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Molecular mechanism underlying pseudopeloria in *Habenaria radiata* (Orchidaceae)

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SUMMARY

Habenaria radiata (Orchidaceae) has two whorls of perianth, comprising three greenish sepals, two white petals, and one lip (labellum). By contrast, the pseudopeloric (decreasing of the degree of zygomorphy) mutant cultivar of *H. radiata*, 'Hishou', has a shift in the identity of the dorsal sepal by a petaloid organ and the two ventral sepals by lip-like organs. Here, we isolated four *DEFICIENS*-like and two *AGL6*-like genes from *H. radiata*, and characterized their expression. Most of these genes revealed similar expression patterns in the wild type and in the 'Hishou' cultivar, except *HrDEF-C3*. The *HrDEF-C3* gene was expressed in petals and lip in the wild type but ectopically expressed in sepal, petals, lip, leaf, root, and bulb in 'Hishou'. Sequence analysis of the *HrDEF-C3* loci revealed that the 'Hishou' genome harbored two types of *HrDEF-C3* genes, one identical to wild type *HrDEF-C3*, and the other carrying a retrotransposon insertion in its promoter. Genetic linkage analysis of the progeny derived from an intraspecific cross between 'Hishou' and the wild type demonstrated that the mutant pseudopeloric trait was dominantly inherited and was linked to the *HrDEF-C3* gene carrying the retrotransposon. These results indicate that the pseudopeloric phenotype is caused by retrotransposon insertion in the *HrDEF-C3* promoter, resulting in ectopic expression of *HrDEF-C3*. Since the expression of *HrAGL6-C2* was limited to lateral sepals and lip, overlapping expression of *HrDEF-C3* and *HrAGL6-C2* are likely responsible for the sepal to lip-like identity in the lateral sepals in 'Hishou' cultivar.

SIGNIFICANCE STATEMENT

Unlike wild type *Habenaria radiata* flowers which have a single modified medial petal into a lip, the mutant cultivar 'Hishou' flowers exhibit two additional lip-like organs replacing the lateral sepals. Here, we identified *Hret2* retrotransposon insertion in the *HrDEF-C3* gene promoter as the cause of the pseudopeloric phenotype of 'Hishou'. Based on *DEF*- and

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AGL6-like genes expression patterns in wild type and 'Hishou', the differential dorsoventral expression of *HrAGL6-C2* gene is correlated with the lateral sepals to lip-like structures.

Keywords: *DEFICIENS*-like gene, floral homeotic mutant, Orchidaceae, pseudopeloric mutation, retrotransposon.

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INTRODUCTION

In the past two decades, molecular mechanisms of flower development have been extensively investigated in *Arabidopsis thaliana* and *Antirrhinum majus* (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994; Schwarz-Sommer et al., 1990 ; Theissen et al., 2000). Studies have shown that MADS-box transcription factors are key regulators of floral organ specification and development. According to the well-known 'ABCE model' of flower development (Theissen and Saedler, 2001; Bowman *et al*., 1991a; Soltis *et al*., 2007), four classes of MADS-box genes specify the formation of distinct floral organs in four whorls: the A- and E-class genes specify sepals formation in whorl 1; A-, B-, and E-class genes specify petals formation in whorl 2; B-, C-, and E-class genes determine stamen formation in whorl 3; and C- and E-class genes specify carpel development in whorl 4. The expression of A-class genes is required for the establishment of floral meristem and for specifying sepals and petals identity (Irish and Sussex 1990; Mandel *et al*., 1992; Bowman *et al*., 1993). The B-class floral homeotic genes comprise two major clades, *APETALA3 (AP3)*/*DEFICIENS (DEF)*-like and *PISTILLATA (PI)*/*GLOBOSA (GLO)*-like genes (Zahn et al., 2005), and are responsible for specifying petals and stamen identity. The loss of expression of B-class genes in *Arabidopsis* results in the conversion of petals to sepals and stamens to carpels (Goto and Meyerowitz 1994; Jack *et al*., 1992). Ectopic expression of *AP3* in *Arabidopsis* causes a partial conversion of carpels to stamens, whereas ectopic expression of *PI* causes a partial transformation of first-whorl sepals to petals (Jack *et al*., 1994). The C-class gene *AGAMOUS (AG)* is important for the proper development of stamens and carpels (Bowman *et al*., 1991b).

Orchidaceae is the largest family of flowering plants, and contains more than 25,000 species in approximately 880 genera. Orchid flowers exhibit zygomorphy of perianth organs: three sepals in whorl 1, three petals with the ventral one being strongly modified into a lip in whorl 2, a column is a compound structure formed by the fusion of one functional stamen with the three stigmas in whorl 3 and 4. The sepals and petals in an orchid flower show

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almost similar phenotype; however, the lip has a different size and more complex shape than the remaining perianth segments (Rudall and Bateman, 2002).

To date, genes in the ABCE model have been characterized in several orchid genera, including *Phalaenopsis* (Tsai *et al*., 2004, 2005, 2008; Chen *et al*., 2007; Su et al., 2013; Pan *et al*., 2014), *Oncidium* (Hsu and Yang, 2002; Hsu *et al*., 2003; Chang *et al*., 2009, 2010), *Dendrobium* (Yu and Goh, 2000; Skipper *et al*., 2005; Xu *et al*., 2006), and *Erycina* (Lin *et al*., 2016). To explain distinct tepal formation in orchids, two hypotheses have been proposed: a revised 'orchid code' and 'P code'. According to the revised 'orchid code', combinatorial expression patterns of duplicated *DEF*-like genes determine orchid perianth development (Mondragón-Palomino and Theißen, 2011). The expression of clade-1 and -2 genes and lack of expression of clade-3 and -4 genes leads to the development of sepals. Higher expression of clade-1 and -2 genes and lower expression of clade-3 and -4 genes is associated with the development of petals. By contrast, lower expression of clade-1 and -2 and higher expression of clade-3 and -4 genes specifies the development of the lip. According to the 'P-code' model, conserved competitive expression patterns of different *AP3(DEF)/AGL6* homologs are associated with the formation of sepals, petals and lip in orchids (Hsu *et al*., 2015); higher-order heterotetrameric SP complex (OAP3-1/OAGL6-1/OAGL6-1/OPI) specifies sepals and petals formation, whereas the L complex (OAP3-2/OAGL6-2/OAGL6-2/OPI) is required exclusively for lip formation (Hsu *et al*., 2015).

The genus *Habenaria* contains approximately 800 species and is one of the largest genera in orchids (Yokota, 1990). *Habenaria radiata* grows in wetlands in East Asia and is one of the popular orchids in Japan. Flowers of *H. radiata* are consisted of three greenish sepals (whorl 1), two white petals and a lip (whorl 2), and a column (whorls 3 and 4). In *H. radiata*, several mutant cultivars are known, such as 'Ryokusei' and 'Hishou'. Cultivar 'Ryokusei' has greenish flowers. The petals and lip are greenish and the column changed to greenish sepal-like organs. Recently, we isolated and characterized C- and E-class genes in

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the wild type and 'Ryokusei' (Mitoma and Kanno, 2018). Our results showed that the expression of *HrSEP-1* gene, which is one of E-class genes, was reduced in 'Ryokusei'. Furthermore, analysis of the genomic structure of *HrSEP-1* in the wild type and 'Ryokusei' shows that exon 1 of *HrSEP-1* in 'Ryokusei' harbors a retrotransposon *Hret1*, which suggests that the greenish mutant cultivar is caused by the insertion of retrotransposon in the *HrSEP-1* coding sequence (Mitoma and Kanno, 2018). Thus, our data show that *HrSEP-1* plays a key role in tepal and column development in *H. radiata*. Another mutant cultivar 'Hishou' has a white petaloid sepal and two white lip-like sepals instead of green sepals (Figure 1a). In orchid, there are peloric (actinomorphic mutant) and pseudopeloric (decreasing of the degree of zygomorphy) mutant (Bateman and Rudall, 2006). The flower of 'Hishou' looks like that of 'Hua-Guang-Die' which is a pseudopeloric mutant in *Cymbidium sinense* (Su et al., 2018). Among the pseudopeloric mutants, 'Hishou' seems to belong to Type D pseudopeloric although half of lateral sepals change to lip-like structures (Mondragón-Palomino and Theißen, 2009). Previously, we isolated and characterized the expression of a *DEF*-like gene (*HrDEF*) and two *GLO* genes (*HrGLO-1* and *HrGLO-2*), all of which are B-class genes (Kim *et al*., 2007). Our results showed that *HrGLO-1* and *HrGLO-2* exhibit similar expression patterns in the wild type and 'Hishou'. However, the expression pattern of *HrDEF* differs between the wild type and 'Hishou'; in the wild type, *HrDEF* is expressed in petals and lip, whereas in 'Hishou', *HrDEF* is expressed not only in petals and lip, but also in sepals. These results suggest that the floral phenotype of 'Hishou' is related to the wider range of *HrDEF* gene expression (Kim *et al*., 2007). However, there is no direct evidence of the relationship between *HrDEF* gene expression and the pseudopeloric phenotype of 'Hishou' flowers.

According to 'orchid code' and 'P code' mentioned above, *DEF*-like and *AGL6*-like genes regulate the development of distinct tepals in orchid flowers. Thus, we isolated these genes from *H. radiata* and characterized their expression in the wild type and 'Hishou' mutant cultivar. We also analyzed the genetic inheritance of the pseudopeloric phenotype

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among the progeny of an intraspecific cross between the wild type and 'Hishou'. Genetic linkage analysis revealed that a mutation in the *DEF*-like gene of 'Hishou' causes the pseudopeloric phenotype. We also investigated the molecular mechanism of the homeotic conversion of lateral greenish sepals to lip-like structures in 'Hishou'.

RESULTS

Isolation of *DEF*-like genes from *H. radiata*

In general, orchid genomes sequenced so far harbors four *DEF*-like genes. We isolated these four *DEF*-like genes from *H. radiata* by rapid amplification of cDNA ends (RACE) using gene-specific primers. Phylogenetic analysis using maximum-likelihood method showed that these genes clustered into four phylogenetic clades of orchid *DEF*-like genes, clade-1, -2, -3 and -4, and were named *HrDEF-C1, HrDEF-C2, HrDEF-C3* and *HrDEF-C4*, respectively (Figure 1b). *HrDEF-C1, HrDEF-C2, HrDEF-C3* and *HrDEF-C*4 encode four putative MADS proteins with 227, 220, 223 and 233 amino acids, respectively (Fig. 2). Amino acid sequence alignments of *HrDEF*-like proteins with other MADS-box proteins showed that three *HrDEF* proteins, *HrDEF-C1, HrDEF-C3* and *HrDEF-C4,* harbored the conserved MADS, K and C domains with the conserved PI-derived and paleo AP3 motifs. Although HrDEF-C2 harbored the conserved MADS and K domains, the end of C domain was not conserved among Orchid *DEF*-clade2 genes because many of them do not have PI-derived motif and paleoAP3 motif (Figure S1).

Expression analysis of B-class genes in *H. radiata*

We examined the expression of four *HrDEF*-like genes in the floral organs of the wild type and 'Hishou' using real time polymerase chain reaction (qRT-PCR) (Figure 1c). In the wild type, *HrDEF-C1* and *-C2* transcripts were detected in all floral organs, and these genes were

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highly expressed in petals. *HrDEF-C3* was expressed in petals, lip, and column, but not in sepals. Expression of *HrDEF-C4* was detected only in the petals and column.

In 'Hishou', *HrDEF-C1* was expressed in all floral organs, and its expression level in the petaloid sepal was higher than that in lip-like sepals. The *HrDEF-C2* gene was predominantly expressed in petals, and its expression level in the petaloid sepal was similar to that in lip-like sepals. The *HrDEF-C3* transcripts were detected in all floral organs, and *HrDEF-C4* was expressed in the petals and column. Expression patterns of *HrDEF-C1*, *-C2*, and *-C4* were similar between wild type and 'Hishou' flowers, whereas the expression of *HrDEF-C3* in 'Hishou' was also detected in whorl 1. These results indicate that differential expression of *HrDEF-C3* may be responsible for the homeotic conversion of sepals into petaloid sepal and lip-like sepals in 'Hishou'.

