Seasonal Abscisic Acid Signal and a Basic Leucine Zipper Transcription Factor, DkbZIP5, Regulate Proanthocyanidin Biosynthesis in Persimmon Fruit[1][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; Mellway 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]; Mellway et al., 2009), and grapevine (*Vitis vinifera* [VvMYBPA1 {Bogs et al., 2007} and VvMYB5a and VvMYB5b {Deluc

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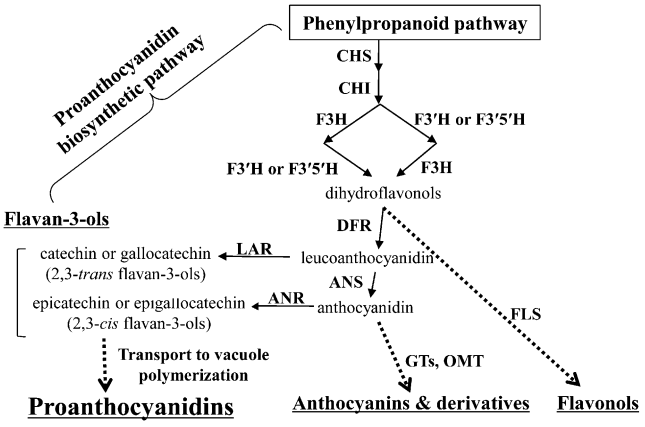

Figure 1. Representative scheme of the PA biosynthetic pathway. CHS, Chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′5′-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; GTs, glycosyltransferases; OMT, *O*-methyltransferase.

et al., 2008}; VvMYBPA2 {Terrier et al., 2009}]). Despite these findings on basic PA regulatory mechanisms, few reports have provided insight into the molecular basis for the phenological response of Myb TFs to internal or external environments.

In general, the biosynthesis of flavonoids, including PAs, is highly responsive to environmental conditions and can be regulated by seasonal signals. This is important because the phenological expression of some flavonoid-derived traits also affects the quality of commercial crops. Early progress has been made in the study of the phenological regulation of anthocyanin biosynthesis, which plays a role in the coloration of Rosaceae and *Vitis* species by regulating the expression of Myb TFs (Kobayashi, 2009; Lin-Wang et al., 2010) in response to seasonal environmental factors, such as light (Takos et al., 2006; Koyama and Goto-Yamamoto, 2008; Matus et al., 2009) or temperature, coincident with abscisic acid (ABA) concentration (Spayd et al., 2002; Yamane et al., 2006). Although the responses of anthocyanin accumulation to environmental conditions have been discussed, the fundamental mechanisms that regulate the expression of Myb TFs in response to environmental factors need to be further elucidated. In contrast to the progress in the study of anthocyanin biosynthesis regulation, few reports have demonstrated the mechanisms that regulate PA biosynthesis in response to environmental factors, despite the fact that PA and anthocyanin pathways share almost all enzymatic genes. The seasonal expression patterns of three Myb TFs (VvMYBPA1, VvMYB5a, and VvMYB5b) and their response to light conditions were demonstrated in grape skin and seeds (Bogs et al., 2007; Gagné et al., 2009; Matus et al., 2009; Terrier et al., 2009). In general, certain biotic and abiotic stresses affect *MYB134* expression levels in leaves of certain plants (Mellway et al., 2009). However, the signaling pathways involved in these responses remain to be elucidated.

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_r 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 *C1* Myb TF, which is a flavonoid regulator in maize (*Zea mays*). Hattori et al. (1992) demonstrated that *C1* expression and the resultant anthocyanin biosynthesis in seeds are regu-

lated by ABA signals and the ABA-regulated *Viviparous1* TF. To date, a large number of ABA signaling components have been identified (Hirayama and Shinozaki, 2007; Wasilewska 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; Wasilewska 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 μM 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-

