RESEARCH ARTICLE

Antisense transcription from stress-responsive transcription factors fine-tunes the cold response in Arabidopsis

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Short title: Detection of cold-responsive transcription factors

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ABSTRACT

Transcription of antisense long noncoding RNAs (lncRNAs) occurs pervasively across eukaryotic genomes. Only a few antisense lncRNAs have been characterized and shown to control biological processes, albeit with idiosyncratic regulatory mechanisms. Thus, we largely lack knowledge about the general role of antisense transcription in eukaryotic organisms. Here, we characterized genes with antisense transcription initiating close to the Poly(A) signal (PAS genes) in Arabidopsis (*Arabidopsis thaliana*). We compared plant native elongation transcript sequencing (plaNET-seq) with RNA sequencing (RNA-seq) during short-term cold exposure and detected massive differences between the response in active transcription and steady-state levels of PAS gene-derived mRNAs. The cold-induced expression of transcription factors *B-BOX DOMAIN PROTEIN28 (BBX28)* and *C2H2-TYPE ZINC FINGER FAMILY PROTEIN5 (ZAT5)* was detected by plaNET-seq, while their steady-state level was only slightly altered due to high mRNA turnover. Knockdown of *BBX28* and *ZAT5* or of their respective antisense transcripts severely compromised plant freezing tolerance. Decreased antisense transcript expression levels resulted in a reduced cold response of *BBX28* and *ZAT5*, revealing a positive regulatory role of both antisense transcripts. This study expands the known repertoire of noncoding transcripts. It highlights that native transcription approaches can complement steady state RNA techniques to identify biologically relevant players in stress responses.

INTRODUCTION

Widespread long non-coding transcription from the complementary DNA (antisense) strand is present at thousands of protein-coding gene loci in eukaryotic organisms. Recent research in plants has evidenced antisense transcription for 30% of expressed genes in the model plant Arabidopsis (*Arabidopsis thaliana*) and for 60% of the genes in rice (Chen et al., 2019; Kindgren et al., 2019). Our current understanding is that the majority of antisense transcripts, also called *cis*-natural antisense transcripts (*cis*-NATs),

are long non-coding transcripts (>200 nucleotides in length, lncRNA), but their general functional significance remains elusive (Reis and Poirier, 2021). Complementarity between *cis*-NATs and the sense transcript, coupled with the overlapping spatiotemporal expression of sense-antisense pairs, endows them with the potential to engage in the formation double-stranded RNA (dsRNA). dsRNA could undergo subsequent detection by the factors of RNA silencing machinery such as Dicer or Dicer-like (DCL) and Argonaute (AGO) family proteins. However, there is weak evidence that endogenous small interfering RNAs (siRNAs) are derived from NAT-sense pairs (Henz et al., 2007; Reis and Poirier, 2021). Only a handful of studies from different plant species describe a negative role of NATs over the sense transcription (Borsani et al., 2005; Katiyar-Agarwal et al., 2006; Swiezewski et al., 2007; Held et al., 2008; Wan et al., 2016). So far, few *cis*-NATs in plants have been experimentally characterized with mechanistic insights, thereby limiting our understanding of their general function (Wierzbicki et al., 2021). Thus, we only have rudimentary knowledge of the roles of antisense transcription, and its widespread prevalence is certainly an enigma in modern plant research.

Our global numerical comprehension of non-coding transcription has been mostly based on sequencing technologies that fundamentally use the steady-state RNA detection principle. For example, in Arabidopsis, 70% of mRNAs were postulated to form sense-antisense pairs based upon RNA-sequencing (RNA-seq) experiments (Wang et al., 2014). However, non-coding transcription is far more pervasive and complex than visualized by canonical steady-state analyses. A major challenge in the field is the detection of antisense and other non-coding transcription due to the low abundance and high turnover rate (Mayer et al., 2015; Kindgren et al., 2019; Thieffry et al., 2020). Classical steady-state level detection methods, such as RNA-seq, are ill-suited to investigate non-coding transcription. In contrast, a technique that investigates active transcription (i.e., Native Elongation Transcript sequencing (NET-seq)) is better suited for detecting rapidly degraded RNA species (Mayer et al., 2015). NET-seq in plants (plaNET-seq or pNET-seq) captures actively transcribing RNA Polymerase II (RNAPII) complexes and enables strand-specific sequencing of the RNA molecules associated with the captured RNAPII complexes before any degradation can occur, thereby

uncovering all transcription events genome-wide (Zhu et al., 2018; Kindgren et al., 2019). Case-in-point, plaNET-seq detected ~8,000 unannotated long non-coding transcripts, many of them being antisense transcripts initiating from the host gene's 3′-end (Poly(A) antisense genes, PAS genes) (Kindgren et al., 2019).

Emerging evidence indicates that antisense transcription is essential for plant development and stress responses (Matsui et al., 2008; Thieffry et al., 2020). However, the underlying molecular mechanism(s) involved in sense/antisense transcriptional crosstalk often seem idiosyncratic with few common components involved (reviewed in (Lucero et al., 2021)). Nevertheless, antisense RNAs have been characterized to control seed germination (Fedak et al., 2016), phosphate starvation (Jabnoune et al., 2013), flowering control (Swiezewski et al., 2007; Henriques et al., 2017), hormonal regulation (Ariel et al., 2020), and cold acclimation (Kindgren et al., 2018). Cold acclimation, initiated by transcriptional processes, allows plants to adapt and eventually withstand freezing. At present cold stress is the sole abiotic stress monitored by plaNET-seq (Kindgren et al., 2019). Remarkably, in plaNET-seq datasets, antisense transcription at PAS genes responds rapidly to cold with a global downregulation, suggesting a putative role in the cold-response (Kindgren et al., 2019). Thus, exposure to cold temperature is an excellent environmental cue for studying antisense transcription's role in Arabidopsis.

In this study, we use plaNET-seq and RNA-seq data to show an earlier hidden layer of genes involved in cold acclimation. We show that RNA-seq data poorly captures the cold stress induced transcriptional changes of protein coding genes. On the contrary, plaNET-seq efficiently uncovers these transcriptional changes since transcription is captured prior to their degradation. Among those, we identified two cold-responsive transcription factors that responded natively as crucial for cold acclimation in Arabidopsis. Importantly, experimental validation demonstrated that their antisense transcription is positively correlated to the stress responsiveness of the sense transcription, suggesting a role for antisense transcription in assisting certain transcription factors' stress responses, such as cold exposure.

RESULTS

PAS genes are enriched in stress responsive transcription factors with high active transcription

Genes that host PAS transcription (PAS genes) represent over 3000 genes in Arabidopsis and were recently detected by plaNET-seq (Kindgren et al., 2019). PAS genes are defined as protein coding genes with antisense transcription initiating in the 3′-half or 20% of the gene length downstream of the sense gene's PAS (Figure 1A, Supplemental Data Set S1). Characterization of PAS genes has not been done rigorously in Arabidopsis, so we first investigated which biological processes and molecular functions were overrepresented in the list of all PAS genes (Figure 1B-C, Supplemental Data Set S2). Interestingly, there was a clear enrichment of stress-responsive genes that are involved in DNA binding (in particular, transcription factors (TFs)). Therefore, for our further analysis, we decided to divide the PAS genes into PAS TFs (*n = 294*) and PAS non-TFs. We also included TFs without PAS (TFs non-PAS) as a control group. An example of a known stress-responsive gene encoding a TF with an antisense transcript that responds natively to cold temperature was *WRKY48* ((Xing et al., 2008), Figure 1D).

Overall, PAS genes tended to be shorter than other expressed genes and TFs non-PAS (Figure 2A) and the steady-state levels of PAS non-TFs or PAS TFs gene mRNA were not significantly different from those of all expressed genes (Figure 2B). Surprisingly, PAS gene mRNA had a greater turnover rate compared to all expressed genes when measured by RNA-seq after treatment with the transcription inhibitor, cordycepin (alpha decay) (Figure 2C) (Sorenson et al., 2018). TFs non-PAS also showed a significantly higher turnover rate, suggesting that the rapid degradation of mRNA in these classes of genes are independent of antisense transcription. We confirmed these results with data from transcription inhibition with another inhibitor, actinomycin D (Supplemental Figure S1A) (Narsai et al., 2007). Both PAS non-TFs and PAS TFs genes displayed high active RNAPII transcription at the genome-wide level compared to expressed genes and TFs non-PAS (Figure 2D, Supplemental Figure S1B). In addition, PAS-TFs showed a higher RNAPII occupancy compared to PAS non-

TFs (Supplemental Figure S1B). Thus, the higher expression level of PAS genes and high turnover rate explained the small effects on RNA steady state-levels. Taken together, PAS genes are enriched in stress responsive transcription factors with high transcriptional and post-transcriptional regulation. Moreover, the high turnover rate seems to be a general feature of TF mRNAs and not a consequence of PAS TF antisense transcription.

The expression levels of cold responsive genes detected by plaNET-seq correlate poorly with RNA-seq

The high transcription activity of PAS genes and rapid turnover of their mRNA made us interested in comparing the genome-wide steady state levels of mRNA to how genes are actively transcribed (plaNET-seq). Thus, we performed RNA-seq using samples from seedlings grown similarly to the plaNET-seq experiment (10 days in long day conditions at 22°C and cold stress samples from 3h at 4°C and 12 h at 4°C). In our cold-treated RNA-seq dataset, many mRNAs drastically changed their steady-state level (Figure 3A, Supplemental Data Set S3). When we compared DE genes (fold change) between our RNA-seq and plaNET-seq datasets, we saw that RNA-seq poorly reflected the genes differentially expressed determined by plaNET-seq (Kindgren et al., 2019). The overlap was only 616 of the up-regulated (UP) genes and 270 of the down-regulated (DOWN) genes in the same direction after 3h at 4°C (Figure 3A). After 12h at 4°C, we observed increased overlap, 1819 of UP genes and 1674 of DOWN genes (Figure 3A). In addition, we saw a low correlation between the DE gene fold change at both time points (Figure 3B-C). This suggests that there is a clear discrepancy between the active transcriptional changes (plaNET-seq) and changes to the steady-state levels of mRNA (RNA-seq) occurring during cold stress, especially early in the cold response.

Focusing on the PAS genes, we saw a similar pattern as in all DE genes (Figure 3D-F). Astoundingly, we found that 2214 (71%) PAS genes changed their expression significantly to cold (either in plaNET-seq or RNA-seq data) at one or both cold time points, corroborating their responsiveness to stress (Supplemental Data Set S4). Out of these genes, 229 were PAS TFs (78% were responsive to cold, Supplemental Data Set S4). To further confirm that PAS genes are highly responsive to cold, we compared

PAS genes UP after 3h 4°C with UP genes without antisense transcription (determined by plaNET-seq). At 22°C, PAS genes showed an increased RNAPII stalling around the +1 nucleosome, a hallmark for stress-responsiveness compared to other UP and non-DE genes (Figure 4A) and an overall high active transcription (Figure 4B). After 3h 4°C, PAS UP genes showed a more extreme response to cold temperature compared to other UP genes (Figure 4C-D), suggesting that, indeed, PAS genes have an enhanced responsiveness to cold compared to other genes. In contrast, antisense transcription for upregulated PAS genes remained similar throughout the cold response (Supplemental Figure S2). Taken together, these data strengthen the hypothesis that PAS genes are inclined to respond to cold temperatures, but there is no correlation between PAS host gene expression and antisense expression during the initial cold response. In addition, the discrepancy between plaNET-seq and RNA-seq argues that some transcription factors might have been overlooked as cold-responsive genes and highlight the use of combining nascent RNA methods with steady-state levels of mRNA to fully understand the cold response in an organism.

