The Auxin-induced Maize Gene ZmSAUR2 Encodes a Short-lived Nuclear Protein Expressed in Elongating Tissues*

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Received for publication, December 10, 2002, and in revised form, April 11, 2003
Published, JBC Papers in Press, April 14, 2003, DOI 10.1074/jbc.M212585200

By differential screening of a cDNA library from auxin-induced maize coleoptiles we have isolated and characterized a SAUR gene, designated ZmSAUR2, belonging to a not yet characterized subtype of the SAUR family. ZmSAUR2 encodes a 15.3-kDa protein and is specifically induced by auxin in elongating coleoptile tissue but not in primary leaves or in roots. The transcript level rapidly increased within minutes and preceded auxin-stimulated elongation of coleoptile segments. Cycloheximide also induced ZmSAUR2 transcription, as has been shown for other early auxin-induced genes, whereas abscisic acid, brassinolide, ethylene, gibberellic acid, kinetin, and methyl jasmonate did not provoke an increase in ZmSAUR2 mRNA abundance. In pulse-chase experiments using auxin-induced coleoptiles and an anti-ZmSAUR2 antibody we were able to precipitate a protein of the expected molecular mass and to determine a half-life of about 7 min, which is among the shortest known in eukaryotes. In gel shift assays binding of calmodulin to ZmSAUR2 was demonstrated, suggesting the possibility of post-transcriptional regulation. Upon transformation of onion epidermal cells with a ZmSAUR2::GUS construct the corresponding chimeric protein was detected in the nucleus. The results suggest that ZmSAUR2 encodes a short-lived nuclear protein that might be involved in auxin-mediated cell elongation.

The plant hormone auxin influences a variety of processes during plant growth and development, including cell elongation, cell division, differentiation, control of tropisms, and apical dominance. How auxin mediates and controls these effects on a molecular and cellular level is still far from being understood. Besides modulating membrane function it has clearly been demonstrated that auxin is able to cause rapid changes in the expression of a selected set of genes. Several sequence elements within the promoters of auxin-responsive genes have been described that are up-regulated by auxin (for review, see Refs. 1–3). Of major interest are genes that are specifically induced within minutes after auxin application in the absence of protein synthesis and are referred to as early or primary auxin-responsive genes. Three major classes are known: Aux/IAA, SAUR, and GH3 families (for review, see Ref. 3). It was shown that apart from auxin, Aux/IAA and SAUR genes can also be induced by cycloheximide, and it is assumed that a pool of repressors, rapidly turning over, is involved in their transcriptional regulation. The very low abundance of the derived proteins, their short half-lives (as far as analyzed), their nuclear localization, and the absence of a catalytic domain point to an involvement in auxin signal transduction and in control of secondary downstream genes. Whereas for Aux/IAA proteins increasing evidence emerges that they act as repressors, including repressing their own transcription (for review, see Ref. 4), the function of SAURs still remains obscure, and even the experimental proof at the protein level is missing.

SAUR genes (for small auxin up RNA) were originally identified and characterized in soybean (5) and found to encode a set of unstable transcripts that are induced by auxin within minutes. SAURs are abundant in the zone of elongation in soybean hypocotyls and are expressed most strongly in epidermal and cortical cells (6). Meanwhile SAURs have been identified in a variety of organisms, in Vigna radiata (7), Pisum sativum (8), Arabidopsis thaliana (9), Nicotiana tabacum (10), Raphanus sativus (11), Malus domestica (12) and more recently also in Zea mays (13). Genome analysis in A. thaliana reveals more than 70 SAUR homologs, which mostly are found in clusters (3). However, it is not known how many of them are really expressed and are auxin-inducible. With one exception (AtSAUR11), all SAUR genes appear to lack introns (3). SAURs encode unstable mRNAs (14, 15), and their high turnover rate may be due to a conserved downstream (DST)* element in the 3′-non-translated region of the mRNA (16, 17) as well as elements within the coding region (9, 18). There is evidence that SAURs are not only transcriptionally regulated but are also regulated at the post-transcriptional (19) and post-translational levels (13). Recently it was demonstrated that SAUR proteins bind calmodulin in vitro, pointing to a possible “cross-talk” between the Ca2+/calmodulin (CaM) second messenger system and auxin signal transduction (13).

