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GENETICS AND GENOMICS

Characterization of a family of *Arabidopsis* receptor-like cytoplasmic kinases (RLCK class VI)

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Abstract The receptor-like cytoplasmic protein kinases (RLCKs) are plant-specific proteins encoded by almost 200 genes in the *Arabidopsis* genome. Despite of their high number, the available information on the potential function of RLCKs is very limited. In this report, the sequence analysis and the gene expression pattern of 14 members of one of the *Arabidopsis* RLCK families (RLCK class VI) are described. Sequence comparison indicated that gene duplication played a significant role in the formation of the kinase family and that several members carry an N-terminal "universal stress protein" (UspA) domain. In order to gain insight into the potential function of the RLCK VI kinases, real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to determine the relative transcript levels in the various organs of the *Arabidopsis* plant as well as under a series of abiotic stress/hormone treatments in seedlings. The obtained data revealed the differentially regulated expression of the genes in agreement with a high variability of sequence elements in their promoters. The divergent expression patterns indicate that the encoded kinase proteins may be involved in a wide variety of signal transduction pathways related to plant development and stress responses. The significance of gene duplication and expression divergence in the extension of the *Arabidopsis* RLCK VI family during evolution is discussed.

Keywords Abiotic stress treatment · *Arabidopsis thaliana* · Gene expression · Gene duplication · Organs · Real-time quantitative PCR · qRT-PCR · UspA domain

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Introduction

Plants have to respond and adapt to a variety of continuously changing environmental factors in order to establish an appropriate developmental strategy to ensure survival. There are ample data showing that protein phosphorylation/dephosphorylation plays a central role in cellular signal transduction in all organisms (Herrmann et al. 2006; Stone and Walker 1995). For example, protein kinases possessing an extracellular receptor domain (receptor kinases) can relay external signals into the cells by protein phosphorylation (Becraft 2002; Kruijt et al. 2005). In plants, two different types of transmembrane receptor kinases are known, including receptor-like serine/threonine (Ser/Thr) kinases (receptor-like kinases RLKs; Shiu and Bleecker 2001, 2003; Shiu et al. 2004; Walker 1994) and receptor histidine (His) kinases (Grefen and Harter 2004; Mizuno 2005; Urao et al. 2000).

In *Arabidopsis*, RLKs belong to a large, monophyletic gene family with more than 610 members (Shiu and Bleecker 2001, 2003; Shiu et al. 2004). These types of kinases represent nearly 2.5% of the protein-coding genes in *Arabidopsis*. Most of the RLKs have a receptor configuration with an extracellular domain, a transmembrane domain, and an intracellular kinase domain. However,

kinases, with the catalytic domain similar to RLKs but without extracellular or transmembrane domains are also represented by a high number in the RLK family (approximately 25% of RLKs). Based on their putative intracellular localization, these kinases are named receptor-like cytoplasmic protein kinases (RLCKs).

Studies regarding the function of RLKs have revealed their roles in a wide range of signal responses, such as in hormone perception (BRI1; Li 2005; Li and Chory 1997; Scheer and Ryan 2002), pollen–pistil interaction (SRK; Kachroo et al. 2002; Takayama and Isogai 2005), disease resistance (Xa 21; Kruijt et al. 2005; Song et al. 1995) and in the organization of the shoot apical meristem (CLAVATA1; Clark et al. 1997; Sharma et al. 2003). However, we have only very limited information on the potential role of RLCKs in signal transduction pathways. In tomato, Pto (RLK) confers resistance to the pathogen *Pseudomonas syringae* (Tang et al. 1996) and it is mediated by the RLCK, Pto-interacting1 (Pti1, RLCK VIII) (Zhou et al. 1995). More interestingly, Pti1-like proteins have recently been shown to play a role in phospholipid and oxidative stress signaling downstream of PDK and OXI1 kinases (Anthony et al. 2006). Pti1-like kinases have also been identified in maize and one of them proved to be specifically expressed in the pollen (Herrmann et al. 2006). In *Arabidopsis*, the RLCK kinase PBS1 (class VII), and the RLK kinase RPS5, are both required to recognize the *P. syringae* strains that express the avirulence gene *avrPphB* (Swiderski and Innes 2001). Self-incompatibility is mediated by the SRK kinase (RLK) the function of which is linked to the presence of MLPK (RLCK VII) (Murase et al. 2004). The overexpression of the *Constitutive Differential Growth 1* gene, which encodes a RLCK VII-subfamily protein kinase, results in abnormal elongation growth following organ differentiation in *Arabidopsis* (Muto et al. 2004). The RLCK VII subfamily member Esi47 protein of wheat has been identified as an "early salt inducible" gene product with a role in the transduction of hormonal (gibberellin) signals (Shen et al. 2001). However, little is known about the precise functions of RLCKs in the above processes. Based on these few examples, it is very likely, that they play major roles in the perception and transmission of external signals perceived by RLKs. Therefore, the transcriptional and posttranslational regulation of their activity can also be of high significance considering receptor signalling in plants.

Here the expressional profiling and sequence analysis of one of the *Arabidopsis* RLCK families (class VI) is reported highlighting their potential functional diversity. The importance of gene duplication events in the evolution of the *Arabidopsis* RLCK VI family is also discussed.

