Transgenic Plants Producing the Bacterial Pheromone *N*-Acyl-Homoserine Lactone Exhibit Enhanced Resistance to the Bacterial Phytopathogen *Erwinia carotovora*

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Bacterial pheromones, mainly different homoserine lactones, are central to a number of bacterial signaling processes, including those involved in plant pathogenicity. We previously demonstrated that *N*-oxoacyl-homoserine lactone (OHL) is essential for quorum sensing in the soft-rot phytopathogen *Erwinia carotovora*. In this pathogen, OHL controls the coordinate activation of genes encoding the main virulence determinants, extracellular plant cell wall degrading enzymes (PCWDEs), in a cell density-dependent manner. We suggest that *E. carotovora* employ quorum sensing to avoid the premature production of PCWDEs and subsequent activation of plant defense responses. To test whether modulating this sensory system would affect the outcome of a plant–pathogen interaction, we generated transgenic tobacco, producing OHL. This was accomplished by ectopic expression in tobacco of the *E. carotovora* gene *expI*, which is responsible for OHL biosynthesis. We show that *expI*-positive transgenic tobacco lines produced the active pheromone and partially complemented the avirulent phenotype of *expI* mutants. The OHL-producing tobacco lines exhibited enhanced resistance to infection by wild-type *E. carotovora*. The results were confirmed by exogenous addition of OHL to wild-type plants, which also resulted in increased resistance to *E. carotovora*.

Additional keywords: autoinducer, elicitor, systemic resistance.

Quorum sensing is central to a number of physiological responses in bacteria, including bioluminescence, conjugation, production of secondary metabolites, and pathogenicity (Fuqua et al. 1996; Salmond et al. 1995; Swift et al. 1996). The bacteria are able to monitor their population density by producing diffusible pheromones, usually different acyl-homoserine lactones. The accumulation of such a pheromone (autoinducer) over a threshold level at a certain cell density (quorum) will subsequently trigger the regulons controlled by

this particular pheromone and result in the bacterial response (Fuqua et al. 1994; Kaplan and Greenberg 1985; Pierson et al. 1998; Von Bodman and Farrand 1995).

We previously demonstrated that the pheromone *N*-(3-oxohexanoyl)-homoserine lactone is an essential component controlling virulence of the soft-rot phytopathogen *Erwinia carotovora* (Pirhonen et al. 1993). Accumulation of OHL is required for the coordinate activation of genes encoding the main virulence determinants of this pathogen, extracellular plant cell wall degrading enzymes (PCWDEs) (Jones et al. 1993; Pirhonen et al. 1991; Pirhonen et al. 1993). The *expI* gene of *E. carotovora* directs the synthesis of *N*-(3-oxohexanoyl)-homoserine lactone and related OHLs in *E. carotovora*. The synthesis of OHLs is from the common intermediates of cellular metabolism in prokaryotes and eukaryotes as well as S-adenosyl methionine and acyl-ACP or acyl-CoA (Jiang et al. 1998; Val and Cronan 1998). Homoserine lactone synthases such as LuxI in *Photobacterium fischeri* and the *expI* gene product in *E. carotovora* directs the conversion of these metabolites into OHL (Salmond et al. 1995; Sitnikov et al. 1995). Mutations in the *expI* gene result in an avirulent phenotype as a result of the lack of OHL because this pheromone is required for the coordinated production of PCWDEs at high cell densities (Pirhonen et al. 1993). The mutant phenotype can be complemented extracellularly by the addition of OHL to plants (Pirhonen et al. 1993).

We have shown that PCWDEs of *E. carotovora* release pectic fragments from the plant cell wall that elicit plant defense. Such elicitors trigger the activation of plant defense-related genes and the development of systemic resistance to *E. carotovora* in plants, independent of salicylic acid but involving ethylene and jasmonic acid signaling (Norman-Setterblad et al. 2000; Palva et al. 1993; Vidal et al. 1997; Vidal et al. 1998).

