Seasonal Abscisic Acid Signal and a Basic Leucine Zipper Transcription Factor, DkbZIP5, Regulate Proanthocyanidin Biosynthesis in Persimmon Fruit1[C][W][OA]

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Proanthocyanidins (PAs) are secondary metabolites that contribute to plant protection and crop quality. Persimmon (Diospyros kaki) has a unique characteristic of accumulating large amounts of PAs, particularly in its fruit. Normal astringent-type and mutant nonastringent-type fruits show different PA accumulation patterns depending on the seasonal expression patterns of DkMyb4, which is a Myb transcription factor (TF) regulating many PA pathway genes in persimmon. In this study, attempts were made to identify the factors involved in DkMyb4 expression and the resultant PA accumulation in persimmon fruit. Treatment with abscisic acid (ABA) and an ABA biosynthesis inhibitor resulted in differential changes in the expression patterns of DkMyb4 and PA biosynthesis in astringent-type and nonastringent-type fruits depending on the development stage. To obtain an ABA-signaling TF, we isolated a full-length basic leucine zipper (bZIP) TF, DkbZIP5, which is highly expressed in persimmon fruit. We also showed that ectopic DkbZIP5 overexpression in persimmon calluses induced the up-regulation of DkMyb4 and the resultant PA biosynthesis. In addition, a detailed molecular characterization using the electrophoretic mobility shift assay and transient reporter assay indicated that DkbZIP5 recognized ABA-responsive elements in the promoter region of DkMyb4 and acted as a direct regulator of DkMyb4 in an ABA-dependent manner. These results suggest that ABA signals may be involved in PA biosynthesis in persimmon fruit via DkMyb4 activation by DkbZIP5.

Flavonoids are a family of plant secondary metabolites. This family includes many groups of compounds and contributes to various important plant functions. Flavonoids generally contribute to protection against biotic or abiotic stresses, such as grazing, elevated light intensities, UV irradiation, and pathogen infection (Harborne and Grayer, 1993; Winkel-Shirley, 2001; Peters and Constabel, 2002; Mellow et al., 2009). Proanthocyanidins (PAs) are colorless phenolic polymers that result from the polymerization of flavan-3-ol units and are synthesized via the anthocyanin/PA branch of the flavonoid biosynthetic pathway (for representative structures, see Fig. 1; Dixon et al., 2005; Lepiniec et al., 2006). The presence of PAs in forage plants is considered a beneficial trait that protects ruminants from pasture bloat and enhances ruminant nutrition (Lees, 1992). In addition, PAs contribute to human health (Bagchi et al., 2000; Dixon et al., 2005) and to the quality of many important plant products, such as tea, some berries, and especially wine (Aron and Kennedy, 2008). Thus, insights into the artificial modification of PA biosynthesis or accumulation in plant cells will greatly contribute to many aspects of future agricultural programs.

Regulatory steps in PA biosynthesis, including its transcriptional regulation, have been well characterized, although the final steps in the transportation or polymerization of PAs remain to be elucidated (Zhao et al., 2010). With regard to enzymatic steps, an important finding in the analysis of PA biosynthesis was the identification of anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR), which act in the production of flavan-3-ol, having 2,3-cis- or 2,3-trans-stereochemistry of the heterocyclic C ring (Fig. 1; ANR by Xie et al., 2003; LAR by Tanner et al., 2003). With regard to transcriptional regulation, some Myb transcription factors (TFs) regulating PA pathway genes have been identified in Arabidopsis (Arabidopsis thaliana; TRANSPARENT TESTA2 [TT2]; Nesi et al., 2001; Lepiniec et al., 2006), poplar (Populus trichocarpa [MYB134]; Mellow et al., 2009), and grapevine (Vitis vinifera [VvMYBPA1 [Bogs et al., 2007] and VvMYB5a and VvMYB5b [Deluc
Persimmon (*Diospyros kaki*), one of East Asia’s major fruit crops, accumulates large amounts of PA in its fruit flesh. PAs in persimmon fruit constitute more than 1% of its fresh weight (Taira et al., 1998). PAs are high-M, polymers with a mean degree of polymerization above 30 at the middle developmental stages, during which the accumulation of PA terminates in certain cultivars (Matsuo and Ito, 1978; Akagi et al., 2009a), which causes very strong astringency. Wild-type astringent-type (A-type) persimmon fruit accumulates PAs for a long term during its developmental stages, which ranges from the stage before full anthesis (April in Japan) to before the mature stage (August in Japan; Akagi et al., 2009a). In fruits with a spontaneous mutant phenotype, PA accumulation is terminated at an early fruit development stage and astringency is not expressed (Ikegami et al., 2005; Akagi et al., 2009b). This mutant non-astringent (NA) phenotype is also called the pollination constant NA type (Yonemori et al., 2000). It has been suggested that a single gene known as the *ASTRINGENCY* (*AST*) locus controls the allelotype of normal A- and mutant NA-type fruits, and the NA genotype expression requires six homozygous recessive alleles (*ast*) at the *AST* locus (Yamada and Sato, 2002; Akagi et al., 2009c). However, the *AST* locus has not been identified, and the genetic bases for the A/NA phenotypes have been partially elucidated. On the other hand, mRNA profiling provides some clues that coordinate reduction in the expression of the PA and shikimate pathway genes in NA-type fruits is involved in the substantial reduction of PA content (Akagi et al., 2009a). It has been suggested that NA-type fruit-specific down-regulation of *DkMyb4*, which is a Myb TF regulating PA and shikimate pathway genes, may reduce the expression of these genes, with a corresponding reduction in PA content in NA-type fruits (Akagi et al., 2009b). In addition, low temperatures affect the phenological expression of *DkMyb4* and the resultant PA accumulation in NA-type cultivars, particularly in the early developmental stage, accompanied by a significant increase in ABA concentration, although this was not observed in the A-type cultivar (Akagi et al., 2011). These results suggest that further characterization of the phenological response of PA biosynthesis in persimmon would provide a good understanding of the signaling pathways regulating PA accumulation in response to seasonal and environmental factors.

