# Kiwifruit *EIL* and *ERF* Genes Involved in Regulating Fruit Ripening[1][W]

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Kiwifruit (*Actinidia deliciosa*) is a climacteric fruit sensitive to low concentrations of ethylene. To investigate the transcriptional mechanisms underlying kiwifruit ethylene response, transcription factors encoding four EIN3-Like (*EILs*) and 14 Ethylene Response Factors (*ERFs*) were cloned from kiwifruit. Expression of these transcription factors was examined during fruit development. The expression of transcripts of most *AdERFs* was higher during early fruit development, with the exception of *AdERF3*, which increased with maturity. Several *AdERFs* were apparently down-regulated by ethylene, as they were affected by the ethylene inhibitor 1-methylcyclopropene and by antisense suppression of *ACO* (for 1-aminocyclopropane-1-carboxylic acid oxidase) in the fruit. In contrast, *AdEILs* were constitutively expressed during fruit development and ripening. The transcription factors *AdEIL2* and *AdEIL3* activated transcription of the ripening-related genes *AdACO1* and *AdXET5* (xyloglucan endotransglycosylase gene) and, when overexpressed in Arabidopsis (*Arabidopsis thaliana*), stimulated ethylene production. The potential repressor *AdERF9* suppressed this promoter activity. These results support a role for kiwifruit *EILs* and *ERFs* in transcriptional regulation of ripening-related genes and in the regulation of kiwifruit fruit-ripening processes.

Plant tissues, including fruit, respond to ethylene through an increasingly well-defined signal transduction pathway. The transcriptional response to ethylene includes the EIN3-Like (*EIL*) and Ethylene Response Factor (*ERF*) families of transcription factors (TFs). Members of both families act as positive controllers of the ethylene response and potentially are involved in cross talk with other signals (Guo and Ecker, 2004).

*EILs* encode a small TF gene family (Chao et al., 1997; Riechmann et al., 2000; Tieman et al., 2001; Rieu et al., 2003). Members of this family have been shown to directly bind to the primary ethylene response element (PERE) in the Arabidopsis (*Arabidopsis thaliana*) *ERF1* promoter to regulate *ERF1* expression. This was first verified by Solano et al. (1998), and subsequently, a consensus binding sequence (EIL conserved binding sequence [ECBS]) was also found for tobacco (*Nicotiana benthamiana*) *TEIL*, comprising A[A/C]G[A/T]A[A/C]CT

(Kosugi and Ohashi, 2000). However, there is a need for more information on the interaction of EIN3/EILs and promoters of potential target genes.

Unlike *EIN3/EILs*, *ERFs* constitute one of the largest TF gene families in plants, with 122 members in Arabidopsis and 139 members in rice (*Oryza sativa*), containing a conserved DNA-binding domain (ERF domain; Nakano et al., 2006). *ERFs* were first identified from tobacco by interaction with a GCC box (core sequence is AGCCGCC; Ohem-Takagi and Shinshi, 1995). Besides the GCC box, a small group of *ERFs* (DREBs) also specifically bind to dehydration-responsive elements (DRE), with a core sequence of CCGAC (Hao et al., 2002; Sun et al., 2008).

These GCC and DRE motifs have been identified in many stress-related gene promoters associated with ethylene-mediated, and ethylene-independent, stress responses. Examples include *CBF/DREB1* (for C-repeat-binding factor/dehydration responsive element-binding factor 1) genes in cold response (Novillo et al., 2004) and *DREB2* in drought and heat stress response (Sakuma et al., 2006; Qin et al., 2008). Overexpression of sugarcane (*Saccharum officinarum*) *SodERF3* has been shown to increase plant salt and drought tolerance (Trujillo et al., 2008), and wheat (*Thinopyrum intermedium*) *TiERF1* is involved in disease resistance (Chen et al., 2008b). In addition to involvement in stress response, *ERF* genes have other distinct functions, such as involvement in lipid, cutin, and wax biosynthesis (Aharoni et al., 2004; Kannangara et al., 2007; Taketa et al., 2008).

*EIN3/EIL* genes show functional redundancy in modulating ethylene response (Tieman et al., 2001),

despite the fact that transcripts have been found to be little affected by ethylene in some plants (Lee and Kim, 2003; Mao et al., 2006). Ethylene appears to modulate EIN3/EIL at the protein level; treatment with 1-aminocyclopropane-1-carboxylic acid (ACC) results in the accumulation of EIN3 protein in Arabidopsis (Yanagisawa et al., 2003). Recently, tobacco *TEIL* has been found to have some relationship with the *basic PR* gene, as *basic PR5* was induced in *TEIL*-overexpressed plants; *TEIL* is also involved in flower development (Hibi et al., 2007). An important role for ethylene is in fruit ripening, and *EIL* genes have been isolated from fruit such as tomato (*Solanum lycopersicum*; Tieman et al., 2001; Yokotani et al., 2003) and kiwifruit (*Actinidia deliciosa*; Yin et al., 2008, 2009). In banana (*Musa acuminata*) fruit, *MA-EIL2* is a ripening- and ethylene-inducible gene, unique within the *EIL* gene family (Mbéguié-A-Mbéguié et al., 2008). In kiwifruit, *EIL* genes have been shown to be induced by low temperature, suggesting an involvement in low temperature response (Yin et al., 2009). Tomato *LeEIL* genes show some functional redundancy and can modulate ethylene response and fruit development (Tieman et al., 2001). However, there has been little research on *EIL* function in transcriptional regulation in fruit.

Fruit *ERF* genes have been isolated from apple (*Malus domestica*) and plum (*Prunus salicina*; Wang et al., 2007; El-Sharkawy et al., 2009). In tomato, *LeERF* genes have GCC box DNA-binding activity and differential responses to ethylene and other stimuli; *LeERF2* is induced by ethylene and suppressed in ripening-inhibited mutants (Wu et al., 2002; Tournier et al., 2003). Overexpression of *LeERF1* shortens fruit postharvest life (Li et al., 2007), and *LeERF2* regulates ethylene production in tomato and tobacco by modulating ethylene biosynthesis genes (Zhang et al., 2009b), transcriptional regulation being achieved by interaction of *LeERF2* with promoters of *NtACS3* and *LeACO3*. Autoregulation of ethylene biosynthesis is only one consequence of ethylene response in ripening fruit. Ethylene is involved to varying degrees in different fruit species in ripening-related processes such as softening (cell wall loosening), membrane breakdown, and aroma generation, but the involvement of the TFs of the *EIL* and *ERF* families in these processes is largely unknown.

Kiwifruit provides a good model for investigating ethylene response in fruit ripening, since it is very sensitive to ethylene (McDonald and Harman, 1982). Ripening-related genes have been isolated from kiwifruit, including those associated with ethylene biosynthesis (e.g. *ACS* and *ACO*; Whittaker et al., 1997; Xu et al., 1998), cell wall metabolism and fruit softening (e.g. polygalacturonase gene, *PG*; xyloglucan endotransglycosylase/hydrolase gene, *XTH*; expansin gene, *EXP*; Atkinson and Gardner, 1993; Yang et al., 2007; Atkinson et al., 2009), and membrane metabolism and aroma generation (e.g. lipoxygenase gene, *LOX*; Zhang et al., 2006, 2009a). We have also recently shown differential responses of ethylene receptors as well as *CTR* (for constitutive triple response) and *EIL* genes to ripening

stimulation and low temperature in kiwifruit (Yin et al., 2008, 2009).

The *Actinidia* EST database (Crowhurst et al., 2008) provides an opportunity to investigate further the ethylene response during ripening of kiwifruit by selecting *ERF* genes and analyzing their expression. In this study, three *AdEILs* (data for a fourth, *AdEIL1*, have been published by Yin et al. [2008]) and 14 *AdERF* genes were isolated from kiwifruit ESTs. Tissue- and time-specific gene expression in ripening kiwifruit was analyzed, and the interaction of *AdEIL* and *AdERF* genes with target gene promoters was investigated.