Materials and methods

Sequence analysis

RLCK VI and control cDNA/protein sequences have been downloaded from the *Arabidopsis* Biological Resource Center (ABRC) at http://www.arabidopsis.org/abrc/. The accession numbers and the used nomenclature are shown in Fig. 1. Sequence alignment has been made using the ClustalW algorithm (Thompson et al. 1994). Phylogenetic analysis was carried out by the Phylip 3.67 program package (http://www.evolution.genetics.washington.edu/phylip.html). Potential gene duplication events during the evolution of the RLCK VI family have been analysed by an

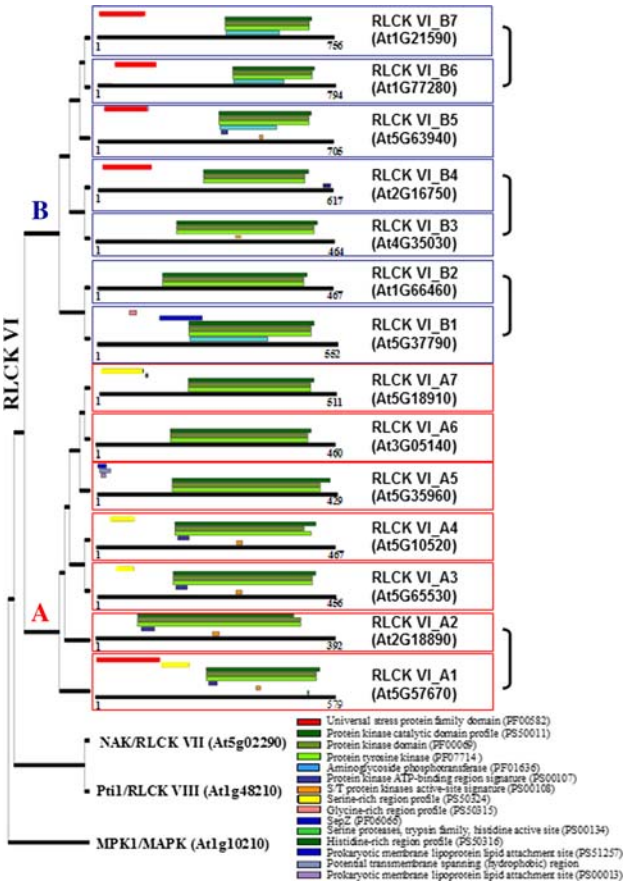

Fig. 1 Phylogenetic relationship and domain organization of *Arabidopsis* RLCK VI protein kinases. The protein sequences of the 14 RLCK VI kinases and three other *Arabidopsis* kinases have been compared. The cladogram on the *left* indicates the phylogenetic relationship of the investigated proteins. The RLCK VI kinases fall into two groups (*A*, *B*) and were designated accordingly. Domain composition is shown according to the "PlantsP Kinase Classification" database (http://www.plantsp.genomics.purdue.edu/html/family/class.html). The *hooks* at the *right* indicate duplicated genes (paralogons) residing in well-recognisable duplicated chromosome regions based on the search of paralogons in the *Arabidopsis* genome (http://www.wolfe.gen.tcd.ie/athal/dup)

online tool maintained by Ken Wolfe's laboratory at the Genetics Department, Trinity College, Dublin (http://www.wolfe.gen.tcd.ie/athal/dup). Domain information was collected from the PlantsP database (http://www.plantsp.genomics.purdue.edu/html/). The promoter elements of the corresponding genes were identified by the "ATHENA" tool (O'Connor et al. 2005; http://www.bioinformatics2.wsu.edu/Athena). Microarray expression data were extracted from the Genevestigator database (Zimmermann et al. 2004; https://www.genevestigator.ethz.ch/at/). The heat map of expression data has been made by the heat mapper tool at http://www.bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_heatmapper.cgi.

Plant material

To analyse the gene expression in different *Arabidopsis thaliana* (Columbia ecotype) organs, roots, rosette leaves, cauline leaves, inflorescence stems, flower buds, open flowers and green siliques were harvested from plants 60 days after sowing. All plant materials were frozen in liquid nitrogen immediately after harvesting.

For abiotic stress treatments, *Arabidopsis* seedlings have been germinated on nylon meshes wetted by water in Petri dishes. Three-week-old seedlings have been subjected to various stress/hormone treatments by moving the filters along with the plantlets to new dishes with water supplemented with the stress/hormone agents. RNA samples were isolated from whole seedlings 6 h after the treatments. The treatments were as follows: polyethylene glycol (30%), glucose (300 mM), sucrose (8%), NaCl (300 mM), paraquat (25 μM), abscisic acid (100 μM), etephon (10 mg/l), salicylic acid (10 mM). Seedlings were also transferred to pure water as control. Cold (4°C) treatment as well as the corresponding control (22°C) was carried out without transfer to new medium. All the treatments were repeated three times.

Nucleic acid isolation

Total RNA has been extracted from the frozen plant material using the acidic phenol/guanidium method (Chomczynski and Sacchi 1987). To avoid genomic DNA contamination, each RNA preparation was treated with RNAse-free DNAse according to the manufacturer's instructions (Sigma, St-Louis, USA). RNA was then quantified measuring absorbance at 260 nm using a spectrophotometer (NanoDrop Technologies, Wilmington, USA) and loaded on a denaturing agarose gel to check concentration and integrity.

