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Novel interaction of selenium-binding protein with glyceraldehyde-3-phosphate dehydrogenase and fructose-bisphosphate aldolase of *Arabidopsis thaliana*

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Abstract. The metabolic role and regulation of selenium, particularly in plants, is poorly understood. One of the proteins probably involved in the metabolic regulation of this element is the selenium-binding protein (SBP) with homologues present across prokaryotic and eukaryotic species. The high degree of conservation of SBP in different organisms suggests that this protein may play a role in fundamental biological processes. In order to gain insight into the biochemical function of SBP in plants we used the yeast two-hybrid system to identify proteins that potentially interact with an *Arabidopsis thaliana* (L.) Heynh. homologue. Among the putative binding partners of SBP, a NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a fructose-bisphosphate aldolase (FBA) were found as reliable positive candidates. The interaction of these proteins with SBP was confirmed by *in vitro* binding assays. Previous findings in *Escherichia coli*, demonstrated the direct binding of selenium to both GAPDH and aldolase. Therefore our results reveal the interaction, at least in pairs, of three proteins that are possibly linked to selenium and suggest the existence of a protein network consisting of at least SBP, GAPDH and FBA, triggered by or regulating selenium metabolism in plant cells.

Keywords: FBA, fructose-bisphosphate aldolase, GAPDH, GST, glutathione S-transferase, glyceraldehyde-3-phosphate dehydrogenase, selenium, selenium-binding protein.

Introduction

Selenium is an essential micronutrient for animals and microorganisms but in elevated concentrations it can result in toxic effects. In plants, the essentiality of selenium is still an open question (Lauchli 1993; Terry *et al*. 2000). The importance of selenium for normal cellular function can be explained, at least in part, by its incorporation as selenocysteine in a unique class of enzymes named selenoproteins, such as glutathione peroxidase, selenophosphate synthetase and formate dehydrogenase (Gladyshev and Kryukov 2001; Driscoll and Copeland 2003; Kryukov *et al*. 2003; Kryukov and Gladyshev 2004). In photosynthetic organisms, however, such essential selenoproteins have been identified only in the unicellular green algae (Fu *et al*. 2002; Novoselov *et al*. 2002), but not in higher plants. Although the mechanism of specific incorporation of selenium into proteins in the form of selenocysteine has been studied extensively in microorganisms and in animals, little is known about other possible metabolic role(s) of selenium or its physiological regulation in plants and animals.

One of the proteins that is likely to be involved in selenium metabolism is the selenium-binding protein (SBP, originally termed SBP56), which was originally identified by labelling experiments in mouse liver as one of the main cytosolic proteins able to bind radioactive selenium (Bansal *et al*. 1989, 1990), by an as yet unidentified mechanism. Homologues of SBP exist in many eukaryotic and prokaryotic organisms, sharing a significantly conserved amino acid sequence, comparable to that of the histone

Abbreviations used: FBA, fructose-bisphosphate aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; SBP, selenium-binding protein.

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(Thatcher and Gorovsky 1994) or actin (Egelman 2003) protein families. Although the physiological function of SBP remains unknown, studies based primarily on animal systems associate SBP with the inhibition of cell proliferation and anti-carcinogenic growth regulation (Lanfear *et al*. 1993; Yang and Sytkowski 1998; Chen *et al*. 2004), with acetaminophen-induced hepatotoxicity (Bartolone *et al*. 1992; Pumford *et al*. 1992; Lanfear *et al*. 1993; Qiu *et al*. 1998; Ishida *et al*. 2004), interaction with other minerals (Jamba *et al*. 1997; She *et al*. 2003), detoxification (Ishii *et al*. 1996*a*, *b*; Ishida *et al*. 1998, 1999), intra-Golgi protein transport (Porat *et al*. 2000), peroxisome proliferation (Giometti *et al*. 2000; Chu *et al*. 2004), senescence (Cho *et al*. 2003), rapid cell outgrowth and motility (Miyaguchi 2004) and oxidative stress response (Yang *et al*. 1998; Gracey *et al*. 2001; Casey *et al*. 2002; Fajardo *et al*. 2004).

In plants, processes related to root nodule formation and function during the symbiosis between *Lotus japonicus* and *Mesorhizobium loti* seem to require up-regulation of SBP, while under non-symbiotic conditions it is constitutively expressed in various tissues of the uninfected plants (Flemetakis *et al*. 2002). In rice, overexpression of SBP leads to enhanced resistance to different pathogens but it is also involved in responses to environmental stress (Sawada *et al*. 2003, 2004). Analyses in various plant species have also shown that SBP is transcriptionally regulated during biotic and / or abiotic stress (Schenk *et al*. 2000; Desikan *et al*. 2001; Liu and Baird 2003; Zhu-Salzman *et al*. 2004). Furthermore, our recent studies indicate that in *Arabidopsis thaliana* SBP plays a role in protection against selenium toxicity, and give extra support to the implication of SBP in selenium metabolism and its interaction with low concentrations of selenium or selenium metabolites (Agalou *et al*. 2005).

To study the function of SBP in plants we used the yeast two hybrid system to screen an *A. thaliana* cDNA library for proteins potentially interacting with SBP. Among the clones we identified in this manner were a NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a fructose-bisphosphate aldolase (FBA). Since GAPDH and a prokaryotic aldolase (deoxyribose-5-phosphate aldolase; DPA) were shown to bind selenium in *Escherichia coli* (Lacourciere *et al*. 2002), our results on the interaction of three proteins that are possibly linked to selenium suggest the existence of a novel biochemical network that is possibly part of the physiological regulation and metabolism of this element.

