Reduction of G-box binding factor DNA binding activity, but not G-box binding factor abundance, causes the downregulation of *RBCS2* expression during early tomato fruit development

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Abstract The downregulation of *RBCS2* promoter activity during tomato fruit development has been investigated by transient gene expression. A major drop in promoter activity occurs between 5 and 25 mm fruit diameter, corresponding to the late cell division to early cell enlargement phase. This drop is abolished by a mutation of the single G-box element necessary for high *RBCS2* promoter activity in young tomato fruit. The G-box binding activity of fruit nuclear and total protein extracts drops concomitantly with the reduction of *RBCS2* promoter activity while G-box binding factor expression is not affected. The data indicate that the developmental signal that downregulates the *RBCS2* promoter acts on the regulation of DNA binding activity of constitutively expressed G-box binding factors.

Key words: *RBCS* promoter; Fruit development; G-box binding factor; Tomato

1. Introduction

The family of *RBCS* genes encodes the small subunit of ribulose-1,5-bisphosphate carboxylase, the key enzyme in photosynthetic carbon dioxide fixation. All five tomato *RBCS* genes are active in leaves and light-grown cotyledons, but only *RBCS1* and *RBCS2* are strongly transcribed in young tomato fruits [1], while no *RBCS* promoter activity or *RBCS* mRNA has been detected in mature green fruit. Thus, the *RBCS1* and *RBCS2* promoters are downregulated during fruit development and before the onset of ripening. This downregulation might correlate with loss of the net photosynthetic activity of fruit [2], but its precise developmental stage, or the signals involved, have not been investigated.

A detailed *cis*-analysis of the *RBCS2* promoter has shown that a single G-box element at position −156 is responsible for 80% of the *RBCS2* promoter activity in young tomato fruit [3]. In contrast, the G-box and an adjacent I-box contribute equally to the promoter activity in leaves, indicating that organ-specific differences exist in the activation of the *RBCS2* promoter in different green tissues [3]. G-box binding factors (GBFs), a family of related bZIP proteins which specifically recognize the CACGTG motif of the G-box, have been identified from several plant species [4–7]. In tomato, GBFs have been detected by DNAse I protection experiments with *RBCS* promoters in nuclear extracts from both young (3–8 mm) and mature red tomato fruit [8]. Three bZIP proteins (GBF4, GBF9 and GBF12) that bind to the G-box in the *RBCS1*, *RBCS2* and *RBCS3A* promoters have been cloned from tomato and are expressed in young tomato fruits [9]. Their expression pattern during fruit development has not been investigated.

The importance of the G-box for *RBCS2* promoter activity in young tomato fruit leads to the question whether the developmental regulation of the *RBCS2* promoter is caused by a loss of GBF activity. Alternatively, additional factors, binding elsewhere on the *RBCS2* promoter, could be involved in its downregulation in the continuous presence of GBF. To investigate this question, we have used a recently developed transient gene expression system for tomato fruit [3] to follow the activity of the *RBCS2* promoter during fruit development and to identify the element(s) involved in its downregulation. The activity and expression pattern of tomato GBFs during fruit development has been characterized and correlated to *RBCS2* inactivation.

2. Materials and methods

2.1. Plant material

Tomato cultivar VFNT cherry LA 1221 was grown under greenhouse conditions. Fruit stages 5 mm, 15 mm, 25 mm correspond to approximately 5, 14 and 35 days post-pollination, respectively, and fruit stage 40 mm corresponds to mature green fruit.

2.2. Reporter gene constructs, particle bombardment, luciferase and β-glucuronidase (GUS) assays

All reporter gene constructs are described in [3]. Particle bombardment, luciferase and GUS assays were carried out as described [3], with the following modifications. To avoid quenching of GUS activity in extracts of 5 mm fruit, 80 μl extract of unbombarded orange fruit was routinely added to 40 μl extract of bombarded 5 mm fruit and 20 μl EGL reaction buffer [3]. Under these conditions, unbombarded fruit extracts of all fruit stages, that were spiked with commercially available GUS (Sigma, St. Louis, MO, USA), showed the same measurable GUS activity (data not shown).

