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Full Length Article

Identification and Expression Analysis of Leucine-Rich Repeat Receptor-Like Kinase Family Reveals the Roles of Resistance Proteins During Formation of Replant Disease in *Rehmannia glutinosa*

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Abstract

Leucine-rich repeat receptor-like protein kinases (LRR-RLKs) are one of the core members of plant pathogen-associated molecular pattern (PAMP)-triggered immune system s. Preliminary studies found that LRR-RLK proteins were involved in formation of replant disease in *Rehmannia glutinosa* Libosch. This study screened and identified the genes encoding LRR-RLK proteins based on *R. glutinosa* transcriptome, and profiled their expression patterns during formation of replant disease using real-time fluorescence quantitative PCR (qRT-PCR) in fields and controlled condition. The expression of the crucial genes in normally grown and replanted plants was further validated at protein level by Western blotting (WB). Of 40 LRR-RLK proteins identified in *R. glutinosa*, 10 were down-regulated throughout the process, 27 were slightly increased at first but decreased in later stages, and remaining proteins were not changed in process of the death of replanted *R. glutinosa*. Twelve genes selected randomly from 27 genes were found to down-regulate under replanted stress in controlled condition. The LRR-RLK 19 protein and LRR-RLK 29 protein that were inhibited in replanted *R. glutinosa* were further selected to use for polyclonal antibodies preparation and detection using WB. As a result, their protein abundance decreased obviously in replanted *R. glutinosa*. These data showed that replant practice might provoke or disrupt the normal functions of critical LRR-RLKs in replanted *R. glutinosa*, followed by destroyed immune system of replanted *R. glutinosa* and induced replant disease.

Keywords: *Rehmannia glutinosa*; Replant disease; LRR-RLKs; Polyclonal antibody preparation

Introduction

Rehmannia glutinosa Libosch belongs to the Scrophulariaceae family and is a distinguished medicinal plant in China as a source of herbal medicine that has been used for more than 1000 years. However, *R. glutinosa* is sensitive to replant disease during cultivation. Because of an abysmal replant problem, *R. glutinosa* could not be planted normally during a period of approximately 8 to 10 years (Zhang *et al*., 2013). The replanted *R. glutinosa* could be easily infected by different diseases, leading to a significant decrease in the yield and quality and the disruption of the normal growth of the plants (Zhang *et al*., 2013; Chen *et al*., 2016). In addition to *R. glutinosa*, the replant problem exists widely in various medicinal plants with tuberous roots, such as *P. ginseng*, *P. quinquefolius*, *P. notoginseng*, and *A. sinensis* (Guo *et al*., 2006; Huang *et al*., 2013; Zhang *et al*., 2013; Chen *et al*., 2016; Wang *et al*., 2017). The replanted problem has gradually become an urgent problem in the production of medicinal plants (Lin *et al*., 2011; Huang *et al*., 2013; Wu *et al*., 2013; Yang *et al*., 2014).

The formation of the replant problem involves complex interactions among plants, rhizosphere soil microbes, and allelopathic autotoxins (Zhang and Lin, 2009). Currently, the interactions among plants, allelopathic autotoxins and microbes in the rhizosphere soils in plants are considered to be the core factors that resulted in the formation of the replant disease problem (Guo *et al*., 2006; Zhang *et al*., 2013; Chen *et al*., 2016). Of these factors, the interaction between autotoxic substances and microbes has been comprehensively clarified in different studies, and the identical results found that allelopathic autotoxins induce the shift in the microbes from beneficial microbes to harmful ones (Li *et al*., 2012; Chi *et al*., 2013; Bigeard *et al*., 2015; Zhao *et al*., 2016). Among these interaction factors, the interaction between the soil and microbes that promoted the induction of replant disease have been widely studied. However, how the rhizosphere microbes, especially the community levels, attack and infect replanted plants remained largely unknown. Previous studies revealed that

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the critical 'molecular events' that are closely involved in the immune response were significantly induced in replanted *R. glutinosa*, including immune signals, Ca^{2+}, MAPK and ethylene signals (Li *et al*., 2013; Yang *et al*., 2014, 2015; Wu *et al*., 2016; Li *et al*., 2017). And there are 45 NB-ARC proteins in *R. glutinosa* that have been found to be involved in the formation of the *R. glutinosa* replant problems (Chen *et al*., 2018). Moreover, some LRR-RLKs in the immune systems were found to be significantly regulated on both the transcriptome and protein levels in the replanted *R. glutinosa* (Li *et al*., 2017). These results suggested that proteins in the immune system might have important significance to the induction of replant disease.

The immune system of plants is primarily comprised of two-layers of defence, effector-triggered immunity (ETI) and PAMP-triggered immunity (PTI). The first layer, PTI, could effectively recognize pathogen-associated molecular patterns (PAMPs) located in the cell walls using conserved regions of proteins (Macho and Zipfel, 2014). ETI, the second layer, senses the effectors released by the fungal intercellular cytoplasm (Boller and He, 2009). In comparison to ETI, PTI is considered to be a basic and crucial defence system to resist the attacks from various harmful microbes (Boller and He, 2009; Peng *et al*., 2017). LRR-RLK proteins are the core components sensed microbes and PAMPs from the microbes (Braun and Walker, 1996; Boller and He, 2009; Wu *et al*., 2013; Peng *et al*., 2017; Jamieson *et al.*, 2018). The LRR-RLKs primarily include three parts, the extracellular sensing region, intracellular kinase domain and transmembrane proteins. A remarkable characteristic of the extracellular domain of the LRR-RLKs is having different numbers of tandem-parallel repeats, the numbers or types of which determine the specificity of LRR-RLKs to the different pathogens (Arbona and Gómezcadenas, 2015; Dalio *et al*., 2017). When the plant cells encounter PAMPs, the extracellular sensing region transmits the transmembrane signal, followed by the activated intracellular kinase domain that further initiates the expression of specific genes (Jung *et al*., 2004; Shi *et al*., 2014). Numberous studies have proved that LRR-RLKs played the crucial roles in plant-pathogen interactions (Braun and Walker, 1996; Nürnberger and Kemmerling, 2006; Boller and He, 2009; Peng *et al*., 2017; Jamieson *et al*., 2018). Replant disease formation is essentially the result from interaction of pathogenic microbes drived by allelopathic autotoxins with replanted plants. Consequently, LRR-RLK proteins in replant *R. glutinosa*, as one of important defence systems, must have important roles during replant disease formation. To deeply undertake the relationship between LRR-RLKs and replant disease, this study in detail identified LRR-RLK family proteins in *R. glutinosa*, and profiled their expression patterns during the formation of replant disease on transcript and protein level. This study was aimed at providing an effective method to strengthen the immune response system, improving the resistance mechanism of replanted *R. glutinosa*, and serving as a reference to conquer the replant disease problem.