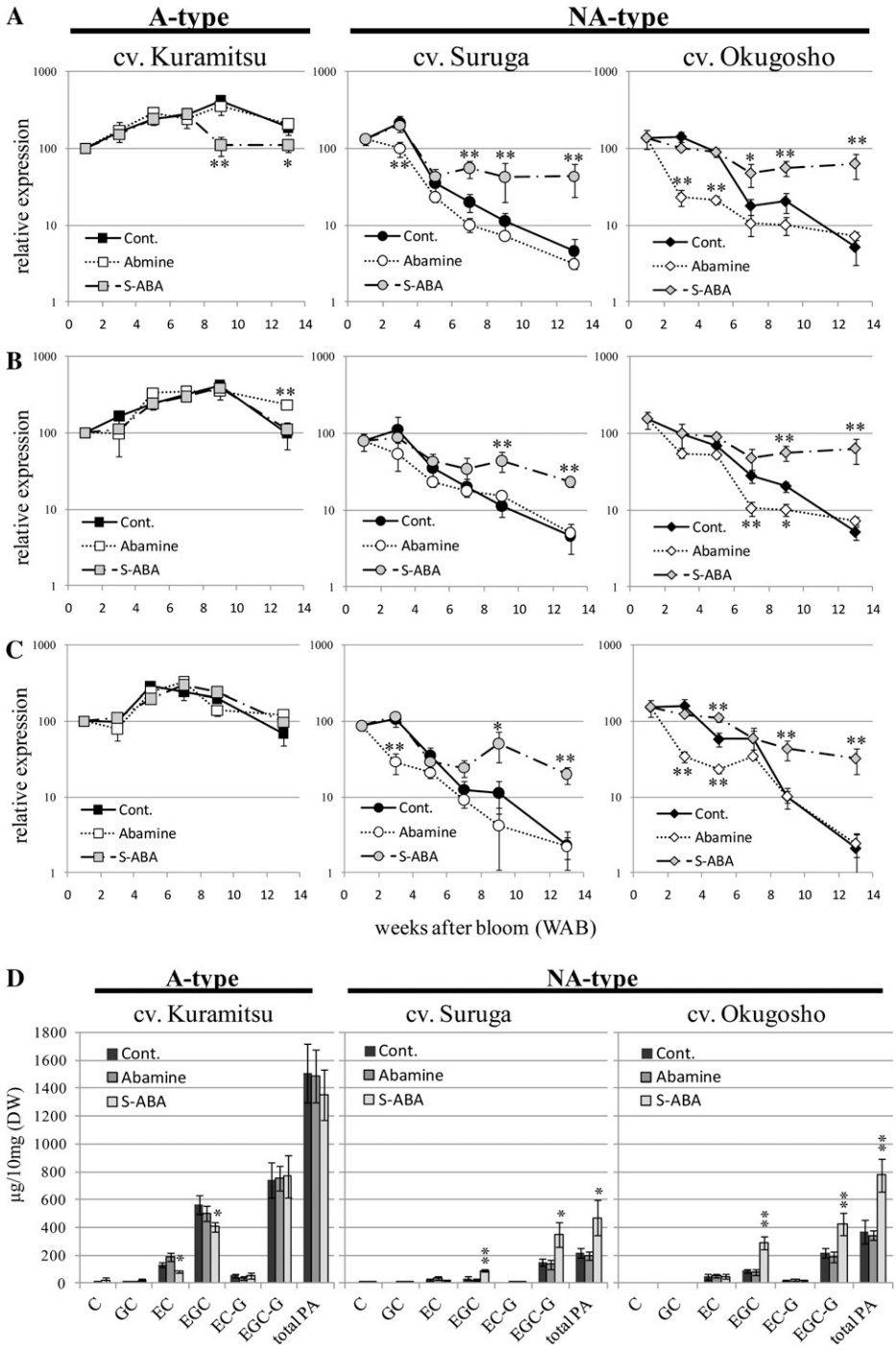

Figure 2. ABA treatment affects the expression patterns of genes regulating PA biosynthesis and PA concentration in persimmon. A to C, qRT-PCR analysis of gene expression in the control fruit and fruits treated with abamine and S-ABA. A, *DkMyb4* expression. B, *DkANR* expression. C, *DkF3′5′H* expression. 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 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), gallocatechin (GC), epicatechin (EC), EGC, 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.

