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TROUBLESHOOTING OF CLASS III PEROXIDASE GENE FROM THE MOSS *DICRANUM SCOPARIUM*

Onele A.O., Chasov A.V., Minibayeva F.V.

Kazan Institute of Biochemistry and Biophysics
FRC KazSC RAS, Kazan, Russia
E-mail: donjay.ao@gmail.com

Peroxidases (PRX) are a class of enzymes present in the tissues of plants, animals, and microorganisms. These enzymes serve to catalyze oxidoreduction between H_2O_2 and various reductants. PRX has been studied extensively in higher plants and has been implicated in a variety of physiological processes including lignin biosynthesis, extensin polymerization, disease and pathogen response, wound healing, and response to air pollutant stress. In this work, we isolated and sequenced a complementary DNA (cDNA) of class III PRX gene in the *Dicranum scoparium* Hedw. and designated as *DsPRX*. BlastN in the EnsemblPlants database showed that *DsPRX* displayed 100% identity with a homologous *PRX* of *Physcomitrella patens*. Cloning of plant *PRX* could help in elucidating the physiological role of individual PRX isoforms during plant normal development and in response to various environmental factors.

Keywords: coding sequence (CDS), **c**omplementary DNA (cDNA), *Dicranum scoparium*, peroxidase, *Physcomitrella patens*, Sanger sequencing

ПОИСК И УСТРАНЕНИЕ НЕИСПРАВНОСТЕЙ ГЕНА ПЕРОКСИДАЗЫ КЛАССА III ИЗ МХА *DICRANUM SCOPARIUM*

Онеле А.О., Часов А.В., Минибаева Ф.В.

Казанский институт биохимии и биофизики
ФИЦ КазНЦ РАН, г. Казань, Россия
E-mail: donjay.ao@gmail.com

Пероксидазы (PRX) представляют собой класс ферментов, присутствующих в тканях растений, животных и микроорганизмов. Эти ферменты служат для каталитического окисления с помощью H_2O_2 различных соединений. PRX широко изучена на высших растениях и был вовлечен в различные физиологические процессы, включая биосинтез лигнина, полимеризацию экстензина, реакцию на болезни и патогены, заживление ран и реакцию на стресс, вызванный загрязнением воздуха. В данной работе мы выделили из *Dicranum scoparium* Hedw. и секвенировали комплементарную ДНК (кДНК) PRX III класса, ген был обозначен как *DsPRX*. Была показана 100% идентичность *DsPRX* с гомологичным *PRX Physcomitrella patens* с помощью BlastN в базе данных EnsemblPlants. Клонирование *PRX* растений может помочь в выяснении физиологической роли отдельных изоформ PRX во время нормального развития растений и в ответ на различные факторы окружающей среды.

Ключевые слова: кодирующая последовательность (CDS), комплементарная ДНК (кДНК), *Dicranum scoparium*, пероксидаза, *Physcomitrella patens*, секвенирование по Сэнгеру

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INTRODUCTION

Plant *PRXs* are multigene family and have diverse functions in abiotic and biotic stress tolerance, lignification, cellular growth, fruit ripening and plant senescence [Yoshida et al., 2003; Passardi et al., 2005]. During the last decade, several molecular biology approaches have been developed to isolate, characterize and study the expression of *PRX* in plants. In *A. thaliana*, since the characterization of the first genes [Intapruk et al., 1991], numerous genes encoding PRXs have been identified through the different expressed sequence tag (EST) sequencing projects and have already been reported [Capelli et al., 1996; Østergaard et al., 1998; Tognolli et al., 2000].

