Ethylene Regulates Apple (*Malus × domestica*) Fruit Softening Through a Dose × Time-Dependent Mechanism and Through Differential Sensitivities and Dependencies of Cell Wall-Modifying Genes

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In fleshy fruit species that have a strong requirement for ethylene to ripen, ethylene is synthesized autocatalytically, producing increasing concentrations as the fruits ripen. Apple fruit with the ACC OXIDASE 1 (ACO1) gene suppressed cannot produce ethylene autocatalytically at ripening. Using these apple lines, an ethylene sensitivity dependency model was previously proposed, with traits such as softening showing a high dependency for ethylene as well as low sensitivity. In this study, it is shown that the molecular control of fruit softening is a complex process, with different cell wall-related genes being independently regulated and exhibiting differential sensitivities to and dependencies on ethylene at the transcriptional level. This regulation is controlled through a dose × time mechanism, which results in a temporal transcriptional response that would allow for progressive cell wall disassembly and thus softening. This research builds on the sensitivity dependency model and shows that ethylene-dependent traits can progress over time to the same degree with lower levels of ethylene. This suggests that a developmental clock measuring cumulative ethylene controls the fruit ripening process.

**Keywords:** Cell wall • Cumulative ethylene • *Malus* • Ripening • Softening.

**Abbreviations:** ACO1, ACC OXIDASE1; AFase, ARABINOFURANOSIDASE; BGAL, β-GALACTOSIDASE; Chr, chromosome; EXP, EXPANSIN; 1-MCP, 1-methylcyclopropene; N, Newton(s); PG, POLYGALACTURONASE; QTL, quantitative trait locus; qRT-PCR, quantitative real-time PCR.

**Introduction**

Fleshy fruits undergo considerable physiological changes during ripening that ultimately lead to the production of an edible organ attractive to seed-dispersing animals. This change is mediated through changes in phytohormones such as ethylene, ABA and auxin, with different fruits showing different responses to each (McAtee et al. 2013). Fruits that have a strong requirement for ethylene to ripen (climacteric fruit) include *Solanum lycopersicum* (tomato), *Malus × domestica* (apple), *Actinidia chinensis* (kiwifruit), *Cucumis melo* (melon) and *Prunus persica* (peach). When ethylene is suppressed in these fruits, either through the use of chemical inhibitors such as 1-methylcyclopropene (1-MCP) (Watkins 2006) or through transgenic approaches (Oeller et al. 1991, Guis et al. 1997, Dandekar et al. 2004, Schaffer et al. 2007, Pech et al. 2008, Atkinson et al. 2011), a considerable reduction in ripening is observed.

In all fleshy fruits, a key ripening event is the softening of flesh tissue. The degree of flesh softening depends on the species, with extensive softening observed in species such as tomato (Giovannoni 2004), melon (Pech et al. 2008) kiwifruit (Richardson et al. 2011) and peach (Brummell et al. 2004), and only moderate softening in species such as apple (Johnston et al. 2001). In these climacteric fruits, softening is correlated with an increase in ethylene, and in transgenic fruit suppressed for the ethylene biosynthesis gene, ACC OXIDASE1 (ACO1), the addition of exogenous ethylene can re-initiate softening (Pech et al. 2008, Johnston et al. 2009, Tacken et al. 2010, Atkinson et al. 2011). These experiments demonstrate the central role of ethylene in fruit softening. However, a small consistent amount of softening has been observed to occur independently of ethylene in both melon and apple (Guis et al. 1997, Pech et al. 2008, Johnston et al. 2009). Application of a range of ethylene concentrations to apple and melon has shown that individual ripening traits, such as color change, volatiles and softening, have different sensitivities to and dependencies on ethylene (Pech et al. 2008, Johnston et al. 2009). Apple fruit softening was observed to have low sensitivity to ethylene, but was highly dependent on ethylene to progress (Johnston et al. 2009; **Supplementary Fig. S1**). It was also observed that treatment with 0.1 and 1 μl L⁻¹ for 14 d led to incomplete softening, and it...
is not known whether this was due to slower softening or because a certain ethylene threshold had not been reached.

