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Pathogen-inducible *CaUGT1* is involved in resistance response against TMV infection by controlling salicylic acid accumulation

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ABSTRACT

***Capsicum annuum* L. *Bugang* exhibits a hypersensitive response against *Tobacco mosaic virus* (TMV) P_0 infection. The *C. annuum UDP-glucosyltransferase 1* (*CaUGT1*) gene was upregulated during resistance response to TMV and by salicylic acid, ethephon, methyl viologen, and sodium nitroprusside treatment. When the gene was downregulated by virus-induced gene silencing, a delayed HR was observed. In addition, free and total SA concentrations in the *CaUGT1*-downregulated hot pepper were decreased by 52% and 48% compared to that of the control plants, respectively. This suggested that the *CaUGT1* gene was involved in resistance response against TMV infection by controlling the accumulation of SA.**
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1. Introduction

Plant defense response involves the orchestrated transcriptional activation of multiple genes and the accumulation of secondary metabolites. It entails the hypersensitive response (HR) and the systemic acquired resistance (SAR) in the plant against pathogenic infections such as TMV [1,2]. These events require elevated levels of salicylic acid (SA) to limit infection to a number of cells, and undergo programmed cell death [3]. Plant rapidly metabolizes SA to sugar conjugate SA glucoside (SAG) during HR [4]. UDP-glucosyltransferases (UGTs) constitute a large family of enzymes that catalyze the transfer of a glucose unit from UDP-glucose to a specific substrate/acceptor [5]. Several UGTs involved in the glycosylation of various metabolites have been isolated, including potato glucosyltransferase induced by wounding [6] and tobacco SA glucosyltransferases (SA GTases) induced by pathogen [7].

In the present study, *Capsicum annuum UDP-glucosyltransferase 1* (*CaUGT1*) was isolated. This gene was induced by resistance response against TMV pathotype P_0 and/or *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) infection. SA, ethephon, and methyl viologen (MV), which are important signal molecules in the defense response, also triggered the expression of *CaUGT1* [8]. In addition, virus-induced gene silencing (VIGS) was performed to observe the knockdown phenotype of the *UGT1* gene in hot pepper and tobacco plants.

2. Materials and methods

2.1. Isolation of full-length CaUGT1 cDNA clone

Hot pepper RNA was isolated from TMV-P_0-inoculated plants as described by Ausubel et al. [9]. The cDNA was synthesized from total RNA using a cDNA amplification kit (Clontech, USA) according to the manufacturer's instructions. For performing RACE (rapid amplification of cDNA ends), two specific primers of the *CaUGT1* gene (*CaUGT GSP1*; 5′-TTCCCATCAAAACCGTTGGTCCTCTGTTC-3′ and *CaUGT GSP2*; 5′-AGGTCCTACATCTAGTGATGTCCAAGGTG-3′) that corresponded to the highly conserved sequence were used for polymerase chain reaction (PCR) amplification.

2.2. Plant cultivation, pathogen inoculation, and chemical treatments

Hot pepper (*C. annuum* L.) cultivar *Bugang*, which is resistant to the TMV-P_0 pathotype but susceptible to the TMV-$P_{1.2}$ pathotype, was used. The plants were grown in a greenhouse at 25 °C with a 16 h light photoperiod cycle. Leaves from 2-month-old plants were used for pathogen inoculation and nucleic acid extraction. To inoculate plants, virus-containing tobacco leaf sap was applied to the surface of leaves with carborundum (Hayashi Chemical, Japan). Two other cultivars of pepper, Early Calwonder (ECW) and

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ECW-20R were used for infiltration with *Xcv*. *Xcv* cultures were grown in nutrient broth for 18 h at 28 °C. Cells were centrifuged at 4000×g, 15 min and resuspended in sterile distilled water. The concentration of bacterial cells was adjusted to an OD_{600} of 0.3 ($2–5 \times 10^8$ CFU/ml) with the WPA spectrophotometer (UK). Bacterial suspensions were diluted to a concentration of 1×10^5 CFU/ml, and were infiltrated with a hypodermic syringe in pepper leaves. For the chemical treatment, hot pepper leaves were sprayed with a solution of 10 mM SA, 20 mM ethephon, 500 μM MV, and 3 mM sodium nitroprusside (SNP), respectively, and then harvested at the indicated time-points.

