Plant Biotechnology 35, 349–356 (2018)
DOI: 10.5511/plantbiotechnology.18.0820a

Original Paper

Cloning and characterization of a *Mimulus lewisii* NPR1 gene involved in regulating plant resistance to *Rhizoctonia solani*

Xia He[1], Yancai Shi[2,*]

[1]College of Forestry and Landscape Architecture, South China Agricultural University, Guangzhou 510642, PR China;
[2]Guangxi Institute of Botany, The Chinese Academy of Sciences, Guilin 541006, PR China
*E-mail: shiyancainan@163.com Tel: +86-15-907876676 Fax: +86-773-3550067

Received June 18, 2018; accepted August 20, 2018 (Edited by T. Kobayashi)

Abstract The monkey flower *Mimulus lewisii* is a new emerging model plant for the study in corolla tube formation, pigmentation patterns and pollinator selection, etc. However, the cultivation and management of this plant are difficult due to its susceptibility to a wide range of pathogens and the lack of rigid varieties with high levels of resistance to pathogens. In this regard, genetic engineering is a promising tool that may possibly allow us to enhance the *M. lewisii* disease resistance against pathogens. Here, we reported the isolation and characterization of non-expressor of pathogenesis related gene 1 (*NPR*1) gene from *M. lewisii*. The phylogenetic tree constructed based on the deduced sequence of *MlNPR*1 with homologs from other species revealed that *MlNPR*1 grouped together with other known *NPR*1 proteins of Scrophulariaceae family, and was nearest to *Mimulus guttatus*. Furthermore, expression analysis showed that *MlNPR*1 was upregulated after SA treatment and fungal infection. To understand the defensive role of this gene, we overexpressed *MlNPR*1 in *M. lewisii*. The transgenic lines showed slight phenotypic abnormalities, but constitutive expression of *MlNPR*1 activates defense signaling pathways by priming the expression of antifungal PR genes. Moreover, *MlNPR*1 transgenic lines showed enhanced resistance to *Rhizoctonia solani* there was delay in symptoms and reduced disease severity than non-transgenic plants. Altogether, the present study suggests that increasing the expression level of *MlNPR*1 may be a promising approach for development of monkey flower cultivars with enhanced resistance to diseases.

Key words: disease resistance, *Mimulus lewisii*, *MlNPR*1 gene, *Rhizoctonia solani*, systemic acquired resistance.

Introduction

The genus *Mimulus* (monkey flowers) contains over 150 species with tremendous phenotypic variation and has served as a classic model system in evolutionary and ecological functional genomics (Wu et al. 2008). In particular, the species *Mimulus lewisii* is emerging as an excellent member among this genus to elucidate the genes and developmental pathways underlying ecologically important floral traits, largely due to its rapid generation time (~3 months), high fecundity (1000 seeds per fruit), and relatively small genome size (~500 Mb) (Lafountain et al. 2015; Yuan et al. 2013a). In the past decade, researches of *M. lewisii* in genetic related to corolla tube development, pigment spot formation (Ding et al. 2017; LaFountain et al. 2017; Sagawa et al. 2016), and in ecological related to habitat and pollinator selection have been carried out (Bradshaw et al. 2003; Yuan et al. 2013a). However, *M. lewisii* is susceptible to damages from biotic stress (e.g., insects and diseases) and abiotic stress (e.g., high temperature) that limit its habitat and cultivation. In the past two year in our greenhouse, *M. lewisii* has been suffered from the devastating diseases several times. The causes were fungal pathogens such as *Thielaviopsis* and *Rhizoctonia* leading to leaf blight, stem rot and powdery mildew, etc. Generally, *M. lewisii* lack sufficient innate resistance to these fungal diseases and development of resistant varieties through conventional breeding is difficult due to unavailability of disease resistant germplasm. Present management is based principally on intensive use of chemical control, representing a substantial cost to researchers, and creating a basis for potential environmental and health concerns. In this regard, transgenic manipulation of *M. lewisii*'s innate defense signaling pathways may offer an alternative approach to generate resistance or tolerance to various pathogens and diseases.

Plants are often exposed to a variety of biotic stresses, and thus have evolved multidimensional defense systems to survive. Systemic acquired resistance (SAR) is one such system that is induced in response to a localized infection and confers long-lasting protection against a broad spectrum of pathogens (Durrant and Dong 2004). In previous studies, SAR has been well characterized in

This article can be found at http://www.jspcmb.jp/
Published online December 13, 2018

