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Differential Induction of *PepTLP* Expression via Complex Regulatory System against Fungal Infection, Wound, and Jasmonic Acid Treatment during Pre- and Post-Ripening of Nonclimacteric Pepper Fruit

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Ripe fruit of pepper (*Capsicum annuum*) showed resistance to *Colletotrichum gloeoporioides*, but unripe fruit was susceptible. We previously isolated the *PepTLP* gene that induced in both unripe and ripe fruit by fungal infection and wound, and only in ripe fruit by jasmonic acid (JA) treatment. To examine further regulation of *PepTLP*, the action of specific agonist and antagonists of known signaling effector on the *PepTLP* expression by fungal infection, wound, and JA was investigated. A similar dephosphorylation event negatively activated all the *PepTLP* expression in the ripe fruit by fungal infection, wound, and JA. The induction of *PepTLP* expression by wound is differentially regulated via phosphorylation and dephosphorylation step during pre- and post-ripening, respectively. In addition, the induction of *PepTLP* expression in the ripe fruit by wound and JA is differentially regulated via dephosphorylation and phosphorylation step, respectively. Only both wound and JA treatment has synergistic effect on the *PepTLP* expression in the unripe fruit. Both SA and JA treatments on the unripe fruit, and both wound or JA and SA on the ripe fruit could not do any effect on the expression of *PepTLP*. These results suggest that the induction of *PepTLP* expression is differentially regulated via complex regulatory system against fungal infection, wound, and JA treatment during pre- and post-ripening of pepper fruit.

Keywords : *Colletotrichum gloeosporioides*, okadaic acid, pepper fruit, signaling pathway, staurosporine

Colletotrichum gloeosporioides causes severe anthracnose disease on pepper fruits. This fungus was classified into G and R strain (Kim et al., 1986). The G strain infected both unripe and ripe fruit and the R strain did only ripe fruit. We have established that an isolate of *C. gloeosporioides* has susceptible and resistant interactions with pepper fruits during pre- and post-ripening, respectively (Oh et al., 1998; Kim et al., 1999; Oh et al., 1999a). In this pathosystem, the incidence of fungal appressorium and infection hyphal formation was lower in the ripe fruit than in the unripe fruit.

Ripe fruits generally exhibit increased susceptibility to pathogen infection (Swinburn, 1983; Prusky et al., 1991). However, in several nonclimacteric fruits such as cherry, grape, and pepper, resistance against phytopathogens increases during ripening (Fils-Lycaon et al., 1996; Robinson et al., 1997; Tattersall et al., 1997; Oh et al., 1998; Salzman et al., 1998). These studies suggest that fruits as a reproductive organ have their own protection mechanism against pathogens to maintain their integrity during seed maturation. We have been interested in elucidating a molecular mechanism by which resistance is induced against fungal infection during ripening of nonclimacteric pepper fruit. We have previously isolated several genes, *PepCYP*, *PepThi*, and *PepTLP*, that are highly expressed during the resistance of infected ripe fruit (Oh et al., 1999b, 1999c; Kim et al., 2002).

Thaumatin-like protein (TLP) was accumulated during ripening of banana (Clendennen and May 1997), cherry (Fils-Lycaon et al., 1996), pepper (Meyer et al., 1996), and tomato (Pressey, 1997). In addition, TLPs and *TLP* genes are being induced in various organs of plants upon pathogen infection (Ruiz-Medrano et al., 1992; Rodrigo et al., 1993), abiotic stress (Zhu et al., 1993), and chemical stimuli (King et al., 1988). Although the precise function of TLP is not yet understood, the antifungal activity of TLP appears to act by permeabilizing fungal membranes (Robert and Selitrennikoff, 1990; Vigers et al., 1992).

We demonstrated that there is a correlation among

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PepTLP gene expression, disease resistance, and sugar accumulation with beginning of fruit ripening of the nonclimacteric pepper (Kim et al., 2002). The expression of *PepTLP* gene was induced in pepper fruits during ripening and in both unripe and ripe fruit by fungal infection and wound. JA treatment induced accumulation of the *PepTLP* transcripts only in the ripe fruit, but not in the unripe fruit. These results suggest that the *PepTLP* is up-regulated during ripening, fungal inducible in pepper fruits, and is expressed via JA-independent wound signaling pathways in the unripe fruit. In order to further examine regulation of *PepTLP*, the action of specific agonist and antagonists of known signaling effector on the *PepTLP* expression by fungal infection, wound, and JA was investigated.

