

Article

Over-Expression of Soybean *GmSAUL1* Enhances Disease Resistance in *Nicotiana tabacum*

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Abstract: E3 ubiquitin ligases play essential roles in plant defense responses. However, their roles in other plant species have not been investigated extensively. Here, we used a gain-of-function approach to interrogate the function of *GmSAUL1* (Senescence-Associated E3 Ubiquitin Ligase 1) homologs in soybeans. Ectopic over-expression of *GmSAUL1a* in *Nicotiana tabacum* resulted in autoimmune responses that could be suppressed by high temperature, which is a hallmark of NLR (nucleotide-binding leucine-rich repeat protein)-mediated resistance. Interestingly, an elevated activation of *NtMPK6*, but reduced activation of *NtMPK4*, responding to flg22 treatment, was observed in these *GmSAUL1a*-over-expressing tobacco lines, suggesting that over-expressing *GmSAUL1a* activates immune signaling through activating *NtMPK6*, but suppressing *NtMPK4* kinase activity. Collectively, these results demonstrated that *GmSAUL1* plays a positive and pivotal role in soybean immunity that is likely governed by NLR proteins.

Keywords: E3 ligase; *Glycine max*; cell death; immune responses; MAPK



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1. Introduction

Plants fight against pathogens via two layers of defense: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) [1]. Upon sensing and detection of PAMPs by plasma-membrane-localized receptor-like kinases (RLKs) or receptor-like protein (RLPs), PTI is activated [2,3]. Specific recognition of effectors secreted from pathogens into plant cells by host NLRs (nucleotide-binding leucine-rich repeat proteins) activates a stronger and long-lasting ETI [1]. Recently, it has been shown that PTI and ETI share common components and activate a set of overlapping responses [4,5]. In addition, PTI and ETI mutually regulate each other to achieve stronger immunity [6,7].

Ubiquitination is a conserved protein modification in regulating protein stability in eukaryotes [8]. Ubiquitination plays a wide range diverse functions in plants. Protein ubiquitination reactions are sequentially catalyzed by three family of enzymes: an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin ligase (E3) [8,9]. The substrate specificity is determined by the E3 ligases [10]. Over 1400 E3 ligase-encoding genes have been identified in the Arabidopsis genome [11], which can be classified into five families: HECT, SCF, RING-finger, U-box, and APC [12,13]. It has been shown that U-box E3 ligases play a critical role in plant immunity [12–14].

Senescence-associated E3 ubiquitin ligase 1 (SAUL1) in Arabidopsis was initially identified as a U-box E3 ubiquitin ligase, whose function was to suppress premature senescence or spontaneous cell death [15]. Loss-of-function of SAUL1 led to elevated disease resistance to different biotrophic pathogens [16–20]. This activated autoimmunity is SA-dependent [16–18], and SOC3 served as a guard for monitoring SAUL1 levels (suppressors of *chs1-2*, 3) [19,21]. Surprisingly, SOC3 was also required for the auto-immune responses

induced by overexpression of *SAUL1* [19]. Interestingly, Liang et al. [20] recently found that SOC3 pairs with two different truncated TIR-NB (TN) proteins, CHS1 or TN2, to monitor the loss and over-expression of *SAUL1*, respectively.

Mitogen-activated protein kinase (MAPK) signaling pathways are major components downstream of PTI and ETI [22–25]. It has been established that, whereas MPK3 and MPK6 function as positive regulators in immunity [25–28], MPK4 plays both positive and negative roles in Arabidopsis immunity [29–32]. We have previously proved that *GmMPK4* plays a negative role, and *GmMPK6* plays both positive and negative roles, respectively, in soybean immunity [33,34].

BPMV-mediated VIGS is the most widely used approach in the interrogation of gene functions in soybean [35,36]. Using this approach, we have successfully silenced multiple genes associated with defense responses [33,34,37–41]. Recently, we showed that silencing two homologous *SAUL1* genes simultaneously in soybean (*GmSAUL1a* and *GmSAUL1b*) activated defense responses [42]. To further understand the role of *GmSAUL1* in immunity, we generated transgenic plants ectopically expressing *GmSAUL1a* in *Nicotiana tabacum*. We found that ectopic over-expression of the *GmSAUL1a* in *N. tabacum* activated immune responses that could be partially suppressed by high temperature, indicating that the *GmSAUL1a* plays a positive role in immunity. Interestingly, over-expression of the *GmSAUL1a* in *N. tabacum* led to activation of *NtMPK6*, but it led to repression of *NtMPK4*. Taken together, our results indicated that *GmSAUL1a/1b* plays a positive role in regulating cell death and immunity.

