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RESEARCH PAPER

Structure and expression profile of the sucrose synthase multigene family in *Arabidopsis*

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Abstract

The release of the complete genome sequence of *Arabidopsis* enabled the largest sucrose synthase family described to date, comprising six distinct members, for which expression profiles were not yet available, to be identified. Aimed at understanding the precise function of each AtSUS member among the family, a comparative study of protein structure was performed, together with an expression profiling of the whole gene family using the technique of real-time quantitative reverse transcriptase-polymerase chain reaction. Transcript levels were analysed in several plant organs, including both developing and germinating seeds. A series of treatments such as oxygen deprivation, dehydration, cold treatment, or various sugar feedings were then carried out to characterize the members of the family further. The *AtSUS* genes exhibit distinct but partially redundant expression profiles. Under anaerobic conditions, for instance, both *AtSUS1* and *AtSUS4* mRNA levels increase, but in a distinct manner. *AtSUS2* is specifically and highly induced in seeds at 12 d after flowering and appears as a marker of seed maturation. *AtSUS3* seems to be induced in various organs under dehydration conditions including leaves deprived of water or submitted to osmotic stress as well as late-maturing seeds. *AtSUS5* and *AtSUS6* are expressed in nearly all plant organs and do not exhibit any transcriptional response to stresses. These results add new insights on the expression of *SUS* genes and are discussed in relation to distinct functions for each member of the *AtSUS* family.

Key words: *Arabidopsis*, functional genomics, seed development, stress response, sucrose synthase, transcriptional profiling.

Introduction

Sucrose synthase (SUS; UDP-glucose: D-fructose 2-glucosyltransferase, EC 2.4.1.13) is a key enzyme involved in sucrose metabolism. This enzyme catalyses the reversible conversion of sucrose and UDP to UDP-glucose and fructose. Its activity has been studied in various plants and has been shown to play a major role in energy metabolism, controlling the mobilization of sucrose into various pathways important for the metabolic, structural, and storage functions of the plant cell (Hesse and Willmitzer, 1996; Sturm and Tang, 1999, for a review). First of all, SUS activity associated with vascular tissues appears to play a key role in supplying energy in the companion cells for phloem loading by providing substrate for respiration (Fu and Park, 1995; Hänggi and Fleming, 2001). SUS is also proposed to supply UDP-glucose for cell wall biosynthesis in association with the cellulose synthase complex (Delmer and Amor, 1995; Haigler *et al*., 2001; Ruan *et al*., 2003). SUS cleavage activity correlates with the sink strength of storage organs, providing substrates for starch synthesis in potato tubers, maize kernels, or pea embryos (Sun *et al*., 1992; Zrenner *et al*., 1995; Déjardin *et al*., 1997). *SUS* expression is also associated with important processes such as nodule development and metabolism in legumes (Craig *et al*., 1999; Hohnjec *et al*., 1999) or apical meristem functioning in tomato (Pien *et al*., 2001). Several pieces of evidence indicate that SUS exists both free in the cytosol and in association with the plasmalemma (Amor *et al*., 1995; Carlson and Chourey, 1996). Moreover, a tonoplast-associated form of SUS has

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Abbreviations: DAF, days after flowering; RT-PCR, reverse transcriptase-polymerase chain reaction.

been described recently in red beet (Etxeberria and Gonzalez, 2003). *In vitro* experiments suggest that the phosphorylation status of the enzyme could determine the extent to which SUS is soluble or membrane-bound (Winter *et al*., 1997), but the mechanisms that act *in vivo* remain highly debated.

SUS genes have been isolated primarily from starch-storing and sucrose- or hexose-storing plants (Sturm and Tang, 1999). Enhanced expression level in sink tissues is a widespread observation emerging from diverse *SUS* expression studies. *SUS* gene induction under stress conditions is also often described in roots and/or shoots. For instance, increased *SUS* transcript levels are thought to be involved in meeting the increased glycolytic demand during O_2 deficiency (Ricard *et al*., 1998; Déjardin *et al*., 1999). Cold treatment, dehydration, or osmotic stress are also reported to induce *SUS* expression (Hesse and Willmitzer, 1996; Déjardin *et al*., 1999; Kleines *et al*., 1999). In the same manner, *SUS* expression is regulated by externally provided sugars (Koch, 1996; Déjardin *et al*., 1999). The vast majority of *SUS* genes characterized to date belong to small multigene families, both in monocot and dicot species (Komatsu *et al*., 2002). Due to partial redundancy in expression patterns, elucidating the precise function of each isoform in a family, if any, is far from being obvious. Moreover, the multigene families studied to date might be incomplete (Carlson *et al*., 2002), thus hindering the overall understanding of the family.

In the model plant *Arabidopsis thaliana*, the complete sequencing of the genome reveals six putative members in the *SUS* gene family (Barratt *et al*., 2001). The complete *AtSUS* family, which is the largest *SUS* family described to date, appears as a good system to elucidate the function of each isoform, which is mostly unknown. *AtSUS1* (At5g20830) was shown to be expressed in the phloem of leaves and in roots (Martin *et al*., 1993). *AtSUS2* (At5g49190) was cloned and characterized by Chopra *et al*. (1992). The two genes were found to be strongly and differentially regulated in leaves exposed to environmental stresses (Déjardin *et al*., 1999). To date, no data relative to *AtSUS3* (At4g02280), *AtSUS4* (At3g43190), *AtSUS5* (At5g37180), or *AtSUS6* (At1g73370) are available. Aimed at analysing the six *AtSUS* genes, the real-time quantitative reverse transcriptase (RT)-PCR technique appears both as a highly sensitive and specific method (Bustin, 2000). Because it allows the specific detection of each member of the family, the real-time RT-PCR technique is indeed a good tool to carry out an analysis of the expression level of genes belonging to a very conserved gene family. This was illustrated in *A. thaliana* on the members of a cell wall enzyme gene family (Yokoyama and Nishitani, 2001), on the NRT2 nitrate transporter family (Orsel *et al*., 2002), and on the shaggy-like kinase multigene family (Charrier *et al*., 2002).

This article reports on the comprehensive expression profile of the six *AtSUS* genes in various organs of *A. thaliana*, including time-scale studies on developing and germinating seeds. A series of abiotic treatments such as O_2 deprivation, dehydration, cold treatment, or various sugar feedings was then considered to characterize the members of this multigene family further. Finally, the role of the different AtSUS isoforms will be discussed in the light of their expression patterns, which appear to be original.

Materials and methods

Plant material and growth conditions

Seeds of *A. thaliana* ecotype Wassilewskija (WS) were surface-sterilized and germinated on Murashige and Skoog (MS) medium (M02 555, pH 5.6; Duchefa, Haarlem, The Netherlands) solidified with 0.7% (w/v) agar. After a cold treatment of 48 h, at 4 °C, in the dark, the plates were transferred to a growth chamber and incubated at 21/15 °C day/night, under a 16/8 h light/dark regime. After 15 d, the plantlets were transferred to sterilized compost in individual pots, grown under the same conditions as above and irrigated twice a week with a complete mineral nutrient solution (Lesaint and Coïc, 1983). Primary shoots only were used and secondary shoots were removed. To harvest siliques of defined developmental stages, individual flowers were tagged using coloured tape on the day of flowering. For time-course studies during the seed maturation process, all the developing seeds of one shoot were harvested 3–4 weeks after the onset of flowering: siliques ranging from 6–18 d after flowering (DAF) were opened and the corresponding seeds removed and subsampled. To obtain root material, seedlings were grown in vertical plates and maintained in growth chamber for 4–5 weeks. For time-course study during the germination process, sterilized seeds were germinated on 0.8% (w/v) agar in the same conditions as described above. For stress treatments, germinating seeds and plantlets were incubated at 21/18 °C day/night, under a 8/16 h light/dark regime to avoid flowering. After 6–7 weeks, some plants were either deprived of water, transferred to a cold chamber (cold-stressed plants; 5 °C, same day/light regime), or submerged into degassed water. For the feeding experiments (sugar treatments), leaves were detached from intact plants, pre-adapted to the dark for 5 h, and placed into containers for 16 h with water or sugars/osmotica in 10 mM MES/KOH, pH 6.25. All the plant material were immediately frozen in liquid nitrogen after harvesting and stored at –80 °C prior to RNA extraction.