At the cellular level, fruit softening is associated with swelling of the cell wall and depolymerization of polysaccharides; however, in apples, cell wall swelling is minimal, consistent with maintenance of a crisp texture (Redgwell et al. 1997). In part, softening is due to the activity of a suite of cell wall-modifying proteins which solubilize (loosen) and depolymerize (break down) the carbohydrate matrix of the cell wall (Brummell 2006), including cell wall-loosening expansins (Cosgrove et al. 2002) and many families of cell wall hydrolases (Brummell and Harpster 2001). There is considerable literature demonstrating ethylene regulation of these key cell wall-modifying genes in many climacteric fruits, and these genes are differentially regulated as the fruit ripens (Bennett and Labavitch 2008). Differential cell wall-related gene expression changes have been observed by manipulating ethylene levels through 1-MCP or exogenous ethylene treatment, as well as indirect treatments such as heat or controlled atmosphere (Girardi et al. 2005, Bustamante et al. 2012). In addition, the use of natural mutants in tomato and transgenics has further linked ethylene with the regulation of cell wall-associated genes (Smith and Gross 2000, Brummell and Harpster 2001). There is considerable literature demonstrating ethylene regulation of these key cell wall-modifying genes in many climacteric fruits, and these genes are differentially regulated as the fruit ripens (Bennett and Labavitch 2008).

In apple, cell wall-modifying genes shown to increase in expression during softening include EXPANSIN1 (EXPA1), EXPA2 and EXPA3 (Wakasa et al. 2003, Goulao et al. 2008, D. Chagné et al. unpublished data) and the cell wall hydrolases β-GALACTOSIDASE-1 (BGAL1) (Ross et al. 1994), POLYGLUCOSYLASE/HYDROLASE-1 and -2 (XTH1/2) (Goulao et al. 2008), and ARABINOFURANOSIDASE-1 and -3 (AFase1/3) (Goulao et al. 2008, Nobile et al. 2011). Differential regulation of these genes between cultivars highlights a possible mechanism for the range of cultivar-specific textures and softening rates observed in different apple fruits (Wakasa et al. 2006). One of the key players in apple softening is PG1 as it has been mapped to a dominant quantitative trait locus (QTL) for softening (Costa et al. 2010), and suppression of the gene results in apples that are firmer than controls (Atkinson et al. 2012). Significantly, PG1-suppressed apples still undergo a significant degree of softening, which demonstrates the importance of other cell wall-modifying genes in this process and is consistent with studies in tomato that have shown how multiple cell wall genes need to be suppressed in order to affect fruit firmness (Powell et al. 2003).

In apples, many of these cell wall-modifying genes have been shown to be regulated in an ethylene-dependent manner; their response has often been measured in apples that were either treated with 1-MCP or were undergoing a climacteric response with increasing ethylene. In this study, transgenic ACO1-suppressed (ACO1as) apple fruit were used to study mechanisms of ethylene regulation in softening. ACO1as fruits are ideal to test these mechanisms as they are unable to produce autocatalytic ethylene, but respond to ethylene treatment with normal ripening and the concentration of ethylene within the fruit is the same as that applied (Schaffer et al. 2007, Johnston et al. 2009). By using non-ethylene-producing, ethylene-responsive apples, the ethylene response can be dissected in detail at the physiological and molecular level.

## Results

### Elements of early fruit softening show low dependence for ethylene

Johnston et al. (2009) demonstrated that the control of fruit softening in ACO1as apples is strongly dependent on ethylene; however, a small but significant drop in firmness (~5 Newtons (N)) was observed in the absence of ethylene during their 14 d study (Johnston et al. 2009; Supplementary Fig. S1). To establish whether ACO1as fruit would undergo further softening with time in the absence of ethylene, apples were harvested over two consecutive seasons and assessed for firmness every 2 weeks over 6 and 8 week periods, respectively (Fig. 1A). Similar to the results observed in Johnston et al. (2009), in season 1, an approximately 5 N drop in firmness was observed following 2 weeks at 20°C, after which no further softening was observed. In season 2, the initial 5 N drop was not observed, but the at-harvest firmness (~49 N) suggested the fruit had undergone this early softening step prior to harvest; in season 2, no drop in firmness was observed over the 8 week period (Fig. 1A). This demonstrates that while some early softening events are regulated in an ethylene-independent or low dependence manner, the majority of fruit softening has an absolute requirement for ethylene.