The discrepancy between active transcription and steady state levels can partly be explained by mRNA turnover rates

Next, we focused on the differentially expressed TFs with antisense transcription. We aimed to identify additional biologically important TFs in the cold response. A special emphasis was put on the expression pattern in plaNET-seq as the distinct dynamics of PAS TFs mRNAs (Figure 2) might have masked cold responsive TFs in earlier studies. We reasoned that rapidly responsive TFs had a high probability to be involved in cold acclimation since this expression pattern mirrors that of known assigned TFs in the cold response, such as C-REPEAT BINDING FACTOR2 (CBF2, At4g25470) (Figure 5A). *CBF2* is massively induced and transiently peaks after 3h at 4°C. Out of the 242 PAS TFs that responded to cold, 25 showed a similar plaNET-seq expression pattern to *CBF2* (UP after 3h and DOWN between 3 and 12h) (Supplemental Data Set S5). Only 9 of the 25 genes showed up-regulation by RNA-seq after 3h (Supplemental Data Set S5). An example of a gene that was DE in plaNET-seq but not in RNA-seq was *HY5-HOMOLOG* (*HYH*, At3g17609) (Figure 5B).

Due to the RNA degradation characteristics of PAS TFs, we first tested the mRNA turnover rates (by cordycepin incubation) for 5 randomly chosen genes from the 25 genes with convincing expression patterns in plaNET-seq and no or slight upregulation by RNA-seq. MYB DOMAIN PROTEIN-47 (MYB47, At1g18710) has a potential role in drought and hormone signaling (Ding et al., 2014; Marquis et al., 2022). CONSTANS-LIKE 7 (COL7, At1g73870) is involved in the shade avoidance response (Wang et al., 2013). The C2H2-type zinc finger family protein, *C2H2-TYPE ZINC FINGER FAMILY PROTEIN-5* (ZAT5 , At2g28200) and SCARECROW-LIKE-8 (SCL8, At5g52510), are uncharacterized proteins and B-BOX DOMAIN PROTEIN-28, (BBX28, At4g27310) has been characterized for its involvement in flowering and photomorphogenesis (Song et al., 2020; Cao et al., 2022). *MYB47, SCL8,* and *BBX28* showed a slight up-regulation in the RNA-seq data. However, neither BBX28, SCL8, MYB47 or any of the other chosen proteins have a known function in the cold response of Arabidopsis. Our assay control *EUKARYOTIC TRANSLATION INITIATION FACTOR-4A1* (*EIF4A1*), a stable mRNA at 22°C, showed similar stability at 4°C as compared to 22°C (Figure 5C). In addition, a recent study showed increased stability in cold for a key TF in the cold response, *CBF1* (Zacharaki et al., 2023). In contrast, all the PAS TF candidate genes' mRNAs, except *SCL8*, showed a significantly decreased transcript stability at 4°C compared to 22°C (Figure 5D-H). Thus, induced transcriptional activity at gene loci responding to cold may remain partly undetected by steady state methods due to rapid mRNA decay at 4°C. Again, our results highlight that nascent transcription methods can complement RNA steady state level methods to identify stress responsive genes, in particular those with highly dynamic regulation and mRNA turnover.

ZAT5 ***and*** **BBX28** ***are involved in cold acclimation***

Can mRNAs with minor differences in steady state levels during stress have an important biological role in the stress adaptation for the plant? To answer this, we focused on two candidate genes for our continued analysis, *ZAT5* and *BBX28*. Both genes responded rapidly to cold temperatures, as detected by plaNET-seq (Figure 6A-B). We could not detect any significantly increased steady-state level of *ZAT5* after

exposure to 4°C with RT-qPCR, although a positive trend was observed early in the cold response (Figure 7A). We did see a significant down-regulation from control levels starting at 8 h of 4°C (Figure 7A). For *BBX28*, we could not detect any significant up- or down-regulation, but a positive trend early in the cold response was observed (Figure 7B). The discrepancy between our RT-qPCR and RNA-seq results may suggest that the up-regulation of *BBX28* and *ZAT5* is slight and not always consistently statistically significant depending on separate cold treatments and the variation between replicates. However, since both *BBX28* and *ZAT5* have been found to be diurnally expressed (Romanowski et al., 2020), we also checked their steady state levels with controls (seedlings kept at 22°C) taken at the same time points as the cold samples (3h and 12 at 4°C). Here, we did see a small but significant increase of both *BBX28* and *ZAT5* after 3h (Supplemental Figure S3A-B).

To test their biological importance in the cold acclimation process, we isolated two independent T-DNA lines disrupting the two genes and subjected these lines to a freezing test together with wild type (Figure 7C-D). In the freezing test, leaf discs of non-acclimated and cold-acclimated plants (4 days in 4°C) are in contact with water and exposed to decreasing freezing temperatures and measured for plasma membrane disruption (i.e., leakage of electrolytes). Therefore, measuring electrolyte leakage is a measurement of how well the cells can survive freezing temperatures. Indeed, we found that both mutant lines were impaired in their acclimation to cold. For *zat5-1*, the freezing tolerance was significantly lower (−3.7±1.7°C, $p<0.0001$) compared to −6.1±0.2°C for WT. The freezing tolerance for *bbx28-1* was also significantly lower (−5.7±0.2°C, $p=0.0022$) compared to -6.5±0.2°C for WT. We could not detect any difference in non-acclimated plants (Supplemental Figure S3C-D), suggesting that both *ZAT5* and *BBX28* have a specific role in the cold acclimation process in Arabidopsis. Overall, these results indicate that changes to the transcription activity with minor changes to the mRNA steady state levels can have a significant biological role.

***Antisense transcription is required for proper regulation of* ZAT5 *and* BBX28**

Next, we turned to our second question; what is the role of antisense transcription along the *ZAT5* and *BBX28* gene body? Our plaNET-seq data revealed that both *ZAT5* and

BBX28 had antisense transcription affected by cold temperature, although the differences were small compared to sense expression (Figure 8A-B, upper panel). To identify the 5´ and 3′-end of *asZAT5* and *asBBX28*, we used available Cap Analysis of Gene Expression sequencing (CAGE) (Thieffry et al., 2020) and Direct RNA Sequencing (DRS) data (Schurch et al., 2014) (Figure 8A-B, middle and lower panels). Interestingly, both antisense transcripts are targets of the nuclear exosome (see CAGE data for the exosome mutants *hen2-2* and *rrp4-2*). Consequently, 5′-ends are more prominent in the exosome mutants, suggesting that the transcripts are degraded rapidly after their synthesis. We could see that there was no precise start or end to the antisense transcription, but rather a window at both ends. Antisense transcription starts well beyond the poly(A)-site of their host genes and navigates until at least 1 kb upstream of the host gene's transcription start site (TSS).

To investigate the role of the antisense transcription at the translational level, firefly luciferase (*LUC*) reporter constructs for *ZAT5* were generated, using GreenGate cloning system (Lampropoulos et al., 2013), with and without the 1403 bp DNA sequence that harbors *ZAT5* 3' UTR, associated PAS antisense transcript and putative promoter i.e. *ProZAT5:ZAT5-LUC-UTR-ASProZAT5* and *ProZAT5:ZAT5-LUC-tNOS*. The *Agrobacterium tumefaciens* cultures carrying these plasmids were used for infiltration of *Nicotiana benthamiana* leaves for transient expression assay (Supplemental Figure S4A-B). The first construct included the endogenous promoter and cDNA fused to the *LUC* gene. Downstream of the *LUC* gene was the endogenous untranslated 5′-region of *ZAT5* and the AS promoter. In the second construct, the *ZAT5* promoter and cDNA were fused to LUC followed by the strong tNOS terminator. The tNOS terminator diminishes any antisense transcription over the *LUC* and *ZAT5* gene body (Kindgren et al., 2018). The endogenous construct (construct 1 in Supplemental Figure S4) showed an induction after cold exposure, confirming that the *ZAT5* promoter is cold responsive and corresponds with positive transcriptional regulation of *ZAT5* by its antisense (Supplemental Figure S4C). At both 22°C and 4°C, the tNOS construct showed lower LUC activity compared to the full-length construct (Supplemental Figure S4D-E). The overall cold responsiveness decreased from 2.5-fold in the full-length construct to 2.0-fold in the tNOS construct, suggesting that the antisense transcription

over *ZAT5* has a positive role for the transcription level and stress responsiveness of the gene.

To further elaborate their role, the promoter region of the antisense transcription was targeted with the CRISPR-Cas9 approach to minimally alter and knockout only parts of their regulatory sequence and/or the 5′-end of the TSS window. That way, the direct interference with sense transcription would be minimized. For *ZAT5*, we were able to retrieve a mutant with a 283 bp deletion i.e. 392 bp from the start of the TSS window of *asZAT5* and 710 bp from the poly(A) site for *ZAT5* (Figure 9A). We named this mutant *aszat5-1*. For *BBX28*, we aimed to delete the 5′-end of the TSS window of *asBBX28*. We retrieved a 392 bp deletion that included a deletion of 220 bp of the TSS window (*asbbx28-1*, Figure 9A). The deletion is 193 bp from the poly(A) site of sense *BBX28*. In both mutants, we did not alter the stability of the respective mRNA, suggesting that we did not interfere with transcription termination (Supplemental Figure S5A-B).

In *aszat5-1*, there was a significant down-regulation of the nascent transcription of *asZAT5* at 22°C but we could not detect any difference after 3h 4°C (Figure 9B). For *ZAT5*, the mutant showed lower nascent transcription at both time points (Figure 9B). The steady-state levels of *ZAT5* showed a decreased level after cold treatment (Figure 9B). These results corroborate the results from the *ZAT5* constructs and suggests two key regulatory aspects, antisense transcription over the *ZAT5* locus is required for proper cold induction and that CRISPR-Cas9 targeted *asZAT5* promoter sequences contain regulatory elements in the antisense promoter to regulate the initiation of *asZAT5* transcription. To show a biological role for *asZAT5*, we performed cold acclimation and freezing test in the *aszat5-1* mutant (Figure 9C). We found a significantly lower freezing tolerance for the *aszat5-1* (−4.8±0.2°C, *p=0.0021*) compared to WT (−5.9±0.2°C). In *asbbx28-1*, we detected a significant down-regulation of the nascent transcription of *asBBX28* at 22°C and lower active transcription of *BBX28* at both 22°C and 3h 4°C (Figure 9D). The steady state level of the sense *BBX28* transcript was also lower in this mutant (Figure 9D), corroborating that antisense transcription had a positive role on sense *BBX28* transcription as it had on *ZAT5* transcription.

Additionally, we could see a significant decrease in the freezing tolerance in the mutant (−4.0±1.1°C, *p=0.0015* for *asbbx28-1* and −5.9±0.2°C for WT, Figure 9E).

Our results suggest that antisense transcription of *ZAT5* and *BBX28* has a positive role in priming the sense transcription for stress response and that the characterized antisense transcription has an important biological role in the cold acclimation process. All in all, our study shows that antisense transcription can play a crucial role in priming certain plant stress-responsive transcription factors.

DISCUSSION

A remarkable finding in our study is that the nascent transcriptional response to cold differs from the one detected by steady-state level measurements (Figure 3). Additionally, we show that plaNET-seq complements steady-state methods (RNA-seq) to detect genome-wide transcriptional changes in the cold response (Figure 3). A similar concept has earlier been proposed for heat stress in Arabidopsis (Liu et al., 2021). Thus, these reports highlight the importance of taking into account both active transcription and steady state levels of mRNA to fully understand the response to stress (Sidaway-Lee et al., 2014). Changes in the active transcription of a gene measured by plaNET-seq have biological relevance, even though the steady-state level of the gene's mRNA is only slightly different to control conditions after cold exposure (Figure 4-5). This report takes active transcription into account to find cold responsive genes in Arabidopsis.

