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Pathogen resistance of transgenic tobacco plants producing caffeine

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Abstract

Caffeine (1,3,7-trimethylxanthine) is a typical purine alkaloid, and produced by a variety of plants such as coffee and tea. Its physiological function, however, is not completely understood, but chemical defense against pathogens and herbivores, and allelopathic effects against competing plant species have been proposed. Previously, we constructed transgenic tobacco plants, which produced caffeine up to 5 μg per gram fresh weight of leaves, and showed them to repel caterpillars of tobacco cutworms (*Spodoptera litura*). In the present study, we found that these transgenic plants constitutively expressed defense-related genes encoding pathogenesis-related (PR)-1a and proteinase inhibitor II under non-stressed conditions. We also found that they were highly resistant against pathogens, tobacco mosaic virus and *Pseudomonas syringae*. Expression of *PR-1a* and *PR-2* was higher in transgenic plants than in wild-type plants during infection. Exogenously applied caffeine to wild-type tobacco leaves exhibited the similar resistant activity. These results suggested that caffeine stimulated endogenous defense system of host plants through directly or indirectly activating gene expression. This assumption is essentially consistent with the idea of chemical defense, in which caffeine may act as one of signaling molecules to activate defense response. It is thus conceivable that the effect of caffeine is bifunctional; direct interference with pest metabolic pathways, and activation of host defense systems.
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Keywords: Caffeine; *Nicotiana tabacum*; Solanaceae; Pathogenesis-related genes; *Pseudomonas syringae* pv. *glycinea*; Tobacco mosaic virus

1. Introduction

Plants have developed a variety of defense mechanisms to cope with microbial pathogens and herbivores. Defense response includes programmed cell death to isolate pathogens, production of antimicrobial compounds and cell wall fortification. One of the initial actions of the defense reaction is triggered by increasing the expression of a number of pathogenesis-related (PR) genes (Leon et al., 1993). For example, systemic induction of PR proteins is associated with systemic acquired resistance (SAR), which provides defense against subsequent attacks by a broad spectrum of microbial pathogens and herbivores (Ryals et al., 1996). SAR has been shown to be induced by salicylic acid (SA) and phytoalexins (Hammond-Kosack and Jones, 1996; Durrant and Dong, 2004), the latter typical is being secondary metabolites. This suggested that secondary metabolites may play a certain role in defense response, but detailed information is limited.

Plants produce more than 50,000 secondary metabolites including alkaloids, anthocyanins, flavonoids, quinines, lignans, steroids, and terpenoids, among which more than 12,000 are alkaloids (Croteau et al., 2000). The physiological functions of alkaloid are not completely understood, but they are considered to participate in plant chemical defenses (Ashihara and Crozier, 1999). A typical example is caffeine (1,3,7-trimethylxanthine) (**5**), which is found in seeds and leaves of certain plant species such as coffee and tea (Ashihara and Crozier, 1999). Exogenously applied caffeine (**5**) was shown to be effective not only as repellant and pesticide for tobacco hornworms (Nathanson, 1984), but also to interfere reproductive potentials of several moth species (Mathavan et al., 1985). We also showed that transgenic tobacco plants, which produced caffeine (**5**) *in planta*, were highly resistant against feeding of tobacco cutworms

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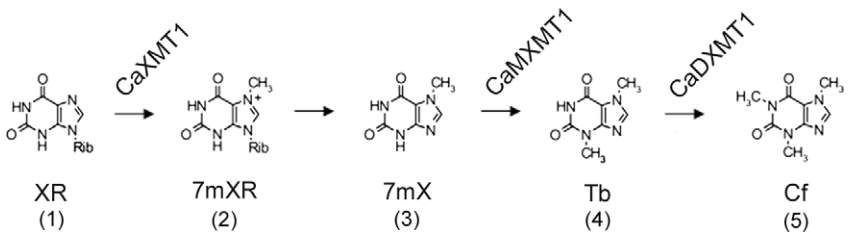

Fig. 1. Caffeine biosynthetic pathway in coffee plants. Caffeine (**5**) is successively synthesized through three methylation steps catalyzed by *N*-methyltransferases, *CaXMT1*, *CaMXMT1*, and *CaDXMT1*, and by removal of ribose moiety by ribonucleosidase. XR, xanthosine (**1**); 7mXR, 7-methylxanthosine (**2**); 7mX, 7-methylxanthine (**3**); Tb, theobromine (**4**); Cf, caffeine (**5**).

