Journal of Experimental Botany, Vol. 55, No. 406, pp. 2251–2259, October 2004
DOI: 10.1093/jxb/erh252 Advance Access publication 10 September, 2004

Journal of Experimental Botany

RESEARCH PAPER

SOR1, a gene associated with bioherbicide production in sorghum root hairs

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Received 12 March 2004; Accepted 16 July 2004

Abstract

Sorghum [*Sorghum bicolor* (L.) Moench] roots exude a potent bioherbicide known as sorgoleone, which is produced in living root hairs and is phytotoxic to broadleaf and grass weeds at concentrations as low as 10 μM. Differential gene expression was studied in sorghum (*S. bicolor*× *S. sudanense*) cv. SX17 between roots with abundant root hairs and those without root hairs using a modified differential display approach. A differentially expressed gene, named *SOR1*, was cloned by using Rapid Amplification of the 5′ ends of cDNA (5′-RACE). Real-time PCR analysis of multiple tissues of sorghum SX17 revealed that the *SOR1* transcript level in root hairs was more than 1000 times higher than that of other tissues evaluated, including immature leaf, mature leaf, mature stem, panicle, and roots with hairs removed. Semi-quantitative RT-PCR revealed that *SOR1* was expressed in the sorgoleone-producing roots of sorghum SX17, shattercane [*S. bicolor* (L.) Moench], and johnsongrass [*S. halepense* (L.) Pers.], but not in the shoots of sorghum or in the roots of sweet corn (*Zea mays* L.) 'Summer Flavor 64Y', in which sorgoleone production was not detected by HPLC analysis. Similarity searches indicated that *SOR1* probably encodes a novel desaturase, which might be involved in the formation of a unique and specific double bonding pattern within the long hydrocarbon tail of sorgoleone.

Key words: Allelopathy, differential display, gene cloning, real-time PCR, root exudates, root hair, sorghum, sorgoleone.

Introduction

Among the pesticides registered for use in the USA, herbicides account for the greatest use in volume and expenditure (National Research Council (US), 2000). Weed management with herbicides, although effective, can be costly and is increasingly problematic due to public concerns about health and environmental issues (Cheema and Khaliq, 2000). Most recently, weed management in modern agriculture relies on the use of biotechnologically-derived herbicide-resistant crops, especially for key agronomic species (Gressel, 2000). The use of herbicide-resistant transgenic crops, although increasing the producer's flexibility in the timing and cost-effectiveness of weed management, may result in additional problems due to the potential for the development of herbicide-resistant weeds as a result of gene introgression from crops to weed species (Gressel, 2000; Ellstrand, 2001). Although no compelling scientific arguments have been found to demonstrate that genetically engineered (GE) crops are innately different from non-GE crops (Dale *et al*., 2002), the use of herbicide resistance in agriculture remains controversial from a public perspective (Senior and Dale, 2002). By contrast, allelopathy offers potential for biorational weed control through the production and release of allelochemicals by plants themselves, either as weed-suppressive plant residues or direct release of volatiles or root exudates (Weston, 1996; Bertin *et al*., 2003; Weston and Duke, 2003). Allelochemicals can be defined as secondary plant products released by one plant species which are toxic to neighbouring vegetation. Various plant species are known to suppress other species by the production of allelochemicals from leaves, flowers, seeds, stems, and roots of living or decomposing plant materials (Nimbal *et al*., 1996; Chou, 1999; Gressel, 2000; Weston and Duke, 2003). Researchers have recently

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proposed genetically engineering allelochemical production into plants for the development of transgenic crops that produce their own natural defences against weeds (Gressel, 2000; Duke *et al*., 2001).

Sorghum [*Sorghum bicolor* (L.) Moench] roots exude large quantities of allelochemicals which are biologically-active hydrophobic substances. One major bioactive component of sorghum root exudates is sorgoleone, characterized as 2-hydroxy-5-methoxy-3-[(8′Z, 11′Z)-8′,11′, 14′-pentadecatriene]-*p*-benzoquinone (Chang *et al*., 1986; Netzly and Butler, 1986; Nimbal *et al*., 1996). Sorgoleone is phytotoxic to broadleaf and grass weeds at concentrations as low as 10 μM in hydroponic assays (Einhellig and Souza, 1992; Nimbal *et al*., 1996). Post-emergent foliar application of sorgoleone, at a similar concentration to labelled field rates of atrazine (0.6 kg ai ha^{-1}), inhibited growth of most 14-d-old weed species evaluated, especially small-seeded broadleafs (Czarnota *et al*., 2001). Pre-emergence soil applications were also toxic to certain small-seeded weed species (Weston and Czarnota, 2001).

Sorgoleone is a potent inhibitor of both photosynthetic (Einhellig *et al*., 1993; Gonzalez *et al*., 1997; Rimando *et al*., 1998) and mitochondrial electron transport (Rasmussen *et al*., 1992; Einhellig, 1995; Czarnota, 2001). In addition, Meazza *et al*. (2002) reported that sorgoleone inhibited hydroxyphenylpyruvate dioxygenase (HPPD), at concentrations equivalent to those of synthetic herbicides which act as plastoquinone biosynthesis inhibitors. HPPD is the novel target site for the new family of triketone herbicides. Inhibition of this enzyme disrupts the biosynthesis of carotenoids and results in foliar bleaching (loss of chlorophyll), which is also observed in sorgoleone-treated seedlings (Nimbal *et al*., 1996). The herbicidal and allelopathic properties of sorgoleone make isolation of the genes responsible for its biosynthesis desirable, as manipulation of those genes in sorghum or their introduction into other plant species could provide a better understanding of the role of sorgoleone in plant–plant interaction and enhance the natural weed control provided by sorghum and its residues (Dayan *et al*., 2003). Microscopic evidence was obtained to suggest that sorgoleone is synthesized in association with the endoplasmic reticulum (ER) and, through a cellular transport mechanism, is eventually deposited between the cell wall and the plasmalemma (Czarnota *et al*., 2003*a*). Recently, environmental modifications were developed using a mist or mat growth system to produce either sorghum roots with abundant root hairs, or those without any root hairs (Yang *et al*., 2004). As no mutants for sorgoleone production were discovered (Nimbal *et al*., 1996), the choice was made to utilize differential display technology (Liang and Pardee, 1992) in order to analyse differential gene expression between sorghum roots with and without root hairs, and eventually to isolate and identify key genes associated with sorgoleone biosynthesis.

