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Organ-Specific Differential Regulation of a Promoter Subfamily for the Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Small Subunit Genes in Tomato[1]

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The tomato (*Lycopersicon esculentum*) gene family for the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*RBCS*) has been investigated to determine the role of promoter regions and DNA-protein interactions in the differential organ-specific transcription of individual genes. Transgenic plants expressing *RBCS*-promoter-β-glucuronidase fusion genes have confirmed that promoter fragments ranging from 0.6 to 3.0 kb of the *RBCS1*, *RBCS2*, and *RBCS3A* genes were sufficient to confer the temporal, organ-specific, and differential expression pattern observed for the endogenous genes. The individual temporal and organ-specific β-glucuronidase enzyme activities closely reflect the qualitative and quantitative transcription activities of the respective *RBCS* genes, including the strongly reduced activity of *RBCS3A* (L.A. Wanner, W. Gruissem [1991] Plant Cell 3: 1289–1303). In particular, tissue-specific activity of all three promoters is similar in developing fruit, with high activity in the locular tissue and extremely reduced activity in the pericarp. This specific pattern of gene activity was further substantiated by in situ analysis of *RBCS* mRNA levels. Together, the data suggest an interesting correlation between *RBCS* gene activity and sink strength in different fruit tissues. DNA-protein interaction studies have revealed a novel fruit-specific DNA-binding protein called FBF that specifically interacts with a sequence element directly upstream of the G-box in the *RBCS3A* promoter. FBF binding thus correlates with the reduced activity of this promoter in developing tomato fruit, rendering it a candidate for a fruit-specific negative regulator of transcription in tomato.

The nuclear gene family for the SSU of Rubisco is a paradigm for the control of gene expression in higher plants by developmental programs and environmental factors. In most plants, the multigene family comprises 4 to 13 individual *RBCS* genes (Manzara and Gruissem, 1988; Dean et al., 1989b). Although the amino acid sequences of the mature SSU proteins can differ among members of a multigene family, no functional difference between the protein isoforms has been described (Manzara and Gruissem, 1988). In several plant species examined, the temporal mRNA expression pattern of *RBCS* gene family members is coordinated, with one or few genes contributing the majority of *RBCS* transcripts (Coruzzi et al., 1985; Dean et al., 1985, 1987a, 1987b, 1989a, 1989b; Fluhr et al., 1986). In certain plants such as tomato (*Lycopersicon esculentum*) and *Lemna gibba*, however, individual genes have different organ-specific and temporal expression patterns (Sugita and Gruissem, 1987; Silverthorne and Tobin, 1990; Silverthorne et al., 1990; Wanner and Gruissem, 1991).

In tomato the *RBCS* gene family consists of five members at three chromosomal loci. Three genes (*RBCS3A*, *RBCS3B*, and *RBCS3C*) are arranged in tandem array within 10 kb, whereas *RBCS1* and *RBCS2* are located on different chromosomes (Sugita et al., 1987). The *RBCS* family encodes three different SSU protein isoforms, of which all locus 3 genes encode isoform 3. The mRNAs from all five genes accumulate to similarly high levels in leaves and light-grown cotyledons. Only *RBCS1*, *RBCS2*, and *RBCS3A* mRNAs accumulate in dark-grown cotyledons and in water-stressed leaves. In young tomato fruit *RBCS1* and *RBCS2* mRNAs accumulate, the *RBCS3A* mRNA level is strongly reduced, and *RBCS3B* and *RBCS3C* mRNAs are not detectable. No *RBCS* mRNAs can be detected in roots and mature tomato fruit (Sugita and Gruissem, 1987; Wanner and Gruissem, 1991). These differences in mRNA accumulation reflect different activities of the individual *RBCS* promoters in various organs (Wanner and Gruissem, 1991).

Based on the presence or absence of several conserved DNA sequence motifs together with their spatial arrangements, the five tomato *RBCS* promoters can be divided into two groups (Manzara and Gruissem, 1988). The first group consists of the *RBCS1*, *RBCS2*, and *RBCS3A* promoters that share the DNA sequence motifs "1," I-box, and G-box in a similar spatial arrangement. Promoters in the second group (*RBCS3B* and *RBCS3C*) lack these elements and contain instead the DNA sequence elements "5," "2," "8," "9," and "10" (Manzara and Gruissem, 1988; Manzara et al., 1991, 1993; Carrasco et al., 1993). The difference in DNA se-

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Abbreviations: GBF, G-box-binding factor; *GUSA*, GUS gene; *RBCS*, gene for small subunit of Rubisco; SSU, small subunit; X-Gluc, 5-bromo-4-chloro-3-indolyl-β-glucuronic acid.

quence motifs correlates with the activity of the promoters in the two groups in response to developmental and environmental signals (Sugita and Gruissem, 1987; Bartholomew et al., 1991; Wanner and Gruissem, 1991). The exception is the reduced activity of the *RBCS3A* promoter in young fruit compared to the higher activity of the *RBCS1* and *RBCS2* promoters (Wanner and Gruissem, 1991). Thus, in young tomato fruit the activity of the *RBCS3A* promoter is coordinated with the linked *RBCS3B* and *RBCS3C* genes.

We are using *RBCS* gene expression in tomato as a model system to investigate the molecular mechanisms of differential temporal and spatial promoter activation. The work in this report focuses on the expression patterns of the *RBCS1*, *RBCS2*, and *RBCS3A* genes for two reasons. First, their promoters share conserved *cis* elements such as the I-box and the G-box that have already been functionally characterized in other plant promoters (Donald and Cashmore, 1990; Gilmartin et al., 1990). Second, the uncoupling of *RBCS* promoter activities in young tomato fruit provides an important experimental system in which to identify elements involved in organ-specific transcriptional activation or inactivation in promoters that share a similar organization. As a first step in determining whether organ-specific signals act on *RBCS* transcription through immediate upstream sequences, we have transformed tomato with *RBCS* promoter-*GUSA* fusions and analyzed GUS expression in different organs of these plants. In addition, we describe a DNA-binding protein (FBF) that specifically interacts with the *RBCS3A* promoter in tomato fruit. Binding of FBF is closely correlated with the fruit-specific reduction in *RBCS3A* promoter activity. We discuss the possible function of FBF as an organ-specific, negative transcriptional regulator.

MATERIALS AND METHODS

Plant Material

For GUS assays T_2 seeds from transgenic plants were surface sterilized by a 15-min incubation in 2% sodium hypochloride followed by extensive rinsing in sterile H_2O. Five seeds each were placed onto Murashige and Skoog agar (Sigma) in Magenta boxes and incubated in a growth chamber at 22°C for 16 h of light and 8 h of dark. For mature F_2 plants, seedlings were transferred to soil and grown in the greenhouse under controlled conditions. Tomato (*Lycopersicon esculentum* cv VFNT Cherry) plants for in situ hybridization experiments and preparation of nuclear extracts were grown in the greenhouse under controlled conditions. Tomato seedlings for the preparation of nuclear extracts were grown as described by Manzara et al. (1991).

DNA Constructs

The pMLJI vector (De Block et al., 1984) for tomato transformation was modified in three steps. First, the two original *Hin*dIII sites in pMLJ1 were destroyed by filling in and a *Hin*dIII linker was inserted at the original *Sma*I site to give pML6–1-1. Second, the *RBCS* promoter-*GUSA* fusions were constructed in pBI101.1 (Jefferson et al., 1987) by inserting the *RBCS* promoters as *Hin*dIII/*Nhe*I fragments between the *Hin*dIII site and the *Xba*I site of pBI101.1. Third, the *Hin*dIII/*Eco*RI fragments from the intermediary pBI101.1 plasmid derivatives containing the *RBCS-GUSA*-nopaline synthase gene 3′ fusion were inserted between the *Eco*RI and the newly created *Hin*dIII site of pML6–1-1. For *RBCS1* and *RBCS3A* the *Hin*dIII cloning sites correspond to the upstream *Hin*dIII site shown by Sugita et al. (1987). For *RBCS2* the upstream sequence extends to the first *Sau*3A site at approximately −600, and the *Hin*dIII site in the construct is part of the pUC19 polylinker.

Plant Transformation

Tomato cv T5 was transformed essentially as described by Deikman and Fisher (1988). Instead of excised cotyledons, roots of 5- to 7-d-old seedlings, grown on plates set at an angle of 30° to 45°, were used. T_2 seedlings and mature F_2 plants derived from two *RBCS1-GUSA*, one *RBCS2-GUSA*, and two *RBCS3A-GUSA* T_1 plants were used for the histochemical and fluorometric GUS assays.

Histochemical GUS Assays

Histochemical staining for GUS activity was performed as described (Jefferson, 1987) with the following modifications. Leaflets and fruit from greenhouse-grown plants were surface sterilized by a 5-min incubation in 2% sodium hypochloride followed by five rinses in sterile H_2O. Fruit were shock frozen in liquid nitrogen, thawed on ice, and cut into 2- to 3-mm slices for vacuum infiltration in 50 mM $NaPO_4$, pH 7.0, 1 mM X-Gluc, 1 mM EDTA, 0.05% Triton X-100. Seedlings and leaves were used directly for vacuum infiltration. Incubation at 37°C was followed visually and terminated when sufficient color had developed. Plant material was then destained from Chl by subsequent incubation in 30% ethanol for 1 h, 70% ethanol for 1 h, and 100% ethanol overnight.

Fluorometric GUS Assays

Fluorometric GUS assays were performed as described by Montgomery et al. (1993) with the following modifications. Individual leaflets or fruit from two to five transgenic T_2 plants for each construct were ground in liquid nitrogen, transferred to disposable cuvettes, and homogenized in 2 mL extraction buffer/g leaf material and 1 mL extraction buffer/g fruit material. Samples were centrifuged in 1.5-mL microtubes for 15 min at 12,000*g* and 4°C and the supernatant was directly used for the enzyme assay and protein determination.

