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Molecular cloning of a 14-3-3 protein gene from *Lilium regale* Wilson and overexpression of this gene in tobacco increased resistance to pathogenic fungi

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ABSTRACT

14-3-3 proteins exist in all eukaryotes and act as important regulators to modulate diverse signaling pathways associated with a wide range of biological processes. Besides, they are involved in molecular networks protecting plant from damages caused by the biotic and abiotic stresses, and it has been reported that many 14-3-3s play an important role in defense response against pathogens. To isolate 14-3-3 genes involved in self-defense during pathogenic infection, a novel gene *Lr14-3-3* was isolated from the root of *Lilium regale* Wilson which is a kind of wild lily species with high resistance to phytopathogens, viruses, and drought. The *Lr14-3-3* was expressed at relatively low level in root, stem, and leaf of *L. regale* under normal development, by contraries, *Lr14-3-3* was significantly up-regulated in an incompatible interaction between *L. regale* and *Fusarium oxysporum* as well as in a compatible interaction between susceptible *Lilium* Oriental Hybrid 'Siberia' and *F. oxysporum*. However, the transcription level of *Lr14-3-3* in *L. regale* was evidently higher than that in 'Siberia' during *F. oxysporum* infection. Moreover, the gene expression of *Lr14-3-3* was responded to ethylene treatment. Furthermore, in order to verify the function of *Lr14-3-3*, the constitutive plant expression vector of *Lr14-3-3* was constructed and transferred into tobacco (*Nicotiana tabacum* L. cv Xanthi). The following quantitative reverse transcription-PCR (QRT-PCR) analysis demonstrated that the *Lr14-3-3* gene was steadily expressed in the T1 transgenic tobacco lines, and compared with the WT, the disease resistance-related genes including *N* gene, osmotin, MYC, and NADPH oxidase gene were up-regulated in the transgenic lines. Moreover, the antioxidant enzymes, superoxide dismutase (SOD), glutathione S-transferase (GST), and ascorbate peroxidase (APX), showed significantly higher activities in the T1 transgenic lines than that in WT. The antifungal activity of *Lr14-3-3* transgenic tobacco was performed *in vitro* plates and *in vivo* inoculation, respectively, and the results showed that the transgenic tobacco plants evidently resisted the infection of *Botryosphaeria dothidea*, *Phomopsis* sp., and *F. oxysporum*.

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1. Introduction

Plants are constantly threatened with a variety of stresses during growth and development, such as drought, salinity, heavy metals, fungal pathogens, bacterial pathogens, viruses, and so on. It is noteworthy that the plant diseases caused by pathogenic fungi are the major obstacle to plant growth and productivity worldwide. Serious outbreak of the fungal pathogens negatively affects the yield and quality of crops and ornamental plants, and sometimes they even lead to no yield and death of the infected plants. To survive under the unfavorable conditions, plants have evolved a series of defense strategies including perceiving external stimuli, transducing the stress signals for activation of the optimal response to different type of stresses, and strengthening resistance. Plants protect themselves from fungal infection through lignin deposition, hypersensitive reactions, activation of R genes and other physiologic changes to limit pathogen infection (Melchers and Stuiver, 2000). Moreover, the effective resistance of plant was originated from the induction of specific stress-related genes and synthesis of proteins with defense roles (Vinocur and Altman, 2005). The sophisticated expression regulations of stress-related genes by the transcription factors, kinases, and other regulatory proteins function in multiple pathways and facilitate crosstalk between different signaling pathways (Yamaguchi-Shinozaki and Shinozaki, 2006).

The 14-3-3 proteins are highly conserved regulatory proteins encoded by multiple gene family in eukaryotes (Ferl et al., 2002). The phylogenetic analysis of the core region of 14-3-3s from different species showed that plant 14-3-3s fall into two groups, the epsilon and the non-epsilon groups (Ferl et al., 2002). In *Arabidopsis*, the 14-3-3 epsilon group has 5 members (mu, pi, iota, omicron,

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epsilon) and the non-epsilon group has 8 members (kappa, lambda, omega, phi, chi, psi, nu, upsilon). It has become clear that the 14-3-3 protein are involved in the molecular networks protecting plant cells from damages of abiotic stresses and play an important role in defense response against pathogens (Chelysheva et al., 1999; Roberts et al., 2002; Chen et al., 2006; Denison et al., 2011). The 14-3-3 proteins are known to function as regulators of a wide range of target proteins and play an important role in stress responses via regulating complex environmental signaling pathways and networks as well as allowing crosstalk between different pathways. For instance, a tomato (*Solanum lycopersicum*) 14-3-3 protein (*TFT7*) was positively implicated in R-protein-mediated resistance and programmed cell death (PCD) in response to effector virulence proteins from *Pseudomonas syringae* pv. *tomato* (Oh and Martin, 2011). Y_2H (yeast two-hybrid) and co-immunoprecipitation assays of *TFT7* transfected *Nicotiana benthamiana* cells demonstrated that the *TFT7* coordinately interacted with the client protein MAPKKKα protein kinase and its downstream kinase MAPKK for efficient signal transfer and PCD induction (Oh et al., 2010). In *Arabidopsis*, a 14-3-3λ protein encoded by *AtGRF6* has been indicated to be a positive regulator to recognize *Golovinomyces* spp. which causes powdery mildew disease (Yang et al., 2009). Additionally, the accumulations of *14-3-3* and other defense-related transcripts were identified during the hypersensitive reaction in soybean (*Glycine max*) after inoculation with *P. syringae* pv. *glycinea* (Seehaus and Tenhaken, 1998).

