Induction of Kranz Anatomy and $C_4$-like Biochemical Characteristics in a Submerged Amphibious Plant by Abscisic Acid

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The amphibious leafless sedge Eleocharis vivipara develops $C_4$-like traits as well as Kranz anatomy under terrestrial conditions, but it develops $C_3$-like traits without Kranz anatomy under submerged conditions. When submerged plants are exposed to aerial conditions, they rapidly produce new photosynthetic tissues with $C_4$-like traits. In this study, experiments were performed to determine whether abscisic acid (ABA), a plant stress hormone, could induce the formation of photosynthetic tissues with Kranz anatomy and $C_4$-like biochemical traits under water in the submerged form. When the submerged plants were grown in water containing 5 μM ABA, they developed new photosynthetic tissues with Kranz anatomy, forming well-developed Kranz (bundle sheath) cells that contained many organelles. The ABA-induced tissues accumulated large amounts of phosphoenolpyruvate carboxylase, pyruvate orthophosphate dikinase, and NAD–malic enzyme at the appropriate cellular sites. The tissues had 3.4 to 3.8 times more $C_4$ enzyme activity than did tissues of the untreated submerged plants. Carbon-14 pulse and carbon-12 chase experiments revealed that the ABA-induced tissues fixed higher amounts of carbon-14 into $C_4$ compounds and lower amounts of carbon-14 into $C_3$ compounds as initial products than did the submerged plants and that they exhibited a $C_4$-like pattern of carbon fixation under aqueous conditions of low carbon, indicating enhanced $C_4$ capacity in the tissues. This report provides an example of the hormonal control of the differentiation of the structural and functional traits required for the $C_4$ pathway.

INTRODUCTION

The leaves of $C_4$ plants have specialized structural and biochemical features that differ from those of $C_3$ plants. The leaves of $C_4$ plants are composed of two types of photosynthetic cells: the mesophyll cells and the Kranz or bundle sheath cells (Brown, 1975; Nelson and Langdale, 1992). $C_4$ photosynthesis requires the coordination of biochemical functions between the two types of cells and the cell type–specific expression of the enzymes involved in $C_4$ photosynthesis (Edwards and Walker, 1983; Hatch, 1987). Phosphoenolpyruvate carboxylase (PEPCase) and pyruvate orthophosphate dikinase (PPDK) are localized in the mesophyll cells, whereas $C_4$ acid-decarboxylating enzymes, such as NAD–malic enzyme (NAD-ME) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), are in the Kranz cells. In contrast, leaves of $C_3$ plants contain only a single type of photosynthetic cell, which is the mesophyll cell. The bundle sheath cells of $C_3$ leaves are poorly developed and contain only a few organelles (Brown and Hattersley, 1989). Although the biochemical and physiological features of $C_4$ photosynthesis have been elucidated in considerable detail, genetic and developmental aspects of this process remain to be fully characterized (Nelson and Langdale, 1992; Furbank and Taylor, 1995).

Recently, $C_3$–$C_4$ intermediate species have been found in certain genera such as Flaveria and Moricandia. Flaveria includes $C_3$, the $C_3$–$C_4$ intermediate, and $C_4$-like and $C_4$ species, which show a continuous gradation in photosynthetic and photorespiratory traits. These plants seem to be of significance for elucidating the evolutionary and developmental processes of $C_4$ photosynthesis (Edwards and Ku, 1987; Rawsthorne, 1992; Rosche et al., 1994; McGonigle and Nelson, 1995).

In general, plants exploit one mode of photosynthesis in their leaves, but some plants can alter the mode of photosynthesis in response to changes in environmental conditions. Well-known examples for such photosynthetic plasticity are a change from $C_3$ to Crassulacean acid metabolism (CAM) in some succulent plants (Winter, 1985) and a change from $C_3$ to
to C₄-like metabolism without Kranz anatomy in Hydrilla verticillata (Bowes and Salvucci, 1989). In these cases, however, the changes in the photosynthetic mechanism are not accompanied by structural changes in the photosynthetic tissues. By contrast, a dramatic change in the photosynthetic mechanism with accompanying structural changes has been found in a freshwater amphibious leafless sedge, Eleocharis vivipara (Ueno et al., 1988; Ueno, 1996a, 1996b). The terrestrial form of this plant has C₃-like biochemical traits of the NAD-ME type and Kranz anatomy, whereas the submerged form has C₄-like biochemical traits and non-Kranz anatomy (Ueno et al., 1988). A unique pattern of cellular distribution of C₃ and C₄ enzymes and changes in the extent of accumulation of these enzymes appear to be the main factors responsible for the expression of the different photosynthetic traits of the two distinct forms (Ueno, 1996b). In addition, structural changes in the photosynthetic tissues might account to some extent for the differences in photosynthetic traits (Ueno, 1996a). Thus, E. vivipara is an attractive plant with which to study the relationships between genetic and developmental aspects of C₃ and C₄ traits can be studied (Agarie et al., 1997).

