Biochemical and Biophysical Research Communications 393 (2010) 38–44

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A thaumatin-like protein gene involved in cotton fiber secondary cell wall development enhances resistance against *Verticillium dahliae* and other stresses in transgenic tobacco

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ARTICLE INFO

Article history:
Received 9 January 2010
Available online 25 January 2010

Keywords:
GbTLP1
Verticillium dahliae
Fusarium oxysporum
Thaumatin-like protein

ABSTRACT

For the first time, a sea-island cotton (*Gossypium barbadense* L.) thaumatin-like protein gene (*GbTLP1*) with a potential role in secondary cell wall development has been overexpressed in tobacco to elucidate its function. The presence of the transgene was verified by Southern blotting and higher expression levels of *GbTLP1* in transgenic tobacco plants were revealed by reverse-transcription and quantitative real-time polymerase chain reaction analyses. Transgenic plants with constitutively higher expression of the *GbTLP1* showed enhanced resistance against different stress agents, particularly, its performance against *Verticillium dahliae* was exceptional. Transgenic tobacco plants also exhibited considerable resistance against *Fusarium oxysporum* and some abiotic stresses including salinity and drought. In this experiment, transgenic plants without *GbTLP1* expression were also used as controls, which behaved similar to non-transgenic control plants. Overexpression of *GbTLP1* had no significant deleterious effect on plant growth except that flowering was delayed for 3–5 weeks. The apparent pleiotropic effect of this novel gene has given us insight to the plant defense mechanism.

Introduction

Understanding the defense mechanisms of plants is of prime importance to induce fundamental resistance under different stress conditions. Naturally, plants are subjected to different biotic and abiotic stress conditions that affect their growth and yield. To cope with these stress conditions, plants respond by using an innate immune system [1]. It initiates a series of physiologic changes at the site of infection, including hypersensitive response (programmed cell death), the production of pathogenesis-related (PR) proteins and reactive oxygen species, accumulation of both free benzoic acid and salicylic acid, and synthesis of antimicrobial phytoalexins [2]. In pathologic and physiologic stress, PR proteins are of significant importance because they are induced and coded by the host plant. Accumulation of some PR proteins is associated with the onset of systemic-acquired resistance that enables plants to survive against a broad spectrum of pathogens [3].

So far, PR proteins have been divided into 17 distinct unrelated families that function as part of the plant defense system [4]. Among these, plant thaumatin-like proteins (TLPs) belong to the PR-5 family which share a high homology with intensely sweet-tasting protein thaumatin (about 100,000 times sweeter than sucrose), isolated from the fruits of the West African rain forest shrub *Thaumatococcus danielliifrom* [5]. The thaumatin-like proteins have been reported to be involved in plant resistance against different fungi in many transgenic crops [6,7]. Along with biotic stress, TLPs are also expressed and resist abiotic stress conditions [8]. All these studies make the TLP gene an excellent choice to induce biotic and abiotic stress resistance in different transgenic plants.

Previously, we isolated two TLP genes (*GbTLP1* and *GbTLP2*) from sea-island cotton (*Gossypium barbadense* L.) fiber cDNA library, which had 97% protein sequence identity to each other [9] (GenBank: DQ912960, DQ912961). In this study, we inserted full-length *GbTLP1* cDNA into pCAMBIA 2300S vector under the control of CaMV 35S promoter and transferred it into tobacco to determine its role in different biotic and abiotic stress conditions. Study of this gene against *V. dahliae* was of special interest for us because, *in vitro*, TLPs have previously been tested as growth inhibitors of *V. dahliae* [5]. *Verticillium* wilt, caused by fungi of genus *Verticillium* affects more than 400 plant types, including cotton and tobacco [10]. We also studied the behavior of the *GbTLP1* gene against *Fusarium oxysporum* and other major abiotic stresses such as drought and salinity.

Materials and methods

Vector construction and transformation of tobacco. To construct an overexpression vector, plasmid containing *GbTLP1* cDNA (GenBank: DQ912960) was extracted. Sense primer (5′ GCGGAG

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doi:10.1016/j.bbrc.2010.01.069

CTCTTCACAAGGCAGTAATGGCG 3′) and antisense primer (5′ CCGCTGCAGCATGGGTTATCAATCAAATCAAG 3′) with added *Sac* I and *Pst* I restriction sites, respectively (underlined), were used to amplify *GbTLP1*. The amplified product was ligated into a modified pCAMBIA 2300S vector (Fig. 1A). *Agrobacterium*-mediated transformation of tobacco (*Nicotiana tabacum* L.) was performed according to the standard protocol [11]. The T_0 plants were self-fertilized and T_1 seeds were germinated on kanamycin selection medium. Surviving plants were transferred to the soil for further study.

