INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
20–0031/2020/24–3–591–602
DOI: 10.17957/IJAB/15.1476
http://www.fspublishers.org

Full Length Article

Identification of MAPK Cascade Genes Response to Consecutive Monoculture Stress in *Rehmannia glutinosa*

Li Gu[1,2], Yunfang Wu[1], Meigui Lin[1], Feiyue Yuan[1], Shangyu Zhan[1], Fengji Wang[1,2], Jianming Wang[1,2], Mingjie Li[1,2*] and Zhongyi Zhang[1,2*]

[1]*College of Agriculture, Fujian Agriculture and Forestry University, Fuzhou 350002, China*
[2]*Key Laboratory of Ministry of Education for Genetics, Breeding and Multiple Utilization of Crops, Fujian Agriculture and Forestry University, Fuzhou 350002, China*
[*]For correspondence: xinyuzszj@163.com; zyzhang@fafu.edu.cn
Received 06 January 2020; Accepted 10 April 2020; Published 11 July 2020

Abstract

The consecutive monoculture problem leads to serious yield reduction, quality deterioration, and disease aggravation in the production of *Rehmannia glutinosa*. To comprehensively analyze the immune response of mitogen-activated protein kinases (*MAPKs*) involved in consecutive monoculture stress, the MAPK cascade family proteins and their typical changes were identified and analyzed in detail. Based on the highly conserved characteristics of MAPK cascade gene families, we have identified and obtained 34 MAPK (*RgMAPKs*), 7 Mitogen-activated protein kinase kinase (*RgMAPKKs*), and 32 mitogen-activated protein kinase kinase kinases (*RgMAPKKKs*) in *R. glutinosa* by the pre-constructed protein library. The results of multiple comparisons of protein sequences and construction of a phylogenetic tree indicated that 34 *RgMAPKs* and 7 *RgMAPKKs* families could be divided into 4 groups, while the family of 32 *RgMAPKKKs* was divided into 3 subtypes: MEKK, RAF, and ZIK. Additionally, the MAPK cascade family proteins were widely involved in the response of consecutive monoculture stress, prominently reflected in the 40, 60, and 80 days after planting and consistent with the physiological response result. According to the differential expression of MAPK cascade family genes in three key growth stages, the key candidate genes responding to consecutive monoculture stress were screened, including 27 *RgMAPK*s, 7 *RgMAPKK*s, and 24 *RgMAPKKK*s. In this study, the MAPK cascade family genes were identified for the first time, and the basic process of which involved in consecutive monoculture stress was initially analyzed. Meanwhile, this study provided a theoretical and data foundation for further study of the immune response mechanism to consecutive monoculture stress. © 2020 Friends Science Publishers

Keywords: *Rehmannia glutinosa*; MAPK cascade; Consecutive monoculture stress; Signal transduction

Introduction

Rehmannia glutinosa is one of the most famous medicinal herbs with a long cultivation history in China. It has multiple functions, such as immune regulation, anti-aging, anti-tumor and blood sugar reduction effects (Fan *et al*. 2012; Zhang *et al*. 2013). However, consecutive monoculture problems are widespread in *R. glutinosa* production, which leading to yield reduction, quality deterioration, poor growth status, and disease aggravation (Zhang *et al*. 2010; Chen *et al*. 2018). Notably, these effects affect only *R. glutinosa* and can persist for 8–10 years before *R. glutinosa* can be replanted (Gu *et al*. 2013; Zhang *et al*. 2013). Much research has focused on the dose-effect relationship in the "plant-microbial-soil" system at different levels, and it is believed that microecological imbalance mediated by allelopathic substances may be the main cause of the consecutive monoculture problem (Zhang *et al*. 2010; Li *et al*. 2016; Zhang *et al*. 2016). According to the recent research, plant immune system abnormalities are the initial characterization of rhizosphere microecological imbalance (Chen *et al*. 2018, 2019; Xie *et al*. 2019), while mitogen-activated protein kinase (MAPK) cascades play an important role in this process (Yang *et al*. 2015; Tian *et al*. 2017). However, the MAPK cascades and their involvement in immune responses to consecutive monoculture stress in *R. glutinosa* remain unclear.

The MAPK cascades have a highly conserved three-level cascade response mode, including Mitogen-activated protein kinase kinase kinase (MAPKKK), Mitogen-activated protein kinase kinase (MAPKK) and MAPK, which play a crucial role in the growth and development of plants and the signal transduction of various biotic and abiotic factor stress responses (Asai *et al*. 2002; Wang *et al*. 2018), such as cell division (Jiménez *et al*. 2007), growth and development (Xu and Zhang 2015), hormone response

To cite this paper: Gu L, Y Wu, M Lin, F Yuan, S Zhan, F Wang, J Wang, M Li, Z Zhang (2020). Identification of MAPK cascade genes response to consecutive monoculture stress in *Rehmannia glutinosa*. *Intl J Agric Biol* 24:591–602

(Tena *et al*. 2001), pathogen infection (Pitzschke *et al*. 2009), drought, salt stress (Suarez and Fernandez 2010) and ultraviolet radiation (Galletti *et al*. 2011). During a eukaryote nuclear reaction, MAPK cascades work as a common signal transduction pathway and connect different receptors or sensors (Tena *et al*. 2001). Therefore, to further study how the MAPK cascades are involved in the immune response to consecutive monoculture stress, we systematically analyzed the MAPK cascade families by searching the protein library (Li *et al*. 2017), which were constructed by the colleagues and the differential expression patterns of the coding genes under consecutive monoculture stress. This research provides an important data-based foundation and theoretical basis to further understand how the immune mechanism of *R. glutinosa*'s responds to consecutive monoculture stress.

Materials and Methods

Identification of *R. glutinosa* MAPK cascade family proteins

First, the known *Arabidopsis* MAPK cascade family proteins were obtained from a public database (https://www.arabidopsis.org/). Feature extraction and model construction of these *MAPKs*, *MAPKKs* and *MAPKKKs* sequences were carried out through a Hidden Markov Model-based method. Subsequently, the protein sequences of putative MAPK cascades in *R. glutinosa* were extracted from the *R. glutinosa* protein library (Li *et al*. 2017) by the constructed model. Finally, candidate MAPK cascade family proteins were annotated and further screened by Blast2GO software to obtain candidate *RgMAPKs*, *RgMAPKKs*, and *RgMAPKKKs*.

Analysis of physicochemical properties of *R. glutinosa* MAPK cascade family proteins

The ProtParam (https://web.expasy.org/protparam) online tool was used to predict the sequence length, protein molecular weight and isoelectric point, instability index, and aliphatic index of *RgMAPKs*, *RgMAPKKs* and *RgMAPKKKs*.

Analysis of structure and conserved domain of *R. glutinosa* MAPK cascade family proteins

Phylogenetic trees of *RgMAPKs*, *RgMAPKKs* and *RgMAPKKKs* were constructed by molecular evolutionary genetics analysis (MEGA6.06) software using a neighbor-joining method with 1000 bootstrap replicates. At the same time, the multiple comparisons of MAPK cascade protein sequences corresponding to *R. glutinosa* and *Arabidopsis* were carried out using Clustalx 2.1. In addition, the conserved domains of *RgMAPKs* and *RgMAPKKs* were analyzed using the MEME online tool (https://meme-suite.org).

Test setup and collection of plant materials

The field experiments for this study were arranged at the Wenxian Agricultural Institute in Jiaozuo City, Henan Province, China. The field experiments were divided into two groups. One group was the first year to plant with *R. glutinosa* in the fields (FP). The other group was continuous planting of *R. glutinosa* in the field the second year (SP). Sowing time for both groups was April 25, harvested on November 28, 2017. *R. glutinosa* planted in both groups was 1000 plants, with row spacing of 30 cm × 30 cm. We collected fresh tuber roots under 40, 60, 80, 100 and 120 days after planting (DAP), which were transferred into liquid nitrogen and stored at a refrigerator at -80°C until use. Three biological replicates were collected for all samples.

Measurement of root activity and the physiological index

To determine the activities of superoxide dismutase, peroxidase, catalase, and the contents of malondialdehyde, we used the corresponding kit (Nanjing Jiancheng Bioengineering Institute) to obtain their respective absorbances (Gu *et al*. 2018). Meanwhile, measurement of root activity and the hydrogen peroxide content were conducted per the methodology described by (Chen *et al*. 2019). Finally, the root activity and the hydrogen peroxide content were calculated from the absorbance values measured at 415 nm and 390 nm, respectively.

