Transcription Factor AREB2 Is Involved in Soluble Sugar Accumulation by Activating Sugar Transporter and Amylase Genes[1]

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Sugars play important roles in plant growth and development, crop yield and quality, as well as responses to abiotic stresses. Abscisic acid (ABA) is a multifunctional hormone. However, the exact mechanism by which ABA regulates sugar accumulation is largely unknown in plants. Here, we tested the expression profile of several sugar transporter and amylase genes in response to ABA treatment. *MdSUT2* and *MdAREB2* were isolated and genetically transformed into apple (*Malus domestica*) to investigate their roles in ABA-induced sugar accumulation. The MdAREB2 transcription factor was found to bind to the promoters of the sugar transporter and amylase genes and activate their expression. Both MdAREB2 and MdSUT2 transgenic plants produced more soluble sugars than controls. Furthermore, MdAREB2 promoted the accumulation of sucrose and soluble sugars in an *MdSUT2*-dependent manner. Our results demonstrate that the ABA-responsive transcription factor MdAREB2 directly activates the expression of amylase and sugar transporter genes to promote soluble sugar accumulation, suggesting a mechanism by which ABA regulates sugar accumulation in plants.

Sugars are major products of carbon and energy during photosynthesis. In plants, they act not only as an essential source of carbon but also as signaling molecules in response to the integration of information from environmental signals as well as developmental and metabolic cues (Lastdrager et al., 2014). Therefore, they are very important for growth and development. In addition, when sugars are transported into and accumulate in the vacuole, they become crucial players in the development of fruit quality and stress tolerance.

In higher plants, high sugar levels are accumulated in sinks, depending on the expression of various genes associated with sugar biosynthesis, metabolism, and transportation. The genes *SuSy* and *AINV* encode Suc synthase and vacuolar acid invertase, respectively. They play an important role in the regulation of sugar accumulation because they are involved in the regulation of Suc decomposition through which Suc is generally broken down into Glc, Fru, or UDP-Glc in fruit (Yamaki, 2010). In addition, Suc transporters also play an important physiological role in the regulation of fruit development by stimulating sugar accumulation in fruit and, thus, improving fruit yield and quality (Lu et al., 2000). In grapevine (*Vitis vinifera*), the Suc transporters VvSuc11 and VvSuc12 regulate soluble sugar accumulation by controlling Suc transport (Lecourieux et al., 2014). The transcript level of *CitSUT1* increases with the development and ripening of citrus fruits. Its expression pattern is similar to those of *VvSuc11* and *VvSuc12*, and its overexpression increases Suc accumulation in transgenic citrus lines (Zheng et al., 2014; Islam et al., 2015). A Suc uptake transporter (*SUT*) gene is highly expressed in pollen, which leads to the accumulation of Suc in tobacco (*Nicotiana tabacum*; Chung et al., 2014). When the *SUT1* gene is symplasmically isolated during fruit development, soluble sugars accumulate at a high level through an apoplasmic phloem-unloading pathway in apple (*Malus domestica*) fruit (Zhang et al., 2004). In addition, ectopic expression of the *MdSUT2* gene confers enhanced stress tolerance, early flowering time, and increased plant size in transgenic Arabidopsis (*Arabidopsis thaliana*; Ma et al., 2016).

Notably, sugar accumulates at a relatively high level in vacuoles and produces high turgor pressure. Therefore, sugar also is involved in the response to abiotic stresses by affecting osmotic potentials (Gibson, 2005). Soluble sugars respond to environmental stresses,

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including water stress and salinity (Moustakas et al., 2011; Rasheed et al., 2011). In Arabidopsis, soluble sugars, anthocyanins, and Pro all increase together under drought stress (Sperdouli and Moustakas, 2012). Previous studies have indicated that the Suc and fructan levels increased together in salt-stressed wheat (*Triticum aestivum*; Kafi et al., 2003). Transgenic cotton (*Gossypium hirsutum*) plants with a vacuolar H^+-PPase gene are more resistant to NaCl than nontransgenic plants, which may be attributed to their enhanced sugar levels upon exposure to salt stress (Lv et al., 2008; Yao et al., 2010). The levels of compatible solutes, in particular of Glc, Fru, mannitol, and several amino acids, were increased in the halophyte *Thellungiella halophila* upon exposure to high salt levels. Abiotic stresses such as salt, cold, and drought severely impact crop performance and productivity at least in part because they affect the sugar supply (Khan et al., 2017).

As mentioned above, various environmental stimuli such as drought stress promote sugar accumulation, which is very important for stress tolerance and fruit quality, by inducing the expression of genes that are associated with sugar biosynthesis and transport (Ferrandino and Lovisolo, 2014; Medici et al., 2014). However, the exact mechanism through which environment signals induce the expression of those genes is not particularly clear. Abscisic acid (ABA) is well known as a key hormone that acts in response to various abiotic stresses. It regulates the accumulation of soluble sugars by affecting the transcript levels of genes that are associated with sugar synthesis and transport in plants (Gibson, 2004). The expression of *TREHALOSE 6-PHOSPHATE SYNTHASE*, which plays a key role in modulating trehalose 6-phosphate levels in the vegetative tissues of Arabidopsis, is regulated by ABA. It is involved in regulating the accumulation of soluble sugars (Gómez et al., 2010). ABA induces the expression of the cellulose synthase gene *AtCesA8/IRX1*, which plays a role in sugar metabolism. *AtCesA8/IRX1* mutant plants accumulate more ABA, Pro, and soluble sugars than the wild type (Chen et al., 2005). The transcripts of an ADP-Glc pyrophosphorylase gene in rice (*Oryza sativa*) called *OsAPL3* accumulate significantly in response to increased levels of Suc and ABA in the medium (Akihiro et al., 2005). In addition, a high concentration of ABA reduces Suc transport into the grains and lowers the ability of the grains to synthesize starch (Bhatia and Singh, 2002). However, an appropriate concentration of ABA enhances Suc synthase activity and increases the expression of genes related to sugar metabolism (Akihiro et al., 2005; Tang et al., 2009).

Some evidence shows that plant monosaccharide transport proteins such as the hexose transporter (HT) also are regulated by ABA. *VvHT1* is transcriptionally regulated by *VvMSA*, the expression of which is induced by ABA, but only in the presence of Suc (Rook et al., 2006). *VvHT5* expression is regulated by ABA during the hexose transition from source to sink in response to infection by biotrophic pathogens (Hayes et al., 2010). ABA also plays a vital role in regulating the key enzymes that are involved in fructan and Suc metabolism in wheat (Yang et al., 2004). Although genetic analyses have revealed that sugar transporters may be involved in interactions between the sugar signaling pathways and the ABA plant hormone (Gibson, 2004; Rolland et al., 2006), the exact mechanism by which ABA regulates sugar transport and accumulation is largely unknown.

In this study, the tonoplast monosaccharide transporters MdTMT1 and MdSUT2 were found to be noticeably induced at the transcriptional level by ABA hormone and were involved in the accumulation of soluble sugars. The transcription factor MdAREB2 was bound directly to the ABA-responsive element (ABRE) recognition site in the promoter regions of several genes encoding sugar transporters and amylase, and it positively regulated their expression. This discovery shed light on the molecular mechanism by which ABA regulates sugar accumulation in apples.

RESULTS

The Sugar Transporter Genes *MdTMT1* and *MdSUT2* Are Crucial for the ABA-Induced Accumulation of Soluble Sugars

To determine whether ABA affects the soluble sugar content, in vitro shoot cultures of cv Gala apples at the same size were treated with 100 μM ABA. Starch staining demonstrated that ABA treatment markedly reduced the starch contents of the shoot cultures (Fig. 1, A and B). Simultaneously, the soluble sugar, Suc, and Glc contents increased noticeably with ABA treatment. However, ABA treatment did not significantly influence the Fru content (Fig. 1, C and D).

Generally, *TMT* genes are responsible for monosaccharide (such as Glc) transport, while SUTs are responsible for Suc transport (Barker et al., 2000; Slewinski, 2011). To find out the *MdTMT* and *MdSUT* genes in the apple genome, a BLAST search against the apple genome database (The Apple Gene Function & Gene Family DataBase version 1.0) was performed using Arabidopsis AtTMT1 and AtSUT2 as queries, respectively. As a result, there are a total of five *MdTMT* and four *MdSUT* genes: *MdTMT1* (MDP0000381084), *MdTMT2* (MDP0000212510), *MdTMT3* (MDP0000250194), *MdTMT4* (MDP0000868028), *MdTMT5* (MDP0000250193), *MdSUT1* (MDP0000426862), *MdSUT2* (MDP0000277235), *MdSUT3* (MDP0000584979), and *MdSUT4* (MDP0000275743; Supplemental Fig. S1, A and B). Their expression in response to ABA treatment was examined by quantitative real-time (qRT)-PCR. The result showed that only the transcript levels of the *MdTMT1* and *MdSUT2* genes were noticeably induced (Fig. 1E). In addition, the amylase *MdAMY* and *MdBAM* genes also were examined. A BLAST search (https://www.rosaceae.org/tools/ncbi) was performed using Arabidopsis AtAMY1 and AtBAM1 as queries, respectively. The results showed that the expression of *MdAMY1*, *MdAMY3*, *MdBAM1*,

