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ORIGINAL ARTICLE

Expression analysis of proteinase inhibitor-II under *Os*RGLP2 promoter in response to wounding and signaling molecules in transgenic *Nicotiana benthamiana*

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Abstract
Proteinase inhibitor-II (PI-II) genes are important defense related genes that play critical regulatory roles in plant growth and development. In the present study, the expression of tomato *PI-II* gene was investigated under the control of a wound-inducible *Os*RGLP2 (*Oryza sativa* root germin like protein 2) promoter in transgenic tobacco plants after wounding, ABA and MeJA applications. Transcript level of target gene in transgenic plants was confirmed by quantitative real time PCR (qPCR). In response to ABA treatment at different concentrations, *PI-II* gene was strongly induced under *Os*RGLP2 promoter at higher concentration (100 µM), while considerable level of target gene expression was observed with MeJA application at 50 µM concentration. Upon wounding, relatively high *PI-II* gene expression was observed after 36-h treatment. Correspondingly, high *GUS* activity was detected at 36 h with histochemical assay and microscopic analysis in the vascular regions of leaves, stem and roots in wounded transgenic plants. This inducibility of *PI-II* gene by wounding, ABA and MeJA indirectly indicates its role in plant defense mechanism against biotic and abiotic stresses. Moreover, it was also suggested that ABA and MeJA dependent signaling pathways are involved in stimulation of *PI-II* gene. To the best of our knowledge, this is the first report describing the induction of *PI-II* gene under the regulation of *Os*RGLP2 promoter under stress conditions. The results of present research are useful for potential role of *PI-II* gene to improve stress tolerance in transgenic crops. Thus, efficacy of this gene can potentially be exploited to test the responses of different plants to various environmental stresses.

Keywords Proteinase inhibitor-II · Wounding · GUS activity · Signaling pathways · Stress tolerance

Introduction

Usually plants growing in nature are continuously exposed to biotic and abiotic stresses. Plants secure themselves by triggering several defense mechanisms which control or reduce the effects of such stresses. The plant proteinase inhibitors (PIs) are one of the important defensive proteins that play significant role to resist different kinds of environmental stresses. Most of plant PIs work to act against the herbivores and pathogens by interacting the active sites of their target proteases (Kim et al. 2009; Srinivasan et al. 2009), thereby forming a stable inhibitory complex. Some PIs have also been up-regulated in response to abiotic factors such as salt and drought stresses (Pernas et al. 2000; Gaddour et al. 2001; Huang et al. 2007; Rehman et al. 2017a, b). A variety of PIs are known in plants that have been divided into many families or classes. Among these PIs, serine proteinase inhibitors constitute the largest and well-studied family (Haq and Khan 2003), that have been characterized for their crucial role in various physiological and defense processes in plants. The members of this family are universal throughout the plant kingdom and mostly present as storage proteins in seeds and tubers or also accumulate in vegetative organs of plants.

The wound-inducible proteinase inhibitor-II (PI-II) proteins are one of the important members of serine proteinase inhibitors that have been reported to inhibit trypsin,

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chymotrypsin, oryzin, subtilisin, elastase and pronase E (Antcheva et al. 1996). They have been identified and characterized from many Solanaceous plant species including tomato, potato, tobacco and pepper, etc. (Bryant et al. 1976; Plunkett et al. 1982; Keil et al. 1986; Pearce et al. 1988, 1993; Tamhane et al. 2009; Mishra et al. 2012). The expression of *PI-II* genes have been found to be regulated by variety of stresses including wounding and associated signaling molecules. For example, there are many reports on involvement of phytohormones like abscisic acid (ABA) and jasmonic acid (JA) in triggering the local and systemic induction of *PI-II* genes in many plants including tomato, potato, and tobacco (Farmer et al. 1992; Hildmann et al. 1992; Peña-Cortés et al. 1995; Peña-Cortés and Willmitzer 1995; Wasternack and Parthier 1997). This local and systemic induction of inhibitory genes was apparently to occur via octadecanoid (OD) pathway (Koiwa et al. 1997).

Similarly, it has been established that many defensive traits are expressed in plants distal to the site of injury as a result of wounding (Green and Ryan 1972). Previously, it was reported that wounding of tomato and potato leaves has increased the expression level of *PI-II* genes (Pena-Cortes et al. 1988). Moreover, endogenous level of ABA and JA was found to elevate in response to mechanical wounding which in turn induces rapid accumulation of PI-II mRNA. In addition, synthesis of *PI-II* was also reported to be induced by systemin and oligosaccharide elicitors in potato and tomato (Doares et al. 1995). A range of wound-inducible promoters have been identified from many plants, which are involved in regulating the gene expression of many defense related genes under certain stress conditions. *Os*RGLP2 is a robust promoter that could mediate rapid gene responses by several agents, including wounding, salt, dehydration and pathogenic infection in transgenic plants (Mahmood et al. 2013; Munir et al. 2016; Shah et al. 2017). Based on this information, a recombinant construct was designed by ligating a tomato *PI-II* gene downstream to *Os*RGLP2 promoter, which was further investigated for expression analysis in response to wounding, ABA and MeJA treatments after transformation in tobacco plants.

