

# Patterns of *Dwarf* expression and brassinosteroid accumulation in tomato reveal the importance of brassinosteroid synthesis during fruit development

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## Summary

Brassinosteroids (BRs) are essential for many physiological functions in plants, however little is known concerning where and when they are synthesized. This is especially true during flower and fruit production. To address this we have used a promoter-GUS reporter fusion and RT-PCR to determine the relative expression levels of the tomato *Dwarf* (*D*) gene that encodes a BR C-6 oxidase. In young seedlings GUS reporter activity was observed mainly in apical and root tissues undergoing expansion. In flowers GUS activity was observed in the pedicel joints and ovaries, whereas in fruits it was strongest during early seed development and was associated with the locular jelly and seeds. RT-PCR analysis showed that tissue-specific expression of *Dwarf* mRNA was consistent with that of the *Dwarf*:GUS fusion. In good correlation with the high local *Dwarf* activity, quantitative measurements of endogenous BRs indicated intense biosynthesis in developing tomato fruits, which were also found to contain high amounts of brassinolide. Grafting experiments showed the lack of BR transport indicating that BR action occurs at the site of synthesis.

**Keywords:** tomato *Dwarf* gene expression, brassinosteroids, GUS, fruit, grafting.

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## Introduction

The process by which plants optimize their growth and development for reproduction is highly complex. Unravelling this complexity, especially in crops, will enable the generation of enhanced varieties that are required for sustaining future food production. As plant hormones coordinate numerous physiological responses, their relative levels have been targeted for manipulation in order to improve crop performance. Brassinosteroids (BRs) are steroidal hormones that are essential for normal plant development, thereby offering approaches to improve crop productivity

(Bishop, 2003). When exogenously applied to plants, BRs elicit many physiological responses, including the increase of cell elongation, pollen tube growth, leaf bending/expansion, ethylene biosynthesis, xylem differentiation, enhancement of stress resistance and delayed senescence (for recent review see: Sasse, 2003). The importance of this group of hormones is demonstrated by the fact that many of the mutants defective in BR biosynthesis and signalling are severely dwarfed with dark green leaves, reduced fertility, an increased lifespan and abnormal skotomorphogenesis

(Altmann, 1999; Fujioka and Yokota, 2003; Yokota, 1997). The BR-deficient mutant phenotype can be restored to wild-type (WT) by exogenous application of brassinolide (BL), the most bioactive BR. However, such phenotypic rescue is not possible in the case of BR-insensitive mutants. Application of BRs and BR analogues to crops in the field has also been shown to increase yield (Mandava, 1988), suggesting that the manipulation of BR biosynthesis or signalling in plants provides an additional methodology by which crop productivity can be improved.

Tomato is a highly valuable crop and has been adopted as the model species for studying fruit growth, development and ripening (Giovannoni, 2001, 2004). Several mutants are available that are altered in these processes, including those deficient in the biosynthesis and signalling of phytohormones. For example the *Never ripe (Nr)* mutant is defective in the signalling of the gaseous plant hormone ethylene that is required in many plant processes, especially in fruit ripening (Lanahan *et al.*, 1994; Wilkinson *et al.*, 1995). The mutants defective in the biosynthesis of the plant growth promoting hormone gibberellin (GA) are severely dwarfed and require exogenous GA application for flower and fruit development (Groot *et al.*, 1987; Nester and Zeevaart, 1988). By contrast, tomato mutants defective in BR synthesis are able to produce flowers and fruits but have a reduced habit. Mutations at the *Dwarf (D)* and *Dumpy (Dpy)* loci cause BR deficiency (Bishop *et al.*, 1999; Koka *et al.*, 2000), whereas those at the *Curl3* locus affect BR signalling (Koka *et al.*, 2000; Montoya *et al.*, 2002). The *Dwarf* gene was identified by transposon tagging and shown to encode a cytochrome P450 monooxygenase (CYP or P450) (Bishop *et al.*, 1996). Further characterization of *Dwarf* indicated that it catalyses the C-6 oxidation of 6-deoxocastasterone (6-deoxo-CS) to castasterone (CS), the immediate precursor of BL, the most bioactive BR (Bishop *et al.*, 1999). Intermediate feeding rescue of the *dpy* mutant implicated the *Dpy* gene product in the C-23 hydroxylation and the synthesis of 6-deoxoteasterone (Koka *et al.*, 2000). The allelic *curl3* and *abs* mutants are defective in a leucine-rich repeat receptor-like kinase tBRI1 (Montoya *et al.*, 2002), an orthologue to Arabidopsis BRASSINOSTEROID INSENSITIVE 1 (BRI1) that is an essential component of BR signalling (Li and Chory, 1997).

