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Alternatively spliced, spliceosomal twin introns in *Helminthosporium solani*

Norbert Ág [a], Michel Flipphi [a,*], Levente Karaffa [a], Claudio Scazzocchio [b,c], Erzsébet Fekete [a]

[a] Dept. of Biochemical Engineering, Faculty of Science and Technology, University of Debrecen, H-4032 Debrecen, Hungary
[b] Dept. of Microbiology, Imperial College, London SW7 2AZ, United Kingdom
[c] Institut de Biologie Intégrative de la Cellule (I2BC), UMR CEA/CNRS/Université Paris-Sud, 91405 Orsay Cedex, France

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ABSTRACT

Spliceosomal twin introns, "stwintrons", have been defined as complex intervening sequences that carry a second intron ("internal intron") interrupting one of the conserved sequence domains necessary for their correct splicing via consecutive excision events. Previously, we have described and experimentally verified stwintrons in species of Sordariomycetes, where an "internal intron" interrupted the donor sequence of an "external intron". Here we describe and experimentally verify two novel stwintrons of the potato pathogen *Helminthosporium solani*. One instance involves alternative splicing of an internal intron interrupting the donor domain of an external intron and a second one interrupting the acceptor domain of an overlapping external intron, both events leading to identical mature mRNAs. In the second case, an internal intron interrupts the donor domain of the external intron, while an alternatively spliced intron leads to an mRNA carrying a premature chain termination codon. We thus extend the stwintron concept to the acceptor domain and establish a link of the occurrence of stwintrons with that of alternative splicing.

1. Introduction

In Eukaryotes, almost all introns present in primary transcripts of the nuclear genomes belong to the spliceosomal class. These introns are processed by a two step mechanism involving a specialised RNA–protein complex, the spliceosome. After site-specific cutting upstream of a 5′ "donor sequence" and subsequent cutting downstream of the 3′ "acceptor sequence" the intron is released as a lariat, where the donor guanosine is covalently joined to an adenosine included in a third conserved, internal sequence domain, the "lariat branch point sequence", often located closely upstream of the acceptor (for recent reviews, see e.g.: Chen and Cheng, 2012; Han et al., 2011; Horowitz, 2012; Roca et al., 2013; Will and Lührmann, 2011). Concomitantly with the release of the lariat RNA, the exonic sequences bordering the intron are joined, to yield the mature, translatable mRNA. Most spliceosomal introns belong to the majority U2 class. A minority U12 class involving a different set of small nuclear RNAs in the spliceosome is present in many eukaryotic lineages, including metazoans and plants, but it is absent in the Ascomycota and probably in all Dikarya (Turunen et al., 2013). Dikarya contain typically short U2 introns (40–250 nt) in most of their genes (see e.g., Kupfer et al., 2004), the paucity of introns in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* being an anomaly. Alternative splicing leading to different protein products (for a recent review, see: Lee and Rio, 2015) has been described in fungi (e.g.: Grützmann et al., 2014; Kempken, 2013; Mekouar et al., 2010; Sibthorp et al., 2013; Wang et al., 2010; Zhao et al., 2013).

We have recently described a new class of introns, spliceosomal twin introns (in short, "stwintrons") in species of the Sordariomycetes class and of the Botryosphaeriales order of the Dothideomycetes class (Ascomycota, subphylum of Pezizomycotina) (Flipphi et al., 2013). These are complex introns where one of the U2 canonical sequence domains necessary for proper processing of the primary transcript is itself interrupted by a canonical U2 intron. The stwintron is thus processed in two consecutive splicing events: the first involving the excision of the "internal" U2 intron interrupting one of the canonical intron domains of the "external" intron, essential for the subsequent excision of the thus generated canonical ("external") intron. The stwintron concept is illustrated in Fig. 1.

The stwintrons characterised by us in two species of Hypocreales (Sordariomycetes) carried an internal intron interrupting the donor of the external intron between the first and second

* Corresponding author at: Dept. of Biochemical Engineering, Faculty of Science and Technology, University of Debrecen, PO Box 64, H-4032 Debrecen, Hungary.
E-mail address: drir.michelflipphi@gmail.com (M. Flipphi).