In this study, attempts were made to exploit the effect of ABA signals on phenological PA accumulation in persimmon fruit. As described, coincident increases in ABA concentration and Myb TF expression regulating anthocyanin/PA biosynthesis in grape and/or persimmon fruit suggest some relationship between them. This hypothesis would be supported by some reports on the ABA-associated regulation of Myb TF expression in Arabidopsis (Urao et al., 1993; Abe et al., 2003) and also by the regulatory mechanism for CI Myb TF, which is a flavonoid regulator in maize (*Zea mays*). Hattori et al. (1992) demonstrated that CI expression and the resultant anthocyanin biosynthesis in seeds are regu-
lated by ABA signals and the ABA-regulated *V. rous* TF. To date, a large number of ABA signaling components have been identified (Hirayama and Shinozaki, 2007; Wasilewskia et al., 2008). Among them, one of the major TFs involved in ABA signaling is the basic leucine zipper (bZIP) class TF (Jakoby et al., 2002). bZIP TFs binding ACGTG-containing motifs, which are known as ABA-responsive elements (ABREs), constitute the ABF/AREB/ABI5 subfamily (Choi et al., 2000, 2005; Uno et al., 2000; Wasilewskia et al., 2008). bZIP TFs have a regulatory function in ABA and/or stress responses. These factors can activate an ABRE-containing promoter in an ABA-dependent manner (Uno et al., 2000). However, the involvement of the ABRE-binding bZIP TFs in Myb TF expression regulating flavonoid biosynthesis has not yet been demonstrated, although bZIP TFs have been suggested to directly affect the expression of some flavonoid pathway genes such as *FLS* in Arabidopsis and grapevine (Hartmann et al., 2005; Czemmel et al., 2009; Hichri et al., 2011). Our study showed that ABA signals can act as a regulator of *DkMyb4* and the resultant PA biosynthesis via the activity of ABF/AREB/ABI5-like bZIP TFs in specific developmental stages of persimmon fruit. A good understanding of the fundamental mechanisms underlying PA regulation, such as those cued by environmental factors and endogenous or exogenous plant hormones, will enable us not only to modify PA-derived traits in many crops but also to shed light on the possibility of the phenological regulation of PA biosynthesis.

**RESULTS**

**Effects of ABA and Its Biosynthesis Inhibitor on PA Accumulation in Persimmon Fruit**

To exploit the possibility that the modification of ABA concentration in persimmon fruit affects PA regulation, fruit flesh and calyx parts of cv Kuramitsu (A type), Okugosho (NA type), and Suruga (NA type) were sprayed with Miyobi, which is a fertilizer containing 10.0% (w/w) S-ABA (natural-type ABA), at a concentration of 4,000 µg L\(^{-1}\) (400 µg L\(^{-1}\) [approximately 1.5 mM] S-ABA). In addition, fruit and leaves from these three cultivars were sprayed with abamine, which is a competitive inhibitor of 9-cis-epoxycarotenoid dioxygenase (Kitahata et al., 2006), at a concentration of 100 mM to inhibit ABA biosynthesis in fruit flesh. These treatments started from the preflowering developmental stage and continued until the midfruit stage, at which PA accumulation in A-type cultivars was terminated (Akagi et al., 2009b). ABA concentration in persimmon fruit gradually decreases after anthesis until the middle fruit development stage. Similar tendencies are shown in A- and NA-type cultivars (Akagi et al., 2011; Supplemental Fig. S1).

In control sections of A- and NA-type cultivars, *DkMyb4* apparently showed an NA-type-specific down-regulation 3 weeks after bloom (WAB), which corresponded to previous reports (Akagi et al., 2009b). The expression of *DkMyb4* in NA-type fruit treated with S-ABA was significantly up-regulated after 7 WAB until 13 WAB (Fig. 2A; *P* < 0.01). Note that the up-regulated expression level of *DkMyb4* in NA-type fruit was still significantly less than that in the control A-type fruit (Fig. 2A; *P* < 0.01). On the other hand, *DkMyb4* expression in NA-type fruit treated with abamine tended toward a slight down-regulation after 3 WAB, ranging up to approximately 6-fold lower than that of the control (*P* < 0.01 for cv Suruga at 3 WAB and cv Okugosho at 3 and 5 WAB). In contrast to NA-type fruit, *DkMyb4* expression in A-type cv Kuramitsu showed the opposite response to the S-ABA treatment at 9 WAB (*P* < 0.01). The expression levels of *DkMyb4* in fruits treated S-ABA were approximately 4- and 2.5-fold lower than those in the control at 9 and 13 WAB, respectively (*P* < 0.01), but the abamine treatment did not significantly affect the expression of *DkMyb4* at these sampling points (Fig. 2A; *P* > 0.05). Two PA pathway genes, *DkANR* and *DkF3', 5'-H, were analyzed. These genes are controlled by *DkMyb4* expression and affect substantial PA concentration and composition in persimmon fruit (Akagi et al., 2009b). Analysis showed significant differences in expression between S-ABA, abamine, and control sections in NA-type cultivars, which correlated with those in the expression of *DkMyb4* (Fig. 2, B and C, for *DkANR* and *DkF3', 5'-H, respectively). On the other hand, in A-type fruit, no significant differences were observed in the expression patterns of *DkANR* and *DkF3', 5'-H among the three sections (*P* > 0.05), except the abamine section at 13 WAB (Fig. 2B; *P* < 0.01). The expression of *DkMyb2*, which is another TF involved in PA biosynthesis in persimmon (Akagi et al., 2010a), and four other Myb TFs expressed in persimmon fruit (Akagi et al., 2009b) did not show a significant difference between S-ABA, abamine, and control sections in A- and NA-type cultivars (*P* > 0.05 for all sampling points; Supplemental Fig. S2). By performing HPLC analysis following acid catalysis in the presence of excess phloroglucinol (Kennedy and Jones, 2001; Akagi et al., 2010b), we detected a significant increase in the total PA concentration in NA-type cultivars with S-ABA treatment (*P* < 0.05 for cv Suruga, *P* < 0.01 for cv Okugosho), mainly because of an increase in the concentrations of epigallocatechin (EGC) and epigallocatechin-gallate (EGC-G; Fig. 2D). This result corresponds with the gene regulation following S-ABA treatment, because the expression of *DkMyb4* in persimmon fruit mainly contributes to the concentrations of EGC and EGC-G (Akagi et al., 2009b). However, abamine treatment could not have significant effects on PA accumulation in A- and NA-type fruit, presumably because of slight differences in PA pathway gene expression (Fig. 2, B and C).

