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Identification of Genes Differentially Expressed in a Resistant Reaction to *Fusarium Oxysporum* in *Lilium Regale* by SSH

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Abstract

Fusarium wilt, caused by the fungal phytopathogen *Fusarium oxysporum*, is the major disease of lily (*Lilium* L.). However, the interaction between lily and *F. oxysporum* is still poorly understood. In order to identify of genes differentially expressed in a resistant reaction to *F. oxysporum* in *L. regale*，a rare lily genetic resource with high resistance to *F. oxysporum*, a cDNA library of *L. regale* root infected by *F. oxysporum* was constructed using the method of suppression subtractive hybridization (SSH). In total, 585 unique expressed sequence tags (ESTs) were obtained after sequence analysis of 1101 clones, and homology analysis showed that 342 unique sequences were assigned to no significant homology. On the base of function classification established in model plant, the functional categories of the 243 ESTs which had significant homology with known proteins were assigned, and they were classified into 14 putative cellular functions. The largest group contained genes involved in disease/defense including *PR3* (chitinase), *PR10*, glutathione S-transferase (GST), cytochrome P450, catalase (CAT), peroxidase and so on. Moreover, the signal transduction related genes and important transcription factors participated in defense response against phytopathogens were included in the cDNA library of present study. Briefly, the genes differentially expressed in the resistant reaction to *F. oxysporum* in *L. regale* revealed the partial molecular mechanism associated with the resistance of *L. regale* against *F. oxysporum*.

Keywords: Lilium regale; Fusarium oxysporum; defense response; differentially expressed genes; suppression subtractive hybridization

1. Introduction

Plants usually face threats of biotic and abiotic stresses during their growth and development. Plant diseases in large numbers caused huge losses of both the crop yield and quality (Yevtushenko et al., 2005). The fungal diseases are important parts of plant diseases, such as Fusarium Head Blight infected by several pathogenic fungi of *Fusarium* sp. is the most serious disease of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). Similarly, the pathogen of cotton (*Gossypium*) Fusarium wilt disease is *F. oxysporum* f. sp. *vasinfectum* which causes considerable fiber losses in many cotton

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planting regions worldwide (Wang and Roberts, 2006). Lily (*Lilium* L.) is one of the major ornamental crops which product cut flowers, and the lily plants are often endangered by some fungal diseases during productions of fresh cut flowers and bulbs. Thereinto, the fungus *F. oxysporum* is one of the most crushing pathogens of lily, which causes the basal rot of lily plants. The infection of *F. oxysporum* causes some commonly known symptoms in plants including wilt, root rot, and yellowing (Diener and Ausubel, 2005; Lee et al., 2010).

In order to develop cultivars with desirable resistance to *F. oxysporum*, genetic resources showed high-level resistance play important roles in modern plant breeding. As for the lily, none cultivar or wild *Lilium* species is immune from the infection of *F. oxysporum*, however, there are some cultivars and wild lilies with high resistance against *F. oxysporum*, such as *Lilium* Asiatic Hybrids and *L. regale* (Lim et al., 2002). Among the lily cultivars, *Lilium* Oriental Hybrids are most susceptible to *F. oxysporum*. *L. regale* is a special and wild lily species of China, and it distributes in the river basin of Minjiang of Si Chuan province. *L. regale* was found to have extremely high resistance to fungal pathogens, viruses, and drought stress, therefore, it has been widely applied in lily breeding worldwide, and some lily cultivars with desirable traits were developed. However, the molecular mechanisms on resistance of *L. regale* against *F. oxysporum* are still unclear. To understand the molecular mechanisms on the resistance of *L. regale* against *F. oxysporum*, a cDNA library was constructed from *L. regale* under the stress of *F. oxysporum* by suppression subtractive hybridization (SSH) in this study. Isolation and analysis of resistance-related genes to *F. oxysporum* in *L. regale* supplied some theoretic knowledge for disease resistance breeding in *Lilium*.

2. Materials and methods

2.1. Plant materials and pathogen infection

A highly aggressive *F. oxysporum* strain kept in our laboratory was activated on potato dextrose agar (PDA) plates for 5-7 days at 28 °C. The spores were then washed from the plate with sterile distilled water. The concentration of liquid suspension was diluted to 10^6 spores per mL with sterile distilled water before infection. The sterile bulblets of *L. regale* were infected with spores of *F. oxysporum* using the method of root dip inoculation. In parallel, a treatment with sterile distilled water, termed mock-inoculation, was performed as control. Root of the bulblets was collected at 2, 4, 8, 12, 24, 36, 48, 72 hours post-inoculation (hpi), respectively. All plant tissues were treated with liquid nitrogen then stored at −80 °C until nucleic acid isolation.

