Tissue-Specific Expression of the Alternative Oxidase in Soybean and Siratro

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ABSTRACT

Alternative oxidase activity (cyanide-insensitive respiration) was measured in mitochondria from the shoots, roots, and nodules of soybean (Glycine max L.) and siratro (Macroptilium atropurpureum) plants. Activity was highest in the shoots and lowest in the nodules. Alternative oxidase activity was associated with one (roots) or two (shoots) proteins between 30 and 35 kilodaltons that were detected by western blotting with a monoclonal antibody against Sauromatum guttatum alternative oxidase. No such protein was detected in nodule mitochondria. Measurements of oxygen uptake by isolated soybean root and nodule cells in the presence of cyanide and salicylhydroxamic acid indicated that alternative oxidase activity was confined to the uninfected cortex cells of the nodule. Immunoprecipitation of translation products of mRNA isolated from soybean shoots revealed a major band at 42 kilodaltons that is assumed to be the precursor of an alternative oxidase protein. This band was not seen when mRNA from nodules was treated in the same fashion. The results indicate that tissue-specific expression of the alternative oxidase occurs in soybean and siratro.

The resistance of plant respiration to cyanide has been observed since the turn of the century. This resistance is now known to involve a bypass of the last two complexes of the conventional electron transport chain, with electrons traveling from reduced ubiquinone to oxygen through an enzyme known as the alternative oxidase (11, 21, 30). However, little is known of the mechanism of action of the alternative oxidase or of its role in general plant metabolism.

Much of what we do know about the alternative oxidase has come from studies of the floral spadices of thermogenic plants such as Sauromatum guttatum and Symplocarpus foetidus. Floral development in these plants involves periods when the energy of electron transport within the spadix is released mainly as heat to volatilize compounds that attract pollinating insects, and the alternative oxidase appears to be involved here (21, 23). Elthon and McIntosh (12) identified components of the alternative oxidase of S. guttatum mitochondria, which intensified during development of the floral spadix, coincident with the dramatic increase in alternative oxidase activity. Three protein bands between 35 and 37 kD were thus identified, and antibodies against them were produced (13). These were used to show that salicylic acid can induce the alternative oxidase during floral development in Sauromatum (14).

The antibodies raised against S. guttatum alternative oxidase cross-react with proteins of mitochondria isolated from other cyanide-resistant tissues (14, 20) and have been used in studies of alternative oxidase expression in potato tubers (16) and soybean (25). Aging of potato tuber tissue induces expression of a single 36-kD mitochondrial polypeptide, whereas in soybean mitochondria, either one or two protein bands react with the antibody, depending on the age of the tissue. In Neurospora, alternative oxidase proteins are only synthesized when chloramphenicol is used to inhibit mitochondrial protein synthesis, indicating a nuclear origin for the enzyme (20). More recently, a cDNA clone encoding the alternative oxidase was isolated from S. guttatum and sequenced (29).

Despite these studies, the role and regulation of the alternative oxidase in nonthermogenic plant tissues remain poorly understood. We reported previously (9) that mitochondria from soybean nodules lack alternative oxidase activity, in contrast to their root and cotyledon counterparts, and soybean, therefore, offers a useful system for the study of alternative oxidase expression. Here, we have used the Sauromatum antibodies in such a study and show that alternative oxidase proteins between 32 and 35 kD are expressed to different extents in shoots and roots of both soybean and siratro. Immunoprecipitation of mRNA translation products indicate that the oxidase is made as a 43-kD precursor. In nodules, the oxidase is not synthesized in the infected cells but is in the cortex. Possible regulatory mechanisms are discussed.

MATERIALS AND METHODS

Reagents

Percoll and low mol wt standards for SDS-PAGE were purchased from Pharmacia Biochemicals Inc. (Uppsala, Sweden). Other electrophoresis reagents were purchased from Bio-Rad (Richmond, CA). Otherwise, reagents were purchased from Sigma. Rabbit reticulocyte lysate was purchased from Promega (Madison, WI) and [35S]methionine from Amer sham International (Sydney, Australia).

