Down-regulation of DR12, an auxin-response-factor homolog, in the tomato results in a pleiotropic phenotype including dark green and blotchy ripening fruit

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Summary

Following differential screening of gene expression during tomato fruit development, we isolated developmentally regulated (DR) clones, including several putative transcription factors. Based on sequence homology, DR1, DR3, DR4 and DR8 are members of the Aux/IAA family, and DR12 belongs to the auxin response factor (ARF) family of transcription factors. Importantly, mRNA accumulation for the *Aux/IAA*-like genes was regulated by ethylene in tomato fruit but not in the leaves, indicating that these putative auxin response components also participate to the ethylene-dependent regulation of gene expression in a tissue-specific manner. The functional significance of *DR12*, the *ARF*-like gene, was investigated by cellular biology and reverse genetics approaches. Heterologous protein targeting studies, carried out using a *DR12–GFP* gene fusion construct, revealed specific nuclear localization of the DR12-encoded protein, in accordance with its putative function as a transcriptional regulator. Transgenic plants over- and under-expressing *DR12* were generated in order to explore the physiological role of the gene. Both antisense and sense co-suppressed DR12-inhibited lines displayed a pleiotropic phenotype that included dark-green immature fruit, unusual cell division in the fruit pericarp, blotchy ripening, enhanced fruit firmness, upward curling leaves and increased hypocotyl and cotyledon growth. While a perturbation of the response to auxin may explain some of the phenotypes, surprisingly, the expression of members of four classes of early auxin-regulated genes was unaffected in the DR12-inhibited plants. The involvement of this ARF-like encoded protein in mediating the auxin response is discussed along with the possibility that it might affect responsiveness to other phytohormones in the tomato.

Keywords: *Licopersicon esculentum*, transcription factor, auxin, ethylene, fruit ripening, GFP tagging.

Introduction

Major alterations occur throughout fruit ontogeny in the levels of several classes of phytohormones (Gillaspy *et al.*, 1993), suggesting a dynamic involvement in the fruit developmental program. It is well established that ripening of climacteric fruit such as the tomato is triggered by ethylene (Lelievre *et al.*, 1997). The plant hormone auxin plays a role in cell division and cell expansion during fruit development (Abel and Theologis, 1996). Auxin is also involved in the fruit ripening process as exogenous applications of the hormone delay fruit ripening in many crop species (Cohen, 1996; Vendrell, 1985) suggesting a role for endogenous auxins in regulating the capacity of fruit to ripen. Auxin modulates plant growth and development through transcriptional regulation of specific genes (Ulmasov *et al.*, 1999a). Functional analyses of promoter regions of auxin-regulated genes resulted in the identification of a conserved auxin-responsive *cis*-element (AuxRE) known as the TGTCTC box (Ulmasov *et al.*, 1995) or the TGTCCCAT element (Oeller *et al.*, 1993). Subsequent research identified auxin response factors (ARFs) that bind with specificity to this element (Ulmasov *et al.*, 1997a). ARF proteins have been shown to activate or suppress transcription in an

auxin-dependent manner (Ulmasov *et al*., 1999a) and to interact with short-lived nuclear proteins belonging to the Aux/IAA family of auxin-responsive transcription factors (Abel *et al*., 1994; Kim *et al*., 1997; Ulmasov *et al*., 1997b). It has been suggested that the myriad spatial and temporal responses to auxin are, at least in part, determined by interactions between members of these two gene families (Kim *et al*., 1997).

We isolated differentially expressed genes in pre-ripening tomato fruit in an attempt to identify factors that act in concert with ethylene in determining the tomato fruit developmental program. Among the developmentally regulated (DR) genes, several were identified that putatively coded for auxin transcription factors. The first of these to be further characterized were *DR12*, a tomato ARF homolog, and *DR1, DR3, DR4, DR8* that showed sequence similarity with *Aux/IAA* genes from several species. Here, we describe the characterization of these genes in tomato fruit. Strikingly, we found that the expression of many of these putative auxin transcription factors was regulated by ethylene as well as by auxin, suggesting a novel point of convergence between the responses to the two hormones. A reverse genetics approach was used to study the function of *DR12* in the tomato. Several aspects of the pleiotropic phenotype resulting from DR12 inhibition indicate a role for the encoded protein in the response either to auxin or to cytokinin. However, given the regulation of DR12 transcript accumulation by ethylene, these aspects of physiology and morphology are more likely under the control of several interacting factors.

Results

Isolation of ARF *and* Aux/IAA *homologs in the tomato*

A combination of differential display screening (Zegzouti *et al*., 1997) and RT-PCR using gene–family-specific degenerate primers was used to identify genes differentially expressed during pre-ripening tomato fruit development. Among the DR clones isolated, we identified a 315-bp differential display fragment (DR12) which, when compared to GenBank sequences, showed 57% amino acid identity with the *Arabidopsis thaliana ARF4* gene. It has been proposed that in *Arabidopsis*, the auxin response is controlled, at least in part, by interactions between ARF and Aux/IAA transcriptional regulator proteins (Kim *et al*., 1997; Ulmasov *et al*., 1997b). Four full-length tomato *Aux/IAA* homologs were also isolated on the basis of their differential expression during fruit ripening. DR1, DR3, DR4 and DR8 were found to correspond to the partial GenBank clones *LeAux/IAA1*, *LeAux/IAA3*, *LeAux/IAA4*, and *LeAux/IAA8,* respectively.

Sequence analysis of the DR clones

DR12 shares strong sequence similarity with *Arabidopsis ARF* genes that are known to code for DNA-binding transcription factors that regulate auxin-dependent gene expression (Guilfoyle *et al*., 1998). The DNA-binding domain (DBD) of ARF-encoded proteins binds to a conserved auxin response element (AuxRE) (TGTCTC) found in the promoters of a variety of early/primary auxin-responsive genes, including members of the *Aux/IAA*, *SAUR* and *GH3* families (Ulmasov *et al*., 1999b). A PCR-based approach (Zegzouti *et al*., 1999) was used to isolate the full-length cDNA of DR12. The deduced polypeptide showed 57% amino acid identity with *ARF4* over the entire sequence and 95% amino acid identity over the predicted DBD (Figure 1a). With the exception of ARF3 and one other clone, *Arabidopsis* ARF proteins also contain carboxy-terminal domains (CTDs) related to domains III and IV in members of the *Aux/IAA* family (Guilfoyle *et al*., 1998). DR12 was shown to contain a nuclear localization signal (PSORTII) and 94 and 87% amino acid identity with ARF4 over the domains III and IV, respectively. This strong sequence identity is indicative of a similar function in the tomato protein. Recent work (Ulmasov *et al*., 1999b) has indicated that ARF4 is capable of binding, either as a homo- or heterodimer with ARF1, to the single copy palindromic sequence (TGTCTCCCAAAGGGAGACA).

