DkMyb4 is a Myb transcription factor involved in proanthocyanidin biosynthesis in persimmon (*Diospyros kaki* Thunb.) fruit

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ABSTRACT

Proanthocyanidins (PAs) are secondary metabolites that contribute to the protection of the plant and also to the taste of the fruit, mainly through astringency. Persimmon (Diospyros kaki) is unique in being able to accumulate abundant PAs in the fruit flesh. Fruits of the non-astringent (NA) type mutants lose their ability to produce PA at an early stage of fruit development, while those of the normal astringent (A) type remain rich in PA until fully ripened. The expression of many PA pathway genes was coincidentally terminated in the NA-type at an early stage of fruit development. The five genes encoding the Myb transcription factor were isolated from an A-type cultivar (cv. Kuramitsu). One of them, DkMyb4 showed the expression pattern synchronous to that of the PA pathway genes in A- and NA-type fruit flesh. The ectopic expression of DkMyb4 in kiwifruit induced PA biosynthesis, but not anthocyanin biosynthesis. The suppression of DkMyb4 in persimmon calluses caused a substantial down-regulation of the PA pathway genes and PA biosynthesis. Furthermore, analysis of the DNA-binding ability of DkMyb4 showed that it directly binds to the MYBCORE cis-motif in the promoters of the some PA pathway genes. Our all results indicate that DkMyb4 acts as a regulator of PA biosynthesis in persimmon, and therefore, suggest that the reduction in the DkMyb4 expression causes the NA-type-specific down-regulation of PA biosynthesis and resultant non-astringent trait.
INTRODUCTION

Proanthocyanidins (PAs, also called condensed tannins) are phenolic oligomers that result from the condensation of flavan-3-ol units and are synthesized via the flavonoid pathway (Fig. 1 for representative structures) (Dixon et al., 2005; Lepiniec et al., 2006). Flavonoids generally have protective functions in plants, particularly against herbivores and UV irradiation (Harborne and Grayer, 1993; McMahon et al., 2000; Peters and Constabel, 2002; Winkel-Shirley, 2001). They act as antioxidants with beneficial effects for human health including protection against free radical, cardiovascular, and metabolic diseases (Bagchi et al., 2000; Cos et al., 2004; Aron and Kennedy 2008). As a final product of the flavonoid pathway, PA also contributes to the quality of many important plant products, such as wine, teas, some berries, and cocoa (Aron and Kennedy 2008). PA biosynthesis is controlled by structural genes, which encode enzymes that directly participate in the formation of the biochemical structure, and transcription factors that control the expression patterns of structural genes (Koes et al., 2005; Lepiniec et al., 2006). The genetics and biochemical functions of some structural genes in the flavonoid pathway have been well characterized in some plant species (Holton and Cornish, 1995), and recently, much progress has been made in elucidating PA biosynthesis (Aron and Kennedy, 2008; Dixon et al., 2005; Lepiniec et al., 2006).

Recently, Xie et al. (2003) demonstrated that BANYULS (BAN) functions as an anthocyanidin reductase (ANR), which converts anthocyanidins to 2,3-trans-flavan-3-ols from Arabidopsis (Arabidopsis thaliana), and Tanner et al. (2003) reported the cloning and biochemical characterization of another key enzyme leucoanthocyanidin reductase (LAR), which converts leucoanthocyanidins to 2,3-cis-flavan-3-ols from Desmodium uncinatum. In Arabidopsis, the transparent testa (tt) and tannin-deficient seed (tds) mutants, which have an altered seed coat color, define many reactions in PA biosynthesis and accumulation (Abrahams et al., 2002; Lepiniec et al., 2006; Shirley et al., 1992), and five structural genes of the flavonoid pathway, namely chalcone isomerase (CHI), chalcone synthase (CHS), dihydroflavonol 4-reductase (DFR), flavanone 3-hydroxylase (F3H), flavanone 3′-hydroxylase (F3′H), and ANR, were identified in these mutants (Lepiniec et al., 2006). Other genes involved in PA accumulation, such as TT12 encoding a multidrug and toxic compound extrusion family transporter (Debeaujon et al., 2001), TT19 encoding a glutathione S-transferase (GST) (Kitamura et al., 2004) and AUTO-INHIBITED
H+-ATPase ISOFORM (AHA10) encoding a proton pump involved in vacuolar biogenesis (Baxter et al., 2005) were identified in the Arabidopsis mutant lines. Furthermore, some transcription factors such as the Myb-like protein, which regulates transcription of the structural genes involved in PA biosynthesis, have been identified in a few plant species. In Arabidopsis, TT2, a Myb transcription factor that controls the transcription of ANR, DFR, TT12, and AHA10 has been identified (Baudry et al., 2004; Lepiniec et al., 2006; Nesi et al., 2001; Sharma and Dixon, 2005). It was reported that TT2-like Myb-TFs in Brassica napus (Wei et al., 2007) and Lotus japonicas (Yoshida et al., 2008) are involved in PA biosynthesis. Furthermore, in the grapevine (Vitis vinifera), it was demonstrated that the function and expression patterns of three Myb-like transcription factors VvMYBPA1, VvMYBPA2, and VvMYB5b were involved in PA biosynthesis (Bogs et al., 2007; Deluc et al., 2008; Terrier et al., 2009). In contrast to these findings, however, few spontaneous genetic mutants in PA biosynthesis or accumulation have been reported, although there are many mutants for the biosynthesis of other flavonoids, mainly anthocyanin, in plant species, such as apple (Espley et al., 2007, 2009; Takos et al., 2006), grapevine (Kobayashi et al., 2004), maize (Lloyd et al., 1992; Mol et al., 1998), and petunia (Brugliera et al., 1999; Holton et al., 1993).

The oriental persimmon (Diospyros kaki Thunb.; 2n = 6x = 90) is one of the major fruit crops in East Asia. It has a unique character in that it accumulates high-molecular weight PAs that cause strong astringency in fresh fruits, and therefore it is not edible without artificial treatment to remove astringency (Taira, 1996). PAs are overwhelmingly the most abundant phenolic compounds in persimmon fruit and can constitute more than 1% of fruit flesh weight in the mature stage (Taira et al., 1998). There is a spontaneous mutant phenotype that terminates the accumulation of PAs at an early stage of fruit development (Akagi et al., 2009; Ikegami et al., 2005; Yonemori et al., 2000). This non-astringent (NA) mutant phenotype, also called the pollination constant and non-astringent (PCNA) type loses its astringency naturally on the tree during fruit development, so that fruits are edible without any artificial treatment after harvest. It has been reported that the allelotype of astringent (A) and non-astringent (NA) was controlled by a single gene known as the AST/ast allele, and expression of the NA genotype requires homozygous recessive alleles (ast) at the AST locus (Yamada and Sato, 2002; Kanzaki et al., 2001). However, the molecular mechanisms determining the A/NA phenotypes are not fully understood. This situation may be mainly due to technical difficulties caused by the prolonged life cycle, and genetic complexity of the hexaploid persimmon.

Previously, using mRNA expression profiling between the A- and NA-type cultivars or
breeding lines, it was demonstrated that some structural genes of the PA pathway were synchronously down-regulated in the NA-type with coincident termination of PA accumulation (Akagi et al., 2009; Ikegami et al., 2005). In addition, Akagi et al. (2009) suggested that the coincidental down-regulation of the two PA pathway genes, \( DkF3 \) and \( DkANR \) were mainly involved in termination of PA biosynthesis in the NA-type fruit. These results suggest that there is a common regulatory factor controlling the transcription of the PA pathway genes including the \( DkF3 \) and \( DkANR \), and it is possibly involved in substantial PA biosynthesis in persimmon fruit. In this study, we aimed to identify the transcription factors involved in PA biosynthesis during fruit development using segregated BC1-like offspring, which are considered to be a genetically homogeneous population. We isolated five Myb homologs from persimmon fruit and found that one of them \( DkMyb4 \), which notably showed the highest expression level among them, was down-regulated in the NA type, as well as many PA pathway genes. To this end, we indicated that \( DkMyb4 \) acts as a regulatory gene for PA biosynthesis in developing persimmon fruit. These insights into the molecular mechanism of seasonal PA regulation in this non-model plant will contribute to future progress in the study of PA accumulation.

