Apple EIN3 BINDING F-box 1 inhibits the activity of three apple EIN3-like transcription factors

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Abstract

Background and aims

Fruit ripening in Malus × domestica (apple) is controlled by ethylene. Work in model species has shown that following the detection of ethylene, the ETHYLENE INSENSITIVE 3 (EIN3) transcription factor is stabilized, leading to an increase in transcript accumulation of ethylene-responsive genes, such as POLYGALACTURONASE1 (PG1). In the absence of ethylene, the EIN3 BINDING F-box (EBF) proteins rapidly degrade EIN3 via the ubiquitination/SCF (Skp, Cullin, F-Box) proteasome pathway. In this study, we aim to identify and characterize the apple EBF genes, and test their activity against apple EIN3-like proteins (EILs).

Methodology

The apple genome sequence was mined for EBF-like genes. The expression of EBF-like genes was measured during fruit development. Using a transient assay in Nicotiana benthamiana leaves, the activity of three apple EILs was tested against the PG1 promoter, with and without ethylene and EBF1.

Principal results

Four EBF-like genes in apple were identified and grouped into two sub-clades. Sub-clade I genes had constant expression over fruit development while sub-clade II genes increased in expression at ripening. EBF1 was shown to reduce the transactivation of the apple PG1 promoter by the EIL1, EIL2 and EIL3 transcription factors in the presence of ethylene.

Conclusions

The apple EBF1 gene identified here is likely to be a functionally conserved EBF orthologue, modulating EIL activity in apples. The activity of EBF1 suggests that it is not specific to a single EIL, instead acting as a global regulator of apple EIL transcription factors.

Introduction

Ethylene is involved in a wide range of developmental processes in plants including seed germination, cell elongation, sex determination, fruit ripening, senescence and leaf abscission, as well as biotic and abiotic stress responses (Abeles and Biles 1991; Barry and Giovannoni 2007; Lin et al. 2009). The ethylene response pathway can be briefly summarized as follows: the pathway is thought to be predominantly linear, consisting of ethylene receptors, which in the absence of ethylene constitutively repress the activity of the MAP kinase CONSTITUTIVE TRIPLE RESPONSE1 (CTR1); this modulates the activity of

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ETHYLENE INSENSITIVE 2 (EIN2), which results in the destabilization of the ETHYLENE INSENSITIVE 3 (EIN3) transcription factors. In the presence of ethylene this pathway is repressed and EIN3 is stabilized, initiating a transcriptional cascade leading to an ethylene response (Chen et al. 2005).

In the absence of ethylene, EIN3 is short-lived with a half-life of <30 min due to rapid degradation through the ubiquitin/Skp, Cullin, F-Box degradation pathway (Guo and Ecker 2003; Yanagisawa et al. 2003). In Arabidopsis, two redundant nuclear localized F-box proteins, EIN3-BINDING F BOX PROTEIN1 (AtEBF1) and AtEBF2, were shown to target EIN3 and a functional homologue EIN3-like 1 (EIL1) for degradation (Guo and Ecker 2003; Potuschak et al. 2003; Binder et al. 2007). Loss-of-function ebf1 ebf2 double mutants had high levels of EIN3 protein and consequently exhibited a constitutive ethylene response (Chao et al. 1997; Potuschak et al. 2003). While EBF1 and EBF2 in Arabidopsis are constitutively expressed over plant development, they both show an increase in expression with exogenously added ethylene, and over-expression of EBF1 resulted in reduced EIN3 levels leading to an ethylene-insensitive phenotype. These results suggest that the EBF-like genes are controlled, at least in part, at the transcription level (Potuschak et al. 2003).

