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Overexpression of a Tonoplast Malate Transporter Gene Leads to Enhanced Anthocyanin Biosynthesis in Apple

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ABSTRACT

Anthocyanins, a group of secondary metabolites synthesised in the phenylpropanoid pathway, largely determine the peel colour of fleshy fruits, but it is not known if their synthesis is linked to vacuolar malate accumulation that underlies fruit acidity. Here, we show that when the coding sequence of *Ma1* (*cMa1*), encoding a tonoplast malate transporter for controlling apple fruit acidity, is overexpressed in 'Royal Gala' apple, anthocyanin biosynthesis in the fruit peel is enhanced, corresponding to the downregulation of the expression of *MdMYB73*, a transcriptional activator for *Ma1*. RNAi suppression of *MdMYB73* expression increases anthocyanin biosynthesis whereas its transient overexpression decreases anthocyanin biosynthesis in apple fruit peel. MdMYB73 binds to all 7 MYB-sites in the promoter of the gene encoding UDP-glucose: flavonoid-3-O-glucosyltransferase (UFGT), the enzyme that catalyses the last step in anthocyanin synthesis, to repress its expression. When *MdMYB73* expression is suppressed by RNAi, *MdUFGT* expression is enhanced, leading to more anthocyanin synthesis, but this effect is blocked by RNAi suppression of *MdUFGT* expression. In addition, MdMYB73 competes with MdMYB1, a key transcriptional activator of anthocyanin synthesis, by binding to the same MYB-sites in the promoter of *MdUFGT*. These results indicate that, in addition to being a transcriptional activator for *Ma1*, MdMYB73 negatively regulates anthocyanin biosynthesis via repressing *MdUFGT* expression and competing with MdMYB1 for binding to the *MdUFGT* promoter in apple peel. In *cMa1*-OE fruits, downregulation of *MdMYB73* releases *MdUFGT* from MdMYB73 repression, which allows more MdMYB1 to bind to the promoter of *MdUFGT*, leading to enhanced anthocyanin biosynthesis.

1 | Introduction

Anthocyanins are a group of flavonoids responsible for fruit coloration in many fleshy fruits. In commercial fruit production, the intensity and extent of the peel red colour are often used as an indicator for fruit ripeness and quality. In addition to attracting pollinators and aiding seed dispersal, anthocyanins are involved in plant stress resistance and human health due to their antioxidant effects (Chalker-Scott 1999; Castañeda-Ovando et al. 2009; He and Monica Giusti 2010; Ghosh and Konishi 2007). Anthocyanins are synthesised in the phenylpropanoid pathway via a series of reactions catalysed by chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), leucoanthocyanidin dioxygenase (ANS) and UDP-glucose: flavonoid-3-*O*-glucosyltransferase (UFGT) (Koes et al. 2005; Holton and Cornish 1995). After being synthesised, they are transported into the vacuole for storage by multi-drug resistance proteins (MRPs), glutathione s-transferases (GSTs), multi-antimicrobial extrusion (MATE)-like and ATP-binding cassette transporters (Goodman et al. 2004; Conn et al. 2008; Hu et al. 2016). Overexpression or suppression of any single gene involved in

anthocyanin synthesis or transport could alter its accumulation in target tissues (Griesser et al. 2008; Han et al. 2012; Zhao et al. 2012).

Many transcription factors including MYB, bHLH (basic helix–loop–helix) and WD40 have been identified to regulate anthocyanin biosynthesis. In plants, MYBs either directly bind to the promoters of the structural genes or interact with bHLH and WD40 to form an MYB-bHLH-WD40 (MBW) complex regulating anthocyanin biosynthesis (Mol et al. 1998; Jaakola 2013; Ramsay and Glover 2005). Depending on the number of conserved MYB domains, the MYB protein family is categorised into four subclasses: 4R-MYB (R1/R2-like repeats), 3R-MYB (R1R2R3-type MYB), 2R-MYB (R2R3-MYB) and 1R-MYB (R1/2, R3-MYB). R2R3-MYB is the largest subclass, which is further divided into 25 subgroups in *Arabidopsis* based on the conserved DNA binding domain and amino acid motifs in the C-terminal (Stracke et al. 2001; Dubos et al. 2010). Many R2R3 MYBs have been reported to play a role in anthocyanin biosynthesis, such as *ZmC1* in maize (Paz-Ares et al. 1987), AtMYB75 in *Arabidopsis thaliana* (Borevitz et al. 2000), MdMYB1/A/10 in apple (Takos et al. 2006; Matus et al. 2008; Espley et al. 2009) and PcMYB10 in pear (Wang et al. 2013). Many of the MYBs characterised so far are transcriptional activators for key structural genes in anthocyanin biosynthesis (Albert et al. 2014; Hu et al. 2016; Yao et al. 2017; Liu et al. 2016). However, some MYBs are transcriptional repressors for anthocyanin biosynthesis, such as MdMYB6, MdMYB15L, MdMYB16 and MdMYB17 in apple (Gao et al. 2011; Xu et al. 2017, 2018; Wang et al. 2022), MYB-LIKE2 in Arabidopsis (Xing et al. 2024), CsMYB3 in Citrus (Huang et al. 2020) AmMYB308 in *Antirrhinum majus* (Tamagnone et al. 1998), FaMYB1 in strawberry (Aharoni et al. 2001; Salvatierra et al. 2013), RH2 (RED HEART2) in *Medicago truncatula* (Wang et al. 2021), PhMYB27 in petunia, VvMYBC2-L1/3 and VvMYB4-like in grapevine (Cavallini et al. 2015; Pérez-Díaz et al. 2016) and PpMYB17-20 in peach (Zhou et al. 2016). These MYB repressors have the ethylene-responsive element binding factor-associated amphiphilic repression (EAR) domain (Ohta et al. 2001; Ma and Constabel 2019), which allows for the binding of co-repressors (Szemenyei et al. 2008; Shyu et al. 2012), suppressing the expression of their target genes in anthocyanin synthesis. However, it remains unclear if any of the R2R3 MYB transcriptional repressors also act as transcriptional activators for genes involved in another process.

Fruit acidity is a major contributor to the taste and flavour of fleshy fruits. Malic acid accounts for most of the acidity in apple and many other fleshy fruits (Zhang et al. 2010; Etienne et al. 2013). Malic acid accumulation in the vacuole of plant cells is mediated by two tonoplast malate transporters, aluminium-activated malate transporter 9 (ALMT9) and tonoplast dicarboxylate transporter (tDT), via an acid trap mechanism (Emmerlich et al. 2003; Etienne et al. 2013; Kovermann et al. 2007). Vacuolar H^+-ATPase (VHA) and H^+-pyrophosphatase (VHP) pump protons from the cytosol into the vacuole, lowering its pH. Upon entering the vacuole, malate is immediately protonated to maintain its concentration gradient for continuous diffusion into the vacuole. Acidity and anthocyanin accumulation are co-regulated in plant species such as apple, petunia and citrus. The MBW complex that regulates anthocyanin biosynthesis also modulates vacuolar acid accumulation in plants. MYB1 transcriptionally activates the expression of genes encoding B subunits and E2 subunits of VHA, VHP1, MATE-Like 1 and tDT1 to facilitate vacuolar acidification and the transport of both malate and anthocyanins into vacuoles in apple and Arabidopsis (Hu et al. 2016). In petunia, PH4, an R2R3 MYB, interacts with ANTHOCYANIN 1 (AN1), a bHLH transcription factor, to control both anthocyanin synthesis and vacuolar acidification by transcriptional regulation of the structural genes in anthocyanin synthesis and those encoding tonoplast proton pumps (Spelt et al. 2000, 2002; Quattrocchio et al. 2006). The physical interaction of PH1, a tonoplast P_{3B}-ATPase and PH5, a tonoplast H^+-pumping P_{3A}-ATPase, boosts the H^+ transport activity of PH5, which acidifies the vacuole and keeps the flowers in red or violet colour; failure of vacuolar acidification leads to the blue petal colour in Petunia mutants, ph 1 to ph 7 (Faraco et al. 2014). In citrus, Ruby, an R2R3 MYB transcriptional activator, is essential for anthocyanin biosynthesis (Butelli et al. 2012, 2017). However, despite having a functional Ruby, some citrus accessions show extremely low acidity in fruit (acidless) as well as a lack of anthocyanins in leaves and flowers and a lack of proanthocyanidins in seeds. It turns out that the *Noemi* gene, a bHLH transcription factor, controls both fruit acidity and flavonoid biosynthesis by interacting with Ruby, and the acidless trait is genetically associated with large deletions or insertions of retrotransposons in the *Noemi* gene (Butelli et al. 2019). Analogous to petunia, citrus homologues, *CitPH1* and *CitPH5* are expressed in accessions with high acidity, but their transcript levels are dramatically reduced in acidless genotypes due to mutations that disrupt the expression of MYB, bHLH and/or WRKY transcription factors (Strazzer et al. 2019). However, it is not known if a malate or citrate transporter is part of the molecular network that regulates fruit colour in addition to fruit acidity.

Ma1 underlies the *Ma* locus, a major quantitative trait locus controlling fruit acidity in apple (Visser and Verhaegh 1978; Liebhard et al. 2003; Bai et al. 2012). *Ma1* encodes ALMT9 in the tonoplast (Bai et al. 2012; Li et al. 2020), with its expression activated by MdMYB73 (Hu et al. 2017, 2025), an R2R3 MYB transcription factor that is a homologue of AtMYB73 in subgroup 22 with an EAR domain at its N-terminal (Figure S1; Stracke et al. 2001). *Ma1* undergoes alternative splicing, generating two isoforms: Ma1β being 68 amino acids shorter with much lower expression than the full-length Ma1α. Ma1β does not have malate transport function but interacts with the functional Ma1α to form heterodimers, creating synergy with Ma1α for malate transport when Ma1β is equal to or exceeds the threshold value 1/8 of Ma1α (Li et al. 2024). Overexpression of the coding sequence of *Ma1* (*cMa1* or *Ma1α*) in 'Royal Gala' apple (of *Ma1ma1* genotype) triggers feedback inhibition on the native *Ma1* gene expression via *MdMYB73*, lowering the Ma1β/Ma1α ratio below the threshold that leads to a significant reduction in *Ma1* function and malic acid accumulation. We observed that the *cMa1*-OE fruits exhibited enhanced red colour development. In this work, we show that MdMYB73 acts as a negative regulator for anthocyanin biosynthesis by repressing the expression of *MdUFGT* and competing with MdMYB1 for binding to the *MdUFGT* promoter. In *cMa1*-OE fruits, downregulation

of *MdMYB73* releases *MdUFGT* from its repression, which allows more MYB1 binding to *MdUFGT* promoter, leading to upregulation of anthocyanin biosynthesis.

2 | Results

2.1 | Overexpression of *Ma1* Increases Anthocyanin Biosynthesis in Apple Fruit Peel

When the transgenic lines of 'Royal Gala' overexpressing the coding sequence of *Ma1* (*cMa1*-OE), L6, L14 and L16 produced the first fruits, we observed that all the transgenic fruits had redder coloration over a larger surface area of the fruit compared with the wild-type (WT) control (Figure S2). This suggests that *cMa1*-OE somehow enhances anthocyanin biosynthesis in apple fruit peel. In the subsequent growing season, we took fruit peel samples from the 3 *Ma1*-OE lines along with WT at four developmental stages from 50 days before harvest (DBH) when red colour began to develop to fruit harvest (50, 25 and10DBH and at harvest). Peel colour photos were taken, and anthocyanin concentrations were measured (Figure 1). Concentrations of anthocyanins increased from 50 DBH to fruit harvest for all genotypes, but compared with WT, *cMa1*-OE fruits had significantly higher concentrations of anthocyanins at each developmental stage and a larger percentage of the surface with red colour at harvest (Figure S3A). Consistent with our earlier report (Li et al. 2024), the *cMa1*-OE fruits had about 1/3 of the WT titratable acidity and slightly higher soluble solids at harvest (Figure S3B,C).

