Veinal Necrosis Induced by *Turnip mosaic virus* Infection in *Arabidopsis* Is a Form of Defense Response Accompanying HR-Like Cell Death

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In the pathosystems of *Turnip mosaic virus* (TuMV) with Brassicaceae crops, various symptoms, including mosaic and necrosis, are observed. We previously reported a necrosis-inducing factor *TuNI* in *Arabidopsis thaliana*, a model species. In this study, we show that the necrotic symptom induced by *TuNI*, observed along the veins, was actually a form of defense response accompanying a hypersensitive reaction (HR)-like cell death in the veinal area. The virus is often localized in the necrotic region. The necrotic response is associated with the production of H₂O₂, accumulation of salicylic acid (SA), emission of ethylene, and subsequent expression of defense-related genes. Additionally, this HR-like cell death is eased or erased by a shading treatment. These features are similar to the HR-associated resistance reaction to pathogens. However, unlike HR, two phytohormones—SA and ethylene—are involved in the necrosis induction, and both SA- and ethylene-dependent pathogenesis-related genes are activated. We concluded that the veinal necrosis induced by TuMV is regulated by a complex and unique network of at least two signaling pathways, which differs from the signal transduction for the known HR-associated resistance.

Additional keywords: cryptochrome, phytochrome

In Brassicaceae crops infected by *Turnip mosaic virus* (TuMV), a member of the genus *Potyvirus*, symptoms such as mosaic, necrosis, or a combination of symptoms develop (Smith 1972). Mosaic often is accompanied by leaf distortion and stunting, while necrotic symptoms such as veinal necrosis or necrotic spot cause not only developmental arrest but also leaf or plant death. These TuMV symptoms result in loss of yield and poor product quality depending on the growth stage of infected plants. Necrosis severely damages product appearance, especially for vegetables such as Chinese cabbage. Because complete control of the aphid vector is not easy and the genetic resources for TuMV resistance also are limited in some Brassicaceae crops (Walsh and Jenner 2002), the development of methods to manage the symptoms is important. However, we do not fully understand what pathogen and host factors determine the symptoms (Jenner et al. 2003; Kaneko et al. 2004; Suehiro et al. 2004). We previously reported that the

type of symptom in *Arabidopsis thaliana*–TuMV interaction was determined by the *TuNI* locus (Kaneko et al. 2004). The necrosis starts from the base of the petiole and expands along veins in the uninoculated upper leaves, but the spread of virus from phloem to mesophyll is often prevented. Because the necrotic symptom is induced by a host factor and the virus is localized in the tissues in which the cells eventually die, the necrotic response was suggested to be an active response of *Arabidopsis* to TuMV; namely, a hypersensitive reaction (HR) rather than a passive damage from TuMV infection.

HR, a form of programmed cell death (PCD) associated with resistance to pathogens, generally is induced at infected sites or within defined areas surrounding the infected sites and limits pathogen growth. It also is accompanied by accumulation of salicylic acid (SA), an endogenous phytohormone in a defense signaling pathway that leads to expression of defense-related genes and production of phytoalexins (Enyedi et al. 1992; Scheel 1996). PCD induced along veins has been described for the lesion-mimic *Arabidopsis* mutant *vad1* (Lorrain et al. 2004). Cell death in the *vad1* mutant was induced spontaneously in a light-dependent manner near vascular bundle tissues at a relatively late stage in the vegetative phase. Because generation of reactive oxygen species (ROS), accumulation of SA, defense-related gene expression, and increased resistance to *Pseudomonas syringae* pv. *tomato* were accompanied with a necrotic lesion in the *vad1* mutant, this cell death along veins has been considered to be an HR-like PCD. *VAD1* encodes a putative membrane protein containing a GRAM domain and was proposed to negatively regulate cell death associated with the cell membrane (Lorrain et al. 2004).

Some endogenous phytohormones (SA, jasmonic acid [JA], and ethylene) are known to act as a signal molecule in defense responses (Kunkel and Brooks 2002). However, their involvement in HR is not well understood. SA accumulation was required for HR-like PCD in some lesion-mimic mutants such as *lsd1* and *vad1* mutants (Dietrich et al. 1997; Lorrain et al. 2004). However, correlation between SA and PCD has not been shown in the pathogen-induced HR (Chandra-Shekara et al. 2004; Dewdney et al. 2000; Mur et al. 1997; Nawrath and Métraux 1999), although it was reported recently that the accelerated HR conferred by overexpression of AtMYB30, a positive regulator of the HR, was dependent on SA accumulation (Raffaële et al. 2006). The contribution of JA and ethylene to HR also has been unclear; however, ethylene biosynthesis but not ethylene itself was shown to be necessary to form HR lesions during blast fungus resistance in rice (Iwai et al. 2006). In cases of PCD aside from HR, ethylene together with SA was reported to be associated with the initiation of ROS-

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dependent PCD by ozone exposure (Van Breusegem and Dat 2006). By contrast, JA can induce PCD when the initial ROS signal is singlet oxygen (Danon et al. 2005); JA antagonistically acts in superoxide-dependent PCD (Overmyer et al. 2005).

