Multiple trans-splicing events are required to produce a mature nad1 transcript in a plant mitochondrion

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The mitochondrial gene encoding NADH dehydrogenase subunit 1 (nad1) in Petunia hybrida is split into five exons, a, b, c, d, and e. With the use of a complete restriction map of the 443-kb Petunia mitochondrial genome, we have cloned these exons and mapped their location. Exon a is located 130 kb away from and in the opposite orientation from exons b and c. Exon d maps 95 kb away and in the opposite orientation from exons b and c. Exons d and e are separated by 190 kb. By performing the polymerase chain reaction on Petunia cDNAs, we have shown that transcripts from these five exons are joined via a series of cis- and trans-splicing events to create a mature nad1 transcript. In addition, we have found 23 C → U RNA edit sites in Petunia nad1. RNA editing changes 19 of the amino acids predicted by the genomic sequence.

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Several different RNA processing events can occur during the maturation of a protein-coding plant mitochondrial transcript. These events distinguish plant mitochondria from their mammalian counterparts and include RNA editing, cis-splicing and, as we will describe here, trans-splicing.

RNA editing has been shown to occur in many, if not all, plant mitochondrial protein-coding genes; specific cytidines in the DNA sequence are altered in the RNA and appear as thymidines in the cDNAs that are isolated from these genes [Covello and Gray 1989; Gualberto et al. 1989; Hiesel et al. 1989]. This is consistent with a process that alters cytidine into uridine residues, although uridine in place of cytidine in edited RNAs has not been directly demonstrated. The extent of RNA editing varies from gene to gene. Ten cytidines are edited in 75 codons of Petunia hybrida atp9 [Wintz and Hanson 1991], whereas only 2 are edited within 99 codons of Oenothera rps14 [Schuster et al. 1990]. RNA edits usually alter the protein sequence from that predicted from the DNA sequence.

Introns have been described in several plant mitochondrial genes. A group II intron splits the coxl gene in several, but not all, plant species that have been examined [Fox and Leaver 1981; Hiesel and Brennicke 1983; Turano et al. 1987; Pruitt and Hanson 1989]. The Oenothera nad5-coding sequence is separated by two introns, one of which is clearly of the group II intron class [Wissinger et al. 1988]. In maize [Hunt and Newton 1991] and Petunia [C. Sutton et al., unpubl.], the proposed rps3 gene also contains an intron.

The higher plant mitochondrial gene that encodes NADH-dehydrogenase subunit I (nad1) also contains introns. In contrast, nad1 is encoded by a single open reading frame (ORF) in the mammalian [Chomyn et al. 1985], Drosophila [Clary et al. 1984], and Chlamydomonas [Boer and Gray 1988] mitochondrial genomes. In Neurospora mitochondria, nad1 is interrupted by a group I intron [Burger and Werner 1985]. Four exons that encode different regions of the NAD1 protein have been described in higher plant mitochondrial genomes. Two exons that encode the central portion of NAD1 were found in watermelon. These nad1 exons are separated by a group II intron of ~1450 bp [Stern et al. 1986]. The first of these two exons was also described in tobacco and maize [Bland et al. 1986]. Two additional nad1 exons, predicted to encode the carboxy-terminal portion of nad1, were recently reported in the broad bean mitochondrial genome. Two group II intron-like domains were found upstream of the first of these two exons. Within the group II intron that separates these broad bean nad1 exons, a maturase-like gene [mat-r] was found. The protein sequence predicted from this gene is similar to the maturases encoded within fungal mitochondrial introns [Wahleithner et al. 1990]. As yet, it is not known whether the plant mat-r sequences actually specify a protein with maturase activity.

We commenced to clone the P. hybrida nad1 exons using probes from both watermelon and broad bean. In addition to the exons described above, we cloned and mapped an exon that encodes the amino-terminal portion of NAD1 with the use of a probe from the equivalent wheat exon [Chapdelaine and Bonen 1991]. A complete restriction map of the Petunia mitochondrial ge-
We were able to map the location and orientation of the nad1 exons on the Petunia mitochondrial genome with the use of this restriction mapping data. Our mapping data indicate that the five nad1 exons are dispersed around the 443-kb Petunia mitochondrial genome. With the use of the polymerase chain reaction (PCR) on reverse-transcribed mitochondrial RNA, we have shown that a series of trans- and cis-splicing events, in addition to RNA editing, are necessary to create a mature nad1 transcript. nad1 is the first example of a trans-spliced mitochondrial gene.

