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The cyclin-dependent kinase G group defines a thermo-sensitive alternative splicing circuit modulating the expression of Arabidopsis ATU2AF65A

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SUMMARY

The ability to adapt growth and development to temperature variations is crucial to generate plant varieties resilient to predicted temperature changes. However, the mechanisms underlying plant response to progressive increases in temperature have just started to be elucidated. Here, we report that the cyclin-dependent kinase G1 (CDKG1) is a central element in a thermo-sensitive mRNA splicing cascade that transduces changes in ambient temperature into differential expression of the fundamental spliceosome component, ATU2AF65A. CDKG1 is alternatively spliced in a temperature-dependent manner. We found that this process is partly dependent on both the cyclin-dependent kinase G2 (CDKG2) and the interacting co-factor CYCLIN L1 (CYCL1), resulting in two distinct messenger RNAs. The relative abundance of both CDKG1 transcripts correlates with ambient temperature and possibly with different expression levels of the associated protein isoforms. Both CDKG1 alternative transcripts are necessary to fully complement the expression of ATU2AF65A across the temperature range. Our data support a previously unidentified temperature-dependent mechanism based on the alternative splicing (AS) of CDKG1 and regulated by CDKG2 and CYCL1. We propose that changes in ambient temperature affect the relative abundance of CDKG1 transcripts, and this in turn translates into differential CDKG1 protein expression coordinating the AS of ATU2AF65A.

Keywords: alternative splicing, thermo-sensitivity, cyclin-dependent kinases, Arabidopsis.

INTRODUCTION

Plants adapt quickly to changes in ambient temperature, optimizing diverse physiological and developmental processes. Understanding the mechanisms underlying this adaptation becomes ever more critical for agriculture in times of climate change (Solomon, 2007; Porter et al., 2014). Crops are a vital source of food supply, and small changes in either the average temperature or extreme heat events could have significant impacts on yields (Lobell et al., 2008). While heat waves have dramatic effects on productivity of many plant species including cereals (Barnabás et al., 2008; Schlenker and Roberts, 2009; Bita and Gerats, 2013; Hatfield and Prueger, 2015), adaptation to moderate fluctuations in temperature, thermo-morphogenesis, still presents many open questions (Quint et al., 2016). Identification of the molecular components involved in temperature modulation of plant development is a primary aim in breeding thermo-tolerant crop varieties.

Although the coordination of gene expression in response to fluctuating temperatures seems to be conserved across eukaryotes (Kumar and Wigge, 2010), it is less clear whether the sensing mechanisms are also conserved. In mammals, external temperature sensing is mediated by well-defined thermo-sensors (Vriens et al., 2014), but no similar system has been reported for plants. Strikingly, phytochromes have recently been implicated as thermo-sensors, specifically regulating plant morphogenesis in response to temperature variations (Jung et al., 2016; Legris et al., 2016).

Several processes in plants are strongly influenced by temperature at the molecular level, including flowering time and the circadian clock mechanism (Troncoso-Ponce and Mas, 2012; Gu et al., 2013; Song et al., 2013; Marshall et al., 2016). Moreover, plants are able to integrate environmental perturbations directly by transcription of stress-responsive genes (Staiger and Brown, 2013; Samanta and...
Thakur, 2015) and, recently, regulation of pre-mRNA splicing per se was proposed to be part of a molecular thermometer adjusting the transcriptome to specific conditions (Capovilla et al., 2015). Several genes undergo temperature-sensitive mRNA maturation (Posé et al., 2013; Streitner et al., 2013; Airoldi et al., 2015; Sureshkumar et al., 2016), including splicing regulators (Reddy and Shad Ali, 2011; Verhage et al., 2017), and may represent a general mechanism by which temperature modulates gene expression.

Splicing is the removal of intronic (mostly non-coding) sequences from the primary transcript to form the mature messenger RNA (mRNA). This takes place within a multimegadalton complex, the spliceosome, that assembles on the pre-mRNA and contains five small nuclear ribonucleoprotein particles (snRNPs U1, U2, U4, U5 and U6) and approximately 100 non-snRNP proteins, including SR proteins and hnRNPs (Krämer, 1996; Graveley, 2000; Zhou et al., 2002; Noguès et al., 2003; Jurica et al., 2004). Multiple transcripts may be produced from a single pre-mRNA through the combinatorial usage of distinct splice sites in a process called alternative splicing (AS). In mammals and plants, AS affects more than 90% and 63% of intron-containing genes, respectively (Mollet et al., 2010; Zhang et al., 2015). Phosphorylation and dephosphorylation of various spliceosomal components play a central role in regulating formation, activation and inactivation of the spliceosome and in the control over constitutive and AS (Gui et al., 1994; Xiao and Manley, 1997; Misteli et al., 1998; Wang et al., 1998; Graveley, 2000; Shin and Manley, 2004; Shepard and Hertel, 2008; Kralovicova et al., 2011).

