

WRKY40, WRKY70, and Downy Mildew Resistant 6 (DMR6)-Like Oxygenase 1 are universal marker genes for the salicylic acid pathway in bananas

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Abstract

Background: Banana, an important cash and staple crop worldwide, suffers from various biotrophic and hemi-biotrophic pathogens. In plants' defense against these pathogens, the phytohormone salicylic acid (SA) plays a key role in the regulation of immune response. Using a specific set of SA-responsive genes as markers is frequently adopted to monitor the onset of SA-mediated immune response. However, reliable SA-responsive genes marker genes have not been well established in bananas.

Results: From the transcriptome analysis of SA-treated 'Pei-Chiao' banana roots, we identified 19 up-regulated and 3 down-regulated genes. Four of the up-regulated genes previously reported to play crucial roles in SA-mediated immunity in other species were further analyzed for their applicability in different tissues and cultivars of bananas using real-time quantitative reverse-transcription PCR. The analysis showed that *WRKY40*, *WRKY70*, and *Downy Mildew Resistant 6 (DMR6)-Like Oxygenase 1 (DLO1)* were significantly induced upon SA treatment in both the leaves and roots of 'Pei-Chiao' (AAA genome), 'Pisang Awak' (ABB genome), and 'Lady Finger' (AA genome) bananas.

Conclusions: The uncovering of common marker genes *WRKY40*, *WRKY70*, and *DLO1* for SA response in different banana genome types provides the stepping stone for studies towards understanding of SA-mediated immune response in bananas.

Background

Bananas and plantains are cash and stable crops crucial to the economic and food security for millions of people worldwide [1]. The global production of banana and plantains accounts for more than 100 million tons annually and is valued at more than \$38 billion USD [1].

Most commercial bananas are triploid derived from inter- and intra-specific crosses of diploid *Musa acuminata* and *M. balbisiana* [2]. These commercial cultivars are seedless and parthenocarpic; therefore, agricultural cultivation is largely limited to vegetative propagation [3, 4]. Although many different banana cultivars are grown locally around the world, the Cavendish cultivar currently dominates the world's banana trade market, and accounts for 47% of global banana production [5]. The limited genetic diversity and monoculture practices make the sustainability of banana especially vulnerable to mass destruction in face of growing adverse abiotic and biotic stressors [3, 6].

As sessile organisms, plants are prone to pathogens, insects, and changing environment without the ability to move away from incoming stresses. Thus, plants have evolved repertoire of mechanisms tuned by phytohormones in response to stresses [7]. Among the various identified phytohormones, the phenolic compound salicylic acid (SA) is critical for plant defense against a broad spectrum of pathogens. It is the major defense hormone in the interactions with biotrophic and hemi-biotrophic pathogens [8–12].

The plants' immune response can be stimulated from the recognition of the invading pathogen, triggering the increase in SA concentration that is both required and sufficient to activate plant defense [10, 11, 13]. The basal resistance involves the perception of conserved pathogen molecular signatures, pathogen- or microbe-associated molecular patterns (i.e. PAMPs or MAMPs), which activates pattern-triggered immunity (PTI) [14].

However, plant pathogens have evolved the ability to deliver counteractive effector proteins that suppresses PTI [15–18]. In turn, plants have evolved disease resistance (R) proteins, which directly or indirectly detects pathogen effectors, and activate effector-triggered immunity (ETI) [19]. Activation of both PTI and ETI is associated with increase in SA accumulation, which is important for resistance against pathogens [20–22]. In addition to the activation of PTI and ETI upon primary pathogen infection, plants have evolved systemic acquired resistance (SAR) that also requires SA to confer resistance in the uninfected systemic (distal) organs in response to prior (primary) infection [23–25]. Since endogenous SA accumulation can trigger defense reprogramming and activate a specific set of genes in response to pathogen attack [26], using these SA-modulated genes as markers to monitor the onset of SA-mediated immune response is commonly adopted in the study of plant immunity [27, 28].

Banana suffers from various biotrophic and hemi-biotrophic pathogens including viruses [29] and the important soil-borne disease caused by *Fusarium oxysporum* f. sp. *cubense* [3, 4]. In order to expedite the research on the important SA-signaling in banana, identification of the SA marker genes with expression levels highly correlated with endogenous SA concentration will greatly help to the understanding of the banana immune response and banana-pathogen interaction in detail.

In the dicotyledonous model plant *Arabidopsis*, the NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) has been identified as a central regulator in SA-mediated plant defense [30, 31]. Upon SA treatment or pathogen infection, NPR1 translocates to the nucleus where it interacts with TGA transcription factors, to coordinate the transcriptional activation of downstream immune responsive genes such as genes encoded PATHOGENESIS-RELATED proteins (PRs). Among PRs, PR-1 gene has been used as a canonical marker to monitor the onset of SA-mediated pathway [32, 33]. In bananas, a gene closely similar to *Zea mays* PR-1 (GenBank accession no. AAC25629), and two NPR1 homologs, MNPR1A (GenBank accession no. DQ925843), and MNPR1B (GenBank accession no. EF137717) have been shown to be induced by exogenous SA treatment or pathogen challenge [34, 35]. However, a recent transcriptome analysis with treatment of a synthetic SA analogue, benzothiadiazole (BTH), identified the induction of PR-1 homolog (GenBank accession no. XM_009419475.2) [36] instead of the PR-1 (AAC25629.1) [34]. Moreover, NPR1 homolog was not recovered from the analysis of genes induced by the BTH treatment [36]. The discrepancy among the previously reported SA marker genes could be caused by the differences in growth conditions, stages plants for treatment, the SA treatment methods, the banana cultivars, and sampling time for expression profiling.

In this report, the transcriptome analysis recovered 3 reliable SA-responsive marker genes, MaWRKY40, MaWRYK70, and Downy Mildew Resistant 6-Like Oxygenase 1 (MaDLO1) from ‘Pei-Chiao’ (a popular Cavendish banana cultivar with AAA genome grown in Taiwan). The expression level of all three genes was highly correlated with the concentration of SA in both ‘Pei-Chiao’ roots and leaves. Moreover, all of the 3 SA-responsive marker genes were also robustly induced in the roots and leaves of banana cultivars belonging to the genomic groups other than AAA genome, namely ‘Pisang Awak’ (ABB genome) and ‘Lady Finger’ (AA genome). The core SA responsive genes uncovered in this study can serve as the stepping stone for the studies of SA-mediated signaling in banana.

