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Impact of low temperature on splicing of atypical group II introns in wheat mitochondria

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ABSTRACT

To investigate the impact of cold on group II intron splicing, we compared the physical forms of excised mitochondrial introns from wheat embryos germinated at room temperature and 4 °C. For introns which deviate from the conventional branchpoint structure, we observed predominantly heterogeneous circularized introns in the cold rather than linear polyadenylated forms arising from a hydrolytic pathway as seen at room temperature. In addition, intron-containing precursors are elevated relative to mature mRNAs upon cold treatment. Our findings indicate that low temperature growth not only reduces splicing efficiency, but also shifts the splicing biochemistry of atypical group II introns to novel, yet productive, pathways.

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1. Introduction

Group II introns belong to a family of retrotransposable elements that are defined by a distinctive and highly conserved secondary structure comprised of six helical domains (dI–dVI) that radiate from a central core (Bonen and Vogel, 2001; Lambowitz and Zimmerly, 2011). Splicing of classical group II intron ribozymes is carried out by two consecutive transesterification reactions that culminate in ligated exons and a lariat-shaped excised intron. The initiating nucleophile is the 2′-OH of a bulged adenosine located 7–8 nts upstream of the 3′ end of the intron within dVI. A second pathway which involves first-step hydrolysis and results in a linear excised intron has been observed *in vitro* for bacterial group IIC (Toor et al., 2006) and mitochondrial group IIB introns (Li et al., 2011a), as well as several instances *in vivo* in plant chloroplasts (Bonen and Vogel, 2001) and mitochondria (Li-Pook-Than and Bonen, 2006). The linear introns in organelles were also seen to possess short adenosine tails, a hallmark of bacterial-type tagging of linear molecules for RNA degradation (Lange et al., 2009). In addition to lariat and linear structures, full-length circular intron molecules have also been observed *in vivo* (Li-Pook-Than and Bonen, 2006; Molina-Sanchez et al., 2006, 2011) and *in vitro* (Murray et al., 2001).

In flowering plants, certain mitochondrial group II introns lack characteristic structural features; for example, a number of them exhibit weak domain V/VI helices (Bonen, 2008). In some cases, C-to-U type RNA editing can restore proper folding by converting A–C mispairs to canonical A–U pairs, and several such sites have been demonstrated through mutational analysis to be crucial for splicing (Castandet et al., 2010; Farré et al., 2012). This reinforces the notion that RNA editing is not only important for generating the correct coding sequences (Maier et al., 1996) but also for appropriate precursor RNA structure. It should be noted, however, that not all A–C mispairs in core helical regions of introns undergo editing and there are numerous mispairs (such as pyrimidine–pyrimidine) that are not candidates for correction (Carrillo and Bonen, 1997). In an earlier study, we demonstrated that several introns with non-conventional dV/VI structures exhibited unusual heterogeneous circularized forms of excised introns in wheat mitochondria pointing to the use of novel biochemical pathways in splicing (Li-Pook-Than and Bonen, 2006). Notably, although plant mitochondrial introns belong to the group II ribozyme family, none have been observed to self-splice *in vitro*. Their excision is dependent on nucleus-encoded machinery, whose complexity is now beginning to be elucidated (*cf.* de Longevialle et al., 2010 and references therein; Francs-Small et al., 2012; Keren et al., 2012; Koprivova et al., 2010; Köhler et al., 2010; Kühn et al., 2011; Liu et al., 2010; Zmudjak et al., 2013).

Relatively little is known about the response of group II intron splicing to environmental stresses such as cold temperature. Because these introns, which typically are about 1–2 kb in length, have very intricate folding and must undergo precise conformational shifts during splicing, this might be compromised in the cold. If misfolding occurs or regions are trapped in non-competent structures, this might well compound other limitations expected in the cold, like reduced enzymatic activity and import of the nuclear-encoded splicing machinery. In keeping

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with this view, analysis of *cox2* intron splicing in wheat and rice seedlings which had been grown in the cold, revealed elevated levels of intron-containing precursor RNAs and reduced editing at certain exon sites (Kurihara-Yonemoto and Handa, 2001; Kurihara-Yonemoto and Kubo, 2010).