Results

Cloning the Petunia nad1 exons

Four of the five nad1 exons encoded by the P. hybrida line 3704 mitochondrial genome were found by virtue of their similarity to nad1 exons encoded by other higher plant mitochondrial genomes. Determination of the complete sequence of Petunia nad1 was begun by cloning nad1 exon c from a cosmid library of Petunia mitochondrial DNA with the use of a random hexamer-labeled watermelon URF1 (exon c) probe, kindly provided by D. Stern. By restriction enzyme and sequence analysis, nad1 exon b was found upstream of exon c, in an arrangement very similar to that of the analogous nad1 exons described in watermelon [Stern et al. 1986]. The sequence of Petunia nad1 exons b and c, along with the intron that separates them, is shown in Figure 1. The DNA sequence of these Petunia nad1 exons is 100% and 99% identical to that of watermelon exons b and c published previously [Stern et al. 1986]. The sequence of the 986-bp intron that separates exons b and c in Petunia is quite similar to the analogous intron in watermelon, except that a 459-bp insertion is present in the watermelon intron relative to Petunia. The position of this insertion is noted in Figure 1.

DNA probes from the broad bean terminal exon and the mat-r gene were then utilized to clone these coding regions from a Petunia mitochondrial cosmid library with the help of D.R. Wolstenholme. The sequence of the Petunia nad1 terminal exon (exon c) is shown in Figure 1. At the DNA level, this Petunia exon is 95% identical to the broad bean terminal exon published previously [Wahleithner et al. 1990]. The sequenced regions of the Petunia mat-r gene are nearly identical to the broad bean mat-r sequence [C. Conley and J. Hampton, unpubl.].

Comparison of the amino acid composition of the protein products predicted from nad1 exons b and c and the terminal exon of Petunia to the nad1 protein from human mitochondria [Anderson et al. 1981] made it evident that protein sequences in addition to those predicted by the cloned Petunia exons were necessary to encode a full-length NAD1 protein. A small region of protein sequence carboxy-terminal to exon c and the amino terminus of NAD1 were not encoded by exons b and c and the terminal exon [comparison not shown].

To obtain the Petunia nad1 exon(s) that encode the portion of the NAD1 protein between exon c and the terminal exon, a PCR on reverse-transcribed transcripts was undertaken. Two oligonucleotides were made as primers: The first was homologous to a sequence at the 5′ end of exon b [primer b], and the second was homologous to a sequence downstream of the terminal exon [primer c]. The exact sequence and orientation of these primers is noted in Figure 1. A PCR amplification was carried out on random hexamer-primed cDNAs from purified suspension cell mitochondrial RNA. The amplification product obtained using the exon b/e primer set was ~650 bp [data not shown]. This PCR-amplified cDNA was subcloned and sequenced. A small exon [exon d] was found spliced in-frame between exons c and the terminal exon [exon e]. This small exon found in Petunia is 98% identical at the DNA level to a small nad1 exon found upstream of mat-r in broad bean [Wahleithner et al. 1990]. The sequence of Petunia nad1 exon d is presented in Figure 1.

Between the Petunia nad1 exon d and the mat-r sequence, an unidentified ORF was found that is 96% identical to the ORF85 found downstream of the equivalent exon in broad bean nad1 [Wahleithner et al. 1990]. The Petunia ORF [ORF85P] contains 92 codons in contrast with the 85 codons in the broad bean ORF.

While the work in Petunia nad1 was in progress, an exon [exon a], which encodes the amino terminus of NAD1, was isolated in both the wheat [Chapdelaine and Bonen 1991] and Oenothera [Wissinger et al. 1991] mitochondrial genomes. An oligonucleotide homologous to the wheat exon a [sequence provided by L. Bonen] was used as a probe to clone Petunia nad1 exon a from the cosmid library of Petunia mitochondrial DNA. The sequence of the Petunia nad1 exon a is shown in Figure 1.

