Analysis of Temperature Modulation of Plant Defense Against Biotrophic Microbes

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Plant-pathogen interactions are known to be affected by environmental factors including temperature; however, the temperature effects have not been systematically studied in plant disease resistance. Here, we characterized the effects of a moderate increase in temperature on resistance to bacterial pathogen *Pseudomonas syringae* and two viral elicitors in *Arabidopsis thaliana* and *Nicotiana benthamiana*. Both the basal and the resistance (*R*) gene-mediated defense responses to *Pseudomonas syringae* are found to be inhibited by a moderately high temperature, and hypersensitive responses induced by *R* genes against two viruses are also reduced by an increase of temperature. These indicate that temperature modulation of defense responses to biotrophic and hemibiotrophic pathogens might be a general phenomenon. We further investigated the roles of two small signaling molecules, salicylic acid and jasmonic acid, as well as two defense regulators, *EDS1* and *PAD4*, in this temperature modulation. These components, though modulated by temperature or involved in temperature regulation or both, are not themselves determinants of temperature sensitivity in the defense responses analyzed. The inhibition of plant defense response by a moderately high temperature may thus be mediated by other defense signaling components or a combination of multiple factors.

Plant-pathogen interactions are modulated by environmental factors that could affect both pathogenicities and host defense responses (Browder 1985; Colhoun 1973). Temperature is known to influence disease resistance to bacteria, fungi, virus, and insects, and different host-pathogen interactions respond differently to different temperature ranges (Garrett et al. 2006). A high temperature very often inhibits disease resistance (Dropkin 1969), although a low temperature is found to reduce plant defense as well (Szittyá et al. 2003).

Extensive studies on plant disease resistance has revealed that it has multiple layers (Chisholm et al. 2006; Jones and Dangl 2006) and can be largely divided into basal and resistance (*R*) gene-mediated resistance. The largest family of *R* proteins are nucleotide binding-leucine rich repeat (NB-LRR) molecules with either a coiled-coil (CC) domain or a toll-interleukin-1 receptor (TIR) domain at the N terminus (Dangl and Jones 2001; Martin et al. 2003). *EDS1* (*ENHANCED DISEASE SUSCEPTIBILITY1*) and *PAD4* (*PHYTOALEXIN DEFICIENT4*) are required for defense responses mediated by the TIR type of

R genes as well as basal disease resistance (Wiermer et al. 2005). *EDS1* and *PAD4* are lipase-like proteins and they physically interact with each other to function. The salicylic acid (SA) pathway is an essential part in the defense response network (Durrant and Dong 2004). SA is upregulated during the hypersensitive response (HR) to induce expression of defense effector molecules such as *PR-1*. While SA is required for both local and systemic defense response to biotrophics that feed on live host cells, jasmonic acid (JA) and ethylene are regulators of plant defense responses against necrotrophic pathogens that kill host cells (Glazebrook 2005). A number of JA signaling components have been identified. *COI1* mediates almost all JA responses and it encodes an F-box protein that targets JAZ repressors for degradation for JA signaling (Chini et al. 2007; Thines et al. 2007; Turner et al. 2002). *JAR1* encodes a JA-amino synthetase that may generate the active form of JA (Staswick et al. 2002). Extensive interactions between SA and JA pathways have been observed, with most of them antagonistic and some synergistic (Beckers and Spoel 2006; Kunkel and Brooks 2002). The mutual inhibition between SA and JA signaling results in an apparent tradeoff between plant defenses against biotrophic and necrotrophic pathogens. However, this antagonism is regulated both temporally and spatially to reduce undesirable tradeoff when plants encounter multiple pathogen attacks (Spoel and Dong 2008; Spoel et al. 2007).

Heat sensitivity has been found in some defense responses against biotrophic and hemibiotrophic microbes. The tobacco *N* gene mediates resistance to *Tobacco mosaic virus* (TMV), and the resistance is reversibly inactivated at elevated temperatures of 28°C or above (Samuel 1931). This temperature-sensitivity property of *N* is retained in tomato (Whitham et al. 1996). The *Mi-1* gene in tomato confers resistance to root-knot nematodes, and the resistance is inactive above 28°C (Hwang et al. 2000; Jablonska et al. 2007). The *Arabidopsis* *RPW8* gene against powdery mildew induces HR-like lesions when expressed as a transgene, and these lesions are suppressed by environmental variations including a temperature above 30°C (Xiao et al. 2003). The NB-LRR type of *R*-like gene *SNC1*, when activated, induces defense responses at 22°C but not at a higher temperature of 28°C in *Arabidopsis* (Yang and Hua 2004). *Cf4* and *Cf9* are tomato *R* genes against fungal pathogen *Cladosporium fulvum*, and HR mediated by these two genes can be suppressed at 33°C (de Jong et al. 2002). Thus, moderately elevated temperature could inhibit defense responses, including HR mediated by a number of *R* genes.

