In plant cells, ascorbate is a major antioxidant that is involved in the ascorbate-glutathione cycle. Monodehydroascorbate reductase (MDAR) is the enzymatic component of this cycle involved in the regeneration of reduced ascorbate. The identification of the intron-exon organization and the promoter region of the pea (Pisum sativum) MDAR 1 gene was achieved in pea leaves using the method of walking polymerase chain reaction on genomic DNA. The nuclear gene of MDAR 1 comprises nine exons and eight introns, giving a total length of 3,770 bp. The sequence of 544 bp upstream of the initiation codon, which contains the promoter and 5′ untranslated region, and 190 bp downstream of the stop codon were also determined. The presence of different regulatory motifs in the promoter region of the gene might indicate distinct responses to various conditions. The expression analysis in different plant organs by northern blots showed that fruits had the highest level of MDAR. Confocal laser scanning microscopy analysis of pea leaves transformed with Agrobacterium tumefaciens having the binary vectors pGD, which contain the autofluorescent proteins enhanced green fluorescent protein and enhanced yellow fluorescent protein with the full-length cDNA for MDAR 1 and catalase, indicated that the MDAR 1 encoded the peroxisomal isoform. The functional analysis of MDAR by activity and protein expression was studied in pea plants grown under eight stress conditions, including continuous light, high light intensity, continuous dark, mechanical wounding, low and high temperature, cadmium, and the herbicide 2,4-dichlorophenoxyacetic acid. This functional analysis is representative of all the MDAR isoforms present in the different cell compartments. Results obtained showed a significant induction by high light intensity and cadmium. On the other hand, expression studies, performed by semiquantitative reverse transcription-polymerase chain reaction demonstrated differential expression patterns of peroxisomal MDAR 1 transcripts in pea plants grown under the mentioned stress conditions. These findings show that the peroxisomal MDAR 1 has a differential regulation that could be indicative of its specific function in peroxisomes. All these biochemical and molecular data represent a significant step to understand the specific physiological role of each MDAR isoenzyme and its participation in the antioxidant mechanisms of plant cells.
protein and the associated gene responsible for each activity have not been investigated. Very recently, it has been reported that Arabidopsis thaliana has five genes of MDAR, and one of them has multiple transcription starts that cause a dual targeting to chloroplasts and mitochondria (Obara et al., 2002; Chew et al., 2003).

In chloroplasts, MDAR could have two physiological functions: the regeneration of reduced ascorbate from modehydroascorbate and the mediation of the photo¬duction of dioxygen to superoxide radicals when the substrate modehydroascorbate is absent (Miyake et al., 1998).

Peroxisomes are single membrane-bound subcellular organelles with an essentially oxidative type of metabolism and a simple morphology that does not reflect the complexity of their enzymatic composition (Tabak et al., 1999; Corpas et al., 2001). The main functions described for peroxoisomes in plant cells are the photorespiration cycle, fatty acid β-oxidation, the glyoxylate cycle, and the metabolism of reactive oxygen species and ureides (Huang et al., 1983; Baker and Graham, 2002). These roles indicate that peroxisomes are involved in distinct metabolic networks, mainly by establishing interconnections between different cell compartments (Corpas et al., 2001; Igamberdiev and Lea, 2002; Minorsky, 2002). Different lines of evidence have shown that leaf peroxisomes can be responsible for a variety of induced oxidative stress situations (del Río et al., 1996, 1998). The activity of the ascorbate-glutathione cycle enzymes has been demonstrated in pea (Pisum sativum) leaf peroxisomes (Jiménez et al., 1997, 1998), but with the exception of APX, there is no molecular information on the other enzymatic components of this cycle in these organelles.

In this article, we report the isolation and characterization of a full-length genomic clone encoding a monodehydroascorbate reductase (MDAR 1) containing a putative peroxisomal targeting signal type 1 (PTS1) in the C terminus that was demonstrated to be localized in peroxisomes. Transcriptome analysis of peroxisomal MDAR 1 under different abiotic stress conditions showed a differential regulation.

RESULTS

Full-Length Genomic Clone of an MDAR from Pea Leaves

Using the PCR walking strategy, we isolated the complete gene of the MDAR 1, which comprises nine exons and eight introns, giving a total length of 3,770 bp. The sequence of 544 bp upstream of the initiation codon, which contains promoter and 5’ untranslated region, and 190 bp downstream of the stop codon were also determined.

Bioinformatic analysis was undertaken to identify conserved motifs found in other eukaryotic promoters and to find putative cis-elements that could be operative in the regulation of MDAR gene expression. Table I shows the promoter sequence containing several putative regulatory elements. Additionally, the comparison of the pea MDAR 1 promoter regions with that of the Arabidopsis putative peroxisomal MDAR (At3g52880) showed a TATA box (positions −410 and −250) in the Arabidopsis gene and many identical cis-elements in the pea MDAR 1 promoter (Table I).

