Impact of Low Temperature Growth on RNA Splicing of Aberrant Mitochondrial Group II Introns in Wheat Embryos

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Abstract

A subset of mitochondrial group II introns of flowering plants has, over evolutionary time, lost characteristic features and employs unconventional splicing pathways. Given the potential impact of cold treatment on RNA folding, as well as on enzymatic activity and import of nuclear-encoded splicing machinery, I have examined the physical excised forms of aberrant introns from wheat embryos subjected to 4^{o}C. My findings suggest a shift in biochemistry with cold treatment to novel splicing pathways that generate heterogeneous *in vivo* circularized forms for *nad1* intron 2, *nad2* intron 1 and the *cox2* intron, in contrast to predominantly linear excised intron forms at room temperature. Interestingly, the highly degenerate *nad1* intron 1, which due to DNA rearrangement has been broken into two halves that interact for splicing in *trans*, is excised exclusively by first-step hydrolysis at room temperature and under cold treatment. In this case, splicing culminates in two distinct linear half introns that appears correlated with an unusual 5' terminal insert. This represents the first *in vivo* demonstration of hydrolytic *trans*-splicing. Based on northern analysis, cold treatment was further associated with reduced splicing efficiency for all introns surveyed. Moreover, study of precursor transcripts of the *nad1a*-intron 1a locus suggests the efficiency of end-maturation, including processing of the cotranscribed tRNA-Pro gene, is also reduced in the cold. My findings demonstrate a temperature-sensitivity of transcript maturation, particularly for RNA splicing, providing new insight into the impact of cold growth conditions on plant mitochondrial gene expression.

Résumé

Au fil du temps évolutif, un sous-ensemble d'introns mitochondriaux de type II chez les plantes à fleurs on perdu des caractéristiques typiques de l'épissage et emploient des voies d'épissage non conventionnelles. Compte tenu de l'impact potentiel du traitement à basse température sur le repliement de l'ARN, ainsi que sur l'activité enzymatique et l'importation de la machinerie d'épissage codée par le noyau, j'ai examiné les formes physiques excisées d'introns aberrants à partir d'embryons de blé soumis à une température de 4^{o}C. Mes résultats suggèrent un changement biochimique produit par la basse température et qui mène à de nouvelles voies d'épissage génèrant des formes hétérogènes circulaire *in vivo* pour l'intron 2 de *nad1*, l'intron 1 de *nad2* et l'intron de *cox2*, contrairement aux formes lineares d'introns excisés à température ambiante. L'intron 1 dégénéré de *nad1*, qui, grâce à un réarrangement de l'ADN a été divisé en deux moitiés qui interagissent pour un épissage en *trans*, est excisé exclusivement par hydrolyse lors de la première étape à température ambiante et à basse température. Dans ce cas, l'épissage culmine en deux introns demi-linéaires distincts qui corrèlent avec un insert terminal inhabituel en 5'. Il s'agit de la première démonstration *in vivo* d'un *trans*-épissage par hydrolyse. Basé sur l'analyse northern, un traitement à basse température a été associé à une baisse d'efficacité de l'épissage de chaque intron testé. De plus, l'analyse des transcrits précurseurs de l'intron 1a du locus *nad1a* suggère que l'efficacité de la maturation terminale, y compris le traitement du gène co-transcrit ARNt-Pro, sont réduits à basse température. Mes résultats démontrent une sensibilité de la maturation des transcripts à la température, en particulier pour l'épissage de l'ARN, ce qui offre un nouvel aperçu de l'impact des conditions de stress abiotique sur l'expression des gènes mitochondriaux de la plante.

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List of abbreviations

snRNA: small nuclear RNA
tRNA: transfer RNA
rDNA ribosomal DNA
ATP: adenosine triphosphate
dI-dVI: group II introns domains I-VI
EBS: exon binding site
IBS: intron binding site
nt: nucleotide(s)
Mg: magnesium
ORF: open reading frame
IEP: intron encoded protein
RT: reverse transcriptase
OH: hydroxyl
cDNA: complementary DNA
NADH: nicotinamide adenine dinucleotide (reduced form)
UTR: untranslated region
CRM: chloroplast RNA splicing and ribosome maturation
PORR: plant organelle RNA recognition
PPR: pentatricopeptide repeat
TIM: translocase of the inner membrane
TOM: translocase of the outer membrane
ROS: reactive oxygen species
DIUF: deionized ultrafiltered
NaCl: sodium chloride
EtOH: ethanol
kb: kilobases
mya: million years ago
RT-PCR: reverse transcriptase polymerase chain reaction
CR-RT-PCR: circularized reverse transcriptase polymerase chain reaction
M-MLV: Moloney murine leukemia virus
SSII: superscript II
NCBI: National Center for Biotechnology Information
nad: NADH dehydrogenase subunit genes
atp: adenosine triphosphate synthase subunit genes
cox: cytochrome bc1 oxidoreductase subunit genes
rps: protein of the small ribosomal subunit
rpl: protein of the large ribosomal subunit

Chapter 1: Literature Review

1.1 *Intron classes*

RNA splicing refers to the post-transcriptional event in which introns, non-coding segments imbedded within an RNA transcript, are removed. All introns presently characterized in the tree of life can be placed into one of four major classes: spliceosomal, tRNA/archaeal, group I and group II (Haugen *et al.,* 2005). Spliceosomal introns are widely distributed throughout the nuclear genomes of eukaryotes and their excision is mediated by the spliceosome, a massive ribonucleoprotein complex comprised of 5 small nuclear RNAs (snRNAs) and nearly 300 proteins (Rogozin *et al*., 2012). Meanwhile, splicing of tRNA and archaebacterial introns is carried out by a unique "cut-rejoin" mechanism involving ATP and endonucleases (Lykke-Anderson *et al*., 1997). Group I introns are frequently found in fungal mitochondrial genomes, nuclear rDNA genes in protists and certain plant organellar genomes (Haugen *et al*., 2005). They are comprised of 9 domains (P1-P9) organized as three helical stacks and are capable of self-splicing *in vitro*. Group II introns belong to a family of retro-transposable elements capable of autocatalytic splicing that have been found in bacteria as well as in organellar genomes of plants, fungi and protists (reviewed in Lambowitz and Zimmerly, 2011). Similarities in structure and splicing between group II introns and nuclear spliceosomal introns have led to the belief that there is an evolutionary relationship between them, with the former representing the ancestral state (Rogozin *et al*., 2012). The presence of group II introns in a diverse variety of eubacteria as well as in mitochondria and chloroplasts of eukaryotes compared to the infrequency of group II introns in archaebacteria suggests that they originated in eubacteria and, following endosymbiosis, were passed on to eukaryotes. These mobile genetic elements are thought to have then invaded the nucleus and proliferated

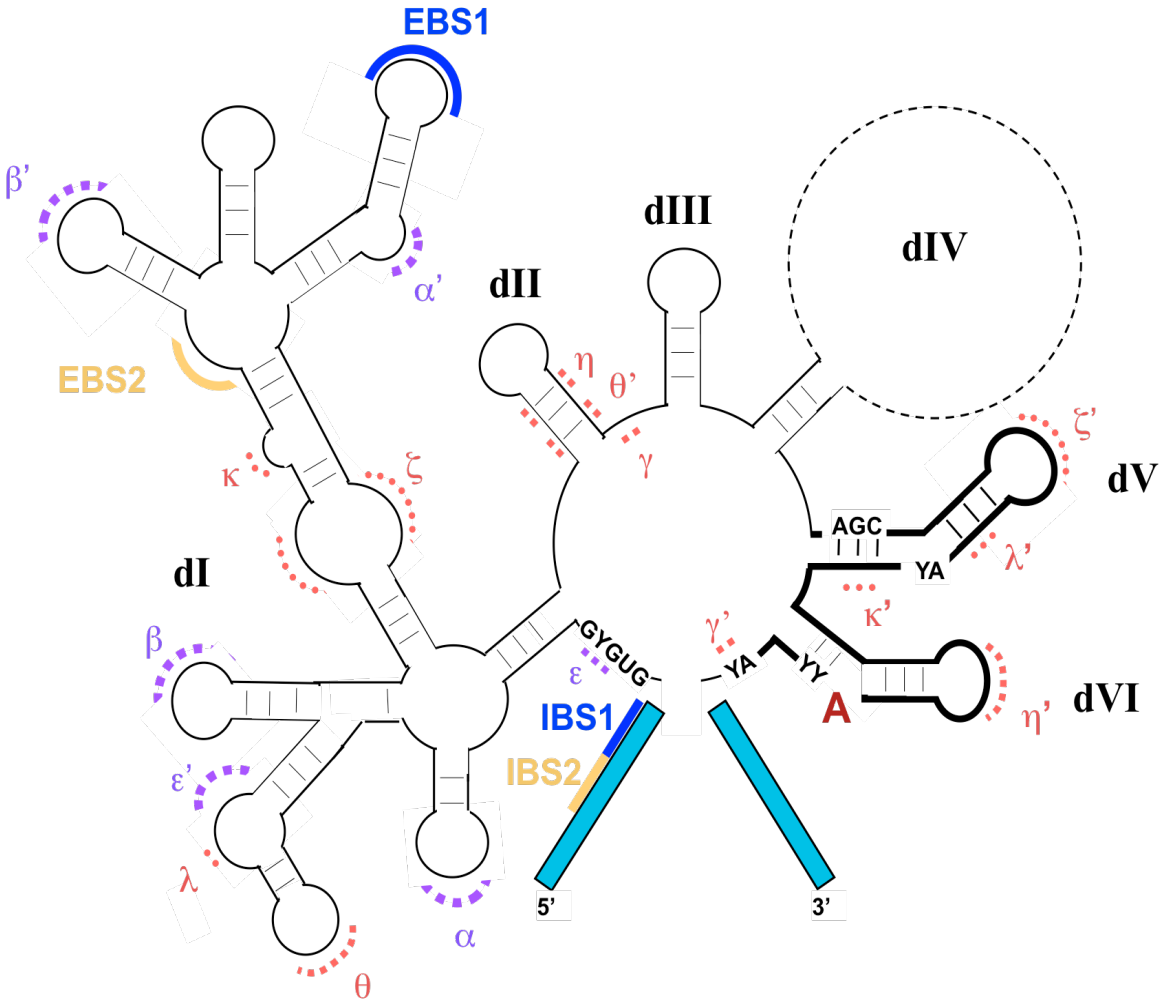
throughout the host genome where they eventually degenerated and fragmented, the segments (snRNAs) now functioning in *trans* to generate the spliceosome. The introduction of group II introns in the host nucleus has been proposed as a driving force for the formation of the nuclear membrane to prevent translation of unspliced RNAs (Koonin, 2006).

1.2 *Group II introns*

Group II introns are characterized by a highly conserved secondary structure comprised of six helical domains (dI-dVI) that branch out from a central "hub" (Fig 1.1) (Michel and Ferat, 1995; Bonen and Vogel, 2001). Essential to intron excision is the formation of the "active site" that is facilitated by multiple long-range Watson-Crick interactions that bring together core domains to initiate splicing. Key elements in the formation of the active site are exon-binding sites (EBSs) found in dI that associate with complementary sequences in the upstream exon referred to as intron-binding sites (IBSs). Base-pairing between these respective sites, together with multiple intra-domain interactions of dI, establishes the scaffold structure on which the other 5 domains then fold to generate the complex tertiary architecture that has been well characterized by RNA crystallography (Chan *et al*., 2012). This elaborate folding of dI also serves to position the conserved 5' terminal sequence GUGYG at the active site to form a "sharp kink" that makes the 5' exon-intron junction accessible for first-step catalysis (Marcia and Pyle, 2012). At the catalytic core of the active site is the highly conserved 34 nt dV comprised of 9+5 or 8+6 base pairs and a distal 5'- GNRA-3' tetraloop (Michel and Ferat, 1995). DV also contains two divalent metal ion-binding sites, the invariant AGC triad and AY bulge, that specifically bind Mg^{++} ions to initiate folding of the domain into the active site (Lambowitz and Zimmerly, 2011). Additional Mg^{++} ion binding sites are present in dVI that catalyze positioning of the

Figure 1.1: Schematic of the conventional RNA secondary structure of a group II intron.

Group II introns are characterized by six helical domains (dI-dVI) that radiate from a central hub. Elaborate tertiary folding for splicing is mediated by multiple long-range interactions (greek letters) as well as exon-binding sites (EBS1 and EBS2) within dI that basepair with complementary intron-binding sites (IBS1 and IBS2) at the 3' end of the upstream exon. Positions of classically invariant sequences are shown (Y = pyrimidine) along with the conventional bulged adenosine nucleophile in dVI. Figure adapted from Michel and Ferat (1995) and Bonen and Vogel (2001).


EBS1
β'
α'
dII
dIII
dIV
EBS2
η
θ'
γ
ζ'
dV
κ
ζ
AGC
YA
λ'
dI
κ'
β
GYGUG
γ'
YA
YY
ε
IBS1
A
η'
dVI
IBS2
ε'
λ
5'
3'
α
θ

branchpoint nucleotide residue, typically a bulged adenosine, located 7-8 nt upstream of the 3' intron-exon junction into the active site where it acts as the attacking nucleophile that initiates conventional lariat splicing (see subsection 1.2.2) (Hertwick and Mueller, 2001). DII and dIII do not contribute to the active site but are important structurally and have secondary roles in splicing, with dII participating in the conformational change required between splicing steps and dIII acting as a "catalytic effector" that increases the efficiency of splicing kinetics (Fedorova *et al*., 2003).

In classic self-splicing group II introns, dIV contains an open reading frame (ORF) within its loop region that encodes an intron-encoded protein (IEP) that is crucial for intron mobility (Lambowitz and Zimmerly, 2004). IEPs, also known as maturases, typically have four conserved domains: 1) the reverse-transcriptase (RT) domain comprised of seven subdomains (RT1-7); 2) the X or "maturase" domain that contains an RNA-binding domain shown to be important for splicing (Mohr *et al*., 1993); 3) the D (DNA-binding) domain that associates with target DNA for intron mobility; and, 4) the En (DNA endonuclease) domain which functions in DNA cleavage during intron mobility to generate the 3'OH of the target DNA that is used as a primer in the subsequent reverse transcription of the inserted intron RNA (Matsuura *et al*., 2001). In flowering plants, only a single maturase-encoding gene is present in each organellar genome (de Longevialle *et al*., 2010). In mitochondria, *matR* is found in dIV of *nad1* intron 4 and in chloroplasts, *matK* is found in dIV of the *trnK* intron.

1.2.1 *Group II intron subgroups*

Based on the base-pairing interactions used in recognition of flanking exons to generate the active site, group II introns can be further categorized into group IIA and IIB, subdivided into IIA1, IIA2, IIB1 and IIB2 classes based on RNA secondary structure, and

IIC (http://webapps2.ucalgary.ca/~groupii/secondary/secondarystructure.html; Dai *et al*., 2003; Lambowitz and Zimmerly, 2011). Group IIA introns associate with the upstream exon using dI base-pairing interactions EBS1 and EBS2 and use the δ – δ' long-range interaction to associate with the downstream exon (Fig 1.1). Group IIB introns use the same IBSs for recognition of the upstream exon but use IBS3 for the downstream exon while group IIC introns use only IBS1 and IBS3 for the upstream and downstream exons, respectively. Group II introns in flowering plant mitochondria are either IIA or IIB type that can be further distinguished by the position of the bulged adenosine in dVI that sits 7 or 8 nt upstream of the 3' intron-exon junction, respectively (Michel *et al*., 1989). Group IIC introns have thus far only been found in bacteria (Toor *et al*., 2001). An additional subgroup found in the plastid genome of *Euglena*, formerly referred to as group III introns, is a highly degenerate group II type (Copertino and Hallick, 1993). These introns are approximately 100 nt in length and lack domains II-V.

1.2.2 *Splicing pathways of group II introns*

Conventional splicing is carried out by two consecutive transesterification reactions that culminate in excision of the intron in a lariat physical form and the ligation of flanking exons (Fig 1.2A) (Bonen and Vogel, 2001). Central to this pathway is the 2'OH of a bulged adenosine within dVI that initiates splicing by attacking the 5' splice site at the exon-intron junction. Formation of the lariat-3'exon intermediate results in the liberation of the upstream exon and dissolution of the active site through the disassociation of bound Mg^{++} ions from the AGC and AY binding sites within dV (Lambowitz and Zimmerly, 2011). Removal of the active site triggers the formation of new long-range interactions that initiate a conformational change in intron tertiary structure that, in turn, creates a "pocket" at the 3' intron-exon

junction that is selectively accessible to the terminal 3'OH of the liberated upstream exon. Subsequent attack of the 3' splice site results in the ligation of the flanking exons and release of the lariat intron (Bonen and Vogel, 2001).

In addition to the bulged adenosine residue in dVI, water can act as the initial attacking nucleophile of the 5' splice site in what is referred to as "hydrolytic splicing" (Lambowitz and Zimmerly, 2011). As there is no branch formation between dVI and the 5' terminus of the intron in hydrolytic splicing, the intron is excised as a linear molecule rather than in a lariat form (Fig 1.2B). Analysis of *in vivo* splicing of yeast mitochondrial group II intron mutants has determined that removal of the branchpoint adenosine or altering its three-dimensional orientation, such that it is no longer in a bulged conformation, abolishes conventional lariat splicing leading to exclusive use of the hydrolytic splicing pathway (Chu *et al*., 1998; Podar *et al*, 1998; Chu *et al*., 2001). Interestingly, shortening of the dVI region of a self-splicing brown algal mitochondrial group II intron showed a similar effect *in vitro* despite no mutagenesis of the bulged adenosine (Li *et al*., 2011) and suggests that the distal stem-loop may play a structural role in lariat-splicing. Moreover, weakening of the dVI structure of an *Arabidopsis* mitochondrial group II intron by mutagenesis at Mg^{++} ion-binding sites also shifts splicing to hydrolysis *in vitro* and is likely due to a reduced capacity for positioning the nucleophile at the active site (Hertweck and Mueller, 2001).

In flowering plants, hydrolytic splicing has been observed *in vivo* for several wheat mitochondrial group II introns that lack an unpaired adenosine at the conventional position for lariat splicing and/or are poorly structured in dVI (Li-Pook-Than and Bonen, 2006). In such cases, additional full length or 3' heterogeneous *in vivo* circularized forms were also observed. Group II introns excised as full circles have also been detected in yeast mitochondria and are thought to be generated by the nucleophylic attack of the 3' splice site

by the 3'OH of an external liberated upstream exon in what is referred to as the spliced exon reopening (SER) model (Fig 1.2C) (Murray *et al*., 2001). Subsequent attack of the 5' splice site by the 3' terminal 2'OH of the intron leads to excision in a full circle form. Meanwhile, *in vivo* circularized 3' heterogeneous excised intron molecules are consistent with the employment of internal nucleophiles at variable positions within dV and dVI that are capable of correctly recognizing the 5' splice site (Li-Pook-Than and Bonen, 2006).

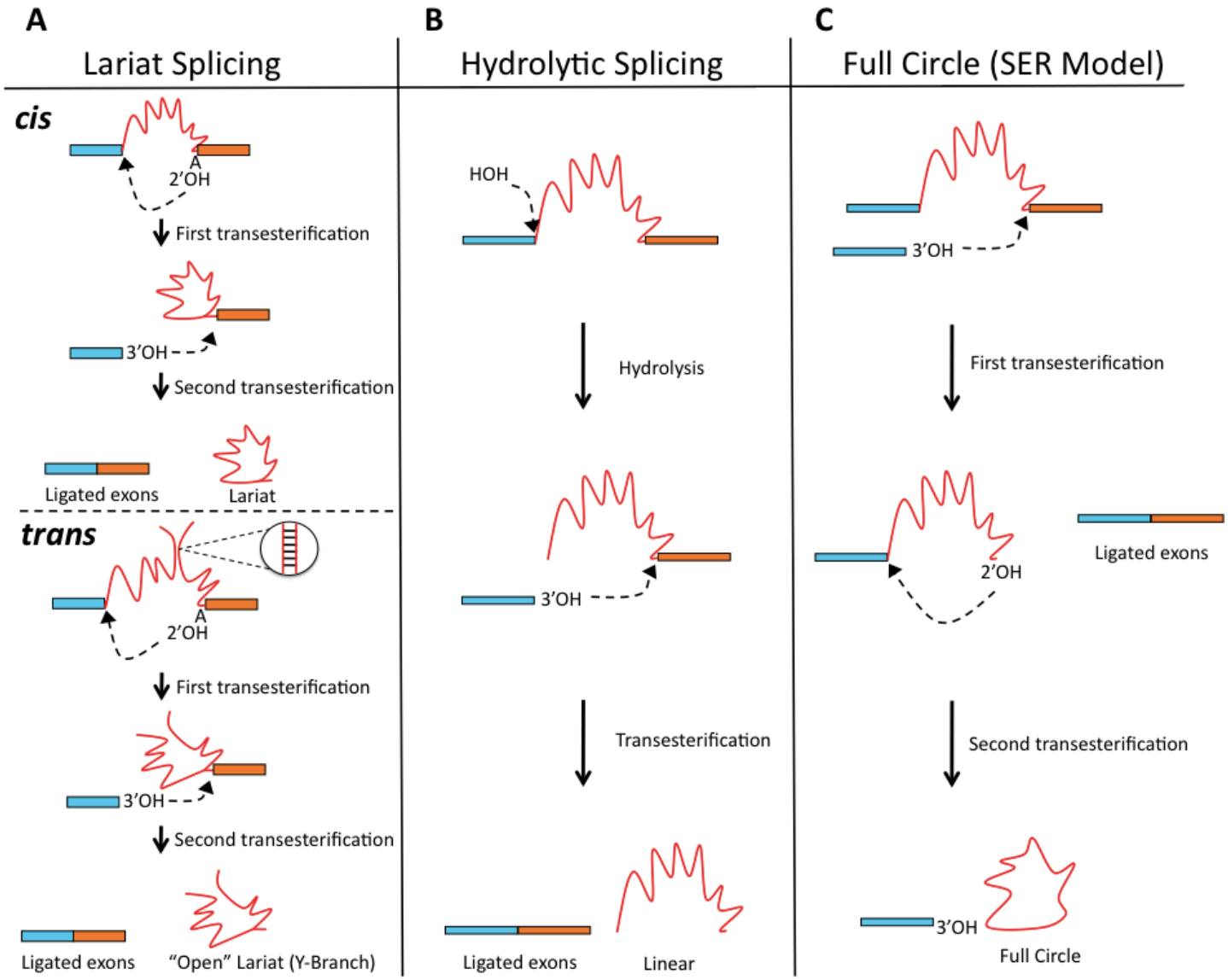
1.2.3 *Mitochondrial group II introns in flowering plants*

To date, there have been 26 distinct group II introns identified in plant mitochondria and none are observed to self-splice *in vitro* (Bonen, 2008). These introns are predominantly found inside *nad* genes that encode subunits of complex I (NADH dehydrogenase) (ex. wheat – 19 of 23) suggesting a regulatory role of RNA splicing. Of the plant mitochondrial genomes that have been sequenced, no single species has been found to contain a full set and among flowering plants the complement of mitochondrial group II introns is quite stable (Hepburn *et al*., 2012). The variability that is observed between species is a result of sporadic intron loss by retroprocessing in which cDNA reverse-transcribed from a spliced mRNA transcript is integrated into the genome.

While most mitochondrial group II introns in flowering plants are intact at a single locus and undergo *cis*-splicing, there are 6 cases of introns found in *nad1*, *nad2* and *nad5* that have been fragmented into 2 or more pieces around the genome that are independently transcribed (Bonen, 2008). Characterization of *cis*-splicing homologues in non-flowering plant families such as ferns, fern allies and mosses has determined that intron fragmentation is associated with expansion of dIV that is then susceptible to DNA rearrangement (Malek *et*

Figure 1.2: Comparison of group II intron splicing pathways

[A] Conventional group II intron splicing is initiated by the nucleophylic attack of the 5' exon-intron junction by a bulged adenosine residue within dVI. Mediated by two consecutive transesterification reactions, splicing culminates in a lariat form of the excised intron and ligation of flanking exons. In *trans*, basepairing between halves of dIV contribute to assembly of competent splicing structure. As the 3' and 5' ends of the respective half-introns are non-contiguous, the intron is excised in an "open" lariat (Y-branch) form. **[B]** In hydrolytic splicing, water acts as the attacking nucleophile resulting in excision of the intron in a linear form. **[C]** Full-circle excised introns can also be generated by the spliced-exon recognition (SER) model that involves nucleophylic attack of the 3' intron-exon junction by an external liberated upstream exon (Murray *et al*., 2001).


A
Lariat Splicing
B
Hydrolytic Splicing
C
Full Circle (SER Model)
cis
A
2′OH
First transesterification
3′OH
Second transesterification
Ligated exons
Lariat
trans
A
2′OH
First transesterification
3′OH
Second transesterification
Ligated exons
"Open" Lariat (Y-Branch)
HOH
Hydrolysis
3′OH
Transesterification
Ligated exons
Linear
3′OH
First transesterification
2′OH
Ligated exons
Second transesterification
3′OH
Full Circle

al., 1997; Pruchner *et al*., 2001). All *trans*-splicing group II introns in flowering plant mitochondria are broken within dIV with the exception of the tri-partite *nad5* intron 3 in *Oenothera* that has a second break within dI (Knoop *et al*., 1997). Interestingly, study of *trans*-splicing *in vivo* of Ll.LtrB group II intron mutants in the Gram-positive bacterium *Lactococcus lactis* has demonstrated astonishing tolerance for fragmentation throughout most of the intron secondary structure (Belhocine *et al*., 2008). Only in cases where mutants were fragmented within EBSs, dV, at 5' and 3' splice sites or in close proximity to the bulged adenosine in dVI was *trans*-splicing not observed. The mechanism by which group II intron segments associate with one another for *trans*-splicing has not been fully elucidated but, in flowering plants, it is believed to involve base-pairing between the respective halves of dIV that is further mediated by RNA chaperones (Bonen, 2008). Association between dIV segments is thought to facilitate RNA folding in the same fashion observed for *cis* introns but, since the ends of these segments are non-contiguous, splicing culminates in an "open lariat" or "Y-branch" physical form (Bonen, 1993). Notably, *trans*-splicing of *nad5* intron 2 in *Brassica napus*, generates multiple mis-spliced forms containing variable lengths of upstream exon sequence in addition to the Y-branch form (Elina and Brown, 2010). These nonproductive splicing products are thought to be due to inappropriate 5' splice site selection at cryptic positions within the upstream exon as result of folding constraints placed on this intron by an additional neighbouring *trans*-intron and provide a clear demonstration of the potential impact of RNA misfolding on gene expression. In chloroplasts of flowering plants, two introns of *rps12* are the only documented cases of *trans*-splicing in the plastid genome (Perron *et al*., 2004).

A subset of mitochondrial introns in flowering plants deviates from the conventional structure of classic group II introns (Bonen, 2008). The most striking examples are those

with nonconventional dV and dVI regions that include A-C mispairs, indels and/or are missing the branchpoint adenosine entirely (Carrillo *et al*., 2001; Li-Pook-Than and Bonen, 2006). Moreover, the vast majority of flowering plant mitochondrial group II introns no longer encodes maturases that promote their excision, the sole exception being *matR* within dIV of *nad1* intron 4 (Farré and Araya, 1999). Consistent with the loss of intron mobility, *matR* lacks a DNA-binding domain and is missing segment 1 of its RT domain.

1.3 *Splicing of nuclear spliceosomal and group I introns*

At the core of the spliceosome that mediates excision of nuclear spliceosomal introns are snRNAs U1, U2, U4, U5 and U6 several of which are similar in structure and function to particular domains of group II introns (Rogozin *et al*., 2012). For instance, U1 and U2, much like dI, act as the scaffold that brings together the 5' splice site and the 3' end of the intron that contains a bulged adenosine ~100 nt upstream of the 3' intron-exon junction and, together with U6 that has a similar structure to dV, forms the active site (Schmitzová and Pena, 2012). As in group II intron splicing, spliceosomal introns are excised through two consecutive transesterification reactions that culminate in a lariat intron (Rogozin *et al*., 2012). In contrast, splicing of group I introns is initiated by an exogenous guanosine (exoG) that binds to the G-site located within a pocket of P7 and, in turn, attacks the 5' splice site (Nielson and Johansen, 2009). The first splicing step liberates the upstream exon and transfers the exoG nucleophile to the 5' intron terminus. Similar to hydrolytic splicing of group II introns, the 3' terminal 3'OH of the liberated upstream exon attacks the 3' splice site resulting in excision of the group I intron in a linear form and ligation of flanking exons (Haugen *et al*., 2005). Interestingly, a third transesterification event typically occurs following excision in which the 3' terminal nucleotide, almost invariably a G, attacks the 5'

end of the linear intron either precisely at the terminus, generating a full-length circular form, or at variable positions downstream, resulting in 5' truncated circular forms. While group I introns are common to bacteria and the organellar genomes of fungi and non-vascular plants, there is only a single documented case in flowering plant mitochondria (Cho *et al*., 1998). This particular group I intron, which is of fungal origin, has been found in only a select number of distantly related flowering plant species suggesting it has invaded the plant kingdom and since proliferated within it through horizontal gene transfer.