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Plants

Siratro (Macroptilium atropurpureum) seeds were covered with concentrated H$_2$SO$_4$ for 10 min and then washed with five rinses of distilled water before planting. Soybean (Glycine max [L] Merr.) cv Bragg seeds were surface sterilized in sodium perchlorite (10%, w/v). For etiolated cotyledons, soybean or siratro seeds were sown in trays of vermiculite and grown at 25°C without light and were harvested after 5 to 7 d. For both green cotyledons and leaves, plants were propagated in a glasshouse in trays of vermiculite and were harvested 7 to 9 and 10 to 14 d after sowing, respectively. For siratro shoots, plants were maintained in a glasshouse for 7 to 9 d before the emergent leaves were harvested. Nodules were picked from plants that had grown in a glasshouse for 63 to 70 d in pots of sand and provided with nitrogen-free Herridge nutrient solution (10) three times a week. Soybean plants were inoculated with Bradyrhizobium japonicum strain ANU289. Roots were harvested from plants grown in trays of sand in a glasshouse for 7 to 9 d.

Mitochondrial Isolation

Mitochondria were isolated from all tissues by the method of Day et al. (8), except for nodules for which the method of Day et al. (9) was followed.

Nodule Cell Isolation

Uninfected nodule cortex rings and infected cells were isolated by the method of Kouchi et al. (18).

Assays

O$_2$ consumption was measured in a Rank Bros (Cambridge, United Kingdom) electrode at 25°C in 2 mL of reaction medium (0.3 mM sucrose, 10 mM Tes buffer [pH 7.0], 5 mM KH$_2$PO$_4$, 10 mM NaCl, 2 mM MgSO$_4$, 0.1% [w/v] BSA). For assays involving nodule cells, 3 mL of reaction medium was used. The protein content of samples was estimated by the method of Lowry et al. (22).

Gel Electrophoresis

As much as 60 mg of mitochondrial proteins was solubilized in ≤50 mL of sample buffer (2% [w/v] SDS, 62.5 mM Tris [pH 6.8], 10% [v/v] glycerol, 0.002% [w/v] bromphenol blue, 50 mM DTT) and were boiled for 3 to 5 min. Electrophoresis was then carried out in a manner similar to that of Laemmli (19) using a 5% polyacrylamide stacking gel and a 12% polyacrylamide resolving gel (3, 8).

Immunological Probing

Antibodies against alternative oxidase proteins of Sauromatum guttatum were raised in mice as described by Elthon et al. (15). Polyclonal antibodies against isolated complex I from red beet (31) were raised in rabbits. A modified version of the method of Towbin et al. (32) was used for western blotting. Bands were visualized using alkaline phosphatase and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

RNA Isolation and Translation

Total RNA was isolated according to the method of Kirby (17) using phenol extraction of fresh plant material frozen in liquid nitrogen immediately after harvesting and ground in a coffee grinder to a fine dust. Poly(A$^+$) RNA was isolated according to the method of Aviv and Leder (2) using oligo(dT)-cellulose chromatography. The resulting RNA was translated in a rabbit reticulocyte lysate in the presence of [$^{35}$S]methionine (26). Immunoprecipitations were carried out according to the procedure of Anderson and Blobel (1). Translation products were boiled in Laemmli sample buffer to dissociate them and then diluted 10-fold in 1% Triton X-100, 100 mM NaCl, 10 mM Tris (pH 7.0), 0.1 mM PMSF. Pre-washed protein-A Sepharose was added to the mixture, which was incubated for 1 h and centrifuged to pellet the Sepharose. Antibodies were added to the translation mixture, which was incubated for 2 h at 4°C. In the case of monoclonal antibodies to the alternative oxidase, a second antibody, namely, rabbit anti-mouse IgG was added before sequestering with protein-A Sepharose. In the case of complex I antibodies raised in rabbit, sequestration was carried out directly with protein-A Sepharose. After washing, the protein-A antibody complex was dissociated by boiling in gel sample buffer and analyzed by SDS-PAGE and fluorography.

