**AHL10 phosphorylation determines RRP6L1 chromatin association and growth suppression during water stress.**

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Introduction, Methods, Results and Discussion: 4,417 words

**Summary**

•Phosphorylation of AHL10, one of the AT-hook family of plant-specific DNA binding proteins, is critical for growth suppression during moderate severity drought (low water potential, $\psi_w$) stress. To understand how AHL10 phosphorylation determines drought response, we identified putative AHL10 interacting proteins and further characterized interaction with RRP6L1, a protein involved in epigenetic regulation.

• RRP6L1 and AHL10 mutants, as well as *ahl10-1rrp6l1-2*, had similar phenotype of increased growth maintenance during low $\psi_w$. Conversely, loss of AHL13, which is homologous to AHL10 and phosphorylated at similar C-terminal site, blocked the enhanced growth maintenance of *ahl10-1*.

•Chromatin precipitation demonstrated that RRP6L1 chromatin association increased during low $\psi_w$ stress and was dependent upon AHL10 phosphorylation. Transcriptome analyses showed that AHL10 and RRP6L1 have concordant effects on expression of stress- and development-related genes.

•Stress signaling can act via AHL10 phosphorylation to control the chromatin association of the key regulatory protein RRP6L1. AHL10 and RRP6L1 interaction in meristem cells is part of a mechanism to down-regulate growth during low $\psi_w$ stress. AHL10 and AHL13 are not redundant but rather have distinct roles, likely as part of AHL hetero-complexes.

**Keywords:** drought stress, phosphorylation, nuclear localization, chromatin-associated proteins, At-Hook, RRP6 Like protein

## Introduction

Protein phosphorylation and de-phosphorylation is a central feature of signaling pathways that control plant responses to environmental stimuli. The Clade A of the Type 2C Protein Phosphatases (PP2Cs) have established roles in stress signaling (Cutler et al., 2010; Danquah et al., 2014; Bhaskara et al., 2019). However, there is diversity of physiological function among the Clade A PP2Cs despite their close phylogenetic relatedness. HAI1, in particular, has relatively weak effect on ABA sensitivity of seed germination, the canonical phenotype used to define the ABA signaling function of other Clade A PP2Cs, but strong effect on growth and osmotic adjustment during low water potential (low $\psi_w$) stress (Bhaskara et al., 2012; Wong et al., 2019). At the molecular level, it has also been shown that HAI1, but not the Clade A PP2Cs ABI1 or ABI2, could directly inactivate MPK3 and MPK6 (Mine et al., 2017).

Phosphoproteomic analysis of *hai1-2* identified putative HAI1-regulated phosphorylation sites on nuclear-localized proteins (Wong et al., 2019), consistent with the predominant nuclear localization of HAI1 (Antoni et al., 2011; Bhaskara et al., 2012). These included S313 and S314 of AT-Hook Like10 (AHL10). Further experiments demonstrated that HAI1 could directly dephosphorylate the AHL10 C-terminal region containing S313 and S314 and that AHL10 S314 phosphorylation was critical for suppression of growth during low $\psi_w$ stress (Wong et al., 2019). The phosphoproteomic data of Wong et al. (2019) also contained a phosphopeptide from the equivalent site on one of the most closely related AHL10 homologs (AHL13 S376) (Wong et al., 2019). In a separate study, phosphoproteomic analysis of chromatin-associated proteins found that AHL13 S376 was phosphorylated by Mitogen Activated protein kinases (MPKs) in response to the pathogen elicitor flg22 (Rayapuram et al., 2021). They found that AHL10 S314 was also a putative MPK6 phosphorylation site but was not responsive to flg22 treatment. Thus, phosphorylation of the C-terminal region of AHL10 and AHL13 may be regulated by MPKs and HAI1 (both directly and by HAI1 suppression of MPK3 and MPK6 activity); however, the two AHLs differ in how their phosphorylation status is regulated in response to specific environmental stimuli. AHL10 and AHL13 are Clade B AHLs (AHL1-AHL14), which are less studied than the Clade A AHLs (AHL15-AHL29; Zhao et al., 2013). The C-terminal regions of the Clade B AHLs are highly variable and the MPK-HAI1 regulated phosphorylation sites identified in AHL10 and AHL13 are not conserved among Clade B AHLs (Wong et al., 2019).

AHL10 and AHL13 are members of the AT-Hook family of plant-specific DNA binding proteins. AHLs bind AT-rich DNA sequences via their N-terminal AT-hook domain (Fujimoto et al., 2004; Zhao et al., 2013). AHLs also contain a Plant and Prokaryote Conserved (PPC)/DUF296 domain which is involved in self-interaction, or cross-interaction among AHLs, to form trimers (Fujimoto et al., 2004; Seo and Lee, 2021). In contrast to many other types of transcriptional regulators, AHLs bind the minor groove of DNA and it has been proposed that binding of AHL complexes to AT-rich sites can modify DNA architecture to bring to together loops of DNA and place otherwise distal elements in close proximity to induce the formation of transcriptional regulatory complexes (Zhao et al., 2013; Franco-Zorrilla et al., 2014).

AHLs are not thought to have transcriptional regulatory activity of their own. Rather, the highly variable AHL C-terminal domain may recruit other transcriptional regulators or may mediate interactions that determine AHL sub-nuclear localization. Varied AHL protein interactions may explain observations that even though different AHLs bind to essentially identical AT-rich sequences (Franco-Zorrilla et al., 2014), they can either induce or repress gene expression. For example, ChIP-RNA sequencing of AHL29 found that binding motifs of TCP and bHLH transcription factors were more highly enriched than AT-rich sequences directly bound by AHL29 (Favero et al., 2020), consistent with previous data showing that AHL29 interacts with TCP transcription factors (Zhao et al., 2013). Such observations raise the question of whether AHLs have sufficient sequence specificity of their own to recognize specific genomic sites or whether it is the AHLs that are recruited by other proteins and then interact with nearby AT-rich sequences in a more non-specific manner. For, AHL10 specifically, we are aware of only one study of AHL10 examining its cellular function (other than the phosphoproteomic studies mentioned above). AHL10 was found to interact with Admetos (ADM), a protein involved in hybrid seed lethality after interploidy hybridization, and with the SET domain-containing SU(VAR)3–9 homolog SUVH9, a protein involved in deposition of the heterochromatin mark H3K9me2 (Jiang et al., 2017). Together these results illustrate how functional diversity among AHLs is likely due to their interaction with a wide range of proteins via their variable C-terminal domains. Although AHLs are sometimes referred to as transcription factors, there is still much unclarity about how they function in gene regulation.

To better understand why AHL10 S314 phosphorylation is so critical for its function to restrict growth during drought stress, we searched for AHL10-interacting proteins. One of those proteins, RRP6L1, was found to also be a suppressor of growth during low $\psi_w$ stress.