Genomic DNA has been extracted from cultured *A. thaliana* var. Columbia cells using the modified CTAB method (Aldrich and Cullis 1993) and was treated with RNase (Sigma).

cDNA synthesis

A measure of 2.5 μg total RNA was reverse transcribed using oligo dT primers and reverse transcriptase (RevertAid M-MuLV, Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. The cDNAs were diluted to 200 μl with sterile H_2O.

Real-time quantitative RT-PCR (qRT-PCR)

Appropriate primer pairs allowing the differential identification of the 14 members of the *Arabidopsis* RLCK VI subfamily (Table 1) have been designed by the Primer Express software (Applied Biosystems, Foster City, USA).

Real-time quantitative RT-PCR was assayed on an ABI Prism 7700 sequence detection system and the accumulation of PCR products was measured in real time as the increase in SYBR Green fluorescence using ROX (glycine conjugate of 5-carboxy-X-rhodamine, succinimidyl ester) as a passive reference dye according to the manufacturer's (Applied Biosystems) instruction as described elsewhere in detail (Otvos et al. 2005; Szucs et al. 2006).

Real time PCR reactions were carried out at least in duplicate with 6 μl cDNA samples. Two actin genes (actin2 and 8 transcripts coded by the At3g18780 and At1g49240 genes, respectively, and detected together by the same primer pair; Charrier et al. 2002) have been used as internal controls for data normalization (geNorm method, Vandesompele et al. 2002). As further controls, the relative expression of one housekeeping gene coding for a glycerinaldehyde-3-phosphate dehydrogenase enzyme (GAPDH) (At1g13440; Czechowski et al. 2005) and one stress-induced gene RD29A (At5g52310; Yamaguchi-Shinozaki and Shinozaki 1993; for the used primers see Charrier et al. 2002) has also been determined in the appropriate samples, respectively.

In order to allow the comparison of the absolute gene expression levels of the 14 RLCK VI genes, genomic DNA have been used as the standard. Genomic DNA was quantified and a range of 3 dilutions was prepared representing 200, 2,000 and 20,000 *Arabidopsis* genomes in 6 μl of water, respectively. Steady state transcript levels were expressed as genome equivalents per ng initial RNA for each gene.

Results

Sequence analysis

The family of class VI receptor-like cytoplasmic kinases (RLCK VI) of *Arabidopsis* consists of 14 members.

Table 1 Genes and oligonucleotides used in the real-time RT-PCR experiments

Sequences of the primers used	AGI code
5′-GCTAAAATGGGCAAAGCTACAAGTT-3′	At1g21590
5′-CCGCAATTTCTCGTCCTTGA-3′	
5′-CGGCTTCGATGGAGCGTAT-3′	At1g66460
5′-CTTCGTGATTCCTCATTGATCACA-3′	
5′-ACAACAATAATGATGACCAAATGCA-3′	At1g77280
5′-CTAGCTTGAGGACTACGTCGAATACA-3′	
5′-GACGAGGTTTACCCAAATTCGAA-3′	At2g16750
5′-ACAGAGTCATTATCCTCCACGTCAA-3′	
5′-GCAACAACTTCATCGCATTGC-3′	At2g18890
5′-TGGAAGGTCGACATAGAGAAGAAGAT-3′	
5′-CGGACATCCTGCCCTTGA-3′	At3g05140
5′-GCTTTTCTCTCTAGCAACGGCTTA-3′	
5′-ACGAAGCCGGGAAATGGA-3′	At4g35030
5′-CGGGTAAACCTCGTCATCGA-3′	
5′-GCAATGCGACCTGACATGACT-3′	At5g10520
5′-TCACCTGCCTTCTGCTGTAGCT-3′	
5′-CCTGCTTCTCGAGCTCATTACC-3′	At5g18910
5′-AGCCCACATGACAATGCTATGTT-3′	
5′-GAGATATCAAAGCCGCAAACATTC-3′	At5g35960
5′-TCGCAAGCCCAAAGTCACAT-3′	
5′-GGTTCAAATGGAGCGGATGAT-3′	At5g37790
5′-GGTCTTCGGGACTCCTCATTG-3′	
5′-GAACGTTTGGTTATCTAGCTCCAGAGT-3′	At5g57670
5′-CGAATGCGTAGATGTCGGTTTT-3′	
5′-GCTGCCACGCTATGCATCA-3′	At5g63940
5′-ATCTTTAGGACAAGGCCAATTTGC-3′	
5′-CGAGATGAAACGAGTGATGCAA-3′	At5g65530
5′-TCATGTCAGGACGCATAGTGGAT-3′	
5′-GGTAACATTGTGCTCAGTGGTGG-3′	At3g18780 (ACTIN2) and At1g49240 (ACTIN 8)
5′-AACGACCTTAATCTTCATGCTGC-3′	
5′-ATTGGAAAATTGACCGGAATGT-3′	At1g13440 (GAPDH)
5′-CGGTGAGATCAACAACTGAGACA-3′	

Sequence comparison (Supplementary material 1) and phylogenetic analysis (Fig. 1), revealed that these proteins are highly homologous to each other, especially at the kinase domain, but are divergent from the related kinase families. It could also be determined that the 14 RLVK VI kinases form 2 groups with 7 members each. The RLCK VI family members have been designated accordingly as RLCK VI_A1–A7 and B1–B7 (see Fig. 1 for details). Furthermore, within these groups, there are well recognisable pairs of sequences with high similarity to each other, possibly as the result of gene duplication events.

