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PII: S1674-2052(22)00219-2
DOI: https://doi.org/10.1016/j.molp.2022.06.014
Reference: MOLP 1384

To appear in: MOLECULAR PLANT

Received Date: 21 December 2021
Revised Date: 28 April 2022
Accepted Date: 27 June 2022


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Regulation of jasmonate signaling by reversible acetylation of TOPLESS in Arabidopsis

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ABSTRACT
The plant hormone jasmonate regulates plant immunity and adaptive growth through orchestrating a genome-wide transcriptional program. Key regulators of jasmonate-responsive gene expression include the master transcription factor MYC2, which are repressed by the conserved Groucho/Tup1-like co-repressor TOPLESS (TPL) in the resting state. However, the mechanisms underlying TPL-mediated transcriptional repression of MYC2 activity and hormone-dependent switching between repression and de-repression remain enigmatic. Here, we report the regulation of TPL activity through reversible TPL acetylation. The histone acetyltransferase GCN5 mediates TPL acetylation, which enhances its interaction with the NOVEL-INTERACTOR-OF-JAZ (NINJA) adaptor and promotes its recruitment to MYC2 target promoters, facilitating repression. Conversely, TPL deacetylation by the histone deacetylase HDA6 weakens TPL–NINJA interaction and inhibits TPL recruitment to MYC2 target promoters, facilitating activation. In a resting state, the opposing activities of GCN5 and HDA6 maintain TPL acetylation homeostasis, promoting TPL repression activity. In response to jasmonate elicitation, HAD6 expression is transiently induced, decreasing TPL acetylation and repressor activity, facilitating target gene activation. Thus, the GCN5–TPL–HDA6 module maintains the homeostasis of acetylated TPL, thereby determining the transcriptional state of jasmonate-responsive genes. Our findings uncovered a mechanism by which the TPL co-repressor activity in jasmonate signaling being actively tuned in a rapid and reversible manner.

Keywords: Jasmonate signaling, transcriptional repression, corepressor, TOPLESS, GCN5, HDA6, acetylation, deacetylation, histone acetylation

Short summary:
The histone acetyltransferase GCN5-mediated TPL acetylation enhances TPL–NINJA interaction and TPL recruitment to MYC2 target promoters, facilitating gene repression. On the contrary, histone deacetylase HDA6-mediated TPL deacetylation weakens TPL–NINJA interaction and TPL recruitment to MYC2 target promoters, facilitating gene activation. Thus, the GCN5–TPL–HDA6 module maintains the homeostasis of acetylated TPL, thereby determining the transcriptional state of jasmonate-responsive genes.
INTRODUCTION
Jasmonate (JA), a lipid-derived hormone, plays a central role in the regulation of plant defense against a broad spectrum of biotic and abiotic stresses. In addition, JA acts as a growth regulator by repressing vegetative growth and promoting reproductive development (Browse, 2009; Chini et al., 2016; Goossens et al., 2016; Howe et al., 2018; Wasternack and Hause, 2013). Underlying these juxtaposing physiological functions, JA orchestrates a genome-wide transcriptional program controlling resource allocation between defense- and growth-related processes, thus optimizing plant fitness according to an ever-changing and often hostile environment.

Decades of studies have elucidated a core JA signaling pathway consisting of multiple interconnected functional modules that govern the transcriptional state of hormone-responsive genes (Howe et al., 2018; Zhai et al., 2020). Among the best-studied JA-responsive transcription factors (TFs) are the basic helix-loop-helix protein MYC2 and related MYC TFs (Boter et al., 2004; Du et al., 2017; Fernandez-Calvo et al., 2011; Liu et al., 2019; Lorenzo et al., 2004). In the Arabidopsis (Arabidopsis thaliana) and tomato (Solanum lycopersicum) model systems, MYC2 acts as a master regulator and orchestrates a hierarchical transcriptional cascade that differentially regulates diverse aspects of defense- and growth-related JA responses (Chen et al., 2011; Dombrecht et al., 2007; Du et al., 2014; Du et al., 2017; Kazan and Manners, 2013; Liu et al., 2019; Zhai et al., 2020). In the resting (i.e., repressed) state of JA signaling, MYC TFs are repressed by a group of JASMONATE ZIM DOMAIN (JAZ) repressors (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). JAZ-mediated repression of MYC TFs is achieved through multiple mechanisms. First, JAZ proteins compete with the MED25 subunit of the Mediator coactivator complex for interaction with MYC TFs (An et al., 2017; Liu et al., 2019; Zhang et al., 2015). Second, JAZ proteins recruit TOPELESS (TPL), a conserved Groucho/Thymidine uptake 1 (Gro/Tup1) family corepressor, either directly through binding of TPL to the ethylene-response factor-associated amphiphilic repression (EAR) motifs of a subset of JAZ proteins (Shyu et al., 2012), or indirectly through binding of TPL to the EAR motif of the adaptor protein NOVEL-INTERACTOR-OF-JAZ (NINJA) (Pauwels et al., 2010). Third, JAZ proteins directly interact with several chromatin-associated Polycomb proteins to mediate repressive chromatin modification (H3K27me3) at JA-responsive genes, thus repressing their transcription (Li et al., 2021).
In response to internal or external cues, plants produce high levels of bioactive jasmonoyl-isoleucine (JA-Ile), which acts as molecular glue and promotes the formation of a coreceptor complex consisting of JAZ, JA-Ile and CORONATINE INSENSITIVE1 (COI1), the F-box subunit of the E3 ubiquitin ligase SCF\textsuperscript{COI1}. The SCF\textsuperscript{COI1}-dependent proteolysis of JAZ repressors leads to the liberation of MYC TFs (Sheard et al., 2010; Yan et al., 2009), which, in turn, recruit MED25 to activate the transcription of JA-responsive genes (An et al., 2017; Cevik et al., 2012; Chen et al., 2012; Zhai et al., 2020; Zhai and Li, 2019). Thus, transcriptional repression or hormone-dependent switching between repression and de-repression (i.e., activation) is a key regulatory mechanism underpinning JA signaling.

Despite these advances, how TPL represses JA-responsive gene expression and switches its activity between repression and de-repression in the absence or presence of JA, respectively, remains enigmatic. It is presumed that TPL epigenetically represses MYC TFs by recruiting repressive histone deacetylases (HDACs), including HDA6 and HDA19 (Howe et al., 2018; Pauwels et al., 2010; Wasternack and Hause, 2013; Zhu et al., 2011). However, this presumption contradicts previous findings of HDA6 and HDA19 playing a positive role in regulating JA-responsive gene expression (Wu et al., 2008; Zhou et al., 2005). Thus, the current understanding by which TPL represses JA signaling should be re-evaluated.

Here, we report on the mechanistic role of post-translational acetylation of TPL in the regulation of JA signaling. We show that histone acetyltransferase (HAT) GENERAL CONTROL NONREPRESSED 5 (GCN5)-mediated TPL acetylation enhances TPL–NINJA interaction and TPL recruitment to MYC2 target promoters, thereby facilitating gene repression. Conversely, HDA6-mediated TPL deacetylation weakens TPL–NINJA interaction and TPL recruitment to MYC2 target promoters, facilitating gene activation. Thus, the GCN5–TPL–HDA6 module maintains the homeostasis of acetylated TPL, thereby determining the transcriptional state of JA-responsive genes. Our findings provide new perspectives to the current understanding of corepressor/coactivator functions as follows: (1) TPL and TPL-related (TPR) proteins likely dictate transcriptional repression through a variety of mechanisms in different transcriptional programs; and (2) the substrates of various HAT transcriptional coactivators or HDAC transcriptional corepressors could be non-histone proteins, depending on the biological context.
RESULTS

HDA6 and HDA19 positively regulate JA-responsive gene expression independent of their HDAC activity

To re-evaluate the function of HDA6 and HDA19 in JA signaling, we examined the effect of mutations in *HAD6* and *HAD19* on the expression of several JA-inducible marker genes, including *JAZ8*, wounding-responsive *LOX2* and *VSP1* genes, and pathogen-responsive *ERF1* and *PDF1.2* genes (An et al., 2017; Chen et al., 2012; Dombrecht et al., 2007; Lorenzo et al., 2004; Zhai et al., 2013). The basal- and methyl jasmonate (MeJA)-induced expression levels of these marker genes were significantly reduced in both *HDA6* and *HDA19* loss-of-function mutants, *axe1-4* (Murfett et al., 2001) and *hda19-3* (Choi et al., 2012), respectively, compared with the corresponding wild types (WTs) (Figure 1A and Supplemental Figure 1A), confirming that HDA6 and HDA19 are indeed positive regulators of JA signaling. Moreover, we found that the expression of *HDA19* itself was transiently induced by MeJA (Supplemental Figure 1B) and that MeJA-induced gene expression was significantly lower in the *axe1-4 hda19-3* double mutant compared to its parental single mutants (Supplemental Figure 1C), indicating that HDA6 and HDA19 act redundantly to regulate JA-responsive gene expression.

We then evaluated whether the positive effect of HDA6 and HDA19 on JA-responsive gene expression depends on their HDAC activities. Given that both *axe1-4* and *hda19-3* showed increased global levels of histone H3 lysine 9 (K9) acetylation (H3K9ac) and H3K14ac (Supplemental Figure 1D and 1E), which are typical chromatin marks for gene activation (Benhamed et al., 2006; Earley et al., 2007), we examined whether the impaired expression of JA-responsive genes in these mutants was correlated with reduced H3K9ac and H3K14ac levels. Chromatin immunoprecipitation-quantitative polymerase chain reaction (ChIP-qPCR) assays revealed that the basal- and MeJA-induced levels of H3K9ac and H3K14ac in these JA-responsive marker genes were comparable between the two mutant lines and their corresponding WTs (Figure 1B and 1C, Supplemental Figure 1F and 1G), suggesting that the function of HDA6 and HDA19 in regulating JA signaling is independent of their HDAC activities.