Materials and methods

Selection of plant material and transformation

The tobacco species *Nicotiana benthamiana* was used for stable transformation with Agrobacterium strain *EHA*101 carrying p1391Z_*Os*RGLP2::PI-II vector fused with *GUS* reporter gene using leaf disc method (Horsch et al. 1985).

Confirmation of transgenic plants

DNA extraction from leaves of transgenic plants was carried out using DNeasy Plant Mini Kit. PCR of transgenic plants was done using PI-II and hygromycin resistant gene primers. The sequences of these primers are PI-II F: 5′TAT CCATCATGGCTGTCCAC3′ and PI-IIR: 5′AACACACAA CTTGATCCCCACA3′ and Hygro F: 5′ GCTCCATACAAG CCAACCAC 3′ Hygro R: 5′ CGAAAAGTTCGACAGCGT CTC 3′. For amplification, 25 µL of amplification reaction containing 25 pmol of each primer, 2.5 µL of 10× PCR buffer, 1.5 µL of 25 mM $MgCl_2$, 1.5 µL of 2.0 mM dNTPs, 45 ng/µL of genomic DNA, and 1.5 U Taq polymerase was prepared. Gradient Multigene Thermal Cycler (Labnet) was used to run amplification reaction for 35 cycles of denaturing at 94 °C for 40 s, 55 °C for 40 s, 72 °C for 45 s, and a final extension step at 72 °C for 20 min.

In vitro germination and selection of transgenic seeds

T_0 seeds obtained from wild type (WT) and transgenic plants (TL1, TL2 and TL3) were surface sterilized according to method described by Srinivasan et al. (2009). The sterilized seeds were selected on hygromycin (50 mg/mL) media and maintained in a growth room at 27 °C with a photoperiod of 16:8 light/dark cycles. T_1 progeny was selected on the basis of hygromycin resistance.

ABA and MeJA treatments

For ABA and MeJA stress treatments, ABA and MeJA solutions (10, 50, and 100 µM), were sprayed on 15 days old T_1 transgenic lines (TL1, TL2 and TL3) and wild type (WT). Samples were taken after 24 h of spray and immediately frozen in liquid nitrogen and stored at − 80 °C for RNA extraction. Each experiment was repeated at least three times.

Wounding stress

Wounding stress was applied to the young leaves of T_1 transgenic plants growing on MS media for 12, 24, and 36 h by injuring the leaves with pre-sterilized forceps. Unstressed transgenic plants were used as control. Wounded leaves were collected, frozen in liquid nitrogen and further processed for RNA isolation and qPCR analysis.

Histochemical assay

GUS histochemical analysis was performed on T_1 transgenic seedlings following the procedure of Jefferson (1989).

Briefly, plant tissues were incubated at 37 °C in GUS reaction solution containing 50 mM sodium phosphate buffer (pH 7.0) and 2 mM X-Gluc. After incubation, stained tissues were washed with 70% ethanol to remove the chlorophyll and observations were recorded.

RNA isolation and cDNA synthesis

For RT-PCR, ABA and MeJA and wound treated samples were processed for total RNA isolation using Trizol reagent (invitrogen). For complementary DNA (cDNA) synthesis, 1 µg of DNase-treated RNA was reverse transcribed in a 20 µl reaction using M-MuLV reverse transcriptase along with oligo (dT) primers. The total reaction mixture was incubated for 1 h at 42 °C followed by 70 °C for 10 min to stop the reaction. To check the contamination of genomic DNA, a control was run without the reverse transcriptase enzyme. Quantity and quality of cDNA was confirmed through NanoDrop method and PCR using housekeeping gene (actin) primers. Finally cDNA was stored at − 20 °C until further use.

qPCR

For expression analysis, qPCR was performed with Stratagene Mx3005P QPCR System using ten times diluted cDNA, 1× EvaGreen master mix and gene specific primers: 5′-TTCGGGATATGCCCACGTTC-3′ (forward) and 5′-AGG TGCAAGCATTTGGCCTT-3′ (reverse). The *N. benthamiana* actin primers 5′-GATGAAGATACTCACAGAAAGA-3′ (forward) and 5′-GTGGTTTCATGAATGCCAGCA-3′ (reverse) were used as internal reference. The qPCR programme was carried out in a 40 cycle reaction under the following conditions: 94 °C (30 s), 56 °C (60 s), and 72 °C (10 s). The resultant data were analyzed according to the formula $2^{-\Delta\Delta Ct}$.

