The Role of Ethylene and Cold Temperature in the Regulation of the Apple *POLYGALACTURONASE1* Gene and Fruit Softening[1][W][OA]

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Fruit softening in apple (*Malus* × *domestica*) is associated with an increase in the ripening hormone ethylene. Here, we show that in cv Royal Gala apples that have the ethylene biosynthetic gene *ACC OXIDASE1* suppressed, a cold treatment preconditions the apples to soften independently of added ethylene. When a cold treatment is followed by an ethylene treatment, a more rapid softening occurs than in apples that have not had a cold treatment. Apple fruit softening has been associated with the increase in the expression of cell wall hydrolase genes. One such gene, *POLYGALACTURONASE1* (*PG1*), increases in expression both with ethylene and following a cold treatment. Transcriptional regulation of *PG1* through the ethylene pathway is likely to be through an ETHYLENE-INSENSITIVE3-like transcription factor, which increases in expression during apple fruit development and transactivates the *PG1* promoter in transient assays in the presence of ethylene. A cold-related gene that resembles a *COLD BINDING FACTOR* (*CBF*) class of gene also transactivates the *PG1* promoter. The transactivation by the *CBF*-like gene is greatly enhanced by the addition of exogenous ethylene. These observations give a possible molecular mechanism for the cold- and ethylene-regulated control of fruit softening and suggest that either these two pathways act independently and synergistically with each other or cold enhances the ethylene response such that background levels of ethylene in the ethylene-suppressed apples is sufficient to induce fruit softening in apples.

Apple (*Malus* × *domestica*) fruit softening is likely to be controlled by a complex interaction between developmental and environmental factors. The importance of ethylene as a developmental driver for ripening is well known, but the role of environmental stimuli such as cold temperatures is yet to be understood. While flesh softening in apples is highly dependent on ethylene (Johnston et al., 2009; Wang et al., 2009), softening can also partially occur in the absence of ethylene. There is evidence for a strong cold requirement to initiate ethylene-related ripening in some apple and pear cultivars such as Granny Smith and Passe-Crassane, while other apple cultivars such as Royal Gala produce ethylene without prolonged cold exposure (Larrigaudiere et al., 1997; El-Sharkawy et al., 2004). However, it is not known if exposure to cold can initiate ripening independently from ethylene.

Loss of flesh firmness in fleshy fruit is achieved by a suite of cell wall-related enzymes (Goulao and Oliveira, 2008). Reduction in the levels of a single enzyme often has only minor effects on the maintenance of fruit firmness (Sheehy et al., 1988; Smith et al., 1990). Softening in apples is associated with an increase in the expression of a number of cell wall-related genes such as *POLYGALACTURONASE1* (*PG1*), *B-GALACTOSIDASE*, and *XYLOGLUCAN ENDOTRANSGLYCOSYLASE1* (Atkinson, 1994; Goulao and Oliveira, 2007). The best characterized of these genes is *PG1*. Down-regulation of *PG1* expression in tomato (*Solanum lycopersicum*) had little effect on fruit firmness (Sheehy et al., 1988; Smith et al., 1990), while in strawberry (*Fragaria* species), suppression of PG led to firmer fruit (Quesada et al., 2009). In apple, *PG1* expression levels have been associated with softening patterns in a range of cultivars (Wakasa et al., 2006). Transgenic apple plants overexpressing *PG1* have reduced cell-to-cell adhesion in the leaves (Atkinson et al., 2002), and suppression of *PG1* results in firmer fruit (Atkinson et al., 2008). While these PG-suppressed apples were firmer than the controls, they were significantly softer than the *ACC OXIDASE1* (*ACO1*)-suppressed apples, suggesting that also in apples a suite of enzymes is required for fruit softening. Fusions of the *PG1* promoter to the

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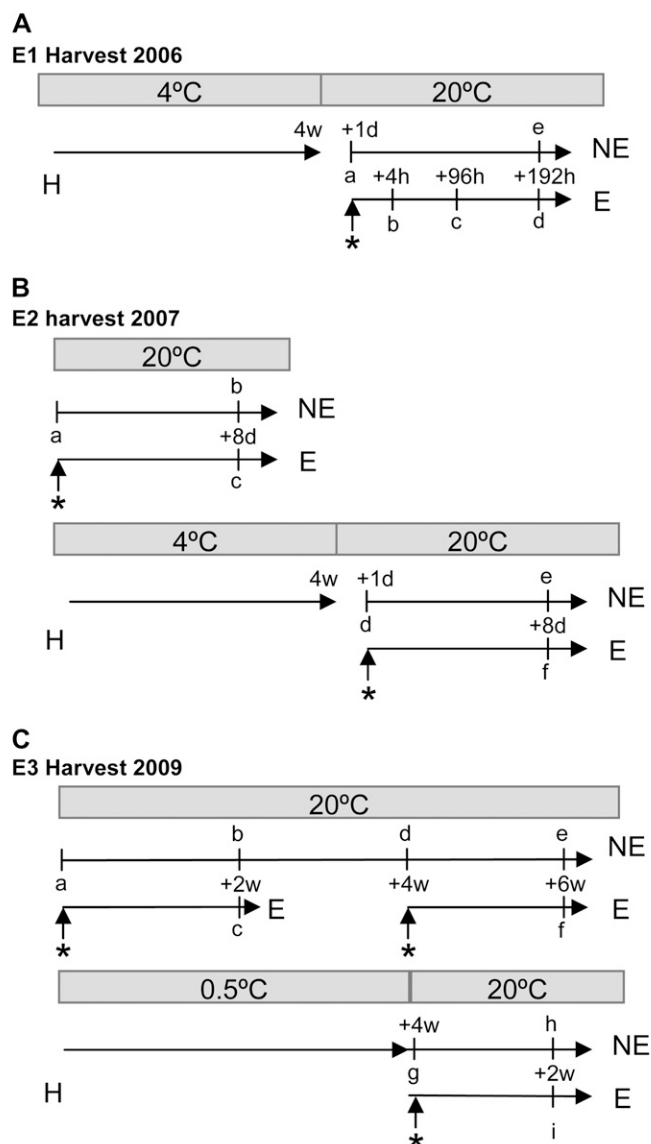

Figure 1. Postharvest regimes for *ACO1*-suppressed apples. Asterisks mark the point of 100 μL L^{-1} ethylene treatment. NE, No ethylene treatment; E, ethylene treatment; H, harvest; w, weeks. Lowercase letters represent sampling points. A, For the 2005 harvest (E1), apples were harvested and stored at 4°C for 1 month before they were warmed to 20°C for 24 h, and half were treated with 100 μL L^{-1} ethylene. Apple samplings are labeled a to e as follows: a, just before ethylene treatment; b, 4 h after ethylene treatment; c, 4 d (96 h) after ethylene treatment; d, 8 d (192 h) after ethylene treatment; e, 8-d no-ethylene control. Six apples were sampled at a time. B, For the 2007 harvest (E2), apples were sampled either immediately following ethylene treatment or after being store in the cold for 4 weeks. Sample times are labeled a to f as follows, with six apples in each group: a, sampled immediately; b, stored in an ethylene-free environment for 8 d before being sampled; c, sampled after being treated with 100 μL L^{-1} ethylene for 8 d; d to f, stored at 4°C for 4 weeks and then transferred to 20°C for 1 d as follows: d, sampled immediately; e and f, sampled following an 8-d treatment either with or without 100 μL L^{-1} ethylene. C, For the 2009 harvest (E3), apples were sampled following a room temperature treatment or a 0.5°C treatment. Sampling times are labeled a to i, with eight apples sampled at each time, as follows: a, sampled immediately; b, d, and e, stored in an ethylene-free environment at 20°C and sampled after 2 weeks of storage (b), after 4 weeks of storage (d), and after 6 weeks of storage (e); c, treated with 100 μL L^{-1} ethylene for 2 weeks; f, treated with 100 μL L^{-1} ethylene for 2 weeks after 4 weeks of storage at 20°C; g, h, and i, stored at 0.5°C in an ethylene-free environment for 4 weeks, then g was sampled immediately and h and i were transferred to 20°C and sampled after 2 weeks either with or without treatment with 100 μL L^{-1} ethylene.