(*Spodoptera litura*) (Uefuji et al., 2005). These observations indeed pointed to caffeine (**5**) having a powerful defense role against herbivores. In the present study, we found that caffeine (**5**) is also effective in pathogen resistance, supporting the idea that it commonly functions in chemical defense. Unexpectedly however, the effect appeared not to be direct by causing toxicity on pathogens, but indirectly by activating the host defense system. Hence we propose that one of the functions of caffeine (**5**) is to serve as a sort of signaling substance to modulate the plant stress response.

2. Results

2.1. Transgenic tobacco producing caffeine

We previously constructed transgenic tobacco plants, in which three genes encoding *N*-methyltransferases involved in caffeine (**5**) synthesis were simultaneously expressed (Uefuji et al., 2005). In the present study, we used two T_2 progeny (lines #3 and #5), in which expression of the introduced three genes were confirmed by RT-PCR (Fig. 2a). Accumulation of caffeine (**5**) in leaves was also confirmed by HPLC, showing a 1.8 μg per gram fresh weight (ca. 10 μM) in the line #3, and 2.3 μg (ca. 13 μM) in the line #5 (Fig. 2b). The wild-type plants did not produce caffeine (**5**) (Fig. 2b). Since parental plants of these lines exhibited resistance against herbivore feeding (Uefuji et al., 2005), we speculated that some biochemical pathways might have changed. Subsequently, we examined expression of the defense-related genes encoding pathogenesis-related protein 1a (PR-1a) (X06361) and proteinase inhibitor II (PI-II) (Z29537) in healthy leaves of T_2 plants by RT-PCR, and found that their transcripts constitutively accumulated under non-stressed conditions (Fig. 2c). Control plants showed no accumulation (Fig. 2c). These results suggested that caffeine (**5**) produced *in planta* stimulated expression of these defense-related genes.

2.2. Antiviral effects

PR-1a is typically expressed in tobacco leaves upon infection by pathogens such as tobacco mosaic virus (TMV) (Ward et al., 1991; Cordelier et al., 2003). Its constitutive expression has thus implicated some changes in the pathogen response of T_2 plants. When healthy leaves of wild-type (WT) and transgenic lines (#3 and #5) were inoculated with TMV and kept at 30 °C, plants did not recognize infection and the virus particles propagated. Upon shifting to 23 °C, the hypersensitive response (HR) took place, and a series of defense systems begins to operate. Physiologically, these responses can be visibly estimated by formation and development of necrotic lesions (Fig. 3a). In WT plants, lesions appeared 48 h after the temperature shift, there further developing up to 48 h. In the transgenic line #3, lesions were similarly formed 48 h after the temperature shift, but did not develop further. In the case of transgenic line #5, lesions hardly developed before 48 h after temperature shift (Fig. 3a). In addition to slow lesion formation, their size in transgenic lines was much smaller than in the control WT lines (Fig. 3b). The total number of lesions was also fewer in transgenic lines; 34 per leaf in #3 and 11 in #5, versus 148 in WT (Fig. 3c). These differences were apparently due to elevated resistance after the temperature shift, since TMV equally propagated in all samples before temperature shift as confirmed by an equal accumulation of coat protein transcripts (Fig. 3d). During the HR, transcripts for *PR-1a* and *PR-2* (M59443), which encodes acidic β-1,3-glucanase (Hammond-Kosack and Jones, 1996), were induced in both WT and transgenic plants (Fig. 3d). However, their level in the transgenic lines was 2- to 5-fold higher than that of WT (Fig. 3d). These results suggested that endogenously produced caffeine (**5**) activated expression of PR genes, and increased resistance against TMV infection.