To get deeper insights into the genomic structure of the pea MDAR1 gene, this was compared with the MDAR genes found in the Arabidopsis genome. Table II shows the five MDAR genes and the eight deduced proteins found in the Arabidopsis genome with the number of exons/introns. A comparative analysis of the intron position in the protein sequence between

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**Table I. Promoter elements identified in the 5’ untranslated region of the MDAR 1 gene**

<table>
<thead>
<tr>
<th>Category</th>
<th>Co-Acting Element</th>
<th>Sequence</th>
<th>Position</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initiation</td>
<td>INRNTPSABD</td>
<td>YTCANTYY</td>
<td>−63, +52</td>
<td>Nakamura et al. (2002)</td>
</tr>
<tr>
<td>Light</td>
<td>ASF1MOTIFCAMV</td>
<td>TGACGG</td>
<td>−172*</td>
<td>Terzaghi and Cashmore (1995)</td>
</tr>
<tr>
<td></td>
<td>BOXIIPCHS</td>
<td>AGTGGC</td>
<td>−176* +12</td>
<td>Block et al. (1990)</td>
</tr>
<tr>
<td></td>
<td>CIACADIANLELHC</td>
<td>CAANNNNATC</td>
<td>−226, −109, +18*</td>
<td>Piechulla et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>GATABOX</td>
<td>GATA</td>
<td>+87</td>
<td>Lam and Chua (1989)</td>
</tr>
<tr>
<td></td>
<td>IBOX</td>
<td>GATAAG</td>
<td>−226</td>
<td>Giuliano et al. (1988)</td>
</tr>
<tr>
<td></td>
<td>LRENPCABE</td>
<td>AGCTGGCA</td>
<td>−174*</td>
<td>Castresana et al. (1998)</td>
</tr>
<tr>
<td>Abscisic acid/drought</td>
<td>ABRELATERD1*</td>
<td>AGCTG</td>
<td>−174*</td>
<td>Simpson et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>ACCTGTERD1*</td>
<td>ACCT</td>
<td>−173, −173*, −89</td>
<td>Simpson et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>MYBIAT*</td>
<td>WAACCA</td>
<td>+45*</td>
<td>Abe et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>MYBATRD22</td>
<td>CTAAACCA</td>
<td>−90</td>
<td>Abe et al. (1997)</td>
</tr>
<tr>
<td>Cold/freeze</td>
<td>MYCONSENSUSAT</td>
<td>CANNTG</td>
<td>+41, +41*</td>
<td>Chinnusamy et al. (2003)</td>
</tr>
</tbody>
</table>

*Cis-acting elements found in the promoter region (650 bp analyzed) of the Arabidopsis putative peroxisomal MDAR (At3g52880): BOXIIPCHS (positions −325 and −169*), GATA BOX (positions −570, −516, −193, −31, −5, −455*, −334*, and −152*), IBOX (position −336), LRENPCABE (position −325 and −170*), ABRELATERD1 (positions −325, −166, and −167*), ACCTGTERD1 (positions −560, −325, −166, −560*, −325*, and −166*), MYBIAT (positions −50 and −121*), and MYCONSENSUSAT (positions −638, −475, −464, −452, −357, −317, −167, −638*, −475*, −464*, −452*, −357*, −317*, and −167*).
pea MDAR1 (AAU11490) and the eight Arabidopsis MDARs is shown in Figure 1. The existence of a pattern in the position of the introns in the different MDARs was observed. In the case of the pea putative peroxisomal MDARs, it was found that six introns had identical positions to the Arabidopsis putative peroxisomal MDAR (NP_190856) since both contain a putative PTS1. On the contrary, in At3g09940 (NP_566361), which has the same number of exons/introns as pea MDAR1, only one intron was found with identical position.

Analysis of the Deduced Amino Acid Sequence of Pea MDAR 1

The MDAR 1 cDNA contained an open reading frame of 1,302 bp that coded for a protein of 433 amino acids. The deduced protein had a theoretical molecular mass of 47,351 D and a pI of 5.79. The total number of negatively charged residues (Asp + Glu) was 56, and the positively charged residues (Arg + Lys) were 50. The instability index is computed to be 27.30, which classifies the protein as stable (Guruprasad et al., 1990). This protein showed a 78% identity with the MDAR of C. sativus (BA0A5408) and Lycopersicon esculentum (T06407), a 76% identity with Mesembryanthemum crystallinum (CAC82727) and Arabidopsis (NP_190856), and a 75% identity with Oryza sativa (BAA14934) and Brassica oleracea (BAD14934).

The analysis of the protein sequence also showed some characteristic motifs found in other MDARs (Murthy and Zilinskas, 1994; Sano and Asada, 1994; Grantz et al., 1995). Thus, the residues Lys-6 to Phe-23 (KYILIGGGVSAGYAAREF) and Ile-35 to Ala-40 (IISKEA) seem to be involved in the binding of FAD and the residues Lys-164 to Leu-181 (KAVVVG-GYIGLELSAVL) and Met-190 to Glu-194 (MVYPE) in the binding of NAD(P)H. Additionally, there is an 11-amino acid domain between the residues Thr-286 to Asp-296 (TSVPDVYAVGD), which is important in the binding of the flavin moiety of FAD. The deduced amino acid sequence of the C terminus is Ser-Lys-Ile (SKI), probably a PTS1. The phylogenetic tree of the deduced protein of the pea MDAR 1 (Fig. 2) associated this protein with the group of other putative peroxisomal MDARs, which were well separated from the groups of isoforms that must be localized in other cell compartments, such as chloroplasts/mitochondria and cytosol. Table III shows the predicted pI and M_r of each group of MDARs.

