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Chymotrypsin protease inhibitor gene family in rice: Genomic organization and evidence for the presence of a bidirectional promoter shared between two chymotrypsin protease inhibitor genes

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ABSTRACT

Protease inhibitors play important roles in stress and developmental responses of plants. Rice genome contains 17 putative members in chymotrypsin protease inhibitor (ranging in size from 7.21 to 11.9 kDa) gene family with different predicted localization sites. Full-length cDNA encoding for a putative subtilisin–chymotrypsin protease inhibitor (OCPI2) was obtained from Pusa basmati 1 (*indica*) rice seedlings. 620 bp-long *OCPI2* cDNA contained 219 bp-long ORF, coding for 72 amino acid-long 7.7 kDa subtilisin–chymotrypsin protease inhibitor (CPI) cytoplasmic protein. Expression analysis by semi-quantitative RT-PCR analysis showed that *OCPI2* transcript is induced by varied stresses including salt, ABA, low temperature and mechanical injury in both root and shoot tissues of the seedlings. Transgenic rice plants produced with *OCPI2* promoter-gus reporter gene showed that this promoter directs high salt- and ABA-regulated expression of the *GUS* gene. Another *CPI* gene (*OCPI1*) upstream to *OCPI2* (with 1126 bp distance between the transcription initiation sites of the two genes; transcription in the reverse orientation) was noted in genome sequence of rice genome. A vector that had GFP and GUS reporter genes in opposite orientations driven by 1881 bp intergenic sequence between the *OCPI2* and *OCPI1* (encompassing the region between the translation initiation sites of the two genes) was constructed and shot in onion epidermal cells by particle bombardment. Expression of both GFP and GUS from the same epidermal cell showed that this sequence represents a bidirectional promoter. Examples illustrating gene pairs showing co-expression of two divergent neighboring genes sharing a bidirectional promoter have recently been extensively worked out in yeast and human systems. We provide an example of a gene pair constituted of two homologous genes showing co-expression governed by a bidirectional promoter in rice.

1. Introduction

Protein turnover machinery involves three basic components namely proteases, their substrate proteins and protease inhibitors (Lopez-Otin and Overall, 2002). Genes encoding for PIs have been cloned and characterized from a varied range of plant species (Habib and Fazili, 2007). Primarily, PIs are considered important in endogenous as well as exogenous defense against various pathogenic organisms (Habib and Fazili, 2007). Some insects and many of the phyto-pathogenic microorganisms produce enzymes causing proteolytic digestion of host proteins. Plants fight against these pathogens through PIs that act against the proteolytic enzymes. Additionally, plant PIs have been shown to be involved in various physiological and developmental responses. For instance, PIs have been considered important in the phenomenon of programmed cell death (Wang et al., 2008). Jimenez et al. (2007) recently implicated *CaTPI-1* gene (encoding a Kunitz-type PI from *Cicer arietinum*) in the elongation of seedling epicotyls and radicles and in vascular cell development. Plant PIs are important component in osmotic stress response too (Kawasaki et al., 2001; Dombrowski 2003). Gosti et al. (1995) identified Kunitz family of PIs in salt-treated radish and drought-stressed *Arabidopsis thaliana*. A Kunitz-type PI gene has been shown to be induced by drought and heat stress in *Brassica* (Satoh et al., 2001). Pernas et al. (2000) showed that cysteine-PI gene was induced by low temperature, salt and heat stress in chestnut. Further, PI genes are subjected to regulation by intercellular signaling molecules such as jasmonic acid (Farmer and Ryan, 1992), salicylic acid (Doares et al., 1995) and systemin (Constabel et al., 1998). Botella et al. (1996) noted that the transcript levels of soybean cysteine-PI gene respond to wounding and methyl jasmonate (MJ). In rice, a PI gene named *OsBBP1* is reported to respond to wounding, MJ and ethylene (Rakwal et al., 2001).

Abbreviations: ABA, Abscisic acid; CAMBIA, Centre for Application of Molecular Biology to International Agriculture; CPI, Chymotrypsin protease inhibitor; EST, Expressed sequence tag; GFP, Green fluorescent protein; ORF, Open reading frame; RT-PCR, Reverse Transcription Polymerase chain reaction.

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A large number of chymotrypsin protease inhibitor (*CPI*) genes have been isolated from different dicotyledonous plants. *CPI* genes are however, poorly analyzed in monocots (Casaretto et al., 2004; Wang et al., 2008). Full-length cDNA designated as *Oryza sativa* chymotrypsin protease inhibitor-like gene (*OCPI1*) has been reported from rice (Huang et al., 2007). Transgenic rice plants expressing GUS reporter gene driven by *OCPI1* promoter showed strong GUS expression in response to drought, salt and ABA stresses. In the course of analyzing salt stress response of rice, we identified a cDNA clone that encodes for a PI protein (Sahi et al., 2003; 2006). We note that this PI gene (designated as *OCPI2*) actually lies upstream of *OCPI1* gene in a head-to-head orientation. Using *GUS* reporter gene, we found that promoter of *OCPI2* gene is highly-active in a stress-regulated manner in rice callus and seedling tissues. Based on semi-quantitative RT-PCR results, we find that *OCPI2* and *OCPI1* genes are expressed in an almost identical manner. By constructing a vector with two different reporter genes (i.e. GUS and GFP), we observed that the intergenic region between *OCPI2* and *OCPI1* genes serves as a bidirectional promoter.

2. Materials and methods

2.1. Rice chymotrypsin protease inhibitor genes

We have earlier reported construction of three subtracted cDNA libraries employing *indica* rice cultivars namely Pusa basmati 1