Inheritance of the pseudopeloric mutation in *H. radiata*

To investigate the inheritance of the pseudopeloric flower trait of 'Hishou', we performed intraspecific crosses between the wild type and 'Hishou' (Figure 2a). Since the female reproductive organ is sterile in 'Hishou' because the stigma is underdeveloped, crosses were made using the wild type plant ([WT] phenotype) as the female parent and 'Hishou' ([H] phenotype) as the male parent. A total of 230 F_1 hybrids were obtained from the intraspecific cross. In the F_1 generation, 186 plants produced flowers, of which 102 plants produced flowers with the mutant phenotype (F_1 [H]), and 84 plants produced flowers with the wild type phenotype (F_1 [WT]). Since F_1 [H] of the 'Hishou' type flower was female-sterile, like the 'Hishou' cultivar, we used F_1 plants with 'Hishou' type flower phenotype as the male parent and backcrossed them with the wild type as the female parent to produce the BC_1 progeny. The BC_1 progeny comprised 208 plants, of which 134 plants produced flowers. Of these, 70 plants produced 'Hishou' type flowers, and 64 plants produced wild type flowers. Additionally, F_1 plants with wild type flower phenotype were self-pollinated, which produced

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268 F_2 plants. Of the 268 F_2 plants, 94 plants produced flowers, all of which were wild type. These results suggest that the pseudopeloric mutation of 'Hishou' is a dominant gain-of-function mutation. Moreover, both F_1 and BC_1 populations showed 1:1 segregation for the 'Hishou' type and wild type flower phenotype. This suggests that the mutant allele is heterozygous.

PCR-restriction fragment length polymorphism (RFLP) analyses

Our results showed that the pseudopeloric trait of 'Hishou' was correlated with ectopic expression of *HrDEF-C3* and 'Hishou' character was inherited dominantly. To verify the relation between genetic inheritance of the pseudopeloric trait and *HrDEF-C3* gene expression, we performed PCR-restriction fragment length polymorphism (RFLP) analyses. In a previous study (Kim *et al*., 2010), we identified seven sequences in the C-terminal region of *HrDEF-C3* cDNA that were polymorphic between the wild type and 'Hishou'; these sequences likely represent cultivar-specific polymorphisms. Therefore, we used the C-terminal region of *HrDEF-C3* for PCR-RFLP analyses with *Hin*1II restriction enzyme (Figure S2), which cleaves homozygous wild type (WT/WT) DNA into two fragments (123 and 258 bp), homozygous 'Hishou' (H/H*) DNA into four fragments (102, 21, 180, and 78 bp), although the 21 and 78 bp fragments could not be detected in 2% agarose gel (Figure 2b), and heterozygous F_1 (WT/H) DNA into four fragments (102, 123, 180, and 258 bp). H* shows allele which cause 'Hishou' character. Genotyping the BC_1 progeny revealed that plants with 'Hishou' flowers were heterozygous at the *HrDEF-C3* locus (WT/H*), whereas plants with WT flowers carried the WT allele of *HrDEF-C3* in the homozygous state (WT/WT). The F_2 progeny showed three genotypes at the *HrDEF-C3* locus: WT/WT, WT/H, and H/H (Figure 2a, b). These data suggest that *HrDEF-C3* is associated with the 'Hishou' flower phenotype.

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Structural analysis of the *HrDEF-C3* gene

To identify the cause of ectopic expression of *HrDEF-C3* in 'Hishou', we compared the promoter sequences of *HrDEF-C3* between the wild type and 'Hishou'. Approximately 2,500 bp sequence upstream of the start codon (ATG) of *HrDEF-C3* was isolated with Genome Walker using *HrDEF-C3* promoter-specific primers. PCRs using these primers produced one band of approximately 400 bp in the wild type, but two bands of approximately 400 bp and 5.4 kb in 'Hishou' (Figure 3a). Sequences of the 400 bp fragments in 'Hishou' and the wild type were identical. However, the 5.4 kb fragment carried an insertion (Figure 3b and S1). The sequence of this insertion showed the typical features of *Ty1/Copia*-like retrotransposon, and we named this insertion *Habenaria* retrotransposon 2 (*Hret2*). The *Hret2* retrotransposon was 5,052 bp long, and included a target site duplication (TSD; 6 bp, AGAGAT), followed by a long terminal repeat (LTR; 306 bp), group-specific antigen (GAG; 419 bp), integrase (IN; 284 bp), reverse transcriptase (RT; 728 bp), ribonuclease H (RH; 446 bp), LTR (306 bp), and TSD (6 bp). Since two types of *HrDEF-C3* promoters were identified in 'Hishou', *HrDEF-C3* with the wild type promoter and *HrDEF-C3* with *Hret2*-containing promoter are hereafter referred to as *HrDEF-C3*^{W} and *HrDEF-C3*^{P}, respectively.

To analyze the relationship between *Hret2* insertion in the *HrDEF-C3* promoter and 'Hishou' type flower phenotype, we investigated the association between the flower phenotypes and *HrDEF-C3* genotypes among F_1 (72 individuals), F_2 (76), and BC_1 (128) populations by PCR using *HrDEF-C3* promoter-specific primers (F1 and R1) (Figure 3c). Both *HrDEF-C3*^{P} and *HrDEF-C3*^{W} promoters were amplified from progeny with 'Hishou' type flowers, but only *HrDEF-C3*^{W} promoter was amplified from progeny with wild type flowers (Figure 3c). Since all progeny harboring the *HrDEF-C3*^{P} gene produced 'Hishou' type flowers, we conclude that the pseudopeloric mutation is caused by the insertion of retrotransposon in the *HrDEF-C3* promoter.

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Expression of *HrDEF-C3^P* in 'Hishou'

We investigated the expression of *HrDEF-C3^W* and *HrDEF-C3^P* in floral organs, leaf, root, and bulb of the wild type and 'Hishou' using semi-quantitative reverse-transcription PCR (RT-PCR). In the wild type, expression of *HrDEF-C3^W* was detected in the petals, lip, and column, but not in other organs (Figure 4). By contrast, *HrDEF-C3^W* expression in 'Hishou' was detected in all floral organs but not in other organs. Interestingly, *HrDEF-C3^P* transcripts were detected in all floral organs as well as in leaf, root, and bulb (Figure 4). These results showed that *HrDEF-C3^W* was expressed not only in whorls 2, 3, and 4, but also in whorl 1 in 'Hishou', and *HrDEF-C3^P* was expressed in all organs of 'Hishou'.

Isolation and characterization of *AGL6* genes in *H. radiata*

Although the *HrDEF-C3* gene was expressed in all floral organs and some vegetative organs in 'Hishou', only two lateral sepals were changed to lip-like structures in this cultivar. Since *AGL6*-like genes play an important role in the distinctive tepal morphology (Hsu *et al*., 2015), we isolated two *AGL6*-like genes, *HrAGL6-C1* (LC424959) and *HrAGL6-C2* (LC424960), from wild type *H. radiata* (Figure 5a). Full-length cDNAs of *HrAGL6-C1* (953 bp) and *HrAGL6-C2* (912 bp) encoded 243 and 240 aa proteins. These genes contain the MADS-, I-, K- and C-domains. In addition, both HrAGL6-C1 and HrAGL6-C2 harbored the AGL6-I and -II motifs at the C-terminal ends (Fig. S4, Ohmori et al., 2009). Amino acid sequences of HrAGL6-C1 and HrAGL6-C2 share 66% identity.

Next, we analyzed the expression patterns of *HrAGL6-C1* and *HrAGL6-C2* genes in floral organs of the wild type and 'Hishou' using qRT-PCR. In the wild type, *HrAGL6-C1* showed a strong expression in dorsal and lateral sepals but weak expression in the petals, lip, and column, whereas *HrAGL6-C2* was expressed in the lateral sepals, lip, and column (Figure 5b). In 'Hishou', the expression of *HrAGL6-C1* was detected in dorsal sepal, lateral sepals, petals, and column, with higher expression in the column than in other organs, whereas

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HrAGL6-C2 expression was detected in lateral sepals, lip, and column, with higher expression in lateral sepals than in other organs.

DISCUSSION

Expression patterns of *HrDEF*-like and *HrAGL6*-like genes were consistent with 'orchid-code' and 'P-code' models

According to the 'orchid code' and 'P code', *DEF*- and *AGL6*-like genes play important roles in the morphological differentiation of tepals in orchids (Mondragón-Palomino and Theißen, 2011; Hsu *et al*., 2015). Among orchid species, the expression pattern of four *DEF*-like genes is almost conserved. The *DEF*-like genes in clade-1 and -2 are expressed in all floral organs (Tsai *et al*., 2004; Mondragón-Palomino and Theißen, 2011). The clade-3 *DEF*-like genes are expressed in petals, lip, and column, but not in sepals (Tsai *et al*., 2004; Xu *et al*., 2006; Mondragón-Palomino and Theißen, 2011; Hsu *et al*., 2015; Kim *et al*., 2007), whereas clade-4 *DEF*-like genes are specifically expressed in the lip (Mondragón-Palomino and Theißen, 2011; Xiang *et al*., 2017). In this study, we isolated four *DEF*-like genes (*HrDEF-C1*, *-C2*, *-C3*, and *-C4*) from *H. radiata* and investigated their expression pattern in wild type *H. radiata* and mutant cultivar 'Hishou'. The expression of *HrDEF-C1* was predominantly in petals than in sepals and lip in wild type and 'Hishou', these results suggest that *HrDEF-C1* is important for the development of petaloid organs. The expression of the *HrDEF-C2* gene was detected in all floral organs in the wild type and 'Hishou', suggesting that *HrDEF-C2* gene has pleiotropic roles in tepal development. The *HrDEF-C3* was not expressed in sepals in wild type, whereas *HrDEF-C3* was expressed in petaloid sepal and lip-like sepals in 'Hishou'. This expression pattern of the *HrDEF-C3* gene is consistent with our previous report (Kim et al., 2007). Expression patterns of *HrDEF-C1*, *-C2*, and *-C3* in the wild type were consistent with those in other orchid species, and these expression data almost fit the 'orchid code' (Mondragón-Palomino andTheißen, 2011). The expression of *HrDEF-C4* was

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detected in petals and column in the wild type and 'Hishou', indicating that *HrDEF-C4* is not required for the establishment of lip identity in *H. radiata*. Among the four *HrDEF*-like genes, the most remarkable difference in the expression pattern was observed in *HrDEF-C3*, which showed ectopic expression in 'Hishou'; *HrDEF-C3* is most likely associated with the pseudopeloric mutation.

On the other hand, according to the 'P-code' hypothesis, the identity of perianth organs depends on the expression levels and interactions among B- and E-class genes (Hsu *et al*., 2015). The L quartet (OAP3-2/OAGL6-2/OAGL6-2/OPI) specifies lip formation, whereas the SP quartet (OAP3-1/OAGL6-1/OAGL6-1/OPI) determines sepals/petals formation. Expression patterns of *HrAGL6-C1* and *-C2* genes in wild type *H. radiata* were similar to those of their orthologs in *H. ciliolaris* and *H. rhodocheila* (Hsu *et al*., 2015). Additionally, expression patterns of *HrAGL6-C1* and *HrAGL6-C2* in wild type *H. radiata* were consistent with those in other orchid species, and almost fit the 'P-code' model. Comparative expression analyses of *HrAGL6-C1* and *HrAGL6-C2* suggested that *HrAGL6-C1* is important for the establishment of greenish sepals but not of petals and lip, whereas *HrAGL6-C2* gene is important for the formation of lip-like structures.

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Pseudopeloric mutation is caused by the retrotransposon insertion in the *HrDEF-C3* promoter

Intraspecific cross between the wild type and 'Hishou' demonstrated that the pseudopeloric mutation was inherited dominantly, and the locus responsible for the pseudopeloric mutation was most likely heterozygous in 'Hishou' (Figure 2). In our previous study (Kim et al. 2010), we obtained intraspecific hybrids between wild-type and 'Hishou' in order to investigate the inheritance of pseudopeloric phenotype ('Hishou' characters). Since F_1 progeny had two types of flower with 'Hishou' type and wild-type plants, we suggested that pseudopeloric phenotype inherited dominantly (Kim et al., 2010). In this study, we investigated the

inheritance of pseudopeloric phenotype and we obtained F_2 and BC_1 generations. Since the half of the BC_1 had wild-type flowers and the other half had pseudopeloric phenotype, and all F_2 plants performed by self-pollination of F_1 [WT] plants had wild type flower, the locus of pseudopeloric mutation was considered to be heterozygous in 'Hishou'. As shown in Fig. 2, PCR-RFLP analyses revealed that *HrDEF-C3* is linked to the pseudopeloric phenotype.

