A long noncoding RNA functions in high-light-induced anthocyanin accumulation in apple by activating ethylene synthesis

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Abstract
Anthocyanin production in apple (Malus domestica) fruit and their consequent coloration can be induced by high-light treatment. The hormone ethylene is also essential for this coloration, but the regulatory relationships that link ethylene and light with anthocyanin-associated coloration are not well defined. In this study, we observed that high-light treatment of apple fruit increased anthocyanin accumulation more than moderate-light treatment did and was the main contributor of induced ethylene production and activation of anthocyanin biosynthesis. A transcriptome study of light-treated apple fruit suggested that a long noncoding RNA (lncRNA), MdLNC610, the corresponding gene of which is physically located downstream from the 1-aminocyclopropane-1-carboxylate oxygenase (ACO) ethylene biosynthesis gene MdACO1, likely affects anthocyanin biosynthesis under high-light treatment. Expression and promoter β-glucuronidase reporter analyses further showed that MdLNC610 upregulates expression of MdACO1 and so likely participates in high-light-induced ethylene biosynthesis. Overexpression of MdACO1 and MdLNC610 in apple fruit and calli indicated that a major increase in MdLNC610 expression activates MdACO1 expression, thereby causing an increase in ethylene production and anthocyanin levels. These results suggest that MdLNC610 participates in the regulation of high-light-induced anthocyanin production by functioning as a positive regulator to promote MdACO1 expression and ethylene biosynthesis. Our study provides insights into the relationship between mRNA and IncRNA networks in the ethylene biosynthetic pathway and anthocyanin accumulation in apple fruit.

Introduction
Fleshy fruit ripening is typically associated with changes in texture, aroma, and nutritional value, but another common feature is a change in color due to the biosynthesis of pigments, such as carotenoids and anthocyanins (Adams-Phillips et al., 2004; Klee and Giovannoni, 2011). A notable
example of the latter is exhibited by apple (*Malus domestica*), a fruit crop that is widely cultivated in temperate zones. Anthocyanins contribute to the red color of apple peels and are also a key indicator of fruit maturity and quality, as well as being associated with their positive dietary attributes (Lancaster, 1992; Ma et al., 2014; Vimolmangkang et al., 2014).

The mechanism of anthocyanin synthesis and its regulation has been elucidated in many plant species such as *Arabidopsis* (*Arabidopsis thaliana*) (Kubasek et al., 1992), maize (*Zea mays*) (Wienand et al., 1986), petunia (*Petunia × hybrida*) (Britsch and Grisebach, 1986), and snapdragon (*Antirrhinum majus* L.) (Martin et al., 1991). It represents a branch of the flavonoid pathway, and the catalytic enzymes include those involved in early anthocyanin biosynthesis, such as chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), and flavonoid 3′-hydroxylase, and late biosynthesis, such as dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and flavonoid 3-O-glycosyl-transferase (UGFT) (Ju et al., 1997; Ban et al., 2007; Lister and Lancaster, 2015; TiAn et al., 2017; Wang et al., 2000). In addition to these biosynthetic enzymes, a MYB DOMAIN PROTEIN (MYB)/basic helix-loop-helix (bHLH)/WD REPEAT PROTEIN 40 (WD40) (MBW) complex has been identified, comprising MYB transcription factors (TFs), bHLH TFs, and WD-repeat proteins, which acts as a core regulator of anthocyanin accumulation (Ban et al., 2007; Espley et al., 2007; Ma and Constabel, 2019).

Environmental stimuli, such as temperature, light, water, and nutrient levels in the soil can also contribute to the regulation of anthocyanin biosynthesis (Winkel-Shirley, 2001, 2002; Carbone et al., 2009; Jaakola, 2013; Honda and Moriya, 2018). Of these, light is perhaps the most important as outlined in several studies (Takos et al., 2006; Jaakola, 2013). TFs whose expression is induced by light and that activate the expression of anthocyanin biosynthetic genes, and hence anthocyanin accumulation, have been studied in several fruit species (Ubi et al., 2006; Gong et al., 2015; Henry-Kirk et al., 2018; Zhang et al., 2018; An et al., 2019; Fang et al., 2019; Ni et al., 2019). One example from apple is *MdMYB1*, which is allelic to *MdMYB10* and *MdMYBA*, the expression of which increases within 1 day of fruit exposure to sunlight (Takos et al., 2006). Functional assays have further shown that *MdMYB1* promotes anthocyanin accumulation and fruit coloration by direct binding to the DFR and UFGT promoters (Takos et al., 2006). In addition, the basic leucine zipper TF, *MdhHY5* (ELONGATED HYPOCOTYL 5), was demonstrated to promote anthocyanin accumulation by regulating *MdMYB1* expression and that of downstream anthocyanin biosynthesis genes during light conditions (An et al., 2017). Moreover, in apple fruit, the B-Box (BBX) protein, *MdBBX37*, suppresses light-induced anthocyanin biosynthesis by interacting with two inducers of anthocyanin biosynthesis, *MdMYB1* and *MdMYB9* (An et al., 2020a). A recent study showed that higher light intensities induced anthocyanin biosynthesis in apple fruit by upregulating the expression of anthocyanin biosynthesis-related genes to a greater degree than low- and moderate-light treatment (An et al., 2020b).

In the same study, the TEOSINE BRANCHED1/CYCOIDEA/PROLIFERATING 46 (*MdTCP46*), was shown to participate in responses to high-light exposure and promote anthocyanin biosynthesis by interacting with *MdMYB1*, thereby enhancing its binding to its target genes.

Other regulatory systems are involved in pigment formation in ripening fruit. Notably, the gaseous phytohormone ethylene promotes the conversion of fleshy climacteric fruits from the immature to the fully ripe state (Bapat et al., 2010; Klee and Giovannoni, 2011). Ethylene biosynthesis has been extensively studied in climacteric fruit, such as apple, which show a burst of ethylene production coincident with ripening (Yue et al., 2020; Brumos, 2021). Ethylene is derived from S-adenosyl methionine, which is converted into 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS) and ACC is then oxidized by ACC oxidase (ACO) to form ethylene (Adams and Yang, 1981; Wang et al., 2002). In apple, three ACS genes are expressed at different stages of fruit development and ripening: *MdACS6* expression is initiated immediately after the first day of full bloom; *MdACS3a* expression occurs before fruit ripening; and *MdACS1* is specifically expressed during ripening (Li et al., 2015; Dong et al., 1992a, 1992b; Ross et al., 1992; Zhu et al., 1995; Bolitho et al., 1997; Atkinson et al., 1998; Tan and Bangerth, 2000; Cin et al., 2005; Park et al., 2006; Wang et al., 2009a, 2009b). However, *MdACS1* expression is deficient in the “Red Fuji” cultivar (Sunako et al., 1999; Kakusa et al., 2006). *MdACO1* is expressed during fruit development, and functional assays have suggested that decreased *MdACS1* and *MdACO1* transcription leads to very low levels of ethylene production during ripening (Danskek et al., 2004; Schaffer et al., 2007).

A previous study of apple ripening showed that the onset of anthocyanin accumulation coincides with the start of rapid ethylene production (Faragher and Brohier, 1984), and treatment with the ethylene precursor, ethephon, was observed to promote anthocyanin biosynthesis (An et al., 2018). More recently, several TFs have been shown to affect anthocyanin accumulation in apple by regulating ethylene biosynthesis. *MdMYB1/10* was observed to activate *ETHYLENE RESPONSE FACTOR 3* (*MdERF3*), expression in apple callus, resulting in increased ethylene emission and anthocyanin production (An et al., 2018). *MdACO1* can activate *ACS* and *ACO* expression in apple, possibly via upregulation of *ERF* genes linked to anthocyanin concentration and ethylene production (Espley et al., 2019). High expression of the *MdHLH3* gene, which encodes a MYB1/10 partner in the MBW complex, promotes ethylene production by activating *MdACO1* and *MdACS1* and *MdACSSA* expression to drive ethylene production and anthocyanin accumulation (Hu et al., 2019). However, it is not known whether light and ethylene regulate anthocyanin biosynthesis in apple fruit in a coordinated manner.

