Research Note

Oligogalacturonide-Mediated Induction of a Gene Involved in Jasmonic Acid Synthesis in Response to the Cell-Wall-Degrading Enzymes of the Plant Pathogen *Erwinia carotovora*

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Identification of *Arabidopsis thaliana* genes responsive to plant cell-wall-degrading enzymes of *Erwinia carotovora* subsp. *carotovora* led to the isolation of a cDNA clone with high sequence homology to the gene for allene oxide synthase, an enzyme involved in the biosynthesis of jasmonates. Expression of the corresponding gene was induced by the extracellular enzymes from this pathogen as well as by treatment with methyl jasmonate and short oligogalacturonides (OGAs). This suggests that OGAs are involved in the induction of the jasmonate pathway during plant defense response to *E. carotovora* subsp. *carotovora* attack.

Additional keyword: elicitors.

Erwinia carotovora subsp. *carotovora* and related soft-rot *Erwinia* spp. constitute one of the most important groups of plant-pathogenic bacteria affecting a wide range of plant species (Pérombelon and Kelman 1980). The virulence of these bacteria is correlated with the ability to produce and secrete a large array of cell-wall-degrading enzymes, including pectinases, cellulases, and proteases, that macerate plant tissue and release nutrients for bacterial growth (Collmer and Keen 1986; Kotoujansky 1987; Pirhonen et al. 1991). These enzymes, in particular pectinases, appear to play a dual role in the interaction between *E. carotovora* subsp. *carotovora* and plants; besides being the main virulence determinants of the pathogen they also induce defense responses in plants (Davis et al. 1984; Palva et al. 1993; Vidal et al. 1997, 1998). Inoculation of *Arabidopsis thaliana* plants with *E. carotovora* subsp. *carotovora* results in the development of soft-rot symptoms that can be reproduced by treatment of plants with culture filtrates (CF) from *E. carotovora* subsp. *carotovora* containing the secreted plant cell-wall-degrading enzymes of the pathogen. Treatment with CF has also been shown to induce the expression of a number of previously characterized,

defense-related genes in *A. thaliana* (C. Norman, S. Vidal, and E. T. Palva, *unpublished*).

To elucidate the *A. thaliana* defense response to *E. carotovora* subsp. *carotovora*, we wanted to identify new CF-induced genes. The mRNA differential display (Liang and Pardee 1992) was employed to identify such genes. Differences in mRNA populations from untreated (control) and CF-treated plants were characterized in *A. thaliana* ecotype C24. A 421-bp cDNA fragment present in higher amounts in CF-treated samples was isolated (Fig. 1A), cloned, sequenced, and used as a probe in RNA-gel blot hybridization experiments to confirm the differential expression pattern (Fig. 1B). The cDNA fragment apparently corresponded to a gene that was transiently induced upon CF treatment. A search in data bases revealed that the nucleotide sequence of the cDNA fragment was identical to bases 1230 to 1651 of an *A. thaliana* sequence identified as an expressed sequence tag (TC14169, TIGR data base; The Institute for Genomic Research, Rockville, MD). The deduced amino acid sequence of this gene shows extensive similarity to allene oxide synthase (AOS) (EC 4.2.1.92, hydroperoxide dehydratase), an enzyme involved in the biosynthesis of jasmonates (Vick and Zimmerman 1987). Alignment of the deduced amino acid sequence of the isolated AOS-like gene (designated *Ataol*) with other AOS proteins showed 46% sequence identity to a previously identified *A. thaliana* AOS (Laudert et al. 1996) and 58, 47, and 48% amino acid identity when compared with the pepper, guayule, and flaxseed AOS, respectively. Figure 1C shows the alignment of partial amino acid sequence of *Ataol* with other known plant AOS sequences. Highly conserved regions such as a heme-binding domain and an oxygen-binding pocket characteristic of cytochrome P450 enzymes and present in all known AOS are also evident in *Ataol* (boxed). This suggests that *Ataol* encodes a novel, AOS-like enzyme from *A. thaliana*.

AOS is the first enzyme after lipxygenase in the biosynthetic pathway of jasmonates. Jasmonates originate from linolenic acid via the inducible octadecanoid pathway consisting of several enzymatic steps (Mueller 1997). AOS

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catalyzes the conversion of fatty acid hydroperoxides to unstable allene epoxides (Song and Brash 1991), which are converted into the jasmonate precursor 12-oxophytodienoic acid through the action of allene oxide cyclase. Jasmonates constitute a large family of compounds, of which jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA) are the major physiologically active substances (Sembdner and Parthier 1993). Jasmonates are produced in plants upon wounding (Creelman et al. 1992), elicitor treatment (Gundlach et al. 1992; Doares et al. 1995), and pathogen attack (Penninckx et al. 1996).

