The Pea SAD Short-Chain Dehydrogenase/Reductase: Quinone Reduction, Tissue Distribution, and Heterologous Expression\textsuperscript{1[W][OA]}

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The expression of the small pea (\textit{Pisum sativum}) gene family encoding short-chain alcohol dehydrogenase-like proteins (SAD-A, -B, and -C) is increased dramatically by low-intensity UV-B radiation (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 and stems. The different localization patterns of SAD suggest functions both in development and in responses to environmental stimuli. Finally, the pea SAD-C promoter was shown to confer heterologous wound-induced expression in Arabidopsis (\textit{Arabidopsis thaliana}), which confirmed that the inducibility of its expression is regulated at the transcriptional level.

\textsuperscript{1}This work was supported by the Helge Ax:son Johnson Foundation (to N.S.), the Carl Trygger Foundation (to Å.S.), and the Faculty for Science and Technology at Örebro University (to Å.S.). The Strategic Network for Swedish Plant Biotechnology (SSF) funded J.R.G.’s stay in Sweden with a grant to Å.S. This work was conducted as part of the European Union COST action FA 0906 “UV4 growth-UV-B radiation: A specific regulator of plant growth and food quality in a changing climate,” of which Å.S. and M.B. are members, representing Sweden and Estonia, respectively.

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\textsuperscript{[W]}The online version of this article contains Web-only data.

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www.plantphysiol.org/cgi/doi/10.1104/pp.111.173336
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, aluminum 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 (sensitivity of 55%; Ringer et al., 2005), Δ3-3β-hydroxyxysteroid dehydrogenase (HS; 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 (Arabidopsis thaliana) contains at least eight different genes homologous to the pea SAD genes, with similarity scores of at least 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-Guzmán et al., 2002). For two of the other Arabidopsis SAD gene homologs (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 (17β-HSD; Ghosh et al., 1995) or homotetrameric (3α,20β-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 enzymes were incubated with a large number of potential substrates and were shown to catalyze the reduction of carboxyls and quinones at a low rate (M. Brosché and A. Strid, unpublished data). This suggested that the SAD proteins are catalysts in the metabolism of quinones and/or carboxyls in response to environmental stimuli. In order to test this hypothesis, the catalytic function of the SAD-C enzyme has 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 pea and the expression of pea SAD promoter/reporter constructs in Arabidopsis have also been examined in nonexposed or UV-B-exposed tissue and 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 (data not shown). Second, for unknown reasons, the yield of the Escherichia coli-produced recombinant SAD-C protein was much greater than that of SAD-A. However, for the studies described in Supplemental 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 A340 upon incubation with a variety of putative carbonyl- or hydroxyl-containing substrates (Supplemental Table S1), including gibberellins, brassinosteroids, other steroids, alcohols, sugars, flavonoids, quinones, and aromatic carboxyls. Activity was found only with quinones and aromatic carboxyls. 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, 2,6-dimethoxybenzoquinone, duroquinone (DQ), thymoquinone, 1,4-naphthoquinone (1,4-NQ), and menadione (K3) were all reduced (Fig. 1A). In addition, the aromatic carboxyl 4-nitrobenzaldehyde was reduced. Other quinones with long isoprenoid side chains, such as menaquino- none, phyloquinone, ubiquinone, and decylplastoquinone, were not reduced (Fig. 1B; Supplemental Table S1). Furthermore, the three-membered ring compound anthraquinone failed to function as a substrate for this enzyme. Other carbonyl-containing chemical species, such as the furocoumarins 8-methoxypsoralen 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 (data not shown).