Cyclin-dependent kinases (CDKs) are an evolutionarily conserved family of serine/threonine kinases in Eukaryotes. Initially, CDKs were characterized for their role in cell cycle (Strausfeld et al., 1996; Rane et al., 1999; Sherr and Roberts, 1999; Kozar et al., 2004), but they are also involved in other regulatory pathways like DNA replication and repair (Caspari and Hilditch, 2015; Chadha et al., 2016). Moreover, CDKs have been implicated in pre-mRNA processing through the interaction with several spliceosomal components (Ko et al., 2001; Hu et al., 2003; Loyer et al., 2005; Even et al., 2006; Cheng et al., 2012). In particular, human CDK11 was shown to affect both pre-mRNA splicing and apoptosis, while retaining a primary role in cell cycle (Loyer et al., 2005).

In plants, the CDKG group is the most closely related to human CDK11 (Menges et al., 2005; Umeda, 2005). CDKGs are also phylogenetically linked to the enigmatic Ph1 kinase cluster that participates in chromosome pairing in wheat (Doonan and Kitsios, 2009). In Arabidopsis, CDKG proteins are encoded by two closely related genes, CDKG1 and CDKG2. CDKG1 has a role in splicing of a pollen cell wall gene (Huang et al., 2013), and in chromosome pairing and recombination at high ambient temperature (Zheng et al., 2014). CDKG2 was reported as a negative regulator of flowering and abiotic stress response (Ma et al., 2015), but not previously implicated in mRNA regulation.

Given the previously described roles of CDKG1 in splicing (Huang et al., 2013) and meiosis (Zheng et al., 2014), both inherently temperature-sensitive processes, we asked whether CDKG1 plays a role in mRNA thermo-response. Here, we show that CDKG1 is the central element in a thermo-sensitive cascade that transduces changes in ambient temperature into differential transcript abundance of the target gene ATU2AF65A. This mechanism requires the AS of CDKG1 itself in order to maintain the proper expression pattern of ATU2AF65A across the temperature range. Finally, CDKG2 and the associated CYCL1 activate the circuit by adjusting CDKG1 AS to ambient temperature.

We propose that CDKG1, together with CDKG2 and CYCL1, is part of a molecular thermometer fine-tuning environmental information into AS of target genes.

RESULTS

CDKG1 is required for ATU2AF65A AS at ambient temperature

Several Arabidopsis CDKs have been previously shown to be involved in pre-mRNA processing (Kitsios et al., 2008; Hajheidari et al., 2013; Huang et al., 2013). On the other hand, splicing factor transcripts are themselves subjected to temperature-dependent AS (Reddy and Shad Ali, 2011; Verhage et al., 2017). Using a panel of genes representing more than 80 potential alternative transcripts, we performed a reverse transcriptase-polymerase chain reaction (RT-PCR) screen for splicing events dependent on functional CDKG1 (Table S1). We found that AS of ATU2AF65A (U2 Auxiliary Factor 65A; Figure 1a for scheme), a constitutive spliceosomal component involved in flowering time regulation (Verhage et al., 2017), was affected in the null allele cdkg1-1 (Figure 1b). ATU2AF65A has a complex splicing pattern of intron 11: intron splicing (mRNA1) and partial (mRNA2) or complete intron retention (mRNA3). Specifically, lack of CDKG1 decreased splicing efficiency with a consequent increase in intron 11 retention in the mature mRNA (mRNA3; Figure 1b).

Because CDKG1 was previously known to have a temperature-dependent function in meiosis and to be involved in mRNA maturation (Huang et al., 2013; Zheng et al., 2014), we asked whether its function was required along the environmental temperature range, using ATU2AF65A as a gene model. Wild-type Col-0 and cdkg1-1 mutant seedlings were grown for 2 weeks at 22°C, and then transferred to 12°C or 27°C for 48 h to test the effect of temperature on target pre-mRNA processing. We included mutant lines for CDKG2 and CYCL1 (cdkg2-1; cycl1-1; Zheng et al., 2014; Ma et al., 2015) to reveal possible compensatory functions or cross-regulations: CDKG2 is the only
paralogue of CDKG1 and CYCL1 is the cognate cyclin in Arabidopsis for both kinases (Van Leene et al., 2010).