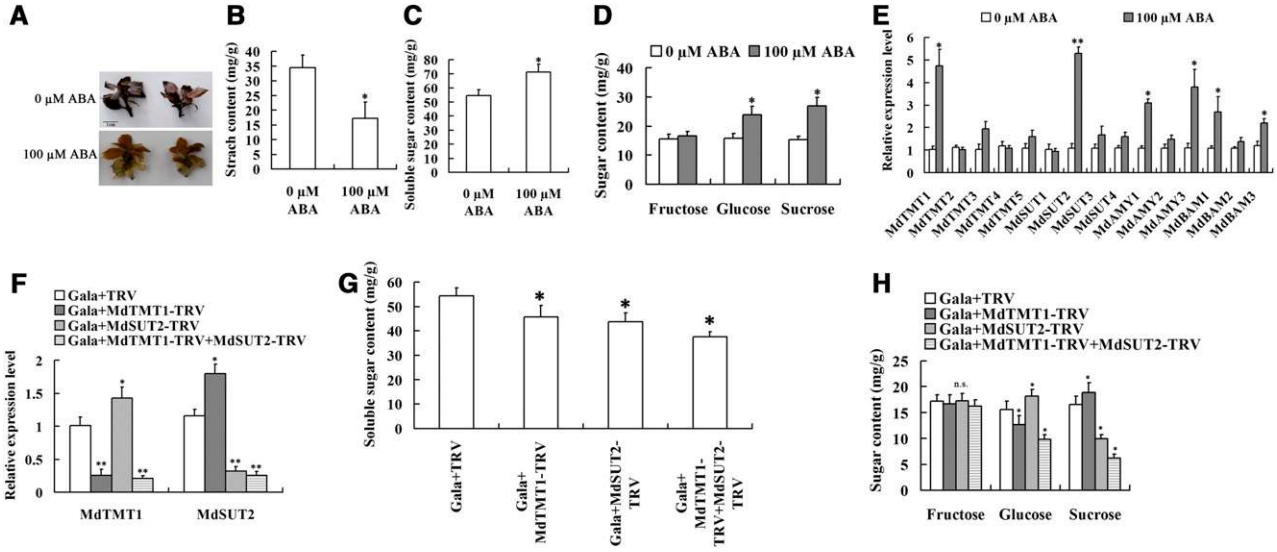


Figure 1. ABA promotes the accumulation of soluble sugars and increases the expression of genes encoding sugar transporters and amylases. A, Starch accumulation effect of 100 μM exogenous ABA on the in vitro shoot cultures of cv Gala apples. B to D, Starch (B), soluble sugar (C), and Fru, Glc, and Suc (D) contents of cv Gala apples without or with ABA. E, ABA effect on the gene expression of *MdTMTs*, *MdSUTs*, *MdAMYs*, and *MdBAMs*. F, Gene expression of MdTMT1 and MdSUT2 in the in vitro shoot cultures of cv Gala apples that were transiently infected by the TRV system. MdTMT1 and MdSUT2 were cloned into the TRV vector and used for suppression. The empty vectors were used as controls. G and H, Contents of soluble sugar (G) and Fru, Glc, and Suc (H) in the MdTMT1-TRV- or MdSUT2-TRV-infected shoot cultures with 100 μM ABA treatment. n.s. indicates a non-significant difference ($P > 0.01$); asterisks indicate statistically significant differences (*, $P < 0.01$ and **, $P < 0.001$). The values are means ± SD ($n = 3$ independent experiments).

and *MdBAM3* was increased markedly with ABA treatment (Fig. 1E). In addition, it was found that those genes were constitutively expressed in different organs, such as root, stem, leaf, flower, and fruit (Supplemental Fig. S2, A and B).

To elucidate the functions of sugar transporter genes in apple, the Suc transporter MdSUT2 was selected and cloned by PCR. According to the phylogenetic tree, MdSUT2 (MDP0000277235) was located together with the Arabidopsis Suc transporter AtSUT2, supported by a bootstrap value of 100%. The *MdSUT2* gene was located on apple chromosome 3. A gene structure analysis showed that there were 14 exons and 13 introns in the MdSUT2 open reading frame (Supplemental Fig. S3A). Hydrophobicity plots showed that MdSUT2 has an N-terminal domain that is approximately 30 amino acids longer than the apple Suc transporters MdSUT1, MdSUT3, and MdSUT4. AtSUT2 and MdSUT2 were overlaid, and the extended central cytoplasmic loop is approximately 50 amino acids longer at amino acid residue 319 (Supplemental Fig. S3B). They had a very high similarity in terms of the predicted three-dimensional protein structure (Supplemental Fig. S3C). In addition, the position of the *MdTMT1* gene was found to be at apple chromosome 6, which has five exons and four introns in the MdTMT1 open reading frame (Supplemental Fig. S3D).

To examine if *MdTMT1* and *MdSUT2* genes are involved in regulating ABA-induced soluble sugar accumulation, a viral vector-based method was used to alter their expression. The tobacco rattle virus (TRV) vector was used to suppress gene expression. The two viral constructs MdTMT1-TRV and MdSUT2-TRV were obtained. They were separately or simultaneously transformed into in vitro shoot cultures of cv Gala apples, while the empty TRV vector was used as the control. An expression analysis demonstrated that the empty TRV infection did not influence the expression of *MdTMT1* and *MdSUT2* genes (Supplemental Fig. S4, A–C). The transcript levels of *MdTMT1* and *MdSUT2* were reduced markedly in MdTMT1-TRV-, MdSUT2-TRV-, and MdTMT1-TRV + MdSUT2-TRV-infected shoot cultures (Fig. 1F), suggesting that the TRV viral vector-based method worked well in this study.

The MdTMT1-TRV-, MdSUT2-TRV-, or MdTMT1-TRV + MdSUT2-TRV-infected shoot cultures were subsequently used to check if *MdTMT1* and *MdSUT2* genes are involved in ABA-induced Glc and Suc accumulation with 100 μM ABA treatment, respectively. The result indicated that the MdTMT1-TRV-mediated suppression of the *MdTMT1* gene successfully counteracted the ABA-induced increase in the Glc content, while the MdSUT2-TRV-mediated suppression of the *MdSUT2* gene did the same for the Suc content (Fig. 1H). As a result, both MdTMT1-TRV- and MdSUT2-TRV-infected shoot cultures produced lower amounts of soluble sugars than the empty TRV control (Fig. 1G). In addition, simultaneous suppression of two genes (i.e. MdTMT1-TRV + MdSUT2-TRV double infection) resulted in decreased amounts of Glc, Suc, and soluble sugar (Fig. 1, G and H). Therefore, ABA-induced Glc

and Suc accumulation required the functions of MdTMT1 and MdSUT2, respectively.

MdSUT2 Functions as a Suc Transporter

The *MdSUT2* gene was chosen subsequently for further investigation. To characterize the function of MdSUT2 in planta, an expression construct called 35S::MdSUT2 was constructed and genetically transformed into the cv Gala apple. As a result, nine independent transgenic lines of MdSUT2 were obtained. Expression analysis demonstrated that the transgenic lines MdSUT2-1, MdSUT2-2, and MdSUT2-5 produced much greater amounts of *MdSUT2* transcripts than the wild-type control (Fig. 2, A and B; Supplemental Fig. S5). And it seems that they grew faster than the wild-type control (Fig. 2B). The three transgenic lines accumulated more MdSUT2 proteins and exhibited higher Suc transport activity than the wild-type control (Fig. 2, C and D). They accumulated more total soluble sugars and Suc than the wild-type control (Fig. 2, E and F). As a result, it seems that they grew faster than the wild-type control (Fig. 2B).

The ABRE cis-Element in the Promoter Region of the MdSUT2 Gene Is Required for Its ABA-Induced Expression

In our previous study, the expression of *MdSUT2* was found to be induced by ABA (Ma et al., 2016). To explain why its expression is induced by ABA, the promoter region of the *MdSUT2* gene was analyzed. The MdSUT2 promoter was found to contain various cis-elements (Supplemental Table S1). Among them, there is an ABRE that has a CACGTC core sequence (Fig. 3A). To examine if the transcription activity of the MdSUT2 promoter is induced by ABA, a GUS reporter gene was fused downstream from the promoter. The resulting pMdSUT2::GUS construct was then genetically transformed into the apple calli. GUS staining demonstrated that ABA treatment noticeably increased the GUS activity, indicating that the promoter is ABA responsive (Fig. 3, B and C).

To examine if the ABRE core GCACGTCC sequence is crucial for the ABA response, a mutant MdSUT2 promoter that contains CTGGAT but not CACGTC was artificially constructed and used for the GUS analysis. In this case, the pMdSUT2::GUS(m) construct was transformed into the apple calli. When the pMdSUT2::GUS(m) transgenic calli were treated with ABA, it was found that ABA treatment did not influence the GUS activity (Fig. 3, B and C). At the same time, pMdSUT2::GUS and pMdSUT2(m)::GUS were genetically transformed into Arabidopsis. The transgenic Arabidopsis showed the same GUS phenotype as the transgenic apple calli (Fig. 3D). In other words, the mutation in the core sequence destroyed the responsiveness of the transgenic calli to the ABA treatment. These results indicated that the ABRE cis-element plays a crucial role in the ABA response.

