HISTONE DEACETYLSACE 15 and MOS4-associated complex subunits 3A/3B coregulate intron retention of ABA-responsive genes

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Abstract
Histone deacetylases (HDAs) play an important role in transcriptional regulation of multiple biological processes. In this study, we investigated the function of HDA15 in abscisic acid (ABA) responses. We used immunopurification coupled with mass spectrometry-based proteomics to identify proteins interacting with HDA15 in Arabidopsis (Arabidopsis thaliana). HDA15 interacted with the core subunits of the MOS4-associated complex (MAC), MAC3A and MAC3B, with interaction between HDA15 and MAC3B enhanced by ABA. hda15 and mac3a/mac3b mutants were ABA-insensitive during seed germination and hyposensitive to salinity. RNA sequencing analysis demonstrated that HDA15 and MAC3A/MAC3B co-regulate ABA-responsive intron retention (IR). Furthermore, HDA15 reduced the histone acetylation level of genomic regions near ABA-responsive IR sites and the association of MAC3B with ABA-responsive pre-mRNA was dependent on HDA15. Our results indicate that HDA15 is involved in ABA responses by interacting with MAC3A/MAC3B to mediate splicing of introns.

Introduction
Posttranslational modifications of histones including methylation, acetylation, phosphorylation, ubiquitination, and SUMOylation play important roles in modulating gene expression involved in many essential biological processes (Chen and Tian, 2007; Kim et al., 2015; Ueda and Seki, 2020). Histone acetylation catalyzed by histone acetyltransferases (HATs) relaxes chromatin structure, which is essential for gene activation (Fisher and Franklin, 2011). Histone deacetylases (HDAs or HDACs) remove the acetyl groups from histones, resulting in chromatin compaction and gene repression. Most studies have focused on histone H3 and H4 acetylation since H3 and H4 are more preferable substrates for HATs and HDAs. At least four lysine residues (K9, K14, K18, and K23) on H3 and 5 lysine residues (K5, K8, K12, K16, and K20) on H4 can be acetylated by HATs and...
HDA15 and MAC3A/MAC3B coregulate IR

In Arabidopsis (*Arabidopsis thaliana*), HDAs are grouped into three families: the Reduced Potassium Dependency 3 (RPD3)/HDA1 superfamily, Silent Information Regulator 2 family, and HD2 family (Pandey et al., 2002; Liu et al., 2014).

HDA15, a RPD3/HDA1-type HDA, has been reported to mediate different biological processes including photomorphogenesis, abscisic acid (ABA) response, and stress tolerance. HDA15 associates with PIF3 and is involved in the regulation of chlorophyll biosynthesis and photosynthesis by reducing histone H4 acetylation levels in the dark (Liu et al., 2013). HDA15 also interacts with PIF1 to regulate the transcription of the light-responsive genes involved in multiple hormonal signaling pathways and cellular processes in germinating seeds (Gu et al., 2017). Furthermore, HDA15 acts cooperatively with the NC-YC transcriptional factors to regulate light control of hypocotyl elongation by co-regulating the transcription of the light-responsive genes (Tang et al., 2017). In addition, HDA15 also interacts with ELONGATED HYPOCOTYL 5 involved in repressing hypocotyl cell elongation during photomorphogenesis (Zhao et al., 2019). More recent studies indicate that HDA15 is also involved in abiotic stress responses. HDA15 and LONG HYPOCOTYL 5 cooperating in regulation of pre-mRNA splicing of introns. HDA15 interacts with MAC3A/MAC3B coregulate IR.

In Arabidopsis, the Reduced Potassium Dependency 3 (RPD3)/HDA1 superfamily includes proteins such as HDA15 and HDA15. SIN3-LIKE 2 (SNL2), a core subunit of the SIN3-HDAC complex (Gonzalez et al., 2007), was considered to be the potential interacting proteins. Proteins extracted from 10-day-old Col-0 plants treated with or without (mock) ABA were used for immunopurification using an anti-HDA15 antibody. Proteins that were only identified in WT immunoprecipitation (IP) samples but not in the control (hda15-1) were considered to be the potential interacting proteins of HDA15. SIN3-LIKE 2 (SNL2), a core subunit of the SIN3-HDAC complex (Gonzalez et al., 2007), was found to be co-purified with HDA15 in both mock and ABA treatment. Interestingly, the core subunits of MAC, MAC3B, MAC5A, and CDC5 (Palma et al., 2007; Monaghan et al., 2009, 2010). More recently, it has been found that MAC3A/MAC3B interacts with three other splicing factors, SKI-INTERACTING PROTEIN, PRL1, and SPliceosomal TIMEKEEPER LOCUS1, to mediate the splicing of pre-mRNAs encoded by circadian clock- and abiotic stress response-related genes (Li et al., 2019).

The involvement of histone modifications in splicing regulation has been reported in yeasts and animals (Pajoro et al., 2017). Two models have been proposed to explain how histone modifications regulate AS (Rahhal and Seto, 2019). In the kinetic coupling model, condensed chromatin caused by histone deacetylation slows down the transcription elongation rate. Therefore, the splicing factors are recruited to the weak splicing sites, causing exon inclusion. In contrast, acetylated histones lead to looser chromatin and a faster elongation rate. As a result, the splicing factors are recruited to the strong splicing sites, leading to exon skipping (ES) (Rahhal and Seto, 2019). In the chromatin-adapter model, chromatin-binding proteins read the histone marks and recruit different splicing regulators. For example, the heterochromatin 1 protein HP1 recognizes the H3K9me3 mark to facilitate inclusion of the alternative exons via decreasing transcriptional elongation rates in human (Saint-André et al., 2011). In addition, deletion of the yeast HDACs Hos3 and Hos2 reduces the recruitment of Prp19 and small nuclear ribonucleoproteins (snRNPs) to pre-mRNA, suggesting that histone acetylation is coupled with spliceosome dynamics (Gunderson et al., 2011). However, the role of HDAs in splicing regulation in plants remains elusive.

