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Sink- and Vascular-Associated Sucrose Synthase Functions Are Encoded by Different Gene Classes in Potato

Hongyong Fu and William D. Park[1]

Department of Biochemistry and Biophysics, Texas A & M University, College Station, Texas 77843-2128

Two differentially regulated classes of sucrose synthase genes, *Sus3* and *Sus4*, were identified in potato. They cannot be classified as *Sus1* and *Sus2* types based on sequence homology and appear to have evolved after the divergence of the major families of dicotyledonous plants but before the divergence of tomato and potato. The potato sucrose synthase clones *Sus3*-65 and *Sus4*-16 share an 87% nucleotide identity in the coding regions, and both are interrupted by 13 introns, including a long leader intron. Potato *Sus3* genes are expressed at the highest levels in stems and roots and appear to provide the vascular function of sucrose synthase. In contrast, *Sus4* genes are expressed primarily in the storage and vascular tissue of tubers and appear to facilitate sink function. The genes are differentially regulated in root tips, with *Sus3* expressed at high levels in the cell division zone and *Sus4* expressed at high levels in the meristem and cap.

INTRODUCTION

Sucrose synthase (EC 2.4.1.13) catalyzes the reversible conversion of sucrose and UDP into UDP-glucose and fructose. Sucrose synthase is ubiquitous in higher plants (Avigad, 1982) and plays a variety of important roles. It is the predominant sucrose cleavage enzyme in cereal endosperm and potato tuber and provides substrates for starch synthesis in these and other storage organs (Chourey and Nelson, 1976; Claussen et al., 1985; Dale and Housley, 1986; Sung et al., 1989; Sun et al., 1992; Wang et al., 1994). Sucrose synthase is also involved in meeting the increased glycolytic demand during anaerobic and cold stress as well as in supplying UDP-glucose for cell wall biosynthesis (Springer et al., 1986; Hendrix, 1990; Maas et al., 1990; Maraña et al., 1990; Ricard et al., 1991; Martin et al., 1993).

In addition, sucrose synthase appears to play a key role in supplying energy for loading and unloading in phloem by providing substrate for respiration. Sucrose synthase activity has been shown to be associated with vascular tissues in a number of species (Hawker and Hatch, 1965; Lowell et al., 1989; Tomlinson et al., 1991) and is localized specifically in the companion cells in maize leaves and citrus fruits (Nolte and Koch, 1993). Expression of sucrose synthase in phloem tissues of transgenic tobacco plants has been observed by using the maize *Shrunken1* (*Sh1*) promoter (Yang and Russell, 1990); it has also been observed recently in the vascular tissue by using an Arabidopsis sucrose synthase promoter (Martin et al., 1993). Recent experiments in which phloem-specific removal of pyrophosphate in transgenic tobacco resulted in sugar accumulation in source leaves and stunted growth are also consistent with a key role for sucrose synthase in phloem function (Lerchl et al., 1995).

In monocotyledonous species, such as cereals, sucrose synthase is encoded by two differentially expressed nonallelic loci, *Sus1* and *Sus2* (Werr et al., 1985; McCarty et al., 1986; Maraña et al., 1988a, 1988b; Chourey et al., 1991; Sánchez de la Hoz et al., 1992; Wang et al., 1992; Yu et al., 1992; Shaw et al., 1994). In maize, for example, the *Sus2* gene, *Sh1*, is expressed primarily in endosperm, whereas *Sus1* is expressed in the embryo, aleurone, and basal endosperm transfer cells, in sink leaves, and in the shoots and roots of seedlings (McCarty et al., 1986; Chen and Chourey, 1989; Heinlein and Starlinger, 1989; Nguyen-Quoc et al., 1990). Expression of the two maize genes is also modulated differentially by sugar levels (Koch et al., 1992) and by anaerobiosis (McCarty et al., 1986; Springer et al., 1986). Expression of *Sh1* is inducible under anaerobic conditions, whereas *Sus1* is relatively unaffected.

A sucrose synthase cDNA clone from potato tubers has been isolated and characterized by Salanoubat and Belliard (1987). They have shown that the steady state level of sucrose synthase transcripts is highest in developing tubers and is very low in other organs (Salanoubat and Belliard, 1989). Although transcripts were not detectable in normal leaves, they could be detected after incubation in high concentrations of sucrose. Potato sucrose synthase is also regulated by wounding and anaerobiosis (Salanoubat and Belliard, 1989).

Individual sucrose synthase cDNAs have been sequenced from carrot (Šebková et al., 1995), mung bean (Arai et al., 1992), broad bean (Heim et al., 1993), and tomato (GenBank accession number L19762). However, despite the characterization of these clones and the isolation of two divergent

[1] To whom correspondence should be addressed.

sucrose synthase genomic clones from Arabidopsis (Chopra et al., 1992; Martin et al., 1993), it is not clear whether dicots also contain differentially expressed classes of sucrose synthase genes as do monocots and, if so, how their patterns of expression would compare.

Here, we report the isolation and characterization of two classes of sucrose synthase genes from potato and demonstrate by RNA gel blot analysis and by expression in transgenic potato plants that they have different patterns of expression. Based on sequence homology with other published sucrose synthase clones, the *Sus3* and *Sus4* classes of potato sucrose synthase genes appear to have evolved after the divergence of the major dicot families but before the divergence of tomato and potato.

RESULTS

Presence of Two Classes of Sucrose Synthase Genes in Potato

Forty-four clones were isolated by screening a λ genomic DNA library (1.5 × 10^6 plaque-forming units) at low stringency with a polymerase chain reaction (PCR)-amplified genomic product corresponding to the region from positions 1495 to 2462 of the potato sucrose synthase cDNA *Potssyn* (Salanoubat and Belliard, 1987). Five of these clones, which contained the entire coding region plus at least 1.0 kb of 5′ flanking sequence, were characterized further by restriction enzyme mapping.

As shown in Figure 1, these five clones can be grouped into two classes, *Sus3* and *Sus4* (previously designated V and T classes, respectively; Fu et al., 1991), based on their restriction patterns. The two *Sus3* clones, *Sus3*-9 and *Sus3*-65, appear to be from different genes or alleles because their overlapping regions differ at one SstI site. The *Sus4* clones share restriction sites with the published potato tuber sucrose synthase cDNA *Potssyn*. Clones *Sus4*-1 and *Sus4*-16 may come from the same gene because they possess an identical restriction pattern in the overlapping regions. *Sus4*-34 has a different restriction pattern, indicating that it comes from a different *Sus4* class gene or allele.

The presence of two classes of sucrose synthase genes was confirmed by the detection of distinct restriction fragments when genomic DNA blots were probed at high stringency with fragments containing the coding region of either *Sus3*-65 or *Sus4*-16 (Figure 2). The restriction fragments detected agreed with those predicted from the restriction maps shown in Figure 1, except that additional bands were observed with both probes. The additional bands may be the result of the presence of other uncharacterized *Sus3* and *Sus4* genes or alleles in this tetraploid variety. The presence of additional classes of sucrose synthase genes cannot be ruled out. However, all bands detected by *Sus3* and *Sus4* probes were also detected at lower stringency (final washing at 65°C in 2 × SSC) by either probe alone (data not shown).

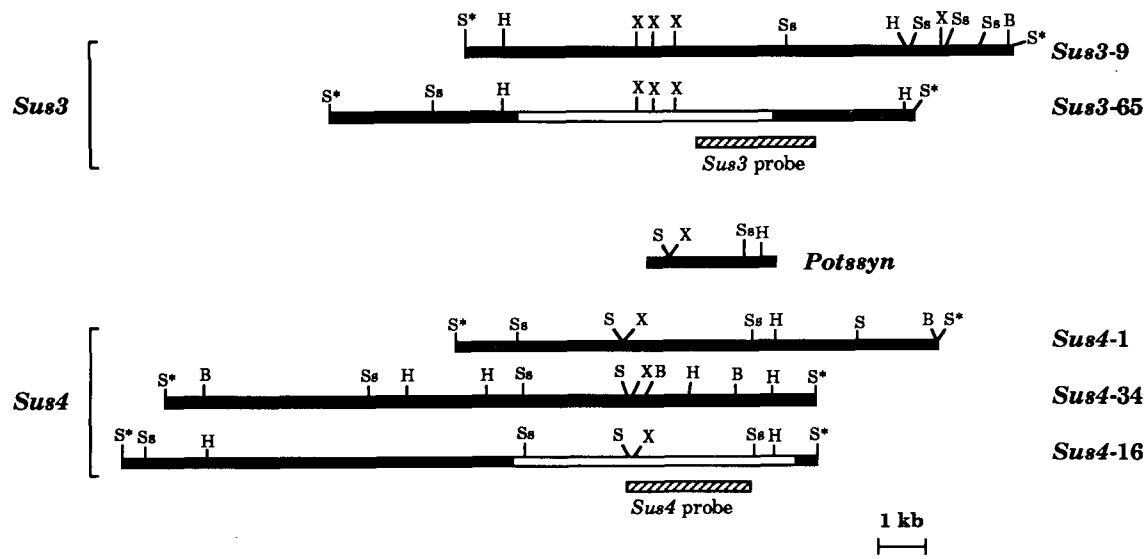

Figure 1. Restriction Maps of Potato Sucrose Synthase Genomic Clones.

Two classes of potato sucrose synthase genomic clones are displayed. Clone designations appear at right. Also shown is the published potato tuber sucrose synthase cDNA *Potssyn*. Open boxes indicate deduced transcription units. Positions of class-specific probes for DNA gel blot analysis are shown as hatched boxes. The *Sus3* class probe is a 2.5-kb EcoRI fragment of *Sus3*-65 (nucleotides 3737 to 6256), and the *Sus4* class probe is a 2.6-kb SalI-SstI fragment of *Sus4*-16 (nucleotides 2462 to 5089). Asterisks indicate sites from the λ vector. B, BamHI; H, HindIII; S, SalI; Ss, SstI; X, XhoI.

Genomic reconstruction experiments suggest that there are eight and four copies of the *Sus3* and *Sus4* class genes, respectively, per tetraploid genome (Figure 2).

