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Two SNP Markers Identified Using Genotyping-by-Sequencing Are Associated with Remontancy in a Segregating F1 Population of *Syringa meyeri* 'Palibin' × *S. pubescens* 'Penda' Bloomerang®

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Abstract. **Lilacs (*Syringa* sp.) have been used as ornamental plants since the mid-16th century and remain important in modern gardens due to their attractive and fragrant flowers. However, a short flowering season is a critical drawback for their ornamental value. Breeders have identified remontancy (reblooming) in dwarf lilac (*Syringa pubescens*), and have tried to introgress this trait into related species by interspecific hybridization. Molecular tools for lilac breeding are limited because of the shortage of genome sequence knowledge and currently no molecular markers are available to use in breeding for remontancy. In this study, an F_1 population from crossing *Syringa meyeri* 'Palibin' × *S. pubescens* 'Penda' Bloomerang® Purple was created and subjected to genotyping-by-sequencing (GBS) analysis and phenotyped for remontancy. Plants were categorized as remontant, semi-remontant, and nonremontant based on the relative quantity of inflorescences during the second flush of flowers. A total of 20,730 single-nucleotide polymorphism (SNP) markers from GBS were used in marker-trait association to find remontant-specific marker(s) without marker position information. Two SNP markers, TP70580 (A locus) and TP82604 (B locus), were correlated with remontancy. The two loci showed a partial epistasis and additive interaction effects on the level of remontancy. Accumulation of recessive alleles at the two loci was positively correlated with increased reblooming. For example, 87% of aabb plants were remontant, and only 9% were nonremontant. In contrast, 100% of AaBB plants were nonremontant. These two SNP markers associated with remontancy will be useful in developing markers for future breeding and demonstrate the feasibility of developing markers for breeding woody ornamental taxa that lack a reference genome or extensive DNA sequence information.**

Lilacs (*Syringa* sp.) have been used as hardy ornamental plants by humans throughout history because of their showy, fragrant flowers and winterhardiness (Dirr, 2009; Juntheikki-Palovaara et al., 2013). Lilacs are an economically important ornamental crop in the United States. In 2014, sales in excess of $20 million were reported (U.S. Department of Agriculture, 2016). Breeders have improved many ornamental traits in lilacs such as larger flowers, double flowers, and fragrance (Dadpour et al., 2011; Hu et al., 2009; Zheng et al., 2015). However, the short flowering season during spring is one of the major drawbacks of lilacs. Summer remontancy was reported in dwarf lilac (*Syringa pubescens*), and has come to dominate the lilac market in the form of introductions such as Bloomerang® Purple (Fiala and Vrugtman, 2008). Remontancy in lilacs has become one of the most emphasized traits of modern lilac breeders because of the obvious market value (Fiala and Vrugtman, 2008).

Based on morphological characters and molecular analysis, *Syringa* is divided into six series: *Pubescentes*, *Villosae*, *Ligustrae*, *Ligustrina*, *Pinnatifoliae*, and *Syringa* (Kim and Jansen, 1998; Li et al., 2012). Dwarf lilacs, which are included in series *Pubescentes*, are compact and many produce prolific numbers of flowers (Fiala and Vrugtman, 2008). Remontancy, disease resistance, and growth habit are key traits targeted for improvement in modern dwarf lilac cultivar breeding programs. Whereas *S. pubescens* provides a source for remontancy, *S. meyeri* 'Palibin' has moderate to complete resistance to all three major lilac pathogens: powdery mildew (*Podosphaera xanthii*), bacterial blight (*Pseudomonas syringae*), and foliar blight (*Alternaria alternata*) (Mmbaga et al., 2005, 2011). Intraspecific hybridization, interspecific hybridization, and ploidy manipulation have all been successfully used in lilac cultivar development (Fiala and Vrugtman, 2008). For dwarf lilac breeding, *S. meyeri* and *S. pubescens* were found to have high cross-compatibility (Lattier and Contreras, 2017).