In this study, we found that HDA15 interacts with the core subunits of MAC, PEAT in plants in ABA and drought stress responses (Lee and Seo, 2019). In addition, HDA15 can be phosphorylated in vivo and HDA15 phosphorylation results in the loss of enzymatic activity and functions (Chen et al., 2017). MOS4-associated complex (MAC), a highly conserved complex in eukaryotes, is the Arabidopsis orthologous complex of the NINETEEN COMPLEX (NCT) or Prp19 complex (Prp19C) in yeast and humans (Palma et al., 2007). The NCT/Prp19C function is essential for the pre-mRNA splicing reaction and is involved in the progression of spliceosome rearrangement (Chan et al., 2003; Hogg et al., 2010). The Arabidopsis MAC complex consists of over 20 proteins, and many of them are involved in alternative splicing (AS) of mRNA (Monaghan et al., 2009, 2010; Zhang et al., 2014). MAC also controls miRNA levels through modulating pri-miRNA transcription, processing, and stability (Jia et al., 2017; Li et al., 2018a). The core subunits of the MAC complex, MOS4, CELL DIVISION CYCLES (CDC5), PLEIOTROPIC REGULATORY LOCUS 1 (PRL1), MAC3A/3B, and MAC5A/5B/5C are required for plant immunity, since their loss-of-function mutations result in plants exhibiting enhanced susceptibility to pathogen infection (Palma et al., 2007; Monaghan et al., 2009, 2010).}

**Results**

**HDA15 interacts with MAC3A and MAC3B in vivo and their interaction is enhanced by ABA**

A previous study has shown that HDA15 interacts with the transcription factor MYB96 to regulate the gene expression involved in ABA responses (Lee and Seo, 2019). To further investigate the function of HDA15 in ABA responses, we performed immunopurification coupled with mass spectrometry-based proteomics to identify the HDA15 interacting proteins. Proteins extracted from 10-day-old Col-0 wild-type (WT) and hda15-1 plants treated with or without (mock) ABA were used for immunopurification using an anti-HDA15 antibody. Proteins that were only identified in WT immunoprecipitation (IP) samples but not in the control (hda15-1) were considered to be the potential interacting proteins of HDA15. SIN3-LIKE 2 (SNL2), a core subunit of the SIN3-HDAC complex (Gonzalez et al., 2007), was found to be co-purified with HDA15 in both mock and ABA treatment. Interestingly, the core subunits of MAC, MAC3B, MAC5A, and CDC5 (Palma et al., 2007; Monaghan et al., 2009, 2010).
et al., 2009, 2010), were identified only in ABA treatment conditions. Several splicing regulators such as PRP40B, ABH1, and SmD3b (Kanno et al., 2017; Hugouvieux et al., 2001, Deng et al., 2016) were also identified (Table 1).

Previous studies indicate that MAC3A and MAC3B function redundantly in plant innate immunity (Monaghan et al., 2009) and salt tolerance (Li et al., 2019). The interaction between MAC3A/MAC3B and HDA15 was further confirmed by bimolecular fluorescence complementation (BiFC) assays and co-IP assays. As shown in Figure 1A, both MAC3A and MAC3B interacted with HDA15 in the nucleus in Arabidopsis protoplasts in BiFC assays.

For Co-IP assays, MAC3A or MAC3B fused with GFP (Pro35S:GFP-MAC3A or Pro35S:GFP-MAC3B) was transformed into Arabidopsis protoplasts. An anti-HDA15 antibody was used for IP and an anti-GFP antibody was then used for immunoblot analysis. GFP-MAC3A and GFP-MAC3B proteins could be precipitated by the anti-HDA15 antibody in Arabidopsis protoplasts (Figure 1B), supporting that MAC3A and MAC3B interact with HDA15 in vivo.

Moreover, we also examined the interaction of HDA15 and MAC3B under ABA treatment conditions by Co-IP assays and split-luciferase complementation (SLC) assays. In the Co-IP assay, the Pro35S:GFP-MAC3B/mac3a mac3b (mac3a3b) transgenic plants expressing GFP-MAC3B driven by the 35S promoter in the mac3a/mac3b background were treated with or without ABA. Although GFP-MAC3B was coimmunoprecipitated by endogenous HDA15 in both mock and ABA-treated conditions, the interaction between HDA15 and MAC3B was increased 2.6- to 4.1-fold in two independent biological replicates after ABA treatment (Figure 1C and Supplemental Figure S1). In the SLC assay, HDA15 also interacted with MAC3B in Nicotiana benthamiana leaves and ABA treatment promoted this interaction (Figure 1, D and E). Taken together, these experiments indicated that ABA promotes the interaction between HDA15 and MAC3B.

