Enhanced Formaldehyde Detoxification by Overexpression of Glutathione-Dependent Formaldehyde Dehydrogenase from Arabidopsis

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The ADH2 gene codes for the Arabidopsis glutathione-dependent formaldehyde dehydrogenase (FALDH), an enzyme involved in formaldehyde metabolism in eukaryotes. In the present work, we have investigated the potential role of FALDH in detoxification of exogenous formaldehyde. We have generated a yeast (Saccharomyces cerevisiae) mutant strain (sfa1Δ) by in vivo deletion of the SFA1 gene that codes for the endogenous FALDH. Overexpression of Arabidopsis FALDH in this mutant confers high resistance to formaldehyde added exogenously, which demonstrates the functional conservation of the enzyme through evolution and supports its essential role in formaldehyde metabolism. To investigate the role of the enzyme in plants, we have generated Arabidopsis transgenic lines with modified levels of FALDH. Plants overexpressing the enzyme show a 25% increase in their efficiency to take up exogenous formaldehyde, whereas plants with reduced levels of FALDH (due to either a cosuppression phenotype or to the expression of an antisense construct) show a marked slower rate and reduced ability for formaldehyde detoxification as compared with the wild-type Arabidopsis. These results show that the capacity to take up and detoxify high concentrations of formaldehyde is proportionally related to the FALDH activity in the plant, revealing the essential role of this enzyme in formaldehyde detoxification.

Formaldehyde is a toxic compound produced during plant one-carbon (C1) metabolism. Most formaldehyde does not exist in vivo in a free state but is bound to endogenous nucleophiles, such as glutathione or tetrahydrofolate (Sardi and Tyihak, 1994; Chen et al., 1997). The main sources of formaldehyde in plants are dissociation of 5,10-methylene-tetrahydrofolate and oxidation of methanol, derived mainly from pectin demethylation (Fall and Benson, 1996). Formaldehyde can also arise from other oxidative demethylation reactions (Sardi and Tyihak, 1994), from glyoxylate decarboxylation (Prather and Sisler, 1972), and from P-450-dependent oxidation of herbicides (Clejan and Cederbaum, 1993). Furthermore, formaldehyde might have an exogenous origin from industrial waste, being a polluting agent of residual waters and of air (Wolverton et al., 1989; Giese et al., 1994). It is one of the main indoor air pollutants, present in tobacco smoke, furniture, industrial adhesives, and varnishes. It has been classified as a mutagen and suspected carcinogen (Wippermann et al., 1999) and as causing headaches and irritation of the mucosa. Formaldehyde is considered a toxic compound because of its ability to react with proteins, nucleic acids, and lipids. The World Health Organization has established an air quality guideline of 100 µg m⁻³ for formaldehyde (European Commission, 1994).

Several studies have demonstrated that exogenous formaldehyde can be incorporated into the metabolism of photosynthetic cells and be used as a carbon source. For example, feeding the common spider plant (Chlorophytum comosum) with ¹⁴C-formaldehyde resulted in ¹⁴C-labeled products derived from the C1 metabolism (Giese et al., 1994). Formaldehyde can occur by different in vivo pathways, through the formation of adducts with glutathione, Arg, Asn, and tetrahydrofolate (for a review on C1 metabolism in higher plants, see Hanson and Roje, 2001). However, biochemical and genetic studies in several eukaryotes indicate that the main enzyme responsible for the metabolism of formaldehyde is the glutathione-dependent formaldehyde dehydrogenase (FALDH). This enzyme is universally present in animal and plant tissues and catalyzes the following reaction:

\[ \text{S-hydroxymethylglutathione} + \text{NAD} \rightarrow \text{S-formylglutathione} + \text{NADH} + \text{H}^+ \] (1)
where S-hydroxymethylglutathione is a nonenzymatically formed adduct of glutathione and formaldehyde.

S-formylglutathione is then hydrolyzed to formate and glutathione by S-formylglutathione hydrolase:

\[
\text{Hydrolase} \quad \text{Formate + Glutathione} \quad (2)
\]

Formate can give rise to C1 folates, but a quantitatively more important fate is oxidation to CO₂ mediated by formate dehydrogenase (Cossins, 1964). NAD-dependent formate dehydrogenase has been cloned from Arabidopsis, which is predicted to have a mitochondrial and a chloroplastic localization (Olson et al., 2000). As is the case in the photosynthetic bacteria Rhodobacter sphaeroides (Barber and Donohue, 1998), it might be that in plants the Calvin cycle assimilates in part the products of FALDH activity.