Identification of the Coding Sequences in Representative *Sus3* and *Sus4* Class Genes

The nucleotide sequences of the *Sus3* genomic clone *Sus3*-65 and the *Sus4* genomic clone *Sus4*-16 are shown in Figures 3 and 4, respectively. The protein coding sequences for both genes were identified by comparison with the potato sucrose synthase cDNA *Potssyn* (Salanoubat and Belliard, 1987). All splicing junctions obey the GT/AG boundary rule and conform to the consensus sequences for splicing junctions in plant genes (Brown, 1986).

The deduced protein coding sequences of *Sus3*-65 and *Sus4*-16 are both 2415 bp in length. They could encode proteins of 805 amino acids with calculated molecular masses of ~92 kD, which is consistent with the estimated size of 90 kD for potato sucrose synthase (Ross and Davies, 1992). *Sus3*-65 shares 86.9% nucleotide identity in the coding region and 91.8% amino acid identity with *Potssyn*. *Sus4*-16 shares 98.6 and 99.3% nucleotide and amino acid identity, respectively, with *Potssyn*.

Characterization of the 5′ Untranslated Regions and Assignment of Transcription Start Sites

The presence of a long leader intron in the 5′ untranslated region is a typical feature of sucrose synthase genes (Werr et al., 1985; Chopra et al., 1992; Wang et al., 1992; Yu et al., 1992; Shaw et al., 1994). A 1612-bp leader intron with a GT/AG splicing boundary was easily identified in *Sus4*-16 by comparing its sequence with that of the 5′ untranslated region of the potato sucrose synthase cDNA *Potssyn* (Figure 4). However, comparison of the 5′ untranslated regions of *Potssyn* with *Sus3*-65 did not reveal regions of significant homology. To determine whether *Sus3*-65 also has a long leader intron and to map the transcription start sites for both genes, a collection of 5′ sucrose synthase cDNAs was isolated from potato tubers using a rapid amplification of cDNA ends protocol (Frohman et al., 1988).

Eighteen of the 5′ cDNAs that we isolated were either identical to or highly homologous with *Sus4*-16. These cDNAs could be divided into four groups: *Sus4*-5′a, *Sus4*-5′b, *Sus4*-5′c, and *Sus4*-5′d, which are shown, along with the published cDNA *Potssyn* and *Sus4*-16, in Figure 5B. Three to six clones were obtained for each of these groups. The sequences of *Sus4*-5′a and *Sus4*-5′b, which differ in length by 3 bp at the 5′ end, are identical to *Sus4*-16. The sequences of *Sus4*-5′c and *Sus4*-5′d, which also differ in length by 3 bp at the 5′ end, differ from *Sus4*-16 only by insertions of 13 and 3 bp in the untranslated region and by 1 or 2 mismatched bases. The deduced lengths of the 5′ untranslated regions for the four groups of 5′ cDNAs

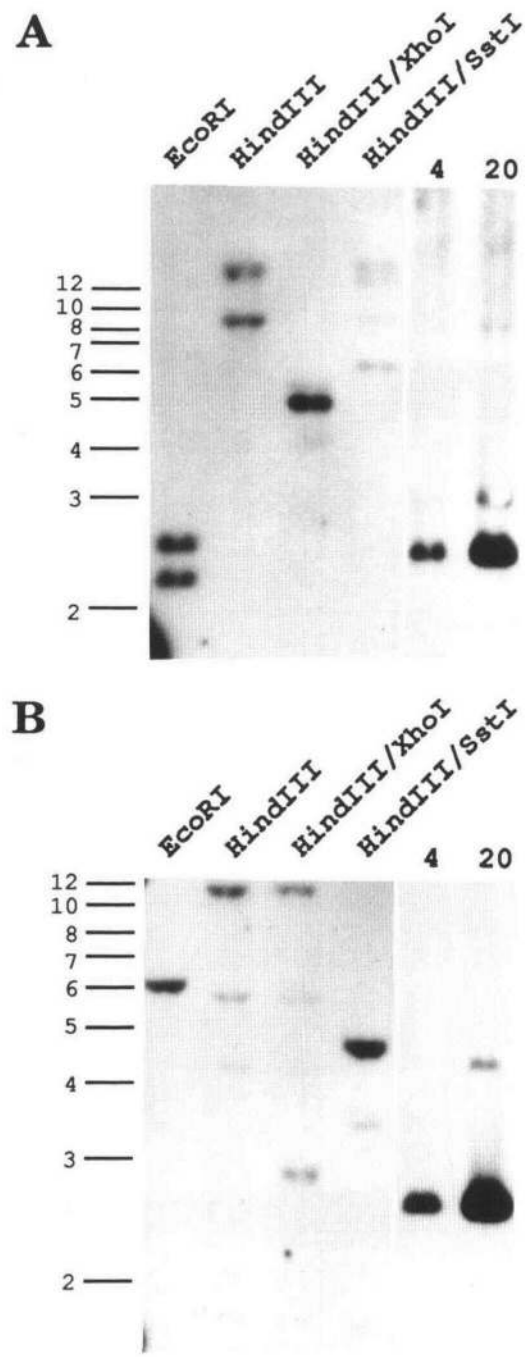

Figure 2. DNA Gel Blot Analysis of Potato Sucrose Synthase Genes.

Genomic DNA (10 μg per lane) was digested with restriction enzymes, shown above each lane, and probed under stringent conditions (final washing at 65°C in 0.1 × SSC). For reconstruction experiments, either four or 20 copies of cold probe were loaded individually. Probe positions are indicated in Figure 1. Molecular length standards are indicated at left in kilobases.

(A) Gel blot probed with the *Sus3* class probe.

(B) Gel blot probed with the *Sus4* class probe.

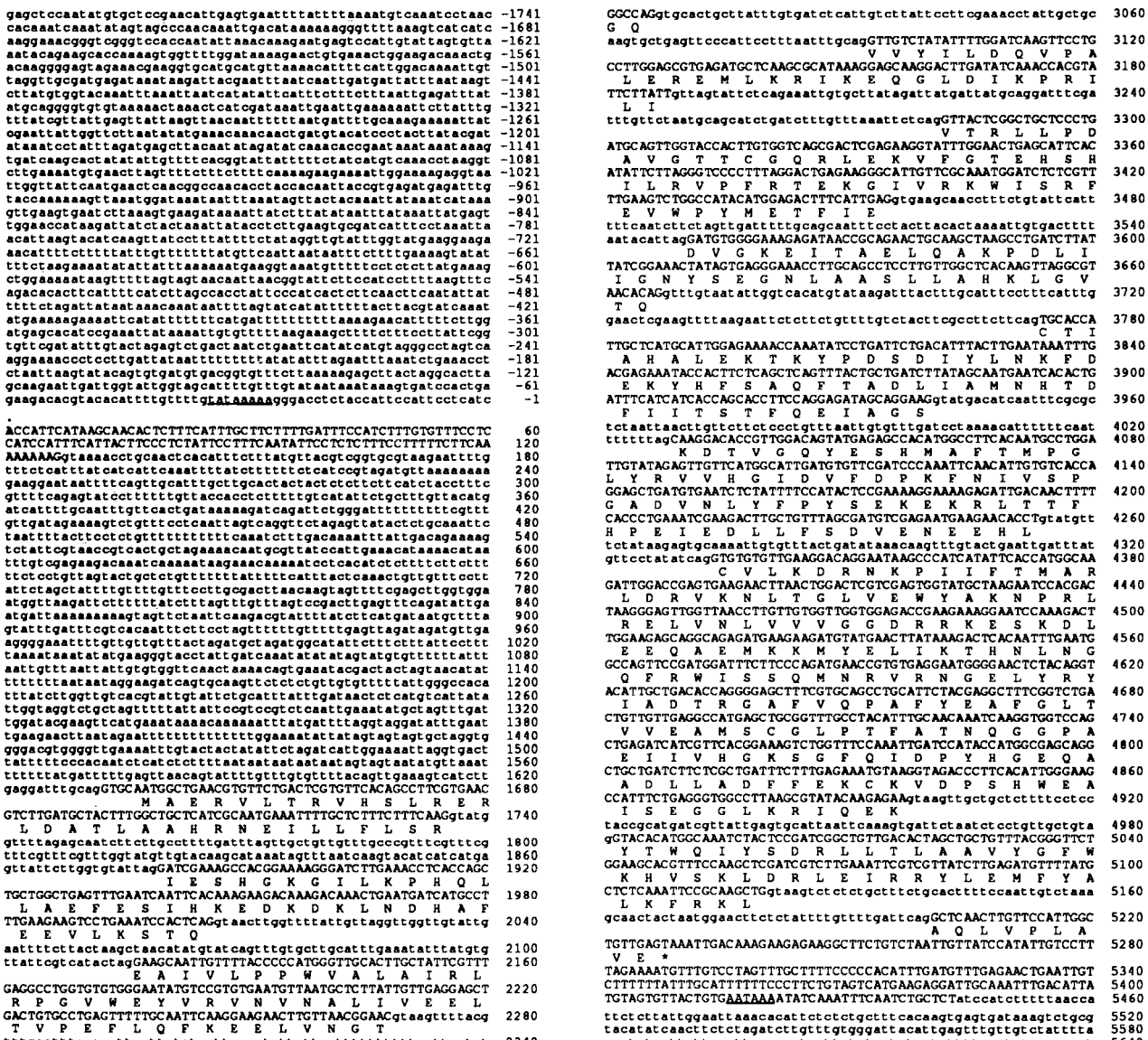

Figure 3. Nucleotide and Deduced Amino Acid Sequences of the *Sus3* Class Gene *Sus3*-65 (GenBank accession number U24088).

Exons are shown in uppercase letters; flanking regions and introns are shown in lowercase letters. The putative transcription start site is indicated by a boldface letter with a dot on top. Nucleotide sequences are numbered by referring to the transcription start site as +1. The TATA box and polyadenylation signal are underlined. The asterisk indicates the stop codon.