This study characterizes genes with antisense transcription that initiates from the 3′-end of genes in Arabidopsis (Figure 1-2). Notably, we find a stark contrast in how the Arabidopsis genome implicates antisense transcription compared to other eukaryotes. In human cells, antisense transcription is most prevalent from early exon-intron junctions of genes (Brown et al., 2018). This type of antisense transcription is generally associated with low active transcription (Mayer et al., 2015), albeit with higher stability of the sense transcript (Brown et al., 2018). In Arabidopsis, we see almost the opposite scenario. Antisense transcription is most prevalent from the 3′-end of genes and their

host genes are associated with high active transcription and fast turnover rates (Figure 2) (Kindgren et al., 2019). In agreement, accelerated transcript degradation is suggested as an advantageous evolutionary strategy to facilitate genome wide swift responses during cold stress in Arabidopsis (Chiba et al., 2012). It is likely that plants, being sessile organisms, have evolved distinct ways of gene regulation compared to other eukaryotes, especially when responding to biotic and abiotic stresses. This hypothesis is corroborated by the fact that many genes with antisense transcription in Arabidopsis are stress-regulated transcription factors (Figure 1), proteins that are required to kick-start stress responses.

Our data paints a picture of how plants keep their stress-responsive transcription factors in a constant “on” mode to prime their response to stress situations. A tempting hypothesis would be that antisense transcription is involved in sense transcript degradation, a generalized regulatory role that was widely postulated for several thousands of NATs in Arabidopsis and other plant species (Borsani et al., 2005; Held et al., 2008). However, our data does not endorse the concept of ‘universal gene silencing roles of NATs’ but rather indicates that antisense transcription could have a more positive regulatory role on sense transcription (Reis and Poirier, 2021). When antisense transcription is down-regulated after cold exposure (Figure 6, Supplementary Figure 2), sense transcript stability is lower compared to 22°C (Figure 5) and in our CRISPR-Cas9 deletion lines that exhibit reduced antisense transcription, we detected decreased sense mRNA levels at 4°C (Figure 9). In addition, our data from *ZAT5* and *BBX28* highlight that even a marginal reduction of their antisense transcription can impair the cold responsiveness and ability of plants to acclimate to cold temperatures (Figure 9).

Thus, an outstanding question and an important avenue for future research is how antisense transcription could relay a positive role to mRNA steady-state levels. A possible mechanism could be that the antisense transcription increases the stability of the sense transcript and assists in the translation of the sense transcript, as shown for the rice lncRNA, *cis-NAT PHOSPHATE1;2 (cis-NAT PHO1;2*) (Jabnoune et al., 2013). However, this is unlikely to be a general mechanism since most antisense transcripts are short lived and degraded soon after their synthesis (Figure 8) (Kindgren et al., 2019;

Thieffry et al., 2020) and in our CRISPR lines, we did not see any effect of the mRNA stability (Supplemental Figure S5). Another, more likely mechanism, could be antisense-promoted changes to the local chromatin environment which could be important in priming the optimal transcriptional response, resembling the example of *NUCLEAR ENRICHED ABUNDANT TRANSCRIPT 1* (*NEAT1*) lncRNA in animals where the act of transcription itself at the *NEAT1* locus was shown to be sufficient for biological function (Mao et al., 2011). It is possible that sense-antisense promoters can act autonomously both during developmental transitions and in stress conditions if uncoupled from the genomic context of each other, as shown for e.g., *asDOG1*, *COOLAIR* and *SVALKA* (Swiezewski et al., 2009; Fedak et al., 2016; Kindgren et al., 2018). In contrast, the strong influence of native antisense transcription in *cis* over *ZAT5* and *BBX28* suggests that genomic proximity of the sense-antisense pairs could be crucial for the cold acclimation process (Figure 9). In fact, genome-wide native transcriptional analysis in *Saccharomyces cerevisiae* reinforces the idea that the dynamic chromatin structures could be central in determining the landscape of eukaryotic sense-antisense transcription (Murray et al., 2015).

In Arabidopsis, the H3K27 demethylase RELATIVE OF EARLY FLOWERING-6 (REF6) has been proposed to recruit a chromatin remodeling complex that includes BRAHMA to regulate antisense transcription (Li et al., 2016; Archacki et al., 2017). Other repressing chromatin mechanisms have been described for *COOLAIR*, *AGAMOUS INTRONIC RNA-4 (AG-incRNA4)*, and *AUXIN PROMOTER REGULATED LOOP (APOLO)*, albeit their mechanisms of action are distinct (Ariel et al., 2014; Csorba et al., 2014; Wu et al., 2018; Ariel et al., 2020). In a parallel manner, it is possible for lncRNAs to mediate deposition of activating marks in histones by recruiting other sets of modifiers, as has been shown for the antisense transcript of *MADS AFFECTING FLOWERING 4 (MAF4)* and *MARNERAL SILENCING (MARS)* in Arabidopsis (Zhao et al., 2018; Roulé et al., 2022). Similar modes of action have been reported in other plant species as well for example an antisense lncRNA, transcribed from *DgTCP1* (*CLASS I TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING [TCP]* transcription factor), arbitrates histone modification depositions at the sense promoters playing a positive role in cold tolerance in *Chrysanthemum morifolium* (Li et

al., 2022). It will be central to focus on different histone marks and histone variants in future studies of antisense genes to elucidate noncoding transcription and its broader role(s) in plants.

Our discoveries take the first steps to a broader role of antisense transcription in plants and support the notion that transcription from the complementary strand modulates the responsiveness of stress genes. Furthermore, our study moves away from the paradigm that antisense transcription has a prominent silencing role in plants instead supporting a more positive regulatory function.

MATERIALS AND METHODS

Plant growth, mutants and CRISPR-Cas9 mutant generation

For the wild-type background Arabidopsis (*Arabidopsis thaliana*) *Col-0* or Columbia accession was employed. For the growth of plants, seeds were surface sterilized and stratified for 2-4 days at 4º C in the dark and either transferred to soil directly or plated on ½ Murashige and Skoog (MS) basal medium supplemented with 1% (w/v) sucrose. Plants were grown in long day conditions (16h light, 8 dark, ~100 µE, SciWhite LEDs (Percival)) for 10 days. Biological replicates in all experiments represent approximately 20-30 seedlings grown on separate plates. Cold treatment (4º C, ~25 µE) was initiated at ZT4 to replicate the conditions set by the plaNET-seq dataset. T-DNA insertional mutant lines viz: *zat5-1 (SALK_041934), bbx28-1 (SAIL_412_A09), hen2-2 (GABI_774H07, (Lange et al., 2014))* were genotyped and confirmed for homozygosity by PCR. For the CRISPR-Cas9 mutants, guide RNAs (gRNAs) were designed using the CHOPCHOP webserver (http://chopchop.cbu.uib.no/) and a 2gRNA fragment was amplified using DT1T2 plasmid (Xing et al., 2014) template using Phusion DNA polymerase (Thermo Fisher Scientific). Oligos used can be found in Supplementary Data Set 6. The PCR product was electrophoresed, and gel purified followed by Green Gate reaction into a modified pHSE401 binary vector as described before (Xing et al., 2014). In the modified pHSE401 vector, the hygromycin resistance has been replaced by a *GFP* seed coat expression cassette for faster screening. Final plasmids were

verified by sequencing and transformed into wild type *Col-0* plants by *Agrobacterium tumefaciens* (*GV3101*) floral dip. T1 seeds were first selected by visual screening for *GFP* expression followed by PCR genotyping. Further selection of the T2 lines was performed by picking seeds lacking the GFP signal, and then homozygous plants were confirmed by PCR for the genomic deletion and the absence of the Cas9 construct. Seeds from homozygous plants were used in experiments.

Generation of reporter constructs

We used GreenGate cloning system for generation of firefly luciferase (LUC) gene reporter constructs (Lampropoulos et al., 2013). To construct *ProZAT5:ZAT5-LUC-UTR-ASProZAT5* and *ProZAT5:ZAT5-LUC-tNOS*, 2438 bp fragment upstream (1408 bp promoter and 1030 CDS of *ZAT5* without stop codon) was PCR amplified from genomic DNA . A separate PCR amplification was carried out for 1402 bp long *UTR-*$_{AS}$*ZAT5*$_{prom}$ fragment using proofreading Phusion DNA polymerase using genomic DNA template. LUC and tNOS terminator (tNOS) were also separately PCR amplified in similar manner. Subsequently different PCR products were cloned into respective GreenGate entry modules by employing BsaI/T4 DNA ligase and using reaction conditions as described earlier (Lampropoulos et al., 2013). All Greengate entry plasmids were confirmed by restriction digestion and DNA sequencing. Finally, the GreenGate reaction was performed for the assembly of 6 entry modules to create final destination plasmids i.e. *ProZAT5:ZAT5-LUC-UTR-ASProZAT5* and Pro*ZAT5:ZAT5-LUC-tNOS* using pGGZ003 as backbone vector according to protocol reported earlier (Lampropoulos et al., 2013). Oligos used can be found in Supplemental Data Set S6.

Transient agroinfiltration and luciferase assay

Agrobacterium strain *GV3101* was separately transformed with plasmids harboring $_{prom}$*ZAT5::ZAT5-LUC-UTR-ASZAT5*$_{prom}$ and $_{prom}$*ZAT5::ZAT5-LUC-tNOS* constructs and plated on LB media containing 10 µg/ml rifampicin, 25 µg/ml gentamicin, 75 µg/ml spectinomycin. After 48 hours of growth positive colonies selected and grown in 5 ml of LB medium supplemented with antibiotics. Additionally, presence of constructs in *Agrobacteria* confirmed by colony PCR. After overnight growth at 30°C, 30 ml of fresh induction media (LB supplemented with 10 mM MES pH 5.6, 20 µM acetosyringone, 25

µg/ml, gentamicin, 75 µg/ml spectinomycin, 10 µg / ml rifampicin) inoculated. When OD_{600} reached 0.5, cells were harvested by centrifugation and resuspended in infiltration media (LB supplemented with 10 mM MES pH 5.6, 10 mM MgCl 2 and 150 µM acetosyringone without antibiotics) to obtain an OD_{600} value of 1 followed by incubation of bacterial culture at room temperature for minimum of 3 hours. Finally, leaves from 6-week-old *N. benthamiana* plants were infiltrated with bacterial suspension and the area was delimited and marked with a marker.

72 hours post agroinfiltration, half of the randomly selected *N. benthamiana* plants (a minimum of 5 independent plants each with 2-3 infiltrated leaves for individual constructs) were subjected for cold stress treatment (3h cold 4°C) at ZT4. Control plants were maintained at 22°C. Immediately after 3 hours of cold stress, previously infiltrated leaves with marked area from cold treated and control plants were carefully and quickly re-infiltrated with 5 mM working solution of D-Luciferin (GoldBio®). The ratio of 5mM D-Luciferin in 0.01% (v/v) triton X100 and sterile water was kept at 1:3 during re-infiltration of leaves. Further, several 1 cm leaf discs were prepared from marked re-infiltrated area and subjected for luminescence measurement after 5-10 minutes of incubation at respective temperatures by GloMax® Navigator Microplate Luminometer. Data extracted and analyzed using Excel and GraphPad® software.

Electrolyte leakage assay

Electrolyte leakage measurements were carried out according to previous report (Kindgren et al., 2015). In short, plants were grown in short days (8h light /16 h dark cycle) for 4 weeks. For the cold acclimation experiments, WT and mutant plants were transferred to a cold chamber set at 4 ºC for 4 days without changing photoperiodic conditions. Randomized leaf discs of 1 cm diameter, for each genotype in triplicates from several similar sized leaves, were prepared using a cork borer for acclimated or non-acclimated plants and carefully placed horizontally in a manner to avoid floating in clean glass tubes filled with 200 µl deionized distilled water. The tubes containing two leaf discs were then transferred to a programmable freezing bath (FP51, Julabo, Germany) set at -2º C. After 45 minutes, icing was induced manually in each tube with the help of liquid N_2 and a metallic stick. Temperature decrease occurred at the rate of

−1 °C per 30 mins, and samples were taken out at designated temperature point(s) followed by incubation on ice for at least 1 hour in the cold room (4 ºC). Soon after the collection and 1 hour ice-incubation of tubes, 1.3 ml of water was added to each tube and placed on a shaker overnight at 4º C and conductivity was measured using a conductivity cell (CDM210, Radiometer, Denmark) on the next day. Finally, all tubes were subjected to flash freeze using liquid N_2 and left on a shaker overnight at room temperature. To obtain the total electrolyte content from leaf discs, conductivity was measured again, and the % of electrolyte leakage was calculated using the formula – (conductivity before flash freeze/ conductivity after flash freeze) *100. Data were fitted into a sigmoidal dose-response curve using GraphPad Prism software and significant differences of the fit were determined with an extra sum-of-squares F test.