RNA extraction and cDNA preparation

Frozen tissues were ground in liquid nitrogen and total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The extracts were treated with 30 units of Rnase-free Dnase I (Qiagen) to eliminate the residual genomic DNA present in the preparation and eluted with 40 µl of diethyl pyrocarbonate-treated water. For the expression study in plant organs, two independent extractions per organ were performed, while three independent extractions were carried out for each data point in stress studies. For reverse transcription (RT)-PCR studies, DNA-free RNA was converted into first strand cDNA using the SuperScript preamplification system for first-strand cDNA synthesis (Gibco BRL, Cergy Pontoise, France) with oligo$(dT)_{22}$. In all cases, three independent RT were performed.

Table 1. *Oligonucleotides for real-time RT-PCR experiments*

Gene	Forward primer	Reverse primer
AtSUS1	5′-CTC GTC CAA CAC TCC ACA A-3′	5′-TCT TCA TAC AGC GTT TCG G-3′
AtSUS2	5′-AGG GTG TAC CAA ATC TCA T-3′	5′-CAT AGT GAA AGC TGT GTG G-3′
AtSUS3	5′-GAG ATA CCG CAG GGA GAG TT-3′	5′-CAG CAT TTC AGT CTC AAG GG-3′
AtSUS4	5′-CAA GTG TAA GCA TGA CCC-3′	5′-AGG AAC AGC TTG AGC CAG-3′
AtSUS5	5′-CGA ACA AGA AAC GGT GAA CT-3′	5′-TTC GTT TAT GCG TTG TAG CC-3′
AtSUS6	5′-GAT GAA GAT TGC GTA TGG AC-3′	5′-AGC CGA GTT AGC ACC AAA G-3′
EF	5′-CTG GAG GTT TTG AGG CTG GTA T-3′	5′-CCA AGG GTG AAA GCA AGA AGA-3′

Table 2. *Identity/similarity matrix for the six predicted AtSUS amino acid sequences*

		Similarity					
		AtSUS1	AtSUS2	AtSUS3	AtSUS4	AtSUS5	AtSUS6
	MM (kDa)	92.98	92.05	91.98	92.98	95.77	106.86
Identity	AtSUS1	–	81.1	81.8	95.3	67.4	62.8
	AtSUS2	66.3	–	86.2	81.0	69.0	62.0
	AtSUS3	67.9	74.2	–	82.4	70.7	62.4
	AtSUS4	89.0	67.2	68.2	–	66.4	61.9
	AtSUS5	50.6	50.2	52.2	48.9	–	69.5
	AtSUS6	47.4	46.6	48.0	46.9	57.7	–

Real-time quantitative RT-PCR

The reaction was performed on the LightCycler Instrument (Roche, Meylan, France) with the LightCycler-FastStar DNA Master SYBR Green I kit for PCR (Roche) according to the manufacturer's protocol. Each reaction was performed with 5 µl of 1:10 to 1:100 dilution of the first cDNA strands in a total volume of 20 µl. The reactions were incubated at 95 °C for 8 min to activate the hot start recombinant *Taq* DNA polymerase, followed by 45 cycles of 10 s at 95 °C, 6 s at 55 °C, and 20 s at 72 °C (Orsel *et al*., 2002; Baud *et al*., 2003). Specific primer sets were designed for each gene (Table 1). The specificity of the PCR amplification was checked with a heat dissociation protocol (from 65 °C to 95 °C) following the final cycle of the PCR; PCR products were then purified and sequenced. The efficiency of the primer sets was calculated by performing real time PCR on several dilutions of first strands. Efficiencies of the different primer sets used were checked to be almost similar. The specificity of each primer set was checked by sequencing PCR products. The results obtained for the different tissues analysed were standardized to the constitutive *EF1A4*α gene expression level (Liboz *et al*., 1990).

Construction of the distance tree

For the construction of the rooted tree, only the glucosyl-transferase domains of each selected sucrose synthase protein were used. The MultAlin program (Corpet, 1988) was used to create an alignment of the sequences that was then submitted to a Neighbor–Joining analysis to generate a branching pattern thanks to the ClustalX package (Thompson *et al*., 1997). Bootstrap analysis with 1000 replicates was performed to assess the statistical reliability of the tree topology and nodes with bootstrap score of <50% were discarded. The consensus tree was drawn using the TreeView program (version 1.5.3, Roderic DM Page, University of Glasgow, UK).

Results

Structure of the AtSUS *family*

The *A. thaliana* genome sequence was searched with genomic and cDNA sequences of *AtSUS1* (Martin *et al*., 1993) and *AtSUS2* (Chopra *et al*., 1992) using the BLAST program: four new putative *AtSUS* genes were isolated (Barratt *et al*., 2001). A complete alignment of the deduced amino acid sequences, beginning and ending at the putative start and stop codons, respectively, was performed using the Needle program (Needlman and Wunsch, 1970; Table 2). Three distinct AtSUS couples arose from this analysis (Fig. 1). The highest percentage of amino acid identity/similarity was found between AtSUS1 and AtSUS4 (89% and 95%, respectively). AtSUS2 and AtSUS3 then appeared to be closely related as well (74% and 86%). AtSUS5 and AtSUS6 are two isoforms very different from the four other AtSUS. A schematic representation of the *AtSUS* gene structures is presented in Fig. 1. The predicted exon/intron structure of these genes was analysed between the start and stop codons, regardless of the presence (*AtSUS2*; Chopra *et al*., 1992) or absence (*AtSUS1*; Martin *et al*., 1993) of a non-coding leader exon 1. The six genes shared the two characteristic sucrose synthase and glucosyl-transferase domains. The *AtSUS1*/*AtSUS4* couple was characterized by the presence of a 6th/5th 336 bp long and a 12th/11th 564/567 bp long unsplit