DNA extraction, PCR, and Southern blotting analysis. Genomic DNA of transgenic and wild-type tobacco plants was extracted as described previously [12]. Gene-specific (*GbTLP1*) primers (mentioned above) were used to confirm the presence of transgene. Presence of *NPT* II (neomycin phosphotransferase II) was verified by amplification with *NPT* II-f (5′ CGTAAAGCACGAGGAAGCG 3′) and *NPT* II-r (5′ GGCACAACAGACAATCGGC 3′) primers. Positive transgenic plants were selected for gene copy number analysis. DNA (20 μg) from each positive and one control sample were digested by *Eco*RI restriction endonuclease, electrophoresed, and transferred to Hybond-N^+ membrane (Amersham Life Sciences, UK). Hybridization and signal detection were performed following a validated protocol [13].

RNA extraction, reverse-transcription PCR, and quantitative real-time PCR analysis. To determine the expression level of *GbTLP1* in transgenic tobacco and normal cotton plants (both *Gossypium hirsutum* and *Gossypium barbadence*); RNA was extracted using a modified guanidine thiocyanate method [14]. These RNA samples (3 μg) were reverse-transcribed to cDNA by using the Superscript III RT (Invitrogen). The expression levels of *GbTLP1* were quantified from cDNA of both transgenic tobacco and cotton by using specific primers (forward 5′ GCAGTCAAGGCAGTTGGTGGTA 3′, reverse 5′ GCAGTCAAGGCAGTTGGTGGTA 3′). As an internal control, actin (GenBank: X69885) primer pair (forward 5′ GCTTGCTTACATTGCT CTCGAC 3′, reverse 5′ ACCTCAGGACAACGGAAACG 3′) was used for tobacco and ubiquitin (GenBank: AY375335) primer pair (forward 5′ GAAGGCATTCCACCTGACCAAC 3′, reverse 5′ CTTGACC TTCTTCTTCTTGTGCTTG 3′) was used for cotton. For reverse-transcription PCR, the program was: denatured at 94 °C for 3 min; followed by 28 cycles consisting of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s; finally extended at 72 °C for 5 min. Real-time PCR analysis was performed in three duplicates in an optical 96-well plate using 7500 Real-Time PCR System (Applied Biosystems). PCR mixture was prepared as described earlier [15]. Thermal cycling was performed with an initial denaturation step of 1 min at 95 °C, followed by 40 cycles consisting of 95 °C for 15 s, 57 °C for 15 s, and 72 °C for 45 s.

Evaluation of disease resistance. Infection of detached leaf with *V. dahliae* (strain 6sy2-37) and *F. oxysporum* (strain hn-1) was performed as described previously [16]. To calculate the area of infection, a small hole was made in the leaves and 30 μL of filtered spores at a specific concentration (10^7 spore mL^{-1}) was applied. Infected leaves were placed at 25–28 °C. The area of infection was measured 7 d after infection. In the whole-plant inoculation assay, 100 mL (same filtered solution) of both fungi was applied weekly to the soil of transgenic and non-transgenic plants for 4 consecutive weeks. Disease severity, expressed as percentage of diseased leaves over the total number of leaves per plant, was recoded 6 weeks after first inoculation [17].

Salt and oxidative stress assay. Leaf disks (1 cm in diameter) of both transgenic and control plants were kept in NaCl (0, 0.4 M and 0.8 M) and H_2O_2 (1% and 2%) for 3 d. Resistance of leaf disks to these stresses was compared by measuring chlorophyll contents using a reliable protocol [18]. To further assess the effect of salinity on plant growth and development, wild-type and transgenic plants of the same size and age (2 month old) were watered weekly with 200 mM NaCl and kept at 21–25 °C for 6 weeks. After 6 weeks,

