ORIGINAL PAPER

CsPDR8 and *CsPDR12*, two of the 16 pleiotropic drug resistance genes in cucumber, are transcriptionally regulated by phytohormones and auxin herbicide in roots

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Abstract There are 15 and 23 members of the pleiotropic drug resistance (PDR) proteins, a subfamily of ATP-binding cassette (ABC) transporters, in *Arabidopsis thaliana* and *Oryza sativa*, respectively. Until recently, only a few members of *Arabidopsis thaliana* PDRs (AtPDRs) had been characterized in detail but growing reports indicate that proteins of this family may participate in growth regulator-mediated signaling and phytohormone transport. The profile of *Oryza sativa PDRs* (*OsPDRs*) expression in response to different stimuli also suggests that some rice PDRs are associated with a phytohormone-mediated response to environmental changes. Due to the lack of full genome resources, only individual members of PDRs in other plants (*Nicotiana*, *Oryza*, *Glycine* or *Spirodela*) have been studied. We have previously cloned two full cDNA sequences encoding for cucumber homologs of *Arabidopsis* PDR8 and PDR12. Here, we present the genomic organization of 16 cucumber *PDRs* and the phylogeny of the predicted *Cucumis sativus* PDR (CsPDR) proteins. Further analysis of *CsPDR8* and *CsPDR12* reveal that these cucumber genes are constitutively expressed mainly in roots of young seedlings and flowering plants (*CsPDR8* and *CsPDR12*), and in flowers (*CsPDR12*). In roots, the transcript of *CsPDR8* decreased in response to ABA and markedly raised in the presence of ACC, salicylic acid or jasmonic acid. In comparison, the expression of *CsPDR12* was not affected by ACC and significantly increased upon the addition of jasmonic acid, salicylic acid, 2,4-D, kinetin or ABA to the growth media. These data suggest that CsPDR8 and CsPDR12 may be involved in a phytohormone-mediated response of plants to different stimuli by sharing different signaling pathways.

Abbreviations

ACC	1-AminoCyclopropane-1-Carboxylic acid
IAA	Indole-3-Acetic Acid
ABA	Abscisic Acid
SA	Salicylic Acid
JA	Jasmonic Acid
GA_3	Gibberellic Acid
RACE-PCR	Rapid Amplification of cDNA Ends

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Introduction

In plants, ABC transporters constitute a large family of proteins exhibiting ATP hydrolyzing activity associated with the presence of the conservative ATP-binding cassette (ABC) domain. The family comprises different subfamilies separated according to the distinct order of their cytoplasmic and membrane-spanning domains. The full-size transporters contain two ABC (NBD) domains that can be differentially organized in relation to transmembrane

domains (TMDs) of the proteins. They can be subdivided into three families with the following NBD-TMD composition: $(TMD-NBD)_2$ in multidrug resistance proteins MDR/ABCB, $TMD-(TMD-NBD)_2$ in multidrug resistance-associated proteins MRP/ABCC and $(NBD-TMD)_2$ in pleiotropic drug resistance proteins PDR/ABCG (Crouzet et al. 2006). The half-size ABC proteins contain one TMD domain followed by one ABC domain (TAP, ATH, PMP and ATP subfamilies) with the exception of white-brown complexes (WBC), showing an ABC domain followed by TMD (Sanchez-Fernandez et al. 2001).

Of all the ABC subfamilies, PDR transporters have recently received particular attention since they have been found in plants and fungi but not in animal or prokaryote species. Besides PDRs, only WBC proteins show reverse topology of NBD and TMD domains, so a specific relation between the two subfamilies was somewhat expected. Indeed, sequence analyses of yeast and plant PDRs suggest that PDRs arose by duplication of genes encoding for the half-sized WBC proteins (Dassa and Bouige 2001; Crouzet et al. 2006). They also revealed that the diversity of the PDR family within yeast and plants appeared after the separation of fungi and plant ancestors; therefore, the individual yeast and plant PDR homologs can fulfil different functions (Crouzet et al. 2006). Detailed functional characterization of many of the yeast PDRs revealed that the proteins confer resistance to large number of diverse toxic compounds, including fungicides, herbicides, pesticides, antibiotics and detergents (Crouzet et al. 2006). Hence, yeast PDRs are now regarded as proteins involved predominantly in cell detoxification and cell resistance (Bauer et al. 1999; Rogers et al. 2001). Until recently, it was assumed that plant PDRs are also involved in the cell response to abiotic and biotic stress. *Arabidopsis thaliana* contains 15 genes encoding members of the PDR family but only a few have been characterized in detail (Table 1). Recently, growing reports reveal that plant PDR proteins might be important not only for the efflux of toxic and harmful compounds from the cell but also for the proper trafficking of the crucial signaling molecules, including hormones that are essential for the regulation of plant growth, development and response to environmental stimuli. It has been shown that AtPDR12/AtABCG40 mediates the uptake of phytohormone ABA in guard cells and other types of plant cells, contributing to the timely closure of stomata in response to drought stress, normal seed germination and lateral root development (Kang et al. 2010). Other plant PDRs, AtPDR9/AtABCG37 and its homolog AtPDR8/AtABCG36 transport a range of synthetic auxinic compounds as well as the endogenous auxin precursor indole-3-butyric acid (IBA) out of the cells

(Strader and Bartel 2009; Růžička et al. 2010). In addition, transcriptional profiles of *PDR* genes in rice reveal that the expression of plant PDRs is significantly induced by jasmonates, salicylic acid and ABA (Moons 2008; Table 1). Also the expression of soybean homolog of *At-PDR12/AtABCG40*, *GmPDR12*, is rapidly induced by salicylic acid and methyl jasmonates (Eichhorn et al. 2006).

Altogether, the data indicate a rather novel and significant role for plant PDR proteins in the transport and homeostasis of plant growth regulators and suggest a possible connection between the PDRs and jasmonates and salicylic acid in the plant response to pathogens. The questions arise whether PDR proteins contribute to the signaling pathways induced by other phytohormones essential for the proper function of plant cells and whether PDR proteins in other plants are functionally similar to their *Arabidopsis* homologs. The availability of *PDR* sequences in other plants is still limited though, due to the lack of full genomic resources.

In this study, we have identified and analyzed the expression profile of two cucumber genes, *PDR8* and *PDR12*, in different organs at two stages of plant development and under various plant growth regulators. We also present the molecular and phylogenetic characterization of *CsPDR8*, *CsPDR12* and 14 other cucumber *PDR* homologs of *AtPDRs* that have been identified through investigation of the recently sequenced cucumber genome (Huang et al. 2009).

Materials and methods

Plant material

Cucumber plants (*Cucumis sativus*, var. Krak) were grown hydroponically on 3-fold diluted Hoagland solution, pH 6.0, as described earlier (Migocka and Papierniak 2010). The nutrient solution was filter-sterilized, permanently aerated and exchanged twice a week. For the assays of organ expression analyses, roots, hypocotyls or stems, cotyledons, leaves, flowers and fruits were collected after 1 week or 8 weeks of plants cultivation. For the assays including short-term treatment of plants with plant growth regulators, 7-day-old seedlings were transferred on the fresh nutrient solutions containing ABA, IAA, 2,4-D, kinetin, salicylic acid, GA_3, ACC or jasmonic acid and kept for 4 h. The final concentrations of each regulator are given in the figure captions. For each treatment, four root samples of 50 mg from four different plants were taken for RNA extraction and immediately frozen in liquid nitrogen before storage at $-80\,°C$.

