Acta Physiologiae Plantarum (2018) 40:95
https://doi.org/10.1007/s11738-018-2672-1

ORIGINAL ARTICLE

Identification of *Rehmannia glutinosa* L. NB-ARC family proteins and their typical changes under consecutive monoculture stress

Aiguo Chen[1,2] · Li Gu[1] · Mingjie Li[1,3] · Na Xu[2] · Fajie Feng[1] · Guangliang Liu[2] · Bao Zhang[1] · Daping Gong[2] · Junyi Zhang[1,4] · Hongyan Liu[1] · Zhongyi Zhang[1,3]

Received: 10 July 2017 / Revised: 14 April 2018 / Accepted: 21 April 2018 / Published online: 27 April 2018

Abstract

NB-ARC proteins are critical to effector-triggered immunity and play important roles in effector recognition and signal transduction in healthy plant growth. However, their primary protein traits, functions and roles remain incompletely understood in *Rehmannia glutinosa*. Here, we identified 45 NB-ARC protein sequences from the protein sequence sets and transcriptome of *R. glutinosa*. The CC type was the main one, accounting for 84.44% of these sequences. The most conserved motif was a 288 aa ADP-binding sequence. This motif belongs to the disease-resistance proteins. Differential expression of 36 expressed *NB-ARC* genes revealed that *NB-ARC* genes were rarely expressed 30 days after planting and were frequently expressed approximately 60 days after planting. To further understand the function of NB-ARC in replanted *R. glutinosa*, the genes encoding NB-ARC domains were profiled using qRT-PCR under the different stress states involved in the formation of consecutive monoculture problems. The results showed that *NB-ARC* genes might play a role in the formation of *R. glutinosa* consecutive monoculture problems. This study is the first to identify NB-ARC genes in *R. glutinosa* and to reveal their roles in consecutive monoculture problems in *R. glutinosa*. These findings provide insights into the mechanism of formation of consecutive monoculture problems.

Keywords *Rehmannia glutinosa* L. · Consecutive monoculture problems · NB-ARC · Expression profile

Introduction

Consecutive monoculture is the dominant agricultural practice in modern agricultural production due to limited land resources (Lin et al. 2016). However, this mode typically triggers the formation of consecutive monoculture problems, leading to crop yield reduction, quality deterioration, poor growth status, and disease aggravation. The formation of consecutive monoculture problems involves complex interactions among soil-borne diseases, allelochemicals and soil quality deterioration (Zhang and Lin 2009). Consecutive monoculture problems are widespread in crop plant production, especially in medicinal crops.

Rehmannia glutinosa, which belongs to *Scrophulariaceae*, is an important Chinese medicinal herb. *R. glutinosa* plants that are cultivated for consecutive years on the same land typically exhibit negative characteristics, including abnormal growth and death during production. Notably, these effects affect only *R. glutinosa* and can persist for 8–10 years before *R. glutinosa* can be replanted (Wen et al. 2001). Given these typical characteristics, *R. glutinosa*

Communicated by Z.-L. Zhang.

Aiguo Chen and Li Gu have contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11738-018-2672-1) contains supplementary material, which is available to authorized users.

✉ Zhongyi Zhang
zyzhang@fafu.edu.cn

[1] Key Laboratory of Ministry of Education for Genetics, Breeding and Multiple Utilization of Crops, Fujian Agriculture and Forestry University, Fuzhou 350002, China

[2] Tobacco Research Institute, Chinese Academy of Agricultural Sciences, Qingdao 266101, China

[3] Key Laboratory of Crop Ecology and Molecular Physiology of Fujian Universities, Fujian Agriculture and Forestry University, Fuzhou 350002, China

[4] Department of Horticulture, Huaqiao University, Xiamen 361021, China

is an ideal material for studying the mechanism of formation of consecutive monoculture problems (Li et al. 2015; Wen et al. 2002). Previous studies have indicated that some biotic and abiotic stress factors involved in the formation of consecutive monoculture problems in *R. glutinosa* (Tian et al. 2017; Wu et al. 2015). In consecutive monocultures of *R. glutinosa*, root exudates selectively attract pathogenic microbes, which colonize the root surface in consecutive monocultures of *R. glutinosa*. This effect ultimately causes the proliferation of harmful microorganisms in the rhizosphere and induces adverse chemotaxis from "bacterial" to "fungal" types (Wu et al. 2013, 2015, 2016; Zhang et al. 2011; Zhang and Lin 2009). A specific pathogenic *Fusarium oxysporum* screened from rhizosphere soil microorganisms has been identified as *Fusarium oxysporum* f.sp. *R. glutinosa*. The strain can specifically invade *R. glutinosa* and this has been successfully simulated in the laboratory (Wu et al. 2016). Previous studies also screened and identified allelochemicals, such as ferulic acid, from the exudates of rhizosphere in *R. glutinosa*, which successfully induced the characteristics of consecutive monoculture problems in pot experiments (Zhang et al. 2016). Approximately 70 percent of consecutive monoculture problems can in fact be attributed to soil-borne diseases (Sun et al. 2008). The results suggested that the imbalance of the rhizosphere microecology probably is one important cause for the formation of consecutive monoculture problems in *R. glutinosa*. Modern plant immunology and molecular biology studies have showed that pathogen infection is closely related to the false response of host plant immune defence system (Smith et al. 2014; Kushalappa et al. 2016). Previous studies have demonstrated that core biological processes, including DNA replication, RNA transcription and protein translation, are significantly damaged in consecutive monocultures of *R. glutinosa*. The expression of some genes is closely related to fibre root formation and replant disease. In addition, Ca^{2+}, MAPK and ethylene signals and chromatin modification are specifically up-regulated in consecutive monocultures of *R. glutinosa* (Li et al. 2013; Wu et al. 2016; Yang et al. 2013, 2014, 2015). Therefore, we infer that the immune defence system in *R. glutinosa* could respond incorrectly to consecutive monoculture stress to aggravate abnormal growth and even lead to death.

The plant immune system consists of PTI (pathogen-associated molecular pattern (PAMP)-triggered immunity) and ETI (effector-triggered immunity) (Jones and Dangl 2006; Stael et al. 2015). The pattern recognition receptors (PRRs) of PTI systems act on the cytomembrane and can recognize conserved PAMP features of different species or genera. The receptors of ETI systems located in the cytoplasm can specifically and robustly respond to pathogen effectors through NB-LRR (nucleotide-binding-leucine-rich repeat) domain-mediated perception (Bigeard et al. 2015; Dodds and Rathjen 2010; Groll et al. 2008; Marone et al. 2013; Thomma et al. 2011). NB-LRR mainly consists of a carboxy-terminal LRR domain, NB-ARC (nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4) and amino-terminal TIR (toll/interleukin-1 receptor/coiled coil) or CC domains. LRRs are important for effector recognition, and TIR/CC transmits signals of pathogen invasion to downstream proteins, including EDS (enhanced disease susceptibility) and NDR (non-race-specific disease resistance), to induce a hypersensitive response (Ma et al. 2013). In the plant immune response to effectors derived from invading pathogens, NB-LRR domians determine whether the ETI can be properly initiated (Gassmann and Bhattacharjee 2012; Pajerowska-Mukhtar et al. 2013). NB-ARC proteins are encoded by numerous polymorphic *R* family genes (Yue et al. 2011), whereas the protein properties of NB-ARC and their response mechanisms in consecutive monocultures of *R. glutinosa* remain largely unknown. Here, we identified *R. glutinosa* NB-ARC proteins and analysed their differential expression profiling at different developmental stages. The stress factors involved in the formation of consecutive monoculture problems in *R. glutinosa* were used to assess the roles of NB-ARC proteins in consecutive monoculture problems. These findings provide valuable information for understanding the roles of NB-ARC in the formation of consecutive monoculture problems.