2.3. Total RNA isolation and Northern blot hybridization analysis

One gram of plant material was ground in liquid nitrogen. The fine powder was added with 10 ml of RNA extraction buffer (0.2 M Tris–HCl, pH 8.0, 0.4 M $LiCl_2$, 25 mM EDTA, and 1% SDS). Ten milliliters of water-saturated phenol were added and vortexed. The samples were centrifuged at 10 000×*g* for 20 min at 4 °C. The supernatant was transferred to new tubes with 10 ml of chloroform. After centrifugation at 10 000×*g* for 10 min at 4 °C, the supernatant was ethanol precipitated. The pellet was dissolved in 2 M $LiCl_2$. After re-precipitation, the pellet was dissolved in water. For RNA blot analysis, 15 μg of total RNA was electrophoretically resolved on a 1.0% agarose gel containing 6% formaldehyde in MOPS buffer (pH 7.0) and transferred onto Nytran Plus membrane (Schleicher and Schuell, USA). Hybridization, washing, and the exposure were performed according to a previous study [10].

2.4. ELISA and RT-PCR

Total proteins were extracted in 200 μl of extraction buffer (0.1 M Tris–HCl with 1% sodium sulfite, pH 7.4) and quantitated using the Bio-Rad protein assay kit (USA). ELISA plates were activated with protein extract (60 μg) at 4 °C. After washing with TBST buffer (0.01 M Tris–HCl containing 0.85% NaCl and 0.05% Tween 20, pH 7.4), the plates were blocked for 1 h with 1% bovine serum albumin (BSA) in 0.1 M Tris–HCl with 0.85% NaCl, pH 7.4. Then anti-TMV-coat protein (CP) antibody was diluted 1:500, and the dilute (200 μl) was applied to the plates for 2 h. The plates were rinsed and incubated with alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit IgG; Sigma, USA; 1:2000) for 2 h. The plates were developed with 100 μl of substrate solution containing *p*-nitrophenyl phosphate (1 mg/ml) (Sigma) in 0.2 M carbonate buffer, pH 9.8 and absorbance was read at 405 nm using the Bio-Rad ELISA Reader.

Semi-quantitative RT-PCR was executed as described [10]. First-strand cDNA was synthesized using 1 μg of total RNA, oligo (dT) primer and SuperScript Reverse Transcriptase (Promega, USA). For RT-PCR, primers that anneal outside the region targeted for silencing were used to ensure that only the endogenous gene was tested. The *CaActin* gene was used as an internal control for RNA quantity. The PCR-generated fragments were analyzed and quantified using the Bio-Rad Gel Doc 2000 and Quantity One Version 4.3 software.

2.5. VIGS and ion leakage measurement

Vectors for VIGS have previously been described in Liu et al. [11]. For pTRV2::*CaUGT1*, a 451-bp cDNA fragment corresponding to bases 1183–1634 of the *CaUGT1* gene was PCR-amplified using primer pairs 5′-GAATTCAACTCTAGAAGCACTTTC-3′ and 5′-GAATTCACGGCGAAAGAAGTGAGA-3′. For pTRV2::*NbUGT1*, a 451-bp conserved region of *UGT1* fragment was amplified from *Nicotiana benthamiana* cDNA using the primers used to amplify the *CaUGT1* gene.

For the VIGS, pTRV1 or pTRV2 and its derivatives were introduced into an *Agrobacterium* strain, EHA105. The culture was inoculated into 50 ml LB medium containing 50 mM kanamycin, 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) with 10 mM $MgSO_4$ and 20 μM acetosyringone, and incubated for 16 h in a shaking incubator at 28 °C. Harvested *Agrobacterium* cells were resuspended in infiltration media (10 mM $MgSO_4$, 10 mM MES, 100 μM acetosyringone) with OD_{600} of 0.3 and incubated at room temperature for 3 h. They were infiltrated using a needleless syringe and the plants were grown at 25 °C in a growth chamber under 16 h light/8 h dark cycle with 60% humidity. For the measurement of ion leakage, 1 cm diameter leaf disks were placed in 50 ml tubes containing 20 ml of Milli-Q water and incubated at room temperature for 2 h. The ion conductivity was measured using the Thermo Orion conductivity meter (USA).