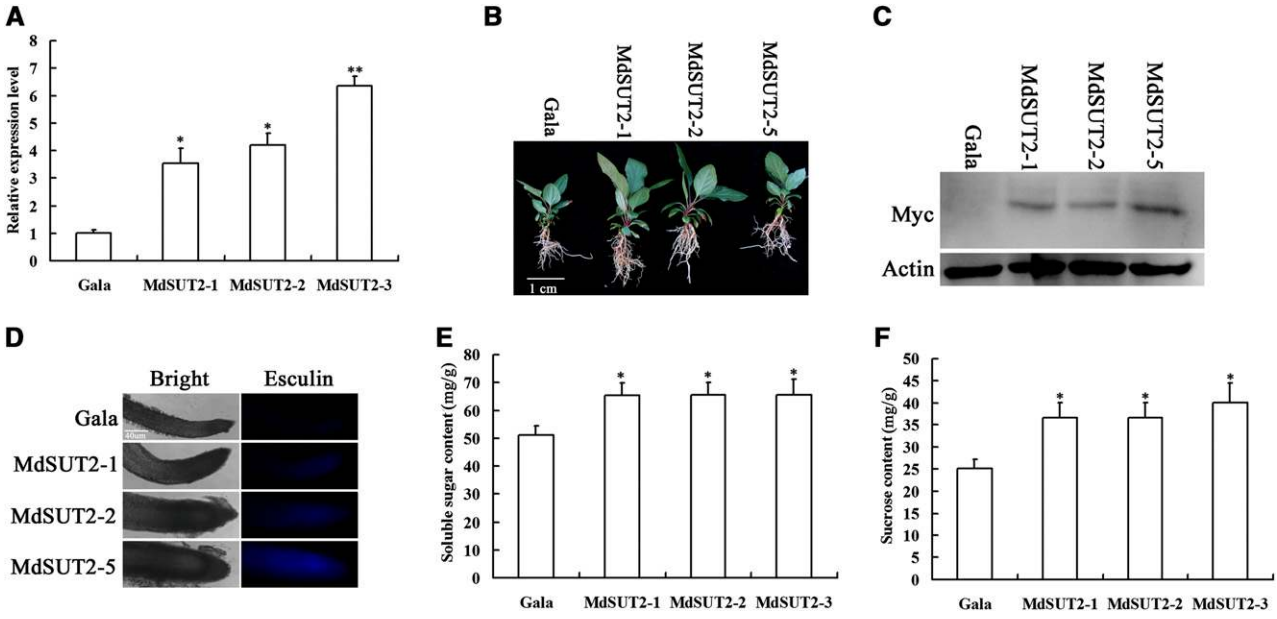


Figure 2. MdSUT2 functions as a Suc transporter. A, qRT-PCR was used to examine the transcription levels of the three overexpression lines MdSUT2-1, MdSUT2-2, and MdSUT2-5 compared with cv Gala. B, Two-month-old cv Gala plants and three MdSUT2 overexpression lines (MdSUT2-1, MdSUT2-2, and MdSUT2-5). C, Western-blot examination of the MdSUT2 protein abundance in 35S::MdSUT2-Myc transgenic plants. D, Suc activity in the roots of transgenic plants. E and F, Soluble sugar (E) and Suc (F) contents. Asterisks indicate statistically significant differences (*, $P < 0.01$ and **, $P < 0.001$). The values are means ± SD ($n = 3$ independent experiments).

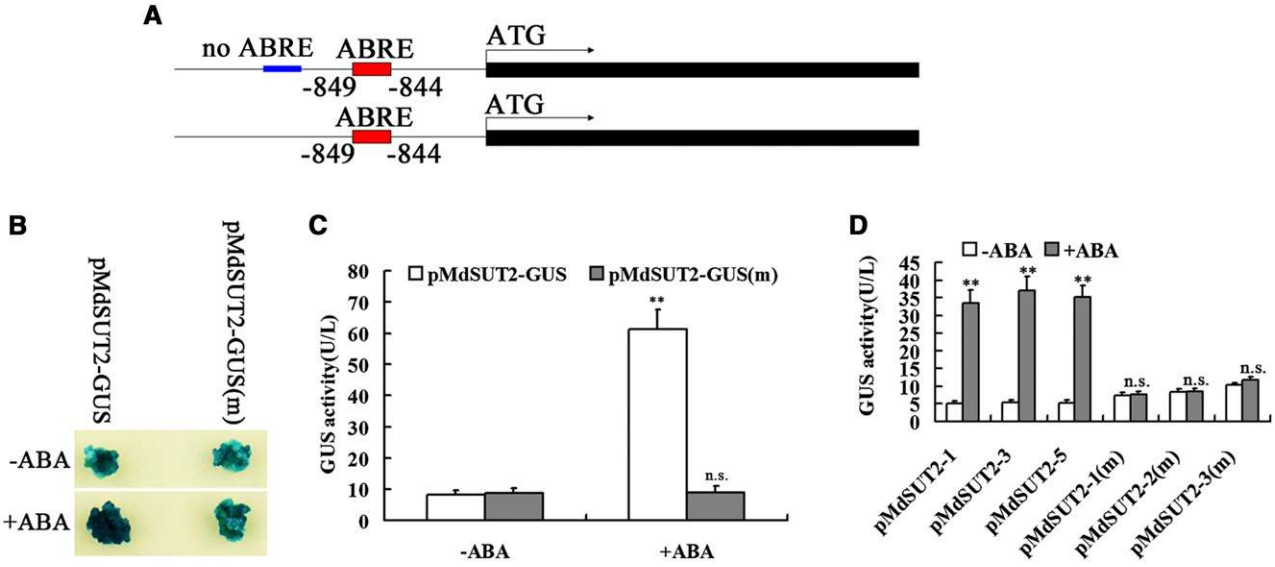


Figure 3. The expression of MdSUT2 was induced by ABA. A, Schematic diagram showing the cis-element in the MdSUT2 promoter as analyzed by PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Red boxes indicate the ABRE in the MdSUT2 promoter. ABRE(m) indicates the site mutants in which the ABRE core sequence CTGCAC was changed to TAGGTC. The blue box represents sequence without the ABRE core. B, GUS-stained pMdSUT2-GUS and pMdSUT2-GUS(m) transgenic apple calli in treatments with or without ABA. C, Quantitative statistics of GUS activity in apple calli. D, Quantitative statistics of GUS activity in pMdSUT2-GUS and pMdSUT2-GUS(m) Arabidopsis seedlings. n.s. indicates nonsignificant differences ($P > 0.01$); asterisks indicate statistically significant differences (**, $P < 0.001$). The values are means ± SD ($n = 3$ independent experiments).

The ABA-Responsive Transcription Factor MdAREB2 Binds to the Promoters of the Sugar Transporter and Amylase Genes in Response to ABA in Apple

To identify the potential transcription factors that recognize and bind to the ABRE cis-element, the oligonucleotide probe TCCATACAGCACGTCCTA-GTTTGATTTTTAGCACTCGTGAATTA with the ABRE core sequence underlined was designed on the basis of the MdSUT2 promoter. The resulting probe was used for DNA-affinity trapping and electrophoretic mobility shift assay (EMSA). When the nuclear protein extracts were incubated with biotin-labeled MdSUT2 promoter oligonucleotides, a DNA-protein complex with a slower mobility in EMSA was observed (Fig. 4A). Subsequently, a mass spectrometry analysis was performed to identify the proteins that bind to the oligonucleotide. The result showed that the protein encoded by the MDP0000248567 gene was a candidate from among the liquid chromatography-mass spectrometry data (Supplemental Appendix S1). To identify MDP0000248567, a phylogenetic tree was conducted with MEGA 5.0 software. It was found that MDP0000248567 was located together with the Arabidopsis transcription factor AtAREB2, supported by bootstrap values of 100% (Supplemental Fig. S6A). Therefore, MDP0000248567 was named MdAREB2. The *MdAREB2* gene is localized to chromosome 5 and has four exons and three introns (Supplemental Fig. S6B). The expression analysis demonstrated that the transcript level of the *MdAREB2* gene was markedly induced by ABA, drought, and polyethylene glycol (Supplemental Fig. S6C), indicating that it is an ABA-responsive gene.

To determine whether MdAREB2 actually binds to the ABRE recognition site of the MdSUT2 promoter, EMSA was performed using MdAREB2-GST fusion proteins. A specific DNA-MdAREB2 protein complex was detected when the CACGTC-containing oligonucleotide was used as a labeled probe. The formation of these complexes was reduced by increasing the amounts of the unlabeled ABRE competitor probe with the same sequence. This competition was not observed when using the mutated version. This competition specificity confirmed that the specific binding of MdAREB2 to the MdSUT2 promoter required the ABRE recognition sequence (Fig. 4B).

To verify the specific in vivo binding of MdAREB2 to the *MdSUT2* promoter, chromatin immunoprecipitation (ChIP)-PCR assays were conducted using pAREB2::MdAREB2-GFP and pAREB2::GFP transgenic apple calli treated with 50 μM ABA. The ABRE-containing promoter regions of *MdSUT2*, but not those of *MdSUT1* and *MdSUT4*, were enriched by ChIP in the pAREB2::MdAREB2-GFP transgenic calli compared with the pAREB2::GFP control (Fig. 4C; Supplemental Fig. S7, A and B). Another primer without ABRE core sequence based on the promoter sequence of the *MdSUT2* gene was designed. ChIP-PCR showed that MdAREB2 failed to bind to the sequence (Fig. 4C). It showed that MdAREB2 specifically bound to the ABRE cis-acting element on the promoter of MdSUT2.