Comparative sequence analysis of the *HrDEF-C3* gene in the wild type and 'Hishou' revealed the insertion of *Hret2* retrotransposon in the *HrDEF-C3* promoter in 'Hishou'. *Hret2* is a *Ty1/Copia*-like retrotransposon, similar to the *Hret1* retrotransposon isolated from the greenish flower mutant cultivar 'Ryokusei' (Mitoma and Kanno, 2018). *Hret2* (5,052 bp) is longer than *Hret1* (4,534 bp), and PCR analysis showed that both retrotransposons exist in the wild type genome (Figure S3). Our results showed that the 'Hishou' genome harbors two types of allelic *HrDEF-C3* genes: *HrDEF-C3*^{W}, which is identical to the wild type gene, and *HrDEF-C3*^{P}, which carries *Hret2* in its promoter (Figure 3a and 3b). Genotyping the wild type, 'Hishou' and their progeny using promoter-specific primers revealed that 'Hishou' and all progeny exhibiting 'Hishou' flowers were heterozygous at the *HrDEF-C3* locus (*HrDEF-C3*^{W}/*HrDEF-C3*^{P}), whereas the wild type and all progeny with wild type flowers were homozygous for the wild type allele of *HrDEF-C3* (*HrDEF-C3*^{W}/*HrDEF-C3*^{W}) (Figure 3c). These results suggest that the pseudopeloric mutation is linked to *HrDEF-C3*^{P}, indicating that pseudopeloric mutation is caused by *Hret2* insertion in the promoter region of *HrDEF-C3*.

Molecular mechanism of pseudopeloria in 'Hishou' cultivar

The expression of *HrDEF-C3* in whorl 1, in addition to other whorls, in 'Hishou' implied that *Hret2* insertion might affect the expression of the *HrDEF-C3* gene. To explore the relationship between retrotransposon insertion and the expression pattern of *HrDEF-C3*, we performed RT-PCR with *HrDEF-C3*^{W}- and *HrDEF-C3*^{P}-specific primers to examine the expression of these genes in the wild type and 'Hishou' (Figure 4). Transcripts of *HrDEF-*

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C3^{W} were detected in petals, lip, and column in the wild type, whereas in 'Hishou', *HrDEF-C3*^{P} transcripts were detected in all floral organs as well as in leaf, root, and bulb. These results suggest that a part of the *Hret2* sequence might work as a promoter of *HrDEF-C3*, resulting in the ectopic expression of *HrDEF-C3*. Notably, *HrDEF-C3*^{W} in 'Hishou' was expressed in whorl 1, whorl 2, and column but not in vegetative organs. This expression pattern of *HrDEF-C3*^{W} in 'Hishou' might be related to the autoregulation of GLO and DEF proteins (Saedler and Huijser, 1993). The GLO and DEF proteins heterodimerize and bind to CArG sequences in the promoter regions of *GLO* and *DEF* genes, thus upregulating their own expression. In *H. radiata*, two *GLO*-like genes, *HrGLO-1* and *HrGLO-2*, are expressed in all floral organs (Kim *et al*., 2007). Since the expression of *HrDEF-C3*^{P} was expanded to whorl 1 in 'Hishou', it is possible that HrDEF-C3^{P} forms a heterodimer with HrGLO proteins and induces the expression of the *HrDEF-C3*^{W} gene in sepals.

Although *HrDEF-C3* expression was expressed in all floral organs and in some vegetative organs in 'Hishou', only two lateral sepals and dorsal sepal were transformed into lip-like and petal-like structures, respectively. The effect of the ectopic expression of *HrDEF-C3* in 'Hishou' on the homeotic conversion of three sepals is intriguing. According to the 'P-code' model, the higher-order heterotetrameric SP complex (OAP3-1/OAGL6-1/OAGL6-1/OPI) specifies sepals/petals formation, whereas the L complex (OAP3-2/OAGL6-2/OAGL6-2/OPI) is exclusively required for lip formation (Hsu *et al*., 2015). Here, we isolated and characterized two *AGL6*-like genes, *HrAGL6-C1* and *-C2*, from *H. radiata*, and showed that *HrAGL6-C2* was expressed in lateral sepals and lip but not in the petals and dorsal sepal (Figures 5 and 6). These expression patterns suggest that HrAGL6-C2 forms the L complex with HrDEF-C3 in lateral sepals, resulting in homeotic change from lateral sepals to lip-like structure in 'Hishou', whereas HrDEF-C1, HrAGL6-C1, and HrDEF-C3 likely form the SP complex in dorsal sepal, resulting in homeotic change from greenish dorsal sepal to petaloid sepal in 'Hishou'.

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It is generally assumed that extant orchids originate from a recent common ancestor that lived in the Late Cretaceous (76–84 million years ago) and fast increase in diversity occurred at around 65 million years ago (Ramírez et al., 2007). The number of orchid *DEF*-like genes are generally four members (Mondragón-Palomino and Theißen, 2011). In contrast, analysis of Asparagales species showed that there are two *DEF*-like genes (Miura et al., 2019). It is possible that the four *DEF*-like genes in Orchidaceae were increased by a result of the whole-genome duplication and gene duplications via 62 million years ago (Mondragón-Palomino et al., 2009), after that four *DEF*-like genes caused the sub- and neo-functionalization in Orchidaceae. In this study, we clarified that *DEF*-clade3-like *HrDEF-C3* gene involved with development of lip. In addition, we suggested L complex (HrDEF-C3/HrAGL6-C2/HrAGL6-C2/HrGLO) is necessary for lip formation. Our results strongly support the four *DEF*-like genes have acquired different functions in the course of evolution.

In conclusion, we showed that the pseudopeloric trait in *H. radiata* is caused by the insertion of *Hret2* retrotransposon in the *HrDEF-C3* promoter. This insertion altered the spatial expression pattern of *HrDEF-C3*, causing it to be expressed in some vegetative organs as well as in the floral organs. Since *HrAGL6-C2* expression was limited to lateral sepals and lip, homeotic conversion to lip-like structure occurred only in lateral sepals. We proved that pseudopeloric mutation occurs as a result of ectopic expression of *HrDEF-C3*.

EXPERIMENTAL PROCEDURES

Plant materials

Habenaria radiata ‘Aoba’ and ‘Ginga’ (wild type cultivars) and ‘Hishou’ (pseudopeloric mutant cultivar) were used in this study. These cultivars were grown in a greenhouse at the Graduate School of Life Sciences, Tohoku University, Japan. Tissues were collected from flower buds (0.7–1.0 cm) and stored at -80 °C, until needed for RNA extraction. For gene

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expression analysis, sepals, lip-like sepals, petaloid sepals, petals, lip, and column were dissected from 5–10 flowers of the wild type and 'Hishou'.

Cloning and characterization of *DEF* and *AGL6*-like genes from *H. radiata*

Total RNA was isolated from the entire flower buds of wild type cultivars using RNeasy Plant Mini Kit (QIAGEN). Poly $(A)^+$ mRNA was extracted from the total RNA using Dynabeads mRNA Purification Kit (Life Technologies). First strand cDNA was synthesized from mRNA by AMV Reverse Transcriptase (Roche) using oligo dT primers (P019HA and P019HH) for the wild type and 'Hishou', respectively. The *HrDEF* and *HrAGL6* cDNAs were isolated by 3′RACE using degenerate primers specifically targeting the MADS domain. The amplification products were checked by agarose gels and purified using QIAquick Gel Extraction Kit (QIAGEN). Purified PCR products were then cloned into the pGEM-T Easy Vector (Promega). The 5' region of transcripts was obtained by 5' RACE method using 5'/3'RACE Kit, 2nd Generation (Roche). Primers used for the isolation of MADS-box genes are listed in Table S1.

Phylogenetic analysis

Predicted amino acid sequences of known MADS-box genes were downloaded from the EMBL/DDBJ/GenBank DNA database (Table S2 and S3). Full-length amino acid sequences were aligned using the ClustalW method. The phylogenetic analysis of *DEF*- and *AGL6*-like genes nucleotide sequences was constructed by maximum likelihood tree under 500 of bootstrap replicates with MEGA v7.0.26 software (Kumar et al., 2016).

Expression analysis of *HrDEF* and *HrAGL6* genes

Total RNA was isolated from sepals, petals, lips, and columns of wild type cultivars, and from the petaloid sepals, lip-like sepals, petals, lips, and columns of 'Hishou', and used for

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cDNA synthesis, as described above. The expression patterns of *HrDEF-C1*, *HrDEF-C2*, *HrDEF-C3*, *HrDEF-C4*, *HrAGL6-C1*, and *HrAGL6-C2* were examined by qRT-PCR using a MiniOpticon Real-time PCR Detection System with CFX Manager software (Bio-Rad) and gene-specific primers (Table S1). The cycling program was as follows: preheating at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 64 °C for 1 min, and lastly melt curve analysis (60–90 °C). All qRT-PCR experiments were performed in triplicate. *Eukaryotic translation elongation factor 1A* (*eEF1A*) was used as an internal control for standardization.

Isolation of the *HrDEF-C3* promoter from the wild type and 'Hishou' by genome walking

The modified hexadecyl trimethylammonium bromide (CTAB) method was used to obtain genomic DNA from *H. radiata* leaves. Genomic DNA was digested with four blunt-end restriction enzymes (*Dra*I, *Eco*RV, *Pvu*II, and *Stu*I) at 37 °C overnight. The digested DNA was ligated to a custom-designed adaptor from Genome Walker Kit (Clontech) at 16 °C overnight to generate genomic DNA libraries. The primary PCR amplification was conducted with each constructed genomic DNA libraries using the outer adaptor primer (AP1) provided in the kit and a *HrDEF-C3*-specific primer (GSP1). The nested adaptor primer (AP2) and a nested *HrDEF-C3*-specific primer (GSP2) were used for the secondary PCR with the primary PCR products. The secondary PCR products were cloned into the pGEM-T Easy Vector and sequenced. Primers used for sequencing the *HrDEF-C3* promoter are listed in Table S1.

For sequence analysis of retrotransposon-like structure in *HrDEF-C3* promoter, we performed genomic PCR. Genomic PCR was performed on genomic DNA from the leaves of wild type and 'Hishou' after adjusting the concentration as 100ng/µl. For genomic PCR, we used Tks Gflex DNA Polymerase (TaKaRa Bio Inc.) in a 25 µL reaction mixture containing 50 ng total DNA and P1-P4 primers (Table S1, 50 pmol of each primer) with a TaKaRa PCR Thermal Cycler Dice (TaKaRa Bio Inc.). The PCR consisted of an initial incubation step for

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1 min at 94°C, followed by 35 cycles at 98°C for 10 sec, 67°C for 15 seconds, and 68°C for 3 min.

Expression analysis of *HrDEF-C3^{W}* and *HrDEF-C3^{P}*

Total RNA was extracted from floral organs, leaf, root, and bulb, and cDNAs were synthesized as described above. *HrDEF-C3^{W}* gene specific primer pair was designed between the promoter and exon 1. The specific primer pair for *HrDEF-C3^{P}* was designed between *Hret2* and exon 1. PCR was performed in a 25 µl reaction containing an adjusted amount of first-strand cDNA, 10 pmol each of forward and reverse gene-specific primers, 0.5 mM dNTPs, 2.5 ml of 10× PCR buffer, and 0.5 units of Ex*Taq* DNA polymerase (Takara). The PCR conditions were as follows: preheating at 96 °C for 2 min, followed by 32 cycles of denaturation at 96 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The *eEF1A* gene was used as a control for the internal standardization.

PCR-RFLP analyses of intraspecific hybrids

Genomic DNA was extracted from leaves of the wild type, 'Hishou', and their progeny, as described by Honda and Hirai (1990). PCR was performed using Ex*Taq* and gene-specific primers designed in the C-terminal region of *HrDEF-C3* (Kim *et al*., 2010). The PCR conditions were as follows: denaturation at 96 °C for 2 min, followed by 30 cycles of denaturation at 96 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were digested with 5 units of *Hin*1II at 37 °C for 1 h. The digested samples were separated by electrophoresis on a 2% agarose gel to visualize DNA fragments.

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The *DEF*- and *AGL6*-like genes, *HrDEF-C1, C2, C4*, *HrAGL6-C1* and *C2* sequences have been deposited in the GenBank database with accession numbers LC424956, LC424957, LC424958, LC424959 and LC424960, respectively.

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AUTHOR CONTRIBUTIONS

M.M. and A.K. designed the study; M.M., Y.K., R.H., M.E., S.K., and A.K. performed the experiments; M.M., Y.K., and S.K. analyzed the data; M.M. and A.K. wrote the paper.

SUPPORTING INFORMATION

Figure S1. Alignment of the deduced amino acid sequence of clade 2 *DEF*-like genes. Positions with strictly conserved amino acids are highlighted in black and similar residues is denoted by gray. Boxes indicate the MADS domain, I region and K domain.

