Hormonal and Environmental Regulation of the Carrot 
lea-Class Gene Dc3

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ABSTRACT

Dc3 is a carrot lea-class gene belonging to a small gene family that encodes Dc3 and Dc3-like RNA sequences. We have examined the responsiveness to water deficit and abscisic acid (ABA) of the promoter/enhancer complex of Dc3 fused to a β-glucuronidase (GUS) reporter gene in vegetative cells of transgenic tobacco. In 56-d tobacco, GUS expression in leaves increased about 200-fold during a 3-d drying cycle, during which there were small decreases (3 atmospheres or less) in leaf water potential and a 16-fold increase in free ABA. These effects were reversed by rewatering. Changes in GUS activity were closely paralleled by changes in GUS transcript levels during the desiccation/watering cycle, indicating transcriptional regulation of GUS gene expression. The Dc3 promoter responds to exogenous ABA; the effect is time and concentration dependent, with greater than 10-fold induction in 8 h with 10 μM ABA. Histochemical visualization of GUS activity in seedlings induced by water deficit or exogenous ABA revealed Dc3-driven GUS expression in all organs of transgenic tobacco seedlings. We suggest that the Dc3/GUS reporter system is a sensitive analytical tool to study various environmental effects on plant growth and development.

Overall patterns of plant growth and development are directed by two classes of regulatory signals: (a) ontogenic signals that are programmed by the genome and that act early during embryogenesis and throughout development to establish the fundamental morphogenetic patterns, and (b) environmental signals that provide external cues for the continued action of the ontogenic signals throughout the life cycle of the plant. Although the precise mechanism by which these two classes of regulatory signals act is not clear, plant growth regulators must play an important role in integrating these two classes of regulatory cues. ABA is strongly implicated in the signal transduction pathway controlling some classes of plant genes in response to changes in water potential and in response to selected developmental cues, but the mechanism is unknown. ABA accumulates in vegetative cells of higher plants in response to water deficit, and it is thought to act as a signal for the initiation of processes involved in adaptation to this and other environmental stresses (1, 3, 26, and references therein).

In addition, ABA appears to function during "normal" plant development and growth, regulating expression of specific genes in embryos of rape, cotton, soybean, wheat, rice, barley, and maize, among others (reviewed in ref. 22; also see ref. 10). Cotton embryos that are exposed to exogenous ABA accumulate quantities of late embry-abundant (lea) mRNAs (9). Dure et al. (8) speculated that phylogenetically conserved lea peptide domains functioned to protect cellular structures during seed desiccation. Genes encoding lea or lea-like polypeptides were characterized based on their relative sequence similarity, separating them into three major groups. Group I includes the wheat Em gene; group II includes the rice RAB21 gene; group III includes the carrot Dc3 and Dc8 genes and the barley phVal gene (8).

The expression patterns of lea-class genes are complex, and the relationship between individual members of the preceding structural groups and their induction by ABA and water deficit per se and their "normal" expression during embryogenesis is unresolved. Exposure of nonembryonic rice cells to exogenous ABA induces an endogenous lea-like gene RAB21 (19), and a GUS reporter gene is expressed under the control of 5' upstream elements of a wheat lea-like gene (Em) in ABA-treated rice protoplasts following DNA transformation (18). In the case of the rice RAB21 gene, water and salt stress also were shown to induce expression. Similarly, in barley aleurone cells, exogenous ABA or water deficit stimulates the expression of lea-like mRNAs (4, 13). However, not all lea-like genes are inducible in vegetative cells; another carrot embryonic gene, Dc8, is not inducible in nonembryonic cells by desiccation or ABA (12).