GUS reporter gene were cloned into tomato, and the first 1.6 kb was found to have an expression pattern corresponding to tomato ethylene fruit ripening, while a larger 2.6-kb promoter did not and was hypothesized to contain an element that caused inhibition of expression (Atkinson et al., 1998).

While the transcription factors that regulate the expression of various apple fruit-ripening events are largely unknown, there is a considerable amount known about both the transcriptional regulation of the ethylene response pathway and the cold response pathway from the model plants Arabidopsis (*Arabidopsis thaliana*) and tomato (Alonso and Stepanova, 2004; Chen et al., 2005). In these systems, it has been shown that the ethylene signal cascade ultimately leads to stabilization of the transcription factor ETHYLENE INSENSITIVE3 (EIN3; Solano et al., 1998), which has been shown to bind and activate other transcription factors such as ETHYLENE RESPONSE FACTOR1 (ERF1; Solano et al., 1998). In Arabidopsis, the *AP2/ERF*-like genes belong to a large transcription factor family of 147 genes (Feng et al., 2005; Nakano et al., 2006), several of which are up-regulated by ethylene (Alonso and Stepanova, 2004). This family also includes the key cold induction genes *COLD-BINDING FACTOR1* (*CBF1*) to *CBF4*, which are known to bind to and activate a number of cold response genes (Stockinger et al., 1997).

While fruit softening in apples has been previously tightly linked to ethylene, there is currently little understanding of the role of cold in this process. This study used the previously published *ACO1*-suppressed apples (Schaffer et al., 2007; Johnston et al., 2009), which produce levels of ethylene that are insufficient to cause a ripening response in apples (Johnston et al., 2009), to determine the role of cold in fruit ripening in the absence of exogenously added ethylene. Using the ripening-induced cell wall hydrolase, PG1, as a marker for fruit softening, we investigated the transcriptional control of this gene by the *EIN3-like* (*EIL*) and *AP2* domain-containing transcription factors in response to both cold and ethylene.

RESULTS

Cold Alone Is Sufficient to Induce Apple Fruit Softening

To test the effect of cold on fruit softening, *ACO1*-suppressed apples (A03 lines described by Schaffer et al., 2007) were treated with combinations of cold and ethylene treatments over three independent har-

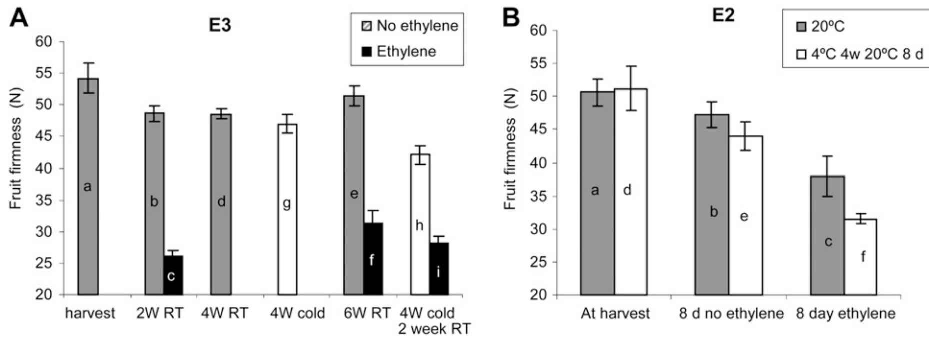

Figure 2. Flesh firmness (N) of *ACO1*-suppressed apples following different treatments. A, Harvest E3. Letters represent sampling times, gray bars represent a no-ethylene treatment at 20°C, white bars represent a no-ethylene treatment after a 0.5°C treatment, and black bars represent apples treated with 100 μL L^{-1} ethylene for 2 weeks (W). RT, Room temperature. B, Harvest E2. Apples were either treated at 20°C (gray bars) or stored at 4°C for 4 weeks before being transferred to 20°C (white bars). Firmness was assessed either immediately or following an 8-d treatment either with or without 100 μL L^{-1} ethylene. Error bars represent SE (n = 6).

vests (Fig. 1). In all cases, ethylene treatment of the *ACO1*-suppressed apples induced the greatest change in firmness, irrespective of storage time or temperature. Apples harvested in 2009 (E3; Fig. 1C) were either left at 20°C for a 4-week period or cold stored (0.5°C) for 4 weeks. After this time, the apples that had been cold stored showed no significant difference in firmness compared with the apples stored at room temperature (Fig. 2A, bars d and g). However, cold-treated apples transferred to 20°C for a further 2 weeks softened by a further 7.6 N (36% of softening observed with an ethylene treatment) compared with apples of the same age that had not had a cold treatment (Fig. 2A, bars e and h). This suggests that cold treatment alters the *ACO1*-suppressed apples in such a way that subsequent storage at 20°C is sufficient to cause fruit