**HDA6 positively regulates JA-responsive gene expression through TPL**
Next, we focused on HDA6 and tested whether its positive effect on JA signaling requires TPL. Given that the original dominant negative mutant tpl-1 was sensitive to temperature and displayed severe developmental defects at early seedling stage (Long et al., 2006), we generated an axel-4 tpl tpr1 tpr4 quadruple mutant by crossing axel-4 with the tpl tpr1 tpr4 triple mutant lacking TPL and two TPR genes (Zhu et al., 2010). In contrast to axel-4 that showed an impaired JA response, basal- and MeJA-induced expression levels of JA-responsive genes were elevated in the tpl tpr1 tpr4 triple mutant compared to the WT (Figure 1D). Because the axel-4 tpl tpr1 tpr4 quadruple mutant largely resembled the tpl tpr1 tpr4 triple mutant (Figure 1D), these analyses suggest that HDA6 acts through TPL to regulate JA-responsive gene expression.

**HDA6 interacts with and deacetylates TPL**

We then investigated the biochemical mechanism underlying the genetic interplay between HDA6 and TPL during JA signaling. In line with a previous report showing that HDA6 and TPL exist as a protein complex in vivo (Wang et al., 2013), HDA6-GFP interacted with TPL-myc in Agroinfiltrated Nicotiana benthamiana leaves (Supplemental Figure 2A), and HDA6-FLAG interacted with endogenous TPL in HDA6-FLAG #4 transgenic Arabidopsis plants (Supplemental Figure 2B and 2C), as shown by our coimmunoprecipitation (co-IP) assays. The HDA6–TPL interaction was further confirmed by in vitro pull-down assays, as the GST-TPL recombinant protein purified from Escherichia coli could pull down MBP-HDA6 (Supplemental Figure 2D). Together, these results suggest that HDA6 interacts with TPL both in vivo and in vitro.

The above results led us to speculate that HDA6 deacetylates TPL as a non-histone protein. To test this possibility, we investigated whether TPL undergoes in vivo acetylation, which usually occurs at the ε–amino group of lysine (Lys) residues (Narita et al., 2019). Protein extracts of TPL-GFP plants expressing the TPL-GFP fusion gene under the driven of TPL promoter (Long et al., 2006) and 35S::GFP plants were immunoprecipitated with GFP-trap beads and then probed with anti-acetyl-lysine antibody. Two bands with different molecular weight were detected in TPL-GFP plants but not in the 35S::GFP control plants (Figure 2A), suggesting that the TPL-GFP fusion protein was subjected acetylation in Arabidopsis plants. The TPL-GFP acetylation levels increased in TPL-GFP transgenic plants upon treatment with trichostatin A (TSA) (Figure 2B and Supplemental Figure 2E), an inhibitor
of HDACs (Yoshida et al., 1990). We also introduced the TPL-GFP fusion gene into the axe1-4 mutant background by crossing (Supplemental Figure 2F), and found that the basal- and MeJA-induced TPL-GFP acetylation levels were slightly, yet reproducibly, higher in the axe1-4 mutant than in the WT (Figure 2C and Supplemental Figure 2G). Interestingly, when the TPL-GFP fusion gene was transiently expressed in N. benthamiana leaves, only the higher molecular weight band could be detected by the anti-ac-Lysine antibody (Figure 2D), suggesting that, when being ectopically expressed in N. Benthamiana leaves, only the higher molecular weight form of the TPL-GFP fusion protein can be acetylated. The acetylation levels of TPL-GFP in N. benthamiana leaves co-expressing TPL-GFP and HDA6-FLAG were much lower than those in leaves co-expressing TPL-GFP and vector control (Figure 2D). Collectively, these results suggest a critical role for HDA6 in in vivo TPL deacetylation. Similarly, TPL-GFP acetylation levels were higher in the hda19-3 mutant than in the WT (Supplemental Figure 1H), suggesting that, as with HDA6, HDA19 also plays a role for TPL deacetylation in vivo.

To test whether HDA6 deacetylates TPL in vitro, we purified the GST-TPL recombinant protein from E. coli cultured with or without TSA. However, the purified GST-TPL protein showed very low levels of acetylation, and TSA treatment had a minor effect on GST-TPL acetylation (Supplemental Figure 2H). To circumvent the observation that TPL does not show high levels of acetylation in E. coli, we used the TPL-FLAG fusion protein immunoprecipitated from the TPL-FLAG #4 transgenic line (Supplemental Figure 2I) as a substrate for in vitro deacetylation assays, and found that the E. coli-produced GST-HDA6 recombinant protein could deacetylate plant-produced TPL-FLAG (Figure 2E). Taken together, these results confirm that HDA6 deacetylates TPL in vitro.

We then set out to understand how HDA6-mediated TPL deacetylation positively regulates JA signaling. Given that TPL is physically recruited by the NINJA adaptor to MYC2 target promoters (Pauwels et al., 2010), we reasoned that HDA6-mediated TPL deacetylation could affect the TPL–NINJA interaction. To test this, we generated NINJA-FLAG transgenic plants (Supplemental Figure 2J) and crossed the NINJA-FLAG fusion into the axe1-4 mutant background (Supplemental Figure 2J). Co-IP assays indicated that the basal- and MeJA-induced ability of NINJA-FLAG to pull down endogenous TPL was stronger in axe1-4 than in WT (Figure 2F and Supplemental Figure 2K), suggesting that HDA6-mediated TPL deacetylation weakens TPL–NINJA interaction. Consistently,
ChIP-qPCR assays indicated that the basal- and MeJA-induced enrichment of TPL-GFP on promoters of MYC2 direct transcriptional targets \((JAZ8, LOX2, \text{and } ERF1)\) (An et al., 2017) was substantially higher in \(axel-4\) than in WT (Figure 2G), suggesting that HDA6-mediated TPL deacetylation inhibits its recruitment to MYC2 target promoters. Collectively, our results suggest that HDA6-mediated TPL deacetylation weakens the TPL–NINJA interaction and TPL recruitment to MYC2 target promoters, thereby facilitating the release of MYC2 from TPL-orchestrated transcriptional repression.

**GCN5 negatively regulates JA-responsive gene expression through TPL**

HDA6 promotes JA-responsive gene expression by deacetylating TPL, suggesting the existence of acetyltransferase(s) that catalyze(s) the acetylation of TPL, thereby inhibiting JA-responsive gene expression. GCN5 negatively regulates JA signaling, but the underlying mechanism remains unknown (Vlachonasios et al., 2003). To determine this mechanism, we examined JA-responsive gene expression in the \(GCN5\) loss-of-function mutant \(hag1-6\) (Kornet and Scheres, 2009; Long et al., 2006). Both the basal- and MeJA-induced expression of JA-responsive marker genes was significantly elevated in \(hag1-6\) compared with the WT (Figure 3A), confirming that GCN5 is indeed a negative regulator of JA signaling.

To examine the genetic relationship between GCN5 and TPL in regulating JA-responsive gene expression, we generated the \(hag1-6\) \(tpl\) \(tpr1\) \(tpr4\) quadruple mutant by crossing \(hag1-6\) with \(tpl\) \(tpr1\) \(tpr4\). Basal- and MeJA-induced expression levels of JA-responsive genes were largely comparable between the \(hag1-6\) \(tpl\) \(tpr1\) \(tpr4\) quadruple mutant and the \(tpl\) \(tpr1\) \(tpr4\) triple mutant (Figure 3B), indicating that there is no additive effect between GCN5 and TPL in regulating JA-responsive gene expression. In addition, we found that overexpression of the \(TPL\)-GFP fusion gene in the \(hag1-6\) background partially rescued the JA-related phenotype of the mutant (Supplemental Figure 3A and 3B). Collectively, these results support that GCN5, like HDA6, acts through TPL to regulate JA signaling.

Considering that the \(hag1-6\) mutant showed reduced global levels of H3K9ac and H3K14ac (Supplemental Figure 3C), we examined whether the elevated expression levels of JA-responsive genes in this mutant were correlated with increased H3K9ac and H3K14ac levels. ChIP-qPCR assays showed that the basal- and MeJA-induced H3K9ac and H3K14ac levels in the analyzed JA-responsive
genes were significantly lower in *hag1*-6 than in WT (Figure 3C and 3D). Thus, the *hag1*-6 mutant displayed increased expression of JA-responsive genes but decreased H3K9ac and H3K14ac levels on these JA-responsive genes, suggesting that the negative effect of GCN5 on JA-responsive gene expression is independent of its HAT activity.

**GCN5 interacts with and acetylates TPL**

We then investigated the biochemical basis underlying the genetic interplay between GCN5 and TPL during JA signaling. Co-IP assays performed using *N. benthamiana* leaves indicated that GCN5-GFP could pull down TPL-myc (Figure 4A), and those conducted using the *GCN5-GFP* transgenic plants expressing the *pGCN5::GCN5-GFP* fusion gene (Kornet and Scheres, 2009) indicated that GCN5-GFP could pull down endogenous TPL (Figure 4B). We further performed *in vitro* pull-down assays with MBP-GCN5 and GST-TPL recombinant proteins purified from *E. coli*, and found that the GST-TPL fusion, but not GST, could pull down MBP-GCN5 (Figure 4C). These results collectively suggest that GCN5 interacts with TPL both *in vivo* and *in vitro*.

A possible biochemical outcome of the GCN5–TPL interaction is that GCN5 acetylates TPL. Indeed, in *in vitro* acetylation assays, an MBP-GCN5 recombinant protein, but not MBP, acetylated the GST-TPL recombinant protein in the presence of the acetyl donor acetyl-CoA (Figure 4D). In addition, acetylation levels of TPL-GFP in *N. benthamiana* leaves co-expressing *TPL-GFP* and *GCN5-FLAG* were much higher than those in leaves co-expressing *TPL-GFP* and a vector control (Figure 4E). Furthermore, we crossed the *TPL-GFP* fusion gene (Jeff et al., 2006) into the *hag1*-6 mutant background (Supplemental Figure 2F), and found that the basal- and MeJA-induced TPL-GFP acetylation levels were lower in *hag1*-6 than in WT (Figure 4F and Supplemental Figure 3D). Together, these results suggest that GCN5 acetylates TPL both *in vitro* and *in vivo*.