We have examined the relationship between ontogenic and environmental signals in plant growth and development, focusing primarily on the carrot gene Dc3. Dc3 is a group III lea-class gene (8) belonging to a small gene family that encodes Dc3 and Dc3-like RNA sequences (24). Analysis of Dc3 expression during initial phases of induction of embryogenic carrot cultures and in nonembryogenic mutant carrot cell lines indicated that expression of Dc3 is a useful molecular marker for the acquisition of embryogenic potential in plant cell cultures (6, 25). Seffens (23) showed that members of the Dc3 gene family are divergent within the species, but that elements of the family are phylogenetically conserved in both dicots and monocots. Similarities with lea and lea-like genes suggest a role for Dc3 in seed desiccation and more generally in water stress response (8, 24). Analysis of transgenic tobacco

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2 Abbreviations: GUS, β-glucuronidase; DPI, days postimbibition; 4-MU, 4-methylumbelliferone.
containing a GUS reporter gene (15) fused to a 1.5-kb 5′ upstream element of Dc3 defined a promoter/enhancer complex that confers developmentally regulated expression of GUS activity in developing tobacco seeds and environmentally responsive expression in nonembryonic tobacco tissues mediated by ABA (24). Here, we examine in detail the response of Dc3 promoter GUS constructs to changes in plant water potential and to exogenous ABA in vegetative cells of transgenic tobacco.

MATERIALS AND METHODS

Plant Material

Transgenic tobacco containing the promoter region of the carrot gene Dc3, fused to a GUS reporter gene, was produced as described (24). Briefly, plasmids constructed by ligation of a 1.5-kb 5′ upstream fragment of the carrot gene Dc3 to the GUS reporter cassette in pH101 (15) were conjugated into Agrobacterium tumefaciens and used to transform tobacco leaf disks. Transformed shoots and subsequently rooted plantlets were selected on kanamycin medium (2). Putative transformed plants were confirmed by determination of neomycin phosphotransferase II expression, and transgenic plants were transferred to soil.

Transgenic tobacco seeds were surfaced sterilized with 5% (v/v) Chlorox bleach and washed with sterile distilled water several times. Germinating seeds were selected on 400 μg/mL kanamycin and transferred to 3.5-L pots of soil. Growth was in a controlled environment room (Conviron) at 24°C with a 12-h light period, PPFD of 600 μmol photons m⁻²s⁻¹, and RH = 75 to 85%.

Twelve-DPI seedlings were germinated and grown on kanamycin and were transferred to basal media (Murashige and Skoog salts [21], 0.8% inositol, 10 μg/mL thiamine-HCl containing 0, 10, and 100 μM ABA, and GUS activity was determined 1, 2, 3, and 4 d after transfer. Seedlings (12 DPI) were desiccated on sterile Whatman No. 3 filter paper over night at room temperature. Leaf disks of transgenic tobacco plants were taken from a single leaf and were floated on basal media containing 0, 10, and 100 μM or 1 mM ABA for variable times, and triplicate samples were subsequently removed for GUS determination.

Desiccation Treatment and Measurement of Leaf Water Potential

Uniformly sized 56-DPI plants were subjected to desiccation by withholding water. Control plants were irrigated six times a day with 150 mL of water (a total of 900 mL); partially stressed plants received 50 mL of water six times daily (a total of 300 mL); fully stressed plants received no water. There were three independent replications for each treatment. At the end of the 4th d of desiccation, recovery from stress was initiated by rewatering all plants at the control level.

Throughout the experimental period, leaf disks (5 mm diameter) were punched from two selected leaves of each plant, one leaf being a mature, fully expanded leaf, and the other (inserted two or three leaves above it on the stem) almost fully expanded. For each leaf, beginning at the apical end, six disks were cut using a paper hole punch. Three were punched from one side of the main vein (mid-rib) between the lateral veins and placed in sterile microcentrifuge tubes on ice for subsequent determination of GUS and protein. Three further disks were punched from the opposite side of the main vein and immediately placed in a Wescor C-52 sample chamber and sealed. On succeeding days, sets of leaf disks were cut from opposite sides of the main vein at a location closer to the petiole. The average dimensions of the older leaf were 380 mm long × 200 mm broad, and for the younger one, 300 mm × 130 mm. The leaf disks, therefore, constituted a small fraction of the total leaf area. The area surrounding the punched hole did not desiccate or show any sign of abnormality. Water potential measurements were made with Wescor C-52 thermocouple psychrometers and a microvoltmeter after 60 min of equilibration time. A cooling current was passed for 7 s and the water potential determined from the microvolt reading in transient mode with all readings corrected to 25°C. The se expressed as a percent of the mean water potential (n = 3) did not exceed 16% for all measurements.