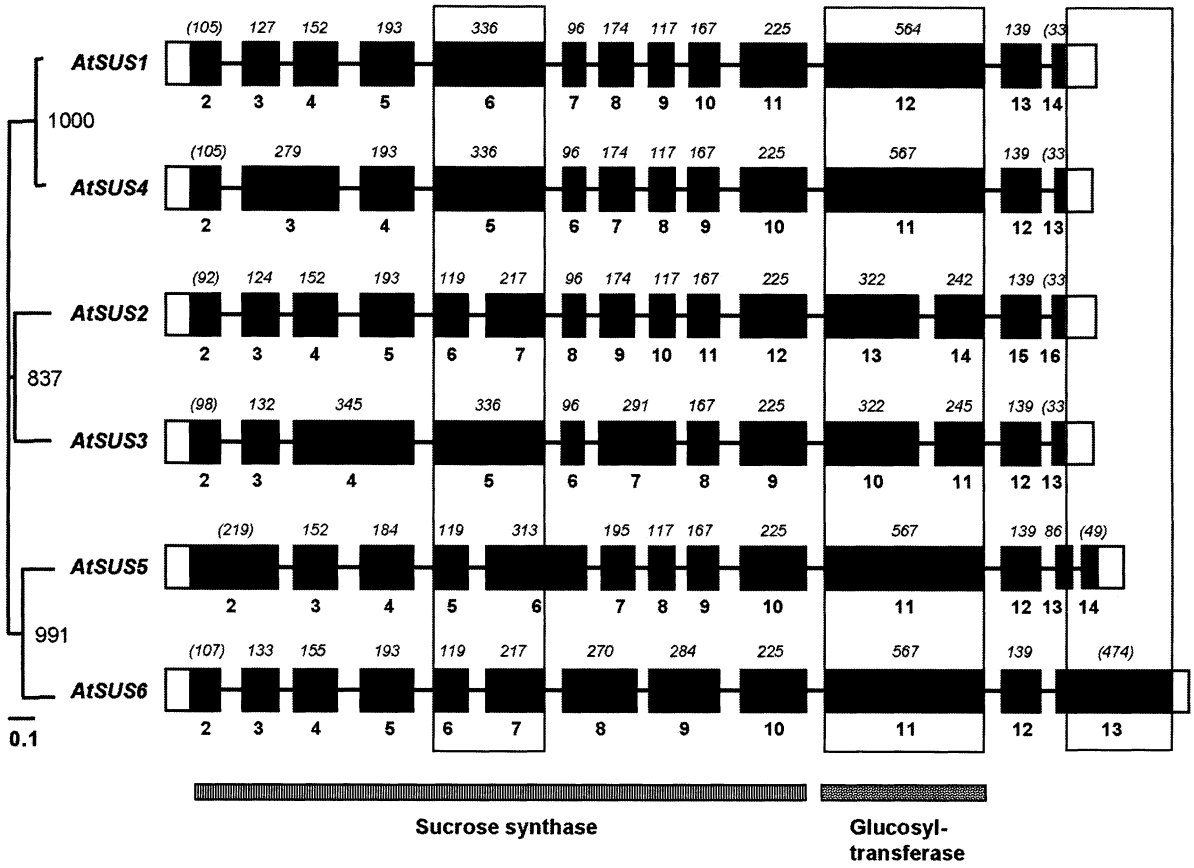


Fig. 1. Comparison of the six *AtSUS* genes. On the left, a distance tree among the glucosyl-transferase (GT) domains from *A. thaliana* sucrose synthase proteins is presented. A bootstrap analysis with 1000 replicates was performed to assess the statistical reliability of the tree topology (bootstrap scores are indicated). On the right, a comparison of the gene structures is presented. Numbered black boxes represent exons and the lines connecting them denote introns; the possible presence of a non-coding exon 1 was not considered. Sizes of exons are given above the structure, and the numbers in parentheses indicate the length of coding sequences. The regions analysed in details in the text are surrounded by vertical open boxes.

exon. In *AtSUS2* and *AtSUS3*, the 564 bp exon was split by an intron. *AtSUS5* and *AtSUS6* were characterized by the presence of an 11th 567 bp unsplit exon, while the 336 bp exon was split by an intron. Moreover, *AtSUS5* and *AtSUS6* genes exhibited a 3′ extension that had never been observed so far among the plant *SUS* genes. As a consequence, the predicted molecular masses of the corresponding proteins (96 and 107 kDa; Table 2) were higher than those calculated for other AtSUS peptides (92/93 kDa in average).

Expression analysis of AtSUS *genes in* A. thaliana *organs*

To date, among the six *AtSUS* genes present in the *A. thaliana* genome, expression analyses were reported for the *AtSUS1* and *AtSUS2* genes (Martin *et al.*, 1993; Déjardin *et al.*, 1999). A set of specific primers was designed to study the expression profile of each member of the family in various organs of the plant. The specificity of each primer was checked by sequencing of the PCR products. Figure 2 illustrates the relative transcript levels of the six *AtSUS* genes standardized to the constitutive *EF1A4α* (*EF*) gene expression level (Liboz *et al.*, 1990). Due to high variations in the relative transcript levels observed between various plant organs for a single gene, a logarithmic scale was chosen to present these results. Each type of transcript was detected, demonstrating that all the members of this gene family were transcriptionnaly active. In the set of organs tested, the *AtSUS1* gene exhibited transcript levels ranging from 0.1% *EF* in flowers to 1.7% *EF* in silique walls at 15 d after flowering (DAF). *AtSUS2* transcripts were barely detectable and never exceeded 0.1% *EF*. The *AtSUS3* expression pattern only differed from the one described for *AtSUS2* in that increased transcript levels were observed in silique walls at 15 DAF (4.5% *EF*). The highest transcript levels for *AtSUS4* were measured in roots (11% *EF*) and stems (3.4% *EF*), as well as in silique walls at 6 DAF (6.4% *EF*), while the lowest expression levels for this gene was observed in flowers and young siliques. Transcript levels measured for *AtSUS5* and

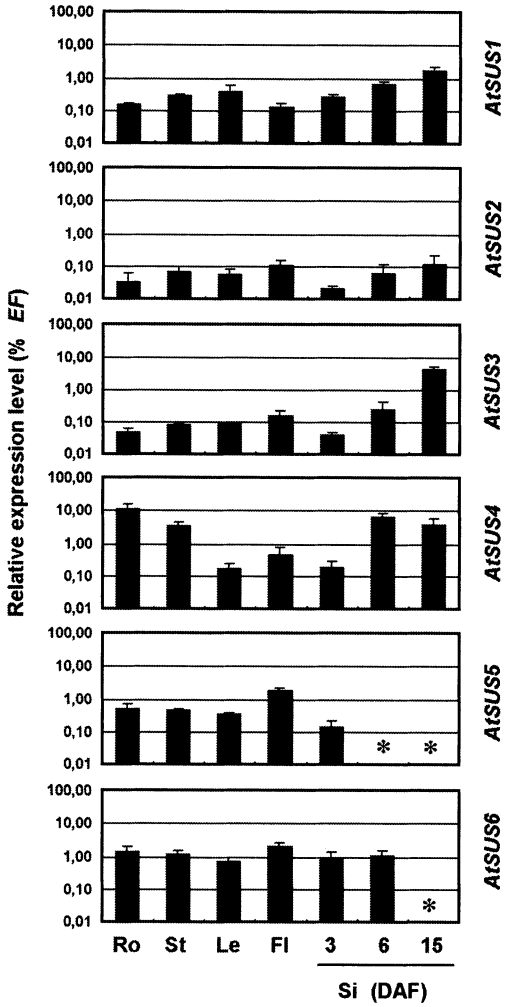


Fig. 2. Relative expression profile of the six *AtSUS* genes in *A. thaliana* organs. Relative transcript levels were measured by real-time RT-PCR and standardized to the constitutive *EF1A4*α (*EF*) gene expression level. The mRNA were extracted from roots (Ro), stems (St), leaves (Le), flowers (Fl), siliques (Si), at 3, 6, and 15 DAF. Note that the whole silique was sampled at 3 DAF, while seeds were removed at 6 and 15 DAF, so that RNAs were extracted from silique walls alone. All values are the means ±SE of at least three measurements. The asterisk indicates that transcripts were not detectable.

