The Zinc Finger Transcription Factor *SlZFP2* Negatively Regulates Abscisic Acid Biosynthesis and Fruit Ripening in Tomato[1]

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Abscisic acid (ABA) regulates plant development and adaptation to environmental conditions. Although the ABA biosynthesis pathway in plants has been thoroughly elucidated, how ABA biosynthetic genes are regulated at the molecular level during plant development is less well understood. Here, we show that the tomato (*Solanum lycopersicum*) zinc finger transcription factor *SlZFP2* is involved in the regulation of ABA biosynthesis during fruit development. Overexpression of *SlZFP2* resulted in multiple phenotypic changes, including more branches, early flowering, delayed fruit ripening, lighter seeds, and faster seed germination, whereas down-regulation of its expression caused problematic fruit set, accelerated ripening, and inhibited seed germination. *SlZFP2* represses ABA biosynthesis during fruit development through direct suppression of the ABA biosynthetic genes *NOTABILIS*, *SITIENS*, and *FLACCA* and the aldehyde oxidase *SlAO1*. We also show that *SlZFP2* regulates fruit ripening through transcriptional suppression of the ripening regulator *COLORLESS NON-RIPENING*. Using bacterial one-hybrid screening and a selected amplification and binding assay, we identified the (A/T)(G/C)TT motif as the core binding sequence of SlZFP2. Furthermore, by RNA sequencing profiling, we found that 193 genes containing the SlZFP2-binding motifs in their promoters were differentially expressed in 2 d post anthesis fruits between the *SlZFP2* RNA interference line and its nontransgenic sibling. We propose that *SlZFP2* functions as a repressor to fine-tune ABA biosynthesis during fruit development and provides a potentially valuable tool for dissecting the role of ABA in fruit ripening.

Abscisic acid (ABA) plays important roles in seed maturation and germination as well as in responses to stresses, such as cold, drought, and salinity (Wasilewska et al., 2008). ABA is derived from apocarotenoids, and the synthetic pathway involves several enzymatic reactions (Nambara and Marion-Poll, 2005; Wasilewska et al., 2008; Hauser et al., 2011). Zeaxanthin derived from β-carotene is first converted to violaxanthin by zeaxanthin epoxidase (ZEP) and then to xanthoxin by 9-cis-epoxycarotenoid dioxygenase (NCED) in plastid. Then, xanthoxin is subsequently converted to ABA in the cytoplasm via the intermediate ABA aldehyde by xanthoxin oxidase/short-chain dehydrogenase/reductase and aldehyde oxidase (AO) together with its cofactor molybdenum-cofactor sulfurase (MoCOS). In tomato (*Solanum lycopersicum*), *high pigment-3* (*hp-3*), *notabilis* (*not*), *sitiens* (*sit*), and *flacca* (*flc*) contain mutations in *ZEP*, *LeNCED1*, an *AO*, and a *MoCOS* gene, respectively (Burbidge et al., 1999; Sagi et al., 2002; Galpaz et al., 2008; Harrison et al., 2011). It is well known that the expression of many ABA biosynthetic genes is induced by stresses to increase ABA production (Seo and Koshiba, 2002). For example, *ABA1/ZEP*, *NCEDs*, *AOs*, and *ABA3/MoCOS* are up-regulated by water stress, salt, and high temperature in Arabidopsis (*Arabidopsis thaliana*) (Xiong et al., 2001b, 2002; Barrero et al., 2006; Toh et al., 2008; Frey et al., 2012). Likewise, *NOT/LeNCED1* and *HP-3/ZEP* are induced by drought in tomato leaves and roots (Thompson et al., 2000). ABA biosynthesis is also developmentally regulated, especially during seed maturation and germination (Xiong and Zhu, 2003). In tomato, pollination triggers down-regulated *NOT/LeNCED1* expression in ovary during fruit set, which is likely responsible for the rapid decline in ABA levels after anthesis (Vriezen et al., 2008; Nitsch et al., 2009). Later during fruit development, ABA production is gradually elevated to its maximal level when ripening occurs and then decreases afterward (Gillaspy et al., 1993; Buta and Spaulding, 1994; McAtee et al., 2013).

Despite such insights, the molecular mechanisms whereby the transcription of ABA biosynthetic genes is regulated, especially during plant development, are not well understood. Under stress conditions, the Arabidopsis gene *SOMNUS* promotes ABA biosynthesis by enhancing *ABA1*, *NCED6*, and *NCED9* expression (Kim et al., 2008).

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SUPERSENSITIVE TO ABSCISIC ACID AND DROUGHT1 also is required for ABA production through modulating *ABA3* and Arabidopsis *ALDEHYDE OXIDASE3* (*AAO3*) expression (Xiong et al., 2001a). The exosome subunit Ribonuclease-PH domain subunit RRP41L controls mRNA decay of the ABA biosynthetic genes *NCED3*, *NCED5*, *NCED6*, and *NCED9* (Yang et al., 2013). Overexpression of the salt-inducible RING-H2 zinc finger gene *XERICO* increases *NCED3* expression and produces more cellular ABA in Arabidopsis (Ko et al., 2006). The C_2H_2 zinc finger gene *INDETERMINATE DOMAIN1/ENHYDROUS* negatively regulates ABA biosynthesis in Arabidopsis during seed development, likely through a DELLA-mediated pathway (Feurtado et al., 2011). Nevertheless, there are only several transcription factors identified to directly target ABA biosynthetic genes. The rice (*Oryza sativa*) APETALA2 (AP2)-like gene *OsAP2-39* targets *OsNECD1*, and the Arabidopsis *NECD3* is the direct target of the WRKY transcription factor *ACQUIRED DROUGHT TOLERANCE* and the NAC (for no apical meristem [NAM], Arabidopsis transcription activation factor [ATAF], and cup-shaped cotyledon [CUC]) family member *ATAF1* (Yaish et al., 2010; Jiang et al., 2012; Jensen et al., 2013). Recently, two more transcription factors, the AP2/ETHYLENE RESPONSE FACTOR (ERF) family member *dehydration-responsive element-binding protein 2C* and the NAC-like gene *ACTIVATED BY AP3/PI*, have been shown to directly activate *NCED9* and *AAO3* expression in Arabidopsis during seed germination and leaf senescence (Je et al., 2014; Yang et al., 2014).

Ripening of climacteric fruits, such as tomato, is regulated mainly by the ethylene pathway but also by several transcription factors acting upstream (Klee and Giovannoni, 2011; Seymour et al., 2013). The MADS box gene *RIPENING INHIBITOR* (*RIN*) in the *AP1/FRUITFULL* (*FUL*) subfamily controls the early phase of ripening via both ethylene-dependent and independent mechanisms (Vrebalov et al., 2002). *RIN* induces ethylene production through transcriptional regulation on two developmentally controlled 1-amino-1-carboxylic acid (ACC) synthases, *LeACS1A* and *LeACS4*; it also regulates the expression of *LeACS2* and two ACC oxidases, *LeACO1* and *LeACO3*, that are mainly responsible for ethylene production during ripening (Barry et al., 2000). The known ripening regulators acting upstream of the ethylene pathway also include the NAC transcription factor *NONRIPENING* (*NOR*), the *SQUAMOSA PROMOTER BINDING* box gene *COLORLESS NON-RIPENING* (*CNR*), and an *AP2/ERF* member, *AP2a*, in addition to several other MADS box genes, such as *TDR4/SlFUL1*, *SlFUL2*, *TOMATO AGAMOUS-LIKE1* (*TAGL1*), and *TOMATO AGAMOUS1* (*TAG1*; Vrebalov et al., 2002, 2009; Manning et al., 2006; Itkin et al., 2009; Chung et al., 2010; Karlova et al., 2011; Bemer et al., 2012). In addition, *LeMADS1* (also named *SlMADS1*) has been shown to weaken *RIN* action during fruit ripening; down-regulation of its expression in tomato elevated ethylene production (Dong et al., 2013). An epimutation in the *CNR* promoter inhibits fruit ripening, likely through *AP2a*-mediated negative regulation of ethylene biosynthesis and signaling (Manning et al., 2006; Karlova et al., 2011). Recently, at least 241 direct RIN targets, including *CNR*, *NOR*, and *TDR4/SlFUL1*, have been identified (Martel et al., 2011; Fujisawa et al., 2013). Besides ethylene and the above-mentioned regulators, ABA also is implicated to play a role in the fruit ripening of tomato and other species, including nonclimacteric fruit crops such as strawberry (*Fragaria ananassa*) and grape (*Vitis vinifera*; Jia et al., 2011; Seymour et al., 2013), although the mechanism remains unclear. In tomato, repression of *LeNCED1* decreases the expression of several genes encoding ripening-associated cell wall enzymes and delays fruit ripening, suggesting that ABA promotes ripening (Sun et al., 2012b).

C_2H_2 zinc finger proteins (ZFPs) containing one or more zinc finger motifs constitute a large gene family, and members in this family are transcription factors involved in the transcriptional regulation of diverse biological processes (Englbrecht et al., 2004). Several ZFPs with a single C_2H_2 zinc finger motif in the C1-1i subfamily regulate flowering time, trichome development, and floral organ formation. For example, *SUPERMAN* regulates Arabidopsis stamen development (Sakai et al., 1995) and *LATE FLOWERING* acts as a floral repressor (Weingartner et al., 2011). Arabidopsis trichome development requires several single finger ZFPs, including *GLABROUS INFLORESCENCE STEMS* (*GIS*), *GIS2*, *AtZFP5*, *AtZFP6*, and *AtZFP8*; they likely act on the GA pathway (Gan et al., 2006, 2007; Zhou et al., 2011, 2013). Overexpression in Arabidopsis of the senescence-induced zinc finger gene *AtZFP2* leads to delayed floral organ abscission (Cai and Lashbrook, 2008). Some two-fingered ZFPs in the C1-2i subfamily are known for their roles in stress responses. For example, *SALT TOLERANCE ZINC FINGER/ZINC FINGER OF ARABIDOPSIS10* (*STZ/SAT10*) responds to salt, drought, cold, and ABA treatments, and its high expression enhances drought tolerance in Arabidopsis (Sakamoto et al., 2004). Like *STZ/SAT10*, the expression of *ARABIDOPSIS ZINC FINGER PROTEIN1* (*AZF1*) and *AZF2* also is induced by different stresses and ABA (Sakamoto et al., 2000). Overexpression of *AZF1* and *AZF2* represses a subset of genes regulated by osmotic stress and ABA and also several auxin-responsive genes, indicating that the two genes function as transcriptional repressors to inhibit plant growth under stress conditions (Kodaira et al., 2011). Although many ZFP transcription factors have been identified, DNA-binding sequences have been identified for only a few of them. It has been shown that AZFs and STZ bind to the sequences containing the A(G/C)T motif (Sakamoto et al., 2004), whereas DROUGHT AND SALT TOLERANCE from rice recognizes a cis-element containing TGCTANNATTG found in the promoters of *PEROXIDASE24 PRECURSOR* and the glutathione *S*-transferase *OsGSTU2* (Huang et al., 2009).

There are at least 116 C_2H_2 zinc finger transcription factors in tomato (Tomato Genome Consortium, 2012), but few have been characterized molecularly. In this study, we characterized the role of the single zinc finger

gene *SlZFP2* in plant development. Using a reverse genetics approach, we show that *SlZFP2* negatively regulates fruit ripening and also plays important roles in seed development and seed germination. Furthermore, through biochemical analysis and transcriptome profiling by RNA sequencing (RNA-seq), we identified the (A/T)(G/C)TT motif as the core binding site of SlZFP2 and at least 199 genes as its direct targets during early fruit growth. Our results demonstrate that *SlZFP2* negatively regulates ABA biosynthesis through the direct suppression of several ABA biosynthetic genes and delays ripening through the down-regulation of the ripening regulator *CNR*.

RESULTS

SlZFP2 Is Expressed Mainly in Developing Fruits

Previous microarray analysis of tomato flowers and early-developing fruits revealed that a subset of transcription factors was preferentially expressed in developing fruits at 5 d post anthesis (DPA; Xiao et al., 2009). One of these was the transcription factor *SlZFP2*, named for its high similarity with the Arabidopsis zinc finger protein *AtZFP2* (Supplemental Fig. S1). Consistent with the microarray results, semiquantitative reverse transcription (RT) and quantitative reverse transcription (qRT)-PCR analysis showed that *SlZFP2* was expressed mainly from anthesis to fruit ripening; no or very limited expression was detected in vegetative tissues of tomato 'M82' and its wild relative *Solanum pimpinellifolium* LA1589 (Supplemental Fig. S2, A–D). Publicly available RNA-seq data also confirmed high *SlZFP2* expression in fruits during fruit growth and ripening and further revealed its very low expression in roots, cotyledons, young leaves, and vegetative shoots (Supplemental Fig. S2, E and F).