Little is known concerning the sites of BR synthesis during plant growth and development. Current insights into such locations are based on transcript analyses of BR biosynthesis genes. In Arabidopsis the transcript levels of P450 genes involved in BR biosynthesis have been monitored directly using RT-PCR (Bancos *et al.*, 2002; Shimada *et al.*, 2003) and cDNA microarray experiments (Goda *et al.*, 2002, 2004a,b; Müssig *et al.*, 2002). In addition, promoters of BR-biosynthetic genes have been fused to the  $\beta$ -glucuronidase (GUS) reporter gene and histological staining of GUS activity used as an indicator of gene expression (Mathur *et al.*, 1998). As expected from the pleiotropic dwarf mutant phenotype,

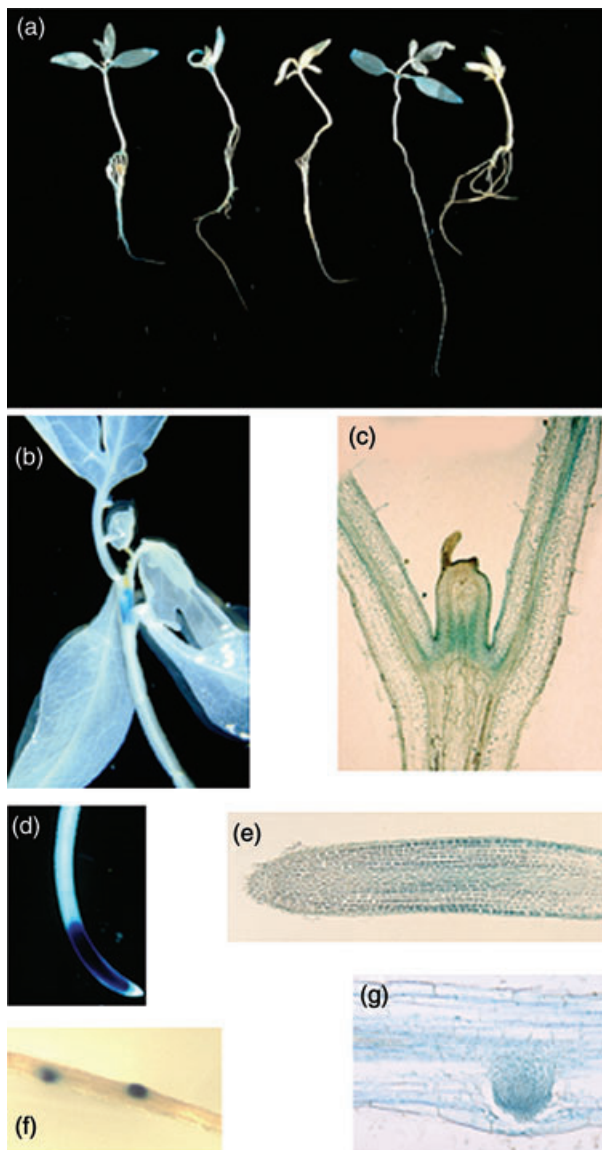
these different approaches revealed that transcripts of the BR biosynthesis genes were detectable in most tissues. But differences in the expression patterns of the particular genes suggested a control mechanism of BR biosynthesis that is based on the differential organ-specificity of these genes (Bancos *et al.*, 2002; Shimada *et al.*, 2003). Interestingly, all Arabidopsis P450s involved in BR synthesis were found to be feedback-regulated by exogenous application of BL. These genes are also upregulated in *bri1* mutants, and their levels are unaffected by exogenous application of BL (Bancos *et al.*, 2002). These data highlight that BR perception by the BRI1 receptor kinase is required for maintaining the homeostasis of BRs via the feedback regulation of their biosynthesis.

