

Running head: Pea SAD Short-Chain Dehydrogenase/Reductase

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The *Pisum sativum* SAD Short-Chain Dehydrogenase/Reductase: Quinone Reduction, Tissue Distribution, and Heterologous Expression¹

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ABSTRACT

The pea (*Pisum sativum*) tetrameric short-chain alcohol dehydrogenase-like protein (SAD) family consists of at least three highly similar members (SAD-A, -B, and -C). According to mRNA data, environmental stimuli induce *SAD* expression (Brosché and Strid (1999) *Plant Physiol* **121**: 479-487). The aim of this study was to characterize the SAD proteins by examining their catalytic function, distribution in pea, and induction in different tissues. In enzyme activity assays using a range of potential substrates, the SAD-C enzyme was shown to reduce one- or two-ring membered quinones lacking long hydrophobic hydrocarbon tails. Immunological assays using a specific antiserum against the protein, demonstrated that different tissues and cell types were shown to contain small amounts of SAD protein that was predominantly located within epidermal or sub-epidermal cells and around vascular tissue. Particularly high local concentrations were observed in the protoderm of the seed cotyledonary axis. Two bow-shaped rows of cells in the ovary and the placental surface facing the ovule also exhibited considerable SAD staining. UV-B irradiation led to increased staining in epidermal and sub-epidermal cells of leaves and stems. The different localization patterns of SAD suggest functions in both development and in responses to environmental stimuli. Finally, the pea *SAD-C* promoter was shown to confer heterologous wound-induced expression in *Arabidopsis thaliana*, which confirmed that the inducibility of its expression is regulated at the transcriptional level.

INTRODUCTION

The expression of the small pea (*Pisum sativum*) gene family encoding short-chain alcohol dehydrogenase-like proteins (*SAD-A*, *SAD-B*, and *SAD-C*) is increased dramatically by low intensity ultraviolet-B radiation (UV-B; 280-315 nm) and by several abiotic stresses (Brosché and Strid, 1999). Compared with other UV-B-regulated pea genes (Brosché et al., 1999, Sävenstrand et al., 2002), the *SAD* genes are induced more rapidly and at significantly lower UV-B levels. Induction of *SAD* mRNA accumulation occurs differently in separate tissues (Brosché and Strid 1999). The exposure of pea plants to UV-B results in *SAD* mRNA accumulation in both epidermal and mesophyll cells of leaves (Kalbin et al., 2001). UV-B treatment also leads to an increase in *SAD* protein levels in both leaf and stem tissue but not in roots (Scherbak et al., 2009).

SAD forms a tetramer in solution and this is the sole oligomeric structure found under non-denaturing conditions (Scherbak et al., 2009). The pea *SAD* proteins are extremely similar: *SAD-A* and *SAD-C* differ by only three out of 268 amino acids (Brosché and Strid 1999). *SAD-B* is more distantly related to the other two isoforms (94% identity to *SAD-A*; Brosché and Strid 1999), with the most important difference being the loss of part (12 amino acids) of the predicted nucleotide-binding site. Unlike the coding regions, the promoters of the *SAD-A* and *SAD-C* genes differ markedly (Gittins et al., 2002 and Supplementary Fig. S1). However, both contain an 11-bp GC-rich motif that was shown to bind nuclear factors in an electrophoretic mobility shift assay (Gittins et al., 2002). This *SAD* promoter binding motif (SPBM) has features in common with a number of previously recognized classes of plant *cis*-elements involved in stress-responsive regulation of gene expression, which may explain the induction of the *SAD* genes by a wide variety of environmental stimuli, including ozone fumigation, wounding, aluminium exposure, and salt treatment (Brosché and Strid 1999).

Sequence database searches using the amino acid sequences encoded by the pea *SAD* cDNAs revealed homologies to the short-chain dehydrogenase/reductase (SDR) superfamily. The highest similarities were seen with sequences from other plants (Scherbak et al., 2009): (-)-isopiperitenol dehydrogenase from *Mentha* species (similarity 55%; Ringer et al., 2005), Δ^5 -3 β -hydroxysteroid dehydrogenase (HSD; EC 1.1.1.145) from *Digitalis lanata* (49%; Finsterbusch et al., 1999) and secoisolariciresinol dehydrogenase from *Podophyllum peltatum* (46%; Xia et al., 2001). The three-

dimensional structure of the *Podophyllum* protein has been solved (Youn et al., 2005), while the *Mentha* protein is the polypeptide with the highest similarity to the pea SADs for which an enzymatic function is known. The model plant *Arabidopsis thaliana* contains at least eight different genes homologous to the pea *SAD* genes, with similarity scores of the translated sequences of approximately 50%. A role in abscisic acid (ABA) biosynthesis has been identified for one of these *Arabidopsis* SAD homologs,, corresponding to gene At1g52340 (enzyme AtABA2; González-Guzman et al., 2002). For two of the other *Arabidopsis* *SAD* gene homologous (At2g47140 and At2g47130), names have been proposed for the corresponding proteins (AtSDR3 and AtSDR4, respectively; Choi et al., 2008), without knowledge of their function.

The SDR superfamily is one of the largest known protein families and comprises thousands of members found in species ranging from bacteria to humans (Oppermann et al., 2003). A large number of SDR X-ray crystal structures have been solved and, typically, these enzymes are either homodimeric (17 β -HSD; Ghosh et al., 1995) or homotetrameric (3 α ,20 β -HSD; Ghosh et al., 1994). The proteins are NAD(H)- or NADP(H)-dependent enzymes with a wide variety of substrates including alcohols, steroids, sugars and xenobiotics (Jörnvall et al., 1981, Kallberg et al., 2002).

The physiological substrates of the SAD proteins have remained elusive. In a pilot study, the purified recombinant SAD-A and SAD-C enzyme was incubated with a large number of potential substrates and was shown to catalyze the reduction of carbonyls and quinones at low rate (Brosché and Strid, unpublished results). This suggested that the SAD proteins are catalysts in the metabolism of quinones and/or carbonyls in response to environmental stimuli. In order to test this hypothesis, the catalytic function of the SAD-C enzyme have now been examined further using different compounds containing these functional groups. In addition, to gain further insight into the role of these proteins, their tissue distribution in *Pisum sativum* and the expression of pea *SAD* promoter/reporter constructs in *A. thaliana*, have also been examined in non-exposed or UV-B-exposed tissue or in wounded leaves.

RESULTS

Substrate specificity and kinetics of the SAD-C protein

The enzymatic activity assays described in this study were all carried out using SAD-C for the following reasons. First, SAD-B, which lacks part of the nucleotide binding site, was not active using the substrates tested (not shown). Second, for unknown reasons, the yield of the *E. coli*-produced recombinant SAD-C protein was much greater than that of SAD-A. However, for the studies described in Supplementary Table S1, which were carried before the SAD-C gene had been identified and cloned, recombinant SAD-A was used.

Substrates for the pea SAD enzyme were sought by measuring the reduction of NAD⁺/NADP⁺ or the oxidation of NADH/NADPH, respectively, by following the change in absorbance at 340 nm upon incubation with a variety of putative carbonyl- or hydroxyl-containing substrates (Supplementary Table S1), including gibberellins, brassinosteroids, other steroids, alcohols, sugars, flavonoids, quinones, and aromatic carbonyls. Activity was found only with quinones and aromatic carbonyls. p-Quinone reducing activity was detected with NADH as the cofactor using compounds with both one- and two-membered rings (Fig. 1a), but not those with three-membered rings (Fig. 1b). Permitted ring substitutions included methyl, methoxy and isopropyl groups. Therefore, compounds such as 2,5- and 2,6-dimethylbenzoquinone (DMBQ), 2,6-dimethoxybenzoquinone (DMoBQ), duroquinone (DQ), thymoquinone (TQ), 1,4-naphthoquinone (1,4-NQ), and menadione (K₃) were all reduced (Fig. 1a). In addition, the aromatic carbonyl 4-nitrobenzaldehyde (NBA) was reduced. Other quinones with long isoprenoid side chains, such as menaquinone (K₂), phylloquinone (K₁), ubiquinone (Q10), and decylplastoquinone (PQ), were not reduced (Fig. 1b and Supplementary Table S1). Furthermore, the three-membered ring compound anthraquinone (AQ) failed to function as a substrate for this enzyme. Other carbonyl-containing chemical species, such as the furocoumarin 8-methoxysoralen, or the monoterpene pulegone, were also not substrates for this enzyme. As expected, the SAD-C protein, NADH, and substrate were all necessary for activity (not shown).

NADPH could be used as the reductant instead of NADH but with a much reduced reaction rate (not shown). Also, catalysis of the reverse reaction could be demonstrated (reduction of NAD⁺ by e.g. duroquinol and reduced K₃) but with lower activity, presumably due to limitation by kinetic or structural factors.

