A tomato HD-Zip homeobox protein, LeHB-1, plays an important role in floral organogenesis and ripening

Zhefeng Lin¹, Yiguo Hong², Mingan Yin³, Chunyang Li², Ke Zhang² and Don Grierson¹

¹Plant Sciences Division, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD, UK,
²Warwick HRI, University of Warwick, Wellesbourne, Warwick CV35 9EF, UK, and
³College of Horticulture, Northwest A & F University, Yangling, Shaanxi 712100, China

Received 24 January 2008; revised 11 March 2008; accepted 17 March 2008; published online 27 May 2008.

Summary
Ethylene is required for climacteric fruit ripening. Inhibition of ethylene biosynthesis genes, 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase, prevents or delays ripening, but it is not known how these genes are modulated during normal development. LeHB-1, a previously uncharacterized tomato homeobox protein, was shown by gel retardation assay to interact with the promoter of LeACO1, an ACC oxidase gene expressed during ripening. Inhibition of LeHB-1 mRNA accumulation in tomato fruit, using virus-induced gene silencing, greatly reduced LeACO1 mRNA levels, and inhibited ripening. Conversely, ectopic overexpression of LeHB-1 by viral delivery to developing flowers elsewhere on injected plants triggered altered floral organ morphology, including production of multiple flowers within one sepal whorl, fusion of sepals and petals, and conversion of sepals into carpel-like structures that grew into fruits and ripened. Our findings suggest that LeHB-1 is not only involved in the control of ripening but also plays a critical role in floral organogenesis.

Keywords: LeHB-1, HD-Zip homeobox protein, LeACO1, floral organogenesis, ripening, tomato.

Introduction
The gaseous hormone ethylene regulates many aspects of plant growth and development, including ripening, senescence, abscission, and responses to biotic and abiotic stresses (Abeles et al., 1992). Ethylene biosynthesis occurs via the Yang pathway (Yang and Hoffmann, 1984) using two key biosynthetic enzymes, 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) and ACC oxidase (ACO) (Kende, 1993), encoded by differentially expressed multigene families (Barry et al., 1996; Holdsworth et al., 1988; Zarembinski and Theologis, 1994). Antisense-mediated RNA silencing of LeACS2 prevents tomato ripening (Oeller et al., 1991), and inhibition of LeACO1 also causes delayed ripening and leaf senescence (Blume and Grierson, 1997; Giovannoni, 2002; Picton et al., 1993). How transcription of the ethylene biosynthesis genes is regulated is therefore critical for our understanding of processes such as ripening, senescence, abscission and responses to stress.

Analysis of the LeACO1 promoter (~1 to ~1855 nucleotides) (accession no: X88732; Blume and Grierson, 1997; ZL and DG, unpublished data) revealed that it contains putative homeobox cis-elements, similar to those to which AthB-1, a class-I homeodomain leucine zipper (HD-Zip) protein from Arabidopsis binds (Sessa et al., 1993). HD-Zip homeobox proteins are defined by the conserved homeodomain (HD) and adjacent leucine zipper motifs (Sessa et al., 1993). They are unique to plants, but are related to other eukaryotic HD proteins (Henriksson et al., 2005). Homeobox genes contain a highly conserved homeobox DNA sequence of 180 bp, encoding a protein which folds into a characteristic DNA binding structure of helix-loop-helix-turn-helix, and are involved in developmental processes. Some HD proteins...
from both animals and plants have been shown to regulate hormone genes. The homeodomain transcription factors Hesx1 and Prop-1 in mammals, for example, are heavily involved in pituitary organogenesis, and both proteins synergistically regulate the follicle stimulating hormone ß subunit gene by binding its promoter region from −852 to −746 bp (Susa et al., 2007). In plants, the knotted-like homeobox (KNOX) proteins function in shoot apical meristems through regulating the production of gibberellin (GA) and cytokinin (Ori et al., 1999). In both Arabidopsis and tobacco, the KNOX proteins directly repress transcription of genes encoding GA 20-oxidases, the enzymes that encode the last step in GA biosynthesis (Hay et al., 2002; Jasinski et al., 2002; Sakamoto et al., 2001).

Since the discovery of KNODD1 in maize (Sinha et al., 1993; Vollbrecht et al., 1991), a large number of plant genes encoding HDs have been isolated, and can be classified into six families, depending on their gene structures, sequences, size, HD location and association with other domains (Ariel et al., 2007). These include HD-Zip (homeodomain associated with a leucine zipper), PHD finger (plant homeodomain associated with a finger domain), Bell (named after the distinctive Bell domain), ZF-HD (zinc finger associated with a homeodomain), WOX (wuschel related homeobox) and KNOX (knotted related homeobox) (Ariel et al., 2007; Henriksson et al., 2005).