2. Results

2.1. Transient Over-Expression of *GmSAUL1a* Induces HR Cell Death on the Leaves of *Nicotiana benthamiana* Plants

We previously showed that simultaneously silencing two homologous *GmSAUL1* genes, *GmSAUL1a* and *GmSAUL1b*, resulted in an autoimmune response in soybean [42]. Over-expression of *AtSAUL1* in Arabidopsis also activates autoimmunity [19]. To test whether over-expressing *GmSAUL1a* results in a similar phenotype, we transiently over-expressed the *GmSAUL1a* driven by a 35S promoter on the leaves of *N. benthamiana* via agro-infiltration. As anticipated, transient over-expression of *GmSAUL1a* induced cell death, similar to what is induced by over-expression of *GmMEKK1* (Figure 1A; [38]). As a negative control, infiltration of an agro-bacterial strain, expressing free GFP, did not induce HR-like cell death. This result demonstrated that the function of the *SAUL1* homologs from different plant species is conserved.

2.2. Stable Transgenic *N. tabacum* Plants Over-Expressing *GmSAUL1a* Displayed an Auto-Immune Phenotype

Soybeans are recalcitrant for transformation. To circumvent the obstacle and to confirm the result from transient over-expression of *GmSAUL1a* (Figure 1A), over 20 independent stable transgenic *N. tabacum* (Samsun, NN) plants that overexpress the *GmSAUL1a* were generated. Genomic PCR and RT-PCR analysis confirmed that the *GmSAUL1a* was successfully transformed and over-expressed in the majority of these transgenic lines (Figure S1). Consistent with the result from transient over-expression of *GmSAUL1a*, spontaneous cell death was displayed on the leaves of all the *GmSAUL1a*-overexpressing plants. Representative images of the whole plants and the leaves from line #9 and #12 are shown in Figure 1B–D. The cell death was observed on the older leaves first and, subsequently, progressed to the younger leaves (Figure 1B,C). HR-like cell death was accompanied by significantly induced expression of multiple *PR* genes (Figure 1E) and over-accumulation of H_2O_2 (Figure 1F), suggesting that cell death is reminiscent of what occurred in the *GmSAUL1a/b*-silenced leaves [42].

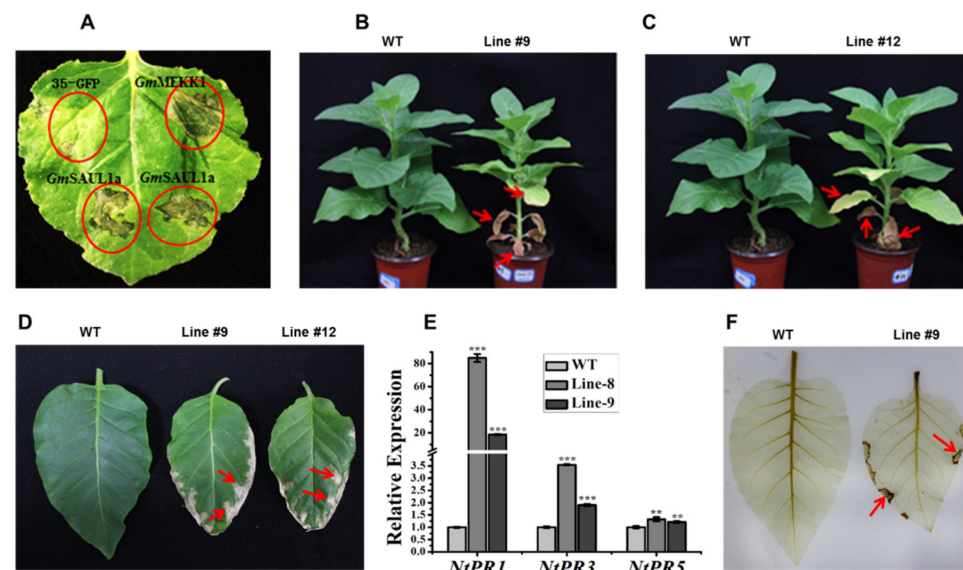


Figure 1. Either transient or stable over-expression of the *GmSAUL1a* in *N. tabacum* cv. Samsun (NN) induces cell death and activates immune responses. (A). Transient overexpression of *GmSAUL1a* via agro-infiltration resulted in HR-like cell death on the leaves of *N. benthamiana*. The 35S::GmMEKK1 was used as a positive control, and the 35S::GFP was used as a negative control. The leaf was photographed at three days post-infiltration; (B–D). Transgenic *N. tabacum* plants over-expressing *GmSAUL1a* induce HR-like cell death on the leaves (see red arrows). Line 8, Line 9, and Line 12 represent three independent transgenic lines; (E). The expressions of three *Pathogenesis-related* (PR) genes, *NtPR1*, *NtPR3*, and *NtPR5*, were highly induced in the *GmSAUL1a*-overexpressing tobacco plants. The *NtUBQ5* gene was used as a reference gene. ** and *** represent significant differences at 0.01 and 0.001 levels, respectively, by Student's *t*-test; (F). H_2O_2 was over-accumulated on the leaves of the *GmSAUL1a*-overexpressing tobacco plants. The oxidized DAB spots are indicated by the red arrows.

