

Full Length Research Paper

Cloning and expression analysis of a blue copper-binding protein gene from *Dasypyrum Villosum*

Huagang He^{1*}, Shanying Zhu¹, Wenbing Wang¹, Tongde Bie² and Peidu Chen³

¹Jiangsu University, Zhenjiang 212013, P. R. China.

²Yangzhou Academy of Agricultural Sciences, Yangzhou 225007, P. R. China.

³Nanjing Agricultural University, Nanjing 210095, P. R. China.

Accepted 20 May, 2011

A differentially expressed fragment EST145 was isolated by suppression subtractive hybridization (SSH) method. Using EST145 as the probe, a blue copper-binding protein gene designated as *DvBCB* was screened from *Dasypyrum villosum* cDNA Library. The *DvBCB* gene was 845 bp in length with an open reading frame (ORF) which encoded a 178-amino acid polypeptide and contained the deduced functional sites: H₆₆, C₁₀₇, H₁₁₂ and M₁₂₁. Northern blot analysis showed that, the expression of *DvBCB* gene was enhanced in leaves after inoculation with *Erysiphe graminis*; reached a peak level at 24 h and decreased to constitutive level at 72 h after inoculation in resistant Gh21 line. The expression level in susceptible mutant M14S line was slightly lower than that in the resistant Gh21 line at all stages after inoculation, and the peak could not appear in M14S line. The function of *DvBCB* gene might include lignification of cell wall or scavenging of reactive oxygen species (ROS) during powdery mildew attack.

Key words: *Dasypyrum villosum*, powdery mildew, suppression subtractive hybridization, blue copper-binding protein gene.

INTRODUCTION

Powdery mildew caused by *Erysiphe graminis* Dc. f. sp. *tritici* Marchal is a serious disease of *Triticum aestivum* (common wheat). *Dasypyrum villosum*, a close relative to common wheat, carries powdery mildew resistance gene *Pm21* which confers effective resistance to all current powdery mildew races (Qi et al., 1995). Cloning resistance or pathogenesis-related genes from *D. villosum* is important to develop the resistance to powdery mildew in common wheat.

Blue copper-binding protein (BCB) can bind a single copper atom (Ryden and Hunt, 1993). The copper binding sites consist of two histidine (H), one cysteine (C) and one methionine (M) or glutamine (Q) (Garrett et al., 1984). BCB proteins can rapidly regulate Ca²⁺, Mg²⁺, Zn²⁺ or other ions in plants (Lin and Wu, 1994), whose functions include reactive reaction (Gysel et al., 1993)

and/or lignification of cell wall (Drew and Gatehouse, 1994). The expression of *BCB* gene was enhanced by UV-light (John et al., 2001), ozone (Miller et al., 1999), aluminum (Richards et al., 1998), wounding (Kim et al., 1994) or other abiotic stresses. It was also reported that the expression of *BCB* gene was up-regulated by pathogens (Luo et al., 2002; Jansen et al., 2005).

A blue copper-binding protein gene (*DvBCB*) from *D. Villosum* was cloned and its expression pattern was revealed for the first time in this study. The results shed light on the resistance mechanism of *D. Villosum* to *E. graminis*.

MATERIALS AND METHODS

Growth and inoculation condition

Wild type *D. villosum* resistant Gh21 line and its susceptible mutant M14S line-induced by EMS were grown separately without pathogens at 20°C for 14 days. The seedlings at two-leaf stage were inoculated with *E. graminis*. The temperature and humidity were kept in order to induce spores to germinate. The leaves were harvested and their surface was cleaned using sterile water. Total RNA was extracted by TRIZOL reagent (Invitrogen, USA). Poly (A)⁺ RNA was purified by PolyATtract® mRNA Isolation Systems

*Corresponding author. E-mail: hghe@ujs.edu.cn.

Abbreviations: SSH, Suppression subtractive hybridization; BCB, blue copper-binding protein; ORF, open reading frame; ROS, reactive oxygen species.

(Promega, USA).

Suppression subtractive hybridization

Samples of total RNA of resistant Gh21 line inoculated by *E. graminis* at 15, 18 and 21 h were mixed with equal amounts and used to extract mRNA as tester, and mRNA of susceptible M14S line as driver. SSH procedures were performed according to the manual of PCR-Select™ cDNA Subtraction kit (Clontech, USA). Purified PCR products were cloned into pGEM-T easy vector (Promega, USA). PCR products of positive clones were denatured using NaOH solution and transferred onto the Hybond-N⁺ nylon membrane (Amersham, UK). Forward and reverse SSH products were labeled by α -³²P-dCTP and used to screen the differentially expressed clones according to the manual of PCR-Select Differential Screening kit (Clontech, USA).