The HD-Zip family consists of a large number of members. In the Arabidopsis genome, there are 47 HD-Zip proteins that can be divided into four groups, I–IV, according to distinctive features of DNA-binding specificities, gene structures, additional common motifs and physiological functions (Ariel et al., 2007; Henriksson et al., 2005). The HD of HD-Zip transcription factors is responsible for the specific binding to DNA, whereas the Zip domain acts as a dimerization motif. HD-Zip proteins bind to DNA as dimers, and the absence of the Zip domain abolishes the binding activity. Proteins in each group recognize different DNA sequences in vitro. Class-I AtHB-1 and class-II AtHB-2, for example, bind to 9-bp DNA sequences with dyad symmetry, CAAT(A/T)ATTG and CAAT(G/C)ATTG, respectively, through the combined HD-Zip domains (Sessa et al., 1993).

Although the HD-Zip proteins have the conserved HD and Zip motifs, their sequences elsewhere are very diverse. The class-I and -II HD-Zip proteins are in general smaller (200–300 aa) than the class-III and -IV proteins (700–800 aa) (Ariel et al., 2007; Henriksson et al., 2005). HD-Zip genes from various plant species are involved in diverse biological functions, including developmental processes in apical meristems and response to light, water stress or ABA (Ariel et al., 2007; Henriksson et al., 2005). The class-III HD-Zip proteins PHABULOSA and PHAVOLUTA have roles in determining radial patterning in shoots (McConnell et al., 2001), and they are targeted by microRNAs (Mallory et al., 2004). AtHB-1 is reported to function as a transcription activator, and affects leaf cell fate when overexpressed in tobacco (Aoyama et al., 1995). More recently, HaHB-4, a class-I HD-Zip gene from sunflower transcriptionally regulated by water availability and ABA, has been reported as a new component of ethylene signalling pathways. Transgenic Arabidopsis plants overexpressing this gene have been shown to exhibit a marked delay in senescence, and are less sensitive to ethylene (Manavella et al., 2006). Expression of this gene has a major repressive effect on genes related to ethylene synthesis, such as ACO, and on genes related to ethylene signalling, such as ERF2 and ERF5 (Manavella et al., 2006). We report here on the identification of a cDNA clone for a previously uncharacterized HD-Zip protein from tomato, LeHB-1, that binds to LeACO1 promoter fragments containing the putative HD-Zip protein binding sequences. Virus-induced silencing of LeHB-1 was shown to inhibit LeACO1 mRNA accumulation and ripening, whereas ectopic overexpression of the gene led to altered flower organ identity and conversion of sepals to fruit-like structures.

Results

LeHB-1 encodes an HD-Zip protein

Important regulatory regions in the ACO1 promoter were identified previously by testing promoter-reporter gene constructs in transgenic plants (Blume and Grierson, 1997). Putative AtHB-1 binding sites were found subsequently in the LeACO1 promoter, and a BLAST search of tomato expressed sequence tag (EST) databases was performed using the AtHB-1 cDNA sequence. Tomato LeHB-1 was identified as the closest match to AtHB-1, and we isolated the corresponding EST clone (tomato EST: TC183162), referred to here as LeHB-1, by RT-PCR using the primers LeHB-1F1/LeHB-1R1 corresponding to the coding sequence (Table 1). Sequencing confirmed that LeHB-1 encodes a 285-aa protein (Figure 1a) with the conserved HD (aa 64–122) and Zip domains (aa 123–165). Sequence alignment indicated that LeHB-1 shares an overall 69% similarity to AtHB-1 (data not shown), and that its HD-Zip domains share 92 and 56% amino acid similarity to those of AtHB-1 and HaHB-4, respectively (Figure 1b). The conserved residues for the HD transcription factors are also present in the three proteins (Figure 1b). There are 17 class-I HD-Zip proteins in Arabidopsis (Henriksson et al., 2005), and 15 have been found in the tomato databases so far (data not shown). Only a small number of them, however, have been characterized. Phylogenetic analysis using the full-length sequences of Arabidopsis class-I HD-Zip proteins, the sunflower HaHB-4, and two tomato class-I HD-Zip proteins VaHox1 and H52 (Mayda et al., 1999; Tomero et al., 1996), together with LeHB-1, indicated that among all these sequences LeHB-1 is most similar to AtHB-1 (Figure 1c).
Table 1 Primers used for this study