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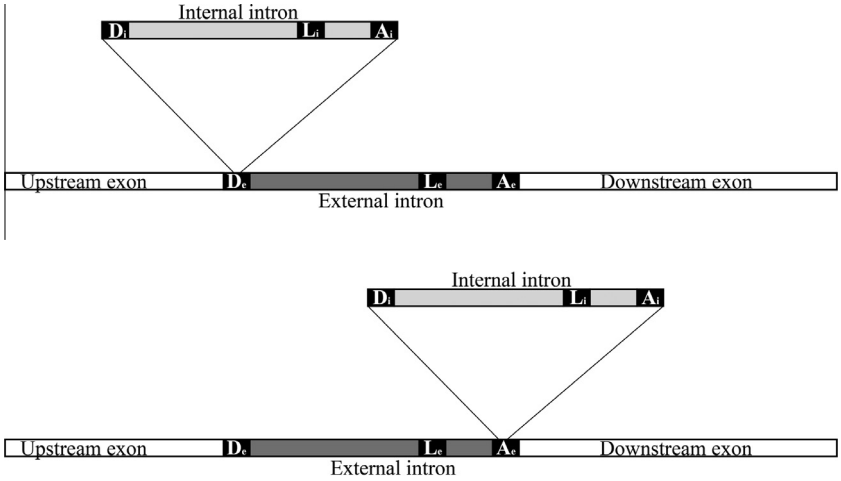

Fig. 1. Schematic representation of spliceosomal twin introns (stwintrons). Top panel: A stwintron where the internal intron interrupts the donor sequence of the external intron. Bottom panel: A stwintron where the internal intron interrupts the acceptor sequence of the external intron. D, donor sequence, L, lariat branch point sequence, A, acceptor sequence. The subscripts "e" and "i" denote "external" and "internal", respectively.

([D1,2]) or the second and third nucleotides ([D2,3]) of the six-nt donor consensus sequence, 5′-GUAUG(U/A), respectively. The stwintron concept predicts specific splicing intermediates, which were experimentally demonstrated. The splice sites of both introns comprising these two different stwintrons are necessarily paired via intron definition (cf. Berget, 1995), where the 5′ and 3′ sites involved in excision are those closest to one another across the intron. Once the stwintron concept is established there is no *a priori* reason why the internal intron of a stwintron could not interrupt either the acceptor or the lariat branchpoint domain.

Twin introns ("twintrons"), where the excision of an internal intron is necessary for the final processing leading to a mature RNA have been described previously only for chloroplast group II and III introns (see, e.g.: Copertino and Hallick, 1991, 1993). A number of complex splicing processes which superficially resemble stwintron splicing, have been discussed in our previous report (Flipphi et al., 2013).

Here we describe two new stwintrons in the potato pathogen *Helminthosporium solani*, the cause of silver scurf (for review: Errampalli et al., 2001). Molecular phylogeny places this organism within the Massarinaceae, a family of the Pleosporales order (class, Dothideomycetes; subphylum, Pezizomycotina) (Olivier et al., 2000; Eriksson and Hawksworth, 2003; Zhang et al., 2012). The demonstration of the formation of all the predicted splicing intermediates extends the concept of the stwintrons to those carrying an internal intron in the acceptor sequence of the external intron. Alternative splicing is shown to occur in both new stwintrons. The existence and characterisation of different types of spliceosomal twin introns may be relevant to the origin of alternative spliced messages, and may contribute to our understanding of spliceosomal intron generation, proliferation, evolution and loss.

2. Materials and methods

2.1. Fungal strain, media and growth conditions

H. solani B-AC-16A (Mattupalli et al., 2014) was used in this study. Its genome sequences are accessible at the National Centre for Biotechnology Information (NCBI) webservers under Whole Genome Shotgun contigs Master Accession AWWW00000000.

H. solani was maintained on malt extract agar (LAB M Limited, UK). Mycelia were grown in 500-mL Erlenmeyer flasks with 100 mL of liquid medium containing 1% (w/v) yeast extract, 0.5% malt extract, 0.3% peptone and 0.3% dextrose (Mimee et al., 2011) seeded with vegetative spore inoculum, at 22 °C in a rotary shaker (Infors HT Multitron) at 200 rotations per min (rpm) for one week.

2.2. Nucleic acid isolation

Fungal mycelia were harvested by medium filtration, washed with distilled water, frozen and ground to powder under liquid nitrogen. For the extraction of genomic DNA, plasmid DNA and total RNA, Macherey–Nagel NucleoSpin kits (NucleoSpin Plant II, NucleoSpin Plasmid EasyPure and NucleoSpin RNA Plant, respectively) were used according to the manufacturer's instructions.

2.3. Reverse transcription PCR (RT-PCR)

Reverse transcription was primed off 1 μg of total RNA with Oligo(dT) as a primer using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). PCR reactions were performed in a 25 μL volume containing 4 μL of single strand cDNA, using gene-specific oligonucleotides (Table S1) as primers and DreamTaq DNA Polymerase (Thermo Scientific). Cycling conditions after initial denaturation at 95 °C for 2 min were: 40 cycles of 95 °C for 30 s, 50–60 °C for 1 min, and 72 °C for 30 s, followed by one post-cyclic elongation at 72 °C for 5 min. Amplified fragments were resolved in a 3% native agarose gel and stained with ethidium bromide.