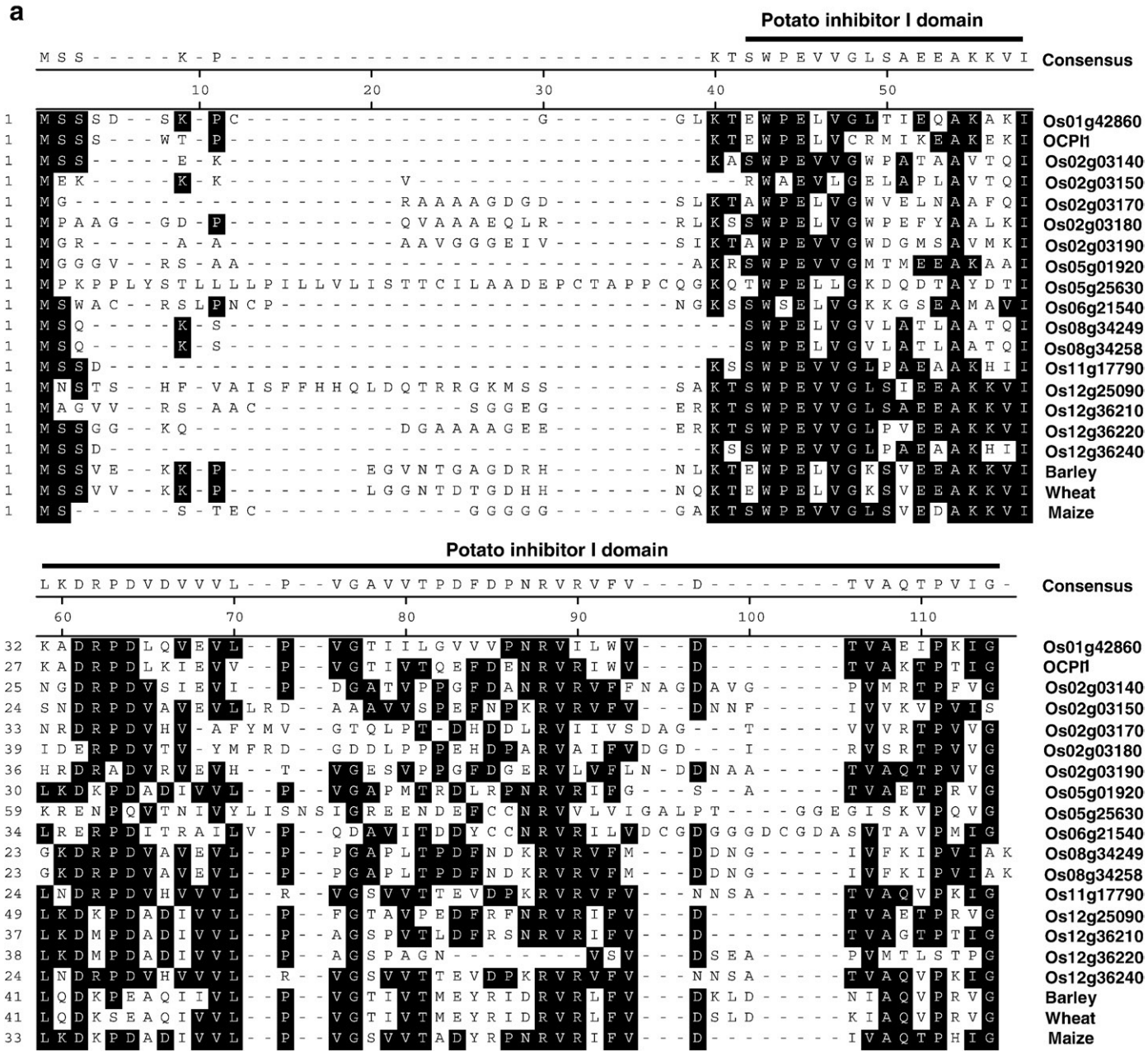

Fig. 1. (a) Alignment profile of the 17 members of the rice CPI protein family. Selected members from other plant species are also included. The bold line above the consensus marks the potato inhibitor I domain. OCPI1 [GenBank: AY878695]; Barley (*Hordeum vulgare*) [GenBank: ABW71776]; Wheat (*Triticum aestivum*) [Swiss-Prot: *P82977*]; Maize (*Zea mays*) [EMBL: *X78988*]. (b) Phylogenetic analysis of CPIs from different plant species. CPIs analyzed for this analysis were same as used for making the alignment profile shown above. The phylogenetic tree was visualized using TreeView program.

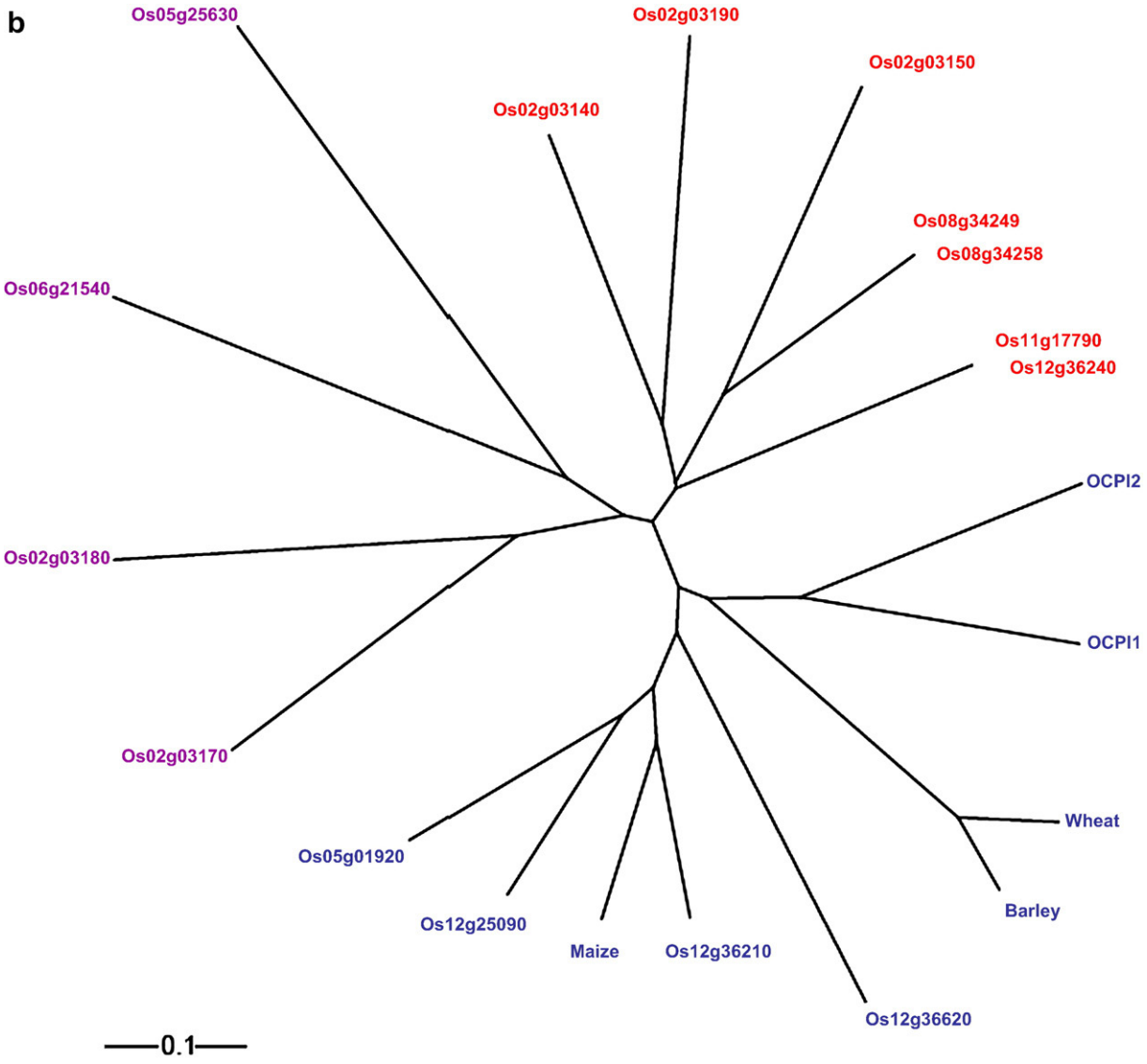

Fig. 1 (*continued*).