| Primer          | Sequences (5′−3′)                                      | Gene or fragment amplified |
|-----------------|--------------------------------------------------------|---------------------------|
| LeHB1F1         | ATGGGATCTGGGTCATATA                                      | LeHB-1 coding sequence    |
| LeHB1R1         | TTAAGACAGAAGACCATCC                                      | LeHB-1 coding sequence    |
| LeHB15UTrF      | CGCCCGTCGCGGAGTCCTTA                                     | LeHB-15-UTR               |
| LeHB15UTrR      | TCCTCTTTTCCACCCAGGAG                                    | LeHB-15-UTR               |
| LeHB1F-Nhe      | GCCCTCTCGTTCCTCCCCTGCTCTCCAC                            | LeHB-1 HD-Zip             |
| LeHB1R-Nhe      | GCGCTAGCTTATCCACCCCTGCTCTCCAC                            | LeHB-1 HD-Zip             |
| F1-1F           | CCTAACAGGTTGAGTGGTT                                      | LeACO1 promoter F1-1      |
| F1-1R           | GGTGGAATATTGGAATAAT                                        | LeACO1 promoter F1-1      |
| F3-1F           | ATCTCATTTGCCGAGTCGTT                                      | LeACO1 promoter F3-1      |
| F3-1R           | ACCTCTCTGAAACATTTCC                                      | LeACO1 promoter F3-1      |
| F4-1F           | CATCTCAAATATTGGAAT                                        | LeACO1 promoter F4-1      |
| F4-1R           | AGATGCTTTAACTTTCTTACC                                     | LeACO1 promoter F4-1      |
| PP82(PVXF)      | CATGTGTTGGCTTGCAACTAG                                     | Viral transient LeHB-1    |
| PP228(PVXR)     | GGGTAAAGTTTCCGTAGTTG                                     | Viral transient LeHB-1    |
| PP402           | AGTTGAATgcATGGGATCTGGGATATATTATTTC                       | Wild-type/mutant LeHB-1   |
| PP403           | AAATTggggcAgACGAGACCACTCGATAGGCTT                        | Wild-type/mutant LeHB-1   |
| PP483           | AGTTGAATgcATGATGATCCTGGGATATATTATTTC                     | Mutant LeHB-1             |
| AC1897f         | TCTCTCAATTTTGATATT                                       | LeACO1                    |
| AC2540r         | GTACTGAGATATGATATGAG                                     | LeACO1                    |

Introduced Clal and PspOMI sites are set in italic, changed nucleotides are set in lower case letters, the start codon and its mutated version are set in bold font and the introduced stop codon is underlined.

LeHB-1 is highly expressed in flowers and developing fruits

Northern blots revealed that LeHB-1 was highly expressed in tomato flower buds, senescing flowers (developing ovary stage), and developing immature and mature green fruits, but that the mRNA declined during ripening and was maintained at a stable but relatively low level in ripe fruits (Figure 2a). The LeHB-1 transcripts were also expressed in emerging young leaves and fully-expanded mature leaves, but wounding had no effect on expression (Figure 2a). The mRNA was much higher in flower buds in comparison with vegetative buds, and was abundant in all floral parts, particularly the sepals and the carpels (Figure 2b).

LeHB-1 binds to the LeACO1 promoter in vitro

To test whether the LeHB-1 protein had the capacity to bind the LeACO1 promoter, we expressed the LeHB-1 HD-Zip polypeptide tagged with glutathione S-transferase (GST) (GST::HD-Zip) (Figure 3a) in Saccharomyces pombe, as the HD-Zip domains have been shown to be essential for the in vitro DNA binding activity of AtHB-1 (Sessa et al., 1993). The free GST and GST::HD-Zip fusion proteins were then purified, and their integrity and purity were examined by SDS-PAGE (Figure 3b).

Three LeACO1 promoter regions (F1-1, F3-1 and F4-1), containing predicted homeobox cis-elements similar to the AtHB-1 binding sequence (CAATA/TATTG) (Figure 3c), were selected for the GST::HD-Zip/DNA binding analysis. The 141-bp F1-1 promoter fragment contains a 10-bp sequence AATA(A)TATT with dyad symmetry, F3-1 (119 bp) has a 9-bp sequence CAAT(A)ATGG, and F4-1 (128 bp) has a 9-bp sequence AATA(A)TATT with dyad symmetry (Figure 3c). These three fragments were PCR amplified and sequenced. Incubation of GST::HD-Zip with either F1-1 or F4-1 resulted in a DNA–protein complex that showed an electrophoretic mobility shift compared with free DNA fragments (Figure 3d).

The formation of the DNA–protein complex was specific, and was out-competed by a 200-fold molar excess of unlabelled starting DNA of each respective promoter region. However, the GST::HD-Zip fusion did not produce a similar complex with the fragment F3-1 (data not shown). No DNA–protein complex was formed between any of the promoter fragments and free GST (Figure 4d). These findings demonstrate that the LeHB-1 HD-Zip is capable of binding to the LeACO1 promoter, probably by recognizing the 9 or 10-bp DNA sequences with dyad symmetry, indicating that LeHB-1 might be involved in transcriptional regulation of LeACO1.