In tomato, two EBF-like genes have also been identified: EBF1 and EBF2 (Yang et al. 2010). Consistent with the results in Arabidopsis, silencing of either gene resulted in plants that were indistinguishable from controls, indicating that they are functionally redundant. The results suggested a feedback mechanism whereby suppression of one EBF gene resulted in an increase in transcription of the second. As in Arabidopsis, a constitutive ethylene response phenotype was observed when both EBF1 and EBF2 were silenced in tomato, including accelerated fruit ripening (Yang et al. 2010). However, unlike Arabidopsis, the expression of tomato EBF1 and EBF2 was not constitutive, with a transient decrease in expression at the onset of ripening (mature green), and consistent with Arabidopsis both showed an increase of expression with ethylene and a decrease with auxin (Yang et al. 2010). Tomato EBF1 appeared to be less affected at the transcriptional level, while EBF2 appeared to be more transcriptionally variable (Yang et al. 2010).

In the fleshy fruiting apple, ethylene plays a key role in the control of fruit ripening. The importance of ethylene in apple fruit ripening was confirmed with the suppression of the ripening-associated ethylene biosynthesis gene ACC OXIDASE 1 (ACO1). In these apples, no ripening-associated flesh softening or aroma volatiles are produced (Schaffer et al. 2007; Johnston et al. 2009). Owing to consumer requirements to maintain a firm texture, many commercial apples have been selected for low ripening-related ethylene. This has been achieved in part through the selection of lines with disrupted ethylene biosynthetic gene ACC SYNTHASE (ACS), leading to longer storage capacity and slower softening (Harada et al. 1997; Costa et al. 2005; Wiersma et al. 2007; Wang et al. 2009). Owing to the importance of ethylene in fruit ripening, much of the molecular biology research conducted in apple has been focused on ethylene biosynthesis and response. One of the earliest genes cloned from apple was the ETHYLENE RESPONSE 1 (ETR1)-like receptor (Lee et al. 1998), along with the ethylene biosynthetic gene ACO1 (Lay-Yee and Knighton 1995). Subsequent work identified four other receptor-like genes, a CTR1-like gene, an EIN2-like gene (Wiersma et al. 2007) and three EIN3-like genes (Tacken et al. 2010). With the release of the complete apple genome sequence (Velasco et al. 2010), there is now a growing literature studying whole gene families (Devoghalae et al. 2012), which has led to the identification of three further receptor genes in apple (Ireland et al. 2012).

While five EIN3-like genes have been identified in Arabidopsis, ethylene signal transduction occurs predominantly through the action of two of them, EIN3 and EIL1. Originally identified through an ethylene-insensitive phenotype, it was proposed that EIN3 acted by binding and activating the promoters of the AP2/ERF class of transcription factors (Solano et al. 1998). Since this study, it has been shown that EIN3-like transcription factors are likely to be involved directly in the activation of a suite of ethylene biosynthesis and response genes (Huang et al. 2010; Tacken et al. 2010; Yin et al. 2010), and transient assays suggest that EIL2 and EIL3 in apple may be involved in the up-regulation of key apple ripening genes such as the cell wall hydrolase endopolygalacturonase 1 (PG1) (Tacken et al. 2010).

Owing to the importance of the EBF class of genes as key controllers of the ethylene signal transduction pathway, this study used the apple genome sequence to identify EBF-like genes. One EBF-like gene (EBF1) was cloned and tested for the ability to inhibit the activity of three EILs in Nicotiana benthamiana transient assay.

Methods

Identification of the apple EBF genes and generation of a phylogeny

EBF-like genes were mined from the predicted peptide models from the apple genome using BLASTP. To verify the DNA sequence of the selected gene models, the
DNA sequence from each EBF-like gene was compared with expressed sequence tag (EST) sequences. Predicted amino acid sequences were aligned in Geneious Pro™ version 4.8.4 (Biomatters, Auckland, New Zealand) (Drummond et al. 2011). Phylogenetic trees were created in Geneious Pro™ using the PHYML substitution method (Guindon and Gascuel 2003) with the JTT model (Jones et al. 1992). A total of 1000 replicates of each tree were used to generate bootstrap data. EBF sequences from other species used to construct the phylogenetic tree were: Fragaria vesca FvEBF1 (strawberry gene model 1520754), FvEBF2 (gene model 1540140) (www.rosaceae.org), the Malus gene models shown in Table 1 and EBF-like protein sequences drawn from published work (Yang et al. 2010; Arabidopsis thaliana AtEBF1 (NP_565597), AtEBF2 (NP_197917), AtFBL4 (NP_567467), AtTIR1 (NP_567135), AtZTL (NP_568855), Brassica oleracea BoF-box (ACB59221), OsF-box (BAD15849), Populus trichocarpa PtEBF3 (EE92188), PtEBF4 (EE92505), PtF-box (EEF03786), Solanum lycopersicum SIEBF1 (ACS44349) and SIEBF2 (ACS44350).