To understand how GCN5-mediated TPL acetylation represses JA signaling, we introduced the *NINJA-FLAG* fusion into the *hag1*-6 mutant background by crossing (Supplemental Figure 2J). Our repeated co-IP assays indicated that the basal- and MeJA-induced ability of NINJA-FLAG to pull down TPL was lower in *hag1*-6 than in the WT (Figure 4G and Supplemental Figure 3E), suggesting that GCN5-mediated TPL acetylation enhances the TPL–NINJA interaction. Consistently, ChIP-qPCR assays indicated that the basal- and MeJA-induced enrichment of TPL-GFP on MYC2 target promoters
was substantially lower in hag1-6 than in the WT (Figure 4H), suggesting that GCN5-mediated TPL acetylation facilitates the recruitment of this corepressor to MYC2 target promoters. Collectively, our results indicate that GCN5-mediated TPL acetylation enhances the TPL–NINJA interaction and TPL recruitment to MYC2 target promoters, thereby facilitating TPL-mediated repression of JA-responsive genes.

**TPL acetylation at K689 enhances its corepressor activity by promoting TPL–NINJA and TPL–JAZ8 interactions**

To identify acetylation sites in TPL, we extracted total protein from TPL-GFP transgenic plants (Long et al., 2006) by affinity purification, and analyzed the acetylation sites of TPL-GFP by mass spectrometry. The potential acetylation sites identified in TPL were K148, K531, and K689 (Figure 5A, Supplemental Figure 4A, 4B and 4C). Because K-to-arginine (R) substitutions are known to block acetylation without affecting the positive charge of the residues (Barlev et al., 2001), we substituted the K residue at each potential TPL acetylation site with R, generating three TPL variant proteins (TPLK148R, TPLK531R, and TPLK689R). Subsequent in vivo acetylation assays in N. benthamiana leaves revealed that each K-to-R substitution considerably reduced TPL acetylation (Figure 5B), confirming that K148, K531, and K689 are all TPL acetylation sites.

To determine the functional significance of amino acid substitutions at the TPL acetylation sites, we examined their effects on TPL interactions with NINJA and the EAR motif-containing JAZ8 protein (Pauwels et al., 2010; Shyu et al., 2012). Split-luciferase complementation imaging (LCI) assays in N. benthamiana leaves (Chen et al., 2008) indicated that the K689R substitution, but neither K148R nor K531R, significantly reduced the TPL–NINJA (Figure 5C) and TPL–JAZ8 (Supplemental Figure 4D) interactions. These results were corroborated by co-IP assays in N. benthamiana leaves (Figure 5D and Supplemental Figure 4E). Together, these results suggest that TPL acetylation at K689 enhances its interaction with both NINJA and JAZ8.

We then used tobacco leaf transient expression assays to evaluate whether and how the K689R substitution affects GCN5-mediated acetylation of TPL. Co-expression of TPL-GFP with GCN5-FLAG led to abundant TPL-GFP acetylation (Supplemental Figure 4F), whereas co-expression of TPLK689R-GFP with GCN5-FLAG only led to marginal TPLK689R-GFP acetylation (Supplemental
Figure 4F), suggesting that the K689R substitution substantially impaired GCN5-mediated acetylation of TPL. These results suggest that GCN5 acetylates K689 of TPL to modulate JA-responsive gene expression.

In a similar approach, we evaluated whether and how the K689R substitution affects HDA6-mediated deacetylation of TPL. Co-expression of HDA6-FLAG with TPL-GFP led to a marked reduction of TPL-GFP acetylation (Supplemental Figure 4G). When TPL\textsuperscript{K689R}-GFP was co-expressed with a vector control, the detected TPL-GFP acetylation was already low (Supplemental Figure 4G), although co-expression of TPL\textsuperscript{K689R}-GFP with HDA6-FLAG led to a further reduction of TPL-GFP acetylation, this reduction is obviously less than that of the WT version of TPL-GFP (Supplemental Figure 4G). These results suggest that HDA6 likely deacetylates K689 of TPL to modulate JA-responsive gene expression.

We then used the well-established dual-luciferase (LUC) reporter system (Hellens et al., 2005) to determine whether TPL acetylation at K689 affects its transcriptional corepressor activity. We compared the effects of TPL and its K-to-R substitutions on MYC2-regulated expression of the \textit{pLOX2::LUC} reporter construct (You et al., 2019). Co-expression of MYC2 with \textit{pLOX2::LUC} in \textit{Arabidopsis} protoplasts significantly increased LUC activity (Figure 5E and 5F), suggesting that MYC2 activates the expression of \textit{pLOX2::LUC}. When TPL was co-expressed with MYC2 and the \textit{pLOX2::LUC} reporter, MYC2-mediated activation of LUC activity was markedly reduced (Figure 5E and 5F), suggesting that TPL inhibits MYC2-mediated activation of \textit{pLOX2::LUC} expression. In parallel experiments, the ability of TPL\textsuperscript{K689R} to inhibit MYC2-induced \textit{pLOX2::LUC} expression was significantly lower than that of TPL (Figure 5E and 5F), whereas the ability of TPL\textsuperscript{K148R} and TPL\textsuperscript{K531R} to repress the MYC2 transcriptional activity was comparable to that of TPL (Figure 5E and 5F). Together, these results suggest that TPL acetylation at K689 enhances its corepressor activity on MYC2.

Next, to test the significance of K689 acetylation for the function of TPL in JA signaling, we introduced the \textit{TPL-GFP} and \textit{TPL\textsuperscript{K689R}-GFP} fusion genes into the \textit{tpl-1} mutant, and examined the developmental phenotype and MeJA response of the resulting transgenic plants (Supplemental Figure 4H). Both \textit{TPL-GFP} and \textit{TPL\textsuperscript{K689R}-GFP} fully rescued the seedling growth defects of the \textit{tpl-1} mutant (Supplemental Figure 4I). In line with the negative role of TPL in JA signaling, \textit{tpl-1} plants displayed
increased JA sensitivity in term of JA-responsive gene expression and JA-mediated root growth inhibition (Figure 5G and 5H). As expected, these JA response defects of the tpl-1 mutant were fully rescued by TPL-GFP but only partially rescued by TPLK689R-GFP (Figure 5G and 5H), indicating that K689 acetylation is important for the function of TPL in regulating different aspects of JA responses.

To evaluate whether the K689R substitution of TPL affects its recruitment to MYC2 target promoters, we performed ChIP-qPCR assays by using the resulted transgenic plants containing the TPL-GFP or TPLK689R-GFP fusion genes in the background of tpl-1 (Supplemental Figure 4H and 4I). Results showed that the K689R substitution significantly reduced the enrichment of TPL-GFP on MYC2 target promoters (Figure 5I). As control, the K689R substitution did not affect the enrichment of TPL-GFP on the promoter of SMXL7 (Figure 5I), which encodes a repressor of strigolactone-responsive gene expression (Plant et al., 2021). These results were consistent with the findings that the K689R substitution weakens TPL-NINJA and TPL-JAZ8 interactions (Figure 5C, 5D, Supplemental Figure 4D and 4E). Collectively, these data revealed that TPL acetylation at K689 is important for its repressive function in JA signaling.

The GCN5–TPL–HDA6 module regulates dynamic TPL acetylation during JA signaling

Our results suggest that GCN5-mediated TPL acetylation constitutes a turn-off switch for JA-responsive gene expression, whereas HDA6-mediated TPL deacetylation constitutes a turn-on switch. To understand how these switches function during JA signaling, we compared MeJA-induced regulation of GCN5 and HDA6 expression with that of TPL acetylation. GCN5 transcript levels were high in the resting state and remained stable upon MeJA treatment (Figure 6A). In contrast, HDA6 transcript levels increased transiently, reaching a peak at 1 h post-MeJA treatment, followed by a gradual decline to basal levels after ca. 24 h (Figure 6B). Protein gel analysis using the HDA6-myc/axe1-4 transgenic plants (expressing the pHDA6::HDA6-myc fusion gene in the background of axe1-4, Li et al, 2015) indicated that the HDA6-myc fusion protein was also transiently up-regulated upon MeJA treatment (Supplemental Figure 5A). Consistently, ChIP-qPCR assays using the HDA6-myc/axe1-4 plants revealed that the enrichment of the HDA6-myc fusion on MYC2 target promoters was significantly elevated upon MeJA treatment (Supplemental Figure 5B). Notably, the MeJA-induced expression pattern of HDA6 correlated with that of TPL acetylation levels, which transiently
decreased at 1 h post-MeJA treatment and then gradually increased, reaching basal levels after ca. 24 h (Figure 6C). These observations suggest that HDA6, rather than GCN5, is the major factor that determines the dynamics of TPL acetylation during the activation of JA signaling.

Next, we investigated whether the GCN5- and HDA6-mediated reversible TPL acetylation is involved in plant response to Botrytis cinerea, a necrotrophic pathogen that triggers JA-mediated plant immunity (Glazebrook, 2005; Howe et al., 2018; Mengiste, 2012; Pieterse et al., 2012). When WT plants were inoculated with B. cinerea, GCN5 transcript levels remained stable, but those of HDA6 showed a transient induction pattern, which correlated well with the pathogen-triggered kinetics of TPL acetylation levels (Figure 6D, 6E and 6F). Consistent with the positive role of HDA6 in regulating JA signaling, B. cinerea-infected axel-4 plants showed reduced defense gene (ERF1 and PDF1.2) expression levels and larger leaf lesions compared with the WT (Figure 6G, 6H and 6I). In contrast, hag1-6 and the tpl tpr1 tpr4 triple mutant displayed elevated defense gene expression levels and smaller leaf lesions compared with the WT (Figure 6G, 6H and 6I). Collectively, these results suggest that the GCN5–TPL–HDA6 module for TPL acetylation functions in plant immunity to B. cinerea infection.

DISCUSSION
Sustained activation of defense responses is metabolically expensive (Lacchini and Goossens, 2020). Therefore, plants, being sessile, have evolved a tight control of defense responses for normal growth and development. A key strategy used to control stress-related gene expression is the application of transcriptional corepressors capable of switching the defense responses between repression and activation in response to the changing environmental conditions. In the current study, we discovered an acetylation-dependent regulatory mechanism governing the function of TPL in JA signaling, which provides insights into how TPL actively switches its activity between repression and activation in a rapid and reversible manner.

