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Exon skipping induced by cold stress in a potato invertase gene transcript

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ABSTRACT

We show that two invertase genes in potato, like most other plant invertase genes, include a very short second exon of 9 bp which encodes the central three amino acids of a motif highly conserved in invertases of diverse origin. This mini-exon is one of the smallest known in plants and pre-mRNA from these genes may be susceptible to alternative splicing, because of a potential requirement for specialized interaction with the splicing machinery to ensure correct processing for the production of a mature mRNA. No evidence of aberrant post-transcriptional processing was observed during normal invertase gene expression in potato. The fidelity of post-transcriptional processing of the pre-mRNA from one of the genes was perturbed by cold stress, resulting in the deletion of the mini-exon from some transcripts. This alternative splicing event occurred under cold stress in both leaf and stem, but was not induced by wounding. This adds an example of exon skipping and the induction of alternative processing by cold stress to the small number of transcripts which have been shown to exhibit alternative splicing in plants. The differential sensitivity of post-transcriptional processing to cold stress observed for the two transcripts examined will permit further dissection of the nucleotide sequence requirements for their accurate splicing.

INTRODUCTION

In plants the cleavage of sucrose to glucose and fructose can be catalysed by invertase (β-fructosidase, EC 3.2.1.26; 1) or sucrose synthase (EC 2.4.1.13; 2). These enzymes are central to sucrose metabolism and so are involved in fundamental physiological processes, at both the whole plant and cellular levels, such as source–sink interaction and carbohydrate partitioning (3,4). Invertases can be divided into neutral (cytosolic) and acid (vacuolar and apoplastic) classes (5). Acid invertases have been purified from a wide variety of plants (for a compilation of references see 6,7) and, with many cDNA and genomic clones also having been isolated, have been shown in some plants to be encoded by a family of related genes (see for example 8,9). Derived amino acid sequences reveal that plant invertase proteins share a number of highly conserved motifs with each other and with invertases from yeast and bacteria (10). These include a potential active site motif (WECPD) and a further highly characteristic motif (NDPNG/A), which may also participate in the catalytic mechanism, near the N-terminal of the protein. This latter motif has been included in protein databases (e.g. PROSITE) as the definitive β-fructosidase motif. Characterization of plant genomic clones encoding invertases has shown that, with a single exception, the central core of this motif (DPN) is encoded by a mini-exon of 9 bp. This is one of the smallest exons known in plants. In animal systems a minimal exon length of 51 bp for the promotion of correct splicing has been proposed, with smaller exons being liable to aberrant splicing resulting from the close juxtaposition of 5′ and 3′ splice sites (11). A significant number of animal, but few plant, exons are shorter than this limit and it has been further proposed that these shorter exons may require splice sites which are ‘stronger’ than normal and/or additional enabling elements for their correct recognition (12). A variety of alternative splicing events (involving intron retention, alternative 3′ or 5′ splice sites, exon skipping, etc.) have been catalogued in animal systems (13). Such events have been shown to result in the synthesis of alternative proteins which may differ in their function or tissue distribution. The widely documented occurrence of alternative splicing has prompted suggestions that it constitutes an alternative mechanism at which the regulation of gene expression can be effected. In plants, only a few examples of alternative splicing have been described. Several examples of alternative 5′ splicing events are known: e.g. in transcripts for rubisco activase in spinach and *Arabidopsis* (14) and in barley (15), for RNA binding protein 1 in tobacco (16) and for chorismate synthase in tomato (17). Alternative 3′ splicing occurs in processing of transcripts from the maize *P* gene (18) and encoding the H protein of *Flaveria trinervia* (19). Alternative processing of the transcript for granule-bound starch synthase in rice can result in the retention of intron I in the mature message (20). Given the small size of the mini-exon in plant invertase genes, their transcripts are candidates for alternative splicing events which would result in omission of sequences encoded by the mini-exon from the mature mRNA and translation to a mutated polypeptide which lacks the core of the β-fructosidase motif. This possibility has led us to survey expression from

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invertase genes in potato for alternative splicing events. We demonstrate that alternative splicing can be induced by cold stress and that a differential effect is observed during expression of the two plant invertase genes examined.