Despite of the considerable similarity of the kinase domain of all RLCK VI members, the overall length (ranging from 392 to 794 aminoacids) and domain composition of the individual proteins are divergent. Six of the RLCK VI kinases, five of them belonging to group B, contain the "universal stress protein" (UspA) domain. In contrast, four RLCK VI group A members have an N-terminal serine-rich region each. As listed in Fig. 1, there are few other regions exhibiting homology to protein domain database sequences in the RLCK VI kinases. The functional significance of these regions still needs to be verified.

Expression analysis

Differential expression in plant organs

Figure 2a allows the comparison of the expression strength (steady state transcript level) of the investigated RLCK VI genes in the plant organs. It can be seen that the average expression of the RLCK VI genes was below 10,000 genome equivalent copies per ng total RNA. For

comparison, the average transcript level of the control GAPDH gene was close to 200,000 g.e. copies/ng RNA. The average expression strength of the duplicated genes (paralogons) was similar, except for *rlckVI_B1* and *B2*.

Despite the relatively low level of expression, most RLCK VI genes exhibited wide range of transcript abundance in the various plant organs. This differential transcript accumulation can be better observed on Fig. 2b, where the relative expression of the 14 genes in the various organs is compared. Relative RLCK expression is shown in relation to the average expression of each individual gene in all the investigated organs. The relative gene expression levels were highly variable among the genes as well as the organs. However, it can be observed that the range of expression (absolute and relative as well, see Fig. 2a and b, respectively) is wider in the case of RLCK VI group A than for group B members (with exception of the *rlckVI_B3* and *B4* gene pair). Only some small changes could be observed in the relative transcript abundance for the genes *rlckVI_B1, B2, B5* and *B6*.

Most of the RLCK VI genes of *Arabidopsis* were only weakly expressed in the exponentially dividing cultured cells and transcripts of six of them could hardly be detected in this sample. Only the genes *rlckVI_A1, A4, A5*, and *B1* exhibited expression levels at or above the average in dividing cells, and for *rlckVI_A1* the relative expression was the highest in this sample. Similarly, only few RLCK VI genes showed increased expression in rosette and/or cauline leaves including *rlckVI_A2, A4, B4*, and *B7*, the latter exhibiting the highest relative expression in this