Autoimmunity, resulting from the activation of *R* genes, is usually suppressed by high temperature [43,44]. To further test if the autoimmunity of the *GmSAUL1a/1b*-silenced plants resulted from NLR activation, the *GmSAUL1a*-overexpressing *N. tabacum* plants were treated at 30 °C. As anticipated, HR-like cell death on the leaves of the *GmSAUL1a*-overexpressing *N. tabacum* plants could be suppressed by high temperature (Figure S2), indicating that cell death could be a result of a NLR activation.

2.3. *GmSAUL1a*-Overexpressing *N. tabacum* Plants Exhibit Enhanced Resistance against Different Biotrophic Pathogens

To examine whether cell death is associated with enhanced resistance, we firstly infected two independent *GmSAUL1a*-overexpressing tobacco plants with *Tobacco mosaic virus* (TMV). Because the tobacco cultivar we used for generating transgenic plants carries the *N* gene, TMV infection induces HR cell death in the leaves of these plants [45]. The size of the HR reflects the extent of TMV replication or movement [46]. As seen in Figure 2A, the number of Trypan blue-stained HR spots formed on the leaves of the *GmSAUL1a*-overexpressing plants upon TMV infection was significantly reduced compared to that on the WT plants (Figure 2A). In addition, the sizes of the HRs on the *GmSAUL1a*-overexpressing plants were significantly reduced compared with those formed on the WT leaves (Figure 2B). Furthermore, RT-qPCR analysis showed that the transcript level of the TMV capsid protein (CP)-encoding gene was significantly lower in the leaves of the *GmSAUL1a*-overexpressing plants than in the leaves of the WT plants (Figure 2C), demonstrating that overexpressing *GmSAUL1a* enhances resistance to TMV in tobacco.