2.2. Total RNA and mRNA isolation

According to the protocol of Liu et al (Liu et al., 2006), the total RNA was isolated from the collected root using the method of guanidine thiocyanate. Furthermore, the RNA was treated with DNase I (Promega, USA) for 1 h to remove DNA contamination. Then mRNA was isolated and purified using NucleoTrap® mRNA Mini Kit (MACHEREY-NAGEL, Germany) with the method described in the manufacturer's user manual.

2.3. SSH cDNA library construction and analysis

The SSH cDNA library was constructed with PCR-Select™ cDNA Subtraction Kit (Clontech, USA) based on the user manual. The mRNA from the infected *L. regale* root collected in the different time points (2, 4, 8, 12, 24, 36, 48 and 72 hpi) were mixed together in equal proportions as the tester, and mRNA from the water-treated root at the same time intervals was as the driver. The subtracted PCR products were ligated into the pGEM-T Easy vector (Promega, USA), then transformed into *Escherichia coli* DH5α competent cells. Based on blue-white spot screening, the positive transformants were picked and cultured overnight in 96-well microplates. The length of each inserts was analyzed through PCR with the specific primers of 1 and 2R. All PCR products were analyzed by 1.2% agarose gelelectrophoresis and clones with inserts < 200 bp or more than one insert were removed, and the other clones were chosen to sequence.

2.4. Sequence and bioinformatics analysis

Colonies were sequenced using the universal M13 sequencing primer (Sangon, China). The sequence analysis was performed with the methods descried by Xu et al (2011). The annotated ESTs were assigned to functional categories according to a catalog system described by Bevan et al (1998).

3. Results and discussion

3.1. Isolation of differentially expressed genes in L. regale after F. oxysporum inoculation by SSH

To enrich the differentially expressed genes in resistant defense response of *L. regale*, a subtracted cDNA library of *L. regale* inoculated with *F. oxysporum* was constructed. The cDNA transcribed from the mRNA of *F. oxysporum* infected root was as the tester, and the cDNA from mock-inoculated root as the driver. Two rounds of subtraction between the tester and the driver were performed, then the pool of differentially expressed gene fragments was amplified with primers of 1 and 2R. Through TA-cloning and blue-white spot screening, 3168 clones were randomly picked.

Totally, 1726 clones were screened out by colony PCR, and the length of cDNA inserts ranged from 200 to 1000 bp with average length of 400 bp. Then 1101 clones were successfully sequenced, and there were 585 unique sequences including 194 contigs and 391 singlets through CAP3 sequence analysis.

3.2. Functional categories of genes responsive to F. oxysporum infection

The 585 unique sequences were then performed homology analysis with BLASTx tool in GenBank. As a result, more than one half of the unique sequences were assigned to no significant homology. The partial result of homology analysis was showed in Table 1. The functional categories of the 243 ESTs which had significant homology with known proteins were assigned, as a result, they were classified into 14 putative cellular functions on the base of categories established in *Arabidopsis* (Fig 1).

Table 1. The list of representative clones of SSH cDNA library from *L. regale* infected by *F. oxysporum*

