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Developmental and Hormonal Regulation of the Arabidopsis *CER2* Gene That Codes for a Nuclear-Localized Protein Required for the Normal Accumulation of Cuticular Waxes[1]

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The previously cloned *CER2* gene is required for the normal accumulation of cuticular waxes and encodes a novel protein. Earlier reports suggested that the CER2 protein is either a membrane-bound component of the fatty acid elongase complex or a regulatory protein. Cell fractionation and immunoblot analyses using polyclonal antibodies raised against a chemically synthesized peptide with a sequence based on the predicted CER2 protein sequence have demonstrated that the 47-kD CER2 protein is soluble and nuclear localized. These results are consistent with CER2 being a regulatory protein. Detailed studies of plants harboring a *CER2* promoter/GUS transgene (*CER2-GUS*), in combination with immunoblot analyses, revealed that *CER2* is expressed and the CER2 protein accumulates in a variety of organs and cell types. Expression is highest early in the development of these organs and is epidermis specific in most tissues. In agreement with the activity of the *CER2* promoter in hypocotyls, cuticular wax accumulates on this organ in a *CER2*-dependent fashion. In leaves *CER2* expression is confined to the guard cells, trichomes, and petioles. However, application of the cytokinin 6-benzylaminopurine induces ectopic expression of *CER2-GUS* in all cell types of leaves that emerge following treatment.

The aerial portions of plants are covered with a continuous extracellular layer of cuticle that is believed to play an important role in protecting these organisms from water loss, UV irradiation, frost damage, and pathogen and insect attack. The major components of the cuticle are cutin and cuticular wax. The former is a biopolymer of hydroxy fatty acids (Kolattukudy, 1984). Cuticular wax is a complex mixture of VLCFAs and their derivatives, such as aldehydes, alkanes, primary alcohols, secondary alcohols, and ketones (for review, see Tulloch, 1976). The precursor of VLCFAs is believed to be stearic acid, a product of de novo fatty acid biosynthesis that occurs in plastids (for review, see Post-Beittenmiller, 1996). In epidermal cells stearic acid is released from the plastidic fatty acid synthase by a specific thioesterase (Liu and Post-Beittenmiller, 1995) and is made available by an unknown mechanism to microsomally located fatty acyl elongases for further elongation (Agrawal et al., 1984; Lessire et al., 1985; Cassagne et al., 1987). The resulting VLCFAs are further modified to form the other wax components (for reviews, see von Wettstein-Knowles, 1993; Kolattukudy et al., 1976).

The amount and composition of the cuticular waxes that accumulate on plant surfaces differ from species to species and organ to organ and vary during development. For example, the surfaces of Arabidopsis stems and siliques are covered by a heavy wax bloom, whereas Arabidopsis leaves produce considerably less wax (Koornneef et al., 1989; Hannoufa et al., 1993; Jenks et al., 1995). The major components of the waxes on Arabidopsis stems and siliques are alkanes, ketones, and alcohols (Hannoufa et al., 1993). In contrast, the waxes on the leaves of Arabidopsis are mainly composed of alkanes, alcohols, and fatty acids (Jenks et al., 1995). Similarly, maize juvenile leaves are covered with a heavy layer of wax bloom, but adult leaves appear glossy because of a reduced level of wax accumulation. Although the waxes of juvenile leaves are composed mainly of alcohols, esters, alkanes, and aldehydes, the major constituents of wax from adult maize leaves are esters, alkanes, acids, and alcohols (Bianchi et al., 1985). Wax deposition is also regulated by environmental signals (for reviews, see Bengtson et al., 1979; von Wettstein-Knowles, 1993; Post-Beittenmiller, 1996) such as light, moisture, and temperature (von Wettstein-Knowles et al., 1979; Baker, 1980; Bianchi et al., 1985; Hadley, 1989).

Mutations that affect the accumulation of cuticular wax can be identified readily with the naked eye by the absence of a wax bloom, which results in a shiny, glossy phenotype. Such mutants have been identified from a variety of plant species, including maize (*Zea mays* L.) (*glossy* or *gl* loci, Bianchi, 1978; for review, see Schnable et al., 1994), Arabidopsis (*ECERIFERUM* or *CER* loci, Koornneef et al., 1989; Hannoufa et al., 1993), barley (*eceriferum* or *cer* loci, von Wettstein-Knowles, 1987), and sorghum (*bloomless* or *bm* loci, Jenks et al., 1995).

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Abbreviations: BAP, 6-benylaminopurine; MS, Murashige-Skoog; SEM, scanning electron micrograph; VLCFA, very-long-chain fatty acid.

Within the last several years, at least six genes involved in cuticular wax accumulation have been cloned, including three Arabidopsis genes (*CER1*, *CER2*, and *CER3*) and three maize genes (*gl1*, *gl2*, and *gl8*) (Aarts et al., 1995; Tacke et al., 1995; Hannoufa et al., 1996; Negruk et al., 1996; Xia et al., 1996; Hansen et al., 1997; Xu et al., 1997; for a review, see Lemieux, 1996). The Arabidopsis *CER2* gene was cloned by two groups using chromosome walking (Xia et al., 1996) and T-DNA tagging (Negruk et al., 1996) and has been shown to be a single-copy sequence in the Arabidopsis genome (Xia et al., 1996). The deduced CER2 protein is distinct from all other known proteins with defined molecular functions. However, it does share 35% amino acid identity (Xia et al., 1996) with the *gl2* gene of maize, which was cloned via transposon tagging (Tacke et al., 1995). The functions of the CER2 and GL2 proteins in cuticular wax accumulation remain to be determined.

Identification of the cellular and subcellular localization of these proteins may provide clues to their functions. In addition, there have been no detailed studies of how cuticular wax genes are regulated in response to developmental and environmental signals that are known to affect cuticular wax accumulation.

In a previous report we used RNA in situ hybridization and transgenic plants harboring a *CER2-GUS* construct in which 1 kb of the *CER2* promoter region was fused with the GUS gene to demonstrate that *CER2-GUS* is expressed in the epidermal cells of young stems and siliques (Xia et al., 1996). These expression patterns are consistent with the visible phenotype associated with *cer2* mutants, indicating that the 1-kb *CER2* promoter fragment used to construct the *CER2-GUS* gene fusion contains all of the elements required for expression of the endogenous *CER2* gene.

In this report we expand on this earlier study and provide a detailed characterization of *CER2-GUS* expression at different development stages, in different organs and tissues, in response to the exogenous application of a variety of growth factors and under several environmental conditions known to affect cuticular wax accumulation. In addition, we raised polyclonal antibodies against the CER2 protein and used these antibodies to determine the subcellular localization of the CER2 protein via cell fractionation and immunoblot analyses.

MATERIALS AND METHODS

Expression of the CER2 Protein in *Escherichia coli*

The entire coding region from a *CER2* cDNA was released from expressed sequence tag 154C7T7 (Xia et al., 1996) with *Sal*I and *Hin*dIII and cloned into the corresponding sites of pGem3ZF(−) (Promega) to generate pGem/CER2C. The insert of pGem/CER2C was released with *Sal*I and *Not*I and cloned into the corresponding sites of the expression vector pET30(a) (Novagen, Madison, WI) to generate pET/CER2C. To express the CER2 protein, pET/CER2C was introduced into *E. coli* strain BL21 (DE3) (Novagen). The expression and purification of the CER2 protein was performed according to the manufacturer's instructions. The CER2 protein accumulated predominantly in inclusion bodies but could be solubilized in 6 M urea. The expressed CER2 protein contains an N-terminal "His-tag" and could therefore be purified using an Ni-affinity column in association with Novagen's denatured purification procedure. The purity of the isolated protein was monitored by SDS-PAGE.