![Fig. 1](https://academic.oup.com/pcp/article-abstract/55/5/1005/2755984/fig1)
Ethylene-regulated softening is dose \( \times \) time dependent

Over a 14 d period it has been shown that continuous low ethylene (1 \( \mu l \) \( l^{-1} \)) results in partial fruit softening in ACO1as fruit, while lower treatments (0.01 and 0.1 \( \mu l \) \( l^{-1} \)) result in softening similar to no-ethylene controls (Johnston et al. 2009; Supplementary Fig. S1). To test whether low ethylene can lead to more complete softening given a longer treatment, two batches of ACO1as fruit were treated with continuous 0.1 and 1 \( \mu l \) \( l^{-1} \) ethylene for 6 weeks. During this time, fruit treated with 1 \( \mu l \) \( l^{-1} \) ethylene softened continuously and after 6 weeks achieved a firmness level similar to that of untransformed, ethylene-producing 'Royal Gala' fruit stored 14 d at room temperature (Fig. 1B; Supplementary Fig. S1). ACO1as fruit treated with 0.1 \( \mu l \) \( l^{-1} \) ethylene showed a small degree of softening after 6 weeks which exceeded that observed in the absence of ethylene after 6 weeks (\( P < 0.01 \); Fig. 1A, B). This demonstrates that fruit softening is achieved in a dose \( \times \) time-dependent manner, with lower ethylene concentrations leading to slower softening, and also suggests that the low doses tested are above the response threshold.

Ethylene receptor expression is dynamic and dependent on ethylene dose

To study the response to ethylene in the fruit, the transcriptional response of ethylene receptors to different concentrations of ethylene was examined. It has been shown that five of the nine putative ethylene receptors in apple exhibit a rapid increase (within 12 h) in expression in ACO1as fruit treated with 100 \( \mu l \) \( l^{-1} \) ethylene (Ireland et al. 2012). This response illustrates the potential of the fruit to detect ethylene and provides a transcriptional marker for the ethylene response in ripening ACO1as fruit. Expression was first analyzed using tissue from the Johnston et al. (2009) study in which ACO1as fruit were treated continuously for 14 d with different concentrations of ethylene. After the 14 d treatment, most receptors showed an increasing dose–response curve, which did not saturate at high concentrations (Fig. 2A). The low doses of 0.01 and 1 \( \mu l \) \( l^{-1} \) were observed to have a significant inhibitory effect on ERS2 and ETR2 expression, whereas 0.1 \( \mu l \) \( l^{-1} \) resulted in enhanced expression in ERS1 and ERS2, suggesting instability in response to low ethylene doses. After 14 d, transcriptional regulation of the receptors exhibited low ethylene sensitivity, estimated as the concentration at which a medium response is observed.

To investigate the dose \( \times \) time response observed in softening (Fig. 1B), a second study of ethylene receptor expression was carried out in a different season with ACO1as fruit treated continuously with 0–10,000 \( \mu l \) \( l^{-1} \) ethylene and assessed over five time points over 8 d (192 h). Two ethylene receptors, ERS2 and ETR102, were selected for further analysis because they exhibited a strong response to ethylene, represent two homologous pairs, ERS1 and ETR2, respectively, and showed differences in the dose–response curves (Fig. 2A; Ireland et al. 2012). The low expression level of ETR5 excluded it from further study.

Consistent with the findings of Ireland et al. (2012), a rapid and strong increase in transcription was observed in all treatments at 12 h (Fig. 2B). Interestingly, transcript levels at this time point showed a maximum transcriptional activation with \( > 1 \) \( \mu l \) \( l^{-1} \) ethylene. For both receptors, the maximum transcriptional response increased with time and dose: 1 \( \mu l \) \( l^{-1} \) at 12 h, to 10 \( \mu l \) \( l^{-1} \) at 48 h, then to 100 \( \mu l \) \( l^{-1} \) at 96 h, which shows both increasing ethylene responsiveness to a single dose and decreasing ethylene sensitivity (higher concentration required for medium response) over time. These results suggest a dose-dependent mechanism of gene regulation whereby low (below saturating) doses result in fluctuating, unstable expression, while high doses produce stable expression that appears to decrease in sensitivity over time, which may help to dampen the overall ethylene response in the presence of increasing autocatalytic ethylene levels.

Expansins are differentially regulated and tend to be expressed early in ripening

