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Characterization of a novel anther-specific gene encoding a leucine-rich repeat protein in petunia

Y.Z. Yue, J. Sun, X. Huang, H. Peng, G.F. Liu and H.R. Hu

Key Laboratory of Horticultural Plant Biology (Ministry of Education),
College of Horticulture and Forestry Sciences,
Huazhong Agricultural University, Wuhan, Hubei Province, China

Corresponding author: H.R. Hu
E-mail: huhuirong@mail.hzau.edu.cn

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ABSTRACT. In *Petunia* x *hybrida* 'Fantasy Red', a leucine-rich repeat (LRR) gene referred to as *PhLRR*, was identified in a flower bud cDNA library. The open reading frame sequence of *PhLRR* was 1251 bp, encoding a putative 46.2-kDa protein of 416 amino acids. The PhLRR protein showed high similarity to members of polygalacturonase inhibitor proteins (PGIPs), contained 11 conserved LRR domains, and was an extracellular localization protein. Phylogenetic analysis showed that PhLRR belonged to the same PGIPs subfamily as SHY, indicating that *PhLRR* may be involved in the development of pollen-like *SHY*. Expression analysis revealed that *PhLRR* was abundantly expressed during early stages of flower bud and anther development, while it was not detected in any other examined organs, such as sepals, petals, pistils, roots, stems, leaves, or open flowers. Furthermore, many cis-acting elements (such as AGAAA and GTGA) related to anther-specific gene expression were identified in the *PhLRR* gene promoter region, indicating that the promoter is also anther-specific. These results suggested that *PhLRR* is a novel anther-specific gene

that may be essential for the early development of anthers.

Key words: *Petunia* x *hybrida* 'Fantasy Red'; Anther-specific gene; Quantitative real-time RT-PCR; Promoter region

INTRODUCTION

Anthers form during a specific period of flowering plant life cycles, carrying pollens, which plays a key role in plant reproduction. Recently, several anther-specific genes related to pollen development have been identified in various plant species through high-throughput genomic studies (Kato et al., 2010; Li et al., 2010; El-Shehawi et al., 2011; Viswanathan et al., 2011). Moreover, the production of fertile pollen depends on a complex regulatory network, as expression of anther/pollen-specific genes may play vital roles in this process, and disruption during development of anther/pollens can result in male-sterile plants (Glover et al., 1998). Interestingly, various cis-acting regulatory/enhancer elements involved in anther/pollen-specific expression have been identified in the promoters of anther/pollen-specific genes, and the 5¢ upstream regions of anther/pollen-specific genes typically exhibit tissue-specific functions (Swapna et al., 2011; Chen et al., 2010, 2012b; Khurana et al., 2013).

Petunia hybrida is one of the most important ornamental plants, and is also a model material for the study of plant development biology. Many anther/pollen-specific genes have been identified through PCR-based screening of organ-specific cDNA libraries in petunia (Kobayashi et al., 1998; Cnudde et al., 2006). However, specific anther development-related genes have not been fully identified or characterized, and the molecular mechanism of anther development is largely unknown in petunia.

Leucine-rich repeat (LRR)-containing proteins from plants have diverse overall structures and functions. The largest group in the LRR superfamily include the adhesive proteins (Kobe and Deisenhofer, 1995). The structure of the PhLRR protein is nearly identical to those of polygalacturonase inhibitor proteins (PGIPs). PGIPs have been identified as defense proteins as their activity increases in response to wounding, elicitors, and fungal infection (De Lorenzo et al., 2001). However, PGIPs of plants may not always function as defense proteins; they also participate in developmental activities. The SHY protein, which plays an important role in mediating pollen tube growth, functions in a signal transduction pathways (Guyon et al., 2004).

In the present study, *PhLRR*, an anther-specific gene containing LRRs, was isolated from flower buds. Expression analysis and promoter cis-acting element prediction suggested that *PhLRR* is an anther-specific gene expressed in flower buds during initial developmental stages. This is the first report regarding the expression and functional analysis of the *PhLRR* gene in plants. Studies examining *PhLRR* may further our understanding of the molecular mechanisms of anther development in petunia.