RESULTS

Temporal PA biosynthesis and the expression of PA pathway genes in A and NA types

It was previously reported that soluble PA concentration was markedly reduced in the NA-type compared to the A-type at an early stage of fruit development (Akagi et al., 2009; Ikegami et al., 2005). In this study, we used 11 individuals of a BC1-like offspring, named the Atf-line, which were derived from cv. Aizu-mishirazu (A-type), cv. Taishu (NA-type), and cv. Fuyu (NA-type) (see Materials and methods) for the analysis of temporal PA accumulation. We confirmed the same tendency for a marked reduction of total PA concentration in the NA-type (Fig. 2A). This NA-type-specific reduction in PA concentration started from five weeks after bloom (5 WAB). During this stage, PA concentrations in A-type individuals increased, whereas all of the NA-type individuals showed a marked reduction. The analysis for total PA content per fruit also indicated that NA-type individuals accumulate PA in their fruits like the A-type until 5 WAB (\( P = 0.351 \) on 3 WAB, \( P = 0.041 \) on 5 WAB), but this stopped in the NA-type individuals after 7 WAB (Fig. 2B, \( P < 0.001 \)). We found no significant difference in fruit weight between A and NA.
types (data not shown, \( P > 0.125 \)).

Expression levels of the 10 genes \( \text{DkPAL, DkCHS, DkCHI, DkF3H, DkF3'H, DkF3'5'H, DkDFR, DkLAR, and DkANR} \) that encode the enzymes of the PA biosynthetic pathway (Fig. 1) were measured in fruit flesh in four individuals each of A and NA types by quantitative real-time PCR (qRT-PCR). We also measured the expression level of \( \text{DHD/SDH} \), which is an enzymatic gene of the shikimate pathway (Fig. 1) and is reported to be involved in PA regulation in persimmon fruit (Akagi et al., 2009; Ikegami et al., 2007). Expression of most of the genes (\( \text{DkPAL, DkCHS, DkCHI, DkF3H, DkF3'5'H, DkDFR, DkANS, and DkANR} \)) were synchronously down-regulated from 5 WAB and were almost below the detection limit after 7 WAB in NA-type individuals (Fig. 3), even though significant differences in their expression levels were not detected at 3 WAB (\( P > 0.2 \)) between A and NA types. Expression in the A-type was gradually down-regulated during the late developmental stages and approached the levels of the NA-type by 13 WAB (Fig. 3). The mean expression levels of these genes were typically ca. 40- to 10000-fold higher in the A-type individuals than those in the NA-type at 7 WAB. The NA-type-specific down-regulation of PA pathway genes was coincident with the reduction in PA content in the NA-type (Fig. 2A and B). This result indicates that the NA-type-specific reduction in PA content is due to the phenological and marked down-regulation of the genes of the PA biosynthetic pathway, as described in previous reports by Akagi et al. (2009) and Ikegami et al. (2005).

We found that the expression of \( \text{DkDHD/SDH} \) was also down-regulated in the NA-type (Fig. 3K). The expression patterns of \( \text{DkDHD/SDH} \) were largely synchronous with the genes of the flavonoid biosynthetic pathway, but the NA-type-specific down-regulation of \( \text{DkDHD/SDH} \) occurred earlier than that with the genes of the flavonoid biosynthetic pathway in this analysis. The shikimate pathway provides phenylalanine for both protein synthesis and as a secondary metabolite in higher plants (Lillo et al., 2007; Nicholson and Hammerschmidt 1992). It has been demonstrated that compromising the shikimate pathway by the transcription inhibition of \( \text{DHD/SDH} \) leads to stunted growth and a reduction in the major second metabolites such as lignin in tobacco plants (Ding et al., 2007). In persimmon, it has been suggested that the down-regulation of \( \text{DkDHD/SDH} \) expression reduced the content of PAs in the NA-type fruit (Akagi et al., 2009; Ikegami et al., 2007, 2009). In addition, intermediates from the shikimate pathway serve as substrates for the biosynthesis of gallic acid (Werner et al. 2004, see Fig. 1). Gallic acid is essential for the formation of galloylated PA, which is a major esterified form of PA in persimmon fruit. Therefore it was
suggested that the down-regulation of \textit{DkDHD/SDH} expression also affected the reduction in the content of galloylated PA in the NA-type fruit, as described in a previous report by Akagi et al. (2009).

By contrast, \textit{F3’H} did not show significant differences in expression patterns between A and NA types from 5 WAB (Fig. 3E). A significant reduction in the expression level of \textit{DkF3’H} was detected only at 11 WAB (\(P < 0.01\)), although the expression levels were lower in NA-type individuals than those in A-type. Furthermore, the expression of \textit{LAR} was considerably up-regulated in the NA-type, particularly after 7 WAB (Fig. 3I, \(P < 0.01\) on all the data points after 7 WAB). The mean expression level of \textit{DkLAR} was ca. 5- to 40-fold higher in the NA-type than in the A-type after 7 WAB. The relative expression levels of \textit{DkLAR}, however, were clearly lower than other genes.

\textbf{Cloning of Myb homolog candidates from persimmon fruit}

Most of the PA pathway genes were coincidentally down-regulated in fruit flesh of the NA-type (Fig. 3) implies one or more transcription factors controlling these PA pathway genes. To exploit this possibility, we prepared cDNA from the fruit of A-type cv. Kuramitsu at the development stage in which PA biosynthesis was specifically down-regulated in the NA-type, and used it as a template for PCR to isolate Myb transcription factors involved in PA regulation. We isolated a partial cDNA fragment of the Myb homolog and screened the cDNA libraries derived from cv. Kuramitsu to obtain the full-length cDNA of this Myb fragment. To this end, we isolated one full-length Myb gene \textit{DkMyb1}, which showed homology in BLAST searches to maize \textit{ZmMYB31}, 42, or subgroup 4 MYBs (Fornalé et al., 2006; Kranz et al., 1998) from other species. We screened \textit{ca.} 500,000 recombinant clones from the same cDNA libraries under a low stringent hybridized condition with the R2R3 conserved region of \textit{DkMyb1} (see Fig. 4A) as a probe and isolated 84 clones of Myb homologs. Their sequence alignment gave five presumably full-length cDNA sequences of \textit{DkMyb1}, \textit{DkMyb2}, \textit{DkMyb3}, \textit{DkMyb4}, and \textit{DksMyb1}. We found that DksMyb1 is a small-type Myb TF containing the R3 conserved region, but not R2, and that others are R2R3-type Myb TFs containing a R2R3 conserved region in their sequences (Stracke et al., 2001). We isolated 59 clones of \textit{DkMyb4}, 12 of \textit{DkMyb2}, six of \textit{DkMyb1}, five of \textit{DkMyb3}, and two of \textit{DksMyb1}.

A phylogenetic analysis of the R2R3 region of these deduced amino acid sequences placed DkMyb2 and DkMyb4 in a cluster of MYB proteins that include the following:
Picea mariana PmMBF (Xue et al., 2003), Vitis vinifera VvMYBPA1 and VvMYBPA2 (Bogs et al., 2007; Terrier et al., 2009), and Arabidopsis thaliana TT2 (Nesi et al., 2001) (Fig. 4B). Three of these Mybs, VvMYBPA1, VvMYBPA2, and TT2 have been characterized as PA regulators (Bogs et al., 2007; Nesi et al., 2001; Terrier et al., 2009). This suggests that DkMyb2 and DkMyb4 also act as regulators of PA biosynthesis. DkMyb2 has 86% identity to VvMYBPA2 and DkMyb4 has 91% to VvMYBPA1 in the R2R3 region (Fig. 4A), but no significant homology was detected in the other coding regions. DkMyb3 was placed in the same clade as Petunia hybrida ODORANT1 (Verdonk et al., 2005) and Arabidopsis thaliana AtMYB15 (Chen et al., 2005), which are regulators of the shikimate pathway. A phylogenetic analysis of the R3 region (data not shown) suggested that DksMyb1 is similar to the R3 type CAPRICE (Lepiniec et al., 2006; Tominaga et al., 2007), which is a repressor of Arabidopsis thaliana AtMYB66 (WEREWOLF).

An alignment of deduced amino acid sequences in the R3 region showed that four DkMybs, DkMyb1, DkMyb2, DkMyb4, and DksMyb1 had the amino acid residues [D/E]Lx2[R/K]x3Lx6Lx3R that specifically interacted with the R-like bHLH protein (Fig. 4A, the residues are indicated in the box) (Grotewold et al., 2000; Zimmermann et al., 2004). These results suggest that specific R-like bHLH cofactors are needed to act as regulatory factors, as well as other plant Myb TFs (Espley et al., 2007; Lepiniec et al., 2006).

