SHORT REPORT
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Molecular cloning and expression analysis of the pathogenesis-related gene *VaPR2* in azuki bean (*Vigna angularis*)

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ABSTRACT: A cDNA sequence including the complete open reading frame was isolated from leaves of flowering azuki bean variety No. 56. The nucleotide sequence was submitted to the NCBI Genbank database. The gene was related to pathogenesis (protein gene resistance to disease) and named *VaPR2*. *VaPR2* was very similar to *PvPR2*, *PvPR1*, and *CpPR3*, with identity ranging from 88.4% to 91.1%. The gene was highly induced after challenging with the soybean mosaic virus and the anthracnose fungus *Colletotrichum lindemuthianum*. Results from RT-PCR analysis showed that the *VaPR2* gene is expressed strongly in stem, leaves, flowers, and pods, but weakly in roots.

KEYWORDS: *PR* gene, PR protein, legume

INTRODUCTION

Azuki bean (*Vigna angularis* (L.) Ohwi & Ohashi; $2n = 2x = 22$) is one of the most important economic legume crops in China, Japan, and Korea[1,2]. A common product made from azuki bean is the red bean paste used as filling in cakes, pastries, and ice cream[3]. Diseases and insect pests are the major problems limiting the cultivation of azuki bean in the East Asia region[4–6].

Pathogenesis-related (PR) proteins are soluble proteins whose expression is usually induced in plants by pathogen infection or treatment with some chemical agents[7–9]. Nearly thirty years ago, PR proteins were first found in tobacco leaves as a result of a hypersensitive response against tobacco mosaic virus (TMV)[10]. Soon after, PR proteins were further observed in the fluid washed from intercellular spaces of the leaves. In 1991, Dore et al[11] found that PR proteins accumulate mainly in cells around the necrotic spots of TMV-induced lesions. Acidic chitinases, beta-(1,3)-glucanases, and thaumatin-like proteins were found to accumulate in extracellular pocket-like vesicles whereas the basic chitinases were found in electron dense inclusion bodies in the vacuoles. Van Eldik et al[12]. reported a pistil-specific gene similarity to the PR-1 proteins which had a possible function in protecting or guiding the pollen tubes through the pistil. PR proteins are widely observed in various plant species as well as in cultured plant cells. To date, studies have mainly focused on purification and characterization of PR proteins in tobacco, tomato, and common bean. However, the regulation of the *PR* gene expression is still undetermined, partly due to inconclusive observations. Ishimoto et al[13] proposed that the synthesis of PR proteins in tobacco is mainly regulated at the translational level, whereas Nakamura et al[14] found that PR1 protein is transcriptionally regulated in tobacco, as well as in cultured parsley cells.

Common bean (*Phaseolus vulgaris* var. Saxa) can be induced to accumulate large amounts of PR proteins when treated with mercuric chloride or infected with alfalfa mosaic virus. De Tapia et al[15] proved that functional mRNA encoding bean PR4 protein is only present when the expression of the gene has been induced. Treatment with mercuric chloride resulted in a rapid induction of PR4 mRNA within 2–3 h, whereas in virus-infected plants this mRNA could only be detected by the second day following the infection. Bean PR4 protein is synthesized in vitro as a 35 kDa precursor protein. This precursor can be processed into a mature polypeptide of 33.5 kDa, as the in vivo PR4 protein, by adding the mRNA

to a cell-free translation system of canine pancreatic microsomal membranes.

PR proteins can be divided into 17 groups on the basis of their sequence characteristics, immunological relationships, and biological activities[16]. However, only 14 distinct families of PR proteins have been found in plants[17]. Not all families are found in any one plant species[18]. Among the diverse PRs, PR-2 is the dominant group, whose function is not well known. Expression of PR proteins could be induced by many stressors such as senescence, callus culture, infection, plasmolysis, exposure to certain chemicals including polyacrylic acid, auxin, cytokinin, arachidonic acid, ozone, hydrogen peroxide, wounding, and environmental conditions. Hence if the signal transduction pathways and the molecular mechanisms of inducing resistance are elucidated, we should be able to genetically manipulate anti-pathogenic processes in plants, and to develop transgenic plant lines which are resistant to pathogens[13].

To understand the roles of the PR2 disease induced protein gene in azuki bean, we designed degenerate primers according to the conserved amino acid sequences of PR proteins in other species. A full length cDNA sequence was amplified and characterized.

MATERIALS AND METHODS

Material

Azuki bean variety No. 56 was collected by the Institute of Vegetable Crops, Jiangsu Academy of Agricultural Sciences, Nanjing, Jiangsu, China.