MATERIAL AND METHODS

Plant materials

Petunia plants (*Petunia* x *hybrida* 'Fantasy Red') were grown in the experimental

field of Huazhong Agricultural University, Wuhan, China. Materials for RNA extraction were sampled from at least 5 individual flowering plants and mixed randomly. RNA was extracted from the flower buds and anthers at 8 developmental stages [bud1 (Bl < 2 mm), bud2 (Bl 3 ± 0.5 mm), bud3 (Bl 5 ± 0.5 mm), bud4 (Bl 10 ± 0.5 mm), bud5 (Bl 15 ± 0.5 mm), bud6 (Bl 20 ± 0.5 mm), bud7 (Bl 25 ± 0.5 mm), and bud8 (Bl 35 ± 0.5 mm)] (Bl indicates the bud length excluding sepal), and from roots, tender stems, fresh leaves, and opening flowers, as well as from 4 whorls of floral organs (sepal, petal, anther, and pistil) in bud2, for use in real-time reverse transcriptase (RT)-polymerase chain reaction (PCR) analysis. Upon harvesting, these materials were immediately frozen in liquid nitrogen and stored at -80°C until RNA was extracted.

DNA/RNA extraction and cDNA synthesis

Genomic DNA was extracted from young petunia leaves using the CTAB method (Murray and Thompson, 1980). Total RNA was extracted from flower buds, anthers, and other tissues using RNAiso Reagent (TaKaRa, Shiga, Japan) according to previously described methods (Yue et al., 2013). RNA quality was evaluated by electrophoresis on 1.2% denaturing agarose gels. First-strand cDNA was synthesized using a TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGene, Beijing, China) according to manufacturer instructions.

Isolation of *PhLRR* and its promoter

Expressed sequence tags (ESTs) of *PhLRR* were acquired from the library by suppression subtractive hybridization. Through the EST BLAST in the genome database of *Petunia hybrida*, we successfully predicted the full-length open reading frame (ORF) of *PhLRR* and the promoter region using the FGENESH tool (http://www.softberry.com). The full-length cDNA and gDNA were amplified using the primers F (5'-GCAGGAAGTTGGCAAAAGATG-3') and R (5'-GTCGCTCAGATAGAAGAAATAAGGC-3'). The primers F (5'-GCGGTTTGAAC TATTGATTTACG-3') and R (5'-TTTGTTTAGACAGCTTTTGAGTGAC-3') were used to clone the promoter region of the *PhLRR* ATG start codon from petunia genomic DNA. These primers were all designed using the Primer 5 software.

Sequence analysis

Sequence character prediction of *PhLRR* was conducted using the ProtParam tool (http://www.expasy.ch/tools/protparam.html). Hydrophobicity of *PhLRR* was analyzed using the ProtScale tool (http://web.expasy.org/protscale/). A phylogenetic tree was constructed using MEGA5 based on the neighbor-joining method with 1000 bootstrap replications (Kumar et al., 2004). Amino acid sequences used in phylogenetic tree construction were obtained from NCBI (http://www.ncbi.nlm.nih.gov/). Sequence of amino acid sequences were aligned using the ClustalW method (www.ebi.ac.uk/clustalw). The putative domain was predicted by Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). NetNGlyc 1.0 Server and NetPhos 2.0 Server were used to predict the N-linked glycosylation sites and phosphorylation sites. PLACE (http://www.dna.affrc.go.jp/PLACE/signalup.html) was used to search for putative cis-acting elements in the promoter region.

Expression analysis by quantitative real-time RT-PCR

Quantitative real-time RT-PCR (qRT-PCR) was performed using an ABI 7500 Fast Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA). The primers F (5'-GAAGGAGATGAATTGGAGGGG-3') and R (5'-CATTAAAAAG ACTGCCAGAGAAAAA-3') were designed using the Primer Premier 5 software. *Beta-Actin* was used as an internal control, and a negative control (no template) was included in each run. Reactions were performed using the SYBR® Premix Ex Taq™ (TaKaRa). Briefly, PCR products were amplified using 1 μL template from the RT reaction mixture, 5 μL 2X SYBR® Premix Ex Taq™, 0.5 μL of each forward and reverse primer (10 μM), and water to a final volume of 20 μL. The thermal cycling conditions were programmed based on previously described methods (Yue et al., 2013). A melting temperature cycle was performed to confirm the presence of a single product. qRT-PCR was performed in 3 replicates for each sample, with the data shown as means ± SD (N = 3).