Expression analysis of Myb homologs

The expression patterns of DkMyb1, DkMyb2, DkMyb3, and DksMyb1 showed no significant differences between the A and NA types, and these tendencies were inconsistent (Fig. 5A). The expression levels of DkMyb1 and DksMyb1 were gradually reduced in both A- and NA-type individuals. The expression of DkMyb2 and DkMyb3 showed sudden up-regulations in some individuals, but this tendency was observed in both A- and NA-type individuals. However, the expression of DkMyb4, whose level was notably the highest of the five Myb-TFs isolated from persimmon fruit flesh, showed NA-type-specific down-regulation from 5 WAB and was almost below the limits of detection after 7 WAB in NA-type individuals (Fig. 5A). This expression pattern of DkMyb4 was accompanied by that of the genes of the PA biosynthetic pathway, which showed NA-type-specific down-regulations (Fig. 3). These results suggest that DkMyb4 acts as a regulator of the PA biosynthesis in fruit flesh of persimmon.
To test this possibility, we analyzed the expression level of *DkMyb4* in A- and NA-type cultivars and their plant organs. We performed qRT-PCR analysis for transcribing *DkMyb4* from the fruit flesh of eight A-type and six NA-type cultivars at 7 WAB when the NA-type-specific down-regulation of *DkMyb4* and PA biosynthesis were detected in the fruit flesh of the Atf-line (Figs. 2A, 3, and 5A). The expression level of *DkMyb4* was ca. 20- to 200-fold higher in all A-type cultivars than in NA-type cultivars, as well as the Atf-line (Fig. 5B). In addition, this remarkable expression difference between A and NA types was observed only in the fruit flesh and no other plant organs (Fig. 5C). The expression level of *DkMyb4* in the leaf, stem, and fruit calyx was considerably lower than that in the fruit flesh, and showed no significant differences between A- and NA-types (*P* = 0.614 for leaf, *P* = 0.542 for stem, and *P* = 0.109 for calyx). In the fruit seeds, *DkMyb4* was highly expressed, but no significant difference in expression was detected between the A- and NA-type cultivars (*P* = 0.655).

**Analysis of PA biosynthesis in CaMV35S-DkMyb4-transformed kiwifruit**

To analyze the function of DkMyb4 in PA biosynthesis in plants, a kiwifruit (cv. Hayward) was transformed with *DkMyb4* under the transcriptional control of the cauliflower mosaic virus 35S promoter (CaMV35S) and a kanamycin selective marker. Koshita et al. (2008) demonstrated the effective functional analysis of grape (*Vitis labrascana*) Myb TFs VlMYBA1-2, the regulator of anthocyanin biosynthesis, by kiwifruit transformation (cv. Hayward). The anthocyanin and PA biosynthetic pathways share almost all structural genes except for the last catalytic steps (see Fig. 1) (Koes et al., 2005; Lepiniec et al., 2006). These facts indicated that the activity of Myb TFs regulating PA biosynthesis could be analyzed by transformation of kiwifruit. The regenerated calluses of kanamycin-positive transformants were pigmented with red two months after the infection (Fig. 6A–F), whereas control plants transformed with an empty vector consistently showed no pigmentation (Fig. 6G and H). DMACA staining (Li et al., 1996) showed high PA accumulation on the surface cells of the transformed callus, while there was only slight PA accumulation in the control callus (Fig. 6I). In addition, we analyzed anthocyanin accumulation with HPLC, comparing the *VlMYBA1*-2-introduced plants which accumulate a quantity of anthocyanin (Koshita et al., 2008) and the *DkMyb4*-introduced plants. The major anthocyanin of *VlMYBA1*-2-introduced plants was detected as cyanidin 3-O-(2-O-β-xylosyl)-β-glucoside as reported by Koshita et al. (2008). On the other hand, no
anthocyanins were detected in DkMyb4-introduced plants (Supplementary Fig. S1), as well as control plants. These results show that the expression of DkMyb4 induces PA biosynthesis in plant cells, and suggests that red pigmentation in the surface of transformants is not due to anthocyanin but presumably oxidized PAs, as in the seed coat of Arabidopsis (Abrahams et al., 2002; Lepiniec et al., 2006; Shirley et al., 1992). Control plants had regenerated shoots seven months after transformation, but the calluses transformed by CaMV35S-DkMyb4 stopped developing and became brown (Fig. 6J).

**Transformation of persimmon with CaMV35S-antisense DkMyb4**

To test the function of DkMyb4 in a homologous system, persimmon (cv. Fujiwaragoshio) was transformed with CaMV35S-sense DkMyb4 or CaMV35S-antisense DkMyb4, and a kanamycin selective marker (see Materials and methods). We preliminarily confirmed that the persimmon callus (cv. Fujiwaragoshio) expresses a quantity of DkMyb4 and markedly accumulates soluble PAs. We therefore introduced sense or antisense DkMyb4 into persimmon calluses to analyze the effect of DkMyb4 expression levels on PA biosynthesis. Some regenerated calluses transformed by CaMV35S-antisense DkMyb4, named AM4-lines, whose expression level of DkMyb4 clearly declined, showed a slight accumulation of PA in the surface cells when stained by DMACA (Li et al., 1996), whereas control plants transformed with an empty vector consistently showed high accumulation of PA (Fig. 7A). The same tendencies were observed in the content of soluble PA with methanol extraction as well as with the DMACA (Li et al., 1996) and Folin–Ciocaltaeu methods (Oshida et al., 1996) (Fig. 7B). Almost all calluses transformed by CaMV35S-sense DkMyb4 turned black and stopped growing immediately after transformation (Fig. 7C). We could not precisely measure the PA content in these transformants by methanol extraction or DMACA staining because of the small quantity of blackened callus.

qRT-PCR expression analysis in the calluses of four AM4-lines (AM4-1, 2, 6, and 8) revealed the down-regulation of DkMyb4 accompanied by a proportional reduction in the expression levels of some structural genes of the PA biosynthetic pathway; DkPAL, DkCHS, DkCHI, DkF3H, DkF3′5′H, DkDFR, and DkANR (Fig. 7D). These are specifically the down-regulated genes in the NA-type fruit (Fig. 3). Expression levels of DkDHD/SDH, which showed the NA-type-specific reduction (Fig. 3K), were also reduced in proportion to the reduction in those of DkMyb4. However, expression levels of DkF3′H and DkLAR,
which did not show the NA-type-specific reduction in the fruit flesh (Fig. 3E for DkF3'H and 3I for DkLAR), were not influenced by the DkMyb4 expression levels (Fig. 7D).

These expression changes of PA pathway genes in AM4-lines were clearly reflected in the composition of PA. HPLC analysis of the PA subunits following acid-catalysis in the presence of excess phloroglucinol (Akagi et al., 2009; Kennedy and Jones, 2001) showed that the content of epicatechin (EC) and especially epigallocatechin (EGC) which are both synthesized via ANR (see Fig. 1) were notably reduced in all AM4-lines, whereas the content of catechin and gallocatechin which are synthesized via LAR (see Fig. 1) were much lower than EC and EGC, and not significantly influenced (Fig. 7E and Supplementary Fig. S2). Galloylated EC and EGC (EC-3-O-gallate and EGC-3-O-gallate) were also detected, but the amounts were much lower than those of other PA components and not were affected in AM4-lines (data not shown). These observations indicate that the reduction in total PA content caused by the suppression of DkMyb4 expression in the AM4-lines (Fig. 7C) was mainly due to the reduction in the contents of EC and EGC, and DkLAR whose expression was not regulated by DkMyb4 did not affect PA biosynthesis in the AM4-lines.

All of these results indicated that DkMyb4 directly or indirectly acts as a regulator for the PA pathway genes and controls the PA biosynthesis in persimmon.

Identification of target motifs and genes of DkMyb4

To identify the motifs or genes directly targeted by the DkMyb4 protein, we performed an electrophoretic mobility shift assay (EMSA) with the recombinant GST-DkMyb4 fusion protein expressed in Escherichia coli. We obtained the GST-DkMyb4 fusion protein from E. coli (BL21) cells transformed by pDEST15-DkMyb4, which encodes an N-terminal in-frame fusion of DkMyb4 with a GST tag (see Materials and methods), and confirmed the solubility of this fusion protein. GST-DkMyb4 was easily soluble when expressed at 18°C (data not shown). We isolated the promoter regions of DkF3'H, DkF3'5'H, DkANS, DkLAR, and DkANR (see Fig. 1) and used their oligonucleotide fragments containing MYB-binding cis-motifs as the DNA probes. The MYB-binding cis-motifs were detected in the database of plant cis-acting regulatory DNA elements (PLACE: Higo et al., 1999). Finally, seven groups of the MYB-binding cis-motif were identified in the promoter regions of the five genes (Fig. 8A). We tested the four groups that were highly conserved for two genes among the five as candidates for the DkMyb4-binding cis-motif. These five groups included the following: WAACCA sequence, the MYB1AT motif; YAACKG (TAACTG), the
MYB2CONSENSUS (MYB2AT) motif; CNGTTR, the MYBCORE motif; MACCWAMC (CCWACC), the MYBPLANT (MYBPZM) motif; and GGATA, the MYBST1 motif (Abe et al., 2003; Planchais et al., 2002; Solano et al., 1995; Tamagnone et al., 1998; Urao et al., 1993).