## RESULTS

### Gene Isolation and Analysis

Fourteen ERF full-length cDNAs were isolated and designated as *AdERF1* to *AdERF14* (GenBank accession nos. GQ869852–GQ869865). Based on a phylogenetic tree, the 14 ERF genes are clustered into six subfamilies (Fig. 1). Within the three main subfamilies, *AdERF4* to *AdERF6* belong to subfamily VII, while *AdERF7* to *AdERF9* and *AdERF10* to *AdERF13* belong to subfamilies VIII and IX, respectively. Four genes were isolated from three other families: *AdERF1* and *AdERF2* (subfamily III), *AdERF3* (subfamily IV), and *AdERF14* (subfamily X; Fig. 1).

The sizes of the deduced amino acid sequences differed substantially (e.g. *AdERF3* has 382 amino acids and *AdERF8* has 157 amino acids). Alignments of full-length deduced proteins of *AdERFs* and *AtERFs* showed conserved motifs, including a DNA-binding domain called the ERF domain (amino acids 57–59; Fig. 2, A and B). The ERF domain is a defining character of the *ERF* transcriptional factor gene family (Fujimoto et al., 2000; Nakano et al., 2006). In subfamily VIII, *AdERF8* and *AdERF9* also had a repressor domain, the ERF-associated amphiphilic repression domain (EAR; Fig. 2, A and C). Unlike the 3′ end location of EAR repressor domains, the location of the ERF domain varied among genes, being near the 5′ end in *AdERF8* and *AdERF9* and near the middle of *AdERF1* and *AdERF2* sequences (Fig. 2A).

Although all *AdERF* genes have the conserved ERF domain, the similarities of the *ERFs* varied from 12% (*AdERF5* and *AdERF11*) to 66% (*AdERF8* and *AdERF9*). Genes in some subfamilies had higher similarity (e.g. *AdERF7* had 61% and 57% similarity with *AdERF8* and *AdERF9*, respectively), although, in contrast, those in subfamily VII were less similar (e.g. *AdERF5* only had 36% and 26% similarity with *AdERF4* and *AdERF6*; Supplemental Table S7).

### Tissue Specificity of *AdEIL* and *AdERF* Gene Expression

*AdEIL* and *AdERF* genes were all differentially expressed in various kiwifruit tissues (Fig. 3). Within the *AdERF* family, only *AdERF2* was not detectable in mature fruit (Fig. 3). Among the different patterns,
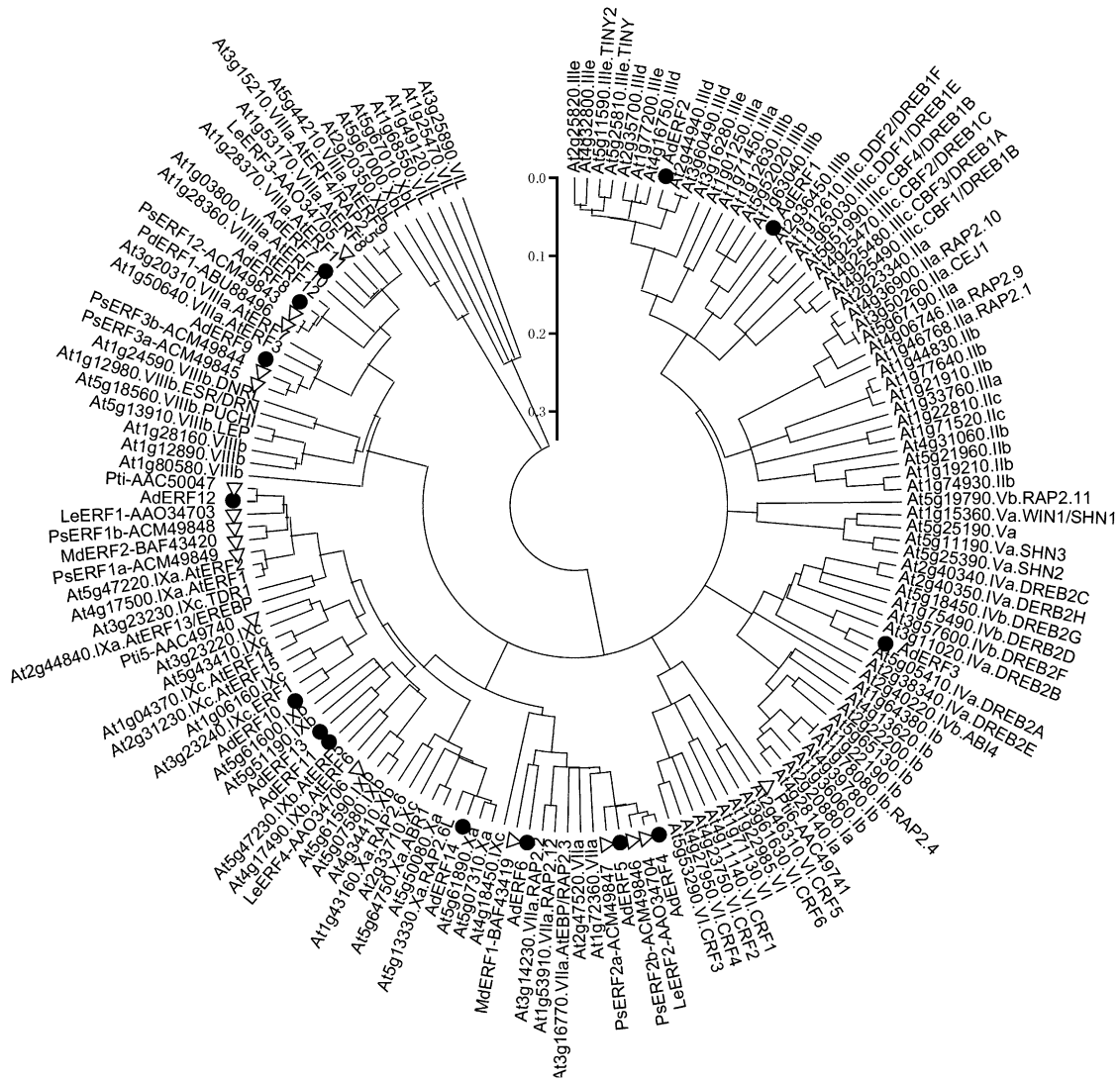
**Figure 1.** Phylogenetic tree of *ERF*s. Fourteen kiwifruit *AdERF*s (black circles) were aligned with the Arabidopsis ERF family and other fruit ERFs (white triangles). The amino acid sequences were obtained from The Arabidopsis Information Resource or the National Center for Biotechnology Information database. The amino acid sequences were analyzed with Vector NTI (version 9.0.0; Invitrogen), and the phylogenetic tree was constructed with MEGA (version 3.1) using a bootstrap test of phylogeny with minimum evolution test and default parameters.

genes such as *AdERF3* and *AdERF5* were constantly expressed in all tissues, those such as *AdERF4*, *AdERF11*, and *AdERF12* were higher in leaves, while *AdERF1* was strongly expressed in flowers and mature fruit. In contrast, *AdERF8* and *AdERF10* were weakly expressed in reproductive tissues. In mature fruit, *AdERF1*, *AdERF3*, *AdERF11*, and *AdERF12* were strongly expressed, while *AdERF6*, *AdERF8*, *AdERF10*, and *AdERF14* were weakly expressed in mature fruit compared with other tissues (Fig. 3). Since *AdERF2* was not detected in mature fruit, it was excluded in subsequent expression analysis of fruit during development and ripening.

Within the *AdEIL* gene family, *AdEIL3* was constitutively expressed in all tissues, and *AdEIL2* and *AdEIL3* had similar expression patterns, with transcripts weak in leaves and flowers (Fig. 3).