Results

Test of previously reported SA-responsive genes in leaves of 'Pei-Chiao' plantlets

In order to identify the salicylic acid (SA) marker genes in banana that would enable the analysis of SA-induced signaling in 'Pei-Chiao', we first analyzed genes that had been previously reported to be SA-responsive (Table 1). Banana plantlets grown in potted soil were treated with buffer (Mock) or SA by foliar spray. Leaves of each treated plant were harvested at two time points, 0 (no-treatment) and 6 hour-post treatment (hpt) for SA concentration and gene expression analyses. We measured SA concentration by high-performance liquid chromatography-mass spectrometry, which revealed a significant increase in SA concentration in SA-treated plants compared to the mock or no-treatment control at 6 hpt (Fig. 1a).

Table 1
Salicylic acid responsive candidate marker genes selected from reference studies.

Name	Description	Reference	Primer Sequence
MNPR1A	NPR1	Endha et al., 2008	F: 5'-GTCGGCATTGTACCAACACA-3' R: 5'-CAGTGCAGGAGTCAGAAAA-3'
MNPR1B	NPR1	Endha et al., 2008	F: 5'-AGGTTGCCGAACAAGAAG-3' R: 5'-TGAGAGGCAACAACTCAGAGAG-3'
MaNPR1B	NPR1	Wang et al., 2015	F: 5'-CCATTCCCAGATTAGATACA-3' R: 5'-TCGACATTCTTCGCAACC-3'
MaNPR1D	NPR1	Wang et al., 2015	F: 5'-CTGAACGCCCTCCTAACAA-3' R: 5'-TGACCTGAACGAAACACC-3'
BanPR1	PR1	Van den Berg et al., 2007	F: 5'-TCCGGCCTTATTCACATT-3' R: 5'-GCCATCTTCATCATCTGCAA-3'
MaTGA1	TGA	Wang et al., 2015	F: 5'-ATGATGACGAGGAAGATAA-3' R: 5'-AATGGACCTAAATGAAGC-3'
MaTGA2	TGA	Wang et al., 2015	F: 5'-ACATTCTGACATCCCTCCAC-3' R: 5'-GCTGATCTGATCCCAACG-3'

We next examined the transcriptional responses of the known SA-responsive genes listed in the Table 1 with real-time reverse transcriptase quantitative PCR (RT-qPCR). Among them, only MaNPR1D showed significant and reproducible induction at 6 hpt of SA (Fig. 1b). Compared to mock treatment, MaNPR1D increased by 2-fold at 6 hpt (Fig. 1b). This gene was selected for further analysis.

Analysis of SA concentration and MaNPR1D expression in leaves and roots of 'Pei-Chiao' plantlet after SA treatment

Roots serve as the initial infection site for several hemi-biotrophic pathogen such as *Fusarium oxysporum* f. sp. *cubense*, *Xanthomonas* spp., and *Ralstonia* spp. [4, 37]. To analyze whether MaNPR1D could serve as a reliable SA marker in roots of 'Pei-Chiao', we first optimized our SA treatment. To this end, we used 'Pei-Chiao' tissue culture plantlets grown on sterilize agar medium to prevent the potential infection of soil microorganism and minimize the damage during root sampling. 'Pei-Chiao' tissue culture plantlets grown in agar were treated with buffer (mock) or SA by foliar spray. Since we do not know whether SA level is increased under the

treatment method of foliar spray, we first quantified the SA concentration in mock- and SA-treated leaves and roots by the use of ultra-performance liquid chromatography chromatography (HPLC)-mass spectrometer (MS)/MS. (Fig. 2). Quantification of SA concentration revealed the increased levels of SA in the SA-treated ‘Pei-Chiao’ leaves and roots harvested at 6 hpt (Fig. 2a and b). Statistical significance was observed for the SA concentration in leaves of SA-treated plantlets at 6 hpt compared to that of no-treatment control or mock-treated plantlets (Fig. 2a); no statistical significance was found between no-treatment (0 hpt) and mock-treated plantlets (6 hpt) (Fig. 2a). In roots, the average SA concentration was also significantly higher in 6 h post SA-treated plantlets compared to no-treatment control plantlets or mock-treated plantlets (Fig. 2b). No statistical significance was found between no-treatment (0 hpt) and mock-treated plantlets (6 hpt) (Fig. 2b).

As expected, real-time RT-qPCR confirmed that MaNPR1D expression positively correlated with higher levels of SA in the leaves of ‘Pei-Chiao’ in repeated experiments (Fig. 2c). A 3.4-fold increase of statistical significance in MaNPR1D mRNA level was detected in the leaves of SA-treated plants compared to mock-treated plants at 6 hpt (Fig. 2c). However, only a slight induction of MaNPR1 gene expression level (2.3-fold) was observed in the roots (Fig. 2d).

Transcriptome Analysis Of Sa Responsive Genes In ‘pei-chiao’ Plantlet

Although MaNPR1 can be induced by SA treatment, only 2.3-fold induction was observed in our repeated experiments. To identify more sensitive SA-responsive genes that could be as reliable SA marker genes in roots of ‘Pei-Chiao’, we conducted 2 sets of RNA-seq for transcriptional response analysis of SA treatment vs. no-treatment at 0 hpt and mock treatment at 6 hpt of roots of banana grown in sterile conditions.

To identify differentially expressed genes under SA treatment, we first mapped the reads to the banana genome database (*Musa acuminata* DH Pahang v2; banana-genome-hub.southgreen.fr) after adaptor trimming and quality filtering. The mapping rates of the processed reads among the samples for RNA-seq analysis were similar and ranged from 83.29%-89.53% (Table 2). We then analyzed differentially expressed genes (DEGs) under SA treatment in ‘Pei-Chiao’ using statistic test with adjusted P value < 0.1 and obtained a total of 22 DEGs (Table 3). Among 19 SA up-regulated gene, *M. acuminata* Pathogenesis-Related protein 1-like (MaPR1-like; Ma04_g29640), WRKY transcription factor 40 (MaWRKY40; Ma07_g16310), WRKY transcription factor 70 (MaWRKY70; Ma07_g23510), and Downy Mildew Resistant 6 (DMR6)-Like Oxygenase 1 (MaDLO1; Ma00_g04490) are known to be important in the SA-mediated immunity pathway [38–42] and showed an induction > 2.5 fold at 6 h post treatment of SA in both replicates (Table 3 and Fig. 3). These 4 genes were selected for further analysis by real-time RT-qPCR (Table 3 and Fig. 3). Expression pattern of MaPR1-1, MaWRKY40, MaWRKY70, and MaDLO1 are shown in Fig. 3.

Table 2
Summary of RNA-seq data for analysis of SA responsive genes in 'Pei-Chiao' plantlet.