Quantification of gene expression
Gene expression levels from a fruit development cDNA series (Janssen et al. 2008) were determined via quantitative polymerase chain reaction (qPCR) using the Lightcycler480™ (Roche, Basel, Switzerland). Primers for tentative polymerase chain reaction (qPCR) using the Lightcycler480™ (Roche, Basel, Switzerland). Primers for qPCR were designed to amplify unique sequences of each EBF gene, and the qPCR products sequenced to verify the amplification of the correct gene.

Table 1 Apple EBF-like genes.

| Gene name | Gene model | Chromosome | Position (Mb) |
|-----------|------------|------------|---------------|
| EBF1      | MDP0000239011 | 8          | 18.66         |
| EBF101    | MDP0000429728 | 15         | 5.98          |
| EBF2      | MDP0000230402 | 15         | 10.55         |
| EBF102    | MDP0000165656 | 2          | 3.02          |

Identification of apple EBF-like genes
The protein sequences of Arabidopsis EBF1 and EBF2 were used to identify EBF-like genes within the predicted peptide models from the apple genome (Velasco et al. 2010) using BLASTP. Six gene models with a high BLAST score (P < e-150) were selected. The next highest apple model (MDP0000224875) had a considerably lower BLAST score (P < e-37) and only showed
homology in the N-terminal F-box region, suggesting that this was unlikely to be within the EBF group of F-box proteins. When these proteins were aligned each was found to have the expected F-box region, and leucine-rich repeats were found in EBF-like genes (Fig. 1). Reciprocal BLASTP comparisons of the six apple peptide models with the Arabidopsis proteins selected EBF1 and EBF2 as the most similar Arabidopsis proteins. The six apple peptide models aligned to four unique chromosomal locations: two on chromosome 15, one on chromosome 2 and one on chromosome 8 (Table 1). Two of the chromosomal loci had two models each, suggesting that apple has four EBF-like genes. To test whether the gene models were correctly constructed, the DNA sequences of the four predicted protein sequences were compared with sequences from both an apple EST collection (Newcomb et al. 2006) and short read (100 bp) data from mRNA seq analysis from ripe ‘Royal Gala’ fruit (Schaffer et al. 2012). In two cases the predicted gene models differed from the EST sequences, firstly EBF1 (with two gene models MDP0000314942 and a shorter model MDP0000239011) both extended 3’ beyond the region covered by ESTs. A single clone from a ‘Royal Gala’ cDNA library was fully sequenced, verifying that the gene was shorter in length than the gene models supplied (GenBank JX512439). When this new sequence was translated, the C-terminus was more consistent with the length of the Arabidopsis and tomato EBF genes. Secondly, the model for EBF2 (MDP0000230402)

