



Overexpression of the PAP1 Transcription Factor Reveals a Complex Regulation of Flavonoid and Phenylpropanoid Metabolism in *Nicotiana tabacum* Plants Attacked by *Spodoptera litura*

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Abstract

Anthocyanin pigments and associated flavonoids have demonstrated antioxidant properties and benefits for human health. Consequently, current plant bioengineers have focused on how to modify flavonoid metabolism in plants. Most of that research, however, does not consider the role of natural biotic stresses (e.g., herbivore attack). To understand the influence of herbivore attack on the metabolic engineering of flavonoids, we examined tobacco plants overexpressing the *Arabidopsis* *PAP1* gene (encoding an MYB transcription factor), which accumulated anthocyanin pigments and other flavonoids/phenylpropanoids. In comparison to wild-type and control plants, transgenic plants exhibited greater resistance to *Spodoptera litura*. Moreover, herbivory suppressed the *PAP1*-induced increase of transcripts of flavonoid/phenylpropanoid biosynthetic genes (e.g., *F3H*) and the subsequent accumulation of these genes' metabolites, despite the unaltered *PAP1* mRNA levels after herbivory. The instances of down-regulation were independent of the signaling pathways mediated by defense-related jasmonates but were relevant to the levels of *PAP1*-induced and herbivory-suppressed transcription factors, An1a and An1b. Although initially *F3H* transcripts were suppressed by herbivory, after the *S. litura* feeding was interrupted, *F3H* transcripts increased. We hypothesize that in transgenic plants responding to herbivory, there is a complex mechanism regulating enriched flavonoid/phenylpropanoid compounds, via biotic stress signals.

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Introduction

The production of specialized metabolites in plants has attracted great interest as a result of the successful development of mechanisms that enable metabolic enzymes to be produced [1–3]. To synchronously activate metabolic genes, plants use a large variety of transcription factors. These factors may control multiple genes or orchestrate entire metabolic pathways or their specific branches. PRODUCTION OF ANTHOCYANIN PIGMENT1 (*PAP1*) is a typical R2R3 MYB-type transcription factor in *Arabidopsis thaliana* that is able to ectopically activate the entire array of genes involved in the biosynthesis of phenolic acids and flavonoid compounds (including anthocyanins) in the flavonoid biosynthetic pathway in several plant species, including tobacco, salvia, petunia and rose [4–8]. The overexpression of *Arabidopsis* *PAP1* in transgenic tobacco plants under the control of a constitutive promoter led to the induction of anthocyanins in the

leaves, flowers, stems and roots; the deep red or purple appearance of the plant parts was evidence [5]. Moreover, *PAP1*-overexpressing petunia flowers have been shown to increase not only levels of anthocyanins but also emissions of floral volatiles (benzenoids) [7]. Gene expression profiling and measurements of the levels of pathway intermediates suggest that both increased metabolic flux and the transcriptional activation of scent and color genes underlie the enhancement of petunia flower color and scent production [7]. The coordinated regulation of metabolic steps within or between pathways involved in vital plant functions – for instance, pathways involved in floral display determining plant-pollinator interactions – provides plants with a clear advantage. Indeed, bees and humans can discriminate between the floral scents of *PAP1*-overexpressing and control rose flowers [6]. Therefore, *PAP1* overexpression may find applications in horticulture and human health.

Manipulating flavonoid metabolism, especially in response to biotic or abiotic stresses, may have unpredicted side effects, as these compounds belong to the largest group of specialized metabolites produced by plants [9]. Flavonoids serve as essential components of a number of structural polymers that provide protection; lignin, for instance, due to its antioxidant and free radical scavenging properties, protects plants from ultraviolet light and defends plants against herbivores and pathogens [10]. Anthocyanin accumulation in *Arabidopsis* is enhanced in response to a number of environmental stress factors, such as cold, drought, pathogen attack and nutrient depletion [11]. Moreover, it appears that transgenic tobacco expresses structural genes and regulatory genes involved in flavonoid biosynthesis, and these genes defend the plant against herbivores [12,13]. Therefore, *PAPI*-overexpressing plants that typically accumulate large amounts of flavonoids might possess increased plant resistance to biotic stresses, including herbivores, but to date no practical evidence of this prediction has been provided. Whether the *PAPI* transgene interferes with endogenous TFs and processes involved in plants' defense and development has not been extensively investigated.

A separate branch of flavonoid catabolism controlled by other MYB transcription factors (e.g., MYBJS1) was previously shown to control the accumulation of anti-herbivore compounds, phenolamides (PAs), in *Nicotiana* species [14–17]. In addition, CoA-activated phenolic acids with polyamines in PA production is controlled by the MYBJS1 homologue MYB8 in a herbivory-inducible fashion in wild tobacco, *N. attenuata* [18]. MYB8 (and MYBJS1) also likely contributes to lignin accumulation by targeting the control of the hydroxycinnamoyl-CoA:shikimate/quininate hydroxycinnamoyl transferase (HCT) gene in tobacco [19–21].

Because *PAPI*-overexpressing plants constitutively produce large amount of anthocyanins, we asked if the accumulation of other inducible flavonoids and phenylpropanoids was affected. *PAPI* and MYBJS1 seem to share common gene targets in the pathways they regulate, and so these two transcription factors are likely to engage in complex interactions when *PAPI* is introduced into plants under a constitutive promoter and also, in response to herbivory, when other MYBs are additionally activated upon. It is likely that when *PAPI*-overexpressing plants are damaged by herbivores, the dynamic activation of the metabolic flow via other MYB members competes with activated coumaric acid units used in the constitutive synthesis of anthocyanins by *PAPI*-promoted enzymes. Both transcriptional and post-transcriptional regulations of *PAPI* have been shown to control anthocyanin levels in ubiquitous light/dark rhythms [22]. Moreover, when anthocyanin production is regulated by independent PAP-transcription and environmental stress, anthocyanin levels are reduced [23]. Given that the stress of herbivory could affect *PAPI* function in *PAPI*-overexpressing plants, we asked how efficiently transgenes such as *PAPI* could be expected to perform under natural, uncontrolled environmental conditions.