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 A- and NA-type samples were sampled in vitro for PA accumulation at approximately 1 WAB, at which both types express the same level of *DkMyb4*, and at 9 WAB, at which different levels of *DkMyb4* are expressed, along with the resultant PA accumulation. The results are summarized in Figure 3. Incubation of six Aft line samples that were obtained at 9 WAB in the MS medium containing no ABA (0 µM) and 0.1 µM ABA resulted in *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

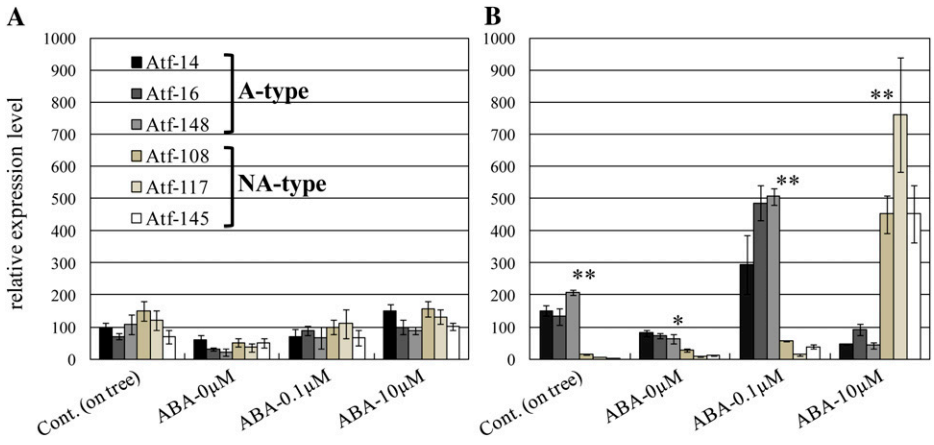

Figure 3. In vitro ABA treatment affects *DkMyb4* expression differentially in A- and NA-type cultivars. Relative *DkMyb4* expression levels in the control fruit (immediately after sampling) and fruits treated with 0, 0.1, and 10 μM ABA are shown. Each of the three A- and NA-type samples from the Atf lines are displayed: 1 WAB (A) and 9 WAB (B). Fruits from three A-type (Atf-14, Atf-16, and Atf-148) and NA-type (Atf-108, Atf-117, and Atf-145) samples from the Atf lines were independently used for expression analysis. The expression levels are given as values relative to those in Atf-14 in the control at 1 WAB, whose expression level is defined as "100." Error bars indicate SD ($n = 3$). Asterisk indicate significant differences (* $P < 0.05$, ** $P < 0.01$) according to Student's *t* test among the A- and NA-type individuals. [See online article for color version of this figure.]

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 Expressed 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 *GBF4*. 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 putative 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