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Successful reciprocal crosses between *S. meyeri* 'Palibin' and *S. pubescens* 'Penda' Bloomerang® Purple were made to build a mapping population for lilac genetic mapping (Lattier, 2017).

Marker-assisted selection (MAS) could make cultivar development of dwarf lilacs more efficient by reducing population size at the seedling stage. Dwarf lilacs, like many other woody perennials, have a long life cycle and can be slow to flower, which makes phenotyping expensive and time-consuming. Dwarf lilacs can take up to 5 years before remontancy can be properly evaluated, as populations will begin sporadic flowering in year 3 or 4 but are not consistent enough to properly phenotype. Although MAS for primary selection at the seedling stage holds great promise for increasing efficiency, few molecular tools have been developed for lilac (Juntheikki-Palovaara et al., 2013; Kochieva et al., 2004; Lendvay et al., 2013; Rzepka-Plevnes et al., 2006), and available markers to date have only been used in taxonomy or cultivar identification but not selection.

Because of a shortage of knowledge about genomics and genome sequences, nonreference genome-based molecular markers such as intersimple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) have been used in polymorphism identification and ascertainment of genetic relationships of *S. vulgaris* cultivars (Juntheikki-Palovaara et al., 2013) and *Syringa* species (Kochieva et al., 2004). A few simple sequence repeat (SSR) markers were transferred from related genera (*Olea* and *Ligustrum*) for use in *S. vulgaris* (De la Rosa et al., 2002) and *S. josikaea* (Lendvay et al., 2013), but the marker densities were too low for selection purposes. More SSR markers of common lilac were developed from ISSR products and sequence walking (Korpelainen et al., 2007). However, the resulting marker density was not sufficient for MAS.

Genotyping-by-sequencing (GBS) is a promising option to generate high-density markers for minor crops such as lilac. GBS is a fast and cost-effective molecular tool that can generate large numbers of single-nucleotide polymorphism (SNP) markers based on next-generation sequencing without the necessity of a reference genome (Elshire et al., 2011). With a double restriction enzyme treatment that reduces genome complexity in sample DNA, GBS has been recommended for species with complex genomes, including polyploids, highly heterozygous genomes, and interspecific hybrids (Elshire et al., 2011; van Nocker and Gardiner, 2014). Dwarf lilacs are highly heterozygous and often derived from an unknown pedigree. However, using GBS, a total of 20,732 SNP markers were identified for an interspecific hybrid dwarf lilac population (*S. meyeri* 'Palibin' × *S. pubescens* 'Penda' Bloomerang® Purple) and used to build a draft linkage map (Lattier, 2017). This SNP marker system could be applied to identify functional markers useful in MAS of dwarf lilacs for important traits like remontancy.

The objectives of this study were to phenotype the previously developed mapping population and use the high-density SNP markers from GBS to look for marker-trait association to identify functional SNP markers for remontancy in lilac. The findings of such work will be valuable to lilac breeders and also demonstrate the power of GBS to develop markers that are useful in breeding minor crops without a reference genome or a priori genomic information.

Materials and Methods

Plant material. A biparental mapping population with 66 F_1 individuals was developed from crosses made during 2012 and 2013 and subjected to no prior selection before use in the current study. The female parent, *S. meyeri* 'Palibin' (10-0209), acquired from Blue Heron Farms Nursery (Corvallis, OR), was pollinated with *S. pubescens* 'Penda' Bloomerang® Purple plants from Garland Nursery (Corvallis, OR). Plants were grown in containers from 2013 to 2017 and field-planted at the Lewis Brown Horticulture Farm (Corvallis, OR).

