Elimination of a group II intron from a plastid gene causes a mutant phenotype

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ABSTRACT

Group II introns are found in bacteria and cell organelles (plastids, mitochondria) and are thought to represent the evolutionary ancestors of spliceosomal introns. It is generally believed that group II introns are selfish genetic elements that do not have any function. Here, we have scrutinized this assumption by analyzing two group II introns that interrupt a plastid gene (ycf3) involved in photosystem assembly. Using stable transformation of the plastid genome, we have generated mutant plants that lack either intron 1 or intron 2 or both. Interestingly, the deletion of intron 1 caused a strong mutant phenotype. We show that the mutants are deficient in photosystem I and that this deficiency is directly related to impaired ycf3 function. We further show that, upon deletion of intron 1, the splicing of intron 2 is strongly inhibited. Our data demonstrate that (i) the loss of a group II intron is not necessarily phenotypically neutral and (ii) the splicing of one intron can depend on the presence of another.

INTRODUCTION

Expression of a number of genes in plant mitochondria and plastids (chloroplasts) is dependent on removal of intervening sequences (introns) that interrupt the coding sequences. Intron excision from the primary transcript by splicing represents a prerequisite for translation of the messenger RNA (mRNA) into the correct full-length protein. The vast majority of introns in the organellar genomes of seed plants are so-called group II introns (1,2), but a few group I introns have also been found (3–5). Both group I and group II introns represent catalytically active RNAs (ribozymes) and are often also referred to as self-splicing introns (6–10). At least some group I (6,8) and group II (11,12) introns have the ability to undergo self-splicing in vitro in the absence of any help from protein factors. However, this appears not to be the case for most, if not all, group II introns in the organellar genomes of vascular plants. Their splicing seems to be strictly dependent on the assistance of proteinaceous splicing factors (13–15). Most splicing factors for group II introns in plant organellar genomes are encoded in the nuclear genome and post-translationally imported into plastids or mitochondria (14,16–21). However, a few splicing factors, also called intron maturases, are encoded by the plastid or mitochondrial genomes themselves (22,23). Typically, these maturase ORFs reside within intronic sequences suggesting that the splicing factor is produced by translation of the excised intron. In addition to their splicing activity, some group II introns behave as mobile genetic elements and are capable of inserting themselves into intron-less alleles, a process referred to as intron homing (24).

Group II introns adopt a typical six-domain secondary structure (1,2,5,26,10) and one assumed function of splicing factors is to help fold the intron RNA into the catalytically active secondary and tertiary structures. Splicing involves two sequential transesterification reactions. First, the 2’OH group of the branch-point nucleotide in domain VI performs a nucleophilic attack on the 5’ splice site forming a circular RNA intermediate called the ‘lariat’. Subsequently, the free 3’OH group of the released 5’ exon performs a nucleophilic attack at the 3’ splice site resulting in exon ligation and release of the intron lariat. This chemical mechanism is conserved in the splicing of spliceosomal introns present in the nuclear genomes of all eukaryotes and, therefore, group II introns are thought to represent the evolutionary ancestors of spliceosomal introns (27,28).

Group II introns are considered to be selfish genetic elements that have no other function besides their own removal from the pre-mRNA. Here we show that this assumption is not generally correct. We demonstrate that elimination of a group II intron from a plastid (chloroplast) gene is associated with a severe decline in plant fitness. Our finding that at least some group II introns have a selective value has important implications for the origin and maintenance of introns in evolution.

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MATERIALS AND METHODS

Plant material and growth conditions

Tobacco plants (Nicotiana tabacum cv. Petit Havana) were grown under aseptic conditions on agar-solidified Murashige and Skoog medium containing 30 g/l sucrose (29). Transplastomic lines were rooted and propagated on the same medium. For seed production and analysis of plant phenotypes, transplastomic and wild-type plants were grown in soil under standard greenhouse conditions. Seedling phenotypes were analyzed by germination of seeds in soil and grown for 21 days at 26°C, before they were transferred to 10°C and further cultivated under a light intensity of 150 µE m⁻² s⁻¹.

