

Expression of Sucrose Synthase Genes Involved in Enhanced Elongation of Pondweed (*Potamogeton distinctus*) Turions under Anoxia

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• **Background and Aims** Overwintering buds (turions) of the monocot aquatic pondweed species (*Potamogeton distinctus*) are highly tolerant to anoxic stress. Sucrose metabolism accompanied by enhanced activity of sucrose synthase (SuSy) operates actively during anaerobic elongation of pondweed turions. The aim of this study is to isolate SuSy genes from the turions and to investigate their transcriptional changes in response to anoxia and other stimuli.

• **Methods** SuSy genes were isolated from pondweed turions by PCR methods and transcript levels of SuSy genes were examined in response to anoxia, sugars and plant hormones. In addition, the effects of anoxia on SuSy activity were examined both in the soluble fraction and in the microsomal fraction.

• **Key Results** cDNAs of two SuSy genes (*PdSUS1* and *PdSUS2*) were cloned from pondweed turions. The levels of *PdSUS1* transcripts increased under anoxia but did not with sugar treatments. Anoxia-stimulated elongation of turions was further enhanced by 2,4-dichlorophenoxyacetic acid (2,4-D) and suppressed by treatments with sorbitol, 2-deoxyglucose (2-dGlc) and abscisic acid (ABA). The levels of *PdSUS1* transcripts were increased by 2,4-D and decreased by sorbitol under anoxia. The levels of *PdSUS2* transcripts were not significantly affected by anoxia and any other treatments. SuSy activity of turions under anoxia was enhanced in the soluble fraction, but not in the microsomal fraction.

• **Conclusions** Up-regulation of *PdSUS1* transcription under anoxia may not be attributed to sugar starvation under anoxia. A positive correlation between stem elongation and the level of *PdSUS1* transcripts was observed in turions treated with anoxic conditions, 2,4-D and sorbitol. The increase in SuSy activity in the cytosol may contribute to sugar metabolism and sustain stem elongation under anoxia.

Key words: Anaerobic elongation, anoxia, gene expression, plant hormone, pondweed, *Potamogeton distinctus*, sucrose, sucrose synthase.

INTRODUCTION

Plant anaerobiosis has been extensively studied, mostly in major crops with an emphasis on various aspects including energy metabolism, maintenance of homeostasis in cells, and the regulation of gene and protein expression (Gibbs and Greenway, 2003; Greenway and Gibbs, 2003). Selection of appropriate plant species and tissues as experimental materials that have notable characteristics such as high tolerance to lack of oxygen is one way to contribute to the development of this field. Many plants exposed to oxygen-deprived conditions cannot grow and will die within a few days. In contrast, *Potamogeton distinctus* (pondweed) can elongate its stem under anoxia (Ishizawa *et al.*, 1999). This anaerobic growth is supported by an active glycolytic pathway (Sato *et al.*, 2002). *Potamogeton pectinatus* has similar characteristics (Summers and Jackson, 1994). Its tubers remain sensitive to auxin and abscisic acid, although not to gibberellin, despite the absence of oxygen (Summers and Jackson, 1996).

Pondweed is a floating-leaved plant and forms turions (overwintering buds) in soil. Rapid starch degradation is accompanied with anoxic growth of turions (Sato *et al.*,

2002; Harada and Ishizawa, 2003). Changes in enzymatic activity of starch degradation, such as amylolytic activity, α -amylase activity and starch phosphorylase activity have not been detected during anoxic growth, but activities of certain isozymes of amylases and starch phosphorylase are induced under anoxia in *P. distinctus* (Harada and Ishizawa, 2003). The incorporation into sucrose of ¹⁴C-glucose fed exogenously to turions was enhanced under anoxia, and it was concluded that more sucrose is synthesized under anoxia than is synthesized in air (Sato *et al.*, 2002). The content of sucrose in the stems of turions decreases rapidly within 1 d of anoxia, but remains constant thereafter. These results indicate that turnover of sucrose is activated by anoxia. The activities of invertase, sucrose phosphate-synthase and sucrose synthase have been shown to increase under anoxia, the increase in the activity of sucrose synthase (SuSy; EC 2.4.1.13) being especially notable (Harada and Ishizawa, 2003). From these results, it was proposed that sucrose metabolism following rapid starch degradation plays an important role in energy production and growth of pondweed turions under anoxia.

SuSy can catalyse both synthesis and degradation of sucrose (Giegenberger and Stitt, 1993), but the degradation dominates *in vivo*. SuSy in the cytosol is thought to supply UDP-glucose and fructose produced by sucrose cleavage for

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glycolysis, and possibly starch synthesis. In addition, SuSy associated with the plasma membrane is considered to contribute to growth by supplying sugars for cellulose synthesis (Amor *et al.*, 1995; Carlson and Chourey, 1996; Winter *et al.*, 1997; Haigler *et al.*, 2001). On the other hand, SuSy is one of the anaerobic proteins (ANPs) that are expressed in maize roots under anaerobic conditions (Sachs *et al.*, 1980; Sachs *et al.*, 1996). Two enzymes, invertase and SuSy, degrade sucrose in plants and the action of SuSy may be more energy-efficient by requiring less ATP. Accordingly, SuSy activation is thought to be a metabolic adaptation to anaerobic conditions (Guglielminetti *et al.*, 1995; Bologna *et al.*, 2003). Regulation of sucrose metabolism under anoxia has been studied in rice, which is the most tolerant among monocotyledonous species in major crops. Starch degradation and sucrose cleavage occur in anoxic rice and SuSy is mainly involved in sucrose degradation (Guglielminetti *et al.*, 1995). Sucrose synthesis mediated by sucrose-phosphate synthase is particularly active in rice seedlings under anoxia, but not in barley, an anoxia-intolerant species (Guglielminetti *et al.*, 1999). In the case of *P. pectinatus*, sucrose accumulates in the stem, which is the major elongating tissue under anoxia (Summers *et al.*, 2000).

In the present study, two different SuSy cDNAs were isolated from pondweed turions in order to explore roles of SuSy in sucrose metabolism and stem elongation under anoxia. Their gene expression was examined in response to various kinds of stimuli including not only anaerobic conditions, but also sugars and plant hormones. It was found that the transcripts of one gene encoding SuSy increased during anaerobic growth in pondweed turions.