Materials and Methods

Plant material and treatment. Unripe and ripe fruits were harvested from pepper plants (cv. Nokkwang) grown in greenhouses. The fruits were treated in 10% Clorox for 3 min, washed in sterile distilled water three times, and after treatments placed on plastic mesh screens in containers (25 cm × 16 cm × 6 cm). Four layers of paper towels moistened with sterile distilled water were placed in the containers to maintain 100% relative humidity (Oh et al., 1999a).

One micro-molar staurosporine (STA), 100 nM okadaic acid (OKA) (Sigma-Aldrich, Korea), 50 μM JA, and/or 1 mM salicylic acid (SA) for chemical applications and wound treatment were applied to both the fruits for 24 h under the above mentioned conditions. Both unripe and ripe fruits were deeply scratched by a knife for the wound treatment. A 10 μl of chemical solutions was applied to both the fruits. After the incubation, samples of 1 cm^2 were excised from the application site of the chemicals, wounding, or fungus inoculation. The samples were then frozen in liquid nitrogen.

Fungal inoculation. Monoconidial isolate KG13 of *C. gloeosporioides* was cultured on potato dextrose agar (Difco Laboratories, MI, USA) in darkness at 28°C for 5 days. Ten milliliters of sterile distilled water were added and the conidia were filtered through four layers of cheesecloth to remove mycelial debris. The conidia were then washed three times in sterile distilled water by centrifugation and adjusted to 5×10^5 conidia/ml with sterile distilled water. Twenty microliters of the conidial suspension were inoculated on pepper fruits (Oh et al., 1999a).

RNA gel-blot analysis. Total RNA (10 μg/lane) from pepper fruits used in this study was separated in 1.2% denaturing agarose gels in the presence of formaldehyde. RNA gel blotting, hybridization, and washing ware conducted as described by the manufacturer of the positively charged nylon membrane employed (Hybond N^+; Amersham Pharmacia Biotech, Buckinghamshire, UK). Radiolabeled probes were prepared with [α^{32}P]dCTP (Amersham Pharmacia Biotech), using a random primer labeling kit (Boehringer, Mannheim, Germany).

Results

***PepTLP* expression by wound, JA, and SA during pre- and post-ripening.** In unripe fruits, the expression of *PepTLP* gene was induced from 6 h after wound treatment, and significantly induced at 24 h (Fig. 1). Neither JA nor SA treatment could induce any *PepTLP* transcripts in the unripe fruit. In ripe fruits, accumulation of *PepTLP* transcripts was detected (Kim et al., 2002). The expression of *PepTLP* gene was significantly increased from 12 h after wound or JA treatment, and reached to maximum level at 24 h. SA treatment couldn't induce any *PepTLP* transcripts in the ripe fruit. Based on these expression analyses, we further examined effect of chemical treatments on regulation of *PepTLP* expression in unripe and ripe fruit at 24 h after treatment.

Effect of OKA and STA on the ripening-specific and fungal-inducible *PepTLP* expression during pre-and

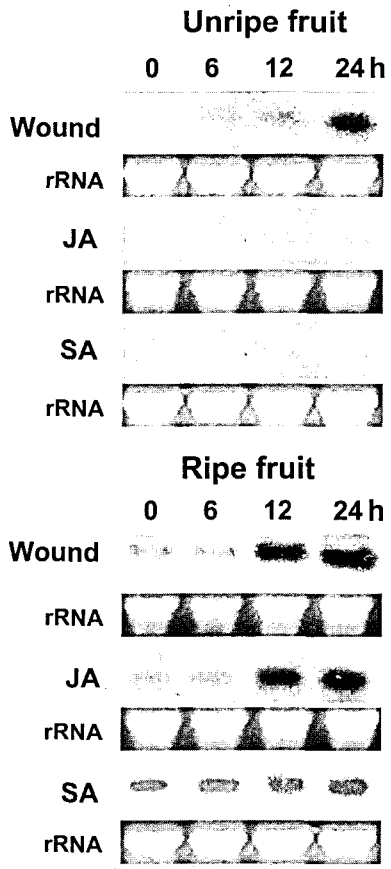

Fig. 1. Expression and induction of *PepTLP* genes by wound, JA, and SA during pre- (Unripe) and post-ripening (Ripe) of pepper fruit. Total RNAs were isolated from the application sites of both unripe and ripe fruit 6, 12, and 24 h after treatments. The RNAs were electrophoresed and allowed to hybridize with *PepTLP* cDNA as a probe. The rRNA bands in Ethidium Bromide-stained gels were shown as a loading control.