(PB1), CSR27 and Pokkali (Sahi et al 2003; 2006). EST clone T4D2-50 clone was isolated from the subtractive library made from mRNA isolated from salt stressed roots of CSR27 and Pokkali rice types. Nucleotide sequencing of the clone was carried out by Sanger's dideoxy method of chain termination employing Thermo-sequenase Cycle Sequencing Kit (Amersham Pharmacea, USA) as well as commercially at Microsynth (Switzerland). Deduced protein sequence of T4D2-50 was used as a query for search at the NCBI (National Centre for Biotechnology Information; www.ncbi.nlm.nih.gov/) database for PIs from other crop species and at TIGR (The Institute for Genomic Research; http://www.tigr.org/tdb/rice) database for rice proteins. The nucleotide and protein sequences were analyzed using DNA analysis software DNASTAR. For protein alignment, Clustal V of MegAlign was used in which clusters were aligned as pairs, then collectively as sequence groups to produce the overall alignment. For phylogenetic analysis, the protein sequences were aligned using ClustalX version 1.83 according to the neighbor joining method clustering strategy (Saitou and Nei, 1987) in ClustalX program and analyzed using TreeView 1.6.5 software (Page, 1996). The locations of the cDNAs were obtained from the rice genome browser (http://www.tigr.org/tigr-scripts/osa1_web/gbrowse/rice/) portal at TIGR database. Sequences were also searched on rice full-length cDNA consortium database (KOME) (Kikuchi et al., 2003; http://cdna01.dna.affrc.go.jp/cDNA/). *In silico* analyses of different proteins was determined by PSORTII (Nakai and Kanehisa, 1992) and TargetP (Emanuelsson et al., 2007) database.

2.2. Growth of rice seedlings and stress treatments

Rice [*Oryza sativa* L. var. Pusa basmati 1 (PB1)] seeds were surface-sterilized by washing first with a mild detergent, then treating with 70% ethanol (for 2 min), then washing thoroughly with water, subsequently treating with 1.2% sodium hypochlorite solution (for 30 min) and finally rinsing several times with water to remove traces of sodium hypochlorite. Seeds were sown on wet cotton, in plastic trays which were placed in dark (for 2 d) and then in light for varied periods under culture room conditions (26±2 °C).

Uniform-sized, 7- to 10-d-old PB1 seedlings were subjected to various stress treatments. Salt stress was imposed by placing the seedlings in beakers containing NaCl solution at indicated dosage. Desiccation stress was induced by 12% PEG. For mechanical injury,

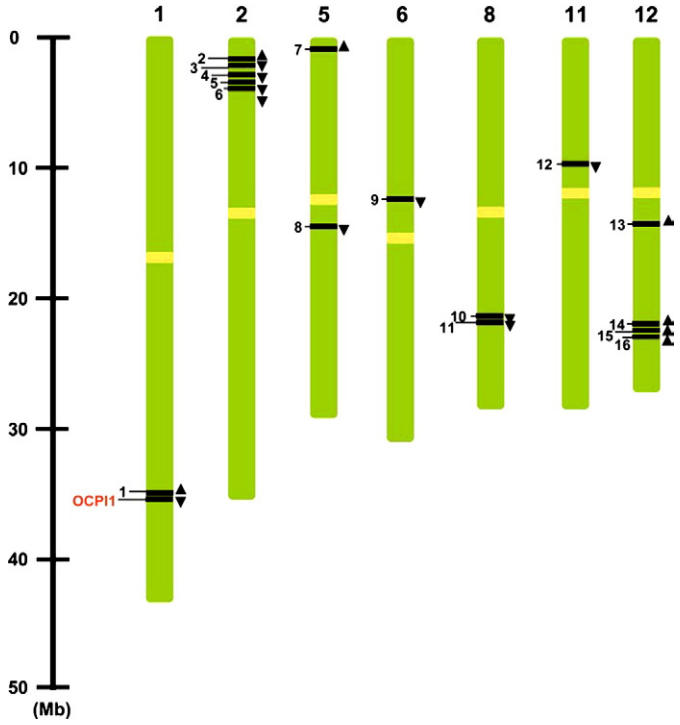

Fig. 2. Location of rice CPI family members on chromosomes based on the genome sequence as visualized at TIGR database. Arrowheads mark the direction of transcription for individual genes. Centromeres are shown by a yellow box. The lengths of the chromosomes are to scale. The chromosome number is indicated at the top of each chromosome. For the numbering 1–17 of the individual genes, refer Table 1.

shoots and roots were cut using a pair of scissors and left in a glass beaker containing water. Seedlings were placed in glass beaker and ABA was added to water. Subsequent to completion of the stress intervals, tissues were harvested, frozen in liquid nitrogen and kept at −80 °C.

2.3. Mapping of the transcription initiation site

5′-end-labelling of primer (5′gatcactcgaaatcttgcgg3′; 10 pmol) was done using 10 U of T4 polynucleotide kinase (Gibco-BRL, USA) and 5 μl of γ^{32}P-ATP (3000 Ci/mmol) according to Sambrook et al. (1989). 50 μg of rice total RNA isolated from ABA-treated (10 μM, 3 h) PB1 roots was used for this reaction.

2.4. Semi-quantitative reverse transcriptase PCR

Total RNA was isolated from the control and stressed tissues from PB1 as per the standard protocol (Chomczynski and Sacchi, 1987). For semi-quantitative reverse transcriptase PCR, complementary DNAs were synthesized from 5 μg of total RNA primed with oligo (dT) primers using M-MLV reverse transcriptase (MBI Fermentas, Lithuania). RT-PCR amplification parameters were optimized to analyze individual target genes and gene specific primers were used (*OCPI2* For 5′cacacacaacttaagaatcgatc3′ and *OCPI2* Rev 5′ttagccaatcttggaatc3′; *OCPI1* For 5′cagagaagaagcatcgatc3′ and *OCPI1* Rev 5′ggtctttgccactgtgtc3′). One of the primers in both the cases was made from the 5′ UTR. Three RT-PCR replicates were conducted using independently isolated RNAs from all the tissues as per the following conditions: 94 °C for 5 min; 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s for 25 cycles; 72 °C for 1 min; and 4 °C. β-actin was amplified as an internal control (Act Fw—5′ccctattgagcatggtattg3′; Rev—5′cagttgttgtaaaggaataa3′).

2.5. Vector construction

For constructing a bidirectional expression module, the vector pCAMBIA1381Z (CAMBIA, Australia) was used as a backbone. GFP along with the terminator was amplified from pCAMBIA1302 using primers containing the sites for EcoRI and BamHI (For 5′cgggatccatggtagatctgactagtaaagg3′ and Rev 5′ggaattccccgatctagtaacatagatgac3′) and cloned in the sites EcoRI and BamHI in pCAMBIA1381Z such that the translation initiation codon for GFP was on the opposite strand as that for GUS. The final vector was designated as pGFP-GUS.

2.6. Cloning of the 1546 bp OCPI2 promoter and the 1881 bp bidirectional promoter

1546 bp region upstream of the translational start codon of *OCPI2* was PCR-amplified using Phusion™ high-fidelity polymerase (Finnzymes, Finland) with primers containing suitable restriction sites (EcoRI in the forward primer and BamHI in the reverse primer; For 5′ gaattccaaatatgccgtgaataag3′; Rev 5′ggatcctctggtcacagcaaattaatg3′) using PB1 genomic DNA as template. PCR product containing the putative promoter was digested with the requisite restriction enzymes and ligated to pCAMBIA1391Z. Recombinant plasmid was mobilized into *Agrobacterium* strain EHA105. For amplifying the 1881 bp intergenic region (encompassing region from translation initiation site of *OCPI2* to translation initiation site of *OCPI1*), specific primers (both primers contained site for BamHI; BamHI For 5′cgggatcccttcgcaaggaaacaatcgagaag3′ and BamHI Rev 5′cgggatcctctggtcacagcaaattaatg3′) were synthesized and Phusion™ high-fidelity polymerase was used. Amplified product was digested with BamHI and cloned in the BamHI cut vector pGFP-GUS. Search for regulatory elements was performed manually as well as at PLACE (Plant *cis*-acting regulatory DNA Elements) database (Higo et al., 1999; http://www.dna.affrc.go.jp/PLACE/).