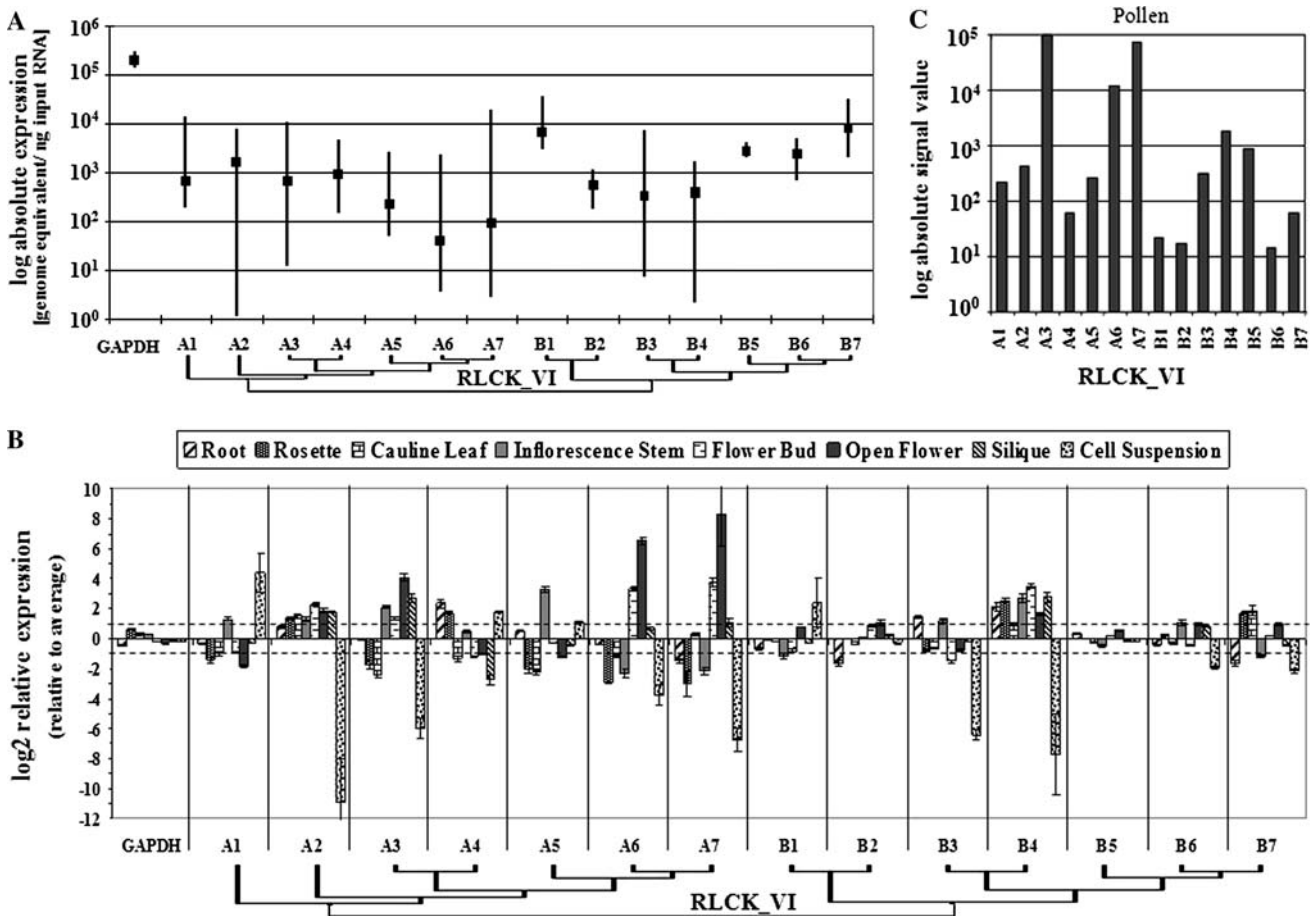

Fig. 2 Transcript levels of the RLCK VI genes in various organs of the *Arabidopsis* plant. qRT-PCR have been used to determine transcript levels of the 14 *Arabidopsis* RLCK class VI genes in various organs of greenhouse plants. **a** Absolute transcript level of the 14 RLCK VI genes and the control At1g13440 (GAPDH) gene normalized to actin2/8 gene expression is expressed as genome equivalent (g.e.) copies of transcripts per ng initial RNA. The *bars* range from the highest to the lowest transcript level and the mean transcript level is shown by *squares*. The cladogram below the histogram shows the phylogenetic relation of the genes. **b** Relative transcript level of the same genes in the various plant organs expressed as the log 2 ratio of actin2/8 normalized transcript levels in a given organ in comparison to the average expression of the gene in all organs tested. Averages and standard deviations of three measurements are shown. *Dashed lines* indicate twofold increase in the transcript level. The cladogram below the histogram shows the phylogenetic relation of the genes. **c** Gene expression of the RLCK VI genes in the *Arabidopsis* pollen as log absolute signal values of microarray hybridization experiments obtained from the Genevestigator database (https://www.genevestigator.ethz.ch/at/)

organ. The genes *rlckVI_ A4*, *B3, and B4* were expressed at a level above average in the root. Several genes exhibited above average expression in the inflorescence stem and the gene *rlckVI_A5* showed the highest expression in this organ.

The *rlckVI_A6* and *A7* genes exhibited a very high relative expression in flower buds and open flowers and the *rlckVI_A3* gene had also its highest relative transcript level in the open flower. Figure 2c shows that this elevated expression detected in the flower indeed indicates strong expression in the pollen as can be deduced from microarray experiment data stored in the Genevestigator database (https://www.genevestigator.ethz.ch; Zimmermann et al. 2004, 2005). In general, the data obtained by qRT-PCR in relation to the gene expression in the *Arabidopsis* organs were in good correlation with similar data available from the Genevestigator database as microarray datasets (Supplementary Material 2). However, in order to have the best match, the microarray data had to go through similar normalization steps used for the analysis of the qRT-PCR results.

In Fig. 2, despite the high general variability in the overall gene expression patterns, it can be recognized that some of the paralogous gene pairs exhibit very similar (e.g. *rlckVI_A6* and *A7*) or rather complementary (e.g. *rlckVI_A1* and *A2, B3* and *B4*) relative gene expression patterns in the various plant organs.

Differential expression in response to stress treatments

Five of the RLCK VI proteins (A1, B4-7) have a domain homologous to the prokaryotic "universal stress protein", UspA (Nystrom and Neidhardt 1994, see Fig. 1). In order to clarify the potential role of these kinases in stress responses, the differential gene expression of all RLCK VI members has been tested in response to various abiotic stress and stress-related hormone (abscisic acid, salicylic acid, ethylene) treatments for 6 h. The normalized (*actin2/8* as reference gene; geNorm method (Vandesompele et al. 2002) stress-related expression of the genes was compared to gene expression in mock (normal culture medium) treated plantlets (Fig. 3a, b). As a positive control, the relative expression of the RD29A gene (Charrier et al. 2002; Yamaguchi-Shinozaki and Shinozaki 1993) was also determined in the same samples (Fig. 3c). The transcript level of the positive control RD29A gene was considerably increased in response to the treatments (from approximately tenfold in response to ethylene up to more than 1,000-fold in response to salt stress or ABA), verifying the effectiveness of the treatments and responsiveness of the seedlings.

As the 14 RLCK genes are considered, only few of them (*rlckVI_A1, A2* and *A3, B2* and *B3*) showed significantly (more than fivefold) increased expression in response to stress/hormone treatments and only one of those carrying the UspA homology domain (*rlckVI_A1*) was found amongst them. Only a moderate (at or slightly above twofold) increase could be observed in the case of the UspA domain-carrying *rlckVI_B4, B5 and B7* genes in response to some of the treatments.