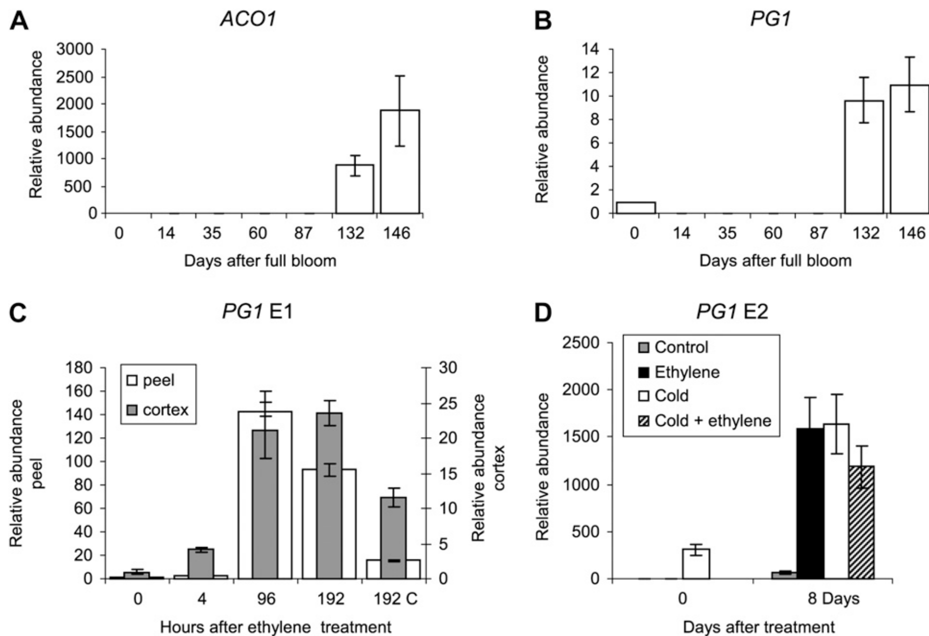

Figure 3. Patterns of gene expression measured by qPCR of either *ACO1* or *PG1*. A and B, *ACO1* expression (A) and *PG1* expression (B) during fruit development of Royal Gala apples, from open flowers (0 DAFB) to eating ripe (146 DAFB). C, Expression of *PG1* in Royal Gala *ACO1*-suppressed mutants from the E1 harvest at 0, 4, 96, and 192 h of 100 μL L^{-1} ethylene treatment or 192 h of no-ethylene treatment. White bars represent expression in peel tissue, and gray bars represent expression in cortex tissue. D, Expression analysis of *PG1* from the E2 harvest. The gray bar represents apples stored at 20°C, the white bars represent apples stored at 4°C for 4 weeks followed by a 20°C no-ethylene treatment, the black bar represents ethylene-treated apples stored at 20°C, and the hatched bar represents apples treated at 4°C followed by a 100 μL L^{-1} ethylene treatment. Error bars represent SE (n = 4).

softening. Apples that had a 2-week ethylene treatment followed by storage at 0.5°C for 4 weeks were of a similar firmness to fruit that had a 2-week ethylene treatment followed by 4 weeks at 20°C (Fig. 2A, bars f and i).

Analysis of the firmness of cold-treated *ACO1*-suppressed apples from the 2007 harvest (E2; Fig. 1B) showed that with a shorter ripening time (8 d instead of 2 weeks), apples prestored at 4°C softened faster with ethylene than ethylene-treated apples that had not been cold stored (Fig. 2B, bars c and f). This suggests, first, that both the E3 ethylene-treated apples and E3 cold- and ethylene-treated apples had reached maximum amounts of softening after 2 weeks, and second, that cold and ethylene have an additive effect on apple fruit softening.

Apple PG1 Is Regulated Late in Fruit Development by Both Ethylene and Cold

Expression patterns of the cell wall gene *PG1* were measured over Royal Gala fruit development (Janssen et al., 2008) using quantitative reverse transcription-PCR (qPCR). There was a small peak of *PG1* expression at full bloom, then there was no detectable expression until late in fruit development, 132 d after full bloom (DAFB), that coincided with the up-regulation of *ACO1* (Fig. 3, A and B). To assess the effect of ethylene and cold on *PG1* expression, *PG1* levels were measured in tissue from *ACO1*-suppressed apple treated as shown for apple harvested in 2005 (E1) and E2 (Fig. 3, C and D). In the E1 harvest, there was a large increase in *PG1* expression in both the peel and cortex of ethylene-treated apple and a smaller increase in the 192-h non-ethylene-treated tissue. This suggests that while ethylene is a dominant activator of PG expression, a 4-week, 4°C treatment followed by storage at 20°C is sufficient to increase the level of *PG1* expression. Interestingly, although the pattern of expression was similar in the two tissues tested, there was considerably higher expression in the peel tissue than the cortex tissue. *PG1* expression in the E2 harvest confirmed that a 4°C cold treatment followed by an 8-d, 20°C treatment in the absence of added ethylene was sufficient to induce or up-regulate transcription of the *PG1* gene. The combination of ethylene and cold did not have an additive effect on *PG1* expression levels at this time point (Fig. 3D).

Characterization of Ethylene- and Cold-Related Transcription Factors in Apple

To identify potential regulators of *PG1*, we examined two classes of transcription factors, the *EIL* genes and the *AP2/ERF* class of genes. The Arabidopsis EIN3 and EIL protein sequences were compared with six frame translations of nonredundant (NR) contiguous sequence *Malus* ESTs (Newcomb et al., 2006; Wisniewski et al., 2008). Three NR sequences showed high homology to the EIN3 protein sequence from Arabidopsis, and the clones from the most 5′ EST of each of these were sequenced and translated into the longest open reading frame and compared with the Arabidopsis protein sequences using cluster analysis. These apple EILs showed higher similarity to *AtEIN3/AtEIL1* than the other *AtEILs*, suggesting a gene duplication in apple (Fig. 4A). These were labeled *EIL1* to *EIL3*. A similar method was used to select a second class of transcription factors containing an *AP2/ERF* domain. This class of transcription factor was chosen because it includes family members involved in both ethylene signal transduction and cold response (Kim et al., 2006; Nakano et al., 2006). Sixty independent apple *AP2/ERF* genes (named APETELA2 DOMAIN [AP2D] hereafter) were

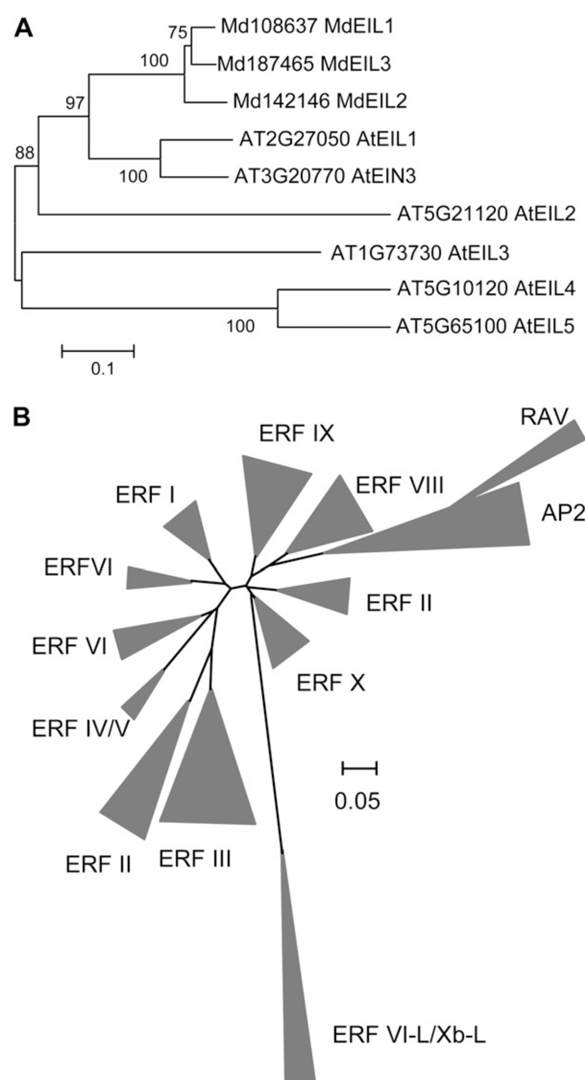

Figure 4. Phylogenetic clustering. A, Phylogenetic relationship among the six Arabidopsis EIL proteins and three apple EIL proteins. B, Phylogenetic relationship among the 147 Arabidopsis AP2 domain-containing proteins and 60 apple AP2 domain-containing proteins. The previously published Arabidopsis cluster groups are shown. For the full cluster and the number of apple and Arabidopsis genes per group, see Supplemental Figure S1 and Supplemental Table S1.