Here we report the distribution of *Dwarf* expression at different stages of tomato development, which was determined using transgenic plants harbouring the *Dwarf* promoter-GUS fusion, as well as RT-PCR assays of the *Dwarf* mRNA. Our expression analyses revealed high gene activity during the development of reproductive organs. In immature fruits we detected high levels of BL indicating that in contrast to earlier expectations this compound is present in tomato plants. The coincidence between the high *Dwarf* gene activity and the accumulation of bioactive BRs during fruit development suggests that *Dwarf* may be preferentially expressed at the sites of active BR synthesis.

## Results

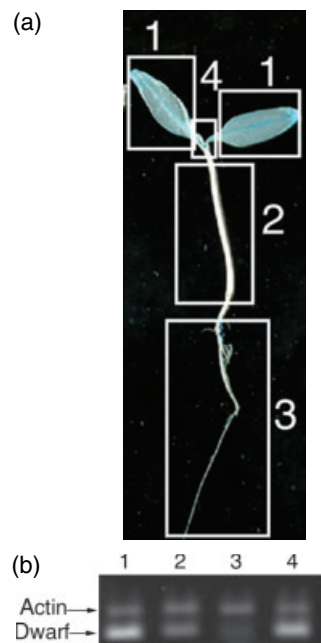
### *Pattern of Dwarf expression in tomato seedlings*

Previously we have used RT-PCR to monitor the expression of *Dwarf* in both WT and mutant plants (Montoya *et al.*, 2002). In order to gain more detailed information as to where and when *Dwarf* is expressed, we generated transgenic lines harbouring *Dwarf* promoter-GUS fusion. GUS expression of young seedlings from five independent transgenic lines can be seen in Figure 1(a). Although these lines varied slightly in the intensity of GUS levels, they exhibited similar patterns of expression, and a representative line was used in further experiments. Maximal expression was observed in shoot apical regions, root tips and in zones undergoing lateral root formation (Figure 1b,d,f). Sectioning of the tissues reporting GUS activity was carried out in order to further characterize the tissue-specificity of *Dwarf* expression. Longitudinal sections of the shoot apex highlighted that staining was predominant in sub-meristematic regions. GUS activity was also observed in vascular tissue (Figure 1c). Longitudinal sections of the root indicated that staining was not present in the root cap but, similar to the shoot apex, maximal activity was observed in the sub-meristematic region (Figure 1d,e). High expression was also observed during lateral root formation (Figure 1f,g).



**Figure 1.** Expression of *Dwarf* in seedlings using GUS reporter gene. Histochemical GUS staining of 7-day-old seedlings harbouring the *Dwarf*:GUS fusion. Seedlings of five independent transgenic lines (a). Close-up (b) and longitudinal section (c) of the apical region highlighting intense staining in the sub-meristematic and vascular tissues. Close-up (d) and longitudinal section (e) of the root tip showing most GUS activity in sub-meristematic regions. Close-up (f) and longitudinal section (g) of the root featuring GUS expression at the sites of lateral root formation.

To confirm that the expression pattern deduced from GUS histochemical data corresponded to *Dwarf* expression, we carried out semi-quantitative RT-PCR analysis of the endogenous *Dwarf* transcript using total RNA isolated from different organs of 10-day-old seedlings (Figure 2). The RT-PCR data indicated that the relative levels of the *Dwarf* transcript are higher in the shoot apex and cotyledons than in the hypocotyls and roots (Figure 2b). These data of



**Figure 2.** Correlation of GUS staining pattern with endogenous *Dwarf* transcript level.

(a) Ten-day-old transgenic seedling stained for GUS activity highlighting regions used for RT-PCR analysis.