Restriction maps of the five Petunia nad1 exons are shown in Figure 2A. A DNA fragment from each of these exons was random hexamer-labeled and used as a probe in Southern blot DNA hybridization. The specific exon probes are shown as lines under the restriction maps of each exon. Purified Petunia line 3704 mitochondrial DNA was digested with BamHI, separated on an agarose gel, and blotted to a nylon membrane. Identical lanes were probed with the five labeled exon probes. The au-
Trans-splicing of Petunia nad1

Figure 1. (See facing page for legend.)
toradiographs from these hybridizations are shown in Figure 2B. It is clear that each nad1 exon is present in a single copy in the Petunia mitochondrial genome.

Mapping of the Petunia nad1 exons

A restriction map of the entire P. hybrida line 3704 mitochondrial genome has been described previously (Folkerts and Hanson 1989). This higher plant mitochondrial genome is 443 kb in size. The location and orientation from 5' to 3' of the five nad1 exons are shown on the Petunia mitochondrial genome in Figure 3. The nad1 exons are dispersed relative to each other around this circular genome: Exon a is located 130 kb and in the opposite orientation of exons b and c; exon d maps 95 kb away and in the opposite orientation from exons b and c; exons d and e are separated by 190 kb.

Trans-splicing of nad1

Because of the relative location and orientation of the nad1 exons, separate transcripts must be trans-spliced to create a mature nad1 mRNA. This model is shown schematically in Figure 4. Three trans-splicing events (denoted by the solid lines) and one cis-splicing event (broken line) are proposed as necessary to create a mature nad1 transcript from four RNA precursors.

The PCR-amplified cDNA described earlier, which contained spliced Petunia nad1 exons b–e, supports this trans-splicing model. This model was tested further by using PCR on reverse-transcribed transcripts to amplify a full-length nad1 cDNA containing all five exons. Two oligonucleotide primers were used: The first was homologous to a sequence just upstream of exon a (primer a), and the second was primer e (described earlier). The exact sequence and orientation of these primers is noted in Figure 1. A PCR amplification was carried out using the primer a/e set on random hexamer-primed cDNAs made from total leaf RNA from Petunia line 3704. Using the a/e primer set, the amplification product was ~1 kb, the expected size for an amplified cDNA containing nad1 exons a–e (data not shown). No amplification product was obtained when either the b/e or a/e primer sets were used to amplify Petunia mitochondrial (genomic) DNA. The PCR-amplified cDNAs were subcloned and sequenced to define the precise splice sites. Five PCR-derived subclones containing the exon a/exon e splice junction, eight containing the exon b/exon c splice junction, and three containing the exon c/exon d and exon d/exon e splice junctions were sequenced. Arrows in Figure 1 indicate the splice sites at each exon/intron junction.

The intron that separates nad1 exon b and c has been identified previously as a group II intron. Group II intron-like domains have also been identified upstream of the broad bean nad1 exons d and e (Wahleithner et al. 1990). The involvement of group II intron domains in trans-splicing in plant organelles is not without precedent. Trans-splicing events that involve group II introns have been reported to occur in the chloroplast genes rps12 (Koller et al. 1987; Zaita et al. 1987; Kohchi 1988) and psaA (Kuck et al. 1987).

Group II introns usually contain a 5'-GYGCG-3' motif just downstream of the 5'-splice site and a 5'-YAY-3' motif just upstream of the 3'-splice site (Michel et al. 1989). At the splice junction of Petunia nad1 exon a and exon b, there is a 2-base redundancy that makes assignment of the exact splice junction impossible from the cDNA sequence alone. It is believed that the splice sites are as shown in Figure 1 because of the occurrence of a 5'-TAT-3' motif present at this proposed 3'-splice site. This same motif is present at the 3'-splice site upstream of exon c. The 5'-GYGCG-3' motif is also present at the 5'-splice site downstream of exons b, c, and d. It is not present downstream of the 5'-splice site of exon a.

Group II introns are composed of six helical domains (Michel et al. 1989). Trans-splicing of the two exons in rps12 in chloroplasts is thought to occur via reconstitution of a group II intron through base-pairing of the two halves of a split domain III (Kohchi et al. 1988). Domain IV is highly variable and has also been found as a split domain in the trans-spliced Chlamydomonas chloroplast gene, psaA (Goldschmidt-Clermont et al. 1991).
Trans-splicing of *Petunia nadl*