Materials and methods

Isolation of the A. thaliana *SBP cDNA (AtSBP)*

RT-PCR was performed with 1 µg of total RNA from 7-d-old wild type *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia-0) seedlings. The set of oligonucleotide primers used for the amplification was designed based on the nucleotide sequence available in the NCBI database for the putative SBP with accession number NM_117478. First-strand synthesis was performed with primer prAtSBP3 (5′-CATAGGCTCGAGTCAAATCCAGATATCGG-3′) and Expand reverse transcriptase according to the supplied protocol (Roche, Mannheim, Germany), and the subsequent PCR amplification with primers prAtSBP1 (5′-GAGAGAGATAGAGAGATGGCGACGG-3′) and prAtSBP3 using Herculase polymerase (Roche). PCR conditions were as follows: 3 min at 94°C, 30 cycles of 30 s at 94°C, 60 s at 54°C and 45 s at 72°C, followed by 10 min at 72°C. After amplification, the full-length SBP cDNA was cloned into the pGEM-easyT vector (Promega, Madison, WI) and sequenced (plasmid pMP7301).

Yeast expression plasmids

For the construction of the bait vector pODB8-AtSBP (pMP7201), two complementary oligonucleotides (prpODB1: 5′-CATGGGGTACCACTAGTC-3′ and prpODB2: 5′-CCGGGACTA GTGGTACCC-3′) with overhanging *Nco*I and *Xma*I sites containing an *Acc*65I restriction site were annealed and cloned into the pODB8 vector (Louvet *et al*. 1997), resulting in plasmid pMP3906. The full-length AtSBP cDNA was introduced as *Bsr*GI / *Xho*I fragment from pMP7301 plasmid to the *Acc65*I / *Xho*I sites of pMP3906 plasmid in translational fusion with the Gal4 DNA-binding domain (plasmid pMP3927). For the bait vector pAS2-1-AtSBP (pMP7202), the full-length AtSBP cDNA was introduced as *Eco*RI fragment from pMP7301 plasmid to the pAS2-1 vector (Clontech, Mountain View, CA) in frame with the Gal4 DNA-binding domain.

Bacterial protein expression plasmids

AtSBP cDNA from pMP7301 plasmid was fused as *Eco*RI fragment to the *C*-terminal of glutathione S-transferase (GST) in the pGEX3X vector (Amersham Biosciences, Diegem, Belgium) resulting in plasmid pMP7203. In order to obtain T7::6× His-tagged proteins of *A. thaliana* GAPDH and FBA (AtGAPDH and AtFBA), the respective coding regions were isolated from plasmids pMP7206 and pMP7209 and ligated into the *Eco*RI / *Xho*I sites of the pET28a and pET28b vectors (Novagen, Madison, WI) resulting in plasmids pMP7204 and pMP7205. All constructs were verified by restriction analysis and DNA sequencing. *E. coli* XL1 Blue was used as host for all plasmid constructions and propagations. BL-21 codon plus cells were used for bacterial expression of the recombinant proteins.

Sequence analysis

The SWISSPROT-TrEMBL, PFAM and GenBank databases were used for essentially all sequence searches. The amino acid sequence of the *A. thaliana* SBP (SWISSPROT accession number O23264) was used to identify homologues in the above database sets by the BlastP algorithm. The sequence of SBP of the scallop was recently published by Song *et al*. (2006). The phylogenetic tree was constructed by the neighbour-joining method (Saitou and Nei 1987) with ClustalW (http://Hypernig.nig.ac.jp; verified 30 May 2006) at the web server of the DNA Data Bank of Japan (DDBJ). ClustalW analysis was performed with default settings, without Kimura's correction. Bootstrap sampling was reiterated 10 000 times. For the matrix table 'blosum' was used. The gap extension penalty was set at 0.2 and the gap distance was set at 8. Trees were printed with the program Treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html; verified 30 May 2006). Genbank or SWISS-PROTein accessions used for the phylogenetic analysis are: *Anopheles gambiae*, EAA05714; *Arabidopsis thaliana* 1, NP_193139; *Arabidopsis thaliana* 2, NP_193140; *Arabidopsis thaliana* 3, NP_189022; *Bos taurus*, XP881423; *Bradyrhizobium japonicum*, BAC53217; *Caenorhabditis briggsae*, CAE71767; *Caenorhabditis elegans*, NP 502691; *Canis familiaris*, XP 533056; *Chlamys farreri*, AAX39709; *Chloroflexus aurantiacus*, ZP_00766800; *Ciona intestinales*, AK173579; *Danio rerio*, XP 707846;

Drosophila melanogaster, NP_650256; *Gallus gallus*, XP 423397; *Glycine max*, CAC67472; *Homo sapiens*, NP_003935; *Lotus japonicus*, CAC67492; *Medicago sativa*, CAC67501; *Mus musculus* 1, NP_033176; *Mus musculus* 2, NP_062287; *Oryza sativa*, NP_914832; *Pongo pygmaeus*, Q5RF48; *Pyrobaculum aerophilum*, AAL64395; *Rattus norvegicus*, AAH74008; *Silicibacter pomeroyi*, AAV95637; *Strongylocentrotus purpuratus*, XP 787563; *Sulfolobus solfataricus*, AAK42968; *Sulfolobus tokodaii*, BAB65016; *Tetraodon nigroviridis*, CAF90090; *Thellungiela halophila*, AAM19706; *Xenopus laevis*, AAH94129; *Xenopus tropicalis*, NP_001016750; *Zea mays*, AY104661.