In the present study, we have examined the status of mitochondrial splicing in cold-treated germinating wheat embryos using RT-PCR and circularized RT-PCR methods to assess the physical forms of excised introns. We have focused in particular on several cis-splicing group II introns that were known to have aberrant domain dVI structures and/or to exhibit non-lariat splicing at room temperature, namely *nad1* intron 2, *nad2* intron 1 and the *cox2* intron (Li-Pook-Than and Bonen, 2006). These introns showed a shift in splicing biochemistry that resulted in novel heterogeneous excised intron forms in the cold compared to linear forms at room temperature. In contrast, introns possessing a classical bulged adenosine in dVI, such as *nad2* intron 4, continued to be excised as lariats in the cold.

2. Materials and methods

2.1. RT-PCR and CR-RT-PCR analysis of the physical form of excised introns

Mitochondrial RNA was isolated from wheat embryos (*Triticum aestivum* cv. FT Wonder, a cold-hardy cultivar) that had been germinated in the dark either at room temperature for 36 h or at room temperature for 18 h followed by 72 h at 4 °C. Prior to imbibition with sterile distilled water, seeds were surface-sterilized with 1% sodium hypochlorite and the bulk of the endosperm tissue was removed by dissection. Mitochondrial RNA isolation was performed as previously described (Li-Pook-Than et al., 2004).

To identify and characterize *in vivo* lariat/circularized intron molecules, RT-PCR was used to amplify excised intron junctions. More specifically, for cDNA synthesis, RNA was incubated with Superscript II reverse transcriptase (Invitrogen) at 42 °C for 2 h, and this was followed by PCR amplification, cloning and sequencing. Circularized (CR)-RT-PCR experiments were performed in parallel to survey the presence of any *in vivo* linear intron molecules. For this step, wheat mitochondrial RNA (~5 μg) was incubated with 0.6 U/μL RNA ligase (New England Biolabs), 20–40 U/μL RNasin (Promega), 50 μg/mL acetylated BSA (Promega) and 0.1 vol 10× RNA ligase buffer (New England Biolabs) for 30 min at 37 °C followed by phenol extraction and overnight ethanol precipitation. Oligomers used in the (CR)RT-PCR analysis of excised introns are shown in Table 1. The introns selected for this study were *cox2* intron, *nad2* intron 1, *nad2* intron 4 and *nad1* intron 2, which are designated as *cox2*i373, *nad2*i156, *nad2*i1282 and *nad1*i477, respectively, in the nomenclature of Dombrovska and Qiu (2004). It should be noted that oligomers used for the analysis of the *cox2* intron and *nad1* intron 2 differ from those in our earlier room temperature studies (Li-Pook-Than and Bonen, 2006). For all the RNA analyses conducted in this study, multiple independent mitochondrial RNA preparations were used.

RT-PCR and CR-RT-PCR products with the expected size of excised introns were gel-purified using UltraClean 15 (MoBio Laboratories Inc.) and after corroboration by nested PCR, they were cloned into pGemT-Easy (Promega) plasmid vectors for sequencing. Sequencing was performed by StemCore Laboratories at the Ottawa Health Research Institute (OHRI). RT-PCR products that were longer than what would correspond to full-length introns were set aside since they would not lead to functional mRNAs. The only other clones that were excluded from further analysis were three *nad2* intron 1 clones which were approximately the correct size but contained flanking upstream and downstream exon sequences. Direct sequencing was used to assess the degree of heterogeneity at exon/exon junctions of RT-PCR products derived from spliced RNAs and precursors, and the oligomers used in this analysis are shown in Table 1.

Table 1
Oligomers for (CR)RT-PCR and northern hybridization analysis. Reverse oligomers (rev) used as primers in cDNA synthesis and/or as probes in northern analysis are designated by (c) and (n), respectively. Forward primers (for) paired with cDNA oligomers in (CR) RT-PCR are designated by (p). Oligomers were designed on the wheat mitochondrial genome (AP008982).