**Figure 3.** Location of the five *nadl* exons and *mat-r* on the 443-kb *Petunia hybrida* line 3704 mitochondrial genome. Arrows indicate the orientation 5'→3' of the *nadl*-coding regions, which are shown in bold print. The three copies of a recombination repeat that exist in this genome are designated by solid boxes. Previously mapped plant mitochondrial genes (Folkerts and Hanson 1989, Conklin and Hanson 1991) are shown for reference.

wheat *nadl*, group II intron domains IV and V have been modeled on the basis of sequences present in the flanking regions downstream of exons a–d and upstream of exons b–e (Chapdelaine and Bonen 1991). Domains IV and V of the cis-spliced group II intron separating the *Petunia* exons b and c are identical at the sequence level to those proposed in wheat and are therefore not shown. In wheat *nadl*, domain IV has been proposed to be the split domain in all three trans-splicing events (Chapdelaine and Bonen 1991). The 5’ or upstream portion of domain IV is encoded by sequences downstream of exons a, c, and d, whereas the 3’ portion of domain IV is present upstream of exons b, d, and e. In *Petunia nadl*, sequences identical to the 3’ portion of the proposed wheat domain IV structures are present. A schematic of the proposed *Petunia nadl* domain V structures present upstream of exons b, d, and c is shown in Figure 5. Unspliced *Petunia nadl* cDNAs that represent primary transcripts of exons b, c, and e contain the group II intron domains described above [data not shown]. An unspliced exon d cDNA has not yet been isolated and sequenced.

The predicted domain V upstream of exon b is aberrant in that it contains 18 nucleotides in the loop rather than the 4 commonly present at this position. The wheat domain V is also unusual in containing 17 nucleotides in this loop (Chapdelaine and Bonen 1991). The circled bases in Figure 5 are those substituted at the indicated position in the wheat sequence. In each of the four cases where the *Petunia* sequence differs from wheat, the stem of domain V is maintained. In addition, these three proposed intron domains all contain a bulging 3'-GC-5' on the 3’ side of the stem. A bulging 3'-GY-5' at this position is common in a subgroup [IIB] of group II introns (Michel et al. 1989). These data strengthen the prediction that these are true group II intron domains.

**Figure 4.** Schematic of the splicing events proposed to occur for the creation of a mature *nadl* transcript. The *nadl* exons are designated by open boxes. The exons that map to different locations on the *Petunia* mitochondrial genome are shown as separate transcripts. Exons b and c are linked and are therefore shown on the same transcript. Solid lines indicate the three proposed trans-splicing events; broken lines indicate the cis-splicing event.

**RNA editing in Petunia nadl**

The sequences from the PCR-amplified cDNAs (described earlier) and from *nadl* cDNAs (not shown; Sutton et al. 1991) were compared to the genomic sequence of *nadl* to determine the extent of RNA editing. All of the sequenced cDNAs were only partially edited. A total of 27 C→U editing sites were found within the five exons of *Petunia nadl*. In addition, one site of editing resides 10 nucleotides upstream of the 3’-splice site of exon e. This C→U change is actually predicted to weaken the stem structure of a proposed group II intron domain VI at this position [not shown]. Of the 27 edits within the coding region, 7 are silent and do not change the predicted amino acid. The remainder of the edited sites change 19 predicted amino acids, or 6% of the *nadl* codons. The 19 codons that are changed because of editing are not randomly distributed throughout the five
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Figure 5. Structure of the group II intron domain V (d5) present upstream of the coding sequence of nadl exon b (a/b), exon d (c/d), and exon e (d/e). The distance of these domains in nucleotides from the 3' splice sites is noted. Nucleotide substitutions present in wheat (Chapdelaine and Bonen 1991) relative to the analogous position in Petunia are circled.

nad1 exons. RNA editing alters ~2% of the codons in exon a, 4% in exon b, 8% in exon c, 10% in exon d, and 9% in exon e. The sites of RNA editing are shown in Figure 1, with the altered amino acids shown above the amino acids predicted by the genomic sequence.

The temporal relationship between both cis- and trans-splicing of Petunia nadl transcripts and RNA editing was reported previously. Briefly, we found that RNA editing can precede the splicing of Petunia nadl transcripts; cDNAs representing splicing intermediates were edited (Sutton et al. 1991).