2.6. Agrobacterium-mediated transient expression in N. benthamiana leaves

AvrPtoB expression constructs in pBTEX were introduced into an *Agrobacterium tumefaciens* strain, GV2260. *Agrobacterium* was grown in LB medium overnight and diluted with induction medium [50 mM MES (pH 5.6), 0.5% (w/v) glucose, 1.7 mM NaH_2PO_4, 20 mM NH_4Cl, 1.2 mM $MgSO_4$, 2 mM KCl, 17 μM $FeSO_4$, 70 μM $CaCl_2$, and 200 μM acetosyringone] until an OD_{600} of 0.5. Bacterial suspensions were infiltrated with a needleless syringe into 7- to 8-week-old *N. benthamiana* leaves.

2.7. Salicylic acid measurement

Hot pepper leaves were inoculated with TMV and detached after 48 h of inoculation along with the mock treatment. Leaves were ground in liquid N_2 and 3 ml of 90% methanol was added to 0.3–0.5 g FW samples, which were sonicated for 20 min and centrifuged for 20 min at 1700×g. From the supernatant, 200 μl was used for the measurement of free SA and 200 μl was taken for hydrolysis with 8 M HCl for 1 h followed by vacuum drying. Ninety percent methanol (200 μl) was added in dried extracts for total SA analysis. SA was analyzed using the Dionex HPLC system on a Hydrosphere C_{18} column (250 × 4.6 mm; YMC Co., USA) at a flow rate of 0.8 ml/min. The linear gradient eluting mobile phase was a combination of solution A (20 mM ammonium acetate pH 7.5) and B (methanol) as follows: A/B (5:95, v/v) to A/B (50:50, v/v) over 30 min. Data were collected at 285 nm using the Dionex UVD170U UV detector, and SA was detected at 10.9 min.

3. Results and discussion

3.1. HR-specific transcriptional regulation of CaUGT1

In previous microarray studies, the *CaUGT1* transcripts were induced by 2.9-fold with TMV-P_0 after 72 h and by 2.8-fold with *Xcv* after 12 h during incompatible responses [10,12]. A full-length cDNA of the *CaUGT1* gene was isolated through 5′- and 3′-RACE PCR of the TMV-inoculated hot pepper cDNA library (GeneBank Accession No. FJ008718). The amino acid sequences of CaUGT1 and UDP-glucose glucosyltrasferase of *Fragaria ananassa* shared 64% identity, whereas only about 35% identity with UDP-Xylose phenolic glycosyltransferase of *Lycopersicon esculentum*.

To determine whether the *CaUGT1* transcripts were induced upon TMV inoculation in HR-specific manner, its expression pattern was monitored after TMV-P_0 and TMV-$P_{1.2}$ inoculation. The *CaUGT1* transcripts accumulated from 12 h until 72 h by TMV-P_0,

but not by TMV-$P_{1.2}$ (Fig. 1a). The induction pattern was slightly earlier than the one obtained with the *CaPR-1* [13].

A pathogenic bacterium *Xcv* was also used to test the *CaUGT1* involvement in HR-specific response generated by other pathogens. The *CaUGT1* transcripts accumulation reached a maximal level at 36 h in *Xcv*-resistant pepper cultivar ECW-20R, but not in susceptible cultivar ECW, upon *Xcv* infection (Fig. 1b). From these results, it was verified that the *CaUGT1* was induced in HR-specific responses directed against viral and bacterial pathogenic infections.