Light is required not only as energy for photosynthesis but also as a signal to regulate various developmental processes in plants (Franklin et al. 2005). Light signals also play an important role in defense responses to pathogens (Chandra-Shekara et al. 2006; Genoud et al. 2002; Guo et al. 1993; Karpinski et al. 2003; Zeier et al. 2004). In the incompatible interaction between *Arabidopsis* and the bacterial pathogen *P. syringae* pv. *tomato*, HR and expression of the pathogenesis-related (PR) protein PR1 were reduced strongly in the dark (Genoud et al. 2002). The suppression of defense responses did not result from a decrease in carbohydrates produced by the chloroplasts, but was dependent on a light signal mediated by the photoreceptors phytochrome A (PHYA) and phytochrome B (PHYB). Resistance controlled by *RPM1* to *P. syringae* pv. *maculicola* in *Arabidopsis* has been shown to include two distinct sets of inducible defense responses, which are regulated light dependently or independently (Zeier et al. 2004). Continuous darkness drastically influenced HR, accumulation of SA, and SA-dependent defense responses, whereas occurrence of the oxidative burst, accumulation of JA, production of camalexin, and expression of myrosinase were not suppressed in the dark. In the *HRT*-mediated resistance of *Arabidopsis* to *Turnip crinkle virus* (TCV), the dark treatment also caused suppression of HR, resistance to TCV, and expression of *PR1* (Chandra-Shekara et al. 2006).

In this study, we characterized the necrotic symptom induced by TuMV infection in an ecotype of *A. thaliana*, *Landsberg erecta* (*Ler*), to elucidate whether the necrosis could be categorized as a form of defense response and the hormonal signals could be involved in that symptom induction. In addition, because development of the necrotic symptom was influenced by light conditions, the association of a light signal with symptom induction also was investigated.

RESULTS

Veinal necrosis is a light-dependent defense response.

When infected by TuMV isolate TuR1, *Ler* plants carrying *TuNI* developed a different symptom from the mosaic on Columbia (Col-0) plants that did not carry *TuNI* (Fig. 1). At 7 to 10 days after inoculation, veinal necrosis started to develop from the base of the petiole in mature rosette leaves of *Ler*, and cell death usually progressed along the primary or secondary veins with slight yellowing (Fig. 2A and B). These symptoms rarely encompassed the entire leaf and sometimes stopped in the middle of the veins. The results of tissue-printing experiments revealed that the spread of TuMV was limited within the necrotic tissues (Fig. 2C). Such localization was never observed in the Col-0 plants with mosaic. Interestingly, *Ler* developed a yellowing symptom with or without weak veinal necrosis in the shade, whereas the mosaic symptom in Col-0 was not altered by shading (Figs. 1 and 2). These results suggest that the necrotic symptom is regulated by light intensity.

Plant cell death in infection sites and pathogen localization in the necrotic tissues were typical features of the HR. Therefore, we investigated whether other features of the HR also were accompanied by the necrotic symptom in TuR1-infected *Ler*. First, generation of ROS, a secondary messenger leading to HR, was examined. To detect H₂O₂, a form of ROS, TuR1-infected leaves were stained with H₂DCFDA (2',7'-dichlorofluorescein diacetate). As expected, H₂O₂ was detected along the necrotic veins in *Ler*, but not in Col-0 with mosaic (Fig. 2D). This cou-

pling of cell death, viral localization, and ROS production strongly suggested that the necrotic symptom in *Ler* was an HR-like PCD. Under shading, the features associated with the necrotic symptom disappeared (Fig. 2). In a general reaction of resistance, SA, JA, or ethylene accumulate as signal molecules to induce defense against pathogens. When the total SA was measured 2 weeks after inoculation, SA had increased significantly in *Ler* in both light and shade treatments compared with the mock-inoculated plant in the same conditions (Fig. 3). The SA level in *Ler* increased approximately five times more than that in Col-0 in the light treatment, while the SA level in *Ler* in the shade was approximately three times more than that in Col-0. On the other hand, there was little difference in the JA level in *Ler* between the mock- and TuR1-inoculated plants in both light and shade treatments (Fig. 3). The JA level in Col-0 was decreased by TuR1 under the light but increased under the shade (Fig. 3). When the ethylene level was measured by a quick-measuring detector tube (GASTEC, Ayase, Japan), only *Ler* with necrosis in the light treatment emitted a high quantity of ethylene (approximately 400 nl h⁻¹ g⁻¹ of fresh mass), approximately 20 times more than that in the mock-inoculated plant (data not shown). Neither *Ler* with yellowing nor Col-0 with mosaic had such an increase in ethylene.