Table 1 The properties of so far characterized plant PDR genes and/or proteins

PDR gene	Species	The responses to phytohormones, growth regulators and other stimuli	Tissue expression	References
AtPDR6	Arabidopsis thaliana	Biotic stress, hormones stress (SA and MJ)	Root	Trombik et al. (2008)
AtPDR8	Arabidopsis thaliana	Response to pathogen infection, auxin precursor-IBA Detoxification of metals (Cd and Pb)	Root	Stein et al. (2006), Kobae et al. (2006), Kim et al. (2007)
AtPDR9	Arabidopsis thaliana	Transmembrane movement of IAA, 2.4-D	Root	Ito and Gray (2006)
AtPDR11	Arabidopsis thaliana	Biotic stress and hormones stress (SA and MJ)	Root, flowers (carpel, ovary)	Trombik et al. (2008)
AtPDR12	Arabidopsis thaliana	Response to pathogen infection, hormones stress (MJ, SA, ethylene) Cd and Pb transport	Shoot root, rosette leaf, cauline leaf	Campbell et al. (2003), Lee et al. (2005), Trombik et al. (2008)
OsPDR1	Oryza sativa	Upregulated by JA	Seeds	Moons (2008)
OsPDR2	Oryza sativa	Growth regulator responses (JA, SA, ACC, IAA), redox perturbation (DTT), weak acid stress CA	Root	Moons (2008)
OsPDR3	Oryza sativa	Upregulated by ABA, JA, SA, IAA, BA, weak acid stress (CA, MA)	Root, leaves	Moons (2008)
OsPDR5	Oryza sativa	Upregulated by BA, JA	Panicles	Moons (2008)
OsPDR6	Oryza sativa	Upregulated by ABA, CA	Panicles	Moons (2008)
OsPDR7	Oryza sativa	Upregulated by JA, ABA	Seeds, pistil	Moons (2008)
OsPDR8	Oryza sativa	Upregulation by JA, BA, redox perturbation (DTT, ASA), weak acid stress (CA LA, MA)	Root, leaves	Moons (2008)
OsPDR9	Oryza sativa	Upregulation by salt stress, hypoxic stress, heavy metals (Zn, Cd) and redox perturbations (H_2O_2, DTT ASA, GSSG, GSH), hormones (IAA, cytokinin, JA)	Root, callus	Moons (2003, 2008) Crouzet et al. (2006)
OsPDR12	Oryza sativa	Upregulated by JA, BA, redox perturbations (H_2O_2, ASA)	Green leaves	Moons (2008)
OsPDR17	Oryza sativa	Upregulated by BA, JA, redox perturbations (ASA, DTT)	Root, leaves	Moons (2008)
OsPDR20	Oryza sativa	Pathogen defense, upregulated by SA, response to DTT, GSSG GSH-induced redox perturbations	Root, leaves	Moons (2008)
OsPDR23	Oryza sativa	Upregulated by CA	Panicles, pistil	Moons (2008)
NpPDR1 (NpABC1)	Nicotiana plumbaginifolia	Secretion of a secondary metabolite, biotic and abiotic stress (sclareol, slareolide, abietic acid) induced by pathogen and MJ	Root, leaf trichome, petal epidermis	Jasiński et al. (2001), Grec et al. (2003), Stukkens et al. (2005), Crouzet et al. (2006), Trombik et al. (2008)
NpPDR2	Nicotiana plumbaginifolia	Pollination process	Root, flowers	Trombik et al. (2008)
NtPDR1	Nicotiana tabacum	Pathogen reaction, regulation by SA and JA	ND	Sosabe et al. (2002), Crouzet et al. (2006)
NtPDR3	Nicotiana tabacum	Upregulation by Fe deficiency, abiotic stress, regulation by MJ, SA, NAA	ND	Ducos et al. (2005), Crouzet et al. (2006)
TaPDR1	Triticum aestivum	Conferring resistance to deoxynivalenol	ND	Shang et al. (2009)
GmPDR12	Glycine max	Regulation by SA and MJ	ND	Eichhorn et al. (2006)
SpTUR2	Spirodella polyrrhiza	Abiotic stresses: cold, salinity, upregulated by ABA	ND	Smart and Flaming (1996), Crouzet et al. (2006)

JA jasmonic acid, *SA* salicylic acid, *CA* citric acid, *ABA* abscisic acid, *MA* malic acid, *BA* 6-benzylaminopurine, *IAA* indole-3-acetic acid, *ACC* 1-aminocyclopropane-1-carboxylic acid, *DTT* dithiothreitol, *GSH* glutathione, *ASA* ascorbic acid, *LA* lactic acid, *GA* gibberellins, *MJ* methyl jasmonate, *2,4-D* (2,4-dichlorophenoxyacetic acid), *NAA* naphtalene acetic acid

Semiquantitative and quantitative RT–PCR analysis

Total RNA was isolated from 50 mg of different cucumber tissues using the TRI Reagent (Sigma-Aldrich) following the manufacturer's instruction. To remove contaminating genomic DNA, RNA samples were treated with DNAse I (Fermentas). DNase-treated RNA samples were used for the semiquantitative RT–PCR or reverse transcribed into cDNA for real-time PCR. Semiquantitative RT–PCR was performed using 200 ng of RNA and Titan One Step System (Roche) under the following conditions: 50 °C for 30 min (1 cycle), 94 °C for 2 min (1 cycle), 94 °C for 30 s, 57 °C for 30 s and 68 °C for 1 min (25 cycles), and 68 °C for 10 min (1 cycle). The reverse transcription of DNAse-treated RNA samples (2,000 ng) was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem) with random primers following the manufacturer's instructions. Quantitative RT–PCR was performed with a Lightcycler 2.0 system (Roche) using SYBR-Green (A&A Biotechnology) to detect gene expression abundance, and $EF\alpha$ gene was used as an internal control. The cDNA reaction mixture was diluted eight times, and 1 μL was used as a template in a 10 μL PCR. Amplifications were carried out in 20 μL capillaries (Roche) after pre-incubation at 95 °C for 30 s, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 57 °C for 10 s and extension at 72 °C for 15 s, with final melting at 65 °C for 15 s. A negative control without cDNA template was included in the same PCR run for each primer pair. To confirm the specificity of amplification, melting curve analysis was performed allowing to identify putative unspecific PCR products (e.g., primer dimers, reaction mix contamination) and the RT–PCR products were sequenced. Successive dilutions of the sample with the lowest Cp were used as a standard curve. Amplification efficiency was around 2. For each of the two independent RNA extractions, measurements of gene expression were obtained in triplicate. The list of primers is shown in Table 2.

