

NaMLP, a new identified Kunitz trypsin inhibitor regulated synergistically by JA and ethylene, confers *Spodoptera litura* resistance in *Nicotiana attenuata*

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Abstract

Upon insect herbivore attack, *Nicotiana attenuata* accumulates trypsin protease inhibitor (TPI) activities as a defense response from different protease inhibitor (PI) coding genes, including WRKY3-regulated NaKTI2, and JA-dependent NaPI. However, whether any other TPI gene exists in *N. attenuata* is still unclear. A miraculin-like protein gene (*NaMLP*) was highly up-regulated in *N. attenuata* after *Alternaria alternata* infection. However, silencing *NaMLP* had no effect on lesion diameter developed on *N. attenuata* leaves after *A. alternata* inoculation. Meanwhile, the transcripts of *NaMLP* could be induced by wounding and amplified by *Spodoptera litura* oral secretions (OS). *S. litura* larvae gained significantly more biomass on *NaMLP*-silenced plants but less on *NaMLP* over-expressed plants. Although NaMLP showed low sequence similarity to NaKTI2, it had conserved reaction sites of Kunitz trypsin inhibitors, and exhibited TPI activities when its coding gene was over-expressed transiently or stably in *N. attenuata*. This was consistent with the worst performance of *S. litura* larvae on *NaMLP* over-expressed lines. Furthermore, *NaMLP*-silenced plants had reduced TPI activities and better *S. litura* performance. Finally, OS-elicited *NaMLP* was dramatically reduced in JA-deficient irAOC and ethylene-reduced irACO plants, and the expression of *NaMLP* could be significantly induced by methyl jasmonate or ethephon alone, but dramatically amplified by co-treatment of both methyl jasmonate and ethephon. Thus, our results demonstrate that in addition to JA-regulated NaPI, and WRKY3/6-dependent NaKTI2, *N. attenuata* plants also up-regulates TPI activities via NaMLP, which confers *S. litura* resistance through JA and ethylene signaling in a synergistic way.

1. Introduction

Plant protease inhibitors (PIs) have been well established to play an important defensive role against insect herbivores and pathogens. They include Kunitz, Bowman-Birk, Potato I and II, Squash, Barley, Cystatins and Miscellaneous type. In wild tobacco *Nicotiana attenuata*, NaPI, which belongs to the potato PI-II family and exhibits trypsin protease inhibitor (TPI) activities, was identified as an effective defense against plant's native herbivores *Manduca sexta* (Zavala, et al. 2004).

Most Kunitz-type trypsin inhibitors (KTIs) have four conserved cysteine residues that form two disulfide bonds, and have been reported to localize in cell wall of elongating seedling organs and vascular tissue (Jimenez, et al. 2007). KTIs are generally strongly induced by injury or insects feeding (Eberl, et al. 2021). *Spodoptera litura* is a serious polyphagous pest, infesting various host plants of economic importance (Bhattacharyya, et al. 2007). Recently, a KTI (*NaKTI2*) is found to be specifically elicited in *N. attenuata* by oral secretions (OS) of *S. litura* and confers *S. litura* resistance (Yin, et al. 2021), indicating *N. attenuata* plants accumulate different PIs, including NaPI and NaKTI2 to defense against insect herbivores.

Plant hormones play key regulatory roles in plant defense responses, especially JA and ET signaling (Chen, et al. 2021). JA is an essential signal positively regulating defense responses of *N. attenuata* to insect herbivores. TPI activities were dramatically reduced in JA-insensitive irCOI1 plants (Paschold, et al. 2007). Insect herbivore-induced *NaPI* was completely dependent on JA signaling, since it was abolished

in JA-deficient irAOC plants (Yin, et al. 2021). However, *NaKTI2* was only slightly regulated by JA signaling, but mainly dependent on transcription factor NaWRKY3 and NaWRKY6 (Yin, et al. 2021).

Alternaria alternata is a notorious necrotrophic fungus causing black spot disease in *Nicotiana* species. When infected by *A. alternata*, *N. attenuata* plants will accumulate certain amounts of phytoalexin scopoletin and scopolin to defend against this pathogen (Li and Wu 2016; Sun, et al. 2014). Feruloyl-CoA 6-hydroxylase 1 (*F6 H1*) is the gene encoding the key enzyme for scopoletin biosynthesis. Both JA and ethylene signaling are required for *A. alternata*-elicited *F6 H1* expression (Sun, et al. 2017; Sun, et al. 2014).

Miraculin is a taste-modifying protein, it has been known for a long time due to its surprising property of transforming sourness into sweetness (Mohammad, et al. 2011). Interestingly, many miraculin-like proteins (MLPs), which have certain sequence similarity to natural miraculin, have been reported to be involved in pest and pathogen resistance. *RlemMLP2*, but not *RlemMLP1* could inhibit the growth of *Alternaria* spores (Tsukuda, et al. 2006). In curry tree, *MKMLP* can resist *Helicoverpa armigera* and *S. litura* by inhibiting the activity of intestinal protease (Gahloth, et al. 2011).

In this study, a miraculin-like protein (*NaMLP*) which is strongly induced in *N. attenuata* after *A. alternata* infection draws our attention. By generation of *NaMLP*-silenced and over-expressed lines, we found that NaMLP was required for the resistance against *S. litura* instead of *A. alternata*. Further sequence alignment and functional tests revealed that NaMLP is a new KTI. Finally we demonstrate that NaMLP is a KTI regulated synergistically by JA and ethylene in *N. attenuata*.

2. Materials And Methods

2.1 Plant and fungal material

An inbred line of *N. attenuata* was used as the wild-type (WT). Stably transformed lines of JA-deficient plant irAOC, JA-insensitive plant irCOI1, ethylene-reduced plant irACO, and ethylene-insensitive plant *Ov-etr1* were provided by Professor Ian T. Baldwin (Max-Planck Institute of Chemical Ecology); Plants silenced with *NaMLP* (irNaMLP line 11 and 12), over-expressed with *NaMLP* (OvNaMLP line 2 and 3) were generated in this study. Seed germination and plant growth were conducted as described (Krügel, et al. 2002; Zhao, et al. 2021). *A. alternata* were grown and used for inoculation as described (Sun, et al. 2014; Sun, et al. 2014).

2.2 *S. litura* performance and leaf treatments

S. litura eggs were purchased from Keyun biological company and hatched in a growth chamber at 26 °C for 16-h light, and 24 °C for 8-h dark as described (Wu, et al. 2013). To investigate how *S. litura* larvae performed on plants, 5 newly hatched neonates were randomly selected and placed directly on node + 3 leaves (0, + 1, + 2 leaves were used for real-time PCR or TPI activity experiments) of plants; 24 plants for each genotype were used for caterpillar performance. The weight of *S. litura* larvae was recorded. The

caterpillar performance experiments were repeated three times with similar tendency, and one of them was presented in this study.

To analyze gene expression and TPI activity, wounding plus water (W + W) or wounding plus oral secretions (W + OS) of *S. litura* larvae treatments were used to mimic herbivory. For simulated herbivory treatments, the source–sink transition leaves (0 leaves) were wounded with a fabric pattern wheel and 20 µl OS of *S. litura* larvae (diluted 3 times) or water were rapidly rubbed into the puncture wounds of each wounded leaf (Halitschke and Baldwin 2003).

2.3 Sequence analysis

Miraculin (*Synsepalum dulcificum*, BAH84844), NaKTI2 (*N. attenuata*, XP_019230715.1) and NtKTI (*N. tabacum*, ACL12055) were used for sequence alignment with NaMLP (*N. attenuata*, XP_019229306.1). Multiple protein alignment was performed by MEGA- and GeneDoc applications. Secondary and tertiary structure predictions were made by JPred and SWISSMODEL, which were used to manually edit and mark in the alignment according to (Yin, et al. 2021).

2.4 Transient over-expression and trypsin protease inhibitor (TPI) activity assay

The open reading frame of *NaMLP* was amplified by primers (CJ09 and CJ10) and insert directly upstream of eGFP under the control of CaMV 35S promoter in pCAMBIA1301. *Agrobacterium tumefaciens* (strain GV3101) cells carrying this construct or empty vector (EV) were inoculated into the leaves of 6-week-old Ov-NahG *N. attenuata* plants for transient expression. Inoculated leaves were collected after 48 h for protein extraction. Total proteins were extracted from inoculated leaves of Ov-NahG plants with W + OS treatments as described (Jongsma 1994). Bradford method (Bradford 1976) was used to determine the protein content of the samples. TPI activities were analyzed with the radial diffusion assay described (Jongsma 1993).