2.4. cDNA sequencing

PCR was primed off the primary RT product using gene-specific oligos (Table S1) with cycling conditions as described above. Double strand cDNA was gel-purified and subsequently cloned (pGEM-T Easy Vector System I, Promega). Three independent clones were sequenced using universal primers hybridising to the vector (MWG-Biotech AG, Ebersberg, Germany). cDNA sequences were deposited at GenBank under accession numbers KT306963 (aldose mutarotase) and KT306964 (alternative oxidase).

3. Results

3.1. A stwintron in the H. solani alternative oxidase transcript is alternatively spliced

While mining the NCBI Whole Genome Shotgun contigs library, we serendipitously encountered a potential stwintron in the unannotated genome sequences of *H. solani* corresponding to a sequence putatively encoding a mitochondrial alternative oxidase [systematic name ubiquinol:oxygen oxidoreductase (non-electrogenic) (EC 1.10.3.11)] of 342 residues included in contig 00674 (Accession number: AWWW01000672). The gene includes an 56 nt-intron which splits the codon of Val69 (GAG) between the first and the second nucleotide. Its position is conserved among most sequenced species of the Dothideomycetes class, including almost all Pleosporales, albeit it is lost in all but one of the Capnodiales. At the Glu145 codon, we find in *H. solani* an additional intron, which will be described and analysed below. No introns are extant at this 3′ intron position in the orthologue gene(s) of any other sequenced members of the Pezizomycotina. We confirmed the excision of both these introns by sequencing both genomic DNA and three cDNA clones of the alternative oxidase gene (GenBank Accession number KT306964).

The 3′-, *H. solani*-specific intron, appears to start with an aberrant GG-doublet (Fig. 2A; see Fig. S1 for its complete sequence). The sequence context of this intron suggests, however, a novel stwintron structure in that two alternative splicing mechanisms, both involving an internal intron, could be proposed. The first and most obvious prediction would be that the donor sequence of the 52-nt-long host intron (5′-G|UGCGU—27-nt—ACUAAC—10-nt—CAG) is interrupted by an 59-nt-long internal intron (5′-GUA GGU—31-nt—GCUUAU—13-nt—CAG) between its first and second nucleotides (Fig. 2A, top scheme; internal and external introns shown as "I", see below), essentially the same structure as we have determined for the stwintron in one of the putative imidine hydrolase genes in some species of *Fusarium*. In line with previous nomenclature, this would be a [D1,2] stwintron (Flipphi et al., 2013). This stwintron splits the GAG Glu145 codon between the second and the third nucleotide.

In the alternative splicing proposal, the G preceding the most upstream, canonical donor (5′-GUAGGU) would be exonic rather than intronic. The acceptor of the alternative external intron (5′-GUAGGU—31-nt—GCUUAU—13-nt—CA|G) would in this case be disrupted between the penultimate and ultimate nucleotides of the acceptor sequence by an alternative internal intron (5′-GUGC GU—27-nt—ACUAAC—10-nt—CAG). The stwintron would end with an aberrant GG doublet and be situated between the codons for Glu145 (GAG) and Ser146 (AGC) (Fig. 2A, lower scheme; shown as "II"). This *H. solani* alternative stwintron would thus be the first example of an [A2,3] stwintron.

To determine which of the alternative modes of internal intron excision occurs, we followed a RT-PCR strategy using pairs of gene-specific primers that allow detection and characterisation of either predicted splicing intermediate. The pair of primers P1 (5′ exonic) and P3 (overlapping the junction of the 3′ exon and the acceptor site of the [D1,2] external intron) would reveal the intermediate predicted by [D1,2] internal intron splicing, while P2 (3′ exonic) and P4 (overlapping the junction of the 5′ exon and the donor site of the [A2,3] external intron) would reveal the intermediate predicted by the [A2,3] internal intron splicing. This strategy would allow determining which (if any) of the two alternative modes of internal splicing were operating. In the event we found that both modes of splicing occurred. Fig. 2B shows that RT-PCR with either primer pairs yields an amplification corresponding to the primary transcript – of the same size as that obtained amplifying off genomic DNA – while additional, shorter bands of a size corresponding to the two different postulated splicing intermediates, were amplified off reverse transcribed single strand cDNA. The two shorter amplification products obtained with each primer pair were cloned and three independent clones of each were sequenced using universal primers hybridising to the bacterial vector used for cDNA cloning.