Expansins have a well documented role in cell wall loosening (Cosgrove et al. 2002) and some demonstrate regulation by ethylene, making this family a candidate for further study of ethylene regulation of softening. Previously, five expansin genes (EXPANSIN-1, -2, -3, -5 and -8 (EXP1–8)) with ripening-related expression were identified in apple (Chagné et al. unpublished data). Expansin gene expression was studied using tissue from the Johnston et al. (2009) study where ACO1as fruit were treated with different ethylene concentrations for 14 d (Fig. 3A). A range of dose–response curves were observed at 14 d: EXP2 and EXP8 expression increased with increasing ethylene concentrations, EXP5 was enhanced by ethylene and saturated at 1 \( \mu l \) \( l^{-1} \) (\( P < 0.01 \)), and EXP1 and EXP3 were suppressed by both low (0.01–0.1 \( \mu l \) \( l^{-1} \)) and high (>10 \( \mu l \) \( l^{-1} \)) ethylene (Fig. 3A). The minimum concentration required to achieve maximum expression, which provides an indication of ethylene sensitivity, was different for each expansin: EXP5 at 1 \( \mu l \) \( l^{-1} \); EXP2 at 100 \( \mu l \) \( l^{-1} \); and EXP8 at 1,000 \( \mu l \) \( l^{-1} \) ethylene. EXP1 and EXP3 were negatively regulated by ethylene at 14 d, resulting in a minimum concentration for maximum inhibition of 0.01 \( \mu l \) \( l^{-1} \) ethylene for both genes (Fig. 3A). Expression was also assessed using tissue from Ireland et al. (2012) in which ACO1as fruit were treated with the physiologically relevant dose of 100 \( \mu l \) \( l^{-1} \) ethylene for 4 d: EXP3/5 were high at harvest and were down-regulated by ethylene, while EXP1/8 and EXP2 were transiently and stably up-regulated by ethylene, respectively (Fig. 3A). Expansin protein abundance was also studied using tissue from the Johnston et al. (2009) study using polyclonal antibodies raised against CsEXPA1 (Rochange and McQueen-Mason 2000).
negative and positive regulation of expansins by ethylene (Fig. 3B). Interestingly, a decrease in expansin protein abundance was observed in fruit stored cold for 14 d. In tomato and pear, cold treatment does not suppress protein abundance (Hiwasa et al. 2003, Rugkong et al. 2010), which may suggest additional controls over expansin protein regulation in cold conditions in apple. To investigate the dose × time response in softening, expression analysis was carried out using ACO1as fruit treated continuously with 0–10,000 μL L⁻¹ ethylene and assessed at five time points from 12 h to 8 d (192 h). EXP1, EXP3 and EXP8 were selected for further analysis as they exhibited expression patterns of interest: EXP1 showed early transient expression with ethylene, EXP3 showed high at-harvest expression that decreased over time, and EXP8 exhibited a more prolonged, but transient induction by ethylene (Fig. 3A). For EXP1, two treatments, 0.1 and 10 μL L⁻¹ ethylene, showed a peak in expression at 48 and 24 h, respectively (Fig. 3C, blue and red bars and trend lines). In combination with the time course experiment at 100 μL L⁻¹ ethylene (Fig. 3A), this suggests a dose × time-dependent, transient induction of EXP1. EXP3 was up-regulated at harvest and decreased in the absence of ethylene, but this decrease was enhanced by ethylene in a dose × time-dependent manner. In addition, similar to ETR102 and ERS2, fluctuating expression was observed at low doses (≤1 μL L⁻¹ ethylene, Fig. 3C). EXP8 was up-regulated by ethylene and exhibited a consistent response over 8 d (Fig. 3C). The results suggest that the expansin gene family is subject to diverse regulatory mechanisms that, with the exception of EXP2, lead to early expression during ripening (Fig. 3A).
Fig. 3  Molecular characterization of expansins of interest in cortex tissue of ACO1as fruit treated with ethylene. (A) qRT-PCR analysis of five expansin genes of interest measured after 14 d continuous ethylene at different concentrations (bar charts) or measured periodically over 96 h continuous ethylene at the physiologically relevant concentration of 100 μl l⁻¹ (line graphs) in pooled tissue (n = 5); * and ** denote significance compared with no-ethylene control at P < 0.05 and P < 0.01, respectively, as determined by Student’s t-test. (B) Western blot analysis of expansin proteins of approximately 28 kDa detected with CsEXP1 antibody (arrow) in untransformed ‘Royal Gala’ (wild-type, WT) and ACO1as fruit, at harvest (H), stored 14 d with no added ethylene at 20°C (S) or 1°C (C), or stored for 14 d with different concentrations of continuous ethylene. (C) qRT-PCR analysis of EXPA1, EXPA3 and EXPA8 measured at five time points over 8 d continuous ethylene at different concentrations in pooled tissue (n = 10); the same letter denotes no significant difference (P < 0.01) between comparisons within the same time point as determined by Student’s t-test; bars of different colors and trend lines indicate patterns of interest. For all qRT-PCRs, expression is relative to ACTIN and is presented as the mean ± SD of four technical replicates.
Expression of key cell wall-modifying genes is regulated in a dose × time-dependent manner