The other hallmark of a resistance reaction to pathogens is the expression of the PR protein genes. The results of Northern blot analysis and quantitative reverse-transcription polymerase chain reaction (RT-PCR) showed that the expression levels of *PR1* and *PR5* genes, which generally are induced by the SA signaling pathway, increased after TuR1 infection in *Ler* (Figs. 4 and 5). By contrast, *PR3* and *PDF1.2*, which are induced by the JA or ethylene signaling pathways, had different expression patterns. Although the expression of *PR3* clearly increased in *Ler* with the necrotic symptom (Figs. 4. and 5), the expression of *PDF1.2* was suppressed as compared with the mock-inoculated plant (Fig. 5). Because the expression level of *PDF1.2* was very low, the signal of *PDF1.2* was not detected in both mock- and TuR1-inoculated plants of *Ler* in Northern blot analysis (data not shown); the difference of the expression level of *PDF1.2* was confirmed by semi-quantitative RT-PCR (Supplemental Figure S1). The shading treatment caused a significant decrease in expression levels of *PR1* and *PR5* in the TuR1-infected *Ler* plants (Fig. 5). The expression level of *PR3* also decreased, whereas the shading treatment did not affect the expression level of *PDF1.2* in the TuR1-infected plants (Fig. 5).

Induction of veinal necrosis is mediated by SA and ethylene.

To clarify which signal molecules, SA, JA, or ethylene, are important for inducing the necrotic symptoms, we made crosses between *Ler* and the SA-deficient *eds5* mutant, the JA-insensitive *coi1*, or the ethylene-insensitive *etr1-3* mutants. From each cross, the F₃ or F₄ lines with genotypes *TuNI TuNI/eds5 eds5*, *TuNI TuNI/coi1 coi1*, and *TuNI TuNI/etr1-3 etr1-3* were selected using DNA polymorphism markers linked to *eds5*, *etr1-3*, and *TuNI* or the cleaved amplified polymorphic sequence (CAPS) markers designed within the sequence of *coi1* (discussed below). These plants were inoculated with TuR1. In the *TuNI/eds5* plants, the necrotic responses obviously weakened, and a yellowing phenotype without veinal necrosis became predominant (Table 1). Viral localization along the vein also was lost. A similar symptom alteration was observed in the *TuNI/etr1-3* plants (Table 1). In both genotypes, 10 to 20% of the plants showed veinal necrosis, indicating partial effects of *eds5* and *etr1-3* mutations on necrosis induction. These results showed that the HR-like cell death induced by TuR1 in *Ler* was mediated by both SA and ethylene. On the other hand, 10% of the plants developed a yellowing symptom in the *coi1/TuNI* plants. However, those

plants showed spindly growth; therefore, it was difficult to determine whether the alteration of symptoms was due to the effect of *coi1* mutation or the spindly growth.

The expression level of *PR1* significantly declined in the *TuNI/eds5* genotype but was not affected in the *TuNI/etr1-3* genotype (Fig. 6). On the other hand, the opposite occurred for *PR5* (Fig. 6). These observations indicate that the signaling pathway for the induction of *PR5* and for *PR1* is different and that the ethylene-signaling pathway is perhaps more important for the *PR5* gene expression. The expression level of *PR3* did not differ significantly among the four genotypes (Fig. 6). The expression of *PDF1.2* was suppressed in three of the genotypes, but not for the *TuNI/eds5* genotype, suggesting that suppression of *PDF1.2* in *Ler* infected by TuR1 may be due to an antagonistic effect by SA (Fig. 6).

Involvement of photoreceptors in the necrotic induction.

Because we observed that the induction of the necrosis was influenced by light, the relationships between the photoreceptor

genes (*Cryptochrome1* [*CRY1*], *Cryptochrome2* [*CRY2*], *PHYA*, and *PHYB*) and HR-like cell death and defense responses were investigated. Because all the photoreceptor mutants used in this study were derived from *Ler*, they were expected to carry *TuNI*. As a result, none of the *cry1*, *cry2*, *phyA*, *phyB*, and *phyA phyB* mutants had any alteration in the necrotic symptoms, indicating that the light signal that induced the HR-like cell death was not mediated by these photoreceptors (Table 2). In *Ler* and the photoreceptor mutants, TuR1 infection increased the expression levels of the *PR* genes except for *PDF1.2*. The level of *PDF1.2* in *Ler* was decreased by TuR1 infection whereas it was critically increased in the photoreceptor mutants (Fig. 7). The suppression of *PDF1.2* by the antagonistic effect of SA seems to be modulated by mutations in those photoreceptor genes.