2.3. Preparation of nuclear and total protein extracts

Nuclear protein extracts were prepared as described [8]. Native total protein extracts for an electrophoretic mobility shift assay (EMSA) were prepared as described [10]. For the isolation of denatured total protein extracts for immunoblot experiments, ca. 0.1 g plant material was frozen in liquid nitrogen, ground to a fine powder and suspended in 50 mM Tris-HCl, 2% SDS, 30% glycerol, 100 mM DTT, pH 6.8. Samples were boiled for 5–10 min, centrifuged for 10 min at 4°C and 12000 rpm in a table top centrifuge 2K15 (Sigma, St. Louis, MO, USA) and the supernatant was transferred to a fresh tube and stored at −70°C. The protein concentration in nuclear extracts and native total protein extracts was determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories). Denatured protein extracts were normalized for immunoblots by staining of protein gels with Coomassie brilliant blue.

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2.4. Protein expression in Escherichia coli

Recombinant GBF4, GBF9 and GBF12 were expressed in *E. coli* as described [9]. Cells of 1 ml culture were harvested, suspended in 100 μl 50 mM Tris-HCl, 2% SDS, 30% glycerol, 100 mM DTT, pH 6.8, and treated as described for denatured total protein extracts.

2.5. Immunoblots

Immunoblots were carried out essentially as described [11]. Enhanced chemiluminescence detection was performed as described by the manufacturer (Amersham, UK). A 1:500 dilution of anti-GBF1 and of the pre-immune serum and a 1:10 000 dilution of peroxidase-coupled goat anti-rabbit IgG (Biogenes, Berlin, Germany) were used.

2.6. EMSA

For EMSA, 33 bp oligonucleotides containing the wild-type G-box motif (CACGTG, G-box) and a mutation of this motif to TCTAGA (GM) [8] were cloned into the *Eco*RV site of pIC20H [12], after filling in the overhanging ends. The polylinker containing the respective oligonucleotide was cut out with *Hin*dIII and labelled with the DIG Gel Shift kit (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. Competitor DNA was obtained by PCR, using the forward and reverse primers on pIC20H. Reactions contained 0.8 ng DIG-labelled DNA fragment, 1.2 μg poly-(dIdC) and different amounts of protein as indicated in the figure legends. Where indicated, reactions contained a 50-fold excess of unlabelled competitor DNA. All reactions were brought to 13 μl with 20 mM HEPES, 40 mM NaCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, pH 7.6. For supershift experiments, 125 μg of anti-GBF1 or of the pre-immune serum were added as indicated. Reactions were incubated for 30 min at room temperature and separated on 5% polyacrylamide gels in 0.5×TBE [11]. For DIG detection, gels were transferred by electroblotting with 0.5×TBE as transfer buffer for 30 min at 12 V to Hybond NX membranes. DIG detection was performed as described by the manufacturer (Boehringer, Mannheim, Germany).

2.7. RNA blots

Total RNA was isolated as described [13]. For labelling of probes, the plasmids pBluescript SK GBF4, pBluescript SK GBF9 and pBluescript SK GBF 12 [9] were linearized at the *Eco*RI site for in vitro transcription with the DIG RNA Labelling kit (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. The 25S rDNA probe was DIG-labelled by PCR. The probe corresponds to the fragment from nucleotide 3089 to nucleotide 3334 of the tomato 25S rRNA. Separation of RNAs on a formaldehyde gel and blotting to a Hybond NX membrane was as described [11]. Hybridization and detection were carried out with reagents of the DIG DNA Labelling and Detection kit (Boehringer, Mannheim, Germany), according to the manufacturer's protocol.

