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Expression of *Pisum sativum* SAD polypeptides in production hosts and *in planta*: Tetrameric organization of the protein

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ABSTRACT

In *Pisum sativum*, the short-chain alcohol dehydrogenase-like protein (SAD) gene family consists of at least three members (*SAD-A*, *-B*, and *-C*). Expression of two of these genes (*SAD-A* and *-C*) in *Escherichia coli* or *Pichia pastoris* resulted in full-length soluble proteins. Purified SAD-A was used as antigen for antibody production in rabbits. With these antibodies the recombinant SAD-C protein (which was most highly expressed of the two isoforms) was shown to be a tetramer consisting of a dimer of dimers. The *SAD* genes are transiently expressed in plants by short exposures to ultraviolet-B radiation (UV-B), as judged by northern blotting. In turn, mRNA accumulation leads to formation of SAD protein in leaf and stem tissue upon prolonged UV-B irradiation.

Introduction

The expression of a small gene family of at least three genes (*SAD-A*, *SAD-B*, *SAD-C*; short-chain alcohol dehydrogenase-like proteins) was found to be up-regulated in pea (*Pisum sativum*) at the mRNA level as the result of a low intensity ultraviolet-B radiation (UV-B; 280–315 nm) treatment and by several other abiotic stresses [1]. Compared with other UV-B-regulated genes, such as those encoding phenylalanine ammonia lyase and chalcone synthase (PAL and CHS, important enzymes in the strongly UV-B-induced phenylpropanoid and flavonoid biosynthetic pathways), the *SAD* genes were on the mRNA level activated more rapidly and at significantly lower UV-B levels. It was also shown in the same study that stress-induced accumulation of mRNA for the *SAD* genes occurred differently in separate tissues [1]. In leaves, exposure of pea plants to UV-B results in *SAD* mRNA accumulation in both epidermal and mesophyll cells [2].

A study of the promoters of the *SAD-A* and *SAD-C* genes identified the presence of a novel 11-bp GC-rich motif that was shown to bind nuclear factors using the electrophoretic mobility shift assay [3]. This *SAD* promoter binding motif (SPBM) has features in common with a number of previously recognized classes of *cis*-elements involved in stress-correlated regulation of gene expression in plants, which may explain the responsiveness of the *SAD* genes to a wide variety of stresses.

Searches of sequence databases using the *SAD-A*, *-B*, and *-C* cDNA sequences (GenBank Accession Nos. AF053638, AF053639, and AF097651, respectively) or translated amino acid sequences revealed homologies to the short-chain dehydrogenase/reductase (SDR)[4] superfamily. The highest similarities were seen with sequences from other plants: Δ^5-3β-hydroxysteroid dehydrogenase (HSD; EC 1.1.1.145), a multifunctional enzyme in steroid metabolism, found in *Digitalis lanata* as well as in *Solanum tuberosum* and *Nicotiana tabacum* [4]; secoisolariciresinol dehydrogenase, found in stem tissue of the dicot *Forsythia intermedia* and involved in biosynthesis of lignans [5]; GAD3 protein of *Lycopersicon esculentum* that was found to be induced after treatment of shoot tissue with gibberellic acid [6]; and the TASSELSEED2 protein that is involved in sex determination in maize [7]. In addition, the model plant *Arabidopsis*

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[4] *Abbreviations used:* MALDI, Matrix assisted laser desorption ionization; MS, mass spectrometry; Q-TOF, quadrupole time-of-flight; SAD, short-chain alcohol dehydrogenase-like protein; SDR, short-chain dehydrogenase/reductase.

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thaliana contains at least eight different genes orthologous to the pea *SADs*, with similarity scores of the translated sequences of between 57% and 68% compared with the pea proteins.

The SDR superfamily contains over 3000 members, present in species from bacteria to humans. These are NAD(H)- or NADP(H)-dependent enzymes with a wide variety of substrates including alcohols, steroids, sugars, and xenobiotics [8,9]. Plant SDR enzyme activity has been identified in several different tissues, including leaf [10], stem [5], flower [7], and tuber [11]. An SDR-related endoribonuclease localized in the chloroplast [12] is present in all green tissues. The distribution of other SDRs is more tissue-specific. Secretory cells of epidermal oil glands in peppermint and spearmint are highly enriched in transcripts for isopiperitenol dehydrogenase, an enzyme involved in monoterpene biosynthesis [13].

The pea SAD proteins were similar to each other, SAD-A and SAD-C only differed by three amino acids out of 268 [1]. SAD-B was more distantly related to the other two isoforms (94% identity to SAD-A; Ref. 1), the most important difference being the loss of part (12 amino acids) of the nucleotide binding site. A sequence alignment of the three pea SAD proteins, together with *Mentha haplocalyx* (−)-isopiperitenol dehydrogenase, *D. lanata* 3β-hydroxysteroid dehydrogenase and *Podophyllum* secoisolariciresinol dehydrogenase, is shown in Fig. 1. The latter protein shares 46% sequence identity with SAD-C and has a known three-dimensional structure [14]. The *Mentha* protein has the highest similarity to the pea SADs of any polypeptide for which an enzymatic function is known (55%; GenBank Accession No. ABR15425) and the *Digitalis* protein also shows high similarity (49%; Ref. [4]).