the model plant *Arabidopsis thaliana* (Boyle et al. 2009; Wu et al. 2012). The activation of SAR requires salicylic acid (SA) as a signal molecule and is associated with accumulation of pathogenesis-related (PR) proteins, which are thought to contribute to resistance (Vlot et al. 2009). Most of the PR proteins such as chitinases, glucanases, defensins, and thaumatins possess antifungal activities and are known to play important role in disease resistance. The NPR1 protein is a key regulator in the SA mediated SAR signal transduction pathway. NPR1 is the receptor for SA pathway in *Arabidopsis* (Wu et al. 2012). *AtNPR*1 is constitutively expressed in *Arabidopsis* at low levels and the coding product is located in the cytosol as an inactive multimeric protein complex. During pathogen infection or SA treatment, the multimeric protein complex is dissociated and turn into active monomer, and then transport into the nucleus where it bind to TGA factors there by inducing PR genes (Rochon et al. 2006; Tada et al. 2008). To explore the defense role of *NPR*1 against viral, bacterial and fungal pathogens, various overexpression studies have been carried out in both model and crop plants. For example, overexpression of *AtNPR*1 in *Arabidopsis* plants confers enhanced disease resistance to bacterial and fungal infections (Friedrich et al. 2001). Transgenic strawberry plants overexpressing *AtNPR*1 gain high disease resistance not only to biotrophs (*Podosphaera aphanis*) but also to hemibiotrophic fungal pathogens (*Colletotrichum acutatum and Colletotrichum gloeosporioides*), respectively (Silva et al. 2015). Ali et al. reported that *Brassica juncea* transgenic plants overexpressing *BjNPR*1 exhibited broad spectrum of disease resistance not only to necrotrophic pathogens but also to biotrophic pathogens (Ali et al. 2017). Additionally, sweet orange plants overexpressing *MhNPR*1 confers resistance to *Xanthomonas citri* as well as induces battery of pathogen related genes (Boscariol-Camargo et al. 2016). In monocots, researches have revealed that ectopic expressing *NPR*1 gene in rice and wheat crops confers broad spectrum of disease resistance against most disastrous pathogens *Rhizoctonia solani*, *Fusarium graminearum*, and *Fusarium oxysporum*, respectively (Molla et al. 2016; Quilis et al. 2008; Yu et al. 2017). These studies strongly suggest the existence of similar defense mechanisms in plants, making *NPR*1 desirable candidate gene for transgenic manipulation in crops for developing disease resistant transgenic crops against multiple pathogens.

In this study, we evaluated the role of *MlNPR*1 in *M. lewisii* for improving disease resistance against fungal pathogens. Characterization of the transgenic plants indicates that ectopic expression of *MlNPR*1 in monkey flower confers resistance to *Rhizoctonia solani*. These findings indicate that increasing the transcript levels of *MlNPR*1 may be a promising approach for development of monkey flower cultivars with enhanced resistance to multiple diseases.

Materials and methods

Plant material and sampling procedures

The plant material used in the present study is *M. lewisii* Pursh inbred line LF10, and plants were grown in pots containing a mixture of organic manure and soil (1:2) in a growth chamber under a 16 h light/8 h dark photoperiod at 25°C. For cDNA library construction, *M. lewisii* plants were sprayed with 2 mM SA (pH 7.0) with 0.01% Tween20 as an emulsifier and control plants were similarly treated with only Tween20 (0.01%) buffer. After processing, leaf samples were harvested for RNA isolation at time points of 0, 2, 4, 8, 12, and 24 h.

Cloning of MlNPR1 gene and sequence analysis

Total RNA was extracted using the SV total RNA isolation kit (Promega, USA), and then treated with amplification grade DNaseI (Sangon, China). First-strand cDNA was synthesized with M-MLV reverse transcriptase (TaKaRa, Japan) according to the manufacturer's instructions, then diluted 10-fold before PCR. Primers (*MlNPR*1_F1: 5′-ACA CTT CAC ACC TGC GTA GC-3′ and *MlNPR*1_R1: 5′-ACC CAC AGA TTA AGA GCC AC-3′) were designed based on the alignment of *MlNPR*1 homologues from *Mimulus guttatus* which is a closely related species to *M. lewisii*, The PCR cycling program consisted of 95°C for 3 min, followed by 28 cycles of a stepped program (95°C, 20 s; 55°C, 20 s; 72°C, 2 min), and terminated by an extension at 72°C for 5 min. The amplification products were analyzed on 1.5% agarose gels, and then the purified product was cloned into the pLB Vector (Tiangen, China) for sequencing. The deduced amino acid sequences of *MlNPR*1 were compared with other plant *NPR*1 protein sequences in NCBI using the BLAST program. Protein sequence alignment was performed using the ClustalX program with genetic distance matrices. The phylogenetic tree was constructed with the MEGA 5.0 program by the Maximum–Likelihood (ML) method with 1000 bootstraps. Conserved domain structure of this protein was analyzed by SMART database (Letunic and Bork 2018).

Vector construction and monkey flower transformation

The pEarleyGate 100 vector was used in this study, as it contains the *bar* gene which encode phosphinothricin acetyltransferase resistance to the Herbicide Basta for plant selection (Earley et al. 2006). The coding region of *MlNPR*1 was amplified from pLB Vector using the primer pair *MlNPR*1_F: 5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAT GGA CAG CCG AAC CGC GGT T-3′) and *MlNPR*1_R: 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CTT CTT AAG AGT GAG CAT AC-3′. The PCR product was cloned into the pDonor207 vector (Invitrogen) and then recombined into the pEarleyGate 100 destination vector (Earley et al. 2006), which

drives transgene expression by the CaMV 35S promoter. The final plasmid constructs were verified by sequencing and then transformed into *Agrobacterium tumefaciens* strain GV3101 for subsequent plant transformation. Briefly, the *Agrobacterium* culture (OD 600=0.6–0.8) was centrifuged at 8,000 rpm for 10 min and the pellet suspended in 300 ml of transformation solution: 300 ml dH_2O, 5% sucrose, 0.1 M Acetosyringone, and 300 μl Silwett. Seven young healthy plants that have branched and have lots of young flower buds (<5 mm) were used for infiltration. Briefly, gently peel back the leaves around the young buds, and spray the young buds with the Agro transformation solution, and then vacuum infiltrate the plants in the vacuum under 20 inches Hg for about 5 min, then quickly release the vacuum to let *Agrobacterium* liquid infiltrate into the flower buds. Artificially pollinate the transfer plants in the following two weeks, and then wait for 3 weeks and collect the mature seeds. Plant the seeds in soil flats. When the seeds start to germinate, begin spraying with 1 : 1,000 Basta every day. After selection, the well-developed resistant plants were transplanted into new soil and placed in a growth chamber with a 12-h photoperiod. After 2 months of growth, well-developed plants were used in the experiments.