Comparison of Petunia nadl with human NAD1

The similarity between Petunia and human NAD1 is increased slightly because of RNA editing. A comparison of the deduced Petunia NAD1 protein with that of human NAD1 (Chomyn et al. 1985) is shown in Figure 6. The 19 amino acids changed as a result of RNA editing are indicated. The amino acid identity of the edited Petunia protein with that of human NAD1 is 46%, whereas that with the unedited Petunia protein is 43%. Of the 19 amino acid changes in Petunia NAD1, 10 result in amino acids that are identical to human NAD1 at the analogous position and 4 result in conservative changes relative to the human protein.

Discussion

We have cloned and sequenced the five nadl exons present in the P. hybrida mitochondrial genome. Unlike the nadl exons that have been identified in other plant mitochondrial genomes, the nadl exons of Petunia could be oriented on a complete restriction map of the mitochondrial genome. The Petunia nadl exons are dispersed over the 443-kb mitochondrial genome, with up to 140 kb separating individual exons.

The arrangement of Petunia nadl exons d and e relative to mat-r is unique in comparison with the reported arrangement of these coding regions in other higher plant mitochondrial genomes. In Petunia, exon d and mat-r are linked and are separated by only a few hundred bases. Petunia exon e is unlinked to exon d and mat-r. In contrast, exon d, mat-r, and exon e are present at a single locus in the broad bean mitochondrial genome with exon e located downstream of mat-r (Wahleithner et al. 1990). The relative locations of exon d, mat-r, and exon e are different in wheat than in both broad bean and Petunia.

Figure 6. An alignment of Petunia NAD1 with that of human NAD1 (Chomyn et al. 1985). The Petunia sequence was deduced from a composite cDNA sequence derived from the sequence of several nadl cDNAs. The 19 amino acids in the Petunia protein that were changed as a result of RNA editing are indicated by asterisks (*). Arrows highlight the exon/exon borders in Petunia NAD1. This alignment was performed by the method of Needleman and Wunsch (1970). Gaps were introduced in human nadl to improve the alignment.
mat-r and exon e are linked and exon d is unlinked [Chapdelaine and Bonen 1991]. The arrangement of these coding regions in *Petunia*, broad bean, and wheat is shown schematically in Figure 7.

Although the relative positions of *nad1* exon d, mat-r, and *nad1* exon e appear to be quite plastic, this does not appear to be the case for the location of *nad1* exons b and c relative to the plant mitochondrial gene, rps13. In *Petunia* (see Fig. 3), wheat [Chapdelaine and Bonen 1991], tobacco, and maize [Bland et al. 1986], rps13 is located just upstream of *nad1* exon b.

Results from the analysis of PCR-amplified cDNAs show that transcripts containing each of the five *nad1* exons are spliced together, in the correct order, to create a mature *nad1* transcript. One cis-splicing and three trans-splicing events are necessary to generate this mature transcript.

In addition to splicing, *Petunia nad1* cDNAs undergo the process of RNA editing. We have shown previously that this editing can occur prior to both cis- and trans-splicing [Sutton et al. 1991]. None of the sequenced *nad1* cDNAs were fully edited at all 28 RNA edit sites. The presence or absence of partially edited transcripts appears to depend on the gene under study. No partially edited *Petunia atp9* transcripts were found [Wintz and Hanson 1991], whereas all of the examined *Oenothera nad3* transcripts are partially edited [Schuster et al. 1990].

RNA trans-splicing has been demonstrated previously in both nuclear and chloroplast systems. In trypanosomes and the nematode *Caenorhabditis elegans*, a short leader RNA is trans-spliced to the 5' end of many, if not all, mRNAs [Murphy et al. 1986; Sutton and Boothroyd 1986; Krause and Hirsch 1987]. In *Marchantia* the rps12 gene is composed of three exons; a transcript containing exon 1 is trans-spliced to exon 2 via a group II intron split in domain III [Kohchi et al. 1988]. In a second example, the *psaA* gene in the *Chlamydomonas reinhardtii* chloroplast is split into three exons [Kuck et al. 1987]. A small RNA encoded by the gene *tscA* has recently been described, which is thought to bridge the two parts of the first intron by base-pairing to the separating exon 1 and exon 2 exons. In the case of exons 2 and 3, trans-splicing is thought to occur via a group II intron split in domain IV [Goldschmidt-Clermont et al. 1991].