Assuming Michaelis-Menten kinetics, which was supported by typical Michaelis-Menten and Hanes plots (not shown), catalytic constants were determined for SAD-C

protein using three of the compounds that were reduced by this protein: DQ, 1,4-NQ, and K₃ (Table 1). The catalytic rate for these substrates was similar, differing only by up to 15% from an average of 85 nmol NADH oxidized per minute and mg protein. However, the affinity of the enzyme for the three substrates differed markedly, with K₃ having a three- and nine-fold lower K_m than 1,4-NQ and DQ, respectively, which was reflected in the k_{cat}/K_m (Table 1). A two-membered ring (K₃ and 1,4-NQ) seemed to facilitate binding compared with a one-membered ring (DQ), as did methyl substituents on the ring that carries the carbonyl oxygens (compare K₃ and 1,4-NQ). Once bound to the active site, catalysis appeared to proceed at a similar rate for all three substrates. Furthermore, the K_m for NADH was determined to 7 μM (not shown).

It also became evident during the course of this study that all steps in the preparation of the recombinant SAD-C had to be performed in one unbroken sequence in order to obtain the fully active enzyme. Leaving a partially purified protein on ice overnight led to uncharacterized events at the molecular level that severely inhibited function.

Detection of SAD protein in pea tissues by immunoblot analysis and verification of the protein identity

Rabbit SAD-A antiserum (which interacts with both SAD-A and SAD-C; Scherbak et al., 2009) was used in immunoblotting to determine the presence of SAD proteins in extracts of different *Pisum sativum* tissues. This analysis showed a distribution of the SAD protein in leaves, stems, tendrils, roots and pea seeds (Fig. 2a). Doublets or multiple bands seen on the western blots at the size of the SAD monomer (28-29 kDa) represent either tissue differences in the expression of different *SAD* genes or more likely variable post-translational modifications. Interestingly, dry and soaked pea seeds contained the largest amount of SAD protein – considerably more than any other tissue (Fig. 2a) – which was in accordance with previous results showing that seeds contained the highest levels of SAD mRNA (Brosché and Strid 1999). After soaking, pea seeds were separated into three parts: the embryonic axis, cotyledon and seed coat. The SAD protein was abundant in the two former parts (Fig. 2b), while the seed coat contained only trace amounts, if any. Significant amounts of SAD protein were also evident in tissue from pods, fresh pea seeds and flowers (Fig. 2c). Since both fresh seeds and dry and

soaked seeds showed distinct bands on the western blots, it is clear that SAD content is not affected by seed storage. In flowers, the protein was evenly distributed in the wing and standard petal tissue, and in the composite keel petal, pistil and stamen (not shown). SAD was found in the tissues of all three cultivars tested (Greenfeast, Greenshaft, and Kelvedon Wonder; also not shown).

In order to confirm the specificity of the SAD-A antiserum, immunoprecipitation was performed using homogenates of pea seed cotyledonary axes. Immunoprecipitated protein was separated by SDS-PAGE and a band of the correct size was excised (Fig. 3a). This was the only band of approximately the right size visible on the gel, demonstrating the specificity of the antibodies. In-gel trypsin digestion and MALDI-TOF MS analysis of this band not only confirmed that the labeled protein was a member of the SAD protein family but also showed that it was the SAD-A isoform (Fig. 3b & Supplementary Fig. S2a & c).

Analysis of SAD protein tissue distribution in UV-B-irradiated and non-irradiated pea plants by immunohistochemistry

In order to identify which cell types in the examined tissues contained the SAD protein, immunohistochemistry (IHC), using the rabbit anti-SAD-A antiserum, was employed to stain formaldehyde-fixed pea tissues. A preliminary experiment using pre-immune serum resulted in no immunostaining (Fig. 4a), confirming that the polyclonal antiserum contained no antibodies cross-reacting with other pea proteins. This pre-immune serum was used as a negative control throughout the IHC experiments.

IHC analysis showed some staining of SAD protein in control pea leaf tissues, i.e. those not exposed to UV-B radiation, or those exposed to UV-A radiation for 2 or 5 days. The distribution pattern was indistinct but the protein appeared to be located in the mesophyll and bundle sheath cells around the xylem and phloem (data not shown). Leaves exposed to UV-B radiation, on the other hand, showed more distinct immunostaining concentrated in the palisade parenchyma cells at the upper surface of the leaf (Fig. 4b), i.e. close to the surface where UV-B radiation impinges on the leaf and therefore has its highest intensity. In order to confirm that the protein labeled in UV-B-exposed leaves was indeed a member of the SAD protein family, immunoprecipitation, SDS-PAGE of precipitated protein, in-gel trypsin digestion of a protein band of the right

size, and MALDI-TOF MS analysis were performed. Although it was not possible to discern whether the UV-B-induced protein was SAD-A or SAD-C, this analysis confirmed that SAD was the UV-B-regulated protein detected by the antiserum (Fig. 3c & Supplementary Fig. S2b & d). As before, no proteins other than SAD were identified by MS analysis of the immunoprecipitate pulled down by the SAD-specific antiserum.

In control stem tissue, minor staining of SAD protein was distributed in the cortex and around the vascular tissue (data not shown). After UV-B irradiation, the SAD protein accumulated in the outer cell layers of the stem as is clearly seen in Fig. 4c. Distinct staining was found in the epidermis or the cell layers below the epidermis and also in the cortex.

The root system, unlike the stems and leaves, showed no noticeable difference in SAD protein distribution between the control tissue and tissue harvested from plants exposed to UV-B (Scherbak et al., 2009). In both cases there was some minor immunostaining in the root cortex (data not shown). Coiling of pea tendrils is a specific effect of UV-B radiation (Brosché and Strid 2000). However, SAD IHC analysis of tendril sections showed no clear difference between treatments (controls, UV-A-exposed, or UV-B-exposed plants; data not shown) with respect to the abundance of this protein.

Since immunoblotting demonstrated that non-UV-exposed pea pods, embryonic axes, and seed cotyledons contained considerable amounts of SAD protein (see above), only the tissues from plants grown under control conditions were analyzed with IHC. In pod tissue SAD protein was detected around the vasculature (Fig. 4d) and in the thick-walled epidermis of the exocarp (Fig. 4e). Immunostaining was also observed in the parenchymatous cells of the mesocarp and in palisade parenchyma cells. The inner epidermal layer of the pod, on the surface facing the seeds, also contained SAD (Fig. 4f).

The seed embryonic axis (separated from the rest of the seed) is composed of three different tissues: the protoderm, ground meristem and procambium. SAD protein immunostaining was extremely strong in the protoderm in seeds soaked for 24 h (Fig. 4g). IHC analysis showed very clear and specific staining located in the outer cell layer of the embryonic axis. These were the most heavily stained cells observed in this study, which implies an important role for SAD protein in this tissue.

In the different types of petals examined by IHC, all showed staining in the epidermal cell layer, as exemplified by keel tissue shown in Fig. 4h. This staining was not observed when pre-immune serum was used instead of SAD antiserum. In the stamen, SAD was predominantly located in epidermal cells (not shown), whereas in the stigma,

this protein appeared to be more uniformly distributed (not shown). Two bow-shaped lines of cells containing SAD protein were apparent in the pistil ovary (Fig. 4i). SAD-containing cells were also identified on the placental surface facing the ovules.

Heterologous wound-induced transcription regulated by the pea *SAD-C* promoter in *Arabidopsis*

The above results indicate that SAD has at least two functions in pea plants. The first requires constitutive expression in certain cell layers of different tissue types, such as epidermal and sub-epidermal cell layers of the cotyledonary axes, pods, stamens and petals (Fig. 4). The second involves a diffuse but clear increase in SAD content (Fig. 4 and Scherbak et al., 2009) in cell layers close to the upper epidermis of leaves (Fig. 4b) and the epidermis of stems (Fig. 4c) in plants exposed to ultraviolet-B radiation. Besides UV-B, other environmental stimuli, primarily wounding, are known to strongly induce *SAD* gene expression in pea (Brosché and Strid, 1999). To more clearly visualize this inducibility of SAD expression and to confirm that the induction is controlled by the *SAD* promoter, *Arabidopsis thaliana* plants were transformed with promoter/reporter constructs comprising the *SAD-C* 0.7-kb proximal promoter (Gittins et al., 2002) fused to either the β-glucuronidase (*gusA*; Fig. 4j and k) or luciferase (*luc*; Fig. 5) reporter gene.

As judged by the luciferase activity measured in the leaves of nine independent *SAD-CP-luc* transgenic lines (Fig. 5), it was clear that the 0.7-kb *SAD-C* promoter drives wound-induced expression in *Arabidopsis*. For whole tissue visualization of this expression, rosette leaves of *SAD-CP-gusA* transgenics were wounded by cutting (Fig. 4j) or pricking with a needle (Fig. 4k). It was evident that wound-induced *gusA* expression, driven by the pea *SAD-C* promoter, was confined to the locality of the wound site and the neighboring cells.

In contrast to the strong wound-induced expression, the 0.7-kb pea *SAD-C* promoter was not activated by UV-B exposure in *Arabidopsis* (data not shown). This indicates either that the 0.7kb promoter fragment used in the construct does not contain the necessary UV-B regulatory elements or that pea uses different regulatory elements compared to *Arabidopsis* to direct UV-B-induced expression.