PCR and real-time quantitative PCR analysis

To verify the presence of the transgene, the *MlNPR*1 gene was amplified by PCR. *M. lewisii* genomic DNA was extracted from leaves using an optimized CTAB method (Yuan et al. 2013b). The *MlNPR*1 transgene was amplified using the primer pair *MlNPR*1_F2: 5′-GCT AGG CCA TCA GAT CTC AC-3′ and attR2_R: 5′-CAC CAC TTT GTA CAA GAA AGC TG-3′ (700 bp). The PCR reactions were performed as the above cloning conditions. Expression of *MlNPR*1 in the first generation transgenic plants was detected by RT-PCR and qRT-PCR. Primers *MlNPR*1_qF: 5′-TAT CCG GAT ATA GCT CAG ATG-3′ and *MlNPR*1_qR: 5′-CTT CTT AAG AGT GAG CAT AC-3′ (200 bp) were used for the RT-PCR confirmation with 1.5 μl of the cDNA sample as the template. The qRT-PCR reactions consisted of a total volume of 20 μl with 1 μl each of 5 μM *MlNPR*1_qF and *MlNPR*1_qR, 10 μl SYBR Green PCR Master Mix (Invitrogen), 1.5 μl cDNA, and 6.5 μl ddH_2O. The experiment was carried out on the LightCycler480 real-time PCR system (Roche, USA). The qPCR program used was as follows: initiation with a 3 min denaturation period at 95°C, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 20 s. *Ml*ACTIN gene was used as an internal reference for normalization using the primer pair *Ml*UBC_F: 5′-GGC TTG GAC TCT GCA GTC TGT-3′ and *Ml*UBC_R: 5′-TCT TCG GCA TGG CAG CAA GTC-3′. We also performed qPCR to monitor the transcript levels of the *Ml*PR1, *Ml*PR2 (β-1,3-glucanse) in the leaf tissue of the transgenic plants. Reactions were run with three biological replicates and two technical replicates. The relative expression levels were calculated using the comparative Ct method.

Subcellular localization studies

The CDS of *MlNPR*1 without stop codon was amplified using gene-specific primers *MlNPR*1_F: 5′-ACG CGT CGC ATG GAC AGC CGA ACC GCG GTT-3′) and *MlNPR*1_R: 5′-GGG GTA CCC TTC TTA AGA GTG AGC ATA C-3′. After *Sal*l and *Kpn*I digestion, the fragment was inserted into a modified binary vector pCAMBIA1300-GFP. The recombinant plasmid pCAMBIA1300-*MlNPR*1-GFP was introduced into the *A. tumefaciens* strain GV3101, and then transferred into leaf epidermal cells of one month old *Nicotiana benthamiana* plants using a 1-ml needleless syringe. GFP fluorescent microscopic analysis was performed through a Nikon A1R confocal laser scanning microscope equipped with a 60X water immersion objective (Plan APO VC 60X/1.20). The light images were captured under a Zeiss Axioskop microscope connected to a QImaging Micro Publisher 3.3 RTV camera (QImaging, Canada)., and the enhanced green fluorescent protein (GFP) for visual selection

Performance of transgenic plants under biotic pressure from Rhizoctonia

Leaves of *M. lewisii* infected with *R. solani* were collected and ground into fine homogenate in an ice bath, and then immersed in Murashige & Skoog liquid medium (MS, pH=6) at a ratio of 1 : 10 (Leaf mass : buffer volume), The supernatant was collected by centrifuging at 3,000 rpm, and used as a source of fungal inoculation. Inoculation was performed by spraying 1 ml supernatant on to the leaf adaxial using pipettor, and then the leaf surface was rubbed using a cotton swab. The inoculated plants were maintained at 100% relative humidity. Disease phenotype was examined after *R. solani* inoculation.

Results and discussion

Expression analysis of MlNPR1 in M. lewisii after SA treatments and fungal infection

The expression pattern of *MlNPR*1 was investigated under SA hormonal stress through real-time PCR. Transcript levels of *MlNPR*1 started to increase at 2 h, and continued gradually to increase until 8 h and then decreased at later time points (Figure 1A). It has been well characterized that NPR1 plays important role in disease resistance in plants. To further study the defensive role of *MlNPR*1, we inoculated wild monkey flower plants with *R. solani* fungal pathogen. After inoculation, the transcript level of *MlNPR*1 was slightly increased at 2 h, and the highest expression levels were observed after 12 to 48 h (Figure 1B). Together, these results indicated that the expression of *MlNPR*1 was induced quickly after treatment with SA and *R. solani* infection, suggesting that *MlNPR*1 may be involved in the defense response against fungal pathogens.