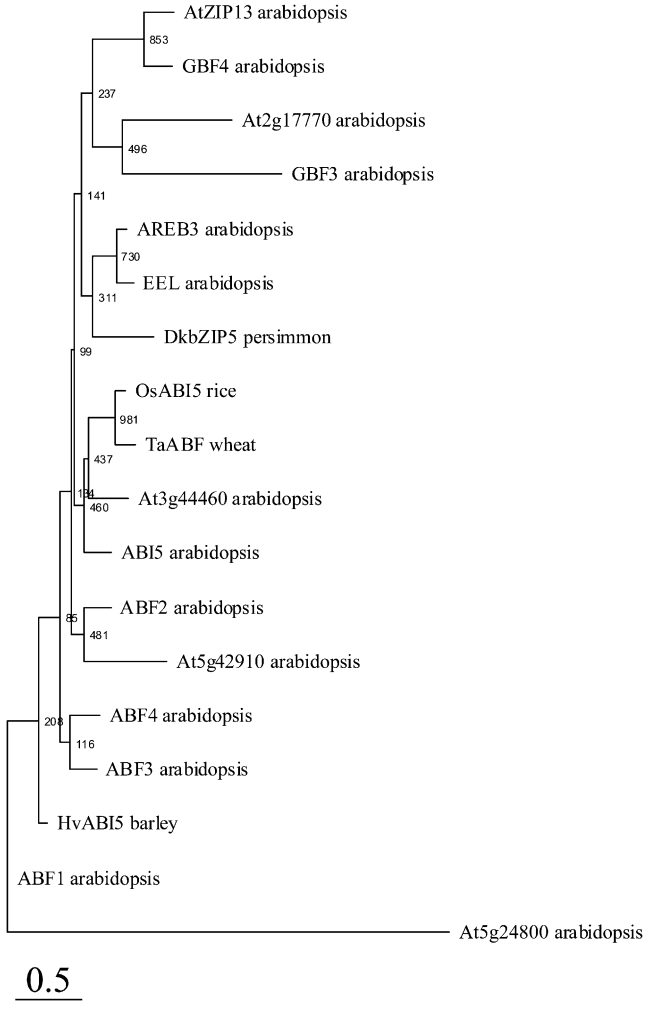

Figure 4. The gene tree of bZIP TFs is organized according to ABRE-binding proteins (AREB). AREB-like bZIP TFs in Arabidopsis selected according to previous reports (Jakoby et al., 2002; Suzuki et al., 2003), AREB-like bZIP TFs in other crops studied previously, and DkbZIP5 were used for constructing the tree. At5g24800, which is located on the subclade adjacent to Arabidopsis AREBs (Suzuki et al., 2003), was used as an outgroup gene. The tree is based on the alignment of each full-length bZIP TF using the ClustalW multiple sequence alignment system (Thompson et al., 1997) and the default parameters of the DNA Data Bank of Japan. The tree is constructed using the neighbor-joining method of TreeView (Page, 1996). The scale bar represents 0.5 substitutions per site, and the numbers next to the nodes are bootstrap values from 1,000 replicates. GenBank accession numbers for bZIP TFs in persimmon are provided in "Materials and Methods."

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-

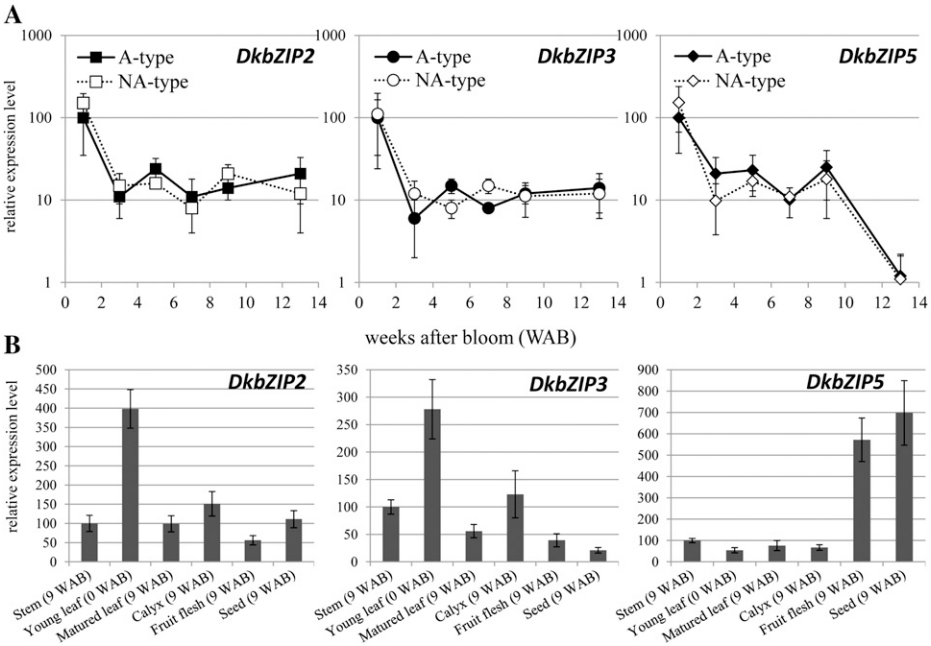

Figure 5. qRT-PCR analysis of the expression levels of three AREB-like *DkbZIPs* isolated from persimmon fruit. A, Temporal expression of *DkbZIP2*, *DkbZIP3*, and *DkbZIP5* in fruits obtained from 1 WAB until 13 WAB is displayed for each of the three A- and NA-type samples from the Atf lines (Atf-14, 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.

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 upregulation 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. It