Virus-induced gene silencing (VIGS) of LeHB-1 delays ripening

To determine whether LeHB-1 regulated LeACO1 gene expression in vivo, we attempted to overexpress or silence LeHB-1 in stably transformed tomato plants using the constitutive 35S promoter, but this failed, indicating that LeHB-1 overexpression or knock-down might be deleterious or lethal. We then employed VIGS (Manning et al., 2006) to inhibit the LeHB-1 gene. The cDNA encoding the full-length LeHB-1 protein, and a non-sense mutant derivative with Met1 to Ile followed by a stop codon, were PCR-amplified and cloned into the potato virus X/Green fluorescent protein...
Figure 1. Sequence analysis of LeHB-1.
(a) LeHB-1 nucleotide and deduced amino acid sequences. The homeodomain (HD) is underlined and the conserved leucine residues are circled.
(b) Comparison of LeHB-1 HD-Zip amino acid sequences with those from Arabidopsis AtHB-1 and Sunflower HaHB-4. Conserved regions are boxed. Conserved sequences in the HD are indicated below the alignment, and the three α helices of the HD and the leucine (or valine) residues are underlined.
(c) A phylogenetic tree generated using the class-I HD-Zip protein sequences from Arabidopsis, HaHB-4 from sunflower, VaHox1 from tomato, and H52 and LeHB-1. AtHB-9 (Ariel et al., 2007), a class-III HD-Zip protein from Arabidopsis, was used as an outgroup.
(PVX/GFP) vector to generate PVX/LeHB1::GFP and PVX/mLeHB1::GFP constructs (Table 1, Figure 4a and Experimental procedures). Viral RNA transcripts were needle-injected into the carpopodium of wild-type Ailsa Craig tomato fruits attached to the plant, and the effects on the fruit were observed 2–4 weeks later. Thirty fruits (out of approximately 90) injected with PVX/LeHB1::GFP or PVX/mLeHB1::GFP produced regions that failed to ripen normally and displayed a distinct green sector, indicative of delayed ripening (Figure 4b, panel 1), or produced yellow (partially ripened) fruits (Figure 4b, panels 2, 3). The green sectors of inhibited ripening on fruit of VIGS LeHB-1 plants eventually turned orange, and showed signs of slow ripening. All control fruits injected with the PVX/GFP vector ripened normally (Figure 4b,Ctl panel). Strikingly, some of the delayed-ripening phenotypes (Figure 4b, panels 2, 3) mimicked the fruits produced by LeACO1 antisense transgenic plants, in which LeACO1 mRNA was inhibited by 95% (Picton et al., 1993).

Northern analysis of total RNA isolated from the VIGS delayed-ripening fruits and the control fruits, at 7 days after the start of colour change, showed that the endogenous LeHB-1 expression was 64–90% downregulated in the delayed-ripening fruits and fruit sectors, compared with the controls (Figure 4c), and that LeACO1 expression was also reduced by 80–86% in the fruits where ripening was delayed by VIGS (Figure 4c), indicating a mechanistic link exists between LeHB-1, LeACO1 and ripening. The viral delivery of the silencing inducer, i.e. the wild-type or the mutated LeHB-1 RNA, was readily detected in the VIGS fruits, but not in the control PVX/GFP (Figure 4c, HB1-tran). Taken together, this evidence supports the suggestion that LeHB-1 functions as a transcription activator in the regulation of LeACO1 expression and ripening.

Ectopic expression of LeHB-1 disrupts flower development
Introduction of recombinant viruses into plants can, in addition to producing local effects, also lead to systemic

Figure 2. Northern analysis of LeHB-1 mRNA.
(a) Expression of LeHB-1 in different tissues. Abbreviations: FB, flower buds; OF, fully open flowers; SF, senescing flowers; EL, emerging leaves; ML, mature leaves; WL, wounded mature leaves; IF, immature green fruit; MF, mature green fruit; Br, fruit at start of colour change; +3 and +14, 3 or 14 days after start of colour change.
(b) Expression of LeHB-1 in floral organs. Abbreviations: VB, vegetative buds; FB, floral buds; Se, sepals; Pe, petals; St, stamens; Ca, carpels. RNA (10 μg) was used for the northern blot. The full-length coding sequence of LeHB-1 was used as the probe. Ethidium bromide stained rRNA (rRNA) indicates equal loading.