In Situ Hybridization

In situ hybridizations were performed essentially as previously described (Cox and Goldberg, 1988; Fleming et al., 1993). Briefly, tomato fruit of 3 to 5 mm were cut in half and then fixed under vacuum in a fixative buffer consisting of 4% (w/v) formaldehyde, 0.25% (w/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.2). The samples were dehydrated through ethanol, exchanged with xylene, and

embedded in paraffin. Sections (7 μm) were cut from paraffin blocks and mounted onto poly-L-Lys-coated slides. After a prehybridization treatment with proteinase K and acidic anhydrate, the slides were hybridized with ^{35}S-labeled riboprobes generated using either T3 or T7 RNA polymerase. The riboprobes used (*RBCS* and *rRNA*) were as described by Fleming et al. (1993).

After hybridization overnight at 42°C, the slides were washed in a series of 4× SSC solutions at room temperature, then in an RNase solution (1 μg/mL) for 30 min at 37°C. After four washes at 37°C in RNase buffer (30 min/wash), the slides were washed in 2× SSC for 30 min at room temperature, then finally at 0.1× SSC for 20 min at 37°C. After drying, the slides were coated with Kodak NTB2 x-ray emulsion and exposed for periods of 1 to 4 weeks before development. The slides were then stained with toluidine blue before analysis by either dark-field microscopy or a combination of bright-field and epifluorescence microscopy. For each gene analyzed, both sense and antisense probes were generated and used in parallel hybridizations with comparable tissue sections. In all cases the signals observed when using the sense probes were not significantly different from background.

Preparation of Nuclear Extracts

Nuclear extracts were prepared from cotyledons of 7-d-old, light-grown tomato seedlings; young, fully expanded tomato leaves; 3- to 8-mm tomato fruit (young fruit); and firm, red tomato fruit (red fruit) according to Manzara et al. (1991).

Gel Mobility-Shift Assays

Standard gel mobility-shift reactions were done in a volume of 12 μL, using 100 pg of ^{32}P-end-labeled, double-stranded oligonucleotides, 1.2 μg of poly(dI-dC), and 1 to 8 μg of nuclear protein in 20 mM Hepes, pH 7.6, 40 mM NaCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT. Unlabeled oligonucleotides as competitors were added as indicated. Binding was for 30 min at room temperature. Reactions were loaded onto 5% polyacrylamide gels having a ratio of 19:1 acrylamide to bisacrylamide in 22 mM Tris base, 22 mM boric acid, 0.05 mM EDTA, pH 8.0. Gel electrophoresis was at 150 V.

DNase I Footprinting Assays

DNase I footprinting reactions and gel electrophoresis were done according to Manzara et al. (1991). For the competition experiment, a 5000-fold molar excess of double-stranded oligonucleotide was added to the binding reaction. Expression of recombinant GBF was as described by Meier and Gruissem (1994).

RESULTS

5′ Upstream Sequences Are Sufficient to Direct Organ-Specific and Differential Expression of *RBCS* Genes

Translational fusions between 5′ upstream regions of the tomato *RBCS1*, *RBCS2*, and *RBCS3A* genes and the bacterial *GUSA* were constructed. The 5′ upstream DNA fragments were 3, 0.6, and 1 kb in size for *RBCS1*, *RBCS2*, and *RBCS3A*, respectively. These fragments contain the entire promoter region, which was mapped for protein-binding sites in DNase I footprint assays (Manzara et al., 1991, 1993; Carrasco et al., 1993), plus additional upstream sequences to avoid the exclusion of potentially important *cis* elements in the fusion constructs. The *Nhe*I site present in the first exon of all tomato *RBCS* genes (Sugita et al., 1987) was used to fuse the DNA promoter fragments containing the first 21 codons for the SSU protein with the GUS protein coding region (Fig. 1). The constructs were introduced into tomato cv T5 via *Agrobacterium*-mediated transformation and GUS activity was assayed in T_2 seedlings and mature T_2 plants.

GUS activity was determined histochemically to determine that the pattern of GUS expression was representative of the pattern of *RBCS* promoter activities for the endogenous genes. Ten-day-old, light-grown seedlings (*RBCS1*, *RBCS2*, and *RBCS3A* promoters active in cotyledons and hypocotyl, no *RBCS* promoter activity in roots), leaflets of mature plants (all three *RBCS* promoters active), and young tomato fruit (only *RBCS1* and *RBCS2* promoters active) were assayed. Figure 2 shows the results of GUS activities for each of the *RBCS-GUSA* constructs. All plants assayed for each construct showed the same pattern of gene expression with some quantitative differences in overall activities (Fig. 3). In light-grown seedlings, strong GUS activity was detected in cotyledons and weaker activity was seen in the hypocotyl. No GUS activity was detected in roots. No significant quantitative or qualitative differences were observed between seedlings carrying the *RBCS1-*, *RBCS2-*, and *RBCS3A-GUSA* fusion genes. GUS activity was evenly distributed in leaflets of mature plants, and no significant difference in activity could be detected between the three *RBCS* promoters. In contrast, in young tomato fruit the *RBCS1-GUSA* and *RBCS2-GUSA* fusion genes were strongly expressed, whereas expression of the *RBCS3A-GUSA* fusion gene was undetectable.

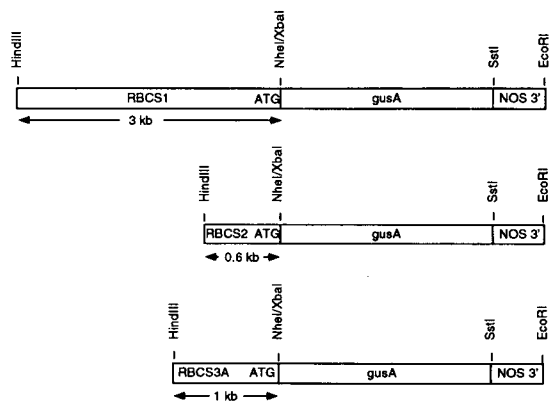

Figure 1. Schematic representation of the constructs used for tomato transformation. Only the inserts between the *Hin*dIII site and the *Eco*RI site of pML6–1-1 are shown. The sizes of the respective 5′ upstream regions of the *RBCS* genes are indicated by the arrows. The *RBCS-GUSA* fusions are translational fusions, including the first 21 amino acids of SSU. The *Nhe*I/*Xba*I fusion site is indicated.

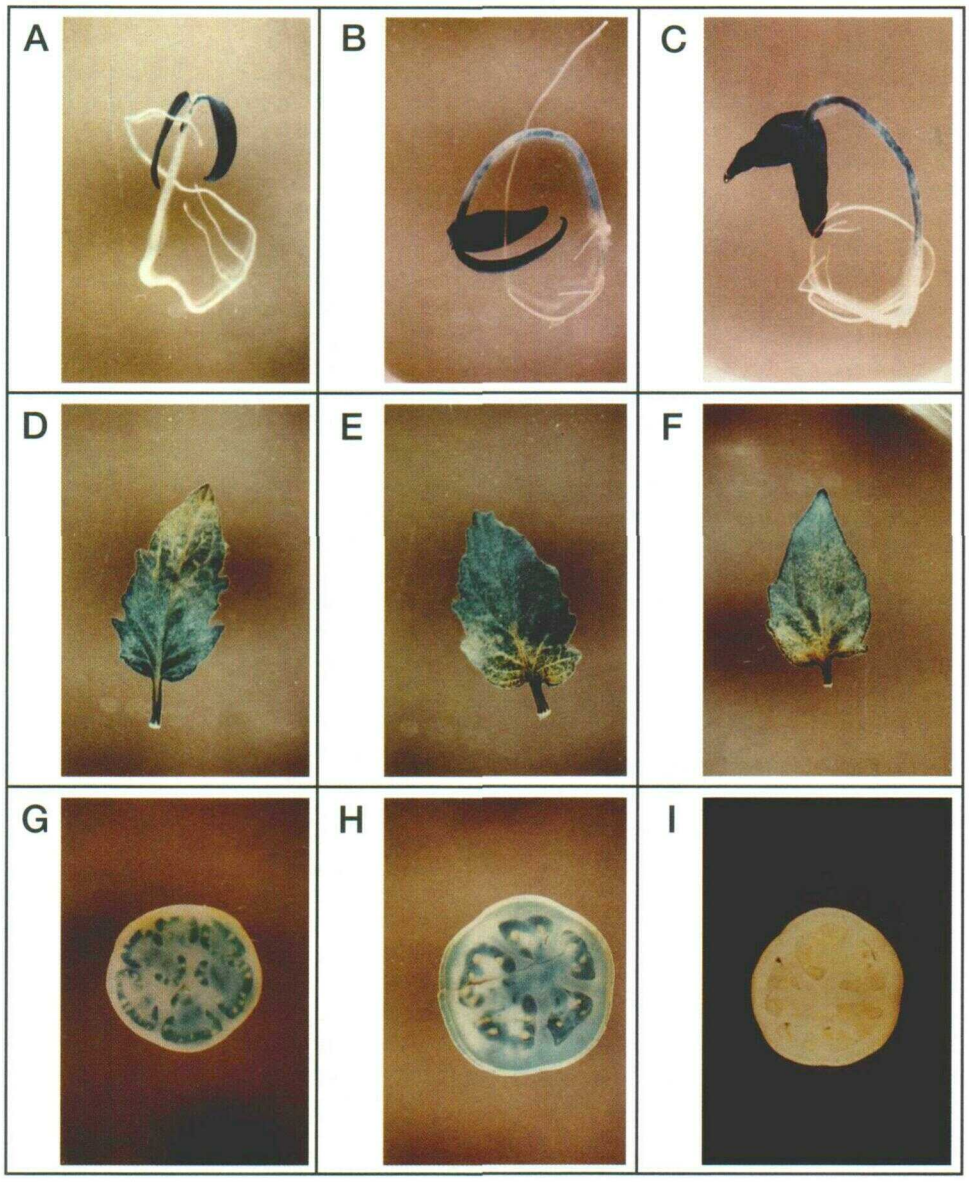

Figure 2. Histochemical detection of GUS activity from representative T_2 plants. A to C, Ten-day-old, light-grown seedlings; D to F, leaflets of mature plants; G to I, cross-sections through fruits of 1.5 cm diameter. A, D, G, *RBCS1-GUSA* plant; B, E, H, *RBCS2-GUSA* plant; C, F, I, *RBCS3A-GUSA* plant.