Phenotypic segregation. Phenotyping of remontancy was conducted during Summer 2017 and Summer 2018, when plants were 4 and 5 years old. The phenotype for initial blooming was recorded in mid-May of each year to confirm that each plant had reached reproductive maturity and prevent phenotyping a plant as nonremontant when it may still be juvenile. Phenotyping of remontancy was repeated three times for each year between mid-July and mid-Aug. 2017 and 2018. Levels of remontancy were also recorded. Plants without any flowers were recorded as nonremontant. Plants with one to three inflorescences were recorded as semi-remontant. Plants with more than three inflorescences were recorded as remontant. Several segregation ratios were examined by chi-square (χ^2) tests.

Genotype and marker-trait association analysis. A total of 20,730 SNP markers were selected and filtered (Lattier, 2017). SNPs were identified using the TASSEL-GBS discovery software pipeline (Glaubitz et al., 2014). Principal component analysis (PCA) and marker–trait association analysis were performed using GAPIT (Lipka et al., 2012). Different PCA depths from three to eight and several statistical models, including Multivariate Linear Models (MLM), Generalized Linear Model (GLM), Regular and Compression MLM (CMLM), and enriched CMLM (ECMLM) were used for marker-trait association analysis.

Results and Discussion

Phenotype and segregation. Of the 66 F_1 plants, only 57 had reached sufficient size to be phenotyped in 2017. In 2018, all 66 F_1 plants were phenotyped: 27 were remontant, 13 were semi-remontant, and 26 were nonremontant (Fig. 1A). Phenotypes from the 2 years showed a good consistency ($\chi^2 = 0.56$, df $= 2$, $P = 0.65$). Of the 57 plants phenotyped in both years, 38 had the same phenotype. Although 19 plants (33.3%) showed different phenotypes between 2017 and 2018, 13 shifted to increased remontancy, including six plants moving from semi-remontant to remontant, four plants moving from nonremontant to semi-remontant, and three plants moving from nonremontant to remontant. Six plants exhibited a reduction in remontancy between years, including two plants that moved from remontant to semi-remontant and four plants that moved from semi-remontant to nonremontant. No plants categorized as remontant in 2017 shifted to nonremontant in 2018. Considering that many plants may not have reached sufficient maturity to express their true remontancy phenotype, only phenotype data from 2018 were used for marker-trait-association analyses.

All possible segregation ratios including single- and two-gene models were tested, including two possible two-gene segregation models. The first two-gene model had an expected segregation ratio of 6:4:6 for nonremontancy,