Materials and methods

Plant materials and treatments

Field experiments with *R. glutinosa* 'Wen 85-5' were arranged at the Wenxian Agricultural Institute in Jiaozuo City, Henan Province, China. One group of seedlings was grown in a field where *R. glutinosa* had not been planted for more than 10 years. Another group was grown in a field where the same cultivar had been grown the previous year (planted on April 20 and harvested on November 27, 2012). For convenience of description, we refer to the former group as first-year plants (FP) and the latter group as second-year plants (SP). The tuberous roots of *R. glutinosa* used for cultivation in two groups were planted with the density of 30 cm × 30 cm on April 10, 2013. A total of 2000 plants were contained in each group. Fields were maintained with locally normal production conditions. The fresh tuberous roots were collected, respectively, at 30, 60, 90, 120, and 150 days after planting, and six plants were taken at each time. Each plant was regarded as a biological replicate. All samples mentioned above were immediately frozen in liquid nitrogen and stored at − 80 °C until use.

Pot experiments were performed under controlled conditions (28 °C, 10,000 lx) at the Institute of GAP for Chinese

Medicinal Materials, Fujian Agriculture and Forestry University. *R. glutinosa* "wen 85-5" was grown in plastic pots of 25 cm diameter and 22 cm height. Four treatments, including first-year planting (FP), second-year planting (SP), ferulic acid chemigation (FA), and invasion of *Fusarium oxysporum* [FO, identified as *Fusarium oxysporum* f.sp. *R. glutinosa* (Wu et al. 2016)], were conducted, and each treatment was assessed in ten pots. The soil used in the FP, FA and FO treatments was collected from land where *R. glutinosa* had not been planted for at least the last 10 years, and the soil used in the SP treatment was collected from land where *R. glutinosa* had been planted in the previous year. Each treatment was conducted as follows. For the FA treatment, FA solution was used for watering until a negative appearance was noted that was similar to the appearance caused by consecutive monoculture problems. In total, 60 ml of FA solution was poured each time in a pot, and the final concentration was 10 mmol L^{-1} (the FA concentration gradient was from 0.3 to 10 mmol L^{-1} in 30 days). For the FO treatment, *F. oxysporum* strains were isolated from the infected *R. glutinosa* tuberous roots and cultured in 100 ml of potato dextrose broth (PDB) in 250 ml Erlenmeyer flasks for 4–5 days at 24 °C, without shaking and in darkness. The mycelia were filtered from the PDB and washed three times with sterile, distilled water. Conidial suspensions of *F. oxysporum* f.sp. *R. glutinosa* were prepared from 5-day-old cultures in PDB, followed by filtering through four layers of cheesecloth and diluting to 1×10^6 conidia/ml with sterilized water. FO suspension solution was irrigated until disease symptoms appeared. The amount of FO solution added to a pot was 60 ml each time. The FP and SP treatments were irrigated with the same volume plain water (60 ml) and assessed 60 days after planting. Each treatment was photographed, and fresh tuberous roots were then collected and stored at − 80 °C after being frozen by liquid nitrogen for qRT-PCR.

Identification of *R. glutinosa* NB-ARC proteins

To identify *R. glutinosa*-specific NB-ARC proteins, HMM files (PF00931) that characterized the conserved properties of the NB-ARC structure were extracted from the Pfam 31.0 database (http://pfam.xfam.org/). HMMER suite (v3.1b2, Finn et al. 2015) was used to identify candidate NB-ARC proteins in the *R. glutinosa* database translated from the *R. glutinosa* transcriptome based on the HMM files. NB-ARC proteins were further analysed using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) and PSORT software (http://psort.hgc.jp/form.html) to predict the transmembrane and subcellular localization, respectively. The proteins located in the cytoplasm with loss of transmembrane structure were identified as candidate NB-ARC proteins. The genes encoding NB-ARC proteins were simultaneously screened from the *R. glutinosa* transcriptome database (Li et al. 2017). NB-ARC functions were further confirmed based on annotation information from the Nr (NCBI non-redundant protein sequences, http://www.ncbi.nlm.nih.gov/), GO (Gene Ontology, http://www.geneontology.org/) and KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) databases.

Protein structure and phylogenetic analysis

The conserved motifs of *R. glutinosa* NB-ARC proteins were identified using the online SMART tool (Marchler-Bauer et al. 2017) and depicted using IBS 1.0.1 software (Liu et al. 2015). The secondary structures of the NB-ARC proteins were identified using DNAMAN8.0 software (Lynnon BioSoft, Quebec, Canada). The tertiary structures of the NB-ARC proteins were analysed based on homology modelling methods using the online SWISS-MODEL software (http://www.swissmodel.expasy.org/) and were displayed using SPDBV 4.10 software (Guex and Peitsch 1997). Phylogenetic relationships among candidate NB-ARC proteins were constructed based on the neighbour-joining method and bootstrap method (1000 replicates) using MEGA6.06 software (Tamura et al. 2013).

Expression profile analysis of *NB-ARC* genes

Total RNA in field experiment was extracted from the tuberous root using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and treated with DNase I (Qiagen) to degrade any possible DNA contamination. 6 μg total RNA was treated with oligo-(dT) magnetic beads to purify the mRNA. The mRNA was then fragmented into short (100–200 bp) pieces using moderate divalent cations under high-temperature conditions. These fragments were reverse transcribed into the cDNA first strand with a random hexamer primer, followed by cDNA second strand generation using DNA polymerase I. The cDNA fragments were enriched using PCR amplification and then purified using magnetic beads. The library products were sequenced on the Illumina HiSeq™ 2000 (Beijing Genomics Institute, Shenzhen, China). After quality control (QC) and further filtration, the clean reads were mapped onto the RNA-Seq reference sequence using the BWA software (Kobayashi et al. 2014). Sequence alignment was controlled in no more than 2 bp mismatches. The gene expression level was calculated using the RPKM (Reads Per Kilobase of transcript per Million mapped reads) method (Mortazavi et al. 2008). The threshold of "FDR (False Discovery Rate) ≤ 0.001 and the absolute value of $\log_2^{Ratio}$ (Ratio = RPKM of SP/RPKM of FP) ≥ 1" was used to assess the significance of the gene expression differences. In 45 *NB-ARC* genes aligned from the *R. glutinosa* transcriptome library, 36 differentially expressed *NB-ARC* genes were used in the following studies based on the expression profiling. A differential expression heatmap of

identified genes was analysed via the hierarchical clustering method using MeV 4.9.0 tool (Saeed et al. 2006).