To examine whether *Arabidopsis SAD* homologs are induced by stimuli, publicly available microarray data were explored. Raw data files were obtained from several databases (see Materials and methods) and RMA normalized. To take into account the sample variation in the microarray experiments, parametric bootstrapping combined with Bayesian hierarchical clustering (Savage et al., 2009, Jaspers et al., 2010) was applied (Fig. 6). Of the *Arabidopsis* genes with highest similarity to pea *SAD-C*, At3g29250 and At2g47130 (corresponding to protein AtSDR4; Choi et al., 2008), were found to be highly and weakly induced by UV-B, respectively, and overall, these two genes form the closest partners in cluster analysis. In addition to UV-B, both of these genes were induced by osmotic and salt stress and infection with *Pseudomonas*. Interestingly, neither of these genes were induced by wounding treatment (not shown).

Since the pea *SAD-C* promoter is wound-inducible (Fig. 5) whereas *Arabidopsis SAD* promoters are not, this is further evidence that the promoters of these homologous genes contain different regulatory elements. This hypothesis was tested by searching the promoter sequences for recognised stress-responsive *cis*-elements (Table 2). Consistent with its induction by several pathogens, the At2g47130 promoter contained several WRKY recognition sites, previously shown to be important in pathogen responses (Eulgem et al., 2000). The SPBM element in the pea *SAD-C* promoter, which binds nuclear proteins (Gittins et al., 2002) was not found in the *Arabidopsis* promoters. The *SAD-C* promoter, but not the promoters of At3g29250 and At2g47130, contained several ACE elements known to be involved in UV/blue light responses (Hartmann et al., 1998). Overall, the promoters of *SAD-C* and those of the UV-B-induced At3g29250 and At2g47130 differ considerably in the nature of the previously characterized promoter elements they contain. To identify any novel promoter elements that may potentially be involved in UV-B responses, the three promoters were analyzed using the Motif sampler program (<http://homes.esat.kuleuven.be/~sistawww/bioi/thijs/Work/MotifSampler.html>; Thijs et al., 2002). A novel element, AANCAATT (Table 2), was found in all three promoters, and may be involved in UV-B responses, although further studies are required to determine its exact role.

Seed-directed promoter elements in the pea *SAD-A* and *SAD-C* promoters

The results of immunoprecipitation and MS analysis (Figs. 3a & b) showed that SAD-A is the SAD isozyme expressed in pea seeds. Therefore, the *SAD-A* promoter was searched for known promoter elements conferring gene expression in seeds (Supplementary Fig. S1a). Interestingly, both an RY repeat element (Chamberland, Daile and Bernier 1992; Reidt et al., 2000) and a TACACAT element (with an allowed one-base mismatch; Josefsson et al., 1987) were found. Unfortunately, the published 0.9 kb sequence of the pea *SAD-C* promoter was too short (Supplementary Fig. S1b) to allow extensive comparison with the *SAD-A* promoter.

DISCUSSION

The aims of this study were to elucidate the catalytic function of the pea short-chain dehydrogenase/reductase SAD-C protein and to examine SAD protein localization, and the properties of their gene promoters. Chromatographic and electrophoretic analysis of purified recombinant pea SAD-C protein has shown that this enzyme is a tetramer consisting of a dimer of dimers (Scherbak et al., 2009). Functionally, one- and two-membered quinones with short side chains (Fig. 1) were bound by the enzyme and reduced by NADH, with two-membered compounds being bound with higher affinity (Table 1). There were no indications in our study that SAD-C is involved in ABA biosynthesis or metabolism (Supplementary Table S1) similar to its *Arabidopsis* homolog AtABA2 (At1g52340) with which it shares 40% identity.

All the compounds that were found to be substrates for SAD-C are non-physiological; the physiological quinones tested, i.e. those containing long hydrophobic aliphatic side chains, did not function as substrates. Several hundred of different benzoquinones and naphthoquinones are found in plants (Thomson 1957; 1971; 1986; 1997) and some of these might function as SAD-C substrates. Unfortunately, none of these are commercially available and they have mainly been sought and found in medicinal plants. Very little is known about the presence of benzoquinones and naphtoquinones, other than K₁, Q10 or PQ, in legumes, such as the pea, or *Arabidopsis*. Therefore, we have so far not been able to exactly pin-point the true physiological substrate(s) for SAD-C, although we now know which classes of compounds are turned-over by the enzyme.

Immunoblotting demonstrated that the SAD protein is present to a greater or lesser extent in all pea tissues examined (Fig. 2). The pea seed (apart from the seed coat which contains only negligible levels of SAD) is the tissue with the largest content of this enzyme. In the seed cotyledonary axis, SAD-A was identified as the isozyme present (Fig. 3b). UV-B irradiation of pea plants results in a clear increase in the content of SAD in leaf and stem tissues, but not in roots (Scherbak et al., 2009). This indicates that increased expression of the *SAD* genes, as a result of UV-B radiation, is limited to the exposed tissues and is a local or semi-local effect rather than a systemic response. The conclusion that SAD is locally expressed after stimuli was supported by the pattern of heterologous expression from the pea *SAD-C* promoter in *Arabidopsis* following wounding. Only the wound site and cells in the vicinity of the wound show activity of a reporter gene driven by this promoter (Fig. 4j and k). It could not be conclusively determined whether SAD-A or SAD-C was the isozyme induced in leaves during UV-B exposure (Fig. 3c).

Immunohistochemical analysis demonstrated that in control tissue (as well as in UV-B-exposed leaves and stems), SAD is predominantly located in epidermal or subepidermal cells (Fig. 4b, c and e-h). SAD (in this case SAD-A) was most abundant in the protoderm of the pea seed cotyledonary axis (Fig. 4g), which also demonstrates the involvement of this enzyme in development in addition to a role in the response to environmental stimuli. However, expression around vascular tissue was also apparent (Fig. 4c, d, and h). Although IHC is not a quantitative method, our results support the previous finding (Scherbak et al., 2009) that UV-B increases the SAD content in leaf and stem tissues and also show that this enzyme is mainly confined to the epidermal and subepidermal cell layers (Fig. 4b and c) that experienced the highest intensities of UV-B radiation [in accordance with previous findings by Kalbina et al. (2001)].

The apparent SAD distribution within the ovary is particularly noteworthy. Fig 4i shows two bow-shaped lines of cells in this organ, indicating a specialized tissue producing SAD. The placental surface facing the ovule also contains cells with considerable SAD-staining. This again implies an important role for SAD in development and/or reproduction. Therefore, SAD appears to be important in *Pisum sativum* for both development and in response to environmental stimuli.

Another interesting finding is that the pea *SAD-C* promoter directs wound-induced but not UV-B-induced expression in *Arabidopsis thaliana*. This was further examined by identifying UV-B-inducible *Arabidopsis* SAD homologs in expression studies using

public Affymetrix chip data (Fig. 6). Of the eight *Arabidopsis* genes most similar to the pea *SAD* genes (AGI codes At1g52340, At2g47120, At2g47130, At2g47140, At3g26770, At3g29250, At3g29260, At3g51680) two, At3g29250 and At2g47130, are induced by UV-B, biotic stress, and osmotic stress. At2g47140 (corresponding to protein AtSDR3; Choi et al., 2008) shows some regulation in response to salt and biotic stress. The other five *Arabidopsis SAD* homologs display only minor responses to environmental stimuli. These different expression patterns exhibited by *Arabidopsis SAD* homologs suggests that separate members of the pea *SAD* gene family may also play different roles in response to stress and/or in development, which will most likely be reflected in the cellular distribution of the corresponding proteins. Alternatively, the fact that *Pisum sativum* apparently only contains three to six *SAD* genes (Brosché and Strid 1999; Gittins and Strid, unpublished observations) compared to at least eight putative *Arabidopsis SAD* genes, may mean that each pea gene has to perform multiple functions unlike more specialized roles played by individual *Arabidopsis* gene.

To test whether the promoter regions of the *Arabidopsis SAD* homologs that were induced by environmental stimuli and the pea *SAD* promoters share any *cis*-regulatory elements, the presence of previously characterized light, stress or pathogen response elements was investigated (Table 2). Unlike the pea *SAD-C* promoter, the *Arabidopsis SAD* sequences contain no elements previously shown to be important for UV-B-induced expression of the *Arabidopsis* chalcone synthase gene (Hartmann et al., 1998). Thus, it is likely that the UV-B induction of At3g29250 and At2g47130 is driven by a novel promoter element, which will require promoter deletion analysis for its identification. Furthermore, the pea *SAD-A* promoter contains elements previously shown to be involved in directing gene expression in seeds (Supplementary Fig. S1a; Chamberland et al., 1992; Josefsson et al., 1987; Reidt et al., 2000).

Whereas the reported immunological studies do not differentiate between *SAD-C* and *SAD-A* (Scherbak et al., 2009), except when immunoprecipitation was followed by MALDI-TOF MS analysis (see above), the data on substrate specificity and enzyme kinetics should be regarded as being valid for *SAD-C* only, although the difference between *SAD-C* and *SAD-A* is only three amino acids, all located towards the C-terminal ends of the proteins (Brosché et al., 1999). These data should be regarded as being of even less relevance for *SAD-B*, which also lacks a substantial part of the N-terminal nucleotide binding site.