Only a few articles have reported the genome-wide identification of plant class III PRX. Tognolli et al. identified 73 class III *PRXs* in *A. thaliana* and analysed gene structures (intron/exon),

gene duplication events, and expression patterns in different tissues (roots, stems, leaves and flowers) [Tognolli et al., 2002]. In 2004, Passardi et al. identified 138 rice class III *PRXs* and classified them into eight subfamilies (I-VIII) [Passardi et al., 2004]. A class III PRX, *GhPOX1* has been identified in cotton (*Gossypium hirsutum*) [Mei et al., 2009]. In the *Populus trichocarpa*, 93 class III *PRXs* were identified and investigated the *PtPRX* expression patterns in five tissues (roots, shoots, leaves, buds, and phloem) and under abiotic stresses (H_2O_2, salicylic acid, salt, and drought) [Ren et al., 2014]. In 2015, Wang et al. identified 119 maize (*Zea mays*) class III *PRXs* and divided them into 18 groups [Wang et al., 2015]. Cao et al. identified 94 pear (*Pyrus bretschneideri*) class III *PRXs* [Cao et al., 2016]. Even though mosses are the second most diverse phylum of land plant with approximately 13,000 species [Goffinet et al., 2009], much is not known about the class III *PRXs* of these species compared to higher vascular plants.

Despite the lack of complete plant genomes, Passardi et al. [2004] also studied the origin and expansion of class III *PRXs* by using EST sequences and found 11–14 putative PRX sequences in *P. patens* (moss). To date, among the only three moss species that had their genome sequenced i.e., *Sphagnum fallax* (DOE-JGI, http://phytozome.jgi.doe.gov/) [Shaw et al., 2016], *P. patens* [Rensing et al., 2008] and *Pleurozium schreberi* [Pederson et al., 2019]. Only 82 *PRXs* of *P. patens* were found in the National Center of Biotechnology Information (NCBI) database, 64 *PRXs* of *S. fallax* were found in the phytozome. No *PRXs* of *P. schreberi* were found in any of the databases because the genome is not fully annotated.

Previously, we demonstrated that moss *D. scoparium* possesses high activity of class III peroxidase [Onele et al., 2018]. We also identified ascorbate peroxidase in the *D. scoparium*, studied enzyme activity and gene expression in response to abiotic stresses [Onele et al., 2020 submitted for publication]. In this present study, we attempted to isolate and sequence class III *PRX* in the *D. scoparium*.

MATERIALS AND METHODS

Plant material

Dicranum scoparium Hedw. was collected from the Aisha forest, Tatarstan, Russia (55°53′21.3″N48°38′14.3″E). Thalli were allowed to air-dry slowly for 2 d between sheets of paper, and then stored refrigerated at 4°C in the dark before use.

Gene cloning and identification

RNA extraction and double strand cDNA synthesis

Total RNA was extracted using GeneJET Plant RNA Purification Mini Kit (Thermo Scientific, Lithuania). RNA concentration and purity were measured using a *NanoDrop® ND-1000* spectrophotometer (Thermo Scientific, Waltham, USA) and the integrity was accessed by gel electrophoresis 1% (m/v) in agarose gel. First strand cDNA and double strand cDNA were synthesized using Evrogen Mint 2 synthesis kit protocols.

Identification of *PRX* in *D. scoparium*

Database searches were performed at the NCBI server (http://www.ncbi.nlm.nih.gov). As the gene that codes for class III PRX from *D. scoparium* had not been sequenced, *P. patens* cDNA *PRX* (*Pp1s55*, identification numbers: Pp1s55_121V6.2, Pp3c3_30190V3.1) was used as a query. Only the coding sequence (CDS) of the *PRX* was used in the analysis. Specific primer sets for *PRX* gene were designed using Vector NTI Suite 9 (Invitrogen, Carlsbad, USA). The designed sequences of *PRX* were as follows:

