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Structure and Temperature Regulated Expression of a Cysteine Proteinase Gene in *Pachysandra terminalis* Sieb. & Zucc.

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Abstract. **A cysteine proteinase gene (DQ403257) with an open reading frame of 1125 base pairs was isolated from *Pachysdandra terminalis*. The primary translated peptide has a predicted length of 374 amino acids, pI (isoelectric point) of 5.70, and molecular mass of 40.9 kDa. The Peptidase_C1 domain is between residue 141 and 367. The proteinase has a conserved motif Gly-Xaa-Thy-Xaa-Phe-Xaa-Asn in the pro region. Sequence comparison shows that the deduced peptide shares 82% identity with the cysteine proteinase RD19a precursor (RD19) (accession P43296) from *Arabidopsis thaliana* (L.) Heynh. Real-time quantitative reverse-transcriptase–polymerase chain reaction revealed that the gene is induced by treatments of 1 to 7 days of darkness, 2 hours and 3 to 7 days at 5 °C, and 3 days at 38 °C.**

Proteinases are the catalyst in the hydrolysis reaction of peptide linkages in oligopeptides or polypeptides (van der Hoorn et al., 2004). The enzymes function to maintain strict protein quality control by degrading specific sets of proteins in response to diverse environmental and developmental stimuli. Proteinases are grouped into four major classes according to their catalytically important residues: serine proteinases (e.g., trysin), cysteine protease (papain), aspartic acid proteinase (pepsin), and metalloproteinases (thermolysin and carboxypeptidase A).

Cysteine proteinases are a class of enzymes requiring the thiol group of a cysteine residue for their catalytic activity. Many cysteine proteinases from animals, plants, and microbial sources have been characterized (Rawlings and Barrett, 1994). Cysteine proteinases have emerged as key enzymes in the regulation of programmed cell death in animals and plants (Solomon et al., 1999). They are induced by environmental stresses such as drought (Guerrero et al., 1990; Harrak et al., 2001; Koizumi et al., 1993; Williams et al., 1994), salt (Koizumi et al., 1993), and temperature (Schaffer and Fischer, 1990). These enzymes are involved in developmental processes of senescence (Drake et al., 1996; Griffiths et al., 1997; Jones et al., 2005; Ueda et al., 2000), seed maturation and germination (Becker et al., 1994; Domoto et al., 1995; Jones et al., 1996; Okamoto et al., 2001; Shintani et al., 1995; Tsuji et al., 2004). Proteinases can affect activity of other enzymes by breaking some of the peptide bonds and cause changes in the quaternary or tertiary structures of targeted proteins. Peroxidase and rubisco-1, 5-bisphosphate carboxylase–oxygenase is found to be activated by proteinases (Chollet and Anderson, 1977; Grzywnowicz et al., 1992, 1993). Cystenine proteinase is involved in disease resistance by activating different disease resistance proteins (Avrova et al., 1999).

We have cloned a full-length cystenine proteinase gene from *P. terminalis* while analyzing its mechanism for cold tolerance. This article reports the structural characterization of the encoded proteinase protein. Recent evidence demonstrates that activation of gene expression constitutes one response to temperature stresses (Provart et al., 2003). Real-time quantitative reverse transcriptase (qRT-PCR) has been used to quantify the level of transcripts present in plant organs (Charrier et al., 2002; Lammers et al., 2001), thus predicting the transcription regulation of the genes. This technique was used to evaluate the regulation of low and high temperature stresses on the cloned gene.

Materials and Methods

cDNA library construction and screening. Total RNA extraction, cDNA library construction, and screening followed the same procedure as previously described (Zhou et al., 2006). Briefly, a cDNA library was constructed with mRNA extracted from cold-treated leaf tissues and packed into λgt11 phagemid using the ZAP-cDNA Synthesis and ZAP- cDNA Gigapack III Gold Cloning Kit (Stratagene, La Jolla, Calif.). The leaf cDNA library was screened by plaque hybridization using the 230-base pair (bp) insert fragment of a PCR clone previously isolated (Zhou et al., 2005). The probes were labeled with ^{32}P-dATP through random priming methods described in the Hot-Prime Kit (Genhunter, Nashville, Tenn.). Through second plague hybridization, the positive clones were isolated and phage DNA was prepared from them. The cDNA was subcloned into pBlueScript SK-phagemid vector.