Another potential regulatory factor in pigment formation is long noncoding RNAs (IncRNAs), which can broadly be
defined as noncoding RNAs longer than 200 nucleotides (Jin et al., 2013) with no protein-coding potential. They may have short open reading frames, which have been shown to affect diverse aspects of plant development, including flowering time, root organogenesis, seedling photomorphogenesis, sexual reproduction, crop yield, and responses to biotic and abiotic stresses (Wang et al., 2014a, 2014b, 2014c, 2018; Zhang et al., 2014). LncRNAs can act through a multitude of mechanisms, including in cis to affect the gene expression of their chromosomal neighborhood and downstream from their location by modulating chromatin conformation, or in trans to affect distant genes when distal genomic locations are brought into close spatial proximity by base pairing with another RNA or interacting with proteins and/or DNA (Ørom et al., 2010; Ariel et al., 2014). They can also recruit epigenetic complexes or act as target mimics (Li et al., 2018; Yang et al., 2019). Studies have also shown that mutants of ripening-related tomato IncRNA, IncRNA1459, have substantially reduced ethylene production and lycopene accumulation, as well lower expression of their associated genes, compared with wild type fruit. It was predicted that IncRNA1459 indirectly regulates gene transcription by interacting with a target protein (Li et al., 2018). Our previous research showed that the LncRNAs, MLNC3.2 and MLNC4.6, function as endogenous target mimics (eTMs) for miR156a in apple fruit and prevent cleavage of MLNC4.6 lncRNA1459 that are brought into close spatial proximity by base pairing with another RNA or interacting with proteins and/or DNA (Ørom et al., 2010; Ariel et al., 2014). They can also recruit epigenetic complexes or act as target mimics (Liu et al., 2015; Yang et al., 2019). Studies have also shown that mutants of ripening-related tomato IncRNA, IncRNA1459, have substantially reduced ethylene production and lycopene accumulation, as well lower expression of their associated genes, compared with wild type fruit. It was predicted that IncRNA1459 indirectly regulates gene transcription by interacting with a target protein (Li et al., 2018). Our previous research showed that the LncRNAs, MLNC3.2 and MLNC4.6, function as endogenous target mimics (eTMs) for miR156a in apple fruit and prevent cleavage of SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE 2 (SPL2-like) and SPL33 by the microRNA, miR156a, during light-induced anthocyanin biosynthesis, thereby promoting anthocyanin accumulation (Yang et al., 2019). This suggests that LncRNAs play an important role in regulating anthocyanin biosynthesis or ethylene biosynthesis; however, little is known about the function of IncRNA in light- or ethylene-regulated anthocyanin accumulation in apple fruit.

In this study, we investigated the putative function of IncRNAs in the coordinated high-light and ethylene-mediated mechanisms that regulate anthocyanin biosynthesis in apple fruit. An ethylene-related gene, MdLNC610, was identified through sequencing data analysis and was shown to be induced by high-light, and to regulate anthocyanin accumulation and fruit ripening by mediating positive regulation of MdACO1 expression. Our findings indicate that MdLNC610 is a positive regulator of anthocyanin accumulation and provide insights into the function of IncRNAs in the synergistic regulation of apple fruit coloration.

Results
High-light treatment promotes anthocyanin biosynthesis in apple fruit by activating ethylene production
Mature ‘Red Fuji’ fruit bagged with double-layered light-impermeable paper bags were harvested 140 d after full bloom (DAFB). The harvested apples were transferred in the dark from their bags to an incubator for different light treatments. We observed that continuous high-light treatment caused higher accumulation of anthocyanins than other treatments (Figure 1A). For the light-transition treatments, debagged apple fruit were transferred to moderate light after 3 d in high light, and these fruit showed significantly less anthocyanin accumulation than those exposed to continuous high light. Debagged apple fruit transferred to high light after 3 d in moderate light also showed significantly higher anthocyanin accumulation than those in continuous moderate light (Figure 1A and B). Thus, high light significantly accelerated fruit coloration compared to moderate light. We also measured ethylene production under different light intensities and observed a significantly greater increase in ethylene production in response to the high-light treatment than to the moderate-light treatment (Figure 1C). Finally, we observed an association between ethylene production and anthocyanin accumulation, suggesting that high light may contribute to ethylene production in apple fruit (Figure 1C).

We next measured the expression of anthocyanin biosynthetic genes (MdCHS, MdF3H, MdDFR, MdANS, and MdUFGT), the anthocyanin-associated regulatory gene MdMYB1, and ethylene biosynthetic genes (MdACO1, MdACO2, MdACS3, MdACS3a, and MdACS6) in fruit exposed to the same light treatments as above. The expression of all these genes was induced by the high-light treatment (Figure 2). These results suggested that high light may promote ethylene production through the expression of ethylene biosynthesis genes and we hypothesized that this light-induced ethylene might in turn induce the expression of anthocyanin-related genes, resulting in fruit coloration.

To investigate the role of ethylene during high-light-induced anthocyanin accumulation, ‘Red Fuji’ apples harvested 140 DAFB were treated with the ethylene inhibitor 1-methylcyclopropene (1-MCP) and stored in a phytotron with high light (300 μmol m–2 s–1, 22°C) for 6 d. Anthocyanin accumulation was inhibited in 1-MCP-treated fruit stored under high-light conditions (Figure 3, A and B). Furthermore, ethylene production and anthocyanin biosynthesis showed similar trends (Figure 3C). We also noticed that the expression of ethylene biosynthetic genes and anthocyanin-related genes was inhibited by high light + 1-MCP treatment (Figure 3, D and E). These results suggested that high-light-induced anthocyanin accumulation mainly depends on the production of ethylene.

MdLNC610 regulates anthocyanin accumulation via MdACO1
In a previous transcriptome study of light-treated apple fruit, we found that LncRNAs likely play important roles in anthocyanin biosynthesis under light treatment conditions (Yang et al., 2019), and we hypothesized that a LncRNA might be involved in regulating the expression of ethylene biosynthesis genes. To test this, we performed a weighted gene co-expression network analysis in our previous transcriptome data and identified 20 distinct modules (Bioproject Number: PRJNA555185, SRX6464003–SRX6464008). An analysis of the
relationship between modules and anthocyanin levels revealed that the "Purple" module (r = 0.8, P = 0.002) highly correlated with ethylene production (Figure 4A). Among these differentially expressed (DE) IncRNAs (Supplemental Table S1), we noted that MSTRG.97610.1 (hereafter named MdLNC610) was located 80,129-bp downstream from the coding region of the ethylene biosynthesis gene, MdACO1 (Figure 4B). Correlation analysis showed that MdLNC610 expression was closely associated with MdACO1 transcript levels (Figure 4C), and we deduced that it might participate in high-light-induced anthocyanin biosynthesis by regulating the expression of MdACO1. Reverse transcription-quantitative PCR (RT-qPCR) was used to assess MdLNC610 expression under different light intensities and in response to 1-MCP treatments. We observed that MdLNC610 expression was significantly higher in response to high-light treatment compared to moderate-light treatment, and had a similar pattern to MdACO1 expression (Figure 4D). The 1-MCP treatments had no obvious effects on MdLNC610 expression (Figure 4D).

To elucidate the potential mechanism by which MdLNC610 and MdACO1 contribute to the light response, 2,000 bp of their respective promoters were cloned from 'Red Fuji' fruit peel, and cis-elements were identified using the PLACE database (http://www.dna.affrc.go.jp/PLACE/) (Supplemental Table S2), with a focus on light response-related elements. No light-responsive elements were found in the MdACO1 promoter; however, a BOX4 element, which is known to be associated with light responses, was identified in the MdLNC610 promoter (Figure 4E; Supplemental
Tables S3 and S4). To investigate the regulation of *MdLN610*, its promoter was fused to the β-glucuronidase (GUS) reporter gene and introduced into apple calli (Figure 4F). We observed that GUS staining was higher under high-light conditions than under moderate-light conditions (Figure 4G) and RT-qPCR analysis confirmed the levels of GUS transcripts were higher in response to high-light in the apple calli (Figure 4H).

**MdACO1** participates in anthocyanin biosynthesis in apple fruit and calli

To investigate whether *MdACO1* participates in ethylene production and high-light-induced anthocyanin biosynthesis, *Agrobacterium tumefaciens* cultures containing pRI-MdACO1 (pRI101, for overexpression) or TRV-MdACO1 (TRV, for silencing) vectors were individually injected into apple fruit, which were then exposed to high-light treatments. Fruit with suppressed *MdACO1* expression showed reduced ethylene production and anthocyanin accumulation, while overexpression of *MdACO1* was accompanied by increased ethylene production and anthocyanin levels, resulting in substantial red coloration of the peels. Fruit transformed with pRI-MdACO1 showed higher anthocyanin accumulation in response to high-light treatment than control fruit (pRI101 vector infiltrated) (Figure 5, A–C). Using RT-qPCR analysis, we saw a significant increase in *MdCHS*, *MdDFR*, *MdANS*, and *MdUFGT*. Anthocyanin regulatory gene: *MdMYB1*. The value for Day 0 was set to 1. RT-qPCR was performed with three technical replicates and three biological replicates. For RT-qPCR standard error bars are calculated from three biological replicates. Different letters above the bars indicate significantly different values (\( P < 0.05 \)), calculated using one-way ANOVA followed by Tukey’s multiple range test.