NADPH could be used as the reductant instead of NADH but with a much reduced reaction rate (data not shown). Also, catalysis of the reverse reaction could be demonstrated (reduction of NAD+ by 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 (data 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 I). The catalytic rate for these substrates was similar, differing only by up to 15% from an average of 85 nmol NADH oxidized min⁻¹ mg⁻¹ protein. However, the affinity of the enzyme for the three substrates differed markedly, with K₃ having a 3- and 9-fold lower Kₘ than 1,4-NQ and DQ, respectively, which was reflected in the kₗ/ₘ (Table I). 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 Kₘ for NADH was determined to 7 μM (data 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 pea tissues. This analysis showed a distribution of the SAD protein in leaves, stems, tendrils, roots, and seeds (Fig. 2A). Doublets or multiple bands seen on the western blots at the size of the SAD monomer (28–29 kD) represent either tissue differences in the expression of different SAD genes or, more likely, variable posttranslational 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 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 (data not shown). SAD was found in the tissues of all three cultivars tested (cv Greenfeast, Greenshaft, and Kelvedon Wonder; data not shown).

In order to confirm the specificity of the SAD-A antiserum, immunoprecipitation was performed using homogenates of 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 matrix-assisted laser-desorption ionization time of flight mass spectrometry (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; Supplemental Fig. S2, A and C).

Analysis of SAD Protein Tissue Distribution in UV-B-Irradiated and Nonirradiated 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 preimmune serum resulted in no immunostaining (Fig. 4A), confirming that the polyclonal antiserum contained no antibodies cross-reacting with other pea proteins. This preimmune 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 d). 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; Supplemental Fig. S2, B and 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 Figure 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.

### 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$^{-1}$ protein) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) |
|-----------|------------|---------------------------------------------|-------------------|---------------------------------|
| DQ        | 180        | 96                                          | 0.046             | 256                             |
| 1,4-NQ    | 66         | 88                                          | 0.042             | 636                             |
| $k_j$     | 22         | 71                                          | 0.034             | 1,554                           |
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 vascular tissue (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 the keel tissue shown in Figure 4H. This staining was not observed when preimmune serum

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**Figure 2.** Western immunoblots showing the presence of SAD protein in different tissues of pea. A, L, Leaves (10.5 μg of material loaded); S, stem (10.5 μg of material loaded); T, tendril (10.5 μg of material loaded); DSS, dry seed soaked for 24 h in tap water (2 μg of material loaded); DS, dry seed not soaked (2 μg of material loaded); C, purified recombinant SAD-C protein (0.05 μg of 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 of protein loaded); G, embryonic axis tissue (5.8 μg of material loaded); COT, cotyledon tissue (5.8 μg of material loaded); SC, seed coat tissue (5.8 μg of material loaded). C, Presence of SAD protein in fresh pea reproductive tissues. P Pods (14 μg of material loaded); FS, fresh pea seeds (14 μg of material loaded); C, purified recombinant SAD-C protein (0.05 μg of protein loaded); F, whole flowers (14 μg of material loaded). Molecular masses of protein standard (M) bands are marked in kD. Asterisks denote the monomeric SAD protein bands (28–29 kD).

**Figure 3.** A, Coomassie Brilliant Blue-stained SDS-PAGE gel of SAD antisem immunoprecipitate of extracts from pea seed cotyledonary axis (SE) and pea leaves exposed to 5 d of low-level supplementary UV-B radiation (UL). H indicates the rabbit IgG H chain, and S marks the bands excised for MALDI-TOF MS analysis. Molecular masses of protein standard (M) bands are marked in kD. B and C, Comparison of SAD isoform protein sequences with peptide sequences obtained by MALDI-TOF MS 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 boldface (Broschel 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 SE in A underlined. The three amino acids discriminating SAD-A from SAD-C are shown in boldface. The peptide pattern obtained does not identify which of the SAD isoforms is induced by UV-B in leaves.
was used instead of SAD antiserum. In the stamen, SAD was predominantly located in epidermal cells (data not shown), whereas in the stigma, this protein appeared to be more uniformly distributed (data 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 subepidermal 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; 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 UV-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 plants were transformed with promoter/reporter constructs comprising the SAD-C 0.7-kb proximal promoter (Gittins et al., 2002) fused to either the GUS (gusA; Fig. 4, J and K) or luciferase (luc; Fig. 5) reporter gene.