To determine that the observed differences in GUS-directed staining patterns between plants carrying the *RBCS1-GUSA*, *RBCS2-GUSA*, or *RBCS3A-GUSA* fusion genes do reflect differences in the activities of the endogenous promoters in a quantitative manner, GUS activities in leaflets and fruit were measured in fluorometric GUS assays (Fig. 3). In leaflets, GUS activities for *RBCS1-GUSA*, *RBCS2-GUSA*, and *RBCS3A-GUSA* were on average 300-, 450-, and 350-fold, respectively, higher than background activities of untransformed control plants. In fruit the activities of the *RBCS1-GUSA* and *RBCS2-GUSA* fusion genes were 1400- and 4000-fold, respectively, higher than background activities, but the activity of the *RBCS3A-GUSA* fusion gene was only 35-fold higher than background activity. Thus, activities in fruit from plants carrying the *RBCS1*- and *RBCS2-GUSA* fusion genes were 40- and 100-fold higher, respectively, compared to fruit expressing the *RBCS3A-GUSA* fusion gene. These results are consistent with results from nuclear run-on transcription experiments, which showed that the endogenous *RBCS1* and *RBCS2* promoters are approximately 30- and 50-fold more active, respectively, than the *RBCS3A* promoter (Wanner and Gruissem, 1991). Thus, the observed GUS activities tightly follow the activities of the endogenous *RBCS* genes.

RBCS Promoters Direct Tissue-Specific Expression in Developing Tomato Fruit

GUS activity was not equally distributed among the different tissues of developing fruit in plants expressing

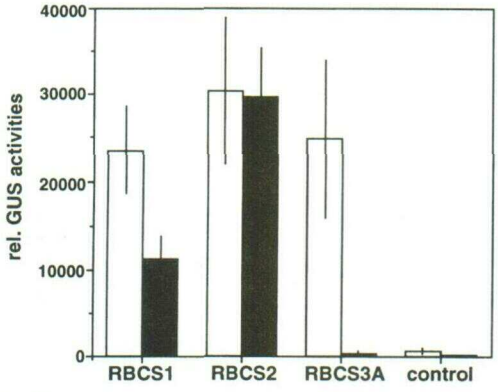

Figure 3. Fluorometric determination of GUS activity. White bars represent activities in leaflets and black bars represent activities in fruit of 1.5 cm diameter. Relative GUS activity is shown as the mean value from assays of six individual leaflets and seven individual fruits from two to five different transgenic plants each. Control indicates results from nontransformed plants.

the *RBCS1-GUSA* and *RBCS2-GUSA* fusion genes. As shown in Figure 2, the highest activity was detectable in the locular tissue for both the *RBCS1-* and *RBCS2-GUSA* fusion genes. Reduced GUS activity was detected for the *RBCS1-GUSA* fusion gene in the inner pericarp and for the *RBCS2-GUSA* fusion gene in both the inner and outer pericarp. The quantitative GUS assays (Fig. 3) showed that the activity of the *RBCS3A* promoter in fruit was significantly reduced, but it was still clearly detectable over GUS background levels in control plants. The weak expression of the *RBCS3A-GUSA* fusion gene could be detected histochemically when the time of the enzymatic assay was extended (Fig. 4). Under these conditions, the observed GUS activity had a similar tissue-specific distribution compared to GUS activities derived from the *RBCS1-* and *RBCS2-GUS* fusion genes, with the highest activity in the locular tissue (Fig. 4B). Under these staining conditions there was still no GUS activity detectable in the outer pericarp of fruit expressing the *RBCS1-GUSA* fusion gene, indicating that the activity of the *RBCS1* promoter is strongly reduced in this tissue (Fig. 4A). No staining was detected with an untransformed tomato fruit (Fig. 4C), which demonstrates that the weak staining detected with the *RBCS3A-GUSA* fusion gene in fruit was not due to endogenous GUS-like enzymatic activities. Together, these results suggest that the three *RBCS* promoters are targets for the same signals in developing tomato fruit that direct their spatial activities, but they appear to respond to the tissue-specific signals with a different degree of transcriptional activity.

RBCS mRNA Accumulates Predominantly in the Locular Tissue of Young Tomato Fruit

To determine that the tissue-specific distribution of GUS activity in developing tomato fruit truly reflects differences in *RBCS* promoter activities and is not caused by artifacts of the GUS assay, we analyzed the distribution of total *RBCS* mRNA by in situ hybridization (Fig. 5). A DNA probe for cytoplasmic ribosomal RNA (rDNA) was used as a standard to assess the quantitative distribution of RNA within the tissue sections. The rDNA probe showed a strong signal in the embryo, the ovule wall, the outermost cell layers of the pericarp, and vascular tissue and showed a moderate signal in the locular tissue and the main body of pericarp (Fig. 5, C and F), most likely reflecting differences in cell size of the different tissues. The *RBCS* cDNA probe showed a very low signal in the embryo, the ovule wall, vascular tissue, and the pericarp and a moderate to high signal in the locular tissue (Fig. 5, B and E). This distribution of *RBCS* mRNA correlates well with the summed pattern of GUS activity observed with the *RBCS-GUSA* fusion genes (Fig. 2). Together, the observed pattern of GUS activity derived from the *RBCS-GUSA* fusion genes truly represents the pattern of mRNA accumulation from the *RBCS1*, *RBCS2*, and *RBCS3A* genes in young tomato fruit.

A DNA-Protein Interaction Immediately Upstream of the G-Box Correlates with the Reduced Activity of the RBCS3A Promoter in Developing Tomato Fruit

DNase I footprint analyses had demonstrated that several sites within the five *RBCS* promoters are protected by nuclear proteins in an organ-specific manner (Manzara et al., 1991, 1993; Carrasco et al., 1993). We have analyzed these data in the context of the differential regulation of the

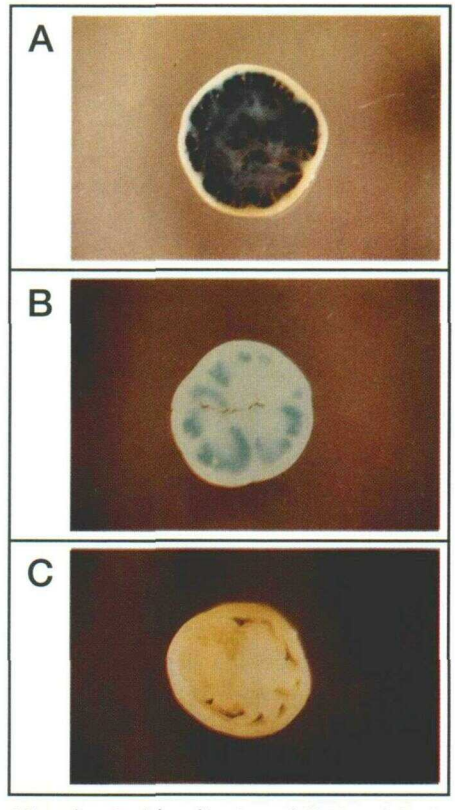

Figure 4. Histochemical localization of GUS activity in cross-sections of fruit of 1.5 cm diameter after 24 h of incubation with the substrate. A, *RBCS1-GUSA* plant; B, *RBCS3A-GUSA* plant; C, untransformed control plant.