Sample	Repeat	Treatment	Raw reads	Clean reads	Mapped reads	Mapping rate (%)
PC-M-0 h	1	Mock	31,419,774	28,078,146	26,592,044	84.63
PC-M-0 h	2	Mock	26,820,728	25,081,892	24,006,856	89.51
PC-M-6 h	1	Mock	26,419,442	23,288,808	22,004,390	83.29
PC-M-6 h	2	Mock	29,018,865	27,243,084	25,981,414	89.53
PC-S-6 h	1	SA	28,817,447	25,838,512	24,202,029	83.98
PC-S-6 h	2	SA	27,345,079	25,654,923	24,428,110	89.33

Table 3
List of differentially expressed genes in 'Pei-Chiao' plantlet at 6 hour post-salicylic acid-treatment.

Gene ID	Fold Change (log2)	Adjusted p- value	Description
SA up-regulated genes (6 hpt)			
Ma00_g02460	2.21	1.94E-03	calmodulin-like
Ma00_g03560	5.78	1.03E-11	probable mannitol dehydrogenase
Ma00_g04490	3.12	2.01E-09	DMR6-like oxygenase 1
Ma01_g07400	1.99	3.43E-03	monothiol glutaredoxin-S9-like
Ma01_g07410	1.62	8.02E-02	pentatricopeptide repeat-containing protein At4g02750
Ma01_g20340	2.50	1.38E-02	Hypothetical protein
Ma02_g01990	1.81	4.68E-02	adenylate isopentenyltransferase 5, chloroplastic-like
Ma03_g11500	4.27	1.16E-04	3-oxoacyl-[acyl-carrier-protein] reductase, chloroplastic-like
Ma03_g12470	2.05	1.33E-02	uncharacterized LOC103978016
Ma04_g29640	1.46	3.42E-02	pathogenesis-related protein 1-like
Ma05_g12600	1.55	5.16E-02	flavanone 3-dioxygenase-like
Ma07_g06750	5.24	6.70E-15	L-lactate dehydrogenase A-like
Ma07_g16310	2.25	1.73E-03	probable WRKY transcription factor 40
Ma07_g23510	1.55	7.50E-02	probable WRKY transcription factor 70
Ma09_g26520	1.90	5.85E-03	Probable glutathione S-transferase parA
Ma10_g12070	2.15	3.35E-03	uncharacterized LOC104000673
Ma10_g28170	2.66	1.73E-03	uncharacterized LOC103969442
Ma11_g04000	1.84	1.38E-02	ankyrin repeat-containing protein At2g01680-like
Ma11_g18240	1.76	2.98E-02	non-specific lipid-transfer protein-like
SA down-regulated gene (6 hpt)			
Ma02_g24600	-2.00	5.16E-02	ribulose bisphosphate carboxylase small chain, chloroplastic
Ma09_g31150	-2.12	2.27E-02	Putative germin-like protein 2 - 1

Gene ID	Fold Change (log2)	Adjusted p- value	Description
Ma11_g03350	-1.55	9.97E-02	methionine gamma-lyase-like

Verification of SA Induction By Real-time Rt-qpcr

Expression levels of 'Pei-Chiao' MaPR1-like, MaWRKY40, MaWRKY70, and MaDLO1 in the roots and leaves of mock- and SA-treated samples by foliar spray were analyzed by real-time RT-qPCR (Fig. 4) with the use of gene specific primers (Table 4). In the roots, all 4 genes exhibited a higher expression level in the SA treatment compared to the mock treatment at 6 hpt, MaPR1-like increased by 2-fold, MaWRKY40 increased by 56-fold, MaWRKY70 increased by 31-fold, and MaDLO1 increased by 487-fold; however, the induced expression of MaPR1-like expression did not reach a statistical significance (Fig. 4a).

Table 4
Primers for real-time RT-qPCR of salicylic acid responsive marker genes in banana

Name	Description	Primer Sequence	Gene ID	NCBI Accession
MaPR1-Like	Pathogenesis-related protein 1-like	F: 5'-GAAGCAGGACTACGACTACAAC-3' R: 5'-GGACGAACGCCACACAA-3'	Ma04_g29640	XM_009400035.2
MaWRKY40	WRKY transcription factor 40	F: 5'-CGGGATGATGTGTACGGTTT-3' R: 5'-TAAGGTTGCAGGTGTGTTC-3'	Ma07_g16310	XM_009411509.2
MaWRKY70	WRKY transcription factor 70	F: 5'-CTGCAGCTTGGACATGGA -3' R: 5'-CCCTCTTAACTGATGTACTCATCG-3'	Ma07_g23510	XM_009412236.1
MaDLO1	DMR6-like oxygenase 1	F: 5'-GAGAGCTTGGGACTTGAGAAG -3' R: 5'-CTGTGGGCATGGTGGATAG-3'	Ma00_g04490	XM_009389881.2

Consistent with the RT-qPCR results of the roots, MaWRKY40, MaWRKY70, and MaDLO1 mRNA levels in the leaves were significantly higher by 92-fold, 34-fold, and 1332-fold, respectively, at 6 hour post-SA-treatment compared to mock-treatment (Fig. 4b). No obvious difference was observed for MaPR1-like expression after the SA treatment in roots (Fig. 4b).

MaWRKY40, MaWRKY70, and MaDLO1 are induced by SA treatment in all tested cultivars

To determine whether MaWRKY40, MaWRKY70 and MaDLO1 could represent a core set of SA-responsive genes across bananas of various genomic groups, we further compared the gene expression profiles of these genes in between mock- and SA-treated samples of 'Pisang Awak' (ABB genome) and 'Lady Finger' (AA genome) by real-time RT-qPCR at 6 hpt (Fig. 5–6).

In concordance with the real-time RT-qPCR result of 'Pei-Chiao', MaWRKY40, MaWRKY70, and MaDLO1 expression was significantly induced (> 20 folds) by SA in 'Pisang Awak' plantlet at 6 hpt (Fig. 5a). In 'Pisang Awak', roots of treatment group compared to mock treatment, MaWRKY40, MaWRKY70, and MaDLO1 significantly increase by 179-fold, 21-fold, and 2672-fold, respectively (Fig. 5a). In the leaves of 'Pisang Awak' plantlet, SA treatment also significantly increased MaWRKY40 (188-fold), MaWRKY70 (25-fold), and MaDLO1 (1650-fold) mRNA levels (Fig. 5b).

Similarly, in the diploid 'Lady Finger' banana, MaWRKY40, MaWRKY70, and MaDLO1 mRNA levels are significantly higher in SA-treated leaves and roots of plantlet compared to that of mock treatment (Fig. 6a and b). In the roots, MaWRKY40, MaWRKY70, and MaDLO1 significantly increase by 11-fold, 7-fold, and 122-fold, respectively, compared to mock treatment (Fig. 6a). MaWRKY40, MaWRKY70, and MaDLO1 significantly increase by 146-fold, 24-fold, and 855-fold, respectively, in SA-treated leaves compared to mock treatment (Fig. 6b).

