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A glutathione *S*-transferase gene from *Lilium regale* Wilson confers transgenic tobacco resistance to *Fusarium oxysporum*

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ABSTRACT

Glutathione *S*-transferases (GSTs) are multifunctional proteins that are encoded by a large gene family. Plant GSTs are involved in host defense against both biotic and abiotic stresses as well as are involved in some physiological processes under normal conditions. In the present study, a novel tau GST gene, *LrGSTU5*, was isolated from *Lilium regale* Wilson. The deduced protein LrGSTU5 shared a high identity with AcGSTU1 from *Allium cepa*. In addition, there was relatively high gene expression of *LrGSTU5* in the roots but the only minimal expression in both the stem and leaf of *L. regale* under normal conditions. The gene expression of *LrGSTU5* was obviously up-regulated after treatment with signaling molecules including salicylic acid and ethylene as well as after inoculation with *Fusarium oxysporum*. Furthermore, in order to verify the function of *LrGSTU5*, a constitutive plant expression vector of *LrGSTU5* was constructed and transferred into tobacco (*Nicotiana tabacum* L. cv Xanthi). Compared to the wild type (WT), several diseases resistance-related genes, including *osmotin*, *PR1b*, *chitinase*, and *MnSOD*, were up-regulated in the transgenic lines. Moreover, three important antioxidant enzymes, GST, superoxide dismutase, and ascorbate peroxidase, exhibited significantly higher activities in the T1 transgenic lines than in the WT after inoculation with *F. oxysporum*. Meanwhile, the rate of superoxidate anion production in the transgenic tobacco lines was significantly lower than in the WT. The antifungal activity of three *LrGSTU5* transgenic tobacco lines was estimated through *in vivo* inoculation, and the results showed that the transgenic tobacco plants enhanced the resistance to *F. oxysporum*.

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1. Introduction

As the global population continues to rapidly grow, food shortage becomes an increasingly important issue that is capable of threatening human survival; therefore, it is paramount to increase crop yields. However, plant diseases remain highly problematic for crop production worldwide, especially those diseases that are caused by innumerable pathogenic fungi (Li et al., 2014). Fortunately, many antifungal genes have been isolated from a variety of plants due to advancements made in molecular biology and biotechnology: such as, chitinases, β-1,3-glucanases, thaumatin-like proteins, and other pathogenesis-related (PR) proteins (Garcia-Casado et al., 2000; Wróbel-Kwiatkowska et al., 2004; He et al., 2014; Kolosovaet al., 2014). These resistance genes are potential resources for developing novel cultivars with a high level of resistance to fungal pathogens through genetic engineering. Obviously, isolating novel resistance-related genes and illustrating their resistant spectrum are crucial for the genetic engineering of disease resistance in plants.

Throughout the long history of plant-pathogen interactions, plants have evolved a series of complex mechanisms to cope with pathogenic infection, including pathogen perception, signal transduction, and the induction of specific resistance-related genes (Vinocur and Altman, 2005). Biotic stresses lead to a battery of defense responses that include changes to cell metabolism, cell wall reinforcement, oxidative burst, and the hypersensitive response (HR) (Dean et al., 2005). In the early stage of the defense response to pathogens, the generation and accumulation of reactive oxygen species (ROS) trigger an oxidative burst. Then, the ROS directly or indirectly cooperates with other molecules and played important roles in activating the defense responses to the host plant (Polkowska-Kowalczyk et al., 2007). However, the excessive amount of ROS results in oxidative stress to the plant cells, such as the peroxidation of lipids, damage to both nucleic acids and cell membranes, and inhibition of DNA and protein synthesis. Therefore, in order to combat excessive ROS and to protect the cells from oxidative damage, plants have evolved a series of antioxidant enzymes and molecules, such as glutathione (GSH), thioredoxin

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(Trx), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), peroxidase (POD), and glutathione *S*-transferase (GST) (Mukherjee et al., 2010). These detoxification enzymes reduce the ROS level through a variety of catalytic mechanisms. As a result, there is a balance maintained between ROS production and elimination, and there exists an enhanced resistance of plant cells to external stimuli.

The aforementioned GSTs are multifunctional proteins that are encoded by a large gene family. At least 53 *GST* genes have been identified in *Arabdopsis* and 59 *GST* genes in rice (*Oryza sativa* L.) (Chi et al., 2011). Generally, the molecular mass of a monomeric GST is 25–27 KDa. Furthermore, GSTs catalyze the conjugation of GSH to electrophilic molecules in cells, and then, the compounds that are potentially harmful to plant cells are further processed and detoxified (Seppänen et al., 2000). The expression of plant GSTs is activated by both abiotic and biotic stresses, such as chilling, hypoxic stress, dehydration, wounding, and pathogenic attack. For example, when inoculated with the spore suspension of late blight fungus (*Phytophthora infestans*), the expression level of a GST gene, *prp1*, as well as GST activity were both evidently increased in potato (*Solanum tuberosum*) leaves (Hahn and Strittmatter, 1994). Moreover, *AtGSTF6* was up-regulated as a result of an infection by the downy mildew pathogen *Peronospora parasitica* in *Arabidopsis* (Wagner et al., 2002). In addition, some GST genes are induced by certain signaling molecules, including ethylene (ET), jasmonic acid (JA), H_2O_2, and salicylic acid (SA) (Jain et al., 2010).