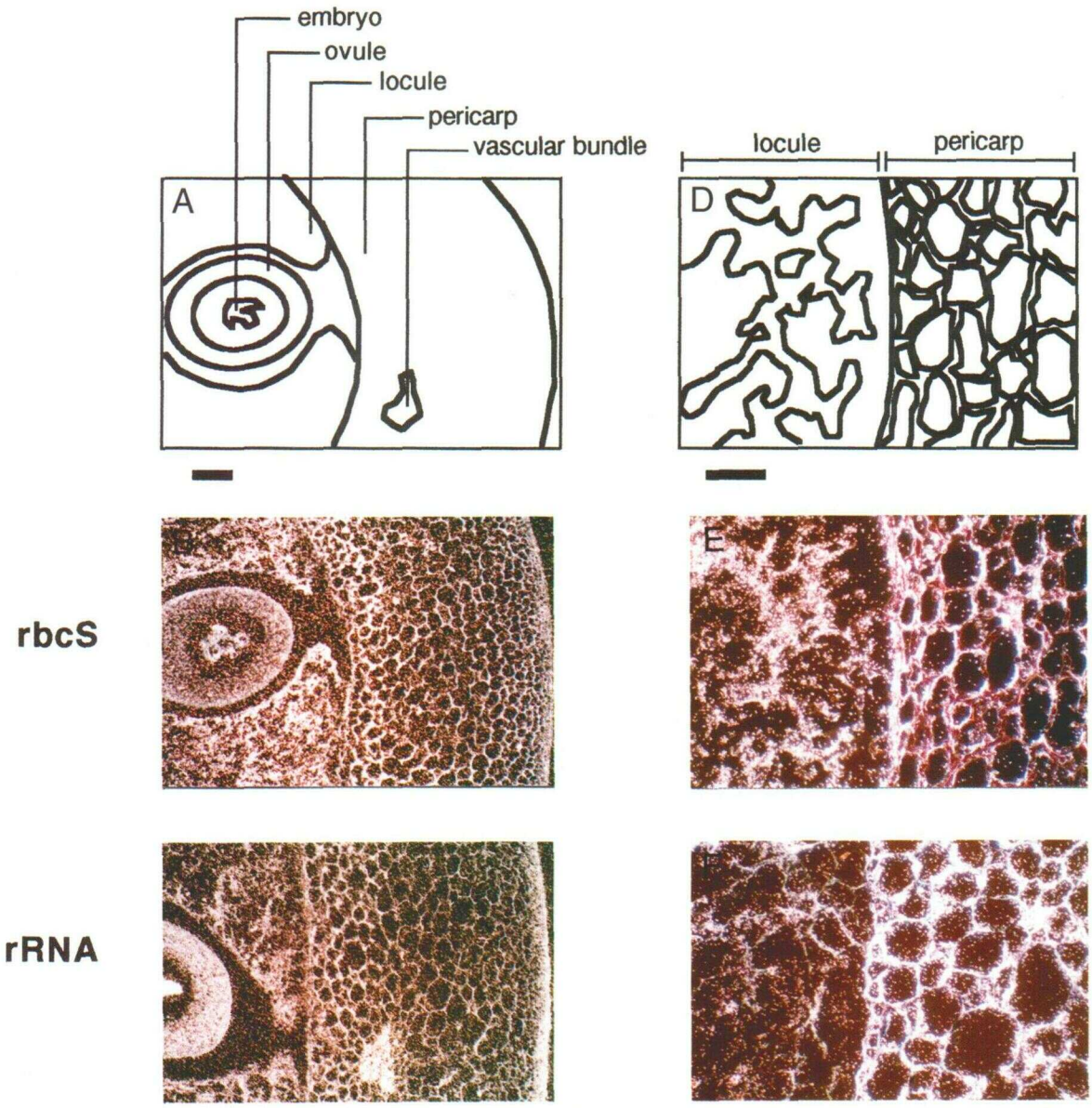

Figure 5. In situ hybridization analysis of *RBCS* expression in fruit tissue. A, Schematic representation of a cross-section through part of a tomato fruit to show the position of the pericarp, locule, vascular tissue, ovule, and developing embryo. Bar = 100 μm. B, In situ localization of *RBCS* transcripts in a section of fruit similar to that shown in A. C, In situ localization of *rRNA* in a section of fruit tissue similar to that shown in A. In B and C the signal (silver grains) has been visualized by dark-field microscopy. D, Schematic representation of a cross-section through a tomato fruit to show the interface of the pericarp and locule. Bar = 50 μm. E, In situ localization of *RBCS* transcripts in a section of fruit tissue similar to that shown in D. F, In situ localization of *rRNA* in a section of fruit tissue similar to that shown in D. In E and F, the signal (silver grains) has been visualized by a combination of bright-field and epifluorescence microscopy using an IGS block (Nikon).

RBCS1, *RBCS2*, and *RBCS3A* genes in young tomato fruit to determine if the binding of one or more proteins may correlate with the various activities of the promoters. One DNase I protection was identified in the *RBCS3A* promoter that fits these criteria. Figure 6A summarizes the organization of the *RBCS1*, *RBCS2*, and *RBCS3A* promoter regions spanning the conserved I- and G-boxes and the DNase I protection pattern detected in these regions (Manzara and Gruissem, 1988). Within this region, the G-box is protected in all three promoters when incubated with a nuclear protein extract from leaves. In addition, the area of the I-box in the *RBCS1* and *RBCS2* promoters is partially protected by leaf nuclear protein(s). With a nuclear protein extract from young fruit, the DNase I protection pattern in these regions of the *RBCS1* and *RBCS2* promoters is similar to that in leaf. In contrast, the DNase I protection in the *RBCS3A* promoter extends further upstream with the young fruit nuclear protein extract and covers approximately half of the I-box as well as the DNA sequence between the I-box and the G-box.

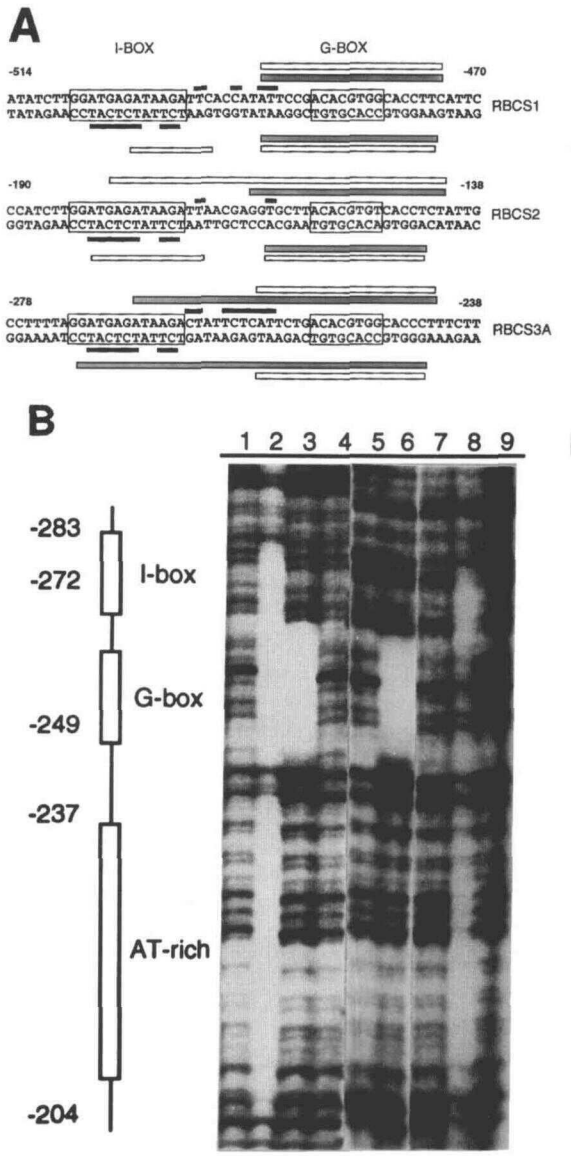

Figure 6. DNase I protection analysis of the I-box/G-box region within the *RBCS1*, *RBCS2*, and *RBCS3A* promoters. A, Schematic representation of the protected regions as described by Manzara et al. (1991). Sequences protected with leaf nuclear extract are indicated by open bars, sequences protected with young fruit nuclear extract are indicated by gray bars. The I-box and the G-box consensus sequences are boxed. Nucleotide positions upstream of the transcriptional start sites are indicated by numbers. Black lines above and below the DNA sequence indicate sequences homologous to the F-box (see text). B, DNase I footprint analysis of the I-box/G-box region within the *RBCS3A* promoter. The bottom strand between position −283 and position −204 upstream of the start site of transcription is shown. Lanes 1, 4, 5, and 9, No protein; lane 2, 9 μg of young fruit nuclear extract; lanes 3 and 6, 4.5 μg of *E. coli* protein extract containing recombinant tomato GBF12 (Meier and Gruissem, 1994); lane 8, 9 μg of young fruit nuclear extract plus 5000 fmol of G-box oligonucleotide; lanes 1 through 4 and lanes 5 through 9, respectively, are derived from two different experiments. The extent of DNA visible on the gel is indicated by the solid line, and the positions of the I-box, the G-box, and an AT-rich region protected by young fruit nuclear extract (Manzara et al., 1991) are indicated by open boxes.

It has been shown previously that GBFs interact with all three *RBCS* promoters in young tomato fruit (Meier and Gruissem, 1994). GBFs have been shown to act as transcriptional activators in a variety of plant promoters (Donald and Cashmore, 1990; Guiltinan et al., 1990; Oeda et al., 1991; Weisshaar et al., 1991; McKendree and Ferl, 1992). Three different cDNAs encoding GBFs were isolated from a young tomato fruit cDNA library. When expressed in *Escherichia coli*, the proteins produce a footprint over the G-box of all three promoters identical to that observed with a leaf nuclear protein extract (Meier and Gruissem, 1994). Therefore, the extended DNase I protection in the fruit nuclear protein extract over the G-box of the *RBCS3A* promoter could either be produced by a different protein with an overlapping binding site or consist of two proteins that bind very closely to each other, one of them being GBF. To distinguish between these possibilities, we performed a DNase I footprint-competition experiment. Figure 6B shows the extended protection pattern observed with a nuclear protein extract from young tomato fruit (lane 2) compared to the protection observed with recombinant GBF alone (lanes 3 and 6). A 5000-fold molar excess of a G-box-containing oligonucleotide (see Fig. 7) specifically eliminates the downstream footprint representing the G-box but does not affect the upstream protection (Fig. 6B, lane 8). This demonstrates that the fruit-specific footprint in the *RBCS3A* promoter consists of two proteins, a GBF-like activity binding to the G-box and a second protein binding independently and immediately upstream of GBF. We named the second DNA-binding activity FBF for fruit-specific binding factor.

The DNase I protection analysis has indicated that FBF does not interact with the *RBCS1* and *RBCS2* promoters in young fruit nuclear extract (Manzara et al., 1991, 1993; this study). It is unlikely, therefore, that FBF represents one of the I-box-binding activities that have been described in nuclear extracts from tomato (Borello et al., 1993), because the I-box is 100% conserved between the three promoters. Close examination of the DNA sequence for the three promoters revealed that part of the I-box sequence in *RBCS3A* is duplicated as a palindrome in the spacer sequence between the I-box and the G-box (indicated by the black bars in Fig. 6A). This palindromic duplication is not present in *RBCS1* and *RBCS2* because the DNA sequence separating the I-box and the G-box is not conserved between the three promoters (Fig. 6A).