Figure S2. Structure of the *HrDEF-C3* gene in the wild type and 'Hishou' showing the location of *Hin*1II restriction sites in the C-terminal region and the insertion of retrotransposon (*Hret2*) in the promoter region. Dark gray boxes represent the *HrDEF-C3* gene. The *Hret2* retrotransposon is shown in a white box. Black triangles indicate PCR primers used in PCR-RFLP analyses. The sizes of *Hin*1II digestion products are indicated for WT/WT genotypes (one *Hin*1II recognition site; 123 and 258 bp products) and H/H* genotypes (three *Hin*1II recognition sites; 102, 21, 180, and 78 bp products).

Figure S3. PCR detection of the *Hret2* retrotransposon in the *HrDEF-C3* promoter.

(a) Schematic diagrams of the structure of *HrDEF-C3* promoter from the wild type and 'Hishou'. White boxes indicate exon 1 of *HrDEF-C3*; the ATG start codon is also shown. 'Hishou' has a *Hret2* retrotransposon in the promoter of *HrDEF-C3*.

(b) PCR analysis of *HrDEF-C3* from the wild type and 'Hishou'. PCR was performed using primer sets which are specific for the promoter region (P1), the first exon of *HrDEF-C3* gene (P2) and retrotransposon (P3 and P4), as shown in Fig. S3(a). Lane M; DNA MW Standard Marker; Lane 1, 'Ginga'; Lane 2, 'Aoba'; Lane 3, 'Hishou'.

Figure S4. Alignment of the deduced amino acid sequence of *AGL6*-like genes. Positions with strictly conserved amino acids are highlighted in black and similar residues is denoted by gray. Boxes indicate the MADS domain, I region, K domain, AGL6-I motif and AGL6-II motif.

Table S1. List of primers used in this study

Table S2. Accession numbers for the DEF-like genes used in the phylogenetic analysis

Table S3. Accession numbers for the AGL6-like genes used in the phylogenetic analysis

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Figure Legends

Figure 1. Floral phenotype and gene expression of *HrDEF*-like genes in wild type cultivar ‘Aoba’ and pseudopeloric mutant cultivar ‘Hishou’ of *Habenaria radiata*. (a) Flowers of the wild type (‘Aoba’), The wild type flower shows three greenish sepals, two white lateral petals, and a lip. (b) ‘Hishou’ (pseudopeloric mutant cultivar). The mutant ‘Hishou’ flower has a white petaloid organ, instead of a green dorsal sepal, and two green lateral sepals are replaced by white lip-like organs. Scale bars: 1 cm. (c) Phylogenetic analysis of *DEF*-like genes. The phylogenetic tree was constructed using the maximum-likelihood method. Genes

isolated from *H. radiata* are outlined in rectangles. Bootstrap values greater than 50% from 500 replicates are shown on the nodes. (d) qRT-PCR analysis of *DEF*-like gene expression in sepals (Se), petaloid sepal (W1P), lip-like sepals (W1L), petals (Pe), lip (Li), and column (Co) of the wild type and 'Hishou'. Data represent mean ± standard error (SEM) (n = 3). Arrows indicates *HrDEF-C3* expression in whorl 1.

Figure 2. Genetic linkage analysis of *HrDEF-C3* in the wild type and 'Hishou'. (a) Phenotypes and genotypes of the wild type and 'Hishou' (parents) and their progeny are shown. The F_1 showed 1:1 ([WT]:[H]) ratio. The F_2 progeny of self-fertilizing F_1 [WT] plants showed wild type phenotype only. The BC_1 progeny derived from the F_1 [H] × wild type cross showed 1:1 ([WT]:[H]) segregation ratio. The number of each progeny and their genotypes is indicated. The H* indicated allele that has 'Hishou' character. (b) PCR-restriction fragment length polymorphism (RFLP) analyses of intraspecific hybrids. PCR fragments of the wild type contained one *Hin*1II recognition site, whereas PCR fragments of 'Hishou' contained three *Hin*1II recognition sites.

Figure 3. Genomic structure and genetic linkage analysis of the *HrDEF-C3* gene in the wild type, 'Hishou', and their progeny. (a) PCR analysis of the *HrDEF-C3* promoter in the wild type and 'Hishou'. The *HrDEF-C3* promoter-specific primers (F1, R1) are indicated below. Multiplex amplification of wild type *HrDEF-C3* promoter (400 bp) and *HrDEF-C3* promoter carrying the retrotransposon insertion (ca. 5.4 kb) were separated by electrophoresis on a 1% agarose gel. (b) Structure of the *HrDEF-C3* promoter in the wild type and 'Hishou'. The genome of 'Hishou' harbors two types of *HrDEF-C3* promoters: wild type promoter (*HrDEF-C3*^{W}) and promoter harboring a retrotransposon (*HrDEF-C3*^{P}). The retrotransposon identified in the *HrDEF-C3* promoter was 5,052 bp long and contained 8 bp target site duplications (AGAGAT) and long terminal repeats (LTR, hatched) at both 5′ and 3′ ends. (c)

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Genetic linkage analysis of retrotransposon insertion in the *HrDEF-C3* gene promoter in the wild type, 'Hishou' and their progeny. A total of 72, 76, and 128 plants in the F_1, F_2, and BC_1 populations, respectively, were genotyped by PCR using *HrDEF-C3* promoter-specific primers (F1 and R1). All plants with 'Hishou' phenotype were heterozygous (*HrDEF-C3*^{W}/*HrDEF-C3*^{P}), and all plants with wild type phenotype were homozygous for the wild type allele (*HrDEF-C3*^{W}/*HrDEF-C3*^{W}).

Figure 4. Retrotransposon insertion is associated with ectopic expression of *HrDEF-C3*. (a) Amplification of *HrDEF-C3*^{W} and *HrDEF-C3*^{P} cDNAs in sepals (Se), petaloid sepals (W1P), lip-like sepals (W1L), petals (Pe), lip (Lip), column (Co), flower (F), leaf (L), root (R), and bulb (Bu) of the wild type and 'Hishou' using *HrDEF-C3*^{W}-specific primers (F1, R1) and retrotransposon-specific primers (F2, R1), as indicated. (b) Schematic representation of the expression pattern of *HrDEF-C3*^{W} and *HrDEF-C3*^{P} in the wild type and 'Hishou'. Organs in which *HrDEF-C3*^{W} or *HrDEF-C3*^{P} was expressed are indicated in yellow.

Figure 5. Phylogenetic and expression analyses of *AGL6*-like genes. (a) A phylogenetic tree was constructed using the maximum-likelihood method. Genes isolated from *H. radiata* are outlined in black. Bootstrap values greater than 50% are indicated on the nodes. (b) qRT-PCR analysis of *AGL6*-like genes in the wild type and 'Hishou'. Total RNAs were isolated from dorsal sepal (Ds), lateral sepals (Ls), petaloid sepals (W1P), lip-like sepals (W1L), petals (Pe), lip (Lip), and column (Co). Data represent mean ± standard error (SEM) (n = 3).

Figure 6. Schematic representation of the expression pattern of *HrDEF-C3* and *HrAGL6-C2* in wild type and 'Hishou'. The expression of *HrDEF-C3* and *HrAGL6-2* is shown in yellow and pink, respectively. Floral organs expressing both genes are shown in red.

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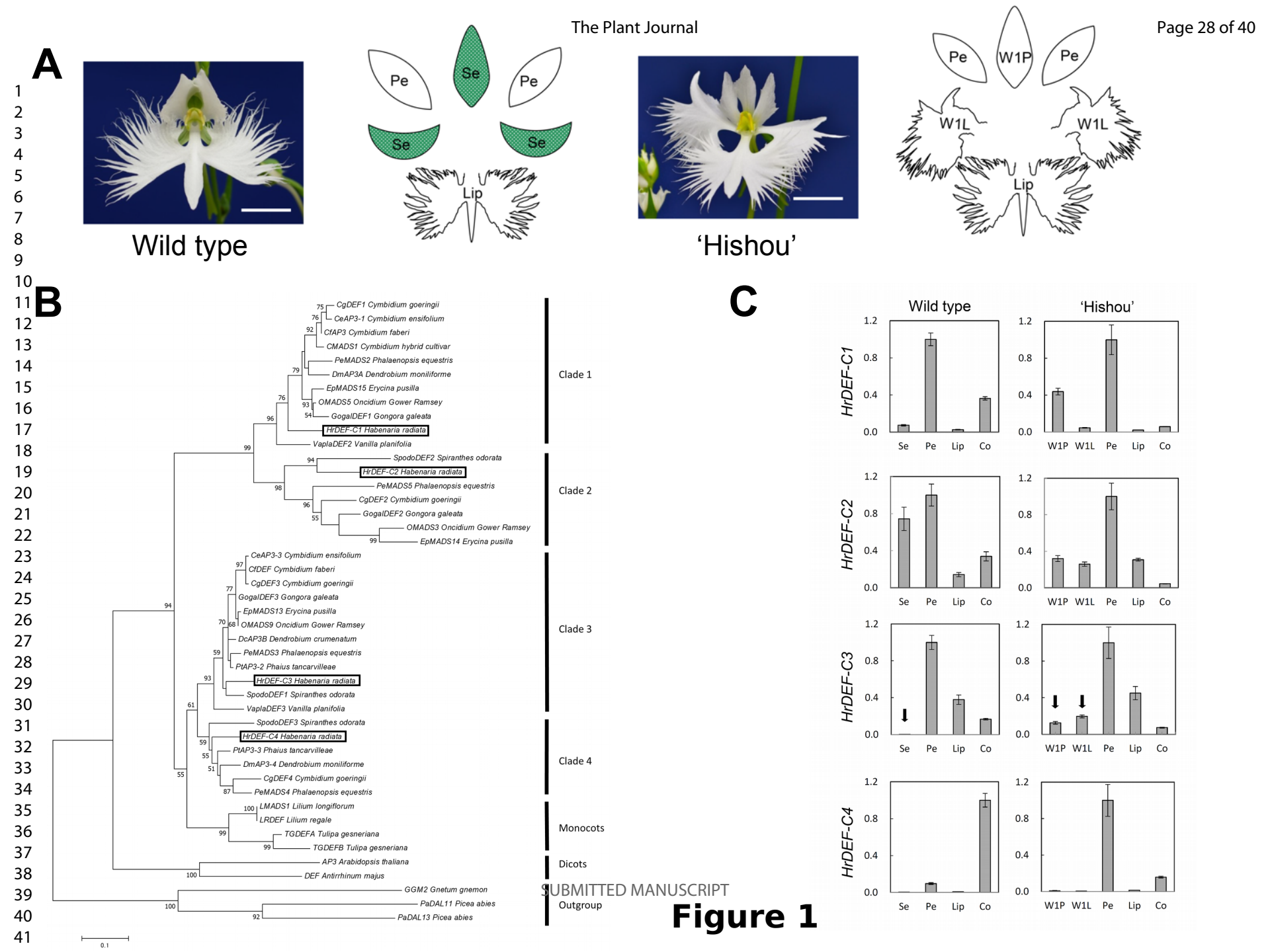