As judged by the luciferase activity measured in the leaves of nine independent SAD-C-P-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-C-P-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 0.7-kb promoter fragment used in the construct does not contain the necessary UV-B regulatory elements or that pea uses different regulatory elements compared with 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 robust mulitarray average 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 was induced by wounding treatment (data 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 recognized stress-responsive cis-elements (Table II). Consistent with its induction by several pathogens, the At2g47130 promoter contained several WRKY recognition sites, previously shown to be important in pathogen responses (Euglem 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

Figure 5. Wound-induced luciferase activity in SAD-CP-luc transgenic Arabidopsis. Luciferase activity per leaf section is shown for nine 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).
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://bioinformatics.psb.ugent.be/webtools/plantcare/html/Motif_Sampler. html; Thijs et al., 2002). The other promoter elements have been described previously: G-box (Menkens et al., 1995); WRKY (Euglem et al., 2000); TGA1 (Schindler et al., 1992); GT-1 (Green et al., 1988); ACE (Hartmann et al., 1998); MRE (Feldbrügge et al., 1997); W-box AtNPR1 (Yu et al., 2001); GT1-SCAM4 (Park et al., 2004); MYBATRD22 (Abe et al., 1997); SPBM (Gittins et al., 2002).

Table II. 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

| Gene            | UV-B Regulation | Motif Name | Motif Sequence | Presence of Motif | Presence of Motif |
|-----------------|-----------------|------------|----------------|-------------------|-------------------|
| Pea SAD-A       | Yes             | G-Box      | (CACGTG)       | WRKY (TTGACY)     | TGA1 (TGACG)      | GT-1 (GGTTAA)     | GT1-SCAM4 (GAAAAA) | ACE (CACGT) |
| Pea SAD-C       | No              | —          | —              | —                 | —                 | —                 | —             | —         |
| At2g47120       | Yes             | —          | —              | —                 | —                 | —                 | —             | —         |
| At2g47130       | No              | —          | —              | —                 | —                 | —                 | —             | —         |
| At2g47140       | No              | —          | —              | —                 | —                 | —                 | —             | —         |
| At1g52340       | No              | —          | —              | —                 | —                 | —                 | —             | —         |
| At3g26770       | No              | —          | —              | —                 | —                 | —                 | —             | —         |
| At3g29260       | No              | —          | —              | —                 | —                 | —                 | —             | —         |
| At3g29250       | Yes             | —          | —              | —                 | —                 | —                 | —             | —         |
| At3g51680       | No              | —          | —              | —                 | —                 | —                 | —             | —         |
| MRE (ACCTA)     | Yes             | —          | —              | —                 | —                 | —                 | —             | —         |
| MYBATRD22 (CTAACCA) | Yes          | —          | —              | —                 | —                 | —                 | —             | —         |
| W-Box AtNPR1 (TGACTTGAC) | Yes    | —          | —              | —                 | —                 | —                 | —             | —         |
| SPBM (GTGGCGCCCAC) | Yes          | —          | —              | (1 with 1 mismatch) | 1                 | 1                 | 1             | 1         |

DISCUSSION

The aims of this study were to elucidate the catalytic function of the pea SDR 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 I). There were no indications in our study that SAD-C is involved in ABA biosynthesis or metabolism (Supplemental 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 nonphysiological; the physiological quinones tested (i.e. those containing long hydrophobic aliphatic side chains) did not function as substrates. Several hundred different benzoquinones and naphthoquinones are found in plants (Thomson, 1957, 1971, 1987, 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
naphthoquinones, other than phyloquinone, ubiqui-
none, or decylplastoquinone, in legumes, such as pea,
or Arabidopsis. Therefore, we have so far not been able
to exactly pinpoint 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 isoform 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 semilocal 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. 4, J and K). It could not be conclu-
sively determined whether SAD-A or SAD-C was the
isoform 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 epider-
mal or subepidermal cells (Fig. 4, B, C, and E–H). SAD
(in this case SAD-A) was most abundant in the proto-
derm of the pea seed cotyledonary axis (Fig. 4G),
which also demonstrates the involvement of this en-
zyme in development in addition to a role in the
response to environmental stimuli. However, expres-
sion around vascular tissue was also apparent (Fig. 4,
C, 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. 4, B and C) that experi-
enced the highest intensities of UV-B radiation (in
accordance with previous findings by Kalbin et al.
[2001]).