Similar to their animal counterparts Gro and transducin-like enhancer of split (TLE), the plant TPL family corepressors are physically recruited by a myriad of transcriptional repressors to repress the expression of genes regulating diverse biological processes steered by developmental and environmental signals (Causier et al., 2012; Kagale and Rozwadowski, 2011; Krogan and Long, 2009;
Lee and Golz, 2012; Liu and Karmarkar, 2008; Plant et al., 2021). Despite the widespread importance of TPL/TPR-mediated repression, relatively little is known about the mechanisms behind it. By analogy to Gro/TLE, TPL family members are generally considered to repress gene expression by recruiting HDACs to gene promoters, resulting in chromatin compaction and reduced accessibility to the transcription machinery (Agarwal et al., 2015; Causier et al., 2012; Kagale and Rozwadowski, 2011; Krogan and Long, 2009; Lee and Golz, 2012; Liu and Karmarkar, 2008; Plant et al., 2021; Turki-Judeh and Courey, 2012). Although this presumption was indirectly supported by the meristem defect of the tpl-1 mutant, which was enhanced by hda19 mutants and suppressed by gcn5 mutants (Long et al., 2006), it was contradicted by early studies showing that HDA6 and HDA19 positively regulate JA signaling (Wu et al., 2008; Zhou et al., 2005), suggesting that TPL family corepressors could repress gene expression through diverse biochemical mechanisms in different transcriptional programs.

Indeed, our study revealed that the TPL protein undergoes post-translational acetylation, and that its acetylation status determines its corepressor activity. In the resting state of JA signaling, TPL exists primarily in an acetylated status to inhibit the activation of JA signaling. The opposing activities of GCN5 and HDA6 maintain TPL acetylation homeostasis to ensure its abundant recruitment to MYC2 target promoters, keeping JA-mediated defense responses tightly in-check (Figure 6J). In response to intrinsic and extrinsic stimuli that lead to elevated hormone levels, HDA6, but not GCN5, is transiently induced, leading to decreased TPL acetylation levels and corepressor activity, which facilitates the activation of JA-mediated defense responses (Figure 6K). When HDA6 expression is restored to basal levels, TPL acetylation and deacetylation reach equilibrium again, and the system returns to the resting state. We further provide evidence showing that acetylation of TPL at the K689 residue enhances its interaction with NINJA and JAZ8, thus increasing its transcriptional corepressor activity.

Our findings show how the TPL acetylation status and corepressor activity are actively and dynamically regulated during JA signaling (Figure 6J and 6K). The transient increase in HDA6 expression following hormone elicitation or pathogen infection is expected to rapidly reduce TPL acetylation to proper levels while preventing excessive reduction of TPL corepressor activity, thereby achieving a swift, but not exaggerated, activation, of JA-mediated defense responses. GCN5 expression stays stable in response to hormone elicitation or pathogen infection, which likely suggests that plants tend to maintain TPL acetylation homeostasis in a resting state, thereby favoring the
repression of JA-mediated defense responses and the maintenance of normal growth and development.

That GCN5 expression keeps stable could provide another layer of tight feedback control, for instance when HDA6 expression goes down again because no more JA is produced (i.e., the stress has gone), then immediately TPL is acetylated again and can operate at maximum capacity to repress defense gene expression. Thus, the GCN5–TPL–HDA6 regulatory module involved in reversible TPL acetylation enables plants to swiftly fine-tune JA-mediated defense responses in response to fluctuating environmental conditions.

Our findings raised the important question of how TPL acetylation affects its corepressor activity. Recent structural studies revealed that the N-terminal TOPILESS Domain (TPD) of the TPL family proteins forms a highly conserved tetramerization interface, which is critical for the interaction of TPL with the EAR repression motif-containing proteins, nucleosomes and histones (Ke et al., 2015; Ma et al., 2017; Martin-Arevalillo et al., 2017). Thus, it is reasonable to speculate that TPL acetylation at K689 may lead to conformational changes in the TPL protein, thereby affecting its binding affinity to the recruited repressors/adaptors, nucleosomes and histones. Alternatively, both yeast TUP1 and TPL have been reported to block the recruitment of the RNA polymerase II transcription machinery, possibly through interacting with the conserved MED21 subunit of the Mediator coactivator complex (Gromoller and Lehming, 2000; Ito et al., 2016; Leydon et al., 2021; Papamichos-Chronakis et al., 2000). In this regard, TPL acetylation could affect its corepressor activity by regulating its interaction with the Mediator coactivator complex. A combination of genetic, molecular, biochemical, and structural studies is expected to provide further insights into how the TPL acetylation status regulates its corepressor activity.

Likewise, our results also raised the question of how the specificity of the role of TPL acetylation is determined. It is known that TPL interacts with a large number of transcriptional repressors, including the auxin signaling repressor IAA12 (Szemenyei et al., 2008) and the strigolactone signaling repressor SMXL7 (Plant et al., 2021). Notably, in our split-LUC assays, none of the three TPL substitutions (i.e., TPL K148R, TPL K531R, and TPL K689R) showed significant effect on TPL interactions with IAA12 (Supplemental Figure 4J) or SMXL7 (Supplemental Figure 4K). In the context that the highly conserved TPD of TPL family proteins is involved in their interaction with the EAR motif of a wide spectrum of repressors, our results suggest that TPD could show distinct binding affinities to
different repressors. In line with this, recent structural studies showed that the binding affinities of OsTPR2 TPD to different Arabidopsis and rice EAR motif peptides were differed by ~100-fold (Ke et al., Science Advances, 2015). This implies that although the three leucine (L) resides of the highly conserved EAR motif (LxLxL) are important for binding, the flanking residues or other parts of repressor proteins are also important contributors to the binding affinities to TPL family proteins. Indeed, it was found that the rice strigolactone signaling repressor D53 contains two EAR motifs: the universally conserved EAR-3 and the monocot-specific EAR-2 (Ma et al., 2017). Structural studies revealed that the monocot-specific D53 EAR-2 motif interacts with two separate binding sites in the TPR2 TPD (Ma et al., 2017). In this perspective, our findings (i.e., TPL acetylation at K689 specifically affects its interaction with NINJA but not IAA12 and SMXL7) suggest that both TPL and IAA12/SMXL7 must contain yet to be identified motifs that are important for corepressor–repressor interaction. Future structural studies by using full length TPL/TPR proteins and their interacting repressors should provide insight into the mechanisms by which TPL/TPR proteins bind context-specific repressors.

Notably, our findings have important implications for the mechanistic roles of the evolutionarily conserved HAT coactivators and HDAC corepressors in transcriptional regulation. It is generally believed that HAT-mediated histone acetylation is directly connected with transcriptional activation, whereas HDAC-mediated histone deacetylation is associated with transcriptional repression (Helmlinger and Tora, 2017; Hollender and Liu, 2008; Kumar et al., 2021; Lee and Workman, 2007; Pandey et al., 2002; Shahbazian and Grunstein, 2007; Spedale et al., 2012). Consistent with this scenario, Arabidopsis GCN5 has been shown to act as a coactivator of multiple transcriptional programs via its intrinsic HAT activity (Chen and Tian, 2007; Dong et al., 2021; Kim et al., 2018; Kornet and Scheres, 2009; Kumar et al., 2021; Li et al., 2019; Vlachonasios et al., 2003). However, the histone acetylation activation presumption alone cannot explain the fact that GCN5 functions as both a positive and negative regulator of transcription (Vlachonasios et al., 2003), suggesting that GCN5 plays HAT-independent roles in transcriptional regulation.

In support of the view that GCN5 performs other functions in addition to its role as a HAT, we demonstrated that GCN5 exhibits potent non-histone protein acetyltransferase activity, and GCN5-mediated TPL acetylation can markedly increase its corepressor activity to inhibit JA-responsive gene
expression, which is opposite to its presumed HAT activity (i.e., coactivator). In a similar manner, we demonstrated that HDA6 possesses a potent non-histone protein deacetylase activity and that HDA6-mediated TPL deacetylation plays a positive role in JA-responsive gene expression, which is opposite to its presumed HDAC activity (i.e., corepressor). The non-histone protein deacetylase activity of HDA6 is further supported by a previous study showing that the GSK3-like kinase BIN2 is deacetylated by HDA6 (Hao et al., 2016). It was recently shown that OsGSK2, the rice BIN2 homolog, interacts with and phosphorylates OsJAZ4 and partially inhibits OsJAZ-OsNINJA interaction, thereby positively regulates JA signaling in rice (He et al., 2020). In the context that HDA6-mediated deacetylation of TPL weakens TPL-NINJA/JAZ interaction and facilitates JA-responsive gene expression, it is of significance to investigate whether and how HDA6-mediated BIN2 acetylation regulates JA signaling in Arabidopsis. In addition, the multi-talented HDA6 can act as a H3K18ac eraser (Wang et al., 2021) and can regulate mRNA polyadenylation (Lin et al. 2020), it is of significance to determine whether and how these multiple biochemical activities of HDA6 regulate JA-responsive gene expression.

Therefore, our findings add an important perspective to the current understanding of the molecular mechanisms employed by acetyltransferase coactivators and deacetylase corepressors to regulate gene transcription, by implicating that histone-independent functions may operate instead of or in addition to their HAT and HDAC functions, respectively. Thus, a presumed HAT or HDAC may be recruited by gene-specific transcriptional regulators (i.e., TFs or cofactors including coactivators and corepressors) to induce (de)acetylation modifications of either the transcriptional regulators themselves or the histones at the local chromatin. In this context, our work reinforces the notion that HAT/HDAC-mediated activation/repression mechanisms must be considered on a case-by-case basis, depending on the specific biological context.

METHODS

Plant materials and growth conditions

Arabidopsis thaliana and Nicotiana benthamiana plants were grown in soil at 22°C and 70% relative humidity under a long-day (LD; 16-h light/8-h dark) photoperiod. The hag1-6 (Vlachonasios et al., 2003), axe1-4 (Murfett et al., 2001), hda19-3 (Choi et al., 2012), and tpl tpr1 tpr4 (Zhu et al., 2010)
mutants in Columbia-0 (Col-0) background have been previously described. The hag1-6 tpl tpr1tpr4 and axe1-4 tpl tpr1 tpr4 quadruple mutants were generated by crossing tpl tpr1tpr4 with hag1-6 or axe1-4, respectively, and homozygous plants were selected by genotyping. The tpl-1 (Long et al., 2006) dominant negative mutant, in the Landsberg erecta (Ler) background and transgenic lines, including pTPL::TPL-GFP (Long et al., 2006), pGCN5::GCN5-GFP (Kornet and Scheres, 2009), pHDA6::HDA6-myc (Li et al., 2015) have been described previously. HDA19-FLAG/hda19-3 plants are transgenic plants in the hda19-3 background expressing a pHDA19::HDA19-FLAG fusion (FLAG-tagged HDA19 driven by a 1.5-kb HDA19 promoter) that can rescue the JA- and salicylic acid-related phenotype of the hda19-3 mutant (Choi et al., 2012). The 35S::TPL-FLAG, 35S::NINJA-FLAG and 35S::HDA6-FLAG constructs were introduced into Col-0 plants to generate TPL-FLAG, NINJA-FLAG, and HDA6-FLAG transgenic plants, respectively. The TPL-GFP (Long et al., 2006), and NINJA-FLAG cassettes were introduced into the axe1-4, hda19-3 or hag1-6 background via crossing. The 35S::TPL-GFP was transformed in hag1-6 heterozygous. Homozygous lines were obtained by genotyping using allele-specific primers (Table S1). The TPL promoter-driven TPL- and TPLK689R-GFP fusion constructs were transformed into the tpl-1 background to generate complementation lines and conduct functional analyses.