Temperature treatments and real-time quantitative reverse transcriptase polymerase chain reaction assay. Seedlings with two mature leaves (dark green) and two fresh leaves (light green and fully expanded) were supplied by Yoder Brothers (Barberton, Ohio). On arrival, these seedlings were incubated at 25 °C and weak light (indoor of the laboratory) for 3 d to recover from stresses induced during transportation

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process. Leaf tissues collected at this stage were considered controls. For temperature treatments, these plants were incubated at 25, 5, and 38 °C in total darkness for 2 h, 4 h, 9 h, 23 h, 2 d, 3 d, 6 d, and 7 d. At the end of each treatment, fresh leaves were harvested from a pool of three plants. Isolation of total RNA and removal of genomic DNA followed the same procedures as described by Zhou et al. (2006).

The oligonucleotide primers specific for the proteinase gene were 5′-GTTCAAAGCGGTTGTTGGAT-3′-forward/5′-TCCCTTTTCTCCCCAACTCT–3′-reverse (amplicon size 110 bp). The housekeeping gene 18S ribosomal RNA (18S rRNA) was selected to normalize the result of q-PCR reactions. Its primers were designed using the GeneBank sequence (accession NC-003071). The forward/reverse primers were 5′-GGGGGCATTCGTATTTCATA-3′/5′-AACATCCT TGGCAAATGCTT –3′ (amplicon size 100 bp). The real-time PCR reaction mixtures contained cDNA (100 pg total RNA), primer mix (250 μM each), and 2× PCR master mix supplied in the SYBR-green PCR mix/RT kit (Applied Biosystems, Foster City, Calif.). The PCR amplification was preformed using a program of 40 cycles of 94 °C, 30 s, and 60 °C, 1 min on a 7000 Real Time PCR System (Applied Biosystems). At the end of the 40 cycles, the data were analyzed with the ABI Prism 7000 SDS software (Applied Biosystems) and reported as cycle threshold (C_T) value. Primer specificity was validated by the dissociation kinetics and separation of PCR products on agarose gels.

In one experiment, three C_T values, corresponding to the absolute transcript levels, were produced for each sample. The experiments were repeated three times independently, and the data were averaged. To minimize errors introduced during handling process, each real-time PCR reaction was repeated two times to produce two C_T values. The results were averaged and considered as the C_T value for that PCR reaction. Data analysis was through the relative quantification using the comparative C_T method (ABI Prism 7700 Sequence Detection System, User Bulletin #2; Applied Biosystems). Values of ΔC_T were calculated to represent the accumulation of gene transcripts. Fold changes were used to compare difference between treatment and control.

Primer design and synthesis, sequence analysis, and database search. DNA sequence analysis was performed on a 3100 Avant Genetic Analyzer using a BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems). All the PCR primers were designed using OligoPerfect Designer (Invitrogen, 2006). The primers and DNA sequence primers were purchased from Invitrogen (Carlsbad, Calif.). Protein translation was performed using ExPaSy translation tool (Swiss Institute of Bioinformatics, 2006). The conserved domains were predicted using MotifScan (Falquet et al., 2002). Signal peptide was predicted with the eukaryotic linear motif resources for functional sites in proteins (Puntervoll et al., 2003).

Results

Cloning and characterization of the putative cysteine proteinase in *P. terminalis*. A 230-bp cDNA clone has been previously isolated from leaf tissues of *P. terminals* that shared high identity with C-terminal sequence of cysteine proteinases (Zhou et al., 2005). Screening of $\approx 2.0 \times 10^5$ independent recombinant clones from a λgt11 cDNA library with this fragment identified two positive clones. Comparison of the nucleotide sequences of the cDNAs revealed that these two positive clones were identical. Both have a length of 1350 bp and an open reading frame of 1125 bp (NCBI accession DQ403257).