### Expression of *AdEIL* and *AdERF* Genes during Fruit Development

Six sampling times ranging from 20 d after full bloom (DAFB) to maturity (181 DAFB) covered the fruit development on the vine. Abundance of most *AdERF* transcripts was relatively high in fruit at the early stages of development (20 DAFB), including *AdERF4*, *AdERF6*, *AdERF7*, *AdERF8*, *AdERF10*, *AdERF12*, and *AdERF14* (Fig. 3). However, expression patterns over time were slightly different, some decreasing through to 181 DAFB or peaking again at later
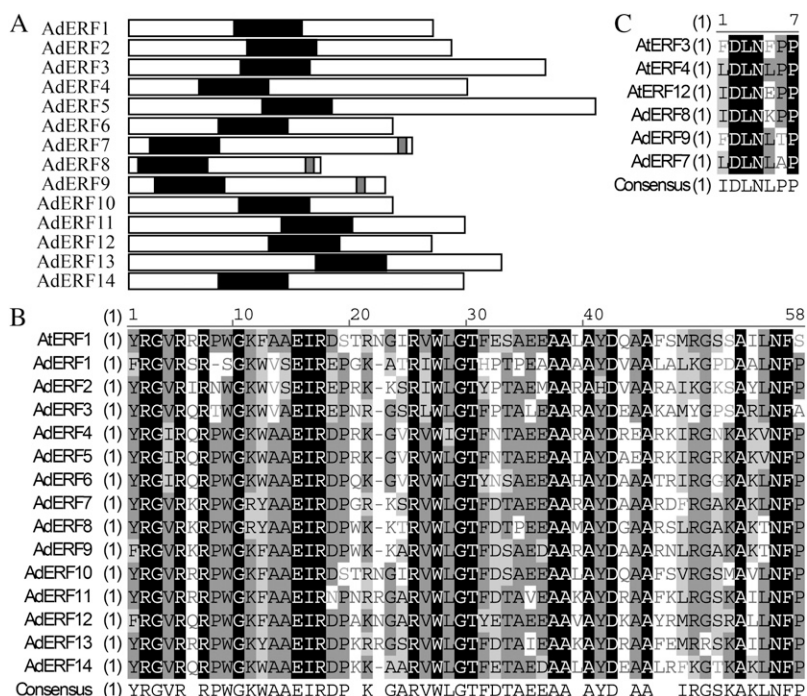
**Figure 2.** Schematic analysis of AdERFs with ERF domains and EAR repressor domains. A, Location of ERF domains (black bars) and EAR repressor domains (gray bars). B, Comparison of ERF domains by deduced amino acids. C, Comparison of EAR repressor domains by deduced amino acids.

stages; *AdERF9* and *AdERF13* had peaks in expression at 97 DAFB. In contrast, *AdERF3*, although at relatively low levels, was the only gene that increased in abundance during fruit development. *AdERF5* was constantly expressed at low levels, while the abundance of *AdERF1* varied during development, with three peaks (20, 97, and 159 DAFB; Fig. 3).

Within the *AdEIL* family, unlike the constitutively expressed *AdEIL1* (Yin et al., 2008), *AdEIL3* and *AdEIL4* had two peaks at 20 and 97 DAFB, and *AdEIL2* was weakly expressed at the earliest stage (Fig. 3).

**Expression of *AdEIL* and *AdERF* Genes in Ripening Kiwifruit**

Fruit physiology data for this experiment have been described in our previous publication (Yin et al., 2008). Ethylene treatment accelerates fruit ripening, promoting a climacteric rise beginning 6 d after harvest (DAH) and peaking at 9 DAH. Control fruit started the ethylene climacteric at 19 DAH and reached a peak at 21 DAH. In contrast, 1-methylcyclopropene (1-MCP), an ethylene receptor inhibitor, delayed fruit ripening, maintaining higher fruit firmness, with no ethylene being detected over the postharvest period.

*AdEIL2* and *AdEIL4* showed some response to ethylene, with expression increasing slightly at the late climacteric stage in control fruit or at the time of the increase in ethylene production in ethylene-treated fruit (Fig. 4). *AdEIL3* showed an early rise in expression in air and also increased at the time that ethylene increased in ethylene-treated fruit. 1-MCP treatment inhibited the ethylene-induced increases in expression (Fig. 4).

Expression patterns of *AdERF* genes were more diverse and can be divided into four patterns. Most of the genes (seven members) followed a decreasing pattern during ripening (Fig. 4). *AdERF1*, *AdERF7*, *AdERF11*, and *AdERF12* had slight increments at 1 DAH in control fruit, and ethylene treatment transiently increased *AdERF1* and *AdERF3* expression levels.

A second group (*AdERF10* and *AdERF14*) showed very strong expression increases, with the increase in ethylene production at about 19 DAH in control fruit and 6 DAH in ethylene-treated fruit (Fig. 4). *AdERF4* and *AdERF6* expression was relatively high at the beginning of storage and again in the postclimacteric stage (25 DAH in control and 12 DAH in ethylene-treated fruit). Expression of *AdERF5* and *AdERF9* was constant and not affected by exogenous ethylene (Fig. 4).

1-MCP treatment was used to confirm the association of gene expression with ethylene production in the control fruit. 1-MCP treatment substantially inhibited the increases in *AdERF4*, *AdERF6*, *AdERF10*, and *AdERF14* transcript levels toward the end of the ripening period and also inhibited the decrease in *AdERF3* and *AdERF13*. 1-MCP also inhibited the 1-d increase in some transcript levels found in the control fruit, again confirming a likely association of the transcript increase and ethylene. 1-MCP treatment had little effect on the decrease in mRNA abundance of *AdERF1*, *AdERF7*, *AdERF8*, *AdERF11*, and *AdERF12* in control fruit (Fig. 4; Supplemental Fig. S5).

We also took the opportunity to follow expression of the ERF genes in *ACO* knockout kiwifruit, which have inhibited ethylene production (R. Atkinson, unpub-
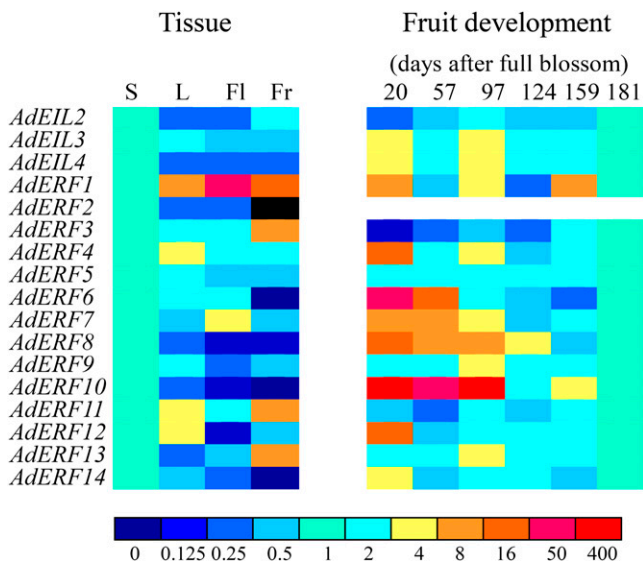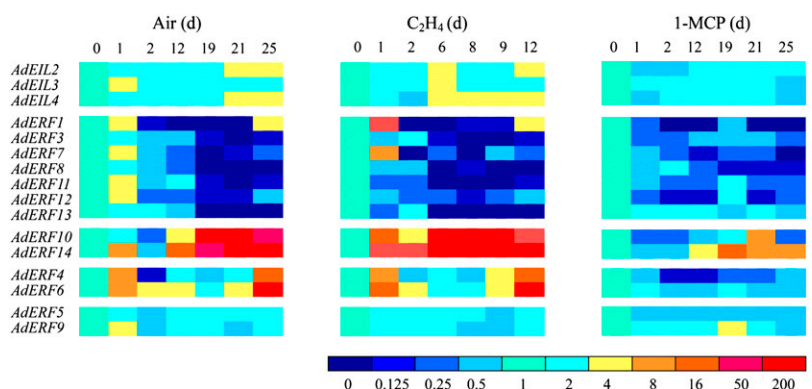
**Figure 3.** Tissue specificity and temporal expression pattern in fruit development. S, Stem; L, leaf; Fl, flower; Fr, mature fruit. Relative mRNA abundance was evaluated by real-time PCR from three biological repeats. Different colors indicate the average of relative mRNA abundance. Stem (S) and 181 DAFB were set as 1. The black area for AdERF2 in Fr means nondetectable. Data with SE from three repeats are provided in Supplemental Figures S2 and S3.

lished data). Although these were of a related species (*Actinidia chinensis*), ethylene treatment of the fruit induced expression of *AdERF1*, *AdERF7*, and *AdERF8* and suppressed that of *AdERF3*, *AdERF11*, *AdERF12*, and *AdERF13* (Supplemental Fig. S6), confirming the above 1-MCP data, showing that decreases of *AdERF3* and *AdERF13* transcripts in ripening fruit were associated with ethylene, while decreases of *AdERF1*, *AdERF7*, *AdERF8*, *AdERF11*, and *AdERF12* transcripts were independent of ethylene.