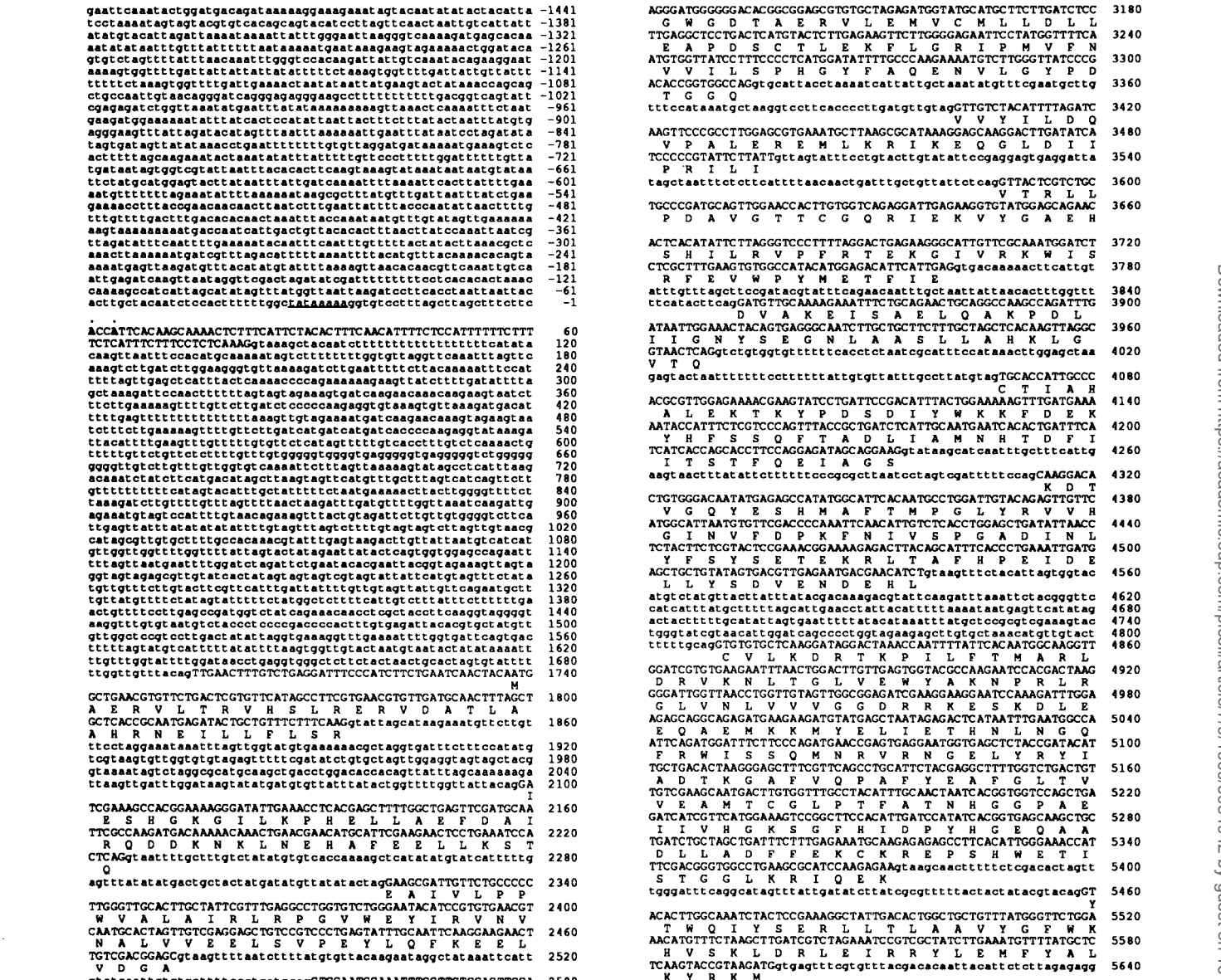

Figure 4. Nucleotide and Deduced Amino Acid Sequences of the *Sus4* Class Gene *Sus4*-16 (GenBank accession number U24087).

The flanking regions, exons, introns, TATA box, polyadenylation signal, and stop codon are designated as given for Figure 3. Two putative transcription start sites are given in boldface letters with a dot on top. Nucleotide sequences are numbered by referring to the first putative transcription start site as +1.

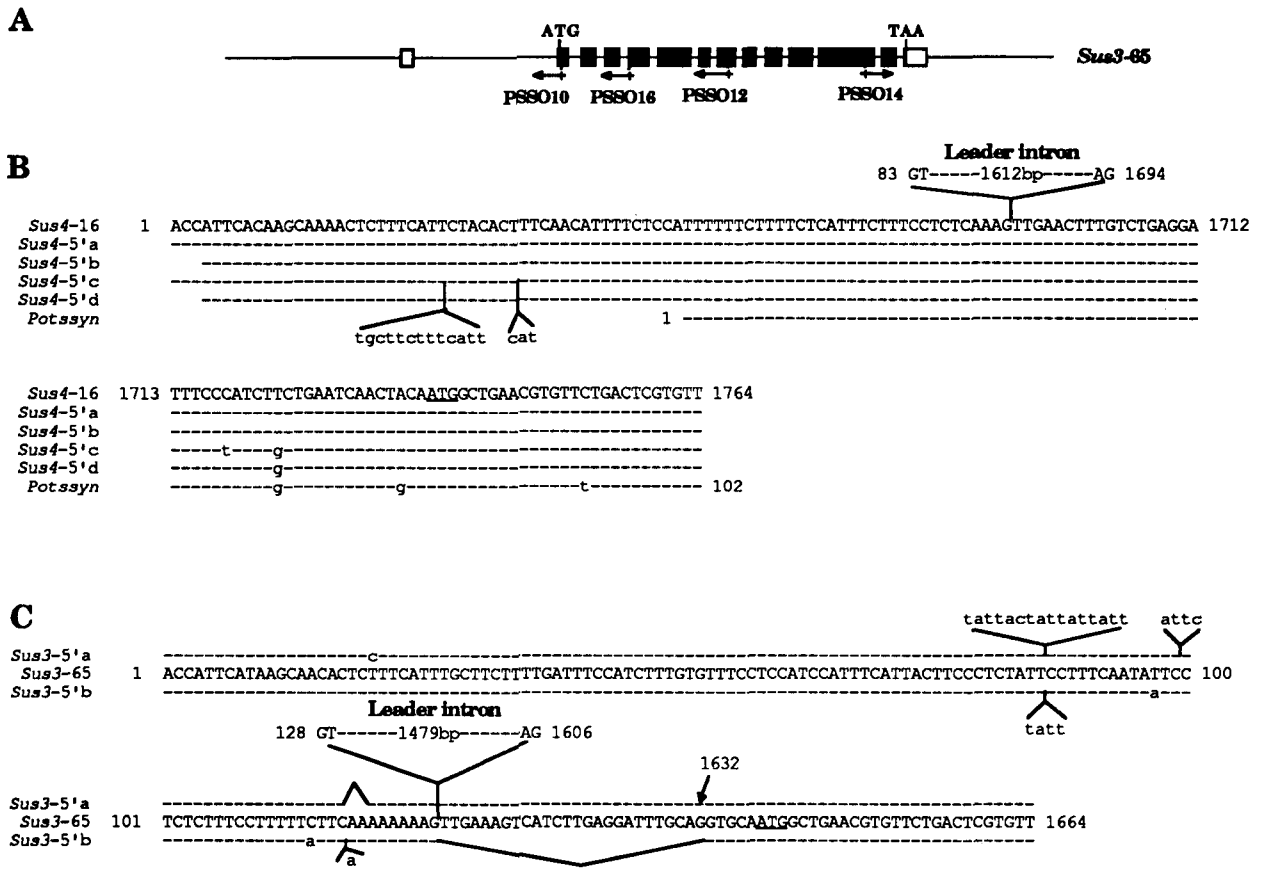

Figure 5. Comparison of 5′ Untranslated Sequences of 5′ cDNAs and Genomic Clones.

(A) The positions of the primers used in the rapid amplification of cDNA ends method, with a schematic diagram of the *Sus3* class gene *Sus3*-65, are shown. Arrows indicate the direction (5′ to 3′) of the primers. Open and solid boxes indicate the untranslated and coding regions, respectively.
(B) Alignment of the 5′ cDNAs *Sus4*-5′a, *Sus4*-5′b, *Sus4*-5′c, and *Sus4*-5′d and the published cDNA *Potssyn* with *Sus4*-16.
(C) Alignment of the 5′ cDNAs *Sus3*-5′a and *Sus3*-5′b with *Sus3*-65.
Identical bases are shown as dashes, and mismatched bases are shown in lowercase letters. Insertions and deletions are indicated above or below the cDNAs. Leader introns for both genes are also indicated. An alternative 3′ splicing site of the leader intron for *Sus3*-65 is indicated by an arrow with the position numbered. The start codons in both *Sus3*-65 and *Sus4*-16 are underlined.

are 125, 122, 141, and 138 bp, respectively, which agree with the four primer extension products seen with tuber mRNA (Figure 6). Based on the primer extension results and the perfectly matched 5′ cDNAs, *Sus4*-5′a and *Sus4*-5′b, two putative transcription initiation sites were assigned to *Sus4*-16 (Figure 4).

The remaining two 5′ cDNAs that we isolated, *Sus3*-5′a and *Sus3*-5′b, were highly homologous with *Sus3*-65 (Figure 5C). Alignment of these two cDNAs with *Sus3*-65 shows clearly that *Sus3* class genes also contain a long leader intron with a conserved GT/AG splicing boundary. It also suggests that alternative 3′ leader intron splicing sites, differing by 26 bp, may have been used by the *Sus3* genes encoding *Sus3*-5′a and *Sus3*-5′b. We assigned the 3′ splicing site of the leader intron to *Sus3*-65 based on *Sus3*-5′b, which has higher sequence homology with *Sus3*-65. This assignment gives a leader intron length of 1505 bp. Based on the two highly homologous 5′ cDNAs, *Sus3*-5′a and *Sus3*-5′b, the putative transcription start site was also assigned to *Sus3*-65 (Figure 3).

The assigned transcription initiation sites for *Sus3*-65 and *Sus4*-16 are both adenines flanked by pyrimidines, in agreement with the consensus for plant genes observed by Joshi (1987). Conserved TATA boxes were found 27 and 25 bp upstream from the transcription start site for *Sus3*-65 and *Sus4*-16, respectively (Figures 3 and 4).

Mapping of 3′ Ends and Assignment of Polyadenylation Signals

To map the 3′ ends of both genes, six 3′ cDNAs were also isolated from tubers by using the rapid amplification of cDNA ends protocol. These cDNAs could be divided into three groups:

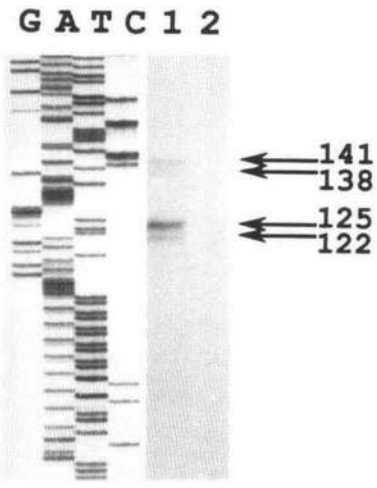

Figure 6. Mapping of Transcription Start Sites of the *Sus4* Genes by Primer Extension.