In wild-type plants, splicing of ATU2AF65A was affected by temperature (Figure 1c), as previously reported (Verhage et al., 2017). At 12°C, the fully spliced form (mRNA1) was the most abundant, while at 27°C the complete intron retention form (mRNA3) was dominant. In cdkg1-1 plants, the intron 11 splicing defect was more prominent at high temperatures (Figure 1c, lower panel). Moreover, cycl1-1 showed a similar splicing pattern to cdkg1-1 with increased intron retention. Interestingly, we did not observe a splicing defect in cdkg2-2 plants (Figure 1c). To validate these results, we analysed the levels of each isoform by quantitative RT-PCR (RT-qPCR). We confirmed that the three ATU2AF65A mRNA variants were differentially regulated by temperature: in wt plants mRNA1 was the most abundant at 12°C, while at 27°C mRNA2 and mRNA3 levels were increased (Figure S1a). Superimposed on the temperature regulation, cdkg1-1 showed impaired levels of partial and total intron retention (mRNA2 and mRNA3, decreased and increased, respectively), while the expression of fully spliced mRNA1 was not different from control plants. In the cycl1-1 mutant, we also observed increased mRNA3 and mRNA2 levels at both temperatures but these were not compensated by equal mRNA1 reduction, suggesting that transcriptional regulation is also involved (Figure S1a).

To quantify the differences between the mutant cdkg1-1 and the wt, we calculated the splicing index (SI) as the ratio mRNA2/[mRNA2 + mRNA3] (partial and total intron 11 retention) in Col-0 and cdkg1-1 seedlings grown at 12°C (White) or at 27°C (Grey). Each mRNA isoform was detected with the primer as pairs indicated in (a). Data represent means ± SD (n ≥ 3). Student’s t-test comparing Col-0 with cdkg1-1: *** P < 0.001.
These results indicate that CDKG1 is an important regulator of ATU2AF65A mRNA splicing, but there is an additional and partially independent effect of ambient temperature.

**CDKG2 and CYCL1 adjust CDKG1 AS to ambient temperature**

To explore this further, we next asked whether CDKG1 mRNA levels themselves fluctuate along the temperature range in order to maintain the proper ratio between ATU2AF65A isoforms. Contrary to our expectations, CDKG1 was constitutively expressed at similar levels both at high and low temperatures in the wild-type, as well as in the single cdkg2-2 and cycL1-1 and in the double cdkg2-2; cycL1-1 mutant lines (Figure S2a). However, we noted the presence of several bands for the CDKG1 transcript in RT-PCR, suggesting alternative mRNA splicing. While CDKG1 does not need to undergo splicing to produce a mature coding mRNA, several introns have been annotated in what was considered an intron-less gene (Figure 2a and Figure S2b; Table S2). Moreover, CDKG1 alternative introns were preferentially spliced in Col-0 at higher temperatures, while largely retained at lower ones (Figure 2b and Figure S2b; Verhage et al., 2017).

Strikingly, CDKG1 intron 1 removal was severely impaired in cdkg2-2, cycL1-1 and further decreased in the double mutant (Figure 2b). We named the alternative mRNA species as CDKG1L (Long) and CDKG1S (Short), depending on whether the intron was retained or spliced, respectively. We speculated that this could be a mechanism to fine-tune CDKG1 transcript/protein abundance in a temperature-dependent manner, under the control of CDKG2 and CYCL1.

To quantify this effect, the SI was calculated as the ratio of CDKG1S/CDKG1L and CDKG1S + CDKG1L. In Col-0, the SI at 27°C was approximately 1.5-fold that calculated for 12°C, while the SI was decreased in cdkg2-2, cycL1-1 (3- and 1.8-fold, respectively) and in the double mutant lines (16-fold; Figure 2c) where the temperature dependence of the SI was almost completely abolished. No appreciable differences in the annotated AS events for CDKG2 or CYCL1 transcripts occurred either at different temperatures or in different backgrounds (Figure S3a), suggesting that temperature regulation was specific for CDKG1 mRNA.

Removal of CDKG1 intron 1, located across the 5′UTR and adjacent coding region, erases the primary start codon (ATG). This shorter transcript could use a downstream in frame start codon to produce an uncharacterized CDKG1 protein lacking the nuclear localization signal (NLS) and two (out of four) of the reported Arg/Ser-rich (RS) interaction domains (Huang et al., 2013), based on database annotations (TAIR 10; Figure 2a).

Many AS events either trigger nuclear retention (Gohring et al., 2014) or premature degradation of mRNAs via the nonsense mediated mRNA decay (NMD) pathway (Kalyna et al., 2012), and the affected mRNAs are unlikely to be translated. To test whether CDKG1L and CDKG1S

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mRNAs were affected by the NMD pathway, transcript accumulation in NMD defective lines upf1-5 and upf3-1 (Hori and Watanabe, 2005; Arciga-Reyes et al., 2006) was compared with Col-0 and neither transcript showed appreciable upregulation in these mutants, indicating that they are not targeted by NMD (Figure 3a).