Cloning and sequence analysis of MlNPR1

The CDS of *MlNPR*1 (GenBank No. MH667319) was

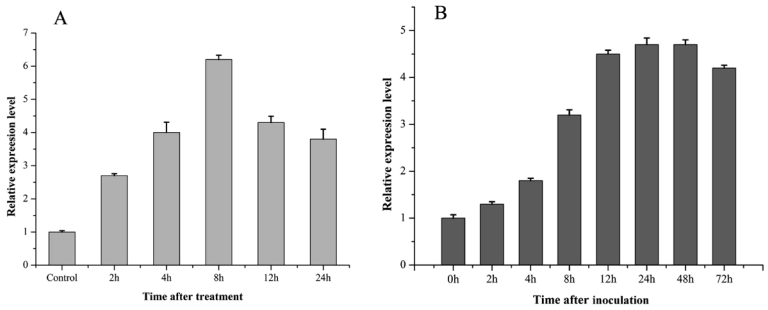

Figure 1. Expression analysis of *MlNPR1* gene after SA treatment and fungal infection. Fifty days old *M. lewisii* were used in these treatment. Leaf samples were collected at different time points for RNA isolation. Control plants were treated with Tween20 (0.01%) buffer. (A) Expression analysis of *MlNPR1* after SA treatment. (B) Expression analysis of *MlNPR1* after *R. solani* infection.

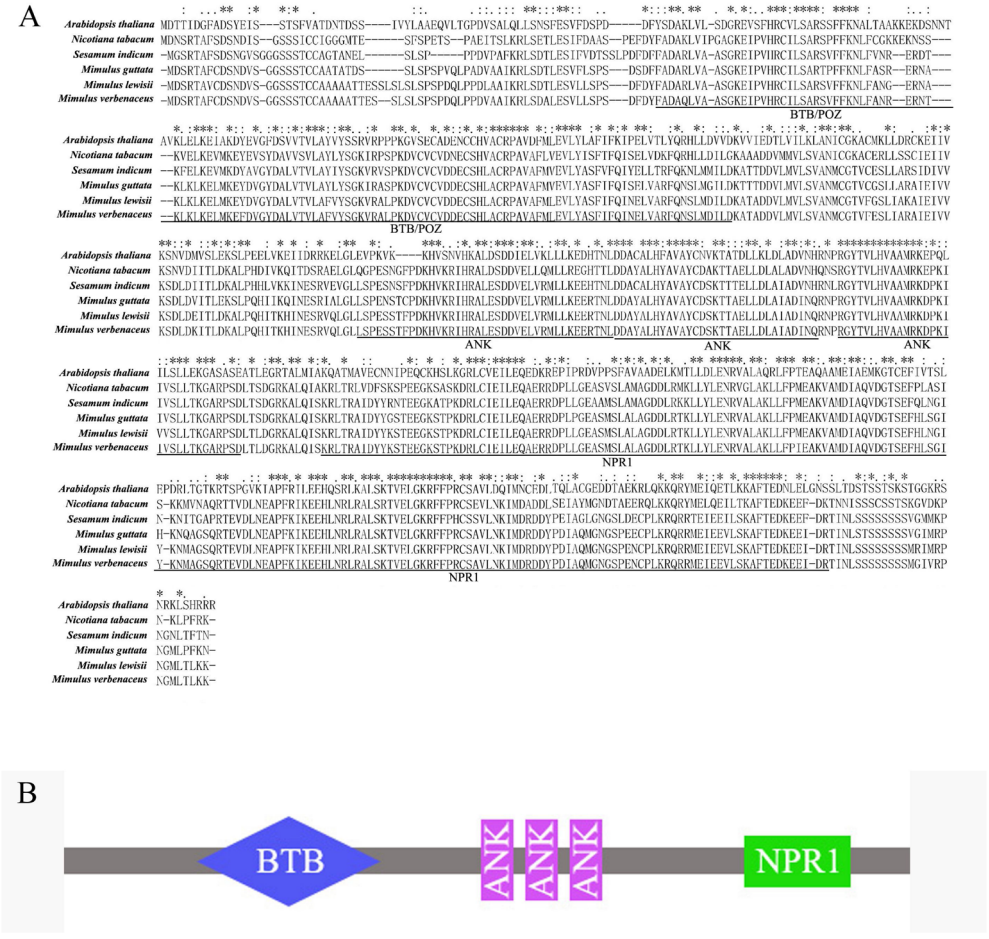

Figure 2. Multiple sequence alignment and structure analysis of *MlNPR1* protein sequence. (A) Sequence comparison of *MlNPR1* and related *NPR1* or *NPR1*-like proteins. The underlined amino acids indicated that there were three functional domains: the BTB domain, the ankyrin repeat sequence, and NPR1 domain. (B) The conserved domains (BTB-ANK-NPR1) of the *MlNPR1* protein after analyzed by SMART.

1,779 bp long coding a protein of 593 amino acids. A comparison between the *MlNPR1* cDNA and the genomic DNA sequence confirmed that *MlNPR1* had four exons (555, 750, 201, and 273 bp). The number and position of the *MlNPR1* introns were conserved as compared with other *NPR1* homologs (data not shown). The analysis using SMART program revealed that a typical BTB/POZ domain (A72 to L193) at the N-terminal, three significant homologies to ankyrin repeats (from K268 to L296, D297 to Q327 and R331 to D360) in the middle and one NPR1-like domain (K373 to R567) located at the C-terminal (Figure 2A). Previous studies have demonstrated that the BTB/POZ domain was a protein–protein interaction motif which is found at the N-termini of several C2H2-type transcription factors as well as Shaw-type potassium channels. The ankyrin repeat was always in proteins referring to the most common protein–protein interaction. More importantly,