Primer extension was performed with antisense primer PSSO10 located in the second exon of *Sus4*-16. Lanes 1 and 2 are primer extension products of total tuber RNA and yeast tRNA, respectively. Lanes G, A, T, and C contain a known sequence and were used as length markers. Four major products from tuber RNA are indicated by arrows. The numbers indicate the lengths (in base pairs) of the untranslated regions.

Sus4-3′a, *Sus4*-3′b, and *Sus3*-3′a. One to three clones were obtained for each of these groups. *Sus4*-3′a showed sequence identity with *Sus4*-16 (Figure 7A) and was used to assign its 3′ end. *Sus4*-3′b was highly homologous with *Sus4*-16 but, like the previously described sucrose synthase cDNA *Potssyn*, was 52 bp shorter at the 3′ end than was *Sus4*-3′a (Figure 7A). *Sus3*-3′a showed sequence identity with *Sus3*-65 and was used to assign its 3′ end (Figure 7B).

A polyadenylation signal, AATAAA, was identified 22 bp upstream from the 3′ end of *Sus3*-65 (Figures 3 and 7B). For *Sus4*-16, the closest putative polyadenylation signal is AATAAG, located 80 bp upstream from the assigned 3′ end (Figures 4 and 7A).

Comparison of *Sus3*-65 and *Sus4*-16

Sus3-65 and *Sus4*-16 have an identical exon/intron organization (Figures 3 and 4). Both genes are composed of 14 exons, including a leader exon in the 5′ untranslated region, and 13 introns, including a long leader intron. Coding regions of corresponding exons are of the same size and are split at identical positions.

Sus3-65 and *Sus4*-16 share 87.4% nucleotide identity in the coding regions and 91.8% amino acid identity. Outside the

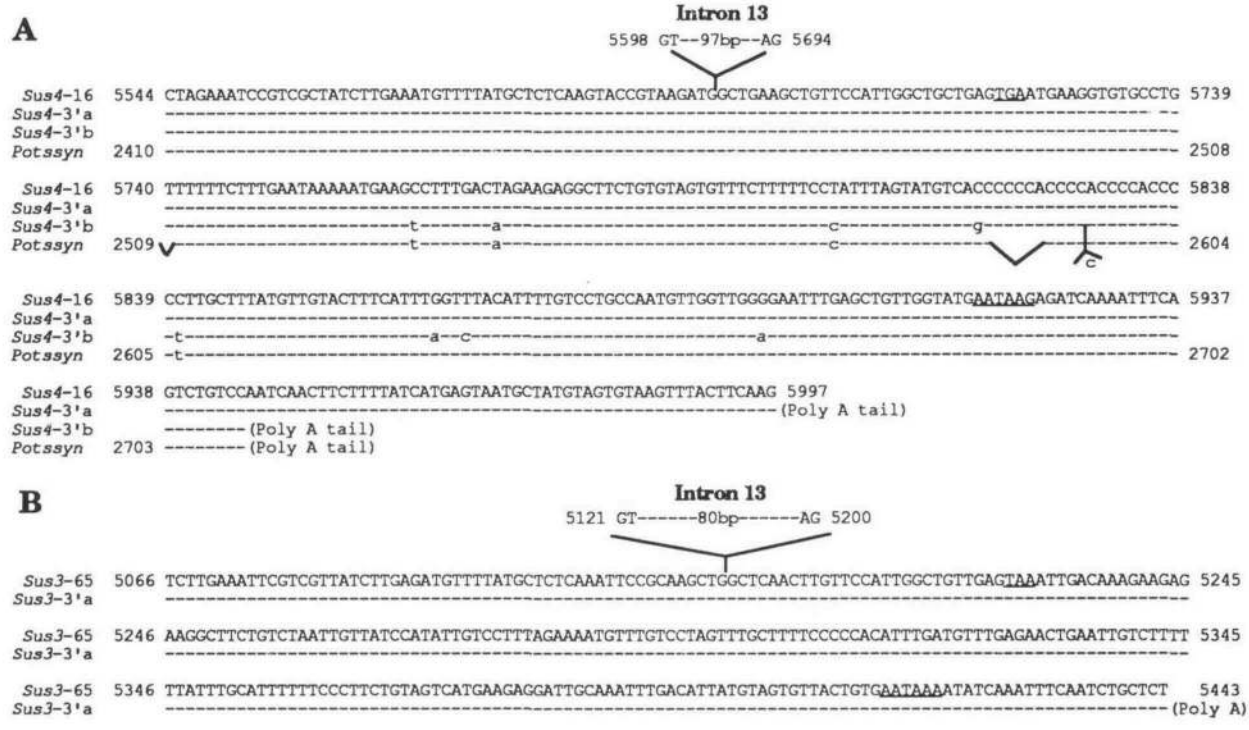

Figure 7. Mapping of the 3′ Ends of the Potato Sucrose Synthase Genes Using 3′ cDNAs.

(A) Alignment of the 3′ cDNAs, *Sus4*-3′a and *Sus4*-3′b, and the published sequence of the cDNA *Potssyn* with *Sus4*-16.
(B) Alignment of the 3′ cDNA *Sus3*-3′a with *Sus3*-65.
Identical and mismatched bases, insertions, and deletions are denoted as given for Figure 5. Intron 13 for both genomic clones is indicated, with the positions numbered. Stop codons and presumed polyadenylation signals are underlined. Polyadenylation tails for all cDNAs are shown.

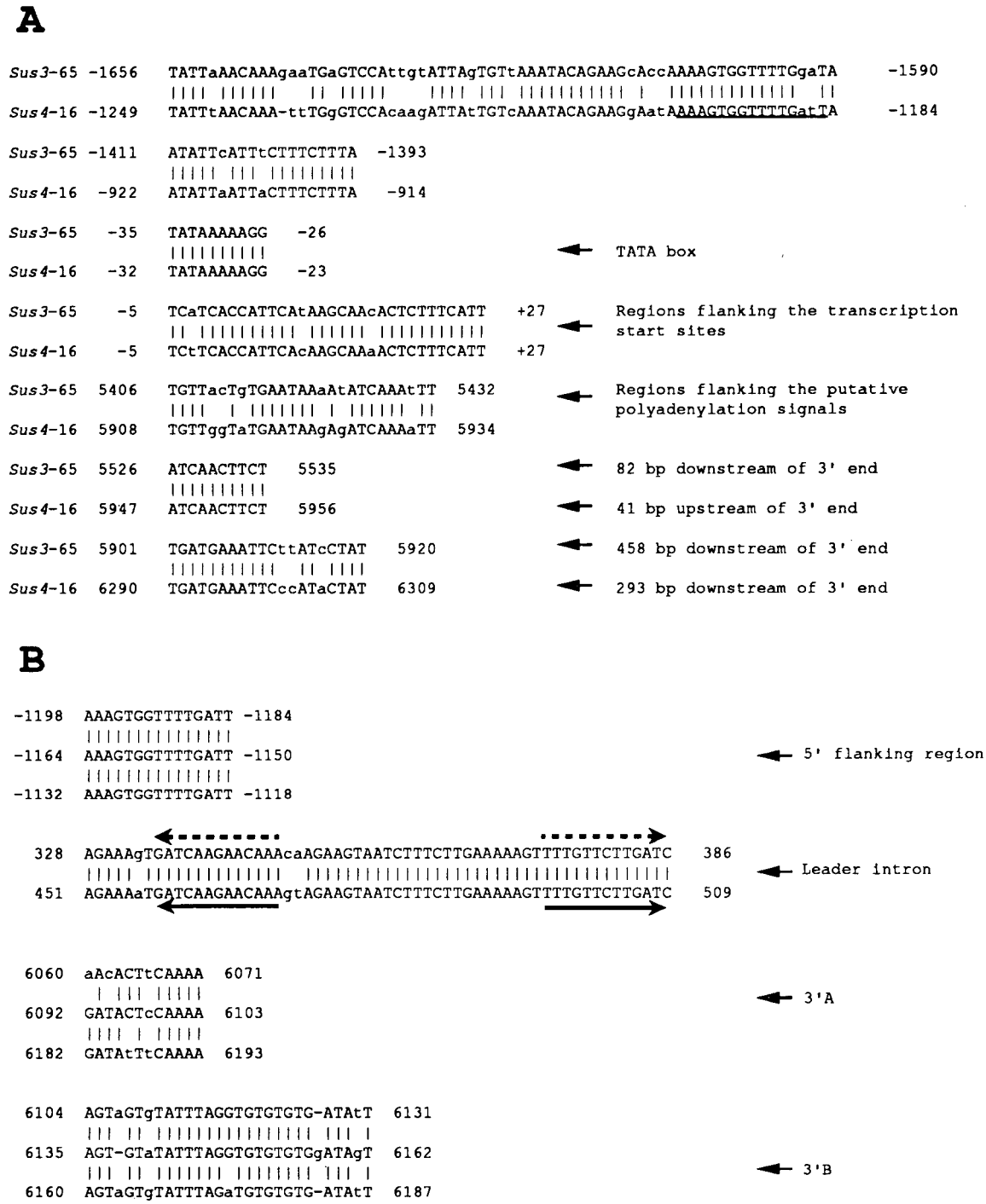

Figure 8. Conserved Regions and Repeated Sequences in Potato Sucrose Synthase Genes.

(A) Conserved regions between *Sus3*-65 and *Sus4*-16.

(B) Repeat sequences identified in *Sus4*-16.

Matched bases are shown in uppercase letters with vertical bars, and mismatched bases are shown in lowercase letters. The underlined sequence is present as a repeat in *Sus4*-16 (shown in **[B]**). The 13-bp inverted repeats present in the leader intron of *Sus4*-16 are indicated as dashed or solid arrows. Sequences are numbered as given for Figures 3 and 4.

coding region, however, the two genes diverge. Most of the limited homology between the 5′ and 3′ flanking region of *Sus3*-65 and *Sus4*-16 was found in several short regions (Figure 8A). The most extensive conserved region extends from nucleotides −1656 to −1590 in *Sus3*-65 and from −1249 to −1184 in *Sus4*-16. Other conserved regions are located around the transcription start sites and polyadenylation sites. Short conserved regions were alsc observed in corresponding introns, especially near the 5′ and 3′ splicing junctions and the putative branching sites (data not shown).