Yeast two-hybrid screening

The bait construct pMP7201 was introduced into *Saccharomyces cerevisae* strain PJ69-4A (MATa *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δgal8Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GASL7-lacZ*) (James *et al*. 1996). The resulting yeast strain PJ69-4A / pMP7201, selected on –Trp medium, was used to screen an *A. thaliana* cDNA library fused to the GAL4-activation domain in the pACT2 vector (Clontech). The library was derived from stem, leaves, and flowers of 6-week-old *A. thaliana* (ecotype L. erecta) plants (kindly provided by Dr J Memelink, IBL, Leiden University). The putative interacting proteins were selected for His auxotrophy on minimal medium (–Trp / –Leu / –His) and subsequently for Ade auxotrophy. 5 mM 3-amino 1,2,4 triazole (3-AT), was always included in the His-deficient medium, in order to increase histidine dependence. Further selections were performed in higher concentrations of 3-AT up to 20 mM. All transformations were performed by the lithium acetate method (Meijer AH *et al*. 2000; Gietz and Woods 2002). The prey plasmids of GAPDH and FBA were transformed into *E. coli*, purified and back-transformed into PJ69-4A simultaneously with pMP7201, pODB80, pMP7202 or pAS2-1 to confirm the interaction. Positive prey plasmids were sequenced with pACT2-specific primers. Sequence homology searches were carried out with the BLAST package of search algorithms at the NCBI databases.

Expression of recombinant proteins and GST pull down assay

Plasmids pGEX3X, pMP7203, pMP7204 or pMP7205 were used to transform *E. coli* BL21 codon plus cells (Stratagene, La Jolla, CA). Cultures were grown to mid-log phase and protein synthesis was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. To obtain recombinant fusion proteins from the soluble fraction of cell lysates, bacterial pellets were, resuspended in NETN buffer [20 mM Tris, pH: 8.0, 100 mM NaCl, 1 mM EDTA, 0.01% NP-40 and Complete protease inhibitors (Roche)] supplemented with 1 mg mL^{-1} lysozyme, sonicated (3 × 10-s bursts; 30 s pause between bursts) and disrupted by French Press at 140 MPa. The supernatant of the bacterial extracts that contained the proteins of interest in a soluble form, as verified by western blotting and HRP conjugated anti-T7 (EMD Biosciences, San Diego, CA) / anti-His (Clontech)-based immunodetection (see also Fig. 3*a*), was used for the *in vitro* binding assays. Additionally, polyhistidine-tagged GAPDH and FBA recombinant proteins were purified from the soluble fraction of bacterial extracts by immobilised metal affinity chromatography with TALON^{TM} metal affinity resin (BD Biosciences, Alphen aan den Rijn, The Netherlands), according to the manufacturer's protocol.

Glutathione-sepharose 4B beads (Amersham Biosciences) were blocked with 0.5% (w / v) milk powder in NETN buffer at 4°C and incubated for 1 h at 4°C with bacterial extracts containing equal amounts of either GST::AtSBP or GST alone after quantification by SDS PAGE. The beads were washed four times with NETN buffer and subsequently incubated for three hours at 4°C with either bacterial cell lysates containing T7::6× His::GAPDH or T7::6× His::FBA, or with the respective purified proteins. The washing stringency varied from 100 to 500 mM NaCl and final samples were equilibrated with NETN. Bound proteins were eluted in SDS-loading buffer, separated by SDS–PAGE and transferred to PVDF membrane (Roche). The GST fused or T7 tagged proteins were detected with anti-GST (Amersham Biosciences) and anti-T7 (EMD Biosciences) horseradish peroxidase-conjugated monoclonal antibodies, respectively.