Subcellular Localization of MDAR by Electron Microscopy and Confocal Laser Scanning Microscopy

The cellular localization of MDAR in pea leaves was studied by electron microscopy (EM) immunocytochemistry (Fig. 3). Using a polyclonal antibody against cucumber MDAR, immunogold particles appeared in chloroplasts and peroxisomes. However, it was also observed in mitochondria and cytosol (data not shown).

To determine if the MDAR1 cDNA coded for the peroxisomal protein, we studied the potential localization of MDAR 1 in comparison with the peroxisomal marker catalase (CAT). We used the pGD binary vector that allowed the transient expression of native and autofluorescent fusion proteins when they were agroinfiltrated into the leaf cells. Figure 4 shows representative images illustrating the confocal laser scanning microscopy (CLSM) detection of the fluorescence in pea leaf cells following infiltration with Agrobacteria carrying the pGD vector with the full length of either CAT or MDAR 1. A red punctuate fluorescence pattern was obtained with the construction pGDY-CAT showing the peroxisomes that appeared as small fluorescent spots within the transformed leaf cells (Fig. 4A). Figure 4B shows the green punctuate fluorescence pattern obtained with the construction pGDG-MDAR 1 in the same leaf sections. The colocalization of the expression of constructions pGDY-CAT and pGDG-MDAR is shown in Figure 4C, where the nearly complete overlapping of the two punctuate patterns indicated that MDAR1 was localized in peroxisomes.
Figure 1. Comparative analysis of the conservation and variability of the intron position of the peroxisomal \textit{MDAR 1} from pea with the different \textit{Arabidopsis} \textit{MDAR}s (see also Table II). The intron positions are indicated in the protein sequences by boxes. Numbers at the right site indicate protein length in amino acids of each protein, and those at the top indicate the relative position among the different amino acid sequences.
Figure 4D shows the bright field of the pea leaf area infiltrated with Agrobacterium.

**Tissue-Specific Expression of MDAR**

To investigate the expression pattern of MDAR in different pea tissues, northern-blot analysis was performed (Fig. 5). The intensity was most intense in fruits, followed by stems and flowers, being the leaves the tissues that contained the lowest levels of transcripts of MDAR.

**Regulation of MDAR Activity, Protein, and mRNA Levels in Response to Various Stress Conditions**

It is widely accepted that diverse environmental conditions can induce oxidative stress. Considering that MDAR is an enzyme of the ascorbate-glutathione cycle, we examined its activity, protein, and mRNA levels under several stress conditions.

Figure 6 shows the analysis of protein expression (top panel) and activity of MDAR in crude extracts of leaves from pea plants exposed to different stress conditions, including continuous light, high light intensity, continuous dark, mechanical wounding, low and high temperature, cadmium, and the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). Among all these stresses, the activity was significantly higher under high light intensity and cadmium and was reduced by the herbicide 2,4-D. Similar responses were observed in the protein expression, but the immunoreactive band was also induced with low and high temperature. These results are representative of the response of all MDAR isoforms present in the leaf because activity assays or antibodies cannot distinguish the different isoforms.

Figure 7 represents the analysis of the expression by semiquantitative RT-PCR of the peroxisomal MDAR 1 under the same stress conditions mentioned above. In this case, the transcript level of the peroxisomal MDAR 1 did not have a similar pattern to the activity and protein content observed in the crude extract analysis. Thus, the highest expression was detected in plants exposed to low temperature, followed by me-

### Table III. Predicted pI and MW of MDAR in different plant species considering the prediction made in Figure 2

<table>
<thead>
<tr>
<th>Subcellular Localization/Plant Species</th>
<th>pI/MW</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxisome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pea</td>
<td>5.79/47,351</td>
<td>AY662655</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>6.41/46,487</td>
<td>NP_190856</td>
</tr>
<tr>
<td>Cucumber</td>
<td>5.29/47,416</td>
<td>BAA05408</td>
</tr>
<tr>
<td>Rice</td>
<td>5.53/46,631</td>
<td>BAA77214</td>
</tr>
<tr>
<td>Rice</td>
<td>5.30/46,685</td>
<td>XP483751</td>
</tr>
<tr>
<td>Tomato</td>
<td>5.77/47,036</td>
<td>Q43497</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>5.20/48,364</td>
<td>NP_566361</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>5.25/47,480</td>
<td>NP_568125</td>
</tr>
<tr>
<td>Cytosol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>8.36/53,526</td>
<td>NP_189420</td>
</tr>
<tr>
<td>Rice</td>
<td>8.88/51,864</td>
<td>XP467388</td>
</tr>
<tr>
<td>Rice</td>
<td>7.57/50,876</td>
<td>XP467387</td>
</tr>
<tr>
<td>Chloroplast/mitochondrion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rice</td>
<td>6.84/52,759</td>
<td>XP480126</td>
</tr>
<tr>
<td>Spinach</td>
<td>6.65/54,011</td>
<td>BAB63925</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>8.11/53,279</td>
<td>NP_84839</td>
</tr>
<tr>
<td>Broccoli</td>
<td>7.60/52,572</td>
<td>BAD14933</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>8.75/45,023</td>
<td>NP_849840</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>7.61/52,115</td>
<td>NP_849841</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>7.06/52,501</td>
<td>NP_564818</td>
</tr>
</tbody>
</table>
The presence of either a TATA box, an Inr, or both seems to contribute to gene regulation (Smale, 1997; Smale et al., 1998; Nakamura et al., 2002). Thus, the Inr at position $-63$ corresponds to the cDNA of the MDAR 1, isolated by Murthy and Zilinskas (1994). The second Inr is situated in the first intron, at position $+52$.

