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Analysis of the floral MADS-box genes from monocotyledonous Trilliaceae species indicates the involvement of *SEPALLATA3*-like genes in sepal-petal differentiation

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ABSTRACT

The evolution of greenish sepals from petaloid outer tepals has occurred repeatedly in various lineages of non-grass monocots. Studies in distinct monocot species showed that the evolution of sepals could be explained by the ABC model; for example, the defect of B-class function in the outermost whorl was linked to the evolution of sepals. Here, floral MADS-box genes from three sepal-bearing monocotyledonous Trilliaceae species, *Trillium camschatcense*, *Paris verticillata*, and *Kinugasa japonica* were examined. Unexpectedly, expression of not only A- but also B-class genes was detected in the sepals of all three species. Although the E-class gene is generally expressed across all floral whorls, no expression was detected in sepals in the three species examined here. Overexpression of the E-class *SEPALLATA3*-like gene from *T. camschatcense* (*TcamSEP*) in *Arabidopsis thaliana* produced phenotypes identical to those reported for orthologs in other monocots. Additionally, yeast hybrid experiments indicated that TcamSEP could form a higher-order complex with an endogenous heterodimer of B-class APETALA3/DEFICIENS-like (TcamDEF) and PISTILLATA/GLOBOSA-like (TcamGLO) proteins. These results suggest a conserved role for Trilliaceae *SEPALLATA3*-like genes in functionalization of the B-class genes, and that a lack of *SEPALLATA3*-like gene expression in the outermost whorl may be related to the formation of greenish sepals.

1. Introduction

Flowers, the reproductive organs of angiosperms, have undergone an extensive diversification during the evolutionary process. Despite their bewildering diversity, the basic architecture of the floral organs comprises four components, sepals, petals, stamens, and carpels, that are usually arranged in circles. Many plants bear sepals at the outermost whorl (W1), petals at the second whorl (W2), stamens at the third whorl (W3), and carpels at the innermost whorl (W4). Flowering plant species have achieved their unique floral morphologies by modifying the shapes and colors of these components and, in some cases, by component rearrangement. One of the current areas of interest for plant evolution research is how genetic changes have led to the diversification of flowers [1]. Studies on floral mutants of *Arabidopsis thaliana* and *Antirrhinum majus* shed light on the genetic regulation of floral development and led to the 'ABC model' [2]. In this elegant model, the identity of each floral whorl is determined by a combination of genes grouped into different functional classes. Genes within the A-class alone specify sepals in W1, a combination of A- and B-class genes specify petals in W2, a combination of B- and C-class genes specify stamens in W3, and C-class genes alone specify carpels in W4. In *A. thaliana*, the A-class genes are *APETALA1* (*AP1*) and *APETALA2* (*AP2*), the B-class genes are *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), and the C-class gene is *AGAMOUS* (*AG*). In *A. majus*, the A-class gene is *SQUAMOSA* (*SQUA*), the B-class genes are *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*), and the C-class gene is *PLENA* (*PLE*). In particular, gene products from both *AP3* and *PI* (*DEF* and *GLO* in *A. majus*, respectively) are required for B-class function. With the exception of *AP2* in *A. thaliana*, all floral identity genes belong to the MADS-box gene family, which encodes DNA-binding transcription factors. MADS-box proteins have a highly conserved MIKC-type domain structure in which the N-terminal MADS (M) domain is followed by intervening (I), keratin-like (K), and C-terminal (C) domains. Subsequent discovery of the D- and E-class genes led to the establishment of the improved 'ABCDE model' [3]. D-class genes, such as *SEEDSTICK* (*STK*) of *A. thaliana*, are mainly expressed in W4 and regulate the

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development of ovules [4,5]. E-class genes are expressed throughout all floral whorls, and their expressed proteins are considered to have a hub-like role in floral development through their binding to other MADS-box proteins and formation of functional multimeric protein complexes [6,7] called 'floral quartets' [8]. It was suggested that these floral quartets bind to two cis regulatory elements (CArG boxes) thereby looping the DNA to regulate target gene expression [8]. In *A. thaliana*, four partially redundant *SEPALLATA* (*SEP*) genes, termed *SEP1*, *SEP2*, *SEP3*, and *SEP4*, are characterized as the E-class genes. Constitutive expression of *SEP3*, together with the B-class (*AP3* and *PI*) genes, can convert leaves into petal-like organs [7]. Conversely, quadruple mutants lacking all four *SEP* proteins showed homeotic conversions of all floral organs into leaf-like organs [9].

Unlike *A. thaliana* and other higher eudicot species, many of the non-grass monocots bear petaloid outer tepals instead of greenish sepals. For instance, lilies, tulips, and many orchids do not show sepal-petal differentiation and bear petaloid tepals at both W1 and W2. To account for these lily-type flowers, a 'modified ABC model' was proposed in which B-class function is present in W1, W2, and W3 [10]. Molecular studies on monocot species with lily-type flowers, such as *Tulipa gesneriana* [11], *Agapanthus praecox* [12], *Muscari armeniacum* [13], *Alstroemeria ligtu* [14], *Crocus sativus* [15], and *Lilium* x *formolongi* [16] successfully detected the expression of both B-class *AP3*/*DEF*- and *PI*/*GLO*-lineage genes in petaloid outer tepals, and confirmed that the extended expression of B-class genes in W1 was responsible for the replacement of sepals with petaloid organs (reviewed in Ref. [17]). Furthermore, analysis of a viridiflora tulip cultivar that showed sepal-like tepals at W1 and W2 detected lower expression of *AP3*/*DEF*-lineage genes in the two outer whorls compared to that in the wild type [18]. Because expression of *PI*/*GLO*-lineage genes in the viridiflora tulip was identical to that in wild type, these data empirically demonstrated that both *AP3*/*DEF*- and *PI*/*GLO*-lineage gene products were required for petal formation. Although members of the grasses (Poales) were also subjected to detailed molecular studies (reviewed in Ref. [19]), the peripheral organs surrounding the sexual organs (lemma, palea, and lodicules) differ substantially from those of the non-grass monocots, making it difficult to make direct comparisons.