Results

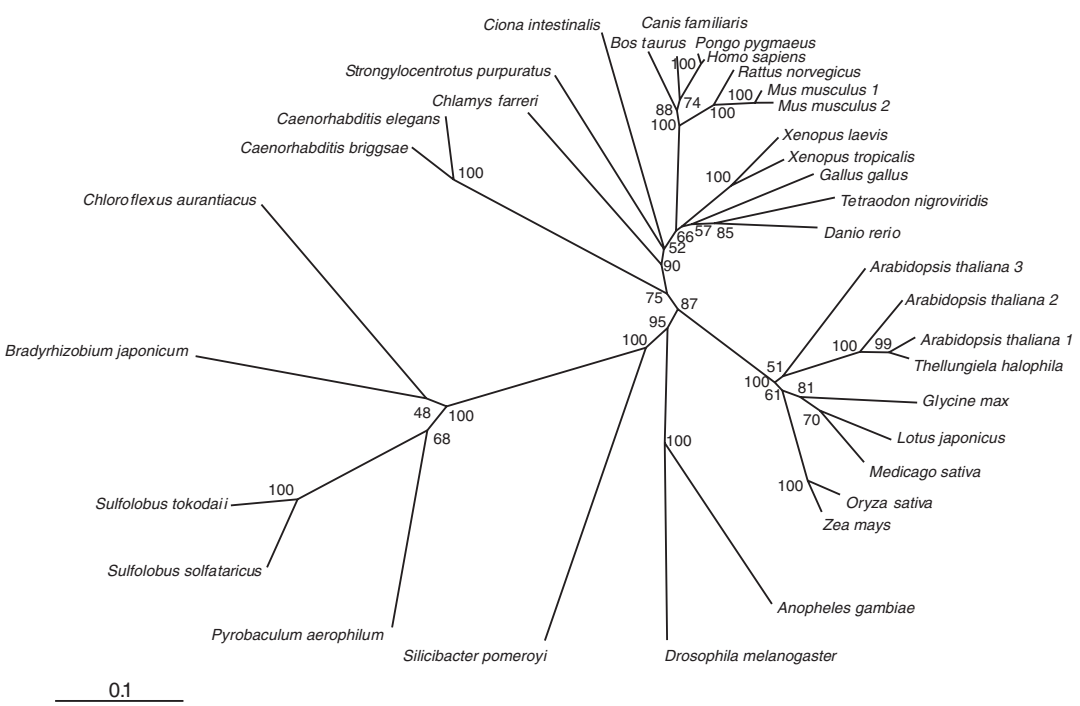
Sequence conservation of SBP

BlastP searches in the SwissProt-TrEMBL and GenBank database sets with the amino acid sequence of the *A. thaliana* SBP (accession SwissProt: O23264) as a query returned hits consisting of proteins from various organisms, ranging from archaea to humans. Alignment of all the SBP homologues indicated a significant degree of conservation across species. The identity level over the full length of the SBP polypeptides ranged from 37% between human and archaeal sequences, to 59% between human and plant sequences. Full-length SBP amino acid sequences were used for subsequent phylogenetic analysis as shown in Fig. 1*a*. The results show that SBP is broadly conserved within all prokaryotes and eukaryotes. The three *A. thaliana* SBP sequences branch off within the plant kingdom, indicating that they result from duplication events after the evolutionary divergence of plants from the other kingdoms. The conservation of the previously identified metal-binding motifs HxxH and HxD as well as the characteristic of redox proteins bis (cysteinyl) motif CSSC (Jamba *et al*. 1997; Liu and Stadtman 1997; Flemetakis *et al*. 2002) are shown in Fig. 1*b* (domains I, II, III). We have also identified putative clathrin-binding boxes built around the consensus sequence pLϕpϕp (p: polar, ϕ: hydrophobic) (Lafer 2002) (Fig. 1*b*, domains III and IV) and a KDEL ER-retention signal (Fig. 1*b*, domain I), whose presence could correlate with the proposed involvement of SBP in membrane trafficking functions (Porat *et al*. 2000). Furthermore, several motifs that could potentially localise SBP to the endoplasmic reticulum or Golgi apparatus could be identified by searching of the ELM database (data not shown). The homology regions I–IV shown in Fig. 1*b* are only indicative of the conservation and motif content of SBP proteins.

Identification of Arabidopsis thaliana *SBP-interacting partners*

In order to identify SBP-interacting proteins, we performed a series of yeast two-hybrid screenings using as bait the full-length polypeptide of SBP encoded by a cDNA clone that was amplified from *A. thaliana*, based on sequence information from the SwissProt accession O23264. A chimeric protein consisting of SBP and the GAL4 DNA-binding domain was presented *in vivo* to a translated *A. thaliana* cDNA library made from leaves, stems and young flowers, fused to GAL4 transcriptional activation domain. Of a total of approximately 10^6 transformants and after exclusion of the false positives and out-of-frame clones we identified 18 different proteins interacting *in vivo* with SBP (Table 1).

a



b

Alias	Accession #	Organism		I KDELHH W CSSC HxD		II HxxH		III pLϕpϕp W HxD		IV pLϕpϕp
1	BAC53217	*Bradyrhizobium japonicum*	[65]	GDEFHHFGWNACSSALS-PLAGH	[135]	RPHTIHCG	[326]	DIDLSMDDRFLYVSCWGTGEMRQ	[434]	KDYFVSFPD--
2	BAB65016	*Sulfolobus tokodaii*	[70]	NDELHHFGWNACSSAL--CPNGK	[139]	RLHTVHCG	[325]	DIDISLDDKFLYLSLWGIGEVRQ	[433]	KEFFVDFGE--
3	NP 650256	*Drosophila melanogaster*	[70]	GDELHHSGWNACSSCYYVDESSK	[140]	APHTTHCL	[321]	DIIISLDDRFLYVNCWRHGDVRQ	[450]	EDFLVDFANEP
4	AAM19706	*Thellungiella halophila*	[19]	GDELHHSGWNSCSSC-----HGD	[71]	YPHTAHCL	[249]	DFLISLDDRFLYFVNWLHGDIRQ	[377]	PNFFVDFGDEP
5	NP 193139	*Arabidopsis thaliana*	[86]	GDELHHSGWNSCSSC-----HGD	[152]	FPHTAHCL	[330]	DFLISLDDRFIYFVNWLHGDIRQ	[458]	PDFFVDFGDEP
6	NP 193140	*Arabidopsis thaliana*	[83]	GDELHHTGWNSCSSC-----HGD	[149]	FPHTSHCL	[327]	DFLISLDDRFFYFVNWLHGDIRQ	[455]	PDFFVDFGDEP
7	NP 189022	*Arabidopsis thaliana*	[76]	EDELHHSGWNSCSSC-----HDK	[142]	YPHQPHCL	[320]	DFLISLDDRFLYCSNWLHGDIRQ	[448]	PNFFVDFGTEP
8	CAC67492	*Lotus japonicus*	[83]	GDELHHTGWNSCSSC-----HGD	[149]	YPHTSHCL	[327]	DFLISLDDRFLYFVNWLHGDIRQ	[455]	PNFFVDFGAEP
9	CAC67501	*Medicago sativa*	[84]	GDELHHTGWNSCSSC-----HGD	[150]	YPHTSHCL	[328]	DFLISLDDRFLYFVNWLHGDIRQ	[456]	PNFFVDFGAEP
10	AY104661	*Zea mays*	[92]	GDELHHSGWNSCSSC-----HGD	[158]	FPHTSHCL	[336]	DFVISLDDRYLYFVNWLHGDIRQ	[465]	PNFFVDFGTEP
11	NP 914832	*Oryza sativa*	[77]	GDELHHSGWNACSSC-----HGD	[143]	FPHTSHCL	[321]	DFVISLDDRYLYLVNWLHGDIRQ	[450]	PNFFVDFGAEP
12	NP 502691	*Caenorhabditis elegans*	[69]	GDEVHHTGWNACSSC-----HDK	[133]	FPHTSHCL	[314]	DILISMDDRFLYVSCWLHGDIRQ	[439]	RDFLIDFGKIE
13	XP 707846	*Danio rerio*	[69]	KDELHHSGWNACSSC-----FDD	[135]	NPHTSHCL	[313]	DILISLDDRFLYFSNWLHGDIRQ	[441]	ENFLVDFGAEP
14	XP 423397	*Gallus gallus*	[105]	KDELHHSGWNACSSC-----FGD	[171]	NPHTSHCL	[348]	DILISLDDRFLYFSNWLHGDIRQ	[476]	TNFLVDFGKEP
15	AAH94129	*Xenopus laevis*	[68]	NDELHHSGWNTCSSC-----YGD	[134]	NPHTSHCL	[312]	DILISLDDRFLYFSNWLHGDIRQ	[440]	PNFLVDFGKEP
16	NP 033176	*Mus musculus*	[69]	KDELHHSGWNTCSSC-----FGD	[135]	SLHTSHCL	[312]	DILLSLDDRFLYFSNWLHGDIRQ	[440]	PNFLVDFGKEP
17	XP 533056	*Canis familiaris*	[206]	RDELHHSGWNACSSC-----FGD	[272]	YVHTSHCL	[449]	DILLSLDDRFLYFSNWLHGDLRQ	[577]	PNFLVDFGKEP
18	NP 003935	*Homo sapiens*	[69]	KDELHHSGWNTCSSC-----FGD	[135]	FLHTSHCL	[312]	DILLSLDDRFLYFSNWLHGDLRQ	[440]	PNFLVDFGKEP

