RESEARCH PAPER

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Involvement of protein kinases in induction of acidic isopolyphenol oxidases in cell walls of tomato by fruit extracts of *Azadirachta indica* A. Juss

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Polyphenoloxidases have a defensive role against biotic stress. Neem fruit extract induces signaling pathway leading to defense response. The inhibitors of tyrosine protein kinases (Lavendustin A), protein serine/threonine kinases (Staurosporine), and protein kinase (K-252a) were utilized to study the role of protein kinase, as components of signaling leading to systemic acquired resistance by neem fruit extract in tomato plants. K252a-sensitive protein phosphorylation is involved in induction of signal transduction leading to appearance of a novel acidic isopolyphenoloxidases (Rf 0.71) ionically bound to cell walls of tomato plants treated with neem fruit extract. Physical pathogen barrier could be generated as a result of cross-linking of oxidized phenolics as well as due to oxidative cross-linking of cell wall proteins and enhanced lignin formation due to enhanced PPO activity. The cell wall bound acidic isopolyphenoloxidases which have a net positive charge may immobilize pathogens by interacting ionically with the negatively charged surfaces of plant pathogens.

Key words : Cell wall proteins, Isopolyphenol oxidases, K-252a, Lavendustin A, Protein kinase, Staurosporine

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INTRODUCTION

Polyphenol oxidases (PPO) have been implicated a role in the phenylpropanoid pathway (Kojima and Takeuchi, 1989) and has a defensive role since it is conspicuously appeared upon wounding, pathogen infection or insect infestation, and due to the inducibility of PPO in response to various abiotic and biotic injuries or signaling molecules (Mayer and Harel, 1979; Constabel *et al*., 1995; Thipyapong and Steffens, 1997; Maki and Morohashi, 2006). PPO acts in disease resistance by hydroxylizing monophenols to o-diphenols and oxidizing these compounds to quiñones, which are often more toxic to the microorganisms than the original phenolic compounds (Gandia-Herrero *et al*., 2005). PPO is multifunctional enzyme that can prevent biological and chemical attacks by raising physical barriers or by counterattacking a pathogen with a high production of free radicals (Passardi *et al*., 2005). In resistant varieties of grapevine, there is not only a higher basal activity of PPO, but also a rapid increase in their activity after pathogen inoculation (Kortekamp and Zyprian, 2003). The induction of a PPO isoform in tomatoes susceptible to *Pseudomonas syringae* pv. *tomato* and *Alternaría solani* suggests that this enzyme plays a role in disease resistance (Thipyapong and Steffens, 1997). Root tissue of the tolerant Goldfinger banana responds to the fungal elicitor through deposition of lignin, preceeded by the induction or activation of the enzyme activities involved in cell wall strengthening i.e. phenylalanine ammonia lyase (PAL), peroxidase (POX) and PPO (De Ascensao and Dubery, 2000). Lignin is highly resistant to attack by microorganisms, and lignified cell walls are an effective barrier to pathogen entrance and spread (Ride, 1983).

The development of an effective systemic acquired resistance (SAR) in any plant system relies, not only in their gene composition and expression, but also on an effective and rapid transduction of signal. Reversible protein phosphorylation is a key mechanism for intracellular signal transduction in eukaryotic cells. Plants harbour histidine and aspartate kinases as part of the two-component signaling system. Ser/Thr-kinases are also abundant and implicated in many signaling events, but classical Tyr-kinases are less well known in plants. It is