Noticeable repeat sequences are present only in *Sus4*-16 (Figure 8B). In one of the conserved regions, located from nucleotides −1249 to −1184 in *Sus4*-16, we found a short sequence (AAAGTGGTTTTGATT), which is repeated tandemly twice more in *Sus4*-16 but not in *Sus3*-65. The 5′ end of the leader intron in *Sus4*-16 also possesses a 59-bp sequence that is repeated twice (328 to 386 and 451 to 509) and is 95% identical in sequence. The repeat contains 13-bp inverted repeats at both ends. In addition, two sequences (3′A and 3′B), which are repeated three times, are found 110 bp downstream of the putative 3′ polyadenylation site. The functional roles, if any, of these repeat sequences remain to be determined.

Sus3 and *Sus4* Genes Are Differentially Expressed

To determine whether the two classes of potato sucrose synthase genes have different patterns of expression, the steady state levels of *Sus3* and *Sus4* class transcripts were assayed by RNA gel blot analysis using gene-specific probes. *Sus4* transcripts were detected at very high levels in developing tubers but at much lower levels in roots and stems (Figure 9A). *Sus4* transcripts were normally not detected in leaves. In contrast, *Sus3* transcripts were detected at low levels in tubers but at much higher levels in roots and stems. *Sus3* transcripts could be detected in leaves but only after long exposure (data not shown).

Sus4 sucrose synthase transcripts could be induced to accumulate in detached leaves by incubation with 60 mM of sucrose and reached higher levels after incubation with 200 mM of sucrose (Figure 9B). In contrast, *Sus3* transcripts were not sucrose inducible and were present at relatively constant low levels after incubation with various concentrations of sucrose.

Effect of Tuber Excision on *Sus4* Class Transcripts

Ross and Davies (1992) have shown that tuber excision causes a rapid decrease in both the amount and activity of sucrose synthase. As expected, we found that the *Sus4* class transcript level decreased dramatically within 6 hr after tuber excision and was almost undetectable after 12 hr (Figure 9C). The steady state level of patatin transcripts also decreased after tuber excision but at an apparently slower rate. Whether the apparently slower decay rate of the patatin transcripts was due at least

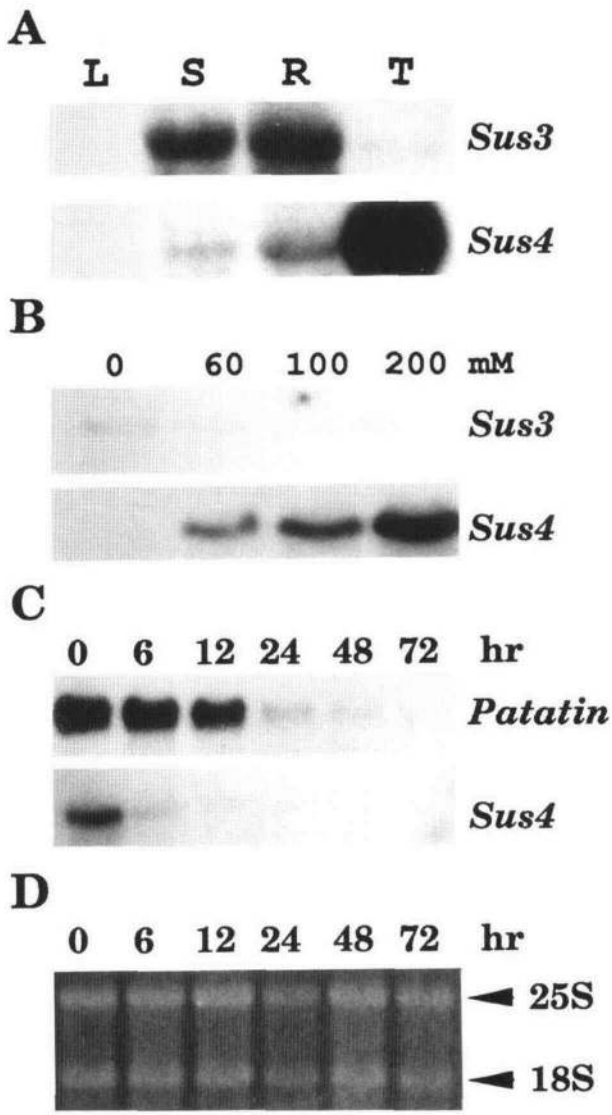

Figure 9. RNA Gel Blot Analysis of Potato Sucrose Synthase Transcripts.

(A) Duplicate samples of total RNA (50 μg) isolated from different organs were subjected to electrophoresis and probed with a 5′ untranslated region from either *Sus3*-65 or *Sus4*-16. L, leaf; S, stem; R, root; T, tuber.
(B) Duplicate samples of total RNA (50 μg) isolated from detached leaves incubated in MS basal medium supplemented with sucrose, as indicated, were electrophoresed and hybridized with the same probes as in **(A)**.
(C) Duplicate samples of total RNA (50 μg) isolated from excised developing tubers (~10 tubers) at different time points were separated by electrophoresis and probed with either patatin cDNA pGM01 (Mignery et al., 1984) or the *Sus4* class sucrose synthase probe.
(D) Ethidium bromide–stained gel showing that approximately equal amounts of total RNA were loaded in each lane.

in part to the higher amount of transcript present before tuber excision is unknown.

β-Glucuronidase Gene Expression in Transgenic Potato Plants

To examine promoter activity, we prepared translational fusions in which the β-glucuronidase (*GUS*) reporter gene was preceded by 3.6 to 3.9 kb of 5′ flanking sequence and followed by 0.6 to 0.7 kb of 3′ untranslated and flanking regions from either *Sus3*-65 or *Sus4*-16 (Figure 10A). Very high levels of *GUS* expression were observed in tubers of transgenic plants containing the *Sus4* construct SS-IV-3.6/3′ (Figure 10B). The average level of *GUS* expression in roots, stems, and leaves was approximately six-, 95-, and 2000-fold lower, respectively, than that in tubers. This pattern of expression was in very good agreement with that predicted from the steady state levels of *Sus4* class transcripts in these organs (Figure 9A). Although GUS activity was very low in normal leaves, it could be induced by exogenous sucrose (Figure 10C), which agrees with our results from mRNA analysis.

Transgenic plants containing the *Sus3* construct SS-III-3.9/3′ had a very different expression pattern (Figure 10B). Roots had the highest levels of expression, and GUS activity was ~11- to 14-fold lower in stems and tubers and 40-fold lower in leaves. Except for stems, this expression pattern agreed with that predicted from the steady state level of *Sus3* transcripts. RNA gel blot analysis showed that the level of *Sus3* class transcripts in stems was approximately the same as the level in roots and was much higher than that in tubers. However, *GUS* expression in stems was lower than in roots and was approximately the same as in tubers. Whether this discrepancy is due to the absence of regulatory sequences in this construct or to the presence of other *Sus3* genes with different expression patterns is not yet known. However, the presence of eight copies of the *Sus3* genes in the tetraploid potato genome agrees with the latter possibility.

The tissue specificity of *GUS* expression in plants containing either SS-III-3.9/3′ or SS-IV-3.6/3′ was examined by histochemical staining (Figure 11). Tubers from *Sus4* construct–containing plants showed strong, relatively consistent levels of staining in all tissues except for the periderm. Tubers from *Sus3* construct–containing plants generally showed a similar pattern; however, the intensity of staining was weaker in all but vascular tissue.

GUS activity was usually not observed in leaves from plants containing the *Sus4* construct SS-IV-3.6/3′, except for short stretches occasionally observed in veins (data not shown). This observation agrees with the low GUS activity seen with fluorometric assays (Figure 10B). In contrast, GUS staining was observed in leaves from plants containing the *Sus3* construct SS-III-3.9/3′. Expression was highest in the primary veins but also was clearly detectable in mesophyll and guard cells and in trichomes (data not shown).

GUS staining was usually not seen in internode cross-sections of stems from plants containing the *Sus4* construct SS-IV-3.6/3′, although weak staining was sometimes detected in the vascular tissues in plants with high overall levels of expression. The only location within stems in which high levels of expression were consistently observed with the *Sus4* construct was at nodes, where expression was associated with the basal vascular tissues of axillary buds and shoots (data not shown). In contrast, plants containing the *Sus3* construct SS-III-3.9/3′ consistently showed high levels of GUS staining in the phloem, particularly in the internal phloem of the stem.

The characteristic pattern of GUS staining in roots of plants containing the *Sus4* construct SS-IV-3.6/3′ involved the cap, apical meristem, and vascular tissues; however, a small portion of roots stained negatively. Consistent GUS staining in roots was observed in plants containing the *Sus3* construct SS-III-3.9/3′. The strongest GUS staining was observed in root tips. However, unlike plants containing the *Sus4* construct SS-IV-3.6/3′, staining occurred in the cell division zone but not in the root cap. GUS staining with the *Sus3* construct was also observed in the root cortex and with stronger intensity in vascular tissues.

DISCUSSION

We isolated and characterized two classes of sucrose synthase genes from potato and showed that they are differentially expressed both by RNA gel blot analysis and by expression of *GUS* constructs in transgenic potato plants. The potato *Sus3* and *Sus4* genes have gene structures similar to those of the two classes of sucrose synthase genes from cereals and generally appear to be functionally analogous but cannot be classified as *Sus1* and *Sus2* types based on sequence homology.

Potato *Sus3*-65 and *Sus4*-16 both contain a long leader intron and have coding regions composed of 13 exons split at identical positions. This overall gene structure is very similar to that of the sucrose synthase genes isolated from maize, rice, and Arabidopsis (Figure 12). However, a few differences exist between species. Coding regions corresponding to exons 6 and 12 of potato sucrose synthase genes *Sus3*-65 and *Sus4*-16 are intact, as in Arabidopsis *Asus1*, but are split by one intron in all other characterized sucrose synthase genes. Also, the Arabidopsis sucrose synthase gene *Asus1* is missing the leader intron (Martin et al., 1993), and both maize and rice *Sus1* (Yu et al., 1992; Shaw et al., 1994) differ from all other characterized sucrose synthase genes in missing the last intron.