DISCUSSION

In this study, we showed that veinal necrosis induced by TuMV in *Arabidopsis* was a form of defense response because

Fig. 1. Symptoms induced by *Turnip mosaic virus* isolate TuR1 in *Arabidopsis thaliana* ecotypes Columbia (Col-0) and Landsberg *erecta* (Ler). Photographs were taken 2 weeks after inoculation. **A**, Mosaic on Col-0 and **B**, veinal necrosis on *Ler* under normal light. **C**, *Ler* grown with a light shade treatment developed yellowing.

i) movement of TuMV from phloem to mesophyll was restricted by the necrotic response accompanying the generation of ROS, and ii) production of SA and ethylene and expression of defense-related genes markedly increased in the leaves with veinal necrosis. In the F_1 plants of a cross between *Ler* and Col-0, veinal necrosis also was observed (Kaneko et al. 2004), but the symptom developed more slowly in the F_1 plants than in *Ler* and eventually the virus spread all over the infected leaves where necrosis had not developed. This observation indicates that the function of *TuNI* is incomplete in the heterozygous state in terms of defense response and also that the HR-like cell death induced by *TuNI* is tissue specific. Such tissue-specific HR-like cell death has been reported in the lesion mimic mutant *vad1* of *Arabidopsis* (Lorrain et al. 2004). Besides the tissue specificity, other features of the *TuNI*-induced necrosis, such as correlation of both SA and ethylene with activation of cell death, expression of SA and JA- or ethylene-

dependent defense-related genes, and dependency on light, also are similar to those in the *vad1* mutant (Bouché et al. 2007; Lorrain et al. 2004). Thus, we speculate that the veinal necrosis induced by TuMV in *Arabidopsis* also may be negatively regulated by *VAD1*.

Role of SA and ethylene in induction of necrosis and defense responses.

Here, at least SA and ethylene were shown to be associated with the necrotic response induced by TuMV in *Arabidopsis*. In both genotypes *TuNIeds5* and *TuNIetr1-3*, the necrotic symptoms were significantly weakened. However, because neither of the genotypes completely suppressed veinal necrosis (Table 1), the HR-like cell death may be regulated by cross-talk between SA- and ethylene-signaling pathways.

Increased levels of SA and ethylene also were observed in the *Ler* leaves showing veinal necrosis (Fig. 3). The SA- and

Fig. 2. Symptoms in Columbia (Col-0) and Landsberg erecta (*Ler*) ecotypes of *Arabidopsis thaliana* after inoculation with isolate TuR1 of Turnip mosaic virus (TuMV) under nonshaded and shaded conditions. **A**, Leaves from a mature rosette of Col-0 grown under both light conditions developed the same mosaic, but *Ler* developed veinal necrosis in the nonshaded and yellowing in the shaded condition. **B**, Evans blue staining of the leaves in A. Cell death was observed only in the nonshaded *Ler* leaf with veinal necrosis. **C**, Tissue print blots of the infected leaves. TuMV was localized along the vein of *Ler* with necrosis under the nonshaded condition. TuMV was not localized in the leaves with either mosaic (Col-0) or yellowing (*Ler*). **D**, H₂O₂ detection by using H₂DCFDA in the leaves in C. H₂O₂ was detected only in the necrotic region (green fluorescence) in the *Ler* leaf where TuMV was localized.

Fig. 3. Total salicylic acid (SA) and jasmonic acid (JA) in leaves of Columbia (Col-0) and Landsberg erecta (*Ler*) ecotypes of *Arabidopsis thaliana* after inoculation with Turnip mosaic virus (TuMV) isolate TuR1 or mock in nonshaded and shaded conditions. The values are the means of three biological replicates, and the error bars are standard errors. The differences between the means of mock- and TuMV-infected plants were evaluated by *t* test; * and ** indicate significance at 5 and 1% level, respectively.

Fig. 4. Defense-related gene expression in Columbia (Col-0) and Landsberg *erecta* (Ler) plants 2 weeks after inoculation with *Turnip mosaic virus* isolate TuR1 and grown in normal light. Expression levels of *PR1*, *PR5*, and *PR3* were determined by RNA gel blot analysis.

ethylene-dependent defense-signaling pathways were activated, resulting in the expression of the *PR* genes. In this study, it was clearly shown that *PR1* expression was SA dependent while *PR5* expression was ethylene dependent (Fig. 6). On the other hand, the relationships between SA, JA, ethylene, and *PR3* expression, which was reported to be regulated by JA- or ethylene-signaling pathways, was unclear (Fig. 6). However, *PR3* expression was found to correlate with SA levels (Figs. 3 and 5), suggesting that *PR3* induction was regulated at least by SA. For *PR3* expression, it is conceivable that more than two signaling pathways including SA may be involved.

Effect of light intensity and quality on the necrotic induction.