Analysis of genes encoding MAPK cascades family proteins by qRT-PCR

Based on the identification and annotation of *RgMAPKs*, *RgMAPKKs* and *RgMAPKKKs*, the expression patterns of these genes at the FP and the SP *R. glutinosa* (40, 60, 80, 100 and 120 DAP) were analyzed. This study used the Prime Script RT Reagent Kit (Takara, Japan) to extract total RNA (1 μg) from each sample and synthesize the cDNA. SYBR Premix Ex Taq (Takara, Japan) was used to conduct the Quantitative real-time PCR (qRT-PCR). 18S was selected to normalize the expression of the validated genes (Li *et al*. 2017). Three biological replicates were performed. Primer pairs are listed in Table 1.

Results

Identification of *R. glutinosa* MAPK cascade family proteins

In order to extract the conservative characteristics of the MAPK cascade family proteins to train Hidden Markov Model for downstream identification of MAPK in *R. glutinosa*, we obtained 110 *Arabidopsis* MAPK cascade family proteins from TAIR10 database (https://www.arabidopsis.org), which including 20 *MAPKs*,

Table 1: qRT-PCR primers used in validating genes and internal references

Style	Gene name	Forward	Reverse
*MAPK*s	*RgMAPK1*	TCAACACGGACATAATACAC	ACATCTCTGCTCCTTCAT
	RgMAPK2	GAATGAAGGAGCAGAGATG	GTGTAGAAGGAGCAGACTA
	RgMAPK3	CTCGAAGCAACTGATACAA	TTGATGGAGACAGACCTT
	RgMAPK4	ATGAAGGAGCAGAGATGT	GTGTATTGGTGTAGAAGGAG
	RgMAPK5	CCATTACCGTAGTACCTCTA	GTCACAAGCATACACAGAT
	RgMAPK6	AAGGTGGAAGCATTATATGTT	GGTAGTTATGGTGTTGTAGG
	RgMAPK7	GTGTTGCTCATTGTCATTAC	AAGAAGGTCCGAAGAGAA
	RgMAPK8	GCTGATGCTGACTGTAAG	GTCCAAGATATTGCTGATGA
	RgMAPK9	TGGTGAAGGAAGTAGATATAGA	GACGAAGAAGCCTAAGAAG
	RgMAPK10	GAATATGGTGAAGGAAGTAGAT	TGACGAAGAAGCCTAAGA
	RgMAPK11	CTCAGAGACAACATTCATCA	GCCATAGGAAGTATAGAAGAC
	RgMAPK12	TATCTTCCTCGCCTTCAT	GCAACAACAGCATCAATAG
	RgMAPK13	CTCAATACGGTGGTCAAG	CTAATACATCCTCGCCATAC
	RgMAPK14	CCTATGACCTCCTGGATT	GGCTGCTGTTGATATTGA
	RgMAPK15	CAATTCCACTGTAATCTCCA	CGTTCACAAGTTCATCTCT
	RgMAPK16	CAATCTCGGTTTCCATTCTA	CATCCTTCCTTCTCAATACAT
	RgMAPK17	TTCTGTGATTCCTAGTGGTA	GTGCTCGTCATTACATAGAT
	RgMAPK18	ATTGTGCGAGTCATCTTC	AGCAGGCATAATGAATAGTC
	RgMAPK19	TATTCCTGTCAGCACTCA	GAACAACAACCTCACCAA
	RgMAPK20	TATTCCTGTCAGCACTCA	TTCCTCCACAATCTATCTCT
	RgMAPK21	CAGTTGGCGTTAATGAGTA	TAAGAGGTCTGAAGTATCTACA
	RgMAPK22	GCTTGATTGGCACATACT	CTGAATGGAAGACGAAGAG
	RgMAPK23	CTCTGCTGTGATAACTACG	GCTACCAAGGATGTTGATAA
	RgMAPK24	TTCAGTTACACCGATCCT	CATTCAGTCTCTTCCGTATT
	RgMAPK25	GCTGTGAGGACTTAATAATCT	GTTATGATGCCGACCAAT
	RgMAPK26	TCGCTCTCAAGGATGTTA	AAGAATGTTGGCAGAATGTA
	RgMAPK27	TTAGCACCACACTACTCA	CGAAGAACAGATGAAGGAG
	RgMAPK28	GCACCTTCCACTGTAATC	ACATCCGACAACTTCCTA
	RgMAPK29	ACGATTCATCCAATACAACA	GTCCTCTTCGCATCAATT
	RgMAPK30	GTTATGAACACGGAGACAA	CTTAATGGAGGAGGAATCAC
	RgMAPK31	TTATTGTTGCCGCTATTAGTA	ATCTCAAGAAGTCTGTAGGA
	RgMAPK32	ATATGAGTTGATGGATACTGATT	CAGGCGGAATGTATGTATT
	RgMAPK33	TTCAGGTTCAAGCAAGTC	GCAATCTCCTCATTAGTCTC
	RgMAPK34	GCAAGACATTAGCGGAAT	CTATGAACTTATGGACACTGAT
*MAPKK*s	*RgMAPKK1*	GTTATCTGATGGCTGAATTAAG	TTCCTCCTCCTGATGAAG
	RgMAPKK2	CCTCCATCCATATACTCCA	TCAATCAGTCATCTCAATCTC
	RgMAPKK3	CATACAATTAGCAAGTAACATCA	CAAGTCCAAGTGTAAGTTCTA
	RgMAPKK4	GTGATAGAATGAGTGATAGCA	AGATGAATATACAGGAGGAGAT
	RgMAPKK5	TTAACTGGACCTTCATTAGC	TCGTAGGAACTGTCACAT
	RgMAPKK6	CCCTCCAATTTGCTGATTA	CATATCCATCGTATTGTCCAT
	RgMAPKK7	CAAGCACACCTCTTCAAT	CATACAGATGGCGATGTC
*MAPKKK*s	*RgMAPKKK1*	CCGTAACAGACCAATCAG	AATACTGCCTCAACCTCT
	RgMAPKKK2	AGAGGTTGAGGCAGTATT	TATCATCGGACGGTAAGG
	RgMAPKKK3	AGCATCCATTGTATGTATCC	AGGTGACGGTAACGAATA
	RgMAPKKK4	TAATTCGGCTCCTCAGAA	AACGGTGATGATGATGATAG
	RgMAPKKK5	ACGCTATCATCATCATCAC	TGGAACTCTATCAGACGAA
	RgMAPKKK6	TGGAGGAGGATGAGATTAC	CGGATAACATTGTCTGCTAT
	RgMAPKKK7	ATTTCACGGCGAAGATTT	CACCTCACATACTTACATTCT
	RgMAPKKK8	ATTGCTTCATCTTCGGATT	CACAGTCAACCAGTCTTC
	RgMAPKKK9	GCGAGTGACTTGAGAATT	GAGCCTATGGTACAGTGA
	RgMAPKKK10	AGAGGAGGATTCGTTGAA	TGTTCTCGTGGAGGTATT
	RgMAPKKK11	ATATTCTCTAAGCCTCCTGT	TCAGCATTATGTCATCTCTATC
	RgMAPKKK12	TCGTCCTACCATCATACAA	TCTCCTTCTTCTGCTTCA
	RgMAPKKK13	GACTTCACATCATTCAACTTAG	GCTTATACAAGGCAGATTCTA
	RgMAPKKK14	GCATACACAAGGCAGATT	TTCACAGAACCACTTACATC
	RgMAPKKK15	TCATCCTTCTTCACTCATTATC	CACTGACCTACTACACATTC
	RgMAPKKK16	TGGCATCACATCCTTATTC	TTCATACGAAGTTCACAAGAT
	RgMAPKKK17	TTGTCCGTCAATCTTATCATT	GAGACTCCACCAATACCT
	RgMAPKKK18	TGGTCTGGCTTACTTACA	TTGAAGGATAGCATTGAAGAA
	RgMAPKKK19	GGATAATGCGAGAACAATAAC	CCGACAGAAGTATAAGATGG
	RgMAPKKK20	CTTCCACAGTATTGAACAATG	AGTGAGAATGGGCAAATG
	RgMAPKKK21	TGCTAATGGACAAGTTAATGA	TCAATGGAGAGGAAGGATT
	RgMAPKKK22	ACTTAGCATCGTCAGAGA	ATCATATTCAACAGGAACATCT
	RgMAPKKK23	GAGGTGAAGAGGAGACAT	GATTATGATATTGGCTGTAGGA
	RgMAPKKK24	GCAGAATCTTATGGATGGAA	GCAGTATTATGGATCGGAAT
	RgMAPKKK25	TTTGAGAAGGATATTTGATGGA	GTTGACATTATGCTACAGATTG
	RgMAPKKK26	TTGGATAATAGGAATGAGGATG	GTCTGAATGGAGTAGTTGAG
	RgMAPKKK27	TTCTTGATTGGTCCTATGC	ACTCCTCTGTATGTCTCTG
	RgMAPKKK28	GTTTATGTGATGATGATGTGTT	AGTGATCCAATTATCTGATGTT
	RgMAPKKK29	ACATTCCTCCTCCTCAAA	GCGAAGGGATTACACAAA
	RgMAPKKK30	GGCTCCTGAAGTTATTGTT	AGATGCTCTGGTATTGGT
	RgMAPKKK31	GCTGGAGGAGGATATTCT	TGGTACTGAAGGTGATGT
	RgMAPKKK32	AAGGAGCATCTTCTGATAATC	GCCGACTGTTCATTAACT
18S		ATGATAACTCGACGGATCGC	CTTGGATGTGGTAGCCGTTT

