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Small changes in ambient temperature affect alternative splicing in *Arabidopsis thaliana*

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Alternative splicing (AS) gives rise to multiple mRNA isoforms from the same gene, providing possibilities to regulate gene expression beyond the level of transcription. In a recent paper in Nucleic Acids Research we used a high resolution RT-PCR based panel to study changes in AS patterns in plants with altered levels of an hnRNP-like RNA-binding protein in *Arabidopsis thaliana*. Furthermore, we detected significant changes in AS patterns between different *Arabidopsis* ecotypes. Here we investigated how small changes in ambient temperature affect AS. We found significant changes in AS for 12 of 28 investigated events (43%) upon transfer of *Arabidopsis* plants from 20°C to 16°C and for 6 of the 28 investigated events (21%) upon transfer from 20°C to 24°C.

Keywords: alternative splicing, RNA-binding proteins, temperature, posttranscriptional regulation

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Primary transcripts or precursor mRNAs (pre-mRNAs) usually contain introns that are removed during splicing. By combinatorial use of alternative splice sites pre-mRNAs can produce multiple transcript isoforms, leading to protein variants with different domains.[1,2] Furthermore, different transcript isoforms originating from the same pre-mRNA can have different fates in the cell due to the presence or absence of miRNA target sites or inclusion of premature termination codons (PTC), channeling the transcript into the nonsense-mediated decay (NMD) pathway.[3-6] Because recent data show that more than 60% of transcripts in *Arabidopsis* are alternatively spliced under regular growth conditions, AS provides an important layer of regulation at the posttranscriptional level.[7] In particular, AS patterns of disease resistance genes have been shown to change upon pathogen infection.[8-10] Furthermore, abiotic stresses such as cold or heat treatment elicit changes in AS patterns.[1,11,12] The use of AS sites is determined by RNA-binding proteins, predominantly SR (serine/arginine)-rich proteins and hnRNPs (heterogenous nuclear ribonucleoproteins) which help to recruit the spliceosome to the splice sites.[13-15]

Previously, we employed a high resolution AS panel based on RT-PCR with fluorescent primers to investigate the impact of the hnRNP-like RNA-binding protein *At*GRP7 (*Arabidopsis thaliana* glycine-rich RNA-binding protein 7)[16,17] on a suite of known AS events in *Arabidopsis*. For 59 out of 288 analyzed AS events (21%) the ratio between the main splice isoforms significantly changed by more than 5% ($p < 0.05$) in plants constitutively overexpressing this RNA-binding protein (*At*GRP7-ox plants) compared with wild type plants.[18] Notably, we found ten AS events that showed a reciprocal change in the ratio of AS forms in plants with elevated levels of *At*GRP7 compared with plants with reduced levels of *At*GRP7. This suggested that *At*GRP7 directly regulates these AS events. Indeed, RNA immunoprecipitation experiments revealed that the majority of these transcripts were bound in vivo by *At*GRP7. In addition, mutation of a single conserved arginine that impairs the *At*GRP7 RNA-binding capability abrogates the effect of *At*GRP7 on AS of these events indicating