It is unclear how prevalent this phenomenon is because no systemic studies have been carried out to specifically investigate temperature effects on different plant-pathogen interactions. If, indeed, plant-pathogen interactions are often modulated by temperature, this temperature sensitivity would pose a challenge to agriculture. The current climate change does

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appear to cause increased range and severity of plant diseases (Evans et al. 2008). Thus, it is both fundamental and practical to understand the scope and the molecular basis for temperature modulation of defense response. A common mechanism may exist for different systems of disease resistance, because many of them share similar signaling molecules and use similar signaling mechanisms. Some of the signaling components are themselves modulated by temperature. SA is found to function in both chilling and heat shock responses in addition to its well-established roles in biotic responses. *Arabidopsis* accumulates much more free and glucosyl SA at 5°C than at 23°C, contributing to cell growth inhibition at the chilling temperature (Scott et al. 2004). SA is differentially required for acquired and basal thermotolerance, depending on the developmental stage in *Arabidopsis* (Clarke et al. 2004; Larkindale et al. 2005), and a heat shock of 38°C induces a moderate increase of SA transiently (Scott et al. 2004). The amount of *EDS1* and *PAD4* transcripts is also temperature dependent, with lower accumulation at 28°C than at 22°C (Yang and Hua 2004). These components are therefore potential temperature sensors mediating temperature modulation of disease resistance. A feedback regulatory loop consisting of the *R* gene, *EDS1*, *PAD4*, and SA may enforce the regulation of defense by temperature (Yang and Hua 2004).

In this study, we characterized the temperature effects on basal and *R*-mediated resistance against *Pseudomonas syringae* and specific viral effectors. All defense responses analyzed are found to be reduced at a moderately elevated temperature. We further tested candidate temperature-responsive regulators of plant defense responses including *EDS1*, *PAD4*, SA, and JA. These molecules, though each regulated by temperature or involved in temperature regulation, do not appear to be responsible for temperature sensitivity in defense response by themselves. Thus, temperature sensitivity is likely a general phenomenon in plant disease resistance against biotrophic and hemibiotrophic pathogens. Further study should identify one or more possible common mechanisms for this temperature sensitivity.

RESULTS

Temperature effects on basal resistance to a bacterial pathogen.

We investigated the interaction between *Arabidopsis thaliana* and the virulent pathogen *Pseudomonas syringae* pv. *tomato* DC3000 under the normal *Arabidopsis* growth temperature of 22°C and a warmer temperature of 28°C. Wild-type 10-day-old seedlings were inoculated with *P. syringae* pv. *tomato* DC3000 by dipping and the bacterial growth in plants were analyzed 3 days postinoculation (dpi). DC3000 grew to 1.3×10^5 CFU per milligram of plant tissue at 22°C and to 1.0×10^6 CFU/mg at 28°C (Fig. 1A). The eightfold more growth of bacteria in plants at 28 than at 22°C indicates a large effect of temperature on this plant-pathogen interaction. Consistent with an increase of pathogen growth, infected plants developed more severe disease symptoms faster at 28 than at 22°C (data not shown).

To determine whether the difference in plants is due to an intrinsic growth rate difference of the bacterium at different temperatures, we analyzed its growth outside the plants. Cell density of *P. syringae* pv. *tomato* DC3000 was monitored after an overnight culture was inoculated in a rich liquid growth medium. From 8 to 18 h postinoculation (hpi), DC3000 grown at 28°C had a slightly greater (no more than 20%) density than at 22°C, while from 18 hpi on, DC3000 grown at 22°C had a greater density than at 28°C (Fig. 1B). None of the timepoints showed differences at the two temperatures exceeding twofold.

Although the rich medium does not mimick the environment of plants, a higher growth rate at 28°C does not appear to be intrinsic for bacteria. As previous studies show a suppression in the secretion of toxins and effector proteins from pathogenic bacteria at 28°C (Budde et al. 1998; Palmer and Bender 1993; Smirnova et al. 2001; van Dijk et al. 1999), the increase of pathogen growth at 28°C in plants can not be explained by a higher virulence of DC3000 at higher temperatures either. Thus, the plant defense responses against DC3000 are likely suppressed by a higher growth temperature.

Temperature effects on *R*-mediated resistance to avirulent bacterial pathogens.

We next analyzed effects of temperature on *R* gene-mediated defense response against avirulent *P. syringae* pv. *tomato* DC3000 strains, using HR responses in the wild-type *Arabidopsis* Col-0 plants. Col-0 plants carry *RPS2*, *RPM1*, and *RPS4*, *R* genes recognizing avirulent proteins AvrRpt2, AvrRpm1, and AvrRps4, respectively, and coding for the CC- and TIR-type of NB-LRR proteins (Bent et al. 1994; Gassmann et al. 1999; Grant et al. 1995) and thus have a HR when encountering avirulent DC3000 strains expressing these effector proteins. Wild-type plants were grown at 22°C for 4 weeks or at 28°C for 3 weeks, until they reached a similar developmental stage before bolting. Bacterial strains were infiltrated into expanded leaves of these plants, and HR progression indicated by leaf collapse was followed at respective temperatures until the completion of HR or for 48 h after infiltration (Fig. 2A). For DC3000 with AvrRpt2, 50% of the leaves underwent HR at 7 hpi in 22°C and at 10 hpi in 28°C, while 80% of leaves showed HR at 12 hpi in 22°C and at 18 hpi in 28°C. For

Fig. 1. Temperature effects on the growth of virulent bacterial pathogens in *Arabidopsis thaliana* and liquid medium. **A**, Growth of *Pseudomonas syringae* pv. *tomato* DC3000 at 22 and 28°C in wild-type *Arabidopsis* plants. Overnight culture (0.1 ml) was inoculated into 100 ml of culture at 0 h. Error bars represent standard deviations of three biological repeats. A *t*-test shows a significant difference in DC 3000 growth at 22 and 28°C ($P = 0.0011$). **B**, Growth curve of DC3000 grown in liquid medium at 22 and 28°C. The experiment was repeated three times, and similar growth curves were obtained.