The apparent SAD distribution within the ovary is
particularly noteworthy. Figure 4I shows two bow-
shaped lines of cells in this organ, indicating a spe-
cialized 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. There-
fore, SAD appears to be important in pea both for
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. This was further examined
by identifying UV-B-inducible Arabidopsis SAD ho-
mologs in expression studies using public Affymetrix
chip data (Fig. 6). Of the eight Arabidopsis genes most
similar to the pea SAD genes (Arabidopsis Genome
Initiative codes At1g52340, At2g47120, At2g47130,
At2g47140, At3g26770, At3g29250, At3g29260, and
At3g51680), two, At3g29250 and At2g47130, are induced
by UV-B, biotic stress, and osmotic stress. At2g47140
(corresponding to protein AtSDR3; Choi et al., 2008)
and At3g51680 show some regulation in response to
biotic and/or salt stress. The other five Arabidopsis
SAD homologs display only minor responses to envi-
ronmental 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 pea apparently only con-
tains three to six SAD genes (Brosché and Strid, 1999;
J.R. Gittins and A. Strid, unpublished data), compared
with 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 genes.

To test whether the promoter regions of the Arabi-
dopsis SAD homologs that were induced by environ-
mental stimuli and the pea SAD promoters share any
cis-regulatory elements, the presence of previously
characterized light, stress, or pathogen response ele-
ments was investigated (Table II). Unlike the pea
SAD-C promoter, the Arabidopsis SAD sequences con-
tain 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 identifica-
tion. Furthermore, the pea SAD-A promoter contains
elements previously shown to be involved in directing
gene expression in seeds (Supplemental Fig. S1A;
Josefsson et al., 1987; Chamberland et al., 1992; 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 toward 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 this study show that the
pea SAD-C protein is capable of reducing benzoqui-
nones and naphthoquinones in vitro. SAD proteins
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 pea plant and in various cell types within the examined tissues. SAD expression was studied by immunoblotting, immunoprecipitation, IHC, and heterologous expression using the pea SAD-C promoter in Arabidopsis. 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* 'Greenfeast') were grown in vermiculite in a growth chamber at 22°C with a 16-h-light/8-h-dark cycle (approximately 80 μmol photons m⁻² s⁻¹). Following leaf appearance, plants were watered every 2 d 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°C ± 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 (*Arabidopsis thaliana*) ecotype Columbia (Col-0) were sown in sterilized compost (70% moss:30% perlite; Weibulls Sår-/Plugggard; Weibull Trädgård). Synchronous germination was promoted by holding at 4°C in the dark for 2 to 3 d. Before transfer to compost, primary transgenic lines (Weibull Tra¨dga˚rd). Synchronous germination was promoted by holding at 4°C in the dark for 2 to 3 d. Before transfer to compost, primary transgenic lines were used for induction of transgenic plants.

**Enzymatic Activity Measurements**

The catalytic activity and kinetics of the recombinant pea SAD protein were determined by following the change in absorbance of NADH at 340 nm. When hydrophobic substrates were used, these were dissolved in 5% albumin, and 0.1% Triton X-100. The amount of bovine pancreas RNase I powder (Roche Diagnostics) 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) as the standard. SDS-PAGE of protein from pea leaf extracts was performed using 10% precast gels (Ready-Gel; Bio-Rad) and transferred by electroblotting onto Hybond-C pure membrane (GE Healthcare). Western blotting with anti-SAD-A protein primary antiseraum (500-fold dilution) was performed according to a standard method (Qagen). After reaction with alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody, immunostained bands were visualized using chromogenic substrate (nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate) according to a standard protocol (Promega). Western blots were repeated at least twice with samples from different 20-d-old plants. Where applicable, SDS gels were silver stained using an automated stainer (GE Healthcare) according to the manufacturer’s protocol.