3.2. Expression pattern of CaUGT1 by elicitors involved in the defense signal transduction cascades

In plant defense response, several elicitors play key roles in developing the signal transduction cascades [14–18]. To determine whether the *CaUGT1* gene was induced by these elicitors, hot pepper was treated with various inducers. When the plants were sprayed with 10 mM SA, expression of the *CaUGT1* gene showed a rapid increase at 4 h (Fig. 1c). As shown in Fig. 1d, the *CaUGT1* transcripts accumulated at 6 h and then increased until 24 h with 10 mM ethephon, an ethylene-releasing compound [14]. Expression of the *CaUGT1* gene was detected from 12 h and sustained until 24 h (Fig. 1e) after spraying with 500 μM MV, an agent that generates superoxide radicals during photosynthesis [15]. Nitrogen oxide (NO) is a gaseous bioactive molecule that acts as a pivotal regulator of plant physiology, and has been associated with the biotic and abiotic stress response [17]. We tested whether NO regulated the expression of the *CaUGT1* gene after treatment of NO-donor, sodium nitroprusside (SNP). The *CaUGT1* transcripts increased from 6 h until 24 h after the treatment, and then decreased after 24 h, whereas the maximal expression level of the *CaPR-1* gene was detected at 48 h (Fig. 1f).

The accumulation kinetics of *CaUGT1* mRNA after TMV infection showed an earlier induction compared to the *CaPR-1* transcripts. The rapid activation of the *CaUGT1* gene suggested a role for this gene in the early events during the resistance response. An important observation was that the induction of *CaUTG1* occurred only during incompatible interactions, suggesting that the *CaUGT1* played an important role in resistance response and that its induction was dependent on the onset of HR. Several studies reported that plant glucosyltransferase transcripts accumulated during HR. For instance, the tobacco *glucosyltransferase* gene was expressed during incompatible interactions of TMV with Samsun NN tobacco [19]. *UGT73B3* and *UGT73B5* mRNAs of Arabidopsis were also induced by avirulent bacteria *Pseudomonas syringae* pv. *tomato* [20]. In addition, the induction response of *CaUGT1* by elicitors was shown at an early stage. It was reported that the early induction of gene transcription served as a function of stress adaptation, intercellular communication, and transcriptional regulation of late genes to coordinate long-term biological responses [21].

3.3. Compromised HR response and changes in SA levels in CaUGT1-silenced hot pepper

To examine the biological function of *CaUGT1*, the VIGS was employed in hot pepper. Suppression of the *CaUGT1* gene resulted in a more delayed HR phenotype against TMV-P_0 inoculation compared to the wild-type or the vector control plants (data not shown). Suppression of the *CaUGT1* gene also resulted in a decreased number of HR lesions after 48 h of inoculation with TMV-P_0 (Fig. 2a). Because of the delayed and reduced HR response, the *CaUGT1*-silenced hot pepper was tested whether *CaUGT1* suppression could influence the expression of *PR* genes. The *CaPR-5* gene was completely down-regulated in the *CaUGT1*-silenced hot pepper (Fig. 2b), but the expression of *CaPR-1, 2, 4* genes was largely unchanged. These results demonstrated that the *CaUGT1* gene was able to regulate the level of the *CaPR-5* gene, which is known to be involved in an HR response.

To further confirm the involvement of the *CaUGT1* gene in TMV resistance in hot pepper, ELISA was performed to analyze the accumulation of the TMV coat protein (CP). TMV-CP level was 50% higher in the *CaUGT1*-suppressed plants compared to the control plants (Fig. 2c). The indicative plant cell death can also be visualized by