**In Vitro ABA Treatments Regulate *DkMyb4* Expression Differentially per A/NA Genotype and Developmental Stage**

To further validate the involvement of ABA signals in PA regulation, the expression response of PA reg-
ulatory genes to ABA was analyzed in vitro using Murashige and Skoog (MS) medium containing varied concentrations of ABA and A/NA-type fruit pieces from the BC 1-like Atf lines (see "Materials and Methods"). The expression levels of each gene are given as values relative to those in cv Kuramitsu at 1 WAB, whose expression level is defined as “100.” Error bars indicate SD (n = 3). D, Concentration of each PA component and total PA of the three cultivars in each treatment at 13 WAB. Main PA components of persimmon fruit, catechin (C), gallicatechin (GC), epicatechin (EC), EC-G, epicatechin-gallate (EC-G), and EGC-G (Akagi et al., 2009a), were detected after depolymerization of PA by acid catalysis in the presence of excess phloroglucinol (see "Materials and Methods"). DW, Dry weight. Error bars indicate SD (n = 4). In all panels, asterisks indicate significant differences (*P < 0.05, **P < 0.01) according to Student’s t test compared with the control sections.

Figure 2. ABA treatment affects the expression patterns of genes regulating PA biosynthesis and PA concentration in persimmon. A to C, qRT-PCR analysis of gene expression in the control fruit and fruits treated with abamine and S-ABA. A, DkMyb4 expression. B, DkANR expression. C, DkF3’5’H expression. Each treatment is shown as a black point and solid line (control), white point and dotted line (abamine), and gray point and dotted/dashed line (S-ABA) in cv Kuramitsu (A type; squares), Suruga (NA type; circles), and Okugosho (NA type; diamonds). Three fruits of each cultivar were sampled at 1, 3, 5, 7, 9, and 13 WAB and independently subjected to qRT-PCR analysis. The expression levels of each gene are normalized as values relative to those in cv Kuramitsu at 1 WAB, whose expression level is defined as “100.” Error bars indicate SD (n = 3). D, Concentration of each PA component and total PA of the three cultivars in each treatment at 13 WAB. Main PA components of persimmon fruit, catechin (C), gallicatechin (GC), epicatechin (EC), EC-G, epicatechin-gallate (EC-G), and EGC-G (Akagi et al., 2009a), were detected after depolymerization of PA by acid catalysis in the presence of excess phloroglucinol (see “Materials and Methods”). DW, Dry weight. Error bars indicate SD (n = 4). In all panels, asterisks indicate significant differences (*P < 0.05, **P < 0.01) according to Student’s t test compared with the control sections.

Figure 3. Incubation of six Atf line samples that were obtained at 9 WAB in the MS medium containing no ABA (0 µM) and 0.1 µM ABA resulted in differing DkMyb4 expression patterns within A- and NA-type samples that were similar to those in natural conditions. Expression levels of DkMyb4 in the A-type samples were different between the different ABA concentrations (Fig. 3B). On the other hand, the MS medium containing 10 µM ABA resulted in differing DkMyb4 expression patterns. These results suggest that responses differ between A- and NA-type cultivars and are dependent on...
ABA concentration. A-type samples showed high levels of DkMyb4 expression in natural condition and in the three ABA concentrations compared with NA-type samples in natural conditions, which exhibited up-regulation of DkMyb4 in only 0.1 μM ABA (P, 0.001). In other words, A-type samples could sustain substantial DkMyb4 expression regardless of the ABA concentration, whereas NA-type samples in natural conditions showed a considerably reduced DkMyb4 expression in low ABA concentrations (0–0.1 μM); the expression was up-regulated proportional to increases in the ABA concentration.

In contrast to differences in DkMyb4 expression for fruit flesh sampled at 9 WAB, the in vitro ABA treatment on fruit flesh sampled at 1 WAB did not have significant expression changes in both A- and NA-type samples (Fig. 3A). The expression of DkMyb4 for both A- and NA-type samples was reduced on incubation with non-ABA-containing MS medium, although there was no significant difference (P > 0.05). Thus, it is suggested that in vitro ABA treatments have different effects on DkMyb4 expression only during the middle developmental stages of the A and NA genotypes. On the other hand, during the early developmental stages, only treatments inducing a reduction in ABA concentration seem to affect DkMyb4 expression. These results clearly correspond to the in vivo results, as described (Fig. 2A).

Identification of ABF-, AREB-, and ABI5-Like bZIP TFs Expresssed in Persimmon Fruit