Enzymes involved in jasmonate biosynthesis can be induced by elicitors of jasmonate production as well as by JA itself. For example, lipoxygenases have been shown to be induced by wounding, pathogen attack, or direct treatment with exogenous MeJA in *A. thaliana* (Bell and Mullet 1993; Melan et al. 1993). Similarly, the previously identified *A. thaliana* AOS was shown to be induced by wounding (Laudert et al. 1996).

To characterize the expression pattern of *Ataol*, we analyzed the temporal mRNA accumulation for this gene by RNA-gel

blot hybridization after treatment of *A. thaliana* plants with CF or MeJA, or by wounding. Accumulation of the *Ataol* transcript was compared with the induction patterns of the previously isolated *A. thaliana* AOS gene (*Aos*) and two other CF-induced genes in *A. thaliana*, encoding a vegetative storage protein (*Atvsp*) and a hevein-like protein (*Hel*). *Atvsp* has previously been shown to be induced by MeJA and wounding (Berger et al. 1995) whereas *Hel* has been shown to be up-regulated by ethylene and turnip crinkle virus infection (Potter et al. 1993). Therefore, these genes were used as markers for the jasmonate and ethylene pathways, respectively.

As seen in Figure 2, CF treatment resulted in accumulation of all four mRNAs studied. Transcript accumulations of *Ataol*, *Aos*, and *Atvsp* showed similar patterns in response to CF treatment. The three transcripts accumulate within 3 to 6 h after treatment, reaching maximum levels within 9 to 12 h and decreasing to almost control levels 24 to 48 h after treatment. In contrast, induction of the ethylene-responsive gene *Hel* by CF was observed 3 h after treatment with CF and continued to increase until 24 h. Forty-eight hours after treatment a de-

Fig. 1. Identification of an *Arabidopsis thaliana* cDNA induced upon treatment with culture filtrates from *Erwinia carotovora* subsp. *carotovora*. **A**, *A. thaliana* ecotype C24 plants were grown axenically for 3 weeks on sterile MS-2 medium (Murashige and Skoog 1962) in a Conviron growth chamber at 22°C with a 16-h light regime (120 to 170 $\mu\text{E s}^{-1} \text{m}^{-2}$). Plants were sprayed with 1 ml of culture filtrate (CF) from *E. carotovora* subsp. *carotovora* SCC3193, prepared as previously described (Vidal et al. 1998), and leaf samples corresponding to 12 plants were harvested 12 h after treatment. Total RNA was prepared from treated (CF) and untreated control (CO) plants as previously described (Vidal et al. 1998) and RNA samples were analyzed by mRNA differential display. Equal amounts of RNA were ensured by spectrophotometrical measurement of OD at 260 nm. A differentially expressed band corresponding to a 421-bp cDNA fragment, indicated with the arrow, was amplified by polymerase chain reaction (PCR) with the T₁₂MC anchored primer and the AP-2 random primer from the RNAmapping kit (GenHunter, Brookline, MA) and cloned into the pCRII-vector (Invitrogen, San Diego, CA). **B**, *A. thaliana* plants grown as described in **A** were sprayed with 1 ml of CF and leaf samples corresponding to 12 plants were harvested at the indicated time points (hours). Total RNA was isolated and poly A⁺ RNA was purified from each sample with oligo dT Dynabeads (Dynal, Oslo, Norway) and analyzed by RNA-gel blot hybridizations. Poly A⁺ RNA (250 ng) was separated on formaldehyde-agarose gels, transferred to nylon membranes, and hybridized to a ³²P-labeled probe corresponding to the isolated cDNA fragment. **C**, Alignment of specific regions (a, b, and c) of several known plant allene oxide synthases (AOSs). The deduced amino acid sequence of *Ataol* (accession no. TC14169) was aligned to AOS sequences from pepper (accession no. U51676), *A. thaliana* (accession no. X92510), flax seed (accession no. P48417), and guayule (accession no. A56377). Boxed are an oxygen-binding pocket (a and b) and a heme-binding domain (c) characteristic of cytochrome P450 enzymes. Identical residues (*); similar residues (•).