Identification of full cDNAs of CsPDR8 and CsPDR12

The partial sequences of *CsPDR8* and *CsPDR12* were amplified using primers targeting conservative regions of *PDR* genes (Table 2). Primers were designed manually basing on the multiple alignment of the known *PDR* ESTs from different plants. PCRs were run using cDNA synthesized from the total RNA isolated from 1-week-old roots of cucumber seedlings and Marathon polymerase (A&A Biotechnology). The obtained PCR products were subcloned into pGEM T-Easy (Promega) and sequenced at least four times. For amplification of unknown 3′and 5′ cDNA ends of *CsPDR8* and *CsPDR12*, RACE-PCRs were performed according to the protocol provided with the GeneRacer Kit (Invitrogen) using 5 μg of total RNA isolated from 1-week-old cucumber roots. For the 3′ end, the first strand synthesis reaction was carried out at 50 °C using GeneRacer OligodT Primer (Table 2). The obtained cDNAs were used in the RACE3′-PCR containing 0.3 μM

Table 2 The list of primers used in all PCRs

Primers	Sequences (5′–3′)
Primers used in RACE-PCR	
GeneRacer Oligo dT Primer	GCTGTCAACGATACGCTACGTAACGGCATGACAGTG(T)$_{24}$
GeneRacer 3′ Primer	GCTGTCAACGATACGCTACGTAACG
GeneRacer 5′ Primer	CGACTGGAGCACGAGGACACTGA
PDR8race3	GGATGCTATAGTAGGGCTACCAGGAG
PDR12race3	GGAAATGAAATCACAAGGCGTTAC
PDR8race5	AAGGAGTTTCGCTTCATCAACAACC
PDR12race5	CGATGGTCCAACCAACATCTC
PDR8race5n	CTCTTGACGCCTTGAGCAGCAGAGGCC
PDR12race5n	GCCCACCGGAGATCCCTCGA
Primers used in RT–PCR and real-time PCR	
CsPDR8for	CTTCCCTTCTTGAATCTG
CsPDR8rev	ATTTCACCTCTAGGAATGATA
CsPDR12for	AGATTCTAGGACTTGAAATCTGTGC
CsPDR12rev	TAAGTTGTCGAACTGTCGAATCCTG
CsEFfor	ACTTTATCAAGAACATGATTAC
CsEFrev	TTCCTTCACAATTTCATCG
Primers targeting regions conservative in PDR genes	
PDRfor	GATATTGATATATTCATGAAGGC
PDRrev	CTTTCTTTGCTCTGTTGACAAACC

GSP primers (PDR8race3 or PDR12race3) and 0.9 μM GeneRacer 3′ Primer (Table 2). For the 5′ end, the RNA was ligated to GeneRacer RNA Oligo Sequence and reverse transcribed using GSP primers: PDR8race5 or PDR12race5 (Table 2). The obtained cDNAs were used in the RACE5′-PCR containing 0.3 μM GSP nested primers (PDR8race5n or PDR12race5n) and 0.9 μM GeneRacer 5′ Primer (Table 2). All PCRs contained 1 U of Marathon polymerase (A&A Biotechnology), 1× Marathon amplification buffer and dNTP solution (0.3 μM each). The RACE-PCR conditions were as follows: 94 °C for 2 min, followed by 5 cycles of 94 °C for 30 s and 72 °C for 1 min/1 kB cDNA, followed by 5 cycles of 94 °C for 30 s and 70 °C for 1 min/1 kB cDNA, followed by 25 cycles of 94 °C for 30 s, 65–68 °C for 30 s and 72 °C for 1 min/ 1 kB cDNA with final extension at 72 °C for 10 min. In all cases, the PCR products obtained were cloned into TOPO vector (Invitrogen) and sequenced (at least three times each). Full cDNAs were submitted to GenBank.

Database searching and sequence analyses

The complete genomic sequence from cucumber has recently been made available to the public in GenBank with accession code ACHR01000000 (Huang et al. 2009). NCBI database (whole-genome shotgun reads) was used for Blastn searches of cucumber sequences with homology to the previously annotated *Arabidopsis thaliana PDR* sequences. Full cDNAs as well as the protein sequences encoded by the newly identified cucumber *PDRs* were generated using FGENESH (Salamov and Solovyev 2000). All protein alignments were performed using ClustalW, and the phylogenetic tree was generated using MEGA5.0 software (Tamura et al. 2011) with bootstraps 1,000. The prediction of subcellular localization was performed using ProtComp v8.0 (softberry.com), whereas TMHMM method (Sonnhammer et al. 1998), based on a hidden Markov model (HMM) approach, was applied to predict membrane topology of two CsPDR proteins.

Accession numbers

Sequence data from this article can be found in the GenBank/ EMBL databases under the following accessions numbers: GQ374243 (*CsPDR8*) and GQ374244 (*CsPDR12*). Additional accession numbers are provided in Table 3.

Statistical analyses

The qPCR data were analyzed by the ΔΔCT method using the LightCycler® Software 4.1 (Roche). Student's *t* test and ANOVA (Excel) were used to confirm the statistical significance of difference in *CsPDRs* expression between control and hormone-treated plants.

Results

Identification of cucumber genes homologous to *Arabidopsis thaliana PDR8* and *PDR12*

The full sequences of two *CsPDRs* were cloned using a RACE-PCR approach and designated *CsPDR8* and *CsPDR12*, according to their homology with *AtPDR* genes. The entire cDNAs of *CsPDR8* and *CsPDR12* contain an uninterrupted open reading frame of 4,428 bp and 4,356 bp long, respectively; hence, the putative proteins encoded by the two cucumber genes contain 1,475 and 1,451 amino acids, respectively. Both proteins have all domains and motifs characteristic of plant pleiotropic drug resistance type ABC transporters (ABCG), for example, two hydrophilic nucleotide binding folds (NBFs) (ABC_PDR_domain1 and ABC_PDR_domain2) and two hydrophobic transmembrane domains (TMD), the first with six, the second with seven transmembrane spanning regions, according to TMHMM 2.0 Server (Fig. 1). The cytosolic loops containing NBFs precede the transmembrane domains, giving a typical reverse configuration, [NBF-TMD]$_2$, specific to all PDR proteins (Fig. 1). Within the two NBF domains, Walker A, Walker B and ABC signature motifs have been identified, while the PDR-associated domain has been recognized within the sixth span of transmembrane domain 1 (Figs. 1, 2). Both CsPDR8 and CsPDR12 exhibit a high degree of homology at the nucleic acid (62 %) and amino acid (58 %) level. Nevertheless, CsPDR8 and CsPDR12 are more similar to their close homologs of *Arabidopsis thaliana*, AtPDR8/AtABCG36 and AtPDR12/AtABCG40, with identity of 69 % at the nucleic acid level (*CsPDR8* with *AtPDR8/AtABCG36* and *CsPDR12* with *AtPDR12/AtABCG40*), and 72 % (CsPDR8 with AtPDR8/AtABCG36) and 70 % (CsPDR12 with AtPDR12/AtABCG40) at the amino acid level.

The PDR protein family of cucumber

The release of the cucumber genome sequence (Huang et al. 2009) allowed for a first inventory of all cucumber genes encoding for PDR proteins. Through the screening of the whole cucumber genome shotgun reads submitted to GenBank, we have identified 16 genes that were homologous to 15 *AtPDRs* available in the Aramemnon database (Table 3). To date, only two cDNAs of cucumber PDR genes remain cloned to their full length: *CsPDR8* and *CsPDR12*, which are described here. Research on the cucumber genome revealed that *CsPDR8* and *CsPDR12*