Figure 3. Binding of LeHB-1 to LeACO1 promoter fragments.
(a) Outlines of the LeHB-1 protein and GST-HD-Zip fusion protein used for DNA binding.
(b) Expression of fusion protein in yeast. Lane 1, GST-LeHB1 HD-Zip fusion; lane 2, GST; lane 3, 1 μg BSA.
(c) Structure of the LeACO1 promoter showing the three regions used for LeHB-1 binding (F4-1, F3-1 and F1-1).
(d) Comparison of Athb-1 binding sequence with similar sequences found in F1-1, F4-1 and F3-1 of the LeACO1 promoter.
(e) Gel retardation assays with F1-1 and F4-1 fragments, and the LeHB-1 GST-HD-Zip fusion protein. Lane 1, free F1-1 probe; lane 2, GST-HD-Zip with F1-1 probe; lane 3, GST with F1-1 probe; lane 4, sample from lane 2 plus a 200-fold molar excess of unlabelled competitor F1-1; lane 5, free F4-1 probe; lane 6, GST-HD-Zip with the F4-1 probe; lane 7, GST with the F4-1 probe; lane 8, sample from lane 6 plus a 200-fold molar excess of unlabelled competitor F4-1.
movement and expression of virus genes elsewhere in developing plants (Chapman et al., 1992). Viral delivery of wild-type LeHB-1 to distant flowers sometimes altered floral organ identity, and caused remarkable flower developmental abnormalities. This was associated with overexpression, not silencing, of the virus-delivered LeHB-1 gene. Flower abnormalities included production of multiple flowers or carpel-like structures within one sepal whorl (Figure 5a, panels 1, 2), fused sepals and petals (Figure 5a, panels 3, 4), and swelling of the base of the sepals (Figure 5a, panels 3, 4, 5). Delivery of the LeHB-1 transgene to the abnormal flowers and its transcription were confirmed by RT-PCR using the primers corresponding to the PVX vector (Figure 5b and Table 1). Furthermore, the endogenous LeHB-1 mRNA in these samples was readily detectable and showed no obvious reduction compared with the control (Figure 5c; note that 10 μg of total RNA was used for the control, and for lanes 1 and 2, and that 5 μg was used in lanes 3 and 4).

These results indicated that overexpression of LeHB-1 was occurring in these organs. In addition, the LeACO1 transcripts were more abundant in the abnormal flowers than in the control (Figure 5c), indicating that ectopic expression of LeHB-1 in vivo enhanced the accumulation of LeACO1 transcripts above their normal level. The fact that these flower phenotypes were not found in the plants infected with the PVX/GFP vector, or the mutated PVX/mLeHB::GFP construct (data not shown), suggested that functional LeHB-1 was required to cause these effects.

**Ectopic expression of LeHB-1 converts sepals into fruits**

Viral delivery of the wild-type LeHB-1 construct into flowers also triggered the production of swollen green structures in the position of the sepals. In several instances, conversion of sepals into carpel-like structures was evident (Figure 6a, panels 1, 2), and these eventually developed into fruit-like structures and ripened (Figure 6a, panels 3, 4). Sometimes twin fruits were produced, or an additional fruit developed from one pedicel (Figure 6a, panels 4, 5). None of these phenotypes was seen in the plants infected with the mutated LeHB-1 construct, the PVX vector control or in non-infected plants. Northern analysis indicated that the LeHB-1 transcripts above their normal level.

**Discussion**

This study demonstrates that LeHB-1 is involved in the regulation of tomato floral organogenesis, carpel development...
and ripening. LeHB-1 encodes a class-I HD-Zip protein that binds to the promoter of LeACO1 (Figures 1, 3). VIGS silencing of LeHB-1 results in a significant delayed-ripening phenotype, which is associated with a great reduction of LeACO1 mRNA (Figure 4). Antisense inhibition of LeACO1 has been shown previously to lead to reduced ethylene synthesis (Hamilton et al., 1990; Picton et al., 1993). These results are consistent with the suggestion that LeHB-1 positively controls LeACO1, and that silencing LeHB-1 represses LeACO1, which consequently leads to delayed ripening. Putative LeHB-1 binding sites are also found in the promoters of a number of ripening-related genes, including, LeACO2, PG1, LeMADS-RIN and NAC-NOR (ZL and DG, unpublished data), and it is possible that LeHB-1 may directly regulate these ripening-related genes. The identification of LeHB-1 marks a further step in our understanding of ripening control, and begins to answer a long-standing and key question about how LeACO1 is regulated. LeHB-1 is highly expressed in mature green fruit and declines at the breaker stage, whereas LeACO1 mRNA increases in mature green fruit and accumulates during ripening (Blume and Grierson, 1997). This difference might be explained by differences between mRNA accumulation and protein turnover, but could also suggest that other factors, in addition to LeHB-1, control LeACO1 mRNA accumulation.