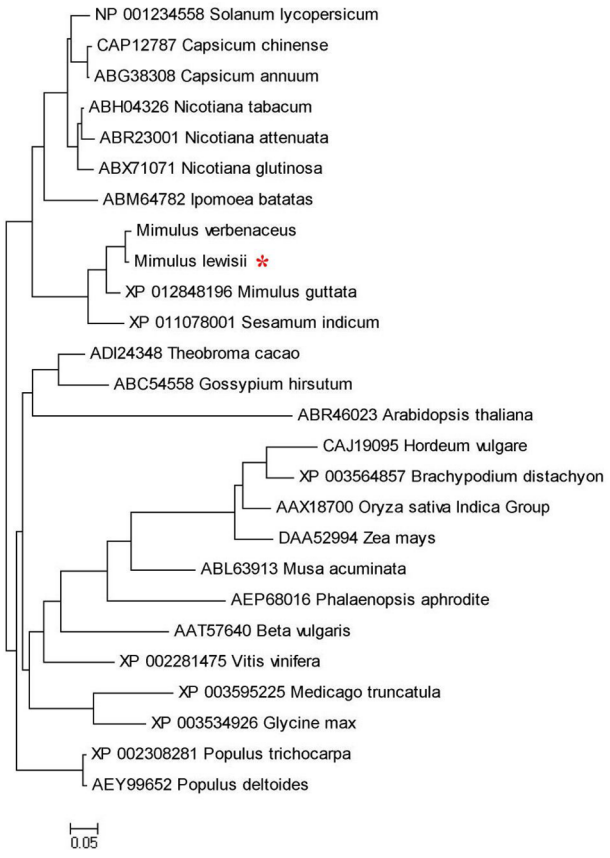

Figure 3. Phylogenetic analysis of *MlNPR*1 with other *NPR*1 proteins from different plant species. The tree was generated using Maximum–Likelihood method with 1,000 bootstrap replicates. The amino acid sequences of NPR1 and NPR1-like proteins used for construction of the tree are derived from the GenBank database.

the NPR1-like domain is a critical motif in mediating the leucine zipper transcription factor TGA binding to the as-1 motif and consequently control the start of SAR system. To know more about the *MlNPR*1 gene, amino acid sequence from other species were extracted from NCBI Genebank. The alignment of sequences indicates the conservation of these five domains among NPR1 gene (Figure 2B). A phylogenetic tree based on ML method was constructed to evaluate the evolutionary relationship between *MlNPR*1 and other *NPR*1-like proteins (Figure 3). *MlNPR*1 and *MgNPR1* from *M. guttatus* belonged to the same cluster, indicating their closely relationship during evolution process.

Molecular analysis of transgenic M. lewisii lines overexpressing MlNPR1

In order to further characterize the defensive role of the *MlNPR*1, transgenic *M. lewisii* lines with constitutive expression of *MlNPR*1 were generated. Full length CDS of *MlNPR*1 was cloned into pEarleyGate 100 binary construct under the control of CaMV35S promoter. Further, *MlNPR*1 transgenic plants were generated using *Agrobacterium*-flowerbud vacuum infiltration transformation method. In the present study, a total of 6 transgenic lines were obtained through Herbicide Basta screening. All these plants showed the amplification of the *MlNPR*1 gene-specific 700 bp PCR product, whereas non-transgenic control plants did not show any amplified bands (Figure 4A). Overexpression of *MlNPR*1 was examined in transgenic lines by qRT-PCR. Among these transgenic plants, line 2 and line 3 showed substantial levels of expression of the transgene with 9.4 and 11.4-fold higher than the level of wild-type plant (Figure 4B,

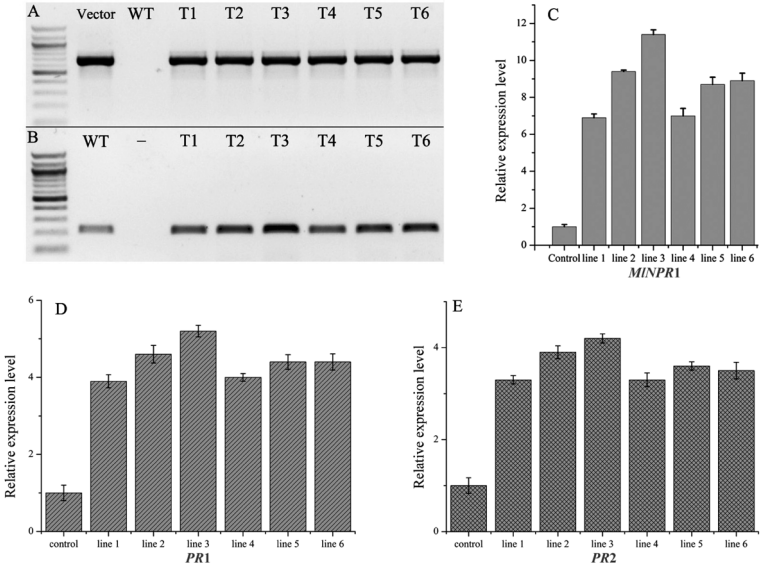

Figure 4. Analysis of transgenic *M. lewisii* expressing *MlNPR*1 and *Ml*PR genes. (A) PCR analyses of *MlNPR*1 gene in the six independent transgenic plants. (B) and (C) RT-PCR and qPCR analyses of *MlNPR*1 gene were carried out in the one month old transgenic plants, respectively. (D) and (E) Relative expression level of PR1 and PR2 in the *MlNPR*1 transgenic plants.

