Development and industrial applications of versatile-usable genes of plant
(식물 유용 유전자의 발굴 및 산업적 응용)

Boung-Jun Oh
Plant & Microbe Co., LTD., Biotechnology Industrialization Center, 880-4 Ansan-Ri, Naju-Si, Jeonnam 520-811, Korea

Fruit ripening represents a genetically synchronized system that involves developmental process unique to plant species. The phenomenon of ripening includes changes in color, texture, respiration rate, flavor, and aroma. Ripe fruits generally exhibit increased susceptibility to pathogen infection. However, fruits as a reproductive organ have their own protection mechanism against pathogens to maintain their integrity during seed maturation. In several nonclimacteric fruits, such as cherry, grape, and pepper, that do not have an ethylene burst during ripening, resistance against phytopathogens increases during ripening. *Colletotrichum gloeosporioides* is a causal agent of anthracnose disease in pepper plants (*Capsicum annuum*). We have established that *C. gloeosporioides* has susceptible and resistant interactions with pepper fruits during pre- and post-ripening stages, respectively. And we have interested in looking for a molecular mechanism that would explain the fungal resistance during ripening of nonclimacteric pepper fruit. In this presentation, a molecular characterization of the pepper esterase gene (*PepEST*) that is highly expressed in the resistant response will be demonstrated as an example of development and industrial applications of versatile-usable genes of plant.

Introduction

Colletotrichum gloeosporioides (Penz.) Penz & Sacc. [teleomorph: *Glomerella cingulata* (Stonem.) Spauld. & von Schrenk] is a causal agent of anthracnose disease in pepper plants (*Capsicum annuum*). This pathogen causes significant losses in pepper fruit quality and yield (Kim *et al.*, 1986), and also produces anthracnose symptoms in other fruit such as blueberry (Daykin, 1984) and mango (Dodd *et al.*, 1991). *C. acutatum*, *C. cocodes*, *C. dematium*, *C. gloeosporioides*, and *G. cingulata* have been identified as fungal species responsible for anthracnose of pepper in Korea (Park and Kim, 1992) and *C. capsici*, *C. gloeosporioides*, and *G. cingulata* in Taiwan (Manandhar *et al.*, 1995). *C. gloeosporioides* is the predominant cause of anthracnose in pepper.

C. gloeosporioides has been classified into G and R strains (Kim *et al.*, 1986). The G strain produces symptoms in both unripe-green and ripe-red fruits of pepper, whereas the R strain produces symptoms only in ripe fruit. We have also recently found an isolate that causes symptoms only in unripe fruit (Kim *et al.*, 1999; Oh *et al.*, 1998). Analysis of these complex pathosystems may provide insight into the interaction between phytopathogen and disease response of fruit.

The fruit is a reproductive organ unique to plants. It undergoes a ripening process involving many physical and biochemical changes (Giovannoni, 1993). Generally, ripe fruits that are rich in macromolecules are susceptible to pathogen infections. However, in several nonclimacteric fruits, such as cherry, grape, and pepper, that do not have an ethylene burst during ripening, resistance against phytopathogens increases during ripening (Fils-Lycaon *et al.*, 1996; Oh *et al.*, 1998; Robinson *et al.*, 1997; Salzman *et al.*, 1998; Tattersall *et al.*, 1997).

We have established the pepper-*C. gloeosporioides* system as a model to examine the mechanism underlying the development of resistance during nonclimacteric fruit ripening (Kim *et al.*, 1999; Oh *et al.*, 1998; 1999a). Several genes that may have important roles in this process have been isolated from infected ripe fruit (Kim *et al.*, 2002; Oh *et al.*, 1999b). In addition, we have produced transgenic pepper plants that showed disease resistance to the anthracnose fungus by using these genes that might have responsible for the resistant interaction.

Carboxylesterases catalyze the hydrolysis of compounds containing an ester bond.