1.4 *Plant mitochondrial gene content and expression*

Of endosymbiotic origin, mitochondria have significantly reduced gene content compared to their α-proteobacteria ancestors in large part due to functional gene transfer to the host nucleus or by functional replacement by preexisting nuclear genes (Gray *et al*. 2001). The consequence is that the vast majority of the mitochondrial proteome, upwards of 99% in certain lineages, is encoded by the host genome and must, in turn, be imported from the cytosol (Baker *et al*. 2007). Many of the genes that typically persist in plant mitochondrial genomes belong to families that encode subunits of four respiratory chain complexes: *nad* (complex I – NADH dehydrogenase), *cob* (complex III – cytochrome bc1 oxidoreductase), *cox* (complex IV – cytochrome oxidase), and *atp* (complex V – ATP synthase) (reviewed in Adams and Palmer, 2003). The remainder of the gene content is comprised of tRNAs, rRNAs, ribosomal protein genes of the small and large subunit (*rps* and *rpl*), and, typically, *ccm* (cytochrome c biogenesis), *mttB* (membrane targeting and translocating gene), and *matR* (see subsection 1.2). Plant mitochondrial gene expression is dependent on nuclear-encoded machinery for transcription and several RNA processing

events required in transcript maturation, including intron splicing, C-to-U editing and RNA cleavage (reviewed in Schmitz-Linneweber and Small, 2008; Small *et al*., 2013).

1.4.1 *Transcription*

Transcription in plant mitochondria is mediated by RpoTm, a nuclear-encoded single subunit RNA polymerase (RNAP) of T3/T7 bacteriophage origin (reviewed in Liere *et al.*, 2011). A second RNAP, RpoTmp, has been characterized in *Arabidopsis* and is dual-targeted to the mitochondria and chloroplasts (Hedtke *et al*., 2000). These RNAPs recognize the primary consensus motif CRTA as well as several alternative motifs. In flowering plants, multiple transcription initiation sites are common and have been proposed to ensure transcription occurs in the event of a genomic rearrangement (Kuhn *et al*., 2005). Indeed, orthologous genes in plant mitochondria often have different transcription profiles owing to the recombinogenic nature of the genomes (Lupold *et al*., 1999). Interestingly, only a weak correlation has been identified between relative promoter activities and steady-state levels of mature mRNAs suggesting gene expression is predominantly regulated at the post-transcriptional level (Giegé *et al*., 2000).

1.4.2 *C-to-U editing*

C-to-U RNA editing involves the deamination of specific cytosine residues to uracil (Castandet and Araya, 2011). Based on the status of precursor RNAs, editing is considered to be an early event in RNA processing of plant mitochondrial transcripts and typically occurs at approximately 300-500 sites across the plant mitochondrial transcriptome, with some extreme cases far exceeding this range (ex. *Isoetes engelmanii*: ~1800 sites) (Giegé and Brennicke, 1999; Mower and Palmer, 2006; Grewe *et al*., 2010). The vast majority of editing

sites are within coding regions with most occurring at the first or second triplet position usually resulting in nonsynonymous changes and in some cases generating a start or stop codon. Notably, the altered amino acid sequence tends to have increased sequence similarity to orthologous proteins in other plant species suggesting C-to-U editing plays an important role in the production of functional proteins (Shikanai, 2006).

Although only limited editing is observed in intron sequences and almost always within highly structured domains important in splicing, including dI, dV and dVI, recent data suggests the extent of C-to-U edits in introns may be greater than previously thought (Picardi *et al*., 2010; Grewe *et al*., 2010). Intron edits typically increase the stability of intron RNA structures by correcting A-C mispairs to Watson-Crick (A-U) and have been shown to be prerequisite to *cis*- and *trans*-intron removal suggesting they facilitate correct RNA folding (Carrillo *et al*., 2001; Farré *et al*., 2012). Notably, RNA editing can also function in restoring canonical base-pairing within stem-loop regions of certain tRNAs and degenerate tRNAs known as "t-elements" (Forner *et al*., 2007). Improved thermodynamic stability of these structures resulting from C-to-U edits are thought to be important in their recognition by *trans*-acting factors (Byers and Bonen, 2012).

1.4.3 *RNA end-maturation*

Despite examples in which the termini of the translated messenger are the same as those of the primary transcript, processing of 5' and 3' UTRs by RNA cleavage is observed for most mRNA transcripts in plant mitochondria (Forner *et al*., 2007; Choi *et al*., 2012). From analysis of the termini of mature mRNAs in wheat and *Arabidopsis*, 3' ends are generally discrete, mapping to a specific nucleotide position, whereas heterogeneity is observed at 5' ends potentially due to multiple *cis*-elements recognized by endonucleases.

End-maturation of tRNAs is also mediated by RNA cleavage. Endonucleases RNase P and RNase Z, the former being protein-based in plant organelles, cleave at the precise 5' and 3' ends of the tRNA with both events believed to occur quite rapidly following transcription (Rossmanith, 2012). Interestingly, degenerate tRNAs known as "t-elements" coincide with the termini of several plant mitochondrial mRNAs suggesting they may contribute to RNA stability as well as transcript maturation following endonucleolyic cleavage (Forner *et al*., 2007).

1.4.4 *RNA splicing machinery*

Trans-factors imported into the mitochondrion from the cytosol that are known to promote intron splicing include nuclear-encoded maturases and members of the DEAD-box, CRM, PORR domain, and PPR protein families (de Longevialle *et al*., 2010) (Table 1.1). In addition to the maturase encoded by *matR* of *nad1* intron 4 in the mitochondrial genome, four maturases have been identified in the nuclear genomes of *Arabidopsis* and rice of which 3 (nMAT1-3) are targeted to the mitochondrion and one (nMAT4) to the chloroplast (Matsuura *et al*., 2001). Knockout of the *nMat1* gene in *Arabidopsis* results in reduced splicing efficiencies of *nad1* intron 1, *nad2* intron 1, and *nad4* intron 2 (Nakagawa and Sakurai, 2006; Keren *et al*., 2012). Meanwhile, knockout of the *nMat2* gene decreased the splicing efficiencies of *nad1* intron 2, *nad7* intron 2, and the *cox2* intron (Keren *et al*., 2009). Notably, all introns in each case, with the exception of *nad7* intron 2, lack a conventional branchpoint adenosine suggesting similar increased structural requirements. Since complete loss of splicing was not observed for any intron, additional *trans*-factors likely contribute to excision (Keren *et al*., 2009). An additional maturase-encoding gene, *matK*, is present in the chloroplast genome of flowering plants. In tobacco, it was shown that the MatK maturase

Table 1.1: Identity and target of known nuclear-encoded splicing factors of plant mitochondrial group II introns

Name	Gene ID	Class	Intron(s)	Reference
nMAT1	At1G30010	Maturase	*nad1* i1	Keren *et al.*, 2012
nMAT2	At5G46920	Maturase	*nad1* i2, *nad7* i2, *cox2*	Keren *et al.*, 2009
PMH2	At3G22330	DEAD-Box	*nad1* i2&3, *nad2* i1,2&4, *nad4* i2&3, *nad5* i1,2&3, *nad7* i1&4, *cox2*, *rpl2*	Kohler *et al.*, 2010
mCSF1	At4G31010	CRM	all except *nad1* intron 1 &4, *nad4* intron 1, *ccmFC* and *rpl2* (*Arabidopsis*)	Zmudjak *et al.*, 2013
WTF9	At2G39120	PORR protein	*rpl2*; *ccmFC*	Francs-Small *et al.*, 2012
OTP43	At1G74900	PPR protein	*nad1* i1	de Longevialle *et al.*, 2007
ABO5	At1G51965	PPR protein	*nad2* i3	Liu *et al.*, 2010
BIR6	At3G48250	PPR protein	*nad7* i1	Koprivova *et al.*, 2010
PpPPR_43	Pp1s446_7V6.1	PPR protein	*cox1* i3	Ichinose *et al.*, 2012

contributes to the splicing of 7 of the 8 group IIA introns (Zoschke *et al*., 2010). A similar study to identify the RNA substrates of the MatR maturase has not been conducted.

DEAD-box proteins represent a large family of RNA helicases that participate in a wide range of RNA-level events (eg. transcription, translation, RNA degradation) and are ubiquitous to eukaryotes and present in most prokaryotes (reviewed in Cordin *et al*., 2006). These proteins are defined by 8 highly conserved motifs, including the Walker B motif that contains the invariable amino acid sequence Asp-Glu-Ala-Asp (D-E-A-D). In *Arabidopsis*, 58 DEAD-box proteins are encoded by the nuclear genome of which at least 2, PMH1 and PMH2, are targeted to the mitochondrion (Köhler *et al*., 2010). While PMH1 does not contribute to RNA splicing, PMH2 participates in excision of *nad2* intron 1, *nad2* intron 2, and *nad2* intron 4 and is thought to function as a RNA chaperone by stabilizing intron secondary structure.

Members of the CRM, chloroplast RNA splicing and ribosome maturation, class of proteins are encoded by 16 and 14 nuclear genes in *Arabidopsis* and rice, respectively, and are predominantly targeted to the chloroplasts where they have documented importance to group II intron splicing (Jenkins *et al*., 1997). Recently, one such protein, mCSF1, was shown to be targeted to the mitochondria and essential to splicing of several group II introns (Zmudjak *et al*., 2013). Knockout of mCSF1 was further correlated with retarded growth during early embryogenesis demonstrating the importance of RNA processing during periods of increased energy demand.

The PORR, plant organelle RNA recognition, proteins are defined by a unique and recently characterized RNA-binding domain (Kroeger *et al*, 2009). Virtually all PORR domain proteins are predicted to be targeted to either the chloroplasts or mitochondria of which 2, WTF1 and WTF9, have been functionally characterized. WTF1 is targeted to the

chloroplast and was demonstrated to promote *in vivo* splicing of many group II introns in maize (Kroeger *et al*., 2009). Meanwhile, WTF9 is targeted to the mitochondria where it was shown to be essential to *in vivo* splicing of the *ccmFC* and *rpl2* group II introns in *Arabidopsis* (Francs-Small, CC. *et al*., 2012).

Of the portion of imported proteins that participate in organelle RNA processing, the vast majority belong to the pentatricopeptide repeat (PPR) family, that is characterized by a canonical 35 amino acid motif present in 30 tandem repeats (Saha *et al*., 2007). While present in all eukaryotes, the PPR family present in plants is greatly expanded (>450 in *Arabidopsis*) suggesting they participate in a wide variety of plant-specific processes. Referred to as a "socket set for organelle expression", PPR proteins have sequence specific RNA binding activity and have a central role in RNA editing and contribute to group II intron splicing and end-maturation (Schmitz-Linneweber and Small, 2008). At present, only 4 PPR proteins have been shown to have a specific role in RNA splicing of plant mitochondrial group II introns. In *Arabidopsis*, individual knockout of OTP43, ABO5 and BIR6 resulted in complete loss of RNA splicing of *nad1* intron 1, *nad2* intron 3 and *nad7* intron 1, respectively (de Longevialle *et al*., 2007; Liu *et al*., 2010; Koprivova *et al*., 2010). Similarly, PpPPR_43 in *Physcomitrella patens* was demonstrated to be essential for splicing of *cox1* intron 3 (Ichinose *et al*., 2012).

1.4.5 *PPR proteins in RNA editing and end-maturation*

All *trans*-factors that have a functionally characterized role in RNA editing in plant mitochondria belong to the PPR protein family (Castandet and Araya, 2011). Mutational analysis of *cis*-elements for recognition by PPR proteins suggest that the 22 nt immediately surrounding an editing site (16 upstream + 5 downstream) are involved, with upstream

nucleotides being most important (Bock *et al*., 1996). For editing sites located near exon-intron junctions, increased levels of C-to-U editing are observed following intron excision suggesting either the PPR proteins are sterically hindered by intron RNA structures or the recognition sequence is generated following ligation of flanking exons (Li-Pook-Than *et al*., 2007). A subset of PPR proteins found only in land plants possess a DYW domain at their C-terminal that contains a conserved amino acid sequence that resembles the active site of editing enzymes that have been characterized in other systems (Salone *et al*., 2007). Moreover, there is a strong correlation between the presence of PPR-DYW proteins and the observance of RNA editing in land plants suggesting they may catalyze C-to-U deamination in plant organelles. However, *in vivo* analysis of *Arabidopsis* mutants has demonstrated that the DYW domain is not essential to the C-to-U deamination reaction suggesting that PPR proteins, in the context of RNA editing, may instead serve as the functional equivalent of guide RNAs for the true, and as-yet-unknown, editing machinery (Okuda *et al*., 2009; Okuda *et al*., 2010). Interestingly, additional functional analysis has uncovered specific RNase activity of the DYW domain that suggests a role of PPR proteins in catalyzing RNA cleavage events in mRNA end-maturation (Nakamura and Sugita, 2008). Notably, the PPR protein RPF1 was shown to be essential to 5' end-processing of *nad4* transcripts in *Arabidopsis* mitochondria (Hölzle *et al*., 2011).

1.4.6 *RNA degradation*

The primary RNA degradation pathway employed in plant mitochondria is a remnant of the organelle's bacterial origin and is the same as that observed in the mitochondria of yeast and humans (Slomovic *et al*., 2005). Meditated by as-yet-unknown poly(A) polymerases, 3' polyadenylation, in contrast to cytosolic mRNAs, identifies RNA species for

degradation (Holec *et al*., 2008). These short polyA tracts are recognized by mtPNPases, 3' to 5' exoribonucleases, including the recently characterized nuclear-encoded AHG2 and AGS1 (Hirayama *et al*., 2013). Poly(A) polymerases in plant mitochondria are thought to be "scavenging" enzymes that indiscriminately add 3' A-tails to 3' terminal OHs (Holec *et al.,* 2008). As such, the stability of RNA species is largely dependent on the accessibility of the 3' end. In the case of plant mitochondrial mRNAs, terminal stem-loops, frequently t-elements, as well as associated PPR proteins involved in end-generation have been implicated in 3' protection for RNA stability (Forner *et al*., 2007; Zhelyazkova *et al*., 2012). While additional turnover mediated by unidentified endonucleases is known to occur, significant accumulation of nonproductive transcripts is observed in mutants lacking mtPNPase suggesting that alternative degradation pathways are minor and cannot compensate for the primary polyadenylation-mediated pathway (Perrin *et al*., 2004; Holec *et al*., 2006).

1.4.7 *Developmental changes in mitochondrial gene expression*

Notable differences are observed in the expression patterns of certain mitochondrial genes over the course of plant development from embryo to seedling. While steady-state levels of mature *nad7*, *cox2* and *rps3* mRNAs in wheat remain rather constant relative to 18S rRNA through this developmental period, markedly higher levels of precursor mRNAs are observed soon after imbibition of dry seeds that then drop off dramatically within 6 days of germination (Li-Pook-Than *et al*., 2004). Early plant germination is understood to be a time of significantly increased energy demand necessitating rapid assembly of respiratory complexes needed in ATP production. As such, increased levels of precursor species suggest that *de novo* transcription during this stage of development far outpaces RNA processing.

Since processing events are dependent on nuclear-encoded machinery, it is conceivable that such a discrepancy may be due, in part, to a lag in protein import by the TIM, translocase of the inner membrane, and TOM, translocase of the outer membrane, complexes. Increased steady-states levels of excised intron RNAs were also observed in wheat during embryonic germination compared to seedling suggesting a lower rate of turnover (Carrillo and Bonen, 1997; Subramanian *et al*., 2001; N.Niknejad, MSc Thesis, University of Ottawa, 2003) that is possibly due to less efficient coupling of 3' polyadenylation and mtPNPase activity.

1.5 *Impact of temperature on plant mitochondrial gene expression*

On account of the sessile nature of plants, abiotic stressors such as drought, high salinity and cold represent major threats to their survival that can impact heavily on plant metabolism. One particular complication associated with abiotic stress conditions is increased levels of reactive oxygen species (ROS) (reviewed in Gill and Tuteja, 2010). Although steady-state levels of ROS are maintained for use in intracellular signaling pathways, high concentrations can cause serious damage to cell structures and DNA. In *Arabidopsis*, nuclear genes *ndb2*, that encodes for an external NAD(P)H dehydrogenase that bypasses complex I, and *ucp1*, an uncoupling protein-encoding gene, are upregulated under cold stress and function to mitigate ROS emissions (Armstrong *et al.,* 2008). Increased expression of NAD(P)H dehydrogenase genes is also observed in mutants that do not generate functional *nad1* messengers (de Longevialle *et al*., 2007). Taken together, this raises interesting questions related to the expression of plant mitochondrial *nad* genes in the cold and the concomitant impact on ATP production. Moreover, mRNA levels for *nad2*, *nad4*, *nad6*, *cox1*, *cox2*, *ccmC*, and *ccmB* in germinating wheat embryos subjected to 4^{o}C

show a 4-fold decrease suggesting that the expression patterns of multiple mitochondrial gene families are impacted (Naydenov *et al*., 2010).

Stemming from evidence of reduced expression of respiratory genes in plant mitochondria during low temperature growth, coupled with strong support for the regulation of plant mitochondrial gene expression being primarily at the post-transcriptional level (Small *et al*., 2013) is the question of whether differences in the efficiencies of RNA processing of precursor mRNAs are a major contributing factor. Investigation of the impact of cold on C-to-U editing of *cox2* transcripts in wheat and rice has uncovered a lower degree of editing at positions immediately adjacent to exon-intron junctions, including one within IBS1, that is correlated with elevated levels of intron-containing precursor mRNAs (Kurihara-Yonemoto and Handa, 2001; Kurihara-Yonemoto and Kubo, 2010). These findings suggest that reduced C-to-U editing during cold growth can impact intron folding and result in a concomitant decrease in splicing efficiency. In the specific context of group II intron splicing at low temperature, little is known, however study of the self-splicing bacterial Ll.LtrB group II intron under heat stress has uncovered a strong correlation between increased growth temperature and the accumulation of unspliced transcripts (Yao *et al*., 2006). Similarly, investigation of heat stress on plant mitochondrial gene expression identified a substantial increase in the steady-state levels of polyadenylated unspliced transcripts of *rpl2* (Adamo *et al*., 2008) that may be the result of reduced efficiency of splicing coupled with increased accessibility of the 3' terminus to poly(A) polymerases. Taken together, these findings indicate a temperature sensitivity of RNA structure that can directly impact group II intron splicing kinetics.

1.5.1 *Wheat as a model system for cold growth studies*

Wheat (*Triticum aestivum*), with over 200 million bushels cultivated annually (USDA 2013: http://www.ers.usda.gov/data-products/wheat-data.aspx#.UiC8OHBkLRo), represents one the most agronomically important crop plant species in Canada and the rest of the world. Cultivars of wheat are typically characterized by their ability to grow at low temperatures (eg. cold sensitive vs. cold hardy) and have been the subject of substantial research on cold stress response in plants (c.f. Winfield *et al*., 2010; Tang *et al*., 2012; Vigeland *et al*., 2013). As such, wheat was selected as the model system for our investigation into group II intron splicing during low temperature growth.

1.5.2 *Objectives*

My work aims to gain greater insight into questions related to intron splicing in wheat mitochondria by determining the physical excised forms of *nad1* intron 1, *nad2* intron 1 and *nad1* intron 2 that belong to a subset of aberrant group II introns in wheat that deviate from the conventional branchpoint structure of dVI and are, therefore, not predicted to employ classic lariat-type splicing. In light of elevated levels of precursor mRNA species observed under cold growth, a primary element of my research is the investigation of the efficiency of RNA processing events at low temperature including intron splicing, particularly in the context of differential biochemistry as inferred from excised intron physical form, and end-maturation of mRNAs. Despite having a bulged adenosine at the correct position, the *cox2* intron was also included on the basis that its dVI is weakly-structured and was previously shown by our lab not to be excised as a lariat under room temperature growth conditions. Major questions addressed are as follows:

- What are the predominant physical forms of excised wheat mitochondrial *nad2* intron1, *nad1* intron 2 and *cox2* intron at room temperature and in the cold? Do any potential differences indicate a shift in splicing biochemistry? Is there a change in RNA splicing efficiency? Do potential changes differ from the impact of cold treatment on splicing of a conventional group II intron? Is RNA stability and turnover of excised intron molecules affected?

- What is the primary mode of splicing for the highly degenerate bipartite wheat mitochondrial *nad1* intron 1? Is *trans*-splicing impacted by cold treatment? Is the efficiency RNA processing, including end-maturation, of the *nad1a*-intron 1a locus different between room temperature and cold?

Chapter 2: Materials & Methods

2.1 *Germination of wheat embryos and mitochondrial RNA isolation*

Wheat seeds (*Triticum aestivum* cv. FT-Wonder) were kindly provided by Dr. R. Pandeya (Agriculture and Agri-Food Canada). FT-Wonder is a cold-hardy winter wheat cultivar that is tolerant of pathogenic species of fungi of the genus *Fusarium*. For each RNA preparation, approximately 50g of dry seeds were surface-sterilized using a 1:6 dilution of Javex in distilled water followed by 10mM HCl and then repeated rinses using autoclaved deionized ultrafiltered (DIUF) water. Prior to imbibition of the sterilized seeds, the majority of the endosperm was removed by dissection in order to reduce polysaccharide contamination during RNA extraction. Dissected seeds were placed in Petri dishes on autoclaved filter paper and imbibed using autoclaved DIUF water. Seeds were grown in the dark (etiolated) for 36 hours at room temperature. For cold studies, seeds were grown in the dark for 18 hours at room temperature and then transferred to 4^{o}C (standard in cold growth studies of wheat) for an additional 72 hours in the dark. Growth conditions for room temperature and cold growth were established in order to reach a comparable phenotype based on length of emerging radicle (Fig 2.1).

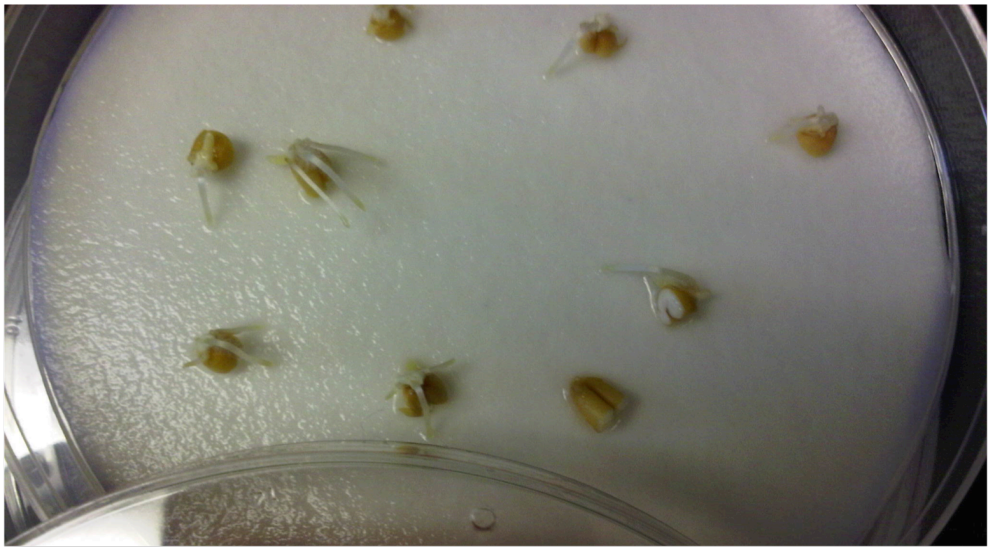
Isolation of mitochondrial RNA from germinating wheat embryos was carried out as previously described (Subramanian *et al*., 2001). Embryos that failed to germinate (<10% on average) were discarded prior to isolation. Crude RNA preparation were purified using 2 phenol extractions (1.5 vol. phenol saturated in TE buffer). Nucleic acids were precipitated using 0.1 vol. 5M NaCl and 2 vol. 95% EtOH.

Figure 2.1: Etiolated germinating wheat (Triticum aestivum cv. FT Wonder) embryos immediately prior to RNA extraction.

Post-imbibition, surface-sterilized wheat seeds were grown in the dark for **[A]** 36 hours at room temperature or, **[B]** 18 hours at room temperature then transferred to 4^{o}C for an additional 72 hours. Excess water shown with cold grown embryos is not representative of differences in the volume of water applied to germinating seeds between temperature conditions.

A

B



2.2 *Mitochondrial RNA analysis*

The quality and concentration of the mitochondrial RNA from each extraction was assessed by ethidium bromide staining of RNA gels. Mitochondrial RNA samples (300uL) together with a 0.5-10kb RNA size ladder (3 ug, Invitrogen) were electrophoresed on a 1.2% agarose/formaldehyde gel for approximately 4 hrs at 60V (~24mA). Subsequent northern transfer to a nylon membrane was carried out using standard procedures.

2.2.1 *Northern hybridization*

Northern analyses were carried out using γ-^{32}P-5'-end-labelled 20 nt oligomers as described in Li-Pook-Than and Bonen (2006) with the exception of the prehybridization of RNA blots which were done overnight. Following overnight hybridization with radiolabelled probe, RNA blots were washed twice with 20mL of wash solution (20X SSC + 0.1% SDS) for 20 min to remove unincorporated probe. Radiography of probed blots was carried out by phosphoimaging (Molecular Imager X – Biorad). Steady-state levels of RNA species identified by northern hybridization were quantified relative to 18S rRNA.

2.2.2 *(CR)RT-PCR*

(CR)RT-PCR was used for analysis of spliced junctions of excised intron species and the termini of precursor and mature mRNAs. Initial RNA ligation of ~5ug of wheat mitochondrial RNA was carried out as previously described (Li-Pook-Than and Bonen, 2006) with the exception of the RNA denaturation, which was conducted at 90^{o}C for 2 min, and the RNA ligase incubation, which was conducted at 37^{o}C for 30 min. RNA samples that did undergo an RNA ligase pretreatment (RT-PCR) were carried through in parallel but with T4 RNA ligase substituted with autoclaved DIUF water. Due to its ability to cross 2'-5'

bonds, Superscript II reverse transcriptase (Invitrogen), incubated at 42^{o}C for 2 hours, was used in cDNA synthesis of excised intron species. M-MLV reverse transcriptase (Invitrogen), incubated at 37^{o}C for 2 hours, was used in cDNA synthesis of mRNA transcripts. (CR)RT-PCR products of the expected size were gel purified using Ultra-Clean 15 (MoBio Laboratories) and were confirmed by nested PCR. The ability to discern between full-length and 3' truncated species was limited by the resolution of the gel system used in this study. Amplicons that exceeded the size of the full-length intron were excluded from further analysis as they could not be generated by a productive splicing pathway. Mitigation of spurious (CR)RT-PCR products was facilitated by "touchdown" PCR conditions, as needed.

2.2.3 *Cloning and sequencing*

(CR)RT-PCR products were vector-ligated using the pGEM T-easy vector system (ProMega) and then cloned using competent TB1 *E.coli*. Recombinant plasmid DNA was isolated using the QIAprep spin Miniprep kit (Qiagen) and the size of the inserts were confirmed by PCR. Sequencing was performed by StemCore Laboratories at the Ottawa Health Research Institute (OHRI). Gel-purified RT-PCR products of spliced mRNA junctions were sent for direct sequencing using custom oligomers in order to assess the degree of heterogeneity in the mRNA population.

2.2.4 *Oligomers*

Synthetic 20 nt oligomers used in northern hybridization, cDNA synthesis, (CR)RT-PCR and sequencing were designed based on the wheat mitochondrial genome (AP008982) and are summarized in Table 2.1. Positions of oligomers are illustrated in Figure 2.2.

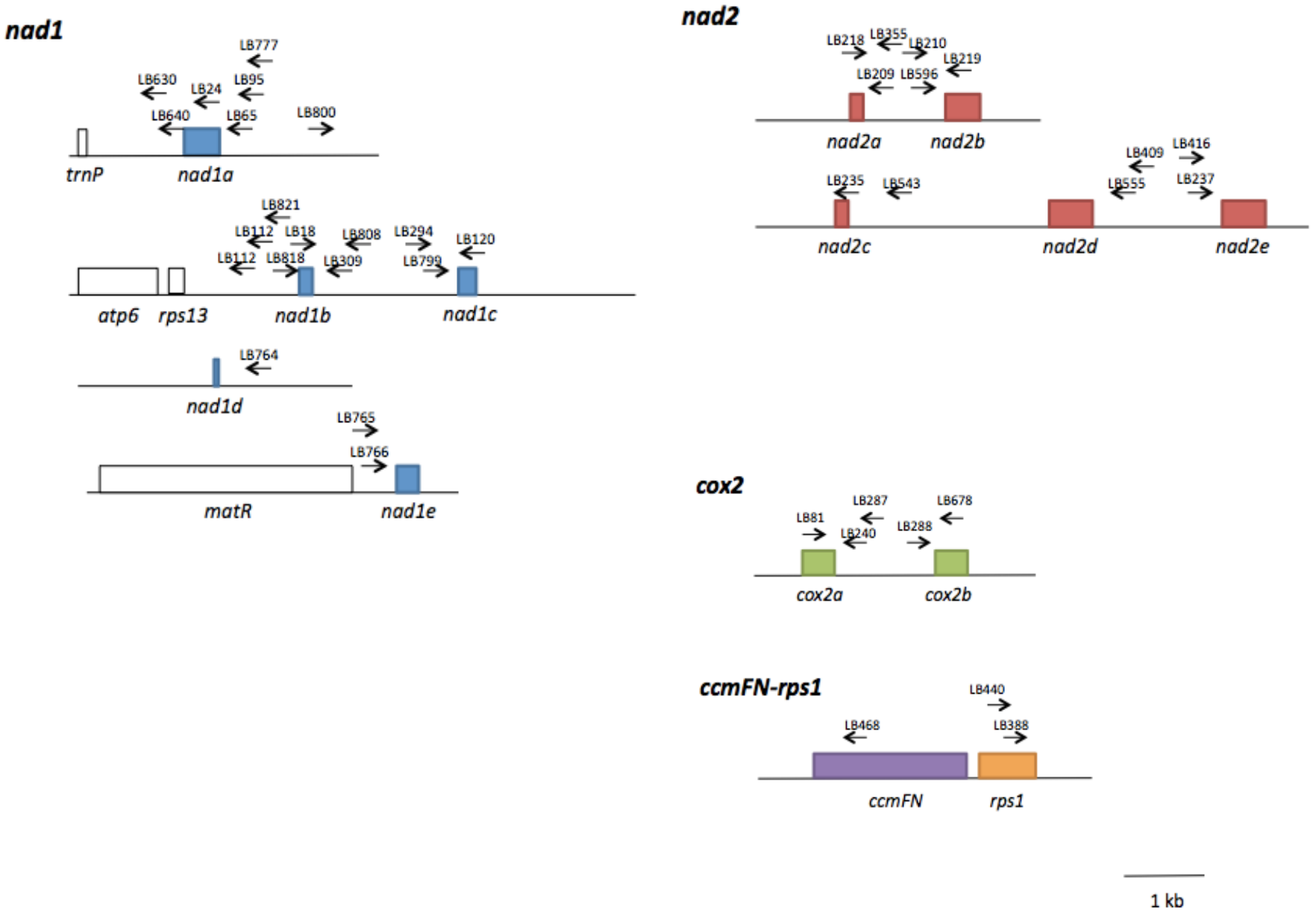
Table 2.1: Oligomers used in this study

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 used in nested PCR are designated by (s). All oligomers were designed on the wheat mitochondrial genome (AP008982).