post-ripening. Expression of *PepTLP* gene was up-regulated during post-ripening of pepper fruit, but not during pre-ripening (Kim et al., 2002; Fig. 1). We analyzed the effect of OKA that is protein phosphatase inhibitor and STA that is protein kinase inhibitor on the ripening-regulated *PepTLP* expression. In the unripe fruit, expression of *PepTLP* gene was not induced by OKA or STA (Fig. 2). In the ripe fruit, the up-regulation of *PepTLP* was highly enhanced by OKA, but not by STA. This suggests that a protein phosphatase, probably type 2A, acts as a negative regulator in the signaling pathway of *PepTLP* expression during ripening. By fungal infection, expression of *PepTLP* gene was induced at a low level in the unripe fruit, but significantly induced in the ripe fruit (Kim et al., 2002; Fig. 1). We analyzed the effect of OKA and STA on the fungal-inducible *PepTLP* expression during pre- and post-ripening. In the unripe fruit, the induction by fungal infection was remained constant by combinational treatment of both fungal infection and OKA or STA (Fig. 2). In the ripe fruit, the induction by fungal infection was highly enhanced by both fungal infection and OKA, but was remained constant by both fungal infection and STA. These results suggest that a protein phosphatase acts as a negative regulator in the signaling pathway of *PepTLP* expression by fungal infection during ripening.

Effect of OKA and STA on the JA- and wound-inducible *PepTLP* expression during pre- and post-ripening. Expression of *PepTLP* gene was not induced in the unripe fruit by JA, but significantly induced in the ripe fruit (Kim et al., 2002; Fig. 1). We analyzed the effect of OKA and STA on the JA-inducible *PepTLP* expression during pre- and post-ripening. In the unripe fruit, expression of *PepTLP* gene was not induced by both JA and OKA or STA (Fig. 3). In the ripe fruit, the expression of *PepTLP* was induced by both JA and OKA. The treatment of both JA and STA enhanced the induction of *PepTLP* compared with that of JA alone and of JA and OKA. These results suggest that a protein kinase acts mainly as a negative regulator in the signaling pathway of *PepTLP* expression by JA treatment during ripening, and a protein phosphatase acts partially as a negative regulator.

By wound treatment, expression of *PepTLP* gene was significantly induced in both the unripe fruit and the ripe fruit (Kim et al., 2002; Fig. 1). We analyzed the effect of OKA and STA on the wound-inducible *PepTLP* expression during pre- and post-ripening. In the unripe fruit, the induction of *PepTLP* was remained constant by both wound and OKA, and the expression level was partially blocked by both wound and STA (Fig. 3). In the ripe fruit, the induction of *PepTLP* was enhanced by both wound and OKA, and remained constant by both wound and STA. These results suggest that a protein kinase act as a positive regulator in the signaling pathway of *PepTLP* expression by wound treatment during pre-ripening, and a protein phosphatase act as a negative regulator during ripening.

Effect of combinational treatment of wound, JA, or SA on *PepTLP* expression during pre- and post-ripening. The wound-inducible *PepTLP* expression has JA-independent signaling pathway in unripe fruit and JA-

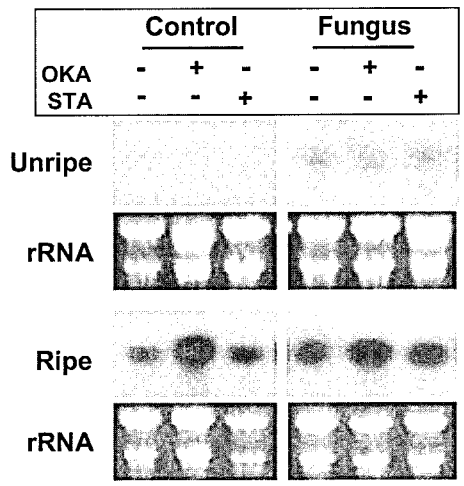

Fig. 2. Effect of okadaic acid and staurosporine on the ripening-specific and fungal-inducible expression of *PepTLP* gene during pre- (Unripe) and post-ripening (Ripe) of pepper fruit. Total RNAs were isolated from both unripe and ripe fruit at 24 h after 1 μM okadaic acid (OKA) and 100 nM staurosporine (STA) treatment. The RNAs were electrophoresed and allowed to hybridize with *PepTLP* cDNA as a probe. The rRNA bands in Ethidium Bromide-stained gels were shown as a loading control.