2.5 Immunoblot analysis

Total proteins from leaves of transiently over-expressed *NaMLP* or EV were extracted as described previously (Jongsma 1994). Three protein extract replicates were separated in 10% SDS-PAGE and transferred to immobilon PVDF membrane (Millipore Corporation) by using the Semi-Dry Transfer Cell system (Bio-Rad). Immuno-reacting proteins were detected by anti-GFP/Actin (1:5000, Abmart Corporation) as the primary antibody and peroxidase-conjugated goat anti-rabbit/goat anti-mouse IgG (1:20,000; Proteintech Corporation) as the secondary antibody. The results were imaged by MicroChem

4.2 Bio Imaging Systems.

2.6 Real-time PCR assay

Total RNA and cDNA and was extracted and synthesized as described (Wu, et al. 2013) Real-time PCR was performed as described by (Sun, et al. 2014) on a CFX Connect qPCR System (Bio-Rad) with iTaq Universal SYBR Green Supermix (Bio-Rad) and gene-specific primers (Supplementary Table). The

unknown samples transcripts were quantified by the method as described (Song, et al. 2019) and the *NaActin* gene, which were unaltered and used as an internal standard (Xu, et al. 2018).

2.7 Generation of *NaMLP*-silenced plants via virus induced gene silencing

Specific 366 bp of the *NaMLP* cDNA fragments were amplified by primers (CJ03 and CJ04) and cloned into pTV00, *Agrobacterium tumefaciens* (strain GV3101) cells carrying above constructs were mixed with those having pBINTRA, and inoculated into three leaves of 4-week-old *N. attenuata* plant, thus generating *NaMLP*-silenced (VIGS *NaMLP*) plants. Phytoene desaturase (*PDS*) was silenced to monitoring the progress of VIGS (Saedler and Baldwin 2004). When the leaves of *NaPDS*-silenced plants began to bleach, the 0 leaves of VIGS *NaMLP* plants and empty vector plants (EV plants) were selected and inoculated with *A. alternata* for further experiments. Around 30 plants were inoculated with each construct, and typically 24 biological replicates per construct exhibiting efficient silencing were used for each experiment. All the VIGS experiments were performed three times.

2.8 Generation of stable *NaMLP*-silenced and over-expressed plants

We silenced *NaMLP* in *N. attenuata* by *Agrobacterium*-mediated transformation (Krügel, et al. 2002; Zhao, et al. 2021) using a pRESC8 vector containing a specific 395bp *NaMLP* fragment (with primers CJ13_F and CJ14_R) in an inverted-repeat orientation. Two independently transformed F2 lines, each harboring a single insertion were selected for all further experiments (irNaMLP lines 11 and 12).

We also generated *N. attenuata* plants with *NaMLP* over-expressed stably via *Agrobacterium*-mediated transformation (OvNaMLP line 2 and 3). The full-length cDNA of *NaMLP* with two hemagglutinin (HA: YPYDVPDYA) epitopes at its C-terminus was cloned into pCAMBIA1301 vector after the 35S promoter with the in-fusion technique (Clontech). *N. attenuata* plants (WT) were transformed by *Agrobacterium tumefaciens* with this construct according to (Krügel, et al. 2002; Zhao, et al. 2021). T1 seeds were screened for single T-DNA inserts (1:3 segregation of hygromycin resistance). Two independently transformed F2 lines (OvMLP line 2 and 3), each harboring a single insertion, were selected and used in this study.

2.9 Methyl jasmonate (MeJA) and ethephon treatments

Methyl jasmonate (MeJA) treatments were conducted as described by (Xu, et al. 2018). When applied, the chemical is quickly de-methylated into jasmonic acid by plant MeJA-esterase (Wu, et al. 2008). A solution of 1 mM MeJA or 5 mM ethephon was prepared by distilled water, and sprayed with a fine mist on the 0 leaves of rosette-staged *N. attenuata* plants (32-day-old) and immediately covered with a plastic bag on the treated plant, sampled after the above process at 1 and 3 h, the plants sprayed with distilled water were used as control treatments.

3. RESULTS

3.1 NaMLP can be strongly induced by *A. alternata* while it is not essential for *A. alternata* resistance in *N. attenuata*

A miraculin-like protein gene (*NaMLP*, XM_019373761), which shared 34.18% amino acid identity to miraculin in *Synsepalum dulcificum*, was found highly up-regulated in *N. attenuata* after *A. alternata* inoculation. Transcriptome analysis (Song, et al. 2019) indicated that this gene was strongly up-regulated in *N. attenuata* leaves at 1 day post inoculation (dpi). To confirm this result, we also quantified *NaMLP* expression by real-time PCR. Compared with mock control, the expression of *NaMLP* was increased to 10.9-fold at 1 dpi, and nearly 388-fold at 3 dpi (Fig. 1a). Interestingly, *A. alternata*-elicited *NaMLP* was dramatically reduced in JA-deficient *irAOC* and ethylene-insensitive *Ov-etr1* plants, suggesting that *A. alternata*-induced *NaMLP* is regulated by both JA and ethylene (Fig. 1b).

We studied the subcellular location of NaMLP by transient over-expression of *NaMLP* fused with *GFP* in *N. benthamiana* leaves. Our results showed that most of the signals were from cell membranes (Fig. 1c). To rule out the possibility of cell wall localization, we also transformed *NaMLP::GFP* to the protoplasts of *N. attenuata*. Indeed, strong signals were detected in cell membranes again (Fig. 1c), indicating NaMLP is a cell membrane protein.

To investigate the role of *NaMLP* during *A. alternata* infection, we generated plants stably silenced with *NaMLP* (*irNaMLP* line 11 and 12). Real-time PCR indicated that *A. alternata*-induced *NaMLP* expression was reduced by 94% in *irNaMLP* line 11, and 89% in *irNaMLP* line 12 at 1 dpi (Fig. 2a). When the source-sink transition leaves (0 leaves) of *irNaMLP* and wild-type (WT) were collected and inoculated with *A. alternata* for 7 d. No significant difference of lesion diameter were observed in two independent experiments (Fig. 2b), suggesting that *NaMLP* does not play an essential role in *N. attenuata* against *A. alternata*.

3.2 NaMLP can be specifically induced by oral secretion (OS) of *S. litura* and is required for the resistance of *S. litura* in *N. attenuata*

Previously, JA signaling has been demonstrated to be an essential signal for both *A. alternata* and herbivore resistance (Paschold, et al. 2008; Sun, et al. 2014). As *A. alternata*-induced *NaMLP* was dependent on JA signaling, but we found that it was not required for this pathogen resistance, we thus speculated that it might be involved in herbivore resistance.

To test whether the saliva of *S. litura* can specifically induce the expression of *NaMLP*, we detected its expression in the 0 leaves of rosette-staged plants, by wounding with a fabric pattern wheel and immediately applying with diluted OS of *S. litura* (Wounding + OS; W + OS) to simulate herbivory, or water (Wounding + Water; W + W) as a control. The expression of *NaMLP* was gradually but significantly increased after time with W + W treatments at all the time points from 0.5 h to 6 h (Fig. 3a). However,

NaMLP transcripts were strongly amplified by W + OS treatments at all these time points (Fig. 3a). These results suggested that *NaMLP* could be specifically induced by the OS of *S. litura*.

Next, we tested whether *NaMLP* is required for herbivore resistance by growing *S. litura* larvae on WT and *NaMLP*-silenced plants generated via VIGS. Our results showed that larvae gained significantly more mass on VIGS *NaMLP* plants indicating that *NaMLP* is required for the resistance to *S. litura* (Fig. 3b).

We also tested caterpillar performance in plants stably silenced with *NaMLP* and plants over-expressed with *NaMLP* (Ov*NaMLP* line 2 and 3). Compared with WT, Os-elicited *NaMLP* expression were significantly increased to 4.5-fold in Ov-*NaMLP* line 2, and 5-fold in Ov*NaMLP* line 3 after W + OS 6 h treatments (Fig. 3c). Our results showed that *NaMLP* was required for *N. attenuata* resistance to *S. litura*, as the insect larvae performed better in *NaMLP*-silenced plants (ir*NaMLP* line 11 and 12) while performed the worst in both lines over-expressed with *NaMLP* (Fig. 3d; Supplementary Figure). These results strongly suggest that *NaMLP* can be specifically induced by OS and confers *N. attenuata* resistance to *S. litura* larva.