Construction of plastid transformation vectors

A 6.6-kb EcoRI/XhoI restriction fragment from a previously constructed vector containing the aadA marker gene and a FLAG-tagged ycf3 (30,31) was cloned into a similarly derived vector (MoBiTec GmbH, Göttingen, Germany), in which a 1-kb EcoRI/MluI fragment had been integrated to extend the homologous region upstream of ycf3, generating plasmid pKMP21. To modify the ycf3 coding region, a ycf3-containing 2.5-kb EcoRI/SacI fragment was cloned into a pBluescript II KS(+) vector (Stratagene). Intron-free fragments of ycf3 were then obtained via polymerase chain reaction (PCR) with primer pairs Pex1EcoRIfor/Pex3BsmBIrev for the 5′-GCC GCGAATTCTAGAATGGA-3′/GATCTTCAA-3′ and Pex1EcoRIfor/Pex3BsmBIrev (5′-GATCTTCAAAC-3′) and P18psaA (5′-CGATTAAGCG GGCTCTGTG-3′) and an 895-bp PCR product generated by amplification of the non-coding region upstream of ycf3 using primers P5ycf3UTR (5′-TCTATGATGATGGAGCAG-3′) and P3/rps4 (5′-GGTCTGGTG TTGAAAATAATG-3′) were used as RFLP probes to verify chloroplast transformation. Total cellular RNA samples (3.5 µg total RNA) were electrophoresed in formaldehyde-containing 1% agarose gels and blotted onto Hybond XL membranes (GE Healthcare) by capillary blotting using standard protocols. A 479-bp PCR product generated by amplification of the psaA coding region using primers PK52psaA (5′-CCAGTTGAGATGGATATGTTGGAATA-3′) and P18psaA (5′-CGATTAAGCG GGCTCTGTG-3′) and an 895-bp PCR product generated by amplification of the non-coding region upstream of ycf3 using primers P5ycf3UTR (5′-TCTATGATGATGGAGCAG-3′) and P3/rps4 (5′-GGTCTGGTG TTGAAAATAATG-3′) were used as RFLP probes to verify chloroplast transformation. Total cellular RNA samples (3.5 µg total RNA) were electrophoresed in formaldehyde-containing 1% agarose gels and blotted onto Hybond XL membranes. Probes for detection of intron 1 (Pyc3intron1for: 5′-CCTCGAGATTAGCGAACC CTC-3′; Pyc3intron1rev: 5′-TCCAGGAATTTGCTAC TTC-3′) or intron 2 (Pyc3intron2for: 5′-ACCTCATAC GGCTCAGCAG-3′; Pyc3intron2rev: 5′-CCGTTAAGAG TCAATAGCGAG-3′) containing transcripts and for ndhA (PndhAforEx1: 5′-GAAGTCTCTATGGGATCAT CATGATGC-3′; PndhArevEx1: 5′-TGATTTTGTGAGCT GCGTGACC-3′) were generated by amplification of the corresponding DNA sequences. An rps12 probe covering all three exons was obtained by PCR amplification with primer pair P184 (5′-CGTATGAATGACATC TAAACCAACC-3′) and P185 (5′-GGAGGCATGAAAG GTGGTCAAGATC-3′) using cDNA as template. PCR-generated probes were purified by agarose gel electrophoresis following extraction of the DNA fragments of interest from excised gel slices using the Nucleospin Extract II kit (Macherey-Nagel) and radiolabeled with α-32P-dCTP using the MegaPrime kit (GE Healthcare). 5′-labeling of single-stranded oligonucleotides with polyuridylate kinase (PNK) was done by incubation (30 min at 37°C) of 10 pmol oligonucleotide, 30 µCi γ-32P-dATP and 5 U T4 PNK. Oligonucleotides for end-labeling were derived from ycf3 exon 1 (5′-CTTCGTATAGTGAAATGCTCCCTTT TTTCCTCGAATTGTCGAGAATATGCTGTAATAA GATATTGGCTCAATAGGAAAAGTTTCTTAAATT CAAAATTCATTATACGTGATCCGGC-3′), ycf3 exon 2 (5′-GGCCATATATTAGATGATAATTTTG AGATCATAGGGATCAATTTCT ACATTAGGATC-3′) and ycf3 exon 3 (5′-CTTCGTATAGTGAAATGCTCCCTTT TTTCCTCGAATTGTCGAGAATATGCTGTAATAA GATATTGGCTCAATAGGAAAAGTTTCTTAAATT CAAAATTCATTATACGTGATCCGGC-3′).