Table I. *Selected genes containing an AP2 binding domain*

Apple Gene	Subgroup	GenBank Accession No.	Gene Expression Measured	Assayed Transiently
AP2D22	Ib	GU732446	Yes	Yes
AP2D28	Ib	GU732452	Yes	Yes
AP2D34	Ib	GU732458		
AP2D51	Ib	GU732475		
AP2D42	IIa	GU732466		
AP2D45	IIa	GU732469		
AP2D6	IIb	GU732430	Yes	Yes
AP2D56	IIb	GU732480		
AP2D23	IIIa	GU732447	Yes	Yes
AP2D33	IIIa	GU732457	Yes	Yes
AP2D16	IIIb	GU732440	Yes	Yes
AP2D54	IIIb	GU732478		
AP2D7/CBF2	IIIc	GU732431	Yes	Yes
AP2D30	IIIc	GU732454	Yes	Yes
CBF1	IIIc	DQ074478		Yes
AP2D9	IIIe	GU732433	Yes	Yes
AP2D39	IIIe	GU732463	Yes	Yes
AP2D44	IIIe	GU732468		
AP2D48	IIIe	GU732472		
AP2D49	IIIe	GU732473		
AP2D38	IVa	GU732462	Yes	
AP2D21	Va	GU732445	Yes	Yes
AP2D35	Va	GU732459	Yes	Yes
AP2D13	VI	GU732437	Yes	Yes
AP2D20	VI	GU732444	Yes	Yes
AP2D1	VIIa	BAF43419	Yes	Yes
AP2D11	VIIa	GU732435	Yes	Yes
AP2D15	VIIa	GU732439	Yes	
AP2D24	VIIa	GU732448	Yes	
AP2D50	VIIa	GU732474		
AP2D55	VIIa	GU732479		
AP2D5	VIIIa	GU732429	Yes	Yes
AP2D10	VIIIa	GU732434	Yes	Yes
AP2D18	VIIIa	GU732442	Yes	Yes
AP2D25	VIIIa	GU732449	Yes	Yes
AP2D37	VIIIa	GU732461	Yes	Yes
AP2D43	VIIIa	GU732467		
AP2D52	VIIIa	GU732476		
AP2D57	VIIIa	GU732481		
AP2D2	IXa	ACT79399	Yes	Yes
AP2D27	IXa	GU732451	Yes	Yes
AP2D41	IXa	GU732465		
AP2D4	IXb	GU732428	Yes	Yes
AP2D8	IXb	GU732432	Yes	Yes
AP2D19	IXb	GU732443	Yes	Yes
AP2D47	IXb	GU732471		
AP2D60	IXb	GU732483		
AP2D29	IXc	GU732453	Yes	Yes
AP2D31	IXc	GU732455	Yes	Yes
AP2D26	Xa	GU732450	Yes	Yes
AP2D32	Xa	GU732456	Yes	Yes
AP2D46	Xa	GU732470		
AP2D17	Xb	GU732441	Yes	Yes
AP2D58	Xb	GU732482		
AP2D3	VI-L	GU732427	Yes	Yes
AP2D14	VI-L	GU732438	Yes	Yes
AP2D53	VI-L	GU732477		
AP2D36	RAV	GU732460	Yes	Yes
AP2D12	AP2	GU732436	Yes	Yes
AP2D40	AP2	GU732464		

identified, and the DNA-binding domains from each of these AP2D genes were aligned with the DNA-binding domains from the 147 Arabidopsis *AP2/ERF*s (Supplemental Table S1). The 12 Arabidopsis subgroups described by Nakano et al. (2006) were clearly represented, except subgroup VI, which was separated into two distinct clades (Fig. 4B; Supplemental Fig. S1). Each full-length apple gene was assigned a name and a subgroup based on the clade it was separated into (Table I).

EIL and AP2D Expression Analysis

To assess whether these transcription factors were expressed at the same time as *PG1*, PCR primers that were able to discriminate the three *EIL* genes from each other were designed and their expression patterns determined during apple fruit development series. *EIL1* and *EIL3* showed no change in expression during fruit development (Fig. 5A). *EIL2*, however, showed a greater than 10-fold induction of expression over fruit development (Fig. 5A).

The expression patterns of 38 apple AP2D transcription factors were assessed during fruit development and in response to ethylene treatment. Two time points from fruit development were selected: (1) 35 DAFB, when *PG1* expression was very low; and (2) 132 DAFB, when *PG1* was first detected (Fig. 2B). Seventeen were predominantly expressed at 35 DAFB compared with 132 DAFB (Fig. 5B), and two were predominantly expressed at 132 DAFB. The remaining genes were either undetectable or showed less than a 2-fold change in expression between these time points. Three or four time points (depending on the gene) were selected to screen for expression changes in the E1 harvest: 0 h, 4 h, 192 h, and 192 h no-ethylene control. These time points were selected as they represent low *PG1* (0 h) and high *PG1* (192 h, ethylene treatment); the 4-h time point was chosen to identify genes that rapidly respond to ethylene treatment (Fig. 6).

For the ethylene-induced experiment (E1), the largest increase in gene expression at 4 h was in subgroup IX, which has been previously associated with increasing expression in response to ethylene such as *AtERF1* (*AP2D29* and *AP2D19*; Fig. 6A). After 192 h of ethylene treatment, genes from many of the subgroups had altered expression profiles (Fig. 6, B and C). Most of the changes observed were similar in both the ethylene-treated and the ethylene-untreated tissue, suggesting a predominantly ethylene-independent effect.

Transient Assays

To test if these transcription factors were involved in the regulation of *PG1*, a transient assay (Hellens et al.,

