Inhibition of Somatic Embryogenesis in Orchardgrass by Endogenous Cytokinin

Communication

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ABSTRACT

Endogenous indoleacetic acid (IAA) and cytokinin concentrations were measured by high performance liquid chromatography in leaf sections of an orchardgrass (Dactylis glomerata L.) genotype which exhibited a high capacity for somatic embryogenesis in vitro and in two genotypes that did not exhibit this capacity. The nonembryogenic genotypes contained 3- to 4-fold higher concentrations of zeatin, zeatin riboside, dihydrozeatin, dihydrozeatin riboside, and total cytokinins than the embryogenic genotype. There were no significant differences in IAA concentrations between genotypes. Cytokinin concentrations between basal and distal sections of embryogenic genotype were not different, but the IAA concentration was significantly greater in basal sections. Somatic embryogenesis was inhibited in the embryogenic genotype by 0.001 micromolar exogenously added zeatin.

Despite recent advances in regeneration of cereals and grasses from protoplasts (1, 5, 15, 22), the development of highly efficient and repeatable regeneration systems remains a major hurdle in the advancement of biotechnological applications, including gene transfer, in these species. Somatic embryogenesis systems developed for Dactylis glomerata L. (orchardgrass or cocksfoot) are among the most advanced currently existing for the Poaceae. These include the formation of embryos directly from mesophyll cells in cultured leaf segments (4, 8) and the full development of somatic embryos in a single liquid medium (6, 7, 19).

Somatic embryogenic capacity in orchardgrass is highly dependent on genotype (9, 13). Genotypic differences for in vitro response, including somatic embryogenesis, have also been reported for other cereal and grass species (2, 11, 18), but these differences have not been related to either endogenous or exogenous PGRs. The requirement for an exogenously added auxin to gramineous cell and tissue cultures has been long established (21); however, the influence of cytokinins is less clear. The objectives of this study were to measure and relate endogenous levels of IAA and cytokinins (Z, DHZ, ZR, DHZR) to the genotypic response for somatic embryogenesis in orchardgrass and to investigate the effect of exogenously added Z to cultured leaf segments of the embryogenic genotype.

MATERIALS AND METHODS

Genotypes of Dactylis glomerata L. used in this study included two clones (designated E1a and E1b) of one that exhibited a very high capacity for somatic embryogenesis (4, 8) and two (designated NE1 and NE2) which exhibited no capacity to produce somatic embryos. Tillers were collected from mature plants grown in an environmentally controlled chamber with a 12 h day/night cycle at 22°/15°C. Individual leaves were separated and basal sections (0–3 cm) of the two innermost (youngest) were lyophilized. The next distal (3–6 cm) sections (nonembryogenic portion) of E1a and E1b were also collected. Plant growth regulators were extracted and purified according to a modification of existing procedures (10, 12) that is outlined in Figure 1. Cytokinins were analyzed by reverse phase HPLC using an octadecylsilica (C18) column with 10% acetonitrile in 0.1 M phosphate buffer (pH 7) and a UV detector (270 nm). Analysis of IAA was by reverse phase ion pair HPLC using a C18 column with 40% methanol in 0.1 M phosphate buffer (pH 6.6) and 0.01 M tetrabutylammonium phosphate with fluorometric detection (excitation 280 nm, emission 360 nm). Peaks were identified by having the same retention times as standard compounds (Sigma) and by enhancement of suspect peaks with these standards.

For the tissue culture experiments, basal sections of the two innermost leaves were split lengthwise along the midvein and surface sterilized as previously described (4). The basal 30 mm portion of each leaf half was cut transversely into six equal segments. Segments from one half of each leaf were plated serially onto solidified SH medium (16) containing 30 μM 3,6-dichloro-o-anisic acid and 0.001, 0.01, or 0.1 μM Z. Corresponding (sister) segments of the other leaf half were explanted onto identical medium but lacking Z. There were 20 or more paired replications for each Z concentration. Incubation was for 4 weeks in the dark at 20°C before transfer to medium without PGRs and culture with a 18 h day/night cycle at 22°/18°C. The number of embryos was estimated as the number of plantlets formed on a plate divided by the embryo germination rate (20).

RESULTS AND DISCUSSION

Mean PGR concentrations for basal (0–3 cm) sections of all plants studied (6 replications each) the next distal 3 cm sections (3 replications each) of E1a and E1b are listed in Table I. Results of linear contrast analyses of these data are presented in Table II. On the average, the nonembryogenic genotypes contained 3.5-fold more endogenous cytokinins than the embryogenic genotype. There were no differences in cytokinin levels between basal and distal sections of the embryogenic genotype; however,
basal leaf sections contained sevenfold more IAA than distal sections.

