Plant Physiol. (1996) 110: 1069–1079

DNA Sequences That Activate Isocitrate Lyase Gene Expression during Late Embryogenesis and during Postgerminative Growth[1]

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We analyzed DNA sequences that regulate the expression of an isocitrate lyase gene from *Brassica napus* L. during late embryogenesis and during postgerminative growth to determine whether glyoxysomal function is induced by a common mechanism at different developmental stages. β-Glucuronidase constructs were used both in transient expression assays in *B. napus* and in transgenic *Arabidopsis thaliana* to identify the segments of the isocitrate lyase 5′ flanking region that influence promoter activity. DNA sequences that play the principal role in activating the promoter during postgerminative growth are located more than 1200 bp upstream of the gene. Distinct DNA sequences that were sufficient for high-level expression during late embryogenesis but only low-level expression during postgerminative growth were also identified. Other parts of the 5′ flanking region increased promoter activity both in developing seed and in seedlings. We conclude that a combination of elements is involved in regulating the isocitrate lyase gene and that distinct DNA sequences play primary roles in activating the gene in embryos and in seedlings. These findings suggest that different signals contribute to the induction of glyoxysomal function during these two developmental stages. We also showed that some of the constructs were expressed differently in transient expression assays and in transgenic plants.

Glyoxysomes are peroxisomes with a specialized function in lipid mobilization. They contain enzymes of the β-oxidation pathway and the glyoxylate cycle that largely account for the ability of plants to utilize lipids as a carbon source (Trelease and Doman, 1984). These organelles play a prominent role in the metabolism of postgerminative seedlings by converting storage lipids into carbohydrates that serve as carbon and energy sources for the growing seedling (Bridenbach et al., 1967; Huang et al., 1983; Trelease, 1984). Functional glyoxysomes are also present in senescing organs, where they are thought to play a similar role in transforming membrane lipids into carbohydrates that can be translocated to other parts of the plant (Gut and Matile, 1988; De Bellis et al., 1990; De Bellis and Nishimura, 1991; Wanner et al., 1991; Graham et al., 1992). The findings that genes encoding the two glyoxysome-specific enzymes, isocitrate lyase and malate synthase, are active during pollen and seed development as well as during postgerminative growth opens the possibility that peroxisomes present at these stages of the life cycle may have a glyoxysomal function (Pais and Feijo, 1987; Charzynska et al., 1989; Comai et al., 1989; Turley and Trelease, 1990; Zhang et al., 1994). Thus, glyoxysomal enzymes are present at several stages of the plant life cycle.

Although genes encoding the key glyoxylate-cycle enzymes are activated in developing pollen and in maturing seed, the pathway's precise metabolic role(s) at these developmental stages is not known. Understanding the mechanisms that induce these enzymes may provide clues about their physiological functions. For example, the activities of isocitrate lyase and malate synthase genes in bacteria and fungi are regulated by metabolite levels; expression of these genes is repressed by carbohydrates or their derivatives and/or activated by acetate or related metabolites (Kornberg, 1966; Lopez-Boado et al., 1987; Chin et al., 1989; Rua et al., 1989; Sanderman and Hynes, 1989; Tolstorukov et al., 1989). Studies have also shown that isocitrate lyase and malate synthase are similarly regulated by metabolite levels in cultured plant cells and protoplasts (Kudielka et al., 1981; Kudielka and Theimer, 1983a, 1983b; Graham et al., 1994b). Thus, the possibility exists that changes in metabolite levels serve as a common signal that activates the genes encoding the glyoxylate-cycle enzymes in developing seed, seedlings, pollen, and senescing organs.

To define the processes that regulate glyoxysomal function, we have investigated the control of isocitrate lyase gene activity during postgerminative growth, a stage when the enzyme has a well-characterized role in lipid mobilization, and during late embryogenesis, when the enzyme's role in metabolism is not known (reviewed by Olsen and Harada, 1991). Isocitrate lyase gene transcription occurs at a high rate in postgerminative seedlings and at low rates in maturing seed and in developing pollen, but it is not detected in seed at the early stages of embryogenesis or in nonsenescent organs of mature plants (Comai et al., 1989;

[1] This work was supported by grants from the National Science Foundation. C.M.S. was supported in part by a fellowship from the North Atlantic Treaty Organization.

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Abbreviation: Luc, luciferase.

Zhang et al., 1994). The modulations in enzyme activities and protein and mRNA levels that occur over the life cycle are largely, but not solely, controlled by these changes in transcription rates, indicating that isocitrate lyase genes are primarily regulated at the transcriptional level (Comai et al., 1989; Ettinger and Harada, 1990; Zhang et al., 1994). Although there are several isocitrate lyase genes in *Brassica napus* L., we showed that at least one gene is active during seed development, postgerminative growth, and pollen maturation (Zhang et al., 1993, 1994). We also demonstrated that the 3.5 kb of DNA upstream of this gene contains sufficient information to specify its expression at each of these diverse developmental stages (Zhang et al., 1993, 1994). Thus, a single isocitrate lyase gene can be expressed at several stages of the life cycle.

In this paper we address the question of whether glyoxysomal activities are induced by a common mechanism during late embryogenesis and during postgerminative growth by analyzing the DNA sequences that regulate the isocitrate lyase gene at these stages. We show that DNA sequences that play a major role in activating the gene during postgerminative growth are located upstream of position −1200 relative to the gene's transcriptional start site. We also present evidence that a DNA fragment that activates the gene in embryos has only a modest effect on the expression of the gene in seedlings. We conclude that DNA sequences involved in activating isocitrate lyase gene expression in seedlings are distinct from those that are sufficient for high-level expression of the same gene in developing seed. In addition, we discuss our results in comparison with the activities of genes in transient expression assays and in transgenic plants.