Auxin-induced cell elongation of maize coleoptiles is one of the fastest phytohormonal responses known, and this organ has been widely used as a model system to study regulation of cell growth and tropisms (20–24). To detect genes involved in the elongation growth of maize, we carried out a differential screening of a cDNA library from auxin-induced coleoptiles and report here the isolation and characterization of a cDNA clone, designated as ZmSAUR2 because of its homology to SAUR genes. ZmSAUR2 shows typical characteristics of primary auxin-responsive genes; it is induced within minutes exclusively by auxin and by the protein biosynthesis inhibitor cycloheximide.

* This work was supported by Deutsche Forschungsgemeinschaft Grant SFB 521 and by a grant from Fonds der Chemie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. This paper is dedicated to Meinhart H. Zenk on the occasion of his 70th birthday.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X79211. 1 To whom correspondence should be addressed. Tel.: 49-941-943-3043; Fax: 49-941-943-3352; E-mail: ludwig.lehle@biologie.uni-regensburg.de.

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In addition, sequence motifs of SAURs, such as nuclear localization (NLS) and DST can be identified. For the first time we succeeded in detecting the corresponding SAUR protein and affinity chromatography according to the manufacturer (New England Biolabs). The C-terminal 566-bp fragment of the coding region of Zm-
SAUR2 was used as a probe and was cloned in-frame into pMAL-c2. Antibodies were raised in rabbits against the fusion protein and purified by affinity chromatography using the fusion protein immobilized on a HiTrap affinity column.

Correlation with ZmSAUR2—[35S]-Methionine/cysteine-labeled Zm-
SAUR2 was produced using the Promega TNT coupled transcription-
translation reticulocyte lysate system. As template 1 μg of pTR2ZDNA (pBS vector with the ZmSAUR2 cDNA as an insert under the control of the T7-promotor) was added and incubated as described in the produc-
ter’s manual. 1 μl of lysate containing 35S-labeled ZmSAUR2 was incu-
bated with 2 and 0.4 μg of bovine CaM as indicated for 1 h at room temperature in 100 mM Tris-HCl (pH 7.2) containing 1 mM CaCl2 in a total volume loaded onto 15% polyacrylamide gels in 375 mM Tris/HCl (pH 8.8) and 1 mM CaCl2. Electrophoresis was performed at a constant current of 25 mA in running buffer consisting of 25 mM Tris/HCl (pH 8.3), 192 mM glycine, and 1 mM CaCl2 followed by autoradiography.

Plant Transformation and GUS Staining—For transformation of onion cells epidermal layers were peeled and placed inside-up on Petri dishes containing Chu basal salt mixture powder (Sigma C1416) supplemented with 3% sucrose and 0.7% agar ultrapure. Plant tissue was bombarded with 1.6-μm gold particles coated with DNA by ethanol precipitation using a Biological PDS-1000He particle gun (Bio-Rad). Screening for GUS activity was performed according to Hall et al. (29).

Other Nucleic Acid Techniques—For construction of pBL221-Zm-
SAUR2-GUS the ZmSAUR2 coding sequence (without the stop codon) was amplified by PCR and cloned in-frame to the GUS gene in pBI 21 (Clontech). Standard procedures for cloning and DNA manipulation were carried out as described by Sambrook et al. (30).