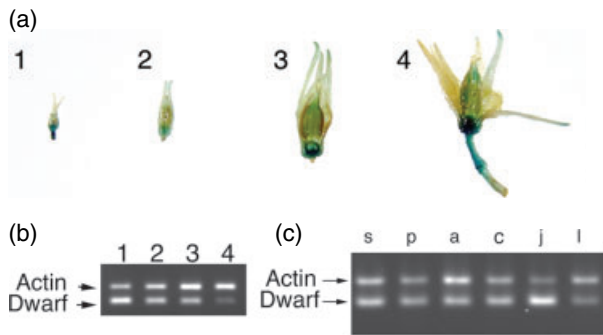
(b) Resulting gel image from RT-PCR using *Dwarf* and *actin* primers. Numbers correspond to cotyledon (1), hypocotyl (2), root (3) and shoot apex (4).

organ-specific *Dwarf* expression are consistent with those obtained from GUS histochemical staining.

#### *Dwarf* expression during flower and fruit development

Tomato is widely used as a model plant for studying fruit growth and development. We were therefore keen to observe the transcriptional regulation of *Dwarf* during flower and fruit differentiation. To achieve this, flowers and fruits of different developmental stages were collected from *Dwarf*:GUS transgenic plants and GUS histochemical staining was carried out. During flowering *Dwarf* expression was observed in most flower parts, with strongest expression detected in the carpels (Figure 3A). Semi-quantitative RT-PCR assays confirmed *Dwarf* promoter activity in all flower parts, and also indicated highest expression in young flower buds and at the joint in the pedicel (Figure 3B,C).

During fruit development maximal GUS expression was associated with early seed development, with higher activity in the locular jelly than in the pericarp and epidermal tissue. However, the vascular tissue of the pericarp also exhibited more prominent GUS activity than other pericarp tissue. Earlier stages of fruit development did not exhibit intense GUS staining, although semi-quantitative RT-PCR indicates the presence of *Dwarf* transcript (Figure 4a,b). This suggests that *Dwarf* function may be important during the cell



**Figure 3.** Analysis of *Dwarf* expression during flower development.

(a) Flowers of transgenic tomato were collected at different stages of development and subjected to GUS staining. 1: flower bud <7 mm, 2: flower bud >7 mm, 3: flower slightly open, 4: flower fully open.

(b) *Dwarf*- and *actin*-specific RT-PCR products obtained from total RNA of flower samples corresponding to the stages shown in panel (a).

(c) *Dwarf*- and *actin*-specific RT-PCR products obtained from total RNA isolated from sepals (s), petals (p), anthers (a), carpels (c) and joints of the pedicel (j) fully developed flowers. (l) Products obtained from leaves.

expansion phase that occurs later in fruit development, rather than cell division phase that occurs earlier.

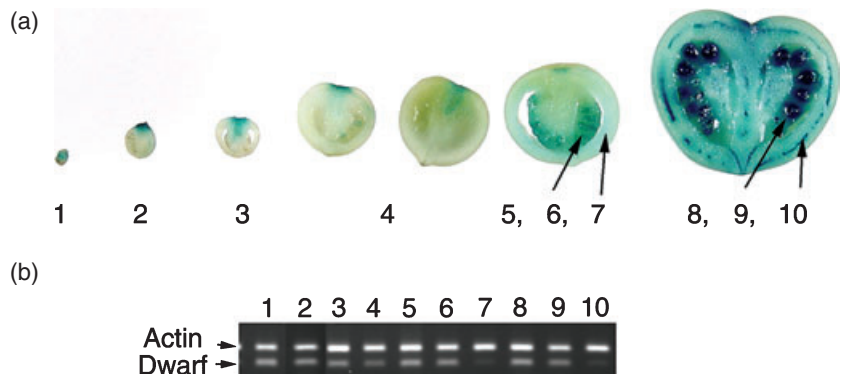
#### Immature tomato fruits accumulate high amounts of BL

Fruit tissue samples, from the same material that was used for RT-PCR assays, were also analysed for endogenous BR content. One of the striking observations from these measurements was the presence of BL at relatively high levels in both the early and later stages of fruit development. Intriguingly, the amounts of CS detected in the fruit samples were much lower than those of BL (Figure 5). As *Dwarf* promoter-driven GUS expression was shown to be stronger in the locular jelly during seed development (Figure 4a), this fruit part was separated from the pericarp and both samples were subjected to BR analysis. The much higher levels of BL measured in the locular jelly including the immature seed fraction coincided with the elevated *Dwarf* promoter activity detected in this region of the fruit. We also found that,