To perform Co-IP and RT-qPCR assays, Arabidopsis seedlings were grown on half-strength Murashige and Skoog (1/2 MS) medium at 22°C under a LD photoperiod for 10 days post-germination. To conduct Botrytis cinerea inoculation assays, Arabidopsis plants were grown in soil (vermiculite:nutrition soil ratio of 1:1) at 22°C under a short-day (10-h light/14-h dark) photoperiod for 4 weeks, and inoculated with B. cinerea as previously described (You et al., 2019).

**Plasmid construction and plant transformation**

To generate constructs for recombinant protein production, coding sequences (CDSs) of GCN5 and HDA6 were cloned into the pMAL-c2X vector (NEB) to create MBP-GCN5 and MBP-HDA6 fusion genes, respectively; TPL and HDA6 CDSs were cloned into the pGEX4T-3 vector (Amersham Biosciences) to create GST-TPL and GST-HDA6 fusion genes, respectively. To generate constructs for split-luciferase assays, CDSs of TPL, NINJA, and JAZ8 were cloned into pCAMBIA1300-nLUC or pCAMBIA1300-nLUC (Chen et al., 2008). To conduct transcriptional repression activity assays in Arabidopsis protoplasts, the LOX2 promoter, MYC2 CDS, and TPL CDS were cloned into the pGreenII
0800-LUC (You et al., 2019), pUC19-35S-HA-RBS (You et al., 2019), and pSuper1300-FLAG (Ni et al., 1995; Yang et al., 2010) vector, respectively. To carry out transient expression studies in N. benthamiana leaves, the GCN5, HDA6, JAZ8, NINJA, and TPL CDSs were cloned into the pSuper1300-FLAG vector (Ni et al., 1995; Yang et al., 2010). The 35S::TPL-GFP construct was generated by the LR reaction into pK7WG2F2.0 (Karimi et al., 2002).

To conduct genetic complementation assays of tpl-1, a 5,821-bp DNA fragment containing the native promoter and CDS of TPL was cloned into the pCAMBIA1300-GFP vector (An et al., 2017). CDSs of GCN5, HDA6, JAZ8, NINJA, and TPL were cloned into the pSuper1300-FLAG vector (Ni et al., 1995; Yang et al., 2010) to generate FLAG-tagged translational fusions.

The constructs were transformed into Agrobacterium tumefaciens strain GV3101, which was used to transform Arabidopsis plants by the floral dip method (Clough and Bent, 1998). Transformants were selected based on their resistance to hygromycin. T3 or T4 homozygous lines were used for further experiments. The TPL\textsuperscript{K148R}, TPL\textsuperscript{K531R}, and TPL\textsuperscript{K689R} substitutions were created by site-directed mutagenesis of the above-mentioned TPL-related constructs. Primers used for plasmid construction and genotyping are listed in Table S1.

**MeJA treatment and gene expression analysis**

Ten-day-old seedlings grown on MS medium were treated with or without 50 µM MeJA for the indicated interval. MeJA treatment of soil-grown plants was conducted as previously described with minor modifications (Liu et al., 2019). Briefly, 4-week-old soil-grown plants were enclosed in a Lucite box (30×30×60 cm) containing 100 µL of 50 mM MeJA applied to cotton wicks that were spaced evenly within the box. Plants exposed to MeJA vapor were harvested at the indicated interval. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen), according to the manufacturer’s instructions. Then, cDNA was prepared from 2 µg total RNA using the PrimeScript\textsuperscript{TM} RT Reagent Kit with gDNA Eraser (Takara) and quantified on a Roche 480 cycler with the SYBR Green I Master (Roche), according to the manufacturer’s instructions. Expression levels of target genes were normalized relative to that of ACTIN7 (At5g09810). Primers used for reverse transcription-quantitative PCR (RT-qPCR) are listed in Table S1.

**Pathogen inoculation and disease resistance assays**
*B. cinerea* inoculation assays were performed as described previously (You et al., 2019). Briefly, *Arabidopsis* plants were grown in a vermiculite:nutrition soil (1:1) mixture at 22°C under a SD photoperiod for 4 weeks. The central vein of leaves of 4-week-old *Arabidopsis* plants was inoculated with a single 5-µL droplet of *B. cinerea* spore suspension (5 × 10⁵ spores/mL). Then, the plants were incubated in a growth chamber with high relative humidity (90%). Photographs were taken at 3 days post-inoculation (dpi), and the lesion size was measured and recorded. Twelve lesion-bearing leaves were harvested for RT-qPCR to examine the expression levels of defense-related genes.

**Protein expression and *in vitro* pull-down assays**

GST- and MBP-tagged recombinant proteins were expressed in *E. coli* strain BL21, and affinity-purified with GST-Bind™ resin (Millipore) and amylose resin (NEB), respectively, according to the manufacturer’s instructions. To conduct *in vitro* pull-down assays, 200 µg GST or GST-TPL was bound to the GST resin and incubated with MBP-GCN5 or MBP-HDA6 in pull-down buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol [DTT], and 0.2% TritonX-100) at 4°C for 1 h. Each sample was washed three times with pull-down buffer. Then, the washed amylose resin was resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and boiled at 95°C for 5 min. The samples were separated by SDS-PAGE and detected by immunoblot using anti-MBP antibody.

**Bacterial acetylation assays**

*E. coli* BL21 cells transformed with the *TPL-GST* construct was grown at 37°C on LB medium supplemented with or without 5 nM TSA. When the optical density of the cultures at 600 nm absorbance (OD₆₀₀) reached a value of 0.6, protein production was induced by the addition of 250 µM isopropyl-β-thiogalactopyranoside (IPTG), and the cultures were grown at 18°C for 6 h. The GST-TPL recombinant protein was purified using Glutathione Sepharose 4B (GE Healthcare), according to the manufacturer’s instructions. After denaturation, protein samples were separated by SDS-PAGE and detected by immunoblot using anti-acetyl-Lysine (anti-ac-Lys) antibody (Cell Signaling).

**In vitro acetylation and deacetylation assays**

To determine whether GCN5 acetylates TPL *in vitro*, MBP-GCN5 and GST-TPL fusion proteins produced in *E. coli* strain BL21 were purified as described above. The purified MBP-GCN5 and GST-
TPL fusion proteins were incubated at 22°C for 1 h in HAT buffer (50 mM HEPES [pH 7.5], 10% glycerol, 5 mM MgCl₂, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mM TSA) supplemented with or without acetyl-CoA (20 μM). The acetylation level of GST-TPL was detected by immunoblotting with anti-ac-Lys antibody. To test whether HDA6 deacetylates TPL in vitro, the TPL-FLAG fusion protein affinity-purified from TPL-FLAG stable transgenic plants was used as a substrate. Plant-produced TPL-FLAG and E. coli-produced GST-HDA6 were incubated in HDAC buffer (50 mM Tris-HCl [pH 8.0], 10% glycerol, 150 mM NaCl, 1 mM DTT, 1 mM PMSF, and 5 mM ZnCl₂) at 22°C for 1 h. The acetylation level of TPL-FLAG was detected by immunoblotting with anti-ac-Lys antibody.

Co-IP assays
Co-IP assays were performed as described previously (You et al., 2019), with slight modifications. Briefly, A. tumefaciens strain GV3101 containing the indicated constructs were co-infiltrated into the fully expanded leaves of N. benthamiana plants. After incubation for 48 h, the infiltrated leaves were harvested and homogenized in protein extraction buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 0.2% NP-40, and 20 mM MG132 with Roche protease inhibitor cocktail). The extract was centrifuged twice at 13,000 × g for 10 min. After protein extraction, 20 μL anti-FLAG antibody-bound agarose beads (Sigma) or GFP-trap (ChromoTek) was added to the samples and rotated at 4°C for 3 h. The precipitated samples were centrifuged at 1,000 × g for 3 min at 4°C. The beads were washed three times with IP buffer, and proteins were eluted by boiling the beads in SDS protein loading buffer for 5 min. Protein samples were separated by SDS-PAGE and analyzed by immunoblotting. Co-IP assays in Arabidopsis were performed as described above using proteins extracted from 10-day-old seedlings of the indicated genotypes. The co-immunoprecipitated TPL protein was detected by immunoblotting with anti-TPL antibody.

LCI assays
LCI assays were carried out as described previously (Chen et al., 2008), with slight modifications. A. tumefaciens strain GV3101 carrying the indicated constructs was infiltrated into the fully expanded leaves of N. benthamiana plants. After 48 h of incubation, leaf discs were excised from the infiltrated leaves and transferred to a 96-well plate containing 200 mL water and 1 mM luciferin in each well. The leaf discs were incubated in luciferin for 10–15 min, and luminescence of each sample was
captured with the GLOMAX 96 microplate luminometer or SpectraMax plate reader with the LUM module.

**Transient transcriptional repression assays**

To perform transient transcriptional activity assays, the desired constructs were transfected into *Arabidopsis* mesophyll protoplasts as described previously (You et al., 2019). LUC and REN activities were measured using the Dual-LUC Reporter Assay System (Promega), according to the manufacturer’s instructions, and LUC:REN ratios were calculated.