Cell wall-modifying hydrolase genes with well-documented roles in ripening were also examined, namely BGAL, AFase and PG. High expression of BGAL1 (L29451; MDP0000416548; chromosome (Chr) 15) during ripening was reported by Ross et al. (1994). However, upon examination of an expressed sequence tag (EST) database (Newcomb et al. 2006), a second gene (KC107807; MDP0000127542; Chr 2), homeologous to BGAL1, was found to be more highly expressed in 'Royal Gala' apple fruit. Using the naming convention proposed in Devoghalare et al. (2012), this gene has been named BGAL101. Due to sequence similarity (91.5% identity in aligning regions at the nucleotide level), it is unlikely that Northern blot analysis (Ross et al. 1994) would distinguish between the two genes. After 14 d in different ethylene concentrations, BGAL101 and AFase1 showed increasing expression with increasing ethylene concentration, with BGAL101 showing higher ethylene dependency and lower ethylene sensitivity (a medium response to ethylene, which was also the saturating dose observed for regulation of PG1, also at 100 μl/L) compared with AFase1 (Fig. 4A). After the same 14 d period, BGAL protein abundance paralleled expression, with bands detectable in ACO1as fruit held for 14 d in the absence of ethylene and in all ethylene treatments, with greater band intensity at higher concentrations, and was also detectable in untransformed 'Royal Gala' fruit both at harvest and after storage for 14 d (Fig. 4B), providing support for both developmental and ethylene-based regulation. In the time course experiment with 100 μl/L ethylene, AFase1 and BGAL101 transcripts increased moderately with time but were enhanced by ethylene treatment (Fig. 4A), thereby demonstrating both ethylene-dependent and -independent gene regulation, consistent with moderate ethylene dependency of these genes. In the dose × time experiment, expression of BGAL101 showed different responses to ethylene depending on the concentration applied (Fig. 4C). Over the first 4 d, expression levels appeared to saturate at 10 μl/L ethylene, which was also the saturating dose observed for softening after 14 d of ethylene (Johnston et al. 2009). At concentrations below saturation, dose × time-dependent regulation was observed which resulted in a near-linear additive effect on expression levels over time (P < 0.05). This was in contrast to the expression patterns of ETR102 and ERS2 at these concentrations (rapid up-regulation, followed by fluctuating expression) and suggests regulation by different pathways. Ethylene regulation of PG1 expression showed some differences compared with BGAL101 and AFase1. PG1 displayed stronger ethylene dependency and lower ethylene sensitivity (Fig. 5A), and PG1 protein abundance was detected only in ethylene treatments ≥ 10 μl/L 1 for 14 d (Fig. 5B), suggesting close correlation of transcription and translation. Over the 4 d time course at 100 μl/L ethylene, PG1 expression was not induced by ethylene until the 24 h time point, suggesting a time-dependent mechanism of regulation that delays induction of this gene. In the dose × time experiment, it was found that PG1 and BGAL101 shared a similar near-linear dose × time-dependent regulation of expression at low concentrations. The major difference between BGAL101 and PG1 expression was delayed induction of PG1, which also displayed dose × time dependence, with expression induced earlier with increasing concentrations of ethylene. The flavor volatile gene ALPHA FARNESENE SYNTHASE-1 (AFS1; Fig. 5C) is also strongly regulated by ethylene (Schaffer et al. 2007) and after an initial period of unstable expression exhibits strong and consistent up-regulation by high ethylene doses, similar to PG1. Interestingly, a trend of increasing ethylene sensitivity (lower doses required for medium response) was observed for regulation of PG1 over time.

Discussion

Fruit softening has been shown to be regulated by ethylene in many plant species, but the mechanisms by which the fruit translate the ethylene signal into phenotypic changes are largely unknown. This study shows that ethylene regulates softening in a co-ordinated manner both through a dose × time-dependent mechanism capable of measuring cumulative ethylene, and through differential sensitivities to and dependencies on ethylene, resulting in differential transcriptional regulation of each cell wall-modifying gene. Together these mechanisms allow for a temporal progression of gene expression that is governed by increasing levels of autocatalytic ethylene observed as ripening progresses. At the physiological level, ethylene regulates fruit softening in a dose × time-dependent manner whereby lower doses result in slower softening, and doses at and above saturation lead to a maximum rate of softening (Fig. 1; Johnston et al. 2009).