Materials and Methods

Plant Materials and Treatments

In this field experiment, two treatments were established: first-year planting (FP) and second-year planting (SP) *R. glutinosa* (Fig. 1). The first-year planted areas had not been planted for ten years, and the replanted areas had grown *R. glutinosa* in 2016. Field experiments with *R. glutinosa* 'Wen 85-5' were arranged at the Wenxian Agricultural Institute in Jiaozuo City, Henan Province, China. Tuberous roots of *R. glutinosa* of the same size (approximately 3 to 4 cm) used for cultivation were planted with a density of 30 cm×30 cm on 4 May, 2017, the other cultivation managements were identical. The fresh tuberous roots were collected at the seedling, elongation, pre-expansion, mid-expansion, late-expansion, and maturity stages. All the samples were immediately frozen in liquid nitrogen and stored at −80°C until use.

Identification and Analysis of the *R. glutinosa* LRR-RLKs Proteins

To identify specific LRR-RLK proteins in *R. glutinosa*, the kinase region HMM files (PF00069.16) and the LRR inclusion region HMM files (PF00560.24) that characterized the conserved properties of the LRR structure were extracted from the Pfam 31.0 database (http://pfam.xfam.org/) based on the exclusive conserved domains (LRR structural domain and protein kinase domain). The HMMER suite (http://www.hmmer.org/) was used to identify candidate LRR-RLK proteins in the *R. glutinosa* database translated from the *R. glutinosa* transcriptome (Li *et al*., 2017) based on the HMM files. The LRR-RLK proteins were further analysed using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) and SignalP (Nielsen, 2017) (http://www.cbs.dtu.dk/services/SignalP/) to predict the transmembrane regions and signal peptides, respectively. The protein sequences without the transmembrane and signal peptide were excluded, and the reserved sequences were used as the sequences of the *R. glutinosa* LRR-RLK proteins. The genes encoding the LRR-RLK proteins were simultaneously reverse screened from the *R. glutinosa* transcriptome database.

The nucleotide sequences of *LRR-RLK* genes were analysed by Blast2 GO software after blastx alignment in cloud database, and then the different biological processes were analysed by WEGO 2.0 (http://wego.genomics.org.cn/). The phylogenetic relationships among candidate LRR-RLK proteins were constructed based on the Neighbour-Joining and bootstrap methods (1000 replicates) using MEGA6.0 (Tamura *et al*., 2013) software. All the results of the conserved motifs of the LRR-RLK proteins identified in *R. glutinosa* were identified using the online SMART tool (Bauer *et al*., 2017) (http://smart.embl-heidelberg.de/), and the corresponding structure charts of the LRR-RLK proteins were drawn using IBS (Illustrator of Biological Sequences) 1.0 software (Liu *et al*., 2015).