Fig. 2C shows the sequence of the shorter P1/P3 amplification product (band A, 348 bp – sequence given without the gene-specific oligos on either side) from which the predicted internal intron of the [D1,2] stwintron ("I" in Fig. 2A) had been removed while a canonical donor (5′-GUGCGU; underlined nt) for the predicted external intron sequence (lower case letters) has resulted upon the first of the sequential splicing events. Fig. 2D depicts the sequence of the shorter P4/P2 amplification product (band B, 289 bp – sequence minus the oligos), similarly showing the absence of the predicted internal intron of the [A2,3] stwintron ("II" in Fig. 2A) and the assembly of a canonical acceptor domain (5′-CAG; underlined nt) for the predicted external intron (lower case letters), that remained present in the splicing intermediate probed with primer pair P4/P2. As both amplifications were carried out with the same cycling protocol (see Section 2.3 for further details) on the same template material, we may conclude that both alternative, sequential splicing routes occur *in vivo* without an obvious preference for either of them. The *H. solani* alternative oxidase stwintron can thus be excised as a [D1,2]- or as a [A2,3] stwintron, both mechanisms leading to the same coding mRNA sequence.

3.2. A second alternatively spliced stwintron in the H. solani transcriptome

In the primary alternative oxidase transcript described above, the two alternative internal introns overlap by one nucleotide, a G that could serve as the final nt in the acceptor sequence of the 5′ internal intron ("I" in Fig. 2A and Fig. S1) and as the first nt of the donor of the 3′ internal intron ("II" in Fig. 2A and Fig. S1). The first G of the 5′ GG doublet could function either as the first nucleotide of the stwintron or as the last of the upstream exon, while similarly, the second G of the 3′ GG doublet could function either as the last nucleotide of the stwintron or the first of the downstream exon. This sequence context leads to identical mRNA coding sequences whichever stwintron splicing reaction occurs.

Another unconventional intron of *H. solani* permitted us to address the question whether there would be a preferred splicing mechanism when only one splicing pathway leads to a functional translation product. This intervening sequence is present in a putative gene for aldose mutorotase (aldose 1-epimerase, EC 5.1.3.3) on contig 00130 (Accession number: AWWW01000130). This gene contains two putative introns. The 3′ one is a standard U2 intron which splits the codon of Ser162. The 5′ intron, which splits the UAC codon of Tyr39 between the first and second nucleotides, shows the sequence characteristics of a stwintron. Its complete sequence is given in Fig. S1.

In this 154 nt stwintron, a 51-nt long internal intron (5′-GUAU AG—30-nt—ACUAAU—6-nt—CAG) appears to disrupt the donor domain of the 103-nt long external intron (5′-G|UAAGU—75-nt—GCUAAU—13-nt—CAG) between the first and the second nucleotide (Fig. 3A, top of the panel; internal and external introns labelled "I"). This is analogous to the 5′ internal intron "I" of the alternative oxidase stwintron described in the previous subsection (Fig. 2A) and to the [D1,2] stwintron of the *Fusarium verticillioides* putative imidine hydrolase gene (*pih2*, Flipphi et al., 2013). Sequencing of both genomic DNA and three independent cDNA clones (GenBank KT306963) confirmed the splicing of this stwintron, restoring the Tyr39 codon and a cognate 987 bp-long open reading frame for a peptide of 328 residues.