Homeobox genes were originally discovered in Drosophila, and were shown to function as transcriptional regulators that control embryonic morphogenesis. They regulate diverse aspects of morphogenesis (Graba et al., 1997), and are now known to play a role in the control of hormones in plants and animals (Sakamoto et al., 2001; Susa et al., 2007). The first plant homeobox gene to be identified was KNOTTED 1 (Vollbrecht et al., 1991), and this and related genes are involved in the control of GA and cytokinin (Hay et al., 2002; Jasinski et al., 2002; Ori et al., 1999; Sakamoto et al., 2001). Several HD-Zip genes have also been implicated in the control of, or responses to, other hormones, such as ABA (Ditzer and Bartels, 2006; Lee et al., 2001; Söderman et al., 1996, 1999), auxin (Baima et al., 1995; Plesch et al., 1997), red/far-red light effects on cell expansion (Carabelli et al., 1996; Steindler et al., 1999), de-etiolation (Aoyama et al., 1995) and blue-light signalling (Wang et al., 2003).
sunflower HD-Zip gene HaHB-4, which is induced by ABA, has been implicated in senescence and ethylene signalling (Manavella et al., 2006), but is distinct from LeHB-1 (Figure 1). The present studies demonstrate that LeHB-1 is not only involved in ethylene and ripening, but also in flower and fruit development.

Altered floral organ identity (Figure 5) and conversion of the sepals into carpel-like structures (Figure 6), caused by transient overexpression of LeHB-1 in planta, highlight a crucial role for LeHB-1 in floral organogenesis, which is consistent with the abundance of LeHB-1 transcripts in floral organs and developing fruits (Figure 2). Ripening-related changes in sepals can be induced by low temperature in VFNT cherry tomatoes (Bartley and Ishida, 2003). The cells swell, turn red, express ripening-related genes (Bartley and Ishida, 2003) and accumulate mRNA for transcription factors TAG1, TMG, LeAP2 and VaHox1 (Bartley and Ishida, 2007).

Interestingly, ethylene is required in order for mRNA accumulation, except for VaHox1, a tomato class-I HD-Zip gene (Tomero et al., 1996; Figure 1). A role for ethylene in flower and fruit development has been suggested by previous studies. For example, ethylene is known to stimulate female flower development in cucumber (Yamasaki et al., 1996; Figure 1). Ethylene is involved in floral organogenesis, fruit development and ripening. Identification of the targets for LeHB-1 should generate new insights into the hormonal control of floral organ identity and early fruit development.

In the present experiments, ectopic overexpression of LeHB-1 altered floral organ identity and triggered the formation of carpel-like structures from sepals, which was associated with increased accumulation of LeACO1 mRNA (Figure 5). It seems unlikely that enhanced LeACO1 mRNA alone could result in these developmental changes, as they are not found when LeACO1 is overexpressed in tomato under the control of the 3S promoter (DG and Y. Han, unpublished data), or in plants overexpressing ACC synthase (H. Klee, personal communication). It is proposed that the unscheduled synthesis of LeHB-1 in cells of floral organs affects a series of genes that leads to altered floral development and ectopic carpel formation. It has yet to be established whether this occurs during normal development or only as a result of ectopic overexpression of LeHB-1.

This study provides evidence for the control of the hormone ethylene by an HD-Zip homeobox gene, and suggests a link between LeHB-1 and the regulation of floral organogenesis, fruit development and ripening. Identification of the targets for LeHB-1 should generate new insights into the hormonal control of floral organ identity and early fruit development.

Experimental procedures

Preparation of the GST::LeHB-1 HD-Zip fusion protein

The partial LeHB-1 cDNA encoding the combined homeobox and leucine zipper domain (HD-Zip, aa 34–165) was amplified by PCR using a pair of primers LeHB1F-Nhe and LeHB1R-Nhe (Table 1). The PCR product was digested with Nhel and cloned into the Nhel site of vector pESP-2 (Stratagene, http://www.stratagene.com). The construct was confirmed by sequencing and then transformed into yeast S. pombe strain SP-Q01. The fusion protein GST::HD-Zip was expressed in yeast and purified. 

Figure 6. Development of carpel-like structures from sepals in fruit of PVX/LeHB1::GFP-injected plants.
(a) Panel 1: a fruit-like structure (fr*, arrow) arising from the sepal of a PVX/LeHB1::GFP-infected flower. Panel 2: 5x enlarged image of panel 1. Panel 3: multiple ripe fruit-like structures (fr*) and flowers (fl*) developed from the sepals of one original, much larger, fruit after injection with PVX/LeHB1::GFP. Panel 4: a second fruit developed from the elongated pedicel of the original fruit following injection with PVX/LeHB1::GFP. Panel 5: twin fruits developed from one pedicel injected with PVX/LeHB1::GFP. Ctl: control fruit injected with PVX/GFP.
(b) Northern analysis of endogenous LeHB-1 (Endo) and the LeHB-1 transgene mRNA (Trans) in abnormal and control fruits. RNA samples were isolated from the lower part of the fruit, shown in panel 1 (lane 1), or the upper parts of the same fruit, including the mini fruits and the floral structures, shown in panel 3 (lane 2), and control fruit. Ethidium bromide stained rRNA (rRNA) shows RNA loading.
together with the free GST protein were purified on GST affinity resin following the manufacturer’s instructions (Stratagene), and were then examined by SDS-PAGE and visualized by Coomassie blue staining (CBB R250).