In summary, the results of the present study show that the pea SAD-C protein is capable of reducing benzoquinones and naphthoquinones *in vitro*. SADs are induced *in vivo* after environmental stimuli, both at the mRNA and protein levels. SAD antibodies raised against the recombinant protein permitted the localization of this enzyme in different parts of the *Pisum sativum* plant and in various cell types within the examined tissues. *SAD* expression was studied by immunoblotting, immunoprecipitation, IHC, and by heterologous expression using the pea *SAD-C* promoter in *A. thaliana*. These findings indicate that SAD proteins function in both development (seeds, pods, placenta) and in response to environmental stimuli, such as UV-B radiation and wounding.

MATERIALS AND METHODS

Plant material

Pea plants (*Pisum sativum* cv. Greenfeast) were grown in vermiculite in a growth chamber at 22°C with a 16 h light/8 h dark cycle (approx. 80 µmol photons m⁻² s⁻¹). Following leaf appearance, plants were watered every two days with Hoagland nutrient solution (Hoagland and Arnon 1950) until they were used for experimentation. In the experiments where flowers, pods and fresh seeds were studied, the plants were moved to an open growth room and placed under Philips IP54, HPI-T 400 W lamps, at 25±2°C with a 16 h light/8 h dark cycle. Two other pea cultivars (Greenshaft and Kelvedon wonder) were also used to check for variation between varieties. These plants were grown under the same conditions as Greenfeast.

Seeds of *Arabidopsis thaliana* Col-0 were sown in sterilized compost (70% moss/30% perlite; Weibulls Så-/Pluggjord; Weibull Trädgård AB, Hammenhög, Sweden). Synchronous germination was promoted by holding at 4°C in the dark for 2-3 days. Before transfer to compost, primary transgenic lines derived from Col-0 were selected by germination on MS basal medium (Murashige and Skoog 1962) with 0.75% agar (pH 5.7) containing 20 mg/l hygromycin. All *Arabidopsis* seedlings were propagated in a growth room at 22°C with a 16 h light/8 h dark cycle. Water and occasional feeding with Hoagland nutrient solution were given during growth.

Purification of recombinant SAD protein

E. coli strain BL21(DE3)pLysS carrying the SAD-C over-expression construct was grown and protein expression induced as described by Scherbak et al. (2009). Three hours after induction, bacteria were harvested, frozen in liquid nitrogen, and stored at -20°C until required. The cells were broken and the SAD protein was purified as previously described (Scherbak et al., 2009). The protein concentration was determined using a Bradford protein assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA).

Enzymatic activity measurements

The catalytic activity and kinetics of the recombinant pea SAD protein were determined by following the change in absorbance at 340 nm (A_{340}) caused by the disappearance or appearance of NADH (depending on whether reduction or oxidation was studied) at room temperature. The final reaction mixture (1 ml) typically contained 50 mM Tris buffer at pH 7.5, 0.4 mM NADH or NAD⁺, 0.3 mg purified recombinant SAD-C, and potential substrates at appropriate concentrations. When hydrophobic substrates were used, these were dissolved in 5% dimethylformamide. In certain experiments, NADPH or NADP⁺ were used in place of NADH or NAD⁺ to test co-factor specificity. For determination of the Km for NADH, 0.1 mM menadione was used.

UV-B exposure

Twenty days after sowing, pea plants were exposed to UV-A or UV-A+B radiation, added to their normal illumination, by using Philips TL40/12UV fluorescent tubes. Control plants were exposed to UV-A (0.64 W m^{-2}) obtained from the same tubes by using mylar film filter with a wavelength cutoff at 315 nm. For the UV-A+B exposures, a cellulose acetate film filter was used with a wavelength cutoff at 292 nm. Plants were exposed to UV radiation for either 2 or 5 days (daily exposure equaled 6 h of UV-B irradiation centered around noon) with $\text{UV-B}_{\text{BE},300} = 0.3 \text{ W m}^{-2}$, where $\text{UV-B}_{\text{BE},300}$

was the biologically effective radiation normalized to 300nm according to Caldwell (1971) and Green, Sawada and Shettle (1974). The irradiation was quantified using an Optronics 754 portable high accuracy UV-visible spectroradiometer (Optronic Laboratories Inc, Orlando, FL). Following irradiation, tissue samples were collected, weighed, frozen in liquid N₂ and held at -80°C until required.

Electrophoresis, western blotting and staining

Plant tissue (0.2-0.3 g) was ground and mixed with 0.1 ml 50 mM MES buffer (2-[N-morpholino]ethanesulfonic acid), pH 6.3, and centrifuged for 10 min at 13,000 g. The supernatant was collected and placed on ice. To the tissue supernatant obtained from the embryonic axis (which contains large amounts of nucleic acids) a small amount of bovine pancreas RNase I powder (Roche Diagnostics, Indianapolis, IN) was added. The seed coat, which was difficult to solubilize by the above method, was sonicated on ice for a few minutes to promote protein release. The protein concentration in each tissue extract was determined using a Bradford protein assay according to the manufacturer's instructions (Bio-Rad) with ovalbumin (Sigma-Aldrich, St.Louis, MO) as the standard. SDS-polyacrylamide gel electrophoresis of protein from pea leaf extracts (10 µg) was performed using 10% pre-cast gels (Ready-Gel, Bio-Rad, CA) and transferred by electroblotting onto Hybond-C pure membrane (GE Healthcare, Uppsala, Sweden). Western blotting with anti-SAD-A protein primary antiserum (500-fold dilution) was performed according to a standard method (Qiagen, Hilden, Germany). After reaction with alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody, immunostained bands were visualized using chromogenic substrate (nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate) according to a standard protocol (Promega, Madison, WI). Western blots were repeated at least twice with samples from different 20-day-old plants. Where applicable, SDS gels were silver-stained using an automated stainer (GE Healthcare) according to the manufacturer's protocol.

Immunoprecipitation

One-hundred μ l of pea leaf or pea seed homogenate was mixed with 50 μ l of antiserum and 350 μ l RIPA buffer [10 mM Tris-HCl, pH 7.2; 160 mM NaCl; 1% (v/v) Triton X-100; 1% (w/v) Na-deoxycholate; 0.1% (w/v) SDS; 1 mM EGTA; 1 mM EDTA] and incubated for 4 h at 4°C with agitation. Then, 20 μ l of Protein G+-Sepharose bead suspension (Santa Cruz Biotechnology, Santa Cruz, CA) were mixed with the sample/antiserum solution and incubated overnight at 4°C with agitation. The mixture was centrifuged for 1 min at 1000g at 4°C and the pelleted Protein G+-Sepharose beads washed three times in 1 ml PBS. After the final wash, the pellet was taken up in 50 μ l PBS and mixed with an equal volume Laemmli buffer containing 10% 2-mercaptoethanol and heated at 100°C for 10 min. After centrifugation at 1000g for 1 min, the supernatant was separated on a 10 % SDS-polyacrylamide gel as described above and the gel stained with Coomassie Brilliant Blue R-250. The band corresponding to the precipitated protein was excised from the gel and analyzed by in-gel trypsin digestion and MALDI-TOF MS as described by Scherbak et al. (2009).

Immunohistochemistry

Pisum sativum tissue samples were placed in cassettes and immersed in Histofix fixation solution (5% formaldehyde in NaPi buffer, pH 7.2, effective osmolarity 300 mOsmol; Histolab AB, Göteborg, Sweden) for 6-24 h. When more than 6 h of fixation was required, the cassettes were held at 4°C. Fixed sections of roots, leaves, tendrils, stems, embryonic axes and pods were dehydrated by washing in a graded series of ethanol (70%, 90%, 95%) followed by xylene (30%, 10%, 5%), then embedded in paraffin at 60°C and cut into 4 μ m-thick sections using a HM 355 S Rotary Microtome (MICROM International GmbH, Walldorf, Germany). The sections were floated on distilled water at room temperature and heat-stretched in distilled water at 50°C, then mounted on positively charged slides (Superfrost plus, Menzel-Gläser, Braunschweig, Germany). Before immunostaining, the sections were heated to 60°C for 30 min and cooled to room temperature. Subsequently, the paraffin was removed by treatment with xylene, followed by rehydration using a graded ethanol series (95%, 90%, 75%) and finally the sections were immersed in PBS.