**Figure 4.** Analysis of *Dwarf* expression during early fruit development.

(a) Transgenic fruits of different developmental stages were sectioned and stained for GUS activity. 1: fruit 3 mm, 2: fruit 8 mm, 3: fruit 1 cm, 4: fruit 2–2.5 cm, 5: fruit 3.2 cm, 6: locular jelly of fruit 3.2 cm fruit, 7: pericarp and epidermis of fruit 3.2 cm, 8: fruit 5 cm, 9: fruit 5 cm locular jelly and immature seed, 10: fruit 5 cm pericarp and epidermis.

(b) *Dwarf*- and *actin*-specific RT-PCR products obtained from using total RNA isolated from the fruits or fruit parts identified in panel (a).



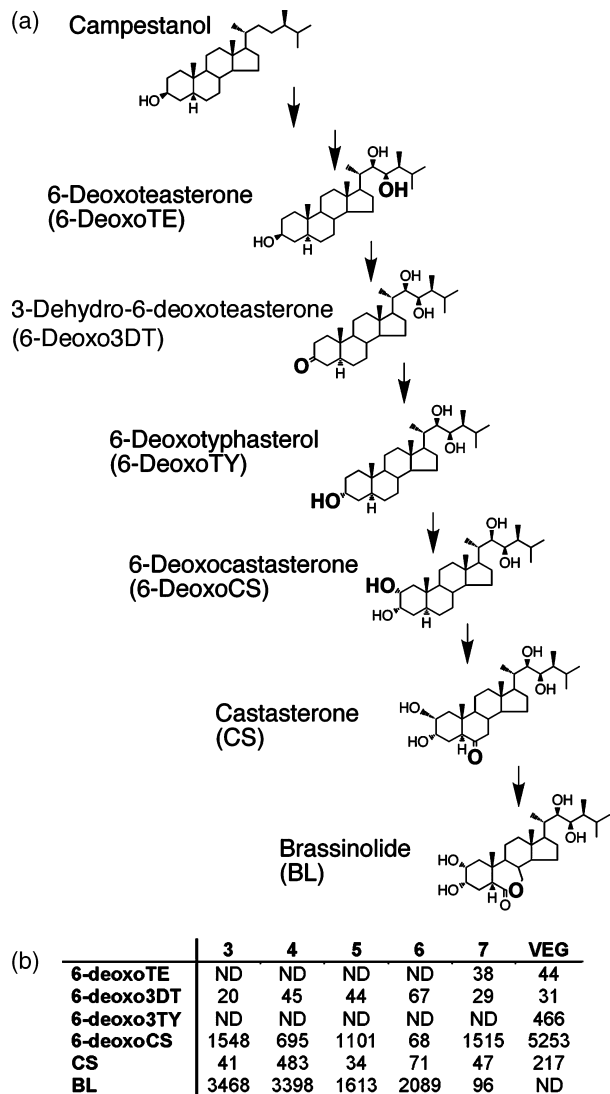
compared to the pericarp tissues, the much higher BL levels of the locular jelly were associated with the lower 6-deoxo-CS content. Such depletion of the 6-deoxo-CS pool also seems to be in good agreement with the strong *Dwarf* expression in the locular jelly and immature seed fraction.

#### Grafting indicates lack of BR transport

The presence of BL in tomato fruit and its apparent absence in the vegetative organs suggested the lack of BL transport from fruits. This is possibly the consequence that fruits are sink organs. However, to test whether the lack of BR transport in tomato was similar to that observed in pea (Symons and Reid, 2004) we carried out reciprocal grafting experiments between WT and  $d^x$  plants. As shown in Figure 6, no phenotypic recovery of the  $d^x$  phenotype was observed when either WT or  $d^x$  was used as the stock or scion.