RESULTS

Alternative Oxidase in Isolated Mitochondria

Mitochondria were isolated from different organs of soybean and siratro plants, and alternative oxidase activity was measured as KCN-insensitive O$_2$ uptake (Table I). Although some variation was seen in different preparations, generally, alternative oxidase activity in soybean cotyledon, leaf, and root mitochondria was 40 to 60% of state 3 succinate oxidation. Mitochondria from shoots and roots of siratro seedlings also displayed substantial alternative oxidase activity (Table I). However, mitochondria from the nodules of both species were almost completely inhibited by KCN (Table I).

Mitochondrial proteins from the different tissues were separated by SDS-PAGE and probed by western blotting with a monoclonal antibody against the S. guttatum alternative oxidase protein (13). Initial experiments showed that this antibody recognized three proteins of 35 to 37 kD in S. guttatum spadix mitochondria, as reported previously (13). In soybean and siratro, on the other hand, only one or two proteins were recognized (Figs. 1 and 2). In soybean, two bands at 35 and 33 kD were seen in the cotyledon and leaf mitochondria (Fig. 1B, lanes a–c). In root mitochondria, only one band at 35 kD was observed (Fig. 1B, lane e). Likewise, in siratro shoot mitochondria, the antibody reacted with two proteins (33 and 32 kD; Fig. 2B, lane c) but reacted with only one in the mitochondria from roots (Fig. 2B, lane b). In nodule mitochondria,

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2 Abbreviations: poly(A$^+$) RNA, polyadenylated RNA; IgG, immunoglobulin G; SHAM, salicylhydroxamic acid.
Table I. Alternative Oxidase Activity in Mitochondria from the Different Tissues of Soybean and Siratro

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Oxygen Consumption*</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (+KCN)</td>
<td></td>
</tr>
<tr>
<td>Soybean</td>
<td>nmol min⁻¹ mg⁻¹ protein</td>
<td>%</td>
</tr>
<tr>
<td>Etiolated cotyledons</td>
<td>111 ± 14 66 ± 7</td>
<td>60</td>
</tr>
<tr>
<td>Green cotyledons</td>
<td>99 ± 6 43 ± 2</td>
<td>43</td>
</tr>
<tr>
<td>Leaves</td>
<td>177 ± 22 83 ± 14</td>
<td>47</td>
</tr>
<tr>
<td>Roots</td>
<td>153 ± 5 66 ± 3</td>
<td>43</td>
</tr>
<tr>
<td>Nodules</td>
<td>86 ± 15 6 ± 3</td>
<td>6</td>
</tr>
<tr>
<td>Siratro</td>
<td>Shoots 76 19</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Roots 28 14</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Nodules 70 3</td>
<td>4</td>
</tr>
</tbody>
</table>

*Measured in the presence of 10 mM succinate and 1 mM ADP. KCN added at 0.5 mM: the insensitive rate was inhibited by n-propylgallate. Values shown are means of three to five separate measurements from different experiments (± st). Averages of two separate experiments are shown.

Figure 2. Analysis of siratro mitochondrial proteins. Mitochondrial proteins were separated by SDS-PAGE (A) and then immunobotted (B) with antibodies raised against alternative oxidase proteins from S. guttatum mitochondria. A, Coomassie blue-stained mitochondrial polypeptides from shoots (lane a), roots (lane b), and nodules (lane c). B, Immunoblot of mitochondrial polypeptides from nodules (lane a), roots (lane b), and shoots (lane c). Molecular mass (MW) is expressed in kD.