To evaluate possible nuclear retention of the CDKG1L and CDKG1S transcripts, cell fractionation of Col-0 protoplasts was followed by RT-PCR to compare the cytoplasmic and nuclear fractions. Both CDKG1L and CDKG1S transcripts were exported to the cytoplasm, although to different extents (Figure 3b, top panel). Only the fully spliced form of a control transcript, SEF factor (AT5G37955), was found in the cytoplasm (Figure 3b, middle panel), as would be expected (Gohring et al., 2014). The purity of the fractions was confirmed by Western blot (Figure 3b, bottom panel).

These data suggested that both CDKG1L and CDKG1S transcripts are exported to the cytoplasm where they could be translated into proteins. Confirming that the short transcript can be translated, CDKG1S (AT5G63370.2) was found in the ribosomal fractions in RNA-seq analyses (Aubry et al., 2014; Juntawong et al., 2014).

Taken together, our data suggest that CDKG1L, CDKG1S, and CYCL1 participate in a cascade of AS events that amplifies ambient temperature effects on target gene expression.

**CDKG1L and CDKG1S show distinct subcellular localization**

To define the subcellular distribution of the two CDKG1 proteins in planta, CDKG1L and CDKG1S were transiently expressed in Nicotiana benthamiana leaf cells as cDNA-GFP fusion proteins. We also generated a splicing-competent variant (365:CDKG1SC-GFP) consisting of CDKG1 5’UTR containing the full intron 1 region and the coding sequence that allows for the production of both S and L mRNA isoforms.

As previously reported (Huang et al., 2013), the CDKG1L-GFP protein localized to the nucleus while CDKG1S, lacking the NLS, showed both nuclear and cytoplasmic GFP signal (Figure 4a). Moreover, CDKG1L-GFP co-localized strongly with the nuclear splicing factor RSp34 (Lorković et al., 2004a), and was associated with nuclear speckles (Figure S4a and b; Huang et al., 2013), as has been described for other spliceosome-associated components (Lorković et al., 2004b).

Although temperature did not affect the cellular distribution of either CDKG1L or CDKG1S, CDKG1SC-GFP-containing cells showed a clear temperature-dependent change in signal localization (Figure 4a): at low temperature, the GFP signal was detected only in the nucleus, while at higher temperatures GFP localized both to the cytoplasm and the nucleus (Figure 4a) as expected from the temperature-dependent production of the CDKG1S transcript (described in Figure 2). The preferential localization of GFP signal to the cytoplasm at higher temperature was not due to the cleavage of the GFP moiety, as shown by Western blot (Figure S4c and d). We have also quantified relative nuclear to cytoplasmic fluorescence in stably transformed lines (see below). There were no changes in the percentage of nuclear GFP fluorescence for CDKG1L-GFP and for CDKG1S-GFP, while for CDKG1SC-GFP the ratio of nuclear to cytoplasmic fluorescence was lower at 27°C (Figure S5a), as expected for increased AS of the

![Figure 3](attachment:figure3.png)

**Figure 3.** CDKG1L and S are exported to the cytoplasm and not degraded by the nonsense mediated mRNA decay (NMD) pathway.

(a) Reverse transcriptase-polymerase chain reaction (RT-PCR) products of CDKG1 long and short mRNA isoforms in wt and in the NMD defective upf1-5 and upf1-3 mutant backgrounds. Analysis of the splicing pattern showed no accumulation for any of the transcripts, suggesting no sensitivity to degradation by the NMD pathway.

(b) Upper panel, gel separation of RT-PCR products of CDKG1 splice variants (L and S) in fractionated cell extracts. Middle panel, gel separation of RT-PCR products of the SEF factor splice variants in fractionated cell extracts. FS, fully spliced; ir1, intron 1 retention; ir1+2, retention of introns 1 and 2. Lower panel, polyacrylamide gel electrophoresis separation of protein extracts from the same cell fractionation. The cytoplasmic fraction is free of histone 3, and the nuclear fraction is free of the cytoplasmic isoform of fructose-1,6-bisphosphatase. T, total RNA/protein fraction; C, cytoplasmic RNA/protein fraction; N, nuclear RNA/protein fraction.

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transcript. Although expression is driven by a strong promoter, these data together suggest that CDKG1 is not directly regulated by temperature at the protein level, and support the idea that temperature modulation acts on the transcript level, affecting the balance between $L$ and $S$ forms.