2.3. Antibacterial effects

Resistance against microbial pathogens was then examined. Healthy leaves of WT and transgenic line #3 plants were inoculated with *P. syringae* pv. *glycinea*, which is a incompatible pathogen of tobacco plants, and lesion development was periodically examined up to 48 h (Fig. 4a). In WT plants, distinct lesions were formed 24 h after infection and consistently developed into severe necrosis up to 48 h. In contrast, lesion development was remarkably inhibited in the line #3 even 48 h after inoculation (Fig. 4a). The number of propagated bacteria was lower in the transgenic line than in the control, showing 6.3×10^7 in WT after 48 h infection, while 1.6×10^7 in the line #3, 1/4 that of the control (Fig. 4b). These results pointed to that caffeine (**5**) was also effective to activate defense system against bacterial pathogens.

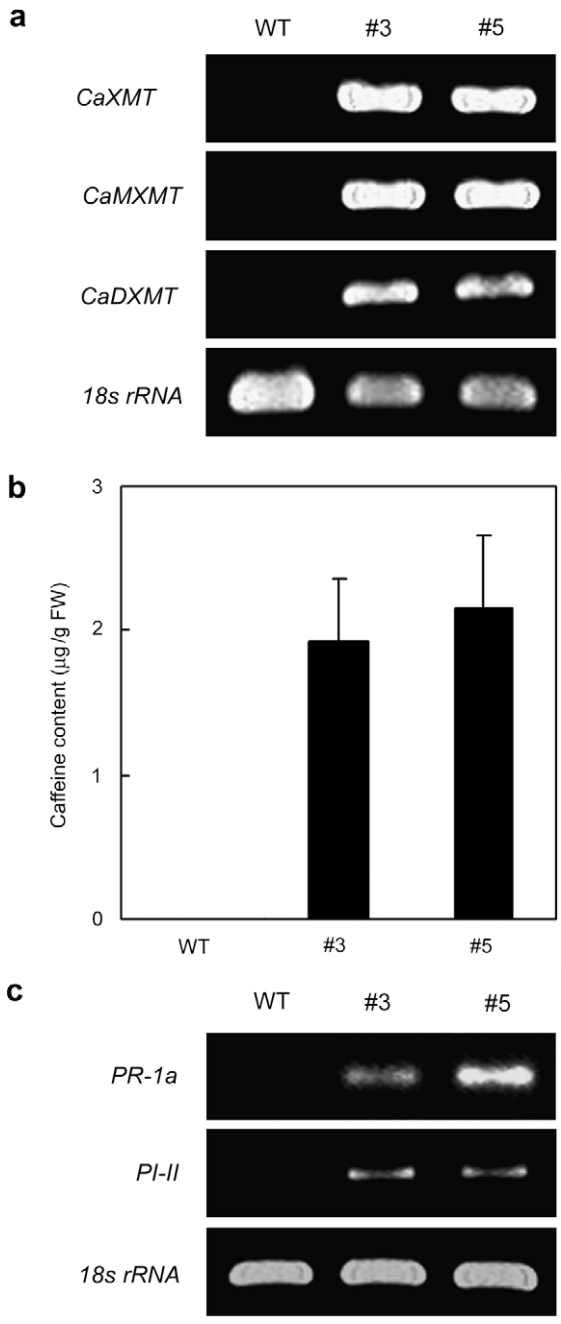

Fig. 2. Properties of transgenic tobacco plants. (a) Expression of introduced genes. Accumulation of transcripts for three *N*-methyltransferases (*CaXMT1*, *CaMXMT1* and *CaDXMT1*) in wild-type (WT) and transgenic lines (#3 and #5) was measured by RT-PCR. Each leaf sample was harvested from 2-month-old plants. As internal controls, 18*S* rRNA was used. (b) Production of caffeine (**5**). Leaf samples from wild-type (WT) and transgenic lines (#3 and #5) were processed to isolate and measure caffeine contents. Data are mean values with standard deviation obtained from three independent plants. (c) Constitutive expression of PR genes. Leaves from wild-type (WT) and transgenic lines (#3 and #5) were harvested from 2-month old plants, and total RNA was used for RT-PCR with specific primers for *PR-1a* and *PI-II*. As internal controls, 18*S* rRNA was used.