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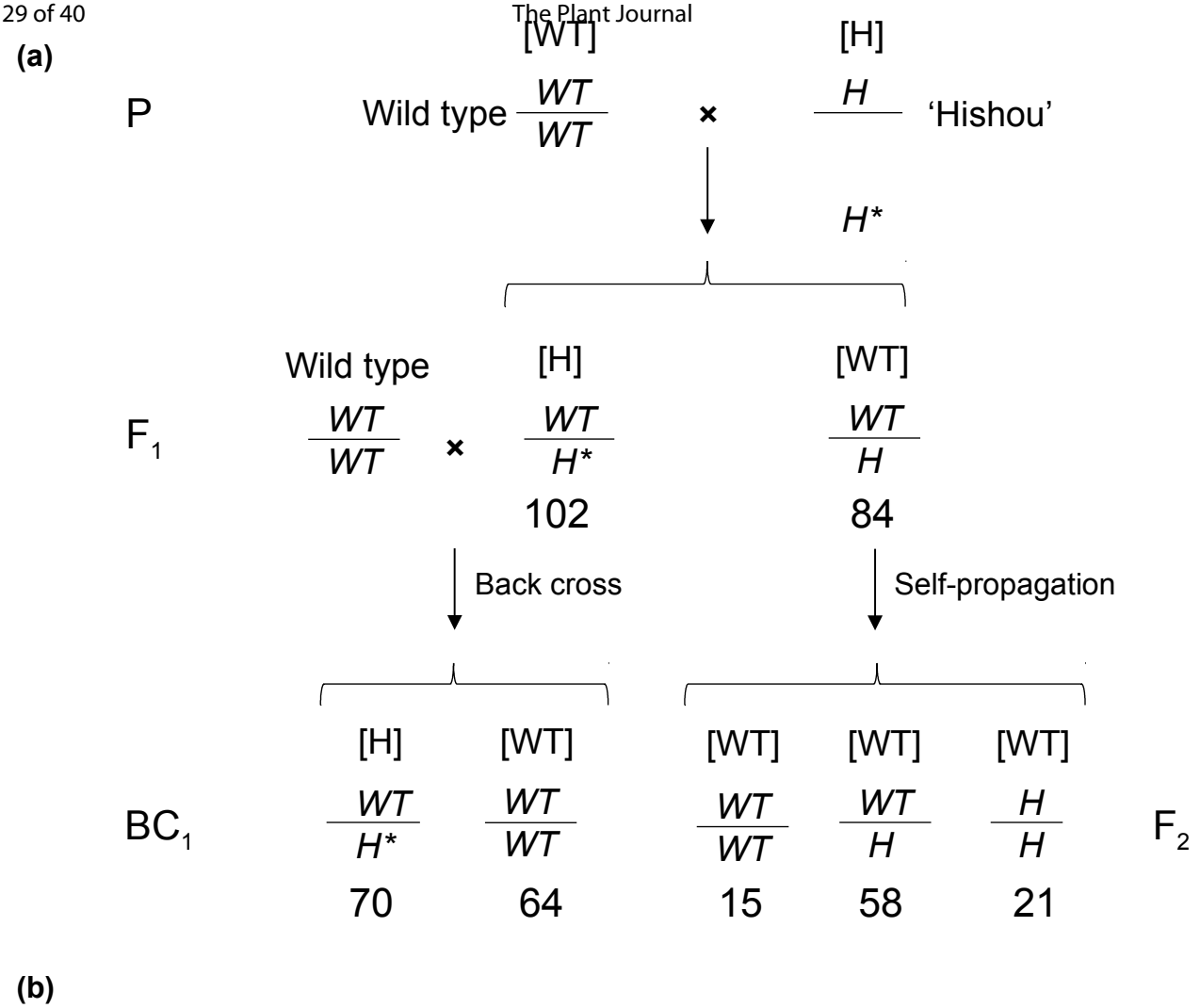
Figure 1


(a)
P
Wild type
[WT]
WT / WT
×
[H]
H
'Hishou'
H*
F1
Wild type
WT / WT
×
[H]
WT / H*
102
[WT]
WT / H
84
Back cross
Self-propagation
BC1
[H]
WT / H*
70
[WT]
WT / WT
64
F2
[WT]
WT / WT
15
[WT]
WT / H
58
[WT]
H / H
21

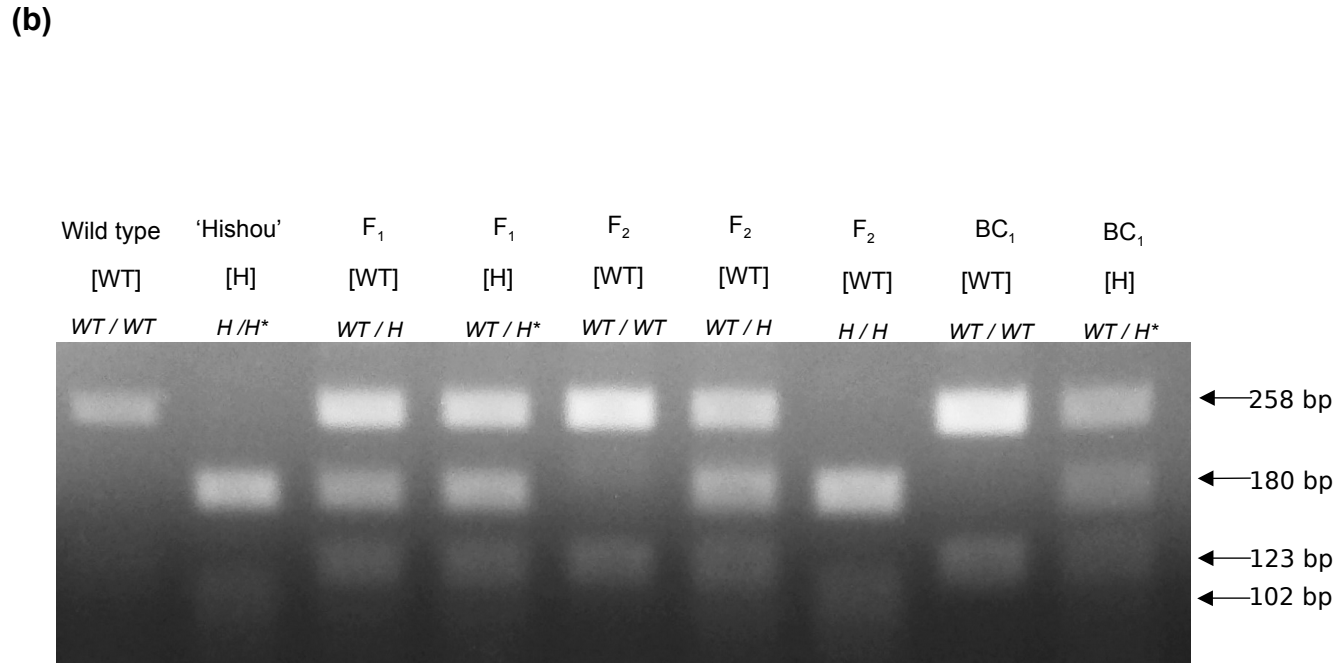

(b)
Wild type
[WT]
WT / WT
'Hishou'
[H]
H /H*
F1
[WT]
WT / H
F1
[H]
WT / H*
F2
[WT]
WT / WT
F2
[WT]
WT / H
F2
[WT]
H / H
BC1
[WT]
WT / WT
BC1
[H]
WT / H*
258 bp
180 bp
123 bp
102 bp