RRP6L1 (also referred to as Atrimmer1) is an RNA binding protein with several proposed functions related to small RNA production and RNA-directed DNA Methylation (RdDM) (Zhang et al., 2014, Ye et al., 2016; Wendte et al., 2017). Both AHL10 and RRP6L1 act by being recruited to specific sites in the genome; DNA binding sites in the case of AHL10 or sites where specific RNAs are being transcribed in the case of RRP6L1 (Shin and Chekanova, 2014; Zhang et al., 2014; Wendte et al., 2017). However, in both cases it is unclear how this occurs as neither protein is thought to have sequence specific binding. Surprisingly, we found that RRP6L1 chromatin recruitment depended upon AHL10 and AHL10 S314 phosphorylation. We also made the unexpected discovery that AHL10 and AHL13 are not functionally redundant, despite their close homology and common regulation by HAI1 and MPKs.

**Methods**

Yeast two-hybrid screening was performed according to the manufacturer's instructions using the ProQuest$^{TM}$ Two-hybrid system (Invitrogen) and a previously described library prepared from low $\psi_w$-treated seedlings (Chong et al., 2019). Ratiometric Bi-molecular fluorescence complementation assays using the vector system described by (Grefen and Blatt, 2012) and transient expression in seedlings of an Arabidopsis line with Dexamethasone (Dex) inducible AvrPto expression (Tsuda et al., 2012). Low $\psi_w$ treatment on PEG-infused agar plates and soil drying experiments were performed as described previously (Verslues et al., 2006; Wong et al., 2019). Isolation of total nuclear, nucleoplasmic and chromatin-bound protein was conducted following the procedure of (Wu et al., 2016) with some modifications as described in Supplemental Methods. The RNA sequencing of *rrp6l1-2* was conducted in the same set of experiments as previously reported data of wild type and *ahl10-1* and followed the previously described procedures for sample processing, and data analysis (Wong et al. 2019). Chromatin Immunoprecipitation (ChIP) assays were conducted according to Yamaguchi et al. (2014) with minor modifications. Full experimental details can be found in Methods S1-S10.

## Results

### AHL10-interacting proteins, including RRP6L1, identified by yeast two-hybrid screening.

To identify AHL10 interacting proteins, we used full length AHL10 as bait to screen a yeast two-hybrid library prepared using cDNA isolated from low $\psi_w$-treated seedlings (the library has been described in Chong et al., 2019). The screening identified several clones of AHL1, AHL3, AHL10 and AHL13 that showed strong interaction with AHL10 (Supplemental Fig. S1A). This indicated that these four Clade B AHLs can form complexes with each other and is consistent with the results of Jiang et al. (2017) where TAP tagging and immunoprecipitation of ADM also found these four AHLs. Among other nuclear-localized proteins identified in the screen, AHL10 strongly interacted with RRP6L1 and the cell-cycle regulator CDC20.2 and had weaker putative interaction with RNA-binding (RBP45A), DNA-binding (WRKY1, Histone 3.1) and chromatin associated (SGO2) proteins. We focused further attention on RRP6L1 because of its strong interaction in the yeast-two hybrid assays (Fig. 1A). The interacting clone detected in the yeast two-hybrid screen contained the C-terminal 76 amino acids of RRP6L1. Retransformation assays confirmed AHL10 interaction of this clone as well as a longer clone containing the C-terminal 192 amino acids. However, neither the C-terminal 338 amino acids nor full length RRP6L1gave a positive interaction signal with AHL10 (Fig 1A). It is possible that the longer RRP6L1 clones interfered with reporter activation in the yeast assays.

### AHL10-RRP6L1 interaction

Ratiometric Bi-molecular fluorescence complementation (rBiFC) assays found that AHL10-RRP6L1 generated a strong fluorescence signal in seedlings exposed to low $\psi_w$ stress but not in unstressed seedlings (Fig. 1B and C). As a control for the rBiFC interaction assays, we also tested AHL10 interaction with AHL27 and AHL29. In the low $\psi_w$ treatment, AHL27 and AHL29 had lower rBiFC signal compared to the AHL10-RRP6L1 interaction (Fig. 1B). This lower rBiFC signal may represent non-specific background, perhaps due to high level of transient expression of the proteins, or may represent a limited interaction of RRP6L1 with other AHLs. We also found that AHL10 had a relatively strong rBiFC signal with WRKY1 in both unstressed and low $\psi_w$ -treated plants (Supplemental Fig. S1B), indicating that AHL10 expression was not the cause of the lower AHL10-RRP6L1 rBiFC signal in

unstressed plants. Together these data demonstrated that RRP6L1 interacted with AHL10 in the nucleus and that this interaction may be stimulated by low $\psi_w$ stress.

AHL10-RRP6L1 interaction was also detected using *in vitro* pull-down assays. RRP6L1 was divided into the N-terminal 300 amino acids (N300) and C-terminal 337 amino acids (C337) as full length RRP6L1 could not be expressed in *E. coli*, consistent with previous report (Zhang et al., 2014). The N300 fragment of RRP6L1 contains the 3'-5' exonuclease domain while the C337 fragment contains a Helicase and RNase D C-terminal (HRDC) nucleic acid-binding domain. Interestingly, both halves of RRP6L1 interacted with AHL10; however, the pull-down band intensity was significantly greater for the C337 fragment of RRP6L1 than for N300 (Supplemental Fig. S2A and C). Phosphomimic mutations at the S313 and S314 phosphorylation sites previously found to be critical for AHL10 function (Wong et al., 2019) increased the pull-down band intensity of the N300 fragment but had no significant effect on interaction of C337 (Supplemental Fig. S2B and C). These data confirmed AHL10-RRP6L1 interaction and indicated that AHL10 phosphorylation at S313 or S314 is unlikely to block interaction with RRP6L1; however, we cannot rule out that phosphorylation affects the affinity of AHL10-RRP6L1 interaction or affects how AHL10 interacts with the different domains of RRP6L1.

**AHL10 and RRP6L1 accumulate in meristematic tissue**

We generated transgenic lines where a genomic fragment containing the AHL10 promoter and gene body up to the stop codon was fused with YFP ($AHL10_{genomic}$*:YFP*) and used to complement *ahl10-1* (Supplemental Fig. S3). Imaging of these transgenic lines found that AHL10-YFP accumulated in the root quiescent center, developing stele and columella cells under both control and low $\psi_w$ stress conditions (Fig. 1D). AHL10-YFP also accumulated to high levels in the shoot meristem region as well as in the meristem region of lateral roots and sites of lateral root emergence (Supplemental Fig. S4). The same AHL10 native promoter construct was produced for phosphonull AHL10 ($AHL10^{S313A}$, $AHL10^{S314A}$) and phosphomimic AHL10 ($AHL10^{S313D}$, $AHL10^{S314D}$) and transformed into *ahl10-1*. Consistent with previous results $AHL10^{S313D}$ and $AHL10^{S314D}$ complemented *ahl10-1* while $AHL10^{S314A}$ did not complement. $AHL10^{S313A}$ was hyper-functional and suppressed growth below the wild type level during low $\psi_w$ stress (Supplemental Fig. S3). $AHL10^{S314A}$ was diffusely localized in the nucleoplasm and lacked the nuclear foci seen for wild type AHL10, $AHL10^{S313A}$ and phosphomimic AHL10 (Supplemental Fig. S5). Note that the nucleoplasm

localization of AHL10-YFP matches previously reported localization of RRP6L1 (Lange et al., 2008; Zhang et al., 2014).