The different modes of ethylene regulation observed here result in a temporal transcriptional response having distinct waves of transcription observed from rapid expression of ethylene receptors through to delayed expression of the potent cell wall enzyme, PG1. Transcriptional waves were recently observed by Chang et al. (2013) whereby an initial wave was highly variable and represented a general response to the perturbation, whereas later waves were less variable and represented a more specific response to ethylene. Similarly, it is observed here that ethylene receptors, and some expansin genes, are expressed in an initial first wave and exhibit highly variable expression, whereas some cell wall genes are stably expressed in second (e.g. BGAL101 and AFase1) and third (e.g. PG1) waves. This study builds on the data of Chang et al. (2013) showing that the response is dependent on ethylene dose, with, for example, ethylene receptors showing unstable and fluctuating expression at low doses and strong saturating expression at high doses, and, similarly, strongly ethylene-regulated cell wall-modifying genes show dose × time dependence at low doses but switch to strong consistent expression at high doses. This suggests that multiple pathways of ethylene regulation may be in operation depending on ethylene dose, such that as
Cumulative ethylene regulates apple softening

autocatalytic ethylene levels increase with ripening, a switch occurs from a partial, less committed, ethylene response to a stronger, more stable response that reflects increased commitment to the ripening outcome. Interestingly, the potent cell wall-modifying gene PG1 is subject to delayed expression in a dose × time-dependent manner, which may suggest that a negative feedback loop, similar to that which involves AP2a in tomato (Chung et al. 2010, Karlova et al. 2011), may play a role in apple softening and apple ripening as a whole.

The two modes of ethylene regulation of dose × time dependence and differential sensitivities to and dependencies on ethylene of cell wall-modifying genes lead to a model for the progression of softening-related transcriptional regulation by ethylene (Fig. 6). As autocatalytic ethylene increases throughout ripening, cumulative ethylene exposure is monitored through a dose × time-dependent mechanism, that when coupled to different dependencies on and sensitivities to ethylene results in a temporal transcriptional response of appropriate magnitude for each gene. This suggests that expansins, particularly EXPAS/S, may be responsible for the ethylene-independent 5 N drop in firmness (Fig. 1; Supplementary Fig. S1). In addition, when expression patterns are aligned to the softening curve, it would suggest that the early and rapid softening (Johnston et al. 2001) is more associated with the EXPA class of genes, while the later and slower softening events are associated with BGAL101 and PG1. This observation would agree with mapping data linking a softening QTL on Chr1 that includes EXPA2, as well as EXPA6 and EXPA7 genes.

Fig. 4 Molecular characterization of AFase1 and BGAL101 in cortex tissue of ACO1as fruit treated with ethylene. (A) qRT-PCR analysis of AFase1 and BGAL101 measured after 14 d continuous ethylene at different concentrations (bar charts) or measured periodically over 96 h continuous ethylene at the physiologically relevant concentration of 100 μL L⁻¹ (line graphs) in pooled tissue (n = 5); * and ** denote significance compared with no-ethylene control at P < 0.05 and P < 0.01, respectively, as determined by Student’s t-test. (B) Western blot analysis of BGAL1/101 protein of approximately 76 kDa detected with polyclonal antibody for apple BGAL in untransformed ‘Royal Gala’ (wild-type, WT) and ACO1as fruit, at harvest (H), stored for 14 d with no added ethylene at 20°C (S) or 1°C (C), or stored for 14 d with different concentrations of continuous ethylene; bands between 51 and 64 kDa represent non-specific binding of the polyclonal antibody. (C) qRT-PCR analysis of BGAL101 measured at five time points over 8 d continuous ethylene at different concentrations in pooled tissue (n = 10); the same letter denotes no significant difference (P < 0.01) between comparisons within the same time point as determined by Student’s t-test; bar colors depict the degree of response to ethylene: white, no response; gray, partial; black, maximum. For all qRT-PCRs, expression is relative to ACTIN and is presented as the mean ± SD of four technical replicates.
Fig. 5 Molecular characterization of PG1 in cortex tissue of ACO1as fruit treated with ethylene. (A) qRT-PCR analysis of PG1 measured after 14 d continuous ethylene at different concentrations (bar charts) or measured periodically over 96 h continuous ethylene at the physiologically relevant concentration of 100 μl·l⁻¹ (line graphs) in pooled tissue (n = 5). * and ** denote significance compared with no-ethylene control at P < 0.05 and P < 0.01, respectively, as determined by Student’s t-test. (B) Western blot analysis of PG1 protein of approximately 45 kDa detected with polyclonal antibody raised against recombinant apple PG1 (arrow) in untransformed ‘Royal Gala’ (wild-type, WT) and ACO1as fruit, at harvest (H), stored for 14 d with no added ethylene at 20 °C (S) or 1 °C (C), or stored for 14 d with different concentrations of continuous ethylene. (C) qRT-PCR analysis of PG1 and the related ripening gene AFS1 measured at five time points over 8 d continuous ethylene at different concentrations in pooled tissue (n = 10); the same letter denotes no significant difference (P < 0.01) between comparisons within the same time point as determined by Student’s t-test; bar colors depict the degree of response to ethylene: white, no response; gray, partial; black, maximum. For all qRT-PCRs, expression is relative to ACTIN and is presented as the mean ± SD of four technical replicates.