Figure 1. Analysis of soybean mitochondrial proteins using antibodies raised against alternative oxidase. Mitochondrial proteins (20 mg of total protein per lane) were separated by SDS-PAGE and then blotted and incubated with antibodies raised against the alternative oxidase of S. guttatum. Apparent molecular masses (MW) are expressed in kD. A, SDS-PAGE; mitochondrial proteins isolated from soybean leaves, green cotyledons, etiolated cotyledons, nodules, and roots (lanes a–e, respectively). B, Western blot; mitochondrial proteins isolated from soybean leaves, green cotyledons, etiolated cotyledons, nodules, and roots (lanes a–e, respectively).

In soybean, the polypeptide profiles of cotyledon and leaf mitochondria were very similar, but differences were visible between these and the root and nodule mitochondria (Fig. 1A). Likewise, in siratro, polypeptide patterns of roots, nodules, and shoots were obviously different from one another (Fig. 2A). The purity of the different soybean preparations is well established (3, 7, 9), and the differences shown in Figures 1 and 2, therefore, reflect real differences in the protein complement of mitochondria from the different tissues.

Alternative Oxidase Activity in Nodule Cells

Infected cells in the center of mature soybean nodules were separated from the uninfected cells of the cortex enzymatically (18). The isolated infected cells were easily discernible by their large size, pink color, and irregular shape (18) and were <10% contaminated by uninfected cells (judged by microscopy). The cortical rings that remained were also essentially free from cells of the infected zone. Uninfected, interstitial cells from the infected zone were lost during this preparation.

Oxygen uptake by the two cell types was measured (Table II). The respiration of infected cells using both added substrate (experiment 1) and endogenous reserves (experiment 2) was almost entirely cyanide sensitive, and the residual respiration in each case was SHAM resistant. This indicates that these cells contain no measurable alternative oxidase activity. In
contrast, the respiration of cortex cells showed considerable cyanide resistance (>50% of the uninhibited rate) in both experiments, almost identical with other soybean tissues. This cyanide-resistant respiration was almost all SHAM sensitive, suggesting that it was alternative oxidase activity. This part of the study also supports previous suggestions (27) that, when mitochondria are isolated from the nodules of soybean, cells of the infected zone constitute the primary source of these mitochondria because the isolated mitochondria show little alternative oxidase activity (Table I).

Synthesis of Alternative Oxidase

In an attempt to identify the level of control of alternative oxidase expression (i.e. transcriptional or posttranscriptional), immunoprecipitations were carried out on products formed by in vitro translation of mRNA from green cotyledon and nodule tissues. To test whether the alternative oxidase antibody would recognize the cytoplasmic precursor of the alternative oxidase, immunoprecipitations were first carried out with mRNA isolated from green cotyledon tissue (Fig. 3). A major protein with an apparent molecular mass of 43 kD was precipitated by the alternative oxidase antibodies (Fig. 3, lane 3). Some additional minor bands were also evident that probably represent aggregated proteins and incomplete translation products due to premature termination of translation. Immunoprecipitation with protein-A Sepharose and rabbit anti-mouse IgG gave rise to no immunoprecipitated products (Fig. 3, lane 1). In addition, immunoprecipitation with a monoclonal antibody raised against a nonplant antigen also gave a negative result (Fig. 3, lane 2). These results indicate that the products seen with the alternative oxidase antibody (Fig. 3, lane 3) were due to a specific interaction between that antibody and the translated proteins.

When a similar experiment was carried out with mRNA isolated from nodule tissue, no products were evident (Fig. 4, left, lane 2), suggesting an absence of translatable mRNA for the alternative oxidase in nodule tissue; in the control with cotyledon tissue (Fig. 4 left, lane 1), a band at 43 kD and several lower molecular mass bands (presumably incomplete translation products) were seen again (the band at the top of the gel was due to aggregated protein that did not enter the resolving gel). If the immunoprecipitated products from nodule tissue were exposed for several weeks, a weak signal could be detected (data not shown). This signal was presumably due to the presence of some mRNA for alternative oxidase in the cortex cells of the nodule because the cortex showed alternative oxidase activity (Table II).