In order to more closely study the pea SAD proteins, the present study was conducted. The different SAD isoforms were over-produced as recombinant proteins in *Escherichia coli* and *Pichia pastoris* for antibody production and in order to characterize their oligomeric structure. Northern blotting and immunoblotting was used after UV-B induction to study gene expression patterns *in planta*.

Materials and methods

Cloning of the SAD genes for expression in E. coli and P. pastoris

The pea *SAD-A* (AF053638), *SAD-B* (AF053639), and *SAD-C* (AF097651) open reading frames were amplified by PCR utilizing primers introducing a XhoI and a MluI restriction enzyme site at the 5′ and 3′ ends of the open reading frame, respectively. After restriction, the genes were cloned into the *E. coli* expression vector pET8c [16], which permits overexpression of the proteins under control of the T7 promoter and adds a His_6-tag to the N-termini of the proteins. The correct sequence of all constructs was verified by DNA sequencing.

The *SadC* open reading frame was amplified by PCR utilizing primers introducing a SfuI and a ApaI restriction enzyme site at the 5′ and 3′ ends of the open reading frame, respectively. After restriction, the gene was cloned into the vector pPICZ-A (Invitrogen, Breda, The Netherlands). This construct was restricted with SacI and transformed into *P. pastoris* strain X33.

Purification of recombinant SAD protein

Escherichia coli strain BL21(DE3)pLysS containing the new constructs was grown in LB-medium containing 2.5 mM betaine until the absorbance of the bacterial culture at 600 nm (A_{600}) had reached 0.7. Then, protein expression was induced by addition of isopropyl-β-D-thiogalactoside to a final concentration of 0.1 mM. Three hours after induction the culture was harvested and frozen in liquid nitrogen and stored at −20 °C until further use. The cells were broken in an X-press (AB Biox, Göteborg, Sweden) and resuspended in 50 mM sodium phosphate (pH 8.0), 200 mM NaCl, 5 mM L-histidine, 0.05% Tween 20, 1 mM phenylmethyl sulphonyl fluoride and sonicated on an ice bath for 10 min. Unbroken cells were removed by centrifugation and the membrane and cytoplasmic fractions were separated by ultracentrifugation (Beckman 70 Ti, 45,000 rpm, 45 min, 4 °C). The cytosolic fraction was mixed with Ni-NTA resin (Qiagen, Hilden, Germany) and incubated on rotating rolls for 1 h and then transferred to a chromatography column. The column was washed with the same buffer as above and the recombinant protein eluted with the same buffer that had been supplemented with 150 mM L-histidine. The protein concentration was determined by using the Bio-Rad protein assay, based on the Bradford method, and according to the manufacturer's instructions (Bio-Rad, Hercules, CA).

Pichia pastoris cells containing the *SAD-C* gene under control of the alcohol oxidase promoter was grown at 30 °C from a single colony in 25 ml of MGYH media (1.34% yeast nitrogen base, 1% glycerol, 4×10^{-5}% biotin, and 0.004% histidine) overnight. The cells were harvested and resuspended into 1 L of MMH media (1.34% yeast nitrogen base, 0.5% methanol, 4×10^{-5}% biotin and 0.004% histidine), with methanol added every 24 h to keep the final concentration at 0.5% and thereby to maintain expression. The cells were harvested after 48 h and frozen in liquid nitrogen. The *P. pastoris* cells were broken and the recombinant SAD-C protein was purified as described for the recombinant protein expressed in *E. coli*.

Antibodies and Western blotting

Recombinant SAD-A protein was further purified by anion-exchange chromatography on Whatman DE32 according to the manufacturer's instructions (Brentford, UK). Then, either native or SDS-denatured protein was used to inject rabbits for production of antisera. Immunisation, maintenance of rabbits and collection of serum was carried out by Davids Biotechnologie (Regensburg, Germany) and antiserum against SAD-A can now be obtained from F:a Rubrum (Örebro, Sweden). Protein from pea leaf extracts (14 μg) was separated by SDS–PAGE on pre-cast gels (Ready-Gel, Bio-Rad, CA, USA) and the proteins transferred by electroblotting onto Hybond-C pure membrane (Amersham Biosciences, Uppsala, Sweden). Western blotting using the SAD protein antibodies was performed according to a standard method described in the manual from Qiagen (Hilden, Germany). Visualisation was then obtained by using alkaline phosphatase-conjugated anti-rabbit IgG goat antibodies yielding stained bands on the immunoblot when catalyzing the reaction between nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) according to the standard protocol (Promega, Madison, WI). The purification of recombinant protein was sometimes also visualized using penta-His-tag antibodies according to the manufacturer's instructions (Qiagen, Hilden, Germany). For analysis of the content of SAD protein in plant tissue, the loading of the wells was checked using a second antibody towards a house-keeping protein (ATPsynthase β subunit; AgriSera AB, Vännäs, Sweden). After the use of the SAD antibody the membrane was incubated in stripping solution (2% SDS; 62.5 mM Tris–HCl, 6.7; 100 mM 2-mercaptoethanol) at 50 °C for 30 min. The membrane was thereafter washed 2 × 10 min in TBS-T before application of the ATPsynthase β subunit antibody (dilution 1:2500). For detection of this antibody, the ECL detection system was used: Blots were washed in TBS-Tween and incubated in secondary horseradish peroxidase (HRP)-conjugated goat-anti-rabbit IgG antibody at a dilution 1:2500 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h. After a final wash in TBS-T, HRP was detected using the Immun-Star HRP Substrate Kit from Bio-Rad and a