Isolation of *VaPR2* gene

Azuki bean seeds (variety No. 56) were grown in pots (250 mm diameter) filled to a depth of 200 mm with soil at the Institute of Vegetable Crops, Jiangsu Academy of Agricultural Sciences. The pots were placed 1.5 m apart in a greenhouse. The plants started flowering after 45 days. Then, 5 plants each were inoculated with soybean mosaic virus (SMV) or the anthracnose fungus (*Colletotrichum lindemuthianum*) to induce disease symptoms. Then, 7 days later, the roots, stem, leaves, flowers, and pods were collected from the inoculated and non-inoculated plants and kept for further use.

Trizol reagent (Invitrogen) was used to extract total RNA from azuki bean leaves. cDNA templates were prepared according to the SMART RACE cDNA Amplification Kit Users' Manual (Clontech, USA). Two pairs of primers, beanpr2f: 5′-ATGGC(C/T)GTTTTCACATTCGA(G/A)C-3′ and beanpr2r: 5′-TCA(A/G)G(C/G)(A/G)TTGG-CCAA(A/T)(A/T)GGTAAGC-3′, were designed for RT-PCR according to conserved amino acid regions of a cowpea pathogenesis related gene *PvPR2* gene (GenBank accession no. CAA43636), *PvPR1* gene (GenBank accession no. CAA43637), *CpPR3* (GenBank accession no. BAA77691), and the *PR10* gene (GenBank accession no. AAX19889).

The PCR was performed in a 25 µl volume containing 1 µl of the synthesized floral bud cDNA, 2.5 µl 10 × PCR buffer, 5 µmol/l of each of the primers, 0.2 µM each dNTP, 1.5 µM $MgCl_2$, and 1U rTaq polymerase (Takara, Japan). The starting temperature was 94 °C for 10 min without polymerase, followed by 33 cycles of 94 °C for 30 s, 50 °C for 30 s, and at 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR products were electrophoresed on a 1.2% agarose gel. The amplified products were cloned into the pGEM-T Easy vector (Promega) using standard procedures. Positive clones were identified and sent for sequencing at Boya Company, Shanghai.

Expression and verification of *VaPR2* gene

To verify the expression of the isolated *VaPR2* gene during pathogenesis, RNA was extracted from leaves of inoculated and control azuki bean plants by Trizol (Invitrogen). The RNA was reverse transcribed to cDNA. A primer pair named beanpr30: 5′-CAC-TTCTCCTGTGGCTCCTGCTA-3′ and beanpr450: 5′-GTAAGCCTCAATGACCTTGAAAAGA-3′ were used to amplify the cDNA extracted from different leaves. A fragment of the actin gene, a general housekeeping gene, was amplified as an internal control using the primers, actin378 (bean): 5′-TGAGA-CCTTCAACACTCCTGCTA-3′ and actin709 (bean): 5′-CTGAGCTAGTCTTGGCAGTTTCC-3′. A PCR product of 330 bp was obtained, as expected.

Sequence analysis of the pathogenesis-related gene

DNASSIST version 2.0 was used for sequence alignment and open reading frame (ORF) identification through NCBI BLAST (www.ncbi.nlm.nih.gov). The protein molecular weight and isoelectric point were predicted from the database ExPASy (cn.expasy.org). The phylogenetic relationship between *VaPR2* and the other homologues were determined using the CLUSTALW program (www.ebi.ac.uk/Tools/clustalw) and TREEVIEW program (version 1.6.6)[19]. Previously published *PR* gene sequences were retrieved from the NCBI database, and protein numbers are: P25986, CAA65727, BAA77691, AAX19889, CAA67200, P25985, ABF13312,

P26987, ACD39391, AAP37978, AAP57943, AAK09428, CAA56142, CAA69931, CAC37691, and ABB73065.

RESULTS AND DISCUSSION

Isolation of the full-length *VaPR2* gene from azuki bean

Using the degenerate primers, a sequence of approximately 500 bp was amplified from cDNA of azuki bean cv. No. 56, including a 468 bp complete ORF. DNA sequence analysis showed that the sequence is a homologue of *PR2* in *Vigna angularis*, putatively encoding a protein with 156 amino acids, giving a molecular weight of 16.5 kDa and an isoelectric point at 4.62. The sequence was assigned the name *VaPR2* and submitted to GenBank as accession no. EU046566.

