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Characterization of Tomato Proteinase Inhibitor-II Gene in Response to Salicylic Acid and H_2O_2 Signaling Molecules in Transgenic Tobacco

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ABSTRACT

Proteinase Inhibitors (PIs) are distributed throughout the plant kingdom. They are characterized as natural plant defense proteins as their genes are induced by biotic and abiotic stresses and other stress prone circumstances (insect mastication, mechanical injuries, pathogen infestation, and drought and UV exposure). Expression of such stress responsive genes can be modulated with the help of signaling molecules. In this study, transgenic tobacco (*Nicotiana benthamiana*) generated through leaf disc method harboring *PI-II* gene under OsRGLP2 promoter. The effect of Hydrogen Peroxide (H_2O_2) and Salicylic Acid (SA) were checked on *PI-II* gene expression in transgenic *N. benthamiana*. The maximum 10.4 fold induction was observed with 20 mM H_2O_2 and 8.05-fold with 15 mM SA. This H_2O_2 and SA signaling inducibility of *PI-II* is a strong indicative of its role in plant defense against abiotic stresses through SA and H_2O_2 dependent pathways. These results can be useful in studying a possible potential of *PI-II* gene to improve abiotic stress tolerance in plants.

Keywords: Proteinase inhibitor-II; OsRGLP2 promoter; Hydrogen peroxide; Salicylic acid; Expression; Abiotic stress

INTRODUCTION

PIs are naturally occurring proteins which are responsible for the inhibition of proteases. PIs have been characterized in microorganisms, animals and plants with a large number from storage organs of plants [1,2]. PIs families consist of serine PIs (serpin), kunitz type, bowman-birk and cysteine PIs (cystatins) [3]. The most widespread are serine PIs that are abundant in rhizomes, leaves and seeds of a large number of members of the Fabaceae, Solanaceae and Poaceae [4]. The plant's defense against insects [5], bacterial and viral toxicities [6], fungal pathogens [7], as well as definite abiotic stresses [8] has been reported due to potential involvement of plant *PI-II* gene. Solanaceae is the only family, from which *PI-II* members

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have been reported [9]. It has been reported that PI-IIa and PI-IIb regulate the progressive Programmed Cell Death (PCD) and floral development [10]. Moreover, the metabolic rates and protein turnover is regulated *via* PIs to adjust the endogenous level of proteinases before and after seed germination [11]. It has been revealed that *PI-II* gene responds to system in, methyl jasmonate, polyethylene glycol, salt, Abscisic Acid (ABA), cold stress and electric current in numerous Solanaceae plants like *Nicotiana*, *Capsicum annum* and *Solanum* sp. [12]. In was also reported that the *PI-II* gene is induced by wounding in tomato (*S. esculentum* L.) [13]. While chymotrypsin, trypsin, elastase, pronase, oryzin and subtilisin are inhibited by *PI-II* gene [14]. Their maximum abundance has been observed in tobacco (*N. naalata*) flowers and tomato (*S. lycopersicum*) [15, 16].

Abiotic stress conditions induce ABA in plants while SA, Jasmonic Acid (JA) and ethylene are specially synthesized in biotic stresses [17]. In transgenic plants, PIs were found effective against abiotic factors explained by many research groups [18]. The level of many secondary metabolites was elevated in plants with SA and JA treatments [19]. Many physiological activities like respiration, cell development, stomata opening, seed propagation, seedlings growth and high temperature tolerance were signaled by SA in plants [20]. H_2O_2 mediates a suitable response to many stresses, a part of signaling molecules in plants which regulates the down-stream gene expression during stress. The gene expression in plants is regulated through signal transduction pathway which can be tested by exogenous application of such signals leading to the stimulation or suppression of down-stream gene expression. *PI-II* gene is expressed in response to exogenous application of some signaling molecules in transgenic plants. Therefore, current study was carried out to observe the expression of *PI-II* gene (ligated downstream to OsRGLP2 promoter) in response to some signaling molecules (H_2O_2 and SA) in transgenic tobacco plant (*N. benthamiana*).