Screening of cDNA library

The cDNA library from the leaves of *D. villosum* infected by *E. graminis* was constructed according to the manual of Superscript cDNA synthesis kit (Invitrogen, USA). A differentially expressed EST145 and a homolog of blue copper-binding protein gene, was used as probe labeled by α -³²P-dCTP to screen this cDNA library. The method was southern blot (Sambrook et al., 1989).

Sequence analysis

DNA sequencing of the positive clone was finished by Bioasia Biotechnological Company Ltd (Shanghai, China). Analysis of homology and open reading frame (ORF) were respectively based on BLAST program and ORF Finder program in GenBank. Multiple alignment analysis was performed by clustalW program and the result was visualized by BOXSHADE 3.21 software. Phylogenetic tree was constructed using neighbor-joining method by MEGA3.1 software.

Northern blot analysis

The resistant Gh21 line and its susceptible mutant M14S line were inoculated at 0, 6, 12, 24, 48 and 72 h, respectively. Total RNA was extracted by TRIZOL reagent and detected by agarose gel electrophoresis and UV-spectrophotometer at the wave length of 260 and 280 nm. 15 μ g of the total RNA in each lane was separated by electrophoresis on 1.0% formaldehyde agarose gel and blotted on Hybond-N⁺ nylon membrane. The full-length *DvBCB* cDNA was labeled by α -³²P-dCTP using random primer method. Northern blot was performed at 42°C and the details were according to Sambrook et al. (1989).

RESULTS

Cloning and sequence analysis of *DvBCB* gene

In order to clone defense-related genes to powdery mildew in *D. villosum*, a suppression subtractive hybridization library was constructed from the leaves infected by *E. graminis* and 218 differentially expressed DNA fragments were screened between the resistant Gh21 line and its susceptible mutant M14S line. EST145, 255 bp in

length, was a homolog of blue copper-binding protein gene. EST145 was employed as probe to screen a cDNA library from the leaves of *D. villosum* infected by *E. graminis* and a positive cDNA clone was obtained.

The full-length cDNA, designated as *DvBCB* (accession number: EU070903), was 845 bp in length with an ORF which encoded a 178-amino acid polypeptide (Figure 1). The molecular weight of this deduced *DvBCB* protein was about 17.6 kDa and the pI was about 5.05. *DvBCB* protein contained a 25-amino acid signal peptide in the N-terminal and a 24-amino acid transmembrane region in the C-terminal. According to Garrett et al. (1984), this *DvBCB* protein also contained the possible copper-binding sites which were H₆₆, C₁₀₇, H₁₁₂ and M₁₂₁.

Blast analysis showed that the deduced BCB protein of *D. villosum* and the ones of wheat, barley, rice and *Arabidopsis thaliana* had 75, 66, 58 and 45% identity, respectively (Figure 2). Phylogenetic analysis of *DvBCB* and other plant BCB proteins indicated that *DvBCB* protein might share the same origin with the ones of wheat, barley and rice which belongs to grass (Figure 3).

Northern blot analysis of *DvBCB* gene

Northern blot analysis showed that *DvBCB* gene was constitutively expressed and greatly induced by *E. graminis* in resistant Gh21 line and its susceptible mutant M14S line (Figure 4). In Gh21 line, the expression of *DvBCB* gene reached a peak level at 24 h after inoculation and decreased to constitutive level at 72 h. In M14S line, the level was a little lower than that in Gh21 line at all stages after inoculation and the peak could not appear.