Discussion

We initially aimed to use reported SA marker gene for monitoring the SA-mediated immune response in our selected banana cultivar; however, only MaNPR1D are consistently induced by SA treatment in our analysis. MaNPR1D can be induced by SA treatment in leaves of 'Pei-Chiao' (3.4-fold) and in the roots (2.3-fold), which is similar to reported NPR1 in dicotyledonous model plants *Arabidopsis thaliana* and tobacco. NPR1 play an important role in sensing SA-induced changes in the cellular redox state, and lead to the translocation of NPR1 move into the nucleus, and function as transcriptional coactivator to activate the transcriptional reprogramming of genes including the activation of downstream responses genes such as PR1 [43–45]. As post-translational regulation of NPR1 is the major regulation mode to trigger immune response, NPR1 transcription is only moderately (2–3 times) induced upon pathogen or SA treatment [46, 47]. Therefore, it is not common to use NPR1 as a marker for monitoring the onset SA-mediated immune response.

In contrast to NPR1, the induction of PR1 genes has been reported to be 500–1000 fold higher at 6 hour post-SA treatment in *Arabidopsis* [48], thus PR1 is commonly considered as a reliable SA marker gene. However, previously report indicates the discrepancy of PR1 homolog as SA marker genes in different banana cultivars [34, 36]. Moreover, MaPR1-like (NCBI accession: XM_009400035.2) that was identified through transcriptome analysis (Table 3) in 'Pei-Chiao' was only mildly induced in the roots but not leaves (Fig. 5).

Our result for the induction of PR1 homolog genes by SA treatment is not consistent with *Arabidopsis thaliana* and tobacco, but in some monocotyledonous plants similar results have been reported. Kouzai et al. (2016) reported that of the 11 PR1 gene homologs in *B. distachyon*, only 1 (Bradi1g57590) was identified to be induced by SA treatment at 3 hpt but not other PR1 homologs[49]. In contrast, in a separate study, another PR1 homologs, Bradi1g57580, exhibited higher induction after 24 and 48 hours post SA treatment. In the monocotyledonous rice plant, variable results of PR1 expression have also been reported. In the characterization of selected 12 rice PR1 family genes, Mitsuhashi et al. (2008) found several PR1 homologous genes that were induced by SA treatment; however, Yang et al. (2004) reported exogenous application of SA as a poor activator of PR gene expression [50, 51]. These results suggest differences in PR1 gene as SA markers between monocots and dicots.

From our study, we identified 2 WRKY transcription factors, MaWRKY40 and MaWRKY70 that robustly corresponded to elevated SA concentration in banana leaves and roots (Fig. 5–7). WRKY transcription factors have been reported to be induced by SA treatment in both dicotyledonous and monocotylendous plants [52, 53]. It was reported that the induction of WRKY70 transcripts occurred earlier and accumulated to higher levels upon pathogen infection or SA treatment in Arabidopsis. In addition, overexpression of WRKY70 resulted in the constitutive expression of SA-responsive PR genes and enhanced resistance to the biotrophic and hemi-biotrophic pathogens, while repressing jasmonic acid (JA) response [54, 55]. Moreover, in the previous study of Arabidopsis [54], WRKY40 is also induced by SA and suppresses the expression of ABA-responsive genes ABI4 and ABI5 [39, 41, 56, 57]. Hence, WRKY70 and WRKY40 function as a node of convergence for integrating signals from SA and JA-dependent pathways or SA- and ABA-dependent pathways, respectively.

The most sensitive SA-responsive gene observed in this study was DLO1, which showed strongest induction (> 100 folds) in different cultivars (Fig. 5–7). In Arabidopsis, DLO1 is strongly activated during hemi-biotrophic *Hyaloperonospora arabidopsidis* attack and following BTH treatment [42]. Studies showed that DLO1 catabolizes SA by converting SA to 2,3-dihydroxybenzoic acid (2,3-DHBA) and is a component of a negative feedback regulation system of SA levels during senescence [58]. Similar hydroxylation mechanisms to control levels of other plant hormones jasmonate and auxin have also been observed [59, 60]. Since the induction of DLO1 in the negative feedback regulation of cytoplasmic SA levels may be critical to maintain the homeostasis of SA concentration. Our data indicates that DLO1 serve as a suitable marker gene to monitor the fine tuning of SA signaling.

Conclusion

In conclusion, we conducted a comprehensive analysis of SA-responsive genes in bananas and identified MaWRKY40, MaWRKY70, and MaDLO1 as common and robust SA marker genes. Given the critical roles of SA in plant defense, the uncovering of a core set of SA responsive genes in the banana cultivars of different genomic groups may facilitate the studies in SA-mediated banana biology.

Methods

Plant material and growth conditions

Tissue-cultured 'Pei-Chiao' (Cavendish banana, AAA genome), 'Pisang Awak' (ABB genome), and 'Lady Finger' (AA genome) plantlets in jars were purchased from Taiwan Banana Research Institute (Pingtung, Taiwan) and placed in growth chamber for at least seven days for acclimation at 28°C under 16 h light/8 h dark cycle with 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ illumination. For potted banana plants, sterilized soil containing 6 parts peat, 1 part perlite, and 1 part vermiculite, and grown in insect-proof growth chamber at 28°C under 16 h light/8 h dark cycle with 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ illumination.

Salicylic Acid Treatment

Plants were treated with 5 mM salicylic acid (SA; Sigma-Aldrich) in 0.5% EtOH and 0.025% L-77. Control mock plants were treated with 0.5% EtOH and 0.025% L-77. For treatments of potted plants, SA was foliar sprayed on 8 weeks old plants until imminent runoff. The 2nd, 3rd, and 4th leaves (the most upper leaf designated as

1st leaf) of 3 biological plants were harvested at time points 0 hpt (no treatment) and 6 hpt, harvested and snap frozen in liquid nitrogen for subsequent analysis.

For tissue cultured banana jar and transcriptome samples, 1 ml per tissue-culture Cavendish ‘Pei-Chiao’ plantlets were foliar sprayed with 5mM SA in 0.5% EtOH and 0.025% L-77. Control mock plants were treated with 0.5% EtOH and 0.025% L-77. Roots of 3-5 biological plants were harvested at time points 0 hpt (no treatment) and 6 hpt, harvested and snap frozen in liquid nitrogen for subsequent analysis.