The purified GST-DkMyb4 fusion protein was examined for its ability to bind to an oligonucleotide containing the MYB-binding cis-motifs by EMSA. To analyze the specificity of the cis-motif binding activity, we used non-labeled mutated oligonucleotides and non-labeled normal oligonucleotides as probe inhibitors. The mutated oligonucleotides contained two or three point mutations in the MYB-binding cis-motifs, as shown in Fig. 8B. The GST-DkMyb4 fusion protein bound to an oligonucleotide containing the MYBCORE motif occurred in the \( \text{DkF3}'5'H \) promoter region (Fig. 8C). The non-labeled oligonucleotides containing the normal MYBCORE motif competed with the labeled MYBCORE probe and reduced the binding signal between GST-DkMyb4 and MYBCORE probes in proportion to its concentration. However, a quantity of non-labeled oligonucleotides containing the mutated MYBCORE motif did not significantly reduce the binding signal. This inability of GST-DkMyb4 to bind to oligonucleotides containing the mutated MYBCORE motif suggests that DkMyb4 recognizes the MYBCORE motif in the probe sequences. DkMyb4 also showed an ability to bind to other MYBCORE motifs in the promoter regions of \( \text{DkANR} \), and \( \text{DkANS} \), as well as \( \text{DkF3}'5'H \) (Fig. 8C), but not to the other four MYB-binding cis-motifs, MYB1AT, MYB2CONSENSUSAT (MYB2AT), MYBPLANT (MYBPZM), and MYBST1 in any promoter regions (Fig. 8D). The promoter regions of \( \text{DkLAR} \) have MYB1AT and MYBST1 motifs, but not the MYBCORE motif at least up to 1773 bp upstream from the ATG start codon (Fig. 8A). This suggests that DkMyb4-independent expression levels of \( \text{DkLAR} \) in the fruit and callus (see Figs. 3I and 5A for fruit, and 7D for callus) are due to the inability of DkMyb4 to bind to the promoter region of \( \text{DkLAR} \). Also, we could not find the MYBCORE motif in the promoter region of \( \text{DkF3}'5'H \), where expression showed no significant correlation with the \( \text{DkMyb4} \) expression (see Figs. 3E and 5A for fruit, and 7C for callus). Taken together, these results suggest that DkMyb4 directly binds to the MYBCORE cis-motif in the promoter regions of the PA pathway genes, and also that the presence of MYBCORE cis-motifs in the promoter region is possibly responsible for the DkMyb4-dependent activation of their expression in persimmon fruit.
DISCUSSION

DkMyb4 acts as a regulator of PA pathway genes

Myb-TFs generally act as regulators for the second metabolite pathway in plant species. Myb-TFs have been studied through coloration due to anthocyanin biosynthesis in many plant species, such as Arabidopsis (Borevitz et al., 2000), apple (Espley et al., 2007, 2009; Takos et al., 2006), grapevine (Kobayashi et al., 2002, 2004), maize (Lloyd et al., 1992; Mol et al., 1998), petunia (Mol et al., 1998; Quattrocchio et al., 1999), and strawberry (Aharoni et al., 2001). However, only a few Myb-TFs have been reported to regulate PA biosynthesis (Bogs et al., 2007; Mellway et al., 2009; Nesi et al., 2001; Terrier et al., 2009). In this study, we showed that DkMyb4, a Myb-TF, functions as PA regulator by transformation in kiwifruit and persimmon (Figs. 6 and 7). The correlation between the expression level of DkANR, which is a PA-specific gene, and the expression level of DkMyb4 (Figs. 3K for DkANR and 5A for DkMyb4, and 7D) with the ability to bind to MYBCORE motifs in the promoter region of DkANR and DkMyb4 (Fig. 8C) suggests that DkMyb4 directly activates DkANR transcription. The expression correlation and binding ability with DkMyb4 (DkMyb4) were also observed in DkANS and DkF3′5′H (Fig. 3G for DkF3′5′H, 3I for DkANS, 5A for DkMyb4, and 8C for binding ability with DkMyb4); these were shared with anthocyanin, flavonol, and the PA biosynthetic pathway (see Fig. 1). This result indicates that DkMyb4 directly activates the PA-specific genes and also other genes of the flavonoid pathway.

On the other hand, one of the two PA specific genes, DkLAR showed the expression completely no correlated to that of DkMyb4 in persimmon fruit (Figs. 3I for DkLAR and 5A for DkMyb4). As described, the expression of VvMYBPA1 in grapevine activates only one of the two LAR genes (Bogs et al., 2007). In addition, in Medicago truncatula, slight activation of LAR expression was observed when compared to ANR expression after overexpression of a Myb transcription factor, TT2 (Pang et al., 2008), and the expression level of LAR did not correlate with PA accumulation (Pang et al., 2007). These results support the possibility that DkLAR is not regulated by DkMyb4 in persimmon.

The coincident and synchronous down-regulation of DkMyb4, and many structural genes of the flavonoid pathway including DkDHD/SDH in the NA-type (Figs. 3 and 4) suggested that DkMyb4 acts as a direct or indirect regulator of them. Ectopic suppression of
DkMyb4 expression in persimmon calluses causing a proportional reduction in the expression levels of the identical flavonoid pathway genes (Fig. 7D) indicated at least indirect regulation by DkMyb4. This is supported by the results showing that VvMYBPA1, which is a grapevine Myb-TF phylogenetically close to DkMyb4 (Fig. 4), regulates the expression of VvANR and one of the two LAR genes in grapevine, VvLARI, which are PA-specific genes, and the expression of other structural genes of the flavonoid pathway (Bogs et al., 2007) and also the phenylpropanoid and/or shikimate pathways including DHD/SDH (Terrier et al., 2009). However, we have not identified the promoter regions of the early flavonoid pathway genes, such as PAL or CHS, and therefore could not confirm whether DkMyb4 directly regulates their expression. The early flavonoid pathway, including the phenylpropanoid and/or shikimate pathways, can affect the biosynthesis of a diverse class of secondary metabolites (i.e., pigment, lignin, phytoalexins, and plant hormones) (Hermann, 1995; Lillo et al., 2008). Hence, applications of transcription factors that can regulate the general flavonoid pathway will contribute to studies on the metabolic engineering of plants for both plant and human health, as discussed previously (Dixon, 2005; Sharma and Dixon, 2005; Yu and McGonigle 2005).

**MYBCORE, the target motif of DkMyb4, is a general key cis-element for PA biosynthesis in plant species**

There are various target motifs of plant Myb-TFs, such as MYBCORE (Urao et al., 1993; Solano et al., 1995), MYBPLANT (Grotewold et al., 1994; Tamagnon et al., 1998), MYB2CONSENSUSAT (Abe et al., 2003), in the PLACE database for plant cis-motifs (Higo et al., 1999). The ability of Myb-TF binding to them depends on the sequences of the R2 region (Koes et al., 2005). Therefore, it is suggested that the Myb-TFs with conserved homologous R2 sequences have the same binding ability. The R2 regions of Myb-TFs phylogenetically placed in the same cluster of DkMyb4, such as VvMYBPA1, VvMYBPA2, or TT2 (AtMYB123) (see Fig. 4B) showed high sequence identity to DkMyb4, 88% to VvMYBPA1, 65% to VvMYBPA2, and 66% to TT2 (Fig. 4A). Their detailed target motifs have not been identified. Our results suggest that they can bind to the MYBCORE cis-motif as well as DkMyb4. Their functions were partially or entirely consistent, and all of them acted as a regulator for ANR, which is a PA-specific gene (see Fig. 1) (Bogs et al., 2007; Nesi et al., 2001; Terrier et al., 2009). It has also been reported that VvMYBPA1 acts as a
regulator of ANR in Arabidopsis (Bogs et al., 2007). Their conserved functions among plant species suggest that these Myb-TFs have the same DNA-binding ability. Furthermore, the promoter of ANR contains the same MYB-binding cis-motifs targeted by them. The comparative analyses of the promoter regions of ANR orthologs in persimmon (DkANR, accession no. 195284), grapevine (VvANR, accession no. CAD91911), and Arabidopsis (AtANR, accession no. AT1G61720) using the PLACE database showed that they have many MYBCORE cis-motifs that are highly conserved (Fig. 9). In addition, only the MYBCORE cis-motif among the MYB-binding cis-motifs was conserved in all the three promoter regions of ANR at 1 kb upstream from the ATG start codon. Taken together, these results suggest that the regulation of ANR by Myb-TFs depends on the presence of the MYBCORE cis-motif in the promoter region of ANR and the ability of Myb-TFs to bind to the MYBCORE cis-motif in plant species. This hypothesis was supported by a previous study on the promoter of AtANR in Arabidopsis. Debeaujon et al. (2003) demonstrated that a minimal promoter of AtANR necessary for expression in PA accumulating cells contains only the MYBCORE cis-motif among the MYB-binding cis-motifs. Furthermore, in grapevine, all promoter regions of the genes activated by VvMYBPA1, which regulate PA biosynthesis in grapevine, VvANR, VvCHI, VvLAR1, VvF3′5′H, and VvLDOX, contain the MYBCORE cis-motifs. These results are consistent with our results that DkANR, DkANS, and DkF3′5′H, which contain the MYBCORE cis-motifs in the promoter region, are regulated by DkMyb4, but DkF3′H and DkLAR that do not contain the MYBCORE cis-motifs in the promoter region are not regulated by DkMyb4. Therefore, it is suggested that the MYBCORE cis-motif is widely conserved in the promoter regions of general PA pathway genes in plant species, and this cis-motif is a key factor in transcription activation by the Myb-TFs regulating the PA biosynthetic pathway.