RESULTS

Cloning of *PhLRR*

To identify new genes encoding proteins that regulate flower bud development in petunia plants, we constructed a suppression subtractive hybridization cDNA library. A cDNA fragment of 663 bp appeared to accumulate specifically in the flower bud. The sequence BLAST results (BLASTX) showed that the putative amino acid sequence encoded by this fragment was homologous to LRR proteins. Thus, EST BLAST of the genome database of *Petunia hybrida* successfully predicted the full-length ORF of the LRR protein using the FGENESH tool (http://www.softberry.com). A sequence with putative ORFs were obtained using RT-PCR and designated as *PhLRR* (GenBank accession No. KF146986).

Sequence analysis

The 1355-bp *PhLRR* cDNA contains a 1251-bp ORF, encoding a 416-amino acid protein (Figure 1). The deduced molecular mass of the mature PhLRR protein was 46.2 kDa, with a predicted pI of 7.08. The amino acid sequence displayed 17 possible phosphorylation sites and 4 putative glycosylation sites for N-linked glycosyl chains (NXS/T, where X denotes any amino acid).

The region spanning from amino acids 90-344 contained 11 tandem repeats with an average size of 24 amino acids: 13 of the 24 residues were found to be conserved (Figure 2). The composition of the repeats was very similar to the LRR motif, which was observed in several other proteins (Figure 2). Alignment of LRRs revealed a 24-amino acid repeat matching the external LRR consensus (Figure 2), LxxLxxLxxLxLxxNxLxGxIPxx, as observed in the tomato Cf-2 and Cf-9 genes (Jones and Jones, 1997). Using GenomeNet CLUSTALW, multiple sequence alignment was obtained for 14 accessions of the PhLRR/PGIP/LRP/SLRP gene family. Phylogenetic analysis showed that different types of LRR proteins were classified into the same groups. *PhLRR* and *SHY* were clustered in the same clade (Figure 3).

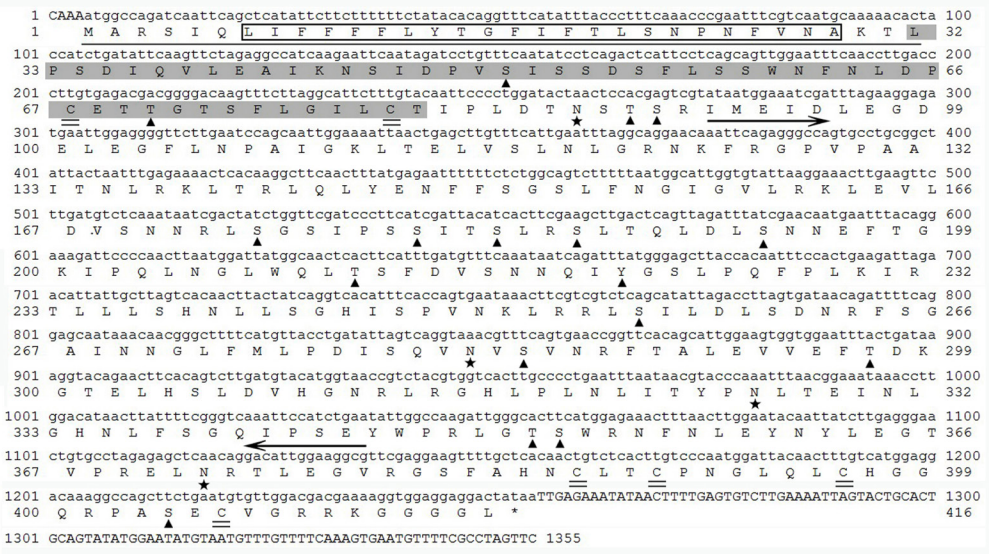

Figure 1. cDNA and deduced amino acid sequence of the *PhLRR* gene. The small asterisk at position 1253 indicates the translation termination codon. The putative signal peptide for secretion is underlined. Putative N-glycosylation sites and the phosphorylation sites are shown by black triangles and by big asterisks, respectively. The putative leucine zipper domain is represented by shaded box. The predicted transmembrane sequence is boxed. The LRR region is marked by arrows.