Esterase and lipase enzymes have been studied mainly in mammals and microbes (Feller et al., 1991; Kok et al., 1993; Langin et al., 1993; Contras et al., 1996; Osterund et al., 1997). In plant, *hsr203J*, the tobacco esterase gene, was isolated from hypersensitive reaction (HR) against the phytopathogenic bacterium *Ralstonia solanacearum* (Pontier et al., 1994; Baudouin et al., 1997). A suggested putative role for the HSR203J is as a detoxification effector to oxidative stress during HR or a signaling component. Recently, two *Arabidopsis* genes *EDS1* and *PAD4* that required for disease resistance were cloned (Parker et al., 1996; Aarts et al., 1999; Falk et al., 1999; Jirage et al., 1999). These predicted proteins encode enzymes homologous to esterases and lipases containing a lipase motif and a putative catalytic triad. These data suggested that the enzyme activity of esterases or lipases in plants is involved in defense mechanisms.

In this study, we report characterization of the *PepEST* that is highly expressed in the resistant response against *C. gloeosporioides* infection during fruit ripening. To our knowledge, *PepEST* is the first fruit-specific esterase gene. Expression of *PepEST* was studied in the context of fungal morphogenesis and symptom development during infection process. Over-expression of *PepEST* in transgenic *Arabidopsis* plants resulted in restricting colonization of *Alternaria brassicicola* and constitutive upregulation of *PR-2* and *PR-4* genes, suggesting that *PepEST* could be involved in the resistance of ripe pepper fruit against *C. gloeosporioides*. And a possible role of *PepEST* for the defense mechanism of transgenic *Arabidopsis* plants against fungal infection is discussed.

Results

Sequence analysis and characterization of *PepEST*

Full-length *PepEST* cDNA (GenBank AF072533) was previously cloned (Kim et al., 2001). The nucleotide sequence contained an open reading frame of 328 amino acids. The *PepEST* for pepper esterase was named after enzymatic assay.

Deduced amino acid sequence of *PepEST* cDNA is homologous to esterases and

lipases of mammals and microbes. PepEST protein contained pentapeptides -His-Gly-Gly-Gly-Phe- (-HGGGF-; amino acids 86-90) found in many esterases and lipases, and -Gly-Ser-Ser-Cys-Gly- (-GSSCG-; amino acids 163-167), the consensus motif -GXSXG- found in many members of serine hydrolase family (Feller et al., 1991; Kok et al., 1993; Langin et al., 1993) (Fig. 1). The PepEST also contained a potential catalytic triad, Asp, His, and the conserved Ser in the -GXSXG-, characteristic of lipases.

Homology search (Fig. 1) revealed that PepEST shows 37% and 35% identity to two esterases of *Arabidopsis*, respectively (F17K2.14 and F17K2.13 in GenBank AC003680, unpublished data). Next sequence identity was 32% to HSR203J protein, the tobacco esterase induced from HR (Pontier et al., 1994) and 26% to a lipaselike enzyme of an aerobic bacterium *Alcaligenes eutrophus* (Valentin et al., 1995).

Expression analysis of *PepEST*

To examine *PepEST* gene expression in various organs and its inducibility by fungal inoculation or wounding, RNA gel blot analysis was performed with total RNA populations extracted from fruits, leaves, stems, and roots of pepper plants at 24 h after treatments. Induced expression of *PepEST* was observed in both unripe and ripe fruits by fungal inoculation, and only in the ripe fruit by wounding (Fig. 2A). However, in leaves, stems, and roots, the *PepEST* mRNA did not accumulate.

To further examine *PepEST* expression in response to various inducers, RNA gel blot analysis was performed with both unripe and ripe fruits treated with exogenous abscisic acid (ABA), ethephone, jasmonic acid (JA), and salicylic acid (SA) for 24 h. *PepEST* mRNA was detected only in the ripe fruit treated with JA at 40 μM (Fig. 2B). However, neither of ABA, ethephone, and SA did affect the *PepEST* expression. These results suggest that *PepEST* expression is fruit-specific in response to *C. gloeosporioides* and up-regulated by JA application or wounding during fruit ripening.