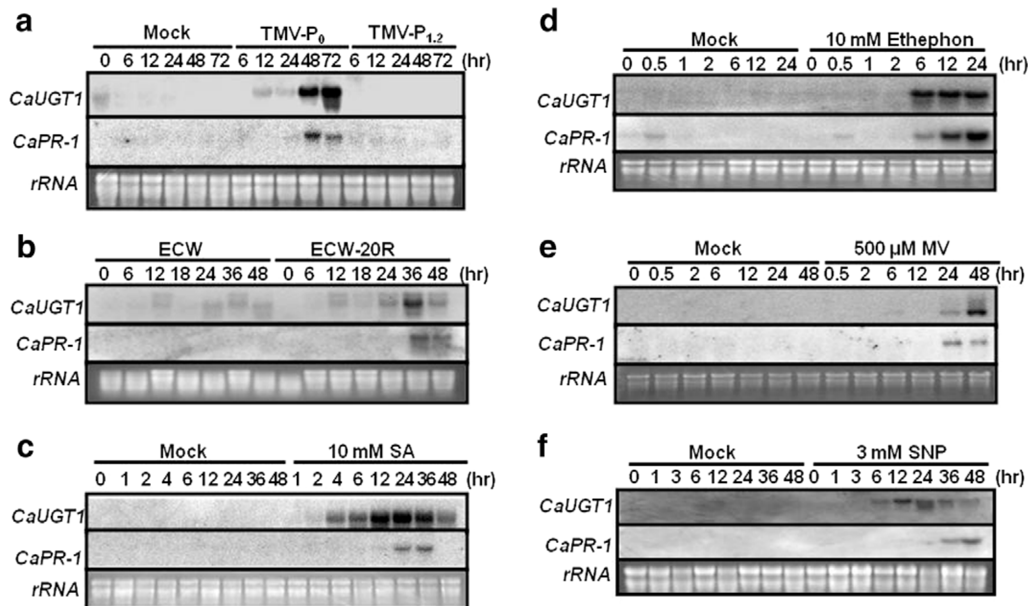

Fig. 1. The *CaUGT1* gene expression by pathogen inoculation and treatments with various chemicals. (a) Expression pattern of *CaUGT1* upon inoculation with TMV-P_0 or TMV-$P_{1.2}$. As a control, leaves were mock-inoculated with inoculation buffer and carborundum only. (b) Expression pattern of the *CaUGT1* upon inoculation of *Xcv*. *Xcv*-susceptible cultivar ECW and -resistant cultivar ECW-20R, were infiltrated with the *Xcv* culture. (c–f) Expression pattern of *CaUGT1* by 10 mM SA (c), 10 mM ethephon (d), 500 μM MV (e), and 3 mM SNP (f) treatment at the time periods indicated. The hybridization was carried out with the ^{32}P-labeled 3′-UTR part of the *CaUGT1* cDNA as a specific probe under high stringency conditions. *CaPR-1* was used as positive controls. Total RNA were stained with ethidium bromide as a loading control.