Materials and Methods

Plant material

For differential display analysis, sorghum (*Sorghum bicolor*×*S. sudanense*) cv. SX17 seeds were sterilized with 15% bleach containing 5.25% sodium hypochlorite for 10 min and washed subsequently three times with purified water. Sterilized seeds were germinated in the dark at room temperature (24±1 °C) using two rooting environments (Yang *et al*., 2004). Briefly, a mist system with air flow at 15 l min^{-1} was used to produce roots with abundant roots hairs and a mat system with water movement at 1 ml min^{-1} was used to produce hairless roots. After 60 h, root segments (~1 cm) were excised from a region 0.5 cm behind the root tip of the sorghum seedlings, frozen in liquid nitrogen, and stored at −80 °C prior to RNA isolation. For multiple-tissue real-time PCR analysis, immature leaves were taken from 10-d-old sorghum SX17 seedlings produced in a growth chamber at 29 °C under ~400 μmol m^{-2} s^{-1} light intensity. Mature leaf tissue (fully expanded), mature stem tissue (the first internode), panicle tissue (emerging panicle bearing florets just prior to anthesis), and the entire root system were collected from 12-week-old sorghum SX17 plants grown in a greenhouse in Stoneville, MS, USA under natural lighting and temperatures ranging from 25–35 °C. Root hairs were removed from roots by using the method of Bucher *et al*. (1997) from 5-d-old seedlings grown on a capillary mat system (Czarnota *et al*., 2001). Tissue was frozen in liquid nitrogen and stored at −80 °C until use. For Southern blot analysis, seeds of sorghum SX17 were germinated in the dark at room temperature (24±1 °C) on six layers of paper towel moistened with purified water in a storage box. After 7 d, leaves were harvested, frozen in liquid nitrogen, and stored at −80 °C prior to DNA isolation.

Differential display

Total RNA was isolated from the root segments with the RNAqueous™-4PCR kit (Ambion, Inc., Austin, TX, USA). RNA preparations were subjected to DNase I treatment according to the manufacturer's instructions. Reverse transcription (RT) was performed with the GeneAmp® Gold RNA PCR Reagent Kit (Applied Biosystems, Foster City, CA, USA). Equal amounts of 1.6 μg total RNA each were transcribed to cDNA in 80 μl reactions containing 1× RT-PCR buffer, 2.5 mM $MgCl_2$, 250 μM of each dNTP, 40 U of RNase inhibitor, 10 mM DTT, 60 U of MultiScribe reverse transcriptase, and 625 nM of 3′ anchor primer DA (5′-XTTTTTTTTTTTTTTTA-3′), DC (5′-XTTTTTTTTTTTTTTTC-3′), or DG (5′-XTTTTTTTTTTTTTTG-3′); where X=GTAATACGACTCACTATAGGGAAGC. RT reaction was performed at 25 °C for 10 min, then at 42 °C for 12 min.

cDNA (2 μl) was amplified with the GeneAmp® Gold RNA PCR Reagent Kit (Applied Biosystems, Foster City, CA, USA) in 25 μl reactions containing 1× RT-PCR buffer, 1.75 mM $MgCl_2$, 200 μM of each dNTP, 2.5 U of AmpliTaq Gold DNA Polymerase, 0.8 μM 3′ anchor primer (DA, DC, or DG), and 0.08 μM 5′ primer. Eight 5′ primers were used in this study: B3TA [5′-Y$(N)_{10}$CGCCGTA-3′], B3TC [5′-Y$(N)_{10}$CGCCGTC-3′], H1GA [5′-Y$(N)_{10}$GGGCGGA-3′], H4AT [5′-Y$(N)_{10}$CGGCCAT-3′], H4AG [5′-Y$(N)_{10}$CGGCCAG-3′], Y2GA [5′-Y$(N)_{10}$AGGGTGA-3′], L1GT [5′-Y$(N)_{10}$CCCCTGT-3′], and L1GG [5′-Y$(N)_{10}$CCCCTGG-3′]; where Y=CCAATTAACCCTCACTAAAGGGAGGATCC. PCR was performed with one round at 95 °C for 10 min; 5 touch-down cycles: 94 °C for 30 s, 66 °C (−4.0 °C/cycle) for 30 s, 72 °C for 1 min; 23 cycles: 94 °C for 30 s, 68 °C for 1 min; and a final step at 72 °C for 7 min. PCR products (2.1 μl) were further amplified with the Advantage® 2 PCR Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) in 35 μl reactions containing 1× Advantage 2 PCR buffer, 400 μM of each dNTP, 1× Advantage 2 polymerase mix, 0.6 μM 3′ primer