As shown in Figure 1, in situ hybridization analysis conducted on LA1589 revealed that, during vegetative growth, *SlZFP2* was weakly expressed in shoot meristem but highly expressed in axillary buds and young leaves. Later during reproductive development, *SlZFP2* expression was detected in floral meristems, ovule primordia and anthers, and particularly in ovules/seeds and their connective placental regions of anthesis flowers and 5-DPA fruits. This expression pattern suggests that *SlZFP2* may play a role in bud growth and fruit development.

SlZFP2 Regulates Fruit Ripening and Seed Development

To investigate the role of *SlZFP2* in fruit development, we generated its overexpression and RNA interference (RNAi) lines in cv M82 and LA1589. Compared with their corresponding nontransgenic plants, overexpression of the *SlZFP2* coding sequence fused to the epitope tag hemagglutinin (*HA-SlZFP2*) driven by the cauliflower mosaic virus 35S promoter in cv M82 and LA1589 led to increased branching, shorter plants, and early flowering (Fig. 2, A and H). We also found that the *HA-SlZFP2* overexpression lines from LA1589 had thinner stems and fewer roots (Fig. 2, B and C), while

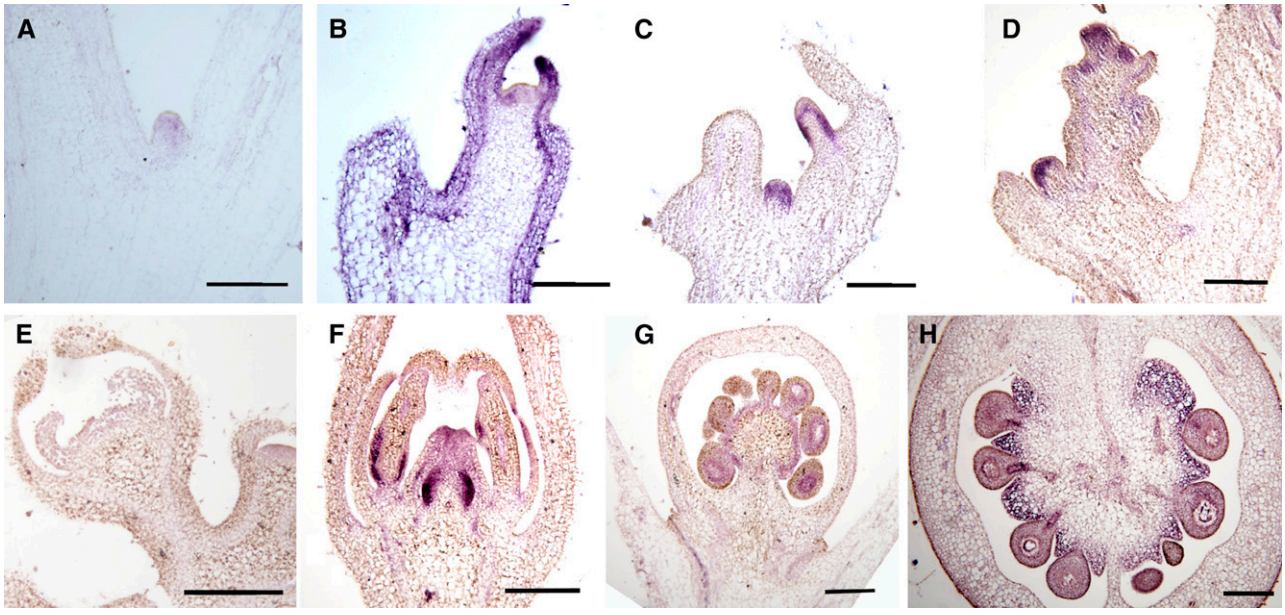

Figure 1. *SlZFP2* is expressed in both vegetative and reproductive tissues. In situ hybridization using sense (A and E) and antisense (B–D and F–H) probes of *SlZFP2* was performed on apical meristems and young leaves (B), axillary buds (A and C), floral meristems (D), flower buds (E and F), ovaries at anthesis (G), and developing fruits (H) at 5 DPA from LA1589 plants. Bars = 200 μm.

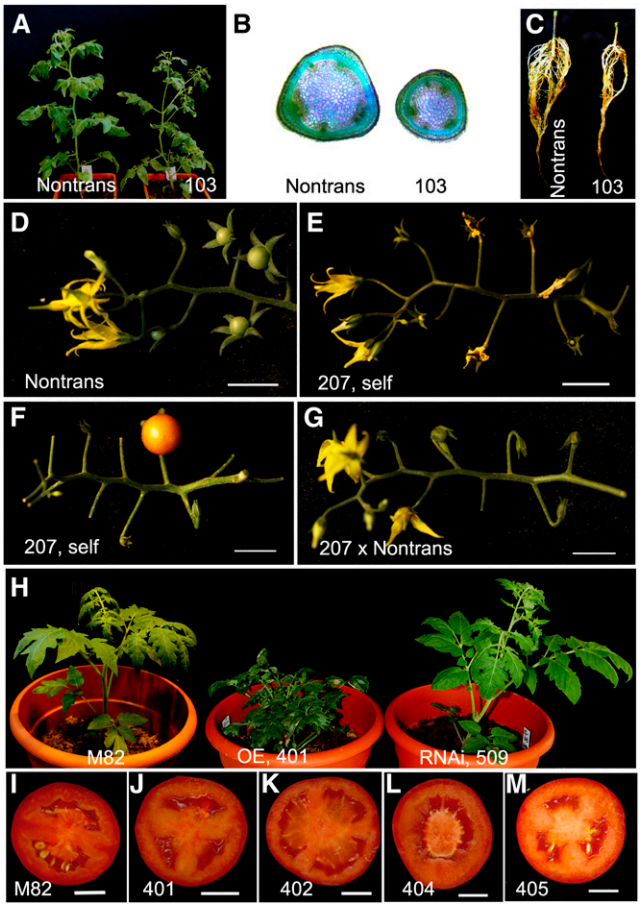

Figure 2. Phenotypes of *SlZFP2* overexpression and RNAi lines. Representative transgenic lines from LA1589 (A–G) and cv M82 (H–M) show multiple phenotypes during vegetative growth and fruit development. Overexpression of *HA-SlZFP2* in both LA1589 (A–C) and cv M82 (F and G) displayed increased branching, shorter plant stature, and early flowering (A and H) and also led to thinner stems (B) and few roots (C). Overexpression of *HA-SlZFP2* in cv M82 severely affected seed development (I–M), whereas the *SlZFP2* RNAi line of LA1589 showed severely affected fruit set (E and F) compared with its nontransgenic sibling (D). Fruit set in the RNAi line was rescued by pollination with nontransgenic pollen (G). The fruit images of cv M82 overexpression lines (J–M) were from primary (T0) transgenic plants; others were taken from T2 plants. Nontrans, Nontransgenic siblings; OE, overexpression line. Bars = 1 cm.

the *SlZFP2* RNAi lines of LA1589 and cv M82 grew normally during the vegetative phase. However, the RNAi lines of LA1589 barely set fruits when pollinated with their own pollen, while those from cv M82 set fruits normally (Fig. 2, D–F). The problematic fruit set in these RNAi lines of LA1589 was improved by pollination with nontransgenic pollen (Fig. 2G). During fruit development, fruit weight and seed number were apparently not affected in the *HA-SlZFP2* overexpression lines of LA1589, although some lines produced smaller fruits, but their seed weights were consistently reduced (Supplemental Fig. S3). More severely, overexpression of *HA-SlZFP2* in cv M82 produced fruits with very few and even no seeds (Fig. 2, I–M). Thus, overexpression of *HA-SlZFP2* affects vegetative growth and seed weight, whereas down-regulation of *SlZFP2* by RNAi impacts fruit set.

Given its high expression at later stages of fruit development, we investigated whether *SlZFP2* plays a role in the regulation of fruit ripening. Indeed, fruit ripening was significantly delayed by 5 to 7 d in four homozygous *HA-SlZFP2* overexpression lines of LA1589 compared with their nontransgenic siblings (Fig. 3, A and B). To determine whether the delay could be attributed to *SlZFP2* expression, we further generated and analyzed transgenic lines of LA1589 constitutively expressed *SlZFP2* alone under the control of the 35S promoter (*p35S*:*SlZFP2* lines). We observed that the *p35S*:*SlZFP2* fruits also required more days from anthesis to the turning stage, confirming that high *SlZFP2* expression delays ripening (Fig. 3C). Due to the defective seed development of the *HA-SlZFP2* overexpression lines from cv M82, their fruit ripening was not recorded. Although we failed to obtain homozygous plants for all *SlZFP2* RNAi lines from LA1589, fruit ripening was accelerated in the heterozygous plants of the three lines investigated (Fig. 3, D and E). In addition, fruit ripening was significantly shortened in two of four cv M82 homozygous RNAi lines (Fig. 3F). Taken together, these results suggest that high *SlZFP2* expression delays fruit ripening, whereas down-regulation of its expression promotes ripening.

SlZFP2 Regulates Fruit ABA Production and Seed Germination

Since overexpression of *SlZFP2* affected seed development, we further tested whether seed germination was impacted in *SlZFP2* overexpression and RNAi lines. Freshly harvested seeds, and dry seeds stored for 5 d and 1 month under room temperature from four *HA-SlZFP2* overexpression lines, germinated 12 to 24 h earlier than their nontransgenic siblings (Fig. 4A; Supplemental Fig. S4, A and B). Similarly, three of four LA1589 lines overexpressing *SlZFP2* alone also had faster seed germination (Supplemental Fig. S4C). In addition, two of three *HA-SlZFP2* overexpression lines of cv M82 showed early seed germination (Fig. 4B). In contrast, seeds from all *SlZFP2* RNAi lines except one cv M82 line germinated slower (Fig. 4, C and D). For those *SlZFP2* RNAi lines of LA1589, only 70% to 80% of seeds germinated after 2 weeks, contrasting with the nearly 100% germination rate of their nontransgenic siblings. These results indicate that high *SlZFP2* expression promotes seed germination, while down-regulation of its expression inhibits germination.

To test whether the sensitivity of seed germination to ABA was affected in *SlZFP2* overexpression lines, we germinated seeds from three LA1589 lines overexpressing *HA-SlZFP2* and their nontransgenic siblings on one-half-strength Murashige and Skoog medium supplemented with 0, 1, 2.5, and 5 μM ABA. The seed germination of the *HA-SlZFP2* overexpression lines and their nontransgenic siblings was not substantially impaired by 1 and 2.5 μM ABA, but 5 μM ABA reduced