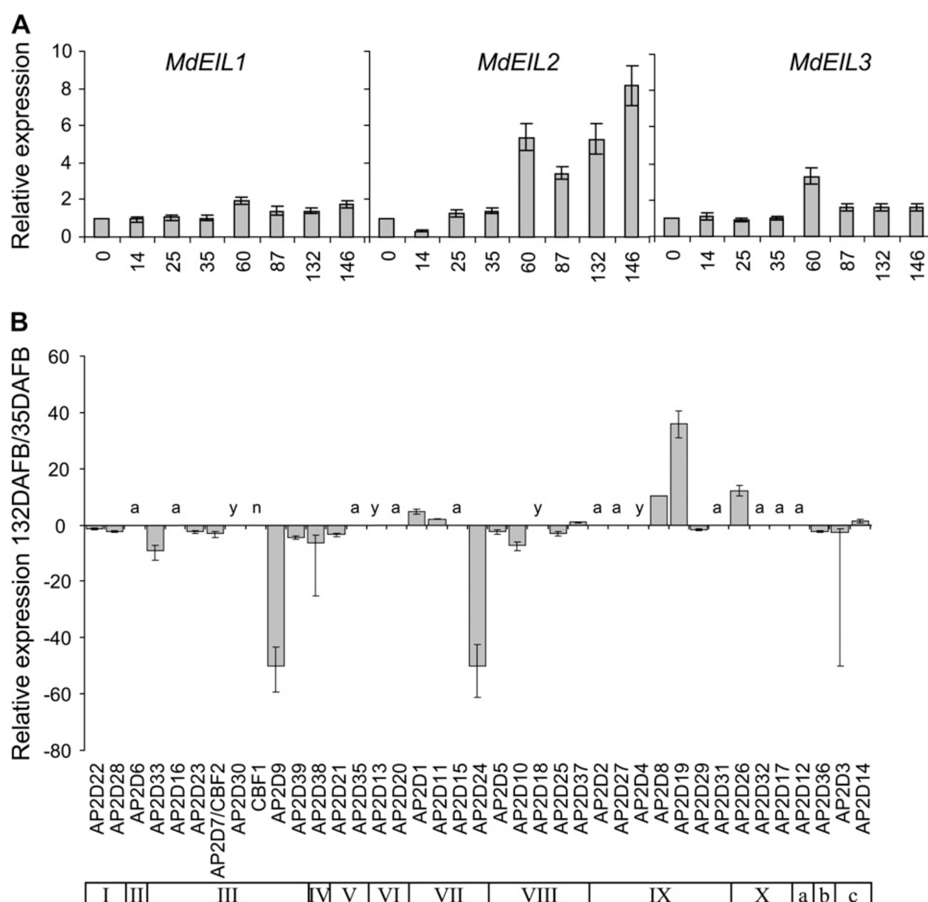

Figure 5. Expression analysis of *EILs* and *AP2D* genes over fruit development in Royal Gala apples, from open flowers (0 DAFB) to eating ripe (146 DAFB). A, *EIL1*, *EIL2*, and *EIL3*. B, Relative expression patterns of 38 apple AP2 domain genes comparing 35 with 132 DAFB. A high ratio represents high expression late in fruit development. The letter a represents undetectable qPCR expression, y represents genes for which the error was too great to provide meaningful information, and n represents genes that were not assayed for this time point. The bar underneath represents the cluster groups for each of the AP2D genes; on this bar, a represents AP2 class genes, b represents RAV class genes, and c represents the VI-L class genes. Error bars represent SE ($n = 4$).

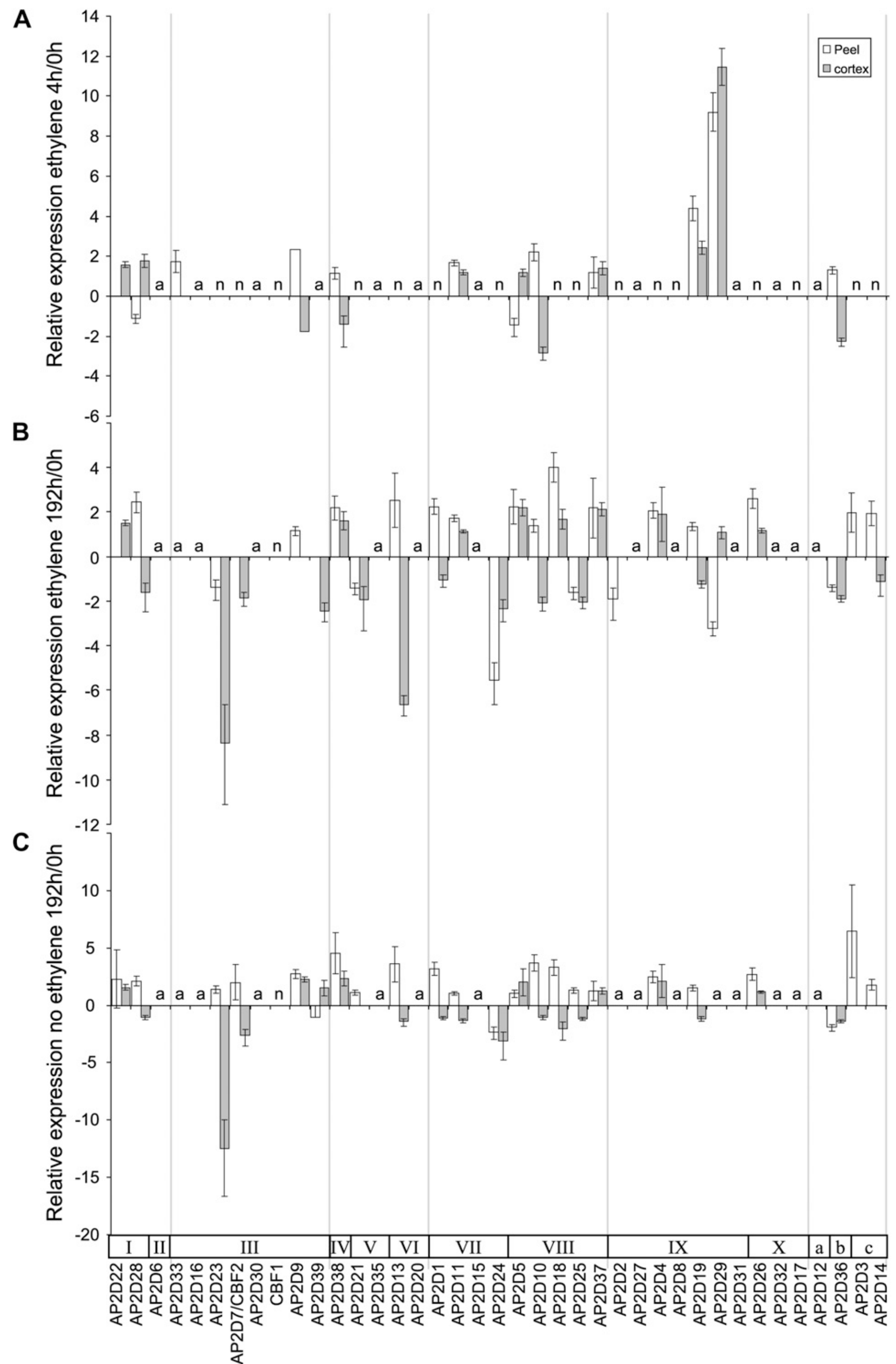

Figure 6. Relative expression analysis of apple AP2 genes in the E1 harvest of *ACO1*-suppressed apples that have been induced with ethylene. Values represent expression relative to the 0-h sample. A, Expression after a 4-h, 100 μL L^{-1} ethylene treatment. B, Expression after a 192-h, 100 μL L^{-1} ethylene treatment. C, Expression after 192 h in an ethylene-free environment. The genes are arranged in order of cluster groupings. Other features are as described in Figure 5.

2005) was used to measure the levels of transactivation each transcription factor had on the 2.8-kb *PG1* promoter (Atkinson et al., 1998). A fragment of the *PG1* promoter, including the ATG start codon and extending 2.8 kb upstream, was amplified from genomic DNA from cv Granny Smith apples, sequenced, and cloned as an ATG fusion into the pLUC 0800 transient assay cassette (Hellens et al., 2005) and named PG1-Luc. The sequence information showed a very high level of similarity with only six polymorphisms in the 2.8-kb fragment between the Granny Smith and previously published Royal Gala *PG1* promoter sequences (Supplemental Fig. S2). To ascertain the levels of autoactivation of this promoter, PG1-Luc was infiltrated into the leaves of tobacco (*Nicotiana benthamiana*) and exposed to either 100 μL L^{-1} ethylene for 24 h or cold (4°C) for 3 d. Both these treatments showed only a weak transactivation of the luciferase

reporter gene (Fig. 7A). Tobacco plants infiltrated with PG1-Luc and subjected to cold for 24 h followed by 2 d at 21°C also showed no significant activation (Fig. 7A).

Two EIL and 36 AP2D transcription factors were cloned, as a fusion to the cauliflower mosaic virus 35S promoter, into a binary vector (Hellens et al., 2005) and coinfiltrated into tobacco with PG1-Luc. EIL3 showed a small but significant transactivation of the *PG1* promoter (Fig. 7B). As Arabidopsis *EIL* proteins are stabilized by ethylene, the transient assay was repeated, but 3 d after agroinfiltration the plants were treated with 100 μL L^{-1} ethylene. Under these conditions, EIL2 strongly transactivated the *PG1* promoter (Fig. 7B). The highest activator of expression was a CBF-like transcription factor (*AP2/ERF* group III), AP2D7. Neither the closely related CBF1 gene, nor any of the group IX transcription factors, transactivated the *PG1* promoter (Fig. 7C).