GUS Assays

Fluorometric analysis of GUS was as described (15) using leaf discs homogenized in lysis buffer containing 50 mM sodium phosphate (pH 7.0), 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% Triton X-100, and 0.1% sarkosyl. The homogenate was centrifuged at 14,000 rpm in a microfuge for 15 min and the supernatant was used for GUS assay. For fluorometric reactions, 100 μL of supernatant was used. Duplicate reactions were started by addition of 10 μL of 1 mM 4-methylumbelliferyl glucuronide (Research Organics, Inc., Cleveland, OH) and incubated at 37°C (a total of 200 μL). One reaction was terminated at time zero and the second at 60 min with the addition of 800 μL of 0.2 M Na₂CO₃ to each reaction. Fluorescence of the product 4-MU was then measured in a Hoefffer TKO-100 minifluorometer as described by Jefferson (14). All fluorescence values were corrected for background fluorescence by subtracting the zero time value from the corresponding 60-min value. Protein determination was by the Bradford method (Bio-Rad). The se expressed as percent of the mean GUS value (n = 3) did not exceed 30% for all GUS measurements.

Histochemical GUS analysis was essentially as described by Jefferson (14). Seedlings selected on 400 μg/mL kanamycin were used at 12 DPI. One batch of seedlings was treated with 10 μM ABA overnight, and the other batch was desiccated at room temperature overnight on sterile Whatman No. 3 filter paper. The following day, the seedlings were immersed in a histochemical reaction mixture containing 5-bromo-4-chloro-3-indolyl-β-D glucuronic acid (X-Gluc, Research Organics, Inc) in 50 mM sodium phosphate buffer (pH 7.0). The histochemical reaction, carried out in the dark at 37°C overnight, was stopped by washing the samples several times with the same buffer without X-Gluc. To allow better visualization of stained tissue, samples were cleared with an ethanol series and mounted in 80% glycerol for photomicrography.

Endogenous ABA Determinations

Leaf samples were collected from Dc3 transgenic tobacco plants during desiccation treatment, frozen immediately in
liquid nitrogen, and lyophilized. The extraction and determination were by GLC as described by Creelman et al. (5). An internal standard of \(^3\)H(\pm)-ABA was added for the determination of ABA recovery.

**RNA Gel Blot Analysis**

During the stress and recovery treatments, leaf tissue was collected and frozen in liquid nitrogen for later analysis. To isolate total RNA, leaf tissue was ground in liquid nitrogen using mortar and pestle, homogenized in buffer containing 0.1 M Tris-HCl, pH 9.0, 0.1 M NaCl, 0.001 M EDTA, and 0.5% SDS using a polytron homogenizer, and was extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol. The supernatant from the phenol extraction was precipitated first with lithium chloride and potassium acetate and then with ethanol. Twenty micrograms of total RNA from each sample was resolved by electrophoresis on 1.2% agarose formaldehyde gels as described by Maniatis et al. (17). RNA was transferred to nylon membranes (Gene Screen, New England Nuclear DuPont) according to the manufacturer’s specifications and was subsequently hybridized at 42°C in 5 × SSC, 50% formamide, 1 × Denhardt’s solution, and 10% dextran sulfate overnight using a \(^32\)P-labeled 1.8-kb GUS fragment as probe.

**RESULTS**

Fluorometric and histochemical methods were used to analyze Dc3-driven GUS expression in transgenic tobacco in response to desiccation and to exogenous ABA. In well-watered tobacco, with midday leaf water potentials (\(\psi_l\)) in the range of −1 to −2 atm (Fig. 1A), Dc3-driven GUS expression was at a low, basal level. When leaves were fully stressed (Fig. 1B) by withholding water, \(\psi_l\) declined to −4 atm at 2 d, and GUS expression rose appreciably. At day 4, \(\psi_l\) had declined below −5 atm, and GUS expression had increased some 200-fold above the basal level. With one-third watering (Fig. 1C), \(\psi_l\) remained at or a little below −2 atm, but GUS expression was strongly enhanced, reaching about half that of the fully stressed leaves at 4 d. Rewatering began after sampling on day 4, and by day 6, \(\psi_l\) had returned to control level, whereas GUS expression did not return to the low, basal level until day 9, at least 3 d after the restoration of \(\psi_l\). Essentially identical responses to desiccation were found in the younger and older leaves. Results for only the former are presented here. During desiccation (full stress) treatments, endogenous free ABA in the leaves rose steeply above the initial control level at 1 d (Fig. 2), and at day 3, reached an apparent maximum some 16-fold above the initial level.