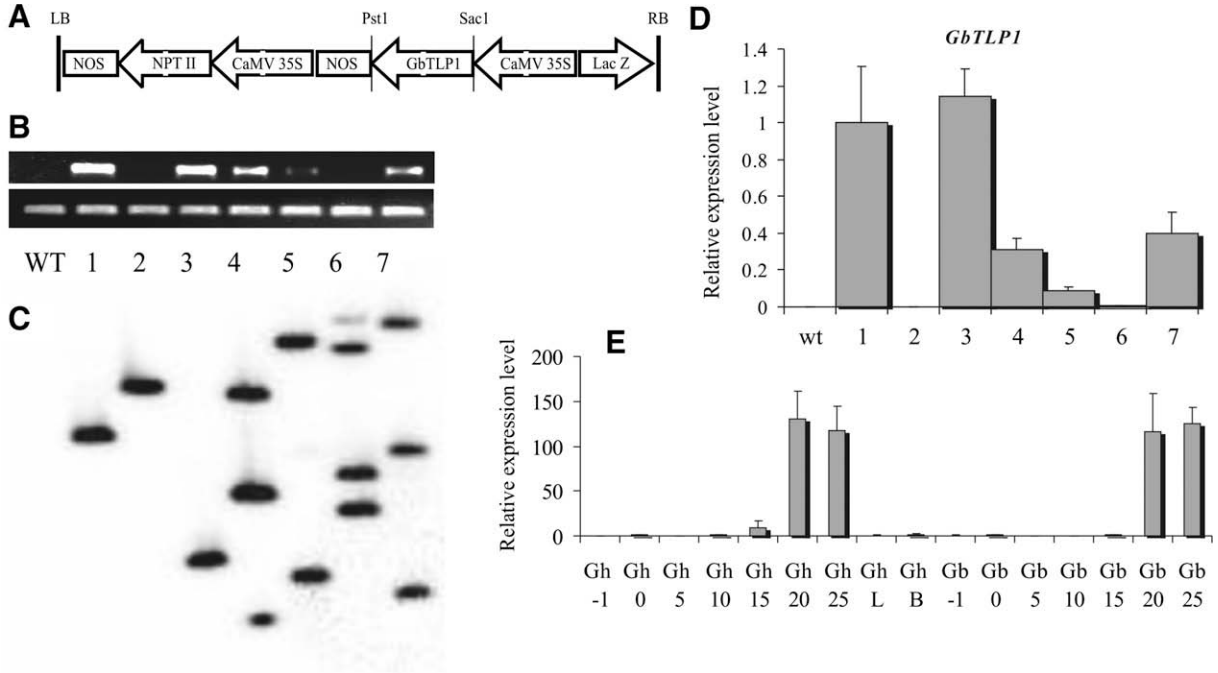

Fig. 1. Production of transgenic tobacco plants overexpressing *GbTLP1*. (A) Partial diagram of the pCAMBIA 2300S transformation vector with *GbTLP1* under the control of CaMV 35S promoter. (B) Expression profile of *GbTLP1* by RT-PCR. Actin is used as an internal control. (C) Southern blotting analyses to determine the transgene copy numbers in transgenic tobacco plants. DNA of wild-type tobacco plant (WT) was used as control. (D) Relative expression of *GbTLP1* obtained through real-time PCR analysis (1–7 in panels B, C, and D are 7 independent transgenic tobacco lines). (E) Relative expressions of *GbTLP1* in cotton at different fiber developmental stages were revealed by real-time PCR. Gh and Gb symbolize *Gossypium hirsutum* and *Gossypium barbadence*, respectively. Numbers represented days post-anthesis (DPA); L and B indicated leaf and bud.

proline and Na^+ and K^+ contents of each plant were determined [19,20].

Whole plant drought tolerance test. Transgenic and wild-type plants of the same size and age were selected and subjected to drought stress by omitting watering for 21 d. Relative leaf water contents (RLWC%) were measured to determine the water status of plants stressed by drought [21]. Furthermore, lipid peroxidation was assayed from leaf samples of all plants by determining the amount of malondialdehyde (MDA) [22]. After 21 d, plants were photographed and re-watered for recovery. All plants were kept under the same controlled condition and pods were counted for comparison.

Bioinformatics studies of GbTLP1 and other proteins of the same family. Protein sequences of *GbTLP1* and other already reported genes of the same family were obtained by using their available accession numbers at the NCBI network service (http://www.ncbi.nlm.nih.gov/). The common thaumatin domain (accession number PF00314) was manually checked using the Pfam database (http://www.sanger.ac.uk/Software/Pfam/). We used the ExPASy ProtParam tool (http://www.expasy.org/tools/protparam.html) to get basic information on the protein sequences. Computational subcellular localization of protein was predicted by using SubLoc v1.0 program (http://www.bioinfo.tsinghua.edu.cn/SubLoc/eu_predict.htm).