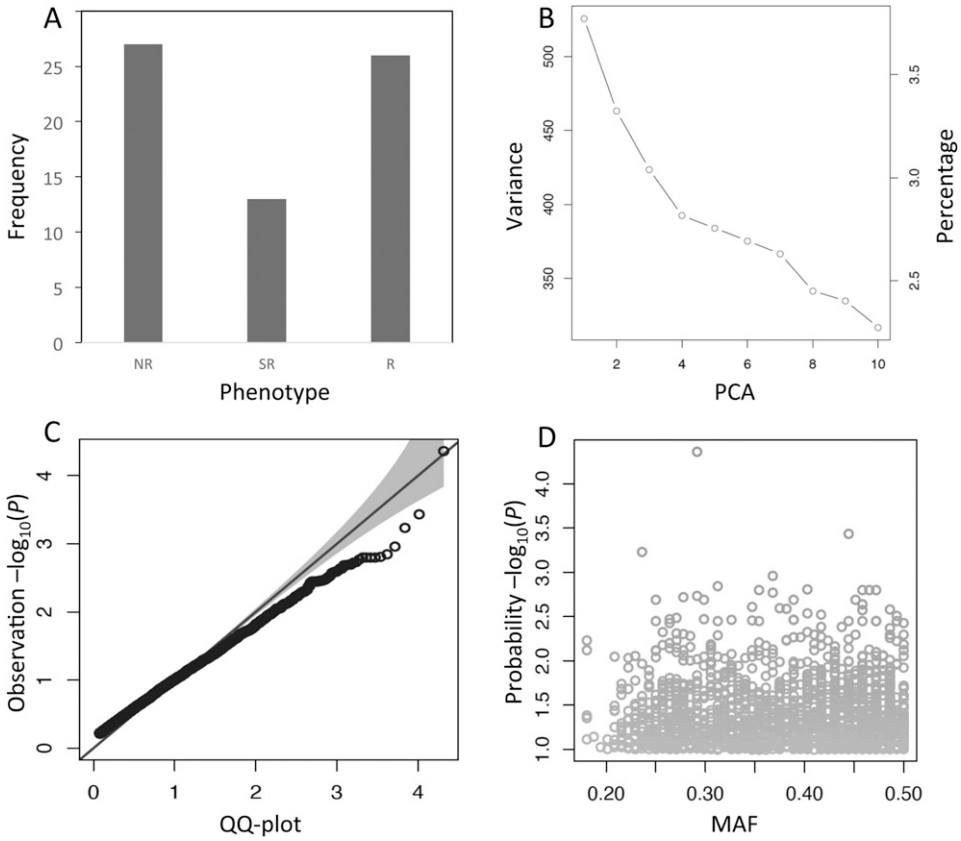

Fig. 1. Marker–trait association among a total of 66 hybrid plants of *Syringa meyeri* 'Palibin' × *S. pubescens* 'Penda' Bloomerang® phenotyped for reblooming (remontancy) with 20,730 single-nucleotide polymorphism (SNP) markers used in the GAPIT analysis (Lipka et al., 2012). Results from phenotyping and analysis include the following: (**A**) genotype distributions of remontancy, nonremontant (NR), semi-remontant (SR), and remontant (R), among the population; (**B**) principal component analysis (PCA) eigenvalues from 1 to 10; (**C**) quantile-quantile (QQ) plot analysis; and (**D**) the method of arbitrary functions (MAF) plot.

semi-remontancy, and remontancy ($\chi^2 = 5.44$, df = 2, $P = 0.76$). Another possible segregation ratio, 3:5 for nonremontancy and the sum of semi-remontancy and remontancy ($\chi^2 = 0.05$, df = 1, $P = 0.82$), was also a two-locus segregation model that aggregates semi-remontant and remontant phenotypes together as one ''remontancy'' category. For possible single-gene segregation ratios of remontant semi-remontant and nonremontant, the probability values of χ^2 goodness-of-fit tests of 1:1 and 1:3 were 0.082 and 0.007, respectively. The results showed that the phenotype fit two-gene models better than single-gene models based on χ^2 goodness-of-fit tests; however, at this juncture, it is not possible to indicate if this is due to dominance or recombination. Furthermore, with our small population size it is not possible to completely rule out a single-gene model.

Marker-trait association. After trimming the genotype and phenotype datasets, 20,730 markers for 66 plants were included in the genome-wide association study (GWAS) analysis. In the marker-trait association analysis, results of the analysis with different PCAs were very similar; the same markers were discovered with slightly different probability values. Because of their similarity, only a PCA with a result of 8, which might obtain the most information, was reported (Fig. 1B). Results of different statistical models, including MLM, GLM, CMLM, and ECMLM, were similar; therefore, only the results of MLM are presented. We further used a quantile-quantile (QQ) plot and method of arbitrary functions (MAF) plot to examine the MLM model fitness for three levels of the trait of interest (remontant, semi-remontant, and nonremontant). The top one to three markers may be associated with remontancy (Fig. 1C and D). Without using marker location information, GAPIT found two SNP markers, TP70580 and TP82604, associated with remontancy with $P < 0.0001$. TP70580 was a T/C SNP marker, and both parents were heterozygous T/C. Genotypes of C/C, T/C, and T/T of TP70580 were found in the F_1 population. TP82604 was a T/A SNP marker. The genotype of the remontant parent, *S. pubescens* Bloomerang®, was T/T and the genotype of the nonremontant parent was T/A. Genotypes of T/A and T/T of TP82604 were found in the F_1 population. For convenience, we expressed TP82604 and TP70580 as an AB two-loci model. T/A and T/T alleles of TP82604 are presented as Aa and aa, and C/C, T/C, and T/T of TP70580 alleles are presented as BB, Bb, and bb in this study. Genotypes of the remontant parent *S. pubescens* Bloomerang® and the nonremontant parent *S. meyeri* 'Palibin' were Aabb and AaBb, respectively. The genotypes of seedlings were AABb, AaBb, AAbb, aaBb, Aabb, and aabb (Fig. 2).