3.3 *NaMLP* is a new KTI exhibiting TPI activity

To understand the reason why *NaMLP* conferred the resistance to *S. litura*, we performed protein sequence blast in Gene Bank. Homologs were found in Solanaceae plants, with the highest similarity proteins (with 93–100% identity) in *Nicotiana* species. Interestingly, several conserved reaction sites of KTI were identified in *NaMLP*. Thus, NaKTI2 and NtKTI, two KTIs identified previously, were selected for sequence alignment with *NaMLP*. The protein sequences of NaKTI2 exhibited 53.55% similarities to NtKTI, while *NaMLP* only exhibited 33.16% similarities to NtKTI (Fig. 4). Yet, *NaMLP* had typical structural features of KTIs, including trypsin inhibitor domain (Kunitz legume family PF00197 in Pfam database), two disulphide bonds, a single reactive site and a number of β -trefoil folds (Fig. 4).

Although *NaMLP* showed lower sequence similarity to NaKTI2, the fact that *NaMLP* had conserved reaction sites of KTIs lead us hypothesized that *NaMLP* is a KTI with TPI activity. We transiently over-expressed *NaMLP-eGFP* under the control of CaMV 35S promoter in Ov-NahG leaves. Western blot analysis by using GFP antibody revealed that the *NaMLP-eGFP* protein was highly expressed in three Ov-NahG leaves independently transformed with 35S::*NaMLP-eGFP*, but was not present in leaves transformed with EV (Fig. 5a). Our results showed that the TPI activities of crude protein increased nearly 6.8-fold in Ov-NahG leaves over-expressed with *NaMLP-eGFP* when compared with those leaves transformed EV (Fig. 5a).

We next investigated the TPI activities in leaves of WT and *NaMLP* VIGS plants at 3 dpi of *A. alternata*. TPI activity were significantly reduced in 0 leaves of VIGS plants when *NaMLP* was successfully silenced (Fig. 5b).

We also investigated the OS-elicited TPI activities in leaves of ir*NaMLP* and Ov*NaMLP* plants. Our results showed that after treated with W + OS 3 d, significantly lower levels of TPI activity were detected in 0

leaves of both irNaMLP lines of 11 and 12, but higher levels were in OvNaMLP line 2 and 3 (Fig. 5c). Thus, our data showed that NaMLP has not only typical KTI structural features, but also confers *N. attenuata* resistance to *S. litura* larva through TPI activities.

3.4 Synergetic induction of *NaMLP* by JA and ethylene.

As *A. alternate*-induced *NaMLP* expression was significantly reduced in irAOC and Ov-etr1 plants (Fig. 1b), we speculate that Os-elicited *NaMLP* may also be regulated by JA and ethylene. Indeed, the induction of *NaMLP* expression by OS of *S. litura* was dramatically reduced in ethylene-reduced plants (irACO) and JA-deficient irAOC plants (Fig. 6a).

Since the induction of the *NaMLP* is dependent on the JA and ET signaling, we next investigated whether exogenously applied MeJA and ethephon could also induce the expression of the *NaMLP*. *NaMLP* transcripts were highly induced by MeJA or ethephon. The expression levels of *NaMLP* could reach to 886-fold that of the control after 3 h of MeJA treatment, and could reach nearly 26-fold after ethephon treatments (Fig. 7a). Interestingly, co-treatment with MeJA and ethephon for 3 h led to a much higher induction of *NaMLP* to 11235-fold (Fig. 7a).

Unlike *NaMLP*, Os-elicited *NaPI* was solely dependent on JA as it was only dramatically reduced in JA-deficient irAOC plants but not in ethylene-reduced irACO or WRKY3-silenced plants (Fig. 6b). When treated with MeJA or ethephon, *NaPI* could only respond to MeJA at both 1 and 3 h (Fig. 7b). In terms of *NaKTI2*, OS-elicited *NaKTI2* was largely reduced in WRKY3-silenced plants, but only slightly reduced JA-deficient irAOC plants (Fig. 6c). When treated with MeJA or ethephon, neither treatment could induce *NaKTI2* expression (Fig. 7c).

To further confirm the synergistic induction of the *NaMLP* by MeJA and ethephon is dependent on endogenous JA and ET signaling, 0 leaves of WT, irAOC, and irCOI1 plants were co-sprayed with MeJA and ethephon. *NaMLP* was significantly induced in WT and irAOC, but not in irCOI1 plants under the co-application of MeJA and ethephon (Fig. 8a), suggesting that endogenous JA perception is required for the synergistic induction of *NaMLP*. Similarly, in the presence of MeJA and ethephon, the *NaMLP* was significantly induced in WT and irACO, but not in Ov-etr1 plants (Fig. 8b), suggesting that endogenous ethylene signaling transduction is also required for the synergistic induction of *NaMLP*.

Thus, our data strongly indicates that *N. attenuata* plants accumulated different PIs through different signaling after herbivory, and *NaMLP* is synergistically induced by JA and ethylene signaling in *N. attenuata* plants when attacked by insect herbivores.

4. Discussion

NaMLP was not required for *A. alternata* resistance but required for herbivore resistance in *N. attenuata*

Miraculin is known for its property of transforming sourness into sweetness (Mohammad, et al. 2011). Several miraculin-like proteins (MLPs) are reported to be involved in pathogen resistance. *RlemMLP2* could inhibit the growth of *Alternaria* spores in rough lemon (Tsukuda, et al. 2006). In *N. attenuata*, *NaMLP* was highly elicited after *A. alternata* infection (Fig. 1). However, our data did not support that *NaMLP* played an essential role in *A. alternata* resistance, as no significant difference of lesion diameter were observed in WT and both *NaMLP*-silenced lines (Fig. 2).

Instead, our results strongly indicated that *NaMLP* conferred herbivore resistance. *NaMLP* transcripts responded strongly and specifically to the OS of *S. litura* (Fig. 3). Importantly, larvae grown better on plants silenced with *NaMLP* with both virus induced-gene silencing and stable transformation with RNAi construct (Fig. 3). In addition, we also generated stable transformation lines with *NaMLP* over-expressed, and *S. litura* larvae performed the worst in these plants comparing to WT and *NaMLP*-silenced plants (Fig. 3). Similar cases that MLP was involved in herbivore resistance were also found in curry tree (Gahloth, et al. 2011) and eggplant (Lopez-Galiano, et al. 2017).

NaMLP is a new KTI, exhibiting TPI activities

MKMLP in curry tree has the TPI activities to inhibit the activity of intestinal protease of *Helicoverpa armigera* and *S. litura* (Gahloth, et al. 2011). In eggplant, MLP can inhibit the activity of protease in the intestinal juice of potato beetle larvae (Lopez-Galiano, et al. 2017). However, it is not clear whether *NaMLP* has TPI activities.

Sequence blast and alignment analysis showed that although *NaMLP* has low sequence similarity to NtKTI, it has typical KTI domains, including two disulphide bonds, a single reactive site, a number of β -trefoil folds, and the trypsin inhibitor domain (Oliva et al. 2010; Bendre et al. 2018). Further analysis did show that *NaMLP* has TPI activities. When *NaMLP* was over-expressed, no matter transiently in Ov-NahG or stably in *N. attenuata*, plant leaves had higher TPI activities (Fig. 5). However, when *NaMLP* was silenced via VIGS or stable transformation lines with RNAi constructs, plant leaves had lower TPI activities (Fig. 5).

Thus, our study revealed that *NaMLP* is a new KTI with typical KTI structural features, and required for *N. attenuata* resistance to *S. litura* larva through its TPI activity.

NaMLP, a new TPI regulated synergistically by JA and ethylene in *Nicotiana attenuata*

Upon insect herbivore attack, host plants will usually accumulate large amount of TPIs to suppress the digestive machinery of insects. Thus such inhibitors were considered as an important part of plant defense arsenal against herbivores.

Currently, two different inhibitors, including NaPI and NaKTI2 were found in wild tobacco *N. attenuata* after herbivory. NaPI, which belongs to potato PI-II family with 77 known PI-II repeat domains, was demonstrated to be an effective defense against hornworm *Manduca sexta* (Zavala, et al., 2004). OS-elicited *NaPI* was strongly reduced in JA-deficient *irAOC* plants but not in WRKY3-silenced plants or ethylene-reduced *irACO* plants (Fig. 6), indicating that JA signaling played a key role in OS-elicited *NaPI*. This result was further supported by the strong elicitation of *NaPI* by exogenous MeJA treatments (Fig. 7). However, supplied ethephon had not effect on *NaPI* expression (Fig. 7). *NaKTI2* was a Kunitz trypsin inhibitor gene recently identified as a defense gene against *S. litura* (Yin, et al. 2021). Unlike *NaPI*, OS-elicited *NaKTI2* was mainly depended on transcription factors NaWRKY3 and NaWRKY6 (Yin, et al. 2021). *NaKTI2* transcripts were not altered after MeJA or ethephon treatments (Fig. 7).