10 *MAPKKs* and 80 *MAPKKKs*, respectively. A total of 1407 putative *R. glutinosa* MAPK cascade family proteins were obtained by scanning with the homologous sequences with protein library of *R. glutinosa* based on the constructed

model. After removing redundancies and annotating these putative protein sequences using Blast2GO, we identified a total of 73 candidate *R. glutinosa* MAPK cascade family proteins, including 34 *MAPKs* (*RgMAPK1~RgMAPK34*), 7 *MAPKKs* (*RgMAPKK1~RgMAPKK7*) and 32 *MAPKKKs* (*RgMAPKKK1~ RgMAPKKK32*), respectively. In addition, a series of parameters including the sequence length, protein molecular weight, isoelectric point, instability index and aliphatic index of *R. glutinosa* MAPK cascade family proteins were predicted through ProtParam (https://web.expasy.org/protparam) (Table 2). For 34 *RgMAPKs*, the length ranged from 178 to 622 bp, and the molecular weight ranged from 20508.7 to 69862.2 Da. The isoelectric point of the protein ranged from 5.04 to 9.27, and the instability index ranged from 22.39 to 49.05. Instability indexes from 41.18% of *RgMAPKs* were greater than 40. The fat index ranged from 77.60 to 102.52. For seven *RgMAPKKs*, which have the shortest and maximum length was 128 bp and 392 bp, respectively. The molecular weight ranged from 14557.9 to 43709.1Da. Their protein isoelectric point (pI) ranged from 5.52 to 9.24. The instability index ranged from 38.84 to 61.68, of which 5 *RgMAPKKs* (accounting for 71.43%) were greater than 40. The aliphatic index ranged from 82.61 to 115.55. For 32 *RgMAPKKKs*, the sequence length ranged from 113 to 883 bp and the molecular weight ranged from 12967.5 to 95354.0Da. Their protein isoelectric point (pI) ranged from 4.65 to 9.80. The instability index ranged from 37.41 to 74.86, of which 28 *RgMAPKKKs* (accounting for 87.5%) were greater than 40. The aliphatic indexes ranged from 56.02 to 96.36.

The construction of phylogenetic trees of *R. glutinosa* MAPK cascades family proteins

MEGA 6.06 was used to construct the phylogenetic trees from the corresponding protein sequences of the *R. glutinosa* and *Arabidopsis* MAPK cascade family based on the Neighbor-Joining Tree model. The results indicated that both *RgMAPKs* and *RgMAPKKs* were divided into four subtypes, named as A, B, C and D, respectively. *RgMAPKKKs* were divided into three subtypes, named MEKK, RAF and ZIK (Fig. 1a–c). Meanwhile, most of the *R. glutinosa* MAPK cascade family proteins could match the corresponding proteins in *Arabidopsis*. For MAPK family (Fig. 1a), *RgMAPK6*, *RgMAPK7*, and *RgMAPK8* from *RgMAPKs* and ATMK9 from *Arabidopsis MAPKs* were grouped into one branch of D subtype (Fig. 1a). *RgMAPK1*, *RgMAPK2*, *RgMAPK3*, *RgMAPK4*, and *RgMAPK5* from *RgMAPKs* and ATPK17 from *Arabidopsis MAPKs* were classified into another branch of the D subtype (Fig. 1a). While *RgMAPKK5* in *RgMAPKKs* and *ATMAPKK3* in *Arabidopsis MAPKKs* were classified into the branch of the B subtype (Fig. 1b), which confined the highly conserved features of the MAPK cascade family proteins. The conservative features also supply a reference for studying the biological function of the MAPK cascade family proteins.

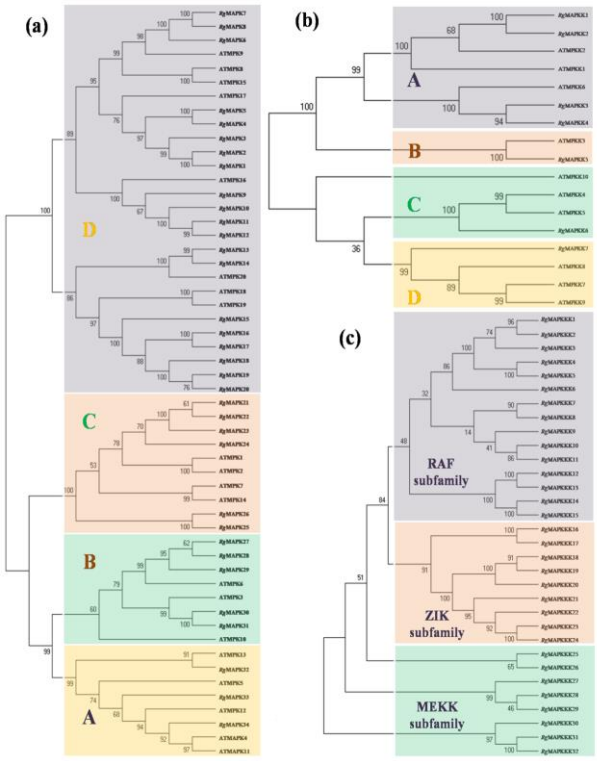

Fig. 1: Construction of the phylogeny trees of the MAPK cascade family proteins in *R. glutinosa* and *Arabidopsis*. (**a**) construction of the phylogeny tree of *MAPKs* in the *MAPK* cascades of *R. glutinosa* and *Arabidopsis*; (**b**) construction of the phylogeny tree of *MAPKKs* in the *MAPK* cascades of *R. glutinosa* and *Arabidopsis*; (**c**) construction of the phylogeny tree of *MAPKKKs* in the *MAPK* cascades of *R. glutinosa*

Comparative analysis of amino acid sequences of *R. glutinosa* MAPK cascade family proteins

Multiple sequence alignment of the amino acid sequences of 34 *RgMAPKs* and 20 *Arabidopsis MAPKs* by ClustalX2.1 presented highly similar amino acid motifs of these *MAPKs* ranging from 270 to 440 aa. TDY or TEY structure ranging from 270 to 440 aa in each *MAPKs* protein are conserved motifs which was the specific structure for recognizing the *MAPKs* family. In addition, in the 421–430aa position, there was a CD domain defined as (LH)DXXDE(P)X, which was found only in the C and D subtypes and excluded in the A and B subtypes of *RgMAPKs* (Fig. 2a). Meanwhile, the conserved motifs of *RgMAPKs* predicted by the MEME online tool indicated that most of the same subtypes of *RgMAPKs* had similarly conserved motifs (Fig. 2b); especially, motif 2 presented this motif in all the subtype of *RgMAPKs*.

Multiple sequence alignment of the protein sequences of 7 *RgMAPKKs* and 10 *Arabidopsis MAPKKs* were carried out using ClustalX 2.1 and a highly similar motif was found in the sequence of these *MAPKKs* ranging from 200 to 260 aa. For example,

Table 2: Analysis of physicochemical properties of *R. glutinosa* MAPK cascades family proteins