Table 1. Details of the AS events analyzed

primer pair	AGI	Gene description	AS event	5' primer	3' primer
12	At1g72320	APUM23 (ARABIDOPSIS PUMILIO 23); RNA binding	3′SS	CGT CAA CTG TGT TTT TGC ATC C	CATC AAA TCC ACG GTT ACC C
36	At4g12790	ATP-binding family protein	3′SS	GGT TTT GAG AGC AAA GAA AAA CG	GGT TAA CAA CAT GCA TTG TTC G
59	At5g66010	RNA-binding (RRM/RBD/RNP motifs) family protein	3′SS	GGC GGC AGG TCA TGT ACG G	GGG AAG ACC CCT GAG GCG AAC G
72	At2g04790	similar to unnamed protein product [Vitis vinifera]	5′SS	CCC TGA AAG CAT AGA AGC AGC	CCC ATG ACT TAT TAA ACT CC
75	At2g36000	mitochondrial transcription termination factor-related	5′SS	CTC GTT TAG TTT GGA GAA TC	CTT CAT CAG CAT TCA TTA C
87	At4g35450	AKR2 (ANKYRIN REPEAT-CONTAINING PROTEIN 2)	5′SS	CCA CCA CAA CAT TGT CTT TTC	CCA GCG TTA GGA ATA GAT CTC
118	At2g02960	zinc finger (C3HC4-type RING finger) family protein	3′SS	GGG GAG CTT TCA CCA ATT AG	GCC TTA TCA TTA ACC ACC GG
148	At1g76510	ARID/BRIGHT DNA-binding domain-containing protein	5′SS	CCG TTT CTC GCT TCT TTT TCT C	CCT CTA CAA CAC CTT TGG TAC C
179	At5g48150	PAT1 (PHYTOCHROME A SIGNAL TRANSDUCTION 1)	ES	CCT TGT CTC CGA CAA CTT TC	CCT AAG CTT CTC AAC AGA GTT AG
181	At5g05550	sequence-specific DNA binding transcription factors	ES	GGA GAA GCA GAG AAT GGA AG	GGA TCC TCC AAT TTC AAT GAG
187	At5g02470	DPA; transcription factor	5′SS	CAG TTT GTT TGT TTG TTT ATA G	CCA ATT TCA GAA TCA TCA TC
189	At5g43270	SPL2 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 2)	5′SS	CCT CTGGGATCCATAAGTTTTG	CCA TCA ATT TCC CAC TCC ATT G
227	At4g24740	AFC2 (ARABIDOPSIS FUS3-COMPLEMENTING GENE 1)	ES	CCT CAT ACT CAC ATG GAT CGT CGT CC	CCA TTT CCT TCC TCT CCC TAT CCC
243	At2g33830	dormancy/auxin associated family protein	3′SS	CCG GAC CTA AAC CGG AGC ATG GCC	CCG ATC CTG GCG TCG TCG GAG TTC C
261	At4g10100	CNX7/SIR5	5SS	CTC ATG TGT GTG GTA TTC ACC	CAG TGT TAG ATC AGG CAC ACC
268	At1g03457	RNA-binding (RRM/RBD/RNP motifs) family protein	3′SS	CCG TTG CAA GTT AAG TAT GC	CCC TCT TAG AAT CTG TAG ATC C
285	At3g19840	pre-mRNA-processing protein 40C	5′SS	CCA TAT TCT GGT TCT CAT CC	CCA GGC ATC TAA CCG ATT TCC
288	At3g12570	FYD	3′SS	CCA TGT GTT GTA CTA GTG CC	CCA TGG ATA GCA GTG TTG AC
295	At2g02390	ATGSTZ1 (GLUTATHIONE S-TRANSFERASE 18)	3′SS	GGC TTG ATT ATG AGT ATA TAC CAG	GGT AAC AAA GGT GGC TCA GGG
309	At5g65060	MAF3 (MADS AFFECTING FLOWERING 3)	3′SS	CAA GGA GTT ACT AGA AAT AGT CC	CCC GTG ACA TTC CTC TGT CAC C
310	At5g65070	MAF4 (MADS AFFECTING FLOWERING 4)	5′SS	CCG TCG CTC TTA TCA TCA TCT C	CCG GTG ACA TTG CTC TTG CAT CC
314	At2g43410	FPA	IR	GGG CTG GCT CTT ACG ATA ACA G	GAT GGC CTC CTC CAG TTT GG
322	At2g33480	ANAC041 (Arabidopsis NAC domain containing protein 41)	5′SS	CCG ATG TTT GTA AAT CCG ATC C	CTG TCT CTT TCT CAT TCT CC
324	At5g43270	SPL2 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 2)	5′SS	GGG ATC CAT AAG TTT TGA G	GCT TGA AGA ATA CAG AGA GG
327	At5g59950	RNA and export factor-binding protein, putative	IR	CTG CTC CAT ACC AAT CAG CC	CCA CTT CTA TCA AAA TGA AC
343	At3g29160	AKIN11 (ARABIDOPSIS SNF1 KINASE HOMOLOG 11)	5′SS	CCT GAC TCA GCT CTG CGT CAC C	CCC AAT TCC AAG AGT TTT ACC
378	At3g62190	DNAJ heat shock N-terminal domain-containing protein	3′SS	CCT GAT GAT CAG AAG CTT GTT GCC	CCG AGG AAC CCC AGT CTT GAC
380	At5g08185	npcRNA 78; MIR162a	ES	GTC CAT TTG GTT TCA TAA GG	GCT TTC CCA GAA AAG TAA TCG G

The number of the primer pair,[6,20] *Arabidopsis* gene identifier, gene description, AS event (3′SS, alternative 3' splice site; 5′SS, alternative 5' splice site; ES, exon skipping; IR, intron retention) and sequences of the primers are indicated.