A quorum sensing mechanism controlling the expression of virulence genes could facilitate the establishment of a successful infection. We reasoned that the introduction of the pheromone synthesis into plants would alter the situation to the benefit of the plant host by triggering PCWDE synthesis, which is already at low cell densities. This could lead to the premature induction of plant defense and compromise the infection. To test this hypothesis, we generated transgenic

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tobacco expressing the *E. carotovora* gene *expI*, which is responsible for the pheromone biosynthesis in the pathogen. We show that *expI*-positive transgenic tobacco lines produce a biologically active pheromone, resulting in enhanced resistance to infections by *E. carotovora*.

RESULTS

Ectopic expression of *expI* in tobacco results in OHL production.

To achieve production of the OHL bacterial pheromone in plants, the *expI* gene of *E. carotovora* was first cloned under control of the *Cauliflower mosaic virus* (CaMV) 35S promoter (Fig. 1A), which is known to provide high-level expression of chimeric genes in plants. This chimeric *expI* gene was introduced in tobacco (*Nicotiana tabacum* cv. Samsun) by standard Agrobacterium-mediated transformation as well as through selection for kanamycin resistance. The presence of the chimeric *expI* gene in 15 kanamycin-positive, morphologically normal tobacco lines was verified by Southern hybridization and/or polymerase chain reaction (PCR) amplification (data not shown). Expression of the chimeric *expI* gene in the transgenic lines was subsequently characterized by Northern blot hybridization and reverse-transcription (RT)-PCR (Fig. 1B). The majority of the transgenic plant lines but none of the vector-transformed control plants appeared to accumulate a specific *expI*-related transcript that was slightly larger than the *expI* transcript in bacteria. On the basis of the

expI transcript in bacteria. On the basis of the RT-PCR and Northern hybridization results, we selected two plant lines (22 and 39) for a more detailed analysis. Both lines appeared to accumulate clearly detectable amounts of *expI*-related transcripts.

To study whether the tested *expI* expressing lines also produced an active OHL synthase and, as a consequence, a biologically active pheromone, organic extractions of transgenic and control plants were performed. The presence of OHL and related pheromones in these extracts was characterized by measuring bioluminescence of the *E. carotovora expI* mutant (SCC3065) harboring the *lux* indicator plasmid pHV200-I (Gray and Greenberg 1992; Pearson et al. 1994) (Fig. 2). The indicator plasmid carries all the *lux* genes from *P. fischeri* but is mutated for the pheromone synthase gene *luxI*. Thus, light production is achieved only if OHL is added to the system. As a positive control, we employed similar extracts from the OHL-producing wild-type *E. carotovora* strain SCC3193. The results show that *expI* expressing transgenic tobacco produced substantial amounts of the biologically active pheromone (Fig. 2), whereas there was no biological activity in the vector-transformed control plants. Complementation of the *expI* mutant strain SCC3065 on enzyme indicator plates suggests that the plant-derived OHL preparation also was able to complement the PCWDE-negative phenotype of this mutant in vitro.

Partial complementation of *expI* mutants in OHL-producing tobacco.

The level of OHL production observed in transgenic plants (about 1 µg per g of fresh weight) suggested that there might

Fig. 1. The chimeric *expI* gene and its expression in transgenic tobacco plants. **A**, The chimeric *expI* gene construct in pAG6 introduced into tobacco contains the coding region of *expI* from the wild-type *Erwinia carotovora* strain SCC3193 under control of the *Cauliflower mosaic virus* 35S promoter, followed by the 3' untranslated part of the *nos* gene from T-DNA. **B**, Analysis of *expI* expression in the transgenic tobacco lines by reverse transcription-polymerase chain reaction. Total RNA was isolated from each plant, and 0.5 µg of this RNA was amplified with *expI*-specific primers. Numbers 4, 5, 6, 7, 12, 13 indicate individual transgenic plants from vector-transformed control lines (VC) and lines 22 and 39 transformed with the chimeric *expI* gene. Plasmid DNA (ExpI) harboring the *expI* gene from *E. carotovora* strain SCC3193 was used as a positive control. The negative reaction control is indicated by a (-).