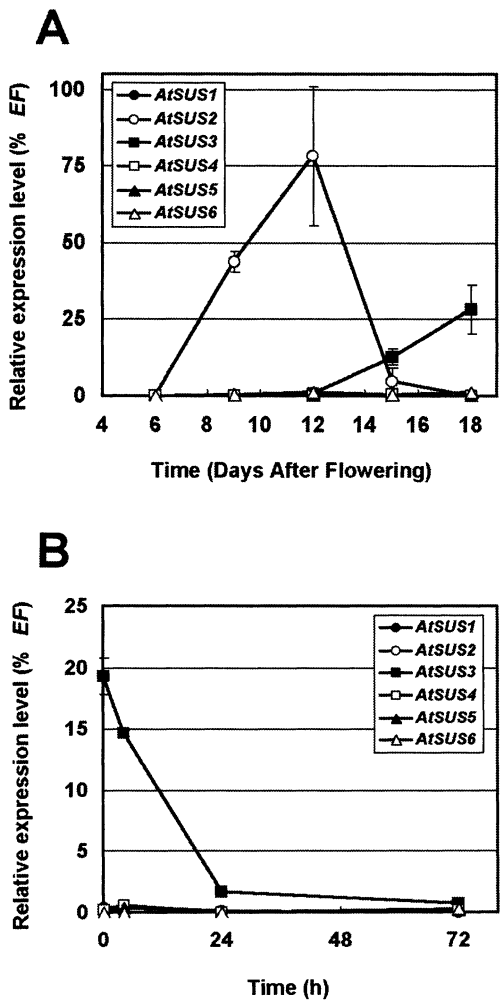


Fig. 3. Relative expression profile of the six *AtSUS* genes in developing (A) and germinating (B) seeds. (A) Seeds were harvested at 6 (early embryo morphogenesis), 9, 12, 15 (maturation), and 18 d after flowering (late maturation). (B) After a 48 h long stratification period on germinating medium, seeds were exposed to the light in a growth chamber (0 h). Germinating seeds were harvested 4, 24, and 72 h after the first exposure to light. Note that root protrusion was effective at 48 h. In all cases, RNAs were then extracted and the transcript level of each *AtSUS* gene was studied by real-time RT-PCR. Relative transcript levels were standardized to the constitutive *EF1A4*α (*EF*) gene expression level. Each point is the mean ±SE of at least three independent measurements.

AtSUS6 were fairly constant in all organs studied, except for the oldest silique wall samples, where they could not be detected.

Expression analysis of AtSUS *genes in developing and germinating seeds*

The relative expression profile of the six members of the *AtSUS* gene family was investigated in developing seeds. For each gene, a five-point kinetic was performed (Fig. 3A) that was representative of the three developmental stages of *A. thaliana* seed development (Baud *et al.*, 2002): early embryo morphogenesis at 6 DAF, maturation at 9, 12, and 15 DAF, and late maturation at 18 DAF. The highest and most significant variations in relative *AtSUS* transcript levels observed during seed development were those encountered by *AtSUS2* and *AtSUS3*. The *AtSUS2* transcript level displayed a 2600-fold increase between 6 and 12 DAF, peaking at almost 80% *EF*, before falling sharply at 5% *EF* at 15 DAF. The *AtSUS2* transcripts were not detectable anymore at 18 DAF. Conversely, the transcript level of *AtSUS3* was lower than 1% *EF* until 12 DAF, then it increased steadily to reach almost 30% *EF* at 18 DAF. During all the seed development process, *AtSUS1*, *AtSUS4*, and *AtSUS5* displayed a similar, fairly constant, and weak expression pattern, with no relative transcript level

exceeding 0.5% *EF*. *AtSUS6*, though exhibiting a low expression profile too, peaked at 12 DAF, reaching 1.4% *EF*.

The expression pattern of the six *AtSUS* genes was also analysed in germinating seeds. For each gene, a four-point kinetic was performed (Fig. 3B) from 0–72 h after the first exposure to light (fully germinated plantlets). Root protrusion was effective at 48 h. The *AtSUS3*-relative transcript level observed at 0 h (almost 20% *EF*) was comparable to the one measured in desiccating seeds at 18 DAF. It then decreased regularly until 24 h, reaching 1.6% *EF*, and then remained almost stable. During all the germination process, *AtSUS1*, *AtSUS2*, *AtSUS4*, *AtSUS5*, and *AtSUS6* exhibited low and almost constant expression profiles, with no level exceeding 0.6% *EF*.

Expression analysis of AtSUS *genes in response to O_2 deficiency*

Induction of *SUS* genes by hypoxia or anoxia is a widely described phenomenon in dicot and monocot species, both in roots and shoots (Marana *et al*., 1990; Ricard *et al*., 1998; Zeng *et al*., 1998). To characterize the expression pattern of *AtSUS* genes in response to O_2 deficiency, 6–7-week-old plants were submerged into degassed water for 2 d. *AtSUS* mRNA levels were analysed in the rosette leaves after 24 h and 48 h of treatment. The results showed that the *AtSUS* genes are differentially regulated by O_2 deprivation (Fig. 4). While *AtSUS2*, *AtSUS3*, *AtSUS5*, and *AtSUS6* mRNA levels were unaffected by the stress, a significant induction was observed for *AtSUS1* and *AtSUS4*. After 1 d of treatment, their transcript levels exhibited a very significant 3-fold and 2-fold induction, respectively. This induction appeared to be transient for *AtSUS1* and was not observed further after 2 d of stress. In the case of *AtSUS4*, this induction level rose steadily at 48 h, but this was not observed for all the leaves treated, thus explaining the relatively low level of significance of the variation observed.

Expression analysis of AtSUS *genes in response to dehydration, cold treatment, and sugar feedings*

The relative transcript level of the six *AtSUS* genes was analysed in leaves of plants undergoing a gradual dehydration treatment. For each gene, a four-point kinetic was performed that began at the onset of water deprivation and ended with a quite drastic dehydration of the plant, 11 d later (Fig. 5). An increase in both *AtSUS1* and *AtSUS3* transcript levels was observed during the last days of treatment. While *AtSUS3* mRNA level exhibited a 40-fold induction, the transcript level of *AtSUS1* increased up to 8 times in response to water deprivation. In both cases, statistical analyses demonstrated that inductions observed in stressed leaves were significant. An increase in *rab18* transcript level (Lang *et al*., 1994) was specifically observed 11 d after the onset of water deprivation (data not shown). Osmotic potential measurements confirmed that dehydration was not serious before day 11 (data not shown).

The relative transcript level of the *AtSUS* genes was then analysed in leaves of plants undergoing a cold treatment (4 °C during 48 h). While *AtSUS2*, *AtSUS4*, *AtSUS5*, and *AtSUS6* mRNA levels were unaffected by the stress, 5.5- and 24.5-fold inductions were measured for *AtSUS3* and *AtSUS1*, respectively (Fig. 6). Statistical analyses showed that the increase observed in *AtSUS1* mRNA level was very significant.