Expression analysis of *PhLRR*

Expression analysis was performed to examine the space and time expression profile of *PhLRR*. In flower buds in different stages, this gene was highly amplified in bud1 to bud2 and largely decreased in bud3 (Figure 4). *PhLRR* was found to be expressed only in anthers, but not detected in sepals, petals, pistils, roots, tender stems, fresh leaves, or open flowers (Figure 5). Since the anther is only a small component of the petunia flower organ, particularly in stages bud4 to bud8, the expression of this gene may not be detected during late-stage flower buds. Hence, to explore the expression of this gene in different stages of anther development, anthers (an1 to an8) were stripped from bud1 to bud8, respectively, and subjected to time expression analysis. The results showed that *PhLRR* was highly expressed during the initial stages of anther development (Figure 6).

Promoter analysis of *PhLRR*

PhLRR was particularly abundant in anthers. To gain further insight into the regulation mechanism of *PhLRR* expression, a 1254-bp promoter upstream of the *PhLRR* ATG start codon from petunia genomic DNA was cloned (Figure 7). Various regulatory motifs (Swapna et al., 2011) related to anther/pollen-specific expression were observed in the *PhLRR* promoter region, such as ACGT MOTIF (ACGT), DOFCOREZM (AAAG), POLLENILELAT52 (AGAAA), GTGA MOTIF (GTGA), LAT enhancer element (TGTGA, TGTGG), and LAT52 quantitative element (TGGTTA) (Figure 7).

A

	. . . . . I MEI DLEGDELEG. FLNPA	1
	I GKLTELVSLNLGRNKFRG. PVPAA	2
	I TNLRKLTRLQLYENFFSG. SLFNG	3
	I GVLRKLEVLDVSNNRLSG. SI PSS	4
	I TSLRSLTQLDLSNNEFTG. KI PQ.	5
	LNGLWQLTSFDVSNNQI YG. SLPQF	6
	. . . PLKI RTLLLSHNLLSG. HI SP.	7
	VNKLRRLSI LDLSDNRFSG. AI NNG	8
	LFMLPDI SQVNVSVNRFTALEVVEF	9
	TDKGTELHSLDVHGNRLRG. HLPLN	10
	LI TYPNLTEI NLGHNLFSG. QI PSE	11
Consensus	LxxLxxLxxLDLSxNxFSG xI Pxx	

B

PROTEIN	CONSENSUS SEQUENCE	LRRs	REFERENCE
	LxxLxxLxxLDLxxNxLxGxI Pxx		
PhLRR	LxxLxxLxxLDLSxNxFSGxI Pxx	11	Yue, this work
BRI1	FxxFxxLxxLxLSxNxFSGxI Pxx	25	Li and Chory, 1997
CALRR1	LxxLxxLxxLxLxxNxLxGxI Pxx	5	Jung et al., 2004
Cf-2.1	LGNLxxLxxLxLxxNxLxGSI Pxx	36	Dixon et al., 1996
Cf-9	LxxLxxLxxLDLSSNNLxGxI PSx	28	Jones et al., 1994
CLAVATA1	FxxFxxLxxLxFxxNxFTGxI Pxx	23	Clark et al., 1997
FIL2	LxxLxxLxSLxLSxNxLxGxI Pxx	10	Steinmayr et al., 1994
LRPKm1	LxxLxxLxxLDLxxNxLSGxI Pxx	23	Komjanc et al., 1999
N	xxxLxxLxxLDLxxxxxLxxLPxL	14	Whitham et al., 1994
NtLRR1	LxxLxxLxxLxLxxNxLxGxI Pxx	9	Xu et al., 2009
NtLRR2	LxxLxxLxxLxLxxNxLxGxI Pxx	3	Xu et al., 2009
PGIP(bean)	LxxLxxLxxLDLSxNxLxGxLPxx	10	De Lorenzo et al., 1994
RLK5	LxxLxxLxxLxLxxNxLSGxI Pxx	21	Walker et al., 1993
RPM1	PxxLxxLxxLDL. xxxxLxxLPxL	8	Grant et al., 1995
RPS2	LxxLxxLxxLDLxxxxxLxxLPxx	11	Bent et al., 1995
SHY	LxxLxxLxxLxLxxNxLxGxI PxS	10	Guyon et al., 2004
Xa21	LxxLxxLxxLDLSSNNLxGxI Pxx	23	Song et al., 1995
ZmSERK1	LGxLxxLxxLDLxNNNLSGxI Pxx	5	Baudino et al., 2001
ZmSERK2	LGNLxxLxxLDLxNNNLSGxI Pxx	5	Baudino et al., 2001

Figure 2. Characteristics of the LRR region of PhLRR. **A.** Alignment of the 11 LRRs of PhLRR. Numbers to the right indicate the specific LRR number. The bottom line indicates the consensus sequence for the PhLRR LRR. "x" stands for any amino acid. **B.** Comparison of the LRR consensus sequence of PhLRR and other LRR proteins.