Fig. 2. Transgenic plants produce a biologically active bacterial pheromone. Pheromone activity was characterized in organic extracts of transgenic *expI*-expressing tobacco plants. The presence of *N*-oxoacyl-homoserine lactone (OHL) and related pheromones in these extracts was determined by measuring bioluminescence of *Erwinia carotovora* strain SCC3065 harboring the *lux* indicator plasmid pHV200-I. Emission of light was measured at times indicated and expressed as relative light units per optical density at 600 nm. The extract from transgenic plants is indicated by filled squares (■). As a positive control, we used an OHL extract prepared from the *E. carotovora* wild-type strain SCC3193 (▲). As a negative control, we used OHL extract prepared from the vector-transformed plants (□). The control culture (SCC3065 pHV200-I) contained no additions and shows the background bioluminescence produced by the test system (○). Results show the means of three independent experiments. Standard deviation is indicated.

be sufficient amounts of OHL to affect the bacterial phenotype in planta. To test this, we first characterized whether the OHL-negative phenotype of the *expl* mutant SCC3065 could be complemented in planta. This mutant is avirulent (Pirhonen et al. 1991; Pirhonen et al. 1993) essentially because of the lack of OHL-dependent PCWDE production required for plant-tissue maceration and that it is not able to multiply in planta. To assess the complementation by in planta-produced OHL, control and transgenic plants were inoculated with this mutant. The results show that the *expl* mutant phenotype was complemented partially by ectopic expression of the chimeric *expl* gene in transgenic tobacco (Fig. 3). As expected, the mutant SCC3065 did not grow in control plants harboring the empty vector but its numbers were reduced rapidly. In contrast, when the mutant was inoculated into the transgenic tobacco seedlings, producing OHL, growth was transiently restored (Fig. 3). Interestingly, the complementation of the avirulent phenotype was not complete, although the bacterial growth was initially restored, the bacterial numbers were reduced at later time points. We did not observe tissue maceration in the transgenic *expl* plants. This is in contrast to infection with wild-type strain SCC3193, which resulted in clear tissue maceration (Fig. 4). In conclusion, the results indicate that there is sufficient OHL production by the transgenic plants to affect bacterial behavior in planta.

Enhanced disease resistance in OHL-producing tobacco.

We previously suggested that *E. carotovora* employs the quorum sensing system to avoid premature detection by the plant defense response (Pirhonen et al. 1993). To test whether OHL production in planta would facilitate plant resistance, we compared the ability of wild-type *E. carotovora* (SCC 3193)

to cause disease on vector-transformed control plants and its ability on OHL-producing transgenic tobacco (Fig. 4). The ability of the pathogen to cause disease was tested on tobacco seedlings. Two subsequent generations of axenically grown seedlings were used for these experiments, giving essentially similar results. The ability of *E. carotovora* to cause disease was reduced to about half in OHL-producing transgenic lines as compared with the plant line harboring only the vector (Fig. 4). Even after prolonged incubation (48 h) with the pathogen, the OHL-producing lines were clearly more tolerant to the initiation of infection. If the infection was established and tissue maceration started, however, we could not detect significant differences in bacterial growth between the OHL-producing and control plants (data not shown). Similarly, increasing the inoculum size by a factor of four or more could overwhelm the enhanced tolerance to *E. carotovora* infection in OHL-producing tobacco (data not shown). Taken together, these data suggest that production of a bacterial pheromone in transgenic plants will modulate the balance in a plant-pathogen interaction in favor of the plant.

Fig. 3. Complementation of the avirulent *expl* mutant phenotype in planta. Transgenic tobacco lines 22 (dotted bars) and 39 (open bars) as well as vector-transformed control plants (solid bars) were inoculated locally with 3×10^6 colony forming units of the *expl* mutant strain SCC3065 of *Erwinia carotovora*. Bacterial growth in locally inoculated tobacco leaves was determined after 0, 24, 48, and 72 h by a viable count from leaf extracts. Each value is a mean of six plants inoculated, and the standard deviation is indicated. Results were analyzed statistically by means of a Student's *t* test. *P* values for the differences between vector control and transgenic lines inoculated with SCC3065 at 24 h postinoculation were 0.000015 for line 22 and 0.000012 for line 39, respectively.