MATERIALS AND METHODS

Incubation of pondweed turions and growth analysis

Turions of pondweed were collected in a greenhouse of our laboratory and stored at 4 °C. Stem segments, 15 mm in length, were prepared by cutting off the base and the apex from turions with a razor blade after stripping off scaly leaves. Intact turions and stem segments were placed on moist filter paper and pre-incubated at 25 °C in the dark for 1 d. Then, these segments or intact turions were stood in a small vial (10 mm in diameter) containing 500 µL of incubation medium. Distilled water was the usual incubation medium, but for treatments with sugars, sorbitol, 2,4-dichlorophenoxyacetic acid (2,4-D), abscisic acid (ABA) and 2-deoxyglucose (2-dGlc), a 10 mM MES buffer (pH 7.5) was used as the incubation medium. Twenty-five µg of carbenicillin and 250 µg of vancomycin were added to the incubation medium in the case of sugar treatments to inhibit microbial growth. The vials were placed in a 100 mL glass vessel that was connected to a continuous supply of water-saturated air or pure N₂ gas (Ishizawa *et al.*, 1999). After incubation in aerobic or anaerobic conditions at 25 °C in the dark, stem segments were plunged into liquid nitrogen for extraction of RNA immediately after length measurement.

RNA isolation

Frozen turion segments (100 mg) were ground with liquid nitrogen in a pestle and mortar. The frozen powder was

thoroughly mixed with 500 µL of extraction buffer containing 1 % SDS, 50 mM Tris/HCl (pH 7.5), 50 mM EDTA and 500 µL of water-saturated phenol. The mixture was centrifuged at 1300 g at 4 °C for 15 min. The supernatant was thoroughly mixed with 250 µL of water-saturated phenol and 250 µL of chloroform. The mixture was then centrifuged at 1300 g at 4 °C for 10 min, the supernatant was thoroughly mixed with the same amount of chloroform and, after centrifugation at 1300 g at 4 °C for 10 min, the supernatant pH was adjusted to below 6.0. RNA was then precipitated with isopropanol at –20 °C overnight. Following centrifugation at 7000 g at 4 °C for 15 min, the RNA was dried with 70 % ethanol and solubilized in 50 µL of water. RNA was further purified by precipitation with 2 M LiCl at 4 °C for 2 h, collected by centrifugation at 7000 g at 4 °C for 15 min, dried with 70 % ethanol, and solubilized in 30 µL of water. An amount of total RNA was determined by absorbance at 260 nm.

Subcloning and sequencing of cDNAs

For 3'RACE, first strand cDNAs were synthesized from total RNA isolated from stem segments incubated in various conditions with Oligo (dT)₂₀-M4 Adaptor primer (Takara, Shiga, Japan) and SuperScript II (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then RT-PCR was conducted with an oligonucleotide primer (5'-CCC AAG TTC AAC ATY GTS TCT CCT GG-3'), which was designed from a highly conserved region of nucleotide sequences of plant SuSy genes, M13 primer M4 (Takara) and Taq DNA Polymerase (Fermentas, Vilnius, Lithuania). Amplified DNA fragments were ligated into pGEM-T Easy Vectors (Promega, Madison, WI, USA) according to the manufacturer's instructions. Plasmids were introduced to competent cells by heat shock of 42 °C for 50 s. After checking insertion of gene fragments to plasmids by PCR, the plasmids for determination of nucleotide sequences were extracted by the alkali-SDS method. For 5'RACE, SMART™ Oligo cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) was used. First-strand cDNAs were synthesized with SMART II A Oligonucleotide, 5'RACE cDNA Synthesis Primer and Power Script Reverse Transcriptase (Clontech). Then RT-PCR was conducted with gene-specific primers (5'-CAG CTC AGC CTG TTC TTC-3' for *PdSUS1* and 5'-GGA GTG CAG TAA GTC TC-3' for *PdSUS2*) designed from the partial nucleotide sequences of the genes, Universal Primer A Mix, and Advantage 2 Polymerase (Clontech). Amplified fragments were subcloned by the same methods as 3'RACE. Nucleotide sequences of DNA were determined by the dideoxynucleotide chain termination method using DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Little Chalfont, UK). Sequence analysis was performed with 373A DNA Sequencing System (Applied Biosystems, Foster City, CA, USA), GENETIX-Mac Ver 8.0. and ClustalW.

RT-PCR analysis

First-strand cDNAs were synthesized from RNA isolated from stem segments with SuperScript II or SuperScript III

(Invitrogen). The synthesized cDNAs were diluted 10-fold and then used for RT-PCR. The PCR mixture contained 75 mM Tris/HCl buffer with 20 mM $(\text{NH}_4)_2\text{SO}_4$, 200 mM dNTP, 250 μM MgCl_2 , 200 nM each two different primers, 5'-GCA GTT CGT GCT TCT AGT GAA CGA TCG-3', which was located in the beginning of 3'-UTR region in the predicted mRNA, and 5'-GTC GGT GCT TCT TTA TTC ACT TGC CC-3', which correspond to the complementary sequence of the ending part in the 3'-UTR, for *PdSUS1* or 5'-GGT GCT AAT GGT GCA GGC CAT CAC TG-3', which was located at the end of the ORF, and 5'-CAC GGA AAT TTG GCA TAT CGG CC-3', which corresponds to the complementary sequence of the ending part in the 3'-UTR, for *PdSUS2*, 0.2 units of Taq DNA Polymerase (Fermentas) and 2 μL of diluted first strand cDNA in a final volume of 10 μL . PCR was performed for various cycles to determine a logarithmic phase of DNA amplification. The most suitable reaction for PCR analysis was selected as pre-heating at 94 °C for 4 min and then 21 cycles of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 2 min. The expression of 26S rRNA gene was used as an internal control to determine the efficiency of RT-PCR among different samples. Eight μL of each PCR product were used for 1 % agarose gel electrophoresis analysis. DNA was stained with 0.5 $\mu\text{g mL}^{-1}$ ethidium bromide solution.

Northern blot analysis

Ten μg of total RNA was analysed by electrophoresis in 1.0 % agarose gel containing 2.2 M formaldehyde and 20 mM MOPS (pH 7.0), 8 mM sodium acetate and 1 mM EDTA (pH 8.0). The gel was blotted onto Nylon Membranes, Positively Charged (Roche, Basel, Switzerland). The digoxigenin (DIG)-labelled RNA probe was made from the 3'-UTR of *PdSUS1* using the same primer set as used for RT-PCR according to the manufacturer's instructions (Roche). The membrane was hybridized with the probe at 68 °C overnight. The membrane was washed twice with a solution containing 2 \times SSC and 0.1 % SDS for 5 min at room temperature and then twice with a solution containing 0.1 \times SSC and 0.1 % SDS for 15 min at 68 °C. The DIG Luminescent Detection Kit (Roche) was used for signal detection.

Enzyme extraction and activity measurement

After incubation under various conditions, intact turions were ground in an ice-cold mortar and pestle with extraction buffer containing 100 mM Tris/HCl (pH 7.5), 1 mM EDTA, 10 mM dithiothreitol, 150 mM NaCl, and 10 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 7000 *g* at 4 °C for 20 min to remove debris. The supernatant was centrifuged at 95 000 *g* at 4 °C for 1 h. The microsomal fraction was resuspended in a small amount of the extraction buffer. The soluble fraction and microsomal fraction were dialysed twice against dialysation buffer containing 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, and 10 mM β -mercaptoethanol for 2 h for each. Activity of SuSy in the soluble fraction and microsomal fraction was determined by the same method as described by Harada and Ishizawa (2003). Protein contents were measured by BCA reagent (Pierce, Rockford, IL, USA).