MATERIALS AND METHODS

Genomic PCR

DNA was isolated from leaf of *Solanum tuberosum* (cv. Cara) by extraction including an alkaline SDS lysis step (21). Aliquots of 30 ng were used as template in a PCR assay of a total volume of 50 µl containing 1 µM each of 5′ and 3′ primers [for CD111, AMWT1 (CCAGGGTTGCAATCTACAAGCACGG) and AMW6 (GTCGGTTGAAACTGAATGGGCCCAA) respectively; for CD141, AMWT4 (CCATGTTGATGTTAGCAAGGTCCAT) and AMW2 (GCCCCATATCGCTCCCTTTGGGTTG)], 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl and 20 mM Tris–HCl, pH 8.4, with 1 U *Taq* polymerase (Gibco-BRL). PCR was conducted for 40 cycles (95°C/1 min, 65°C/1 min, 72°C/3 min per cycle) with a final extension at 72°C for 5 min. Reaction products were analysed by agarose gel electrophoresis and the single products obtained were cloned into pGEM-T (Promega) and sequenced (22) in both directions.

RT–PCR

Total RNA was isolated from leaf and stem of *Solanum tuberosum* (cv. Cara) following the procedure of Schröder *et al.* (23). Contaminating DNA was removed by incubation with 1 U RQ1 DNase (RNase-free; Promega) in 100 µl 10 mM Tris–HCl, pH 8, 1 mM EDTA containing 72 U RNase inhibitor (RNAguard; Pharmacia Biotechnology) for 20 min at 37°C followed by RNA precipitation and repetition of this treatment. Aliquots of 2 µg total RNA (DNA-free) were reverse transcribed with 200 U Superscript reverse transcriptase (Gibco-BRL) in a final volume of 25 µl of 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 50 mM Tris–HCl, pH 8.3, at 42°C for 45 min in the presence of 1 mM dNTPs and 0.5 µg of the appropriate 3′ primer [for CD111, 111R (GATAGACACCATTATAGTACATTGG); for CD141, 141R (GATACACTCCATTGTAATACATTGG)]. Samples of 5 µl from this reaction were used to provide the cDNA template for PCR using the same 3′ primer with the appropriate 5′ primer [for CD111, 111F (GCACGGTGATGAGAAAAATGTTCA); for CD141, 141F (GCCATGTGATGTTAGCAAGGTCCA)] end-labelled with ^{32}P and buffer as described above. PCR conditions were 35 cycles of 94°C/1 min, 60°C/2 min and 72°C/3 min followed by a final extension at 72°C for 5 min. RT–PCR products were resolved by electrophoresis in 6% polyacrylamide–10% formamide gels and detected by autoradiography. Products obtained using CD111 primers were eluted from the polyacrylamide gel and used as substrate for PCR assays using these primers with conditions as for the genomic PCR except that an annealing temperature of 60°C and 35 cycles were used. Single products from these reactions were cloned into pGEM-T and sequenced (22) in both directions.

Stress conditions

Stress experiments were conducted on plantlets in tissue culture grown on MS-30 medium (24) under a 16/8 h day/night cycle at 25°C. Three weeks after subculture of apical nodes, plantlets were stressed by either cold or wounding. For cold stress, plantlets were transferred to a growth cabinet and held at 4°C, with other conditions similar to those previously described. Plantlets were harvested after 0 (control), 6, 24 and 48 h. For recovery, after 48 h at 4°C, plantlets were returned to 25°C for 24 h prior to harvest. For wounding stress, leaves on the plantlets were punctured with a sharp sterile scalpel over the entire surface and harvested after 24 h.

RESULTS

Organization of invertase genes in plants

Genomic clones encoding invertase enzymes have now been isolated from carrot, *Arabidopsis*, maize and tomato (9,25–29). The intron/exon organization of all of these (Fig. 1a) is characterized by the presence of a major exon of ~1 kb encoding the majority of the polypeptide, including the proposed active site. Downstream of this some variation in the size of introns is evident, with their absence in some cases (e.g. one *Arabidopsis* gene where the major exon incorporates sequences represented by two further exons in the other genes). Upstream of the major exon an unusual feature of these genes is the presence of a very small exon, only 9 bp in size, which is present in all the genes except in one carrot gene, where it is incorporated into the major exon. Both introns flanking the mini-exon show variability in length in these plant species. The 9 bp exon encodes the central three amino acids of the β-fructosidase motif (DPN), which has been proposed to be involved in the formation of the catalytic site of the enzyme, a function consistent with its high degree of evolutionary conservation.