In addition to formaldehyde, FALDH can also oxidize long-chain alcohols and ω-hydroxy fatty acids, such as octanol and 12-hydroxydocosanoic acid. Furthermore, it has been demonstrated recently that it is very active in the reduction of S-nitrosoglutathione, the condensation product of glutathione and NO (Jensen et al., 1998; Liu et al., 2001; Sakamoto et al., 2002).

FALDH from Arabidopsis has been characterized, and its cDNA has been cloned (Martínez et al., 1996). This enzyme exhibits features similar to those of their animal counterparts and shows a structure highly conserved in plants and animals. FALDH is expressed in low amounts in Arabidopsis (5 × 10⁻³ units mg⁻¹; Martínez et al., 1996), which is approximately the same amount that has been observed in dry pea (Pisum sativum) seeds (4 × 10⁻³ units mg⁻¹; Shafqat et al., 1996) and about twice the amount present in the spider plant (Giese et al., 1994). The gene is expressed at similar levels in all plant organs, as assessed by northern-blot analysis (Martínez et al., 1996), in analogy with the ubiquitous distribution of the corresponding enzyme in animal tissues (Smith, 1988). FALDH is encoded in the Arabidopsis genome by a single gene, which maps in chromosome 5 (Dolferus et al., 1997).

In the present work, we have investigated the potential role of FALDH in detoxification of exogenous formaldehyde. We have generated a yeast (Saccharomyces cerevisiae) mutant strain (sfa1Δ) by in vivo deletion of the SFA1 gene that codes for the endogenous FALDH. We have investigated the effect of the overexpression of Arabidopsis FALDH in this mutant, regarding its capacity to detoxify exogenous formaldehyde. To investigate the role of the enzyme in plants, we have generated transgenic Arabidopsis lines transformed with either sense or antisense constructs of FALDH gene, and we have measured the rates for formaldehyde uptake and detoxification by both transgenic plants overexpressing FALDH and plants with reduced FALDH levels. Our results confirm the central role of FALDH in formaldehyde metabolism in plants.

RESULTS

Construction of a Yeast sfa1Δ Strain

To explore the physiological role of the Arabidopsis FALDH, we made complementation experiments by transforming a yeast strain lacking FALDH activity. This strain was constructed by in vivo deletion of the SFA1 gene, coding for the yeast glutathione-dependent FALDH (Wehner et al., 1993). A linear fragment containing the HIS3 gene flanked by the flanking sequences of SFA1 gene was used to transform a haploid strain (His⁻), derived from the diploid W303D, to prototrophy (His⁺). Isoelectrofocusing analysis stained by activity confirmed that none of the His⁺ transformants contained glutathione-dependent FALDH activity (Fig. 1A, lane 3). The sfa1::HIS3 replacement was also confirmed by Southern analysis, using as probe a PstI/NcoI fragment containing the SFA1 gene (Fig. 1, B and C).

![Figure 1](https://plantphysiol.org)

**Figure 1.** Analysis of the yeast sfa1Δ strain (sfa1::HIS3). A, Isoelectric focusing and FALDH activity staining on homogenates of wild-type W303D (lane 1) and sfa1::HIS3 strain transformed with (lane 2) and without (lane 3) the ADH2-pYes2 plasmid. B, Southern-blot analysis of genomic DNA digested with PstI and EcoRI from sfa1::HIS3 (lane 1) or W303D (lane 2) strain. The HIS3 gene contains an internal PstI restriction site that is not present in the SFA1 gene. C, Schema of the homologous recombination procedure used to construct the sfa1Δ yeast strain. The PstI/NcoI fragment containing the SFA1 gene (at the bottom) was used as probe for Southern analysis.
Overexpression of Arabidopsis FALDH in Yeast. Purification of the Enzyme and Generation of Specific Antibodies