RNA isolation and reverse transcription

Total RNA in pot experiment was extracted from different samples of fresh *R. glutinosa*. Approximately 50 mg of tissue was collected and subjected to RNA extraction using TRIzol reagent (Invitrogen). Total RNA samples were treated extensively with RNase-free DNase I (Invitrogen) to remove any contaminating genomic DNA. RNA concentration was measured using a spectrophotometer, and RNA integrity was ensured via analysis on a 1.5% (w) agarose gel. First strand cDNA was synthesized in a 20 µL of mixture containing 2 µg of total RNA, 2 µL of 50 µM oligo-(dT)12–18 primers, 1 µL of RNase inhibitor and 1 µL of 40 U/µL M-MLV reverse transcriptase (Invitrogen). The mixture was incubated at 37 °C for 50 min and then heated to 70 °C for 15 min.

qRT-PCR analysis

For qRT-PCR, NB-ARC PCR primers (Additional file 1) were designed using Beacon designer 8.0 software (Premier Biosoft International, Palo Alto, CA, USA). A fragment of gene encoding 18S rRNA (Additional file 1) was used as a reference. PCR was performed using a Bio-Rad IQ5 instrument (Bio-Rad, Hercules, CA, USA) based on SYBR Green to detect transcript abundance. Each 25 µL of reaction contained 0.5 µM of each primer and approximately 0.5 U enzymes, cDNA and SYBR Green. Negative control reactions contained no cDNA. Fivefold dilutions of the cDNA template were tested under identical conditions. The PCR programme included an initial denaturing step (95 °C for 10 s); 40 cycles at 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 15 s; and a final stage at 55–95 °C to determine the dissociation curves of the amplified products. All reactions were at least replicated for three times. The data were analysed using Bio-Rad IQ5 Optical System Software v2.1. The data were normalized on the basis of the 18S rRNA threshold cycle (Ct) value. The samples in the FP treatment were used as controls, and their normalized Ct values were set to 1. The relative gene expression of the SP, FA and FO was calculated using the$2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The expression data of the different treatments were statistically analysed with SAS 9.1 statistical software using one-factor ANOVA and Duncan multiple comparisons.

Results

Identification of the NB-ARC protein family in *R. glutinosa*

A set of 68 NB-ARC protein sequences were separated from the *R. glutinosa* protein sequence sets obtained from our previous study using the Hidden Markov model method (Li et al. 2017). Subsequently, 45 sequences were further confirmed in Nr and KEGG using blastp. To assess the NB-ARC homology with other species, *R. glutinosa* NB-ARC sequences were aligned using the NCBI database, resulting in 19 sequences highly homologous (38–89%) to *Sesamum indicum*, 21 sequences highly homologous (51–85%) to *Erythranthe guttata*, and 5 sequences highly homologous (45–59%) to *Nicotiana tomentosiformis, Capsicum annuum, Arachis ipaensis, Nicotiana tabacum* and *Cynara cardunculus* (Table 1). Further functional annotation on basis of KEGG and GO analyses of the candidate NB-LRR proteins revealed that all sequences were resistance proteins involved in the interaction between plant and pathogen (Additional files 2, 3).

Protein structure analysis of the NB-ARC sequences in *R. glutinosa*

In general, NB-ARC proteins in the ETI system typically lack a transmembrane region. To assess the subcellular localization of 45 NB-ARC sequences, the corresponding transmembrane trait was predicted using the online TMHMM server 2.0 and PSORT software. As a result, all candidate NB-LRR proteins were located in the cytoplasm and lacked a transmembrane region.

According to the prediction, the conserved domains of the 45 NB-ARC sequences and their lengths are presented in Fig. 1a. In total, 45 NB-ARC sequences contained at least one conserved domain of LRR, NB-ARC or TIR/CC. Of these, seven proteins, including CL343.Contig1_All, CL4723.Contig1_All, CL6508.Contig2_All, CL6748.Contig1_All, CL7829.Contig2_All, Unigene907_All and Unigene12365_All, contained NB-ARC, LRR and TIR/CC. NB-ARC sequences with a CC type amino terminal were the main type, accounting for 84.44% of the total NB-ARC sequences. Moreover, the 45 NB-ARC sequence lengths ranged from 195 to 1310 aa.

Based on the conserved motifs in the NB-ARC domain (Additional file 4), an ADP (adenosine diphosphate)-binding element of 288 aa in length was the most conserved motif, containing 12 α-helixes and 4 β-strands. Of these structures, 4 parallel β-strands located at Ser24–Gly30, Leu55–Val61, Leu105–Asp109, and Ile135–Thr139 in the