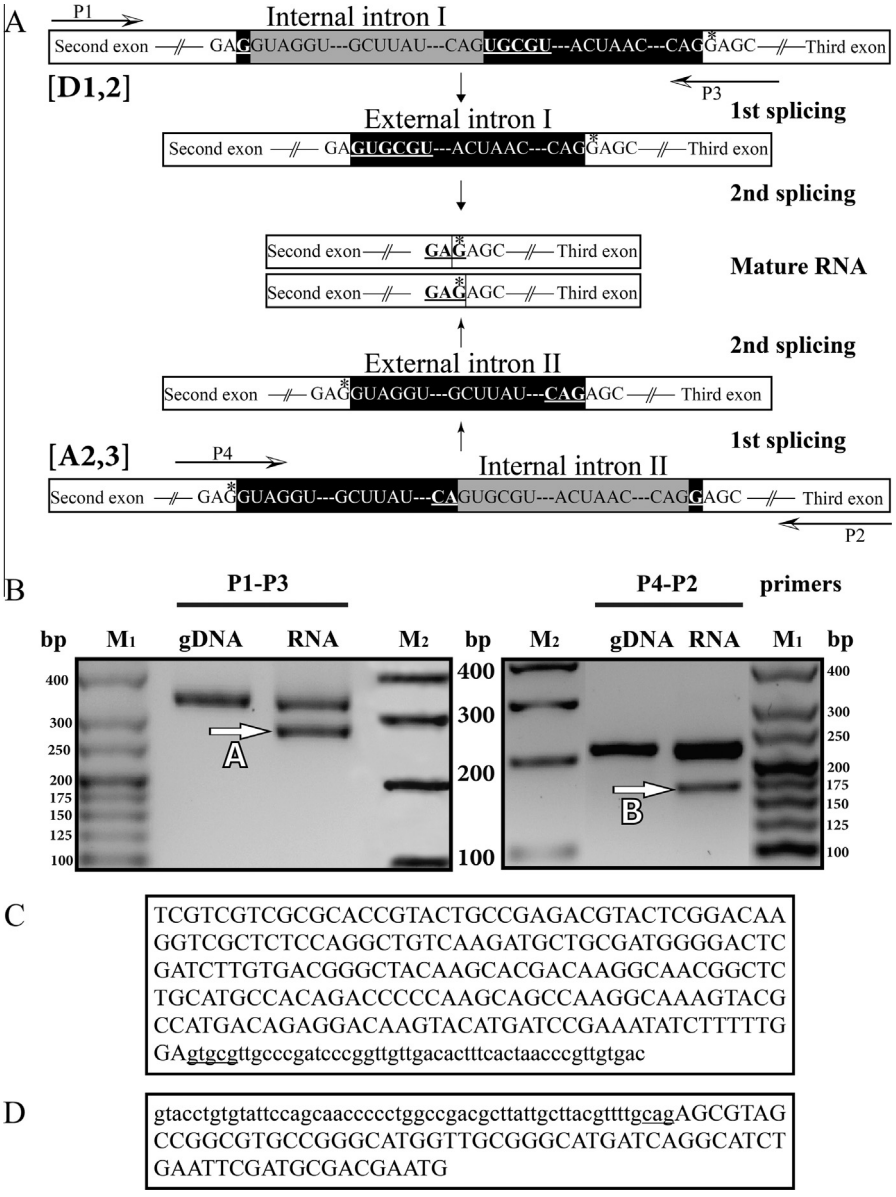

Fig. 2. The alternatively spliced [D1,2]/[A2,3] stwintron of the alternative oxidase gene of *H. solani*. (Panel A) Schematic representation of the alternative two-step stwintron excision pathways; top half: [D1,2] (downward arrows); bottom half: [A2,3] (upward arrows). The identical end products of each pathway ("mature RNA") are juxtaposed at the median of the panel. The pathway-specific internal- and external introns are labelled "I" for the [D1,2]- and "II" for the [A2,3] splicing pathway, respectively. The | sign represent the definitive exon fusion sites. The origin of the exonic G at the third position of the GAG codon in the mature messenger is tagged ∗. The positions of the primers used to detect the pathway-specific splicing intermediates are indicated above and below the schematic representations of the primary transcript. (Panel B) Experimental results. Primer pair P1/P3 was used to demonstrate the [D1,2] excision pathway at the left, P2/P4 to evidence the [A2,3] excision pathway at the right. Hollow arrows indicate the fragments amplified off RNA template corresponding in size to the predicted splicing intermediate – marked "A" (for [D1,2]) and "B" (for [A2,3]), respectively. "M1" and "M2": molecular size markers; "gDNA": genomic DNA template; "RNA": single strand cDNA template. (Panel C) Predicted and actual sequence of the [D1,2] splicing intermediate fragment A. Exonic sequences are printed in capitals, intronic sequences in lower case letters. The newly generated donor sequence of the [D1,2] external intron (5′-GTGCGT) is underlined. (Panel D) Predicted and actual sequence of the [A2,3] splicing intermediate fragment B. The newly generated acceptor sequence of the [A2,3] external intron (5′-CAG) is underlined.

The sequence of this intervening sequence, however, permits in principle an alternative splicing reaction, in which the excision of the sequence (5′-GUAAGU—75-nt—GCUAAU—13-nt—CAG) (labelled alternative intron "II" in Fig. 3A) from the primary transcript would leave the remainder of the stwintron as a sequence featuring canonical donor- and lariat branchpoint domains but lacking a consensus acceptor (51 nt: 5′-GGUAUAG—30-nt—ACUAAU—6-nt—CA) and thus leading to a processing dead end. As the first exonic nucleotide behind the [D1,2] stwintron is an A, rather than a G, the shifting of the whole unconventional intron by one nt, as it occurs in the alternative oxidase stwintron described in the previous subsection, is precluded. The net result of this alternative splicing reaction in the mutarotase primary transcript would by a mRNA species with a premature ochre codon (shaded in Fig. 3A), coding for a small, C-terminally truncated product of 50 residues.