Differential expression during fungal infection

C. gloeosporioides infects fruits through conidium germination, appressorium

formation and infection hypha formation on fruit surface (Bailey et al., 1992). In previous studies (Oh et al., 1998; Kim et al., 1999), we found that higher incidence of appressorium and infection hypha formation was observed on unripe fruit than on ripe fruit at 12 and 24 HAI. Only on the unripe fruit, initial anthracnose symptoms were detected after 48 h, and typical sunken necrosis symptoms occurred within 120 h. Thus, we examined if time-course accumulation of *PepEST* mRNA during fungal infection was related with critical events in fungal morphogenesis and symptom development. RNA gel blot analysis was performed with both unripe and ripe fruits at 0, 3, 6, 12, 24, 48, and 72 HAI (Fig. 3). Accumulation of *PepEST* mRNA was detected both in the unripe and ripe fruits from 24 HAI. The *PepEST* mRNA accumulation was increased to high levels only in the ripe fruit at 48 and 72 HAI, but maintained in the unripe fruit. However, with water inoculation without fungal spores as a mock inoculation, *PepEST* mRNA was not accumulated in both fruits.

Recombinant PepEST protein was harvested from *E. coli* by transforming bacteria with *PepEST* cDNA (Chung et al., 1989; Kim et al., 2001). A purified 36 kDa protein was used to obtain specific antibodies against the PepEST protein (Fig. 4A).

Western blot analysis revealed that PepEST accumulation was detected both in the unripe and ripe fruits from 24 HAI (Fig. 4B). After that time, the PepEST was highly accumulated only in the ripe fruit, but not in the unripe fruit. However, with water inoculation as a mock inoculation, PepEST was not accumulated in both fruits. These results suggest that *PepEST* gene and its gene product are fungal-inducible and differentially expressed in ripe versus unripe fruit.

Localization of PepEST protein during fungal infection

To examine localization and accumulation of PepEST during early infection, we performed immunohistochemical examination of transverse-sections. The sections were prepared from infected unripe and ripe fruits at 0, 6, 24, 48 and 72 HAI, respectively. PepEST accumulation was not detected in both fruits at 0 (Fig. 5, A and F) and 6 HAI (Fig. 5, B and G). In the unripe fruit at 24 HAI, the accumulation of PepEST was localized rarely in outer epidermal cells that were highly vacuolated, but not in cortical cell layers (Fig. 5C). And even at 48 and 72 HAIs (Fig. 5, D and E), the PepEST

accumulation was still low and localized only in the epidermal cell layers.

In the ripe fruit, PepEST accumulated only in epidermal cells at 24 HAI (Figure 5H). However, at 48 HAI (Fig. 5I), the PepEST began accumulating in cortical parenchyma cells. Finally, at 72 HAI (Fig. 5J), the PepEST was detected throughout both the epidermal cells and the cortical parenchyma cells. Control experiments using pre-immune serum did not show any reactions (Fig. 5, A-J). These results suggest that PepEST accumulation is differentially localized in tissues of ripe versus unripe fruits in response to fungal infection with time course.

Effect of esterase inhibitor 3.4-dichloroisocoumarin

Since amino acid sequence of PepEST contained the consensus motif of serine hydrolase, we examined if the serine residue is essential for PepEST enzyme activity by using specific inhibitor, 3.4-dichloroisocoumarin. The PepEST enzyme activity was spectrophotometrically measured by the rate of hydrolysis of *p*-nitrophenyl butyrate (Kim et al., 2001). The 3.4-dichloroisocoumarin inhibited PepEST enzyme activity and thereby the PepEST protein could not effectively degrade the substrate (Fig. 6). This result suggests that the serine residue is important for the PepEST enzyme activity as a member of serine hydrolase family.

Enhanced resistance of *Arabidopsis* plants expressing *PepEST* to *A. brassicicola*

To investigate if *PepEST* gene is involved in the resistance of ripe fruit, we constructed sense *Arabidopsis* transgenic lines with cauliflower mosaic virus (CaMV) 35S promoter and *PepEST*. Transformants were identified from their resistance to kanamycin. Of 10 transgenic lines produced, a transgenic line 3 (P3) that had intermediate level of expression of *PepEST* mRNA and protein was chosen for further study (Fig. 7, A and B). This P3 line had single copy of *PepEST* gene that confirmed by Southern hybridization (data not shown).