Arabidopsis FALDH cDNA was cloned into the yeast pYes2 expression vector, under the control of the GAL1-GAL10 promoter (ADH2-pYes2 plasmid), and introduced into the sfa1::HIS3 strain. The transformed strain was grown in the presence of Gal as carbon source and using His and uracil as markers. The selected colonies exhibited FALDH activity in their crude extracts, confirmed by the presence of a band of the expected size (45 kD) in electrofocusing gels stained by activity (Fig. 1A, lane 2), indicating both that it was correctly expressed and that it was functional in this heterologous system. To purify the enzyme from this source, we used three consecutive chromatographic steps. From 500 g of yeast cell pellet, 2.24 mg of purified protein was obtained that appeared as a single band of $M_r$ 45,000 on SDS-PAGE (Fig. 2A). This band was recognized by an anti-rat FALDH antiserum (result not shown). The specific activity of the purified enzyme against S-hydroxymethylglutathione was found to be 15 units mg$^{-1}$. Fold purification and recovery of the process are shown in Table I.

The purified enzyme was inoculated into rabbits to raise polyclonal antibodies. The antibodies recognized a single band of the expected size (45 kD) in Arabidopsis crude protein extracts that was not detected using the pre-immune sera (Fig. 2B, lanes 2 and 3, respectively). Those immunoreactions were specific because the binding of the antibodies could be competed with the protein used for immunization (Fig. 2B, lanes 4–9).

Enzymatic Properties of Arabidopsis FALDH

The Arabidopsis enzyme expressed in yeast showed a $K_m$ value of 7 $\mu$M. The deduced turnover number ($K_{cat}$) was 1,351 min$^{-1}$, calculated for a $M_r = 90,000$ (dimer), and the catalytic efficiency ($K_{cat}/K_m$) was 193,000 mm$^{-1}$ min$^{-1}$. The enzyme exhibited high activity at pH 10 toward several plant alcohols, such as farnesol and geraniol. However, at more physiological pH (pH 7.5), only farnesol showed a significant activity, with a $K_{cat}/K_m$ of 780 mm$^{-1}$ min$^{-1}$. Other medium-chain alcohols and $\omega$-hydroxy fatty acids were also substrates of the enzyme, though their activity was much lower (Table II).

Overexpression of Arabidopsis FALDH in Yeast Greatly Improves Its Ability to Grow in the Presence of Formaldehyde

The yeast sfa1::HIS3 strain could grow at the same rate as the wild type in rich medium but could not grow in the presence of 0.6 mm formaldehyde. In contrast, the wild-type yeast strain could grow in the presence of 0.6 mm formaldehyde, though after a long lag phase (data not shown). These results confirm the essential role of FALDH for yeast formaldehyde metabolism. The sfa1::HIS3 strain transformed with the ADH2-pYes2 plasmid was then grown both in the absence and in the presence of different concentrations of formaldehyde added exogenously. Figure 3A shows that both the wild type and the transformed strains grew equally well in the absence of formaldehyde, reaching saturation at around 35 h after subculturing. In contrast, at 1 mm formalde-

### Table I. Purification of Arabidopsis recombinant FALDH

<table>
<thead>
<tr>
<th>Step</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific Activity</th>
<th>Purification</th>
<th>Yield</th>
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<tr>
<td></td>
<td>units</td>
<td>mg</td>
<td>units mg$^{-1}$</td>
<td>-fold %</td>
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<td>510</td>
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<td>DEAE-Sepharose</td>
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<td>4.4</td>
<td>7.6</td>
<td>40</td>
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<td>62</td>
<td>4.1</td>
<td>15</td>
<td>25.8</td>
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</table>

Activity was measured in 0.1 M sodium phosphate (pH 8) at 25°C using 1 mm formaldehyde, 1 mm glutathione, and 4 mm NAD.
hyde, yeast wild type was unable to grow during the time of the experiment (60 h), whereas the transformed strain showed the same growth rate as in the absence of formaldehyde. At 2 mM formaldehyde, the transformed strain still grew very efficiently.