**Application of ectopic DkMyb4 expression**

The ectopic overexpression of VvMYBPA1 in Arabidopsis plants induced elevated biosynthesis of PA in the roots, hypocotyls, and apical meristems (Bogs et al., 2007). These transformed plants, however, died before they fully developed their first leaves because of the accumulation of excess PAs. Similar toxic effects of PA accumulation were observed in Arabidopsis by constitutive coexpression of TT2, PAP1, and the maize regulatory gene leaf color, which resulted in PA formation in roots and leaves, and eventual death of the plants (Sharma and Dixon, 2005). In our study, the activation of PA biosynthesis in kiwifruit.
leaves and regenerated calluses in vitro by ectopic \textit{DkMyb4} expression caused termination of callus growth and adventitious bud regeneration (Fig. 6). These results are presumably due to both the toxic effects of PA and disturbance by PA oxidization because previous reports showed that phenolic compounds and their oxidant cause the browning of plant tissue and physically disturb regeneration (Johkan et al., 2008; Madhusudhanan and Rahiman, 2000; Tang and Newton, 2004). The transformation of many fruit crops, such as apple, grapevine, kiwifruit, and persimmon depends on adventitious bud regeneration from leaves or calluses in vitro (Koshita et al., 2008; Maheswaran et al., 1992; Matsuta et al., 1993; Scorza et al., 1995; Tao et al., 1997). However, our results suggested that it is difficult to regenerate transgenic plants with \textit{DkMyb4} via adventitious buds in vitro. It would be interesting to determine whether the ectopic expression of \textit{DkMyb4} in forage plants, such as alfalfa and clover (\textit{Trifolium repens}) can induce PA accumulation in leaves because there is great interest in engineering PAs in forage crops to reduce the risk of pasture bloat in ruminants (Dixon et al., 2005).

\textbf{Involvement of \textit{DkMyb4} in the astringency trait in persimmon fruit flesh}

Our results suggested that \textit{DkMyb4} acts as a regulator of the PA pathway genes and controls PA biosynthesis in persimmon. Therefore, this suggests the possibility that the fruit flesh-specific down-regulation of \textit{DkMyb4} expression in the NA-type causes a significant reduction in PA content and the resultant non-astringent trait in fruits. Previously, it was reported that the expression levels of \textit{DkF3'5'H} and \textit{DkANR} substantially affect the PA content and composition of developing fruits of the A- and NA-type, whereas that of \textit{DkLAR} does not (Akagi et al., 2009). Our results suggest the direct regulation of \textit{DkF3'5'H} and \textit{DkANR} transcription by \textit{DkMyb4}, but no regulation of \textit{DkLAR} transcription, with the suppression of \textit{DkMyb4} in persimmon calluses (Fig. 7D) and EMSA (Fig. 8). Furthermore, the significant reduction in EGC content caused by suppression of \textit{DkMyb4} in AM4-lines (Fig. 8E) is consistent with that in NA-type fruits, which is the main reason for the non-astringent trait of the NA-type (Akagi et al., 2009). These all support the possibility that the down-regulation of \textit{DkMyb4} expression causes the suppression of \textit{DkF3'5'H} and \textit{DkANR} expression resulting in a significant reduction in EGC content in fruit flesh of the NA-type.

In this study, we also isolated \textit{DkMyb2}, another MYB-TF which is in a phylogenetic cluster containing MYB proteins regulating the PA pathway (Fig. 4B). However, we
detected no correlation between the expression level of DkMyb2 and PA biosynthesis (Figs. 2 and 5A). In addition, we could not detect the ability of DkMyb2 to bind to the MYBCORE cis-motif (T. Akagi, unpublished data). This implied that DkMyb2 could not act as a direct regulator of DkF3\(\sim5\sim H\) because the promoter region of DkF3\(\sim5\sim H\) contained only the MYBCORE cis-motifs among the MYB-binding motifs detected using the PLACE database (Fig. 8A). These results suggest that the expression level of DkMyb2 is not involved in the substantial PA accumulation in developing persimmon fruit. Other Myb-TFs or transcription factors, such as basic helix-loop-helix (bHLH) and/or WD-repeat may also possibly regulate the PA pathway genes in persimmon fruit flesh, and are involved in the astringency trait. In the future, further analysis, such as ectopic expression of DkMyb4 in NA-type fruit flesh, will show whether the involvement of phenological activity of DkMyb4 in the astringency trait in persimmon fruit.

CONCLUSION

To determine the nature of the genetic basis of PA biosynthesis in persimmon fruit, we analyzed temporal expression levels of the structural genes of the PA biosynthetic pathway. Almost all of their expression specifically showed coincident down-regulation in the NA-type. We identified a Myb-TF DkMyb4 that showed a synchronous expression pattern to the genes of the PA biosynthetic pathway. DkMyb4 recognized the MYBCORE cis-motif in the EMSA analysis and could act as a regulator for PA pathway genes in the transformation of kiwifruit and persimmon. These results indicate that DkMyb4 acts as a direct regulator of the PA pathway genes and controls PA biosynthesis in persimmon. This suggests the possibility that phenological down-regulation of DkMyb4 causes the NA-type-specific down-regulation of PA biosynthesis and determines the non-astringent trait. An insight into the phenological mechanisms regulating PA biosynthesis will greatly contribute to the modification of the PA-derived trait in fruits by controlling environmental conditions.
MATERIALS AND METHODS

Plant materials

To analyze temporal PA accumulation and gene expression in persimmon fruit, the fruit flesh of 11 individuals in a BC₁-like offspring named the Atf-line were collected in 2009. The Atf-line was derived from a cross between cv. Fuyu (NA-type) and 275-13 (A-type). The A-type parent 275-13 was derived from a cross between cv. Taishu (NA-type) and cv. Aizu-mishirazu (A-type). The offsprings were grown and maintained in the orchard of Kyoto University, Kyoto, Japan and the Department of Grape and Persimmon Research, National Institute of Fruit Tree Science, Akitsu, Hiroshima, Japan. Three fruits of each individual were sampled at two weekly intervals from three weeks after full bloom (3 WAB), until 11 or 13 weeks after full bloom (11 or 13 WAB) in which PA accumulation in the fruit is almost completed as reported previously (Akagi et al., 2009; Ikegami et al., 2005).

To isolate Myb-TF homologs in persimmon fruit, we used the fruit of an A-type cv. Kuramitsu sampled on 5 and 7 WAB in 2007 in the orchard of Kyoto University, Kyoto, Japan. For additional expression analysis on DkMyb4, we sampled fruits fresh and leaves of 14 cultivars of the A-type ( cvs. Akadu, Hiratanenashi, Kuramitsu, Miyazakimukaku, Nikura, Saijyo, Sangokuichi, and Yokono) and the NA-type ( cvs. Fuyu, Hanagosho, Jiro, Mikado, Okugosho, and Suruga) on 7 WAB in the orchard of Kyoto University, Kyoto, Japan.

In these all experiments, fruit flesh was diced into small pieces (ca. 1 cm × 0.7 cm × 0.5 cm), frozen in liquid nitrogen, and stored at −80°C until analyzed.

Analysis for PA and anthocyanin accumulation

Soluble PAs were extracted from 50 mg of finely ground dried materials in 25 ml of 80% methanol at room temperature. Insoluble PAs were extracted from the residue in 25 ml of 1% (v/v) HCl-methanol for 1 h at 60°C according to the previous report by Taira et al. (1997, 1998). We examined the PA content using the DMACA method (Li et al., 1996). We also examined soluble phenolic compounds using the Folin–Ciocalteau method (Oshida et al., 1996). PAs or phenolic compound concentration per fresh weight was expressed as (+)-catechin equivalents.

The presence of PAs in transformed plant callus tissue was detected by staining the tissues with DMACA solution (1% DMACA and 1% 6 N HCl in methanol) according to the previous report by Li et al. (1996). Plant callus tissue was decolorized in 5 ml of 30% acetic
The tissues were washed with 75% ethanol and then stained blue in DMACA solution for 1 min.