Figure 2

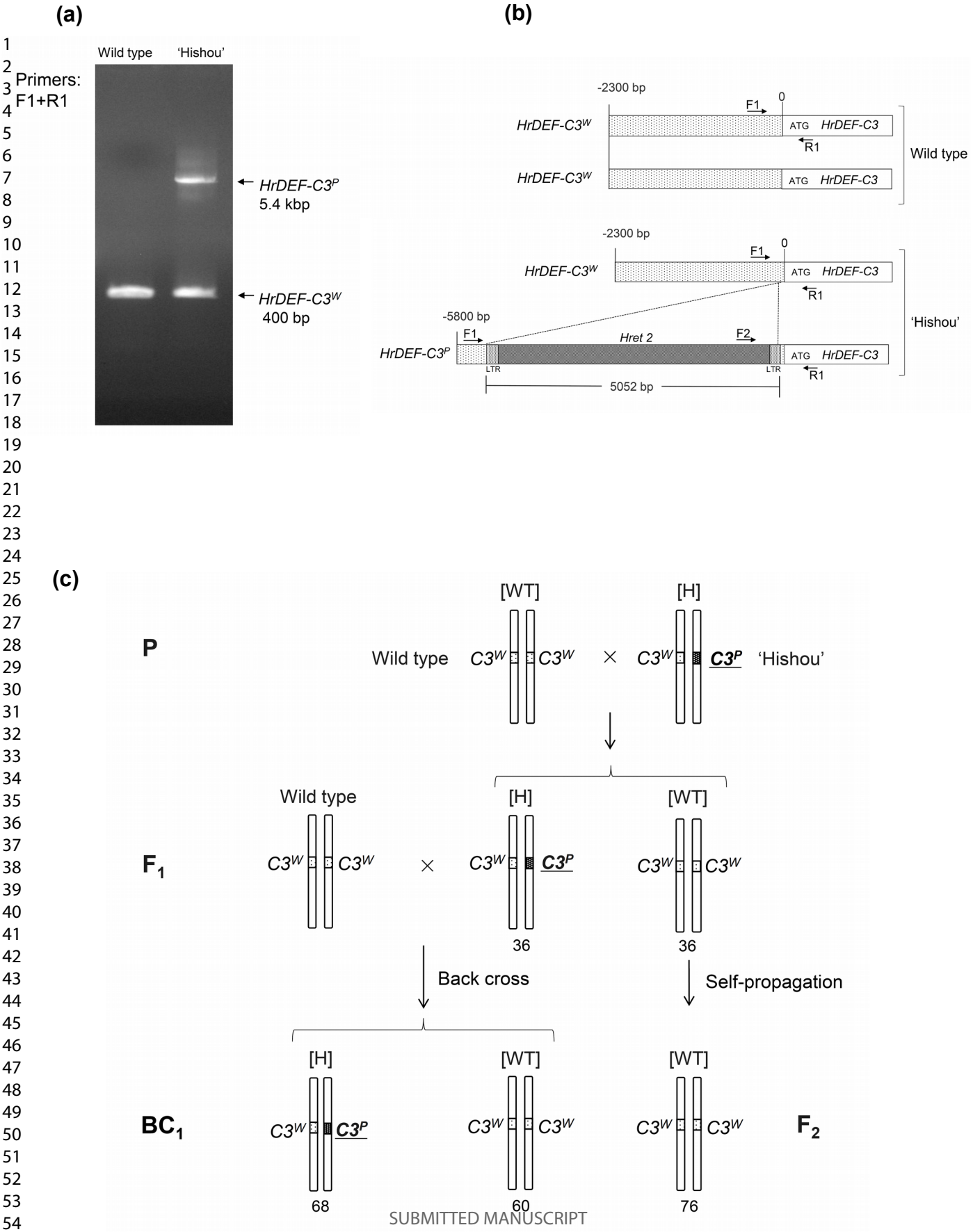


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Figure 3

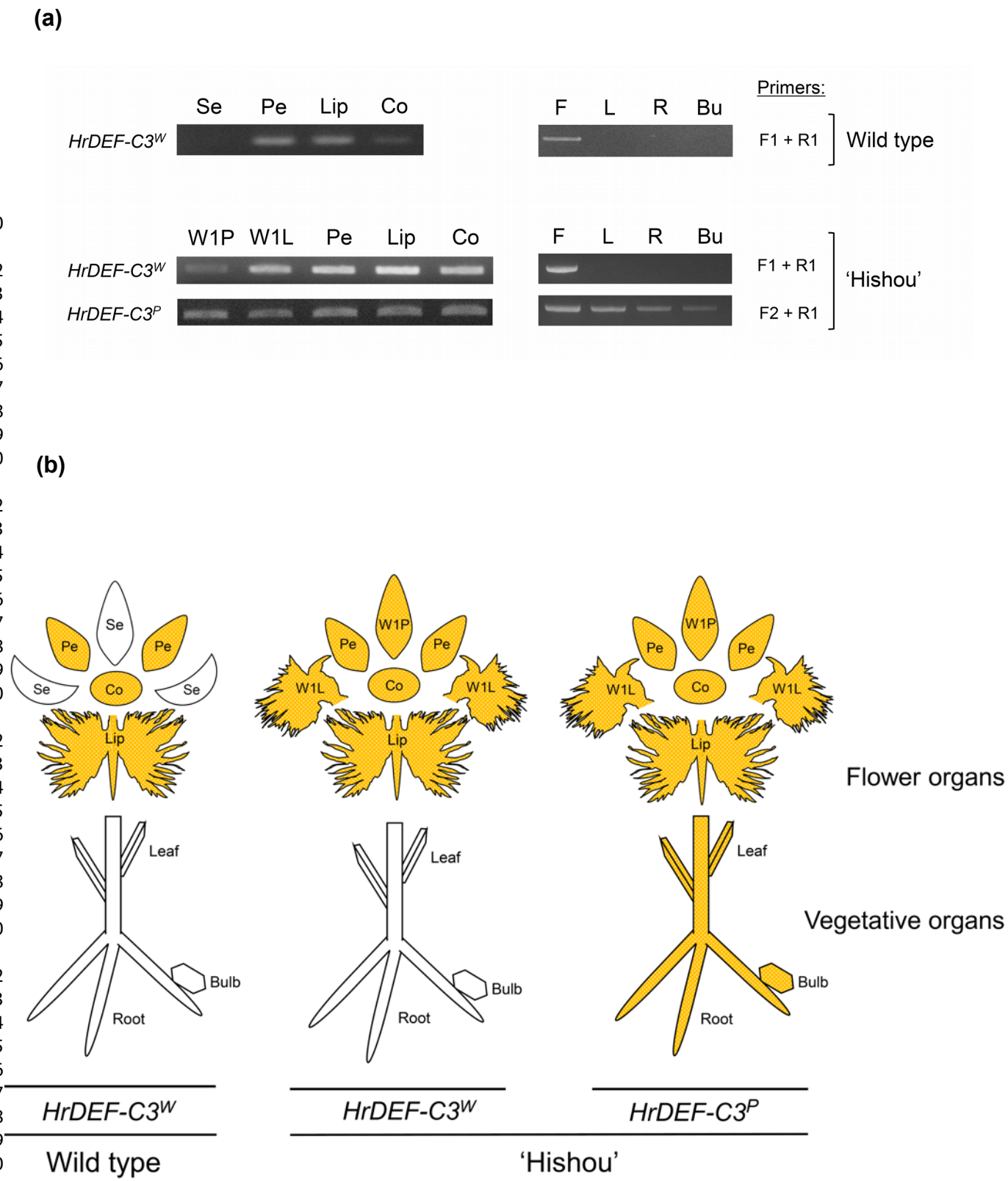
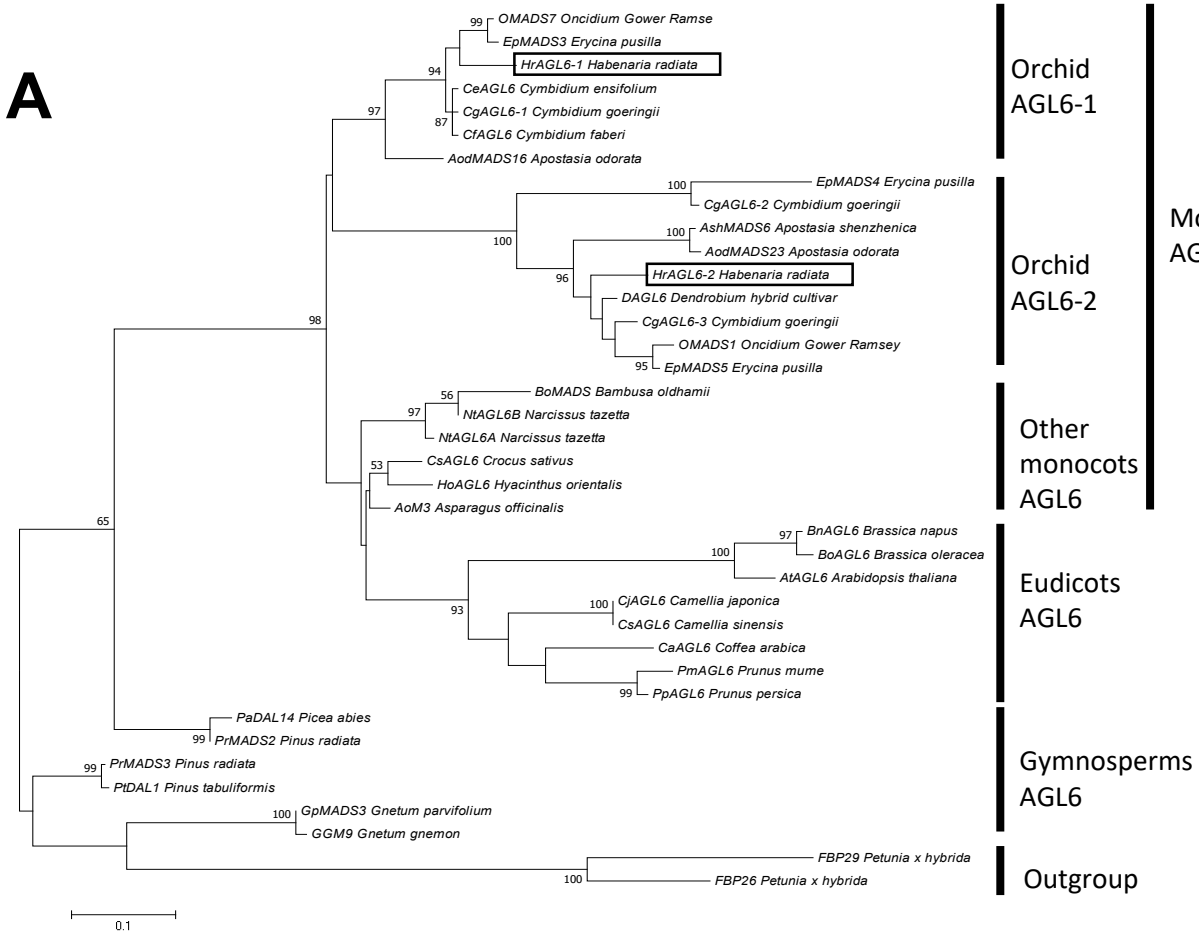

(a)
Primers:
Se Pe Lip Co
F L R Bu
HrDEF-C3W
F1 + R1
Wild type
W1P W1L Pe Lip Co
F L R Bu
HrDEF-C3W
HrDEF-C3P
F1 + R1
F2 + R1
'Hishou'
(b)
Se Pe Pe Se Co Se Lip
W1P Pe Pe W1L Co W1L Lip
W1P Pe Pe W1L Co W1L Lip
Flower organs
Leaf Bulb Root
Leaf Bulb Root
Leaf Bulb Root
Vegetative organs
HrDEF-C3W
HrDEF-C3W
HrDEF-C3P
Wild type
'Hishou'