We also determined the kinetics of formaldehyde elimination from the culture medium (Fig. 3B). Our results show that exogenous formaldehyde disappeared very rapidly in the case of the transformed yeast strain and was completely metabolized in 50 to 60 h, which is consistent with the cell’s growth rate shown in Figure 3A. However, in the case of the wild-type strain, the concentration of formaldehyde in the culture medium remained constant for long periods of time, which proves its inability to metabolize it efficiently and, as a consequence, to grow in the presence of this toxic compound.

Detoxification of Exogenous Formaldehyde by Transgenic Arabidopsis Plants

We addressed the question of whether plants over-expressing FALDH might be able to cope better with moderately high concentrations of environmental formaldehyde. We generated transgenic Arabidopsis lines transformed with FALDH cDNA under the control of the 35S cauliflower mosaic virus promoter. A total of 40 independently transformed plants were obtained (T₁ progeny) after infiltration of Columbia plants. The T₁ plants were self-fertilized, and 16 individuals of the T₂ progeny showing a Mendelian segregation of kanamycin resistance were brought to homozygosis (T₃ generation). Western-blot analysis of seven individuals from the T₃ generation is shown in Figure 4A. One of them is a cosuppression line (line 13), whereas the others show from a moderate to a high increase of FALDH protein levels. In the same figure, it can be seen that the increase in FALDH levels correlated well with increased values of FALDH enzymatic activity.

To test the ability of the transgenic plants to metabolize exogenous formaldehyde, we selected three of the homozygous lines (T₃ generation) showing the highest expression of the transgene (lines 1, 4, and 6). Equal amounts of seeds (3 mg) were germinated and grown in liquid medium (5 ml), and formaldehyde at the desired concentration was added to 6-d-old plantlets. Subsequently, aliquots were taken to measure the variation of formaldehyde concentration.

### Table II. Kinetic properties of Arabidopsis FALDH

Activity versus S-hydroxymethylglutathione was measured in 0.1 M sodium phosphate (pH 8). Activity with other substrates was measured in 0.1 M sodium phosphate (pH 7.5) or in 0.1 M glycine buffer (pH 10). In all cases, 2.4 mM NAD was used.

<table>
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<tr>
<th>Substrate</th>
<th>kₘ</th>
<th>kₘcat</th>
<th>kₘcat/kₘ</th>
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<tr>
<td>S-hydroxymethylglutathione (pH 8)</td>
<td>7.3</td>
<td>1,351</td>
<td>193,000</td>
</tr>
<tr>
<td>Farnesol (pH 7.5)</td>
<td>7.7</td>
<td>6</td>
<td>780</td>
</tr>
<tr>
<td>Geraniol (pH 7.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cinnamylalcohol (pH 7.5)</td>
<td>22,000</td>
<td>324</td>
<td>15</td>
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<td>12-Hydroxydecanoic acid (pH 7.5)</td>
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<tr>
<td>Farnesol (pH 10)</td>
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<tr>
<td>Geraniol (pH 10)</td>
<td>800</td>
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<td>1,500</td>
</tr>
<tr>
<td>Cinnamylalcohol (pH 10)</td>
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<td>1,220</td>
<td>350</td>
</tr>
<tr>
<td>Octanol (pH 10)</td>
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<td></td>
</tr>
<tr>
<td>12-Hydroxydecanoic acid (pH 10)</td>
<td>4,700</td>
<td>335</td>
<td>70</td>
</tr>
</tbody>
</table>

* n.s., Non-saturation reached with this substrate.

Figure 3. Growth kinetics and formaldehyde detoxification by yeast strains expressing Arabidopsis FALDH. Growth (A) and formaldehyde concentration (B) were measured in culture medium containing no added formaldehyde (circles) or 1 (triangles) or 2 (squares) mM formaldehyde. White symbols, Yeast strain overexpressing the Arabidopsis FALDH; black symbols, W303D yeast strain.
during a time course. A control of liquid medium with formaldehyde but without plants was also performed to discard losses of formaldehyde by evaporation. At least three independent experiments were carried out with each line.