A search in the Arabidopsis databank revealed the presence of five putative MDAR genes in chromosomes 1, 3, and 5, and the number of exons/introns in these genes was different (Table II). The comparative analysis of gene organization (exon/intron) between the pea peroxisomal MDAR1 gene and the Arabidopsis genes evidenced that both putative peroxisomal MDARs have six conserved intron positions (Fig. 1), which could indicate a certain degree of conservation between both genes.

**MDAR 1 cDNA Codes for a Peroxisomal Isoform**

Although there is some evidence indicating that MDAR 1 cDNA could code for a putative peroxisomal isoform (Murthy and Zilinskas, 1994), to our knowledge there are no reports demonstrating that a specific MDAR cDNA from a plant species codes for a peroxisomal isoform. This is in contrast with the abundant biochemical data available establishing the presence of MDAR activity in these organelles (del Río et al., 2003).

The peptide SKI is a putative PTS1, and its presence at the C terminus of the pea MDAR1 could suggest that this enzyme has a peroxisomal localization. In fact, experiments in tobacco cell cultures transformed with several variations of the SKL motif appended to the C terminus of chloramphenicol acetyltransferase, demonstrated that the SKI motif functioned as a type 1 peroxisomal targeting signal (Mullen et al., 1997). In this context, Lingard et al. (2004) recently reported preliminary results of the analysis of some peroxisomal targeting signals of several Arabidopsis MDARs. The designated AtMDAR47a (NP_190856) has a putative type 1 matrix peroxisomal targeting signal (PTS1; C-terminal Ala-Lys-Ile) that is relatively inefficient. However, the AtMDAR54 (NP_189420) has a putative type 2 membrane PTS (PTS2; C-terminal membrane-spanning domain and basic cluster) and seems to target directly to peroxisomes via a C-terminal membranePTS composed of a predicted transmembrane domain adjacent to a cluster of five basic amino acid residues (http://abstracts.aspb.org/pb2004/public/P58/7532.html). However, these data are not strictly contradictory with the results described in this article, and they only reflect the complex nature of the peroxisomal targeting signals that have not been fully characterized yet (Baker and Graham, 2002; Reumann, 2004; Reumann et al., 2004). Moreover, this also indicates that the peroxisomal protein import system in pea and Arabidopsis has some differences. For instance, the typical peroxisomal marker enzyme, CAT, in Arabidopsis has three genes and six isoforms, and none of the genes have a typical PTS1 because the
Figure 4. Colocalization of MDAR 1 and CAT in pea leaves. Representative images illustrating the CLSM detection of fluorescence in pea leaf cells following infiltration with Agrobacteria carrying the pGD vector. A, Pattern obtained with the construction pGDY-CAT, the red spots corresponding to peroxisomes. B, Spot pattern obtained with the construction pGDG-MDAR 1. C, Overlap of the punctuate patterns obtained in (A) and (B). D, Bright field. E and F, Fluorescence controls of leaves not infiltrated with Agrobacterium observed with the corresponding filters for fluorescence proteins EYFP and EGFP, respectively. Bar = 80 μm.
SRL residues are not at the C terminus (Frugoli et al., 1996). However, in the case of pea CAT, the PTS1 are the PSI residues, and they are localized at the C terminus (Isin and Allen, 1991).

An additional clue was the analysis of the pI and molecular weight (MW) values of the MDARs localized in the different cell compartments obtained from the phylogenetic tree (Table III). Thus, all putative peroxisomal MDARs including the pea MDAR1 had predicted average pI/MW values of 5.68/46,934. On the other hand, the cytosolic and mitochondrial/chloroplastic isozymes had a more basic pI and higher MW values, 8.27/52,083 and 7.59/51,528, respectively. Therefore, the predicted difference in pI and MW values between the peroxisomal MDARs and the isoenzymes localized in the cytosol, chloroplasts, and mitochondria could be the result of different chemical environments and might serve as a diagnostic character for distinguishing between the amino acid sequences of peroxisomal MDARs and the isoenzymes localized in other cell compartments.

The immunolocalization of MDAR by EM obtained in this study (Fig. 3) clearly confirmed previous data of the presence of MDAR activity in chloroplasts, cytosol, mitochondria, and peroxisomes (Hossain et al., 1984; Dalton et al., 1993; Murthy and Zilinskas, 1994; Jiménez et al., 1997). In the case of peroxisomes, the gold particles were present in both membrane and matrix, indicating that the MDAR protein was not exclusively located in the membrane as it was previously inferred from biochemical data (Jiménez et al., 1997; López-Huertas et al., 1999; Corpas et al., 2001). Moreover, depending on the program used for the determination of the hydropathic profile, sometimes we found the presence of transmembrane regions that could imply that pea MDAR 1 was localized both in the membrane and matrix of peroxisomes.