RNA extraction, cDNA synthesis and RT-qPCR

Total RNA extraction from plant material was carried out using RNeasy Plant Mini Kit (QIAGEN) as per suppliers' instructions. The extracted RNA was additionally treated with dsDNase (Thermo Fisher Scientific) for the elimination of genomic DNA contamination. Successively, complementary DNA (cDNA) synthesis was carried out using Superscript IV® (Invitrogen) reverse transcriptase as per manufacturer instructions using strand-specific RT primers carrying a sequence tag (GACTGGAGCACGAGGACACT) at 5' end (Parent et al., 2015; Kindgren et al., 2018) together with a reference gene. Quantitative real-time PCR (RT-qPCR) was performed on CFX96 and CFX384 Real-Time PCR detection systems (BioRad) using SYBR premix (Bio-Rad), cDNA, reverse primer (aligning to tag sequence) and appropriate forward primers at the concentration of 10 pmol/µl with the PCR cycler following initial denaturation at 95 º C for 30s, standard 40 cycles of 94 º C for 10 s, 60 º C for 30s. The specificity of RT-qPCR products was assessed from the single peak melt curves. For the data analysis, the C_q values from a minimum of 3 biological replicates with 2-3 technical replicates were averaged and $\triangle C_q$ was obtained as C_q (gene of interest)-C_q (reference gene). Final calculations were performed by following with $2^{\wedge(-\Delta c_q)}$ or $2^{\wedge(-\Delta\Delta c_q)}$, adjusted to experimentally determined primer efficiency for determination of fold change

in gene expression levels. Statistical significant differences were calculated with Student's t-test. Primers used are listed in Supplemental Data Set S6.

Measuring nascent transcription

Nuclei were isolated from around 3 grams of 12-day old seedlings with Honda buffer (0.44 M Sucrose, 1.25% Ficoll, 2.5% Dextran T40, 20 mM Tris –HCl pH 7.4, 10 mM MgCl2, 0.5% Triton-X, Prot. inhibitor tablet, RNase inhibitor, 5 mM DTT). The nuclear lysis and RNAPII-IP were done according to (Kindgren et al., 2018) with small modifications. Briefly, after lysis and DNAse I treatment, the supernatant was mixed with protein G magnetic beads (Thermo Scientific) coupled to an endogenous RNAPII antibody (8WG16, Sigma Aldrich) for 2h in 4°C. The beads were washed 4 times with wash buffer (0.3 M NaCl, 20 mM Tris-HCl pH 7.5, 5 mM MgCl2, 5 mM DTT, proteinase inhibitor tablet and RNase inhibitor (20 U/ml)). To disrupt the RNAPII complexes, QIAzol was added, and RNA was isolated using the miRNeasy kit from Qiagen. RNA concentration was measured with Nanodrop and approximately 100 ng was used for cDNA synthesis with gene specific primers and Superscript IV (Invitrogen) according to manufacturer's instructions. Oligos used can be found in Supplemental Data Set S6.

RNA sequencing and analysis

RNA was isolated from 10-day old Arabidopsis Col-0 seedlings grown on ½MS medium. Briefly, seeds were stratified for 2-4 days at 4ºC in the dark, followed by growth in long day (16h light/8h dark, 22ºC Day/18ºC night) conditions and ~100µEm^{-2}s^{-1} light. On the 12th day, seedlings were subjected to cold stress (4ºC) for 3h and 12h with ~20-25µEm^{-2}s^{-1} light. Total RNA was isolated using RNeasy Plant Mini Kit (QIAGEN) according to manufacturer's instructions. RNA thus obtained was treated with TURBO® DNAse (Thermo Fischer) according to the standard protocol. Three biological replicates from each time point of RNA samples were sent to Novogene Europe where strand specific libraries were prepared and sequenced using Illumina's NovaSeq 6000 platform. Libraries were sequenced to a depth of 40-60 million raw reads (6G raw data per sample). For data analysis, the guidelines previously established at UPSC were followed (Delhomme et al., 2023). Pre-processing of data was done using FastQC v0.11.9 (quality control of the raw data) and SortMeRNA v4.3.4 ((Kopylova et al., 2012);

filter and remove rRNA contamination). Thereafter, Trimmomatic v0.32 (Bolger et al., 2014) was used to trim the adapter sequences and FastQC was performed again to ensure data integrity. Salmon v1.6.0 (Patro et al., 2017) was used to determine the read counts with ARAPORT11 as a reference. R-package DESeq2 (Love et al., 2014) was used to perform the differential expression analysis. Statistically significant genes were filtered using the following parameter: false discovery rate (fdr) < 0.05 and $\log_2$ fold change ≥ 0.5. RNA-seq data have been deposited on GEO (GSE252832). To investigate the rate of transcript degradation of control genes vs PAS-host genes we used the Decay Rate (alpha estimate) from Sorenson RS and Deshotel MJ et al (Table S2 from cited paper) (Sorenson et al., 2018), which was originally determined by cordycepin treatment followed by RNA-seq. In addition, we used available data to estimate the decay rate for mRNAs after actinomycin D treatment (Narsai et al., 2007). GO-term enrichment was done at TAIR (https://www.arabidopsis.org/tools/go_term_enrichment.jsp).

RNA stability assay

RNA stability measurements to determine the half-life ($t_{1/2}$) of transcripts were performed according to previous report (Fedak et al., 2016). In summary, 10 days old wild-type seedlings were grown in long day photoperiod (16h light/8h dark, 22ºC Day/18ºC night) (CLF Plant Climatics cabinet) over ½ MS medium supplemented with 1% sucrose (w/v). On day 10, seedlings were transferred to liquid ½ MS media and acclimatized while maintaining 22ºC or 4ºC growth temperatures for respective sample sets under the same light conditions. Further, samples from 22ºC or 4ºC were transferred to incubation buffer (1 mM PIPES at pH 6.25, 1 mM trisodium citrate, 1 mM KCl and 15 mM sucrose) in 12-well plates for 30 minutes followed by addition of 150 mg/l cordycepin (3'-deoxyadenosine, Sigma Aldrich). Immediately after cordycepin addition, samples were vacuum infiltrated for (5 min x 2 times). 15 seedlings for each of samples in triplicates were collected at 0, 15, 30, 60, 120 minutes after cordycepin treatment. Subsequently, total RNA extraction and strand-specific RT-qPCR analyses were carried out by using cDNA as a template synthesized with SuperScript IV Reverse Transcriptase (Invitrogen™) and gene specific primers. *EUKARYOTIC TRANSLATION*

INITIATION FACTOR4A1 (*EIF4A1*, *AT3G13920*) (Perea-Resa et al., 2012) was used as an internal assay control. C_q values at 15, 30, 60, 120 minutes were normalized with C_q at 0 minute and RNA degradation curve obtained following [$C_q(n) = (\ln (C_q/C_q (0)) *(-10)]$ equation. Finally, $t_{1/2}$ of transcripts was calculated from obtained slope [$t_{1/2} = (\ln_2)/\text{slope}$]. Oligos used can be found in Supplemental Data Set S6.

Genome-wide analyses

Detailed bioinformatic methods can be accessed at https://github.com/peterkindgrengroup/Meena_et_al_2024. Briefly, a control set of genes without PAS was curated from all expressed genes (22°C RNA-seq data from this study). In order to better define the gene coordinates of both controls and PAS-host genes, we used available TSS-seq data (S4 Table from cited paper) (Nielsen et al., 2019) in combination with the TTS from TAIR10. Gene length was calculated from these curated genomic features. RNAPII datasets were retrieved (GSE95301 at Gene Expression Omnibus) (Liu et al., 2018). Bedgraphs from samples GSM2522253 for PolII_WT were converted to bigwig (Kent et al., 2010). Deeptools (Ramírez et al., 2016) was used to compute ChIP-seq score matrices and to generate metaplots along the gene body. Differentially expressed genes from plaNET-seq were extracted from Table S2 from (Kindgren et al., 2019). To build the plaNET-seq metaplots, the raw sequences (SRR9117170-SRR9117181) were downloaded and processed as indicated in the previously mentioned GitHub repository. Shortly, after the quality control of raw reads, bam files were generated by aligning the sequence reads against the Arabidopsis genome using STAR 2.7.10a (Dobin et al., 2012) and used on ngs.plot (Shen et al., 2014) to generate the meta gene profiles using the in-built TAIR10 genome and the gene lists of interest. A few existing datasets were remapped for this study. They include DR-seq (Schurch et al., 2014), CAGE (Thieffry et al., 2020), and plaNET-seq (Kindgren et al., 2019).

DATA AVAILIBILITY

RNA Seq data for wild type is available online at NCBI under accession number GSE252832. All new code is available at https://github.com/peterkindgrengroup/Meena_et_al_2024. Statistical data are provided in Supplementary Data Set S7.

ACCESSION NUMBERS

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are as follows: At1g18710 for *MYB47*, At1g73870 for *COL7*, At2g28200 for *ZAT5*, At5g52510 for *SCL8*, and At4g27310 for *BBX28*.

SUPPLEMENTARY DATA

Supplementary Figure S1. Half-life and RNAPII occupancy of PAS genes.

Supplementary Figure S2. PAS transcription in response to cold temperature.

Supplementary Figure S3. RNA level of *ZAT5* and *BBX28* with control at different ZT and freezing test of non-acclimated *zat5-1* and *bbx28-1* plants.

Supplementary Figure S4. LUCIFERASE assay for *ZAT5* constructs.

Supplementary Figure S5. RNA stability of *ZAT5* and *BBX28* in CRISPR mutants.

Supplementary Data Set S1. List of PAS genes.

Supplementary Data Set S2. Full list of GO terms.

Supplementary Data Set S3. Differentially expressed genes in the RNA-seq experiment.

Supplementary Data Set S4. Differentially expressed PAS genes.

Supplementary Data Set S5. PAS genes with similar expression profile to *CBF2*.

Supplementary Data Set S6. Oligos used in this study.

Supplementary Data Set S7. Statistical data.

FUNDING INFORMATION

This research was funded by the Swedish research council (2018-03926), FORMAS (2021-01065), Carl Trygger foundation (20:224), and grants from the Knut and Alice Wallenberg Foundation and the Swedish Governmental Agency for Innovation Systems [KAW 2016.0355 and 2020.0240, VINNOVA 2016-00504].

ACKNOWLEDGMENTS

We would like to thank members of the Kindgren lab for their critical reading of the manuscript. A special thanks to Linda Allo, Julia Viklander and Tim te Morsche for assistance with the CRISPR-Cas9 constructs. We would like to thank the greenhouse personnel at Umeå Plant Science Centre for plant maintenance and Nicolas Delhomme for bioinformatic help.

AUTHOR CONTRIBUTIONS

designed the research (SKM, SSB, VZ, PK); performed research (SKM, CV, SSB, VZ, PK); analyzed data (SKM, MQ, CV, SSB, VZ, SMN, PK); wrote the paper (SKM, MQ, CV, SSB, VZ, SMN, PK).

FIGURE LEGENDS

Figure 1. Poly(A) antisense (PAS) genes are overrepresented by stress-responsive transcription factors.

A) Graphical representation of the definition of PAS genes. Abbreviations: TSS: transcription start site, AS: antisense.

B) GO term enrichment of PAS genes (molecular function). The number in bars indicates found number of genes/expected number of genes.

C) GO term enrichment of PAS genes (biological processes). The number in bars indicates found number of genes/expected number of found genes.

D) Example of a PAS gene (*WRKY48*, At5g49520). Screenshots showing plaNET-seq expression profile from datasets at 22° C, 3 hours, and 12 hours post cold treatment. Elevated transcriptional activity indicated by higher peaks density and amplitude.