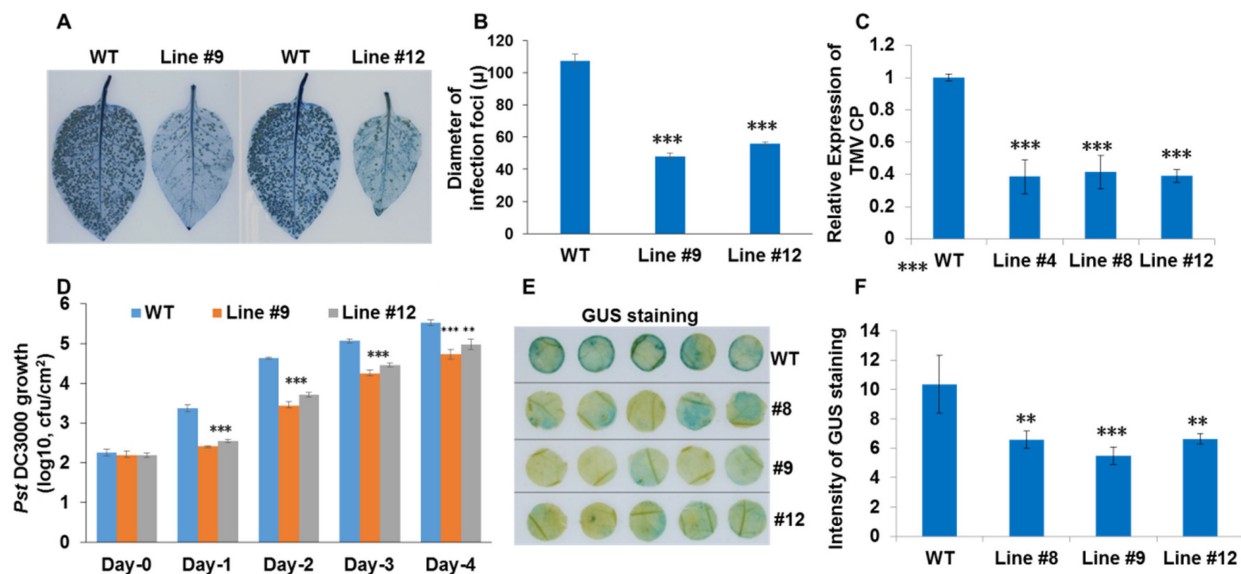


Figure 2. Transgenic tobacco plants over-expressing *GmSAUL1a* exhibit enhanced resistance to different types of pathogens. (A). Trypan blue-stained HR lesions on the leaves of the WT and the *GmSAUL1a*-overexpressing *N. tabacum* Samsun (NN) plants upon TMV infection. The photos were taken at 3 dpi; (B). Comparison of the diameters of the HR lesions on the TMV-infected WT and *GmSAUL1a*-overexpressing *N. tabacum* Samsun (NN) plants, shown in (A). The diameters of the HR lesions were measured using Image J; (C). RT-qPCR analysis showed that the transcript of the TMV capsid protein encoding gene (CP) was significantly reduced in three independent *GmSAUL1a*-overexpressing *N. tabacum* Samsun (NN) plants, relative to the WT plants. The *NtActin* gene was used as an internal reference gene; (D). Comparison of the colony forming units on the leaves of the control *N. tabacum* Samsun (NN) plants and the *GmSAUL1a*-overexpressing lines at different days post inoculation (dpi) of *Pst* DC3000 infection. The leaves of indicated 45-day-old tobacco lines were injected with *Pst* DC3000 to determine the colony forming units at different days post infiltration; (E). Overexpression of *GmSAUL1a* in *N. tabacum* leads to increased resistance to an agrobacterium strain (GV3101), carrying a construct of the pBI121-GUS; (F). The relative intensity of GUS staining was quantitated by Image J. ** and *** represent significant differences at 0.01 and 0.001 levels, respectively, by Student's *t*-test.

Next, WT and two independent *GmSAUL1a*-overexpressing plants were inoculated with a bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC 3000 (*Pst* DC3000). As anticipated, the multiplication of the *Pst* DC3000 on the leaves of the *GmSAUL1a*-overexpressing plants was significantly lower than that on the leaves of the WT plants (Figure 2D), indicating that overexpression of *GmSAUL1a* in tobacco enhances resistance to *Pst* DC3000.

Lastly, we examined the effect of over-expression of *GmSAUL1a* on the resistance to an *Agrobacterium* strain carrying a 35::GUS construct. The agro-bacterial strain was infiltrated in the leaves of the WT and the *GmSAUL1a*-overexpressing tobacco plants. At 2 dpi, the leaf discs from the infiltrated area were stained for GUS activity, which reflects the infectivity of the *Agrobacterium* strain. As shown in Figure 2E,F, the intensity of the GUS staining on the leaf discs collected from the *GmSAUL1a*-overexpressing plants was significantly reduced relative to that of the WT plants, indicating that overexpressing *GmSAUL1a* in tobacco enhances its resistance to *Agrobacterium*. Collectively, these results indicated that overexpressing *GmSAUL1a* in tobacco enhances resistance to multiple biotrophic pathogens with different lifestyles and infection strategies, and *GmSAUL1a* is a positive regulator of immunity.

2.4. Enhanced Disease Resistance of *GmSAUL1a*-Overexpressing Tobacco Plants Is Correlated with Elevated Callose Deposition

To understand the molecular mechanism of the enhanced resistance of the *GmSAUL1a*-overexpressing tobacco plants, we performed the callose deposition assay on the leaves of WT and the *GmSAUL1a*-overexpressing plants. It was found that callose deposition of the *GmSAUL1a*-overexpressing plants was significantly increased relative to the WT plants (Figure 3A,B), suggesting that the enhanced callose deposition is one of the mechanisms explaining the elevated resistance in the *GmSAUL1a*-overexpressing plants.