To evaluate level of disease resistance, T3 progenies of P3 line were inoculated with *A. brassicicola*, a necrotrophic fungal pathogen (Thomma et al., 1999). Wild-type *Arabidopsis* plants (ecotype Ws-0) showed the typical susceptible symptoms in which

spreading lesions were formed (Fig. 7C) and fungal hyphae eventually grew to the border of leaf (data not shown). However, in the P3 line, fungal colonization was arrested around the infection sites. And the average diameter of lesions was significantly reduced in the P3 line compared with in the wild-type plant at 5 days after inoculation, and was the shortest in the Col-0 plants as a resistant control (Fig. 7D).

To verify if the restricted symptom of P3 line is caused by inhibited fungal colonization, spore production on infected plants was measured. Spore production was significantly reduced in the P3 line compared with the wild-type plant, and was the lowest in the Col-0 plant (Fig. 7E). This observation is consistent with the results described above that P3 line showed restriction of fungal colonization.

Constitutive expression of *PR-2* and *PR-4* genes

JA/ethylene-regulated resistance in *Arabidopsis* plants leads to protection against *A. brassicicola* (Thomma et al., 1998, 1999). To investigate if activation of the JA/ethylene-regulated defense involves in the defense response, expression of different classes of *PR* genes was examined in transgenic plants. Transgenic plants showed constitutive upregulation of SA-regulated *PR-2* and JA/ethyleneregulated *PR-4* genes (Penninckx et al., 1998; Thomma et al., 1998, 1999), but control plants did not (Fig. 8). And SA-regulated *PR-1* transcript and JA/ethylene-regulated *PDF1.2* transcript were not detected in the transgenic lines, and SA-regulated *PR-5* transcript was detected at quite low level or not detected.

Summary

The present study showed that *PepEST* mRNA and protein were differentially accumulated in ripe versus unripe fruit in response to fungal colonization. Over-expression of the *PepEST* gene resulted in enhanced defense response of transgenic *Arabidopsis* plants to *A. brassicicola* infection, and caused constitutive upregulation of *PR-2* and *PR-4* genes. Although PepEST inhibited fungal appressorium

formation *in vitro*, the esterase activity of PepEST may be involved in the resistance of ripe fruit to inhibit fungal colonization. It remains to be elucidated how *PepEST* contributes to effective defense in pepper fruits against fungal infection.

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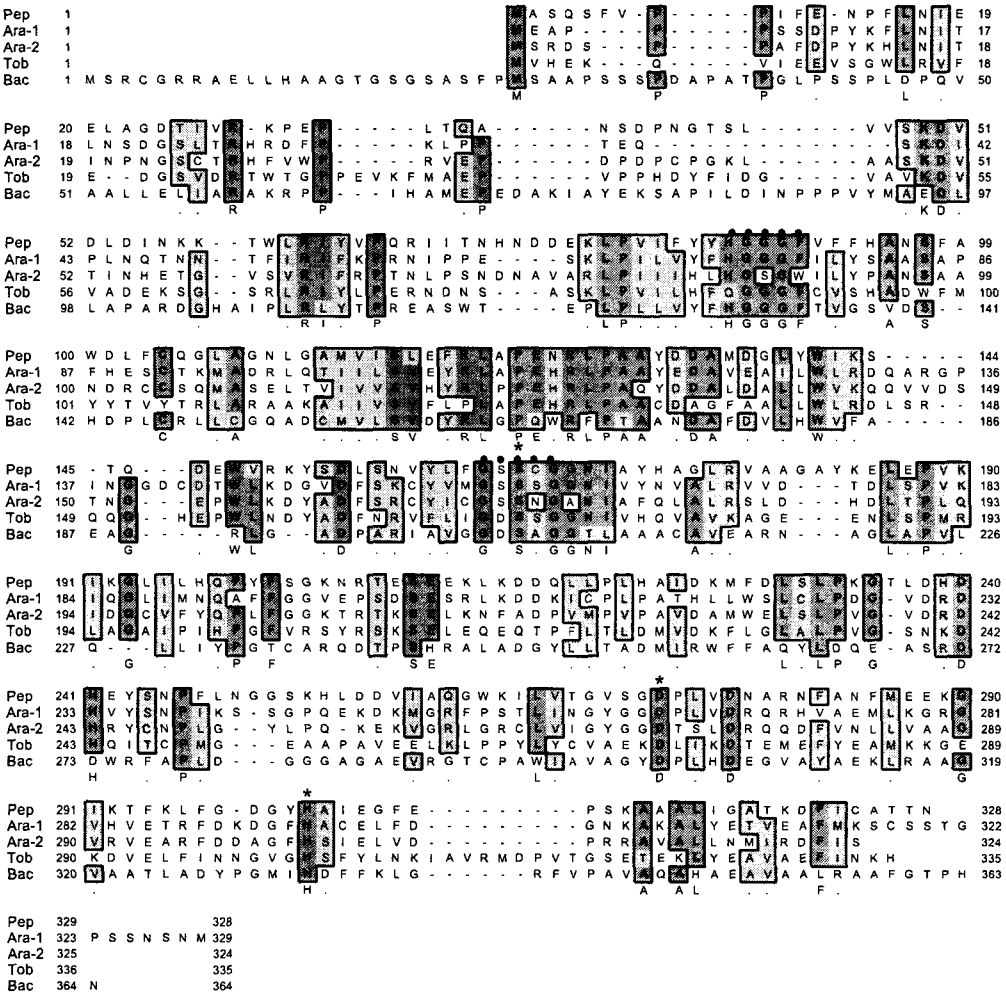
Figure 1