MATERIALS AND METHODS

For the present study, seeds of T1 transgenic *N. benthamiana* plants developed through *Agrobacterium* mediated transformation harboring *PI-II* gene were used. In these plants the target *PI-II* gene was ligated to downstream of OsRGLP2 promoter in a pCAMBIA vector.

Plant Material

Transgenic seeds of *N. benthamiana* were sterilized, dried and spread on solid MS media for germination. Petri plates containing sterilized seeds were placed in growth chamber at conditions of 16/8 light and dark cycle at 25°C. Seeds began to germinate within a week and mature plants were obtained after 40 days which were multiplied using nodes and internodes. The well-established mature plants with proper vigor were used for expression analysis.

Expression Analysis

Expression analysis of *PI-II* gene was done by applying signaling molecules (SA and H_2O_2) on transgenic *N. benthamiana* through qRT-PCR. The fresh plants were used for each treatment while untreated transgenic plants were used as control.

H_2O_2 Stress

For experiment related to oxidative stress, two months old transgenic plants were shifted to solid MS media containing different concentrations of H_2O_2 (10 mM, 15 mM, and 20 mM) and plants were analyzed for expression analysis after different time intervals (6 h, 12 h and 24 h). Leaves were collected after 6, 12 and 24 h from each treatment and immediately stored in liquid nitrogen for total RNA isolation which was used for later experimentation.

SA Stress

Two months old transgenic plants were shifted to solid MS media containing 10 mM, 15 mM and 20 mM of SA. The leaves were collected from stressed plants after 6 h, 12 h and 24 h for each treatment and preserved in liquid nitrogen for further studies.

RNA Extraction and cDNA Synthesis

The harvested leaf tissue (100 mg) from untreated transgenic plants (control) and stressed transgenic plants with H_2O_2 and SA, was homogenized by grinding in liquid nitrogen. Afterwards, total RNA was extracted by manual method using DNase (RQ1 RNase-free DNase; Promaga M6101). The quality and quantity of isolated RNA was confirmed by Nanodrop (Nanodrop 1000 spectrophotometer, Thermoscientific). The cDNA was synthesized by adding Oligo (dT)18 primer 25 pM (Gene Link TM: e-oligos) and revert aid reverse transcriptase (15 U) using 1 µg of total RNA and cDNA samples were stored at -80°C.

Quantitative Real Time PCR (qRT-PCR)

Quantitative PCR was performed for each stress to analyze the expression of *PI-II* gene in comparison to house-keeping gene. The whole procedure comprised of the following steps; Using the MyGo-Pro RT-PCR System, 'SYBR' was used as a reporter dye. The total reaction volume was 12 µl comprising 2 µl cDNA (1:5 dilution), 6 µl (Invitrogen) SYBR Green master mix, 2 µl (Forward and Reverse) Primer mix (25 pM) and 2 µl Nuclease free water. A particular set of primers for gene of interest (*PI-II*) and housekeeping gene (Actin) were used. The RT-PCR reaction was carried with pre-denaturation at 95°C for 10 minutes, 40 cycles of denaturation at 95 °C for 10 seconds, annealing at 57.3°C for 45 seconds and extension at 72°C for 15 seconds steps.

Data Analysis

In terms of *PI-II* gene expression, raw data was collected for qRT-PCR data analysis. Mathematical model CT (2-ΔΔCT) using

MyGo-Pro qRT-PCR software was used to analyze the data. For 2-ΔΔCT method, an internal control and calibrator was selected. In each reaction, the level of *PI-II* transcript driven by OsRGLP2 promoter as compared to control (untreated) was varied significantly by relative quantification of the samples. The fold change was calculated, and the graphical form of data generated in response to each stress.

RESULTS

Transgenic tobacco plants were used to check the expression of *PI-II* gene in response to H_2O_2 and SA.

Induction of *PI-II* gene in Response to H_2O_2

To evaluate the expression of *PI-II* gene, T1 transgenic plants were subjected to H_2O_2 stress. Overall the *PI-II* gene activity was found highly responsive with 20 mM H_2O_2 after 24 hours. The *PI-II* gene response showed an increase trend with the increase of H_2O_2 concentration. At 20 mM H_2O_2 after 6 hours, the induction of *PI-II* gene increased up to 7.36 fold and reached the maximum 10.4 fold after 24 hours. Furthermore, with the increase of H_2O_2 concentration (10 mM-20 mM), the *PI-II* gene expression also increased and reached to maximum at 20 mM. While the high expression (10.4 fold) was observed at 15 mM after 24 while with 10 mM the expression was not significant rather remained almost same near to 7 fold (**Figure 1**). It was observed that an overall increase in expression pattern of *PI-II* gene in response to H_2O_2 was remarkable.