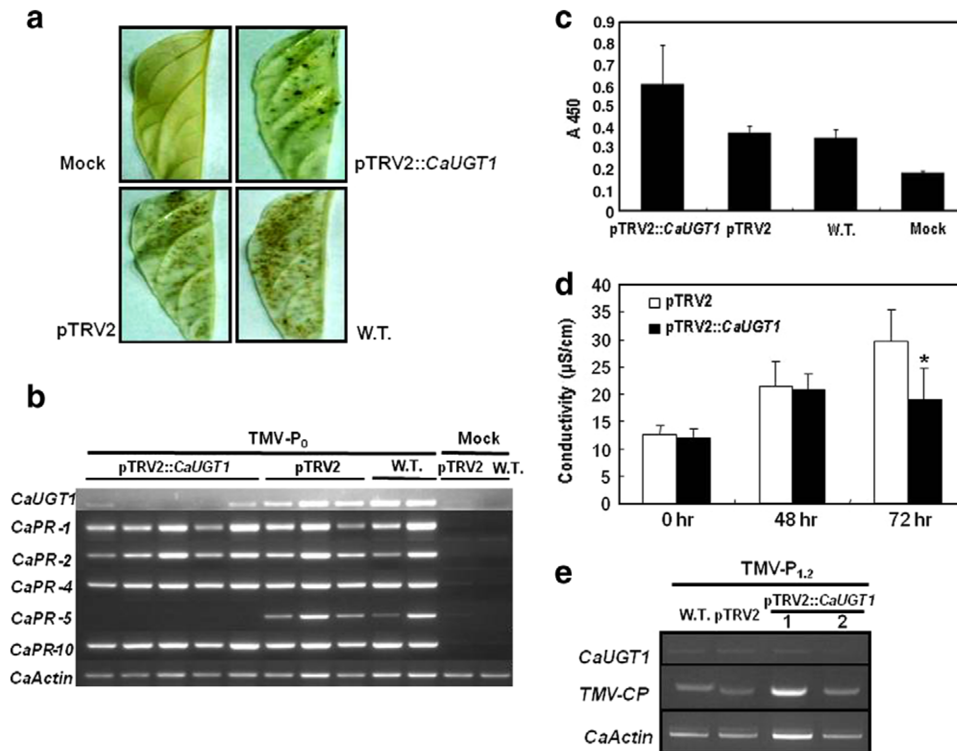

Fig. 2. Phenotypes of *CaUGT1*-silenced hot pepper by TMV treatment. (a) Both *CaUGT1*-silenced hot pepper and vector control plants were treated with TMV-P_0. After 48 h of treatment, half of the leaves were decolorized with 100% ethanol and the HR lesions remained brown. (b) After *CaUGT1*-silenced hot pepper was inoculated with TMV-P_0, RNA was extracted from the other half of leaves. Vector control and wild-type plants were used as a positive control and the mock-inoculated plants were used as the negative control. The different lanes are biological replicates. (c) After TMV-P_0 treatment, leaves from *CaUGT1*-silenced hot pepper, vector and wild-type control plants were used for extraction of proteins to measure TMV-P_0 CP accumulation. Anti-TMV-P_0 IgG antibody was used for TMV-P_0 CP detection by ELISA, measured as absorbance at 405 nm (A405) per 60 μg of total protein. Error bars indicate standard deviations. (d) After TMV-P_0 treatment, leaves from *CaUGT1*-silenced and vector control plants were measured for cellular ion leakage. Asterisk represents significant difference between members of a pair (Student's *t* test; $^*P < 0.05$). (e) TMV-$P_{1.2}$ was inoculated in *CaUGT1*-silenced hot pepper, vector control and wild-type control plants for 48 h. *CaActin* was used for the RNA control. *CaUGT1*-silenced hot pepper, pTRV2::*CaUGT1*; Vector control plant, pTRV2; Wild-type control plant, W.T.

measuring the ion conductivity after TMV-P_0 inoculation. At 72 h post-TMV inoculation, the amount of ion conductivity in the *CaUGT1*-silenced plant was lower than that of the control plants, confirming a compromised HR response (Fig. 2d). The *CaUGT1* transcripts were not increased by TMV-$P_{1.2}$ (Fig. 1a) and no change in the accumulation of TMV-$P_{1.2}$ CP was observed compared to the control plants (Fig. 2e). In summary, these results showed that the *CaUGT1* gene participated in incompatible responses involved in the HR process against TMV infection of the hot pepper.

The importance of SA as a signaling molecule in defense responses has been demonstrated in numerous reports [22–25]. Thus, we measured free and total SA levels in the *CaUGT1*-silenced plants. In the control plant, the concentration of free and total SA, 48 h after TMV inoculation, was 3.9 ± 0.05 μg/g FW and 5.0 ± 0.26 μg/g FW (Fig. 3). The concentration of free and total SA in the vector control plant was 3.9 ± 0.91 μg/g FW and 7.5 ± 0.39 μg/g FW, while that of the *CaUGT1* knockdown plant was 2.3 ± 0.12 μg/g FW and 3.2 ± 0.29 μg/g FW, respectively. It was therefore speculated that the concentration of SA was greatly decreased because of the reduced activity of CaUGT1 in the *CaUGT1*-silenced plant.

The concept of HR limiting pathogen growth is still argued. However, it is accepted that HR contributes to resistance, depending on the specific plant–pathogen interaction. For instance, TMV-infected tobacco cells beyond necrotic lesions still contain infectious virus particles. Therefore, cells adjacent to HR cells are required to obtain defense mechanisms such as SAR to limit the spread of the pathogen [23]. SA was produced in vivo during the HR induced by TMV infection [24]. SA inhibited TMV replication in compatible interactions with tobacco [25]. Thus, the higher TMV-CP level in *CaUGT1*-silenced plants may be a consequence of the reduced SA level, which would allow enhanced TMV replication and reduced HR lesion-formation in infected leaves. In the tobacco plant, the purified SA glucosyltransferase (GTase) has broad substrate specificity for SA as well as its precursors [7]. It is suggested that SA GTase may play a role in regulating the biosynthesis