Table 1 NB-ARC family proteins identified in the *R. glutinosa* transcriptome

Gene ID	Amino acid length (aa)	Blast results (query cover, *E* value, identities, accession no., description, [species])
CL343.Contig1_All	1310	100%, 0, 79%, XP_011089052.1, disease resistance protein At3g14460 [*Sesamum indicum*]
CL698.Contig4_All	476	100%, 9E−166, 59%, XP_012841530.1, late blight resistance protein homologue R1A-3 [*Erythranthe guttata*]
CL788.Contig4_All	760	99%, 0, 64%, XP_012854094.1, disease resistance protein RGA4 [*Erythranthe guttata*]
CL1151.Contig3_All	626	100%, 0, 60%, XP_012857645.1, late blight resistance protein homologue R1A-3 [*Erythranthe guttata*]
CL1654.Contig1_All	854	99%, 0, 59%, XP_012853175.1, late blight resistance protein homologue R1A-3 [*Erythranthe guttata*]
CL3079.Contig2_All	489	100%, 1E−179, 59%, XP_012853244.1, late blight resistance protein homologue R1A-10 isoform X2 [*Erythranthe guttata*]
CL4021.Contig1_All	519	100%, 2E−162, 50%, XP_009617577.1, disease resistance protein At5g66900 [*Nicotiana tomentosiformis*]
CL4439.Contig1_All	233	100%, 2E−110, 71%, XP_012854177.1, disease resistance protein RGA1 [*Erythranthe guttata*]
CL4447.Contig1_All	862	99%, 0, 88%, XP_011080937.1, uncharacterized protein LOC105164077 [*Sesamum indicum*]
CL4723.Contig1_All	888	100%, 0, 85%, XP_012855653.1, late blight resistance protein homologue R1A-10 [*Erythranthe guttata*]
CL6508.Contig2_All	891	100%, 0, 88%, XP_011070823.1, late blight resistance protein homologue R1B-17 [*Sesamum indicum*]
CL6748.Contig1_All	811	100%, 0, 63%, XP_012849297.1, disease resistance protein RPM1-like isoform X1 [*Erythranthe guttata*]
CL7604.Contig1_All	306	100%, 2E−119, 60%, XP_011098143.1, disease resistance protein RPM1-like [*Sesamum indicum*]
CL7774.Contig1_All	867	100%, 0, 59%, XP_012829248.1, late blight resistance protein homologue R1A-3 [*Erythranthe guttata*]
CL7829.Contig2_All	918	100%, 0, 88%, XP_011073815.1, disease resistance protein At1g50180 [*Sesamum indicum*]
CL8221.Contig1_All	268	98%, 3E−51, 38%, XP_011078622.1, disease resistance protein At1g50180 [*Sesamum indicum*]
CL8782.Contig1_All	610	100%, 0, 59%, XP_012855535.1, late blight resistance protein homologue R1A-10 [*Erythranthe guttata*]
CL9919.Contig1_All	721	99%, 0, 65%, XP_012853175.1, late blight resistance protein homologue R1A-3 [*Erythranthe guttata*]
CL10007.Contig4_All	195	100%, 4E−68, 61%, XP_012856179.1, late blight resistance protein homologue R1B-16 [*Erythranthe guttata*]
Unigene283_All	327	100%, 0, 85%, XP_011070551.1, late blight resistance protein homologue R1B-14 [*Sesamum indicum*]
Unigene907_All	935	100%, 0, 73%, XP_011072600.1, disease resistance protein RGA2-like [*Sesamum indicum*]
Unigene2179_All	443	100%, 2E−172, 55%, XP_016547057.1, disease resistance protein TAO1-like isoform X2 [*Capsicum annuum*]
Unigene2278_All	539	100%, 9E−180, 51%, XP_012833863.1, disease resistance RPP13-like protein 3 [*Erythranthe guttata*]
Unigene4299_All	217	100%, 5E−102, 76%, XP_011078960.1, disease resistance protein RGA1 [*Sesamum indicum*]
Unigene4345_All	535	100%, 0, 81%, XP_011080807.1, disease resistance RPP13-like protein 2 [*Sesamum indicum*]
Unigene4363_All	901	99%, 0, 59%, XP_011091324.1, disease resistance protein At1g58602 [*Sesamum indicum*]
Unigene6172_All	247	98%, 2E−122, 73%, XP_011083960.1, late blight resistance protein homologue R1A-10 isoform X2 [*Sesamum indicum*]
Unigene6303_All	226	100%, 3E−107, 72%, XP_011089745.1, late blight resistance protein homologue R1A-10 [*Sesamum indicum*]
Unigene7113_All	722	99%, 0, 56%, XP_012829191.1, late blight resistance protein homologue R1A-10 [*Erythranthe guttata*]
Unigene7646_All	626	99%, 0, 59%, XP_012857645.1, late blight resistance protein homologue R1A-3 [*Erythranthe guttata*]
Unigene7647_All	619	100%, 0, 61%, XP_012857645.1, late blight resistance protein homologue R1A-3 [*Erythranthe guttata*]
Unigene7657_All	794	100%, 0, 58%, XP_012829191.1, late blight resistance protein homologue R1A-10 [*Erythranthe guttata*]
Unigene9801_All	435	100%, 5E−124, 45%, XP_016178697.1, disease resistance protein RGA3 [*Arachis ipaensis*]
Unigene10109_All	334	100%, 5E−118, 56%, XP_012847840.1, late blight resistance protein homologue R1A-3 [*Erythranthe guttata*]
Unigene12365_All	1183	99%, 0, 63%, XP_011088145.1, disease resistance protein RGA3 [*Sesamum indicum*]
Unigene12477_All	820	100%, 0, 89%, XP_011083793.1, disease resistance protein At4g33300 [*Sesamum indicum*]
Unigene13465_All	873	100%, 0, 68%, XP_011079215.1, disease resistance RPP13-like protein 1 [*Sesamum indicum*]
Unigene13744_All	251	99%, 5E−98, 59%, XP_016512278.1, disease resistance protein TAO1-like [*Nicotiana tabacum*]
Unigene17274_All	424	100%, 0, 83%, XP_012850882.1, disease resistance protein At4g27220 [*Erythranthe guttata*]
Unigene21268_All	252	100%, 1E−86, 60%, XP_011072560.1, late blight resistance protein homologue R1A-3 [*Sesamum indicum*]
Unigene23508_All	220	100%, 1E−49, 45%, KVH92583.1, disease resistance protein [*Cynara cardunculus var. scolymus*]
Unigene25475_All	889	99%, 0, 53%, XP_011071970.1, late blight resistance protein homologue R1A-10 [*Sesamum indicum*]

Table 1 (continued)

Gene ID	Amino acid length (aa)	Blast results (query cover, *E* value, identities, accession no., description, [species])
Unigene25774_All	722	99%, 0, 56%, XP_012829191.1, late blight resistance protein homologue R1A-10 [*Erythranthe guttata*]
Unigene26135_All	877	99%, 0, 51%, XP_011091064.1, late blight resistance protein homologue R1B-16 [*Sesamum indicum*]
Unigene26472_All	375	100%, 7E−163, 63%, XP_012856361.1, late blight resistance protein homologue R1B-17 [*Erythranthe guttata*]

NB-ARC domain sequence enclosed the core barrel structure of the subunit (Fig. 1b).

Phylogenetic analysis of the *R. glutinosa* NB-ARC proteins

To identify the NB-ARC sequence domains of *R. glutinosa* and their homology with *Arabidopsis thaliana*, an unrooted phylogenetic tree was constructed using sequence alignment via the neighbour-joining method. In total, the 45 candidate sequences were divided into 6 groups. Of these, group VI included 25 NB-ARC proteins with relatively complete ARC motifs. Groups I, II, IV and V consisted of six, two, four and seven proteins, respectively. Group III had only one sequence (Fig. 2a). These data were consistent with stable binding of 12 LRR monomers, which require longer NB-ARC domains (Ma et al. 2013).

Rehmannia glutinosa NB-ARC sequences were also aligned with *Arabidopsis thaliana* sequences. Eighteen homologous NB-ARC protein sequences were present in *R. glutinosa*. Highly conserved motif traits and similar protein functions were presented according to the node on the phylogenetic tree. Of these proteins, six NB-ARC proteins, Unigene17274_All, Unigene2179_All, CL7829.Contig2_All, CL343.Contig1_All, Unigene13465_All, and Unigene9801_All, in *R. glutinosa* exhibited high similarity with six NB-ARC proteins, AT4G27220.1, AT4G27190.1, AT4G09360.1, AT3G14470.1, AT3G14460.1 and AT1G50180.1, in *Arabidopsis thaliana* (Fig. 2b).

Expression of *NB-ARC* genes at different developmental stages in FP and SP *R. glutinosa*

The gene expression pattern can provide important clues regarding gene function. To clarify the response characteristics of *NB-ARC* genes in consecutive monocultures of *R. glutinosa*, 45 *NB-ARC* genes were matched from the *R. glutinosa* differential expression profiles using RNA-Seq. The expression profiles were evaluated using Euclidean distance hierarchical clustering (Fig. 3a, b, additional file 5).