Alternative splicing of the *Ma1* gene generates two transcripts, *Ma1α* and its alternative splicing isoform *Ma1β* (Li et al. 2024). To confirm the effect of *Ma1*-OE on anthocyanin biosynthesis, we transiently overexpressed *Ma1α*, *Ma1β* and genomic *Ma1* (*gMa1*) and suppressed *Ma1* via RNAi in the 'Zestar' apple peel (Figure 2A). 'Zestar' was chosen because of its high sensitivity to light for anthocyanin synthesis. Overexpression of *Ma1α*, *Ma1β* or *gMa1* significantly increased, whereas transient *Ma1*-RNAi significantly decreased the peel red colour and anthocyanin concentrations (Figure 2A,B). We measured the overall transcript level of *Ma1* along with the anthocyanin biosynthesis key regulator *MdMYB1* and structural genes *MdCHS*, *MdCHI*, *MdF3H*, *MdDFR*, *MdANS* and *MdUFGT* in the transient *Ma1*-OE and *Ma1*-RNAi peels (Figure 2C–J). Overexpression of *Ma1α*, *Ma1β* or *gMa1* significantly increased the expression levels of *Ma1* and the structural genes *MdUFGT*, *MdDFR* and *MdANS*, whereas RNAi of *Ma1* significantly decreased their expression levels, with those of *MdCHS*, *MdCHI* and *MdF3H* unaltered.

2.2 | *MdMYB73* Expression Is Decreased in the Peel of *Ma1*-OE Fruits

To identify the molecular link between *Ma1* overexpression and enhanced anthocyanin synthesis, we analysed the expression levels of key structural genes in anthocyanin biosynthesis, *MdCHS*, *MdCHI*, *MdF3H*, *MdDFR*, *MdANS* and *MdUFGT*, during fruit colour development (Figure 3A). Many of these genes showed higher expression levels in *cMa1*-OE fruits at the early stages of colour development, consistent with higher anthocyanin biosynthesis. At harvest, *MdDFR*, *MdANS* and *MdUFGT* remained at significantly higher expression levels in *cMa1*-OE lines compared with WT. As *MdMYB1* is a key transcriptional activator for both *MdDFR* and *MdUFGT* genes in anthocyanin synthesis in apple (Takos et al. 2006; Xie et al. 2017; Ma et al. 2019), we analysed its expression level and found that *MdMYB1* expression was significantly enhanced in *cMa1*-OE fruit peels compared with WT (Figure 3A). Based on these data,

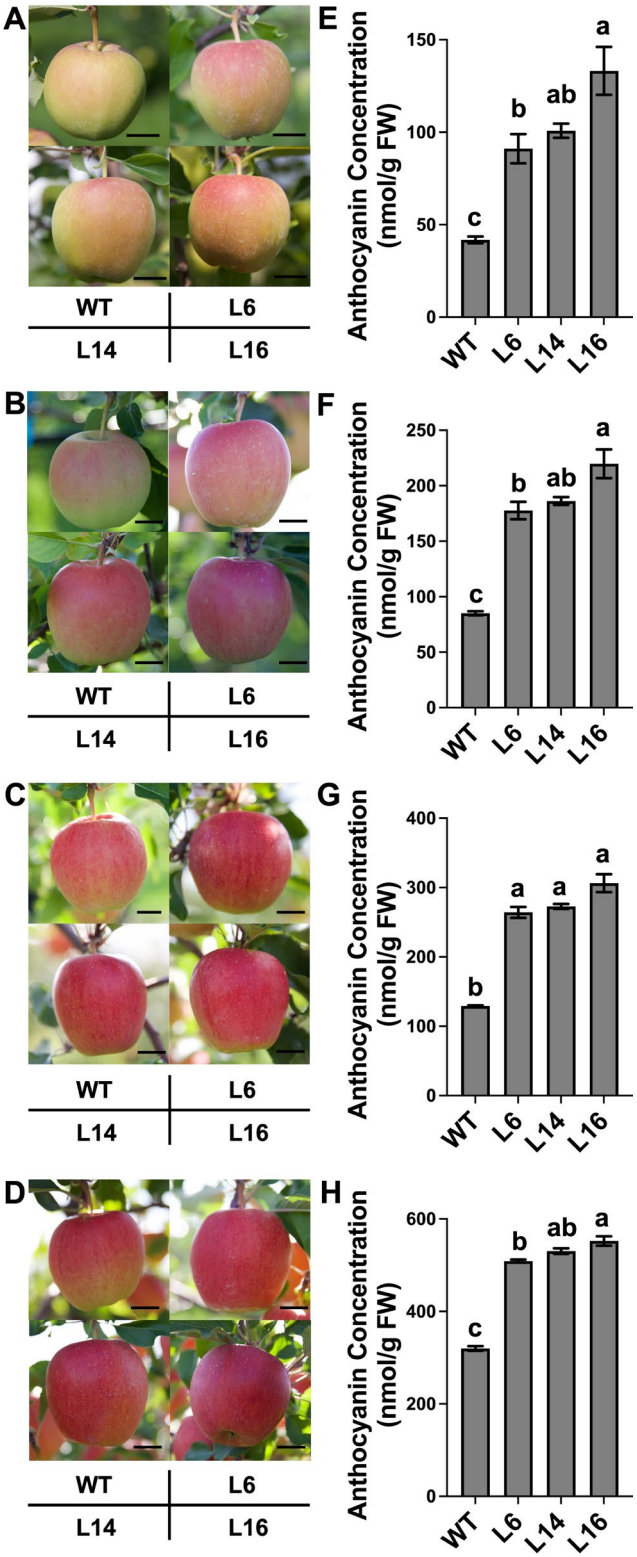

FIGURE 1 | Overexpression of the coding sequence of *Ma1* (*cMa1*) increases anthocyanin concentrations in apple fruit peel. (A–D) Apple peel colour in wild-type (WT) and *cMa1*-overexpression lines of 'Royal Gala', L6, L14 and L16 at 50, 25, 10 days before harvest (DBH), and at harvest, respectively. Bar = 2 cm. (E–H) Anthocyanin concentrations of WT, L6, L14 and L16 apple peels at 50, 25, 10. DBH, and at harvest, respectively. Data are mean ± SE of three biological replicates with four fruits per replicate. Different letters (a, b and c) indicate significant differences between genotypes using Tukey's HSD test at $p < 0.05$ after ANOVA.

a plausible explanation for the enhanced anthocyanin biosynthesis in *cMa1*-OE fruits is that *cMa1*-OE somehow increases *MdMYB1* expression, leading to up-regulation of anthocyanin biosynthesis. However, we found that the expression level of *MdMYB73*, a transcription factor that activates the expression of *Ma1* as well as two other genes encoding vacuolar proton pumps *MdVHA-A* and *MdVHP1* for vacuolar acidification in apple (Hu et al. 2017), was significantly decreased in *cMa1*-OE lines compared with WT (Figure 3B), consistent with that detected in the bulk fruit earlier (Li et al. 2024). This suggests a feedback regulatory loop between *MdMYB73* and *Ma1*. Overexpression of *Ma1α*, *Ma1β* or *gMa1* also significantly decreased *MdMYB73* expression levels, whereas *Ma1*-RNAi significantly increased *MdMYB73* transcript levels (Figure 3C). Considering the findings in Citrus that fruit acidity and anthocyanin synthesis are regulated by the same MBW complex (Butelli et al. 2012, 2017, 2019), we explored if *MdMYB73* plays a role in regulating anthocyanin biosynthesis as well as fruit acidity in apple.

2.3 | MdMYB73 Negatively Regulates Anthocyanin Biosynthesis in Apple Peel

As anthocyanin biosynthesis is negatively associated with the expression level of *MdMYB73* (Figures 1, 2 and 3B,C), we reasoned that *MdMYB73* would act as a repressor if it has any role in anthocyanin biosynthesis. To explore this idea, we constructed an RNAi vector of *MdMYB73* and transformed it into WT 'Royal Gala' apple. Of the 10 independent transgenic lines generated, we selected three transgenic lines, Z3, Z7 and Z8, with significantly lower *MdMYB73* expression levels (Figure S4A) in leaves, grafted them onto G.11 rootstock and grew the trees until they produced the first fruits for peel colour analysis. We took peel samples at harvest and analysed peel anthocyanin concentrations along with gene expression levels of *MdMYB73*, *Ma1*, *MdCHS*, *MdCHI*, *MdF3H*, *MdDFR*, *MdANS* and *MdUFGT*. Peel anthocyanin concentrations were significantly higher in the *MdMYB73*-RNAi lines compared with WT (Figure 4B). *MdMYB73*-RNAi significantly decreased the expression levels of *MdMYB73* and *Ma1* but increased the expression levels of *MdDFR*, *MdANS* and *MdUFGT* (Figure 4C–G), with those of *MdCHS*, *MdCHI* and *MdF3H* unaltered (Figure S4E–G).

To confirm the in-planta function of *MdMYB73*, we also used virus-induced gene silencing (VIGS) and virus-based gene expression to suppress and overexpress *MdMYB73*, respectively, in the 'Zestar' apple peel (Figure S5). *MdMYB73*-RNAi significantly increased peel anthocyanin biosynthesis whereas *MdMYB73*-OE decreased peel red colour (Figure S5A,B). The expression level of *Ma1* in the peel was significantly decreased and increased by *MdMYB73*-RNAi/OE, respectively, consistent with MdMYB73 being a transcriptional activator for *Ma1* (Hu et al. 2017; Li et al. 2024). However, the expression levels of *MdDFR*, *MdANS* and *MdUFGT* were significantly increased and decreased by transient *MdMYB73*-RNAi and *MdMYB73*-OE, respectively (Figure S5C). We subsequently conducted VIGS suppression of *MdMYB73* in the WT 'Royal Gala' fruit peel and virus-based gene overexpression in *cMa1*-OE fruit peel (Figure S6). RNAi suppression of *MdMYB73* significantly increased anthocyanin biosynthesis in WT apple peel whereas overexpression of *MdMYB73* significantly decreased anthocyanin biosynthesis in L6, L14 and L16 fruit peel. These data are consistent with those obtained on stably transformed *MdMYB73* RNAi lines and confirm that MdMYB73 is a repressor for anthocyanin biosynthesis in apple.