RESULTS

Isolation of ZmSAUR2 cDNA and Sequence Analysis—In an effort to isolate genes that may be involved in auxin-mediated cell elongation, a cDNA library in Agt10 was constructed from poly(A)-mRNA of subapical excised segments of etiolated maize coleoptiles that were depleted of auxin for 60 min and subse-
quently induced by 10 μM IAA for 2 h. This library was differen-
tially screened with radiolabeled cDNA probes synthesized from mRNA of excised coleoptile segments floated in 10 μM IAA and water, respectively. Of 50,000 plaques, 42 showed a differen-
tial response. After two rounds of rescreening and Southern blot analysis, eight positive clones were left. One of them was further characterized and is designated as ZmSAUR2 because of its homology to the SAUR gene family and its inducibility by auxin. The sequence of the cDNA clone is shown in FIG. 1. The ZmSAUR2 open reading frame of 432 nucleotides (bp 64—495) encodes a polypeptide of 144 amino acid residues with a calcu-
lated molecular mass of 15.3 kDa and an isoelectric point of 7.3.
The sequence flanking the first possible ATG is consistent with the consensus context for eukaryotic translation initiation (31). Cell-free translation of an *in vitro* transcript of ZmSAUR2 in a wheat germ extract produced a polypeptide of the expected size (see Fig. 5, lane 1). ZmSAUR2 has neither large hydrophobic domains nor an N-terminal ER signal sequence but has a putative bipartite nuclear localization signal (NLS) of the type KK\_X\_5KK\_X\_7RRR (32). Also a conserved casein kinase II phosphorylation site at Ser77 can be identified. In the 3'-non-translated region a putative DST element is present that was shown to be responsible for the rapid decay of SAUR transcripts (17, 33).

A comparison of the predicted amino acid sequence of Zm-SAUR2 with other protein sequences compiled in databases reveals that ZmSAUR2 can be grouped into a cluster separate from SAURs previously characterized (Fig. 2). In *Arabidopsis* eight of them are localized to chromosome I (e.g. At1g29430, At1g29450, At1g29460), but also other chromosomes contain members of this subgroup (e.g. At5g27780). Within the AP005862 locus of *Oryza sativa* an open reading frame can be identified (between bp 54216 and 54651) that has the highest homology to ZmSAUR2, 67%. Most members of this subgroup are composed of about 144 amino acids and are, therefore, larger than the SAURs characterized earlier (e.g. GmSAUR-10A5 with 93 amino acids). The larger size of the ZmSAUR2 subtype is due to N-terminal and C-terminal extensions. In the N-terminal region the identity within the ZmSAUR2 subgroup (Met1-Arg24, numbers refer to ZmSAUR2) amounts to 82%. This domain is not present in the subtype represented by GmSAUR-10A5 (Fig. 2). ZmSAUR1, the only other described SAUR from maize, is as large as ZmSAUR2 and also comprises a long N-terminal extension, but its homology to ZmSAUR2 within this region is rather low. Therefore, it might cluster to a third subgroup. The C-terminal region (67% identity between Asp\_5Val\_144) is a further characteristic for the ZmSAUR2 subtype, although some of its motives are only found in members from *Arabidopsis*. In contrast, the middle region between Lys\_45 and Pro\_93 is not only conserved within the ZmSAUR2 subgroup (77% identity) but also in the subtype represented by GmSAUR-10A5 (50%) and in ZmSAUR1 (40%). Especially, the EEE(Y/F)G motif (between amino acids 78 and 83) as well as amino acids that might be important for secondary structure, such as Pro, Glu/Asp, or Arg, are highly conserved.

**Auxin Induction and Tissue-specific Expression of ZmSAUR2**

**Time Dependence**—Northern blot analysis of mRNA from freshly harvested coleoptile segments gives a weak ZmSAUR2 signal that after 60 min of incubation in water is no longer detectable, presumably due to depletion of endogenous auxin (Fig. 3A, lanes 1 and 2). Subsequent addition of 10 \( \mu \)M IAA to the medium leads to a strong increase of the ZmSAUR2 transcript level. Already after 10 min of induction (the earliest time point taken in this experiment) it is higher than the *in situ* level and increases further within the following 60 min. Berghfeld et al. (34) show that exogenous application of IAA to auxin-depleted coleoptile segments reinduces elongation after a lag period of about 16 min. Thus, ZmSAUR2 transcripts appear before the segments start to elongate. In lane 6, RNA is analyzed from coleoptile segments incubated for an additional 60 min as in lane 5 but in the absence of auxin, and a reappearance of ZmSAUR2 mRNA is observed. This correlates with
the restoration of elongation of auxin-depleted coleoptile segments that occurs within 2.5–3 h after excision and is caused by the regeneration of the physiological tip (35).