## Interaction of *AdEIL* and *AdERF* Genes with Promoters of Ripening-Related Genes

Promoters of six ripening-related genes were isolated from kiwifruit by genome walking with primers designed against existing sequences. By searching for

cis-acting regulatory elements, some ethylene-related motifs were found in the promoters (Fig. 5A; Supplemental Fig. S1). However, a GCC box was only found in the promoter of *AdEXP1* (Fig. 5A; Supplemental Fig. S1). An internal *Nco*I site in the promoter of *AdPG* meant that there was a 55-bp pGEM-T Easy vector sequence interval between the *AdPG* promoter and firefly luciferase (LUC; Fig. 5A).

In vivo interactions between TFs and promoters were estimated by transient assays in tobacco leaves. *AdEIL2* and *AdEIL3* acted as activators inducing the *AdACO1* and *AdXET5* promoters, while no significant changes were found with other promoters (Fig. 5, B and C). Although the promoter of *AdEXP1* had a GCC box, *AdERF* genes failed to affect promoter activity (data not shown). However, *AdERF9* significantly suppressed *AdXET5* promoter activity (Fig. 5D), and this was confirmed in a separate experiment described below. The other *AdERF*s had little effect on the *AdXET5* promoter activity.

## Promoter Deletions to Study Different Functions of PERE and ECBS Motifs and Possible Effective Regions for *AdERF9*

In order to study the functions of different motifs in the promoter of *AdXET5*, four deletions were made targeting four EIN3/EIL-related motifs (Fig. 6A). There was only a small effect of deletions on *AdXET5* promoter activity, with 78.8% and 69.0% activity remaining for deletions P2 and P4. With respect to *AdEIL2* and *AdEIL3*, the first deletion, P1, significantly reduced TF and promoter interaction, as there was only around 70% activity remaining for both *AdEIL2* and *AdEIL3* (Fig. 6B). The third deletion, P3, also significantly affected interaction of *AdEIL2* and the *AdXET5* promoter, resulting in only 40.1% and 60.1% of the LUC/*Renilla* luciferase (REN) ratio of P0 and P2, respectively (Fig. 6B). Nevertheless, except for P1, deletion P2 was also important for *AdEIL3*, with 49.6% and 71.4% compared with the LUC/REN ratio of P0 and P1 (Fig. 6B).

As there was no GCC box in this promoter region, some unknown effective motif or region of the *AdXET5* promoter might be involved in the interac-

**Figure 4.** Expression of the *AdEILs* and *AdERFs* in kiwifruit flesh during fruit ripening at 20°C. Gene transcript accumulation was evaluated by real-time PCR. Fruit were held at 20°C and treated with air (Air), 100 $\mu$L L$^{-1}$ ethylene (C$_2$H$_4$), or 0.5 $\mu$L L$^{-1}$ 1-MCP (1-MCP) for 24 h. Relative mRNA abundance was evaluated by real-time PCR from three biological repeats. Different colors indicate the average of relative mRNA abundance. Harvest time (0 d) was set at 1. Data with SE from three repeats are provided in Supplemental Figure S4.
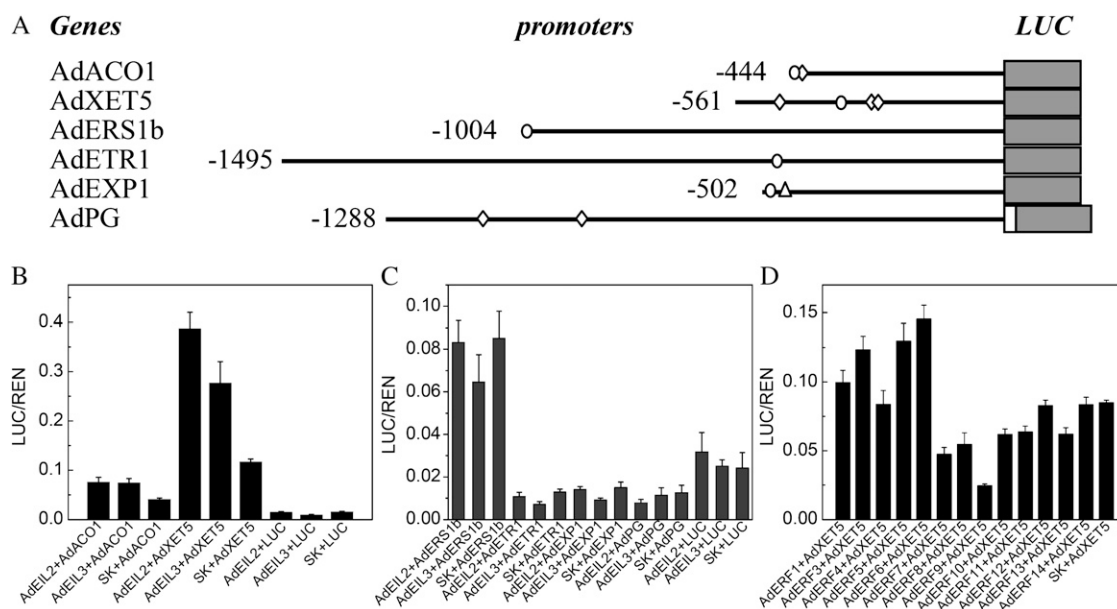
**Figure 5.** In vivo interaction of transcriptional factors and ripening-related gene promoters. A, Schematics of promoters are indicated with lines (promoter length), diamonds (PERE motif), circles (ECBS motif), and triangles (GCC box). B to D, In vivo associations of TFs and promoters were obtained from transient assays in tobacco leaves. Error bars indicate SE from at least four replicates.

tion with *AdERF9*. *AdERF9* constantly suppressed activity of the *AdXET5* promoter, regardless of the different deletions (Fig. 6B), which suggests that unknown effective motifs or regions for *AdERF9* exist at positions −265 to −1 of the *AdXET5* promoter.

## Overexpression of *AdEIL2* and *AdEIL3* in Relation to Ethylene Production in Arabidopsis

Since *AdEIL2* and *AdEIL3* can activate the promoter of *AdACO1*, the full-length sequences of *AdEIL2* and *AdEIL3* were introduced into Arabidopsis to study the relationship of ethylene production and *AdEIL2* and *AdEIL3*. Transformed Arabidopsis plants were selected by kanamycin resistance and were verified by end-point PCR (Fig. 8). Long-term ethylene treatment was used to stimulate ethylene production of Arabidopsis 15-d-old seedlings. Overexpression of *AdEIL2* and *AdEIL3* significantly induced ethylene production in Arabidopsis. Generally, *AdEIL2*-overexpressed plants had higher ethylene production rates than equivalent *AdEIL3* plants (Fig. 7). In order to analyze changes in ethylene biosynthesis genes in transformed Arabidopsis, *AtACS* and *AtACO* gene family members were chosen and transcript levels analyzed. The gene expression results show that both *AtACS* and *AtACO* genes are up-regulated in plants overexpressing *AdEIL2* or *AdEIL3* (Fig. 8, B and C). In addition, *AtXTH* genes were also up-regulated in transformed plants, with *AtXTH17* and *AtXTH18* most strongly affected by the transgene (Fig. 8D).