DC3000 with AvrRpm1, 50% of the leaves underwent HR at 5 hpi in 22°C and at 8 hpi in 28°C, while 80% of leaves showed HR at 7 hpi in 22°C and at 15 hpi in 28°C. For DC3000 with AvrRps4, the difference at the two temperatures was more dramatic. At 22°C, 50% of leaves showed HR at 15 hpi and all showed HR at 36 hpi. In contrast, it took 48 h for 50% of leaves to show HR in 28°C.

Correlating with a delay of HR in plants at 28°C, bacterial growth in plants was increased at 28°C as compared with that at 22°C. Wild-type Col-0 seedlings grown at 22 and 28°C to a similar developmental stage were dip-inoculated with avirulent DC3000 strains. The propagation of bacteria was monitored in plants kept growing at these two temperatures. None of the three avirulent DC3000 strains had any growth at 3 dpi over 0 dpi at 22°C (Fig. 2B). In contrast, each amplified at least 10× at 28°C. The growth of DC3000 with AvrRps4 was most affected by a warm condition and had almost 100× amplification over 3 days at 28°C (Fig. 2B).

Temperature effects on plant defense responses to viruses.

We next investigated the temperature effects of *R* gene-mediated defense against viruses using HR as a readout. The *Rx* gene from potato confers resistance to *Potato virus X* (PVX),

and it encodes a CC-NB-LRR type of *R* protein (Bendahmane et al. 1999). The *N* gene from tobacco mediates resistance to TMV, and it encodes a TIR-NB-LRR-type of *R* protein (Whitham et al. 1994). Both of them can trigger HR when they are coexpressed with their respective elicitors, the PVX coat protein (CP) and the TMV helicase fragment (p50) (Bendahmane et al. 1995; Erickson et al. 1999; Whitham et al. 1994).

Agrobacterium strains containing *Rx* and its elicitor CP were infiltrated into fully expanded leaves of *N. benthamiana*. These plants were grown in greenhouses with limited temperature control before acclimation at 22°C on lab bench for 1 day. Infiltrated plants were kept on the bench for 6 h before they were moved to controlled growth chambers. All other environmental conditions for these chambers were the same, except that the temperatures were set at 22, 28, and 30°C. At 22°C, HR induced by *Rx* and CP started to develop at 24 hpi and was completed at 48 hpi (Fig. 3A). In contrast, no HR could be seen at 30°C for up to 4 days (Fig. 3A). Interestingly, HR usually developed at about the same rate at 28 as at 22°C, although occasionally a delay at 28°C was observed.

Disease resistance conferred by the *N* gene is known to be temperature sensitive in *N. tabacum* (Samuel 1931) and tomato (Whitham et al. 1996). In *N. benthamiana*, we found a similar temperature sensitivity of *N*-mediated responses. HR was induced by the *N* gene and its elicitor p50 at 22°C but not at 28 (Fig. 3B) or 30°C (data not shown). Thus both *Rx*- and *N*-mediated HR against viral proteins are suppressed by an elevated temperature in *N. benthamiana*, and the lower end of temperature necessary to inhibit HR appears to be slightly different for *Rx* and *N*.

Analysis of the involvement of SA, *EDS1*, and *PAD4* in high-temperature inhibition of resistance to virulent *P. syringae* pv. *tomato* DC3000.

We asked whether effects of temperature on the accumulation of SA and transcripts of *EDS1* and *PAD4* confer tempera-

Fig. 2. Temperature effects on disease resistance to avirulent *Pseudomonas syringae* pv. *tomato* DC3000. **A**, Progression of hypersensitive responses (HR) in wild type (wt) Col-0 to DC3000 strains expressing AvrRpm1, AvrRpt2, and AvrRps4 at 22 and 28°C. Shown are the percentages of leaves exhibiting HR derived from at least 20 leaves. The experiments were repeated twice and similar results were obtained. **B**, Growth of DC3000 expressing AvrRpm1, AvrRpt2, and AvrRps4 in wild-type *Arabidopsis* Col-0 at 22 and 28°C. Error bars represent standard deviations of three biological repeats. The *t*-test shows that there is a significant amplification of these avirulent strains in Col-0 at 28°C but not at 22°C.