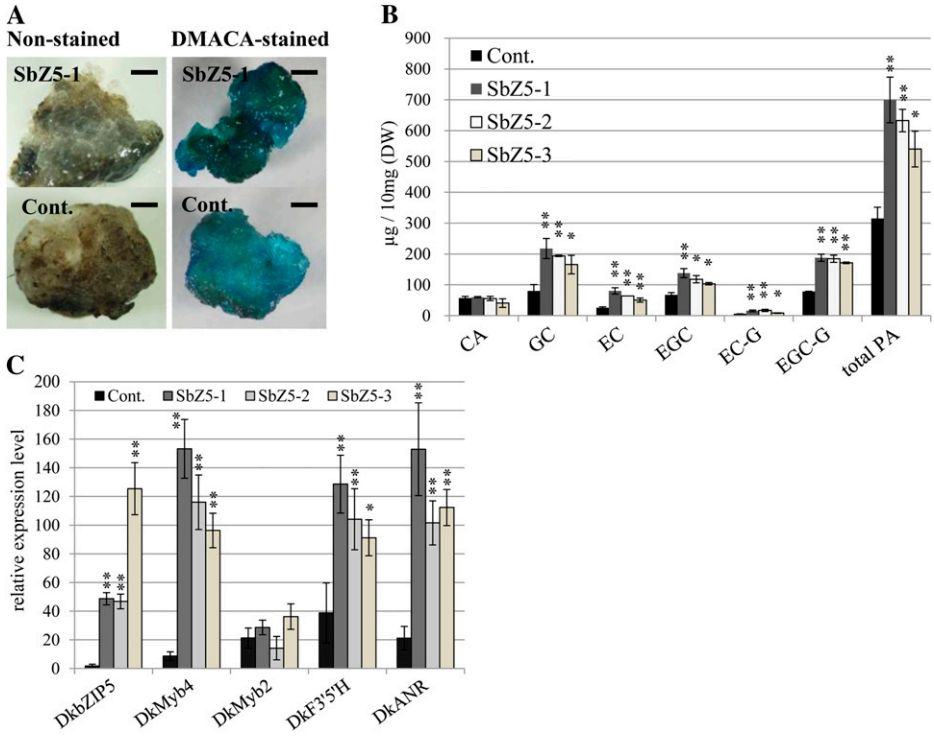

Figure 6. Functional analysis of DkbZIP5 with persimmon transformation. Some of the persimmon calluses in which sense DkbZIP5 had been introduced were regenerated and designated SbZ5 lines. A, Calluses of SbZ5-1, one of the SbZ5 lines, and control calluses at 6 months after infection of leaves with *Agrobacterium* (left) and stained with DMACA (right). DMACA staining of the SbZ5-1 calluses tended to have a deep blue color that differed from the control calluses, which indicates a higher PA concentration in the surface cells of the SbZ5-1 calluses. Bars = 2 mm. B, Concentration of each PA component and total PA in the calluses of three SbZ5 and control lines. Error bars indicate SD (n = 4–6). Abbreviations are as in Figure 2D. C, qRT-PCR analysis of gene expression in the calluses of the SbZ5 and control lines. *DkbZIP5*, *DkMyb4*, *DkMyb2*, and two of the structural genes of the PA biosynthetic pathway is given. Expression levels of other structural genes of the PA pathway are shown in Supplemental Figure S4. The expression levels are given as values relative to those in fruit flesh of cv Kuramitsu sampled at 9 WAB, whose expression level is defined as "100." Using qRT-PCR, we analyzed each of the four individuals for all transgenic lines in three technical replicates. Error bars indicate SD (n = 4 for biological replicates). In all figures, asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$) according to Student's t test, in comparison with the control lines. [See online article for color version of this figure.]