mac3a/mac3b plants are hyposensitive to salt stress and ABA

Since HDA15 is involved in abiotic stress and ABA responses (Lee and Seo, 2019), we also investigated whether MAC3A and MAC3B are also involved in abiotic stress and ABA responses by comparing the phenotypes of the hda15, mac3a, and mac3b single mutants as well as the mac3a/hda15, mac3b/hda15, and mac3a/mac3b double mutants. After salt stress treatment, the survival rates of hda15-1 and mac3a/mac3b plants ranged from 50% to 60%, whereas the survival rate of WT plants was ~30% (Figure 2, A and B). The water loss rates of detached leaves in hda15-1 and mac3a/mac3b plants were also higher compared with WT (Figure 2C). Furthermore, hda15 and mac3a/mac3b seeds had higher germination rates on the medium containing ABA (Figure 2D). We also generated Pro35S:GFP-MAC3A/mac3a3b and Pro35S:GFP-MAC3B/mac3a3b transgenic plants, in which GFP-MAC3A and GFP-MAC3B driven by the 35S promoter were transformed into the mac3a mac3b double mutant, respectively. Both Pro35S:GFP-MAC3A/mac3a3b and Pro35S:GFP-MAC3B/mac3a3b could rescue the ABA hyposensitive phenotype of mac3a/mac3b (Figure 2E).

These results indicate that similar to HDA15, MAC3A and MAC3B are also involved in abiotic stress and ABA responses. However, no obvious phenotype difference was observed in the mac3a and mac3b single mutants compared with WT. Furthermore, the phenotypes of mac3a/hda15 and mac3b/ hda15 double mutants were similar to that of the hda15 single mutant in salt and ABA responses as well as leaf transpiration rates (Figure 2). These observations support the notion that MAC3A and MAC3B function redundantly in abiotic stress and ABA responses.

Transcriptome analysis of hda15-1 and mac3a/mac3b mutants

To further characterize the role of HDA15, MAC3A, and MAC3B in ABA responses, we analyzed HDA15 and MAC3A/MAC3B regulated transcriptome changes. Ten-day-old Col-0, hda15-1, and mac3a/mac3b plants treated with or without (mock) ABA were used for RNA-sequencing (RNA-seq) analysis. Two independent biological replicates for each condition were performed. After low-quality read trimming, more than 94% of the reads for every replicate were mapped to Araport11 (Supplemental Table S1). Compared
Figure 1  HDA15 interacted with MAC3A and MAC3B. A, HDA15 fused with the YFP N-terminal fraction (HDA15-YFP\(^N\)) and MAC3A/MAC3B fused with the YFP C-terminal fraction (MAC3A-YFP\(^C\), MAC3B-YFP\(^C\)) were cotransformed into Arabidopsis protoplasts. BiFC signals were observed by a confocal microscope. Nuclear localization was detected by NLS-mCherry. MYB78-YFP\(^C\) and YFP\(^C\) are negative controls. Scale bar, 10 μm. B, Co-IP analysis from the transient expression of GFP-MAC3A or GFP-MAC3B in Col-0 Arabidopsis protoplasts. Anti-HDA15 was used for IP and endogenous HDA15 was detected by anti-HDA15. C, Co-IP analysis of interaction between HDA15 and MAC3B in Pro35S:GFP-MAC3B transgenic plants in mock and ABA treatment. Col-0 and hda15-1 were used as negative controls of immunoblot. Anti-HDA15 was used for IP, and endogenous HDA15 was detected by an anti-HDA15 antibody. The numbers below indicate the quantitative results normalized to mock and calculated using ImageJ software. The arrow indicates GFP-MAC3B. D, ABA promoted interaction between HDA15 and MAC3B in split-luciferase complementary assays. Nicotiana benthamiana leaves treated with or without ABA (20 μM) were transformed by infiltration of GV3101 with indicated construct combinations. Luc images were captured by cooled CCD imaging apparatus. RLU indicates relative light units. E, Relative Luc activities were defined as the ratio of RLU to infiltrated leaf area and normalized by ImageJ software. Error bars represent ± (t test, \( n = 3 \), *\( P < 0.05 \)).
with WT in mock conditions, 78 and 705 genes displayed higher transcript levels (fold change ≥ 1.5 with \( P < 0.05 \) in \( hda15-1 \) and \( mac3a/mac3b \) plants, respectively (Supplemental Figure S2 and Supplemental Data Set S1).

Among these upregulated genes, only nine of them (Fisher’s exact test, \( P < 6.9 \times 10^{-6} \)) were overlapping genes that were upregulated in both \( hda15-1 \) and \( mac3a/mac3b \) (Supplemental Figure S2 and Supplemental Data Set S2).
also identified 115 and 965 downregulated genes in hda15-1 and mac3a/mac3b plants, respectively (Supplemental Figure S2 and Supplemental Data Set S1). Among these downregulated genes, 18 of them (Fisher’s exact test, \( P < 1.8 \times 10^{-10} \)) were overlapping genes (Supplemental Figure S2 and Supplemental Data Set S2). The stress-responsive genes RESPONSIVE TO DESICCATIO...supplemental data.}

Under ABA treatment, 108 and 1,058 genes were upregulated (fold change \( \geq 1.5 \) with \( P < 0.05 \)) in hda15-1 and mac3a/mac3b, respectively (Supplemental Figure S2 and Supplemental Data Set S1). Among these upregulated genes, 30 of them (Fisher’s exact test, \( P < 5.8 \times 23 \)) were upregulated genes that were upregulated in both hda15-1 and mac3a/mac3b (Supplemental Figure S2 and Supplemental Data Set S2). There were 137 and 1,334 downregulated genes in hda15-1 and mac3a/mac3b, respectively (Supplemental Figure S2 and Supplemental Data Set S1), and 34 of them (Fisher’s exact test, \( P < 6.5 \times 21 \)) were overlapping genes (Supplemental Figure S2 and Supplemental Data Set S2). The salt stress-responsive genes SODIUM HYDROGEN EXCHANGER 3 and HSP22 (Li et al., 2018a, 2018b; Ma et al., 2018) were affected in both hda15 and mac3a/mac3b plants under ABA treatment (Supplemental Data Set S2).