In the current study, we found that the defensive properties of *PAPI*-overexpressing plants increased upon herbivore attack, and addressed the issue of metabolic fluxes and transcriptional regulation in the flavonoid/phenylpropanoid pathways that are co-regulated by powerful *PAPI*-activators and herbivory. Since jasmonic acid (JA) and its derivatives (referred as jasmonate [JAs]) coordinate the expression of regulatory and structural genes in the flavonoid/phenylpropanoid pathways under stress, we also assessed the role of JAs (and other phytohormones) in the herbivory- and *PAPI*-mediated regulation of phenylpropanoid metabolism. Finally, we discuss the implications of enhancing the production of

specialized metabolites in plants under natural environmental conditions.

Materials and Methods

Production of transgenic tobacco plants

The full-length coding region of *Arabidopsis PAPI* (At1g56650) cDNA was inserted into the binary vector pSMABR35SsGFP by replacing the GFP reporter gene of the vector [24]. The resulting plasmid, pSMABR-35SPAPI, was transformed into *Agrobacterium tumefaciens* strain EHA101 by electroporation. *PAPI*-overexpressing tobacco (*N. tabacum* cv. SR1) was produced by *Agrobacterium*-mediated transformation as described previously [24]. After rooting and acclimatization, the regenerated plants were grown in a controlled greenhouse to set seeds. Nine lines of transgenic T₁ seeds were tested for germination on Murashige and Skoog medium supplemented with 5 mg l⁻¹ bialaphos at 25°C under a 16-h photoperiod at a light intensity of ca. 30 μE m⁻² s⁻¹. T₂ seeds harvested from each T₁ individual plant that showed ca. 3:1 segregation ratio were tested for bialaphos-resistance again. Finally, T₂ and T₃ homozygous plant lines were used for further analyses. A homozygous tobacco T₂ line transformed with the binary plasmid pSMABR-35SpGUS (in which the reporter *GFP* gene of pSMABR35SsGFP was replaced by β-glucuronidase [*GUS*] gene), expressing bialaphos resistance [*bar*] and *GUS* genes, was used as a control.

Three representative *PAPI*-overexpressing plants (*PAPI* lines), constitutively expressing *Arabidopsis PAPI* under the control of the cauliflower mosaic virus (CaMV) 35S promoter, were used. The *PAPI* lines exhibited substantial expression of *PAPI* based on their increased pigmentation throughout their development, in comparison to wild-type (WT) and transgenic plants expressing *uidA* (coding for the *GUS* reporter gene and serving as a control) (Figures S1 and S2). Except for having increased pigmentation, *PAPI* lines developed and matured in a similar manner to their control plants, as shown in previous studies [5,6] (Figure S2).

Plants and caterpillars

Tobacco plants were grown in plastic pots in a growth chamber at 25°C (16 h photoperiod at a light intensity of 80 μE m⁻² s⁻¹) for about 5 weeks. Eggs of *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) were obtained from Sumika Technoservice Co. Ltd. (Takarazuka, Japan), and the hatched larvae were reared on artificial diet (Insecta LF, Nihon Nosan Kogyo Ltd., Tokyo, Japan) in the laboratory at 25°C.

Chemical and herbivore treatments and leaf sample preparation

For chemical treatment, methyl jasmonate (MeJA, 0.1 mM, Wako Pure Chemical Industrials, Ltd., Osaka, Japan) in 2 mL of 0.1% ethanol solution was sprayed onto intact plants in plastic pots. Ethanol solution (0.1%) was used as the control. For herbivore treatment, four third-instar larvae were incubated with a whole tobacco plant in a pot (Figure S3). All herbivory bioassays were carried out in a climate-controlled chamber at 25°C (16 h photoperiod). For the determination of phytochemical concentrations and RNA extractions, leaf tissue samples were harvested into the liquid nitrogen, flash-frozen, and kept at -80°C prior to extraction.

Performance of *S. litura* larvae

Larvae were reared on artificial diet until the assay started, as described above. Second-instar *S. litura* larvae were weighed prior to release onto a potted plant (ranging from 0.7 to 1.2 mg) and

released one at a time. Each larva was allowed to move on the whole plant in a climate-controlled room at 25°C under a 16-h photoperiod. Twenty-six to thirty-two larvae were analyzed for each line.

Analysis of phenolic compounds

Phenylpropanoids and flavonoids in tobacco leaves were determined using high-performance liquid chromatography (HPLC) with a slight modification of the method described by Galis et al. (2013) and Matsuba et al. (2010) [25,26]. Replicated analyses were conducted with five independent leaf samples.

For anthocyanin (cyanidin-3-rutinoside) determination, the ground leaf samples (300 mg each) were further homogenized in the liquid nitrogen and extracted with 3 ml of 80% methanol containing 1% trifluoroacetic acid (TFA) at 4°C overnight. After passing through a 0.22 µm syringe filter (Millipore, Billerica, MA, USA), filtrates (10 µl) were subjected to an HPLC (L-6320 Intelligent pump, L-4200 UV-VIS detector, AS-4010 Intelligent Autosampler, L-5025 column oven, Hitachi, Tokyo, Japan) equipped with a COSMOSIL 5C₁₈-MS-II packed column (4.6 mm×50 mm; Nacalai Tesque, Kyoto, Japan). The separation was performed by the linear gradient elution of 10–70% (v/v) methanol in 1.5% aqueous phosphoric acid for 5 min at a flow rate of 1.5 ml min⁻¹ at 35°C. The elution profiles were monitored at 528 nm and recorded using the Chromato-Pro (Runtime Instruments, Kanagawa, Japan). The authentic compound used for quantification was cyanidin-3-rutinoside (Funakoshi Co. Ltd., Tokyo, Japan).