In this context, to corroborate if the pea MDAR 1 cDNA coded for the peroxisomal isoform, pea leaves were transformed with the full-length cDNA of MDAR 1 using CAT cDNA as control (Fig. 4). The colocalization obtained with CAT clearly indicated that this cDNA encoded the peroxisomal MDAR. These results suggest that the MDARs from other plants reported to have a SBI motif in the C terminus of its sequence, such as those from cucumber and tomato, very probably also have a peroxisomal localization.

**MDAR Activity Has a Differential Response under Oxidative Stress Conditions**

In pea plants, the eight different types of abiotic stress used in this study have previously been demonstrated to produce oxidative stress due to the induction of imbalances in the antioxidative systems (Sandalo et al., 2001; Leterrier et al., 2004; Romero-Puertas et al., 2004). In our experimental conditions, the MDAR activity was up-regulated under high light intensity and cadmium and was reduced by the herbicide 2,4-D. These data contrast with other activity data reported in the literature. Thus, the increase of MDAR activity has been described in several stress conditions, for instance in tomato by salinity (Mittova et al., 2003) and high light intensity (Gechev et al., 2003), in rice by low temperature (Oidaira et al., 2000), and in Arabidopsis by UV-B radiation (Kubo et al., 1999). However, in Arabidopsis, stresses such as high temperature (30°C), enhanced light intensity (200 μE m^{-2} s^{-1}), water deficiency (water deprivation for 2 d), and low temperature (5°C) did not affect the activity of MDAR (Kubo et al., 1999). Additionally, there are also some data about MDAR activity in other physiological processes that are related to oxidative stress. For instance, during senescence of pea leaves, a simultaneous decrease in MDAR and APX activities was observed (Jiménez et al., 1998). During maturation of pepper (Capsicum annuum) fruits, an increase of ascorbate content has been described that can be attributed to an increase in ı-galactono-γ-lactone dehydrogenase and MDAR activities (Imahori et al., 1998). During cold acclimation of Scots pine (Pinus sylvestris) seed-
Mechanical Wounding, and 2,4-D Stress

corresponding cell compartment. The contribution and function of each isoenzyme in its specific cell compartment and the enzymatic production of NO from L-Arg (nitric oxide synthase activity) has also been demonstrated in pea leaf peroxisomes (Barroso et al., 1999; Corpas et al., 2004). This has prompted to propose that plant peroxisomes could act as subcellular indicators or sensors of plant stress and senescence by releasing the signaling molecules NO, O₂⁻, and H₂O₂ to the cytosol and triggering specific gene expressions (Corpas et al., 2001, 2004; del Río et al., 2003).

**Peroxisomal MDAR 1 Is Up-Regulated by Cold, Mechanical Wounding, and 2,4-D Stress**

Some data are available concerning MDAR mRNA expression, but much less is known on the expression of specific MDAR isoforms. In tomato, MDAR mRNA accumulates after wounding or mechanical stimulation (Grantz et al., 1995; Ben Rejeb et al., 2004), which is in agreement with the up-regulation of the transcript of the peroxisomal MDAR 1 described in this article. In Conyza bonariensis, the mRNA of MDAR is up-regulated by paraquat (Ye and Gressel, 2000) and in Brassica campestris is up-regulated by treatment with H₂O₂, salicylic acid, and paraquat (Yoon et al., 2004).

The up-regulation in transcripts observed with cold and mechanical wounding could be related to the presence of a promoter in the MDAR 1 gene that responds to cold and ABA (Table I). In the latter case, the implication of ABA in wounding and the stress by the herbicide 2,4-D could be the reason.

On the other hand, the difference observed between the activity and protein expression data obtained in crude extracts, which represent the total MDAR of all cell compartments, and the expression of the specific peroxisomal MDAR 1, does not mean that they are contradictory and could indicate that the response of each isoenzyme depends on its specific cell compartment.

MDAR, in conjunction with APX, DHAR, and GR, forms part of the ascorbate-glutathione cycle that is present in leaf peroxisomes (Jiménez et al., 1997) and whose main function is the removal of H₂O₂ (Noctor and Foyer, 1998). MDAR and APX were initially found localized in the peroxisomal membranes, while the other cycle enzymes were in the soluble fraction of peroxisomes (matrix) (Jiménez et al., 1997, 1998; López-Huertas et al., 1999; del Río et al., 2003). However, EM immunocytochemical results reported in this work show that MDAR also occurs in the peroxisomal matrix. In peroxisomal membranes, three integral polypeptides (PMPs) with molecular masses of 18, 29, and 32 kD have been demonstrated to generate superoxide radicals (O₂⁻) using NAD(P)H as electron donor (López-Huertas et al., 1997, 1999). These PMPs were characterized, and the polypeptide of 32 kD was identified as MDAR (López-Huertas et al., 1999). The additional presence of MDAR in the peroxisomal matrix, described in this work, implies the existence of a new superoxide-generating enzymatic system that adds to xanthine oxidase, the characteristic producer of superoxide radicals in peroxisomal matrices (del Río et al., 2003). In addition to the generation of O₂⁻ radicals, the enzymatic production of NO from L-Arg (nicotinic oxide synthase activity) has also been demonstrated in pea leaf peroxisomes (Barroso et al., 1999; Corpas et al., 2004). This has prompted to propose that plant peroxisomes could act as subcellular indicators or sensors of plant stress and senescence by releasing the signaling molecules NO, O₂⁻, and H₂O₂ to the cytosol and triggering specific gene expressions (Corpas et al., 2001, 2004; del Río et al., 2003).