Antibody Production and Purification

A peptide of 15 amino acids, PEEDLAKLKEEVTNC, was synthesized and purified at the Protein Facility of Iowa State University (Ames). The first 14 amino acids of this peptide correspond to amino acids 405 to 418 of the deduced CER2 protein sequence (Xia et al., 1996). The terminal Cys was added for the purpose of conjugating the peptide to maleimide-activated BSA (Pierce, Rockford, IL). Polyclonal anti-CER2 antibodies were generated by injecting 0.6 mg of the CER2 peptide-BSA conjugate emulsified in Freund's incomplete adjuvant into individual rabbits, which were subsequently challenged with two additional injections of 0.3 mg of the conjugate emulsified in Freund's incomplete adjuvant. The resulting antisera were affinity-purified using the purified, expressed CER2 protein (Sambrook et al., 1989). The purified CER2 protein was subjected to SDS-PAGE and electrophoretically transferred to a nitrocellulose filter. The region of the filter that contained the CER2 protein was preincubated with blocking buffer (SuperBlock, Pierce) and then incubated with the crude antisera. The filter was then washed in TBS buffer (25 mM Tris-HCl, 137 mM NaCl, and 2.7 mM KCl, pH 7.4) and the bound antibodies were eluted by incubating the filter in low-pH buffer (0.2 M Gly and 1 mM EGTA, pH 2.8). The resulting solution was quickly neutralized with 0.1 volume of 1 M Tris base and stored at 4°C in the presence of 0.02% sodium azide.

Preparation of Protein Extracts and Cell Fractions

Protein extracts were prepared by quickly freezing and then pulverizing the indicated tissues in liquid N_2 and then homogenizing them in extraction buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM β-mercaptoethanol, and 0.5 mM PMSF). Cell debris were removed by subjecting the resulting homogenate to centrifugation in a microcentrifuge for 10 min, after which the supernatant was retained.

To separate the membrane and soluble fraction, tissues (entire shoots including stems, cauline leaves, and attached inflorescence before seed set) were pulverized in liquid N_2 and homogenized in extraction buffer. The homogenate was filtered through 159- and 60-μm mesh nylon netting (Spectrum, Houston, TX). An aliquot of the resulting filtrate was reserved for later analysis, and the remainder was centrifuged at 100,000*g* for 1 h. The resulting pellet and supernatant were collected as the membrane (P100) and soluble (S100) fractions, respectively.

Nuclei isolations were performed according to the method of Beach et al. (1985) with some modifications. Frozen tissues (entire shoots including stems, cauline leaves, and attached inflorescence before seed set) were pulverized in liquid N_2 using a mortar and pestle and

homogenized in Honda's buffer (2.5% Ficoll 400, 5.0% dextran T40, 0.4 M Suc, 25 mM Tris-HCl, pH 7.4, and 10 mM $MgCl_2$; β-mercaptoethanol and PMSF were added to 10 and 0.5 mM, respectively, immediately prior to use). The homogenate was filtered through four layers of cheesecloth and then through 159- and 60-μm mesh nylon netting. Triton X-100 (20%) was added to the filtrate to a final concentration of 0.5% and the solution was slowly mixed by swirling. An aliquot of this total extract was retained for later analysis, and the remainder was centrifuged at 1500*g* for 5 min. The supernatant fraction was saved, and the pellet was washed once with Honda's buffer containing 0.1% Triton X-100. After the wash, the pellet was resuspended in Honda's buffer and centrifuged at 50*g* for 1 min to remove cell debris, cell wall fragments, and starch grains. The supernatant was centrifuged at 1500*g* for 5 min to pellet the nuclei. The quality of each nuclei preparation was monitored by light microscopy after it was stained for 1 min with Azure B (0.1% in 20 mM Tris-HCl, pH 7.2, and 0.4 M Suc).

As a control for the nuclear isolation procedure, the DNA concentration of each fraction was determined according to the diphenylamine procedure (Durham and Bryant, 1983). Ten microliters of each extract was mixed with 2 mL of 5% perchloric acid. The mixture was incubated at 70°C for 40 min, and 4 mL of diphenylamine reagent (0.75 g of diphenylamine in 50 mL of glacial acid, 0.75 mL of sulfuric acid, and 0.25 mL of acetylaldehyde) was added to the mixture, which then was incubated at 30°C for 18 h. DNA concentrations were measured at A_{600}.

SDS-PAGE and Immunoblotting

SDS-PAGE and immunoblotting were conducted according to standard procedures (Sambrook et al., 1989). Protein samples were fractionated by SDS-PAGE and transferred to a nitrocellulose filter using a semidry electrophoretic transfer system (Trans-Blot SD, Bio-Rad). Following a preincubation in blocking buffer (SuperBlock, Pierce) to block nonspecific binding sites, the filter was incubated with affinity-purified antibodies for 1 to 2 h. After the filter was washed with TBS it was incubated with the secondary antibody, anti-rabbit IgG-alkaline phosphatase conjugate (Sigma, 1:30,000 dilution). The antigen-antibody complexes were visualized via the alkaline phosphatase reaction assayed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Sigma). Biotin-containing proteins were detected by incubating filters with ExtrAvidin-alkaline phosphatase conjugate (Sigma, 1:200,000 dilution). Protein concentrations were measured using the Bio-Rad protein assay kit according to the manufacturer's instructions.

Transgenic Arabidopsis and Other Plant Materials

Transgenic Arabidopsis plants harboring a *CER2-GUS* construct were generated as previously described (Xia et al., 1996). Histochemical determinations of GUS activity were conducted on tissues fixed in 0.5% paraformaldehyde as described previously (Jefferson, 1987; Xia et al., 1996) using a modification of the GUS-staining buffer (50 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 2 mM potassium ferrocyanide and potassium ferricyanide, and 1 mg/mL 5-bromo-4-chloro-3-indoyl-β-D-glucuronide cyclohexylamine salt) as described by Sundaresan et al. (1995), which substantially increases the sensitivity of these assays. Seeds of the Arabidopsis ecotype Landsberg *erecta* (*Ler*) were obtained from Drs. S. Rodermel and D. Voytas (Iowa State University). Arabidopsis plants were grown at 23°C under 16 h of illumination and 8 h of dark unless otherwise noted.

Four transgenic lines (HB2, HB4, HB5, and HB7) harboring an in-frame fusion of positions −1009 through +234 of the *CER2* gene to GUS (*CER2-GUS*) and representing at least three independent transformation events (Xia et al., 1996) were examined for GUS activity by histochemical staining. These transgenic lines exhibited somewhat different staining intensities, perhaps due to positional effects of transgene insertion. For example, HB7 showed the lowest level of GUS activity in almost all organs examined. A high variation of staining intensity was observed among the plants derived from HB5 lines, which could have been due to segregation of different transgene loci. Transgenic lines HB2 and HB4 (which represent two independent transformation events) showed higher levels of GUS activity and smaller variation of staining intensity. However, all four lines exhibited similar expression patterns.

Treatments of Plant Materials

All seeds were vernalized at 4°C for 3 d prior to germination. To grow plants under tissue culture conditions, seeds were germinated and grown on MS medium (1× MS basal salt, 1% Suc, 0.5 g/L Mes, and 7% Bacto-agar, pH 5.7). Etiolated seedlings were obtained by germinating seeds as described above, with the exception that the plates were covered with aluminum foil. To break dormancy, either 2 μM GA_3 was provided in the medium or the seeds were exposed to light for 15 min following vernalization. Heat and cold shocks were performed by exposing plants for 4 h to 42 and 4°C, respectively. Following the treatments, the plants were placed at 23°C for 12 h prior to the collection of samples. Plants were exposed to high salinity conditions (high osmotic pressure) by saturating the soil in which they were growing with 0.2 M NaCl. Plants were subjected to drought conditions by withholding water until they wilted (approximately 6 d). Tissues were wounded by cutting them into 0.2- to 0.5-mm slices. One-half of the slices were frozen or fixed immediately to serve as unwounded controls. The remaining slices were incubated on 3MM (Whatman) paper soaked with water for 24 h.