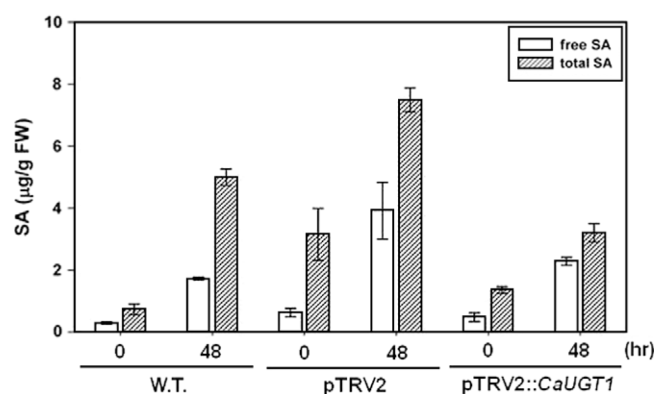

Fig. 3. SA contents of *CaUGT1*-silenced hot pepper. Total and free SA levels in wild-type, vector control and *CaUGT1*-silenced hot pepper plant at 0 and 48 h after TMV-P_0-inoculation were measured. Results are means of three samples and error bars indicate standard deviation. Blank bars designate free SA, and striped bars, total SA. *CaUGT1*-silenced hot pepper, pTRV2::*CaUGT1*; Vector control plant, pTRV2; Wild-type control plant, W.T.

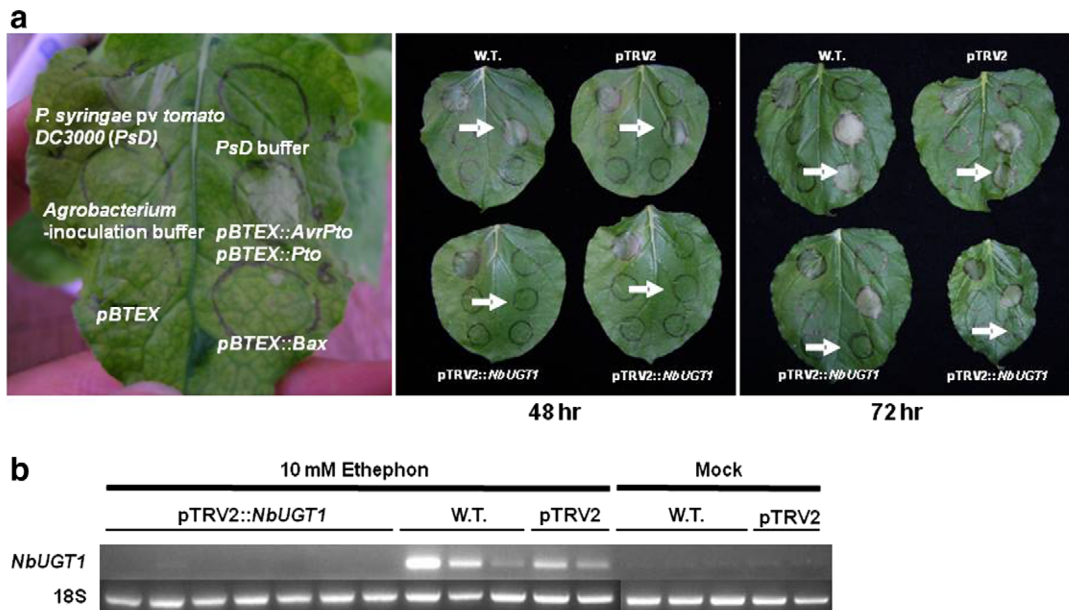

Fig. 4. Phenotypes of *NbUGT1*-silenced *N. benthamiana*. (a) Inoculation of *P. syringae* pv. *tomato* DC3000, co-expression of pBTEX::*AvrPto*, pBTEX::*Pto* clones, and *Agrobacterium* containing *Bax* in *N. benthamiana*. Leaves were agro-infiltrated within the marked circles, and photos were taken after 2 or 3 days. *NbUGT1*-silenced leaves were compared to the wild-type or vector control leaves (right panel). The arrows indicate comparable regions of HR by *AvrPto/Pto*- and *Bax*-mediated PCD. (b) For RT-PCR, tobacco plants were treated with 10 mM ethephon to induce *NbUGT1*. Wild-type and vector control plants were used as a positive control while the mock-treated plants were used as a negative control. *NbUGT1*-silenced *N. benthamiana*, pTRV2::*NbUGT1*; Vector control plant, pTRV2; Wild-type control plant, W.T.