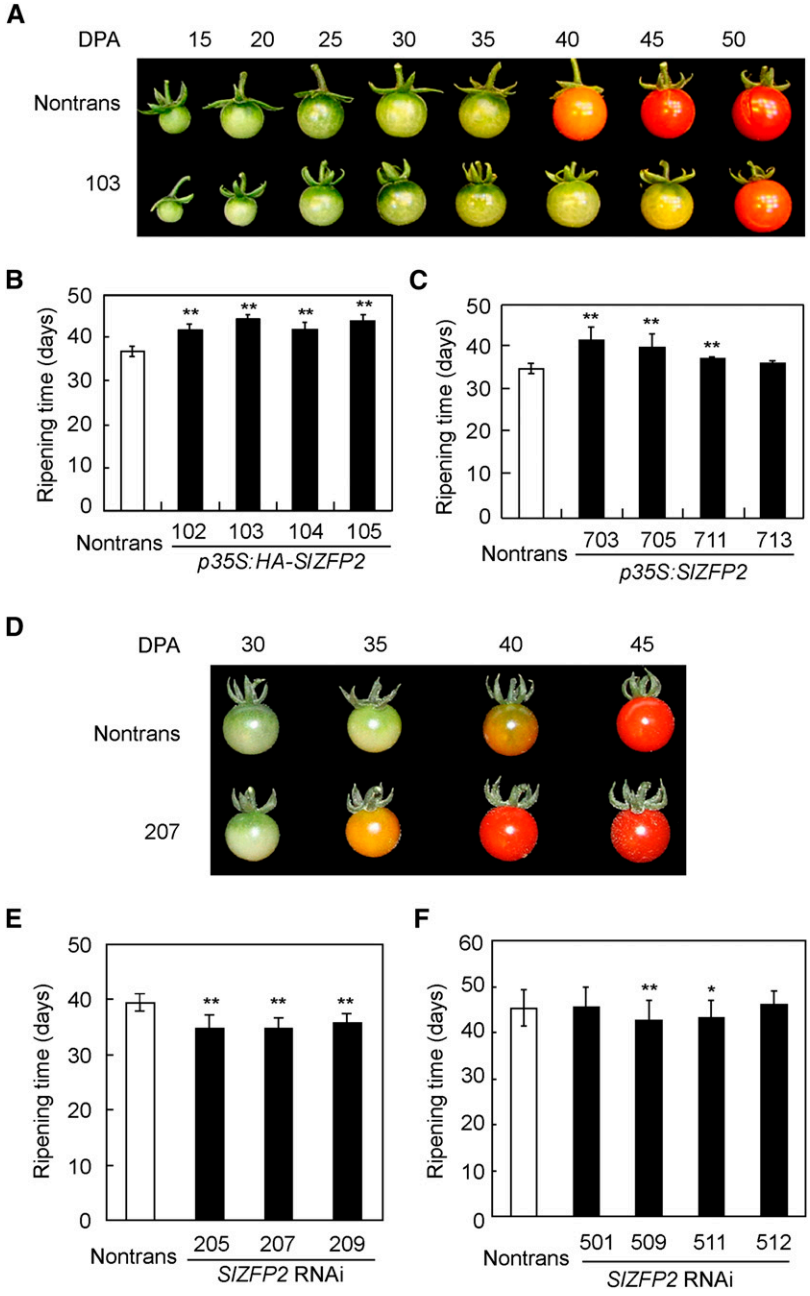

Figure 3. Fruit ripening of *SlZFP2* overexpression and RNAi lines. A, Fruit ripening process of a representative *HA-SlZFP2* overexpression line (103) compared with its nontransgenic sibling (Nontrans). B, Quantification of fruit ripening by days to reach the turning stage in four *HA-SlZFP2* overexpression lines of LA1589. Fifteen to 50 fruits per plant from a total of three to five plants for each line were used to quantify ripening time. C, Quantification of fruit ripening in four *SlZFP2* overexpression lines of LA1589. D, Fruit ripening of a representative *SlZFP2* RNAi line (207) compared with its nontransgenic sibling. E, Quantification of fruit ripening in three *SlZFP2* RNAi lines of LA1589. F, Quantification of fruit ripening in four *SlZFP2* RNAi lines of cv M82. For cv M82 transgenic lines, five to 10 fruits were assessed. Statistical significance was based on Student's *t* test: *, $P < 0.05$; and **, $P < 0.01$. Data are means ± SD; $n = 45$ to 100.

the germination rate of nontransgenic seeds to about 20%, whereas more than 70% of seeds of the three *HA-SlZFP2* overexpression lines still germinated (Fig. 4E). When constitutively expressing *HA-SlZFP2* in the ABA-deficient mutant *sit*, viviparous seeds within fruits were more frequently observed (Fig. 4F). This further suggests that ABA signaling can be impaired by overexpression of *HA-SlZFP2*. A defect in ABA signaling resulting from high *SlZFP2* expression also was suggested by the observation that the leaf stomata of the *HA-SlZFP2* overexpression lines opened wider and were less sensitive to 1 and 5 μM ABA (Supplemental Fig. S5). Thus, ABA biosynthesis and/or signaling can be inhibited by high *SlZFP2* expression.

ABA levels in fruits change dynamically after anthesis (Buta and Spaulding, 1994). In agreement with their results, we also found that ABA production decreased after pollination and then increased after 10 DPA, reaching their maxima at 20 DPA, in both the small fruits of LA1589 and the relatively large ones of cv M82 (Fig. 5A; Supplemental Fig. S6). When overexpressing *HA-SlZFP2* in LA1589, ABA levels in anthesis flowers and mature green (MG) fruits were lower, especially the latter, which contained only about two-thirds the amount of ABA in their nontransgenic siblings (Fig. 5B). Overexpression of *HA-SlZFP2* also repressed ABA biosynthesis in leaves, especially in mature and old leaves (Supplemental Fig. S7A). If *SlZFP2* is required for the suppression of ABA

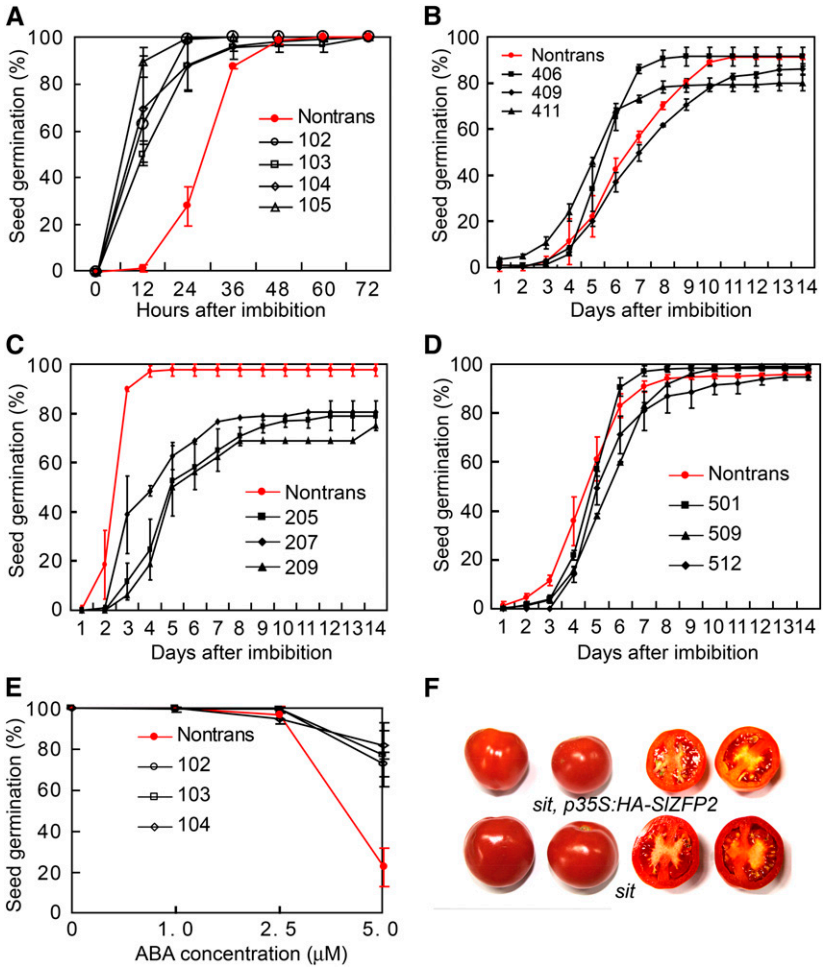

Figure 4. *SlZFP2* promotes seed germination. A, Seed germination of four *HA-SlZFP2* overexpression lines of LA1589 and their nontransgenic siblings (Nontrans). Germination based on radicle emergence was determined on seeds harvested from plants grown in the same seasons. B, Seed germination of three *HA-SlZFP2* overexpression lines from cv M82 and their nontransgenic siblings. C, Seed germination of three *SlZFP2* RNAi lines from LA1589 and their nontransgenic siblings. D, Seed germination of three *SlZFP2* RNAi lines from cv M82 and their nontransgenic siblings. E, ABA sensitivity of three *HA-SlZFP2* overexpression lines from LA1589 during seed germination. Seeds harvested from the same batch of plants were germinated on one-half-strength Murashige and Skoog medium with ABA supplemented at different concentrations as indicated. F, Fruits of the *sit* mutant with or without the *HA-SlZFP2* transgene. Except for RNAi seeds from line 209, for which fewer than 50 seeds were assayed, at least 150 seeds were tested in triplicate. Seeds used for germination assays were dried for 5 d at room temperature. Data are means ± SD.

biosynthesis during fruit development, it would be expected that more ABA was accumulated in young fruits of its RNAi lines due to the released suppression. Indeed, down-regulation of *SlZFP2* expression in LA1589 caused higher ABA levels to be accumulated in fruits at 5 and 10 DPA (Fig. 5C). These results confirm that *SlZFP2* is required to repress ABA biosynthesis during early fruit growth.

Since ABA is derived from apocarotenoids, impairing ABA biosynthesis often affects fruit carotenoid accumulation, as shown in the fruits of the ABA-deficient mutant *hp-3* (Galpaz et al., 2008). Similarly, fruits overexpressing *HA-SlZFP2* also accumulated more β-carotene and lycopene, whereas *SlZFP2* RNAi flowers contained less of these metabolites, further indicating a role of *SlZFP2* in the regulation of the ABA biosynthesis pathway (Supplemental Table S1). Collectively, the results suggest that *SlZFP2* negatively regulates ABA biosynthesis in tomato during fruit development.

SlZFP2 Negatively Regulates the Transcription of ABA Biosynthetic Genes

To understand the mechanism whereby *SlZFP2* regulates ABA biosynthesis, we tested the possibility that it represses the transcription of ABA biosynthetic genes. Consistent with the ABA levels in nontransgenic leaves at different developmental stages, the ABA biosynthetic genes *NOT*, *SIT*, *SlAO1*, and *FLC* showed age-dependent expression patterns, as revealed by qRT-PCR; they all had higher expression in mature leaves (Supplemental Fig. S7B). In a representative *HA-SlZFP2* overexpression line (line 103) of LA1589, these ABA biosynthetic genes were repressed in mature leaves to varying degrees (Supplemental Fig. S7B). Although in anthesis flowers, overexpression of *HA-SlZFP2* did not affect the transcription of the ABA biosynthetic genes analyzed, *SIT*, *FLC*, and *SlAO1* were substantially down-regulated in MG fruits from the four *HA-SlZFP2* overexpression lines of LA1589 investigated (Fig. 6, A and B). *NOT* expression also was repressed in three of the four overexpression lines investigated.

During fruit development, ABA is synthesized in both seeds and their surrounding parental tissues (Frey et al., 2004; Nambara and Marion-Poll, 2005). We then assessed the expression of the above-mentioned ABA biosynthetic genes in seeds extracted from fruits at MG, breaker (Br), and red ripe (B10, breaker plus 10 d) stages of the four *HA-SlZFP2* overexpression lines in the LA1589 background. *NOT* and *FLC* were repressed in MG and Br seeds by overexpression of *HA-SlZFP2* in LA1589, whereas *SIT*

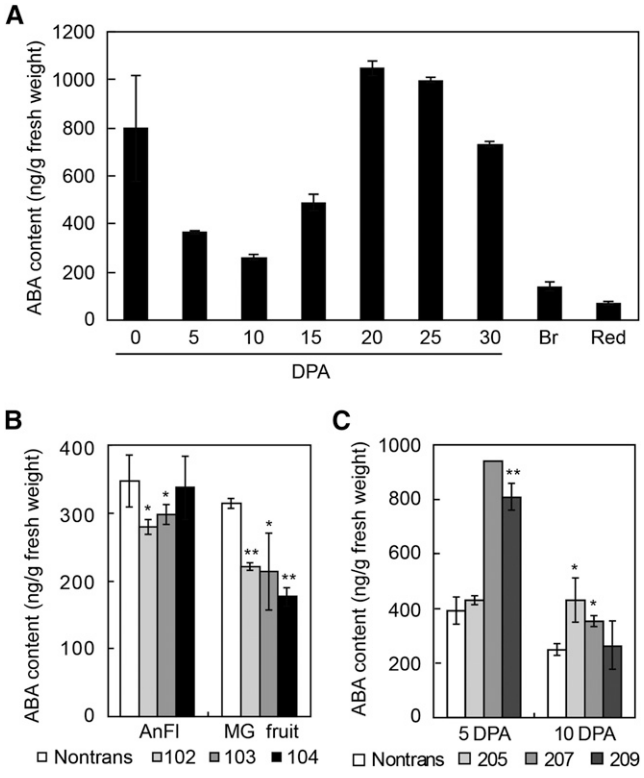

Figure 5. ABA levels in *SlZFP2* overexpression and RNAi lines. A, Dynamic changes of ABA levels in the fruits of LA1589 after anthesis. Red, Red ripe. B, ABA levels in anthesis flowers (AnFl) and MG fruits from three *HA-SlZFP2* overexpression lines and their nontransgenic siblings (Nontrans). C, ABA levels in the fruits at 5 and 10 DPA of three *SlZFP2* RNAi lines and nontransgenic siblings. The three *HA-SlZFP2* overexpression lines used for ABA measurements were homozygous T2 plants, while the three RNAi lines were T2 heterozygous plants. Three biological replicates were conducted, except for the measurement in the 5-DPA fruits of the RNAi line 207, which was done on the pooled samples from three replicates due to few fruits being obtained. Data represent means ± SD. Statistical significance was based on Student's *t* test: *, $P < 0.05$; and **, $P < 0.01$.

expression was elevated (Supplemental Fig. S8). Compared with their transcript levels in leaves, *SlT* and *SlAO1* were either not expressed or expressed at very low levels in fruits and seeds at MG and later stages (Fig. 6B; Supplemental Fig. S8), suggesting that the two genes play minor roles in ABA production during the fruit-ripening process. Overall, the results indicate that overexpression of *HA-SlZFP2* also represses the expression of ABA biosynthetic genes in MG or Br seeds. Thus, overexpression of *HA-SlZFP2* can repress the transcription of several ABA biosynthetic genes during fruit development.