To ensure that the faint signal seen with the nodule tissue was not a result of the isolation of ineffective mRNA or due to a general lack of mitochondrial message in nodule cells, antibodies to complex I of the respiratory chain were used in immunoprecipitations with mRNA isolated from both cotyledon and nodule tissues (Fig. 4, right). In both cases, several proteins were precipitated, showing that the nodules contained substantial quantities of complex I mRNA. We, therefore, conclude that the weak signal seen with alternative oxidase immunoprecipitation in nodules was due to a specific lack of translatable alternative oxidase mRNA in that tissue.

Discussion

Previous results with soybean have shown that mitochondria isolated from nodules possess very little alternative oxidase activity, whereas their root and shoot counterparts display high rates. The present study confirms those observations and extends them to the siratro-Bradyrhizobium symbiosis. The possible implications of this for nitrogen fixation have been discussed previously (4, 6).

In both plants, alternative oxidase activity in the shoots and
roots was correlated with the expression of one or two proteins between 32 and 35 kD. The lack of alternative oxidase protein in nodule mitochondria was reflected in much lower levels of translatable mRNA for the oxidase in that tissue.

Measurements of the respiration of cells isolated from different parts of the nodule indicated that some alternative oxidase expression does occur in the cortex, which presumably is not disrupted as easily as the infected cells during mitochondria and RNA isolation (27). (It should also be noted that infected cells are more numerous than uninfected cells in a mature soybean nodule.) These results point to oxygen as a possible regulatory factor in alternative oxidase expression. An oxygen diffusion barrier has been shown to reside in a layer or two of cells in the inner nodule cortex, restricting O₂ supply to the nitrogenase-containing infected cells (33). Oxygen concentrations in the central zone fall well below the Kₘ of the alternative oxidase (5), and, therefore, it makes sense that expression of this protein, abundant elsewhere in the plant, is curtailed in the infected cells. Lack of the non-phosphorylating alternative oxidase also fits well with the high energy demand of the infected cells. The involvement of O₂ in the regulation of alternative oxidase expression in potato tubers has also been suggested, on the basis of an increase in protein levels upon slicing the tissue (16).

It is interesting to note that whereas alternative oxidase levels are low in nodule mitochondria, cytochrome oxidase activity is higher, compared with that in other soybean tissues (9, 28), suggesting that expression of the two enzymes can be differentially regulated. This has also been shown in *Sauro-

**Figure 4.** Immunoprecipitation of products from translation of mRNA from different tissues, with alternative oxidase and complex I antibodies. A, Immunoprecipitation with alternative oxidase antibodies. Lane 1, mRNA isolated from green cotyledon tissue was translated and immunoprecipitated using alternative oxidase antibodies; lane 2, as in lane 1 except that mRNA was isolated from nodule tissue. B, As in A except that antibodies raised against complex I were used to immunoprecipitate translation products. Molecular mass (right lane) is expressed in kD.

matum* spadices, where cytochrome oxidase synthesis is reduced and alternative oxidase activity increases during thermogenesis (14).

More subtle variations in alternative oxidase expression were also observed in soybean and siratro. In both species, only one alternative oxidase protein was observed in root mitochondria, whereas in cotyledon and leaf mitochondria, two proteins were evident. Yet, alternative oxidase activity was similar in these tissues. This suggests that the presence of only one of the alternative oxidase proteins (35 kD) is required for activity. In cotyledons, we noticed that the relative intensities of the two bands varies according to the age of the seedling (data not shown), as reported also by Obenland et al. (25).

Immunoprecipitation of *in vitro* translation products made using soybean poly(A⁺) RNA suggested that the alternative oxidase was synthesized as a 43-kD precursor. Although in some experiments smaller sized, additional proteins were also precipitated, these did not correspond in size to any of the mature proteins seen in the isolated mitochondria, and it is likely that only a single alternative oxidase protein is synthesized in soybean (the smaller products being the result of incomplete translations). This has also been found in *S. gattatum*, in which a 42-kD precursor protein has been observed and a nuclear gene identified (29). In the yeast *Han-

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**

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