When apple fruit harboring pRI-MdACO1 was transferred to high-light conditions after 1-MCP treatment, coloration was reduced, while ethylene treatment of *MdACO1*-silenced fruit restored coloration (Figure 5A). High pressure liquid chromatography (HPLC) analysis of anthocyanin accumulation and RT-qPCR analysis of *MdACO1* and anthocyanin...
biosynthesis genes were consistent with the fruit color phenotypes, in that 1-MCP treatment suppressed MdACO1-mediated anthocyanin accumulation and ethylene treatment promoted coloration when MdACO1 expression was reduced (Figure 5, B–E). These results further indicated that MdACO1 participates in anthocyanin accumulation by regulating ethylene biosynthesis.

To further elucidate MdACO1 function, an MdACO1 overexpression vector (OE-MdACO1) and an MdACO1 RNAi vector (RNAi-MdACO1) were constructed and transformed into wild type apple calli. Three transgenic lines harboring each construct were selected for subsequent assays. Overexpression of MdACO1 significantly increased anthocyanin accumulation compared with the wild-type, and there was no obvious color variation in the RNAi-MdACO1 calli compared to wild-type (Figure 6A). HPLC and gas chromatography–mass spectrometry (GC–MS) analyses revealed higher anthocyanin accumulation and ethylene production in MdACO1-overexpressing calli than in the wild-type controls and RNAi-MdACO1 calli (Figure 6, B and C). In addition, RT-qPCR analysis showed that overexpression of MdACO1 increased the expression of anthocyanin biosynthetic genes (MdCHS, MdCHI, MdF3H, MdDFR, MdANS, MdUFGT, and MdMYB1), while the opposite results were observed in RNAi-MdACO1 calli (Figure 6D). We also noticed that the expression of MdLNC610 was not affected by the transcription variation of MdACO1 (Figures 5, D, E and 6, D).

Functional MdLNC610 assays of apple fruit and calli
We next used a transient assay of apple fruit to determine whether MdLNC610 regulates the expression of MdACO1 and participates in ethylene production and light-induced anthocyanin biosynthesis during fruit coloration. Agrobacterium cultures harboring either the MdLNC610 overexpression vector (pRI-MdLNC610) or virus-induced gene silencing (VIGS) vector (TRV-MdLNC610) were injected into ‘Red Fuji’ apple epidermis samples. The injection sites were observed after high-light treatment. As shown in Figure 7A, overexpression of MdLNC610 promoted anthocyanin accumulation, while silencing suppressed epidermal coloration (Figure 7B). We also observed that ethylene production was promoted by overexpression of MdLNC610 and inhibited by VIGS-based silencing of the gene (Figure 7C). RT-qPCR results further revealed that the expression of MdACO1 and anthocyanin-related genes increased in MdLNC610-overexpressing fruit, and was considerably lower in the TRV-MdLNC610 fruit, compared with control fruit (Figure 7, D–G).

MdLNC610-silenced fruit treated with ethylene showed elevated anthocyanin accumulation, while decreased anthocyanin accumulation was observed in MdLNC610-overexpressing fruit after treatment with 1-MCP under high-light conditions, consistent with the involvement of MdLNC610 in ethylene production and anthocyanin accumulation. HPLC analysis and expression assays indicated that
Figure 4  High light may regulate MdACO1 expression via MdLNC610. A, Module-anthocyanin weight correlations and corresponding P-values (in parentheses). B, The location of MdACO1 and MdLNC610 on apple chromosome 10. C, Correlation and expression analysis of MdACO1 and related lncRNA, MdLNC610 by RNA-seq in four fruit coloration stages. D, MdLNC610 expression in apple fruit during light treatments and 1-MCP treatment. E, Cis-element analysis of the MdACO1 and MdLNC610 promoters, identifying the light-response element BOX4 in the MdLNC610 promoter. ABRE, ABA-responsive element; AuxRR-core, auxin-responsive region core; ARE, androgen-responsive element; MBS, MYB-binding site. F, Schematic representation of the GUS reporter vector containing the MdLNC610 promoter for growth under dark, moderate-light and high-light conditions. G, Reporter constructs in binary vectors were introduced into apple calli for GUS activity assays. MdLNC610pro::GUS transgenic apple calli were grown under dark, moderate-light and high-light conditions at 25°C and stained to visualize GUS activity. Apple calli were digitally extracted for comparison. H, Detection of GUS expression by RT-qPCR. RT-qPCR analysis was performed with three biological replicates. In (D) and (H) statistical analyses, error bars indicate the standard error of the mean (±SE) of three replicate measurements. And different letters above the bars indicate significantly different values (P < 0.05), calculated using one-way ANOVA followed by Tukey’s multiple range test.
MdLNC610 overexpression promoted anthocyanin accumulation via a pathway that is ethylene-dependent, and MdLNC610 expression corresponded with the expression level of MdACO1 (Figure 7).

Subsequently, MdLNC610 overexpression (OE-MdLNC610) or suppression (RNAi-MdLNC610) was induced in wild-type apple calli via Agrobacterium-mediated transformation. After light treatments for 15 d, the anthocyanin content of the calli was determined. Overexpression was seen to significantly increase anthocyanin accumulation compared with the wild-type, but there was no obvious color variation between RNAi-MdLNC610 calli and the wild-type (Figure 8, A and B). Furthermore, ethylene production was significantly lower in RNAi-MdLNC610 lines, and significantly higher in OE-MdLNC610 lines (Figure 8C). We also observed that MdLNC610 overexpression significantly increased the expression of MdACO1 and anthocyanin-related genes but that the opposite occurred in RNAi-MdLNC610 calli (Figure 8, D and E). These data suggest that MdLNC610 enhances light-induced anthocyanin biosynthesis by promoting MdACO1 expression.

MdLNC610 activates MdACO1 transcription

Transient luciferase (LUC) imaging assays were used to confirm the activation of MdLNC610 and its effect on the expression of MdACO1. Agrobacterium cultures harboring a plasmid containing MdACO1 fused to the LUC reporter gene (MdACO1pro::LUC) or 35S::MdLNC610 (for overexpression) were co-infiltrated into Nicotiana benthamiana leaves (Figure 9A). Luminescence signals were observed in the co-expression regions but not in the control region in N. benthamiana leaves (Figure 9B), and co-expression of 35S::MdLNC610 with MdACO1pro::LUC resulted in a substantially greater luminescence intensity (Figure 9C). These results indicated that MdLNC610 induces MdACO1 expression.

To verify these results, a GUS transactivation assay was conducted. The MdACO1pro::GUS construct was co-transformed into apple calli with 35S::MdLNC610 (Figure 9D). The transgenic calli containing MdACO1pro::GUS plus 35S::MdLNC610 exhibited significantly higher GUS enzyme activity in a staining assay than those harboring MdACO1pro::GUS alone (Figure 9, E and F), indicating that MdLNC610 activates GUS transcription driven by the MdACO1 promoter.
To assess the proximity of MdLNC610 and MdACO1, we conducted Hi-C (high-throughput chromosome conformation capture) sequencing of apple fruit. The results showed a strong cross-linking signal between MdACO1 and MdLNC610 (Figure 9G), located in the same topologically associated domain (TAD) region (Figure 9H; Supplemental Figure S1), suggesting that MdLNC610 is physically located close to MdLNC610.

Discussion

Ethylene plays a pivotal regulatory role in the ripening of climacteric fleshy fruits (Giovannoni, 2001; Seymour et al., 2013), which is often accompanied by the accumulation of anthocyanin pigments. However, the regulatory relationships or mechanisms that link ethylene and light with anthocyanin-associated coloration are not well defined. In this study, we identified a regulation mechanism in which IncRNA recognizes distant loci during apple fruit coloration. Our work shows that high-light treatment activates the expression of IncRNA-MdLNC610 to accurately trigger the transcription of ethylene biosynthesis gene MdACO1. Gradually, ethylene production leads to the accumulation of anthocyanin in apple fruit.

Regulation of ethylene production can have profound physiological consequences (Guo and Ecker, 2004) and ethylene synthesis is enhanced by a variety of external factors, including wounding, viral infection, elicitors, hormone treatment, chilling injury, drought, Cd2+ and Li+ ions, O3, SO2, CO2, and especially light (Thomma et al. 1999; Yang and Hoffman 1984; Bleecker and Kende 2000; Lin et al. 2009). Light affects many aspects of plant growth and development (Leivar and Monte, 2014; Inoue et al., 2017; Pham et al., 2018); however, the role of light in regulating ethylene biosynthesis, and specifically whether it enhances or inhibits ethylene biosynthesis, remains controversial for both vegetative tissues and fruits. In tomato (Solanum lycopersicum L. cv. Micro-Tom), light was found to suppress ethylene metabolism in ripening fruit, as indicated by the light-triggered reduction in ACC content, ACO activity and ethylene emission (Cruz et al., 2018). In another study, supplementary red light increased expression of the ripening regulator SlRIN (RIpening INHIBITOR), before inducing the expression of other ripening-related genes and promoting ethylene biosynthesis and signaling, resulting in earlier tomato fruit ripening (Zhang et al., 2020). It has also been reported that light does not significantly influence ethylene production rate in ‘Hakuho’ peach (Prunus persica Batsch) fruit (Li et al., 2007). However, blue light exposure induced fruit ripening, as evidenced by an accelerated decline in fruit firmness and epidermal color changes. This was associated with an increase in ethylene production, as a consequence of upregulated expression of PpLOX (LIPoxyGENASE) genes and ethylene biosynthetic genes (Gong et al., 2015). However, the nature of the interplay between light and ethylene in apple fruit has remained unclear. Here, we confirmed the important role of light in regulating ethylene biosynthesis in apple fruit. High-light treatment significantly promoted ethylene production in apple fruit through upregulated expression of ethylene biosynthesis gene, and that their expression was inhibited by 1-MCP application in high-light treated fruit (Figure 3). We suppose that the light quality and light intensity may be a key factor for the role of light in regulating ethylene biosynthesis in plant species and related mechanism remains to be studied.