Since down-regulation of *SlZFP2* expression in LA1589 led to more ABA accumulated in young fruits, we also investigated the transcript levels of these ABA biosynthetic genes in anthesis flowers and early developing fruits. *SlT* and *FLC* were slightly up-regulated in anthesis flowers of the three *SlZFP2* RNAi lines investigated, but expression of *NOT* and *SlAO1* was less affected (Fig. 6C). Furthermore, by analysis of their expression in 2-DPA fruits, we showed that all four ABA biosynthetic genes were up-regulated and that the transcript levels of *NOT* and *SlAO1* were increased by more than 2-fold in the three RNAi lines compared with their nontransgenic siblings (Fig. 6D).

In addition, we found that *SlZFP2* was positively regulated by ABA, because its expression was consistently lower in ovaries at anthesis and fruits at 5 and 10 DPA of the three ABA-deficient mutants *not*, *sit*, and *flc* compared with the wild type (Fig. 6E). This suggests that ABA activates *SlZFP2* and the latter in turn represses ABA biosynthesis during fruit development.

SlZFP2 Regulates Fruit Ripening through *CNR*

In addition to its role in seed germination, *SlZFP2* also regulates fruit ripening (Fig. 3). To understand how this transcription factor regulates ripening, we analyzed the transcript levels of several ethylene biosynthetic genes and ripening regulators in *SlZFP2* transgenic lines of LA1589 by qRT-PCR. Other than *LeACS2*, which was up-regulated in Br and B10 fruits, other ethylene biosynthetic genes, *S-ADENOSYL-L-METHIONINE SYNTHETASE1* (*SAM1*), *LeACS6*, *LeACO1*, and *LeACO3*, were only down-regulated in B10 fruits by overexpression of *HA-SlZFP2* (Fig. 7). Expression of the ethylene signaling component *ETHYLENE INSENSITIVE3-LIKE3* (*EIL3*) was not affected. This indicates that *SlZFP2* may not directly regulate ethylene production at the onset of ripening. Moreover, fruit ripening in tomato also is regulated by several transcription factors that act upstream of ethylene production (Vrebalov et al., 2002; Giovannoni, 2004; Itkin et al., 2009; Chung et al., 2010; Bemer et al., 2012; Seymour et al., 2013). Compared with their nontransgenic siblings, the ripening regulator *CNR* was consistently repressed in MG, Br, and B10 fruits by overexpression of *HA-SlZFP2*, whereas *RIN* and *NOR* were mainly repressed in B10 fruits (Fig. 7). In addition, *TDR4/SlFUL1* and *TAGL1* were down-regulated in Br and B10 fruits of the four HA-SlZFP2 overexpression lines. Recently, *LeMADS1*, which can form heterodimers in vitro with *RIN*, has been implicated to weaken the latter's action on ripening regulation (Dong et al., 2013). However, *LeMADS1* expression was only slightly down-regulated in MG fruits and not affected at the Br and B10 stages (Fig. 7).

Because fruit ripening was shortened in *SlZFP2* RNAi lines, we also monitored the transcription of the above-mentioned ripening genes in three RNAi lines from LA1589. In addition to the three ripening stages (MG, Br, and B10), more time points (10, 15, 20, 25, and 28 DPA) were included because of the high *SlZFP2* expression throughout the entire fruit development and its repressive role on the expression of several fruit-ripening genes. For the ethylene biosynthetic genes, *SAM1* expression was enhanced in the RNAi fruits from 15 DPA until the MG stage. Expression of the two ACC oxidase genes, *LeACO1* and *LeACO3*, was increased in

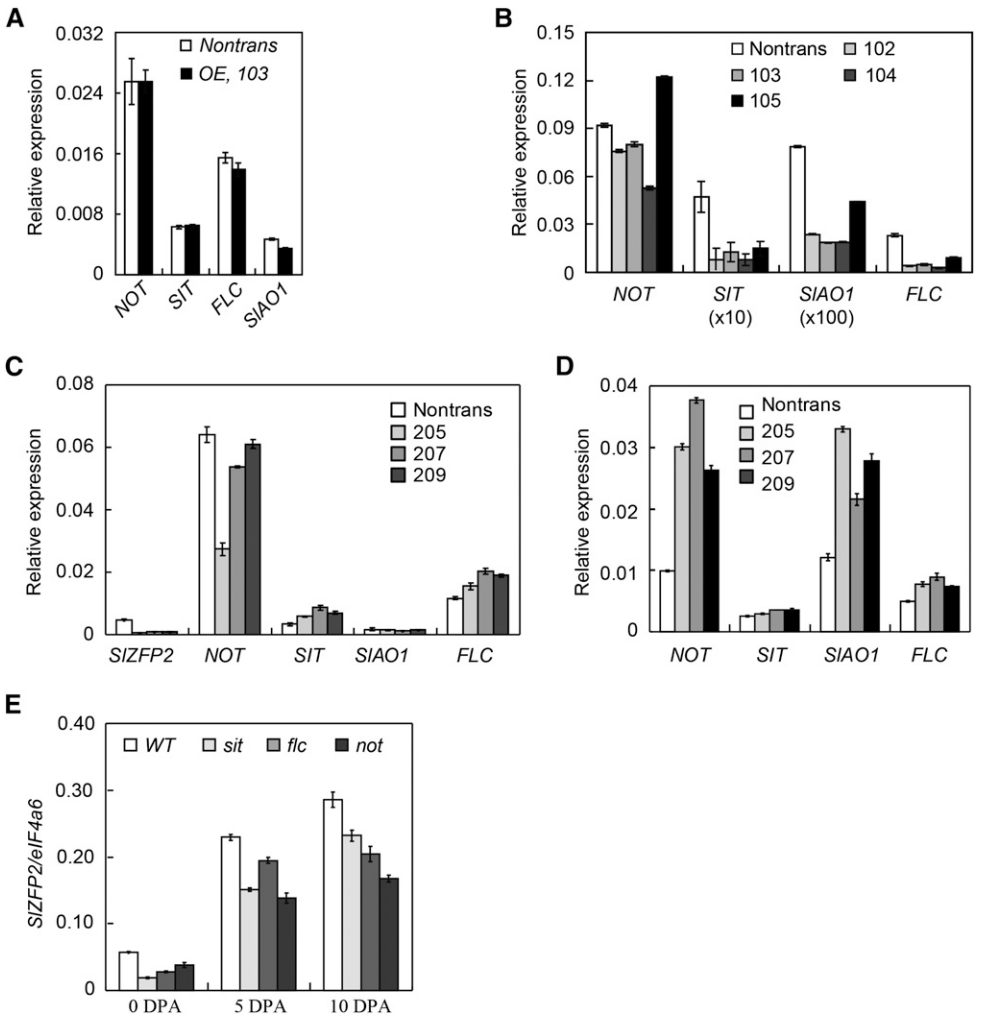

Figure 6. *SlZFP2* regulates the expression of ABA biosynthetic genes during fruit development. A, Transcript levels of ABA biosynthetic genes in anthesis flowers of a representative *HA-SlZFP2* overexpression line (103) and its nontransgenic sibling (Nontrans). B, Transcript levels of ABA biosynthetic genes in mature green fruits of four *HA-SlZFP2* overexpression lines and their nontransgenic siblings. C, Transcript levels of ABA biosynthetic genes in anthesis flowers of three *SlZFP2* RNAi lines and their nontransgenic siblings. D, Transcript levels of ABA biosynthetic genes in 2-DPA fruits of three *SlZFP2* RNAi lines and their nontransgenic siblings. E, *SlZFP2* expression in developing fruits of the ABA-deficient mutants *not*, *sit*, and *flc*. WT, Wild type. Data represent means ± SD; $n = 3$.

the MG fruits of the three *SlZFP2* RNAi lines, but the two ACC synthase genes, *LeACS2* and *LeACS6*, were not affected. However, the ripening regulators *CNR* and *TDR4/SlFUL1* were activated much earlier; their expression started to increase as early as 15 DPA, in contrast to very low expression before the MG stage in their nontransgenic lines (Fig. 8). This indicates that *SlZFP2* represses the expression of *CNR* and *TDR4/SlFUL1* in developing fruits before entering the ripening process. In addition, decrease in *LeMADS1* expression during fruit development was delayed in these RNAi fruits, whereas *RIN* and *NOR* were not impacted by the down-regulation of *SlZFP2* expression. Taken together, these results suggest that *SlZFP2* regulates fruit ripening through the modulation of *CNR* expression.

SlZFP2 Binds to (A/T)(G/C)TT-Containing Sequences

We investigated the subcellular localization of SlZFP2 to confirm its presence in the nucleus, using a transient expression assay in *Nicotiana benthamiana* leaves. Fluorescent signals were detected only in the nuclei of leaf epidermal cells transiently expressing an SlZFP2-YFP (for yellow fluorescent protein) fusion protein driven by the 35S promoter (Supplemental Fig. S9), indicating that SlZFP2 is likely a nucleus-localized protein. Then, two approaches were applied to identify the SlZFP2-binding sequences: the selected amplification and binding (SAAB) assay and a bacterial one-hybrid (B1H) screen. Following six rounds of selection, as part of the SAAB assay using a purified GST-SlZFP2 fusion protein expressed in *Escherichia coli*, together with a synthetic random 14-mer oligonucleotide library, we recovered 89 DNA fragments. Of these, 64 unique sequences were identified; all of these contained one to four AGTT/AACT repeats, while one-third contained one or two extra ACTT/AAGT and a few contained TCTT/AAGA or TGTT/AACA (Table I). As a complement to this approach, B1H screening resulted in the identification of 24 unique sequences from a total of 63 clones. Except for one clone that contained a partial TCTT/AAGA sequence, the others contained at least one of the four repeats identified by SAAB. For the 24 unique sequences, 14 contained one or two TCTT/AAGA repeats, and 10 and seven had AGTT/AACT and ACTT/AAGT, respectively (Table I). These