**Nuclear protein extraction and global histone acetylation analysis**

Nuclei were extracted from 10-day-old WT, *hag1-6, axe1-4*, and *hda19* seedlings using the nuclei isolation buffer (0.25 M sucrose, 15 mM PIPES [pH 6.8], 5 mM MgCl₂, 60 mM KCl, 15 mM NaCl, 1 mM CaCl₂, 0.9% Triton X-100, 1 mM PMSF, and 1× Roche protease inhibitor cocktail). The solution was filtered through two layers of Miracloth and centrifuged. To isolate nuclear proteins, the resultant pellet was resuspended in nuclei lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF, and 1× Roche protease inhibitor cocktail). To determine the substrate and site specificity of GCN5, HDA6, and HDA19, protein samples were subjected to immunoblot analysis with anti-H3 (Abcam), -H3K9ac (Abcam), -H3K14ac (Millipore), and -H3K18ac (Abcam) antibodies.

**ChIP-qPCR assays**

ChIP assays were performed as described previously (Zhu et al., 2012), with minor modifications. Briefly, 10-day-old seedlings were treated with or without 50 μM MeJA for the indicated interval. Then, 2 g of each sample was harvested and crosslinked in 1% (v/v) formaldehyde at room temperature for 10 min, followed by neutralization with 0.125 M glycine. The chromatin complex was isolated, resuspended in lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF, and 1× Roche protease inhibitor cocktail), and sheared by sonication to reduce the average DNA fragment size to approximately 500 bp. The sheared chromatin was pre-cleared with protein G magnetic beads (Invitrogen), and 10 μL of the pre-cleared chromatin was removed for use as an input control. Chromatin was immunoprecipitated overnight at 4°C with anti-GFP (Abcam), anti-H3 (Abcam), anti-H3K9ac (Abcam), and anti-H3K14ac
(Millipore) antibodies. The immunoprecipitated chromatin complex was incubated with protein G magnetic beads (Invitrogen) and then sequentially washed with low-salt buffer (20 mM Tris-HCl [pH 8.0], 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, and 0.2% SDS), high-salt buffer (20 mM Tris-HCl [pH 8.0], 2 mM EDTA, 500 mM NaCl, 0.5% Triton X-100, and 0.2% SDS), LiCl buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.25 M LiCl, 0.5% NP-40, and 0.5% sodium deoxycholate), and TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA). After washing, the immunoprecipitated chromatin was eluted with elution buffer (1% SDS and 0.1 M NaHCO₃). The protein–DNA crosslinks were reversed by incubating the immunoprecipitated complexes at 65°C overnight. DNA was recovered using the QIAquick PCR Purification Kit (Qiagen) and analyzed by qPCR. Enrichment of DNA (expressed as % input) was calculated by determining the apparent immunoprecipitation efficiency at the target loci using the following equation:

\[ DNA\,enrichment = \frac{Amount\,of\,immunoprecipitated\,DNA}{Normalized\,amount\,of\,starting\,material} \times 100 \]

H3K9ac and H3K14ac levels are indicated as the ratio of the amount of immunoprecipitated DNA assembled with H3K9ac to the amount of immunoprecipitated DNA assembled with H3. Primers used for qPCR are listed in Table S1.

**TPL purification and acetylation site identification**

Ten-day-old pTPL::TPL-GFP (Long et al., 2006) seedlings (2 g) were harvested, and TPL-GFP was immunoprecipitated as described above. The precipitate was eluted by adding 2× SDS loading buffer and separated by 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue staining buffer (1% Coomassie Brilliant Blue R250 in 40% methanol and 10% acetic acid), and washed with double distilled water. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis was performed as described previously (You et al., 2019). The gel slice containing the TPL-GFP protein was excised and de-stained in buffer containing 25 mM NH₄HCO₃ and 50% acetonitrile. Proteins were reduced with 10 mM DTT at 37°C for 1 h, and alkylated with 25 mM iodoacetamide at room temperature for 1 h in darkness. In-gel trypsin digestion was performed at 37°C. The resulting peptides were extracted from the gel with buffers containing 5% trifluoroacetic acid and 50% acetonitrile, via two rounds of ultrasonication. The supernatant was freeze-dried in a SpeedVac, and the peptides were resolubilized in 0.1% formic acid. Then, the peptides were filtered through a 0.45-μm centrifugal filter and analyzed using a Triple TOF 5600 mass spectrometer (AB SCIEX) coupled online to an Eksigent
nanoLC Ultra HPLC system in Information Dependent Mode. The LC gradient (A = 0.1% formic acid in water; B = 0.1% formic acid in acetonitrile) was 5–90% B for 120 min at a flow rate of 300 nL/min. The peptides were identified from the MS/MS spectra using the ProteinPilotTM software v4.2 by searching against the TAIR 10.0 database. The following peptide modifications were allowed during the search: carboxyamidomethylation of cysteine residues (fixed), oxidation of methionine (variable) and acetylation of lysine (variable). Trypsin was specified as the proteolytic enzyme, with two missed cleavages allowed. Mass tolerance was set at 0.05 Da, and the maximum false discovery rate for proteins and peptides was set at 1%.

**Antibody generation**
The recombinant GST-TPL fusion protein purified from *E. coli* BL21 cells was used to raise polyclonal antibodies in mice. The resulting anti-TPL antibody was used in protein gel blotting at a final dilution of 1:3000. Primers used for plasmid construction are listed in Table S1.

**Quantification and statistical analysis**
Statistical analysis was performed in Prism 7. The mean and standard deviation (SD) were calculated. Significance (*P*-value) was determined using two-tailed Student’s *t*-test in Microsoft Excel. Immunoblot signal intensity was measured using the ImageJ software. All experiments were performed at least three times, and representative results are shown.

**FUNDING**
This work was supported by the National Natural Science Foundation of China (32161133018, 31730010, 31991183, 31900243) and the Strategic Priority Research Program of the CAS (XDPB16). Chunpeng An was supported by the National Postdoctoral Program for Innovative Talents (BX20180355) and the China Postdoctoral Science Foundation.

**AUTHOR CONTRIBUTIONS**
C.L. conceived and supervised the overall research; C.A., D.L., H.Z., Y. Y., F.W. and Q.Z. performed the experiments; C.A., A.G. and C. L. analyzed the data. C. L., C.A. and D.L. wrote the manuscript with input from all authors.
ACKNOWLEDGMENTS
We thank the Proteomics Facility for performing mass spectrometry analysis, and the Animal Center of Institute of Genetics and Developmental Biology, Chinese Academy of Sciences (CAS) for generating antibodies. We also thank Dr. Jeff A. Long for sharing the tpl-1 and pTPL::TPL-GFP seeds; Dr. Yuelin Zhang for sharing the tpl tpr1 tpr4 seeds; Dr. Ben Scheres for sharing the GCN5-GFP seeds; Dr. Shunong Bai for sharing pHDA6::HDA6-myc/axe1-4 seeds, Dr. Bosl Noh for sharing the hda19-3 and HDA19-FLAG/hda19-3 seeds, and Dr. Jingbo Jin for sharing the pBI121-TPL-myc construct.

DECLARATION OF INTERESTS
The authors declare no competing interests.

FIGURE LEGENDS
Figure 1. HDA6 positively regulates JA-responsive gene expression through TPL.
(A) MeJA-induced expression of JA-responsive marker genes in WT and axe1-4 seedlings. Ten-day-old seedlings were treated with 50 μM MeJA for 1 h (for JAZ8, LOX2 and ERF1), 6 h (for VSP1), or 24 h (for PDF1.2) before sample collection.
(B and C) ChIP-qPCR analysis showing the enrichment of H3K9ac (B) and H3K14ac (C) marks in the transcription start sites (TSSs) of JA-responsive genes. ACTIN7 (ACT7) was used as an unrelated control. Ten-day-old WT and axe1-4 seedlings were treated with 50 μM MeJA for different intervals, as indicated in (A).
(D) MeJA-induced expression of JA-responsive marker genes in the indicated genotypes. Ten-day-old WT and axe1-4 seedlings were treated with 50 μM MeJA for different intervals, as indicated in (A). Data represent mean ± standard deviation (SD; n = 3, three independent biological replications). Different letters indicate significant differences (P < 0.05; two-tailed Student’s t-test).

Figure 2. HDA6-mediated TPL deacetylation weakens the TPL–NINJA interaction.
(A) TPL was acetylated in Arabidopsis. Ten-day-old TPL-GFP seedlings grown on medium were subjected to immunoprecipitation with anti-GFP beads, followed by immunoblotting with anti-acetyl-Lysine (anti-ac-Lys) antibody to detect TPL-GFP acetylation. 35S::GFP transgenic plants were used as a negative control. Arrowheads indicate the positions of GFP and TPL-GFP.
(B) TPL-GFP acetylation in response to the trichostatin A (TSA) treatment. Ten-day-old TPL-GFP seedlings grown on medium supplemented with or without 100 nM TSA were subjected to immunoprecipitation with anti-GFP beads, followed by immunoblotting with anti-acetyl-Lysine (anti-ac-Lys) antibody to detect TPL-GFP acetylation. Also shown in Supplemental Figure 2E.

(C) TPL-GFP acetylation in WT and axel-4 plants. Ten-day-old seedlings were treated with or without 50 μM MeJA for 1 h. Total proteins were subjected to immunoprecipitation with anti-GFP beads, followed by immunoblotting with anti-ac-Lys antibody to detect TPL-GFP acetylation. Numbers indicate arbitrary densitometry units of TPL-GFP acetylation normalized to mock-treated WT. Also shown in Supplemental Figure 2G.

(D) HDA6-FLAG deacetylates TPL-GFP. N. benthamiana leaves co-expressing either TPL-GFP and empty vector or TPL-GFP and HDA6-FLAG were subjected to immunoprecipitation with anti-GFP beads, followed by immunoblotting with anti-ac-Lys antibody to detect TPL-GFP acetylation.

(E) HDA6 deacetylates TPL in vitro. TPL-FLAG protein immunoprecipitated from transgenic plants was incubated with purified GST or GST-HDA6 protein. TPL-FLAG acetylation was determined by immunoblotting with anti-ac-Lys antibody.

(F) Mutation of HDA6 enhances TPL–NINJA interaction. Ten-day-old WT and axel-4 seedlings were treated with or without 50 µM MeJA for 1 h. Total proteins were subjected to immunoprecipitation with anti-FLAG antibody, followed by immunoblotting with anti-TPL antibody to detect endogenous TPL. Numbers indicate arbitrary densitometry units of TPL normalized to mock-treated WT. Also shown in Supplemental Figure 2K.