Together, our genome-wide expression analyses suggest that HDA15 and MAC3A/MAC3B coregulate a subset of genes involved in ABA responses. In addition, a large number of differentially expressed genes that are not overlapped in hda15-1 and mac3a/mac3b plants indicate that HDA15 and MAC3A/MAC3B also function independently in gene regulation.

**HDA15 and MAC3A/MAC3B are involved in AS in ABA responses**

MAC3A and MAC3B are the core subunits of MAC, which plays a critical role in pre-mRNA splicing (Palma et al., 2007; Monaghan et al., 2009, 2010). We further explored whether HDA15 and MAC3A/MAC3B are involved in the coregulation of pre-mRNA splicing by analyzing genome-wide AS. AS events can be classified into three types: alternative donor and/or acceptor (AltD/A), ES, and intron retention (IR). Compared with Col-0 in mock treatment, 1,050 and 15,260 additional IR events were identified in hda15-1 and mac3a/mac3b, respectively (Figure 3A and Supplemental Data Set S3). In ABA treatment, IR events were increased to 2,177 and 26,555 in hda15-1 and mac3a/mac3b, respectively (Figure 3A and Supplemental Data Set S4). Since IR is the major mode of AS events, we further analyzed the patterns of the IR events by monitoring relative IR levels. The IR level for a given intron was defined as the read coverage depth of the intron divided by that of the two neighboring exons (Shih et al., 2019). hda15-1 and mac3a/mac3b had higher IR levels than Col-0 both in mock and ABA treatment (Figure 3B).

We further analyzed the coregulated IR events in hda15-1 and mac3a/mac3b. The Venn diagram showed that among the 591 coregulated IR events in mock treatment, 441 of them (74.6%) were co-enhanced as shown in the quadrant plot (Figure 4A). In ABA treatment, among the 1,450 coregulated events, 1,027 of them (70.8%) showed a similar enhancement pattern in hda15-1 and mac3a/mac3b (Figure 4B). In the co-enriched IR events, 418 and 1,014 events were specifically occurred in mock and in ABA treatment, respectively (Figure 4C). The Gene Ontology (GO) analysis showed that “response to salt stress” and “response to wounding” were enriched in both mock and ABA treatment (Figure 4D). Interestingly, ABA- and stress-related GO terms such as “response to salt stress, cold, and ABA” were highly increased in ABA treatment (Figure 4D). Taken together, these results indicate that HDA15 and MAC3A/MAC3B coregulate IR of ABA- and stress-related genes in ABA response.

**HDA15 and MAC3A/MAC3B affect ABA-responsive IR**

To further uncover the involvement of HDA15 and MAC3A/MAC3B in ABA-responsive IR, we analyzed the “ABA-responsive IR events” in Col-0, hda15-1, and mac3a/mac3b. The IR events with more than two-fold enrichment (\( P < 0.005 \)) after ABA treatment in Col-0 were defined as ABA-responsive IR events, and 2,252 ABA-responsive IR events were identified (Figure 5A and Supplemental Data Set S5). We further examined whether HDA15 and MAC3A/MAC3B regulate these ABA-responsive IR events. After ABA treatment, 266 (12%) and 236 (11%) of these IR events were not altered in hda15 (HDA15-specific) and mac3a/mac3b (MAC3A/3B-specific), respectively. 144 (6%) of these IR events were also affected in hda15 and mac3a/mac3b (HDA15 or MAC3A/3B-unrelated) similar to Col-0. Interestingly, 1,606 (71%) of these IR events were not changed by ABA in both hda15 and mac3a/mac3b mutants (HDA15 and MAC3A/3B-codependent), indicating that HDA15 and MAC3A/MAC3B co-regulate these ABA-responsive IR events under ABA treatment (Figure 5A).

Moreover, we compared the IR levels of the ABA-responsive IR events in Col-0 with those in hda15-1 and mac3a/mac3b. The ABA-responsive IR levels were slightly increased in Col-0, hda15-1, and mac3a/mac3b when treated with ABA (Figure 5B). Intriguingly, the IR level of mac3a/mac3b in mock or ABA was higher than that of Col-0, suggesting that MAC3A/MAC3B are important for ABA-responsive IR. We further divided the ABA-responsive IR events into the ABA-enhanced or ABA-reduced IR events. In the ABA-enhanced IR events, the IR level was increased 3.7-fold in Col-0 after ABA treatment, compared with 1.5-fold increases in hda15-1 and mac3a/mac3b (Figure 5C). In the ABA-reduced IR events, the IR level was decreased to 4.8-
fold in Col-0 after ABA treatment, whereas the IR level was not affected in hda15-1 and mac3a/mac3b (Figure 5D). Taken together, these data suggest that HDA15 and MAC3A/MAC3B play important roles in modulating ABA-responsive IR, especially in the ABA-reduced IR.