MATERIALS AND METHODS

Plant Material

Brassica napus L. (rapid cycling base population, CrGC5) embryos and seedlings were grown as previously described (Harada et al., 1988). *Arabidopsis thaliana* (L.) Heynh plants were grown in a controlled-temperature room at 22°C under continuous light. For the particle-gun experiments, staged *B. napus* embryos at approximately 45 d postanthesis were dissected from their seed coats and placed on modified Monnier embryo culture medium with 12.5% sorbitol, 10 μM ABA, and 0.8% agar (Monnier, 1976). The embryos were used in experiments on the same day. For particle-gun experiments with seedling cotyledons, *B. napus* seeds were sterilized prior to germination. The seeds were first rinsed in 95% ethanol for 2 min, blotted dry with tissues, stirred in 1% sodium hypochlorite containing 1 drop of Tween 20 for 45 min, and rinsed three times with sterile distilled water. The seeds were germinated on plates containing one-half strength MSO medium (2.2 g/L Murashige and Skoog salts [Murashige and Skoog, 1962] and 50 μg/mL *myo*-inositol, 5 μg/mL thiamine HCl, 0.5 μg/mL nicotinic acid, 0.5 μg/mL pyridoxine-HCl [Gamborg et al., 1968]) with 0.8% agar and kept at 25°C in the dark for 3 d. The cotyledons were then excised and placed on one-half strength MSO medium and used for bombardment on the same day. For the leaf-bombardment experiments, the second or third leaves of *B. napus* plants grown in the greenhouse were surface sterilized with 0.1% sodium hypochlorite with 1 drop of Tween 20 for 10 min, rinsed three times with sterile distilled water, and placed with their adaxial surface down on the one-half strength MSO medium. The leaves were either used the same day or left at 25°C overnight before bombardment.

DNA Sequencing

Nucleotide sequences were determined as described previously using both a nested set of deletion constructs and primers generated to specific regions (Zhang et al., 1993). Both strands of the DNA sequence were determined.

Plasmid Construction

The constructs, which are based on the *−3500IL/GUS/IL* fusion gene described previously as *IL/GUS/IL*, were prepared by taking advantage of appropriate restriction sites or fragments generated in exonuclease III experiments (Zhang et al., 1994). The following 5′ deletion constructs are designated by the approximate length of the 5′ flanking region of the *ILA* isocitrate lyase gene from *B. napus*: *−2700IL/GUS/IL*, *−1700IL/GUS/IL*, *−1200IL/GUS/IL*, *−800IL/GUS/IL*, *−590IL/GUS/IL*, *−390IL/GUS/IL*, *−350IL/GUS/IL*, *−330IL/GUS/IL*, *−80IL/GUS/IL*, and *−30MPIL/GUS/IL*. The precise lengths of the 5′ flanking regions are 2801, 1700, 1185, 812, 590, 390, 356, 329, 81, and 30 bp, respectively. Constructs containing internal deletions within the −2700-bp *ILA* 5′ flanking region are designated by the approximate positions of the region removed as follows: *−2700Δ(1200–30)IL/GUS/IL*, *−2700Δ(1200–590)-IL/GUS/IL*, *−2700Δ(590–350)IL/GUS/IL*, and *−2700Δ(350–30)IL/GUS/IL*. Additionally, the gain-of-function construct, *−(590–350)MPIL/GUS/IL*, was prepared by fusing the −590 to the −350 fragment with the *−30MPIL/GUS/IL* gene, which contains the *ILA* minimal promoter. Detailed descriptions of the construction strategies are available upon request.

Particle-Gun Experiments

Transient assays were done with a particle gun (Flowtech Engineering, Alameda, CA). The speed of the microprojectiles was controlled by pushing the macroprojectiles to a specific depth in the gun barrel. The depth (in 0.5-cm increments) that resulted in the highest GUS activity in tobacco suspension culture cells was used for all the experiments (Klein et al., 1988). The procedures for precipitating plasmid DNA on the microprojectiles were as described by Klein et al. (1988). After bombardment, the plant material was incubated at 25°C overnight on the same plates. Leaves were incubated under a 16-h light period, and embryos and seedling cotyledons were incubated in the dark. The plant material was ground in a glass grinder with extraction buffer containing 50 mM KPO_4, 1 mM EDTA, 10 μM β-mercaptoethanol (pH 7.0). For the Luc assay, 20 μL of extract was first mixed with 200 μL of reaction buffer containing 25 mM Tricine, 15 mM $MgCl_2$, 5 mM ATP, 0.5 mg/mL BSA,

pH 7.8, and left at room temperature for 15 min (de Wet et al., 1987). One hundred microliters of 0.5 mM luciferin (Sigma, diluted from a 10 mM stock in 1 mM Tricine, pH 7.8) was injected into the reaction mix, and the amount of light emitted within the first 10 s after luciferin injection was recorded using a Moonlight 2010 luminometer (Analytical Luminescence, San Diego, CA). GUS assays were performed as described by Zhang et al. (1994).

Generation and Analysis of Transgenic Arabidopsis

Arabidopsis was transformed using the root explant procedure described by Valvekens et al. (1985) with the transformation vector GA482 (An, 1987). T_1 seed from T_0 plants (the original transformants) were collected and they were germinated on medium containing kanamycin. The kanamycin-resistant seedlings were transplanted to soil, from which T_2 seeds were collected. Only those transgenic plants that contained at least one copy of the transgene that did not have obvious rearrangements based on DNA gel blot experiments were chosen for further study. GUS assays were done on T_2 dry seed, T_2 seedlings grown for 2 d in the dark after cold-temperature treatment of seeds that had imbibed, and young leaves from T_2 plants grown for 18 d in continuous light. Dry seed and seedlings dissected from their seed coats were stained for GUS activity overnight as described by Zhang et al. (1994). Samples were photographed with a Zeiss SV-II stereomicroscope and Kodak Gold 100 film.

Mann-Whitney *U* Test

Tests were conducted as described by Pollard (1977). Briefly, GUS specific activity values from all lines containing the two constructs being compared were combined and ranked in order of increasing size. *U* values were calculated as follows:

$$U_1 = (n_1 n_2) + (0.5)(n_1)(n_1 + 1) - R_1,$$

$$U_2 = (n_1 n_2) + (0.5)(n_2)(n_2 + 1) - R_2,$$

where R_1 is the sum of the ranks assigned to GUS specific activities from construct 1, whose sample size is n_1, and R_2 is the sum of the ranks assigned to GUS specific activities from construct 2, whose sample size is n_2. The larger of the *U* values is then compared with a small-sample critical-region table.

RESULTS

Identification of DNA Sequences That Activate the Isocitrate Lyase Promoter in Transient Expression Assays

We showed previously that the 3.5-kb 5′ flanking and untranslated region of an isocitrate lyase gene from *B. napus*, *ILA*, is sufficient to control the expression of a reporter gene in transgenic tobacco in a manner similar to that of the endogenous isocitrate lyase genes (Zhang et al., 1994). To identify the DNA sequences in the 5′ flanking region that are needed to activate the isocitrate lyase promoter, we fused different lengths of the *ILA* 5′ flanking region as well as the 5′ untranslated region with the coding region of the *Escherichia coli uidA* gene encoding GUS (Jefferson et al., 1987). These constructs were joined with the *ILA* 3′ untranslated and flanking regions to generate the *IL/GUS/IL* fusion genes. These 5′ deletion constructs, shown diagrammatically in Figure 1, are designated by the approximate lengths of their 5′ flanking region appended to the name of the fusion gene. For example, the *−3500IL/GUS/IL* gene contains approximately 3500 bp of the *ILA* 5′ flanking region attached to the *GUS* reporter gene. The exact sizes of the 5′ flanking regions are given in "Materials and Methods."