Figure 4



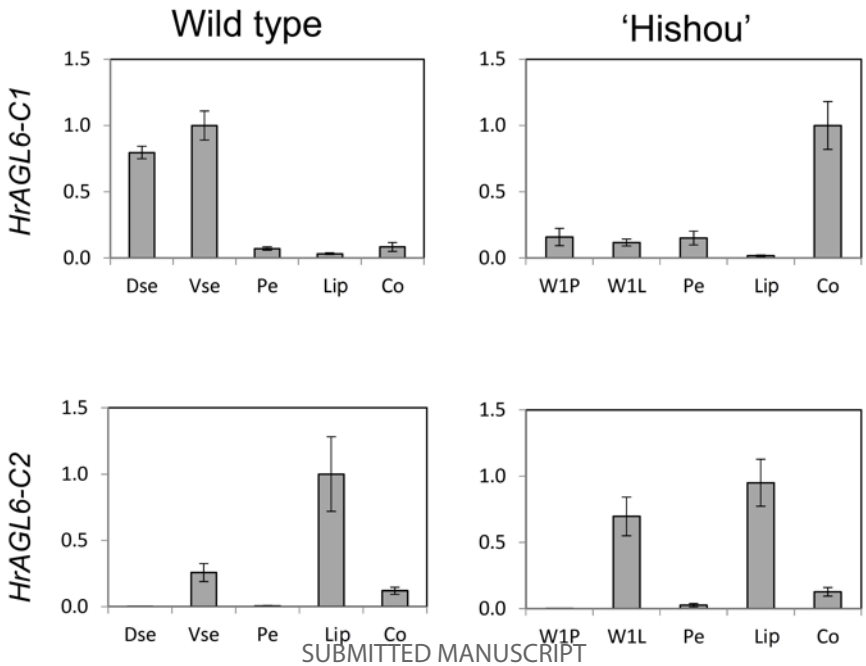


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Figure 5

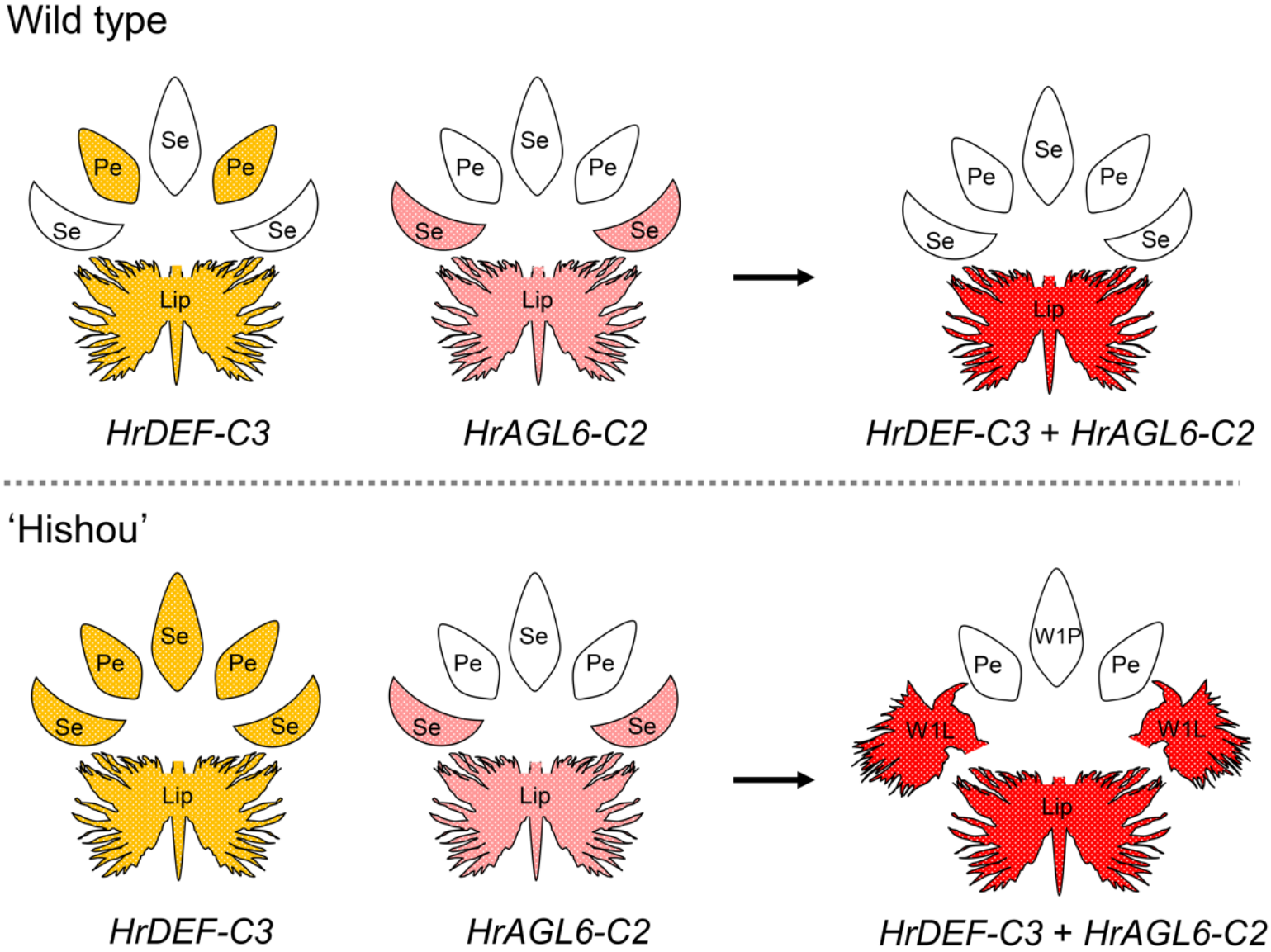
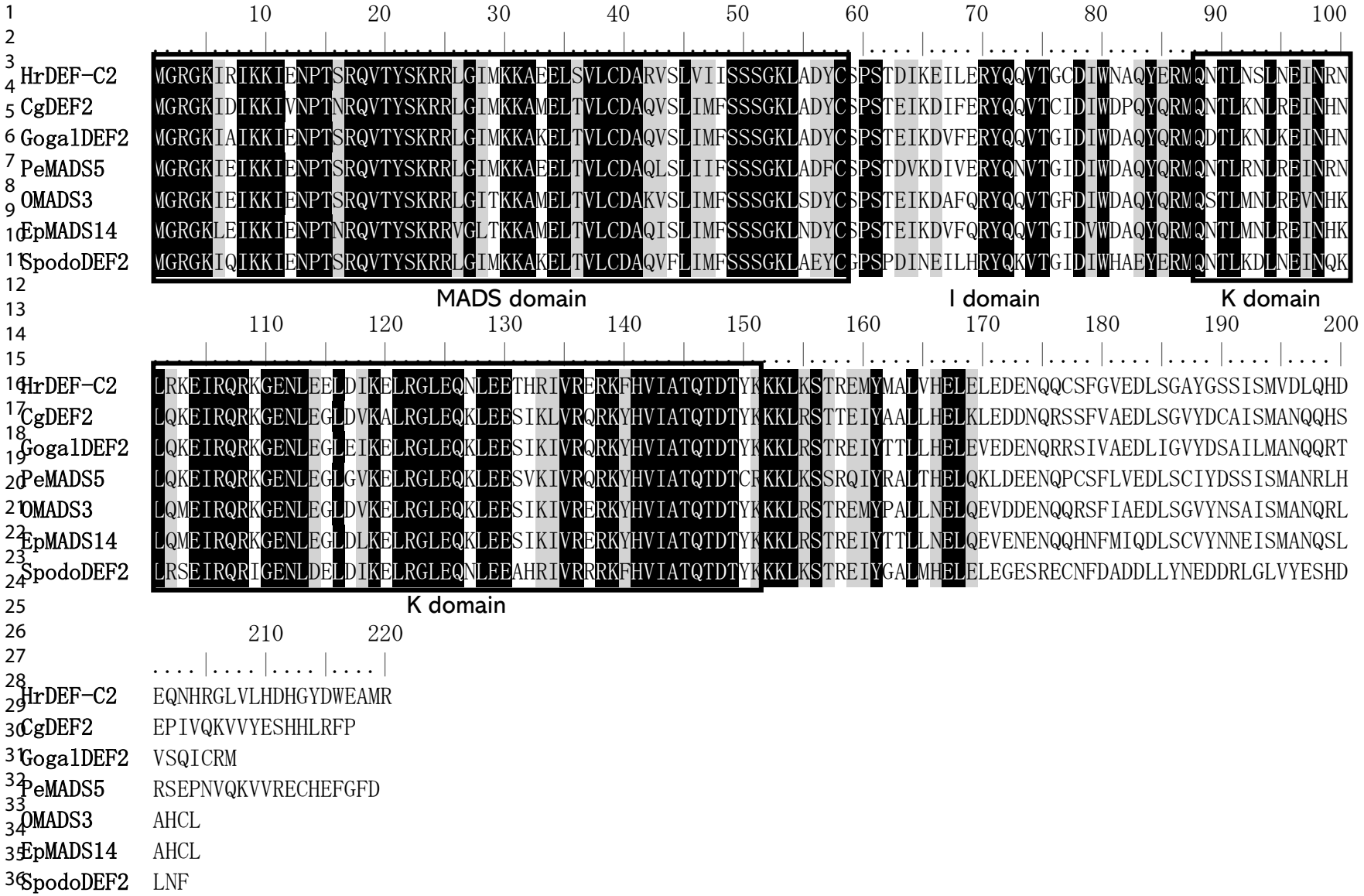
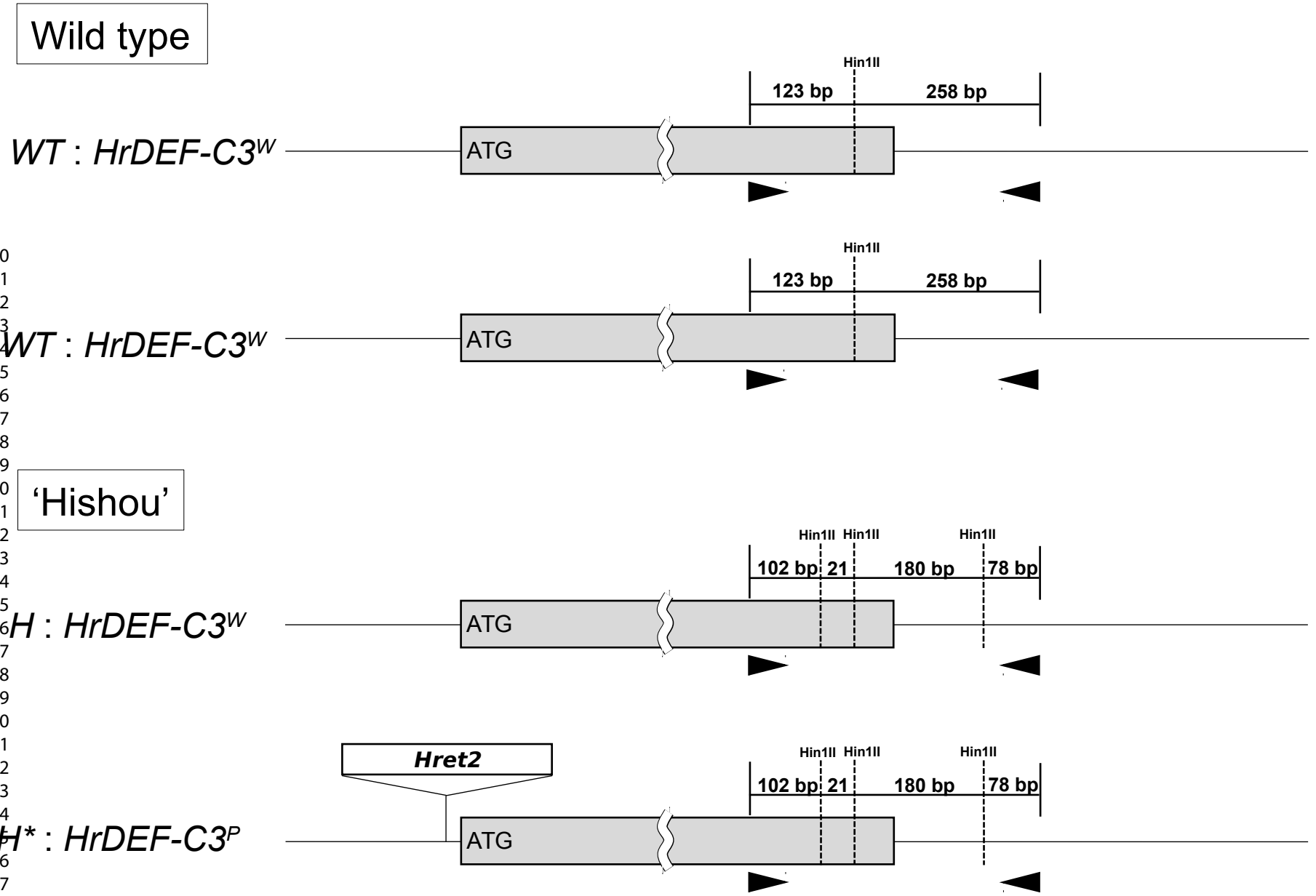

Wild type
Se
Pe
Pe
Se
Se
Lip
HrDEF-C3
Se
Pe
Pe
Se
Se
Lip
HrAGL6-C2
Se
Pe
Pe
Se
Se
Lip
HrDEF-C3 + HrAGL6-C2
'Hishou'
Se
Pe
Pe
Se
Se
Lip
HrDEF-C3
Se
Pe
Pe
Se
Se
Lip
HrAGL6-C2
W1P
Pe
Pe
W1L
W1L
Lip
HrDEF-C3 + HrAGL6-C2

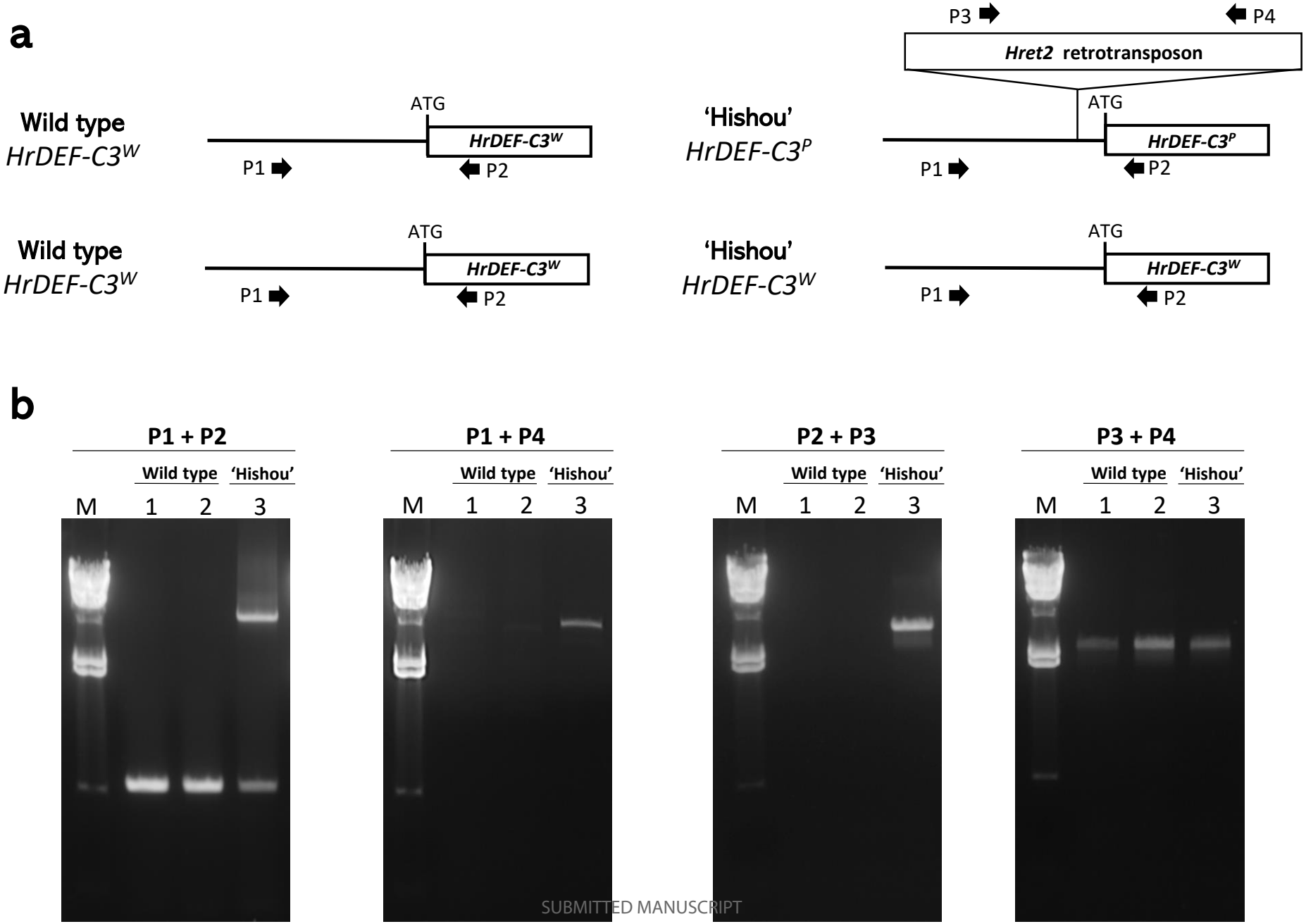
Figure 6



Supplementary Fig. S1



Supplementary Fig. S2



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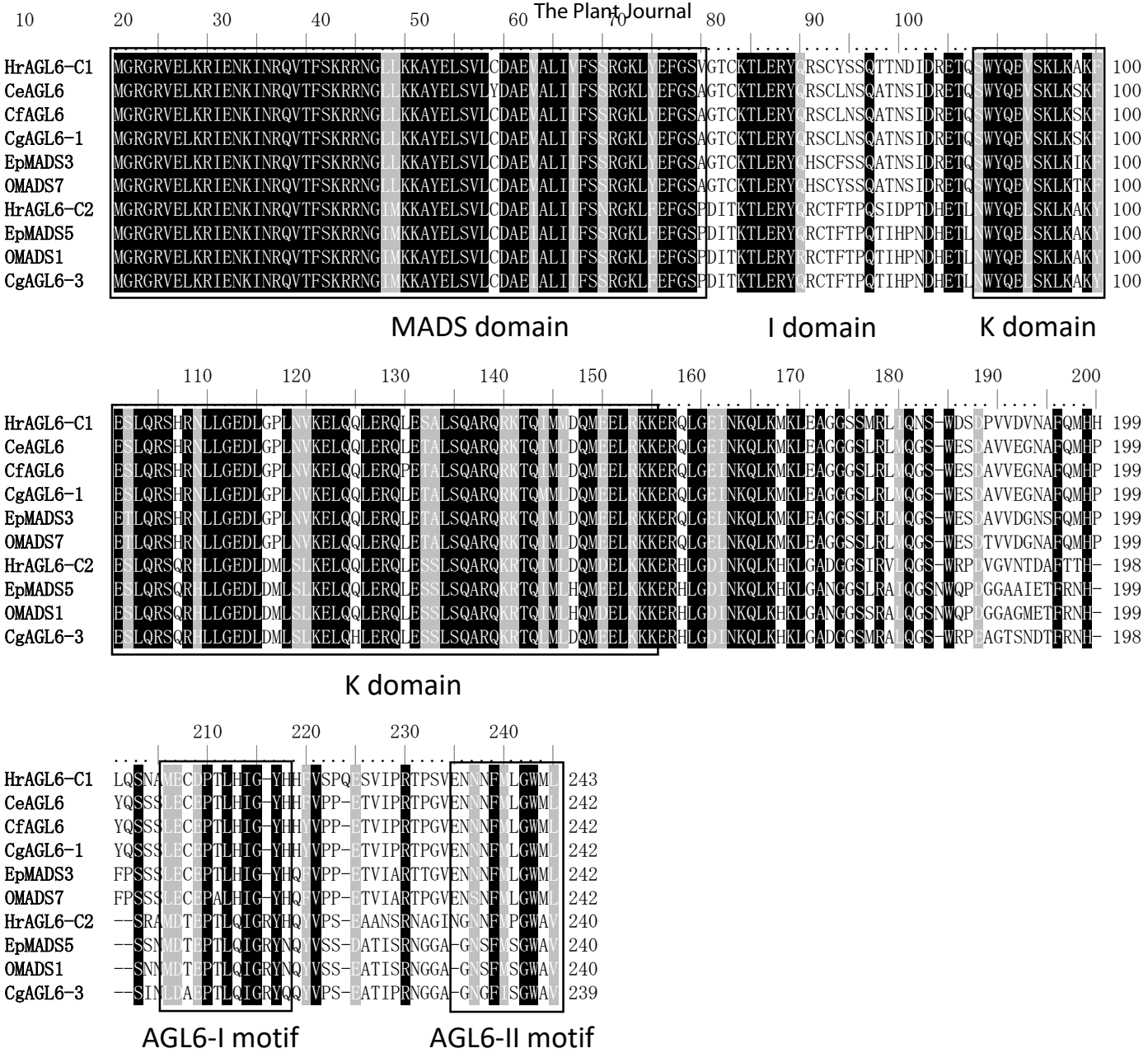
Supplementary Fig. S3



Supplementary Fig. S4

Supplementary Table S1. List of primers used in this study.