In this study, we revealed a new TPI after herbivory, NaMLP. The expression of this gene is controlled by both JA and ethylene pathways in a synergistic way. OS-elicited *NaMLP* was strongly reduced in both JA-deficient *irAOC* plants and ethylene-reduced *irACO* plants but not in WRKY3-silenced plants (Fig. 6). Furthermore, *NaMLP* transcripts could be significantly elicited by exogenous MeJA or ethephon treatments alone, but it levels were dramatically amplified when treated with MeJA and ethephon simultaneously, suggesting a synergistic induction of *NaMLP* by JA and ethylene pathways.

Thus, we concluded that once the leaves of *N. attenuata* were treated with OS, three signaling were activated, including JA, ethylene and WRKY3, which in turn regulated *NaPI* expression through JA signaling, *NaKTI2* via WRKY3, and *NaMLP* by JA and ethylene in a synergistic way; finally all three TPIs, including NaPI, NaKTI2 and *NaMLP*, contributed to the OS-elicited TPI activities and to herbivore resistance (Fig. 9). The findings in this study elucidate a new source of TPI activities, and provide new insights to the complexity of the regulation of TPI-based defense.

Collectively, our results demonstrated that *NaMLP* is a new KTI coding gene regulated by JA and ethylene in a synergistic way, and confers *S. litura* resistance in *N. attenuata*.

Declarations

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Jinsong Wu conceived and designed the project; Jinsong Wu and Mao Yang prepared the manuscript; Junbin Cheng cloned *NaMLP* and performed VIGS experiments, tested TPI activities of NaMLP in VIGS

plants and Ov-NahG plants, and found the synergistic induction of NaMLP by JA and ethylene; Min Yin generated RNAi plants and found the different regulation of TPIs; Mao Yang screened stable over-expression plants and performed caterpillar performance. All authors contributed to manuscript revision, read and approved the submitted version.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

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Figures

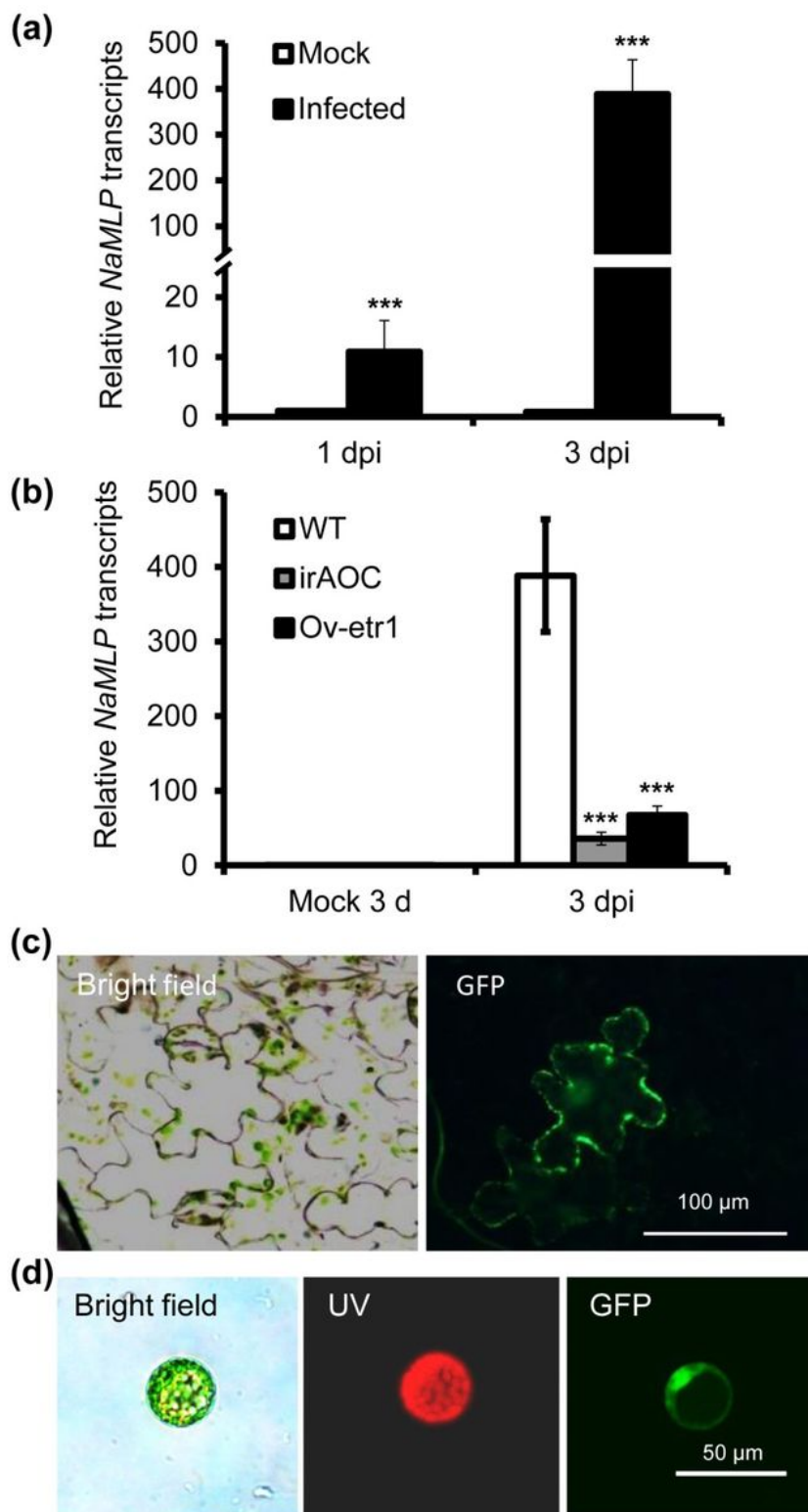


Figure 1

Elicitation of *NaMLP* transcripts by *A. alternata* and the subcellular location of *NaMLP*.

(a): *NaMLP* transcripts were measured by real-time PCR in the source-sink transition leaves (0 leaves) of WT plants treated with mock or with *A. alternata* at 1, and 3 days post inoculation (dpi). All transcriptional levels were normalized with a housekeeping gene *NaActin*. Values are means $\pm$ SE for five biological

replicates. Asterisks indicate the level of significant difference between WT plants with the different treatments (Student's *t*-test: ***, $p < 0.005$).

(b): *NaMLP* transcripts were measured by real-time PCR in 0 leaves of WT, *irAOC* (JA-deficient) and *Ov-etr1* (ethylene-insensitive) plants treated with mock or with *A. alternate* at 3 days post inoculation (dpi). Asterisks indicate the level of significant difference between WT plants with the different treatments (Student's *t*-test: ***, $p < 0.005$).

(c): 35S::*NaMLP-eGFP* was transiently expressed in *N. benthamiana* leaves. Bar = 100 μm . GFP signals were detected in cell membranes. Images were taken in bright field (left) and GFP channels (right).

(d): Transient expression of *NaMLP-eGFP* in protoplast of *N. attenuata*. Bar = 50 μm . GFP signals were found around cell membranes. Images were taken in bright field (left), UV (middle) and GFP channels (right).