According to the *R. glutinosa NB-ARC* gene expression profiles in response to two treatments of FP and SP, 36 expressed genes could be divided into three groups (Fig. 3a, additional file 5). Groups I (Unigene12477_All and Unigene 907_All) and II (Unigene 25475_All and CL4439.Contig1_All) contained two genes respectively, all of which presented higher expression abundance in comparison to other group during entire growth period of FP and SP treatments. The other genes belonged to group III had the characteristics of lower sustained expression ability. Most of the *NB-ARC* genes were up-regulated in consecutive monocultures of *R. glutinosa* (Fig. 3b, additional file 5). The results indicated that consecutive monoculture stress mediates increased expression of *NB-ARC* genes in the ETI system.

Among the *NB-ARC* genes expressed at the SP1-SP5 developmental stages in consecutive monocultures of *R. glutinosa*, the up-regulated genes accounted for 8.33, 91.67, 38.89, 66.67 and 97.22%, respectively. *NB-ARC* genes were rarely expressed in the SP1 stage (1–30 days after planting) under consecutive monoculture stress, whereas *NB-ARC* genes were frequently expressed in the SP2 (31–60 days after planting) and SP5 (121–150 days after planting) stages. The changes were consistent with normal growth at the SP1 stage and large-scale plant deaths during the SP2 stage in consecutive monocultures of *R. glutinosa*.

Therefore, the SP2 might represent a critical developmental stage in which the immune resistance balance maybe altered in response to pathogen invasion, and the seedling stage may represent a key stage for the formation of the immune resistance system.

Expression profiles of *NB-ARC* genes under different stress factors involved in the formation of consecutive monoculture problems

To identify *NB-ARC* genes involved in the formation of consecutive monoculture problems in response to different stress factors, an in vitro test experiment was conducted under SP, FA, and FO stresses. Expression profiles of different stress factors are presented in Fig. 4. Pearson's correlation coefficients are shown in Table 2.

Compared with the FP control, each treatment caused an abnormal growth phenomenon (Fig. 4a). For different stress factors, FO and FA treatments significantly increased the expression of nine *NB-ARC* genes, and five of the nine *NB-ARC* genes exhibited increased expression upon SP treatment compared with FA (Fig. 4b). Significant positive correlation was only between FO and SP treatments (Table 2).

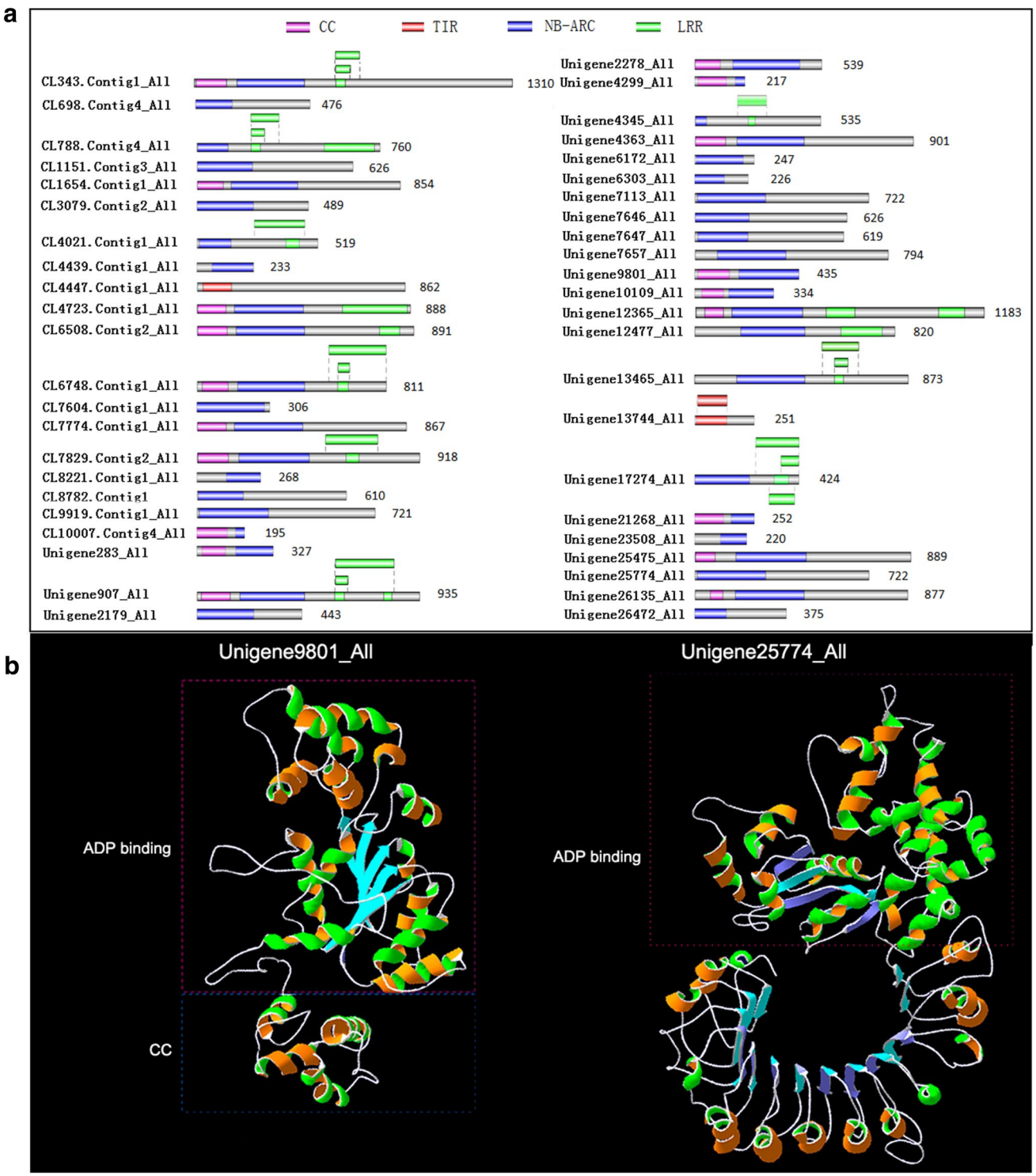

Fig. 1 Schematic diagram of the NB-ARC protein conserved domain in *R. glutinosa*. **a** Four putative domains are represented by a number on the coloured box. Names of all members and domain sizes are presented on the left and right side of the figure, respectively. **b** Tertiary structure of two representative NB-ARC proteins Unigene9801_All and Unigene25774_All