2.7. Rice transformation

Scutellum-derived embryogenic calli from mature seeds of the rice cultivar PB1 were used for *Agrobacterium*-mediated transformation as described previously (Katiyar-Agarwal et al., 2003).

Table 1
Characteristics of the rice CPI genes.

S. No.	TIGR Locus	Mol. Wt. (kDa)	p*I*	No. of amino acids	ORF (nt)	Localization	
						(PSORT)	(TargetP)
1.	Os01g42860	7.72	5.12	72	219	Cytoplasm	Cytoplasm
2.	Os02g03140	7.48	4.56	71	216	Cytoplasm	Cytoplasm
3.	Os02g03150	7.68	8.61	69	210	Cytoplasm	Cytoplasm
4.	Os02g03170	8.15	4.56	76	231	Cytoplasm	Cytoplasm
5.	Os02g03180	9.17	4.45	83	252	Chloroplast	Cytoplasm
6.	Os02g03190	8.46	4.78	81	246	Cytoplasm	Cytoplasm
7.	Os05g01920	7.50	9.98	71	216	Cytoplasm	Cytoplasm
8.	Os05g25630	11.9	4.49	110	333	Extracellular	Extracellular
9.	Os06g21540	9.11	4.68	86	261	Chloroplast	Chloroplast
10.	Os08g34249	7.25	6.49	67	204	Cytoplasm	Cytoplasm
11.	Os08g34258	7.25	6.49	67	204	Cytoplasm	Cytoplasm
12.	Os11g17790	7.21	8.68	67	204	Cytoplasm	Cytoplasm
13.	Os12g25090	9.98	8.58	89	270	Chloroplast	Chloroplast
14.	Os12g36210	8.02	5.14	77	234	Cytoplasm	Cytoplasm
15.	Os12g36220	7.37	4.17	73	222	Nucleus	Cytoplasm
16.	Os12g36240	7.21	8.68	67	204	Cytoplasm	Cytoplasm
17.	OCPI1	7.69	6.59	67	204	Extracellular	

For generating the details regarding molecular weight, isoelectric point (p*I*), number of amino acids and length of ORF, DNASTAR was used. Localization predictions were done using PSORT (*http://wolfpsort.org/*) and TargetP 1.1 (*http://www.cbs.dtu.dk/services/TargetP/*).

2.8. Particle bombardment of onion epidermal cells

For particle bombardment (BioRad, biolistic PDS-1000/He system), procedure was followed as described by Nigam et al. (2008). After bombardment, the explants were kept in dark at 25 °C for 16–18 h. GFP expression was monitored with a Nikon Fluorescence microscope (Zeiss Corp., Germany). Photographs were taken by digital camera using the UV2A filter (330–380 nm excitation, 400 nm beam splitter and 420 nm long pass). After overnight treatment with GUS staining solution, photographs were taken in bright field.

2.9. Histochemical and fluorometric determination of GUS

Histochemical and fluorometric GUS staining was carried out at 37 °C in dark as described by Jefferson et al. (1987). GUS staining solution consisted of 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.3% (v/v) Triton X-100, 0.1 M phosphate buffer (pH 7.0) and 0.3% (w/v) X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide). Ground rice tissue (50–100 mg) was homogenized in 200 µl of GUS extraction buffer. The protein content of extracts was measured using the Bradford method (Bradford, 1976). The subsequent assay was performed by incubation of 20 µg with 300 µl of 2 mM 4-methylumbelliferyl glucuronide at 37 °C for 16 h in dark, and the reaction was terminated with 0.2 M Na_2CO_3. The GUS activity assayed using DyNAQuant 200 Fluorometer (Hoefer Instruments, USA) was expressed as nmol 4-methylumbelliferyl per mg of protein.

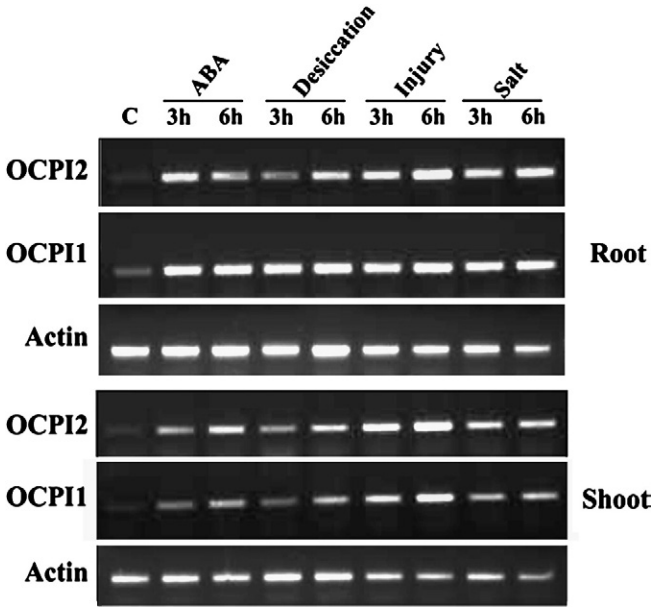

Fig. 4. Stress-regulated transcript expression of *OCPI2* and *OCPI1* genes analyzed by semi-quantitative RT-PCR reaction. 10-d-old PB1 rice seedlings were subjected to different stresses for the indicated time periods. Gene specific primers as shown in text were used for amplification. Actin was amplified using specific primers as an internal control. C—unstressed control seedlings.

3. Results

3.1. Complexity of rice CPI gene family

Genome-wide analysis of rice *CPI* gene family was carried out on the basis of complete rice genome sequence (IRGSP, 2005), its updated versions and KOME full-length cDNA database. Analysis at the various database shows that there are overall 17 CPI genes in rice (Fig. 1a). Phylogenetic analysis of putative rice CPIs and a few CPIs from other species suggests that plant CPIs are largely-diversified (Figs. 1a, b). Potato inhibitor I domain sequence noted in different subtilisin–chymotrypsin PI proteins was found to be conserved in rice CPIs too (Fig. 1a). Rice *CPI* genes are scattered onto different chromosomes but were noted to be conspicuously absent from chromosome numbers 3, 4, 7, 9 and 10. Analysis of chromosomal locations based on the genome sequence showed that several of the rice *CPI* genes are present in clusters in the genome (Fig. 2). Within a region of 17 kb on chromosome number 2, 5 *CPI* genes (i.e. Os02g03140, Os02g03150, Os02g03170, Os02g03180 and Os02g03190) and a Ty1-copia subclass retrotransposon (Os02g03160) were noted. This analysis also showed that OCPI1 (TIGR ID not known) and OCPI2 (Os01g42860) as well as Os02g03140 and Os02g03150 gene pairs were arranged in a head-to-head orientation with respect to each other. Detailed characteristics of rice CPIs are provided in Table 1. *In silico* analysis at MEROPS database (http://merops.sanger.ac.uk) showed that all rice CPIs belong to the inhibitor family I13 from the clan IG.