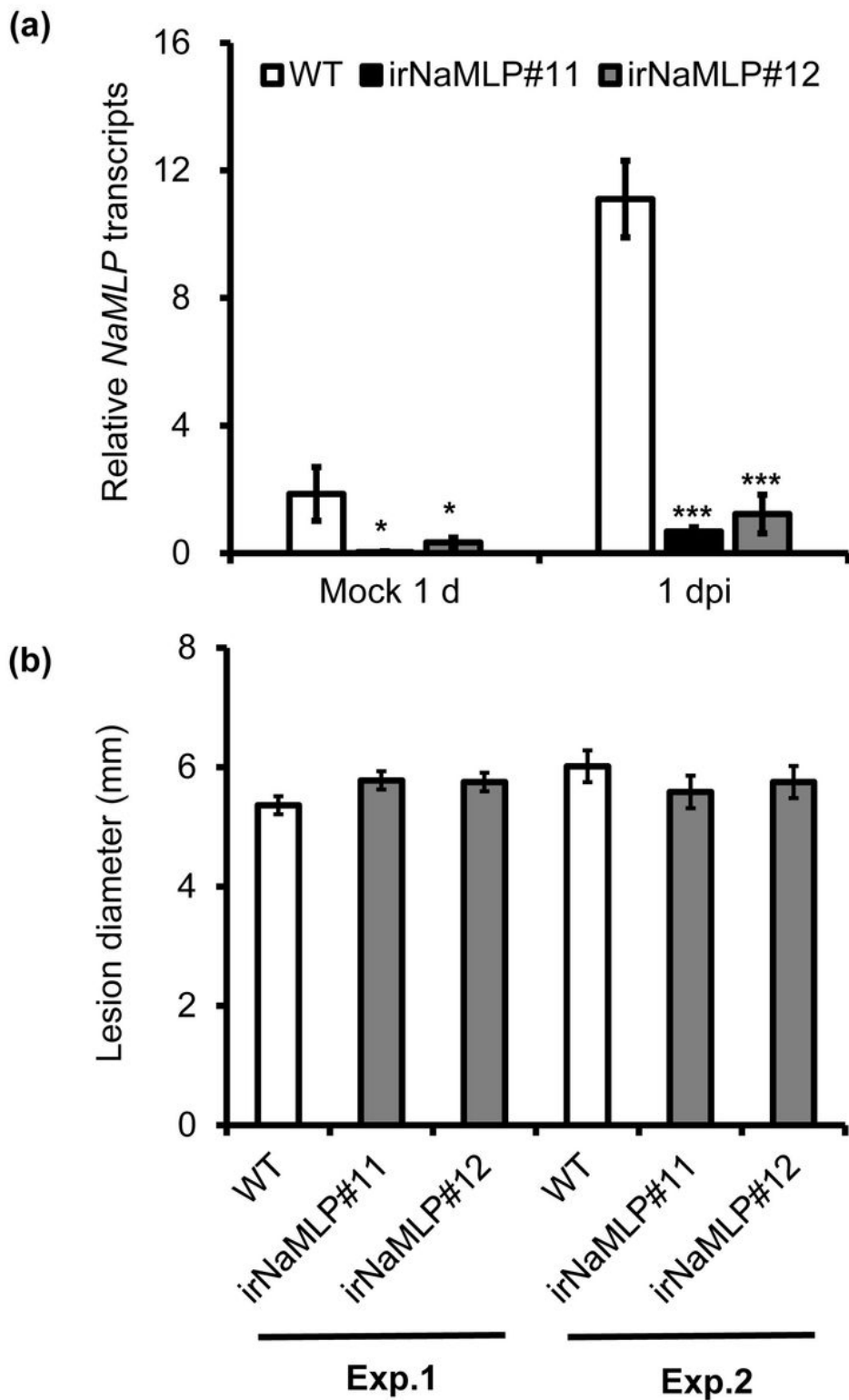


Figure 2

NaMLP is not essential for *A. alternata* resistance in *N. attenuata*

(a): Mean ($\pm$ SE) *NaMLP* transcripts were measured by real-time PCR in 0 leaves of four biological replicates in WT, irNaMLP line 11 and 12 after *A. alternata* inoculation at 1 dpi. Asterisks indicate the level

of significant difference between WT and irNaMLP plants with the same treatments (Student's *t*-test: *, $p < 0.05$; ***, $p < 0.005$).

(b): Silencing *NaMLP* had no effect on lesion diameter developed on *N. attenuata* leaves after *A. alternata* inoculation at 7 dpi.

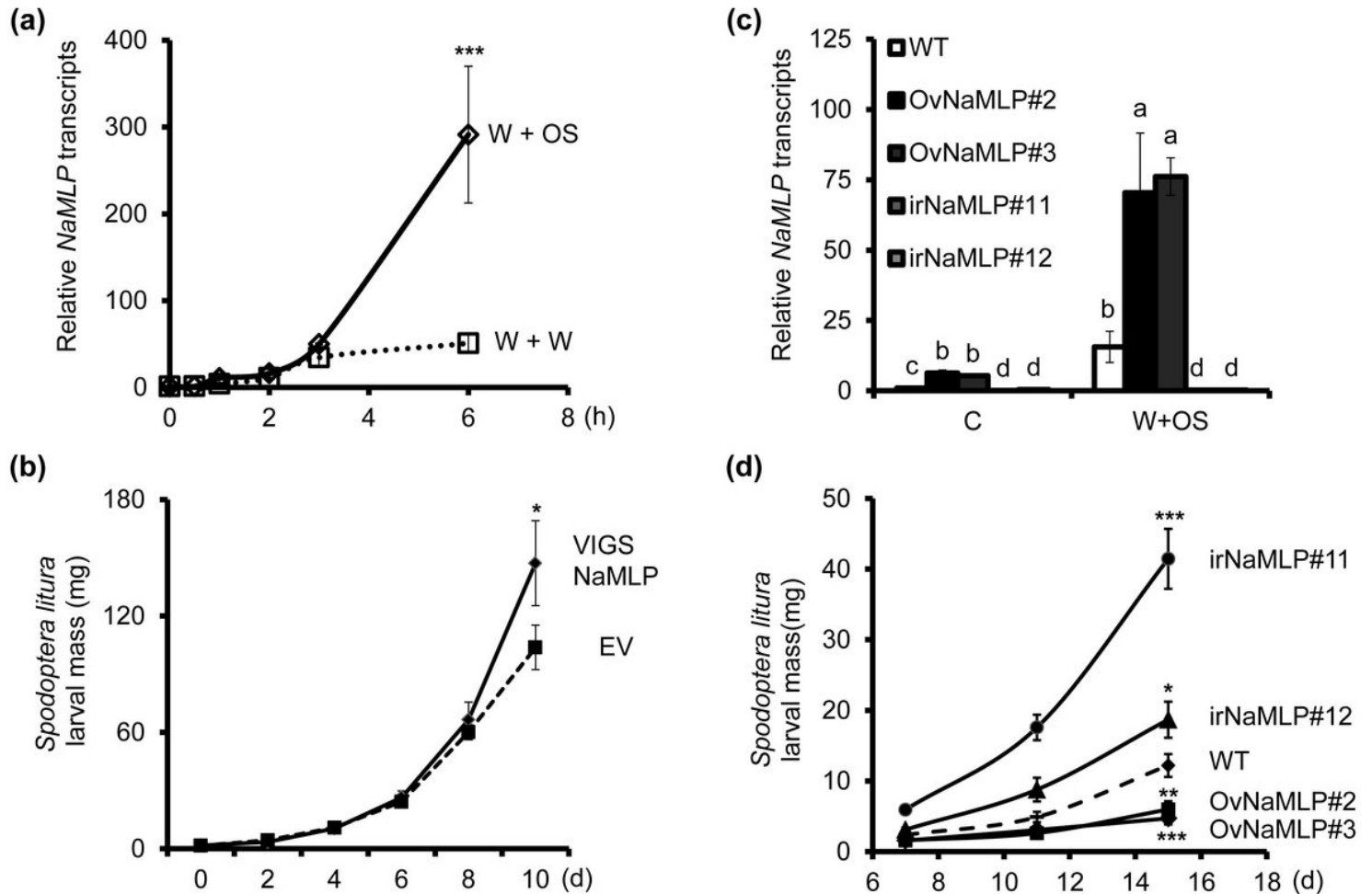


Figure 3

Elicitation of *NaMLP* by *Spodoptera litura* larval oral secretion in WT plants and *NaMLP* enhances plant resistance to *Spodoptera litura*.

(a): Mean ($\pm$ SE) *NaMLP* transcripts were measured by real-time PCR in five biological replicates of 0 leaves in WT treated with Wounding + OS and Wounding + Water at 0, 0.5, 1, 2, 3, and 6 h. Asterisks indicate the level of significant difference between W+W and W+OS (Student's *t*-test: ***, $p < 0.005$).

(b): Mean ($\pm$ SE) weight of *S. litura* larval of fifteen replicates grew on EV and VIGS *NaMLP* plants at 0, 2, 4, 6, 8 and 10 d. Asterisks indicate the level of significant difference between EV and VIGS *NaMLP* plants (Student's *t*-test: *, $p < 0.05$).

(c): Expression of *NaMLP* in 0 leaves of WT, irNaMLP and OvNaMLP plants after induction with *S. litura* oral secretions for 6 h. Lowercase letters in panel indicate significant differences by one-way ANOVA followed by least significant difference post hoc test ($P < 0.05$).

(d): Mean ($\pm$ SE) weight of *S. litura* larval of 24 replicates grew on WT, irNaMLP and OvNaMLP plants at 7, 11, and 15 d. Asterisks indicate the level of significant difference between WT and transgenic plants at the same time point (Student's *t*-test: *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.005$).