#### Discussion

A key objective has been to identify potential sites of active BR biosynthesis within plants. Seeds and pollen are known to accumulate BRs, but it was unclear whether the hormone detected in these reproductive structures is synthesized locally. More direct evidence has been obtained by monitoring the transcript levels of the BR-biosynthetic P450 genes of Arabidopsis in various plant organs using RT-PCR (Bancos *et al.*, 2002; Shimada *et al.*, 2003). Furthermore, expression of the *CPD* gene, encoding the CYP90A1 BR C-23 hydroxylase, was studied using transgenic Arabidopsis carrying the *CPD*:GUS reporter fusion (Mathur *et al.*, 1998). These expression studies revealed distinct spatial expression patterns of the genes involved in BR synthesis. However, *CPD* and *BR6ox2/CYP85A2*, one of the two Arabidopsis C-6 oxidase genes, showed preferential activity both around the apical meristem and in flower organs (Mathur *et al.*, 1998; Shimada *et al.*, 2003). More recently, reporter fusions generated using the Arabidopsis C-6 oxidase promoters indicated that *CYP85A2* has higher gene activity than *CYP85A1*. These studies, consistent with RT-PCR and



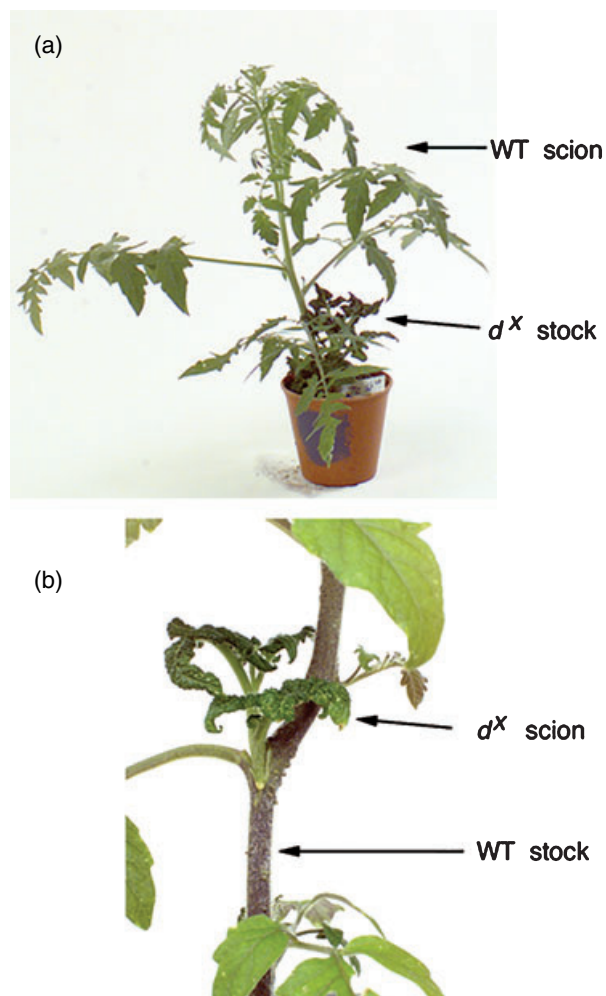
**Figure 5.** Changes of BR content during fruit development.

(a) BR biosynthesis pathway. Intermediate structures are shown with larger font size moieties that change at each conversion step.

(b) BR content in fruits. The samples (3–7) from which the endogenous BR levels (ng kg<sup>-1</sup> fresh weight) were determined correspond to the fruit stages or parts 3–7 of Figure 4, respectively. VEG refers to BR content data of vegetative tissues previously reported (Bishop *et al.*, 1999). ND: not detected.

microarray data, also showed that the expression pattern of *CYP85A2* in *Arabidopsis* was similar to that of *Dwarf*, i.e. associated with the apical and root meristems (Castle *et al.*, 2005). The C-23 hydroxylase and C-6 oxidases encoded by these genes function in the latter steps of BR synthesis and therefore the location of their expression can be regarded as a likely indicator of active steroid hormone synthesis.