Statistical analysis

All the data were subjected to analysis of variance (ANOVA) using a PROC GLM procedure of SAS 9.4. The mean within each treatment were compared using the least significant difference (LSD) test with a threshold probability of $P < 0.01$.

Results

Effect of wounding

To investigate the induction of *PI-II* gene under *Os*RGLP2 promoter in response to wounding, the transgenic plants were wounded at three experimental time periods (12, 24 and 36 h). Overall, transcript level of *PI-II* gene was found to increase after 24 h reaching to maximum level (6.5-fold) at 36 h in wounded transgenic plants (Fig. 1). The present results were also supported by histochemical assay of *GUS* reporter gene under *Os*RGLP2 promoter. The GUS staining revealed that expression pattern of *GUS* gene was similar to that of *PI-II* gene (Fig. 2), and was significantly induced at 36 h in transgenic plants after wounding. *GUS* activity detected by microscopic studies showed strong *GUS* expression in the vascular bundles of leaf, stem and root after wounding. In leaves, *GUS* expression was observed in all parts such as leaf epidermis, guard cells, mesophyll cells, and midrib (Fig. 3). After 12 h of injury, diffused expression was noted in vascular tissues and mesophyll cells of leaf which become intense with the passage of time. In stem, high level of *GUS* activity was detected in vascular bundles and relative low *GUS* expression was observed in outer cells after 36 h of injury (Fig. 4). In roots, microscopic analysis revealed that *GUS* activity was mainly associated with vascular bundles, root epidermal layer, and root hairs. Roots showed prominent *GUS* expression in vascular region than cortex after 24 h. However, *GUS* activity increased after 36 h and become more uniform in cortex as well as in vascular bundles, while low expression was also detected in outer root epidermal layer (Fig. 5).

Induction of PI-II in response to ABA

The relative quantification data showed that *PI-II* gene expression was triggered significantly at higher level under *Os*RGLP2 promoter in three independent transgenic lines (TL1, TL2 and TL3) than WT under three different ABA concentrations (10, 50, and 100 µM) after 24 h (Fig. 6a). After 10 µM ABA treatment, *PI-II* gene was initially

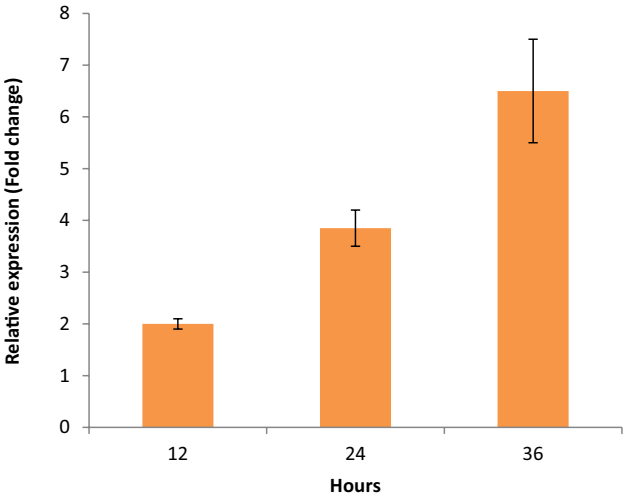

Fig. 1 Expression profile of *PI-II* gene in transgenic plants in response to wounding at different time intervals (12, 24 and 36 h)