OCPI2 was obtained as one of the clones from subtractive libraries which were constructed earlier in the lab (Sahi et al 2003, 2006). Nucleotide sequence of *OCPI2* (EMBL: AJ601438) completely matched with a KOME clone [DDBJ: AK062495]. Deduced OCPI2 protein consisted of 72 aa (Fig. 1a) with predicted molecular mass of 7.7 kDa, showing 100% alignment to a region on chromosome number 1 of the rice genome which coincides with the locus LOC_Os01g42860.

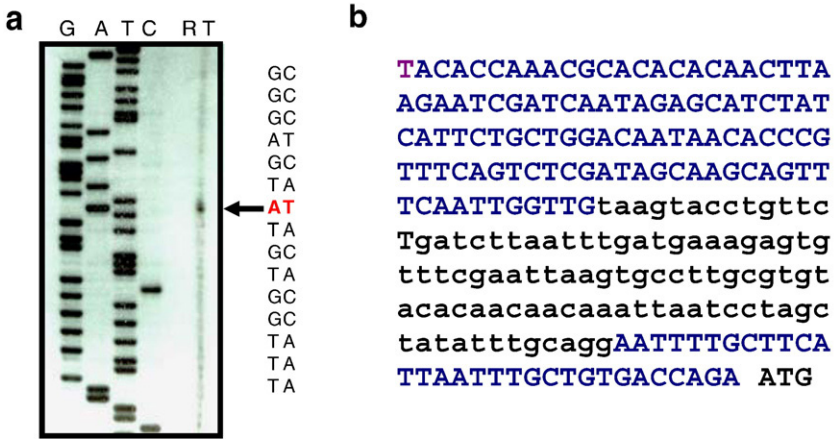

Fig. 3. (a) Mapping of transcription initiation site of *OCPI2*. End-labeled gene specific reverse primer corresponding to the 5′ UTR of *OCPI2* gene was used for primer extension. The same primer was used to sequence the promoter fragment and the different reaction mixes were loaded as G, A, T and C. The final extended product (RT) was run along with the sequencing reaction on 6% polyacrylamide gel. (b) DNA sequence corresponding to the 5′ UTR along with the intron. Upper case sequence shows the exon while the lower case sequence marks the intron. Translation initiation codon (ATG) is shown in bold.

Analysis of the conserved domains of the protein using the NCBI Conserved Domain Search (Marchler-Bauer and Bryant, 2004) revealed that *OCPI2* belongs to the potato inhibitor I family of proteins. Mapping of the transcription initiation site showed that the transcription for the *OCPI2* gene starts 138 bp upstream of the translation start site (Fig. 3a). The comparison of the cDNA sequence with the genomic sequence revealed that a 97 bp intron is present in the 5′ UTR region of this gene (Fig. 3b).

3.2. Expression analysis of OCPI2 and OCPI1 transcripts

In semi-quantitative reverse-transcription PCR analysis, transcripts of both these genes showed almost identical expression profiling. Low levels of *OCPI2* and *OCPI1* transcripts were noted in root and shoot tissues from PB1 under unstressed conditions. Imposition of ABA, desiccation, injury and salt treatments resulted in significant up-regulation of the levels of transcripts for both the genes (Fig. 4).

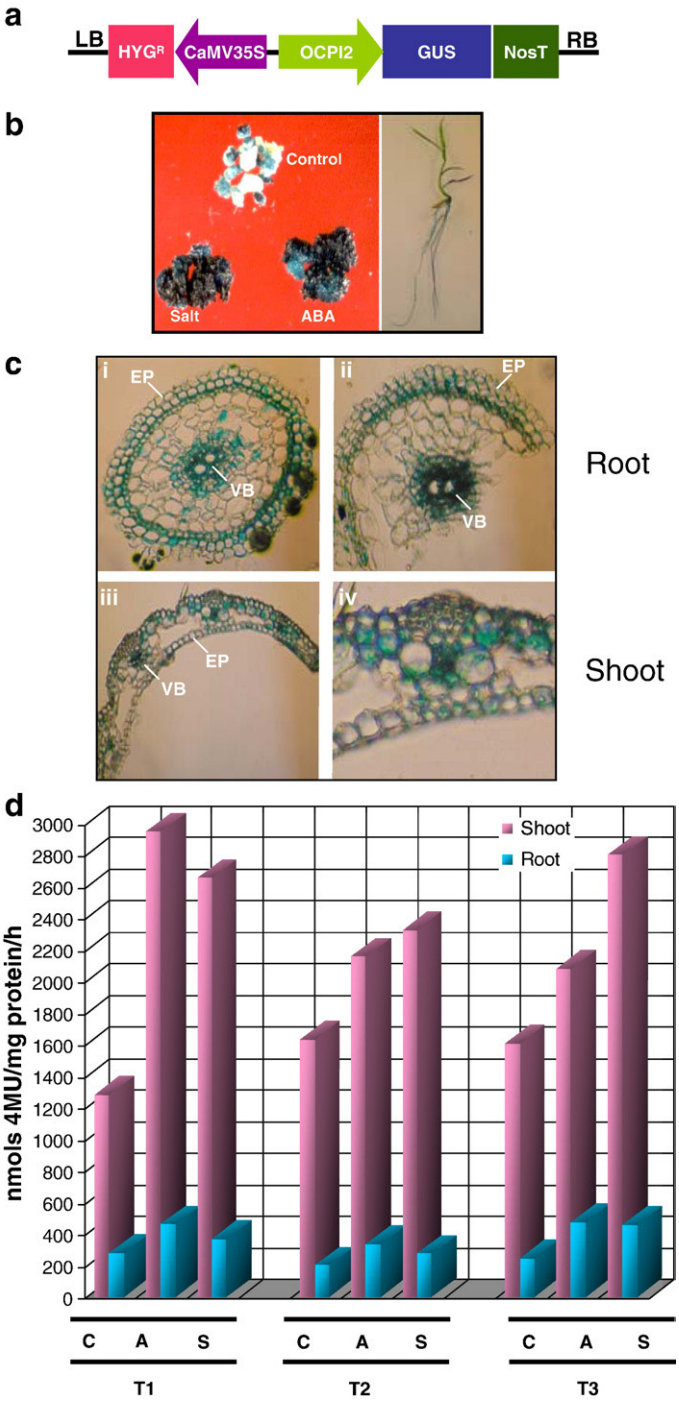

Fig. 5. (a) Diagrammatic representation of T-DNA of pCAMBIA1391Z. Linear map of the T-DNA of pCAMBIA1391Z plasmid containing 1546 bp upstream region of *OCPI2* gene, constructed for expression of *OCPI2* promoter in rice. (b) Stress-induced profiling of *OCPI2* promoter. Selected white calli were treated with ABA (10 μM, 3 h) or 200 mM NaCl for 6 h and then stained for GUS protein. Calli were photographed after 24 h. Unstressed calli (control) were also checked for basal GUS expression levels in transgenic rice calli (top). (c) Histochemical analysis of transgenic plants expressing GUS under the control of *OCPI2* promoter. Cross-section of 10-d-old seedlings: (i) root at its initiation point from the seed, (ii) root at its tip region, (iii) shoot and (iv) enlarged view of panel iii. EP: epidermis; VB: vascular bundle. (d) Fluorometric assay for GUS activity. For fluorometric assay, seedlings from three independent transgenic lines (T1, T2 and T3) were analyzed. C: control; A: 100 μM ABA for 6 h; S: 200 mM NaCl for 6 h.