## DISCUSSION

A model for ethylene signal transduction has been well established, with at least five gene families involved, including a receptor family, *CTR1, EIN2, EIN3/EILs*, and *ERFs* (Kendrick and Chang, 2008). While most of the research on this pathway has been done in Arabidopsis, analysis of the pathway has been extended to fruit species, particularly tomato (Barry and Giovannoni, 2007). Within the pathway, *EIN3/EILs* and *ERFs* are plant-specific nucleus-localized proteins, serving as TFs that bind conserved motifs in promoter regions of target genes, thus providing a route for activation of the ethylene signal at the level of target gene transcription.

Kiwifruit are generally accepted as being more physiologically sensitive to ethylene than other fruit, such as tomato, apple, or peach (*Prunus persica*; Wills et al., 2001). Unlike many other fruit, softening and ethylene production are temporally separated, as fruit initially soften, followed by a rise in ethylene production and rapid senescence. Because of this, the species provides a good model for investigating molecular responses to ethylene and fruit softening and to fruit ripening in general.

In previous work, we isolated members of three gene families, including five ethylene receptor genes, two CTR1-like genes, and four *EIN3/EILs* (Yin et al., 2008, 2009). Our analysis of the *Actinidia* EST database provided at least 36 nonredundant *ERF*-related sequences (R. Hellens, unpublished data). The 14 *AdERF*s cloned, characterized by the conserved ERF
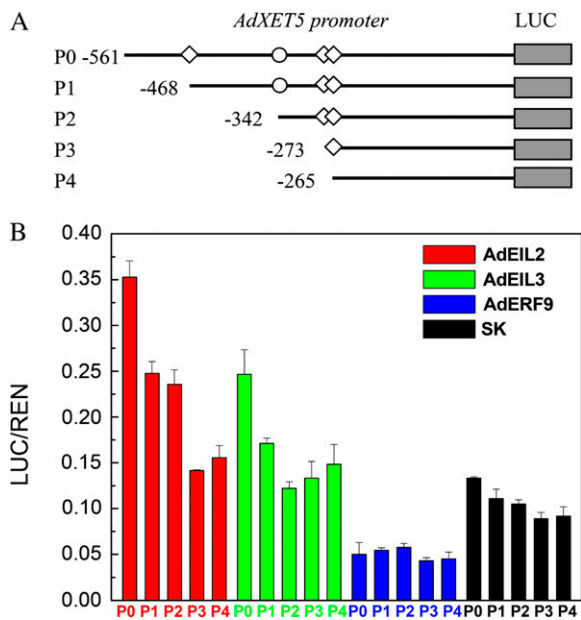
**Figure 6.** *AdXET5* promoter deletions. A, Four deletions were designed to remove four EIN3/EIL-related motifs. B, The in vivo interactions between *AdEIL2*, *AdEIL3*, *AdERF9*, and *AdXET5* promoters were tested with transient assays in tobacco leaves. SK represents empty vector. Error bars indicate SE from at least four replicates.

domain (Fujimoto et al., 2000; Nakano et al., 2006), were chosen because of their clustering into clades across six subfamilies. However, they were highly diverse in size, ranging from 157 to 382 amino acids, and therefore shared low sequence similarities, with the lowest at 12% and the highest at 66%. In subfamily VIII, *AdERF7*, *AdERF8*, and *AdERF9* also had an EAR repressor domain, suggesting that these three members might act as transcription repressors (Ohta et al., 2001).

## Spatial and Temporal Regulation of *AdEIL* and *AdERF* Expression

Among the three *AdEILs* and 14 *AdERFs*, only *AdERF2* was undetectable in fruit tissue, while the others were widely expressed in various kiwifruit plant tissues. Three *AdEILs* were constitutively expressed in various tissues and during fruit development, with slightly higher abundance of *AdEIL3* and *AdEIL4* early in fruit development, as we found in a previous study of *AdEIL1* (Yin et al., 2008).

As might be expected from the spread across subfamilies, the *AdERFs* were differentially expressed in various tissues, with, for example, *AdERF1* and *AdERF7* more highly expressed in flowers. Such differential expression patterns have also been found in other fruit species (Tournier et al., 2003; Wang et al., 2007) and match the multiple functions of ethylene in higher plants (Bleecker and Kende, 2000). The *AdERFs* were also differentially expressed during fruit devel-

opment. Most of them, including *AdERF4*, *AdERF6*, *AdERF7*, *AdERF8*, *AdERF10*, and *AdERF14*, were expressed more strongly in early stages of development, as has been found with ERFs in plum (El-Sharkawy et al., 2009) and in tomato *LeERF3b* (Chen et al., 2008a), consistent with kiwifruit ethylene receptor and CTR1-like genes (Yin et al., 2008). This agrees with our knowledge of ethylene-dependent activity in the early stages of organ development. Generally, the first 10 weeks of kiwifruit development is a rapid growth stage, with about two-thirds of the fruit volume and weight gained in this period (Beever and Hopkirk, 1990). This was shown with the fruit used in our work, with kiwifruit having mean weights of 61.27 g at 97 DAFB and 87.16 g at 181 DAFB, indicating faster fruit growth before 97 DAFB. These genes appear to be associated with this rapid growth period.

## Differential Expression of *AdEILs* and *AdERFs* during Fruit Ripening

The *ERF* gene family is the third biggest TF family in Arabidopsis (Riechmann et al., 2000). Subfamilies III and IV have been associated with the stress response (Novillo et al., 2004; Sakuma et al., 2006; Qin et al., 2008), while subfamilies VII, VIII, and IX contain members that are ethylene responsive (Gu et al., 2000; Tournier et al., 2003; Yang et al., 2005; Oñate-Sánchez et al., 2007; Champion et al., 2009). Subfamily VII genes have been particularly associated with fruit ripening; for example, the tomato *LeERF2* (Tournier et al., 2003), apple *MdERF1* (Wang et al., 2007), and
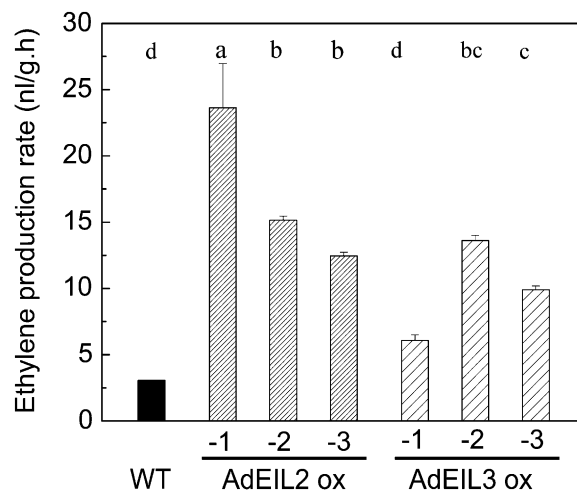