Genetic model of remontancy in lilacs. By observing the phenotypes of each genotype, both A and B loci affect remontancy by epistasic and additive interaction. The A locus (TPTP82604) is critical to remontancy and shows a semi-dominant or dominant effect. Of recessive homozygous aa plants, only 17% were nonremontant and 83% were remontant. For plants with the Aa genotype, 79% of plants were nonremontant and 21% of plants were remontant. The relative impact on levels of remontancy at the A locus is aa > Aa. Similar results were observed with TP70580, with a comparatively smaller effect on remontancy. For plants with the genotype BB, 73% were nonremontant and 27% were semi-remontant. No plant with genotype BB was remontant. For plants with the Bb genotype, 42%, 29%, and 29% of plants were nonremontant, semi-remontant, and remontant, respectively. In contrast, for plants with the bb genotype, individually, 17%, 10%, and 72% of plants were nonremontant, semi-remontant, and remontant, respectively.

The results showed that the genotypes of the two loci had a cumulative effect on remontancy. None of the plants with genotype AABb was remontant. Furthermore, 91% of plants with the genotype aabb were either remontant or semi-remontant. For other genotypes, including AAbb, AaBb, Aabb, and aaBb, the frequency of remontant, semi-remontant, and

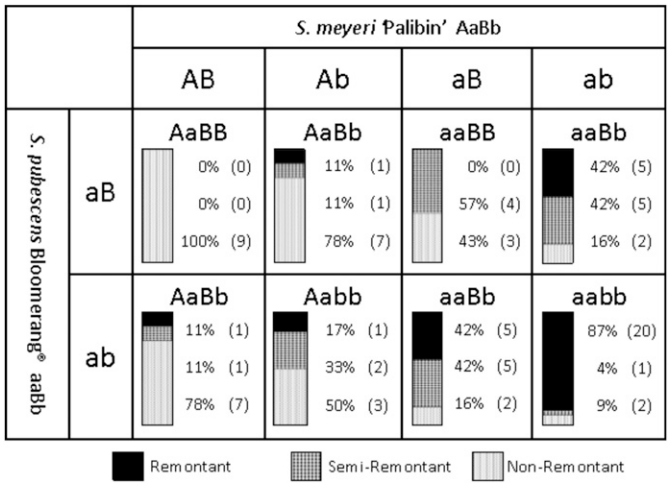

Fig. 2. Genotypes and phenotypes among a total of 66 plants resulting from the cross of *Syringa meyeri* 'Palibin' × *S. pubescens* 'Penda' Bloomerang® included in the study without prior selection. Values are the percentages of plant phenotypes (with an original number) in each genotype. The order of the number from top to bottom represents plants with phenotypes of remontant, semi-remontant, and nonremontant. Genotypes Aa and aa represent T/A and T/T at single-nucleotide polymorphism (SNP) marker TP82604; BB, Bb, and bb represent C/C, T/C, and T/T at SNP marker TP70580 marker. To illustrate the phenotype percentage trend among genotypes, genome types aaBb and AaBb are shown twice in the figure.

nonremontant plants varied, but the frequency of remontant and semi-remontant plants increased as more recessive alleles were present at the two loci (Fig. 2).