2.4 | MdMYB73 Binds to the *MdUFGT* Promoter to Repress Its Expression

As *MdUFGT* is a key structural gene in anthocyanin biosynthesis that is transcriptionally regulated by MYB1 (Xie et al. 2017), and its expression was significantly increased in the peel of *cMa1*-OE fruits (Figure 3A) and responded to RNAi suppression and overexpression of *MdMYB73* in opposite directions (Figure 4G; Figures S5C and S6G), we first explored the possibility that MdMYB73 may regulate anthocyanin biosynthesis via *MdUFGT* in apple fruit peel. We analysed the 2000-bp promoter region of *MdUFGT* and found seven putative MYB binding sites (U1–U7) (Figure 5A). We cloned the 2000-bp promoter of *MdUFGT* into the pHis2 vector and performed a yeast one-hybrid assay between pHis2-*MdUFGT pro* and pGAD424-*MdMYB73* (Figure 5B). Significantly better yeast growth was obtained on the 100 mM 3-AT selection SD plate, confirming the interaction between MdMYB73 and the *MdUFGT* promoter. We next conducted a chromatin immunoprecipitation (ChIP)-PCR assay to examine the interaction between MdMYB73 and different MYB binding sites in the *MdUFGT* promoter (Figure 5C). As U1 and U2, U3 and U4, U5 and U6 MYB binding sites were too close to each other to be separated into individual segments, we targeted four regions (U1 + U2, U3 + U4, U5 + U6 and U7) for qPCR. All four regions were found to be significantly enriched in the MYB73-DNA complex obtained from apple calli expressing MYB73-GFP by immunoprecipitation using a GFP antibody compared with those expressing GFP alone. Finally, we performed electrophoretic mobility shift assay (EMSAs) using biotin-labelled oligonucleotide probes designed specifically for U1–U7. Clear bands of biotin-labelled probes bound to MYB73 were detected for all the binding sites (Figure 5D), consistent with the ChIP-PCR result. These bands were attenuated when cold probes were added in the presence of corresponding biotin-labelled probes. No band was detected when mutant probes and control MBP protein were used. Taken together, these in vitro and in vivo tests confirm that MdMYB73 binds to the promoter region of *MdUFGT*.

To determine the transcriptional regulation of MdMYB73 on *MdUFGT*, we did a dual luciferase (Luc) assay in *Nicotiana benthamiana* leaves (Figure 5E). *MdMYB73* CDS was cloned into

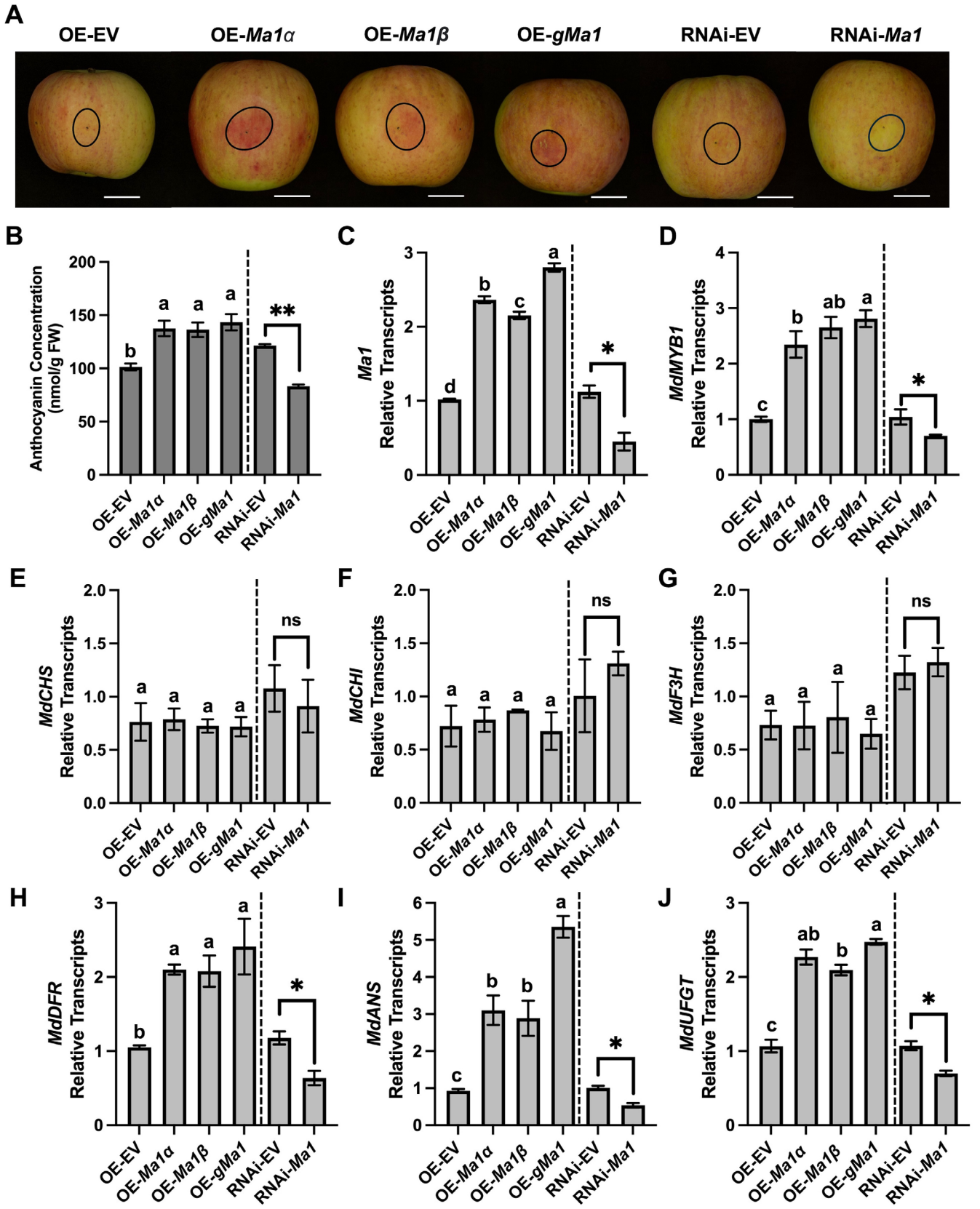

FIGURE 2 | Legend on next page.

the 62SK vector while the *MdUFGT* promoter was cloned into the LUC vector. The *MdMYB73*-62SK + $_{pro}$*MdUFGT*-LUC constructs were introduced into tobacco leaves via agroinfiltration while 62SK + $_{pro}$*MdUFGT*-LUC were used as the control. A significantly reduced luminescent intensity was detected in the region injected with *MdMYB73*-62SK + $_{pro}$*MdUFGT*-LUC compared with the control, demonstrating that MdMYB73 represses the expression of *MdUFGT*.

FIGURE 2 | Transient overexpression and RNAi of *Ma1* in 'Royal Gala' apple peel increase and decrease anthocyanin biosynthesis, respectively. (A) Fruit peel phenotypes after transient overexpression of two isoforms of *Ma1* generated via alternative splicing (*Ma1α* and *Ma1β*), genomic *Ma1* (*gMa1*) and RNAi of *Ma1*. The black circles indicate the injection areas. Bar = 2 cm. (B) Anthocyanin concentrations in fruit peel after transient overexpression of *Ma1α*, *Ma1β*, *gMa1* or RNAi of *Ma1*. (C-J) Expression levels of *Ma1*, *MdMYB1*, *MdCHS*, *MdCHI*, *MdF3H*, *MdDFR*, *MdANS* and *MdUFGT* after transient overexpression of *Ma1α*, *Ma1β*, *gMa1* or RNAi of *Ma1*. Data are mean ± SE of three biological replicates with three fruits per replicate. For the *Ma1*-OE experiment, different letters (a, b, c and d) indicate significant differences between genotypes using Tukey's HSD test at $p < 0.05$ after ANOVA. For the *Ma1*-RNAi experiment, * and ** represent significant differences using Student's *t*-test at $p < 0.05$ and 0.01, respectively.

As the expression of two other key structural genes *MdDFR* and *MdANS* was also up-regulated in *cMa1*-OE fruits at harvest (Figure 3A), we used ChIP-PCR to determine if MdMYB73 binds to their promoters in vivo (Figure S7). None of the promoter regions of the two genes were enriched in the MdMYB73-DNA precipitated from the calli expressing MdMYB73-GFP using GFP antibody compared with GFP alone, indicating MdMYB73 does not bind to their promoters.

2.5 | MdMYB73 Negatively Regulates Anthocyanin Synthesis by Repressing *MdUFGT* Expression in Apple

To verify that MdMYB73 regulates anthocyanin biosynthesis via acting on *MdUFGT*, we conducted a VIGS experiment in 'Zestar' apple peel at harvest. Agrobacteria harbouring RNAi-*MdMYB73* and RNAi-*MdUFGT* vectors were infiltrated into the 'Zestar' apple peel alone or in combination, with an empty RNAi vector as the control. RNAi suppression of *MdMYB73* enhanced peel red colour development as observed before (Figures 4 and 6A,B; Figures S5 and S6) while RNAi suppression of *MdUFGT* inhibited red colour formation (Figure 6A,B). The effect of *MdMYB73*-RNAi on peel red colour development was largely blocked by *MdUFGT*-RNAi (Figure 6A,B). RT-qPCR assay showed that *MdMYB73*-RNAi not only decreased the expression of *MdMYB73* and *Ma1* but also increased *MdUFGT* expression (Figure 6C–E; Figures S5C and S6G). *MdUFGT*-RNAi significantly decreased its own expression and abolished the effect of *MdMYB73*-RNAi on *MdUFGT* expression in the *MdMYB73* + *MdUFGT* combination (Figure 6C–E). This indicates that *MdUFGT* is downstream of *MdMYB73* and MdMYB73 represses *MdUFGT* to negatively regulate anthocyanin biosynthesis in apple. Furthermore, we noticed that *MdMYB1* expression levels were increased in *MdMYB73*-RNAi peels and decreased in *MdUFGT*-RNAi peels. *MdMYB73*-RNAi + *MdUFGT*-RNAi did not alter the *MdMYB1* expression level (Figure 6F). When *MdUFGT* was transiently overexpressed in 'Zestar' apple peel, both anthocyanin synthesis and *MdMYB1* expression were enhanced (Figure S8B–D). *Ma1* expression did not respond to *MdUFGT*-RNAi (Figure 6E).