To study RRP6L1 protein accumulation, and compare it to that of AHL10, we generated antisera recognizing the N-terminal domain of RRP6L1. Immunoblotting of wild type and two previously described RRP6L1 knockout mutants, *rrp6l1-1* (Lange et al., 2008; Zhang et al., 2014; Ye et al., 2016; Wendte et al., 2017) and *rrp6l1-2* (Hsu et al., 2014), found that the antisera recognized a 77 kD band which corresponded to the expected molecular weight of RRP6L1 and which was absent in both of the *rrp6l1* alleles (Fig. 1E). It was also consistently observed that RRP6L1 protein level was moderately increased by low $\psi_w$ stress. Samples of shoot and root meristem (and surrounding tissue) had substantially higher levels of RRP6L1 and AHL10-YFP than whole seedling samples (Fig. 1F). These data indicated that AHL10 and RRP6L1 proteins accumulate in the same parts of the plant, consistent with their physical interaction. Immunoblots also showed that RRP6L1 protein level did not substantially differ between wild type and *ahl10-1*. Likewise, AHL10 protein level was not substantially affected by *rrp6l1-2* (Fig. 1E and F). Thus, the purpose of the AHL10-RRP6L1 interaction was not related to one protein affecting the abundance of the other protein.

**RRP6L1 and AHL10, but not AHL13, restrict growth at low $\psi_w$.**

We subjected both *rrp6l1* alleles to a moderate severity low $\psi_w$ (-0.7 MPa) that decreases, but does not completely inhibit, growth of wild type (Wong et al., 2019). Both *rrp6l1* mutants maintained a higher level of growth than wild type during this moderate severity low $\psi_w$ stress while having minimal or no effect on growth in the unstressed control, similar to *ahl10-1* (Fig. 2A, Supplemental Fig. 6A, wild type data used for normalization can be found in Supplemental Fig. S6B). An *ahl10-1rrp6l1-2* double mutant had similar increased growth maintenance as either single mutant. *rrp6l1-2* also had enhanced growth maintenance during controlled soil drying assays where plants were exposed to an extended period of partial soil drying that slowed the increased in rosette size but did not lead to wilting (Fig 2B, C). Note that the *ahl10-1* data from these soil drying experiments has been previously reported (Wong et al., 2019) and is shown again here to allow better comparison between all genotypes.

AHL13 is closely related to AHL10, including conservation of the C-terminal phosphorylation site (Wong et al., 2019; Rayapuram et al., 2021), and likely forms

heteromeric complexes with AHL10 (Supplemental Fig. S1). However, *ahl13-1* did not differ from wild type in growth maintenance at low $\psi_w$ (Fig 2A). Interestingly though, loss of AHL13 blocked the increased growth of *ahl10-1* as *ahl10-1ahl13-1* grew similarly to wild type in both PEG-plate (Fig. 2A) and soil drying assays (Fig 2B). Transformation of *ahl10-1ahl13-1* with *35S:AHL13* restored the *ahl10-1* phenotype (Supplemental Fig. 6C), validating that AHL13 is required for the increased growth maintenance of *ahl10-1*. These data indicate that AHL10 and AHL13 do not have redundant function. Rather AHL13 acts genetically downstream of AHL10, consistent with the formation AHL10-AHL13 heterocomplexes.

**AHL10 S314 phosphorylation is required for RRP6L1 chromatin association.**

AHLs and RRP6L1 are both hypothesized to require additional interacting factors to correctly target and associate with specific genomic sites or transcripts (Zhao et al., 2013; Wendte et al., 2017; Favero et al., 2020). Thus, we used chromatin precipitation to test whether AHL10 or RRP6L1 affected one another's partitioning to the nucleoplasm or chromatin-associated fractions of nuclear extracts and whether this partitioning was affected by low $\psi_w$ stress. In these assays, centrifugation of nuclear extracts was used to separate soluble nuclear proteins (Soluble Fraction, labeled as "S.F." in Fig. 3) from chromatin-associated proteins (labeled as "C.B." in Fig. 3). Sonication then allowed the chromatin-associated proteins to be solubilized and separated from the remaining insoluble material (pellet after sonication, labeled as "P.S." in Fig. 3).

RRP6L1 presence in the chromatin-associated fraction was strongly increased by low $\psi_w$ and was dependent upon AHL10 S314 phosphorylation. RRP6L1 chromatin association was reduced to background level in *ahl10-1* (compare Fig. 2B and C to Fig. 2E where a line expressing GFP alone was used as a negative control). RRP6L1 presence in the chromatin-associated fraction was also strongly reduced by the AHL10$^{S314A}$ phosphonull mutation (Fig 2B and D). This reduced RRP6L1 chromatin association in plants expressing AHL10$^{S314A}$ correlates with the altered sub-nuclear localization of AHL10$^{S314A}$ (Supplemental Fig. S5) and with its inability to complement *ahl10-1* (Supplemental Fig. S3). Interestingly, the opposite effect was observed for AHL10$^{S313A}$ where the portion of RRP6L1 in the chromatin-associated fraction was high and similar to the high level seen in the AHL10 phosphomimic mutants (AHL10$^{S313D}$ and AHL10$^{S314D}$). The high level of chromatin-associated RRP6L1 in plants expressing AHL10$^{S313A}$ correlates with the strong growth suppression activity of AHL10$^{S313A}$ (Supplemental Fig. S3; Wong et al., 2019) and is consistent with the idea that

blocking AHL10 S313 phosphorylation directs more phosphorylation to the critical S314 site (Wong et al., 2019). These effects of AHL10 on RRP6L1 chromatin association were more apparent in the low $\psi_w$ stress treatment, consistent with the low $\psi_w$-induced increase of AHL10-RRP6L1 association seen in rBiFC assays (Fig. 1B).

Conversely, the proportion of wild type AHL10 (labeled as N.M., for Non-Mutated) in the chromatin-associated fraction was not affected by *rrp6l1-2*, even though it was significantly increased by low $\psi_w$ stress; (Fig. 3A and C). We also found that the $AHL10^{S313A}$, $AHL10^{S313D}$, and $AHL10^{S314D}$ phosphomimic and null mutations did not affect AHL10 presence in the chromatin-associated fraction (Fig. 3A and D), consistent with phosphorylation of the C-terminal domain having no direct effect on DNA binding activity of the N-terminal AT-hook domain. $AHL10^{S314A}$ may have been slightly less present in the chromatin-associated fraction at low $\psi_w$, however, the effect was marginally non-significant ($P = 0.06$) and thus should be interpreted with caution.

The remaining pellet after chromatin-associated proteins were released by sonication did not contain detectable AHL10 or RRP6L1, suggesting that neither protein was part of other, non-chromatin, insoluble protein aggregates in the nucleus. Histone 3 (H3) was used as a control chromatin-associated protein (Fig. 3C). Note that our assays indicated that low $\psi_w$ stress led to an increased level of H3 in the soluble nuclear fraction but had little effect on the chromatin bound fraction. This indicated that the increased chromatin association of AHL1- and RRP6L1 in the stress treatment was a specific effect and not a general effect of the low $\psi_w$ treatment on all nuclear proteins.