Three treatment regimes were performed to study the effects of a variety of exogenous phytohormones and salicylic acid on *CER2-GUS* expression: (a) the transgenic plants were germinated and grown on MS medium with or without a hormone supplement; (b) after germination on MS media, the seedlings were transferred onto media with or without a hormone supplement; and (c) soil-grown transgenic plants were sprayed with water or solutions containing a hormone supplement or sodium salicylate. Arabidopsis seed germination was inhibited on media sup-

plemented with 4 μM ABA or 0.5 mM sodium salicylate (data not shown). Therefore, for ABA and sodium salicylate, only the last two treatment regimes (b and c) were conducted.

SEMs

SEMs of cuticular wax crystals were obtained at the Bessey Microscopy Facility of Iowa State University. Samples were frozen in liquid N_2 using a cryosystem (model SP2000A, Emscope Laboratories, Kent, UK) and observed using an scanning electron microscope (model JSM-5800LV, Jeol) at 3 kV. All samples were collected when the plants had just begun to produce seeds.

RESULTS

Detection of the CER2 Protein

Affinity-purified anti-CER2 antibodies detected a polypeptide of approximately 47 kD in immunoblot analyses of protein extracts from the stems of L*er* plants (Fig. 1B, left lane). The molecular mass of this immunologically detected protein is in agreement with that predicted from the CER2 sequence (Xia et al., 1996). This 47-kD protein was not detected in crude protein extracts from *cer2–2* mutant stems (Fig. 1B, right lane). Because the *cer2–2* mutation contains a premature stop codon that is upstream of the peptide used to immunize the rabbits (Xia et al., 1996), this allele cannot encode normal protein. Therefore, these results establish that the 47-kD protein detected immunologically is the CER2 protein.

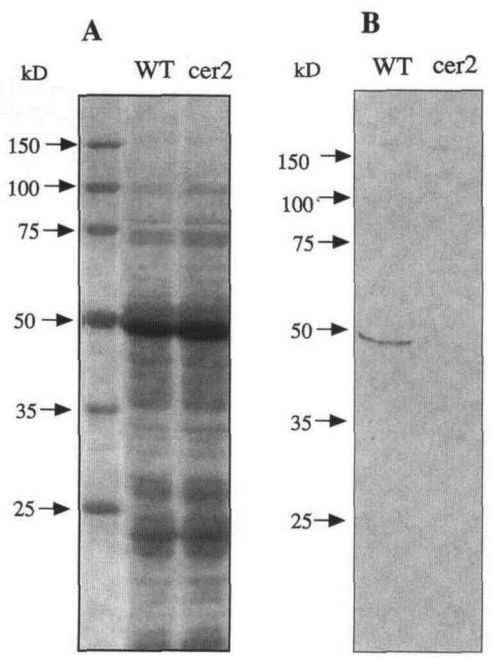

Figure 1. Immunodetection of CER2 protein. A, Protein extracts isolated from stems of L*er* (WT, 30 μg) and cer2–2 mutant (cer2, 40 μg) plants were fractionated by SDS-PAGE and stained with Coomassie brilliant blue. The left lane contains molecular mass markers. B, Proteins from a gel identical to that shown in A were transferred to a nitrocellulose filter. The CER2 protein was detected with affinity-purified anti-CER2 antibodies as described in "Materials and Methods."

Subcellular Localization of the CER2 Protein

Based on the chemical phenotype associated with *cer2* mutations, it has been suggested that the CER2 protein is either a regulatory protein (Jenks et al., 1995; Xia et al., 1996) or a fatty acid elongase (McNevin et al., 1993). These hypotheses would predict that the CER2 protein is located in either the nucleus or the microsomal fraction, respectively (Agrawal et al., 1984; Lessire et al., 1985; Cassagne et al., 1987; Liu and Post-Beittenmiller, 1995). To determine whether the CER2 protein is a membrane-bound or a soluble protein, its distribution between the P100 and S100 fractions was determined. As shown in Figure 2A, the CER2 protein was detected only in the S100 fraction, indicating that the CER2 protein is soluble. This result provides strong evidence that CER2 is not an integral part of a fatty acid elongase.

To determine the subcellular location of the CER2 protein, extracts of stems were subjected to fractionation by differential centrifugation to isolate organelles. These fractionations resulted in the recovery of the CER2 protein in a fraction enriched in nuclei (i.e. pelleted at 1500*g*; data not shown). To confirm that CER2 is a nuclear-localized protein, three fractions were examined: the total extract (T, Fig. 2, B–E), the nuclear extract (N, Fig. 2, B–E), and the supernatant fraction (S, Fig. 2, B–E) that was recovered after the nuclei were removed by centrifugation from the initial extract. The distribution of the CER2 protein was determined among these three fractions and compared with the distribution of DNA, which was used as the nuclear marker. The ratio of DNA to protein concentrations was 3.5-fold higher in the nuclear fraction than in the total extract or the supernatant fraction.

As shown in Figure 2C, although the amount of protein loaded in the lane containing the nuclear fraction was only about 30% of that loaded in the lane containing the total extract (Fig. 2B), there was a 3-fold higher concentration of the CER2 protein in the former. Therefore, compared with the total extract, the CER2 protein was enriched approximately 10-fold in the nuclear fraction. The amount of CER2 protein detected in the supernatant fraction was about the same as that observed in the total fraction; this is likely the result of the expected contamination of the supernatant fraction with broken nuclei, because nuclei recovery by this procedure ranges from 2.3 to 46% (Durham and Bryant, 1983). Because the distribution of the CER2 protein among these fractions closely parallels the distribution of DNA, the nuclear marker, these results suggest that the CER2 protein is localized in the nucleus.

To assess the purity of the nuclear fraction, streptavidin was used to determine the distribution of three biotin-containing polypeptides (Wurtele and Nikolau, 1990) among the fractions. These polypeptides are a 33-kD subunit of the plastidic ACCase (*CAC1*, Choi et al., 1995), a 78-kD subunit of the mitochondrial methylcrotonyl-CoA carboxylase (Weaver et al., 1995), and a 250-kD subunit of