**Figure 7.** Different ethylene production rates of wild-type (WT) and transformed (*AdEIL*) Arabidopsis. Three different independent overexpression (ox) lines were used. Fifteen-day-old plants on 1/2 MS medium were treated with around 80 $\mu$L L$^{-1}$ ethylene for 24 h and then were exposed to ethylene-free air for 4 h. Before ethylene measurement, plants were sealed in air-tight jars for 18 h. Error bars indicate SE from three replications. Different letters above the columns represent significant differences ($P < 0.05$) between different constructs and lines.
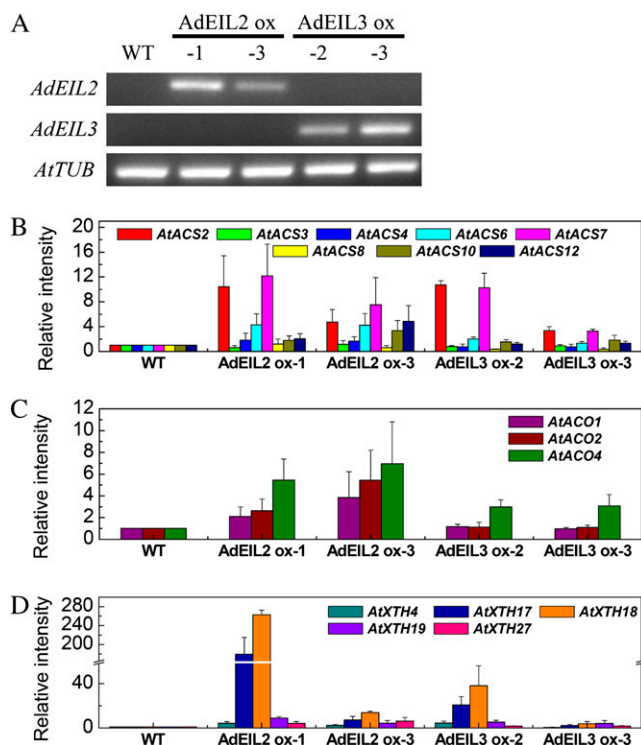
**Figure 8.** Gene expression in transgenic Arabidopsis overexpressing (ox) *AdEIL2* or *AdEIL3*. The Arabidopsis plants were germinated and grown in soil without any additional hormone or stress treatments. A, End-point PCR tests of *AdEIL2* and *AdEIL3* presence in transgenic Arabidopsis plants. B to D, Real-time PCR analysis changes of gene family members of *AtACS* (B), *AtACO* (C), and *AtXTH* (D) in transgenic EIL plants. Error bars indicate SE from three biological replicates. WT, Wild type.

plum *PsERF2a* and *PsERF2b* (El-Sharkawy et al., 2009) genes accumulate during fruit ripening. This supports the hypothesis that there might be a group-specific expression pattern in subfamily VII. However, we found no clearly consistent pattern of expression in the kiwifruit *ERF*s across the three subfamilies (VII, VIII, and IX). For example, in subfamily VII, *AdERF4* and *AdERF5* transcripts decreased while *AdERF6* accumulated at the late ripening stage. However, as with other fruit, there were four genes, *AdERF4*, *AdERF6*, *AdERF10*, and *AdERF14*, that generally increased during fruit ripening. *AdERF10* and *AdERF14* showed a strong association with the late rise in ethylene production, which was confirmed by a reduction in expression with the 1-MCP treatment and the positive response to external ethylene. *AdERF4* and *AdERF6* also increased during fruit ripening, but not concomitantly with the rise in ethylene, and were markedly stimulated at the later senescence stage. Thus, *AdERF10* and *AdERF14* might be ripening related, while *AdERF4* and *AdERF6* are more likely associated with senescence or other fruit processes.

One of the most interesting of our observations was the decrease in expression of some *ERF* genes with

ripening. Most reported fruit *ERF*s have been shown to be induced with ripening or ethylene treatment, including seven from plum, apple *MdERF1*, and tomato *LeERF1*, *LeERF2*, *LeERF4*, *JERF3*, and *Pti4* (Thara et al., 1999; Tournier et al., 2003; Huang et al., 2004; Wang et al., 2007; El-Sharkawy et al., 2009). However, *AdERF1*, *AdERF7*, *AdERF11*, and *AdERF12* transcripts were slightly higher directly after harvest in control fruit and then declined over ripening. An apparent stimulation of *AdERF1* and *AdERF7* at 1 DAH in control fruit could be related to trace ethylene accumulation over 24 h in the controls for the ethylene and 1-MCP treatments. The decreasing expression patterns of these genes might not depend on ethylene, confirmed by 1-MCP treatment and *ACO* knockout fruit. 1-MCP treatment inhibited the reduction in *AdERF3* and *AdERF13* expression, while the other five were less affected by 1-MCP. Similarly, in *ACO* knockout fruit, only *AdERF3* and *AdERF13* were suppressed by long-term ethylene treatment and maintained mRNA levels without ethylene (external and internal ethylene). Therefore, the decline in *AdERF3* and *AdERF13* is likely to be associated with ethylene, while the decline in *AdERF1*, *AdERF7*, *AdERF8*, *AdERF11*, and *AdERF12*, while ripening specific, is independent of ethylene. There is only one other report of such a decline in *ERF* expression, where tomato *LeERF3b* transcripts decreased during ripening of wild-type fruit, and in *Neverripe* mutant and *ACO1* cosuppression lines (Chen et al., 2008a). Expression patterns that paralleled the ethylene rise, such as *AdERF10* and *AdERF14*, might only be ethylene associated. These genes might not necessarily be involved in controlling ripening but perhaps in other fruit processes. In contrast, the five ripening-specific ERF genes that decreased could be involved in specific ripening-related processes. A decline in any repressor activity will result in increased gene activation of the TF targets. However, the roles of these five ERF genes need to be further investigated.

### *AdEIL2* and *AdEIL3* Act as Transcriptional Activators by Interaction with Ripening-Related Genes

*AdEIL* genes showed less response to ethylene and 1-MCP and were constitutively expressed during fruit development and ripening, similar to findings with *EIN3/EIL*s in other plants (Shinshi, 2008). However, the *EIN3/EIL* gene family is considered to have a role in transcriptional regulation of target genes, such as Arabidopsis *ERF1* (Solano et al., 1998) and tobacco basic PR (Hibi et al., 2007). In order to explore the relation between *AdEIL* genes and kiwifruit ripening-related genes, six such genes were selected for promoter isolation: *AdACO1* (ACC oxidase, similar to the gene used in the *ACO* knockout kiwifruit; Xu et al., 1998), *AdXET5* (xyloglucan endotransglycosolase; Schröder et al., 1998; Atkinson et al., 2009), *AdEXP1* (Yang et al., 2007), *AdPG* (the promoter of *AdPG* was confirmed in tomato; Atkinson and Gardner, 1993), and *AdERS1b* and *AdETR1* (ethylene receptor genes;

Yin et al., 2008). All these genes are associated with fruit ripening and softening. As described previously, the PERE and ECBS motifs are two different EIN3-interactive motifs that have been identified in Arabidopsis and tobacco, respectively (Solano et al., 1998; Kosugi and Ohashi, 2000). Despite the constant expression levels of the kiwifruit *AdEIL* (Fig. 4), these genes were confirmed as TFs, especially as transcriptional activators, inducing activities of the promoters of *AdACO1* and *AdXET5*. A recent publication on melon (*Cucumis melo*) fruit shows that *CmEIL1* and *CmEIL2* can activate the *CmACO1* promoter in leaf discs (Huang et al., 2010). Such activation of *EIL* genes from both kiwifruit and melon fruit confirms that this gene family is active in regulating genes associated with ripening, such as those involved in softening and ethylene synthesis.

### *AdEIL2* and *AdEIL3* Triggered Ethylene Production in Overexpressed Arabidopsis

Since both *AdEIL2* and *AdEIL3* were shown to activate the promoter of *AdACO1*, the effect of both genes on ethylene biosynthesis was tested. As kiwifruit transformation, through to fruit production, is a long process, *AdEIL2* and *AdEIL3* were overexpressed in Arabidopsis. Because of low basal levels of ethylene production in Arabidopsis, external ethylene was used to stimulate synthesis, following reports showing that ACC treatment can accelerate Arabidopsis ethylene production rates (Pieterse et al., 1998) and inhibit EIN3 protein degradation (Potuschak et al., 2003). The external ethylene treatment increased plant ethylene production. Moreover, gene expression results using transformed Arabidopsis showed that *AdEIL2* and *AdEIL3* were not only involved in the regulation of *ACO* gene expression but also up-regulated *ACS* genes. The increased ethylene production in transgenic Arabidopsis and transcriptional regulation of ethylene biosynthesis genes confirms the suggestion that *AdEIL* genes function within the signal transduction pathway connecting ethylene signaling and ripening processes.