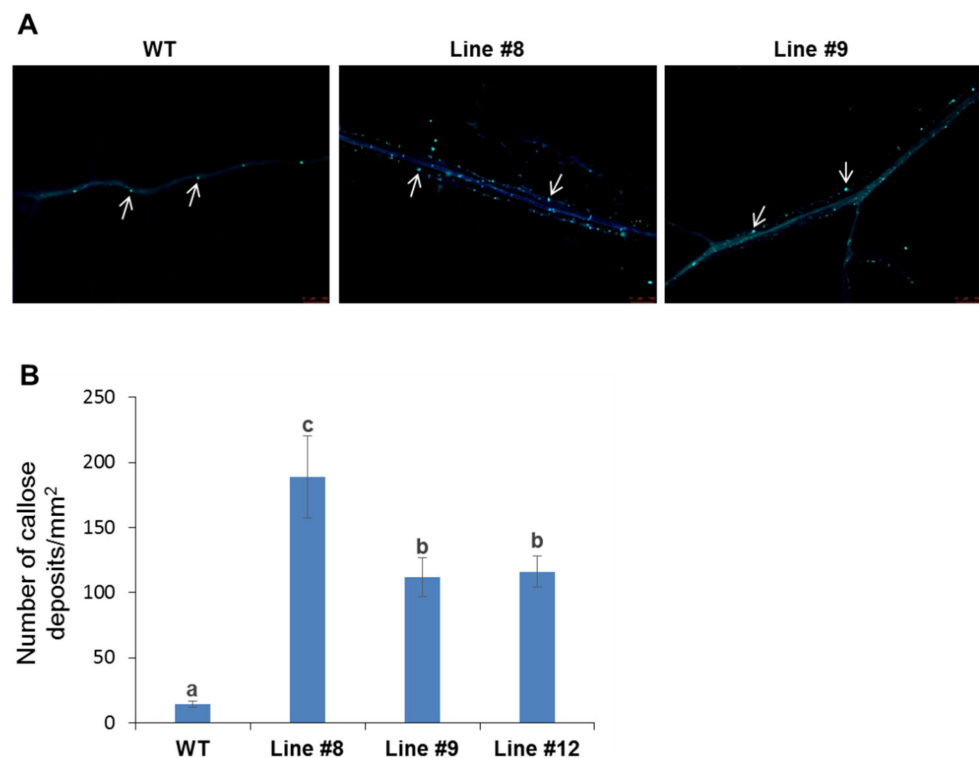


Figure 3. Over-expressing *GmSAUL1a* in *N. tabacum* cv. Samsun (NN) enhances the flg22-induced callose deposition. (A). The callose deposition of two-week-old WT and *GmSAUL1a*-overexpressing tobacco plants was visualized by staining with Aniline blue. The representative callose spots were pointed out by white arrows; (B). Comparison of the number of callose foci per mm² of leaf surface area visualized by Aniline blue staining between WT and different *GmSAUL1a*-overexpressing lines. The significant differences were determined by an ANOVA with a post hoc Duncan's test, as indicated by different letters ($p < 0.05$, $n > 10$).

2.5. Over-Expressing *GmSAUL1a* in Tobacco Has Opposite Effects on the Activation of *NtMPK4* and *NtMPK6* Responding to flg22 Elicitation

To test the effect of the *GmSAUL1a* overexpression on the activation of the MAPK signaling pathways, MAPK kinase assay was performed using a Phospho-p44/42 MAPK Erk1/2 antibody. As shown in Figure 4, the activation of *NtMPK6* in response to flg22 elicitation was dramatically elevated in the *GmSAUL1a*-overexpressing plants compared with the WT plants. Interestingly, contrary to *NtMPK6*, the activation of *NtMPK4* was drastically reduced in the *GmSAUL1a*-overexpressing plants relative to the WT plants. The activation of *NtMPK3* was barely detectable on the *GmSAUL1a*-overexpressing leaves, but it was only visible on the WT leaves at 60 min of flg22 elicitation. Together, these results indicated that over-expression of the *GmSAUL1a* exerts opposite effects on the activation of *NtMPK6* and *NtMPK3/4*.

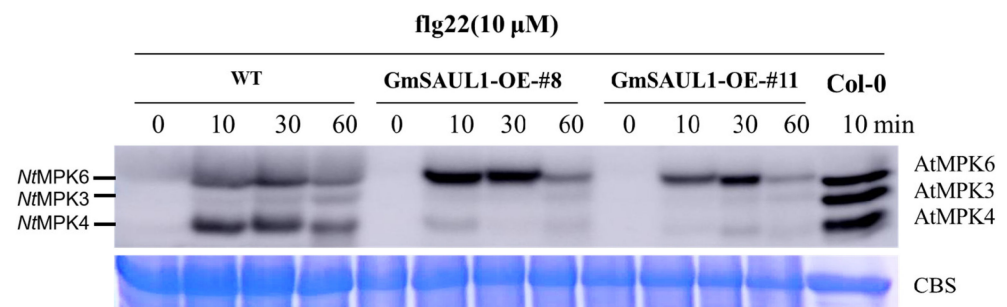


Figure 4. Over-expressing *GmSAUL1a* in *N. tabacum* cv. Samsun (NN) enhances the activation of NtMPK6, but it reduces the activation of NtMPK4. Protein samples were prepared, as described previously [38]. Western blotting was performed using a Phospho-p44/p42 MAP Erk1/2 antibody. An Arabidopsis sample treated with 10 μM flg22 for 10 min was used as a positive control. Coomassie blue-stained gel (CBS) was used as a loading control.

2.6. Subcellular Localization of *GmSAUL1a*

To visualize the subcellular localization of *GmSAUL1a*, the 35S::GFP-*GmSAUL1a* construct was transiently expressed in *N. benthamiana* leaves via agroinfiltration. Green fluorescence was observed, not only in the cytosol, but also in the nucleus (Figure 5). Interestingly, we frequently observed the fluorescent patches at the plasma membrane (PM) (Figure 5, see white arrows). These GFP patches may represent the sites for tethering the tonoplast and/or MVBs to the PM, as described in Arabidopsis [47–49]. In addition, mobile vesicles were also observed in the cytoplasm (Figure 5, see red arrows), suggesting that the function of SAUL1 homologues might be conserved in different plant species.