Fig. 1. (*a*) Unrooted phylogenetic tree of SBP proteins from representative organisms of different taxonomic groups. The tree was constructed by neighbour-joining analysis based on the full-length amino acid sequences. The numbers indicate the occurrence of nodes during bootstrap analysis. The bootstrap values are given as percentages of 10 000 reiterations. The scale bar indicates the branch length. For accession numbers of the sequences used see Materials and methods. (*b*) Amino acid sequence alignment of conserved regions in selenium-binding proteins containing putative functional motifs. Above each homology block the respective motif is indicated. The asterisks (*) indicate highly conserved histidine or tryptophan residues and the short vertical lines (|) indicate the core motifs. It should be noted that in general SBP polypeptides have a high content of histidines, and several Golgi- and endoplasmic-reticulum-related signals but for practical reasons are not shown in this figure. Conserved motifs shown are: KDEL (region I), CXXC (region I), HxD (region I and region II, HxxH (region II) and two putative clathrin-binding boxes (regions III and IV). In the clathrin box motifs (p) and (ϕ) represent polar and hydrophobic amino acids, respectively. Shaded amino acids represent identity to the motif.

The majority of the deduced polypeptides fell in two broad categories related to biochemical networks involved either in vesicle trafficking and membrane synthesis [CAX (for cation exchanger)-interacting protein 1, CTP synthase, cysteine protease, lipase], or in the control of the redox state of the cell (CAX interacting protein 1, acireductone dioxygenase, dirigent protein). Other potential SBP interacting partners that we identified in our screenings include proteins related to energy production, photosynthesis, carbohydrate metabolism, nucleic acid processing, and pollen germination.

Table 1. List of SBP-interacting partners identified by two-hybrid screening

NCBI accession numbers (Acc no.) that resulted from Blast search of each putative binding partner of SBP presented next to the description of the genes and their predicted function. The number of independent clones identified in the screenings is also indicated. Proteins related with vesicle trafficking and membrane synthesis are underlined and proteins related with oxidation / reduction control of the cells, are in bold characters

Acc no.	Description	No. of clones	Function	pMPs
NM_201797	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	3	Aldehyde dehydrogenase activity	pMP7206–pMP7208
NM_120057 / NM_127705	Fructose-bisphosphate aldolase	2	Fructose-bisphosphate aldolase activity / pentose-phosphate shunt	pMP7209–pMP7210
NM_102819	CTP synthase-like protein / UTP ammonia ligase	1	CTP synthase activity	pMP7220
NM_117715	Putative cysteine proteinase	1	Cysteine-type peptidase / proteolysis and peptidolysis	pMP7217
NM_128607	Lipase (class 3)	1	Triacylglycerol lipase activity / lipid metabolism	pMP7212
NM_115347	**CAX-interacting protein 1**	1	Antiporter activity / cation transport	pMP7215
NM_123752	**Acireductone dioxygenase (ARD / ARD′)**	1	Metal ion binding	pMP7216
NM_112211	**Dirigent protein-related / disease resistance response protein-related**	1	Defence / immunity protein activity / defence response / lignan biosynthesis	pMP7219
NM_121081	**Arabinogalactan-protein**	1	Carbohydrate transport and metabolism	pMP7211
NM_119019	Photosystem I reaction centre subunit IV / PSI-E (PSAE1)	1	Electron transport	pMP7213
NM_101280	Oxygen evolving enhancer 3 (PsbQ)	1	Lumenal oxygen-evolving activity of Photosystem II	pMP7214
NM_116938	Pollen Ole e 1 allergen and extensin	1	Pollen hydration and germination	pMP7218
NM_102574	Polynucleotide adenylyltransferase	1	RNA binding / RNA processing	pMP7221
NM_106464	Methyltransferase-related (At1 g78140) mRNA	1	DNA methylation	pMP7222
NM_121221	Hypothetical protein	1	?	pMP7223

Most of these clones were only found once. One clone had no homology to known proteins and is referred to as a hypothetical protein. We chose to further analyse two of the 18 in-frame positive clones from the yeast two-hybrid screen based on the following criteria: both clones had a published link related to selenium binding, they were selected more than once and were identified in independent screenings. The first cDNA clone, a NADP-dependent GAPDH was found in three independent screenings and the second one, an FBA, represented by two different clones, was found twice in independent screenings.