Our results indicated that there are two SNPs associated with remontancy segregating in our population of lilacs; however, the precise mode of genetic control is still unknown. Additional research with larger populations and/or the use of reference genome sequences will help build a fuller understanding of the genetic control of remontancy in lilacs. In this study, the phenotype segregation ratio fits in the two-gene model better than single-gene models, and the character of remontancy trait was more like a quantitative trait; however, the two discovered markers were linked ($\chi^2 = 31.88$, df = 5, $P < 0.0001$). Therefore, even though the two markers found by this study were significantly associated with remontancy, we cannot confirm whether this was due to dominance or recombination.

Two markers suitable for selection of remontant lilacs. The two SNP markers, TP70580 (A locus) and TP82604 (B locus), were strongly associated with remontancy and could be used for MAS. Simultaneously using these two recommended markers could efficiently predict remontancy (Fig. 2). The additive effect of the two markers on remontancy was observed such that as recessive alleles at the two loci accumulate, the more likely the phenotype is remontant. In our population, 91% of plants with the genotype aabb were either remontant (87%) or semi-remontant (4%). Of the two candidate loci, the A locus showed a stronger effect on remontancy. If one locus was homozygous recessive and the other was heterozygous, then the relative impact of the two loci were as follows: 84% of plants with genotype aaBb were remontant; however, only 50% of plants with Aabb genotype were remontant.

In addition to the accumulated effect of the two loci, some level of epistasis between the two loci might be present by the following mechanism: recessive homozygous aa determines whether a plant exhibits any level of remontancy (on or off), and the b allele of the semi-dominant B locus determines the level of remontancy (semi-remontant or remontant). Comparing the phenotype proportion of aaBb and aabb supported the presence of epistasis. High percentages of both aaBb (84%) and aabb (91%) plants were semi-remontant or remontant; however, 87% of aabb plants were remontant and only 42% of aaBb plants were remontant.

Using a three-level phenotype and two-loci model effectively explained remontancy in our population. Selection targeting the greatest expression of remontancy is a desired strategy because of the improved stability of the trait in these plants. Nursery producers have reported instability or unimpressive flower production during the second flush in some remontant cultivars, likely due, at least in part, to environmental influence or production practices. Plants that have stable, consistent, and high rates of remontancy are valued. Misleading low levels of remontancy may lead to false-positive phenotyping. Low levels of remontancy were observed in dwarf lilac cultivars generally regarded as nonremontant according to nursery producers (personal observation). Some inconsistent phenotypes between 2017 and 2018 were observed, including eight plants that shifted between semi-remontant and nonremontant phenotypes.

The double recessive aabb is the best genotype for selecting remontant phenotypes and we suggest simultaneously using the two markers for the best results. Although the remontancy level prediction could be affected by a variety of causes, including the environment, other possible minor genes, and distance of the genes to discovered markers, 87% of the aabb plants were remontant. If selection is made by only one marker, then many semi-remontant plants will be falsely selected. For example, using SNP marker TP70580 exclusively would lead to selection of 71% aa plants exhibiting some level of remontancy, but more than 50% of those were semi-remontant. When selecting plants with valuable remontancy, the breeder should only select those plants with a remontant type that is stable.

GBS for ornamental plant breeding. Like many other minor crops, the lack of a reference genome and the shortage of resources limit breeding strategies for woody ornamental plants. In the present research, heterozygosity and interspecific hybridization that are often challenging to molecular marker development were present in our population. However, GBS successfully identified high-density genetic markers for MAS without relying on a reference genome. The complexity and lack of a reference genome are common in most clonally propagated ornamental plants. In addition, because of the long lifecycle or long juvenile phase, MAS for early-stage selection could reduce costs and increase efficiency. Our research demonstrated the potential practicality of GBS for breeding projects of such ornamental plants. Two SNP markers from GBS were found to relate to remontancy in the dwarf lilac population and appear sufficient for early-stage selection.