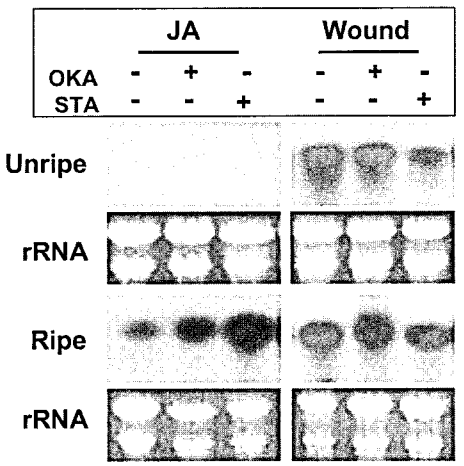

Fig. 3. Effect of okadaic acid and staurosporine on the JA- and wound-inducible expression of *PepTLP* gene during pre- (Unripe) and post-ripening (Ripe) of pepper fruit. Total RNAs were isolated from both unripe and ripe fruit at 24 h after 1 μM okadaic acid (OKA) and 100 nM staurosporine (STA) treatment. The RNAs were electrophoresed and allowed to hybridize with *PepTLP* cDNA as a probe. The rRNA bands in Ethidium Bromide-stained gels were shown as a loading control.

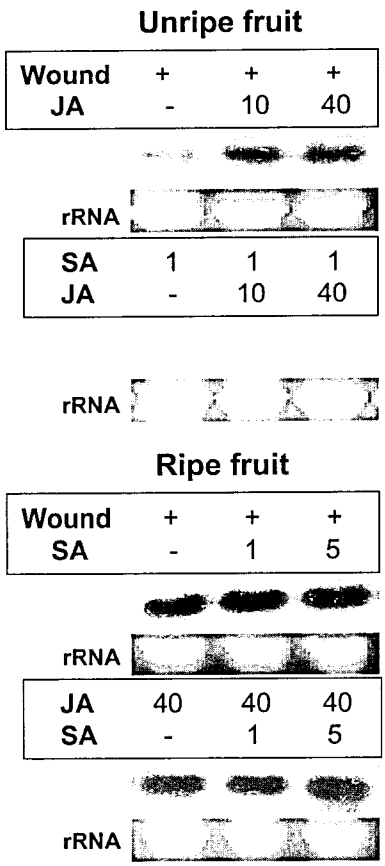

Fig. 4. Effect of combinational treatment of wound, JA, and SA on the expression of *PepTLP* gene during pre- (Unripe) and post-ripening (Ripe) of pepper fruit. Total RNAs were isolated from both unripe and ripe fruit at 24 h after combinational treatment of wound, SA (1 or 5 mM), and JA (10 or 40 μM). The RNAs were electrophoresed and allowed to hybridize with *PepTLP* cDNA as a probe. The rRNA bands in Ethidium Bromide-stained gels were shown as a loading control.

dependent in ripe fruit (Kim et al., 2002; Fig. 1). To examine if JA or SA treatment has synergistic or antagonistic effect on the expression of *PepTLP* via wound and JA-dependent signaling pathway, we performed RNA gel blot analysis with fruit during pre-and post-ripening. The *PepTLP* expression in the unripe fruit was not induced by SA and JA treatments (Kim et al., 2002; Fig. 1). The accumulation of *PepTLP* mRNA in the unripe fruit was induced by wound alone and significantly enhanced by both wound and JA (Fig. 4). However, both SA and JA treatments in the unripe fruit, and both wound or JA and SA couldnt any effect on the expression of *PepTLP*.