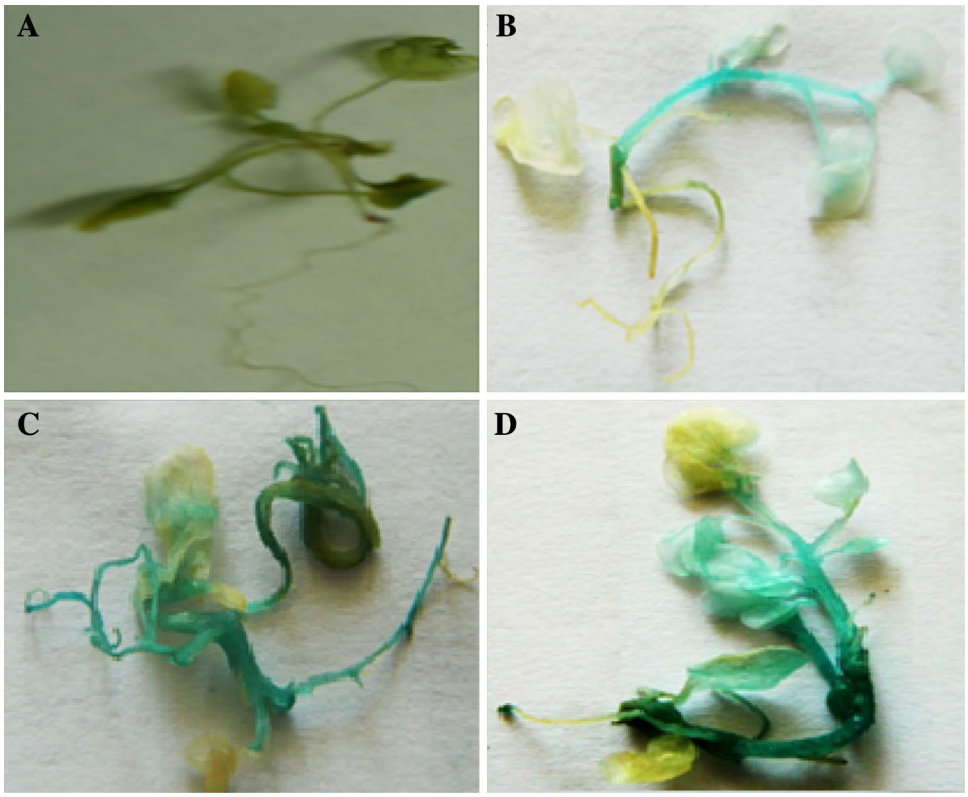

Fig. 2 *GUS* expression of transgenic seedlings in response to wounding. **a** Untreated transgenic seedling as control, **b** *GUS* expression after 12 h, **c** *GUS* expression after 24 h, **d** *GUS* expression after 36 h

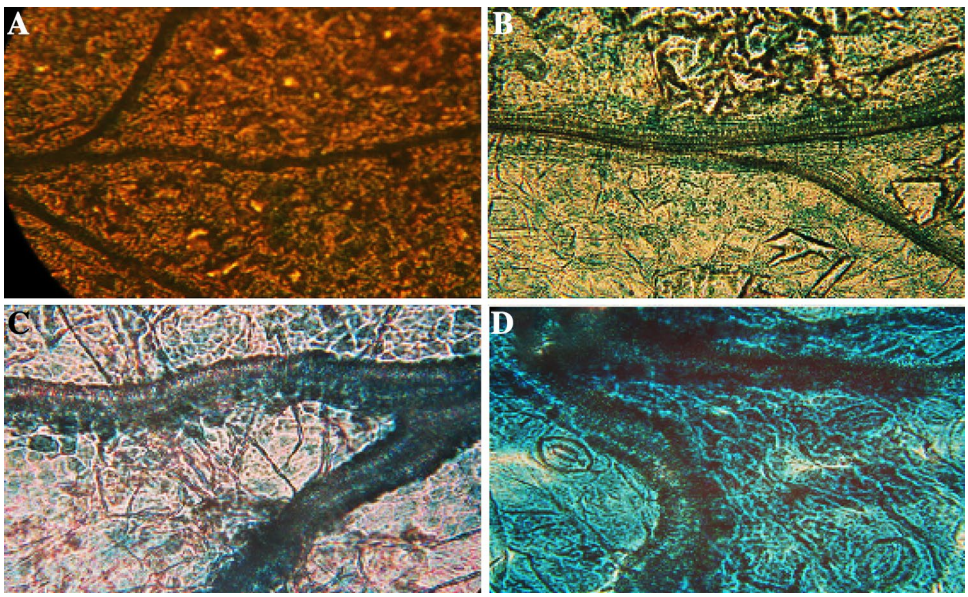

Fig. 3 *GUS* expression of transformed tobacco leaf tissues after wounding. **a** No *GUS* expression in control plant leaf, **b** low diffused *GUS* expression in midrib and surrounding tissues after 12 h, **c**, **d** high *GUS* activity in vascular bundles and stomatal guard cells after 24 and 36 h (magnification, 40×)

induced at very low level in all treated plants. The transcript level was found to increase and differ significantly ($P < 0.01$) between transgenic lines versus WT plants with an increase in ABA concentrations (Table 1). However, transgenic lines displayed comparatively higher expression than WT (Fig. 6a). When treated with 100 µM ABA, TL1, TL2, TL3 showed higher fold change of 9.5, 8.4 and 5.2, respectively, when compared with WT. Moreover, the transcript level of TL1 line was highest among all the other transgenic lines and control plants at all concentrations. The present data indicated that *PI-II* gene is responsive to ABA application which suggested that ABA might play an important role in the induction of *PI-II* gene under abiotic stress conditions.

