Splicing regulation of a potato invertase mini-exon

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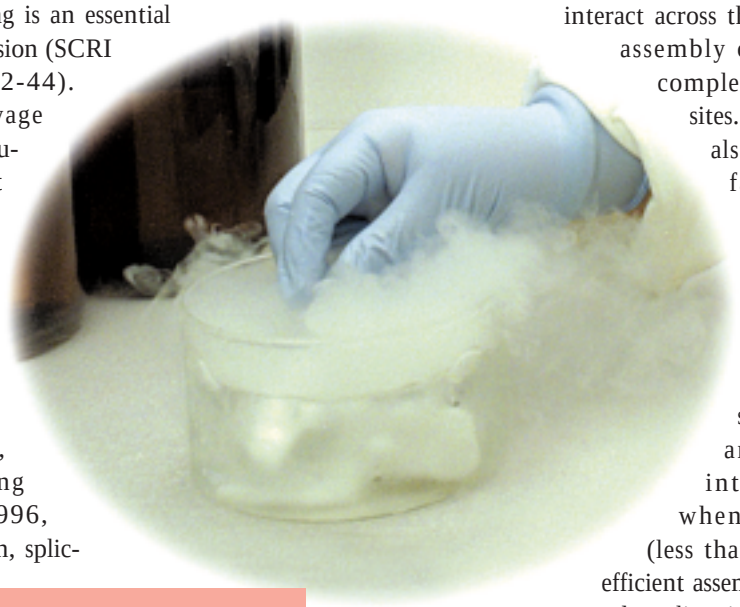
The removal of non-coding intron sequences from precursor messenger (pre-mRNA) transcripts by the process of splicing is an essential step in plant gene expression (SCRI Ann. Rep. 1991, 42-44). Selection of the cleavage (splice) sites must be accurate to retain the correct translational open reading frame. One mechanism aiding splice site selection is exon definition (Fig. 1a), which has been demonstrated in vertebrate and, more recently, plant intron splicing (SCRI Ann. Rep. 1996, 102). In exon definition, splicing components associate at the 3' splice site region and 5' splice site bordering an exon, and interact across the exon to stabilise assembly of splicing factor complexes at each of the sites. This process may also be mediated by factors bound to the exon itself. Following exon definition, interactions across introns occur to initiate spliceosome formation and removal of introns. However, when exons are small (less than 50 nucleotides), efficient assembly of splicing factors at the splice sites may be adversely affected due to steric constraints, precluding the involvement of exon definition in splice site selection.



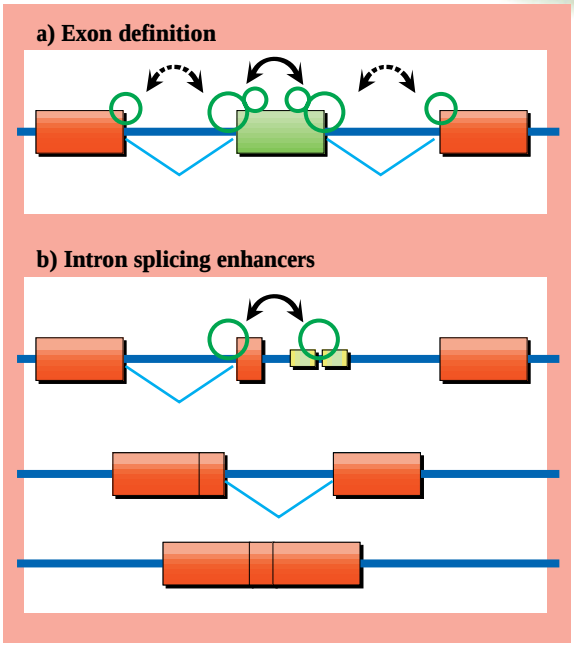


Figure 1 Model of exon definition and mini-exon/intron enhancers. (a) Splicing factors (circles) associate with splice sites at the ends of or on exons (boxes) and interact across the exon prior to intron (solid line) removal (dashed lines). (b) Factors associate with intron splicing enhancers (small boxes within intron) stabilising factor assembly on the mini-exon, permitting splicing.

Different mechanisms have been proposed to explain inclusion of short, mini-exons (as small as three nucleotides) in vertebrates. Firstly, sequential splicing may occur whereby one intron is removed, splicing factors dissociate from the mRNA and the spliceosome reassembles on the second intron, which is then removed. Such a mechanism has been described for the seven nucleotide long, troponin I, mini-exon 3 from chicken. Secondly, mini-exons may be flanked by strong splice sites, improving their inclusion in mature mRNA transcripts. In some cases, however, this is not sufficient to promote inclusion, and other elements/factors are required. Thirdly, and most commonly, intron splicing enhancers (ISEs) have been described which promote the selection of splice sites (Fig. 1b). ISEs are usually found downstream of the mini-exon, are diverse in sequence and often consist of a series of direct repeats. Binding of specific protein factors to ISEs is thought to recruit splicing factors to the exon to initiate splicing (Fig. 1b).