Figure 4. Effect of temperature on the localization of CDKG1-GFP protein isoforms.
(a) Subcellular localization of the different CDKG1 isoforms as C-terminal GFP fusion proteins in *Nicotiana benthamiana* leaves. Infiltrated plants were kept at 12°C or 30°C as indicated for 48 h before leaves were imaged using confocal microscopy. Left-hand-side images GFP channel, right-hand-side brightfield images. Inset images show nucleus detail.
(b) Localization of CDKG1-GFP protein isoforms in Arabidopsis roots grown at 22°C. Roots from Arabidopsis plants stably expressing the different CDKG1 isoforms as GFP fusions as indicated were imaged using confocal microscopy. Cell walls were stained with propidium iodide (PI; red).
Temperature-dependent AS of CDKG1 is necessary to compensate expression of ATU2AF65A along the temperature range

To test the functions of the different CDKG1 isoforms, cdkg1-1 mutants were stably transformed with the three different CDKG1-GFP fusion constructs. As observed in transient assays, plants expressing CDKG1L showed exclusive nuclear GFP fluorescence, while CDKG1S-GFP was found both in the nucleus and cytoplasm of root epidermal cells (Figure 4b). As for CDKG1SC-GFP, we observed strong fluorescence in the nucleus with some faint cytoplasmic localization at normal growth temperature (22°C). To address whether CDKG1 variants could have different functions in splicing of ATU2AF65A, 2-week-old transgenic seedlings (35S::CDKG1L-GFP, 35S::CDKG1S-GFP, 35S::CDKG1SC-GFP) were incubated at 12°C or 27°C for 48 h prior to RNA isolation. As previously observed, ATU2AF65A SI increased in Col-0 control plants from 12°C to 27°C in RT-qPCR (Figures 1d and 5a).

The full-length CDKG1L could restore mRNA processing of ATU2AF65A at low temperatures but, surprisingly, showed severely reduced efficiency at high temperatures (Figure 5a). In relative terms, we calculated that ATU2AF65A splicing in CDKG1L-GFP-expressing plants was severely reduced by the temperature shift from 12°C to 27°C (0.65-fold; Figure 5b). While the efficiency of splicing intron 11 was also reduced in comparison to Col-0 at 27°C (0.67-fold), at low temperature all CDKG1L lines had somewhat higher splicing compensation than the control (1.2-fold; Figure 5b). Expression of CDKG2-GFP could not restore the splicing of ATU2AF65A in the cdkg1 mutant line (Figure 5a), but did cause a small increase in the fully spliced ATU2AF65A mRNA1 (Figure 5c).

In contrast, the splice-competent variant CDKG1SC-GFP was able to rescue the ATU2AF65A splicing phenotype at both temperatures, and a similar result was observed for the lines expressing CDKG1S-GFP (Figure 5a).

These data show that splicing of CDKG1 is necessary to maintain the correct balance between the ATU2AF65A splice forms across the temperature range (Figure 6).

DISCUSSION

We report a temperature-sensing module involving CDKG1, CDKG2 and CYCL1 that transduces ambient temperature information to downstream targets by modulation of AS (Figure 6). In particular, this module impinges on the AS of ATU2AF65A, a splicing factor already known to be environmentally sensitive (Pettillo et al., 2014; Verhage et al., 2017), and thus provides a mechanism to integrate temperature variation with the gene expression machinery.

Splicing is a conceptually attractive mechanism for integrating the thermal response in a flexible and sensitive manner. The spliceosome is one of the largest cell molecular complexes and its temperature dependency has been widely documented (Reddy and Shad Ali, 2011; Staiger and Brown, 2013; Streitner et al., 2013; Schlaen et al., 2015; Verhage et al., 2017). The RNA secondary structures required for splicing are inherently thermo-sensitive, and could be tuned either on the individual gene basis for specific responses or could even affect gene expression on a genome-wide scale. A mechanism based on this general principle operates during the heat shock response in budding yeast Saccharomyces cerevisiae (Meyer et al., 2011) and on the thermal-sensitive splicing of a clock gene, period-3, in Drosophila melanogaster, potentially providing an adaptive mechanism across different climatic zones (Low et al., 2008). In plants, the spliceosome itself may be directly involved mediating temperature responses as the LSM2-8 complex, the protein moiety of the U6 snRNP, is required in Arabidopsis to correctly couple spliceosome activity to environmental conditions, including temperature (Carrasco-López et al., 2017). Our results indicate that protein kinases associated with the spliceosome are capable of modulating its activity, providing an additional route for environmental information to affect gene expression. It will be interesting to determine whether this is a widespread mechanism across species.

Severe temperature changes clearly affect AS, but the thermodynamic effects of ambient temperature fluctuations are more subtle and likely to require augmentation by associated factors.