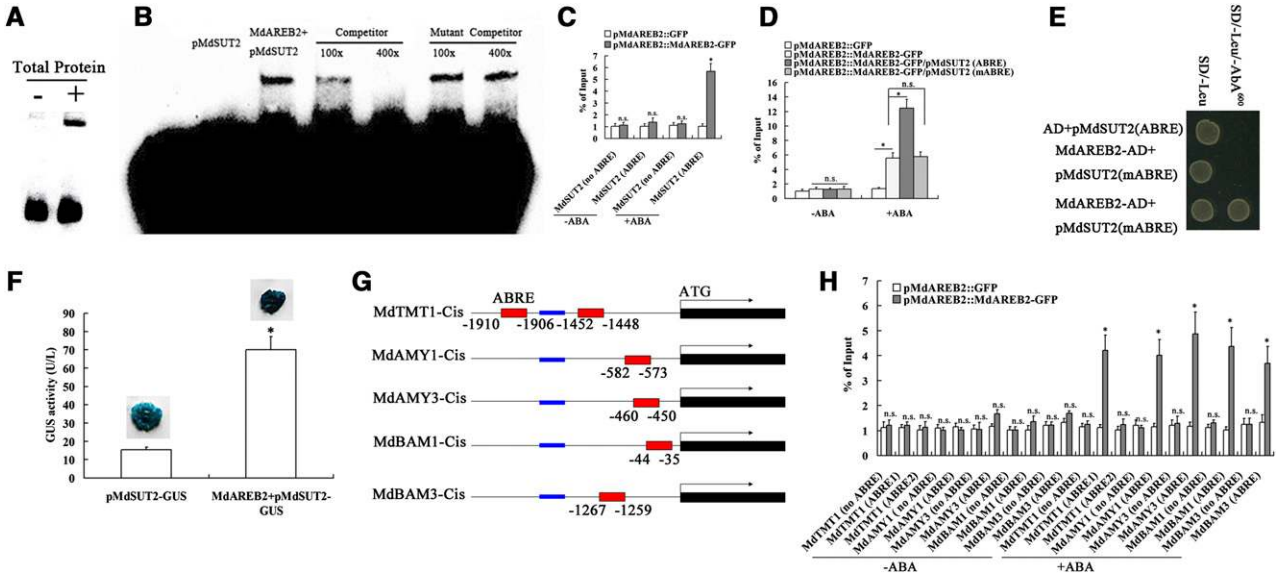


Figure 4. The transcription factor MdAREB2 binds to the cis-element of the sugar metabolism promoter. A, Identification of the MdAREB2 TF proteins that bind to the cis-element of the MdSUT2 promoter with EMSA. + and − represent samples with and without the addition of total protein extracted from the apple plants, respectively. B, Interaction of MdAREB2 protein with labeled DNA probes for the cis-elements of the MdSUT2 promoter in the EMSA. pMdSUT2 was used as a negative control without the MdAREB2 protein. C, Relative enrichment of the MdSUT2 promoter fragments. The MdAREB2-DNA complex was coimmunoprecipitated from MdAREB2-GFP transgenic apple calli using an anti-GFP antibody. GFP was used as a negative control. D, ChIP analysis of MdAREB2 binding to pMdSUT2(ABRE) and pMdSUT2(mABRE) with or without ABA. E, The promoter of MdSUT2 was fused to the pAbAi vector, and the MdAREB2 gene was fused to the activation domain in yeast for yeast one-hybrid assays. F, GUS activity in the transgenic apple calli as labeled. G, Schematic diagram showing the cis-element in *MdTMT1*, *MdAMY1(MDP0000210170)*, *MdAMY3(MDP0000281786)*, *MdBAM1(MDP0000193684)*, and *MdBAM3(MDP0000397284)* promoters as analyzed by PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Red boxes indicate the ABRE in the promoter of each gene. Blue boxes represent sequence without the ABRE core. H, ChIP-PCR showed that MdABRE2 binds specifically to the ABRE of the *MdTMT1*, *MdAMY1*, *MdAMY3*, *MdBAM1*, and *MdBAM3* promoters in vivo. n.s. indicates nonsignificant differences ($P > 0.01$); asterisks indicate statistically significant differences (*, $P < 0.01$). The values are means ± SD ($n = 3$ independent experiments).

In addition, ChIP-PCR was performed using pMdAREB2::MdAREB2-GFP, pMdAREB2::MdAREB2-GFP/pMdSUT2(ABRE)::GUS, and pMdAREB2::MdAREB2-GFP/pMdSUT2(mABRE)::GUS coexpressed apple protoplasts treated with or without ABA. Apple isolated protoplasts from pMdAREB2::GFP were used as a control. The result showed that the enrichment of the *MdSUT2* promoter region increased in the protoplasts treated with ABA compared with those without ABA treatment. When the ABRE of the *MdSUT2* promoter was mutated, however, the enrichment of the *MdSUT2* promoter region did not increase in protoplasts even under ABA treatment. These results indicated that ABA increased the specific binding of MdAREB2 to the ABRE of the *MdSUT2* promoter (Fig. 4D).

For the yeast one-hybrid assays, the MdSUT2 promoter was fused to the pAbAi vector and the MdAREB2 gene was fused to the activation domain (AD). When fused pMdSUT2-AbAi was coexpressed with MdAREB2-AD in yeast, the strain was able to grow on a synthetic dextrose (SD)/-Leu/plus 600 ng mL^{-1} aureobasidin A plate. No growth was observed in the negative control expression in which the MdSUT2 promoter was mutated (Fig. 4E). These results provided in vivo evidence for the specific binding of MdAREB2 to the MdSUT2 promoter.

GUS assays were conducted to examine if MdAREB2 influences the transcription activity of the MdSUT2 promoter in response to ABA. The 35S::MdAREB2 construct was genetically transformed into the pMdSUT2::GUS transgenic calli. The GUS analysis showed that the transgenic calli containing pMdSUT2::GUS plus 35S::MdAREB2 exhibited a much higher GUS activity than those harboring pMdSUT2::GUS alone (Fig. 4F), indicating that MdAREB2 specifically activated *GUS* transcription as driven by the *MdSUT2* promoters in response to ABA.

The *MdTMT1* promoter was found to have two ABRE cis-acting elements, ABRE1 and ABRE2 (Fig. 4G; Supplemental Table S1). ChIP-PCR demonstrated that MdAREB2 binds specifically to ABRE1 but not to ABRE2 (Fig. 4H), suggesting that *MdAREB2* also may be involved in the transcriptional regulation of *MdTMT1*. Furthermore, the genes *MdTMT3*, *MdAMY1*, *MdAMY2*, *MdAMY3*, *MdBAM1*, *MdBAM2*, and *MdBAM3* also have one ABRE cis-acting element (Fig. 4G; Supplemental Table S1; Supplemental Fig. S7A). ChIP-PCR assays showed that MdAREB2 could bind to the promoters of the α-amylase genes *MdAMY1* and *MdAMY3* as well as the β-amylase genes *MdBAM1* and *MdBAM3* but not those of MdAMY2 and MdBAM2, which suggested that MdAREB2 also is involved in regulating starch degradation (Fig. 4H;

Supplemental Fig. S7C). In addition, we designed another primer without the ABRE core sequence based on the promoter sequence of *MdTMT1*, *MdAMY1*, *MdAMY3*, *MdBAM1*, and *MdBAM3* genes. ChIP-PCR showed that MdAREB2 failed to bind to these sequences (Fig. 4H).

MdAREB2 Promotes the Accumulation of Suc and Soluble Sugars in Response to ABA

To characterize the function of MdAREB2, the gene was introduced into the pCXMyc-P plant transformation vector downstream of the cauliflower mosaic virus 35S promoter and then transformed into cv Gala apples. As a result, eight transgenic lines of MdAREB2 were obtained. The three independent transgenic lines MdAREB2-1, MdAREB2-2, and MdAREB2-4 were used for the functional analysis. The RT-PCR analysis showed that the transcript levels of three transgenic lines increased noticeably compared with the cv Gala control (Fig. 5B; Supplemental Fig. S8). A western blot with an anti-Myc antibody indicated that the MdAREB2-Myc protein was detected in the transgenic plants (Fig. 5C). The overexpression of the *MdAREB2* gene markedly up-regulated the expression levels of the *MdTMT1*, *MdSUT2*, *MdAMY1*, *MdAMY3*, *MdBAM1*, and *MdBAM3* genes in response to ABA in the three transgenic lines compared with the wild-type control (Fig. 5D). The transcript levels of the other MdTMTs, MdSUTs, and amylase genes barely changed. As a result, three transgenic lines accumulated less starch but much more Glc, Suc, and soluble sugars than the wild-type control (Fig. 5, A and E–G).

In addition, TRV-based gene suppression demonstrated that the suppression of *MdAREB2* down-regulated the expression of *MdSUT2*, *MdTMT1*, *MdAMY1*, *MdAMY3*, *MdBAM1*, and *MdBAM3* genes (Fig. 6B), indicating that MdAREB2 positively regulates their expression. As a result, the MdAREB2-TRV transiently transgenic shoot cultures accumulated more starch but less soluble sugars than the empty vector control under ABA treatment (Fig. 6, A, C, and D), while no significant difference for starch and soluble sugar contents was found in these materials without ABA treatment (Supplemental Fig. S9). Furthermore, HPLC assays showed that the Fru, Glc, and Suc contents were noticeably reduced in MdAREB2-TRV shoot tissues (Fig. 6E). Therefore, MdAREB2 is required for ABA-induced sugar accumulation in apple.

MdAREB2-Promoted Suc Accumulation under ABA Treatment Partially Needs MdSUT2

To examine if MdAREB2 regulates Suc accumulation in response to ABA in an MdSUT2-dependent manner, a virus-induced gene silencing method was performed using the in vitro leaves of three MdAREB2 transgenic apple lines. The MdSUT2-TRV vector was used to suppress the *MdSUT2* gene. It was transiently transformed into the transgenic lines MdAREB2-1, MdAREB2-2, and MdAREB2-4, while the empty TRV vector was used as a control. The resulting leaves that were infected with empty MdSUT2-TRV and TRV vectors were then transferred onto plates containing Murashige and Skoog (MS) medium for 4 d under normal light at room temperature. The expression analysis demonstrated that the MdSUT2-TRV infection successfully suppressed the expression level of MdSUT2 in three MdAREB2 transgenic lines (Fig. 7A).

The Suc and soluble sugar contents were determined subsequently. The result showed that infection with the TRV empty vector barely influenced the function of MdAREB2 in regulating soluble sugar and Suc accumulation (Fig. 7, B and C). However, infection with the MdSUT2-TRV vector at least partially, if not completely, inhibited MdAREB2 function. In addition, sugar content was detected in cv Gala apple leaves infected with empty vector TRV, MdAREB2-TRV, MdAREB2-TRV/MdSUT2-IL60, and MdAREB2-TRV/MdSUT2-TRV. MdAREB2-TRV infection noticeably decreased Suc content compared with TRV injection. MdAREB2-TRV/MdSUT2-TRV double injection further decreased Suc content, while MdAREB2-TRV/MdSUT2-IL60 double injection restored Suc content to the TRV control level (Supplemental Fig. S10). Therefore, MdAREB2 regulates Suc and sugar accumulation in response to ABA at least partially in an MdSUT2-dependent manner.