E. vivipara develops new photosynthetic tissues with either the C₃-like traits or the C₄-like traits, depending on environmental conditions (Ueno et al., 1988). It seems likely that specific environmental stimuli induce differentiation of these traits. In some succulent plants, salt stress and water stress induce the expression of CAM (Winter, 1985). Thus, in E. vivipara, osmotic stress also might be involved in the C₃ and C₄ differentiation. When the submerged form is exposed to air, the photosynthetic tissues die as a result of rapid desiccation. Within several days, however, the plants begin to develop new photosynthetic tissues, which have Kranz anatomy and C₄-like biochemical traits (Ueno et al., 1988; O. Ueno, unpublished data).

It is well known that the leaves of some freshwater amphibious plants exhibit dimorphism between the terrestrial form and the submerged form. This phenomenon is known as heterophyll (Sculthorpe, 1967; Smith and Hake, 1992) and is controlled by various environmental and hormonal factors (Johnson, 1967; Anderson, 1978; Goliber and Feldman, 1989). Although abscisic acid (ABA) is considered to be a stress hormone in plants (Hartung and Davies, 1991), there is evidence that it is involved in the determination of leaf identity in some heterophyllic aquatic plants (Anderson, 1978; Goliber and Feldman, 1989) as well as in the induction of CAM in some succulent plants (Ting, 1981; Chu et al., 1990; McElwain et al., 1992; Dai et al., 1994; Taybi et al., 1995). Thus, we might expect that ABA could also induce the expression of some C₄ traits in E. vivipara.

In this study, we attempted to evaluate the possible effects of ABA on the development of photosynthetic traits in the submerged form of E. vivipara. The results demonstrate that ABA can induce the formation of photosynthetic tissues with Kranz anatomy and C₄-like biochemical traits in the submerged form of this plant, thereby regulating C₄ differentiation.

RESULTS

Anatomy of Terrestrial versus Submerged Form

E. vivipara lacks leaf blades, and the culms perform all photosynthetic functions (Ueno et al., 1988). A brief summary is provided here for the anatomical features of the culms of the terrestrial and submerged forms before we present the results of exposing the submerged form to ABA. The culms of the terrestrial form have an unusual Kranz-type anatomy, which is characterized by semiradially arranged mesophyll cells and three kinds of bundle sheath (Figures 1A and 1B; Ueno, 1996a). The outermost sheath consists of parenchyma sheath cells with small chloroplasts, and it basically functions as mesophyll cells do (Ueno, 1996b). The middle sheath is a mesophyll sheath without chloroplasts. The innermost sheath consists of Kranz cells (bundle sheath cells) that contain large numbers of organelles (Figures 2A and 2B). The chloroplasts have well-developed grana. The mitochondria are larger than those in the two other types of photosynthetic cells, the parenchyma sheath cells and the mesophyll cells. These structural features reflect the fact that the terrestrial form has biochemical traits of the NAD-ME C₄ type (Ueno, 1996a).

The culms of the submerged form have a layer of mesophyll cells inside the epidermis and large air cavities (Figures 1C and 1D). The mesophyll cells appear circular in transverse sections and differ strikingly from those in the terrestrial form. The vascular bundles are smaller and less densely distributed than are those of the terrestrial form (Figures 1C and 1D). Although the submerged form also has three kinds of bundle sheath cells, the innermost and outermost sheath cells are modified. The Kranz cells are reduced in number and size (Figure 1D) and contain only small numbers of organelles (Figures 2C and 2D). The chloroplasts of the Kranz cells are also smaller than those in the terrestrial form (Figures 2C and 2D). The parenchyma sheath cells are larger than those of the terrestrial form and contain chloroplasts (Figures 1C and 1D). Such anatomy is typical of submerged aquatic plants; traits typical of Kranz-type anatomy are absent (Ueno, 1996a). Stomata are not present in the epidermis (Figures 1C and 1D), except at the tips of the culms.