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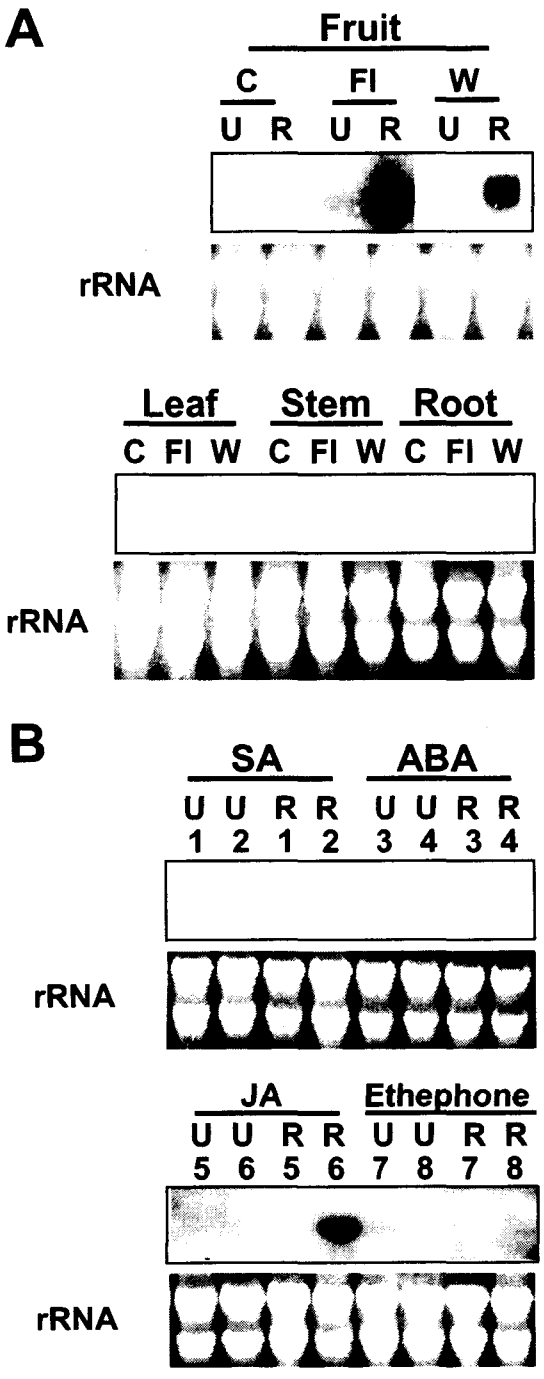
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W
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rRNA
Leaf
Stem
Root
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SA
ABA
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JA
Ethephone
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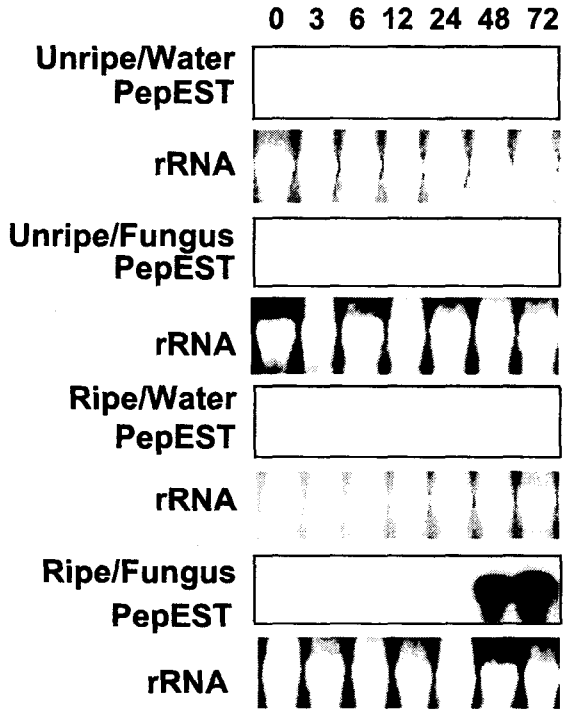
rRNA