We initially used transient assays, in which the fusion genes were delivered into *B. napus* tissues with a particle gun, as a rapid means to assess the consequences of altering the 5′ flanking region. For these experiments, plasmids containing the isocitrate lyase fusion genes were co-precipitated onto tungsten microprojectiles along with a firefly *Luc* gene that was fused with the 35S promoter (Fromm et al., 1986; de Wet et al., 1987). Because this 35S promoter is active in virtually all tissues of the plant, Luc activity serves as an internal control of the bombardment efficiency. The ratios of GUS and Luc activities, measured 16 h after bombardment, represent the normalized activities of the isocitrate lyase promoters. Thus, the deletion of a positively or negatively acting DNA sequence should result in a decrease or increase in the activity ratio, respectively.

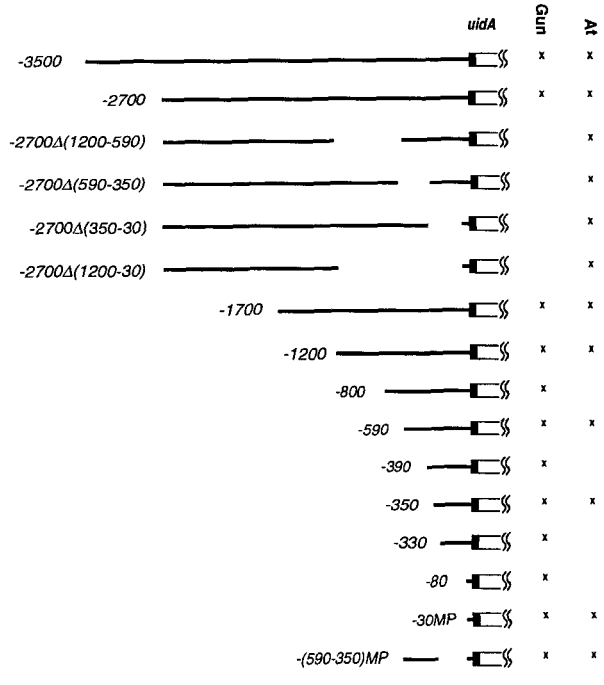

Figure 1. Isocitrate lyase-GUS fusion gene constructs. Diagrammatic representation of the *IL/GUS/IL* constructs used in the transient expression assays and in the transgenic plant experiments are shown. Filled and open rectangles represent the 5′ untranslated region of the *ILA* gene and a portion of the *uidA* gene encoding GUS, respectively. Lines indicate regions from the isocitrate lyase 5′ flanking regions. Construct designations are given to the left of the constructs. Gun and At indicate the constructs used in the transient expression assays in *Brassica* and the transgenic Arabidopsis experiments, respectively.

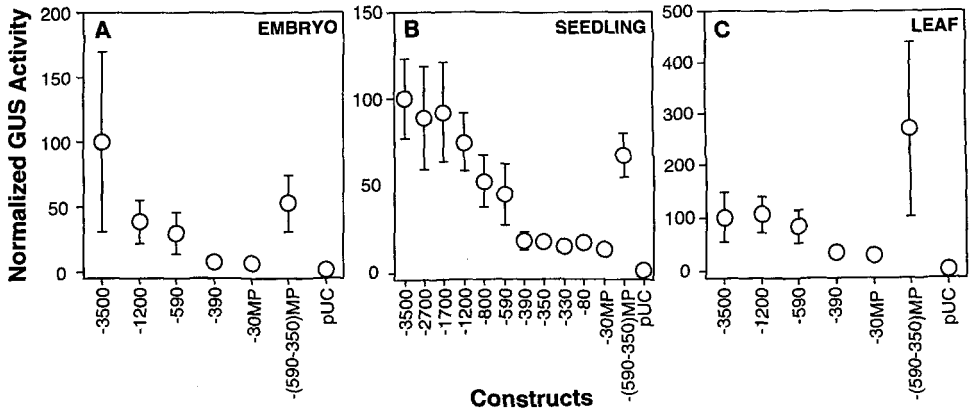

Figure 2. Analysis of the isocitrate lyase gene 5′ flanking region in transient expression assays. GUS and Luc activities were measured in extracts from embryos (A), seedling cotyledons (B), and leaves (C) of *B. napus* that were bombarded with the *IL/GUS/IL* fusion genes. Constructs are indicated by the approximate lengths of their 5′ flanking regions with the exception of the *−(590–350)MPIL/GUS/IL* gene, which is abbreviated as *−(590–350)MP*. GUS/Luc activity ratios were calculated, and the mean values were divided by the value obtained with the *−3500IL/GUS/IL* gene and multiplied by 100 to normalize for variations between experiments. The mean normalized ratios and SE values are shown. The number of bombardments that were averaged to obtain the embryo, seedling, and leaf values were 3, 3 to 24, and 3 to 6, respectively. Average GUS activity obtained with the *−3500IL/GUS/IL* gene in embryos, seedlings, and leaves were 4,040, 15,500, and 1,130 nmol h^{-1}, respectively. GUS activity obtained with a positive control for the leaf bombardment experiments, a *35S/GUS/NOS* gene, averaged 7,500 nmol h^{-1}. Background GUS activity obtained with bombardment of the pUC plasmid DNA averaged 160, 90, and 30 nmol h^{-1}, respectively, in embryos, seedlings, and leaves.

We first tested the activities of the 5′ deletion constructs in the cotyledons of *B. napus* seedlings grown for 3 d after the start of imbibition. Figure 2B shows that sequential reductions in the length of the *ILA* 5′ flanking region between −3500 and −390 resulted in successive decreases in the GUS/Luc activity ratios, suggesting that DNA sequences that positively activate the gene in seedlings are located throughout this region. Removal of the −590 to −390 region reduced promoter activity to between 33 and 50% of that obtained with the *−590IL/GUS/IL* gene, whereas further deletions downstream of −390 did not affect the promoter activity appreciably. These experiments identified the region between −590 and −390 as the DNA sequences closest to the gene that activated the promoter in bombarded seedlings.