2.6 | MdMYB73 Competes With MdMYB1 for Transcriptional Regulation of *MdUFGT* in Anthocyanin Biosynthesis

So far, we have shown that MdMYB73 is a negative regulator for anthocyanin biosynthesis by transcriptionally repressing *MdUFGT* expression. As MdMYB1 has been reported as a key transcriptional activator for *MdUFGT* and its expression increased in *cMa1*-OE fruit peels (Figure 3A), we explored the possibility that MdMYB73 directly regulates *MdMYB1* expression in addition to *MdUFGT*. We analysed 2000 bp of the *MdMYB1* promoter region and found three putative MYB binding sites. However, MdMYB73 did not bind to any of the 3 sites as demonstrated by ChIP-PCR (Figure S7A,D). Overexpression of *MdMYB1* via virus-based gene overexpression increased anthocyanin synthesis but did not alter *MdMYB73* or *Ma1* expression (Figure S9). Based on these data, we reasoned that MdMYB73 may compete with MdMYB1 for binding to the promoter of *MdUFGT* in regulating anthocyanin biosynthesis. To test this hypothesis, we performed an EMSA that confirmed MdMYB1 binds to all U1-U7 MYB binding sites in the promoter of *MdUFGT* (Figure S8A). Then, we did all factorial combinations of *MYB73*-RNAi/OE and *MYB1*-RNAi/OE via VIGS and virus-based gene overexpression in 'Zestar' apple peel, with empty vectors as control: RNAi-Empty Vector (EV) + OE-EV, RNAi-*MYB73* + OE-*MYB1*, RNAi-*MYB73* + RNAi-*MYB1* + OE-EV, RNAi-EV + OE-*MYB73* + OE-*MYB1* and OE-*MYB73* + RNAi-*MYB1* (Figure 7A). Anthocyanin concentrations and expression levels of *MdMYB73*, *MdMYB1*, *MdUFGT* and *Ma1* were then analysed (Figure 7B–F). Among these combinations, RNAi-*MYB73* + OE-*MYB1* resulted in the highest *MdUFGT* expression and anthocyanin concentration whereas OE-*MYB73* + RNAi-*MYB1* led to the lowest *MdUFGT* expression and anthocyanin concentration; RNAi-*MYB73* + RNAi-*MYB1* and OE-*MYB73* + OE-*MYB1* did not alter the transcript level of *MdUFGT* or the anthocyanin concentration (Figure 7B,F). These data indicate that repression of *MdMYB73* and activation of *MdMYB1* on *MdUFGT* expression and anthocyanin biosynthesis is largely blocked by RNAi-*MYB1* and OE-*MYB73*, respectively. We next conducted Luc assays in *N. benthamiana* leaves to confirm the competitive relationship between MdMYB73 and MdMYB1 in transcriptionally regulating *MdUFGT* (Figure 7G). *MdMYB1*-62SK + $_{pro}$*MdUFGT*-LUC and *MdMYB73*-62SK + $_{pro}$*MdUFGT*-LUC yielded the highest and lowest luminescence intensity, respectively, with a significantly reduced luminescence intensity detected in *MdMYB73*-62SK + *MdMYB1*-62SK + $_{pro}$*MdUFGT*-LUC relative to *MdMYB1*-62SK + $_{pro}$*MdUFGT*-LUC. Simultaneous transient overexpression of *MdMYB73* and *MdMYB1* significantly reduced the expression of *MdUFGT* compared with *MdMYB1*-OE alone in 'Royal Gala' leaves and 'Orin' calli (Figure 7H,I). These data confirm that MdMYB73 is a competitor of MdMYB1 in transcriptionally regulating *MdUFGT* in anthocyanin biosynthesis. As MdbHLH3 interacts with MdMYB1, enhancing MdMYB1's transactivation activity (Xie et al. 2012), we explored the possibility that MdMYB73 may also compete with MdMYB1 for interacting with MdbHLH3. However, no interaction was detected between MdMYB73 and MdbHLH3 by the yeast two-hybrid assay (Figure S10).

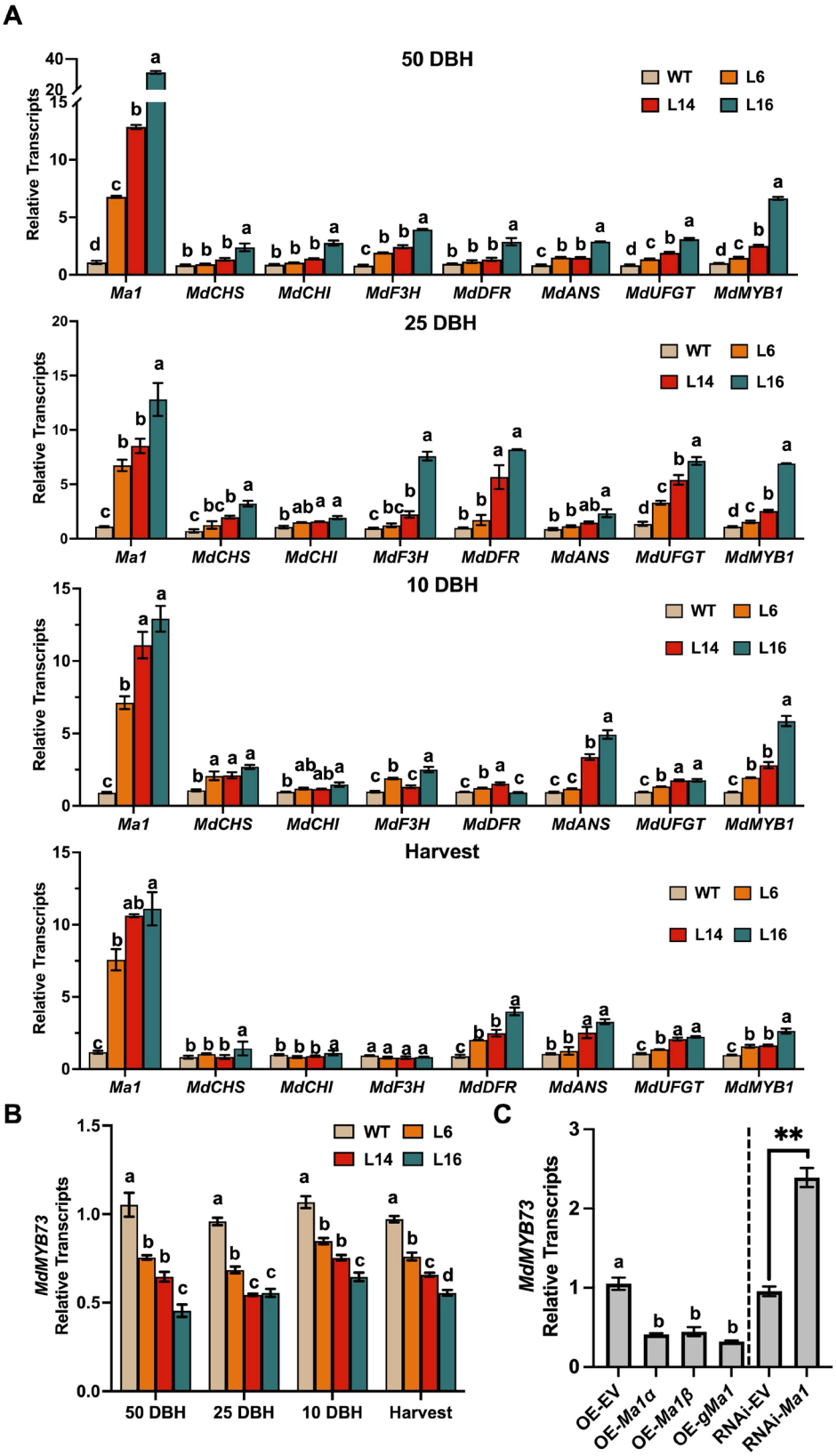

FIGURE 3 | Legend on next page.

3 | Discussion

Apple fruit acidity is largely controlled by *Ma1*, which encodes the tonoplast malate transporter ALMT9 for malate transport into the vacuole (Bai et al. 2012; Li et al. 2020), with its expression transcriptionally activated by MdMYB73 (Hu et al. 2017; Li et al. 2024). In this work, we show that overexpression of the coding sequence of *Ma1* (*cMa1*) in the 'Royal Gala' apple

FIGURE 3 | Transcript levels of *Ma1*, structural genes in anthocyanin biosynthesis, and transcriptional factors in the peel of *Ma1*-OE or *Ma1*-RNAi fruit. (A) Transcript levels of *Ma1*, structural genes in anthocyanin biosynthesis and *MdMYB1* in c*Ma1*-OE lines L6, L14 and L16 compared with the wild-type (WT) at 50, 25 and 10 days before harvest (DBH), and at harvest, respectively. Ma1, aluminium-activated malate transporter 9; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, UDP-glycose: Flavonoid-3-*O*-glycosyltransferase; MYB1, MYB transcription factor 1. (B) Expression levels of *MdMYB73* in the peel of *cMa1*-OE lines L6, L14 and L16 compared with WT at 50, 25 and 10 DBH, and at harvest. (C) Expression levels of *MdMYB73* after transient overexpression of *Ma1α*, *Ma1β*, *gMa1* or RNAi of *Ma1* in 'Zestar' apple peel. In (A) and (B), the expression level of each gene in WT at each developmental stage was normalised to 1. Data are mean ± SE of three biological replicates with three fruits per replicate. Different letters (a, b, c and d) indicate significant differences between treatments using Tukey's HSD test at $p < 0.05$ after ANOVA. For the *Ma1*-RNAi experiment, ** represent significant differences using Student's *t*-test at $p < 0.01$.

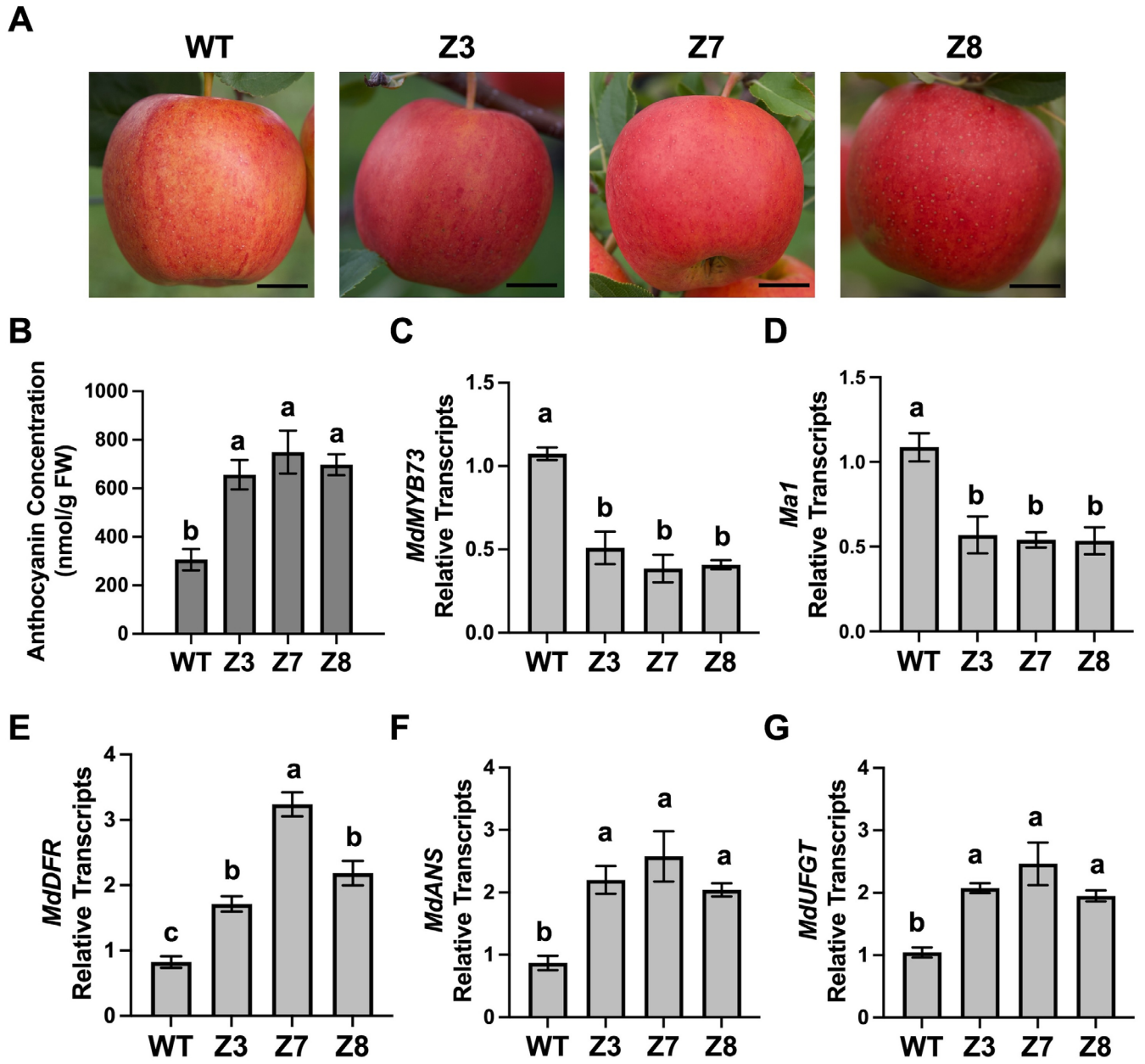

FIGURE 4 | MdMYB73 negatively regulates anthocyanin synthesis in the 'Royal Gala' apple peel. (A) Apple peel colour in wild-type (WT) and *MdMYB73*-RNAi lines of 'Royal Gala' (Z3, Z7 and Z8) at fruit harvest. Bar = 2 cm. (B) Anthocyanin concentrations in WT, Z3, Z7 and Z8 apple peels at fruit harvest. (C–G) Expression levels of *MdMYB73*, *Ma1*, *MdDFR*, *MdANS* and *MdUFGT* in WT, Z3, Z7 and Z8 apple peels at fruit harvest. Data are mean ± SE of three biological replicates with three fruits per replicate. Different letters (a, b and c) indicate significant differences between genotypes using Tukey's HSD test at $p < 0.05$ after ANOVA.