To characterize the primary structure of the gene, the DNA sequence was translated into protein using the ExPaSy translation tool. The deduced polypeptide has a predicted length of 374 amino acids, molecular mass of 40.9 kDa, and a pI of 5.70. The polypeptide showed an identity of 82% to the cysteine proteinase RD19a precursor (RD19) in *A. thaliana* (P43296), 80% to the cysteine proteinase 1 precursor in *Zea mays* L. (Q10716D), and 75% to the cysteine proteinase 15A precursor in *Pisum sativum* L. (P25804, Turgor-responsive protein 15A). Further structural analysis revealed that the primary translation product of the cloned gene (DQ403257) has three regions: a signal sequence, a pro region, and mature proteinase domain (Fig. 1), similar to most of the cysteine proteinases (Kassell and Kay, 1973; Neurath, 1984; van der Hoorn et al., 2004).

Identification of the conserved motif in the pro region of cysteine proteinase from different plant species. The signal sequence and pro regions are variable among cysteine proteinases. Vernet et al. (1995b) aligned 56 cysteine proteinases and identified a conserved amino acid motif of a stretch of seven residues located between -42 and -36 (papain numbering) in the pro region. This sequence motif is Gly-Xaa-Asn-Xaa-Phe-Xaa-Asp (GxNxFxD). It appeared in all noncathepsin B and C members of papain group and, in the case of kinetoplastids, the place of Asn is replaced by Thr and the motif becomes Gly-Xaa-Try-Xaa-Phe-Xaa-Asp (GxTxFxD). Table 1 shows the conserved motif of cysteine proteinases from different plant species. The GxTxFxD motif is conserved in *P. terminalis*, *A. thaliana, Phaseolus vulgaris* L., *Ipomoea batatas* (L.) Lam., *Vigna mungo* (L.) Hepper, *Z. mays, Phaseolus vulgaris* L., *P. sativum*, *Lycopersicon esculentum* Mill, *Brassica napus* L., and *Solanum tuberosum* L. Some of the plant species contain cysteine proteinases that have either the motif of GxNxFxD or GxTxFxD. In the case of *A. thaliana*, seven of the cysteine proteinases have the conserved motif as GxTxFxD (AAM96982, T13022, E85435, AAK92229, JN0719, BAB02464, BAB02463). Three proteinases have the motif GxNxFxD (AAM65468, AAB67626, T13023). It also has the motif as DINEFSD (Xaa-Xaa-N-Xaa-F-Xaa-D). The Xaa-Xaa-N-Xaa-F-Xaa-D format also exists in *Nicotiana tabacum* L. (78,660), *Mesembryanthemum crystallinum* L. *(*AAA74430), and *Oryza sativa* L. (CAA56844).

MDLLSRFVLL LFSSSLVFAA TASTVSSDES DDLLIRQVVAGADDHDNDDL

LLNAEHHFSSFKKRFGKAYTSCDEHDRRFGVFKANLRRAKRNQILDPSAV

HGVTQFFDLTPAEFRRTYLGLKRLRLPAD*THEAPILPTNDLPADFDWRDH*

GAVTPVKNQGSCGSCWSFSATGALEGANFLATGKLVSLSEQQLVDCDHVC

DSEDPSSCDSGCNGGLMTSAFEYTLKAGGLEREEDYPYTGTDHSKCKFDK

TKIAVSASNFSVVSLDENQIAANLVTNGPLAIGINAMFMQTYIGGVSCPY ICSKRLLDHGVLLVGYGSAG

FAPIRFKEKPYWIIKNSWGESWGEKGYYKI CRGRNICGMDSMVSAVAAAVPTVN

Fig. 1. Primary structure of deduced cysteine proteinase peptide (accession DQ403257) in *Pachysandra terminalis.* The polypeptide has a predicted length of 374 amino acids. The signal sequence (1–19) is marked in bold. The Peptidase_C1 domain is italicized (141–367). Protein translation was performed using ExPaSy translation tool (Swiss Institute of Bioinformatics, 2006). The conserved domains were predicted using MotifScan (Falquet et al., 2002). Signal peptide was predicted with the eukaryotic linear motif resources for functional sites in proteins (Puntervoll et al., 2003).