RESULTS

Isolation of two distinct SuSy cDNAs from pondweed

Two different cDNA fragments of SuSy were isolated from pondweed tissues. One was from etiolated stem segments incubated under anoxia in the dark and the other was from green leaves of turions grown under aerobic condition in the light. cDNA from the former tissues was 2842 bp long and consisted of a 5'-untranslated region (UTR) of 108 bp, an open reading frame (ORF) of 2442 bp and a 3'-UTR of 292 bp. cDNA from the latter tissues was 2771 bp long and consisted of a 5'-UTR of 22 bp, an ORF of 2526 bp and a 3'-UTR of 223 bp. Figure 1 shows the amino acid sequences predicted from those two pondweed SuSy genes (*PdSUS1* and *PdSUS2*). The nucleotide sequences revealed that *PdSUS1* and *PdSUS2* encode ORFs of 814 and 842 amino acid residues, respectively, with predicted molecular masses of 92.1 and 95.0 kDa, respectively. The amino acid sequences of *PdSUS1* showed 74.5 % identity to those of *PdSUS2*. Both the amino acid sequences showed from 70–82 % identity with plant SuSys reported by others. *PdSUS1* and *PdSUS2* had a serine (Ser) residue in the N-terminal region (Ser 11). The Ser residue in the N-terminal region corresponding to Ser 11 of *PdSUS1* and *PdSUS2* is conserved in most SuSys from various species of plants (Winter and Huber, 2000) and is reported to be phosphorylated in maize (Ser 15, Huber *et al.*, 1996). *PdSUS1* and *PdSUS2* consisted of two common parts, the characteristic N-terminal extension of sucrose synthase and glycosyl transferases group 1 domain. *PdSUS2* had an extended alanine-rich region in the C-terminal region.

Figure 2 shows a phylogenetic tree generated with amino acid sequences of SuSys from various plants, *PdSUS1* and *PdSUS2*. They are divided into four groups, the dicot SUS1 group, the dicot SUSA group, the monocot SUS group and the new group according to classification by Komatsu *et al.* (2002). Recently, a new SuSy gene named *ZmSUS3* was found in maize (Carlson *et al.*, 2002) and it belongs to the dicot SUSA group. Until now, three SuSy genes have been reported in rice (Wang *et al.*, 1992; Huang *et al.*, 1996). The databases of the TIGR Rice Genome Project (<http://www.tigr.org/tdb/e2k1/osa1/>) and the Rice Genome Annotation Database (<http://ricegaas.dna.affrc.go.jp>) were searched to identify SuSy genes in the rice genomes. Three new SuSy genes were found and named *OsSUS4*, *OsSUS5* and *OsSUS6*. *OsSUS4* is very similar to *ZmSUS3* and the remaining pair (*OsSUS5* and *OsSUS6*) are placed in the new group, including *AtSUS5* and *AtSUS6*. *PdSUS1* and *PdSUS2* were placed in the monocot SUS group. *PdSUS1* most resembled *TgSUS2* from tulip (75.5 % identity, Balk and Boer, 1999) and *PdSUS2* had highest identity to *ZmSUS1* from maize (82.5 % identity, Huang *et al.*, 1994). SuSys from Poaceae are apparently divided into two groups, one containing *ZmSH1*, the other including *ZmSUS1*. *PdSUS2* was closer to the latter group than *PdSUS1*. However, more information is required about amino acid sequences of SuSy genes in monocotyledonous plants. Moreover, comparison between structures of exons and introns of their genes (Komatsu *et al.*, 2002) is necessary for phylogenetic analysis of *PdSUS1* and *PdSUS2*.

<i>PdSUS1</i>	1:	MATRSLTRVLSMTTERIGDSLGAYPEDLSTLFTKYIEHGKGM LQRHEILAEFEALTKGGDK	60
<i>PdSUS2</i>	1:	MPERKRLARLHSIKEQLSDSLAAHPNELLAIFSRFVHHGKGM LQPHELLAEFEAVIPDGDK	60
<i>PdSUS1</i>	61:	DHLKNGAFGEVLMAAQEAIVLPPMVA M TVRPRPGVWGYIRVHVNDLSVESMNVTDY LKFK	120
<i>PdSUS2</i>	61:	EKL RDGAI GEL LKAAQEAIVLPPWVALA I RPRPGVWEYIRVNVNELAVENMTIPEYLQFK	120
<i>PdSUS1</i>	121:	EQLI-DGCD SNFTLELDFEPFNASFP RPTLSKSIGNGVEFLNKHLS SKLFDKDSIFPLV	179
<i>PdSUS2</i>	121:	EELVGE GIDNNFVLELDFEPFNVSFP RPTLSKSIGNGVQFLNRHLS SKLFDKESMYPLL	180
<i>PdSUS1</i>	180:	DFLRNHNYKGT SIMLNVKIQSVSALESALRKAEEHLLS I PLDTPYSDFDAKFRDLGLEKG	239
<i>PdSUS2</i>	181:	NCLRQHNYNGM TMMLNDRIRSLDALQAALRKAEEHLLLF PGDTPSSEFDHRFQELGLEKG	240
<i>PdSUS1</i>	240:	WGNTAKRVL ESIHLLLDLLEAPDPCTFEKFLS IIPMVFN VVILA PHGYFAQANVLGYPDT	299
<i>PdSUS2</i>	241:	WGDCAQRVH ENIHLLLDLLEAPDPCTLEKFLGT IIPMVFN VVILS PHGYFAQDNVLGYPDT	300
<i>PdSUS1</i>	300:	GGQVVYILDQVRALENEMLLRIKQQGLD IVPKILVVTRLLPDAVGTTC CELVEPVEGTVH	359
<i>PdSUS2</i>	301:	GGQVVYILDQVRALENEMLLRIQQGLD ITPKILIVTRLLPDAVGTTC GQRLRKFLGTEH	360
<i>PdSUS1</i>	360:	TNII RIPFRTEEGILRKWIS RFDVWPYLETYAEDCIKEVTKQLQAKPDLIIIGNYSDGNLV	419
<i>PdSUS2</i>	361:	THILRV PFRTEENGILRKWIS RFDVWPYLETYTEDVANEIAGALQAKPDLIIIGNYSDGNLV	420
<i>PdSUS1</i>	420:	ASLMAHKL E V TQCTIAHALEKTKY P NSDLYWKKLDDHYHFSCQFTADL LAMNHADFIITS	479
<i>PdSUS2</i>	421:	ATLLAHKL G V TQCTIAHALEKTKY P NSDLYWKEKESHYHFSCQFTADL I AMNHADFIITS	480
<i>PdSUS1</i>	480:	TYQEIAGSKNTV GQYESHIAFTMPGLYRVVHGINVFDPKFNIVSPGADM DIYFPYTEKEK	539
<i>PdSUS2</i>	481:	TFQEIAGSKD TVGQYETHAFTMPGLYRVVHGINVFDPKFNIVSPGADM SIFSPYTE DSE	540
<i>PdSUS1</i>	540:	RLVHLHPEIEELLYSQVDNTEHKFALADKTKPIIFSMARLDRVKNL TGLVELYGKNARLR	599
<i>PdSUS2</i>	541:	RLTALHPEIEELLF SQVENADHKFVLNDRNKPIIFSMARLDRVKNL TGLVELYGKNQRLR	600
<i>PdSUS1</i>	600:	ELANLVIVCGDHGKESKDKEEQAE LKMFSLIEEHLKLNQIRWISAQMDRVRNGELYRVI	659
<i>PdSUS2</i>	601:	ELVNLVIVCGDHGKESKDKEEQAE LA KMYNLIETHNLNQIRWISAQMNRVRNGELYRYI	660
<i>PdSUS1</i>	660:	ADSGGVFVQPAFYGAFGLTVVESMTCGLPTFATVHGGPGEIIVNGVSGFHIDPYLGDKVS	719
<i>PdSUS2</i>	661:	CDAKGA FVQPAFYEA FGLTVVEAMTCGLPTFATVHGGPGEIIVDGVSGFHIDPYHGDKVS	720
<i>PdSUS1</i>	720:	EILVNF FEKSKADP SHWKAISQGLKRIYEKYTWKLYSERLLT LTTIYGFWKHVSNLERR	779
<i>PdSUS2</i>	721:	EILLVNF FEKCKVDPT HWVNISQGLKRIYEKYTWKLYSERLMTL SGVYGFWKYVSNLDRR	780
<i>PdSUS1</i>	780:	ETRRYLEM FYGLKYRPLADKVPLAVDAKPTTNGSA-----	814
<i>PdSUS2</i>	781:	ETRRYLEM FYALKYRNLA KSVPLAVDGA PALNGSKAAAVNGAKAALNGAEAALNGANGAG	840
<i>PdSUS1</i>	815:		
<i>PdSUS2</i>	841:	HH	842