	5' - 3' sequence	Position
nad1abc* and *nad1de		
LB777	CGAGGCTAGGCAAACTCAGC (rev, c, n)	5' end of *nad1* intron 1a
LB65	GTAGATCGTTCAACACCTGC (rev, c)	5' end of *nad1* intron 1a
LB95	GACTTCCACTTCTCGATCCC (rev, c, s)	5' end of *nad1* intron 1a
LB800	TGATGATGTCTGCTGGTTGG (for, p)	3' end of *nad1* intron 1a
LB24	AAGGCTACTCCTAGTAGAAG (rev, n)	5' end of *nad1a*
LB640	CACTTGACTAAGCATAAGCG (rev, c)	5' *nad1* UTR
LB630	CCATCTTGACTCGTTTTCGG (rev, s)	5' *nad1* UTR
LB103	GAAGCTGTCGCTTGACGGAC (rev, c)	3' end of *nad1* intron 1b
LB112	TGAACAGTGTGATTGATCAG (rev, s)	3' end of *nad1* intron 1b
LB821	TATCCGCTTGGGACTGATAG (rev, c, n)	3' end of *nad1* intron 1b
LB818	TGGGTATGATATTCTCGTGG (for, p)	3' end of *nad1* intron 1b
LB120	ATATTCTACATTATAGCCTG (rev, c, n)	5' end of *nad1*c
LB18	ATGGTCCCTTATGAAGTCTC (for, p)	mid-region of *nad1b*
LB808	TTGTCAGAGTGGATTCGGAC (rev, c)	5' end of *nad1* intron 2
LB294	CAGCTTACTCACCCTACTCC (for, p)	3' end of *nad1* intron 2
LB309	CTCAAAATGAGCCTTGCGAC (rev, n, s)	5' end of *nad1* intron 2
LB799	GCTCTGAACACGAAAGTTTG (for, s)	3' end of *nad1* intron 2
LB764	GGTCACCACTACTGAGGATC (rev, c, n)	5' end of *nad1* intron 4
LB765	GATTAGTTGAGTAGGCTTGC (for, p)	3' end of *nad1* intron 4
LB766	AGGCTTCGCTATCGCTCATG (for, s)	3' end of *nad1* intron 4
nad2abc* and *nad2de		
LB355	ATGCACAGGTACCTACGTAG (rev, c)	5' end of *nad2* intron 1
LB210	GTAGATGATAATGCGAAAGG (for, p)	5' end of *nad2* intron 1
LB596	AGTTATCACGGACGAGCCAC (for, p, s)	3' end of *nad2* intron 1
LB209	CGCACATTCATAATAGCGTT (rev, n, s)	5' end of *nad2* intron 1
LB235	AATTGATCGAAGTGGGTAGC (rev, c)	5' end of *nad2c*
LB543	CCTTACGAGGTAGTGATGAG (rev, c)	5' end of *nad2* intron 3
LB218	GCTCATTCATGGAGTTGTAT (for, p)	mid-region of *nad2a*
LB219	CTTCCGTGGAAAATTCAGAC (rev, n)	3' end of *nad2b*
LB555	CGTGTAGTGATTGTGGACTC (rev, c)	5' end of *nad2* intron 4
LB416	GGGATGGATAAAGTGGGCAA (for, p)	3' end of *nad2* intron 4
LB237	GAGAGGACTCAGCTGTTAGT (for, s)	3' end of *nad2* intron 4
LB409	GGCTGTATCACATCGAGATG (rev, n)	5' end of *nad2* intron 4
cox2		
LB287	CTGTGGTGCCGATAGATTCA (rev, c, n)	5' end of intron
LB240	CCGAGAAGAGGTATGTGTAC (for, s)	5' end of intron
LB288	ATAAGAGTAGGCGTGGAGAG (for, p)	3' end of intron
LB678	AAGTCACTGCTTCTACGACG (rev, c, n)	3' end of *cox2b*
LB81	CAACACCTATGATGCAAGG (for, p)	5' end of *cox2a*
ccmFN-rps1		
LB468	TGAGACATTATGGCTTTGGG (rev, c)	5' end of *ccmFN*
LB440	TCAGACAAAACCGAGCTTGG (for, p)	3' end of *rps1*
LB388	TGAAGAAGATTTGGCGAACG (for, s)	3' end of *rps1*

Figure 2.2: Schematics illustrating positions of oligomers used in this study

Genes of interest are identified by coloured boxes. Direction of oligomers designated by arrows with oligomer name above.


nad1
LB777
LB630
LB24
LB95
LB640
LB65
LB800
trnP
nad1a
LB821
LB112
LB18
LB808
LB294
LB120
LB112
LB818
LB309
LB799
atp6
rps13
nad1b
nad1c
LB764
nad1d
LB765
LB766
matR
nad1e
nad2
LB218
LB355
LB210
LB219
LB209
LB596
nad2a
nad2b
LB409
LB416
LB235
LB543
LB555
LB237
nad2c
nad2d
nad2e
cox2
LB287
LB678
LB881
LB240
LB288
cox2a
cox2b
ccmFN-rps1
LB440
LB468
LB388
ccmFN
rps1
1 kb

2.3 *Bioinformatics*

Plant mitochondrial genomes used in sequence comparisons with wheat were accessed from the NCBI databank using the following accession numbers: Tobacco (*Nicotiana tabacum*) – NC_006581; Clubmoss (*Huperzia squarrosa*) – NC_017755; Cycad (*Cycas taitungensis*) – NC_010303. Characterization of RNA secondary structures was carried out using mfold v3.2 (http://mfold.rna.albany.edu/?q=mfold).

Chapter 3 Results: Impact of low temperature on splicing of atypical group II introns in wheat mitochondria

3.0 Comments

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3.1 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^{o}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.

Keywords: wheat, mitochondria, RNA splicing, group II intron, cold, ribozyme, intron evolution

3.2 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 (Vogel and Börner, 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* (Molina-Sanchez *et al*., 2006; Li-Pook-Than and Bonen, 2006) 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; Köhler *et al*. 2010; Koprivova *et al*. 2010; Liu *et al*. 2010; Kühn *et al*. 2011; Keren *et al*., 2012; Francs-Small *et al*., 2012; 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 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.

3.3 Materials and methods

3.3.1 *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 hours or at room temperature for 18 hours followed by 72 hours at 4 ^{o}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^{o}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 (~5μg) 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 10X RNA ligase buffer (New England Biolabs) for 30 min at 37 ^{o}C followed by phenol extraction and overnight ethanol precipitation. Oligomers used in the

(CR)RT-PCR analysis of excised introns are shown in Table 3.1. 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 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 3.1.

3.3.2 ***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);

Table 3.1: 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

5’ CTCAAAATGAGCCTTGCGAC 3’ (*nad1* intron 2); 5’ CTTCCGTGAAAATTCAGAC 3’ (*nad2* exon 2); 5’ CGCACATTCATAATAGCGTT 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).

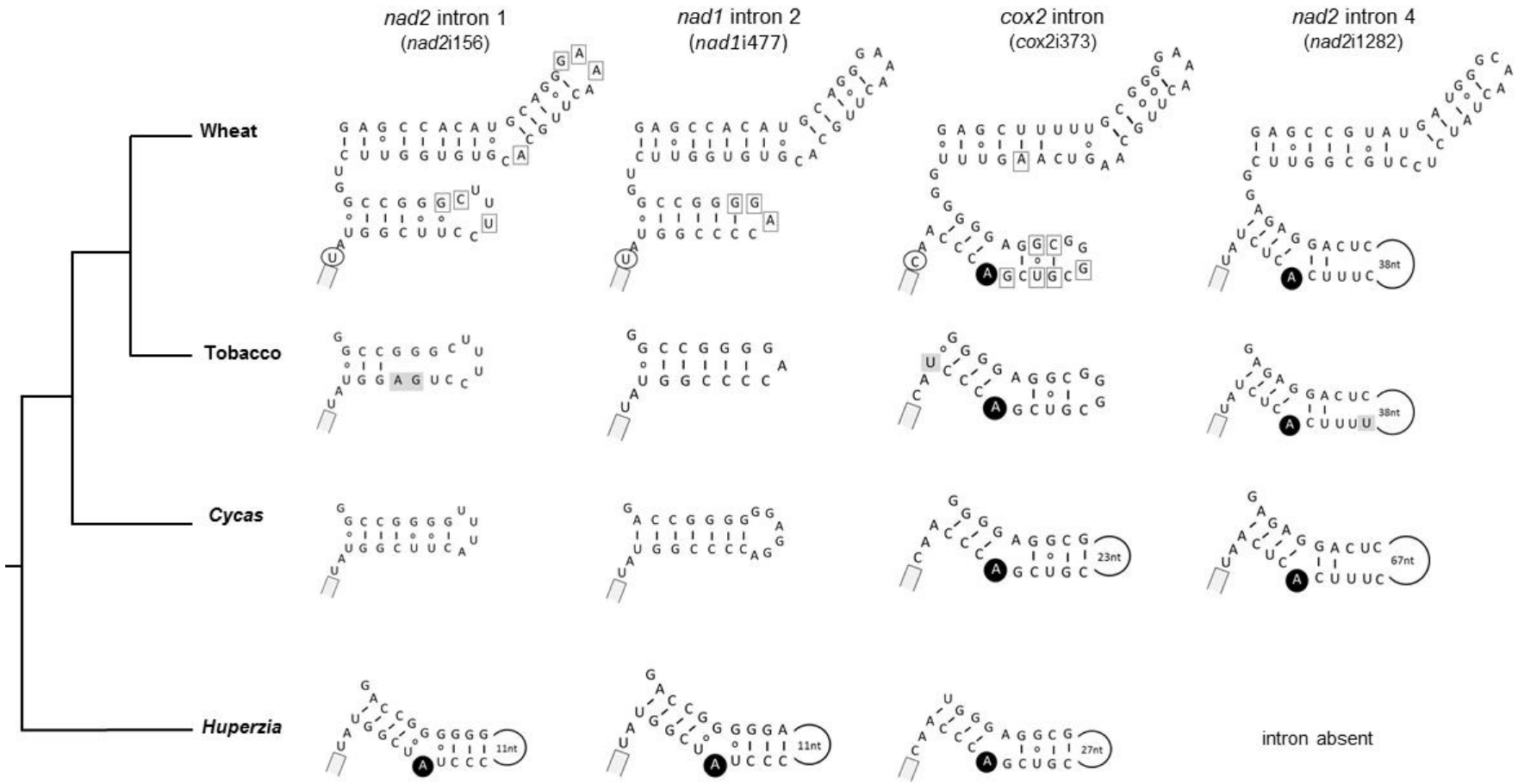
3.4 Results

3.4.1 *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 3.1). 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 3.1, 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) in the nomenclature of Dombrovska and Qiu, 2003) but it is not an orthologue of the wheat one (*cox2*i373). Figure 3.1 also shows the domain VI regions from the orthologous introns in the gymnosperm *Cycas taitungensis* and a lycophyte (club moss) *Huperzia squarrosa*. Notably, the introns in *Huperzia squarrosa*,

Figure 3.1: Domain V/VI secondary structure models for the four wheat mitochondrial group II introns in this study and comparison with orthologues from divergent vascular plant lineages.

Schematic of relationships among wheat (monocot), tobacco (eudicot), *Cycas* (gymnosperm) and *Huperzia* (lycophyte, club moss) are shown at left. Bulged adenosines at the conventional position 7-8 nt upstream of the 3' splice site are shown with solid black circles. Shaded nucleotides on the tobacco sequence identify positions that differ from wheat. Boxed nucleotides denote extent of 3' region missing from excised intron molecules and circled pyrimidines denote full-length introns (see Table 2 and text). Data bank sources of the nucleotide sequences are AP008982 (wheat), BA000042 (tobacco), AP009381 (*Cycas*), and JQ002659 (*Huperzia*). The intron nomenclature of Dombrovska and Qiu (2004) is shown below the commonly-used terms. Orthologues of *nad2* intron 1 and the *cox2* intron are present in the more distantly related non-vascular plant *Physcomitrella patens* (AB251495) and they conform to the conventional domain VI structure.


nad2 intron 1
(nad2i156)
nad1 intron 2
(nad1i477)
cox2 intron
(cox2i373)
nad2 intron 4
(nad2i1282)
Wheat
Tobacco
Cycas
Huperzia
11nt
11nt
23nt
38nt
38nt
67nt
27nt
intron absent

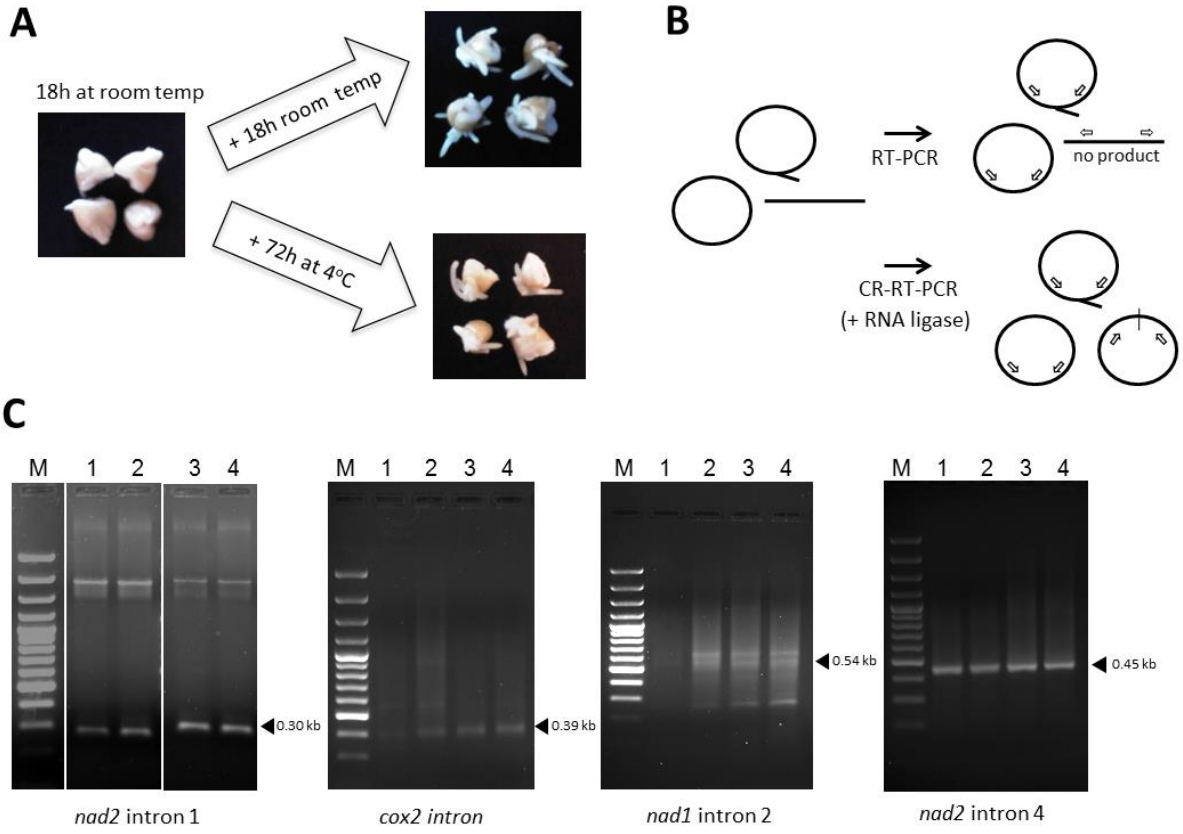
which belongs to an early-diverging vascular plant lineage, possess classical group II intron dVI features with a branchpoint adenosine located at the expected position. This comparison suggests that early in vascular plant evolution, certain mitochondrial group II introns were no longer constrained to the classical ribozyme structure and acquired various idiosyncratic features. Interestingly, *nad2* intron 1 and *nad1* intron 2 share considerable sequence similarity throughout their core domains I-VI (Lippok *et al*., 1994). This includes ~50 identical nucleotides stretching from the dIV helix into dVI, so it seems possible that gene conversion events during evolution may have contributed to their unusual domain VI features.

3.4.2 *Shift to novel forms of excised mitochondrial introns upon cold exposure of wheat embryos*

To assess whether splicing biochemistry is affected by environmental temperature, we examined the behaviour of these three introns in wheat embryos germinated in the dark at room temperature and 4^{o}C, and we also included *nad2* intron 4, which has a classical dVI structure (Fig 3.1) and thus was expected to use lariat-type splicing. The embryo developmental stage was chosen because our earlier studies on *nad7* splicing had shown that excised intron levels were relatively higher in embryos than in seedlings (Carrillo and Bonen, 1997). Cold growth conditions were selected so that embryos would reach a comparable morphological stage to ones maintained at room temperature for 36 h, as judged by the emerging radicle length. More specifically, after 18 h at room temperature, embryos were transferred to 4^{o}C for an additional 72 h (Fig 3.2A). To distinguish between excised introns that have a linear rather than circularized structure *in vivo* and to determine the nature of the junction sequences, we used (CR)RT-PCR strategies (Fig3.2B) as in our earlier studies

Figure 3.2: RT-PCR and CR-RT-PCR products for excised intron junctions from wheat embryos germinated at room temperature or subjected to cold treatment.

A. Dissected germinating embryos showing stage of mitochondrial RNA isolation. **B.** Schematic of (CR)RT-PCR strategy. By using RT-PCR, *in vivo* circularized/lariat intron RNA molecules can be identified, whereas if the RNA is pretreated with RNA ligase (CR-RT-PCR), linear intron molecules can also be detected. Arrows designate primers for cDNA synthesis and PCR. **C.** Representative gels of RT-PCR and CR-RT-PCR products for *nad2* intron 1, *cox2*, *nad1* intron 2 and *nad2* intron 4. In each case, lane 1: room temp RT-PCR, lane 2: room temp CR-RT-PCR, lane 3: cold RT-PCR, lane 4: cold CR-RT-PCR, and M: DNA size ladder. Black arrowheads designate approximate size expected for excised introns. Other amplicons were identified as spurious products by nested PCR experiments and excluded from further study.


A
18h at room temp
+ 18h room temp
+ 72h at 4°C
B
RT-PCR
no product
CR-RT-PCR
(+ RNA ligase)
C
M 1 2 3 4
0.30 kb
nad2 intron 1
M 1 2 3 4
0.39 kb
cox2 intron
M 1 2 3 4
0.54 kb
nad1 intron 2
M 1 2 3 4
0.45 kb
nad2 intron 4

(Li-Pook-Than and Bonen, 2006). By pre-treating samples with RNA ligase, linear excised introns can thus be detected. Two or more independent RNA preparations were used for each environmental condition and representative profiles for RT-PCR (without RNA ligase) and CR-RT-PCR (pretreatment with RNA ligase) are shown in Figure 3.2B. Although only very low amounts of RT-PCR products could be seen in the case of *cox2* and *nad1* intron 2 at room temperature (Fig3.2C,lanes 1), they were gel-purified and subjected to further analysis.

The resulting sequence data from clones of the RT-PCR and CR-RT-PCR products are shown in Table 3.2. In brief, it can be seen that *nad2* intron 1, *nad1* intron 2 and the *cox2* intron are not excised as lariats, whereas *nad2* intron 4, exhibits the expected lariat form under both environmental conditions, as exemplified by the absence of 6 nts at the 3' end for both ligase-treated and untreated RNAs. All the *nad2* intron 4 clones have T instead of A at the branchpoint reflecting mis-incorporation at the 2'-5' bond position during cDNA synthesis, as is commonly observed for group II intron lariats (Vogel and Börner, 1997). In contrast, for the three introns with non-conventional dVI structures, although the correct 5' splice site is present in all cases, the 3' regions show heterogeneity. For room temperature conditions, virtually all the CR-RT-PCR clones contain non-encoded adenosines at the intron junction consistent with a hydrolytic pathway in which linear molecules are subsequently 3' tagged with adenosines for the bacterial-type degradation pathway (Lange *et al*., 2009). Because these excised introns are all full-length, it suggests that polyadenylation is a rapid step. In contrast, for cold-treated embryos, very few clones have such adenosine tracts, and there is a higher representation of ones lacking heterogeneous lengths at the 3' end, as well as full-length *in vivo* circularized forms.

For *nad2* intron 1, most of the excised introns detected in cold-treated embryos are circularized molecules lacking heterogeneous stretches (10-61 nts) at the 3' end. Their

Table 3.2: Sequence analysis of cloned (CR)RT-PCR intron junction regions for mitochondrial nad2 intron 1, cox2, nad1 intron 2 and nad2 intron 4 from room temperature and cold-treated wheat embryos.

RNA-ligase pre-treated or untreated samples are denoted by +L and –L, respectively. The Δ values reflect number of nucleotides missing from 3' terminus. Underlined regions correspond to lariat tail length expected for conventional group II introns. Genomically-encoded sequences within shaded rows are shown in bold. Lower case nucleotides indicate flanking nad1c exon sequence.

Intron	Growth Condition	Clone	Ligase	3′ Terminus	Non-encoded nucleotides	5′ Terminus	Growth Condition	Clone	Ligase	3′ Terminus	Non-encoded nucleotides	5′ Terminus
***nad*2 intron 1**				**…GCCGGGCTTTCCTTCGGTAT**		**GCGCGCCTAGGA…**				**…GCCGGGCTTTCCTTCGGTAT**		**GCGCGCCTAGGA…**
	Room Temp	2/8	-L	Δ41		GCGCGCCTAGGA…	**Cold**	3/8	-L	Δ40		GCGCGCCTAGGA…
		2/8	-L	Δ39		GCGCGCCTAGGA…		1/8	-L	Δ39		GCGCGCCTAGGA…
		1/8	-L	Δ32		GCGCGCCTAGGA…		1/8	-L	Δ32		GCGCGCCTAGGA…
		2/8	-L	…GCCGGG		GCGCGCCTAGGA…		1/8	-L	…GCCGGGC		GCGCGCCTAGGA…
		1/8	-L	…GCCGGGCTTT		GCGCGCCTAGGA…		2/8	-L	…GCCGGGCTTT		GCGCGCCTAGGA…
		1/11	+L	…GCCGGGC		GCGCGCCTAGGA…		1/8	+L	Δ61		GCGCGCCTAGGA…
		2/11	+L	…GCCGGGCTTTCCTTCGGTAT	A	GCGCGCCTAGGA…		1/8	+L	Δ41		GCGCGCCTAGGA…
		1/11	+L	…GCCGGGCTTTCCTTCGGTAT	AA	GCGCGCCTAGGA…		2/8	+L	Δ41	CCTAC	GCGCGCCTAGGA…
		1/11	+L	…GCCGGGCTTTCCTTCGGTAT	AAA	GCGCGCCTAGGA…		1/8	+L	…GCCGGGC		GCGCGCCTAGGA…
		1/11	+L	…GCCGGGCTTTCCTTCGGTAT	AAAA	GCGCGCCTAGGA…		2/8	+L	…GCCGGGCTTTCCTTCGGTAT		GCGCGCCTAGGA…
		1/11	+L	…GCCGGGCTTTCCTTCGGTAT	AAAAAA	GCGCGCCTAGGA…		1/8	+L	…GCCGGGCTTTCCTTCGGTAT	AAAAA	GCGCGCCTAGGA…
		2/11	+L	…GCCGGGCTTTCCTTCGGTAT	AAAAAAA	GCGCGCCTAGGA…						
		1/11	+L	…GCCGGGCTTTCCTTCGGTAT	AAACAAA	GCGCGCCTAGGA…						
		1/11	+L	…GCCGGGCTTTCCTTCGGTAT	AAAAAAAA	GCGCGCCTAGGA…						
***cox*2**				**…GAGGCGGGCGTCGACCCAAC**		**GTGCGCCTCTTA…**				**…GAGGCGGGCGTCGACCCAAC**		**GTGCGCCTCTTA…**
	Room Temp	1/5	-L	…GAGG		GTGCGCCTCTTA…	**Cold**	1/8	-L	…GAGG		GTGCGCCTCTTA…
		2/5	-L	…GAGGC		GTGCGCCTCTTA…		2/8	-L	…GAGGCGGG		GTGCGCCTCTTA…
		2/5	-L	…GAGGCGGGCGTCGACCCAAC		GTGCGCCTCTTA…		2/8	-L	…GAGGCGGGCG		GTGCGCCTCTTA…
								1/8	-L	…GAGGCGGGCGT		GTGCGCCTCTTA…
								2/8	-L	…GAGGCGGGCGTCGACCCAAC		GTGCGCCTCTTA…
		2/5	+L	…GAGGCGGGCGTCGACCCAAC	AAAA	GTGCGCCTCTTA…		1/8	+L	Δ29		GTGCGCCTCTTA…
		1/5	+L	…GAGGCGGGCGTCGACCCAAC	AAAAAA	GTGCGCCTCTTA…		1/8	+L	…GAGGCGGG		GTGCGCCTCTTA…
		1/5	+L	…GAGGCGGGCGTCGACCCAAC	AAAAAAAAA	GTGCGCCTCTTA…		1/8	+L	…GAGGCGGGCGT		GTGCGCCTCTTA…
		1/5	+L	…GAGGCGGGCGTCGACCCAAC	AAAAAAAAAA	GTGCGCCTCTTA…		1/8	+L	…GAGGCGGGCGTCG		GTGCGCCTCTTA…
								3/8	+L	…GAGGCGGGCGTCGACCCAAC		GTGCGCCTCTTA…
								1/8	+L	…GAGGCGGGCGTCGACCCAAC	AAA	GTGCGCCTCTTA…
***nad*1 intron 2**				**…CTGGCCGGGGACCCCGGTAT**		**GTGCGCCTTGTG…**				**…CTGGCCGGGGACCCCGGTAT**		**GTGCGCCTTGTG…**
	Room Temp	3/8	-L	…CTGGCCGGGGACCCCGGTAT		GTGCGCCTTGTG…	**Cold**	1/8	-L	…CTGGCCGGG		GTGCGCCTTGTG…
		5/8	-L	…CTGGCCGGGGACCCCGGTAT	actgt	GTGCGCCTTGTG…		3/8	-L	…CTGGCCGGGG		GTGCGCCTTGTG…
								1/8	-L	…CTGGCCGGGGA		GTGCGCCTTGTG…
								3/8	-L	…CTGGCCGGGGACCCCGGTAT		GTGCGCCTTGTG…
		3/8	+L	…CTGGCCGGGGACCCCGGTAT	A	GTGCGCCTTGTG…		5/8	+L	…CTGGCCGGGGACCCCGGTAT		GTGCGCCTTGTG…
		3/8	+L	…CTGGCCGGGGACCCCGGTAT	AAA	GTGCGCCTTGTG…		1/8	+L	…CTGGCCGGGGACCCCGGTAT	AA	GTGCGCCTTGTG…
		1/8	+L	…CTGGCCGGGGACCCCGGTAT	AAAA	GTGCGCCTTGTG…		1/8	+L	…CTGGCCGGGGACCCCGGTAT	AAA	GTGCGCCTTGTG…
		1/8	+L	…CTGGCCGGGGACCCCGGTAT	AGAAAA	GTGCGCCTTGTG…		1/8	+L	…CTGGCCGGGGACCCCGGTAT	AAAAAAA	GTGCGCCTTGTG…
***nad*2 intron 4**				**…GGTGGACCCTTTCACTCTAT**		**GGGCGGCCGGAA…**				**…GGTGGACCCTTTCACTCTAT**		**GGGCGGCCGGAA…**
	Room Temp	4/4	-L	…GGTGGACCCTTTCT		GGGCGGCCGGAA…	**Cold**	4/4	-L	…GGTGGACCCTTTCT		GGGCGGCCGGAA…
		4/4	+L	…GGTGGACCCTTTCT		GGGCGGCCGGAA…		4/4	+L	…GGTGGACCCTTTCT		GGGCGGCCGGAA…

circularized status *in vivo* as well as abundance in the RNA population is supported by the (CR)RT-PCR gel profiles (Fig 3.2B), and contrasts with the room temperature experiments where linear, oligoadenylated excised intron forms predominate. For the ligase-treated samples from cold-grown embryos, 3 out of 8 clones represented full-length introns, but only one had non-encoded adenosines compared to 10/11 at room temperature. Potential splicing pathways to generate these novel excised intron forms are discussed below.

The *cox*2 intron in cold-treated embryos also showed a shift away from the hydrolytic splicing pathway seen at room temperature to the generation of *in vivo* circularized molecules that either lack heterogeneous lengths (9-29 nts) at their 3' ends or are full-length (Table 3.2). Notably for the ligase-untreated samples, full-length forms were seen under both environmental conditions (2/5 clones and 2/8 clones, respectively) reflecting their presence as circles *in vivo*. For the ligase-treated samples from the cold, 4/8 clones represented full-length introns, one of which can be attributed to a hydrolytic pathway since it contained non-encoded adenosines. The other 3 clones could be the result of *in vivo* circularization and/or a hydrolytic pathway in which the linear excised intron did not undergo subsequent polyadenylation. The absence of lariats and the presence of heterogeneous *cox2* excised intron forms at room temperature is in keeping with our previous analysis (Li-Pook-Than and Bonen, 2006).