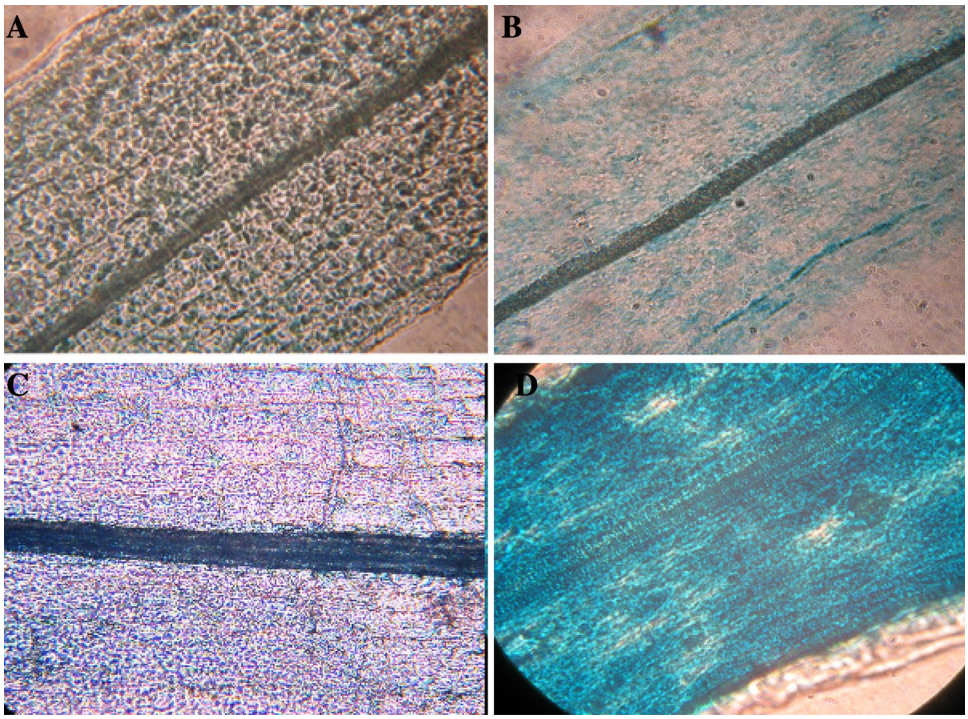

Fig. 4 *GUS* expression of transformed tobacco stem. **a** No *GUS* expression in control plant stem, **b** diffused *GUS* expression in stem 12 h, **c**, **d** *GUS* activity in stem after 24 and 36 h (magnification, 40×)

Induction of PI-II in response to MeJA

To verify the role of MeJA in the induction of *PI-II* gene, the relative expression of target gene was analyzed in selected transgenic lines (TL1, TL2 and TL3) and WT by qPCR. The results showed significant difference in expression level of *PI-II* gene in transgenics and WT following the exogenous MeJA treatment at certain particular concentrations (Fig. 6b; Table 1). Overall, the transcript levels of transgene were transiently induced by MeJA and vary significantly with an increase in concentrations of MeJA in transgenic plants and WT ($P < 0.01$). After increasing the concentration from 10 to 50 µM MeJA, the expression levels of all lines gradually increased that reached to the maximum level at 50 µM MeJA and then declines at 100 µM MeJA. Compared to WT, a significant higher expression level was noted at 50 µM MeJA in all transgenic lines that was about 8.2-fold for TL1, 7.8-fold for TL2 and 4.5 fold for TL3, while 10 µM MeJA and 100 µM MeJA treatments resulted in decreased *PI-II* activity (Fig. 6b). However, transcript levels of all transgenic lines were comparatively higher at 100 µM MeJA than at 10 µM MeJA. Moreover, among all transgenic lines tested, TL1 was highly responsive to MeJA treatment at all concentrations. This transcriptional activation of *PI-II* gene in response to MeJA treatment clearly indicates the positive role of MeJA in the regulation of stress responsive genes.