Induction of Kranz Anatomy by ABA

When plants of the submerged form were grown in an aqueous solution of 5 µM ABA, they began to develop new culms within a week. These culms were erect, with a firm appearance, and were similar to the culms of the terrestrial form. The mesophyll cells were arranged semiradially and were of a shape similar to those of the terrestrial form (Figures 1E and 1F). The vascular bundles were larger than those of the submerged form (Figures 1E and 1F). The bundle sheaths...
Figure 1. Anatomical Structure of Culms of the Two Growth Forms of E. vivipara and of Culms Induced in 5 μM ABA Solution.

(A), (C), and (E) show hand-cut sections of fresh materials. (B), (D), and (F) show sections prepared from materials embedded in Spurr’s resin and stained with toluidine blue O.

(A) and (B) Cross-sections of culms of the terrestrial form.
(C) and (D) Cross-sections of culms of the submerged form.
(E) and (F) Cross-sections of ABA-induced culms.

Arrowheads indicate stomata. AC, air cavity; KC, Kranz cell; MC, mesophyll cell; MSC, mestome sheath cell; PSC, parenchyma sheath cell. Bars in (A), (C), and (E) = 100 μm. Bars in (B), (D), and (F) = 50 μm.
Figure 2. Ultrastructure of Kranz Cells in Culms of the Two Growth Forms of E. vivipara and in Culms Induced in 5 μM ABA Solution.

(A) and (B) Kranz cells in the terrestrial form.
(C) and (D) Kranz cells in the submerged form.
(E) and (F) Kranz cells in ABA-induced culms.

C, chloroplast; mt, mitochondrion; P, phloem; other abbreviations are as given in the legend to Figure 1. Bars in (A), (C), and (E) = 4 μm. Bars in (B), (D), and (F) = 0.5 μm.
also resembled those in the terrestrial form. The Kranz cells were well developed and contained large numbers of organelles, such as chloroplasts, mitochondria, and peroxisomes (Figures 2E and 2F), as did the Kranz cells of the terrestrial form. The chloroplasts had grana, and the chloroplasts themselves were larger than those in the Kranz cells in the submerged form (Figures 2E and 2F). The mitochondria were well organized and larger than those in the other two types of cells (Figures 2E and 2F). The quantitative and qualitative traits of these organelles tended to resemble those in the Kranz cells of the terrestrial form. The shapes of the parenchyma sheath cells resembled those of the terrestrial form to some extent, and the cells contained small chloroplasts, as did those of the terrestrial form (Figures 1E and 1F). The mestome sheath cells lacked chloroplasts, however. Moreover, there were many stomata in the epidermis (Figures 1E and 1F). Thus, it is evident that exogenously applied ABA induced new culms with Kranz anatomy, which resembled the terrestrial form, in the submerged form.

The culms that had been formed before ABA treatment gradually became brownish during their treatment with ABA and finally died. Although almost all of the culms that were induced in 5 μM ABA had the anatomical features described above, the anatomy of some of the culms was intermediate between that of the submerged and terrestrial forms. When plants of the submerged form were grown in aqueous solutions of ABA at lower concentrations, they tended frequently to develop various intermediate types of anatomy (data not shown). When plants were grown with ABA at concentrations >5 μM, they also developed culms with Kranz anatomy, but the growth rate was reduced.