**RRP6L1 and AHL10 affect the expression of an overlapping set of low $\psi_w$-responsive genes**

We conducted transcriptome analysis of *rrp6l1-2* under unstressed conditions and after four-day acclimation to moderate severity low $\psi_w$ stress (-0.7 MPa). This relatively long term low $\psi_w$ treatment was used to find genes that undergo sustained induction or repression and thus are more likely to be associated with the differences in growth between *rrp6l1* versus wild type at low $\psi_w$. The *rrp6l1-2* transcriptome data were collected in the same set of experiments as *ahl10-1* and wild type data previously reported (Wong et al., 2019). In comparison of unstressed wild type to unstressed *rrp6l1-2*, we found 147 genes having increased expression and 147 genes having decreased expression, including *RRP6L1*

itself (log2 fold-change ≥ 0.5, adjusted P ≤ 0.05; Supplemental Table S1). Genes up- or down-regulated in *rrp6l1-2* had the same direction of change in *ahl10-1* in nearly all cases (Supplemental Fig. S7A), albeit that the *ahl10-1* effect was weaker in many cases. This was consistent with AHL10 and RRP6L1 acting together in gene regulation. Many of the genes with altered expression in unstressed *rrp6l1-2* were stress responsive in wild type (Supplemental Fig. S7A). Particularly, genes with increased expression in unstressed *rrp6l1-2* were enriched for GO terms related to abiotic stress or environmental stimuli (Supplemental Table S2), suggesting that RRP6L1 has a role in preventing aberrant expression of these genes in unstressed conditions.

In the low $\psi_w$ stress treatment, relatively fewer genes were differentially expressed in *rrp6l1-2* (11 genes had increased expression, 11 genes had decreased expression including *RRP6L1*; log2 fold-change ≥ 0.5 versus wild type at low $\psi_w$, adjusted P ≤ 0.05; Supplemental Table S3). There was again substantial concordance between gene expression changes seen in *rrp6l1-2* and *ahl10-1* (Fig. 4A). This concordance could be seen even more clearly if we considered a slightly relaxed cutoff for differential expression (log2 fold-change ≥ 0.3, adjusted P ≤ 0.05) as shown in Supplemental Fig. S7B. The closest concordance between *rrp6l1-2* and *ahl10-1* was for genes that had decreased expression in both mutants (Fig. 4A; Supplemental Fig. S7B). We validated the decreased expression of a number of these genes in low $\psi_w$-treated *rrp6l1-2* and *ahl10-1* by QPCR (Fig. 4B). Interestingly, given the proposed functions of *rrp6l1-2* in RNA-directed DNA methylation (Wendte et al., 2017), *RNA-Dependent RNA Polymerase 3* (*RdR3*) and *RdR4* had decreased expression in both *rrp6l1-2* and *ahl10-1*. These two RdRs are of unclear physiological function (Willmann et al., 2011). The large number of genes with relatively small change in expression in *rrp6l1-2* and *ahl10-1* (Supplemental Fig. S7B) was not unexpected given the tissue specific accumulation of both proteins (Fig. 2) such that localized changes in gene expression may have been diluted in the whole seedling samples used for RNAseq.

AHL10 has been previously associated with AT-rich transposons (Jiang et al., 2017). Our transcriptome analysis of *ahl10-1* found altered RNA levels of several transposons (Wong et al., 2019) and many of these had similarly altered expression in *rrp6l1-2*. For example, the copia-like retrotransposon *At5g35935* and hAT-like transposase family gene *At3g29120* were the two most highly upregulated genes in the low $\psi_w$ treatment for both *ahl10-1* and *rrp6l1-2* (Fig. 4A; Supplemental Table S3). *At5g35935* is adjacent to the promoter region of *At5g35940* which was down-regulated in both *rrp6l1-2* and *ahl10-1*.

ChIP assays found low $\psi_w$-induced enrichment of AHL10 at several AT-rich sites in the region between *At5g35935* and *At5g35940* (Fig. 4C). This stress-induced association was not observed for a nearby region in the *At5g35940* gene body or a distal off-site (EIF4a promoter). A similar enrichment pattern was observed for AHL10$^{S314A}$, consistent with the idea that the phosphonull mutation in the C-terminal region of AHL10 does not interfere with the DNA binding region in the N-terminal part of the protein. We also conducted ChIP assays for AT-rich promoter regions within 1 kb of the transcriptional start site for several other genes having decreased expression in *ahl10-1* and *rrp6l1-2* (Supplemental Fig. S8). However, we did not find enrichment of AHL10 on those sites. Possibly AHL10 is associated with more distal sites which influence expression of those genes by formation of chromatin loops which bring AHL10 (and RRP6L1) in closer proximity to the gene or its newly transcribed RNA.

Two studies have previously identified regions of reduced methylation in *rrp6l1-2* (Zhang et al., 2014; Wendte et al., 2017) or conducted transcriptome analysis of *rrp6l1-2* (Zhang et al., 2014). However, we found little overlap between *rrp6l1-2* differentially expressed genes identified by Zhang et al. (2014) and those identified in our analysis (Supplemental Fig. S9A) or between *rrp6l* DEGs identified in any study and *rrp6l1*-related demethylated regions (Supplemental Fig. S9B). The limited overlap in differentially expressed genes and demethylated regions between these data sets suggests a strong effect of environmental conditions on RRP6L1 action in transcriptional regulation.

## Discussion

Our results demonstrate that RRP6L1 is involved in low $\psi_w$ response, that AHL10 and RRP6L1 interact and, that AHL10 S314 phosphorylation is required for chromatin association of RRP6L1. Both RRP6L1 and AHL10 protein levels were high in meristem tissue indicating that this is where AHL10-RRP6L1 interaction mainly occurs and where AHL10 and RRP6L1 exert their effects on growth during low $\psi_w$ stress. High levels of AHL10 and RRP6L1 in meristem can explain their effects on expression of meristem-specific genes such as *STM* and the auxin-related genes such as *WES1* and *DFL1*. In the context of previous work, our results indicate that control of AHL10 phosphorylation by competing action of MPKs versus Clade A PP2C (particularly HAI1) in meristems is a new connection

of stress-related phospho-signaling to growth suppression during low $\psi_w$ stress and gene regulation.

The lack of RRP6L1 chromatin recruitment in *ahl10-1* during low $\psi_w$ stress is surprising as it indicates that AHL10 has a controlling influence on RRP6L1 compared with other RRP6L1-interacting proteins such as the C-Terminal Domain (CTD) of NRPE1, the largest subunit of RNA Polymerease V (Wendte et al., 2017). In that study it was stated that RRP6L1 affects only about 20% of RdDM-regulated loci and the authors speculated that RRP6L1 interaction with the CTD is transient or involves only a small portion of Pol V. Our data suggest that the converse may also be true: CTD interaction may involve only a small portion of RRP6L1, particularly during low $\psi_w$ stress. Alternatively, RRP6L1 interaction with AHL10 could precede, or be required for, CTD interaction (or other interactions) that position RRP6L1 at specific chromatin locations. Zhang et al. (2014) noted that it is not known what factors determine RRP6L1 preference for certain methylation sites over others. Determining whether AHL10 binding is competitive with CTD binding, or whether AHL10 binding can influence the 3'-5' exonuclease activity of RRP6L1 (Zhang et al., 2014) will be questions of interest for future studies. It should also be kept in mind that previous studies of RRP6L1 (Lange et al., 2008; Shin and Chekanova, 2014; Zhang et al., 2014; Ye et al., 2016; Wendte et al., 2017) have only observed RRP6L1 function under unstressed conditions and mainly focused on RRP6L1 effects on non-coding RNAs. Thus, it is unclear how their proposed mechanisms of RRP6L1 function may be altered by low $\psi_w$ stress and whether RRP6L1 affects protein coding genes via distinct mechanisms.