DISCUSSION

Cell wall is the first physical block when pathogens invade plant hosts. Lignification can fortify cell wall and increase the resistance to pathogens in plants (Moerschbacher et al., 1990). It was reported that the blue copper-binding protein of pea is correlated with lignin deposition in pod endocarp (Drew and Gatehouse, 1994). Under aluminium stress, *AtBCB* protein of *A. thaliana* was localized to cell membrane region by transient expression in onion epidermal cells. In transgenic *A. thaliana*, in which *AtBCB* gene was over-expressed, lignin production was constitutive in the whole roots and could be increased by aluminium stress in the root-tip region (Ezaki et al., 2005). In this research, the deduced BCB protein of *D. villosum* contained a transmembrane domain. So we proposed that *DvBCB* protein might be localized to cell membrane and be correlated with lignin deposition in the invaded epidermal cells of *D. villosum*. Reactive oxygen species (ROS) play crucial dual roles during defense against pathogens in plants

1 cagcaacacct ccct t ct at ct cct ct gct tt gcat t cct cct t ccagt t ccaagcgcgaat ca
 64 at ggct gccat gaagat caccct cct t gccgt ggccgcgaat ct cggcagt ct t gct aggcaccgcg cggcggcc
 M A A M K I T L L A V A A I S A V L I G T A S A A
 139 acct acggg gt cggcgagccggccggcgcgt ggacact cagcaccgact acagcacct gggg ggccgcgaagcag
 T Y G V G E P A G A W T L S T D Y S T W V A D K K
 214 tt caacgt gggg gat gaaat cgt ct t caagt act cgccct cgt cgcacgacgt ggt cgaggt cagcaagccggc
 F N V G D E I V F K Y S P S S **H** D V V E V S K A G
 289 t acgact cct gt agcaccgcggcggccat caacacgt t caagacaggcaacgacgt t at ccccd caacgt cacc
 Y D S C S T A G A I N T F K T G N D V I P L N V T
 364 ggcacccgct act t cat ct gcggcat cact ggccact gcagt cccaccgaagccgccagcat gaaggt cgt gat c
 G T R Y F I **C** G I T G **H** C S P T E A A S **M** K V V I
 439 gacgt cgcct cgggct cct ct t cgccat cct caccat gccagccgcaggt cct ggcgcgagcaat ct cccccg
 D V A S G S S S P S S P M P A A G P G A S N S P P
 514 gcgccacct cct ccgccgccacct ccgt cggg gccacagcaggat t t ggcct t gt cgcct act ggcggccggg
 A P P S S A A T S V G A T A G F G L V A L L A A G
 589 ct cat ggct t gaacacat at at t gat t agct gccgcgagct t gt at gagct t at at at at t gt t aact act act c
 L M A *
 664 t agt t at t at caccgagt gaat acgt gt at t ct t t t t agagt gagaat gt gat agt t act t gt at ct at caccg c
 739 t at at t t aat ct gcaggcct t cat gt ct t t t at at aaal aat t ct agaggat t at t aaaaaaaaaaaaaaaaaa
 814 aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

Figure 1. The nucleotide sequence of *BCB* gene and its deduced amino acid sequence. Each amino acid is below its correspondent codon. The asterisk represents the stop codon. The predicted signal peptide is underlined and the transmembrane region is boxed. The four amino acid residues shade form the possible copper-binding site.

(Vandenabeele et al., 2000). On one hand, ROS can trigger hypersensitive response at the local site invaded by pathogens and induce systemic acquired resistance in the whole plant. On the other hand, the accumulation of ROS can harm the host cells. So, plants have developed a series of enzymes as ROS-scavengers for defense against ROS stress. According to the conserved domain, Gysel et al. (1993) identified a blue copper-binding protein (AtBCB) of *A. thaliana* as a cupredoxin-like protein which could catalyze oxygenic Cu^{2+} to reactive

Cu^{+} , and proposed that *AtBCB* gene might be involved in electron transfer reactions in the cell membrane region. Ezaki et al. (2005) found that the concentration of lipid peroxides was rather low at the site of lignin accumulation which was considered to be related to BCB protein. It is also considered that oxidative burst appears at early stage after inoculation (Lamb and Dixon, 1997). In our research, the expression of *DvBCB* gene was rapidly induced and reached the peak level at 24 h after inoculation. This result indicated that the expression