**Cellular Accumulation of C₃ and C₄ Enzymes in ABA-Induced Tissues**

Immunogold labeling and electron microscopy were used to determine the cellular accumulation of representative C₃ and C₄ enzymes in culms having Kranz anatomy that were induced in 5 μM ABA. Plants of the terrestrial and submerged forms that were used as controls had patterns of cellular accumulation of Rubisco, PEPCase, and PPDK that were similar to those reported in a previous study (Ueno, 1996b). Therefore, only a few electron micrographs are shown here for the control plants (Figures 3D, 3E, and 3G) so that the results with the ABA-induced culms can be compared with them (Figures 3A to 3C, 3F, 3H, and 3I). In this study, cellular accumulation of NAD-ME, a C₄ acid decarboxylating enzyme, was further investigated (Figure 4). The details of the cellular accumulation of the photosynthetic enzymes in the three types of photosynthetic cells, namely, the mesophyll cells, the parenchyma sheath cells, and the Kranz cells, are summarized in Table 1. Virtually no labeling specific for the photosynthetic enzymes was found in the mestome sheath cells of the two growth forms or the ABA-induced culms.

Both the terrestrial and submerged forms accumulated the large subunit of Rubisco in the chloroplasts in all three types of photosynthetic cells (Table 1). Thus, the terrestrial form differed from typical C₄ plants in the cellular distribution of Rubisco (Ueno, 1996b). This cellular distribution of Rubisco resembles that in the C₄-like species of Flaveria (Cheng et al., 1988; Moore et al., 1989). Among the three types of cells, the density of labeling specific for the large subunit of Rubisco was lowest in the chloroplasts of the parenchyma sheath cells (Table 1). The chloroplasts of the three types of cells in ABA-induced culms also accumulated high levels of the large subunit of Rubisco, and the density of labeling was similar in all of them (Figures 3A to 3C and Table 1). The terrestrial form accumulated PEPCase in the cytosol of both the parenchyma sheath cells (Figure 3D) and the mesophyll cells but not in the Kranz cells. The density of labeling specific for PEPCase was higher in the parenchyma sheath cells than in the mesophyll cells (Table 1). In the submerged form, densities of labeling specific for PEPCase were lower than in the terrestrial form (Figure 3E and Table 1).

The pattern of labeling specific for PEPCase in ABA-induced culms was similar to that in the terrestrial form. Dense labeling specific for PEPCase was found in the cytosol of the mesophyll cells and the parenchyma sheath cells (Figure 3F). The density of labeling specific for PEPCase in the ABA-induced culms was significantly higher than in the submerged form (Table 1). The cellular localization of PPDK in E. vivipara is unusual (Ueno, 1996b). In the terrestrial form, labeling specific for PPDK was seen in the cytosol as well as in the chloroplasts of the mesophyll cells and the parenchyma sheath cells but not in the Kranz cells. The density of labeling in the chloroplasts was somewhat higher in the parenchyma sheath cells than in the mesophyll cells (Table 1). In the submerged form, the density of labeling specific for PPDK in the chloroplasts was lower than in the terrestrial form (Table 1). In addition, labeling specific for PPDK was noted in the cytosol of the Kranz cells (Table 1) as well as in that of the other two types of cells (Figure 3G).

In ABA-induced culms, the mesophyll cells and parenchyma sheath cells showed a pattern of labeling specific for PPDK that was similar to the terrestrial form (Figure 3H and Table 1). However, PPDK was also labeled in the cytosol of the Kranz cells (Figure 3I). The terrestrial form accumulated NAD-ME only in the mitochondria of the Kranz cells (Figure 4C) and not in the mesophyll cells (Figure 4A) or the parenchyma sheath cells (Figure 4B). The submerged form also showed the same cellular pattern of accumulation of NAD-ME (Figures 4D and 4E). However, densities of labeling specific for NAD-ME in the mitochondria of the Kranz cells were somewhat lower in the submerged form than in the terrestrial form (Table 1). In ABA-induced culms, only the mitochondria of the Kranz cells accumulated NAD-ME, and the density of labeling was similar to that in the terrestrial form (Figures 4F and 4G and Table 1). In ABA-induced culms, therefore, the pattern of cellular accumulation of photosynthetic enzymes was basically similar to that found in the
Figure 3. Immunogold Localization of the Large Subunit of Rubisco, PEPCase, and PPDK in Photosynthetic Tissues.
terrestrial form. However, the pattern of accumulation of PPDK appeared to be a combination of the patterns in the terrestrial and submerged forms.