### Nonredundant Roles of Different Motifs in TF and Promoter Interaction

Although *AdEIL2* and *AdEIL3* modulated *AdACO1* and *AdXET5* promoters and ethylene production in Arabidopsis, there were also four promoters that did not respond. Furthermore, another 15 promoters, selected from a resource created by Dare et al. (2008), containing PERE and/or ECBS motifs, were also tested using the transient assay, but none could be induced by *AdEIL2* or *AdEIL3* (data not shown). These results suggest that the motifs are not equal in the interaction between TFs and promoters, both between and within promoters. *AdXET5* promoter deletions indicated different functions in relation to the *AdEILs*; for *AdEIL2*, the first and third PERE motifs were important, while

for *AdEIL3*, the first PERE and ECBS motifs were more active (Fig. 6).

### *AdERF9* Acts as a Transcriptional Repressor, and the Possible Non-GCC Motif Is Located Near the Start Code Region

In the above six kiwifruit promoters, only *AdEXP1* had a GCC box, which is one of the targets for ERF genes. However, there was no interaction between *AdERFs* and *AdEXP1*. Surprisingly, *AdERF9* significantly suppressed activity of the *AdXET5* promoter, which does not have a GCC or DRE box. This result might be explained by recognizing that *AdERF9* is a repressor TF. In tomato, *Pti4*, an ERF-like TF, could regulate defense-related gene expression via GCC and non-GCC boxes (Chakravarthy et al., 2003), suggesting that there are potential unknown motifs in relation to *ERFs*. In order to identify unknown motifs in the *AdXET5* promoter, the four deletions, based on PERE and ECBS motifs, were also applied to *AdERF9*. The transient assay results confirmed the suppression of *AdERF9* on the *AdXET5* promoter. The possible effective motif for *AdERF9* is probably in the −265 to −1 region of the *AdXET5* promoter, but the specific region needs to be further investigated. Alternatively, GCG boxes may exist farther upstream from the transcriptional start site in the *AdXET5* promoter.

### CONCLUSION

Four EIN3-like genes and 14 ERF genes isolated from kiwifruit encoded two separate levels of the ethylene signaling transduction pathway and have been shown to function as TFs; *AdEIL2*, *AdEIL3*, and *AdERF9* could interact with promoters of several ripening-related genes. Expression studies of these genes revealed different behavior patterns in various tissues and during fruit development and ripening. In particular, five *AdERF* genes had reduced expression with the progression of fruit ripening, confirmed by 1-MCP treatment. These may be more important in fruit ripening than other ethylene-associated *AdERFs*. Transient assays of *AdEIL2* and *AdEIL3* verified that these genes were transcriptional activators in vivo, while *AdERF9* acted as a transcriptional repressor. Although *AdEIL* genes were constitutively expressed during fruit ripening, we could show that they up-regulated promoters of *AdACO1* and *AdXET5* and also up-regulated *AtACS* and *AtACO* gene expression, thus potentially triggering ethylene production in *AdEIL2*- and *AdEIL3*-overexpressed Arabidopsis. In contrast, *AdERF9* suppressed the *AdXET5* promoter, although no GCC box was identified in this promoter. *AdERF9* constantly suppressed the activity of the *AdXET5* promoter, with the region from −256 to −1 being important for this interaction. Future research will focus on cis-elements for these TFs and the broader function of *ERFs* in kiwifruit responses. This research

has taken us a step farther in understanding the nature of transcriptional regulation involved in the later stages of the ethylene signal transduction pathway in ripening fruit.

## MATERIALS AND METHODS

### Plant Material

To allow analysis of tissue-specific gene expression, different kiwifruit (*Actinidia deliciosa* var *deliciosa* 'Hayward') plant tissues, including stem, leaf, and whole flowers, were collected from the Plant and Food Research Orchard, Te Puke, New Zealand, over the 2006 to 2007 growing season.

For a fruit development series, Hayward kiwifruit at different developmental stages were picked from the same site. Full blossom was on November 15, 2006, and there were six subsequent sampling times: 20, 57, 97, 124, 159, and 181 DAFB (Yin et al., 2008).

To test postharvest treatments, kiwifruit with a mean soluble solids concentration (SSC) of 6.7% (commercial maturity) were harvested from a commercial orchard in Patumahoe, South Auckland. Three postharvest treatments, ethylene treatment (100 $\mu$L L$^{-1}$, 24 h), 1-MCP treatment (0.5 $\mu$L L$^{-1}$, 24 h; SmartFresh), and a control treatment (air), were performed as described previously (Yin et al., 2008). After treatment, fruit were held at 20°C until reaching a firmness of less than 2 N and development of the ethylene climacteric, although the latter did no occur in 1-MCP-treated fruit. At each sampling time, ethylene production, fruit firmness, and SSC were recorded, as described by Yin et al. (2008). For ethylene production, eight replicates (two fruit for each replicate) were performed, and 10 replicates (one fruit for each replicate) were performed for fruit firmness and SSC. After measurement, fruit flesh (without skin, seeds, or core) was bulked for the 10 fruit, frozen in liquid nitrogen, and stored at −80°C for further use.

### RNA Extraction and cDNA Synthesis

Total RNA was isolated from the frozen tissues following the protocol described by Chang et al. (1993b). Approximately 1.8 or 0.5 g of tissue was used for fruit flesh or vegetative and flower tissues, respectively. Total RNA was treated with TURBO DNase (Ambion) to remove contaminating DNA. After DNase treatment, 2.5 $\mu$g of RNA was used for cDNA synthesis with the SuperScript III First Strand Synthesis Kit (Invitrogen) following the manufacturer's protocol. In order to reduce the between-fruit or between-tissue variability, for each time point, three batches of RNA were isolated as three biological repeats for separate cDNA synthesis.

### Gene Isolation

Fourteen ERF genes were isolated from the Plant and Food Research EST database of *Actinidia* species using the BLAST algorithm (Crowhurst et al., 2008). Five of the 14 ERF genes were full-length EST sequences in the database, with complete start and stop codes, while full-length sequences of the other nine ERF genes were obtained by RACE with the SMART RACE cDNA Amplification Kit (Clontech). The details of the primers used for RACE are described in Supplemental Table S1. Full-length proofreadings were performed by the primers described in Supplemental Table S2 and by PCR product resequencing confirmation. Four EIN3-like genes, *AdEIL1* to *AdEIL4*, were also isolated from the EST database, and these have been described in our previous publications (Yin et al., 2008, 2009). Alignments were carried out on Vector NTI (version 9.0.0 [Invitrogen], using ClustalW), and a phylogenetic tree was generated with MEGA version 3.1 (Kumar et al., 2004).

### Real-Time PCR

For real-time PCR, oligonucleotide primers were designed according to each gene's untranslated region with Primer3 (version 0.4.0; http://frodo.wi. mit.edu/cgi-bin/primer3/primer3_www.cgi). The sizes of all PCR products ranged from 118 to 196 bp. All primers were tested with melting peaks and dissociation curves to confirm that there was only one product for each pair of primers. In order to verify that primers were specific amplifications of target genes, all PCR products were purified and resequenced. The sequences of all

primers used for real-time PCR analysis are described in Supplemental Table S3.

The PCR mixture (10-$\mu$L total volume) comprised 5 $\mu$L of 2× LightCycler 480 SYBR Green I Master Mix (Roche), 1 $\mu$L of each primer (5 $\mu$M), and 3 $\mu$L of diluted cDNA. PCR was performed on a LightCycler 480 instrument (Roche), initiated by 5 min at 95°C, then followed by 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 15 s, and completed with a melting curve analysis program. No-template controls and melting curve analyses were included in every reaction. Kiwifruit actin was used as the housekeeping gene to quantify cDNA abundance.