Previous study demonstrated that fruit coloration is accompanied by rapid ethylene production during fruit ripening in apple fruit (Faragher and Brohier, 1984), which confirmed the important role of ethylene in fruit anthocyanin accumulation. Furthermore, the molecular mechanism
of how ethylene promotes anthocyanin accumulation during fruit ripening still need to be further elucidated. Recently, studies also showed that MdbHLH3, MdERF3, and MdMYB1 regulate fruit coloration by activating the expression of \textit{MdACS} and \textit{MdACO1} (An \textit{et al.}, 2018; Espley \textit{et al.}, 2019; Hu \textit{et al.}, 2019). In this research, our finding raised a mechanism by which ethylene regulates fruit ripening through the lncRNA regulation. We found that the \textit{MdACO1} transcript level significantly increased and was accompanied with the massive expression of \textit{MdLNC610}, thereby promoting ethylene production in ‘Red Fuji’ (Figures 2 and 4). Overexpression of \textit{MdLNC610} increased ethylene production and anthocyanin accumulation in apple fruit and calli by activating the expression of \textit{MdACO1}, while silencing \textit{MdLNC610} had the opposite effect (Figures 7 and 8). Here we show that \textit{MdLNC610} modulates the expression of genes related to ethylene synthesis, thus providing a framework for future investigations of the involvement of lncRNAs in the ethylene production and fruit ripening.

LncRNAs are known to coordinate gene expression via a number of complex mechanisms, acting as scaffolds for chromatin-modifying complexes, or as eTMs to block miRNA cleavage of their target mRNAs. Several studies have shown that lncRNAs coordinate gene expression in both a cis- and trans manner (Fatica and Bozzoni, 2014). For example, the expression of the apple lncRNA, \textit{MSTRG.85814} is...
induced by Fe deficiency. One MSTRG.85814 splice variant (MSTRG.85814.11) positively modulates its cis target mRNA derived from the small auxin upregulated gene SMALL AUXIN UP RNA 32 (SAUR32), and this in turn promotes the expression of SAUR32 and a plasma membrane proton ATPase, AHA10 (Sun et al., 2020). In A. thaliana, the IncRNA ELF18-INDUCED LONG-NONCODING RNA1 (ELENA1) enhances plant resistance to bacterial pathogens by activating the transcription of the PATHOGENESIS-RELATED 1 gene (Seo et al., 2017), and in tomato, IncRNA33732 can induce the expression of RESPIRATORY BURST OXIDASE, which increases H2O2 accumulation and promotes resistance to the oomycete pathogen Phytophthora infestans (Cui et al., 2019). Here, we showed that the light-induced IncRNA, MdLNC610, encodes a positive regulator and activates the expression of MdACO1 (Figures 4, 7–9), thus providing a framework for future investigations of the involvement of IncRNAs in fruit ripening. We also observed, through transiently co-expressing 35S::MdLNC610 with the MdACO1pro::LUC reporter in N. benthamiana leaves (Figure 9, B and C), that expression of the MdACO1 promoter was induced by MdLNC610, which suggested that MdLNC610 regulates the expression of MdACO1 in a trans manner. We propose that MdLNC610 may recruit a regulatory protein complex (histone modification proteins, a phosphorylation protein, or DNA methylation proteins) to other regulatory genes expression by influencing epigenetic modification in a variety of genomic contexts to coordinate MdACO1 expression (Wang et al., 2014a, 2014b, 2014c; Long et al., 2017; Zhao et al., 2018). However, Hi-C data indicate that MdLNC610 is physically located close to MdLNC610, and combined with the transgenic data, could support that MdLNC610 is a cis or trans regulator. Notably, a recent study showed that the AUXIN-REGULATED PROMOTER LOOP (APOLO) IncRNA is able to recognize multiple distant independent loci in the A. thaliana genome by chromatin isolation by RNA purification sequencing and RNA-seq. And Hi-C data suggested that APOLO recognizes its target locus by mediating R-loop formation. So APOLO IncRNA coordinates the expression of distant unrelated auxin-responsive genes in a trans manner (Ariel et al., 2020). We propose that a similar regulatory mechanism may underlie the regulation of MdACO1 expression by MdLNC610, which MdLNC610 may recognize the sequence of MdACO1 by sequence complementarity and the formation of R-loops. The above exemplifies high-light positive relationships in expression between DE-IncRNAs and their target genes. However, the mechanistic basis for alterations in IncRNA expression, or their modulation of the expression of ethylene biosynthesis genes, and as a consequence fruit coloration, will be the target of future studies.

In our previous study (Yang et al., 2019), we found that IncRNAs are also involved in anthocyanin biosynthesis. In apple, MLNC3.2 and MLNC4.6 act as miR156a eTMs via a direct complementary interaction with miR156a, thereby suppressing miR156a-mediated cleavage of SPL2-like and SPL33, and their participation in SPL2-like and SPL33-mediated anthocyanin accumulation (Yang et al., 2019). Meanwhile, we also construct a MdWRKY1-MdLNC499-MdERF109 transcriptional cascade in which MdWRKY1 is activated by light to increase the transcription of MdLNC499, which in turn induces MdERF109. The MdERF109 protein induces the expression of anthocyanin-related genes and the accumulation of
Figure 9 MdLNC610 activates the transcription of MdACO1 to enhance its expression. A, Schematic representation of the LUC reporter vector containing the MdACO1 promoter and the effector vector containing MdLNC610. B, The dual LUC assay shows promoter activity expressed as a ratio of promoter LUC to 35S::Renilla. Error bars are the SE for three replicate reactions. C, Transient LUC imaging assays showing that MdLNC610 activates the expression of MdACO1prom::LUC. Representative images of LUC activity in N. benthamiana leaves 72 h after infiltration were shown. D, Schematic diagrams of the effector (35S::MdLNC610) and reporter vectors (MdACO1prom::GUS) that were used for transient expression analysis. E, The effector and reporter constructs in the binary vectors were introduced into apple calli for GUS reporter activity assays. MdACO1prom::GUS transgenic apple calli with or without the 35S::MdLNC610 effector, were grown at 25°C in the dark and stained to visualize GUS activity. Apple calli were digitally extracted for comparison. F, Detection of GUS expression by RT-qPCR. RT-qPCR analysis was performed with three biological replicates. G, Hi-C interaction map (5-kDa bins) within the region of Chr10: 40,500,000–41,000,000. The color intensity represents the frequency of contact between two 5-kb loci. The black squares surround the modules that indicate the frequency of contact between two locus of MdACO1 and MdLNC610. H, Snapshot of identified TADs within the region of Chr10: 40,500,000–41,000,000 and delimited TAD-interior regions (internal lines), TAD boundaries (two-line endpoints) and location of MdACO1 and MdLNC610. Error bars indicate the standard error of the mean (±SE) of three replicate measurements. Different letters above the bars indicate significantly different values (P < 0.05), calculated using one-way ANOVA followed by Tukey’s multiple range test.
anthocyanins in the early stages of apple coloration (Ma et al., 2021). In this study, our results indicate that IncRNA MdLNC610 coordinates the expression of distal ethylene biosynthesis gene MdACO1 during high-light induced anthocyanin biosynthesis in apple fruit (Figures 2, 4, and 9). Taken together, we deduced that IncRNAs can act through a multitude of mechanisms to participate in apple fruit coloration.

Overall, our results provide insights that the IncRNA-MdLNC610 exerts an important role of transcriptional fine-tuning of trans targets in response to light treatment, expanding the possibilities about how IncRNAs shape fruit quality formation. These findings suggest a possible strategy for promoting anthocyanin accumulation by high-light or ethylene treatments, which will be usefully for developing biotechnological strategies to create new fleshy fruits with improved fruit quality and differing degrees of ripeness.