**Induction of C₄-like Biochemical Characteristics by ABA**

Activities of C₃ and C₄ photosynthetic enzymes were measured in culms induced in 5 μM ABA solution and compared with those in the terrestrial and submerged forms (Table 2). The ABA-induced culms had higher levels of enzyme activity than did the submerged form (Table 2). The extent of the increase was more evident in the case of the C₄ enzymes, PEPCase, PPDK, and NAD-ME (3.4 to 3.8 times the activity) than in that of the C₃ enzyme Rubisco (1.6 times). However, the activities of these C₄ enzymes were still lower than those in the terrestrial form (Table 2). The activity of Rubisco in the ABA-induced culms was higher than in both the terrestrial and submerged forms. Consequently, the ratio of the activity of Rubisco to that of PEPCase in the ABA-induced culms
was intermediate between the ratios in the terrestrial and submerged forms (Table 2).

To determine whether the anatomical features and the increases in levels of the C4 enzymes in the ABA-induced culms were reflected by changes in photosynthetic carbon metabolism, pulse–chase experiments were performed (Figure 5). When the submerged form was exposed to 0.5 mM NaH14CO3 for 10 sec, 70 and 19% of the total radiolabel were incorporated into C3 compounds (phosphate esters) and C4 compounds (malate and aspartate), respectively. During the chase with carbon-12, the radiolabeling in C3 compounds decreased rapidly, and the label was transferred to sucrose (Figure 5A), indicating that the C3 pathway is in operation (Ueno et al., 1988). When culms induced in 5 μM ABA solution were examined under the same conditions, the pattern of labeling was similar to that in the submerged form (Figure 5B). However, the rate of incorporation of the radiolabel into C3 compounds (49%) was lower and that into C4 compounds (38%) was higher than those rates in the submerged form. The radiolabeling of malate increased somewhat during a 2-min chase with carbon-12, whereas that of aspartate showed a slight tendency to decrease during a 4-min chase (Figure 5B). When ABA-induced culms were exposed to a lower concentration of NaH14CO3 (0.05 mM) for 10 sec, almost all of the radiolabel (97%) was incorporated into C4 compounds (Figure 5D). The radiolabeling of aspartate decreased gradually from 62 to 33% during a 4-min chase, whereas that of malate decreased slightly during the chase period. The radiolabeling of C3 compounds increased gradually during a 2-min chase, accompanying an increase in the labeling of sucrose (Figure 5D).

In the submerged form, by contrast, 63 and 29% of the total radiolabel was incorporated into C4 and C3 compounds, respectively. The labeling patterns of aspartate and malate showed a reflected image of each other. The labeling of C3 compounds decreased from 28 to

### Table 1. Summary of the Cellular Accumulation of C3 and C4 Photosynthetic Enzymes in Culms of the Two Growth Forms of E. vivipara and in ABA-Induced Culms

<table>
<thead>
<tr>
<th>Plant and Enzyme</th>
<th>MC</th>
<th>PSC</th>
<th>KC</th>
</tr>
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<tbody>
<tr>
<td>Rubisco LSU</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>PEPCase</td>
<td>--</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>PPDK</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>NAD-ME</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Rubisco LSU</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>PEPCase</td>
<td>--</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>PPDK</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>NAD-ME</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Rubisco LSU</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>PEPCase</td>
<td>--</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>PPDK</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>NAD-ME</td>
<td>--</td>
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</tr>
</tbody>
</table>

a (+) and (−) refer to the relative intensities of labeling, with (+ + +) indicating heavy labeling and (−) indicating little or no labeling. MC, mesophyll cells; PSC, parenchyma sheath cells; KC, Kranz cells; Chlt, chloroplasts; Mit, mitochondria; Cyt, cytosol; LSU, large subunit.

### Table 2. Activities of C3 and C4 Photosynthetic Enzymes in Culms of the Two Growth Forms and in ABA-Induced Culms

<table>
<thead>
<tr>
<th>Plant</th>
<th>Activity (μmol mg⁻¹ Chl hr⁻¹)</th>
<th>Ratio (Rubisco/PEPCase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terrestrial form</td>
<td>Rubisco 524 ± 186</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>PEPCase 1044 ± 163</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PPDK 107 ± 16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NAD-ME 215 ± 33</td>
<td></td>
</tr>
<tr>
<td>Submerged form</td>
<td>Rubisco 673 ± 179</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>PEPCase 259 ± 69</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PPDK 10 ± 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NAD-ME 34 ± 10</td>
<td></td>
</tr>
<tr>
<td>ABA-induced culms</td>
<td>Rubisco 1058 ± 262</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>PEPCase 872 ± 62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PPDK 38 ± 11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NAD-ME 125 ± 34</td>
<td></td>
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</tbody>
</table>

aData are means ± SD (n = 3 to 5). Chl, chlorophyll.
Induction of C₄ Characteristics