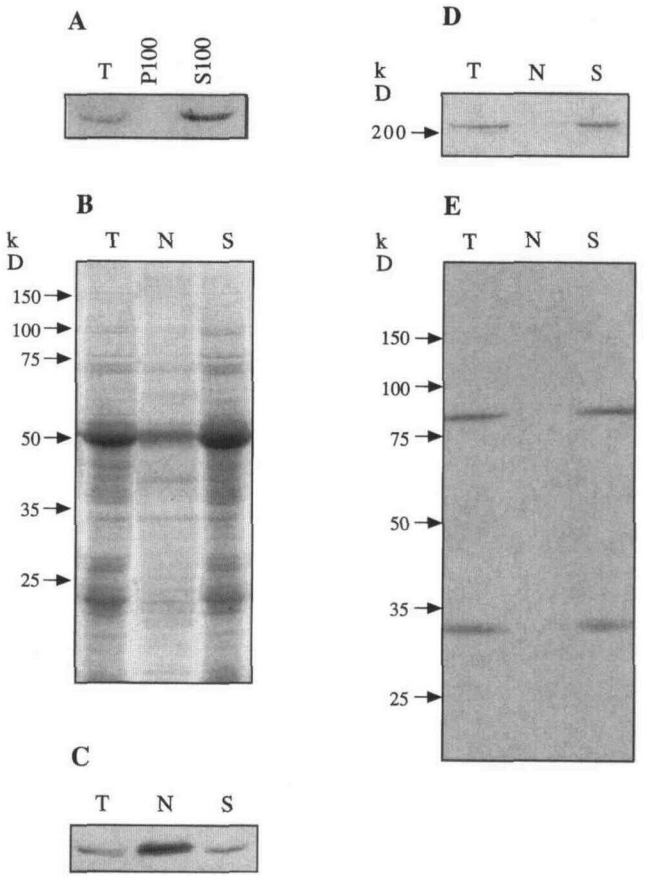

Figure 2. Subcellular localization of the CER2 protein. A, Protein extact from stems (T) was fractionated by centrifugation into the P100 and S100 fractions as described in "Materials and Methods." Aliquots of each fraction were subjected to SDS-PAGE (10% acrylamide). The CER2 protein was immunologically detected with affinity-purified anti-CER2 antibodies as described in "Materials and Methods." Each lane contained 35 μg of protein. B, Fractions obtained from L*er* stems via the nuclear isolation procedure described in "Materials and Methods" were fractionated by SDS-PAGE and stained with Coomassie brilliant blue. The positions of the molecular mass standards are indicated. T, 35 μg of protein from the initial extract; N, 10 μg of protein from the nuclear extract; S, 35 μg of protein from the supernatant extract. C, Proteins from a gel identical to that shown in B were transferred to a nitrocellulose filter. The CER2 protein was detected with affinity-purified anti-CER2 antibodies as described in "Materials and Methods." D, Proteins from a gel identical to that shown in B (but containing 6% acrylamide) were transferred to a nitrocellulose filter. Biotin-containing polypeptides were detected with ExtraAvidin-alkaline phosphatase conjugate as described in "Materials and Methods." Only a portion of the gel is shown. E, Proteins from a gel identical to that shown in B (10% acrylamide) were transferred to a nitrocellulose filter. Biotin-containing polypeptides were detected with ExtraAvidin-alkaline phosphatase conjugate as described in "Materials and Methods."

the cytosolic ACCase (Roesler et al., 1994; Yanai et al., 1995), respectively. As shown in Figure 2, D and E, the 33-, 78-, and 250-kD biotin-containing polypeptides were each detectable in the total extract and the nuclear supernatant fraction but only barely detectable in the nuclear fraction. This indicates that the nuclear fraction was fairly free of plastid, mitochondrial, and cytosolic contamination. Therefore, in combination, these results establish that the CER2 protein accumulates in the nucleus.

Developmental Regulation of Organ- and Tissue-Specific Expression of the *CER2* Gene

Seedlings

Transgenic seeds of four transgenic lines were germinated on MS medium or soil and stained for GUS activity at different stages of development. The same expression patterns were observed in the tissue-culture- and soil-grown seedlings (data not shown). GUS activity was most pronounced on hypocotyls as soon as they emerged and elongated (approximately 14 h after germination; Fig. 3A and data not shown). The staining of hypocotyls was greatly decreased in 5-d-old seedlings (Fig. 3B) and had become undetectable in 12-d-old seedlings (Fig. 3F and data not shown). GUS staining became apparent in guard cells, initially at the tips of the cotyledons 1 d after germination and later extended to the guard cells of the entire cotyledons (Fig. 3, A–C). GUS activity was also readily detected on the petioles of cotyledons approximately 3 to 4 d after germination, corresponding to the time at which the petioles began to elongate (Fig. 3B and data not shown), and declined with the age of the seedling (Fig. 3F). No GUS activity was observed in roots (Fig. 3A and data not shown). Consistent with the observation of GUS activity in germinating seedlings, CER2 protein was detected in crude protein extracts of 3-d-old L*er* seedlings germinated on wet 3MM paper (Fig. 4B).

The prior observation that *CER2-GUS* is expressed in stems and siliques (Xia et al., 1996) is consistent with the fact that wild-type Arabidopsis plants accumulate cuticular waxes in their stems and siliques and that this accumulation is reduced on *cer2* mutant plants. Although, to our knowledge, there are no prior reports of cuticular wax accumulation in Arabidopsis hypocotyls, the observation that *CER2-GUS* is expressed in hypocotyls suggested that *CER2* might also play a role in cuticular wax accumulation on this organ. To test this hypothesis, SEMs of hypocotyls from L*er* and *cer2–2* plants were examined. A pronounced wax bloom was observed on the surfaces of hypocotyls from L*er* plants (Fig. 5E) but not on those from *cer2–2* mutant plants (Fig. 5F).

In stems and siliques *CER2* expression is confined to epidermal cells (Xia et al., 1996). To study the tissue-specific pattern of *CER2-GUS* expression in young seedlings, GUS activity was assayed in cross-sections of stained hypocotyls and petioles. GUS activity was restricted mainly to the epidermal cells of the hypocotyls and petioles (Fig. 3, D and E). However, in those regions of the hypocotyls and petioles that exhibited the strongest GUS activity, staining often extended into inner cells (data not shown).

Juvenile Plants

Transgenic juvenile plants grown in both tissue culture conditions and in soil were also examined for GUS activity.