Materials and methods

Plant materials and treatments
Previous studies have shown that light treatment of debagged apple (M. domestica) fruit (apple fruit were debagged with double-layered light-impermeable paper bags and then exposed to sunlight) can trigger rapid anthocyanin accumulation, so this represents a potentially excellent system for studying the molecular mechanisms that regulate light-induced anthocyanin accumulation (Li and Cheng, 2008; Feng et al., 2013; Yang et al., 2019). Bagged ‘Red Fuji’ apple fruit were harvested 140 d after blooming. For ethylene treatments, debagged apples were exposed to 10 μL·L⁻¹ ethylene for 12 h at room temperature in an airtight container. The containers were stored in a phytotron with continuous high light (300 μmol·m⁻²·s⁻¹; 22°C). For 1-MCP treatments, the apples were exposed to 1 μL·L⁻¹ 1-MCP for 12 h at room temperature in an airtight container. After 1-MCP treatment, the airtight container was transferred to a phytotron with constant high light (300 μmol·m⁻²·s⁻¹; 22°C) for 8 d. Anthocyanin contents and ethylene production were measured at the given times. The two groups of fruit were treated simultaneously, and the tests were conducted on three biological replicates (three different apple fruit in each group).

For the high-light treatment, debagged apple fruit were exposed to high-light (300 μmol·m⁻²·s⁻¹; 22°C) conditions and harvested at the indicated time points. For the moderate-light treatment, debagged apple fruit were exposed to moderate-light (150 μmol·m⁻²·s⁻¹; 22°C) conditions and harvested at the indicated time points. For the light-transition treatments, debagged apple fruit were transferred to moderate light after 3 d of high-light treatment, or debagged apple fruit were transferred to high light after 3 d of moderate-light treatment, before being harvested at the indicated time points. Light measurements were made using a spectroradiometer (SPIC-200, Everfine Co., Hangzhou, China). Fruit were harvested at 0, 3, 5, 8, and 12 d after treatment and fruit epidermal samples were collected by peeling them such that the peels had < 1 mm of adhering cortical tissue. All samples were frozen in liquid nitrogen and stored at −80°C.

The apple calli were induced from the sarcocarp of ‘Orin’ apples (M. domestica Borkh.) (Horsch et al., 1988). They were grown on MS medium plus 0.5 mg·L⁻¹ IAA and 1.5 mg·L⁻¹ 6-BA at 25°C in the dark. The calli were subcultured three times with a 2-week interval before being used for genetic transformation and other assays.

Identification of apple peel IncRNAs
Transcriptome sequencing data from a previous study investigating light-induced anthocyanin accumulation in ‘Red Fuji’ apple peels (Yang et al., 2019; Bioproject Number: PRJNA555185, SRX6464003-SRX6464008) were used here. Differentially expressed gene (DEGs) analysis and correlation analysis of DEGs (Yang et al., 2019) were used to identify light-induced or ethylene-induced IncRNAs.

HPLC analysis
Frozen apple fruit peel and calli samples (~0.8–1.0 g fresh weight) were ground in 10 mL extraction solution (methanol: water: formic acid: trifluoroacetic acid = 10:22:21:1) and incubated at 4°C in the dark for 72 h, shaking the samples every 6 h. The supernatant was passed through filter paper and then through a 0.22 μm Millipore filter (Billerica, MA, USA). For the HPLC analysis, trifluoroacetic acid: formic acid: acetic acid: water (0.1: 2: 97.9) was used as mobile phase A and trifluoroacetic acid: formic acid: acetonitrile: water (0.1: 2: 48: 49.9) was used as mobile phase B. The gradients used were as follows: 0 min, 30% B; 10 min, 40% B; 50 min, 55% B; 70 min, 60% B; 30 min, 80% B. A high performance liquid chromatography (Agilent 1100, Agilent Technologies Inc., Santa Clara, CA, USA) with a 150-mm column was used for determination of anthocyanin content. When the retention time was ~5.9 min, the peak represented Cyanidin-3-O-glucoside (Revilla and Ryan, 2000; Li et al., 2020). And the peak area (x) was used to figure up anthocyanin content by following formula.

\[
\text{Anthocyanin content} = (R \cdot x + a) \cdot v/m
\]

“R” and “a” are the coefficients of the standard curve as measured by Cyanidin-3-O-glucoside standard samples. “x” is the peak area value, “v” is extraction solution, and “m” is weight of samples. And the representative chromatogram was shown as Supplemental Figure S2.

Measurements of ethylene production
Ethylene production by the apple fruit and apple calli was determined as described in Tan et al. (2013) and Li et al. (2016), respectively. Apple fruit were placed in individual containers and stored at room temperature for 3 h before measurement: between five and ten fruit were used. Apple calli were placed in individual bottles with agar and stored at room temperature for 3 h before measurement. One-
milliliter syringes were used to collect the gas samples and ethylene concentration was determined by GC–MS (Fiehn et al., 2000).

A gas chromatograph (Agilent 6890, Agilent Technologies Inc.) with capillary column (P/N 19091J-433, HP-5) was used for determination of ethylene production. N2 gas was used for eluant gas, H2 gas was used for make-up gas, and passed into the machine before action. The oven protocol was set as: the start temperature is 40°C; as 10°C/min heating rate up to 90°C, then as 15°C/min heating rate up to 170°C, and keep for 4 min. The detector is TCD, heater is set at 300°C. Each simple repeated three times, 2 mL gas was taken out from reservoir bag by manual GC syringes, pushed in the inlet for determination. When the time of intake was ~1.75 min, the peak represented ethylene, and the peak area (x) was used to figure up ethylene concentration by the following formula.

Ethylene concentration = R · x + a

“R” and “a” are the coefficients of the standard curve as measured by ethylene multi component calibration gas.

The ethylene production of simples was figured up by the following formula.

Ethylene production = (c · v)/(m · t)

“c” is the ethylene concentration of the measured gas, “v” is gas solution about storage gas, and “m” is weight of apples, “t” is storage time. And the representative chromatogram was shown as Supplemental Figure S3.

RNA extraction and RT-qPCR analysis
Total RNA was extracted from apple peels using an RNA Extraction Kit (Aidlab, Beijing, China) according to the manufacturer’s instructions. DNase I (TaKaRa, Ohtsu, Japan) was added to remove genomic DNA and the samples were converted to cDNA using the Access RT-PCR System (Promega, Madison, WI, USA), according to the manufacturer’s instructions. PCR primers (Supplemental Table S5) were designed using Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). RT-qPCR analysis was carried out in a total volume of 20 μL, containing 9 μL of 2 × SYBR Green qPCR Mix (TaKaRa, Ohtsu, Japan), 0.1 μM specific primers (each), and 100 ng of template cDNA. The reaction mixtures were heated to 95°C for 30 s, followed by 39 cycles at 95°C for 10 s, 50–59°C for 15 s, and 72°C for 30 s. A melting curve was generated for each sample at the end of each run to ensure the purity of the amplified products. MdActin (GenBank ID LOC103453508) was used as the internal control for DE-InCRNAs and mRNA, and M. domestica 5.8S ribosomal RNA (GenBank ID LOC114826676) was used as the internal control for DE miRNAs in these experiments. The $2^\Delta(\Delta C_t)$ analysis method was used to calculate expression levels (Livak and Schmittgen, 2001).

Construction of expression vectors and stable transformation of apple calli
To make the overexpression vectors, the full-length MdLNC610 and MdACO1 coding sequences were amplified by PCR from ‘Red Fuji’ apple peel cDNA and individually ligated in frame with the sequence encoding enhanced green fluorescent protein in the pGFPUSPLUS plant transformation vector (Vickers et al., 2007), using the XbaI restriction site. MdLNC610 (461 bp) and MdACO1 (174 bp) gene fragments were cloned into the pTCK303 plant transformation vector for RNA interference using the BamHI and KpnI restriction sites. MdLNC610-antisense and MdACO1-antisense sequences were cloned into the pTCK303 vector using the XbaI site. All primers used in this study are listed in Supplemental Table S5. Transformation of ‘Orin’ calli was performed as previously described (Li et al., 2020).

Transient expression in apple fruit
The pRI101 plant transformation vector (Li et al., 2016) was used for transient gene overexpression in apple fruit. The MdACO1 and MdLNC610 cDNAs were cloned into the pRI101 vector using the Ndel and XbaI restriction sites. For the VIGS assay, 371 bp MdLNC610 and 343-bp MdACO1 fragments were cloned into the pTRV2 vector (Liu et al., 2002) using the EcoRI and KpnI sites. Agrobacterium tumefaciens (GV3101) cells were resuspended in 10-mM MES, 10-mM MgCl2, and 200-μM acetosyringone solution to a final optical density of 1.5 at 600 nm, and then incubated at room temperature for 3–4 h without shaking.