10%, whereas that of sucrose increased from none to 16% during the chase period (Figure 5C). These data indicate that at higher concentrations of carbon, the C₃ pathway still dominates in the ABA-induced culms, as occurred in the submerged form. At lower concentrations of carbon, the C₄ pathway, resembling that in the terrestrial form (Ueno et al., 1988), operates in the culms.

The incorporation of carbon-14 into C₃ and C₄ compounds was examined in culms that were induced in aqueous solutions containing various concentrations of ABA (Figure 6). The culms formed in the solutions were exposed to 0.1 mM NaH¹⁴CO₃ for 10 sec. The incorporation of radiolabel into C₄ compounds increased and that into C₃ compounds decreased, with an increase in concentration of ABA from 0.1 to 5 μM. However, no significant difference was found between culms induced in 5 and 20 μM ABA (Figure 6).

**DISCUSSION**

This study shows clearly that exogenously applied ABA induced Kranz anatomy in entirely submerged plants of E. vivipara. The ABA-induced culms had the features of organelles of Kranz cells that are typical of the NAD-ME type. It is evident that ABA induced the enhanced development of
organelles and the cellular arrangement of photosynthetic tissues that are similar to those of the terrestrial C₄ form. Many amphibious plants exhibit heterophylly. This intriguing morphological response is controlled not only by environmental stimuli, such as osmoticum, temperature, light quality, and photoperiod, but also by several plant hormones, including ABA (Johnson, 1967; Anderson, 1978; Goliber and Feldman, 1989). Thus, it was not totally unexpected that ABA was able to induce the anatomical traits of the terrestrial form in the submerged form of \textit{E. vivipara}. Nonetheless, this study provides clear evidence of the hormonal regulation of the development of Kranz anatomy.

To date, the molecular mechanisms that lead to the differentiation of Kranz anatomy have been poorly understood (Nelson and Langdale, 1992; Langdale and Kidner, 1994; Hall and Langdale, 1996; Roth et al., 1996; Schultes et al., 1996). The results of this study imply that in \textit{E. vivipara}, the activities of key C₄ enzymes increased compared with those in the submerged form. The activity of Rubisco also increased in the ABA-induced culms, but the extent of the increase was smaller than that of the C₄ enzymes. The enzymatic activities in the ABA-induced culms coincided with the results of determinations of cellular levels of the photosynthetic enzyme proteins that were obtained in the immunolabeling study.

In leaves of C₄ plants, the expression of genes for photosynthetic enzymes is coordinated with the differentiation of two types of cells (Langdale and Nelson, 1991; Nelson and Langdale, 1992; Hall and Langdale, 1996). In \textit{E. vivipara}, the patterns of accumulation of photosynthetic enzymes in ABA-induced culms are similar to those in the terrestrial form. The intracellular positions of these enzymes also correspond closely to those in the terrestrial form. The difference between the ABA-induced culms and the terrestrial form was the presence of PPDK in the cytosol of the Kranz cells of the ABA-induced culms. The accumulation of PPDK in the cytosol of these cells was also observed in the submerged form. Therefore, it appears that the cellular pattern of distribution of PPDK in ABA-induced culms is a combination of that found in both the terrestrial and submerged forms. The physical environment under water, as well as ABA, might influence the cellular pattern of expression of PPDK. The ABA-induced culms accumulated Rubisco at similar levels in the chloroplasts of all three types of cells. This pattern of labeling differed somewhat from those in both the terrestrial and submerged forms. With these exceptions, it seems that ABA can induce the expression of photosynthetic enzymes in the submerged form at cellular positions that are found in the terrestrial form.