We investigated the occurrence of the two alternative splicing reactions with a strategy analogous to the one employed above, in which the pair of primers P1 and P3 would detect the splicing intermediate extant after the removal of the [D1,2] internal intron ("I" in Fig. 3A) while primers P2 and P4 would detect the dead-end product obtained after the excision of alternative intron ("II" in

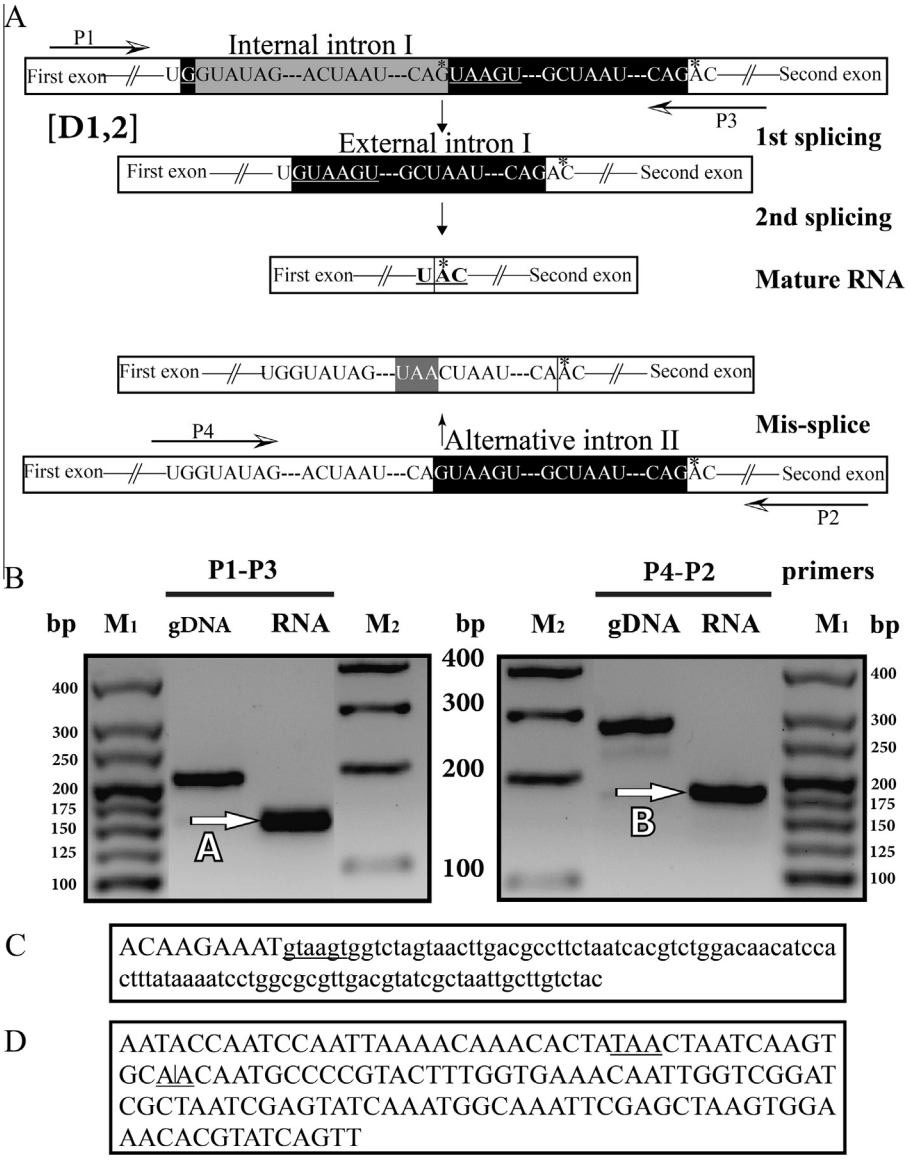

Fig. 3. Internal intron excision from the [D1,2] stwintron of the aldose mutarotase primary transcript of *H. solani* and alternative generation of an mRNA carrying a premature termination codon. (Panel A) Schematic representation of the *bona fide* stwintron excision and the stwintron mis-splice event. Top half: the [D1,2] pathway (downward arrows); bottom half: the stymied [A2,3] pathway (upward arrow). The exonic A at the second position of the UAC codon in the mature messenger as well as in the faulty splicing product is tagged ∗. Other annotation as in Fig. 2. (Panel B) Experimental results. P1/P3 was used to demonstrate the [D1,2] excision pathway at the left, P2/P4 to evidence the mis-splicing event when the alternative intron "II" is removed, at the right. Hollow arrows indicate the fragments amplified corresponding to the predicted splicing intermediate of the [D1,2] excision pathway (fragment "A") or the end product of the stymied [A2,3] excision pathway (fragment "B"). "M1", "M2, "gDNA" and "RNA": track labels as in Fig. 2B. (Panel C) Predicted and actual sequence of the [D1,2] splicing intermediate fragment A after removal of internal intron "I". The newly generated donor sequence of the [D1,2] external intron (5′-GTAAGT) is underlined (Panel D) Predicted and actual sequence of the RT-PCR fragment B corresponding to the splicing end product, after excision of the alternative intron "II". The exon fusion site is indicated with a | sign. The premature, in frame stop codon (TAA) is underlined.