Table 1. The conserved motif within the pro region of cysteine proteases of different plant species.

Plant species	Gene accession	Propeptide motif	Gene expression
Arabidopsis thaliana	AAM96982	GVTQFSD[z]	N/A
A. thaliana	AAD23687	GVTQFSD[z]	N/A
A. thaliana	B84601	GVTQFSD[z]	Wilt inducible
A. thaliana	JN0718	GVTQFSD[z]	Drought-inducible
Phaseolus vulgaris	CAB17077	GVTQFSD[z]	N/A
Ipomoea batatas	AAK27969	GVTQFSD[z]	N/A
Vigna mungo	BAA92495	GVTKFSD[z]	Seed germination
Zea mays	BAA08244	GVTKFSD[z]	Seed germination and ripe
Pisum sativum	P25804	GITKFSD[z]	Turgor-responsive
Lycopersicon esculentum	CAA78403	GITQFSD[z]	N/A
Pachysandra terminalis	DQ403257	GVTQFFD[z]	Chilling-inducible
Brassica napus	ABA71355	GVNQFTD[z]	N/A
Solanum tuberosum	CAB53515	GLTKFAD[z]	Disease resistance
A. thaliana	T13022	GLTGFAD[z]	Drought-inducible
A. thaliana	E85435	GLTKFTD[z]	N/A
A. thaliana	AAK92229	GLTKFTD[z]	Seed germination
A. thaliana	JN0719	GLTRFAD[z]	Drought-inducible
A. thaliana	BAB02464	GLTRFAD[z]	Unknown
A. thaliana	BAB02463	GLTRFAD[z]	Unknown
L. esculentum	CAA88629	GINEFTD[y]	Leaf senescence-inducible
L. esculentum	AAM19209	GMNEFAD[y]	Disease resistance
Nicotiana tabacum	BAA96501	GVNEFTD[y]	Circadian and senescence-enhanced
A. thaliana	AAB67626	GVNEFAD[y]	N/A
P. sativum	CAA92583	GVNHFAD[y]	Seed germination
P. vulgaris	CAB17074	GLNKFAD[y]	Seed germination
N. tabacum	CAB16317	GLNQFAD[y]	Seed germination
Z. mays	CAA68192	GINRFAD[y]	Senescence-enhanced
Z. mays	BAA08245	GINRFAD[y]	Seeds ripe and germination
I. batatas	AAK27968	GINAFAD[y]	Senescence
P. vulgaris	CAB17076	GLNRFAD[y]	Seed germination
A. thaliana	T13023	GLNRFAD[y]	Drought-inducible
A. thaliana	AAM65468	GLNKFSD[y]	N/A
Oryza sativa	CAA56844	ALNKFAD[x]	Seed germination
N. tabacum	78660	KLNKFAD[x]	N/A
A. thaliana	AAD15594	DINEFSD[x]	N/A
Mesembryanthemum crystallinum	AAA74430	RLNQFGD[x]	Drought-inducible

[z]Motif = Gly-Xaa-Try-Xaa-Phe-Xaa-Asp^{-36} (GxTxFxD).
[y]Motif = Gly-Xaa-Asn-Xaa-Phe-Xaa-Asp^{-36} (GxNxFxD).
[x]Motif = Xaa-Xaa-Asn-Xaa-Phe-Xaa-Asp^{-36} (XxTxFxD).
N/A = Not available.