Figure 2. PAS genes are highly expressed, but their mRNA is degraded rapidly.

A) Violin plot of the length of PAS non-TF genes, TFs non-PAS, PAS TF genes and all expressed genes. Center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range. *p* value was calculated with Mann-Whitney U test and $p<0.05$ was considered significant.

B) Violin plot of the steady-state level of PAS non-TF genes, TFs non-PAS, PAS TF genes and all expressed genes. Center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range. *p* value was calculated with Mann-Whitney U test and $p<0.05$ was considered significant.

C) Violin plot of the decay rate of PAS non-TF genes, TFs non-PAS, PAS TF genes and all expressed genes after transcriptional inhibition by cordycepin. Center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range. *p* value was calculated with Mann-Whitney U test and $p<0.05$ was considered significant.

D) Metagene analysis of plaNET-seq data of PAS non-TF genes (light blue), TFs non-PAS (grey), PAS TF genes (blue) and all expressed genes (black). The shaded area shows a 95% confidence interval for the mean.

Figure 3. Discrepancy between plaNET-seq and RNA-seq.

A) Number of total genes differentially expressed in RNA-seq and plaNET-seq after 3 and 12 hours of 4°C exposure. The overlap between the two sequencing techniques is shown in darker grey.

B-C) Correlation plots between differentially expressed genes in RNA-seq and plaNET-seq (UP: grey squares, DOWN: grey diamonds) after **B)** 3 hours of 4°C and **C)** 12 hours of 4°C. The genes plotted come from the overlap seen in **A)**.

D) Number of PAS genes differentially expressed in RNA-seq and plaNET-seq after 3 and 12 hours of 4°C exposure. The overlap between the two sequencing techniques is shown in blue.

E-F) Correlation plots between differentially expressed PAS genes in RNA-seq and plaNET-seq (UP: blue squares, DOWN: blue diamonds) after **E)** 3 hours of 4°C and **F)** 12 hours of 4°C. The genes plotted come from the overlap seen in **D)**.

Figure 4. PAS genes have enhanced cold responsiveness.

A) Metagene analysis of the plaNET-seq signal (at 22°C) in a 500 bp window centered at the +1 nucleosome. PAS genes that will be UP after 3h at 4°C are shown in blue, genes without antisense transcription but UP after 3h at 4°C are shown grey, and non-DE genes are shown in black. The shaded area shows a 95% confidence interval for the mean.

B) Metagene analysis of plaNET-seq data (at 22°C). PAS genes that will be UP after 3h at 4°C are shown in blue, genes without antisense transcription but UP after 3h at 4°C are shown in grey, and non-DE genes are shown in black. The shaded area shows a 95% confidence interval for the mean.

C) Metagene analysis of the plaNET-seq signal (after 3h at 4°C) in a 500 bp window centered at the +1 nucleosome. PAS UP genes after 3h at 4°C are shown in blue, genes without antisense transcription but UP after 3h at 4°C are shown in grey, and non-DE genes are shown in black. The shaded area shows a 95% confidence interval for the mean.

D) Metagene analysis of plaNET-seq data (after 3h at 4°C). PAS UP genes after 3h at 4°C are shown in blue, genes without antisense transcription but UP after 3h at 4°C are

shown in grey, and non-DE genes are shown in black. The shaded area shows a 95% confidence interval for the mean.

Figure 5. *ZAT5* and *BBX28* mRNA show decreased stability at 4°C.

A) At4g254970 (*CBF2*). Screenshots are from plaNET-seq and RNA-seq datasets. Elevated transcriptional activity indicated by higher peaks density and amplitude.

B) At3g17609 (*HYH*). Screenshots are from plaNET-seq and RNA-seq datasets. Elevated transcriptional activity indicated by higher peaks density and amplitude.

C-H) Transcript stability assays for **C)** *EIF4A1*, **D)** *ZAT5,* **E)** *BBX28,* **F)** *COL7* **G)** *MYB47*, and **H)** *SCL8* after transcriptional inhibition with cordycepin at 22°C and 4°C. Half-life ($t_{1/2}$) was determined from the slope of degradation curves that were obtained after RT-qPCR analysis of cordycepin treated seedlings at indicated time points. Each data point is the mean of three biological replicates. Error bars represent ± SD.

Figure 6. Examples of cold-responsive PAS genes.

A) *AT2G28200* (*ZAT5*). Screenshots are from plaNET-seq and RNA-seq datasets. Elevated transcriptional activity indicated by higher peaks density and amplitude.

B) *AT4G27310* (*BBX28*). Screenshots are from plaNET-seq and RNA-seq datasets. Elevated transcriptional activity indicated by higher peaks density and amplitude.

Figure 7. *ZAT5* and *BBX28* are important for cold acclimation.

A) The relative steady-state level of *ZAT5* measured by RT-qPCR following cold exposure (4°C). Steady-state levels were normalized to WT levels at 22°C. The mean values are from three biological replicates. Error bars represent ± SEM. Statistical significance was calculated with Student's t-test (* $p<0.05$).

B) The relative steady-state level of *BBX28* measured by RT-qPCR following cold exposure (4°C). Steady-state levels were normalized to WT levels at 22°C. The mean values are from three biological replicates. Error bars represent ± SEM. Statistical significance was calculated with Student's t-test (* $p<0.05$).

C) Electrolyte leakage in wild-type and *zat5-1* of cold-acclimated (4 days of 4°C) plants. Each data point represents the mean from at least 3 biological replicates (±SEM). The dashed line represents the threshold value, 50%. The dotted lines represent the curve fit. Statistical significance was calculated with an extra sum-of-squares F test and the p value is shown in the figure.

D) Electrolyte leakage in wild-type and *bbx28-1* of cold-acclimated (4 days of 4°C) plants. Each data point represents the mean from at least 3 biological replicates (±SEM). The dashed line represents the threshold value, 50%. The dotted lines represent the curve fit. Statistical significance was calculated with an extra sum-of-squares F test and the p value is shown in the figure.

Figure 8. Characterization of *asZAT5* and *asBBX28*.

Screenshots from plaNET-seq (upper panel), CAGE (middle panel), and DRS-seq (lower panel) for **A)** *ZAT5*, and **B)** *BBX28*. 0 indicates the TSS of the sense transcript. Elevated transcriptional activity indicated by higher peaks density and amplitude.

Figure 9. *asZAT5* and *asBBX28* are important for proper regulation of their host gene.

A) Graphical representation of the *ZAT5* and the *BBX28* loci showing the location of sequence targeted by CRISPR-Cas9 to generate an *asZAT5* knockdown line (*aszat5-1*) and an *asBBX28* knockdown line (*asbbx28-1*). Antisense transcription start site (asTSS) window and distances of knocked out genomic sequence from 3′-end are marked with dotted lines. Location of RT-qPCR probes for sense and antisense are shown.

B) The relative nascent and steady-state level of *asZAT5* and *ZAT5* in wild-type and *aszat5-1* measured by RT-qPCR at 22°C and following 3h of cold exposure (4°C). All levels were normalized to WT levels at 22°C. The mean values are from three biological replicates. Error bars represent ± SEM. Statistical significance was calculated with Student's t-test (* $p<0.05$).

C) Electrolyte leakage in wild-type and *aszat5-1* of cold-acclimated (5 days of 4°C) plants. Each data point represents the mean from at least 3 biological replicates (±SEM). The dashed line represents the threshold value, 50%. The dotted lines represent the curve fit. Statistical significance was calculated with an extra sum-of-squares F test and the p value is shown in the figure.

D) The relative steady-state level of *asBBX28* and *BBX28* in wild-type and *asbbx28-1* measured by RT-qPCR at 22°C and following 3h of cold exposure (4°C). All levels were normalized to WT levels at 22°C. The mean values are from three biological replicates. Error bars represent ± SEM. Statistical significance was calculated with Student's t-test (* $p<0.05$).

E) Electrolyte leakage in wild-type and *asbbx28-1* of cold-acclimated (5 days of 4°C) plants. Each data point represents the mean from at least 3 biological replicates (±SEM). The dashed line represents the threshold value, 50%. The dotted lines represent the curve fit. Statistical significance was calculated with an extra sum-of-squares F test and the p value is shown in the figure.

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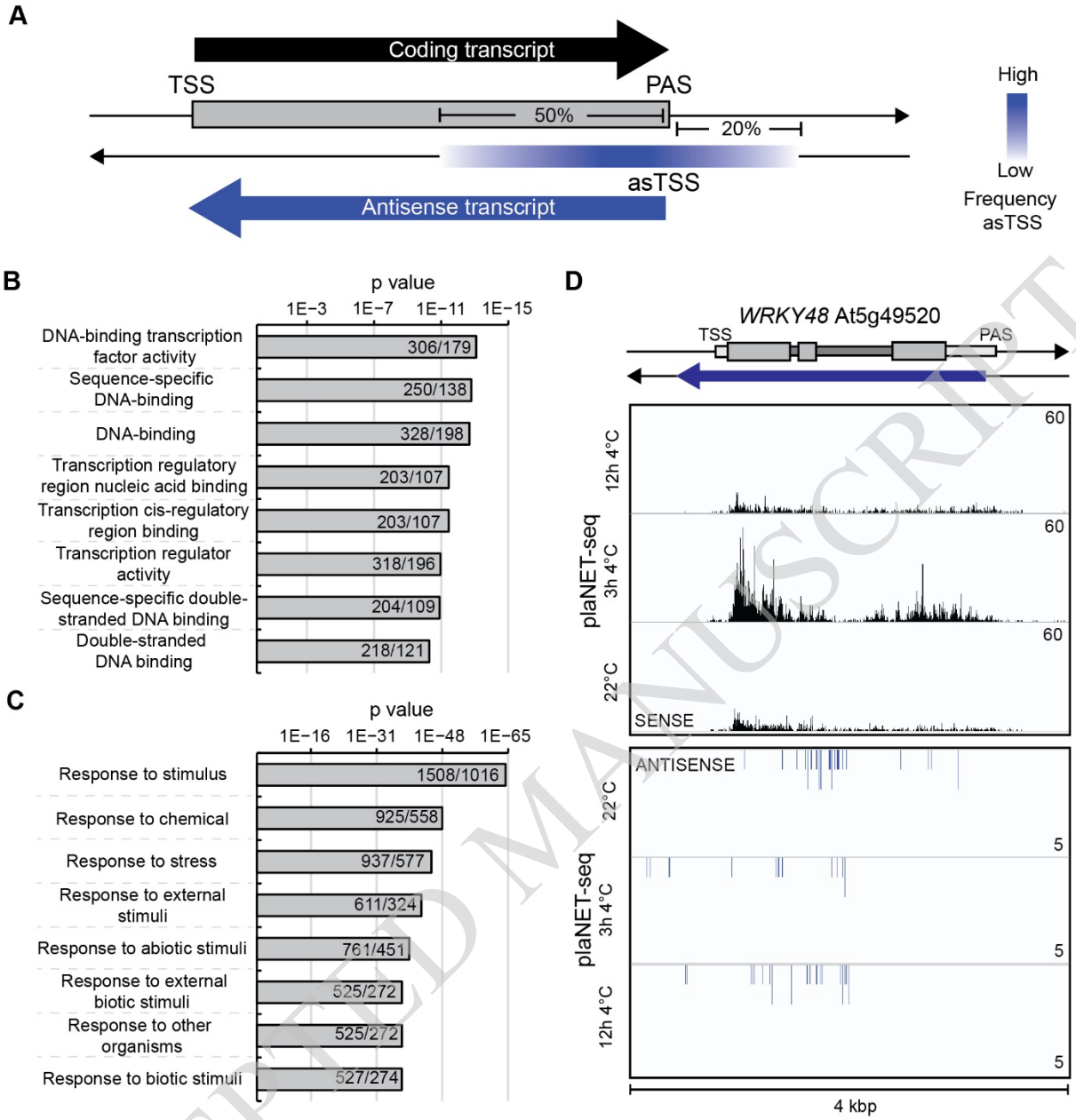

Figure 1. Poly(A) antisense (PAS) genes are overrepresented by stress-responsive transcription factors.

