The Role of Ethylene and Cold Temperature in the Regulation of the Apple POLYGALACTURONASE1 Gene and Fruit Softening*[^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-INSSENSITIVE3-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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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 (AO3 lines described by Schaffer et al., 2007) were treated with combinations of cold and ethylene treatments over three independent har-
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
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) contig 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.](image-url)
| Apple Gene  | Subgroup | GenBank Accession No. | Gene Expression Measured | Assayed Transiently |
|------------|----------|-----------------------|--------------------------|---------------------|
| AP2D22     | lb       | GU732446              | Yes                      | Yes                 |
| AP2D28     | lb       | GU732452              | Yes                      | Yes                 |
| AP2D34     | lb       | GU732458              |                         |                     |
| AP2D51     | lb       | 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              |                         |                     |
| AP2D9      | IIle     | GU732433              | Yes                      | Yes                 |
| AP2D39     | IIle     | GU732463              | Yes                      | Yes                 |
| AP2D44     | IIle     | GU732468              |                         |                     |
| AP2D48     | IIle     | GU732472              |                         |                     |
| AP2D49     | IIle     | GU732473              |                         |                     |
| AP2D38     | IVa      | GU732462              |                         |                     |
| 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              |                         |                     |
| AP2D24     | VIIa     | GU732448              |                         |                     |
| AP2D50     | VIIa     | GU732474              |                         |                     |
| AP2D55     | VIIa     | GU732479              |                         |                     |
| AP2D5      | VIIa     | GU732429              | Yes                      | Yes                 |
| AP2D10     | VIIa     | GU732434              | Yes                      | Yes                 |
| AP2D18     | VIIa     | GU732442              | Yes                      | Yes                 |
| AP2D25     | VIIa     | GU732449              | Yes                      | Yes                 |
| AP2D37     | VIIa     | GU732461              | Yes                      | Yes                 |
| AP2D43     | VIIa     | GU732467              |                         |                     |
| AP2D52     | VIIa     | GU732476              |                         |                     |
| AP2D57     | VIIa     | 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/ERFs (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).
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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 (Hollens 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).
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

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**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).

**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 (Mbegue-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 palindromic 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 (A103 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; Circul-Aire) to absorb ethylene and lime to absorb CO2. 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 CO2, 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). It 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...
Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers ACT79099, 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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Phylogenetic Lineups
ESTs that contained an AP2 domain were selected based on 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.
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