In vitro gel retardation assay

The LeACO1 promoter fragments F1-1, F3-1 and F4-1 were amplified by PCR using various sets of specific primers (Table 1), cloned into pGEM-T-Easy vector (Promega, http://www.promega.com) and sequenced. Radiolabelled fragments were prepared using the Rediprime II random prime labelling system (Amersham Pharmacia Biotech, http://www.gelifesciences.com). Double-stranded 32P-labelled DNA (3 ng) was incubated with 1 μg purified GST:HD-Zip fusion protein or GST in binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 2 mM EDTA), which contained 1 μg of double-stranded poly (dl-dC) (Amersham Pharmacia Biotech). Binding reactions were incubated in a volume of 20 μl for 0.5 h at 4°C, with gentle shaking. After incubation, the mixture was immediately loaded on to 4.5% polyacrylamide gels. Electrophoresis was carried out in 0.5 x TAE (2.42 g Tris base, 0.571 ml glacial acetic acid and 1 ml 0.5 M acetic acid, pH 8.0, per litre) for 2.5 h at 14 mA, the gel was then dried and subjected to autoradiography at -80°C.

RNA isolation and northern analysis

Flower RNA was isolated using the RNeasy plant mini kit (Qiagen, http://www.qiagen.com) following the manufacturer’s instructions. A 2-μg portion of total RNA was used for reverse transcription in a reaction volume of 20 μl using SuperScriptII Reverse Transcriptase (Invitrogen, http://www.invitrogen.com). A 2-μl volume of this RT mixture was then used for PCR using primers pp82/pp228 (Table 1).

ACKNOWLEDGEMENTS

We thank D. Baulcombe for providing the original PVX vector. This work was in part supported by the UK Biotechnology and Biological Sciences Research Council (BBSRC) grant to DG, and by Warwick HRI-BBSRC core funding to YH.

REFERENCES

Abeles, F.B., Morgan, P.W. and Salvetet, M.E., Jr (1992) Ethylene in Plant Biology. 2nd edn. New York: Academic Press.

Achard, P., Baghour, M., Chappelle, A., Hedden, P., Van der Straeten, D., Geschick, P., Moritz, T. and Harberd, N.P. (2007) The plant stress hormone ethylene controls floral transition via DELLA-dependent regulation of floral meristem-identity genes. PNAS, 104(15), 6484–6489.

Ayama, T., Dong, C.H., Wu, Y., Carabelli, M., Sessa, G., Ruberti, I. and Chua, N.H. (1995) Ecopptic expression of the Arabidopsis transcriptional activator Athb-1 alters leaf cell fate in tomato. Plant Cell, 7(11), 1773–1788.

Ariel, A.D., Manavella, P.A., Dezar, C.A. and Chan, R.C. (2007) The true story of the HD-Zip family. Trends Plant Sci., 12, 419–426.

Baima, S., Nobili, F., Sessa, G., Lucchetti, S., Ruberti, I. and Morelli, G. (1995) The expression of the Athb-8 homeobox gene is restricted to provascular cells in Arabidopsis thaliana. Development, 121(12), 4171–4182.

Barry, C.S., Blume, B., Bouzayen, M., Cooper, W., Hamilton, A.J. and Grieron, D. (1996) Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. Plant J. 9, 525–535.

Bartley, G.E. and Ishida, B.K. (2003) Developmental gene regulation during tomato fruit ripening and in-vitro sepal morphogenesis. BMC Plant Biol. 3, 4. (http://www.biomedcentral.com/1471–2229/ 3/4).

Bartley, G.E. and Ishida, B.K. (2007) Ethylene-sensitive and insensitive regulation of transcription factor expression during in vitro tomato sepal ripening. J. Exp. Bot., 58(8), 2043–2051.

Becker, A. and Theißen, G. (2003) The major clades of MADS-box genes and their role in the development and evolution of flowering plants. Mol. Phylo. Evo. 29, 484–499.

Blume, B. and Grieron, D. (1997) Expression of ACC oxidase promoter·GUS fusions in tomato and Nicotiana plumbaginifolia regulated by developmental and environmental stimuli. Plant J. 12, 731–746.