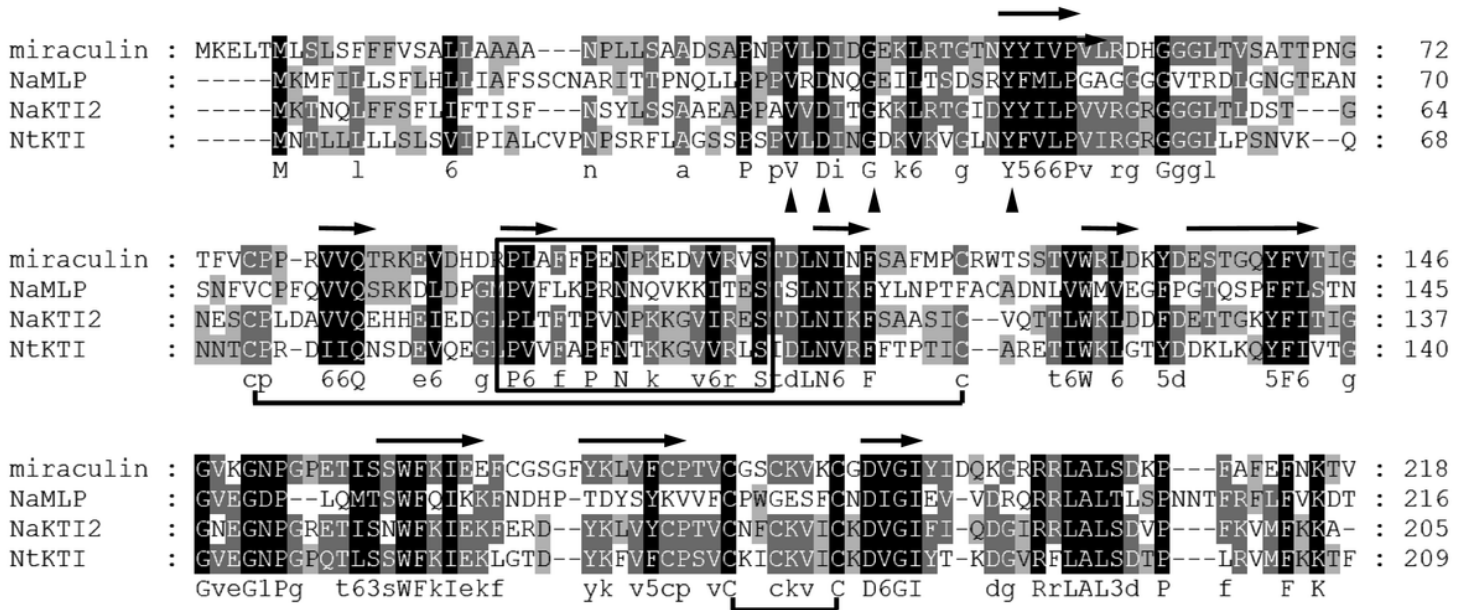


Figure 4

Sequence alignment of NaMLP with known KTIs.

Sequences were retrieved from the National Center for Biotechnology Information. NaMLP (*N. attenuate*, XP_019229306) were used for sequence alignment with Miraculin (*Synsepalum dulcificum*, BAH84844), NaKTI2 (*N. attenuate*, XP_019230715.1), and NtKTI (*N. tabacum*, ACL12055). Conserved and similar amino acid residues were shown with black and gray shading. The brackets indicated the disulfide bonds, triangles denote the Kunitz motif, a boxed region (full line) indicates the reactive site, and the arrows represented β -sheets.

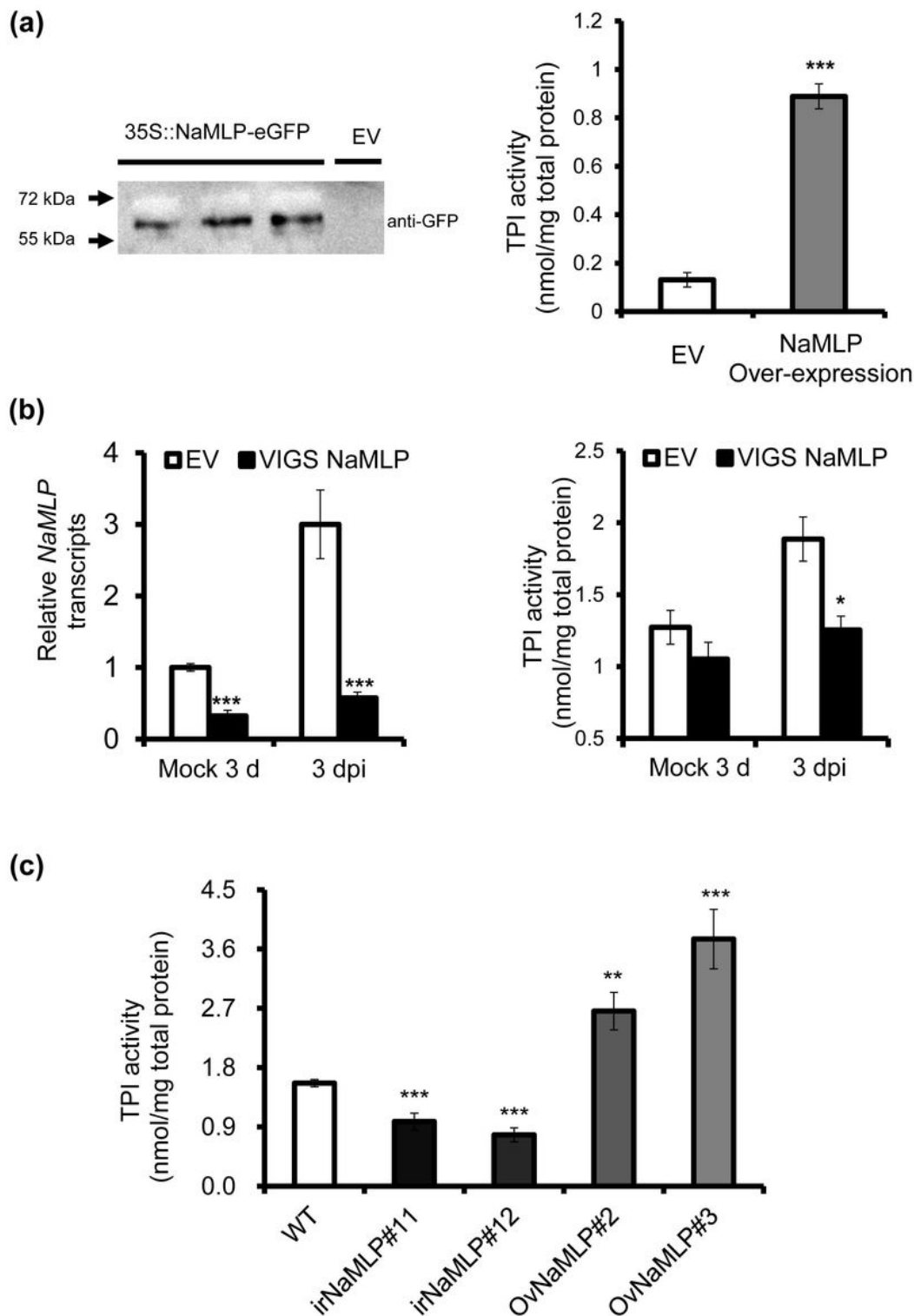


Figure 5

NaMLP exhibits Trypsin protein inhibitor activity

(a): Western blot analysis of protein extracts in leaves of Ov-NahG transiently over-expressed with NaMLP-eGFP or empty vector (EV). Three NaMLP-eGFP over-expressed samples were separated in lanes 1, 2 and 3, and EV samples in lanes 4. GFP antibody was used to detect NaMLP-eGFP (expected around

59 kDa, in the left panel). TPI activities as detected by radial diffusion experiment of total proteins (three biological replicates) in over-expression of NaMLP-eGFP leaves. Asterisks indicate level of significant difference between EV and 35S::NaMLP-eGFP protein samples (Student's *t*-test: ***, $P < 0.001$).

(b): *A. alternata* induced *NaMLP* expression and TPI activities were detected in five biological replicates of 0 leaves of EV and VIGS NaMLP plants at 3 dpi. Asterisks indicate level of significant difference between EV and VIGS NaMLP (Student's *t*-test: *, $P < 0.05$; ***, $P < 0.001$).

(c): Mean ($\pm$ SE) trypsin protein inhibitor in 8 biological replicates of + 1 leaves of WT, stable *NaMLP*-silenced plants and stable *NaMLP* over-expressed plants. Asterisks indicate level of significant difference between EV and VIGS NaMLP (Student's *t*-test: **, $P < 0.01$; ***, $P < 0.001$).