*Aux/IAA*s are early/primary auxin-responsive genes encoding short-lived, nuclear-localized proteins (Abel *et al*., 1994; Kim *et al*., 1997; Ulmasov *et al*., 1997b). Three of the full-length Aux/IAA clones, DR1, DR3 and DR8, were of similar length, whereas DR4 was significantly longer. However, all four predicted polypeptide sequences contained the domains I–IV that define Aux/IAA proteins (Figure 1b). At the protein level, DR1, DR3, DR4 and DR8 derived polypeptides are most closely related to *Arabidopsis* IAA4, IAA3, IAA9 and IAA17, respectively.

ARF *and* Aux/IAA *homologs are differentially regulated by ethylene and during fruit development*

RT-PCR analysis of DR12 expression indicated that mRNA levels increased throughout tomato fruit development, with the highest levels in early red-stage fruit (Figure 2a). As with *Arabidopsis ARF* genes, DR12 mRNA accumulation does not alter with auxin treatment (Figure 2b). Analysis of the spatial expression of DR12 indicated that its transcripts were most abundant in leaves and hypocotyls but are undetectable in roots and are found at similar levels in reproductive tissues (flowers, seeds, and fruit pericarp) (Figure 2c).

In the tomato, autocatalytic ethylene production leads to high levels of endogenous ethylene during fruit ripening (Figure 3e). Elevated levels of transcripts at this stage indicated a potential involvement of endogenous ethylene

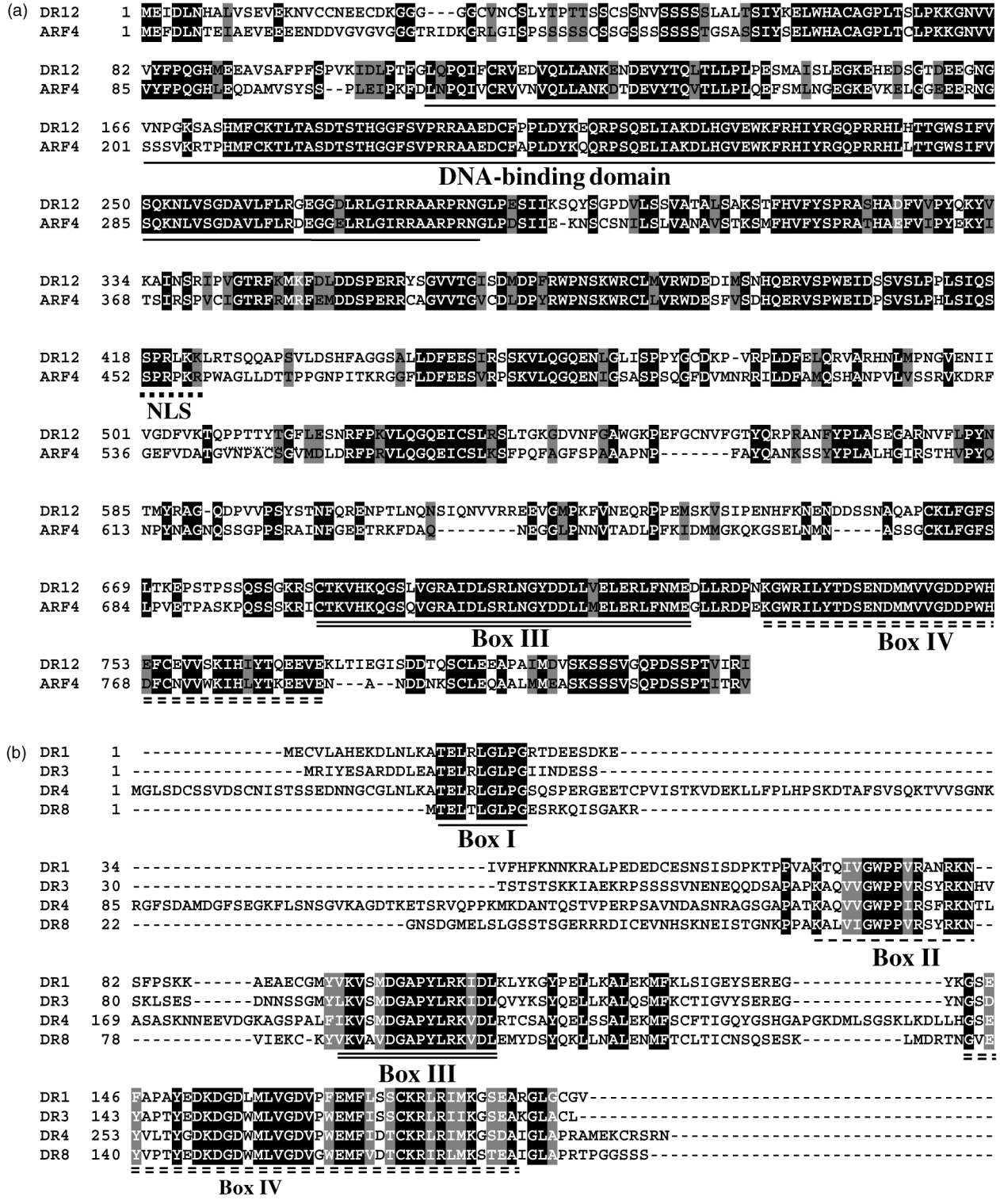

Figure 1. (a) Predicted amino acid sequence alignments of DR12 and *Arabidopsis* ARF4. The sequence predicted to constitute the DNA-binding domain is underlined and the domains related to the sequences III and IV of Aux/IAA proteins double underlined in continuous and broken lines, respectively. (b) Sequence alignment of the DR1, DR3, DR4 and DR8 predicted polypeptide sequences. The sequences predicted to constitute the four conserved domains in Aux/IAA proteins, I–IV are underlined in single, single broken, double, and double broken lines, respectively. The numbers at the left of the sequences indicate the amino acid residue number. Identical and similar amino acids are shaded in black and grey, respectively. Amino acid sequences were deduced from cDNA sequences.