FIG. 1. Predicted amino acid sequences of *PdSUS1* and *PdSUS2*. The alignment was made using GENETIX-MAC Ver. 8.0. Identical amino acids are shaded and gaps are indicated by dashes. An arrowhead shows a conserved serine residue (see text). The characteristic N-terminal extension of sucrose synthase (thin underline) and a glycosyl transferases group 1 domain (broken underline) were identified by NCBI Conserved Domain Search. *PdSUS2* has an alanine-rich region at the C-terminal region (bold underline). A primer for 3' RACE of SuSy genes was designed from nucleotides encoding amino acid residues, indicated by an arrow.

Levels of *PdSUS1* and *PdSUS2* transcripts under anoxia

Levels of *PdSUS1* and *PdSUS2* transcripts in stem segments incubated in air or under anoxia were examined by RT-PCR (Fig. 3). Transcripts of *PdSUS1* remained at a low level during a 3-d incubation in air, whereas it increased 1 d after anoxic incubation and then remained at the same

increased level by 3 d. Enzymatic activity of SuSy in pondweed turions was not enhanced until the third day after the start of anoxic incubation (Harada and Ishizawa, 2003). These results indicate that enhancement of *PdSUS1* expression under anoxia precedes the increase in SuSy enzyme activity. In contrast to *PdSUS1*, the transcript levels of *PdSUS2* changed little during incubation either in air

or under anoxia. Thus, unlike *PdSUS1*, *PdSUS2* is not up-regulated by anoxia.

Effects of exogenous sugars on stem elongation and levels of PdSUS1 and PdSUS2 transcripts

In many plant tissues, the expression of SuSy genes is responsive to sugars, especially glucose and sucrose (Koch *et al.*, 1992; Koch, 1996). We examined whether exogenous glucose and sucrose affect the elongation of turion segments (Fig. 4A) and transcript levels of *PdSUS1* and *PdSUS2* (Fig. 4B, C). Neither glucose nor sucrose at 50 mM or 100 mM significantly affected the elongation both in air and under anoxia (Fig. 4A). Figure 4B shows effects of glucose and sucrose on the levels of *PdSUS1* and *PdSUS2* transcripts analysed by RT-PCR. Anoxia increased the levels of *PdSUS1* transcripts, but neither glucose nor sucrose had clear effects on it. The level of *PdSUS2* transcripts was not much affected by anoxia, or glucose or sucrose. The conclusion was that glucose and sucrose had little effect on the level of *PdSUS1* and *PdSUS2* transcripts in air and under anoxia. Stem segments were also treated with sorbitol to examine effects of osmotic stress. Sorbitol at 100 mM did not affect elongation, but sorbitol at 300 mM strongly inhibited the elongation both in air and under anoxia (Fig. 4A). Sorbitol at 300 mM also showed an inhibitory effect on the level of *PdSUS1* transcripts under anoxia, but it did not have clear effects on the level of *PdSUS2* transcripts (Fig. 4B). On the other hand, 2-deoxyglucose (2-dGlc), a glucose analogue, slightly stimulated the elongation in air but significantly suppressed the elongation under anoxia (Fig. 4A). 2-dGlc increased the level of *PdSUS1* transcripts remarkably in air but not under anoxia (Fig. 4B). 2-dGlc had little effect on the level of *PdSUS2* transcripts although 2-dGlc seemed to slightly increase the *PdSUS2* transcripts in air.

We also examined the effects of anoxia and 300 mM sorbitol on the level of *PdSUS1* transcripts by Northern blot analysis (Fig. 4C). An intense signal for *PdSUS1* was detected after incubation in anoxia for 3 d, but not after the same period in air. Three hundred mM sorbitol depressed the increased level of *PdSUS1* transcripts under anoxia.