F: ATGGAATCAGCCGCAGGAAGAAC

R: TTACAAAGGAGAAGGGGCCTCCAC

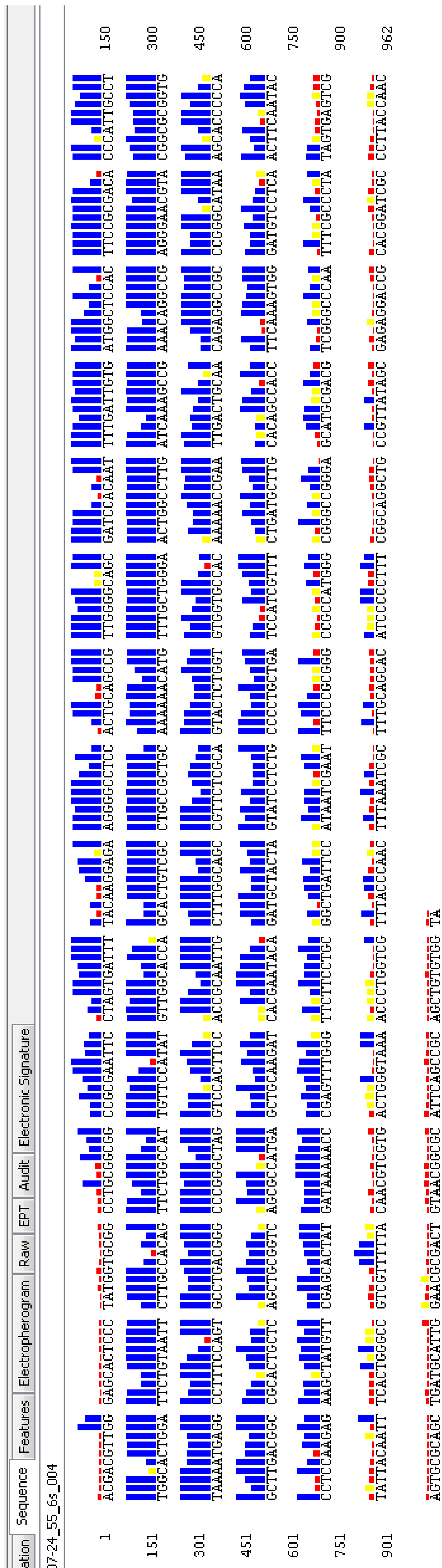

Fig. 1. ***DsPRX*** **sequences from DNA Sequencer, analysed in AB (Applied Biosystems) Sequencing Analysis Software v5.3.1.**