In our results, the necrotic response induced by TuMV also was regulated by light intensity as well as other resistance reactions previously reported (Chandra-Shekara et al. 2006; Genoud et al. 2002; Guo et al. 1993; Zeier et al. 2004). To elucidate the factors mediating light signals in the necrotic induction pathway, the *Ler* mutants for the four photoreceptor genes *CRY1*, *CRY2*, *PHYA*, and *PHYB* were tested for their response to TuR1. Because none of the mutants had any changes in symptoms, we believe that the HR-like cell death induced by TuMV is independent of at least these four photoreceptor genes. Other photoreceptors, such as cryptochrome (*CRY3*), phytochromes (*PHYC*, *PHYD*, and *PHYE*), phototropins (*PHOT1* and *PHOT2*), and even the chloroplast, may be involved in the signal generation for the necrotic responses to TuMV. When defense-related genes were induced, the expres-

Fig. 5. Effect of light shading on defense-related gene expression in Landsberg *erecta* (Ler) ecotype of *Arabidopsis thaliana* 2 weeks after inoculation with *Turnip mosaic virus* (TuMV) isolate TuR1. Expression levels of *PR1*, *PR5*, *PR3*, and *PDF1.2* were determined by quantitative reverse-transcription polymerase chain reaction and are the means of three biological replicates. Error bars represent standard errors.

Table 1. Type of symptom in the *Turnip mosaic virus* isolate TuR1-infected plants carrying *TuNI* with the mutations of *eds5*, *coi1*, or *etr1-3*

Ecotype name or line number	Genotype	Number of plants			Total
		Veinal necrosis	Yellowing	Mosaic	
Landsberg <i>erecta</i> (Ler)	<i>TuNI TuNI</i>	50	0	0	50
Columbia (Col-0)	<i>tuni tuni</i>	0	0	50	50
F ₃ lines (no. 22, 25, 27, 31)	<i>TuNI TuNI / eds5 eds5</i>	4	36	0	40
F ₃ lines (no. 6, 30, 32, 36)	<i>TuNI TuNI / coi1 coi1</i>	44	5	0	49
F ₄ lines (no. 42, 45, 157)	<i>TuNI TuNI / etr1-3 etr1-3</i>	22	78	0	100

sion levels of *PR1*, *PR3*, and *PR5* did not decrease in any photoreceptor mutants (*cry1*, *cry2*, *phyA*, *phyB*, and *phyA phyB*) (Fig. 7). However, the expression of *PDF1.2*, which was suppressed in the infected *Ler* plants, increased in all the mutants (Fig. 7). Because the level of *PDF1.2* expression did not change in the infected *Ler* plants in the shade (Fig. 5), light quality, such as the proportion of blue and red light, rather than light intensity may be responsible for the change in *PDF1.2* expression. Because the suppression of *PDF1.2* in the infected *Ler* plants under normal light is considered to be due to negative cross-talk between the SA- and ethylene-signaling pathways, it is conceivable that the antagonistic effect of SA on the ethylene signal transduction is affected by light quality (Fig. 8).

Management of symptom and biological meaning of lethal necrosis.

The knowledge obtained from this *Arabidopsis* study provides ideas to control the type of symptom induced by TuMV in agriculturally important Brassicaceae crops. For example, veinal necrosis induced by TuMV infection was influenced by external factors such as light in *Arabidopsis*. Actually, the type of symptom induced by TuMV in Chinese cabbage was dependant on temperature and the necrotic symptom was suppressed under high temperatures (Kasuyama et al. 1978). These

facts suggest that development of the necrotic symptom induced by TuMV in Brassicaceae crops fluctuates considerably depending on environmental conditions. This might make it difficult to identify the host factor responsible for the necrotic symptom under field conditions even if inheritance of the symptom determinant is simple. On the other hand, fluctuation of the necrotic symptom with environmental conditions means that it is possible to suppress necrosis by modifying cultivation methods. For instance, a temporal shading treatment might effectively suppress the necrotic symptom induced by TuMV.

We showed that the veinal necrosis induced by TuMV in *Arabidopsis*, previously considered to be a susceptible reaction, was actually a defense response. Considering this biological meaning of TuMV-induced lethal necrosis, we discuss the following points: i) why the defensive response results in lethal necrosis in the TuMV-infected *Arabidopsis* and ii) why a similar system is widely conserved among many Brassicaceae species. Veinal necrosis is likely to prevent long-distance movement of the virus (Lorrain et al. 2004). In this respect, the system that induces the necrosis might still be evolving. Otherwise, death of the diseased plants could contribute to elimination of the TuMV inocula from the field if the genotype with the lethal necrosis is predominant in the population. Moreover, because larvae of a vector insect aphid likely starves to death