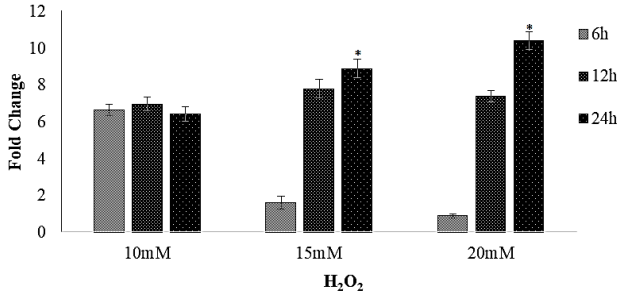

Figure 1: Quantification of *PI-II* gene in response to 10, 15 and 20 mM H_2O_2 stress treatment. One-month old T1 transgenic Nicotiana lines were subjected to 10, 15 and 20 mM H_2O_2 growing on MS media and real-time PCR was performed after 6, 12 and 24 h to detect fold induction of *PI-II* gene. Figure data is mean ± of three independent readings (P<0.01, n=3).

Induction of *PI-II* Gene in Response to SA

Different concentrations of SA (10, 15 and 20 mM) were used to check the expression profile of *PI-II* gene which was found to be up-regulated up to maximum 8.05 fold with 15mM SA treatment (**Figure 2**). At 20 mM SA, the maximum induction of *PI-II* (6.9 fold) was significant after 12 h. Unlike H_2O_2, lower level of *PI-II* gene expression was observed with 10 mM SA treatment. The OsRGLP2 showed a response to SA signaling.

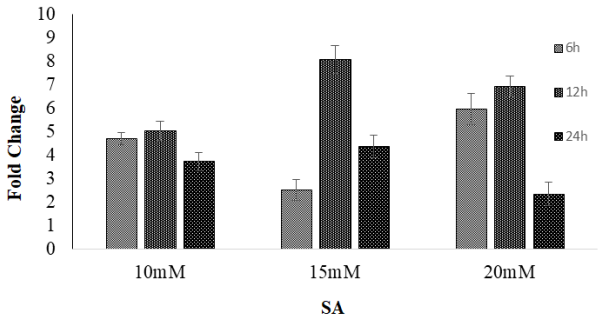

Figure 2: RT-PCR analysis of *PI-II* gene expression in response to 10, 15 and 20-mM SA application. Quantitative real-time PCR was carried out to detect fold induction of *PI-II* gene in one-month old T1 transgenic Nicotiana lines growing on MS media. Figure data is mean ± of three independent readings (P<0.01, n=3).

DISCUSSION

PIs are natural defense proteins and various signaling molecules such as JA, SA, ABA and ethylene are related to defense responses against phytopathogens, abiotic stresses and regulation of developmental stages, senescence and fertility. Both H_2O_2 and SA involve in Hypersensitive Response (HR), Systemic Acquired Response (SAR) and PAMP-Triggered Immunity (PTI) in plants. SA is categorized as biotic stress-responsive hormone in biochemical reactions and physiological responses. H_2O_2 is highly responsive to Pathogen-Associated Molecular Patterns (PAMPs), a part of recognition of pathogen infection as well as stable component of Reactive Oxygen Species (ROS). In the present study, the expression of *PI-II* gene in response to H_2O_2 and SA in T1 transgenic tobacco indicated that *PI-II* gene is highly responsive to these signaling molecules. The PIs expression was enhanced by abiotic stress factors such as ABA and H_2O_2. PIs induced defense related genes in excised tomato treated with glucose oxidase-glucose (G/GO), a H_2O_2 generating system, which contributes a continuous production of H_2O_2 within the applets leading to 80% accumulation of *PI-I* within 2 hour. H_2O_2 in bacterial pathogen infestation induced a plasma membrane intrinsic gene (At PIP1; 4) in *A. thaliana* leaf aquaporin. It was demonstrated that H_2O_2 may lead to enhance tolerance against pathogens, high temperature and oxidative stress in wild tomato (*Solanum pennellii*) cells. Furthermore, H_2O_2 also regulate the plant defense and HR related genes.