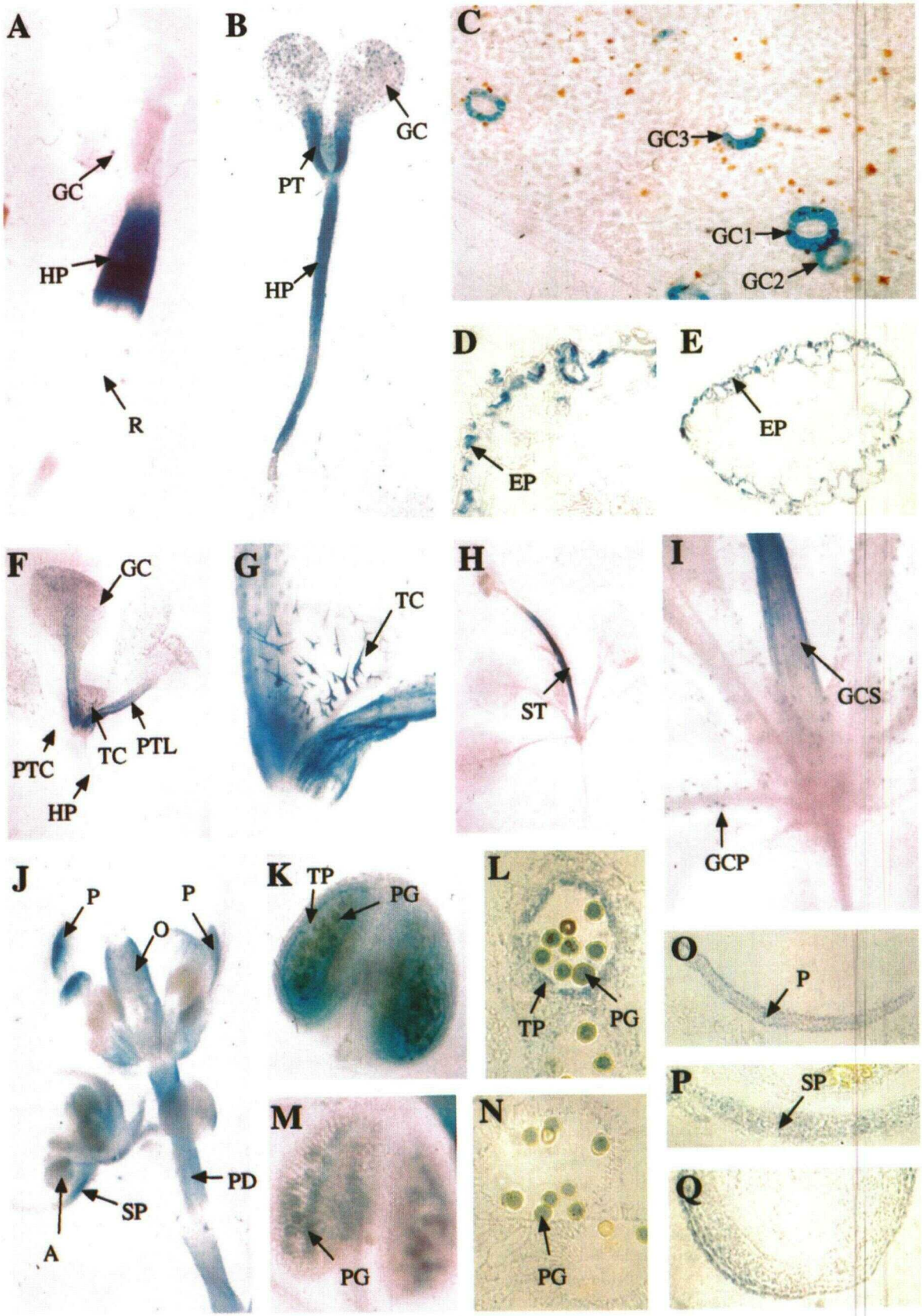

Figure 3. (Figure continues on facing page.)

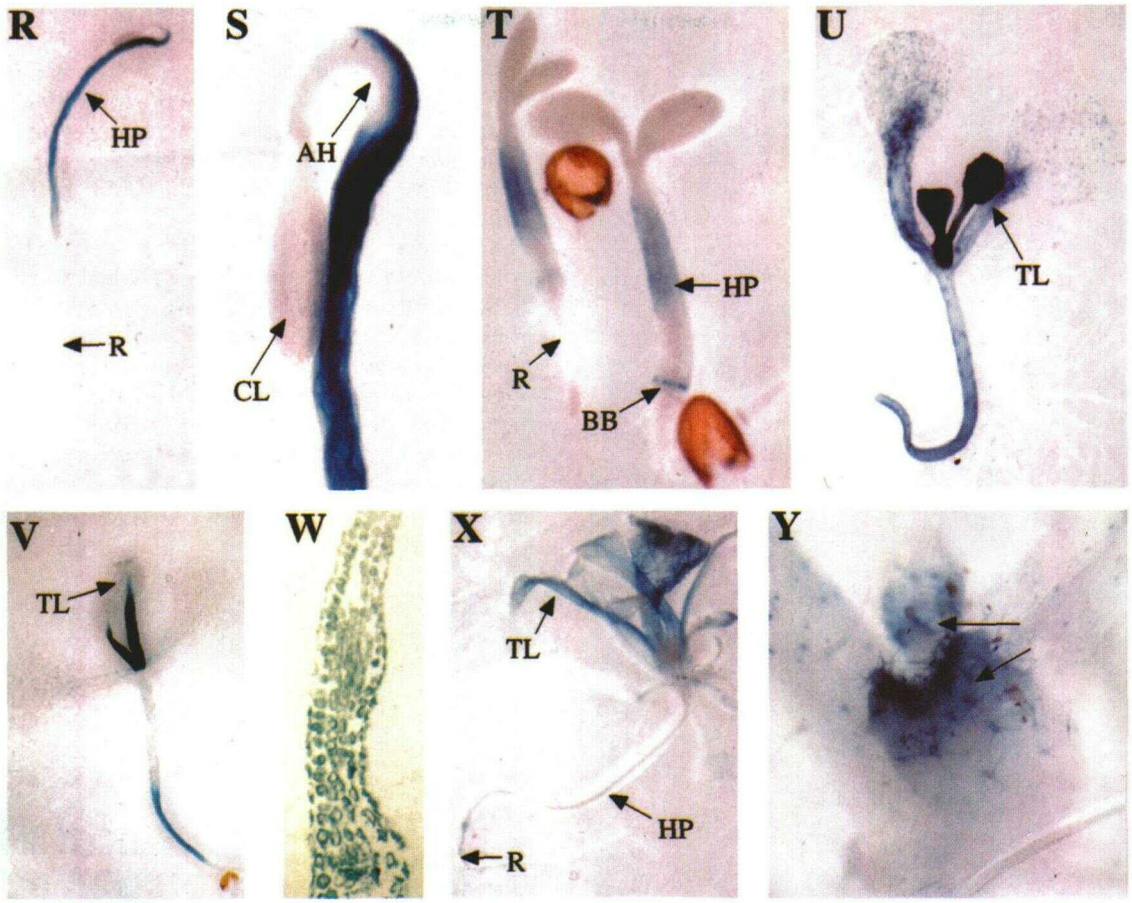

Figure 3. (Figure continued from facing page.) Expression of the *CER2-GUS* transgene. GUS staining was conducted as described in "Materials and Methods." A, One-day-old seedling germinated on MS medium. GUS activity is evident on the hypocotyl (HP) and guard cells (GC). No GUS activity is observed in the root (R). B, Five-day-old seedling grown on MS medium. GUS activity is present in hypocotyl (HP), petiole (PT), and guard cells (GC). C, Close-up of a cotyledon from a seedling similar to that shown in B. GUS activity is evident in guard cells of adaxial (GC1) and abaxial (GC2) epidermes. In some cases, only one member of a pair of guard cells (GC3) stained. D, Cross-section (12 μm thick) of a hypocotyl obtained from a 5-d-old seedling similar to that shown in B. GUS activity is observed in the epidermis (EP). E, Cross-section (12 μm thick) of a cotyledonary petiole obtained from a 5-d-old seedling similar to that shown in B. GUS activity is localized mainly in the epidermis (EP). F, Twelve-day-old soil-grown juvenile plant. GUS activity is detected in the petioles of leaves (PTL), guard cells (GC) of cotyledons and leaves, and developing trichomes (TC). At this stage GUS activity is no longer detected in the hypocotyls (HP) and the petioles of cotyledons (PTC). G, Close-up of a portion of the plant shown in G. GUS activity is prominent in trichomes (TC). H, Twenty-two-day-old soil-grown plant. GUS activity was detected in the stem (ST). I, Close-up view of a portion of the plant shown in H. GUS activity is detected in guard cells of the petioles (GCP) and stems (GCS). J, Developing inflorescence. GUS activity is detected in the petals (P), sepals (SP), anthers (A), ovaries (O), and pedicels (PD). The flowers were surgically opened to enhance the visibility of the floral structures. K, Stage 12 anther. GUS activity was detected in the tapetal layer (TP) and pollen grains (PG). L, Cross-section (12 μm) of a stage 12 anther. GUS activity is located mainly in the tapetal layer (TP) and pollen grains (PG). M, Stage 13 anther. GUS activity was only detected in pollen grains (PG). N, Cross-section (12 μm) of a stage 13 anther. GUS activity was detected only in pollen grains (PG). O, Transverse section of a petal (P). GUS staining was most intense in the epidermal cells; weaker staining was observed in the inner cells. P, Cross-section (12 μm) of a sepal (S). All cells were almost equally stained. Q, Cross-section (12 μm) of an ovary. GUS activity was located mainly in the epidermal cells; weaker staining was observed in a few of the inner cell layers. R, Two-day-old etiolated seedling. GUS activity was observed in the hypocotyl (HP). No GUS activity was observed in roots (R). S, Portion of an etiolated seedling. Within the apical hook region (AH), only those cells outside the hook exhibited GUS staining. Those cells inside the hook did not stain. CL, Cotyledon. T, Two 2-d-old seedlings germinated on MS medium for 12 h and then transferred onto MS medium supplemented with 0.5 mM sodium salicylate. HP, Hypocotyl; R, root; and BB, basal band. U, Seven-day-old seedling germinated and grown continuously on MS medium supplemented with 4 μM BAP. Intense staining was observed in all cells of the leaves (TL). V, Seven-day-old seedling germinated and grown continuously on MS medium without BAP supplementation. In contrast to U, staining was observed only in the petioles and guard cells of leaves (TL). W, Cross-section (12 μm) of a leaf from a plant similar to that shown in U. GUS activity was evident in all cell types. Compare with C. X, Fifteen-day-old plant germinated and grown continuously on BAP-supplemented medium. The staining in the leaves (TL) was reduced compared with those shown in U. Portions of the roots (R) of some plants stained. HP, Hypocotyl. Y, Plant germinated and grown on basic MS medium for 5 d and then moved for 5 d to MS medium supplemented with 4 μM BAP. In the newly emerging leaves, both trichomes and pavement epidermal cells stained (arrows).