Apples agro-infiltrated with pRL, pRL-MdACO1 or pRL-MdLNC610 were exposed to high-light (300 μmol m−2 s−1; 22°C) conditions and harvested 3 d postinfiltration. Before infiltration, A. tumefaciens cultures containing combinations of pTRV1 (acting as an assistant vector responsible for virus replication and for allowing systemic movement throughout the host) (Liu et al., 2002) and pTRV2, or its derivatives, were mixed in a 1:1 ratio. The infiltration protocol and culturing methods for transient expression were adapted from previously described methods (Tian et al., 2015; Zhang et al., 2016). The TRV, TRV-MdACO1, and TRV-MdLNC610 infected apples (‘Red Fuji’) were exposed to high-light (300 μmol m−2 s−1; 22°C) conditions and harvested 8 d postinfiltration. All peel samples were frozen in liquid nitrogen upon collection and stored at −80°C.

Transient expression assays in N. benthamiana
The transient expression assays were performed using N. benthamiana leaves (Yang et al., 2019). MdACO1 (1,678 bp) and MdLNC610 (2,000 bp) promoter fragments were cloned into the pGreenII 0800-LUC (Hellens et al., 2005; Xiang et al., 2015) vector using the KpnI and BamHI sites to generate the reporter constructs (MdACO1pro-LUC and MdLNC610pro-LUC). The effector (35S: MdLNC610) was generated by
recombining the MdLNC610 gene into the pGreenII 62-SK vector using the SacI and KpnI sites. The recombinant plasmids were transformed into A. tumefaciens GV3101. The bacteria were mixed and infiltrated into N. benthamiana leaves. A live imaging apparatus (NightSHADE LB 985, Berthold Technologies, Germany) was used for luminescence detection.

GUS staining assays for transient expression in apple calli
The MdLNC610 promoter fragment (2,000 bp) was cloned into a modified pB1101 vector with a minimal 35S promoter using the HindIII and BamHI sites (Guerrero et al., 1990). Apple calli overexpressing the proMdACO1::GUS construct were placed in the dark for 3 d.

The full-length MdLNC610 sequence was cloned into the pB1121 vector using the XbaI and BamHI sites, and the MdACO1 promoter sequence was cloned into the pB1101 vector using the HindIII and BamHI sites. Agrobacterium tumefaciens-mediated transient expression assays with apple calli were carried out as previously described (Li et al., 2012), as was GUS staining (Jefferson, 1987). All experiments were carried out with three biological replicates, 0.3–0.5 g apple calli were used in each biological replicates.

Construction of Hi-C interaction maps
The Hi-C interaction maps were constructed following the methods described in a previous study (Wang et al., 2015). The final Hi-C contact map was displayed using Juicebox (Durand et al., 2016). Hi-C sequencing data that support the findings of this study have been deposited in NCBI Bioproject database under accession number (PRJNA713418).

Statistical analyses
All experiments were conducted three times with consistent results, and data are presented for one representative experiment. Experimental results were analyzed using the IBM SPSS Statistics version 25 software by one-way analysis of variance and leastsignificant difference (LSD) post-hoc tests at the 5% significance level.

Accession numbers
Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers MdACO1 (MDP0000195885), MdACO2 (MDP0000200737), MdACO3 (MDP0000725984), MdCHS (MDP0000686666), MdCHI (MDP0000759336), MdF3H (MDP0000323864), MdDFR (MDP0000494976), MdANS (MDP0000240641), MdUGFT (MDP0000405936), MdMYB1 (MDP0000259614), MdAC5a (MDP0000145123), and MdACS6 (MDP000259614).

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

Supplemental Figure S1. Hi-C interaction map within Chromosome 10 of M. domestica.

Supplemental Figure S2. HPLC chromatogram and peak area.

Supplemental Figure S3. GC chromatogram of air, 400-ppm ethylene, and three technical replicates from a sample showing data processing for ethylene determination.

Supplemental Table S1. List of DE-lncRNAs from the Purple module.

Supplemental Table S2. DNA sequences used in this study.

Supplemental Table S3. Cis-element analysis of the ‘Red Fuji’ apple MdACO1 promoter.

Supplemental Table S4. Cis-element analysis of the ‘Red Fuji’ apple MdLNC610 promoter.

Supplemental Table S5. DNA primers used in this study.

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References
Adams DO, Yang SF (1981) Ethylene the gaseous plant hormone: mechanism and regulation of biosynthesis. Trends Biochem Sci 6: 161–164
Adams-Phillips L, Barry C, Giovannoni J (2004) Signal transduction systems regulating fruit ripening. Trends Biochem Sci 9: 331–338
An JP, Liu YJ, Zhang XY, Bi SQ, Wang XF, You CX, Hao YJ (2020b) Dynamic regulation of anthocyanin biosynthesis at different light intensities by the BT2-TCP46-MYB1 module in apple. J Exp Bot 71: 3094–3109
An JP, Fu J, Yao JF, Wang XN, You CX, Wang XF, Hao YJ (2017) The bZIP transcription factor MdHY5 regulates anthocyanin accumulation and nitrate assimilation in apple. Hortic Res 4: 17023
An JP, Wang XF, Espley RV, Lin WK, Bi SQ, You CX, Hao YJ (2020a) An apple B-Box protein MdBBX37 modulates anthocyanin biosynthesis and hypocotyl elongation synergistically with MdMYBs and MdHY5. Plant Cell Physiol 61: 130–143
An JP, Wang XF, Li YY, Song LQ, Zhao LL, You CX, Hao YJ (2018) EIN3-LIKE1, MYB1, and ETHYLENE RESPONSE FACTOR3 act in a regulatory loop that synergistically modulates ethylene biosynthesis and anthocyanin accumulation. Plant Physiol 178: 808–823
An JP, Wang XF, Zhang XW, Bi SQ, You CX, Hao YJ (2019) MdBBX2 regulates UV-B-induced anthocyanin biosynthesis through regulating the function of MdHYS and is targeted by MdBT2 for 265 proteasome-mediated degradation. Plant Biotechnol J 17: 2231–2233

Ariel F, Jegu T, Latrasse D, Romero-Barrios N, Christ A, Bennhamed C, Crespi M (2014) Noncoding transcription by alternative RNA polymerases regulates an auxin-driven chromatin loop. Mol Cell 55: 385–396

Ariel F, Lucero L, Christ A, Mammarella MF, Jegu T, Veluchamy A, Mariappan K, Latrasse D, Blein T, Liu C, et al. (2020) R-loop mediated trans action of the APOL0 long noncoding RNA. Mol Cell 77: 118

Atkinson RG, Bolitho KM, Wright MA, Itrurraga-Gotia-Bueno T, Reid SJ, Ross GS (1998) Apple ACC-oxidase and polygalacturo- nase: ripening-specific gene expression and promoter analysis in transgenic tomato. Plant Mol Biol 38: 449–460

Ban Y, Honda C, Hatsuyma Y, Igarashi M, Bessho H, Moriguchi T (2007) Isolation and functional analysis of a MYB transcription factor gene that is a key regulator for the development of red coloration in apple fruit. Plant Cell Physiol 48: 958–970

Bapat V, Trivedi P, Ghosh A, Sane V, Ganapathi T, Nath P (2010) Ripening of fleshy fruit: molecular insight and the role of ethylene. Biotechnol Advances 28: 94–107

Bleecker AB, Kende H (2000) Ethylene: a gaseous signal molecule in plants. Annu Rev Cell Dev Biol 16: 1–18

Bolitoh KM, Lay-Yee M, Knighton ML, Ross GS (1997) Antisense apple ACC-oxidase RNA reduces ethylene production in transgenic tomato fruit. Plant Sci 122: 91–99

Britsch L, Grisebach H (1998) Purification and characterization of (2S)-flavanone 3-hydroxylase from Petunia hybrida. Eur J Biochem 156: 569–577

Brumos J (2021) Gene regulation in climacteric fruit ripening. Curr Opin Plant Biol 63: 102042

Carbone F, Preuss A, De VR, D’AMICO E, Perrotta G, Boyo AG, Martens S, Rosati C (2009) Developmental, genetic and environmental factors affect the expression of flavonoid genes, enzymes and metabolites in strawberry fruits. Plant Cell Environ 32: 1117–1131

Cin VD, Danesin M, Boschetti A, Dorigoni A, Ramina A (2005) Ethylene biosynthesis and perception in apple fruitlet abscission (Malus domestica L. Borck). J Exp Bot 56: 2995–3005

Cruz AB, Bianchetti RE, Alves FRR, Purgatto E, Peres LEP, Rossi M, Freschi L (2018) Light, ethylene and auxin signaling interaction regulates carotenoid biosynthesis during tomato fruit ripening. Front Plant Sci 9: 1370

Cui J, Jiang N, Meng J, Yang GL, Liu WW, Zhou XX, Ma N, Hou XX, Luan YS (2019) LncRNA33732-respiratory burst oxidase module associated with WRKY1 in tomato-Phytophthora infestans interactions. Plant Cell 97: 933–946

Dandekar AM, Teo G, Delfilippi BG, Urrutx S, Passey AJ, Kader AA, Stow JR, Colgan RJ, James DJ (2004) Effect of down-regulation of ethylene biosynthesis on fruit flavor complex in apple fruit. Transgenic Res 13: 373–384