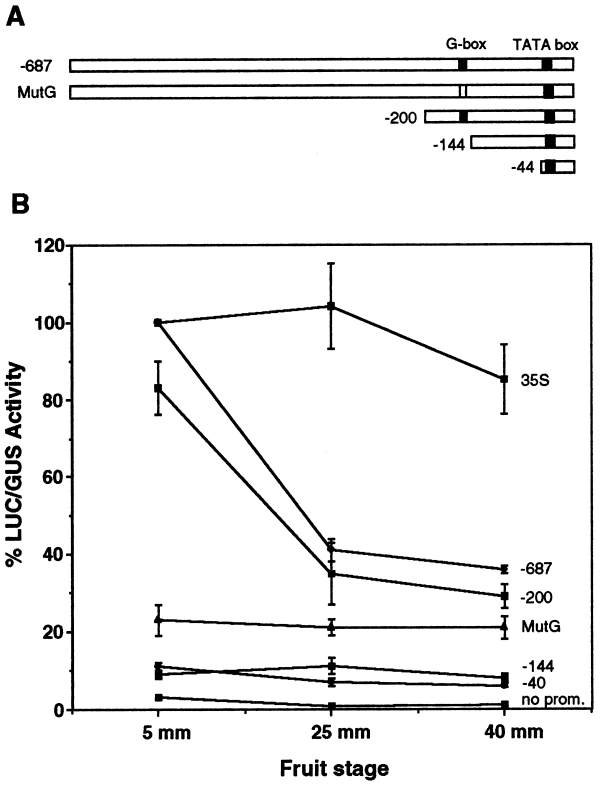

Fig. 1. Analysis of *RBCS2* promoter activity at different stages of fruit development. (A) Schematic representation of the *RBCS2* promoter fragments used for transient transformation. Numbers indicate the last nucleotide of the respective promoter fragment upstream of the start site of transcription. The G-box and the TATA box are indicated by filled boxes. The open box indicates a mutated G-box, in which the sequence CACGTG has been exchanged to TCTAGA. (B) LUC/GUS activities obtained for different promoter constructs in three different fruit stages (see Section 2). The respective promoters are indicated on the right. Activities obtained for all *RBCS2* promoter constructs are expressed as percentage of the activity obtained with the −687 *RBCS2* promoter construct in 5 mm fruit. Activities obtained with the 35S promoter are expressed as a percentage of the activity obtained with the 35S promoter in 5 mm fruit. Mean values and S.D.s of six independent transformations are shown.

3. Results and discussion

3.1. Downregulation of RBCS2 promoter activity during fruit development is a G-box dependent process

RBCS2 promoter activity was measured during fruit development using biolistic transient transformation of fruit slices [3]. The three different developmental stages used were green fruits of 5 mm, 25 mm and 40 mm diameter (corresponding to 5 and 35 days post-pollination and to mature green fruit). The fruit slices were simultaneously transformed with two reporter gene plasmids, one containing the respective *RBCS2* promoter fused to the firefly luciferase gene (LUC) and a second control plasmid containing the *E. coli* GUS gene driven by the cauliflower mosaic virus 35S promoter. All values were expressed as LUC/GUS activity. Fig. 1A shows the *RBCS2* promoter constructs used. The LUC/GUS activities of control transformations with a 35S-LUC and a 35S-GUS plasmid were comparable in all three fruit stages (35S in Fig. 1B), indicating that under our assay conditions, the three fruit stages lead to a comparable gene expression and enzyme activities. Under the same conditions, the activity of the longest *RBCS2* promoter construct (−687) dropped to about 40% between fruit stage 5 mm and fruit stage 25 mm and declined slightly further to about 36% in 40 mm fruit. A very similar drop in activity was observed for the −200 promoter construct. In contrast, the MutG construct, in which the core 6 bp of the G-box (CACGTG) have been replaced by TCTAGA in the context of the −687 promoter, had a constant activity in all fruit stages. This activity was about 20% of the activity observed with the −687 promoter construct in 5 mm fruit. The −144 and −40 constructs both showed a low and constant activity in all fruit stages of about 10% of the maximal activity observed with the −687 promoter.

These data show that the major drop in *RBCS2* promoter activity happens relatively early during fruit development, between stage 5 mm and stage 25 mm. This corresponds to the late cell division to early cell enlargement phase of cherry tomato fruits [14] and clearly precedes the drop in chlorophyll accumulation, CO_2 fixation rate and rubisco enzyme activity, which all happen at the mature green stage [2,15]. The downregulation of *RBCS* promoters is therefore a very early step in

the developmental switch of tomato fruits from a photosynthetically active tissue to a sink tissue.