The prepared slides were placed in TE buffer (10 mM Tris-HCl, pH 9; 1 mM EDTA) which was boiled by heating in a microwave oven at 650 W for 30 min. The slides were then cooled to room temperature and placed in 0.1 M PBS. The tissue was blocked by incubation for 1 h at room temperature in a blocking buffer (0.1 x TBS, pH 7.6; 1 g/l BSA; 0.1% Triton X-100). Primary anti-SAD-A antiserum, routinely diluted 1:8000 with DAKO ChemMate antibody diluent (Dako Cytomation Norden AB, Solna, Sweden), was added to the slides, which were then incubated for 1 h at room temperature. Preimmune rabbit serum was used at similar concentrations as a negative control to determine the degree of non-specific binding. Following three washes with 0.1 M PBS, horseradish peroxidase-linked biotinylated goat anti-rabbit IgG secondary antibody (DAKO) diluted 1:1 in DAKO ChemMate antibody diluent, was added to the slides, which were then incubated for 1 h at room temperature. After being overlaid with avidin peroxidase complex, the slides were incubated for a further 1 h at room. Development was performed using a diaminobenzidine (DAB)/peroxidase detection kit (DAKO ChemMate). DAB plus chromogen was diluted 1:51 in substrate buffer containing hydrogen peroxide and added to the slides, which were incubated at room temperature. After the appearance of black stain (approx. 2 min), the slides were washed in PBS, dehydrated using a graded ethanol series (75%, 90%, 95%), cleared in xylene and mounted with Pertex (Histolab). Positive immunostaining produced an insoluble brown precipitate in the tissues.

Pea seeds (dry, or soaked in tap water for 24 h) and fresh seeds harvested directly from pods, were covered with cryo-embedding compound (Histolab), and frozen in isopentane and dry ice. Sections of 6 µm were cut using a cryostat at -20°C, then mounted on a slide and stored at -20°C before processing. The frozen sections were immersed in acetone for 10 s and dried at room temperature for 12-24 h. The sections were then blocked and incubated with antisera as described above.

Preparation of SAD promoter-reporter gene constructs, transformation of *Arabidopsis*, and detection of wound-induced SAD expression

SAD gene-specific primers were used to amplify fragments from pea DNA following the PCR-based GenomeWalker method (Clontech, BD Bioscience, Franklin

Lakes, NJ; Siebert et al., 1995). One amplified fragment of approximately 1 kb contained the 5' non-coding and promoter regions of the *SAD-C* gene (Gittins et al., 2002). A 0.7 kb subfragment carrying the *SAD-C* proximal promoter was cloned into binary vectors pPCV814luc and pPCV812 (Koncz et al., 1994) to drive expression of the luciferase (*luc*) and β-glucuronidase (*gusA*) reporter genes, respectively. These constructs were introduced into *Arabidopsis* Col-0 by *Agrobacterium tumefaciens* using the vacuum infiltration procedure (Clough and Bent 1998).

The effect of wounding was tested on leaves from primary *SAD-CP-luc A. thaliana* transgenics. Rosette leaves were cut along the mid-vein, one half was frozen in liquid N₂ while the other was wounded (by pricking with a needle) and held for 3 h before freezing. Extracts made from these samples were tested using a luciferase assay system (Promega). For the *SAD-CP-gusA* transgenics, rosette leaves were wounded, either by cutting with a blade or pricking with a needle, then held for 3 h, frozen and subsequently stained for GUS activity with X-Gluc (Jefferson 1987).

Analysis of public *Arabidopsis* Affymetrix chip data

Affymetrix raw data was downloaded from NASCArrays
<http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl> (accession numbers
 NASCARRAYS-143 – paraquat; NASCARRAYS-353 – ZAT12; NASCARRAYS-176 –
 ABA time course experiment 1; NASCARRAYS-192 – Ibuprofen), ArrayExpress
<http://www.ebi.ac.uk/microarray-as/ae/> (accession numbers E-GEOD-12856 – *Blumeria*
graminis sp. hordei; E-GEOD-5684 – *Botrytis cinerea*; E-GEOD-5743 – 2,4-
 dichlorophenoxyacetic acid (2,4-D); E-ATMX-13 - methyl jasmonate; E-MEXP-550 –
 polychromatic radiation with decreasing short-wave cut-off in the UV range (UV-B
 experiment); E-MEXP-739 – syringolin A; E-MEXP-1797 – rotenone), Gene Expression
 Omnibus <http://www.ncbi.nlm.nih.gov/geo/> (accession numbers GSE5615 – elicitors
 LPS, HrpZ, Flg22 and NPP1; GSE5685 – virulent and avirulent *Pseudomonas syringae*;
 GSE9955 – BTH experiment 1; GDS417 – *E. cichoracearum*; GSE5530 – H₂O₂;
 GSE5621 – cold time course experiment; GSE5622 – osmotic stress time course
 experiment; GSE5623 – salt time course experiment; GSE5624 – drought time course
 experiment; GSE5722 – O₃; GSE12887 – norurazon; GSE10732, OPDA and

phytoprostane; GSE7112 – ABA experiment 2) and The Integrated Microarray Database System <http://ausubellab.mgh.harvard.edu/imds> (Experiment name: BTH time course, BTH experiment 2). Raw data files were RMA normalized and analyzed as previously described (Jaspers et al., 2010).

Sequence data relevant to this study can be found in GenBank data libraries under the following accession numbers: AF053638 (*SAD-A* cDNA), AF053639 (*SAD-B* cDNA), AF097651 (*SAD-C* cDNA), AF242183 (*SAD-A* promoter), and AF242182 (*SAD-C* promoter).

Supplemental Data

The following materials are available in the online version of this article.

Supplementary Figure S1. Potential *SAD* promoter elements conferring gene expression in seeds.

Supplementary Figure S2. MALDI-TOF MS analysis chromatograms and analysis reports for the SAD content of immunoprecipitates from pea seeds and leaves of pea plants irradiated with UV-B radiation

Supplementary Table S1. Substrates tested in enzymatic activity assays with recombinant SAD-A and SAD-C proteins.

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LITERATURE CITED

- Abe H, Yamaguchi-Shinozaki K, Urao T, Iwasaki T, Hosokawa D, Shinozaki K** (1997) Role of Arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression. *Plant Cell* **9:** 1859-1868
- Brosché M, Fant C, Bergkvist SW, Strid H, Svensk A, Olsson O, Strid, Å.** (1999) Molecular markers for UV-B stress in plants: alteration of the expression of four classes of genes in *Pisum sativum* and the formation of high molecular mass RNA adducts. *Biochim Biophys Acta* **1447:** 185-198
- Brosché M, Strid Å** (1999) Cloning, expression, and molecular characterization of a small pea gene family regulated by low levels of ultraviolet B radiation and other stresses. *Plant Physiol* **121:** 479-487
- Brosché M, Strid Å** (2000) Ultraviolet-B radiation causes tendril coiling in *Pisum sativum*. *Plant Cell Physiol* **41:** 1077-1079
- Caldwell MM** (1971) Solar UV irradiation and the growth and development of higher plants. In AC Giese, ed, *Photobiology (Currents topics in photobiology and photochemistry)*, Vol 6. Academic Press, New York, pp 131-177
- Chamberland S, Daile N, Bernier F** (1992) The legumin boxes and the 3' part of a soybean β -conglycinin promoter are involved in seed gene expression in transgenic tobacco plants. *Plant Mol Biol* **19:** 937-949
- Choi HW, Lee BG, Kim NH, Park Y, Lim CW, Song HK, Hwang BK** (2008) A role for a menthone reductase in resistance against microbial pathogens in plants. *Plant Physiol* **148:** 383-401
- Clough SJ, Bent AF** (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* **16:** 735-743
- Eulgem T, Rushton PJ, Robatzek S, Somssich IE** (2000) The WRKY superfamily of plant transcription factors. *Trends Plant Sci* **5:** 199-206
- Feldbrügge M, Sprenger M, Hahlbrock K, Weisshaar B** (1997) PcMYB1, a novel plant protein containing a DNA-binding domain with one MYB repeat, interacts in vivo with a light-regulatory promoter unit. *Plant J* **11:** 1079-1093
- Finsterbusch A, Lindemann P, Grimm R, Eckerskorn C, Luckner M** (1999) $\Delta(5)$ - 3β -Hydroxysteroid dehydrogenase from *Digitalis lanata* Ehrh. - a multifunctional enzyme in steroid metabolism? *Planta* **209:** 478-486

- Ghosh D, Pletnev VZ, Zhu D-W, Wawrzak Z, Duax WL, Pangborn W, Labrie F, Lin S-X** (1995) Structure of human estrogenic 17 β -hydroxysteroid dehydrogenase at 2.20 Å resolution. *Structure* **3**: 503-513
- Ghosh D, Wawrzak Z, Weeks CM, Duax WL, Erman M** (1994) The refined three-dimensional structure of 3 α ,20 β -hydroxysteroid dehydrogenase and possible roles of the residues conserved in short-chain dehydrogenases. *Structure* **2**: 629-640
- Gittins JR, Schuler MA, Strid Å** (2002) Identification of a novel nuclear factor-binding site in the *Pisum sativum sad* gene promoters. *Biochim Biophys Acta* **1574**: 231-244
- González-Guzmán M, Apostolova N, Bellés JM, Barrero JM, Piqueras P, Ponce MR, Micol JL, Serrano R, Rodríguez PL** (2002) The short-chain alcohol dehydrogenase ABA2 catalyzes the conversion of xanthoxin to abscisic aldehyde. *Plant Cell* **14**: 1833-1846
- Green AES, Sawada T, Shettle EP** (1974) The middle ultraviolet reaching the ground. *Photochem Photobiol* **19**: 251-259
- Green PJ, Yong MH, Cuozzo M, Kano-Murakami Y, Silverstein P, Chua NH** (1988) Binding site requirements for pea nuclear protein factor GT-1 correlate with sequences required for light-dependent transcriptional activation of the rbcS-3A gene. *EMBO J* **7**: 4035-4044
- Hartmann U, Valentine WJ, Christie JM, Hays J, Jenkins GI, Weisshaar B** (1998) Identification of UV/blue light response elements in the *Arabidopsis thaliana* chalcone synthase promoter using a homologous protoplast transient expression system. *Plant Mol Biol* **36**: 741-754
- Hoagland DR, Arnon DI** (1950) The water-culture method for growing plants without soil. In California Agricultural Experiment Station Circular 347. The College of Agriculture, University of California, Berkeley, CA, USA
- Jaspers P, Overmyer K, Wrzaczek M, Vainonen JP, Blomster T, Salojärvi J, Reddy RA, Kangasjärvi J** (2010) The RST and PARP-like domain containing SRO protein family: analysis of protein structure, function and conservation in land plants. *BMC Genomics* **11**: 170
- Jefferson RA** (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol Biol Rep* **5**: 387-405