The GUS reporter fusion to the *Dwarf* promoter allows detailed localization of promoter activity during plant development. Because our semi-quantitative RT-PCR analysis of the *Dwarf* transcript shows good agreement with the GUS



**Figure 6.** Grafting of *d<sup>x</sup>* and WT tomato plants.

Reciprocal grafts of WT and *d<sup>x</sup>* plants highlighting the lack of phenotypic rescue of *d<sup>x</sup>* mutant tissue.

(a) *d<sup>x</sup>* stock with WT scion, showing the lack of basipetal BR transport.

(b) WT stock with *d<sup>x</sup>* scion, indicating the lack of acropetal BR transport.

activity results obtained in *Dwarf:GUS* transgenic tomato, we therefore believe that this reporter fusion is expected to reflect *Dwarf* expression faithfully. The reliability of this *Dwarf* reporter system is further corroborated by the coincidence of high expression level and BL accumulation in developing fruits.

*Dwarf* catalyses the oxidative conversion of 6-deoxo-CS to CS (Bishop *et al.*, 1999), which is thought to be a major bioactive BR in tomato (Bishop *et al.*, 1999; Yokota *et al.*, 1997). The formation of CS has been suggested to be one of the tightly controlled rate-limiting steps of BR biosynthesis not only in tomato, but also in *Arabidopsis* and pea (Nomura *et al.*, 2001). This implicates BR synthesis in apical regions and is consistent with the *in situ* data identifying *Dwarf* as an early marker for leaf primordia formation (Pien *et al.*, 2001). These results, taken together with the GUS staining, are

consistent with the severely dwarfed phenotype exhibited in  $d^x$  plants (Bishop *et al.*, 1999; Nadzhimov *et al.*, 1988).

The lack of long-distance transport of BRs provides further supportive evidence that the regions of GUS activity are the likely loci of BR action. Evidence for the lack of long-distance transport of BRs is derived from the observation of phenotypic variegation, resulting from localized revertant sectors, in the transposon-generated dwarf mutants (Bishop *et al.*, 1996), and also recent reciprocal grafting experiments in pea (Symons and Reid, 2004). In the grafting experiments WT pea stocks and scions failed to rescue the BR-dwarf mutant phenotype (Symons and Reid, 2004). The reciprocal grafting experiments in tomato, using  $d^x$  and WT plants, showed that the  $d^x$  phenotype was not restored to WT in either of these types of graft. Both sets of experiments indicated that neither basipetal nor acropetal transport of BRs has a major role in plant growth and development and that synthesis is most likely associated with the sites of BR action.

The very strong GUS staining associated with the locular jelly of immature fruit is expected to indicate intense *Dwarf* expression in and/or around the developing seeds. Because the texture and pH of the locular jelly is very different from those of other tomato tissues, these conditions may influence the efficiency of the histochemical reaction. To avoid the possibility that altered pH levels may have on GUS activity staining was repeated in higher concentrations of phosphate buffer to increase the buffering capacity, and similar staining patterns were obtained (data not shown). Although high local peroxidase levels might enhance GUS staining by facilitating oxidative dimerization of the Xglucuronide-derived indol product (Jefferson *et al.*, 1987), both our RT-PCR data showing increased amounts of the *Dwarf* transcript and BR analyses revealing BL accumulation are in good agreement with the detected *Dwarf* expression in this fruit region.

One surprising result of our studies was that immature tomato fruits contain high amounts of BL. Earlier it was assumed that BL is not synthesized in this species, because it could not be detected in vegetative organs (Nomura *et al.*, 2001; Yokota *et al.*, 1997), including those of *Dwarf*-over-expressing transgenic plants (Bishop *et al.*, 1999) or BR-insensitive mutants that accumulate BRs (Montoya *et al.*, 2002). The apparent lack of BL in the vegetative parts and its presence in the fruit raises the possibility that BL may have a specific role in the development of fruits. It is exciting to speculate that the high level of BL in fruits is in part due to the expression of *Dwarf*. However, *Dwarf* has only been shown to be involved in the synthesis of CS. BL synthesis in fruits will therefore require the fruit-specific expression of an enzyme catalysing the lactone formation in the steroid B ring.