To determine phenylpropanoids (chlorogenic acid) and flavonoids (kaempferol 3-*O*-rutinoside and rutin), the ground leaf samples (100 mg each) were briefly homogenized in the liquid nitrogen at room temperature in 0.75 ml of 84 mM acetate buffer (adjusted to pH 4.8 with ammonia) in 40% methanol (v/v) using a FastPrep FP120 instrument (Thermo Savant, Thermo Fisher Scientific, Pittsburgh, PA, USA) in the presence of 2.3-mm diameter zirconia beads. Samples were centrifuged for 20 min at 16,100 g, 4°C, and supernatants were collected. Pellets were re-extracted in 0.75 ml buffer containing 80% (v/v) methanol by shaking at RT for 15 min and centrifuged as before; supernatants were pooled with the first extracts. Aliquots (20 µl) of each sample were analyzed on a Shimadzu Prominence HPLC system equipped with a COSMOSIL 5C₁₈-MS-II packed column (4.6 mm×50 mm; Nacalai Tesque, Kyoto, Japan) in a gradient setup: solvent A (0.1% formic acid, 0.1% ammonia in water, pH 3.6) and solvent B (methanol); time (min)/B (%) gradient conditions: 0/2, 10/80, 15/80, 16/95, 21/95, 24/2, followed by a 6-min equilibration step. Flow rate was set to 1 ml/min and the column was maintained at 35°C. Chromatographic peaks of chlorogenic acid isomers and rutin were detected by an SPD-M20A diode array detector and compared with external calibration curves of authentic chlorogenic acid (Sigma-Aldrich) and rutin (Ishizu Pharmaceuticals Co. Ltd., Osaka, Japan) standards, respectively. Kaempferol 3-*O*-rutinoside content was estimated based on rutin external calibration.

Identification of phenolic acid and flavonoid compounds

Leaf extracts of *PAP1* lines originally prepared for HPLC analyses were used to identify the major UV-absorbing peaks in chromatograms. A 20-µl aliquot of leaf sample was first separated on HPLC as above, and peak fractions of major UV-absorbing compounds were collected using an automated fraction collector (FRC-10A, Shimadzu, Kyoto, Japan). Fractions from 10 runs were pooled, evaporated, re-dissolved in 70% methanol in water (v/v) and spun shortly before analysis. A 10-µl aliquot of each fraction

was subjected to measurement on a triple quadrupole liquid chromatography-tandem mass spectrometry (MS) system (LC-MS/MS 6410, Agilent Technologies, Santa Clara, CA, USA) equipped with a Zorbax SB-C18 column (2.1 mm id×50 mm, (1.8 µm), Agilent Technologies), maintained at 40°C and coupled to an MS detector operating in positive scan mode (*m/z* 100–1000; fragmentor at 135 V). Samples were passed through a UV-PDA detector before entering the MS electrospray ion source for reference and later identification of the peaks. Solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) were used in time (min)/B (%) gradient mode: 0/5, 0.5/5, 2/40, 6/40, 10/95, 15/95, 16/5, 20/5 at a constant flow rate of 0.4 ml/min. The mass spectra corresponding to UV-absorbing peaks in the chromatograms were analyzed and used to identify each compound. Only the rutin peak could be assigned against an authentic rutin standard (Ishizu Pharmaceutical Co., Ltd., Osaka, Japan), while the remaining peaks were putatively assigned based on their *m/z*, fragmentation properties and characteristic UV absorbance spectra.

Phytohormone analysis

Leaf hormone concentrations were determined according to the method described by Fukumoto et al. (2013) [27]. Approximately 100 mg of the ground leaf samples was further homogenized in liquid nitrogen and extracted in 1 ml of ethylacetate spiked with internal standards (IS): 25 ng d3-JA; 5 ng d3-JA-Ile; 10 ng d6-ABA and 10 ng d4-SA. After brief homogenization in a FastPrep instrument as above, samples were centrifuged at 4°C, 20 min at 16,100 g. Supernatants were stored and pellets re-extracted in 0.5 ml of ethylacetate without IS. After centrifugation, pooled supernatants were evaporated under reduced pressure in a rotary vacuum concentrator and re-suspended in 250 µl of 70% methanol/water (v/v). Ten-microliter aliquots were analyzed on a triple quadrupole LC-MS/MS 6410 equipped with a Zorbax SB-C18 chromatographic column [2.1 mm id×50 mm, (1.8 µm), Agilent Technologies] protected by a narrow bore guard column Zorbax SB-C8 [2.1 mm id×12.5 mm, (5 µm)]. Samples were chromatographically resolved using solvents A [0.1% formic acid in water (v/v)] and B [0.1% formic acid in acetonitrile (v/v)] used in the following gradient: time (min)/B (%) 0/15, 4.5/98, 12/98, 12.1/15, 18/15. Flow rate was set to 0.4 ml/min and column temperature maintained at 40°C. Mass transitions for each compound [hormone/Q1 precursor ion (*m/z*)/Q3 product ion (*m/z*)] were detected in the ESI-negative mode of the mass spectrometer: JA/209/59; JA-Ile/322/130; ABA/263/153; SA/137/93; d3-JA/212/59; d3-JA-Ile/325/130; d6-ABA/269/159; d4-SA/141/97. Hormone amounts were calculated from the ratio of endogenous hormone peak and known amount of IS spike, and adjusted to the exact fresh mass (FM) of the sample used for extraction. Replicated analyses were conducted with five independent leaf samples.

cDNA synthesis and quantitative PCR

Approximately 100 mg of the ground leaf samples was homogenized in liquid nitrogen, and total RNA was isolated from leaf tissues using NucleoSpin RNA Plant (Machery-Nagel, Düren, Germany) following the manufacturer's protocol. First-strand cDNA was synthesized using a ReverTra Ace qPCR RT Master mix (Toyobo, Osaka, Japan) and 0.5 µg of total RNA at 37°C for 15 min. Real-time PCR was performed on a Light Cycler Nano system (Roche Applied Science, Indianapolis, IN, USA) using FastStart Essential DNA Green Master (Roche Applied Science) and gene-specific primers (Table S1). The following protocol was used: initial polymerase activation: 60 s at 95°C; 40 cycles of 15 s

at 95°C and 45 s at 60°C; and then melting curve analysis preset by the instrument. Relative transcript abundances were determined after normalization of raw signals with housekeeping transcript abundances of *N. tabacum* *ELONGATION FACTOR 1 α* (*NtEF1 α* ; GenBank D63396). Replicated analyses were conducted with four to five independent leaf samples.

Statistical analysis

All experiments were repeated at least four times, and statistical analyses were performed using a Tukey's HSD test after a one-way ANOVA. A *P* value < 0.05 was considered statistically significant.