Table 3 Pleiotropic drug resistance (PDR) transporters genes identified in the whole cucumber genome shotgun reads database (GenBank)

| Arabidopsis gene abbreviation	Cucumis source gene[a] (gene abbreviation)	Ortholog locus	Position of predicted *CsPDR* genes	Length of gene (bp)	Length of protein (aa)	Number of predicted exons	Number of predicted introns	Identity[b] %
AtABCG29 (*AtPDR1*)	ACHR01006946 (+) (*CsPDR1*)	At3g16340	24870–33617	4,302	1,433	20	19	67
AtABCG30 (*AtPDR2*)	ACHR01001960 (+) (*CsPDR2*)	At4g15230	11014–20385	4,521	1,506	25	24	48
AtABCG31 (*AtPDR3*)	ACHR01006356/ ACHR01006357 (+) (*CsPDR3*)	At2G29940	14846–26115	3,903	1,300	23	22	72
AtABCG32 (*AtPDR4*)	ACHR01002610 (−) (*CsPDR4*)	At2g26910	70024–78384	4,230	1,409	24	23	77
AtABCG33 (*AtPDR5*)	ACHR01000871/ ACHR01000872 (+) (*CsPDR5*)	At2g37280	2921–13445	4,287	1,428	23	22	62
AtABCG34 (*AtPDR6*)	ACHR01006492 (−) (*CsPDR6*)	At2g36380	7299–15082	4,260	1,419	20	19	70
	ACHR01001600 (−) (*CsPDR16*)	At2g36380	7299–15082	4,260	1,419	20	19	66
AtABCG35 (*AtPDR7*)	ACHR01001369 (−) (*CsPDR7*)	At1g15210	19090–25244	4,131	1,376	21	20	50
AtABCG36 (*AtPDR8*)	ACHR01014054 (−) (*CsPDR8*)	At1g59870	1819–8895	4,428	1,475	22	21	72
AtABCG37 (*AtPDR9*)	ACHR01000873/ ACHR01000874 (+) (*CsPDR9*)	At3g53480	325–5088	3,795	1,264	21	20	61
AtABCG38 (*AtPDR10*)	ACHR01012132 (+) (*CsPDR10*)	At3g30842	2736–10659	4,356	1,451	24	23	59
AtABCG39 (*AtPDR11*)	ACHR01008429 (−) (*CsPDR11*)	At1g66950	46106–54045	4,332	1,443	19	18	68
AtABCG40 (*AtPDR12*)	ACHR01010243 (+) (*CsPDR12*)	At1g15520	6599–14203	4,356	1,451	23	22	70
AtABCG41 (*AtPDR13*)	ACHR01004252 (+) (*CsPDR13*)	At4g15215	907–24813	3,792	1,263	21	20	60
AtABCG42 (*AtPDR14*)	ACHR01010133 (+) (*CsPDR14*)	At4g15233	5472–15168	4,170	1,389	26	25	48
AtABCG43 (*AtPDR15*)	ACHR01000615 (−) (*CsPDR15*)	At4g15236	9751–16365	4,272	1,423	24	23	59

[a] Accession number of contigs containing *CsPDRs* and orientation of strand encoding PDR proteins (+ for direct or − for complementary)

[b] The identities of cucumber and Arabidopsis orthologs were calculated using ClustalW

sequences can be found in the contigs ACHRO10014054 and ACHRO10010243, respectively, and allowed for a more profound analysis of the DNA sequence of both genes. It was found that *CsPDR8* has 22 exons (each 54–369 bp long) and 21 introns, whereas the slightly longer *CsPDR12* has 23 exons (each 54–393 bp long) and 22 introns (Table 3, Fig. 3A). The 5′ and 3′ untranslated regions in *CsPDR8* have 249 and 302 bp, respectively, whereas *CsPDR12* 5′UTR and 3′UTR regions are 672 and 142 bp long, respectively. According to ProtComp v. 9.0

prediction, the proteins encoded by *CsPDR8* and *CsPDR12* localize to the plasma membrane (Fig. 3b). Both CsPDR8 and CsPDR12 as well as the other predicted CsPDR protein sequences were used to construct the updated phylogenetic tree of plant PDR proteins using the neighbor-joining method (Fig. 4). The arrangement of sequences in the phylogenetic tree suggests four main clusters. Cluster I includes the majority of *Arabidopsis* PDRs (PDR2, PDR5, PDR9-10, PDR13-15), four cucumber PDRs (PDR5, PDR9-10 and PDR13), 5 rice PDRs (PDR1-2, PDR14,