The lack of RRP6L1 chromatin recruitment in *ahl10-1* is also surprising given that one may have expected a greater degree of redundancy among the closely related Clade B AHLs. Also surprising is our observation that AHL10 and AHL13 are not genetically redundant, despite their over high similarity and conserved C-terminal phosphorylation site (Wong et al., 2019; Rayapuram et al., 2021). Instead, *ahl13-1* blocked the *ahl10-1* mutant phenotype of increased growth maintenance during low $\psi_w$ stress. While they are not redundant, AHL10 and AHL13 have common regulatory targets. For example, both affect the regulation of JA-related genes (*AOC1, AOS*) in response to low $\psi_w$ (for *ahl10-1*) or pathogen infection (for *ahl13-1;* Wong et al., 2019; Rayapuram et al., 2021). These seemingly contradictory results can be interpreted in light of previous evidence that AHLs act as trimers (Zhao et al., 2013). It is presumably these trimeric complexes that recruit other transcriptional regulators; albeit, this hypothesis awaits direct testing *in planta*. AHL13 may

act as an anchor for different AHL complexes, thus explaining why it is genetically downstream of AHL10. In this case, the combination of AHL13 with AHL10 gives specificity of recruiting additional regulators such as RRP6L1. Knocking out one of these genes disrupts the composition of AHL complexes and thus disrupts recruitment of interacting proteins and downstream phenotypes in ways more complex than simple redundancy among AHLs.

**Data Availability**

RNAseq data has been deposited to Gene Expression Omnibus and is available under accession number GSE101197. All other data supporting the findings of this study are in the paper and supplemental material.

**Supplemental Data**

Supplemental Figure S1: AHL10 interactors.

Supplemental Figure S2: *In-vitro* pull down experiments confirm direct interaction of AHL10 and RRP6L1.

Supplemental Figure S3: Complementation *of ahl10-1* with AHL10 phosphonull and phosphomimic mutants under control of the *AHL10* native promoter.

Supplemental Figure S4: AHL10 accumulates in root and shoot meristems.

Supplemental Figure S5: Effect of phospho-null and phospho-mimic mutations on AHL10 sub-nuclear localization pattern.

Supplemental Figure S6: Effect of *RRP6L1, AHL10* and *AHL13* mutants on growth during moderate severity low water potential stress.

Supplemental Figure S7: Gene expression changes in *rrp6l1-2* and *ahl10-1* compared to low water potential stress-induced changes in wild type.

Supplemental Figure S8: ChIP assay of AT-rich promoter sites for other genes with decreased expression in *ahl10-1* and *rrp6l1-2* under low water potential stress.

Supplemental Figure S9: Comparison of our *rrp6l1-2* transcriptome data to genes in or near hypomethylated regions in previously reported analyses.

Supplemental Table S1: Genes up or down regulated in *rrp6l1-2* compared to Col-0 wild type in the unstressed control treatment.

Supplemental Table S2: Gene ontology analysis of genes up or down regulated in *rrp6l1-2* in the unstressed control treatment.

Supplemental Table S3: Genes up or down regulated in *rrp6l1-2* compared to Col-0 wild type in the low water potential stress treatment (-0.7 MPa, 96 h).
Supplemental Table S4: Primers used for cloning, genotyping, mutagenesis, gene expression analysis and ChIP.

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**Author Contributions**
P.E.V. conceived research, M.M.W., Y.-C.B. and P.E.V. designed experiments, M.M.W., X.-J..H and Y.-C.B. performed experiments, M.M.W., X.-J..H, Y.-C.B. and P.E.V. analyzed data, P.E.V. wrote the manuscript with assistance from M.M.W. All authors read and approved the manuscript.