Results

Transgenic tobacco plants constitutively expressing *PAP1* genes are better able to defend themselves against herbivores than are non-transgenic tobacco plants

We evaluated the resistance of *PAP1* lines, constitutively expressing *Arabidopsis PAP1*, to the arthropod herbivore *S. litura*. Larvae were applied onto the leaves of potted WT, GUS and *PAP1* lines for 2 days (Figure 1). Larvae on the PAP-5 and PAP-8 lines had markedly lower weight gain compared to those on WT or GUS lines (*P* < 0.05). Larvae on another *PAP1* line (PAP-2) exhibited slightly, but not significantly, less weight gain compared to those on WT or GUS lines (*P* > 0.05).

Biosynthesis of flavonoids/phenylpropanoids in *PAP1* lines

Since *PAP1* has been shown to activate the phenylpropanoid/flavonoid pathway [28], we measured endogenous levels of their metabolites in *PAP1* lines. According to HPLC analysis supported by identification of the most abundant compounds by LC-MS/MS, there was increased accumulation of an anthocyanin compound (cyanidin-3-*O*-rutinoside) as well as of two other flavonoid compounds (kaempferol 3-*O*-rutinoside and rutin) and of phenylpropanoid compounds (chlorogenic acid isomers 1 and 2) in leaves of *PAP1* lines, relative to WT and GUS lines (Figure 2). PAP-8 leaves accumulated all of the above five compounds at the highest levels, unsurprisingly as this line was best able to defend itself against *S. litura* larvae (see Figure 1). Interestingly, *S. litura* infestation substantially diminished the increase of the concentration of these compounds in *PAP1* lines, whereas no significant

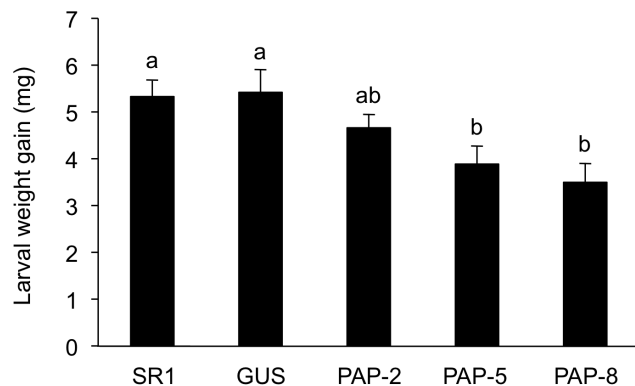


Figure 1. The net body weight that *Spodoptera litura* larvae gained during 4 days on wild-type (WT), GUS control and *PAP1* lines. Data are shown as the mean+standard errors (*n* = 26–32). Means followed by different small letters are significantly different (*P* < 0.05). doi:10.1371/journal.pone.0108849.g001

change (either increase or decreased) of those compounds was observed in both WT and GUS lines (Figure 2).

We therefore assessed the transcriptional pattern of flavonoid/phenylpropanoid pathway genes in leaves of *PAP1* lines not infested or infested with *S. litura* for 2 days (Figure 3). In comparison to GUS lines, PAP-8 showed significantly increased transcript levels of eight genes (*PALs*, *CHS*, *CHI*, *F3H*, *F3'H*, *ANS* and *DFR*) involved in phenylpropanoid and flavonoid biosynthesis. The increase of the transcript level was, however, diminished by 34, 60, 29, 39 and 29% of the initial increase in five cases, *CHS*, *CHI*, *F3H*, *ANS* and *DFR*, respectively, in *PAP1* lines in response to herbivory. *PAP1* was expressed similarly between uninfested and infested PAP-8 leaves. Moreover, we also determined the transcript levels of four genes responsible for producing endogenous transcription factors: MYBJS1 (another MYB transcription factor sharing transcriptional targets with *PAP1* in the phenylpropanoid pathway [18]), An2 (*PAP1* homologue MYB transcription factor [29]), and An1a and An1b (NtAn2-interacting, basic helix-loop-helix (bHLH) transcription factors [30]). An1a, An1b and An2 are known to be responsible for anthocyanin pigmentation in tobacco flowers [29,30]. The transcript of *MYBJS1* was induced similarly between GUS and PAP-8 leaves indicates that the *MYB* gene was not under the direct control of *PAP1*. *An2* was not expressed in either GUS or PAP-8 leaves, confirming what was previously known, namely, that this transcription factor is abundantly expressed in flowers but not in leaves [29]. On the other hand, although PAP-8 increased transcript levels of *An1a* and *An1b* transcript levels of GUS lines, the increase of the transcript levels was diminished by herbivory; this result was also observed in the structural genes mentioned above (see Figures 3 and S4 for 2 days and 4 days of exposure, respectively).

Upstream and downstream JA signaling for *PAP1*-activated genes

Since JA signaling along with other phytohormone signaling plays a central role in defense responses against chewing herbivores [31], it might be expected that induced JA production and signaling would be relevant to the low concentration of flavonoid/phenylpropanoid pathway products in *PAP1* lines attacked by *S. litura*. To assess whether *PAP1* overexpression affects JA biosynthesis and signaling, we analyzed endogenous levels of jasmonoyl-L-isoleucine (JA-Ile, an active form of JAs [32]) and of two other phytohormones: abscisic acid (ABA, involved in protective wound-healing processes [33]) and salicylic acid (SA, involved in pathogenesis and herbivore responses [34–36]). As shown in Figure 4A, JA and JA-Ile increased and accumulated at similar rates in the infested PAP-8 leaves compared to in the leaves of infested GUS lines. Those data indicate that *PAP1* is not an upstream transcription regulator for the biosynthesis of JA. Moreover, neither ABA nor SA was increased in leaves of GUS or PAP-8 lines upon infestation, indicating that neither of those two hormones is directly linked by *PAP1* actions.

Next, we investigated the gene expression levels in leaves of GUS and PAP-8 lines by applying an exogenous solution of MeJA (the commonly used active form of JAs [37]: 0.1 mM). Even though the *PAP1*-regulated transcript levels of *An1a*, *An1b* and *F3H* are negatively affected by herbivory (as shown in Figure 3), MeJA did not reduce their transcript levels to the same extent that it reduced transcript levels of *PAP1* (Figure 4B). The MeJA concentration used for our treatment was undoubtedly sufficient to induce defense responses in leaf tissues, as the transcript levels of a typical MeJA-inducible protease inhibitor (PIs [38]) and *MYBJS1* genes were substantially up-regulated in the same treated samples.