Fig. 1 Topology prediction of CsPDR8 and CsPDR12 according to TMHMM 2.0 Server (Sonnhammer et al. 1998)
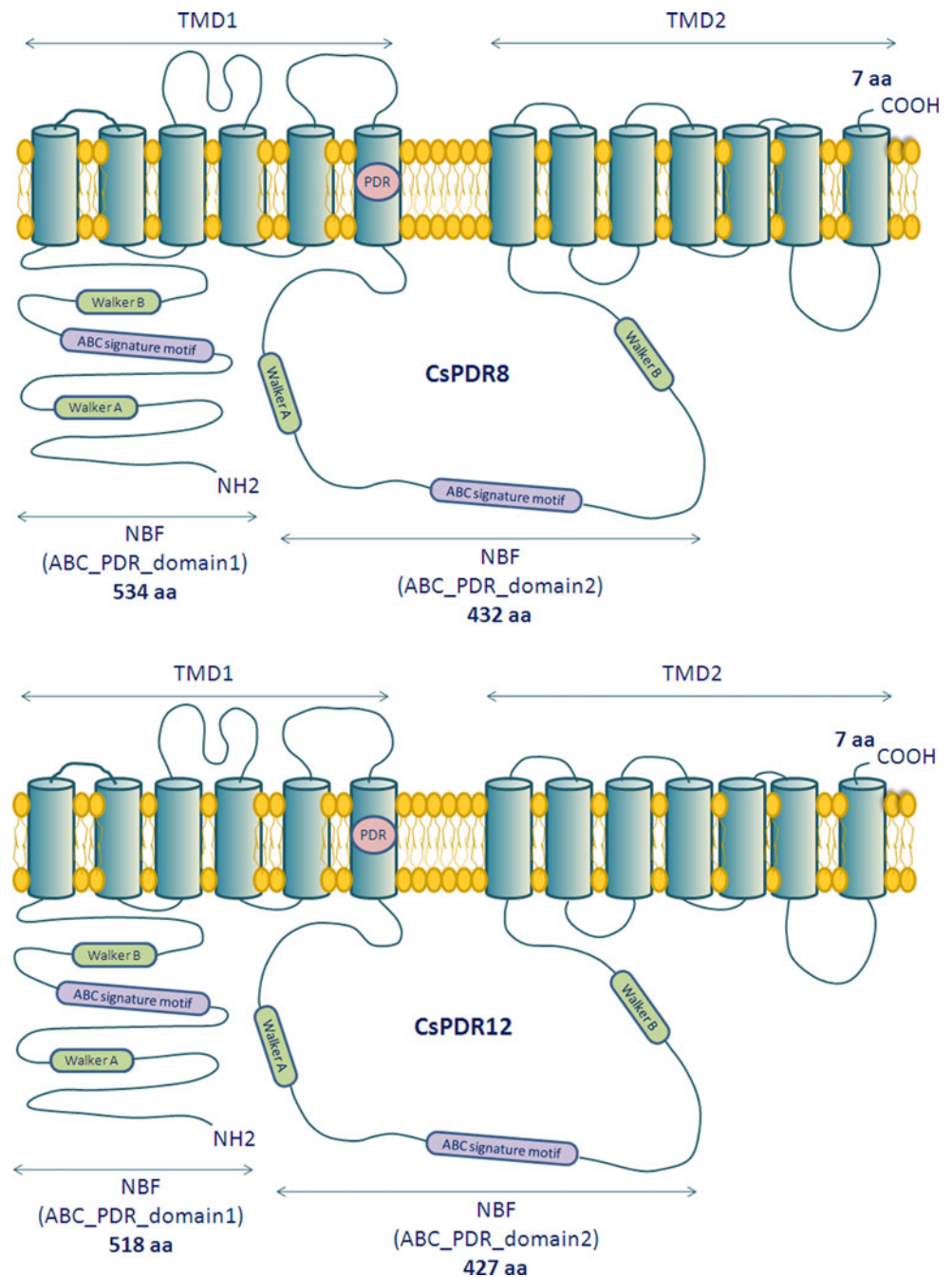
PDR22-23) and PDR3 of *Nicotiana tabacum* (Fig. 4). Another five cucumber PDRs (PDR4, PDR6, PDR11 and PDR15-16) and three AtPDRs (PDR4, PDR6 and PDR11) belong to cluster II together with three PDRs from rice (OsPDR3, OsPDR5-6), two from *Nicotiana plumbaginifolia* (Np40786 and NpPDR2), one from *Vitis vinifera* and one from *Triticum aestivum* (Fig. 4). Cluster III contains four *Arabidopsis* PDRs (PDR1, PDR3 and PDR7-8) together with 4 homologous cucumber PDRs (PDR1, PDR3 and PDR7-8), six rice PDRs (PDR9-13 and PDR15), two PDRs of *Populus* and two PDRs of *Vitis* (Fig. 4). The remaining three cucumber PDR proteins (PDR2, PDR12 and PDR14) belong to cluster IV, which contains *Arabidopsis* PDR12, the majority (8) of rice PDRs (PDR4, PDR7-8, PDR16-17 and PDR19-21), two *Populus* PDRs, three *Nicotiana* PDRs and one PDR from *Glycine max* and *Spirodela polyrhiza* (Fig. 4). Since CsPDR8 and CsPDR12 belong to two different clusters, it may be suggested that both proteins are involved in different physiological reactions of cucumber cells. Indeed, further analyses of organ expression of the genes encoding for CsPDR8 and CsPDR12, as well as their transcriptional profile under different plant growth
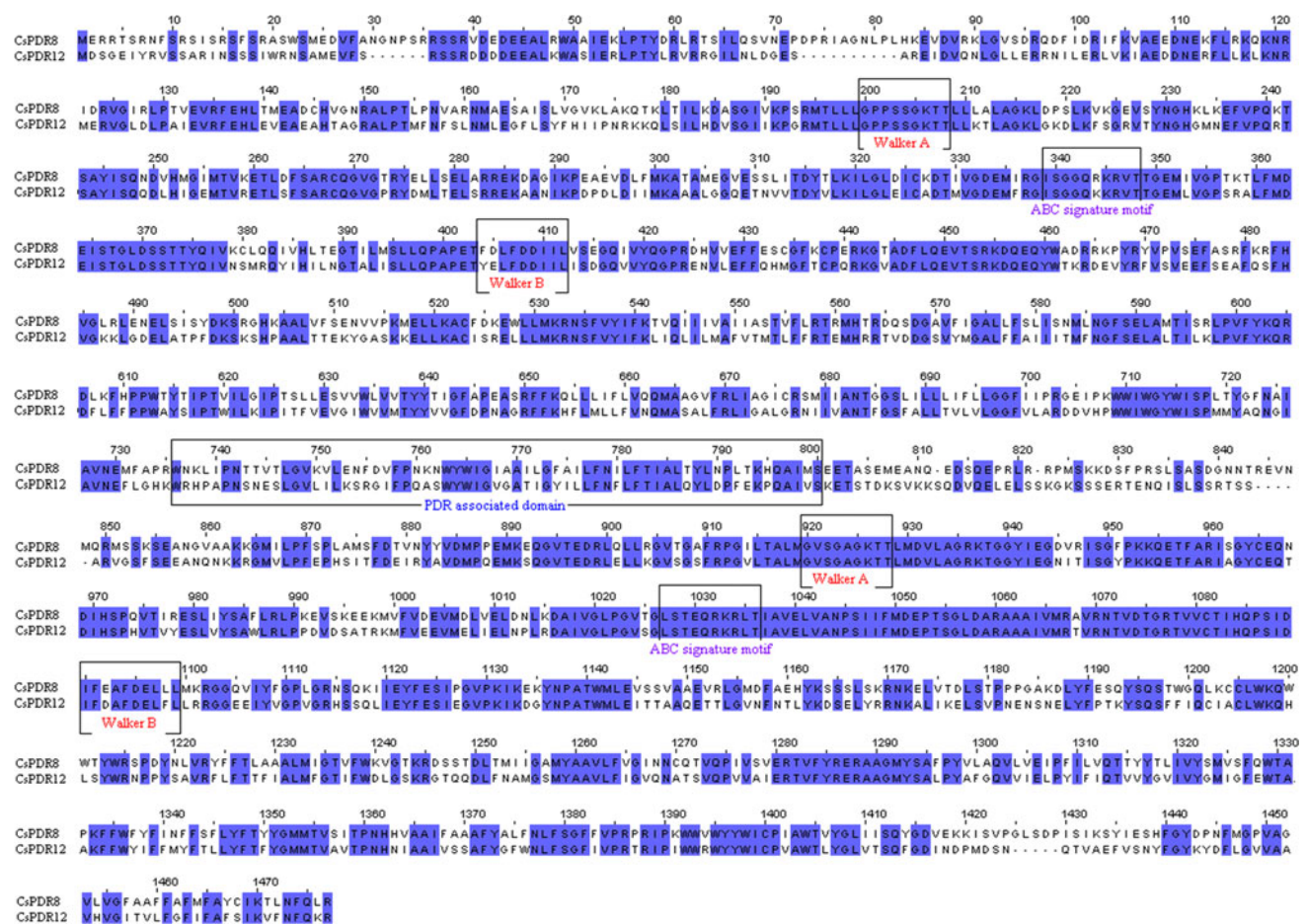
Fig. 2 The alignment of CsPDR8 and CsPDR12 protein sequences with ClustalW (Higgins et al. 1996). ABC signature, Walker A and Walker B motifs as well as PDR-associated domain are presented in boxes

regulators, revealed differences in the function/regulation of both proteins within plant cells.

Transcriptional analysis of *CsPDR8* and *CsPDR12* in various cucumber organs at two different developmental stages

To address the questions whether CsPDR8 and CsPDR12 function in the same or different tissue types or whether they are associated with a broad range of tissues or are specifically expressed in a particular tissue type, we assessed their organ expression patterns using both vegetative organs and inflorescence (Fig. 5). According to RT–PCR analysis, the highest level of *CsPDR8* expression was observed in roots of 1-week-old seedlings (Fig. 5). At this stage of development, a small amount of *CsPDR8* mRNA was also detected in cotyledons and petioles of the first leaves (Fig. 5). Similar to *CsPDR8*, the expression of *CsPDR12* in young seedlings was also enhanced in roots, and in addition, a low level of transcript was detectable in petioles but not in cotyledons (Fig. 5). In 8-week-old

plants, the level of *CsPDR8* mRNA was slightly detectable only in roots, whereas *CsPDR12* mRNA was the most abundant in roots and significantly lower but clearly detectable in male perianth, female perianth and pistils (Fig. 5). Roots of 1-week-old seedlings were chosen for further analysis of the expression of *CsPDR8* and *CsPDR12* under plant growth regulators including mainly phytohormones.