**Table IV. Oligonucleotides used for gene cloning and for the semiquantitative PCR analysis**

Underlined sequences correspond to enzyme restriction sites. F, Forward oligonucleotide; R, reverse oligonucleotide.

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotide Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adaptor primer 1</td>
<td>CTAATACGACTCACTATAGGGT</td>
</tr>
<tr>
<td>Adaptor primer 2</td>
<td>Ph-ACCTCCCC-NH</td>
</tr>
<tr>
<td>MDAR-GEN-F</td>
<td>TTAGGGGGGAATATACACTAGTTCC</td>
</tr>
<tr>
<td>MDAR-BamH1-F</td>
<td>TTATAGGATCCGGATCGCATCCGTC</td>
</tr>
<tr>
<td>MDAR-EcoR1-F</td>
<td>TTAGGGGAATTCTACGATCGATCTGTC</td>
</tr>
<tr>
<td>MDAR-SQ-F</td>
<td>AAGCTTGTGTTGAGG</td>
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<tr>
<td>MDAR-SQ-R</td>
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<td>MDAR-GSP2</td>
<td>TGCTTACGACGGAAACTCTC</td>
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</tr>
<tr>
<td>R-MDAR-Bam</td>
<td>TACAGGATCTACCTACTCCGAAGA</td>
</tr>
<tr>
<td>MDAR-GEN-R</td>
<td>AAAGCGATGACCTGCAATTCCAC</td>
</tr>
<tr>
<td>ACT-SQ-F</td>
<td>AGCAAGATCCAAACGAAAGAGA</td>
</tr>
<tr>
<td>ACT-SQ-R</td>
<td>AAGCTGGAAGGCTGGATTTG</td>
</tr>
</tbody>
</table>
The presence of MADR and APX in leaf peroxisomal membranes has suggested a dual complementary function of these antioxidant enzymes in peroxisomal metabolism. The first role could be the reoxidation of endogenous NADH by MDAR to maintain a constant supply of NAD⁺ for peroxisomal metabolism (Fang et al., 1987). A second function of these antioxidative enzymes could be to protect against H₂O₂ leaking from peroxisomes, particularly when the CAT activity of peroxisomes is depressed and, as a result of it, the endogenous level of H₂O₂ is enhanced (del Río et al., 2003). As hydrogen peroxide can easily permeate the peroxisomal membrane, an important advantage of the presence of APX in the membrane would be the degradation of leaking H₂O₂ as well as the H₂O₂ that is being continuously formed by dismutation of the O₂⁻ generated in the NAD(P)H-dependent electron transport system of the peroxisomal membrane (del Río et al., 2003). This membrane scavenging of H₂O₂ could prevent an increase in the cytosolic H₂O₂ concentration during normal metabolism and under certain plant stress situations, when the level of H₂O₂ produced in peroxisomes can be substantially enhanced (del Río et al., 1996, 2002).

In summary, the data reported in this work demonstrates that in pea leaves, the MDAR 1 cDNA encodes the peroxisomal isozyme that has a differential response to abiotic stress conditions, indicating the probable involvement of peroxisomes in these toxic situations. This evidence emphasizes the importance of studying the MDAR isoenzyme of each cell compartment that could provide more information on the specific subcellular function of MDAR. This consideration perhaps could be extended to other antioxidative enzymes that are located in different subcellular sites.

**Materials and Methods**

**Plant Material and Growth Conditions**

Pea seeds (*Pisum sativum*) cv Phoenix, supplied by Südwestdeutsche Saatzucht, Rastatt, Germany, and cv Lincoln (obtained from Royal Sluis, Enkhuizen, Holland) were used. Seeds were germinated in vermiculite for 14 d and then grown in aerated optimum-nutrient solutions under greenhouse conditions (28°C to 18°C, day-night temperature: 80% relative humidity). For the stress by the herbicide 2,4-D, seedlings were grown for 21 to 28 d and then leaves were sprayed with 22.6 mM 2,4-D and grown for 4 d. For cadmium stress, seedlings were grown for 14 d, and then the nutrient solutions were supplemented with 50 μM CdCl₂ and plants were grown for another 14 d. For the other stress conditions, pea seedlings of 2 to 3 weeks were exposed to continuous light (275 μmol m⁻² s⁻¹) for 4 h), continuous dark (48 h), and high temperature (38°C for 4 h), mechanical wounding, low temperature (8°C for 4 h), and high temperature (38°C for 4 h).

**Crude Extracts of Plant Tissues**

The tissues were ground to a powder in liquid N₂ with a mortar and pestle. Then, they were suspended in 50 mM Tris-HCl buffer, pH 7.8 (1:4, w/v), containing 0.1 mM EDTA, 5 mM dithiothreitol, 10% (w/v) glycerol, and 0.2% (v/v) Triton X-100. Homogenates were centrifuged at 27,000 g for 20 min, and supernatants were used for protein and activity analyses.