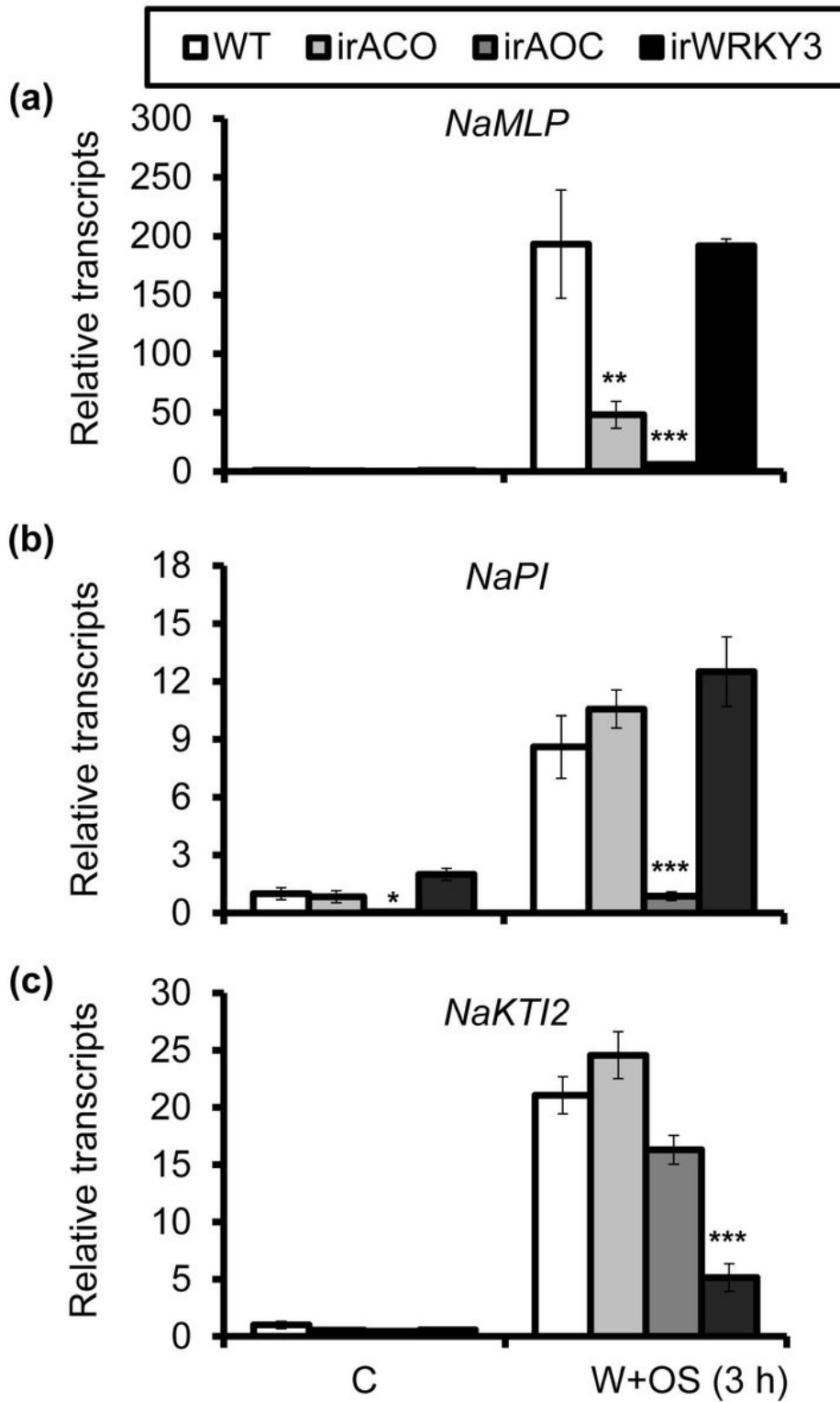


Figure 6

NaMLP, *NaPI* and *NaKT12* transcripts were measured by real-time PCR in the 0 leaves of WT, *irACO*, *irAOC* and *irWRKY3* plants treated with mock or OS 3h. Asterisks indicate level of significant difference between EV and transgenic plants (Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

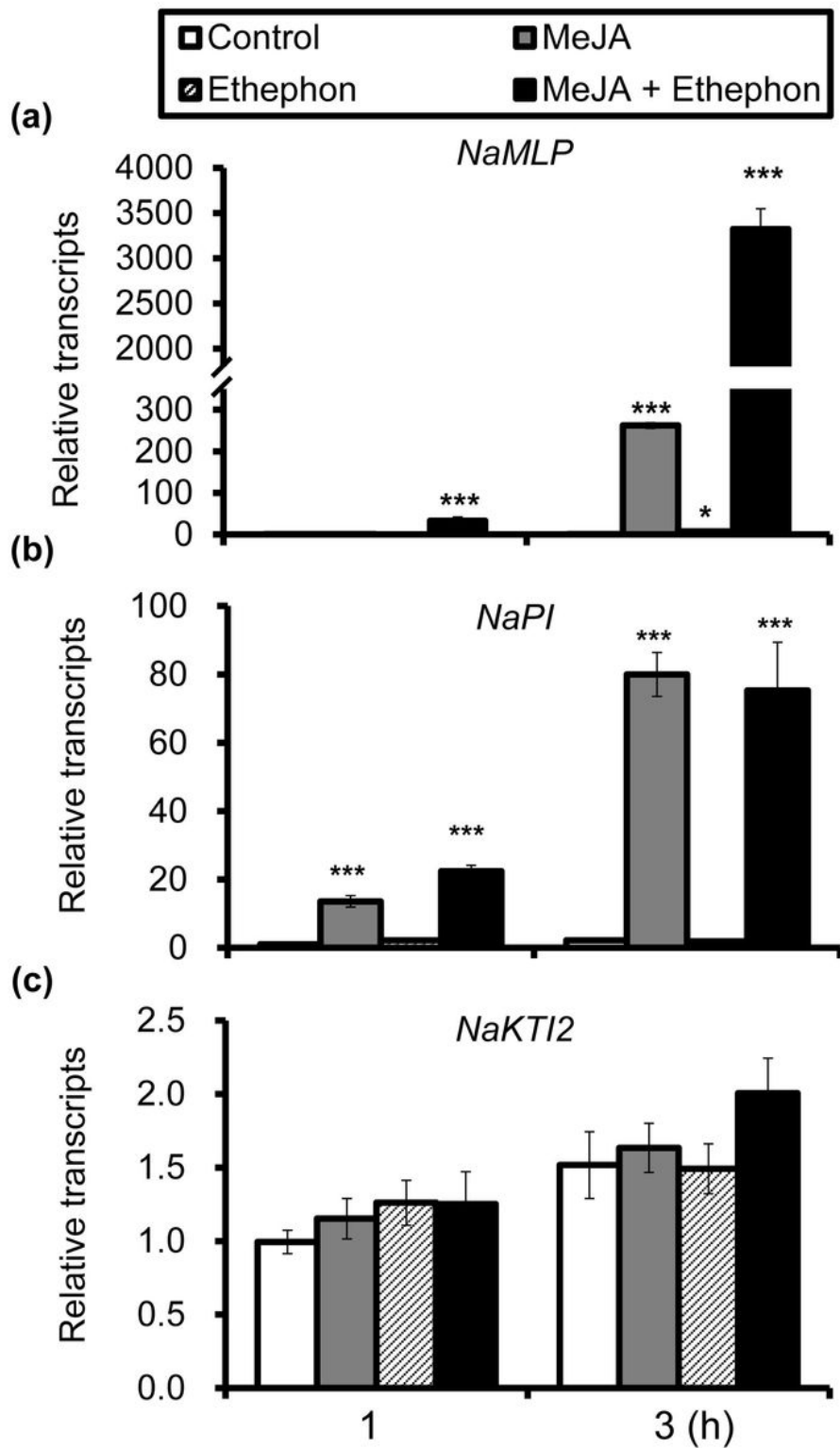


Figure 7

NaMLP, *NaPI* and *NaKTI2* transcripts were measured by real-time PCR in five replicates of 0 leaves treated with water (as control), MeJA and Ethephon individually and simultaneously at 1, and 3 h. Asterisks indicate the level of significant difference between control and treated leaves (Student's *t*-test: *, $p < 0.05$; ***, $p < 0.005$).