(Costa et al. 2008, Trujillo et al. 2012), and a transgenic study where PG1 was suppressed in apples resulting in little difference in firmness at harvest (Atkinson et al. 2012). In the same study, the PG1-suppressed apples were found to be significantly firmer later in fruit softening compared with controls, which is consistent with PG1 mapping to a dominant QTL for fruit softening (Costa et al. 2010), and suggests that due to its potency PG1 may be subject to additional controls over gene regulation compared with other cell wall-modifying genes, such as delayed induction and changes in ethylene sensitivity over time.

The temporal progression of transcription is controlled in part by differential ethylene dependencies and sensitivities (estimated from dose–response curves generated after 14 d ethylene treatment) summarized in Table 1. Taken together with the model presented by Johnston et al. (2009), this would allow for a progression of cell wall-modifying gene expression such that, as autocatalytic ethylene increases over time with ripening, high sensitivity/moderate dependency genes (e.g. APhase1 and BGAL101) are expressed before low sensitivity/high dependency genes (e.g. PG1 and EXP2). The situation is more complex for expansins due to early and/or transient expression, but in general the results indicate an early role for expansins in ripening, with some occurring independently of ethylene. This model for ethylene-regulated transcription aligns with the model for the progression of cell wall disassembly of solubilization, loss of side chains, then depolymerization (Brummell 2006, Schroder and Atkinson 2006), through the sequential and combined action of first expansins, then side chain hydrolases BGAL101 and APhase1, and finally endo-acting PG1. Several studies have shown differential temporal
expression of cell wall-modifying genes throughout ripening, with ‘early’ genes switched off by ethylene and ‘late’ genes positively regulated by autocatalytic ethylene (Brummell and Harpster 2001, Trainotti et al. 2003). Similarly, in melon, Hadfield et al. (1998) demonstrated strong ethylene dependency for CmPG1, and Nishiyama et al. (2007) observed ethylene independency of CmEXP1. Through the use of transgenic fruit suppressed for ACO1, this study builds on the work by showing that ethylene regulation of cell wall-related genes is under finer controls that are dependent on dose, cumulative exposure to ethylene and on the differential sensitivity and dependency of each gene to ethylene. To the authors’ knowledge, regulation by cumulative exposure to ethylene has not been demonstrated since the idea was first proposed by Yang (1987) in the context of young fruit being induced to ripen. In addition to the finer controls mentioned above, ethylene sensitivity was observed to change over time for some genes (ETR102 and ERS2 decreased, while PG1 increased); this may allow for an additional level of control over ethylene-regulated pathways in the presence of increasing autocatalytic ethylene. Johnston et al. (2009) demonstrated that total volatile production in apple ripening exhibited low sensitivity/high dependency for ethylene, while some of the individual volatiles that contribute to the phenotype exhibit differential sensitivities to and dependencies on ethylene. A similar effect is observed here in the regulation of genes involved in softening and suggests that this high degree of ethylene regulation may also govern other ripening traits.

It is also worth noting that there are further complexities to this model. It has been shown that less mature apple fruit require higher amounts of ethylene for ripening to occur (Knee et al. 1987), such that, as the fruit matures, less ethylene is needed to promote ripening. In addition to the capability to monitor cumulative ethylene, developmental or environmental factors may also influence the response. From an environmental perspective, as the fruit matures, typically the seasonal temperature drops. Recent reports have shown that cold can promote ripening in apple either independently of ethylene or by making the apples more sensitive to ethylene (Tacken et al. 2010). Cold may therefore be an additional factor influencing ethylene sensitivity of ripening responses.

While the degree of fruit softening in apples is small compared with that of many fruits, it is evident that even in apples there is considerable complexity around the regulation of the softening process. This complexity is evident in the numbers and types of cell wall-modifying genes and the different ways in which they are regulated, both at the developmental level and temporarily through the action of ethylene. This study has only focused on genes previously shown to be up-regulated during fruit ripening. Further work is needed to establish if other cell wall-modifying genes are up-regulated during the fruit ripening process. This work also suggests that other ripening traits (e.g. volatile production and color change) may be regulated in an equally complex manner.