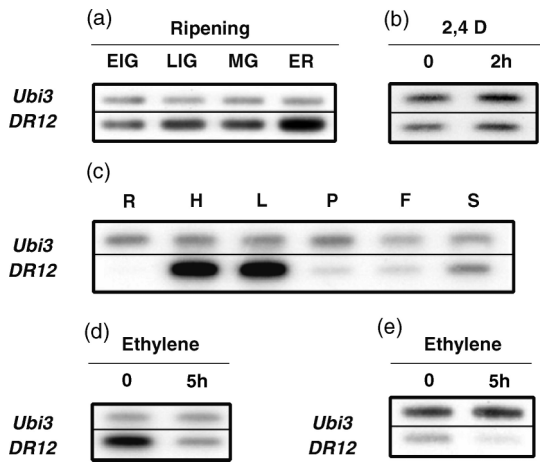

Figure 2. RT-PCR analysis of DR12 transcript accumulation.
(a) RT-PCR analysis of DR12 transcript accumulation during tomato fruit development, at stages: early immature green, EIG; late immature green, LIG; mature green, MG; and early red, ER.
(b) Transcript accumulation in LIG fruit at zero (0) or after 2 h (2 h) auxin treatment (10 μM).
(c) Transcript accumulation in: roots, R; hypocotyls, H; leaves, L; pericarp, P; flowers, F; and seeds, S.
(d) Transcript accumulation in fully expanded leaves at time zero (0) or after 5 h (5 h) ethylene treatment(50 $\mu l\, l^{-1}$).
(e) Transcript accumulation in LIG fruit at time zero (0); or after 5 h (5 h) ethylene treatment (50 $\mu l\, l^{-1}$). In each experiment the internal reference *ubi3* was co-amplified with the DR12 cDNA as described in the Materials and Methods. Results are representative of three separate experiments.

in DR12 mRNA accumulation. To investigate the effect of ethylene on DR12 transcript accumulation, leaves and late immature green fruit, both tissues with low endogenous ethylene production, were treated with exogenous ethylene (50 ppm, 15 min or 5 h). Surprisingly, ethylene treatment in both fully expanded leaves (Figure 2d) and immature fruit lead to a decrease in *DR12* transcript levels (Figure 2e).

We then examined mRNA accumulation for the tomato *Aux/IAA* homologs. Ethylene rapidly and differentially affected transcript accumulation for all four clones in late immature fruit. DR3 was strongly and positively regulated by ethylene whereas DR1, DR4 and DR8 were negatively regulated (Figure 3a). Interestingly, ethylene failed to alter the expression of these tomato *Aux/IAA*-like genes in leaves uncovering a tissue-specific ethylene regulation (Figure 3b). In contrast to DR12, transcript accumulation for each of the *Aux/IAA* homologs altered during fruit ripening (Figure 3c) in accordance with expectations given their ethylene responsiveness and endogenous levels of the hormone (Figure 3e). DR3 mRNA accumulated strongly in early red-stage fruit when autocatalytic ethylene was high, while DR1, DR4 and DR8 showed a decrease in transcript accumulation (Figure 3c). Under the present treatment conditions, only DR3 and DR8 transcripts increased in abundance in response to exogenous auxin (Figure 3d).

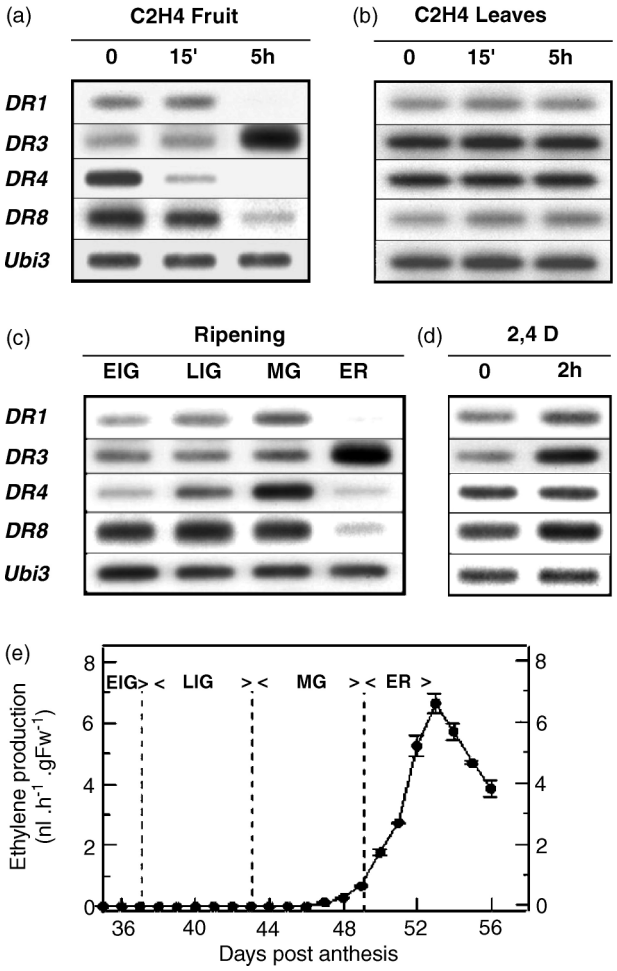

Figure 3. RT-PCR analysis of transcript accumulation of tomato *Aux/IAA* homologs. Transcript accumulation of DR1, DR3, DR4 and DR8 in late immature green fruit (a) or green leaves (b) treated with ethylene (50 $\mu l\, l^{-1}$) for 15 min (15′) and 5 h (5 h) or untreated (0).
(c) Transcript accumulation of DR1, DR3, DR4 and DR8 during tomato fruit development at stages: early immature green, EIG; late immature green, LIG; mature green, MG; and early red, ER.
(d) Transcript accumulation of DR1, DR3, DR4 and DR8 in late immature green fruit treated with auxin (10 μM) for 2 h (2 h) or untreated (0). In each experiment the internal reference *ubi3* was co-amplified with the DR cDNAs as described in the Materials and Methods. The RT-PCR data presented in this figure are representative of three independent experiments.
(e) Endogenous ethylene production during tomato fruit ripening. Ethylene measurement were performed as described previously (Ayub *et al.*, 1996). Data are representative of three independent experiments.