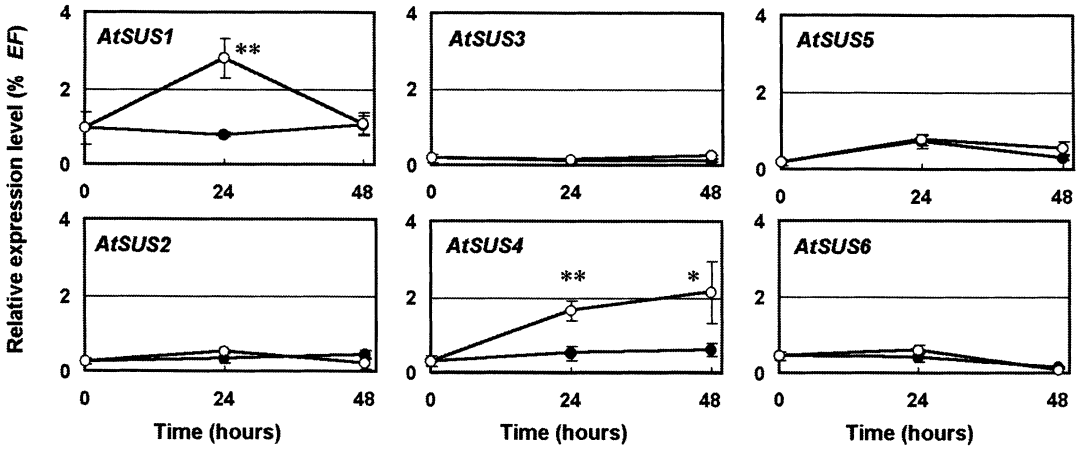


Fig. 4. Effects of O_2 deficiency on the expression of the six *AtSUS* genes. RNAs were extracted from rosette leaves of plants submerged into degassed water (O_2-deficient plants; open circles) and from rosette leaves of control plants (closed circles), after 0, 24, or 48 h of treatment. The transcript level of each *AtSUS* gene was studied by real-time RT-PCR. Relative transcript levels were standardized to the constitutive *EF1A4*α (*EF*) gene expression level. Each point is the mean ±SE of at least three independent measurements. Statistical analysis of the results was performed using the Student *t*-test, each point being compared to the corresponding t_0 value. One asterisk, significant difference at the 95% level; two asterisks, very significant difference at the 99% level.

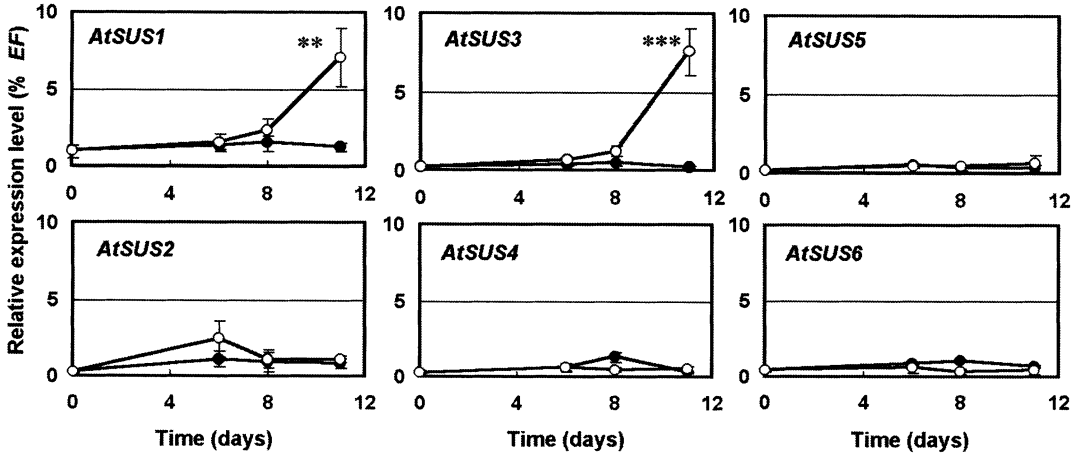


Fig. 5. Effects of water deprivation on the expression of the six *AtSUS* genes. RNAs were extracted from rosette leaves of plants deprived of water (open circles) and from leaves of plants regularly irrigated (control plants; closed circles), after 0, 4, 8, or 12 d of treatment. The transcript level of each *AtSUS* gene was studied by real-time RT-PCR. Relative transcript levels were standardized to the constitutive *EF1A4*α (*EF*) gene expression level. Each point is the mean ±SE of at least three independent measurements. Statistical analysis of the results was performed using the Student *t*-test, each point being compared to the corresponding t_0 value. Two asterisks, very significant difference at the 99% level; three asterisks, highly significant difference at the 99.9% level.

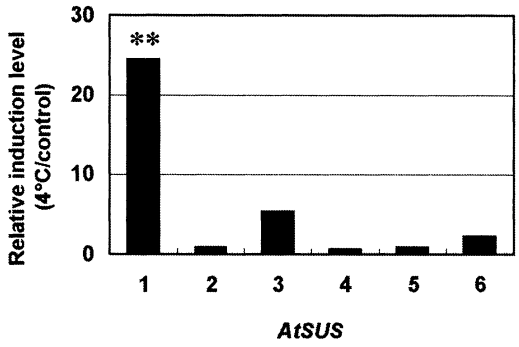


Fig. 6. Effects of cold treatment on the expression of the six *AtSUS* genes. RNAs were extracted from rosette leaves of plants transferred to a cold chamber during 48 h (cold-stressed plants) and from leaves of plants maintained at normal temperature (control plants). The transcript level of each *AtSUS* gene was studied by real-time RT-PCR. Relative transcript levels were standardized to the constitutive *EF1A4*α (*EF*) gene expression level. The results obtained for each *AtSUS* gene were averaged and a statistical analysis was performed using the Student *t*-test, cold-stressed plants being compared to control plants. Two asterisks, very significant difference at the 99% level. For each *AtSUS* gene, the relative induction level (cold-stressed plants to control plants average ratio) is presented.

Sugars are known to affect *SUS* gene expression levels in various species (Koch, 1996). Moreover, sugar contents are liable to change in leaves during stress exposure. As a consequence, the putative effect of exogenously provided sucrose and mannitol in the regulation of *AtSUS* gene expression was investigated. Before treatments, *A. thaliana* plants were kept in the dark for 5 h to lower the internal level of soluble sugars. The relative expression profile of the six members of the *AtSUS* family was analysed in darkened cut leaves fed for 14 h with sugar/osmotica (Fig. 7). The *AtSUS1* mRNA level exhibited a slight (2.5-fold), but significant, increase in response to the two sucrose concentrations tested. In the same manner, the 4-fold (250 mM mannitol) and 7-fold (sucrose+mannitol) inductions observed in response to mannitol (penetrating osmotic agent) were statistically significant. An even stronger and more significant induction (up to 37-fold) due to mannitol was measured for the *AtSUS3* transcript level, while no induction was observed with sucrose alone. Conversely, under the various feeding experiments tested, *AtSUS2*, *AtSUS4*, *AtSUS5*, and *AtSUS6* displayed a fairly constant expression pattern.

Discussion

The AtSUS *genes exhibit distinct but partially overlapping expression patterns*

Recent investigations (Sturm *et al*., 1999; Komatsu *et al*., 2002) as well as the publication of the *A. thaliana* genome sequence support the idea that *SUS* genes can be classified into four distinct groups. The comparison of predicted amino acid sequences as well as the intron/exon structure of the *SUS* genes corroborate this classification. The *AtSUS* family comprises six different genes distributed in three of these groups (Figs 1, 8A). *AtSUS1* and *AtSUS4* can be classified among the vast dicot SUS1 group, characterized at the gene structure level by the presence of two specific unsplit exons (Fig. 1; Komatsu *et al*., 2002). *AtSUS2* and *AtSUS3* belong to the SUSA group. Their 564 bp long exon is split by an intron in the same manner as for genes of the SUSA and monocot SUS1 groups (Fig. 1; Komatsu *et al*., 2002). *AtSUS5* and *AtSUS6* constitute a separate group on their own. The six predicted amino acid sequences exhibit a typical sucrose synthase organization with, in particular,