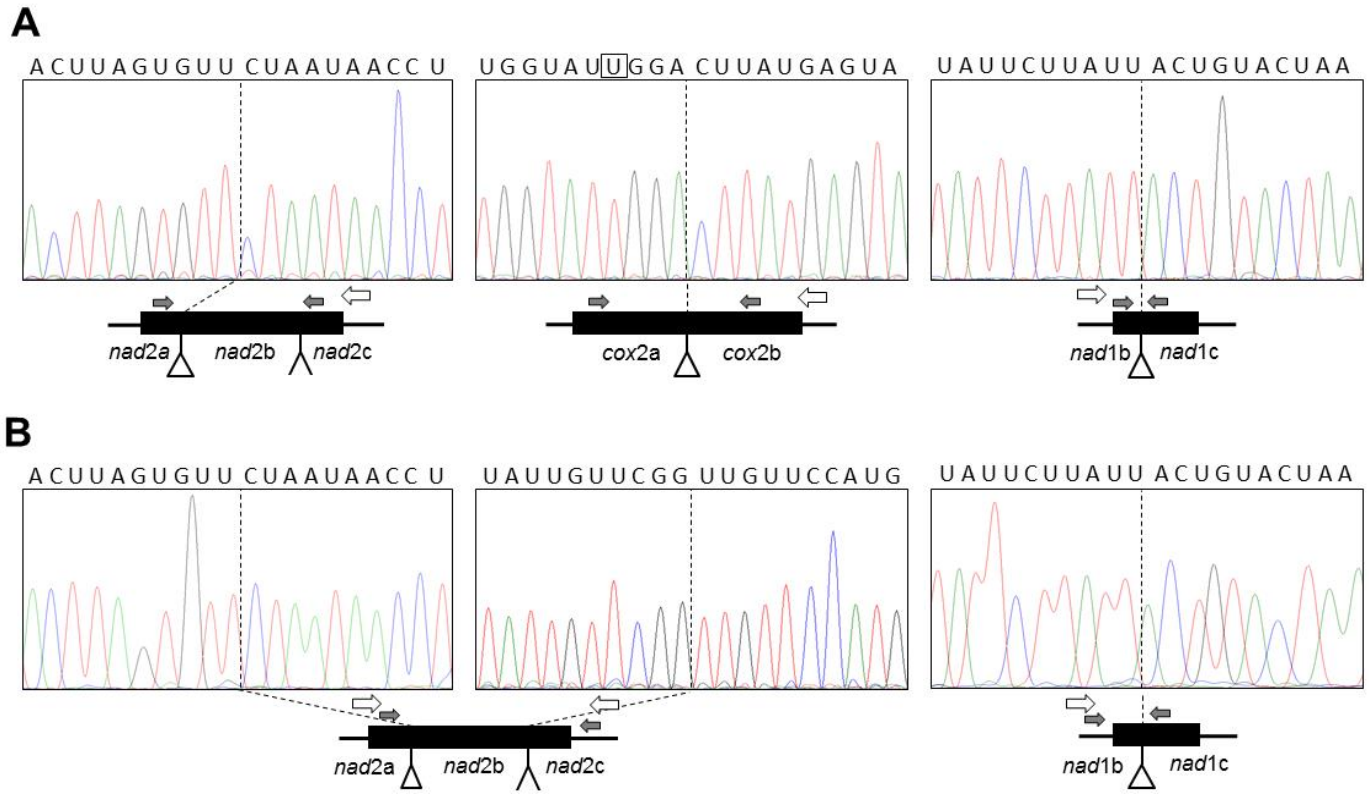
In the case of *nad1* intron 2, which has a short tight dVI helix, a somewhat less pronounced shift in physical form was seen between the environmental conditions. Clones from ligase-treated RNA revealed full-length linear introns with non-encoded adenosines in both cases, namely 8/8 clones at room temperature and 3/8 with cold treatment. Full-length *in vivo* circularized forms are also present in the population, based on the outcome of the non-ligase-treated RNA experiments (3/8 clones in both cases). However, the latter are

expected to be a very minor species in the room temperature population since the amount of RT-PCR product obtained was consistently very low (Fig 3.2B) and none were seen in the ligase-treated RNA experiments (Table 3.2). The remaining 5/8 clones from room temperature experiments contained not only the full intron but also 5 nt of the downstream exon so must be nonproductive mis-spliced products, as previously noted (Li-Pook-Than and Bonen, 2006). In contrast, for the cold-treated embryos, the remaining 5/8 clones were *in vivo* circularized forms that lack 9-11 nts from the 3' end of the intron, that is, they map to the distal end of the dVI loop (Fig 3.1, boxed nts).

As a test of the possibility that the heterogeneous 3'-incomplete introns arose from mis-spliced products in which intron stretches were incorporated between the two exons, we monitored the status of the spliced exon RNA population by direct sequencing of RT-PCR products generated using primers mapping to flanking exons (Fig 3.3A). Only correctly spliced exon junctions were observed under both environmental conditions (room temperature data not shown) and no visible differences in the level of background "noise" were seen on opposite sides of the junction position. This was also the case for our tests of partially-spliced *nad2* and *nad1* transcripts (Fig 3.3B). Defective molecules may however be present at very low levels because of rapid selective turnover. The chromatograms for *cox2* also illustrate complete editing in the RNA population of a site 4 nts upstream of the exon-exon junction under both environmental conditions (Fig 3.3, boxed nt). This site is known to be important for splicing through its base-pairing interaction with IBS1 (intron binding site) in domain I of the intron (Farré and Araya, 2002) and was shown to be under-edited in *cox2* precursor molecules from wheat and rice seedlings grown in the cold (Kurihara-Yonemoto and Handa, 2001; Kurihara-Yonemoto and Kubo, 2010; Choi and Bonen, unpublished observations).

Figure 3.3: Chromatograms of directly-sequenced RT-PCR products for nad2, cox2 and nad1 exon/exon junctions from cold-treated wheat embryos.

A. Status of splice junctions using primers mapping to exons. **B.** Status of spliced junctions in nad1 and nad2 precursor RNAs using intron primers. Vertical dashed lines denote exon-exon junctions. Schematics illustrate positions of primers (grey arrows) used to generate RT-PCR products and direction of sequencing (white arrows). Triangles and Y-shaped symbols denote *cis*- and *trans*- introns, respectively. Boxed nucleotide represents editing site (-4) within cox2 IBS1 (see text).

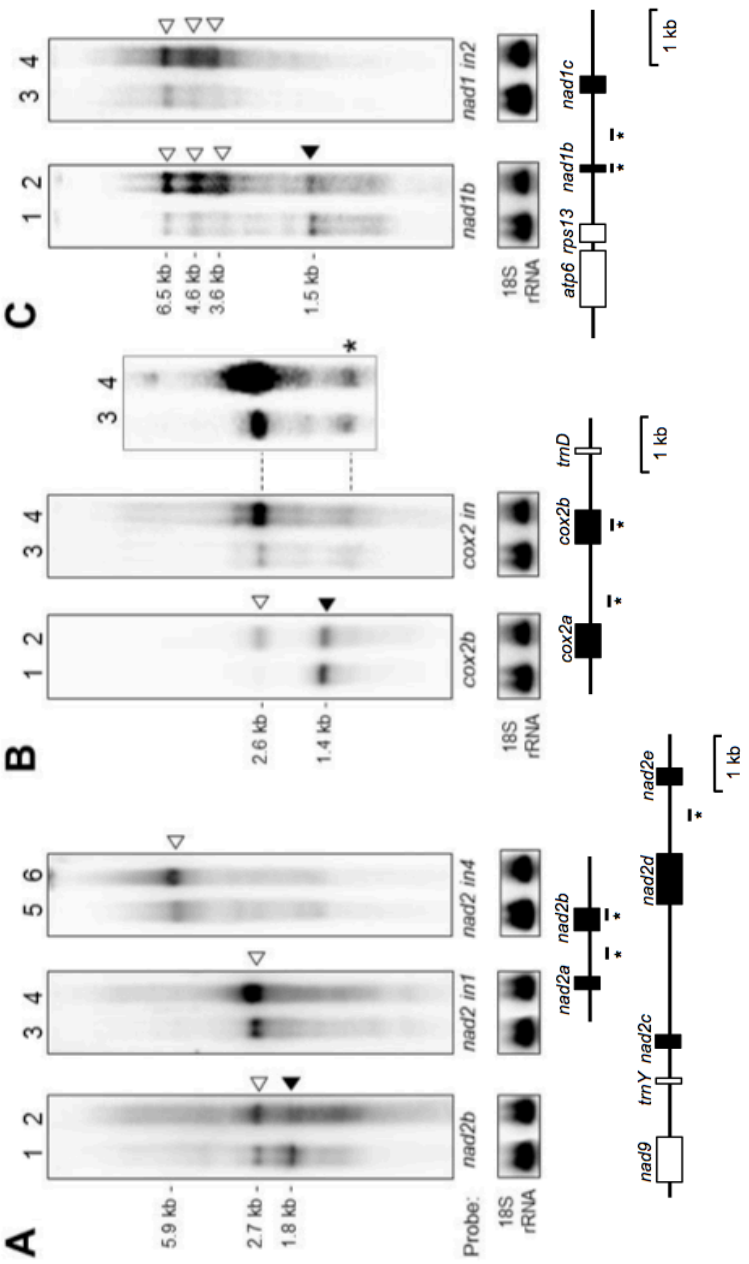

A
A C U U A G U G U U C U A A U A A C C U
U G G U A U U G G A C U U A U G A G U A
U A U U C U U A U U A C U G U A C U A A
nad2a
nad2b
nad2c
cox2a
cox2b
nad1b
nad1c
B
A C U U A G U G U U C U A A U A A C C U
U A U U G U U C G G U U G U U C C A U G
U A U U C U U A U U A C U G U A C U A A
nad2a
nad2b
nad2c
nad1b
nad1c

3.4.3 *Intron-containing precursor RNAs are elevated in the cold*

The steady-state levels of *nad1*, *nad2*, and *cox2* intron-containing species and their respective mRNAs were examined by northern hybridization as shown in Figure 3.4. For all three genes, intron-containing precursors are notably higher in the cold compared to mature mRNA levels. This suggests that there is a greater bottleneck at the post-transcriptional than transcriptional level with cold treatment. Because *nad1* and *nad2* are complex genes, with several cis- and trans-introns, it is not unexpected that high molecular weight precursors are elevated in the cold as the kinetics of bimolecular reactions would be expected to be reduced. The sizes of mature mRNAs shown in Figure 3.4 are in keeping with previous estimates for *nad2* (Morawala-Patell *et al*., 1998), *nad1* (Farré *et al*. 1999; Choi *et al*., 2012), and *cox2* (Bonen *et al*., 1984; Choi *et al*., 2012). In the case of the *nad2* trans-splicing gene, both loci give rise to elevated levels of a single major precursor transcript in the cold, and the 5.9 kb transcript is predicted to include the *trnY* gene located upstream from the third exon of *nad2* (Fig 3.4A). Similarly, the *nad1b*-intron2-*nad1*c region is co-transcribed with the upstream *atp6-rps13* genes and undergoes subsequent end-processing (cf. Choi *et al*., 2012 and 6.5 kb RNA species in Figure 3.4C). For all three genes, the levels of excised introns are not easily detected in northerns, although with longer exposure, signals can be seen for *cox2* (Fig 3.4B, asterisk). The low level of *nad2* intron 4 which has a conventional lariat excised intron (Table 3.2) contrasts with the abundant signals seen for *nad7* excised introns from wheat embryo RNA germinated at room temperature (Carrillo *et al*., 2001). It is worth noting that there is a heterogeneous background signal at the position of the mature *nad1* and *nad2* mRNAs, even though direct sequencing of exon-exon RT-PCR products (Fig 3.3) showed no

Figure 3.4: RNA blot analysis for the wheat mitochondrial genome regions containing the introns in this study for room temperature and cold-germinated embryos.

A. *nad2* introns 2 and 4 **B**. *cox2* and **C**. *nad1* intron 2. Room temperature RNA (odd lanes) and cold RNA (even lanes) are shown with size markers on the left and re-probing of blots with mitochondrial 18S rRNA oligomer below. Black and white arrowheads denote mRNA and intron-containing precursors, respectively. Schematics show exons of interest (black boxes) as well as neighbouring genes (white boxes). The positions of 20'mer hybridization probes are indicated by asterisks. Longer exposure of *cox2* intron hybridization is shown in panel B inset. Three representative blots were successively probed (blot 1: *nad2* intron 1 and *nad2b*; blot 2: *cox2b*, *cox2* intron and *nad1b*; blot 3: *nad2* intron 4 and *nad1* intron 2). Note: Position of *nad1b* probe has been corrected in schematic compared to Dalby and Bonen, 2013. Identical profiles are observed from hybridizations of *nad1b* and *nad1c* probes (Y. Chapdelaine, Ph.D. Thesis).


A
B
C
1 2
3 4
5 6
5.9 kb
2.7 kb
1.8 kb
2.6 kb
1.4 kb
6.5 kb
4.6 kb
3.6 kb
1.5 kb
Probe:
18S rRNA
nad2b
nad2 in1
nad2 in4
cox2b
cox2 in
nad1b
nad1 in2
nad9
trnY
nad2c
nad2a
nad2b
nad2d
nad2e
cox2a
cox2b
trnD
atp6
rps13
nad1b
nad1c
1 kb

evidence for mis-spliced RNA species, at least for the junctions under study here, and this raises the possibility that altered mRNA structure might compromise stability.

3.5 Discussion

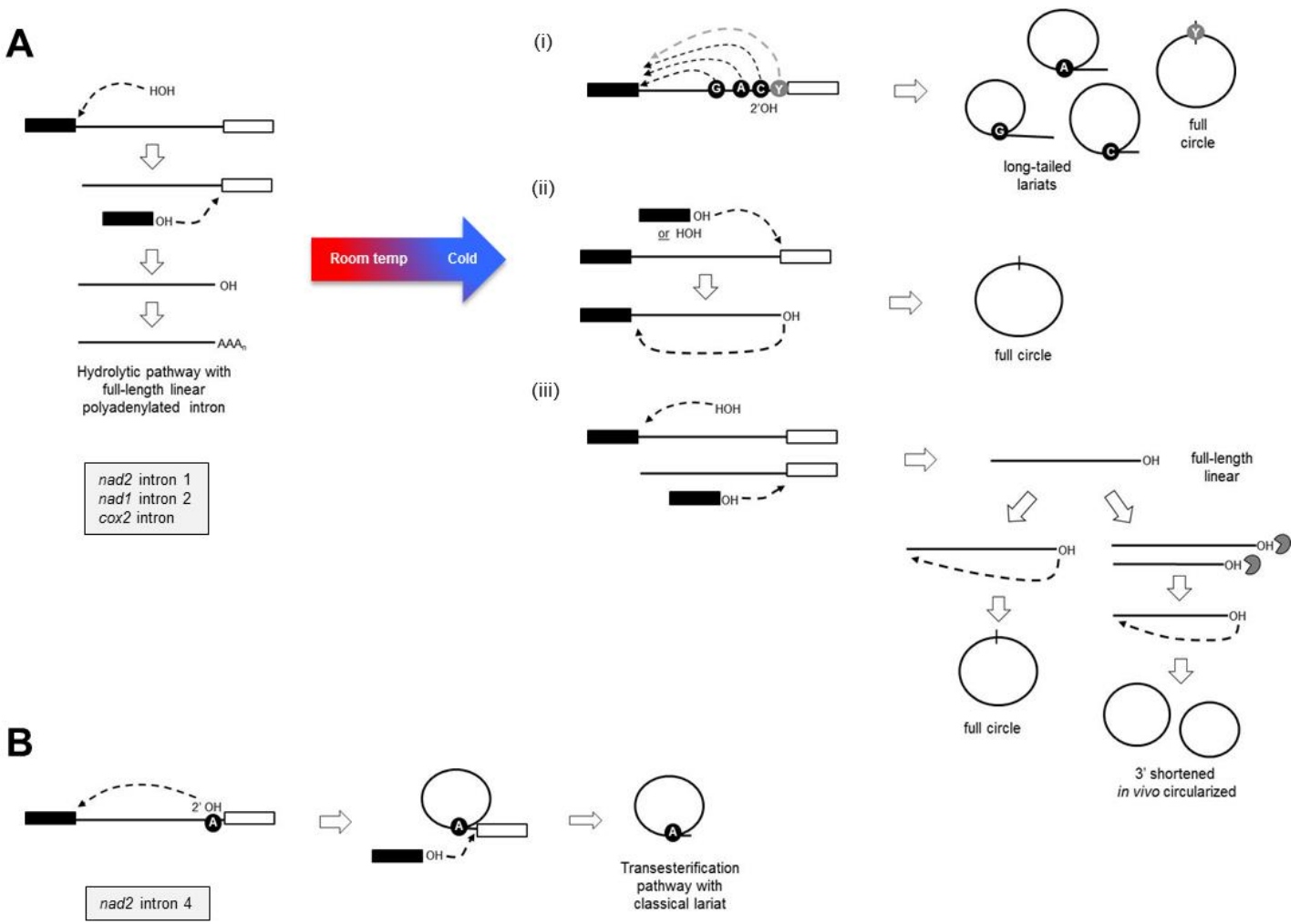
Our investigation of the impact of cold treatment on the splicing of wheat mitochondrial group II introns has revealed pronounced differences between the behaviour of introns with a conventional branchpoint structure and those that have acquired unusual domain VI features over evolutionary time. The former, which includes *nad2* intron 4 (Table 3.2) as well as others (eg. *nad4* introns 1 and 3, data not shown), exhibited classical lariat-type splicing regardless of whether embryos had been germinated at room temperature or in the cold. In contrast, the three non-conventional introns examined in this study (*nad2* intron 1, *nad1* intron 2 and *cox2*) shifted from a predominantly hydrolytic pathway at room temperature to alternative pathways in the cold. The majority of excised intron forms are consistent with *in vivo* circularized molecules that are either full-length or lack heterogeneous stretches at their 3' ends. Interestingly, the latter map primarily to unpaired sites within the distal loops of domains V or VI (Fig 3.1, boxed nts) suggesting accessibility of such positions in the splicing reaction. It seems unlikely that these excised intron species reflect mis-splicing by-products since direct sequencing of RT-PCR products across exon-exon junctions revealed no evidence for the presence of heterogeneous extra stretches (Fig 3.3), in contrast to what was seen for certain *nad5* splicing pathways (Elina and Brown, 2010). A markedly lower proportion of clones from the cold-treated embryos contained non-encoded adenosines, and this could reflect an increased efficiency of the bacterial-type degradation pathway. In this regard, it is notable that Arabidopsis plants show elevated expression of the mitochondrial PNPase (polynucleotide phosphorylase) gene when subjected to cold treatment, while mitochondrial splicing factors appear to exhibit variable responses (cf. www.ebi.ac.uk/gxa/).

That said, the abundance of RT-PCR products in the absence of RNA ligase treatment (Fig 3.2C) supports a model in which there is a shift to a higher frequency of full-length in vivo circularized molecules in the cold.

Taken together, our observations indicate novel, yet productive, splicing pathways used by these aberrantly-structured introns in the cold (Fig 3.5A) unlike those with conventional bulged adenosine in dVI (Fig 3.5B). A scenario in which the first step of splicing is mediated by nucleophiles that are located upstream of the conventional branchpoint position and attack the 5' splice site (Fig 3.5A) would result in "long-tailed" lariats. Similarly, full-length circular introns would be generated if the 3' terminal nucleotide of the intron provided the reactive nucleophile, as documented for nuclear group I introns (Nielsen *et al*., 2003). In keeping with this model, the invariant presence of the correct 5' splice site for all three unusual introns examined in this study (Table 3.2, total of 93 clones) suggests that it assumes a reactive conformation which has been described as a sharp "kink" (Marcia and Pyle, 2012) and is correctly recognized, albeit apparently by a variety of different nucleophiles which vary between the two environmental conditions. For such a model, however, it is somewhat curious that the genomically-encoded nucleotide was always observed at the positions of the putative 2'-5' junctions (Table 3.2), unlike the situation for classic lariats where the bulged A is typically mis-read as T by reverse transcriptase (Vogel and Börner, 2002). An alternative model compatible with the absence of heterogeneous stretches from the 3' end of the intron involves a hydrolytic pathway where the linear excised introns are vulnerable to 3' exoribonuclease prior to *in vivo* circularization. Such a model however invokes reduced activity for polyadenylation enzymes but not for exonucleases. On the other hand, if one were to consider a scenario analogous to group I intron splicing, a liberated linear intron might be circularized if attacked by the 2'OH at the 3' end of the intron (Nielsen and

Figure 3.5: Models of major splicing pathways for wheat mitochondrial group II introns in this study.

A. Hydrolytic pathway in which linear excised introns are polyadenylated at room temperature. In the cold, heterogeneous physical forms are seen and may arise through pathways using (i) cryptic internal nucleophiles within domains V and VI to yield long-tailed lariat (black circles) or full-length (grey circles) *in vivo* circularized excised forms, (ii) external nucleophiles that attack the 3' splice site to yield full-length circles *in vivo* (eg. liberated upstream exon, Murray et al., 2001), and/or (iii) the same hydrolytic pathway as at room temperature, but where linear molecules are not polyadenylated, and/or undergo *in vivo* circularization, which if preceded by 3' exonuclease attack would yield shortened circular molecules. **B.** Conventional two-step transesterification pathway in which excised introns have a lariat structure at room temperature and in the cold. Non-productive pathways (i.e. excised *nad1* intron 2 containing downstream exon nts, Table 2) are not included in this figure.


A
HOH
OH
OH
AAAn
Hydrolytic pathway with full-length linear polyadenylated intron
nad2 intron 1
nad1 intron 2
cox2 intron
Room temp
Cold
(i)
G A C Y
2'OH
long-tailed lariats
full circle
(ii)
OH
or HOH
OH
full circle
(iii)
HOH
OH
OH
full-length linear
OH
OH
OH
OH
full circle
3' shortened in vivo circularized
B
2' OH
A
OH
A
Transesterification pathway with classical lariat
nad2 intron 4

Johansen, 2009; Fig 3.5A). Group II intron circles are also observed in yeast mitochondria and have been attributed to a liberated upstream exon attacking the 3' splice site (Murray *et al*., 2001; Fig 3.5A). Given our observation that variable lengths of dV/VI are missing in the excised introns, perhaps it is also pertinent to note that *in vitro* studies of a bacterial self-splicing group IIC intron revealed the autocatalytic removal of sequences within domain VI by the highly reactive intron core, and such "biting off its own tail" was attributed to the dynamic behaviour of domain VI (Pyle, 2010).

Our study provides the first *in vivo* demonstration of the consequences of cold growth conditions on the splicing of naturally-occurring group II introns which have atypical domain VI structures. Their behaviour at room temperature is in keeping with earlier insights gained from *in vitro* self-splicing studies using mutated group II ribozymes. For example, mutagenesis within domain VI of a yeast mitochondrial group IIB intron established that alterations such as deletion of the branchpoint adenosine or removal of its bulged conformation led to hydrolytic, instead of lariat splicing (Chu *et al*., 1998; Chu *et al*., 2001). Moreover, mutational analysis of a brown algal mitochondrial self-splicing group IIB intron has shown that even if the bulged adenosine is present, but the distal region of domain VI is truncated, the pathway shifts from lariat to hydrolysis (Li *et al*., 2011a). In this regard, it is notable that the domain VI lengths of the mitochondrial introns in this study are considerably shorter than their orthologues in distantly-related plants like the club moss *Huperzia* (Fig 3.1) or compared to conventional group II introns elsewhere in wheat mitochondrial genes (cf. Fig 3.1, *nad2* intron 4). Plant mitochondrial group II introns are particularly well-known for their unusual evolutionary pathways, but there are also reports of other naturally-occurring group II introns that lack a bulged adenosine in domain VI and whose splicing pathway has been examined. For example, the plant chloroplast *trnV* intron is spliced by a

hydrolytic pathway (Vogel and Börner, 2002), as are bacterial group IIB1 introns which possess an insert preceding the conventional 5'GUGYG 3' consensus sequence at the 5' end of the intron (Li *et al*., 2011b).

For the germination of seeds at low temperature, it is expected that the kinetics of mitochondrial mRNA maturation steps will be less efficient due to lower activity of the enzymes involved, in addition to the reduced import of nuclear-encoded machinery into the mitochondrion. This is supported by the relative increase in intron-containing precursor RNAs compared to mature mRNAs seen in the cold. In addition, low temperature may well impede folding of introns into the elaborate, yet dynamic, conformational structures needed for splicing competency. It is anticipated that poorly-structured introns require additional splicing machinery to stabilize folding and compensate for defective features, so that splicing in the cold may be further compromised. Other factors that may contribute to elevated levels of precursor RNAs in the cold include reduced effectiveness of bimolecular associations needed for trans-splicing, the status of distal introns within a common transcript, or other RNA processing events such as mRNA end-maturation or intron editing. It is worth noting that none of the introns in this study possess candidate editing sites to restore helical structure within domain V/VI. If abnormal structures are favoured in the cold, this might also decrease accessibility of the 3' termini of linear introns to exonucleases or to adenosine-tailing for degradation, consistent with our observations that the degree of polyadenylation is reduced in the cold. Interestingly, this is reminiscent of a conceptually-related phenomenon seen at high temperature, where there was increased 3' tailing of mitochondrial mRNAs in Arabidopsis (Adamo *et al*., 2008).

Finally, it is intriguing that the vast majority of the group II introns in flowering plant mitochondria are within genes encoding subunits of NADH dehydrogenase, that is, complex

I of the respiratory chain, which can be bypassed by alternative pathways under stress conditions. For example, under cold stress conditions, nuclear-encoded external NAD(P)H dehydrogenase transcripts are up-regulated (Armstrong *et al*., 2008). This raises the possibility that post-transcriptional events in the mitochondrion, like splicing, play a regulatory role in plants under stress conditions.

3.6 Acknowledgements

We thank Dr. R. Pandeya (Agriculture and Agri-Food Canada, Ottawa) for kindly providing the wheat seeds used in this study, and the financial support of Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged.

3.7 Chapter 3 addendum

3.7.1 *Shift in splicing biochemistry at low temperature is not attributed to developmental differences*

Given the impact of low temperature on the rate of germination, as evidenced by the increased time required for cold-treated wheat embryos to reach the same phenotype as those at room temperature, an important consideration was whether the observed shift in the predominant splicing pathway employed by aberrant mitochondrial group II introns was indeed caused by the cold treatment and not simply due to differences in stage of development. This was a particularly critical question to resolve in light of apparent differences in the efficiency of RNA processing observed for certain wheat mitochondrial genes during early germination when *de novo* transcription seems to outpace mRNA maturation (Li-Pook-Than *et al*., 2004). To address this issue, I examined the physical forms of the excised *nad2* intron 1 from mtRNA isolated 4 hours post-imbibition from wheat

embryos germinated at room temperature for comparison with data from my earlier cold study.

(CR)RT-PCR products of 4 hour wheat *nad2* intron 1 are shown in Figure 3.6A. Consistent with 36 hour room temperature data, appreciably less RT-PCR product is generated than by CR-RT-PCR (+RNA ligase) suggesting the latter corresponding RNA species are more abundant. Sequences of clones of these products are shown in Figure 3.6B. All 4 clones of the CR-RT-PCR products are full-length with variable lengths of 3' non-encoded A tails. Of the 4 clones of the RT-PCR products, all are 3' heterogeneous missing lengths between 11 and 18 nt. These results mirror those for 36 hour room temperature wheat embryos and support the cold as having a direct impact on splicing biochemistry.

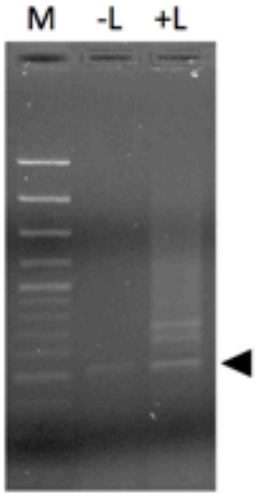
3.7.2 *Activity of polyadenylation enzymes does not appear impacted by cold treatment*

Increased detection at low temperature of excised forms of *nad2* intron 1, *nad1* intron 2 and the *cox2* intron by CR-RT-PCR that were either full-length or were missing heterogeneous stretches from the 3' end and lacked non-encoded A tails raises the possibility that the activity of polyadenylation enzymes is reduced by cold treatment. Given that reduced 3' accessibility of intron structure may be a compounding factor, I approached this question by examining the 3' terminus of the mitochondrial *ccmFN-rps1* bicistronic mRNA in the cold that has been shown to have short A-tails in room temperature germinated wheat embryos (Calixte and Bonen, 2008). Non-encoded 3' A tails identified from clone sequence analysis (Fig 3.6C) suggests that the activity of polyadenylation enzymes is the same in the cold as it is at room temperature. These findings, taken together with the detection of 3' heterogeneous species by RT-PCR (-RNA Ligase; Chapter 3), supports these species as being the product of an alternative splicing pathway that generates *in vivo* circularized forms.

Figure 3.6: Examination of the physical form of excised nad2 intron 1 from 4-hour room temperature germinated wheat embryos and 3' polyadenylation status of ccmFN-rps1 bicistronic mRNA in the cold

[A] Representative gel of RT-PCR (-L) and CR-RT-PCR products for the *nad2* intron 1 excised intron junction. M denotes DNA size ladder. Black arrow designates position of correct product. Additonal amplicons were identified as spurious by nester PCR and excluded from further study. Primers used in generation of *nad2* intron 1 excised junction products differ from those used in Chapter 3. **[B]** Sequence analysis of clones of RT-PCR (-L) and CR-RT-PCR (+L) products. Genomic DNA sequence shown in bold. **[C]** Sequence analysis of 5' and 3' terminal ends of *ccmFN-rps1* bicistronic mRNA from cold-treated wheat embryos.