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                                  (1) 1        10        20        30        40        50        60        70        80        90        100       110       120       133
          Dicranum scoparium Pp1s55 (1) -----------------ACGACGTTGGGAGCACTCCCA-TATGGT--------G--CGGC--CTGCAG-----GGCGGCCCGCGAATTCACTAGTGATTTTACAAAGGAGAAGGGGCCTCCACTGCAGCCGT
Pp1s55_121V6.2_Pp3c3_30190V3.1 CDS (1) ATGGAATCAGCCGCAGGAAGAACACAGAGAGCACTGGCAGTCTGGTTGGTCATTGCGCAGCTGCTGCAGATAGTGGCAGCGCAGGACTTG-CAAGTGGATTT-CTATGGTG---GGACCTGTCCTTCGGCGGA
                          Consensus (1)                  A  AC   G GAGCACT  CA T TGGT        G  C GC  CTGCAG     GGC GC C  GA TT  C AGTG  TTT C A GG G   GG CCT   CT C GC G
                                (134) 134   140       150       160       170       180       190       200       210       220       230       240       250       266
         Dicranum scoparium Pp1s55 (98) TGGGGCAGCGATCCACAATTTTGA--------TTGTGATGGCTC----CACTTCCGCGACACCCATTGCCTTGGCACTGGATTCTGTAATTCTTGCCACAGTTCTGGCCATTGTTCCATATGTTGGCACCAGC
Pp1s55_121V6.2_Pp3c3_30190V3.1 CDS (129) GAAGATAGTGAGAGACGCTGTGGAAGCTGCCGTGGCGAAGGATCATGGCAATGCGCCGGGCCTGATT-CGTCTCCACTTTCATGACTGCTTCGTGCGAGGGTGCGATGCGTCAGTCCTTTTGGACGGACCGAA
                        Consensus (134)   G  AG GA    AC  T T GA        T G GA GG TC    CA T C  CG    C  ATT C T   CACT     T    T  TTC TGC A  GT C     C T    TCC T TG    G ACC
                                (267) 267        280       290       300       310       320       330       340       350       360       370       380       399
        Dicranum scoparium Pp1s55 (219) ACTGTCGCC--TGCCGCTGCAAAAAACATGTTTGCTGGGAACTGGCCTTGA--TCAAAGCCGAAACAGGCCGAGGGAACGTACGGCGCGGTGTAAAATGAGGCCTTTCCAGTGCCTGACGGGCCCGGGCTAGG
Pp1s55_121V6.2_Pp3c3_30190V3.1 CDS (261) ATCGGAGAAGGTGGCGTCGCCCAACTTTAGCTTGCGAGGATTCGAAGTTGTAGACGCGGCCAAAGCAGAGCTCGAGAAACAGTGTCCTGGAATCGTTTCCTGCGCCGACATT-CTTGCCTTTGCCGCTCGTGA
                        Consensus (267) A  G  G      TG CG  GC  AA      G TTGC  GGA   G    TTG      C    GCC AA CAG  C  G GAA     G C  GG  T     T    GC      CA T C TG C      CCG  C  G
                                (400) 400       410       420       430       440       450       460       470       480       490       500       510       520       532
        Dicranum scoparium Pp1s55 (348) TCCACTTCCA--------CCGCAATTGCTTTGGCAGCCGTTCTCGCAGT--ACTCTGGTGTGGTGCCACAAAAACCGA---A--TTGACTGCAACAGAGGCCGCCCGGGCATAAAGCACCCCCAGCTTGACGG
Pp1s55_121V6.2_Pp3c3_30190V3.1 CDS (393) TTCCATTGAATTGACTGGCGGAAAGAGATGGGAGGTTCCTGCGGGTAGACGAGACGGCAACGTGTCCATCAATGCCGAGGCAGAAGCAATGCTGCCGAGCCCGCA-GCTCAATGTGCAACAGTTGACTGACAG
                        Consensus (400) T C  TT  A        C G AA  G T  G     C T C  G AG   A  C G     G    CCA  AA  CCGA    A     A TGC  C GAG CCGC  G  CA      GCA C     G  TGAC G
                                (533) 533    540       550       560       570       580       590       600       610       620       630       640       650       665
        Dicranum scoparium Pp1s55 (466) CCGCACT-GCTCAGC-TGCGGTCAGCGCCATGAGCTG-CAAGATCACGAATACAGATGCTAC-TAGTATCCTCTGCCCCTGC-TGATCCATCGTTTCTGATGCTTGCACAG--CC-ACCTTCAAAGTGGAT-
Pp1s55_121V6.2_Pp3c3_30190V3.1 CDS (525) CTTCACTCGCAAAGGATTATCTCAAAGCGATATGATCACATTATCGGGTGCGCACACGATTGGTCGAATCCATTGTTCGACCGTCGTGGCGAGATTGT-ATCCTGAGACAGATCCCAGCCTCGACGAGGATTT
                        Consensus (533) C  CACT GC  AG  T    TCA  GC AT  G T  CA  ATC  G    CA A G T   T G ATCC  TG  C    C T  T      G TT  T AT CT    ACAG   CC A C TC A G GG  T
                                (666) 666          680       690       700       710       720       730       740       750       760       770       780       798
        Dicranum scoparium Pp1s55 (590) -GTCCTCAACTTCAATACCCTCCAAGAGAAGCTATGTTCGAGCACTATGATAAAAACCCGAGTTTGGGTTCTTCCTGCGGCTGATTCCATAATCGA-ATTTCCCGCGGGC-CGCCATGGGCGGGCCGGGAGCA
Pp1s55_121V6.2_Pp3c3_30190V3.1 CDS (657) GGCTGTGCAGTTGAAGACCCTCTGTCCTCAAGTCGGCGGGAGTTC-ATCCTCAACCTTCAACTT--GGATCCCACGACGCCAGAGCTCTTCGACAACATGTACTACTCGAACCTCTTCAGCGGCAAGGGAGT-
                        Consensus (666)  G   T  A TT AA ACCCTC         A  T  G    GAG   C AT   T AA      C A TT   GG TC     C  CG C GA    C T    C A AT T C   C   G   C   C T   GCGG    GGGAG
                                (799) 799       810       820       830       840       850       860       870       880       890       900       910       920       931
        Dicranum scoparium Pp1s55 (720) TGCGACGTCG-----------------------------------------------------------------------------------------------------------------------------
Pp1s55_121V6.2_Pp3c3_30190V3.1 CDS (786) TCTGCAATCGGACCAGATTCTGTTCGAAAGCTGGTCCACGAAGCTTCCTACAATGTTCAACGTCTTGTCCACAACATCGTTCACTTCGTCATTTGCAGACTCCATGCTCACCATGAGTCAGATCGAAGTGAAG
                        Consensus (799) T  G    TCG
                                (874) 0      940       950       960       970       980       990       1006
        Dicranum scoparium Pp1s55 (730) ---------------------------------------------------------------------------
Pp1s55_121V6.2_Pp3c3_30190V3.1 CDS (861) ACGGGCTCAGAGGGCGAGATTCGTCGCAACTGTAGGGCTGTGAATCCCGTGGTGGAGGCCCCTTCTCCTTTGTAA
                        Consensus (874)
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Identity