A) Graphical representation of the definition of PAS genes. Abbreviations: TSS: transcription start site, AS: antisense.

B) GO term enrichment of PAS genes (molecular function). The number in bars indicates found number of genes/expected number of genes.

C) GO term enrichment of PAS genes (biological processes). The number in bars indicates found number of genes/expected number of found genes.

D) Example of a PAS gene (*WRKY48*, At5g49520). Screenshots showing plaNET-seq expression profile from datasets at 22° C, 3 hours, and 12 hours post cold treatment. Elevated transcriptional activity indicated by higher peaks density and amplitude.

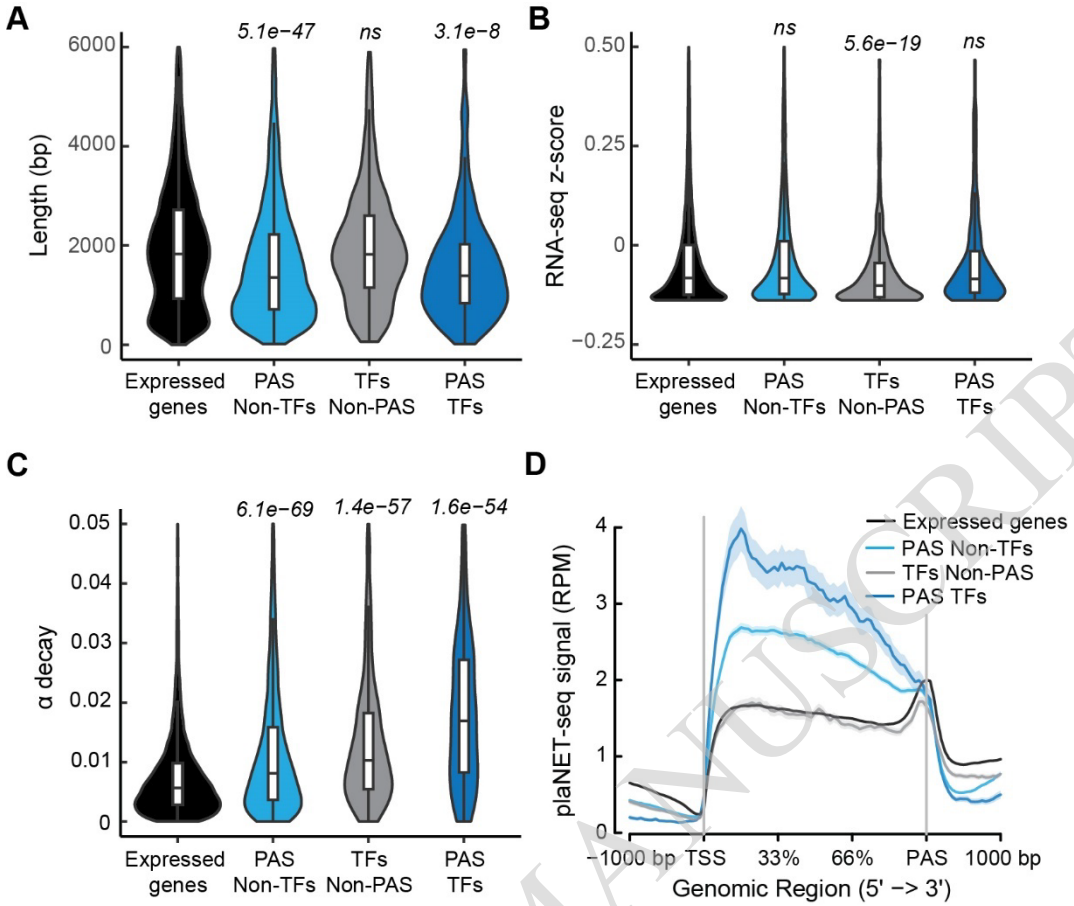

Figure 2. PAS genes are highly expressed, but their mRNA is degraded rapidly.

A) Violin plot of the length of PAS non-TF genes, TFs non-PAS, PAS TF genes and all expressed genes. Center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range. *p* value was calculated with Mann-Whitney U test and p<0.05 was considered significant.

B) Violin plot of the steady-state level of PAS non-TF genes, TFs non-PAS, PAS TF genes and all expressed genes. Center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range. *p* value was calculated with Mann-Whitney U test and p<0.05 was considered significant.

C) Violin plot of the decay rate of PAS non-TF genes, TFs non-PAS, PAS TF genes and all expressed genes after transcriptional inhibition by cordycepin. Center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range. *p* value was calculated with Mann-Whitney U test and p<0.05 was considered significant.

D) Metagene analysis of plaNET-seq data of PAS non-TF genes (light blue), TFs non-PAS (grey), PAS TF genes (blue) and all expressed genes (black). The shaded area shows a 95% confidence interval for the mean.

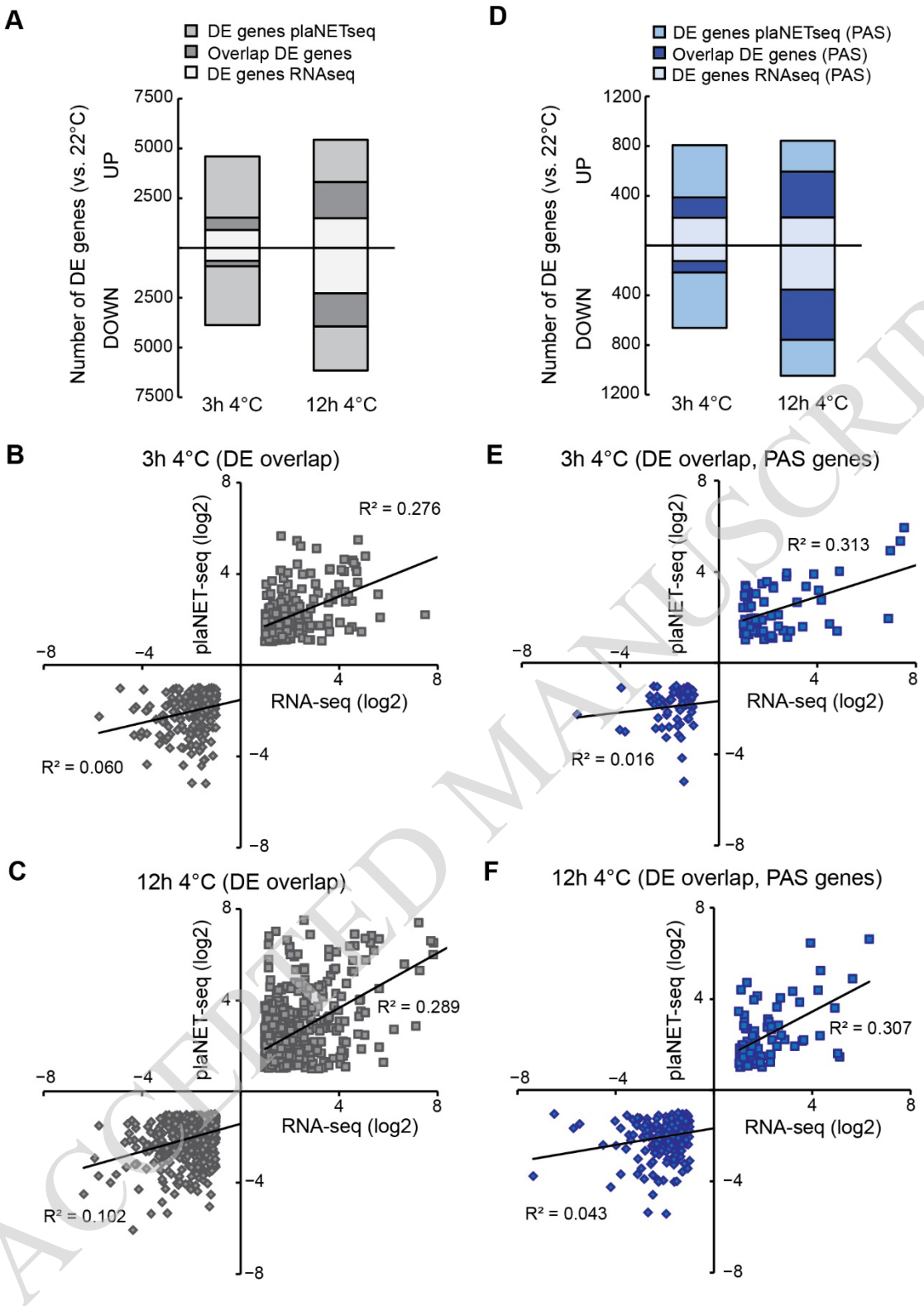

Figure 3. Discrepancy between plaNET-seq and RNA-seq.

A) Number of total genes differentially expressed in RNA-seq and plaNET-seq after 3 and 12 hours of 4°C exposure. The overlap between the two sequencing techniques is shown in darker grey.

B-C) Correlation plots between differentially expressed genes in RNA-seq and plaNET-seq (UP: grey squares, DOWN: grey diamonds) after **B)** 3 hours of 4°C and **C)** 12 hours of 4°C. The genes plotted come from the overlap seen in **A)**.

D) Number of PAS genes differentially expressed in RNA-seq and plaNET-seq after 3 and 12 hours of 4°C exposure. The overlap between the two sequencing techniques is shown in blue.

E-F) Correlation plots between differentially expressed PAS genes in RNA-seq and plaNET-seq (UP: blue squares, DOWN: blue diamonds) after **E)** 3 hours of 4°C and **F)** 12 hours of 4°C. The genes plotted come from the overlap seen in **D)**.

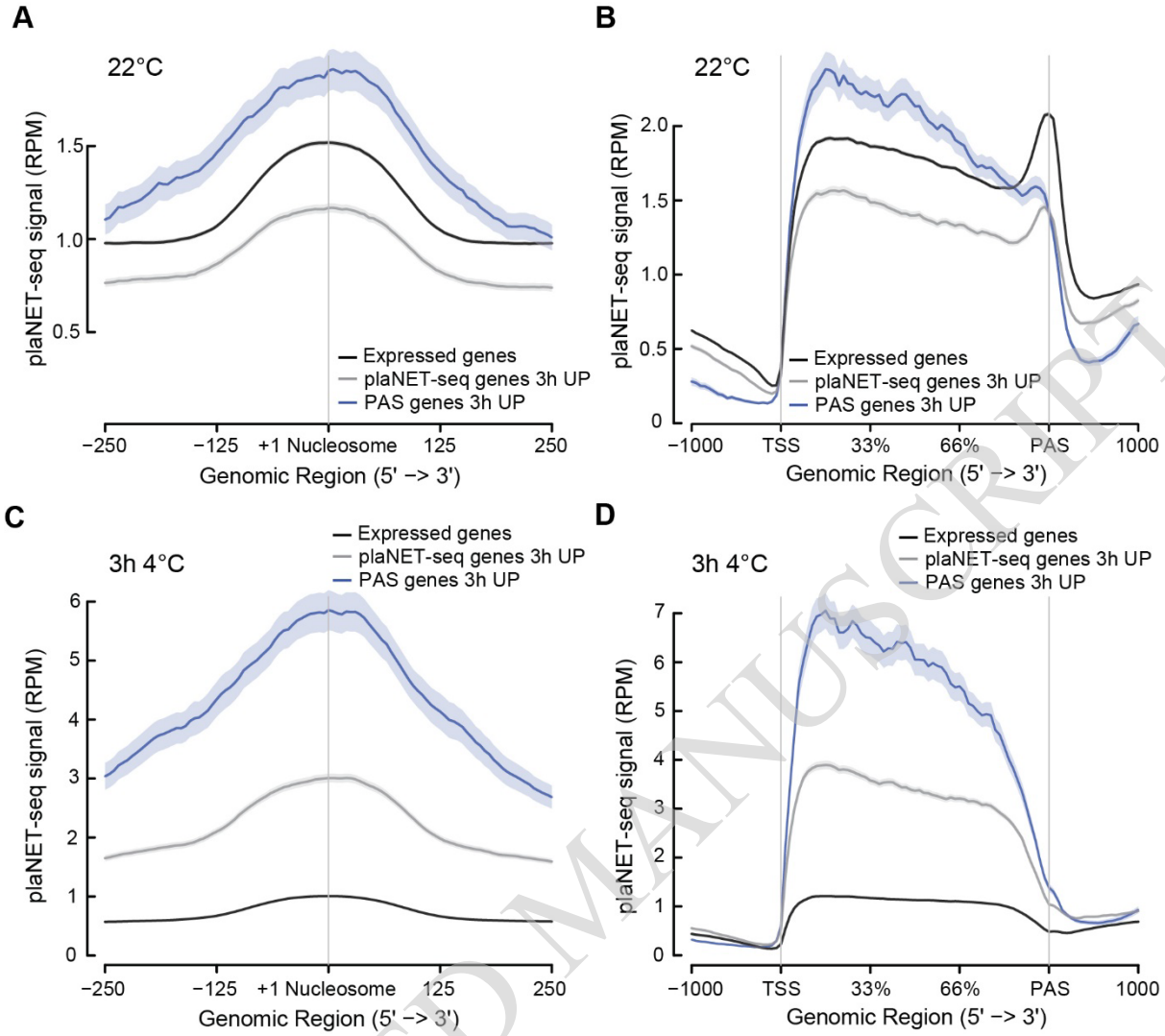

Figure 4. PAS genes have enhanced cold responsiveness.