Fig. 4. Transgenic plants expressing *expl* show enhanced resistance to bacterial infection. **A**, The development of disease symptoms (tissue maceration) caused by locally inoculated SCC3193 was assessed visually and scored as no maceration (on the left) or maceration (on the right). **B**, Transgenic tobacco seedlings of lines 22 (dotted bars) and 39 (open bars) as well as those of vector-transformed controls (solid bars) were inoculated locally with 2×10^6 CFU per plant of the *Erwinia carotovora* wild-type strain SCC3193. Symptoms were assessed 24 and 48 h after inoculation, and plants showing detectable maceration were scored as infected. The data are from two independent experiments, and each data point represents 30 to 40 individual plants. Results were analyzed statistically by a Student's *t* test. *P* values for the differences between vector control and transgenic lines at 24 h postinoculation were 0.00018 for line 22 and 0.0001 for line 39, respectively.

Exogenous OHL triggers enzyme gene expression and enhances resistance of tobacco.

One possibility for the enhanced resistance observed in transgenic tobacco is that the presence of exogenous OHL would trigger production of PCWDEs before sufficient population of *E. carotovora* has been established. To test this possibility, we first characterized the effect of added OHL on enzyme gene expression at low cell densities in vitro. To facilitate this analysis, we used a fusion construct where the reporter gene *uidA* was placed under control of the gene for polygalacturonase *pehA*. The results of this analysis (Fig. 5) showed a rapid induction of *pehA* expression after OHL addition, although the cells were still in early logarithmic growth phase and did not normally show significant expression of this gene.

To confirm that the production of OHL was the probable cause of resistance in the transgenic tobacco plants, we tested whether the exogenous addition of OHL to wild-type inoculum would affect the outcome of the infection. This was done by local inoculation of axenically grown vector control plants by wild-type strain SCC3193. Whereas the untreated control inoculum of SCC3193 resulted in disease development (tissue maceration) in most of the plants, the addition of OHL clearly reduced the number of plants developing maceration symptoms (Fig. 6 and Table 1).

DISCUSSION

Our data demonstrates that it is possible to produce bacterial pheromones in transgenic plants by ectopic expression of a pheromone synthase gene (*expI*) and that this production can alter the plant-pathogen interaction. Similar results to ours were published by Fray et al. (1999), although they used a

Fig. 5. Induction of *pehA::uidA* expression by OHL in vitro. The *Erwinia carotovora* subsp. *carotovora* strain SCC6014 (*pehA::gusA*) was propagated and diluted continuously in L medium, as described in text. The bacterial culture resuspended in fresh medium was subsequently divided into two parts after 2 h (arrow). One part was supplemented with *N*-oxoacyl-homoserine lactone (OHL) at 5 µg per ml (♦), whereas the other part received no OHL addition (■). Samples were taken at times indicated, and β-glucuronidase activities were determined. Values are the means of four different measurements, and the standard deviation is indicated.

different, but related, pheromone synthase gene (*yenI*). Both of these studies clearly show production of biologically active bacterial pheromones in plants as determined by different bioassays. Interestingly, Fray et al. (1999) observed pheromone production only if the synthase was targeted to the chloroplast, not if it was localized in the cytoplasm. In contrast, our studies showed that synthase localized in the cytoplasm was able to direct the synthesis of substantial amounts of the active pheromone. The reason for this difference is not clear but could be the result of the different promoters used to drive the pheromone synthase gene and/or the different synthase genes used. The *expI* gene employed in our studies does produce high amounts in OHL in the bacteria (Pirhonen et al. 1993).