Table 1: Primer sequences used in this study

NO.	*R. glutinosa* Gene Name	Primer sequences (5'-3')	Tm (°C)
CL5019.Contig1_All	*RehLRR1*	F:GCCAGTTAGCCAATCTTG	59.9
		R:GTCCCGTTAAGGAAATTCG	60.1
CL811.Contig2_All	*RehLRR2*	F:CTGCCTCTGAACAACAAC	60
		R:GGTCTTGGAGTTGATGATTG	60.2
CL2027.Contig1_All	*RehLRR3*	F:TAATCCCGAGCAGTCTTG	60.2
		R:TAGGAATCTCACCACTAAGC	60.2
CL344.Contig2_All	*RehLRR4*	F:CAGAGAATGGCACAAACC	60.1
		R:CACGGCTGTTGATACTAATC	60.1
CL344.Contig3_All	*RehLRR5*	F:CAAGAACCTCGCCTTTATAG	59.7
		R:CATATTGGTGGAGCCTAATG	59.8
CL3379.Contig2_All	*RehLRR6*	F:GGAGGGTTTGTTCTATTTGG	60.2
		R:TGGACGAGCAGATACTTG	60.2
CL814.Contig1_All	*RehLRR7*	F:CGTAGGGCTAATTCCAAAC	59.8
		R:CAACCACACCAAACAGAG	60
CL2741.Contig2_All	*RehLRR8*	F:GTGGAATACCGAAGGAGAT	60.1
		R:CACTTGGAGGAACAGAGT	60
CL2741.Contig3_All	*RehLRR9*	F:GCATAGGCACATTGTTAGG	60.2
		R:TGATGAAGATAGCAGAGACC	60.3
Unigene8599_All	*RehLRR10*	F:GCAGAAGTTCGGATATTGG	59.9
		R:GATGGACTGAACCTGAGAT	60
CL3286.Contig1_All	*RehLRR11*	F:GTCAGCAGTAGTAAGCATTC	60
		R:CGGCACATCCATTGATATAC	60.2
CL4576.Contig1_All	*RehLRR12*	F:GGCACTAAGTCCATGTCT	60.1
		R:TCACTCTTCTCGTTCACC	60
CL5906.Contig1_All	*RehLRR13*	F:GTGGAAGACACGAAAGAAG	60
		R:CTGCGAATAATGGAGAACC	59.9
CL2040.Contig2_All	*RehLRR14*	F:GGCTAAGATTCCGAACCT	60.2
		R:CGCTGAGATTACACATTCC	59.9
CL3712.Contig2_All	*RehLRR15*	F:CCAGACTGCTACCTATGTT	60
		R:CTCTTACCTGTGACAACCT	60.2
CL3025.Contig1_All	*RehLRR16*	F:GGAACTGGATTGGTATCAAC	59.9
		R:TTGCCTCATAGTCCTCATC	60.1
Unigene4796_All	*RehLRR17*	F:GCCGATTAGACATCAGATTG	60.2
		R:CCGTTGTGGTAGAATCCT	60.1
Unigene11464_All	*RehLRR18*	F:GCCATTCTGCTTCTCTTG	59.9
		R:CAATCTTGACCGTGTTATCC	60.3
Unigene6620_All	*RehLRR19*	F:GTCTGAACTGTGGTGGTA	60
		R:CCTGGTCCTACTAACAACA	59.9
CL9971.Contig1_All	*RehLRR20*	F:CAAGGTTCTTCTGTTGTCTC	60.1
		R:GCGGTAACTGATATGGAATC	59.9
CL9971.Contig2_All	*RehLRR21*	F:GGTTCTTCTGTTGTCTCTG	59.3
		R:CGGTAACTGATATGGAATCG	59.6
CL2114.Contig1_All	*RehLRR22*	F:TCTCCACTCTCATCAATCTC	60
		R:CGGTGAAGTTGTTGTGAG	60
CL4747.Contig1_All	*RehLRR23*	F:TATCATCCTCCAAGTGCTC	60.1
		R:CTCTATGCTCGGTCTATCAT	59.9
CL6634.Contig1_All	*RehLRR24*	F:CCGATTGCTCACTATTCAC	59.9
		R:GGAGTTCACCGTACAGTT	60.4
Unigene11572_All	*RehLRR25*	F:CTTCTTCAGATGTGGAGTTG	59.9
		R:AGGAGGATAGCAGTAGGT	59.8
Unigene2775_All	*RehLRR26*	F:TCTCTTCTCCAACTCAACC	60.2
		R:GCAAATCAATCCCTTCCTC	60.1
CL3961.Contig2_All	*RehLRR27*	F:AGAATTGGACAGGCTACC	60.1
		R:AGGTGCTCATAGAGTGTTC	60.4
Unigene12254_All	*RehLRR28*	F:CGGACACAGCAATAACAC	60.2
		R:CCACCTCTGCCTATTACAT	60.1
CL891.Contig3_All	*RehLRR29*	F:GGATGAAGACTCTGTGGAT	60
		R:ACCTGATAAACCCTGACTAG	59.9
Unigene5011_All	*RehLRR30*	F:CCCGAGTATTATCAGACCTT	60
		R:CAATGACCAGAGCACCTA	60.1
CL959.Contig2_All	*RehLRR31*	F:CCACCTCTGCCTATTACAT	60.1
		R:CGGACACAGCAATAACAC	60.2
Unigene2209_All	*RehLRR32*	F:TGGACTTGCCGATAGATG	60.3
		R:GGACGAGGATAACTCTTGT	60
Unigene6203_All	*RehLRR33*	F:ATGGTAGTGTGGCATCTAG	60.1
		R:CGAAGTCATCATCCAACAG	59.8
Unigene15318_All	*RehLRR34*	F:CACCAATGCCTCTATCCT	59.9
		R:TGCTTATGTCTCCACCAG	60.1
CL5325.Contig2_All	*RehLRR35*	F:TACAAGTTGCTCTCCTCTG	60.3
		R:CGCCTTCTAACATTCTCAC	59.8
CL4534.Contig1_All	*RehLRR36*	F:GACTTGAAGAAGAGGAGGAT	60.2
		R:GCGACTTGTAGAATCATCTC	59.8
CL3933.Contig3_All	*RehLRR37*	F:CGATAGTAGCGTGGAGATAT	60
		R:ATTCTTCTTCCTGGACCTC	59.9
CL9126.Contig1_All	*RehLRR38*	F:GTGACGGCAGTAACAATG	60.2
		R:GGAGTGTATTCAGCATCTTC	59.7
CL9126.Contig2_All	*RehLRR39*	F:CCTGCTTATCGGAGTCAT	60
		R:CGGTATTCCAATCTGTTAGC	60.1
Unigene9578_All	*RehLRR40*	F:TGGTAGATTGAGGCATCC	59.9
		R:GGCTCTTGATCCGTGTAT	60.3

Tm: melting temperature

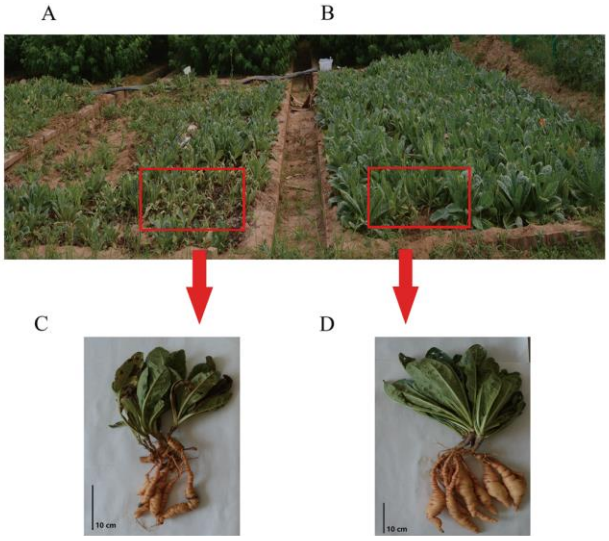

Fig. 1: First-year planting (FP) and second-year planting (SP) *R. glutinosa* in the field. Comparisons between the population (**A**, **B**) and underground part (**C**, **D**) of FP (**B**, **D**) and SP *R. glutinosa* (**A**, **C**)

Spatiotemporal Expression of the *LRR-RLK* Genes during Replanted and Normally Growth *R. glutinosa* in Field

To compare and analyse the expression patterns of the *LRR-RLK*-related genes in the FP and SP *R. glutinosa*, fluorescence quantitative primers (Table 1) were designed using Beacon Designer software 8.0 (http://beacon-designer.software.informer.com/8.0/), and the *LRR-RLK*-related gene expression levels during development were verified using real-time fluorescence quantitative PCR (qRT-PCR) in the FP and SP *R. glutinosa*.