Results

Characteristic features of GbTLP1

Full-length cDNA of *GbTLP1* was 966 bp long with 735 bp open reading frame and encoded a polypeptide of 244 amino acids. *GbTLP1* protein had a molecular weight of 25.44 kDa and an isoelectric point (pI) of 4.78. This protein sequence contained the thaumatin domain (PF00314) like other members of this family. *GbTLP1* and other proteins under study demonstrated characteristic molecular weights and extracellular protein localization and showed resistance against a variety of fungi (Table 1). Relative *GbTLP1* expressions were quantified by real-time RT-PCR in both *G. barbadence* (donor species) and *G. hirsutum*. The expression levels of *GbTLP1* increased significantly in the fiber of both cotton species at 20 and 25 d after anthesis (Fig. 1E). These results indicated the possible involvement of *GbTLP1* in secondary cell wall development.

Table 1
General information on proteins produced by thaumatin-like protein genes of different crops.

Gene name	Accession number (protein)	Donor crop	Length (AA)	Domain sequence		Molecular weight (kDa)	Isoel ectric point	Predicted subcellular localization	Reference
				Start	Stop				
GbTLP1	ABL86687	Cotton	244	32	242	25.44	4.78	Extracellular (84%)[a]	This paper
Hv-TLP8	AAK55326	Barley	233	32	233	24.31	7.83	Extracellular (97%)[a]	[27]
Ta-TLP	AAK60568	Wheat	173	27	173	17.58	5.03	Extracellular (~100%)[a]	[6]
Tlp-1	CAL48262	Barley	172	27	172	17.49	4.33	Extracellular (~100%)[a]	[28]
VVTL-1	AAB61590	Grape Vine	222	31	222	23.96	5.09	Extracellular (97%)[a]	[29]
ATLP-1	AAB71214	A. thaliana	245	30	242	25.37	4.94	Extracellular (94%)[a]	[30]
Zlp	AAA92882	Maize	227	28	227	23.99	7.84	Extracellular (91%)[a]	[7]

[a] Expected accuracy.