The evidence for active pheromone production in *expI* expressing transgenic plants is twofold. First, we obtained organic extracts from the transgenic plants that contained pheromone activity when tested in a pheromone bioassay. This bioassay detects OHL and related pheromones and is based on the external complementation of a *luxI* mutant in the *lux* operon of *P. fischeri* (Gray and Greenberg 1992; Kaplan and Greenberg 1985). The *luxI* gene directs OHL synthesis and controls bioluminescence in this marine bacterium. Second, we show that the avirulent *expI* mutant of *E. carotovora* can multiply transiently in the pheromone-producing transgenic plants but not in the vector-transformed controls. These studies demonstrate that a biologically active pheromone is produced in these plants and that the pheromone is synthesized in sufficient quantities to affect the infecting *E. carotovora*.

Our result provides the first evidence that it is possible to improve plant resistance by engineering bacterial pheromone production into plants. The OHL-producing transgenic lines exhibited enhanced resistance to bacterial infection. This resistance was manifested in the clearly reduced ability of the bacteria to establish infection, suggesting that the early events in this interaction have been altered. If the infection and tissue maceration was initiated, however, the OHL production appeared to be of less value because we could not observe any significant difference in bacterial growth between *expI* transgenic and control plants. The results are in accordance with and support our hypothesis for the role of OHL production in pathogenicity, in which *E. carotovora* employs quorum sensing to avoid the premature production of PCWDEs and subsequent activation of plant defense responses (Pirhonen et al. 1993). According to this model (Fig. 7), *E. carotovora* is initially able to colonize the plant host without triggering plant defense. At low cell densities, OHL does not accumulate in

Table 1. Exogenous *N*-oxoacyl-homoserine lactone (OHL) enhances plant resistance^a

Inoculum	Plants inoculated	Plants macerated
SCC3193 control	33	32
SCC3193 + OHL	33	3

^a Effect of exogenous OHL on virulence of *Erwinia carotovora* wild-type strain SCC3193. Vector-transformed control plants were inoculated locally with 2×10^5 CFU of SCC3193 that was grown overnight in L medium, and cells were pelleted, washed once with 0.9% NaCl, and resuspended in 0.9% NaCl. OHL containing inoculum was supplemented with 5 µg of this compound per ml, whereas the control inoculum contained 0.25% of ethanol (used to solubilize OHL). The maceration symptoms were scored 24, 48, and 72 h postinoculation.

sufficient quantities to activate significant expression of PCWDE genes (Fig. 7A) and few elicitor molecules are released from the plant cell wall. Therefore, the plant is not alerted to the presence of the invading bacteria and allows the bacteria to reach a substantial population size, without being hampered by the plant defense response. When the bacteria reach a critical population density (quorum), the OHL concentration increases above the threshold and turns on the production of PCWDEs, which are required for plant tissue maceration and disease progression. Although the PCWDEs at this stage will release elicitors of plant defense, the bacterial population is large enough to overwhelm plant defenses (Fig. 7A).

Hence, pheromone production is part of the delicate balance governing the outcome of a plant–pathogen interaction, and modulating this balance will provide novel strategies for improving the disease resistance of plants. Our results suggest that in OHL-producing plants, this balance is tilted in favor of the plant (Fig. 7B). Plant-derived pheromone production will result in PCWDE production at low bacterial population densities, eliciting a plant defense when the bacterial population is not sufficient to suppress this response. The likely consequence is that the infection is aborted. This model is supported by the data showing that OHL-producing plants are more resistant than control plants to bacterial infection at lower cell densities, yet this resistance is overcome if the inoculum size is increased. Additionally, the results showing that the transgenic *expI* lines do not exhibit enhanced resistance once the infection is established and the disease is progressing are in agreement with the model.

The model is further supported by *in vitro* studies showing that adding pheromone to exponentially growing *in vitro* cultures can trigger PCWDE gene expression at low cell densities (Fig. 5). Similar results were described previously in Flego (1994). Thus it is likely that premature enzyme gene expression takes place in OHL-producing transgenic plants. Reconstitution experiments with exogenously added OHL strongly indicates that the observed effects on enhanced disease resistance were, indeed, caused by the pheromone (Fig. 6 and Table 1).