MdAREB2 Activated the Expression of MdSUT2, Which Affects the Soluble Sugar Contents of Apple Fruits

To examine if ABA regulates starch and sugar accumulation in fruit, the fruits of the apple cv Red Delicious were treated with 0, 10, 20, and 50 μM ABA. The sugar transport genes *MdTMT1*, *MdTMT4*, *MdSUT1*, *MdSUT2*, and *MdSUT4* were increased under ABA treatment. In addition, *MdAMY1*, *MdAMY3*, *MdBAM1*, and *MdBAM3* were induced significantly by ABA (Fig. 8B). This result showed that ABA treatment markedly reduced the starch content but it increased the contents of Suc and soluble sugars (Fig. 8, A and C–E), just as it did in the apple shoot cultures and callus.

To investigate the function of MdAREB2 in regulating MdSUT2 in apple fruits, these two genes were connected to the IL60 vector and the TRV vector, which were used for transient gene overexpression and transient gene silencing, respectively. The empty vectors were used as controls. The qRT-PCR results showed that MdAREB2-IL60 and MdSUT2-IL60 generated higher transcript levels, while MdAREB2-TRV and MdSUT2-TRV had lower transcription levels (Fig. 8F). These results indicated that MdAREB2-TRV and MdSUT2-TRV reduced the soluble sugar and Suc contents (Fig. 8, G and H). MdAREB2-IL60 and MdSUT2-IL60 showed the opposite trend. These results showed that MdAREB2 positively activated the expression of MdSUT2, increasing the soluble sugar contents of apple fruits.

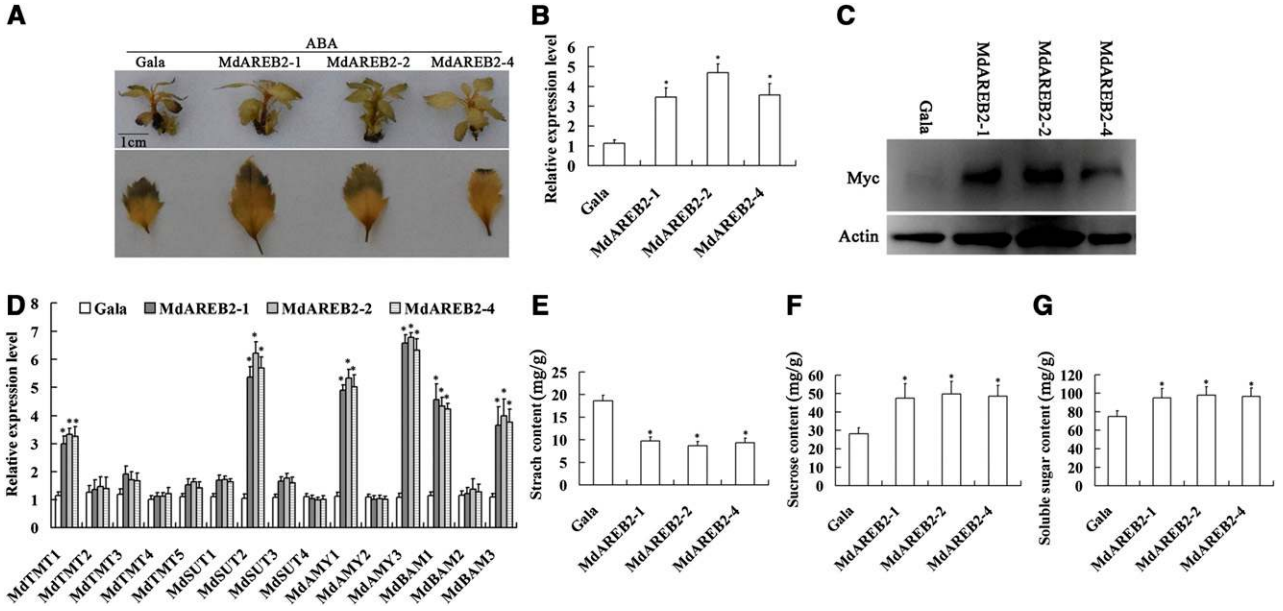


Figure 5. MdAREB2-overexpressing plants show increased accumulations of Suc and soluble sugars. A, Starch staining of MdAREB2-overexpressing plants. B, qRT-PCR was used to examine the transcription level of the three transgenic lines MdAREB2-1, MdAREB2-2, and MdAREB2-4. C, Western blot to check the MdAREB2 protein content. D, qRT-PCR was used to examine the transcription level of *MdTMTs*, *MdSUT2*, *MdAMYs*, and *MdBAMs* in the three transgenic lines MdAREB2-1, MdAREB2-2, and MdAREB2-4. E to G, Contents of starch (E), soluble sugar (F), and Suc (G). Asterisks indicate statistically significant differences (*, $P < 0.01$). The values are means ± SD ($n = 3$ independent experiments).

DISCUSSION

Crops and other plants under natural conditions are routinely affected by a broad range of abiotic and biotic stresses that act simultaneously. One of the pivotal events in abiotic stress responses is the rapid accumulation of ABA, which regulates the expression of ABA-responsive genes that protect plants from damage (Lee and Luan, 2012). Simultaneously, different environmental stimuli increase sugar accumulation by regulating the expression of sugar metabolism genes. In this study, the ABA-induced transcription factor known as MdAREB2 was found to regulate sugar accumulation by directly binding to the promoters of several genes that encode sugar transporters and amylases and by activating their expression.

SUT Mediates Stress-Induced Sugar Accumulation in the Vacuole

Sugars are synthesized from not only photosynthetic carbon fixation but also starch. Starch is the most abundant form in which plants store carbohydrates. Starch metabolism is regulated by various abiotic stresses (Valerio et al., 2011; Zanella et al., 2016), and the resulting accumulation of starch metabolites, including sugars, lowers the water potential of the cell, which promotes water retention in the plant (Verslues and Sharma, 2011; Krasensky and Jonak, 2012). Starch is degraded by a set of glucan-hydrolyzing enzymes (including α-amylases and β-amylases). In Arabidopsis, α-amylase genes such as *AMY3* and *BAM1* are involved in stress-induced starch degradation (Thalmann et al., 2016). In apple, *MdAMY1*, *MdAMY3*, *MdBAM1*, and *MdBAM3* are homologous to Arabidopsis *AMY3* and *BAM1*. Their expression is induced by ABA (Fig. 1). It is reasonable to speculate that these genes may contribute to ABA-induced starch degradation and subsequent sugar accumulation (Fig. 5). The results indicated that these genes were expressed in different tissues (Supplemental Fig. S2). Sugar accumulation at least partially comes from starch degradation in response to ABA in all tissues.

Sugars not only act as osmotic adjustment substances but also help in stabilizing proteins and cell structures under stress conditions (Sami et al., 2016). In addition, they also reduce the effect of stress-induced reactive oxygen species accumulation (Hu et al., 2012). Sugars as osmotic adjustment substances accumulate mainly in the vacuole. The vacuole is involved in the long-term or temporary storage of sugars (Martinoia et al., 2007). Various vacuolar sugar transporters are responsible for the transportation of sugars into the vacuole. TMTs are vacuolar carrier proteins that have transport activity for monosaccharides such Glc (Wormit et al., 2006). Suc transporters (SUTs/SUCs) are proton-coupling Suc uptake transporters that are responsible for the transportation of Suc into the vacuole (Schneider et al., 2012; Shitan and Yazaki, 2013). Both TMTs and SUTs/SUCs abundantly work together to modulate soluble sugar