2.4. Effects of exogenously applied caffeine

In order to confirm that above results are indeed due to endogenously produced caffeine, two additional experiments were carried out: first by measuring plant resistance to pathogenic bacteria in the presence of exogenously applied caffeine (**5**), second by estimating bacterial growth on the medium containing caffeine (**5**). Healthy leaves of WT plants were submerged either in water (control), or in 1 mM caffeine (**5**) solution (ca. 0.02%) for 48 h and then inoculated with *P. syringae* pv. *glycinea*. In the control sample, necrosis was distinct 24 h after inoculation, and further developed up to 48 h (Fig. 5a). In caffeine (**5**)-treated leaves, necrotic lesions were scarce at 24 h, and slowly developed by 48 h (Fig. 5a), indicating that exogenously applied caffeine (**5**) could induce resistance against bacterial attack. Transcripts of defense-related genes, *PR-1a*, acidic *PR-2*, and *PI-II,* also began to accumulate 6 to 12 h after infection, reaching maximal level 48 h later (Fig. 5b). To see whether or not caffeine (**5**) directly affects bacterial growth, *P. syringae* pv. *glycinea* was cultivated on medium containing different concentrations of caffeine (**5**). Results showed that bacterium apparently grew normally in the presence of up to 1 mM caffeine (**5**), and that, in order to completely suppress the growth, 2 mM (ca. 0.4 mg g^{-1} or 0.04%) of caffeine (**5**) was necessary (Fig. 5c). These findings confirmed the idea that, while caffeine (**5**) is directly toxic to bacteria at a high concentration, its effect is indirect at low concentrations by activating plant defense systems.

3. Discussion

Caffeine (**5**) has long been considered to constitute a chemical defense against biological stressors (Ashihara and Crozier, 2001). For example, slugs fed significantly less on cabbage leaves sprayed with 0.01% caffeine (**5**) solution, and topical treatment with over 0.1% caffeine (**5**) solution was lethal to the snails (Hollingsworth et al., 2002). We also found that 1.25 mM (ca. 0.05%) caffeine (**5**) was effective to repel *Pieris rapae* larvae from feeding on Chinese cabbage leaves (Kim et al., 2006). Lethal effects on *S. litura* larvae were also observed at over 50 mM in artificial food (ca. 10 mg per gram fresh weight) (Uefuji et al., 2005). These observations indicated that a relatively high dose of caffeine (**5**) application was necessary to affect pests, ranging from 0.5 mM to 50 mM, approximately corresponding to 100 μg (0.01%) to 10 mg (1%) per gram fresh weight of food. However, in the case of transgenic tobacco plants, which endogenously produced caffeine (**5**), much lower dose at 2 μM (0.4 μg per gram fresh weight) was sufficient to repel insects (Uefuji et al., 2005). This discrepancy in dose-effect has been puzzling, raising a question as to the mode of the chemical defense.

The present study suggested that repelling effects were possibly not due to caffeine (**5**) itself, but due to stimulation of host defense system by inducing expression of defense-related genes such as *PI-II* that encodes proteinase inhibitor II. This finding partly explains the repellent effects, since proteinase inhibitor II is known to cause digestion dysfunction in larvae guts, and eventually resulting in