Although we believe that the proposed model provides the most likely explanation for the results obtained, we cannot exclude other possibilities. Pheromone production at low cell density might, for example, affect the developmental program of the pathogen such that an essential pathogenicity factor required at the onset of infection is not produced. Additional work is required to distinguish conclusively among the different possibilities.

The results of this study describe a new strategy to improve disease resistance of plants by increasing the plant's ability for pathogen recognition. Production of OHL and related pheromones in plants help the plant to demask pathogens that use pheromone-controlled quorum sensing as a strategy to invade host plants. It is very interesting, in this respect, that Teplitski et al. (2000) demonstrated recently that pea plants naturally produce substances that mimic bacterial pheromones and may determine the outcome of different plant–bacterium interactions.

The results presented in this work and by Fray et al. (1999) clearly suggest novel strategies for modulating plant–microbe interactions. We demonstrated that it is possible to engineer plant resistance to pathogenic bacteria by introducing bacterial

pheromone production into plants. The same strategy can be expanded to other plant–microbe interactions, including enhancement of beneficial interactions. In conclusion, it is likely that similar approaches of bacterial pheromone engineering will find many applications in agriculture.

MATERIALS AND METHODS

Plant material, culture conditions, and transformation.

Samsun was employed in all experiments. Greenhouse plants were grown in soil at 22°C under 16 h of light at 150 $\mu\text{mol m}^{-2}$ per s. For axenic growth, seeds were surface sterilized in 7.5% sodium hypochlorite solution containing 0.1% Tween 20 and rinsed in sterile distilled water. Sterilized seeds were plated in 24-well tissue culture plates containing 1 ml of agar-solidified MS medium (Murashige and Skoog 1962) supplemented with 2% sucrose (MS-2). Plates were thereafter transferred to a growth chamber at 22°C with 16 h of light at 250 $\mu\text{mol m}^{-2}$ per s.

For *Agrobacterium tumefaciens*-mediated transformation, leaf discs were isolated from axenically grown *N. tabacum*. Transformation procedures and tissue culture conditions were essentially the same as described previously (Holmström et al. 2000; Mandal et al. 1993). Transgenic plants were selected on kanamycin-containing media and verified by Southern hybridization or PCR amplification of the chimeric transgene. The identity of the PCR fragments was confirmed by DNA sequencing.

To assess bacterial virulence, surface-sterilized seeds from T₂ and T₃ generations were germinated and grown axenically on solidified selective MS-2 medium for 9 weeks before use in virulence assays.

Bacterial strains, plasmids, media, culture conditions, and chemicals.

Escherichia coli host strains used for plasmid maintenance and DNA preparation were DH5 α (Hanahan 1983) and XL1-blue (Stratagene, La Jolla, CA, U.S.A.). The *E. carotovora* subsp. *carotovora* wild-type strain SCC3193 (Pirhonen and Palva 1988) and the *expI* mutant strain SCC3065 (Pirhonen et al. 1991; Pirhonen et al. 1993) have been described. The *E. carotovora* subsp. *carotovora* strain SCC6014 is a derivative of SCC3193 harboring a kanamycin-resistant (Km^R) *pehA::uidA* insertion in the chromosome. Plasmids pTK806-1 and pTK806-6 were described by Pirhonen et al. (1993), and plasmid pHV200-I was described by Pearson et al. (1994). The transfer of plasmids was performed by standard transformation techniques or electroporation with a gene pulser (Bio-Rad, Richmond, CA, U.S.A.). *E. coli* was propagated in L medium (Miller 1972) at 37°C, and *E. carotovora* at 28°C. Added to the media was 25 μg of Km and 150 μg of ampicillin per ml, when required.

To obtain exponentially growing cultures with a low OHL level, an overnight culture of *E. carotovora* strain SCC6014 was diluted into fresh L medium to an optical density at 600 nm of 0.02. The culture was allowed to grow to OD₆₀₀ = 0.1, and cells were pelleted by centrifugation and resuspended into fresh L medium to OD₆₀₀ = 0.02. The culture was then grown until it reached OD₆₀₀ = 0.05, and the cells were collected as described above and resuspended in the same volume of fresh L medium. After growth, the cells were collected

as described above and resuspended in fresh L medium. OHL was added to 5 µg of media per ml, as indicated.