The coding regions of *Sus3*-65 and *Sus4*-16 are highly homologous with those of sucrose synthase cDNAs and genes from other species (Figure 13). However, unlike the sucrose synthase genes from cereals, the *Sus3* and *Sus4* class sucrose synthase genes from potato cannot be classified as *Sus1*

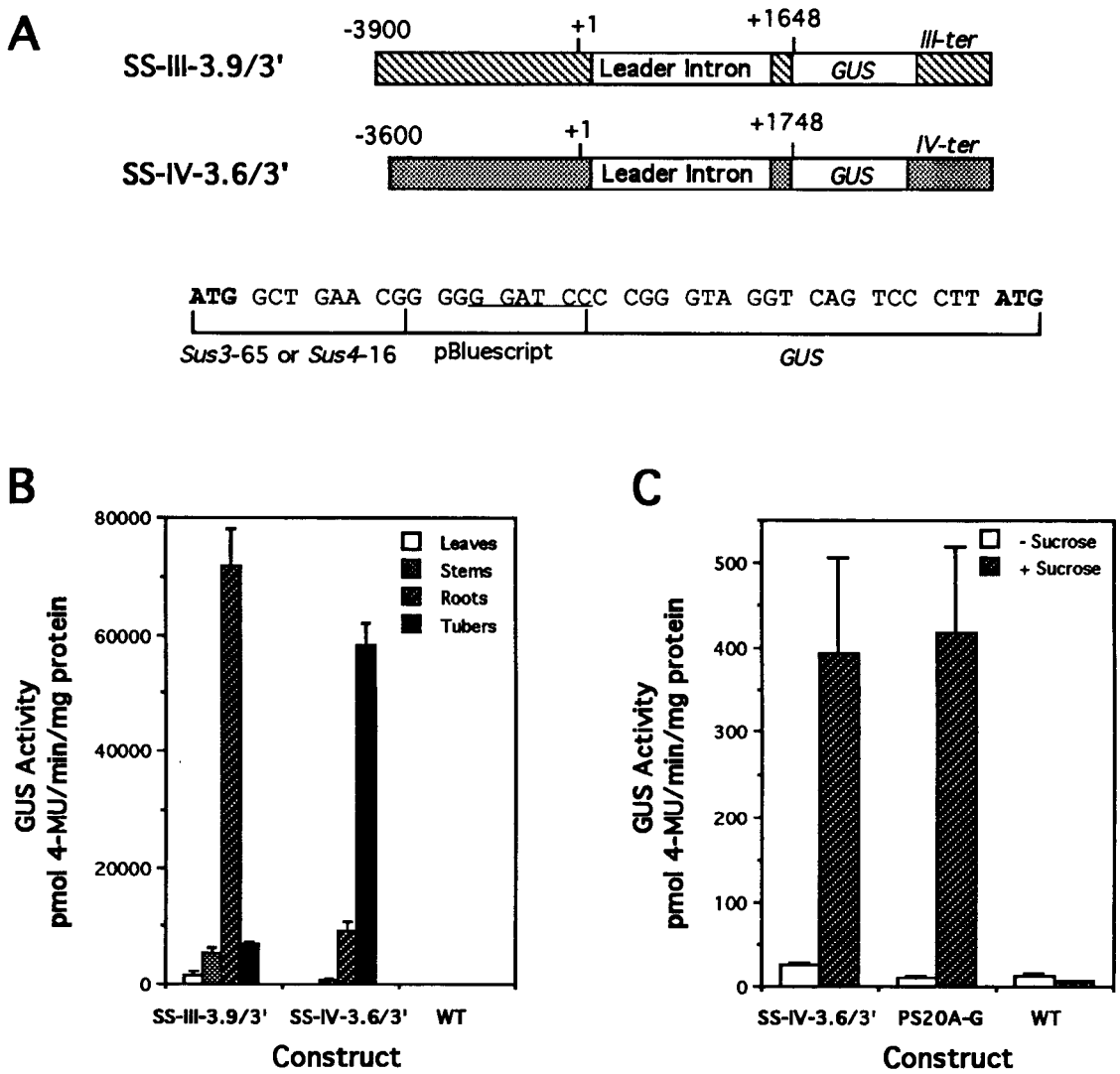

Figure 10. Expression of Potato Sucrose Synthase Genes in Transgenic Potato Plants.

(A) Schematic diagram of constructs SS-III-3.9/3′ and SS-IV-3.6/3′. *III-ter* (hatched rectangle) and *IV-ter* (stippled rectangle) are the 3′ sequences of *Sus3*-65 and *Sus4*-16, respectively. The junction sequences between the sucrose synthase genes and the *GUS* gene are indicated. The sequence derived from pBluescript SK+ is also shown. The start codons of potato sucrose synthase genes and *GUS* are shown in boldface letters. The underlined sequence is the BamHI site used in joining the potato sucrose synthase genes and *GUS* gene.

(B) GUS activity (in picomoles of 4-methylumbelliferone [MU] per minute per milligram of protein) in various organs of transgenic plants containing either SS-III-3.9/3′ or SS-IV-3.6/3′. The histograms represent the average GUS activity of either seven independent transformants containing SS-III-3.9/3′ or eight independent transformants containing SS-IV-3.6/3′. Standard errors are shown as bars. WT, wild-type control.

(C) The effect of sucrose on GUS activity in leaves of SS-IV-3.6/3′–containing plants. The histograms show the average GUS activity (in picomoles of 4-methylumbelliferone [MU] per minute per milligram of protein) after incubation in either 0 (−) or 250 (+) mM sucrose. Bars are standard errors. As controls, GUS activity in leaves from wild-type (WT) plants and plants containing a class I patatin–*GUS* construct (PS20A-G; Wenzler et al., 1989a) were measured.

or *Sus2* types based on DNA sequence homology. *Sus3* and *Sus4* class genes from potato are more homologous with each other (87.4% nucleotide identity) than with any of the sucrose synthase genes isolated from cereals. Furthermore, each shows a very similar homology with both *Sus1*- and *Sus2*-type genes. For example, *Sus3*-65 shows 71 to 72% homology with both *Sus1* and *Sus2* genes from maize, rice, or barley. *Sus4*-16 shows 70 to 71% homology with both *Sus1* and *Sus2* genes.

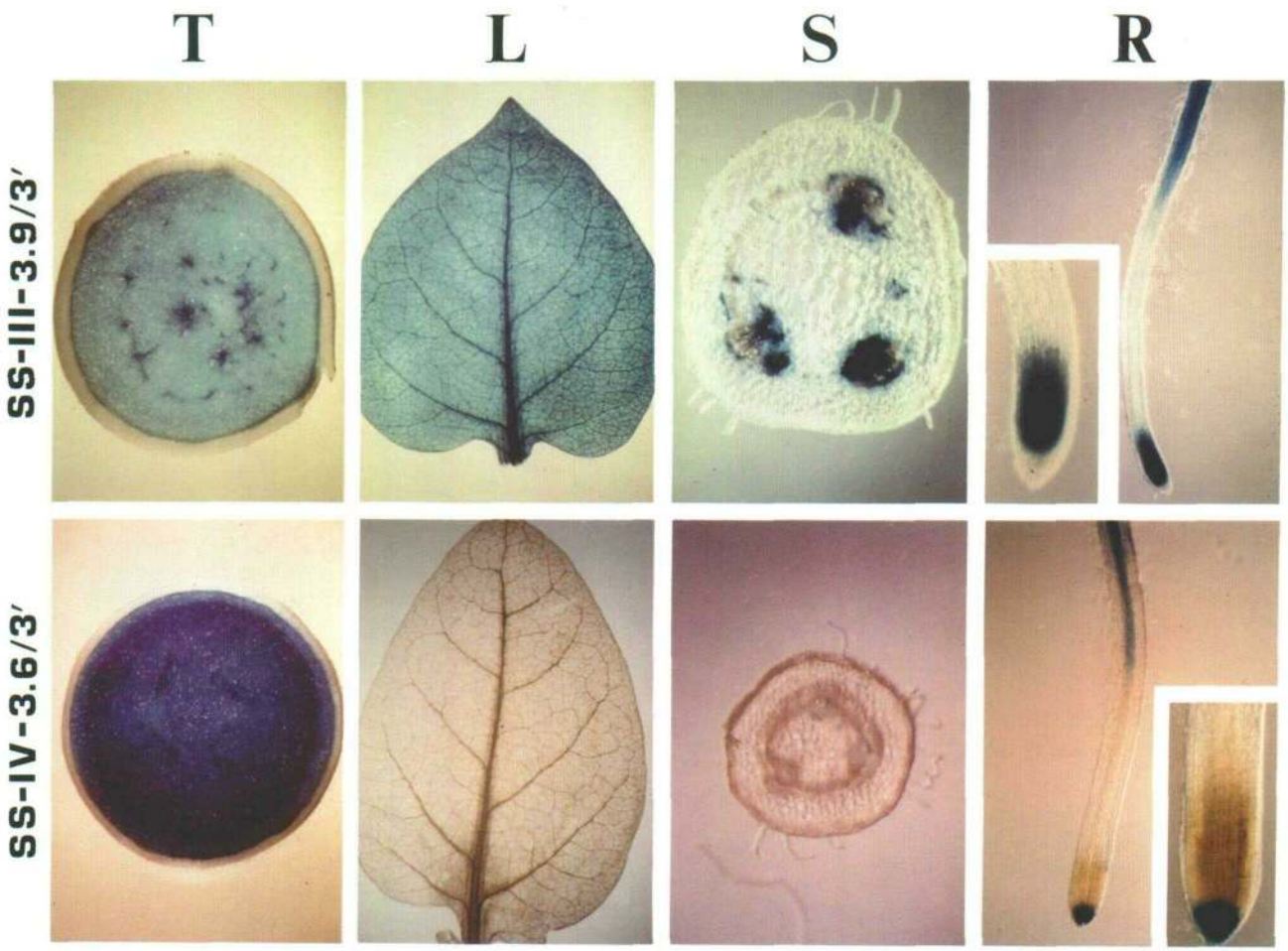

Figure 11. Histochemical Localization of GUS Activity in Various Organs of Transgenic Potato Plants.