- Jörnvall H, Persson B, Jeffery J** (1981) Alcohol and polyol dehydrogenases are both divided into two protein types, and structural properties cross-relate the different enzyme activities within each type. *Proc Natl Acad Sci USA* **78:** 4226-4230
- Josefsson LG, Lenman M, Ericson ML, Rask L** (1987) Structure of a gene encoding the 1.7 S storage protein, napin, from *Brassica napus*. *J Biol Chem* **262:** 12196-12201
- Kalbin G, Hidema J, Brosché M, Kumagai T, Bornman JF, Strid Å** (2001) UV-B-induced DNA damage and expression of defence genes under UV-B stress: tissue-specific molecular marker analysis in leaves. *Plant Cell Environ* **24:** 983-990
- Kallberg Y, Oppermann U, Jörnvall H, Persson B** (2002) Short-chain dehydrogenases/reductases (SDRs). Coenzyme-based functional assignments in completed genomes. *Eur J Biochem* **269:** 4409-4417
- Koncz C, Martini N, Szabados L, Hrouda M, Bachmair A, Schell J** (1994) Specialized vectors for gene tagging and expression studies. In SB Gelvin, RA Schilperoort, DPS Verma, eds, *Plant Molecular Biology Manual*, Vol. B2. Kluwer Academic, Dordrecht, The Netherlands, pp. 1-22
- Menkens AE, Schindler U, Cashmore AR** (1995) The G-box: a ubiquitous regulatory DNA element in plants bound by the GBF family of bZIP proteins. *Trends Biochem Sci* **20:** 506-510
- Murashige T, Skoog F** (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol Plant* **15:** 473-497
- Oppermann U, Filling C, Hult M, Shafqat N, Wu X, Lindh M, Shafqat J, Nordling E, Kallberg Y, Persson B, Jörnvall H** (2003) Short-chain dehydrogenases/reductases (SDR): the 2002 update. *Chemico-Biol Interact* **143-144:** 247-253
- Park HC, Kim ML, Kang YH, Jeon JM, Yoo JH, Kim MC, Park CY, Jeong JC, Moon BC, Lee JH, Yoon HW, Lee SH, Chung WS, Lim CO, Lee SY, Hong JC, Cho MJ** (2004) Pathogen- and NaCl-induced expression of the SCaM-4 promoter is mediated in part by a GT-1 box that interacts with a GT-1-like transcription factor. *Plant Physiol* **135:** 2150-2161
- Reidt W, Wohlfarth T, Ellerström M, Czihal A, Tewes A, Ezcurra I, Rask L, Bäumlein H** (2000) Gene regulation during late embryogenesis: the RY motif of maturation-specific gene promoters is a direct target of the FUS3 gene product. *Plant J* **21:** 401-408

- Ringer KL, Davis EM, Croteau R** (2005) Monoterpene metabolism. Cloning, expression, and characterization of (-)-isopiperitenol/(-)-carveol dehydrogenase of peppermint and spearmint. *Plant Physiol* **137**: 863-872
- Savage RS, Heller K, Xu Y, Ghahramani Z, Truman WM, Grant M, Denby KJ, Wild DL** (2009) R/BHC: fast Bayesian hierarchical clustering for microarray data. *BMC Bioinformatics* **10**: 242
- Sävenstrand H, Brosché M, Strid Å** (2002) Regulation of gene expression by low levels of ultraviolet-B radiation in *Pisum sativum*: Isolation of novel genes by suppression subtractive hybridisation. *Plant Cell Physiol* **43**: 402-410
- Scherbak N, Brosché M, Ala-Häivälä A, Strid H, Öhrfelt A, Nilsson F, Strid Å** (2009) Expression of *Pisum sativum* SAD polypeptides in production hosts and *in planta*: Tetrameric organization of the protein. *Prot Expr Purif* **63**: 18-25
- Schindler U, Beckmann H, Cashmore AR** (1992) TGA1 and G-box binding factors: two distinct classes of *Arabidopsis* leucine zipper proteins compete for the G-box-like element TGACGTGG. *Plant Cell* **4**: 1309-1319
- Siebert PD, Chenchik A, Kellogg DE, Lukyanov KA, Lukyanov SA** (1995) An improved PCR method for walking in uncloned genomic DNA. *Nucleic Acids Res* **23**: 1087-1088
- Thijs G, Marchal K, Lescot M, Rombauts S, De Moor B, Rouzé P, Moreau Y** (2002) A Gibbs sampling method to detect over-represented motifs in upstream regions of co-expressed genes. *J Comput Biol* **9**: 447-464
- Thomson RH** (1957) Naturally occurring quinones. Academic Press, London.
- Thomson RH** (1971) Naturally occurring quinones, 2nd Edition. Academic Press, London.
- Thomson RH** (1987) Naturally occurring quinones III. Recent advances. Chapman & Hall, London.
- Thomson RH** (1997) Naturally occurring quinones IV. Recent advances. Blackie Academic & Professional, London.
- Xia Z, Costa MA, Pélassier HC, Davin LB, Lewis NG** (2001) Secoisolariciresinol dehydrogenase purification, cloning and functional expression. *J Biol Chem* **276**: 12614-12623
- Youn B, Moinuddin SG, Davin LB, Lewis NG, Kang C** (2005) Crystal structures of apo-form and binary/ternary complexes of *Podophyllum* secoisolariciresinol

dehydrogenase, an enzyme involved in formation of health-protecting and plant defense lignans. *J Biol Chem* **280**: 12917-12926

Yu D, Chen C, Chen Z (2001) Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. *Plant Cell* **13**: 1527-1540

FIGURE LEGENDS

Figure 1. (a) Structures of quinones and aromatic carbonyls that function as substrates for recombinant SAD-C protein in the presence of NADH as co-factor; (b) Structures of quinones and cyclic and aromatic carbonyl compounds that do not function as substrates for the recombinant SAD-C protein in the presence of NADH as co-factor.

Figure 2. Western immunoblots showing the presence of SAD protein in different tissues of pea. (a) L – leaves (10.5 µg material loaded); S – stem (10.5 µg material loaded); R – root (10.5 µg material loaded); T – tendril (10.5 µg material loaded); DSS – dry seed soaked for 24 h in tap water (2 µg material loaded); DS – dry seed not soaked (2 µg material loaded); C – purified recombinant SAD-C protein (0.05 µg protein loaded). (b) Presence of SAD protein in different parts of the pea seed, after soaking for 24 h in tap water. C – purified recombinant SAD-C protein (0.05 µg protein loaded); G – embryonic axis tissue (5.8 µg material loaded); COT – cotyledon tissue (5.8 µg material loaded); SC – seed coat tissue (5.8 µg material loaded). (c) Presence of SAD protein in fresh pea reproductive tissues: P – pods (14 µg material loaded); FS – fresh pea seeds (14 µg material loaded); F – whole flowers (14 µg material loaded); C – purified recombinant SAD-C protein (0.05 µg protein loaded). Molecular weights of protein standard (M) bands are marked in kDa. Asterisks denote the monomeric SAD protein bands (28-29 kDa).

Figure 3. (a) Coomassie Brilliant Blue-stained SDS-PAGE gel of SAD antiserum immunoprecipitate of extracts from pea seed cotyledonary axis (SE) and pea leaves exposed to 5d of low level supplementary UV-B radiation (UL). H indicates the rabbit IgG heavy chain and S marks the bands excised for MALDI-TOF mass spectrometry analysis. Molecular weights of protein standard (M) bands are marked in kDa. (b,c) Comparison of SAD isoform protein sequences with peptide sequences obtained by MALDI-TOF mass spectrometry analysis of immunoprecipitated bands. (b) SAD-A sequence with the peptide sequences obtained by MALDI-TOF MS of band S from lane SE in (a) underlined. The three amino acids discriminating SAD-A from SAD-C are shown in bold (Brosché and Strid 1999). The C-terminal peptide proves that the seed-localized SAD is the SAD-A isoform; (c) SAD-C sequence with the peptide sequences obtained by MALDI-TOF MS of band S from lane UL in (a) underlined. The three amino

acids discriminating SAD-C from SAD-A are shown in bold. The peptide pattern obtained does not identify which of the SAD isoforms is induced by UV-B in leaves.