The transcriptional profiling of *CsPDR8* and *CsPDR12* in roots in response to phytohormones and plant growth regulators

Using real-time PCR, we performed transcriptional profiling of *CsPDR8* and *CsPDR12* in roots of 1-week-old cucumbers in response to plant growth regulators. The regulators included hormones involved in the plant response to biotic (SA, JA) and abiotic (ABA) stress as well as molecules crucial for plant growth and development (IAA, kinetin, GA3), phytotoxic herbicide (2,4-D) and ethylene precursor (ACC). Jasmonic acid and salicylic
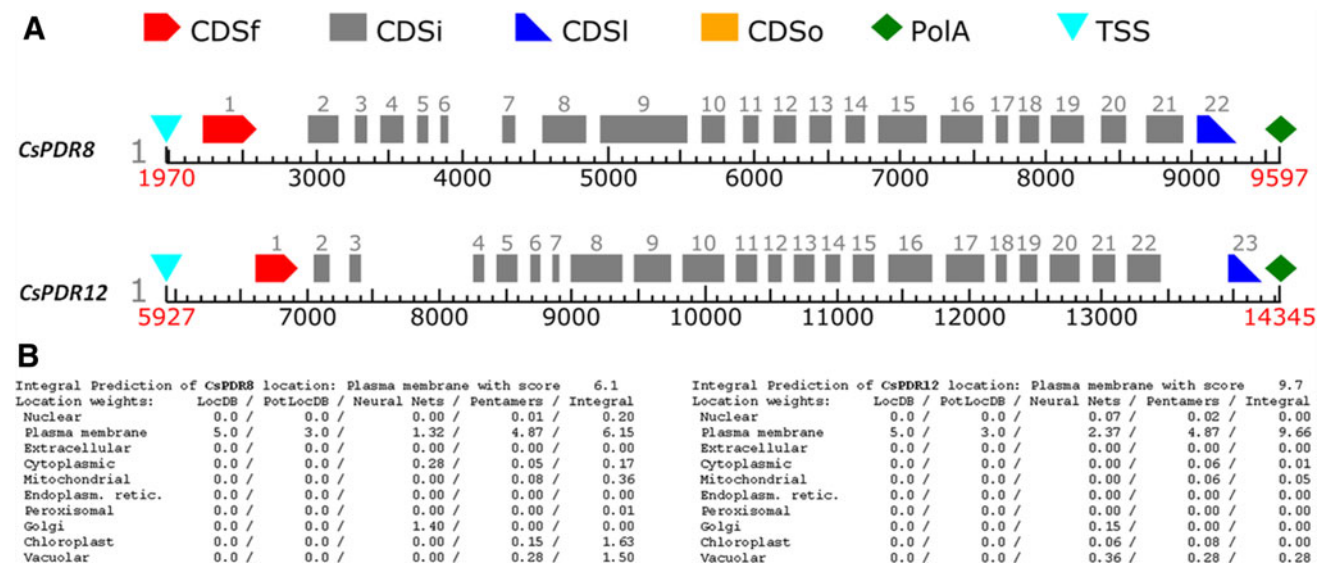
Fig. 3 The genomic organization (**a**) of *CsPDR8* and *CsPDR12* and subcellular localization (**b**) of the proteins encoded by two cucumber genes. The cDNAs were previously sequenced and the whole gene structures were predicted using FGENESH software (softberry.com). Plasma membrane localization of two CsPDRs was predicted using ProtComp v. 9.0 (software (sofberry.com). *CDSf* first coding segment (starting with start codon), *CDSi* internal exon, *CDSl* last coding segment (ending with stop codon), *TSS* position of transcription start (TATA-box position)

acid proved to be the main stimulators of *CsPDR8* and *CsPDR12* expression, respectively. 5–20 μM JA increased the level of *CsPDR8* and *CsPDR12* mRNAs up to 4-fold and over 5-fold, respectively (Fig. 6a, b). The effect of SA on both genes was clearly dependent on phytohormone concentration. *CsPDR8* mRNA level was markedly increased only at the highest (10–20 μM) SA concentrations, whereas *CsPDR12* expression increased significantly in the SA range tested (5–20 μM). Nevertheless, *CsPDR8* expression was enhanced over 2-fold by salicylic acid, whereas *CsPDR12* mRNA increased up to 14-fold in response to the hormone (Fig. 6a, b). The expression of *CsPDR8* was more enhanced by the 100–200 μM ethylene precursor ACC, which did not affect *CsPDR12* transcript (Fig. 6a, b). ACC stimulated *CsPDR8* expression over 3-fold (Fig. 6a). In contrast, the treatment of plants with ABA, another stress-related hormone, resulted in a significant decrease in *CsPDR8* mRNA (up to 50 %) causing a concentration-dependent increase (up to twofold) in *CsPDR12* transcription (Fig. 6a, b). Besides hormones associated with the plant response to stress, the synthetic auxin 2,4-D markedly (6-fold) upregulated *CsPDR12* expression when used in the highest (5 μM) concentration (Fig. 7b). However, the auxinic compound had no influence on *CsPDR8* mRNA level. Of all other hormones used, only 5–50 μM concentrations of kinetin caused a concentration-dependent, up to 4-fold, increase in *CsPDR12* transcript, but similar to 2,4-D had no effect on *CsPDR8* expression (Fig. 7a, b). Only auxin and GA3 did not

significantly influence the transcription of both cucumber *PDRs* (Fig. 7a, b).

Discussion

In this work, we present the first identification of *PDR* genes as well as predicted PDR proteins in cucumber. Two of the *PDRs* had been previously sequenced and submitted to GenBank as homologs of *AtPDR8/AtABCG36* and *AtPDR12/AtABCG40*, according to their structural similarity to *Arabidopsis PDRs*. In addition to that, cucumber genome-wide screening revealed the presence of the remaining 14 homologs of *Arabidopsis PDRs* in cucumber (Table 3). The predicted proteins of the CsPDR family were subjected to phylogenetic analysis revealing that CsPDR8 and CsPDR12 belong to two different clusters, III and IV, respectively (Fig. 4). CsPDR8 clusters with its closest *Arabidopsis* homolog, AtPDR8/AtABCG36, which has been shown to contribute to the response to pathogen infection, detoxification of heavy metals (Cd and Pb) in plant cells and transmembrane transport of herbicides (2,4-D) and IBA (Kobae et al. 2006; Kim et al. 2007; Strader and Bartel 2009). The transcription of *CsPDR8* was markedly upregulated by jasmonic acid, ethylene precursor and, to a lesser extent, by salicylic acid (Fig. 6a). In contrast, ABA significantly reduced the rate of gene expression (Fig. 6a). Since other plant growth regulators did not influence *CsPDR8* transcript, it may be suggested that the
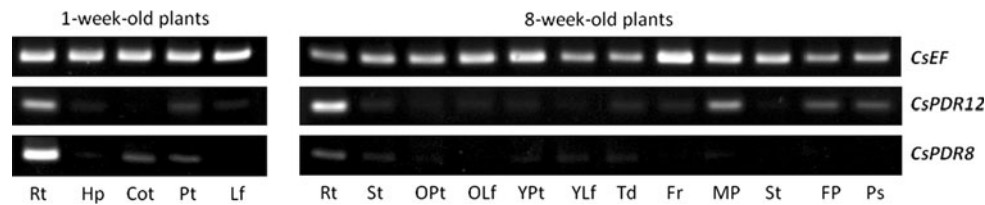
Fig. 4 Phylogenetic relationships of the 16 predicted PDR-like transporters identified in cucumber and PDR-like representatives from other plants. The unrooted tree was constructed by the MEGA5.1 software (Tamura et al. 2011) using the neighbor-joining method with 1.000 bootstrap replicate trees. The accession numbers are as follows: *Arabidopsis thaliana*, AtPDR1 (DAA00870), AtPDR2 (DAA00869), AtPDR3 (DAA00871), AtPDR4 (DAA00872), AtPDR5 (DAA00873), AtPDR6 (DAA00874), AtPDR7 (DAA00875), AtPDR8 (DAA00876), AtPDR9 (DAA00877), AtPDR10 (DAA00878), AtPDR11 (DAA00879), AtPDR12 (DAA00880), AtPDR13 (DAA00881), AtPDR14 (DAA00882), AtPDR15 (DAA00883); *Oryza sativa*, OsPDR1 (CAD59576 or Q8GU82), OsPDR2 (CAD59575 or Q8GU83), OsPDR3 (CAD59574 or Q8GU84), OsPDR4 (DAA00887), OsPDR5 (Q8GU86), OsPDR6 (Q8GU87), OsPDR7 (Q8U88 or CAD59570), OsPDR8 (Q8GU89 or CAD59569),