It could also be observed that if a gene proved to be stress-induced it was a rather general response, although the response to osmotic, sugar, and salt stresses, were the most prominent. The gene *rlckVI_B2* showed increased expression in all treatments except the cold and paraquat (oxidative stress), *rlckVI_B3* was unresponsive only to cold and salicylic acid while *rlckVI_A1* and *rlckVI_A3* only to cold. On the other hand, the gene *rlckVI_A2* exhibited approx. eightfold increased transcript level only in ABA-treated samples and this gene proved to be the more cold responsive (approx. threefold increase). ABA-induced expression could be observed for six RLCK VI genes (*rlckVI_A1, A2*, and *A3, B1, B2*, and *B3*) and four of them also responded positively to salicylic acid and ethylene (*rlckVI_A1, A3, B2*, and *B3*). No paralogous gene pair was found where both members exhibited strong stress response.

The genes not mentioned above either responded to the treatments with decreased gene expression (e.g. *rlckVI_A7*) or exhibited only less than twofold increase in their transcript level. These genes include those having the highest relative transcript level in the reproductive organs, and therefore their stress/hormone responsiveness might not be the best investigated at the seedling stage, although they have significant relative expression in vegetative tissues, e.g., in the root, as well (see Fig. 2b). The transcript level of the *rlckVI_A5* gene did not reach the detection level in whole seedlings, even following the stress treatments, in accordance with its generally low expression level in all organs except inflorescence stem (Fig. 2b).

Analysis of the promoter sequences

In order to investigate whether the observed differences in gene expression patterns can be related to promoter structure, the combination of promoter elements in the 1,000-bp upstream region of the 14 RLCK VI genes has been compared (Fig. 4). The promoter structures were obtained from the ATHENA database (http://www.bioinformatics2.wsu.edu/Athena; O'Connor et al. 2005). It can be established that the combination of transcription factor binding sites in the promoters of the RLCK VI genes are highly variable in accordance with their diverse expression patterns. Therefore, the expression patterns could not be clearly related to the representation of promoter elements.