Figure 4. (a-i) Immunohistochemical staining of tissue cross-sections from 20-day-old pea plants. Sections were incubated with preimmune serum (a) or primary polyclonal SAD-A antiserum (b-i) diluted 1:8000 in all cases except in a) and b) where the dilution was 1:16000. Specific antibody binding was identified after treatment with biotinylated HRP-conjugated secondary antibody (diluted 1:1) and avidin peroxidase complex, by reaction with DAB substrate. (a) Negative immunostaining control – leaf from a pea plant exposed to 5d of UV-B, stained with preimmune serum; (b) Leaf tissue from a plant exposed to 5d of UV-B. Note the stained palisade parenchyma cells (arrow); (c) Stem tissue from a pea plant exposed to 5d UV-B. The arrowhead indicates stained cell layers below the stem surface; (d) Pod vascular tissue with surrounding parenchyma cells; (e) Tissue from the outer part of the pod epidermis. The arrow indicates epidermal cells; (f) Tissue from the inner part of the pod epidermis. The arrow indicates the surface against the ovary cavity; (g) Embryonic axis tissue from pea seeds soaked for 24 h showing the protoderm and the ground meristem. Note the obvious difference between the highly stained protoderm cells (arrow) and cells of the ground meristem, which have little or no SAD present; (h) Keel petal tissue. The arrow indicates the stained epidermal cell layer; (i) Ovary and ovule (indicated by an asterisk). The arrows indicate the two bow-shaped lines in the ovary in which SAD-containing cells are found, in addition to cells on the placental surface facing the ovules; (j-k) GUS activity in *SAD-CP-gusA* transgenic *Arabidopsis thaliana*. Rosette leaves were wounded either by cutting (j) or by pricking with a needle (k) then held for 3 h and frozen before staining for GUS activity with X-Gluc.

Figure 5. Wound-induced luciferase activity in *SAD-CP-luc* transgenic *Arabidopsis*. Luciferase activity per leaf section is shown for 9 different transformant lines before (white columns) and after (gray columns) wounding. The numbers below the X-axis denote the independent transgenic lines. AIR indicates the background reading of the luminometer (black column).

Figure 6. Transcript profiles of *Arabidopsis SAD* homologs. Bootstrapped Bayesian hierarchical clustering of the *A. thaliana* *SAD* homologous genes in plants subjected to

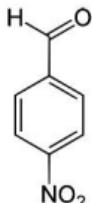
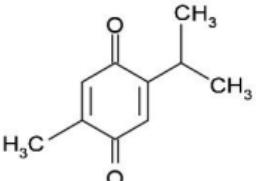
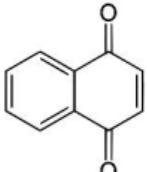
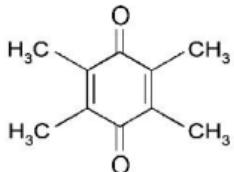
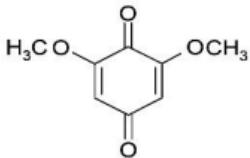
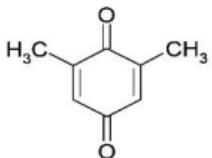
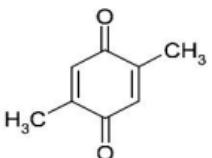
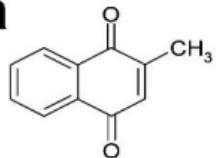
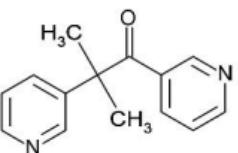
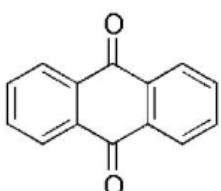
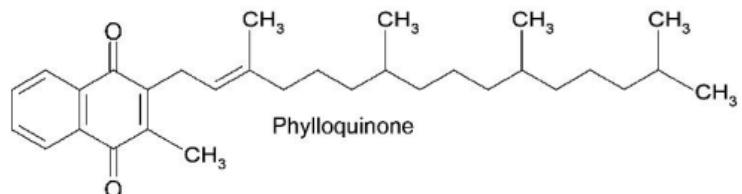
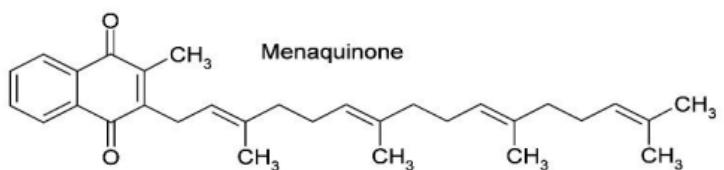
various treatments compared with normal growth conditions. Red and green indicate increased or decreased expression compared with untreated plants, respectively. The intensity of the colors is proportional to the absolute value of the fold difference.

Table 1. Michaelis-Menten kinetic constants for the SAD-C protein with three different substrates.

Substrate	K _m [μM]	V _{max} [nmol NADH min ⁻¹ (mg protein) ⁻¹]	k _{cat} [s ⁻¹]	k _{cat} /K _m [M ⁻¹ s ⁻¹]
Duroquinone (DQ)	180	96	0.046	256
1,4-Naphthoquinone (1,4-NQ)	66	88	0.042	636
Menadione (K ₃)	22	71	0.034	1554

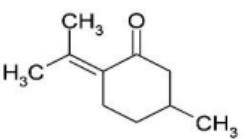
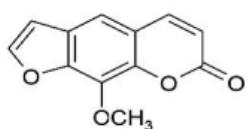
Table 2. Occurrence of promoter elements, previously implicated in either pathogen or light/UV-B responses in the pea *SAD-A* and *SAD-C* promoters, and in the promoters of the eight homologous genes from *Arabidopsis thaliana*. The pea and *Arabidopsis* promoter sequences were analyzed with text searches on both strands for the indicated motif consensus sequences. No mismatches were allowed except for the SPBM-element where 1 mismatch was allowed. An additional novel promoter element (indicated by *) was identified using the web-based program MotifSampler (<http://www.esat.kuleuven.ac.be/~thijs/Work/MotifSampler.html>; Thijs et al., 2002). The other promoter elements have been described previously: G-box (Menkens, Schindler and Cashmore 1995); WRKY (Eulgem et al., 2000); TGA1 (Schindler, Beckman and Cashmore 1992); GT-1 (Green et al., 1988); ACE (Hartmann et al., 1998); MRE (Feldbrügge et al., 1997); W-box AtNPR1 (Yu, Chen and Chen 2001); GT1-SCAM4 (Park et al., 2004); MYBATRD22 (Abe et al., 1997); SPBM (Gittins et al., 2002).

