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Overexpression of Trypsin Protease Inhibitor in Transgenic Tobacco Plants Exhibit Oxidative Stress Tolerance and Fungal Resistance

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ABSTRACT

Protease inhibitors (PIs) are known for their biotic stress tolerance and this has been proved through many transgenic plant studies. Further, the PIs were reported to be induced in abiotic stress conditions and later it also proven to confer abiotic stress tolerance. A PI was isolated and expressed in tobacco plants which have exhibited salinity, pH and osmotic stress tolerance (Srinivasan et al 2009) along with insect resistance. In this communication, we show that these transgenic plants also exhibit enhanced the oxidative stress tolerance and fungal resistance. The trichome number were also enhanced which might be further helping the transgenic plants in conferring resistance to pest and pathogens.

Keywords
trypsin protease inhibitor, transgenic, tobacco plants, oxidative stress, fungal

INTRODUCTION

Protease inhibitors belonging to PR- 6 family are induced by jasmonic acid and are part of Induced Systemic Resistance. Wounding induced *PI* gene expression was detected in leaves of many plant species and they are mainly known for their role in defense against bacteria, fungi and insects (Telang et al., 2003). This was proved by transgenic plant studies also. PIs are also induced in various abiotic stress conditions (Capiatiet al.,2006). Therefore, the role of PIs in mitigating the abiotic stress was also studied through transgenics (Huang et al., 2007). The induction of PIs in various biotic and abiotic stress conditions is confirmed in multiple studies and thus constitutively expression of PI can elevate the fitness of the plants is all the challenging conditions.

Our group has earlier cloned a protease inhibitor (NtPI) from tobacco and generated its transgenic tobacco plants. These transgenics were studied for few stress tolerance possibilities. They have exhibited tolerance to salinity at seed, seedling and plant stages upto 300mM of NaCl. The *NtPI*trangenics could survive in wide range of pH conditions. They could resist and grow in pH range of 4-8 beyond which they bleached. As osmotic stress is an integral part of the above two stress conditions, the transgenic plants were tested for the osmotic stress tolerance. The transgenics could survive upto 300mM concentration of sorbitol. The PIs are known for their toxicity towards larvae. Finally, the *NtPI*transgenics were also tested for their resistance against *S.litura* and *H.armigera* where they exhibited resistance against both the larvae at varied levels (Srinivasan et al 2009).

The oxidative stress is a common stress in all biotic and abiotic stress conditions. As the *NtPI* transgenic plants have exhibited multiple stress tolerance, it is assumed that they might also exhibit tolerance to oxidative stress. Further, these transgenic plants which are effective against certain pests might be also be effective against pathogens. Hence, in the present investigation, we report the impact of these transgenic plants to oxidative stress and fungal pathogens

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MATERIALS AND METHODS

*NtPI*transgenic plants

The generation and characterization of *NtPI* tobacco lines were reported in detail in our earlier study (Srinivasan et al. 2009). The high expression lines (1 and 13) along with a low expression line (6) were used in further experimentation.

Stress treatments to seedlings

The healthy seeds of wild type (WT) and transgenic plants ($T_{1)}$ were washed with tap water. Subsequently, they are surface-sterilized with 70% ethanol, followed by 2% sodium hypochlorite for 8 min. They were later washed multiple times with sterile distilled water. The half strength MS medium was used for the growth of both WT and transgenic seeds. 100 mg L^{-1} kanamycin was also supplemented to the medium of transgenic seeds. They were allowed to grow for 15d. The conditions maintained in the culture room are 27 ± 1°C, photoperiod of 16/8 h light and dark conditions. The seedlings thus developed were analyzed for oxidative stress tolerance. ½ MS media with 10 and 20 mM H_2O_2 was employed for seed germination, similarly ½ MS media with 20 and 30 mM H_2O_2 were used for seedling stage assessment.

Leaf disc senescence assay

The healthy leaves of WT as well as transgenic plants (6 wk-old) were used to excise leaf discs of 1.0 cm diameter.They were added to 20-mL of the H_2O_2 (10, 20 and 30 mM) for oxidative stress tolerance. The treatments were performed under white light at 27 ± 1°C . The leaf discs were observed regularly for any variations.

Detached-leaf assay for disease resistance

The pathogens *Alterneriaalterneta* and *Rhizoctaniasolani* were cultured on PDA medium at 24°C for 2 to 3 d. Fully expanded leaves of 8 wk- old plants (WT, transgenic lines 1, 6 and 13) were used for the detached-leaf assay and a 0.5 cm plug of the mycelium was placed on the adaxial leaf surface. These innoculated leaves were maintained in 110mm sterile Petri plates with 16/8 h light and dark period in a BOD incubator at 24°C.