The effect of ABA on IR of five selected ABA-responsive genes was further analyzed. Two genes with the ABA-enhanced IR events including SUCROSE NONFERMENTING 1-RELATED PROTEIN KINASE 2.3 (SNRK2.3), and SDIR1-INTERACTING PROTEIN1 (SDIRIP1), and three genes with ABA-reduced IR events including PYRIMIDINE 1 (PYD1), ABA INSENSITIVE RING PROTEIN 2 (AIRP2), and ENHANCED DOWNY MILDEW 1 (EDM1), were validated by reverse transcription quantitative PCR (RT-qPCR). The SnRK2.3 protein kinase is an important positive regulator of ABA signaling (Fujita et al., 2009). AIRP2, a RING-type E3 ubiquitin ligase, modulates ABA signaling by ubiquitination of the substrate SDIRIP1 (Oh et al., 2017). EDM1, which may act as a scaffold protein to connect HSC70/HSP90 functions (Catlett and Kaplan, 2006), plays a role in ABA-mediated stomatal closure and seed germination (Clément et al., 2011). PYD1, which promotes the degradation of pyrimidine nucleobases, is a dihydropyrimidine dehydrogenase as well as an ABA-responsive gene (Cornelius et al., 2011). The relative IR levels were shown in Supplemental Figure S3. As shown in Figure 6, the IR forms of SNRK2.3 and SDIRIP1 were significantly increased in ABA treatment in Col-0 compared with hda15 and mac3a/mac3b. The IR forms of PYD1, AIRP2, and EDM1 were significantly decreased in ABA treatment in Col-0 compared with hda15 and mac3a/mac3b. Together, these results indicate that HDA15 and MAC3A/MAC3B coregulate IR of ABA-responsive genes under ABA condition.

HDA15 affects the histone acetylation levels near the ABA-responsive IRs

Since HDAs remove acetyl groups from ε-N-acetyl lysine residues of histones, we further investigated whether HDA15 and MAC3A/MAC3B affect the histone acetylation levels at the nearby introns of the ABA-responsive IR events by chromatin immunoprecipitation (ChIP) assays. We examined the H3K9ac levels of ~250 bp upstream and downstream regions of the corresponding introns (P1 and P2), and 750 bp downstream regions as the control (P3). The H3K9ac
levels near the third intron of SNRK2.3, the first intron of SDIRIP1, the second intron of EDM1, the third intron of AIRP2, and the fourth intron of PYD1 were analyzed. We found that the P1 and P2 regions of the corresponding introns were increased in hda15 compared with Col-0 (Supplemental Figure S4). Furthermore, ABA also increased the H3K9ac levels of these regions compared with mock conditions. Although the H3K9ac level of P3 region was also increased in hda15-1 compared with Col-0, the signal was lower than that of P1 and/or P2 regions in both mock and ABA treatment. However, there was no significant difference in the H3K9ac levels in mac3a/mac3b compared with Col-0 (Supplemental Figure S4). These results show that HDA15 affects the histone acetylation level of the regions near ABA-responsive IRs, and ABA treatment enhances this effect. However, MAC3A/MAC3B do not affect the histone acetylation level of the regions near ABA-responsive IRs.

The ABA-enhanced association of MAC3B with pre-mRNA depends on HDA15
Since deletion of the yeast HDACs Hos3 and Hos2 reduces the recruitment of Prp19 to pre-mRNA (Gunderson et al., 2011), we investigated whether HDA15 and MAC3A/MAC3B associate with the pre-mRNA of the genes with ABA-responsive IR events. ProHDA15:HDA15-GFP/hda15-1, Pro35S:GFP-MAC3A/mac3a, and Pro35S:GFP-MAC3B/mac3b transgenic plants were used for RNA-immunoprecipitation (RIP) assays and determined the RNA
We propose that HDA15 removes H3K9ac of the regions near ABA-responsive IRs and facilitates MAC3B binding to pre-mRNA. Our previous study has revealed that the amino acid residues H277 and D313 are important for the catalytic activity of HDA15 (Zhao et al., 2019). To investigate whether the deacetylase activity of HDA15 is essential for its function in pre-mRNA splicing, the transgenic plants expressing GFP-HDA15 bearing mutations in these active sites (H277A and D313A) in the hda15 mutant (Zhao et al., 2019) were used to analyze the effect of ABA on IR of five selected ABA-responsive genes. Both WT and mutated versions of GFP-HDA15 could rescue the IR level of the hda15 mutant (Supplemental Figure S6), indicating that the HDA activity is not necessary for HDA15 in regulation IR of ABA-responsive genes under ABA treatment.