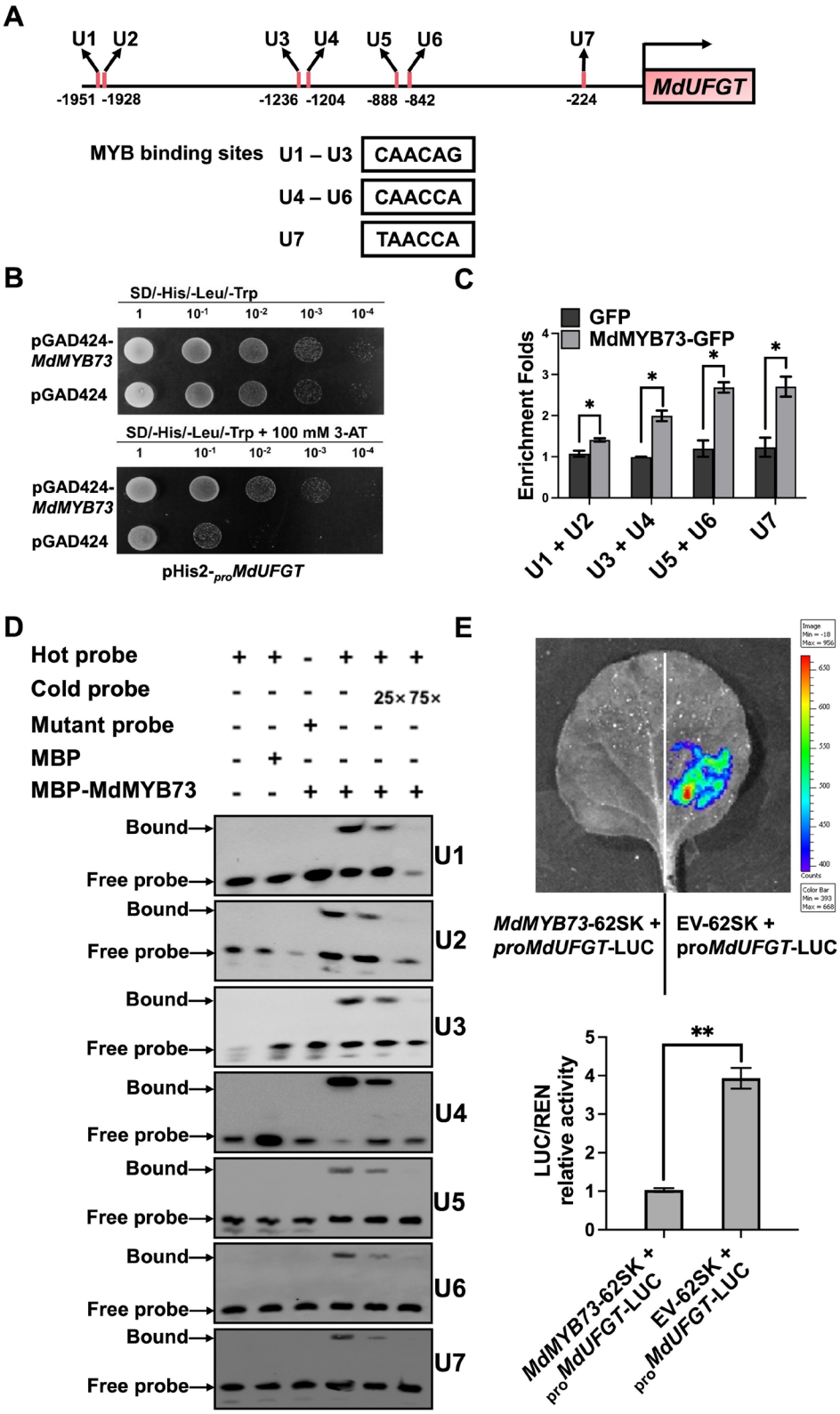

FIGURE 5 | Legend on next page.

results in a decrease in the expression of *MdMYB73* and enhanced red colour development in fruit peel. MdMYB73 represses the expression of *MdUFGT* by binding to its promoter. Downregulation of *MdMYB73* expression in *cMa1*-OE fruits releases *MdUFGT* from transcriptional repression by MdMYB73, leading to higher *MdUFGT* expression. In addition, MdMYB73 competes with MdMYB1, a key transcriptional activator for *MdUFGT*. The release of *MdUFGT* from transcriptional repression by MdMYB73 allows more MdMYB1 proteins to bind to its promoter for transcriptional activation. These combined effects lead to the upregulation of anthocyanin biosynthesis. In addition to being a transcriptional activator for vacuolar malate

FIGURE 5 | MdMYB73 binds to the promoter of *MdUFGT* to repress its expression. (A) Putative MYB binding sites U1 to U7 in the 2000-bp *MdUFGT* promoter. Numbers indicate the positions of MYB *cis*-acting elements upstream of the coding sequence of *MdUFGT*, with the first nucleotide of its start codon designated as 0. The MYB *cis*-acting elements were mutated to AAAAAA in all the mutant probes. (B) Yeast one-hybrid assay of the binding of MdYB73 to the *MdUFGT* promoter, with. pGAD424 + pHis-$_{pro}$*MdUFGT* as a negative control. (C) ChIP-PCR assay of the binding of MdMYB73 to the MYB binding sites in the *MdUFGT* promoter. The MdMYB73-DNA complex was co-immunoprecipitated from MdMYB73-GFP transgenic apple calli using a GFP antibody, with empty GFP vector transgenic apple calli as a negative control. Data are mean ± SE of three biological replicates with calli grown in one petri dish per replicate. * represents significant differences using Student's *t*-test at $p < 0.05$. (D) EMSA assay of the binding of MdMYB73 to the MYB binding sites in the *MdUFGT* promoter. (E) Luciferase assay in *N. benthamiana* leaves showing the repression of MdMYB73 on the *MdUFGT* promoter activity. Data are mean ± SE of three biological replicates with three leaves per replicate. ** represents significant differences using Student's *t*-test at $p < 0.01$.

transport, MdMYB73 acts as a transcriptional repressor for anthocyanin synthesis, linking peel red coloration to fruit acidity.

MdMYB73 as a transcriptional repressor for anthocyanin synthesis is supported by several lines of evidence. First, decreased expression of *MdMYB73* is associated with enhanced anthocyanin synthesis in *cMa1*-OE fruits (Figures 1 and Figure 3B). Second, RNAi suppression and overexpression of *MdMYB73* increases and decreases *MdUFGT* expression and anthocyanin synthesis in apple peel, respectively (Figure 4; Figures S5 and S6). Third, MdMYB73 binds to the promoter of *MdUFGT* as demonstrated in ChIP-PCR, Y1H assay and EMSA, and this binding transcriptionally represses the expression of *MdUFGT* as shown in the luciferase assay (Figure 5). Finally, the enhanced expression of *MdUFGT* by RNAi suppression of *MdMYB73* is blocked by *MdUFGT*-RNAi (Figure 6). MdMYB73 is an R2R3 MYB with an EAR motif at its N-terminal instead of the more common C-terminal (Kazan 2006; Ma and Constabel 2019). Further work is needed to identify its co-repressors binding to the EAR motif of MdMYB73 for triggering the repression of *MdUFGT*.

Catalysing the last step in the anthocyanin biosynthesis pathway, MdUFGT is known to play a key role in anthocyanin biosynthesis. Higher expression of Md*UFGT* is associated with higher anthocyanin levels in apple fruit peel (Kim et al. 2003; Zhao et al. 2012). In this work, we confirmed the role of *MdUFGT* in anthocyanin synthesis in apple by both RNAi suppression and overexpression (Figure 6; Figure S8B–D). Like MdMYB1 (Takos et al. 2006; Espley et al. 2009; Xie et al. 2017), MdMYB73 binds to the same 7 MYB-sites in the promoter of *MdUFGT* as MdMYB1, but the binding leads to repression of its expression instead of activation (Figure 5; Figure S8A). Interestingly, overexpression of *MdUFGT* increases *MdMYB1* expression (Figure S8D). This suggests a feedforward regulation of *MdMYB1* by *MdUFGT*. How this mechanism is triggered is not clear, but its operation is expected to make the regulation of the entire anthocyanin biosynthesis pathway more effective. This provides a possible explanation of why the expression of *MdMYB1* and several structural genes in anthocyanin biosynthesis is up-regulated in *Ma1*-OE fruits although MdMYB73 does not directly target them (Figure 3A; Figure S7).

cMa1-OE fruits have a lower expression of *MdMYB73* (Figure 3B) in the peel as detected in the bulk fruit earlier (Li et al. 2024). This decreased expression of *MdMYB73* is at least partially, if not completely, responsible for the enhanced anthocyanin synthesis observed in *cMa1*-OE fruits. RNAi suppression of *MdMYB73* increases *MdUFGT* expression and anthocyanin synthesis in 'Royal Gala' and 'Zestar' fruits (Figure 4; Figures S5 and S6) whereas overexpression of *MdMYB73* decreases *MdUFGT* expression and anthocyanin synthesis in 'Zestar' and *cMa1*-OE fruits (Figures S5 and S6). MdMYB73 competes with MdMYB1 for binding to the promoter of *MdUFGT*, co-regulating its expression (Figure 7). So, these data support the following scenario when *cMa1* is overexpressed. *cMa1*-OE triggers a feedback inhibition on *MdMYB73* expression via an unidentified mechanism, lowering its expression and activity. As a result, *MdUFGT* expression is released from *Md*MYB73 repression. This enhances the expression of *MdUFGT*, and in addition, it allows more MdMYB1 proteins to bind to the *MdUFGT* promoter for transcriptional activation. The initial increase in the expression and activity of MdUFGT up-regulates the entire anthocyanin biosynthesis pathway by enhancing *MdMYB1* expression. In this scenario, MdMYB1 contributes to the upregulation of anthocyanin biosynthesis only after *MdUFGT* is released from MdMYB73 repression. It is possible that *MdMYB1* expression is somehow enhanced by *cMa1*-OE at the same time when *MdMYB73* is suppressed. Even in this scenario, the release of *MdUFGT* from MdMYB73 repression is essential for more MdMYB1 proteins to bind to the promoter of *MdUFGT*, leading to higher *MdUFGT* expression and anthocyanin synthesis in *cMa1*-OE fruits. A third scenario where *cMa1*-OE somehow triggers up-regulation of *MdMYB1* expression first and this up-regulation suppresses *MdMYB73* expression can be excluded because transient *MdMYB1*-OE did not alter *MdMYB73* expression in fruit peel (Figure S9C).