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## Figures and Legends

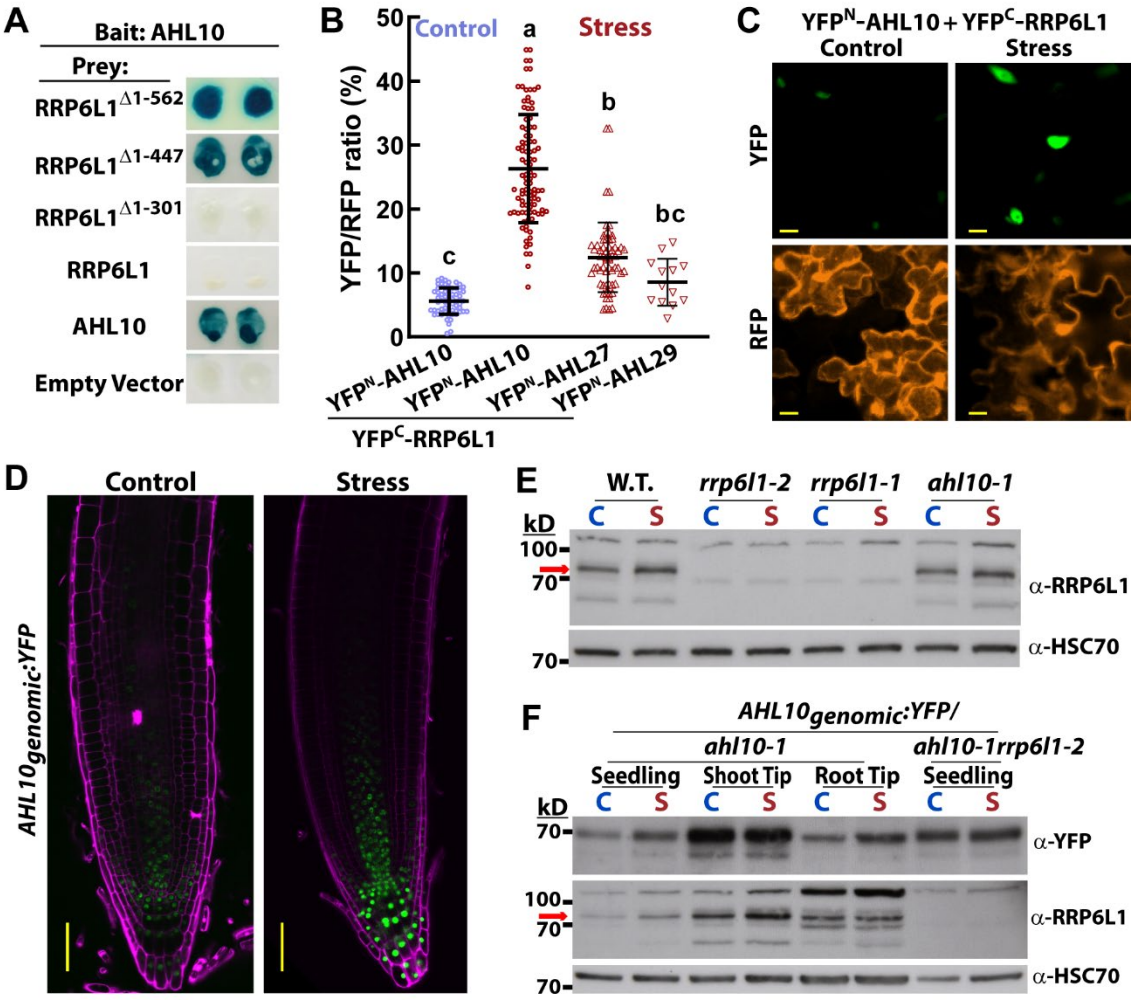


**Figure 1: AHL10 interacts with RRP6l1 and both proteins accumulate in root and shoot meristem regions.**

A. β-galactosidase staining colony lift assays show the interaction of AHL10 with clones encoding the C-terminal portion of RRP6L1. RRP6L1$^{\Delta 1\text{-}562}$ is the clone found in the original yeast two hybrid library screening. Other truncations and full length *RRP6L1* clones were used to confirm the interaction. AHL10 self-interaction is shown for comparison as another strong interaction detected in the yeast two hybrid screen. Empty prey vector is a negative control. Additional proteins found in the yeast two hybrid screen are listed in Supplemental Fig. S1A.

B. Quantification of interaction intensity for rBIFC assays conducted using unstressed Arabidopsis seedlings or seedlings exposed to low $\psi_w$ stress (-0.7 MPa for 48 h).

Individual data points represent the YFP/RFP fluorescence intensity ratio of individual cells. Data are combined from three independent experiments (n = 13 - 97). Different letters indicate statistically significant differences (ANOVA, corrected $P \leq 0.05$).

C. Representative images of rBiFC data presented in B. Scale bars indicate 10 μm.

D. Localization of AHL10-YFP expressed under control of its native promoter (genomic clone of the *AHL10* gene body, 5; UTR and 2.1 kb promoter region with C-terminal YFP fusion). Images show the primary root tip of 9-day-old seedlings growing control media or four days after transfer to low $\psi_w$ (-0.7 MPa). Scale bars indicate 50 μm.

E. Validation of polyclonal antisera raised against the C-terminal region of RRP6L1. Red arrow marks major band corresponding to the molecular weight of RRP6L1 (74 kD) which is absent in both *rrp6l1* mutants. 35 μg of protein was loaded in each lane. A non-specific band was detected at approximately 110 kD. The blot was stripped and re-probed with anti-HSC70 as a loading control. The experiment was repeated with essentially identical results. C = Control; S = Stress (-0.7 MPa for four days). The blot was stripped and re-probed with anti-HSC70 as a loading control.

F. Detection of RRP6L1 and AHL10-YFP in whole seedling, shoot meristem-enriched (shoot tip), and root meristem-enriched (root tip) samples. 35 μg of protein was loaded in each lane. The RRP6L1 blot was stripped and re-probed with anti-HSC70 as a loading control. The experiment was repeated three times with consistent results each time.

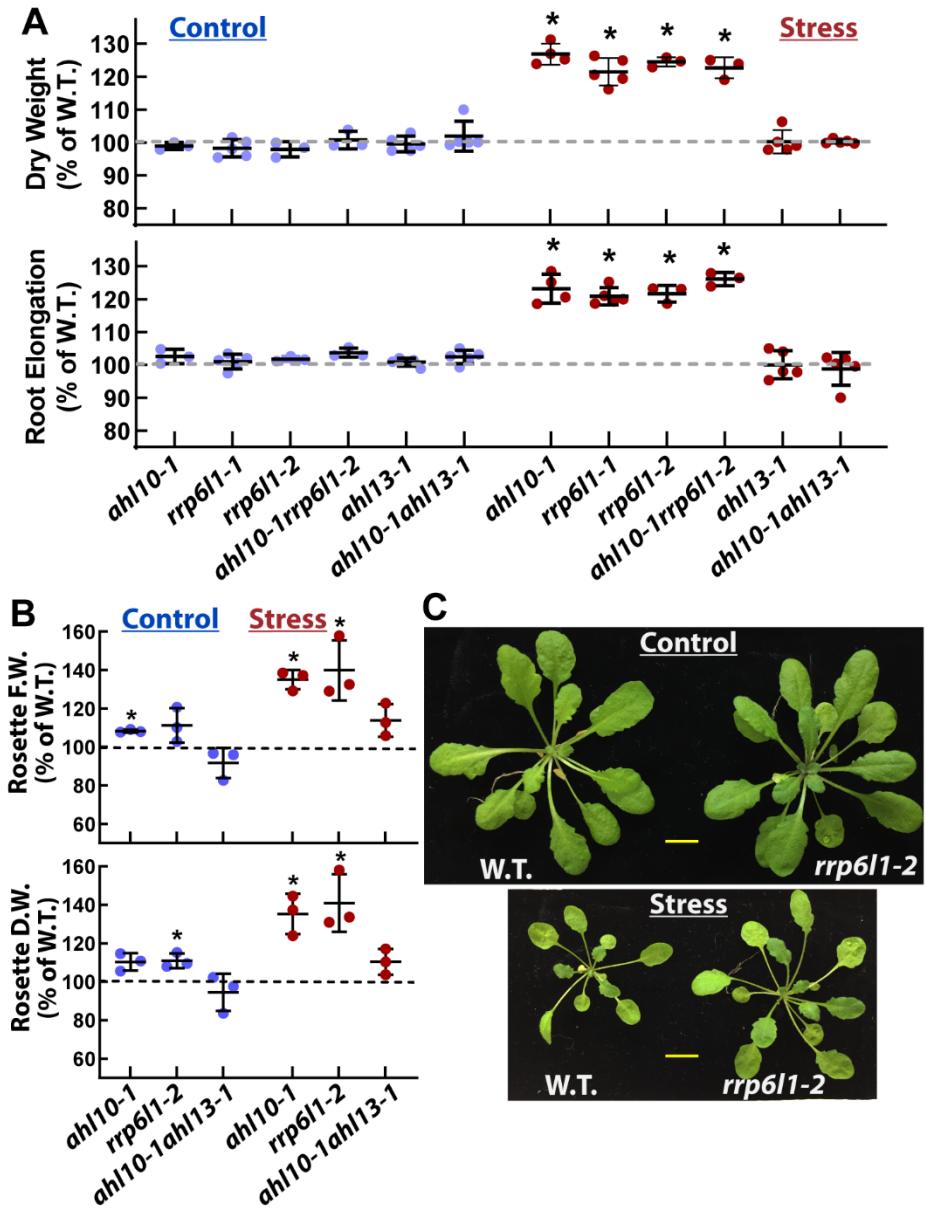


**Figure 2: *RRP6L1* is a negative regulator of growth during moderate severity low $\psi_w$ stress and *ahl10-1* effect on growth depends upon *AHL13*.**

A. Seedling dry weight and primary root elongation in control and stress treatments. For control, five-day-old seedlings were transferred to fresh control agar plates with root length increase and total seedling dry weight measured five days after transfer. For stress treatment, five-day-old seedlings were transferred from control media to PEG-infused agar plates (-0.7 MPa) and dry weight and root elongation measured 10 days after transfer. Data are expressed relative to wild type measured in the same plate as the test genotype. Pictures of representative seedlings in the stress treatment are shown in Supplemental Fig. S6A. Wild type dry weight and root elongation data used for normalization are shown in Supplemental Fig. S6B. Data are means

± S.D. for 3-5 replicate experiments. Asterisks (*) indicate significant difference from 100% (wild type level) by one sample T-test.