Table 2. Transcripts with changes in AS patterns upon moderate temperature shifts

A		Changes upon transfer of Col plants from 20°C to 16°C					Total transcript/ RPL12C Levels 16°C	Total transcript/ RPL12C Levels 20°C	Fold change of total transcript upon transfer to 16°C	
			Product sizes [bp]	Small product [%] at 16 °C	Small product [%] at 20 °C	P-value 20-16				P-value
Primer pair	AGI	Description								
12	At1g72320	APUM23	141/150/215	26	18	0.00175	0.0445	0.0608	0.73	0.40274
72	At2g04790	unknown protein	167/190	85	77	0.03573	0.5868	0.7230	0.81	0.36801
75	At2g36000	mitochondrial transcription termination factor-related	150/254	28	33	0.00014	0.1326	0.3745	0.35	0.16424
118	At2g02960	zinc finger (C3HC4-type RING finger) family protein	197/203/222	58	65	0.00737	0.0351	0.0424	0.83	0.39887
148	At1g76510	ARID/BRIGHT DNA-binding domain-containing protein	189/212	44	32	0.01842	0.0754	0.1275	0.59	0.00808
189	At5g43270	SPL2 SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 2	160/244	43	64	0.04011	0.0201	0.0271	0.74	0.83124
243	At2g33830	dormancy/auxin associated family protein	140/146	8	13	0.00902	1.8394	2.8719	0.64	0.07320
285	At3g19840	pre-mRNA-processing protein 40C	171/207	46	64	0.01337	0.0042	0.0070	0.60	0.47984
288	At3g12570	FYD	159/188	44	39	0.05332	0.0240	0.0428	0.56	0.02980
314	At2g43410	FPA	409/538	20	24	0.00774	0.0580	0.0851	0.68	0.08719
322	At2g33480	ANAC041 Arabidopsis NAC domain containing protein 41	321/399	10	15	0.00555	0.0579	0.0733	0.79	0.49148

Changes in the ratio of AS isoforms with $p < 0.05$ and changes > 4 % are considered significant. In case more than two splice isoforms are generated the ones considered are underlined. The total transcript level of all alternative splice forms is expressed relative to the RPL12c transcript level. The fold-change between the levels observed at the different temperatures and the corresponding p-values are indicated.

that the effect of *At*GRP7 on AS relies on RNA binding.[18,19] Comparison with the analysis of plants with altered levels of SR proteins uncovered several events that are controlled by both, *At*GRP7 and SR proteins.[20] Recently, the hnRNP-like polypyrimidine tract binding proteins (PTBs) also have been shown to globally impact AS in *Arabidopsis*. Around 450 AS events react to changes in PTB1 and PTB2 levels.[21] It is not yet known how many of the transcripts are directly bound by the PTBs.

In the course of monitoring AS in two independent transgenic *At*GRP7-ox lines generated in two different ecotypes, we also compared a suite of AS events between the Col and the C24 ecotypes themselves. We found that around 30% of the AS events changed significantly (> 5%, $p < 0.05$) between the ecotypes. We did not find single nucleotide polymorphisms in the splice sites per se but in intronic or exonic sequences near the splice sites that may cause ecotype-specific usage of the AS sites in either ecotype.[18]

Ratios of AS Isoforms are Altered in Response to Small Temperature Changes

Several studies have shown that exposure to temperature stresses influences AS patterns in plants.[1,11,22-24] However, the impact of small changes in ambient temperature has not been addressed. To investigate how a temperature step as small as 4°C affects AS, *Arabidopsis* plants were grown at 20°C and subsequently transferred to either 16°C or 24°C for one day in three biological replicates. The corresponding cDNAs were analyzed on the RT-PCR based AS panel. Of the 59 AS events previously investigated in detail[18] we selected 28 AS events in transcripts encoding RNA processing factors, transcription factors