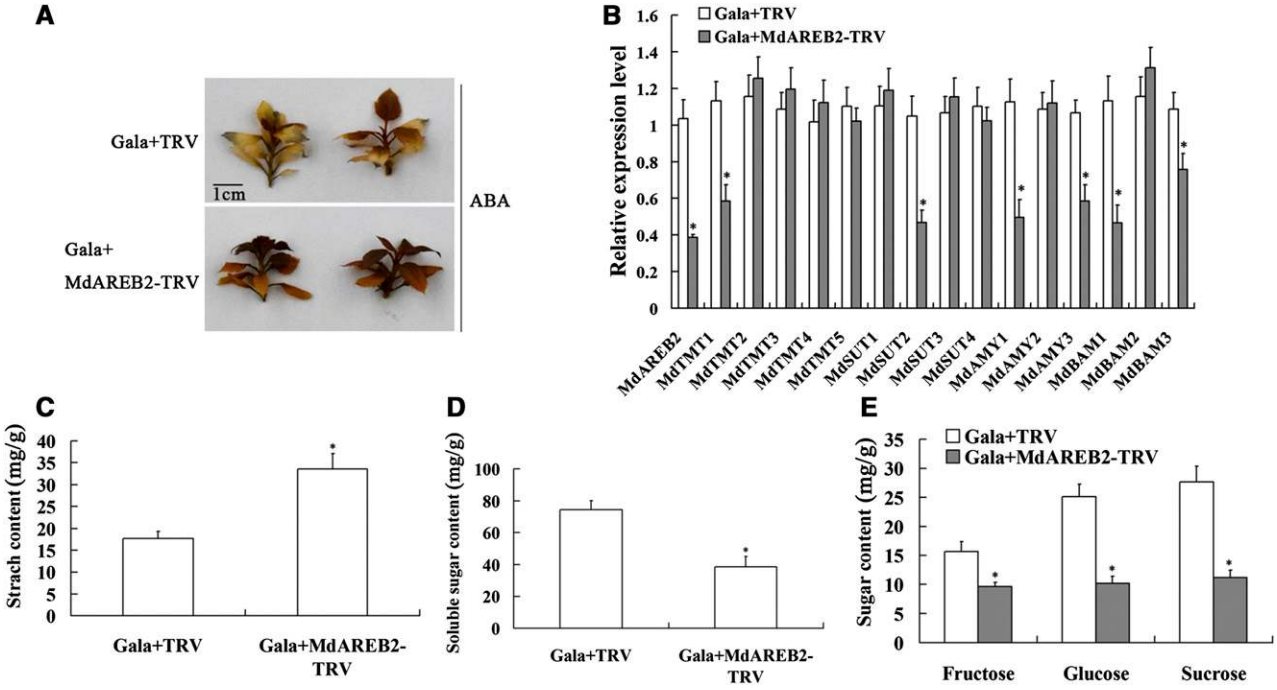


Figure 6. The transient gene-silencing of MdAREB2 through viral vector-based transformation alters the contents of starch and soluble sugar in apple in vitro shoot cultures. A, Starch staining of MdAREB2-TRV transiently infected cv Gala apple in vitro shoot cultures under ABA treatment. The empty vectors were used as controls. B, Gene expression of *MdTMTs*, *MdSUT2*, *MdAMYs*, and *MdBAMs*. C to E, Starch (C), soluble sugar (D), and Fru, Glc, and Suc (E) as treated in plants in A. Asterisks indicate statistically significant differences (*, $P < 0.01$). The values are means ± SD ($n = 3$ independent experiments).

accumulation in the vacuole (Reuscher et al., 2014). As a result, the expression levels of *TMT1* genes were up-regulated, maybe in a feedback regulatory manner, in Arabidopsis mutant *suc2* and apple MdSUT2-TRV shoot cultures (Fig. 1F; Supplemental Fig. S11A). Meanwhile, MdSUT2 overexpression decreased the expression level of the *MdTMT1* gene in transgenic apple plantlets (Supplemental Fig. S11B). Similarly, apple MdTMT1-TRV shoot cultures generated more *MdSUT2* transcripts than the TRV control (Fig. 1F). Therefore, MdSUT2-TRV shoot cultures accumulated more Glc, and MdTMT1-TRV shoot cultures accumulated more Suc, than the TRV control, although both of them accumulated less soluble sugars than the TRV control (Fig. 1G). In addition, transient overexpression of the *MdTMT1* gene produced more soluble sugar and Glc in apple leaves than the empty vector control (Supplemental Fig. S12), suggesting that MdTMT1 acted as a functional Glc transporter.

In addition, all three distinct SUT subfamilies (type SUT1, SUT2, and SUT4) have 12 predicted transmembrane domains in Arabidopsis (Sun et al., 2008). Type SUT2 also has two additional domains, the extended N-terminal and 30- to 50-amino acid center loop domains, compared with type SUT1 and SUT4 (Stolz et al., 1999; Supplemental Fig. S3B). In apple, MdSUT2 contains those conserved domains and exhibits Suc transporter activity (Ma et al., 2016; Supplemental Fig. S3).

Vacuolar sugar transporters are regulated by abiotic stresses. In the apoplasm, the *TMT1* and *TMT2* genes are induced by salt, drought, and cold stresses (Wormit et al., 2006). Arabidopsis *AtSUC1* and *AtSUC2* and rice *OsSUT2* transcripts are up-regulated in response to low temperature, drought, and salinity stresses (Lundmark et al., 2006; Ibraheem et al., 2011). The ectopic overexpression of an apple *MdSUT2* gene improves tolerance to abiotic stresses in apple calli and Arabidopsis (Ma et al., 2016). Therefore, vacuolar sugar transporters play crucial roles in the response to various abiotic stresses by promoting sugar accumulation.

The ABA-Induced Transcription Factor MdAREB2 Regulates Sugar Accumulation

The endogenous ABA level in plant cells increases with osmotic stresses such as drought and high salinity, leading to the expression of stress-responsive genes (Rook et al., 2006). The AREB transcription factors play an important role in ABA signaling. The AREB/ABF genes encode basic-domain Leu zipper (bZIP) transcription factors and belong to a group A subfamily, which comprises nine homologs in the Arabidopsis genome, and they harbor three N-terminal and one C-terminal conserved domains (Yoshida et al., 2015). Among them, AREB1/ABF2, AREB2/ABF4, and ABF3

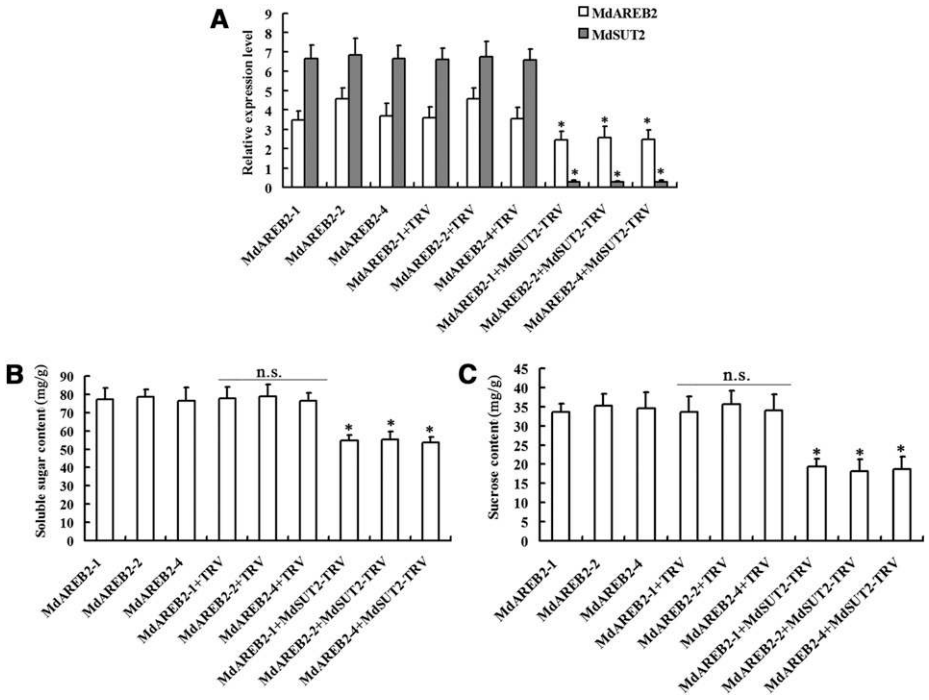


Figure 7. MdAREB2 promotes the accumulation of Suc and soluble sugars by MdSUT2. A, qRT-PCR analyses of *MdAREB2* and *MdSUT2* transcripts in transgenic plants. A virus-induced gene silencing method was performed using the in vitro leaves of three MdAREB2 transgenic apple lines. The MdSUT2-TRV vector was used for MdSUT2 gene suppression, and it was transiently transformed into the transgenic lines MdAREB2-1, MdAREB2-2, and MdAREB2-4. The TRV empty vector was used as a control. B and C, Soluble sugar (B) and Suc (C) contents as treated in plants in A. n.s. indicates nonsignificant differences ($P > 0.01$); asterisks indicate statistically significant differences (*, $P < 0.01$). The values are means ± SD ($n = 3$ independent experiments).

are induced by dehydration, high salinity, or ABA treatment in vegetative tissues (Fujita et al., 2005; Kim et al., 2011). The *areb1areb2abf3* triple knockout mutant shows the impaired expression of ABA and osmotic stress-responsive genes, resulting in increased sensitivity to drought and decreased sensitivity to ABA in primary root growth (Yoshida et al., 2010). Arabidopsis (ABI5, AREB1, and AREB2), rice (TRAB1 and OREB1), and wheat (TaABF) ABF family members have been reported to be crucial for the regulation of ABA-responsive gene expression (Johnson et al., 2002; Kagaya et al., 2002; Yoshida et al., 2010).

In addition, the AREB transcription factors also are involved in sugar accumulation. In Arabidopsis, AREB transcription factors are suggested to activate the expression of *BAM1* and *AMY3* genes through an ABA-dependent SnRK2 signaling pathway (Thalmann et al., 2016). In ABA-treated mosses, the concentration of soluble sugars increased significantly, which may promote cytoplasm vitrification and protect membranes (Mayaba et al., 2001). In carrot (*Daucus carota*), a Suc-upregulated bZIP transcription factor called *CAREB1* responds to the ABA and sugar signal (Guan et al., 2009). *ABI5* overexpression confers increased sensitivity to the Glc inhibition of seedling growth, indicating that ABI5 mediates the sugar response (Finkelstein and Gibson, 2002). In this study, the expression of the *MdAREB2* gene also was found to be induced by ABA (Supplemental Fig. S6), and *MdAREB2* overexpression increased the soluble sugar contents in transgenic apple plantlets in response to ABA (Supplemental Fig. S5).

As is well known, AtSUT2 shows a similar structure to those of the yeast sugar sensors RGT2 and SNF3 (Özcan et al., 1998). MdSUT2 and AtSUT2 exhibited highly similar amino acid sequences and three-dimensional structures to each another (Supplemental Fig. S1A and S3C). This suggests the hypothesis that both of them may function as sugar sensors and influence the expression levels of the related genes. This hypothesis is supported by the fact that the transcript levels of *AREB2* genes decrease in Arabidopsis mutant *suc2* and apple MdSUT2-TRV shoot cultures (Fig. 7A; Supplemental Fig. S13A) but increase in *MdSUT2* transgenic apple plantlets (Supplemental Fig. S13B). Therefore, MdSUT2 may function as a sugar sensor to positively regulate the expression of the *AREB2* gene, although the exact mechanism is unknown. In addition, it was found that the expression level of the *MdAREB2* gene and the content of Suc were reduced in the MdSUT2-TRV leaves of three MdAREB2 apple transgenic plantlets (Fig. 7, A and C). MdSUT2 was overexpressed in MdAREB2-TRV transgenic plants, which could increase Suc content (Supplemental Fig. S10). This showed that *MdAREB2* promotes sugar accumulation at least partially, if not completely, via MdSUT2, which increases SUT2 activity directly by itself and indirectly by enhancing *MdAREB2* expression.