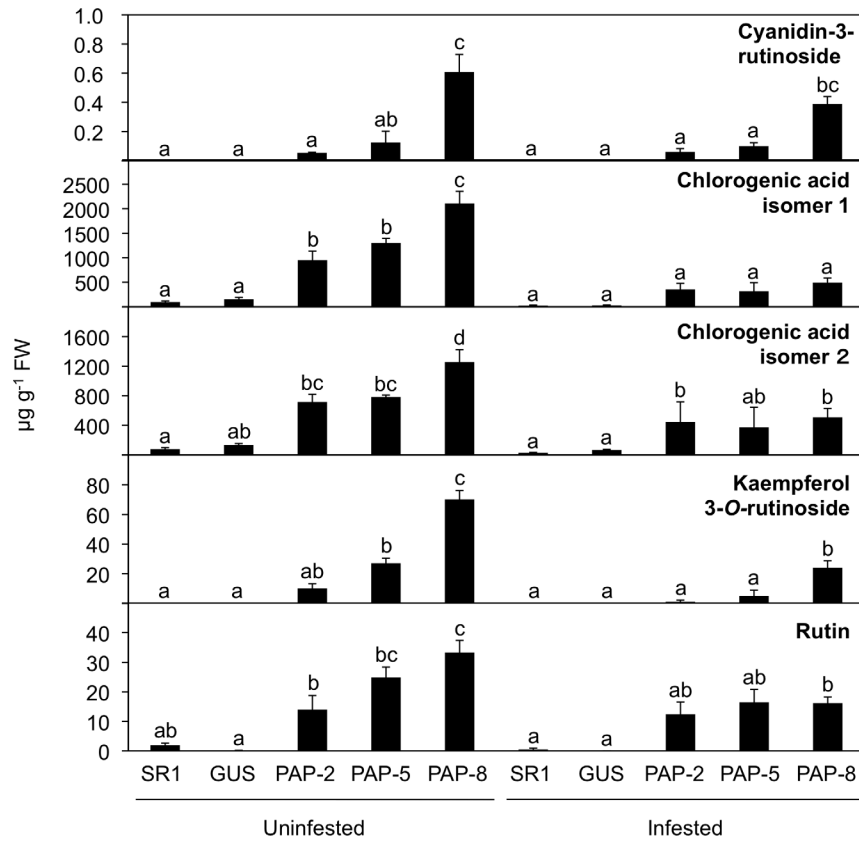


Figure 2. Endogenous accumulation of flavonoids/phenylpropanoids. Values for the major anthocyanin compound (cyanidin-3-O-rutinoside), other flavonoid (kaempferol 3-O-rutinoside and rutin) and phenylpropanoid (chlorogenic acid) compounds were determined in leaves of wild-type (WT), GUS control and *PAP1* lines not infested or infested with *Spodoptera litura* for 2 days. Data are shown as the mean+standard error ($n=5$). Means followed by different small letters are significantly different ($P<0.05$). doi:10.1371/journal.pone.0108849.g002

We therefore conclude that the down-regulation of *PAP1*-activated genes after herbivory is closely linked not to the JA signaling and biosynthesis cascades but, rather, to other herbivory-associated signals discussed below.

Reversible and transient regulatory patterns of *F3H* and *PI2* during herbivory and post-herbivory periods

To clarify the temporal regulation of *PAP1*-regulated genes, we analyzed the temporal patterns of the expression levels of *F3H* (*PAP1*-regulated) and *PI2* (JA-inducible) not only during 48 h after the first exposure to *S. litura* but also for up to 48 h after the larvae were removed (post-herbivory recovery; Figure 5). Consistent with the data shown in Figure 3, the regulation of the expression levels of *An1a*, *An1b* and *F3H* by *PAP1* was suppressed 48 h after the first exposure to *S. litura*. After the larvae were removed, the expression levels remained low during the first 8 h but then increased 24 h and 48 h after the damage had ended. In contrast, *PI2* and *PAP1* displayed different expression profiles: the expression level of *PI2* reached a maximum at 48 h after first exposure to *S. litura* but dropped to the basal level 8 h, 24 h and 48 h after the damage had ended, consistent with the defense function of the *PI2* protein; the expression level of *PAP1* was unchanged through the experimental period. Collectively, these results suggest that *F3H* might be regulated positively by an additional, JA-independent regulator(s) during the post-herbivory period.

Discussion

To date, light stimuli and abiotic stress have been reported to modulate anthocyanin concentrations in several plant taxa. For instance, in grape berries, and flowers of rose and *Brunfelsia calycina*, anthocyanin accumulation appears to be suppressed in response to high temperature [39–41]. Most impressively, Rowan et al. (2009) reported that abiotic stress treatments of high temperature and low light cause reductions in the concentrations of anthocyanins and down-regulation of the genes involved in anthocyanin biosynthesis in leaves of *PAP1*-overexpressing *Arabidopsis* plants [23]. The inhibition of transcription occurs independently of *PAP1*, and the loss of anthocyanins is not the result of an obvious catabolic enzyme regulated at the transcription level. The authors addressed whether the decreased flux of anthocyanins into the vacuole occurred as a consequence of a decline in the expression of biosynthetic genes by reducing the expression of three genes (*TT8*, *TTG1* and *EGL3*) of the *PAP1* transcriptional complex and enhancing the expression of the potential transcriptional repressors *AtMYB3*, *AtMYB6* and *AtMYBL2*. Those results especially reflect to our finding that transcript levels of both biosynthetic genes and endogenous transcription factor (positive regulator) genes were decreased in infested *PAP1* plants compared to those in the uninfested *PAP1* plants (Figure 3). *An1a* and *An1b* function by interacting with *An2* (*PAP1* homologue) to regulate anthocyanin biosynthesis [30], thus implying that those two factors can also interact with *Arabidopsis* *PAP1*. In fact, *An1a* and *An1b* transcripts were