Isolation of nucleic acids and hybridization procedures

Total plant DNA was isolated from fresh leaf tissue by a rapid cetyltrimethylammoniumbromide-based mini-prep procedure (33). RNA was extracted using the peqGOLD TriFast™ reagent (Peqlab, Erlangen, Germany) according to the manufacturer’s protocol. For Southern blot analysis, DNA samples (5 µg total DNA) were digested with the restriction enzymes KpnI and SacI, separated by gel electrophoresis in 1% agarose gels, and transferred onto Hybond XL membranes (GE Healthcare) by capillary blotting using standard protocols. 479-bp PCR product generated by amplification of the psaA coding region using primers PK52psaA (5′-CCAGTTGAGATGGATATGTTGGAATA-3′) and P18psaA (5′-CGATTAAGCG GGCTCTGTG-3′) and an 895-bp PCR product generated by amplification of the non-coding region upstream of ycf3 using primers P5ycf3UTR (5′-TCTATGATGATGGAGCAG-3′) and P3/rps4 (5′-GGTCTGGTG TTGAAAATAATG-3′) were used as RFLP probes to verify chloroplast transformation. Total cellular RNA samples (3.5 µg total RNA) were electrophoresed in formaldehyde-containing 1% agarose gels and blotted onto Hybond XL membranes. Probes for detection of intron 1 (Pyc3intron1for: 5′-CCTCGAGATTAGCGAACC CTC-3′; Pyc3intron1rev: 5′-TCCAGGAATTTGCTAC TTC-3′) or intron 2 (Pyc3intron2for: 5′-ACCTCATAC GGCTCAGCAG-3′; Pyc3intron2rev: 5′-CCGTTAAGAG TCAATAGCGAG-3′) containing transcripts and for ndhA (PndhAforEx1: 5′-GAAGTCTCTATGGGATCAT CATGATGC-3′; PndhArevEx1: 5′-TGATTTTGTGAGCT GCGTGACC-3′) were generated by amplification of the corresponding DNA sequences. An rps12 probe covering all three exons was obtained by PCR amplification with primer pair P184 (5′-CGTATGAATGACATC TAAACCAACC-3′) and P185 (5′-GGAGGCATGAAAG GTGGTCAAGATC-3′) using cDNA as template. PCR-generated probes were purified by agarose gel electrophoresis following extraction of the DNA fragments of interest from excised gel slices using the Nucleospin Extract II kit (Macherey-Nagel) and radiolabeled with α-32P-dCTP using the MegaPrime kit (GE Healthcare). 5′-labeling of single-stranded oligonucleotides with polyuridylate kinase (PNK) was done by incubation (30 min at 37°C) of 10 pmol oligonucleotide, 30 µCi γ-32P-dATP and 5 U T4 PNK. Oligonucleotides for end-labeling were derived from ycf3 exon 1 (5′-CTTCGTATAGTGAAATGCTCCCTTT TTTCCTCGAATTGTCGAGAATATGCTGTAATAA GATATTGGCTCAATAGGAAAAGTTTCTTAAATT CAAAATTCATTATACGTGATCCGGC-3′), ycf3 exon 2 (5′-GGCCATATATTAGATGATAATTTTG AGATCATAGGGATCAATTTCT ACATTAGGATC-3′) and ycf3 exon 3 (5′-CTTCGTATAGTGAAATGCTCCCTTT TTTCCTCGAATTGTCGAGAATATGCTGTAATAA GATATTGGCTCAATAGGAAAAGTTTCTTAAATT CAAAATTCATTATACGTGATCCGGC-3′).
RESULTS