Effects of 2,4-D and ABA on stem elongation and levels of PdSUS1 and PdSUS2 transcripts

We examined whether plant hormones, which influence elongation of pondweed stem segments, also affect the expression of SuSy genes. 2,4-D at 1 and 10 μ M stimulated stem elongation in air, but only 2,4-D at 10 μ M stimulated elongation in the absence of oxygen (Fig. 5A). 2,4-D also slightly enhanced the level of *PdSUS1* transcripts under anoxia when the transcriptional levels were examined by RT-PCR (Fig. 5B). Northern blot analysis also showed the promotion of the level of *PdSUS1* transcripts by 2,4-D under anoxia (Fig. 5C). The level of *PdSUS2* transcripts was not affected by 2,4-D both in air and under anoxia (Fig. 5B). On the other hand, ABA significantly inhibited the elongation under anoxia (Fig. 5A) and slightly decreased the level of *PdSUS1* transcripts under anoxia (Fig. 5B). The amount of *PdSUS2* transcripts was not much affected by ABA

(Fig. 5B). Then, the effects of ABA on the transcription of *PdSUS1* were examined by Northern blot analysis, but none were apparent (Fig. 5C).

Localization of SuSy activity in cell fractions in turions incubated under anoxia

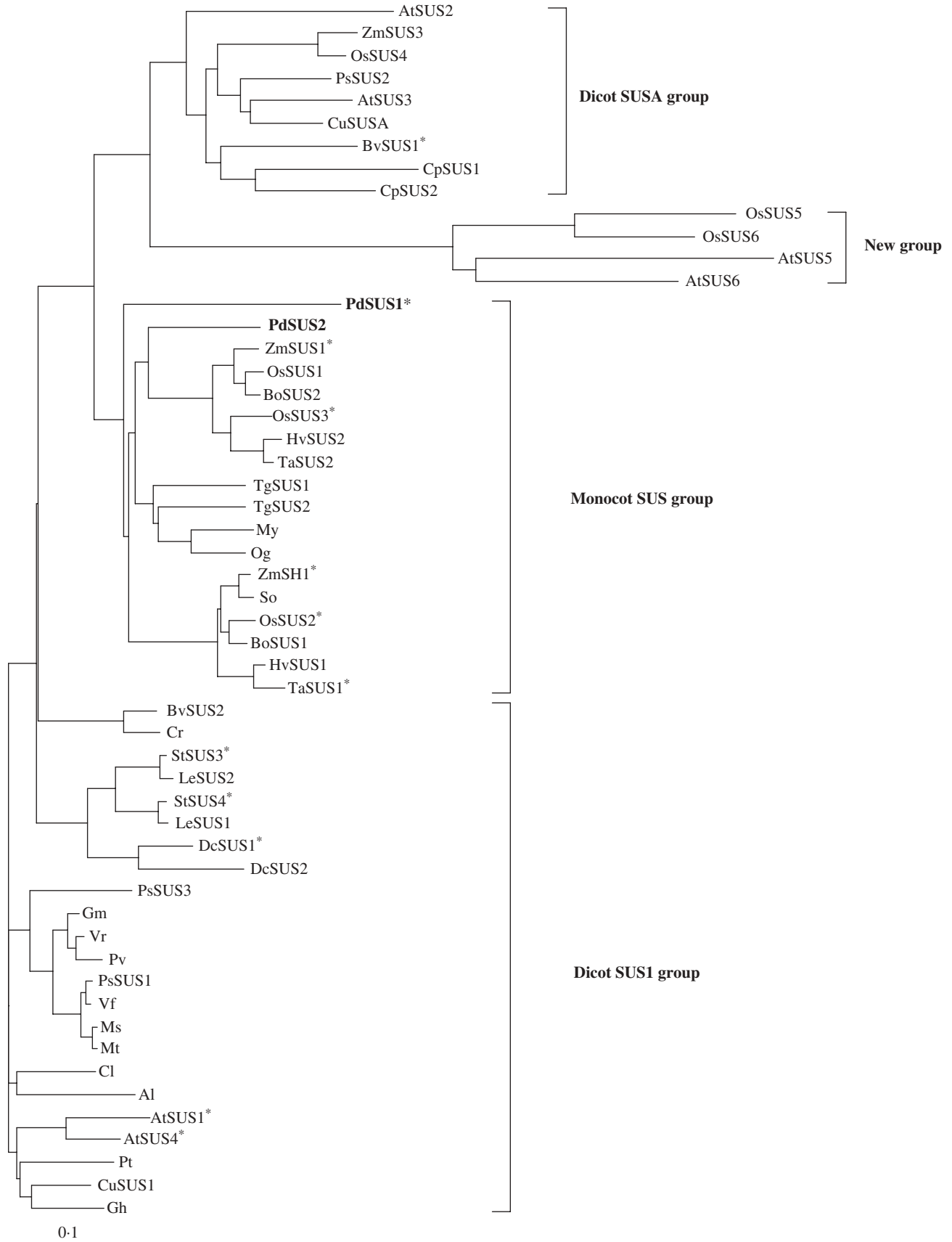
SuSy can be active in the cytosol or in a membrane-bound form. The plasma membrane-bound form has been linked to cellulose synthesis in numerous species and organs (Amor *et al.*, 1995; Carlson and Chourey, 1996; Winter *et al.*, 1997; Haigler *et al.*, 2001). We examined whether soluble SuSy and membrane-bound SuSy were differentially affected by anoxia (Fig. 6). Total activity of SuSy extracted from turions under anoxia was significantly higher than that in air. SuSy activity in the soluble fraction was twice as high under anoxia as in air. However, there was no significant difference between SuSy activity in the microsomal fraction separated from pondweed turions in air and that under anoxia. These results suggest that enhancement of SuSy activity under anoxia is due to activation of SuSy in the cytosol.

DISCUSSION

We isolated two cDNAs encoding SuSy from pondweed turions (Fig. 1). In many cases, two or three SuSy genes have been isolated from one plant species. But six SuSy genes were recently found in the *Arabidopsis thaliana* genome (Komatsu *et al.*, 2002; Baud *et al.*, 2004). In the process of this study, three new genes were found from the rice genome database in addition to the three already reported (Wang *et al.*, 1992; Huang *et al.*, 1996). These newly discovered rice genes are unique in that they locate remotely from other rice SuSy genes in the phylogenetic tree (Fig. 2). These findings suggest a wider divergence of SuSy genes in mono- and dicotyledonous plants than was hitherto recognized. Expression of SuSy genes has been reported to be enhanced under hypoxic or anoxic conditions in several plant species including rice (Ricard *et al.*, 1991; Guglielminetti *et al.*, 1995), maize (Springer *et al.*, 1986; Sachs *et al.*, 1996), *A. thaliana* (Martin *et al.*, 1993; Baud *et al.*, 2004) and potato (Bologa *et al.*, 2003), which differ in their tolerance to anoxia. Such genes are identified by asterisks in Fig. 2 and appear scattered in the phylogenetic tree. Thus, phylogenetic relationships based on amino acid sequences are not important so far as which SuSy gene is responsive to hypoxic or anoxic stress.