Amongst different stresses tested, mechanical injury appeared to exert maximum up-regulation of both the transcripts in root as well as shoot tissues.

3.3. OCPI2 promoter analysis

1546 bp genomic sequence upstream to the translation start site of *OCPI2* was PCR-amplified using PB1 genomic DNA. Nucleotide sequence of this region was determined and it completely matched with the published rice genome sequence (IRGSP, 2005). *In silico* analysis at PLACE database revealed that this region contains several *cis*-acting elements required for optimum promoter activity (Higo et al., 1999) (Fig. 7b). As marked in Fig. 7b, *OCPI2* promoter has a putative TATA box which falls approximately at −30 position from the transcription start site 'T'. As many as eight different elements involved with ABA-regulated gene expression were marked in the promoter sequence. This included ACGT boxes which constitute the core ABREs (abscisic acid response elements). Apart from these, elements like CATGTG (MYCATERD), CACATG (MYCATRD22) and CATGCA (RY motif) were also identified in the promoter region.

OCPI2 promoter-GUS plasmid (Fig. 5a) was employed for raising stably-transformed rice transgenic plants. Transformed unstressed calli showed low level GUS expression (Fig. 5b). Following treatment with ABA (10 μM, 3 h) and salt (200 mM NaCl, 6 h), significant increase in the GUS expression was observed in transformed calli. Plantlets arising from the transformed calli were grown to maturity. Primary screening of the transgenic plants was carried out by PCR using primers specific to *GUS* gene (data not shown). 7-d-old putative T_1 seedlings were further examined for GUS expression. Histochemical staining of the T1 seedlings showed that the expression was ubiquitous (right panel in Fig. 5b). Histochemical analysis of root sections showed that OCPI2 is expressed rather strongly in the epidermal layer and vascular bundles (Fig. 5c-i). In case of shoots, the expression was primarily localized in the vascular bundles (Fig. 5c-iii). Flourimetric analysis showed that there was an increase in the levels of GUS expression in response to 100 μM ABA and 200 mM NaCl both in the root and shoot tissues in all the three transgenic lines tested (Fig. 5d).

3.4. Analysis of the 1881 bp intergenic region for the bidirectionality of the promoter activity

For the construction of a bidirectional promoter module, GFP along with the terminator region was amplified from the vector pCAMBIA1302 and cloned in the vector pCAMBIA1381Z. This vector was designated as pGFP-GUS (Fig. 6a). Further 1881 bp fragment

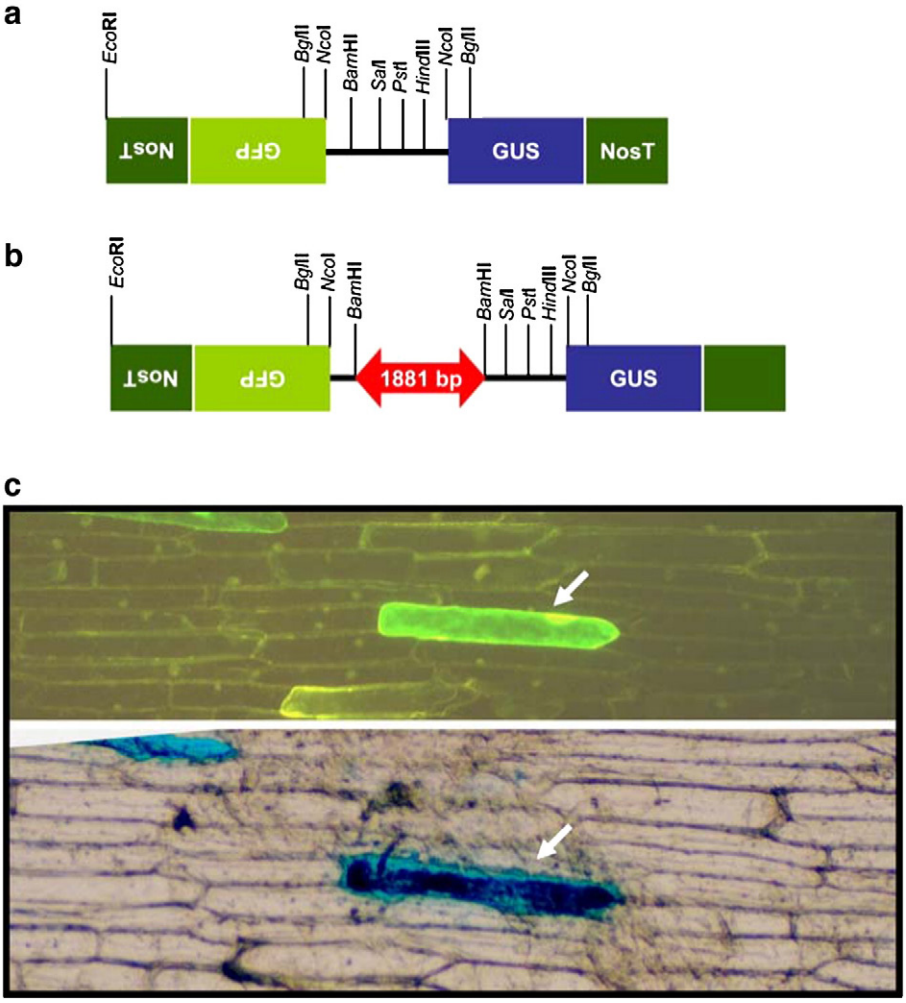

Fig. 6. (a) Linear map of the plasmid pGFP-GUS. (b) Linear diagram of pGFP-1881-GUS construct. Diagrammatic representation of the T-DNA region of pGFP-1881-GUS construct harboring the 1881 bp genomic region as described in text. (c) GFP and GUS expression. Fluorescence expression profile of onion scale epidermal cells shot with pGFP-1881-GUS construct. The signal emanating from the GFP was analyzed using fluorescence microscope. The arrows indicate fluorescence arising because of GFP (upper panel) and blue stain because of GUS reaction (lower panel) in the same onion epidermal cell. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