Using the TRIzol Kit (Invitrogen, U.S.A.), the quality of the total RNA extracted from the materials was verified. The ratio of the 28S and 18S of the total RNA were detected using 1.0% agarose gel electrophoresis (180 V, 0.5 h). The absorbance of the total RNA was measured at 260 nm/280 nm, and its concentration and purity were calculated. Real-time fluorescence quantitative PCR (qRT-PCR) was

performed using a RT-PCR (reverse transcription) kit (RevertAid^{TM} First Strand cDNA Synthesis Kit) (Fermentas, U.S.A.). The corresponding genes and internal reference genes were used for qRT-PCR, and each reaction was repeated three times. qRT-PCR was performed using a Bio-Rad IQ5 instrument (Bio-Rad, Hercules, C.A., U.S.A.) based on SYBR Green to detect the transcript abundance. The reaction system was 25 μL: SYBR Premix Ex Taq II (Tli RNase H Plus) 12.5 μL, 2 μL for the cDNA template, 8.5 μL for ddH_2O, and 1 μL for each primer. The PCR reaction procedure was as follows: 95.0ºC 2 min, 39 cycles at 95.0ºC for 5 s, 60.0ºC for 30 s, 72ºC for 30 s; the dissolution curve detection conditions are 95ºC for 1 min, 60ºC for 1 min, and 0.5ºC s^{-1} speed to 95ºC, and the continuous detection of fluorescence signals. The relative quantitative analysis of the gene expression was based on the reference gene 18S (DQ469606) (Fan *et al*., 2012; Wang *et al*., 2013; Yang *et al*., 2014; Wang *et al*., 2015; Chen *et al*., 2018). The $2^{-\Delta\Delta Ct}$ method (Livak and Sehmittgen, 2001) is widely used in relative quantitative sampling. The Ct value of the target gene of the same sample was calculated, and the average number of Ct values was corrected by 3 parallel holes. The data were normalized on the basis of the 18S rRNA threshold cycle (Ct) value. The samples in the SP and mature stage of the *R. glutinosa* treatment were used as controls, and their normalised Ct values were set to 1. The relative gene expression of the data was calculated using the $2^{-\Delta\Delta Ct}$ method.

Expression Characteristics of the *LRR-RLK* genes in *R. glutinosa* under Different Replanted Levels Made by Different Contents of Replant Soils in Controlled Condition

Four treatments, including different ratio of the first-year planted soils (FPS) and second-year planted soils (SPS) (FPS:SPS=3:0, FPS:SPS=2:1, FPS:SPS=1:2, FPS:SPS=0:3), were studied to further analyse the relationship between replanted *R. glutinosa* and *LRR-RLK* genes, and each treatment was assessed in ten pots. Tissue culture seedlings of *R. glutinosa* wen '85-5' which has been exercised were grown in plastic pots of 25 cm diameter and 22 cm height. Pot experiments were performed under controlled conditions (25°C, 10,000 lx) at the Institute of GAP for Chinese Medicinal Materials, Fujian Agriculture and Forestry University. The FPS used in the treatments was collected from land where *R. glutinosa* had not been planted for at least the last 10 years, and the SPS used in the treatments was collected from land where *R. glutinosa* had been planted in the previous year. Each treatment was conducted as follows. The four treatments were irrigated with the same volume plain water (60 mL) and assessed some days after planting when their appearance was noted that was similar to the appearance caused by replant disease. Fresh fibrous roots were collected when the seedlings were planted after 3 days (DAP3), which was the critical period of respond to replant disease. Meanwhile, the samples in DAP0 were collected as controls, and all samples were stored at −80°C after being frozen by liquid nitrogen for qRT-PCR.

To explore the expression patterns of the *LRR-RLK*-related genes in replanted *R. glutinosa* incontrollable condition, a total of 12 LRR-RLKs genes that high-expressed in SP *R. glutinosa* were choosed randomly, using qRT-PCR to expound the changes of *LRR-RLK*-related gene expression under different degrees of replanted stress. The samples in DAP0 were used as controls, and their normalised Ct values were set to 1. The other methods of qRT-PCR were the same as the above.

Full Length ORF Cloning of the *RehLRR19* and *RehLRR29* Gene and Encoded Protein Information

Forward and reverse primers were designed using Primer Premier 5 software ($F_{RehLRR19}$: 5′-CAGAGGGAGTGACCTTTAGT-3′, $R_{RehLRR19}$: 5′-ACTGCCTGAGCAGAAGTAG-3′. $F_{RehLRR29}$: 5′-CGAAGTGTATGAATCAAATGT-3′, $R_{RehLRR29}$: 5′-TCTTCATTCCATCTTTCATAGT-3′). Using the synthesized cDNA as a template, the PCR reaction was performed with the following system. The reaction system was 25 μL: 5 × PrimeSTAR Buffer (Mg^{2+} plus): 5 μL, dNTP Mixture (2.5 m*M*): 2 μL; Primer F (10 μ*M*): 1 μL; Primer R (10 μ*M*): 1.0 μL; cDNA template: 1 μL (<200 ng=120.6 ng; PrimeSTAR HS DNA Polymerase (2.5 U/μL): 0.25 μL; ddH_2O: 14.75 μL. The PCR reaction conditions were as follows: pre-denaturation at 98ºC for 3 min, 98ºC for 10 s, 55ºC for 15 s, 72ºC for 10 s for 34 cycles, and extension at 72ºC for 5 min. The PCR products were analysed using 1.0% agarose gel electrophoresis (100 V). The DNA fragments were recovered using the DNA recovery kit and linked with the pMD18-T vector overnight at 16ºC. The conjugated product was transformed into *E. coli* Trans 5α, and the target plasmid was identified by PCR and sequenced at the Shanghai Biotechnology Company.

Preparation of Polyclonal Antibodies against RehLRR19 and RehLRR29

After sequencing the two genes, the antigen sequences were analysed and designed (Table 2). Simultaneously, the restriction enzymes *BamH*I and *Eco*RI were used for double digestion to construct the linearized pET32a vector (Shanghai Abmart Pharmaceutical Technology Co., Ltd.). Homologous recombination (ClonExpress Ultra One Step Cloning Kit Nanjing Vazyme Biotech Co., Ltd) was used to construct the pET32a-RehLRR19 and pET32a-RehLRR29 recombinant vector. The recombinant vectors were transformed into *E. coli* BL21 (DE3), and IPTG induced their expression. A sodium dodecyl sulfate polyacrylamide