The DR12-encoded protein is targeted to the nucleus

Given its strong sequence homology with *Arabidopsis* transcription factors and the presence of a putative nuclear localization signal in the predicted sequence (Figure 1) the DR12-encoded protein was expected to be targeted to the nucleus. To analyze the subcellular localization of the DR12 protein, we fused green fluorescent protein (GFP) to the COOH-terminus of the DR12 protein expressed under the

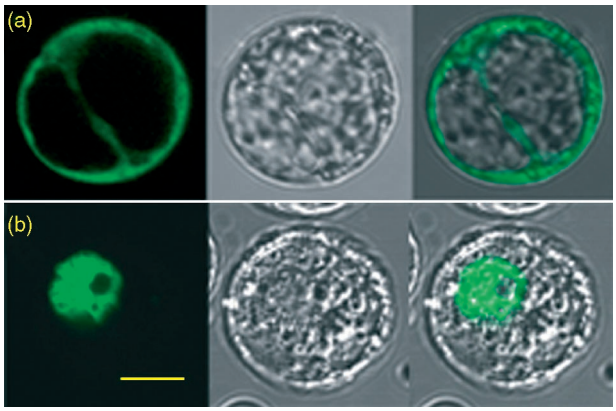

Figure 4. Nuclear localization of DR12 protein fused to GFP tag. Constructs consisting of either control 35S::GFP (a) or 35S::DR12–GFP (b) were used to transiently transform tobacco protoplasts. The subcellular localization of the DR12–GFP fusion protein or GFP alone were analyzed by confocal laser scanning microscopy (left panel). Light micrographs (medium panel) and fluorescence (left panel) images are merged (right panel) to illustrate the different location of the two proteins. The length of the bar corresponds to 10 μm.

control of the 35S promoter. Plasmids bearing either the *GFP* gene alone or the DR12–GFP fusion were transiently transformed into tobacco protoplasts. Fluorescence microscopy analysis associated with image overlay techniques demonstrated that control cells transformed with GFP alone displayed fluorescence distributed throughout the cytoplasm, in accordance with the expected cytosolic localization of the GFP protein (Figure 4a). In contrast, the DR12–GFP fusion protein was exclusively localized to the nucleus (Figure 4b) indicating that DR12 was able to fully redirect the GFP from the cytosol to the nucleus. This is in agreement with a putative transcriptional regulation function.

Exploring the role of DR12 through a reverse genetics approach

To further address the functional significance of the DR12-encoded protein and to explore its physiological role, transgenic tomato lines were generated expressing either sense or antisense constructs of the gene. Eight independent antisense lines and four sense co-suppressed lines were found to be down-regulated for DR12. Two lines, one antisense and the other sense suppressed, showing the strongest inhibition of the *DR12* gene (Figure 5a) were selected for further molecular and physiological characterization. In addition, five independent sense over-expressing heterozygous lines were identified on the basis of their ability to accumulate significantly higher amount of DR12 transcript than wild type (data not shown).

DR12-inhibited lines, either antisense or sense co-suppressed, exhibited a visible pleiotropic phenotype. The fruit phenotype included: (i) a dark-green coloration in unripe

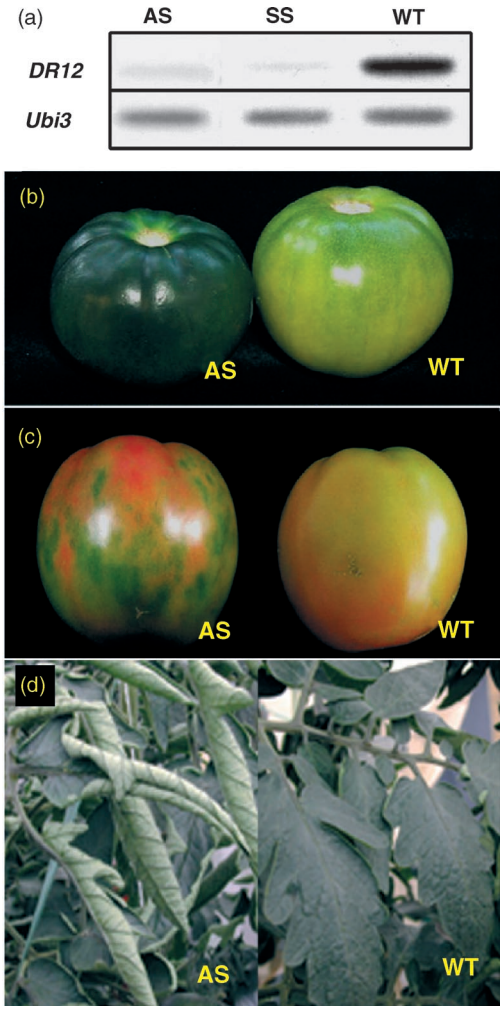

Figure 5. Altered phenotypes of DR12-inhibited plants.
(a) Down-regulation of DR12 in transgenic knockout lines by RT-PCR analysis. DR12 transcript accumulation were analyzed using RNA samples extracted from pericarp tissue of late immature green tomato fruit produced in antisense suppressed (AS), sense suppressed (SS) and wild-type (WT) lines. In each PCR reaction, the internal reference *ubi3* was co-amplified with the *DR12* gene as described in the experimental procedure.
(b) Dark-green phenotype of DR12 antisense fruit (AS) at anthesis + 30 days compared to wild-type fruit (WT) at the same stage.
(c) Blotchy ripening phenotype of DR12 antisense fruit (AS) at breaker + 3 days compared to wild-type fruit (WT) at the same stage.
(d) Close-up view showing the upward curled leaf phenotype of DR12-inhibited lines. The leaves of DR12-suppressed lines exhibit severe in-rolling along the longitudinal axis of the leaf (AS) when compared to wild-type plants (WT) grown in the same conditions.

fruit (Figure 5b); (ii) a patchy or 'blotchy' ripening (Figure 5c); and (iii) an enhanced fruit firmness at the overripe stage (data not shown). In addition to a role during fruit development, down-regulation of DR12 also resulted in severe leaf upward curling along the longitudinal axis of the leaf (Figure 5d) and increased hypocotyl and cotyledon growth. When measured after germination and growth for 7 days in the darkness, etiolated antisense hypocotyls were