Dong JG, Fernández-Maculet JC, Yang SF (1992a) Purification and characterization of 1-aminocyclopropane-1-carboxylic acid oxide from apple fruit. Proc Natl Acad Sci USA 89: 9789–9793

Dong JG, Olson D, Silverstone A, Yang SF (1992b) Sequence of a cDNA coding for a 1-aminocyclopropane-1-carboxylic acid oxidase homolog from apple fruit. Plant Physiol 98: 1530–1531

Durand NC, Robinson JT, Shamim MS, Machol I, Mesirov JP, Lander ES, Aiden EL (2016) Juicebox provides a visualization system for Hi-C contact maps with unlimited zoom. Cell Syst 3: 99–101

Espley RV, Hellens RP, Putterill J, Stevenson DE, Kutty-Amma S, Allan AC (2007) Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. Plant J 49: 414–427

Espley RV, Hellens RP, Plunkett B, McGhie T, Henry-Kirk R, Hall M, Johnston JW, Punter MP, Bolding H, Nardozza S, et al. (2019) Red to brown: An elevated anthocyanic response in apple drives ethylene to advance maturity and fruit flesh browning. Front Plant Sci 10: 1248

Fang H, Dong Y, Yue X, Hu J, Jiang S, Xu H, Wang Y, Su M, Zhang J, Zhang Z, et al. (2019) The B-Box zinc finger protein MdBBX2O integrates anthocyanin accumulation in response to ultraviolet radiation and low temperature. Plant Cell Environ 42: 2090–2104

Faragher JD, Brohier RL (1984) Anthocyanin accumulation in apple skin during ripening: regulation by ethylene and phenylalanine ammonia-lyase. Sci Hortic 22: 89–96

Fatica A, Bozzone I (2014) Long non-coding RNAs: new players in cell differentiation and development. Nat Rev Genet 15: 7–21

Feng FJ, Li MJ, Ma FW, Cheng LL (2013) Phenylpropanoid metabolites and expression of key genes involved in anthocyanin biosynthesis in the shaded peel of apple fruit in response to sun exposure. Plant Physiol Bioch 69: 54–61

Fiehn O, Kopka J, Trethewey RN, Willmitter L (2000) Identification of uncommon plant metabolites based on calculation of elemental compositions using gas chromatography and quadrupole mass spectrometry. Anal Chem 72: 3573–3580

Giovannini J (2001) Molecular biology of fruit maturation and ripening. Annu Rev Plant Biol 52: 725–749

Gong D, Cao S, Sheng T, Shao J, Song C, Wu F, Chen W, Yang Z (2015) Effect of blue light on ethylene biosynthesis, signalling and fruit ripening in postharvest peaches. Sci Hortic 197: 657–664

Guerrero FD, Crossland L., Smutzer GS, Hamilton DA, Mascalierens JP (1990) Promoter sequences from a maize pollen-specific gene direct tissue-specific transcription in tobacco. Mol Gen Genet 224: 161–168

Guo HW, Ecker JR (2004) The ethylene signaling pathway: new insights. Curr Opin Plant Biol 7: 40–49

Hellens RP, Allan AC, Frien EL, Bolitoth K, Grabton K, Templeton MD, Karunairetnam S, Gleave AP, Laing WA (2005) Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. Plant Methods 1: 13

Henry-Kirk RA, Plunkett B, Hall M, McGhie T, Allan AC, Wargent JJ, Espley RV (2018) Solar UV light regulates flavonoid metabolism in apple (Malus x domestica). Plant Cell Environ 41: 675–688

Honda C, Moriya S (2018) Anthocyanin biosynthesis in apple fruit. Horticult J 87: 305–314

Horsch RB, Fry JE, Hoffmann NL, Wallroth M, Eichholtz D, Rogers SG (1988) A simple and general method for transferring genes into plants. Science, 227: 1229–1231

Hu DG, Yu QJ, Han PL, Xie XB, Sun CH, Zhang QY, Wang JH, Hao YJ (2019) The regulatory module MdPUB29-MdBH33 connects ethylene biosynthesis with fruit quality in apple. New Phytol 211: 1966–1982

Inoue K, Nishihama R, Kohchi T (2017) Evolutionary origin of phytochrome responses and signaling in land plants. Plant Cell Environ 40: 2502–2508

Jaakola L (2013) New insights into the regulation of anthocyanin biosynthesis in fruits. Trends Biochem Sci 18: 477–483

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol 5: 387–405

Jin JJ, Liu J, Wang H, Wong L, Chua NH (2013) PLnCDB: plant long non-coding RNA database. Bioinformatics 29: 1068–1071

Ju ZG, Yuan Y, Liu C, Wang Y, Tian X (2017) Dihydroflavonol reductase activity and anthocyanin accumulation in ‘Delicious’, ‘Golden Delicious’ and ‘Indo’ apples. Sci Hortic 224: 315–323

Klee HJ, Giovannoni JJ (2011) Genetics and control of tomato fruit ripening and quality attributes. Annu Rev Genet 45: 41–59

Kubasek WL, Shirley BW, Mckillop A, Goodman HM, Briggs W, Ausubel FM (1992) Regulation of flavonoid biosynthetic genes in germinating Arabidopsis seedlings. Plant Cell 4: 1229–1236

Lancaster JE (1992) Regulation of skin color in apples. Crit Rev Plant Sci 10: 487–502
Leivar P, Monte E (2014) PIFs: systems integrators in plant development. Plant Cell 26: 56–78

Li B, Jia HJ, Okamoto G (2007) Effects of post-harvest light conditions on quality and aromatic volatile formation in 'Hakuho' peach (Prunus persica Batsch) fruits. J Plant Physio Mol Biol 33: 205–12

Li H, Li Y, Yu JX, Wu T, Zhang J, Tian J, Yao YC (2020) MdMYB8 is associated with flavonol biosynthesis via the activation of the MdFLS promoter in the fruits of Malus crabapple. Hortic Res 7: 19

Li PM, Cheng LL (2008a) The shaded peel of apple fruit becomes more sensitive to high-light damage as fruit develops. HortScience 43: 1121–1123

Li R, Fu DQ, Zhu BH, Luo YB, Zhu HL (2019) MYB Repressors as Regulators of CO2 assimilation, ribulose-1,5-bisphosphate carboxylase/oxygenase, apple. Plant Physiol 160: 712–723

Martin C, Prescott A, Mackay S, Bartlett J, Vrijlandt E (2018) How do the shaded peel of apple fruit becomes more sensitive to high-light damage as fruit develops. HortScience 43: 1121–1123

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^(-ΔΔCT) method. Methods 25: 402–408

Long YC, Wang XY, Youmans DT, Cech TR (2017) How do IncRNAs regulate transcription? Sci Adv 3: eaao2110

Ma D, Constable CP (2019) MYB Repressors as Regulators of Phenylpropanoid Metabolism in Plants. Trends Plant Sci 24: 275–289

Ma C, Lu Y, Bai SL, Zhang WN, Duan XW, Meng D, Wang ZG, Wang AD, Zhou ZS, Li TZ (2014) Cloning and characterization of miRNAs and their targets, including a novel miRNA-targeted NBS-LRR protein class gene in apple (Golden Delicious). Mol Plant 7: 218–230

Ma HY, Yang T, Li Y, Zhang J, Wu T, Song TT, Yao YC, Tian J (2021) The long noncoding RNA MdINcRNA9 bridges MdWRKY1 and MdERF109 function to regulate early-stage light-induced anthocyanin accumulation in apple fruit. Plant Cell 33: 3309–3330

Martin C, Prescott A, Mackay S, Bartlett J, Vrijlandt E (1991) Control of anthocyanin biosynthesis in flowers of Antirrhinum majus. Plant J 1: 37–49

Ni J, Zhao Y, Tao R, Yin L, Gao L, Strid A, Qian M, Li J, Li Y, Shen J. et al. (2019) Ethylene mediates the branching of the jasmonate-induced flavonoid biosynthesis pathway by suppressing anthocyanin biosynthesis in red Chinese pear fruits. Plant Biotechnol J 18: 1223–1240

Ørom UA, Derrien T, Beringer M, Gumireddy K, Gardini A, Bussotti G, Lai F, Zytnicki M, Notredame C, Huang QH, et al. (2010) Long noncoding RNAs with enhancer-like function in human cells. Cell 143: 46–58

Park S, Sugimoto N, Larson MD, Beaudry R, Nocker VS (2006) Identification of genes with potential roles in apple fruit development and biochemistry through large-scale statistical analysis of expressed sequence tags. Plant Physiol 141: 811–824

Pham VN, Kathare PK, Huq E (2018) Dynamic regulation of PIFs by COP1-SPA complex to optimize photomorphogenesis in Arabidopsis. Plant J 96: 260–273

Revilla E, Ryan JM (2000) Analysis of several phenolic compounds with potential antioxidant properties in grape extracts and wines by high-performance liquid chromatography-photoelectro array detection. J Chromatogr A 881: 461–469