PCR analyses were performed to identify bZIP TF candidates that control both DkMyb4 expression and PA biosynthesis using ABA signals. The analyses involved the use of degenerate primers that target a conserved bZIP domain and cDNA that was synthesized from cv Kuramitsu fruit flesh. Sampling occurred at four points during the fruit developmental stages (3, 5, 7, and 9 WAB). A partial sequence of DkZIP1 was identified, which was confirmed by BLAST searches to be homologous to the Arabidopsis bZIP TF GFB4. Approximately 300,000 recombinant clones were screened from the cDNA libraries, which were derived from cv Kuramitsu fruit flesh under low-stringency hybridized conditions, using the bZIP domain sequence of partial DkZIP1 as a probe. This screening yielded 22 bZIP TF homolog clones. Their sequence alignments resulted in seven cDNA sequences, DkbZIP1 to -7. The alignments suggest that a putative functional full-length DkbZIP5 was isolated. However, the other six DkbZIP sequences were not isolated as canonical architectures that missed at least one of the three or four conserved C-subdomains (Wasilewska et al., 2008). The phylogenetic tree constructed with an alignment of deduced amino acid sequences showed that tentative partial sequences of DkbZIP2, DkbZIP3, and full-length DkbZIP5 were included in group A of the bZIP TFs (Jakoby et al., 2002). This group is composed of bZIP TF subclades that recognize the ABRE cis-motifs involved in ABA signaling (Fig. 4). Full-length DkbZIP5 holds one conserved BZIP domain, which is required for binding to target promoters. Each individual Ser or Thr residue, within the three conserved C-subdomains, is a phosphorylation target required for bZIP TF activation (Supplemental Fig. S3; Choi et al., 2005; Wasilewska et al., 2008). Together, the alignment results suggest that DkbZIP5 contains all conserved domains and the residues are putatively responsible for the transcriptional activation of downstream genes.

Expression Analysis of ABF-, AREB-, and ABI5-Like bZIP TFs in Persimmon

The expression levels of DkbZIP2, DkbZIP3, and DkbZIP5 in fruit flesh from the described Atf line
individuals show no significant differences between the A and NA types (Fig. 5A). These three genes generally showed constant expression during the developmental stages of the fruits, except for just after anthesis (1 WAB), in which all three genes showed remarkably higher expression levels than those from other sampling points (3–13 WAB; Fig. 5A). Only DkbZIP5 expression was reduced at 13 WAB, when PA accumulation was terminated in A-type fruit flesh (Fig. 5A). Furthermore, DkbZIP2 and DkbZIP3 tended to have high expression levels, especially in young leaves (Fig. 5B), whereas DkbZIP5 showed high expression levels in both fruit flesh and seed. DkMyb4 was highly expressed in fruit flesh and seed compared with other plant organs (Akagi et al., 2009b), although it was expressed in any analyzed organ to some extent.

In the above-described ABA/abamine-treated samples (Fig. 2), no significant activation in DkbZIP2, DkbZIP3 (data not shown), and DkbZIP5 expression was detected (Supplemental Fig. S2D). This does not mean that these bZIP TFs are not ABA signaling genes, because the activation of ABRE cis-elements by ABA signals seems to be induced not because of bZIP TF transcript activation but because of bZIP TF phosphorylation signaling (for details, see “Discussion”).

Overexpression of DkbZIP5 Induces DkMyb4 Expression with a Resultant PA Accumulation in Transgenic Persimmon Callus

To identify the molecular function and test the effect of DkbZIP5 overexpression, persimmon (cv Fujiwaragosho) was transformed using cauliflower mosaic virus (CaMV) 35S-DkbZIP5, since normal persimmon calluses show a considerably lower expression of DkbZIP5. Only three transgenic calluses could be retrieved, because most transgenic lines turned black and died before development. Instances of transformation resulted in Myb TF-induced PA accumulation together with DkMyb2 or DkMyb4 expression (Akagi et al., 2009b; 2010a). The three surviving transgenic lines that showed significant DkbZIP5 expression also died before full development. Therefore, this study could not examine gene regulation and PA biosynthesis induction by the presence of DkbZIP5 in developing calluses.

Regenerated calluses that were transformed via CaMV35S-DkbZIP5, herein named the SbZ5 lines, showed an increase in PA accumulation in the surface cells when stained with p-dimethylamino-cinnamaldehyde (DMACA; Li et al., 1996), whereas the four control lines that had been transformed with an empty vector consistently showed lower PA accumulation (Fig. 6A). The same tendencies were observed with concentrations of total PA by performing HPLC analysis following acid catalysis in the presence of excess phloroglucinol (Kennedy and Jones, 2001; Akagi et al., 2010b; Fig. 6B). Most of the main components of PA in persimmon calluses (Akagi et al., 2009a) showed significant increases in concentration (Fig. 6B; P < 0.01 for gallocatechin, epicatechin, epicatechin-gallate, EGC, and EGC-G); only catechin did not show a significant change in the three transgenic SbZ5 lines (P > 0.1). DkMyb4 expression in the SbZ5 line calluses that clearly showed DkbZIP5 overexpression was increased to levels as high as those in A-type fruit (cv Kuramitsu) sampled at 9 WAB (Fig. 6C). Correlating with the up-regulation of DkMyb4, DkANR and DkF3'5'H showed significant up-regulation in the SbZ5 lines (Fig. 6C). Other flavonoid pathway genes regulated by DkMyb4 (Akagi et al., 2009b) showed a similar tendency toward DkANR and DkF3'5'H expres-
sion, whereas the expression of \( DkF3'H \) and \( DkLAR \), which are not regulated by \( DkMyb4 \), showed no significant changes (Supplemental Fig. S4). This corresponds with the PA composition results (Fig. 6B), since the biosynthesis of catechin requires the expression of both \( F3'H \) and \( LAR \). These results suggest that the up-regulation of PA pathway genes from the overexpression of \( DkbZIP5 \) was mediated by the activation of \( DkMyb4 \) expression. In contrast to the expression of \( DkMyb4 \), expression of another Myb TF that regulates PA biosynthesis in persimmon, \( DkMyb2 \), a putative ortholog of \( TT2 \) in Arabidopsis (Akagi et al., 2010a), was not significantly affected in the SbZ5 lines (Fig. 6C). These changes in expression and PA accumulation correspond to persimmon fruit having undergone ABA treatment (Fig. 2).