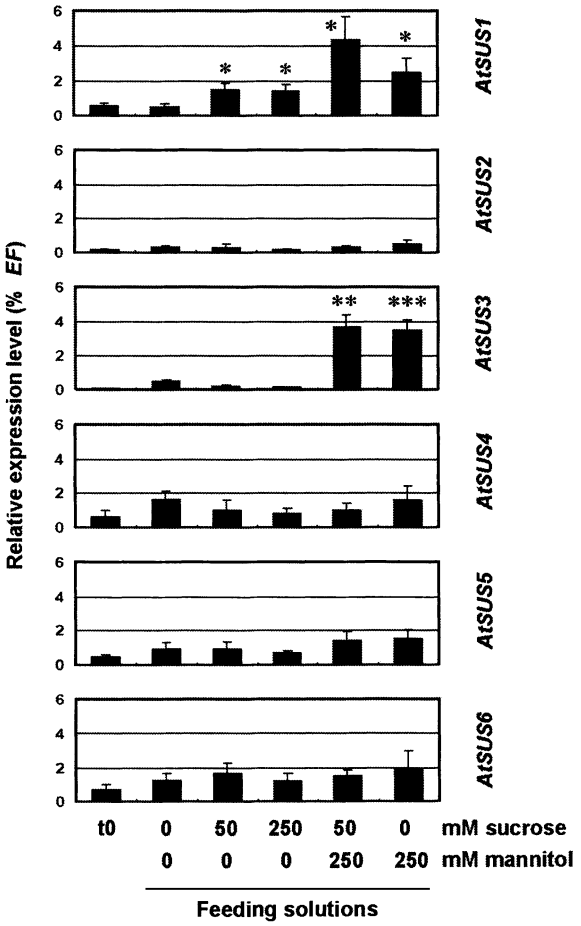


Fig. 7. Effects of sugar/osmoticum on the expression of the six *AtSUS* genes. Rosette leaves were detached from 6–7-week-old plants (pre-adapted to darkness for 5 h) and fed for 16 h in the dark with 10 mM MES/KOH, pH 6.25, containing sucrose and/or mannitol. Total RNAs were isolated from leaves and analysed using a real-time RT-PCR strategy. Relative transcript levels were standardized to the constitutive *EF1A4*α (*EF*) gene expression level. Each value is the mean ±SE of at least three independent measurements. t_0, RNAs extracted from leaves pre-adapted to darkness. Statistical analysis of the results was performed using the Student *t*-test, each point being compared to the water treatment. One asterisk, significant difference at the 95% level; two asterisks, very significant difference at the 99% level; three asterisks, highly significant difference at the 99.9% level.

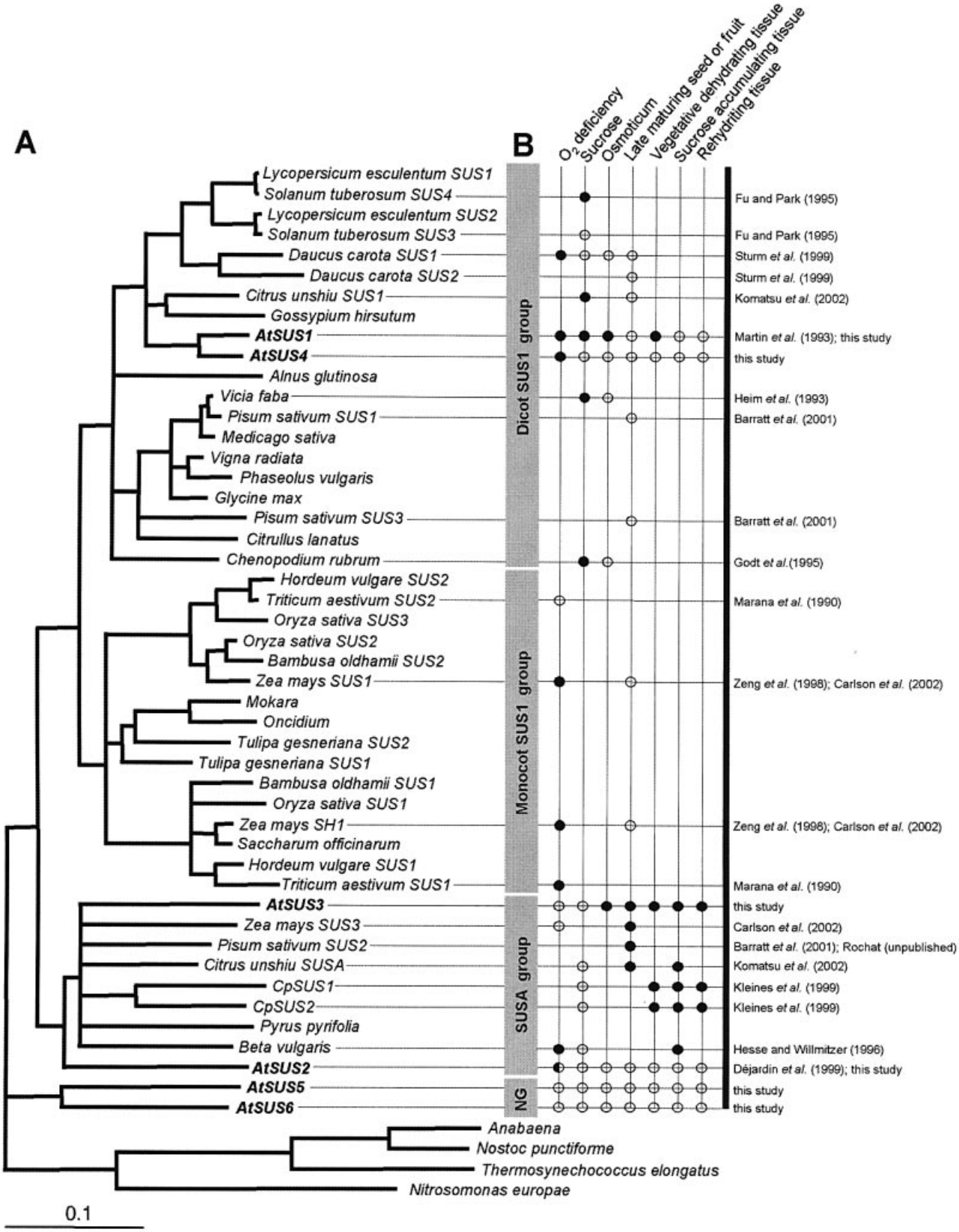
the sucrose synthase and the glucosyl-transferase domains identified in the Pfam database (Bateman *et al*., 2002).

In an attempt to elucidate the role of each *AtSUS* member, the expression profile of each of the six *AtSUS* genes was specifically described in various plant organs, including developing and germinating seeds, thanks to the sensitivity and the reliability of the real-time RT-PCR technique. To characterize the members of this family further, the relative expression profile of each *AtSUS* gene was investigated in plants undergoing various abiotic treatments. It was possible to show that there is no pseudogene in the *AtSUS* family and that the six *AtSUS* genes exhibit distinct but partially overlapping expression patterns. Such a high level of gene redundancy in both structure and expression profiles in a plant with the smallest genome size is remarkable. Thus, these results add a significant new contribution to the current understanding of the genome complexity in plants.