**Discussion**

In yeast and mammals, HDACs exist as components of multiprotein complexes (De Ruijter et al., 2003; Sengupta and Seto, 2004). These HDAC multiprotein complexes interact with a wide variety of transcription repressors and corepressors, providing flexibility and specificity in modulating chromatin structure and transcription (Sengupta and Seto, 2004; Grzenda et al., 2009; McDonel et al., 2009). Mammalian class I HDACs, HDAC1, HDAC2, and HDAC3, are found in four different multiprotein complexes including Sin3, NuRD, CoREST, and NCoR/SMRT complexes (Hayakawa and...
The Sin3 and NuRD complexes are highly conserved from yeast to humans. Six Sin3 homologs, SNL1 (Sin3-Like1), SNL2, SNL3 (AtSin3), SNL4, SNL5, and SNL6, are found in *Arabidopsis thaliana* (Bowen et al., 2010). SNL1 and SNL2 are involved in seed dormancy through regulating the ABA-ethylene antagonism (Wang et al., 2013). Furthermore, SNL1 can also interact with HDA19 in vitro (Wang et al., 2013). Using immunopurification coupled with mass spectrometry-based proteomics, we found that HDA15 interacts with SNL2, indicating that HDA15 is also a component of the Sin3-HDAC complex. HDA15 also interacts with the core subunits of the MAC complex, MAC3A and MAC3B. Interestingly, the interaction between HDA15 and MAC3B is enhanced by ABA. Moreover, the increased association of MAC3B to the ABA-responsive premRNAs of *SNRK2.3*, *SDIRIP1*, and *PYD1* depends on HDA15. Taken together, these results indicate that HDA15 and MAC3A/MAC3B interaction is involved in ABA responses.

**Figure 6** ABA-responsive IR events defects in *hda15-1* and *mac3a/mac3b* with ABA treatment. The levels of ABA-responsive IR events in Col-0, *hda15-1*, and *mac3a/mac3b* plants with ABA treatment were analyzed by RT-qPCR. Gray boxes represent UTRs, and black boxes represent exons. The 3rd intron of *SNRK2.3*, 2nd intron of *SDIRIP1*, 8th intron of *EDM1*, 3rd intron of *AIRP2*, and 4th intron of *PYD1* are the retained introns in RNA-seq. Dash and black lines indicate the PCR products of IR events and total transcripts in RT-qPCR, respectively. *UBQ10* was used as an internal control. The levels of IR isoforms were normalized to the total transcript level and calculated the relative IR level with the mock treatment. Error bars represent SD of three biological repeats (*t* test, *P* < 0.05, **P** < 0.01).
Figure 7 The ABA-enhanced association of MAC3B with pre-mRNA depends on HDA15. A, Schematic diagrams of genes with amplicons (indicated as P and I) used for RT-qPCR were shown. Gray boxes represent UTRs, and black boxes represent exons. The 3rd intron of SNRK2.3, 2nd intron of SDIRIP1, and 4th intron of PYD1 are the introns that were retained in hda15-1 and mac3a/mac3b in RNA-seq. B and C, RIP assays followed by RT-qPCR showing the enrichment of HDA15 (B) and MAC3B (C) on PYD1, SDIRIP1, and SNRK2.3 pre-mRNA in response to ABA. Ten-day-old Col-0, ProHDA15:HDA15-GFP/hda15-1, Pro35S:GFP-MAC3B, and Pro35S:GFP-MAC3B/hda15-1 treated with or without (mock) ABA were used for RIP assays using an anti-GFP antibody. The amounts of DNA were calculated by dividing the amount of DNA immunoprecipitated from Col-0 plants and compared with input DNA. Three biological replicates were performed, and three technical repeats were carried out for each biological replicate. Representative results from one biological replicate were shown. The values shown are means ± SD. Different letters above the error bars indicate statistically significant difference (P < 0.05, by the post hoc Tukey’s honestly significant difference (HSD) test).
The spliceosome is a large dynamic macromolecular complex, which removes the intron in the processes of AS (Lee and Rio, 2015). Two U1 snRNPs, AtU1A and LUC7, are found to be required for abiotic stress tolerance. The atu1a mutant showed a hypersensitive phenotype in salt stress responses. RNA-seq analysis indicated that AtU1A regulates AS in many genes by modulating recognition of 5’ splice sites (Gu et al., 2018). Similar with the atu1a mutant, the luc7 triple mutants also exhibited hypersensitivity in salt stress responses in root length. Interestingly, LUC7 preferentially promotes in the removal of a subset of terminal introns (De Francisco Amorim et al., 2018). Our study indicated that hda15 and mac3a/mac3b mutants are ABA-insensitive in seed germination and hyposensitive to salt stress. RNA-seq and RT-qPCR analysis indicated that the IR form of SDIRIP1 was reduced in hda15-1 and mac3a/mac3b mutants, which may result in more functional SDIRIP1. By contrast, the IR form of AIRP2 was increased in the mutants, which may generate more nonfunctional AIRP2. In ABA signaling, SDIRIP1, a substrate of AIRP2, selectively regulates the expression of the bZIP transcription factor ABI5 (Oh et al., 2017). Increased SDIRIP1 functions might attenuate ABI5 activity to affect ABA responses in hda15-1 and mac3a/mac3b mutants.