No genomic clone encoding potato invertase has been described to date. However, we have cloned two different cDNAs (CD111 and CD141) from potato leaf representing two genes which are differentially expressed during leaf maturation (8,30). From the assumption that the intron/exon organization of the potato genes encoding these cDNAs resembled that of the invertase genes previously described, we designed primers to amplify from the potato genome a region spanning from exon I to the major exon for each gene. PCR products generated from both sets of primers were cloned and their nucleotide sequence determined. In the two gene fragments corresponding to the two potato cDNAs, an organization similar to the majority of the other plant invertase genes was found (Fig. 1b), with each gene including a 9 bp mini-exon, flanked in both cases by one large and one small intron.

Alternative splicing of potato invertase genes induced by cold stress

To assay for alternative splicing events involving the mini-exon of each of the potato genes we used two further sets of primers designed specifically to amplify, by RT–PCR, the same region (exon I–exon III, including exon II and its flanking introns) of the transcripts derived from the two potato invertase genes. In both cases, if transcripts were correctly spliced a product of 105 bp would result, while if exon II was skipped in an alternative splicing event an alternative product of 96 bp would be generated. RNA isolated from tissues where both genes are expressed (leaf and stem) was used for RT–PCR, but no evidence of alternative splicing events was observed, with only a single product of 105 bp representing accurate splicing being detected (results not

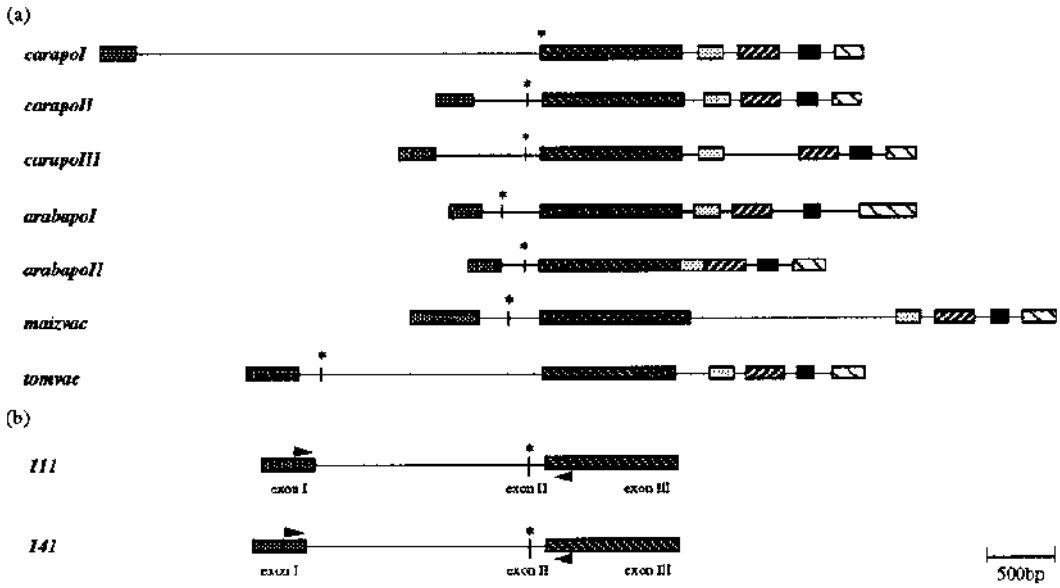

Figure 1. Diagrammatic representation of invertase genomic sequences of (**a**) carrot carapoI (25) and carapoII (9), *Arabidopsis* arapoI (26) and arabapoII (27), maize maizvac (28) and tomato tomvac (29) and (**b**) isolated from potato by PCR. Boxes represent exons (with corresponding sequences hatched) and lines represent introns. Sequences encoding the core (DPN) of the β-fructosidase motif are asterisked. Arrows (not to scale) indicate the position of primers used for RT–PCR.

shown). It is known that stress, e.g. heat shock, can induce alternative splicing in animals (31). In potato, invertase enzymes have been implicated in the accumulation of reducing sugars (low temperature sweetening) in tubers undergoing cold storage (32). For this reason the potential for alternative splicing of invertase transcripts induced by cold stress is of particular interest in potato. We exposed plantlets of potato in tissue culture to a time course of cold exposure and a subsequent recovery period. Leaf RNA isolated from each time point was used as substrate for RT–PCR with the same sets of primers previously described. With primers against CD111 transcripts, after 6 h under cold stress two major products were detected (Fig. 2), one of 105 bp and a second product of 96 bp, representing a potential product of alternative splicing. The amount of this second product increased with the time of exposure to cold stress. However, no evidence of this smaller product was detected after the recovery period. A further product was also detected between the two major products in all samples. All three products were cloned and sequenced to check their correspondence to the products from the accurate and alternative splicing events. Both the 105 bp and intermediate products correspond to the accurate splicing product as predicted from the cDNA sequence and the intermediate product may therefore represent a denaturation artefact generated from the 105 bp product. The 96 bp product was confirmed as an alternatively spliced product which lacked the 9 bp corresponding to exon II (Fig. 3). With primers for CD141 transcripts, no evidence of an alternative spliced product was detected even after 48 h under cold stress (result not shown).