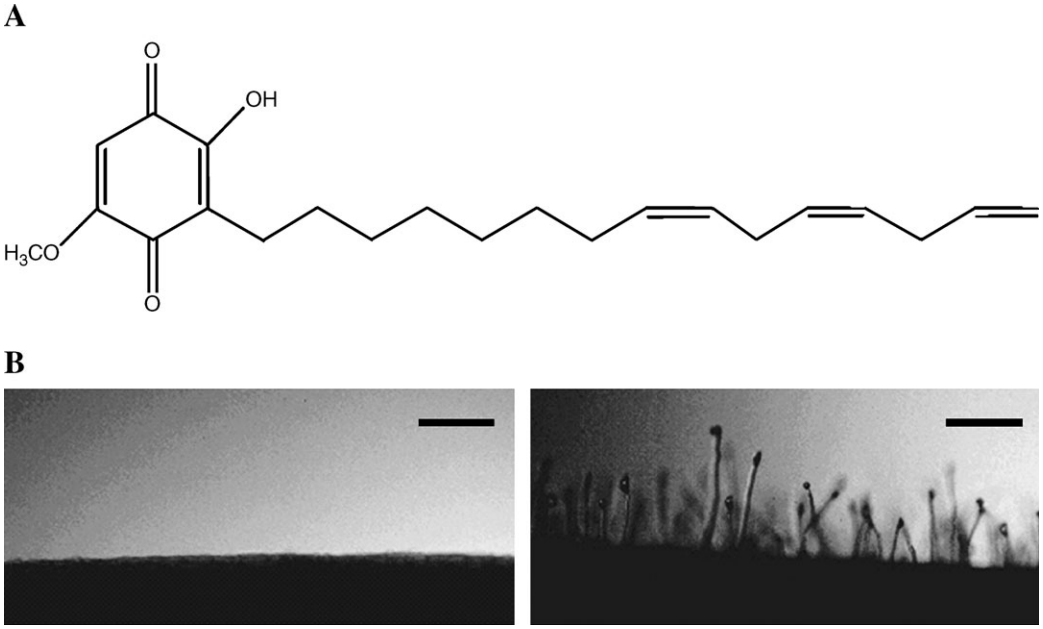

Fig. 1. Sorgoleone structure and characteristics of sorghum roots grown under different environments. (A) The structure of sorgoleone, a potential bioherbicide produced by sorghum root hairs. (B) Hairless (left) and hair-bearing roots (right) of sorghum SX17 produced by using the mat and mist systems, respectively, were used for analysis of differential gene expression associated with sorgoleone production. The droplets around the tip of root hairs contain predominantly sorgoleone. Scale bar=200 μm.

(the same as the preliminary PCR reaction), and 0.6 μM 5′ primer P29 (5′- CAATTAACCCTCACTAAAGGGAGGATCC-3′). PCR was performed with one round at 95 °C for 1 min; 30 cycles: 95 °C for 30 s, 68 °C for 1 min; and a final step at 68 °C for 1 min.

Final PCR reactions were electrophoresed on 6% (W/V) denaturing polyacrylamide gels by using Sequi-Gen GT/PowerPac 3000 System (21×40 cm; Bio-Rad Laboratories, Hercules, CA). After 150 min of electrophoresis, gels were stained with Silverstar staining kit (BioNexus Inc., Oakland, CA, USA) and gel images were documented with AlphaImager™ 2200 (Alpha Innotech Corporation, San Leandro, CA, USA).

Reamplification and sequencing of differential cDNA fragments

Differential cDNA fragments were reamplified and selected according to Yoshikawa *et al*. (1998). Briefly, the region of interest showing high expression in the root hair-plus lane ('plus') was cut out of the polyacrylamide gel, as was the corresponding region showing low expression or no expression in the root hair-minus lane ('minus'). Excised gels were eluted in 50 μl of TE buffer at 100 °C for 10 min. Both plus and minus elutions (2 μl each) were reamplified separately in the same reaction mixture as above for the final PCR reactions. Then, 10 μl of each reamplification product was loaded side-by-side on a 2% agarose gel containing 1 unit ml^{-1} of base-specific DNA ligand H.A.Yellow (GeneScan Europe AG, Freiburg, Germany) to differentiate DNA fragments of identical length which possess different base sequences. After electrophoresis, the cDNA fragments with higher intensity in the plus lane than in the minus lane were recovered from the plus lane. The recovered fragments were reamplified once more and purified with agarose gel by using QIAquick gel extraction kit (Qiagen Inc., Valencia, CA, USA). Gel-purified DNA fragments were sequenced by using the Applied Biosystems Division Automated 3700 DNA Analyzer with Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase (Cornell University DNA sequencing facility, Ithaca, NY, USA).

Semi-quantitative RT-PCR

A multiplex RT-PCR assay, performed by co-amplification of the mRNA species of interest with 18S rRNA over a range of cycles followed by agarose gel electrophoresis, was conducted according to Spencer and Christensen (1999). Specifically, 1.6 μg RNA was reverse-transcribed with the RETROscript kit (Ambion, Inc., Austin, TX, USA) in 20 μl reactions containing 5 μM random decamers. cDNA (0.9 μl) was amplified using the Advantage® 2 PCR Kit (CLONTECH Laboratories, Inc., Palo Alto, CA, USA) in 15 μl reactions containing 1× Advantage 2 PCR buffer, 200 μM of each dNTP, 1× Advantage 2 polymerase mix, 0.4 μM of each gene-specific primer (forward, 5′-GGGCTGACGACGGTGGACA-3′; reverse, 5′-CGAGAGCGCGAGGTATTCAACA-3′), and 0.5 μM of internal standard primer mixture (Universal 18S internal standard:18S PCR Competimers=2:3; Ambion, Inc., Austin, TX). PCR was performed as follows: one round at 95 °C for 1 min; 8 cycles: 95 °C for 30 s, 59 °C for 30 s, 68 °C for 45 s; and a final step at 72 °C for 1 min. The amplified products (8 μl each) were separated on a 2% agarose gel, stained with ethidium bromide, and documented with AlphaImager™ 2200 (Alpha Innotech Corporation, San Leandro, CA, USA). The relative levels of gene expression were represented by gel band intensities normalized for 18S band intensities.

Real-time PCR analysis

Two RNA samples were prepared separately from each type of tissue. RNA was extracted with TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA, USA), and re-purified with RNeasy Midi Kit (Qiagen, Valencia, CA, USA), including an 'on-column' DNase I treatment to remove residual DNA contamination. RNA purity was determined spectrophotometrically, and quality was determined by examining rRNA bands on agarose gels. Real-time PCR was performed in two biological replicates (i.e. two RNA samples from different plants, with three PCR reactions on each RNA sample) for each tissue using an ABI PRISM™ 5700 Sequence Detector (Applied Biosystems, Foster City, CA) with primers specific to *SOR1*

(forward, 5′-GTGAAGTCAGTTGGTGCAATGTATTC-3′; reverse, 5′-TGCATAATATATAGGCCACGAACAG-3′) and 18S rRNA (forward, 5′-GGCTCGAAGACGATCAGATACC-3′; reverse, 5′-TCGGCATCGTTTATGGTT-3′).