To investigate whether the activity of the *ILA* promoter in embryos is controlled by the same DNA sequences that control its activity in seedlings, the deletion constructs were also bombarded into embryos isolated at approximately 45 d postanthesis. To maintain embryonic development in culture and to prevent embryos from germinating precociously, the bombarded embryos were cultured on medium containing a high concentration of osmoticum and ABA (see "Materials and Methods"; Finkelstein et al., 1985; Finkelstein and Crouch, 1986). Comparison of Figure 2, A and B, shows that the deletion of specific sequences flanking the *ILA* gene had similar effects on the GUS/Luc activity ratios in embryos and seedlings. As observed with seedlings, deletion of the −590 to −390 region decreased the normalized activities in bombarded embryos to approximately 25% of the value obtained with the *−590IL/GUS/IL* gene, whereas the removal of sequences downstream of −390 did not cause further changes in the activity ratios. This result suggested that the same DNA sequences are involved in regulating the gene in both embryos and seedlings.

The isocitrate lyase promoter is not normally active in mature organs but, as shown in Figure 2C, we were able to detect fusion gene activity in bombarded leaves. It is possible that these measurable ratios represented residual pro-

Table I. *Pair-wise comparisons of fusion gene activities in transgenic Arabidopsis using the Mann-Whitney U test (5% level)*

Construct	Seed	Seedling
−3500IL/GUS/IL versus *−2700IL/GUS/IL*	Different	Same
−2700IL/GUS/IL versus *−1700IL/GUS/IL*	Different	Different
−1700IL/GUS/IL versus *−1200IL/GUS/IL*	Different	Different
−1200IL/GUS/IL versus *−590IL/GUS/IL*	Different	Different
−2700IL/GUS/IL versus *−2700Δ(1200−590)IL/GUS/IL*	Different	Different
−2700IL/GUS/IL versus *−2700Δ(590−350)IL/GUS/IL*	Different	Same
−2700IL/GUS/IL versus *−2700Δ(350−30)IL/GUS/IL*	Same	Same
−2700IL/GUS/IL versus *−2700Δ(1200−30)IL/GUS/IL*	Different	Same
−(590−350)MPIL/GUS/IL versus *−30MPIL/GUS/IL*	Different	Different

moter activity, although it is impossible to compare directly the GUS/Luc activity ratios in embryos, seedlings, and leaves because the relative bombardment efficiencies of these tissues and the comparative activities of the 35S promoter are not known. Nevertheless, removal of the −590 to −390 region also resulted in a decrease in the GUS/Luc activity ratio. Thus, the region between −590 and −390 contained DNA sequences that activate the gene in embryos, in seedlings, and in leaves.

To determine the sufficiency of this −590 to −390 region, a similar fragment was fused with the gene containing the isocitrate lyase minimal promoter, *−30MPIL/GUS/IL*, to create the *−(590–350)MPIL/GUS/IL* construct (Fig. 1). This *ILA* minimal promoter contains a putative TATA box but lacks the upstream DNA sequences required for its activity. Figure 2 shows that the fusion gene containing this −590 to −350 fragment was active in embryos, seedling cotyledons, and, to our surprise, leaves. We confirmed this result by showing that this fragment activated a different minimal promoter in leaves. The GUS/Luc activity ratios of the 35S minimal promoter (−46 to +8 fragment) fused to the GUS reporter gene was 20, and the activity of this minimal promoter construct fused with the −590 to −350 fragment was 70. One interpretation of these results is that the −590 to −350 fragment contains DNA sequences that are sufficient to activate the minimal promoter constitutively. Implicit in this interpretation is that other sequences in the 5′ flanking region must repress the promoter's activity in leaves.

Localization of DNA Sequences That Activate the Isocitrate Lyase Promoter during Postgerminative Growth of Transgenic Arabidopsis

In parallel experiments to identify the DNA sequences that regulate the *ILA* promoter during late embryogenesis and during postgerminative growth, we initially evaluated the expression of the following fusion genes in transgenic Arabidopsis: *−3500IL/GUS/IL*, *−2700IL/GUS/IL*, *−1700IL/GUS/IL*, *−1200IL/GUS/IL*, *−590IL/GUS/IL*, *−350IL/GUS/IL*, *−30MPIL/GUS/IL*, and *−(590–350)MPIL/GUS/IL* (see Fig. 1). For a given construct, we typically measured GUS specific activities in approximately 10 independent T_2 transformants that each contained at least one copy of the transgene that was not detectably rearranged as determined by DNA gel blot analyses (see "Materials and Methods").

The success of this approach relied on our ability to determine whether there were significant differences between the expression of two constructs in transgenic plants. Uncertainties in this type of analysis can arise because there are large variations in the levels of expression of a single transgene in a population of plants that have been attributed to the position at which a transgene is integrated into the genome (Jones et al., 1985). Because expression levels in independent transformants containing the same transgene typically do not assume a normal distribution, a parametric method of data analysis is not valid (Clarke, 1994). Therefore, we employed a distribution-free statistical test, the Mann-Whitney *U* test, which is based on median values rather than means, to evaluate differences observed in the expression of two gene constructs (Siegal, 1956). A summary of these comparisons indicating differences at the 5% level is shown in Table I.

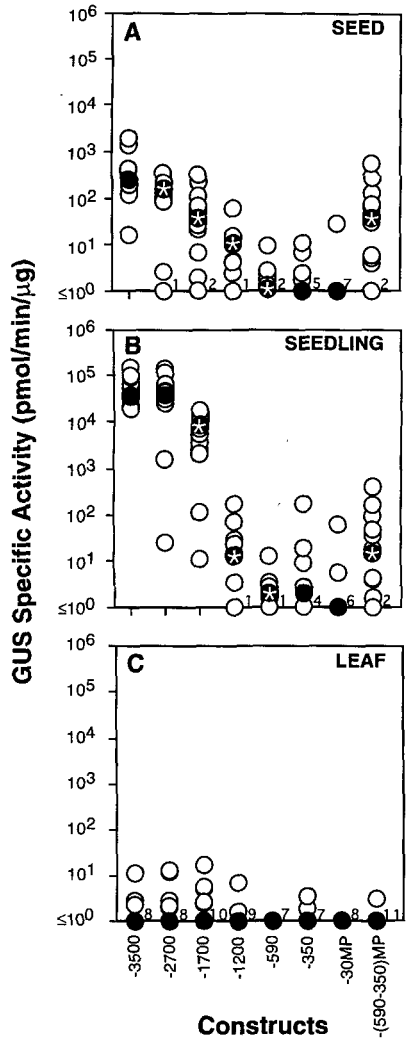

Figure 3. Effects of 5′ deletions of the isocitrate lyase gene 5′ flanking region in transgenic Arabidopsis. GUS specific activities were measured in extracts from dry seed (A), seedlings grown for 2 d (B), and young leaves (C) of plants containing the indicated fusion genes. Constructs are abbreviated as in Figure 2. Each circle represents GUS activity from an independent transformant, and filled circles denote the median value. Filled circles with an asterisk indicate gene constructs whose expression was determined to be significantly different at the 5% level from that of the preceding gene construct (see Table I). Numbers to the right of the circles indicate the number of plant lines without significant GUS activity.