Elimination of introns from the plastid ycf3 gene

The ycf3 gene is present in the plastid (chloroplast) genome of green algae and all vascular plants and encodes an essential assembly factor for photosystem I (PSI;30). Its reading frame is interrupted by two group II introns, whose removal is essential for synthesis of a functional Ycf3 polypeptide and thus for photosynthetic activity. The introns do not contain a reading frame for an intron maturase (splicing factor) as some other group II introns in plant organellar genomes do (40).

To test if the introns have any function besides their own removal from the pre-mRNA, we constructed three intron deletion alleles of ycf3 (Figure 1A): an allele lacking intron 1 (∆intron 1), an allele lacking intron 2 (∆intron 2) and an allele lacking both introns (∆intron 1+2). The mutant alleles were linked to a selectable marker gene for plastid transformation (aadA) and, as a control, an additional construct carrying the aadA and the wild-type ycf3 allele (aadA control; Figure 1A) was generated. To facilitate immunological detection of the Ycf3 protein, the sequence for a C-terminal FLAG epitope tag was fused to the ycf3 gene. Previous work has shown that tethering the FLAG sequence to the C-terminus of Ycf3 is phenotypically neutral and does not alter Ycf3 function or stability (31).

All four constructs were introduced into tobacco plants by stable transformation of the chloroplast genome (32). Transgene integration into the plastid genome by homologous recombination was confirmed by DNA gel blot analyses (Figure 1B) and homoplasy (i.e. the absence of residual wild-type copies of the highly polymorphic plastid genome) was additionally confirmed by inheritance assays (Figure 1C; 32,41). For each construct, two independently generated plastid-transformed (transplastomic) lines were selected for in-depth analysis. To exclude the presence of secondary mutations, all transformation vectors and the manipulated region in the plastid genomes of the transplastomic lines were resequenced.

Mutant phenotype of transplastomic plants lacking ycf3 intron 1

For phenotypic analysis, transplastomic plants and control plants were raised from seeds and grown side by side in a controlled-environment chamber. Surprisingly, plants lacking ycf3 intron 1 (∆intron 1) displayed a pronounced mutant phenotype (Figure 2A). Compared to the wild type and all other transplastomic lines, they were strongly retarded in growth and had light-green leaves, suggesting that the ∆intron 1 plants are impaired in photosynthesis. In contrast, none of the ∆intron 2 and ∆intron 1+2 lines showed any discernable phenotype. Interestingly, the phenotype of the ∆intron 1 mutants was much less pronounced under high-light conditions (Figure 2B), indicating that the presence of intron 1 is especially beneficial, when light availability limits plant growth.