Plants have several classes of splicing factors, and some exist as multigene families that may help maintain transcript homeostasis across environmentally relevant temperatures (Wang et al., 2012; Schlaen et al., 2015; Marshall et al., 2016) and as well as in more extreme conditions (Ciuzan et al., 2015; Kim et al., 2017). Ambient temperature variations are also known to alter the availability of specific splicing factors in grape leaves where their abundance correlates with the level of AS (Jiang et al., 2017). Moreover, splicing factors are subjected to various forms of post-translational modification that could modulate splice site selection. Protein phosphorylation is one of these modifications, and the LAMMER kinase (Savaldi-Goldstein et al., 2000) and CDKG1 (Huang et al., 2013; Zheng et al., 2014) are proposed to have this activity in plants.

Based on our results, CDKG1 is required for the AS of ATU2AF65A across the tested temperature range, contrary to its reported role in meiosis where it is only required at high ambient temperature (Zheng et al., 2014). It will be interesting to determine if the meiosis specific effect is through the regulation of AS. Loss of ATU2AF65A affects flowering time at high and low temperatures (Verhage et al., 2017), and this combinatorial cascade composed of
Figure 5. CDKG1 protein variants show temperature-dependent effects on splicing.
(a) Relative levels of ATU2AF65A estimated by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). Splicing index (SI) represents the ratio of mRNA2/mRNA2 + mRNA3 in Col-0 and transgenic lines in the cdkg1-1 background at 12°C or at 27°C. Three independent lines expressing the long (L), the short (S), or the splicing competent (SC) CDKG1-GFP fusion constructs are shown. Data represent means ± SD (n ≥ 3). Overexpression of CDKG2-GFP fusion showed no compensation of ATU2AF65A splicing pattern in two independent lines. Student’s t-test comparing the respective line with Col-0 at the same temperature: *** P < 0.001; ** P < 0.01; * P < 0.05. Primer position is reported on the respective splicing variant in Figure 1a.
(b) Normalized levels of ATU2AF65A in Col-0 and in the overexpression lines CDKG1L-GFP. SI represents the ratio mRNA2/mRNA2 + mRNA3. Expression levels of Col-0 at 12°C have been set to 100% and the other values been normalized accordingly for each line. Student’s t-test: ** P < 0.01.
(c) RT-qPCR analysis of ATU2AF65A mRNA1 in Col-0, cdkg1-1 and overexpression lines. The fully spliced mRNA1 levels are not significantly different between the control, the mutant and the CDKG1L, S or SC lines. CDKG2 overexpression lines show a statistically significant increase in mRNA1 level in comparison to Col-0 at both temperatures. Data represent means ± SD (n = 3). * P < 0.05.
protein kinases and AS could provide a sensitive means of transducing ambient temperature or other signals into altered gene expression and growth, providing an interesting paradigm to regulate plant development in response to environmental changes.

CDKG2 and CDKG1 are not functionally redundant with regard to mRNA splicing, although they may occasionally affect the same target as CDKG2 increases the level of fully spliced \(\text{ATU2AF65A}\) mRNA. While relative expression does not change along the temperature gradient, CDKG1 is alternatively spliced in a temperature-sensitive manner – as has also been observed for other splicing-related genes (Reddy and Shad Ali, 2011; Verhage et al., 2017). CDKG1 probably originated from a retrotransposition event early in the Arabidopsis lineage with the loss of the original intron sequences (Zhang, 2005). However, CDKG1 still carries memories of splice sites and exon enhancer sequences that enable coding and non-coding sequences to be spliced out, an event that was termed exontron (exonic intron) splicing if occurring in coding regions (Marquez et al., 2015). The fact that splicing events are still present in the retrotransposed CDKG1 gene suggests a strong evolutionary pressure on these splicing events and underscores the importance of post-transcriptional modifications for this gene. Particularly, the presence of the first intron sequence is essential for proper expression and subcellular location of CDKG1 variants along the temperature range as well as for the processing of a downstream target. Our hypothesis was supported by the fact that while ATU2AF65A intron 11 was inefficiently removed in the presence of the full-length CDKG1 at high temperature, both the short CDKG1S and the splicing competent CDKG1SC were able to complement splicing.

The role of this group of CDKs in AS could be evolutionarily conserved across animals and plants, at least partially. The large isoform of mammalian CDK11, p110 activates while the short isoform represses splicing/AS in tissue culture cells (Loyer et al., 2008). While the C-terminal kinase domain is highly conserved between CDKG and CDK11, the large N-terminal domain in plant proteins suggests functional divergence. This divergence may involve ambient temperature sensing as CDKG1S and CDKG1L proteins also differ in their N-terminal domains. Ambient temperature sensing/response is of particular interest in terms of organisms that do not regulate their body temperature. A phylogenic functional comparison CDK11/CDKG function in poikilotherms as compared with homeotherms could be informative as how gene expression networks adapt to ambient temperature.