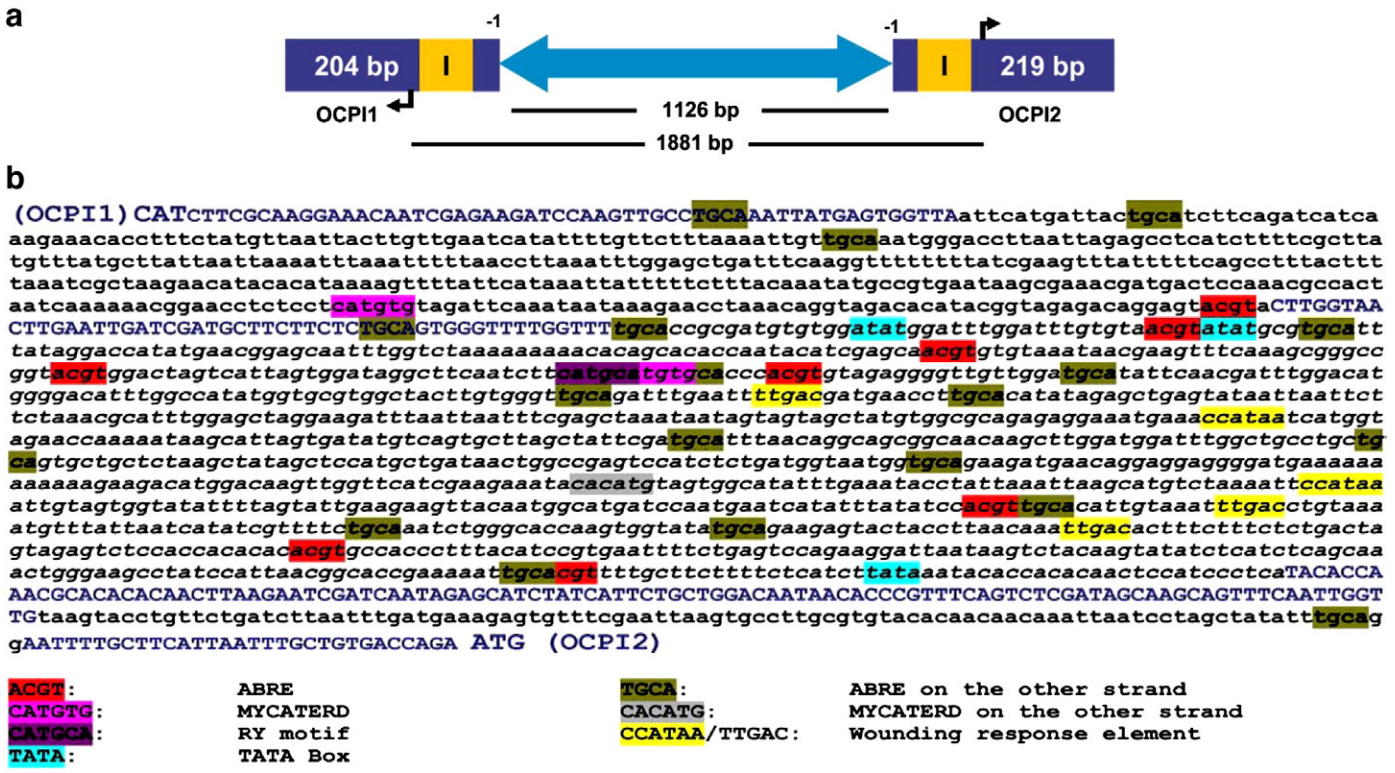

Fig. 7. (a) Gene structure of *OCPI2* and *OCPI1*. Diagrammatic representation showing physical relationship between *OCPI2* gene isolated in this study and *OCPI1* gene studied by Huang et al. (2007).−1 positions in either orientation represent the transcription initiation sites of the two genes which are separated by 1126 bp. The translation start sites of the two genes are shown by arrows and are separated by 1881 bp sequence. (b) *cis*-elements present in the bidirectional promoter. Sequence of the bidirectional promoter obtained from PAC P0410E01 is shown in italics. The putative elements are indicated in color. ABRE on+ and − strands are shown in red and deep yellow color, respectively. MYCATERD elements on the+ and − strands are depicted in pink and grey color, respectively. RY motif is shown in violet. Wounding response element is shown in yellow while putative TATA boxes are marked in turquoise. The exonic regions have been shown by upper case letters.

corresponding to the intergenic region (Fig. 7a) was cloned in BamHI site of pGFP-GUS vector (Fig. 6b; bidirectional plasmid designated as pGFP-1881-GUS). pGFP-1881-GUS plasmid DNA was shot into onion epidermal cells using particle bombardment gun. GFP fluorescence and GUS stain were seen to be emanating from the same cell in case of the pGFP-1881-GUS DNA (Fig. 6c; marked by white arrows). This indicated that the 1881 bp fragment drives transcription in both the directions in the same cell.

4. Discussion

Plant PIs are small-sized proteins which are richly-localized in storage tissues such as tubers and seeds but have also been found in the aerial parts of plants (DeLeo et al., 2001; Habib and Fazili, 2007; Huang et al., 2007). MEROPS database (Rawlings et al., 2004; 2008; http://merops.sanger.ac.uk) assigns the rice PIs into 8 families namely, I3, I4, I6, I12, I13, I25, I29 and I51. Our study showed that CPI group in rice genome (constituted of 17 genes; Fig. 1A) corresponds to I13 family. These members have variable molecular weights, p*I* points, number of amino acids, size of the ORFs and organellar localization sites (Table 1). Whereas different CPI proteins appear to be well-conserved, phylogenetic tree analysis showed that there are fine variations in their phylogeny: except Os08g34258, Os08g34249, Os11g17790 and Os12g36240 CPI genes, all other rice PI genes formed individual branches of the phylogenetic tree presented in Fig. 1B. The phylogenetic tree also suggests that the genes which are closely related to each other and present on the same chromosomes may have diverged recently. It also suggests that such genes may have generated by tandem duplication events (Long et al., 2003). Interestingly, several of the rice CPI genes are observed to be clustered (Fig. 2); while five genes are noted to be clustered on chromosome 2, three are located on chromosome 12. Two genes [namely *OCPI2* (Os01g42860) and *OCPI1* ([GenBank: AY878695] but has not been assigned any locus ID in TIGR database but assigned locus ID Os01g0615050 in RAP-DB; http://rapdb.dna.affrc.go.jp/) present on chromosome 1 showed head-to-head arrangement. Likewise, two genes present on chromosome 2 were arranged in a head-to-head arrangement. Three genes on chromosome 2, two on chromosome 8 and three on chromosome 12 were noted to be arranged tandemly. It is notable that, as many as seven genes representing the Bowman–Birk inhibitors protein family, are clustered on chromosome 1 (four of which are present tandemly; data not shown).

Full-length *OCPI2* cDNA (Os01g42860) obtained in this study showed 219 nucleotides- and 72 aa-long ORF. Primer extension experiment showed that the 5′ UTR of *OCPI2* gene is 138 bases-long (Fig. 3a). Comparison of *OCPI2* cDNA sequence with the rice genomic sequence revealed that there is a 97 bp-long intron in the 5′ UTR (Figs. 3b, 7b). Similarly, comparison of the genomic and cDNA sequences of *OCPI1* suggests presence of an intron in its 5′ UTR region (Fig. 7a). The presence of an intron in the 5′ UTR has been shown to enhance the rate of translation of the downstream genes in several cases (Rose, 2002; Sivamani and Qu, 2006). It will be interesting to see whether the same holds true for *OCPI2* and *OCPI1* introns by carrying out deletion experiments in subsequent studies.