To characterize the physiological basis of the mutant phenotype of the ∆intron 1 plants, chlorophyll content, photosynthetic electron transport and the contents of the components of the photosynthetic electron transport chain (photosystem II: PSI, cytochrome b_{6}f complex: cyt b_{6}f, plastocyanin: PC, photosystem I: PSI) were determined (Table 1; Figure 3). ∆Intron 1 mutants grown under low-light conditions reached only ~70% of the chlorophyll content of the control plants, explaining...
Figure 1. Elimination of introns from the chloroplast ycf3 gene. (A) Physical map of the ycf3-containing region in the tobacco plastid genome (Nt-ptDNA) and the four plastid transformation vectors constructed in this study. Genes above the line are transcribed from the left to the right, genes below the line are transcribed in the opposite direction. ycf3 exons are shown as gray boxes, introns as open boxes. Relevant restriction sites, hybridization probes and sizes of DNA fragments appearing in RFLP analyses are indicated. (B) RFLP analysis of transplastomic lines. Fragment sizes are given in kilobasepairs and correspond exactly to the expected sizes [cf. (A)]. Two independently generated transplastomic lines are shown for each construct. (C) Seed assays confirming homoplasmy of the transplastomic lines and maternal inheritance of the plastid-encoded spectinomycin resistance conferred by the aadA transgene. Seeds were germinated on medium containing 500 μg/ml spectinomycin.
Moreover, strongly increased non-photochemical quenching (qN) already at low light intensities (Figure 3A) suggested impaired photochemistry in \( /C1 \) intron 1 mutants. Determination of the chlorophyll fluorescence parameter qL (correlating with the fraction of PSII reaction centers that are ‘open’, 36) in dependence on the light intensity revealed that, already at low light intensities, the qL values in the \( /C1 \) intron 1 mutants were drastically reduced compared to the wild type and the two other intron deletions (Figure 3B), indicating strong overreduction of the PSII acceptor side and pointing to a deficiency in one of the downstream components of the electron transport chain. As expected, the differences in qN and qL between the \( /C1 \) intron 1 mutant and the wild type were less pronounced in plants grown under low-light conditions.

Figure 2. Phenotypes of transplastomic plants. (A) Phenotype under low-light conditions (30 \( \mu \text{E m}^{-2}\text{s}^{-1} \)). (B) Phenotype under high-light conditions (1000 \( \mu \text{E m}^{-2}\text{s}^{-1} \)). Note the less pronounced phenotype of the \( /C1 \) intron 1 mutant, which is solely visible by a slightly delayed development and senescence (i.e. a darker color of the old leaves).

Table 1. Photosynthetic parameters of \( /c3 \) intron deletion mutants grown at low-light conditions (30 \( \mu \text{E m}^{-2}\text{s}^{-1} \); upper part) or high-light conditions (1000 \( \mu \text{E m}^{-2}\text{s}^{-1} \); lower part)

| Parameter                      | Wild type     | \( aadA \) control | \( \Delta \) Intron 1 | \( \Delta \) Intron 2 | \( \Delta \) Intron 1+2 |
|-------------------------------|---------------|--------------------|-----------------------|-----------------------|------------------------|
| Chlorophyll per leaf area (mg m\(^{-2}\)) | 209.2 ± 9.7  | 221.5 ± 18.7       | 142.6 ± 9.1           | 208.2 ± 13.3          | 210.5 ± 24.8           |
| Leaf absorbance (%)            | 77.1 ± 1.1    | 77.8 ± 2.4         | 73.0 ± 2.8            | 79.5 ± 2.0            | 78.6 ± 2.9             |
| \( F_v/F_M \)                  | 0.80 ± 0.01   | 0.79 ± 0.01        | 0.74 ± 0.04           | 0.78 ± 0.01           | 0.78 ± 0.01            |
| Chlorophyll/leaf area (mg m\(^{-2}\)) | 423.9 ± 59.5 | 447.2 ± 34.9       | 346.1 ± 32.7          | 438.7 ± 18.7          | 416.1 ± 32.1           |
| Leaf absorbance (%)            | 89.0 ± 1.6    | 88.5 ± 1.3         | 86.5 ± 1.4            | 88.0 ± 1.4            | 88.5 ± 1.3             |
| \( F_v/F_M \)                  | 0.77 ± 0.02   | 0.77 ± 0.02        | 0.75 ± 0.01           | 0.77 ± 0.01           | 0.76 ± 0.03            |

The values represent averages from at least five independent measurements ± standard deviation. \( F_v/F_M \): maximum quantum efficiency of PSII.