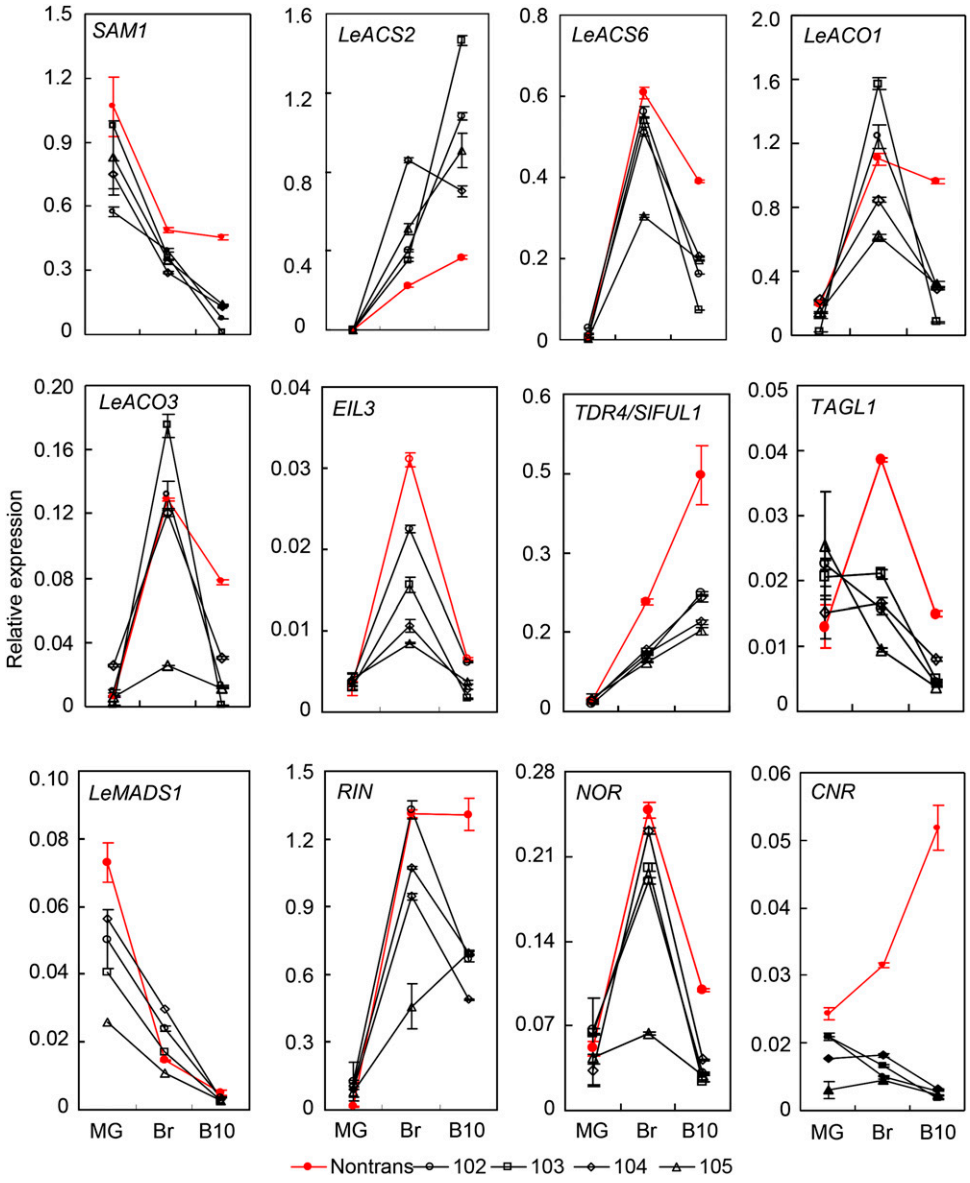

Figure 7. Transcript levels of fruit-ripening genes in the *HA-SlZFP2* overexpression lines of LA1589. Total RNA was isolated from whole fruits at the MG, Br, and B10 stages of the *HA-SlZFP2* overexpression lines and their nontransgenic siblings (Nontrans). Expression levels relative to tomato *eukaryotic initiation factor 4A6* (*SleIF4a6*) were determined for each ripening-related gene by qRT-PCR in three technical replicates (three pooled samples from the same set of plants). Data are means ± SD; $n = 3$.

results indicate that SlZFP2 binds in vitro to DNA sequences containing (A/T)(G/C)TT.

To identify putative SlZFP2 targets in the tomato genome, we performed BLAST searches against a database made from 1.5-kb upstream sequences of the predicted tomato coding sequences retrieved from the Sol Genomics Network database (version SL2.4) using the 24 unique DNA sequences from the B1H assay as queries. DNA sequences from the SAAB screening were not included in the BLAST queries because all these sequences contain multiple (A/T)(G/C)TT repeats. After manual removal of sequences with mismatches on the (A/T)(G/C)TT core sequences, we identified 2,338 genes containing at least one binding site in their 1.5-kb promoter regions. Although *CNR* and the ABA biosynthetic genes *NOT*, *SIT*, *FLC*, and *SlAO1* regulated by *SlZFP2* are not in the list, by manual check we identified multiple (A/T)(G/C)TT motifs within their 1-kb promoter regions (Supplemental Fig. S10). In addition, SlZFP2-binding sites also were found in the *SlAO2* promoter. Thus, there are at least 2,344 genes potentially targeted by SlZFP2 in the tomato genome.

Direct Target Genes of the Transcription Factor SlZFP2

We then applied RNA-seq to investigate how many of these putative target genes in the list were differentially expressed in 2-DPA fruits between a representative RNAi line from LA1589 (line 207) and its nontransgenic sibling. Reads from three biological replicates were mapped to the tomato genome sequences (version 2.4) by Tophat (Trapnell et al., 2009). Then, differentially expressed genes were picked by Cufflinks (Trapnell et al., 2010). In total, 2,722 differentially expressed genes (adjusted $P < 0.05$) were identified (Supplemental Table

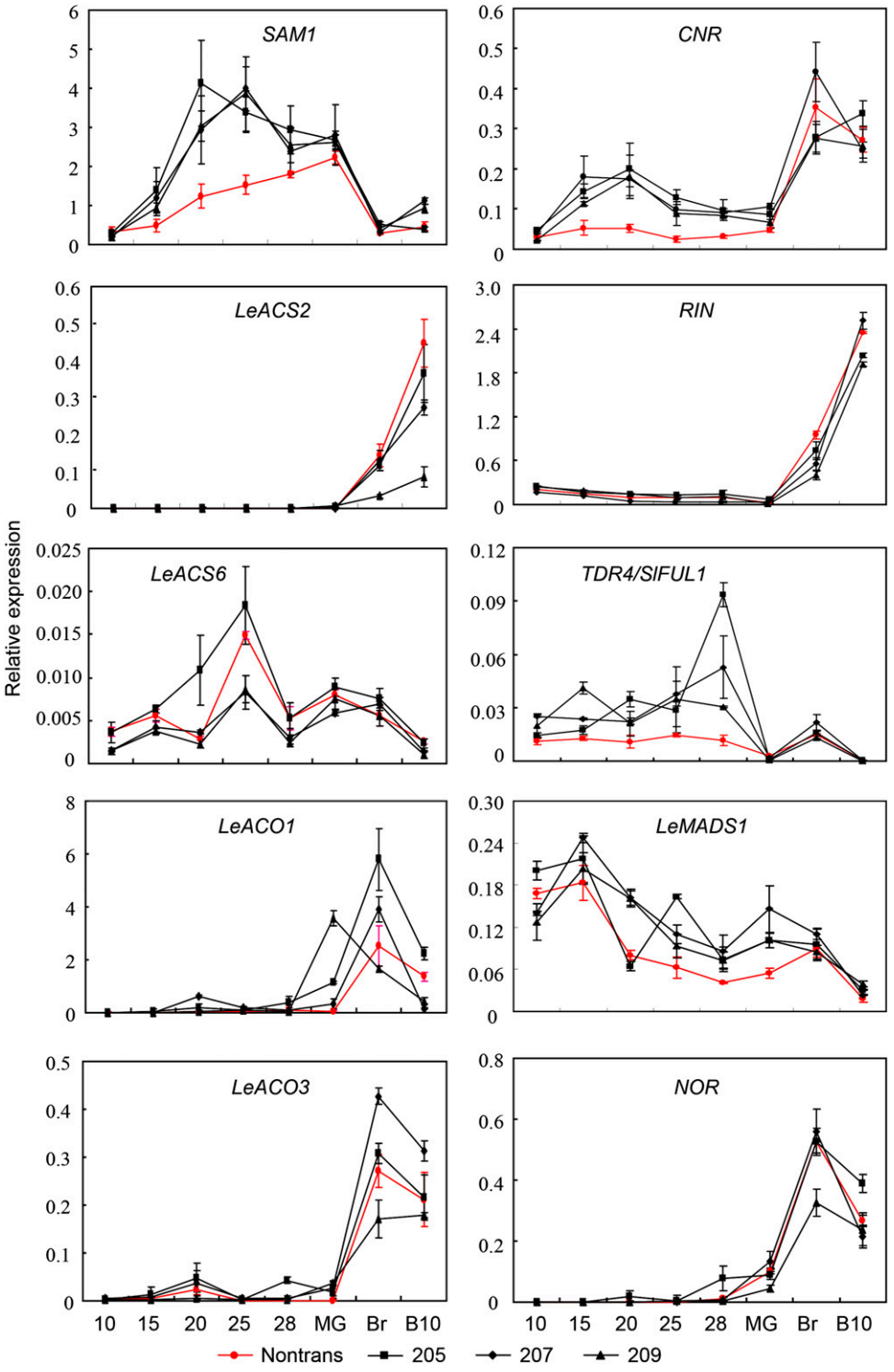

Figure 8. Transcript levels of fruit-ripening genes in the *SlZFP2* RNAi lines of LA1589. Total RNA was isolated from whole fruits at stages 10, 15, 20, 25, and 28 DPA, MG, Br, and B10 of three *SlZFP2* RNAi lines and their nontransgenic siblings (Nontrans). Expression levels relative to *SleIF4a6* were determined for each ripening-related gene by qRT-PCR in three technical replicates (three pooled samples from the same set of plants). Data are means ± SD; $n = 3$.

S2). A total of 193 out of these differentially expressed genes are in the list of putative SlZFP2 target genes identified by the above-mentioned BLAST search (Supplemental Table S3). Expression of the ABA biosynthetic gene *SlAO2* was increased significantly in the 2-DPA fruits of the *SlZFP2* RNAi line 207. Expression of other ABA biosynthetic genes, *NOT*, *SIT*, and *SlAO1*, also was increased by more than 2-fold in the *SlZFP2* RNAi line 207, although not selected by Cufflinks (Supplemental Table S3). *FLC* did not show differential expression by RNA-seq, but its slightly elevated expression was independently detected by qRT-PCR in all three *SlZFP2* RNAi lines of LA1589 (Fig. 6D). *CNR* expression was detected in 2-DPA fruits by RNA-seq and

Table I. *DNA-binding motifs of SlZFP2 identified by SAAB and B1H*

Method	Frequency of DNA Repeats Found in Sequenced Clones					
	AGTT/AACT	TGTT/AACA	ACTT/AAGT	TCTT/AAGA	Total	No. of Clones
SAAB[a]	161	2	34	2	199	64
B1H[b]	24 (10)	21 (7)	3 (3)	60 (14)	108 (34)	63 (24)

[a]All oligonucleotides enriched by SAAB contain at least two repeats of the four kinds of tetramer. [b]The numbers of unique clones sequenced are indicated in parentheses.

was increased slightly by the down-regulation of *SlZFP2* expression. Therefore, there are at least 199 genes in total directly targeted by the transcription factor SlZFP2.

To verify the binding ability of SlZFP2 to the promoters of these ABA biosynthetic genes, we performed chromatin immunoprecipitation (ChIP) assays on four *HA-SlZFP2* overexpression lines and their nontransgenic siblings. Quantitative PCR (qPCR) quantification of the precipitated chromatin DNA fragments by HA antibody revealed that the *NOT*, *SIT*, and *FLC* promoter regions containing multiple clusters of (A/T)(G/C)TT motifs were highly enriched in leaf samples from the four *HA-SlZFP2* overexpression lines of LA1589 (Fig. 9A). There also was an obvious enrichment of the *SlAO1* promoter in line 105. As verification of the ChIP-qPCR results, binding to the *SlAO1* promoter was further confirmed by electrophoretic mobility shift assay (EMSA) using the GST-SlZFP2 fusion protein expressed in *E. coli* (Fig. 9B). Furthermore, using a transient expression assay with protoplasts isolated from Arabidopsis leaves, we demonstrated that SlZFP2 functions as a repressor to inhibit the expression of the ABA biosynthetic genes *NOT*, *SIT*, *SlAO1*, and *FLC*. The transient expression assay was conducted in three biological replicates, using the *GUS* reporter gene driven by each promoter (0.8–2 kb) of the four ABA biosynthetic genes as reporters and the *p35S*:*SlZFP2* construct as an effector. The expression of the luciferase (*LUC*) reporter gene driven by the 35S promoter was used as an internal control. Compared with empty vector controls, cotransformation with the effector plasmid significantly decreased the GUS expression driven by the four gene promoters in protoplasts based on the GUS activities relative to LUC activity (Fig. 9C). We conclude that *SlZFP2* represses the expression of ABA biosynthetic genes through direct binding to their promoters.

DISCUSSION

ABA facilitates plant adaptation to biotic and abiotic stresses and plays indispensable roles in many developmental processes. Accordingly, several ABA biosynthetic genes have been shown to be up-regulated transcriptionally under stress conditions (Nambara and Marion-Poll, 2005). Less is known about how ABA biosynthesis is regulated developmentally. Recently, ABA was implicated in fruit set and ripening in tomato (McAtee et al., 2013), but the underlying molecular mechanism remained elusive. In this study, we identified a tomato transcription factor, *SlZFP2*, that was up-regulated after anthesis. By analysis of transgenic lines constitutively expressing or suppressing *SlZFP2*, we demonstrated that SlZFP2 is responsible for the negative regulation of ABA biosynthesis during fruit development through direct binding to promoters of several ABA biosynthetic genes containing (A/T)(G/C)TT motifs. We also showed that *SlZFP2* delays the onset of fruit ripening mainly through transcriptional repression of the ripening regulator *CNR*.