For real-time RT-qPCR analysis of candidate genes in ‘Pei-Chiao’, ‘Pisang Awak’, and ‘Lady Finger’ were foliar sprayed with 5mM SA in 0.5% EtOH and 0.025% L-77. Control mock plants were treated with 0.5% EtOH and 0.025% L-77. Roots of 3-5 biological plants were harvested at time points 0 hpt (no treatment) and 6 hpt, harvested and snap frozen in liquid nitrogen for subsequent analysis

SA Extraction and Quantification

To extract SA, we used a method following previously described [61]. The LC system used for analysis was ultra-performance liquid chromatography (UPLC) system (ACQUITY UPLC, Waters, Millford, MA). The sample was separated with ACQUITY UPLC HSS T3 column (1.8µm particle size, 2.1 × 100 mm, Waters). The flow rate was 0.3 mL/min, the injection volume of 5 µL, the column temperature of 30°C. The composition of mobile phase A was water containing 0.1% acetic acid, B was methanol containing 0.1% acetic acid, and gradient: 0min-90%A, 1min-60%A, 3min-50%A, 5min-40%A, 7min-0%A, 8min-0%A, 9.5min-99.5%A, 12.0min-99.5%A, 12.5min-90.0%A, 15.0min-90.0%A. The UPLC system was coupled online to the Waters Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, USA). ¹³C₆- salicylic acid (¹³C₆-SA) was used as internal standards. Characteristic MS transitions were monitored using negative multiple reaction monitoring (MRM) mode for salicylic acid (m/z, 137>93) and ¹³C₆-SA (m/z, 143>99). Data acquisition and processing were performed using MassLynx version 4.1 and TargetLynx software (Waters Corp.).

RNA-seq profiling

Two biological replicates of SA and mock treatments grown independently were harvested for sequencing on the Illumina NovaSeq 6000 platform following standard protocols. For each replicate of the SA or mock treatment, total RNA of plantlets from 3 tissue culture jar was extracted with PureLink Plant RNA Reagent (Thermo Fisher Scientific, Waltham, MA, USA) for RNA-seq library construction. The RNA-seq libraries were prepared using the NEBNext Ultra RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA).

Analysis of RNA-seq data

After trimming adapter sequence and low-quality bases with a Phred quality score ≤20 by the trimomatic v0.36, RNA-seq reads shorter than 100 nt were removed . The clean reads were mapped to the genome of *Musa acuminata* DH Pahang downloaded from Banana Genome Hub (<http://banana-genome-hub.southgreen.fr; v2>) using STAR v2.7.0f. The reads with more than one genomic hit were discarded. The abundance of each gene was quantified by RSEM v1.3.0 and normalized into transcripts per million (TPM). The differential expression analysis between SA treatment and mock groups was performed using DESeq2 1.26.0. Benjamini and Hochberg’s method was applied to adjust the p-values for correcting the false discovery

rate for multiple testing. The genes with an adjusted p-value < 0.1 were considered as differentially expressed genes (DEGs) for further data analysis.

Real-time reverse transcription quantitative PCR

Total RNAs were extracted using Purelink Plant Reagent (Thermo Fisher Scientific) from 100 mg of leaf or root samples from 3-5 plants, and treated with Turbo DNase (Thermo Fisher Scientific) to remove genomic DNA. cDNA for qRT-PCR was synthesized from 1 μ g of DNA-free RNA and oligo (dT) using PrimeScript RT Reagent Kit (Takara Bio), following the manufacturer's instructions.

Quantification of *MaWRKY40*, *MaWRKY70*, and *MaDLO1* were carried out using SYBR Premix EX Taq II Kit (Takara Bio Inc., Kusatsu, Shiga, Japan) with ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). Banana actin, *MaACT* (NCBI Accession: XM_009412781), was used as a control to calculate the relative target gene mRNA level. All primer pairs are listed in Table 4.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

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Authors' contributions: YT, H-HY conceived the study and designed the experiments. C-PC maintained and provided experimental plants used in this study. YT, AP-C, and EGC carried out the experiments and performed the statistical analysis. B-HH, S-MT and H-MC performed transcriptome analysis. H-MC and W-CS helped supervise this study. YT and H-HY drafted the manuscript. H-HY coordinated the study and agrees to serve as the author responsible for contact and ensures communication. All authors read and approved the final manuscript.

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Figures

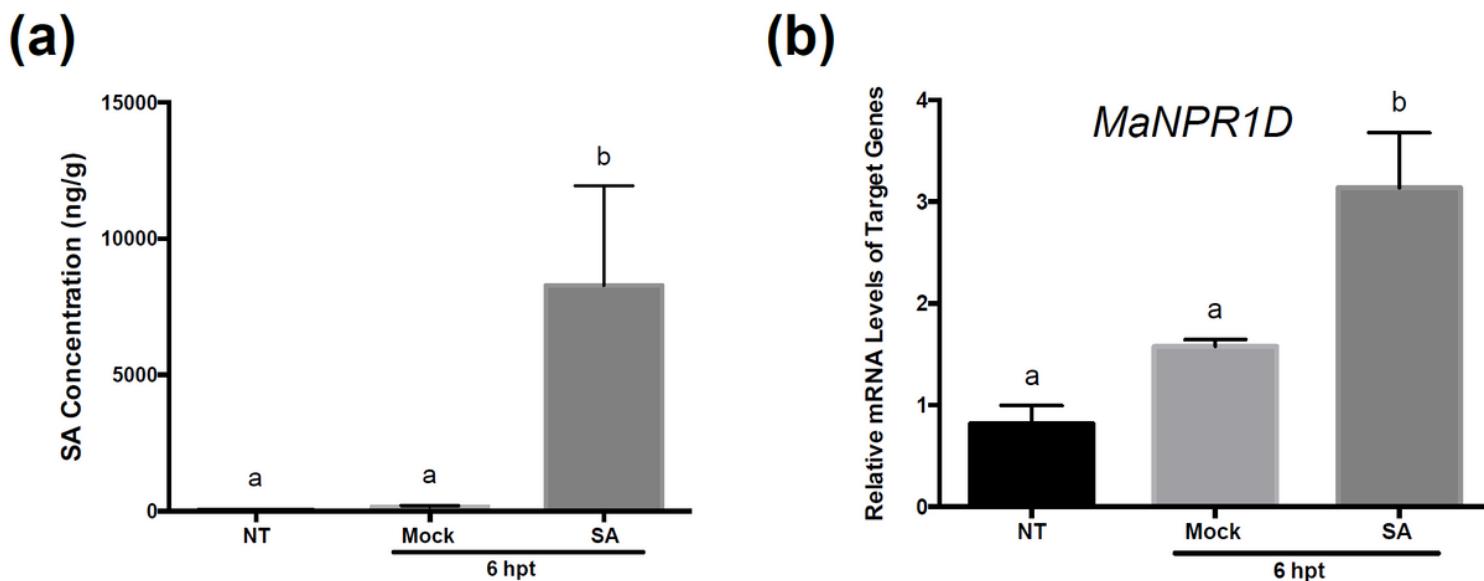


Figure 1

Analysis of SA concentration and MaNPR1D expression in leaves of SA treated 'Pei-Chiao' plantlet. Leaves collected from plantlet without treatment (NT), mock or SA treated plantlet were extracted for the analysis of

SA concentration (a) or the expression of MaNPR1D (b). Data represents mean \pm SD. Different letters indicate statistically significant difference analyzed by one-way analysis of variance (ANOVA) Tukey's test ($P<0.05$).