### Identification of a Target cis-Motif of DkbZIP5

To test whether DkbZIP5 can act as an ABRE-binding protein together with other bZIP TFs involved in ABA signaling, we performed an electrophoretic mobility shift assay (EMSA) with the recombinant GST-DkbZIP5 fusion protein expressed in *Escherichia coli* (see “Materials and Methods”). EMSAs were performed using \( DkMyb4 \) promoter region sequences that had been isolated from cv Kuramitsu along with the GST-DkbZIP5 fusion protein. The \( DkMyb4 \) promoter region contains 1,874 bp with three ABRE-like cis-motifs (Fig. 7A; motifs containing ACGTG/ACGYG sequences [Simpson et al., 2003; Kaplan et al., 2006; Nakashima et al., 2006]) with the PLACE database (Higo et al., 1999). These ABREs were conserved in the \( DkMyb4 \) promoter regions of both the A and NA types. Any mutated sequence specific to the NA type was not observed in the promoter region (data not shown).

The purified GST-DkbZIP5 fusion protein was examined via EMSA for its ability to bind to an oligonucleotide containing the ABRE cis-motifs. To analyze the specificity of the cis-motif-binding activity, we used nonlabeled mutated oligonucleotides and nonlabeled normal oligonucleotides as probe inhibitors. The mutated oligonucleotides contained two point mutations in the ABRE cis-motifs, as shown in Figure 7B. The GST-DkbZIP5 fusion protein bound to a 37-bp oligonucleotide fragment containing the ABRE motif located 447 bp upstream of the \( DkMyb4 \) start codon, at pH 6.5 (Fig. 7C). The nonlabeled oligonucleotides containing the normal ABRE motif competed with the labeled ABRE probe and reduced the binding signal between GST-DkbZIP5 and ABRE probes in proportion to its concentration. However, a quantity of nonlabeled oligonucleotides containing the mutated ABRE motif did not significantly reduce the binding signal. The inability of GST-DkbZIP5 to bind to oligonucleotides that contain the mutated ABRE motif suggests that DkbZIP5 recognizes the ABRE cis-motif in the 37-bp probe sequences.

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Figure 5. qRT-PCR analysis of the expression levels of three AREB-like DkbZIPs isolated from persimmon fruit. A, Temporal expression of DkbZIP2, DkbZIP3, and DkbZIP5 in fruits obtained from 1 WAB until 13 WAB is displayed for each of the three A- and NA-type samples from the Atf lines (Atf-14, Atf-16, and Atf-148 for A type and Atf-108, Atf-117, and Atf-145 for NA type). Mean values of the expression levels in these three individuals are provided for the A- and NA-type cultivars. The expression levels are given as values relative to those in the A type at 1 WAB, whose expression level is defined as "100." B, Expression levels of DkbZIP2, DkbZIP3, and DkbZIP5 in the stem (9 WAB), young leaf (0 WAB), mature leaf (9 WAB), calyx (9 WAB), fruit flesh (9 WAB), and seed (9 WAB) of the A-type cultivar. Mean values of the expression levels in these three individuals are given for the A- and NA-type cultivars. The expression levels are given as values relative to those in stem, whose expression level is defined as "100." Error bars indicate SD.
would introduce the novel possibility that a Myb TF involved in PA regulation is regulated by an ABRE-binding bZIP TF.

DkbZIP5 Activates the DkMyb4 Promoter in an ABA-Dependent Manner

To examine the activation of DkMyb4 by DkbZIP5 with ABA signals, a transient reporter assay with a dual luciferase system (see “Materials and Methods”) was performed in leaves of Nicotiana benthamiana. Activation of certain DkMyb4 promoter fragments (Fig. 8A) was detected with and without the overexpression of DkbZIP5 and ABA (10 or 100 μM), with standardization via Renilla luciferase gene activity (Bogs et al., 2007; Espley et al., 2007).

The whole DkMyb4 promoter was activated in the presence of ABA, without the ectopic overexpression of DkbZIP5 (approximately 2.5-fold; \( P = 0.012 \)), but the activation did not coordinate with ABA concentration (Fig. 8B). This result suggests that without DkbZIP5 expression, ABA concentrations used in this analysis were above the threshold value for the induction of DkMyb4 promoter activity in N. benthamiana leaves. On the other hand, with the ectopic overexpression of DkbZIP5, the DkMyb4 promoter seemed to be slightly activated in the absence of ABA (by approximately 2.5-fold), although it was not statistically significant (\( P > 0.05 \)). Expression was further activated in the presence of ABA, which correlated with ABA concentrations (approximately 4-fold in 10 μM ABA and 7-fold in 100 μM ABA; Fig. 8B). We could detect significant activation patterns by using the short fragments of the DkMyb4 promoter, such as ABRE located 447 bp upstream of the DkMyb4 start codon (Fig. 8A), as well as the whole promoter region (Fig. 8C; \( P < 0.01 \)). Fragments of the DkMyb4 promoter without ABRE, however, were not significantly activated with ectopic overexpression of DkbZIP5 regardless of the ABA concentration (Fig. 8C; \( P > 0.05 \)). In addition, significant differences were not detected in activation levels when using the whole DkMyb4 promoter of the A-type cv Kuramitsu and NA-
type cv Fuyu (Fig. 8C; \( P > 0.1 \)). Considering the results obtained from this transient reporter assay and the described EMSAs, it was suggested that DkbZIP5 binds to the ABRE motif located 447 bp upstream of the DkMyb4 start codon and directly regulates the expression of DkMyb4 in an ABA-dependent manner.

**DISCUSSION**

**Differences in ABA Signaling and Phenological PA Regulation for Two Persimmon Genotypes**

The treatment of ABA and ABA biosynthesis inhibitors affects the expression of genes involved in the PA regulation of NA-type persimmon fruit only during specific developmental stages (Figs. 2 and 3). Previous results demonstrate a temperature-dependent change that coincides with the endogenous ABA concentration and PA accumulation in NA-type fruit (Akagi et al., 2011). This explains why exogenous ABA and ABA inhibitors are effective only during specific periods, because the endogenous ABA concentrations are high only during early fruit development and are considerably down-regulated in the middle developmental stage (Kojima et al., 1999; Akagi et al., 2011; Supplemental Fig. S1). Thus, in the early developmental stage, ABA biosynthesis inhibitors have a distinct effect on ABA accumulation and the resultant PA regulation. However, exogenous ABA treatment would not have an apparent effect, probably because of saturation from internal ABA concentrations. On the other hand, after the middle developmental stage, we can postulate the opposite effects of ABA/abamine treatments on PA regulation. This corresponds with the results given in Figure 2. This hypothesis, however, may not explain the results of in vitro ABA treatment of 1-WAB fruits (Fig. 3A) that show no significant changes in DkMyb4 expression, regardless of ABA concentration.