Discussion

Fruit ripening represents a genetically synchronized system that involves developmental process unique to plant species (Giovannoni, 1993). The feature that ethylene hastens ripening distinguishes climacteric fruit such as apple and tomato from nonclimacteric fruit such as pineapple and strawberry, in which the progress of ripening appears to be independent of ethylene. There have been few studies on disease resistance during ripening of nonclimacteric fruits. We have reported anthracnose developed on unripe fruit of pepper, but not on ripe fruit (Oh et al., 1998; Kim et al., 1999). This pathosystem and other pathosystems of nonclimacteric fruits (Fils-Lycaon et al., 1996; Robinson et al., 1997; Tattersall et al., 1997) generate significant information on the interaction between fruit development and disease resistance.

We showed that there is a correlation between *PepTLP* gene expression, disease resistance, ripening, and sugar accumulation in pepper fruits (Kim et al., 2002). In addition, JA could induce *PepTLP* expression only in the ripe fruit, but not in the unripe fruit. Exogenous application of JA was reported to protect *Arabidopsis* plants against *Alternaria brassicicola* infection (Thomma et al., 1998). A few studies in which methyl JA triggers the ripening process of climacteric fruits (Czapski and Saniewski, 1992; Saniewski et al., 1987a, 1987b). However, JA applications couldnt protect both unripe and ripe fruit against *C. gloeosporioides* infection and hasten ripening of fruit (Unpublished results). The role of JA in ripening or defense of nonclimacteric fruit has not been well studied. Taken together, these data suggest that JA may not be the hormone primarily related to disease resistance or ripening of pepper fruit, although the *PepTLP* gene expression correlates to disease resistance and ripening.

Pharmacological approaches have proved useful in the study of signal transduction pathways in eukaryotes. The availability of specific inhibitors of protein kinases and phosphatase (MacKintosh and MacKintosh, 1994) has allowed the elucidation of the roles of their target enzymes in signal transduction pathways of lower eukaryotes, and in animal and plant systems (Rojo et al., 1998). Staurosporine is a broad range serine-threonine protein kinase inhibitor (MacKintosh and MacKintosh, 1994), and okadaic acid is a potent inhibitor of protein serine-threonine phosphatase types1 (PP1) and 2A (PP2A) (Cohen et al., 1990). We have undertaken a pharmacological approach by using staurosporine and okadaic acid to examine regulation on the signaling pathway of *PepTLP* expression by fungal infection, wound, and JA during pre-and post ripening of pepper fruit. Previous study showed that the expression of *PepTLP* gene was induced in pepper fruits during ripening, in both unripe and ripe fruit by fungal infection and wound, and only in the ripe fruit by JA. Here, we further revealed that, in the ripe fruit, the expression of *PepTLP* was induced by OKA alone and both wound, JA, or fungal infection and

OKA (Figs. 2, 3). These data suggest that a similar protein phosphatase activity negatively regulated the *PepTLP* expression in the ripe fruit by wound, JA, and fungal infection, during ripening. The induction of *PepTLP* expression by wound was partially blocked by STA in the unripe fruit, and enhanced by OKA in the ripe fruit (Fig. 3). These data suggested that the *PepTLP* expression is regulated in the wound signaling pathway via phosphorylation and dephosphorylation step during pre- and post-ripening, respectively. In the ripe fruit, the induction of *PepTLP* expression by wound was affected by OKA (Fig. 2), and that by JA was by STA (Fig. 3). These data suggested that the *PepTLP* expression in the ripe fruit is regulated in a single signaling pathway of wound and JA via dephosphorylation and phosphorylation step, respectively.

The primary interaction between SA and JA signaling appears to be mutual antagonism (Kunkel and Brooks, 2002). JA plays a role in plant responses to wounding (Creelman et al., 1992). However, there is also evidence that wound responses include JA-independent effects (Rojo et al., 1998). We showed that only both wound and JA treatment had synergistic effect on *PepTLP* expression in the unripe fruit. And any combinational treatments of wound, SA, or JA didn't have any effect on *PepTLP* expression in both unripe and ripe fruit. These results suggest that although the wound-inducible *PepTLP* expression in the unripe fruit is via JA-independent pathway, both wound and JA signals may share a common part of both pathways. There may not be existed a cross-talk or interaction between wound or JA and SA in the unripe fruit, and among wound, SA or JA in the ripe fruit on the expression of *PepTLP*.

Acknowledgments

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