Protein family	Protein name	Length (bp)	MW (Da)	pI	Instability index	Aliphatic index
MAPKs	*RgMAPK1*	484	55177.4	8.35	40.65	85.85
	RgMAPK2	488	55686.9	8.19	41.08	86.54
	RgMAPK3	372	43052.5	6.30	35.38	92.31
	RgMAPK4	178	20508.7	6.79	22.39	99.16
	RgMAPK5	254	29708.1	6.15	23.81	94.80
	RgMAPK6	586	66925.8	6.41	40.22	77.87
	RgMAPK7	571	65335.2	7.13	37.24	82.64
	RgMAPK8	484	55734.6	8.09	37.16	87.25
	RgMAPK9	190	22136.5	6.71	28.5	95.95
	RgMAPK10	562	64269.8	9.07	38.55	80.75
	RgMAPK11	470	54297.5	8.61	40.24	83.04
	RgMAPK12	566	64782.3	8.93	40.17	77.60
	RgMAPK13	598	68295.2	9.10	35.14	85.00
	RgMAPK14	346	40257.6	9.22	33.21	89.65
	RgMAPK15	213	25077.2	9.18	28.44	92.49
	RgMAPK16	592	67579.6	9.27	48.05	81.55
	RgMAPK17	607	68936.2	9.22	47.16	80.99
	RgMAPK18	607	68176.4	9.21	45.94	84.05
	RgMAPK19	607	68428.6	9.17	44.69	82.75
	RgMAPK20	622	69862.2	9.09	45.54	82.17
	RgMAPK21	369	42487.2	6.70	39.24	95.93
	RgMAPK22	369	42431.1	6.54	40.89	95.66
	RgMAPK23	369	42503.3	6.54	39.35	96.72
	RgMAPK24	314	36191.9	6.33	49.05	102.52
	RgMAPK25	370	42403.2	7.58	28.55	98.30
	RgMAPK26	370	42375.2	7.56	29.43	98.30
	RgMAPK27	383	43893.2	5.52	38.89	91.20
	RgMAPK28	391	44965.5	5.55	41.68	91.10
	RgMAPK29	190	21439.5	6.57	34.06	96.58
	RgMAPK30	372	42792.1	5.60	37.58	92.07
	RgMAPK31	316	36362.8	6.48	37.83	90.76
	RgMAPK32	368	42192.2	5.04	47.76	94.59
	RgMAPK33	376	43009.2	5.80	38.77	94.39
	RgMAPK34	370	42447.4	6.50	39.05	91.16
MAPKKs	*RgMAPKK1*	353	39093.8	5.59	42.20	97.00
	RgMAPKK2	351	38790.4	5.60	39.60	94.42
	RgMAPKK3	202	22710.0	5.48	47.94	91.58
	RgMAPKK4	128	14557.9	6.34	38.84	115.55
	RgMAPKK5	392	43709.1	5.52	46.52	90.84
	RgMAPKK6	353	39164.7	9.24	61.68	82.61
	RgMAPKK7	308	34559.8	8.01	57.89	85.78
MAPKKKs	*RgMAPKKK1*	288	31838.3	5.71	49.01	79.24
	RgMAPKKK2	341	37677.7	5.02	45.24	80.65
	RgMAPKKK3	359	39881.2	5.24	46.85	78.22
	RgMAPKKK4	363	40086.2	4.75	46.76	79.48
	RgMAPKKK5	363	40181.4	4.80	46.60	80.55
	RgMAPKKK6	395	43840.4	4.99	39.39	74.00
	RgMAPKKK7	207	22633.5	5.83	48.56	81.11
	RgMAPKKK8	351	38763.4	4.65	47.87	79.26
	RgMAPKKK9	154	16230.5	6.82	39.10	96.36
	RgMAPKKK10	285	31303.4	6.63	51.33	94.07
	RgMAPKKK11	136	15025.5	9.41	51.23	75.22
	RgMAPKKK12	581	65064.1	5.27	54.49	78.35
	RgMAPKKK13	581	65041.0	5.22	53.37	78.52
	RgMAPKKK14	621	68578.0	5.35	40.78	83.46
	RgMAPKKK15	620	68447.8	5.22	41.12	83.29
	RgMAPKKK16	678	75229.4	9.28	53.45	67.40
	RgMAPKKK17	654	72265.1	8.92	68.17	74.59
	RgMAPKKK18	629	68648.6	9.29	53.47	70.56
	RgMAPKKK19	628	68181.0	9.28	53.70	69.44
	RgMAPKKK20	120	13088.8	8.42	56.56	82.83
	RgMAPKKK21	230	25230.5	9.35	37.57	78.00
	RgMAPKKK22	215	23766.9	9.39	49.73	73.95
	RgMAPKKK23	883	95354.0	9.54	71.01	66.75
	RgMAPKKK24	533	57099.5	9.73	74.86	56.02
	RgMAPKKK25	166	18722.5	9.34	46.67	78.13
	RgMAPKKK26	190	21093.4	8.85	45.22	92.79
	RgMAPKKK27	151	17681.9	5.44	37.41	75.43
	RgMAPKKK28	275	30370.3	5.22	45.50	76.55
	RgMAPKKK29	113	12967.5	4.75	63.35	83.72
	RgMAPKKK30	423	46975.4	5.33	56.31	65.22
	RgMAPKKK31	624	67777.4	9.49	57.75	70.98
	RgMAPKKK32	316	34775.5	9.80	60.91	81.80

MW: molecular weight; pI: isoelectric point

conserved residual active sites D (L/I/V) K of lysine (K) and aspartic acid (D) were presented in each sequence at positions 218–220 aa. At the same time, there was a highly conserved phosphorylation target site domain S/T-X5-S/T of the *MAPKKs* at positions 246–252aa (Fig. 3a). In addition, the conserved motifs of *RgMAPKKs* were

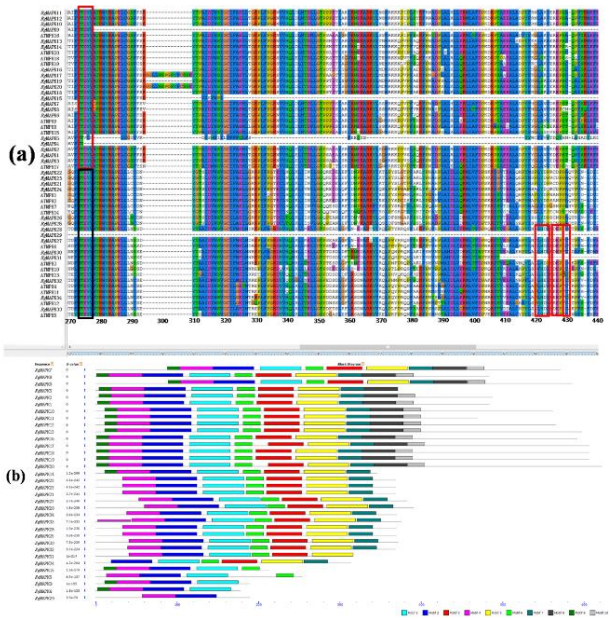

Fig. 2: Multiple comparisons and analysis of conserved *MAPKs* domain. (**a**) multiple comparative analysis of *MAPKs* protein sequences, the boxed portion was obtained by clustalX 2.1; (**b**) the conserved domain of the *RgMAPKs* sequences obtained by MEME, wherein boxes with different colors represent different domains and their corresponding positions in the protein sequence

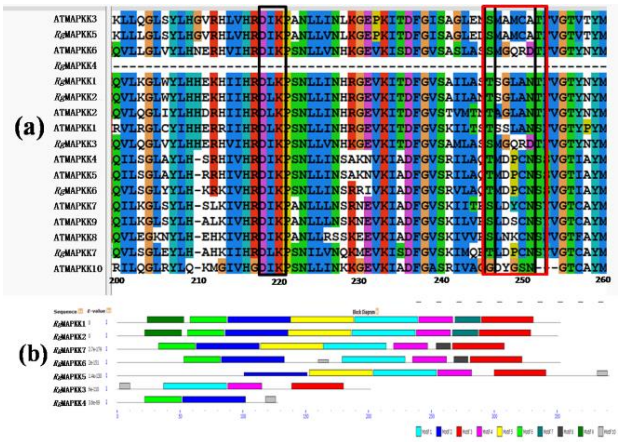

Fig. 3: Multiple comparisons and analysis of the conserved *MAPKKs* domain. (**a**) multiple comparative analysis of the *MAPKKs* protein sequence. The boxed portion was obtained by the clustalX 2.1; (**b**) the conserved domain of the *RgMAPKKs* obtained by the online MEME, wherein boxes with different colors represent different domains and its corresponding positions in the protein sequence

analyzed by the MEME online tool and similar motifs were found in the same subtype (Fig. 3b).

The physiological response of *R. glutinosa* under consecutive monoculture stress

To determine the effects of consecutive monoculture stress on *R. glutinosa*, the physiological indexes in the roots of FP and SP *R. glutinosa* were assessed (Fig. 4). The results showed that catalase activity in SP *R. glutinosa* was significantly higher than that of FP *R. glutinosa* from 40 DAP. At the same time, this significant difference persists during subsequent growth. Moreover, superoxide dismutase, peroxidase, hydrogen peroxide, and malondialdehyde showed the significant differences from 60 DAP. Among them, the activity of superoxide dismutase and peroxidase showed an increasing trend in FP *R. glutinosa*, while the hydrogen peroxide and malondialdehyde content showed a decreasing trend. However, the root activity showed a significant difference between FP and SP *R. glutinosa* after 80 DAP. These findings indicated that the antioxidant enzyme system of SP *R. glutinosa* was triggered to eliminate the oxidative damage caused by replant disease. However, finally, with increasing of replant disease level, SP *R. glutinosa* encountered the serious stress, leading to root vitality decline was still unavoidable.