crease in transcript accumulation was evident. Treatment with MeJA or wounding resulted in a strong induction of *Ataol*, *Aos*, and *Atvsp* transcripts whereas a very weak or no induction of *Hel* was observed. However, the pattern of induction differed between the *Ataol*, *Aos*, and *Atvsp* genes in response to MeJA or wounding. The rapid transcript accumulation of *Aos* reached its highest levels within 3 h after MeJA treatment and within 1 h after wounding, then started to decline by 6 h after treatment. In comparison, the *Atvsp* gene was induced within 3 h of either treatment and the high level of expression was maintained for at least 12 h. A third pattern of expression in response to MeJA or wounding was seen for the *Ataol* transcript. Accumulation of *Ataol* transcripts started 3 h after treatment, reached maximum levels 9 to 12 h after treatment and rapidly declined to background levels at 24 h. In conclusion, both the *Ataol* and *Aos* genes are responsive to all three treatments although they show different induction patterns. An interesting observation is that the expression of the *Ataol* gene showed a similar induction pattern after treatment with CF or MeJA. This differed clearly from the induction of the *Aos* gene, which showed a similar induction pattern after wound or MeJA treatments. Furthermore, *Ataol* was induced by CF to a higher extent than by wounding, while *Aos* was found to be more responsive to wounding than to CF. Taken together, these results suggest that *Ataol* could be pathogen regulated, while *Aos* may represent a more wound-responsive gene.

The data provided in Figure 2 suggest that the induction pattern of *Ataol* seen in CF-treated plants could be mediated

Fig. 2. Accumulation of *Ataol*, *Ataol*, *Ataol*, *Hel*, and *Atvsp* transcripts after treatment with culture filtrates from *Erwinia carotovora* subsp. *carotovora*, methyl jasmonate, and wounding. *Arabidopsis thaliana* plants were sprayed with 1 ml of culture filtrates (CF) from *E. carotovora* subsp. *carotovora*. Treatments with methyl jasmonate (MeJA) were done by placing a cotton stick containing 46 nmol of MeJA (Bedoukian Research, Danbury, CT) diluted in 96% ethanol, in a 300-ml jar. Wounding was done by crushing the leaves with forceps. Leaf samples corresponding to 12 plants were harvested at the indicated time points; total RNA was isolated and analyzed by RNA-gel blot hybridizations. Ten micrograms of RNA was separated on formaldehyde-agarose gels, transferred to nylon membranes, and hybridized to the following ³²P-labeled probes: the isolated 421-bp cDNA fragment corresponding to *Ataol*, the polymerase chain reaction (PCR)-generated DNA fragments corresponding to bases 131 to 595 from the cDNA sequence of an *A. thaliana* hevein-like gene (*Hel*) (Potter et al. 1993), to bases 3 to 236 of the clone ATTSO751 (Höfte et al. 1992) encoding an *A. thaliana* vegetative storage protein acid phosphatase (*Atvsp*), and to bases 379 to 854 from the cDNA sequence of the previously isolated *A. thaliana* allene oxide synthase gene (*Aos*) (Laudert et al. 1996). To ensure equal loading of the RNA samples the filters were hybridized with a probe against ribosomal RNA.

by the recognition of signals from the pathogen leading to the induction of genes coding for enzymes in the octadecanoid pathway. At the same time, treatment with MeJA could lead to a feedback amplification of JA synthesis through the induction

Fig. 3. Expression of *Ataol*, *Atvsp*, and *Hel* upon treatment with short oligogalacturonides. **A**, Time courses of mRNA accumulation for *Ataol*, *Atvsp*, and *Hel* were analyzed by RNA-gel blot hybridizations after *Arabidopsis thaliana* plants were sprayed with 1 ml of the following: culture filtrate from *Erwinia carotovora* subsp. *carotovora* (CF), the PehA endopolygalacturonase from *E. carotovora* subsp. *carotovora* with an activity similar to that present in the CF (7 units per ml) (one unit = amount of enzyme necessary to produce 0.1 μ mol of glucose equivalent per hour), prepared as previously described (Vidal et al. 1998), 0.1 mM di-galacturonic acid (dimers), 0.1 mM tri-galacturonic acid (trimers), 0.5% polygalacturonic acid digested during 30 min with 7 U of PehA and later autoclaved (PGA/PehA), and 10 mM MgCl₂. PGA, dimers, and trimers were purchased from Sigma (St. Louis, MO). Ten micrograms of total RNA was hybridized to ³²P-labeled probes corresponding to *Ataol*, *Atvsp*, and *Hel* as described in the legend of Figure 2. **B**, Transcript accumulation of *Ataol* and *Hel* was analyzed by RNA-gel blot experiments after *A. thaliana* plants were sprayed with 1 ml of the following: water control (CO), culture filtrate from *E. carotovora* subsp. *carotovora* (CF), oligogalacturonides at a concentration of 0.1 mM with a degree of polymerization (DP) ranging from 2 to 8 (kindly provided by Sean D. Simpson, The Plant Laboratory, Department of Biology, University of York, UK), and 0.5% PGA. Treatments were done for 10 h. Ten micrograms of total RNA was hybridized to ³²P-labeled probes corresponding to *Ataol*, *Atvsp*, and *Hel*. To ensure equal loading of the RNA samples the filters were hybridized with a probe against ribosomal RNA.

of *Ataol*. This feedback amplification has been shown for *Aos* (Laudert and Weiler 1998) and for lipoxygenase, another enzyme involved in JA synthesis (Melan et al. 1993). Since JA appears to be necessary for both the wound and pathogen defense response of plants (Howe et al. 1996; Vijayan et al. 1998), it is conceivable that different AOSs might modulate the increase in jasmonate levels in response to different stimuli such as pathogens or wounding.