Further research is needed to make the two SNP markers practical. Theoretically, the two-marker screening method for remontancy could be used in a larger population from the same crossing combination or, potentially, in populations with parents with a similar genotype and phenotype. However, sequencing all plant materials can be expensive and impractical. In addition, the same alleles may not be present. Therefore, converting the SNP marker to a polymerase chain reaction (PCR)-based marker is necessary to make these two markers

useful for MAS. There are two possible strategies: finding SSR markers near the SNPs or directly designing SNP primers. Without a reference genome, finding an SSR marker in close proximity to the SNP marker is difficult. However, the raw sequence data might be used in a draft genome assembly. With a draft reference genome sequence, discovering genes of the sequence near the SNPs is not impossible using GBS alone. However, directly designing primers with PCR products containing the SNP site by using sequence walking might be another viable method. With this method, only short sequences near the SNPs are required to design the primers. Another method to convert the discovered SNPs into PCR-based markers is the use of a high-resolution melting (HRM) marker to directly detect the genotype of the SNP by designing primers around it (Mehta et al., 2013). If an HRM is not available, then designing a pair of primers that have a 5′-end on the SNP marker to generate a new PCR-based dominant marker is another option. However, scoring a dominant marker result can be problematic because homozygous recessive and failed PCR would both yield the same apparent results, leading to false-negative results.

Remontancy in perennial plants. In the present study, two recessive loci were identified as selection targets for remontancy in dwarf lilacs. This result implied that at least two genes (or loci) control dwarf lilac remontancy; however, the genes that control lilac remontancy remain unknown. Studies of the mechanism of remontancy in other perennial plants have found two models of genetic control: 1) *Terminal Flower 1* (*TFL1*) deficiency (Freiman et al., 2012; Kotoda et al., 2006; Wang et al., 2012; Zhu et al., 2015), and 2) a multigene model in which vernalization, gibberellic acid (GA), and photoperiod response genes all might be involved (Jia et al., 2014; Zhou et al., 2013). *TFL1* maintains juvenility and prevents floral initiation in *Arabidopsis thaliana* (Hanano and Goto, 2011). Reduced *TFL1* function is related to remontancy or continuous flowering in several perennial plants like apple [*Malus* ×*domestica* (Kotoda et al., 2006)], pear [*Pyrus communis* (Freiman et al., 2012)], rose [*Rosa* (Iwata et al., 2012; Wang et al., 2012)], strawberry [*Fragaria* ×*ananassa* (Iwata et al., 2012)], and daylily [*Hemerocallis* (Zhu et al., 2015)]. Multigene regulation may regulate remontancy of tree peonies [*Paeonia* sp. (Zhou et al., 2013)], and a tropical fruit species, *Dimocarpus longan* (Jia et al., 2014).

The multiple gene model best explains dwarf lilac remontancy. The results of our research indicated that two major loci with epistasis and additive interactions contributed to the remontancy of the hybrid dwarf lilac population. Without a reference genome, predicting or identifying the exact gene(s) that result in remontancy of hybrid dwarf lilacs is difficult. The two loci could be associated with either two homologous *TFL1* genes or two other genes in a multiple-gene control model. However, when considering prior findings for other perennial plants, we believe that remontancy in lilac is controlled by multiple genes. First, reports of rose, strawberry (Iwata et al., 2012; Wang et al., 2012), and daylily (Zhu et al., 2015) indicated that only one *TFL1* homolog affects remontancy but others did not. Second, remontancy in dwarf lilac appears to be quantitative instead of qualitative and interactions of the two loci were observed. In the *TFL1* deficiency model, remontancy was a simple qualitative trait. In our case, the two loci showed different impacts on remontancy and the QQ plot analysis implied that more minor genes might exist. These phenomena indicated there might be other unknown factors involved in remontancy rather than a single-gene model. We believe the multiple genetic control model explains remontancy in dwarf lilacs better than a single-gene model. However, further research and a reference genome are needed to fully reveal the genetic control of remontancy in lilac.

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