Enumeration of trichomes

The leaf and stem parts were excised from 40 day old plants and are observed under the optical microscope, which was actually mounted on a confocal microscope from Leica Micro systemsTCS SP2 ABOS type. The imaging has been carried out in transmission mode.

Statistical Analysis

The experiments were repeated thrice and data were analyzed with Sigma plot (9.0). Significance of difference was analyzed by repeated measures analysis of variance (ANOVA) and Tukey's test.

RESULTS

Oxidative stress tolerance

Oxidative stress is a common phenomenon in all stress treatments. As the transgenics have shown tolerance to different stresses, we analyzed the impact of oxidative stress on the transgenics using hydrogen peroxide (H_2O_2).

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The transgenic and WT seeds were able to germinate on 10mM H_2O_2, but the wild seeds exhibited slower germination and retarded growth (Fig 1 Aa). On 20mM H_2O_2 transgenic seed germinated but the WT could not germinate even after 10 days (Fig 1 Ab). Similarly, seedlings of transgenics and WT were exposed to 20 and 30 mM H_2O_2. The WT seedlings bleached in both the concentrations of H_2O_2 with one or two escapes. In transgenics, some chlorosis was observed in later stages of growth, but most of the seedlings survived and grew well in 20 mM H_2O_2 (Fig 1 B). When exposed to 30mM H_2O_2, the WT seedlings bleached within 36 hrs of transfer. But the transgenics survived the treatment (Fig.1 C). The leaf discs of the mature plant were also tested with 10, 20 and 30 mM H_2O_2 for4 days. The WT leaf discs bleached and transgenics retained higher amount of chlorophyll. This was confirmed by quantification of total chlorophyll content and lipid peroxidation levels. (Fig. 2)

Enhanced fungal resistance exhibited by transgenics

Protease inhibitors are known to act against necrotrophs and hence *NtPI*transgenicswere challenged with *Alterneriaalternata*and *Rhizoctoniasolani*. The appearance of disease symptoms started after 2 days and were distinct by 5 d inoculation. High expression lines could resist the growth of fungi. The necrotic lesions were comparatively larger in low expression lines than high expression lines. The WT leaves were severely affected compared to transgenics (Fig 3). Complete chlorosis of the WT leaf along with the necrotic lesion was observed in *A. alternata* infection and transgenics exhibited smaller lesion at the site of inoculation (Fig 3 a). *R. solani* caused large necrotic lesions on WT leaf, whereas the damage in transgenics was negligible (Fig 3 b). These results suggest that transgenic plants show enhanced resistance to fungal infections.

Enhancement of trichome number

Trichomes are known to be in the first line of defense in the plants particularly against insect predators. The trichomes substantially enhance the resistance against various biotic stresses and it is also reported that the trichomes are influenced by overexpression of the protease inhibitor. The trichomes are enumerated through confocal imaging of fresh explants. The trichomes on the leaf were found to enhance by 33 % and that of stem by a maximum of 40% (Fig 4). The transgenic stem and leaf trichomes were longer in length than in the WT.

DISCUSSION

The seed germination as well as early seedling stages are very vulnerable to any stress condition and *NtPI*transgenics have shown ample tolerance to oxidative stress by germinating and growing comfortably on H_2O_2 containing media. The leaf disc assay for oxidative stress tolerance proved that these transgenics exhibited reduced TBARS and higher chlorophyll levels in comparison to WT. The whole plant assay for oxidative stress has shown variations with the age of plant. The transgenics expressing *NtPI* showed increased oxidative stree tolerance at all stages.

The necrotrophic fungi like *Rhizoctoniasolani* and *Alterneriaalterneta*were affectively controlled by *NtPI*transgenics. It is also well reported that JA pathway genes restrict necrotrophic fungi (Mur et al., 2006). The fungi generally secrete a variety of proteolytic enzymes (proteases) for the digestion of plant cell wall and thus released amino acids will be utilized for their survival (De Lucca et al., 1998). The constitutively expressed *NtPI*might inhibit the proteases secreted by the fungi and thereby limit their growth. Further, another surprise factor that is in favour of the

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antifungal activity might be the enhanced number of trichomes will impart protective and defense function against the pathogens (Karabourniotis et al 2020).