The two FBA clones differed in only 10 of the 126 amino acids, but database searches showed that they represent two independent FBAs with accession numbers NM_120057 and NM_127705. Indicative of the interaction between SBP and the deduced polypeptides of these particular clones is the fact that although they are not full length, they both correspond to the *C*-terminal domain of two different FBAs. For GAPDH, one clone was 99.8% and two clones were 99.3% identical to the 436 *C*-terminal amino acid residues, of an *Arabidopsis* putative NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (accession number NM_201797).

In the yeast strain PJ69-4A (James *et al*. 1996), the HIS3 reporter gene conferring histidine auxotrophy is controlled by the GAL4 transcriptional activator. Since the GAL4 DNA-binding domain / AtSBP exerted a certain degree of transactivation of its own, selection during screenings was carried out in the presence of 5 mM 3-aminotriazole (3-AT), a competitive inhibitor of the HIS3 gene product. After the initial selection, interactions were verified with increased concentration (up to 20 mM) of 3-AT that was used to estimate the strength of the activation of the HIS3 gene and consequently the strength of the interaction (Fig. 2).

In our experimental conditions the use of the pODB8 vector resulted in significant background growth with several prey clones (data not shown) and also with FBA (Fig. 2, panel 2). It has been shown that with this particular vector, compared with other vectors, such as pAS2-1, up to 5-fold increase in the production of chimeric protein can be achieved, making the screening more sensitive (Louvet *et al*. 1997). For this purpose we also tested by vector swapping, the pAS2-1 vector carrying the GAL4 DNA-binding domain in an SBP bait construct. The fact that AtSBP, when expressed from the pAS2-1 vector, can still bind GAPDH and FBA further confirms that these proteins have a strong interaction (Fig. 2).

In vitro *interactions between AtSBP, GAPDH and FBA*

To analyse the nature of the interaction between SBP and the identified *A. thaliana* GAPDH and FBA, we performed

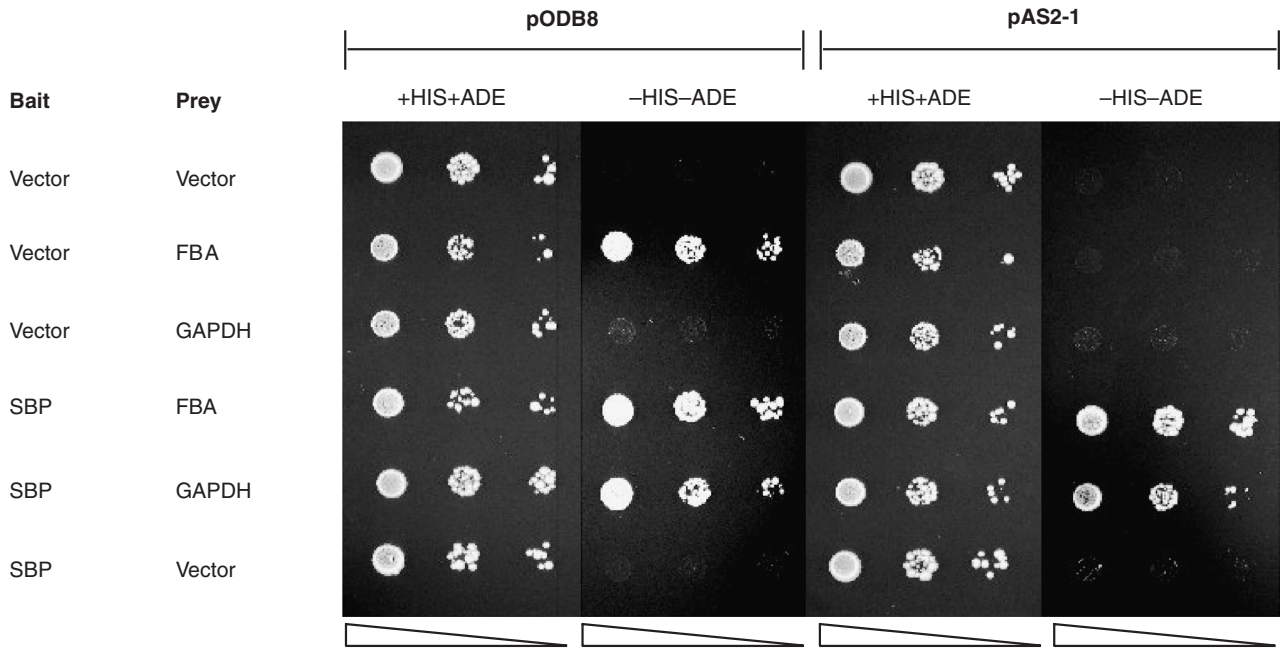


Fig. 2. *In vivo* interaction of SBP with GAPDH and FBA in yeast. The combination of bait and prey constructs that were used for co-transformations in yeast are shown on the left. To confirm histidine and adenine auxotrophy, transformants were spotted in three 10-fold dilutions (indicated by a triangle at the lower part of the picture) on selective plates. Owing to background growth in the interaction of SBP with FBA, attributed to pODB8 vector, AtSBP was cloned also in pAS2-1. The bait vector used in the co-transformations is shown at the top. Notice the absence of growth in the second row of panel 4 compared with the respective row of panel 2.