Specificity of Auxin Induction—To test the specificity of ZmSAUR2 induction, coleoptile segments were incubated with abscisic acid, kinetin, ethylene, gibberellic acid, brassinolide, or methyl jasmonate as well as with the synthetic, physiologically active auxin analogs /H9251-naphthalene-acetic acid and 2,4-dichlorophenoxyacetic acid and the physiologically inactive 3,5-dichlorophenoxyacetic acid. As shown in Fig. 3B, ZmSAUR2 is only induced by IAA, /H9251-naphthalene-acetic acid, and 2,4-dichlorophenoxyacetic acid, but not by 3,5-dichlorophenoxyacetic acid or any of the other phytohormones. As in the case of other auxin-inducible early genes an accumulation of ZmSAUR2 transcripts could be observed in the presence of the protein synthesis inhibitor cycloheximide (CHX, lane 16). A stop of protein synthesis is assumed to cause the disappearance of a putative repressor of transcription or of a labile nuclease responsible for RNA degradation (1). Alternatively it is also discussed that the translational arrest could prevent the nucleolytic breakdown of the transcripts by a mechanism not clearly understood (36). It has not yet been tested as to whether mRNA stabilization or activation of transcription is responsible for the increase in ZmSAUR2 transcript level. Indeed, there is evidence for the existence of both mechanisms among SAUR genes (18, 19).

Tissue Expression Pattern of ZmSAUR2—As shown in Fig. 4, ZmSAUR2 transcripts could be detected only in the coleoptile and in the mesocotyl but not in the root and in the primary leaf of maize seedlings. Furthermore, the amount of ZmSAUR2 transcripts correlated well with the light-dependent elongation growth of the coleoptile (Fig. 4C, lane 1–3). Thus, Northern blot analysis of RNA from coleoptiles and mesocotyls grown for 5 days in the light or 4 days in the dark plus 1 day in the light displays a decreased ZmSAUR2 mRNA level compared with seedlings that were kept for 5 days exclusively in the dark. This is consistent with the growing behavior of maize coleoptiles and mesocotyls (Fig. 4D). In the first 4 days growth of coleoptiles is independent of exogenous light. After this period light-grown coleoptiles stop elongating, and the primary leaf breaks through the coleoptile, whereas in dark-grown coleoptiles further elongating takes place. In contrast mesocotyl growth is inhibited by light from the beginning.

ZmSAUR2 Protein Is Very Short-lived—Metabolic labeling experiments allowed us for the first time to detect a protein of the SAUR gene family in vivo. Auxin-induced coleoptiles were pulse-labeled with [35S]methionine/cysteine and subsequently chased for different times with nonradioactive amino acids as indicated. Coleoptiles were lysed, and the soluble protein fraction, obtained upon centrifugation, was immunoprecipitated by an antiserum raised against ZmSAUR2 and analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 5, the protein precipitated by the ZmSAUR2 antiserum has an extremely short half-life of about 7 min, similar as the auxin-
inducible PS-IAA4 and PS-IAA6 proteins, members of the Aux/IAA family (37). An in vitro transcribed and translated ZmSAUR2 migrated with the same mobility (Fig. 5, lane 1). Therefore, we assume that the precipitated, short-lived protein most probably is ZmSAUR2 (see “Discussion”).