Ross GS, Knighton ML, Lay-Yee M (1992) An ethylene-related cDNA from ripening apples. Plant Mol Biol 19: 231–238

Schaffer RJ, Friel EN, Soulourey EJF, Bolitcho K, Thodey K, Ledger S, Bowen JH, Ma JH, Nain B, Cohen D, et al. (2007) A genomics approach reveals that aroma production in apple is controlled by ethylene predominantly at the final step in each biosynthetic pathway. Plant Physiol 149: 899–912

Seo JS, Sun HX, Park BS, Huang CH, Yeh SD, Jung C, Chua NH (2017) ELF18-INDUCED LONG-NONCODING RNA associates with mediator to enhance expression of innate immune response genes in Arabidopsis. Plant Cell 29: 1024–1038

Seymour GB, Østergaard L, Chapman NH, Knapp S, Martin C (2013) Fruit development and ripening. Annu Rev Plant Physiol 64: 219–241

Sun YQ, Hao PG, Lv XM, Tian J, Wang Y, Zhang XZ, Xu XF, Han ZH, Wu T (2020) A long non-coding apple RNA, M57RC.85814.11, acts as a transcriptional enhancer of SAUR32 and contributes to the Fe-deficiency response. Plant J 103: 53–67

Sunako T, Sakuraba W, Senda M, Akada S, Ishikawa R, Niizeki M, Harada T (1999) An allele of the ripening-specific 1-aminocyclopropane-1-carboxylic acid synthase gene (ACS1) in apple fruit with a long storage life. Plant Physiol 119: 1297–1304

Takos AM, Jaffe FW, Jackson SR, Bogs J, Robinson SP, Walker AR (2006) Light-induced expression of a MYB gene regulates anthocyanin biosynthesis in red apples. Plant Physiol 142: 1216–1232

Tan D, Li T, Wang A (2013) Apple 1-aminocyclopropane-1-carboxylic acid synthase genes, MdACS1 and MdACS3a, are expressed in different systems of ethylene biosynthesis. Plant Mol Biol Rep 31: 204–209

Tan T, Bangerth F (2000) Regulation of ethylene, ACC, MACC production, and ACC-oxidase activity at various stages of maturity of apple fruit and the effect of exogenous ethylene treatment. Gartenbauwissenschaft 65: S121–S128

Thomma BP, Eggemont K, Tierens KS, Broekaert WF (1999) Requirement of functional ethylene-insensitive 2 gene for efficient resistance of Arabidopsis to infection by Botrytis cinerea. Plant Physiol 121: 1093–1102

Tian J, Chen MC, Zhang J, Li KT, Song TT, Zhang X, Yao YC (2017) Characterizations of dihydroflavonol 4-reductase gene promoters from different leaf colored Malus crabapple cultivars. Hort Res 4: 17070

Tian J, Peng Z, Zhang J, Song TT, Wan HH, Zhang ML, Yao YC (2015) MCMYB10 regulates coloration via activating MCF3H and later structural genes in ever-red leaf crabapple. Plant Biotechnol J 13: 948–961

Ubi BE, Honda C, Besho H, Kondo S, Wada M, Kobayashi S, Moriguchi T (2006) Expression analysis of anthocyanin biosynthetic genes in apple skin: effect of UV-B and temperature. Plant Sci 170: 571–578

Vickers CE, Schenk PM, Li D, Mullineaux PM, Gresshoff PM (2007) pGFPGUSPlus, a new binary vector for gene expression studies and optimising transformation systems in plants. Biotechnol Lett 29: 1793–1796

Vimolmangkang S, Zheng DM, Han YP, Khan MA, Soria-Guerra RE, Korban SS (2014) Transcriptome analysis of the exocarp of...
apple fruit identifies light-induced genes involved in red color pigmentation. Gene 534: 78–87

Wakasa Y, Kudo H, Ishikawa R, Akada S, Senda M, Niizeki M, Harada T (2006) Low expression of an endopolygalacturonase gene in apple fruit with long-term storage potential. Postharvest Biol Tec 39: 193–198

Wang AD, Tan DM, Tatsuki M, Kasai A, Li TZ, Saito H, Harada T (2009a) Molecular mechanism of distinct ripening profiles in 'Fujii' apple fruit and its early maturing sports. Postharvest Biol Tec 52: 38–43

Wang AD, Yamakake J, Kudo H, Wakasa Y, Hatsuyama Y, Igarashi M (2009b) Null mutation of the MdACS3 gene coding for a ripening-specific 1-aminocyclopropane-1-carboxylate synthase leads to long shelf life in apple fruit. Plant Physiol 151: 391–399

Wang C, Liu C, Roqueiro D, Grimm D, Schwab R, Becker C, Lanz C, Weigel D (2015) Genome-wide analysis of local chromatin packing in Arabidopsis thaliana. Genome Res 25: 246–256

Wang H, Chuang PJ, Liu J, Jang IC, Kean MJ, Xu J, Chua NH (2014a) Genome-wide identification of long noncoding natural antisense transcripts and their responses to light in Arabidopsis. Genome Res 24: 444–453

Wang HQ, Arakawa O, Motomura Y (2000) Influence of maturity and bagging on the relationship between anthocyanin accumulation and phenylalanine ammonia-lyase (PAL) activity in 'Jonathan' apples. Postharvest Biol Tec 19: 123–128

Wang KL, Li H, Ecker JR (2002) Ethylene biosynthesis and signaling networks. Plant Cell 14: S131–S151

Wang P, Xue Y, Han Y, Lin L, Wu C, Xu S, Jiang Z, Xu J, Liu Q, Cao X (2014c) The STAT3-binding long noncoding RNA Lnc-DC controls human dendritic cell differentiation. Science 344: 310–313

Wang Y, Luo XJ, Sun F, Hu JH, Zha XJ, Su W, Yang JS (2018) Overexpressing IncRNA LAIR increases grain yield and regulates neighbouring gene cluster expression in rice. Nat Commun 9: 3516

Wang YQ, Fan XD, Lin F, He GM, Terzaghi W, Zhu DM, Deng XW (2014b) Arabidopsis noncoding RNA mediates control of photomorphogenesis by red light. Proc Natl Acad Sci U S A 111: 10359–10364

Wienand U, Weydemann U, Niesbach-Klösgen U, Peterson PA, Saedler H (1986) Molecular cloning of the C2 locus of Zea mays, the gene coding for chalcone synthase. Mol Genet Genomics 203: 202–207

Winkel-Shirley B (2001) Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol 126: 485–493

Winkel-Shirley B (2002) Biosynthesis of flavonoids and effects of stress. Curr Opin Plant Biol 5: 218–223

Yang T, Ma H, Zhang J, Wu T, Song T, Tian J, Yao Y (2019) Systematic identification of long noncoding RNAs expressed during light-induced anthocyanin accumulation in apple fruit. Plant J 100: 572–590

Yang SF, Hoffman NE (1984) Ethylene biosynthesis and its regulation in higher plants. Annu Rev Plant Physiol 35: 155–189

Yue PT, Lu Q, Liu Z, Lv TX, Li XY, Bu HD, Liu WT, Xu YX, Yuan H, Wang AD (2020) Auxin-activated MdARF5 induces the expression of ethylene biosynthetic genes to initiate apple fruit ripening. New Phytol 226: 1781–1795

Zhang J, Tian J, Tai DQ, Li KT, Zhu YJ, Yao YC (2016) An optimized TRV-based virus-induced gene silencing protocol for Malus crabapple. Plant Cell Tiss Org 126: 499–509

Zhang JY, Zhang YT, Song SW, Su W, Hao YW, Liu HC (2020) Supplementary Red light results in the earlier ripening of tomato fruit depending on ethylene production. Environ Exp Bot 175: 104044

Zhang Y, Jiang L, Li Y, Chen Q, Ye Y, Zhang Y, Luo Y, Sun B, Wang X, Tang H (2018) Effect of red and blue light on anthocyanin accumulation and differential gene expression in strawberry (Fragaria × ananassa). Molecules 23: 820

Zhang YC, Liao JY, Li ZY, Yu Y, Zhang JP, Li QF, Qu LH, Shu WS, Chen YQ (2014) Genome-wide screening and functional analysis identify a large number of long noncoding RNAs involved in the sexual reproduction of rice. Genome Biol 15: S12

Zhao X, Li J, Lian B, Gu H, Li Y, Qi Y (2018) Global identification of Arabidopsis IncRNAs reveals the regulation of MAF4 by a natural antisense RNA. Nat Commun 9: 5056

Zhu J, Gardiner SE, Lay-Yee M (1995) Physical mapping of three fruit ripening genes: endopolygalacturonase, ACC oxidase and ACC synthase from apple (Malus × domestica) in an apple rootstock A106 (Malus sieboldii). Cell Res 5: 243–253