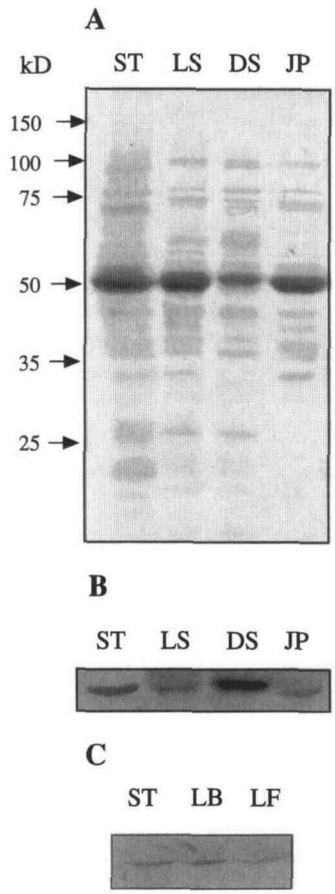

Figure 4. Accumulation of CER2 protein at different developmental stages and in response to light irradiation. A, Total protein extracts from L*er* stems (ST, 30 μg), entire 3-d-old seedlings germinated under light (LS, 30 μg) or dark (DS, 20 μg) conditions, and the aboveground portion of juvenile plants at the four- to six-leaf stage (JP) were fractionated via SDS-PAGE and transferred to nitrocellulose filter. The gel was stained with Coomassie brilliant blue after the transfer. The positions of the molecular mass standards are indicated on the left. B, CER2 protein transferred to the filter described in A was detected using affinity-purified anti-CER2 antibodies. C, Total protein extract from stems (ST, 35 μg) and the upper two-thirds of leaves from 8-d-old plants germinated and grown continuously on media with and without BAP (4 μM) supplement (LB and LF, respectively, 30 μg) were fractionated via SDS-PAGE (8% acrylamide) and transferred to a nitrocellulose filter. CER2 protein was detected using affinity-purified anti-CER2 antibodies.

No differences in staining patterns were observed between the two growth conditions. As shown in Figure 3, F and G, strong *CER2*-directed GUS staining was detected in trichome cells of young leaves but became undetectable as these leaves matured (data not shown). As was true in cotyledons, the guard cells, but not the other epidermal cells of young leaves, expressed *CER2-GUS* (Fig. 3F). In both cotyledons and leaves a careful examination revealed that not all guard cells exhibited GUS activity. In some cases only one of a pair of guard cells stained (Fig. 3C and data not shown). GUS activity was present in guard cells throughout leaf development but became less evident as the leaves aged (Fig. 3, H and I). GUS activity was also detected in developing petioles of leaves (Fig. 3F). This staining also became noticeably weaker as the associated leaves matured and the petioles stopped elongating (Fig. 3H). As was true for the petioles of cotyledons, GUS activity in the petioles of leaves was located primarily in the epidermal cells (data not shown).

The above data indicate that the *CER2* gene is expressed in juvenile plants. Consistent with this conclusion, the CER2 protein was immunologically detected in extracts from 15-d-old plants (at the four–six-leaf stage), although its accumulation was less than that observed in extracts from stems and seedlings (Fig. 4B). In addition, CER2 protein accumulation was detected in extracts from the upper two-thirds of leaf blades (Fig. 4C). The only cells in the samples described above that expressed GUS activity were the guard cells (by this stage of development GUS activity was no longer detectable in trichomes). Therefore, this finding supports the view that guard cells accumulate CER2 protein.

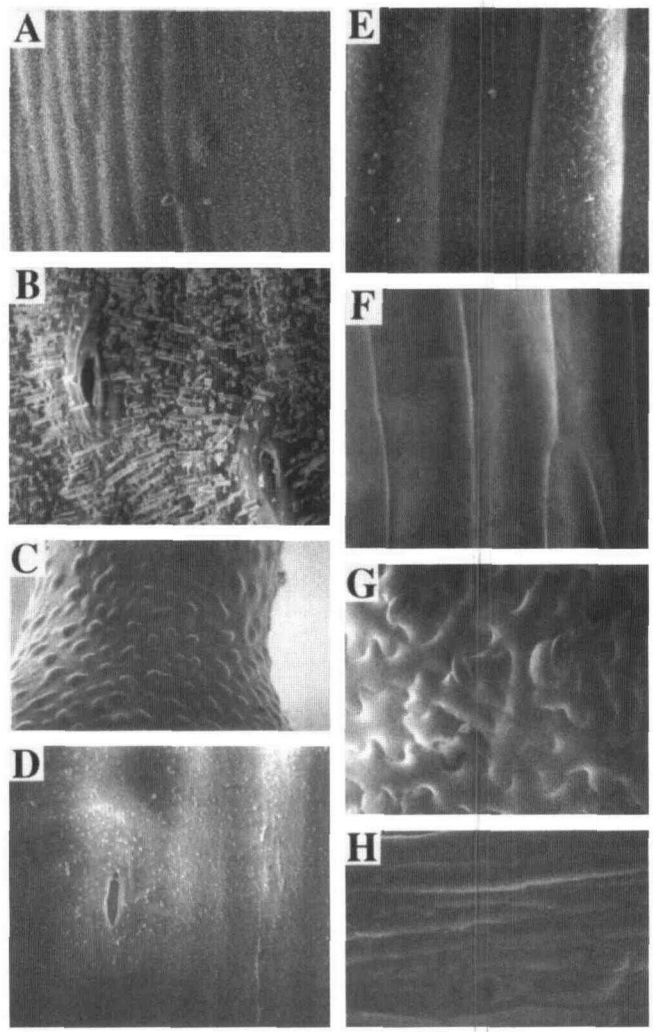

Figure 5. SEMs of cuticular wax crystals on the surfaces of various organs. A, L*er* stem, ×500. B, L*er* stem, ×3000. C, Portion of a L*er* stem trichome, ×3000. D, *cer2–2* stem, ×3000. E, L*er* hypocotyl, ×1000. F, *cer2* mutant hypocotyl, ×1000. G, L*er* leaf, ×500. H, L*er* petiole, ×500.

Adult Plants

When the stems of the transgenic plants emerged and reached approximately 2 cm in height, intense *CER2*-directed GUS staining was detected in the rapidly elongating regions of stems beginning at a position approximately 2 to 5 mm below the shoot apical primordia (Fig. 3H). In cauline leaves GUS staining was restricted to the trichomes and guard cells (data not shown). By this stage GUS staining in the rosette leaves had become nearly undetectable with the naked eye (Fig. 3H). However, GUS activity was still evident in the guard cells of petioles and leaf blades (Fig. 3I and data not shown).