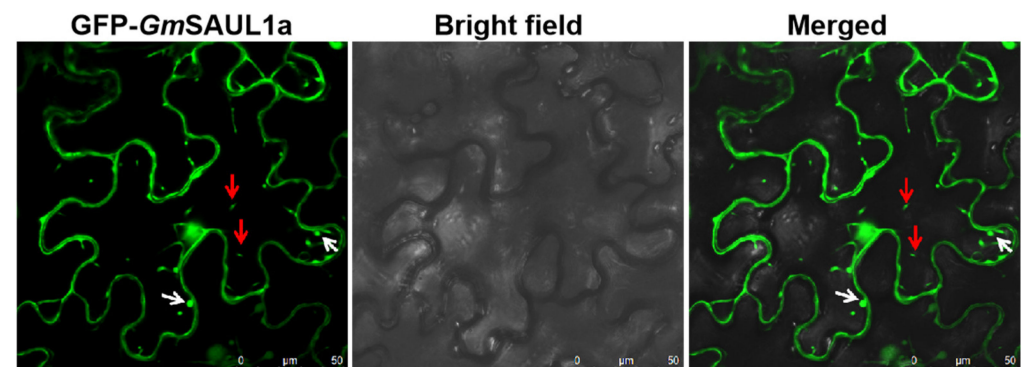


Figure 5. Subcellular localization of GFP-*GmSAUL1a*. GFP-*GmSAUL1a* localizes in the cytoplasm, nucleus, as well as at plasma membrane (PM) (see white arrows) and cytoplasmic vesicles (see red arrows) in the *N. benthamiana* epidermal cells. Bar = 50 μm.

3. Discussion

3.1. The Function of SAUL1 Homologs Is Conserved across Plant Species

In Arabidopsis, either loss function of SAUL1 or ectopic over-expression of SAUL1 results in auto-immune phenotypes [16,17,19,20]. We showed that either ectopic over-expression of *GmSAUL1a* in *N. tabacum* (Figures 1–3) or silencing *GmSAUL1a/1b* in soybean [42] led to similar autoimmunity. The autoimmunity of the Arabidopsis *saul1-1* mutant is dependent on PAD4 and EDS1 [16,17], suggesting that the autoimmunity of *saul1-1* mutant depends on the SA pathway. The elevated levels of both free and conjugated forms of SA in the *GmSAUL1a/1b*-silenced soybean plants suggest that the autoimmunity displayed in these plants is also dependent on SA [42]. Consistent with the auto-immune phenotypes, the tobacco *GmSAUL1a*-overexpressed plants exhibited an increased resistance to different biotrophic pathogens (Figure 2). These results indicated that the function of SAUL1 homologs is highly conserved in different plant species [47]. The previously

published results, together with our results, strongly indicated that the homeostasis of SAUL1 must be properly maintained within a certain threshold range to prevent activation of immunity. A level of SAUL1 exceeding the higher end or below the lower end of the threshold range will result in the activation of immune responses.

In Arabidopsis, the Toll interleukin 1 receptor (TIR)-type NLR (TNL), as well as SOC3 (Suppressors of *chs1-2, 3*), which associates with SAUL1, serve as guards to monitor the homeostasis of SAUL1. Either loss or overexpression of SAUL1 triggers SOC3-mediated autoimmunity [19]. Later, the same group showed that SOC3 pairs with two atypical truncated TIR-NB (TN) proteins to guard the homeostasis of SAUL1 [20]. Over-accumulation of SAUL1 is guarded by the SOC3-TN2 pair, and knocking out or knocking down of SAUL1 is guarded by the SOC3-CHS1 (Chilling Sensitive 1) pair [20]. Autoimmunity, resulting from NLR gene activation, is usually suppressed at high temperature [43,44]. The fact that the auto-immune phenotypes of the *GmSAUL1a*-overexpressing tobacco plants were significantly alleviated by high temperature treatment (Figure S2) suggest that *GmSAUL1a/1b* is similarly guarded by a NLR(s), and the auto-immune phenotype might be a result of a NLR activation. It remains to be examined whether the SOC3-TN2 and SOC3-CHS1 homologues function the same way as in Arabidopsis to guard SAUL1 in soybean or tobacco.

3.2. The Roles of *GmSAUL1s* in PTI and ETI

An enhanced resistance to virulent pathogens was observed in the *GmSAUL1a*-overexpressing tobacco lines, which suggests that PTI is activated in these plants (Figure 2). If the homeostasis of *GmSAUL1* is similarly guarded by SOC3-TN2 and SOC3-CHS1 homologous pairs in soybean [19,20], the activated immunity observed in the 35S::*GmSAUL1a* tobacco plants (Figure 1) actually results from activated NLRs. High temperature rescued the autoimmune phenotype of the *GmSAUL1a*-overexpressing plants (Figure S2), suggesting that certain NLR(s) likely act as guard(s) to monitor the level of *GmSAUL1s* in soybean or tobacco plants. Because PTI and ETI share overlapping components and are mutually required to maintain a prolonged and stronger immunity [6,7], it is not surprising that the enhanced disease resistance observed in the *GmSAUL1a*-overexpressing tobacco plants (Figure 2) could be a consequence of the activated ETI.