(G) ChIP-qPCR assay showing enhanced TPL recruitment to MYC2 target promoters in the axel-4 mutant. SMXL7 was used as an unrelated control. Ten-day-old seedlings were treated with or without 50 µM MeJA for 1 h. Chromatin was immunoprecipitated with anti-GFP antibody. The precipitated DNA was quantified by qPCR, and DNA enrichment was calculated as a percentage of input DNA. Data represent mean ± SD (n = 3, three independent biological replications). Different letters indicate significant differences (P < 0.05; two-tailed Student’s t-test).

Figure 3. GCN5 negatively regulates JA-responsive gene expression through TPL.

(A and B) MeJA-induced expression of JA-responsive marker genes in the indicated genotypes. Ten-
day-old seedlings were treated with 50 μM MeJA for 1 h (for JAZ8, LOX2, and ERF1), 6 h (for VSP1), and 24 h (for PDF1.2) before sample collection.

(C and D) ChIP-qPCR analysis showing the enrichment of H3K9ac (D) and H3K14ac (E) marks in the TSS regions of JA-responsive marker genes. ACT7 was used as an unrelated control. Ten-day-old WT and hag1-6 seedlings were treated with 50 μM MeJA for different intervals, as indicated in (A). Chromatin immunoprecipitated with anti-H3, -H3K9ac and -H3K14ac antibodies. The precipitated DNA was quantified by qPCR, and H3K9ac and H3K14ac levels were normalized relative to the H3 level.

Data represent in A, B, C and D mean ± SD (n = 3, three independent biological replications). Different letters indicate significant differences (P < 0.05; two-tailed Student’s t-test).

Figure 4. GCN5-mediated TPL acetylation enhances the TPL–NINJA interaction.

(A) Co-IP of GCN5 and TPL. N. benthamiana leaves expressing TPL-myc and GCN5-GFP were subjected to immunoprecipitation with anti-GFP antibody, followed by immunoblotting with anti-myc antibody to detect TPL-myc.

(B) Co-IP of GCN5 and TPL in Arabidopsis. Total proteins extracted from 35S::GFP and GCN5-GFP transgenic plants were subjected to immunoprecipitation with anti-GFP antibody, followed by immunoblotting with anti-TPL antibody to detect endogenous TPL.

(C) In vitro pull-down analysis of GCN5 and TPL. The MBP-GCN5 protein purified from E. coli was incubated with GST or GST-TPL, and detected by immunoblotting using anti-MBP antibody. Arrowheads indicate the positions of GST and GST-TPL recombinant proteins (bottom).

(D) GCN5 acetylates TPL in vitro. The GST-TPL recombinant protein purified from E. coli was incubated with MBP or MBP-GCN5 in HAT buffer for 1 h. GST-TPL acetylation was determined by immunoblotting with anti-ac-Lys antibody.

(E) GCN5-FLAG acetylates TPL-GFP. N. benthamiana leaves expressing either TPL-GFP with empty vector or GCN5-FLAG were subjected to immunoprecipitation with anti-GFP beads, followed by immunoblotting with anti-ac-Lys antibody to detect TPL-GFP acetylation.

(F) TPL-GFP acetylation in WT and hag1-6 plants. Ten-day-old seedlings were treated with or without 50 μM MeJA for 1 h. Total proteins were subjected to immunoprecipitation with anti-GFP antibody,
followed by immunoblotting with anti-ac-Lys antibody to detect TPL-GFP acetylation. Also shown in Supplemental Figure 3C.

(G) Mutation of GCN5 weakens TPL–NINJA interaction. Ten-day-old seedlings were treated with or without 50 µM MeJA for 1 h. Total proteins were subjected to immunoprecipitation with anti-FLAG antibody, followed by immunoblotting with anti-TPL antibody to detect endogenous TPL. Also shown in Supplemental Figure 3D.

(H) ChIP-qPCR showing the weakened recruitment of TPL to MYC2 target promoters in the hagl-6 mutant. SMXL7 was used as an unrelated control. Ten-day-old seedlings were treated with or without 50 µM MeJA for 1 h. Chromatin was immunoprecipitated with anti-GFP antibody. The precipitated DNA was quantified by qPCR, and DNA enrichment was calculated as a percentage of input DNA. Data represent mean ± SD (n = 3, three independent biological replications). Different letters indicate significant differences (P < 0.05; two-tailed Student’s t-test).

Figure 5. TPL acetylation at K689 enhances its corepressor activity and interaction with NINJA.

(A) Schematic representation of TPL showing the conserved domains and three potential acetylation sites.

(B) Amino acid substitutions (K148R, K531R, and K689R) reduce TPL acetylation. N. benthamiana leaves expressing the indicated versions of TPL-GFP fusion constructs were subjected to immunoprecipitation with anti-GFP antibody, followed by immunoblotting with anti-ac-Lys antibody to detect TPL-GFP acetylation.

(C) LCI assays showing that the K689R substitution impairs NINJA–TPL interaction. Split-luciferase assays were conducted using the indicated constructs, with TPL-nLUC and cLUC-HAC1 serving as negative controls. Values represent mean relative luminescence units (RLU) ± SD (n = 8, 8 leaves). Different letters indicate significant differences (P < 0.05; two-tailed Student’s t-test).

(D) Co-IP assays showing that the K689R substitution impairs TPL–NINJA interaction. N. benthamiana leaves co-expressing NINJA-FLAG and the indicated versions of the TPL-GFP fusion constructs were subjected to immunoprecipitation with anti-FLAG antibody, followed by immunoblotting with anti-GFP antibody to detect TPL-GFP.

(E) Schematic diagram showing the constructs used in the transient expression assays summarized in
(F) Transient expression assays in Arabidopsis protoplasts showing that the effect of indicated TPL substitutions on the ability of TPL to repress MYC2-mediated activation of the LOX2 promoter. The LUC/REN ratio represents the activity of firefly luciferase (LUC; driven by the LOX2 promoter) relative to that of Renilla luciferase (REN; internal control; driven by the 35S promoter).

(G) MeJA-induced expression of JA-responsive marker genes in the indicated genotypes. Four-week-old soil-grown plants were treated with 50 μM MeJA for 1 h (for JAZ8, LOX2 and ERF1), 6 h (for VSP1), and 24 h (for PDF1.2) before sample collection.

(H) JA-mediated root growth inhibition of indicated genotypes. Primary root length of ten-day-old seedlings grown in 1/2 MS medium with or without 5 μM JA were measured.

(I) ChIP-qPCR showing that the K689R substitution of TPL weakened TPL recruitment to MYC2 target promoters. SMXL7 was used as an unrelated control. Ten-day-old seedlings of the indicated genotypes were treated with or without 50 μM MeJA for 1 h. Chromatin was immunoprecipitated with anti-GFP antibody. The precipitated DNA was quantified by qPCR, and DNA enrichment was calculated as a percentage of input DNA. Data are mean ± SD (n = 3, three independent biological replications in F, G; n = 20 seedlings in H, three independent experiments with similar results). Different letters indicate significant differences (P < 0.05; two-tailed Student’s t-test). All experiments were performed at least three times with similar results.

Figure 6. Reversible acetylation and deacetylation of TPL as a switch for JA signaling.

(A and B) MeJA-induced expression of GCN5 (A) and HDA6 (B). Ten-day-old WT seedlings were treated with 50 μM MeJA for the indicated duration. Gene expression levels were normalized relative to ACTIN7, and relative gene expression in untreated plants was set at 1.

(C) Dynamics of TPL acetylation in response to the MeJA treatment. Ten-day-old TPL-GFP seedlings were treated with 50 μM MeJA for the indicated duration. Total proteins were subjected to immunoprecipitation with anti-GFP antibody, followed by immunoblotting with anti-ac-Lys antibody to detect TPL-GFP acetylation.

(D and E) B. cinerea-induced expression of GCN5 (D) and HDA6 (E). Leaves of 4-week-old soil-
grown plants were inoculated with B. cinerea, and gene expression was analyzed at the indicated time points. Gene expression levels were normalized relative to ACTIN7, and relative gene expression in untreated plants was set at 1.

(F) Dynamics of TPL acetylation in response to B. cinerea infection. Leaves of 4-week-old soil-grown plants were inoculated with B. cinerea. Total proteins were extracted from the inoculated plants at the indicated time points, subjected to immunoprecipitation with anti-GFP antibody, and then immunoblotted with anti-ac-Lys antibody to detect TPL-GFP acetylation.

(G and H) Botrytis cinerea-induced expression of ERF1 (G) and PDF1.2 (H) in the indicated genotypes. Leaves of 4-week-old soil-grown plants were inoculated with B. cinerea, and gene expression was analyzed 24 h post-inoculation.

(I) Resistance of the indicated genotypes to B. cinerea. Leaves of 4-week-old soil-grown plants were inoculated with B. cinerea, and lesion size was analyzed at 3 days post-inoculation.

(J and K) Model showing GCN5- and HDA6-mediated control of TPL acetylation homeostasis. TPL exists as an acetylated and deacetylated protein in plant cells. The acetylated TPL protein shows greater corepressor activity than its deacetylated form. The acetylated and deacetylated pools of TPL exist in equilibrium and are reversibly regulated by GCN5 and HDA6/19. In the resting state, GCN5-mediated acetylation and HDA6/HAD19-mediated deacetylation maintain TPL acetylation homeostasis to ensure its recruitment to MYC2 target promoters and the repression of JA-responsive genes (J). Upon hormone elicitation, HDA6, but not GCN5, is transiently induced, which rapidly decreases TPL acetylation and its recruitment to MYC2 target promoters, promoting gene activation (K).

Data represent mean ± SD (n = 3 in A, B, D, E, G and H, three independent biological replications; n = 12 leaves in I). Different letters indicate significant differences (P < 0.05; two-tailed Student’s t-test).

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. HDA19 positively regulates JA-responsive gene expression independent of its HDAC activity.