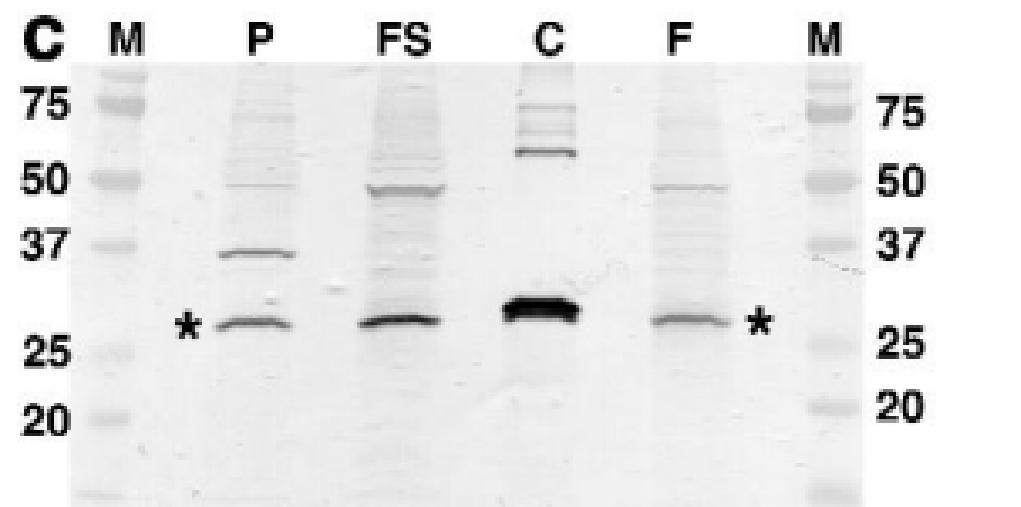
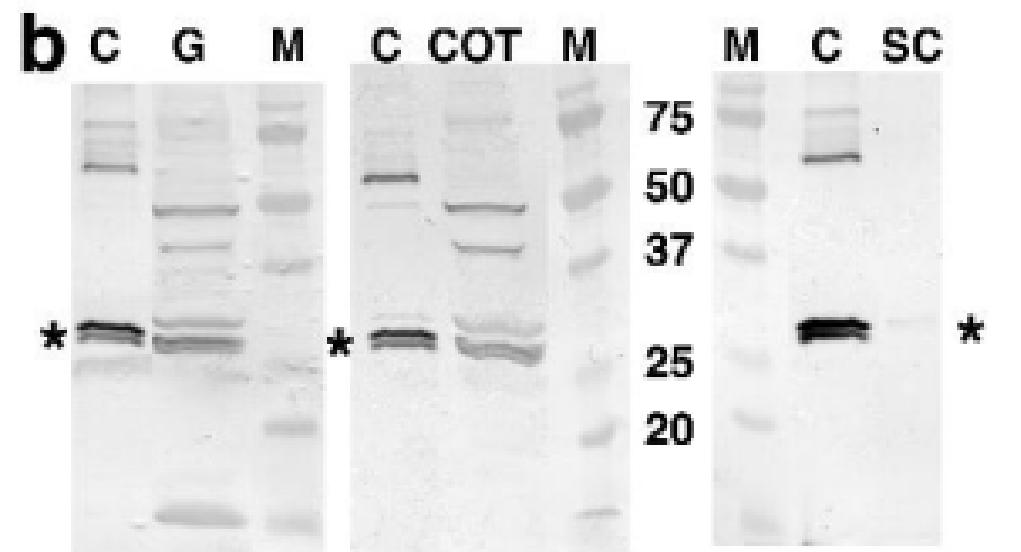
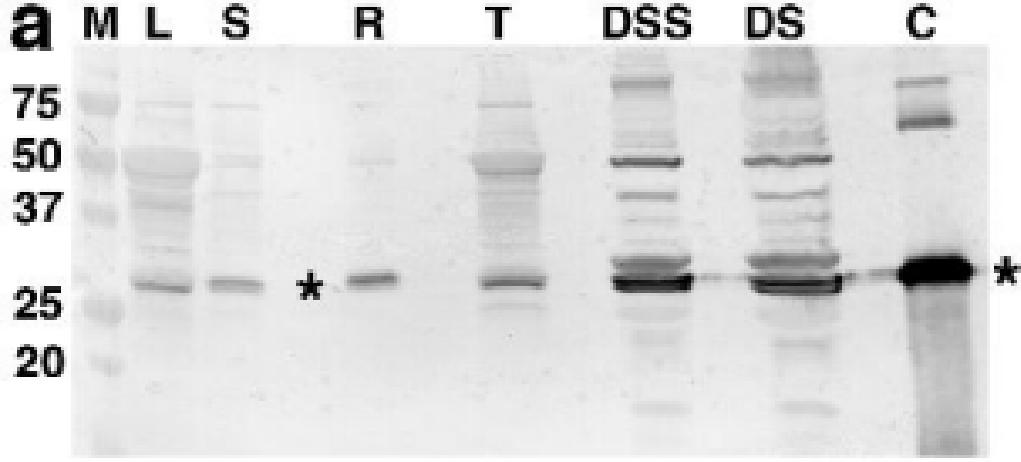
Gene	UV-B regulation	Motif name:	G-Box	WRKY	TGA1	GT-1	GT1-SCAM4	ACE
		Motif sequence:	CACGTG	TTGACY	TGACG	GGTTAA	GAAAAA	CACGT
Pea <i>SAD-A</i>	Yes		-	-	4	1	2	1
Pea <i>SAD-C</i>	Yes		1	-	1	-	1	3
At2g47120	No		-	-	-	2	5	1
At2g47130	Yes		-	5	-	-	1	1
At2g47140	No		-	2	-	-	1	-
At1g52340	No		-	1	2	-	2	2
At3g26770	No		-	2	-	-	-	-
At3g29260	No		-	-	-	2	-	-
At3g29250	Yes		-	-	-	-	1	-
At3g51680	No		-	-	-	-	3	-
		Motif name:	MRE	MYBATRD22	W-box	AtNPR1	SPBM	*
Gene	UV-B regulation	Motif sequence:	ACCTA	CTAACCA	TTGACTTGAC	GTGGCGCCAC	AANCAATT	
						1 (with 1 mismatch)		1
Pea <i>SAD-A</i>	Yes		-	-	-	1 (with 1 mismatch)		1
Pea <i>SAD-C</i>	Yes		-	-	-	1		3
At2g47120	No		-	1	-	-	-	-
At2g47130	Yes		-	1	1	-	-	2
At2g47140	No		-	-	-	-	-	1
At1g52340	No		1	-	-	-	-	-
At3g26770	No		2	-	-	-	-	-
At3g29260	No		1	-	-	-	-	1
At3g29250	Yes		2	1	-	-	-	2
At3g51680	No		-	-	-	-	-	-

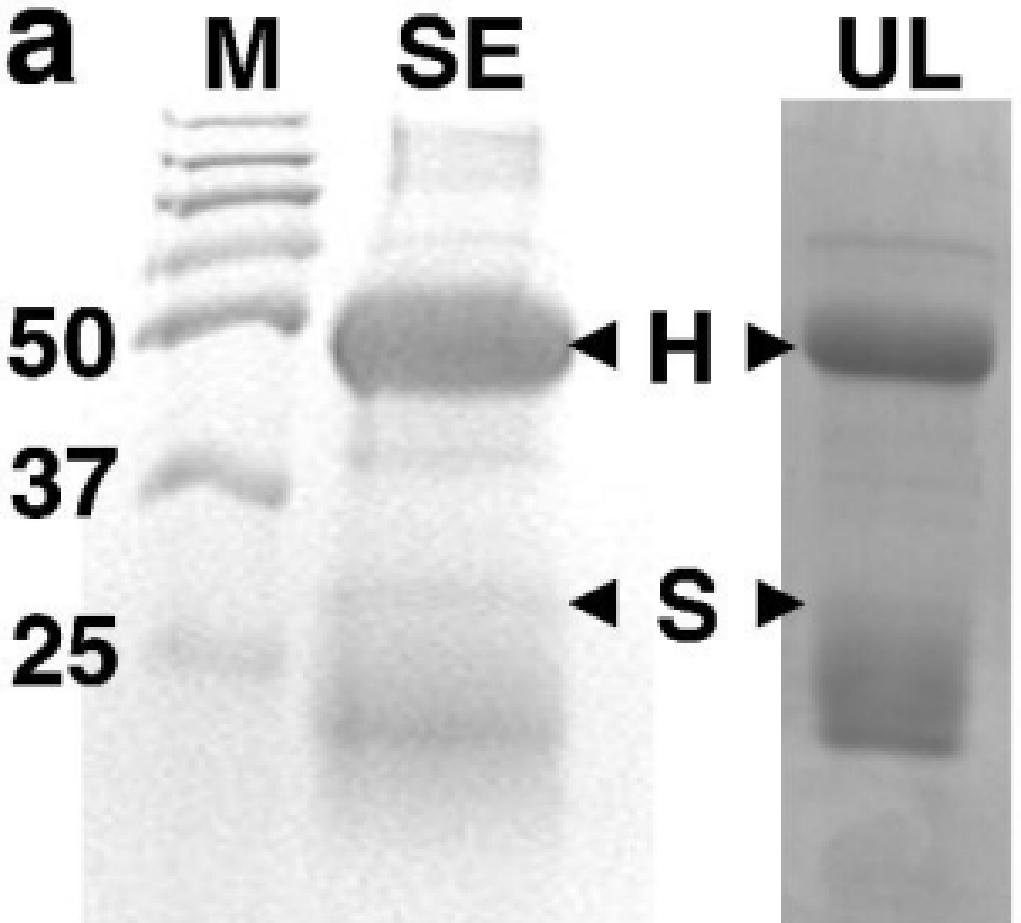
a**b**

Anthraquinone

Metyrapone







b

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1 MAESSSTKIG LRLAGKVAAV TGGASGIGKE TAHLFANQAA RNVVIADIQD
51 ELGIQVAESI GTDRCTFIREC DIRIEDDVKN LVQSTVDTYG QIDITHCNAG
101 IISPSDQTLL ELDVSQANGV FATNAIGTAL CVKHAARAMV DGKVRGSIVC
151 TASISASYCV TTGTDTYSMSK HAVLGLMRSA SVQLAKYGIR VNSVSPNGLA
201 TPLTEKLLDA DAKTVEEIFS KFSMLKGUVL RTNHVADAVL FLASNDSDFV
251 TGFDLDRVDCN YITSHAVI

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c

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1 MAESSSTKIG LRLAGKVAAV TGGASGIGKE TAHLFANQAA RNVVIADIQD
51 ELGIQVAESI GTDRCTFIREC DIRIEDDVKN LVQSTVDTYG QIDITHCNAG
101 IISPSDQTLL ELDVSQANGV FATNAIGTAL CVKHAARAMV DGKVRGSIVC
151 TASISASYCV TTGTDTYSMSK HAVLGLMRSA SVQLAKYGIR VNSVSPNGLA
201 TPLTEKLLDA DAKTVEEIFS KFSMLKGUVL RTNHVADAVL FLASNDSDFV
251 TGFDLDRVDCN YITSDAVI

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