Interestingly, although both the *saul1-1* mutant and the 35S::*SAUL1* transgenic lines display autoimmunity [19,20], macroscopic lesions were only seen on the leaves of *saul1-1* plants, but not on the leaves of the 35S::*SAUL1* plants [20]. Different from the case in Arabidopsis, massive cell death was observed on the leaves of 35S::*GmSAUL1a* plants (Figure 1B–D). This disparity is probably caused by differences in the downstream signaling pathways between Arabidopsis and tobacco.

3.3. Silencing *GmSAUL1a/1b* in Soybean and Over-Expressing *GmSAUL1a* in *N. tabacum* Activate Immunity through Different Downstream MPKs

Silencing *GmSAUL1a/1b* caused a reduced activation of *GmMPK6* in soybean [42], whereas over-expression of *GmSAUL1a* results in an elevated activation of *NtMPK6* in *N. tabacum* (Figure 4), indicating that the positive effect of *GmSAUL1* on MPK6 activation is consistent between soybean and tobacco. Silencing *GmSAUL1a/1b* has contrasting effects on the flg22-induced activation of *GmMPK3* and *GmMPK6* (Figure 4). In Arabidopsis, the enhanced resistance is usually correlated with the enhanced MPK3/MPK6 activity [25]. In Arabidopsis, MPK3 and MPK6 can compensate for each other's function in *mpk3* and *mpk6* mutants, respectively [50].

Over-expressing *GmSAUL1a* in tobacco enhanced the flg22-induced activation of *NtMPK6*, but it reduced the activation of *NtMPK4* (Figure 4). The elevated resistance is usually correlated with the enhanced MPK3/MPK6 activity and reduced MPK4 activity, respectively [22–32,51,52]. Consistent with this, silencing *GmMPK4* results in cell death and activated immunity in soybeans [33]. Either silencing *GmMPK6* in soybean or overexpressing *GmMPK6* in Arabidopsis resulted in cell death and activation of immune responses, indicating that *GmMPK6* plays both positive and negative roles in immune responses [34].

Based on these studies, it is reasonable to postulate that the cell death and the activated immune responses observed in the *GmSAUL1a*-overexpressing tobacco plants are a result of combined effects of the reduced activation of *NtMPK4* and enhanced activation of *NtGMPK6*. Collectively, our results demonstrated that silencing *GmSAUL1a/1b* and over-expressing *GmSAUL1a* activate immune responses through different downstream MAPK modules (Figure 6, [42]). Instead of through a simple linear MAPK pathway, the SAUL1 regulates cell death and immunity through a MAPK network consisting of different downstream MAPKs. It remains to be determined whether the differential activation and repression of downstream MAPK modules resulted from silencing *GmSAUL1a/1b* in soybean and overexpression of *GmSAUL1a* in tobacco are associated with the activation of the SOC3-TN2 and SOC3-CHS1, respectively.

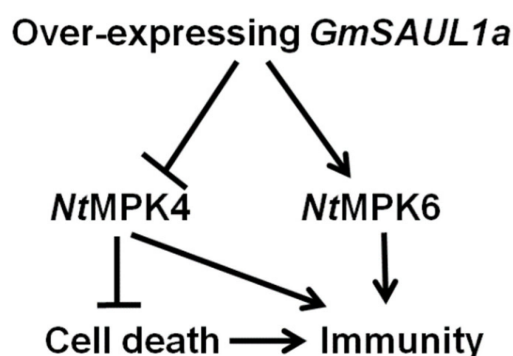


Figure 6. A proposed model for how *NtMPK6* and *NtMPK4* are involved in the *GmSAUL1a*-triggered immunity in tobacco. Overexpression of *GmSAUL1a* in *N. tabacum* triggers cell death and activated immune responses through combined effects of the reduced activation of *NtMPK4* and enhanced activation of *NtGMPK6*.

4. Materials and Methods

4.1. Plant Materials

Nicotiana tabacum cv. Sumsun (NN) and *Nicotiana benthamiana* were used in this study. Tobacco plants were grown in the growth room or growth chamber at 22 °C with a 16-h light/8-h dark photoperiod. High temperature treatment was performed at 30 °C.

4.2. MAPK Activity Assay

After flg22 treatment, protein samples were prepared from tobacco leaf discs in extraction buffer containing 50 mM Tris-MES, pH 8, 0.5 M Suc, 1 mM MgCl₂, 10 mM EDTA, and 5 mM DTT, as described [38,39]. Proteins were separated by 10% SDS-PAGE gel and transferred to PVDF membranes (Millipore, Burlington, MA, USA) by using semidry electrotransfer equipment (Bio-Rad, Hercules, CA, USA). Western blot analysis was performed using the anti-phospho-p44/p42 MAPK (anti-pTEpY) (Cell Signaling Technology, Danvers, MA, USA). The bands were visualized using chemiluminescent horseradish peroxidase (HRP) substrate (Millipore). Coomassie Blue stained gel (CBS) was used as loading control.

4.3. Histochemical Assays

Plant cell death on the infected leaves was visualized by staining with trypan blue in lactophenol and ethanol, as described [53].