First-strand cDNAs were synthesized from 2 μg of total RNA in a 100 μl reaction volume using a TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, Foster City, CA, USA) and random hexamers as primer. For PCR reactions using *SOR1*-specific primers, the cDNA was diluted 50-fold and 2.5 μl (~0.5 ng cDNA) was used for a 25 μl PCR reaction. For PCR reactions using 18S rRNA-specific primers, the cDNA was diluted 50 000 times and 2.5 μl (~0.5 pg cDNA) was used for a 25 μl PCR reaction.

The real-time PCR reactions were performed with the SYBR Green PCR Master Mix Kit (Applied Biosystems, Foster City, CA, USA) with denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. For the 18S rRNA assays, the primers were at 50 nM each and for the *SOR1* assays, the primers were at 900 nM each. The changes in fluorescence of SYBR Green I dye in every cycle were monitored by the ABI 5700 system software and the threshold cycle (C_T) for each reaction was calculated. The relative amount of PCR product generated from each primer set was determined based on the C_T value. 18S rRNA was used for the normalization of RNA quantity. The C_T value of 18S rRNA was subtracted from that of *SOR1* to obtain a ΔC_T value, and then the ΔC_T value for the stem was subtracted from that for each of the other five tissues to obtain a $\Delta\Delta C_T$ value. The gene expression level in a tissue relative to that in the stem was expressed as $2^{-\Delta\Delta CT}$.

Rapid amplification of 5′ ends of cDNA (5′-RACE)

The 5′-RACE was achieved with a GeneRacer kit (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions.

Plant DNA extraction and Southern analysis

Genomic DNA was prepared with a DNAzol ES kit (Molecular Research Center, Inc., Cincinnati, OH, USA). For Southern blot analysis, genomic DNA was digested with the indicated enzymes, separated on a 0.8% agarose gel, and transferred onto a Biodyne® B nylon membrane (Pierce Biotechnology, Inc., Rockford, IL, USA) by the alkaline method according to Ausubel *et al*. (1999). Probe was amplified from *SOR1* cDNA by PCR with *SOR1*-specific primers (forward: 5′-CACCTCAACGACACTACTACTGATGATGCTC-AT-3′; reverse, 5′-CGAACAGATGGAATACATTGCACCAACT-GA-3′). Probe labelling, hybridization, and chemiluminescent signal detection were performed with the North2South Direct HRP Labeling and Detection Kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Hybridization was carried out at 55 °C followed by washing using 0.1× SSC with 0.1% (w/v) SDS at the same temperature.

High Performance Liquid Chromatography (HPLC) analysis

Seeds of sorghum SX17, johnsongrass (*S. halepense* (L.) Pers.), shattercane (*S. bicolor* (L.) Moench), and sweet corn (*Zea mays* L.) 'Summer Flavor 64Y' were germinated in the dark at room temperature (24±1 °C) on six layers of paper towel moistened with purified water in a storage box. After 3 d of germination, sorghum shoots and roots of sorghum, johnsongrass, shattercane, and sweet corn were extracted and analysed by HPLC as described by Yang *et al*. (2004).

Analysis of the sequencing data

Similarity searches were carried out with the NCBI PSI-Blast program (http://www.ncbi.nlm.nih.gov/), and alignment of amino acid sequences was performed with the Clustal W program in the Lasergene software package (DNASTAR, Madison, WI). Prediction of subcellular localization was analysed by the programs iPSORT (http://psort.nibb.ac.jp/) and TargetP v1.01 (http://www.cbs.dtu.dk/services/TargetP/).

Results

Characteristics of sorghum roots grown under different environments

A mist system and a mat system were used to prepare plant materials for differential display analysis of gene expression associated with sorgoleone production in sorghum root hairs. The roots of 60-h-old sorghum seedlings grown in the mist system produced abundant root hairs which exuded large quantities of sorgoleone in oily droplets from the root hair tips. By contrast, the roots of sorghum seedlings grown in the water-flowing mat system produced no root hairs (Fig. 1). Consequently, roots produced using the mat system did not exude sorgoleone.

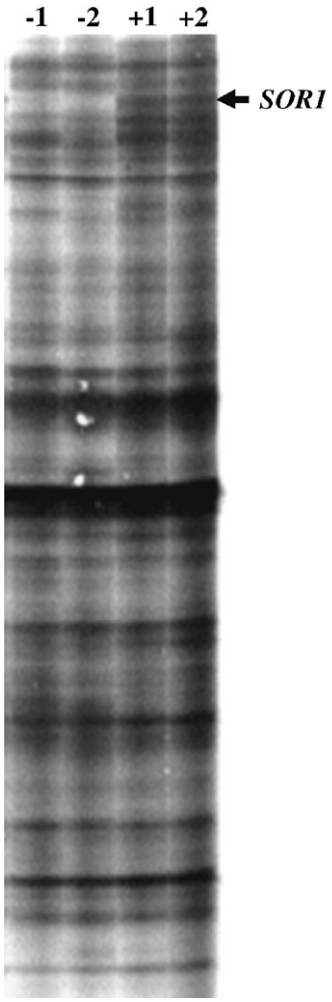

Fig. 2. Gene expression pattern revealed by differential display (DD) PCR with 5′ primer L1GG and 3′ primer DC. The band indicated by arrow represent differential expression of *SOR1* between the RNA samples prepared from roots with abundant root hairs (+1 and +2) and those prepared from roots without root hairs (−1 and −2).

SOR1 *is differentially expressed between root hair-plus and -minus sorghum roots*

To detect genes that were predominantly expressed in the root hairs of sorghum, a modified differential display (DD) method was used. Total RNA prepared from roots with and without root hairs was reverse-transcribed and then cDNAs were amplified by PCR with a combination of eight different 5′ primers (B3TA, B3TC, H1GA, H4AT, H4AG, Y2GA, L1GT, and L1GG) and three different 3′ primers (DA, DG, and DC). A total of seven cDNA fragments representing genes predominantly expressed in roots with abundant root hairs were revealed by DD analysis. The characterization of *SOR1* which corresponds to one of the seven differential cDNA fragments is reported here. The DD PCR with 5′ primer L1GG and 3′ primer DC revealed differential expression of *SOR1* between sorghum roots with abundant root hairs and those without root hairs (Fig. 2). The differential pattern of *SOR1* expression was confirmed by semi-quantitative RT-PCR with eight cycles of amplification and 18S rRNA as an internal standard (Fig. 3A). Even after 35 cycles of PCR amplification, *SOR1* was not detected in the cDNA sample obtained from sorghum roots without root hairs whereas a very strong signal of *SOR1* was detected in the cDNA sample obtained from sorghum roots with abundant root hairs (Fig. 3B). This indicates that, in sorghum roots, *SOR1* is preferentially expressed in root hairs.