B. Rosette fresh and dry weights for soil grown plants kept under well-watered conditions or exposed to partial soil drying. Data are expressed relative to wild type plants grown together in the same pot. Data are means ± S.D. for 3-5 replicate experiments (2-3 biological replicates per experiment). Asterisks (*) indicate significant difference from 100% (wild type level) by on sample T-test.

C. Pictures of representative wild type and *rrp6l1-2* plants from the experiments presented in B. Scale bars indicate 1 cm.

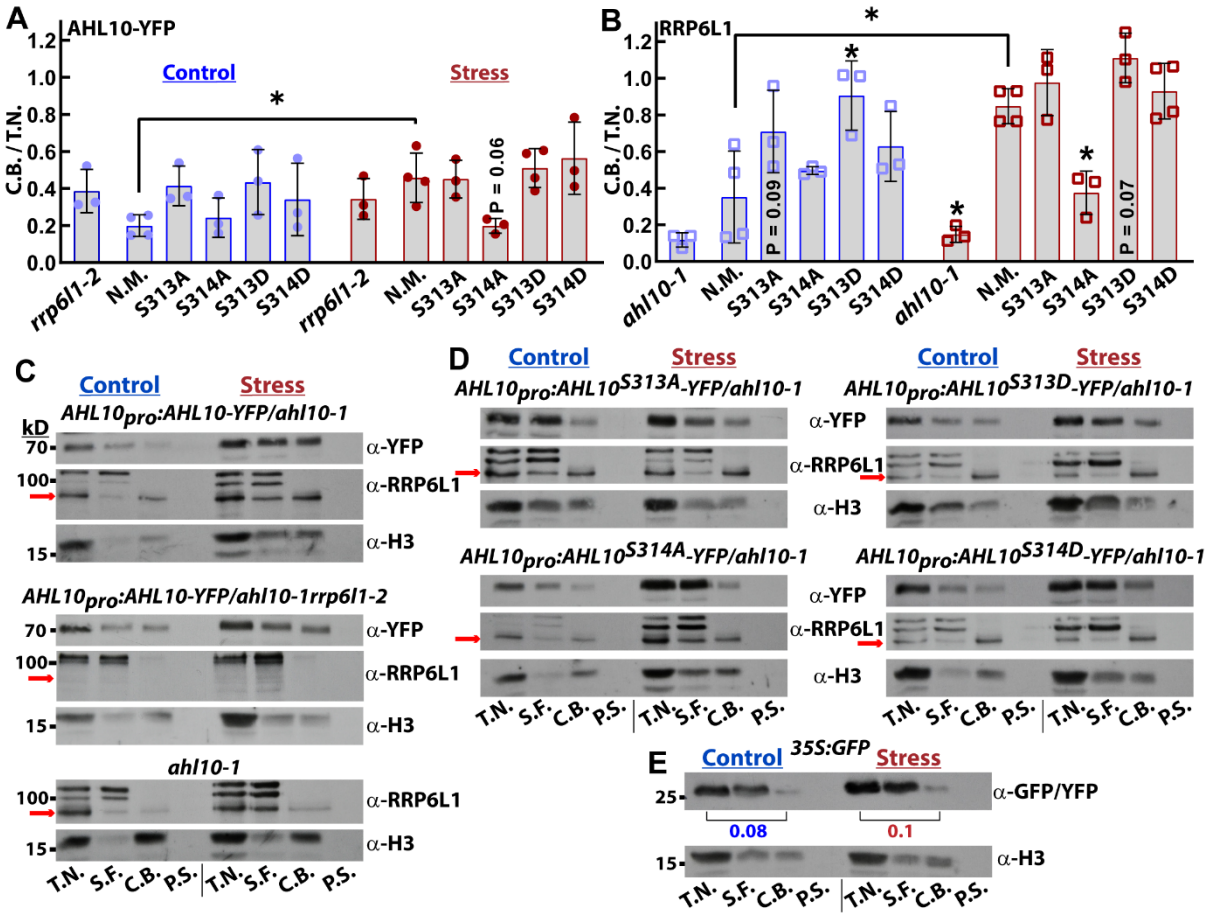


**Figure 3: RRP6L1 chromatin association depends upon AHL10 S314 phosphorylation.**

A. Portion of AHL10-YFP associated with chromatin, measured as the ratio of chromatin bound (C.B.) versus total nuclear (T.N.) immunoblot band intensities for *rrp6l1-2* or *ahl10-1* complemented with non-mutated AHL10 (N.M), phosphonull AHL10 (S313A, S314A) or phosphomimic AHL10 (S313D, S314D). Representative blots used for quantitation can be seen in C and D. Samples were collected for 11-day-old seedlings kept under unstressed control conditions or transferred to low $\psi_w$ stress treatment (-0.7 MPa for four days). Data are means ± S.D. for three or four independent experiments (one replicate per experiment). Asterisks or indicated P value at the bars in the graph indicate the results of ANOVA comparing all genotypes within the stress or control treatments to the wild type AHL10 (N.M.) data for that treatment. The wild type AHL10 (N.M.) data were further compared between control and stress treatment using T-test (Asterisk indicates significant difference, $P \leq 0.05$).

B. Portion of RRP6L1 associated with chromatin measured as the ratio of chromatin bound (C.B.) versus total nuclear (T.N.) immunoblot band intensities for *ahl10-1* or

*ahl10-1* complemented with non-mutated AHL10 (N.M), phosphonull AHL10 (S313A, S314A) or phosphomimic AHL10 (S313D, S314D). Representative blots used for quantitation can be seen in C and D. Sampling conditions and statistical analysis are as described in A. Asterisks above bars indicate a significant difference compared to AHL10 N.M. in that treatment ($P \leq 0.05$).

C. Representative blots used for band intensity quantitation shown in A and B. The AHL10 genomic fragment with 5' UTR and 2.1 kb promoter region was fused to YFP and expressed in the *ahl10-1* or *ahl10-1rrp6l1-2* genetic backgrounds to detect AHL10 chromatin association in the presence of absence of RRP6L1. The *ahl10-1* mutant was used to detect RRP6L1 chromatin association in the absence of AHL10. Red arrow indicates the RRP6L1 specific band of molecular weight corresponding to the full-length protein which was used for quantification. T.N. = Total Nuclear; S.F. = Soluble Fraction of nuclear proteins; C.B. = Chromatin Bound; P.S. = Pellet after Sonication (to check for remaining insoluble protein). Detection of Histone3 (H3) was performed as a control for the purity of the S.F. and C.B fractions. However, note that we consistently observed that stress treatment caused an increase in the amount of H3 in the soluble fraction which was not related to any technical aspects of the chromatin precipitation procedure.