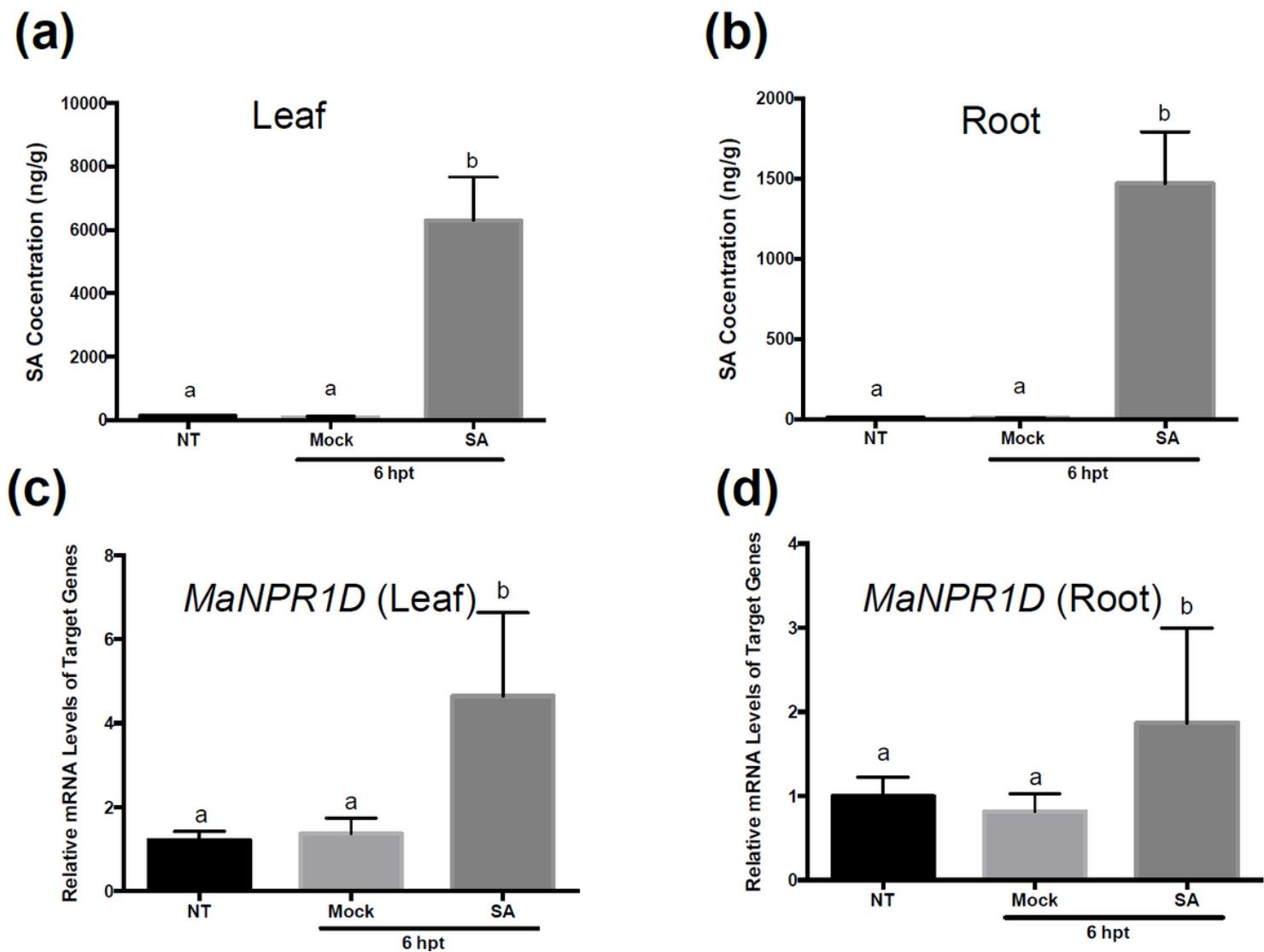


Figure 2

Analysis of SA concentration and MaNPR1D expression in leaves and roots of SA treated 'Pei-Chiao' plantlet. Leaves (a and c) and roots (b and d) collected from banana plantlet without treatment (NT), mock or SA treated plantlet were extracted for the analysis of SA concentration (a and b) or the expression of MaNPR1D (c and d). Data represent mean \pm SD. Different letters indicate statistically significant difference analyzed by one-way analysis of variance (ANOVA) Tukey's test ($P<0.05$).