Differential expression pattern of *R. glutinosa* MAPK cascade family genes under consecutive monoculture stress

To explore the expression pattern of *R. glutinosa* MAPK cascade family genes in process of consecutive monoculture stress, qRT-PCR was used to measure the expression of the genes at different growth stages (40, 60, 80, 100, and 120 DAP). The results showed that there were significant differences in expression between FP and SP *R. glutinosa* at key growth stages for MAPK cascade family genes. According to expression differences between the FP and SP *R. glutinosa* at 40, 60, and 80 DAP, a set of 34 *RgMAPK*s could be roughly divided into three categories, among which 20 *RgMAPK*s have higher expression in SP than FP. Of the 20 *RgMAPK*s up-regulated in replanted *R. glutinosa*, 2 (*RgMAPK2* and *RgMAPK15*) were significantly up-regulated at the 40 DAP and 15 genes were significantly up-regulated at the 60 DAP. One (*RgMAPK26*) showed significant up-regulation at the 80 DAP, while the other two *RgMAPK*s (*RgMAPK18* and *RgMAPK23*) were significantly down-regulated at the 40 DAP and significantly up-regulated at the 60 and 80 DAP (Fig. 5a). Among the seven *RgMAPK*s down-regulated in replanted *R. glutinosa*, except for *RgMAPK28* and *RgMAPK34*, the other five indicated a down-regulated trend in whole growth process of FP and SP *R. glutinosa* (Fig. 5b). At the same time, the *RgMAPK*s showed down-regulated expression in replanted *R. glutinosa*, which were also prominently expressed from 40 DAP to 60 DAP. In addition, *RgMAPK30* in the whole reproductive process of SP *R. glutinosa* showed a significant down-regulated trend compared with FP. There were no significant expression differences among the seven *RgMAPK*s, except for *RgMAPK21*, the other six showed almost the same expression trend in FP and SP *R. glutinosa* (Fig. 5c).

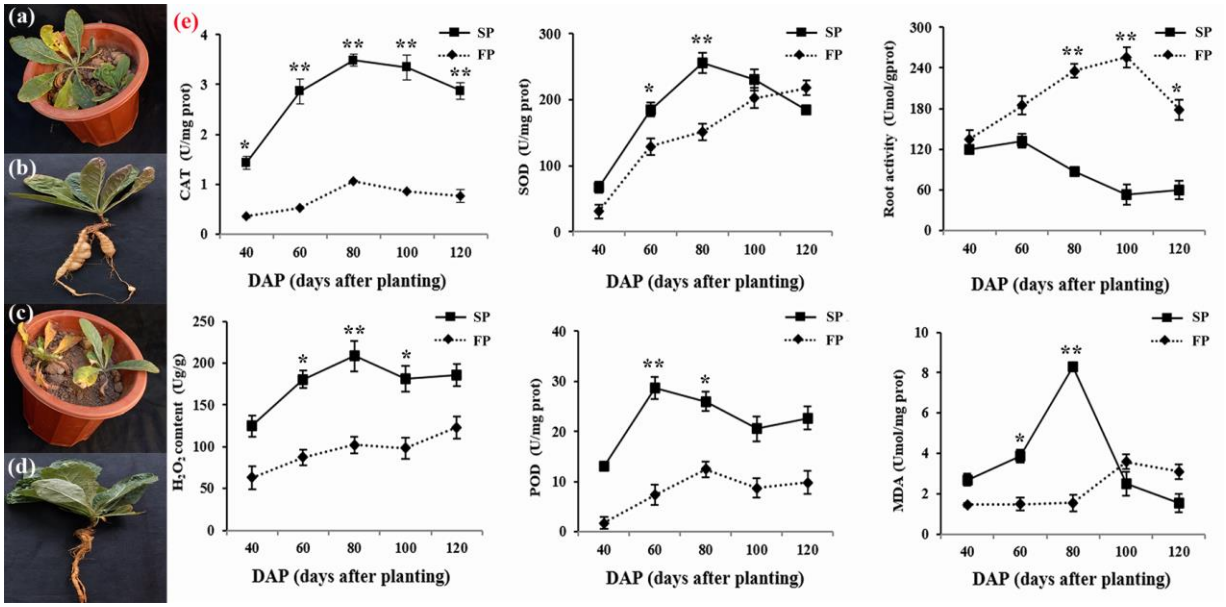

Fig. 4: The contents of physiological indexes in the FP and SP *R. glutinosa* roots. (**a**) The first planted *R. glutinosa*; (**b**) the morphological characteristics of the FP *R. glutinosa*; (**c**) the second planted *R. glutinosa*; (**d**) the morphological characteristics of the SP *R. glutinosa*; (e) the contents of physiological indexes. FP: first planting; SP: second planting. *indicates significant differences ($P < 0.05$; t test), and**indicate significant differences ($P < 0.01$; t test)

Among the seven *RgMAPKK*s identified in this study, two were up-regulated during formation of replanted disease compared with the FP *R. glutinosa*, and five showed a down-regulated expression trend (Fig. 6). For example, the expression of *RgMAPKK1* and *RgMAPKK5* at the 40, 60, and 80 DAP of the SP *R. glutinosa* were higher than those of the FP and reached a significant and extremely significant degree at the 40 and 80 DAP, respectively. The down-regulated five *RgMAPKK*s in SP and FP *R. glutinosa*, reached significant or extremely significant differences at the whole growth stages. For example, *RgMAPKK2*, *RgMAPKK4*, and *RgMAPKK7* indicated significant differences at the 40 DAP, while *RgMAPKK3* and *RgMAPKK6* showed significant differences at the 60 DAP. In addition, compared to the FP, the expression of *RgMAPKK4* in the whole growth process of the SP *R. glutinosa* showed a trend of down-regulation trend and reached the significant or extremely significant differences at the 40 and 100 DAP, respectively.

According to the expression pattern of *MAPKKKs* in the FP and SP at the 40, 60 and 80 DAP, a set of 32 *RgMAPKKKs* could be roughly divided into three categories, of which the expression of 8 *RgMAPKKK*s were significantly higher in the SP *R. glutinosa* than that in the FP, 16 *RgMAPKKK*s were significantly lower in the SP *R. glutinosa* than that in the FP, and eight *RgMAPKKK*s showed no significant difference (Fig. 7). Among the eight *RgMAPKKK*s up-regulated in the SP *R. glutinosa*, four *RgMAPKKK*s (*RgMAPKKK*2, *RgMAPKKK*17, *RgMAPKKK*18 and *RgMAPKKK*30) were significantly up-regulated at the 40 DAP and *RgMAPKKK15* and *RgMAPKKK12* were significantly up-regulated from the 60 DAP and 100 DAP, respectively. *RgMAPKKK5* and *RgMAPKKK11* were significantly down-regulated at the 40 DAP and significantly up-regulated at the 60 and 80 DAP (Fig. 7a). Sixteen *RgMAPKKK*s downregulated in FP *R. glutinosa*, were sharply expressed at the 80 DAP. There were also some genes, such as *RgMAPKKK3*, *RgMAPKKK4* and *RgMAPKKK20*, which shown differentially expressed covering almost the entire reproductive process of *R. glutinosa* (Fig. 7b).

Discussion

According to statistics, more than 70% of roots and rhizomes herbs have consecutive monoculture problems, which seriously restrict the development of modern Chinese medicine agriculture (Huang *et al*. 2013; Zhang *et al*. 2013; Chen *et al*. 2016). Preliminary studies indicated that the immune system abnormalities of *R. glutinosa* may be the initial characterization of the consecutive monoculture problem obstacles (Chen *et al*. 2018, 2019; Xie *et al*. 2019). However, *MAPKs* have been widely recognized as the major protein phosphorylation cascade involved in signal transduction and gene regulation in plants (Tena *et al*. 2001; Lindemose *et al*. 2013). Therefore, the recognition of the expression pattern of MAPK cascade family proteins and its encoding genes responding to replanted *R. glutinosa* becomes a key to comprehend the signal transduction of its immune system abnormalities. In this study, the protein sequences of 34 *RgMAPKs*, 7 *RgMAPKKs* and 32 *RgMAPKKKs* in the MAPK cascades of *R. glutinosa* were