At 2 mM formaldehyde, we observed significant differences in the rate for formaldehyde uptake (Fig. 5A). The three transgenic lines tested were able to completely detoxify the formaldehyde in 48 h, whereas Arabidopsis wild-type metabolized it at a slower rate (80% of the total in 48 h). In view of the fact that the plants could cope well with this concentration of exogenous formaldehyde (no visible defects were observed as a consequence of the addition of this compound), we decided to perform the same experiment using a concentration of 5 mM formaldehyde. As can be seen in Figure 5B, more significant differences were observed in this case. Line 1, showing the highest rate for formaldehyde uptake, could achieve a 50% decrease in formaldehyde concentration in the medium after 48 h (in contrast to the 25% by the Arabidopsis wild type). This higher rate was evidenced very early (at 6 h, there was already a 13% differential rate), and subsequently increased (at 24 and 48 h, there was a 25% differential rate).

To corroborate the above data that suggest a correlation between the amount of FALDH expressed by the plant and its ability to detoxify exogenous formaldehyde, we performed the same experiments with transgenic lines showing reduced levels of FALDH. One line showing a cosuppression phenotype (line 13, Fig. 4A) and three lines bearing antisense constructs (lines 5a, 10a, and 17a, Fig. 4B) were incubated with 2 mM formaldehyde added exogenously. Aliquots from the culture medium were removed during a time course to measure the formaldehyde concentration. The results in Figure 5A demonstrate that the lines with reduced levels of FALDH show a 20% decrease in their ability to detoxify exogenous formaldehyde.

DISCUSSION

Plant FALDHs have been isolated from pea seeds (Uotila and Koivusalo, 1979; Shafqat et al., 1996), Arabidopsis (Martínez et al., 1996), and maize (Zea mays; Wippermann et al., 1999). Their molecular and kinetic properties are remarkably similar to those found in the homolog enzymes from mammals, invertebrates, and microorganisms, providing evidence of the high degree of structural and functional conservation through evolution. The enzyme shows very low expression levels in Arabidopsis plants (Martínez et al., 1996); thus, a system to produce a recombinant enzyme was desirable. We have used a yeast expression system to purify the enzyme to homogeneity and to study its functional significance in the detoxification of exogenous formaldehyde.

Arabidopsis FALDH, purified as a recombinant enzyme from yeast, has a specific activity of 15 units mg\(^{-1}\) and a \(K_m\) value of 7 \(\mu\)M. The polypeptide chain during a time course. A control of liquid medium with formaldehyde but without plants was also performed to discard losses of formaldehyde by evaporation. At least three independent experiments were carried out with each line.

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of the enzyme has a molecular mass of 45 kD, calculated by SDS-PAGE, which is consistent with both the molecular mass deduced from the cDNA sequence and with that of the enzyme purified from Arabidopsis plants (Martínez et al., 1996). The FALDH purified from a variety of different sources consists of two identical subunits and, thus, is a homodimer (Uotila and Koivusalo, 1989). Based on a dimeric structure for Arabidopsis FALDH, we calculated a $K_{cat}$ value of 1,351 min$^{-1}$ and a $K_{cat}/K_m$ of 193,000 mM$^{-1}$ min$^{-1}$. This last value, which is the catalytic efficiency, is comparable with those of pea and other eukaryotes (Shafqat et al., 1996; Fernández et al., 1997).

SFA1 deletion in yeast is not lethal but impairs its growing in the presence of formaldehyde (Fernández et al., 1999; this work). We have demonstrated in this work that Arabidopsis FALDH is able to complement a knockout yeast mutant that has the SFA1 gene replaced by the HIS3 marker gene. Our results show that overexpression of Arabidopsis FALDH in this mutant results in high resistance to formaldehyde, demonstrating the functionality of the Arabidopsis enzyme and its essential role in formaldehyde metabolism. This effect was quite remarkable because at 1 mM formaldehyde, yeast wild type was unable to grow, whereas the strain overexpressing Arabidopsis FALDH grew at the same rate as in the absence of formaldehyde. At 2 mM formaldehyde, the growing rate of the transformed strain still was only slightly affected.