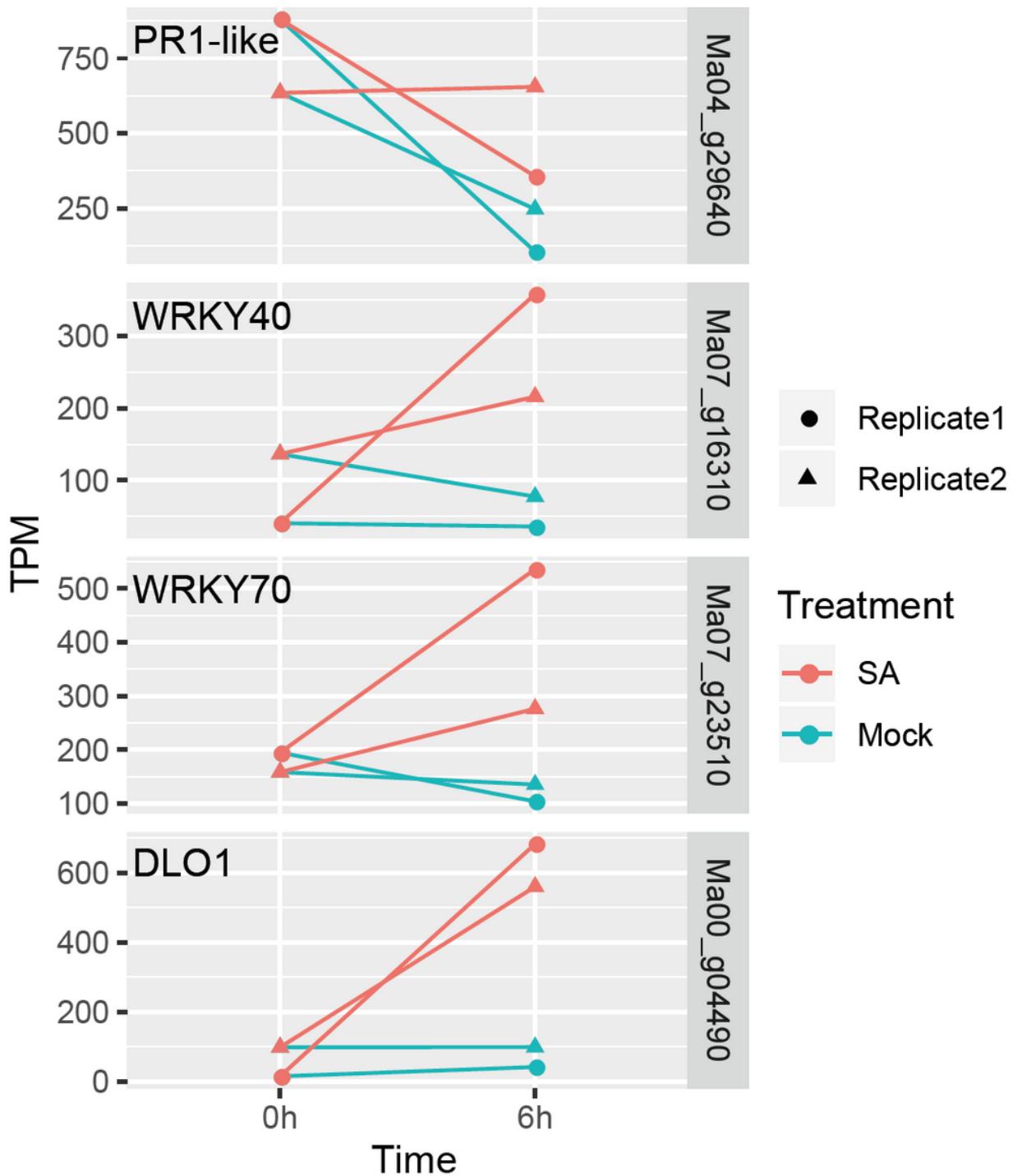
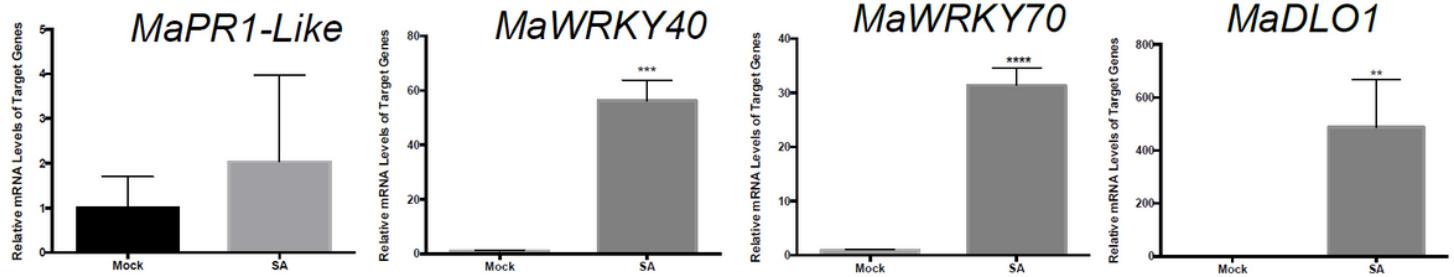


Figure 3
RNA-seq data of selected SA up-regulated genes in 'Pei-Chiao' plantlet. RNA-seq data of PR1-like, WRKY40, WRKY70, and DLO1 are represented. Red line: SA treatment; Blue line: Mock; Dot: Replicate 1; Triangle: Replicate 2; TPM: transcripts per million.

(a) Roots



(b) Leaves

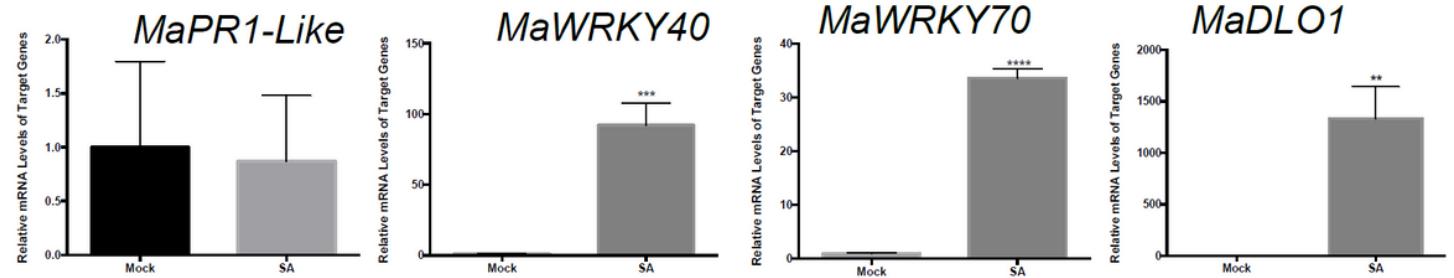
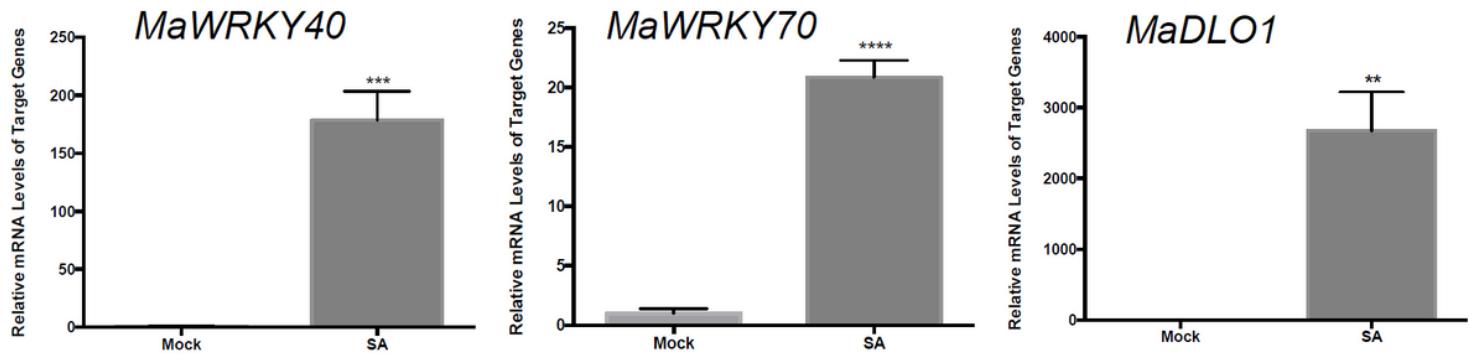


Figure 4

Gene expression of MaPR1-like, MaWRKY40, MAWRKY70, and MaDLO1 in mock- or SA-treated 'Pei-Chiao' plantlet. Data represents mean \pm SD relative mRNA levels of specified target gene; n = 3 biological replicates; **, P \leq 0.01; ***, P \leq 0.001; ****, P \leq 0.0001; Student's t-test compared to mock treatment. The RNA level of mock treated plants was set to 1.