Clone no.	Numbers of identical clones	Accession no. of matching sequence	Matching sequence from database	Source of matching sequence	E-value
			Disease/defense		
F03D11	7	AAD17336.1	intracellular pathogenesis-related protein PR-107	*Lilium longiflorum*	5.00E-43
F28A03	14	AAF21623.1	intracellular pathogenesis-related protein PR-104	*Lilium longiflorum*	5.00E-54
F16A02	7	AEV89264.1	pathogenesis-related protein	*Musa acuminata*	3.00E-41
F21F04	2	ACF06558.1	germin A	*Elaeis guineensis*	8.00E-41
F10D12	1	ACG41245.1	germin-like protein subfamily 1 member 17	*Zea mays*	3.00E-18
F06H12	1	XP_003563447.1	lipid transfer-like protein VAS-like	*Brachypodium distachyon*	6.00E-12
F19C02	13	CAA39535.1	chitinase	*Oryza sativa*	6.00E-54
F18A04	8	AAA32640.1	chitinase, partial	*Allium sativum*	4.00E-64
F03G03	1	XP_002521689.1	class I chitinase, putative	*Ricinus communis*	0.005
F11A08	1	BAG38685.1	chitinase A	*Ananas comosus*	3.00E-09
F04A10	1	AAX83262.1	class II chitinase	*Triticum aestivum*	2.00E-27
F13D09	2	XP_002516746.1	14-3-3 protein, putative	*Ricinus communis*	1.00E-51
F15F01	3	XP_002511298.1	cytochrome P450, putative	*Ricinus communis*	4.00E-32
F07F12	8	ABO20848.1	cytochrome P450-like TBP protein	*Lilium longiflorum*	2.00E-54
F03B11	1	ACF06485.1	cytochrome b5	*Elaeis guineensis*	2.00E-09
F16H06	1	BAK22528.1	tau glutathione S-transferase	*Allium cepa*	5.00E-73
F25H07	1	NP_001236903.1	glutathione S-transferase GST 23	*Glycine max*	6.00E-17
F15A06	1	XP_002273830.1	glutathione S-transferase U17 isoform 1	*Vitis vinifera*	2.00E-81
F06C12	1	ACF06490.1	glutathione-S-transferase Cla47	*Elaeis guineensis*	4.00E-21
F03F10	1	XP_002263386.1	glutathione S-transferase F13	*Vitis vinifera*	0.037
F14H08	1	ADB11335.1	phi class glutathione transferase GSTF7	*Populus trichocarpa*	1.00E-16
F13E08	1	AAK29077.1	mannan-binding lectin	*Crocus sativus*	9.00E-13
F10D10	2	BAH37015.1	NtRab11D	*Nicotiana tabacum*	6.00E-62
F20G08	2	ADD09573.1	dehydrin b	*Trifolium repens*	5.00E-08
F18E02	2	ABO42703.1	putative ADP-ribosylation factor	*Jacobaea maritima*	7.00E-59