We first broadly localized the DNA sequences that activate the isocitrate lyase promoter in seedlings by measuring GUS specific activities in transgenic plants containing these deletion constructs. The results, summarized in Figure 3B and in Table I, showed that the removal of DNA sequences between −2700 and −1200, primarily the elimination of the −1700 to −1200 fragment, caused the largest decrease in promoter activity. These results also showed that deletion of the −1200 to −590 fragment caused a slight but significant decrease in GUS specific activity and that

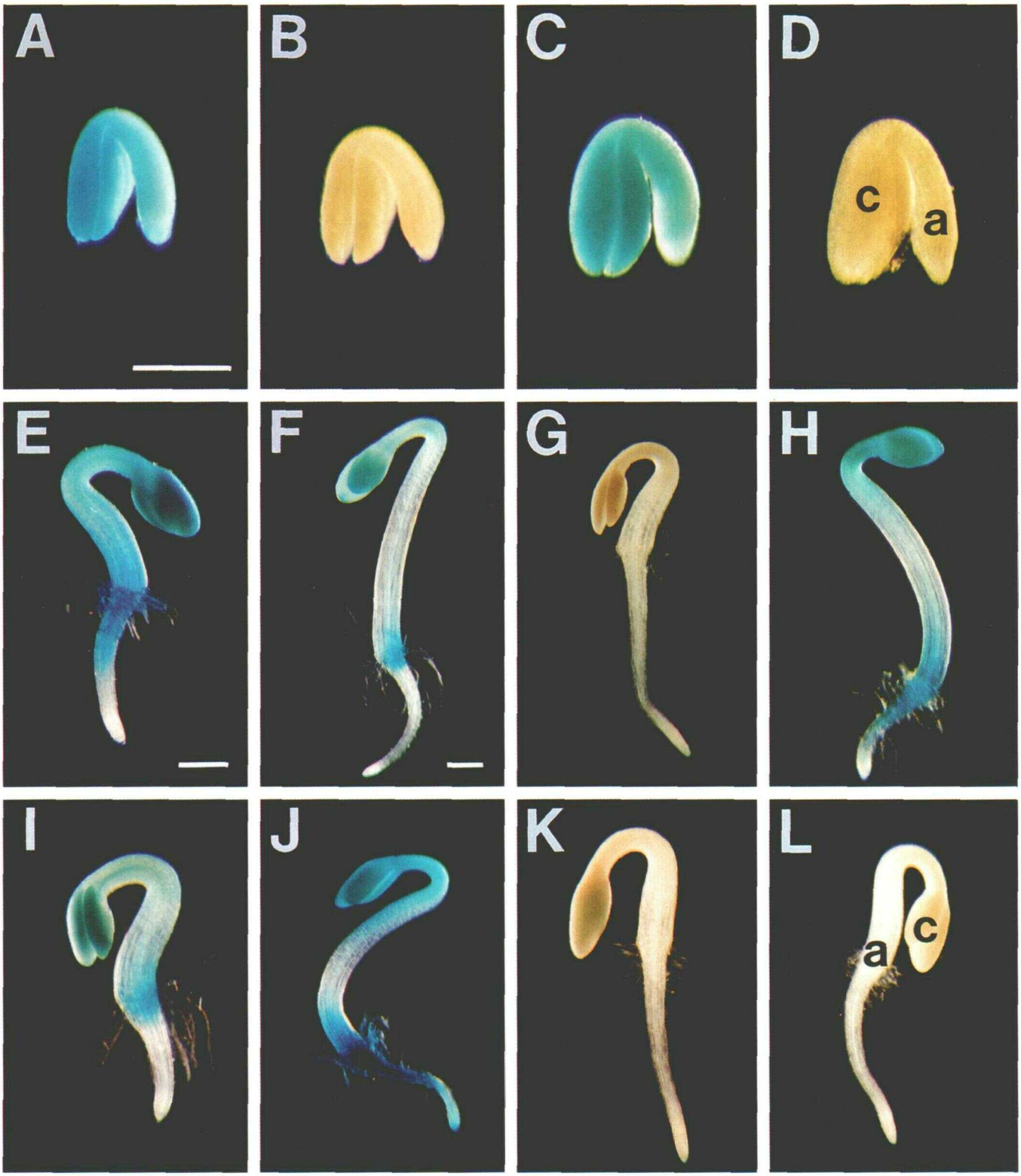

Figure 4. Distribution of GUS activity in mature embryos and seedlings of transgenic Arabidopsis. Panels show representative embryos dissected from dry seed (A–D) and seedlings grown for 2 d in the dark (E–L) that were stained histochemically for GUS activity. The plants contained the following transgenes: *−2700IL/GUS/IL* (A and E); *−2700(590–350)IL/GUS/IL* (B and J); *−(590–350)MPIL/GUS/IL* (C and K); none (not transformed) (D and L); *−1700IL/GUS/IL* (F); *−1200IL/GUS/IL* (G); *−2700Δ(1200–30)IL/GUS/IL* (H); and *−2700Δ(1200–590)IL/GUS/IL* (I). Size bars = 300 μm. The following panels contain photographs at the same magnification: A through D; E, H, and I; and F, G, J, K, and L. a, Axis; c, cotyledon.

there were no significant differences in the activities of seedling populations containing the *−590IL/GUS/IL* gene, the *−350IL/GUS/IL* gene, and the *−30MPIL/GUS/IL* gene. Seedlings from each of the independent transgenic lines containing the *−2700IL/GUS/IL* and the *−1700IL/GUS/IL* genes were stained histochemically to visualize the spatial distribution of GUS activity. For each construct, all seedlings displayed qualitative patterns of staining similar to those shown in Figure 4, E and F, except those from the two lines possessing the lowest GUS activities, which did not stain. Thus, no obvious changes in the spatial distribution of promoter activity resulted from deletion of the −2700 to −1700 fragment. Essentially identical results were obtained with seedlings containing the *−3500IL/GUS/IL* gene (data not shown). By contrast, GUS activity was not consistently detected in these staining experiments in seedlings from six lines containing the *−1200IL/GUS/IL* gene (Fig. 4G), including the line with the highest level of GUS activity as determined in fluorometric assays. We have observed this apparent lack of correspondence between the detection of GUS activity in extracts and in situ (D.L. Laudencia-Chingcuanco and J.J. Harada, unpublished results). The result may indicate that the enzyme is not localized to specific regions but is distributed at a low level throughout the seedling. Seedlings containing the other fusion genes with shorter 5′ flanking regions did not stain, nor did nontransformed seedlings (Fig. 4L; data not shown). These results indicate that the DNA sequences that play a major role in regulating the promoter during postgerminative growth are located upstream of position −1200 and that the −1200 to −30 fragment was not sufficient to confer a high level of fusion gene activity. These results differ from those obtained with the transient assays.