Table 2: Antigen sequences of RehLRR19 and RehLRR29

Name	Antigen sequences
RehLRR19	QIPGFVSLNCGGTDNFTDELGLTWGPDNDMISGEVANISAANETRKQYKTLRFFPADNNKYCYTLNVVSRTRYLIRATFLYGNFDS
	NNVYPKFDISFGPTHWATIVISDANTIESQELIFLATDSTISVCLSNATTGQPFISTLELRQFNGSIYFNQFENQYFLSVSARINFGAESD
	APVRYPDDPFDRIWQSDSLKQANYLVDVAPGTEKISTRMPIDVSRDERPPQKVMQTAVVGRNGSLTYRLNLDGFPGFGWAFTYFA
	EIEDLGPSDVRKFRLVLPGDPDLSKAVVNIQENAQGKYRLYEPGYYNISLPFVLSFRFGKTSDSTLGPLLNAMEINK
RehLRR29	KNILGAGGFGNVYRGKLGDGTLVAVKRLKDLTGTTGESQFRTELEMISLAVHRNLLRIIGYCATTNERLLVYPYMPNGSVASRLR
	GKPALDWQTRKRIAIGAARGLLYLHEQCDPKIIHRDVKAANVLLDDYFEAVVGDFGLAKLLDHAESHVTTAVRGTVGHIAPEYLS
	TGQSSEKTDVFGFGILLLELITGMRALEFGKSVNQKGAMLEWVKKIQQEKKIELLADRELGINYDPIEVGEMLQVALLCTQYLPSH
	RPKMSEVVRMLEGDGLAEKWAASHNYIHTPAKFSCNNSKSRSNEDIDHDQSSMFGMTTMTMDDDYDAHCMELSGPR

gel electrophoresis (SDS-PAGE) showed that there were target protein bands. The proteins were used as antigens to stimulate New Zealand rabbits. After the seventh immunization, rabbit antiserums were obtained. Biochemical methods were used to qualitatively and quantitatively analyse the antiserums. The specific antibody titers were checked using the enzyme-linked immunosorbent assay (ELISA) method. Finally, the highly specific and highly potent polyclonal antibodies were prepared.

RehLRR19 Protein and RehLRR29 Protein Expression Patterns using Western Blotting (WB)

The total protein of the FP and the SP *R. glutinosa* from different periods were extracted. After SDS-PAGE using 10% separating gel and 4% stacking gel on 80 V, the loading quantity of each sample is 15 μg (the volume loading different samples were determined by protein concentration). The protein bands isolated from the gel were transferred to a PVDF membrane using transfer electrophoresis. A β-actin (molecular weight 43 Kd) was incubated with a primary antibody (dilution ratio=1:1000), and horseradish peroxidase was linked to the secondary antibody and served as the internal reference and blank control. The prepared polyclonal antibodies were incubated and tested as the primary antibody (dilution ratio=1:300), and horseradish peroxidase were linked to the secondary antibodies. Images were captured using a gel imaging system (Tanon 2500), and the grey values were analysed using Image J software after exposure and film development.

Results

Screening and Identification of the *R. glutinosa* LRR-RLK Proteins

A total of 40 genes encoded LRR-RLK proteins were identified from the *R. glutinosa* transcriptome database using bioinformatics methods. All the *LRR-RLK* genes contained the entire open reading frame (ORF) region that ranged from 1839 bp to 4459 bp with an average length of 2478 bp. The longest protein encoded by the *LRR-RLK* ORF was 1203 amino acids (aa); the shortest was 605 aa, and the average length was 825 aa (Table 3). Based on the HMM model to the LRR-RLKs, the structural analysis for the *R. glutinosa* LRR-RLK proteins based on conserved domains specific to the LRR-RLKs showed that all the *R. glutinosa* LRR-RLKs possessed the typical characteristics, including the plasma membrane, transmembrane regions, transmembrane helices and kinase domains. However, there were significant differences in the number of LRR helices for the different LRR-RLKs, including 1 to 5 LRR regions for 23 proteins, 6 to10 LRR regions for 13 proteins, and 10 LRR regions for 4 proteins. RehLRR22, RehLRR30, RehLRR33, and RehLRR40 contained one LRR region each, and RehLRR13 contained 16 LRR regions (Fig. 2A and B). To identify the homology of *R. glutinosa* LRR-RLK with other species, an unrooted phylogenetic tree was constructed by sequence similarity using the neighbour-joining (NJ) method. The evolutionary analysis of the *R. glutinosa* LRR-RLKs showed that the *R. glutinosa* LRR-RLK proteins could be divided into four groups, in which there were 9 LRR-RLKs in the first group; 10 in the second group; 19 LRR-RLKs in the third group, and 2 LRR-RLK proteins in the others (Fig. 2C). To fully undertake the function of *R. glutinosa* LRR-RLKs, all LRR-RLKs were aligned to GO terms on basis of BLASTX. The results indicated that, for cellular component terms, *R. glutinosa* LRR-RLK proteins were filled into symplast, cell junction, cell part, cell, organelle, membrane part, organelle part, membrane-enclosed lumen, binding, and catalytic activity. In their molecular function mainly related to terms that including signal transducer activity, development process and regulation of biological process. The cellular processes were mostly involved in terms that including growth, multicellular organismal process, biological regulation, negative regulation of biological process, metabolic process, cellular process, reproduction, reproductive process, response to stimulus, signalling, positive regulation of biological process, cellular component organization or biogenesis, multi-organism process, immune system process and localization. GO analysis indicated *R. glutinosa* LRR-RLKs widely participated in various celluar process, such as plant growth, development, stress response. It reflected that the LRR-RLK proteins covered nearly whole composition of *R. glutinosa* PTI systems (Fig. 3).

The Expression of *R. glutinosa LRR-RLK* Genes during the Formation of Replant Disease in the Fields

To further screen and identify the *LRR-RLK* genes specifically expressed in response to replanted stress, the expression pattern of the *LRR-RLKs* in the replanted *R.*