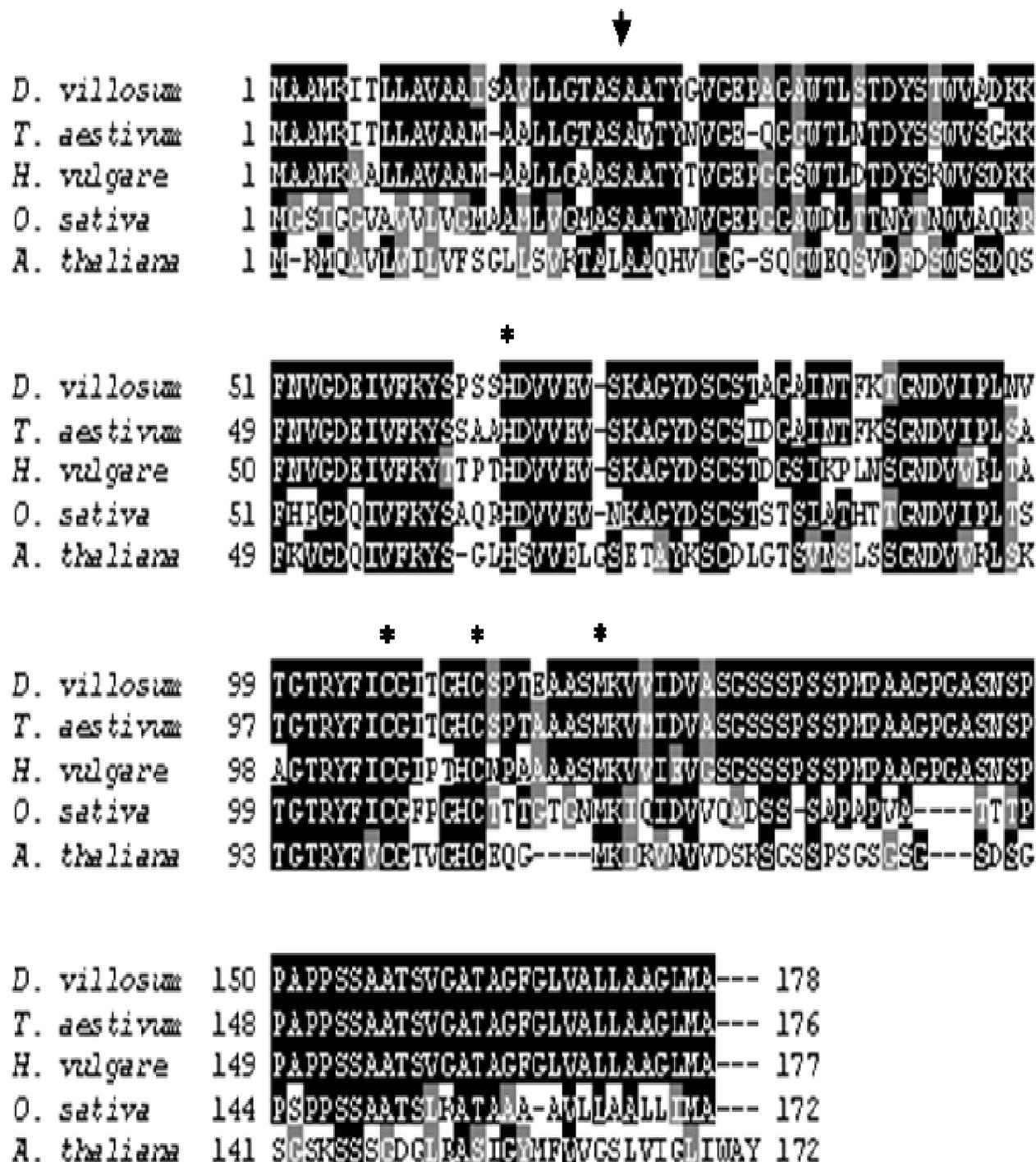


Figure 2. Amino acid sequence alignments of the deduced blue copper-binding protein (BCB) in *D. villosum* with other known plant BCBS. The accession numbers of these BCBS are shown in Figure 3. The arrowhead indicates the putative signal peptide cleavage site. Amino acid residues involved in copper binding are indicated by asterisks.

pattern of *DvBCB* gene in resistant Gh21 line was in accordance with the accumulation pattern of ROS. So, we report that *DvBCB* protein might be involved in the scavenging of ROS to protect host cells during defense

against powdery mildew in *D. villosum*.

