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Short Communication

The phenylalanine pathway is the main route of salicylic acid biosynthesis in *Tobacco mosaic virus*-infected tobacco leaves

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Abstract In plants, salicylic acid (SA), a molecule important for resistance to pathogens, is synthesized from phenylalanine or isochorismate. Although SA is reportedly synthesized via the isochorismate pathway in pathogen-infected *Arabidopsis*, the predominant pathway in pathogen-infected tobacco has not been known. To determine the pathway in tobacco, we studied the gene expression and enzyme activity of phenylalanine ammonia-lyase (PAL) and isochorismate synthase (ICS) in tobacco leaves infected with *Tobacco mosaic virus* (TMV). Two days after TMV inoculation, necrotic lesions had appeared, and the levels of SA, PAL activity, and transcripts of *PAL A* and *PAL B* had increased substantially. In contrast, no ICS activity was detected, and the levels of *ICS* transcript did not increase, after the formation of necrotic lesions caused by TMV infection. These results suggest that SA is synthesized mainly by the phenylalanine pathway in TMV-infected and necrotic-lesion-bearing tobacco leaves.

Key words: Hypersensitive reaction, isochorismate synthase, phenylalanine ammonia-lyase, *Tobacco mosaic virus*.

In some plants, pathogen infection induces a hypersensitive reaction (HR) in the infected area to restrict the pathogen to the site (Greenberg 1997; Heath 2000). The HR accompanies the formation of necrotic lesions and induces local and systemic resistance against subsequent pathogen attacks. During the HR, high levels of salicylic acid (SA) accumulate to induce the production of defense proteins, including pathogenesis-related proteins, and these SA levels are correlated with the level of resistance (Malamy et al. 1990; Malamy et al. 1992; Wildermuth et al. 2001). Transgenic tobacco and *Arabidopsis* that overexpress a gene for a bacterial salicylate hydroxylase, which degrades SA to an inactive form, do not accumulate SA and are susceptible to pathogen infection (Gaffney et al. 1993; Delaney et al. 1994). These results suggest that SA plays a central role in pathogen resistance in plants.

Until recent years, in tobacco, potato, and *Arabidopsis* SA was thought to be synthesized from the phenylalanine pathway via *trans*-cinnamic acid and benzoic acid (BA) (Leon et al. 1993; Yalpani et al. 1993; Mauch-Mani and Slusarenko 1996; Coquoz et al. 1998; Ribnicky et al. 1998; Figure 1). Phenylalanine ammonia-lyase (PAL) catalyzes the synthesis of *trans*-cinnamic acid from phenylalanine (Bate et al. 1994; Howles et al. 1996). In transgenic tobacco plants in which expression of the endogenous *PAL* gene is suppressed, the level of SA after *Tobacco mosaic virus* (TMV) inoculation is lower than that in non-transgenic plants (Pallas et al. 1996). Application of 2-aminoindan-2-phosphonic acid, which is an inhibitor of PAL, inhibits SA accumulation in pathogen-infected *Arabidopsis* and elicitor-treated potato (Mauch-Mani and Slusarenko 1996; Coquoz et al. 1998). These reports suggest that PAL is an important enzyme in SA synthesis.

In bacteria, SA synthesis proceeds from chorismate via isochorismate (Serino et al. 1995), and isochorismate synthase (ICS) is the rate-limiting enzyme for SA synthesis in *Pseudomonas aeruginosa* (Gaille et al. 2003). Wildermuth et al. (2001) demonstrated that this pathway is also active in pathogen-infected *Arabidopsis* (Figure 1); the *salicylic acid-deficient mutant 2* (*sid2*) could not accumulate SA even after pathogen attack, and this mutant was found to have a defective *ICS1* gene.

We have suggested that in ozone-exposed tobacco, SA is synthesized via the phenylalanine pathway rather than

Abbreviations: BA, benzoic acid; BA2H, benzoic acid 2-hydroxylase; ICS, isochorismate synthase; PAL, phenylalanine ammonia-lyase; RT, reverse transcription; SA, salicylic acid; TMV, *Tobacco mosaic virus*.
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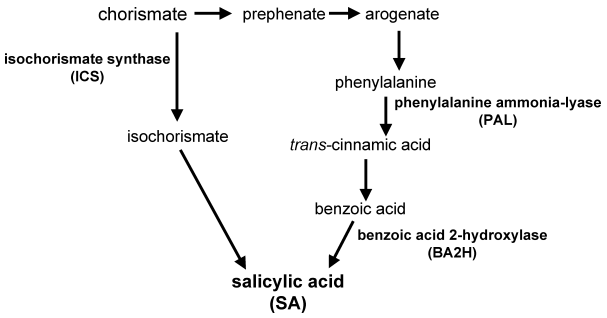

Figure 1. Proposed pathway for biosynthesis of SA.