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-

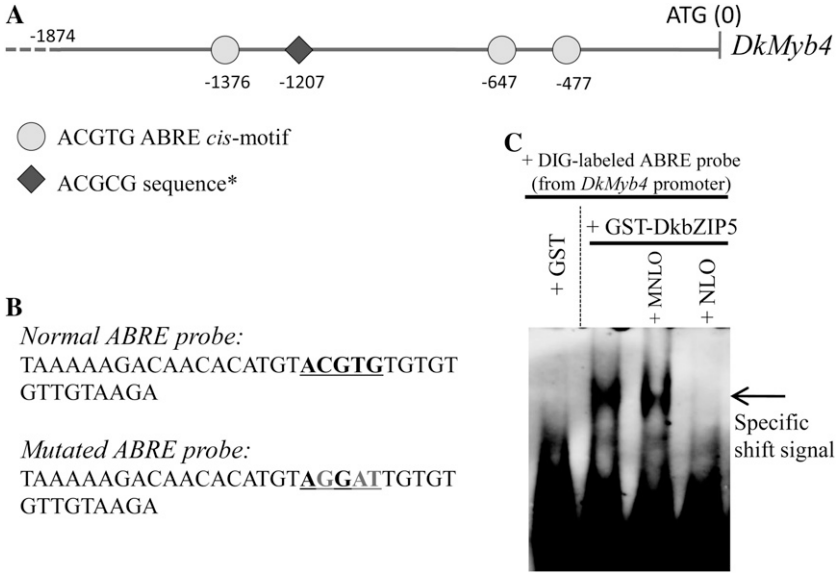

Figure 7. ABRE cis-motifs in the *DkMyb4* promoter region in persimmon (cv Kuramitsu) and the ability of the DkbZIP5 fusion protein to bind to them. A, ABRE (ACGTG) motifs in the *DkMyb4* promoter region detected using the PLACE database (Higo et al., 1999). * The sequence of ACGCG, another possible ABRE motif, is also given. B, Sequences of oligonucleotides used in EMSA. The ABRE motif from the *DkMyb4* promoter region is shown as boldface and underlined nucleotides. The mutated oligonucleotides are shown in red (ACGTG to AGGAT in the ABRE motif). C, EMSA performed using the GST-DkbZIP5 fusion protein and ABRE probe derived from the *DkMyb4* promoter region. The binding signals are below the detection level with 150-fold excess nonlabeled oligonucleotide (NLO) of the ABRE motif in comparison with the DIG-labeled oligonucleotide probe; however, the 150-fold excess mutated nonlabeled oligonucleotide (MNLO) of the ABRE motif does not significantly decrease the binding signal.

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 *VvMYBA1* 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, *VvMYB5* and *VvMYBPA1* (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/

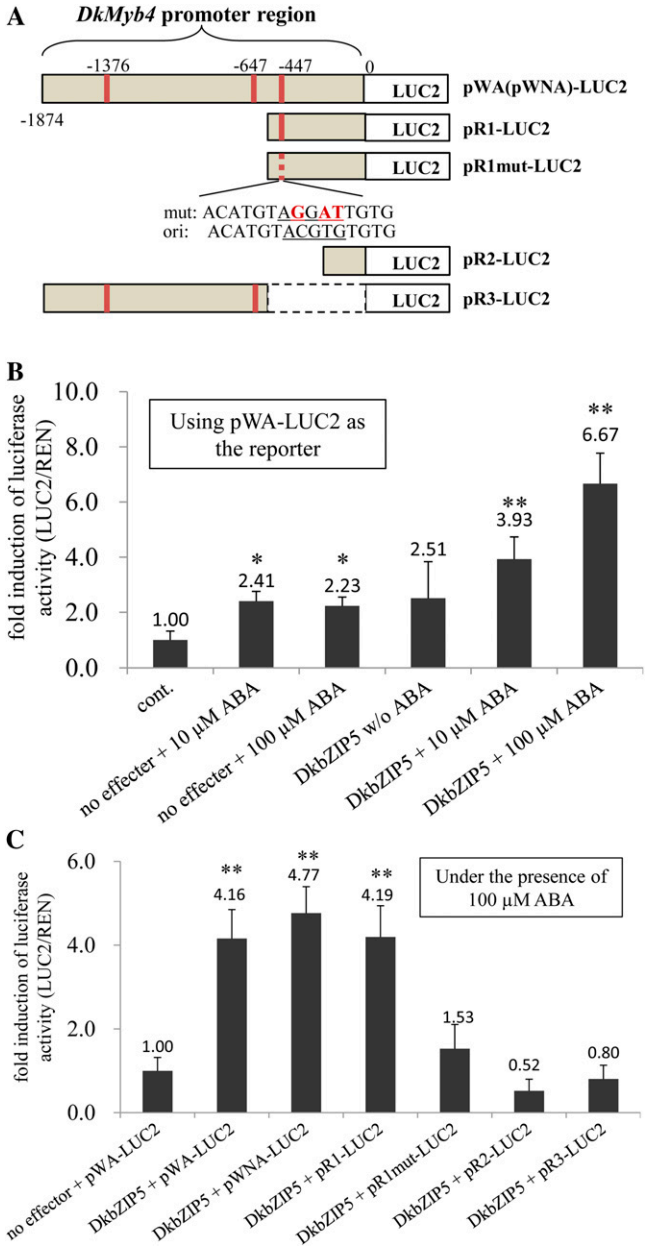

Figure 8. DkbZIP5 and the ABA signal activate the *DkMyb4* promoter. A, Structures of reporter constructs using the transient assay. pWA-LUC2 and pWNA-LUC contain the whole promoter region (1,874 bp) of *DkMyb4* from A-type cv Kuramitsu and NA-type cv Fuyu, respectively, which comprises some single-nucleotide 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.]

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-

cific 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 PYR1-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 phenylpropanoid/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 subclade 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 subclade 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 astringency 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 Planta

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 mM] 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 phloroglucinol (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. Hybridization temperature was set within a range of 53°C to 65°C to isolate heterogenous orthologs.