Another plausible scenario is that the persimmon fruit has PA regulatory mechanisms that respond to external environmental factors, including those of exogenous ABA, in a season- and organ-specific manner. Previous reports on the phenological regulation of flavonoid biosynthesis in persimmon fruit and other tree crops, such as grapevine, would support this possibility. Artificial cool temperatures have an apparent effect on anthocyanin regulation in grape skin (Yamane et al., 2006) and on PA regulation in persimmon fruit flesh (Akagi et al., 2011) during specific developmental stages. Exogenous ABA treatment applied just before and after véraison promotes anthocyanin biosynthesis in grape skin through the up-regulation of VoMYBA1 expression (Ban et al., 2003; Jeong et al., 2004). Such ABA treatment also seems to affect PA biosynthesis in the earlier developmental stages of grape skin, with expression changes in at least two Myb TFs, VoMYB5 and VoMYBPA1 (Lacampagne et al., 2010).

Notwithstanding seasonal changes in the expression of PA regulatory genes that respond to NA-type ABA signaling, A-type fruit showed a constant expression of DkMyb4 until the end of the middle developmental stage, without any significant effects from ABA/
abamine treatments. This difference in A- and NA-type fruits would be because of the AST/ast genotypes. Considering that there are no sequence differences in the cis-motifs of DkMyb4 promoter regions among the A and NA types (data not shown) and that they have the same ability to activate via ABA signal the expression of DkbZIP5 (Fig. 8B), the following three hypotheses are postulated: functional AST (1) complements ABA signals that act as DkMyb4 regulators at low ABA concentrations, (2) can regulate the expression of DkMyb4 independent of ABA signaling, or (3) regulates some epigenetic factors on the DkMyb4 promoter regions. The first hypothesis could be supported by the results from in vitro ABA treatment (Fig. 3). This result demonstrates that the DkMyb4 expression levels in A-type fruit differ with each ABA concentration, therefore suggesting the possibility that functional AST and ABA signals share some upstream signaling pathways for the induction of the expression of DkMyb4.

**Molecular Mechanisms for DkMyb4 Promoter Activation by ABA Signal and DkbZIP5 Expression**

Our results suggest that DkbZIP5 directly regulates DkMyb4 expression, which is dependent on ABA signals. Considering the constant expression of DkbZIP5 in persimmon fruit flesh (Fig. 5; Supplemental Fig. S2D) and the ABA concentration-dependent activation of the DkMyb4 promoter under the constitutive expression of DkbZIP5 (Fig. 8), ABA signals with DkbZIP5 would activate DkMyb4 expression without transcriptional activation of DkbZIP5. Some ABF/AREB/ABI5 genes whose expression is not activated by ABA treatment have been reported to have proper functions for ABA signaling also in Arabidopsis (Suzuki et al., 2003). In addition, preliminary results from the following two sets of suppression subtractive hybridization analyses, (1) cDNA pools from 0.1 μM ABA-treated samples as the tester and nontreated (0 μM ABA) as the driver samples for A-type lines and (2) cDNA pools from 10 μM ABA-treated samples as the tester and nontreated (0 μM ABA) as the driver for NA-type lines (Fig. 3), showed no significant changes in gene expression, except for DkMyb4 expression and its downstream genes (Supplemental Table S1). Although we cannot reject the possibility that our suppression subtractive hybridization analyses did not capture all genes showing expression changes, we are led to the hypothesis that activation of the DkMyb4 promoter by ABA signals with DkbZIP5 requires no change in the expression of upstream DkMyb4 genes.

In transcript activation by ABRE-binding bZIP TFs, many reports suggest that the phosphorylation of spe-

**Figure 8.** DkbZIP5 and the ABA signal activate the DkMyb4 promoter. A, Structures of reporter constructs using the transient assay. pWA-LUC2 and pWNA-LUC contain the whole promoter region (1,874 bp) of DkMyb4 from A-type cv Kuramitsu and NA-type cv Fuyu, respectively, which comprises some single-nucleotide polymorphisms but no differences in their ABRE sequences. Solid red lines in the DkMyb4 promoter regions indicate ABRE cis-motifs. The dotted red line in pR1-LUC2 indicates mutated ABRE, whose changed nucleotides are indicated by boldface. B, Effect of ABA concentration and DkbZIP5 overexpression on the activation of the whole DkMyb4 promoter, using pWA-LUC2 as the reporter. The control indicates analysis using an empty vector as the effector without ABA treatment. C, Effect of the DkMyb4 promoter sequences (presence of each ABRE cis-motif) on activation under DkbZIP5 overexpression and the presence of 100 μM ABA. In all analyses, fold induction that was normalized by LUC2/REN (firefly luciferase activity relative to Renilla luciferase activity) is given. Each column represents the mean value of the three plants containing each of the six technical replicates, for a total of 18 replications, with error bars indicating sd. Asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$) according to Student’s $t$ test from the control (B) or the noneffector + pWA-LUC2 (C). [See online article for color version of this figure.]
specific residues in the C subdomains is indispensable (Choi et al., 2005; Hirayama and Shinozaki, 2007; Wasilewska et al., 2008). The phosphorylation/dephosphorylation of specific bZIP TF residues needs some members of the protein kinase/phosphatase family, such as calcium-dependent protein kinases (Choi et al., 2005; Yu et al., 2006) and protein phosphatase type 2C (PP2C). In addition, the ABA receptors RCARs, Pyrobactin Resistant1 (PYR), and PYRI-like that physically interact with ABA and PP2C were recently identified (Ma et al., 2009; Park et al., 2009). Other than these proteins, some components have been reported to be involved in the signal transduction between ABA and ABRE cis-motifs in several plant species (Hirayama and Shinozaki, 2007; Wasilewska et al., 2008). Referring to an EST database of persimmon fruit (Nakagawa et al., 2008), some homologs of these components are expressed in the developmental stages of the fruit, although they do not show significant changes in their expression levels not only in the developmental stages but also in A- and NA-type fruits (data not shown). The possibility was suggested that such components transducing the ABA signal to ABRE cis-motifs are involved in DkMyb4 promoter activation by DkbZIP5 in persimmon, although, in this study, we could not examine whether these components are involved in the PA regulation of different A- and NA-type fruits.