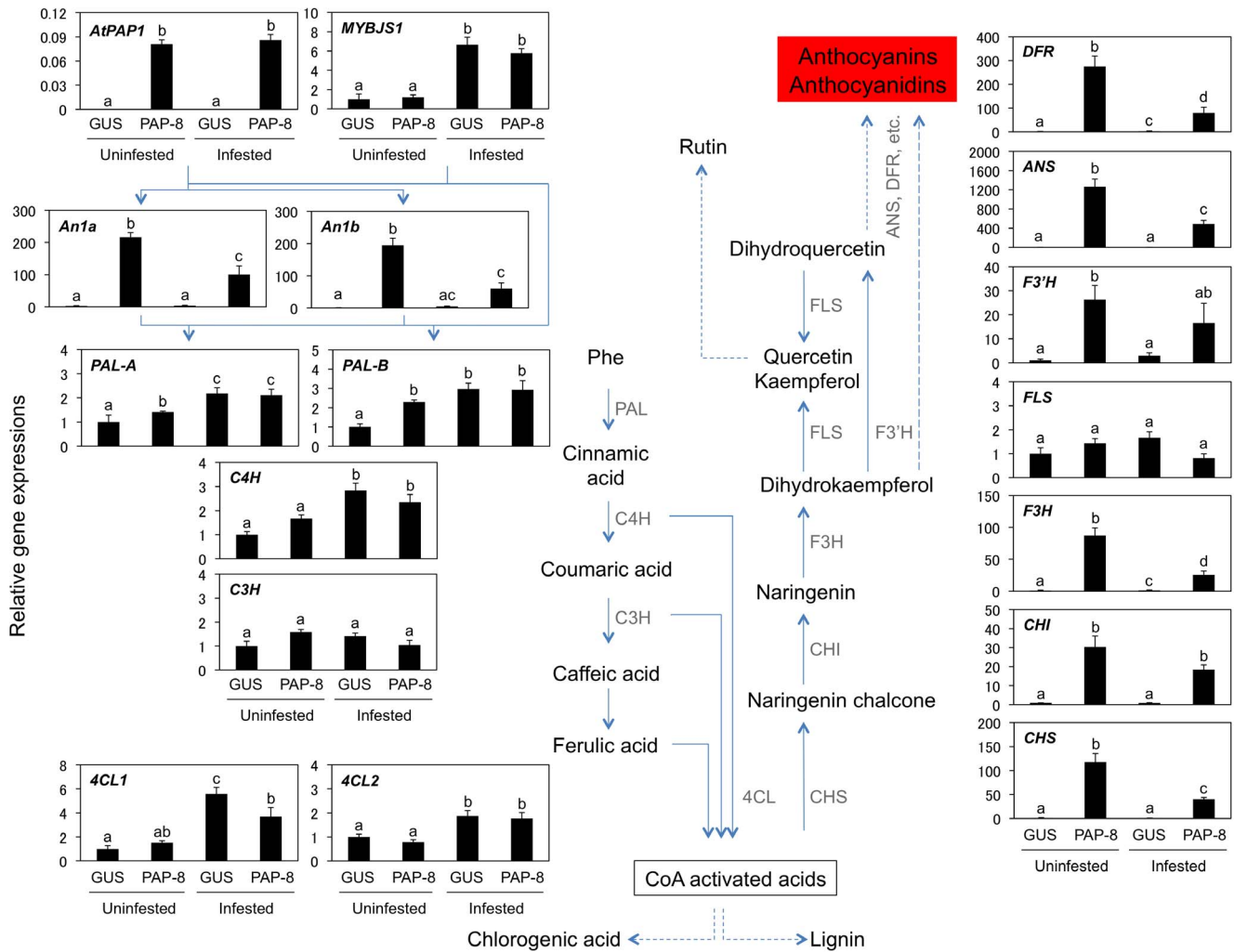


Figure 3. Expression of genes for transcription factors PAP1 and MYBJS1, An1a, An1b and structural genes involved in the flavonoid/phenylpropanoid pathway. Relative transcript levels of genes were determined in leaves of GUS control and PAP1 lines (PAP-8) not infested or infested with *Spodoptera litura* for 2 days. Transcript levels of genes were normalized by those of *NtEF1α*. Data are shown as the mean ± standard errors ($n=4-5$). Means followed by different small letters are significantly different ($P<0.05$). Expression of genes in GUS and PAP-8 leaves after 4 days of damage are presented in Figure S4. ANS, anthocyanidin synthase; C3H, cinnamate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; 4CL, 4-coumarate coenzyme A ligase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; FLS, flavonol synthase; PAL, phenylalanine ammonia-lyase. doi:10.1371/journal.pone.0108849.g003

enhanced in the overexpression of *PAP1*, suggesting that those two bHLH proteins are able to interact with foreign PAP1 genes and are involved in the feedback regulation by the MYB/bHLH complexes as reported in *Arabidopsis* [42]. The diminished expression of those two genes suppress the expression of structural genes – genes that are also suppressed by herbivory – and regulates the flux of flavonoid/phenylpropanoid (Figures 2 and 3). Presumably, in return, plants gain energy and the resources required for induced defense responses (so-called homeostasis and feedback regulation), which might eventually make plants better able to withstand to generalist herbivores.

We present here two important findings, namely, that *PAP1* lines that accumulated great amounts of flavonoid/phenylpropanoid pathway products defend against the generalist herbivore *S. litura*, and that the *PAP1*-stimulated accumulations of those compounds are attenuated by herbivory (Figure 6). The first finding is in line with a previous report that transgenic tobacco plants expressing *Arabidopsis* transcription factor *AtMYB12*

exhibit more resistance to *S. litura* and *Helicoverpa armigera* than do non-transgenic plants, most probably due to these plants' enhanced accumulation of rutin [13], a chemical that is toxic for those herbivores [43]. As rutin is one of predominant products in *PAP1* lines (Figure 2), this flavonoid compound is predicted to be a major anti-herbivore agent in our case as well.