Carabelli, M., Morelli, G., Whitelam, G. and Ruberti, I. (1996) Twilight-zone and canopy shade induction of the Athb-2 homeobox gene in green plants. PNAS, 284(1), 133–141.

Chapman, S., Kavanagh, T. and Baulcombe, D. (1998) Potato virus X as a vector for gene expression in plants. Plant J. 2(4), 549–557.

Ditzer, A. and Bartels, D. (2006) Identification of a dehydration and ABA-responsive promoter regulation and isolation of corresponding DNA binding proteins for the group 4 LEA gene CpC2 from C. plantagineum. Plant Mol. Biol. 61(4–5), 643–663.
Manning, K., Torö, M., Poole, M., Hong, Y., Thompson, A.J., King, G.J., Giovannoni, J.J. (1999) Leaf senescence is delayed in tobacco plants expressing the maize homeobox gene knotted1 under the control of a senescence-activated promoter. Plant Cell, 11(6), 1073–1080.

Picton, S., Barton, S.L., Bouzyen, M., Hamilton, A.J. and Grierson, D. (1993) Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene-forming enzyme transgene. Plant J. 3, 489–491.

Plesch, G., Störmann, K., Torres, J.T., Walden, R. and Somssich, I.E. (1997) Developmental and auxin-induced expression of the Arabidopsis prh homeobox gene. Plant J. 12(3), 635–647.

Sakamoto, T., Kamiya, N., Ueguchi-Tanaka, M., Iwahori, S. and Matsuoka, M. (2001) KNOX homeodomain protein directly suppresses the expression of a gibberellic biosynthetic gene in the tobacco shoot apical meristem. Gene Dev. 15, 581–590.

Sessa, G., Morelli, G. and Ruberti, I. (1993) The AthB-1 and AthB-2 HD-ZIP domains homodimerize forming complexes of different DNA-binding specificities. EMBO J. 12(9), 3507–3517.

Sinha, N.R., Williams, R.E. and Hake, S. (1993) Overexpression of the maize homeobox gene, KNOTTED-1, causes a switch from determinate to indeterminate cell fates. Gene. Dev. 7, 787–795.

Söderman, E., Mattsson, J. and Engström, P. (1996) The Arabidopsis homeobox gene AthB-7 is induced by water deficit and by abscisic acid. Plant J. 10(2), 375–381.

Söderman, E., Hjelström, M., Fahlson, J. and Engström, P. (1999) The HD-Zip gene ATHB6 in Arabidopsis is expressed in developing leaves, roots and carpels and up-regulated by water deficit conditions. Plant Mol. Biol. 40(6), 1073–1083.

Steindler, C., Matteucci, A., Sessa, G., Weimar, T., Ohgishi, M., Aoyama, T., Morelli, G. and Ruberti, I. (1999) Shade avoidance responses are mediated by the AthB-2 HD-ZIP protein, a negative regulator of gene expression. Development, 126(19), 4235–4245.

Susa, T., Nakayama, M., Kitahara, K., Kimoto, F., Kato, T. and Kato, Y. (2007) Homeodomain transcription factor Hext1/Rpx occupies Prop-1 activation sites in porcine follicle stimulating hormone (FSH) β subunit promoter. Biochem. Biophys. Res. Comm. 357, 712–717.

Tomero, P., Conejero, V. and Vera, P. (1996) Phloem-specific expression of a plant homeobox gene during secondary phases of vascular development. Plant J. 9(5), 639–648.

Vollbrecht, E., Veit, B., Sinha, N. and Hake, S. (1991) The developmental gene Knotted-1 is a member of a maize homeobox gene family. Nature, 350(6315), 241–243.

Wang, Y., Henriksson, E., Söderman, E., Henriksson, K.N., Sundberg, E. and Engström, P. (2003) The Arabidopsis homeobox gene, ATHB16, regulates leaf development and the sensitivity to photoperiod in Arabidopsis. Dev. Biol. 264(1), 228–239.

Wezel, R., Dong, X., Liu, H., Tien, P., Stanley, J. and Hong, Y. (2002) Mutation of three cysteine residues in tomato yellow leaf curl virus-china C2 protein causes dysfunction in pathogenesis and posttranscriptional gene-silencing suppression. Mol. Plant-Microbe Interact. 15, 203–208.

Yamasaki, S., Fuji, N. and Takahashi, H. (2003) Characterization of ethylene effects on sex determination in cucumber plants. Sex. Plant Reprod. 16(3), 103–111.

Yang, S.F. and Hoffmann, N.E. (1994) Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. 35, 155–189.

Zarembinska, T.I. and Theologis, A. (1994) Ethylene biosynthesis and action: a case of conservation. Plant Mol. Biol. 26(5), 1579–1597.