The full-length sequence of SOR1 *cDNA*

The full-length cDNA of *SOR1* was obtained using 5′-RACE technology. The *SOR1* cDNA (GenBank accession number AY566285) is 1494 bp, encoding 389 amino acids. A database search using the NCBI PSI-Blast program (http://www.ncbi.nlm.nih.gov/) revealed that the deduced amino acid sequence of *SOR1* showed significant similarity to omega-3 fatty acid desaturases in bread wheat (*Triticum aestivum*), rice (*Oryza sativa*), castor bean (*Ricinus communis*), sesame (*Sesamum indicum*), potato (*Solanum tuberosum*), common tobacco (*Nicotiana tabacum*), and tomato (*Lycopersicon esculentum*) (Fig. 4). However, an alignment of the deduced amino acid sequence of *SOR1* with the most closely related omega-3 fatty acid desaturase sequences revealed 12 amino acid changes in the SOR1 protein in positions that are conserved in all of the seven most closely related omega-3 fatty acid desaturases (Fig. 5). This alignment also shows a pair of predicted conserved histidine-rich motifs (HXXHH) for the family of integral membrane desaturases (Buchanan *et al*., 2000), indicating that *SOR1* probably encodes an integral membrane desaturase. No signal, mitochondrial targeting, or chloroplast

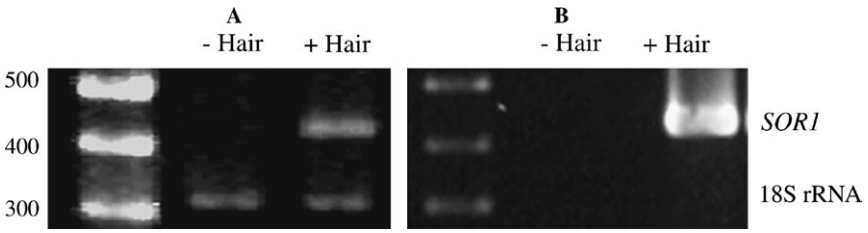

Fig. 3. Confirmation of differential gene expression. (A) Semi-quantitative RT-PCR analysis of *SOR1* expression in root hair-bearing (+Hair) and hairless (−Hair) sorghum roots. 18S rRNA was used as an internal standard; PCR cycle number was 8. (B) RT-PCR analysis of *SOR1* expression in hair-bearing (+Hair) and hairless (−Hair) sorghum roots; PCR cycle number was 35. The size (bp) of DNA markers are indicated.

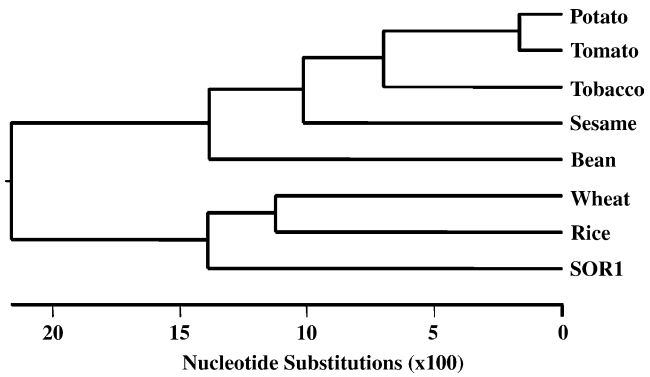

Fig. 4. Phylogenetic tree of the SOR1 protein (GenBank accession number AY566285) and related omega-3 fatty acid desaturases with the highest amino acid sequence identity in bread wheat (*Triticum aestivum*; GenBank accession number: T06238), rice (*Oryza sativa*; T03923), castor bean (*Ricinus communis*; P48619), sesame (*Sesamum indicum*; P48620), potato (*Solanum tuberosum*; T07685), common tobacco (*Nicotiana tabacum*; T03029), and tomato (*Lycopersicon esculentum*; AAP82170). The tree was constructed by using the Clustal W program in DNASTAR.

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SOR1        MAA------- ---------- ---------- ---------- ---------- ---------- -------TDH EVEEAVAKAR
Wheat       MAP------- ---------- ---------- ---------- ---------- ---------- -------AMR PEQEASCKAT
Rice        MAA------- ---------- ---------- ---------- ---------- ---------- -------SAT --QEADCKAS
Bean        MAAGWVLSEC GLRPLPRIYS RPRIGFTSKT TNLLKLRELP DSKSYNLCSS FKVSSWSNSK QSNWALNVAV PVNVSTVSGE
Sesame      MAS-WVLSEC GLRPLPRVYP KPRTGHPLLN SNPTKL--RF SRTDLGNGSS FCLSSGILRE KN-WALRVSA PLRVLQVEEE
Potato      MAS-WVLSEC GLRPLPRIYP KPRIGSSS-- ---------V CATNLN-LRR ISPSP-IRTD RNCWALRVSA PLRIQIAGEE
Tobacco     MAS-WVLSEC GLRPLPRIYP KPRTGAATSA SSHIKL--RI SRTDLS-GSA ISLSN-TNKE RN-WALRVSA PLRIQTVGEE
Tomato      MAS-WVLSEC GLRPLPKIYP KPTIGFSSSV S--------V SATSLN-LRR ISPSP-IRTD RNCWALRVSA PLRIQIAGEE
Consensus   **