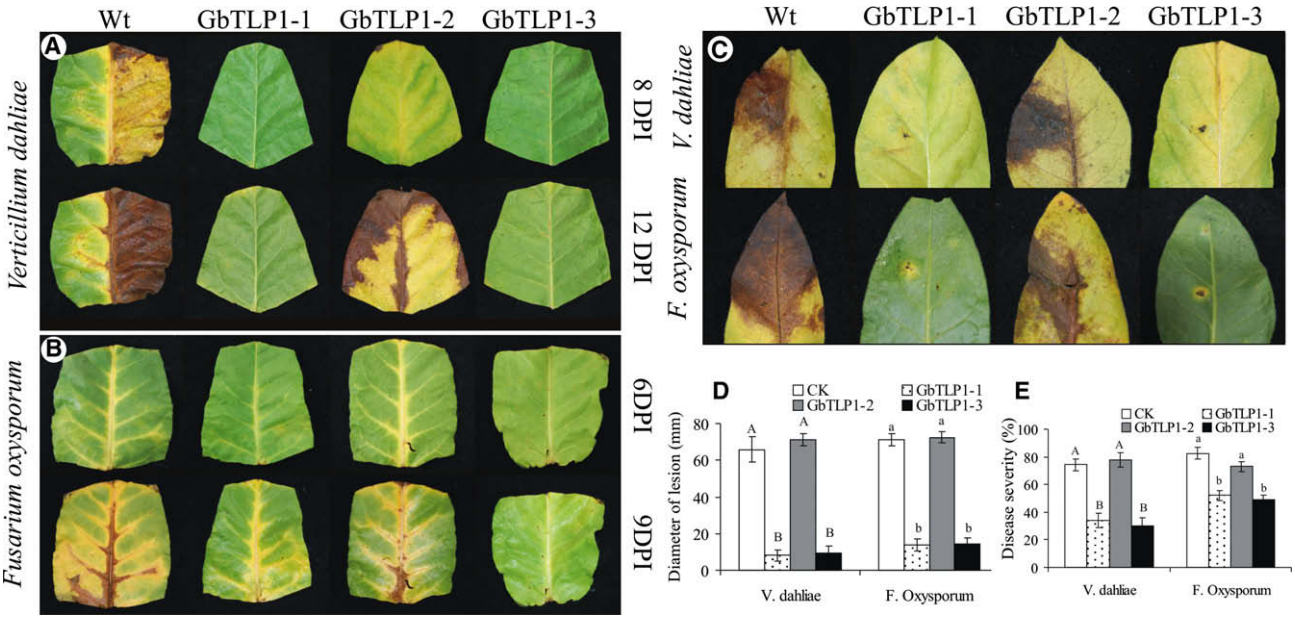

Fig. 2. Evaluation of *GbTLP1* transgenic plants resistance against *Verticillium dahliae* and *Fusarium oxysporum*. Leaves were immersed in conidial suspensions. Disease symptoms were observed at 8 and 12 d after infection (DPI) with *Verticillium dahliae* (A) and at 6 and 9 d after infection with *Fusarium oxysporum* (B). The infected leaf area after point infection was observed 7 d after infection (C) and measured (D). Disease severity was recoded 6 weeks after first inoculation (E). Values followed by the same letter were not significantly different from each other at $P < 0.05$ by DMR test. Vertical bars represented standard error of means ($n = 9$ for D, $n = 3$ for E).

Transformation and regeneration of transgenic tobacco plants

After transformation, 17 transgenic lines were produced and transferred to the soil. Of these, 12 transgenic tobacco lines produced seeds. All transgenic lines were similar in growth and morphologic characteristics, but flowering was delayed for 3–5 weeks in transgenic tobacco plants in both T_0 and T_1 generations. Southern blotting analysis revealed that only three of seven transgenic lines contained single copies of the *NPT* II gene in their genomes (Fig. 1C). Variation in the expression level of *GbTLP1* gene was observed in different transgenic lines by RT-PCR and real-time PCR analysis. Of the seven transgenic lines, line 2 and line 6 showed no expression of *GbTLP1*, as did the non-transgenic tobacco plant (Figs. 1B–D). *GbTLP1* expressions were found in all other five transgenic lines. Both RT-PCR and real-time PCR analysis revealed that the expressions of *GbTLP1* in line 1 and line 3 were higher among these five lines (Figs. 1B–D). On the basis of single copy number and higher *GbTLP1* transcriptional expression level, three lines (lines 1, 2, and 3) were selected to determine their biotic and abiotic stress tolerance and labeled as GbTLP1-1, GbTLP1-2, and GbTLP1-3, respectively. Transgenic line GbTLP1-2 was selected as a control because it had the same genetic composition as other transgenic lines but had no expression of *GbTLP1*.

Disease resistance in GbTLP1 transgenic plants

Detached leaf inoculation by immersion in conidial suspensions showed characteristic disease symptoms on the leaves of transgenic and non-transgenic plants for both fungi. Prominent symptoms appeared after 6 d and became severe after 9 d in *F. oxysporum* (Fig. 2B). In *V. dahliae* characteristic symptoms appeared after 8 d and became severe after 12 d (Fig. 2A). The point infection method was extremely useful to quantify necrotic areas (Fig. 2C). The area of infection was significantly decreased in transgenic lines with higher gene expression for both fungi (Fig. 2D). In the whole-plant inoculation assay, disease symptoms appeared slowly and