ABA-Induced Sugars Contribute to Plant Development and Fruit Quality

Soluble sugars (Suc, Glc, and Fru) play an important role in maintaining the growth and development of plants. The soluble sugars trigger the proliferation of organs and produce larger and thicker leaves, increasing the size and number of tubers and adventitious roots. For example, high sugar concentrations stimulate the formation of adventitious roots in Arabidopsis

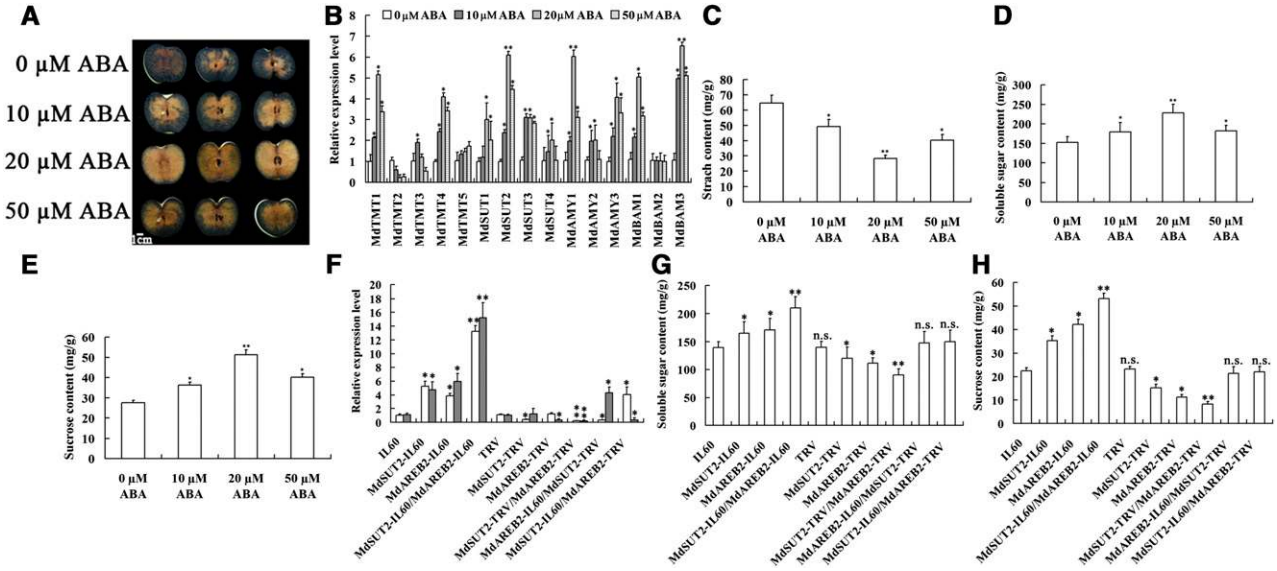
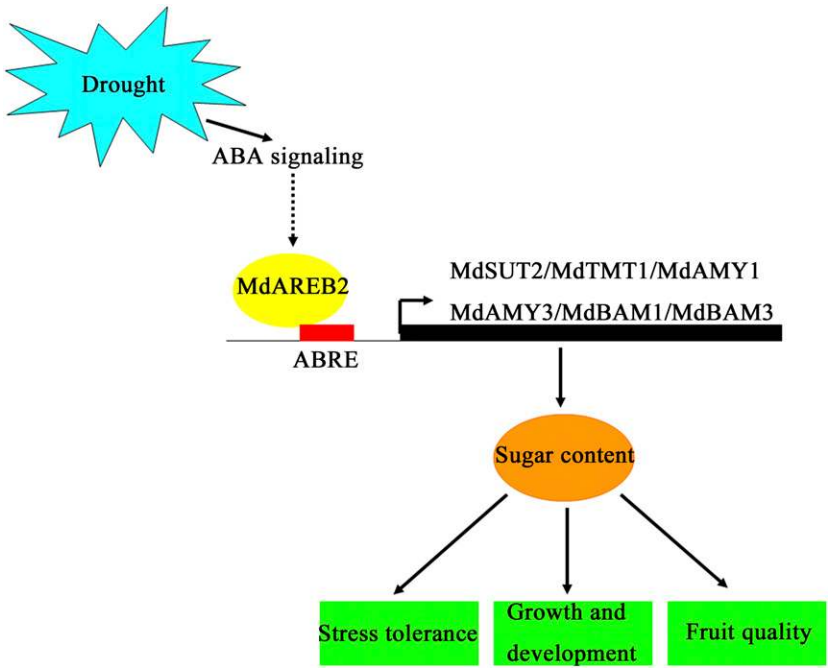


Figure 8. ABA promotes the accumulation of soluble sugars and increases the expression of sugar transporter genes. The apple fruits of cv Red Delicious were treated with 0, 10, 20, and 50 μM ABA. A, Starch accumulation effect of exogenous ABA on apple fruits. B, Expression analysis of *MdTMT*, *MdSUT2*, *MdAMY*, and *MdBAM* genes. C to E, Starch (C), soluble sugar (D), and Suc (E) contents. F to H, The transient expression of MdAREB2 and MdSUT2 via viral vector-based transformation alters the soluble sugars and Suc contents of apple fruits. The MdSUT2-IL60 and MdAREB2-IL60 vectors were used for the overexpression of MdSUT2 and MdAREB2 genes, while the MdSUT2-TRV and MdAREB2-TRV vectors were used for their suppression. F shows the expression analysis of *MdAREB2* and *MdSUT2* genes in each transient expression fruit, while G and H show the soluble sugar (G) and Suc (H) contents. n.s. indicates nonsignificant differences ($P > 0.01$); asterisks indicate statistically significant differences (*, $P < 0.01$ and **, $P < 0.001$). The values are means ± SD ($n = 3$ independent experiments).

(Gibson, 2005), and they lead to an increase in the number of tubers (Sami et al., 2016). The SUT1 mRNA and protein levels can control leaf senescence. The antisense *SUT1* plants delay plant growth (Lalonde et al., 2004).

In addition, sugar acts as a signaling molecule that is involved in plant growth. During germination, Glc is known to be a very potent modulator in activating genes involved in ABA biosynthesis (Price et al., 2003). A higher concentration of sugars triggers the repression

Figure 9. A model for the ABA regulation of soluble sugar accumulation.



of genes associated with photosynthesis (Hammond et al., 2011).

Moreover, sugar molecules also act as nutrients and substrates that contribute to fruit or crop quality characteristics such as sweetness. *SUT2* has a major impact on the sugar contents of potato (*Solanum tuberosum*; Barker et al., 2000). Similarly, *MdSUT2* overexpression increases the soluble sugar and Suc contents in fruits (Fig. 8). In addition, sugar also regulates anthocyanin biosynthesis. In Arabidopsis, Suc induces the expression of the *MYB75/PAP1* gene, which activates the expression of structural genes associated with anthocyanin synthesis (Solfanelli et al., 2006).

Finally, a model for the ABA regulation of soluble sugar accumulation is established. It shows that ABA induces the expression of *MdAREB2*, which subsequently binds to the promoters of the sugar transport genes *MdSUT2* and *MdTMT1* as well as amylase genes including *MdAMY1*, *MdAMY3*, *MdBAM1*, and *MdBAM3*. This signal then transcriptionally activates the expression of *MdSUT2* (and maybe other *MdAREB2*-regulated genes), finally resulting in soluble sugar accumulation to influence abiotic stress tolerance, fruit quality, and plant growth and development (Fig. 9). In fruit, this regulatory pathway sheds light on why ABA and related stresses such as drought promote fruit quality and, thereby, provides theoretical guidance for developing new cultivation techniques to improve fruit quality.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and ABA Treatments

The in vitro shoot cultures of apple (*Malus domestica* 'Gala') were grown on MS medium supplemented with 1 mg L^{-1} naphthyl acetate and 0.5 mg L^{-1} 6-benzylaminopurine at 25°C under long-day conditions (16 h of light/8 h of dark). They were subcultured at 30-d intervals. For rooting, in vitro shoot cultures were grown on MS medium supplemented with 0.5 mg L^{-1} indole-3-acetic acid. Finally, the rooted apple plantlets were transferred to pots containing a mixture of soil and perlite (1:1) and grown in the greenhouse under a 16-h/8-h light/dark and 25°C day/night cycle.

For ABA treatment, apple shoot cultures were cultivated on MS medium supplemented with 0.5 mg L^{-1} indole-3-acetic acid, 1.5 mg L^{-1} 6-benzylaminopurine, and 100 μM ABA for 2 d. Apple calli were cultivated on MS medium supplemented with 1.5 mg L^{-1} 6-benzylaminopurine, 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid, and 100 μM ABA for 1 d. Arabidopsis (*Arabidopsis thaliana*) seedlings were cultivated on MS medium containing 50 μM ABA for 1 d. Fruits of apple cv Red Delicious were sprayed with 0, 10, 20, and 50 μM ABA in the dark for 4 d.

Construction of the Expression Vectors and Genetic Transformation into Apple

To construct *MdSUT2* and *MdAREB2* overexpression vectors, the full-length DNAs of *MdSUT2* and *MdAREB2* were isolated from cv Gala apples using PCR. All the DNAs were digested with *Eco*RI/*Bam*HI and cloned into the *MYC* plant transformation vectors downstream of the cauliflower mosaic virus *35S* promoters. All the primers used here are listed in Supplemental Table S2. These vectors were genetically introduced into apple plants using *Agrobacterium tumefaciens*-mediated transformation as described by Zhao et al. (2016).