The mechanism by which extracellular enzymes of *E. carotovora* subsp. *carotovora* induce defense responses in plants has been proposed to involve the release of oligogalacturonide (OGA) elicitors from plant cell wall pectin (Davis et al. 1984; Yang et al. 1992; Palva et al. 1993). OGAs induce a wide variety of responses in plants, which in most cases depend on the degree of polymerization (DP). These responses include rapid changes at the cell surface such as phosphorylation of plasma membrane-associated proteins (Farmer et al. 1991; Reymond et al. 1995), accumulation of phytoalexins and lignification (Ebel and Cosio 1994), induction of proteinase inhibitors (Ryan 1988), JA production (Doares et al. 1995), and the biosynthesis of ethylene (Tong et al. 1986). Although most reports show that OGAs with a DP of 9 and greater induce defense responses against pathogens (Darvill et al. 1992), smaller oligomers have also been shown to activate defenses in plants. For example, OGAs with a DP 2 to 4 have been shown to induce resistance against *E. carotovora* subsp. *atroseptica* in potato (Weber et al. 1996; Wegener et al. 1996). Furthermore, OGAs with a DP less than 8 can trigger ethylene production (O'Donnell et al. 1996; Simpson et al. 1998), and the accumulation of proteinase inhibitors in tomato (Moloshok et al. 1992; O'Donnell et al. 1996).

Pectolytic enzymes from several *Erwinia* spp. have been shown to digest the polygalacturonate from plant cell walls into short uronate oligomers, predominantly dimers and trimers (Forrest and Lyon 1990; Preston et al. 1992; Bartling et al. 1995). This prompted us to study the role of short OGAs in the CF-induced expression of plant defense-related and JA-responsive genes. Transcript accumulation of *Ataol*, *Atvsp*, and *Hel* was analyzed by RNA-gel blot hybridizations after treatment of plants with CF, the PehA endopolygalacturonase (one of the major pectolytic enzymes from *E. carotovora* subsp. *carotovora*) (Saarilahti et al. 1990), dimers, trimers of polygalacturonic acid (PGA), and PehA-digested polygalacturonic acid (PGA/PehA) (Fig. 3A). The results clearly indicate that dimers and trimers of PGA as well as the digested PGA induce the expression of *Ataol* and *Atvsp* with induction levels and kinetics similar to those resulting from treatment with CF or PehA. In contrast, although *Hel* was strongly induced by CF or PehA, very low or no induction of *Hel* gene expression was observed upon treatment with short OGAs. As mentioned above, OGAs with a DP of 8 and shorter have been shown to induce ethylene production in tomato plants by 30 min after treatment (O'Donnell et al. 1996; Simpson et al. 1998). If this is true for *A. thaliana*, treatment with these short OGAs should trigger the expression of ethylene-responsive genes such as *Hel*. To elucidate the role of the DP of OGAs in *Hel* and *Ataol* expression, we treated plants with OGAs with a DP of 2 to 8 and characterized the induction of *Hel* and *Ataol* by RNA-gel blot hybridization (Fig. 3B). All treatments induced the expression of *Ataol* and maximum levels of transcript accumulation were observed after treatment with CF

as well as with OGAs with a DP ranging from 2 to 5. However, accumulation of *Hel* transcripts was only marginally induced after treatment with OGAs and did not reach the levels observed upon treatment with CF. Taken together, the results suggest that short OGAs released by the action of pectinolytic enzymes mediate the activation of JA-responsive genes upon *E. carotovora* subsp. *carotovora* attack. Application of OGAs with a DP of 4 to 6 to tomato plants through the transpiration stream has been shown to induce ethylene production (Simpson et al. 1998). However, in our system, treatment with short OGAs by spraying *A. thaliana* plants may not lead to a concentration of ethylene high enough to induce expression of the *Hel* gene. This suggests that the production of short cell wall fragments is not the only way that hydrolytic enzymes from *E. carotovora* subsp. *carotovora* elicit defense responses in plants.

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