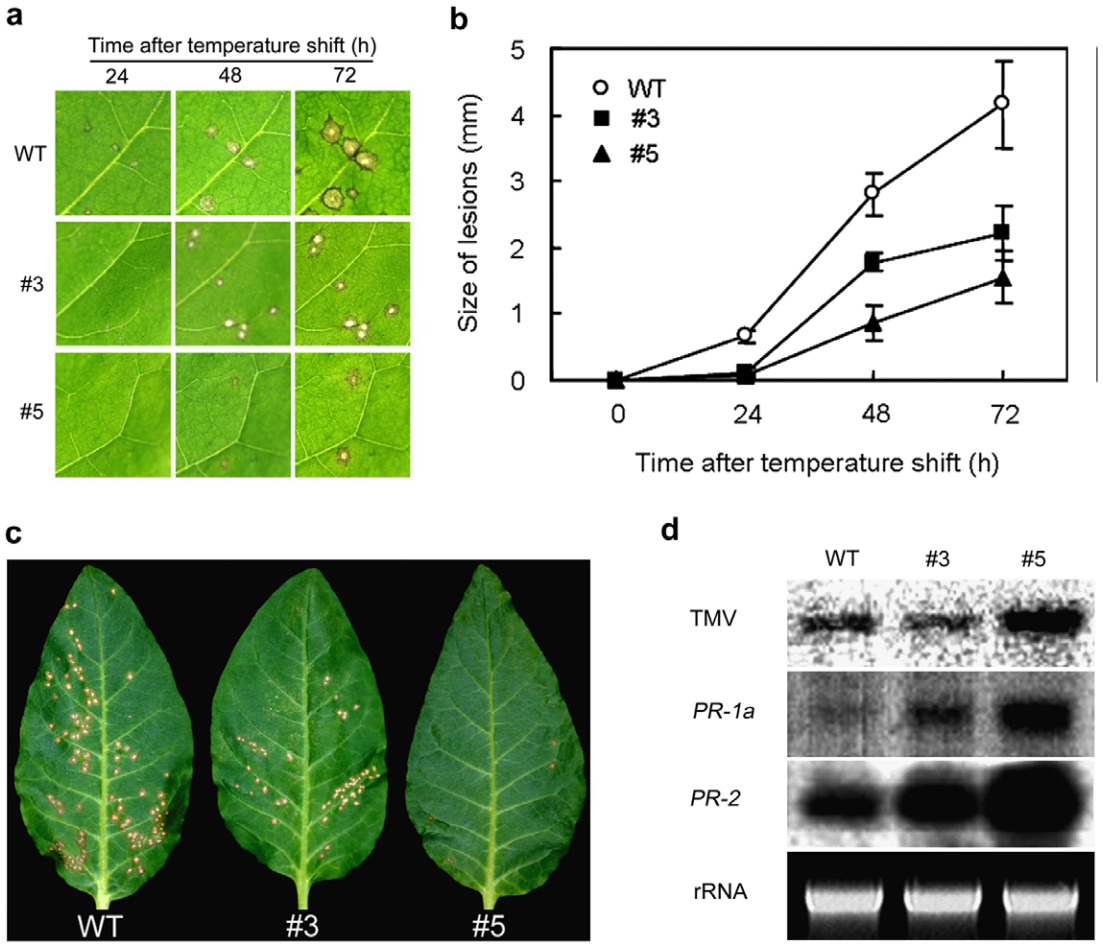

Fig. 3. Resistance against tobacco mosaic virus infection. (a) Lesion development. Healthy leaves from wild-type (WT) and transgenic lines (#3 and #5) were treated with tobacco mosaic virus (TMV), kept at 30 °C for 48 h and then transferred to 20 °C (temperature shift). Lesions were observed at indicated time points after temperature shift. (b) Lesion size. The size of lesions was measured under a microscope at indicated time points after temperature shift. Values are mean from 30 lesions with standard deviations. (c) View of necrotic lesions. Whole leaf was photographed 72 h after temperature shift. (d) Expression of defense-related genes. Total RNA was isolated from infected leaf samples 6 h after temperature shift, and a 10 μg aliquot per lane was fractioned and hybridized with radioactively labeled probes for *PR-1a* and acidic *PR-2*. As the equal loading control, rRNA was used. Infection efficiency was estimated by measuring transcripts for TMV coat protein (top panel).

repelling pests (Green and Ryan, 1972). The present study also showed that pathogenesis-related (*PR*) genes encoding PR-1a and PR-2 (acidic β-1,3-glucanase) were transcriptionally activated by endogenous caffeine, resulting in conferring the host plant resistance against infection by the tobacco mosaic virus (TMV) and the bacterial pathogen, *Pseudomonas syringae*. Thus caffeine (**5**) appears to commonly activate the defense system of plants against broad-range of biotic stresses.

3.1. Concluding remarks

The molecular mechanism for defense gene activation by caffeine (**5**) is not clear. It is conceivable that caffeine (**5**) directly triggers gene expression, although secondary metabolites are generally considered not to affect gene expression by themselves. Alternatively, caffeine (**5**) possibly stimulates production or translocation of signaling molecules such as salicylic acid, which activates *PR* genes, and jasmonic acid, which activates wound-responsive genes including *PI-II*. In either case, the system is novel, and further investigation will determine the underlying molecular mechanisms.

Finally it should be noted that caffeine (**5**) production may provide a novel technology to produce biotic stress-tolerant crops. Since nearly 40% of world agricultural production is lost by herbivores and diseases (Baker et al., 1997; Jouanin et al., 1998), production of a low amount of caffeine (**5**) in agriculturally important crops could be generally effective to control both pest insects and pathogens.