suggested that dual-specificity kinases called as serine/threonine/tyrosine (STY) protein kinase have a relatively high tyrosine phosphorylating activity and are responsible for the observed Tyr-phosphorylation activities in plants (Hirayama and Oka, 1992; Mayrose *et al*., 2004; Rudrabhatla and Rajasekharan, 2004). Staurosporine has also been used to establish the participation of protein kinase C (PKC)-like proteins in potato (Yoshioka *et al*, 2001). Staurosporine effect indicates that a serine/threonine kinase is involved in the signaling cascade of ascorbate-modulated redox regulation of 2-Cys peroxiredoxins (heme-free peroxidases) expression (Horling *et al*., 2001). Bach and Seitz (1997) observed the blocking of PAL and peroxidase activities by staurosporine. Grosskopf *et al.* (1990) observed the blocking of PAL induction and ethylene biosynthesis in tomato cell cultures using K-252a, a known inhibitor of mammalian protein kinases. Ortega *et al.*(2002) observed 55 per cent reduction of PAL induction in lemon seedlings treated with 7.5 nM lavendustin five minutes before inoculation with *Alternaria alternata* suggesting that a protein tyrosine kinase could be involved in signal transduction in the lemon system. Earlier work in our lab demonstrated that fruit extract *Azadirachta indica* A. Juss. (neem) induces SAR which results in *de novo* enhancement of PAL, PPO, POX and isoenzymes of PPO and POX in tomato plants against *Pseudomonas syringae* pv *tomato* (Bhuvaneswari *et al.,* 2009). A number of studies have shown that PPO and its isoform clearly plays a role in the resistance of tomato to *P. syringae* upon elicitor treatment (Thipyapong *et al*., 2004) but no data are available on the effects of the elicitor on specific cell wall proteins, in particular on signalling mechanisms related to these processes. In order to understand the mechanism by which neem fruit extract protects the tomato plant from pathogen attack through SAR induction, it is important to know the involvement of protein kinases in the associated signaling processes. The present aim was to study the effect of inhibitors of tyrosine protein kinases (Lavendustin A), protein serine/threonine kinases (Staurosporine), and protein kinase (K-252a) on isoforms of ionically bound acidic cell wall PPO in neem fruit extract treated tomato plants. The data is expected to reveal the involvement of kinases in the signaling cascade induced by fruit extracts of *A.indica* A.Juss.

RESEARCH METHODOLOGY

All the chemicals used were of analytical grade. This experiment was conducted in 2011.

Sowing of seeds:

Seeds of F_1 hybrid (omni 2525) variety of *L. esculentum* were sown in soil rite in plastic trays (35 cm x 25 cm x 6 cm; L x W x H). Plants were raised in sterile culture room at 25±1°C with a relative humidity (RH) of 70% and 12 h photoperiod. Trays were watered daily with sterile distilled water and once a week with 100 per cent sterilised Hoagland's nutrient solution. Seven week old seedlings were used for this study.

Preparation of neem fruit extract:

About 5g of fully mature but green neem fruits (fruit coat green but seed hard) were washed twice with sterilized distilled water and dried under aseptic condition. Seeds were macerated in 20 ml of sterilized type I water in a pre-chilled sterilized pestle and mortar. The extract thus obtained was filtered through four folds of muslin cloth then centrifuged at 8000 xg at 4°C for 30 min. The supernatant obtained was filtered through 0.45 µm membrane filter and used as neem fruit extract.

Pretreatment with pathway inhibitor before neem treatment in tomato :

Experiments were conducted in a completely randomised fashion. Eight groups of plants having 3 replicates each were treated as following. Each replicate had 52 tomato plants. First group of plants were sprayed with 0.1% dimethyl sulphoxide (DMSO) and used as control. Second group of plants were pre-treated with staurosporine (2 mM in 0.1% DMSO) 5minutes before spraying with sterile type I water and designated as staurosporine control plants. Third group of plants were pre-treated with lavendustin A (7.5 nM in 0.1% DMSO) 5minutes before spraying with sterile type I water and designated as lavendustin A control plants. Fourth group of plants were pre-treated with K-252a (2 mM in 0.1% DMSO) 5minutes before spraying with sterile type I water and designated as K-252a control plants. Fifth group of plants were sprayed with neem fruit extract only and designated as neem fruit extract treated plants. Sixth group of plants were pre-treated with staurosporine (2 mM in 0.1% DMSO) 5minutes before spraying with neem fruit extract and designated as staurosporine neem treated plants. Seventh group of plants were pre-treated with lavendustin A (7.5 nM in 0.1% DMSO) 5minutes before spraying with neem fruit extract and designated as lavendustin A neem treated plants. Eighth group of plants were pre-treated with K-252a (2 mM in 0.1% DMSO) 5minutes before spraying with neem fruit extract, and designated as K-252a neem treated plants.

Spraying was done using atomizer to produce fine spray droplets. All the above operations were carried out under aseptic conditions. For all analysis leaves of 3rd node from the base were sampled from control and treated plants at 0h, 24h, 48h, 72h, 96h, 120h and 144h after treatment. Immediately after sampling the leaves were frozen in liquid nitrogen, stored at -80°C and used for extraction of ionically bound cell wall proteins followed by detection of acidic isopolyphenol oxidases (iso-PPO).