Fusarium oxysporum is a major pathogenic fungus of lily (*Lilium*), and there are not any lily cultivar or species that are totally immune to *F. oxysporum* infection; however, a few wild lily species including *Lilium regale* Wilson possesses a high-level resistance to *F. oxysporum* (Lim et al., 2003). *L. regale* is a wild lily species and is native to China's western Sichuan province. Moreover, it is worth mentioning that *L. regale* also possesses a high level of resistance to viruses and drought. In addition, there have been a few reports that have studied the mechanisms of *L. regale* resistance against *F. oxysporum* (Li et al., 2014; Rao et al., 2014). In this study, based on a *GST* expression sequence tag (EST) from a suppression subtractive hybridization (SSH) cDNA library of *F. oxysporum* -infected *L. regale* (Rao et al., 2014), the full-length cDNA of the *GST* was obtained through the rapid amplification of the cDNA end (RACE). Furthermore, the *GST* gene, named *LrGSTU5*, was overexpressed in tobacco (*Nicotiana tabacum* L. cv Xanthi), and the following physiological and antifungal tests of the *LrGSTU5* transgenic tobaccos were performed in order to verify its function.

2. Materials and methods

2.1. Plant materials, inoculation, and treatments

The sterile bulblets of *L. regale* were cultured in our laboratory. An *F. oxysporum* f. sp. *lilii* strain was isolated from *Lilium* Oriental Hybrid 'Siberia' plants with Fusarium wilt and was kept in a potato dextrose agar (PDA) plate at 4 °C.

In order to analyze the expression pattern of the GST gene, the roots, stem, and leaf tissues were collected from healthy *L. regale* plants. The roots of *L. regale* plants were infected with fresh spores of *F. oxysporum* (10^6 spores mL^{-1}) using the root-dip inoculation method. Meanwhile, a treatment with sterile water, which is termed mock-inoculation, was performed to serve as the control. Then, the infected roots were harvested at 2, 4, 12, 24, 28, and 72 h post-inoculation (hpi), respectively. For the signaling molecule treatment, the roots of *L. regale* plants were respectively dipped into 5 mM L^{-1} SA, 100 μM L^{-1} JA, 1 mM L^{-1} ET, and 1 mM L^{-1} H_2O_2 for 30 min, and then, the roots were collected after treatment for 12 h. All of the plant samples were frozen in liquid nitrogen and stored at −80 °C until nucleic acid extraction.

2.2. RACE

Previously, we isolated a *GST* EST (GenBank accession no. JZ390986) from a SSH cDNA library of *L. regale* inoculated with *F. oxysporum* f. sp. *lilii* (Rao et al., 2014). To investigate the roles of *L. regale GST* in response to *F. oxysporum* f. sp. *lilii*, the full-length cDNA of the *GST* was cloned with RACE. Because the EST contains the end codon and the 3′ UTR, the 5′ terminal of *GST* was cloned through 5′ RACE, which was performed using the SMART RACE cDNA Amplification Kit (Clontech, USA) with the gene-specific primer (5′GGTCTTGTCTCCAAGCTCAGACTCCAGC3′). The mRNA was isolated from 100 μg total RNA of *L. regale* roots using the NucleoTrap® mRNA Midi kit (MACHEREY-NAGEL, Germany). Both the cDNA synthesis and RACE-PCR were performed according to the manufacturer's protocol (Clontech). The PCR product was cloned into the pMD-18T Vector (TaKaRa, Japan) and then transformed into *Escherichia coli* DH5α. Recombinant clones were chosen and sequenced. The overlapping and assembly of the cDNA fragment with the *GST* EST was performed with the bl2seq tool in GenBank (http://www.ncbi.nlm.nih.gov/). The open reading frame (ORF) of *GST* was amplified using the 5′-RACE-Ready cDNA as the template with the Advantage™ 2 PCR Enzyme System (Clontech) and gene-specific primers targeting the noncoding regions.

2.3. Bioinformatics analyses

The methods used in the bioinformatics analyses were identical to those used in Li et al. (2014).

2.4. Construction of LrGSTU5 binary vector for tobacco transformation

In the present study, the modified vector named pCAMBIA2300s (Liu et al., 2013a) was used as a plant overexpression vector. The ORF of *LrGSTU5* was obtained through a double digestion of pMD-18T-*LrGSTU5* with *Pst* I and *Bam* HI and was ligated into the digested pCAMBIA2300s with the same two restriction endonucleases; thus, pCAMBIA2300s-*LrGSTU5* was constructed. The ligation product was then transferred into *E. coli* DH5α, and the positive clones that contained pCAMBIA2300s-*LrGSTU5* were screened out through PCR.

2.5. Tobacco transformation and screening of transgenic tobacco

The vector of pCAMBIA2300s-*LrGSTU5* was transferred to *Agrobacterium tumefaciens* LBA4404 *via* the freeze–thaw method (Holsters et al., 1978). The clones on the LB plate with kanamycin were confirmed by PCR with gene-specific primers of *LrGSTU5*. Then, the positive clones were used to transform leaf discs of tobacco (Horsch et al., 1985). Transgenic plants were selected with 50 mg L^{-1} kanamycin in the MS medium (Murashige and Skoog, 1962). The genomic DNA of regenerated tobacco plants was isolated by the CTAB method (Allen et al., 2006), and the positive transgenic tobacco plants were screened out for the presence of *LrGSTU5* with gene-specific primers, and the WT plants served as the control.

2.6. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The transcription levels of *LrGSTU5* in the roots, stem, and leaf of *L. regale* under normal conditions as well as the transcription levels in the roots after treatment with SA, JA, ET, H_2O_2, and *F. oxysporum* f. sp. *lilii* were estimated by qRT-PCR. Moreover, the expression levels of *LrGSTU5* and five diseases resistance-related genes in the

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