4. Experimental

4.1. Plant materials and chemical treatments

WT and transgenic tobacco plants (*Nicotiana tabacum* cv Xanthi) were grown in a growth chamber at 23 °C under a 14/10 h light/dark cycle. Transgenic lines were prepared as

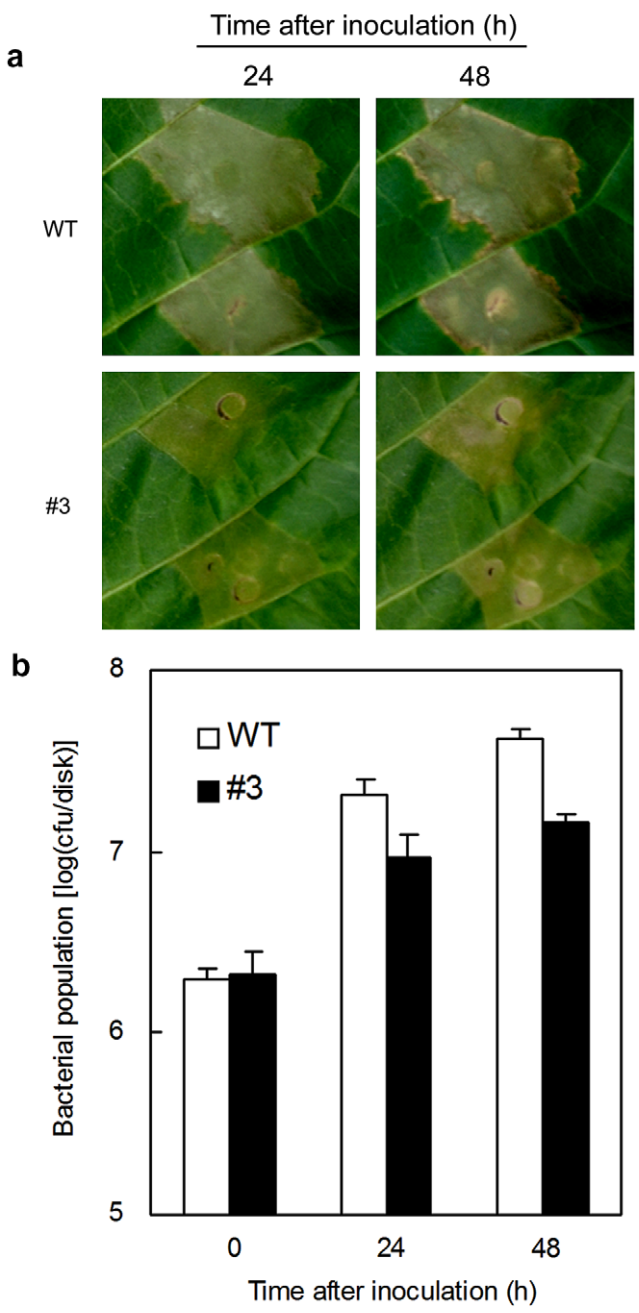

Fig. 4. Resistance against *P. syringae* pv. *glycinea* infection. (a) Development of necrotic lesions. Healthy leaves from wild-type (WT) or transgenic (#3) tobacco plants were inoculated with *P. syringae* pv. *glycinea* and necrotic lesion was observed at indicated time points. (b) Quantification of *P. syringae* pv. *glycinea* cells. Leaf discs were collected at indicated time points after infection and propagated bacterial cells were counted in wild-type plants (WT, open bar) and transgenic line #3 (#3, closed bar). Values are means of three replicates with standard deviations and expressed in logarithmic scale.