The PA subunit composition in transformed plant callus tissue was analyzed using HPLC following acid catalysis in the presence of excess phloroglucinol (Kennedy and Jones, 2001) according to the previous report by Akagi et al. (2009). Briefly, soluble PAs were extracted from 10 mg of finely ground dried materials in 1 ml of 70% acetone containing 0.1% ascorbic acid for 24 h at room temperature. The supernatant was used for acid-catalyzed cleavage of the PAs in the presence of excess phloroglucinol according to the method of Downey et al. (2003). These reactions were stopped with 200 mM sodium acetate (twice the amount of the phloroglucinol reagent), and then a vanillin solution (1 mg vanillin in 5 mL 1% (v/v) HCl/MeOH) of the same volume as the reagent was added as an external standard. Samples were run on a reverse-phase HPLC LC2010 (Shimadzu, Tokyo, Japan) using a Wakosil-II 5C18 RS analytical column (4 mm × 250 mm) protected by a guard column containing the same material. Elution was performed with two solvents, 0.2% v/v aqueous acetic acid (solvent A) and MeOH (solvent B), using the following elution programme: initially 1% B for 30 min, increasing to 15.5% for 35 min, then to 45% for 35 min, followed by washing with 100% B for 15 min, and a return to the initial conditions (1% B). The analysis was performed by detecting absorbance at 280 nm at 30°C with a flow rate of 1 mL/min. For each measurement, three replications of each sample were analyzed.

Anthocyanins in kiwifruit plants were extracted with 5% aqueous acetic acid. We analyzed anthocyanin content according to the previous report by Koshita et al. (2008) with some modifications. Samples were run on a reverse-phase HPLC LC2010 using a Shim-pack VP-ODS analytical column (4.6 mm × 150 mm; Shimadzu, Kyoto, Japan) at 40°C with a flow rate of 1 mL/min by detecting absorbance at 240–580 nm. A linear gradient of 10–50% of solvent B (1.5% H₃PO₄, 40% acetonitrile, 50% acetic acid) in solvent A (1.5% H₃PO₄) was run over 40 min.

Identification of Myb-TF homologs

Total RNA was isolated from 0.2 g of frozen flesh tissue using the hot borate method (Wan and Wilkins, 1994). cDNA was synthesized from 1 μg of total RNA using a SMART™ PCR cDNA Synthesis Kit (Clontech, Mountain View, CA, USA). The degenerate primers for Myb-TFs isolated from persimmon fruit were designed by an alignment of R2R3 nucleotide sequences of the Myb-TFs involved in flavonoid regulation in other plant species. The full-lengths of Myb-TFs were obtained by screening the cDNA
libraries of cv. Kuramitsu. The cDNA libraries were constructed using the SMART™ cDNA Library Construction Kit (Clontech) using 2.0 μg of total RNA of cv. Kuramitsu sampled on 5 or 7 WAB in 2007, according to the manufacturer’s instructions and positive clones were screened with DIG-labeled probes (Roche Diagnostics, Basel, Switzerland).

The phylogenetic analysis of the R2R3 conserved region was performed using the Clustal W multiple sequence alignment system (Thompson et al., 1997) using the default parameters of DNA Data Bank of Japan (DDBJ), and the tree was constructed using the neighbor-joining method of TreeView (Page, 1996).

**Expression analysis by quantitative real-time PCR**

Expression analysis was performed by qRT-PCR. Total RNA was isolated as described above. cDNAs were synthesized from ca. 1 μg of total RNA using SuperScript III transcriptase (Invitrogen Carlsbad, CA) and adopter primer. The primer pairs for amplification were designed based on the conserved sequences of five isolated Myb-TFs (DkMyb1, DkMyb2, DkMyb3, DkMyb4, and DksMyb1) and the structural genes of the flavonoid biosynthetic pathway (DkANR, DkANS, DkCHI, DkCHS, DkDFR, DkF3H, DkF3′H, DkF3′5′H, and DkLAR) (Akagi et al., 2009; Ikegami et al., 2005, 2007; Nakagawa et al., 2008). We also detected DHD/SDH, which is reportedly involved in PA regulation (Akagi et al., 2009; Ikegami et al., 2007). All primers were designed using conserved sequences of them with the Primer Express software (ver 2.1; Applied Biosystems, Tokyo, Japan), and the sequences are listed in Supplementary Table S1. An aliquot of 1:5 diluted pools of cDNA was used in the PCR reaction as a template. Expression levels were assayed using an ABI Prism 7900HT (Applied Biosystems, Tokyo, Japan) with the SYBR Green system and SYBR Pre-mix Ex-Taq (TaKaRa, Tokyo, Japan). All reactions were performed in a total volume of 25 μl/well, comprising 12.5 μl of SYBR-Pre mix, 9 μl of sterilized distilled water, 1 μl each of detection primer (5 μM), 0.5 μl of Rox dye, and 1 μl of template cDNA. The standard amplification protocol consisted of an initial denaturing step at 95°C for 30 s, followed by 40 cycles at 95°C for 10 s, 57°C for 5 s, and 72°C for 15 s.

For each expression, the average threshold cycle (Ct) was automatically determined by ABI Prism 7900HT as the default state. Ct was defined as the point at which fluorescence rises appreciably above the background. For each measurement, independent standard curves were constructed and at least three replications of each sample were analyzed. The mean Ct of three replications was used for each sample. Standard curves for the target genes and housekeeping gene (DkActin) were obtained by the amplification of a
serially diluted mixture of cDNA samples with six dilution points. The gene quantification method was based on the relative expression of the target gene versus reference gene (Actin). In addition, the relative expression levels among target genes were determined approximately by using equal quantities of the PCR products of the target genes for standardization.

**Plant transformations with DkMyb4**

The ORF of DkMyb4 was amplified by PCR from persimmon (cv. Kuramitsu) cDNA derived from fruit flesh sampled on 7 WAB in 2007 using Prime STAR Ex Taq (TaKaRa) polymerase, with the two primer sets DkMyb-startF-TOPO (5′-CACCATGGGAAGAGCTCCTTGTTGT-3′) and DkMyb4-stopR (5′-GGATCCGAAACTACAAGATTCAGAG-3′) for cloning of the sense strand DkMyb4, or DkMyb4-startF (5′-CGTCACAAATATCAGAAGAAGATG-3′) and DkMyb4-stopR-TOPO (5′-CACCGAAACTACAAGATTCAGAG-3′) for cloning of the antisense strand DkMyb4. The sequence in bold (CACC) is the additional sequence necessary for directional cloning in the following procedure. The two PCR fragments generated were subcloned into the entry vector pENTR™/D-TOPO® (Invitrogen) by TOPO® R. The cloning procedure followed the manufacturer’s instructions. Finally, we cloned the sense or antisense DkMyb4 ORF into pGWB2 (Nakagawa et al., 2007) to obtain pGWB2-SSDkMyb4 and pGWB2-ASDkMyb4 where the sense and antisense DkMyb4 were under the control of the CaMV35S promoter, using the Gateway® Cloning System with LR Clonase™ (Invitrogen). The nucleotide sequences were determined using CEQ8000 ver 7.0 (Beckman Coulter, Tokyo, Japan). pGWB2-SSDkMyb4 and pGWB2-ASDkMyb4 were transformed into the *Agrobacterium tumefaciens* strain C58C1 by electroporation.

Transformations of persimmon (cv. Fujiwaragosho) and kiwifruit (*Actinidia deliciosa* cv. Heyward) were performed with *Agrobacterium tumefaciens* according to the methods reported previously by Tao et al. (1997) for persimmon, and by Koshita et al. (2008) and Matsuta et al. (1993) for kiwifruit. Pieces of leaf were infected with the transformed *Agrobacterium tumefaciens* and callus tissue was regenerated on solidified MS media containing kanamycin (50 mg l−1) as a selective antibiotic.

**Isolation of cis-motifs in the promoter region of structural genes of the flavonoid pathway**
The genomic fosmid library derived from *Diospyros lotus*, which is one of the closest diploid relatives of persimmon (*D. kaki*) (Yonemori et al., 2008), was constructed using the CopyControl™ Fosmid Library Production Kit (Epicentre, Madison, WI, USA) by a modified version of the manufacturer’s protocol. Randomly sheared and end-repaired genomic DNA (20–30 µg) was run on a 0.3% agarose gel. Thereafter, DNA fragments of about 40 kb in length were extracted using RECOCHIP (TaKaRa). The extracted size-selected DNA was then purified by chloroform extraction and ligated into pCC1FOS. The inserts in the plasmids were then packaged into phage lambda (Epicentre). We screened clones containing the homologous ORF of *DkANS*, *DkANR*, *DkF3' H*, *DkF3'5'H*, and *DkLAR* from the genomic fosmid library. The sequences of each promoter region were determined and then specific primers amplifying them were designed (Supplementary Table S2). The promoter regions in persimmon (*D. kaki*, cv. Kuramitsu) were amplified using PrimeSTAR® HS (TaKaRa) and directly sequenced using Exo-Sap IT (GE Healthcare Bio-Sciences) and CEQ8000 ver 7.0 (Beckman Coulter, Tokyo, Japan). Many single nucleotide polymorphisms were detected in these promoter sequences because the hexaploid persimmon (2*n* = 6x = 90) has six alleles at each locus. We only detected cis-motifs in their consensus sequences. Analysis of the promoter regions was performed using the PLACE database (http://www.dna.affrc.go.jp/PLACE/signalup.html) (Higo et al., 1999).