Table 2. Transcripts with changes in AS patterns upon moderate temperature shifts (continued)

343	At3g29160	AKIN11 ARABIDOPSIS SNF1 KINASE HOMOLOG 11	159/307	45	34	0.00804	0.1125	0.1437	0.78	0.24488
B		**Changes upon transfer of Col plants from 20°C to 24°C**					**Total transcript/ RPL12C levels 20°C**	**Total transcript/ RPL12C levels 24°C**	**Fold change of total transcript upon transfer to 24°C**	
			Product sizes [bp]	**Small product [%] at 20 °C**	**Small product [%] at 24 °C**	**P-value 20-24**				**P-value**
Primer pair	**AGI**	**Description**								
12	At1g72320	APUM23	141/150/215	18	14	0.04286	0.0608	0.0406	0.67	0.27551
36	At4g12790	ATP-binding family protein	212/338	41	46	0.03911	0.2433	0.2031	0.83	0.18972
59	At5g66010	RNA-binding (RRM/ RBD/RNP motifs) family protein	105 /182	30	34	0.05018	0.0649	0.0719	1.11	0.62294
72	At2g04790	unknown protein	167/190	77	84	0.03198	0.7230	1.1533	1.60	0.00739
75	At2g36000	mitochondrial transcription termination factor-related	150/254	33	45	0.00003	0.3745	0.3893	1.04	0.89047
118	At2g02960	zinc finger (C3HC4-type RING finger) family protein	197/203/222	65	71	0.04727	0.0424	0.0419	0.99	0.95003

Changes in the ratio of AS isoforms with $p < 0.05$ and changes > 4 % are considered significant. In case more than two splice isoforms are generated the ones considered are underlined. The total transcript level of all alternative splice forms is expressed relative to the RPL12c transcript level. The fold-change between the levels observed at the different temperatures and the corresponding p-values are indicated.

and other predicted regulatory proteins[20] (**Table 1**).

For 12 of 28 investigated AS events (43%), the ratio of the two splice isoforms changed more than 4% ($p < 0.05$) in wild type plants transferred to 16°C relative to control plants kept at 20°C (**Table 2A**, **Fig. 1**). For 6 of 28 investigated AS events (21%) the ratio showed a significant change in wild type plants transferred to 24°C relative to control plants kept at 20°C (**Table 2B**, **Fig. 1**). Four transcripts showed AS changes upon transfer from 20°C to both 16°C and 24°C (**Table 2**, **Fig. 1**). For At1g72320 encoding APUM23,[25] a member of the PUMILIO family of RNA-binding proteins, alternative 3' splice sites at the 5' UTR intron lead to transcripts which either encode the authentic protein (according to gene models from TAIR) corresponding to a 215 nt PCR product or an N-terminally truncated protein corresponding to a 150 nt PCR product due to removal of part of exon2 including the authentic start codon (**Fig. 1A**). Upon increasing the temperature an increase in the relative proportion of the 215 nt form corresponding to the authentic protein and a concomitant decrease in the 150 nt form is observed. For At2g3600 encoding a mitochondrial transcription termination factor-related protein (mTERF) the use of the authentic 5' splice site of the intron in the 3' UTR leads to the mRNA (corresponding to a 254 nt PCR product) which gives rise to the authentic protein (according to gene models from TAIR) (**Fig. 1B**). The use of an alternative 5' splice site toward the end of the coding region gives an AS variant (corresponding to the 150 nt product) which would lead to a C-terminally truncated protein. With decreasing temperature there is a shift toward the form encoding the authentic protein (**Fig. 1B**). The primers for *FYD* (At3g12570) cover an alternative 3' splice site in intron 2 in the 5' UTR (**Fig. 1C**). Upon decreasing temperature from 20°C to 16°C, a reduction of the large variant retaining part of intron 2 is observed. This transcript isoform is stabilized in plants treated with cycloheximide, suggesting that it is an NMD substrate.[6] Finally, for *AKIN11* encoding a catalytic subunit of Snf1-related protein kinase, primers detect the use of an alternative 5' splice site in intron 1 in the 5' UTR. Upon decreasing temperature from 20°C to 16°C the proportion of the longer splice variant retaining part of the intron is reduced (**Fig. 1D**).