The suppression of flavonoid/phenylpropanoid pathway products by herbivory is likely to reflect the phenomena described above, such as the response of *Arabidopsis* plants to abiotic stress, namely, the overexpression of *PAP1* [23]. In addition to the proposed reason for suppression (see above), we should consider an alternative (or additional) explanation, such as that PAP1 protein could be post-transcriptionally degraded under herbivory even though normal transcript levels of *PAP1* in PAP-8 leaves are maintained during herbivory (Figure 3). A similar mechanism is consistent with the repression of anthocyanin in the dark; in this case, the COP1/SPA ubiquitin ligase plays a critical role in the degradation of PAP1 proteins [22]. Although we conducted

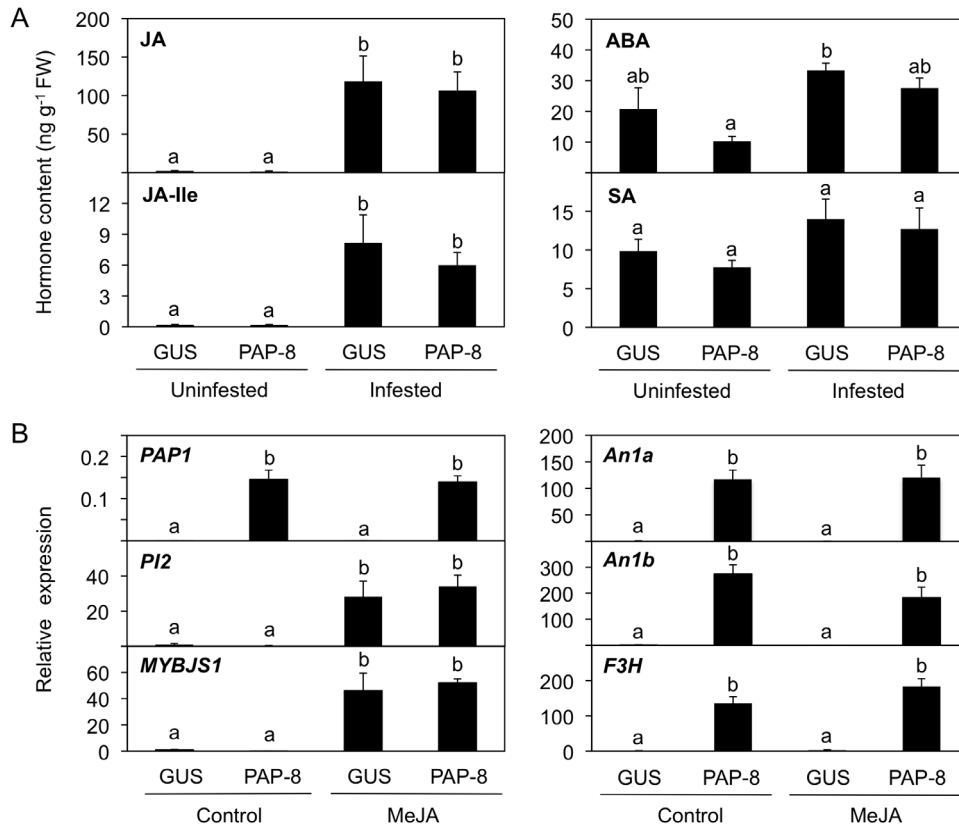


Figure 4. Upstream and downstream transcription regulation of PAP1-activated genes. A, Endogenous phytohormone levels were determined in leaves of GUS control and PAP1 lines (PAP-8) not infested or infested with *Spodoptera litura* for 2 days. ABA, abscisic acid; JA, jasmonic acid; JA-Ile, jasmonoyl-L-isoleucine; SA, salicylic acid. B, The effect of treatment with methyl jasmonate (MeJA) on gene expression was determined in leaves of GUS and PAP-8 lines treated with 0.1 mM MeJA (in 0.1% ethanol solution) for 24 h. Plants treated with 0.1% ethanol solution for 24 h served as controls. Transcript levels of genes were normalized by comparing them to those of *NtEF1 α* . Data are shown as the mean±standard error ($n=4-5$). Means followed by different small letters are significantly different ($P<0.05$). F3H, flavanone 3-hydroxylase; PI2, proteinase inhibitor 2. doi:10.1371/journal.pone.0108849.g004

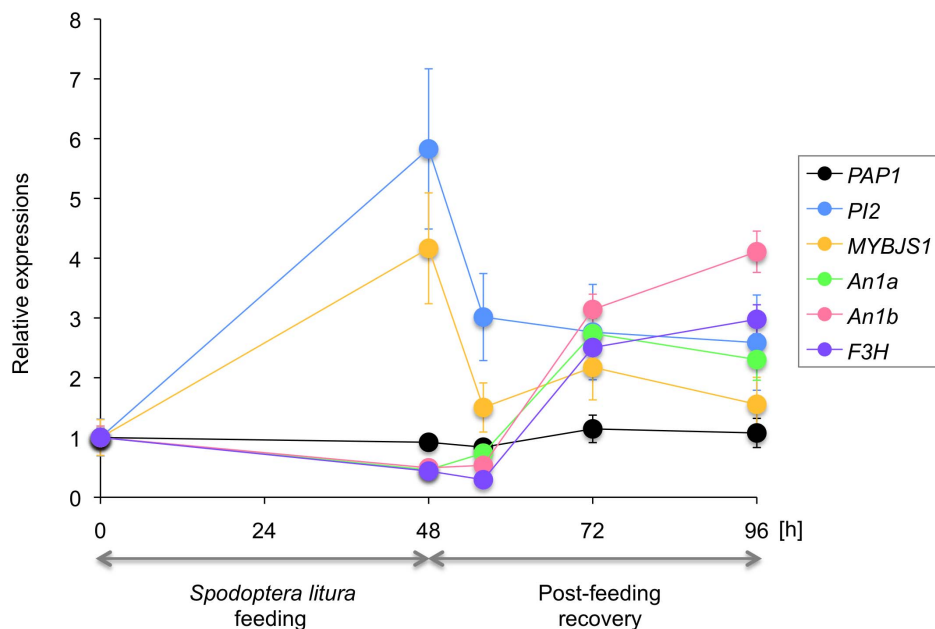


Figure 5. Temporal patterns of F3H and PI2 gene regulation in leaves of PAP1 lines (PAP-8) plants during exposure to *Spodoptera litura* for 48 h and for up to another 48 h after removal of the larvae (recovery period). Transcript levels of genes were normalized by comparing them to those of *NtEF1 α* . Data are shown as the means ± standard errors ($n=4-5$). doi:10.1371/journal.pone.0108849.g005