Because palindromic sequences are known to often represent protein-binding sites, we investigated the binding of factors in nuclear extracts to an oligonucleotide containing the *RBCS3A* I-box palindrome using gel mobility-shift assays. Figure 7A shows the DNA sequence of the *RBCS3A* promoter spanning the I-box, the G-box, and the palindromic duplication of the partial I-box sequence (F-box), as well as the sequence of the different DNA fragments used in the gel mobility-shift assays. The DNA fragment F formed a complex that was specific for nuclear protein extracts from fruit (Fig. 7B), but did not form with nuclear protein extracts from leaves and light-grown cotyledons, organs in which the *RBCS3A* promoter is active. A complex

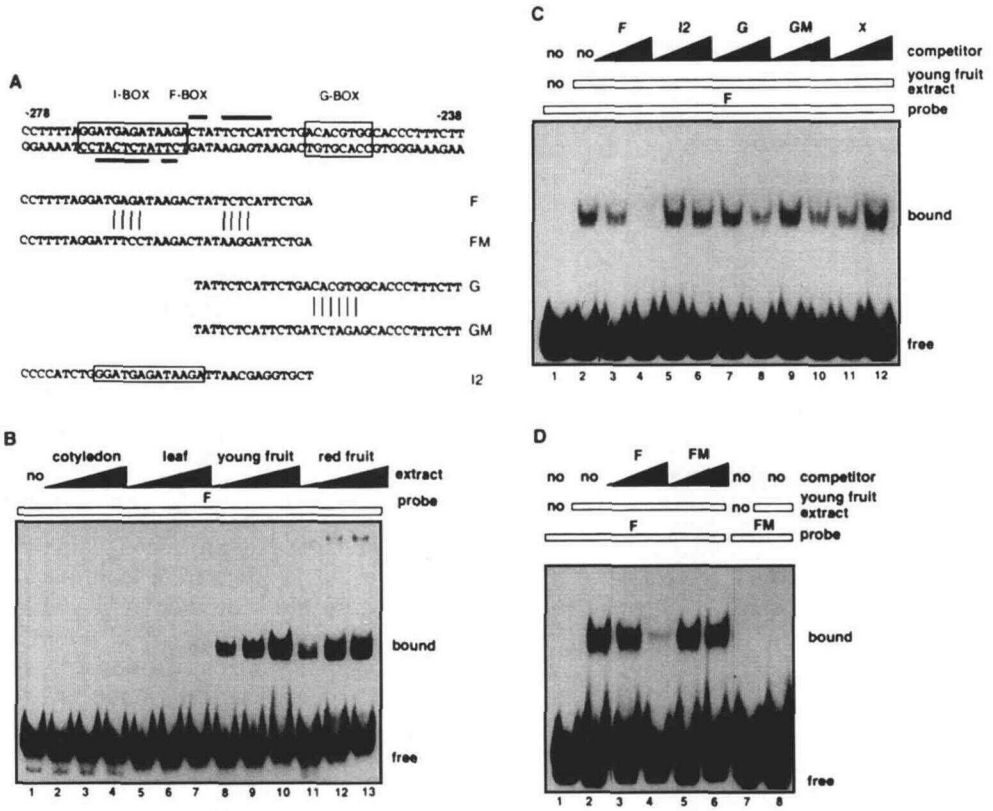

Figure 7. A fruit-specific DNA-binding activity interacting with the *RBCS3A* promoter. A, Nucleotide sequence of the I-box/G-box region of the *RBCS3A* promoter. The consensus sequence of the I-box and G-box are boxed. The palindromic F-box is indicated by the black bars above and below the sequence. The top strand of the oligonucleotides used in the gel mobility-shift assays are shown below the sequence. Nucleotides altered in the mutated oligonucleotides are indicated by the vertical lines. The I-box in the oligonucleotide I2 is boxed. B, Mobility-shift assay with the F-oligonucleotide and nuclear extracts from cotyledons, leaves, young tomato fruit, and red tomato fruit. Within each group of three lanes, the protein amounts were 1, 2, and 4 μg, respectively. C, Mobility-shift competition experiment. Lanes 2 through 12 contain 1 μg of young fruit nuclear protein. Each pair of lanes 3 through 12 contains a 100-fold and a 1000-fold molar excess of the indicated oligonucleotides as competitors. D, Mobility-shift experiment with the F and FM oligonucleotides. Four micrograms of young fruit nuclear extract was added in lanes 2 through 6 and lane 8. Lanes 3 and 4 contain a 100-fold and a 1000-fold excess of oligonucleotide F, respectively. Lanes 5 and 6 contain a 100-fold and a 1000-fold excess of oligonucleotide FM, respectively.

of similar mobility and intensity as fragment F was also detected in nuclear protein extracts from mature red fruit, indicating that the binding activity is present throughout fruit development. Figure 7C shows that the binding was specifically competed for by the F fragment but not by DNA fragments containing the I-box of the *RBCS2* promoter (I2), the G-box (G), a mutated G-box (GM), and an unrelated sequence (X). This confirms that the binding activity is not an I-box-binding protein and shows that the sequence shared between the F fragment and the G fragment does not contain the entire binding site. To determine if the palindrome is part of the binding site, we introduced a 4-bp block mutation on both sides of the palindrome (Fig. 7A). The resulting DNA fragment, FM, does not compete for the binding to the F fragment and is unable to form a complex in the fruit nuclear protein extract. Thus, we conclude that FBF is a fruit-specific nuclear-binding activity, that it selectively interacts with the F-box region of the *RBCS3A* promoter, and that the palindromic duplication of the sequence GAGA is part of its binding site.

GBF and FBF Bind Adjacent to Each Other on the *RBCS3A* Promoter but Do Not Show Cooperative Interaction

The binding of FBF correlates with the reduced activity of the *RBCS3A* promoter in tomato fruit (Fig. 3). Its direct proximity to the transcriptional activator GBF raises the interesting possibility that FBF might directly interact with GBF, thereby interfering with the function of GBF and repressing transcription of *RBCS3A*. One of several possible mechanisms for this interaction, which can be easily tested, is the reduction of the binding constant of the GBF-DNA complex by the binding of FBF to the F-box. To test this possibility, we monitored complex formation in gel mobility-shift assays with a short DNA fragment containing the I-box, the F-box, and the G-box, as well as

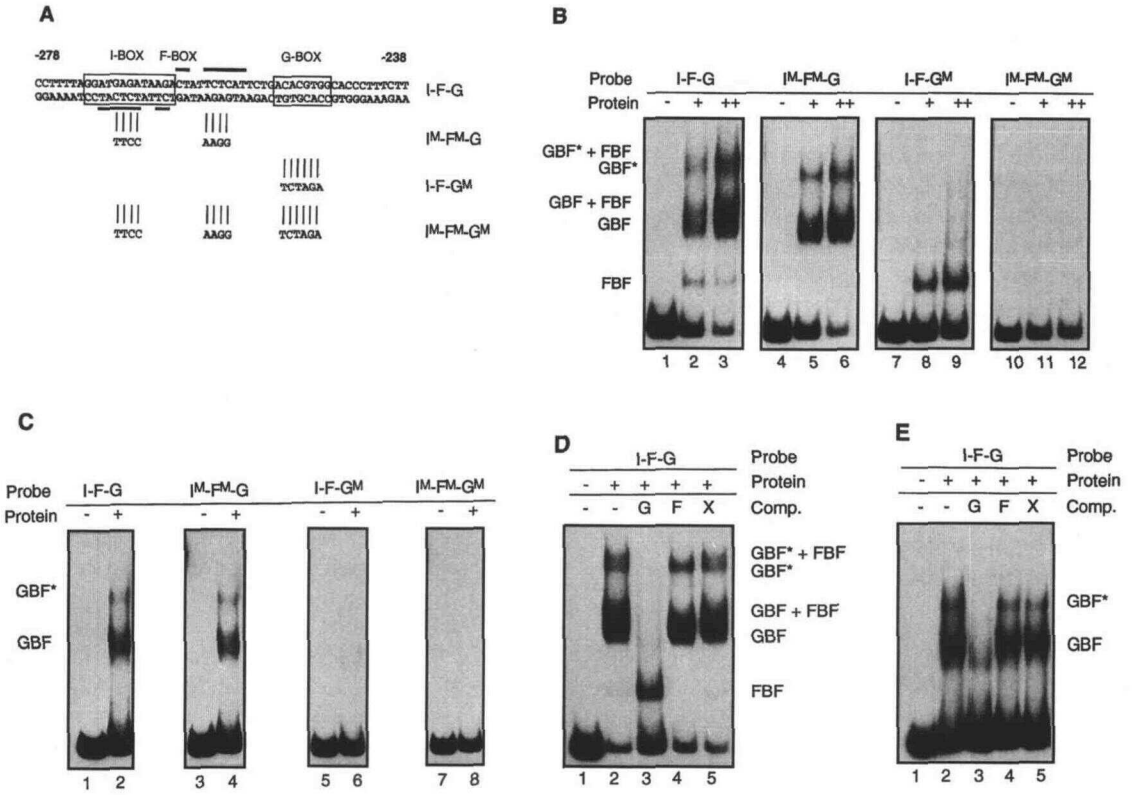

Figure 8. GBF and FBF bind independently to their sites on the DNA. A, Oligonucleotides used for gel mobility-shift experiments. The sequence of the oligonucleotide I-F-G containing the I-box, the F-box, and the G-box is shown. The sequence of the top strand of the base pair substitutions in the mutants IM-FM-G, I-F-GM, and IM-FM-GM is indicated below the vertical lines. B, Gel mobility-shift assay with the four oligonucleotides and young fruit nuclear extract. Lanes 2, 5, 8, and 11 contain 0.6 μg and lanes 3, 6, 9, and 12 contain 1.2 μg of young fruit nuclear proteins. The proteins present in the individual protein-DNA complexes are indicated on the left. C, Gel mobility-shift assay with leaf nuclear extract. In lanes 2, 4, 6, and 8, 0.8 μg of leaf nuclear protein was added. The two different GBF-DNA complexes are indicated on the left. D, Gel mobility-shift competition assay with young fruit nuclear extract. In lanes 2 through 5, 0.6 μg of young fruit nuclear protein was added. Lanes 3 through 5 contain 100 ng of unlabeled oligonucleotides as indicated. E, Gel mobility-shift competition assay with leaf nuclear extract. In lanes 2 through 5, 0.8 μg of leaf nuclear protein was added. Lanes 3 through 5 contain 100 ng of unlabeled oligonucleotides as indicated.