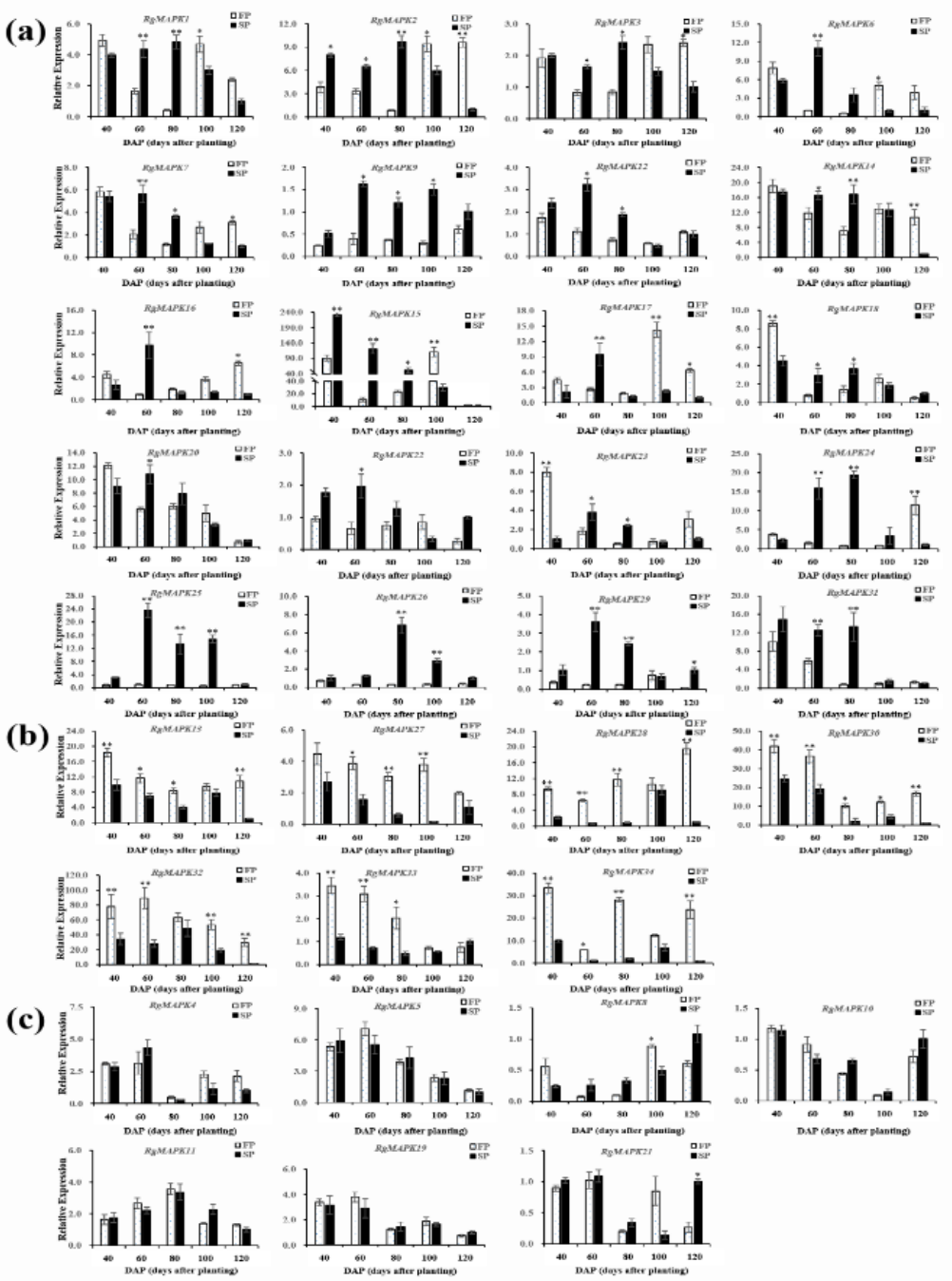

Fig. 5: Validation of expression of the *RgMAPK*s at different growth stages of FP and SP *R. glutinosa* using qRT-PCR. (**a**), (**b**) and (**c**) represent three different types of expression trends. FP: first planting; SP: second planting. The 120 days after planting (DAP) of SP was used as the reference to obtain the expression of different periods, and $2^{-\triangle Ct}$ was used as the relative expression of each gene. * indicates significant differences ($P < 0.05$; t test), and ** indicate significant differences ($P < 0.01$; t test)

initially identified, which provided a data-based foundation for studying the molecular mechanism of *R. glutinosa* MAPK cascades responding to consecutive monoculture stress.

By sequence alignment and motif analysis of the *R. glutinosa* MAPK cascade family proteins, we found that these protein sequences were highly similar and conserved with the homologue sequences in *Arabidopsis*, offering a possibility to explore the "perception" and "receiving" pathways for consecutive monoculture problem obstacle signals. For example, at the 274–276 aa of the *R. glutinosa MAPKs* protein sequence, the conserved motifs TDY and TEY of *MAPKs* were found, which was an essential

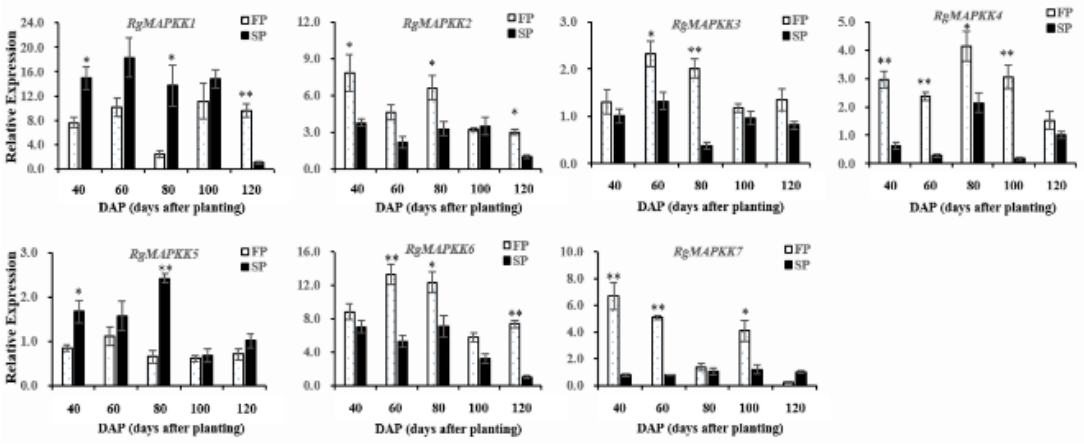

Fig. 6: Validation of expression of the *RgMAPKK*s at different growth stages of FP and SP *R. glutinosa* using qRT-PCR. FP: first planting; SP: second planting. The 120 days after planting (DAP) of SP was used as the reference to obtain the expression of different periods, and $2^{-\triangle Ct}$ was used as the relative expression of each gene. * indicate significant differences ($P < 0.05$; t test), and ** indicates significant differences ($P < 0.01$; t test)

condition to accurately identify the *R. glutinosa MAPKs* cascade family protein members. In addition, at the 421–430 aa of *MAPKs*, there was a CD domain defined as (LH)DXXDE(P)X (Fig. 2a), which might be an action site of *MAPKKs*. It had been shown that the adjacent acidic residues D (aspartate) and E (glutamate) played an important role in the interaction of the K (lysine) and R in *MAPKKs* (Tanoue *et al*. 2000). However, this CD domain only existed in the C and D subtypes of *MAPKs*, exclusive from the A and B subtypes (Fig. 2a), which was consistent with the research in *Brachypodium distachyon* (Chen *et al*. 2012). For another example, a highly conserved phosphorylation target site domain S/T-X5-S/T was found at the 246–252 aa of *RgMAPKK*s, which worked as the recognition site in the activation of the MAPK cascade and was published on other plants, such as *Arabidopsis* (Chen *et al*. 2012; Liang *et al*. 2013). In addition, the reason why the individual MAPK cascade pathway protein sequence differs greatly from the conserved domain of the same subtype may be that the protein sequence was not full length and failed to render its conserved domain.

The expression pattern of all of the obtained MAPK cascade proteins was verified by qRT-PCR. The results revealed that a large number of MAPK cascades family genes significantly differentially expressed in the SP and FP *R. glutinosa*. It is speculated that the effect of consecutive monoculture on the reproductive process of *R. glutinosa* was multifaceted and the MAPK cascade was widely involved. Overall, the differential expression of *MAPK*s, *MAPKK*s, and *MAPKKK*s in FP and SP *R. glutinosa* mainly occurred at the 40, 60 and 80 DAP, which is consistent with the physiological response result (Fig. 4). At the same time, according to the differential expression of these encoding genes at the three key stages, 34 *RgMAPK*s and 32 *RgMAPKKK*s can be divided into three categories: up-regulation, down-regulation, and no significant differential expression (Fig. 5 and 7). The seven down-regulated *RgMAPK*s genes, except *RgMAPK28* and *RgMAPK34*, showed a downward trend in both FP and SP *R. glutinosa*, indicating that these genes play a negative regulatory role. However, the expression of these genes showed a significant downward trend in the SP *R. glutinosa*. We speculated that the consecutive monoculture induced the down-regulation of these genes, accelerating the whole reproductive process of SP *R. glutinosa* and leading to premature senescence and even death (Yang *et al*. 2015). In addition, the expression of seven *RgMAPKK*s was significantly different in the key reproductive processes of FP and SP *R. glutinosa* (Fig. 6). However, the fertility stages at which these genes significant differentially expressed were found to be inconsistent in FP and SP *R. glutinosa*. Some individual genes even significant differentially expressed at other growth stages except these three critical periods, indicating that the same gene plays different functions at different growth and development stages.