AtSUS5 *and* AtSUS6 *constitute an independent group although the functions remain unknown*

This study shows that *AtSUS5* and *ATSUS6*, two genes composing a separate and new group on their own, are ubiquitously expressed in the plant (Fig. 2). Comparison of predicted amino acid sequences anchors these two genes on an independent branch of the distance tree, located between other plant SUS and bacterial SUS (Fig. 8A). Alignment of amino acid sequences then shows that the RXXS consensus sequence recognized by Ser/Thr protein kinase and classically located in the N terminal region of SUS proteins was modified in T/KXXS in AtSUS5 and AtSUS6. According to recent results obtained on the rice enzyme (Asano *et al*., 2002), this may suggest that AtSUS5 and AtSUS6 are not phosphorylated at this site. *AtSUS5* and *AtSUS6* putatively encode for proteins of relatively high molecular weight, the kinetic properties of which are not yet determined. This aspect is particularly striking in the case of *AtSUS6*, which encodes for a 107 kDa protein with a predicted pI of 8.1, the 3′ extension of this particular

Fig. 8. Comparison of deduced amino acid sequences and expression patterns of sucrose synthases. (A) Distance tree among the glucosyl-transferase (GT) domains from sucrose synthase proteins. A bootstrap analysis with 1000 replicates was performed to assess the statistical reliability of the tree topology and nods with bootstrap score <50% were discarded. EMBL database accession numbers of the displayed SUS are as follows: *Alnus glutinosa*, X92378; *Anabaena*, AJ010639; *Bambusa oldhamii* SUS1, AAL50571; *Bambusa oldhamii* SUS2, AAL50570; *Beta vulgaris*, X81974; *Chenopodium rubrum*, X82504; *Citrullus lanatus*, BAA89232; *Citrus unshiu* SUS1, AB022092; *Citrus unshiu* SUSA, AB022091; *Craterostigma plantagineum* SUS1, AJ131999; *Craterostigma plantagineum* SUS2, AJ132000; *Daucus carota* SUS1, X75332; *Daucus carota* SUS2, Y16091; *Glycine max*, AF030231; *Gossypium hirsutum*, U73588; *Hordeum vulgare* SUS1, X65871; *Hordeum vulgare* X69931; *Lycopersicum esculentum* SUS1, L19762; *Lycopersicum esculentum* SUS2; AJ011319; *Medicago sativa*, AF049487; *Mokara* 'yellow' orchid, AF530568; *Nitrosomonas europeae*, ZP_00002943; *Nostoc punctiforme*, AJ316589; *Oncidium* cv. 'Goldiana' orchid, AAM95943; *Oryza sativa* SUS1, X64770; *Oryza sativa* SUS2, X59046; *Oryza sativa* SUS3, L03366; *Phaseolus vulgaris*, AF315375; *Pisum sativum* SUS1, AJ012080; *Pisum sativum* SUS2, AJ001071; *Pisum sativum* SUS3, AJ311496; *Pyrus pyrifolia*, BAB20799; *Saccharum officinarum*, AF263384; *Solanum tuberosum* SUS3, U24088; *Solanum tuberosum* SUS4, U24087; *Thermosynechococcus elongatus*, NP_681838; *Triticum aestivum* SUS1, AJ001117; *Triticum aestivum* SUS2, AJ000153; *Tulipa gesneriana* SUS1, X96938; *Tulipa gesneriana* SUS2, X96939; *Vicia faba*, X69773; *Vigna radiata*, D10266; *Zea mays* SH1, X02400; *Zea mays* SUS1, L22296; *Zea mays* SUS3, AY059416. (B) Comparison of *SUS* expression patterns and related references. Empty circles represent unaltered mRNA levels in response to the stress under consideration or an absence of induction in a specific tissue when compared to the rest of the plant. Full circles represent an induction in the transcription level, with the exception of rehydrating tissues, where they correspond to a decreased expression level.


A
B
O_2 deficiency
Sucrose
Osmoticum
Late maturing seed or fruit
Vegetative dehydrating tissue
Sucrose accumulating tissue
Rehydriting tissue
Lycopersicum esculentum SUS1
Solanum tuberosum SUS4
Lycopersicum esculentum SUS2
Solanum tuberosum SUS3
Daucus carota SUS1
Daucus carota SUS2
Citrus unshiu SUS1
Gossypium hirsutum
AtSUS1
AtSUS4
Alnus glutinosa
Vicia faba
Pisum sativum SUS1
Medicago sativa
Vigna radiata
Phaseolus vulgaris
Glycine max
Pisum sativum SUS3
Citrullus lanatus
Chenopodium rubrum
Hordeum vulgare SUS2
Triticum aestivum SUS2
Oryza sativa SUS3
Oryza sativa SUS2
Bambusa oldhamii SUS2
Zea mays SUS1
Mokara
Oncidium
Tulipa gesneriana SUS2
Tulipa gesneriana SUS1
Bambusa oldhamii SUS1
Oryza sativa SUS1
Zea mays SH1
Saccharum officinarum
Hordeum vulgare SUS1
Triticum aestivum SUS1
AtSUS3
Zea mays SUS3
Pisum sativum SUS2
Citrus unshiu SUSA
CpSUS1
CpSUS2
Pyrus pyrifolia
Beta vulgaris
AtSUS2
AtSUS5
AtSUS6
Anabaena
Nostoc punctiforme
Thermosynechococcus elongatus
Nitrosomonas europae
0.1
Dicot SUS1 group
Monocot SUS1 group
SUSA group
NG
Fu and Park (1995)
Fu and Park (1995)
Sturm et al. (1999)
Sturm et al. (1999)
Komatsu et al. (2002)
Martin et al. (1993); this study
this study
Heim et al. (1993)
Barratt et al. (2001)
Barratt et al. (2001)
Godt et al.(1995)
Marana et al. (1990)
Zeng et al. (1998); Carlson et al. (2002)
Zeng et al. (1998); Carlson et al. (2002)
Marana et al. (1990)
this study
Carlson et al. (2002)
Barratt et al. (2001); Rochat (unpublished)
Komatsu et al. (2002)
Kleines et al. (1999)
Kleines et al. (1999)
Hesse and Willmitzer (1996)
Déjardin et al. (1999); this study
this study
this study

gene containing some low complexity regions (Bateman *et al*., 2002). The two genes do not exhibit any transcriptional response to the stresses tested. Specific tissue localization and/or monitoring response to other types of stresses could help to improve current understanding of the specific role of these two particular genes. Moreover, post-transcriptional and post-translational data relative to these two particular genes would be of particular interest.

AtSUS1 *and* AtSUS4 *are closely related but exhibit distinct expression profiles*

AtSUS1 and *AtSUS4*, members of the dicot *SUS1* group, exhibit the highest percentage of amino acid identity/similarity among the AtSUS family (Table 2). Transcripts of the corresponding genes are detected in the whole plant, and expression of *AtSUS1* is fairly constant in all organs analysed, while *AtSUS4* appears to be more expressed in roots, stems, and silique walls (Fig. 2). The relative expression profile observed in response to oxygen deficiency induced by water flooding (Fig. 4) then demonstrates that both genes exhibit significant induction after one day of treatment. *AtSUS1* and *AtSUS4* transcripts indeed increased by 3- and 2-fold, respectively, after 24 h of stress, and these data are in agreement with previous results. In *A. thaliana* transgenic plants, the *AtSUS1* promoter transcriptionally fused to the β-glucuronidase gene, was shown to be inducible by the same anaerobic treatment (Martin *et al*., 1993). Likewise, a cDNA microarray study, characterizing the expression profile of *A. thaliana* root cultures in response to low-oxygen treatment, pointed out the induction of both *AtSUS1* and *AtSUS4* in the same order of amplitude (2- and 5-fold, respectively; Klok *et al*., 2002). It was not possible to measure any induction or repression of the other *AtSUS* genes in response to anoxia induced by water flooding, in contrast to Déjardin *et al*. (1999). Using a northern blot strategy, these authors describe a strong increase in *AtSUS2* mRNA level in leaves in response to O_2 deficiency while they do not measure any increase in *AtSUS1* transcript level. In other plant species, gene induction in response to oxygen deficiency was demonstrated for *Daucus carota SUS1*, *Triticum aestivum SUS1*, *Beta vulgaris SUS*, and *Zea mays SUS1* and *SH1* (Fig. 8B). In maize, *SUS1* and *SH1* are up-regulated preferentially by hypoxia (3% O_2) and anoxia (0% O_2), respectively (Zeng *et al*., 1998). Thus, it appears that fine differences in oxygen might have a strong effect on the expression of oxygen-responsive genes.