The pulse–chase experiments with a higher concentration of carbon-14 in the submerged form confirm previous results showing that the C₃ pathway operates in the submerged form (Ueno et al., 1988). At a lower concentration of carbon-14, considerable amounts of carbon-14 were incorporated into C₄ compounds as well as into C₃ compounds. It seems that the submerged form has somewhat higher levels of activity of C₄ enzymes than do typical C₃ plants. In addition, it accumulated NAD-ME only in the mitochondria of the Kranz cells, although the quantity of mitochondria was clearly lower than that in the Kranz cells of the terrestrial form. Thus, it appears that the submerged form possesses C₃-like intermediate traits rather than typical C₃ traits. It would be more appropriate to say that the terrestrial form...
also possesses C₄-like traits, as in the C₄-like species of Flaveria (Cheng et al., 1988; Moore et al., 1989), because Rubisco is located in the mesophyll cells as well as in the Kranz cells.

The pulse-chase experiments with carbon-14 in the ABA-induced culms demonstrated that the structural and enzymatic responses were reflected in the metabolic pattern of photosynthesis. At 0.5 mM NaHCO₃, the difference between the ABA-induced culms and submerged form in the rate of incorporation of carbon-14 into C₃ and C₄ compounds was not very large. At 0.05 mM NaHCO₃, however, the ABA-induced culms incorporated almost all of the carbon-14 into C₄ compounds. The free CO₂ concentrations under the former and the latter conditions approximate CO₂ conditions not very large. At 0.05 mM NaHCO₃, the ABA-induced culms demonstrated that the structural and enzymatic responses were reflected in the metabolic pattern of photosynthesis. At 0.5 mM NaHCO₃, the difference between the ABA-induced culms and submerged form in the rate of incorporation of carbon-14 into C₃ and C₄ compounds was not very large. At 0.05 mM NaHCO₃, however, the ABA-induced culms incorporated almost all of the carbon-14 into C₄ compounds. The free CO₂ concentrations under the former and the latter conditions approximate CO₂ conditions not very large.

No evidence for a single gene capable of setting in motion the entire C₄ photosynthetic machinery was found in earlier hybridization studies of species with different modes of photosynthesis: C₄ photosynthesis thus appears to be a combination of independently inherited traits (Brown and Bouton, 1993). Regulatory genes that control C₄ patterns of differentiation have not yet been identified. Superficially, it appears that ABA may stimulate a series of programs of gene expression specific for the differentiation of both the anatomical and biochemical characteristics of C₄ photosynthesis in the submerged form of E. vivipara. At present, however, it is unclear whether ABA stimulates one single signaling system that leads to the entire differentiation of such characteristics or separate signaling systems that are responsible for the individual differentiation of the anatomical and biochemical characteristics. Nevertheless, it is clear that this amphibious plant is a useful resource in the study of the differentiation of C₄ characteristics.

**METHODS**

**Plant Material**

Eleocharis vivipara plants were collected from a creek near Tampa, FL (Ueno et al., 1988), and cultivated in a greenhouse at the National Institute of Agrobiological Resources (Tsukuba, Japan). Some shoots of the terrestrial form were transplanted to 1-liter pots that contained soil, and they were grown in the greenhouse for several months. The plants were watered daily and supplied with standard Hoagland nutrient solution at weekly intervals. The submerged form was induced from the terrestrial form by submergence, and plants were grown in aquariums (50 cm deep) in the greenhouse for at least 6 months, as described previously (Ueno, 1996a). The water in the aquariums was allowed to overflow by the addition of tap water at a slow rate to prevent the growth of epiphytes. The pH of the water in the aquariums was ~6.9. The total inorganic carbon concentration was ~0.9 mM, as determined by titration.

The plants were then transferred to water tanks (30 liters) that had been set up in a growth chamber. The temperature in the growth chamber was maintained at 25°C during the light period (14 hr) and at 20°C during the dark period (10 hr) of every day. Photon irradiance was provided by metal–halide lamps at ~400 μmol m⁻² sec⁻¹ (400 to 700 nm). The water tanks were filled either with water that contained 5 μM abscisic acid (ABA) (cis, trans-isomers; Sigma) or with water (control). The water was the same as that used for the aquariums in the greenhouse. The pH of water in the tanks was ~6.9. The total inorganic carbon concentration was ~0.8 mM. Nutrient solution was not added to the water to prevent the growth of epiphytes.