Group II introns also seem to be involved in the trans-splicing of *nad1*. Group II intron domains V and part of IV have been identified upstream of exons b, d, and e in wheat [Chapdelaine and Bonen 1991] and are also present in *Petunia*. Although single-base changes between *Petunia* and wheat occur in the proposed domain V structure, the changes all preserve the integrity of these domains. The two intron halves that separate exons a/b, c/d, and d/e in *Petunia nad1* may form a complete (split) group II intron, but we have not yet demonstrated the presence or absence of domains I, II, III, and the 5' half of domain IV downstream of exons a, c, and d. It is possible that a small RNA (or RNAs) similar to the product of *tscA* in *Chlamydomonas* may contribute to form complete group II introns in the trans-splicing of the *nad1* exons. Several gene products may be involved in the maturation of the *nad1* transcript. At least 14 nuclear genes are involved in *Chlamydomonas psaA* RNA trans-splicing in addition to the chloroplast-encoded *tscA* [Goldschmidt-Clermont et al. 1991].

The temporal progression of the trans- and cis-splicing events that occur to form a mature *nad1* transcript will be interesting to determine. We have begun to study this question with the information gained from cDNAs that represent intermediates in the splicing process. A *nad1* cDNA has been isolated that contains exons b and c and the cis-spliced intron that separates them, and exon d trans-spliced to exon c. In a second cDNA, exons b and c were cis-spliced, yet exon d was not trans-spliced to exon c (data not shown). This information suggests that the trans-splicing of exon d to exon c can occur before or after the cis-splicing of exons b and c. Additional cDNAs must be examined to determine whether this lack of a specific splicing progression is generally true for all the splicing events that occur to form a mature *nad1* transcript. As yet, we have not detected any cDNAs exhibiting aberrant exon order. Characterization of additional cDNAs should also reveal whether the correct exon order is rigorously maintained during trans-splicing.

Materials and methods

Oligonucleotides

The following oligonucleotides were utilized in the analysis of *Petunia nad1*: primer a, 5'-CCTCCCTTTAATCTATGATTGGTGTC-3'; oligo a3', 5'-CCTGCTATAATTATTCCATAAACA-3'; primer b, 5'-GTTGGGAACCTTTCACTC-3'; primer e, 5'-CTTCCATTACTAAGGGC-3'.

Cloning and mapping of *Petunia nad1* exons

Cosmid DNAs that cover the *P. hybrida* line 3704 mitochondrial genome [Folkerts and Hanson 1989] were digested with *BamH*I and separated on an agarose gel. The DNAs were then blotted to a nylon membrane using standard procedures [Maniatis et al. 1982] and probed with a random hexamer-labeled watermelon URF1 [exon c] fragment (kindly provided by D. Stern) at 60°C overnight in 5× SSC, 0.02% SDS, and 2× Denhardt’s solution. The membrane was then washed at 60°C in 0.05× SSC/0.1% SDS. A 1.8-kb *BamH*I fragment containing exon c was cloned from cosmid 488. In addition, the following *SstI* restriction fragments from this cosmid containing portions

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**Figure 7.** A schematic of the relative arrangement of *nad1* exons d and e and mat-r in three different plant mitochondrial genomes: *Petunia*, broad bean [Wahllethner et al. 1990], and wheat [Chapdelaine and Bonen 1991]. Coding regions that are closely linked are shown on one contiguous line; unlinked coding regions are shown separated by a gap.
of the *Petunia nadi* exons b and c were isolated and subcloned: 0.9-, 4.2-, and 7.5-kb SstI.

A nylon membrane containing cosmids DNAs from the *Petunia* mitochondrial genome [as described above] was sent to D.R. Wolstenholme who kindly probed the blot with radioactively labeled and used to probe the cosmid blot described above. After hybridization overnight at 65°C, the blot was washed in 2× SSC, 0.1% SDS, at 65°C. A 6.0-kb *BamHI* fragment from cosmid 3B9, which contains exon e, and a 0.8-kb *BamHI* fragment from cosmid 3B9, which contained *mat-r*, were subsequently subcloned for further analysis. A 360-bp SstI-[EcoRV] cDNA subclone of exon d [as well as part of exon c and all of exon e] and derived from a PCR-amplified cDNA described below was utilized to clone the genomic *Petunia nadi* exon d. This subclone was random hexamer-labeled and used to probe the cosmid blot described above. After hybridization overnight at 65°C, the blot was washed in 2× SSC, 0.1% SDS, at 65°C. A 6.0-kb *BamHI* fragment from cosmid 3B9, which contains exon d, was then subcloned.