Fig. 6. Expression pattern of defense-related genes in the F₃ or F₄ lines derived from the crosses between the Landsberg *erecta* (*Ler*) ecotype of *Arabidopsis thaliana* and mutants *eds5*, *coi1*, and *etr1-3*. *Ler* and the three genotypes *TuNI/eds5* (F₃ lines no. 22, 25, and 27), *TuNI/coi1* (F₃ lines no. 6 and 30), and *TuNI/etr1-3* (F₄ lines no. 42, 45, and 157) were grown under the normal light condition and expression levels were determined by quantitative reverse-transcription polymerase chain reaction 2 weeks after mock and Turnip mosaic virus (TuMV) isolate TuR1 inoculation. Values are the means of two or three lines for each genotype and three biological replicates for each line. Error bars represent standard errors. The difference between the means of each infected genotype was evaluated with Duncan's new multiple range test. Different lowercase letters on error bars indicate a significant difference at 5% level between the means.

Table 2. Type of symptom observed in the Turnip mosaic virus isolate TuR1-infected photoreceptor mutants derived from Landsberg *erecta*

Ecotype name or accession number	Genotype	Number of plants		Total
		Veinal necrosis	Mosaic	
Landsberg <i>erecta</i> (<i>Ler</i>)	<i>TuNI TuNI</i>	31	0	31
Columbia (<i>Col-0</i>)	<i>tuni tuni</i>	0	31	31
CS70	<i>TuNI TuNI / cry1 cry1</i>	21	0	21
CS108	<i>TuNI TuNI / cry2 cry2</i>	19	0	19
CS6219	<i>TuNI TuNI / phyA phyA</i>	21	0	21
CS6213	<i>TuNI TuNI / phyB phyB</i>	16	0	16
CS6224	<i>TuNI TuNI / phyA phyA / phyB phyB</i>	19	0	19

on plants with veinal necrosis, we may expect that the viral transmission also will decrease. Therefore, the defense system based on PCD against both the virus and the vector insect could be very effective in maintaining the fitness of a natural population.

MATERIALS AND METHODS

Plant materials and growth conditions.

The ecotypes of *A. thaliana*, *Ler* and *Col-0*, and mutant lines *eds5*, *coi1*, *etr1-3*, *cry1*, *cry2*, *phyA*, *phyB*, and *phyA phyB* were used in these experiments. The SA-deficient mutant *eds5*, the JA-insensitive mutant *coi1*, and the ethylene-insensitive mutant *etr1-3* were derived from *Col-0* (Chang et al. 1993; Glazebrook et al. 1996; Xie et al. 1998), while the rest of the photoreceptor mutants were derived from *Ler*. The mutants *eds5*, *coi1*, and *etr1-3* were crossed with *Ler*, and the individuals or lines carrying the *TuNI* allele and each mutant allele at the homozygous states were selected from the F₃ or F₄ progenies. The genotype at the *TuNI* locus was determined based on the genotypes of the two CAPS marker loci, mT8L23 and mF23H11, flanking the *TuNI* locus. The sequence of the PCR primers and the restriction enzyme used to generate polymorphism for the CAPS markers mT8L23 and mF23H11 were as described by Kaneko and associates (2004). The procedures for determining the genotypes at the *eds5*, *coi1*, and *etr1-3* loci followed those of Takahashi and associates (2002). Seed were sown directly into peat pellets and grown in a MLR-350 growth chamber (SANYO, Tokyo) at 21°C with a 12-h photoperiod (150 $\mu\text{mol}/\text{m}^2/\text{s}$). For the shade treatment (10 $\mu\text{mol}/\text{m}^2/\text{s}$), plants were covered with white sulfated paper 1 day before inoculation with TuMV.

Viral inoculation and detection.

Three or four fully expanded leaves of 4-week-old plants were rub inoculated using Carborundum with the sap inocula from turnip leaves infected by the TuMV isolate TuR1 (Kaneko et al. 2004) that were prepared in a 10-fold volume of 0.1 M phosphate buffer (pH 7.1). For investigating the distribution of virus in infected plants or leaves, tissue print blots were prepared and the virus was detected with an anti-TuMV antibody according to the methods described by Kaneko and associates (2004).

Tissue staining.

For localizing cell death, leaves were vacuum infiltrated overnight with 0.25% Evans blue solution. The samples then were destained in water and incubated in 70% ethanol solution

Fig. 8. Working hypothesis for induction of the necrotic symptom observed in the Landsberg *erecta* (*Ler*) ecotype of *Arabidopsis thaliana* after infection by Turnip mosaic virus (TuMV) isolate TuR1. The interaction between the product of the *TuNI* gene and the TuMV factor induces accumulation of salicylic acid (SA) and emission of ethylene leading to hypersensitive reaction (HR)-like cell death. *PR1* and *PR5* also are activated through the SA- and ethylene-dependent pathways, respectively. In this model, light acts upstream of SA- and ethylene-signaling pathways. The pathways for these light signals are independent of the photoreceptors cryptochrome 1 (CRY1), cryptochrome 2 (CRY2), phytochrome A (PHYA), and phytochrome B (PHYB). The expression of *PDF1.2* is suppressed in the induction of the necrotic symptom by the antagonistic effect of SA, which is dependent on photoreceptors CRY1, CRY2, PHYA, and PHYB.