While both the −687 and the −200 constructs were affected, the residual activity of the MutG construct was constant in all fruit stages assayed, indicating that the downregulation affects the G-box dependent but not the G-box independent part of the *RBCS2* promoter activity. Hence, the loss of the G-box renders the *RBCS2* promoter unresponsive to the signals causing its downregulation, indicating that those signals act through the G-box element.

3.2. Reduction of the total G-box binding activity correlates with downregulation of the RBCS2 promoter

The simplest explanation for these data is that the reduction of *RBCS2* promoter activity is due to a loss of transcriptional activation by GBFs. This could be caused either by a downregulation of GBF expression or by a loss of DNA binding activity and/or ability to activate transcription of constitutively expressed GBFs. It has previously been shown that young immature as well as mature tomato nuclear extracts contain G-box binding activity [8], but the abundance of GBFs has not been followed quantitatively through fruit development. Here, we compared, in EMSAs, the amount of G-box binding activity in different fruit stages. EMSAs were performed with an oligonucleotide containing the G-box sequence ACACGTGG [9] and equal amounts of nuclear extracts from different fruit stages (Fig. 2A). Two complexes with a shifted mobility were detected (C1 and C2 in Fig. 2A), consistent with previous results for young fruit (3–8 mm diameter) [9]. The amount of shifted complexes was high at fruit stages 5 and 15 mm, dropped to low levels at fruit stage 25 mm and remained low in mature green (40 mm) and orange fruit. To determine if the complexes in all fruit stages represent G-box-specific binding activities, competition experiments were performed with the G-box fragment and an identical fragment in which the 6 bp core CACGTG of the G-box was replaced by TCTAGA (GM) [9]. The nuclear extracts were titrated to show approximately equal amounts of total shifted complexes (data not shown) and the so determined amounts of nuclear extracts were used for the competition experiment. The ratio of C1 to C2 was found to be variable between extracts as well as between experiments. The reason for this variability is not known, but it might indicate that the complexes represent different modified forms of GBFs and not different GBF proteins. Fig. 2B shows that C1 and C2 are both competed by a 50-fold excess of the G-box fragment, but not the GM fragment, in all five extracts, confirming that all complexes are caused by GBF binding.

These data show that the pool of GBF binding activity in tomato fruit nuclei drops significantly between fruit stage 15 mm and 25 mm. This timing is consistent with the drop in *RBCS2* promoter activity, that was observed between fruit stage 5 mm and 25 mm, and suggests that the downregulation of the *RBCS2* promoter is caused by a loss of binding of GBF to the G-box.