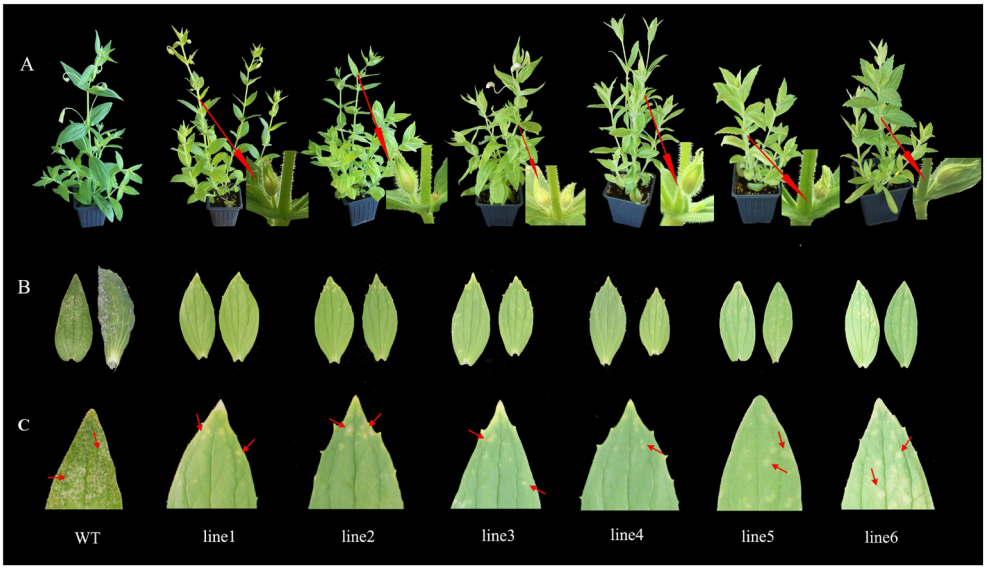

Figure 5. Development traits of wild type and *MlNPR*1 transgenic plants, and their resistant ability to *R. solani*. (A) Morphology of fifty days-old plants of the control and six independent transgenic lines with different transcription levels of *MlNPR*1. (B) Leaves were inoculated with *R. solani* and photos were taken 7 days after inoculation. (C) Magnified photographs of the inoculated leaves from (B). The infected spots are shown with red arrow.

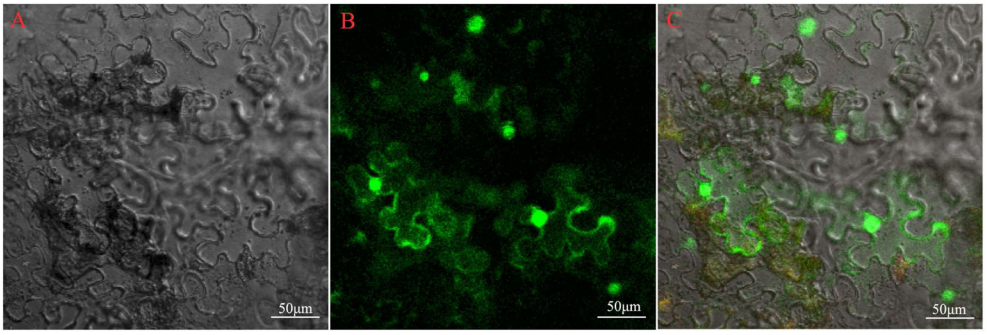

Figure 6. Localization of *MlNPR*1 in transient transformed *N. tabacum* leaf epidermal cells. The expression and subcellular distribution of *MlNPR*1 proteins was examined under a bright microscope or fluorescence microscope and then merged. (A) Pavement cells observed under the bright channel. (B) the same cells under GFP light. (C) The merged image of (A) and (B).

4C). In addition, we examined whether overexpressing *MlNPR*1gene in *M. lewisii* will lead to induction of PR genes. PR genes are known as molecular signatures of the SA and MeJA signaling pathways and widely used as diagnostic markers in pathogen resistance assays. We detected the expression of SA signaling diagnostic genes (i.e., *MlPR*1 and *MlPR*2) in the transgenic lines. As expected, the expression levels *MlPR*1 and *MlPR*2 in transgenic lines was found to be comparatively higher than in non-transgenic plants (Figure 4D, 4E). These results indicated that the constitutive expression of MlNPR1 was associated with a stronger activation of PR genes through which to confer disease resistance to multiple pathogens in *M. lewisii*.

Ectopic expression of MlNPR1 in monkey flower influences plant growth

After the transgenic lines grown up, comparative analyses on phenotypic abnormalities in *MlNPR*1 transgenic plants were systematically evaluated. The stem of transgenic plants was shorter than the non-transformed controls, the length of the leaf and the concentration of chlorophyll tend to be inversely correlated with the expression levels of *MlNPR*1 (Figure 5A, 5B). Although the transgenic lines could generate normal flower buds, some of them could not grow up and develop into normal flower (Figure 5A). Especially when the transgene was expressed at high levels, as in the transgenic lines 2 and 3, flower number was reduced by approximately 20%. These abnormal phenotypes have also been reported in other plants after overexpressing NPR1 gene (Chern et al. 2005; Silva et al. 2015). These results indicate that ectopic expression of *MlNPR*1 in monkey flower has collateral effects on plant growth and development.

Subcellular localization of MlNPR1

Sequence analysis released that *MlNPR*1 contains a

bipartite nuclear localization signal, suggested that *MlNPR*1 protein would be mostly located in the nucleus. To confirm the subcellular distribution of *MlNPR*1 protein, we constructed CaMV35S-*MlNPR*1-GFP to transfer *N. benthamiana* plants. Confocal imaging of GFP fluorescence showed that green fluorescence was predominantly located in the nucleus and, to a lesser extent, in the cytoplasm (Figure 6). This result indicated that *MlNPR*1 belonged to the nuclear localization protein.