Only *PdSUS1* transcripts increased under anoxia (Fig. 3) and the increase was detected 1 d after anoxic incubation, preceding a rise in activity of SuSy by 2 d (Harada and Ishizawa, 2003), suggesting that the increase in *PdSUS1* transcripts contributes to the increase in the activity of SuSy. Since only two genes of SuSy were isolated from pondweed turions in this study, a possibility of involvement of other SuSy genes is not excluded in the enhancement of SuSy activity by oxygen exclusion.

There are reports concerning the mechanism of transcriptional regulation in SuSy gene expression. *Arabidopsis ADH1* encoding alcohol dehydrogenase has anaerobic



responsive elements (ARE) in its promoter region (Hoeren *et al.*, 1998). The induction of *ADH1* under hypoxic conditions is triggered by binding of a transcription factor, AtMYB2, to the ARE. A similar mechanism operates in the induction of *ASUS1* encoding an arabidopsis SuSy (Hoeren *et al.*, 1998). On the other hand, SuSy genes and other genes encoding enzymes that are involved in carbohydrate metabolism are reported to be sugar-responsive (Koch *et al.*, 1992; Koch, 1996). For example, the induction of rice α -amylase in germinating rice occurs in response to sugar starvation brought about under anoxic conditions (Loreti *et al.*, 2003). The contents of soluble sugars and sucrose in pondweed turions rapidly decrease under anoxic conditions (Sato *et al.*, 2002; Harada and Ishizawa, 2003). Therefore, sugar starvation could be a trigger to increase the level of *PdSUS1* transcripts under anoxic conditions. However, Fig. 4 shows that glucose and sucrose have no repressing effect on the level of *PdSUS1* transcripts, which increases under anoxic conditions regardless of sugar additions. These results suggest that sugar starvation is not involved in the regulation of the *PdSUS1* gene under anoxic conditions. Further study is necessary to explore the mechanisms regulating the expression of the *PdSUS1* gene and how anoxia is sensed in pondweed turions.

SuSy is believed to play an important role in cell growth by providing UDP-glucose and fructose through sucrose cleavage for energy metabolism and for cell wall biosynthesis. The correlations between stem elongation of pondweed turions and the levels of SuSy transcripts were examined (Figs 4 and 5). Anoxic conditions enhanced shoot elongation and also increased the level of *PdSUS1* transcripts. Ten μ M of 2,4-D also enhanced both of them under anoxia. Three hundred mM sorbitol inhibited not only the shoot elongation but also the level of *PdSUS1* transcripts under anoxic conditions. 2-dGlc stimulated stem elongation in air and enhanced the level of *PdSUS1* transcripts. These cases showed a positive correlation between the level of *PdSUS1* transcripts and shoot elongation. However, such a positive correlation between them was invalid in two cases. ABA significantly inhibited the anoxic elongation but it did not have clear effects on the

level of *PdSUS1* transcripts. In the case of 2-dGlc, stem elongation was severely restricted under anoxia but the level of *PdSUS1* transcripts under anoxia was not affected. One possible explanation regarding the two results is that breakdown of the *PdSUS1* transcripts was inhibited by ABA and 2-dGlc, and it resulted in hiding the inhibitory effects on the transcriptional level of the *PdSUS1*. Alternatively, these results may reflect a different contribution of SuSy to the regulation of stem elongation. At present, it is difficult to explain the reason why 2-dGlc stimulates shoot elongation in air but inhibits it under anoxia and how 2-dGlc increases the level of *PdSUS1* transcripts. 2-dGlc may disturb carbohydrate metabolism as an analogue of glucose (Herold and Lewis, 1977) or act as an elicitor of sugar-sensing through hexokinase (Jang *et al.*, 1997). Use of sugar analogues such as 2-dGlc as an experimental tool may be useful to investigate roles of SuSys in the control of plant growth.

SuSys in plant cells exist in the cytosol in soluble form and in association with the plasma membrane, Golgi body, tonoplast and actin filaments as a bound form (Koch, 2004). Figure 6 shows that the activity of SuSy in the soluble fraction is enhanced under anoxic conditions. SuSy in the cytosol serves to supply sugars to glycolysis under O₂-deprived conditions (Guglielminetti *et al.*, 1995). There is a strong possibility that sucrose degradation mediated by SuSy consumes less ATP than degradation by invertase (Bologa *et al.*, 2003). These workers showed that O₂-deprived conditions enhance SuSy activity in the cytosol of potato tubers but inhibit one associated with the plasma membrane. Bologa *et al.* (2003) suggest that an energy crisis under O₂-deprived conditions is diminished by activating the SuSy pathway in glycolysis and by reducing the activity of membrane-bound SuSy engaging in biosynthesis demanding high energy costs. Ricard *et al.* (1998) reported that the expression of SuSy is essential for tolerance to anaerobic stress in maize, a moderately tolerant species. In pondweed turions under anoxic conditions, adenylate energy charge remains as high as in air (Ishizawa *et al.*, 1999). It is likely that SuSy in the cytosol serves to maintain high energy status by supplying the necessary amounts of substrates to glycolysis activated in pondweed turions under

FIG. 2. Phylogenetic tree of plant SuSy proteins. Predicted amino acid sequences were aligned using Clustal W. SuSys from *Arabidopsis* were named according to Baud *et al.* (2004). EMBL accession numbers (AGI ID for SuSys from *Arabidopsis thaliana* and gene number assigned by RiceGAAS for OsSUS4, OsSUS5 and OsSUS6) are given in parentheses. Al (X92378) from *Alnus glutinosa*; AtSUS1 (At5g20830), AtSUS2 (At5g49190), AtSUS3 (At4g02280), AtSUS4 (At3g43190), AtSUS5 (At5g37180) and AtSUS6 (At1g73370) from *Arabidopsis thaliana*; BoSUS1 (AF412039) and BoSUS2 (AF412037) from *Bambusa oldhamii*; BvSUS1 (X81974) and BvSUS2 (AY457173) from *Beta vulgaris*; Cl (AB018561) from *Citrullus lanatus*; CpSUS1 (AJ131999) and CpSUS2 (AJ132000) from *Craterostigma plantagineum*; Cr (X82504) from *Chenopodium rubrum*; CuSUS1 (AB022092) and CuSUS2 (AB022091) from *Citrus unshiu*; DcSUS1 (X75332) and DcSUS2 (Y16091) from *Daucus carota*; Gh (U73588) from *Gossypium hirsutum*; Gm (AF030231) from *Glycine max*; HvsUS1 (X65871) and HvsUS2 (X69931) from *Hordeum vulgare*; LeSUS1 (L19762) and LeSUS2 (AJ011319) from *Lycopersicon esculentum*; Ms (AF049487) from *Medicago sativa*; Mt (AJ131943) from *Medicago truncatula*; My (AF530568) from *Mokara 'Yellow'*; Og (AF530567) from *Oncidium 'goldiana'*; OsSUS1 (X59046), OsSUS2 (X64770), OsSUS3 (L03366), OsSUS4 (OSJNBb0050D18_1-01), OsSUS5 (OSJNBb0026I12-04) and OsSUS6 (OJ1149_C12-08) from *Oryza sativa*; PdSUS1 (AB193515) and PdSUS2 (AB193516) from *Potamogeton distinctus* (this study); Pt (AY341026) from *Populus tremuloides*; Pv (AF315375) from *Phaseolus vulgaris*; PsSUS1 (AJ012080), PsSUS2 (AJ001071) and PsSUS3 (AJ311496) from *Pisum sativum*; So (AY118266) from *Saccharum officinarum*; StSUS3 (U24088) and StSUS4 (U24087) from *Solanum tuberosum*; TaSUS1 (AJ001117) and TaSUS2 (AJ000153) from *Triticum aestivum*; TgSUS1 (X96938) and TgSUS2 (X96939) from *Tulipa gesneriana*; Vf (X69773) from *Vicia faba*; Vr (D10266) from *Vigna radiata*; ZmSH1 (X02400), ZmSUS1 (L22296) and ZmSUS3 (AY124703) from *Zea mays*. SuSys that are reported to be enhanced in gene or protein expression at low oxygen concentrations are indicated by asterisks according to Baud *et al.* (2004), except for OsSUS2 and OsSUS3 in Huang *et al.* (1996) and StSUS3 and StSUS4 in Bologa *et al.* (2003).