Clone no.	Numbers of identical clones	Accession no. of matching sequence	Matching sequence from database	Source of matching sequence	E-value
F15H05	7	CAI77769.1	kunitz trypsin inhibitor	*Populus tremula*	2.00E-30
F06E08	1	NP_176059.1	GDSL esterase/lipase LIP-4	*Arabidopsis thaliana*	2.00E-06
F01E04	1	ABR19827.1	cysteine proteinase	*Elaeis guineensis*	2.00E-77
F01G01	2	XP_003625315.1	Disease resistance response protein	*Medicago truncatula*	2.00E-07
F07B08	1	AEX55236.1	late embryogenesis abundant protein lea14-a	*Allium sativum*	4.00E-22
F12B06	2	EFY91418.1	calcofluor white hypersensitive protein	*Metarhizium acridum*	2.00E-12
F13H07	1	BAM28967.1	phenylalanine ammonia lyase	*Lilium*	7.00E-33
F15D09	1	BAM28965.1	phenylalanine ammonia lyase, partial	*Lilium*	1.00E-140
F16E08	1	ACZ60130.1	abscisic stress ripening	*Musa paradisiaca*	6.00E-04
F26E01	1	XP_003635036.1	heat shock cognate protein 80-like	*Vitis vinifera*	1.00E-49
F18B12	1	AAR14052.2	catalase	*Solanum tuberosum*	2.00E-08
F13H04	2	ADF43731.1	monodehydroascorbate reductase	*Lilium longiflorum*	6.00E-81
F23A07	2	XP_002276789.2	peroxidase 5-like	*Vitis vinifera*	2.00E-22
F20F05	6	XP_002266365.2	cationic peroxidase 1-like	*Vitis vinifera*	5.00E-41
F28G11	3	CAD67479.1	peroxidase	*Asparagus officinalis*	2.00E-128
F16B10	2	XP_002518482.1	Peroxidase 63 precursor, putative	*Ricinus communis*	9.00E-12
F14F02	4	XP_003558949.1	peroxidase 5-like	*Brachypodium distachyon*	6.00E-24
F26F12	1	XP_002529755.1	glutathione peroxidase, putative	*Ricinus communis*	6.00E-22
F28C12	1	ADX86748.1	peroxidase PX5	*Cinnamomum micranthum*	2.00E-09
F23E03	1	XP_002269918.1	Peroxidase 4	*Vitis vinifera*	2.00E-76
F10G01	3	NP_001237535.1	thioredoxin h1	*Glycine max*	8.00E-23
F13D01	5	ABX79345.1	thioredoxin h	*Vitis vinifera*	7.00E-47
F22G08	1	ACA13182.1	type II peroxiredoxin	*Xerophyta viscosa*	4.00E-54
F20D05	3	AAW58111.1	polyphenol oxidase	*Prunus salicina*	7.00E-23
F10H06	1	AFI08583.1	polyphenol oxidase IVa	*Malus x domestica*	8.00E-06
F04F11	1	AFX61785.1	manganese superoxide dismutase	*Musa acuminata*	1.00E-09
F17C06	1	NP_001147645.1	oxidoreductase	*Zea mays*	1.00E-70
			Signal transduction		
F02G10	4	AAD10247.1	calmodulin	*Phaseolus vulgaris*	1.00E-27
F07D10	1	XP_003557972.1	serine/threonine-protein kinase AtPK2/AtPK19-like	*Brachypodium distachyon*	2.00E-74
F16G08	2	XP_002528537.1	serine-threonine protein kinase	*Ricinus communis*	8.00E-70
F27G03	4	AEX55233.1	cold-regulated gibberellin-regulated protein 1 LTCOR12	*Allium sativum*	2.00E-27
F03D05	2	ABB47623.1	auxin-induced protein PCNT115	*Oryza sativa*	2.00E-70
F20D02	3	XP_002533935.1	pyruvate kinase, putative	*Ricinus communis*	9.00E-19
F27E07	2	ACF06501.1	SKP1	*Elaeis guineensis*	6.00E-30
F09A03	1	BAC57816.1	putative syntaxin SYP111	*Oryza sativa*	1.00E-26
F14A01	1	ACF78996.1	catalytic/ hydrolase	*Zea mays*	2.00E-11
F25E02	1	ADK56125.1	purple acid phosphatase	*Phaseolus vulgaris*	7.00E-13
			Transcription		
F18F10	3	XP_002272040.1	probable WRKY transcription factor 33-like	*Vitis vinifera*	3.00E-05
F27E12	3	ABB17073.1	bZIP transcription factor	*Capsicum annuum*	3.00E-19
F12F06	2	AEB35702.1	MYC2	*Helianthus annuus*	9.00E-10
F01B03	2	XP_002277951.1	transcription elongation factor 1 homolog isoform 1	*Vitis vinifera*	4.00E-37

Clone no.	Numbers of identical clones	Accession no. of matching sequence	Matching sequence from database	Source of matching sequence	E-value
F04E05	3	ACZ95473.1	disulfide isomerase	*Gloeospermum blakeanum*	2.00E-07
F02F10	1	AAQ56324.1	putative poly(A)-binding protein	*Oryza sativa*	2.00E-28
F07G11	1	NP_179895.6	RING/U-box domain and ARM repeat-containing protein	*Arabidopsis thaliana*	5.00E-07
F12D07	1	ABB47998.1	Zinc finger, C3HC4 type family protein	*Oryza sativa*	5.00E-10
F17C02	1	ABQ42139.1	longevity factor-like protein	*Sonneratia apetala*	4.00E-15
F18A05	1	AFP57452.1	RNA-binding glycine-rich protein	*Nicotiana repanda*	2.00E-04
F18A10	1	XP_003535166.1	zinc finger CCCH domain-containing protein 30-like	*Glycine max*	0.001
F24A02	1	NP_173499.1	calcium-binding EF hand-containing protein	*Arabidopsis thaliana*	5.00E-14
F27C11	1	ACB59195.1	2-isopropylmalate synthase	*Brassica oleracea*	2.00E-09