Spliceosome assembly is strongly coupled with histone modifications including acetylation and deacetylation (Gunderson and Johnson, 2009; Hnilicová et al., 2011). It has been shown that the activity of HDACs modulates the AS in about 700 human genes (Hnilicová et al., 2011). Moreover, inhibition or depletion of HDAC1 increased histone H4 acetylation surrounding the alternative exon (Hnilicová et al., 2011). The yeast histone HAT Gcn5 is required for the association of U2 snRNP to the splicing branchpoint (Gunderson and Johnson, 2009). Mutation or deletion of Gcn5-targeted histone H3 residues leads to the intron accumulation of the Gcn5 target genes (Gunderson et al., 2011). In yeast, Prp19 is a core component of the Prp19C, also known as NTC, which functions in splicing and stabilizes U5/U6 snRNP in the spliceosomal complex (Chan et al., 2003). The deletion of the yeast HDACs Hos3 and Hos2 reduces the recruitment of Prp19 to pre-mRNA (Gunderson et al., 2011). MAC3A and MAC3B are the plant orthologs of Prp19 (Liu et al., 2013). The interaction of MAC3A and MAC3B with HDA15 indicates that they may be functionally associated. However, we found that the H3K9 acetylation levels of SNRK2.3, SDIRIP1, EDM1, AIRP2, and PYD1 are increased in the hda15 mutant but not in the mac3a/mac3b mutant, suggesting that the splicing mediated by MAC3A/MAC3B may not be dependent on histone deacetylation. However, the association of MAC3B with ABA-responsive pre-mRNA is dependent on HDA15, suggesting that HDA15 facilitates the function of MAC3B in splicing. Our genome-wide expression analyses suggest that HDA15 and MAC3A/MAC3B coregulate a subset of genes involved in ABA responses. It is possible that HDA15 and MAC3A/MAC3B also affect ABA-signaling, which may induce both increased and reduced IR events.

Materials and methods

Plant materials and growth conditions

The T-DNA insertion mutants, hda15-1 (SALK_004027), mac3a (SALK_089300), and mac3b (SALK_050811), were described previously (Monaghan et al., 2009; Liu et al., 2013) and all of them are in the Col-0 background. Arabidopsis (Arabidopsis thaliana) plants were grown under long-day conditions (16-h light/8-h dark cycle) at 23°C after seeds were subjected to a 3-day stratification period. To generate Pro35S:GFP-MAC3B transgenic lines, MAC3B complementary DNA (cDNA) was cloned into the pK7WG2F binary vector. The transgenic plants were generated using the floral dip method (Clough and Bent, 1998).

LC-MS/MS

Ten-day-old Col-0 WT and hda15-1 were treated with or without 50 μM ABA for 3 h. Total proteins were extracted in an extraction buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10% [v/v] glycerol, 1% [v/v] Igepal CA-630, and 1 mM phenylmethylsulfonyl fluoride) containing a protease inhibitor cocktail (Roche). The HDA15-associated proteins were immunoprecipitated by rabbit polyclonal anti-HDA15 (Liu et al., 2013). After trypsin digestion and desalting, peptides were used to perform LC–MS/MS by Thermo Orbitrap Elite Mass Spectrometer and for Mascot analysis. hda15-1 was used as a negative control for excluding the proteins that bind non-specifically to the HDA15 antibody in immunopurification. Two biological replicates for each sample were performed independently.

BiFC assays

HDA15, MAC3A, and MAC3B were cloned into the pCR8/GW/GW vector and then recombined into the YFPN (pEarleyGate201-YFPN) and YFPc (pEarleyGate202-YFPc) vectors (Lu et al., 2010). Constructed vectors were transiently transformed into Arabidopsis protoplasts by polyethylene-glycol (PEG)-mediated transfection (Yoo et al., 2007). Nuclear localization was detected by NLS-mCherry (Liu et al., 2013). The florescence was observed using Leica TCS SP5 confocal microscope. YFP florescence was excited by 514 nm of an argon laser and observed at 525 to 565 nm. mCherry florescence was excited by 561 nm of an argon laser and observed at 575–620 nm. Chloroplast autofluorescence was detected at 650–700 nm.

SLC assays

HDA15 and MAC3B were cloned into nLuc and cLuc vectors, respectively (Supplemental Table S2). SLC assay was performed as described previously (Chen et al., 2008; Liang et al., 2021). Constructed vectors were transformed into Agrobacterium tumefaciens GV3101 which was injected into leaves of Nicotiana benthamiana. Six hours before observation, 20 μM ABA or H2O were injected into corresponding...
leaves. After 48-h infection of GV3101, Luc activity was measured after 1 mM luciferin was sprayed onto the leaves. Luc images were captured by cooled CCD imaging apparatus. Relative Luc activities were defined as the ratio of relative light units (RLUs) to infiltrated leaf area and normalized by ImageJ software (https://imagej.nih.gov/ij/).

Co-IP assays
MAC3A or MAC3B cDNA was cloned into the pK7WG2 vector for transient expressing GFP-MAC3A or GFP-MAC3B by PEG-mediated transfection in Col-0 Arabidopsis protoplasts. Ten-day-old Col-0, hda15-1, and Pro35S:GFP-MAC3B transgenic plants were treated with or without 50 μM ABA for 3 h. Total proteins were extracted in the extraction buffer containing protease inhibitor cocktail (Roche) as described above. Protein extracts were incubated with a rabbit polyclonal anti-HDA15 antibody and protein G Mag Sepharose beads (GE Healthcare) overnight at 4°C. The beads were washed with a wash buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10% [v/v] glycerol, and 1% [v/v] Igepal CA-630). Immunoblotting was carried out by using the rabbit polyclonal anti-HDA15 antibody, anti-GFP antibody (Abcam, ab290), and the secondary antibody CleanBlot IP Detection Reagent (Thermo Fisher Scientific, 21230).