4.4. SA Quantification

SA was measured, as described [54], using an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA).

4.5. Generation of Transgenic *N. tabacum* Samsun (NN) Plants Over-Expressing *GmSAUL1a*

The full-length cDNA of *GmSAUL1a* was amplified using following pair of primers with KpnI and XmaI sites (in bold) attached to the 5' end and 3' end, respectively:

GmSAUL1a-pE1776-F: aagGGTACCATGATGGCTGCGAGCTG

GmSAUL1a-pE1776-R: ttgCCCGGGTTCATCCCATGTTTGAAAGATT

The amplified fragment was double-digested with KpnI and XmaI and subsequently cloned into the pE1776 vector [55] predigested with the same pair of restriction enzymes. The pE1776-*GmSaul1a* was transformed into the *Agrobacterium* strain GV3101 and then transformed into *N. tabacum* Samsun (NN), as described [56–58]. Briefly, tobacco leaves were sequentially sterilized with 70% ethanol and 0.5–2% NaClO₃, respectively. Leaf discs from the sterilized leaves were pre-cultivated in MS medium for 3 days. After this, the leaf discs were co-incubated with *Agrobacterium* (GV3101) suspension solution for about a half hour, followed by cultivating in MS medium containing 1 mg/L Indole-3-acetic acid (IAA) and 1 mg/L 6-BA for 2 days. Afterward, these leaf discs were transferred to MS medium containing 1 mg/L IAA, 1 mg/L 6-BA, 50 mg/L Hygromycin, and 25 mg/L rifampicin. The shoots emerging from the callus were subsequently transferred to rooting medium containing IAA (1 mg/L), 50 mg/L Hygromycin, and 25 mg/L Timentin. After rooting, the seedlings were transferred to soil.

4.6. The *Pst* DC3000 Infection

The *Pst* DC3000 of OD₆₀₀ = 0.00001 was infiltrated into the *N. tabacum* leaves, as described previously [59]. A colony-forming unit (cfu) at different days of infiltration (dpi) was counted and calculated, as described [59].

4.7. RNA Isolation and RT-qPCR

RNA isolation and RT-qPCR were performed, as described [34]. The RT-qPCRs were performed with three biological replicates and three technical replicates using an ABI550 Real-Time PCR machine (Applied Biosystems, Thermo Fisher Scientific, Austin, TX, USA) and the 2× SYBR Green qPCR Mix (Aidlab, Beijing, China). The annealing temperature for the RT-qPCRs was 60 °C. The comparative CT method was used for analysis.

The primers used for RT-qPCR are:

NtPR1-F: GCTGAGGGAAGTGGCGATTTC

NtPR1-R: CCTAGCACATCCAACACGAACC

NtPR3-F: GACCCATCCAATTGACAAACCAA

NtPR3-R: CCTGTGGTGTTCATCCAGAACC

NtPR5-F: GGCGATTGTGGCTCAAACC

NtPR5-R: GAAATCTTGCTTCGTACCTGAGA

NtUBQ5-F: CCTAACGGGTAAAACAATCAC

NtUBQ5-R: AGCCATAAAAGTTCCAGCAC

4.8. Callose Deposition

Callose deposition in *N. tabacum* leaves was stained 24 h, as described by [59]. Callose deposits were visualized by ultraviolet epifluorescence using a fluorescence microscope (Olympus, Center Valley, PA, USA).

4.9. GUS Staining

GUS staining was performed, as described by [60]. A stereo microscope (Olympus SZH10, Center Valley, PA, USA) was used to take photographs of the GUS-stained leaves.

4.10. Sub-Cellular Localization of GFP-*GmSAUL1a*

The full-length cDNA of *GmSAUL1a* was amplified by RT-PCR by using the following pair of primers:

pGDG-F-SalI: aaaGTC GAC ATGATGGCTGCGAGCTGG

pGDG-R-BamHI: ttgGGA TCC TCATCCCATGTTTGAAAGATTTC

The PCR product was double-digested with SalI and BamHI and cloned into pGDG vector [61] predigested with the same set of restriction enzymes to generate the GFP–

GmSAUL1a fusion construct. This 35S:: GFP-*GmSAUL1a* construct was infiltrated onto *N. benthamiana* leaves, as described [62]. Images were captured with a confocal laser-scanning microscope (Carl Zeiss, LSM 880, Jena, Germany).

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy13061533/s1>, Figure S1: Screening of transgenic tobacco lines that ectopically expressing GmSAUL1. Figure S2: The cell death phenotype of the *GmSAUL1a*-overexpressing tobacco line is suppressed at 30 °C.

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Abbreviations

SAUL1: senescence-associated E3 ubiquitin ligase 1; PAMPs, pathogen-associated molecular patterns; MAPK, mitogen-activated protein kinase; HR, hypersensitive response; EDS1, enhanced disease susceptibility 1.

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