Fig. 3. High temperature inhibits the hypersensitive responses (HR) induced by *N* and *Rx* in *Nicotiana benthamiana*. **A**, Temperature effects on *Rx*-induced HR. Shown are representative leaves 3 days after agroinfiltration with *Rx* and CP at 22°C, 28°C, and 30°C, respectively. **B**, Temperature effects on *N*-induced HR. Shown are representative leaves 3 days after agro-infiltration with *N* and p50 at 22 and 28°C. Infiltrated areas were circled with black or red markers. The experiments were repeated at least three times and similar results were obtained.

Fig. 4. Involvement of *EDS1* and *PAD4* in temperature modulation of defense response. **A**, *Pseudomonas syringae* pv. *tomato* DC3000 growth in wild type (wt), *nahG*, *sid2*, *pad4*, and *eds1* at 22 and 28°C. The *t*-test shows significant differences between growth at 22 and 28°C for wt and *nahG* ($P = 0.004$ and 0.0003 , respectively), and no significant differences between growth at 22 and 28°C for *sid2*, *pad4*, and *eds1* ($P = 0.04$, 0.054 , and 0.034 , respectively). **B**, The expression of *EDS1* in wt and the *EDS1*-OE line at 28°C, analyzed by an RNA blot. **C**, Growth of DC3000 in wt and the *EDS1*-OE line at 22 and 28°C. The *t*-test shows no significant difference in growth at 3 days postinoculation between wt and *EDS1*-OE at 22 or 28°C ($P = 0.12$ and 0.23 , respectively). **D**, The expression of *PAD4* in wt and the *EDS1*-OE;*PAD4*-OE line at 28°C analyzed by an RNA blot. **E**, Growth of DC3000 in wt and *EDS1*-OE;*PAD4*-OE at 22 and 28°C. The *t*-test shows no significant differences in growth at 22 and 28°C between wt and *EDS1*-OE;*PAD4*-OE ($P = 0.47$ and 0.21 , respectively). Error bars in the growth of DC3000 represent standard deviations of three biological repeats.

ture sensitivity in disease resistance against *P. syringae* pv. *tomato* DC3000. First, the growth of virulent DC3000 at 22 and 28°C was compared in the wild-type Col-0, Col *eds1*-2 (Bartsch et al. 2006), *pad4* (Glazebrook et al. 1996), *sid2* (Dewdney et al. 2000), and the *nahG* transgenic plant (Bowling et al. 1997). The *nahG* transgenic contains a gene encoding a SA-degrading enzyme and the *sid2* mutant is deficient in SA accumulation after pathogen invasion (Wildermuth et al. 2001). Compared with the wild type, all three mutants and the *nahG* transgenic supported more bacterial growth at both temperatures, indicating an involvement of SA, *EDS1*, and *PAD4* in basal defenses at both temperatures. The DC3000 strain grew better at 28°C than at 22°C in *nahG* and *sid2*, retaining the same temperature sensitivity as in the wild type. In contrast, it grew to the same extent at 22 and 28°C in the *eds1* and *pad4* mutants (Fig. 4A). These results suggest that *EDS1* and *PAD4* but not SA contribute to temperature sensitivity in basal defense responses to bacterial pathogen. Alternatively, the pathogen growth facilitated by defects in *eds1* and *pad4* plants has reached the maximum capacity allowed in the plants at 22°C, so that a further growth by the effect of a higher temperature is not detected. In this case, growth of bacteria in *eds1* and *pad4* would appear to be temperature insensitive.

To further investigate the role of *EDS1* and *PAD4* in temperature modulation of plant defense, we tested temperature

Fig. 5. Effects of overexpression of *EDS1* and *PAD4* on the *bon1* phenotype. **A**, Growth phenotype of *bon1*;EDS1-OE and *bon1*;EDS1-OE;*PAD4*-OE at 22°C. Shown are wild type (wt), *bon1*-1, and two representative 25-day-old transgenic plants. **B**, Growth phenotype of *bon1*;EDS1-OE and *bon1*;EDS1-OE;*PAD4*-OE at 28°C. Shown are wt, *bon1*-1, and two representative 20-day-old transgenic plants. **C**, Growth of *Pseudomonas syringae* pv. *tomato* DC3000 in wt, *bon1*, and *bon1*;EDS1-OE at 28°C. Error bars represent standard deviations of three biological repeats. The *t*-test shows a significant difference in growth at 3 days postinoculation between wt and *bon1* ($P = 0.0002$) but not between *bon1* and *bon1*;EDS1-OE ($P = 0.33$).

sensitivity of *Arabidopsis* plants expressing *EDS1* at similar levels at two temperatures. The endogenous *EDS1* genes have a lower expression at 28 than at 22°C (Yang and Hua 2004), and therefore, a strong constitutive 35S promoter of *Cauliflower mosaic virus* was used to drive the expression of an *EDS1* transgene. The *p35S::EDS1* transgenic lines generated in Col-0 were subjected to RNA blot assay, and only one line out of seven was found to have an elevated *EDS1* transcript at 28°C as compared with the wild type (Fig. 4B). This line has a comparable level of *EDS1* transcripts at two temperatures, reversing the temperature regulation of the endogenous *EDS1* genes (Fig. 4B). We named this line *EDS1*-OE and used it for further analysis. The *EDS1*-OE plant did not exhibit any morphological defects (data not shown). Further pathogen-growth testing revealed that it supported the same amount of growth of DC3000 as the wild type at both 22 and 28°C (Fig. 4C), suggesting that transcriptional overexpression of *EDS1* in this line is not sufficient to confer temperature insensitivity to basal defense response against DC3000.