OsPDR9 (AAQ02685, Q8GU90 or CAD59568), OsPDR10 (Q7PC80 or DAA00884), OsPDR11 (Q8GU92 or CAD59566 or DAA00885), OsPDR12 (Q5Z9S8 or CAD59565), OsPDR13 (Q8S628 or CAD59564), OsPDR14 (AJ535214), OsPDR15 (Q7FMW4 or CAD59563), OsPDR16 (AAQ01165), OsPDR17 (Os08g29570), OsPDR19 (EAZ44308), OsPDR20 (EU682752 or EAZ44307), OsPDR21 (Os09g16290), Os-PDR22 (Os12g13720), OsPDR23 (Os12g32820); *Populus trichocarpa*, Pt83045 (EEE83045), Pt8240 (EEE8240), Pt82928 (EEE82928), Pt8123 (XP_002298123); *Nicotiana plumbaginifolia*, NpPDR1 (CAC40990), NpPDR2 (AJ831424), Np40786 (CAH40786); *Nicotiana tabacum*, NtPDR1 (BAB92011), NtPDR2 (BAD07484), NtPDR3 (CAH39853); *Triticum aestivum*, (TaFJ185035); *Glycine max*, GmPDR12 (AM261476); *Spirodela polyrhiza*, SpTUR2 (CAA94437); *Vitis vinifera*, VV9155 (XP_002279155, VV4885(XP_002284885), VV8313 (XP_002278313)

Fig. 5 Semiquantitative RT–PCR analysis of the organ expression pattern of *CsPDR8* and *CsPDR12* in 1-week-old and 8-week-old cucumber plants. Gene encoding for elongation factor (EFα) was used as an internal control. *Rt* roots, *Hp* hypocotyls, *Cot* cotyledons, *Pt* pistil, *Lf* leaf, *S* stem, *OPt* old pistil, *OLf* old leaf, *YPt* young pistil, *YLf* young leaf, *Td* tendril, *Fr* fruit, *MP* male perianth, *St* stamen, *FP* female perianth, *Ps* pistil

Fig. 6 Quantitative real-time PCR analysis of *CsPDR8* (**a**) and *CsPDR12* (**b**) expression in roots of 1-week-old cucumbers treated with ABA (2, 5, 10, 50 μM), SA (5, 10, 20 μM), ACC (100, 150, 200 μM and JA (5, 10, 20 μM) for 4 h. Control plants were treated with the equivalent amount of methanol (the solvent of JA and ABA) or water. The expression data were analyzed using reference gene encoding for elongation factor (EFα) as an internal control. *Asterisks* indicate significant differences between control and hormone-treated plants (*t* test; *$P < 0.05$)
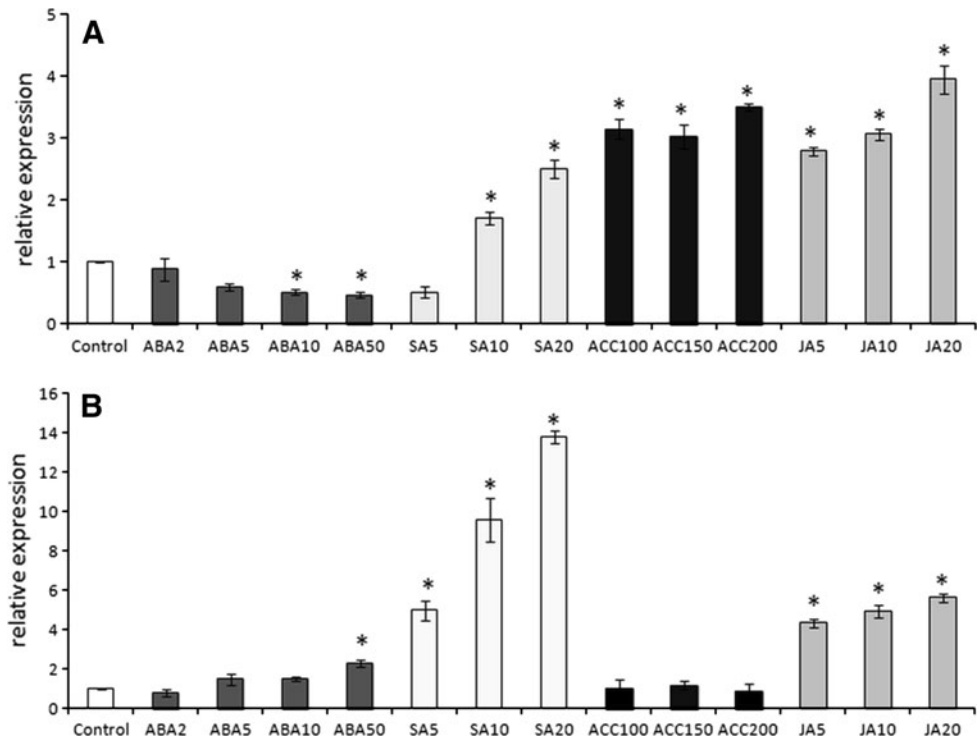

function of CsPDR8 may be specifically related to the plant responses to biotic or abiotic stress which are mediated by salicylic acid, jasmonic acid, ethylene and/or ABA (Fig. 6a). JA and SA are known to control the local apoptotic hypersensitivity response (HR) and induce systemic acquired resistance (SAR) during pathogen invasion. JA is important in response to wounding and for development processes including growth inhibition, senescence, flower development and leaf abscission (Delker 2006). SA also contributes to the responses to some abiotic stresses such as low and high temperature, UV-B irradiation, ozone and heavy metals (Janda et al. 2007). Ethylene is another plant hormone associated with senescence and response to stress. Plants produce higher levels of ethylene under biotic or abiotic stress, such as pathogen attack, salt, wounding, drought, heat, flooding, low phosphorus (Borch et al. 1999) and low iron (Romera et al. 1999). It has been previously suggested that the compounds transported by PDRs

(AtPDR6 and AtPDR11) include antifungal and antimicrobial chemicals that are required for plant protection against pathogen attack (Jasinski et al. 2001; Stukkens et al. 2005; Kobae et al. 2006; Stein et al. 2006; Badri et al. 2007). Like *AtPDR6/AtABCG34* and *AtPDR11/AtABCG39*, cucumber *PDR8* is constitutively expressed in root tissue (Fig. 5), which may suggest the possible involvement of the protein in the secretion of exudates in response to external stimuli. CsPDR8 clusters together with OsPDR9 (Fig. 4), which has been shown to be upregulated by phytohormones IAA, cytokinin and JA at the level of mRNA transcription (Moons 2003; Table 1). The different responses of rice and cucumber *PDR* to IAA and cytokinin suggest that structural homologs of PDRs in monocot and dicot plants are subjected to different modes of regulation by plant hormones.