A



B

Ligase	Clone	3' Terminus	Non-encoded nucleotides	5' Terminus
		GCCGGGCTTTCCTTCGGTAT		**GCGCGCCTAGGAGGGC**
-L	1/4	GC		GCGCGCCTAGGAGGGC
-L	1/4	GCCGGG		GCGCGCCTAGGAGGGC
-L	1/4	GCCGGGCTT		GCGCGCCTAGGAGGGC
-L	1/4	GCCGGGCTTT		GCGCGCCTAGGAGGGC
+L	1/4	GCCGGGCTTTCCTTCGGTAT	A	GCGCGCCTAGGAGGGC
+L	1/4	GCCGGGCTTTCCTTCGGTAT	AA	GCGCGCCTAGGAGGGC
+L	1/4	GCCGGGCTTTCCTTCGGTAT	AAAAAAA	GCGCGCCTAGGAGGGC
+L	1/4	GCCGGGCTTTCCTTCGGTAT	AAAAAAAA	GCGCGCCTAGGAGGGC

C

Clone	5' end . . .	. . . 3' end	Non-encoded nucleotides
	ATTGGGATCATCCTGTGGT	**CCACCTTAATCCATTTTGT**	
1/2	ATTGGGATCATCCTGTGGT	CCACCTTAATCCATTTTGT	AAA
1/2	ATTGGGATCATCCTGTGGT	CCACCTTAATCCATTTTGT	AAAAA

Chapter 4 Results: Hydrolytic *trans*-splicing of the wheat mitochondrial *nad1* intron 1 correlated with unusual 5' terminal insert

4.0 Comments

This chapter has been written in manuscript format for future publication. Following observations of an apparent shift in splicing biochemistry with cold treatment of atypical *cis*-splicing introns *nad1* intron 2, *nad2* intron 1 and the *cox2* intron, it was of further interest to examine the physical excised forms of the highly degenerate *trans*-splicing *nad1* intron 1 in the cold and at room temperature. To expand my analysis of the impact of low temperature growth on RNA processing, end-maturation events of the *nad1a*-intron 1a locus were investigated through end-mapping of precursor transcripts that are elevated relative to mature mRNA by cold treatment. This work demonstrates an idiosyncratic response in splicing biochemistry of aberrant plant mitochondrial group II introns and provides evidence for reduced efficiency of mRNA end-maturation in the cold.

4.1 Abstract

The *trans*-splicing *nad1* intron 1 in wheat mitochondria deviates from the conventional dI and dVI structure of group II introns. We identify and characterize two distinct linear halves (*nad1* intron 1a + *nad1* intron 1b) consistent with hydrolytic splicing in *trans*. Notably, although germination of wheat embryos at 4^{o}C reduced the efficiency of splicing compared to room temperature as seen in earlier studies, heterogeneous Y-branch and/or "broken circle" physical forms consistent with alternative splicing pathways previously characterized for other aberrant wheat mitochondrial group II introns were not detected. We further characterize precursor transcripts of the *nad1a*-intron 1a locus that are elevated in the cold relative to processed mRNA and provide evidence for decreased efficiency of end-maturation, including processing of an upstream tRNA-Pro gene, at low temperature. Our study of *nad1* intron 1 *trans*-splicing suggests idiosyncratic plasticity of splicing biochemistry among atypical wheat mitochondrial group II introns.

Keywords: *trans*-splicing, group II intron, cold, plant mitochondria, end-maturation

4.2 Introduction

Fragmentation of plant mitochondrial genes by DNA rearrangements within group II introns is one characteristic of the high level of recombination observed for these organellar genomes (Bonen, 2008). In such cases, generation of mature mRNAs requires splicing of the bipartite intron in *trans*. Excision of group II introns is mediated by elaborate folding of six highly conserved domains (dI-dVI) facilitated by many long-range basepair interactions (Lambowitz and Zimmerly, 2010). Critical to splicing are dI, the scaffold that is the foundation of the intron tertiary structure that further contains the conserved 5' terminal GUGYG sequence and exon-binding sites (EBSs) that recognize flanking exons, and dV, that is the crux of the catalytic core and contains the AGC triad and AY bulge that bind catalytically important Mg^{++} ions. An additional central feature of conventional splicing is a branchpoint adenosine residue within dVI that is positioned at the catalytic active site to attack the 5' exon-intron junction and initiate the first of two sequential transesterification reactions culminating in an excised lariat intron. Alternatively, water can act as the initiating nucleophile and results in excision of the intron in a linear form (Vogel and Börner, 2002). In *trans*-splicing, long-range basepair interactions are thought to facilitate folding between the respective "half-introns" to generate competent splicing structures that may be further mediated by RNA chaperones (Bonen, 2008). Given that the *trans*-intron is not contiguous, classic splicing culminates in a Y-branch physical form.

In flowering plants, respiratory genes *nad1*, *nad2* and *nad5,* that encode subunits of complex I (NADH dehydrogenase), all contain bipartite introns that are broken within dIV, with the exception of the tri-partite *nad5* intron 3 in *Oenothera* that has an additional break in dI (Knoop *et al*., 1997). As demonstrated by *in vivo* study of the Ll.LtrB group II intron in the Gram-positive bacterium *Lactococcus lactis*, group II introns have a high tolerance for

fragmentation throughout the six domains with breaks within EBSs, dV, at 5' and 3' splice sites or in close proximity to the bulged adenosine as the only cases in which *trans*-splicing in not observed (Belhocine *et al*., 2008). Of the bipartite introns in flowering plants, the fragmentation of *nad1* intron 1 is thought to have been the earliest *trans*-event during evolution (Malek and Knoop, 1998). Interestingly, *nad1* intron 1 also has the distinction of being the most highly variable within dVI among flowering plants orthologues of any mitochondrial group II intron, and a bulged adenosine is absent at the expected position (Carrillo *et al*., 2001). In wheat mitochondria, *in vivo* splicing of *nad1* intron 2 and the *cox2* intron, that deviate from the conventional branchpoint structure of dVI, predominantly generates linear molecules consistent with first-step hydrolysis, in addition to several novel *in vivo* circularized forms (Li-Pook-Than and Bonen, 2006). Interestingly, the frequency of these latter forms appears to increase during low temperature growth conditions indicative of a shift in splicing biochemistry to alternative splicing pathways (Dalby and Bonen, 2013). Variability between *nad1* intron 1 flowering plant orthologues further extends to the editing status of dVI, in which a position 6 nt upstream of the 3' terminus is edited in wheat, pea and soybean but not in *Arabidopsis* or tobacco (Carrillo *et al*., 2001). Intron editing within dVI of the wheat *nad1* intron 4 and *nad5* intron 2 is essential to *trans*-splicing and thought to confer increased stability on the secondary structure required in proper positioning of the bulged adenosine for first-step catalysis (Farré *et al*., 2012).

In this present work, we characterize the physical form of the *trans*-spliced mitochondrial *nad1* intron 1 from wheat embryos germinated at room temperature and at 4^{o}C. Two distinct linear half-introns were identified that provide the first *in vivo* demonstration of hydrolytic *trans*-splicing of a group II intron. Notably, no heterogeneous Y-branch or "broken circle" forms consistent with alternative splicing pathways were

detected under either growth condition. Moreover, the frequency of *nad1* intron 1b molecules with 3' non-encoded A tails were virtually identical between room temperature and 4^{o}C that suggests the bacterial-type degradation pathway is not impacted by cold treatment. Polyadenylation of the 3' terminus of the predominant species detected for *nad1* intron 1a was not observed indicating limited accessibility at this end.

4.3 Methods

Prior to imbibition, wheat embryos (*Triticum aestivum* cv. FT Wonder, a cold-hardy winter wheat cultivar) were surface-sterilized with 1% sodium hypochlorite and dissected to remove the majority of the endosperm tissue. Embryos were then germinated in the dark at room temperature for 36 hours or at room temperature for 18 hours followed by 72 hours at 4 ^{o}C. Isolation of mitochondrial RNA was performed as previously described (Li-Pook-Than *et al*., 2004).

For detection of potential linear, heterogeneous Y-branch and/or open circle forms, (CR)RT-PCR strategies were performed as in our earlier studies (Li-Pook-Than and Bonen, 2006; Dalby and Bonen, 2013). CR-RT-PCR was further used to identify and characterize precursor transcripts of the *nad1a*-intron 1a locus. For all the RNA analyses conducted in this study, multiple independent mitochondrial RNA preparations were used.

CR-RT-PCR products of precursor transcripts and excised half introns were gel-purified using UltraClean 15 (MoBio Laboratories Inc) and corroborated by nested PCR. Spurious amplicons were further mitigated by use of "touchdown" PCR conditions. Products were subsequently cloned into pGemT-Easy (Promega) plasmid vectors for sequencing. Sequencing was performed by StemCore Laboratories at the Ottawa Health Research Institute (OHRI). Oligomers used in this study are shown in Table 4.1.

Table 4.1 : Oligomers used in (CR)RT-PCR and northern 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 1**		
	CGAGGCTAGGCAAACTCAGC (rev, c, n)	5' end of intron
	GTAGATCGTTCAACACCTGC (rev, c)	5' end of intron
	GACTTCCACTTCTCGATCCC (rev, c)	5' end of intron
	TGATGATGTCTGCTGGTTGG (for, p)	3’ end of intron 1a
	GAAGCTGTCGCTTGACGGAC (rev, c)	3’ end of intron 1b
	TATCCGCTTGGGACTGATAG (rev, c, n)	3’ end of intron 1b
	TGGGTATGATATTCTCGTGG (for, p)	3’ end of intron 1b
***nad1a*-intron 1a**		
	AAGGCTACTCCTAGTAGAAG (rev, n)	5' end of *nad1a*
	CACTTGACTAAGCATAAGCG (rev, c)	5' *nad1* UTR
	TGATGATGTCTGCTGGTTGG (for, p)	3' end of intron 1a

4.4 Results

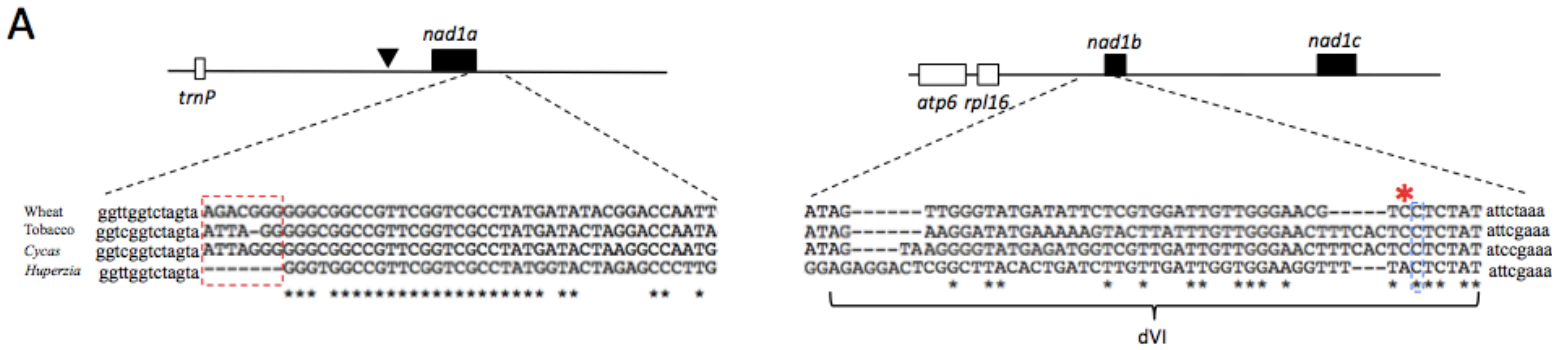
4.4.1 *Hydrolytic splicing of nad1 intron 1 in wheat is consistent with aberrant nature of domain VI*

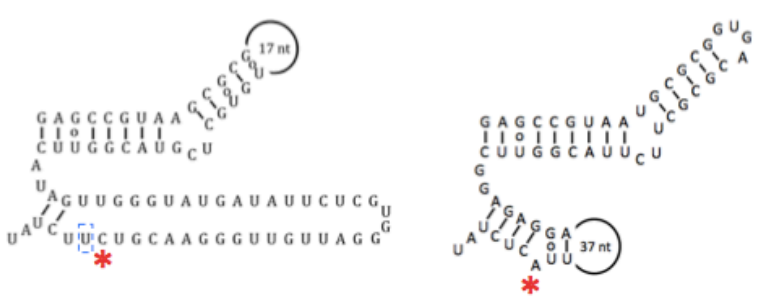
Orthologues of *nad1* intron 1 from available flower plant mitochondrial genomes as well as that of the gymnosperm *Cycas taitungensis* are *trans*-splicing in contrast to that of the early-diverging lycophyte, *Huperzia squarrosa*, which is a *cis*-splicing intron. Alignment of core domains identified a highly unusual 6-7 nt insert located at the 5' splice site in all *trans*-splicing orthologues that is absent in *H.squarrosa* (Fig 4.1A). Furthermore, a branchpoint adenosine within a conventional domain VI helix is still present in *H.squarrosa*, in contrast to the other species surveyed. The correlation between the presence/absence of the 5' insert and branchpoint adenosine has been recently observed for a subset of group IIB1 introns in fungal mitochondria (Li *et al*., 2012). A comparison of the structural features of domains V and VI between wheat and *H.squarrosa* (Fig. 4.1B) identified that, while the highly conserved dV is typical in both species save for an abnormally large distal loop in wheat (17 nt vs 4 nt classically), basepairing within the core region of dVI surrounding the classic position of the branchpoint adenosine is virtually absent in the wheat orthologue. This stands in contrast to other aberrant group II introns in wheat that have retained variable degrees of stability within the proximal stem of dVI (Li-Pook-Than and Bonen, 2006).

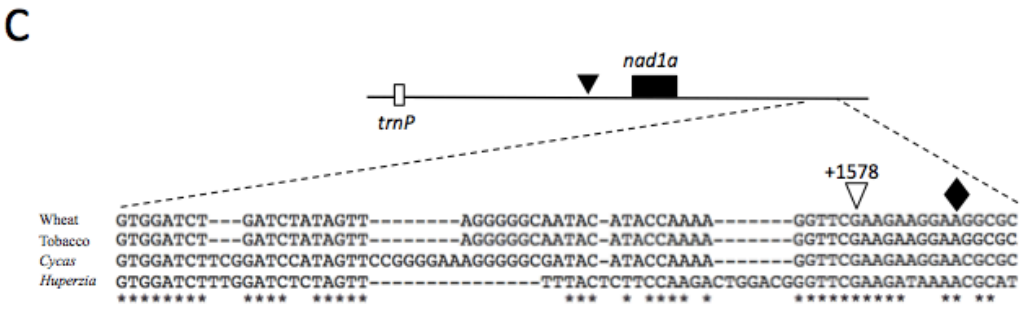
Sequence data from clones of CR-RT-PCR products for the excised *nad1* intron 1 is shown in Table 4.2. As demonstrated in Figure 4.2A, RT-PCR products indicative of heterogeneous Y-branch or "broken circle" forms were not observed even with a 5-fold increase in RNA template. RT-PCR was repeated using 3 different oligomers in cDNA synthesis and several different mtRNA preparations and no products were generated (data

Figure 4.1: Comparison of the 5' and 3' ends of nad1 intron 1 plant orthologues.

A. Sequence alignment of the 5' splice site and dVI among wheat, tobacco, *Cycas taitungensis* and the lycophyte *Huperzia squarrosa*. Conserved nucleotides and position of conventional branch adenosine designated by black and red asterisks, respectively. 5' insert shown in red dashed box. Blue dashed box denotes C-U editing site present in some plants (Carrillo *et al*., 2001). Flanking exon sequence is shown in lower case. **B**. Domain V/VI secondary structures of *nad1* intron 1 in wheat and *Huperzia*. **C**. Sequence alignment of 3' terminal region of *nad1* intron 1a. Position of 3' end of major species observed and breakpoint in homology between orthologues designated by white triangle and black diamond, respectively. Schematics are based on the wheat mitochondrial genome and show *nad1* exons (black boxes) as well as neighbouring genes (white boxes). Position of the 5' end of *nad1* mRNA designated by black triangle in panels A and C.


A
nad1a
trnP
nad1b
nad1c
atp6 rpl16
Wheat ggttggtctagtaAGACGGGGGGCGGCCGTTCGGTCGCCTATGATATACGGACCAATT
Tobacco ggtcggtctagtaATTA-GGGGGCGGCCGTTCGGTCGCCTATGATACTAGGACCAATA
Cycas ggtcggtctagtaATTAGGGGGGCGGCCGTTCGGTCGCCTATGATACTAAGGCCAATG
Huperzia ggttggtctagta-------GGGTGGCCGTTCGGTCGCCTATGGTACTAGAGCCCTTG
ATAG------TTGGGTATGATATTCTCGTGGATTGTTGGGAACG-----TCCTCTAT attctaaa
ATAG------AAGGATATGAAAAAGTACTTATTTGTTGGGAACTTTCACTCCTCTAT attcgaaa
ATAG----TAAGGGGTATGAGATGGTCGTTGATTGTTGGGAACTTTCACTCCTCTAT atccgaaa
GGAGAGGACTCGGCTTACACTGATCTTGTTGATTGGTGGAAGGTTT---TACTCTAT attcgaaa
dVI


B
17 nt
37 nt


C
nad1a
trnP
+1578
Wheat GTGGATCT---GATCTATAGTT--------AGGGGGCAATAC-ATACCAAAA-------GGTTCGAAGAAGGAAGGCGC
Tobacco GTGGATCT---GATCTATAGTT--------AGGGGGCAATAC-ATACCAAAA-------GGTTCGAAGAAGGAAGGCGC
Cycas GTGGATCTTCGGATCCATAGTTCCGGGGAAAGGGGGCGATAC-ATACCAAAA-------GGTTCGAAGAAGGAACGCGC
Huperzia GTGGATCTTTGGATCTCTAGTT--------------TTTACTCTTCCAAGACTGGACGGGTTCGAAGATAAAACGCAT

not shown). It is worth noting that our inability to detect these species does not entirely preclude their existence and may speak to their rapid turnover or low employment of alternative splicing pathways. On the other hand, detection of two linear halves (*nad1* intron 1a + *nad1* intron 1b; Fig. 4.2B) of the bipartite intron points to hydrolytic *trans*-splicing as the primary mode of splicing at room temperature and in the cold. For *nad*1 intron 1a, of the 16 clones analyzed from the two growth conditions, all but 1 have the correct 5' splice site. Notably, sequence analysis at the 3' end of the intron 1a half identified a high frequency of clones (12/16 between room temperature and cold) that mapped to precisely the same position 1578 nt downstream (+1578) of the 5' exon-intron junction. The 4 additional clones all mapped to within 7 nt downstream of this position. No instances of 3' polyadenylation consistent with bacterial-type tagging for degradation were observed. Sequence comparison with orthologues showed that this region corresponds to a breakpoint in homology between flowering plants, *C. taitungensis* and *H. squarrosa* (Fig. 4.1C: black diamond). Moreover, a 10 nt block that spans from +1573 to +1582 nt is highly conserved and suggests there is some form of functional constraint in this region.

For *nad*1 intron 1b using an oligomer in cDNA synthesis that maps 173 nt upstream of the 3' splice site, the 5' ends of 10/16 clones from room temperature and cold mapped to a position 241 nt upstream of *nad1b* while the remaining 6 clones mapped to within 6 nt downstream of this position. Alignment of this region with orthologous introns, however, did not identify any sequence conservation (data not shown). Greater heterogeneity was observed at the 3' end with 10/16 clones lacking 8-26 nt from dVI and possessed variable lengths of non-encoded terminal adenosines consistent with 3' exonuclease activity and A-tailing. Of clones that had a full-length dVI, 3/6 were polyadenylated including 2 from room temperature and 1 from the cold. Taken together, these comparable levels of bacterial-type

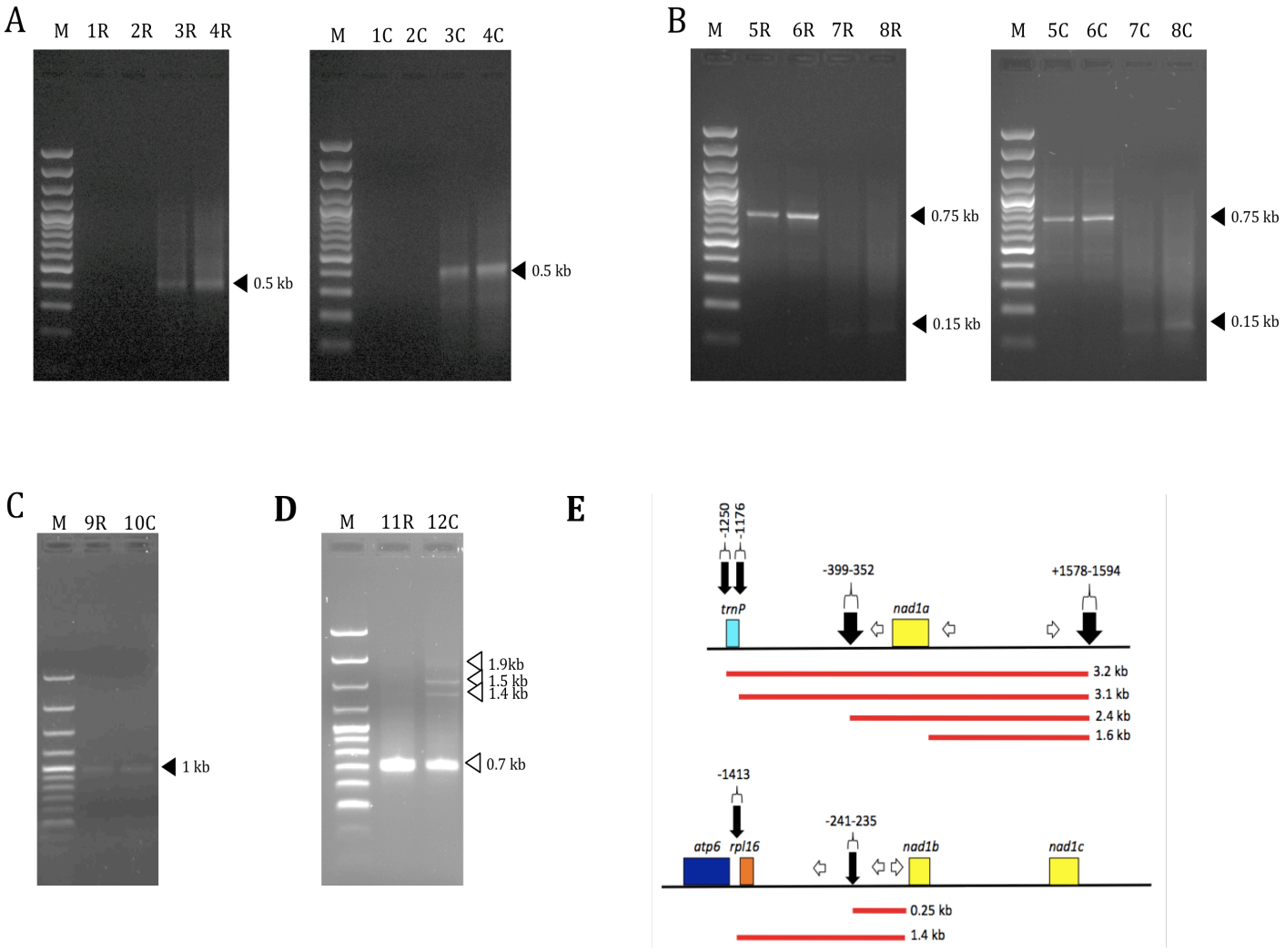
Table 4.2: Sequence analysis of cloned CR-RT-PCR intron junction regions for mitochondrial nad1 intron 1a and nad1 intron 1b from room temperature and cold-treated wheat embryos.

Genomically-encoded sequences are shown within shaded rows. Sequence coordinates relative to flanking nad1 exons are shown in bold.

			+1 5′ end . . .	. . . 3′ end +1578	non-encoded
***nad1* intron 1a**			**A**GACGGGGGGCGGCCGTTCGGTCGCCTATGATATACGGACC . . .	. . . TACATACCAAAAGGTTC**G**AAGAAGGAAGGCGC	
	Room Temp	1/8	(Δ29) CTATGATATACGGACC . . .	. . . TACATACCAAAAGGTTCG	
		2/8	AGACGGGGGGCGGCCGTTCGGTCGCCTATGATATACGGACC . . .	. . . TACATACCAAAAGGTTCGA	
		5/8	AGACGGGGGGCGGCCGTTCGGTCGCCTATGATATACGGACC . . .	. . . TACATACCAAAAGGTTCG	
	Cold	1/8	AGACGGGGGGCGGCCGTTCGGTCGCCTATGATATACGGACC . . .	. . . TACATACCAAAAGGTTCGAAGAAGG	
		7/8	AGACGGGGGGCGGCCGTTCGGTCGCCTATGATATACGGACC . . .	. . . TACATACCAAAAGGTTCG	
			-241 5′ end . . .	**. . . 3′ end -1**	
***nad1* intron 1b**			**G**ACCGGA**C**TTCCACTAGATTTTTATAGGGGTCAGGAGGGAA . . .	. . . TGATATTCTCGTGGATTGTTGGGAACGTCCTCTA**T**	
	Room Temp	1/8	GACCGGA**C**TTCCACTAGATTTTTATAGGGGTCAGGAGGGAA . . .	. . . TGATATTCTCGTG	AAAAAA
		1/8	CGGA**C**TTCCACTAGATTTTTATAGGGGTCAGGAGGGAA . . .	. . . TGATATTCTCGTGGAT	AA
		1/8	GACCGGA**C**TTCCACTAGATTTTTATAGGGGTCAGGAGGGAA . . .	. . . TGATATTCTCGTGGATTGTTG	A
		1/8	CCGGA**C**TTCCACTAGATTTTTATAGGGGTCAGGAGGGAA . . .	. . . TGATATTCTCGTGGATTGTTGGGAA	AAAA
		1/8	ACCGGA**C**TTCCACTAGATTTTTATAGGGGTCAGGAGGGAA . . .	. . . TGATATTCTCGTGGATTGTTGGGAACGTCTTC	AAAAA
		2/8	GACCGGA**C**TTCCACTAGATTTTTATAGGGGTCAGGAGGGAA . . .	. . . TGATATTCTCGTGGATTGTTGGGAACGTCTTCTA**T**	
		1/8	A**C**TTCCACTAGATTTTTATAGGGGTCAGGAGGGAA . . .	. . . TGATATTCTCGTGGATTGTTGGGAACGTCTTCTA**T**	AA
	Cold	1/8	CGGA**C**TTCCACTAGATTTTTATAGGGGTCAGGAGGGAA . . .	. . . TGATATTCT	AAAA
		1/8	GACCGGA**C**TTCCACTAGATTTTTATAGGGGTCAGGAGGGAA . . .	. . . TGATATTCTCGTGGATTGT	AAAAAAA
		1/8	GACCGGA**C**TTCCACTAGATTTTTATAGGGGTCAGGAGGGAA . . .	. . . TGATATTCTCGTGGATTGT	AAAA
		1/8	GGA**C**TTCCACTAGATTTTTATAGGGGTCAGGAGGGAA . . .	. . . TGATATTCTCGTGGATTGTTGG	AA
		1/8	GACCGGA**C**TTCCACTAGATTTTTATAGGGGTCAGGAGGGAA . . .	. . . TGATATTCTCGTGGATTGTTGGGAACG	AAAAA
		1/8	GACCGGA**C**TTCCACTAGATTTTTATAGGGGTCAGGAGGGAA . . .	. . . TGATATTCTCGTGGATTGTTGGGAACGTCTTCTA**T**	
		1/8	GACCGGA**C**TTCCACTAGATTTTTATAGGGGTCAGGAGGGAA . . .	. . . TGATATTCTCGTGGATTGTTGGGAACGTCTTCTA**T**	AAA
		1/8	GACCGGA**C**TTCCACTAGATTTTTATAGGGGTCAGGAGGGAA . . .	. . . TGATATTCTCGTGGATTGTTGGGAACGTCTTCTA**T**	AAAAAAAA
			-1413 5′ end . . .	**. . . 3′ end -1**	
***nad1* intron 1b**			**C**AGCTGCGCTTTTGCAGCACTGAACATGGTTCCGGGTACTCA . . .	. . . TGATATTCTCGTGGATTGTTGGGAACGTCCTCTA**T**	
	Room Temp	1/4	CAGCTGCGCTTTTGCAGCACTGAACATGGTTCCGGGTACTCA . . .	. . . TG	AA
		1/4	CAGCTGCGCTTTTGCAGCACTGAACATGGTTCCGGGTACTCA . . .	. . . TGATATT	AAAAA
		1/4	CAGCTGCGCTTTTGCAGCACTGAACATGGTTCCGGGTACTCA . . .	. . . TGATATTCTCGTGGAT	AA
		1/4	CAGCTGCGCTTTTGCAGCACTGAACATGGTTCCGGGTACTCA . . .	. . . TGATATTCTCGTGGATTGTTGGGAACGTCTTCTA**T**	A
	Cold	1/4	CAGCTGCGCTTTTGCAGCACTGAACATGGTTCCGGGTACTCA . . .	. . . TGATATTCTCGTGGATTG	AAAAAAAAA
		1/4	CAGCTGCGCTTTTGCAGCACTGAACATGGTTCCGGGTACTCA . . .	. . . TGATATTCTCGTGGA	AAAAA
		1/4	CAGCTGCGCTTTTGCAGCACTGAACATGGTTCCGGGTACTCA . . .	. . . TGATATTCTCGTGGATTGTTGGGAACGTCTTCTA**T**	AA
		1/4	CAGCTGCGCTTTTGCAGCACTGAACATGGTTCCGGGTACTCA . . .	. . . TGATATTCTCGTGGATTGTTGGGAACGTCTTCTA**T**	AAAAAAA

Figure 4.2: Representative gels of (CR)RT-PCR experiments for nad1 intron 1 and precursor transcripts of the nad1a-intron 1a locus.