mutants of this fragment as shown in Figure 8A. With the wild-type I-F-G fragment and a nuclear protein extract from young tomato fruit, five complexes were formed that migrated as two doublets and a single fast mobility band (Fig. 8B, lanes 2 and 3). Mutations in the F-box sequence eliminate the fast-migrating complex and the upper band of each doublet (Fig. 8B, lanes 5 and 6). Mutation of the G-box sequence eliminates the two doublets but not the fast-migrating complex (Fig. 8B, lanes 8 and 9), and mutation of both boxes eliminates all specific protein-DNA interactions (Fig. 8B, lanes 11 and 12). Competition with the G-box DNA fragment alone eliminates the two doublets, whereas the F-box DNA fragment competes only for the upper band of each doublet and the fast-migrating complex (Fig. 8D). Thus, the fast-migrating complex consists of FBF bound to the F-box, the lower band of each doublet represents occupation of the G-box by GBF, and the upper bands of the doublets represent the simultaneous occupation of F-box and G-box by FBF and GBF, respectively.

These data show that FBF and GBF bind independently of each other. Comparison of the intensities of the individual bands shows that there is no strong positive or negative cooperativity in the binding of FBF and GBF to their respective binding sites, suggesting that interaction of FBF with the F-box sequences does not significantly destabilize the GBF-DNA complex. The physical difference of the two GBF-DNA complexes detected in this experiment (marked by GBF and GBF* in Fig. 8) is not known. They may represent different GBFs, protein-protein interactions between GBFs or GBF and additional proteins in the reduced mobility complex, or modifications of the GBF protein complex.

An analogous experiment with a nuclear protein extract from tomato leaves reveals formation of two complexes with the wild-type I-F-G fragment (Fig. 8C, lane 2) that are not influenced by mutations in the F-box (Fig. 8C, lane 4), but which are eliminated by mutations in the G-box alone (Fig. 8C, lanes 6 and 8). This result confirms that the two different complexes represent GBF-DNA interactions. Both complexes are competed for by the G-box DNA fragment but not by the F-box DNA fragment or an unrelated DNA fragment X (Fig. 8E). The formation of a weak fast-migrat-

ing complex detected in lane 3 represents a nonspecific binding activity present in the leaf nuclear protein extract. The results demonstrate that, unlike in fruit, no FBF-like activity can be detected in a leaf nuclear protein extract with a DNA fragment capable of binding both FBF and GBF. From these data we conclude that the *RBCS3A* promoter region containing the I-box, the F-box, and the G-box binds one or more GBF(s) in fruit as well as in leaf. In addition, FBF binds to the F-box adjacent to GBF(s) in fruit. Under the experimental conditions, the two proteins show no significant positive or negative cooperativity for binding to their respective sites on the DNA. FBF is the only factor, however, whose binding in the *RBCS3A* promoter region correlates with the reduced transcription of the gene during tomato fruit development.

DISCUSSION

RBCS Promoter Regions Are Sufficient for Correct Temporal and Spatial Regulation

The differential expression of individual members of the *RBCS* gene family in tomato is controlled by signals that specify organ identity and organ development, as well as by external factors such as light (Sugita and Gruissem, 1987; Wanner and Gruissem, 1991). In the work presented here we have addressed two questions raised by the complex *RBCS* gene expression pattern in tomato. First, do the diverse signals that control the *RBCS* expression patterns act exclusively through regulatory DNA sequences located in the promoter regions of the five genes, or do other elements such as introns, downstream sequences, or extended domains in the chromatin at the respective chromosomal locations contribute to the regulation of transcription? And second, are specific DNA-protein interactions in the *RBCS* promoter regions correlated with the differential transcription of their genes? The analysis was focused on *RBCS1*, *RBCS2*, and *RBCS3A* because these three genes share a similar promoter structure, therefore providing a good experimental basis to investigate potential differences in the molecular mechanisms that control the transcription of these genes.

Our transformation experiments in tomato with *RBCS* promoter-*GUSA* fusion genes have shown that as little as 600 bp of promoter region (as in the case of the *RBCS2* promoter) is sufficient to control the correct temporal and spatial expression of GUS activity compared to the activity of the endogenous promoter. This result is in agreement with several other plant promoter studies in which promoter regions in the range of several hundred base pairs to about 1 kb have been found to reproduce faithful expression patterns of reporter genes in vivo (Schulze-Lefert et al., 1989; Ballas et al., 1993; Montgomery et al., 1993), whereas smaller fragments often lose some aspect of the regulation (Kuhlemeier et al., 1989). In the case of the tomato *RBCS1*, *RBCS2*, and *RBCS3A* genes, their promoter fragments contain regions that were previously shown to interact with proteins (Manzara et al., 1991, 1993) and additional sequences upstream of these protein-binding sites. These promoter regions are apparently sufficient to reproduce GUS activity in tomato seedlings, leaves, and fruit in a qualitative as well as quantitative manner compared to the in vivo accumulation of mRNAs for the three genes (Sugita and Gruissem, 1987) and to their relative transcriptional activities (Wanner and Gruissem, 1991). Although the number of transformed plants analyzed in this study was small, the precise correlation between the qualitative and quantitative GUS activity pattern in transgenic plants and the well-characterized expression pattern of the endogenous *RBCS* genes allows us to conclude that the observed activities truly reflect activities of the respective promoter fragments that are not altered by position effects. This strongly suggests that all information required for the temporal and spatial regulation of promoter activities is contained within the promoter fragments used in this study.

Unlike dark-grown cotyledons, where transcription of *RBCS3A* is uncoupled from *RBCS3B* and *RBCS3C*, transcription activity of the linked locus 3 genes is coordinately and quantitatively down-regulated in developing tomato fruit as compared to *RBCS1* and *RBCS2* both in vivo (Sugita and Gruissem, 1987; Wanner and Gruissem, 1991) and at the level of GUS enzyme activity. The fact that *RBCS3A*, *RBCS3B*, and *RBCS3C* are tandemly arranged in a chromosomal location of less than 10 kb and separate from *RBCS1* and *RBCS2* made it tempting to speculate that their silencing in developing fruit could be the consequence of an organ-specific chromatin reorganization that results in the inactivation of the locus 3 genes. Such a mode of regulation has been demonstrated, e.g. for the tissue-specific silencing of genes in *Drosophila* via local heterochromatin formation (for review, see Shaffer et al., 1993). Although we have not mapped the position of the inserted *RBCS3A-GUSA* fusion gene in the transgenic lines, it is unlikely that integration has occurred in or near the chromosomal location of the endogenous *RBCS3A* gene. Thus, the similar spatial and temporal regulation of the *RBCS3A-GUSA* fusion gene argues against chromatin rearrangement as a mechanism for the coordinated regulation of the locus 3 *RBCS* genes. Instead it points toward a mechanism involving transcriptional regulators directly interacting with the promoter DNA of the *RBCS* genes. Regulatory proteins could thereby either repress transcription of *RBCS3A* and the other locus 3 genes or specifically activate transcription of *RBCS1* and *RBCS2* in developing tomato fruit. Comparison of the five *RBCS* promoter sequences did not reveal sequence elements shared either by *RBCS1* and *RBCS2* or by the three locus 3 genes only (Manzara and Gruissem, 1988; I. Meier and W. Gruissem, unpublished results), indicating that the mechanism can be expected to be more complex than one involving a single regulatory protein-DNA interaction.

Cellular Sink Activity and *RBCS* Promoter Activity Are Exclusive

Based on the GUS enzyme activity pattern and the distribution of *RBCS* mRNAs, we also conclude that the activities of the *RBCS1* and *RBCS2* promoters are differentially regulated between tomato fruit tissues. The highest activity of both promoters was detected in the locular

tissue. The *RBCS1* promoter has an additional low activity in the columnella and placental tissues, whereas the *RBCS2* promoter has an additional low activity in columnella, placental tissue, and pericarp tissues. Based on the close correlation between the distribution of endogenous *RBCS* mRNAs and GUS enzyme activities in transgenic plants, it is unlikely that this differential *RBCS1* and *RBCS2* expression pattern in cross-sections of developing fruit is due to unequal permeability of the different cells for the GUS substrate, other possible artifacts of the histochemical GUS assay, or position effects in the transgenic plants. The physiological relevance of this pattern of *RBCS* promoter activity in developing fruit is not known at present. The locular tissue and the pericarp of young tomato fruit contain chloroplasts (Laval-Martin, 1975). Physiological experiments have demonstrated that both the pericarp and the combined locular, columnella, and placental tissues are capable of CO_2 fixation (Laval-Martin et al., 1977). The photosynthetic activity of young tomato fruit is significantly lower compared to that of leaves, and the fruit acts as a sink organ at a very early developmental stage. It is not known, however, whether the different tissues of tomato fruit have different sink status at early stages of development. Wang et al. (1994) have analyzed the pattern of Suc synthase mRNA distribution in developing tomato fruit as a marker for sink tissue activity. Interestingly, the highest level of Suc synthase mRNA and the highest concentration of starch were found in the pericarp and columnella tissues. In contrast, no Suc synthase mRNA accumulation or starch accumulation were detected in the locular tissue. This pattern of Suc synthase gene expression is approximately reciprocal to the expression of the *RBCS* genes based on GUS enzyme activity and *RBCS* mRNA accumulation. It has been demonstrated previously that Suc synthase gene activity can be induced by high concentrations of Suc in potato leaves and petioles (Salanoubat and Belliard, 1989), whereas *RBCS* gene activity is repressed by Suc in mesophyll protoplasts (Sheen, 1990). Therefore, the expression patterns of the tomato *RBCS* and Suc synthase genes might indicate the distribution of Suc in the different tissues of developing fruit and may serve as useful markers for the physiological status and sink activity of different fruit tissues and cell types.