In addition, the temperature-dependent AS splicing of CDKG1 has wider implications in terms of genomic recombination and stability. CDKG1 was shown to be involved in chromosome pairing and recombination during meiosis at high ambient temperature (Zheng et al., 2014). CDKG1 has previously been shown to be involved in the splicing of the CaIm5 gene in the tapetal cells affecting pollen fertility (Huang et al., 2013). In addition, a recent survey of transcripts in the male sexual lineage has shown that mis-splicing of the MPS1 gene leads to meiotic defects (Walker et al., 2018). Taken together, these observations suggest that splicing is an important regulatory mechanism during meiotic cell divisions. Temperature-dependent splicing could, at least in part, explain the temperature sensitivity of the male germ line. Therefore, a detailed characterization of AS in meiocytes along the temperature range could be informative.

Our data support a model where CDKG1 mRNA acts as a thermo-switch that is activated by CDKG2 and CYCL1 (Figure 6). The mechanisms and the pattern of interactions remain to be determined, but our data suggest a clear, previously unidentified link between temperature, CDKs and AS. In addition, the observation that CDKG1 is itself regulated at the mRNA level by both temperature and the CDKG2-CYCL1 complex reveals a multi-layered AS-kinase cascade.
EXPERIMENTAL PROCEDURES

Plant material and growth conditions

The wild-type Columbia (Col-0) and mutant stocks used in this study were obtained from the Nottingham Arabidopsis Stock Centre. The cdkg1-1, cdkg2-2, cycl1-1, upf1-5 and upf3-1 mutant lines have been previously described (Hori and Watanabe, 2005; Arciga-Reyes et al., 2006; Zheng et al., 2014; Ma et al., 2015). Moist sterilized seeds were pre-treated for 3 days at 4°C in the dark and germinated on Petri-plates. Plants transferred to soil after 1 week or sown direct to soil were grown in standard greenhouse conditions at 22°C supplemented with 16 h light and 8 h dark. For analysis of ATU2AF65A splicing plants were grown in plant medium plates (0.5 × MS salts and vitamins, pH 5.8, 0.7% plant agar) for 2 weeks at 22°C in constant light conditions to minimize the reported light effect on ATU2AF65A splicing (Pettillo et al., 2014). Plants were transferred to 12°C or 27°C for 2 days and collected for mRNA.

Construct generation and plant transformation

CDKG1SC, CDKG1L, CDKG1S and CDKG2 sequences were cloned in frame with GFP sequence at the 3′-end by fusion PCR. The entire cassette was cloned into the pB7WG2.0 (Karimi et al., 2002) destination vector by Gateway™ cloning and transformed into Agrobacterium tumefaciens strain GV3101 by electroporation. Arabidopsis cdkg1-1 plants were transformed by floral dip (Clough and Bent, 1998). Single-insertion, BASTA-resistant plants were selected, and a minimum of three lines per construct were analysed.

For transient expression in N. benthamiana leaves, the CDKG1x-GFP cassette was cloned into the pEOAH-DEST2 vector transformed into A. tumefaciens LBA4404 strain. Leaf infiltration was performed as described (Sainsbury et al., 2009). After 5 days, leaves were harvested for confocal imaging of GFP or for protein extraction.

RNA extraction, RT and qPCR

Total RNA was extracted from whole rosettes using the RNeasy Plant Mini kit (Qiagen); 1 μg total mRNA was used to generate cDNA using the SuperScript™ III First-strand Synthesis Kit (Invitrogen); 50 ng cDNA was used for the PCR reaction. The primers used for the analysis of the AS of the different genes are listed in Table S3. qPCRs were performed using the LightCycler 480 (Roche). Typically 10 ng of cDNA was used in a 20 μl reaction containing 0.25 μM of each primer and 10 μl LightCycler® 480 SYBR Green I Master (Roche). Each sample was done in triplicate, and expression of PP2A A3 (AT1G13320) was used as a reference (Czechowski, 2005). Data were analysed using the LightCycler® 480 Software (Roche).