A. RT-PCR. Lanes 1/2: *nad1* intron 1, lanes 3/4: *nad2* intron 1 (control). **B.** CR-RT-PCR. Lanes 5/6: *nad1* intron 1a, lanes 7/8: *nad1* intron 1b. **C.** CR-RT-PCR. Lanes 9/10: 3'*atp6-nad1* intron 1b. **D.** CR-RT-PCR. Lanes 11/12: *nad1a*-intron 1a. "R" and "C" denote room temperature and cold, respectively. Products in lanes 4, 6 and 8 were generated with 2X template in cDNA synthesis as products in lanes 1, 3, 5 and 7. 5X template was used in RT-PCR of lane 2. DNA size ladders designated by "M". Precursor mRNA species and predicted sizes of excised intron products designated by white and black arrowheads, respectively. Other amplicons were identified as spurious products by nested PCR experiments and excluded from further study. **E.** Schematic of precursors transcripts of *nad1a*-intron 1a locus identified by end-analysis of CR-RT-PCR amplicons. Black and white arrows denote positions of precursor termini and oligomers used in (CR)RT-PCR, respectively.


A
M 1R 2R 3R 4R
0.5 kb
M 1C 2C 3C 4C
0.5 kb
B
M 5R 6R 7R 8R
0.75 kb
0.15 kb
M 5C 6C 7C 8C
0.75 kb
0.15 kb
C
M 9R 10C
1 kb
D
M 11R 12C
1.9kb
1.5 kb
1.4 kb
0.7 kb
E
-1250
-1176
trnP
-399-352
nad1a
+1578-1594
3.2 kb
3.1 kb
2.4 kb
1.6 kb
-1413
atp6
rpl16
-241-235
nad1b
nad1c
0.25 kb
1.4 kb

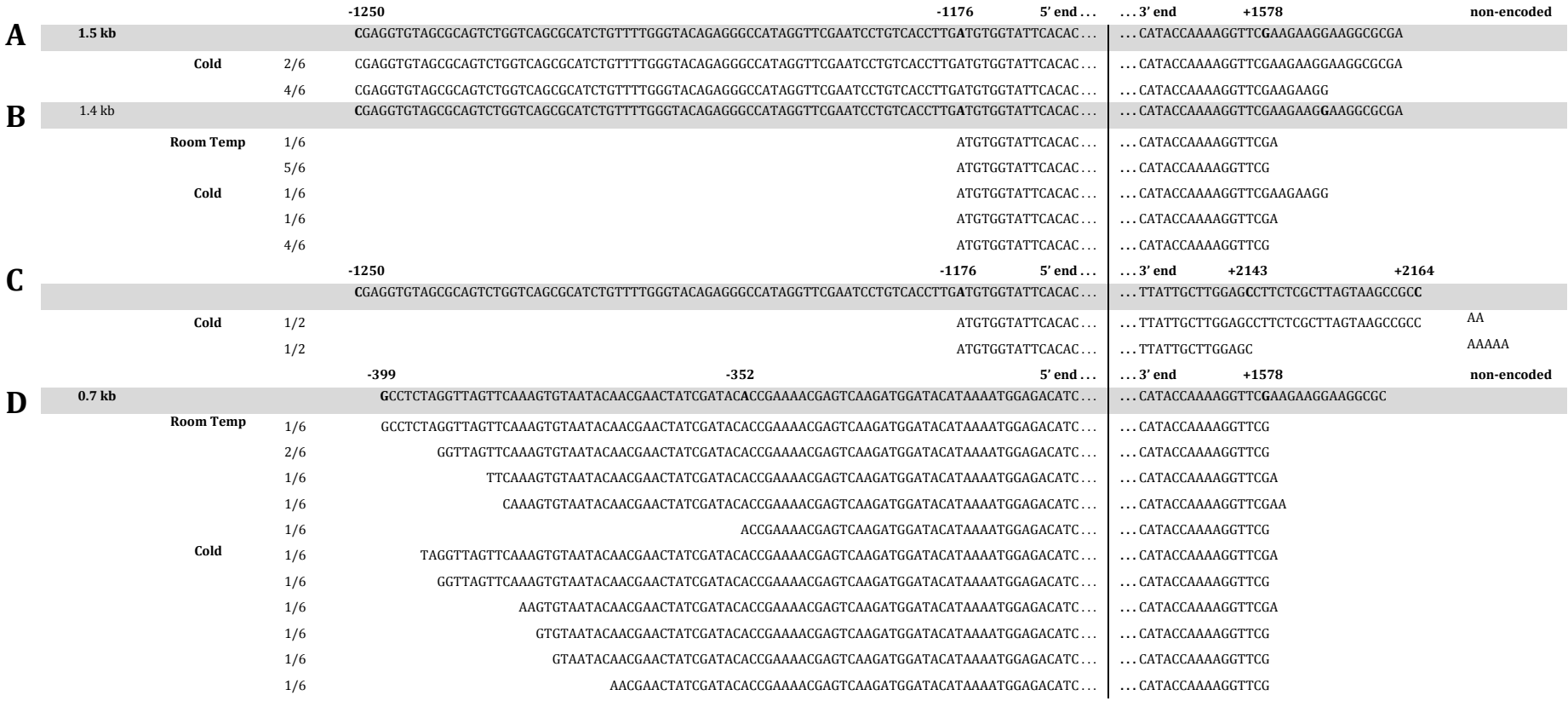
tagging suggest that the accessibility of the 3' end of this particular intron and the activity of the enzymes involved in degradation are unaffected by cold treatment. Interestingly, using a different oligomer in cDNA synthesis that sits 439 nt upstream of the -241 position led to the detection of a higher molecular weight species for this locus (Fig. 2C). All corresponding clones (8/8) had 5' ends that map precisely to the 3' mRNA terminus of the upstream *atp6* and contained variable lengths of 3' non-encoded adenosines of which 5/8 lacked 17-33 nt from the 3' terminus. All *nad1* intron 1b clones that included the C-U editing position located 6 nt upstream of the 3' intron-exon junction were edited suggesting an importance to splicing.

4.4.2 *Cold treatment reduces efficiency of end-maturation*

CR-RT-PCR experiments for end-mapping of RNA transcripts of the *nad1a*-intron 1a locus are shown in Figure 4.2D. From mtRNA isolated from cold-grown wheat embryos, additional high molecular weight amplicons are observed compared to mtRNA from wheat embryos grown at room temperature. These observations are consistent with northern data that show elevated levels of the corresponding RNA species relative to mature mRNA (Fig. 4.3; discussed below). Sequence analysis of clones for these amplicons is shown in Table 4.3. Of the 6 clones for the 1.5 kb product, all 5' ends map precisely to the 5' terminus of the upstream *trnP* gene and, at the 3' end, to either 7 nt (4/6) or 16 nt (2/6) downstream of the end predominantly observed for *nad1* intron 1a (+1578). For the 1.4 kb product, the 5' end of all clones map to the 3' terminus of *trnP* and, at the 3' end, to within 7 nt of +1578, of which 9/12 map directly to this position. The detection of precursor species in the cold that include the upstream *trnP* gene that are not observed at room temperature suggests that the efficiency

Table 4.3: 5' and 3' terminal sequences of the wheat nad1a-intron 1a locus based on CR-RT-PCR data.

Clones represent species shown in Figure 2D: [**A]** 1.5 kb; **[B]** 1.4 kb, **[C]** 1.9 kb, and **[D]** 0.7 kb. Sequence coordinates relative to flanking *nad1* exons are shown in bold.

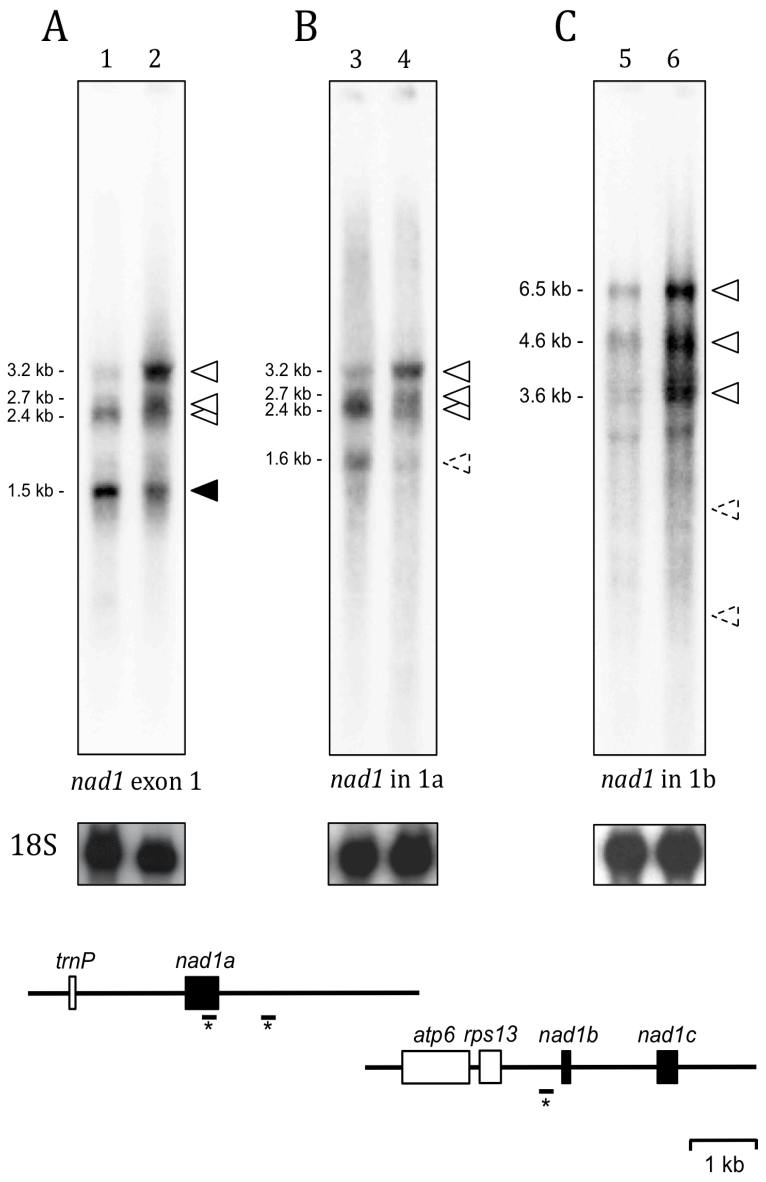

A
-1250
-1176
5′ end . . .
. . . 3′ end
+1578
non-encoded
1.5 kb
CGAGGTGTAGCGCAGTCTGGTCAGCGCATCTGTTTTGGGTACAGAGGGCCATAGGTTCGAATCCTGTCACCTTGATGTGGTATTCACAC . . .
. . . CATACCAAAAGGTTCGAAGAAGGAAGGCGCGA
Cold
2/6
CGAGGTGTAGCGCAGTCTGGTCAGCGCATCTGTTTTGGGTACAGAGGGCCATAGGTTCGAATCCTGTCACCTTGATGTGGTATTCACAC . . .
. . . CATACCAAAAGGTTCGAAGAAGGAAGGCGCGA
4/6
CGAGGTGTAGCGCAGTCTGGTCAGCGCATCTGTTTTGGGTACAGAGGGCCATAGGTTCGAATCCTGTCACCTTGATGTGGTATTCACAC . . .
. . . CATACCAAAAGGTTCGAAGAAGG
B
1.4 kb
CGAGGTGTAGCGCAGTCTGGTCAGCGCATCTGTTTTGGGTACAGAGGGCCATAGGTTCGAATCCTGTCACCTTGATGTGGTATTCACAC . . .
. . . CATACCAAAAGGTTCGAAGAAGGAAGGCGCGA
Room Temp
1/6
ATGTGGTATTCACAC . . .
. . . CATACCAAAAGGTTCGA
5/6
ATGTGGTATTCACAC . . .
. . . CATACCAAAAGGTTCG
Cold
1/6
ATGTGGTATTCACAC . . .
. . . CATACCAAAAGGTTCGAAGAAGG
1/6
ATGTGGTATTCACAC . . .
. . . CATACCAAAAGGTTCGA
4/6
ATGTGGTATTCACAC . . .
. . . CATACCAAAAGGTTCG
C
-1250
-1176
5′ end . . .
. . . 3′ end
+2143
+2164
CGAGGTGTAGCGCAGTCTGGTCAGCGCATCTGTTTTGGGTACAGAGGGCCATAGGTTCGAATCCTGTCACCTTGATGTGGTATTCACAC . . .
. . . TTATTGCTTGGAGCCTTCTCGCTTAGTAAGCCGCC
Cold
1/2
ATGTGGTATTCACAC . . .
. . . TTATTGCTTGGAGCCTTCTCGCTTAGTAAGCCGCC
AA
1/2
ATGTGGTATTCACAC . . .
. . . TTATTGCTTGGAGC
AAAAA
D
-399
-352
5′ end . . .
. . . 3′ end
+1578
non-encoded
0.7 kb
GCCTCTAGGTTAGTTCAAAGTGTAATACAACGAACTATCGATACACCGAAAACGAGTCAAGATGGATACATAAAATGGAGACATC . . .
. . . CATACCAAAAGGTTCGAAGAAGGAAGGCGC
Room Temp
1/6
GCCTCTAGGTTAGTTCAAAGTGTAATACAACGAACTATCGATACACCGAAAACGAGTCAAGATGGATACATAAAATGGAGACATC . . .
. . . CATACCAAAAGGTTCG
2/6
GGTTAGTTCAAAGTGTAATACAACGAACTATCGATACACCGAAAACGAGTCAAGATGGATACATAAAATGGAGACATC . . .
. . . CATACCAAAAGGTTCG
1/6
TTCAAAGTGTAATACAACGAACTATCGATACACCGAAAACGAGTCAAGATGGATACATAAAATGGAGACATC . . .
. . . CATACCAAAAGGTTCGA
1/6
CAAAGTGTAATACAACGAACTATCGATACACCGAAAACGAGTCAAGATGGATACATAAAATGGAGACATC . . .
. . . CATACCAAAAGGTTCGAA
1/6
ACCGAAAACGAGTCAAGATGGATACATAAAATGGAGACATC . . .
. . . CATACCAAAAGGTTCG
Cold
1/6
TAGGTTAGTTCAAAGTGTAATACAACGAACTATCGATACACCGAAAACGAGTCAAGATGGATACATAAAATGGAGACATC . . .
. . . CATACCAAAAGGTTCGA
1/6
GGTTAGTTCAAAGTGTAATACAACGAACTATCGATACACCGAAAACGAGTCAAGATGGATACATAAAATGGAGACATC . . .
. . . CATACCAAAAGGTTCG
1/6
AAGTGTAATACAACGAACTATCGATACACCGAAAACGAGTCAAGATGGATACATAAAATGGAGACATC . . .
. . . CATACCAAAAGGTTCGA
1/6
GTGTAATACAACGAACTATCGATACACCGAAAACGAGTCAAGATGGATACATAAAATGGAGACATC . . .
. . . CATACCAAAAGGTTCG
1/6
GTAATACAACGAACTATCGATACACCGAAAACGAGTCAAGATGGATACATAAAATGGAGACATC . . .
. . . CATACCAAAAGGTTCG
1/6
AACGAACTATCGATACACCGAAAACGAGTCAAGATGGATACATAAAATGGAGACATC . . .
. . . CATACCAAAAGGTTCG

of the RNA cleavage event mediated by RNase Z at the 3' end of the *trnP* is reduced in the cold. Clones of the 0.7 kb product detected from room temperature and cold mtRNA are 5' heterogeneous ranging from -399 to -352 consistent with the 5' end of the mature wheat *nad1* mRNA (Choi *et al*., 2012). The 3' ends of these clones were also consistent with *nad1* intron 1a clone data shown in Table 4.2.

Northern analysis used to examine the steady-state levels of transcripts of the *nad1a*-intron1a locus detected elevated species of sizes 3.2 kb, 2.7 kb and 2.4 kb relative to mature mRNA with cold treatment (Fig. 4.3). The 3.2 kb transcript is consistent in size with mapping from the upstream *trnP* gene to the 3' end of *nad1* intron 1a (+1578; see above) (Fig. 4.3A), while the 2.4 kb transcript is consistent in size with mapping from the 5' end of the *nad1* mRNA to the 3' end of *nad1* intron 1a. The identity of the 2.7 kb species remains to be fully elucidated but is consistent in size with mapping from the 5' end of the mature *nad1* mRNA to the extreme 3' ends observed from 2 clones (+2143 and +2164) in Table 4.3. Hybridization of *nad1* intron 1a detected a steady-state level of the excised intron half that is visibly higher than that observed for other aberrant mitochondrial group II intron in wheat (Dalby and Bonen, 2013) (Fig. 4.3B) indicating this molecule is highly stable. Use of a *nad1* intron 1b specific probe detected precursor transcripts previously characterized by Li-Pook-Than and Bonen, 2006 (Fig. 4.3C) and no species consistent in size with either excised half intron molecule identified by CR-RT-PCR. Given the heterogeneity observed from clone data at the 3' end for the two *nad1* intron 1b species, it is conceivable that they are degraded more rapidly than the predominant species detected for *nad1* intron 1a. In regards to the ~241 nt *nad1* intron 1b species, it is not excluded that due to its relatively small size it may not have been trapped effectively by the nylon membrane during northern transfer and, therefore, escaped detection.

Figure 4.3: Northern analysis for wheat mitochondrial loci of the bipartite nad1 intron 1 for room temperature and cold-germinated embryos.

A. *nad1* exon 1 **B**. *nad1* intron 1a **C**. *nad1* intron 1b. Mitochondrial RNA of room temperature (odd lanes) and cold-germinated (even lanes) wheat embryos are shown with size markers on the left and re-probing of blots with mitochondrial 18S rRNA oligomer shown. Mature mRNA and intron-containing precursors are designated by black and white arrowheads, respectively. Dashed arrows denote excised *nad1* intron 1a and expected migrations of *nad1* intron 1b species detected by CR-RT-PCR. Schematics show *nad1* exons (black boxes) and neighbouring genes (white boxes). The positions of 20'mer hybridization probes are indicated by asterisks. Each panel represents an independent RNA blot.


A
1 2
B
3 4
C
5 6
3.2 kb -
2.7 kb -
2.4 kb -
1.5 kb -
3.2 kb -
2.7 kb -
2.4 kb -
1.6 kb -
6.5 kb -
4.6 kb -
3.6 kb -
nad1 exon 1
nad1 in 1a
nad1 in 1b
18S
trnP
nad1a
atp6 rps13
nad1b
nad1c
1 kb

4.5 Discussion

Our investigation of the *trans*-splicing of *nad1* intron 1 in wheat mitochondria identified two distinct linear excised halves (*nad1* intron 1a + *nad1* intron 1b) that represent the first *in vivo* demonstration of hydrolytic splicing of a bipartite group II intron. We observed elevated levels of precursor transcripts relative to mature mRNA consistent with previous studies of other aberrant group II introns in wheat (Kurihara-Yonemoto and Handa, 2001; Dalby and Bonen, 2013) but did not detect heterogeneous Y-branch or open circle physical forms that would be indicative of a shift to alternative splicing pathways. Clone data for intron 1b suggests that cold treatment does not impact the respective activities of polyadenylation enzymes and 3' exonucleases or accessibility of the 3' end. Interestingly, evidence of 3' polyadenylation and exoribonuclease activity of the predominant *nad1* intron 1a species was not observed for either growth condition and indicates this end is somehow protected.

While hydrolytic splicing is predicted in the absence of a bulged adenosine, as demonstrated for the wheat mitochondrial *nad1* intron 2, *nad2* intron 1 and the *cox2* intron (Li-Pook-Than and Bonen, 2006; Dalby and Bonen, 2013), the absence of heterogeneous Y-branch or open circle forms of excised *nad1* intron 1 consistent with alternative splicing pathways suggests important structural differences to other aberrant plant mitochondrial group II introns that impact on splicing biochemistry. From mutational analysis of the yeast *ai5y* group II intron, the ability to catalyze first-step transesterification *in vivo* is lost when IBS1 is not immediately adjacent to the GUGYG consensus sequence (Michel and Ferat, 1995). As such, the presence of the 5' terminal insert in the wheat mitochondrial *nad1* intron 1 may disrupt the formation of the catalytic core such that branchpoint formation is no longer

possible. Consistent with certain group IIB1 introns in fungi mitochondria (Li *et al*., 2012), the 5' insert may further result in a loss of functional constraint on the branchpoint region of dVI and implicated in the high dVI sequence variability among seed plant orthologues. However, it is not excluded that the lack of heterogeneous Y-branch or open circle forms is a direct result of the degenerate structure of dVI. Mg^{++} ion binding sites identified within dVI are thought to be required in positioning of the bulged adenosine into the active site for conventional lariat-type splicing (Hertweck *et al*., 2001). Examination of the wheat mitochondrial *nad1* intron 1 secondary structure (Fig. 4.1B) suggests that these physical features may have been lost that prevents recruitment of internal nucleophiles in branchpoint formation.

End-analysis of the excised intron halves identified notable differences in 3' accessibility. Virtually all clones of *nad1* intron 1b possess variable lengths of 3' non-encoded A tracts consistent with the polyadenylation-mediated degradation pathway. Many of these clones are also missing lengths from the 3' terminus consistent with 3' exonuclease activity. The observed frequency of these events is nearly the same between growth conditions and suggests that the efficiency of RNA degradation is not affected by cold treatment. Notably, 3' A-tailing of the *nad1* intron 1a species that terminates 1578 nt downstream of the 5' exon-intron junction is not observed that suggests this end is protected from 3' exoribonucleolytic attack. From sequence alignment with orthologous introns, this end is immediately upstream of the breakpoint in homology between flowering plants, the gymnosperm *Cycas taitungensis* and the lycophyte *H.squarrosa* suggesting this region is under functional constraint. Given the location of this end within dIV, it is conceivable that the terminal sequence participates in a long-range interaction with the dIV segment of *nad1* intron 1b that, when bound, is protected from 3' exoribonucleases. Such a scenario may also

explain the detection of the *nad1* intron 1b species that maps to a cryptic position 241 nt upstream of *nad1* exon 2. Alternatively, one could imagine a scenario in which a stem-loop structure in this region, a vestige of its former *cis*-splicing context, fortuitously limits 3' accessibility to exoribonucleases, conceptually analogous to degenerate tRNAs (t-elements) that have been implicated in mRNA stability (Forner *et al*., 2007).

Finally, it is worth noting that using mtRNA isolated from cold treated wheat embryos we successfully amplified *nad1a* precursor transcripts with 5' termini that mapped to the precise 5' end of the upstream *trnP* gene. End-maturation of tRNAs by endonucleolytic cleavage is thought to be an early and rapid processing event (Hanic-Joyce and Gray, 1990; Kunzmann *et al*., 1998) and as evidenced by the detection of such a putatively rare precursor species not observed at room temperature, our findings demonstrate the potential application of low temperature growth conditions to facilitate studies of RNA level events in plant mitochondria, particularly those concerned with early transcript maturation.

4.6 Chapter 4 addendum: RNA splicing of wheat mitochondrial *nad1* intron 4

To have a more complete picture of the variability in splicing pathways employed by wheat mitochondrial group II introns, it was of interest to characterize the physical forms of the excised *nad1* intron 4 at room temperature and in the cold. Aside from being a *trans*-splicing intron, *nad1* intron 4 possesses a conventional branchpoint structure of dVI and in flowering plants, has the distinction of being the only mitochondrial group II intron that contains a maturase-coding gene (*matR*) within dIV.

RT-PCR products of the excised intron junction are shown in Figure 4.4A. These products were generated using nested primers that excluded a major product that was

identified by clone sequence analysis as the result of mis-priming within *nad4d*. The comparable amount of product generated from room temperature and the cold suggests similar RNA stability of the excised *nad1* intron 4. Sequence analysis of the excised intron junction (Fig. 4.4B) shows all clones from room temperature (4/4) and cold (4/4) are missing 6 nt from the 3' end corresponding to the position of the bulged adenosine indicative of classic lariat splicing. As is typically seen for excised lariats, the adenosine nucleophile is mis-read as a T (Vogel and Börner, 2002). These findings are reminiscent of the cold splicing data for the conventional *nad2* intron 4 (Chapter 3), and taken together suggests that introns that undergo lariat splicing at room temperature are less likely to shift to alternative pathways with cold treatment. Given that the RNA samples used in characterization of excised *nad1* intron 4 were not pre-treated with RNA ligase, it cannot be excluded that *trans*-splicing may also be mediated by first-step hydrolysis. It is worth mentioning that C-U editing of a position 10 nt upstream of the 3' intron-exon junction was observed for all clones.

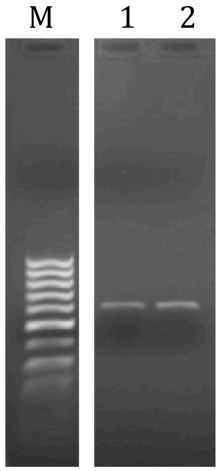
It was of further interest to investigate the impact of cold on the splicing efficiency of *nad1* intron 4 as judged by RNA blot analysis. For the two *nad1* intron 4 loci, higher steady-state levels of precursor transcripts were observed in the cold compared to room temperature relative to 18S rRNA consistent with my previous RNA blot analyses that indicate RNA splicing is slowed by low temperature treatment (Figure 4.5). Notably, one of the elevated RNA species detected by the *nad1* intron 4a probe was 3.8 kb consistent in size with the *Ψrpl2-nad1d-nad6* cotranscript (Haouazine *et al*., 1993; Subramanian *et al.*, 2001). This observation suggests that the efficiency of end-maturation of the downstream *nad6* mRNA is also reduced by cold treatment. Hybridization of a *nad1* intron 4b probe, as shown in Figure 4.5B, led to the detection of three major species of sizes 2.7 kb, 2.2 kb and 1.6 kb. These

species are consistent with those identified by Farré and Araya (1999) of sizes 2.8 kb, 2.4 kb and 1.6 kb., respectively.

Figure 4.4: Analysis of the excised intron junction of wheat mitochondrial nad1 intron 4 at room temperature and in the cold.

[A] Representative gel of nested RT-PCR products of *nad1* intron 4 at room temperature (lane 1) and in the cold (lane 2). M denotes DNA size ladder. Primers used in generation of products were LB764 and LB766. **[B]** Sequence analysis of RT-PCR clones of excised junction of *nad1* intron 4.

A

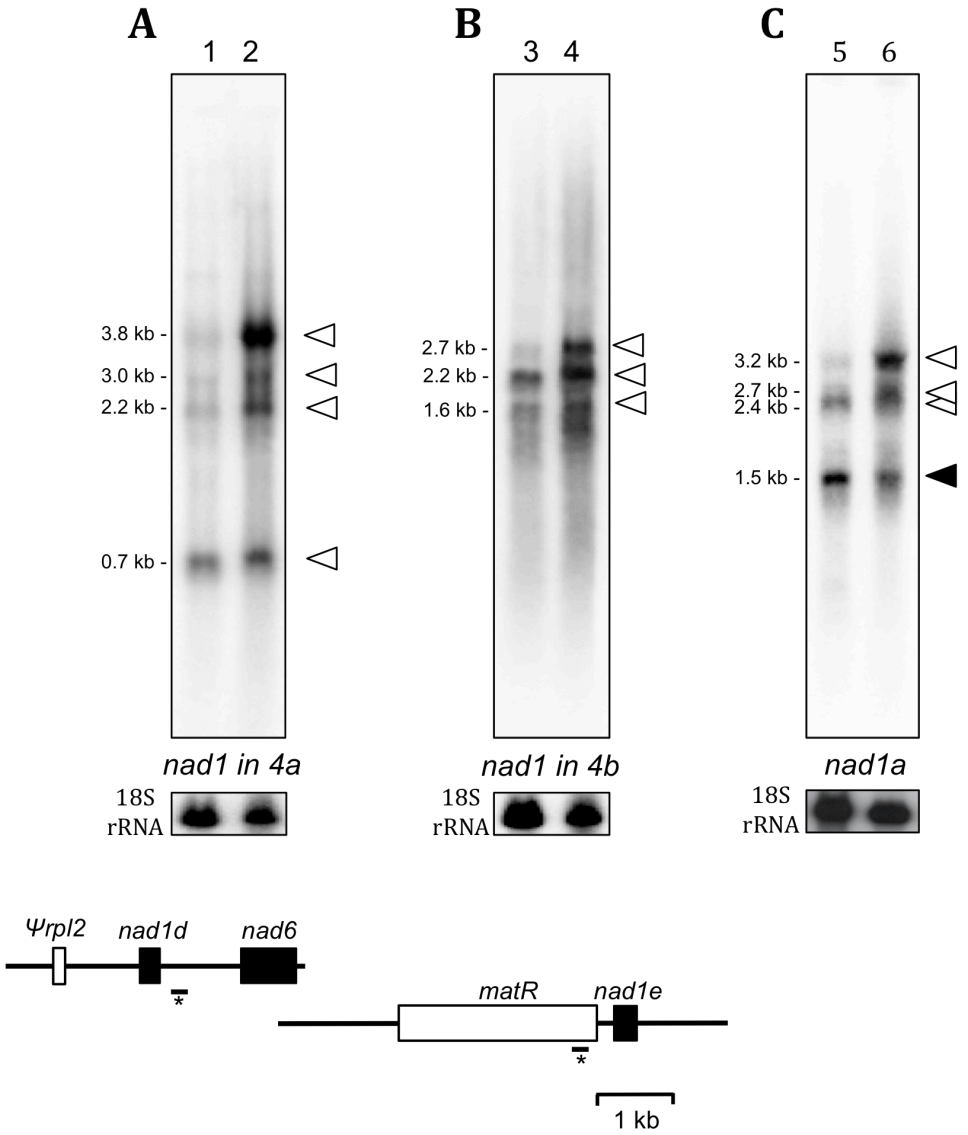


B

Growth Condition	Clone	3' Terminus GCATCCCTACTCACCC	Non-encoded nucleotides	5' Terminus GTGCGGGGCTTTGC
Room Temp	4/4	GCATCCTTT		GTGCGGGGCTTTGC
Cold	4/4	GCATCCTTT		GTGCGGGGCTTTGC

Figure 4.5: RNA blot analysis of wheat nad1 intron 4 loci for room temperature and cold treated embryos.