### Materials and Methods

#### Plant material and growth conditions

Five control apples (Malus × domestica Borkh.) of cultivar ‘Royal Gala’ and seven scion-propagated, transgenic ACO1as, ‘Royal Gala’ background apple trees, all on ‘M9’ root stock were grown in standard glasshouse conditions. At full bloom, flowers were pollinated with ‘Granny Smith’ pollen. Fruit were grown until control apples were mature based on background color and starch clearance (starch pattern index of 2–3 on a 0–6 scale or 3–4 on a 0–7 scale). All fruit were harvested and randomized for tree and size before being divided into batches for treatment and assessment. For fruit treated with different ethylene concentrations and assessed at a single time point after 14 d, samples from fruit assessed in Johnston et al. (2009) were used. For fruit exposed to 100 µl L⁻¹ ethylene
over time, the samples were the same as those described by Ireland et al. (2012). For fruit treated with different ethylene concentrations sampled at multiple time points over 8 d, treatments were conducted in 20 liter ripening bins with continuous air movement and time to absorb CO₂, while the 0.1 μl L⁻¹ treatment contained Campure-8 beads of activated alumina impregnated with potassium permanganate (Camfil Farr) to scrub ethylene. The ethylene concentration within each bin was determined 1 h after application by gas chromatography as described by Johnston et al. (2009). All fruit assessments (starch, internal ethylene, firmness and skin background color) were undertaken as described by Johnston et al. (2009).

**Gene expression analysis**

Total RNA was extracted and cDNA synthesized as described by Schaffer et al. (2007). Quantitative real-time PCR (qRT-PCR) was performed according to Tacken et al. (2010). Expression of each gene was measured relative to the expression of the apple housekeeping gene ACTIN (Espley et al. 2007). Cell wall-modifying genes were selected based on literature reports cited in the text, except the BGAL1 gene, which was replaced in favor of the homeologous gene BGAL1T1. Primers used to measure gene expression (Supplementary Table S1) were designed upon polymorphisms to enable specificity to the homeolog of interest. The qRT-PCR product for all genes was verified by sequencing.

**Western blot analysis**

For PG and β-GAL, crude protein extract was prepared from 100 mg of powdered, liquid N₂-frozen tissue incubated at room temperature in 1 ml of urea/thiourea lysis buffer (Barracough et al. 2004) for 1 h, centrifuged at 14,000 r.p.m. for 15 min, and the supernatant was retained. For expansins, crude protein extract was prepared from 100 mg of powdered, liquid N₂-frozen tissue boiled for 5 min in SDS buffer (Schägger and von Jagow 1987). Total protein concentration was determined using the Protein Assay Kit (Bio-Rad Laboratories) with bovine serum albumin as a standard. Proteins were separated on 12% NuPAGE Bis-Tris pre-cast gels using NuPAGE MOPS SDS running buffer according to the manufacturer’s instructions (Life Technologies). Proteins were electroblotted onto polyvinylidifluoride membrane (Immobilon Transfer Membrane, pore size 0.45 μm, Millipore) using Bjerrum and Schafer Nielsen (BSN) 20% methanol transfer buffer [including 0.1% (w/v) SDS]. A semi-dry transfer cell (Bio-Rad Laboratories) was used for transfer at 5 V for 12 h. Membranes were blocked with TBS buffer [10 mm Tris, 150 mm NaCl, 0.1% (v/v) Tween-20, pH 8] containing 5% non-fat milk powder (Anchor) at room temperature for at least 2 h, followed by standard incubations and washings. Primary antibodies were used at a 1 : 500 ratio in blocking solution and then, after thorough washings, secondary antibody conjugates were used at 1 : 1,000 dilution [Qdot® 655 goat F(ab’)2 anti-rabbit IgG conjugate (H+L)] together with alkaline phosphatase-conjugated affinity-purified anti-rabbit IgG (after confirming that they did not share specific epitopes). Membranes were imaged in a Typhoon 9400 variable mode imager and then followed with immunoprecipitation using 1-Step NBT/BCIP (Pierce). The quantification was carried out using Imagequant 7.0 (Amersham Biosciences). Four antibodies were used in this study: those derived from apple PG1 [1 : 1,000 (v/v) dilution] (Atkinson et al. 2012), apple BGAL1 [1 : 1,000 (v/v) dilution] (JKT. Ng et al unpublished data), cucumber (Cucumis sativus) CSExp1 [1 : 1,000 (v/v) dilution] (Rochange and McQueen-Mason 2000) and apple ACO1 [1 : 1,000 (v/v) dilution] (Binnie and McManus 2009).

**Supplementary data**

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**Disclosures**

The authors have no conflicts of interest to declare.

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