(A) MeJA-induced expression of JA-responsive marker genes in WT, hda19-3 and HDA19-FLAG/hda19-3 plants. Ten-day-old seedlings were treated with 50 μM MeJA for 1 h (for JAZ8, LOX2 and ERF1), 6 h (for VSP1) and 24 h (for PDF1.2) before sample collection.
(B) MeJA-induced expression of HDA19. Ten-day-old WT seedlings were treated with 50 μM MeJA for the indicated durations. Gene expression levels were normalized relative to ACTIN7, and relative gene expression in untreated plants was set at 1.

(C) MeJA-induced expression of JA-responsive marker genes in the indicated genotypes. Ten-day-old seedlings were treated with 50 μM MeJA for different intervals, as indicated in (A).

(D and E) Protein gel analyses showing global H3K9ac, H3K14ac and H3K18ac levels in WT, axe1-4 (D), hda19-3 and HDA19-FLAG/hda19-3 (E). Nuclear proteins were extracted from 10-day-old seedlings and immunoblotted using the indicated antibodies.

(F and G) ChIP-qPCR analysis showing the enrichment of H3K9ac (F) and H3K14ac (G) marks in the TSS regions of JA-responsive marker genes. ACT7 was used as an unrelated control. Ten-day-old WT and hda19-3 seedlings were treated with 50 μM MeJA for different intervals, as indicated in (A). Chromatin of each sample was immunoprecipitated with anti-H3, -H3K9ac and -H3K14ac antibodies. The precipitated DNA was quantified by qPCR, and H3K9ac and H3K14ac levels were relative normalized to the H3 level.

(H) TPL-GFP acetylation in WT and hda19-3 plants. Ten-day-old TPL-GFP/WT and TPL-GFP/hda19-3 seedlings were harvested and total proteins were subjected to immunoprecipitation with anti-GFP beads, followed by immunoblotting with anti-ac-Lys antibody to detect TPL-GFP acetylation. Data represent mean ± SD (n = 3 in A, C, F and G, three independent biological replications). Different letters indicate significant differences (P < 0.05; two-tailed Student’s t-test).

Supplemental Figure 2. HDA6 interacts with and deacetylates TPL.

(A) Co-IP of HDA6 and TPL. N. benthamiana leaves expressing HDA6-GFP and TPL-myc were subjected to immunoprecipitation with anti-GFP antibody, followed by immunoblotting with anti-myc antibody to detect TPL-myc.

(B) RT-qPCR analysis of HDA6 expression in WT and HDA6-FLAG transgenic plants.

(C) Co-IP of HDA6 and TPL in Arabidopsis. Total proteins from WT and HDA6-FLAG transgenic plants were subjected to immunoprecipitation with anti-FLAG antibody, followed by immunoblotting with anti-TPL antibody to detect endogenous TPL.

(D) In vitro pull-down analysis of HDA6 and TPL. The MBP-HDA6 protein purified from E. coli was
incubated with GST or GST-TPL, and detected by immunoblotting using anti-MBP antibody. Arrowheads indicate the positions of GST and GST-TPL recombinant proteins (bottom).

(E) TPL-GFP acetylation in response to the trichostatin A (TSA) treatment. Replication of Figure 2B.

(F) RT-qPCR analysis of TPL expression in TPL-GFP/WT, TPL-GFP/axe1-4, and TPL-GFP/hag1-6 transgenic plants.

(G) TPL-GFP acetylation in WT and axe1-4 plants. Replication of Figure 2C.

(H) Effect of TSA treatment on the acetylation of the GST-TPL recombinant protein. E. coli strain containing the GST-TPL construct was cultured in Luria broth (LB) medium supplemented with or without 5 nM TSA. Acetylation levels of the purified recombinant protein were detected with anti-acetyl-Lysine (anti-ac-Lys) antibody.

(I) RT-qPCR analysis of TPL expression in WT and TPL-FLAG transgenic plants.

(J) RT-qPCR analysis of NINJA expression in NINJA-FLAG/WT, NINJA-FLAG/axe1-4, and NINJA-FLAG/hag1-6 transgenic plants.

(K) Mutation of HDA6 enhances TPL–NINJA interaction. Replication of Figure 2F.

In B, F, I, and J, expression levels of various genes were normalized relative to that of ACTIN7. Relative expression of the indicated genes in untreated WT plants was set at 1. Data represent mean ± SD (n = 3, three independent biological replications). Different letters indicate significant differences (P < 0.05; two-tailed Student’s t-test).

Supplemental Figure 3. GCN5-mediated TPL acetylation enhances TPL–NINJA interaction.

(A) RT-qPCR analysis of TPL expression in WT, hag1-6 and 35S::TPL-GFP/hag1-6 transgenic plants. Expression levels were normalized to ACTIN7. WT plants was set at 1.

(B) MeJA-induced expression of JA-responsive marker genes in the indicated genotypes. Ten-day-old seedlings were treated with 50 μM MeJA for 1 h (for JAZ8, LOX2 and ERF1), 6 h (for VSP1), or 24 h (for PDF1.2) before sample collection. Data represent mean ± standard deviation in A and B (n = 3, three independent biological replications). Different letters indicate significant differences (P < 0.05; two-tailed Student’s t-test).

(C) Protein gel analyses showing global H3K9ac, H3K14ac, and H3K18ac levels in WT and hag1-6 plants. Nuclear protein extracts prepared from 10-day-old seedlings were immunoblotted using the
indicated antibodies.

(D) TPL-GFP acetylation in WT and hagl-6 plants. Replication of Figure 4F.

(E) Mutation of GCN5 weakens TPL–NINJA interaction. Replication of Figure 4G.

Data represent in A and B, mean ± SD (n = 3, three independent biological replications). Different letters indicate significant differences (P < 0.05; two-tailed Student’s t-test).

**Supplemental Figure 4. TPL acetylation at K689 affects its interaction with JAZ8.**

(A–C) Mass spectrometry analysis of the tryptic fragments of the TPL-GFP fusion protein immunoprecipitated from transgenic plants. K148 (A), K531 (B) and K689 (C) were identified as TPL acetylation sites. The C-terminal fragments (y ions) are colored orange, and the N-terminal fragments (b ions) are colored green.

(D) LCI assays showing that the K689R substitution impairs TPL–JAZ8 interaction. Split-luciferase assays were conducted using the indicated constructs, and TPL-nLUC and cLUC-HAC1 served as negative controls. Values represent mean relative luminescence units (RLU) ± SD (n = 8, 8 leaves). Different letters indicate significant differences (P < 0.05; two-tailed Student’s t-test).

(E) Co-IP assays showing that the K689R substitution impairs TPL–JAZ8 interaction. N. benthamiana leaves co-expressing JAZ8-FLAG and the indicated TPL-GFP fusion constructs were subjected to immunoprecipitation with anti-FLAG antibody, followed by immunoblotting with anti-GFP antibody to detect TPL-GFP.

(F) The K689R substitution weakens GCN5-mediated acetylation of TPL. N. benthamiana leaves expressing indicated constructs were subjected to immunoprecipitation with anti-GFP beads, followed by immunoblotting with anti-ac-Lys antibody to detect TPL-GFP acetylation. Empty vector co-infiltrated with TPL-GFP or TPL<sup>K689R</sup>-GFP was used as control.

(G) The K689R substitution impairs HDA6-mediated deacetylation of TPL. N. benthamiana leaves expressing indicated constructs were subjected to immunoprecipitation with anti-GFP beads, followed by immunoblotting with anti-ac-Lys antibody to detect TPL-GFP acetylation. Empty vector co-infiltrated with TPL-GFP or TPL<sup>K689R</sup>-GFP was used as control.

(H) RT-qPCR analysis of TPL expression in the indicated genotypes. Gene expression levels were normalized relative to ACTIN7, and relative gene expression in WT plants was set at 1. Data represent
mean ± SD (n = 3, three independent biological replications). Different letters indicate significant differences (P < 0.05; two-tailed Student’s t-test).

**I** Both TPL-GFP and TPLK689R-GFP rescued the seedling growth defects of tpl-1. Images are 9-day-old seedlings grown on 1/2 MS agar plates. Scale bar, 1 cm.

**J and K** LCI assays showing that the effect of different substitutions on TPL interactions with IAA12 (J) or SMXL7-TPL (K). Split-luciferase assays in J and K were conducted using the indicated constructs, and TPL-nLUC and cLUC-HAC1 served as negative controls. Values represent mean relative luminescence units (RLU) ± SD (n = 8, 8 leaves). Different letters indicate significant differences (P < 0.05; two-tailed Student’s t-test).

**Supplemental Figure 5. Recruitment pattern of HDA6-myc on MYC2 target promoters in response to MeJA treatment.**

**A** MeJA-induced accumulation of the HDA6-myc fusion protein revealed by protein gel analysis. Ten-day-old HDA6-myc/axe1-4 seedlings were treated with or without 50 μM MeJA for the indicated durations. ACTIN was used as loading control.

**B** ChIP-qPCR showing the MeJA-induced enrichment pattern of the HDA6-myc fusion protein on MYC2 target promoters. Ten-day-old seedlings of HDA6-myc/axe1-4 plants were treated with or without 50 μM MeJA for the indicated durations. Chromatin was immunoprecipitated with anti-myc antibody. WT seedlings was used as control. The precipitated DNA was quantified by qPCR, and DNA enrichment was calculated as a percentage of input DNA. Data represent mean ± SD (n = 3, three independent biological replications). Different letters indicate significant differences (P < 0.05; two-tailed Student’s t-test).

**REFERENCES**


**Zhai, Q., Yan, L., Tan, D., Chen, R., Sun, J., Gao, L., Dong, M.Q., Wang, Y., and Li, C.** (2013). Phosphorylation-coupled proteolysis of the transcription factor MYC2 is important for jasmonate-


Figure 1. HDA6 positively regulates JA-responsive gene expression through TPL.
Figure 2. HDA6-mediated TPL deacetylation weakens TPL–NINJA interaction.
Figure 3. GCN5 negatively regulates JA-responsive gene expression through TPL.
Figure 4. GCN5-mediated TPL acetylation enhances TPL-NINJA interaction.
Figure 5. TPL acetylation at K689 enhances its interaction with NINJA and its co-repressor activity.

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Figure 5. TPL acetylation at K689 enhances its interaction with NINJA and its co-repressor activity.

A

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Figure 5. TPL acetylation at K689 enhances its interaction with NINJA and its co-repressor activity.
Figure 6. Reversible acetylation and deacetylation of TPL as a switch for JA signaling.