**Intracellular Localization of ZmSAUR2**—The presence of a putative nuclear localization signal (Fig. 1) raised the prospect that ZmSAUR2 resides in the nucleus. To test this, the GUS reporter gene was fused to the 3′ end of the coding sequence of ZmSAUR2 under the control of the constitutive cauliflower mosaic virus 35 S promoter (pBI-221-ZmSAUR2-GUS). The chimeric gene construct was introduced into onion epidermis mosaic virus 35 S promoter (pBI-221-ZmSAUR2-GUS) under the control of the constitutive cauliflower mosaic virus 35 S promoter (pBI-221-ZmSAUR2-GUS). The results suggest that CaM binding is specific.
The homology of ZmSAUR2 with other characterized SAUR genes is rather low, and e.g. for GmSAUR-10A5 as a typical representative, it amounts to an identity of only 36% (Fig. 2). Surprisingly, this is also true for the other recently identified ZmSAUR1 from maize, which hitherto is the only characterized SAUR from monocots. On the other hand the various sequencing projects reveal putative, not yet characterized SAUR genes such as a rice open reading frame within the AP 005862 locus, which has 67% homology to ZmSAUR2. Also in Arabidopsis, on chromosome I eight open reading frames could be identified in a cluster that are more closely related to ZmSAUR2. This subtype is characterized by N- and C-terminal extensions, which make these SAURs larger than members of the subtype represented by GmSAUR-10A5. ZmSAUR1 was isolated from a corn root cDNA library in a CaM binding screening approach (see below for an additional discussion).

Although it is also larger than GmSAUR-10A5, its extensions are different from ZmSAUR2. In contrast the central portion, most probably important for function, is far more conserved in ZmSAUR1 and the ZmSAUR2 subtype as well as in the best characterized SAURs related to GmSAUR-10A5.

Another important sequence typical for SAURs is the DST element in the 3'-untranslated region. It is also present in ZmSAUR2 and fits well with the SAUR consensus GGAX^_–TAGATX_GTA as shown in Fig. 1. DST sequences were demonstrated to contribute to the instability of SAUR transcripts, which are among the most short-lived mRNAs in higher plants (16, 18, 33). In fact, two mutants (dst1 and dst2), characterized by a defect for DST-dependent degradation, display an elevated level of SAUR-AC1 mRNA (44). Besides the DST element, also the coding region seems to destabilize SAUR mRNA (45).

Despite several attempts SAUR proteins have been failed to be detected (3). This could be due to an instability of SAURs or because of their low abundance. We also failed to detect ZmSAUR2 when using Western blot analysis. But by metabolically labeling excised and auxin-stimulated coleoptile segments with ^35S)methionine/cysteine followed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis, we were able to detect a protein with the same mobility as the in vitro labeled ZmSAUR2. Because it is possible that Z. mays, like Arabidopsis, contains multiple, although not yet identified ZmSAUR2-like proteins (see above), we cannot exclude the possibility that the antibody raised against ZmSAUR2 may cross-react with such closely related proteins. In pulse-chase experiments a half-life of 7 min was calculated for the immuno-precipitated protein, which is as short as that of members of the Aux/IAA family (PS-IAA4, 8 min; PS-IAA6, 6 min (37)) and is among the shortest known for eukaryotic proteins. As for ZmSAUR2, also in the case of the Aux/IAA proteins, immunoblotting was not successful in detecting these short-lived pro-
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teins. Only a mutated Aux/IAA protein could be detected by Western blotting because the mutation caused a stabilization of the protein and led to an increased amount. There is evidence that auxin influences the stability of Aux/IAA proteins (46, 47), and it would be interesting to test whether this is also true for SAUR gene products. An auxin-dependent degradation by a nuclear localized ubiquitin/proteosome system is suggested to play a central role in auxin action. Among others, Aux/IAA proteins are candidates for auxin-regulated destruction (for review, see Ref. 4).

Another feature, which SAURs seem to have in common with Aux/IAAs, is the nuclear localization. Gil et al. (9) already speculate that SAURs may reside in the nucleus, as they identified a bipartite NLS in AtSAUR-AC1. Such NLSs are found in most SAURs, and this motif can also be identified in ZmSAUR2, albeit the N terminus in which it is located is only poorly conserved. Our finding that a ZmSAUR2:GUS fusion protein is predominantly found in the nucleus is the first evidence that these NLSs are functional and SAURs reside in the nucleus.