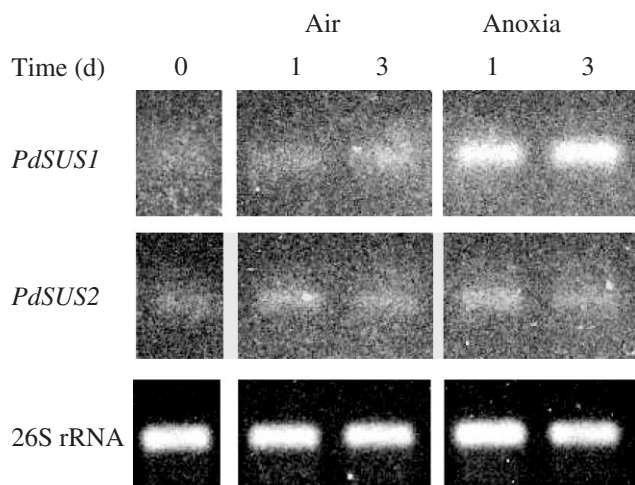


FIG. 3. Effects of anoxia on the levels of *PdSUS1* and *PdSUS2* transcripts. RNA was extracted from turion segments incubated in air or under anoxia at 25°C in the dark for 0d, 1d and 3d. The amount of transcript was determined by RT-PCR. The level of 26S rRNA transcript was used as an internal control. A typical example in three independent experiments is shown.

anoxia with the minimum of ATP consumption. Much starch is stored in the amyloplasts of pondweed turion cells and it is the prime energy source for growth without oxygen. Enhancement of sucrose synthesis and degradation is accompanied by starch degradation in the cells (Sato *et al.*, 2002; Harada and Ishizawa, 2003). We propose that sucrose cleavage mediated by SuSy is an important step regulating sugar metabolism of pondweed turion cells under anoxic conditions.

SuSy associated with the plasma membrane is believed to supply substrates for cell wall formation (Haigler *et al.*, 2001). Callose formation in anoxic maize root tips is accompanied by increases in the activity of SuSy associated with the cell membrane (Subbaiah and Sachs, 2001). The induction of callose formation leads to rapid cell death of root tips, which is closely related to the tolerance of whole maize seedlings to anoxia. Moreover, hypoxia activates not only the activity of SuSy but also cellulose synthesis in wheat roots (Albrecht and Mustroph, 2003). The unexpected increase in cellulose synthesis under hypoxia is understood to enhance the mechanical strength of roots forming aerenchyma. However, no significant increases were found in the activity of SuSy in the microsomal fraction extracted from pondweed turions under anoxia, the activity remaining similar to that in air (Fig. 6). Rapid elongation of pondweed turion cells under anoxic conditions is presumably accompanied by cell wall biosynthesis. Substrates for cellulose biosynthesis are thought to be supplied from the products of sucrose cleavage mediated by SuSy associated with the plasma membrane. We cannot completely exclude a possibility that SuSy associated with the plasma membrane is activated in pondweed turion cells under anoxic conditions. A membrane-associated SuSy in maize roots (SH1) and its activity are enhanced under anoxic conditions (Subbaiah and Sachs, 2001) and its localization is regulated by phosphorylation, and thus it can exchange between the cytosol