SOR1        EDDKSRRQV- ---------D GFDAGKAPPF RIGDVRAAVP EHCWRKSPWR SLWYVVRDVA VVVALGAAAA A----MDSWA
Wheat       EDHRS----- ---------- EFDAAKPPPF RIGDVRAAVP AHCWPQEPPA SLSYVARDVA VVAALAAAAW R----ADSWA
Rice        EDARL----- ---------- FFDAAKPPPF RIGDVRAAIP VHCWRKTPLR SLSYVARDLL IVAALFAAAA SSIDLAWAWA
Bean        DDREREEFNG IVNVDEGKGE FFDAGAPPPF TLADIRAAIP KHCWVKNPWR SMSYVLRDVV VVFGLAAVAA Y----FNNWV
Sesame      EENKEGERV- -----INGGE EFDPGAPPPF KLSDIREAIP KHCWVKDPWR SMGYVVRDVA VVFGLAAVAA Y----FNNWV
Potato      E-EQTTN--- ------NGDE -FDPGASPPF KLSDIKAAIP KHCWVKNPWT SMSYVVRDVA IVFGLAAAAA Y----FNNWL
Tobacco     ESERTNN--- ------SGGE FFDPGAPPPF KLSDIKAAIP KHCWVKNPWK SMSYVVRDVA IVFGLAAAAA Y----FNNWV
Tomato      E-EQTTN--- ------NGDE FFDPGAPPPF KLSDIKAAIP KHCWVKNPWT SMSYVVRDVA IVFGLAAAAA Y----FNNWL
Consensus                           **    ***    *   * *  ***   *   *  ** **    *  * * *         *

SOR1        VWPLYWAVQG TMFWAFFVLG HDCGHGSFSD NATLNSVVGH LLHSFILIPY HGWRISHRTH HQNHGHVDRD ESWHPLTERR
Wheat       LWPLYWAVQG TMFWALFVLG HDCGHGSFSD SGTLNSVVGH LLHTFILVPY NGWRISHRTH HQNHGHIDRD ESWHPITEKV
Rice        -WPLYWARQG TMVWALFVLG HDCGHGSFSD SAMLNNVVGH LLHSFILVPY HGWRFSHRTH HQNHGHIERD ESWHPITEKL
Bean        AWPLYWFCQG TMFWALFVLG HDCGHGSFSN NPKLNSVVGH LLHSSILVPY HGWRISHRTH HQNHGHVEND ESWHPLSEKI
Sesame      VWPLYWFAQS TMFWALFVLG HDCGHGSFSN DPKLNSVVGH ILHSSILVPY HGWRISHRTH HQNHGHVEND ESWHPLSEKI
Potato      VWPLYWFAQS TMFWALFVLG HDCGHGSFSN NHNLNSVAGH ILHSSILVPY HGWRISHRTH HQNHGHVEND ESWHPLSEKL
Tobacco     VWPLYWFAQS TMFWALFVLG HDCGHGSFSN NHKLNSVVGH ILHSSILVPY HGWRISHRTH HQNHGHVEND ESWHPIPEKI
Tomato      VWPLYWFAQS TMFWALFVLG HDCGHGSFSN NHSLNSVAGH ILHSSILVPY HGWRISHRTH HQNHGHVEND ESWHPLSEKL
Consensus    *****  *  ** **:**** *********     ** * **  **  **:**  *** ***** ******   * *****  *:

SOR1        YRRLPPRAKK LRFTPPFPLL LFPLYLFYRS PGKRGSHFLP SSPLFSPKDK GDVILSTTCW CIMLAFLLAM SCAFGPLQVL
Wheat       YQKLEPRTKT LRFSVPFPLL AFPVYLWYRS PGKEGSHFNP SSDLFTPKER RDVIISTTCW FTMIALLIGM ACVFGLVPVL
Rice        YWQLETRTKK LRFTLPFTLL AFP---WYRS PGKTGSHFLP SSDLFSPKEK SDVIVSTTCW CIMISLLVAL ACVFGPVPVL
Bean        FKSLDNVTKT LRFSLPFPML AYPFYLWSRS PGKKGSHFHP DSGLFVPKER KDIITSTACW TAMAALLVYL NFSMGPVQML
Sesame      YKNLDTATKK LRFTLPFPLL AYPIYLWSRS PGKQGSHFHP DSDLFVPNEK KDVITSTVCW TAMLALLVGL SFVIGPVQLL
Potato      YNSLDDITKK FRFTLPFPLL AYPFYLWGRS PGKKGSHFDP SSDLFVASEK KDVITSTVCW TAMAALLVGL SFVMGPLQVL
Tobacco     YNSLDLATKK LRFTLPFPLL AYPFYLWSRS PGKKGSHFDP NSDLFVPSEK KDVMTSTLCW TAMAALLVGL SFVMGPFQVL
Tomato      YNSLDDITKK FRFTLPFPLL AYPFYLWGRS PGKKGSHFDP NSDLFVASEK KDVITSTVCW TAMAALLVGL SFVMGPLQML
Consensus      *   :*   **  **  * : *   : ** *** **** *  * **   :   *   ** **   *  :*       *    *

SOR1        KMYGVPYLVS VMWLDLVTYL HHHGHQERLP WYRGEEWSYL RGGLTTVDRD YGWINSIHHD IGTHVIHHLF PQIPHYHLVE
Wheat       KLYGVPYIVN VMWLDLVTYL HHHGHQD-LP WYRGEEWSYL RGGLTTVDRD YGWINNIHHD IGTHVIHHLF PQIPHYHLVE
Rice        MLYGVPYLVF VMWLDLVTYL HHHGHND-LP WYRGEEWSYL RGGLTTVDRD YGWINNIHHD IGTHVIHHLF PQIPHYHLVE
Bean        KLYGIPYWIF VMWLDFVTYL HHHGHEDKLP WYRGKAWSYL RGGLTTLDRD YGWINNIHHD IGTHVIHHLF PQIPHYHLVE
Sesame      KLYGIPYLGN VMWLDLVTYL HHHGHEDKLP WYRGKEWSYL RGGLTTLDRD YGWINNIHHD IGTHVIHHLF PQIPHYHLIE
Potato      KLYGIPYWGF VMWLDIVTYL HHHGHEDKVP WYRGEEWSYL RGGLTTLDRD YGWINNIHHD IGTHVIHHLF PQIPHYHLVE
Tobacco     KLYGIPYWGF VMWLDLVTYL HHHGHDDKLP WYRGEEWSYL RGGLTTLDRD YGWINNIHHD IGTHVIHHLF PQIPHYHLVE
Tomato      KLYGIPYWGF VIWLDLVTYL HHHGHEDKVP WYRGEEWSYL RGGLTTLDRD YGWINNIHHD IGTHVIHHLF PQIPHYHLVE
Consensus    :** **    * *** **** ***** :  * ****  **** ****** *** *****:**** ********** ******** *