Conversely, *AtSUS1* and *AtSUS4* differ in their response to dehydration (Fig. 5), cold treatment (Fig. 6), or sugar/osmotica feeding (Fig. 7). The *AtSUS4* transcript level remains steady in all the conditions tested. On the contrary, *AtSUS1* is up-regulated by cold treatment (24.5-fold) as already mentioned in other studies (Déjardin *et al*., 1999; Marana *et al*., 1990). An induction by sucrose (2.5-fold) was then reported, but this effect does not seem to be specific to sucrose, since mannitol (a penetrating osmotic agent) led to an even more pronounced induction (4–7-fold). Dehydration also induces an increase in the *AtSUS1* transcript level (8 times). This can be interpreted either as a specific effect or as a side-effect of sugar and/or osmotic increase during stress (data not shown). These results corroborate previous observations (Déjardin *et al*., 1999) showing that *AtSUS1* is up-regulated by a decrease in leaf osmotic potential rather than by the increase in sucrose concentration *per se*. Nevertheless, recent studies strongly suggest that sugars (sucrose, glucose, and mannose) regulate *AtSUS1* via HXK-modulated mechanisms (Cieresko and Kleczkowski, 2002). Sugars are known to be inducers/repressors of gene expression and they have key roles in signal transduction pathways (Koch, 1996; Smeekens, 2000). As a conclusion, the regulation of *AtSUS1* possibly involves two or more transduction pathways depending on the nature and strength of the signal (Cieresko and Kleczkowski, 2002).

AtSUS2 *is a marker of seed maturation*

AtSUS2 transcripts are barely detectable in all the plant organs analysed, with the outstanding exception of seeds undergoing maturation. SUS was classically described as a marker of sink strength in storage tissues such as developing tubers or seeds in which the SUS pathway of sucrose cleavage was usually associated with starch biosynthesis (Zrenner *et al*., 1995; Carlson *et al*., 2002). However, the very strong induction in relative *AtSUS2* transcript level is measured between 6 and 12 DAF, corresponding in the growth conditions to the first period of the seed storage phase (Baud *et al*., 2002). This developmental phase is characterized by a decrease in starch concentration in the seed and by the onset of storage product biosynthesis, including both triglycerides and proteins. This result is consistent with a microarray study that examined changes in gene expression during *A. thaliana* seed development (Ruuska *et al*., 2002). Expression of *AtSUS* clones was indeed reported to increase strongly between 8 and 12 DAF and then coincide with an increase in oil and protein synthesis rather than with the transient starch accumulation in young seeds. Moreover, King *et al*. (1997) reported a peak in SUS activity in developing *Brassica* seeds, emphasizing the role of SUS in providing carbon for oilseed metabolism. In *A. thaliana* seeds, preliminary measurements of SUS activity corroborate the observed expression pattern (data not shown). *AtSUS2* could, consequently, appear as a marker for storage compound accumulation, providing carbon skeletons through the glycolytic pathway for triglyceride and protein synthesis, rather than starch synthesis. *AtSUS2* also appears as specifically expressed in maturing seeds, and is not induced in leaves in response to the various

abiotic stresses tested, including O_2 deficiency, dehydration, or sugar feedings.

AtSUS3 *is a marker of dehydration*

The expression profile of the *AtSUS3* gene is very specific and highly inducible. It is mainly expressed in 15 DAF silique walls (as they began to turn yellowish), in late maturing seeds (the *AtSUS3* transcript level increases while the seed water content steadily decreases; Baud *et al*., 2002), and in early germinating seeds (Fig. 3). Induction of this gene by drought and mannitol, an osmotic agent, is shown to be strong in stressed leaves (Fig. 5). Moreover, abundant *AtSUS3* ESTs were described in dehydration-treated rosette plants (TAIR database). *AtSUS3* also appears as a marker of dehydrating tissues and behaves as an osmoticum- but not sucrose-responsive gene. Likewise, recent studies already suggested, using the *gin1/aba2* mutant, that glucose regulation of some genes was distinct from regulation by osmotic stress (Cheng *et al*., 2002). More experimentation will be required to dissect these regulatory pathways for *AtSUS3*.

Interesting parallels can be drawn between the *AtSUS3* expression pattern and available data concerning different SUS isoforms closely related to *AtSUS3* on a distance tree (Fig. 8). Like *AtSUS3*, citrus *SUSA* (Komatsu *et al*., 2002), pea *SUS2* (Buchner *et al*., 1998; C Rochat, unpublished data), and maize *SUS3* (Carlson *et al*., 2002) transcript levels increase greatly during late maturation in seeds and/or in fruits. The citrus *SUSA* mRNA level was shown to increase with maturation both in edible and peel tissues of the fruit (Komatsu *et al*., 2002). This induction was correlated with sucrose accumulation in both tissues, but sucrose-feeding experiments revealed that the abundance of *SUSA* mRNA was unaffected by sucrose itself, in leaves. The pea *SUS2* transcript level was shown to rise both in the testa and the embryo during late maturation. Likewise, the steady-state level of maize *SUS3* mRNA exhibits a gradual increase during the kernel maturation process (Carlson *et al*., 2002). In the resurrection plant *Craterostigma plantagineum*, two *SUS* genes were isolated, called *CpSSI* and *CpSSII* (Kleines *et al*., 1999). Corresponding mRNA levels were shown to be highly induced in leaves and roots in response to dehydration. This mRNA accumulation correlates with an increase in sucrose concentration in leaf tissues. As a conclusion, a group of closely related *SUS* genes emerges that includes citrus *SUSA*, pea *SUS2*, maize *SUS3*, *AtSUS3*, *Cpss1*, and *Cpss2*. They exhibit resembling expression patterns in addition to strong similarities in predicted amino acid sequences (Fig. 8). These expression data highlight the major contribution that sugar metabolism is thought to play in desiccation-tolerance processes. But the precise function of these SUS isoforms in such a phenomenon remains to be demonstrated. On one hand, an increased glycolytic demand during stress could first be considered (Kleines *et al*., 1999). On the other hand, a massive sugar conversion leading to sucrose accumulation could explain why some of these *SUS* expression patterns correlate with sucrose accumulation although no induction by sucrose itself was observed (Fig. 8). In *A. thaliana*, *AtSUS3* could, consequently, be involved in the response to low moisture content through sustained sucrose cleavage activity in the late-maturing seed and increased glycolytic demand during dehydration. *AtSUS3* could also participate in sucrose and oligosaccharides synthesis and storage in the late-maturing seed (Baud *et al*., 2002), thus allowing acquisition of desiccation tolerance. Finally, as proposed by Kleines *et al*. (1999), transcript accumulation may well be very important for desiccation tolerance in resuming full physiological activity during rehydration, in germinating seeds.

This study adds new significant insights to current understanding of the AtSUS family. These results being focused on correlated changes in transcript profiles and *SUS* genes being well demonstrated instances of both post-transcriptional and post-translational regulations (McElfresh and Chourey, 1988; Chourey and Taliercio, 1994; Zeng *et al*., 1998), consequently, other methods of analysis should now be added to face the complexity of the characterization of the AtSUS functions.

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