Effect of cold and heat stresses on gene expression of the putative cysteine proteinase gene from *P. terminalis*. When the real-time PCR products were separated on 2% agarose gels, one specific band in all the temperature treatments was observed for the cysteine protease (110 bp) and18S rRNA (100 bp) (Fig. 2). These results validated the primer specificity and efficiency of the real-time PCR.

qRT-PCR measures relative abundance of gene transcripts in different tissues. Results from the current study shows that transcript accumulation of the proteinase gene is affected by darkness, cold, and heat stresses. When plants were incubated at 25 °C, 2-h darkness induced a decrease of gene transcripts present in the leaf tissues. The expression level is lower than control (0 h) (Table 2). Extending the darkness to 1 d, 2 d, and 3 d induced a continuous elevation of gene transcript accumulation by 2.2-, 3.9-, and 4.3-fold, respectively. The level of the gene transcripts started to decrease at 6 d and 7 d and the value became approximately twofold compared with control. The interaction of temperature stresses and darkness caused temporal changes in the induction of the proteinase gene. Combination of short-term (2 h) cold (5 °C) and darkness (2 h) induced a 1.83-fold increase. Further exposure to chilling stress delayed the induction of the proteinase gene but increased the multitude. The expression level showed no obvious changes until 3 d, but fold changes increased from fourfold to sevenfold. The most obvious is the 7-d treatment; the cold treatment induced an 8.85-fold increase compared with 2.56-fold at 25 °C. The heat stress functions in a similar pattern. The gene induction delayed until 3 d of treatment, but the intensity was a 9.8-fold increase.

Discussion

The pro peptide regions serve a variety of functions in vivo and in vitro. It is required for the proper folding of the newly synthesized enzyme, the inactivation of the proteinase domain, and stabilization of the enzyme against denaturing at neutral

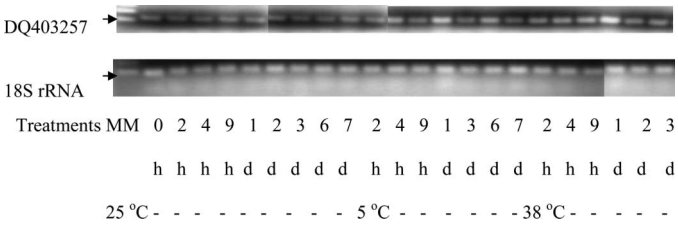

Fig. 2. Agarose gel validation of real-time polymerase chain reactions for a putative proteinase gene and 18S rRNA from leaves of *Pachysandra terminalis.* The DQ403257 is the cloned putative proteinase gene and 18S rRNA (ribosomal RNA) is the housekeeping gene; 2.5% agarose gel was used. One specific band was amplified in the real-time polymerase chain reaction from leaves under all the different temperature treatments. Arrows points to 100 base pairs; MM = DNA molecular marker; h = incubation hours; d = incubation days. Numerals indicate the number of hours or days under treatment. Temperatures are indicated under each treatment period.

to alkaline pH conditions (Vernet et al., 1995a). Amino acid residues within the pro region mediate their membrane association and play a role in the transport of the proenzyme to lysosomes. The variation in the conserved motif of plant proteinases is much bigger than in other eukaryotes species. This phenomenon could be related to species and the range of environment in which plants have evolved. However, no relationship can be identified between the variation of the conserved motif and the possible function of the proteinases as indicated by their involvement in different physiological processes (Table 1). This may be the result of limited availability of information on the specific function of different proteinases and from different plant species.

The temperature treatments were performed under continuous darkness, which is different from the diurnal photoperiod that plants normally experience. Results from the current study indicate that the proteinase gene transcription can be suppressed within 2 h by change of light conditions, or the dark-shock induces degradation of the gene transcripts. Both processes can lower accumulation of gene transcripts in the leaf tissues. The plants will get adjusted to the darkness and maintain the proteinase gene expression similar to control (0 h) in the next 9 h. However, a longer period (>1 d) of darkness can induce a light constraint and thus expression of the stress-related proteinase gene. Changes in gene expression in response to light changes have been documented in other plant species (Wierstra and Kloppstech, 2000).