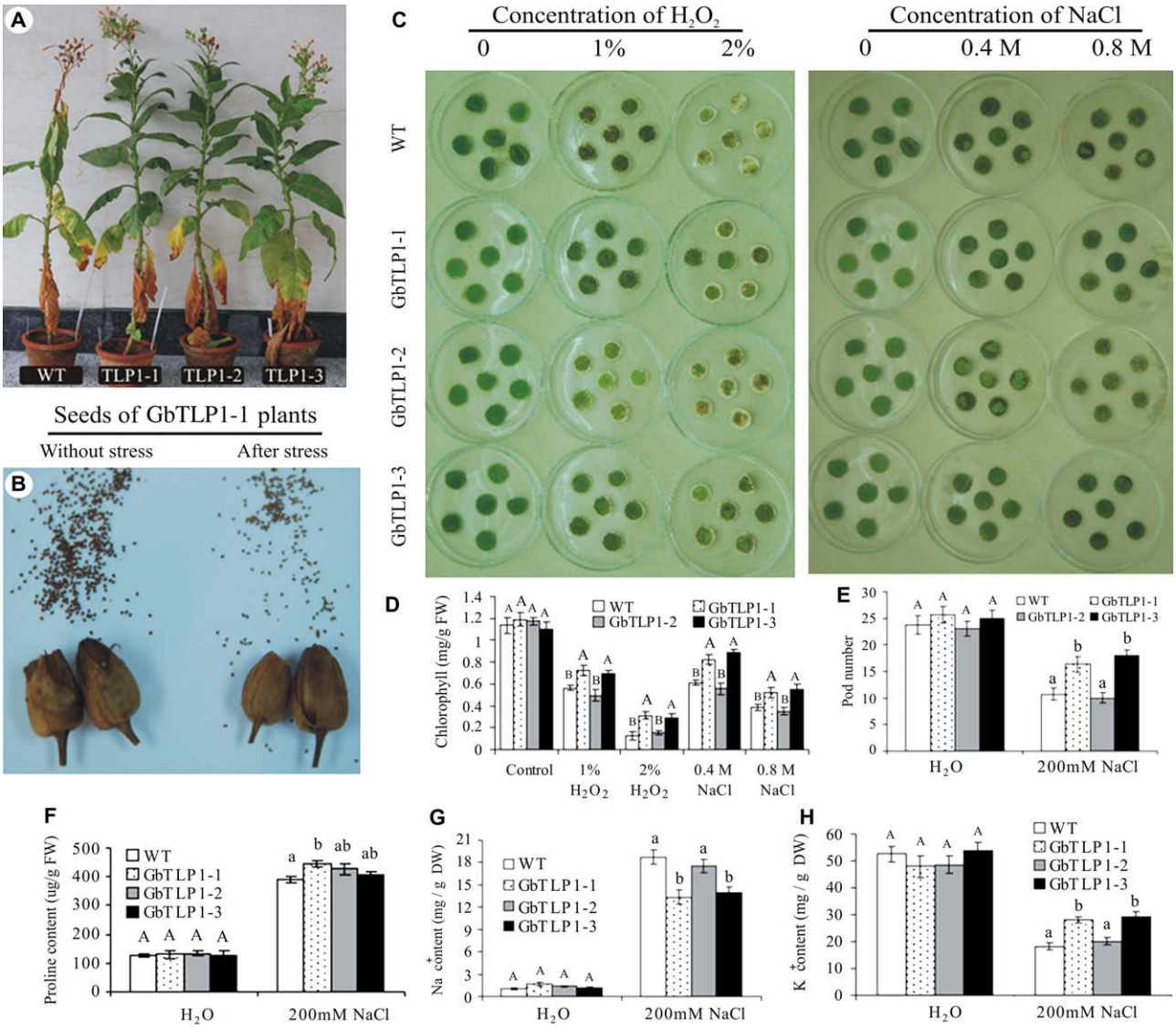

Fig. 3. Salinity tolerance in transgenic tobacco plants. (A) Transgenic and wild-type plants were watered weekly with 200 mM NaCl for 6 weeks. (B) All transgenic and non-transgenic stressed plants produced nonviable and light-colored seeds. (C) Leaf disks of transgenic and control plants after keeping them at different H_2O_2 (1% and 2%) and NaCl (0.4 M and 0.8 M) solutions. (D) Chlorophyll content of leaf disks while disks floating in the water served as control. Average pod numbers (E), proline content (F), Na^+ content (G), and K^+ content (H) of wild-type and transgenic plants after treating them with 200 mM NaCl. Vertical bars represented standard errors of means ($n = 3$). Values followed by the same letter were similar at $P < 0.05$.

leaves started wilting. At 6 weeks after the first inoculation, a smaller percentage of disease severity in *GbTLP1* overexpression lines was observed compared with control plants (Fig. 2E).

Role of GbTLP1 transgenic plants under salinity and oxidative stress

After the application of 200 mM NaCl to the plant soil for 6 weeks, the lower leaves became yellow and gradually died due to salinity. GbTLP1-2 and control plants produced fewer numbers of pods than others (Fig. 3A–E). After salt treatment, all transgenic and non-transgenic plants produced nonviable seeds that were lighter in color (Fig. 3B). We observed a reduced bleaching effect of H_2O_2 on the leaf disks of *GbTLP1* overexpressing plants (Fig. 3C). A significant decrease was noted in the chlorophyll contents of all plants, but this decrease was more severe in wild-type and GbTLP1-2 plants after both 1% and 2% H_2O_2 treatment (Fig. 3D). The same trend of bleaching and decrease in chlorophyll content was observed in leaf disks after keeping them in 0.4 M and 0.8 M NaCl solutions. Transgenic line GbTLP1-3 maintained the highest chlorophyll content, whereas GbTLP1-1 performed much better than other plants (Fig. 3D). A significant but arbitrary increase in proline contents was observed in all salinity-stressed plants (Fig. 3F). Salinity treatment increased Na^+ content and decreased K^+ content in the leaves of all plants; however, constitutively overexpressing *GbTLP1* plants accumulated a lesser Na^+ and a higher K^+ content than other plants (Figs. 3G–H).