SlZFP2 Regulates Fruit and Seed Development in Two Tomato Genetic Backgrounds

Overall, altering *SlZFP2* expression in LA1589 and cv M82 showed similar phenotypes; in both genetic backgrounds, overexpression lines showed increased branching, earlier flowering, and defective seed development, whereas down-regulation of its expression accelerated fruit ripening and inhibited seed germination. Variation in phenotypic severity was observed mainly on fruit set between RNAi lines from LA1589 and cv M82; the LA1589 lines set fruits poorly, whereas fruit set in the M82 lines was largely unaffected. Overexpression of HA-SlZFP2 in cv M82 had a more severe effect on seed development, in which no seed was formed in fruits of several lines. In contrast, when HA-SlZFP2 was overexpressed in LA1589, only the seed weight was affected. The difference in phenotypic severity is likely due to variation in endogenous hormone levels between the two genotypes, because fruit ABA accumulation after anthesis was slightly different between the current tomato LA1589 and cv M82; LA1589 had less ABA per fruit due to its smaller size (approximately 1 g per fruit for LA1589 compared with approximately 50–60 g per fruit for M82 in our growth conditions), and its ABA levels dropped faster after anthesis. It has been shown that auxin and ABA levels as well as their dynamic changes during fruit growth may vary even between cv Pik-red and cv Ailsa Craig (Buta and Spaulding, 1994). It is also likely caused by slightly different expression patterns of *SlZFP2* in the two genetic backgrounds during flower and fruit development. *SlZFP2* was not expressed in LA1589 flower buds; on the contrary, it was weakly expressed at that time in cv M82 (Supplemental Fig. S2, C and D). Another possibility we cannot rule out is the impact on plant growth by the *self-pruning* (*sp*) mutation in cv M82, although this is less likely. *SP* is involved in shoot sympodial development and also interacts with

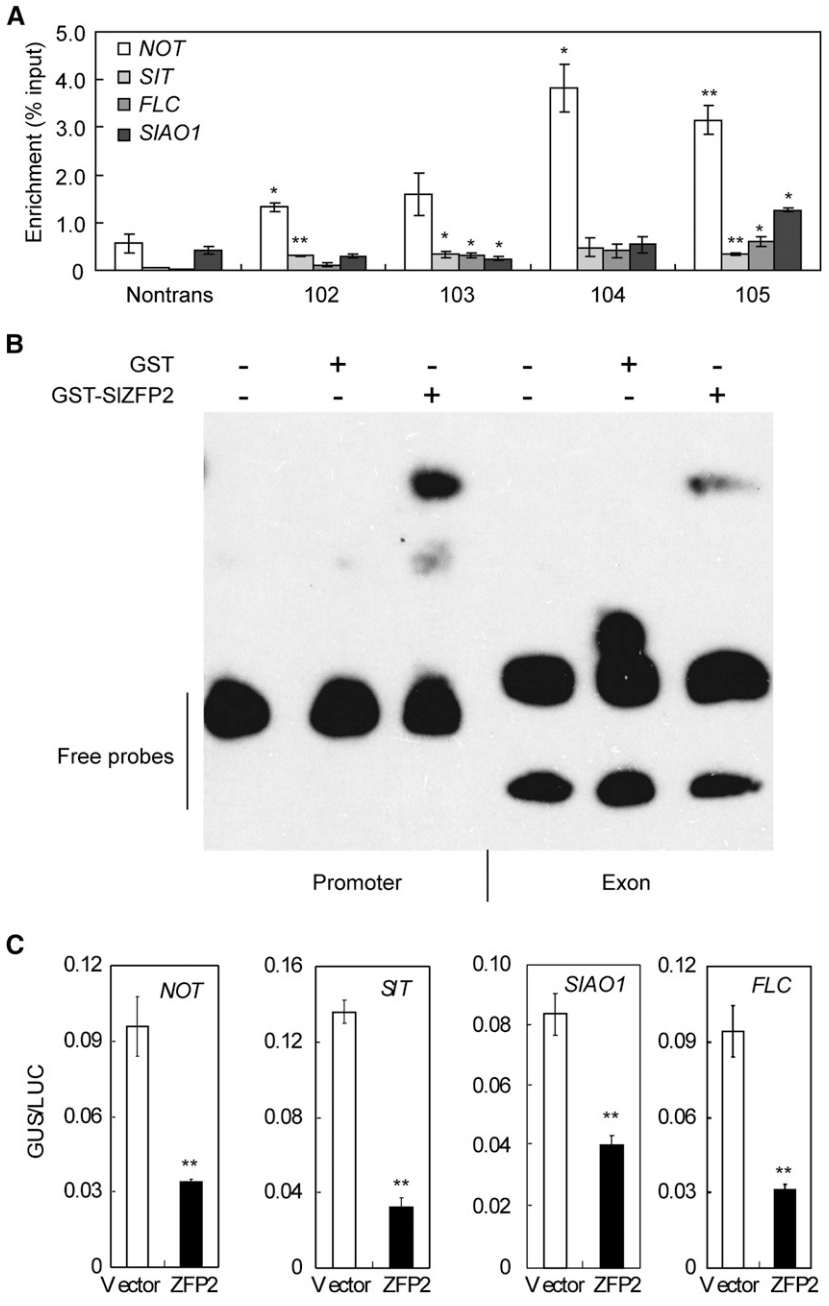

Figure 9. SlZFP2 binds directly to the promoters of ABA biosynthetic genes. A, Promoter enrichment of ABA biosynthetic genes by ChIP assay. Enrichments relative to input were determined by qPCR in triplicate. Nontrans, Nontransgenic siblings. B, In vitro binding of GST-SlZFP2 to the *SlAO1* promoter using EMSA. The probes were amplified by PCR from genomic DNA using the same sets of primers used for the ChIP assay. C, Transient expression analysis of SlZFP2 binding to the promoters of ABA biosynthetic genes. The GUS reporters were driven by the promoters of the ABA biosynthetic genes *NOT* (1,856 bp), *SIT* (1,699 bp), *FLC* (1,959 bp), and *SlAO1* (838 bp). After cotransformation with the internal control *p35:LUC* and the effector *p35:SlZFP2* or *pUC118* (empty vector control), GUS activity normalized to LUC activity (GUS/LUC) of each reporter was compared between the effector (*p35S:SlZFP2*) and the empty vector control (vector). Statistical significance was based on Student's *t* test: *, $P < 0.05$; and **, $P < 0.01$. Data are means ± SD; $n = 3$.

SINGLE FLOWER TRUSS to regulate the transition to flowering and yield heterosis in tomato (Pnueli et al., 1998; Krieger et al., 2010; Lifschitz et al., 2014). Because overexpression of *SlZFP2* accelerates flowering, loss of SP activity may have a substantial effect on plant development also regulated by *SlZFP2*.

Fruit set in tomato is mainly regulated by an interplay between auxin, cytokinin, and GA (Gillaspy et al., 1993; Mariotti et al., 2011; McAtee et al., 2013). Accumulating data from tomato and other plant species has suggested that ethylene and ABA might play a role in fruit set, because their production decreases dramatically right after pollination (Vriezen et al., 2008; Nitsch et al., 2009; Pascual et al., 2009; Wang et al., 2009; Carbonell-Bejerano et al., 2011; Martínez et al., 2013). Our RNA-seq data suggested that the problematic fruit set in the *SlZFP2* RNAi lines seems to result from the disturbed action of multiple hormones, because the expression of many genes involved in the biosynthesis and/or perception of auxin, cytokinin, GA, ABA, and ethylene was affected. In general, the action of auxin and GA, two major hormones that promote fruit set, was attenuated, while the action of ABA and ethylene was enhanced, which is required to be limited for successful fruit set (Supplemental Table S4).

The majority of differentially expressed genes involved in ABA and ethylene pathways were activated by down-regulation of *SlZFP2* expression, suggesting that the transcription factor functions as a repressor to inhibit the two pathways during early fruit development. The altered gene expression involved in auxin and GA pathways may explain the defective seed development observed in the *SlZFP2* transgenic lines, at least in part.

Transcriptional Regulation of ABA Biosynthesis during Fruit Development by *SlZFP2*

The mechanisms by which stresses regulate the transcription of ABA biosynthetic genes are known to be highly complex (Seo and Koshiba, 2002). Our results provide several lines of evidence to show that *SlZFP2* is a transcriptional repressor to limit ABA production during fruit development in tomato. First, *SlZFP2* overexpression lines displayed typical ABA-deficient phenotypes of faster seed germination and altered carotenoid composition. The viviparity frequently observed in the ABA-deficient *sit* mutant constitutively expressing *HA-SlZFP2* further suggests that ABA biosynthesis and/or signaling can be attenuated by high *SlZFP2* expression. Consistently, *SlZFP2* RNAi seeds germinated slower. Second, ABA production is negatively correlated to altered *SlZFP2* expression: lower in MG fruits with high *HA-SlZFP2* expression, while higher in 2-DPA fruits with down-regulated *SlZFP2* expression. Third, the transcription of several ABA biosynthetic genes is regulated by *SlZFP2* during fruit development.

Our analysis of ABA production and the expression of ABA biosynthetic genes in different tissues suggests that *SlZFP2* mainly fine-tunes ABA biosynthesis during fruit development. Unlike the ABA-deficient mutants *not*, *sit*, and *flc*, both *SlZFP2* overexpression and RNAi lines did not show a wilty phenotype under normal growth conditions, suggesting that *SlZFP2* has minor roles, if any, in ABA biosynthesis during vegetative growth. This notion is further supported by the fact that high *SlZFP2* expression represses ABA production mainly in mature leaves but not in young leaves. Furthermore, the MG fruits of the *HA-SlZFP2* overexpression lines, in which ABA production was most affected, still contained two-thirds the amount of ABA of the wild type (their nontransgenic siblings).

Furthermore, it has been shown that *AtZFP2* is induced by ABA in Arabidopsis seedlings using massive parallel sequencing, although no phenotypes in seedlings have been described (Hoth et al., 2002). We also found that *SlZFP2* was down-regulated in anthesis flowers and young fruits of the three ABA-deficient mutants, *not*, *sit*, and *flc*, suggesting that *SlZFP2* is involved in the feedback regulation of ABA biosynthesis during fruit development.

SlZFP2 Negatively Regulates Fruit Ripening through Modulating *CNR* Transcription

Fruit ripening in tomato is known to be influenced by transcription factors acting upstream of ethylene production that constitute a complex regulatory network operating through both ethylene-dependent and independent pathways. Among them, *RIN* is a master regulator that is required for activation of the developmentally controlled transcription of *LeACS1A* and *LeACS4*, which in turn contributes to the initiation of ripening-related ethylene production (Barry et al., 2000). RIN can form heterodimers with TAGL1 and TAG1 and binds to the cis-elements of *NOR* and *TDR4/SlFUL1*, depending on CNR activity (Martel et al., 2011; Fujisawa et al., 2013). Because the *rin* mutation did not abolish *CNR* expression during fruit ripening (Martel et al., 2011), there may be different regulatory mechanisms controlling *CNR* and *RIN* transcription. Our phenotypic and transcriptional analysis demonstrated that *SlZFP2* is a regulatory component of fruit ripening in tomato through the regulation of *CNR* expression, independent of *RIN*. This notion is supported by the fact that *CNR* expression was dramatically repressed by the overexpression of *HA-SlZFP2* from MG to ripe and was activated much earlier in the fruits of the *SlZFP2* RNAi lines. Furthermore, *TDR4/SlFUL1*, acting downstream of *CNR*, showed a similar expression pattern to *CNR* in both the *SlZFP2* overexpression and RNAi lines. *RIN* and its direct target genes *NOR* and *TAGL1* were only down-regulated in ripe fruits of the *HA-SlZFP2* overexpression lines but were not affected by the down-regulation of *SlZFP2* (Figs. 7 and 8), suggesting that *SlZFP2* acts independently of *RIN*.

Because expression of the ethylene biosynthetic genes *LeACS6*, *LeACO1*, and *LeACO3* was not affected before the ripe stage by altered *SlZFP2* expression, *SlZFP2* may not directly regulate ethylene production during the ripening process. The repressed expression of the ethylene-related genes *LeACS6*, *LeACO1*, *LeACO3*, and *EIL3* in ripe fruits by the overexpression of *HA-SlZFP2* may be explained by the down-regulated *RIN* or *CNR* transcription at this stage. However, the reason for the observed increase in *LeACS2* expression in the *HA-SlZFP2* overexpression lines at the Br and ripe stages remains to be determined. One possibility is that the autocatalytic ethylene biosynthesis was less affected by high *SlZFP2* expression, because, unlike the greenish fruits produced by *rin*, *cnr*, and *nor* mutants (Vrebalov et al., 2002; Manning et al., 2006; Giovannoni, 2007), *SlZFP2* overexpression and RNAi lines produced morphologically red ripe fruits (Fig. 3). Thus, *SlZFP2* also function as a transcription repressor to delay the onset of ripening.