Discussion

Leaves of transgenic plants were mechanically wounded to evaluate the activity of *Os*RGLP2 promoter in driving the expression of *PI-II* gene. The present results indicated that mechanical wounding has significantly up-regulated the *PI-II* gene expression (up to 2–6.5-fold) in wounded transgenic plants as compared to their non-wounded counterparts (control). This is indicative of wound-specific activity of *Os*RGLP2 promoter which may control the activation of specific defensive genes including *PI-II* gene. Gulbitti-Onarici et al. (2009) reported similar results by constitutive expression of *Cry1Ac* (insecticidal crystal proteins) gene under the control of wound-inducible AoPR1 promoter in transgenic tobacco plants after wounding. RT-PCR analysis further showed that *PI-II* gene is not an early responsive gene as it was induced by wounding over an extended time period (at 36 h) which is in accordance with the observations of Ryan (2000) and Meyer et al. (2016), suggesting the possible role of *PI-II* gene in late wound signaling pathway and contribution in plant defense against herbivores and pathogens. This notion was also supported by experimental data in transgenic plants over expressing the potato and nightshade *PI-II* genes that resulted in enhanced resistance against *Helicoverpa armigera* and *Spodoptera litura* (Luo et al. 2009; Majeed et al. 2011). Consistent with *PI-II* quantitative data, the transgenic plants showed strong *GUS* expression after 36 h in vascular bundles of leaves, stems and roots by microscopic

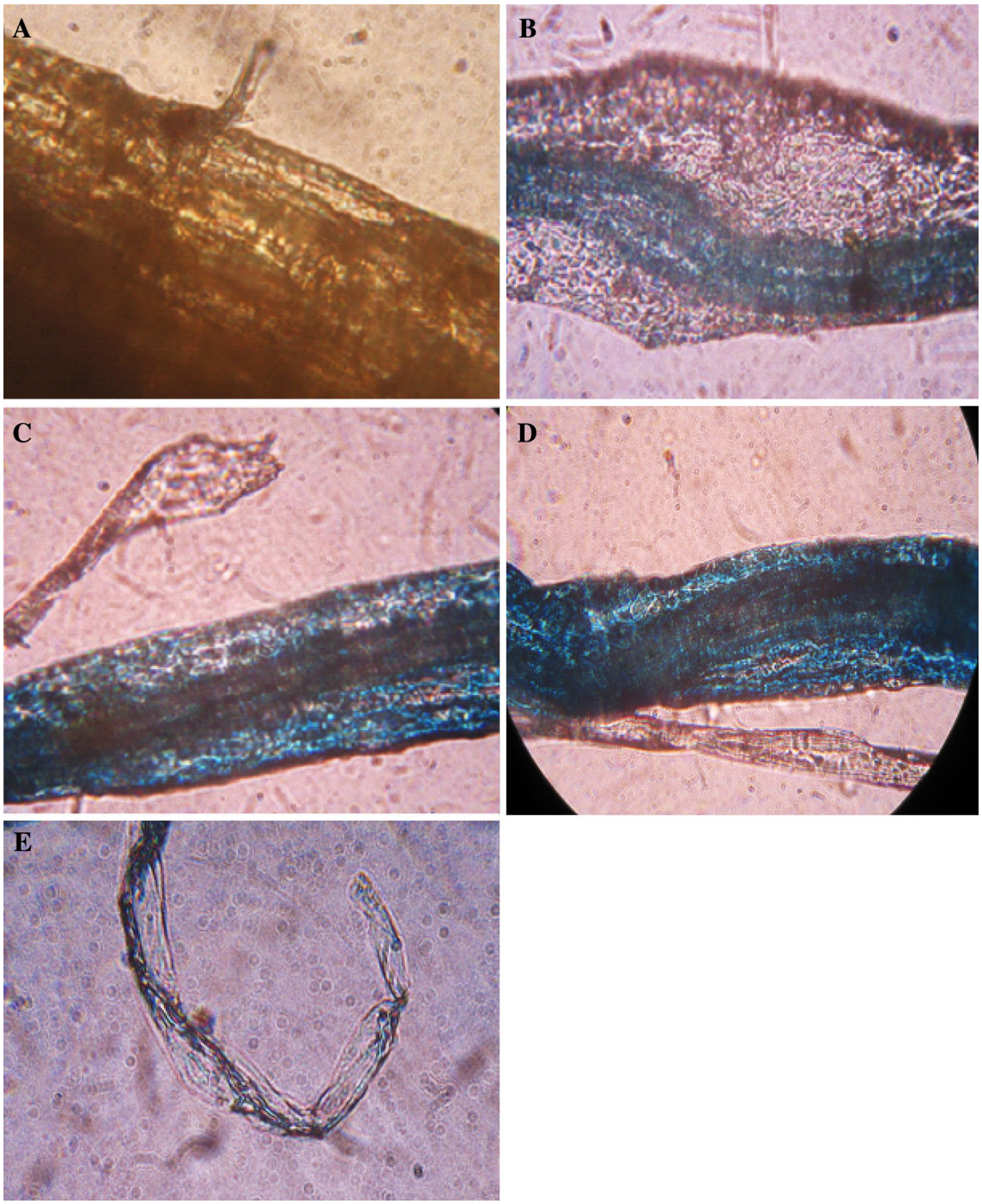

Fig. 5 *GUS* expression of transformed tobacco root. **a** No *GUS* expression in control plant root, **b** *GUS* activity in root showing prominent expression in vascular tissues after 12 h, **c**, **d** *GUS* activity in root after 24 and 36 h, **e** slight *GUS* expression in root hair (magnification, 40×)