Similar to *CsPDR8*, the expression of *CsPDR12* was also stimulated by salicylic acid and jasmonic acid.

Fig. 7 Quantitative real-time PCR analysis of *CsPDR8* (**a**) and *CsPDR12* (**b**) expression in roots of 1-week-old cucumbers treated with IAA (5, 10, 30, 50 μM), 2,4-D (0,5, 2, 5 μM), kinetin (KIN, 5, 20, 50 μM) and GA3 (GA, 5, 20, 50 μM) for 4 h. Control plants were treated with the equivalent amount of methanol (the solvent of IAA and 2,4-D) or water. The expression data were analyzed using reference gene encoding for elongation factor (CsEF) as an internal control. Data are the mean ± SD of at least three independent RNA samples. The results were analyzed by the ΔΔCT method using the LightCycler® Software 4.1 (Roche). *Asterisks* indicate significant differences between control and hormone-treated plants (*t* test; *$P < 0.05$)
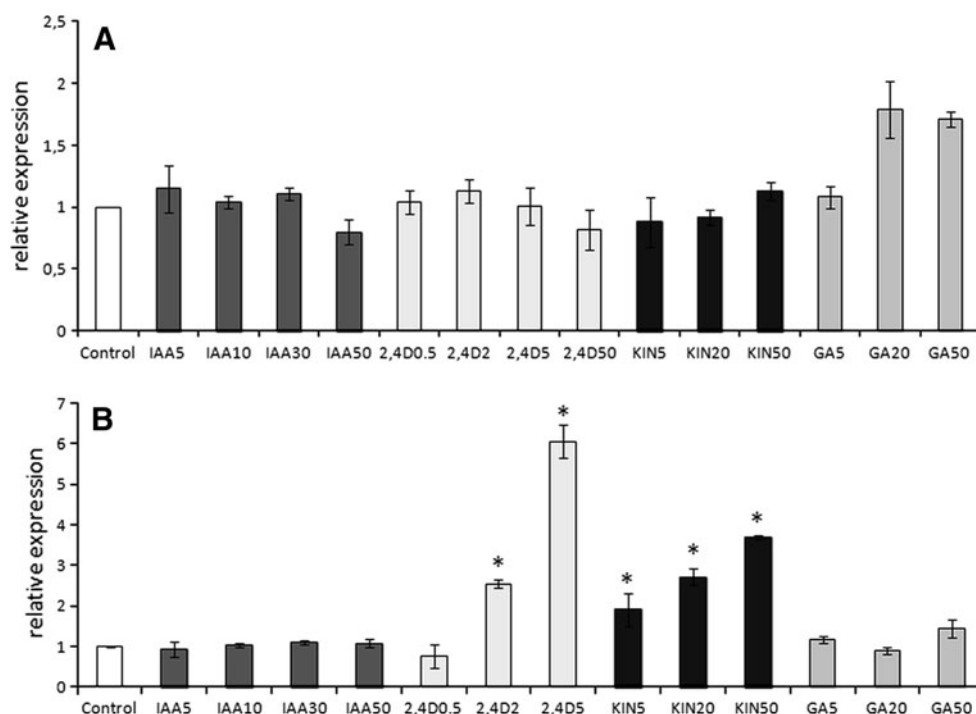
However, SA caused a considerably high increase in the gene transcription when compared with the other growth regulators (Fig. 6b). GmPDR12 and OsPDR20, other proteins that cluster together with CsPDR12 and AtPDR12/AtABCG40, were also markedly induced by both JA and SA or SA and pathogen stress, respectively (Moons 2003; Eichhorn et al. 2006), confirming that PDR12-like proteins fulfill similar functions in different plants. NpPDR1 and NtPDR1, clustering with CsPDR12, have been implicated in pathogen defense (Table 1), possibly through the efflux of antimicrobial and antifungal metabolites out of plant cells (Jasinski et al. 2001; Sasabe et al. 2002; Stukkens et al. 2005). Hence, PDR12-like proteins seem to be key players in the pathogen-induced response. Furthermore, ABA also upregulated *CsPDR12* expression. ABA is known to participate in many physiological processes in plants, including seed germination, stomata closure, seedling growth and lateral root development, resistance to drought and other biotic and abiotic stresses, including salinity and pathogen infection. It has been recently demonstrated that the CsPDR12 homolog AtPDR12/AtABCG40 mediates the closure of stomata in response to water deficit through the active ABA transport into mesophyll cells (Kang et al. 2010). In addition, *Spirodela* PDR clustering together with AtPDR12/AtABCG40 and CsPDR12 (Fig. 4) was also significantly induced by ABA (Smart and Fleming 1996). The *SpTUR2* transcript was also elevated upon salinity and low temperature (Smart and Fleming 1996), suggesting the involvement of *Spirodela* PDR in the ABA-mediated response to these abiotic

stresses. The response of *CsPDR12* to hormones mediating the stress response suggests that cucumber protein is functionally similar to its *Arabidopsis* and *Spirodela* homologs.

Although CsPDR12 seems to be involved in the ABA-mediated response to stress, contrary to CsPDR8, it may not contribute to the ethylene-mediated reaction to environmental stimuli, since ACC did not influence the level of *CsPDR12* mRNA (Fig. 6a, b). Unlike *CsPDR8*, CsPDR12 mRNA was markedly elevated by auxinic herbicide 2,4-D (Fig. 7b). This may suggest that besides ABA, the protein may be capable of transporting auxinic compounds. Indeed, it has been recently demonstrated that PDR proteins may be involved in the transmembrane movement of different auxinic compounds. The direct efflux of IBA or IBA and 2,4-D has been demonstrated using *Schizosaccharomyces pombe* cells and mammalian cells, respectively, expressing *AtPDR9* (Růžička et al. 2010).

The strong stimulation of *CsPDR12* by kinetin was in line with the frequent induction of rice *PDRs* in roots by another cytokinin, 6-benzylaminopurine, and confirmed the need for some of the PDR proteins in root meristematic tissues (Moons 2003, 2008). OsPDR8 and OsPDR17, clustering together with CsPDR12 and AtPDR12/AtABCG40 (Fig. 4), are two of the six OsPDRs significantly stimulated by cytokinin in roots (Table 1). However, all the implications regarding the function of cucumber PDRs require confirmation in further research.

Summing up, we present the first depiction of cucumber PDRs, revealing the presence of at least 16 genes encoding

PDR proteins in *Cucumis sativus*. In addition, we provide evidence for the phytohormone-mediated regulation of at least two CsPDRs, CsPDR8 and CsPDR12. The organ expression pattern of *CsPDR8* and *CsPDR12* suggests that the proteins are specific to roots and, in the case of PDR12, for flowers. The results confirm the previous data demonstrating the involvement of phytohormones (ABA, ethylene, jasmonates, salicylic acid, cytokinins) and plant growth regulators (2,4-D, auxinic compounds) in the regulation of PDR protein synthesis and/or function in plants. Nevertheless, the precise mechanism of interaction between PDRs and phytohormones or their precursors still remains to be clarified. Interestingly, the genes encoding the two cucumber proteins respond to phytohormones in a distinct way, suggesting dissimilar functions of CsPDR8 and CsPDR12 in the response of plant cells to stimuli involving hormone-mediated signaling pathways.

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