Fig. 7. Expression of defense-related genes in photoreceptor mutants of the Landsberg *erecta* (*Ler*) ecotype of *Arabidopsis thaliana* 2 weeks after inoculation with Turnip mosaic virus (TuMV) isolate TuR1 or mock inoculation. *Ler* and cryptochrome (*cry*)1, *cry2*, phytochrome (*phy*)A, *phyB*, and *phyA phyB* mutants were grown in normal light conditions, and expression levels were determined by quantitative reverse-transcription polymerase chain reaction. Values are means of three biological replicates and error bars represent standard errors.

for 1 or 2 days to remove chlorophyll. H_2O_2 generation in leaf tissues was detected by the method of Lorrain and associates (2004). Briefly, leaves first were placed into a small petri dish containing 10 ml of loading buffer (10 mM Tris-HCl and 50 mM KCl, pH 7.2) and 5 μl of 100 mM H2DCFDA dissolved in dimethyl sulfoxide. After vacuum infiltration of H2DCFDA for 3 min, the samples were incubated in the dark for 20 min at room temperature. The samples then were washed two times in fresh loading buffer. Fluorescence was observed using a SZX12 fluorescence stereomicroscope (Olympus, Tokyo) (excitation filter, 460 to 490 nm and barrier filter, 510 nm).

Measurement of SA and JA.

Infected leaves were harvested 2 weeks after inoculation, weighed in plastic bags, and stored at -80°C . The sample (5 to 10 g) was crushed in liquid nitrogen, then soaked in 70% aqueous methanol (aq. MeOH; 1:10, wt/vol) for 48 h. The mixture was filtered to yield a crude extract. Before purification, deuterium-labeled SA (100 ng/g of fresh mass of plants), SA glucoside (200 ng/g of fresh mass of plants), and JA (100 ng/g of fresh mass of plants) were added to the extracts. The volatile components of the extract were removed under reduced pressure, and *n*-hexane (20 ml) was added to the residue. The mixture was extracted with 70% aq. MeOH (2 ml $\times$ 3), and the volatile components of the combined 70% aq. MeOH layers were removed under reduced pressure to give a residue, which was mixed with H_2O (2 ml) and loaded onto the cartridge column of Bond Elut C_{18} (GL Sciences, Tokyo). The column was washed successively with H_2O (2 ml $\times$ 2) and MeOH- H_2O (7:3, vol/vol, 2 ml $\times$ 3). The volatile components of the MeOH- H_2O (7:3) eluents were removed in vacuo, and the residue was dissolved with MeOH (2 ml) and put onto the cartridge column of Bond Elut DEA. The column was washed successively with MeOH (2 ml $\times$ 2) and 1 N AcOH-MeOH (2 ml $\times$ 3). The volatile components of the AcOH-MeOH eluents were removed, and the residue was dissolved in MeOH- H_2O (1:1, 150 μl) and subjected to a YMC-Pack ODS column (300 by 10 mm; YMC, Kyoto, Japan). The column was eluted with a mixed solvent of MeOH with 0.1% AcOH (solvent A) and 0.1% AcOH- H_2O with 0.1% AcOH (solvent B) using a linear gradient mode. The temperature of the column oven was set at 38°C . The flow rate was 2.0 ml min^{-1} in the period from 0 to 25 min whereas, from 25 to 31 min, the flow rate was linearly changed from 2.0 to 2.2 ml min^{-1} , and the flow rate was 2.2 ml min^{-1} from 31 to 37 min. From 0 to 20 min, the combination of A and B was linearly converted from 55:45 to 60:40 whereas, from 20 to 25 min, the combination of A and B was linearly converted from 60:40 to 100:0, and the column was finally eluted with A:B (100:0) from 25 to 37 min. For measuring the relative retention time of the compounds, 4-hydroxy benzoic acid was injected before the injection of samples. The typical retention time for 4-hydroxy benzoic acid is 11.3 min, and the fractions that were eluted during the periods 9 to 11, 19 to 23, and 28 to 33 min were due to SA glucoside, SA, and JA, respectively. Each fraction containing SA and JA was concentrated in vacuo to give a residue, which was dissolved with MeOH (100 μl), treated with an excess amount of CH_2N_2 , and analyzed by gas chromatography-tandem mass spectrometry (GC-MSMS). The fraction containing SA glucoside was concentrated in vacuo and the residue was dissolved in 3 ml of 0.1 N AcONH_4 -AcOH buffer (pH 5.6). β -Glucosidase (10 mg) was added to the mixture, and then the mixture was shaken (100 rpm) at 37°C for 24 h. After the addition of 12 N HCl (150 μl) to the mixture, a solution (6 ml) of ethyl acetate/cyclohexane/2-propanol (50:50:1, vol/vol/vol) was added. The volatile components of the organic layer then were removed and the residue was dissolved in MeOH (100 μl), treated with