With the whole genome sequencing of some plants, a large number of genes and proteins involved in the MAPK cascades pathway were identified and described in some model plants. For example, the first confirmed cascade was the MEKK1-MKK4/5-MPK3/6 cascade in *Arabidopsis*, which played an important role in plant natural immunity (Asai *et al*. 2002; Galletti *et al*. 2011). The MEKK1-MKK2-MPK4 and YDA-MKK4/5-MPK3/6 cascade pathways response to low temperature stress and regulation of stomatal development in *Arabidopsis*, respectively (Eckardt 2007; Furuya *et al*. 2014). The NPK1-NQK1/NtMEK1-NRK1 cascade regulates cytokinesis during meiosis and mitosis (Soyano *et al*. 2003). This study can provide new ideas and possibilities for revealing the mechanism of the consecutive monoculture problem based on revealing the response and transmission of the MAPK

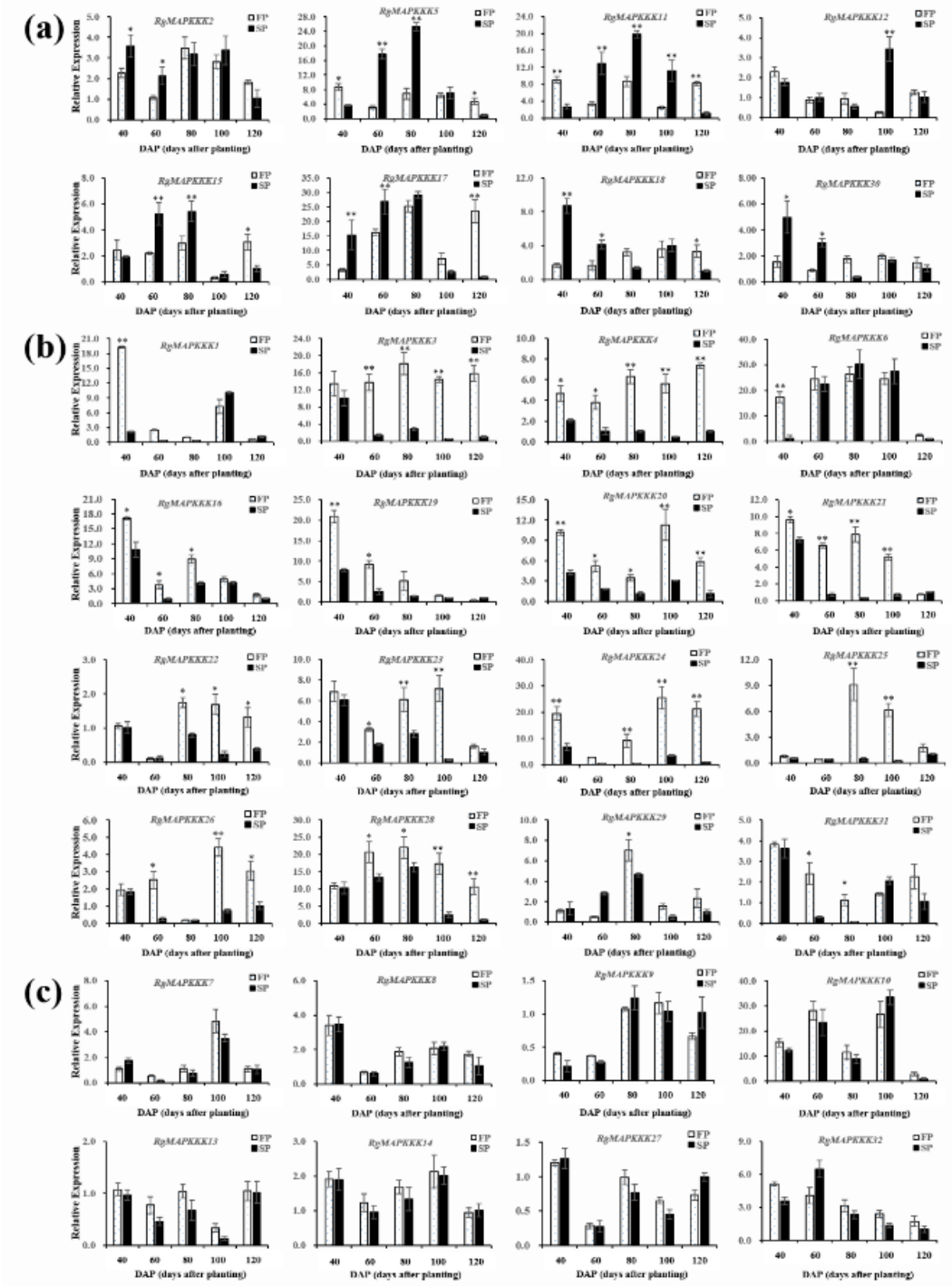

Fig. 7: Validation of expression of the *RgMAPKKK*s at different growth stages of FP and SP *R. glutinosa* using qRT-PCR. (**a**), (**b**) and (**c**) represent three different types of expression trends. FP: first planting; SP: second planting. The 120 days after planting (DAP) of SP was used as the reference to obtain the expression of different periods, and $2^{-\triangle Ct}$ was used as the relative expression of each gene. * indicates significant differences ($P < 0.05$; t test), and ** indicates significant differences ($P < 0.01$; t test)

cascade to consecutive monoculture stress. Combined with the research of MAPK cascades in other plants, the *MAPKK*s family genes are significantly less than the *MAPK*s and *MAPKKK*s family genes. For example, 20 *MAPK*s, 10 *MAPKK*s, and 80 *MAPKKK*s have been found in the *Arabidopsis* genome (Jonak *et al*. 2002; Colcombet and Hirt 2008), while 17 *MAPK*s, 8 *MAPKK*s, and 75 *MAPKKK*s have been found in the rice genome (Rohila and Yang 2007; Rao *et al*. 2010; Wankhede *et al*. 2013). Sixteen possible *MAPK*s, 6 *MAPKK*s, and 89 *MAPKKK*s have been

found in the tomato genome (Kong *et al*. 2012; Wu *et al*. 2014) and at least 14 *MAPK*s, 6 *MAPKK*s, and 59 *MAPKKK*s have been found in the cucumber genome (Wang *et al*. 2015). It is hypothesized that the MAPK cascade should resemble a dumbbell-shaped structure for signal reception and transmission, and the *MAPKK*s family genes may play a central regulatory role. Therefore, with the in-depth study of the small number of sites and specific locations of the *MAPKK*s family genes, this study may become a breakthrough to reveal the MAPK cascade of *R. glutinosa*.

In this study we identified the *R. glutinosa* MAPK cascade family proteins and obtained protein sequences of 34 *RgMAPKs*, 7 *RgMAPKKs*, and 32 *RgMAPKKKs*, respectively. By comparing MAPK cascade protein sequences and analyzing the differential expression pattern of the coding gene in the FP and SP *R. glutinosa*, we initially screened some candidate MAPK cascades family genes that may respond to consecutive monoculture stress (27 *RgMAPK*s, 7 *RgMAPKK*s, and 24 *RgMAPKKK*s), providing a general understanding of the *R. glutinosa* MAPK cascades response to consecutive monoculture stress. In addition, this research complements a new chain of evidence for interpreting the mechanism signal transduction of *R. glutinosa* under consecutive monoculture stress. Finally, the differential expressed genes in this study could be potential target genes for genetic improvement of *R. glutinous* under consecutive monoculture stress.

Conclusion

In this study, the MAPK cascade family proteins of *R. glutinosa* were recognized and identified for the first time, and qRT-PCR was used to quantity-analyze the expression patterns of all of the acquired MAPK cascade proteins at the five growth stages between FP *R. glutinosa* and SP. The MAPK cascades were widely involved in signal transduction, gene regulation and highly conserved characteristics, providing a new way to interpret the immune mechanism of *R. glutinosa* responding to consecutive monoculture stress and adding an important data-based foundation and theoretical basis for consummating the mechanism of the replanted obstacles of *R. glutinosa*.

Acknowledgements

The research was financially supported by the National Natural Science Foundation of China (Nos. 81603243 and 81573538) and National Key Research and Development Program of China (No. 2017YFC700705).