Several recent studies indicate that ABA promotes fruit ripening in tomato, strawberry, and other species (Böttcher et al., 2010; Jia et al., 2011; Sun et al., 2012b). Our results support this hypothesis, because suppression of ABA biosynthesis as a consequence of elevated levels of *SlZFP2* expression results in delayed ripening. However, the mechanism by which ABA regulates ripening remains unclear. One possibility is that ABA promotes ethylene production during fruit ripening, an idea that is supported by the observation that

exogenous application of ABA to tomato MG fruits resulted in increased *LeACO1* and *LeACS2* expression (Zhang et al., 2009; Sun et al., 2012a). Interestingly, silencing of *SlNECD1* caused the down-regulation of several genes involved in ripening-associated cell metabolism, although higher ethylene production was observed in Br and later fruits (Sun et al., 2012b), suggesting that ABA-regulated ripening may be ethylene independent. It has been shown that ABA also functions as a ripening promoter in the nonclimacteric fruit strawberry, where ethylene is not the primary factor triggering ripening (Jia et al., 2011). In our study, except for *LeACS2*, other ethylene biosynthetic genes were not affected at the Br stage in these ABA-deficient lines when overexpressing *HA-SlZFP2*. Therefore, we hypothesize that ripening regulation by ABA in tomato might be perceived through an ethylene-independent pathway that is mediated by *SlZFP2*. Further investigation of the molecular mechanisms underlying the regulation of *CNR* transcription by *SlZFP2* and ABA signaling may shed light on the role of ABA in ripening regulation in tomato and other fruits.

Direct Target Genes of SlZFP2

SlZFP2 belongs to the TFIIIA-type C_2H_2 zinc finger protein family, where DNA binding sequences have been identified for a few members. The stress- and ABA-inducible proteins STZ and AZFs bind to A(G/C)T repeats in vitro (Sakamoto et al., 2004), and this element has been found in promoters of *AZF1*- and *AZF2*-regulated genes (Kodaira et al., 2011). Using a SAAB assay and B1H screening, we identified the (A/T)(G/C)TT motif as the core SlZFP2-binding sequence. Together with transcriptome analysis, we prove that *NOT*, *SlAO1*, *SIT*, and *FLC* are direct targets of SlZFP2 based on the fact that multiple (A/T)(G/C)TT repeats are present in the promoters of the four ABA biosynthetic genes and the confirmed binding ability in vivo and in vitro. *SlAO2* also is likely the direct target of SlZFP2, because its promoter contains the core (A/T)(G/C)TT-binding motifs and its expression was regulated by this transcription factor. Further supportive evidence comes from the similar expression patterns between *SlZFP2* and the ABA biosynthetic genes *NOT*, *SIT*, and *FLC* in 5-DPA fruits by in situ hybridization; they all were expressed in the funiculus and placenta regions (Supplemental Fig. S11). Moreover, *SlZFP2* shares a similar expression pattern with the tomato ABA 8′-hydroxylase gene *SlCYP707A1*, which is responsible for the reduction of ABA levels in pollinated ovary and is predominantly expressed in the ovules and placenta of anthesis flowers (Nitsch et al., 2009). Arabidopsis *NCED3*, *ABA2*, and *AAO3* have been shown to be expressed in the vascular systems of roots, leaves, stems, and inflorescences (Koiwai et al., 2004; Endo et al., 2008). Although the detailed expression patterns in fruits have not yet been determined for most ABA biosynthetic genes, the Arabidopsis *ABA2* gene is primarily expressed in the funiculus and placenta of silique (Cheng et al., 2002). Thus, *SlZFP2* is expressed in regions with high ABA levels, so we reason that *NOT*, *SlAO1*, *SIT*, and *FLC* are direct targets of SlZFP2 during fruit development.

Other than *SlZFP2*, several transcription factors have been shown to directly target the ABA biosynthetic genes *NCEDs* and *AAO3* in rice and Arabidopsis, respectively (Yaish et al., 2010; Jiang et al., 2012; Jensen et al., 2013; Je et al., 2014; Yang et al., 2014). Recently, a tomato C1-2i member of the C_2H_2-type zinc finger proteins SlZF2 also was shown to regulate ABA biosynthesis because of increased ABA production in its overexpression lines (Hichri et al., 2014). But it remains to be determined whether SlZF2 directly targets any ABA biosynthetic genes. All the above-mentioned transcription factors are positive regulators of ABA biosynthesis. On the contrary, *SlZFP2* functions as a repressor to fine-tune ABA production during fruit development. Thus, *SlZFP2* represents a new player in the regulation of ABA biosynthesis.

In addition to the above-mentioned ABA biosynthetic genes, the *CNR* promoter also contains SlZFP2-binding sites, and its expression is regulated by this transcription factor. Therefore, *CNR* is very likely the direct target gene of *SlZFP2* to control fruit ripening. In addition to the above-mentioned six genes, we identified more than 2,000 genes containing SlZFP2-binding sequences in their 1.5-kb promoters, and among them, the transcription of 193 genes was differentially expressed in 2-DPA fruits of a representative *SlZFP2* RNAi line (line 207) and its nontransgenic sibling; they are likely the direct target genes of SlZFP2 at the early fruit growth stage (Supplemental Table S3). Notably, more than half of the 193 target genes were repressed in 2-DPA fruits by down-regulation of *SlZFP2* expression, and many are putatively involved in several cellular processes, including chlorophyll and heme synthesis by chlorophyllase2 (*Solyc12g005300*), protochlorophyllide reductase (*Solyc12g013710*), and porphobilinogen deasminase (*Solyc07g066470*). This indicates that *SlZFP2* not only functions as a repressor to inhibit ABA biosynthesis and fruit ripening but also as a transcription activator or coactivator to promote gene expression involved in other aspects of plant development. It is possible that SlZFP2 targets genes involved in diverse pathways, a hypothesis that will require further investigation.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The wild relative of tomato (*Solanum lycopersicum*), *Solanum pimpinellifolium* LA1589, the ABA-deficient mutants *sit* (LA0574), *not* (LA0617), and *flc* (LA0673), and cv Rheinland Ruhm (LA0535) were obtained from the Tomato Genetics Resource Center at the University of California. The tomato 'M82' was provided by Dr. Daniel Zamir at the Hebrew University of Jerusalem. Plants, including transgenic lines, were grown in phytotrons at 20°C to 25°C under a humidity of 70% to 80%, with illumination for 16 h daily by 150 mE m^{-2} s^{-1} light from metal halide and high-pressure sodium lamps. Plants were fertilized weekly with all-purpose fertilizer and watered as needed. For the

ABA-deficient mutants, 50 μM ABA was foliar sprayed weekly until most of the fruits reached the ripe stage.

Generation of Transgenic Lines

SlZFP2 (Solyc07g006880, unigene Sol Genomics Network-U576689) was identified previously as a differentially expressed gene during floral and early fruit development (TC128959 on the microarray chips; Xiao et al., 2009). The *SlZFP2* complementary DNA (cDNA) was isolated from LA1589 by RT-PCR using primers XP0034 and XP0036 and cloned into the pGEM T-Easy vector (Promega). Information on the primers used in this study is provided in Supplemental Table S5. To overexpress *SlZFP2*, the *p35S:SlZFP2* and *p35S:HA-SlZFP2* constructs were made by placing the full-length *SlZFP2* cDNA with or without an HA tag fused between the cauliflower mosaic virus *35S* promoter and the *NOS* terminator of the binary vector pHX20 derived from pZH01 (Xiao et al., 2003). The *HA-SlZFP2* fusion was made by PCR-based manipulation with primers XP0035 and XP0036. The forward primer XP0035 contains a Kozak cassette followed by the HA coding sequence added in frame to the N terminus of SlZFP2. For construction of the *SlZFP2* RNAi vector, the last 274 bp of the *SlZFP2* coding sequence plus the 127-bp 3′ untranslated region were amplified using primers XP0028 and XP0029 and cloned into the binary vector pFGC5941 in both the sense and antisense directions (Kerschen et al., 2004). The plasmids were then introduced into the *Agrobacterium tumefaciens* strain GV3101. The *p35S:HA-SlZFP2* and *SlZFP2* RNAi constructs were used to transform LA1589 and cv M82, and *p35S:SlZFP2* was transformed into LA1589, as described previously (McCormick, 1991).

Phenotypic Analysis of Transgenic Lines

All the analyses in this study were conducted on homozygous plants of overexpression and RNAi lines using their respective pooled nontransgenic siblings as controls, except RNAi lines of LA1589, for which heterozygous plants were used because of the failure to obtain their homozygous plants. For the selection of homozygous plants of the *SlZFP2* overexpression lines, 50 to 100 5-d-old seedlings from individual T2 plants genotyped were tested for their resistance to 30 mg L^{-1} hygromycin B (Roche). Similarly, 50 to 100 2-week-old seedlings grown in phytotrons were sprayed with 0.02% (v/v) Basta to select homozygous RNAi plants (Shanghai Sangon), and the resistance was recorded when the nontransgenic seedlings were dead. For those segregating transgenic lines, transgenic plants were selected and verified by genotyping with transgene-specific primers: *Hygromycin B resistance gene*-specific primers XP0515 and XP0516 for overexpression lines and *herbicide resistance gene*-specific primers XP0517 and XP0518 for RNAi plants.

Leaf stomata sizes of the *HA-SlZFP2* overexpression lines from LA1589 were measured and analyzed using ImageJ (http://rsbweb.nih.gov/ij/) based on scanning electron microscopy images taken from plastic replicas of abaxial leaf surfaces. Except for some RNAi lines with limited seeds available, seed germination based on radicle emergence was monitored at 25°C in 12-h intervals or daily by placing at least 150 seeds in three replicates on filter paper moistened with distilled water. For the ABA sensitivity assay, seed germination was performed on filter paper moistened with distilled water supplemented with 0, 1, 2.5, and 5 μM ABA. ABA was dissolved in absolute methanol, and the final concentration of methanol was 0.027% (v/v) in all ABA treatments and the mock solution.

Quantification of ABA Content

ABA extraction from leaves, flowers, and fruits was conducted as described previously (Pan et al., 2010). Briefly, samples were ground into a fine powder in liquid nitrogen. For each sample, 50 or 100 mg of ground tissue was extracted twice with 10 volumes of extraction buffer containing 2-propanol:water:concentrated HCl (2:1:0.002, v/v/v) for 30 min at 4°C, followed by two extractions with dichloromethane. After centrifugation, the chloroform phases containing ABA were combined and concentrated using a nitrogen evaporator. Pellets were redissolved in 100 μL of methanol. Before extraction, 100 ng of [$^2H_6$]ABA (Icon Isotopes; catalog no. A101-169-2) was added as an internal standard. The samples were then analyzed on an Agilent liquid chromatography-tandem mass spectrometry device (1200/6520) system equipped with a ZORBAX Eclipse XDB-C18 column as described previously (Yano et al., 2009). ABA levels were determined using the MassHunter qualitative software (Agilent; version B.03.01) based on responsive signals of the internal standard and the sample ABA.

Quantification of Carotenoids

Carotenoids were extracted from 500 mg of fresh fruits and analyzed by HPLC as described previously (Fu et al., 2012). HPLC analysis was carried out using a Waters Alliance 2695 system consisting of a 2695 module and a 2996 photodiode array detector, equipped with a 250- × 4.6-mm i.d., 5-μm, YMC reverse-phase C_{30} column and a 20- × 4.6-mm i.d., YMC C_{30} guard column.

RNA in Situ Hybridization

Shoot, flower buds, ovaries at anthesis, and 5-DPA fruits were collected from LA1589 plants. Tissue fixation, sectioning, and hybridization with digoxigenin-labeled sense and antisense probes were performed as described previously (Coen et al., 1990). For *SlZFP2*, a 401-bp fragment (the same region used for the RNAi construct) was used for the probe template. Probe templates of three ABA biosynthetic genes also were made by PCR amplification using the following primer sets: XP2247 and XP2248 (*NOT*; nucleotides 1,492–1,869), XP2249 and XP2250 (*SIT*; nucleotides 3,200–3,907), and XP2245 and XP2246 (*FLC*; nucleotides 2,324–2,805).