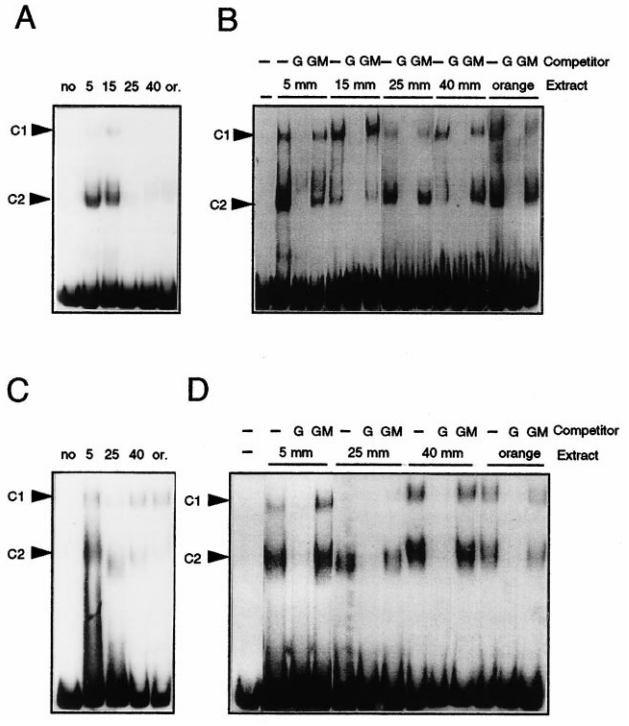

Fig. 2. G-box binding activity in different fruit stages. (A) EMSA with the G-box fragment and 2.5 μg nuclear extract from different fruit stages. no, no extract; 5, 5 mm fruit extract; 15, 15 mm fruit extract; 25, 25 mm fruit extract; 40, 40 mm fruit extract; or., orange fruit extract. (B) EMSA with the G-box fragment and nuclear extracts from different fruit stages. Where indicated, a 50-fold excess of unlabelled G-box fragment (G) or mutated G-box fragment (GM) was added as competitor. Amounts of nuclear extracts added were 1.25 μg 5 mm extract, 2.5 μg 15 mm extract and 5 μg each of 25 mm extract, 40 mm extract and orange extract. (C) EMSA with the G-box fragment and 7.5 μg total protein extract from different fruit stages. no, no extract; 5, 5 mm fruit extract; 15, 15 mm fruit extract; 25, 25 mm fruit extract; 40, 40 mm fruit extract; or., orange fruit extract. (D) EMSA with the G-box fragment and total protein extracts from different fruit stages. Where indicated, a 50-fold excess of unlabelled G-box fragment (G) or mutated G-box fragment (GM) was added as competitor. Amounts of total protein extracts added were 3.8 μg 5 mm extract and 7.5 μg each of 25 mm extract, 40 mm extract and orange extract. C1, complex 1; C2, complex 2 in (A–D).

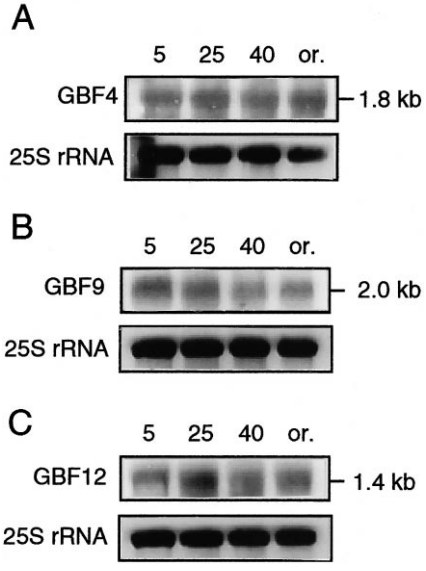

Fig. 3. Tomato GBF mRNA abundance in different fruit stages. 10 μg total RNA from tomato fruit of stages 5 mm, 25 mm, 40 mm and orange was hybridized to gene-specific probes for tomato GBF4 (A), tomato GBF9 (B) and tomato GBF12 (C). Blots were then hybridized with a probe against 25S rRNA as loading control. The approximate size of the GBF mRNAs is indicated on the right.