Abiotic stress tolerance test and seed germination treated with ABA
For the salt tolerance test, 3-week-old plants were watered with 300 mM NaCl for 2 weeks, and the survival rates were measured. For transpiration (water loss) measurements, detached leaves from 5-week-old plants were exposed to white light in a growth chamber at 23°C. Leaves were weighed at various time intervals, and the loss of fresh weight (percentage) was used to indicate water loss. For seed germination test, imbibed seeds were cold treated at 4°C for 3 days, and then sown on a half-strength Murashige and Skoog (MS) medium (pH 5.7) with 1% (w/v) sucrose, 0.8%

RNA-seq
For RNA sequencing, 10-day-old seedlings of Col-0 WT, hda15-1, and mac3a/mac3b were treated with or without 50 μM ABA for 3 h. Total RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer’s protocol, and then used for Illumina NovaSeq 6000 platform to generate paired-end reads of 150 bp. Two biological replicates for each sample were performed independently.

After low-quality read trimming, over 73 million paired-end reads were obtained per sample. More than 94% of the reads for every replicate were mapped to Araport11 using Bowtie version 2 (Langmead and Salzberg, 2012) and BLAT (Kent, 2002). Reads Per Kilobase Million (RPKM) values were calculated using the Rakk software package (http://rackj.sourceforge.net/), and normalized by the TMM method (Robinson and Oshlack, 2010). In this study, differentially expressed genes were defined as P-value < 0.05 (t test) with fold-change $\geq 1.5$.

The major types of AS events, IR, AltD/A, and ES, were analyzed by RackJ as described previously (Kanno et al., 2017). In brief, significant IR changes were defined as P-value < 0.005 (t test) with fold-change > 2. The significant ES and AltD/A events were determined using a method similar to that for IR events following the criteria: P-value < 0.005 (t test) with fold-change > 2; the sum of the read counts in two samples supporting the ES or AltD/A event $\geq 20$, the sum of the splice read counts aligned to the skipped exon $\geq 20$ (for ES); the sum of the splice read count supporting all other junctions of the same intron $\geq 20$ (for AltD/A).

RT-qPCR
Total RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer’s protocol and used to synthesize cDNA. RT-qPCR was performed using iQ SYBR Green Supermix (Bio-Rad) and the CFX96 real-time PCR system (Bio-Rad). The gene-specific primers used for quantitative PCR are listed in Supplemental Table S2. Each sample was quantified at least in triplicate and normalized using Ubiquitin10 (UBQ10) as an internal control.

ChIP-qPCR
ChIP assays were performed as described (Gendrel et al., 2005; Liu et al., 2013). Chromatin was extracted from 10-day-old seedlings. After fixation with 1% (v/v) formaldehyde, the chromatin was sheared to an average length of 500 bp by sonication and then immunoprecipitated with the H3K9ac antibody (Diagenode, C15410004). The cross-linking was then reversed, and the amount of each precipitated DNA fragment was determined by qPCR using specific primers in Supplemental Table S2. Three biological replicates were performed, and three technical repeats were carried out for each biological replicate. Representative results from one biological replicate were shown.

RIP assays
RIP assays were performed as described (Xing et al., 2015). The nuclear extracts were extracted from 10-day-old seedlings with Ribonuclease Inhibitor (Promega, N2515). After fixation with 1% (v/v) formaldehyde, the nuclear extracts were sheared to an average length of 500 bp by sonication and then immunoprecipitated with a GFP antibody (Abcam, ab290). The cross-linking was then reversed, and the RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer’s protocol and used to synthesize cDNA. The amounts of cDNA fragments were determined by RT-qPCR using specific primers in Supplemental Table S2. Three biological replicates were performed, and three technical repeats were carried out for each biological replicate. Representative results from one biological replicate were shown.

Accession numbers
HDA15, AT3G18520; MAC3A, AT1G04510; MAC3B, AT2G33340; SNRK2.3, AT5G66880; SDR1P1, AT5G51110; PYD1, AT3G17810; EDM1, AT4G11260; AIRP2, AT5G01520;
UBQ10, AT4G05320. All RNA-seq data have been uploaded to the Sequence Read Archive database (accession no. PRJNA678483) at the National Center for Biotechnology Information.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Co-IP analysis of interaction between HDA15 and MAC3A in Pro3SS:GFP-MAC3B transgenic plants under mock and ABA treatments.

Supplemental Figure S2. Genome-wide expression analysis in Col-0, hda15-1, and mac3a/mac3b by RNA-seq.

Supplemental Figure S3. ABA-responsive IR event defects in hda15-1 and mac3a/mac3b.

Supplemental Figure S4. H3K9ac levels of the retained introns of SNRK2.3, SDIRIP1, EDM1, AIRP2, and PYD1 in hda15 and mac3a/mac3b.

Supplemental Figure S5. RIP assays followed by RT-qPCR showing the enrichment of HDA15 and MAC3B on ACTIN7 pre-mRNA in response to ABA.

Supplemental Figure S6. ABA-responsive IR events of WT and mutated GFP-HDA15 transgenic plants treated with ABA.

Supplemental Table S1. Read counts and mapping rates in RNA-seq analysis.

Supplemental Table S2. List of primers used in this study.

Supplemental Data Set S1. Genes differentially expressed in hda15-1 and mac3a/mac3b compared with Col-0 under mock and ABA treatments.

Supplemental Data Set S2. Co-regulated genes in hda15-1 and mac3a/mac3b under mock and ABA treatments.

Supplemental Data Set S3. List of AS events in hda15-1 and mac3a/mac3b compared with Col-0 under mock-treated conditions.

Supplemental Data Set S4. List of AS events in hda15-1 and mac3a/mac3b compared with Col-0 under ABA-treated conditions.

Supplemental Data Set S5. List of ABA-responsive IR events in Col-0, hda15-1 and mac3a/mac3b.

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