In view of the nuclear localization and the instability of ZmSAUR2 one may speculate SAURs fulfill their function as transcriptional regulators mediating downstream auxin responses similar to Aux/IAA proteins. A possible target candidate could be the recently identified secondary auxin-induced K⁺ channel ZMK1, which seems to be an essential element of the osmotic motor in auxin-mediated growth. ZMK1 transcripts are differentially expressed in the two coleoptile flanks during gravitropic bending and appear only 30 min after auxin induction (48), i.e., after induction of ZmSAUR2.

The transcriptional regulation of ZmSAUR2 is in accordance with other SAURs and shown to be auxin-specific. From the strong signal of ZmSAUR2 mRNA measured at 10 min and the further time-dependent increase of the transcript level (Fig. 3) one may infer that induction starts earlier than 5 min, similar to soybean SAUR mRNAs. Thus, ZmSAUR2 transcripts precede auxin-induced cell elongation, for which in corn coleoptiles a lag phase of growth of about 15 min was measured (34, 48). Compared with the induction kinetics of the recently identified ZmSAUR1, the ZmSAUR2 gene is more rapidly induced; in addition, ZmSAUR2 is not expressed in roots (13).

With respect to the hormone specificity one should mention that brassinolide, which is also able to cause cell elongation in biological assays developed for auxin (Ref. 49; for review, see Ref. 50), did not induce ZmSAUR2 transcription. Similarly, expansin LeExp2, which is thought to be a target gene for auxin signaling, is also not induced by brassinolide (51). On the other hand in the auxin-insensitive tomato mutant dgt (diaugtropica) brassinolide is able to stimulate elongation growth, suggesting independent signal pathways for auxin- and brassinolide-regulated processes (52).

The tissue-specific expression examined in Northern blot analysis defines ZmSAUR2 only to the coleoptile and the mesocotyl but not to the primary leaf or the roots of maize seedlings. The results are in agreement with the expression pattern of other SAUR genes, which correlates also with auxin-induced cell elongation, although tissue specificity is somewhat variable (Ref. 45; for review, see Ref. 53).

ZmSAUR1 has been isolated by screening an expression library with radioactive CaM (13). Biochemical analysis of the in E. coli heterologously expressed protein showed that the CaM binding site is located at the N-terminal domain that potentially is able to form a basic amphiphilic α-helix. Such a structure can also be identified in ZmSAUR2. In mobility shift assays using [35S]-labeled ZmSAUR2 we were able to demonstrate an interaction with CaM. The CaM binding was specific, because nonradioactive ZmSAUR2 could compete with labeled ZmSAUR2, whereas bovine serum albumin was not able to do so. One may speculate that ZmSAUR2 function may be influenced by a calcium signal. Indeed rapid changes in the cytosolic Ca²⁺ were detected in maize coleoptiles after auxin treatment and after photo- and geotropic stimulation. Such changes were noticeable within 5 min (54, 55). Because no catalytic domain could be identified in SAURs, Ca²⁺/CaM may influence their binding to other factors. It is known that some DNA-binding proteins are able to bind CaM. For transcription factors of the basic-helix-loop-helix group it was reported that Ca²⁺/CaM binding directly inhibits their DNA binding (56). Also for the Arabidopsis transcription factors TGA3 and DL10, an interaction with CaM was shown (57, 58). Therefore, one may speculate that SAURs represent an important integrative element of the cross-talk between the Ca²⁺/CaM messenger system and the auxin signal transduction.

Although the precise function of SAUR genes remains unclear at this time, the demonstration of the existence of a SAUR protein for the first time together with its very short half-life, its nuclear localization, and the rapid induction kinetics of the encoding gene support the notion that SAURs may play an important role in early auxin signal transduction cascades.

Acknowledgments—We thank Dr. A. Hallmann for help in onion cell transformation. We are also grateful to L. Rossini for providing plasmid pRTL2-GUS/NIA.

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