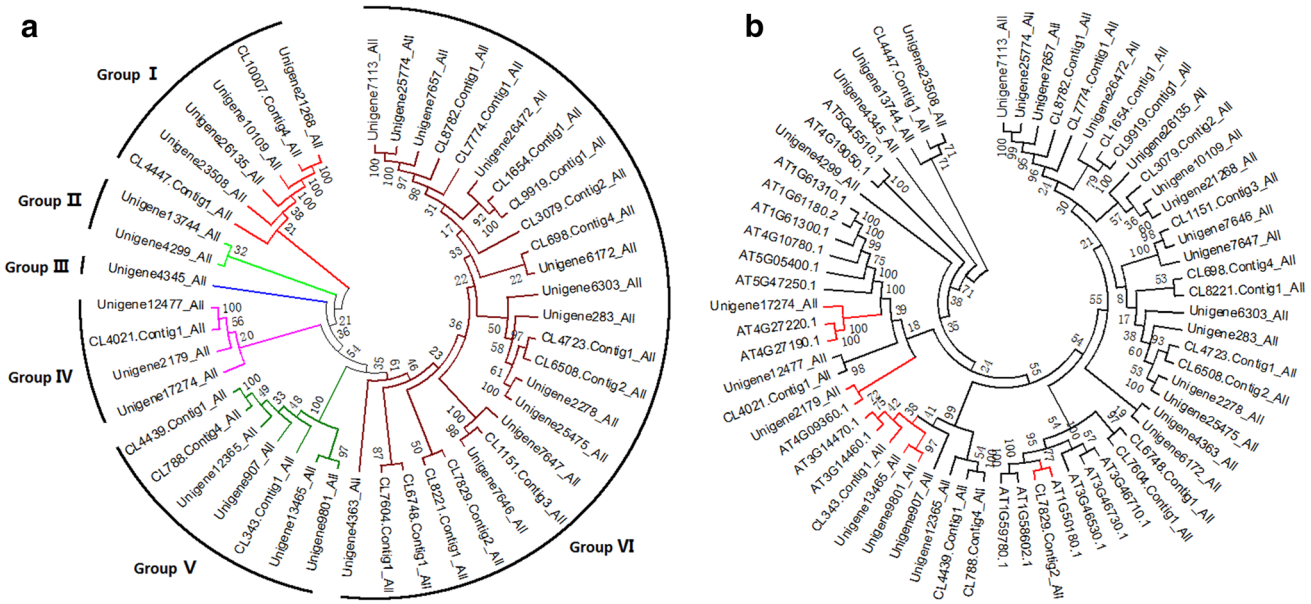

Fig. 2 Phylogenetic tree of *R. glutinosa* NB-ARC protein sequences. Phylogenetic trees of NB-ARC sequences were constructed using the MEGA 6.06 tool. Unrooted neighbour-joining analysis was performed with pairwise deletion and Poisson correction, and the bootstrap values are presented at the corresponding nodes. The same colour represents the same type of homology. **a** Homology analyses for 45 NB-ARC proteins of *R. glutinosa*. **b** Homology analyses for 45 NB-ARC proteins of *R. glutinosa* and alignment with *Arabidopsis thaliana*, in which *R. glutinosa* NB-ARCs remarked by red-coloured line have closely homology with that of *A. Thaliana*

The results showed that SP, FA and FO treatments could induce the up-regulated expression of *NB-ARC* genes, but the induction pattern of *NB-ARC* genes by SP treatment was similar to that by FO treatment. Therefore, *NB-ARC* genes in ETI system were involved in the formation of *R. glutinosa* consecutive monoculture problems.

Discussion

In *R. glutinosa*, consecutive monoculture problems occur in the second year after planting (Zhang et al. 2011). Previous studies have indicated that phenolic acids in root exudates are important allelopathic autotoxins (Li et al. 2012). However, phenolic acids are degraded by soil microorganisms within a week in the field (Zhang et al. 2010). The addition of phenolic acids caused significant increases in the population of pathogenic microorganisms in plate cultures and field experiments (Wu et al. 2017; Zhang et al. 2016). Allelochemicals of root exudates mediated adverse chemotaxis of rhizospheric microbes (Wu et al. 2015; Zhang et al. 2013). The immune systems of *R. glutinosa* are activated and finally compromised due to continuous attack by a large number of proliferating pathogens. (Li et al. 2017). qRT-PCR analysis further demonstrated that *NB-ARC* genes in ETI system are employed in the formation of *R. glutinosa* consecutive monoculture problems.

In the ETI system, the effector is recognized by an NB-ARC protein based on direct and indirect recognizing models (Sekhwal et al. 2015). In NB-ARC proteins, R1A (resistance in linkage group 1A), R1B and RPP13 (resistance to *Peronospora parasitica* 13) can directly recognize effectors (Birch et al. 2008; Du et al. 2015; Kuang et al. 2005; Sekhwal et al. 2015). RPM1 (resistance to *pseudomonas syringae* pv *maculicola*), RPS5 (resistance to *pseudomonas syringae* 5), Prf (for *Pseudomonas* resistance and fenthion sensitivity), RGA (Rx-like proteins), RPS2, TAO1 (target of AvrB operation), RPS4, RPS6, SNC1 (suppressor of npr1-1, constitutive 1) and RPP13 indirectly recognize effectors through interactions with target proteins of RIN4 (RPM1-interacting protein 4), PBS1 (AvrPphB susceptible1), Pto (for *Pseudomonas syringae* pv. *tomato*), RanGAP2 (Ran GTPase-activating protein) and EDS1 (Bittner-Eddy et al. 2000, 2001; Chen et al. 2014; Eitas et al. 2008; Khan et al. 2016; Sacco et al. 2007; Sekhwal et al. 2015; Song et al. 2003; Sun et al. 2013; Zentella et al. 2007). The results of homologous cluster with *R. glutinosa* and *Arabidopsis thaliana* in phylogenetic tree showed that six NB-ARC proteins are closely related to disease resistance. Abnormal growth and death occur in consecutive monocultures of *R. glutinosa*. According to functional predictions, the 45 identified NB-ARC protein sequences in *R. glutinosa* included R1A, R1B, RGA, RPP13, RPM1 and TAO1.

Previous studies have revealed that the expression of pathogenic effectors is closely related to the stage and