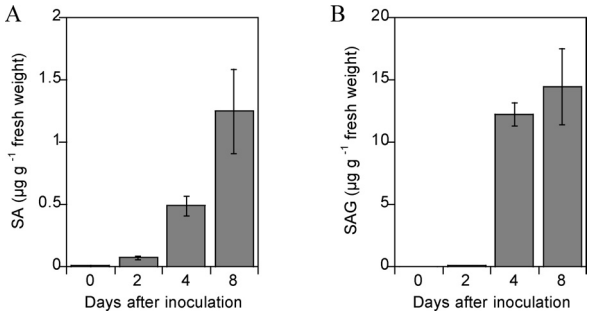

Figure 2. Levels of SA and SAG in TMV-inoculated tobacco. (A) Free SA content. (B) SAG content. Vertical bars represent standard deviations obtained from three replicates.

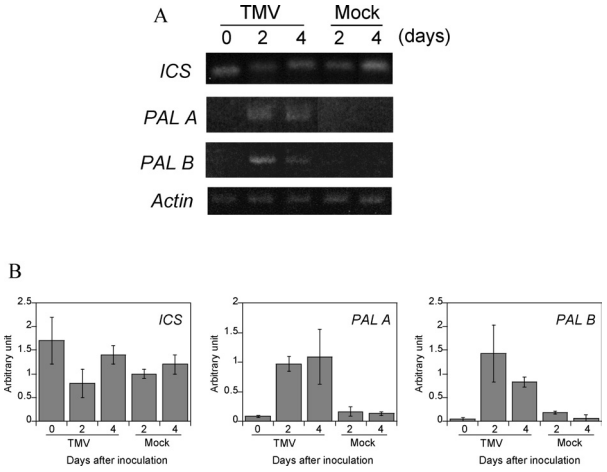

Figure 3. Expression of genes related to SA synthesis. (A) RT-PCR analysis of expression of *PAL A*, *PAL B*, and *ICS*. Total RNAs were obtained from leaves 0, 2, and 4 days after TMV inoculation or mock inoculation. *Actin* was used as a standard for equal loading of RNA. PCR for *ICS*, *PAL A*, *PAL B*, and *Actin* was carried out for 30, 20, 20, and 20 cycles, respectively. Amplified fragments were loaded in 1.3% agarose gel. (B) Levels of transcription of *ICS*, *PAL A*, and *PAL B* were converted to numerical data by using Adobe Photoshop CS (version 8), and arbitrary units for the three genes were calculated by dividing each transcription level into the level of *Actin*. Vertical bars represent standard deviations obtained from three replicates.

the isochorismate pathway; we came to this conclusion from the results of experiments in which we did not observe increases in tobacco ICS activity and *ICS* gene expression after ozone exposure and instead observed a remarkable elevation of PAL activity and *PAL* gene expression (Ogawa et al. 2005). However, although the increase in SA levels in TMV-infected tobacco leaves has been extensively studied (Malamy et al. 1990; Malamy et al. 1992; Seo et al. 1995), the isochorismate pathway has so far not been reported to contribute to SA synthesis in TMV-infected tobacco.

To clarify the contribution of this pathway, we investigated SA biosynthesis in TMV-infected and HR lesion-bearing tobacco plants (*Nicotiana tabacum* cv. Samsun NN) containing the *N* gene for resistance to TMV. Fully expanded upper leaves from 2-month-old 'Samsun NN' tobacco plants were inoculated with a TMV suspension ($2\,\mu g\,ml^{-1}$) and incubated at 20–24°C under a 14-h light/10-h dark cycle at a photosynthetic photon flux density of $100\,\mu mol\,m^{-2}\,s^{-1}$. Necrotic local lesions (1 mm in diameter) caused by the HR were observed 2 days after inoculation, and the lesions continued to grow gradually, reaching a diameter of about 5 mm at 8 days (data not shown). When we determined the SA and salicylic acid β-glucoside (SAG) levels in TMV-inoculated leaves by the method described by Ogawa et al. (2005), the SA level was increased and continued to increase from 0 to 8 days (Figure 2A). High levels of SAG were observed on days 4 and 8 (Figure 2B).