Additional support for the important role of FALDH in detoxifying formaldehyde was obtained by the generation of transgenic Arabidopsis lines. Three independent lines, exhibiting from 11- to 18-fold the wild-type FALDH activity, were used to test the capacity of plants overexpressing FALDH to take up and detoxify exogenous formaldehyde. We observed an increase in the detoxification rate of 25%, both at 2 and 5 mM formaldehyde (64 and 132.5 mL L$^{-1}$, respectively). At 2 mM formaldehyde, transgenic plants could completely detoxify the exogenous formaldehyde in 48 h or less, without any visible damage. These results were corroborated by generating transgenic lines with decreased levels of FALDH enzyme (cosuppression and antisense lines). At 2 mM formaldehyde, these plants showed a 20% decrease in their ability to detoxify formaldehyde, as compared with the wild-type plants.

At 5 mM formaldehyde, which is an extremely toxic concentration, the transgenic lines showed a good rate for formaldehyde uptake during the first 24 h. From this time on, we observed important phytotoxic effects, the leaves starting to display chlorotic patches that finally spread to the whole tissue. At the same time, the rate decreased significantly, and the plants could not achieve a complete detoxification of the exogenous formaldehyde. This phytotoxic effect of formaldehyde at such high concentration is not surprising and might be due to the formaldehyde itself or to the products of its oxidation. The chlorotic phenotype of the plants and the observation of leaf sections under electron microscopy (data not shown) suggest that chloroplasts are seriously damaged after long expositions to 5 mM formaldehyde. This might be due to a process of photooxidation caused by an accumulation of FALDH oxidation products that are toxic and have effects on the glutathione/redox homeostasis.

It has been reported that foliar application of methanol can stimulate plant growth (Nonomura and Benson, 1992) and that methanol is quickly oxidized in most plant tissues successively to formaldehyde, formic acid, and CO$_2$ (Cossins, 1964). Formaldehyde can be incorporated to plant C1 metabolism mainly by two metabolic pathways that involve folate-mediated and -independent reactions (Hanson and Roje, 2001). In the pathway involving folate-mediated reactions, formaldehyde can be converted to 5,10-methylene-tetrahydrofolate by a nonenzymatic reaction with tetrahydrofolate or to formate by the successive reactions catalyzed by FALDH and S-formylglutathione hydrolase. The formate is then incorporated into the C1-tetrahydrofolate pool via the 10-formyl-tetrahydrofolate. In the folate-independent pathway, formaldehyde is successively converted to formic acid and CO$_2$, and CO$_2$ is then assimilated by the Calvin-Benson cycle in the photosynthetic tissues (Cossins, 1964). Formate might also give rise to glyoxylate, in a reaction catalyzed by glyoxylate synthetase that is exclusively found in plants (Janave et al., 1993). Our results showing that the capacity to detoxify exogenous formaldehyde is proportionally related to the level of FALDH activity in the plants strongly suggest that the major metabolic flux of formaldehyde is via formate. However, our data do not allow the elucidation of the final fate of this formate. S-formylglutathione hydrolase and formate dehydrogenase have been cloned from Arabidopsis (Olson et al., 2000; Kordic et al., 2002), demonstrating that all the enzymes necessary for this metabolic pathway are present.

In summary, our results show that overexpression of the enzyme FALDH in plants confer a high resistance to formaldehyde, thus supporting the “green liver” concept of plant xenobiotic metabolism (Sandermann, 1992). The availability of transgenic plants with modified amounts of FALDH will allow us to investigate the mechanisms of formaldehyde toxicity at the cellular level in the future. This knowledge is necessary to improve the detoxification properties of the plants and to better understand C1 metabolism in higher plants.

**MATERIALS AND METHODS**

**Biological Material**

*Yeast (Saccharomyces cerevisiae)* W303D strain was grown at 30°C in Wickerham’s medium (United States Biochemical Corp., Cleveland). Arabi-
dopis ectype Columbus was grown in soil under a 16-h-light/8-h-dark regime at 22°C. For the formaldehyde treatments, plant seeds were surface sterilized and sown in six-well tissue culture clusters (Costar Corp., Cambridge, MA), containing 5 mL well−1 of Murashige and Skoog media (Duchefa, Harlem, The Netherlands) supplemented with 0.5% (w/v) Suc and grown with shaking (150 rpm) under the same light regime as above.