While many of the non-grass monocots possess the above-mentioned lily-type flowers, some groups are known to have flowers with clear sepal-petal differentiation. For instance, *Commelina communis* and *Habenaria radiata* bear greenish tepals (sepals) at W1 and showy petaloid tepals (petals) at W2. These species are distantly related, indicating that the evolution of sepals from petals in W1 occurred independently in multiple monocot lineages. Studies in *C. communis*, *Tradescantia reflexa* (both in Commelinaceae) [20], and *H. radiata* (Orchidaceae) [21] showed that that these sepal-bearing species lacked the expression of B-class *AP3*/*DEF*-lineage genes in their outermost whorls. As shown from the study of viridiflora tulips [18], a lack of *AP3*/*DEF*-lineage genes can diminish the B-function and thus prevent the development of petaloid organs. So far, although the evolutionary origin of sepals in different monocots is considered to be independent, the underlying genetic mechanisms (i.e., the lack of *AP3*/*DEF*-lineage gene expression) were found to be common between the studied taxa [20,21].

Can the lack of *AP3*/*DEF*-lineage gene expression explain the transition of petaloid tepals into sepaloid tepals in all sepal-bearing monocots? An exceptional case was reported in *Sagittaria montevidensis* (Alismataceae), in which expression of both *AP3*/*DEF*- and *PI*/*GLO*-lineage genes was found in the sepals [22]. However, expression of the B-class genes was lower in the sepals than in the petals. Because other classes of genes were not analyzed in *S. montevidensis*, it remains unclear whether the lower expression level of B-class genes is related to sepal formation in this species. It is clear that additional studies in a wider range of taxonomic groups are necessary to assess the generality of the relationship between *AP3*/*DEF*-lineage gene expression and sepal formation in monocots. In addition, as studies have clarified the inter-class interactions of the MADS-box proteins during floral development [2,3,8], analyses of other functional classes, beside the B-class, are crucial. In *A. thaliana*, sepal identity is determined by a tetramer complex composed of two sets of A- and E-class heterodimers, whereas petal identity is determined by a tetramer composed of an A- and E-class heterodimer, and a AP3 and PI heterodimer [8]. Thus, characterization and functional analysis of A-, B-, and E-class genes are necessary to accurately assess the genetic mechanisms for sepal-petal differentiation.

In the present study, we analyzed the genetic mechanisms underlying sepal-petal differentiation in three sepal-bearing monocots: *Trillium camschatcense*, *Paris verticillata*, and *Kinugasa japonica* (Fig. 1). These species represent three of the six genera within Trilliaceae [23,24] (or the tribe Parideae in Melanthiaceae, Liliales [25]) and show sepal-petal differentiation. Unlike other members of the Liliales, including close relatives within Melanthiaceae (e.g., *Heloniopsis*, *Veratrum*, and *Chinographis*), the majority of the species in Trilliaceae show greenish sepals at their outermost whorls. So far, study of functional genes within this group has been limited, with the exception of an analysis of a non-floral specific MADS-box gene from *T. camschatcense* [26]. Floral morphologies within Trilliaceae are somewhat diverged between genera. Species of *Trillium* and *Paris* bear greenish sepals at W1, whereas *K. japonica* bears showy white sepals that eventually turn green after pollination. In addition, species of *Trillium* bear a set of large and showy petals at W2, whereas the equivalent organs in *Paris* and *Kinugasa* are reduced to small filiform structures [24]. While our main objective was to search for the genetic mechanism underlying sepal-petal differentiation, a comparison of expression patterns of floral MADS-box genes between the three species was also of interest. Here, we isolated and characterized MADS-box genes from the three Trilliaceae species and analyzed their expression patterns in each floral organ. For A-, B-, and E-class genes, we examined their conserved functions by *A. thaliana* overexpression and yeast hybrid analysis.

2. Material and methods

2.1. Plant materials

Individuals of *T. camschatcense*, *P. verticillata*, and *K. japonica* were cultivated in a greenhouse at the Graduate School of Life Sciences, Tohoku University. Floral buds and leaves were collected immediately after sprouting of the shoot from the underground rhizome and stored at −80 °C for subsequent RNA extraction. As plants bear only one flower per individual, at least two individuals were sampled for gene isolation and expression analysis. Although a recent study reported the presence of male-sterile female individuals in natural populations of *T. camschatcense* [27], individuals used in the present study were all hermaphroditic.

2.2. Isolation of the MADS-box genes

Total RNA was prepared from the entire floral bud from each of the three species using the RNeasy Plant Mini Kit (QIAGEN, Germany). Poly-A-tailed mRNA was purified from the total RNA using a Dynabeads mRNA Purification Kit (Life Technologies, USA). Synthesis of cDNA was conducted using an AMV Reverse Transcriptase (Promega, USA) with species-specific poly-T-tailed reverse transcription primers P019TC, P019PV, and P019KJ for *T. camschatcense*, *P. verticillata*, and *K. japonica*, respectively (see Table S1 for