Protoplast isolation and subsequent cell fractionation

Mesophyll protoplasts were isolated from 3-week-old Col-0 plants as described by Wu et al. (2009). Subsequent cell fractions were prepared as described by Gohring et al. (2014) with slight modifications. Briefly, 2 × 10^6 Arabidopsis thaliana mesophyll protoplasts were resuspended in 1 ml NiB lysis buffer (10 mM MES-KOH pH 5.5, 200 mM sucrose, 2.5 mM EDTA, 2.5 mM dithiothreitol (DTT), 0.1 mM spermine, 10 mM NaCl, 0.2% Triton X-100, 1 μM RNase (Promega)) and subsequently lysed using a 25-G gauge needle (6–10 passages). Complete lysis was confirmed by light microscopy. For the total fraction, 100 μl of lysed cells was immediately resuspended in 1 ml TRIzol (Ambion) and kept on ice until the remaining fractions were processed. The lysate was pelleted for 10 min at 500 g, and 1 ml of supernatant, which represents the cytoplasmic fraction, was removed and centrifuged for another 15 min at 10,000 g. Then, 800 μl of supernatant was resuspended in 8 ml TRIzol and the pellet, which represents the nuclear fraction, was resuspended in 4 ml NRB (20 mM Tris-HCl pH 7.5, 250 mM sucrose, 10 mM MgCl₂, 0.5% Triton X-100, 5 mM β-mercaptoethanol) and carefully overlaid on top of 500 μl NRB (20 mM Tris-HCl pH 7.5, 1.7 μM sucrose, 10 mM MgCl₂, 0.5% Triton X-100, 5 mM β-mercaptoethanol) and centrifuged at 16,000 g for 45 min. Finally, the nuclear pellet was resuspended in 1 ml TRIzol and RNA as well as proteins of all fractions were isolated following the manufacturer’s instructions. Western blot analyses (see below for details) using anti-H3 and anti-FBPase (1:5000, Agrisera) antibodies were performed to confirm purity of nuclear and cytoplasmic fractions, respectively.

Protein extraction and Western blotting

Total proteins were extracted in RIPA buffer (1 mM sodium phosphate buffer pH 7, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulphate, 1 mM DTT and protease inhibitors cocktail). The extract was filtered through a Celtrix 30-μm filter (Partec) and used for immunoprecipitation using the μMACS GFP Protein isolation kit (Miltenyi Biotec GmbH) following the manufacturer’s instructions. For in vitro expression, proteins were produced in the T7-TNT Quick coupled transcription/translation system using wheat germ extract according to the manufacturer’s instructions (Promega). Proteins were separated in 10% mini-Protein TGX precast gels (Biorad). Western blot was done using anti-GFP monoclonal antibody (Santa Cruz) diluted 1:2500 and horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Sigma, 1:10,000). Detection was done using ECL Select Western Blot Detection Reagent (Amersham) and visualized using Image Quant LAS 4000 (GE).

For the cell fractionation experiments, proteins were separated using 10–16% polyacrylamide gels and the Xcell SureLock Mini cell system (LifeTechnologies), and transferred to PVDF membranes. The primary antibodies used were anti-H3 or anti-FBPase (Agrisera) both at a dilution of 1:5000, and the secondary antibody used was rat anti-rabbit IgG coupled to HRP (Cell Signaling Technology) at a 1:10,000 dilution.

Detection was performed using the ECL Western Blotting Detection Reagent (Amersham), and images were exposed to X-ray films and developed using a Curix80 (AGFA) developer.

Microscopy

Roots stained with propidium iodine or N. benthamiana infiltrated leaves were imaged using a Leica TCS SP5 II confocal laser-scan microscope controlled by Leica LAS-AF software. Multiple plants per line and multiple lines were observed, and representative images are shown.

GFP levels in cells were quantified according to McCloy et al. (2014) with slight modifications. Briefly, a single in-focus plane was acquired. Using ImageJ (v1.51, NIH), an outline was drawn around the sample area and the mean fluorescence was measured, while visible nuclei were selected independently and GFP signal calculated as the sum of all the nuclei intensity.

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Statistical analysis

Statistical analyses were performed using PRISM 6.0 (GraphPad Software, La Jolla) or Excel (Microsoft Office, Microsoft). P-values were calculated using an unpaired, two-tailed Student’s t-test (**P < 0.001; *P < 0.01; *P < 0.05; ns, not significant). Data represent means ± standard deviation (n ≥ 3).

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AUTHOR’S CONTRIBUTIONS

NC, CN, JHD and AB conceived the project and designed research; NC, CN, AF and DD performed research; NC and CN analysed data; NC, CN, JHD and AB wrote the paper.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. RT-qPCR analysis of ATU2AF65A splice variants in Col-0 and cdkg1-1 mutant.

Figure S2. Analysis of CDKG1 AS.

Figure S3. Gel separation of RT-PCR products of CDKG2 and CYCLIN L1 splice variants.

Figure S4. Analysis of CDKG1-GFP protein expression.

Figure S5. Quantification of the GFP signal in 35S-CDKG1 lines.

Table S1. List of the genes investigated in Col-0 and cdkg1-1 mutant lines.

Table S2. List of the transcripts and intron splicing events investigated.

Table S3. List of RT-PCR, RT-qPCR and cloning primers used.

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