Non identity/deletion or insertion

Fig. 2. Alignment of *DsPRX* nucleotides and *P. patens Pp1s55* using Vector NTI Suite 9.

The computer software program Vector NTI was used for comparisons and alignments. Blasting of nucleotide sequences was performed in an online server Ensemblplants: http://plants.ensembl.org/index.html.

RESULTS

Bioinformatic analysis of *DsPRX*

To identify the class III PRX genes in *D. scoparium*, the PCR product (993 bp) was cloned into the pGEM-T vector (Promega) and sequenced using primers designed based on the *Pp1s55* of *P. patens* nucleotide sequence available in the NCBI database. Although good quality of *D. scoparium* DNA sequence was obtained from the automatic DNA analyzer ABI 3130 (Applied Biosystems) after sequencing (Fig. 1), nucleotide sequence comparisons of *DsPRX* with *P. patens Pp1s55* using Vector NTI Suite 9 revealed low percentage identity of 36.4% (Fig. 2). Alignment of nucleotides showed incomplete sequence of *DsPRX* (Fig. 2) because of deletions or insertions of nucleotides in *DsPRX* sequence (Fig. 2). Out of five replicates of Sanger reaction prepared before sequencing, only 729 bp of *DsPRX* was sequenced against the expected 993 bp.

Blasting the nucleotide sequence of *DsPRX* displayed 100% identity with *PRX* (*Pp1s55*) of *P. patens* at chromosome 3 with E-values of 0.0011, 0.0045 and 0.07 (Fig. 3, 4).

Results for BLASTN against Physcomitrella patens Phypa_V3 (Genomic sequence)

Job details

Job name	BLASTN against Physcomitrella patens Phypa_V3 (Genomic sequence)
Species	Physcomitrella patens
Assembly	Phypa_V3
Search type	BLASTN (NCBI BLAST)

Download results file New job

Results table

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Genomic Location	Overlapping Gene(s)	Orientation	Length	Score	E-val	%ID
3:20573895-20573918 [Sequence]	Pp3c3_30190	Reverse	24 [Sequence]	24	0.0011	100.0 [Alignment]
7:16973143-16973173 [Sequence]	Pp3c7_25120	Forward	31 [Sequence]	23	0.0045	93.5 [Alignment]
3:20572680-20572702 [Sequence]	Pp3c3_30190	Reverse	23 [Sequence]	23	0.0045	100.0 [Alignment]
3:23734328-23734348 [Sequence]		Forward	21 [Sequence]	21	0.07	100.0 [Alignment]
11:678595-678618 [Sequence]	Pp3c11_1420	Forward	24 [Sequence]	20	0.28	95.8 [Alignment]
6:17343224-17343243 [Sequence]		Forward	20 [Sequence]	20	0.28	100.0 [Alignment]
23:7734951-7734972 [Sequence]		Reverse	22 [Sequence]	18	4.3	95.5 [Alignment]
17:13681735-13681752 [Sequence]	Pp3c17_20460	Reverse	18 [Sequence]	18	4.3	100.0 [Alignment]
8:10782403-10782420 [Sequence]	Pp3c8_16530	Forward	18 [Sequence]	18	4.3	100.0 [Alignment]

Fig. 3. BlastN result of *DsPRX* against *P. patens* in the EnsemblPlants database with homologous genes. Circled in red are homologous *PRX* of *P. patens*.