**Applicability of ABA and bZIP TFs as Regulators of PA Biosynthesis**

Our results suggest that the regulation of PA biosynthesis by ABA signaling in persimmon fruit is not because of the activation of DkMyb2 expression but of DkMyb4 expression. In addition, transgenic lines that overexpress DkbZIP5 only activate DkMyb4 expression. DkMyb2 and DkMyb4 have almost the same function as transcript regulators for many polyphenol/ flavonoid pathway genes but are apparently located in somewhat different subclades of the MYB family and recognize different cis-motifs (Akagi et al., 2010a). This suggests that ABA signals and bZIP TFs do not contribute to the activation of the general Myb TFs involved in PA biosynthesis but can induce the expression of specific Myb TFs involved in PA biosynthesis. This possibility might be applicable to other plant species. Some Arabidopsis mutants lacking functional bZIP TFs and involved in ABA signaling do not show changes in PA accumulation in the seed coat, which is regulated by a TT2 Myb TF that is not located on the suboclade containing DkMyb4 (Akagi et al., 2010a). In contrast, in grapevine, ABA treatment apparently affects the expression of Myb TFs that are involved in PA biosynthesis, such as VvMYBPA1 (Lacampagne et al., 2010), located on the same suboclade as DkMyb4 (Akagi et al., 2010a). Thus, it would be important to characterize the impact of Myb TFs on ABA signaling if the application of ABA and related bZIP TFs for the modification of PA accumulation is to be considered in other plant species.

**Conclusion**

As a factor providing a clue to the complex accumulation of PA in persimmon fruit, we found that ABA signaling can contribute to seasonal DkMyb4 expression and the resultant PA biosynthesis. This mechanism acts differentially depending on the astrignency genotype (A or NA type) and fruit development stage, suggesting that the AST locus, which controls the A/NA genotype, and/or some other environmental factors interact with ABA signaling in the PA biosynthetic process in persimmon fruit. We identified DkbZIP5, which is highly expressed in persimmon fruit, as one of the AREBs activating DkMyb4. Molecular characterization with EMSA and the transient reporter assay suggest that DkbZIP5 recognizes ABRE in the promoter region of DkMyb4 and acts as its transcript regulator in an ABA-dependent manner. The same holds true for AREBs in other plant species. This suggests that ABA signals affect seasonal PA biosynthesis in persimmon fruit via the activation of DkbZIP5 and also provide the ability for the modification of PA accumulation in fruits by controlling environmental conditions.

**MATERIALS AND METHODS**

**Application of ABA to Persimmon Fruit in Plants**

To exploit the possibility that a modification of the ABA concentration in persimmon (Diospyros kaki) fruit affects PA regulation, fruit flesh and calyx parts of cv. Kuramitsu (A type), Okugosho (NA type), and Suruga (NA type) were treated with Miyobi, which is a fertilizer formulated from 8.0%, 5.0%, 0.90%, 0.05%, 0.03%, and 10.0% (w/w) potassium, phosphorus, magnesium, boron, manganese, and S-ABA (natural-type ABA), respectively. This fertilizer was sprayed at a concentration of 4,000 μL L⁻¹ (400 μL L⁻¹ [approximately 1.5 μM] S-ABA). In addition, fruits and leaves of these three cultivars were sprayed with abamine, which is a competitive inhibitor of 9-cis-epoxycarotenoid dioxygenase (Kitahata et al., 2006), at a concentration of 100 μM to inhibit ABA biosynthesis in fruit flesh. These treatments were performed at 3-d intervals throughout the experiment, starting at 1 WAB, in the experimental orchard of Kyoto University. Three fruits of each cultivar were sampled in 2010 at 1, 3, 5, 7, 9, and 13 WAB, frozen with liquid nitrogen, and stored at −80°C until analysis. Full bloom was determined as the time when 80% of cv. Suruga flower buds had bloomed. The full bloom time of all three cultivars was almost the same in 2010.

**In Vitro ABA Treatment of Persimmon Fruit Flesh**

Experiments were performed using each of the three A- and NA-type samples from the Atf lines (Akagi et al., 2009b; Atf-14, Atf-16, and Atf-148 for A type; Atf-108, Atf-117, and Atf-145 for NA type). The fruits were sampled in 2009 at 1, 3, 5, 7, 9, and 13 WAB. The samples collected at 1, 7, and 13 WAB were used for in vitro ABA treatment. All samples were immediately peeled and diced on a sterile bench into small pieces (approximately 1 × 0.5 × 0.5 cm); the seeds were then removed. The 12 to 15 pieces obtained from each fruit were divided between four 50-mL aliquots of liquid MS medium (pH 5.6–5.8), each containing a different concentration (0, 0.1, 1, and 10 μM) of (+)-ABA (Sigma). After 16 h of incubation, the fruit pieces were stored at −80°C until RNA extraction was performed. Two biological replicates were used for each ABA concentration.

**Analysis of PA Contents and Composition**

Soluble PAs were extracted from 10 mg of finely ground freeze-dried material in 1 mL of 70% acetone containing 0.1% ascorbic acid for 24 h at room temperature. The PA subunit composition was analyzed by HPLC following...
acid catalysis in the presence of excess chlorogluconol (Kennedy and Jones, 2001), as described by Akagi et al. (2010b). Samples were analyzed using reverse-phase HPLC (LC2010; Shimadzu) on the Wakosil-II 5C18 RS analytical column (4.6 × 250 mm).