In floral organs GUS activity was observed in sepals, petals, ovaries, anthers, pollen grains, and pedicels (Fig. 3J). Staining in these organs occurred only within a short developmental window. In anthers GUS staining was detected at about stage 12 (petals level with long stamens; for the definition of the flower developmental stages, see Bowman, 1994). At this stage, GUS activity was localized mainly in pollen grains and the tapetal layer (Fig. 3, K and L). As anthers matured through stage 13 (bud open, petals visible, anthesis), the tapetal cells degenerated and GUS activity was detected only in the pollen grains (Fig. 3, M and N). After anthesis, little GUS activity was detected in anthers except in those with short stamens (Fig. 3J). Even within the same anther, GUS-staining patterns and intensity varied among different pollen sacs (Fig. 3, L and N).

In sepals and petals *CER2*-driven GUS activity was not restricted to epidermal cells, although in the sepals GUS staining was more intense in the epidermal cells (Fig. 3, O and P). Similarly, in young ovaries GUS staining extended into the inner cell layers of the ovary wall (Fig. 3Q). As the ovaries matured this staining tended to become localized in epidermal cells, a result consistent with the report that *CER2-GUS* expression in siliques is epidermis specific (Xia et al., 1996).

Effects of Environmental Factors and Exogenous Applications of Phytohormones on *CER2* Expression

Light

The accumulation of cuticular waxes is light dependent (von Wettstein-Knowles et al., 1979; Bianchi et al., 1985; for review, see von Wettstein-Knowles, 1993). To determine whether this response is mediated by *CER2* expression, the response of *CER2*-driven GUS activity to illumination was assayed. The effect of light on *CER2-GUS* expression patterns in stems and inflorescences was examined by removing the primary shoots from transgenic plants to stimulate secondary bolting. After the first shoots were removed, some plants produced two or more secondary shoots almost simultaneously. One of the secondary shoots was covered with light-tight plastic; the other was covered with light-transparent plastic wrap. Three days after covering, the shoots were collected, fixed in 0.3% paraldehyde, and stained for GUS activity. The expression patterns of *CER2-GUS* in the covered and uncovered stems and inflorescence were indistinguishable, although the staining intensities were slightly weaker in the former (data not shown).

Because the results of this experiment could be confounded by the half-life of GUS activity, the expression of *CER2-GUS* was also examined in dark-germinated seedlings. Dark-germinated seedlings were obtained by covering plates containing seeds with aluminum foil during vernalization and germination. However, because Arabidopsis seeds will not germinate under these conditions, they were exposed to light for 15 min immediately following vernalization to break dormancy and then returned to darkness. GA_3 can substitute for light to break dormancy (Brusslan and Tobin, 1992). Therefore, as a control for the 15-min light exposure, GUS activity was assayed in seedlings that had never been exposed to light but were instead germinated on 3MM paper soaked with 2 μM GA_3.

The results of the two treatments were identical. As shown in Figure 3R, the expression patterns of *CER2-GUS* in transgenic seedlings grown under dark conditions (using both protocols) were similar to those of light-germinated seedlings (Fig. 3A). However, the appearance of GUS staining in the guard cells of cotyledons in etiolated seedlings was delayed relative to light-grown seedlings and appeared approximately 2 d after germination (data not shown). Note that in the apical hook of etiolated seedlings (Fig. 3S) GUS activity is restricted to the cells outside of the hook, further supporting the observation (Xia et al., 1996) that *CER2* expression is highest in cells that are undergoing rapid expansion.

To determine whether the CER2 protein is present in etiolated seedlings, immunoblot analyses were conducted using 3-d-old L*er* seedlings grown under light and dark conditions. Surprisingly, as shown in Figure 4B, the level of CER2 protein accumulation in etiolated seedlings was approximately 5- to 10-fold higher than that in seedlings germinated under light. This can be partly explained because in the etiolated seedlings accumulation of many other proteins including the major 50-kD proteins (probably the large subunit of Rubisco) was reduced, which thereby increased the proportion of the CER2 protein in the total extract. Alternatively, in the etiolated seedlings, the hypocotyls and the cells undergoing rapid elongation, the major organ and cells showing *CER2-GUS* expression in seedlings, may account for a larger proportion of the plant tissues. These results establish unambiguously that light is not required for the expression of the *CER2* gene in germinating seedlings.

Stress Treatments

Water and temperature stress have been reported to enhance the accumulation of cuticular waxes (Bengtson et al., 1979; Baker, 1980; Hadley, 1989). However, stress treatments such as heat and cold shock, drought, high osmotic pressure, wounding, and application of sodium salicylate did not alter *CER2-GUS* expression patterns or increase the intensity of GUS staining (data not shown).

Twelve hours after the plants were sprayed with 0.5 mM sodium salicylate or watered with 0.2 M NaCl, they started to wilt and GUS activity was remarkably decreased (data not shown). Under these conditions growth was retarded and GUS activity was remarkably reduced, and plant death

occurred within 3 d. Figure 3T shows a 2-d-old seedling that was germinated on MS medium for 12 h and then transferred to MS medium supplemented with 0.5 mM sodium salicylate. The nature of the basal band of GUS staining on the hypocotyl is not known.

Exogenous Phytohormones
ABA, GA, and Auxin

Independent treatments with ABA (4 μM) and GA_3 (8 μM) failed to alter the patterns of GUS expression (data not shown). However, the hypocotyls of seedlings germinated on medium supplemented with auxin (2 μM 2,4-D) exhibited delayed appearance of GUS activity (data not shown). In addition, the root and shoot meristems of plants exposed to auxin by treatment regime b underwent dedifferentiation to form callus-like structures that expressed GUS activity (data not shown).

Cytokinin: BAP

Young seedlings germinated on medium supplemented with 4 μM BAP did not exhibit alterations in the patterns of GUS staining, although the staining of hypocotyls and guard cells appeared to be slightly more intense (data not shown). In contrast, as these seedlings matured, a striking change in the pattern of CER2 promoter activity was observed. In 7-d-old plants that had been exposed to BAP, intense GUS staining was apparent in all cell types of the newly emerged leaves (in Fig. 3, compare U and W with V and C). As these leaves matured, this staining became weaker and more restricted to the regions near the veins (Fig. 3X and data not shown). The roots of some but not all of these older (15 d after germination) plants exhibited weak GUS staining (Fig. 3X).

To ascertain the response time of BAP-induced changes in gene expression, 6-d-old seedlings (in which the first pair of leaves had just emerged) that had been germinated on MS medium with or without BAP were transferred onto MS medium supplemented with 4 μM BAP. Twelve hours after transfer, the seedlings were stained for GUS activity. No differences in GUS-staining patterns were observed compared with plants grown continuously on basic MS medium (data not shown), suggesting that ectopic *CER2-GUS* expression in leaves is not rapidly induced by BAP. When examined 5 d after transfer, ectopic expression was observed in parts of some of the newly emerging leaves (Fig. 3Y).

Similarly, 12 h after the plants were sprayed with BAP (treatment regime c), no differences in GUS staining were observed (data not shown). However, after 5 d, ectopic GUS activity was visible on some newly emerging leaves (data not shown). These results further support the view that ectopic *CER2-GUS* expression in leaves is not rapidly induced by BAP. Although we cannot exclude the possibility that BAP is only slowly absorbed by leaf cells, no further changes in the staining patterns were observed after extended spraying with BAP. No changes in *CER2-GUS* expression were observed in the stems or inflorescences of these plants (data not shown).