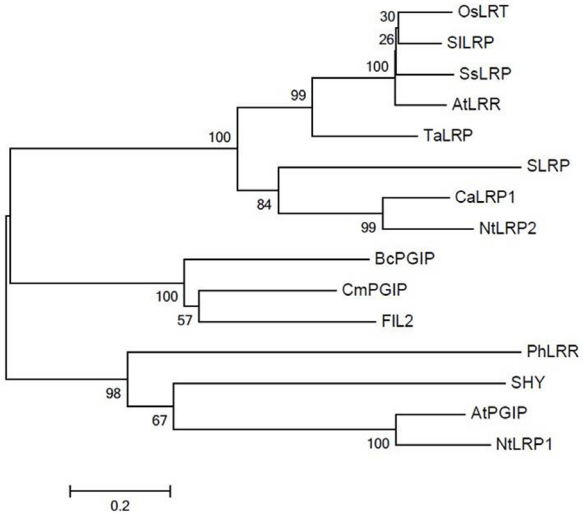

Figure 3. Unrooted phylogenetic tree of PhLRR with other LRR proteins. Phylogenetic tree was constructed by the MEGA5 software. Scale bar represents 0.2 substitutions per site. Numbers on the tree represents confidence values from bootstrap test for 1000 replicates. The accession number of each selected LRR protein is as follows: FIL2: CAA54303; SHY: AF325673; NtLRP1: DQ358108; SLRP: AAC49559; CaLRP1: AAN62015; NtLRP2: EF535611; TaLRP: AAU82111; SsLRP: FJ787729; SILRP: CAA64565; OsLRT: NP_001044578; AtLRR: NP_197608; BcPGIP: AAX68500; CmPGIP: AAP41199; AtPGIP: BAB02490.

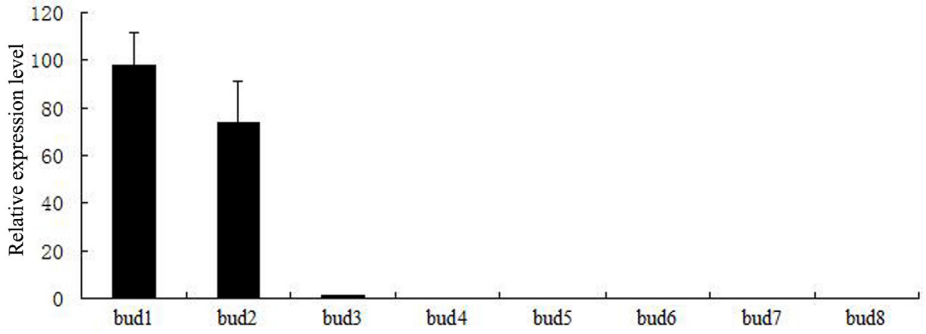

Figure 4. Real-time RT-PCR analysis of the *PhLRR* gene during the flower bud development stages in *Petunia* x *hybrida* 'Fantasy Red'. RT-PCR was performed using RNA isolated from eight different flower bud development stages [bud1 (Bl <2 mm), bud2 (Bl 3 ± 0.5 mm), bud3 (Bl 5 ± 0.5 mm), bud4 (Bl 10 ± 0.5 mm), bud5 (Bl 15 ± 0.5 mm), bud6 (Bl 20 ± 0.5 mm), bud7 (Bl 25 ± 0.5 mm), bud8 (Bl 35 ± 0.5 mm)] (Bl means the bud length excluding sepal).