D. Representative blots of lines expressing phosphomimic or phosphonull AHL10 used for band intensity quantification shown in A and B.

E. Nuclear fractionation and chromatin association of a transgenic line expressing GFP under control of the constitutive *35S* promoter used to check the background level of a non-chromatin associated protein in the chromatin pellet. Numbers under the GFP blot indicate the fraction of chromatin associated GFP based on the ratio of C.B and T.N. band intensities. Data are representative of two biologically independent experiments.

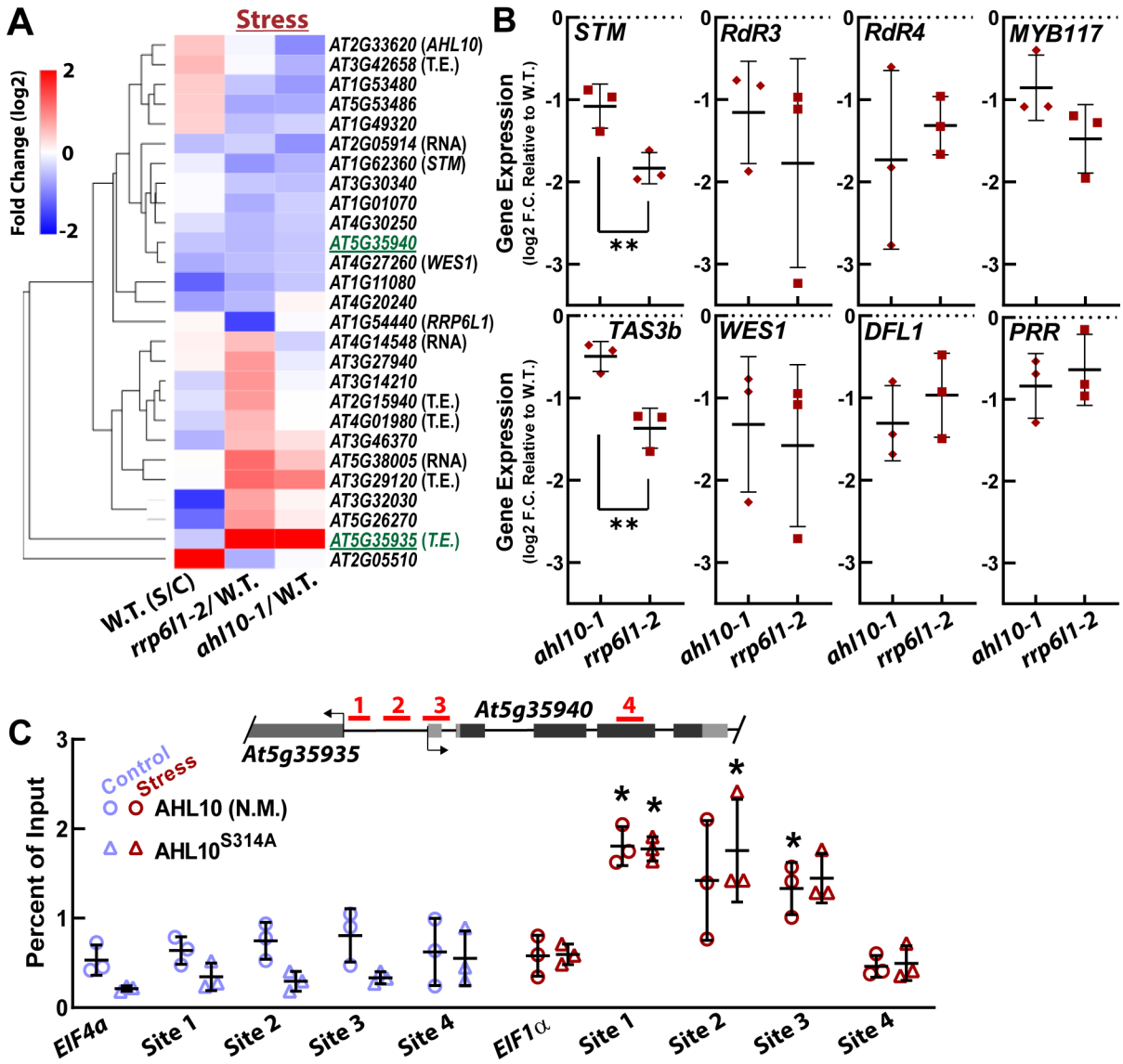


**Figure 4: *rrp6l1-2* and *ahl10-1* have concordant effects on gene expression and low $\psi_w$ stress can increase association of AHL10 with specific AT-rich regions.**

A. Heat map and hierarchical clustering of genes having significantly increased or decreased expression (log2 fold change ≥ 0.5, corrected P-value ≤ 0.05) in stress-treated (transfer to -0.7 MPa for four days) seedlings. Effect of stress treatment on these genes in wild type is also shown for comparison. Underlined genes were used as targets of ChIP assay shown in Fig. 4C. Similar heat map of genes with altered expression in unstressed *rrp6l1-2* or *ahl10-1* is shown in supplemental Fig. S7A. Heat map of genes affected by stress in *rrp6l1-2* or *ahl10-1* at slightly less stringent cutoff (log2 fold change ≥ 0.3, corrected P-value ≤ 0.05) is shown in Supplemental Fig. S7B. Genes labeled as "RNA" are non-coding RNAs. T.E. = Transposable Element.

B. QPCR validation of gene expression differences of *rrp6l1-2* and *ahl10-1* compared to wild type in the stress treatment. Note that some of the genes selected for validation did not meet the cutoff for inclusion in the heat map shown in Fig. 4A but are included in the heat map shown in Supplemental Fig S7B. Data are means ± S.D. for data from 3 independent biological experiments (one sample per experiment, three technical PCR replicates per sample). Data are shown as log2 fold change relative to wild type. Asterisks indicate significant difference between *ahl10-1* and *rrp6l1-2* by T-test (**, $P \leq 0.01$). Note that for some genes (*STM, WES1, DFL1, MYB117* and *PRR*) the QPCR data *ahl10-1* was previously reported in Wong et al. (2019) and is shown again here to facilitate comparison to *rrp6l1-2*.

C. ChIP assays of AT-rich sites lying in the region between *At5g35935* and *At5g35940* (*At5g35940* promoter region) as well as an off-site in exon of *At5g35940* and a distal offsite (*EIF4a* promoter). Samples were collected for unstressed and low $\psi_w$ -treated (-0.7 MPa for four days) seedlings. Data are means ± S.D. for data from 3 independent biological experiments (one sample per experiment, three technical PCR replicates per sample). Asterisks indicate significant differences compared to the *EIF1*$\alpha$ offsite in the stress treatment (ANOVA, $P \leq 0.05$). No significant differences were found in the unstressed control.