of SA precursors, together with displaying a major SAG-forming activity. Similarly, rice SA GTase converted SA to SAG [26]. When plants are infected by invaders such as TMV or *Xcv*, several signal molecules accumulate and pathogen resistance genes are activated [24,27]. This allows the activation of downstream *PR* genes. The results of this study suggest that the *CaUGT1* gene was upstream of *CaPR-5* and regulated the expression level of *CaPR-5*. In previous reports, PR-5 has been shown to exert antifungal activity [28]. Members of this family were also induced during pathogenic infections and associated with HR [29]. Therefore, the suppression of *CaUGT1* may influence reduced expression of *CaPR-5*, which results in lower resistance against TMV infection. It would be interesting to test whether *CaPR-5* silencing phenocopies *CaUGT1*-silenced plants.

3.4. Compromised programmed cell death (PCD) pathway by suppression of NbUGT1 mRNA in tobacco

To further dissect the role of the *UGT1* gene in other general PCD pathways induced by Bax in addition to various *R* gene-mediated pathways, the *NbUGT1* gene, a *CaUGT1* homologue in *N. benthamiana,* was silenced. *P. syringae* pv. *tomato* DC3000, *Agrobacterium* vectors containing *Bax*, and co-expressing *AvrPto* and *Pto* clones were infiltrated into *N. benthamiana* leaves (Fig. 4). At 48 h post-infiltration, wild-type or vector control leaves showed *Pst* DC3000- and *AvPto*/*Pto*-mediated PCD, whereas the *NbUGT1*-suppressed leaves showed only *Pst* DC3000-mediated PCD. The *AvrPto*/*Pto*-mediated PCD was delayed and observed only after 72 h. The expression of *Bax*-mediated PCD was also delayed in *NbUGT1*-silenced plant (Fig. 4). These results suggested that the *UGT1* gene was involved in the *R* gene-mediated PCD pathway and an unknown PCD pathway mediated by Bax, which is a member of the Bcl-2 family of pro-apoptotic proteins and is thought to initiate PCD by localizing to the mitochondria and casing the release of pro-apoptotic factors, including cytochrome *c*. *Pst* DC3000 uses a type III secretion system (TTSS) to directly deliver bacterial effecter proteins into the host cell, which may lead plants to PCD in order to gain resistance against the infecting pathogen [30]. In a gene-for-gene model of plant immunity, disease resistance is initiated by recognition of a pathogen avirulence (Avr) effector by a plant resistance (R) protein. The signaling components necessary for *Pto* (tomato R protein)-mediated PCD are conserved in *N. benthamiana*, because *Agrobacterium*-mediated transient co-expression of AvrPto and Pto causes HR-related cell death [31]. *Ugt73b3* and *ugt73b5* mutants exhibited a loss of resistance to *Pst-AvrRpm1*. Both UGT73B3 and UGT73B5 are assumed to be necessary for redox homeostasis through glucosylation during the HR [20]. *CaUGT1* also might contribute to accumulation of secondary metabolites such as SA during HR. The pro-apoptotic mouse protein Bax has been shown to induce PCD in plants [32]. Bax caused physiological changes in the cell, such as the loss of mitochondrial membrane potential, cessation of cytoplasmic streaming, and consequential organelle destruction, including the loss of membrane permeability, which leads to increased secondary metabolite production [33,34]. Thus, here we propose that the knockdown of *CaUGT1*, which causes delayed PCD after *Bax* overexpression, may have led to instability of hormones and/or metabolites in the cell. It is possible that *CaUGT1* plays a crucial role in controlling the levels of free and active hormones in the cell, such as SA.

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