Table 3: Sequence information of the *LRR-RLK*-related genes in *R. glutinosa*

NO.	Gene Name	Accession No	Length of gene and ORF(bp）	Protein length(aa)
1	*RehLRR1*	CL5019.Contig1_All	4459（3612）	1203
2	*RehLRR2*	CL811.Contig2_All	4155（3414）	1137
3	*RehLRR3*	CL2027.Contig1_All	4223（3288）	1095
4	*RehLRR4*	CL344.Contig2_All	3881（3228）	1075
5	*RehLRR5*	CL344.Contig3_All	3427（3228）	1075
6	*RehLRR6*	CL3379.Contig2_All	3349（3090）	1029
7	*RehLRR7*	CL814.Contig1_All	3550（3084）	1027
8	*RehLRR8*	CL2741.Contig2_All	3594（3054）	1017
9	*RehLRR9*	CL2741.Contig3_All	3800（3051）	1016
10	*RehLRR10*	Unigene8599_All	3555（3063）	1020
11	*RehLRR11*	CL3286.Contig1_All	3504（2973）	990
12	*RehLRR12*	CL4576.Contig1_All	3433（2997）	998
13	*RehLRR13*	CL5906.Contig1_All	3100（2949）	982
14	*RehLRR14*	CL2040.Contig2_All	3386（2934）	977
15	*RehLRR15*	CL3712.Contig2_All	3385（2919）	972
16	*RehLRR16*	CL3025.Contig1_All	3508（2934）	977
17	*RehLRR17*	Unigene4796_All	3315（2994）	997
18	*RehLRR18*	Unigene11464_All	3331（2814）	937
19	*RehLRR19*	Unigene6620_All	3200（2781）	926
20	*RehLRR20*	CL9971.Contig1_All	2625（2163）	720
21	*RehLRR21*	CL9971.Contig2_All	2533（2163）	720
22	*RehLRR22*	CL2114.Contig1_All	2543（2031）	676
23	*RehLRR23*	CL4747.Contig1_All	2601（2058）	685
24	*RehLRR24*	CL6634.Contig1_All	2340（2028）	675
25	*RehLRR25*	Unigene11572_All	2367（2001）	666
26	*RehLRR26*	Unigene2775_All	2576（1980）	659
27	*RehLRR27*	CL3961.Contig2_All	2252（1995）	664
28	*RehLRR28*	Unigene12254_All	2273（1917）	638
29	*RehLRR29*	CL891.Contig3_All	2081（1917）	638
30	*RehLRR30*	Unigene5011_All	2267（1896）	631
31	*RehLRR31*	CL959.Contig2_All	2267（1896）	638
32	*RehLRR32*	Unigene2209_All	2256（1881）	626
33	*RehLRR33*	Unigene6203_All	2872（1878）	625
34	*RehLRR34*	Unigene15318_All	2025（1869）	622
35	*RehLRR35*	CL5325.Contig2_All	2067（1848）	615
36	*RehLRR36*	CL4534.Contig1_All	2337（1863）	620
37	*RehLRR37*	CL3933.Contig3_All	2114（1866）	621
38	*RehLRR38*	CL9126.Contig1_All	2287（1818）	605
39	*RehLRR39*	CL9126.Contig2_All	2180（1824）	607
40	*RehLRR40*	Unigene9578_All	1839（1818）	605

ORF: open reading frame, bp: base pair, aa: amino acid

glutinosa and normal growth ones at different stages were analysed using qRT-PCR. The expression of the *LRR-RLKs* could be divided into 3 groups based on the expression levels of the *LRR-RLKs* of the first-year planting (FP) and second-year planting (SP) *R. glutinosa*. A total of 27 *LRR-RLK* genes of the first group dramatically increased at the first and second stages and sharply decreased in the SP *R. glutinosa* compared with the normally grown *R. glutinosa*, and one gene of the group decreased at all stages in the FP *R. glutinosa* (Fig. 4A). The second group had 10 *LRR-RLK* genes (Fig. 4B), and their expression was identical in the replanted and normally grown *R. glutinosa* through different developmental stages. In addition, the expression of 3 *LRR-RLK* genes in the third group decreased in the replanted *R.*

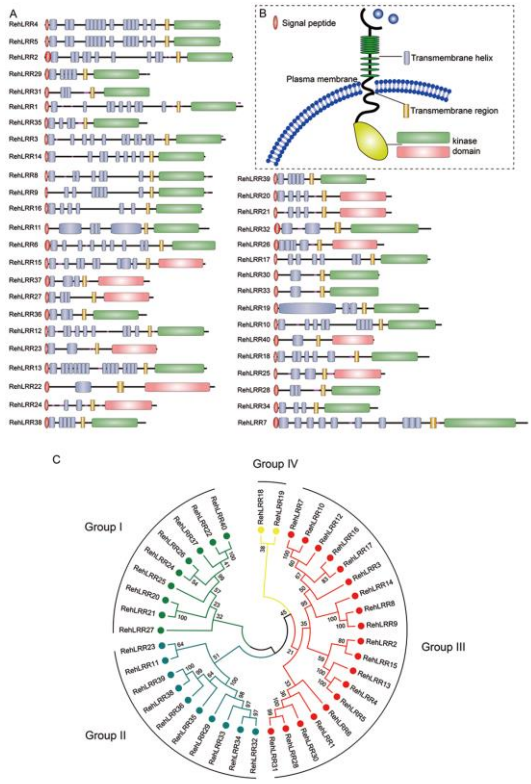

Fig. 2: Conservative motif analysis (**A**, **B**) and phylogenetic tree (**C**) for 40 *R. glutinosa* LRR-RLK proteins

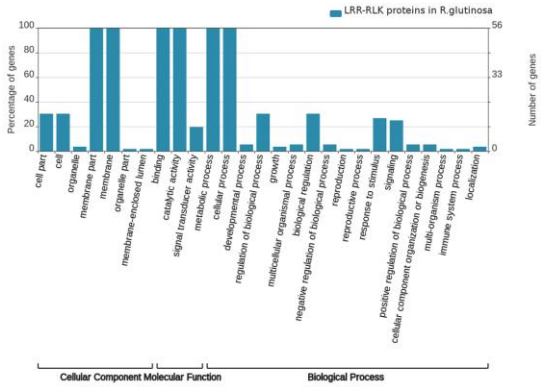

Fig. 3: GO analysis of *LRR-RLK* genes identified in *R. glutinosa*

glutinosa compared with the normally grown *R. glutinosa* at all periods of the plants (Fig. 4C). In summary, the *LRR-RLK* genes in the third group might have been significantly inhibited in the replanted *R. glutinosa*, and the first group was enhanced in the replanted *R. glutinosa.*

The Expression of the *LRR-RLK* Genes under Replanted Stress in Potted *R. glutinosa* under Indoor Condition

Because of the complexity in field environment, it is indefinable to illuminate the expression patterns of *LRR-RLK* genes in replanted *R. glutinosa*. Different ratio of FPS