To determine whether CER2 protein accumulation in leaves increases in response to the application of BAP, the concentration of CER2 protein was assayed in protein extracts from the upper two-thirds of the first pair of leaves from 8-d-old plants germinated and grown continuously in media with and without supplemental BAP. As shown in Figure 4C, the amount of CER2 protein in leaves from the plants grown on BAP-supplemented medium was approximately 2 times that found in control plants.

DISCUSSION

The CER2 Protein Is Localized in the Nucleus

Anti-CER2 polyclonal antibodies generated by immunizing rabbits with a synthetic peptide representing the amino acids 405 to 418 of the deduced CER2 protein sequence (Xia et al., 1996) recognize a protein with the predicted size of the CER2 protein (47 kD) in protein extracts from wild-type but not in *cer2–2* mutant stems. This result demonstrates that the immunologically detected 47-kD protein is CER2.

Using fractionation by differential centrifugation and immunoblot analyses, we demonstrated that the CER2 protein is soluble and is highly enriched in the nuclear fraction. These data suggest that the CER2 protein has a regulatory role in cuticular wax accumulation. The deduced CER2 protein sequence does not contain any of the commonly recognized nuclear localization signals. However, not all nuclear-localized proteins contain such signals (Weighardt et al., 1995). In some cases, this is because they enter the nucleus via heterodimerization with other proteins that do possess nuclear localization signals (Goldfarb and Lewandowska, 1994).

CER2 Expression Is Not Induced by Light or Stress Treatments

It has been reported that the accumulation of cuticular waxes increases in response to high light conditions, drought, and temperature stress (Bengtson et al., 1979; von Wettstein-Knowles et al., 1979; Baker, 1980; Bianchi et al., 1985; Hadley, 1989). However, our results indicate that the expression pattern of *CER2-GUS* is not induced by light. This is further supported by the immunoblot analyses revealing that the CER2 protein accumulates at higher levels in dark-germinated seedlings. The finding that *CER2* transcription is not light dependent is supported by the finding that GenBank contains a *CER2*-derived expressed sequence tag (accession no. Z38078) derived from a cDNA library prepared from RNA isolated from etiolated Arabidopsis seedlings. In addition, *CER2* expression is not induced by wounding, drought stress, salt, salicylic acid, heat or cold shock, or ABA (the stress hormone). Indeed, all factors tested that retard plant growth appear to decrease *CER2* expression.

Spatial and Temporal Expression Patterns of the *CER2* Gene

Using first-generation transgenic plants harboring a *CER2-GUS* construct and in situ RNA hybridization, we

previously demonstrated that the *CER2* gene is expressed in the epidermal cells of Arabidopsis stems and siliques (Xia et al., 1996). In this report we present details of studies of the expression patterns of *CER2-GUS* during other developmental stages. *CER2*-driven GUS staining was observed in hypocotyls, guard cells, petioles, trichomes, stems, sepals, petals, ovaries, siliques, pedicels, the tapetal layer of anthers, and pollen grains. These studies reveal that *CER2* expression occurs early in the development of these organs, with the exception of guard cells and anthers, when they are undergoing rapid cell expansion. Furthermore, in many of these observations GUS staining is transient and was detected at a specific developmental stage of the organ or tissue and subsequently declined. Because GUS is considered to be a very stable protein (Jefferson, 1987), these observations indicate that the expression of the *CER2* gene is highly regulated both spatially and temporally.

As evidenced by the expression of the *CER2-GUS* transgene, the *CER2* promoter is active in the tapetal layer of anthers and in pollen grains. Mutations in some of the *CER* loci, including *cer2* (Preuss et al., 1993; Hulskamp et al., 1995), have been reported to cause male sterility under conditions of low humidity. Two such examples are the *pop1* (allelic to *cer6)* and *cer1* mutants. Pollen from both of these mutants lack the tryphine structure, which may account for their ability to condition male sterility (Preuss et al., 1993; Aarts et al., 1995). However, although the *cer3* mutant is also a conditional male sterile, it does not appear to affect the tryphine structure (Hulskamp et al., 1995). This suggests that there are other mechanisms by which cuticular wax genes might affect male fertility. It remains to be resolved how the *cer2* mutation affects structural features of pollen grains and its specific role in pollen fertility.

Wild-type Arabidopsis plants accumulate substantial wax bloom on their stems and siliques. Mutants at the *CER2* locus block this accumulation but do not appear to affect the accumulation of waxes on rosette leaves (Jenks et al., 1995). The findings that *CER2-GUS* is expressed in hypocotyls, that the CER2 protein accumulates in very young seedlings, and that hypocotyls accumulate cuticular wax in a *CER2*-dependent manner support a role for the *CER2* gene considerably earlier in development than previous reports would suggest.

However, whereas our analyses of the *CER2-GUS* transgene indicated that the *CER2* gene is expressed in a variety of tissues, it was not always clear that this expression was directly involved in cuticular wax accumulation. For example, in sepals and petals expression of *CER2* was not restricted to the epidermal tissue. Additionally, trichomes, guard cells, and petioles of cotyledons and leaves expressed the *CER2* gene. Consistent with this observed GUS activity in leaves, the CER2 protein accumulates in 15-d-old plants and the upper two-thirds of leaf blades, where the guard cells are the only cells that exhibit GUS activity. However, SEMs of these cells and structures did not reveal an accumulation of detectable quantities of wax crystals (although cuticular wax need not form visible crystals). Furthermore, there were no detectable differences in the surface morphological appearance of these cells and organs between wild-type and *cer2* mutant plants. These observations suggest that the *CER2* gene may have additional molecular functions other than its role in cuticular wax deposition. This multifaceted molecular function may be consistent with the suggested regulatory role of the CER2 protein. A similar example of a multifunctional gene is the *TTG* gene of Arabidopsis, which encodes a putative transcription factor that affects anthocyanin biosynthesis and trichome development and determines the developmental fate of root epidermal cells (Galway et al., 1994; Lloyd et al., 1994).

Consistent with this hypothesis, *CER2* expression in guard cells is controlled by a developmental program that is different from that in the other epidermal cells of elongating stems and siliques. This conclusion is based on the observation that early in the development of these organs *CER2* is expressed in all epidermal cells, but later in development, *CER2* expression becomes confined to guard cells.

BAP Induces Ectopic Expression of *CER2-GUS* in Leaves

Another aspect of the complexity of the regulation of *CER2* expression is revealed by the application of exogenous cytokinin. Specifically, the application of 1 μM BAP abolishes the cell specificity of *CER2* expression in leaves, in that it is expressed in all cells. Consistent with the observation of ectopic *CER2-GUS* activity under these conditions is the finding that CER2 protein accumulation increases in response to the exogenous application of BAP. Time-course studies have suggested that the *CER2* promotor is not constitutively receptive to this hormone-dependent derepression. Rather, this hormone affects *CER2* expression only in very young leaves that have not yet elongated at the time of hormone application. In addition, this hormone-dependent derepression of *CER2* expression is restricted to leaf cells, in that it does not occur in the cells of stems, siliques, or cotyledons.

It is tempting to speculate that endogenous *CER2* gene expression is regulated by cytokinins. However, there is little information available concerning the distribution of cytokinins in different organs or tissues in Arabidopsis plants or the concentration of cytokinins at their sites of action. The hypothesis that endogenous cytokinins might be involved in the regulation of cell-type specificity of *CER2* gene expression will be tested via the analysis of *CER2* promoter activity in transgenic plants with elevated cytokinin levels mediated by the isopentenyl transferase (*ipt*) gene from *Agrobacterium tumefaciens* (Medford et al., 1989).

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