The observed changes in the ratios of AS isoforms probably are a consequence of a differential choice of AS sites in response to temperature. However, it is possible that such changes could occur indirectly as a consequence of altered steady-state abundance at the different temperatures potentially reflecting transcription or stability. Thus, the transcript levels were calculated

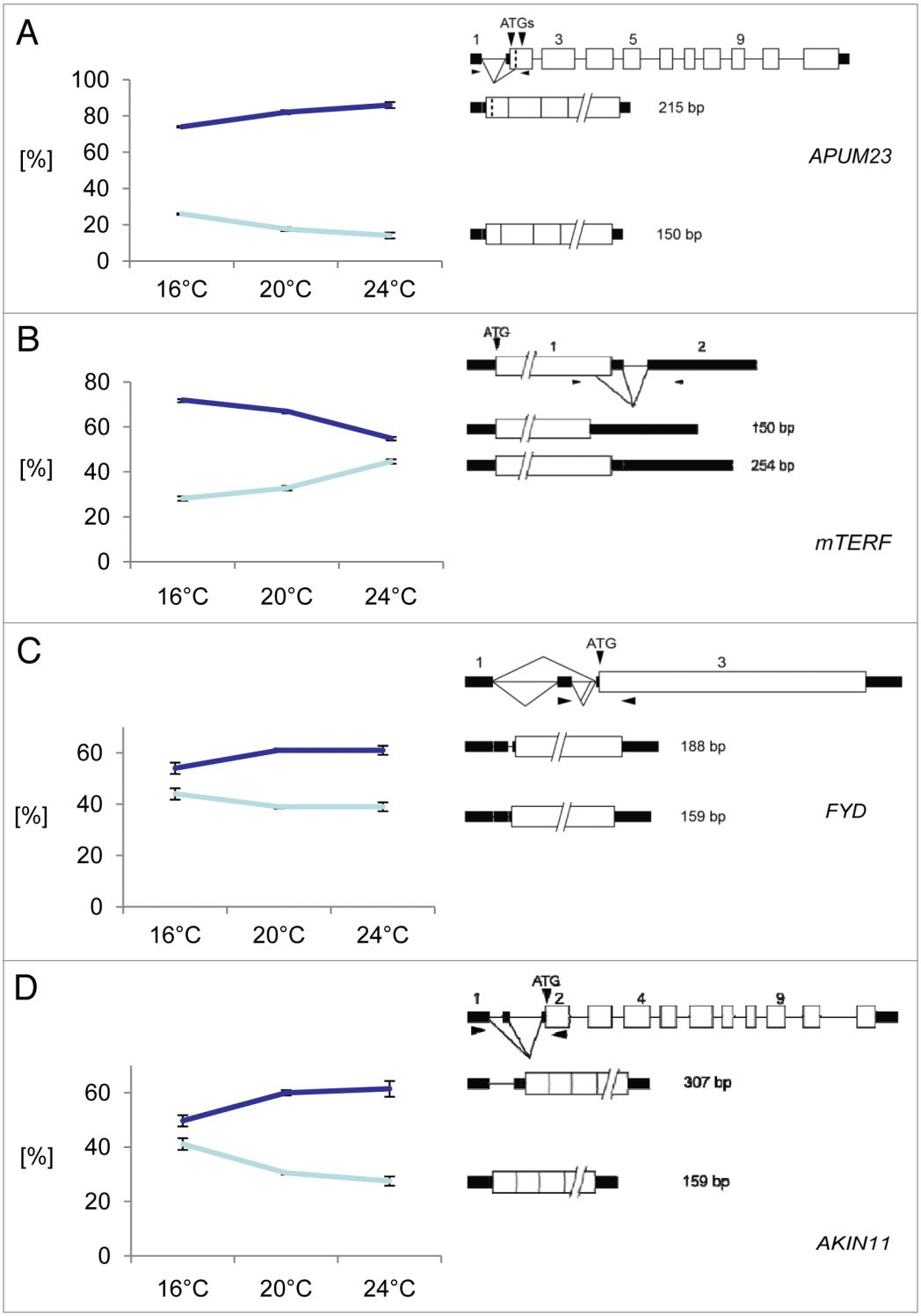

Figure 1. Temperature dependence of AS events that change upon increasing and decreasing ambient temperature. (**A**) APUM23; (**B**) mTERF; (**C**) FYD; (**D**) AKIN11. The gene structure, the structure of the transcript isoforms around the AS event and the ratios of the two splice isoforms at each temperature are shown. On the left side of each panel, the percentage of each splice form +/– s.d. based on three biological replicates is indicated for each temperature. On the right side of each panel, the gene and transcript structures and the AS events are shown schematically. Exons are indicated by open boxes; UTRs, black rectangles; introns, thin lines; splicing events, diagonal lines. The arrowheads denote the approximate position of primers and the sizes of the PCR products from each splice isoform are indicated.

relative to the reference transcript *RPL12c*. None of the affected transcripts showed a statistically significant difference in steady-state abundance of at least 2-fold when plants were transferred to 16°C or 24°C (**Table 2**).

In terms of stability of the alternatively spliced transcripts, some of the transcripts studied here are known to undergo NMD[6] but did not show consistent effects at different temperatures. For example, the transcript represented by the 215 bp product of *APUM23* whose level decreases at 16°C and increases at 24°C is an NMD substrate[6] (**Fig. 1A**); in contrast, the 190 bp product of At2g04790 (unknown protein), also an NMD substrate,[6] is decreased at both 16°C and 24°C (**Table 2**). Similarly, the level of the 159 bp product of *AKIN11*, another NMD substrate,[6] increased at 16°C while other NMD transcripts (e.g., *APUM23*, 259 bp; and At2g04790, 190 bp) decreased at 16°C (**Table 2**, **Fig. 1A and D**). In addition, changes in AS seen in a previous study when plants are transferred to 4°C were not due to effects on NMD.[23] Thus, the observed changes are most likely to reflect alternative splicing.

Overall, our data show for the first time that changes in ambient temperature as small as 4°C can affect many AS events. Previously, larger temperature steps were shown to affect AS. For example, the transcripts encoding core components of the *Arabidopsis* circadian clock, CIRCADIAN ASSOCIATED 1 and LATE ELONGATED HYPOCOTYL undergo AS upon exposure to 4°C.[22-24] Because these AS events can lead to changes in transcript and protein levels they have been implicated in adjusting clock function to reduced ambient temperatures.[23,24]