The upstream location of the major positive regulatory sequences compromised our ability to analyze DNA regions downstream of −1200 in transgenic plants using the 5′ deletion constructs. To circumvent this problem, we made the gene constructions shown diagrammatically in Figure 1, in which specific internal regions were removed from the 2700-bp 5′ flanking region. Figure 5B and Table I show that elimination of the −590 to −350 region, as represented by the fusion gene *−2700Δ(590–350)IL/GUS/IL*, did not significantly affect gene activity. By contrast, median values for the expression of the *−2700Δ(1200–590)IL/GUS/IL* and *−2700Δ(350–30)IL/GUS/IL* genes, in which the −1200 to −590 and the −350 to −30 fragments were removed, respectively, differed from that of the gene without the internal deletion, *−2700IL/GUS/IL*, although the statistical test did not find the latter difference to be significant. These results suggest that the −1200 to −590 fragment, and possibly the −590 to −350 fragment, contain DNA sequences that increase promoter activity in seedlings. We also did not observe significant differences in the expression of the *−2700Δ(1200–30)IL/GUS/IL* gene, which lacks the −1200 to −30 fragment, and the *−2700IL/GUS/IL* gene. This apparent discrepancy between the effect of the mutations in the *−2700Δ(1200–30)IL/GUS/IL* and the *−2700Δ(1200–590)IL/GUS/IL* genes may indicate that the distance of the minimal promoter from the upstream activating DNA sequences influences its activity. Regardless of their effect on the levels of fusion gene expression, none of the internal deletions qualitatively affected the distribution of GUS activity in seedlings. The distribution of GUS activity in the transgenic lines that stained is represented by the seedlings shown in Figure 4 that contain the *−2700Δ(1200–30)IL/GUS/IL* (Fig. 4H), *−2700Δ(1200–590)IL/GUS/IL* (Fig. 4I), and *−2700Δ(590–350)IL/GUS/IL* (Fig. 4J) genes. Together, these results suggest that sequences upstream of position −1200 in the *ILA* promoter play the primary role in regulating its activity during postgerminative growth.

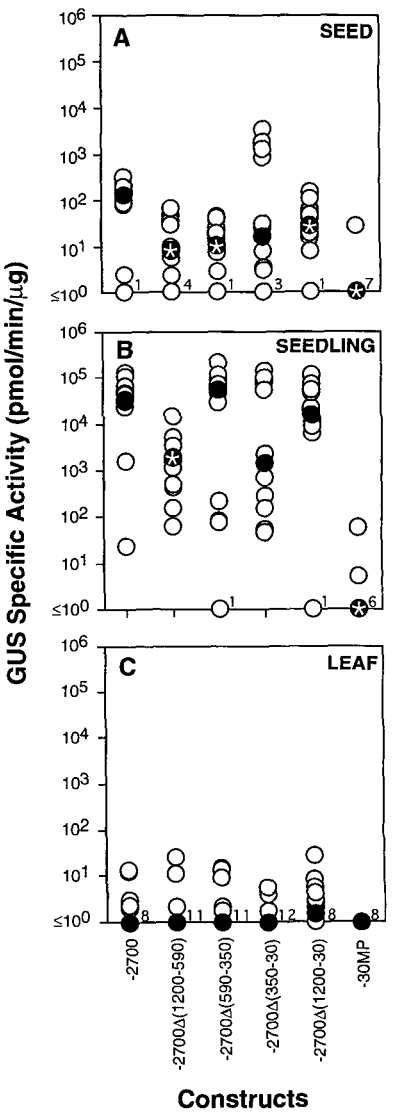

Figure 5. Consequences of internal deletions in the 5′ flanking region in transgenic Arabidopsis. *IL/GUS/IL* fusion genes are designated by the length of the 5′ flanking region and the internal region deleted. GUS specific activities are represented as in Figure 3. Filled circles with the asterisk indicate gene constructs whose expression was determined to be significantly different at the 5% level from that of the *−2700IL/GUS/IL* gene (see Table I). Data for the *−2700IL/GUS/IL* and *−30IL/GUS/IL* genes are taken from Figure 3 and presented for comparison.

Comparison of the DNA Sequences That Activate the Isocitrate Lyase Promoter in Transgenic Embryos and Seedlings

To compare the effects of the mutations in the *ILA* 5′ flanking region on fusion gene expression during late embryogenesis and during postgerminative development, we analyzed GUS activities in dry seed of the transgenic plants. Table I presents the results of the Mann-Whitney *U* tests of the data shown in Figures 3, A and B, and 5, A and B. These analyses indicated that the external 5′ deletion of DNA sequences between −2700 and −1700, between −1700 and −1200, and between −1200 and −590 and the internal deletion of the region between −1200 and −590, caused reductions in fusion gene expression during late embryogenesis and during postgerminative growth that were statistically significant, although, in many cases, the differences are small. The results suggest that the region between −2700 and −590 contains DNA sequences that activate the promoter during both developmental stages. We note that DNA sequences in the −1700 to −1200 fragment have the principal effect on promoter activity in seedlings.

The analysis also identified a DNA region with a primary effect on promoter activity during late embryogenesis. Internal deletion of the −590 to −350 fragment reduced GUS specific activities significantly in developing seed but not in seedlings (Fig. 5, A and B). The statistical test also indicated that elimination of the −3500 to −2700 fragment reduced GUS activity significantly in seed but not in seedlings, although the difference was small. Consistent with the importance of the −590 to −350 region to seed expression was the finding that this fragment, when attached directly with the *ILA* minimal promoter to create the *−(590–350)MPIL/GUS/IL* gene, caused a substantial increase in GUS activity in developing seed and a lesser but significant increase in seedlings (Fig. 3, A and B). Histochemical staining experiments provided in situ evidence consistent with these results. In the *−(590–350)MPIL/GUS/IL* line with the highest GUS activity, we found that embryos from dry seed stained for GUS activity (Fig. 4C) in a pattern similar to that of embryos with the *−2700IL/GUS/IL* gene (Fig. 4A), whereas seedlings from this line stained only weakly in cotyledons (Fig. 4K). GUS activities were highest in dry seed containing the *−(590–350)MPIL/GUS/IL* gene and decreased with time following imbibition (data not shown). The low activity in seedlings may represent residual GUS enzyme synthesized during late embryogenesis. Alternatively, this gene may be expressed at a low level in seedlings. In either case, the results suggest that there are DNA sequences in the −590 to −350 fragment that play a significant role in regulating promoter activity during late embryogenesis.