Specificity of alternative splicing

To examine further the specificity of the alternative splicing event we have looked for its occurrence in a different tissue and under an alternative stress condition. RNA was extracted from stem tissue after 48 h of cold stress and used in RT-PCR with both sets

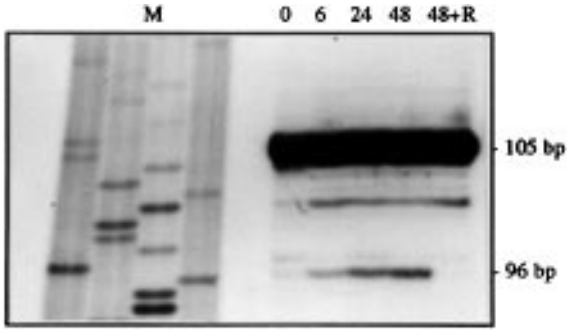

Figure 2. RT–PCR analysis of transcripts from the potato invertase gene encoding CD111 after cold stress of plantlets for the indicated time (h) and subsequent recovery period of 24 h (+R). M, DNA sequence used as size marker.

of primers. Again, for CD111 transcripts an alternatively spliced product was observed after cold stress but not in control plantlets, whereas for CD141 transcripts only the correctly spliced product was detected for both sets of plantlets (Fig. 4). This indicates that the alternative processing event is not specific to leaf but can occur in another tissue where this gene is expressed. To examine an alternative stress condition we have conducted a wounding experiment. RNA was prepared from wounded tissue and used as substrate for RT–PCR, again using both sets of primers. In this case no alternative splicing was observed for either transcript (Fig. 4).

Sequence comparison

We have analysed the nucleotide sequences of both the exons which have been suggested to be important for correct splicing (33) and the introns involved in this splicing event. The relevant

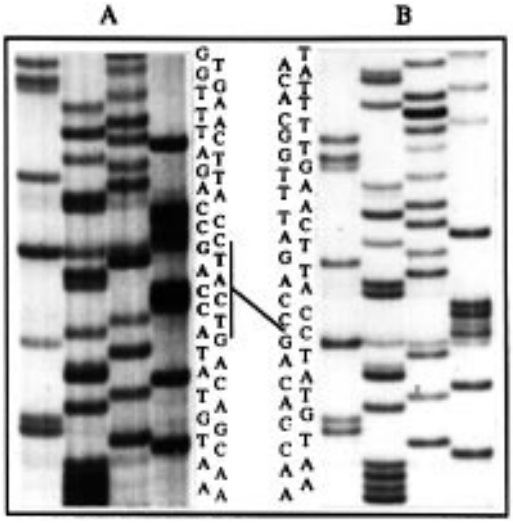

Figure 3. Nucleotide sequence of the correct (105 bp) and alternatively spliced (96 bp) products generated by RT–PCR. Mini-exon sequences in bold.

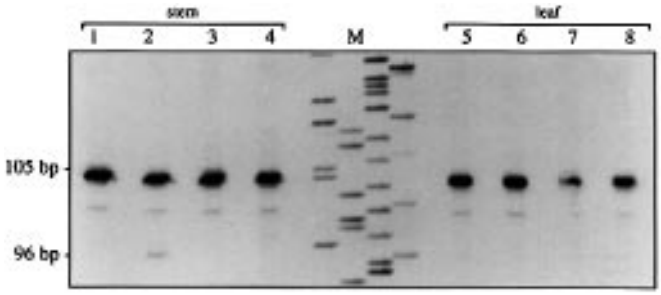

Figure 4. RT–PCR analysis of transcripts from potato invertase genes (**a**) in stem after cold stress (lanes 2 and 4) or untreated (lanes 1 and 3) and (**b**) in leaf after wounding (lanes 5 and 7) or untreated (lanes 6 and 8) using primers specific for transcripts CD111 (lanes 1 and 2 and 5 and 6) and CD141 (lanes 3 and 4 and 7 and 8). M, DNA sequence used as size marker.