analysis which may suggest that wounding signals are transported through vascular bundles (Keil et al. 1989; Xu et al. 1993). Such microscopic observations with *GUS* gene were also reported in both wounded leaves and roots of transgenic dicot plants transformed with pin2/Act1 intron/GUS construct (Keil et al. 1989) and in the leaves of transgenic nightshade plants expressing *SaPIN2b:GUS* construct (Liu et al. 2006) after 24 h of wounding. Collectively, these results indicated that *PI-II* gene was constitutively expressed under *Os*RGLP2 promoter and is considered as a part of defense mechanism in plants due to its induction by wound and herbivore predators.

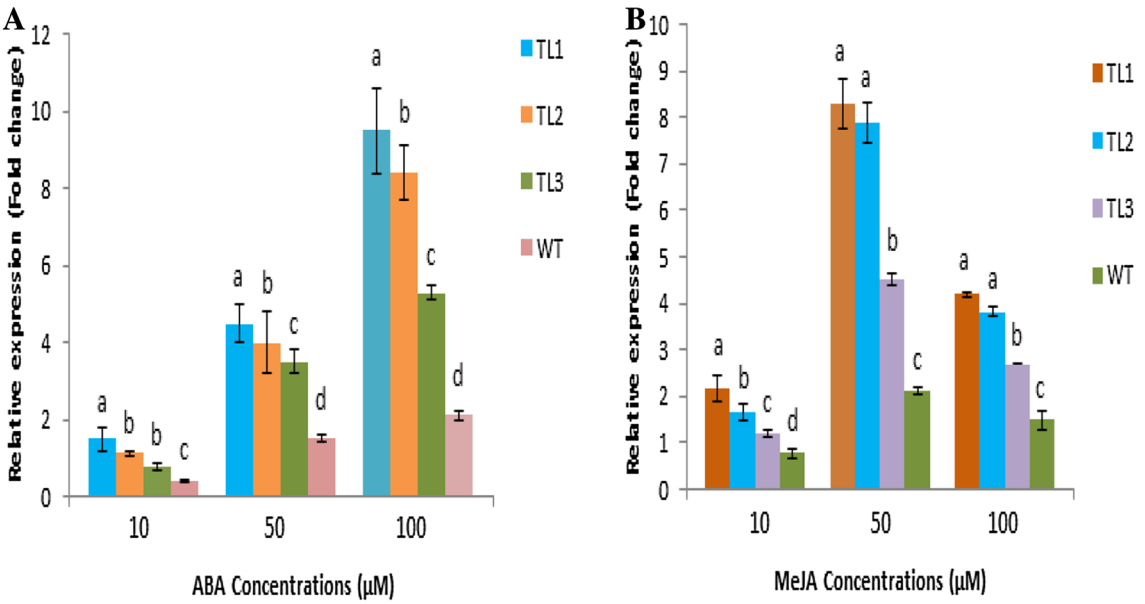

Fig. 6 **a** Expression profile of *PI-II* gene in transgenic lines and WT in response to ABA treatment with different concentrations. The data are the mean ± SE of three replicates ($n = 3$). The letters on each bar within each treatment indicate the significant differences at $P < 0.01$, and bars sharing a common letter are not significantly different. **b** Expression analysis of *PI-II* gene in transgenic lines and WT in response to MeJA treatment with different concentrations. The data are the mean ± SE of three replicates ($n = 3$). The letters on each bar within each treatment indicate the significant differences at $P < 0.01$, and bars sharing a common letter are not significantly different

Table 1 Difference in *PI-II* gene expression level under different concentrations of ABA and MeJA treatments in transgenic lines and WT

5	Lines	Expression level at 10 µM concentration (mean ± SE)	Expression level at 50 µM concentration (mean ± SE)	Expression level at 100 µM concentration (mean ± SE)
ABA	TL1	1.5 ± 0.3^{a}	4.5 ± 0.5^{a}	9.5 ± 1.11^{a}
	TL2	1.14 ± 0.06^{b}	5.3 ± 0.8^{b}	8.4 ± 0.7^{b}
	TL3	0.79 ± 0.09^{b}	3.5 ± 0.3^{c}	5.3 ± 0.2^{c}
	W.T	0.4 ± 0.03^{c}	1.5 ± 0.1^{d}	2.1 ± 0.11^{d}
MeJA	TL1	2.17 ± 0.29^{a}	8.28 ± 0.55^{a}	4.2 ± 0.06^{a}
	TL2	1.67 ± 0.19^{b}	7.89 ± 0.45^{a}	3.82 ± 0.1^{a}
	TL3	1.18 ± 0.08^{c}	4.5 ± 0.12^{b}	2.7 ± 0.01^{b}
	W. T	0.79 ± 0.1^{d}	2.1 ± 0.07^{c}	1.5 ± 0.2^{c}