**Enzyme Assays**

The activity of MDAR was determined spectrophotometrically by measuring the reduction of absorbance a 340 nm according to Hossain et al. (1984), with some modifications. The 1.0-mL assay mixtures contained 50 mM Tris-HCl (pH 7.8), 0.2 mM NADH, 1 mM ascorbate, and sample. The reaction was started by adding 0.2 units of ascorbate oxidase (EC 1.10.3.3 from Cucurbita, Sigma-Aldrich, St. Louis), and the decrease in A₅₅₀ due to NADH oxidation was followed. One milliunit of activity was defined as the amount of enzyme required to oxidize 1 nmol NADH min⁻¹ at 25°C.

**SDS-PAGE and Western-Blot Analysis**

SDS-PAGE was done on 10% polyacrylamide gels, as described by Laemmli (1970). For western-blot analysis, proteins were electroblotted to polyvinylidene difluoride membranes by a semi-dry Trans-Blot cell from Bio-Rad (Hercules, CA). After transfer, membranes were used for cross-reactivity assays with polyclonal antibodies against cucumber MDAR (Sano et al., 1995) at a 1:2000 dilution. For immunodetection, an enhanced chemiluminescence method using luminol (Corpas et al., 1998) was carried out using affinity-purified goat anti-(rabbit IgG) horsedradish peroxidase conjugate (Bio-Rad).

Protein levels were determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

**RNA Isolation and Northern-Blot Analysis**

Total RNA was isolated from leaves, stems, flowers, and fruits by the acid guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (1987) using the Trizol Reagent kit according to the manufacturer’s instructions. Thirteen micrograms of total RNA from the different organs was subjected to electrophoresis on a 1.2% agarose gel containing 2.2 M formaldehyde, transferred to biodyne B membrane (Bio-Rad), and hybridized with a 32P-labeled MDAR cDNA fragment and washed at high stringency, according to the manufacturer’s instructions. The blots were exposed to x-ray film with an intensifying screen.

**Cloning of MDAR Promoter Region**

A region of the MDAR promoter was cloned using the walking PCR method (Devic et al., 1997). The primary PCR reactions (20 μL) contained 1 × PCR buffer 3 (Boehringer-Expand Long template PCR system): 50 mM Tris-HCl, pH 9.2, 14 mM (NH₄)₂SO₄, 2.25 mM MgCl₂, 20% (v/v) DMSO, 1% (v/v) Tween 20, 200 μM each deoxynucleotide triphosphate (dNTPs), 2.5 ng DNA from a walking PCR library, 200 μM Adaptor Primer 1 (see Table IV), and 200 μM MDAR-GSP1 primer (see Table IV), and 0.25 units of Taq DNA polymerase/ Pwo mix (Boehiringer). All PCR reactions were carried out in the Hybaid thermocycler (Ashford, UK). Amplification of the primary PCR was as follows: one denaturation cycle at 94°C for 2 min, 7 cycles of 30 s at 94°C and 4 min at 72°C, 32 cycles of 30 s at 94°C and 4 min at 68°C with a time increment of 10 s/cycle, followed by a final cycle of extension at 68°C for 10 min. The primary PCR was diluted 100-fold prior to a second round of amplification. The secondary PCR reaction was done under the same conditions, but using 0.4 μL of the diluted primary PCR, 200 μL adaptor primer 2, and 200 μM MDAR-GSP2 primer (see Table IV). Amplification of the secondary PCR was as follows: one denaturation cycle at 94°C for 2 min, 5 cycles of 30 s at 94°C and 4 min at 72°C, 25 cycles of 30 s at 94°C and 4 min at 68°C with a time increment of 10 s/cycle, followed by a final cycle of extension at 68°C for 10 min. The PCR reaction was loaded on a 1% agarose gel, and the visualized bands were cut and extracted from the gel (Qiaex II gel extraction kit; Qiagen, Valencia, CA). The purified fragments were cloned into the pBluescript KS+ cut by SnaI and sequenced.

**Isolation of the MDAR Genomic Clone**

The complete MDAR genomic clone was isolated by PCR on genomic DNA from pea leaves (extracted by Qiagen DNA extraction kit) using specific primers designed from the cDNA sequence [accession no. U06164]. The PCR mix contained 1 × PCR buffer 3 Boehringer-Expand Long template PCR system: 50 mM Tris-HCl, pH 9.2, 14 mM (NH₄)₂SO₄, 2.25 mM MgCl₂, 20% (v/v)
The MDAR 1 and actin II cDNAs were amplified by PCR as follows: the produced cDNA diluted 1/20 was added to 250 μM MgCl₂, 1 mM dNTPs, 1× RT buffer, 20 units of RNase II ribonuclease inhibitor, and 15 units of AMV reverse transcriptase (Finnzymes, Espoo, Finland). The reaction was carried out at 42°C for 40 min, by a 5-min step at 98°C, and then by cooling to 4°C.