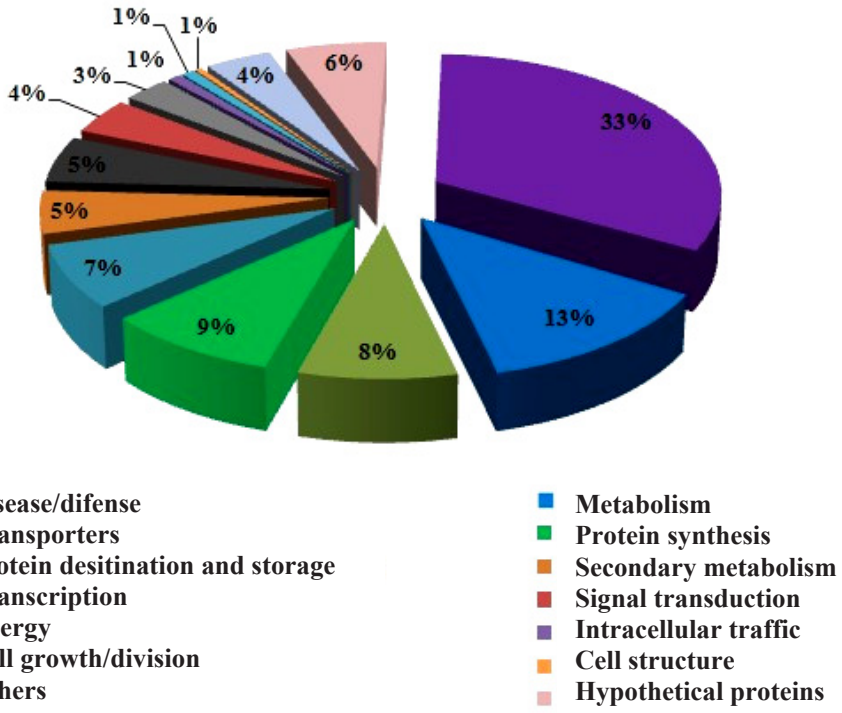

Fig.1. Functional categories of the ESTs isolated from *L. regale* inoculation with *F. oxysporum*

Among the 14 functional categories, the largest proportion belonged to the category "disease/defense" (33%). The genes involved in disease/defense constituted the biggest part of the cDNA collection suggested that the resistance related genes of *L. regale* were enriched in the SSH cDNA library of present study. The majority of the genes included in the category of disease/defense were pathogenesis-related genes and oxidative stress related genes, such as PR3 (chitinase), PR5 (thaumatin-like protein), PR10, glutathione S-transferase (GST), cytochrome P450, catalase (CAT), peroxidise, and heat shock protein (HSP). The above genes were identified as resistance related genes differentially expressed after inoculated pathogens in the previous studies (VAN DEN Berg et al., 2007; Xu et al., 2011). The well-known PR genes, *CHI* and *TLP* have been proven to be part of the defense network in various plant-pathogen interactions (Gao et al., 2012). CHIs and TLPs were involved in the degradation of structural components of some pathogenic fungi (Tobias et al., 2007). Moreover, *GSTs* were identified as toxin catabolic process related genes conferring resistance to fungal pathogens and oxidative stresses (Barthelson et al., 2010).

Besides, the oxidative stress related genes abundantly assembled in root of *L. regale* during response to *F. oxysporum*

infection including the genes encoding glutathione peroxidase, polyphenol oxidase, thioredoxin, CAT, manganese superoxide dismutase, and a series of peroxidases. Oxidative burst is an early defense response after pathogen attack. The generation of reactive oxygen species (ROS) triggers the hypersensitive response (HR) in response to pathogen attack, therefore, it is an important part of the resistance mechanisms. However, excessively releasing of free radicals would damage the cellular components of host plant. The above oxidative stress related genes encoding ROS scavengers play important roles in defense response against phytopathogens (Grimaud et al., 2001). The oxidative stress related genes were highly expressed during the oxidative burst, thus the oxidative damages of plant cells are greatly reduced. Obviously, The oxidative stress related genes should be an important part of defense response in the interaction between *L. regale* and *F. oxysporum*.

What is more, protein-related functional categories (15%) consisted of protein synthesis (8%) and protein destination or storage (7%). About 13% of unique genes were assigned to the metabolism-related functional category, and the percentage of the genes involved in transporting of sugars, amino acids, lipids and ions was 9%. Moreover, there were 5% and 4% of the unique ESTs belonged to the pathways of transcriptional regulation and signal transduction, respectively. These genes involved in intracellular disease resistance signaling pathways and regulation the transcription of resistance related genes including Zinc finger family protein, calmodulin, serine/threonine-protein kinase, bZIP transcription factor, WRKY transcription factor and so on. A serine/threonine kinase gene *Pto* in potato conferred resistance to *Pseudomonas syringae* pv. tomato (Sessa and Martin, 2000), and another serine/threonine kinase gene *Stpk-V* conferred powdery mildew resistance in wheat (Cao et al., 2011). A basic leucine zipper transcription factor (*TabZIP1*) isolated from wheat functioned in resistance to abiotic and biotic stresses (Zhang et al., 2008). The genes in signal transduction pathways as well as the transcription factors were key players in transducing signals generated in response to pathogen infection.