Regulation of AS is determined by the relative abundance and activity of different splicing factors. This was illustrated in *Arabidopsis* by reciprocal changes in AS in plants with increased expression (overexpression lines) or no expression (mutant) of *At*GRP7[18] and PTB proteins.[21] Here, four genes show reciprocal changes in AS in response to small increases and decreases in temperature suggesting temperature-dependent regulation. This most likely reflects altered composition or activity of splicing factors[2] and it is notable that, for example, SR and hnRNP proteins themselves undergo AS in plants exposed to low or high temperatures, suggesting a global impact on downstream transcripts during temperature stress.[1,14,25,26] This is further illustrated in a study on temperature-dependent flower induction, where an enrichment of RNA-processing related gene products was found upon transfer of *Arabidopsis* plants from 16°C to 25°C,[27,28] and recently heat stress-induced AS was found to impact processing of a miRNA precursor located in the intron.[29] Changes in the use of splice sites could also involve temperature dependent secondary structure changes in the introns and adjacent exons. Techniques to assess the regulatory impact of RNA secondary structure at a global scale have recently been applied to *Arabidopsis*.[30]

Conclusion and Perspective

We have shown here that changes in ambient temperature as small as 4°C can have a significant effect on AS events. Thus, plants may acclimate to changing environmental temperatures through adjusting the transcriptome by changes in AS patterns. This can involve altering levels of functional mRNAs able to encode protein by modulating the amount of AS giving rise to non-functional mRNAs, many of which are subject to NMD. Changes in AS can impact the proteome directly through translating AS isoforms into proteins with different domain structures that potentially fulfill temperature-specific functions. Finally, AS can have indirect consequences via interaction with the miRNA regulatory network, e.g., by temperature-conditional interference with processing of intronic miRNA precursors or altered miRNA target site availability.[4,29] Overall, it will be important in future to identify all of the splicing factors involved in AS, what regulates their expression and activity and how they interact to determine the dynamic transcriptome and ultimately phenotype of cells and organisms.[31]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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