Gene Deletion
A null mutant of the SFA1 gene, coding for the yeast FALDH, was constructed by one-step gene replacement (Rothstein, 1983). In brief, an internal fragment containing the SFA1 coding region was removed (from a pBR322-derived construct containing the SFA1 gene) and replaced by the yeast HIS3 gene (Berben et al., 1991). A linear fragment, including the selectable HIS3 gene flanked by sequences homologous to the flanking sequences of the SFA1 coding region, was obtained from the previous construct and used to transform a haploid yeast strain derived from W303. Screening of transformants was performed in Wickerham’s medium without His, and the SFA1 deletion was confirmed by FALDH activity and Southern analysis.

Expression in Yeast
ADH2 cDNA was amplified using two primers based on the reported sequence (Martínez et al., 1996). The upstream primer included the ATG initiation codon and had the following sequence: 5′-GGCGGTACC-ATGGCCGATCAAGTAAAGG-3′. The downstream primer included the termination codon and had the following sequence: 5′-CGCCGTGCAGCTC-ATTTCGGATCTACGG-3′. PCR was carried out in two steps: (a) 95°C for 1 min, 54.5°C for 2 min, and 72°C for 2 min for five cycles, and (b) 95°C for 1 min, 61.5°C for 2 min, and 72°C for 2 min for 25 cycles, followed by an elongation step of 72°C for 10 min. One band of the expected size was obtained, purified, and cloned into the Smal site of pUC18 vector. The insert was completely sequenced by the Sanger method and then subcloned into the KpnI site of the yeast pYes2 expression vector. The recombinant plasmid (ADH2-pYes2) was introduced into the Escherichia coli MC1061 strain to verify insert orientation and into the haploid sfa1:His yeast strain for expression. Screening of transformants was made by growing in the absence of both His and uracil. Transformed yeast strain was grown at 30°C in Wickerham’s medium without His and uracil with 2% (w/v) Gal as carbon source to induce the GAL1-GAL10 promoter and a starting density of 3 × 10^5 cells mL−1.

Purification of Arabidopsis Recombinant FALDH
Yeast sfa1::HIS3 strain transformed with the ADH2-pYes2 plasmid (50 L) was grown until the early stationary phase, and cell pellets were collected by centrifugation and resuspended in buffer A, containing 10 mM Tris/HCl (pH 7.5), 0.5 mM diithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM benzamidine (1:1 [w/v]). Cells were lysed with a bead beater homogenizer (Biospec Products, Inc., Bartlesville, OK), using 0.5-mm diameter glass beads. The homogenate was centrifuged at 29,000g for 1 h, filtered through glass wool, and loaded onto a DEAE-Sepharose CL-6B column (36.5 × 4 cm) equilibrated with the buffer A. A 1,200-mL linear gradient of 0 to 0.2 M NaCl was used to elute the bound enzyme. The active material was concentrated to 60 mL with DiaLoq PM-10 membrane (Amicon, Billerica, MA), dialyzed against 10 mM KH₂PO₄ (pH 6.8), 0.5 mM DTT, 1 mM PMSF, and 1 mM benzamidine, and loaded onto a hydroxyapatite Bio-Gel HT (Bio-Rad Laboratories, Hercules, CA) column (16 × 2 cm) equilibrated with the same buffer. A 1,200-mL linear gradient of 10 to 400 mM KH₂PO₄ was applied, and the active fractions were collected, concentrated to 25 mL, dialyzed against buffer A, and loaded onto a Blue-Sepharose column (15.5 × 1.8 cm) equilibrated with the same buffer. The active material was eluted with a 600-mL linear gradient of 0 to 0.75 mM NADH, concentrated to 16 mL, and the excess of NADH was eliminated with a PD-10 gel filtration column (Amersham-Pharmaica Biotech, Uppsala) equilibrated with 10 mM Tris/HCl (pH 7.5) and 0.5 mM DTT. The active material was equilibrated with 20 mM Tris/acetate buffer (pH 6.5), 0.5 mM DTT, 1 mM PMSF, and 1 mM benzamidine, using a PD-10 gel filtration column, and loaded onto a Protein Pack Q 8HR column (Millipore, Billerica, MA) equilibrated with the same buffer. Bound proteins were eluted with a 40-mL linear gradient of 0 to 0.2 M sodium acetate. Protein concentrations were determined by the Bradford assay (Bio-Rad), using bovine serum albumin as a standard.