Enhanced tolerance in GbTLP1 transgenic plants under drought stress

After 21 d of water stoppage, a significant decrease was found in RLWC% of GbTLP1-2 and wild-type control plants, whereas *GbTLP1* overexpressing lines (GbTLP1-1 and GbTLP1-3) showed higher water-holding ability (Fig. 4D). A remarkable increase in MDA concentration was observed in all stressed plants. This increase in MDA content was significantly higher in control and GbTLP1-2 plants, whereas other transgenic plants suffered less oxidative damage in the form of low MDA accumulation (Fig. 4C). The recovery of plants from drought stress did not affect plant height (Fig. 4A and B), but pod numbers in GbTLP1-2 and non-transgenic control plants were decreased in comparison with transgenic plants (Fig. 4E).

Discussion

Thaumatin-like protein has been isolated and characterized from a variety of monocotyledonous and dicotyledonous plants. In recent years, a constitutively higher level expression of TLPs has been reported to be effective for inducing both biotic and

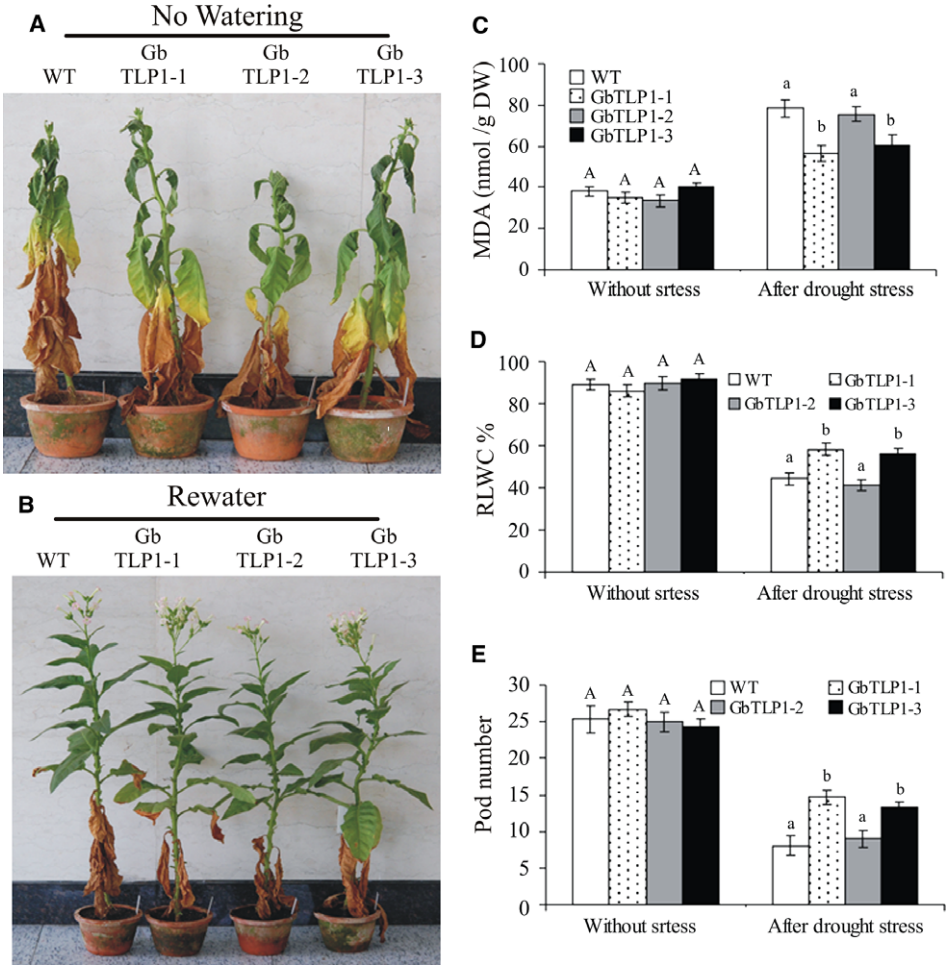

Fig. 4. Effects of drought stress on plant growth and development. (A) Plants were subjected to drought stress by omitting watering for 21 d. (B) After this specific period, plants were re-watered for recovery. (C) Lipid peroxidation was assayed after 21 d of drought stress by determining the amount of malondialdehyde (MDA). (D) Relative leaf water contents (RLWC%) after stress. (E) Effect of drought stress on pod numbers was calculated to determine the role of *GbTLP1*. Values followed by the same letter were similar to each other at $P < 0.05$ by DMR test. Vertical bars represented standard error of means ($n = 3$).

abiotic stress resistance in different transgenic crops [6,8]. We found a TLP (*GbTLP1*) cDNA from sea-island cotton and used it for creating resistance against *V. dahliae* and *F. oxysporum*. Cotton is susceptible to these two fungi and nearly one million hectares of cotton have suffered yearly from *V. dahliae* in China [10].