Amplification of actin II cDNA from pea (X68649) was chosen as a control. The MDAR 1 and actin II cDNAs were amplified by PCR as follows: 1 μL of the produced cDNA diluted 1/20 was added to 250 μM dNTPs, 1.5 mM MgCl₂, 1× PCR buffer, 1 unit of Ampli Taq Gold (PE-Applied Biosystems, Foster City, CA), and 0.5 μM of each primer (MDAR-SQ-F, MDAR-SQ-R, ACT-SQ-F, and ACT-SQ-R) in a final volume of 20 μL. Reactions were carried out in the Hybrid thermocycler. A first step of 10 min at 94°C was followed by 28 cycles of 30 s at 94°C, 30 s at 60°C, and 45 s at 72°C. Amplified PCR products were detected after electrophoresis in 1% agarose gels stained with ethidium bromide. Quantification of the bands was performed using a Gel Doc system (Bio-Rad) coupled with a highly sensitive CCD camera. Band intensity was expressed as relative absorbance units. The ratio between the MDAR 1 and actin II amplification was calculated to normalize for initial variations in expression as relative absorbance units. The ratio between the MDAR 1 and actin II was done with one denaturation cycle at 94°C for 10 min, followed by a final cycle of extension at 68°C for 10 min. The PCR reaction was load on a 0.8% agarose gel. The purified fragment was cloned into the pBluescript KS-

BLAST searches were made with the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov/). Alignments were performed using OMMIGA (2.0) and ClustalW v.1.8 (J.D. Thompson, D.G. Higgins, and T.J. Gibson, 1994; http://www.infobiogen.fr/services/alignseq/cgi-bin/clustalw_in.pl). The phylogenetic tree was made from a protein alignment with ClustalW and then visualized using TREE VIEW v.1.6.6 (R.D.M. Page, 2001; http://taxonomy.zoology.gla.ac.uk/rod/rod.html). Primer design was done with Oligo (Operon Technologies, Alameda, CA) and with Primer3 (S. Rozen and H.J. Skaletsky, 2000; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Localization predictions were realized with PSORT Prediction and iPSORT Prediction (http://psort.nibb.ac.jp). The theoretical molecular mass and pI were estimated using http://expasy.org/cgi-bin/pi_tool.

**Semi-quantitative RT-PCR**

Two micrograms of total RNA from leaves was used as a template for the RT reaction. It was added to a mixture containing 5 mM MgCl₂, 1 mM dNTPs, 0.5 μg oligo(dT) primers, 1× RT buffer, 20 units of RNase II ribonuclease inhibitor, and 15 units of AMV reverse transcriptase (Finnzymes, Espoo, Finland). The reaction was carried out at 42°C for 40 min, by a 5-min step at 98°C, and then by cooling to 4°C.

Amplification of actin II cDNA from pea (X68649) was chosen as a control. The MDAR 1 and actin II cDNAs were amplified by PCR as follows: 1 μL of the produced cDNA diluted 1/20 was added to 250 μM dNTPs, 1.5 mM MgCl₂, 1× PCR buffer, 1 unit of Ampli Taq Gold (PE-Applied Biosystems, Foster City, CA), and 0.5 μM of each primer (MDAR-SQ-F, MDAR-SQ-R, ACT-SQ-F, and ACT-SQ-R) in a final volume of 20 μL. Reactions were carried out in the Hybrid thermocycler. A first step of 10 min at 94°C was followed by 28 cycles of 30 s at 94°C, 30 s at 60°C, and 45 s at 72°C. Amplified PCR products were detected after electrophoresis in 1% agarose gels stained with ethidium bromide. Quantification of the bands was performed using a Gel Doc system (Bio-Rad) coupled with a highly sensitive CCD camera. Band intensity was expressed as relative absorbance units. The ratio between the MDAR 1 and actin II was done with one denaturation cycle at 94°C for 10 min, followed by a final cycle of extension at 68°C for 10 min. The PCR reaction was load on a 0.8% agarose gel. The purified fragment was cloned into the pBluescript KS-

**Construction of Plasmids Used in Colocalization**

All binary vectors used in this study were derived of plasmids pGDG and pGDE containing the self-autofluorescent proteins enhanced green fluorescent protein (EGFP) and enhanced yellow fluorescent protein (EYFP, respectively) (Goodin et al., 2002). MDAR 1 cDNA (without ATG) was amplified by PCR on pGEM-EASY-MDAR 1 containing the full-length cDNA, using the following primers with additional BamHI restriction sites: MDAR-BamHI F- and R-MDAR-Bam (see Table IV). PCR product was cut by BamHI after subcloning in pGEM-T-Easy and then fused in phase with EGFP in the pGDG systems, Wetzlar, Germany) using the recommended filters for fluorescence proteins EGFP (excitation, 488 nm; emission, 508 nm) and EYFP (excitation, 514 nm; emission, 527 nm). Images were captured using the Leica TCS software.

**EM and Immunocytochemistry**

Pea leaf segments of approximately 1 mm² were fixed, dehydrated, and embedded in LR White resin according to Corpas et al. (1994). Immunolabeling was performed as indicated by Sandalio et al. (1997). Ultrathin sections were incubated for 2 h with the antibody against cucumber MDAR diluted 1:500 in TBST [10 mM TrisHCl (pH 7.6), 0.9% (w/v) NaCl, 0.05% (v/v) Tween 20, and 0.02% (v/v) NaN₃] buffer. The sections were then incubated for 1 h with goat anti-rabbit IgG conjugated to 15-nm gold particles (Bio Cell, Cardiff, UK) diluted 1:40 in TBST plus 2% (w/v) bovine serum albumin. Sections were poststained in 2% (v/v) uranyl acetate for 3 min and examined in a Zeiss EM 10C transmission electron microscope (Jena, Germany).

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Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AY662655 for the MDAR 1.
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