To clone *nadi* exon a from *Petunia*, 200 ng of the oligonucleotide a3' (oligo a3') derived from wheat exon a (the sequence for which was provided by L. Bonen) was labeled with the use of T4 polynucleotide kinase [BRL] and utilized as a probe on the *Petunia* mitochondrial cosmid blot described above. Prehybridization, hybridization, and washes were carried out according to a previously outlined procedure [Ausubel et al. 1989]. A 12.3-kb *BamHI* fragment containing *Petunia* exon a was subcloned from cosmid 1B7.

**Sequence analysis**

Fragments of interest were subcloned into Bluescript (Stratagene) for sequencing. Oligonucleotides used as sequencing primers included the standard M13[−20] and M13(reverse) primers as well as primers a and e and oligo a3'. A dideoxy double-stranded sequencing procedure obtained from U.S. Biochemical for Sequenase version 2.0 was used in the sequence analysis. Both strands of the presented genomic sequences for the five *Petunia nadi* exons were sequenced. One strand from ORP8SP and from each of the following PCR-derived cDNA subclones were sequenced: five cDNA subclones containing exons a and b and the first 132 bp of exon c; three containing exon b and the first 132 bp of exon c; two containing the last 56 bp of exons c, d, and e; and three containing the last 210 bp of exon e. In addition, several *nadi* cDNAs were sequenced as described previously [Sutton et al. 1991].

**PCR**

Suspension cell mitochondria from *P. hybrida* line 3704 were isolated following the procedure described previously [Hanson et al. 1986]. Mitochondria were then lysed in 0.5% sarcosyl, followed by phenol/chloroform extraction and ethanol precipitation to recover total nucleic acids. DNA was removed by using DNase Q1 [Promega]. cDNAs were made by adding 5 μg of mitochondrial RNA to 120 ng of random hexamers, 15 units of RNAsin [Promega], 1 mm of each deoxynucleotide, and 1× *Taq* polymerase buffer [Cetus] in the presence of 10 units of AMV–reverse transcriptase [U.S. Biochemical] for 1 hr at 42°C. After 10 min at 65°C, 2.5 units of *Taq* polymerase [Cetus] and 300 ng each of primers b and e were added to the cDNA reaction to a final volume of 100 μl. The PCR was performed on a Hybaid cycler under the following conditions: cycle 1, 2 min at 92°C; cycle 2, 30 sec at 92°C, 1 min at 55°C, and 2 min at 72°C [repeat 25×]; cycle 3, 10 min at 72°C.

PCR amplification of *nadi* cDNAs with primer set a/e was performed as described above with the following change: Total leaf RNA, isolated by a standard method [Ausubel et al. 1989], was used as the starting material. cDNAs from 10 μg of total leaf RNA were made with murine leukemia virus [M-MLV]–reverse transcriptase [BRL] in the buffer recommended by BRL. The total cDNAs were then purified by a phenol–chloroform extraction, followed by ethanol precipitation prior to the PCR.

Control PCRs were performed on total mitochondrial DNA that was isolated as described below. Primer sets a/e and b/e were used in PCR with 1 μg of mitochondrial DNA with the same amplification conditions as were used to amplify the cDNAs.

**Total mitochondrial DNA hybridization**

Mitochondrial DNA was isolated from *P. hybrida* line 3704 following the procedure described previously [Hanson et al. 1986]. After 7.5 μg of mitochondrial DNA was digested with *BamHI*, it was separated into five aliquots and loaded onto five lanes of a 1% agarose gel. After electrophoresis, the digested DNA was blotted onto a nylon membrane. The blot was cut into five strips and each strip was separately probed with the following random hexamer-labeled DNA fragments: 0.8-kb *HincII* (exon a); 0.7-kb *XhoI*–*BamHI* (exon b); 1.2-kb *XhoI*–*BamHI* (exon c); 0.62-kb *XhoI*–*BamHI* (exon d); and 0.3-kb *BglII* (exon e). DNA hybridization was carried out in a standard buffer [Maniatis et al. 1982], and washes were performed at 65°C in 0.2× SSC and 0.1% SDS.

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**Note added in proof**

Sequence data described in this paper have been submitted to the EMBL/GenBank Data Libraries.

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