Figure 3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rRNA
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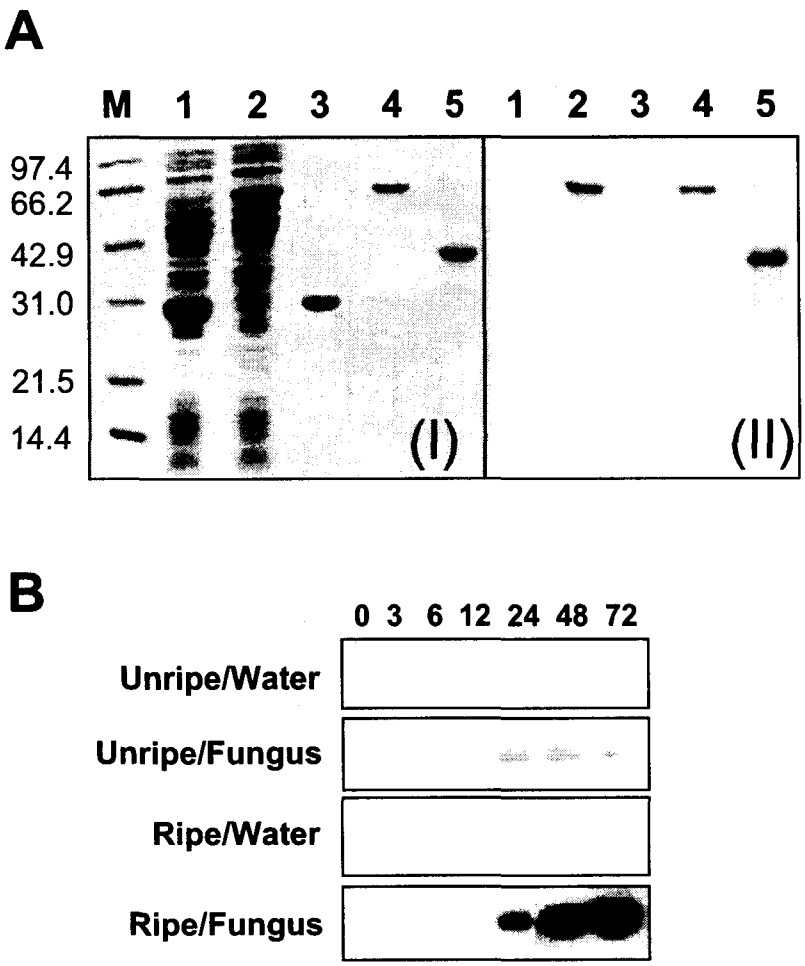
Ripe/Fungus PepEST
rRNA