(Top) *Sus3* construct SS-III-3.9/3′–containing plants.
(Bottom) *Sus4* construct SS-IV-3.6/3′–containing plants.
The insets are enlargements of root tips. T, tubers; L, leaves; S, stems; R, roots.

The fact that corresponding *Sus1* and *Sus2* sucrose synthase genes show higher homology between species rather than between each other within species suggests that they diverged before the speciation of cereals. A similar analysis of the homology relationships between dicot sucrose synthase cDNAs and genes suggests that the *Sus3* and *Sus4* class genes evolved after the speciation of most of the major dicot families. The *Sus3* and *Sus4* class genes are approximately equally homologous with *Asus1* (71 to 72% homology) and *SSA* (66 to 67% homology) from Arabidopsis and to sucrose synthase cDNAs isolated from broad bean (74 to 76%), mung bean (74 to 76%), and carrot (78 to 80%). However, the *Sus3* and *Sus4* class genes appear to have evolved before the divergence of tomato and potato, because *Sus4*-16 is much more homologous with the sucrose synthase cDNA isolated from tomato fruit (97%) than with *Sus3*-65 (87%).

In contrast with their protein coding regions, the 5′ and 3′ flanking regions and introns of the *Sus3* and *Sus4* genes show only very limited homology. Much of this is clustered in regions expected to be important functionally, such as around the TATA box, poly(A) signal, and branching and splicing sites for introns. Other small, conserved sequences found in the 5′ and 3′ flanking region may also be important functionally for the overlapping spatial patterns seen with *Sus3* and *Sus4* constructs in stem and root vascular tissues and in tuber parenchyma. However, as might be expected from the low degree of overall homology outside of protein coding regions, the amount of expression differs dramatically between the *Sus3* and *Sus4* constructs, even in tissues in which their spatial pattern is similar. In other tissues, such as root tips, their spatial pattern differs as well.

Sus4 genes are expressed at highest levels in tubers but are not normally expressed in photosynthetic leaves. Like the maize *Sus2* gene *Sh1*, a key role for *Sus4* genes appears to be to provide substrates for starch synthesis. As in maize endosperm of *Sh1* mutations, reduced starch content has been

observed in potato tubers harboring an antisense sucrose synthase construct (Zrenner et al., 1995). Also like maize *Sh1*, expression of the *Sus4* genes is regulated by anaerobic conditions (Salanoubat and Belliard, 1989).

The high levels of *Sus4* gene expression in tubers may be due partially to induction by the relatively high concentration of sucrose in this organ. However, sucrose is not the only factor responsible for expression of *Sus4* genes in tubers because we have found that deletion constructs that are not sucrose inducible in leaves are still expressed in tubers, although at reduced levels (Fu et al., 1995).

The *Sus4* gene expression observed in vascular tissue, apical meristems of roots, and basal vascular tissues of axillary buds and shoots suggests that *Sus4* genes may also play important roles in sucrose unloading and metabolism in these tissues. In root caps, the high levels of *Sus4* gene expression also suggest a role in starch synthesis similar to that observed in tubers.

Sus3 class potato sucrose synthase genes are expressed at the highest levels in roots and stems and, unlike the *Sus4* genes, are also normally expressed in photosynthetic leaves. This pattern of expression and the vascular localization of GUS staining in transgenic potato plants containing *Sus3* constructs are consistent with the proposed role of sucrose synthase in phloem loading, unloading, and transport (Martin et al., 1993; Nolte and Koch, 1993). The overall pattern of higher expression in roots, stems, and leaves seen in potato with the *Sus3* genes is similar to that seen with maize *Sus1* (Chourey et al., 1986; McCarty et al., 1986; Nguyen-Quoc et al., 1990). Also, the detection of only Sus1 protein in young maize leaves (Nguyen-Quoc et al., 1990) suggests that the observed companion cell–localized sucrose synthase (Nolte and Koch, 1993) is produced by *Sus1*, indicating a similar functional role in phloem for maize *Sus1*.

Like the *Sus1* gene of maize, *Sus3* gene expression predominates in potato stems. However, lower levels of vascular-localized expression in stems were also seen in potato plants containing the *Sus4* construct (data not shown). Although the *Sh1* gene in maize is known to be expressed in the vascular tissue of roots, it is unknown whether it is also expressed in the vascular tissue of stems. Interestingly, a very similar pattern of phloem-associated expression has been observed in the stems of transgenic tobacco plants containing a *GUS* construct under the control of the maize *Sh1* promoter (Yang and Russell, 1990). However, because the *Sh1–GUS* construct examined contained the leader intron, the possibility that the

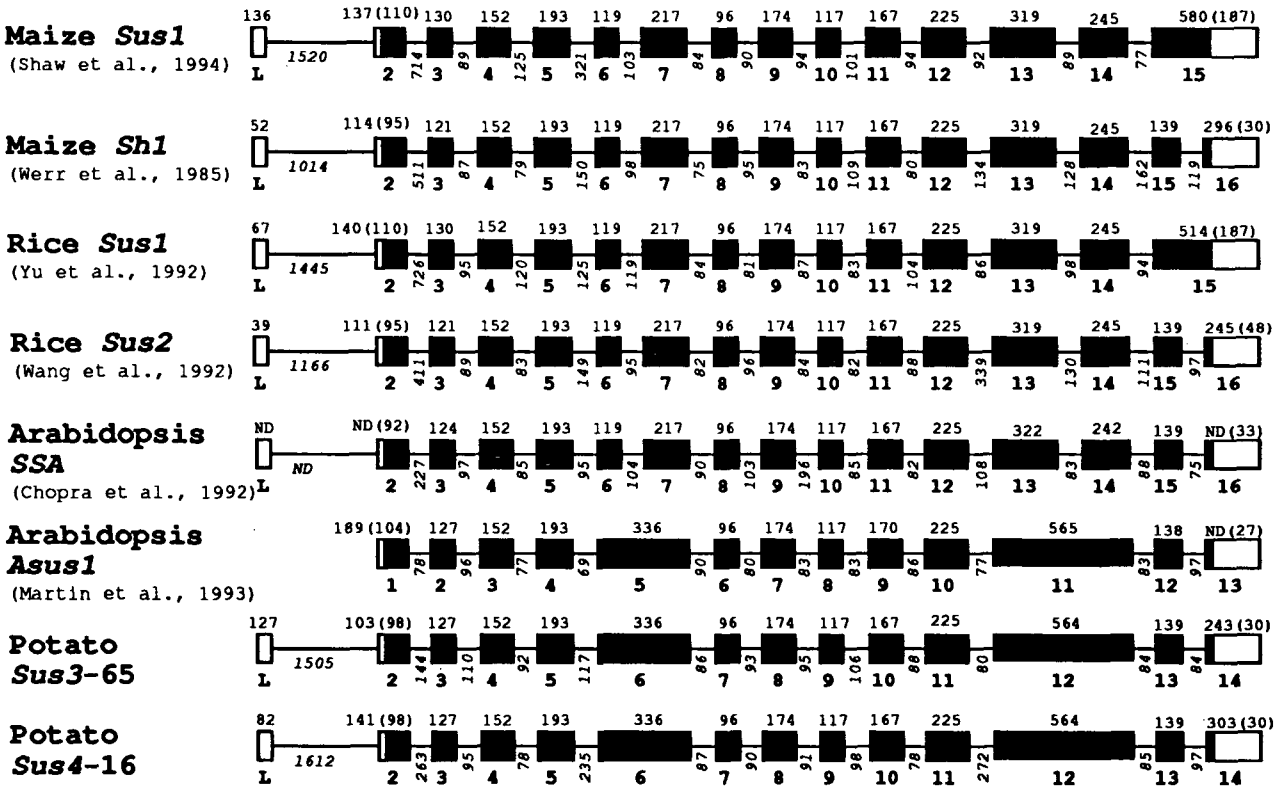

Figure 12. Exon/Intron Structure of Sucrose Synthase Genes.

Exons (boxes) and introns (lines) are drawn schematically, with the lengths in base pairs indicated above or below, respectively. Open boxes are untranslated regions, and solid boxes are coding regions. The numbers in parentheses indicate the lengths of coding sequences. Boldface numbers indicate exons. L, leader exon; ND, length not determined.

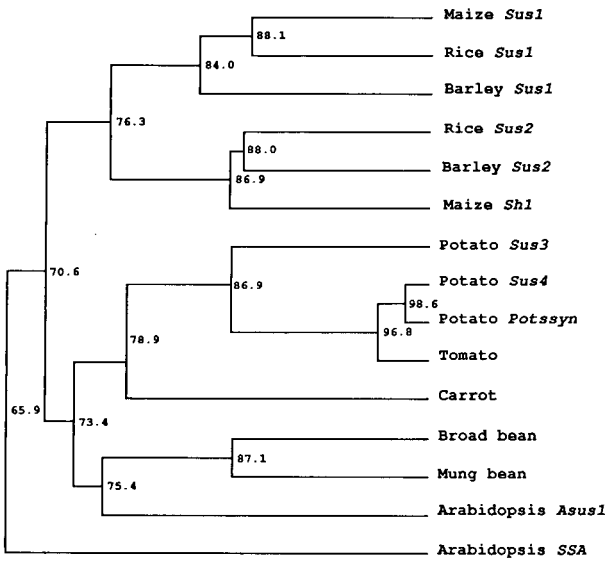

Figure 13. Nucleotide Sequence Similarity in the Coding Regions of Sucrose Synthase Genes.

The dendrogram was generated by the Pileup program from the Genetics Computer Group software package (version 8.0). Numbers are approximate percentages of identities between branched sequences. Sources of maize *Sus1* and *Sh1*, rice *Sus1* and *Sus2*, and Arabidopsis *SSA* and *Asus1* are listed in Figure 12. Other sequence data come from the following sources: barley *Sus1* (Martinez de Ilarduya et al., 1993); barley *Sus2* (Sánchez de la Hoz et al., 1992); broad bean (Heim et al., 1993); carrot (Šebková et al., 1995); mung bean (Arai et al., 1992); potato *Potssyn* (Salanoubat amd Belliard, 1987); and tomato (GenBank accession number L19762).

observed tissue specificity of *Sh1* promoter was due to tissue-specific splicing of the maize leader intron in tobacco cannot be ruled out.