Mature fruit sampled on the harvest day for the ripening fruit series and fruit sampled 181 DAFB for the fruit development series were set as calibrators (set to 1) for calculating relative expression levels. For the tissue-specific experiment, expression in the stem tissue was used as the calibrator. Three separate biological replicates were performed for relative intensities. All relative intensities were calculated from standard curves for each gene.

### Genome Walking

Genomic DNA was extracted from kiwifruit leaves using the DNeasy Plant Mini Kit (Qiagen). Genomic DNA, 2 $\mu$g for each, was digested with eight restriction enzymes (*Dra*I, *Ecl*136II, *Eco*RV, *Hpa*I, *Msc*I, *Stu*I, *Ssp*I, and *Sca*I) separately. After enzyme digestion, all products were cleaned up using a DNA Clean and Concentration Kit (ZYMO Research) and then were ligated to an adaptor (Clontech).

Six known genes were chosen for promoter work: two ethylene receptor genes (*AdETR1* and *AdERS1b*; Yin et al., 2008), one expansin-like gene (*AdEXP1*; Yang et al., 2007), one xyloglucan endotransglycosylase gene (*AdXET5*; Schröder et al., 1998), one polygalacturonase gene (*AdPG*; Atkinson and Gardner, 1993), and one ACC oxidase gene (*AdACO1*; Xu et al., 1998). Among these six genes, the *AdPG* promoter was cloned by Atkinson and Gardner (1993) and the other five 5′ upstream genomic sequences were generated using a GenomeWalker kit (Clontech) with nested PCR. The nested PCR analysis was performed with two sets of primers, including two adaptor primers that were obtained from the kit and two gene-specific primers that were designed by Primer3, as described in Supplemental Table S4.

Conserved cis-element motifs of promoters were predicted using PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) and Plant-CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) databases.

### Promoter Modification

In order to test the interaction between *AdEILs* and motifs, promoter deletions were designed. Four deletions of the *AdXET5* promoter covered four *EIN3/EIL*-related motifs, including three PERE motifs and one ECBS motif. Promoter deletions were conducted with PCR using the primers described in Supplemental Table S5.

### Transient Assay

A transient assay, also known as a dual luciferase assay, was performed with tobacco (*Nicotiana benthamiana*) to study the interaction of TFs and promoters, which has been widely used in our laboratory (Hellens et al., 2005; Espley et al., 2007; Palapol et al., 2009). Two pGreenII vectors were used for the transient assay, pGreenII 0800-LUC vector and pGreenII 0029 62-SK vector, and all related information on the vectors can be found in Hellens et al. (2005). For promoters, primers (Supplemental Table S4) were designed according to the sequences obtained by genome walking, and a *Nco*I site was introduced at the 3′ end of each promoter. PCR products were cloned into pGEM-T Easy vector (Promega) and resequenced to confirm the sequence. Promoters were then cut from the pGEM-T Easy vector and inserted into a pGreenII 0800-LUC vector at the 5′ end of a LUC gene. As the *AdPG* promoter region also contains a *Nco*I site, *Apa*I and *Spe*I were used to digest the vector, so there existed a 55-bp pGEM-T Easy vector sequence between the *AdPG* promoter and LUC. The interaction between TFs and promoters was indicated by luminescence activity. pGreenII 0800-LUC vector carried a REN gene under the control of the 35S promoter as a positive control. The ratios of LUC and REN were expressed as activation or repression of the promoters by the TFs. Full-length cDNAs of the TFs (*AdEILs* and *AdERFs*) were generated by primers (described in Supplemental Table S2) and were cloned into pGreenII 0029 62-SK.

All constructs were electroporated into *Agrobacterium tumefaciens* GV3101 (MP90). The bacterial strains were stored as glycerol stock at −80°C. *Agro-*

*bacterium* was streaked on a Luria-Bertani plate for 2 d from glycerol stock and then was restreaked on a new Luria-Bertani plate for 1 d. *Agrobacterium* cultures were prepared with infiltration buffer (10 mM MgCl$_2$ and 0.5 μM acetosyringone) to an optical density at 600 nm from 0.7 to 1.0. *Agrobacterium* culture mixtures of TFs (1 mL) and promoters (100 μL) were infiltrated into tobacco leaves by needleless syringes. Tobacco plants were grown in a glasshouse, under natural light with daylight extension to 16 h. Three days after infiltration, LUC and REN were assayed using the dual luciferase assay reagents (Promega). The analysis was carried out using an Orion Microplate Luminometer (Berthold Detection System) according to the method described by Palapol et al. (2009), with at least triplicate transient assay measurements for each plant.

## Transformation of Arabidopsis

The constructs with full-length genes and pGreenII 0029 62-SK, as used in the transient assay experiment, were used for transformation in Arabidopsis (*Arabidopsis thaliana*; overexpression). All constructs were electroporated into *Agrobacterium* GV3101 (MP90) and then introduced into Arabidopsis (cv Columbia) by floral dip (T1).

T1 seeds were germinated on half-strength Murashige and Skoog (1/2 MS) medium and were selected with kanamycin resistance. Then, 2- to 3-week-old kanamycin-resistant plants were transferred to soil and grown in a greenhouse under general conditions (T2). Transgene expression was verified by end-point PCR, and the cycles were limited to 35. Changes of transcript abundance of select target genes were studied with real-time PCR using a Bio-Rad C1000 thermal cycler. The methods for end-point PCR and real-time PCR were as described by Yin et al. (2008). The sequences of all primers used for Arabidopsis analysis are described in Supplemental Table S6.

## Ethylene Production of Transformed Arabidopsis

Fifteen-day-old plants (wild type, *AdEIL2* overexpressed, and *AdEIL3* overexpressed; T3, generated from T2 seeds) on 1/2 MS medium were treated with 80 μL L$^{-1}$ ethylene for 24 h and then were exposed to ethylene-free air for 4 h. Before ethylene measurement, plants were sealed in air-tight jars (0.5 L) for 18 h at 23°C. Head-space gas (1 mL) was removed from each jar using a syringe, and ethylene was measured by gas chromatography using a gas chromatograph (Hewlett-Packard 5890 series II) fitted with a flame ionization detector (Philips PU4500; Unicam) and an alumina F1 column (1.5 m × 6 mm). The injector, detector, and oven temperatures were 160°C, 200°C, and 130°C, respectively.

The amount of plant material was determined by the weight of above-ground parts (stems and leaves) without roots. Three technical replicates were performed for ethylene production measurement. The statistical analysis was performed with DPS software (version 3.11).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers GQ869852 to GQ869865.

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Motif analysis of ripening-related gene promoters.

**Supplemental Figure S2.** Tissue specificity expression patterns during fruit development.

**Supplemental Figure S3.** Temporal expression patterns during fruit development.

**Supplemental Figure S4.** Expression of *AdEIL* and *AdERF* genes in kiwifruit flesh during fruit ripening at 20°C.

**Supplemental Figure S5.** Decreasing expression of *AdERF1*, *AdERF3*, *AdERF7*, *AdERF8*, *AdERF11*, *AdERF12*, and *AdERF13* during kiwifruit ripening.

**Supplemental Figure S6.** Study of the relation between decreasing expression of *AdERFs* and ethylene by *ACO* knockout kiwifruit.

**Supplemental Table S1.** Primers for RACE.

**Supplemental Table S2.** Primers for full-length amplification.

**Supplemental Table S3.** Primers for real-time PCR.

**Supplemental Table S4.** Primer sequences (5′–3′) for promoter isolation.

**Supplemental Table S5.** Primers for promoter modification.

**Supplemental Table S6.** Primers for transformed Arabidopsis examination.

**Supplemental Table S7.** Similarity of AdERFs based on deduced amino acids.

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