In this study, the role of the *GbTLP1* gene was studied for the first time. Subsequent detection by Southern blotting hybridization and PCR revealed the presence of a transgene. No physical or physiologic abnormality was observed in transgenic plants. Two transgenic lines showed constitutively high levels of expression, whereas other lines showed varying degrees of expression level, most likely the result of multiple copies of transgene. Southern blotting analysis disclosed that line GbTLP1-2 contains a single copy insertion of transgene like the other two *GbTLP1* overexpressing transgenic lines, but did not show gene expression, possibly due to a position effect. Constitutively overexpressing *GbTLP1* plants displayed resistance against *F. oxysporum* and particularly against *V. dahliae*, which supports our hypothesis. This significant resistance may have been provoked for two possible reasons. The strengthening of plant cell wall might be one possible reason to resist entrance and penetration of fungus into the cell. A higher expression of *GbTLP1* in the fiber of both cotton species at 20 and 25 d post-anthesis (DPA) indicates a possible role of this gene in secondary cell wall development. Fiber development is accomplished in four overlapping stages and secondary wall biosynthesis begins after fiber cell elongation (until 10 DPA) and continues to 25 DPA [23]. This specific expression of *GbTLP1* during secondary cell wall synthesis indicates its particular role in cell wall thickening and rigidity because the secondary cell wall consists mainly of cellulose. Previous studies have also predicted the possible role of *GbTLP1* in secondary cell wall synthesis [9]. The other possible reason is that the protein transcribed by the *GbTLP1* gene may have physically hindered fungal growth because previous studies proved that thaumatin-like proteins inhibit fungal growth conceivably by the degradation of structural components of fungi [24]. Bioinformatics studies predicted that *GbTLP1* and other thaumatin proteins are localized in the extracellular space (Table 1). Infection of fungus involves its penetration through plant cell walls and cell membranes by suppressing the plant's defense system. In this process, extracellular proteins have a vital role in reducing the pathogenic effects of invading fungus [25]. These may potentially be significant reasons for *GbTLP1* protein to be more effective against the invasion and spread of fungus.

A higher level of expression of *GbTLP1* also induced salinity tolerance in transgenic tobacco plants. Overexpression of *GbTLP1* enabled transgenic plants to maintain lower Na^+ and higher K^+ contents, which seems to be a reason for the resistance of transgenic plants, because high Na^+ concentration or low tolerance of the accumulated Na^+ increases the rate of senescence in plants [26]. Accumulation of proline in the cytosol and organelles is important to balance the osmotic pressure and maintain turgor, but determining the proline content did not help us to differentiate resistant and susceptible genotypes, possibly because its accumulation is also affected by variations in the concentration of K^+ and other compatible solutes [26]. We also observed drought tolerance in transgenic plants constitutively overexpressing the *GbTLP1* gene, which had improved cell integrity by avoiding lipid peroxidation resulting in less MDA accumulation.

The main purpose of our study was to determine the role of the *GbTLP1* gene against *V. dahliae*, but we also found its considerable resistance against other biotic and abiotic stresses. We observed the beneficial role of *GbTLP1* simultaneously against key biotic and abiotic stress conditions with different levels of success. These results also provide a ray of hope to minimize the destructive effects of *V. dahliae* on cotton and other crops. Based on these results, experiments are already under way in our laboratory to use this gene for creating fungus resistance in cotton.

In conclusion, we found that the *GbTLP1* gene is helpful for inducing resistance under different stress conditions, particularly against *V. dahliae*. Further studies may also prove this gene to be useful in cotton fiber development and strengthening of the cell wall in different transgenic crops. This gene imposes no deleterious effects on the physiology of transgenic plants, which makes it a possible and useful choice to be genetically engineered in transgenic crops for inducing resistance against variety of stress conditions.

Acknowledgments

We are thankful to Dr. Li Guoqing for providing *Verticillium dahliae* (strain 6sy2-37) and *Fusarium oxysporum* (strain hn-1). This work was financially supported by the national High-tech Project (2008AA10Z102) and the Communal Program Especially for Agricultural Research (3-19). First author is also grateful to the Ministry of Education, Pakistan, for providing the opportunity to pursue studies in China.

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