pull-down assays of recombinant proteins produced in *E. coli*. T7-tagged GAPDH and FBA were incubated with either GST::AtSBP or GST alone in the presence of glutathione sepharose beads. After washing, the associated proteins were immobilised on PVDF membrane by western blot and visualised by antibodies against GST or the T7 tag. The results presented in Fig. 3 indicate that both GAPDH and FBA bind to GST::AtSBP. The stringency of the washes after binding was a critical factor since the optimal salt concentration was different for each protein and buffers with 100, 200, 300 and 500 mM NaCl were tested in each case (data not shown). The GST–AtSBP interaction with GAPDH could occur under all salt concentrations and no unspecific binding of GAPDH to GST was detected. (Fig. 3*a*). FBA, when present in bacterial extracts, exhibited a weak affinity for the GST protein, which resulted in a salt concentration-dependent background. In 300 mM NaCl, binding of FBA to GST alone was significantly less than the binding observed to the GST–AtSBP fusion protein (Fig. 3*a*). Comparable results and complete elimination of the FBA interaction with GST were obtained when the binding studies were performed with purified GAPDH and FBA (Fig. 3*b*).

Discussion

Selenium-binding proteins constitute a highly conserved family with members demonstrated to be present in several eukaryotes and prokaryotes (Fig. 1). Although the two most distant sequences of SBP, those of human and archaeal origin, share an identity of only 37% on the amino acid level, the degree of conservation in roughly the whole length of the polypeptides points towards an important biological role for these proteins. Two of the primary characteristics in the multiple alignment of SBP proteins that gave rise to the tree of Fig. 1*a*, is the conservation of the Cys–Xaa–Xaa–Cys (CXXC) motif, as well as, the overall histidine content with several residues conserved and the presence of metal-binding motifs. The CXXC motif is typical for the broadly occurring thiol disulfide oxidoreductases, which catalyse the oxidation of thiols and the reduction and isomerisation of protein disulfide bonds and it is indispensable for their catalytic role on redox reactions (Walker *et al*. 1996; Chivers *et al*. 1997). It has been suggested (Jamba *et al*. 1997; Liu and Stadtman 1997) that proteins containing this characteristic bis (cysteinyl) motif have a strong affinity for selenium, and many metallochaperones possessing this structural domain are known to have binding activity for metals (DiDonato *et al*. 2000; Wernimont *et al*. 2000; Urvoas *et al*. 2003). The SBP amino acid sequences contain conserved putative metal-binding domains, such as the HxxH and HxD, which could serve as potential sites for selenium binding (Fig. 1*b*, domains I–III).

Clathrins play an important role in receptor-mediated and synaptic vesicle endocytosis and in *trans* Golgi vesicle transport (ter Haar *et al*. 1998; Marsh and McMahon 1999).

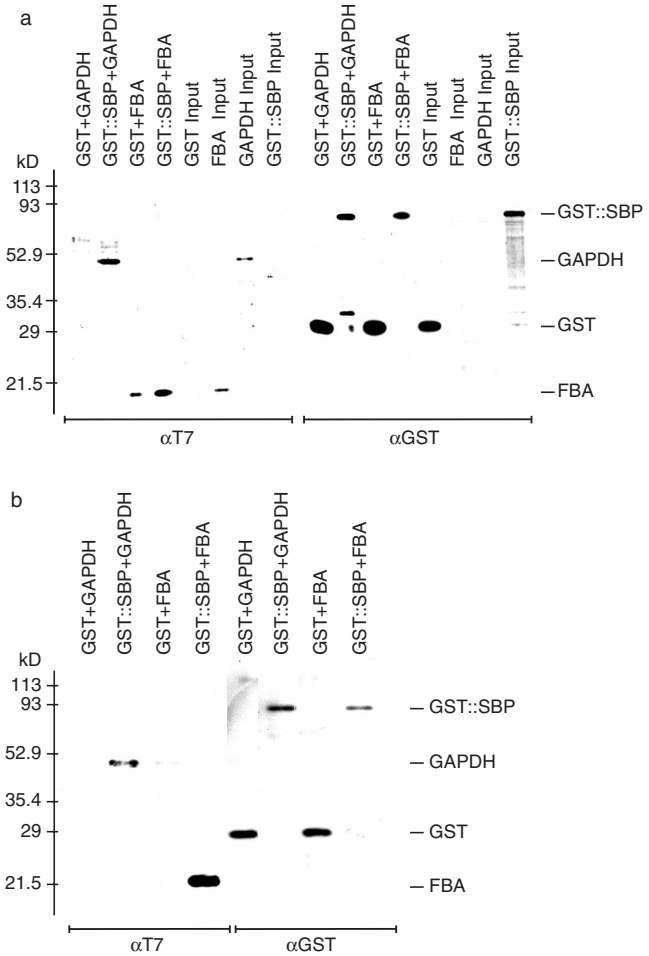


Fig. 3. GAPDH and FBA interact with SBP without the mediation of other factors. GAPDH and FBA were expressed in *E. coli* as T7-tagged proteins and bacterial extracts (*a*) or purified proteins (*b*) were incubated with GST::AtSBP bound to glutathione sepharose 4B beads. Interacting proteins were visualised by immunoblotting with an anti-T7 horseradish peroxidase-conjugated antibody and sepharose bound proteins with an anti-GST horseradish peroxidase-conjugated antibody (indicated in each case under the respective panels in *a* and *b*). Induced bacterial extracts were also immunoblotted, for comparison. The bands of interest are noted on the right-hand side of the figure and the protein combinations analysed in each lane are shown above each blot.