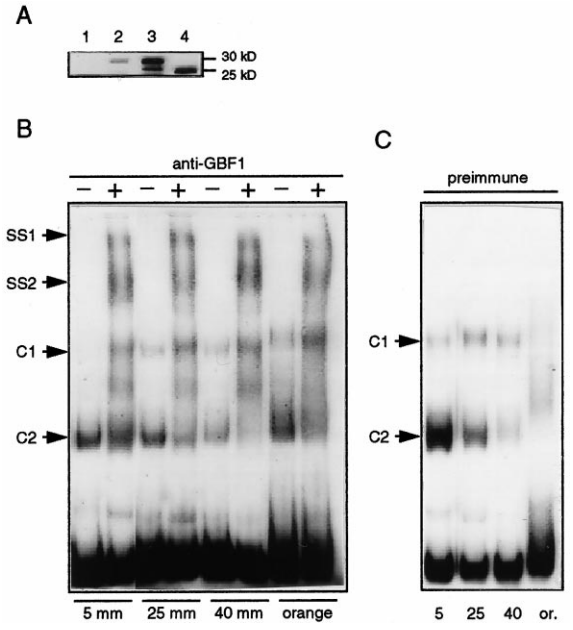

Fig. 4. An antibody against *Arabidopsis* GBF1 recognizes the cloned tomato GBFs and the GBFs present in the fruit nuclear extract complexes. (A) Anti-GBF1 was used in an immunoblot with protein extracts from *E. coli* strains expressing recombinant tomato GBFs. Lane 1, *E. coli* XL1-blue host strain; lane 2, GBF4; lane 3, GBF9; lane 4, GBF12. (B) Supershift experiment using anti-GBF1 and nuclear extracts from different fruit stages. 0.8 ng of G-box fragment was incubated with 1.25 μg nuclear protein extract from the 5 mm fruit stage or 5 μg nuclear protein extract from 25 mm, 40 mm and orange fruit stages and 125 μg of anti-GBF1, where indicated. C1, complex 1; C2, complex 2; SS1, supershift 1; SS2, supershift 2. (C) EMSA with 0.8 ng of G-box fragment and 1.25 μg nuclear protein extract from the 5 mm fruit stage or 5 μg nuclear protein extract from 25 mm, 40 mm and orange fruit stages and 125 μg of pre-immune serum. C1, complex 1; C2 complex 2; 5, 5 mm fruit, 25, 25 mm fruit; 40, 40 mm fruit; or., orange fruit.

In some cases, it has been shown that the amount of nuclear G-box binding activity can be regulated by nuclear import of GBFs [16,17]. To investigate if this might be the case during tomato fruit development, total protein extracts from different fruit stages were compared for their G-box binding activity. Fig. 2C shows that, like for nuclear extracts, two complexes were detected (C1 and C2) and that the highest amount of complexes was detected at fruit stage 5 mm, with lower amounts at fruit stages 25 mm, 40 mm and orange. Fig. 2D shows a competition experiment, demonstrating that all detected complexes are specific. These data demonstrate that between fruit stage 5 mm and 25 mm, the total amount of cellular G-box binding activity drops, indicating that selective nuclear import is not the cause for the reduction of nuclear G-box binding activity.

3.3. *Loss of G-box binding activity is not caused by a downregulation of GBF expression*

To investigate if GBF expression is downregulated during fruit development, the abundance of GBF mRNA at different fruit stages was investigated. Three tomato GBFs have been cloned (GBF4, GBF9 and GBF12) and gene-specific probes are available [9]. Fig. 3 shows a RNA blot experiment with total RNA isolated from four different fruit stages. GBF4, GBF9 and GBF12 mRNAs accumulate to about equal amounts in all four fruit stages, indicating that the three GBFs investigated are not regulated at the level of transcription or RNA stability.

Hence, the loss of G-box binding activity could be either caused by differences in GBF protein synthesis or stability or be due to a regulation of the DNA binding activity of constitutively expressed proteins. In order to determine if GBF protein abundance is regulated in tomato fruit, immunoblot experiments were performed. An antibody against GBF1 from *Arabidopsis* recognizes multiple GBFs in *Arabidopsis*, soybean and parsley [16,17]. To test whether anti-GBF1 also recognizes the three cloned tomato GBFs, partial cDNAs for GBF4, GBF9 and GBF12 were used to express the recombinant proteins in *E. coli*. Fig. 4A shows that anti-GBF1 recognizes a band of the expected size [9] in total protein extracts from *E. coli* strains expressing recombinant GBF4, GBF9 and GBF12 (Fig. 4A, lanes 2–4). No signal was detected in a total protein extract from the *E. coli* host strain (Fig. 4, lane 1). Anti-GBF1 was then used for a supershift experiment with the different fruit nuclear extracts to investigate whether it recognizes the G-box binding activity present in the complexes C1 and C2. Amounts of fruit nuclear extract normalized to show approximately equal binding activities were used for this experiment. Fig. 4B shows that two supershifted complexes (SS1 and SS2) were detected in all four extracts, which are not observed with the pre-immune serum (Fig. 4C). The high amount of supershifted complexes and the equal ratio of supershifted complexes to total complexes in all four extracts show that a significant and comparable amount of GBF