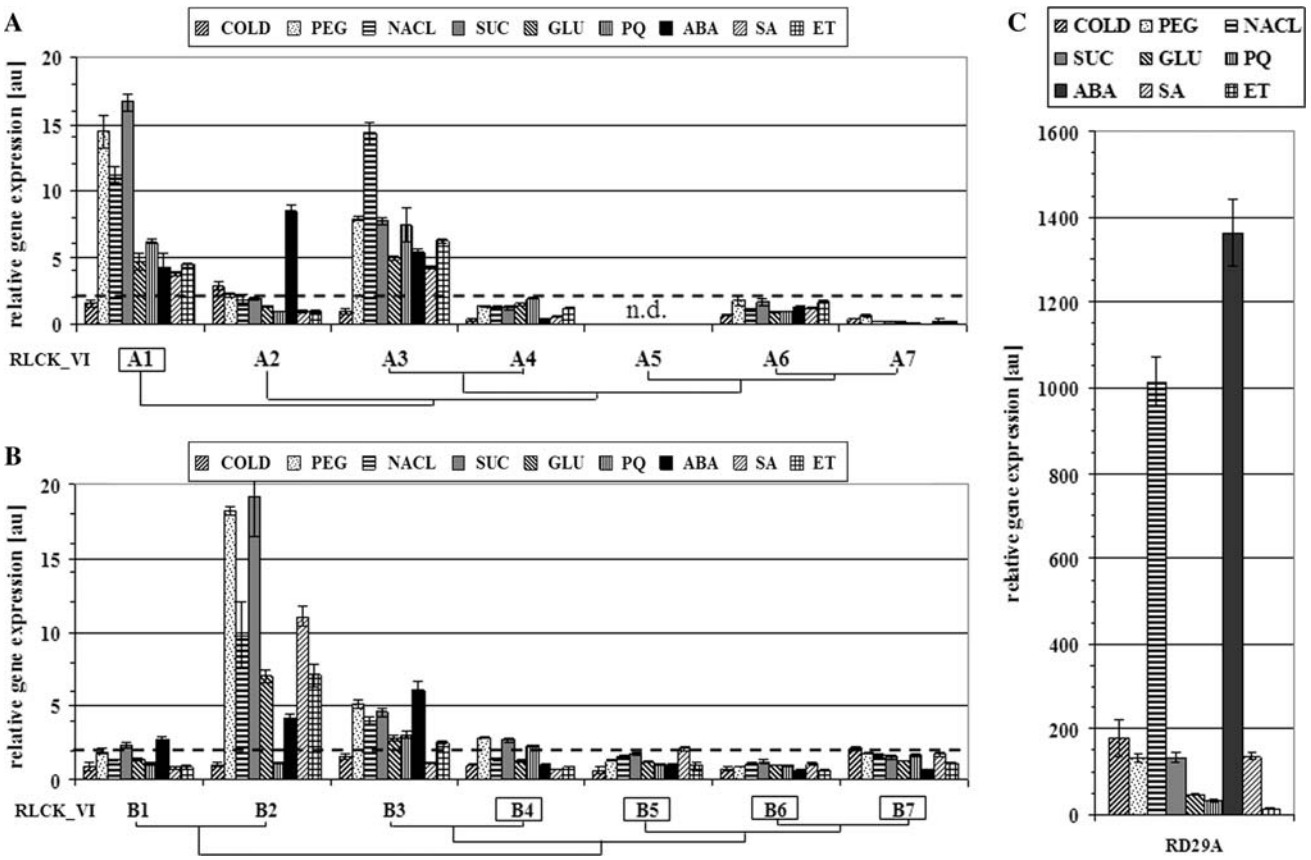

Fig. 3 Relative expression of the *Arabidopsis* RLCK VI genes in response to stress, and stress-related hormone, treatments. Three-weeks-old seedlings were treated by various stress agents or hormones, respectively, during 6 h. Relative gene expression has been determined by qRT-PCR in three repetitions. Transcript levels were normalized to the expression of the actin2/8 gene. The average and the standard deviation values are shown. The mock-treated control was considered to have the relative gene expression value 1. **a** Relative expression of the RLCK VI group A and **b** relative expression of the RLCK VI group B genes in response to the treatments. The UspA domain-containing genes are *boxed*. *Dashed lines* indicate twofold increase in the transcript level. **c** Relative transcript levels of the stress-inducible positive control RD29A gene in the same experiments. *Cold* 4°C; *PEG* 30% polyethylene glycol; *NaCl* 300 mM NaCl; *Suc* 8% sucrose; *Glu* 300 mM glucose; *PQ* 25 μM paraquat; *ABA* 100 μM abscisic acid; *SA* 10 mM salicylic acid; *Et* 10 mg/l etephon

Even paralogous gene pairs with very similar expression patterns according to the present investigations (e.g. *rlckVI_A6* and *A7*; see Figs. 2, 3) have very different promoter composition. Furthermore, although only few genes exhibited strongly increased expression in response to osmotic, cold, salt stresses and/or ABA treatment, all of the genes possess several binding sites (e.g. ABRE, DRE, MYB-binding site, MYC-binding site; Fig. 4) for transcription factors considered to play role in such responses (Ma and Bohnert 2007).

Discussion

The RLK family, comprising more than 600 members in *Arabidopsis*, is thought to have diverged specifically in the plant kingdom, and only related families of small size can be found in animals and in Plasmodium species (Shiu and Bleecker 2003). RLK subfamilies with different domain organizations are the results of ancient gene duplication events, which occurred extensively before the divergence of vascular and nonvascular plants, or even during the early evolution of streptophytes (Sasaki et al. 2007).

The phylogenetic comparison of the *Arabidopsis* RLCK VI sequences revealed that the 14 proteins form two groups and within the groups there is a pair-wise similarity of the sequences (Fig. 1), indicating that gene duplication played a significant role in the formation of this protein family as well. Indeed, at least in four cases, the pairs of corresponding *Arabidopsis* genes (RLCK VI_A1/A2, B1/B2, B3/B4, and B6/B7) can be considered as paralogous genes residing within large duplicated chromosome regions (for the database of *Arabidopsis* paralogons see http://www.wolfe.gen.tcd.ie/athal/dup). The duplication resulting in RLCK VI_A1/A2 likely represents an ancient polyploidization process (at least 100 million years ago) while the

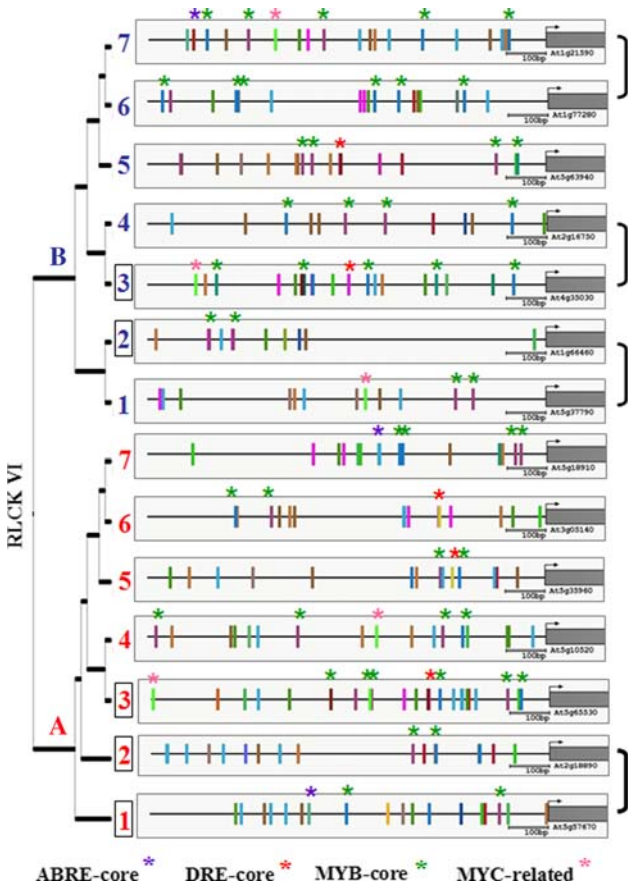

Fig. 4 Promoter structure of the *Arabidopsis* RLCK VI genes. The various putative *cis*-regulatory promoter elements are indicated by differentially *coloured bars* as obtained by the ATHENA promoter analysis tool (http://www.bioinformatics2.wsu.edu/Athena). For simplicity, no details of the promoter elements are given, the comparison is shown only to highlight the diversity of the promoters. The promoter elements potentially involved in stress- or ABA-signalling are indicated by *asterisk* (note that the *bars* with *different colour* may represent different sequences containing the same core motif indicated by the same *colour of the asterisk*). The number of the RLCK VI genes with increased expression in response to stress are *boxed*. The cladogram at the *left* shows the phylogenetic relation of the genes. The *hooks* at the *right* indicate duplicated genes (paralogons) residing in well recognisable duplicated chromosome regions based on the search of paralogons in the *Arabidopsis* genome (http://wolfe.gen.tcd.ie/athal/dup)

other duplication events leading to the formation of the paralogons RLCK VI_ B1/B2, B3/B4, and B6/B7 can be dated back to about 20–24 million years ago (see http://www.wolfe.gen.tcd.ie/athal/dup; for more details see Blanc et al. 2003; Blanc and Wolfe 2004b).