Measurements of gene activity in nonsenescent leaves were consistent with our previous analyses of isocitrate lyase gene expression; none of the deletion constructs were expressed at significant levels in leaves, including the *−(590–350)MPIL/GUS/IL* gene (Fig. 5C). This result was in contrast to the data obtained in the transient assays.

Table II. *Seedling-to-seed ratios of GUS activities in transgenic Arabidopsis*

Construct	Median[a]	*n*[b]
−3500IL/GUS/IL	120	14
−2700IL/GUS/IL	380	13
−2700Δ(1200−590)IL/GUS/IL	220	9
−2700Δ(590−350)IL/GUS/IL	3900	13
−2700Δ(350−30)IL/GUS/IL	47	12
−2700Δ(1200−30)IL/GUS/IL	730	15
−1700IL/GUS/IL	210	16
−1200IL/GUS/IL	2.8	10
−(590−350)MPIL/GUS/IL	0.74	12

[a] The ratio of GUS specific activities in the seedlings and in the seed was determined for each line containing a given construct. The median ratio is presented. [b] Number of lines analyzed.

Although the Mann-Whitney test provides a useful means to evaluate whether two constructs are expressed differently, it does not indicate the magnitude of the differences. To compensate for this deficiency, we calculated the ratio of GUS specific activities in the seedlings and in the seed of each line containing a given construct to obtain an independent assessment of the effects of these changes in the 5′ flanking region. Table II, which shows the median seedling-to-seed ratios from populations containing each construct, confirmed and extended several of our conclusions. First, these ratios provided additional support for the results of the statistical tests, which showed that the −590 to −350 fragment contains DNA sequences that activate the promoter primarily in embryos, and they provided new information by showing that this region plays a primary role in regulating the activity of the gene during late embryogenesis. Table II and Figure 4 show that elimination of this region in the *−2700Δ(590–350)IL/GUS/IL* gene (Fig. 4, B and J) caused a 10-fold increase in the seedling-to-seed ratio of GUS activities relative to that of the *−2700IL/GUS/IL* gene (Fig. 4I). Additional support for this conclusion is the observation that addition of the −590 to −350 fragment to the *ILA* minimal promoter, *−(590–350)MPIL/GUS/IL*, resulted in a seedling-to-seed ratio that is approximately 0.5% of that obtained with the longest 5′ flanking region, *−3500IL/GUS/IL*. We also note that the external deletion of the −3500 to −2700 fragment, which was judged to cause a significant decrease in GUS activity in seed, caused a 3-fold increase in the seedling-to-seed ratio (Tables I and II). Second, although elimination of the region between −1700 and −1200 caused a statistically significant decrease in GUS activities in seed and in seedlings, this fragment appears to primarily affect promoter activity during postgerminative growth. External deletion of the −1700 to −1200 fragment caused a reduction in the seedling-to-seed ratios to 1% of the value for the *−1700IL/GUS/IL* gene (Table II). Together, the results suggest that at least two different DNA sequences located between −1700 and −1200 and between −590 and −350 in the *ILA* 5′ flanking region play roles in regulating promoter activity during late embryogenesis and during postgerminative growth.

DISCUSSION

Several DNA Sequences Regulate the Isocitrate Lyase Gene during Late Embryogenesis and during Postgerminative Growth

We have asked whether the *ILA* isocitrate lyase gene from *B. napus* is controlled by a common mechanism during postgerminative growth and during late embryogenesis to understand how glyoxysomal function is regulated during the plant life cycle. The function of glyoxysomes in the mobilization of storage lipids in postgerminative seedlings has been characterized extensively, but virtually nothing is known about their metabolic role, if any, in embryos (Bridenbach et al., 1967; Huang et al., 1983). The glyoxylate cycle enzymes may be synthesized in preparation of germination to ensure that fatty acids are rapidly converted to carbohydrates that can then serve as a carbon source for the developing seedling. Another potential role for the enzymes may be in the synthesis of carbohydrates that are needed to stabilize membranes as seeds desiccate during late embryogenesis, although the source of substrates for this pathway in embryos is not clear. Defining the signals that activate genes encoding glyoxysomal enzymes may eventually provide insight into their role during late embryogenesis.

The results of our analyses in transgenic Arabidopsis plants are summarized in Figure 6. Sequences upstream of the *ILA* gene between positions −1700 and −1200 appear to be principally responsible for activating the promoter during postgerminative growth, although these sequences also have a lesser, but statistically significant, positive effect on the promoter during late embryogenesis (Fig. 3; Tables I and II). The dual effects of this −1700 to −1200 region on the promoter's activity in embryos and in seedlings could indicate that there is overlap in the developmental specificity of the positive regulatory sequence or that this relatively large fragment contains distinct embryo-specific and seedling-specific regulatory elements. More precise mutations of this fragment will directly distinguish between these possibilities.

DNA sequences that activate the isocitrate lyase promoter primarily in embryos have been localized between positions −590 and −350 (Figs. 3–5; Tables I and II). The role of this −590 to −350 fragment was verified by showing that it is sufficient to activate the *ILA* minimal promoter during late embryogenesis [*−(590–350)MPIL/GUS/IL* gene; Figs. 3 and 4; Tables I and II]. Because GUS activity persists in dry seed and in seedlings, it is unclear whether this fragment also activates the promoter during postgerminative growth. We also showed that other regions of the *ILA* 5′ flanking region between −2700 and −590 affect promoter activity both in developing seed and in seedlings. Potential explanations for the dual roles of these fragments include the possibilities that each contains discrete DNA sequences that activate the promoter in embryos and in seedlings or that each possesses an element(s) that enhances promoter activity only in the presence of other elements that confer developmental-stage specificity (Fromental et al., 1988; Lam et al., 1990).