DNA manipulation, plasmid construction, and determination of the nucleotide sequence.

Unless otherwise stated, the isolation of plasmid DNA or chromosomal DNA, cloning, and gel analysis of plasmid

Fig. 6. Effect of exogenous *N*-oxoacyl-homoserine lactone (OHL) on plant response. Local inoculation of vector-transformed control seedlings with 2×10^5 CFU of the *Erwinia carotovora* wild-type strain SCC3193. The maceration symptoms were documented by photography 72 h after inoculation. The inoculum (1) was supplemented by 5 µg per ml of OHL prior to inoculation, whereas the control (2) did not receive OHL.

DNA were performed by established procedures, as described in Sambrook et al. (1989). The *E. coli* strain DH5α (Hanahan 1983) was used for plasmid amplifications. To fuse the CaMV 35S promoter to the promoterless *expI* gene from *E. carotovora*, the *expI* gene was first amplified by PCR as a *NcoI*–*XbaI* fragment, with the *NcoI* site at the translation initiation codon. This fragment was fused with the 35S promoter amplified by PCR from plasmid pBI121 (Fang et al. 1989) as an *EcoRI*–*NcoI* fragment and cloned into pUC19 (see below for primers used). The resulting *EcoRI*–*XbaI* fragment carrying the chimeric 35S–*expI* gene was subsequently introduced into vector pDE1001 (Denecke et al. 1992). This resulted in the binary plant transformation vector pAG6 that harbored between the T-DNA-derived left and right borders *p35S*–*expI*–*3'nos* and, as a selective marker, the chimeric kanamycin-resistance gene *pnos*–*neo*–*3'ocs*. Before plant transformation, the vector was transferred into *A. tumefaciens* (C58C1Rif^R) containing the nononcogenic Ti plasmid pGV2260 (Deblaere et al. 1985) by electroporation.

The sequencing kit was obtained from United States Biochemicals (Cleveland, OH, U.S.A.); the multiprime DNA labeling kit and radiolabeled compounds were obtained from Amersham International (Little Chalfont, Buckinghamshire, U.K.).

Isolation of chromosomal DNA and total RNA, Southern and Northern blot analysis, and RT-PCR.

Chromosomal DNA was isolated essentially as described by Sambrook et al. (1989), and the plant RNA used for Northern analysis was isolated by the RNeasy kit (Qiagen, Hilden,

Fig. 7. A model for the interaction of *Erwinia carotovora* with control and *expI* transgenic tobacco. **A**, At low cell density, the *N*-oxoacyl-homoserine lactone (OHL) concentration is not sufficient to induce virulence gene expression and plant cell wall degrading enzymes (PCWDE) production in control plants. Therefore, the bacterial population can multiply without triggering a plant defense response (PDR). At high cell density, OHL concentration reaches the threshold level, virulence genes are expressed, and massive PCWDE production results in tissue maceration and disease. **B**, Additional production of OHL by the *expI* transgenic plant leads to the accumulation of OHL to the critical threshold concentration required to activate the virulence genes. This will take place regardless of the bacterial population density. The production of virulence factors, PCWDEs, at low cell density will lead to the release of plant cell wall fragments. These fragments function as elicitors to stimulate plant defense responses and, because the pathogen population density is low, bacteria are not able to overwhelm these defenses and infection is arrested.

Germany). For Southern analysis, chromosomal DNA was digested with restriction enzymes and run on an 0.8% agarose gel. Northern analysis was performed essentially as described (Vidal et al. 1998) with 5 µg of total RNA and a probe specific for *expI*. Probe fragments were labeled with ³²P-dATP by random primer labeling.