Because the *EDS1* protein functions together with the *PAD4* protein, we introduced into the *EDS1*-OE plant *p35S::PAD4* in which the *PAD4* gene is under the control of the strong 35S promoter as well. One out of seven transgenic lines was found to have a higher *PAD4* transcript level at 28°C compared with the *EDS1*-OE line, and we named this line *EDS1*-OE;*PAD4*-OE (Fig. 4D). Resistance to *P. syringae* pv. *tomato* DC3000 was analyzed in this line, and no difference was observed between the *EDS1*-OE;*PAD4*-OE plant and the wild type at 22 or 28°C (Fig. 4E). This indicates that overexpression of *EDS1* and *PAD4* together at the transcriptional level in this transgenic line does not enhance basal resistance at either 22 or 28°C and it does not alter temperature sensitivity of disease resistance to virulent bacterial pathogen DC3000. More *EDS1* and *PAD4* overexpression lines are needed to verify this observation.

Effects of *EDS1* and *PAD4* overexpression on temperature sensitivity of *R*-mediated defense.

We next tested whether transcriptional upregulation of *EDS1* and *PAD4* can reverse the inhibitory effects of 28°C on *R*-mediated defense responses in two systems. The first system is HR mediated by *RPS4*, which exhibits a dramatic temperature sensitivity to *P. syringae* pv. *tomato* DC3000 expressing AvrRps4 (Fig. 2). The *EDS1*-OE;*PAD4*-OE plants grown at 22 and 28°C were infiltrated with DC3000 with AvrRps4, and HR was monitored at the two temperatures. No change in HR progression was observed in this line compared with the wild type at either temperature (data not shown). Thus, transcriptional overexpression of *EDS1* and *PAD4* is not sufficient to reverse the suppression on AvrRps4-triggered defense responses by high temperatures.

The second system in which we analyzed *R*-mediated defense response is the *bon1* (*bonzail*) mutant. The *bon1* mutant has an activation of a TIR-NB-LRR-type of *R*-like gene *SNC1* at 22 but not at 28°C, resulting in a temperature-sensitive dwarf phenotype that can be easily scored. The *EDS1*-OE line was crossed to the *bon1* mutant to generate *bon1*;*EDS1*-OE plants, and *EDS1*-OE;*PAD4*-OE was crossed to *bon1*;*EDS1*-OE to generate *bon1*;*EDS1*-OE *PAD4*-OE. At 22°C, both *bon1*;*EDS1*-OE and *bon1*;*EDS1*-OE *PAD4*-OE plants exhibited a stronger dwarf phenotype compared with *bon1* (Fig. 5A) and this enhanced dwarf phenotype was more dramatic later in development (data not shown). The dwarf phenotype was, however, totally rescued at 28°C, and no phenotypic difference was observed among the wild type, *bon1*, *bon1*;*EDS1*-OE, and *bon1*;*EDS1*-OE *PAD4*-OE (Fig. 5B).

To confirm that disease resistance was indeed suppressed by 28°C in *bon1*;*EDS1*-OE, we compared the growth of DC3000

in this line to those in *bon1* and the wild type at 28°C. Plants were germinated at 22°C for 3 days (to facilitate synchronized germination and establishment of young *bon1*;*EDS1*-OE seedlings) and were transferred to 28°C for 7 days before the pathogen tests were carried out at 28°C. Under this condition, the *bon1* mutant supported slower growth of DC3000 compared with the wild type at 28°C (Fig. 5C). This slight increase of resistance in *bon1* was probably due to an activation of defense responses during the early growth at 22°C, as previous study shows the same growth of DC3000 in *bon1* and the wild type grown constantly at 28°C (Yang and Hua 2004). Nevertheless, the *bon1*;*EDS1*-OE plants behaved similarly to *bon1*

Fig. 6. The role of jasmonic acid in temperature modulation of defense responses. **A**, Growth of *Pseudomonas syringae* pv. *tomato* DC3000 in wild type (wt), *coi1*, and *jar1* at 22 and 28°C. The *t*-test shows a significant difference in growth at 3 days postinoculation (dpi) at 22°C between *coi1* and wt ($P = 0.0001$) and between *jar1* and wt ($P = 0.0007$). There is a significant difference in growth at 3 dpi at 28°C between *coi1* and wt ($P = 0.0004$) but not between *jar1* and wt ($P = 0.02$). **B**, Plant growth phenotype of wt, *bon1*, *coi1*, *bon1coi1*, *jar1*, and *bon1jar1* at 22 and 28°C. **C**, Growth of DC3000 in wt, *coi1*, *jar1*, *bon1*, *bon1coi1*, and *bon1jar1* at 28°C. The *t*-test shows a significant difference in growth at 3 dpi at 28°C between *bon1* and wt ($P = 0.0002$) and between *coi1* and wt ($P = 0.0004$) but not between *jar1* and wt ($P = 0.02$). The *t*-test also shows a significant difference in growth at 3 dpi at 28°C between *bon1coi1* and *bon1* ($P = 0.0001$) and between *bon1coi1* and *coi1* ($P = 0.001$) but no significant difference between *bon1jar1* and *bon1* ($P = 0.02$) and *bon1jar1* and *jar1* ($P = 0.001$). Error bars in the growth of DC3000 represent standard deviations of three biological repeats.