Cold Regulation of AP2D7/CBF2 in Cell Culture

The *AP2D7* gene was not up-regulated late in fruit development or by ethylene (Figs. 5 and 6). As AP2D7 was grouped with the Arabidopsis CBF-like proteins, it suggested that this gene might be regulated by cold. To establish whether *AP2D7* is transcriptionally regulated by cold, apple cell cultures (Wang et al., 2001) were treated with cold (1°C) or cold followed by a 25°C treatment. Expression analysis revealed that AP2D7 was strongly regulated by cold (Fig. 8). Upon the transfer from cold to 25°C, the transcripts rapidly dropped to background levels within 1 h of transfer (Fig. 8A). *PG1* did not increase in expression over this time period, suggesting that other control mechanisms are regulating *PG1* (Fig. 8B). Because of the phylogenic proximity to the *CBF* genes and the rapid increase in expression with cold, we have subsequently named *AP2D7* as *CBF2*.

There Is a Synergistic Effect of CBF2 and Ethylene

To establish whether EIL2 and CBF2 were acting in the same pathway, transient assays were used to measure the transactivation effect of a combination of the two transcription factors with PG1-Luc. These were performed with and without exogenous ethylene added. It was found that the combination of ethylene and/or EIL2 with CBF2 showed a synergistic transactivation of the PG promoter, suggesting that the CBF2 and the ethylene responses are acting on different parts of the promoter (Fig. 9).

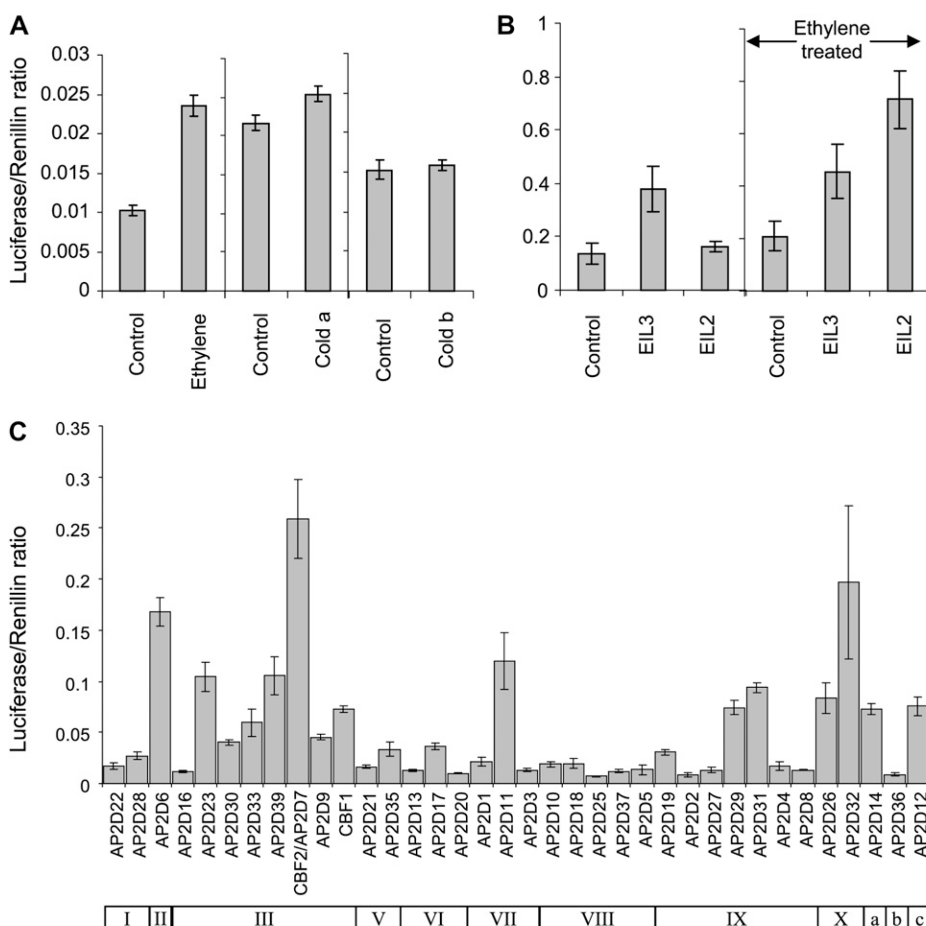

Figure 7. Tobacco transient assay of plants infiltrated with PG1-Luc. Transactivation of the promoter is measured as a ratio of the luciferase signal to the renillin signal. A, PG1-Luc-infiltrated plants with or without 100 μL L^{-1} ethylene for 24 h prior to assay. PG1-Luc-infiltrated plants were kept at either 20°C or 4°C for 24 h prior to assay (cold a) or for 4°C for 24 h followed by 20°C for 2 d (cold b). B, Coinfiltration of PG1-Luc with EIL1 or EIL2 with or without a 24-h, 100 μL L^{-1} ethylene treatment. C, Coinfiltration of PG1-Luc with 37 different apple *AP2* domain-containing genes. The bar underneath represents the cluster groups as defined in Figure 5. Error bars represent SE ($n = 4$).

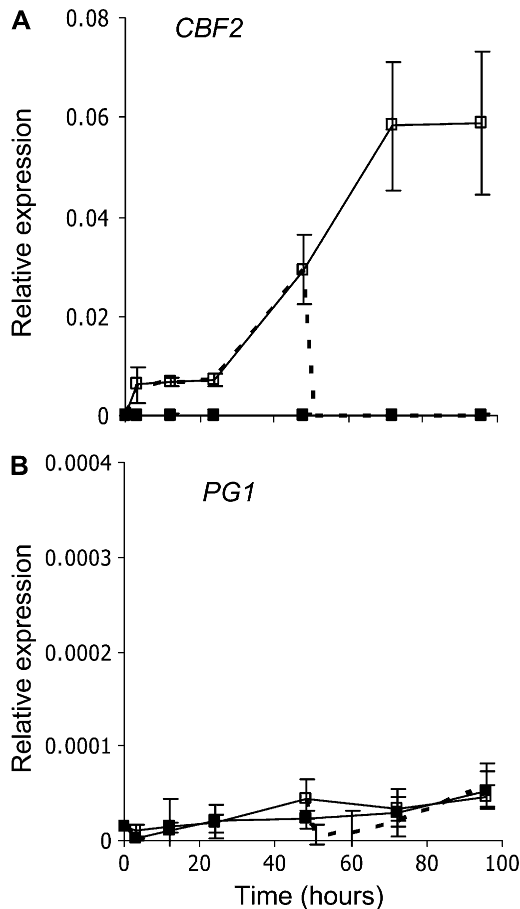

Figure 8. qPCR measuring gene expression in apple culture cells grown at 20°C (solid squares), 1°C (open squares), or 1°C for 2 d followed by 20°C (dashed lines). A, *CBF*-like gene (*AP2D7- CBF2*). B, *PG1*. Error bars represent SE ($n = 4$).