Similarity between azuki bean *VaPR2* with the *PR* genes from the other Legume species

We constructed a molecular phylogenetic tree of *VaPR2* and 16 other PR proteins in the NCBI databases from various organisms. A phylogenetic tree of publicly available full-length PR coding sequences showed that the gene family splits into three major branches (Fig. 1). *VaPR2* showed a high similarity to *PvPR2*[20], *PvPR1*[20], and *CpPR3*, with identity ranging from 88.4% to 91.1%. In addition, analysis of conserved domains within the *VaPR2* amino acid sequence showed that there were several unique

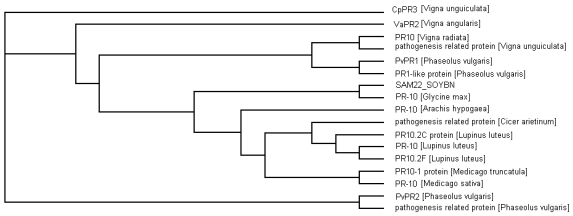

Fig. 1 A phylogenetic analysis of *PR* genes across legume species. The protein numbers are: PvPR2 (P25986), pathogenesis related protein [*Phaseolus vulgaris*] (CAA65727), CpPR3 (BAA77691), PR10 [*Vigna radiata*] (AAX19889), pathogenesis related protein [*V. unguiculata*] (CAA67200), PvPR1 (P25985), PR1-like protein [*P. vulgaris*] (ABF13312), SAM22_SOYBN (P26987), PR10 [*Arachis hypogaea*] (ACD39391), PR10 [Lupinus luteus] (AAP37978), PR10.2F [*Lupinus luteus*] (AAP57943), PR10.2C protein [*L. luteus*] (AAK09428), pathogenesis related protein [*Cicer arietinum*] (CAA56142), PR10-1 protein [*Medicago truncatula*] (CAA69931), PR10 [*M. sativa*] (CAC37691), and PR-10 [*Glycine max*] (ABB73065).

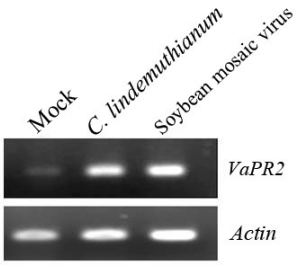

Fig. 2 Expression profiles of *VaPR2* gene among various tissues in azuki bean (*Vigna angularis*). Total RNA was shown as a quantitative control in the lower panel with the same order as in the upper panel. A mixed total RNA from all the tissues was amplified as the positive control.

histidine-rich regions when compare to other members in this family, implicating a possible non-redundant function of *VaPR2* gene in the pathogenesis related gene family.

Expression of *VaPR2* in tissues in vivo

The RT-PCR analysis showed that the *VaPR2* gene was detected in all sampled tissues, i.e.., the roots, stem, leaves, flowers, and pods, indicating a non-tissue-specific expression pattern. Moreover, the expression level among tissues is high, except for a weak profile in the root (Fig. 2). The universal expression pattern strongly suggests that *VaPR2* gene might play roles in non-specific and systemic response to biotic stresses.

VaPR2 is involved with early response to pathogen infection

Until now *PR* genes have commonly been considered to be genes involved in systemic acquired resistance (SAR)[21] which co-express with a wide range of plant defence genes responding to pathogens, environmental stresses, and wounding. In our study, *VaPR2* gene is significantly increased after inoculation with SMV and *C. lindemuthianum* as revealed by RT-PCR (Fig. 3a). The up-regulated expression profile agreed well with the microarray data (Fig. 3b) which indicated involvement of *VaPR2* gene in response to microbe intrusion as a part of SAR. The *VaPR2* showed the highest expression level as early as 7 days post inoculation (DPI), and decreased thereafter, implying that *VaPR2* gene plays roles in response to pathogen invasion only at the early stage.

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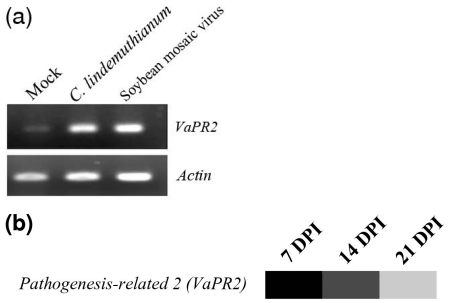

Fig. 3 (a) An up-expressed profile of *VaPR2* gene upon inoculation with *C. lindemuthianum* and soybean mosaic virus (SMV) as revealed by RT-PCR of leaf tissue. *VaPR2* gene is marked in the upper panel. The actin gene in the lower panel was assigned as a quantitative control. (b) A decreasing expression pattern of *VaPR2* gene along with the days post SMV inoculation is displayed by microarray. The black bar represents high expression pattern, the grey bar represents low expression, while the light grey bar represents undetectable expression. DPI (days post inoculation) showed the hybridization signals of gene chips at the specified dates.