P. terminalis is a cold-hardy herbaceous perennial plant species. The plants can survive –6.6 to –1.1 °C (USDA zones 6–9). In this study, we noticed that the leaves appear normal within 2 d when incubated at 38 °C in total darkness. These observations suggest that the plant species is highly tolerant to both high and low temperature extremes. The qRT-PCR assay shows that proteinase gene transcription remains stable for a minimum of 2 d under chilling and heat stresses, suggesting that the plants are maintaining a stable state of metabolism during this time period. However, a few leaves started to wilt and dehydrate after 3 d at 38 °C. Chlorotic spots appeared on some of the young leaves incubated for 6 d at 5 and 25 °C. Under both conditions, the plants are undergoing a degenerative process. Although the leaf samples were collected from normal-looking plants, they may be experiencing or adjusting to the corresponding changes. That can be the reason why the proteinase gene is highly enhanced at this stage.

Table 2. Fold changes of the steady-state transcript levels for the putative proteinase gene (DQ403257) in *Pachysandra terminalis* under darkness, cold, and heat stresses.[z]

Time period[y]	25 °C + dark	5 °C + dark	38 °C + dark
0 h	1.00[x] (0.91–1.10)[w]	1.00 (0.91–1.10)	1.00 (0.91–1.10)
2 h	0.66 (0.56–0.79)	1.83 (1.62–2.06)	1.06 (0.61–1.82)
4 h	1.23 (1.04–1.45)	1.16 (0.77–1.74	1.10 (0.65–1.85)
9 h	0.91 (0.59–1.40)	0.97 (0.73–1.28)	0.72 (0.43–1.21)
1 d	2.22 (1.71–2.89)	1.20 (0.81–1.78)	1.30 (0.80–2.12)
2 d	3.91 (2.29–6.67)	—[v]	1.55 (0.88–2.75)
3 d	4.34 (3.04–6.19)	7.05 (5.09–9.75)	9.82 (8.57–11.25)
6 d	2.36 (1.66–3.37)	3.00 (2.41–3.75)	—
7 d	2.56 (1.81–3.61)	8.85 (5.82–13.45)	—

[z]All the values are compared with 0 h in the same column.
[y]Number of hours (h) or days (d) under treatments.
[x]Fold changes of gene transcripts relative to 0 h and is calculated as $2\text{-}\Delta C_T$ in which ΔC_T is the (proteinase C_T –18S $rRNAC_T)_N$ – (proteinase C_T –18S $rRNAC_T)_0$; N refers to the treatment of N (h/d); 0 refers to 0 h.
[w]Range of the gene expression relative the control calculated using ΔC_{T+s} and ΔC_{T-s}, in which s = the standard deviation of the ΔC_T value. The calculation procedure was following the relative quantification using the comparative C_T method (ABI Prism 7700 Sequence Detection System, User Bulletin #2; Applied Biosystems, Foster City, Calif.).
[v]Quantitative reverse transcriptase–polymerase chain reaction not performed.

In plants and other organisms, proteinase activity can be regulated at different levels: by transcription/translation, by posttranslational processing, and by specific proteinase inhibitor proteins (Bode and Huber, 1992). Cold and heat stresses can induce breakdown of cellular structure (Ilker et al., 1979) and leakage of solutes and electrolytes (Lieberman et al., 1958) that could lead to polypeptide denaturation. The proteinases might function by degrading polypeptides damaged or denatured by exposure to temperature stresses. In tomato (*L. esculentum*) unripe fruit under 4 °C cold treatment, induction of a papain-like thiol proteinase gene expression was detected after 8 h and kept on increasing after 16 and 48 h incubation (Schaffer and Fischer, 1988, 1990). The current study showed that the cold regulation of the putative cysteine proteinase gene was time-dependent. It was enhanced after 2 h followed by a phase of recovering back to the control (before cold treatment) level within 1 d. Compared with the cold-sensitive tomato, *Pachysdandra terminalis* has much higher tolerance to low temperature. Based on the cold tolerance characteristics and gene expression pattern, it is postulated that one of the mechanisms for cold tolerance is that the tolerant plants will respond very fast to the environmental stress by expression of stress-related genes such as the proteinase to eliminate the denatured proteins or to activate other stress-related enzymes. After the initial phase, the plants will return to a normal metabolism state and thus the proteinase gene expression will start to decrease. However, a long period of chilling stresses (>3 d) may induce changes in cellular metabolism system, in which the enhanced expression of the proteinase is one of the components.

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