To determine whether the ICS pathway was used for the SA biosynthesis, we carried out reverse transcription (RT)-PCR to detect the expression of *ICS*, referring to the sequence of the tobacco *ICS* gene, which was originally a single-copy gene in the tobacco genome (Ogawa et al. 2005). The primers 5′-AGTGCATAAGAAGAAAATTGGAG-3′ and 5′-CCCGTGCATCTTCTGTTGGATAC-3′ were used for the RT-PCR analysis. The reaction of RT was performed at 42°C for 30 min, and PCR was carried out in cycles of denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min, and elongation at 72°C for 2 min. The number of cycles was adjusted so that the amplification was within the linear range. The nucleotide sequence of the cDNAs amplified by the *ICS* primers was identical to that of tobacco *ICS* (AB182580) (data not shown). The level of expression of *ICS* decreased 2 days after both TMV inoculation and mock inoculation (Figure 3A, B left panel). Next, we determined the enzyme activity of ICS in TMV- and mock-inoculated tobacco leaves, as described by Ogawa et al. (2005). As a positive control, we used *Arabidopsis* exposed to ozone for 6 h to confirm the peak of isochorismate, which is produced from the chorismate substrate by ICS activity (Ogawa et al. 2005). In tobacco leaves at 0, 2, and 4 days after TMV inoculation or mock inoculation, no ICS activity was found (data not shown).

We investigated the expression of tobacco *PAL* genes (*PAL A* and *PAL B*) using TMV- and mock-inoculated

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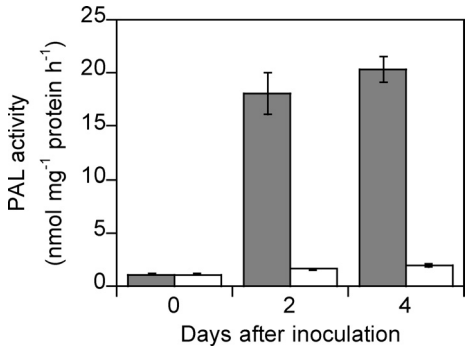

Figure 4. PAL activity after TMV inoculation. Gray and white columns show PAL activity in TMV- and mock-inoculated leaves, respectively. Vertical bars represent standard deviations obtained from three replicates.

tobacco leaves by RT-PCR analysis with the following primer sets: 5′-GCACAAAATGGTCACCAAGAAA-3′ and 5′-AAGCCATTGGGGCGACGTTCTA-3′ for *PAL A*, 5′-CATGTTAATGGAGGAGAAAACT-3′ and 5′-AAGCCATTGTGGAGATGTTCGG-3′ for *PAL B*. The nucleotide sequences amplified by the primers for *PAL A* and *PAL B* were identical to *PAL A* (AB008199) and *PAL B* (AB008120), respectively (data not shown). Expression levels of *PAL A* and *PAL B* markedly increased 2 and 4 days after TMV inoculation, whereas only a slight increase in these levels was found after mock inoculation (Figure 3B, center and right panels). In TMV-infected leaves PAL activity increased significantly. At 2 and 4 days after TMV inoculation, the levels were about 10 times those in the mock-inoculated leaves (Figure 4). This evidence is consistent with the results of Pellegrini et al. (1994). Benzoic acid 2-hydroxylase (BA2H) catalyzes the final step of SA biosynthesis via the phenylalanine pathway. It has been reported that in TMV-inoculated tobacco, levels of BA and SA increase, exogenous BA is metabolized into SA *in vivo*, and BA2H activity is induced (Leon et al. 1993; Leon et al. 1995). Our results and these previous reports suggest that the phenylalanine pathway, not the isochorismate pathway, is the main route of SA biosynthesis in TMV-inoculated tobacco.

We have already reported increments in SA level, *PAL A* and *PAL B* expression, and PAL activity in tobacco exposed to ozone (Ogawa et al. 2005). Treatment of tobacco with TMV also induced these reactions. This result and the results of our previous study indicate that tobacco plants activate expression of the same *PAL* genes and synthesize SA, during the HR and under ozone exposure. It has been already reported that the response to ozone is similar to that to the HR, because both ozone treatment and the HR induce the production of reactive oxygen species and SA accumulation (Schraudner et al. 1998; Rao and Davis 2001). Our report provides new evidence that the response to ozone mimics the HR.

We have shown that SA is synthesized predominantly via the phenylalanine pathway in tobacco during ozone exposure or upon TMV infection. On the other hand, it has been suggested that SA is synthesized via both the isochorismate pathway and phenylalanine pathway in *Arabidopsis* (Mauch-Mani and Slusarenko 1996; Wildermuth et al. 2001). These reports suggest that plants have the ability to synthesize SA via both pathways. The reason for this is not obvious, and to clarify this reason further studies on the pathways of SA biosynthesis are needed.

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