RNA Extraction, RT-PCR, and qRT-PCR Assays

Total fruit RNAs were extracted using the hot borate method as described by Yao et al. (2010). Total RNAs from other tissues were extracted using the Trizol Reagent (Invitrogen Life Technologies). Two micrograms of total RNAs was used to synthesize first-strand cDNA with the PrimeScript First Strand cDNA Synthesis Kit (TaKaRa). For real-time qRT-PCR analysis, the reactions were performed with iQ SYBR Green Supermix in the iCycler iQ5 system (Bio-Rad) according to the manufacturer's instructions. The specific mRNA levels were analyzed by relative quantification using the cycle threshold 2-ΔΔCt method. For all of the analyses, the signal that was obtained for a gene of interest was normalized against the signal that was obtained for the *18s* gene. All of the samples were tested in three to four biological replicates. All of the primers that were used for qRT-PCR are listed. For semiquantitative RT-PCR, the reactions were performed according to the manufacturer's instructions (TransGen) using the following thermal profile: preincubation at 95°C for 5 min followed by 30 cycles of 95°C (30 s), 58°C (30 s), and 72°C (30 s), with a final extension at 72°C for 5 min. The primers are listed in Supplemental Table S2.

Starch Quantification

Starch, which is composed of Glc residues, is a polysaccharide. It can be divided into Glc under acidic conditions by heating and hydrolysis. The chromogenic reagent known as anthrone was used. The 1-g (fresh weight) samples were transferred into a 50-mL volumetric flask, adding 20 mL of hot distilled water, placed in a boiling water bath for 15 min, adding 2 mL of 9.2 mol L^{-1} perchloric acid to extract for 15 min, after cooling, and then filtering with filter paper (or centrifuging at 2,500 rpm for 10 min). The volume was then set with distilled water. This reaction can be subjected to colorimetric determination.

Soluble Sugar Contents

One-gram (fresh weight) samples were ground in 5 mL of 95% (v/v) ice-cold methanol. The methods were described by Hu et al. (2016). Three milliliters of supernatant was passed through a 0.45-μm membrane filter, and the filtrate was then analyzed with HPLC.

Yeast One-Hybrid Assays

Genomic DNAs extracted from apple tissue culture were used as a template to amplify the promoter pMdSUT2(ABRE). In addition, they were also used to generate the mutated *MdSUT2* promoter pMdSUT2(mABRE) with a PCR-based point mutagenesis method. First-stage PCR was performed with mutagenic primers (i.e. pMdSUT2-F1, 5′-GCCATATCAGAGACGGGAAG-3′, and pMdSUT2-R1, 5′-CAAACTAGGACCTACTGTATGGA-3′; the mutant nucleotides are underlined) as well as pMdSUT2-F2 (5′-TCCATACAGTAGGTCCTAGTTTG-3′; the mutant nucleotides are underlined) and pMdSUT2-R2 (5′-GGCGACTCGGTTTCACTGACTCAGT-3′). The resultant PCR products were recovered and purified. They were then mixed 1:1 and used as a template for the second round of PCR amplification with primers pMdSUT2-F1 (5′-GCCATATCAGAGACGGGAAG-3′) and pMdSUT2-R2 (5′-GGCGACTCGGTTTCACTGACTCAGT-3′). The promoter sequence of the *MdSUT2* gene is shown in Supplemental Table S3.

The wild-type and mutated promoters pMdSUT2(ABRE) and pMdSUT2 (mABRE) were ligated into the one-hybrid vector pAbAi (Clontech). The resultant vectors pMdSUT2-pAbAi and pMdSUT2(m)-pAbAi were transferred into yeast one-hybrid strain YM187. And the resulting strain cell was plated on SD/-Ura medium plus 600 ng mL^{-1} aureobasidin A, a competitive inhibitor for yeast growth. The recombinant vector pGADT7-MdAREB2 was constructed and transferred into the yeast strain containing pMdSUT2 (AREB)-pAbAi and pMdSUT2 (mABRE)-pAbAi. The resulting strain cells were grown on SD/-Leu/plus 600 ng mL^{-1} aureobasidin A medium. The plaque indicates that MdAREB2 binds to the MdSUT2 promoter. Otherwise, it does not bind.

ChIP-qPCR Analysis

The transgenic calli pMdAREB2::GFP and pMdAREB2::MdAREB2-GFP were applied to ChIP analysis. Apple protoplasts were isolated from transgenic pMdAREB2::MdAREB2-GFP calli (Hu et al., 2016). The constructed vectors pMdSUT2 (ABRE)::GUS and pMdSUT2 (mABRE)::GUS were expressed in the pMdAREB2::MdAREB2-GFP transformed protoplasts. An anti-GFP antibody (Beyotime) was used for ChIP-quantitative PCR as described by Hu et al. (2016). The immunoprecipitated samples were used as a template for quantitative PCR analysis with primers as listed in Supplemental Table S2.

EMSA

An EMSA was conducted according to Xie et al. (2012). *MdAREB2* was cloned into the expression vector *pGEX4T-1*. The MdAREB2-GST recombinant protein was expressed in *Escherichia coli* strain BL21 and purified using glutathione Sepharose beads (Thermo). An oligonucleotide probe of the *MdSUT2* promoter was labeled with an EMSA probe biotin-labeling kit (Beyotime) according to the manufacturer's instructions. The binding reaction was performed for 20 min at room temperature. The DNA-protein complexes were electrophoresed on 6.5% nondenaturing polyacrylamide gels, electrotransferred, and detected according to the manufacturer's instructions. The binding specificity also was examined by testing its competition with a fold excess of unlabeled oligonucleotides. The primers used are listed in Supplemental Table S2.

GUS Assays

Transient expression assays were conducted using apple calli. The normal and mutant promoters of *MdSUT2* were cloned into pBI121-GUS to fuse with the reporter gene *GUS*. The resulting *MdSUT2*::*GUS* constructs were obtained and genetically transformed into apple calli via an *A. tumefaciens*-mediated method. Subsequently, *35S*::*MdAREB2* was cotransformed into the above-mentioned transgenic calli. Finally, histochemical staining was performed to detect GUS activity in the transgenic calli. It was determined using the method described by Zhao et al. (2016).

Construction of the Viral Vectors and Transient Expression in Apple Fruits

To construct the antisense expression viral vectors, the conserved *MdAREB2* and *MdSUT2* cDNA fragments were amplified with RT-PCR using apple fruit cDNA as the template. The resulting products were cloned into a TRV vector in the antisense orientation under the control of the dual *35S* promoter. The resulting vectors were named MdAREB2-TRV and MdSUT2-TRV, respectively. To generate the viral overexpression vectors, full-length cDNAs of *MdAREB2* and *MdSUT2* were inserted into the IL-60 vector under the control of the *35S* promoter. The resulting vectors were named MdAREB2-IL60 and MdSUT2-IL60. All of the vectors were transformed into *A. tumefaciens* strain GV3101 for inoculation. Apple fruits were collected from a 6-year-old tree of cv Red Delicious. The fruits were bagged at 35 d after flowering and harvested at 140 d. They were debagged before injection. Fruit infiltrations were performed as described by Li et al. (2012).

DNA-Affinity Trapping of DNA-Binding Proteins

DNA promoter fragments of the *MdSUT2*-containing ABRE cis-elements were used to isolate the proteins that were bound to the cis-elements in these promoter fragments. Biotinylated DNA promoter fragments were generated by PCR. Nuclear protein extracts for EMSA were prepared from wild-type apple plants that were grown under natural conditions. The methods were described by Hu et al. (2016).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Phylogenetic tree analysis of sugar transporter genes.

Supplemental Figure S2. qRT-PCR assay for the expression of genes in root, stem, leaf, flower, and fruit.

Supplemental Figure S3. Genome structure analysis of MdSUT2 and MdTMT1.

Supplemental Figure S4. Empty TRV infection did not influence the expression of *MdTMT1* and *MdSUT2* genes.

Supplemental Figure S5. qRT-PCR examination of the transcription levels of the six transgenetic lines MdSUT2-3, MdSUT2-4, MdSUT2-6, MdSUT2-7, MdSUT2-8, and MdSUT2-9.

Supplemental Figure S6. Genome structure analysis of MdAREB2.

Supplemental Figure S7. The cis-element ABRE in the promoters of these genes.

Supplemental Figure S8. qRT-PCR examination of the transcription levels of the five transgenetic lines MdAREB2-3, MdAREB2-5, MdAREB2-6, MdAREB2-7, and MdAREB2-8.

Supplemental Figure S9. Phenotype of transient gene silencing of MdAREB2 plants.

Supplemental Figure S10. Sugar contents in cv Gala apple leaves after infection with empty vector TRV, MdAREB2-TRV, MdAREB2-TRV/ MdSUT2-IL60, and MdAREB2-TRV/ MdSUT2-TRV.

Supplemental Figure S11. Expression of *TMT1*.

Supplemental Figure S12. Sugar contents in cv Gala apple leaves that contained *MdTMT1* transient overexpression vector 35S::MdTMT1-IL60 and empty vector IL60.

Supplemental Figure S13. Expression of AREB2.

Supplemental Table S1. Some important cis-acting regulatory elements in the upstream regulatory sequences of *MdSUT2*, *MdTMT1*, *MdAMY1*, *MdAMY3*, *MdBAM1*, and *MdBAM3* genes.

Supplemental Table S2. Primers used in this study.

Supplemental Table S3. The promoter of MdSUT2.

Supplemental Appendix S1.

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