MdMYB73 regulates vacuolar acidification and malic acid accumulation in apple by transcriptionally activating the expression of *MdVHA-A*, *MdVHP1* and *Ma1* (Hu et al. 2017; Li et al. 2024). In this work, we show that MdMYB73 is a transcriptional repressor for *MdUFGT*, and *cMa1*-OE triggers a feedback inhibition on the expression of *MdMYB73*, thereby up-regulating anthocyanin biosynthesis. So MdMYB73 is a bifunctional transcription factor with opposite transcriptional activities on different genes. This is similar to the Arabidopsis WUSCHEL protein that functions as a transcriptional activator for the *AGAMOUS* gene as well as a repressor for many genes involved in the maintenance of stem cells in shoot apical meristems (Ikeda et al. 2009) and the Arabidopsis MYB44 that transcriptionally activates *AtWRKY70*, modulating the antagonistic interaction between salicylic acid and jasmonic acid signalling in disease resistance and represses the late embryogenesis abundant protein gene *AtLEA4-5* in water deficit tolerance (Shim et al. 2013; Nguyen et al. 2019). This dual function of MdMYB73 directly links fruit peel colour to *Ma1* expression in apple (Figure 8). The regulation of both fruit acidity

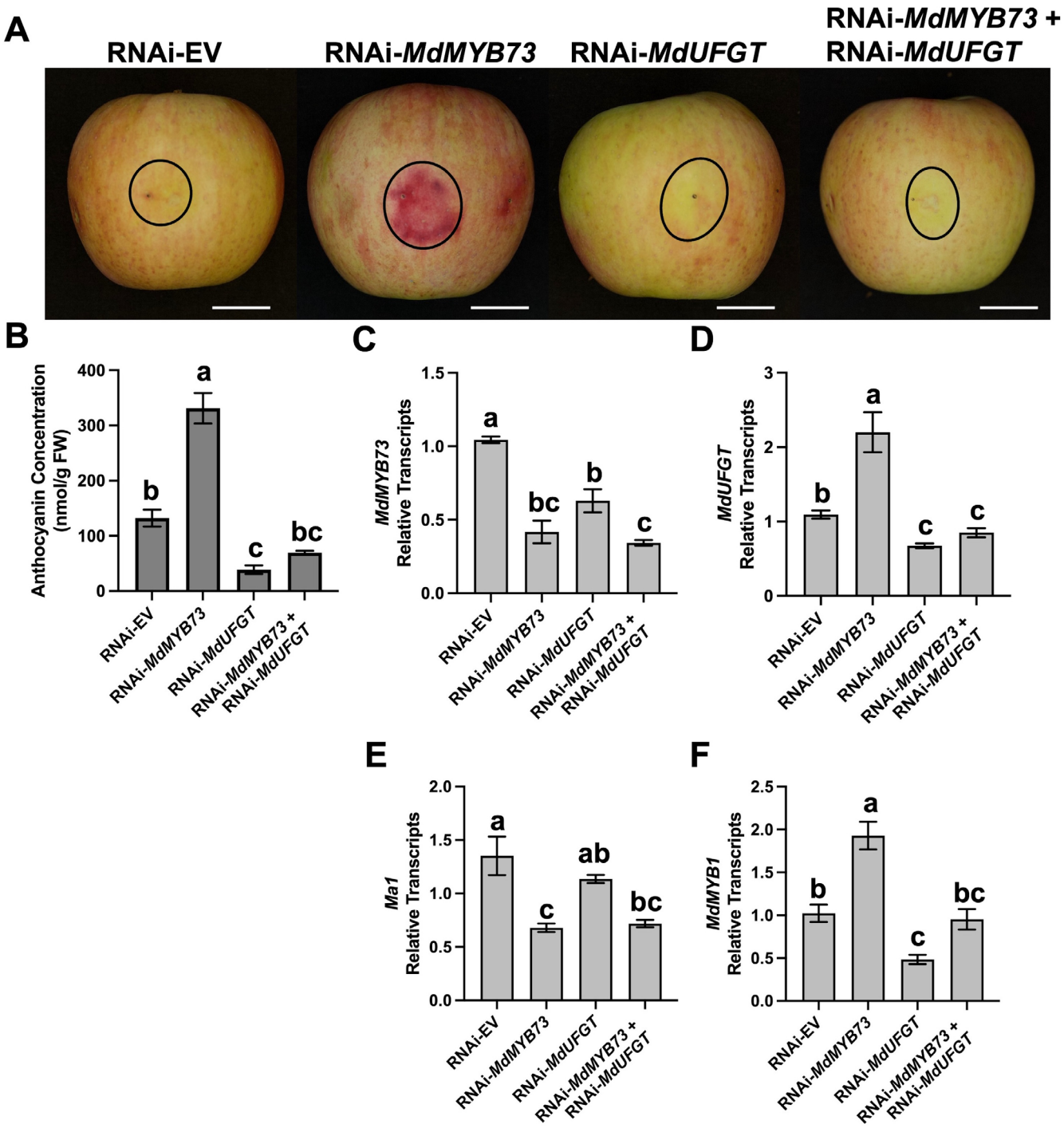

FIGURE 6 | MdMYB73 negatively regulates anthocyanin synthesis by repressing *MdUFGT* expression in apple. (A) Apple peel colour phenotypes in response to transient RNAi suppression of *MdMYB73* and *MdUFGT* alone or in combination in 'Zestar' apple. Black circles indicate the injection areas. Bar = 2 cm. (B) Anthocyanin concentrations in fruit peels as shown in (A). (C–F) Expression levels of *MdMYB73*, *MdUFGT*, *Ma1* and *MdMYB1* in fruit peels as shown in (A). Data are mean ± SE of three biological replicates with three fruits per replicate. Different letters (a, b and c) indicate significant differences between treatments using Tukey's HSD test at $p < 0.05$ after ANOVA.

and peel anthocyanin synthesis by MdMYB73 complements the co-regulation of vacuolar acidification/malate transport and anthocyanin accumulation by MdMYB1/10 characterised earlier (Hu et al. 2016), where MdMYB1/10 activates the expression of the genes encoding vacuolar H^+ pumps (H^+-ATPase subunits VHA-B1, VHA-B2 and VHA-E2, vacuolar H^+-pyrophosphatase VHP1) and transporters for malate and anthocyanins (tDT and MATE-LIKE1). In both petunia and citrus, the interaction between MYB and bHLH transcriptional factors controls anthocyanin synthesis and vacuolar acidification by transcriptionally regulating structural genes in anthocyanin synthesis and those encoding tonoplast proton pumps (Spelt et al. 2000, 2002; Quattrocchio et al. 2006; Faraco et al. 2014; Butelli et al. 2019; Strazzer et al. 2019; Lu et al. 2022), but the involvement of an organic acid transporter has not been demonstrated although overexpression of citrus *MYB73*, *CrMYB73*, in tobacco increases the leaf citric acid level

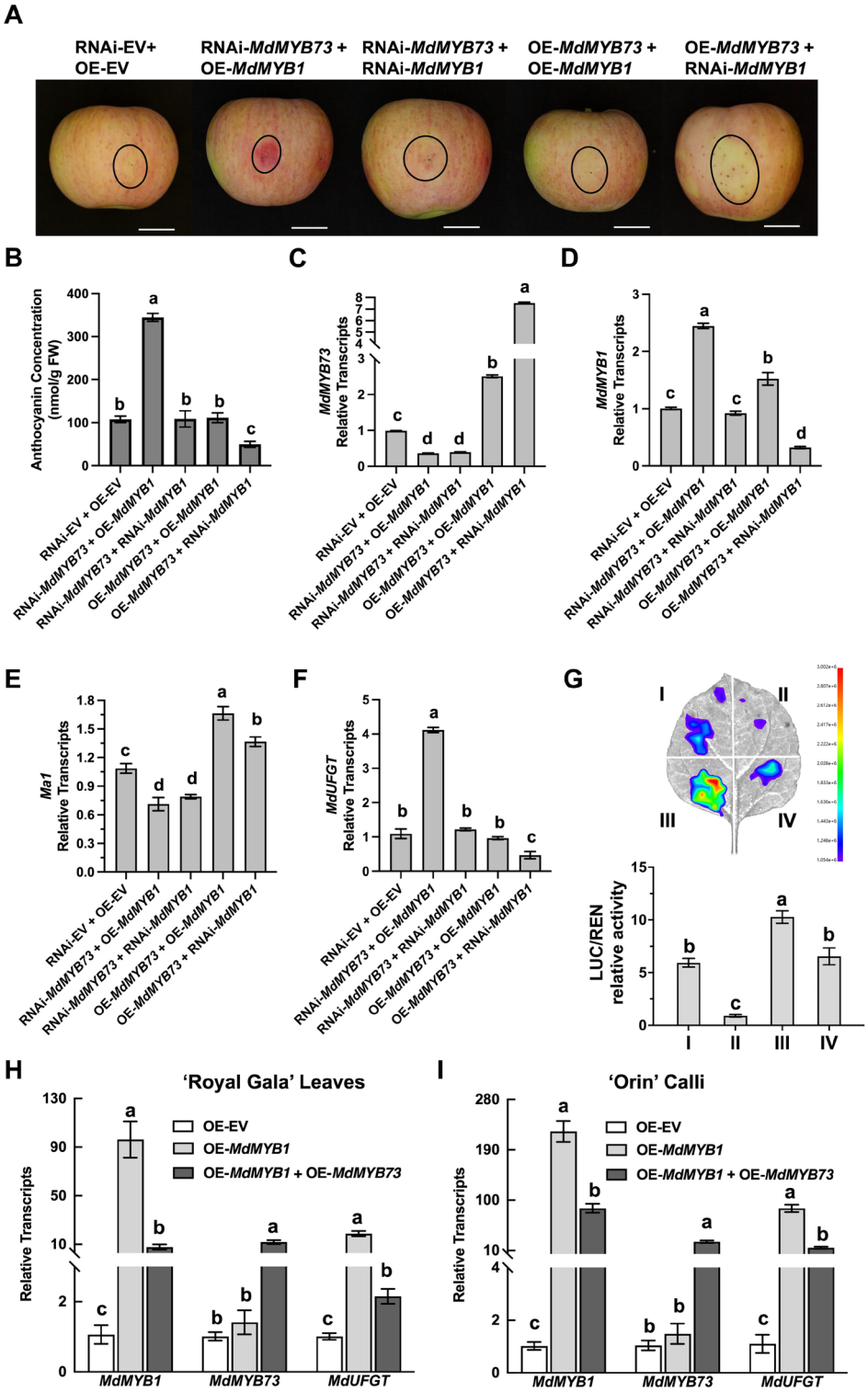

FIGURE 7 | MdMYB73 competes with MdMYB1 for transcriptional regulation of *MdUFGT* in anthocyanin biosynthesis. (A) and (B) Apple peel colour phenotypes and anthocyanin concentrations after transient RNAi or overexpression (OE) of *MdMYB73* and *MdMYB1* in factorial combinations (RNAi-*MdMYB73* + OE-*MdMYB1*, RNAi-*MddMYB73* + RNAi-*MdMYB1*, OE-*MdMYB73* + OE-*Md*MYB1 and OE-*MdMYB73* + RNAi-*MdMYB1*) with RNAi-EV (empty vector) + OE-EV as control in 'Zestar' apple peels. Black circles indicate the injection areas. Bar = 2 cm. (C–F) Expression levels of *MdMYB73*, *MdMYB1*, *Ma1* and *MdUFGT* in fruit peels as described above. (G) Luciferase assay of the competition between MdMYB73 and MdMYB1 on the promoter activity of *MdUFGT* in *N. benthamiana* leaves. I: Blank (Empty vector 62SK + $_{pro}$*MdUFGT*-LUC); II: *MdMYB73*-62SK + $_{pro}$*MdUFGT*-LUC; III: *MdMYB1*-62SK + $_{pro}$*MdUFGT*-LUC; IV: *MdMYB73*-62SK + *MdMYB1*-62SK + $_{pro}$*MdUFGT*-LUC. (H) and (I) Expression levels of *MdMYB1*, *MdMYB73* and *MdUFGT* in 'Royal Gala' leaves and 'Orin' calli in response to transient overexpression (OE) of *MdMYB1* alone or in combination with OE-*MdMYB73*, with empty vector (EV) as control. Data are mean ± SE of three biological replicates with three fruits or leaves per replicate. Different letters (a, b, c and d) indicate significant differences between treatments using Tukey's HSD test at $p < 0.05$ after ANOVA.