References

Asai T, G Tena, J Plotnikova, MR Willmann, WL Chiu, L Gomez-Gomez, T Boller, FM Ausubel, J Sheen (2002). MAP kinase signaling cascade in *Arabidopsis* innate immunity. *Nature* 415:977–983

Chen A, L Gu, N Xu, F Feng, D Chen, C Yang, B Zhang, M Li, Z Zhang (2019). NB-LRRs not responding consecutively to *Fusarium oxysporum* proliferation caused replant disease formation of *Rehmannia glutinosa*. *Intl J Mol Sci* 20:3203–3223

Chen A, L Gu, M Li, N Xu, F Feng, G Liu, B Zhang, D Gong, J Zhang, H Liu, Z Zhang (2018). Identification of *Rehmannia glutinosa* L. NB-ARC family proteins and their typical changes under consecutive monoculture stress. *Acta Physiol Plantarum* 40:95–108

Chen A, M Li, B Zhang, F Wang, L Gu, F Feng, H Liu, Z Zhang (2016). Review on catastrophe mechanism of medicinal plant and its rhizosphere microecosystem mediated by consecutive monoculture. *Modern Chin Med* 18:239–245

Chen L, W Hu, S Tan, M Wang, Z Ma, S Zhou, X Deng, Y Zhang, C Huang, G Yang, G He (2012). Genome-wide identification and analysis of *MAPK* and *MAPKK* gene families in *Brachypodium distachyon*. *PLoS One* 7; Article e46744

Colcombet J, H Hirt (2008). *Arabidopsis MAPKs*: A complex signalling network involved in multiple biological processes. *Biochem J* 413:217–226

Eckardt NA (2007). A complete *MAPK* signaling cascade that functions in stomatal development and patterning in *Arabidopsis*. *Plant Cell* 19:7–8

Fan HM, MJ Li, ZY Zhang, YH Yang, L Gu, FQ Wang, XJ Chen, ZY Zhang (2012). Spatiotemporal expression and analysis of responding consecutive monoculture genes in *Rehmannia glutinosa*. *Chin J Chin Mater Med* 37:3029–3035

Furuya T, D Matsuoka, T Nanmori (2014). Membrane rigidification functions upstream of the MEKK1-MKK2-MPK4 cascade during cold acclimation in *Arabidopsis thaliana*. *FEBS Lett* 588:2025–2030

Galletti R, S Ferrari, GD Lorenzo (2011). Arabidopsis MPK3 and MPK6 play different roles in basal and oligogalacturonide-or flagellin-induced resistance against *Botrytis cinerea*. *Plant Physiol* 157:804–814

Gu L, W Zheng, M Li, H Quan, J Wang, F Wang, W Huang, Y Wu, X Lan, Z Zhang (2018). Integrated analysis of transcriptomic and proteomics data reveals the induction effects of rotenoid biosynthesis of *Mirabilis himalaica* caused by UV-B radiation. *Intl J Mol Sci* 19:3324–3343

Gu L, MM Niu, HY Zheng, JM Wang, LK Wu, ZF Li, ZY Zhang (2013). Effect of continuous cropping of *Rehmannia* on its morphological and physiological characteristics. *J Chin Med Mater* 36:691–695

Huang LF, LX Song, XJ Xia, WH Mao, K Shi, YH Zhou, JQ Yu (2013). Plant-soil feedbacks and soil sickness: From mechanisms to application in agriculture. *J Chem Ecol* 39:232–242

Jiménez C, BR Cossío, CJ Rivard, T Berl, JM Capasso (2007). Cell division in the unicellular microalga *Dunaliella viridis* depends on phosphorylation of extracellular signal-regulated kinases (ERKs). *J Exp Bot* 58:1001–1011

Jonak C, L Ökrész, L Bögre, H Hirt (2002). Complexity, cross talk and integration of plant MAP kinase signalling. *Curr Opin Plant Biol* 5:415–424

Kong F, J Wang, L Cheng, S Liu, J Wu, Z Peng, G Lu (2012). Genome-wide analysis of the mitogen-activated protein kinase gene family in *Solanum lycopersicum*. *Gene* 499:108–120

Li M, Y Yang, F Feng, B Zhang, S Chen, C Yang, L Gu, F Wang, J Zhang, A Chen, W Lin, X Chen, Z Zhang (2017). Differential proteomic analysis of replanted *Rehmannia glutinosa* roots by iTRAQ reveals molecular mechanisms for formation of replant disease. *BMC Plant Biol* 17; Article 116

Li ZF, CL He, Y Wang, MJ Li, YJ Dai, T Wang, WX Lin (2016). Enhancement of trichothecene mycotoxins of *Fusarium oxysporum* by ferulic acid aggravates oxidative damage in *Rehmannia glutinosa* Libosch. *Sci Rep* 6; Article 33962

Liang W, B Yang, BJ Yu, Z Zhou, C Li, M Jia, Y Sun, Y Zhang, F Wu, H Zhang, B Wang, MK Deyholos, YQ Jiang (2013). Identification and analysis of *MKK* and *MPK* gene families in canola (*Brassica napus* L.). *BMC Genomics* 14; Article 392

Lindemose S, C O'Shea, MK Jensen, K Skriver (2013). Structure, function and networks of transcription factors involved in abiotic stress responses. *Intl J Mol Sci* 14:5842–5878

Pitzschke A, A Schikora, H Hirt (2009). MAPK cascade signalling networks in plant defence. *Curr Opin Plant Biol* 12:421–426

Rao KP, T Richa, K Kumar, B Raghuram, AK Sinha (2010). In silico analysis reveals 75 members of mitogen-activated protein kinase kinase kinase gene family in rice. *DNA Res* 17:139–153

Rohila JS, Y Yang (2007). Rice mitogen-activated protein kinase gene family and its role in biotic and abiotic stress response. *J Integr Plant Biol* 49:751–759

Soyano T, R Nishihama, K Morikiyo, M Ishikawa, Y Machida (2003). NQK1/NtMEK1 is a *MAPKK* that acts in the NPK1 *MAPKKK*-mediated MAPK cascade and is required for plant cytokinesis. *Gene Dev* 17:1055–1067

Suarez LC, RR Fernandez (2010). Signalling pathway in plants affected by salinity and drought. *Inform Técn Econ Agrar* 106:157–169

Tanoue T, M Adachi, T Moriguchi, E Nishida (2000). A conserved docking motif in *MAP* kinases common to substrates, activators and regulators. *Nat Cell Biol* 2:110–116

Tena G, T Asai, WL Chiu, J Sheen (2001). Plant mitogen-activated protein kinase signaling cascades. *Curr Opin Plant Biol* 4:392–400

Tian YH, FJ Feng, B Zhang, MJ Li, FQ Wang, L Gu, AG Chen, ZJ Li, WB Shan, XR Wang, XJ Chen, ZY Zhang (2017). Transcriptome analysis reveals metabolic alteration due to consecutive monoculture and abiotic stress stimuli in *Rehamannia glutinosa* Libosch. *Plant Cell Rep* 36:859–875

Wang HB, M Gong, JY Guo, H Xin, Y Gao, C Liu, DQ Dai, LZ Tang (2018). Genome-wide identification of *Jatropha curcas MAPK*, *MAPKK*, and *MAPKKK* gene families and their expression profile under cold stress. *Sci Rep* 8; Article 16163

Wang J, C Pan, Y Wang, L Ye, J Wu, L Chen, T Zou, G Lu (2015). Genome-wide identification of *MAPK*, *MAPKK*, and *MAPKKK* gene families and transcriptional profiling analysis during development and stress response in cucumber. *BMC Genomics* 16; Article 386

Wankhede DP, M Misra, P Singh, AK Sinha (2013). Rice mitogen activated protein kinase kinase and mitogen activated protein kinase interaction network revealed by *in-silico* docking and yeast two-hybrid approaches. *PLoS One* 8; Article e65011

Wu J, J Wang, C Pan, X Guan, Y Wang, S Liu, Y He, J Chen, L Chen, G Lu (2014). Genome-wide identification of *MAPKK* and *MAPKKK* gene families in tomato and transcriptional profiling analysis during development and stress response. *PLoS One* 9; Article e103032

Xie ZM, CY Yang, AG Chen, MJ Li, L Gu, JY Zhang, FJ Feng, B Zhang, SQ Chen, ZY Zhang (2019). 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* Libosch. *Intl J Agric Biol* 22:487–496

Xu J, SQ Zhang (2015). Mitogen-activated protein kinase cascades in signaling plant growth and development. *Trends Plant Sci* 20:56–64

Yang YH, MJ Li, XY Li, XJ Chen, WX Lin, ZY Zhang (2015). Transcriptome-wide identification of the genes responding to replanting disease in *Rehmannia glutinosa* L. roots. *Mol Biol Rep* 42:881–892

Zhang B, XZ Li, FQ Wang, MJ Li, JY Zhang, Li Gu, LJ Zhang, WQ Tu, ZY Zhang (2016). Assaying the potential autotoxins and microbial community associated with *Rehmannia glutinosa* replant problems based on its 'autotoxic circle'. *Plant Soil* 407:307–322

Zhang ZY, MJ Li, XJ Chen, LK Wu, J Li, FQ Wang, ZF Li, GY Guo, WX Lin (2013). Research advancement and control strategy of consecutive monoculture problem of *Rehmannia glutinosa*. *Mod Chin Med* 15:38–44

Zhang ZY, H Chen, YH Yang, T Chen, RY Lin, XJ Chen, WX Lin (2010). Effects of continuous cropping on bacterial community diversity in rhizosphere soil of *Rehmannia glutinosa*. *Chin J Appl Ecol* 21:2843–2848