A) Metagene analysis of the plaNET-seq signal (at 22°C) in a 500 bp window centered at the +1 nucleosome. PAS genes that will be UP after 3h at 4°C are shown in blue, genes without antisense transcription but UP after 3h at 4°C are shown grey, and non-DE genes are shown in black. The shaded area shows a 95% confidence interval for the mean.

B) Metagene analysis of plaNET-seq data (at 22°C). PAS genes that will be UP after 3h at 4°C are shown in blue, genes without antisense transcription but UP after 3h at 4°C are shown in grey, and non-DE genes are shown in black. The shaded area shows a 95% confidence interval for the mean.

C) Metagene analysis of the plaNET-seq signal (after 3h at 4°C) in a 500 bp window centered at the +1 nucleosome. PAS UP genes after 3h at 4°C are shown in blue, genes without antisense transcription but UP after 3h at 4°C are shown in grey, and non-DE genes are shown in black. The shaded area shows a 95% confidence interval for the mean.

D) Metagene analysis of plaNET-seq data (after 3h at 4°C). PAS UP genes after 3h at 4°C are shown in blue, genes without antisense transcription but UP after 3h at 4°C are shown in grey, and non-DE genes are shown in black. The shaded area shows a 95% confidence interval for the mean.

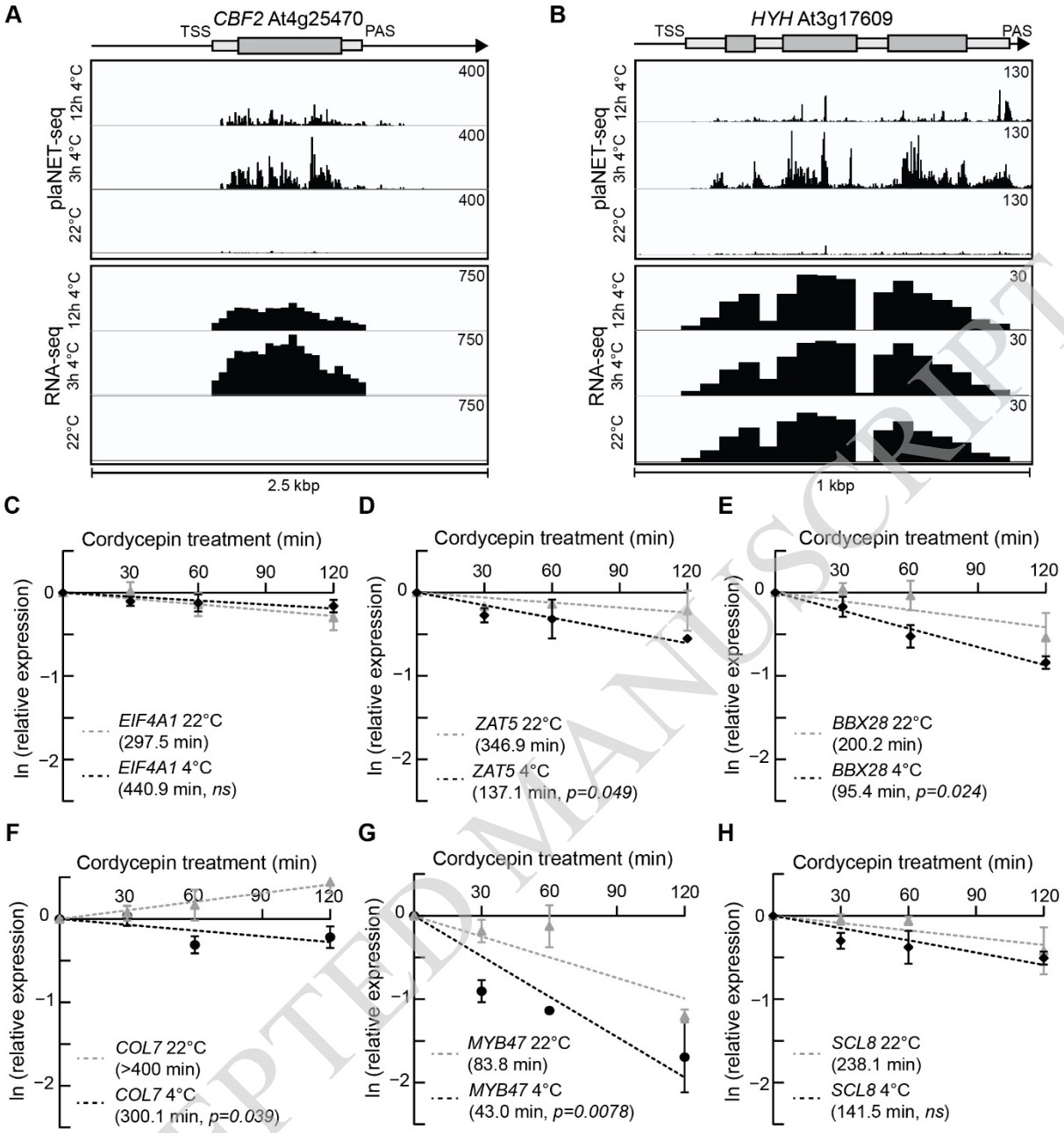

Figure 5. *ZAT5* and *BBX28* mRNA show decreased stability at 4°C.

A) At4g254970 (*CBF2*). Screenshots are from plaNET-seq and RNA-seq datasets. Elevated transcriptional activity indicated by higher peaks density and amplitude.

B) At3g17609 (*HYH*). Screenshots are from plaNET-seq and RNA-seq datasets. Elevated transcriptional activity indicated by higher peaks density and amplitude.

C-H) Transcript stability assays for **C)** *EIF4A1*, **D)** *ZAT5,* **E)** *BBX28,* **F)** *COL7* **G)** *MYB47*, and **H)** *SCL8* after transcriptional inhibition with cordycepin at 22°C and 4°C. Half-life ($t_{1/2}$) was determined from the slope of degradation curves that were obtained after RT-qPCR analysis of cordycepin treated seedlings at indicated time points. Each data point is the mean of three biological replicates. Error bars represent ± SD.

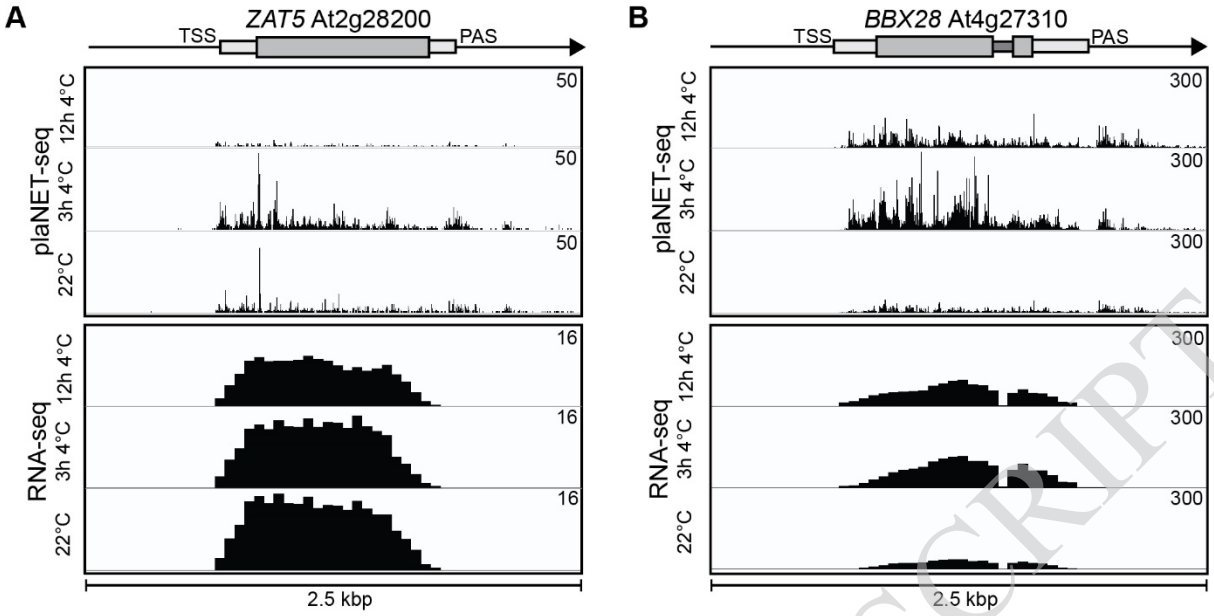

Figure 6. Examples of cold-responsive PAS genes.

A) *AT2G28200* (*ZAT5*). Screenshots are from plaNET-seq and RNA-seq datasets. Elevated transcriptional activity indicated by higher peaks density and amplitude.

B) *AT4G27310* (*BBX28*). Screenshots are from plaNET-seq and RNA-seq datasets. Elevated transcriptional activity indicated by higher peaks density and amplitude.