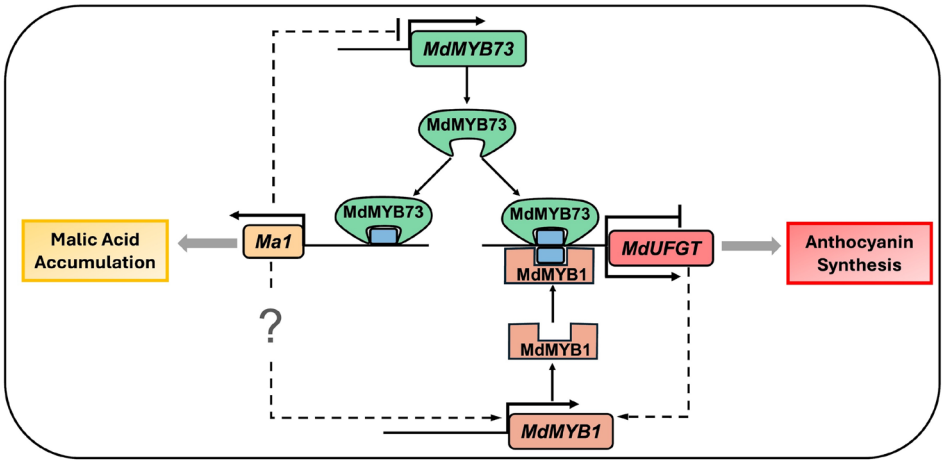

FIGURE 8 | Proposed model for the role of MdMYB73 in linking peel red colour development to fruit acidity in apple. MdMYB73 negatively regulates anthocyanin biosynthesis by repressing the expression of *MdUFGT* in apple peel as well as transcriptionally activating *Ma1* in controlling fruit acidity. The binding of MdMYB73 to the promoter of *MdUFGT* leads to transcriptional repression, whereas its binding to the promoter of *Ma1* results in transcriptional activation (Hu et al. 2017; Li et al. 2024). MdMYB73 binds to the same 7 MYB-sites in the promoter of *MdUFGT* as MdMYB1 does (only one binding site is shown for simplicity). When *Ma1* is overexpressed, it triggers a feedback inhibition on the expression of *MdMYB73*. The reduced MdMYB73 protein level releases *MdUFGT* from transcriptional repression. This also allows more MdMYB1 proteins to bind to its promoter. The combined effects up-regulate anthocyanin synthesis. Dash line "---" indicates an indirect interaction with the mechanism yet to be identified. Question mark "?" with dashed line denotes only a possibility that cannot be excluded at this point.

(Li et al. 2015). Our work on MdMYB73 extends the molecular link between fruit acidity and anthocyanin synthesis to a malate transporter, Ma1. MdMYB73 transcriptionally activates *Ma1* expression but represses *MdUFGT* expression; *cMa1*-OE somehow down-regulates *MdMYB73* expression, releasing *MdUFGT* from MdMYB73 repression for anthocyanin synthesis. *cMa1*-OE, in this case, led to a significant decrease in fruit acidity in 'Royal Gala' because the expression of the native *Ma1* gene was suppressed by the reduced *MdMYB73* transcript level (Li et al. 2024). This lowers the levels of both transcripts generated by alternative splicing of the *Ma1* gene. Although the reduced native *Ma1α* transcript level is overcompensated by *cMa1*-OE, the reduced *Ma1β* transcript level decreases the ratio of Ma1β to Ma1α well below the threshold (*Ma1β* = 1/8 of *Ma1α*) required for Ma1's malate transport function, leading to lower fruit acidity. Transient overexpression of genomic *Ma1* (*gMa1*) increases fruit acidity by proportionally increasing Ma1β and Ma1α for their synergistic interactions as in the untransformed control, leading to downregulation of *MdMYB73* as well (Li et al. 2024). This transient overexpression of *gMa1* enhanced anthocyanin synthesis in apple peel (Figure 2). It would be also interesting to determine if the natural variation in *Ma1* expression is associated with the degree of peel red colour in genotypes with at least one *Ma1* allele and one dominant *MdMYB1* allele across a wide range of Malus germplasm.

Although domestication plays an important role in shaping the association between flower/fruit colour and acidity in fleshy fruits and ornamentals (Allan 2019; Butelli et al. 2019), the molecular/genetic co-regulation of the two traits raises a fundamental question as to the advantages it brings to plant function. Both flavonoids and malate/citrate are stored in the vacuole and their transport into the vacuole relies on the proton gradient generated by vacuolar H^+-ATPase, H^+-pyrophosphatase and/or P-ATPase. As both flavonoids and malate/citrate accumulated in the vacuole play a part in conferring abiotic stress tolerance to cold, salinity and drought (Finkemeier and Sweetlove 2009; Nakabayashi et al. 2014), co-regulation of their synthesis and accumulation may make plants more effective in coping with these environmental stresses, in addition to maintaining cytosol pH homeostasis for plant metabolism (Hu et al. 2016). In the case of MYB73, its involvement in the regulation of vacuolar acidification/malate accumulation is consistent with its role in abiotic stress tolerance. In apple calli, overexpression of *MdMYB73* increases cold stress by activating CBF (C-repeat Binding Factor) expression, which leads to the accumulation of both malate and soluble sugars (Zhang et al. 2017). When wheat *MYB73*, *TaMYB73*, is expressed in Arabidopsis, it enhances salinity tolerance by binding to the promoter regions of salinity stress signalling genes *AtCBF3* and *AtABF3* (He et al. 2012). In addition, MYB73 confers resistance of Arabidopsis to *Bipolaris oryzae* (*Cochliobolus miyabeanus*), a necrotrophic fungal pathogen causing brown leaf spot, via the salicylic acid and jasmonic acid signalling pathway (Jia et al. 2011). Overexpression of *MdMYB73* increases resistance to *Botryosphaeria dothidea*, the fungal pathogen responsible for ring rot in apple (Gu et al. 2020). So, MYB73 is indeed a master regulator for the tolerance of plants to both abiotic and biotic stresses. In this context, uncovering the mechanism that underlies the feedback regulation of *Ma1*-OE on *MdMYB73* expression may allow us to not only gain a better understanding of the molecular link between anthocyanin synthesis and fruit acidity for fruit quality improvement but also enhance plant stress tolerance via targeting *MdMYB73* using biotechnological approaches.

4 | Materials and Methods

4.1 | Plant Materials

Untransformed wild-type (WT) 'Royal Gala' (*Malus domestica*), three *cMa1* overexpression lines (L6, L14 and L16) previously reported (Li et al. 2024), and three *MdMYB73* RNAi lines (Z3, Z7 and Z8) generated in this study were used. Five-year-old trees of WT, L6, L14 and L16 on M.26 rootstock were grown in 20-L

containers with LM-111 medium (Lambert, Quebec, Canada) under natural conditions in Ithaca, New York. These trees were arranged in a completely randomised design in five replicates per genotype with two trees per replicate. Three-year-old trees of WT and three *MdMYB73* RNAi lines on G.11 rootstock were grown in 20-L containers with the LM-111 medium, arranged in a completely randomised design with three single-tree replicates per genotype. During the bloom period, these transgenic trees and their WT controls were moved into a screenhouse for bee exclusion and were hand-pollinated with a mixture of pollen from 'Honeycrisp' and 'Granny Smith'. In addition, WT fruits from 'Zestar' apple trees grown in the field at Cornell Orchards were used for agro-infiltration. All the trees received standard horticultural practices and disease and insect control.

Calli derived from 'Orin' apple were used for ChIP-PCR assay and agro-infiltration. They were cultured on Murashige and Skoog (MS) medium of pH 5.8 with 30 g/L of sucrose, 1.5 mg/L of 2,4-D and 0.4 mg/L of 6-BA at 25°C in the dark.

'Royal Gala' plantlets in vitro were used for agro-infiltration. They were cultured on MS medium in a tissue culture room at 24°C under a 16 h photoperiod at a light intensity of 100 μmol/m^2/s.

Nicotiana benthamiana plants were used for dual luciferase assay. They were grown in Cornell Mix medium in a controlled growth chamber at 24°C with 40%–65% relative humidity under a 16 h photoperiod.

'Royal Gala' and 'Orin' are of *Ma1ma1* genotype and 'Zestar' is of *Ma1Ma1* genotype as detected by the $CAPS_{1455}$ marker (Bai et al. 2012). 'Royal Gala' and 'Zestar' have at least one dominant *MdMYB1* allele detected as a 750-bp red transposon element insertion in its promoter (Zhang et al. 2019) whereas 'Orin' does not, corresponding to their differences in peel colour at harvest (red vs. yellow).

4.2 | Transformation of 'Royal Gala' Apple

A 201-bp fragment of the coding sequence (CDS) of *MdMYB73* from 'Royal Gala' apple was cloned into pDONR221 via BP reaction and then transferred into pGWRNAi via LR reaction (Meng et al. 2018). The resulting *MdMYB73*-RNAi vector was transformed into *A. tumefaciens* strain EHA105 containing an additional virulence plasmid pCH32 (Hood et al. 1993) for subsequent transformation of 'Royal Gala' apple following a protocol as previously described (Meng et al. 2023).

4.3 | Fruit Sampling from *cMa1*-OE and *MdMYB73* RNAi Lines and Measurements of Titratable Acidity and Total Soluble Solids

Fruit peel samples were taken between noon and 2:00 PM at 50, 25 and 10 days before harvest (DBH) and at harvest for WT and *cMa1*-OE lines (L6, L14 and L16), with four fruits per replicate and at harvest for WT and *MdMYB73* RNAi lines (Z3, Z7 and Z8), with three fruits per replicate. The samples were frozen in liquid nitrogen and stored at −80°C for extraction of RNA and anthocyanins. In addition, 20 fruits per replicate were taken at harvest from WT and *cMa1*-OE lines for colour evaluation on a CombiSort system (GREEFA, Tricht, Netherlands). Fruit titratable acidity and total soluble solids were measured at harvest as described (Li et al. 2024).