To determine whether application of ABA alone could induce Dc3-driven GUS expression, transgenic tobacco leaf disks were floated on basal media of different ABA concentrations and periodically sampled for GUS expression. All concentrations of ABA induced detectable GUS expression at 2 h (Fig. 3). From 3 h onward, ABA induction of GUS activity was time and concentration dependent, with GUS expression increasing approximately linearly with time of contact with ABA. The lowest concentration of ABA, 10 \(\mu\)M, was sufficient to increase GUS expression at least 10-fold at 8 h. The apparent lag phase of approximately 2 h is reproducible and may be attributable to the time required for ABA uptake and subsequent metabolic events.

The distribution of GUS expression in 12-DPI seedlings of tobacco was visualized by immersing them in the histochemical stain X-Gluc. Either prior desiccation by withholding water or exposure to exogenous ABA for 15 h induced expression in roots, stems, and leaves, with intense deposition of stain over the leaf veins (Fig. 4). Quantification of GUS expression for tobacco seedlings by assay of the fluorometric product 4-MU (Fig. 5) showed a response similar to that of excised leaf disks (Fig. 3), i.e. a sensitivity to exogenous ABA that was concentration dependent. Likewise, desiccation increased GUS expression in the entire seedling to a similar.

**Figure 1.** Changes in leaf water potential and Dc3-driven GUS expression in transgenic tobacco during water deficit and recovery. Plants were 56 DPI at the initiation of the treatments. The values apply to the younger of two leaves sampled, but comparable results (not shown) were obtained for older leaves. Values are means of three determinations, se expressed as a percentage of the mean did not exceed 16 and 30% for water potential and GUS measurements, respectively. \(\bullet\) Water potential; \(\square\) GUS. The abscissa represents the time in days since treatment began. Vertical arrows represent the time rewatering was initiated.
degree as that induced by exogenous ABA; the precipitous drop in GUS activity at day 4 in desiccated leaf disks is probably a result of proteolysis following desiccation-induced cell death.

In a separate experiment to examine whether the environmental induction of the Dc3-driven GUS reporter was at least in part at the level of transcription, GUS mRNA levels were determined during desiccation and recovery of transgenic tobacco containing the Dc3/GUS reporter. Following treatment, leaf tissue was collected and total RNA was isolated and subjected to northern blot analysis following electrophoresis. Autoradiographs were analyzed by densitometry, and the relative intensity of hybridization with a 32P-labeled 1.8-kb GUS fragment was expressed as a percent of the maximum obtained at 4 d before rewetting began (Fig. 6). There were no detectable GUS transcripts on day zero (not shown). At 1 d of the full water-stress treatment, GUS transcript levels reached 50% of the maximum and then rose less rapidly. During recovery (Fig. 6B), GUS mRNA levels had declined by 8 h following rewetting, but decayed slowly with appreciable levels still at 3 and 4 d of recovery. Comparison between Figures 1 and 6 is tentative because the plants were not treated in the same experimental run; however, it is apparent that GUS activity closely parallels the levels of transcripts both during stress and recovery.