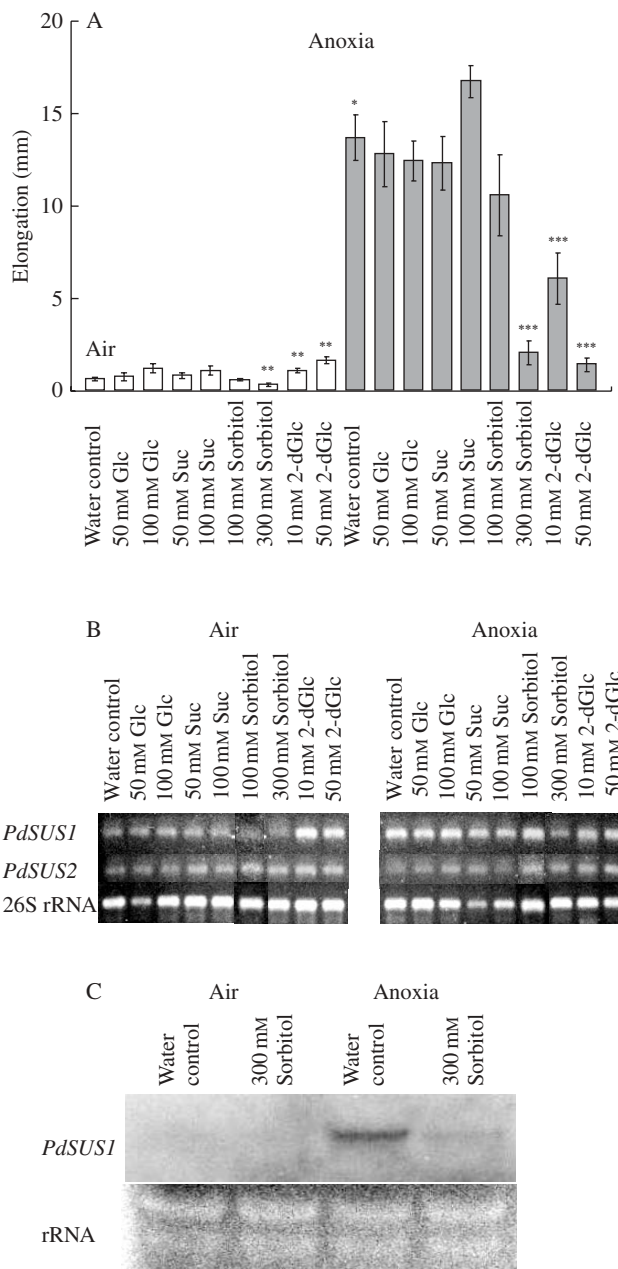


FIG. 4. Effects of exogenous sugars on stem elongation and on the levels of *PdSUS1* and *PdSUS2* transcripts in turion segments. (A) Stem elongation was determined after 3 d of incubation in air (open bars) or under anoxia (shaded bars). Data for elongation are means \pm s.e. of more than six turion segments. (B) The level of transcripts was determined at 3 d of incubation by RT-PCR and an example in three independent experiments is shown. (C) Northern blot analysis of *PdSUS1* transcripts. Total RNA was extracted from turion segments incubated under each condition for 3 d. Ten μ g of total RNA were applied to each lane and hybridized with RNA probes for *PdSUS1*. rRNA stained with ethidium bromide is also shown as a loading control. Significantly different from water control in air at: * $P < 0.0001$, ** $P < 0.01$. Significantly different from water control in anoxia at: *** $P < 0.05$ (Mann-Whitney test).

and the plasma membrane. Phosphorylation of Ser 11 residue conserved in the N-terminal regions of *PdSUS1* and *PdSUS2* may be involved in changes in the localization in cells. Further study of the localization of SuSy, especially

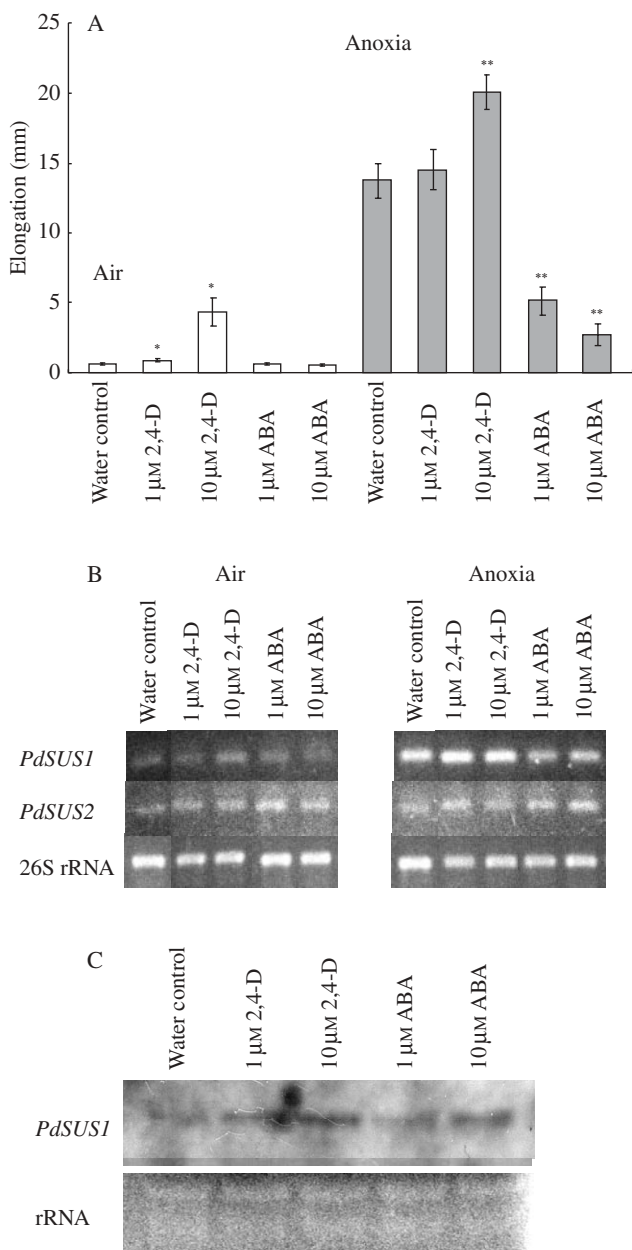


FIG. 5. Effects of auxin and abscisic acid on stem elongation and on the levels of *PdSUS1* and *PdSUS2* transcripts in turion segments. (A) Stem elongation was determined after 3 d of incubation in air (open bars) or under anoxia (shaded bars). Data for elongation are means \pm s.e. of more than 18 turion segments. (B) The level of transcripts was determined at 3 d of incubation by RT-PCR and an example in three independent experiments is shown. (C) Northern blot analysis of *PdSUS1* transcripts. Total RNA was extracted from turion segments incubated under each condition for 3 d under anoxia. Ten μg of total RNA were applied to each lane and hybridized with RNA probes for *PdSUS1*. rRNA stained with ethidium bromide is also shown as a loading control. Significantly different from water control in air at: * $P < 0.05$. Significantly different from water control in anoxia at: ** $P < 0.05$ (Mann-Whitney test).

PdSUS1, is necessary to discuss the role of SuSy in the accelerated site of cell elongation and vigorous anaerobic energy metabolism that characterizes pondweed turions when deprived of all oxygen.

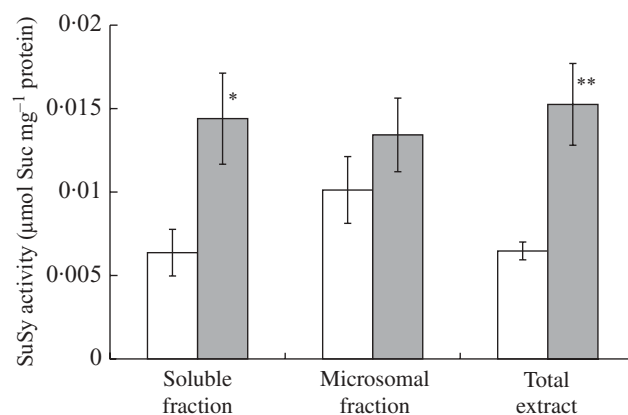


FIG. 6. Effects of anoxic conditions on SuSy activity in soluble and membrane fractions extracted from pondweed turions. SuSy was extracted from pondweed turions incubated at 25 $^{\circ}\text{C}$ in the dark in air (open bars) and under anoxia (shaded bars) for 3 d. Total activity of SuSy was determined in the supernatant after centrifugation of the extract at 7000 g for 20 min. The activity of soluble SuSy and membrane-bound SuSy was determined in the soluble fraction and in the microsomal fraction separated by ultracentrifugation at 95 000 g for 1 h. SuSy activity was measured by the method described in Materials and Methods. Data are means \pm s.e. of three separate replicates. Significantly different from the activity in air at: * $P < 0.1$, ** $P < 0.05$ (*t*-test).

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