Preparation and extraction of cell wall bound proteins:

Frozen leaf tissue sample (1g) was homogenized in 30 ml of cold, low ionic strength 5 mM acetate buffer (pH 4.6) containing 0.4 M sucrose, 0.34% PVP, 5 mM dithiotreitol (DTT), 2 mM phenyl methyl sulfonyl fluoride (PMSF). The homogenate was incubated at 4°C for 30 min with stirring and centrifuged at 1000xg at 4°C for 15min. The supernatant was discarded and the pellet was resuspended in 30ml of 5 mM acetate buffer (pH 4.6) containing 0.6 M sucrose and centrifuged at 1000xg at 4°C for 15min. The supernatant was discarded and the pellet was resuspended in 30 ml of 5 mM acetate buffer (pH 4.6) containing 1M sucrose and centrifuged at 1000xg at 4°C for 15min. The pellet was collected, washed extensively thrice each time with 30 ml of 5 mM acetate buffer (pH 4.6), followed by centrifugation at 4°C at 2000 xg for 20min. The cell wall pellet was homogenized and resuspended with 10 mM Tris maleate buffer, pH 7.3 containing 2 M NaCl, 1 mM EDTA, 2 mM PMSF, vortexed for 10 min, sonicated for 10 min and incubated for 1h at 4°C. The cell wall proteins were extracted twice by centrifuging at 15000 xg at 4°C for 20 min. The proteins were precipitated by overnight incubation with 60% v/v of ice cold acetone at 4°C. The cell wall protein precipitate was obtained by centrifuging at 20000xg at 4°C for 60 min. The final pellet was dissolved in sterile type I water and the cell wall proteins were subjected to native basic PAGE analysis for identification of isoforms of acidic PPO.

Native-Basic PAGE analysis:

The acidic isozyme profile of PPO located in cell wall was examined by native basic polyacrylamide gel electrophoresis (Laemmli, 1970) without SDS. For each sample, 50 µg was loaded onto the native basic polyacrylamide gel. Electrophoresis was carried out for 6 hours at 30 mA/gel and 4°C. After electrophoresis, acidic PPO isoforms were visualized by the modified method of Jayaraman *et al.* (1987), by equilibrating the gel in 0.1% p-phenylene diamine followed by addition of 50 mM catechol in 0.1M sodium phosphate buffer pH 9.0 Isoenzyme pattern in gel were visualized on a transilluminator in white fluorescent light. The relative distance (Rf value) of each isozyme band was calculated from each zymogram using the following equation: (Rf value = Distance migrated by the isoenzyme band from the origin / Distance migrated by tracking dye from the origin). Difference in isoenzyme banding pattern were identified according to the number of bands, their Rf values and their level of expression.

RESULTS AND ANALYSIS

A novel ionically bound cell wall acidic iso-PPO (Rf 0.71) appeared in tomato after 96h of treatment with neem fruit extract. However, the same appeared after 72h itself if neem treatment preceeded with staurosporine / lavendustin. But this novel appearance was completely inhibited on neem treatment preceeded with K-252a (Fig. 1). This clearly indicates that neither protein tyrosine kinase nor protein serine / threonine kinase are involved but K252a-sensitive protein phosphorylation is involved in induction of signal transduction leading to novel appearance of ionically bound cell wall acidic iso-PPO (Rf 0.71) by neem fruit extract.

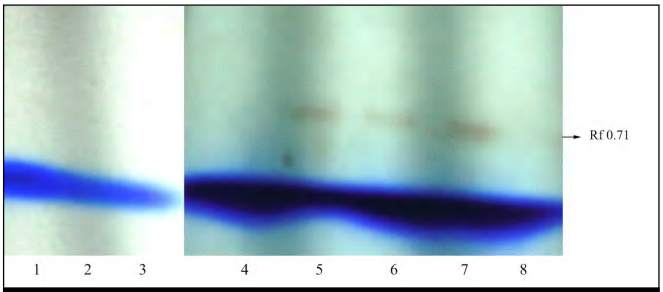

Fig. 1 : Effect of pathway inhibitors on the appearance of ionically bound cell wall isopolyphenol oxidases after 96h of neem fruit extract treatment in F_1 hybrid tomato plants

Lane 1: Control; Lane 2: Staurosporin control; Lane 3: Lavendustin control; Lane 4: K252a control; Lane 5: Neem fruit extract treatment; Lane 6: Staurosporin neem treatment; Lane 7: Lavendustin neem treatment; Lane 8: K252a neem treatment.