Figure 4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42.9
31.0
21.5
14.4
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(II)
B
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Unripe/Water
Unripe/Fungus
Ripe/Water
Ripe/Fungus

Figure 5

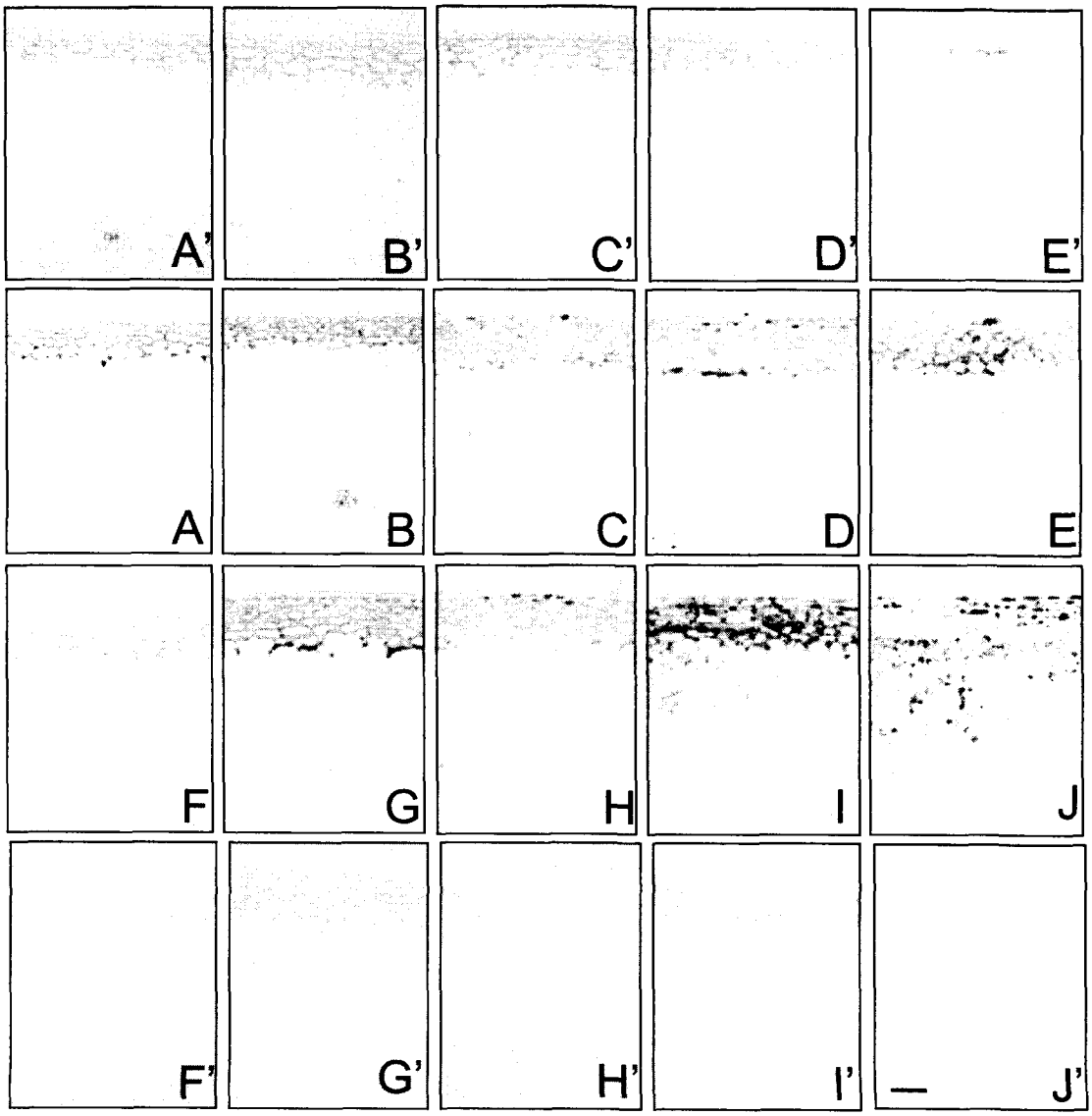


Figure 6

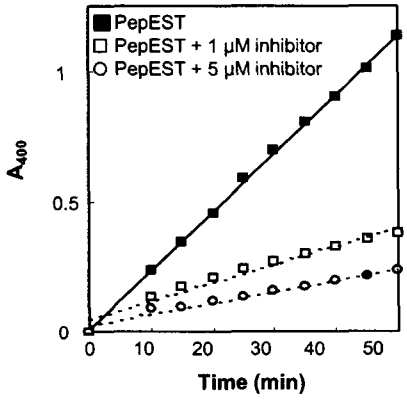