Real-Time qRT-PCR

Total RNA was extracted from various tomato tissues with Trizol reagent (Invitrogen) based on previously described methods (Xiao et al., 2009). Residual genomic DNA in the RNA samples was removed by RNase-free DNase according to the manufacturer's protocol (New England Biolabs). One microgram of DNase-treated total RNA was used to synthesize first-strand cDNA using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific), and real-time RT-PCR was performed with three technical replicates using SYBR Premix ExTaq (Takara Biotech) on an ABI Applied Biosystems StepOnePlus machine (Life Technologies). Transcript levels were calculated as relative expression to *SleIF4α6* (Xiao et al., 2008).

Gene Expression Profiling by RNA-seq

High-throughput RNA-seq was used to quantify genome-wide gene expression regulated by *SlZFP2*. Total RNA was extracted by Trizol reagent (Invitrogen) from 2-DPA fruits of the RNAi line 207 and its nontransgenic sibling (207N) as described previously (Xiao et al., 2009). Paired-end sequencing libraries were created using the TruSeq stranded mRNA kit (RS-122-2101; Illumina) and sequenced on Illumina's Miseq system using the 500-cycles Miseq reagent kit (MS-102-2003). The 250-bp paired-end reads were mapped to the tomato genome using the Tophat program version 2.0.12 (Trapnell et al., 2009). Three biological replicates were conducted. In total, the numbers of read pairs mapped for each replicate were as follows: 1,497,635 (207N, replicate 1; 80.7% mapped), 1,191,334 (207N, replicate 2; 80.6%), 1,279,658 (207N, replicate 3; 83.2%), 573,982 (207, replicate 1; 80.2%), 749,210 (207, replicate 2; 80.2%), and 1,158,542 (207, replicate 3; 80.5%). Then, differentially expressed genes (adjusted *P* of 0.05 or less) were identified by Cufflinks version 2.2.1 (Trapnell et al., 2010). A total of 2,722 differentially expressed genes were identified, and the data set (Supplemental Table S2) was further compared with the list of genes containing (A/T)(G/C)TT motifs in their promoters to select genes directly targeted by SlZFP2 at the early fruit growth stage (Supplemental Table S3). The raw reads and gene expression data have been deposited in the National Center for Biotechnology Information (accession no. GSE63838).

Identification of DNA-Binding Sequences Recognized by SlZFP2

Two approaches were used to identify DNA-binding sequences of SlZFP2: SAAB assay and B1H screening. SAAB was conducted essentially as described previously (Peng et al., 2002). Briefly, a 14-bp randomized oligonucleotide library was prepared by annealing two synthesized oligonucleotides, 5′-GGGAAGACGGATCCATTGCA-N14-CTGTAGGAATTCGGACCCT-3′ and 5′-AGGGTCCGAATTCCTACAG-3′, followed by primer extension at 72°C. The full-length *SlZFP2* coding sequence was amplified by PCR using primers XP0662 and XP0684 and cloned into the pGEX-4T-3 vector. After transformation into *Escherichia coli*, the GST-SlZFP2 fusion protein was expressed and purified using Glutathione Sepharose 4B beads according to the manufacturer's instructions (GE Healthcare Life Sciences). Two micrograms of purified GST-SlZFP2 protein bound to the beads was incubated on ice for 30 min with 0.8 ng of library DNA in a binding buffer containing 20 mM HEPES (pH 7.9), 100 mM KCl, 20 mM EDTA, 20 mM EGTA, 1% (v/v) Nonidet P-40, 1 mM benzamidine, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 mM

dithiothreitol, 20% (v/v) glycerol, 50 ng mL^{-1} bovine serum albumin, and 10 ng μL^{-1} poly(dI•dC). After three washes with the binding buffer without bovine serum albumin, bound DNA was recovered from beads by incubation at 45°C for 1 h with 50 mM Tris-HCl (pH 8), 5 mM EDTA, 100 mM sodium acetate, and 0.5% (w/v) SDS. Then, the selected DNA was amplified by PCR using the PCR primers 5′-GGGAAGACGGATCCATTGCA-3′ and 5′-AGGGTCCGAATTCCTACAG-3′, and the PCR products were further purified by native PAGE. The selection was repeated another five times with a reduced library aliquot down to 0.2 ng of DNA, and the final selected DNA fragments were cloned into the pMD18-T vector (Takara Biotech). In total, 120 clones were picked for sequencing, and 89 sequences of high quality were obtained.

For the B1H assay, the full-length *SlZFP2* sequence was cloned into the pB1H1 vector, and an 18-bp randomized oligonucleotide library was cloned into the pH3U3 vector (Addgene; http://www.addgene.org) as described previously (Meng et al., 2005). Self-activation of the *HIS3* and *URA3* reporter genes in the primary library containing an estimated 2×10^7 clones was avoided by selection with 2 mM 5-fluoroorotic acid. The resulting library DNA was purified and transformed into *US0hisB⁻pyrF⁻* electrocompetent cells containing the bait plasmid pB1H1 with *SlZFP2* coding sequence. Approximately 1×10^8 cells containing the bait and prey library were plated on *HIS⁻* selective minimal medium supplemented with 4 mM 3-amino-1,2,4-triazole at 37°C for 24 h. Plasmid DNA from the surviving colonies was digested with *Xmn*I to remove the bait plasmid. The prey DNA was then purified by the QIAquick PCR purification column (Qiagen) and transformed into the selection strain *US0hisB⁻pyrF⁻* for counterselection with 2 mM 5-fluoroorotic acid. After incubation at 37°C for 24 h, individual colonies were selected for sequencing.

Subcellular Localization of SlZFP2-YFP

The SlZFP2-YFP fusion was made by ligation of a PCR-amplified full-length *SlZFP2* fragment, made by using primers XP0210 and XP0211, to the YFP coding sequence (Clontech). This cassette was cloned into the pHX20 vector. The *A. tumefaciens* GV3101 strain containing *p35S:YFP* or *p35S:SlZFP2-YFP* was infiltrated into *Nicotiana benthamiana* leaves, and transient expression was monitored using a Zeiss LSM510 Meta confocal scanning microscope.

ChIP Assay

ChIP assays were performed on four homozygous *HA-SlZFP2* overexpression lines (102–105) using their nontransgenic siblings as controls. Tissue fixation, cross-linking, and chromatin isolation were performed as described previously (Ito et al., 2012), with minor modifications. Young leaves from 45-d-old plants, including apical meristems, were ground into a fine powder in liquid nitrogen. A total of 1.5 g of powder was suspended in 25 mL of nuclear isolation buffer A (10 mM Tris-HCl [pH 8], 0.4 M Suc, 5 mM KCl, 5 mM $MgCl_2$, 5 mM EDTA, 1% [v/v] formaldehyde, 0.05% [v/v] Triton X-100, and 1 mM PMSF). After 10 min of incubation at room temperature, cross-linking was stopped with Gly at a final concentration of 0.125 M. Lysates were filtered through two layers of Miracloth (Millipore) and cleared by centrifugation at 845*g* for 10 min. The pellets were then washed with ice-cold nuclear isolation buffer B (10 mM Tris-HCl [pH 8], 0.4 M Suc, 5 mM KCl, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM β-mercaptoethanol, and complete Roche Protease inhibitor tablets). The nuclei were lysed in nuclei lysis buffer containing 50 mM Tris-HCl (pH 8), 10 mM EDTA, 1% (w/v) SDS, 1 mM PMSF, 50 μM MG-132 (Sigma-Aldrich), and complete Roche Protease inhibitor tablets. Chromatin DNA was sheared to 500 to 1,000 bp using a 130-W Ultrasonic Processor VXC-130 (Sonics). After removing cell debris by centrifugation, the solution containing chromatin was diluted with ChIP dilution buffer (16.7 mM Tris-HCl [pH 8], 167 mM NaCl, 1.1% [w/v] Triton X-100, 1.2 mM EDTA, 1 mM PMSF, 50 μM MG-132, and complete protease inhibitor mixture tablets), followed by overnight incubation on ice with Dynabeads Protein G (Invitrogen) coupled to HA monoclonal antibody (Sigma-Aldrich). Beads were washed three times with ice-cold low-salt buffer (20 mM Tris-HCl [pH 8], 150 mM NaCl, 0.2% [w/v] SDS, 0.5% [v/v] Triton X-100, and 2 mM EDTA) and three times with ice-cold Tris-EDTA buffer (10 mM Tris-HCl [pH 8] and 1 mM EDTA). DNA subjected to ChIP was eluted with elution buffer (1% [w/v] SDS with freshly added 0.168 g of $NaHCO_3$ per 20 mL) and reverse cross-linked overnight at 65°C by adding 5 M NaCl to a final concentration of 0.2 M. Eluted DNA samples were treated with Protease K (Sigma-Aldrich) at 50°C for 2 h, and DNA pellets were dissolved in 100 μL of Tris-EDTA buffer after further purification by phenol/chloroform extraction and precipitation. Aliquots of 2 μL were used for qPCR.

EMSA

EMSA was performed using *E. coli*-expressed GST-SlZFP2 protein and biotin-labeled DNA fragments containing putative binding sites for SlZFP2 (Thermo Fisher Scientific). The same primers used for the ChIP-qPCR analysis were used for preparing probe templates by PCR. Binding reactions were conducted at room temperature for 20 min in 20 μL of binding buffer containing 10 mM Tris (pH 7.5), 50 mM KCl, 1 mM dithiothreitol, 2.5% (v/v) glycerol, 0.05% (v/v) Nonidet P-40, 5 mM $MgCl_2$, 0.5 mM EDTA, 50 ng mL^{-1} poly(dI•dC), 1.5 μg of purified GST-SlZFP2, and 50 fmol biotin-labeled PCR fragments. Protein-DNA complexes were separated on a 6% (w/v) native polyacrylamide gel in 0.5× Tris-borate/EDTA buffer. After electrophoresis, protein-DNA complexes were transferred onto Hybond-N^+ nylon membranes (GE Healthcare Life Sciences). The protein-DNA binding interaction was detected using the Light Shift Chemiluminescent EMSA Kit (Thermo Fisher Scientific).

Transient Expression Assay in Arabidopsis Protoplasts

The transient expression assay was conducted according to the protocol described by Yoo et al. (2007). A pUC118-based *SlZFP2* expression cassette driven by the cauliflower mosaic virus *35S* promoter (effector) or pUC118 (vector control) was cointroduced into protoplasts isolated from Arabidopsis (*Arabidopsis thaliana*) leaves with GUS reporter plasmids, of which 0.8- to 2-kb promoters of the ABA biosynthetic genes *NOT*, *SIT*, *FLC*, and *SlAO1* were placed upstream of the GUS coding sequences, together with the cauliflower mosaic virus *35S:LUC* plasmid as an internal control. The reporter and internal control plasmids were also pUC118 based. For each assay, three biological replicates were performed, and GUS activity was normalized to the internal control LUC activity measured by the Thermo Scientific Varioskan Flash Multimode Reader (Thermo Fisher Scientific).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. *SlZFP2* encodes a C_2H_2-type zinc finger protein mainly expressed in fruits.

Supplemental Figure S2. *SlZFP2* expression in various tissues.

Supplemental Figure S3. Fruit and seed morphology of *HA-SlZFP2* overexpression lines in LA1589 background.

Supplemental Figure S4. Seed germination of *SlZFP2* overexpression lines.

Supplemental Figure S5. Overexpression of *HA-SlZFP2* affects stomata aperture.

Supplemental Figure S6. Changes of ABA levels in cv M82 fruits after pollination.

Supplemental Figure S7. Repressed ABA biosynthesis in *p35::HA-SlZFP2* leaves.

Supplemental Figure S8. Transcript levels of ABA biosynthetic genes in *p35::HA-SlZFP2* seeds.

Supplemental Figure S9. Subcellular localization of SlZFP2 protein.

Supplemental Figure S10. SlZFP2 binding sites found in the promoters of *NOT*, *SIT*, *SlAO1*, *SlAO2*, *FLC*, and *CNR*.

Supplemental Figure S11. Expression of *SlZFP2* and the ABA biosynthetic genes by in situ hybridization.

Supplemental Table S1. Carotenoid accumulation in anthesis flowers and fruits of *SlZFP2* overexpression and RNAi lines.

Supplemental Table S2. Differentially expressed genes in 2-DPA fruits of *SlZFP2* RNAi line 207 identified by RNA-seq.

Supplemental Table S3. Expression levels (fragments per kilobase of transcripts per million mapped fragments) of SlZFP2 target genes.

Supplemental Table S4. Differentially expressed genes involved in hormone biosynthesis and signaling in 2-DPA fruits of the *SlZFP2* RNAi line 207.

Supplemental Table S5. Primer information used in the study.

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