an excess amount of CH_2N_2 , and analyzed by GC-MSMS on a ZB-Wax capillary column (30 m by 0.25 μm i.d., 0.25-mm film thickness; Phenomenex), using helium as the carrier gas and splitless injection (injection, 150°C ; transfer line, 240°C ; ion source, 200°C). The temperature program was as follows: from 50°C for 0.5 min, to 230°C at $30^\circ\text{C}/\text{min}$, then held at 230°C for 5 min. The ions of m/z 120 and 124 were selected and further ionized on GC-MSMS with an excitation voltage of 4.0 eV for the analysis of SA, and the ions of m/z 224 and 230 with an excitation voltage of 3.0 eV for that of JA. The amounts of endogenous SA and JA were calculated from the peak area ratios of m/z 92:96 and m/z 151:157 on GC-MSMS, respectively.

RNA gel blot analysis and quantitative RT-PCR.

Total RNA was extracted from fresh leaves of plants using Trizol reagent (Invitrogen, Tokyo) following the manufacturer's instructions. For RNA gel blot analysis, 10 μg of total RNA per sample was loaded on 1% formaldehyde-agarose gels, separated by electrophoresis at 50 V, and blotted to Hybond- N^+ (Amersham Biosciences, Little Chalfont, UK) by capillary transfer. DNA probes were generated by PCR with the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Tokyo) using the primers designed based on the sequences of *PR1* (At2g14610), *PR3* (At3g12500), *PR5* (At1g75040), and *PDF1.2* (At5g44420). The expression patterns of *PR1*, *PR3*, *PR5*, and *PDF1.2* also were examined by the eXpress profiling multiplex RT-PCR assay. Briefly, 10 ng of RNA from each sample was reverse transcribed using five reverse primers including the primer for *β -tubulin-4* (At5g44340) as an internal standard. Each reverse primer is chimeric, with the 19 nucleotides of the 5' primer end containing a sequence (5'-GTACGA CTCACTATAGGGA-3') annealed with the universal reverse primers in the PCR. The 3' primer end contains the 20 nucleotides of the gene-specific sequence. Each of the primer pairs were designed to yield PCR products ranging from 137 up to 199 bp using Beckman Coulter eXpress Profiling Analysis Software. The reverse transcription reactions were carried out using the GenomeLAB GeXP Start Kit (Beckman Coulter, Fullerton, CA, U.S.A.) according to the manufacturer's instructions. After some preliminary experiments, the concentration of each primer was adjusted from 1 to 125 nM so that expression of four genes could be monitored simultaneously. The reverse transcription reactions were incubated for 1 min at 48°C , 5 min at 37°C , 60 min at 42°C , and then 5 min at 95°C . Multiplex PCR was performed as follows: 9.3 μl of cDNA from each of the above reverse-transcription reactions was added to the 0.2-ml PCR tube containing 10.7 μl of a PCR reaction mix, including the five forward primers at 200 nM each. Each forward primer is also chimeric, with the 18 nucleotides of the 5' primer end containing a sequence (5'-AGGTGACAC TATAGAATA-3') annealed with the universal forward primers in the PCR reaction. The 3' primer end contains the 20 nucleotides of the gene-specific sequence. Besides the forward primer multiplex, the PCR reaction mix contained 3.5 units of Thermo-Start DNA polymerase (ABgene, Rochester, NY, U.S.A.), 25 mM MgCl_2 (ABgene), and 5 $\times$ PCR buffer prepared in the GenomeLAB GeXP Start Kit, including universal reverse primer, D4 dye-labeled universal forward primer, and dNTPs. The reactions first were subjected to 95°C for 10 min. The following PCR programs consisted of the conditions 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min and cycled 35 times. The PCR products were pre-diluted with 10 mM Tris-HCl (pH 8.0) at 20-fold dilution and prepared for capillary electrophoresis by adding 1 μl of each reaction to the well in a Beckman 96-well CEQ electrophoresis plate (Beckman Coulter) containing 38 μl of CEQ sample loading solution (Beckman

Coulter) and 1 µl of CEQ DNA size standard 400 (Beckman Coulter). The capillary electrophoresis and fragment size analysis were carried out using Beckman CEQ 8800 and eXpress profiling analysis software. The row data of each gene expression were outputted as their peak area, and the normalization was performed by dividing them by the peak area of *β-tubulin-4* as an internal standard.

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