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REFERENCES

1. Xu N, Cheng XZ, Wang SH, Wang LX, Zhao D (2008) Establishment of an adzuki bean (*Vigna angularis*) core collection based on geographical distribution and phenotypic data in China. *Acta Agron Sin* **34**, 1366–73 [in Chinese with English abstract].
2. Jin WL, Pu SJ (2008) Research development of azuki bean in China. *World Agr* **347**, 59–62 [in Chinese with English abstract].
3. Tian J (2008) The industry development of azuki bean. *Process Agr Prod* **3**, 15–7.
4. Pu SJ, Jin WL (2007) Breeding progress and research tendency of azuki bean. *World Agr* **334**, 47–55 [in Chinese with English abstract].
5. Jin WL, Yu SF (1996) Genetic analysis of resistant genes to rust and powdery mildew and brown spot in adzuki bean. *J Beijing Agr Coll* **11**, 1–9 [in Chinese with English abstract].
6. Wang CJ, Shi GS, Sun XF, Yan XD (2003) The sudden happened and seriously damage black shank in azuki bean in Heilongjiang. *Mod Agr* **292**, 12–3. (in Chinese with English abstract)
7. Carr JP, Dixon DC, Nikolau BJ, Volkerding KV, Klessig DF (1987) Synthesis and localization of pathogenesis-related proteins in tobacco. *Mol Cell Biol* **7**, 1580–3.
8. Xie CZ, Liu HY, Li L, Liang XQ (2008) Advances on class 10 pathogenesis-related proteins. *Mol Plant Breed* **6**, 949–53 [in Chinese with English abstract].
9. Herbers K, Meuwly P, Metraux JP, Sonnewald U (1996) Salicylic acid-independent induction of pathogenesis-related protein transcripts by sugars is dependent on leaf developmental stage. *FEBS Lett* **397**, 239–44.
10. Fraser RSS (1982) Are "pathogenesis-related" proteins involved in acquired systemic resistance of tobacco plants to tobacco mosaic virus? *J Gen Virol* **58**, 305–13.
11. Dore I, Legrand M, Cornelissen BJ, Bol JF (1991) Subcellular localization of acidic and basic PR proteins in tobacco mosaic virus-infected tobacco. *Arch Virol* **120**, 97–107.
12. Van Eldik GJ, Wingens M, Ruiter RK, Van Her MMA, Schranwen JAM, Wullens GJ (1996) Molecular analysis of a pistil-specific gene expressed in the stylar cortex of *Solanun tuberosum*. *Plant Mol Biol* **30**, 171–6.
13. Ishimoto M, Sato T, Chrispeels MJ, Kitamura K (1996) Bruchid resistance of transgenic azuki bean expressing seed α-amylase inhibitor of common bean. *Entomol Exp Appl* **79**, 309–15.
14. Nakamura T, Yokoyama R, Tomita E, Nishitani K (2003) Two azuki bean *XTH* genes, *VaXTH1* and *VaXTH2*, with similar tissue-specific expression profiles, are differently regulated by auxin. *Plant Cell Physiol* **44**, 16–24.
15. De Tapia M, Dietrich A, Burkard G (1987) In vitro synthesis and processing of a bean pathogenesis-related (PR4) protein. *Eur J Biochem* **166**, 559–63.
16. Liu Z, Du L, Wan B (2005) Pathogenesis-related proteins in higher plants. *Natur Prod Res Dev* **17**, 229–34.
17. Sarowar S, Kim YJ, Kim EN, Kim KD, Hwang BK, Islam R, Shin JS (2005) Overexpression of a pepper basic pathogenesis-related protein 1 gene in tobacco plants enhances resistance to heavy metal and pathogen stresses. *Plant Cell Rep* **24**, 216–24.
18. Van Loon LC, Van Strien EA (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR1 type proteins. *Physiol Mol Plant Pathol* **55**, 85–97.
19. Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–8.
20. Walter MH, Liu JW, Grand C, Lamb CJ, Hess D (1990) Bean pathogenesis-related (PR) proteins deduced from elicitor-induced transcripts are members of a ubiquitous new class of conserved PR proteins including pollen allergens. *Mol Gen Genet* **222**, 353–60.
21. Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD (1996) Systemic acquired resistance. *Plant Cell* **8**, 1809–19.