SOR1        ATKAAKPVLG RYYREPHKSG PLPLHLLGVL LRSLRVDHFV SDHGDVVYYQ TDHHLNDTTT DDAHKQK
Wheat       ATKAARPVLG RYYREPEKSG PLPMHLITVL LKSLRVDHFV SDVGDVVFYQ TDPSLSGDKW TGTDKQK
Rice        ATKAARPVLG RYYREPEKSG PLPLHLFGVL LRTLRVDHFV SDVGDVVYYQ TDHSLNGTDW AEDAKHK
Bean        ATEAAKPVMG KYYREPKKSG PLPLHLLGSL VRSMKEDHYV SDTGDVVYYQ KDPKLSGIGG EKTE---
Sesame      ATEAAKPVLG KYYREPKKSA PLPFHLLGDL TRSLKRDHYV SDVGDVVYYQ TDPQLTGA-- EKS----
Potato      ATEAAKPVLG KYYKEPKKSG PLPFYLLGYL IKSMKEDHFV SDTGNVVYYQ TDPNLYGS-- EK-----
Tobacco     ATEAAKPVLG KYYKEPKKSG PLPFYLLGVL IKSMKQDHYV SDTGDIVYYR TDPQLSGF-- QK-----
Tomato      ATEAAKPVLG KYYKEPKKSG PLPFYLLGYL IRSMKEDHFV SDTGNVVYYE TDPNLYGS-- KK-----
Consensus   ** ** ** *  ** ** **  ***  *   *       ** * ** *  * *   *  * :
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Fig. 5. Alignment of the deduced amino acid sequence of SOR1 (GenBank accession number AY566285) with related omega-3 fatty acid desaturases in bread wheat (GenBank accession number: T06238), rice (T03923), castor bean (P48619), sesame (P48620), potato (T07685), common tobacco (T03029), and tomato (AAP82170) using the Clustal W program (http://www.ebi.ac.uk/clustalw/). Consensus symbols: 'asterisk' indicates the identical amino acid positions; and 'colon' indicates amino acid changes in the SOR1 protein in positions that are highly conserved in the omega-3 fatty acid desaturases. The two HXXHH motifs are marked with lines.

transit peptides were found in the SOR1 protein sequence by TargetP and iPSORT programs, indicating that SOR1 is not targeted to the endoplasmic reticulum (ER), mitochondrion, or chloroplast.

Spatial pattern of SOR1 *expression*

Relative abundance of *SOR1* mRNA of six different sorghum SX17 tissues was estimated by real-time PCR, and the results revealed tissue-specific differences in the abundance of *SOR1* transcripts between the root hair tissue and the other five tissues (immature leaf, mature leaf, mature stem, panicle, and roots with hairs removed). Specifically, the *SOR1* transcript level in the root hair tissue was more than 1000 times higher than that in other tissues, while *SOR1* transcript levels among immature leaf, mature leaf, mature stem, panicle, and root tissue without root hairs remained near zero (Table 1). This indicates that the expression of *SOR1* is highly specific to root hairs in sorghum SX17.

Table 1. *Relative quantification of* SOR1 *expression in different tissues of sorghum SX17 using the comparative* C_T *method of real-time PCR*

Tissue	Relative expression[a]
Stem	1.0 (0.4–2.5)
Immature leaf	1.3 (0.9–1.8)
Panicle	1.6 (1.0–2.5)
Root with hair removed[b]	4.1 (2.2–7.7)
Mature leaf	4.4 (3.3–5.9)
Root hair	4369.7 (3601.3–5302.1)

[a] The numbers in parenthesis indicated the range of variation.
[b] Root hairs were removed from the roots by the method of Bucher *et al*. (1997).

Gene copy number

For DNA gel-blot analysis, sorghum SX17 genomic DNA was digested with *Bam*HI, *Eco*RI, *Hin*dIII, and *Xba*I. One band was detected in each genomic digestion with *Eco*RI, *Hin*dIII, and *Xba*I while two bands were detected in the *Bam*HI digestion (Fig. 6), which is expected as the probe for hybridization contains an internal *Bam*HI site. These results suggest that *SOR1* is a single-copy gene in sorghum SX17.

Association between SOR1 *expression and sorgoleone production*

Analysis of *SOR1* expression by semi-quantitative RT-PCR revealed a significant difference in the expression of this gene among the root samples of several related species. Specifically, the expression of *SOR1* was not detected in sorghum shoots or sweet corn roots but was detected in the roots of sorghum SX17, shattercane, and johnsongrass (Fig. 7). Sorgoleone production was not detected in sorghum SX17 shoots or sweet corn roots by HPLC analysis (data not shown). The root exudates of *Sorghum* spp. contain mainly sorgoleone, generally in quantities that account for greater than 80% of the total root exudate composition (Nimbal *et al*., 1996; Czarnota *et al*., 2003*b*). In addition, the expression level of *SOR1* was much higher in johnsongrass roots than in sorghum or shattercane roots (Fig. 7). Johnsongrass is a noxious perennial sorghum relative which is currently ranked as one of the world's worst weeds. Earlier studies revealed that the level of root exudate in johnsongrass roots (14.75 mg g^{-1} FW) is ~10 times more than in sorghum SX17 roots (1.55 mg g^{-1} FW), and ~30 times as much as in shattercane roots (0.50 mg g^{-1} FW) (Czarnota *et al*., 2003*b*). These data suggest that *SOR1* is associated with sorgoleone production in the root hairs of sorghum, shattercane, and johnsongrass.