Isolation of bZIP TFs Expressed in Persimmon Fruit

Degenerate PCR primers were designed to isolate major TFs related to ABA signaling, the bZIP TFs (ABF/ABI5/AREB family). The aligned sequences of the genes in Arabidopsis (Arabidopsis thaliana), maize (Zea mays), rice (Oryza sativa), wheat (Triticum aestivum), and apple (Malus × domestica) allowed the amplification of fragments of the corresponding genes in persimmon. The full-length sequences of these genes were obtained by screening the cDNA libraries of cv Kuramitsu. The cDNA libraries were constructed using the SMART cDNA Library Construction Kit (Clontech) and 2.0 µg of total RNA from cv Kuramitsu sampled in 2007 at 5 or 7 WAB. Hyridization temperature was set within a range of 33°C to 65°C to isolate heterogeneous orthologs.

Expression Analysis

Total RNA was isolated from each frozen organ by the hot-borate method (Wan and Winkins, 1994). cDNA was synthesized from 1 µg of total RNA using SuperScriptIII transcriptase (Invitrogen) and an adaptor primer. Expression analysis was performed by quantitative real-time (qRT)-PCR analysis. Primer pairs designed for amplification were based on the conserved sequences of target genes as used in previous reports (Akagi et al., 2009b). Clones containing the homologous sequences of the genes in Arabidopsis (Arabidopsis thaliana), wheat (Triticum aestivum), and apple (Malus × domestica) allowed the amplification of fragments of the corresponding genes in persimmon.

To express the GST-tagged recombinant DbkZIP5 protein in Escherichia coli, the DbkZIP5 sense strand was cloned into the vector pDEST 15 (Invitrogen) using pENTR-DbkZIP5 and the Gateway cloning system with LR Clonase (Invitrogen), as described above. The resultant constructs were transformed into E. coli (BL21) cells. Extraction and purification of the expressed recombinant protein were performed according to the methods reported previously by Urao et al. (1993) and Akagi et al. (2010a).

Oligonucleotide probes containing ABREs were labeled with digoxigenin (Dig)-2'-3'-dideoxyuridine-5'-triphosphate using the DIG Oligonucleotide 3'-End Labeling Kit (second generation; Roche). The DNA-binding reaction was allowed to proceed for 20 min at 25°C in 20 µL of binding buffer (5% glycerol, 4 mM KCl, 5 mM MgCl2, 1 mM EDTA, and 25 mM HEPEs/KOH) at pH 6.5 to 8.5, according to a previous report by Urao et al. (1993). The reaction mixture contained 4 ng of the DIG-labeled oligonucleotide probe and 1,000 ng of purified GST fusion proteins. Competition experiments were performed by adding an unlabeled competitor oligonucleotide at a 150-fold excess versus the DIG-labeled oligonucleotide probe according to the methods reported previously by Akagi et al. (2010a).

Transient Transformation and Dual Luciferase Assay

The pGW82-DkbZIP5 vector described above was used as an effector. To construct the reporter vector, fragments of the 1,874-bp DkMyb4 promoter region were amplified by PCR using PrimeSTAR HS (Takara), genomic DNA from A-type cv Kuramitsu and NA-type cv Fujiwara as templates, and specific primer sets (Supplemental Table S2). The generated PCR fragments were subcloned into the entry vector pENTR/D-TOPO (Invitrogen). Finally, using LR Clonase (Invitrogen), the DkbZIP4 promoter regions were cloned into pGW835, in which the firefly luciferase gene was controlled by the cloned promoter (Nakagawa et al., 2007). pGW82-LucRen, an internal standard vector in which the Renilla luciferase gene was controlled by the CaMV35S promoter, was used according to the study by Akagi et al. (2010a).

Nicotiana benthamiana plants grown under glasshouse conditions were used for infiltration with Agrobacterium. Throughout the experiment, plants were maintained under the constant condition of 22°C to 25°C in natural light. Agrobacterium infiltration and the dual-luciferase assay were performed according to the methods described by Espley et al. (2007) and Akagi et al. (2010b). Light emission was detected using the chemilumino analyzer LAS-3000 mini and semiquantified using Multi Gauge software (Fujifilm). Relative luciferase activity was calculated as the ratio between the firefly and Renilla (standard) luciferase activities. To test the effect of ABA on the induction of the DkbZIP4 promoter, N. benthamiana leaves were sprayed with varied concentrations of (+)ABA immediately after infiltration with recombinant Agrobacterium. Each plant was treated with approximately 20 mL of ABA solution at concentrations of 0 (without ABA), 10, and 100 µM.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: DbkPAL (AB472364), DbkCHS (AB472365), DbkCHI (AB472367), DbkF3H (AB472368), DbkF5' H (AB472369), DbkDFR (AB472371), DbkANS (AB472377), DkLAR (AB472361), DkKIN (AB95284), DkMyb1 (AB503698), DkMyb2 (AB503699), DkMyb3 (AB503700), DkMyb4 (AB503701), DkZIP2 (AB673863), DkZIP5 (AB670684), DkZIP5 (AB670685), and DkActin (AB473616).

Supplemental Data

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

Supplemental Figure S1. Endogenous ABA concentration in fruit flesh of A- and NA-type cultivars.

Supplemental Figure S2. Expression patterns of DkbZIP1, DkbZIP2, DkbZIP3, and DkbZIP5 in ABA/abamine treatments.

Supplemental Figure S3. Sequence alignment and conserved subdomains of ZIP-5-TFs classified to AREB.

Supplemental Figure S4. Expression levels of PA pathway genes in the Sh25 lines.

Supplemental Table S1. Results of the suppression subtractive hybridization analysis for in vitro ABA treatments.

Supplemental Table S2. Primer sequences for the transient reporter assay.
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