PPO located in the cell walls of plants was involved in the polymerization of phenolics leading to lignin synthesis (Cyikrova *et al.*, 2006). Lignification of diseased tissue may be a mechanism of resistance (Vance *et al.,* 1980). In both compatible and incompatible plant - pathogen interactions, PPO may act after cell

decompartmentalization as a component of cell death responses, with quinone production as one of the terminal events in the progression of such responses. Direct toxicity to plant and pathogen cellular macromolecules via quinone-mediated covalent modification or direct anti-microbial toxicity of H_2O_2 in the vicinity of pathogen attack may restrict disease progression. Moreover, protein bioavailability to pathogens may be reduced due to the alkylation of plant proteins. At the same time, a physical pathogen barrier could be generated as a result of cross-linking of oxidized phenolics as well as of oxidative cross-linking of cell wall proteins and enhanced lignin formation that occur as a result of enhanced PPO activity (Thipyapong *et al*., 2007). It has also been proposed that molecules in cell wall which have a net positive charge may immobilize pathogens by interacting ionically with the negatively charged surfaces of plant pathogens (Leach *et al.,* 1982; Mellon and Helgeson, 1982).

Evidence for tyrosine kinase activity in plants has been rapidly increasing in recent years. Serine/threonine/ tyrosine (STY) protein kinase from peanut is induced by abiotic stresses and is regulated by tyrosine phosphorylation (Rudrabhatla and Rajasekharan, 2004). Barizza *et al*. (1999) reported many phosphorylated tyrosine residues in plants. To obtain insight into which classes of kinases are involved in this transactivation, inhibitors of specific classes of kinases were tested. Staurosporine is a general inhibitor of AGC protein kinases at high concentrations, but at lower concentrations it becomes more selective for protein kinase C (PKC) and calcium/ calmodulin depenent protein kinases (CDPK) (Martinez-Noel *et al.,* 2007; Ritsema *et al*., 2009). Martinez-Noel *et al*.(2006) have shown that staurosporine and W7-sensitive protein kinase(s) may participate in sucrose-induced protein phosphorylation which in turn leads to induction of fructan synthesis. Ritsema *et al*. (2009) demonstrated that staurosporine was not able to inhibit sugar-mediated induction of the fructosyltransferase promotor in barley at the low concentrations, suggesting that CDPKs are not important players. Protein kinase inhibitor staurosporine prevents hydroxyproline-rich glycoprotein mRNA accumulation in the presence of elicitor suggesting the involvement of protein phosphorylation in the elicitor transduction pathway (Garcia-Muniz *et al*., 1998). The pattern of protein phosphorylation is altered in plant cells responding to elicitors and protein kinase inhibitors prevent certain elicitor responses such as the induction of PAL activity and ethylene production in tomato cells (Grosskopf *et al*., 1990; Felix *et al*., 1991). Furthermore, staurosporine prevents elicitor-induced changes in protein phosphorylation as well as ethylene biosynthesis (Felix *et al*., 1991) and blocked the elicitor signal transduction pathway that results in an increase of a PR-like gene expression in maize aleurone cells (Raventós *et al*., 1995). Staurosporine and lavendustin A prevented PAL induction as well as scoparone biosynthesis in response to the fungal inoculation, thus allowing us to infer the participation of serine/threonine and of tyrosine protein kinases (TPK) for signal transduction in *Citrus limon* in response to *A. alternata* (Ortega *et al.,* 2002). Lavendustin A, described as a competitive inhibitor for ATP binding to protein tyrosine kinase reduced PAL induction observed in response to fungal inoculation (Umezawa *et al.,* 1990). The general kinase inhibitor K252a reduces the sucrose-induced 6-sft (sucrose:fructan 6-fructosyltransferase) transcription in barley to 20%, indicating that kinase activity is necessary and its role of protein phosphorylation in sugar responses (Ritsema *et al*., 2009). K252a was shown to inhibit the oligogalacturonide-induced accumulation of defense related genes implying the involvement of protein phosphorylation in transducing the elicitor signal in grapevine cells (Aziz *et al*., 2004). K252a-sensitive protein kinases regulate methyl jasmonate-induced cytosolic Ca^{2+} oscillation in guard cells of *Arabidopsis thaliana* (Hossain *et al*., 2011).

Conclusion:

Our results clearly indicates the involvement of K252a-sensitive protein phosphorylation in induction of signal transduction which leads to appearance of a novel acidic iso-PPO (Rf 0.71) ionically bound to cell walls of tomato plants treated with neem fruit extract. This appearance iso-PPO in cell walls may generate physical pathogen barrier due to cross-linking of oxidized phenolics, oxidative cross-linking of cell wall proteins, enhanced lignin formation and their net positive charge may immobilize pathogens by interacting ionically with the negatively charged surfaces of plant pathogens.

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