**Production and purification of DkMyb4-GST fusion proteins**

The sense strand DkMyb4 ORF amplified by the primer set DkMyb-startF-TOPO and DkMyb4-stopR was subcloned into the entry vector pENTRTM/D-TOPO® (Invitrogen) by the TOPO® cloning procedure as described above. To express the GST-tagged recombinant DkMyb4 protein in *E. coli*, the sense strand DkMyb4 was cloned into the vector pDEST™ 15 (Invitrogen) to give pDEST15-DkMyb4, which encodes an N-terminal in-frame fusion of DkMyb4 with the GST tag, using the Gateway® Cloning System with LR Clonase™ (Invitrogen). The resultant plasmid was transformed into *E. coli* (BL21) cells. The transformants were pre-cultured at 37°C for 16 h in LB media containing 100 µg/ml ampicillin. A 3-ml volume of the preculture was inoculated into 300 ml of fresh LB media containing the same antibiotic. After incubation at 37°C, until the absorbance at 600 nm reached 0.2, isopropyl β-D-thiogalactopyranoside was added to the broth to a final concentration of 0.3 mM, followed by further incubation at 18°C for 24 h. The cells were harvested by centrifugation at 4000 rpm for 15 min, and stored at −80°C before further
processing. Collected cells were resuspended in phosphate buffer saline containing 1% Triton-X 100 (PBST) and the insoluble bacterial debris was removed by centrifugation (15000 rpm for 15 min). After dithiothreitol was added to the resulting supernatant to a final concentration of 1 mM, the soluble fraction was loaded onto a 3 ml Glutathione Sepharose™ B column (GE Healthcare Bio-Sciences) that was pre-equilibrated with PBST. The column was washed with 15 column volumes of PBST. The recombinant protein, which was bound to the affinity column, was eluted with 15 ml of 50 mM Tris-HCl buffer (pH 9.5) containing 50 mM of reduced gluthathione. The eluted fraction was collected and concentrated using Y30 membrane (Amicon, Millipore, Billerica, MA, USA) and glycerol was added to a final concentration of 10% (v/v) before being stored at −80°C.

**EMSA analyses**

Oligonucleotide probes were labeled by digoxigenin-ddUTP (DIG-ddUTP) using DIG Oligonucleotide 3′-End Labeling Kit, 2nd Generation (Roche, Basel, Switzerland). The DNA binding reaction was allowed to proceed for 20 min at 25°C in 20 μl of the two binding buffers (5% glycerol, 4 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 25 mM HEPES/KOH pH 7.5, and 5% glycerol, 50 mM KCl, 0.5 mM DTT, 0.5 mM EDTA, 25 mM HEPES/KOH pH 8.0) according to previous reports by Urao et al. (1993) and Hartmann et al. (2005), containing 4 ng of a DIG-labeled oligonucleotide probe and 400 ng purified GST-DkMyb4 fusion protein. Competition experiments were performed by adding unlabeled competitor oligonucleotides at 5-, 15- or 50-fold excess versus the DIG-labeled oligonucleotide probe. The bound complexes were subjected to electrophoresis on native 5% polyacrylamide gels in 0.5% Tris-borate EDTA buffer, pH 8.0, at 90 V for 60–80 min at room temperature, and then transferred to a nylon membrane Biodine-Plus (Pall, East Hills, NY, USA). The DIG-labeled signals were detected using an anti-DIG-alkaline phosphate conjugate, a chemiluminescent substrate CDP-Star (Roche), and a chemiluminometer LAS3000 mini (FUJIFILM, Tokyo, Japan).

**Accession numbers**

DkDHD/SDH (AB472375), DkpAL (AB472364), DkCHS (AB472365), DkCHI (AB472367), DkF3H (AB472368), DkF3'H (AB472369), DkF3'S'H (AB472676), DkDFR (AB472371), DkANS (AB472677), DkLAR (AB472681), DkANR (AB195284), DkMyb1 (AB503698), DkMyb2 (AB503699), DkMyb3 (AB503700), DkMyb4 (AB503701), DksMyb1 (AB503702), DkActin (AB473616)
Supplemental Materials

**Supplemental Table S1.** Information for primers used in qRT-PCR analysis. The accession numbers from public database, Tm-value, and length of amplified fragment were given.

**Supplemental Table S2.** Information for primers used in promoter analysis.

**Supplemental Figure S1.** HPLC chromatogram of anthocyanins from transformed kiwifruit calluses.

**Supplemental Figure S2.** HPLC chromatogram of PA cleavage products from the AM4-1 calluses.
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**FIGURE LEGENDS**

**Figure 1.** Scheme of the PA biosynthetic pathway. DHD/SDH, 3-dehydroquinate dehydratase/shikimate 5-dehydrogenase; PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3\(^{-}\)H, flavanone 3\(^{-}\)-hydroxylase; F3\(^{+}\)H, flavanone 3\(^{+}\)-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; GTs, glycosiltransferases; OMT, O-methyltransferase.

**Figure 2.** Development patterns of fruits in PCNA and non-PCNA individuals of the Atf-line. Round points and dotted lines indicate NA-type individuals, and square points and serial lines indicate A-type individuals. (A) Total PA concentration per fresh weight. (B) Total PA content per fruit. Data collection started at three weeks after full bloom (3 WAB) and continued until 11 or 13 weeks after full bloom (11 or 13 WAB). Error bars indicated standard deviations (SD). We analyzed independent three fruits for each individual of the Atf-line (n = 3).

**Figure 3.** Quantitative real-time PCR (qRT-PCR) analysis for determining the expression levels of the structural genes involved in PA biosynthesis. Flesh from four A-type (Atf-31, Atf-45, Atf-47, and Atf-148) and NA-type (Atf-9, Atf-36, Atf-117, and Atf-145) individual fruits from 3 to 13 WAB are used for the expression analysis. The expression level is shown as the value relative to expression of DkActin (accession no. AB473616) in each sample. Expression of the genes encoding the enzymes A, PAL; B, CHS; C, CHI; D, F3H; E, F3\(^{-}\)H; F, F3\(^{+}\)H; G, DFR; H, ANS; I, LAR; J, ANR; K, DHD/SDH. Error bars indicated standard deviations (SD).

**Figure 4.** Comparison of DkMyb1-4 and DksMyb1 deduced amino acid sequences with other Myb-TFs. A, Protein sequence alignment of the global Myb-TFs by phylogenetic analysis. The R2 and R3 domains are shown and [D/E]L\(_x_3\)[R/K]L\(_x_3\)L\(_x_6\)L\(_x_3\)R motif interacting with R-like bHLH is indicated in the box. B, Phylogenetic analysis displaying the similarities of DkMyb1-4 with other Myb-TFs. The tree is based on the alignment of the 105 amino acids spanning the R2R3 domain with Clustal W multiple sequence alignment system (Thompson et al., 1997) using the default parameters of DNA Data Bank of Japan (DDBJ). The tree is constructed using the neighbor-joining method of TreeView (Page 1996). The scale bar represents 0.1 substitutions per site and the numbers next to the nodes are bootstrap values from 1000 replicates. The numbers indicate bootstrap values. The GeneBank accession numbers of
Myb-TFs are as follows:
AtMYB111 (NP199744), AtMYB12 (NP182268), ZmMYB31 (AM15906), ZmMYB42 (AM15908), DkMYB1 (AB503698), DkMYB4 (AB503671), VvMybPA1 (CAJ90831), PmMBF1 (AAA82943), DkMYB2 (AB503699), VvMybPA2 (EU919682), AtMYB123-TT2 (Q9FJA2), AtMYB15 (Y14207), DkMYB3 (AB503670), ODO1 (AAV98200), AtMyb4 (BAA21619), MdMYB8 (DQ267899), AtMYB23-GL1 (NP189430), AtMYB66-WER (AAF18939), AmROSEA2 (ABB83827), AmROSEA1 (ABB83826), AmVENOSA (ABB83828), CaE (AJ608992), LeANT1 (AAQ55181), PhAN2 (AAF66727), AtMYB90-PAP2 (NP176813), AtMYB75-PAP1 (NP176057), VvMYBA1 (BAD18977), VvMYBA2 (BAD18978), MdMYB10 (ABB84753).

Figure 5. qRT-PCR analysis of expression levels of the Myb-TFs isolated from persimmon fruit. A, Temporal expression for DkMyb1-4 and DksMyb1 from 3 W AB until 13 W AB in the four individuals of A- or NA-type. B, Gene expression of DkMyb4 in the fruit flesh of eight A-type cultivars and six NA-type cultivars at 7 W AB. C, Gene expression of DkMyb4 in fruit flesh, leaf, stem, fruit calyx, and fruit seed of two A- and NA-type cultivars at 7 W AB. Error bars indicated standard deviations (SD).