![Fig. 1 Alignment of the EBF1 protein sequences. The four predicted apple EBF proteins were aligned. The conserved F-box domain (red) and the 13 leucine-rich repeats (LRR—green) are shown underneath.](image-url)
was 25 amino acids longer than the other EBF-like proteins at the N-terminus. Alignment of mRNA seq reads to the apple genome suggested that this model was incorrectly annotated at the 5′ end, with these new data the start codon was consistent with other EBF-like proteins [see Additional information: Supplemental Data 1]. Phylogenetic alignment was conducted with the four predicted apple EBF-like proteins, two genes selected in a similar way from the *Fragaria vesca* (strawberry) genome (Shulaev et al. 2011) and the EBF-like proteins from Yang et al. (2010). The phylogenetic alignment showed that the selected apple proteins fell into the same clade as the *Arabidopsis* (EBF1 and EBF2) and tomato (EBF1 and EBF2) proteins, suggesting that these were likely to be apple EBF orthologues (Fig. 2). The four apple proteins were separated into two proteins per sub-clade, with each sub-clade containing a single strawberry protein. This duplication was consistent with the ancient genome duplication event reported in apple (Velasco et al. 2010). The four selected apple EBF-like genes were assigned gene names as described in Devoghelare et al. (2012). As both *Arabidopsis* EBF1 and EBF2 fell into sub-clade I containing tomato EBF1, the apple genes were named by the closest tomato genes, with strawberry EBF1 and the apple homeologues EBF1 and EBF101 grouping with the tomato EBF1 gene, and strawberry EBF2 and apple homeologues EBF2 and EBF102 grouping with tomato EBF2 in sub-clade II (Fig. 2).

**Analysis of EBF1 expression**

The expression of the EBF genes during apple fruit development was compared with that of known ethylene biosynthesis genes (*ACO1*), potential EBF-like targets *EIL1*, *EIL2* and *EIL3* (Tacken et al. 2010) and the cell wall modifying gene *PG1* (Fig. 3). Expression of EBF1 and EBF101 was similar to that of *EIL1* and *EIL3*, and did not change significantly over the course of fruit development or at the onset of fruit ripening at 132 days after full bloom (DAFB), though a slight increase in expression was observed at 146 DAFB (Fig. 3). The expression of EBF2 and EBF102 was low early in fruit development, increasing as the fruit matured and ripened. This expression was more consistent with that of ethylene-responsive genes such as *ACO1* and *PG1*, which had a significant increase in expression at the onset of fruit ripening (data from Tacken et al. 2010).

**Functional analysis of EBF1 in a transient assay**

It has previously been shown that a 2.6-kb apple *PG1* promoter fused to the *LUCIFERASE* gene can be transactivated when injected into a *N. benthamiana* leaf in the presence of exogenous ethylene (Tacken et al. 2010). When the *EIL2* and *EIL3* transcription factors, driven by a CaMV35S promoter, were co-injected with the *PG1* promoter in the presence of ethylene, an increased transactivation of the *PG1* promoter occurred, especially with *EIL2* (Tacken et al. 2010). To test whether the EBF1 protein can destabilize the apple EIL proteins and thus block their transactivation of *PG1*, the *EIL2* and *EIL3* constructs as well as a construct containing *EIL1* were co-infiltrated with the *PG1* promoter, with and without EBF1. Each assay was performed either in the presence or absence of ethylene. In this study, apple EIL1 trans-activated the *PG1* promoter in the presence of ethylene to a much higher level than *EIL2* and *EIL3* (Fig. 4). When co-infiltrated with the *EBF1* gene, the levels of trans-activation were greatly reduced with all three apple EILs, consistent with the activity of an EBF-like F-box protein. Interestingly, a level of inhibition

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**Fig. 2** Phylogenetic alignment of members of the EIN3 BINDING F-box (EBF) family proteins from different plant species. A phylogenetic tree was generated using PHYML; values given are bootstrap percentages (1000 replicates). EBF-like proteins from apple (*Malus domestica*—Md), strawberry (*Fragaria vesca*—Fv), poplar (*Populus trichocarpa*—Pt), tomato (*Solanum lycopersicum*—Sl), *Brassica oleracea* (Bo), rice (*Oryza sativa*—Os) and *Arabidopsis thaliana* (At) were compared with AtFBL4 and AtTIR used as outgroups.
by the EBF1 was also observed in non-ethylene-treated leaves. This suggests that the act of infiltrating Agrobacterium into the N. benthamiana leaves may elicit an ethylene-induced defence response in the leaves, which by itself can trans-activate the PG1 promoter (Fig. 4).