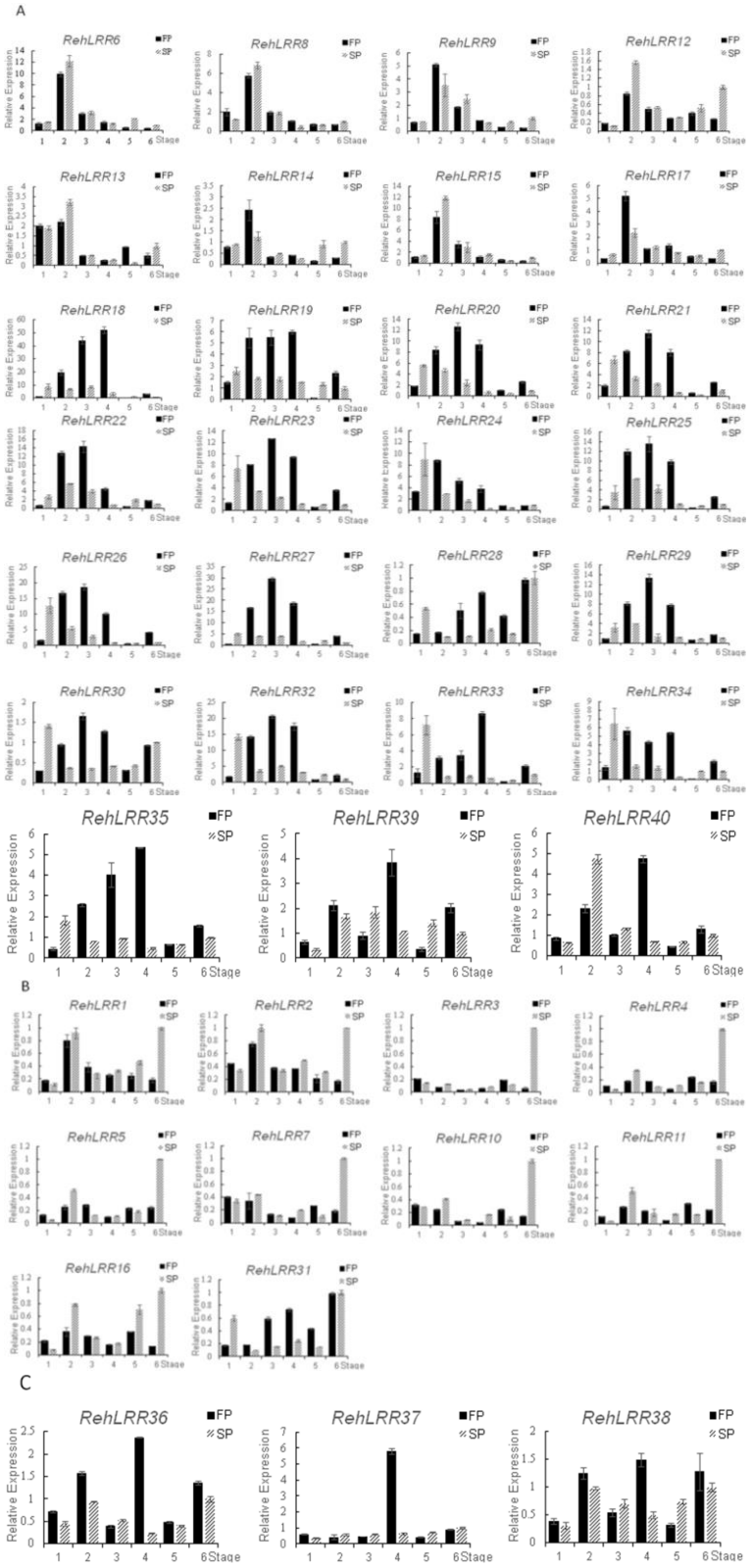

Fig. 4: The expression patterns of the *LRR-RLK* genes according to the qRT-PCR results in *R. glutinosa*. FP1 to FP6 and SP1 to SP6 indicate different developmental stages for first-year planting (FP) and second-year planting (SP) *R. glutinosa*., in which 1 to 6 represent seedling, elongation, pre-expansion, mid-expansion, late-expansion, and maturity stage, respectively. All genes were standarded according to the internal references (18S gene)

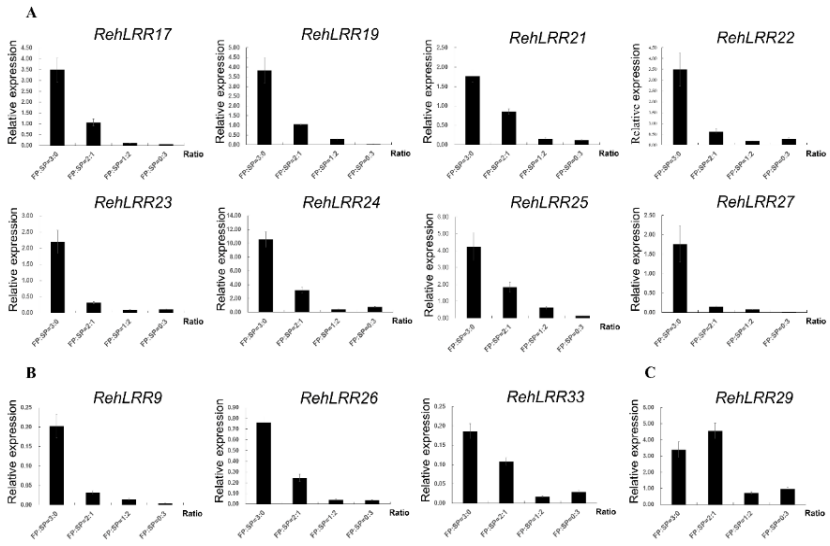

Fig. 5: The expression of the *LRR-RLK* genes under replanted stress in potted *R. glutinosa.* FPS: SPS=3:0, 2:1, 1:2, 0:3 represent the different ratio of the first-year planted soils (FPS) and the second-year planted soils (SPS) respectively. All genes were standardized according to the internal references (18S gene)

and SPS in four treatments (FPS:SPS=3:0, FPS:SPS=2:1, FPS:SPS=1:2, FPS:SPS=0:3) were used in the pot experiment, and a total of 12 genes in the first expression pattern were chose to identify the expression of the *LRR-RLK* genes under four gradient replanted stress in controlled condition. The simulation experimental results showed that the expression of 12 *LRR-RLKs* genes could be divided into 3 groups. The first group included 8 genes, which were high-expressed in FPS but down-regulated and their expression was declining with the augment of SPS (Fig. 5A). Secondly, three genes were inactive under replanted stress and normal condition (Fig. 5B). The last one gene, *RehLRR29*, showed unbinding climb condition in the second treatment and decreased rapidly in the others (Fig. 5C).

Preparation and Expression of the Polyclonal Antibodies against the RehLRR19 Protein and RehLRR29 Protein

To further verify the expression patterns of the LRR-RLKs in the replanted *R. glutinosa* genes (*RehLRR19, RehLRR29*), which were highly expressed specifically in the replanted *R. glutinosa*, were selected and analysed in detail on the protein level. The ORF full-length cloning and gene sequencing were used to construct the recombinant plasmids that were used in the antigen preparation (Table 2).