Fig. 3A) from the primary transcript. Fig. 3 panel B show that both reactions occur, the splicing intermediate after the removal of the internal intron corresponding to fragment A (147 bp) and the splicing dead end product corresponding to fragment B (176 bp). Sequencing of three independent cDNA clones for each amplification of these splicing products (Fig. 3, panels C and D) showed that splicing occurred exactly as predicted: amplification fragment A (147 bp, but in Fig. 3C given without the terminal primer sequences) corresponds exactly to removal of the internal intron ("I" in Fig. 3A) with the formation of a canonical 5′-GUAAGU donor site for the subsequent removal of the [D1,2] external intron, while fragment B (176 bp, in Fig. 3D without terminal primer sequences) corresponds to the predicted dead-end product after excision of intron "II". Note that several independent experiments have failed to reveal amplification of the mutarotase primary transcript with primer pairs P1/P3 and P4/P2 which implies that its steady state concentration is very low relative to the splicing products.

While the 3′ intron, splitting Ser162 in *H. solani* mutarotase is universally conserved among the Pezizomycotina, and beyond, as it is present in the basal species of the Taphrynomycotina, *Saitoella complicata* (cf. Nishida et al., 2011; references therein), the same is not true for the 5′ [D1,2] stwintron described above. This stwintron (or indeed any intron at this position) is, among the available genomes, only present in two close relatives of *H. solani* (Pleosporales, Massarinaceae), *Byssothecium circinans* (Pleosporales, Massarinaceae) and *Lentithecium fluviatile* (Pleosporales, Lentitheciaceae),

both assigned to the sub-order Massarineae (Olivier et al., 2000; Eriksson and Hawksworth, 2003; Zhang et al., 2012). Similarly to *H. solani*, the [D1,2] mutarotase stwintron in *B. circinans* and *L. fluviatile* (for sequences, see Fig. S1) cannot properly be removed via alternative sequential splicing routes. Indeed, EST evidence from *L. fluviatile*, publicly accessible at JGI MycoCosm (Grigoriev et al., 2014), shows both the correct excision of the whole [D1,2] stwintron (EST Contig Locus3050v1rpkm49.35) and the predicted splicing intermediate obtained after the excision of its internal intron (EST Contig Locus9508v1rpkm3.63), corresponding to band A in Fig. 3B and the sequence in Fig. 3C for the processing of the *H. solani* mutarotase stwintron.

4. Discussion

We have demonstrated the existence and investigated the excision of two new spliceosomal twin introns in *H. solani*. These occur in genes encoding respectively a mitochondrial alternative oxidase and an aldose mutarotase. The alternative oxidase gene stwintron (or any intron in the cognate position) is absent in any other ascomycete genome sequenced to date. The stwintron in the mutarotase gene also occurs in two related species of the suborder Massarineae (Dothideomycetes, Pleosporales) to which *Helminthosporium s. str.* belongs (Olivier et al., 2000; Eriksson and Hawksworth, 2003; Zhang et al., 2012). The generic type species of the Massarinaceae family, *Massarina eburnea*, does not have an intron in the cognate position. This suggests that the whole stwintron has been lost since this species diverged from other Massarinaceae, conceivably by "reverse transcriptase-mediated intron loss" (see, e.g., Roy and Gilbert, 2006; Cohen et al., 2012). This contrasts with the situation of the *bioDA* stwintron in the Sordariomycetes, where a canonical U2 spliceosomal intron is extant in the primary transcript of *Nectria haemotococca* at the same site as the [D2,3] stwintron present in all other *bioDA* genes of sequenced Sordariomycetes (Flipphi et al., 2013).

The stwintron in the primary transcript of the terminal oxidase comprises two terminally overlapping U2 internal introns (Fig. S1), excision of either leads to a *de novo* functional U2 intron, which is then subsequently processed. We have shown that both splicing pathways appear to occur with a similar efficiency. In the mutarotase primary transcript, excision of the 5′ internal [D1,2] intron (Fig. S1) would lead to a functional U2 external intron which is then processed to yield the correct messenger, while excision of the 3′ alternative intron is actually a mis-splicing event leading to a faulty RNA end product. Nevertheless, also in this case both pathways are operative.