nucleotide sequences from both genes are deposited in the EMBL nucleotide sequence database under the accession nos X95820 and X95821. One feature apparent in the exon sequences is the presence of a pentanucleotide (YAAYG) which is repeated four times in the gene encoding CD111: at the end of exon I, in exon II and twice at the start of exon III. The number and location of these repeats is conserved in the gene encoding CD141 with the exception of within the mini-exon, where the sequence AAATG is found. An examination of other plant invertase gene exon sequences in this region shows that this pentanucleotide repeat is largely conserved in number and location, although more variation in sequence is observed. The sequences of the smaller intron II of both genes, with A/T contents of 83 and 81%, lie at the upper end of the range of AU content for dicotyledon introns (~60–80%) (34). The AU/GC transitions of intron/exon borders may represent an important signal in plant intron recognition (35) and the high AU content of these introns constitutes a sharp transition in this measure from the neighbouring sequences of intron I and exon II on one side and exon III on the other. Both introns contain long A/T-rich tracts which, notably in the case of CD111, include a 15 bp perfect repeat. Sequences resembling this repeat are also found in the CD141 transcript.

DISCUSSION

The two potato genes whose expression we have studied (8) share with most other plant invertase genes an intron/exon organization which features a mini-exon II of 9 bp (Fig. 1). Under cold stress pre-mRNA from one of these genes (CD111) was subject to alternative splicing which resulted in the omission of sequences corresponding to exon II from some mature transcripts (Figs 2 and 3). These aberrant processing events occurred in both tissues in which this gene is expressed, but accurate splicing was restored on removal of cold stress. No alternative splicing of invertase gene transcripts was induced by wounding (Fig. 4), indicating that it does not result from a generalized response to stress.

It seems unlikely that this alternative splicing results from a non-specific decrease in the efficiency of processing of precursor mRNAs in the cold, since no such effect was observed for CD141 transcripts. This inference is reinforced by control experiments (results not shown) which detected no cold-induced effects on processing of downstream introns in CD111 transcripts. We suggest that accurate splicing of invertase gene transcripts may be mediated by signals within their exon/intron organization and that the difference observed in the processing of CD111 and CD141 transcripts results from differential interaction of the splicing machinery with these under cold stress. The pentanucleotide YAAYG is repeated four times in the exon I border–exon II–exon III border sequence of potato invertase genes and represents a potential signal. These sequences are, however, within coding regions and may be conserved because of a strict requirement for a particular amino acid sequence: this is consistent with the involvement of this region in an essential function at the enzyme level. Alternatively they may, in plants, have a composite function, at both the RNA and protein levels. Within the context of the short but highly AT-rich intron II we have detected a perfect repeat of 15 nt in CD111, with similar sequences in CD141. These repeat sequences may act to signal the presence of the mini-exon in a fashion similar to the repeated hexanucleotides found in animal introns, which are thought to exert an influence on exon selection (36). A wider analysis of the corresponding invertase introns in dicotyledons reveals similar features, with the smaller intron II having a higher A/T content than surrounding sequences and including A/T-rich tracts similar to the repeat sequences found in the potato genes intron II, although the high A/T content precludes definitive assignment as such. Also present are sequences which, on the basis of similarity to consensus plant sequences, are potential 5′ and 3′ splice site signals. Thus a variety of potential signals are present which may mediate correct splicing of the invertase sequences encoded by these mini-exons and the differences which exist between CD111 and CD141 transcript processing under cold stress in potato may reflect altered affinity of one or more splicing factors interacting with these. Sequestration of the mini-exon could also be effected by modification to secondary structures in intron sequences at low temperature. Further analysis of the differential splicing, complemented by a directed mutation approach, might aid dissection of the relative importance of potential splicing signals and the sequence of splicing events.

We have demonstrated that exon skipping induced by cold stress can occur during expression of one of two invertase genes

in leaves and stem of potato. Under the controlled conditions applied in tissue culture the proportion of alternative to correctly spliced transcripts is low. While this may represent either a low level of alternative splicing of CD111 throughout these organs or a higher level of alternative splicing in specific tissues or cell lineages set against a background of correct splicing elsewhere, CD111 expression occurs in young developing leaf tissue where extracellular invertase levels are likely to be critical to the maintenance of sink strength. Further work on the specific expression patterns of these genes and the sensitivity of this to cold stress is required, in addition to a determination of the effect of the deletion of the core of the β-fructosidase motif on the activity and the stability of the enzyme, before the importance of the effects of aberrant splicing of invertase genes to the physiology of the whole plant can be assessed.

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