DISCUSSION

Previous attempts at quantifying the importance of cold in apple fruit softening have been complicated by the presence of increasing endogenous ethylene production during ripening, making it difficult to determine if ripening responses were due to ethylene, cold, or a combination of these factors. This study circumvented this problem by using *ACO1*-suppressed transgenic apples that produce no detectable ethylene-related ripening (Johnston et al., 2009). This system allowed the controlled addition of cold to an apple system devoid of ethylene-related ripening. The results from this study demonstrate the role of cold in modulating fruit softening. However, while cold is a contributing factor to fruit softening, it produces significantly less softening than ethylene.

From this research, there are three models that could explain the action of cold in relation to fruit ripening: model 1 would hypothesize that cold is acting independently of ethylene; model 2 that cold is enhancing the ethylene response to such an extent that the fruit is responding to much lower levels of ethylene that may still be present in the *ACO1*-suppressed apples; and model 3 that cold increases the concentrations of ethylene (El-Sharkawy et al., 2004). Using transient assays, we have shown that the promoter of *PG1* transactivates the promoter of both genes homologous to ethylene signal transduction transcription factors (EIN3) and a cold-regulating transcription factor that clusters in the CBF family (Stockinger et al., 1997). Because of the nature of the transient assays, it is possible that overexpressing a key regulatory transcription factor activates an endogenous signaling pathway in the host plant. Thus, transactivation does not show direct binding but can point to the role of upstream transcription factors that are involved in the regulation of a gene. For example, there is a possibility that CBF2 is acting through the up-regulation of an endogenous tobacco *EIL* that then transactivates *PG1*. However, when CBF2 is coinfiltrated with the *PG1* promoter followed by an ethylene treatment, there is an enhanced transactivation (Fig. 9), suggesting that EIL and CBF2 are acting independently of each other, consistent with model 1. However, it is possible that the other two models are contributing to fruit softening. With recent technical advances, it is now possible to detect ethylene below 1 nL L^{-1}. These studies suggest that very low levels of ethylene may play an important role in plant development (Thain et al., 2004). Using a less sensitive detection system, only background levels of ethylene have been detected in the *ACO1*-suppressed apples. While the independent regulation of softening model best fits the molecular data, there is the possibility that the apples become more sensitive to very low levels of ethylene during a cold treatment and the softening observed is due to the response of fruit to a basal level of ethylene production, or that there is a slight increase in ethylene production through an ACO that is independent of *ACO1*. However, it has been shown that cold activation of ethylene in cv Braeburn apples is mediated at least in part through an increase in both *ACS* and *ACO1* expression (Tian et al., 2002), making it likely

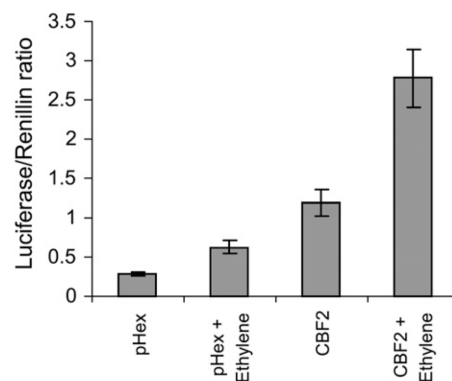

Figure 9. Tobacco transient assays of PG1-Luc coinfiltrated with a combination of empty vector control (pHex) and CBF2 with and without a 24-h, 100 μL L^{-1} ethylene treatment. Error bars represent SE ($n = 4$).

that these two genes are contributing to the cold-induced ethylene effect seen in apples and therefore are not contributing to the softening responses observed in this study.

In Arabidopsis, *CBF2* expression is strongly cold regulated, with an increase in transcript detectable within 1 h of cold treatment and loss of transcript upon removal from cold treatment. In apple tissue culture cells, the up-regulation of *CBF2* does not switch on *PG1*, suggesting that there may be other factors such as a developmentally regulated inhibitor of *PG1* expression that also influences CBF2 action. This is consistent with the observation of an inhibition element in the promoter between −1,460 and −2,356 (Atkinson et al., 1998). While the data presented here show that expression of *CBF2* is not sufficient for *PG1* expression, in a transient system CBF2 can transactivate the *PG1* promoter, and this transactivation is enhanced by ethylene.

During late fruit development, as the apple matures there is an increase in sensitivity to ethylene. Sensitivities to ethylene may be brought about by reducing the numbers of ethylene receptors (Chen et al., 2005) that negatively regulate the ethylene response. However, overexpression of *EIN3* gives an enhanced triple response in Arabidopsis (Chao et al., 1997), suggesting that ethylene sensitivity itself is modulated through levels of *EIN3* expression. Here, we find that, in apple, *EIL2* increases in expression through fruit development, suggesting that this may contribute to ethylene sensitivity during ripening. This is unlike the *EIL* genes in tomato, Arabidopsis, and kiwifruit (*Actinidia deliciosa*), where *EIN3* transcripts have a constant expression level (Chao et al., 1997; Tieman et al., 2001; Guo and Ecker, 2003). However, this is not unique; in banana (*Musa acuminata*), an *EIL* gene shows an increase in transcript accumulation during fruit development (Mbeguie-A-Mbeguie et al., 2008).

The EIL transactivation of the PG1 promoter suggests either a direct binding or binding through an ERF-independent pathway. When the PG1 promoter sequence is analyzed using the motif finder in PLACE (Higo et al., 1999), there are two putative PERE binding sites 538 and 903 bp from the ATG start; these are the minimal motifs thought to be necessary for regulation by *EIL* genes (Kosugi and Ohashi, 2000) rather than the longer inverted palindrome reported by Solano et al. (1998). If the transactivation by the *EILs* is acting through an endogenous tobacco intermediate, then it is unlikely to be a member of the group IX *ERF-like* genes. In our assay, none of the apple genes in this cluster transactivated the PG promoter, even though some of these genes were up-regulated by ethylene (Fig. 6).

During the late summer ripening period of apple, it is conceivable that apple relies on both internal and environmental cues, such as temperature, to time its developmental processes. While apple softening is highly dependent on ethylene, here we have shown that a cold effect can also modulate fruit softening in cv Royal Gala. By applying a molecular approach to identify how temperature may be modulating fruit softening, we have found that both ethylene- and cold-related transcription factors were able to transactivate the cell wall gene *PG1*. These results support a model where both cold and ethylene signals appear to coordinate the ripening process. The combination of these signals enhances ripening. Using both these signals would allow plants to shorten ripening time in colder autumns, allowing fully edible fruit to be produced before freezing temperatures arrive.

MATERIALS AND METHODS

Fruit Growth and Ripening Treatments

Transgenic apple (*Malus* × *domestica*) cv Royal Gala containing an antisense *ACO1* construct (A03 lines; Schaffer et al., 2007) was grown in greenhouse conditions. For the fruit development analysis, tissue from orchard-grown Royal Gala apple was used as described by Janssen et al. (2008). Following harvest, apples were stored in 340-L bins containing Purafil (Multimix MM-1000; Circul-Aire) to absorb ethylene and lime to absorb CO_2. The ambient ethylene concentrations were regularly tested by gas chromatography as described by Johnston et al. (2009). For ethylene treatment, 100 μL L^{-1} ethylene was injected into the fruit-ripening bins containing lime to absorb CO_2, and air was continuously circulated. Ethylene concentrations were tested and adjusted as necessary. Fruit firmness was measured as described by Johnston et al. (2009).