Overexpression of MlNPR1 in M. lewisii plants confers resistance to fungal pathogen

To explore the function of *MlNPR*1 in disease resistance, we evaluated resistance level in *MlNPR*1 transgenic plants against *R. solani* fungal pathogen, which is the most common pathogen in monkey flower and always cause serious blight. For *Rhizoctonia* inoculation, *MlNPR*1 transgenic and control plants were infected and disease scoring was assessed at different time intervals. After inoculation, small necrotic lesions began after 2 days in non-transgenic plants and number of the necrotic lesions increased significantly after disease progression. Furthermore, we also observed that disease severity was very high in non-transgenic plants, and the total plant died after a week. In contrast, *MlNPR*1 transgenic plants showed no obvious infected symptoms compared to non-transgenics (Figure 5C). Interesting, although these six independent transgenic lines shown different expression levels of MlNPR1, they exhibited similar resistance to *R. solani*; this is maybe due to the high expression of PR genes. Therefore, these results demonstrate that overexpression of *MlNPR*1 in *M. lewisii* enhances resistance to the fungal pathogen *R. solani*.

The potential power of MlNPR1 in the genetic improvement of disease resistant monkey flowers

Understanding the role of defense regulatory genes is necessary to breed disease resistant transgenic crops. Manipulation of regulatory genes has many beneficial roles such as activation of multiple defense genes which provides effective and long lasting protection. After pathogen assault, plants produce a variety of phytohormones to resist pathogens. SA is an endogenous phytohormone in the deployment of SAR. Its deployment is monitored through the marker gene PR-1 (Wu et al. 2012), whose activation requires the recruitment of an SA-dependent transcriptional enhanceosome (i.e., the transcriptional coactivator NPR1, and the TGA2 transcription factors) to its promoter (Rochon et al. 2006). NPR1 gene provides a dual function in the enhanceosome owing to its BTB/POZ domain (which could interacts with and negates the function of the TGA2 repression domain) in N-terminal region, and a transactivation domain NPR1 (which is required for the activating function of the enhanceosome) in C-terminal region (Boyle et al. 2009). From the above information, it was postulated that NPR1 is the key regulator of SA in the signaling pathway for defence gene activation. Previous studies have demonstrated that *Arabidopsis* NPR1 enhanced disease resistance in various plants. In this study *MlNPR*1 gene was isolated and characterized. The phylogenetic analysis revealed that *MlNPR*1 grouped into the same clade as other *Mimulus* genus *NPR*1 proteins (Figure 2). Based on structural analysis, *MlNPR*1 protein contains a BTB/POZ domain and three ankyrin repeat domains, which are highly conserved in NPR1 proteins (Cao et al. 1997; Kinkema et al. 2000; Mou et al. 2003). Finally, transgenic plants were generated by overexpressing *MlNPR*1 using 35S promoter through *Agrobacterium*-flowerbud vacuum infiltration transformation. However, although transgenic plants improved resistance to pathogens, overexpression of *MlNPR*1 in *M. lewisii* led to a suite of collateral effects, such as the leaf length and the chlorophyll concentration (Figure 5). It was previously reported that overexpression of *AtNPR*1 homolog *OsNH*1 in rice showed many detrimental effects such as hypersensitive to light, and chlorotic lesions (Chern et al. 2005; Fitzgerald et al. 2004). Those abnormal phenotypes were the result of high basal levels of SA. To address this problem, using different-strength promoters or chemical inducible fusion protein to induce broad-spectrum resistance without any negative impact on growth is our next work.

Conclusion

In summary, overexpression of *MlNPR*1 in *M. lewisii* confers resistance to fungal pathogen *R. solani*, which cause blight diseases in monkey flower. Since transgenic monkey flower plants expressing high levels of *MlNPR*1 displayed abnormal phenotypes that could affect their growth and development. In the future research, plant expressing lower levels of *MlNPR*1 during growth but turn to increase the expression level to resist diseases is expect to obtain. For example, a pathogen or chemical inducible promoter/fusion protein may be employed to activate *MlNPR*1 overexpression when plants encounter pathogens. Any way, our results demonstrated that overexpression of *MlNPR*1 may help in the genetic improvement of disease-resistant monkey flower.

Acknowledgements

This work was supported by Guangxi karst plant conservation and restoration ecology key laboratory construction project (17-259-23) and the Guangxi Institute of Botany start-up fund (Guizhiye 17004).

Conflict of interest

The authors declare that they have no conflict of interest.

Author Contribution Statement

Yancai Shi designed the experiments. Xia He performed them and analyzed the data. Xia He wrote the manuscript. All authors read and approved the final manuscript.