A. *nad1* intron 4a **B.** *nad1* intron 4b **C.** *nad1* exon 1 (same blot as Chapter 4 provided for comparison). Room temperature and cold RNA in odd and even lanes, respectively. Size markers are shown on the left and re-probing of blots with mitochondrial 18S rRNA oligomer below. Black arrowheads denote mRNA and white arrowhead denote intron-containing precursors. Schematics show exons of interest (black boxes) as well as neighbouring genes (white boxes) with positions of 20'mer hybridization probes indicated by asterisks.


A
1 2
3.8 kb -
3.0 kb -
2.2 kb -
0.7 kb -
nad1 in 4a
18S rRNA
B
3 4
2.7 kb -
2.2 kb -
1.6 kb -
nad1 in 4b
18S rRNA
C
5 6
3.2 kb -
2.7 kb -
2.4 kb -
1.5 kb -
nad1a
18S rRNA
Ψrpl2
nad1d
nad6
matR
nad1e
1 kb

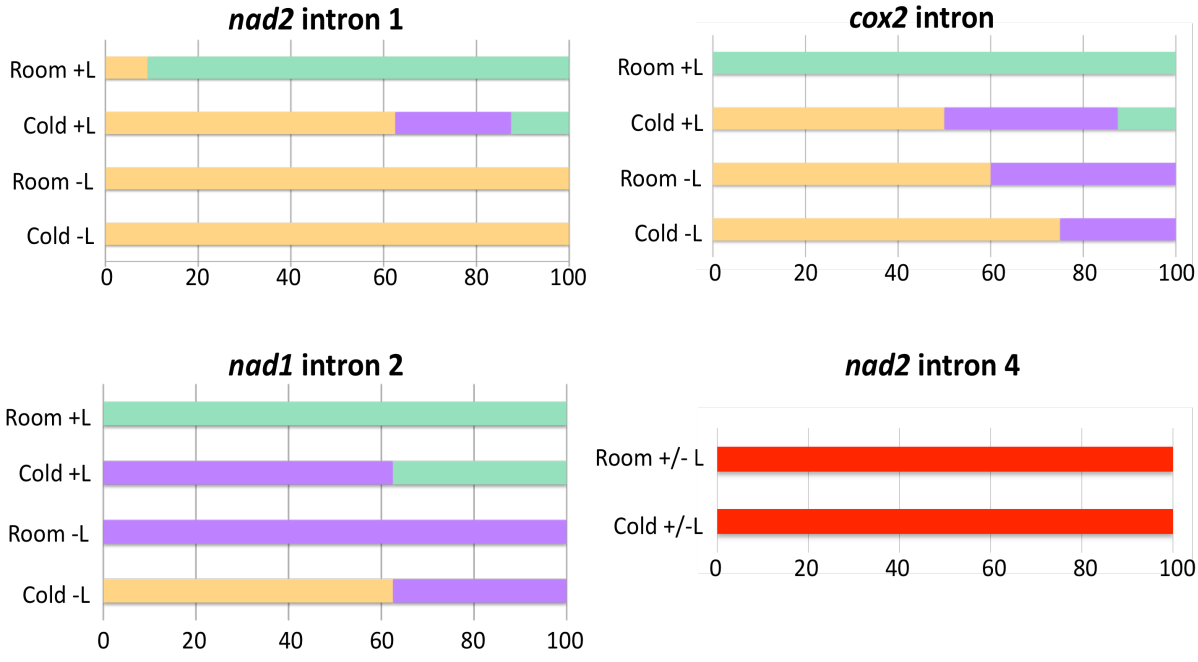
Chapter 5: General Discussion

5.1 *Impact of cold on splicing of aberrant wheat mitochondrial group II introns*

Analysis of RNA splicing of wheat mitochondrial group II introns that deviate from the conventional branchpoint structure of domain VI suggests a shift in biochemistry to alternative splicing pathways with cold treatment, particularly for poorly-structured introns (Fig. 5.1). Notably, this shift is not observed for a classic group II intron or for the highly aberrant *nad1* intron 1. In the latter examples, splicing biochemistry is correlated with an unusual 5' insert that may disrupt the catalytic core such that generation of *in vivo* circularized forms of the excised intron is not possible. RNA splicing of plant mitochondrial group II introns is dependent on nuclear encoded splicing machinery that stabilize secondary structures and assist in folding. Therefore, it is anticipated that introns with poorly structured domains have increased demand for such factors. Due to these requirements, it is further predicted that changes in nuclear gene expression and protein import across the outer and inner mitochondrial membranes will have a direct and serious impact on the elaborate tertiary folding that mediates splicing. Taylor *et al* (2003) previously demonstrated that at low temperature growth conditions the efficiency of translocation of cytosolic proteins to the mitochondrial matrix via the TIM (translocase of the inner membrane) and TOM (translocase of the outer membrane) complexes is significantly reduced. As such, it is conceivable that the apparent shift in splicing biochemistry with cold treatment may be due to reduced stability of intron structures on account of decreased availability of nuclear-encoded splicing machinery. Such a scenario is indicative of the complex integration of organellar processes with nuclear control. It is worth noting that it is not excluded that intron mis-folding could occur despite the presence of splicing factors should the accessibility of RNA structures be

Figure 5.1: Distribution of physical forms of excised nad2 intron 1, nad1 intron 2, cox2 and nad2 intron 4 between room temperature and cold growth from (CR)RT-PCR analysis.

Linear species with 3' A-tails and conventional lariat species are shown in green and red, respectively. Linear species with no 3' A-tails and/or full circle species are shown in purple. Novel *in vivo* circularized long-tailed lariat species are shown in yellow.


nad2 intron 1
Room +L
Cold +L
Room -L
Cold -L
0
20
40
60
80
100
cox2 intron
Room +L
Cold +L
Room -L
Cold -L
0
20
40
60
80
100
nad1 intron 2
Room +L
Cold +L
Room -L
Cold -L
0
20
40
60
80
100
nad2 intron 4
Room +/- L
Cold +/-L
0
20
40
60
80
100

reduced in the cold. Recently, Guan *et al* (2013) demonstrated that the splicing of several nuclear spliceosomal introns is dependent on a cold-induced DEAD-box RNA helicase that is thought to unwind non-competent intron structures that are thermodynamically favoured at low temperature for accessibility of the spliceosome. Given that the RNA-binding activity of group II intron splicing factors, particularly PPR proteins, is highly specific, atypical RNA structures assumed in the cold could impact on their functionality resulting in unconventional tertiary structures that favour alternative splicing pathways. Interestingly, preliminary data of wheat mitochondrial *nad7* intron 3 and *nad7* intron 4 suggests that the RNA editing status of a position within the dV helix is increased in cold grown seedlings (Boyoung Choi, unpublished data) indicating that the accessibility of certain editing factors may actually increase due to mis-folding.

Reduced efficiency of RNA splicing of *nad1*, *nad2* and *cox2* mitochondrial transcripts was observed in the cold as evidenced by elevated levels of intron-containing precursors relative to mature mRNAs. Notably, among these elevated precursors were ones containing *nad1* intron 1 and *nad2* intron 4 that under cold treatment were observed to predominantly employ hydrolytic and lariat-type splicing, respectively. As such, decreased splicing efficiency does not appear correlated with the employment of alternative splicing pathways. While the kinetics of transcript maturation are expected to slow at low temperatures as a result of reduced activities of the enzymes involved, reduced expression, import and/or reduced accessibility of nuclear-encoded processing machinery, that is supported by the observance of alternative splicing pathways, may also explain decreased efficiency of RNA processing. This is particularly true in the context of nuclear-encoded maturases and DEAD-box proteins that function specifically to mediate the efficiency of intron excision (Nakagawa and Sakurai, 2006; Keren *et al*., 2009; Köhler et al., 2010).

More broadly, it is plausible that changes in plant metabolism during abiotic stress conditions could impact on the expression of mitochondrial-encoded respiratory genes. Demonstrated by Armstrong *et al* (2000) in *Arabidopsis*, nuclear-encoded external NAD(P)H dehydrogenases that bypass complex I of the respiratory chain are upregulated in the cold that may be attributed, in part, as a cold response to decreased production of mitochondrial-encoded subunits of complex I, particularly given the disproportionate concentration of group II introns in *nad* genes (ex. 19 of 23 in wheat). It is, however, not excluded that reduced expression of nuclear-encoded subunits in the cold is a major contributing factor. In either case, the concomitant reduction in complex I assembly would directly impact energy production as well as the generation of reactive oxygen species, of which complex I is a primary generator. While the complex mechanisms of retrograde signaling between plant organelles and the nucleus are still being elucidated, levels of reactive oxygen species (ROS) are thought to be central to many cellular signaling pathways and appear to function in initiating several abiotic stress responses (Suzuki *et al*., 2012). As such, it is conceivable that the drop in ROS levels triggers increased expression of genes encoding external NAD(P)H dehydrogenases.

Despite their important role in signaling, ROS, particularly at high concentrations, can cause serious oxidative damage to DNA, lipids, proteins and cell structures (Møller, 2001). Mitigating factors include uncoupling proteins that "short circuit" the respiratory chain by dissipating the proton gradient across the inner mitochondrial membrane and limiting ROS production (Begcy *et al*., 2011). Interestingly, Armstrong *et al* (2000) also detected increased expression of an uncoupling protein coding gene, *ucp1*. Moreover, increased activity of the alternative oxidase (AOX) pathway is also observed at low temperature (Wang *et al*., 2011). AOX further regulates ROS emissions by bypassing

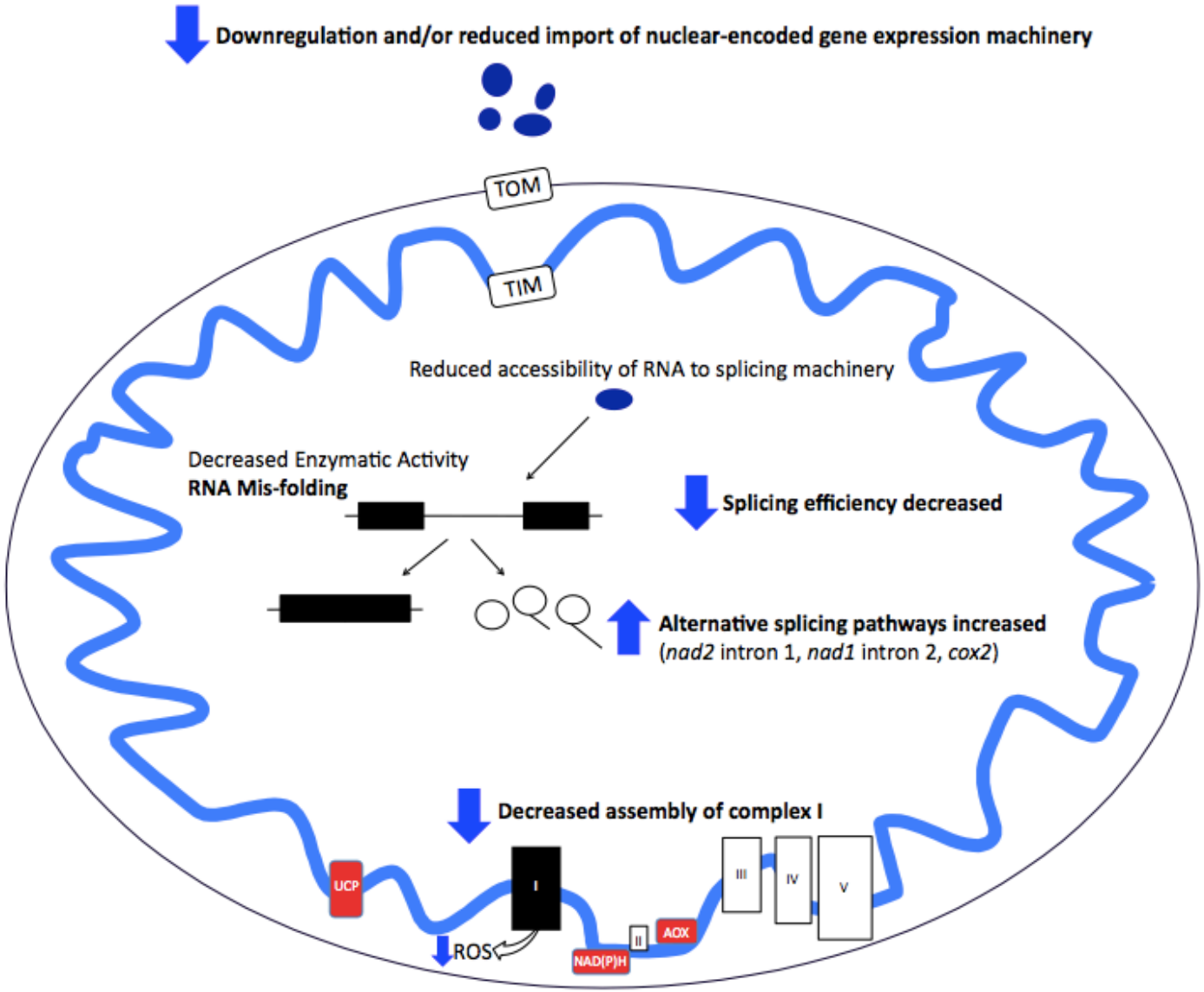
complex III, a second major ROS generator, in control of the redox state of the respiratory chain. Taken together, the induced response of these mechanisms is suggestive of increased ROS levels in the cold. Given that complex I is a major source of ROS, decrease in its assembly would thus be beneficial when levels are too high, such as during cold growth. Therefore, it is conceivable that the high representation of group II introns within *nad* genes, on account of the apparent temperature sensitivity of splicing, may serve an important regulatory role in the mitigation of ROS emissions (Fig. 5.2).

5.2 *Future directions*

The significance of RNA folding to group II intron splicing cannot be understated. In this present study, I have proposed that the decreased efficiency of splicing and the observed shift in splicing biochemistry of aberrant mitochondrial group II introns may be attributed, in part, to unconventional RNA structures favoured in the cold. Accordingly, an important area of future study will be the examination of such structures and how they may differ from those at room temperature. Recent characterization of the crystal structure of a group II intron throughout catalysis has clearly demonstrated the intricate and dynamic 3-dimensional folding that occurs during splicing (Marcia *et al*., 2013) and a comparable analysis for low temperature will be a valuable first-step approach. It will be of further interest to investigate the potential impact cold-favoured structures have on RNA-binding efficiency of stabilizing factors, such as PPR proteins. It is predicted that multiple splicing factors assist in folding of any given intron and that certain structural domains, particularly poorly-structured ones, that require more machinery may represent the "weak link" should mis-folding occur in the cold. While several splicing factors have been determined to be absolutely essential to intron

Figure 5.2: Model for cold-sensitive RNA splicing of plant mitochondrial group II introns.

Reduced expression and/or import of nuclear-encoded splicing machinery and/or reduced accessibility of RNA structures results in atypical intron folding that, together with decreased enzymatic activity, leads to reduced splicing efficiency. For poorly-structured introns, unconventional folding further leads to a shift in biochemistry to alternative splicing pathways. The subsequent reduction in mitochondrial gene expression impacts assembly of respiratory chain complexes, particularly complex I. Concomitant decrease in complex I activity limits ROS emissions that are additionally mitigated by several nuclear-encoded mechanisms (red boxes).


Downregulation and/or reduced import of nuclear-encoded gene expression machinery
TOM
TIM
Reduced accessibility of RNA to splicing machinery
Decreased Enzymatic Activity
RNA Mis-folding
Splicing efficiency decreased
Alternative splicing pathways increased
(nad2 intron 1, nad1 intron 2, cox2)
Decreased assembly of complex I
UCP
I
II
III
IV
V
AOX
NAD(P)H
ROS

excision (reviewed in de Longevialle *et al*., 2010), the precise location of their targeting remains largely unknown and warrants further study.

With increased insight into wheat mitochondrial gene expression at low temperatures come several questions of how these changes fit within the greater context of the plant's response to cold stress conditions. For instance, many mechanisms of cold adaptation are mediated by changes in plastid gene expression and photorespiration (reviewed in Crosatti *et al*., 2012). In fact, nearly twice as many cold responsive nuclear genes are upregulated in cold grown *Arabidopsis* plants when in the presence of light compared to dark (Soitamo *et al*., 2008). Therefore, it is not excluded that the expression of certain *trans* factors that mediate splicing of mitochondrial group II introns is regulated by retrograde signaling from the chloroplasts, particularly those that are dual-targeted to both organelles. A parallel study to the one presented here in which wheat plants are grown in the presence of light would be of interest to identifying any potential impact the chloroplasts may have on RNA processing of mitochondrial transcripts. It is also worth contemplating the potential impact on RNA processing of decreased permeability of the mitochondrial membranes by cold-induced expression of dehydrins (Kosova *et al*., 2011). Dehydrins are very hydrophilic nuclear-encoded proteins that are targeted to numerous subcellular locations, including the outer mitochondrial membrane, to prevent substantial water loss that is associated with abiotic stress. In this regard, it would be interesting to further investigate the potential interplay between cold activation of these particular dehydrin-coding genes and reduced import of nuclear-encoded machinery.

Lastly, an important consideration is the inherent cold tolerance of a winter wheat cultivar, such as the one used in this study (FT-Wonder), compared to a spring wheat cultivar. Interestingly, expression levels of known nuclear cold-responsive genes appear to

relatively equal between cold-hardy and cold-sensitive wheat varieties (Laudencia-Chingcuanco *et al*., 2011). As such, their apparent differences in cold tolerance may relate to variability in the impact of low temperature growth on organellar gene expression. Although preliminary comparative analysis of RNA editing in the cold of the intron-containing mitochondrial gene *ccmFC* between a cold-sensitive wheat cultivar (Glenlea) and FT-Wonder did not identify any appreciable differences (Mathias Fricot, unpublished data) it is not excluded that the impact of cold on intron splicing, RNA editing and end-maturation may somewhat vary among cultivars.

5.3 *Concluding remarks*

My investigation of the temperature-sensitivity of RNA splicing of wheat mitochondrial group II introns has demonstrated remarkable plasticity in splicing biochemistry in the absence of characteristic features that is compounded by cold treatment. Moreover, the extent of variability in splicing pathways employed at low temperature appears correlated with poor stability in the RNA secondary structure of dVI. Intriguingly, in the case of *nad1* intron 1, reduced variability seems connected with the presence of an unusual 5' insert.

What seems clear for the aberrant mitochondrial group II introns examined in this study is that cold treatment is somehow impacting on their ability to fold into conventional RNA structures. Increasing amounts of data are becoming available on nuclear-encoded protein machineries that stabilize intron structures and mediate folding, many of which are intron specific (reviewed in de Longevialle *et al*., 2010). Indeed, several splicing factors have been demonstrated to be essential to the excision of their target introns demonstrating the complex integration of the mitochondrial and nuclear systems that has evolved over time. In

this regard, there is little doubt that changes in expression of genes encoding such splicing factors is a major contributing factor to the observed differences in RNA processing in the cold. Unfortunately, transcriptome data presently available for their expression at low temperature is inconclusive (c.f. Hannah *et al*., 2005; TAIR: www.arabidopsis.org); and will require expansion if we are to move forward with our understanding of nuclear control of mitochondrial gene expression under abiotic stress conditions.

For agronomically important plant species, the study of energy production and abiotic factors that mediate it are of great interest as they are directly correlated with crop yield. In the Canadian context, this is particularly true of wheat and cold growth conditions. As many of the protein-coding genes present in plant mitochondrial genomes encode subunits of respiratory chain complexes essential to their assembly and function, characterization of the impact of cold on mitochondrial gene expression is of critical importance to understanding how low temperature growth affects the generation of ATP and, more broadly, plant metabolism.

References

Adamo, A., Pinney, J.W., Kunova, A., Westhead, D.R., Meyer, P., 2008. Heat stress enhances the accumulation of polyadenylated mitochondrial transcripts in *Arabidopsis thaliana*. PLoS ONE. 3, e2889.

Adams K.L. and Palmer, J.D., 2003. Evolution of mitochondrial gene content: gene loss and transfer to the nucleus. Mol Phylogenet Evol. 29, 380-395.

Armstrong, A.F., Badger, M.R., Day, D.A., Bathet, M.M., Smith, P.M., Millar, A.H., Whelan, J., Atkin, O.K., 2008. Dynamic changes in the mitochondrial electron transport chain underpinning cold acclimation of leaf respiration. Plant Cell Environ. 31, 1156-1169.

Baker, M.J., Frazier, A.E., Gulbis, J.M., and Ryan, M.T., 2007. Mitochondrial protein import machinery: correlating structure with function. Trends Cell Biol. 17, 456-464.

Begcy, K., Mariano, E.D., Mattiello, L., Nunes, A.V., Mazzafera, P., Maia, I.G., Menossi, M., 2011. An Arabidopsis mitochondrial uncoupling protein confers tolerance to drought and salt stress in transgenic tobacco plants. PLoS One. 6(8):e23776.

Belhocine, K., Mak, A.B., and Cousineau, B., 2008. *Trans*-splicing versatility of the Ll.LtrB group II intron. RNA. 14, 1782-1790.

Bock, R., Hermann, M., and Kossel, H., 1996. *In vivo* dissection of *cis*-acting determinants for plastid RNA editing. EMBO J. 15, 5052-5059.

Bonen, L., 1993. *Trans*-splicing of pre-mRNA in plants, animals, and protists. FASEB J. 7, 40-46.

Bonen, L., 2008. Cis- and trans-splicing of group II introns in plant mitochondria. Mitochondrion. 8, 26-34.

Bonen, L., Vogel, J., 2001. The ins and outs of group II introns. Trends Genet. 17, 322-331.

Bonen, L., Boer, P.H., Gray, M.W., 1984. The wheat cytochrome oxidase subunit II gene has an intron insert and three radical amino acid changes relative to maize. EMBO J. 3, 2531-2536.

Byers, E. and Bonen, L., 2012. Potential role of tRNAs in wheat and Lolium mitochondrial *rps7* transcript processing. Genome. 55, 615-621.

Calixte, S. and Bonen, L., 2008. Developmentally-specific transcripts from the *ccmFN-rps1* locus in wheat mitochondria. Mol Genet Genomics. 280, 419-426.

Carrillo, C., Bonen L., 1997. RNA editing status of *nad7* intron domains in wheat mitochondria. Nucleic Acids Res. 25, 403-409.

Carrillo, C., Chapdelaine, Y., Bonen, L., 2001. Variation in sequence and RNA editing within core domains of mitochondrial group II introns among plants. Mol Gen Genet. 264, 595-603.

Castandet, B. and Araya, A., 2011. RNA editing in plant organelles. Why make it easy? Biochemistry (Mosc). 76, 924-931.

Castandet, B., Choury, D., Bégu, D., Jordana, X., Araya A., 2010. Intron RNA editing is essential for splicing in plant mitochondria. Nucleic Acids Res. 38, 7112-7121.

Chan, R.T., Robart, A.R., Rajashankar, K.R., Pyle, A.M., and Toor, N., 2012. Crystal structure of a group II intron in the pre-catalytic state. Nat Struc Mol Biol. 19, 555-557.

Cho, Y., Qiu, Y.L., Kuhlman, P., and Palmer, J.D., 1998. Explosive invasion of plant mitochondria by a group I intron. Proc Natl Acad Sci USA. 95, 14244-14249.

Choi, B., Acero, M.M., Bonen, L., 2012. Mapping of wheat mitochondrial mRNA termini and comparison with breakpoints in DNA homology among plants. Plant Mol Biol. 80, 539-552.

Chu, V.T., Liu, Q., Podar, M., Perlman, P.S., Pyle, A.M., 1998. More than one way to splice an RNA: branching without a bulge and splicing without branching in group II introns. RNA. 4, 1186-1202.

Chu, V.T., Adamidi, C., Liu, Q., Perlman, P.S., and Pyle, A.M., 2001. Control of branch-site choice by a group II intron. EMBO J. 20**,** 6866-76.

Copertino, D.W. and Hallick, R.B., 1993. Group II and group II introns of twintrons: potential relationships with nuclear pre-mRNA introns. Trends Biochem Sci. 18, 467-471.

Cordin, O., Banroques, J., Tanner, N.K., and Linder, P., 2006. The DEAD-box protein family of RNA helicases. Gene. 367, 17-37.

Crosatti, C., Rizza, F., Badeck, F.W., Mazzucotelli, E., and Cattivelli, L., 2013. Harden the chloroplast to protect the plant. Physiol Plant. 147, 55-63.

Dai, L., Toor, N., Olson, R., Keeping, A., and Zimmerly, S., 2003. Database for mobile group II introns. 31, 424-426.

de Longevialle, A.F., Small, I.D., Lurin, C., 2010. Nuclearly encoded splicing factors implicated in RNA splicing in higher plant organelles. Mol Plant. 3, 691-705.

Dombrovska, O., Qiu, Y.L., 2004. Distribution of introns in the mitochondrial gene *nad*1 in land plants: phylogenetic and molecular evolutionary implications. Mol Phylogenet Evol. 32, 246-263.

Elina, H., Brown, G.G., 2010. Extensive mis-splicing of a bi-partite plant mitochondrial group II intron. Nucleic Acids Res. 38:996-1008.

Farré, J.C., Araya, A., 1999. The *mat-r* open reading frame is transcribed from a non-canonical promoter and contains an internal promoter to co-transcribe exons *nad1e* and *nad5III* in wheat mitochondria. Plant Mol Biol. 40, 959-967.

Farré, J.C., Araya, A., 2002. RNA splicing in higher plant mitochondria: determination of functional elements in group II intron from a chimeric cox II gene in electroporated wheat mitochondria. Plant J. 29, 203-213.

Farré, J.C., Aknin, C., Araya, A., Castandet, B., 2012. RNA editing in mitochondrial trans-introns is required for splicing. PLoS ONE. 7, e52644.

Federova, O., Mitros, T., and Pyle, A.M., 2003. Domains 2 and 3 interact to form critical elements of the group II intron active site. J Mol Biol. 330, 197-209.

Forner, J., Weber, B., Thuss, S., Wildum, S., and Binder, S., 2007. Mapping of mitochondrial mRNA termini in Arabidopsis thaliana: t-elements contribute to 5' and 3' end formation. Nucleic Acids Res. 35, 3676-3692.

Francs-Small, C.C., Kroeger, T., Zmudjak, M., Ostersetzer-Biran, O., Rahimi, N., Small, I., Barkan, A., 2012. A PORR domain protein required for rpl2 and *ccmF(C)* intron splicing and for the biogenesis of c-type cytochromes in Arabidopsis mitochondria. Plant J. 69, 996-1005

Giegé, P. and Brennicke, A., 1999. RNA editing in Arabidopsis mitochondria effects 441 C to U changes in ORFS. Proc Natl Acad Sci USA. 96, 15324-15329.

Giegé, P., Hoffmann, M., Binder, S., and Brennicke, A., 2000. RNA degradation buffers asymmeties of transcription in Arabidopsis mitochondria. EMBO Rep. 1, 164-170.

Gill, S.S. and Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem. 48, 909-930.

Gray, M.W., Burger, G., and Lang, B.F., 2001. The origin and early evolution of mitochondria. Genome Biol. 2(6).

Grewe, F., Herres, S., Viehover, P., Polsakiewicz, M., Weisshaar, B., and Knoop, V., 2011. A unique transcriptome: 1782 positions of RNA editing alter 1406 codon identities in mitochondrial mRNAs of the lycophyte Isoetes engelmannii. Nucleic Acids Res. 39, 2890-2902.

Guan, Q., Wu, J., Zhang, Y., Jiang, C., Liu, R., Chai, C., and Zhu J., 2013. A DEAD box RNA helicase is critical to pre-mRNA splicing, cold-responsive gene regulation, and cold tolerance in Arabidopsis. Plant Cell. 25, 342-356.

Hanic-Joyce, P.J. and Gray, M.W., 1990. Processing of transfer RNA precursors in a wheat mitochondrial extract. J Biol Chem. 265, 13782-13791.

Hannah, M.A., Heyer, A.G., and Hincha, D.K., 2005. A global survey of gene regulation during cold acclimation in Arabidopsis thaliana. PLoS Genet. 1(2):e26

Haouazine, N., Takvorian, A., Jubier, M.F., Michel, F., and Lejeune, B., 1993. The *nad6* gene and the exon d of *nad1* are co-transcribed in wheat mitochondria. Curr Genet. 24, 533-538.

Haugen, P., Simon, D.M., and Bhattacharya, D., 2005. The natural history of group I introns. Trends Genet. 21, 111-119.

Hedtke, B., Borner, T., and Weihe, A., 2000. One RNA polymerase serving two genomes. EMBO Rep. 1, 435-440.

Hepburn, N.J., Schmidt, D.W., and Mower, J.P., 2012. Loss of two introns from the Magnolia tripetala mitochondrial *cox2* gene implicates horizontal gene transfer and gene conversion as a novel mechanism of intron loss. Mol Biol Evol. 29, 3111-3120.

Hertweck, M. and Mueller, M.W., 2001. Mapping divalent metal ion binding sites in a group II intron by Mn(2+)- and Zn(2+)-induced site-specific RNA cleavage. Eur J Biochem. 268, 4610-4620.