One of the smallest exons discovered in plants to date is a nine nucleotide mini-exon found within invertase

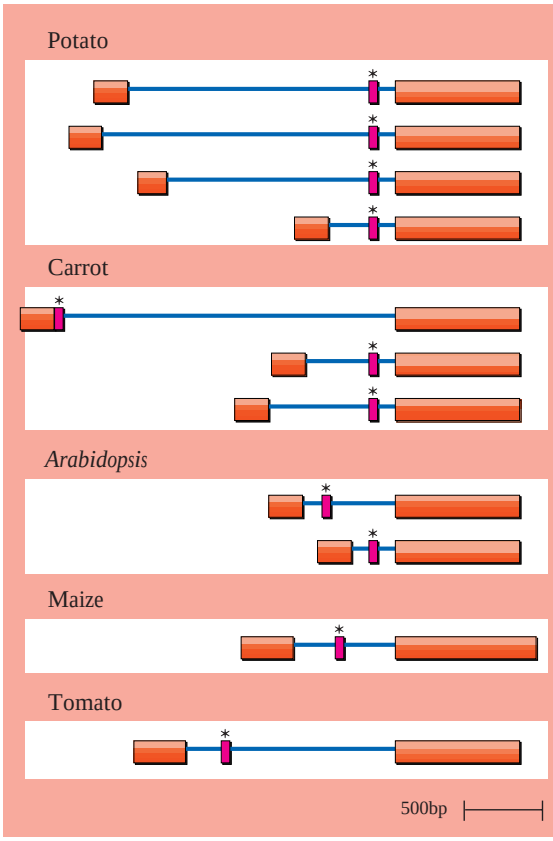


Figure 2 Structure of plant invertase genes. Exons (boxes) and introns (solid lines) are shown for all characterised plant invertase genes. The mini-exon 2 is present in all but one of the carrot genes.

genes and flanked by introns of varying sizes (Fig. 2). Invertase is one of two key enzymes known to catalyse the breakdown of sucrose in plants. This central role in plant carbohydrate metabolism is executed by a variety of invertase isoenzymes specific to particular cell compartments, tissues and developmental stages, encoded by a family of invertase genes. With one exception, all characterised plant invertase genes include the mini-exon which encodes three amino acids of a highly conserved five amino acid motif found in all invertase enzymes and which is therefore likely to be critical to their function. In potato, we have characterised four invertase genes, each of which contains a mini-exon, and we have shown that under cold stress conditions, alternative splicing, which results in skipping of the mini-exon, can occur at low frequency (SCRI Ann. Rep. 1995, 52). The physiological consequences of exon skipping to the function of the resulting enzyme are being examined alongside the mechanism which ensures the accurate inclusion of the mini-exon under normal conditions.

It is likely that inclusion of this mini-exon into mature invertase mRNA will require splicing signals in addition to the flanking splice sites. To identify such signals, we have made a series of intron/mini-exon constructs based on the potato invertase genes, invGE and invGF (SCRI Ann. Rep. 1996, 100-101). All of the constructs were inserted into the expression and splicing analysis vector, pDH515, and their splicing behaviour analysed. Each construct was introduced into tobacco protoplasts and, following expression of the transcripts, RNA was isolated and examined by reverse transcriptase-PCR (RT-PCR) using a labelled primer in the PCR reaction. Primers were designed to amplify from the sequences in pDH515 flanking the intron constructs and mini-exon inclusion or skipping was determined from the sizes of the generated PCR products.

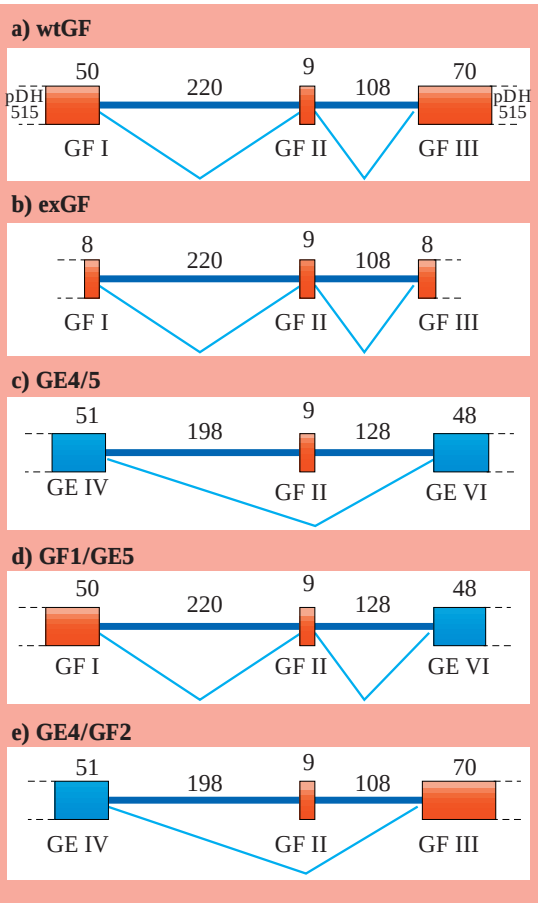


Figure 3 Intron replacement constructs. Schematic diagram showing the region from the invGF gene (red) containing the mini-exon and the modifications where introns from invGE (blue) replace those of invGF. (a) WtGF - wild type. (b) exGF - As a but with reduced sizes of exons 1 and 3. (c) GE4/5 - double intron replacement. (d) and (e) Single intron replacements.