Primer name	Sequence (5' –> 3')	Application
S-HrDEF/Fw	AACTGCGYGGTCTTGAGCAAA	*DEF*-specific primer
S-HrDEF/Rv	AYYADGCRAGRCKDAGATCCTG	*DEF*-specific primer
OrchidAGL6-1/Fw	CTGAAGAGGATTGAGAAC	*AGL6-1*-specific primer
OrchidAGL6-1/Rv	GCATCCACCCAAGCATAA	*AGL6-1*-specific primer
OrchidAGL6-2/Fw	CTCAAGAGGATTGAGAAC	*AGL6-2*-specific primer
OrchidAGL6-2/Rv	GCATCCATCCAAGCATAA	*AGL6-2*-specific primer
P018HA	GACTCGTGACGACATCG	Anchor primer for wild type
P019HA	GACTCGTGACGACATCGATTTTTTTTTTTTTTTT	Anchor primer for wild type
P018HH	GACTCGAGACGTCATCG	Anchor primer for 'Hishou'
P019HH	GACTCGAGACGTCATCGATTTTTTTTTTTTTTTT	Anchor primer for 'Hishou'
HrDEF-C1-full/fw	CAGGGTAAAGAGAGAGAAGG	Full length cloning
HrDEF-C1-full/Rv	CTATCAACCAGACACGCATC	Full length cloning
HrDEF-C2-full/fw	AAAGGAAAGTGCTCGGTGAA	Full length cloning
HrDEF-C2-full/Rv	TGCCTGTCATCATCTACAGAAGT	Full length cloning
HrDEF-C3-full/fw	GCTCTTCCGCTTCTTTTGC	Full length cloning
HrDEF-C3-full/Rv	CAAGACAACTCAAGTGATC	Full length cloning
HrDEF-C4-full/fw	GTCTTTGCTTTCTCTCGG	Full length cloning
HrDEF-C4-full/Rv	GTTTATCCGAAGAAGAAATAAACATTGG	Full length cloning
HrAGL6-1/Fw	GAGAGAGAAGAGTGTGGG	Full length cloning
HrAGL6-1/Rv	CTTGCCATACAGATAGTG	Full length cloning
HrAGL6-2/Fw	GATAAGGAGAGGTTGTGC	Full length cloning
HrAGL6-2/Rv	CAATCAGGGAATGAGAGT	Full length cloning
eEF1A-4/Fw	TAAGTCTGTTGAGATGCACC	Reference gene primer
eEF1A/Rv	CTGGCCAGGGTGGTTCATGAT	Reference gene primer
qrHrDEF-C1/Fw	GGCATACAGAGCTCTAATGCACG	Real time PCR
qrHrDEF-C1/Rv	GGCTGGCTTGGCTGAACAAC	Real time PCR
qrHrDEF-C2/Fw	CGCTACTCAAACTGACACGTACA	Real time PCR
qtHrDEF-C2/Rv	CTCGAGTTCCAGTTCATGC	Real time PCR
qrHrDEF-C3/Fw	GAGCTTAATCCGTGAGCTG	Real time PCR
qrHrDEF-C3/Rv	GTAGGCTGAGTGCGGAAGTAGAG	Real time PCR
qrHrDEF-C4/Fw	GAGCAGCCGGTGTTTG	Real time PCR
qrHrDEF-C4/Rv	GCGTACATCTGATGAGGAG	Real time PCR
rtHrAGL6-1/Fw	CGCCAGCTTGGAGAGATA	Real time PCR
rtHrAGL6-1/Rv	CCATTGCATTTGACTGCA	Real time PCR
rtHrAGL6-2/Fw	CACAAGCTTGGGGCAGAT	Real time PCR
qHrAGL6-2/Rv	CCATGGCCCTTGAGTGAG	Real time PCR
HrDEF-C3-hybrid/Fw	ATGTGGACGAAGATCCAGCAG	Genetic inheritance of pseudopeloric mutation
HrDEF-C3-hybrid/Rv	CAAGACAACTCAAGTGATC	Genetic inheritance of pseudopeloric mutation
HrDEF-C3P-hybrid/Fw	TCTTGTAGCCATTCTACATTAGCC	Genetic inheritance of *HrDEF-C3*^{P}
HrDEF-C3P-hybrid/Rv	CTCTTCGAGTACGTCACTTGCCTGTTC	Genetic inheritance of *HrDEF-C3*^{P}
AP1	GTAATACGACTCACTATAGGGC	Adapter primer for Genome Walker
AP2	ACTATAGGGCACGCGTGGT	Adapter primer for Genome Walker
HrDEF-C3-GSP1	TGAGCTCACTAGCCTTCTTCATGATCC	Isolation of *HrDEF-C3* promoter
HrDEF-C3-GSP2	CTCTTCGAGTACGTCACTTGCCTGTTC	Isolation of *HrDEF-C3* promoter
HrDEF-C3W/Fw	TCTCTTCCGCTTCTTTTGC	Semi-quantitative RT-PCR for *HrDEF-C3*^{W}
HrDEF-C3P/Fw	TCTTGTAGCCATTCTACATTAGCC	Semi-quantitative RT-PCR for *HrDEF-C3*^{P}
HrDEF-C3W and P/Rv	CAAGACAACTCAAGTGACC	Semi-quantitative RT-PCR for *HrDEF-C3*^{W} and *HrDEF-C3*^{P}
P1	GCAGCAGTGTACCACAGTCAA	Mutant gene analysis in wild type and 'Hishou'
P2	CTCTTCGAGTACGTCACTTGCCTGTTC	Mutant gene analysis in wild type and 'Hishou'
P3	TCCCAATCACACAGCCAATA	Mutant gene analysis in wild type and 'Hishou'
P4	GGTTGAATGTCCCCTTCAGA	Mutant gene analysis in wild type and 'Hishou'

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Supplementary Table S2. Accession numbers for the *DEF*-like genes used in the phylogenetic analysis.

Gene	Spesies	Accession number
DEF	*Antirrhinum majus*	AB516402
AP3	*Arabidopsis thaliana*	D21125
AODEF	*Asparagus officinalis*	AB094964
CeAP3-1	*Cymbidium ensifolium*	JQ326261
CeAP3-3	*Cymbidium ensifolium*	JQ326260
CfAP3	*Cymbidium faberi*	HM208536
CfDEF	*Cymbidium faberi*	HM208535
CgDEF1	*Cymbidium goeringii*	HM106983
CgDEF2	*Cymbidium goeringii*	KX347446
CgDEF3	*Cymbidium goeringii*	HM106982
CgDEF4	*Cymbidium goeringii*	KU058678
CMADS1	*Cymbidium* hybrid cultivar	DQ683575
DcAP3B	*Dendrobium crumenatum*	DQ119839
DmAP3A	*Dendrobium moniliforme*	EU056327
DmAP3-4	*Dendrobium moniliforme*	GU132995
EpMADS15	*Erycina pusilla*	KJ002740
EpMADS14	*Erycina pusilla*	KJ002739
EpMADS13	*Erycina pusilla*	KJ002738
GogalDEF1	*Gongora galeata*	FJ804097
GogalDEF2	*Gongora galeata*	FJ804098
GogalDEF3	*Gongora galeata*	FJ804099
HrDEF-C1	*Habenaria radiata*	LC424956
HrDEF-C2	*Habenaria radiata*	LC424957
HrDEF-C3	*Habenaria radiata*	AB232663
HrDEF-C4	*Habenaria radiata*	LC424958
LMADS1	*Lilium longiflorum*	AF503913
LRDEF	*Lilium regale*	AB071378
OMADS5	*Oncidium* Gower Ramsey	HM140840
OMADS3	*Oncidium* Gower Ramsey	AY196350
OMADS9	*Oncidium* Gower Ramsey	HM140841
PtAP3-2	*Phaius tancarvilleae*	EU444051
PtAP3-3	*Phaius tancarvilleae*	EU444052
PeMADS2	*Phalaenopsis equestris*	AY378149
PeMADS5	*Phalaenopsis equestris*	AY378148
PeMADS3	*Phalaenopsis equestris*	AY378150
PeMADS4	*Phalaenopsis equestris*	AY378147
SpodoDEF2	*Spiranthes odorata*	FJ804111
SpodoDEF1	*Spiranthes odorata*	FJ804110
SpodoDEF3	*Spiranthes odorata*	FJ804112
TGDEFA	*Tulipa gesneriana*	AB094965
TGDEFB	*Tulipa gesneriana*	AB094966
VaplaDEF2	*Vanilla planifolia*	FJ804115
VaplaDEF3	*Vanilla planifolia*	FJ804117

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Supplementary Table S3. Accession numbers for the *AGL6*-like genes used in the phylogenetic analysis.

Gene	Spesies	Accession number
AtAGL6	*Arabidopsis thaliana*	NM_130127
AoM3	*Asparagus officinalis*	AY383559
BoMADS	*Bambusa oldhamii*	EF517293
BnAGL6	*Brassica napus*	XM_022719094
BoAGL6	*Brassica oleracea*	KC984301
CjAGL6	*Camellia japonica*	JX657333
CsAGL6	*Camellia sinensis*	KU862281
CaAGL6	*Coffea arabica*	KJ483245
CsAGL6	*Crocus sativus*	EF041505
CeAGL6	*Cymbidium ensifolium*	JN613148
CfAGL6	*Cymbidium faberi*	HM208534
CgAGL6-1	*Cymbidium goeringii*	HM208533
CgAGL6-2	*Cymbidium goeringii*	KX347450
CgAGL6-3	*Cymbidium goeringii*	KU058679
DAGL6	*Dendrobium* hybrid cultivar	KF550139
EpMADS3	*Erycina pusilla*	KJ002728
EpMADS5	*Erycina pusilla*	KJ002730
EpMADS4	*Erycina pusilla*	KJ002729
GGM9	*Gnetum gnemon*	AJ132215
GpMADS3	*Gnetum parvifolium*	AB022665
HrAGL6-C1	*Habenaria radiata*	LC424959
HrAGL6-C2	*Habenaria radiata*	LC424960
HoAGL6	*Hyacinthus orientalis*	AY591333
LeAP1	*Lycopersicon esculentum*	AY306154
NtAGL6A	*Narcissus tazetta*	EU081900
NtAGL6B	*Narcissus tazetta*	EF517294
OMADS7	*Oncidium* Gower Ramsey	HM140845
OMADS1	*Oncidium* Gower Ramsey	HM140843
FBP29	*Petunia x hybrida*	AF335245
FBP26	*Petunia x hybrida*	AF176783
PaDAL14	*Picea abies*	KC347012
PrMADS3	*Pinus radiata*	U76726
PrMADS2	*Pinus radiata*	U42400
PtDAL1	*Pinus tabuliformis*	KJ711020
PmAGL6	*Prunus mume*	XM_016794441
PpAGL6	*Prunus persica*	XM_020557124

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