Their light-green phenotype (Table 1; Figure 2A). Moreover, strongly increased non-photochemical quenching (qN) already at low light intensities (Figure 3A) suggested impaired photochemistry in \( \Delta \) intron 1 mutants.
conditions (Figure 3A and B), due to the earlier saturation of the electron transport system in plants adapted to low light intensities.

Next, we determined the contents of the components of the photosynthetic electron transport chain by spectroscopic methods (39,42). Interestingly, the Δ intron 1 mutants displayed a specific reduction in PSI contents (Figure 3C), raising the possibility that the mutant phenotype is directly related to ycf3 gene function. Consistent with their more severe phenotype, the reduced PSI accumulation in the Δ intron 1 mutants was even more pronounced in low light-grown plants than in high-light grown plants (Figure 3C).

**Defective splicing of ycf3 intron 2 in the absence of intron 1**

To test if lack of intron 1 affects ycf3 transcript processing or mRNA accumulation, we performed RNA gel blot experiments. Interestingly, hybridizations to ycf3 exon-specific probes revealed that, in the Δ intron 1 mutants, the mature 0.7-kb ycf3 transcript was hardly detectable.
and, instead, the mutants accumulated a larger transcript of ~1.5 kb (Figure 4). This larger RNA species corresponds in size to an unspliced precursor that contains intron 2. To test the idea that lack of intron 1 prevents splicing of intron 2, hybridizations with intron-specific probes were conducted. The results confirmed that the Δ intron 1 mutants accumulate the intron 2-containing precursor but not the excised intron 2 and, thus, are defective in intron 2 splicing (Figure 4). The splicing defect was not dependent on the light intensity (Figure 4A and B). This, however, does not contradict the light-dependent phenotype of the mutant plants, because a more severe growth phenotype under low-light conditions is a general property of PSI-deficient mutants (Dominika Bednarczyk, R.B. and M.A.S., manuscript in preparation). Consequently, the phenotypic difference in dependence on the light intensity (Figure 4) is not related to any difference in splicing efficiency, but is solely due to similar reductions in photosystem I levels causing stronger phenotypes in low light than in high light.

As complete lack of intron 2 excision should be equivalent to a ycf3 knock-out, which would be incapable of photoautotrophic growth (30), we suspected that low levels of spliced ycf3 mRNA accumulate in Δ intron 1 mutants. To confirm this assumption, we used a sensitive semiquantitative RT-PCR assay, in which amplification of the small spliced cDNA product is strongly favored over the amplification of large unspliced molecules. The results

Figure 4. Analysis of RNA accumulation and processing patterns of ycf3 in intron deletion mutants and control plants. Northern blots (from denaturing 1% agarose gels) were hybridized to the specific exon or intron probes indicated at the left or right of each autoradiograph. Two independently generated transplastomic lines are shown for each construct. (A) Analysis of plants grown under low-light conditions (30 μE m⁻² s⁻¹). The various RNA species accumulating are indicated by the symbols in the middle. Gray boxes denote ycf3 exons, open boxes introns, the black box represents the aadA (present in dicistronic transcripts originating from read-through transcription; 30) and the curved box indicates the lariat intermediate in group II intron splicing; 1). Note that the circularized intermediate resulting from the first transesterification reaction is detectable for intron 1 but not for intron 2, suggesting that in intron 2 splicing, the second transesterification reaction follows more rapidly than in intron 1 splicing. (B) Analysis of intron 2 splicing in plants grown under high-light conditions (1000 μE m⁻² s⁻¹).
demonstrated that indeed some spliced $ycf3$ mRNA accumulates in the $\Delta$ intron 1 mutants (Figure 5A). Control amplifications with $rpl2$, another plastid gene containing a group II intron, showed that the splicing defect was specific for $ycf3$ intron 2 (Figure 5A), a conclusion that was further corroborated by the analysis of three additional intron-containing genes by northern blotting: $atpF$, $ndhA$ and $rps12$ (Figure 5B).