Hirayama, T., Matsuura, T., Ushiyama, S., Narusaka, M., Kurihara, Y., Yasuda, M., Ohtani, M., Seki, M., Demura, T., Nakashita, H., Narusaka, Y., and Hayashi, S., 2013. A poly(A)-specific ribonuclease directly regulates the poly(A) status of mitochondrial mRNA in Arabidopsis. Nat Commun. 4, 2247.

Holec, S., Lange, H., Dietrich, A., and Gagliardi, D., 2008. Polyadenylation-mediated RNA degradation in plant mitochondria. Methods Enzymol. 447, 429-461.

Holec, S., Lange, H., Kuhn, K., Alioua, M., Borner, T., and Gagliardi, D., 2006. Relaxed transcription in Arabidopsis mitochondria is counterbalanced by RNA stability control mediated by polyadenylation and polynucleotide phosphorylase. Mol Cell Biol. 26, 2869-2876.

Holzle, A., Jonietz, C., Torjek, O., Altmann, Binder, S., and Forner, J., 2011. A RESTORER OF FERTILITY-like PPR gene is required for 5'-end processing of the *nad4* mRNA in mitochondria of Arabidopsis thaliana. Plant J. 65, 737-744.

Ichinose, M., Tasaki, E., Sugita, C., and Sugita, M., 2012. A PPR-DYW protein is required for splicing of a group II intron of *cox1* pre-mRNA in Physcomitrella patens. Plant J. 70, 271-278.

Jenkins, M.E., Kulhanek, D.J., and Barkan, A., 1997. Nuclear mutations that block group II RNA splicing in maize chloroplasts reveal several intron classes with distinct requirements for splicing factors. Plant Cell. 9, 283-296.

Keren, I., Bezawork-Geleta, A., Kolton, M., Maayan, I., Belausov, E., Levy, M., Mett, A., Gidoni, D., Shaya, F., Ostersetzer-Biran, O., 2009. AtnMat2, a nuclear-encoded maturase required for splicing of group-II introns in Arabidopsis mitochondria. RNA. 15, 2299-2311.

Keren, I., Tal, L., des Francs-Small, C.C., Araújo, W.L., Shevtsov, S., Shava, F., Fernie, A.R., Small, I., and Ostersetzer-Biran, O., 2012. nMAT1, a nuclear-encoded maturase involved in the trans-splicing of *nad1* intron 1, is essential for mitochondrial complex I assembly and function. Plant J. 71, 413-426.

Knoop, V., Altwasser, M., and Brennicke, A., 1997. A tripartite group II intron in mitochondria of an angiosperm plant. Mol Gen Genet. 255, 269-276.

Köhler, D., Schmidt-Gattung, S., Binder, S., 2010. The DEAD-box protein PMH2 is required for efficient group II intron splicing in mitochondria of Arabidopsis thaliana. Plant Mol Biol. 72, 459-467.

Koonin, E.V., 2006. The origin of introns and their role in eukaryogenesis: a compromise solution to the introns-early versus introns-late debate. Biol Direct. 1:22.

Koprivova, A., des Francs-Smal,l C.C., Calder, G., Mugford, S.T., Tanz, S., Lee, B.R., Zechmann, B., Small, I., Kopriva, S., 2010. Identification of a pentatricopeptide repeat protein implicated in splicing of intron 1 of mitochondrial *nad7* transcripts. J Biol Chem. 285, 32192-32199.

Kosova, K., Vitamvas, P., and Prasil, I.T., 2011. Expression of dehydrins in wheat and barley under different temperatures. Plant Sci. 180, 46-52.

Kroeger, T.S., Watkins, K.P., Friso, G., van Wijk, K.J., and Barkan, A., 2009. A plant-specific RNA-binding domain revealed through analysis of chloroplast group II intron splicing. Proc Natl Acad Sci USA. 106, 4537-4542.

Kühn, K., Carrie, C., Giraud, E., Wang, Y., Meyer, E.H., Narsai, R., des Francs-Small, C.C., Zhang, B., Murcha, M.W., Whelan, J., 2011. The RCC1 family protein RUG3 is required for splicing of *nad2* and complex I biogenesis in mitochondria of Arabidopsis thaliana. Plant J. 67, 1067-1080.

Kühn, K., Weihe, A., and Borner, T., 2005. Multiple promoters are a common feature of mitochondrial genes in Arabidopsis. Nucleic Acids Res. 33, 337-346.

Kunzmann, A., Brennicke, A., and Marchfelder, A., 1998. 5’ end maturation and RNA editing have to precede tRNA 3’ processing in plant mitochondria. Proc Natl Acad Sci USA. 95, 108-113.

Kurihara-Yonemoto, S., Handa, H., 2001. Low Temperature affects the processing pattern and RNA editing status of the mitochondrial *cox2* transcripts in wheat. Curr. Genet. 40, 203-208.

Kurihara-Yonemoto, S., Kubo, T., 2010. Increased accumulation of intron-containing transcripts in rice mitochondria caused by low temperature: is cold-sensitive RNA editing implicated? Curr Genet. 40, 203-208.

Lambowitz, A.M. and Zimmerly, S., 2004. Mobile group II introns. Annu Rev Genet. 38, 1-35.

Lambowitz, A.M., Zimmerly, S., 2011. Group II introns: mobile ribozymes that invade DNA. Cold Spring Harb Perspect Biol. 1, a003616.

Lange H., Sement F.M, Canaday J., Gagliardi D., 2009. Polyadenylation-assisted RNA degradation processes in plants. Trends Plant Sci. 14, 497-504.

Laudencia-Chingcuanco, D., Ganeshan, S., You, F., Fowler, B., Chibbar, R., and Anderson, O., 2011. Genome-wide gene expression analysis supports a developmental model of low temperature tolerance gene regulation in wheat (Triticum aestivum L.). BMC Genomics. 12:299.

Li, C.F., Costa, M., Michel, F., 2011a. Linking the branchpoint helix to a newly found receptor allows lariat formation by a group II intron. EMBO J. 30, 3040-3051.

Li, C., Costa, M., Bassi, G., Lai, Y., and Michel, F., 2011b. Recurrent insertion of 5'-terminal nucleotides and loss of the branchpoint motif in lineages of group II introns inserted in mitochondrial preribosomal RNAs. RNA. 17**,** 1321-1335.

Li-Pook-Than, J., Bonen, L., 2006. Multiple physical forms of excised group II intron RNAs in wheat mitochondria. *Nucleic Acids Res.* 34, 2782-2790.

Li-Pook-Than, J., Carrillo, C., Bonen, L., 2004. Variation in mitochondrial transcript profiles of protein-coding genes during early germination and seedling development in wheat. Curr. Genet. 46, 374-380.

Liere, K., Weihe, A., and Borner, T., 2011. The transcription machineries of plant mitochondria and chloroplasts: Composition, function, and regulation. J Plant Physiol. 168, 1345-1360.

Lippok, B., Brennicke, A., and Wissinger, B., 1994. Differential RNA editing in closely related introns in Oenothera mitochondria. Mol Gen Genet. 243, 39-46.

Liu, Y., He, J., Chen, Z., Ren, X., Hong, X., Gong, Z., 2010. ABA overly-sensitive 5 (ABO5), encoding a pentatricopeptide repeat protein required for cis-splicing of mitochondrial *nad2* intron 3, is involved in the abscisic acid response in Arabidopsis. Plant J. 63, 749-765.

Lupold, D.S., Caoile, A.G., and Stern, D.B., 1999. Genomic context influences the activity of maize mitochondrial *cox2* promoters. Proc Natl Acad Sci USA. 96, 11670-11675.

Lykke-Andersen, J., Aagaard C., Semionenkov, M., and Garrett, R.A., 1997. Archael introns: splicing, intercellular mobility and evolution. Trends Biochem Sci. 22, 326-331.

Malek, O., Brennicke, A., and Knoop, V., 1997. Evolution of *trans*-splicing plant mitochondrial introns in pre-Permian times. Proc Natl Acad Sci USA. 94, 553-558.

Malek, O. and Knoop, V., 1998. *Trans*-splicing group II introns in plant mitochondria: the complete set of *cis*-arranged homologs in ferns, fern allies, and a hornwort. RNA. 4, 1599-1609.

Marcia, M., Pyle, A.M., 2012. Visualizing group II intron catalysis through stages of splicing. Cell. 153, 497-507.

Maier, R.M., Zeltz, P., Kössel, H., Bonnard, G., Gualberto, J.M., Grienenberger, J.M., 1996. RNA editing in plant mitochondria and chloroplasts. Plant Mol Biol. 32, 342-365.

Matsuura, M., Noah, J.W., and Lambowitz, A.M., 2001. Mechanism of maturase-promoted group II intron splicing. EMBO J. 20, 7259-7270.

Michel, F. and Ferat, J.L., 1995. Structure and activities of group II introns. Annu Rev Biochem. 64, 435-461.

Michel, F., Umesono, K., and Ozeki, H., 1989. Comparative and functional anatomy of group II catalytic introns—a review. Gene. 82, 5-30.

Mohr, G., Perlman, P.S., and Lambowitz, A.M., 1993. Evolutionary relationships among group II intron-encoded proteins and identification of a conserved domain that may be related to maturase function. Nucleic Acids Res. 21, 4991-4997.

Molina-Sanchez, M.D., Martınez-Abarca, F., Toro, N., 2006. Excision of the *Sinorhizobium meliloti* group II intron RmInt1 as circles *in vivo*. J Biol Chem. 281, 28737–28744.

Molina-Sánchez, M.D., Barrientos-Durán, A, Toro, N., 2011. Relevance of the branch point adenosine, coordination loop, and 3' exon binding site for in vivo excision of the *Sinorhizobium meliloti* group II intron RmInt1. J Biol Chem. 286, 21154-63.

Morawala-Patell, V., Gualberto, J.M., Lamattina, L., Grienenberger, J.M., Bonnard, G., 1998. Cis- and trans-splicing and RNA editing are required for the expression of *nad2* in wheat mitochondria. Mol Gen Genet. 258, 503-11.

Mower, J.P. and Palmer, J.D., 2006. Patterns of partial RNA editing in mitochondrial genes of Beta vulgaris. Mol Genet Genomics. 276, 285-293.

Murray, H.L., Mikheeva, S., Coljee, V.W., Turczyk, B.M., Donahue, W.F., Bar-Shalom, A., Jarrell, K.A., 2001. Excision of group II introns as circles. Mol Cell. 8, 201-211.

Nakagawa, N. and Sakurai, N., 2006. A mutation in At-nMat1a, which encodes a nuclear gene having high similarity to group II intron maturase, causes impaired splicing of mitochondrial NAD4 transcripts and altered carbon metabolism in Arabisopsis thaliana. Plant Cell Physiol. 47, 772783.

Nakamura, T., and Sugita, M., 2008. A conserved DYW domain of the pentatricopeptide repeat protein possesses a novel endoribonuclease activity. FEBS Lett. 582, 4163-4168.

Naydenov, N.G., Khanam, S., Siniauskaya, M., and Nakamura, C., 2010. Profiling of mitochondrial transcriptome in germinating wheat embryos and seedlings subjected to cold, salinity and osmotic stresses. Genes Genet Syst. 85, 31-42.

Nielsen, H., Johansen, S.D., 2009. Group I introns: moving in new directions. RNA Biol. 6, 375-383.

Nielsen, H., Fiskaa, T., Birgisdottir, A.B., Haugen, P., Einvik, C., Johansen, S., 2003. The ability to form full-length intron RNA circles is a general property of nuclear group I introns. RNA. 9, 1464-1475.

Okuda, K., Chateigner-Boutin, A.L., Nakamura, T., Delannoy, E., Sugita, M., Myouga, F., Motohashi, R., Shinozaki, K., Small, I., and Shikanai, T., 2009. Pentatricopeptide repeat proteins with the DYW motif have distinct molecular functions in RNA editing and RNA cleavage in Arabidopsis chloroplasts. Plant Cell. 21, 146-156.

Okuda, K., Hammani, K., Tanz, S.K., Peng, L., Fukao, Y., Myouga, F., Motohashi, R., Shinozaki, K., Small, I., and Shikanai, T., 2010. The pentricopeptide repeat protein

OTP82 is required for RNA editing of plastid ndhB and ndhG transcripts. Plant J. 61, 339-349.

Perrin, R., Lange, H., Grienenberger, J.M., and Gagliardi, D., 2004. AtmtPNPase is required for multiple aspects of the 18S rRNA metabolism in Arabidopsis thaliana mitochondria. Nucleic Acids Res. 32, 5174-5182.

Perron, K., Goldschmidt-Clermont, M., and Rochaix, J.D., 2004. A multiprotein complex involved in chloroplast group II intron splicing. RNA. 10, 704-711.

Picardi, E., Horner, D.S., Chiara, M., Schiavon, R., Valle, G., and Pesole, G., 2010. Large-scale detection and analysis of RNA editing in grape mtDNA by RNA deep-sequencing. Nucleic Acids Res. 38, 4755-4767.

Podar, M., Chu, V.T., Pyle, A.M. and Perlman, P.S., 1998. Group II intron splicing *in vivo* by first-step hydrolysis. Nature. 391, 915-918.

Pruchner, D., Nassal, B., Schindler, M., and Knoop, V., 2001. Mosses share mitochondrial group II introns with flowering plants, not with liverworts. Mol Genet Genomics. 266, 608-613.

Pyle, A.M., 2010. The tertiary structure of group II introns: implications for biological function and evolution. Crit Rev Biochem Mol Biol. 45, 215-32.

Rogozin, I.B., Carmel, L., Csuros, M., and Koonin, E.V., 2012. Origin and evolution of spliceosomal introns. Biol Direct. 7, doi: 10.1186/1745-6150-7-11.

Rossmanith, W., 2012. Of P and Z: mitochondrial tRNA processing enzymes. Biochim Biophys Acta. 1819, 1017-1026.

Saha, D., Prasad, A.M., and Srinivasan, R., 2007. Pentatricopeptide repeat proteins and their emerging role in plants.

Salone, V., Rudinger, M., Polsakiewicz, M., Hoffmann, B., Groth-Malonek, M., Szurek, B., Small, I., Knoop, V., and Lurin, C., 2007. A hypothesis on the identification of the editing enzyme in plant organelles. FEBS Lett. 581, 4132-4138.

Schmitz-Linneweber, C. and Small, I., 2008. Pentratricopeptide repeat proteins: a socket set for organelle gene expression. Trend Plant Sci. 13, 663-670.

Schmitzová, J. and Pena, V., 2012. Emerging views about the molecular structure of the spliceosomal catalytic center. RNA Biol. 9, 1311-1318.

Shikanai, T., 2006. RNA editing in plant organelles: machinery, physiological function and evolution. Cell Mol Life Sci. 63, 698-708.

Slomovic, S., Laufer, D., Geiger, D., and Schuster, G., 2005. Polyadenylation and degradation of human mitochondrial RNA: the prokaryotic past leaves its mark. Mol Cell Biol. 25, 6427-6435.

Small, I.D., and Rackham, O., and Filipovska, A., 2013. Organelle transcriptomes: products of a deconstructed genome. Curr Opin Microbiol. doi:pii: S1369-5274(13)00099-4

Soitamo, A.J., Piippo, M., Allahverdiyeva, Y., Battchikova, N., Aro, E.M., 2008. Light has a specific role in modulating Arabidopsis gene expression at low temperature. BMC Plant Biol. 8, 13.

Subramanian, S., Fallahi, M., and Bonen, L., 2001. Truncated and dispersed *rpl2* and *rps19* pseudogenes are co-transcribed with neighbouring downstream genes in wheat mitochondria. Curr Genet. 39, 264-272.

Suzuki, N., Koussevitzky, S., Mittler, R., and Miller, G., 2012. ROS and redox signaling in the response of plants to abiotic stress. Plant Cell Environ. 35, 259-270.

Tang, Z., Zhang, L., Xu, C., Yuan, S., Zhang., F., Zheng, Y., and Zhao, C., 2012. Uncovering small RNA-mediated responses to cold stress in a wheat thermosensitive genic male-sterile line by deep sequencing. Plant Physiol. 159, 721-738.

Taylor, N.L., Rudhe, C., Hulett, J.M., Lithgow, T., Glaser, E., Day, D.A., Millar, A.H., Whelan, J., 2003. Environmental stresses inhibit and stimulate different protein import pathways in plant mitochondria. FEBS Lett. 547, 125-130.

Toor, N., Hausner, G., and Zimmerly, S., 2001. Coevolution of group II intron RNA structure with their intron-encoded reverse transcriptases. RNA. 7, 1142-1152.

Toor, N., Robart, A.R., Christianson, J., and Zimmerly, S., 2006. Self-splicing of a group IIC intron: 5' exon recognition and alternative 5' splicing events implicate the stem-loop motif of a transcriptional terminator. Nucleic Acids Res. 34, 6461-6471.

Vigeland, M.D., Spannagl, M., Asp, T., Paina, C., Rudi, H., Rognli, O.A., Fjellhelm, S., and Sandve, S.R., 2013. Evidence for adaptive evolution of low-temperature stress response genes in a Pooideae grass ancestor. New Phytol. 199, 1060-1068.

Vogel, J., Börner, T., 2002. Lariat formation and a hydrolytic pathway in plant chloroplast group II intron splicing. EMBO J. 21, 3194-3802.

Wang, J., Rajakulendran, N., Amirsadeghi, S., and Vanlerberghe, GC., 2011. Impact of mitochondrial alternative oxidase expression on the response of Nicotiana tabacum to cold temperature. Physiol Plant. 142, 339-351.

Winfield, M.O., Lu, C., Wilson, I.D., Coghill, J.A., and Edwards, K.J., 2010. Plant responses to cold: Transcriptome analysis of wheat. Plant Biotechnol J. 8, 749-771.

Yao, J., Zhong, J., Fang, Y., Geisinger, E., Novick, R.P., and Lambowitz, A.M., 2006. Use of targetrons to disrupt essential and nonessential genes in Staphylococcus aureus reveals temperature sensitivity to Ll.LtrB group II intron splicing. RNA. 12, 1271-1281.

Zhelyazkova, P., Hammani, K., Rojas, M., Voelker, R., Vargas-Suarez, M., Borner, T., and Barkan, A., 2012. Protein-mediated protection as the predominant mechanism for defining processing mRNA termini in land plant chloroplasts. Nucleic Acids Res. 40, 3092-3105.

Zmudjak, M., Colas des Francs-Small, C., Keren, I., Shaya, F., Belausov, E., Small, I., Ostersetzer-Biran, O., 2013. mCSF1, a nucleus-encoded CRM protein required for the processing of many mitochondrial introns, is involved in the biogenesis of respiratory complexes I and IV in Arabidopsis. New Phytol. 199, 379-394.

Zoschke, R., Nakamura, M., Liere, K., Sugiura, M., Borner, T., and Schmitz-Linneweber, C., 2010. An organellar maturase associates with multiple group II introns. Proc Natl Acad Sci USA. 107, 3245-3250.

Appendix A

Chapter 3

Clone	Intron	Env.Condition	Ligase	RNA Prep
SD020	nad2 intron4	Room T	No	Aug 18/11
SD021	nad2 intron4	Room T	No	Aug 18/11
SD024	nad2 intron4	Room T	Yes	Aug 18/11
SD025	nad2 intron4	Room T	Yes	Aug 18/11
SD026	nad2 intron1	Room T	Yes	Aug 18/11
SD027	nad2 intron1	Room T	Yes	Aug 18/11
SD028	nad2 intron1	Room T	Yes	Aug 18/11
SD029	nad2 intron1	Cold	Yes	Oct 21/11
SD030	nad2 intron1	Cold	Yes	Oct 21/11
SD031	nad2 intron1	Cold	Yes	Oct 21/11
SD036	nad2 intron1	Room T	Yes	Aug 18/11
SD038	nad2 intron1	Cold	Yes	Dec 16/11
SD040	nad2 intron1	Cold	Yes	Dec 16/11
SD042	nad2 intron4	Room T	No	Nov 4/11
SD043	nad2 intron4	Room T	No	Nov 4/11
SD045	nad2 intron1	Cold	No	Oct 21/11
SD046	nad2 intron1	Cold	No	Oct 21/11
SD047	nad2 intron1	Cold	No	Dec 16/11
SD048	nad2 intron1	Cold	No	Dec 16/11
SD050	nad2 intron1	Room T	Yes	Nov 4/11
SD051	nad2 intron1	Room T	Yes	Nov 4/11
SD052	nad2 intron1	Room T	Yes	Nov 4/11
SD053	nad2 intron1	Room T	Yes	Nov 4/11
SD054	nad2 intron1	Room T	Yes	Dec 8/11
SD058	nad2 intron1	Room T	No	Aug 18/11
SD059	nad2 intron1	Room T	Yes	Dec 8/11
SD060	nad2 intron1	Room T	Yes	Dec 8/11
SD096	cox2 intron	Room T	No	Jan 25/12
SD097	cox2 intron	Room T	Yes	Jan 25/12
SD098	cox2 intron	Room T	Yes	Jan 25/12
SD100	cox2 intron	Cold	No	Jan 24/12
SD102	cox2 intron	Cold	Yes	Jan 24/12
SD106	nad2 intron4	Cold	No	Dec 16/11
SD107	nad2 intron4	Cold	No	Dec 16/11
SD108	nad2 intron4	Cold	Yes	Dec 16/11
SD109	nad2 intron4	Cold	Yes	Dec 16/11
SD121	cox2 intron	Room T	No	Jan 25/12
SD122	cox2 intron	Room T	No	Jan 25/12
SD123	cox2 intron	Room T	Yes	Jan 25/12
SD124	cox2 intron	Cold	No	Jan 24/12
SD126	cox2 intron	Cold	No	Jan 24/12
SD128	cox2 intron	Cold	Yes	Jan 24/12
SD129	cox2 intron	Cold	Yes	Jan 24/12
SD144	nad2 intron4	Room T	Yes	Nov 4/11
SD145	nad2 intron4	Room T	Yes	Nov 4/11
SD146	nad2 intron4	Cold	No	Jan 24/12
SD147	nad2 intron4	Cold	No	Jan 24/12
SD148	nad2 intron4	Cold	Yes	Jan 24/12
SD149	nad2 intron4	Cold	Yes	Jan 24/12
SD152	cox2 intron	Room T	No	Apr 6/12
SD153	cox2 intron	Room T	No	Apr 6/12
SD154	cox2 intron	Room T	Yes	Apr 6/12
SD155	cox2 intron	Room T	Yes	Apr 6/12
SD158	cox2 intron	Cold	No	Feb 13/12
SD159	cox2 intron	Cold	No	Feb 13/12
SD160	cox2 intron	Cold	No	Feb 13/12
SD161	cox2 intron	Cold	Yes	Jan 24/12

Chapter 4

Clone	Intron	Env.Condition	RNA Prep
SD63	nad1 intron 4	room temp	Apr 6.12
SD64	nad1 intron 4	room temp	Apr 6.12
SD67	nad1 intron 4	cold	Feb 13.12
SD69	nad1 intron 4	cold	Feb 13.12
SD87	nad1 intron 1a	room temp	May 24.12
SD88	nad1 intron 1a	room temp	May 24.12
SD89	nad1 intron 1a	room temp	May 24.12
SD90	nad1 intron 1a	room temp	May 24.12
SD91	nad1 intron 1a	cold	June 12.12
SD92	nad1 intron 1a	cold	June 12.12
SD93	nad1 intron 1a	cold	June 12.12
SD94	nad1 intron 1a	cold	June 12.12
SD111	5'mRNA-nad1i1a	room temp	May 29.12
SD112	5'mRNA-nad1i1a	room temp	May 29.12
SD113	5'mRNA-nad1i1a	room temp	May 29.12
SD114	5'mRNA-nad1i1a	cold	June 22.12
SD115	5'mRNA-nad1i1a	cold	June 22.12
SD116	5'mRNA-nad1i1a	cold	June 22.12
SD117	5'trnP-nad1i1a	cold	June 22.12
SD118	5'trnP-nad1i1a	cold	June 22.12
SD119	5'trnP-nad1i1a	cold	June 22.12
SD120	5'trnP-nad1i1a	cold	June 22.12
SD131	3'trnP-nad1i1a	room temp	May 29.12
SD132	3'trnP-nad1i1a	room temp	May 29.12
SD133	3'trnP-nad1i1a	room temp	May 29.12
SD134	3'trnP-nad1i1a	cold	June 22.12
SD138	3'trnP-nad1i1a	cold	June 22.12
SD139	3'trnP-nad1i1a	cold	June 22.12
SD140	3'atp6-nad1ib	room temp	Sept 18.12
SD141	3'atp6-nad1ib	room temp	Sept 18.12
SD193	3'atp6-nad1ib	room temp	Sept 18.12
SD194	3'atp6-nad1ib	room temp	Sept 18.12
SD195	3'atp6-nad1ib	cold	Sept 1.12
SD196	3'atp6-nad1ib	cold	Sept 1.12
SD197	3'atp6-nad1ib	cold	Sept 1.12
SD198	3'atp6-nad1ib	cold	Sept 1.12
SD199	nad1 intron 1a	room temp	Sept 20.12
SD200	nad1 intron 1a	room temp	Sept 20.12
SD201	nad1 intron 1a	room temp	Sept 20.12
SD202	nad1 intron 1a	room temp	Sept 20.12
SD203	nad1 intron 4	room temp	Sept 3.12
SD204	nad1 intron 4	room temp	Sept 3.12
SD205	nad1 intron 4	cold	Sept 3.12
SD206	nad1 intron 4	cold	Sept 3.12
SD207	short nad1ib	room temp	Sept 20.12
SD208	short nad1ib	room temp	Sept 20.12
SD209	short nad1ib	room temp	Sept 20.12
SD210	short nad1ib	room temp	Sept 20.12
SD211	short nad1ib	cold	Sept 3.12
SD212	short nad1ib	cold	Sept 3.12
SD213	short nad1ib	cold	Sept 3.12
SD214	short nad1ib	cold	Sept 3.12
SD215	5'trnP-nad1i1a	cold	Sept 3.12
SD216	5'trnP-nad1i1a	cold	Sept 3.12
SD217	3'trnP-nad1i1a	room temp	Sept 20.12
SD218	3'trnP-nad1i1a	room temp	Sept 20.12
SD219	3'trnP-nad1i1a	room temp	Sept 20.12

SD162	cox2 intron	Cold	Yes	Feb 13/12	SD220	3'trnP-nad1i1a	cold	Nov 2.12
SD163	cox2 intron	Cold	Yes	Feb 13/12	SD221	3'trnP-nad1i1a	cold	Nov 2.12
SD164	cox2 intron	Cold	Yes	Feb 13/12	SD222	3'trnP-nad1i1a	cold	Nov 2.12
SD165	cox2 intron	Cold	Yes	Feb 13/12	SD223	3'trnP-nad1i1a	cold	Nov 2.12
SD166	nad2 intron1	Room T	No	Sept 20/12	SD224	3'trnP-nad1i1a	cold	Nov 2.12
SD167	nad2 intron1	Room T	No	Sept 20/12	SD225	5'mRNA-nad1i1a	room temp	Nov 29.12
SD168	nad2 intron1	Room T	No	Sept 20/12	SD226	5'mRNA-nad1i1a	room temp	Nov 29.12
SD169	nad2 intron1	Room T	No	Sept 20/12	SD227	5'mRNA-nad1i1a	room temp	Nov 29.12
SD170	nad1 intron2	Room T	No	Sept 20/12	SD228	5'mRNA-nad1i1a	cold	Nov 2.12
SD171	nad1 intron2	Room T	No	Sept 20/12	SD229	5'mRNA-nad1i1a	cold	Nov 2.12
SD172	nad1 intron2	Room T	No	Sept 20/12	SD230	5'mRNA-nad1i1a	cold	Nov 2.12
SD173	nad1 intron2	Room T	Yes	Sept 20/12	SD231	short nad1ib	room temp	Mar 5.13
SD174	nad1 intron2	Room T	Yes	Sept 20/12	SD232	short nad1ib	room temp	Mar 5.13
SD175	nad1 intron2	Room T	Yes	Sept 20/12	SD233	short nad1ib	room temp	Mar 5.13
SD176	nad1 intron2	Cold	No	Sept 3/12	SD234	short nad1ib	room temp	Mar 5.13
SD177	nad1 intron2	Cold	No	Sept 3/12	SD235	short nad1ib	cold	Feb 17.13
SD178	nad1 intron2	Cold	No	Sept 3/12	SD236	short nad1ib	cold	Feb 17.13
SD179	nad1 intron2	Cold	No	Sept 3/12	SD237	short nad1ib	cold	Feb 17.13
SD180	nad1 intron2	Cold	No	Nov 4/11	SD238	short nad1ib	cold	Feb 17.13
SD181	nad1 intron2	Cold	No	Nov 4/11	SD239	nad1 intron 1a	cold	Sept 3.12
SD182	nad1 intron2	Cold	Yes	Sept 3/12	SD240	nad1 intron 1a	cold	Sept 3.12
SD183	nad1 intron2	Cold	Yes	Sept 3/12	SD241	nad1 intron 1a	cold	Sept 3.12
SD184	nad1 intron2	Room T	No	Nov 29/12	SD242	nad1 intron 1a	cold	Sept 3.12
SD185	nad1 intron2	Room T	No	Nov 29/12				
SD186	nad1 intron2	Room T	No	Nov 29/12				
SD186	nad1 intron2	Room T	Yes	Nov 29/12				
SD187	nad1 intron2	Room T	Yes	Nov 29/12				
SD188	nad1 intron2	Room T	Yes	Nov 29/12				
SD189	nad1 intron2	Cold	Yes	Sept 3/12				
SD190	nad1 intron2	Cold	Yes	Sept 3/12				
SD191	nad1 intron2	Cold	Yes	Nov 4/11				
SD192	nad1 intron2	Cold	Yes	Nov 4/11				