References

Ali S, Chandrashekar N, Rawat S, Nayanakantha NMC, Mir ZA, Manoharan A, Sultana M, Grover A (2017) Isolation and molecular characterization of pathogenesis related PR2 gene and its promoter from *Brassica juncea. Biol Plant* 61: 763–773

Boscariol-Camargo RL, Takita MA, Machado MA (2016) Bacterial resistance in AtNPR1 transgenic sweet orange is mediated by priming and involves EDS1 and PR2. *Trop Plant Pathol* 41: 341–349

Boyle P, Le Su E, Rochon A, Shearer HL, Murmu J, Chu JY, Fobert PR, Després C (2009) The BTB/POZ domain of the *Arabidopsis* disease resistance protein NPR1 interacts with the repression domain of TGA2 to negate its function. *Plant Cell* 21: 3700–3713

Bradshaw HD, Schemske DW (2003) Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature* 426: 176–178

Cao H, Glazebrook J, Clarke JD, Volko S, Dong X (1997) The *Arabidopsis* NPR1gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* 88: 57–63

Chern M, Fitzgerald HA, Canlas PE, Navarre DA, Ronald PC (2005) Overexpression of a rice NPR1 homolog leads to constitutive activation of defense response and hypersen-sitivity to light. *Mol Plant Microbe Interact* 18: 511–520

Ding B, Mou F, Sun W, Chen S, Peng F, Bradshaw HD Jr, Yuan YW (2017) A dominant-negative actin mutation alters corolla tube width and pollinator visitation in *Mimulus lewisii. New Phytol* 213: 1936–1944

Durrant WE, Dong X (2004) Systemic acquired resistance. *Annu Rev Phytopathol* 42: 185–209

Earley KW, Haag JR, Pontes O, Opper K, Juehne T, Song K, Pikaard CS (2006) Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J* 45: 616–629

Fitzgerald HA, Chern MS, Navarre R, Ronald PC (2004) Overexpression of *AtNPR*1 in rice leads to a BTH- and environment induced lesion-mimic/cell death phenotype. *Mol Plant Microbe Interact* 17: 140–151

Friedrich L, Lawton K, Dietrich R, Willits M, Cade R, Ryals J (2001) NIM1 overexpression in *Arabidopsis* potentiates plant disease resistance and results in enhanced effectiveness of fungicides. *Mol Plant Microbe Interact* 14: 1114–1124

Kinkema M, Fan W, Dong X (2000) Nuclear localization of NPR1 is required for activation of PR gene expression. *Plant Cell* 12: 2339–2350

LaFountain AM, Chen WJ, Sun W, Chen SL, Frank HA, Ding BQ, Yuan YW (2017) Molecular basis of overdominance at a flower color locus. *G3-Genes, Genom, Genet* 7: 3947–3954

Lafountain AM, Frank HA, Yuan YW (2015) Carotenoid composition of the flowers of *Mimulus lewisii* and related species: Implications regarding the prevalence and origin of two unique, allenic pigments. *Arch Biochem Biophys* 573: 32–39

Letunic I, Bork P (2018) 20 years of the SMART protein domain annotation resource. *Nucleic Acids Res* 46(D1): D493–D496

Molla KA, Karmakar S, Chanda PK, Sarkar SN, Datta SK, Datta K (2016) Tissue-specific expression of *Arabidopsis* NPR1 gene in rice for sheath blight resistance without compromising phenotypic cost. *Plant Sci* 250: 105–114

Mou Z, Fan W, Dong X (2003) Inducers of plant systemic acquired resistance regulate NPR function through redox changes. *Cell* 113: 935–944

Quilis J, Peñas G, Messeguer J, Brugidou C, San SB (2008) The *Arabidopsis* AtNPR1 inversely modulates defense responses against fungal, bacterial, or viral pathogens while conferring hypersensitivity to abiotic stresses in transgenic rice. *Mol Plant Microbe Interact* 21: 1215–1231

Rochon A, Boyle P, Wignes T, Fobert PR, Després C (2006) The coactivator function of *Arabidopsis* NPR1 requires the core of its BTB/POZ domain and the oxidation of C-terminal cysteines. *Plant Cell* 18: 3670–3685

Sagawa JM, Stanley LE, Lafountain AM, Frank HA, Liu C, Yuan YW (2016) An R2R3-MYB transcription factor regulates carotenoid pigmentation in *Mimulus lewisii* flowers. *New Phytol* 209: 1049–1057

Silva KJ, Brunings A, Peres NA, Mou Z, Folta KM (2015) The *Arabidopsis* NPR1 gene confers broad-spectrum disease resistance in strawberry. *Transgenic Res* 24: 693–704

Tada Y, Spoel SH, Pajerowska-Mukhtar K, Mou Z, Song J, Wang C, Zuo J, Dong X (2008) Plant immunity requires conformational charges of NPR1 via Snitrosylation and thioredoxins. *Science* 321: 952–956

Vlot AC, Dempsey DA, Klessig DF (2009) Salicylic acid, a multifaceted hormone to combat disease. *Annu Rev Phytopathol* 47: 177–206

Wu CA, Lowry DB, Cooley AM, Wright KM, Lee YW, Willis JH (2008) *Mimulus* is an emerging model system for the integration of ecological and genomic studies. *Heredity* 100: 220–230

Wu Y, Zhang D, Chu JY, Boyle P, Wang Y, Brindle ID, De Luca V, Després C (2012) The Arabidopsis NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Reports* 1: 639–647

Yu GH, Zhang X, Yao JB, Zhou MP, Ma H (2017) Resistance against fusarium head blight in transgenic wheat plants expressing the ScNPR1 gene. *J Phytopathol* 165: 223–231

Yuan YW, Sagawa JM, Di Stilio VS, Bradshaw HD Jr (2013a) Bulk segregant analysis of an induced floral mutant identifies a MIXTA-like R2R3 MYB controlling nectar guide formation in *Mimulus lewisii. Genetics* 194: 523–528

Yuan YW, Sagawa JM, Young RC, Christensen BJ, Bradshaw HD Jr (2013b) Genetic dissection of a major anthocyanin QTL contributing to pollinator-mediated reproductive isolation between sister species of *Mimulus. Genetics* 194: 255–263