**Ycf3 protein deficiency and stress sensitivity of plants lacking $ycf3$ intron 1**

We next wanted to confirm the significance of inhibited intron 2 splicing in $\Delta$ intron 1 mutants at the protein level. To this end, we used a monoclonal anti-FLAG antibody and determined the amount of Ycf3 protein accumulating in the mutants. The data revealed that $\Delta$ intron 1 mutants accumulate only approximately half the amount of Ycf3 as all other transplastomic lines (Figure 5C). The effect on Ycf3 protein accumulation was much less severe than what could be expected from the strong reduction in mature $ycf3$ mRNA (Figures 4 and 5A), confirming previous reports that, in plastids, translational regulation can largely override changes in mRNA levels (43). Recent work has revealed that the Ycf3 protein acts in a protein complex that plays a crucial role in PSI assembly. Knockdown of the expression of an essential component of this assembly complex to 20–30% of wild-type levels resulted in a similarly strong decline in PSI accumulation (31), suggesting that the observed Ycf3 deficiency (Figure 5C) is sufficiently severe to explain the phenotype of the $\Delta$ intron 1 mutants.

To ultimately confirm the specific PSI deficiency in $\Delta$ intron 1 plants, we performed PSI photoinhibition experiments in the cold. Chilling stress is known to cause photoinhibitory damage to PSI (44–46), presumably caused by inhibited superoxide dismutase (SOD) function. Insufficient SOD activity leads to overaccumulation of the reactive oxygen species that are generated as by-products of PSI activity, which in turn causes irreversible photooxidative damage in PSI. Therefore, reduced

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Analysis of splicing of intron-containing plastid transcripts and Ycf3 protein accumulation in $ycf3$ intron deletion mutants. (A) Semiquantitative RT-PCR assays to detect low-level $ycf3$ intron 2 splicing in $\Delta$ intron 1 mutants. Two independently generated transplastomic lines are shown for each construct. cDNA from the intron-containing $rpl2$ gene was amplified as a control. The specific primers used for RT-PCR are indicated at the left (Ex: exon), sizes of amplification product are given at the right. (B) Analysis of group II intron splicing in the $atpF$, $ndhA$ and $rps12$ transcripts by northern blotting. Major spliced RNA species are labeled (with the cistrons they contain) at the right. Note that the mature $rps12$ mRNA is assembled from three exons (two of which are joined by trans-splicing). (C) Reduced accumulation of the Ycf3 protein in $\Delta$ intron 1 mutants. The FLAG-tagged Ycf3 proteins in the transplastomic lines are detected with a monoclonal anti-FLAG antibody. For quantitative comparison, a dilution series of the protein from an $aadA$ control plant is shown (loaded amounts of thylakoid proteins given in $\mu$g chlorophyll per lane). The upper cross-reacting band is likely to represent a light-harvesting complex protein and can serve as an additional loading control.
amounts of PSI should lead to increased sensitivity to chilling stress. Indeed, when grown under chilling stress at 10°C, Δ intron 1 plants bleached out and the developing leaves nearly fully lost their pigmentation (Figure 6), whereas the old leaves (that had already a fully assembled photosynthetic apparatus prior to cold exposure) were much less affected.

A model for dependence of intron 2 excision on intron 1

How can lack of a group II intron inhibit the splicing of another, distantly located, intron? The two ycf3 introns are separated by an exon of 230 nt. cis-splicing of group II introns is dependent on the typical six-domain secondary structure of the intron and a few adjacent exonic nucleotides, but does not require distant sequence elements (1,2). We, therefore, considered the conceivable possibility that the lack of intron 1 interferes with the proper folding of intron 2, for example, by inducing the formation of an aberrant secondary structure. If this were the case, the disruptive activity should come from the sequence at the junction between exon 1 and exon 2, because this is the only sequence motif not present in the intron 1-containing (wild-type) ycf3 allele. When we searched for complementarities between the junction sequence and sequences in intron 2, we discovered a 6-nt perfect match with a sequence motif in intron domain I (Figure 7; 48,49). We, therefore, propose that intron 1 is required to prevent masking of the ζ motif in intron 2.