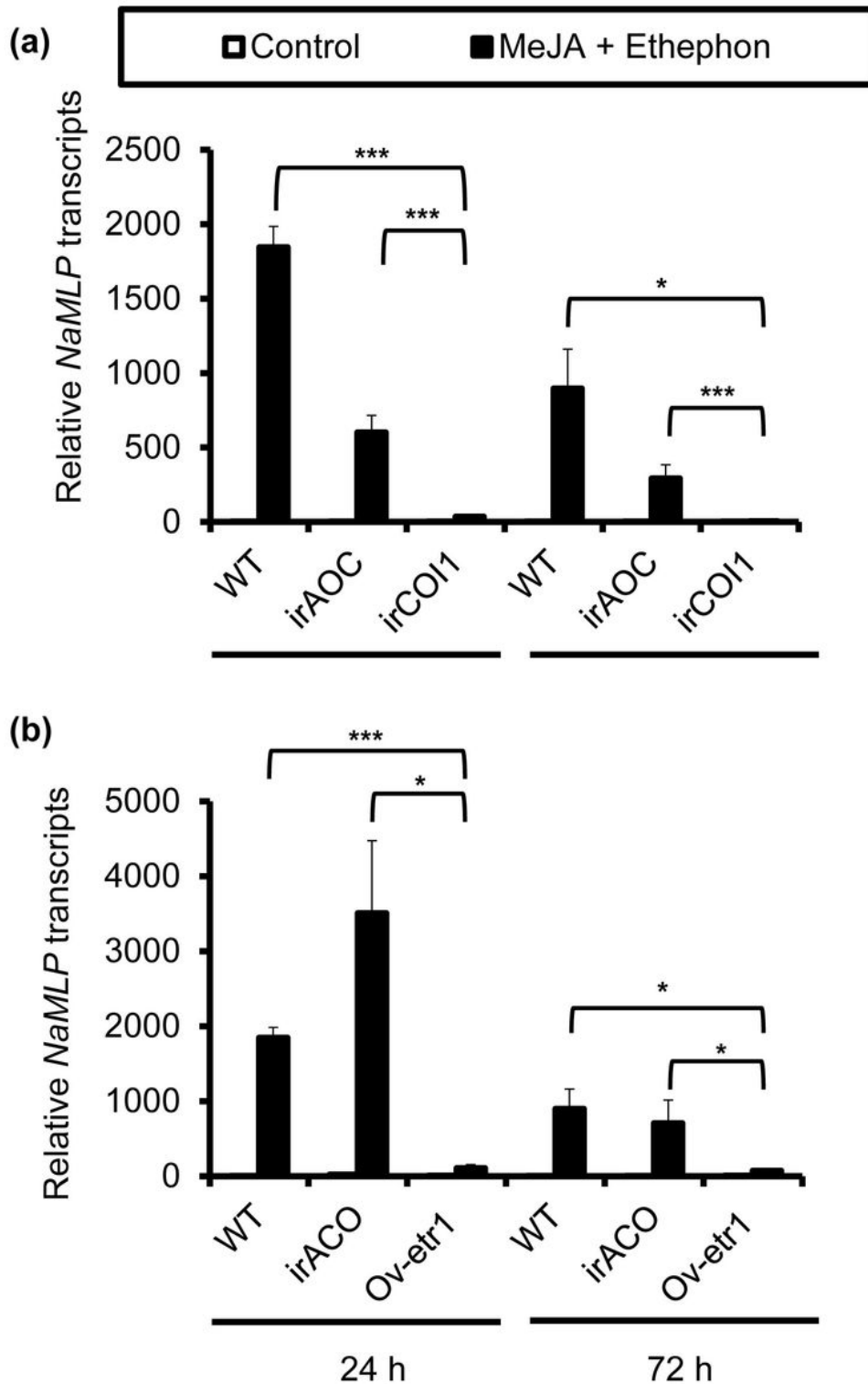


Figure 8

Mean ($\pm$ SE) *NaMLP* transcripts were measured by real-time PCR in five biological replicates of 0 leaves in WT, *irAOC* (JA-deficient), *irCOI1* (JA-insensitive), *irACO* (ethylene reduced) and *Ov-etr1* (ethylene-insensitive) plants co-treated with MeJA and ethephon at 24 and 72 h. Asterisks indicate the level of significant difference between each genotypes co-treated with MeJA and ethephon (Student's t-test: *, $p < 0.05$; ***, $p < 0.005$).

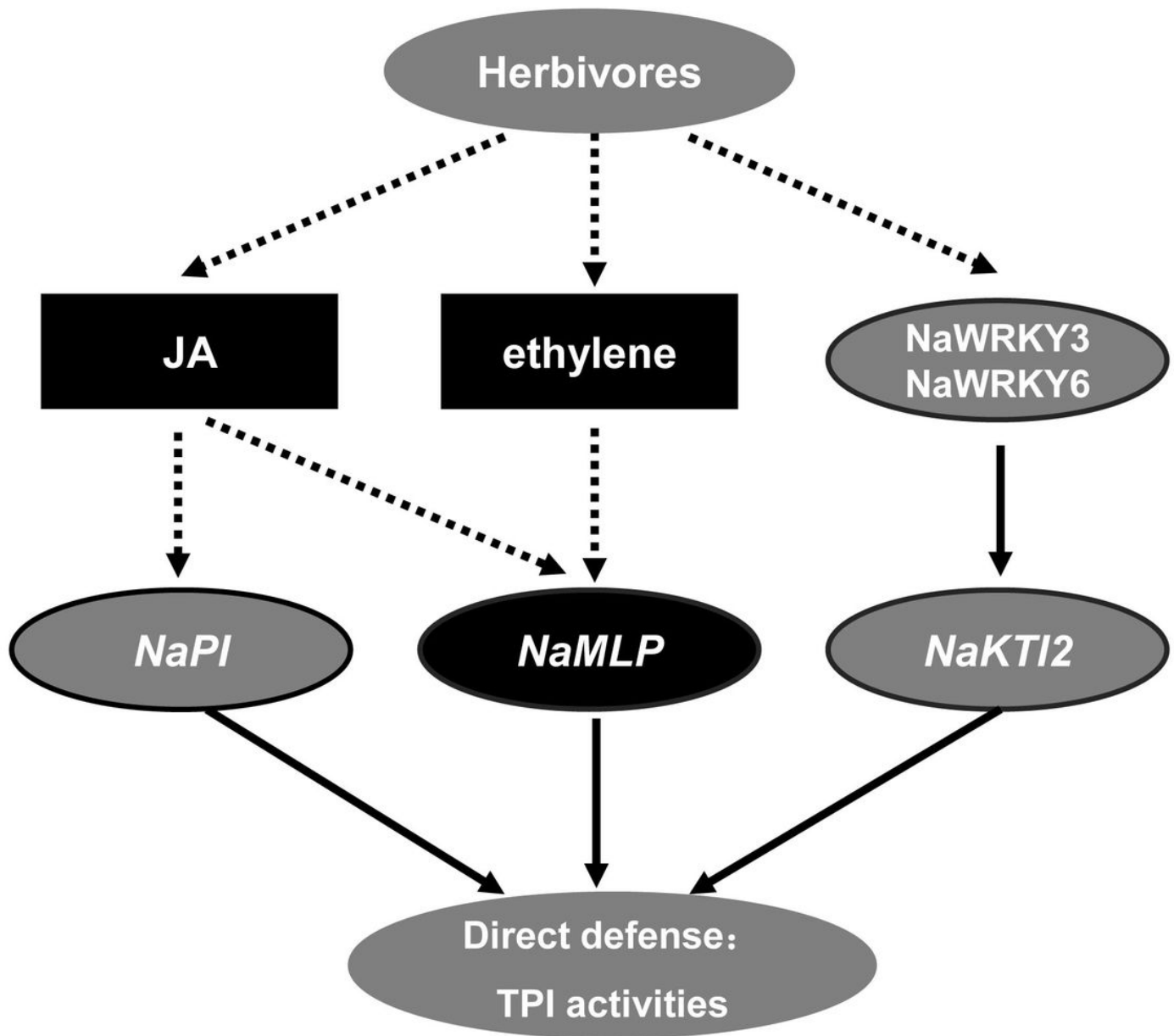


Figure 9

Working model of TPI activities elicited by *S. litura* oral secretions in *N. attenuata*.

When the leaves of *N. attenuata* are attacked by insect herbivores, three independent signaling are activated, including JA, ethylene and transcription factor NaWRKY3. NaPI is regulated solely by JA signaling, while NaKTI2 is mainly by NaWRKY3, and NaMLP is controlled by JA and ethylene in a synergistic way; finally all three TPIs, including NaPI, NaKTI2 and NaMLP, contributed to the insect herbivore-elicited TPI activities, and confer herbivore resistance.

Supplementary Files

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