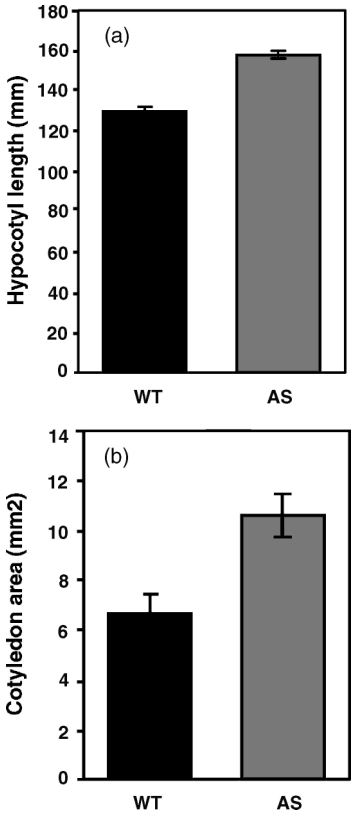

Figure 6. Growth of wild-type and antisense tomato seedlings.
(a) Hypocotyl length after 7 days growth in the dark for wild-type (WT) and DR12-inhibited lines (AS).
(b) Area (mm^2) of wild-type (WT) and antisense (AS) cotyledons after 7 days growth in the light (16/8 day/night). Results represent the average ± SE of 120 individual seedlings. The experiments were repeated three times.

approximately 20% longer than their wild-type counterparts grown under the same conditions (Figure 6a). Furthermore, in light-grown seedlings, at germination + 7 days the average surface area of AS cotyledons was 57% greater than in the wild type (Figure 6b). Root length and mass, fruit size and form, and vegetative growth other than those described above were unaffected by the inhibition of DR12.

The dark green phenotype was observed from around 7 days post-anthesis and the marked difference in color between DR12-inhibited and wild-type fruit persisted up until the fruit were an even red-ripe at breaker + 10 days. Analysis of the chlorophyll content of immature fruit pericarp showed that at anthesis + 20 days, the pericarp of DR12-inhibited fruit had approximately 50% more chlorophyll than the wild type. The difference between DR12-inhibited and wild-type pericarp chlorophyll content markedly increased as the fruit neared the onset of ripening. At anthesis + 30 days (Figure 5b) DR12-inhibited pericarp contained greater than 100% more chlorophyll than the wild type.

No change was observed in the chla:chlb ratio (data not shown). Light micrographs (Figure 7a,b) and a direct count of chloroplast numbers in cells of the outer pericarp of fruit at anthesis + 30 days indicated a three fold higher number of chloroplasts in DR12-inhibited fruit ($88 \pm 9/0.1\,mm^2$) than in wild type ($24 \pm 2/0.1\,mm^2$). In addition, electron microscopy revealed a dramatic increase in grana formation in individual chloroplasts of DR12-inhibited fruit (Figure 7e) as compared to wild-type fruit (Figure 7d). DR12-inhibited lines also showed a consistent 'blotchy' ripening phenotype (Figure 5c) from the breaker through to the red-ripe stage. At breaker + 10 days, both the DR12-inhibited and wild-type fruit had attained a uniform red-ripe appearance. However, commensurate with the increased chloroplast number in immature fruit, DR12-inhibited fruit at breaker + 10 days contained approximately 43% more carotenoids than wild-type fruit at the same stage.

While most aspects of fruit ripening and quality parameters such as Brix, pH, total acidity and time from anthesis to the onset of ripening, were unaffected, DR12-inhibited fruit exhibited an enhanced firmness at the post-breaker stage. The greatest difference in firmness between the antisense and wild-type fruit was observed at breaker + 35 days, when tomato fruit are at the over-ripe stage, normally having undergone severe softening (data not shown).

Unusual cell division in DR12-inhibited pericarp tissue

Cell division in the pericarp of wild-type fruit ceases at the beginning of the expansion phase, 5–7 days after pollination (Gillaspy *et al.*, 1993). Interestingly, light micrographs (Figure 7b) and transmission electron microscopy (Figure 7c) revealed an unusual pattern of cell division in DR12-inhibited fruit tissue that suggested a continuation of division throughout development. Primary cell walls, indicative of continued cell division, were found dividing cells in existing cell files in anthesis + 35 days DR12-inhibited pericarp tissue. These primary cell walls were not present in wild-type fruit of the same age.

Antisense DR12 *does not alter the expression of auxin-responsive genes*

In order to explore, at the molecular level, the potential role of *DR12* in mediating the auxin response, we examined the expression of at least one representative of the three major classes of tomato early/primary auxin-responsive genes. In addition to DR3 and DR8, the two *Aux/IAA*-like genes that showed clear response to auxin (Figure 3d), we have also isolated tomato homologs of the auxin-inducible *GH3* (Liu *et al.*, 1994), *SAUR* (Gil *et al.*, 1994) and *ACC* synthase (Yip *et al.*, 1992) genes. Figure 8 shows that steady-state level of transcript accumulation corresponding to these auxin-responsive genes was similar, in wild-type and DR12-inhibited tissues. Moreover, exogenous auxin treatment