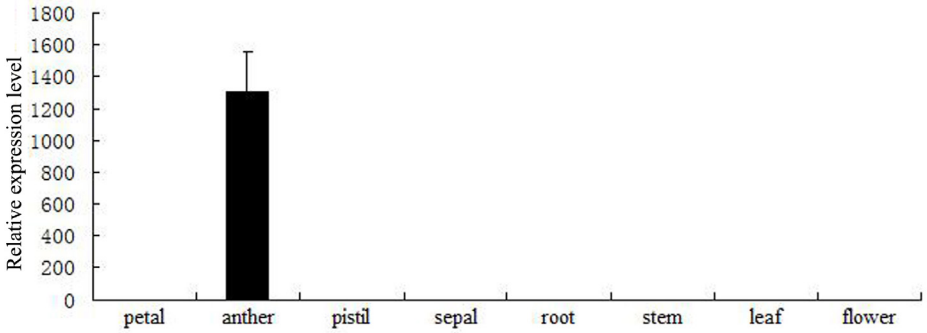

Figure 5. Analysis of the spatial expression pattern of the *PhLRR* gene in different tissues (roots, tender stems, fresh leaves, open flowers and petal, anther, pistil, sepal from bud2) of the *Petunia* x *hybrida* 'Fantasy Red'.

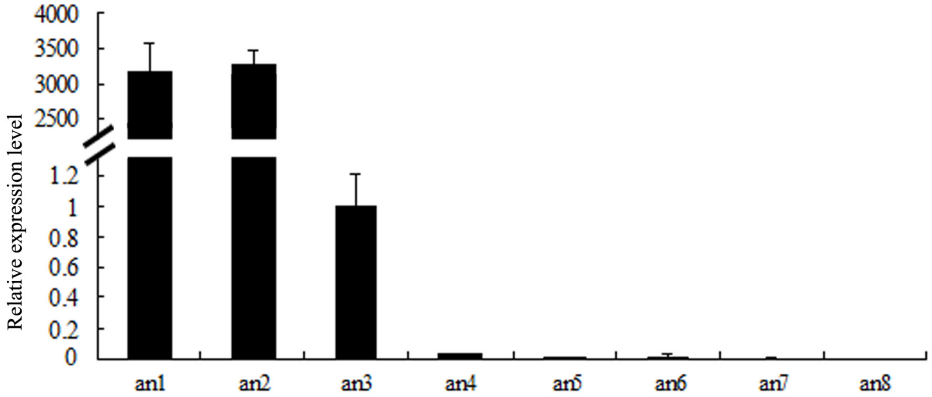

Figure 6. Real-time RT-PCR analysis of the *PhLRR* gene during the anther development stages in *Petunia* x *hybrida* 'Fantasy Red'. RT-PCR was performed using RNA isolated from eight different anther development stages (an1: <2 mm; an2: 3 mm ± 0.5; an3: 5 mm ± 0.5; an4: 10 mm ± 0.5; an5: 15 mm ± 0.5; an6: 20 mm ± 0.5; an7: 25 mm ± 0.5; an8: 35 mm ± 0.5). an: anthers.

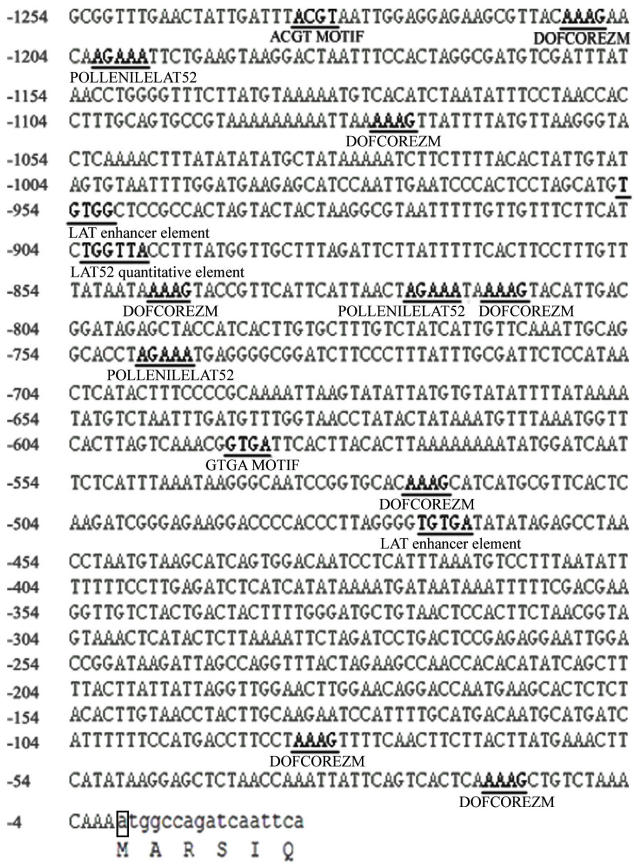

Figure 7. Analysis of putative cis-acting elements of the *PhLRR* promoter. The putative cis-acting elements (such as AGAAA, GTGA, TGTGA, TGTGG, TGGTTA, ACGT, and AAAG) are marked with single underlines. The first base of the translation start site is boxed and assigned as position +1.