DISCUSSION

In this work, we have tested the idea that group II introns can be required for fitness and are maintained by selective pressure. We have shown that, while deletion of one intron (intron 2) from the plastid gene ycf3 has no apparent effect on plant growth and photosynthetic performance, elimination of another intron (intron 1) had severe consequences for fitness, which were especially pronounced under low light (Figure 2) and under cold stress conditions (Figure 6). As the stability of RNA secondary structures can change with temperature and, moreover, the efficiency of group II intron splicing can be dependent on the growth temperature (50,51), it seems conceivable that the aberrant secondary structure of intron 2 in the Δ intron 1 plants (Figure 7) is even more stable under chilling stress conditions, thus additionally contributing to the severity of the phenotype in the cold. However, although our model provides a plausible mechanistic explanation for the dependence of efficient intron 2 removal on the presence of intron 1, we currently cannot definitively rule out the alternative explanations that (i) a sequence in intron 1 exerts a positive effect on intron 2 splicing or (ii) the negative effect on intron 2 splicing in the absence of intron 1 comes from a different interaction (with other RNA sequences or with proteins, such as the LAGLIDADG-type PPR protein OTP51 shown to be involved in ycf3 splicing; 21).

Interestingly, deletion of both introns abrogated the negative effect of the absence of intron 1, suggesting that the combined loss of both introns could be phenotypically neutral. The loss of the mutant phenotype upon additional elimination of intron 2 supports our model of intron 1 function in intron 2 splicing (Figure 7). It also suggests that the correct folding of group II introns may be more sensitive to changes in remote RNA sequences than previously recognized. This could pose serious restrictions on the spreading of group II introns in organellar and bacterial genomes and may in part explain, why spliceosomal introns, which are considerably less dependent upon RNA folding, have been much more successful in evolution than group II (and group I) introns.

The presence of more than one group II intron in transcripts from a single gene or an operon is quite common in both plastid and mitochondrial genomes. It will be interesting to determine how widespread interdependent intron splicing, sequential splicing and, perhaps, other moon-lighting functions of group II introns are. Unfortunately, in the absence of methods for the generation of plants with transformed mitochondrial genomes, stable transformation of the plastid genome is currently the only possible approach to study intron functions in vivo. This involves laborious and time-consuming procedures and thus poses severe restrictions on the systematic probing of group intron function. Preliminary bioinformatics analyses of other pairs of chloroplast introns suggest at least one
additional case that could be analogous to the sequential splicing of the two ycf3 introns (Figure 7). The splicing of intron 2 in the clpP gene (encoding the proteolytic subunit of the Clp protease) could be inhibited by prior removal of intron 1 by a very similar aberrant secondary structural interaction with the sequence arising from ligation of exons 1 and 2 (Figure 7). Circumstantial evidence for splicing events occurring in a specific order has also been obtained for the nad5 mRNA in plant mitochondria (52).

If trans-splicing of exons c and d does not precede the trans-splicing of exons b and c, extensive mis-splicing takes place that results in the production of aberrant RNAs.

Taken together, our data provide an intriguing example of a group II intron that has a function besides its own removal by facilitating the splicing of another intron. This demonstrates that group II introns can have a selective value in that their loss can cause a decline in fitness. The acquisition of functions that go beyond their own excision may have contributed to the evolutionary maintenance of group II introns, because once an intron has adopted such an additional function, it cannot be lost again without entailing an immediate selective disadvantage. Such selective pressures may have been important, and currently underappreciated, forces not only in the preservation of group II introns, but also in the evolution of spliceosomal introns from group II intron progenitors (28).

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