Another experiment using deionized water instead of tap water confirmed that the plants also showed the same anatomical responses and carbon-14 labeling pattern as described in this study. The plants were also grown with ABA at various concentrations to examine the effects of concentration on induction of C₄ traits. The solution of ABA and the water were changed at weekly intervals. After...
2 days of treatment, photosynthetic tissues (culms) were cut off and discarded. Then the plants were grown in the tanks for ~4 more weeks, and newly formed photosynthetic tissues were used for experiments. Plants of the terrestrial form were also grown in the same growth chamber.

**Light and Electron Microscopy and Immunocytochemical Staining**

Light and electron microscopic observations and immunocytochemical staining were performed with sections prepared from culm samples that had been embedded in Spurr’s resin and in Lowicryl K4M resin (Chemische Werke Lowi GmbH, Waldkrubairg, Germany), respectively, as described previously (Ueno, 1996a, 1996b). Immunostaining was performed with antisera raised against phosphoenolpyruvate carboxylase (PEPCase), pyruvate orthophosphate dikinase (PPDK), NAD–malic enzyme (NAD-ME), the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and a suspension of protein A–colloidal gold particles (E.Y. Lab. Inc., San Mateo, CA), as described by Ueno (1996b). Antiserum that had been raised against PEPCase and PPDK from maize leaves were kindly provided by M. Matsuoka (Nagoya University, Nagoya, Japan). Antiserum raised against NAD-ME from leaves of *Eleocharis vivipara* was a generous gift from T. Murata (Iwate University, Morioka, CA), as described by Ueno (1996a). Antiserum that had been raised against Rubisco was kindly provided by M. Matsuoka (Nagoya University, Nagoya, Japan). Antiserum raised against the large subunit of Rubisco from pea leaves was a generous gift from S. Muto (Nagoya University).

**Assays of Enzymatic Activities**

Aliquots of 0.25 g of culms were ground using a mortar and pestle with 0.3 g of sea sand, 25 mg of PVP, and 1 mL of grinding medium (50 mM Hepes–KOH, pH 7.5, 0.2 mM EDTA, 2.5 mM MgCl₂, 2.5 mM MnCl₂, 5 mM DTT, 0.2% Triton X-100, and 0.7% BSA). Homogenates were filtered through gauze, and the filtrates were centrifuged at 10,000 g. The supernatants were used for assays of activity. All enzymes were assayed spectrophotometrically, as described by Ueno et al. (1988) for PEPCase, PPDK, and NAD-ME and as described by Uchino et al. (1995) for Rubisco.

**Carbon-14 Pulse and Carbon-12 Chase Experiment**

Approximately 30 mg of culms was placed in a 20-mL glass tube that contained NaH¹²CO₃ in 25 mM Mes-KOH, pH 6.5. The temperature was maintained at 25°C by immersing the tube in a water bath. The photon irradiance was 500 μmol m⁻² sec⁻¹ at the surface of the tube, and light was provided by metal-halide lamps. After a 15-min preincubation, the segments were transferred to another glass tube that contained NaH¹³CO₃ (0.37 GBq mmol⁻¹) in the same buffer. After a 10-sec pulse of carbon-14, the segments were removed, rinsed quickly in the same buffer, and transferred immediately to a tube that contained NaH¹²CO₃ in the same buffer. At various intervals, segments were removed and stopped by immersion in 80% ethanol at 80°C and then extracted with 80% ethanol at 80°C. Each extract was concentrated in a vacuum and subjected to two-dimensional paper chromatography, as described by Ueno et al. (1988).

**ACKNOWLEDGMENTS**

This study was supported by grants-in-aid from the Ministry of Agriculture, Forestry, and Fisheries of Japan (Enhancement of Center of Excellence) and from the Science and Technology Agency of Japan (Integrated Research Program for the Use of Biotechnological Procedures for Plant Breeding) and from the Science and Technology Agency of Japan (Enhancement of Center of Excellence).

Received December 10, 1997; accepted February 3, 1998.

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Induction of Kranz Anatomy and C₄-like Biochemical Characteristics in a Submerged Amphibious Plant by Abscisic Acid

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*Plant Cell* 1998;10:571-583
DOI 10.1105/tpc.10.4.571

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