The mean with different letter in each column are significantly different at $P < 0.01$ and mean with same letter are not significantly different

± Standard mean of error

The plant hormone, ABA serves as significant signaling molecule that is critical for growth and development of plants, and provides adaptations to wide range of stresses like drought, salinity and cold (Shinozaki and Yamaguchi-Shinozaki 2000; Sah et al. 2016; Ge et al. 2017). With regard to ABA treatment, transgenic plants showed strong *PI-II* gene expression under *Os*RGLP2 promoter than the WT at higher concentration (100 µM) signifying its major role in plants during abiotic stress conditions. This suggests that some critical part of *Os*RGLP2 promoter region contain ABA responsive elements with possible role in directing the expression of *PI-II* gene through ABA signaling under abiotic stress condition. These results correlate well with earlier studies in which an increase in PI-II mRNA level was reported in potato leaves and stem after applying 100 µM ABA to leaves (Pena-Cortes et al. 1989, 1995). In another report, Kim et al. (2001) observed elevated *CaPI2* expression in pepper after 12 h of ABA treatment (50 µM). Similarly, the transcript levels of cysteine proteinase inhibitors from *Panax ginseng* (*PgCPI*) (Jung et al. 2010) and *Glycine soja* (*GsCPI14)* (Sun et al. 2014) were strongly up-regulated by ABA treatment. In a related study, Srinivasan et al. (2009) showed higher transcript level in transgenic tobacco plants over expressing the *N. benthamiana* trypsin inhibitor gene

(*NtPI*) under the regulation of CAM35S promoter in the presence of ABA treatment. In accordance with the previous results, the present data indicated that target gene expression was significantly up-regulated under *Os*RGLP2 promoter in response to ABA stress treatment.

The potential role of JA or MeJA in regulating the expression of proteinase inhibitor genes has been the subject of intense research which has been reviewed in many studies (Sivasankar et al. 2000; Sun et al. 2011; Larrieu and Vernoux 2016; Rehman et al. 2017b). Our results obtained by qPCR data revealed that expression of *PI-II* gene driven by *Os*RGLP2 promoter was induced at considerable level in transgenic plants at 24 h of post treatment of MeJA. These findings suggest that induction of *PI-II* gene is dependent on JA-mediated signaling pathway and can be used to increase plant resistance against biotic stresses. Moreover, cis-acting elements present within the *Os*RGLP2 promoter are involved in mediating the JA-signaling system which might play significant role in up-regulating the *PI-II* gene. Earlier studies concluded that MeJA or JA application has strongly induced the wound-inducible proteinase inhibitors I and II (PI-I, PI-II) in potato, tobacco and alfalfa (Farmer et al. 1992; Peña-Cortés et al. 1995). In a similar study, a marked increase in *Pin2-GUS* gene expression was detected in transgenic *Solanum brevidens* under CaMV35S promoter after 50 µM MeJA treatment (Liu et al. 1996). Moreover, Tian et al. (2014) observed that MeJA has significantly induced the PIN2 gene in def1 (defenseless) mutants and wild type which indicates that MeJA plays an important role in PIN2 induction. Similarly, exogenous application of MeJA (100 µM) to tobacco leaves has increased the transcript level of trypsin inhibitor (*NtPI*) after 24 h (Srinivasan et al. 2009). All these reports clearly indicate that MeJA has positive role in the regulation of proteinase inhibitor genes.

Conclusion

The present work revealed that expression of *PI-II* gene under *Os*RGLP2 promoter was highly up-regulated by mobile wound signals and signaling molecules like ABA and MeJA that are primarily effective for abiotic and biotic responses. These results also provide an evidence for the involvement of signaling cascade-like events in the regulation of inhibitory genes in plants growing under stress conditions. Moreover, fusion of the *Os*RGLP2 promoter to a defense related genes like *PI-II* gene is an effective strategy for engineering crops to cope various forms of environmental stresses. However, further studies are still required for elucidating the mechanism and specific physiological function of *PI-II* gene in response to multiple stresses. In addition, much effort should be focused on multiple signaling molecules to uncover the several features of *PI-II* gene in plant growth and survival. Conclusively, *PI-II* gene is a potential candidate gene for developing transgenic crops tolerant to both biotic and abiotic stresses.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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