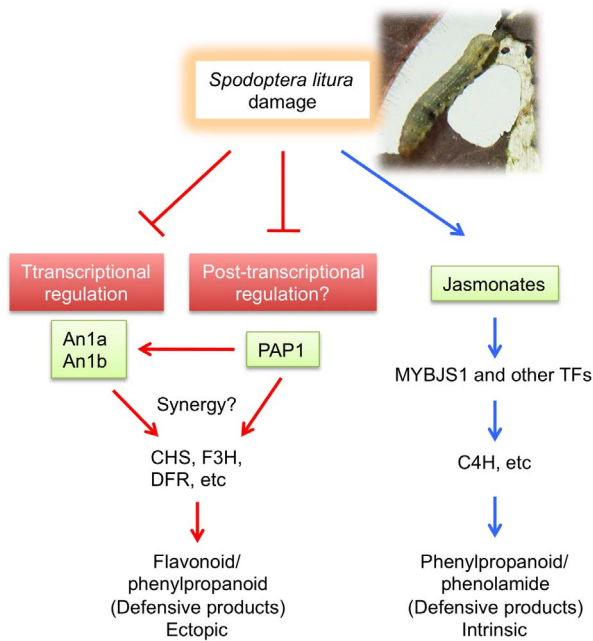


Figure 6. Schematic presentation of effect of *Spodoptera litura* feeding on the PAP1-regulated phenylpropanoid biosynthetic genes in a manner independent of jasmonate signaling. Arrows and bars indicate positive and negative interactions, respectively. TFs, transcription factors.

doi:10.1371/journal.pone.0108849.g006

immunoblot analysis using polyclonal antibodies against partial PAP1 peptide residues ([H]CKIKMKKRDITP[OH]) (produced by Sigma-Aldrich, Ishikari, Japan), our efforts unfortunately failed to detect sufficient PAP1 protein signals extracted from uninfested and infested PAP-8 leaves. Therefore, it remains to be clarified whether PAP1 degradation in response to herbivory is subject to similar mechanisms for light/dark responses observed in *Arabidopsis*.

Alternatively, the possibility that the general decrease observed in the concentration of phenolic compounds after two days of herbivory could be a result of an altered metabolic precursor allocation (competition) cannot be excluded. For example, in transgenic *N. attenuata* plants, the transient silencing of the expression of a hydroxycinnamoyl-CoA:shikimate/quininate hydroxycinnamoyl transferase (HCT) gene involved in lignin biosynthesis dramatically increased herbivory-induced phenolamides produced in the parallel metabolic branch [21]. It was proposed that a strong metabolic tension in the plant, exacerbated during herbivory, exists over the allocation of coumaroyl-CoA units among lignin and other phenolic compounds. This tension could be extended to anthocyanins and flavonoids, as shown in our experiments (see Figure 2), although the branches that are simultaneously enhanced have not yet been identified. In addition, the enhanced catabolism of some phenolics during herbivory stress could be responsible. Unfortunately, very little is known about the anthocyanins, kaempferol-3-*O*-rutinoside and rutin catabolism in plants, and the hypothesis that there is competition among metabolic precursors remains to be tested.

Generally, since JA-mediated defense signaling is predominantly activated when plants are attacked by chewing herbivores [31], it might be expected that induced JA production and signaling would be relevant to the defense responses to *S. litura* attack. Even though *PI2* expression was highly induced during herbivory and

exogenous MeJA application (see Figures 3 and 4), it was likely that the *PAP1*-activated genes were suppressed independently of the induction of JA and JA-Ile (an active form of JA) (Figure 4A). Moreover, since MeJA application also failed to suppress *PAP1*-activated genes in PAP-8 plants (Figure 4B), JA signaling is not likely a part of the down-regulation pathway. Also, in our case SA and ABA signaling pathways seem to be irrelevant in our case (see Figure 4A). Those facts may reflect a recent report that a mitogen-activated protein kinase (MPK4) suppresses a JA-independent defense pathway in *N. attenuata* in response to *Manduca sexta* feeding [44].

Moreover, the reverse mechanism was also likely used, given that *PAP1*-regulated *F3H* expression was dramatically increased in PAP-8 leaves during the post-herbivory period, when JA signaling was already switched off (Figure 5). This up-regulation might be caused by the fine-tuning of the *PAP1* protein level after the cessation of herbivory, resulting in increased *PAP1* levels beyond the basal level in the undamaged condition. Alternatively, additional signal(s) might be facilitated only when JA signaling is turned off, although these speculations require further investigation.

Horticultural ecosystems are very complicated and flexible. Hence, prior to setting genetically modified plants in the field, a wide range of systematic studies should be conducted to understand realistic horticultural ecosystems, where a myriad of plants, animals, and microorganisms interact and coevolve. However, it still remains to be elucidated how other multiple and simultaneous stresses can potentially influence flavonoid/phenylpropanoid catabolism in real agricultural settings.

Supporting Information

Figure S1 Relative mRNA levels of *PAP1* in leaves of wild-type (WT), *GUS* and *PAP1* lines. Transcript levels of genes were normalized by those of *NtEF1 α* . Data are shown as the mean+standard errors ($n = 5$). ND, not detected. (TIF)

Figure S2 Morphology of *PAP1* lines. A, 4-week-old plants; B, 8-week-old plants; C, flowers. (TIF)

Figure S3 *GUS* and *PAP-8* plants infested with *Spodoptera litura* for 2 days (A) and 4 days (B). (TIF)

Figure S4 Expression of genes in *GUS* and *PAP-8* leaves infested with *Spodoptera litura* for 4 days. Relative transcript levels of genes were determined in leaves of *GUS* control and *PAP1* lines (*PAP-8*) not infested or infested with *S. litura* for 4 days. Transcript levels of genes were normalized by comparing them to those of *NtEF1 α* . Data are shown as the mean+standard errors ($n = 4-5$). Means followed by different small letters are significantly different ($P < 0.05$). (TIF)

Table S1 RT-PCR primers used for this study. (XLSX)

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Author Contributions

Conceived and designed the experiments: TM MN IG KF NS KN GA. Performed the experiments: TM MN KA YH KF NS KN GA. Analyzed

the data: TM MN IG KA YH KF NS KN GA. Contributed reagents/materials/analysis tools: MN IG TS GA. Wrote the paper: MN IG GA.

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