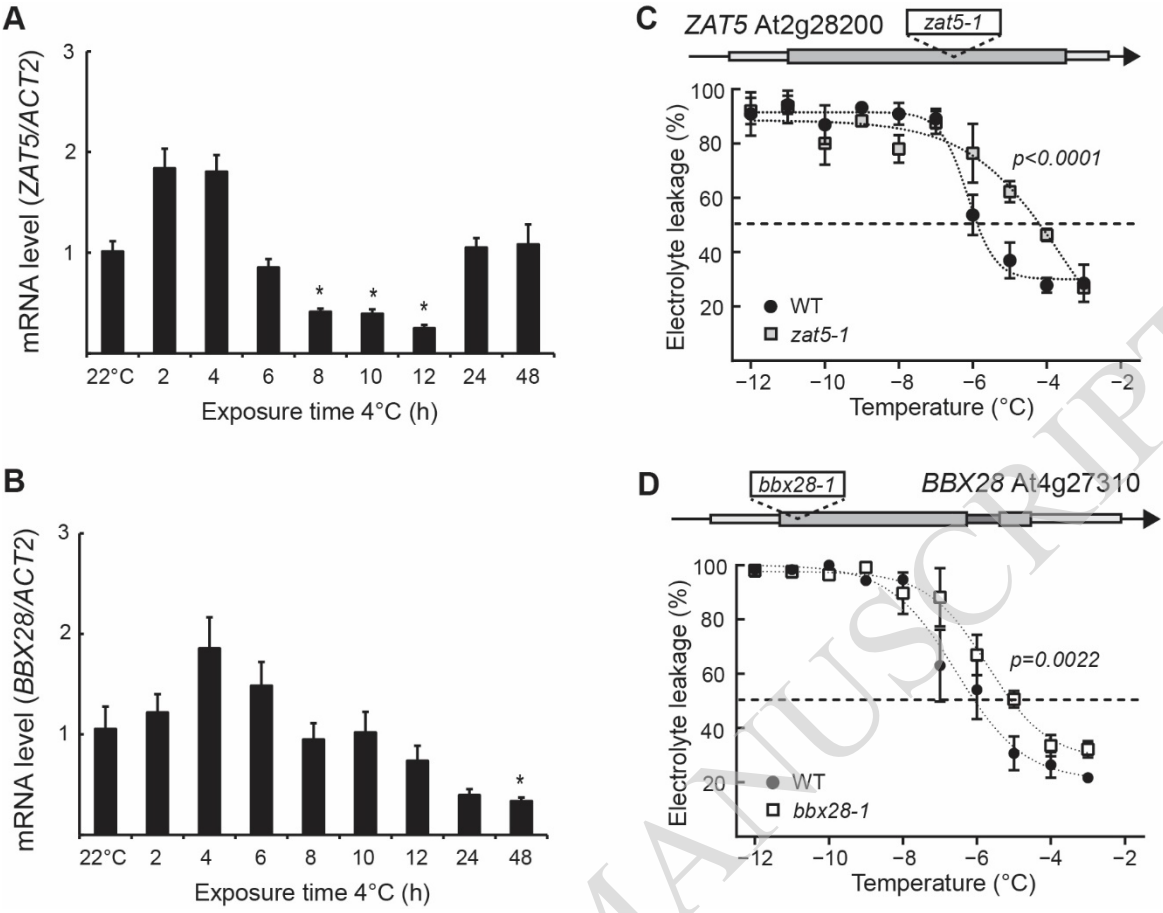

Figure 7. *ZAT5* and *BBX28* are important for cold acclimation.

A) The relative steady-state level of *ZAT5* measured by RT-qPCR following cold exposure (4°C). Steady-state levels were normalized to WT levels at 22°C. The mean values are from three biological replicates. Error bars represent ± SEM. Statistical significance was calculated with Student's t-test (* $p<0.05$).

B) The relative steady-state level of *BBX28* measured by RT-qPCR following cold exposure (4°C). Steady-state levels were normalized to WT levels at 22°C. The mean values are from three biological replicates. Error bars represent ± SEM. Statistical significance was calculated with Student's t-test (* $p<0.05$).

C) Electrolyte leakage in wild-type and *zat5-1* of cold-acclimated (4 days of 4°C) plants. Each data point represents the mean from at least 3 biological replicates (±SEM). The dashed line represents the threshold value, 50%. The dotted lines represent the curve fit. Statistical significance was calculated with an extra sum-of-squares F test and the p value is shown in the figure.

D) Electrolyte leakage in wild-type and *bbx28-1* of cold-acclimated (4 days of 4°C) plants. Each data point represents the mean from at least 3 biological replicates (±SEM). The dashed line represents the threshold value, 50%. The dotted lines represent the curve fit. Statistical significance was calculated with an extra sum-of-squares F test and the p value is shown in the figure.

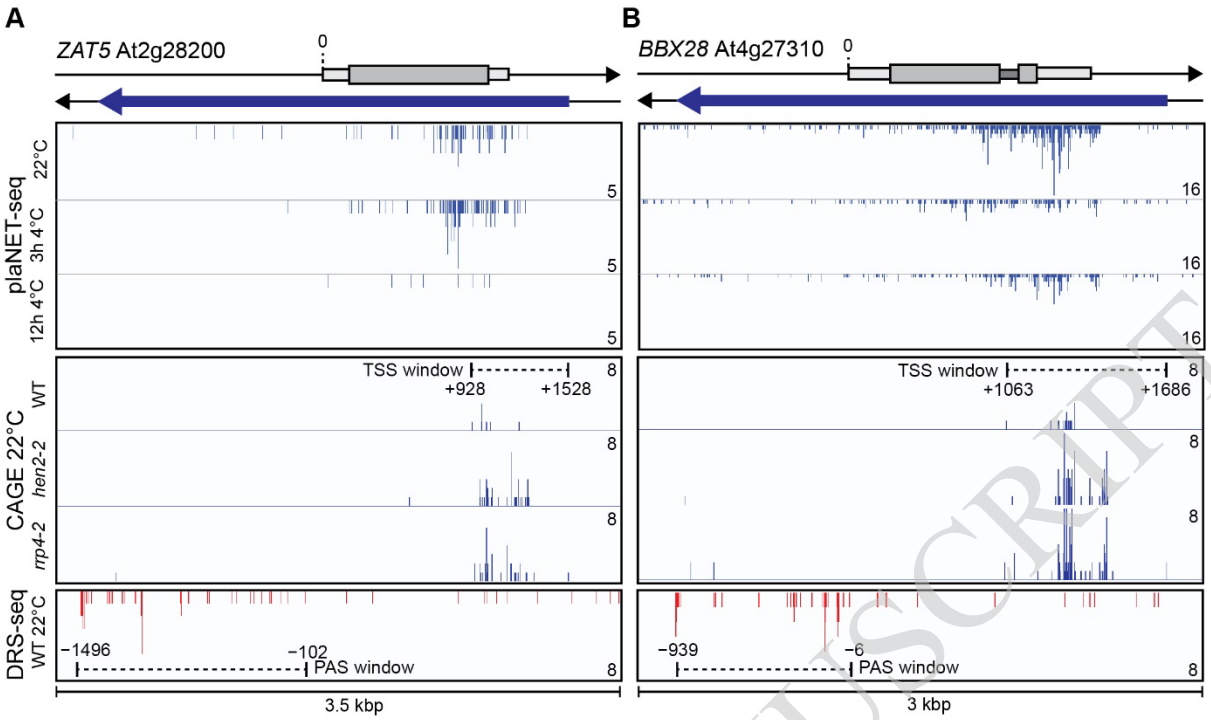

Figure 8. Characterization of *asZAT5* and *asBBX28*.

Screenshots from plaNET-seq (upper panel), CAGE (middle panel), and DRS-seq (lower panel) for **A)** *ZAT5*, and **B)** *BBX28*. 0 indicates the TSS of the sense transcript. Elevated transcriptional activity indicated by higher peaks density and amplitude.

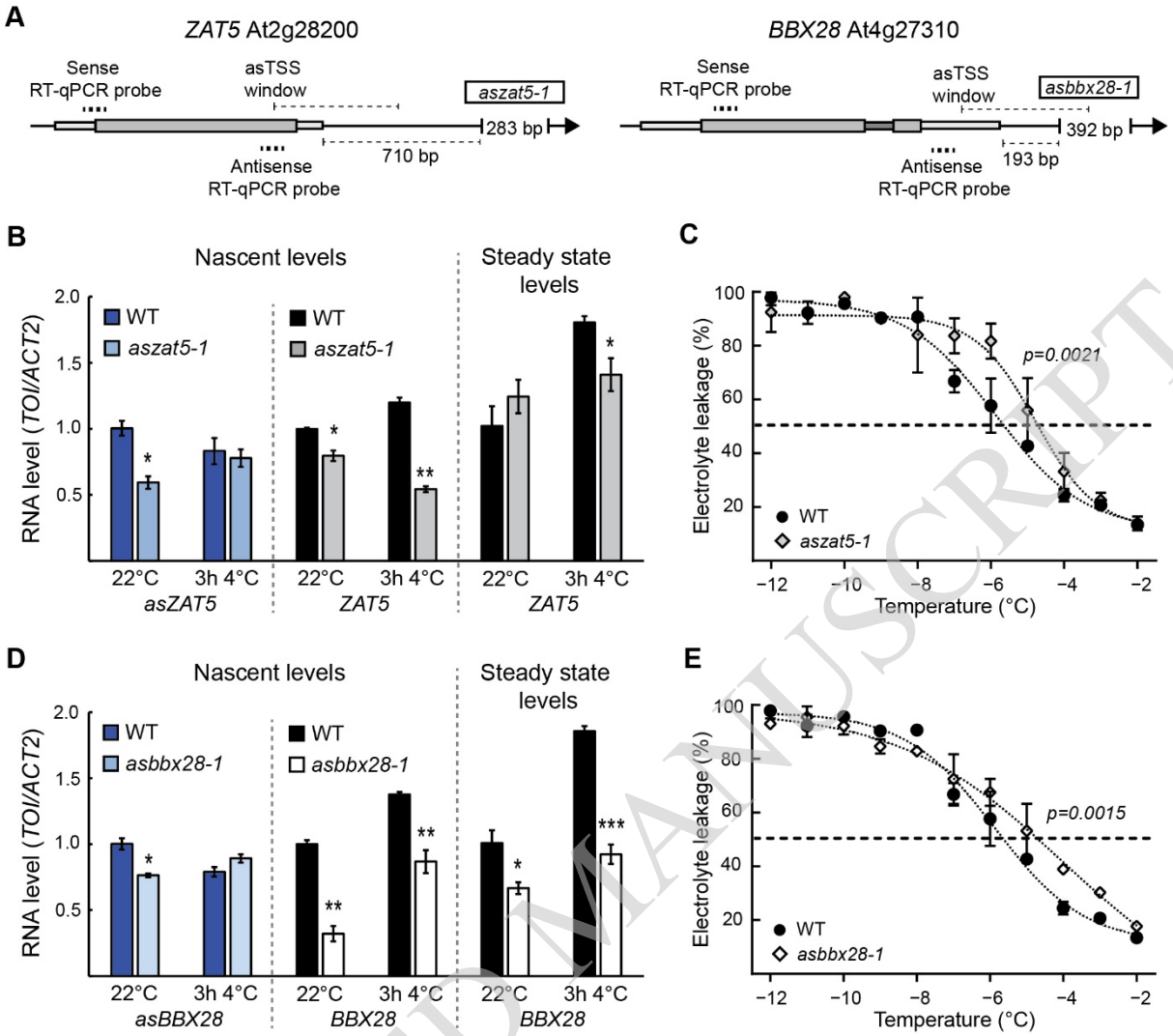

Figure 9. *asZAT5* and *asBBX28* are important for proper regulation of their host gene.

A) Graphical representation of the *ZAT5* and the *BBX28* loci showing the location of sequence targeted by CRISPR-Cas9 to generate an *asZAT5* knockdown line (*aszat5-1*) and an *asBBX28* knockdown line (*asbbx28-1*). Antisense transcription start site (asTSS) window and distances of knocked out genomic sequence from 3'-end are marked with dotted lines. Location of RT-qPCR probes for sense and antisense are shown.

B) The relative nascent and steady-state level of *asZAT5* and *ZAT5* in wild-type and *aszat5-1* measured by RT-qPCR at 22°C and following 3h of cold exposure (4°C). All levels were normalized to WT levels at 22°C. The mean values are from three biological replicates. Error bars represent ± SEM. Statistical significance was calculated with Student's t-test (* $p<0.05$).

C) Electrolyte leakage in wild-type and *aszat5-1* of cold-acclimated (5 days of 4°C) plants. Each data point represents the mean from at least 3 biological replicates (±SEM). The dashed line represents the threshold value, 50%. The dotted lines represent the curve fit. Statistical significance was calculated with an extra sum-of-squares F test and the p value is shown in the figure.

D) The relative steady-state level of *asBBX28* and *BBX28* in wild-type and *asbbx28-1* measured by RT-qPCR at 22°C and following 3h of cold exposure (4°C). All levels were normalized to WT levels at 22°C. The mean values are from three biological replicates. Error bars represent ± SEM. Statistical significance was calculated with Student's t-test (* $p<0.05$).

E) Electrolyte leakage in wild-type and *asbbx28-1* of cold-acclimated (5 days of 4°C) plants. Each data point represents the mean from at least 3 biological replicates (±SEM). The dashed line represents the threshold value, 50%. The dotted lines represent the curve fit. Statistical significance was calculated with an extra sum-of-squares F test and the p value is shown in the figure.

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