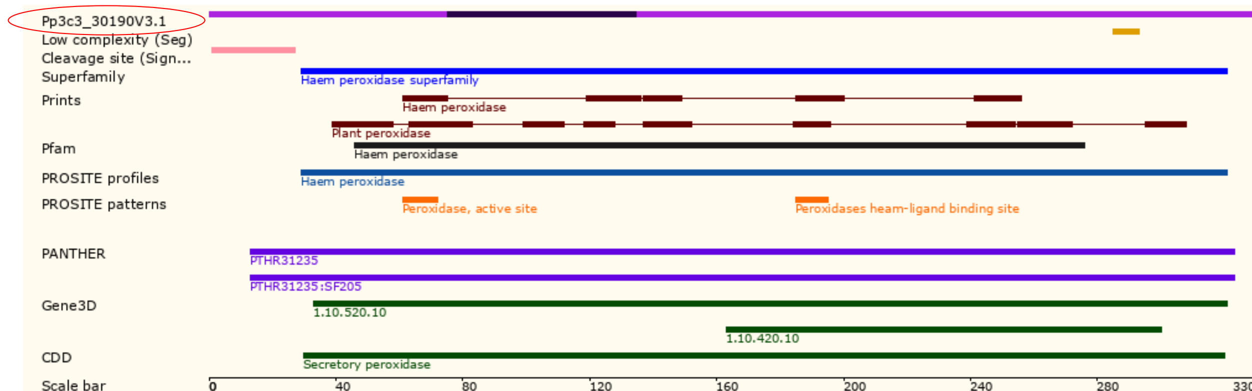

Fig. 4. Homologous *P. patens* PRX gene with *DsPRX*.
Circled in red is the identification number of *P. patens PRX*.

DISCUSSION

From the blast results, other genes different from PRX genes located at different chromosomes shared high nucleotide identity with the cloned *DsPRX* (Fig. 3). It is possible this occurred as a result of the incomplete sequence of *DsPRX* due to deletion or insertion of nucleotides obtained after cloning of this gene, which resulted in the low percentage identity of nucleotide sequence with PRX gene of *P. patens* after alignments (Fig. 2).

However, there are possible factors responsible for this incomplete sequence and shared identity with other genes except only *PRXs*. Firstly, it is possible that PRX primers designed for this cloning are not efficient as both the beginning and ending of *DsPRX* were not sequenced (Fig. 2). Secondly, it is possible that selected clones transformed in the pGEM vector were poorly purified before Sanger amplification. Third factor is probably a poor amplification of Sanger reaction before sequencing. In addition, another factor to consider is the length of the nucleotide that was sequenced. The DNA sequencer ABI PRISM 3700 Genetic Analyser (Applied Biosystems) has the capacity to sequence 600 bp of a DNA length but the length of PRX was 993 bp.

Putting these facts together, further work is needed to sequence the complete nucleotides of *DsPRX*. In order to do that, probably new primers need to be designed. Other cloning methods different from Sanger could be applied in order to clone the full length of PRX genes in the *D. scoparium*.

In conclusion, we believe that continued research of *D. scoparium* and other moss PRX genes could help in elucidating the physiological role of individual PRX isoforms during plant normal development and in response to various environmental factors. Understanding the expression pattern of individual PRX isoforms may lead to understanding their function or may result in findings that are applicable to both nonvascular and vascular plants and therefore lead to a broader and more complete understanding of the evolution of plant defense mechanisms.

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