The gradient for IAA concentration is in agreement with results reported for Pennisetum purpureum (14) and is expected since IAA is synthesized in highly meristematic regions, e.g., basal portions of grass leaves, and is transported basipetally. Although IAA concentration is positively correlated with the gradient response for somatic embryogenesis, it does not appear to be related to genotype differences in orchardgrass. The concentrations in basal portions of embryogenic and nonembryogenic genotypes were not significantly different.

The present results are the first to relate and quantitate the inhibitory effects of endogenous cytokinins to genotype differences for somatic embryogenesis in Poaceae. The finding is supported by results of an experiment in which Z was added exogenously to the medium. Levels as low as 0.001 μM significantly inhibited somatic embryogenesis in the embryogenic genotype (Fig. 2). We had previously shown that exogenously added BA and kinetin inhibited somatic embryogenesis in anther cultures of orchardgrass (17). Cytokinin levels were at least two times higher in nonembryogenic than in embryogenic callus after 10 d culture of Pennisetum purpureum leaf explants (14). The authors suggested that the results may have been largely due to uptake of BA or cytokinins from coconut water. Both BA and coconut water were constituents of their medium. Detached spike culture of wheat (3) removed the explant source from the putative cytokinin source (roots). This technique improved somatic embryogenesis from immature embryo explants of a poor-responding genotype but Z added to the culture medium again suppressed embryogenesis.

The mechanism(s) by which cytokinins regulate or inhibit somatic embryogenesis in the orchardgrass leaf culture system is not understood. However, the discovery of factors influencing genotype differences for somatic embryogenesis in cereals and grasses will aid in our development of improved regeneration

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**Table 1. Mean Values for Cytokinin and IAA Concentrations in Basal, B (0 to 3 cm) Leaf Sections of Two Nonembryogenic (NE2 and NE1) Genotypes and Two Embryogenic Clones (E1a and E1b) of Orchardgrass; PGRs Were Also Measured in the Distal, D (3 to 6 cm) Sections of E1a and E1b**

Cytokinins, ZR, DHZR, Z, and DHZ, were measured by reverse phase HPLC using a C18 column and a UV detector at 270 nm. IAA was measured with fluorometric detection (excitation 280 nm, emission 360 nm).

<table>
<thead>
<tr>
<th>Genotype and Leaf Portion</th>
<th>Mean</th>
<th>ZR</th>
<th>DHZR</th>
<th>Z</th>
<th>DHZ</th>
<th>Total cytokinin</th>
<th>IAA</th>
<th>μg/g dry wt</th>
<th>ng/g dry wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE2 (B)</td>
<td>7.13</td>
<td>0.89</td>
<td>1.81</td>
<td>0.92</td>
<td>10.74</td>
<td>26.25</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>NE1 (B)</td>
<td>4.96</td>
<td>0.88</td>
<td>1.28</td>
<td>0.86</td>
<td>8.06</td>
<td>22.91</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1a (B)</td>
<td>1.05</td>
<td>0.33</td>
<td>0.51</td>
<td>0.19</td>
<td>2.08</td>
<td>18.32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1b (B)</td>
<td>1.74</td>
<td>0.81</td>
<td>0.36</td>
<td>0.08</td>
<td>3.05</td>
<td>20.93</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1a (D)</td>
<td>1.07</td>
<td>1.12</td>
<td>2.15</td>
<td>0.19</td>
<td>4.47</td>
<td>2.98</td>
<td></td>
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</tr>
<tr>
<td>E1b (D)</td>
<td>1.68</td>
<td>0.17</td>
<td>0.17</td>
<td>0.03</td>
<td>2.04</td>
<td>2.66</td>
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</tbody>
</table>

**Fig. 2. Effect of exogenously added zeatin on somatic embryogenesis.** Treatment means designated with an “a” are significantly different from the control at the 0.01 level by a paired t test.

**Table II. Linear Contrast Comparisons of Data Presented in Table I**

<table>
<thead>
<tr>
<th>Contrast</th>
<th>ZR</th>
<th>DHZR</th>
<th>Z</th>
<th>DHZ</th>
<th>Total Cytokinin</th>
<th>IAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE1 (B) versus NE2 (B)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>E1a (B) versus E1b (B)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>E1a (B) + E1b (B) versus NE1 (B) + NE2 (B)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>E1a (B) + E1b (B) versus E1a (D) + E1b (D)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Nonsignificant; * significant at α = 0.01.
systems especially if these factors can be shown to have universal application.

LITERATURE CITED

2. Abe T, Y Fujimura 1986 Genotypic variability for callus formation and plant regeneration in rice (Oryza sativa L.). Theor Appl Genet 73: 3-10