Overall, *DvBCB* gene from *D. villosum* might have multiple functions during infection by *E. graminis*. This study could contribute more to the understanding of the

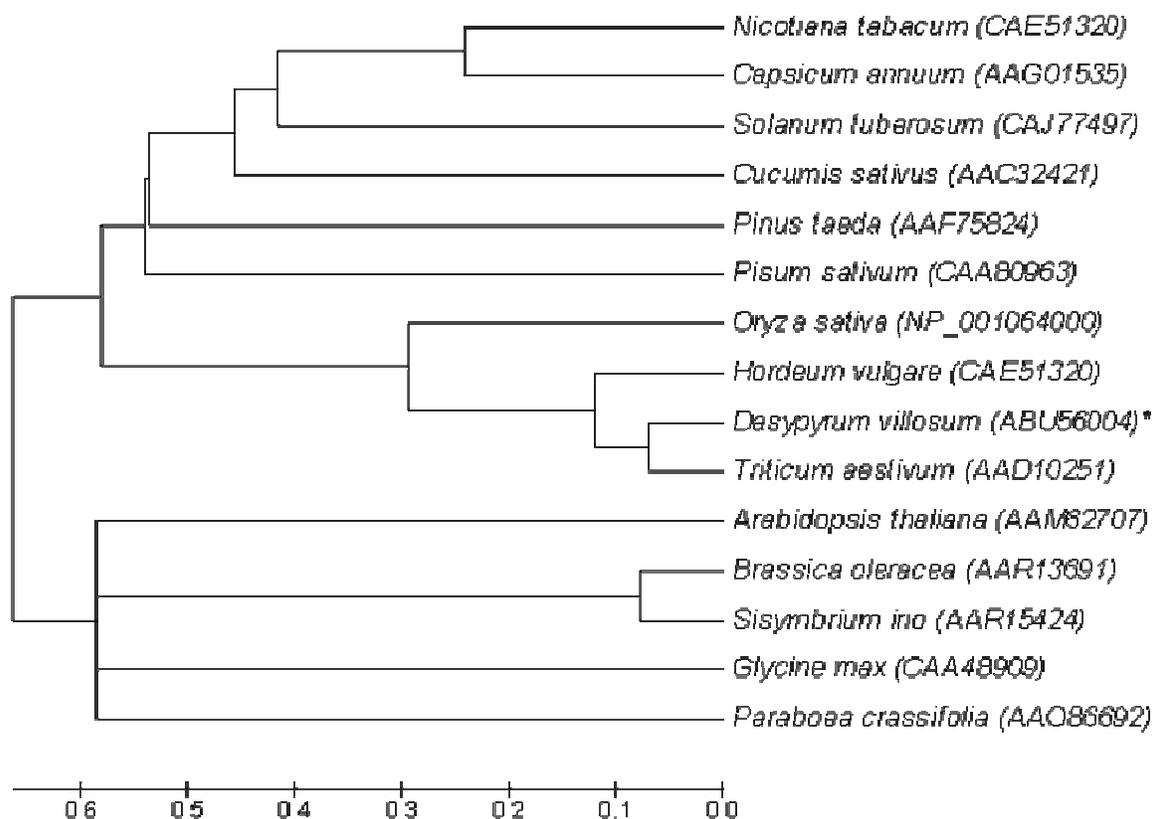


Figure 3. Phylogenetic analysis of the deduced BCB in *D. villosum* with other plant BCBs. The accession numbers of BCBs are shown in the brackets. The DvBCB protein is marked by an asterisk.

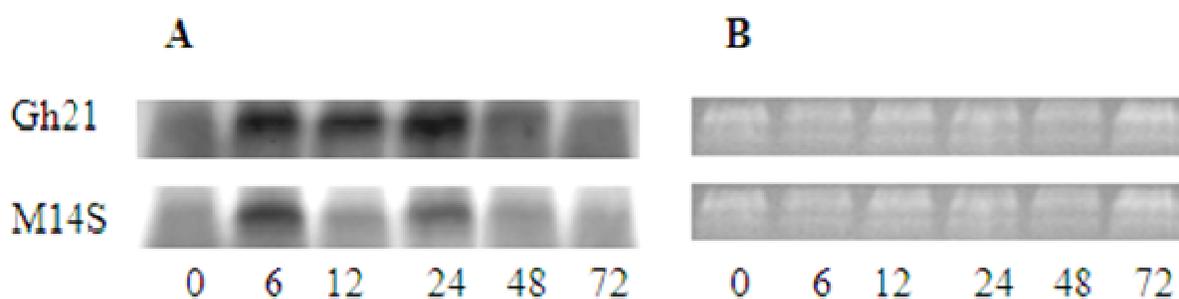


Figure 4. Northern blot analysis of *DvBCB* gene. A, Northern blot results using total RNA of Gh21 and M14S; B, electrophoresis results of 18S rRNA and 28S rRNA of Gh21 and M14S. The resistant Gh21 line and its susceptible mutant M14S line were inoculated at 0, 6, 12, 24, 48 and 72 h, respectively.

resistance mechanism of *D. Villosum* to *E. graminis*.