Expression Analysis

Total RNA was isolated from each frozen organ by the hot-borate method (Wan and Wilkins, 1994). cDNA was synthesized from 1 μg of total RNA using SuperScriptIII transcriptase (Invitrogen) and an adopter 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., 2009a, 2009c, 2010). An aliquot of 1:5 diluted pool cDNA was used as a template for PCR. Expression levels were assayed using ABI Prism 7900HT (Applied Biosystems) with the SYBR Green system and SYBR Premix Ex-Taq (Takara). Three independent measurements were performed, and the mean value with appropriate SD was calculated for the expression data from each of the three technical replicates. Standard curves for target genes and the housekeeping gene *DkActin* were obtained by the amplification of a serially diluted mixture of cDNA samples with six dilution points.

DkbZIP5 Overexpression in Persimmon Callus Cells

The full-length open reading frame (ORF) of *DkbZIP5* from persimmon (cv Kuramitsu) cDNA derived from young fruit flesh sampled in 2009 at 7 WAB was PCR amplified using PrimeSTAR HS (Takara) polymerase with the primer sets DkbZIP5-startF-TOPO (5′-**CACC**ATGGGGTTTCAAACCATGG-3′) and DkbZIP5-stopR (5′-TCAGAATGAAGTTGTTCTTCGTAGCT-3′). The sequence shown in boldface, CACC, is an additional sequence necessary for directional cloning in the following Gateway system. The generated PCR fragments were subcloned into the entry vector pENTR/D-TOPO (Invitrogen) to create the pENTR-DkbZIP5 vector using the TOPO cloning procedure according to the manufacturer's instructions. Using the Gateway cloning system with LR Clonase (Invitrogen), we finally cloned the DkbZIP5 ORF into pGWB2 (Nakagawa et al., 2007) to generate pGWB2-DkbZIP5, in which sense DkbZIP5 was controlled by the CaMV35S promoter. Resultant constructs were transformed into the *Agrobacterium tumefaciens* strain C58C1 by electroporation. Transformation of persimmon (cv Fujiwaragosho) was performed using *Agrobacterium* according to the methods reported previously by Tao et al. (1997). Leaf fragments were infected with the transformed *Agrobacterium* to regenerate callus tissue on solidified MS medium containing kanamycin (50 mg L^{-1}) as the selective antibiotic.

EMSAs

The genomic fosmid library derived from *Diospyros lotus*, which is one of the closest diploid relatives of hexaploid persimmon, was constructed as reported previously (Akagi et al., 2009b). Clones containing the homologous ORF of *DkMyb4* were screened from the library, and the *DkMyb4* promoter region sequence was determined. The homologous promoter regions in persimmon (cv Kuramitsu) were amplified using specific primers and genomic DNA. The regions were directly sequenced using Exo-Sap IT (GE Healthcare Bio-Sciences) and CEQ8000 version 7.0 (Beckman Coulter). Consensus sequences among the alleles were used for detecting ABREs using the PLACE database (Higo et al., 1999).

To express the GST-tagged recombinant DkbZIP5 protein in *Escherichia coli*, the *DkbZIP5* sense strand was cloned into the vector pDEST 15 (Invitrogen) using pENTR-DkbZIP5 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 $MgCl_2$, 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 pGWB2-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 Fuyu 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 *DkMyb4* promoter regions were cloned into pGWB35, in which the firefly luciferase gene was controlled by the cloned promoter (Nakagawa et al., 2007). pGWB2-Luc*Ren*, 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 23°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. (2010a). 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 *DkMyb4* 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: DkPAL (AB472364), DkCHS (AB472365), DkCHI (AB472367), DkF3H (AB472368), DkF3′H (AB472369), DkF3′5′H (AB472676), DkDFR (AB472371), DkANS (AB472677), DkLAR (AB472681), DkANR (AB195284), DkMyb1 (AB503698), DkMyb2 (AB503699), DkMyb3 (AB503700), DkMyb4 (AB503701), DkbZIP2 (AB670683), DkbZIP3 (AB670684), DkbZIP5 (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 *DkMyb1*, *DkMyb2*, *DkMyb3*, and *DkbZIP5* in ABA/abamine treatments.

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

Supplemental Figure S4. Expression levels of PA pathway genes in the SbZ5 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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