**Immunoprecipitation**

One hundred microliters of pea leaf or pea seed homogenate was mixed with 50 μL of antisera and 350 μL of 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 EDTA, and 1 mM EDTA) and incubated for 2 h at 4°C with agitation. Then, 20 μL of Protein G-Sepharose bead suspension (Santa Cruz Biotechnology) was mixed with the sample/antiseraum solution and incubated overnight at 4°C with agitation. The mixture was centrifuged for 1 min at 1,000g at 4°C, and the pelletted Protein G-Sepharose beads were washed three times in 1 mL of phosphate-buffered saline (PBS). After the final wash, the pellet was taken up in 50 μL of PBS and mixed with an equal volume of Laemml buffer containing 10% 2-mercaptoethanol and heated at 100°C for 10 min. After centrifugation at 10,000g for 1 min, the supernatant was separated on a 10% SDS-polyacrylamide gel as described above and the gel was stained with Coomassie Brilliant Blue R-250. The bands corresponding to the precipitated protein were excised from the gel and analyzed by in-gel trypsin digestion and MALDI-TOF MS as described by Scherbak et al. (2009).

**IHC**

Pea tissue samples were placed in cassettes and immersed in Histolfix fixation solution (5% formaldehyde in NaF buffer, pH 7.2; effective osmolarity 300 mosmol; Histolab) for 6 to 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%), embedded in paraffin at 60°C, and cut into 4-μm-thick sections using a HM 355 S Rotary Microtome (Microm International). The sections were floated on distilled water at room temperature, heat stretched in distilled water at 50°C, and then mounted on positively charged slides (Superfrost plus; Menzel-Gla¨ser). 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, and 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% PBS. The tissue was blocked by incubation for 1 h at room temperature in a blocking buffer (0.1% Tris-buffered saline, pH 7.6, 1 g L⁻¹ bovine serum albumin, and 0.1% Triton X-100). Primary anti-SAD-A antiseraum, routinely diluted 1:8,000 with DAKO ChemMate antibody diluent (Dako Cytomation).
Norden), 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 nonspecific 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 temperature. 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 (approximately 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 cryoembedding compound (Histolab) and frozen in isopentane and dry ice. Sections of 6 μm were cut using a cryostat at −20°C and 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. Preimmune rabbit serum was used at similar concentrations as a negative control. 5% bovine serum albumin and 0.1 M PBS were added to the slides, which were then incubated for 1 h.

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 Biosciences; Siebert et al., 1995). One amplified fragment of approximately 1 kb contained the 5′ noncoding 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 pCFV814lac and pCFV812 (Konecz et al., 1994) to drive expression of the lac and 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-C: lac Arabidopsis transgenics. Rosette leaves were cut along the midvein; 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-C: 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 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Jefferson, 1987).

Analysis of Public Arabidopsis Affymetrix Chip Data

Affymetrix raw data were downloaded from NASCArrays (http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl) with the following accession numbers (NASCARRAYS-143, paraquat; NASCARRAYS-353, ZAT12; NASCARRAYS-176, ABA time-course experiment 1; NASCARRAYS-192, α-buprofen); from ArrayExpress (http://www.ebi.ac.uk/microarray-as/aec/) with the following accession numbers (E-GEOD-12856, Blumeria graminis sp. hordei; E-GEOD-5684, Botrytis cinerea; E-GEOD-5743, 2,4-dichlorophenoxoacetic acid; E-ATMX-13, methyl jasmonate; E-MEXP-550, polychlorinated dioxins) with the experiment name BTH time course for BTH experiment 2. Raw data files were robust multiarray average normalized and analyzed as described previously (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.

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

Supplemental 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.

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

ACKNOWLEDGMENTS

We thank Dr. Jarkko Salojärvi for help with the cluster analysis.

Received January 26, 2011; accepted February 20, 2011; published February 22, 2011.

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