Discussion

A rapidly growing number of plant genomes have now been sequenced, giving researchers a valuable insight into these organisms beyond the traditional model species. While these genomes allow researchers to look at features that are unique to different and often commercially important plant species, it is important to translate knowledge gained from model systems to these species of interest. In this study, we build on the growing literature of ethylene-related genes in apple (Lee et al. 1998; Wiersma et al. 2007; Tacken et al. 2010; Ireland et al. 2012) by the characterization of the EBF-like genes. Interestingly, in apple there are four EBF-like genes, consistent with the genome duplication, while the closely related Rosaceae species strawberry has two. In the model species tomato and Arabidopsis, the EBF family is encoded redundantly by at least two genes. In Arabidopsis the two EBF genes fall into sub-clade I, while tomato has one gene in each sub-clade (Fig. 2).

In Arabidopsis, EBF1 and EBF2 mRNA is constitutively expressed (Guo and Ecker 2003; Potuschak et al. 2003) and has been shown to be targeted for degradation by EIN5 (Olmedo et al. 2006), suggesting that mRNA levels are actively regulated. In tomato, EBF1 is constitutively expressed with EBF2 showing considerable changes in expression over development and in different treatments (Yang et al. 2010). From this observation it was suggested that as EBF1 had a more consistent level of expression, it was providing the steady-state level of EBF, and fluctuations of EBF2 allowed the plant to respond to the environment. In apples, the two classes of EBF-like genes appear to follow the same pattern with sub-clade I genes (EBF1 and EBF101) showing little variation in expression, while the sub-clade II genes (EBF2 and EBF102) both increase as the fruit begin to ripen. Although the sub-clade I tomato gene EBF1 had a more consistent level of expression, it did have lower expression in mature green fruit. This

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**Fig. 3** Expression patterns of EBF-like genes over apple fruit development, compared with other ethylene-related and ripening genes. Expression of EBF1, EBF2, EBF3 and EBF4 was measured in cDNA derived from fruit tissue over the course of apple fruit development by qPCR. Expression levels are shown relative to the ACTIN gene. Expression levels of ACO1, PG1 and EIL1-3 are reported in Tacken et al. (2010).
was not observed in the expression pattern of EBF1 in apples, though as this drop was transitory in tomato, there is a possibility that a similar drop in apple would be missed in a less detailed time series experiment (Fig. 3).

In this study, three apple EIN3-like genes were tested in a transient assay for activity against the PG1 promoter. All three apple EILs had reduced activity against the PG1 promoter, in the presence of EBF1, showing that the apple EBF1 was not specific to a single EIL. The non-specific nature of the EBFs is consistent with the Arabidopsis EBF1 and EBF2, where both interact with EIN3 and EIL1, again suggesting a lack of specificity in these F-box proteins to individual EIL proteins.

Conclusions and forward look

An F-box gene EBF1 was identified in apple, the predicted protein product of which clustered with EBF-like proteins involved in the ethylene response in other plant species. EBF1 negatively regulated activation of PG1 by the apple EILs, consistent with the degradation of EIN3 by EBF1 and EBF2 observed in Arabidopsis and tomato. These results also suggest that apple EBF1 acts as a functional EBF upon multiple members of the EIL family of transcription factors. This work suggests that the EBF-like genes in apple are likely to play a crucial role in the control of ethylene-related fruit ripening.

Additional information

The following additional information is available in the online version of this article – Text files of apple EBF DNA sequences and predicted proteins.

Accession numbers

Apple EBF1 GenBank accession no. JX512439.

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Contributions by the authors

The project was conceived, executed and the first draft written by E.J.T. Sequencing, cloning and expression analysis were undertaken by E.J.T. H.S.I. and Y.-Y.W. This work was part of E.J.T.’s PhD project funded by AgMardt PhD scholarship (NZ), supervised by and edited by R.J.S. and J.P.

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Conflict of interest statement

None declared.

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**Appendix**

The complete references with the full list of authors for Shulaev et al. (2011) and Velasco et al. (2010) are as follows:

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