Analysis of transcript profiling is an important tool for deciphering gene functions. Out of the 17 *CPI* genes, ESTs or full-length cDNAs are available only for six of these members (i.e. Os01g42860, Os12g25090, Os12g36210, Os12g36220, Os12g36240 and OCPI1) in public domain database (see Supplementary data 1). There are also indications that

all *CPI* members present on chromosome 2 may not be expressed. RepeatMasker program (http://www.repeatmasker.org/) predicts the presence of several transposons in this region (see Supplementary data 2). This region has a retrotransposon (Os02g03160); however, possible role, if any, of the presence of this retrotransposon element in the silencing of 17.046 kb region with coordinates from 1249053 to 1266099 remains to be addressed to. Semi-quantitative RT-PCR showed that both *OCPI2* and *OCPI1* were maximally induced in response to mechanical injury. Both *OCPI2* and *OCPI1* were also up-regulated in response to salt stress in the root as well in the shoot tissues (Fig. 4). Various wound-induced genes have been noted to be regulated by salt stress in previous studies (Kawasaki et al., 2001; Dombrowski, 2003). Capiati et al. (2006) showed that wounding increased salt-tolerance in tomato plants and conversely that salt stress lead to an enhanced expression of wound-related signaling genes like prosystemin, allene oxide synthase, protease inhibitors etc. In tomato, salt stress activation of PI genes is suggested to work *via* the octadecanoid pathway (Dombrowski, 2003). ABA regulates several aspects of plant development including seed development and desiccation tolerance of seeds, and plays a crucial role in the plant response to abiotic stresses such as salinity, drought and cold (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2002). ABA-insensitive mutants (*abi 1-1*) have also been noted to be defective in wounding-regulated induction of PI genes in tomato. Transcripts of both the *OCPI2* and *OCPI1* genes were significantly up-regulated both in root and shoot tissues in response to ABA treatment (Fig. 4). The induction of both *OCPI2* and *OCPI1* transcripts was further noted in response to desiccation stress. It is thus established that *OCPI2* and *OCPI1* transcripts are induced by wounding, salt, ABA and desiccation in rice seedlings. It remains to be worked out how signaling for induction of these genes is regulated by diverse agents? The likely involvement of reactive oxygen species could be one of the factors (Huh et al., 2002; Xiong and Zhu, 2002). The loss of turgor could be another common factor in elicitation of *OCPI2* and *OCPI1* genes in biotic and abiotic stresses (Reymond et al., 2000). Further, PIs have been implicated in senescence and stress-induced programmed cell death (PCD) in plants (Andersson et al., 2004; Wang et al., in press). Considering that PCD may be a common denominator against different biotic and abiotic stresses, it is possible that the induction of the *OCPI2* and *OCPI1* genes is a stress response involved in PCD induction.

In order to draw parallels between the transcript profiling and the promoter activity, detailed analysis of the *OCPI2* promoter was carried out. 1546 bp *OCPI2* upstream region was cloned driving *GUS* reporter gene in pCAMBIA1391Z and stably-transformed rice progenies were raised (Fig. 5). GUS expression was assayed at T_0 generation in callus tissues and at T_1 generation in intact seedlings. *OCPI2* promoter showed low level expression of GUS protein in unstressed calli. Application of salt stress as well as ABA to calli showed relatively high GUS expression. A constitutively expressing GUS both in shoot and root tissues was noted in T_1 seedlings (Fig. 5). Histochemical analysis showed that the expression was predominantly in the vascular bundles and epidermis in the roots while in the shoots the expression was seen predominantly in the vascular bundles. Fluorometric assay for the GUS activity showed that there was a constitutive expression in both roots and shoots with a clear increase in activity in response to both ABA and NaCl treatments. This corresponds well with the transcript data shown earlier, suggesting that the promoter indeed harbors functional ABA and salt responsive elements. The nucleotide sequence of *OCPI2* promoter showed several *cis*-acting sequences which are implicated in different stress responses. ABA responsive elements (ABREs) function as *cis*-acting element in ABA-dependent regulatory system. ABREs have been shown to be recognized by basic leucine zipper-type proteins known as AREBs/ABFs which have been identified as transcriptional activators in ABA-dependent regulation system (Choi et al., 2000; Uno et al., 2000). It is possible that salt- and dessication-responsive expression of *OCPI2* gene may be regulated by ABA. DREs have been identified as *cis*-acting elements involved in the ABA-independent regulatory systems. DREs have also been shown to function in cold and high salt responsive gene expression. The presence of DRE/C-repeat motif may also have some role to play in the low temperature and salt/dessication induced regulation of *OCPI2*. Induction of *OCPI2* gene by ABA and the *in silico* finding that this promoter has several ABREs and wound response elements supports the possibility that *OCPI2* promoter is a multiple stress-induced promoter. As the expression of *OCPI2* was noted to be predominant in the vascular bundles both in root and shoot tissues and in the epidermis of root, it is likely that stress and developmental cues together are involved in regulation of this promoter. There is a dire need of stress-induced promoters in plant biotechnology research (Grover et al., 1999; Ito et al., 2006). High-level stress-induced expression of *OCPI2* promoter can thus serve as a useful resource in this respect.

Computational studies have shown that more than 10% of human genes are organized in head-to-head manner, suggesting that bidirectional gene organization is a common architectural feature of the human genome (Trinklein et al., 2004). Several large scale analyses of expression data in higher eukaryotes have shown that neighboring genes indeed have similar expression patterns. Zhan et al. (2006) reported that a large number of *Arabidopsis* genes were within large, co-expressed chromosomal regions. Several studies show that natural selection acts to preserve linked pairs of co-expressed genes (Hurst et al., 2002; Singer et al., 2005). Regional similarity in expression has been documented in humans (Lercher et al., 2003), *Drosophila* (Herr and Harris, 2004), yeast (Cohen et al., 2000), *Caenorhabditis elegans* (Lercher et al., 2002) and *Arabidopsis* (Williams and Bowles, 2004). Williams and Bowles (2004) have shown that neighboring genes in *Arabidopsis* are indeed co-expressed. Studies on yeast have shown that divergently transcribed genes have a higher degree of co-expression than genes in convergent orientation (Kruglyak and Tang, 2000). Recent studies show that gene order in eukaryotic genomes is not completely random, but that genes with comparable and/or co-ordinated expression tend to be clustered together (Bellizzi et al., 2007). It has been suggested that the underlying cause for these observations may be due to sharing of common regulatory elements. Importantly, we note in this study that the *OCPI2* and *OCPI1* genes are organized divergently in a head-to-head manner on chromosome 1 (Fig. 7). These two genes also share the fact that their transcripts are coordinately-regulated in terms of constitutive as well as induced expression profiling. We next thought that as the intergenic region between the two genes is rather small (as compared to the whole genome), these genes may be regulated by a bidirectional promoter. We constructed a plasmid which contains two reporter genes namely, GUS and GFP, using pCAMBIA1381Z as a backbone. Using this plasmid system, we did transient transformation of onion epidermal cells *via* particle gun and noted that both GUS and GFP are co-localized (co-expressed) in the same cell.

Bidirectional promoters offer certain structural and functional advantages including providing compact DNA sequence organization; enhancing communication and interplay between enhancer and promoter sequences and transcription factors; and increasing efficacy of transcriptional regulation and gene expression. Although several bidirectional promoters have been found in mammalian genomes (Adachi and Lieber, 2002), few examples have been found in plants (Keddie et al., 1994; Shin et al., 2003). Evidence from our analysis suggests that the intergenic region between *OCPI2* and *OCPI1* functions as a bidirectional promoter, thus providing example of one of the few bidirectional promoters in the rice genome [besides the one shared by a low molecular heat shock protein gene pair; Guan et al. (2005)], which can be of significant use in biotechnological applications such as for gene stacking experiments. In this

study, we thus present evidence for a pair of *CPI* genes which are co-expressed and share promoter. There are other *CPI* genes showing similar head-to-head orientations (for instance, Os02g03140 and Os02g03150 may be another pair of *CPI* genes sharing a bidirectional promoter; these two genes share an intergenic region which is of 747 base pairs in between their translation start codons). In fact, availability of complete genome sequence provides an opportunity to search for such instances across the genome and gene families for unraveling more examples of DNA sequences which can drive transcription in both the directions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2008.09.028.

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