Figure 6. Functional analysis of DkMyb4 with kiwifruit transformation. Ectopic expression of DkMyb4-induced PA accumulation in the kiwifruit callus. Growth of the DkMyb4-introduced callus is shown after the in vitro infection of leaves with Agrobacterium. A, four weeks; B, six weeks; C, eight weeks; D, 10 weeks; E and F, three months. G and H, Control callus with empty vector after three months. I, DkMyb4-introduced and control calluses at three months after infection are stained with DMACA. The strong blue coloration in the DkMyb4-introduced callus indicates the accumulation of a large amount of PA in the surface cells. J, DkMyb4-introduced and control plants at six months after infection. The control callus regenerated, while the DkMyb4-introduced callus is brown and growth has stopped. The scale bar represents 2 mm in all figures.

Figure 7. Functional analysis of DkMyb4 with persimmon transformation. Some of the persimmon calluses into which antisense-DkMyb4 had been introduced are regenerated and designated the AM4-lines. A, The AM4-lines and control callus at two months after the in vitro infection of leaves with Agrobacterium (left) and stained with DMACA (right). Calluses of the AM4-lines clearly showed less blue-staining than controls. This indicates lower PA accumulation in the surface cells of the AM4-line calluses. The scale bars represent 10 mm. B,
Soluble PA content (left) and soluble phenol compounds content (right) in the AM4-line calluses and control callus. C, Sense DkMyb4-introduced persimmon callus. All sense-DkMyb4 lines have stop growing and turned black. The scale bars represent 2 mm. D, Gene expression in the AM4-line and control calluses. The structural genes of the PA biosynthetic pathway and DkDHD/SDH are measured. The transcription levels of the genes showing the NA-type-specific down-regulation (see Fig. 3) were lower in the AM4-lines than those in the controls. Expression of DkLAR was not detected (N.D). E, PA subunit composition in the AM4-line and control callus. The content of epicatechin (EC) and epigallocatechin (EGC) were significantly lower in the AM4-lines than those in the controls. The content of catechin (CA) and gallocatechin (GC) were not changed in the AM4-lines. Error bars indicated standard deviations (SD). We analyzed each individuals with three replications in HPLC (n = 3). *The galloylated EC and EGC were also detected slightly in both AM4-lines and controls, and contained in EC and EGC respectively in the photo E.

Figure 8. MYB-binding cis-motifs in the promoter region of the PA pathway genes in persimmon (cv. Kuramitsu) and the ability of the DkMyb4 fusion protein to bind to them. A, MYB-binding motifs in the promoters of *DkF3* 5′-H, *DkF3* 5′-5′-H, *DkANS*, *DkLAR*, and *DkANR* detected in the sense coding strand using the PLACE database (http://www.dna.affrc.go.jp/PLACE/signalup.html) (Higo et al., 1999). B, Sequences for oligonucleotides used in EMSA analysis. The MYB-binding motifs are shown as the underlined nucleotides. The mutated oligonucleotide sequences of the MYBCORE cis-motif are shown in bold (G and T to C and A respectively). C, EMSA using DkMyb4 fusion protein and the MYBCORE cis-motif derived from the promoter region of *DkF3* 5′-H (above figure). The binding signals are reduced in proportion to the amount of non-labeled MYBCORE inhibitor at 20-, 50-, or 150-fold excess versus the DIG-labeled oligonucleotide probe, but 150-fold excess non-labeled mutated MYBCORE inhibitor does not significantly decrease the binding signal. The binding signals were detected also in EMSA using DkMyb4 fusion protein and the MYBCORE cis-motif derived from the promoter region of *DkANR* and *DkANS* (below figures). D, EMSA using DkMyb4 fusion protein and the MYB1AT, MYB2CONSENSUS, MYBPLANT, and MYB1ST motifs. Significant binding signals are not detected in any of these.

Figure 9. MYB-binding cis-motifs in the promoter region of ANR orthologs from persimmon (*Diospyros kaki*, cv. Kuramitsu), grapevine (*Vitis vinifera*, cv. Pinot noir) and *Arabidopsis* (*Arabidopsis thaliana*). Analysis is performed using the PLACE database as described in the legend to Fig. 8.
Fig. 1

Shikimate pathway

erythrose 4-phosphate + phosphoenolpyruvate

DHD/SDH → shikimate → chorismate → phenylalanine

3-dehydroshikimate

dehydroshikimate, enol form → gallic acid

Flavan-3-ols

catechin or gallocatechin (2,3-trans flavan-3-ols)

epicatechin or epigallocatechin (2,3-cis flavan-3-ols)

polymerization

Proanthocyanidins

Anthocyanins & derivatives

Flavonols

Proanthocyanidin biosynthetic pathway
[D/E]Lx3[R/K]x1Lx6Lx3R motif interacting with R-like bHLH
Fig. 5A

Weeks after bloom (WAB)

relative expression level to DkActin

- **DkMyb1**
  - 3579 levels:
    - DkMyb1
    - DkMyb3
    - DkMyb4
    - DksMyb1

- **DkMyb2**
  - 3579 levels:
    - DkMyb2

- **DkMyb3**
  - 3579 levels:
    - DkMyb3

- **DkMyb4**
  - 3579 levels:
    - DkMyb4

- **DksMyb1**
  - 3579 levels:
    - DksMyb1

Legend:
- Atf-31
- Atf-45
- Atf-47
- Atf-148
- Atf-9
- Atf-36
- Atf-117
- Atf-145

A-type:
- Atf-31
- Atf-45
- Atf-47
- Atf-148

NA-type:
- Atf-9
- Atf-36
- Atf-117
- Atf-145
Fig. 5B-C

**B**

![Bar graph showing relative expression levels of DkActin in NA-type and A-type cultivars.](https://plantphysiol.org)

*Nonaploid cultivars (2n = 9x = 135)*

**C**

![Bar graph showing relative expression levels of DkActin in different tissue types.](https://plantphysiol.org)
Fig. 7A-C

A

Control

AM4-lines

Non-stained

DMACA-stained

B

Soluble PA

Soluble phenolic compound

concentration (FW\%)
B

**MYBCORE (From DkF3’5’H)**
5’- GATAGACGGGGCAGTTAGCCCTCACGTGCGGAGTGGTAGGTGACGGGTAGCA- 3’

**MYBCORE mutated (From DkF3’5’H)**
5’- GATAGACGGGGCAGTTAAGCCCTCACGTGCGGAGTGGTAGGTGACGGGTAGCA- 3’

**MYBCORE (From DkANR)**
5’- CCCACTCGGTTATGCGGAATAAGTCTTCTCAGCCCTT- 3’

**MYBCORE (From DkANS)**
5’- AGAAGGAATTGGGGCAGTTAAGGGAACTCGTGCTTGA- 3’

**MYB1AT (From DkLAR)**
5’- CTCGATGTGCTAAACCAACTACTGTCATTAATTGTGGGATTAT - 3’

**MYB2CONSENSUSAT (From DkANR)**
5’- CTCAACGATGAAATCTTGCATGTAACCTGGATTTCAGTCTCATA - 3’

**MYBPLANT (From DkF3’H)**
5’ - TGCCACGTGCCACCTAACGATCCACTGCTACTCCCCGCCG - 3’

**MYBST1 (From DkLAR)**
5’ - CCACAGTGATAATCAATAACATTATGGGGATAAAAAGACAGATT - 3’

C

**MYBCORE from DkF3’5’H**
+ GST
+ GST-DkMyb4
+ Inhibitor + excess mutant inhibitor

**Specific shift signal**

**Free DNA**

**MYBCORE from DkANR**
+ GST
+ GST-DkMyb4
+ Inhibitor

**Specific shift signal**

**Free DNA**
Fig. 8D

MYB1AT + GST + GST-DkMyb4 + Inhibitor
MYB2CONSUSAT + GST + GST-DkMyb4 + Inhibitor
MYBPLANT + GST + GST-DkMyb4 + Inhibitor
MYBST1 + GST + GST-DkMyb4 + Inhibitor

Free DNA
Fig. 9

DkANR

VvANR

AtANR (AtBAN)

-1802
-1500
-1500
-1000

ATG (0)

-1138
-1332
-1202
-1051
-1008

-929
-1319
-1202
-1051
-837

-794
-824
-719
-685
-568

-795
-568
-427
-238
-550

-686
-642
-392
-218
-227
-120

-794
-824
-686
-218
-120

-1000

MYB1AT
MYB2CONSENSUSAT (MYB2AT)
MYBATRD22
MYBCORE
MYBCOREATCYCB1
MYBGAHV
MYBPLANT (MYBPZM)
MYBST1