Teo *et al*. (2001) have identified a tyrosine kinase ACK1 that associates with clathrin-coated vesicles via a clathrin-binding motif shared by ACK, arrestin3, SBP56 (mouse homologue of SBP), amphiphysin, the AP-3 subunit b3A, epsin 1 and the yeast Ent1p, and showed that *in vitro*, an SBP56 19-mer fused to GST could weakly interact with clathrin. The two potential clathrin-binding boxes we have identified in SBP proteins (Fig. 1*b*, regions III–IV) could be related to the reported role of SBP in vesicle trafficking and intra-Golgi transport (Porat *et al*. 2000; Teo *et al*. 2001). Interestingly, in region III (Fig. 1*b*) a clathrin-binding motif lies in close proximity to a metal-binding motif (HxD), separated by a highly conserved tryptophan residue. Similarly, in region I (Fig. 1*b*) an endoplasmic-reticulum-retention signal (KDEL) followed by two highly conserved histidine residues is also separated from the CXXC motif by a highly conserved tryptophan residue. The significance of these motif arrangements should be further investigated. Although little is known about the physiological role of SBP, the high level of sequence conservation between different organisms (Fig. 1*a*) suggests that this protein could play important role for normal cellular function. Most of the putative partners of SBP that we have identified belong to widely conserved protein families from bacteria to humans and can be classified into two broad groups. The first group consists of proteins related to vesicle trafficking and membrane synthesis, such as CTP synthase, cys protease and lipase. This is consistent with previous studies that demonstrated a function of SBP in intra-Golgi transport and trafficking (Porat *et al*. 2000) but also the immunolocalisation of *Lotus japonicus* SBP at sites of membrane accumulation (Flemetakis *et al*. 2002). The second group of proteins interacting with SBP that we identified by this screening are proteins that can control the oxidation / reduction status in the cell, such as CAX-interacting protein, acireductone dioxygenase and dirigent protein, and this finding can be correlated with the presence of the CXXC motif in the SBP amino acid sequence that is characteristic for redox proteins. However, these putative interacting partners were selected only once during our screening and it remains unclear whether they represent true interacting partners of SBP. Two of the possible interacting partners of SBP, a NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a fructose-bisphosphate aldolase (FBA), were analysed further and the interaction was demonstrated also by *in vitro* binding assays, which indicated a strong direct protein–protein interaction without the mediation of other factors.

Both GAPDH and a prokaryotic aldolase (DPA) have been found to be potential selenium-delivery proteins (Lacourciere *et al*. 2002). These two proteins were found to bind selenium in an *E. coli* strain unable to incorporate selenium from selenite into selenoproteins. The binding observed was retained in denaturing conditions, which points to a strong association. Recently, it was also shown by *in vitro* experiments that GAPDH has great potential as a physiological selenium-delivery protein, as selenodiglutathione (GSSeSG) from the reaction of glutathione with SeO_3^{2-}, was bound in a 4 : 1 ratio to GAPDH, probably by attachment to a protein thiol residue (Ogasawara *et al*. 2005). Unfortunately, for plant proteins such selenium-binding experiments have never been performed. Although neither the nature nor the importance of the selenium binding of SBP is yet known, it was intriguing to find an interaction, at least in pairs, between SBP and two

other proteins binding the same microelement. We therefore hypothesise that the interaction between SBP, GAPDH and aldolase is related, in some way, with selenium metabolism or / and transport.

It has been shown by binding and kinetic studies with rabbit muscle proteins that there is an *in vitro* interaction between NADH-dependent GAPDH and FBA and this interaction can affect the enzyme activity of these proteins (Neuzil *et al*. 1990). Additionally, based on computational simulations and *in vitro* experiments it has been suggested that within a complex formed by GAPDH and FBA their common substrate intermediate, glyceraldehyde-3-phosphate (G-3-P), is channelled between the different active sites (Orosz and Ovadi 1987; Vertessy and Ovadi 1987; Ouporov *et al*. 2001). Furthermore it was proposed that the binding site for G-3-P might also be an ideal binding site for selenium (Lacourciere *et al*. 2002). Therefore if channelling actually exists between these two proteins, selenium might be implicated in the process.

Aldolases and GAPDHs have long been recognised for their central role in energy production, since they are essential enzymes in glycolysis, gluconeogenesis and in the Calvin cycle of photosynthetic organisms. Aldolases catalyse the reversible aldol cleavage to G-3-P and a ketose while GAPDHs catalyse further the oxidation of G-3-P. In both prokaryotes and eukaryotes, several types of GAPDHs exist including phosphorylating and non-phosphorylating varieties that use either NADH or NADPH as a cofactor (Sirover 1999). Aldolases from animals, plants and microorganisms are also divided into different families based on their catalytic mechanism. Each member of the aldolase superfamily can exist in different isoforms that can be organ specific (Marsh and Lebherz 1992). Although the aldolase that we have identified in this study and the bacterial aldolase that was previously reported to bind selenium have different enzymatic activities they both participate in energy and carbon production pathways via cleavage of their substrates to G-3-P. In plants, SBP is expressed in highly energy-demanding tissues, such as young nodules, root tips (*L. japonicus*) and embryos (*A. thaliana*) (Flemetakis *et al*. 2002). In animals, SBP has been shown to play a role in intracellular vesicular transport (Porat *et al*. 2000) and in cell protrusive motility (Miyaguchi 2004), two processes that require large amounts of energy. For the production of the energy needed for these processes, elevated metabolic levels would be required and thus, involvement of metabolic enzymes, such as GAPDH and aldolase, can be expected. Interaction of SBP with these two proteins might be related to their role in energy production pathways.

The present study suggests that a novel protein network for the regulation of selenium metabolism in cells may exist in plant cells. This network includes multifunctional proteins, such as SBP, GAPDH and FBA, which interact with each other in order to serve one or more physiological roles. The conserved nature of these proteins makes it very interesting to further study the role, the importance and the effect of selenium on these proteins with respect to their biochemical and biological function and interaction.

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