In conclusion, lots of differentially expressed genes in *L. regale* response to *F. oxysporum* infection were isolated using the SSH technology in the present study. This is the first large-scale investigation into the interaction between *L. regale* and *F. oxysporum* at the molecular level. Furthermore, the detailed expression and functional analysis of these genes will help us to deeply understand the molecular mechanism of resistant defense response in *L. regale* against *F. oxysporum*.

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References

[1] Yevtushenko DP, Romero R, Forward BS, etc. Pathogen-induced expression of a cecropin A-melittin antimicrobial peptide gene confers antifungal resistance in transgenic tobacco. J Exp Bot, 2005; 56:1685-95.
[2] Wang C, Roberts PA. A Fusarium wilt resistance gene in *Gossypium barbadense* and its effect on root-knot nematode-wilt disease complex. Phytopathology, 2006; 96:727-34.
[3] Diener AC, Ausubel FM. *RESISTANCE TO FUSARIUM OXYSPORUM 1*, a dominant *Arabidopsis* disease-resistance gene, is not race specific. Genetics, 2005; 171:305-21.
[4] Lee H, Damsz B, Woloshuk CP, etc. Use of the plant defense protein osmotin to identify *Fusarium oxysporum* genes that control cell wall properties. Eukaryot Cell, 2010; 9:558-68.
[5] Lim JK, Rhee HK, Kim YJ, etc. Resistance to *Fusarium oxysporum* f.sp. *lilii* in *Lilium*. XXVI International Horticultural Congress. Toronto: ISHS; 2002.
[6] Liu D, Zhang X, Tu L, etc. Isolation by suppression-subtractive hybridization of genes preferentially expressed during early and late fiber development stages in cotton. Mol Biol, 2006; 40:825-34.
[7] Xu L, Zhu LF, Tu LL, etc. Differential gene expression in cotton defence response to *Verticillium dahliae* by SSH. Journal of Phytopathology, 2011; 159:606-15.
[8] Bevan M, Bancroft I, Bent E, etc. Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. Nature, 1998; 391:485-8.
[9] VAN DEN Berg N, BERGER DK, HEIN I, etc. Tolerance in banana to Fusarium wilt is associated with early up-regulation of cell wall-strengthening genes in the roots. Mol Plant Pathol, 2007; 8:333-41.
[10] Gao M, Wang Q, Wan R, etc. Identification of genes differentially expressed in grapevine associated with resistance to *Elsinoe ampelina* through suppressive subtraction hybridization. Plant Physiol Biochem, 2012; 58:253-68.
[11] Tobias DJ, Manoharan M, Pritsch C, etc. Co-bombardment, integration and expression of rice chitinase and thaumatin-like protein genes in barley (*Hordeum vulgare* cv. Conlon). Plant Cell Rep, 2007; 26:631-9.

[12] Barthelson RA, Qaisar U, Galbraith DW. Functional analysis of the *Gossypium arboreum* genome. Plant Mol Biol Rep, 2010; 28:334-43.
[13] Grimaud R, Ezraty B, Mitchell JK, etc. Repair of oxidized proteins. Identification of a new methionine sulfoxide reductase. J Biol Chem, 2001; 276:48915-20.
[14] Sessa G, Martin GB. Signal recognition and transduction mediated by the tomato Pto kinase: a paradigm of innate immunity in plants. Microbes Infect, 2000; 2:1591-7.
[15] Cao AZ, Xing LP, Wang XY etc. Serine/threonine kinase gene *Stpk-V*, a key member of powdery mildew resistance gene *Pm21*, confers powdery mildew resistance in wheat. Proc Natl Acad Sci USA, 2011; 108:7727-32.
[16] Zhang Y, Zhang G, Xia N, etc. Cloning and characterization of a bZIP transcription factor gene in wheat and its expression in response to stripe rust pathogen infection and abiotic stresses. Physiol Mol Plant P, 2008; 73:88-94.