Construction of Transgenic Arabidopsis Plants
To prepare the sense construct, an EcoRI/XbaI restriction fragment containing the Arabidopsis FALDH coding sequence (Martínez et al., 1996) was purified and subcloned downstream of the cauliflower mosaic virus 35S promoter into the SmaI site of pDH51 (Pietrzak et al., 1986). To prepare the antisense construct, a PsI/BglII fragment (425 bp) was purified and subcloned into pDH51 cut with BanHI and PstI. After verifying the correct orientation of the insert, the resulting 35S-ADH2 expression cassettes were excised as EcoRI restriction fragments and subcloned into the EcoRI site of the Agrobacterium tumefaciens binary plasmid pBIN19 (Bevan, 1984). The recombinant plasmids were then introduced into A. tumefaciens C58CI strain by the freeze-thaw method. Arabidopsis plants were transformed by the vacuum infiltration method (Bechtold et al., 1993). Transgenic plants were selected on Murashige and Skoog solid media containing 40 mg L−1 kanamycin and later transferred to soil seedling protocol with pBIN19 empty vector also were produced to be used as controls. Segregation tests were performed with the T₀ seeds obtained by self-fertilization of independent primary transformants. Homozygous, monogenic T₂ seeds were obtained by self-fertilization of T₁ plants using kanamycin resistance as the marker.

Electrophoretic Analysis and Immunological Procedures
SDS-PAGE was performed as described (Laemmli, 1970), with subsequent Coomassie Blue or silver staining (Merrill et al., 1981). Isoelectric focusing was performed on polyacrylamide gels in a pH 3 to 9 gradient (PhastSystem, Amersham-Pharmaica Biotech), and bands were visualized by staining for glutathione-dependent FALDH activity with Nitro Blue tetrazolium/phosphate methosulfate, 4.8 mM formaldehyde, and 1 mM glutathione. For production of the antibodies, Arabidopsis FALDH purified as a recombinant enzyme from yeast was used to inoculate rabbits by conventional methods (Harlow and Lane, 1988). For western blotting, proteins were electrophoresed on 12.6% (w/v) SDS-PAGE gels, transferred to immobilin-P membranes (Millipore), and incubated with the antiserum (dilution 1:3,000 [v/v]). The immunocomplexes were revealed using the Immun-Star detection kit system (Bio-Rad). Loading of equal amounts of proteins was controlled by Bradford analysis and by gel and membrane staining. Competition experiments to prove the specificity of the antibodies were performed by pre-incubation of the antibodies with 30 μg of the same protein used for rabbit inoculation for 1 h at room temperature.

Enzymatic Activity
Enzyme activity was determined spectrophotometrically at 25°C by monitoring the production of NADH at 340 nm (ε₉₄₀ = 6.22 mmol−1 cm−1) with a Hewlett-Packard 8452A diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA). In some experiments, 0.6 mM 3-acetylpyridine adenine dinucleotide (ε₉₄₀ = 9.10 mmol−1 cm−1), Sigma, St. Louis) was used as a cofactor instead of NAD. One unit of activity corresponds to 1 μmol reduced cofactor formed per minute. FALDH activity was measured in 0.1 M sodium phosphate (pH 7.8) as described by Martínez et al. (1996). Kinetic constants for FALDH were determined with S-hydroxymethylglutarate. Concentrations of S-hydroxymethylglutarate were calculated using its dissociation constant (Koivusalo et al., 1989) and keeping free glutathione at a constant concentration of 1 mM, changing accordingly the formaldehyde concentration. Kinetic constants for alcohols were determined for 2.4 mM NAD, in 0.1 M sodium phosphate at pH 7.5, or in 0.1 M Gly buffer at pH 10. Seven substrate concentrations, in duplicate, were used for each kinetic measurement. Kinetic results were analyzed using the nonlinear regression data analysis program Enzfitter (Elsevier, Biosoft, Cambridge, UK), and the curves were expressed in terms of two independent variables. Formaldehyde concentration was determined by the conversion of NAD to NADH in the presence of FALDH from Pseudomonas putida with an excess of NAD.
Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third party owners of all or parts of the material. Obtaining any permission will be the responsibility of the requestor.

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