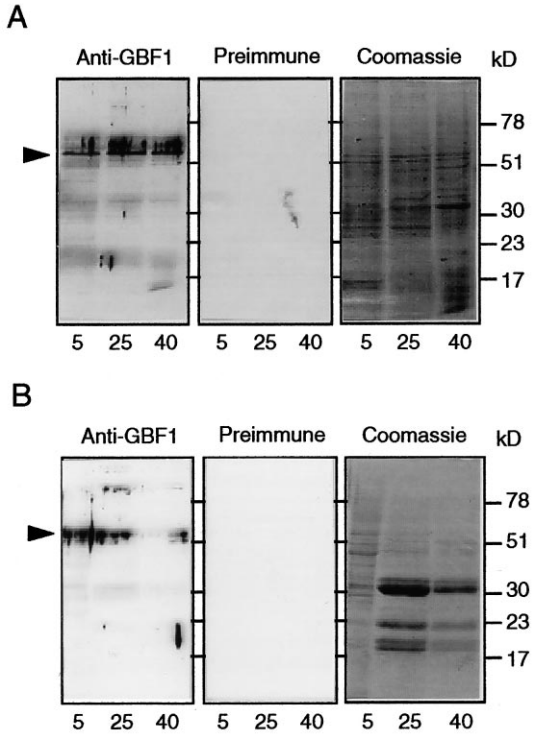

Fig. 5. GBF protein abundance in total protein and nuclear extracts from different fruit stages. Equal amounts of total protein extract (A) or nuclear protein extract (B) from different fruit stages were separated on three replica protein gels and subjected to an immunoblot with anti-GBF1, with the pre-immune serum, or stained with Coomassie brilliant blue. Molecular weight markers are indicated on the right. The arrowheads indicate the most prominent signal. 5, 5 mm fruit; 25, 25 mm fruit; 40, 40 mm fruit.

present in C1 and C2 is recognized by anti-GBF1. The antibody can therefore be used to detect the proteins responsible for the observed DNA binding activity in fruit extracts.

Total and nuclear protein extracts from different fruit stages were compared by immunoblotting. Fig. 5A shows that a major band of equal intensity corresponding to a protein of approximately 55 kDa was detected in total protein extracts of fruits of 5, 25 and 40 mm diameter. This is consistent with the size of the major signal detected with anti-GBF1 in other plant species [16]. No signal was detected with the pre-immune serum. Therefore, the total amount of GBFs detected by anti-GBF1 is not reduced during fruit development. Fig. 5B shows an immunoblot performed with nuclear extracts. The Coomassie brilliant blue staining shows that although equal amounts of protein were loaded according to a Bradford assay, the abundance of individual nuclear proteins varies greatly between different fruit stages. While a somewhat weaker signal was detected with the anti-GBF1 antibody in the 40 mm extract, no difference was observed between 5 and 25 mm, the developmental stage at which the major drop in activity was found. These data confirm that no developmentally regulated nuclear import of the GBF pool occurs at this stage.

Together, the data presented here suggest that the downregulation of *RBCS2* promoter activity during fruit development is caused by a reduction of the GBF pool capable of G-box binding. This reduction is not caused by a reduction in total GBF protein. The supershift experiment demonstrates that a large portion of the G-box binding activity present in the shifted complexes in all fruit stages can be recognized by anti-GBF1. While this activity drops during fruit development, the pool of GBF protein recognized by this antibody remains constant. In addition, no differences in the expression level of the three cloned tomato GBFs have been detected.

Hence, the loss of G-box binding activity during fruit development is most likely caused by a reduction of the DNA binding activity of constitutively expressed GBFs. This could be caused by specific protein modifications that modulate the affinity of GBFs for the G-box. Phosphorylation by casein kinase II has been shown to affect the ability of GBFs to bind DNA in other systems [18]. In our hands, however, no effect on G-box binding activity was observed after treatment of nuclear extracts of four different fruit stages with either casein kinase II or alkaline phosphatase (data not shown). Besides phosphorylation, acetylation has recently been shown to affect the DNA binding activity of transcription factors [19] and could be tested in this system. GBFs have been shown to form homo- and heterodimers [20] and the formation of different GBF dimers at different fruit stages could account for the observed differences in DNA binding activity. Alternatively, GBF DNA binding may be influenced by the association of an additional unknown protein which binds to GBF.

The data presented here show that the downregulation of the *RBCS2* promoter in tomato fruit is a very simple example of developmental regulation of gene expression in plants. A single transcriptional activator is responsible for the majority of promoter activity in a specific organ. The DNA binding activity of this activator is then reduced in response to a developmental signal, that does not influence the expression of the activator itself. The system can now be used to assay the developmental signal causing the reduction of GBF activity.

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