The existence of two mutually exclusive splicing pathways within the same stwintron has implications for the proposed mechanism of spliceosomal intron excision. It has been proposed (Aebi and Weissmann, 1987; recent reviews: Han et al., 2011; Montes et al., 2012; Naftelberg et al., 2015) that introns are removed co-transcriptionally, which implies a strict 5′-to-3′ order of intron excision ("first come, first served"). To accommodate exceptions (see, e.g., Kessler et al., 1993; Attanasio et al., 2003; Denis et al., 2005) the concept has been modified to co-transcriptional commitment ("first come, first committed"), in which spliceosomes assemble at introns along a transcript from 5′ to 3′ although the thus committed introns do not necessarily excise from 5′ to 3′ (Kornblihtt et al., 2013). Even this less stringent concept does not seem to apply to the introns of the stwintrons described in this article. The co-transcriptional hypothesis would predict a clear (or even an exclusive) preference for the excision or commitment of 5′ internal intron of both stwintrons (internal introns "I," see Figs. 2A and 3A), which does not occur. Our results demonstrate a commitment of the splicing complex to one of two exclusive alternatives. After the initial excision of an internal intron, a functional intron must be defined *de novo* in order to excise the remaining stwintron sequences, which for the [A2,3] pathway of the alternative oxidase stwintron lie upstream of the newly assembled (external) intron acceptor site. For the mutarotase stwintron, this blind-choice process leads alternatively to a correct mature mRNA and to a mis-spliced, non-sensical RNA.

Using the exonic PCR primers (P1/P2) for the mutarotase transcript, we detected the amplification of properly spliced, mature mRNA formed via the [D1,2] route (Fig. S2: a fragment of 140 bp) but not that of the end product of the abortive [A2,3] pathway after removal of the alternative intron "II", which should have given a fragment of 191 bp. The intron definition theory would predict that a second excision step, removing the remaining sequences of the original stwintron (with the exception of the guanosine 5′ of the canonical donor) would take place using the most proximal acceptor sequence within the exon downstream. The first AG (5′-GAG) in the downstream sequence is 45 nt away from the exon fusion site of alternative intron "II". Use of the first canonical 5′-AAG (at 70 nt) would restore the open reading frame, albeit resulting in the loss of 24 codons of conserved exonic sequences. However, no fragment smaller than 140 bp was detected with primer pair P1/P2 (Fig. S2) strongly suggesting that a subsequent U2 excision event does not take place.

The mis-spliced RNA species apparently does not accumulate as a splicing end product (Fig. S2). This may result from nonsense mediated mRNA decay (NMD), a consequence of the in-frame UAA stop codon in the 51-nt non-excised (stw)intronic sequences upstream of exon fusion site of the standard 3′ intron, 370 nt 3′ of the stwintron. In *Aspergillus nidulans*, NMD has been observed in transcripts from genes carrying (exonic) nonsense loss-of-function point mutations (Morozov et al., 2006; references therein). Transcript levels are drastically affected by the presence of an premature stop codon (see, e.g.: Oestreicher and Scazzocchio, 1993; Flipphi et al., 2001, 2014). An orthologue of the *A. nidulans nmdA* nuclease-encoding gene, mediating NMD, is present in *H. solani* in contig 00547 (GenBank accession AWWW01000547). In vertebrate models, evidence indicates that alternative splicing often involves the formation of nonsensical mRNA, and that the products from such apparently futile splicing events are turned over by means of NMD. The coupling of alternative splicing and NMD is proposed to function as an additional level of post-transcriptional gene regulation (e.g., Braunschweig et al., 2014; Lykke-Andersen et al., 2014. Reviewed by, e.g.: Hamid and Makeyev, 2014; Ge and Porse, 2013; Kornblihtt et al., 2013; Lareau et al., 2007; Sibley, 2014; Smith and Baker, 2015). This phenomenon has never been studied systematically in Pezizomycotina. Previously, we had verified the formation of a nonsensical splicing product of the *Botrytis cinerea* putative imidine hydrolase (*pih1*) transcript as the result of alternative splicing (Flipphi et al., 2013). This faulty product had accumulated at 15 h of growth on rich medium but appeared almost absent 9 h later. Our current investigation of the mutarotase stwintron appears to confirm that regulation at the level of transcript splicing by coupling alternative splicing with NMD could be a novel regulatory feature in fungi. More specifically, [D1,2]- and [A2,3] stwintrons may have a regulatory role that depends on as yet undefined mechanisms conferring differential splicing preferences to the two constituent introns.

The present work extends the concept of spliceosomal twin introns by demonstrating experimentally the existence of a second class, where the internal intron splits the acceptor domain of the external intron, in addition to that previously demonstrated where the donor sequence of the external intron is interrupted. The demonstration of alternative splicing events within one and the same stwintron, together with its possible coupling with NMD, foresees a novel mode of post-transcriptional regulation in fungi.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2015.10.004.

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