The high levels of expression seen in the cell division zone of roots suggest that *Sus3* genes may also provide substrates for respiration and growth during cell division. A similar pattern of expression in the cell division zone of maize roots has also been observed with *Sus1* (Rowland et al., 1989).

The *Sus3* and *Sus4* class genes of potato are in some ways functionally analogous with maize *Sus1* and *Sh1*, respectively, but there are also a number of differences. For example, *Sus4* gene expression in potato could be induced by high concentrations of sucrose, whereas *Sus1* in maize is preferentially expressed at high sugar concentrations (Koch et al., 1992). Maize *Sus1* also appears to be responsible for expression in root caps, whereas in transgenic potato plants, expression in root caps was only seen with the *Sus4* construct. In addition, *Sus3* genes in potato were expressed in both the vascular tissue and mesophyll cells of leaves, and their expression increased as leaves matured (data not shown). In contrast, *Sus1* expression occurs at the highest level in young sink leaves of maize and is most likely restricted to companion cells (Nguyen-Quoc et al., 1990; Nolte and Koch, 1993).

The expression of *Sus3* sucrose synthase in the veins of potato leaves and its increasing expression as the leaves matured are consistent with its role in phloem loading. However, expression of sucrose synthase in source leaves could lead to a futile cycle of sucrose synthesis/degradation involving sucrose synthase and sucrose phosphate synthase. Such a futile cycle of sucrose synthesis and degradation has been suggested previously for nonphotosynthetic tissues (Geigenberger and Stitt, 1991) and may play a role in regulating sucrose synthesis or export in photosynthetic leaves.

METHODS

Plant Materials

Potato plants (*Solanum tuberosum* cv FL1607) were grown in soil in a growth chamber under a 16-hr photoperiod, at 20/16°C day/night temperatures. Transgenic plants were maintained and propagated axenically in Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962), supplemented with 2% sucrose and 0.8% agar at 25°C under a 16-hr photoperiod. Microtubers were induced by placing nodal cuttings in 0.5 × MS basal medium supplemented with 8% sucrose, 5 mg of kinetin, and 5 mg of ancymidol (Elanco, Indianapolis, IN) per liter of medium.

Construction and Screening of Potato Genomic Library

Genomic DNA was isolated from young potato leaves, according to the method described by Rogers and Bendich (1988). DNA was partially digested with Sau3AI and was size fractionated on a sucrose gradient (Ausubel et al., 1989). Ligation of fractionated DNA (~15 kb in length), into the BamHI site of λ DashII, and packaging were performed according to the manufacturer's instruction (Stratagene). The *Escherichia coli* host for λ DashII was KW251. Screening was performed as described by Sambrook et al. (1989) at reduced stringency (final washing at 65°C in 2 × SSC [1 × SSC is 0.1 M NaCl, 0.015 M sodium citrate]) by using a 1.2-kb potato sucrose synthase genomic fragment cloned by polymerase chain reaction (PCR). The 5′ and 3′ oligonucleotides used for PCR amplification were derived from a published potato tuber sucrose synthase cDNA (Salanoubat and Belliard, 1987) and came from positions 1495 to 1511 and 2444 to 2462, respectively.

DNA Sequence Analysis

DNA sequencing was performed by the dideoxy chain termination method (Sanger et al., 1977) using a Sequenase Sequencing kit (United States Biochemical Corp.). Sequence analyses were performed using computer programs from either Intelligenetics (Release 5.4 for VAX/VMS; Mountain View, CA) or the Genetics Computer Group package (version 8.0; Madison, WI). The nucleotide and amino acid identities between sequences were calculated using the Pileup and Distances programs (option: uncorrected distance) from Genetics Computer Group package (version 8.0).

Primer Extension

Primer extension was performed as described by Sambrook et al. (1989) using 50 μg of total RNA isolated from tubers. Oligonucleotide PSSO10 was used as the primer. The position of this oligonucleotide is shown in Figure 5A.

Isolation of 5′ and 3′ cDNA Ends

The 5′ and 3′ cDNA ends were isolated using a strategy developed by Frohman et al. (1988). Oligonucleotides used for amplification were derived from the *Sus3* class *S. tuberosum* sucrose synthase gene *Sus3*-65. Positions and directions of relevant oligonucleotides are shown in Figure 5A. The names and sequences of relevant primers are listed from 5′ to 3′ as follows (lowercase letters in the first four oligonucleotides are bases that are mismatched with *Sus4*-16): PSSO12, CGAGAGATCCATTTGCGAAC; PSSO10, TCACGAAGgCTgTGAACACGCTCA; PSSO16, CaAAgTTatCgTTgGAa; PSSO14, CTGCTGATCTtCTcGCTGAT; and oligo(dT)-adapter, CCGGATCCGAATTCCCGGGTTTTTTTTTTTTTTTTT.

For amplification of 5′ cDNA ends, first-strand cDNAs were first synthesized by reverse transcription as described by Sambrook et al. (1989) using 0.5 μg poly(A) RNA and 20 pmol of PSSO12 as primer and 20 units of Moloney murine leukemia virus reverse transcriptase (Stratagene). Unincorporated deoxynucleotide triphosphates and primer were removed using a Centricon-100 column (Amicon, Beverly, MA). The first-strand cDNAs were tailed by oligo(dA) and were then used as templates for amplification. PSSO10 and the oligo(dT)-adapter were used to amplify *Sus4* class 5′ cDNAs. For isolation of *Sus3* class 5′ cDNAs, PSSO16 (specific to *Sus3*-65) and the oligo(dT)-adapter were used for amplification. To enrich the amplified *Sus3* class 5′ cDNAs, a second round of PCR was performed using PSSO10 and the oligo(dT)-adapter. For amplification of 3′ cDNA ends, reverse transcription was performed as described earlier, except that oligo(dT) (18-mer) was used as the primer. Oligonucleotides PSSO14 and the oligo(dT)-adapter were used for amplification.

Thermal cycling was performed as described by Frohman et al. (1988). Amplified products were band isolated and cloned into the SmaI site of pBluescript KS+ (Stratagene), after flushing the ends with the Klenow fragment of DNA polymerase I.

RNA Isolation and Gel Blot Analysis

Total RNA was isolated according to the procedure of de Vries et al. (1988). Polyadenylated RNA was selected using an oligo(dT) cellulose column (Sambrook et al., 1989). RNA electrophoresis and gel blotting were performed as described by Ausubel et al. (1989). For detecting either *Sus3* or *Sus4* class sucrose synthase transcripts, the entire 5′ untranslated sequences (minus the leader introns) from *Sus3*-65 and *Sus4*-16 were used as class-specific probes. The specificity of both probes was confirmed by lack of cross-hybridization under the conditions used (data not shown). Preparation of RNA probes and hybridization were performed using instructions from Promega.

Sucrose Induction

For RNA isolation, leaves were isolated from pot-grown plants and were surface sterilized for 10 min in a 10% solution of commercial bleach. The leaves were placed abaxial side up in 15-cm Petri dishes containing 50 mL of MS basal medium supplemented with sucrose and were incubated in the dark at 20°C for 3 days. The leaves were briefly blotted dry and were immediately frozen in liquid nitrogen. Plant material was stored at −80°C until needed. For the β-glucuronidase (GUS) assay, leaves of the same size and position were cut from tissue culture plantlets. Leaves were placed abaxial side up in 24-well tissue culture plates containing 3 mL of MS basal medium supplemented with 250 mM of sucrose in each well and were incubated in the dark at 20°C for 5 days. The leaves were then blotted dry briefly and immediately used in GUS assays.

Construction of Chimeric *GUS* Genes

The construct SS-III-3.9/3′ (Figure 10A) was prepared in two steps in a binary vector pBI101.2 (Jefferson et al., 1987). First, the nopaline synthase 3′ sequence was replaced by the 3′ sequence of *Sus3*-65 (from 19 bp upstream of the stop codon to 333 bp downstream of the polyadenylation site; positions 5209 to 5776) to give pBI101.2-III-ter. Next, a 5.5-kb genomic fragment of *Sus3*-65 (from −3900 to 8 bp downstream of the start codon at position +1648) was cloned into pBluescript KS+ and fused in frame to the *GUS* coding region of the pBI101.2-III-ter, using SalI and BamHI sites.

The construct SS-IV-3.6/3′ (Figure 10A) was also prepared in two steps in the binary vector pBI101.2. First, the nopaline synthase 3′ sequence was replaced by the 3′ sequence of *Sus4*-16, resulting in pBI101.2-IV-ter. The 3′ sequence was prepared by PCR and spans from 2 bp upstream of the stop codon to 400 bp downstream of the polyadenylation site (+5720 to +6397). Next, a 5.3-kb genomic fragment of *Sus4*-16 (from −3600 to 8 bp downstream of the start codon at position +1748) was cloned into pBluescript SK+ and fused in frame to the *GUS* coding region of the pBI101.2-IV-ter, using HindIII and BamHI sites.

Potato Transformation and Regeneration

The modified binary vectors were transferred into *Agrobacterium tumefaciens* LBA4404 by electroporation, using the Gene Pulser (Bio-Rad; Mattanovich et al., 1989). Potato transformation and regeneration were performed according to Wenzler et al. (1989b).

Fluorometric and Histochemical Assays of GUS Activity

Fluorometric assays were performed as described by Jefferson (1987) using 4-methylumbelliferyl β-D-glucuronide as a substrate. The top two fully extended leaves, the whole stem from one or two plantlets, the whole root system, and one or two microtubers (~4 weeks after induction) were assayed. Leaves, stems, and microtubers were ground in 1.5-mL microcentrifuge tubes by using a Kontes pestle (Kontes Scientific Glassware/Instruments, Vineland, NJ). Roots were ground using a mortar and pestle. Protein concentrations in extracts were determined using the method of Bradford (1976); ~20 μg of protein was used per assay.

Histochemical assays of GUS activity were performed as described by Jefferson (1987), using the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc; Biosynth, Staad, Switzerland). The top one or two fully expanded leaves, cross-sections or longitudinal sections of stem, 1 cm of root tip portions, and microtuber cross-sections were placed in 2 mM of X-gluc, vacuum infiltrated for 1 min, and

incubated overnight at 37°C in the dark. After staining, leaves and stem segments were cleared through an ethanol series at room temperature. Photographs were taken with an Olympus (Tokyo, Japan) dissection microscope.

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