The group A of *Arabidopsis* RLCK VI kinases is characterized by the presence of an N-terminal serine-rich region (four out of the seven proteins) while several (four) group B members carry an N-terminal UspA domain (Kerk et al. 2003). The UspA domain is found either in small proteins, or it makes up the N-terminal portion of a larger protein, usually a protein kinase. The precise biological function of the bacterial UspA protein and the UspA domain of eukaryotic proteins is not known; however, it may be involved in nucleotide (ATP)-binding and signal transduction (Zarembinski et al. 1998). The RLCK VI_A1 protein possesses both (UspA and serine-rich) domains and may be the most closely related to the ancestor of this kinase family. It can be supposed that the domain structure of the kinases diverged following the gene duplication events.

Expression divergence, resulting in rapid subfunctionalization (partitioning of functional modules of the ancestral gene), is suggested to contribute substantially to the maintenance of most of the paralogous regulatory and stress-responsive genes in the *Arabidopsis* genome (Haberer et al. 2004; Blanc and Wolfe 2004a; Kim et al. 2005; Duarte et al. 2006; Walther et al. 2007). In agreement with this hypothesis, our gene expression data verified a diverse expression pattern of the 14 investigated RLCK VI genes, in the various plant organs as well as in response to stress/hormone treatments. In certain cases, the paralogous genes still retained a similar relative expression pattern while in other cases (e.g. *rlckVI_A6* and *A7*) they exhibited complementarity in this respect (e.g. *rlckVI_A3* and *A4).* Paralogons, which derive from large-scale duplication events and can still be found in duplicated segments, exhibit more correlated expression patterns as compared to those resulting from small-scale duplications or those residing no longer on duplicated segments (Casneuf et al. 2006). This may explain the difference between RLCK VI group A and group B considering the breadth of their gene expression diversity, since group B genes (at least six out of the seven) reside in large duplicated regions of recent duplications and exhibit only limited differences in their relative transcript levels.

Consistent with their distinct gene expression patterns, the promoter sequences of the *Arabidopsis* RLCK VI genes are also highly divergent (Fig. 4). The faster evolution of the promoter than the protein coding sequences of duplicated genes is a well-accepted phenomenon resulting in the fast diversification of their gene expression (e.g. Haberer et al. 2004; Duarte et al. 2006; Walther et al. 2007). In the case of the *Arabidopsis* RLCK VI genes no clear correlation between the putative promoter elements and the observed gene expression patterns could be established. For example, it was found that only a few RLCK VI genes exhibited stress response in our studies, although all genes contain promoter elements (ABRE/DRE/MYB-core and MYC-like), which may confer ABA and/or osmotic/cold stress responsiveness. In addition, this set of motifs has recently been identified as promoter elements associated with a high breadth of differential gene response (multi-stress response; Walther et al. 2007). Of course, it is

possible that our restricted analysis (young seedlings treated for 6 h) could not reveal the stress-regulated expression of those genes, which may be up- or down-regulated by ABA/stress only under specific conditions or in specific tissues.

The expression pattern of all *Arabidopsis* genes can nowadays be investigated in silico, based on the gene expression datasets derived from experiments with whole genome microarrays. In order to be able to compare our qRT-PCR results to the appropriate microarray datasets, we carried out the same "normalization" of the absolute signal intensity values to the actin 2/8 (At3g18780/At1g49240) genes and related these values to the average intensity. In this way a good correlation of the expression profiles could be observed (Supplementary Materials 2), except a few values obtained by suspension-cultured cells that may indicate the sensitivity of the given genes to certain culture conditions. However, the overall correlation could not have been established between qRT-PCR and microarray data considering the stress response of the genes as the microarray data indicated only moderate (two to fourfold) increase in the case of some of the investigated genes (RLCK VI A1, A2, A4, B1 and B7) in response to one or several treatments (data not shown). This failure in data correlation can be the result of several factors including the differences in the treatments, the way of application, the conditions of the plant material, the low overall expression level of the genes and the lower sensitivity of the microarray hybridization assay, etc. Therefore further investigations including among others time course experiments, are required to elaborate a full picture on the regulation of the expression of RLCK VI genes in response to environmental factors.

The presented data indicate that the activity of several members of the RLCK VI kinase subfamily is regulated at the transcriptional level during plant development as well as in response to environmental stresses. Their tightly regulated and generally low expression may indicate that these proteins are involved in specific rather than general cellular processes. The low expression levels in dedifferentiated cultured cells and diverse distribution in various plant organs suggest that their functions are associated with plant differentiation. In addition, some of them may also be involved in the coordination of plant development and stress responses. Further investigations, among others mutant analysis, transgenic approaches and protein–protein interaction studies only, can verify these hypothetical functions.

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