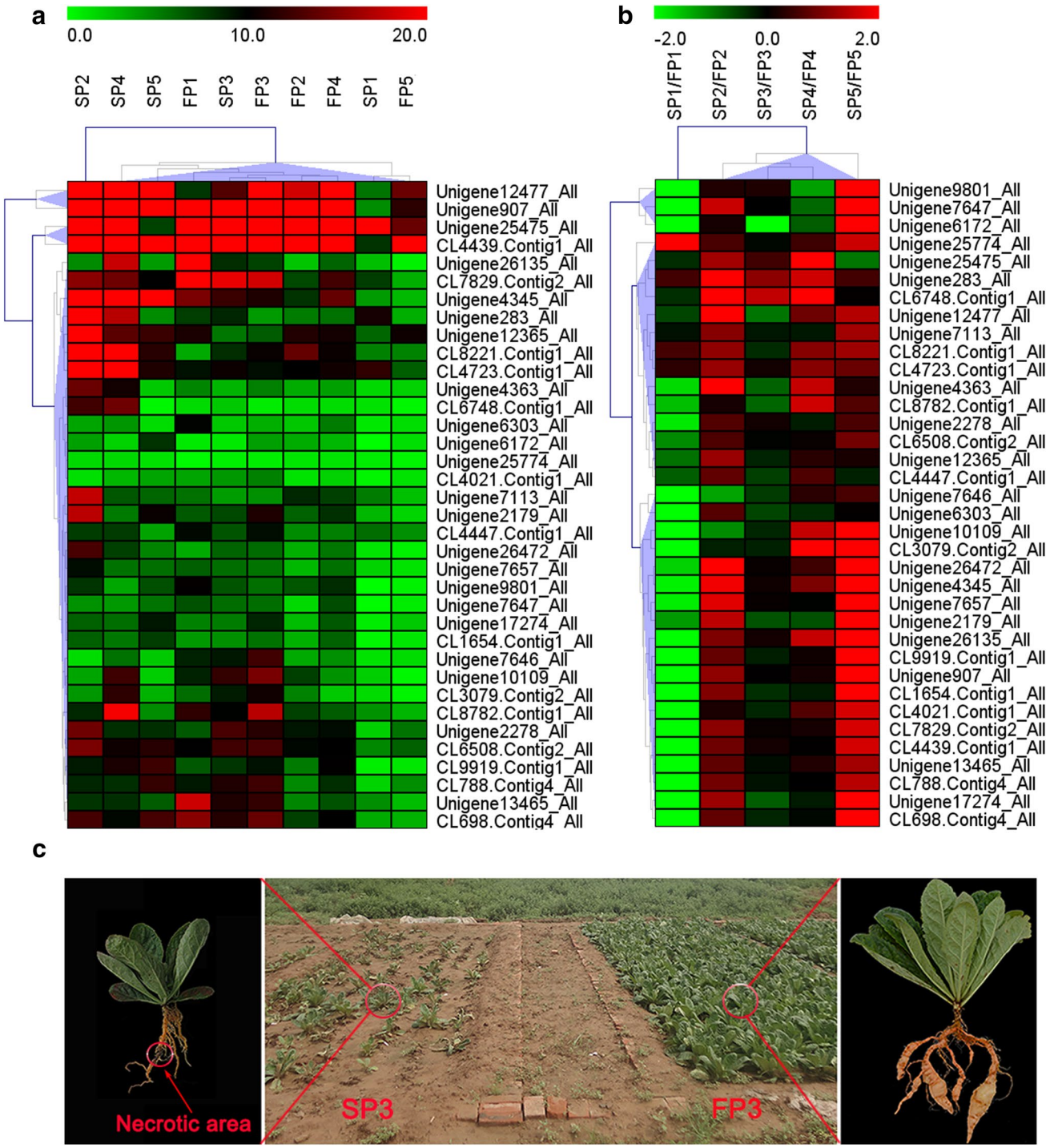

Fig. 3 Expression profiles of 36 expressed *NB-ARC* genes at different developmental stages in *R. glutinosa*. **a** Expression levels (RPKM, reads per kilobase per million mapped reads). **b** Ratios of expression levels. The colour bar presents the gene expression levels. Green indicates low expression, whereas red indicates high expression. Black indicates minimal difference in expression. The five samples represented sampling times of 30, 60, 90, 120, and 150 days after planting. The heat maps were created using MeV 4.9.0 software. **c** Phenotypes of first-year (left) and second-year (right) *R. glutinosa* plants 90 days after planting

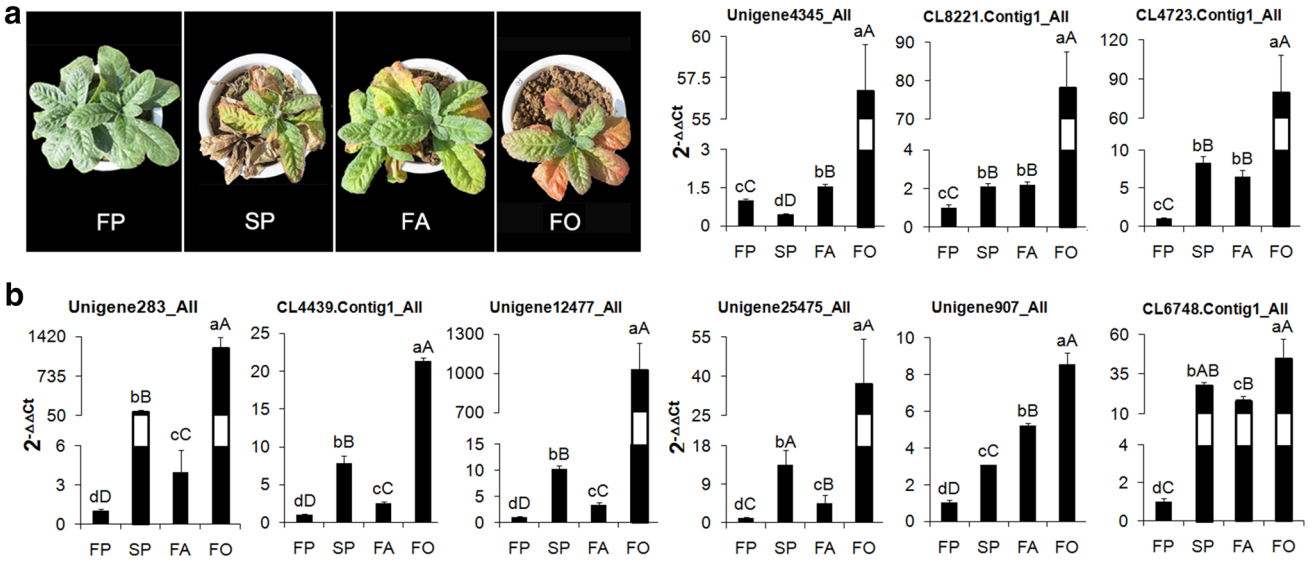

Fig. 4 Nine randomly selected up-regulated *NB-ARC* genes responding to different stress factors involved in the formation of consecutive monoculture problems. **a** Phenotypes of the four different treatments FP, SP, FA and FO. **b** Gene expression levels of the different treatments. FO treatment caused the most significant increases in *NB-ARC* gene expression levels followed by SP and FA treatments. Values are presented as the means ± SD. Lower-case letters indicate significant differences ($P < 0.05$; *t* test), and capital letters indicate significant differences ($P < 0.01$; *t* test). *FP* first-year plant, *SP* second-year plant, *FA* ferulic acid stress, *FO* stress of *Fusarium* invasion (*Fusarium oxysporum* f.sp. *R. glutinosa*)

location of infection (Toruño et al. 2016). For example, the specific effectors, *C. higginsianum* effectors, either inhibit or promote cell death after undergoing threefold increase of expression during infection of *Colletotrichum higginsianum* in *Arabidopsis* (Kleemann et al. 2012). Furthermore, disorder expression of stage-specific effectors significantly reduced *Phytophthora sojae* virulence in *N. benthamiana* and soybean (Wang et al. 2011). In addition, successful expression of *Ustilago maydis* effectors only occurred in the colonized maize organ (Schilling et al. 2014). Therefore, a correct time and place is required when ETI system performs immune function (Smith et al. 2014). Previous studies have demonstrated that immune resistance disorder resulted from inappropriate NB-ARC protein activation (Rodriguez et al. 2015). In this study, the *NB-ARC* genes expression was obviously up-regulated by 91.67% in SP2 stage, and serious death was correspondingly occurred (Fig. 3). The results suggested that consecutive monoculture stress inappropriately triggered *NB-ARC* gene expression, leading to immune response disorders in *R. glutinosa*. qRT-PCR analysis indicated that similar expression patterns of *NB-ARC* genes were observed in SP and FO stress. A possible depict for NB-ARC in ETI-mediated consecutive monoculture stress is presented in Fig. 5.