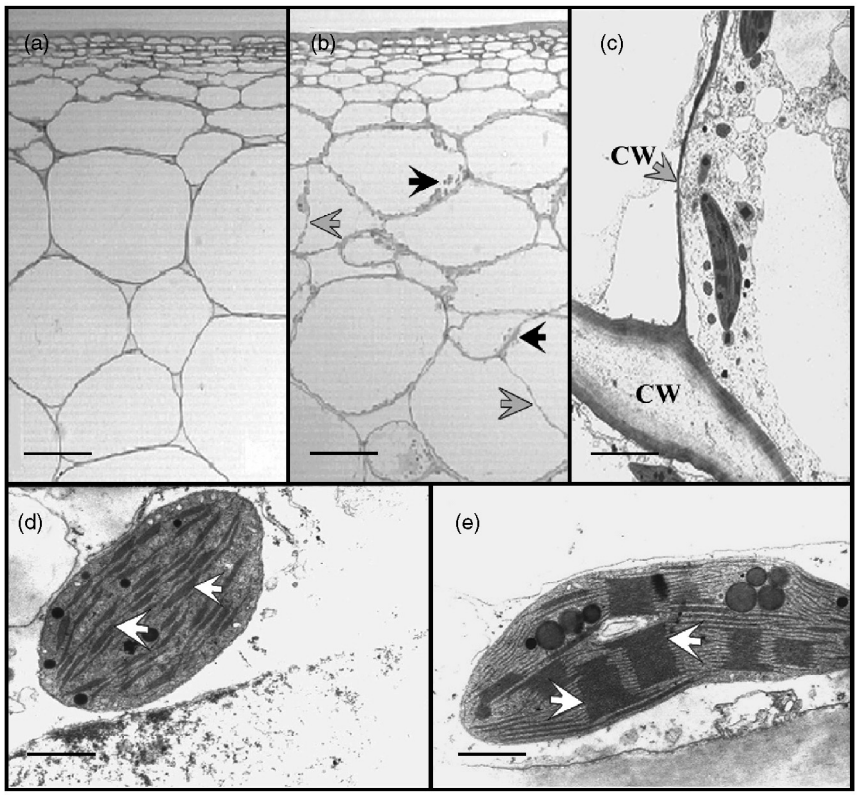

Figure 7. Microscopy analysis of wild-type and antisense fruit tissues at anthesis + 30 days. Light micrograph of exo- and mesocarp tissue from wild-type (a) and DR12 antisense (b) fruit. Black arrows indicate high concentrations of chloroplasts. Grey arrows indicate primary cell walls dividing cells within established cell files observed either by light microscopy (b) or by electron microscopy (c). (d,e) Transmission electron micrograph of chloroplast structure showing less developed thylakoid and grana in mature green wild-type (d) than in DR12 antisense (e) pericarp tissue. Thylakoids are indicated by white arrows. 'a' and 'b', bar = 80 μm; 'c', bar = 10 μm; 'd', bar = 4 μm; 'e' 'and 'f', bar = 0,8 μm. CW = cell wall.

(10 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 2 h) resulted in a similar accumulation of transcripts for these auxin-responsive genes in both wild-type and antisense plants (Figure 8). These data suggest that DR12 does not act through the regulation of early/primary auxin-responsive genes.

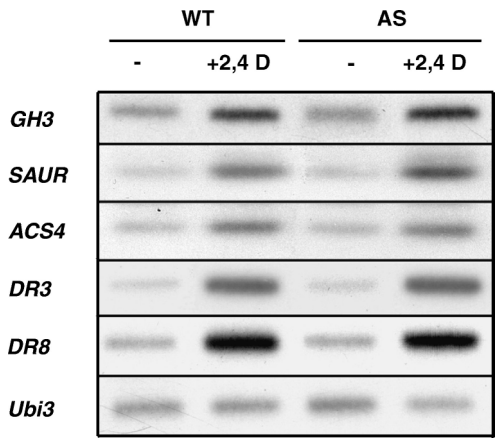

Figure 8. Expression analysis of auxin-responsive genes in *DR12*-inhibited plants. Responsiveness to auxin of early/primary auxin-regulated genes (*GH3, SAUR, ACS4, DR3, DR8*) in wild-type (WT) and DR12-inhibited plants (AS) was analyzed by RT-PCR. The RNA samples were extracted from pericarp tissue of late immature green fruit treated for 2 h with auxin (10 mM 2,4-D) or with water. In each PCR reaction, the internal reference *ubi3* was co-amplified with the target gene as described in the experimental procedure.

DR12 up-regulation perturbs seed development

The five independent DR12 over-expressing lines had fruit with a dramatically reduced number of seeds (Table 1). When germinated on a non-selective medium, germination rates were similar for wild-type and sense over-expressed lines. The ratio of transformed to non-transformed seedlings, determined on the basis of a germination test on selective medium (kanamycin 70 mg l^{-1}) did not exceed 5%, suggesting that ectopic expression of DR12 inhibits seed formation or development. This effect was observed in primary transformants and in R_1 and R_2 generations. All of the germinated DR12 over-expressing seeds were found to be hemizygous for the transgene. As such it was not possible to obtain homozygous DR12 over-expressing lines. For these DR12 over-expressing lines, no other visible phenotype could be observed in vegetative or fruit tissues. In particular, over-expression of DR12 did not result in a lighter green color fruit at the immature stage and did not appear to affect fruit size or the pattern of ripening.

Discussion

The ripening phase of development has been extensively studied in climacteric fruit. In this work, we sought to broaden the study of fruit development through the isolation of genes differentially expressed in pre-ripening tomato fruit. DR12, the first of the isolated genes to be

Table 1 Seed numbers and germination rates in transgenic lines over- and under-expressing DR12

	No. seeds/fruit (g FW)	Germination (%)	Transformants (%)
Wild type	0.90 ± 0.07	84	
DR12 sense	0.30 ± 0.06	80	5
DR12 antisense (homozygous)	0.92 ± 0.07	82	100

Results for the number of seeds are expressed as a number of seeds per gram fresh mass of fruit tissue $\pm$ the standard error of the means. Germination efficiency was assessed on a non-selective medium and the percentage of transformant among the germinated seeds was determined in the presence of the selective agent (kanamycin, 70 mg l^{-1}).

further characterized, was identified as being up-regulated during the course of development and having strong sequence similarity with members of the *Arabidopsis* ARF family of transcription factors.

Mutants have been isolated for three of the *Arabidopsis ARF* genes, *MONOPTEROS/ARF5*, *ETTIN/ARF3* and *NPH 4/ARF7* (Hardtke and Berleth, 1998; Harper *et al*., 2000; Sessions *et al*., 1997). Mutations in these genes result in pleiotropic phenotypes, but each affects a subset of processes consistent with an altered response to auxin. *NPH4/ARF7* null mutants show reduced auxin-responsive gene expression and have multiple differential growth defects, including an altered phototrophic response. Interestingly, the differential growth defects in *NPH4/ARF7* mutants can be suppressed by exogenous ethylene (Harper *et al*., 2000). Ethylene appears to act through an auxin response, as further disruptions to auxin responsiveness in these plants negate the effect. Manifestly, ethylene regulates components of the auxin response in *Arabidopsis*. We have shown here that in the tomato, ethylene regulates transcript accumulation for a number of putative auxin response factors in a tissue-specific manner. This ethylene-regulation of *Aux/IAA* mRNAs contrasts with data obtained so far with *Aux/IAA* in soybean, pea and *Arabidopsis*, where no response to ethylene was reported. It should be noted, however, that none of these studies analyzed the expression in the fruit. In that regard, it is noteworthy that our study also shows no ethylene responsiveness of the *Aux/IAA*-like genes in tomato leaves.