**DISCUSSION**

Plant development is directed by genomically encoded ontogenic signals; however, as plant development and growth continue, environmental cues become increasingly important as modulators of plant developmental programs. In many cases, plant hormones play a central role in the integration of ontogenic and environmental cues. ABA, for example, is highly pleiotropic in its effects, ranging from mediating changes in gene expression in response to lower water potential inhibiting precocious germination (26). To develop tools to distinguish between environmental and ontogenic cues signaled by ABA, we have utilized the GUS reporter gene driven by the promoter of the carrot *lea*-class gene *Dc3* to examine the regulation of this gene by ABA and environmental signals, specifically water deficit. Previously, we demonstrated that in transgenic tobacco, this promoter is responsive to developmental cues and to exogenous ABA (24). Here we demonstrate that the *Dc3* promoter is exquisitely sensitive to exogenous ABA; the effect is time and concentration dependent. However, we cannot be certain that the *Dc3* promoter responds only to ABA during desiccation. For example, Bray (3) noted that in tomato leaves, desiccation-induced ABA clearly induced certain genes, whereas other genes appeared not to be induced by ABA, but by other aspects of leaf desiccation. ABA or desiccation induction of *Dc3*-driven GUS expression occurs in all seedling and mature plant organs examined, with relatively equivalent levels of GUS activity in seedling leaves and roots (Fig. 4).

The relationship between direct induction of *lea*-type genes, like *Dc3*, by ABA, their induction by water deficit, and their normal expression during embryogenesis is still unresolved. However, at the transcriptional level, the response to ABA appears to be mediated by DNA sequences that include elements of the core consensus sequence, CACGTGGC (11, 16, 20). Deletion analysis of the 5' upstream region of *Dc3*/GUS fusions suggests the presence of one or more discrete ABA-responsive cis regulatory sequences, including elements of the preceding core consensus sequence as well as a promoter proximal region (~145, +23) that specifies ABA-independent expression in transgenic tobacco embryos (R. Derose, H. Fu, T.L. Thomas, unpublished results). Interestingly, congeners of this core sequence, sometimes referred to as the G-box, are present in the upstream regulatory sequences of a number of diverse plant genes, including various seed and embryo genes, other stress-induced genes, and various light-regulated genes (7, 11 and references therein).

The results reported here suggest that the *Dc3* GUS reporter gene utilized in this study is extraordinarily sensitive to apparently small changes in plant water status. This is dra-
matically demonstrated in Figure 1C, where only a minor change in leaf water potential without any outward appearance of water deficit of partially stressed plants is accompanied by more than a 10-fold increase in GUS expression. Thus, it seems likely that the Dc3 GUS reporter system 'detects' subtle changes in water potential, presumably transduced by fluctuations in ABA levels, that are apparently below the sensitivity threshold of more conventional analytical approaches. It is unlikely, however, that Dc3-driven GUS expression in our experiments will be related to \( \psi_L \) alone. Experiments with divided root systems ('split roots') have shown that ABA produced by the desiccating part of the root system can translocate to leaves and there induce stomatal closure without concomitant lowering of \( \psi_L \) because of an uninterrupted supply of water to leaves from the well-watered half of the root system (27, 28).

In addition to its apparent utility in studying the effects of ABA and water deficit on plant physiology and gene expression, we speculate that the Dc3 GUS reporter will prove useful in probing the effect of various mutant backgrounds on ABA-mediated signal transduction and possibly the effect of other environmental stresses such as root hypoxia and pathogen infection. To explore the first prediction, we have transferred the Dc3 GUS reporter into wild-type Arabidopsis and tomato, and so far in Arabidopsis, we have found overall similar GUS expression to that in tobacco in response to exogenous ABA and to developmental signals. Furthermore, infection of transgenic tobacco containing Dc3/GUS with a strain of Pseudomonas syringae that elicits a strong hypersensitive response results in rapid GUS expression long before any symptom expression (R. Bostock, T.L. Thomas, unpublished results). These latter results suggest that ABA accumulates at the infection site or that there is increased sensitivity to ABA immediately following infection; they further

Figure 4. Histochemical localization of GUS expression in transgenic tobacco seedlings in response to water deficit and exogenous ABA. A, Water deficit; B, 10 \( \mu M \) ABA; C, control. Transgenic tobacco seedlings were 12 DPI.

Figure 5. Induction of GUS activity in intact seedlings of transgenic tobacco by desiccation and exogenous ABA. At initiation of the experiment, seedlings were 12 DPI. Refer to 'Materials and Methods' and Figure 1 for additional details.
substrate

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LITERATURE CITED