We have unravelled the protein structures, phylogenies, and gene expression patterns of NB-ARC family proteins in *R. glutinosa*. These findings provide insights into the mechanism of formation of consecutive monoculture problems. Moreover, *NB-ARC* genes exhibit molecular polymorphisms. In nature, long-lived plants can renew their composition and ratio of resistance genes (Deng et al. 2017; Kong et al. 2017; Quintin et al. 2014; Santhanam et al. 2015; Tena 2016; Tobias and Guest 2014; Wei et al. 2015). With the development of biotechnological techniques and the modification of resistance genes, some researchers can improve the recognition of pathogen effectors using random single amino acid mutations or protein modification of NB-ARC sequences (Segretin et al. 2014; Wang et al. 2015). These new findings will provide a new method for resolving consecutive monoculture problems in the future.

Table 2 Pearson correlation matrix visualizing *NB-ARC* genes ranked by three different treatments

	SP	FA	FO
SP	1	0.0801	0.7067**
FA		1	− 0.1690
FO			1

**$P < 0.01$

Conclusions

The present study identified 45 NB-ARC protein sequences in *R. glutinosa* and described their corresponding functions, structures and phylogenetic traits. At same time, *NB-ARC* genes were found to be involved in the formation of *R. glutinosa* consecutive monoculture problems that

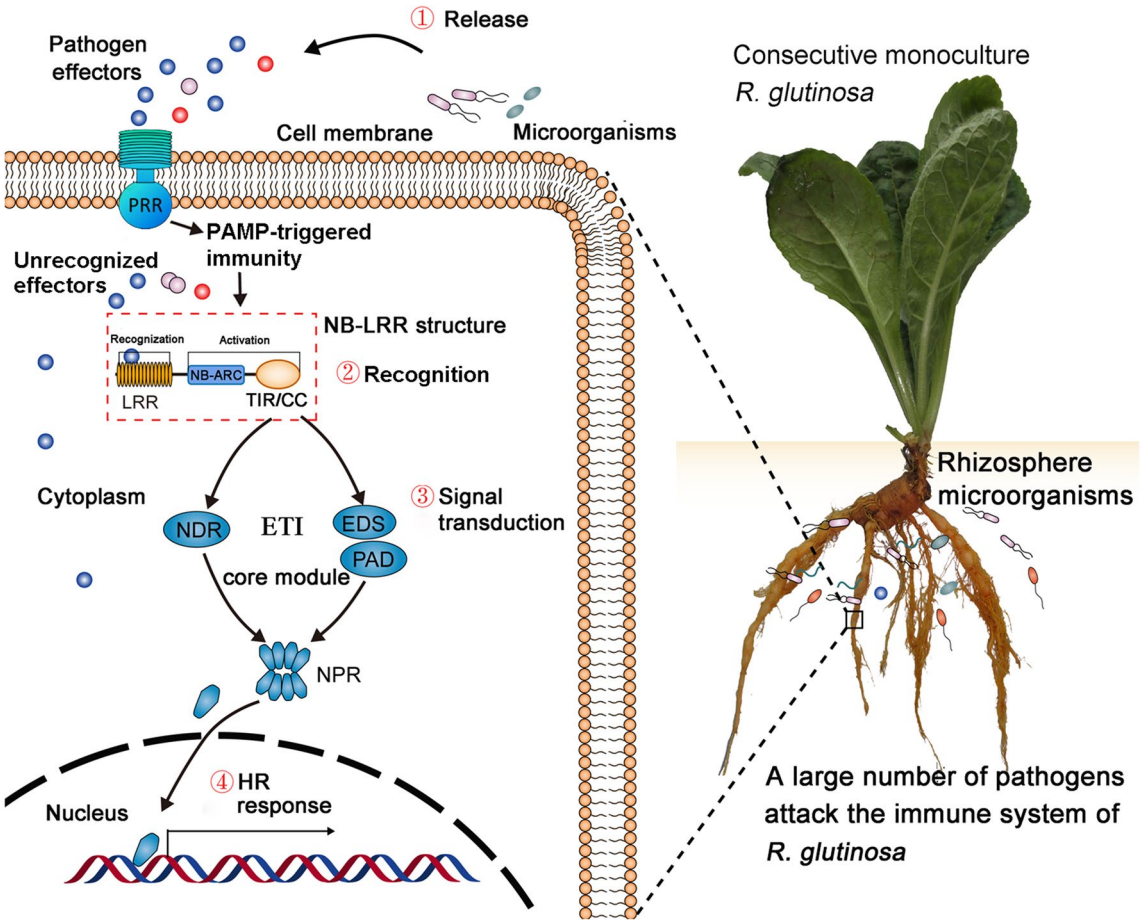

Fig. 5 Sketch map for effector-triggered immunity mediated by consecutive monoculture stress in *R. glutinosa*. A typical ETI system includes NB-ARC, NDR/EDS, and NPR core proteins, in which NB-ARC might recognize effectors from pathogens. NB-ARC protein is thus a critical protein that functions in the ETI system. Allelochemicals released from consecutive monocultures of *R. glutinosa* significantly induce pathogen proliferation. Various effectors from these pathogens are thus continuously released into rhizosphere soils under consecutive monoculture stress. Some effectors might trick the PTI system and successfully enter the cytoplasm. To eliminate these attacks from pathogens as soon as possible, plants can effectively recognize these effectors based on the LRR domain of NB-ARC protein. For a large number of pathogens induced by allelochemicals in consecutive monocultures of *R. glutinosa*, numerous effectors are produced that continuously attack *R. glutinosa* and ultimately result in loss of the recognition ability of the ETI

are rarely expressed 30 days after planting and frequently expressed at approximately 60 days after planting. The findings of this study provide insights into the mechanism of formation of consecutive monoculture problems.

Author contribution statement Conceived and designed the experiments: ML. Performed the experiments: AC, LG, NX, GL, FF, and BZ. Analysed the data and wrote the manuscript: AC, LG, ML, DG, JZ, and HL. Supervisory support: ZZ. All the authors have read and approved the final content of the manuscript.

Acknowledgements In this study, the protein information and the other support data were derived from correspondence author Prof. Zhang Zhongyi, my PhD supervisor in Fujian Agriculture and Forestry University. We gratefully acknowledge all members of Zhang Zhongyi research group.

Funding This study was supported by the National Natural Science Foundation of China (Grant No. 81503193, 81573538, 81603243 and 81403042) and the Key Scientific Research Project of the higher Education Institutions of Fujian Province of China (No. JK2015013).

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