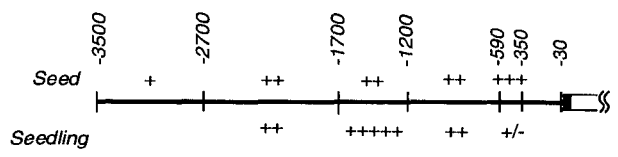

Figure 6. Regulatory DNA sequences in the 5′ flanking region of the ILA isocitrate lyase gene. The horizontal line indicates the 5′ flanking region of the ILA gene. Plus signs represent the relative influence of the indicated fragments on promoter activity in developing seed and seedlings.

The picture that emerges from this study is that the 5′ flanking region of this isocitrate lyase gene comprises a number of elements that act together to regulate the activity of the promoter during several developmental stages. Several regions appear to affect promoter activity in both developing seed and seedlings at a low level, although it is unclear whether these DNA sequences function specifically at these developmental stages. Distinct DNA sequences have been identified that function primarily, but not exclusively, during late embryogenesis and during postgerminative growth. Although more complex interpretations are possible, the results imply that the gene is not regulated equivalently in developing seed and in seedlings, suggesting that distinct physiological signals play roles in activating the gene at different periods of the life cycle.

The relevance of these results to our understanding of the control of glyoxysomal function during plant development concerns the metabolic regulation of isocitrate lyase and malate synthase synthesis. Others have shown that high levels of metabolizable carbohydrates can cause repression in the transcription of these genes in cultured plant cells (Kudielka et al., 1981; Kudielka and Theimer, 1983a, 1983b; Graham et al., 1992, 1994a, 1994b). Because distinct DNA sequences play roles in activating the isocitrate lyase gene in developing seed and seedlings, it seems unlikely that its expression is controlled solely by carbohydrate levels. We do not exclude the possibility that metabolic cues can affect the activity of this gene at either of these stages, as has been shown by others, but suggest that there may be other signals unrelated to the metabolic regulatory system uncovered in cultured cells that are involved in regulating this isocitrate lyase gene during late embryogenesis and during postgerminative growth (Lado et al., 1968; Longo and Longo, 1970).

Comparison of Promoter Activities in a Transient Expression System and in Transgenic Plants

Because transient assays offer a rapid means to analyze the expression of gene constructs, we were interested to determine whether the results obtained were comparable to those generated using transgenic plants. Our results showed that some gene constructs were expressed similarly in transient assays and in transgenic plants and that there were differences in the relative expression of others. Examples of similar results obtained with both assay systems were that the *−(590–350)MPIL/GUS/IL* gene was expressed at significant levels both in bombarded embryos and seedlings and in transgenic seed and seedlings, and

that there was a general decline in fusion gene activity with decreasing length of the 5′ flanking region between positions −1700 and −590. However, two conspicuous differences were noted in the activities of the gene constructs in the two assays. First, the median GUS specific activities of transgenic seedlings and of transgenic seed containing the *−3500IL/GUS/IL* gene were approximately 1800-fold and 50-fold higher, respectively, than the median activities of plants containing the *−1200IL/GUS/IL* gene (Fig. 3). By contrast, transient expression of the same gene constructs in bombarded *B. napus* seedlings and embryos exhibited only a 2- and a 2.5-fold difference, respectively (Fig. 2). Second, in the transient expression system, the *−(590–350)MPIL/GUS/IL* gene was expressed in bombarded but not transgenic leaves (Figs. 2 and 3).

There are several potential explanations for the differences in the relative expression of constructs in the two assay systems. First, the possibility exists that the *ILA* gene is regulated differently in Arabidopsis and *B. napus*, although we think that this is unlikely, in part because both plants are members of the Brassicaceae family. Furthermore, we previously showed that the temporal and spatial expression of the *−3500IL/GUS/IL* gene in transgenic tobacco were similar to those of the endogenous gene in *B. napus* even though tobacco is not as closely related to *B. napus* as is Arabidopsis (Zhang et al., 1994). Second, the large amount of plasmid DNA introduced into plant cells in the transient assays could create conditions that are not physiologically relevant, possibly by titrating out transcription factors. These conditions could potentially affect the expression of the bombarded genes. Third, others who have observed similar discrepancies in expression in transient assays and in transgenic plants have speculated that the lack of chromosomal integration accounts for these differences (Frisch et al., 1995). Fourth, the treatments used for the particle-gun experiments may wound and stress the plant tissues. These conditions may cause alterations in the *ILA* promoter's activity, because studies have shown that the isocitrate lyase gene is activated during organ senescence. We have been unable to induce the activity of this promoter in detached cultured leaves of transgenic plants (Gut and Matile, 1988; De Bellis et al., 1990; De Bellis and Nishimura, 1991; Graham et al., 1992; J.Z. Zhang, D.L. Laudencia-Chingcuanco, and J.J. Harada, unpublished results). Although others have found such transient assays to be reliable methods to measure gene activities, we have relied on the data obtained from the analyses of transgenic plants to study the isocitrate lyase 5′ flanking region because of the potential problems in interpreting the results of the bombardment experiments (Klein et al., 1988, 1989; Bruce et al., 1989; Goff et al., 1990; Rolfe and Tobin, 1991; Twell et al., 1991).

The analysis of data obtained in transgenic plant assays can also be problematic. Although gene-transfer studies provide the opportunity to determine the consequences of foreign gene expression in plants, it has often been difficult to draw quantitative conclusions about transgene activity. Large variation in expression levels obtained in independent transformants containing the same gene construct are frequently observed (see Figs. 3 and 5). These variations have often been attributed to the site at which the transgene is integrated into the genome (Jones et al., 1987). To address this problem, we have used the nonparametric Mann-Whitney *U* test as a means to determine whether there are significant differences in the expression of two gene constructs (Siegal, 1956). This test appears to provide an effective method to assess data from transgenic plant experiments. In some cases, comparisons judged to be statistically different were not obvious from an examination of the data. However, in at least one case an independent treatment of the data provided support for the results of the test. Specifically, the statistical test indicated that elimination of the −3500 to −2700 fragment caused a significant decrease in GUS activity in seed but had no significant effect on GUS activity in seedlings (Fig. 3; Table I). The finding that the seedling-to-seed ratio increased 3-fold supports this conclusion (Table II).

ACKNOWLEDGMENTS

We thank the members of the Harada Lab and Vicki Chandler for their critical comments about the manuscript. We also thank Kelly Matsudaira Yee, Jay Danao, Greg Hartt, Jennifer Hsu, and Nhu Pham for excellent technical assistance, and Judy Callis for the use of her luminometer. Use of the Plant Cell Biology Training Facility at the University of California, Davis, is also acknowledged.

Received August 17, 1995; accepted December 13, 1995.

The accession number for the sequence reported in this article (the 5′ flanking region of the *ILA* gene to position −2801) is U41321.

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