(Fig. 5C), indicating that overexpression of *EDS1* does not alter disease resistance of *bon1* at a higher temperature.

Analysis of the role of the JA in temperature modulation on defense response.

A link between temperature responses and JA signaling was suggested from transcriptional profiling studies in which we compared *Arabidopsis* microarray data of a JA treatment (1 h after 10 μ M of methyl jasmonate) (The Arabidopsis Information Resource [TAIR] accession number 1007965964) and a temperature-shift treatment (from 16 to 25°C for 9 h) (Balasubramanian et al. 2006). Among the 34 genes with the highest induction by JA, 21 responded to the temperature shift treatment with at least twofold induction (Supplemental Table 1). No such coregulation was observed between responses to the temperature shift and responses to other hormones, including auxin, ethylene, and gibberellic acid (TAIR accession numbers 1007965859, 1005823581 and 1005823535, respectively) (data not shown). Because the SA signaling pathway is often antagonistic to the JA pathway, it is possible that a high temperature promotes JA signaling to repress SA signaling leading to a suppression of SA-mediated defense responses.

To test this hypothesis, we analyzed disease resistance to the virulent pathogen *P. syringae* pv. *tomato* DC3000 at 22 and 28°C in *coil* and *jar1*, two mutants defective in JA signaling. The *coil* mutant was shown to exhibit reduced susceptibility to DC3000 under normal growth conditions (Feys et al. 1994; Kloeck et al. 2001). Consistent with these results, we found that both the *coil* and *jar1* mutants were more resistant to DC3000 than the wild type at 22°C (Fig. 6A). At 28°C, *coil* is more disease resistant than the wild type and *jar1* is as resistant as the wild type (Fig. 6A). Both mutants supported more growth of DC3000 at 28°C than at 22°C, indicating that they retain temperature sensitivity in defense responses. As *COI1* mediates almost all JA responses, while *JAR1* is responsible for part of the JA responses, enhanced resistance of the *coil* mutant compared with the wild type at 28°C suggests that JA still regulates defense responses at a higher temperature. Thus, the disease-resistance pathway mediated by JA likely functions in parallel to the temperature-sensitive disease-resistance pathway.

In addition, we analyzed the JA effects on the temperature sensitivity of the *bon1* mutant in which the *R*-like gene *SNC1* is activated. Double mutants *bon1coil* and *bon1jar1* were constructed, and their phenotypes were analyzed at 22 and 28°C. Both double mutants displayed more severe growth defects as compared with the *bon1* single mutant at 22°C. Their dwarf phenotypes were largely rescued at 28°C, although the *bon1jar1* double mutant still displayed a weak dwarf phenotype (Fig. 6B). Their disease-resistance phenotypes were correlated with the growth phenotype. The *bon1* mutant had a slight increase of resistance to DC3000 at 28°C compared with the wild type after 3 days of growth at 22°C followed by a 7-day growth at 28°C. While the *bon1jar1* double mutant supported pathogen growth to the same extent as *bon1*, the *bon1coil* double mutant was more resistant to DC3000 than either the *bon1* or the *coil* single mutants at 28°C (Fig. 6C). Thus, *bon1* and *coil* have additive effects on resistance to DC3000 at 28°C. This again supports the notion that the JA-mediated resistance pathway functions in parallel to the temperature-sensitive resistance pathway.

DISCUSSION

Temperature has been implicated in affecting plant-pathogen interactions, and a higher growth temperature was observed to either increase or decrease disease resistance. This may reflect

a differential influence by the same temperature variation on different plant-pathogen interactions. Alternatively, the observations might have been made at different temperature ranges or other environmental factors may not be well controlled, especially in the field. There appears to be a common theme that *R* gene-mediated disease resistances including HR are often inhibited by an increase in growth temperature. In this study, we investigated the effect of a moderate increase in temperature above the normal on disease resistance in a controlled manner. We show that resistances to *P. syringae* pv. *tomato* DC3000 mediated by three *R* genes were inhibited by a moderate elevation in temperature and the HR response to viral proteins from TMV and PVX were also suppressed by a warm condition. Temperature sensitivity is thus present in defense mediated by different classes of *R* proteins (Martin et al. 2003). The *N*, *SNC1*, and *RPS4* proteins belong to the TIR-NB-LRR class; *RPM1*, *RPS2*, and *Rx* belong to the CC-NB-LRR class; *Cf-4* and *Cf-9* belong to the LRR-TM (trans-membrane domain) class; and *RPW8* is a unique TM-CC protein. In addition, we found that basal resistance to *Pseudomonas syringae* is also compromised by a moderate elevation of temperature. It is, therefore, probably a general phenomenon that disease resistances to biotrophic pathogens are inhibited by a mild elevation of the normal growth temperature.