In transgenic tobacco, the *PI-II* expression after 6, 12 and 24 h with 10, 15 and 20 mM H_2O_2 concentrations which is in accordance with *Arabidopsis* suspension cultures in which 10 mM H_2O_2 resulted in cell death after 6 h reaching the maximum with 20 mM H_2O_2 after 24 h. Similarly, the Vacuolar Processing Enzymes Cysteine Proteinase (γVPECP) expression in *Arabidopsis* suspension cells was also increased with H_2O_2 exposure causing Programmed Cell Death (PCD) with higher *PIs* expression. Plant defense system produces GST (Glutathione S-transferase) mRNA in response of PCD which

initiates defense mechanism by inducing the GSH (Glutathione) and ROS (H_2O_2, O_2.) with maximum expression of defensive gene after 6 h and 24 h which is in consistent with the present up-regulation of *PI-II* gene in response to H_2O_2. The increased expression of *Glycine soja* cysteine (GsCPI14), *Panax ginseng* Cysteine (PgCPI) PI and *Capsicum annum* (CaPI-II) PI were observed against abiotic stresses.

In the present study, *PI-II* gene expression after 6 hours at 10 mM H_2O_2 which is similar to NtPI up-regulation with same concentration of H_2O_2. Salinity tolerance is also induced by activation of enzymatic ant oxidative defense system by exogenous application of H_2O_2. Alscher, Erturk reported that plant protection from superoxide, anion toxicity under oxidative stress and Superoxide Dismutase (SOD) activity plays a vital role through dismutation of superoxide to H_2O_2 and O_2. The oxidative stress is controlled by SOD in peroxisomes, chloroplast and mitochondria organelles. For cell homeostasis, the control of H_2O_2 concentration is crucial in plants.

The SA perception in plants directly interacts with Pathogens Related (PR) defensive gene activation. In tomato and potato plants, both the HR and subsequent expression of SAR are required SA. In the current study, the *PI-II* gene was expressed the maximum after 12 h and then declined after 24 h because at very specific concentration of 20 mM SA. This up-regulation of *PI-II* gene may occur due to the presence of some specific cis-regulatory elements in OsRGLP2 promoter. Exogenous SA also mediates ROS production enhancing the plant defense mechanism.

According to Miura and Tada, during plant development under abiotic stress such as chilling, heat, drought and salt stresses, SA being a phenolic compound confers a significant part. SA induced a variety of *PR* genes responsible for plant defense and in SA-dependent defense signaling, *PR1a* expression is often used as a reporter. In mature tobacco leaves and *Gossypium hirsutum*, the acidic *PR-1* genes were identified and found highly expressed by SA after 12 h also a case in the present *PI-II* gene expression after 12 h of SA application at 15 mM.

Furthermore, *S. lycopersicum* Mitogen-Activated Protein Kinase (*SlMAPK3*) in immune responses were regulated by SA and Methyl Jasmonic Acid (MeJA). This *SlMAPK3* expression by exogenous SA and MeJ increased the level of defense-related genes (*PR1, PR1b, SlPI-I* and *SlPI-II*) after 12 hours which is in agreement with the present higher *PI-II* expression with SA after 12 h.

CONCLUSION

In the present study, the exogenous application of H_2O_2 and SA to T1 transgenic tobacco (*N. benthamiana*) increased the expression profile of *PI-II* gene showing that *PI-II* gene plays key role in plant defense against abiotic stresses. These results can be used for potential understanding of plant responses to such signaling molecules. Conclusively, the *PI-II* is a defense-related gene that can be tested for developing transgenic crops tolerant to abiotic stresses.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR'S CONTRIBUTION

TM conceived and designed research. NKB conducted experiments. NKB, WA, and SR and analyzed data, prepared figures and wrote the manuscript. SLS revised the manuscript. All authors read and approved the manuscript.

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