ACKNOWLEDGEMENTS

This research was supported by grants from Innovation Foundation for Graduate Students of Jiangsu Province (CX09B_211Z), Natural Science Foundation of Jiangsu Province (BK2009175), Modern Agricultural Technology

Research System (nocytx-03) and Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

REFERENCES

Drew JE, Gatehouse JA (1994). Isolation and characterization of a pea pod cDNA encoding a putative blue copper protein correlated with lignin deposition. *J. Exp. Bot.* 45: 1873-1884.

- Ezaki B, Sasaki K, Matsumoto H, Nakashima S (2005). Functions of two genes in aluminium (Al) stress resistance: repression of oxidative damage by the *AtBCB* gene and promotion of efflux of Al ions by the *NtGD1f* gene. *J. Exp. Bot.* 56: 2661-2671.
- Garrett TPJ, Clingeleffer DJ, Guss JM, Rogers SJ, Freeman HC (1984). The crystal structure of poplar apoplastocyanin at 1.8-Å resolution. The geometry of the copper-binding site is created by the polypeptide. *J. Biol. Chem.* 259: 1822-1825.
- Gysel A, Montagu M, Inze D (1993). A negatively light-regulated gene from *Arabidopsis thaliana* encodes a protein showing high similarity to blue copper-binding proteins. *Gene.* 136: 79-85.
- Jansen C, Korell M, Eckey C, Biedenkopf D, Kogel KH (2005). Identification and transcriptional analysis of powdery mildew-induced barley genes. *Plant Sci.* 168: 373-380.
- John CF, Morris K, Jordan BR, Thomas B, A-H-Mackerness S (2001). Ultraviolet-B exposure leads to up-regulation of senescence-associated genes in *Arabidopsis thaliana*. *J. Exp. Bot.* 52: 1367-1373.
- Kim CS, Kwak JM, Nam HG, Kim KC, Cho BH (1994). Isolation and characterization of two cDNA clones that are rapidly induced during the wounding response of *Arabidopsis thaliana*. *Plant Cell Rep.* 13: 340-343.
- Lamb C, Dixon RA (1997). The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48: 251-275.
- Lin SL, Wu L (1994). Effects of copper concentration on mineral nutrient-uptake and copper accumulation in protein of copper-tolerant and copper-nontolerant *Lotus purshianus* L. *Ecotox. Environ. Saf.* 29: 214-228.
- Luo M, Kong XY, Huo NX, Zhou RH, Jia JZ (2002). ESTs analysis of resistance to powdery mildew in wheat at primary infected stage. *Acta Genet. Sin.* 29: 525-530. (in Chinese).
- Miller JD, Arteca RN, Pell EJ (1999). Senescence-associated gene expression during ozone-induced leaf senescence in *Arabidopsis thaliana*. *Plant Physiol.* 120: 1015-1023.
- Moerschbacher BM, Noll U, Gorrichon L, Reisener HJ (1990). Specific inhibition of lignification breaks hypersensitive resistance of wheat to stem rust. *Plant Physiol.* 93: 465-470.
- Qi LL, Chen PD, Liu DJ, Zhou B, Zhang SZ, Sheng BQ, Xiang QJ, Duan XY, Zhou YL (1995). The gene *Pm21*: A new source for resistance to wheat powdery mildew. *Acta Agron. Sin.* 21: 257-261. (in Chinese)
- Richards KD, Schott EJ, Sharma YK, Davis KR, Gardner RC (1998). Aluminum induces oxidative stress genes in *Arabidopsis thaliana*. *Plant Physiol.* 116: 409-418.
- Ryden LG, Hunt LT (1993). Evolution of protein complexity: the blue copper-containing oxidases and related proteins. *J. Mol. Evol.* 36: 41-66.
- Sambrook J, Fritsch EF, Maniatis T (1989). *Molecular cloning: a laboratory manual* (3rd Ed). Cold Spring Harbor Laboratory Press, New York.
- Vandenabeele JDS, Vranova E, Montagu MV, Inze D, Breusegem FV (2000). Dual action of the active oxygen species during plant stress responses. *Cell. Mol. Life Sci.* 57: 779-795.