The *Dwarf* transcript has been shown to be feedback-regulated by BL (Montoya *et al.*, 2002). This raises the question how strong *Dwarf* expression can take place in the

locular jelly containing high amounts of BL. However, the average approximately 5 nM concentration of the hormone, calculated on the basis of fresh weight, might only partially repress *Dwarf* activity. In *Arabidopsis*, 10 nM BL reduces *CPD* transcript level to about 25% of the control, compared with the approximately 10% measured following treatment with 100 nM BL (Mathur *et al.*, 1998). Although in the BR-deficient *det2* mutant 10 nM BL caused a more pronounced decrease in the level of the *CPD* (Goda *et al.*, 2002) and *BR6ox2* transcript (Shimada *et al.*, 2003), in this mutant both the initial amounts of these mRNAs and BR-sensitivity of the seedlings are higher than in WT *Arabidopsis*. Another explanation can be that BRs are sequestered to specific regions or subcellular locations and thus do not influence *Dwarf* expression. These intriguing observations are worthy of further investigation.

## Experimental procedures

### Plant growth and grafting

For histochemical analysis transgenic tomato seedlings harbouring pGB2131 T-DNA were grown in controlled environment conditions, as described previously (Bishop *et al.*, 1999), and growth conditions for glasshouse plants grown for flower and fruit analysis were essentially the same as described by Montoya *et al.* (2002). Simple V-wedge grafts were made between plants at approximately 3–8 weeks old. Cuts were made using a single-edge razor near a region in which a lateral side branch may form, and joints made to similar diameter stem tissue. Joints were covered with petroleum jelly and secured using masking tape. Excess leaf material was removed from scions and stocks. Grafted plants were placed either in custom-built polythene 'tents' or in a misting chamber to maintain high humidity. After a period of time when the grafted plants were stable they were placed in the glasshouse.

### Histochemical staining and sectioning

Tissue was vacuum infiltrated and stained overnight at 37°C in 1 mg ml<sup>-1</sup> X-gluc in 100 mM sodium phosphate buffer (pH 7.0). Plant pigments were removed using 70% ethanol at approximately 60°C. Samples to be sectioned were embedded in Paraplast+ and approximately 10 µm sections were made as described in Rebers *et al.* (1999).

### Generation of GB2131 Dwarf promoter fusion lines

*Dwarf* promoter (1.2 kb) was PCR amplified from the plasmid pGB46-3, containing an approximately 6 kb *XhoI* subclone cosmid GB17-12 (Bishop *et al.*, 1996), using primers DW16 (5'-AGA-AGAAGGCCATGGATGCACCTC) and M13 Universal forward primer. Primer DW16 introduced an *NcoI* site at the ATG of *Dwarf*. The PCR product was digested with *NcoI* and *EcoRI*, then ligated to *NcoI*-*EcoRI*-digested pSLJ4D4 vector containing the GUS reporter (Jones *et al.*, 1992). Sequencing was carried out on the resulting plasmid pGB212-1 to ensure that no PCR errors were introduced. pGB212-1 was then cut by *EcoRI* and *HindIII*, and the *pDwarf*:GUS fusion was gel-purified and ligated into *EcoRI*-*HindIII*-cleaved pSLJ7291 binary vector to give pGB2131. *Agrobacterium*-mediated

transformation of pGB2131 into tomato cultivar Moneymaker was carried out as described (Fillatti *et al.*, 1987).

### BR analysis

Samples were collected and pooled according to size or phase of development. Pooled samples were frozen in liquid nitrogen and ground to a fine powder. Frozen material was freeze-dried and stored until BR analysis. This was performed using deuterated BR internal standards, as described previously (Bishop *et al.*, 1999; Nomura *et al.*, 2001; Yokota *et al.*, 1997).

### Semi-quantitative RT-PCR analysis of Dwarf

Tissue samples were ground to a powder in liquid nitrogen and RNA extracted as described previously (Montoya *et al.*, 2002). Total RNA of fruits was isolated from the same pooled samples used for BR analysis. RT-PCR was carried out using the same method and primers as described in Montoya *et al.* (2002).

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