(a) Roots



(b) Leaves

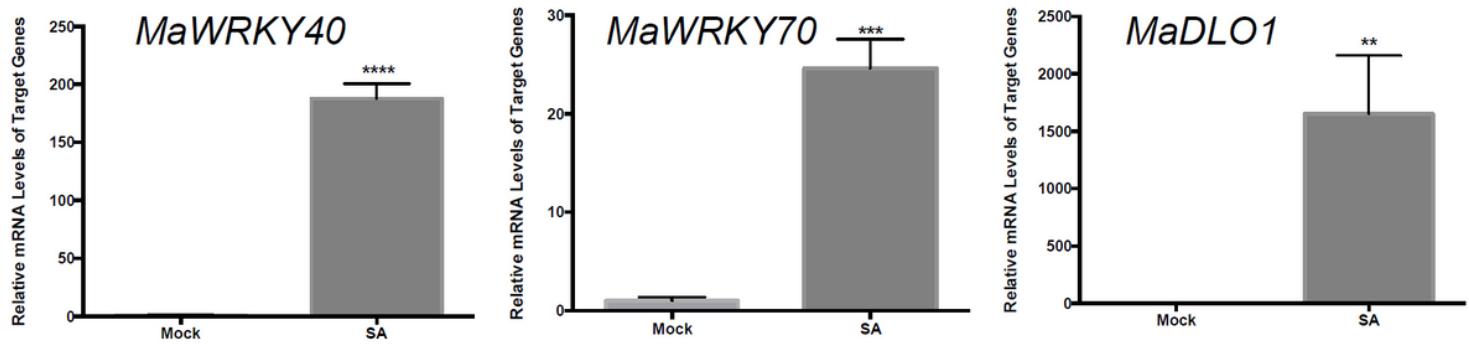
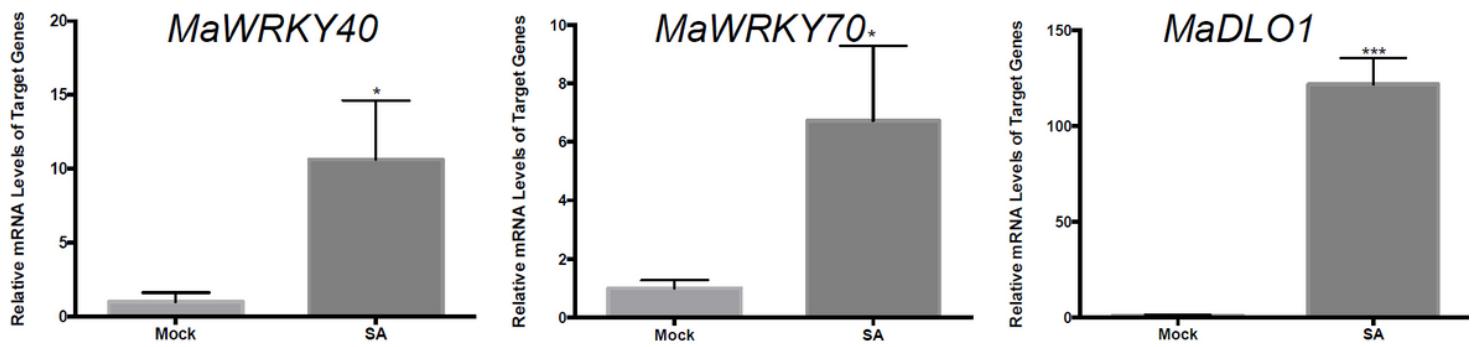


Figure 5

Expression levels of salicylic acid marker genes *MaWRKY40*, *MAWRKY70*, and *MaDLO1* in mock- or SA-treated 'Pisang Awak' leaves (a) or roots (b) at 6 hour post-treatment (hpt). Data represents mean \pm SD relative mRNA levels of specified target gene; n = 3 biological replicates; **, P \leq 0.01; ***, P \leq 0.01; ****, P \leq 0.001; Student's t-test compared to mock treatment. The RNA level of mock treated plants was set to 1.

(a) Roots



(b) Leaves

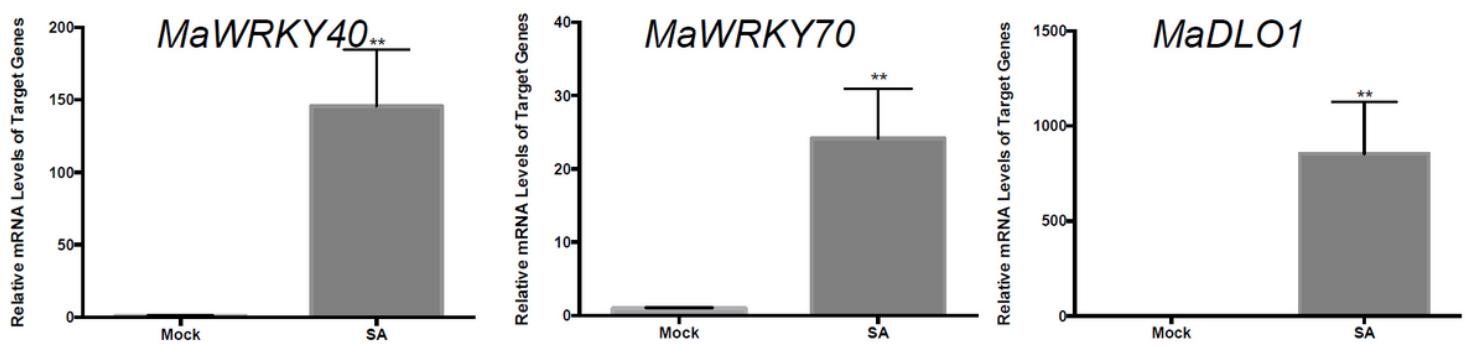


Figure 6

Expression levels of salicylic acid marker genes MaWRKY40, MAWRKY70, and MaDLO1 in mock- or SA-treated 'Lady Finger' leaves (a) or roots (b) at 6 hour post-treatment (hpt). Data represents mean \pm SD relative mRNA levels of specified target gene; n = 3 biological replicates; *, P \leq 0.05; **, P \leq 0.01; ***, P \leq 0.01; Student's t-test compared to mock treatment. The RNA level of mock treated plants were set to 1.