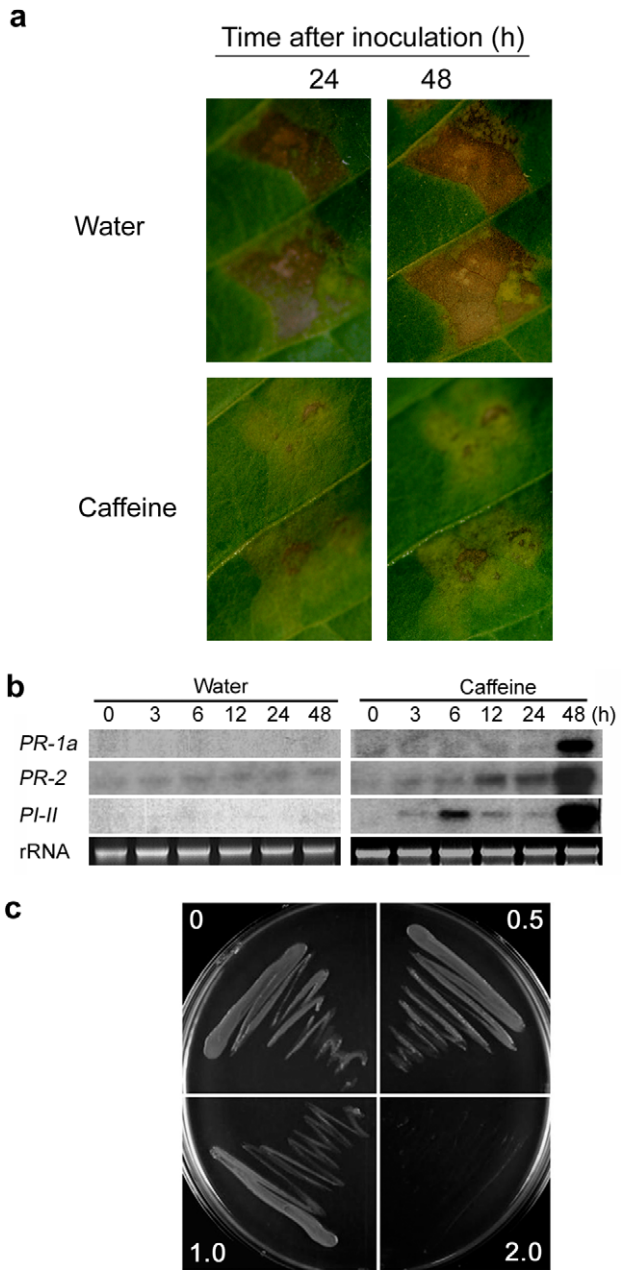

Fig. 5. Effects of exogenously applied caffeine. (a) Disease symptoms. Detached healthy leaves of wild-type plants (WT) were treated with distilled water (Water) or 1 mM caffeine (**5**) (Caffeine) for 48 h, and then infected with *P. syringae* pv. *glycinea*. Disease symptom was observed at indicated time points after inoculation. (b) Accumulation of transcripts of defense-related genes. Total RNA was isolated at the indicated time points after treating leaves from wild-type plants with water (Water) or 1 mM caffeine (Caffeine). A 10 μg aliquot per lane was fractioned by agarose gel electrophoresis and subjected to hybridization with indicated cDNA probes. As the equal loading control, rRNA was used. (c) Caffeine effect on bacterial growth. *P. syringae* pv. *glycinea* was streaked on KB medium containing caffeine at concentration of 0, 0.5, 1.0 or 2.0 mM, incubated at 25 °C for 48 h, and growth was assessed.

previously described, using a multi-gene transformation vector, pBIN-NMT777, containing three coffee *N*-methyltransferases genes, *CaXMT1* (accession number AB048793), *CaMXMT1* (AB048794) and *CaDXMT1* (AB084125) (Uefuji et al., 2005). Among 15 transformants initially obtained, two lines (#3 and #5) were selected, cultivated to maturity and T_2 progeny was used for further experiments after confirmation of caffeine (**5**) production (Fig. 1). Healthy detached leaves, which had been placed in distilled water for 4 h in advance to diminish cutting stress, were

submerged in a solution containing 1 mM caffeine (**5**), incubated at 23 °C under a 14/10 h light/dark cycle, and periodically sampled up to 48 h. Pathogenic bacterium, *P. syringae* pv. *glycinea*, was plated onto KB medium containing appropriate concentration of caffeine (**5**) (0, 0.5, 1.0, and 2.0 mM), and incubated at 25 °C for 48 h.