Fungal diseases were usually controlled by fungicides, but it has to point out that wide application of chemical agents with antifungal activity has resulted in serious environmental pollution and food safety problems. However, genetic engineering using the lots of antifungal genes just supplies a sustainable strategy to control plant fungal diseases. Through genetic transformation, some genes encoding the β-1,3-glucanase, chitinase, thaumatin-like protein, and other proteins with antifungal activities were expressed in different plant species and increased the resistance of transgenic plants against a variety of fungal pathogens (Punja, 2001). The 14-3-3s are involved in molecular network of defense response, and some plant 14-3-3s were isolated as the resistance-related genes. When inoculated with powdery mildew fungus *Golovinomyces* spp., the *Arabidopsis* plants with reduced *GF14λ* expression showed an impaired resistance, whereas overexpression of *GF14λ* increased resistance against powdery mildew and led to hypersensitive response (Yang et al., 2009).

Lilium regale Wilson is a kind of wild lily species and specifically located in western Sichuan of China. It is worth mentioning that the *L. regale* possesses strong resistance to drought, fungal diseases, viruses, and so on. Fusarium wilt caused by the soil-borne pathogen *Fusarium oxysporum* is the major disease of lily, and none lily cultivar or species is totally immune to infection of *F. oxysporum*, but *L. regale* showed very high resistance to *F. oxysporum*. Apparently, it would have large value in isolating the resistant genes from *L. regale*, however, it is regrettable that there is no study on resistance mechanisms of *L. regale* against *F. oxysporum*. In the present study, a novel gene, *Lr14-3-3*, was cloned from the *L. regale* based on an expression sequence tag (EST) encoding 14-3-3 protein from a suppression subtractive hybridization cDNA library of *F. oxysporum* infected *L. regale* (unpublished). In order to investigate the function of *14-3-3* gene involved in defense response of *L. regale* during *F. oxysporum* infection, the *14-3-3* gene was overexpressed in tobacco (*Nicotiana tabacum* L. cv Xanthi). The expression levels of the *Lr14-3-3* gene and several disease resistance-related genes as well as the activities of 3 antioxidative enzymes in the T1 generations of transgenic lines were analyzed. Subsequently, antifungal tests of the *14-3-3* transgenic tobacco were performed to evaluate the antifungal activity regulated by *Lr14-3-3*.

2. Materials and methods

2.1. Plant materials, inoculation and treatments

The sterile bulblets of *L. regale* and *Lilium* Oriental Hybrid 'Siberia' were developed through tissue culture, and then planted in green house using sterile sands as the medium. The young root, stem, and leaf were collected from the plants of *L. regale*. A *F. oxysporum* f. sp. *lilii* strain isolated from 'Siberia' plants with typical symptoms of Fusarium wilt was characterized and preserved in our laboratory. The *F. oxysporum* was activated on PDA plate, then the spores were washed from the plate with sterile distilled water. Subsequently, the roots of *L. regale* and 'Siberia' plants were respectively infected with spores of *F. oxysporum* (10^6 spores mL^{-1}) using the method of root dip inoculation. In parallel, a treatment with sterile distilled water, termed mock-inoculation, was respectively performed in *L. regale* and 'Siberia' as controls. Then the infected roots were harvested at 2, 12, 24 hours post-inoculation (hpi), respectively. The stress-related signaling molecules used for expression analysis were salicylic acid (SA, 5 mM L^{-1}), jasmonic acid (JA, 100 μM L^{-1}), ethylene (ET, 1 mM L^{-1}) and H_2O_2 (1 mM L^{-1}). The treatment method was same as the *F. oxysporum* inoculation, and the root of *L. regale* was collected after treatment for 12 h. All plant samples were frozen in liquid nitrogen and stored at −80 °C until RNA extraction. The sterile seedlings of *N. tabacum* L. cv Xanthi were cultured in climatic cabinet and used for genetic transformation.

There were totally 5 fungi used in antifungal activity assays including *Botryosphaeria dothidea*, *F. oxysporum*, *Phomopsis* sp., *Alternaria* sp. and *Botrytis cinerea*. All the 5 fungi were conserved at −4 °C in our laboratory.

2.2. 5′ and 3′ rapid amplification of cDNA ends (RACEs)

The 5′ and 3′ RACEs were performed using the SMART RACE cDNA Amplification Kit (Clontech, USA) to get the 5′ and 3′ cDNA ends of the *14-3-3*. The gene-specific primers (GSP) for 5′ and 3′ RACEs were designed based on sequence of *14-3-3* EST (GenBank no. JZ390973), and the primer sequences were indicated in Table S1. The poly A^+ RNA was isolated from 100 μg total RNA of *F. oxysporum* infected *L. regale* root with the NucleoTrap® mRNA Midi kit (Macherey-Nagel, Germany). The following cDNA synthesis and RACE-PCR were performed according to the manufacturer's protocol (Clontech). Then the PCR products were cloned into pGEM-T eEasy Vector (Promega, USA). After the vectors were transformed into *Escherichia coli* DH5α, the positive clones were screened out to fully sequence in both directions. The overlapping and assembly of EST and RACE products as well as open reading frame (ORF) finding were completed in NCBI (http://www.ncbi.nlm.nih.gov/). The full-length ORF of *Lr14-3-3* was amplified using the 5′-RACE-Ready as template with the Advantage™ 2 PCR Enzyme System (Clontech) and GSP targeting the non-coding regions.

2.3. Bioinformatics analyses

Except that the phylogenetic neighbor-joining analysis was accomplished with the software of Mega 3.0, the other methods used in the bioinformatics analyses were same as those in Liu et al. (2008, 2013b).

2.4. Construction of Lr14-3-3 binary vector for tobacco transformation

The vector pCAMBIA2300s (Liu et al., 2013a) was used to construct a plant expression vector of *Lr14-3-3*. The full-length ORF of

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