DR12 was isolated on the basis of its differential regulation during fruit development. In climacteric fruit such as the tomato there is a surge of ethylene production commensurate with ripening. DR12 mRNA were found to be most abundant in high ethylene producing red fruit. We then investigated if DR12 transcript accumulation was ethylene-dependent. In contrast to expectations, DR12 transcript accumulation was found to be negatively regulated by exogenous ethylene in leaves and mature green fruit, where ethylene production is low. Treatment of red fruit with 1-methylcyclopropene (1-MCP), a potent inhibitor of ethylene action, did not alter DR12 transcript accumulation (data not shown). This suggests a fundamental shift in ethylene responsiveness for DR12 during the early stages of ripening. Similar changes have been observed for a number of ethylene-responsive genes (Zegzouti *et al*., 1999). In this instance it indicates that a shift in auxin responsiveness may also occur at this developmental juncture.

We have shown for the first time in this study that transcript accumulation for several *Aux/IAA*-like genes are differentially regulated by ethylene. *Aux/IAA* genes encode short-lived nuclear proteins that appear to function as transcriptional repressors (Leyser *et al*., 1996; Tian *et al*., 2002; Tiwari *et al*., 2001; Ulmasov *et al*., 1997b). There are at least 29 *Aux/IAA* genes in *Arabidopsis* (Rogg *et al*., 2001) and all are auxin responsive, although there is a wide diversity in terms of kinetics and dose–response (Abel *et al*., 1995). Of the four tomato *Aux/IAA* genes studied here, only DR3 and DR8 clearly responded to auxin, however, full kinetic and dose–response studies may reveal auxin regulation of the other clones. All four *Aux/IAA* homologs responded to ethylene and, in contrast to DR12, mRNA accumulation during ripening correlated with their ethylene regulation in late immature green fruit. Taken together, the developmental regulation and ethylene and auxin responsiveness of the DR clones isolated in this study strongly suggests that both auxin and ethylene play active/interactive roles throughout tomato fruit development.

To gain more information on DR12 function we analyzed its localization within the cell. The strong homology with ARF transcription factors along with the presence of putative nuclear localization signals (NLS) suggested a nuclear localization for the DR12 protein. Using a protoplast transient expression system, we clearly demonstrated that the GFP-tagged DR12 protein, is efficiently and exclusively targeted to the nucleus. This nuclear subcellular localization is consistent with a putative transcription factor function for DR12. Several *Arabidopsis* ARF proteins contain regions between the DBD and CTD that are either P/S/T or Q/L/S-rich (Ulmasov *et al*., 1999a). In ARF5 through ARF8, the Q-rich middle region acts as an activation domain (AD), enhancing the auxin-induced gene transcription (Ulmasov *et al*., 1999a). In contrast, the middle region of ARF1 is P-rich and its over-expression represses auxin-dependent transcription. In terms of sequence similarity, DR12 is most closely related to ARF4 and the middle regions of both DR12- and ARF4-encoded proteins are enriched in serine

(12%) and proline (10%). In carrot protoplast co-transfection assays, ARF4 did not act as a repressor or activator of auxin-regulated gene expression (Ulmasov *et al.*, 1999a). In accordance with these data, our study shows that down-regulation of the tomato *ARF*-like gene did not alter the expression pattern of *Aux/IAA, GH3, SAUR* and *ACC* synthase genes which are representatives of different classes of early/primary auxin-responsive genes (Abel *et al.*, 1994; Gil *et al.*, 1994; Liu *et al.*, 1994; Yip *et al.*, 1992). Moreover, auxin-inducible tomato homologs such as *Aux/IAA, GH3, SAUR* and *ACC* synthase responded similarly to exogenous auxin treatment in both wild-type and DR12-suppressed plants (Figure 8). These data question the involvement of the DR12-encoded protein in mediating the auxin response. Nevertheless, the phenotypes displayed by both DR12-inhibited and over-expressed plants indicated that appropriate levels of the DR12-encoded protein are important in seed development, seedling growth and fruit cell division processes where auxin is expected to play a role. While a perturbation of the response to auxin may explain several of the phenotypes exhibited by DR12-altered plants, the regulation of DR12 transcript levels by ethylene indicates that changes in the endogenous levels of either or both hormones are involved in regulating these processes in wild-type fruit.

Mutations in some *Arabidopsis ARF* genes indicate that they are important for auxin responsiveness and that the different genes control-specific growth and developmental processes. Inhibiting the expression of DR12 in transgenic plants resulted in an altered phenotype in fruit, leaves and seedlings suggesting that DR12-encoded protein is responsible for modulating several morphological and physiological processes in these tissues. Among the phenotypes exhibited by DR12-suppressed plants, the upwardly curled leaves have been already observed for several gain-of-function *aux/iaa Arabidopsis* mutants (Liscum and Reed, 2002). Similar alterations of leaf morphology have been also reported for *Arabidopsis* early flowering mutants *Curly Leaf* (*clf*) and *Like Heterochromatin Protein 1* (*lhp1*). Both mutations have been shown to affect genes encoding nuclear proteins controlling homeotic genes (Goodrich *et al.*, 1997; Gaudin *et al.*, 2001). It is important to note, however, that down-regulation of DR12 did not appear to affect flowering time or floral organization in transgenic tomato plants. The curled leaf morphology could result from modification of cell division or cell elongation of the adaxial and abaxial epidermis. One of the most dramatic phenotypes in the transgenic plants was the dark-green unripe fruit. This was the result of an augmentation in chlorophyll content associated with a dramatic increase in the number of chloroplasts. Noteworthy, the difference in chlorophyll content between wild-type and DR12 antisense fruit was greater at later stage of fruit development. Because wild type, but not antisense fruit, undergo normal degreening when nearing the onset of ripening, the difference in the green coloring between the two types of fruit becomes more pronounced as the fruit age. Several chlorophyll intensifier mutants have been identified in the tomato. The dark green (*dg*) and high pigment 1 and 2 (*hp1* and *hp2*) mutants have a highly pleiotropic phenotype that includes dark green leaves and immature fruit (Jarret *et al.*, 1984; Sanders *et al.*, 1975). In contrast, the increased chlorophyll in DR12-inhibited plants seems to be confined to the fruit. Furthermore, altered photomorhogenesis in *dg* and *hp* mutations results in a decrease in hypocotyl length (Mustilli *et al.*, 1999) while in DR12-inhibited lines hypocotyls were approximately 20% longer than their WT counterparts. Since auxin is known to promote hypocotyl elongation (Jensen *et al.*, 1998) the phenotype in transgenic plants may be due to an exaggerated response to auxin.