RBCS3A Spatial but Not Quantitative Expression Is Maintained in Developing Fruit

Although the activity of the *RBCS3A* promoter in young tomato fruit is reduced more than 50-fold compared to that of the *RBCS1* and *RBCS2* promoters, the spatial distribution of the remaining *RBCS3A* promoter activity is identical to that of the other two promoters. This similar pattern of promoter activities indicates that, in principal, the same spatial regulatory signals, which are potentially coupled to the physiological status of the cells, act on all three promoters in developing fruit. Only the *RBCS3A* promoter responds to such signals in a quantitatively reduced way. This does not reflect a general lower promoter strength, as can be seen by the equally high activity of all three promoters in cotyledons and leaves. More likely, it points to the action of a negative regulator that is specific for the *RBCS3A* promoter and that quantitatively reduces the response of this promoter to the signals that establish the spatial expression pattern of all three genes in fruit.

RBCS1, *RBCS2*, and *RBCS3A* promoters have similar patterns of conserved DNA sequence elements that differ from the conserved DNA sequence elements shared by the *RBCS3B* and *RBCS3C* promoters (Manzara and Gruissem, 1988; Manzara et al., 1991, 1993; Carrasco et al., 1993). Among other DNA sequence motifs, the *RBCS1*, *RBCS2*, and *RBCS3A* promoters contain a pair of the I-box and G-box DNA sequence elements that were previously reported as DNA sequence motifs present in light-regulated plant promoters (Guiliano et al., 1988). In the tomato *RBCS1*, *RBCS2*, and *RBCS3A* promoters, the I-/G-box elements are highly conserved in sequence as well as in order and spacing (Manzara and Gruissem, 1988). Because *RBCS1*, *RBCS2*, and *RBCS3A* promoters, but not *RBCS3B* and *RBCS3C* promoters, are coordinately activated in dark-grown cotyledons, water-stressed leaves (Bartholomew et al., 1991), and, to a different extent, in developing tomato fruit, it is likely that the I-box and G-box elements are critical for these regulatory processes.

The G-box is currently the best-characterized plant *cis* regulatory DNA sequence element. It is required for full activity of all promoters investigated to date that have the G-box DNA sequence motif (Schulze-Lefert et al., 1989; Donald and Cashmore, 1990; Loake et al., 1992; McKendree and Ferl, 1992). A family of plant basic leucine zipper (bZIP) proteins has been identified that interacts with the G-box element (GBFs) to confer high promoter activity (Schulze-Lefert et al., 1989; Donald and Cashmore, 1990; Loake et al., 1992; McKendree and Ferl, 1992). Their function as general transcriptional activators was proposed based on two lines of evidence. First, the tobacco GBF protein TAF-1 enhances transcription from a chimeric cauliflower mosaic virus "-90" 35S promoter fused to six copies of the TAF-1 binding site (Oeda et al., 1991). Second, fusion of the Pro-rich N-terminal fragment of *Arabidopsis* GBF1 to the DNA-binding domain of the yeast GAL4 protein can activate transcription from a minimal promoter containing the GAL4 binding site in mammalian cells (Schindler et al., 1992).

These results support a model in which plant GBFs have general enhancing activity necessary for full promoter activity by acting as a transcriptional activator through short-range interactions with the basic transcriptional machinery. The G-box is bound by a protein in the *RBCS1*, *RBCS2*, and *RBCS3A* promoters in tomato leaves and developing fruit (Manzara et al., 1991, 1993). cDNAs encoding three GBFs were cloned from young tomato fruit and the three tomato GBFs were shown to bind to the G-box sequence in the *RBCS1*, *RBCS2*, and *RBCS3A* promoters in vitro (Meier and Gruissem, 1994) with a DNase I protection similar to that detected with nuclear proteins (Manzara et al., 1993). Thus, it is likely that the GBF/G-box interaction represents a functional complex necessary for activation of *RBCS* promoters in young tomato fruit as well. Consequently, we

infer that the GBF/G-box interaction in the *RBCS3A* promoter is less productive in developing fruit compared to leaves and compared to the *RBCS1* and *RBCS2* promoters in fruit.

Reduced *RBCS3A* Transcription Is Correlated with FBF Binding

Here we describe a novel fruit-specific DNA-binding activity, the F-box binding factor FBF, that interacts specifically with the *RBCS3A* promoter in nuclear protein extracts from developing fruit and binds within the I-box/G-box region to a sequence element (the F-box) present only in the *RBCS3A* promoter. FBF binding is not detected in nuclear extracts from leaves and light-grown cotyledons, organs in which all three *RBCS* promoters are active. FBF binding thus correlates with the strong reduction of *RBCS3A* promoter activity in fruit compared to its activity in other organs and other *RBCS* gene family members. FBF is therefore a candidate for a negative regulator of *RBCS3A* promoter activity in developing fruit, as postulated from the GUS expression data.

A search of DNA data bases for sequences within other plant promoters that are homologous to the F-box sequence has revealed their presence in two other tomato genes. The sequence 5′-AaGAGATAAGAgTtTTCTtAT-3′ is located at position −575 of the tomato 2A11 promoter and has 17 of 21 bp in common with the *RBCS3A* F-box sequence. The 2A11 gene was isolated as a ripening-related cDNA for a protein of unknown function (Pear et al., 1989). The 2A11 mRNA accumulates to very low levels in young tomato fruit, but the mRNA level increases drastically in mature green fruit before the onset of ripening (Pear et al., 1989). It is possible, therefore, that the F-box homologous element may also contribute to the reduced expression of 2A11 in early fruit development. Although a detailed mutational analysis of the 2A11 promoter has been published (Van Haaren and Houck, 1991, 1993), those experiments were confined exclusively to mature fruit stages. The second sequence, 5′-TATTCTCA-3′, is located at position −1244 of the tomato polygalacturonase gene promoter and is a complete match to the downstream palindromic half-sequence of the *RBCS3A* F-box. Although our gel mobility-shift results indicate that this sequence is not sufficient for the binding of FBF to a short DNA fragment in vitro, it is interesting to note that it is located within a fragment of the polygalacturonase gene promoter that appears to function as a negative regulatory element in tomato fruit (Montgomery et al., 1993). The significance of both findings will have to be evaluated by testing the respective promoter elements for FBF binding.

The binding site of FBF in the *RBCS3A* promoter is located immediately upstream of the G-box, resulting in a DNase I footprint that is entirely contiguous in the F-box/G-box region. This, together with the organ-specific binding of FBF during fruit development when *RBCS3A* promoter activity is reduced, makes FBF a promising candidate for a negative regulator of GBF function. FBF could exert its potentially negative regulation by different mechanisms: (a) FBF and GBF show negative cooperativity, i.e. the binding of FBF destabilizes the GBF/G-box interaction and thereby reduces the productive interaction of GBF with the transcriptional machinery. (b) FBF directly interacts with GBF to block its activation function, e.g. by inhibiting the interaction of the activation domain with other molecules. (c) FBF inhibits a modification of DNA-bound GBF necessary for its function. (d) FBF displaces another protein that binds to the F-box region when *RBCS3A* is transcribed at high levels (and that was not detected in our studies) or interferes with protein-protein interactions of the G-box/GBF complex.

Of these scenarios, our results show that there is no strong cooperativity in the binding of GBF and FBF to their adjacent sites under conditions where the protein concentration is nonsaturating. Thus, it seems unlikely that GBF binding is influenced by FBF directly via DNA-protein interactions. The other scenarios are more difficult to test, but several lines of evidence suggest that GBF action appears to require the presence of at least one other DNA-bound protein (Schulze-Lefert et al., 1989; Donald and Cashmore, 1990; Loake et al., 1992). Preliminary data indicate that a tetramer of the *RBCS3A* I-box/G-box promoter DNA fragment enhances transcription from a minimal cauliflower mosaic virus "-90" 35S promoter in tomato leaves, and mutation of either the I-box or the G-box abolishes this enhancement (data not shown). Although we have not detected an I-box binding activity in our nuclear protein extracts, such an activity from tomato leaves has been reported (Borello et al., 1993). It is possible, therefore, that GBF interacts with an I-box-binding factor in the *RBCS1*, *RBCS2*, and *RBCS3A* promoters when they are fully active, and that binding of FBF to the F-box (which overlaps with the I-box) displaces an I-box-binding factor in the *RBCS3A* promoter in young tomato fruit. This hypothesis is currently being investigated by a site-swapping experiment exchanging the nucleotide sequence between the I-box and the G-box of the *RBCS2* and *RBCS3A* promoters. Because of a lack of availability of a transient expression system allowing the analysis of organ-specific promoter activities in tomato, this analysis will have to await the characterization of the respective mutants in transformed tomato plants.