The mechanism behind the multiple stress tolerance exhibited by *NtPI*transgenics exhibiting is not clearly known. The degradation of protein under stress conditions is a well known phenomenon (Callis, 1995) and accordingly, the plant induces the production of corresponding defense proteins. The enhanced or prolonged exposure to stress would be detrimental to the plant. Essentially, plant protease inhibitors have vacuole targeting signal by which they are translocated and stored in the vacuole. Upon exposure to stress, there might be release of the NtPI from vacuole and this led to higher activity in transgenics. The increase in trypsin protease inhibitor helps in blocking trypsin proteases and thereby, degradation of the respective proteins is inhibited. Thus, the life of undegraded essential proteins is enhanced which would help the transgenics in countering the stress conditions. This can be further corroborated by lower levels of TBARS and elevated levels of chlorophyll, when compared to WT. Further studies for elucidating the specific physiological role of NtPI in mitigating these stress conditions is required.

CONCLUSION

The *NtPI*transgenics have shown enhanced resistance to pests and pathogens. The high expression lines were completely resistant to the initial two instar stages of *S. litura*and *H. armigera,* while the low expression lines were very effective, in case of first instars only. All the transgenics were resistant to the infection of *A.alternata* and *R. solani.* The stem and leaf parts of transgenics showed enhanced number of trichomes. These results convey that NtPImight substantially confer multiple stress tolerance.

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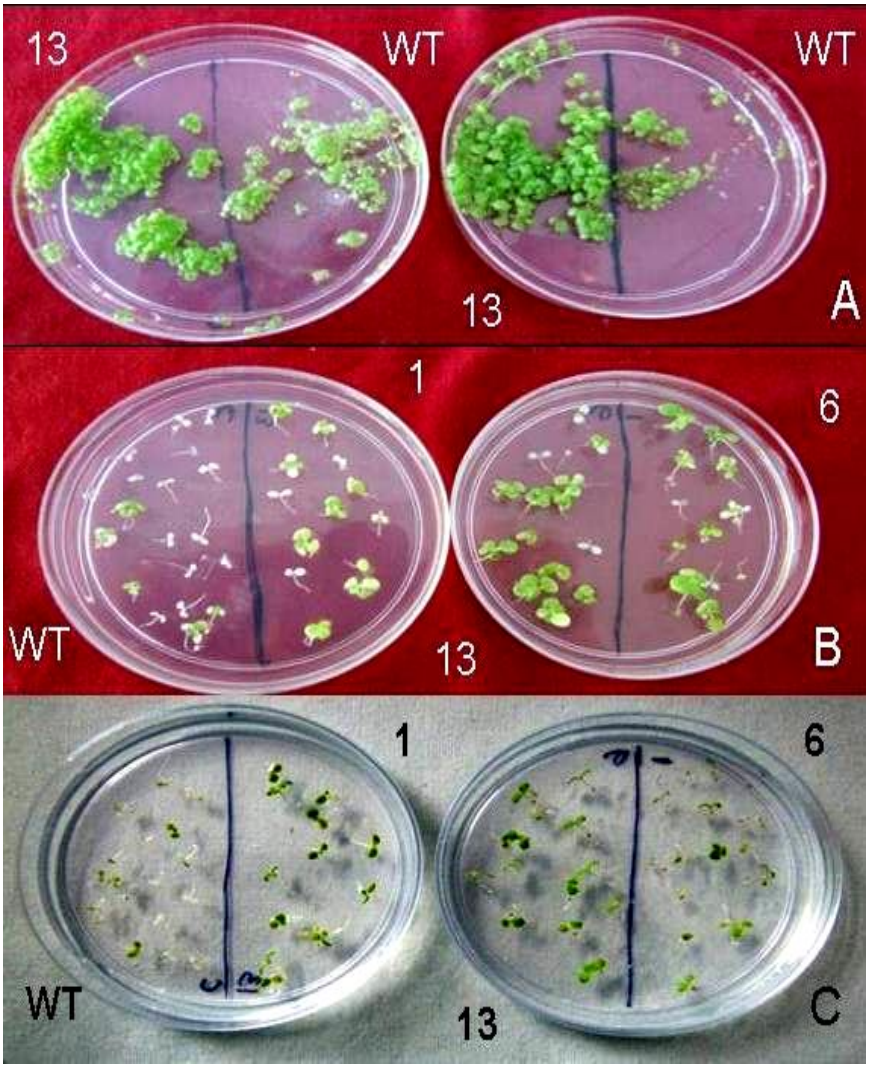

Fig 1: A: Seed germination on (a) 10mM and (b) 20 mM H_2O_2 containing medium. B, C: 15-d seedlings on 20 and 30 mM H_2O_2 respectively.