Harvesting Regimes

ACO1-suppressed transgenic Royal Gala apples (Schaffer et al., 2007) from 3 years were assessed: 2005 harvest (E1), 2007 harvest (E2), and 2009 harvest (E3; Fig. 1). The E1 apples were harvested and stored in 4°C for 1 month before they were warmed to 20°C for 24 h, divided into five groups of six apples each, and all but one group (used as a no-ethylene, 8-d control) were treated with ethylene. These apples had RNA extracted 4 h, 4 d, and 8 d following treatment; for full details, see Schaffer et al. (2007). The E2 apples (Fig. 1B) were randomly divided into six batches of six apples labeled a to f; a was assessed for firmness at harvest, b was stored in an ethylene-free environment for 8 d before being measured for firmness, and c was treated with 100 μL L^{-1} ethylene for 8 d before being measured for firmness. Batches d to f were stored at 4°C for 4 weeks and then transferred to 20°C for 1 d; d was measured for firmness; e and f were either not treated or treated with 100 μL L^{-1} ethylene, respectively, and then assessed for firmness after 8 d of treatment. The E3 harvest (Fig. 1C) apples were randomly separated into nine batches of eight apples labeled a to i. Batch a was assessed for firmness at harvest, and b, d, and e were kept at 20°C in an ethylene-free environment containing Purafil (Multimix MM-1000; Circul-Aire); b was assessed after 2 weeks of storage, d after 4 weeks of storage, and e after 6 weeks. Batch c was immediately treated with 100 μL L^{-1} ethylene at 20°C for 2 weeks and assessed for firmness, and f was treated with 100 μL L^{-1} ethylene for 2 weeks after a 4-week, 20°C storage. Batches g, h, and i were stored at 0.5°C in an ethylene-free environment for 4 weeks, and g was assessed for firmness in the cold; h and i were transferred to 20°C, h was left at 20°C and i was treated with 100 μL L^{-1} ethylene, and both were assessed following a 2-week treatment period.

AP2/ERF Gene Selection

Apple *AP2/ERF* family members were identified by comparing representatives of the 12 Arabidopsis (*Arabidopsis thaliana*) *AP2/ERF* DNA-binding domain subgroups (Nakano et al., 2006) with apple NR sequences as well as the *AP2*-like genes and *RAV*-like genes (Feng et al., 2005; Kim et al., 2006; Nakano et al., 2006). In total, 127 apple NR sequences representing 1,370 ESTs were identified using BLASTX with a *P* value of less than E-05. Eighty-seven of these were represented by at least one EST (Newcomb et al., 2006). The most 5′ EST from this collection was identified, and the representative clone was fully sequenced. One clone was not recoverable, 10 were collapsed into

existing NR sequences, and 12 were deemed not to be full length. Sixty-two full-length sequences were found to code for a Met (ATG) that was at a similar location to the start point of the closest Arabidopsis homolog. Two genes showed DNA sequence identity to other sequenced cDNAs at greater than 97% and were assumed to be allelic. The remaining full-length sequences were labeled *AP2D1* to *AP2D60*. *AP2D59* was shown to be homologous to the already submitted gene *CBF1* and was changed to *CBF1*. *AP2D1* and -2 were named after the previously published *MdERF1* and -2, respectively (Wang et al., 2007).

qPCR

For expression analysis, both new and previously published cDNAs were used. cDNAs from a fruit development series (Janssen et al., 2008) and the E1 harvest regime (Schaffer et al., 2007) were used. For new cDNA, RNA was extracted and cDNA was synthesized as described by Schaffer et al. (2007). qPCR was conducted on a LightCycler 480 (Roche) using the LightCycler 480 SYBR Green 1 Master kit (Roche). Each time point was replicated four times, and a 10-μL reaction comprising 5 μL of Mastermix, 2 μL of 5 μM primers (forward and reverse), and 3 μL of cDNA was used. All expression was normalized to an apple actin gene described by Espley et al. (2007). qPCR primers used are shown in Supplemental Table S2.

Promoter Isolation

Genomic DNA was isolated from cv Granny Smith apple using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Primers PRM4 (5′-TGGTGTCCGTGTATGAAGGATAAGCCCTAG-3′) and RJS019 (5′-AAACTATTTGGACCATTCCGAGCAAGTCTATC-3′) were used to amplify the 2.8-kb PG1 promoter region from this genomic DNA. A 2.6-kb fragment, modified from the ATG start codon to incorporate a *Nco*I restriction site, was then amplified from this 2.8-kb fragment using RJS019 and the *Nco*I-modifying primer RJS018 (5′-CAACTGTGTTTTTAAAGCCATGGATGC-TTTC-3′). PCR was performed using Platinum Taq (Invitrogen) according to the manufacturer's protocol with an annealing temperature of 55°C for 30 cycles. This was cloned first into pGEM T-Easy (Promega), then cut with *Not*I/*Nco*I and cloned into a pGreen 0800-LUC (Hellens et al., 2005), with the modified *Nco*I site producing the ATG start codon for the luciferase reporter gene. All constructs were confirmed by sequencing.

Construction of T-DNAs Overexpressing Apple Transcription Factors

cDNAs from expressed sequenced apple libraries (Newcomb et al., 2006) were cloned either using restriction enzymes into pSAK778 or using Gateway cloning (Invitrogen) into pHEX (Hellens et al., 2005).

Transient Assays

The PG1-Luc plasmid was inserted into *Agrobacterium tumefaciens* GV101 (MP) and tested for transactivation as described by Hellens et al. (2005). For ethylene induction, plants were injected with *Agrobacterium* containing PG1-Luc, left for 2 d, and then transferred to a 340-L container with a circulating fan and a final concentration of 100 μL L^{-1} ethylene for 24 h. Ethylene concentration was checked 1 and 20 h after application using a gas chromatograph to confirm that levels remained consistent. Leaf discs were analyzed for renillin and luciferase activity. For cold induction, instead of a container with ethylene, plants were transferred to a 4°C cold room for 24 h. In the second cold induction experiment, infiltrated plants were transferred into a cold room immediately after infiltration and then warmed to 20°C for 2 d before being assayed. With promoter transcription factor transactivation, one part PG1-Luc was combined with nine parts pHEX transcription factor, coinfiltrated in tobacco (*Nicotiana benthamiana*) leaves, and assayed as described by Hellens et al. (2005).

Phylogenetic Lineups

ESTs that contained an AP2 domain were selected by homology to the Arabidopsis AP2 domain-containing genes. Clones that represented the most 5′ EST (Newcomb et al., 2006) were isolated and fully sequenced. Sixty unique full-length AP2 domain-containing genes have been deposited in GenBank (accession nos. GU732427 to GU732483). The protein sequences of the AP2 domain from the 145 Arabidopsis genes were aligned with the AP2 domain from the 60 apple genes using ClustalW (using an opening penalty of 15 and an extension penalty of 0.3) using the AlignX software in Vector NTI 9.0. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar et al., 2004) using a minimum evolution phylogeny test and 1,000 bootstrap replicates. The *EIL* genes (accession nos. GU732484 to GU732486) were compared similarly.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers ACT79399, BAF43419, DQ074478, and GU732427 to GU732486.

Supplemental Data

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

Supplemental Figure S1. Cluster of *AP2D* genes.

Supplemental Figure S2. Promoter region of the *PG1* gene.

Supplemental Table S1. Class of AP2D proteins.

Supplemental Table S2. qPCR primers.

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