	5′–3′ sequence	Position
nad1 intron 2 (*nad1i477*)		
	TTGTCAGAGTGGATTCGGAC (rev, c)	5′ end of intron
	CAGCTTACTCACCCTACTCC (for, p)	3′ end of intron
	CTCAAAATGAGCCTTGCGAC (rev, n)	5′ end of intron
nad2 intron 1 (*nad2i156*)		
	ATGCACAGGTACCTACGTAG (rev, c)	5′ end of intron
	AGTTATCACGGACGAGCCAC (for, p)	3′ end of intron
	CGCACATTCATAATAGCGTT (rev, n)	5′ end of intron
nad2 intron 4 (*nad2i1282*)		
	CGTGTAGTGATTGTGGACTC (rev, c)	5′ end of intron
	GGGATGGATAAAGTGGGCAA (for, p)	3′ end of intron
	GGCTGTATCACATCGAGATG (rev, n)	5′ end of intron
cox2 intron (*cox2i373*)		
	CTGTGGTGCCGATAGATTCA (rev, c,n)	5′ end of intron
	ATAAGAGTAGGCGTGGAGAG (for, p)	3′ end of intron
nad1b/c exon junction		
	ATATTCTACATTATAGCCTG (rev, c, n)	5′ end of exon 3
	ATGGTCCCTTATGAAGTCTC (for, p)	Mid-region of exon 2
	TGGGTATGATATTCTCGTGG (for, p)	3′ end of *nad1* intron 1b
nad2a/b and nad2b/c exon junctions		
	AATTGATCGAAGTGGGTAGC (rev, c)	5′ end of exon 3
	CCTTACGAGGTAGTGATGAG (rev, c)	5′ end of intron 3
	GCTCATTCATGGAGTTGTAT (for, p)	Mid-region of exon 1
	CTTCCGTGGAAAATTCAGAC (rev, n)	3′ end of exon 2
cox2a/b exon junction		
	AAGTCACTGCTTCTACGACG (rev, c, n)	3′ end of exon 2
	CAACACCTATGATGCAAGG (for, p)	5′ end of exon 1

2.2. Northern analysis

Wheat mitochondrial RNA which had been isolated from dissected seeds germinated in the cold or at room temperature was electrophoresed on 1.2% agarose/formaldehyde gels using standard protocols and transferred to Hybond-N nylon membranes (Amersham). Sequences of the 20′mer probes were: 5′ ATATTCTACATTATAGCCTG 3′ (*nad1* exon 2); 5′ CTCAAAATGAGCCTTGCGAC 3′ (*nad1* intron 2); 5′ CTTCCGTGAAAATTCAGAC 3′ (*nad2* exon 2); 5′ CGCACATTCATAATA GCGTT 3′ (*nad2* intron 1); 5′ AAGTCACTGCTTCTACGACG 3′ (*cox2* exon 2) and 5′ CTGTGGTGCCGATAGATTCA 3′ (*cox2* intron). Oligomers were ^{32}P-end-labelled using T4 polynucleotide kinase (Invitrogen) and after overnight hybridization followed by washes, autoradiography was carried out by phosphoimaging using a Molecular Imager — FX (Bio-Rad).

3. Results

3.1. Absence of conventional domain VI features in certain mitochondrial introns in seed plants

For this study, we selected several cis-splicing wheat mitochondrial introns which exhibit atypical features within domain VI (Fig. 1). Two of them (*nad2* intron 1 and *nad1* intron 2) lack the bulged adenosine which in conventional group II introns acts as the initiating nucleophile in the transesterification reactions that result in the intron being excised as a lariat. In both these introns, the dVI helices are very short, and the latter exhibits a tight helical structure whereas the former has fewer Watson–Crick base-pairs. In addition, *nad2* intron 1 varies in dVI sequence among flowering plants (Fig. 1, shaded nts in tobacco). The *cox2* intron has a bulged adenosine at the appropriate location, but the distal dVI helix/loop is short and weakly-structured, and our earlier studies had shown that this intron does not follow a lariat-generating splicing pathway under room temperature conditions (Li-Pook-Than and Bonen, 2006). It should be noted that in certain plants, such as *Arabidopsis*, there is a single intron in the *cox2* gene (*cox2*i691)

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