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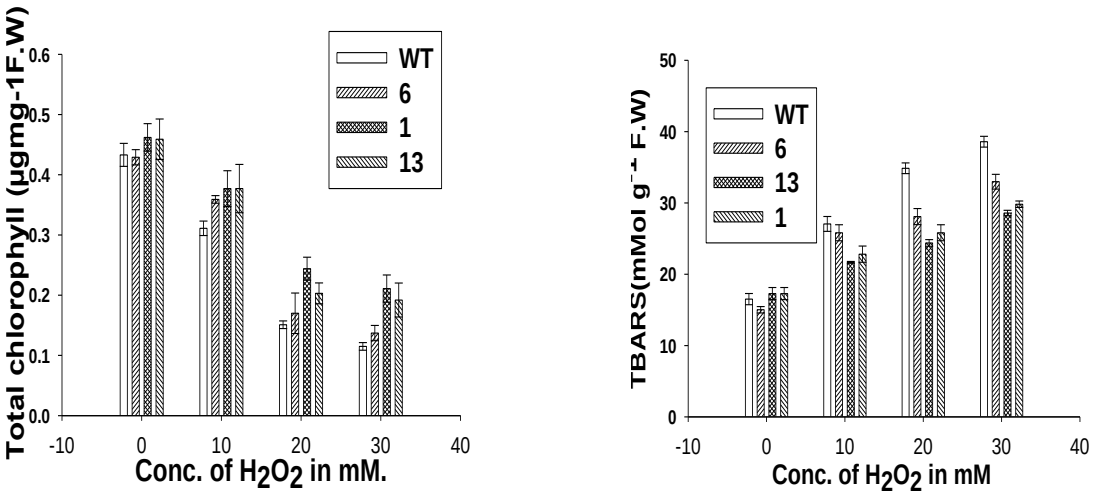

Fig 2: Total chlorophyll and TBARS levels of leaf discs after 4 days of H_2O_2 treatment. Means ± SD was plotted ($p < 0.05$, n = 3).

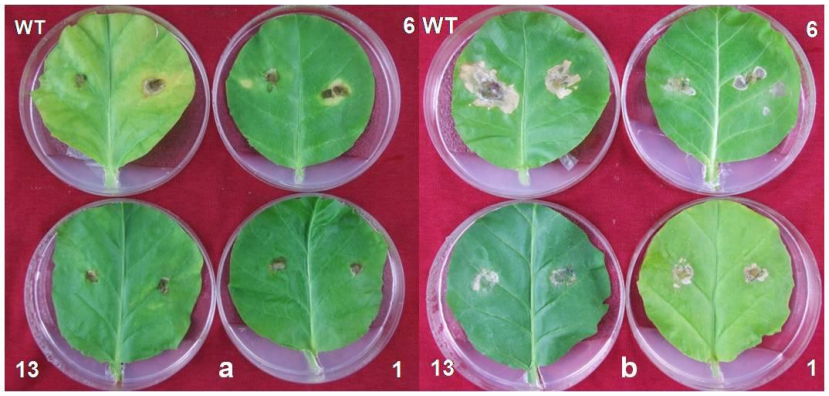

Fig 3: a,bDeatched leaf assay depicting the effect of *A. alternet*aand *R. solani*on*NtPI*transgenics in comparision with the WT.

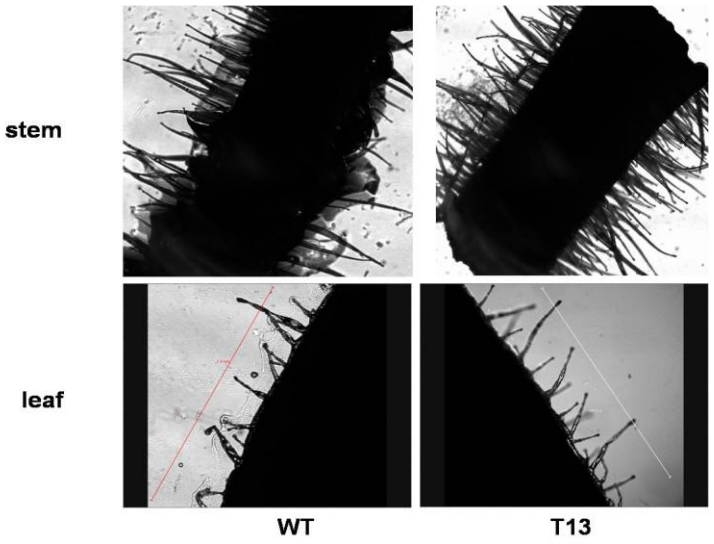

Fig 4: Variation in trichome number on leaf and stem of 40 d old transgenic and WT plants