In conclusion, we have demonstrated that promoter fragments between 3 kb and 600 bp of *RBCS1*, *RBCS2*, and *RBCS3A* from tomato are sufficient to mediate the organ-specific, tissue-specific, and differential expression pattern observed for the endogenous genes. This pattern is independent of the location within the chromatin and is most likely established via the action of organ- and tissue-specific transcriptional regulators interacting with *cis*-acting DNA sequences within the analyzed promoter fragments. The localization of *RBCS* expression within tomato fruit indicates an involvement of the physiological status of the cells in the signaling chain leading to this transcriptional regulation. The low level of activity of the *RBCS3A* promoter together with the identical pattern of tissue-specific activity of all three promoters makes it likely that a negative regulator acting on the *RBCS3A* promoter in fruit is involved. A candidate for such an organ-specific negative

regulator was identified in the fruit-specific DNA-binding protein FBF. The study of its function in the regulation of gene expression in tomato fruit and its potential interaction with the plant transcriptional activator GBF might provide new insights into the mechanisms of organ-specific transcriptional regulation in plants.

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LITERATURE CITED

Ballas N, Wong L-M, Theologis S (1993) Identification of the auxin- responsive element, *AuxRE*, in the primary indolacetic acid-inducible gene, *PS-IAA4/5*, of pea (*Pisum sativum*). J Mol Biol **223:** 580–596

Bartholomew DM, Bartley GE, Scolnik PA (1991) Abscisic acid control of *rbcS* and *cab* transcription in tomato leaves. Plant Physiol **96:** 291–296

Borello U, Ceccarelli E, Guiliano G (1993) Constitutive, light-responsive and circadian clock-responsive factors compete for the different I box elements in plant light-regulated promoters. Plant J **4:** 611–619

Carrasco P, Manzara T, Gruissem W (1993) Developmental and organ- specific changes in DNA-protein interactions in the tomato *rbcS3B* and *rbcs3C* promoter regions. Plant Mol Biol **21:** 1–15

Coruzzi G, Broglie R, Edwards C, Chua N-H (1985) Tissue-specific and light-regulated expression of a pea nuclear gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. EMBO J **3:** 1671–1679

Cox KH, Goldberg RB (1988) Analysis of plant gene expression. *In* CH Shaw, ed, Plant Molecular Biology: A Practical Approach. IRL Press, Oxford, UK, pp 1–34

Dean C, Favreau M, Bond-Nutter D, Bedbrook J, Dunsmuir P (1989a) Sequences downstream of translation start regulate quantitative expression of two petunia *rbcS* genes. Plant Cell **1:** 201–208

Dean C, Favreau M, Dunsmuir P, Bedbrook J (1987a) Confirmation of the relative expression levels of the petunia (Mitchell) *RbcS* genes. Nucleic Acids Res **15:** 4655–4664

Dean C, Pichersky E, Dunsmuir P (1989b) Structure, evolution and regulation of *rbcS* genes in higher plants. Annu Rev Plant Physiol Plant Mol Biol **40:** 415–439

Dean C, van den Eltzen P, Tamaki S, Black M, Dunsmuir P, Bedbrook J (1987b) Molecular characterization of the *RbcS* multi-gene family of petunia (Mitchell). Mol Gen Genet **206:** 465–474

Dean C, van den Eltzen P, Tamaki S, Dunsmuir P, Bedbrook J (1985) Differential expression of the eight genes of the petunia ribulose bisphosphate carboxylase small subunit gene family. EMBO J **4:** 3055–3061

De Block M, Herrera-Estrella L, Van Montagu M, Schell J, Zambryski P (1984) Expression of foreign genes in regenerated plants and in their progeny EMBO J **3:** 1681–1689

Deikman J, Fisher RL (1988) Interaction of a DNA-binding factor with the 5′-flanking region of an ethylene-responsive fruit-ripening gene from tomato. EMBO J **7:** 3315–3320

Donald RGK, Cashmore AR (1990) Mutation of either G box or I box sequences profoundly affects expression from the *Arabidopsis rbcS-1A* promoter. EMBO J **9:** 1717–1726

Fleming AJ, Mandel T, Roth I, Kuhlemeier C (1993) The patterns of gene expression in the tomato shoot apical meristem. Plant Cell **5:** 297–309

Fluhr R, Moses P, Morelli G, Coruzzi G, Chua N-H (1986) Expression dynamics of the pea *RbcS* multigene family and organ distribution of the transcripts. EMBO J **5:** 2063–2071

Gilmartin PM, Sarokin L, Memelink J, Chua NH (1990) Molecular light switches for plant genes. Plant Cell **2:** 369–378

Guiliano G, Pichersky E, Malik VS, Timko MP, Scolnik PA, Cashmore AR (1988) An evolutionarily conserved protein binding sequence upstream of a plant light-regulated gene. Proc Natl Acad Sci USA **85:** 7089–7093

Guiltinan MJ, Marcotte WR, Quatrano RS (1990) A plant leucine zipper protein that recognizes an abscisic acid response element. Science **250:** 267–271

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep **5:** 387–405

Jefferson RA, Kavanagh TA, Bevan MV (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J **6:** 3901–3907

Kuhlemeier C, Strittmatter G, Ward K, Chua N-H (1989) The pea *rbcs3A* promoter mediates light responsiveness but not organ specificity. Plant Cell **1:** 471–478

Laval-Martin D (1975) Chloroplasts of cherry tomato fruits and leaves. Physiological differences and lamellar characteristics. Plant Physiol **56:** 470–474

Laval-Martin D, Farineau J, Diamond J (1977) Light versus dark carbon metabolism in cherry tomato fruits. Plant Physiol **60:** 872–876

Loake GJ, Faktor O, Lamb C, Dixon RA (1992) Combination of H-box (CCTACC(N)$_7$CT) and G-box (CACGTG) *cis* elements is necessary for feed-forward stimulation of a chalcone synthase promoter by the phenylpropanoid-pathway intermediate *p*-coumaric acid. Proc Natl Acad Sci USA **89:** 9230–9234

Manzara T, Carrasco P, Gruissem W (1991) Developmental and organ- specific changes in promoter DNA-protein interactions in the tomato *rbcS* gene family. Plant Cell **3:** 1305–1316

Manzara T, Carrasco P, Gruissem W (1993) Developmental and organ- specific changes in DNA-protein interactions in the tomato *rbcS1*, *rbcS2* and *rbcS3A* promoter regions. Plant Mol Biol **21:** 69–88

Manzara T, Gruissem W (1988) Organization and expression of the genes encoding ribulose-1,5-bisphosphate carboxylase in higher plants. Photosynth Res **16:** 117–139

McKendree WL, Ferl RJ (1992) Functional elements of the *Arabidopsis* and Adh promoter include the G-box. Plant Mol Biol **19:** 859–862

Meier I, Gruissem W (1994) Novel conserved sequence motifs in plant G- box binding proteins and implications for interactive domains. Nucleic Acids Res **22:** 470–478

Montgomery J, Pollard V, Deikman J, Fischer RL (1993) Positive and negative regulatory regions control the spatial distribution of polygalacturonase transcription in tomato fruit pericarp. Plant Cell **5:** 1049–1062

Oeda K, Salinas J, Chua N-H (1991) A tobacco bZIP transcription activator (TAF-1) binds to a G-box motif conserved in plant genes. EMBO J **10:** 1793–1802

Pear JR, Ridge N, Rasmussen R, Rose RE, Houck CM (1989) Isolation and characterization of a fruit-specific cDNA and the corresponding clone from tomato. Plant Mol Biol **13:** 639–651

Salanoubat M, Belliard G (1989) The steady-state level of potato sucrose synthase mRNA is dependent on wounding, anaerobiosis and sucrose concentration. Gene **84:** 181–185

Schindler U, Menkens AE, Beckmann H, Ecker JR, Cashmore AR (1992) Heterodimerisation between light-regulated and ubiquitously expressed *Arabidopsis* and GBF bZIP proteins. EMBO J **11:** 1261–1273

Schulze-Lefert P, Dangl JL, Becker-André M, Hahlbrock K, Schulz W (1989) Inducible *in vivo* DNA footprints define sequences necessary for UV light activation of the parsley chalcone synthase gene. EMBO J **8:** 651–656

Shaffer CD, Wallrath LL, Elgin CR (1993) Regulating genes by packaging domains. Bits of heterochromatin in euchromatin? Trends Genet **9:** 35–37

Sheen J (1990) Metabolic repression of transcription in higher plants. Plant Cell **2:** 1027–1038

Silverthorne J, Tobin EM (1990) Post-transcriptional regulation of organ-specific expression of individual rbcS mRNAs in *Lemna gibba*. Plant Cell **2:** 1181–1190

Silverthorne J, Wimpee CF, Yamada T, Rolfe SA, Tobin EM (1990) Differential expression of individual genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase in *Lemna gibba*. Plant Mol Biol **15:** 49–58

Sugita M, Gruissem W (1987) Developmental, organ-specific, and light- dependent expression of the tomato ribulose-1,5-bisphosphate carboxylase small subunit gene family. Proc Natl Acad Sci USA **84:** 7104–7108

Sugita M, Manzara T, Pichersky E, Cashmore A, Gruissem W (1987) Genomic organization sequence analysis and expression of all five genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase from tomato. Mol Gen Genet **209:** 247–256

Van Haaren MJJ, Houck CM (1991) Strong negative and positive regulatory elements contribute to the high-level fruit-specific expression of the tomato 2A11 gene. Plant Mol Biol **17:** 615–630

Van Haaren MJJ, Houck CM (1993) A functional map of the fruit-specific promoter of the tomato 2A11 gene. Plant Mol Biol **21:** 625–640

Wang F, Smith AG, Brenner ML (1994) Temporal and spatial expression pattern of sucrose synthase during tomato fruit development. Plant Physiol **104:** 535–540

Wanner LA, Gruissem W (1991) Expression dynamics of the tomato *rbcS* gene family during development. Plant Cell **3:** 1289–1303

Weisshaar B, Armstrong GA, Block A, da Costa e Silva O, Hahlbrock K (1991) Light-inducible and constitutively expressed DNA-binding proteins recognizing a plant promoter element with functional relevance in light responsiveness. EMBO J **10:** 1777–1786