCI2C*, and *AtRCI2H* genes are able to complement the *PMP3* deletion in yeast and, therefore, may function in membrane potential homeostasis. The fact that AtRCI2D, AtRCI2E, and AtRCI2F are unable to substitute the the absence of PMP3 is particularly striking. The main difference between these proteins and AtRCI2A–C and AtRCI2H is that they contain an extra C-terminal hydrophilic tail of around 20 amino acids. Interestingly, although this tail does not contain any sequence resembling reported functional or targeting motives, when it is removed from AtRCI2D the truncated protein acquires the capacity to substitute for the lack of PMP3, suggesting it can have a role in AtRCI2D function. Considering that extra C-terminal tails are not restricted to some RCI2 proteins from *Arabidopsis* but are present in other AtRCI2-related proteins (see above), it is tempting to propose that they may modulate the function of RCI2 proteins by modifying either their stability or activity. In summary, the results reported in this work indicate that different members from the RCI2 protein family have unique functions. It has been demonstrated that different AtRCI2 proteins play particular roles in the *Arabidopsis* response to abiotic stresses. Genetic analysis through the identification and characterization of mutants affected in *AtRCI2* and *AtRCI2*-related genes will certainly contribute to understand further the functional redundancies and specificities of RCI2 proteins.

Supplementary data

Supplementary data in three tables and four figures can be found at *JXB* online.

Supplementary Table 1. Genebank accession numbers of the identified ESTs encoding AtRCI2-related proteins.

Supplementary Table 2. Primers used for *AtRCI2* probes.

Supplementary Table 3. Primers used to generate *AtRCI2* cDNAs and *GFP-AtRCI2* fusions, and to perform RT-PCR expression analyses in yeast.

Supplementary Fig. 1. Hydropathy profiles and transmembrane predictions for AtRCI2-related proteins.

Supplementary Fig. 2. Hydropathy profiles and transmembrane predictions for OsRCI2 proteins.

Supplementary Fig. 3. Hydropathy profiles and transmembrane predictions for CeRCI2 proteins.

Supplementary Fig. 4. Subcellular localization of GFP–AtRCI2 fusion proteins in *Arabidopsis* protoplasts.

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