For RT-PCR, RNA was isolated from tobacco leaf tissue as described by Verwoerd et al. (1989). DNA was removed by treatment with RNase-free DNase I (Amersham Pharmacia Biotech, Uppsala, Sweden). The RT reaction was performed with 0.5 µg of DNase-treated RNA, 2 pmols of oligo(dT)₁₈, 400 pmols of deoxynucleoside triphosphate (dNTP), 4 µl of enzyme buffer, and water to 19 µl, which were incubated in a thermocycler programmed as 65°C for 5 min, 37°C for 60 min, 95°C for 5 min, and 4°C. After 10 min at 37°C, 1 µl of Moloney murine leukemia virus reverse transcriptase was added.

PCR was performed with 1 µl of transcribed RNA, 10 µmoles of dNTPs, and 10 pmoles of each primer in 30 µl of volume. The amplification cycle was 94°C for 1 min; 30× at 94°C for 30 s, 52°C for 90 s, and 72°C for 90 s; and 72°C for 5 min. The cDNAs were amplified with *expI*-specific primers *expI1* (CGTTCAAGGATAGGCTGAATTGG) and *expI2* (CTCGAAAGACAGTGGCCACTC).

Extraction and assay of OHL.

Fresh leaves (2 g) from control or OHL-producing plants were homogenized with 20 ml of 96% ethanol. The mixture was filtered (0.8 µm pore size), and the plant residue was extracted in the same way with 80% ethanol. The combined filtrate was evaporated to dryness in a rotary evaporator, dissolved in 10 ml of water, and filtered through an Isolute C-18 (KEBO, Stockholm, Sweden) column (25 × 25 mm). The column was then eluted with 50% methanol, and the collected fractions evaporated to dryness. The resulting material was dissolved in 1.0 ml of 100 mM phosphate buffer and stored at -20°C.

The *E. carotovora* strain SCC3193 was cultured at 28°C in 30 ml of L medium to OD₆₀₀ = 3.0, after which cells were removed by centrifugation at 4°C and 16,000 × *g* for 30 min, and then the residual cells were removed by filtration (0.2 µm pore size). These culture filtrates were extracted with an equal volume of ethyl acetate and evaporated to dryness. The residue was dissolved in 3.0 ml of 100 mM phosphate buffer and stored at -20°C.

To assay OHL extracted from plants or bacteria, 100 µl of the corresponding OHL extract was mixed with 100 µl of a sensor strain (*E. carotovora* SCC3065 harboring the pHV200-I⁻ plasmid). Emission of light was measured during a time course (0 to 18) every h by multilabel counter VICTOR1420² (Wallac, Turku, Finland). Results were calculated as slopes of relative light units (RLU), and final values were defined as RLU/OD₆₀₀ (RLU × ml⁻¹ × 10²/OD₆₀₀ × ml⁻¹).

Enzyme assay.

β-Glucuronidase activity was assayed by p-nitrophenyl β-D-glucuronidase as substrate (Novel et al. 1974). The degradation product, p-nitrophenol (pNP), was detected at 405 nm, and β-glucuronidase-specific activities were expressed as pNP liberated per min (OD₆₀₀ unit)⁻¹.

Assay for bacterial virulence and bacterial growth in planta.

The bacteria used in these experiments were propagated overnight in L medium, pelleted by centrifugation, washed

once, and diluted in 0.9% NaCl before inoculation. Bacterial multiplication in planta was determined by locally inoculating 9-week-old seedlings of Samsun with 5 µl of bacterial suspension containing the indicated number of colony forming units, usually 10⁶. The number of viable bacteria in plant extracts was determined at the described previously times indicated (Palva et al. 1994; Vidal et al. 1998).

Bacterial virulence was determined with an infectivity assay and scoring the development of disease symptoms. The indicated amounts of *E. carotovora* propagated and harvested as described above were used to locally inoculate plant leaves (5 µl of inoculum). When used, the addition of exogenous OHL (5 µg of final concentration per ml) was made to the bacterial inoculum prior to inoculation. The 9-week-old axenic tobacco seedling used were grown as described under plant material. After inoculation, plant growth was continued under the same conditions. The development of disease symptoms (leaf maceration) was scored visually at 24, 48, and 72 h after inoculation, documented by photography, and the ratio of infected over inoculated plants was calculated.

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