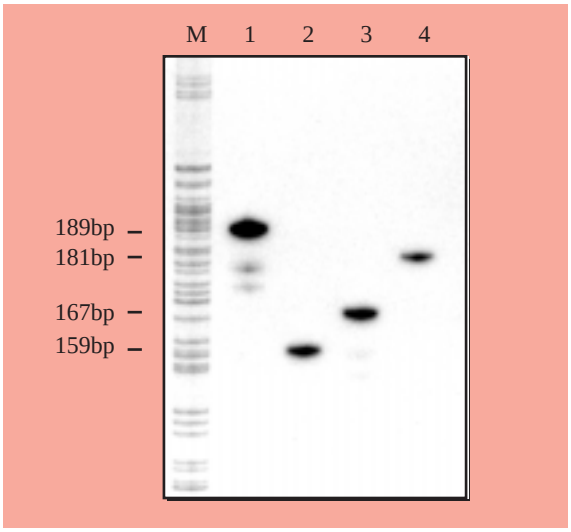


Figure 4 RT-PCR analysis of splicing of intron replacement constructs. Electrophoretic separation of labelled RT-PCR products from wtGF (lane 1), GE4/5 (lane 2). GF1/GE5 (lane 3) and GE4/GF2 (lane 4).

To demonstrate that correct mini-exon splicing (inclusion) occurs in the tobacco protoplast experimental system, a region of invGF containing 50 bp of exon 1, intron 1 (220 bp), exon 2 (the 9 bp mini-exon), intron 2 (108 bp) and 70 bp of exon 3 was isolated by PCR amplification (Fig. 3a; wtGF). In this construct, the potato invGF mini-exon is flanked by its authentic introns: introns 1 and 2, both of which are UA-rich (72% and 84% UA respectively), an important signal in plant intron splicing. RT-PCR analysis of the wtGF transcript (Fig. 3a) revealed a fully spliced product of 189 bp which shows that the mini-exon was included constitutively in the mature mRNA transcript when flanked by its authentic introns and exons (Fig. 4, lane 1). Although it was likely that the signals for mini-exon inclusion lay in the flanking introns, to exclude the possibility that exons 1 and 3 were needed for correct splicing, a construct was made where the exons in wtGF were reduced to 8 bp each (Fig. 3b; exGF). Splicing analysis of this construct showed that the mini-exon was present in the mature transcript, demonstrating that these flanking exon sequences do not influence mini-exon inclusion (results not shown).

The first construct designed to show that at least one of the introns flanking the mini-exon was important for its correct splicing, was a double intron replacement construct. Introns 1 and 2 in wtGF were replaced by introns 4 and 5 (and their flanking exons) of the second invertase gene (invGE). These introns were selected as they were similar in size and UA content to the introns which normally flank the mini-exon. This construct therefore contained the invGF mini-exon flanked by two introns derived from a completely different region of invGE (Fig. 3c; GE4/5). In this case, the mini-exon was no longer included (i.e. it was skipped), as reflected by the spliced product of 159 bp (Fig. 4; lane 2). Therefore, inclusion of the mini-exon in the mature mRNA required its authentic flanking intron(s).

Finally, to determine whether one or both introns were necessary for normal splicing, single intron replacement constructs were made. Firstly, the intron downstream of the mini-exon (invGF intron 2) was replaced by invGE intron 5 (Fig. 3d; GF1/GE5). Secondly, the upstream intron (invGF intron 1) was replaced by invGE intron 4 (Fig. 3E; GE4/GF2). Splicing of GF1/GE5 in tobacco protoplasts resulted in the inclusion of the mini-exon in the final transcript as shown by a 167 bp RT-PCR product (Fig. 4, lane 3). However, splicing of GE4/GF2, always resulted in skipping of the mini-exon as shown by a 181 bp RT-PCR product (Fig. 4; lane 4). Thus, the presence of the authentic upstream intron 1 was essential for the inclusion of the mini-exon and the downstream intron 2 was not required for correct splicing.

Clearly intron 1, upstream of the mini-exon, regulated inclusion of the mini-exon into invertase mRNA. However, intron 1 does not contain sequences similar to known intron splicing enhancers from vertebrate introns nor are there obvious direct repeat sequences, apart from U-rich regions. Therefore, a systematic deletion analysis of this intron is currently underway to identify the signals responsible for mini-exon inclusion. To date, a polyuridine tract and the branch-point have been shown to be required for exon inclusion. Ultimately, it may be feasible to develop biotechnological applications in targeted regulation of gene expression, by controlling the inclusion or exclusion of mini-exon containing genes, resulting in the alteration in the functional capacity of alternatively spliced proteins.