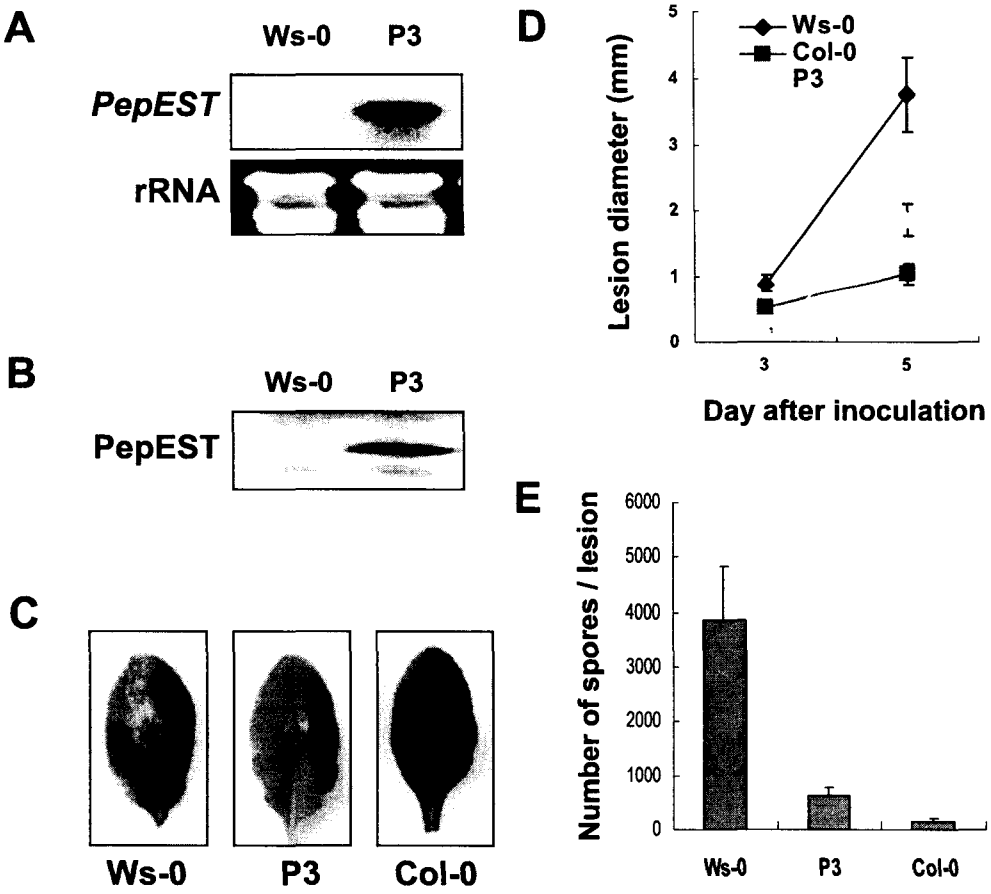

PepEST
PepEST + 1 μM inhibitor
PepEST + 5 μM inhibitor
A400
1
0.5
0
0
10
20
30
40
50
Time (min)

Figure 7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Col-0
P3
Lesion diameter (mm)
0
1
2
3
4
5
3
5
Day after inoculation
E
Number of spores / lesion
0
1000
2000
3000
4000
5000
6000
Ws-0
P3
Col-0

Figure 8