DISCUSSION

It is very important to investigate the genes that participate in pollen development, particularly those that are anther/pollen-specific ones. This information will not only increase the understanding of the molecular mechanism of sexual propagation in flowering plants, but may also be useful in agriculture for hybrid seed production.

N-linked glycosylation was found to play a key role in protein folding, membrane targeting, and substrate binding (Xu et al., 2009). Several potential N-linked glycosylation sites (NXS/T) were found in the PhLRR protein (Figure 1), indicating that the PhLRR protein may be glycosylated post-translationally and then translocated to the cell wall and membranes. Additionally, many putative serine/threonine/tyrosine substrate residues were found in the PhLRR gene, supporting that PhLRR is phosphorylated post-translationally (Figure 1). Therefore, *PhLRR* may be a receptor protein involved in early stages of signal transduction in the cell wall and plasma membranes and mediate protein interactions in various types of biological processes. Sequence analysis of *PhLRR* using bioinformatic tools revealed that *PhLRR* shares

significant sequence similarity to some predicted LRR proteins, whereas the function or expression patterns of these genes remain unclear. Thus, we suggest that *PhLRR* is a novel gene of unknown function. PhLRR contains a conserved LRR domain, which has a highly identity to PGIPs. The LRR domain is characterized by tandem arrays of a leucine-rich consensus sequence and a structurally conserved LRR interaction surface (Kobe and Deisenhofer, 1994; Ho et al., 2002). The PhLRR protein is composed of 11 tandem repeat LRR motifs (Figure 2), of which the diversity of LRR domains may be related to specific molecular characteristics.

According to phylogenetic analysis, PhLRR and SHY are clustered in the same clade with other genes encoding PGIPs (Figure 3). PGIPs, which play a role in plant defense and possibly during development, are also LRR proteins (Williams et al., 1992; Jones and Jones, 1997). *SHY* is a pollen-specific gene, encodes an LRR protein that has been further studied in petunia, and the structure of SHY protein is nearly identical to PGIPs of plants. However, experiments indicate that the SHY protein is not a defense protein that inhibits fungal endopolygalacturonases, suggesting that *SHY* functions in a signal transduction pathway controlling pollen tube growth (Guyon et al., 2004). Thus, *PhLRR* may also function in a signal transduction pathway and mediate plant anther/pollen development.

Recently, many anther/pollen-related or anther/pollen-specific genes have been isolated, characterized, and analyzed. Some of these genes were found to be expressed during early stages of male organ development (Zhang et al., 2011, 2012), some during late stages, and some during the entire period of male organ development (Robert et al., 1993; Fourgoux-Nicol et al., 1999; Honys and Twell, 2004; Kato et al., 2010; Chen et al., 2012a). Expression analysis showed that *PhLRR* was detected only in bud1-3, with expression peaking during the bud1 stage and then decreasing to a minimum during the 3rd stage. Similar expression patterns were observed during the various stages of anther development (Figures 4 and 6). Additionally, *PhLRR* was expressed at an extremely high level in the anthers of petunia, but was not detected in any other tissues (Figure 5). In our study, several different cis-element motifs involved in anther/pollen-specific expression were detected in the *PhLRR* promoter region (Figure 7), suggesting that this promoter may be an anther/pollen-specific promoter. Among the various motifs, ACGT and AAAG, which are the core binding sites of the sperm cell-specific AtGEX2 and OsGEX2 promoters, were shown to be target sequences of the bZIP DNA-binding regulatory proteins and Dof transcription factors (Williams et al., 1992; Yanagisawa and Schmidt, 1999). Remarkably, there were 7 copies of AAAG motifs and 1 copy of an ACGT motif in the *PhLRR* promoter region, indicating that expression of this gene is highly influenced by *bZIP* and *Dof* genes. These results imply that *PhLRR* is an anther-specific gene and plays a critical role in the initial stages of petunia anther development.

However, the precise physiological function of the *PhLRR* gene in anther remains to be elucidated. Further studies examining *PhLRR* in petunia should be conducted using RNAi.

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