4.2. Pathogen infection

Healthy leaves were inoculated with tobacco mosaic virus (TMV) and subjected to the temperature-shift assays as described (Yap et al., 2002). Briefly, detached leaves from 2-month-old tobacco plants were mechanically inoculated with or without TMV (10 μg ml^{-1}) in 10 mM sodium phosphate buffer (pH 7.0) using carborundum (600 mesh) and incubated at 30 °C under continuous light for 48 h, and then transferred to 20 °C to allow plants to recognize TMV. Two to three leaves from each plant were assayed for one series of experiments, and in total at least five series were performed. Development of lesions was assessed at 24, 48 and 72 h from the time of the temperature shift. For bacterial treatment, healthy detached leaves were inoculated with *P. syringae* pv. *glycinea* 801 (absorbency at 600 nm was approximately 0.5) by the infiltration method using a syringe without a needle, and incubated at 23 °C for an appropriate time period up to 48 h. Bacterial growth was estimated by counting bacterial numbers in leaf discs (1 cm^2), which were collected immediately after infection (time 0), and periodically up to 48 h, and homogenized in 10 mM $MgSO_4$ solution. After appropriate dilution, bacteria were plated onto KB agar and incubated at 25 °C for 1 day, and number of colonies was counted.

4.3. Transcript analyses

Transcript accumulation was estimated by RNA blot hybridization and RT-PCR. Total RNA was isolated according to the hot-phenol method (Chomczynski and Sacchi, 1987) and RNA blot hybridization was performed as previously described (Sugimoto et al., 2004). Briefly, a 10 μg aliquot of total RNA was fractionated on a 1% formaldehyde gel and blotted onto a nylon membrane (Hybond–N, Amersham). After immobilization by UV irradiation (RPN 2501, Amersham), the blot was hybridized with appropriate ^{32}P-labeled probes in solution containing 1 mM EDTA, 0.5% SDS, 50 mM Tris–HCl pH 7.5, 1 × Denhardt's solution, 3 × SSC, 50% formamide and 10% dextran sulfate at 42 °C for 16 h. After washing with 0.5 × SSC containing 0.1% SDS at 60 °C several times, the membrane was used to expose either BAS (Fuji Photo Film, Tokyo, Japan) or X-ray film (Eastman-Kodak, Rochester, NY). The probes were radioactively labeled using [$\alpha^{32}P$]dCTP using a *Bca*BEST™ labeling kit (Takara, Kyoto, Japan). RT-PCR was performed using an RNA PCR Kit (Takara, Kyoto, Japan). First-strand cDNA was amplified with sets of gene specific primers. Primer sets were 5′-ATCAACTGGTTCTCGCCAAG-3′/5′-CTGCTCTAACGGAAGATGCA-3′ (for *CaXMT1*); 5′-TCCTACAATCTGGCTCTTGC-3′/5′-TGCTTTAATTTGTTCATGGGATC-3′ (for *CaMXMT1*); 5′-TCATTCTACAATCTGTTTCTCATCAG-3′/5′-TATGGAATTCGGGTTCTCGA-3′ (for *CaDXMT1*). 5′-TTGAAACGACCTACGTCCATTTC-3′/5′-AAGCTTGTTCCTAAGGCATGTAG-3′ (for *PR-1a*); 5′-ATCCAAATGATAGGGGCACA-3′/5′-GCCCTCTGATCAGGAGAAAA-3′ (for acidic *PR-2*); 5′-TTTATAATAAAGAAGCAACCATGTA-3′/5′-CTTCACAAACAAACTCGCCATCC-3′ (for *PI-II*). PCRs were carried out for 25 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min. After fractionation on agarose gel electrophoresis, products were identified by visualization with ethidium bromide staining.

4.4. Quantification of caffeine (5) content

Leaf samples of WT and transgenic plants were obtained at 6 weeks after regeneration of cuttings. The samples were dried at 80 °C for 24 h, and caffeine (**5**) were extracted and purified from dried plant parts as described (Baumann et al., 1995). Suspension of caffeine (**5**) in ultra pure water was dried, dissolved in MeOH–H_2O (1:3, v/v in 200 μl) methanol, and subjected to HPLC using a Puresil C18 column (Waters). The column was developed at a flow-rate of 1 ml/min with the solvent by multisolvent delivery system (Waters) and reaction products were densitometrically monitored at 270 nm with a tunable absorbance detector (Waters) (Uefuji et al., 2005).

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