We show that this temperature sensitivity lies mostly in the plant defense systems. The increase of bacterial growth in plants at elevated temperature can not be explained by variations in intrinsic bacterial growth rate or secretion of effector proteins at different temperatures. A slow HR is also unlikely to be caused by a slow accumulation of bacteria or effector proteins leading to a slower detection by plants. In fact, *P. syringae* pv. *tomato* DC3000 strains grow faster in plants at elevated temperature. That bacterial pathogens may secrete fewer effector proteins to trigger HR at a high temperature may contribute to the slow HR. Even so, it does not totally account for the slow or no HR at elevated temperatures. With expression of effector proteins directly in the plant cells, HR mediated by *N* and *Rx* is also inhibited by a higher temperature, indicating a deficiency in the plant defense system at elevated temperatures.

The evolutionary rationale for such an inhibition of disease resistance by an elevated temperature is unclear. This could be a result of coevolution of plants and their pathogens, as some pathogens are less virulent at a higher temperature. Almost all virulent genes of plant-pathogenic bacteria studied with respect to temperature exhibit increased transcription at temperatures below the respective growth optima; and these virulence determinants include phytoalexins, cell wall-degrading enzymes, and the bacterial type III secretion system (Smirnova et al. 2001). For instances, the production of coronatine by *Pseudomonas syringae* is maximal at 18°C and minimal at 28°C (Palmer and Bender 1993; Weingart et al. 2004) and several bacterial effector proteins are secreted most abundantly at temperatures between 18 to 22°C (van Dijk et al. 1999). It should also be noted that the resistances studied here are mostly against biotrophic pathogens living on live plants and it is yet to be tested whether plants have a higher resistance to necrotrophic pathogens at elevated temperatures, as host resistance to pathogens of these two lifestyles are often antagonistic. The temperature modulation might reflect a balance of defense against pathogens with different virulence at different temperature ranges.

Alternatively, this phenomenon might reflect a complex interaction between biotic and abiotic responses. Both heat shock protein 90 (HSP90) and heat shock cognate 70 (HSC70) are involved in plant defense responses, probably by performing chaperone functions together with SGT1 (Noel et al. 2007; Schulze-Lefert 2004). Temperature variation, even a moderate

one, could change the expression of HSP and HSC and, therefore, affect the defense responses by altering R protein conformation or activities. Plants may also recruit these chaperon proteins to more essential cellular functions at elevated temperatures and thus compromise plant defense responses. Interestingly, overexpression of HSC70 is shown to enhance heat tolerance and to disable both basal and *R*-mediated disease resistance (Noel et al. 2007), supporting the hypothesis of a trade-off between biotic and abiotic responses.

This temperature sensitivity suggests that plant disease resistance could break down at elevated temperatures and an increase of a few degrees Celsius in temperature is sufficient to inhibit defense responses. It is not uncommon for plants in the fields to experience elevated temperatures (such as 28°C) during the middle of the day. Whether that would be sufficient to inhibit disease resistance still waits to be determined, because our current studies are carried out under constant temperatures. Is there a window of time during early events in plant-pathogen interaction critical for elevated temperature to break down resistance or is a sustained high temperature required for this inhibition? Applying an elevated temperature for different lengths of time and at different phases of plant-pathogen interaction will be needed to resolve this issue. In any case, elevated temperatures will likely facilitate the propagation of a number of plant diseases and it is important to understand the scope and the mechanisms of this temperature sensitivity.

It is not known how temperature modulates host disease resistance or whether similar mechanisms apply to its modulation of basal and *R*-mediated resistance. Does temperature affect recognition of the pathogen or does it affect the activities of defense signaling components? Temperature sensitivity of *N*-mediated HR is shown to lie in the early event of HR, and elevated temperature is suggested to weaken the interaction between the virus and the host surveillance mechanism (Padgett et al. 1997). This is an attractive hypothesis that could potentially apply to both Avr recognition and pathogen-associated molecular pattern recognition. Consistent with this model, the temperature threshold required to inhibit HR or defense is different among disease resistances mediated by different *R* genes. A temperature of 28°C is sufficient to suppress defense activated by *SNCI* and *N* but not *Rx*, which requires 30°C for the inhibition. The same temperature of 28°C is more effective to inhibit growth of DC3000 through *RPS4* than through *RPM1* or *RPS2*. This might reflect a difference in temperature sensitivity of R protein recognition or activation. However, the molecular details of this early event remain to be identified and it needs to be tested whether it is, indeed, a common mechanism for temperature sensitivity.

It is equally plausible that some shared signaling components are responsible for temperature sensitivity, because disease resistances mediated by different classes of R proteins are modulated by temperature. This study tested the role of *EDS1* and *PAD4*, which have a temperature-dependent transcript level, by elevating their expression at a high temperature with a strong 35S promoter. However, very few transgenic plants with overexpression constructs achieved a high expression of these two genes at 28°C. It remains to be determined whether few transgenic plants overexpress *EDS1* and *PAD4* at 22°C as well. If so, their overexpression might have an adverse effect on plants and are therefore selected against in the transgenic generating process. If not, the *EDS1* and *PAD4* RNA transcripts themselves might be subject to temperature regulation. In this case, the *EDS1-OE* and *PAD4-OE* lines might have escaped this regulation by acquiring additional regulatory elements close to the transgene insertion site. Nevertheless, the transcripts of *EDS1* and *PAD4* are overexpressed in the line

analyzed and their overexpression did not alter disease resistance at a higher temperature. Although more overexpression lines are needed for further analysis, this study suggests that temperature regulation of the transcript level of *EDS1* and *PAD4* is not sufficient to cause temperature sensitivity of defense responses. However, we can not eliminate the possibility that the *EDS1* protein activity (including its interaction with *PAD4*) could still be regulated by temperature to induce temperature sensitivity of defense.