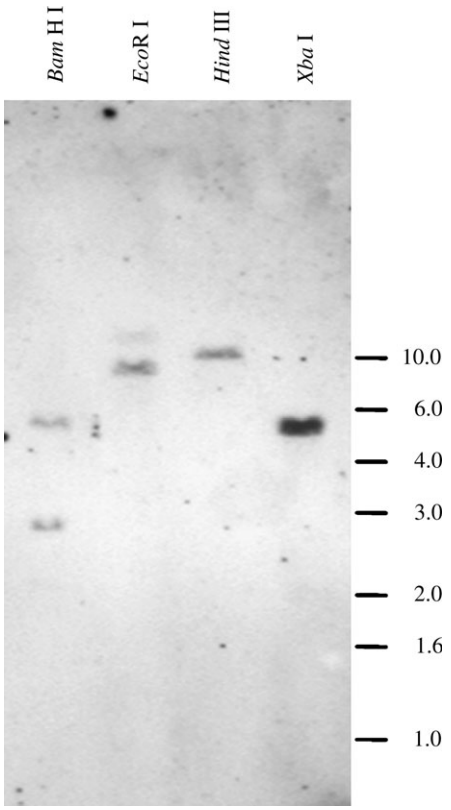

Fig. 6. Southern blot analysis of the sorghum genomic DNA (3 μg) digested with the indicated enzymes. The position and size (kb) of DNA markers are indicated.

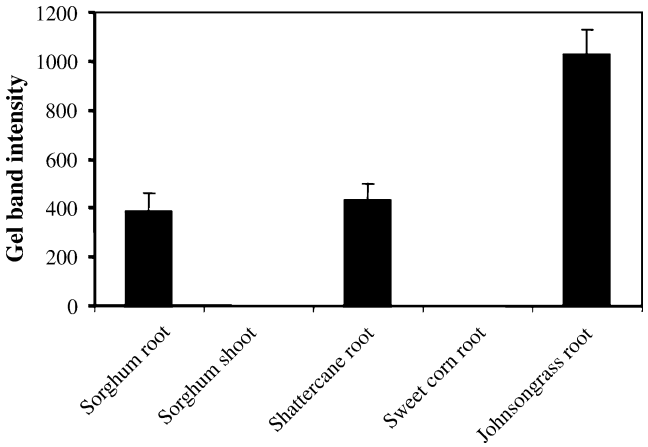

Fig. 7. Comparative analysis of gene expression by semi-quantitative RT-PCR among sorghum and related species. The relative level of gene expression was represented by gel band intensities normalized for 18S band intensities. Values are the average of three replicates ±SE.

Discussion

Sorgoleone is the major component among a mixture of related natural products exuded from living sorghum root hairs (Nimbal *et al*., 1996; Czarnota *et al*., 2001). This study focused on the isolation and identification of the genes related to sorgoleone production in sorghum root hairs. Semi-quantitative RT-PCR revealed that the isolated *SOR1* gene was expressed only in the roots of closely

related *Sorghum* spp., including sorghum SX17, johnsongrass, and shattercane, but not in the roots of distantly related corn (Fig. 7). Using extraction techniques to evaluate root exudates, sorgoleone was not detected in extracts of the living roots of corn (data not presented). Multiple-tissue real-time PCR analysis indicated that the expression of *SOR1* is highly specific to root hairs in sorghum SX17 (Table 1). *SOR1* was clearly expressed most strongly in root tissues of johnsongrass (Fig. 7), which produce proportionately greater quantities of root exudate containing sorgoleone than other related *Sorghum* spp. (Czarnota *et al*., 2003*b*). All of these findings strongly suggest that the newly identified *SOR1* gene is associated with sorgoleone production.

Fate and Lynn (1996) found that [^{13}C]-labelled acetate was incorporated into the aromatic moiety of a dihydroquinone derivative of sorgoleone, suggesting that sorgoleone is synthesized via a polyketide pathway. They suggested that the quinone portion is biosynthetically added onto a pre-existing (16-carbon) fatty acid. Recently, Dayan *et al*. (2003) elucidated the biosynthetic pathway of sorgoleone using retrobiosynthetic NMR analysis. Their studies indicate that unknown fatty-acid desaturases are involved in the formation of the unusual Δ9,12 C16:2 and especially Δ9,12,15 C16:3 desaturation pattern of the aliphatic tail of sorgoleone (Fig. 1A).

Similarity searches revealed that the amino acid sequence of *SOR1* is similar to that of omega-3 fatty acid desaturases (Fig. 4). It is believed that the SOR1 protein is specifically responsible for the unique desaturation pattern occurring in the long hydrophobic tail of sorgoleone (Fig. 1A). Currently, none of the known fatty acid desaturases can form double bonds in the exceptionally unique terminal position within the sorgoleone tail structure (Dayan *et al*., 2003). An alignment of amino acid sequences revealed 12 amino acid changes in the SOR1 protein in positions that are highly conserved in closely related omega-3 fatty acid desaturases (Fig. 5). It is thought that it is likely that these sequence changes result in the altered ability of this desaturase to form the three terminal sequential double bonds in the hydrocarbon side chain of sorgoleone.

Currently, an attempt is being made to test the functionality of the *SOR1* gene in *Arabidopsis*-based systems. However, additional experiments could be designed to evaluate SOR1 activity further by using RNAi (Guo *et al*., 2003) to silence *SOR1* or attempting to overexpress *SOR1* in *Sorghum* spp. Further, studies are also underway to compare the fatty acid composition in sorghum roots with and without root hairs. By understanding more about gene regulation of root exudate biosynthesis in higher plants, one may be able to enhance root exudate production for additional weed suppression or even transfer key genes for the biosynthesis of sorgoleone into important crop species which do not currently produce bioactive root exudates.

Acknowledgements

We are very grateful to Dr Scott Baerson (USDA-ARS-NPURU, National Center for Natural Products Research, Oxford, MS, USA) for providing RNA samples, facility, and technical expertise for Real-time PCR analysis of multiple tissues of sorghum SX17, Dr Susheng Gan for valuable suggestions and comments related to manuscript preparation, and Roselee Harmon for her technical support in differential display and HPLC analysis.

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