Scanning electron microscopy of chloroplasts in immature fruit showed an increase in thylakoid development suggestive of an altered response to light in DR12-inhibited fruit. Alternatively, interactions between auxin and cytokinin responses are well documented. Exogenous cytokinin can mimic the *hp* mutant phenotype (Mustilli *et al.*, 1999) and cytokinin-hypersensitive *Arabidopsis* mutants show a combination of increased chloroplast development and cell proliferation (Kubo and Kakimoto, 2000). The phenotypes observed in DR12-inhibited fruit may be analagous to those observed in the cytokinin-hypersensitive mutants. Furthermore, the blotchy ripening phenotype is reminiscent of transgenic tomatoes with ectopic expression of the *ipt* gene from the Ti plasmid of *Agrobacterium tumifaciens* (Martineau *et al.*, 1994). Fruit from these tomatoes had 10–100-fold higher cytokinin levels than control plants and during ripening, exhibited altered phenotype with green patches remaining within a deep red background. It is possible that the inhibition of DR12 affects the response to endogenous cytokinins either directly or through alterations in the responsiveness to other hormones.

Because DR12-inhibited lines exhibited some auxin-associated phenotypes, it cannot be excluded that DR12 regulates the expression of late or secondary rather than early/primary auxin response genes. In that context, the responses to inhibition of DR12 suggest that the DR12-encoded protein functions as a negative regulator of the auxin response. Alternatively, given the ethylene responsiveness of the clone and despite the strong similarity with *Arabidopsis* ARFs, DR12 may also bind to sites in the promoters of genes not directly involved in the auxin response. Nevertheless, the phenotypes observed in DR12 transgenic lines indicate that the gene is implicated in physiological processes where both auxin and ethylene have been shown to play roles. Future work will center on unravelling the molecular basis of the DR12 phenotypes. In particular, large scale gene expression analysis by DNA-microarray technology will be used to identify the classes of

genes altered in DR12-transgenic lines and thereby the signaling pathways they might affect.

Experimental procedures

Plant material

Lycopersicon esculentum (Mill. cv. Kemer) plants were grown under standard greenhouse conditions. Hypocotyl and cotyledon measurements were made on seedlings grown on nutrient agar medium for 7 days in darkness or light, respectively. The stages used for the RT-PCR analysis of developmental regulation of the clones were: EIG, early immature green; LIG, late immature green; MG, mature green; and ER, early red. *A. tumifaciens*-mediated transformation was carried out using the pGA643 binary vector according to Bird *et al*. (1988) with minor changes: 6 days old cotyledons were used for the transformation; the duration of subcultures for shoot formation was reduced to 15 days; and the kanamycin concentration was increased to 70 mg l^{-1}. Sense and antisense constructs were under the transcriptional control of the cauliflower mosaic virus 35S promoter (CaMV 35S) and the nopaline synthase (Nos) terminator. The antisense construct consisted of a 645-bp nucleotide sequence corresponding to the less well-conserved middle region of the gene and the sense construct consisted of the full length coding sequence of DR12.

Isolation of differentially expressed clones

The differential display screening was carried out as previously described (Zegzouti *et al*., 1997) and applied to four developmental stages of tomato fruit: EIG, LIG, MG and ER. The Aux/IAA homologs were isolated using an RT-PCR-based screening of a cDNA library using degenerate primers based on conserved sequences in *Arabidopsis* and *Pisum sativum Aux/IAA* genes. The sequence extension and RT-PCR analysis were as described in Zegzouti *et al*., (1999).

Hormone treatment

Ethylene treatment was carried out as described in Zegzouti *et al*., (1999). For the auxin treatment, tomato fruit were harvested and treated for 2 h with 2,4-D (10 μM). After the treatments, tissues were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction. RNA was extracted according to Hamilton *et al*. (1991).

RT-PCR analysis

The expression studies were carried out by RT-PCR analysis of transcript accumulation according to the procedure previously described (Zegzouti *et al*., 1999). The endogenous tomato ubiquitin cDNA *ubi3* was used as internal control and co-amplified with the target cDNA in each PCR reaction.

Microscopy

For conventional microscopy, samples were fixed 2.5 h at 4°C in 2.5% glutaraldehyde and 3% *para*formaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) mixture (Olmos and Hellín, 1996). Tissue was post-fixed with 1% osmium tetroxide for 2 h in the same buffer. The samples were dehydrated in a graded alcohol series and embedded in Spurr's resin. Blocks were sectioned on a Reichert ultramicrotome. Thin sections for transmission electron microscopy were picked up on copper grids and stained with uranyl acetate followed by lead citrate. Ultrastracture of tissue was observed with a Zeiss EM10 and Zeiss EM109. Semithin sections (0.5 mm) were stained with toluidine blue and examined with a Leica light microscope. Confocal fluorescent images were obtained by confocal laser scanning microscopy (Leica TCS SP, Leica DM IRBE; Leica Microsystems, Wetzlar, Germany). Samples, consisting of transfected protoplasts incubated at 25°C for 16 h, were illuminated with an argon ion laser (448 nm wavelength) for GFP fluorescence.

Protoplast isolation and transient expression of DR12–GFP fusion protein

The coding sequence of DR12 was cloned in frame with the GFP into the pGreen vector (Hellens *et al*., 2000) as a C-terminal fusion expressed under the control of cauliflower mosaic virus 35S promoter. Protoplasts used for tranfection were obtained from suspension-cultured tobacco BY-2 cells according to the method described previously (Bouzayen *et al*., 1989). Protoplasts were transfected by a modified polyethylene glycol method as described by (Abel and Theologis, 1994). Typically, 0.2 ml of protoplast suspension (0.5 × 106) was transfected with 50 μg of shared salmon sperm carrier DNA and 30 μg of either 35S::DR12–GFP or 35S::GFP (control) plasmid DNA. Transfected protoplasts were incubated at 25°C for 16 h and were analyzed for GFP fluorescence by confocal microscopy. All transient expression assays were repeated at least three times.

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