4.4 | Anthocyanin Extraction and Analysis

Anthocyanins were extracted and measured as described by Lee and Wicker (1991) with modifications. Briefly, 0.5-g samples were incubated in 1.5 mL 1% (v/v) methanol-HCL solutions overnight in the dark at room temperature, and the extracts were centrifuged at 15000 *g* for 10 min, and the supernatants were measured at 530, 620 and 650 nm on a Shimadzu 1600 spectrophotometer.

4.5 | Identification and Promoter Analysis of *MdUFGT*, *MdANS*, *MdDFR* and *MdMYB1*

BLAST searches were performed using GeneBank (www.ncbi.nlm.nih.gov). Coding sequences and annotation for *MdUFGT*, *MdANS*, *MdDFR* and *MdMYB1* were retrieved from the doubled haploid 'Golden Delicious' genome (https://iris.angers.inra.fr/gddh13/), and their promoter sequences were obtained from the Genome Database for the Rosaceae family (https://www.rosaceae.org/) and analysed via the PlantCare website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

4.6 | RNA Extraction and RT-qPCR

Total RNA was extracted from 0.5 g fruit peel samples following the CTAB method (Gasic et al. 2004). The quantity of RNA was analysed by a spectrophotometer (NanoDrop) at 230, 260 and 280 nm, and the integrity of RNA was verified by running samples on 1% denaturing agarose gels. Two micrograms of total RNA was reverse transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). RT-qPCR was performed on an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories), using iQ SYBR Green Supermix Kit, with *MdActin* as an internal reference gene. Each reaction was repeated three times per biological replicate and the relative expression of each gene was calculated using the $2^{-\Delta C_T}$ method described by Udvardi et al. (2008).

4.7 | Yeast One-Hybrid (Y1H) and Two-Hybrid (Y2H) Assays

The Matchmaker One-Hybrid System from Clontech (Takara Bio USA Inc) was used for the Y1H assay. The promoter (2000 bp) of *MdUFGT* was cloned into the pHis2 vector using the primers listed in Table S1. The resulting construct was transformed into the yeast strain YH18, and positive clones were selected through PCR to determine the minimal inhibitory concentration of 3-AT. The CDS of MdMYB73 was cloned into the pGAD424 vector and transformed into YH18 competent cells carrying the pHis2-*MdUFGT* promoter. Co-transformed positive clones were selected and grown in a series of dilutions (1:1, 1:10, 1:100, 1:1000,

1:10000) on SD/-T-H-L plates with or without 100 ng/mL 3-AT at 30°C for 3–4 days.

The Y2H assay was performed as previously described (Wang et al. 2024). The coding sequences of *MdMYB73* and *MdbHLH3* were cloned into pGADT7 and pGBKT7 vectors, respectively, using the primers listed in Table S1. The pGADT7-MdMYB73 and pGBKT7-MdbHLH3 constructs were transformed into the yeast strain Y2HGold from Clontech and cultured on SD/=L/-T medium. Positive clones were selected and grown in a series of dilutions (1:1, 1:10, 1:100, 1:1000) on SD/−T/−L/−H/−A medium at 30°C for 3–4 days.

4.8 | Electrophoretic Mobility Shift Assay (EMSA)

The CDS of *MdMYB73* and *MdMYB1* were cloned into the pDEST-MC2 vector (Yuan 2019). The MdMYB73-MBP and MdMYB1-MBP recombinant proteins were expressed in *Escherichia coli* and purified with the MBPTrap HP column (Cytiva, Marlborough, MA, USA). The Biotin 3′ End DNA Labeling Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for labeling the oligonucleotide probes for the *MdUFGT* promoter. Each recombined protein with the MBP tag was incubated with 10 × binding buffer, 1 μg μL^{-1} poly (dI-dC) and 400 fmol biotin-labelled double-stranded binding consensus oligonucleotides (total volume 20 μL) using a LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific). The binding reaction was performed at room temperature for 20 min. The protein-DNA samples were then separated on 6.5% (W/V) polyacrylamide gels and then transferred to Biodyne B Nylon Membranes (Thermo Fisher Scientific). The membranes were blocked and washed with the Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher Scientific). Signals were captured with a ChemiDoc XRS (Bio-Rad). All the primers used for EMSAs are listed in Table S1.

4.9 | Apple Calli Transformation and ChIP-PCR Assay

Apple calli transformation was performed following An et al. (2012) with minor modifications. *MdMYB73* CDS was cloned into pGWB451 vector with a C-terminal GFP tag through the Gateway cloning system (Thermo Fisher Scientific). pGWB451-*MdMYB73* and empty pGWB451 vector were transformed into Agrobacterium GV3101, and positive clones were selected via colony PCR.

Three-week-old 'apple cv Orin' calli were incubated in the liquid medium of Agrobacterium GV3101 harbouring pGWB451-*MdMYB73* or pGWB451 empty vector for 15 min with shaking (140 rpm) at 25°C. The calli were co-cultured on solid MS medium (4.43 g/L of MS, 1 mg/L of 6-BA, 1 mg/L of 2,4-D, 30 g/L of sucrose and 7 g/L of agar at pH 5.8) for 2–3 days at 25°C in the dark and then transferred onto the selection medium (MS solid medium with 250 mg/L carbenicillin and 50 mg/L of kanamycin) by washing three times with sterile water.

ChIP-PCR was performed using Pierce Agarose ChIP Kit (Thermo Fisher Scientific). One-g calli of *MdMYB73*-GFP or *empty*-GFP control were used for crosslinking in 1% (v/v) formaldehyde. The protein-DNA complex was isolated by immunoprecipitation with the GFP antibody. DNA fragments were purified from the protein-DNA complex and then quantified by qPCR using primers listed in Table S1.

4.10 | Vector Construction and Agro-Infiltration of Apple Fruits, Leaves and Calli

The CDS of *MdMYB73*, *MdUFGT* and *MdMYB1* was cloned into the PHB-GFP vector for transient overexpression whereas ~150 bp coding region of *MdMYB73*, *MdUFGT* and *MdMYB1* was cloned into the TRV2 vector for transient RNAi with the TRV1 vector as a helper plasmid. The CDS of MdMYB1 and MdMYB73 was also cloned into the pPZP211 vector carrying a Flag tag and the pCAMBIA2300 vector carrying a GFP tag, respectively (primers listed in Table S1). The constructs were transformed into *A. tumefaciens* strain GV3101. The agrobacteria were grown on liquid Luria-Bertani (LB) medium containing 100 μg/mL rifampicin and 50 μg/mL kanamycin for 24 h at 30°C, centrifuged at 6000 rpm and re-suspended in the infiltration solution (10 mM $MgCl_2$, 10 mM MES and 150 μM acetosyringone).

Fruits of 'Zestar' and WT 'Royal Gala' and *cMa1*-OE lines (L6, L14 and L16) were taken 1 week before harvest for infiltration. A hypodermic needle (26G × ½ inch) was used to puncture the fruit surface to make a hole of 1–2 mm deep, parallel to the surface around the equator, with two holes per fruit on the shaded side of the fruit. Then a needleless syringe was used to inject approximately 100 μL agrobacterium solution (OD_{600} ~ 0.6) slowly into each hole. After injection, the fruits were kept at room temperature (22°C–23°C) for 24 h and then moved to a growth chamber with a 16 h light/8 h dark cycle at 16°C for 1 week before sampling.

Agro-infiltration of 'Royal Gala' plantlets and 'Orin' calli was performed as described (Ma et al. 2021). They were immersed in 50 mL of the infiltration solution and shaken at 150 rpm at room temperature for 15 min, and then transferred to a sealed chamber for vacuum infiltration at −80 to −100 kPa for 5 min. Subsequently, they were placed onto MS solid medium and incubated in the dark for 72 h followed by 24 h of light exposure before sampling.

4.11 | Luciferase Assay

The luciferase assay was performed as described by Xie et al. (2012) with minor modifications. The CDS of *MdMYB73* and *MdMYB1* were cloned into the pGREENII 62-SK vector separately while the 2000 bp promoter region of *MdUFGT* was cloned into the pFREENII 0800-LUC vector. The constructs were transformed into *Agrobacterium* LBA4404 and incubated in the infiltration buffer (10 mM MES, 0.15 mM acetosyringone and 10 mM $MgCl_2$). The agrobacteria were harvested and resuspended to the final concentration at an OD_{600} value of 0.2. The infiltration of *Nicotiana benthamiana* leaves was made after the agrobacterium was incubated in the dark for 2 h at room temperature. The relative luminescence units (RLU) were detected

by GloMax20/20 Luminometer (Turner Biosystems, Sunnyvale, CA, USA).

4.12 | Statistical Analysis

Analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference (HSD) tests or Student's *t*-test was performed using R v.4.3.2 software.

4.13 | Accession Numbers

Genes can be found with the following accession numbers: *MdCHS* (MD04G1003300), *MdCHI* (MD07G1186300), *MdF3H* (MD15G1246200), *MdDFR* (MD15G1024100), *MdANS* (MD03G1001100), *MdUFGT* (MD01G1234400), *Ma1* (MD16G1045200), *MdMYB73* (MD08G1107400), *MdMYB1* (MD09G1278600).

Author Contributions

L.C. and M.Z. planned and designed the experiments. E.B.W. generated plant material. M.Z., J.Z., N.W., D.G.H. and L.C. performed the experiments and analysed the data. M.Z. and L.C. wrote the manuscript with inputs from all the other authors.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data available upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Phylogenetic analysis of MdMYB73 with four Arabidopsis MYBs belonging to subgroup 22 R2R3 MYBs. **Figure S2:** Peel colour of *cMa1*-overexpressing lines L6, L14 and L16 compared with the wild-type (WT) of 'Royal Gala' apple at fruit harvest in 2018. **Figure S3:** Percentage of fruit surface with red colour, titratable acidity and total soluble solids in wild-type 'Royal Gala' (WT)

and *cMa1*-OE lines (L6, L14 and L16) apples at fruit harvest. **Figure S4:** Gene expression, titratable acidity and total soluble solids in the leaves or fruits of wild-type (WT) and *MdMYB73*-RNAi lines of 'Royal Gala' (Z3, Z7 and Z8). **Figure S5:** Transient RNAi/overexpression of *MdMYB73* increases/decreases anthocyanin biosynthesis in 'Zestar' apple peel. **Figure S6:** Transient RNAi or overexpression of *MdMYB73* alters anthocyanin biosynthesis in wild-type (WT) and *cMa1*-OE lines (L6, L14 and L16) of 'Royal Gala' apple. **Figure S7:** Binding assays of MdMYB73 to the promoters of *MdMYB1*, *MdDFR* and *MdANS*. **Figure S8:** MdMYB1 binds to all seven MYB binding sites in the promoter of *MdUFGT* and overexpression of *MdUFGT* increases anthocyanin biosynthesis and the expression of *MdMYB1*. **Figure S9:** Transient overexpression of *MdMYB1* does not alter the expression levels of *MdMYB73* or *Ma1*. **Figure S10:** Yeast two-hybrid assay showed no interaction between MdMYB73 and MdbHLH3. **Table S1:** List of primers used in this paper.