This study also tested the role of two signaling molecules, SA and JA, in this temperature modulation, using mutants defective in their biosynthesis or signaling, and found that neither of them is sufficient to induce temperature-sensitivity by itself. Rather, it appears that SA fully mediates the temperature-sensitive pathway in disease resistance and JA functions in another independent pathway. Other shared signaling components in plant host defense could be candidates for such a 'temperature sensor.' Molecules like chaperone proteins and plant hormones such as abscisic acid and auxins could be tested. In addition, genetic screens for mutants defective in this modulation can be carried out to dissect this process. These studies to identify essential regulators should reveal the molecular mechanisms underlying temperature sensitivity of defense response and contribute to the understanding of temperature modulation of plant growth and development in general.

MATERIALS AND METHODS

Plant growth.

Arabidopsis seeds were sown on soil or petri dishes containing half-strength Murashige Skoog medium (Sigma, St. Louis) with 2% sucrose and 0.7% agar. *Arabidopsis* plants were grown in growth chambers (Conviron, Pembina, ND, U.S.A.) under fluorescent light (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and 50 to 60% relative humidity with controlled temperatures as specified. A photoperiod of 12-h light and 12-h darkness was used for disease resistance testing and a constant light condition was used otherwise. *Nicotiana benthamiana* plants were grown in the greenhouse for 3 to 4 weeks, followed by 1 day of growth on a lab bench at 22°C before being used for transient expression assays.

Molecular biology manipulation.

Basic molecular manipulations were carried out according to Sambrook and associates (1989). RNA was extracted from 3-week-old plants using Tri Reagent solution (Molecular Research Center, Cincinnati). Total RNAs of 20 or 30 μg each were separated on gel and were transferred to Hybond nylon membrane (Amersham, Piscataway, NJ, U.S.A.). A ^{32}P -labeled single-stranded antisense fragment was used for RNA blot hybridization. rRNA stained by ethidium bromide was used as a loading control.

Generation of transgenic plants.

To produce transgenic plants, the coding regions of the cDNAs of *EDS1* and *PAD4* were isolated by reverse transcription-polymerase chain reaction (RT-PCR) on total RNAs of the wild-type *Arabidopsis*. The cDNAs were then cloned into the pGreen0229 vector under the control of the strong promoter of 35S promoter (pGreen website). The *p35S::EDS1* and *p35S::PAD4* overexpression constructs were transformed into *Agrobacterium* GV3101 (Koncz and Schell 1986) and, subsequently, into *Arabidopsis* plants by the floral dipping method (Clough and Bent 1998).

Disease resistance assays.

Bacterial growth in *Arabidopsis* was analyzed as described with some modifications (Tornerio and Dangl 2001). *Pseudo-*

monas syringae pv. *tomato* DC3000 was grown overnight on King's B medium and was resuspended at 10^8 CFU/ml⁻¹ in a solution of 10 mM MgCl₂ and 0.02% Silwet L-77. Seedlings were grown at a defined temperature for 10 to 14 days, were dip-inoculated with bacteria, and were covered with a plastic dome for 1 h at room temperature (22°C) before returning to their original growth temperature uncovered for 3 days. The amount of bacteria in plants was analyzed 1 h after dipping (day 0) and 3 days after dipping (day 3). The aerial parts of three inoculated seedlings were pooled as one sample, and three samples were collected for each genotype at one time-point. Seedlings were ground in 1 ml of 10 mM MgCl₂, and serial dilutions of the ground tissue were used to determine the number of CFU per gram of leaf tissues.

HR test.

For the HR test in *Arabidopsis*, plants were initially grown at 22°C with a 12-h day and 12-h night for 3 weeks before they were acclimated at 22 and 28°C, respectively, for 1 week. *P. syringae* pv. *tomato* DC3000 with avirulent genes were resuspended at 10^8 CFU/ml and were infiltrated into expanded leaves of these plants. Infiltrated leaves were monitored for symptoms of cell collapse at 22 and 28°C, respectively. For the HR test in *N. benthamiana*, *Agrobacterium* infiltrations were performed as described (Bendahmane et al. 2000; Burch-Smith et al. 2007) with minor modification. Cells containing Rx and CP were each resuspended to a final concentration of an optical density at 600 nm (OD₆₀₀) = 0.2. Cells containing N and p50 were resuspended to OD₆₀₀ = 1.8 and OD₆₀₀ = 1.0, respectively. Infiltrated plants were left on a bench at 22°C for 6 h before they were moved to different temperatures for HR monitoring.

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