The antigen sequences were designed and prepared using a rabbit antibody based on the sequence of the RehLRR19 and RehLRR29 protein. WB was used to verify the protein expression abundance in *R. glutinosa*. As a result, the expression of the RehLRR19 and RehLRR29 protein gradually increased with the developmental process in normally grown *R. glutinosa* and decreased dramatically after the mid-expansion stage. The RehLRR19 and

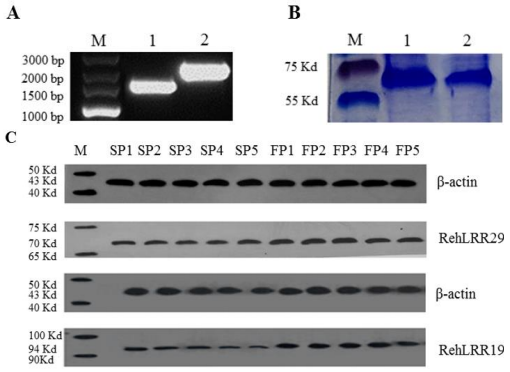

Fig. 6: A: Gel electrophoresis of the *RehLRR19* and *RehLRR29* gene amplification; **B**: SDS-PAGE of antigens (1: vector; 2: enzyme-digested product; M: Marker). **C**: The expression of the RehLRR19 and RehLRR29 protein during the developmental process of the FP and SP *R. glutinosa.* M: marker. FP1 to FP5 and SP1 to SP5 indicated different developmental stages for first-year planting (FP) and second-year planting (SP) *R. glutinosa*, in which 1 to 5 represent seedling, elongation, pre-expansion, mid-expansion and late-expansion, respectively

RehLRR29 protein showed an identical trend at both the protein and transcriptome levels (Fig. 6).

Discussion

Numerous studies in plants owned the property of replant disease have demonstrated that the interaction among plants, microbes and allelopathic autotoxins promoted the formation of replant disease (Guo *et al*., 2006; Zhang *et al*., 2013; Chen *et al*., 2016; Wu *et al*., 2018). Of which, the

allelopathic autotoxins released from plants induced the microbe shift from the beneficial to the pathogenic ones in rhizosphere soils (Mazzola, 1998; Zhang *et al.*, 2010; Lin *et al.*, 2015). These pathogens contained in rhizosphere continously attacked the replanted plants, finally causing to the death of replanted plants (Li *et al*., 2012; Chi *et al*., 2013; Bigeard *et al*., 2015; Zhao *et al*., 2016). Previous studies have confirmed that the PTI related proteins in replanted *R. gltuinosa* were disordered during formation of replant disease (Li *et al*., 2013; Yang *et al*., 2014, 2015; Wu *et al*., 2016). LRR-RLKs in PTI were the most important defenders prevented pathogen infection, and their effectiveness determined the plant disease resistance. For example, overexpression of rice *OsSERK1* led to an increase resistance to the blast fungus, and silencing of *XIK1* decreased the plant resistance to *Xanthomonas oryzae* pv. *oryzae* (Hu *et al*., 2015). In *Nicotiana benthamiana*, *StLRPK1* overexpression could enhance disease resistance against *Phytophthora infestans* (Wang *et al*., 2018). In this study, 40 *R. glutinosa* LRR-RLKs with typic structural characteristic that confirmed in other model plants, were identified. These proteins could be divided into 3 groups based on their expression levels in the normal grown and replanted *R. glutinosa*. Of which, 27 *LRR-RLKs* were upregulated in early stages, and inhibited at later stages of replant disease formation. The expression pattern of these *LRR-RLKs* was almost identical to the symptom changes of replanted *R. glutinosa*, majority of whcih begun to rapidly die at later stages of replant disease (Zhang *et al*., 2013; Chen *et al*., 2016). That implied that the resistance levels of *R. glutinosa* contributed by LRR-RLK proteins might not effectively work in replanted *R. glutinosa*.

Some studies have indicated that the resistance of LRR-RLKs against pathogens was related to the species and number of pathogens and the time scale for pathogen infection. Such as, mRNA levels of rice *OsSERK1* were lower at three days after infection (Hu *et al*., 2005). *Arabidopsis LRR-RLKs* were downregulated at late stages of infection by downy mildew disease (Hok *et al*., 2011). *VfRLK2947* expression in Grapevines was repressed in later time points against different pathogens (Islam *et al*., 2015). In general, LRR-RLK proteins gradually lost the ability recognized the pathogens with increasing of level and time of biotic stresses. In comparsion to common biotic stresses, due to the drive of allelopathic autotoxins, the number and proliferation rate of pathogens presented in rhizosphere soils substantially increased during the formation of replant disease (Zhang *et al*., 2010, 2013; Manici *et al*., 2013; Wu *et al*., 2018). Inhibition of above *LRR-RLKs* expression in replanted *R. glutinosa* might be largely attributed to continous profileration of pathogens in rhizosphere of replanted plants. At same time, further experiments on protein level proved that *R. glutinosa* LRR-RLK proteins were inhibited at later stages of replant disease formation. Furthermore, *LRR-RLKs* in tissue culture seedlings of *R. glutinosa* planted in replanted soils were found to be rapidly repressed under indoor condition. Hence we primarily thought that the resistance losses of LRR-RLKs in replanted *R. glutinosa* might be one of the crucial factors resulted in replant disease.

Although the research on replant disease problem of different crops has such a long history, and many processes have been proposed to alleviate it, a large amount of yield reduction of *R. glutinosa* still occurs. The fundamental reason is that there is no definite objective. In the situation of unknown reasons for replant disease, a new concept is to determine the source of the origin of the replant disease. Alleviating the deleterious nature is necessary to reduce the loss due to replant disease. *LRR-RLK* genes identfied in this study might effected directly or indirectly the formation of replant disease. Further study and analysis of these genes have significance to completely clarify the formation mechanism of replant disease. Simultaneously, it also provides a possible way to alleviate the problems of replant disease at the molecular level.

Conclusion

The present study identified 40 LRR-RLKs proteins in *R. glutinosa*, and described their corresponding functions, structures and phylogenetic traits. At same time, *LRR-RLK* genes were found to be involved in the formation of replant disease in *R. glutinosa*. The findings of this study provided insights into the mechanism of formation of replant disease.

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