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Identification of molecular markers associated with the double flower trait in *Petunia hybrida*

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ABSTRACT

The flower architectural trait of doubleness is a highly important breeding characteristic in the ornamental species petunia (*Petunia hybrida*). Here, we described the development of a near-isogenic line of a petunia population that segregated for single and double flower types. Sequence-related amplified polymorphism (SRAP) and simple sequence repeat (SSR) techniques, combined with bulked segregant analysis (BSA) were used to develop molecular markers linked to this floral trait. From a survey of 170 SRAP and 38 SSR primer combinations, one SRAP marker and one SSR marker were found to be linked to the target trait, each leading to the amplification of a specific band in double flower petunia. The two markers were verified in a population of 1065 plants, including 478 single flower and 587 double flower petunia individuals, and were shown to be tightly linked to the double flower locus with no recombination events observed. Hence, these markers have the potential for application in the positional analysis of the double flower locus and, ultimately, may contribute towards determining the genetic basis of flower type.

1. Introduction

The double flowered trait is associated with increased numbers of petals and larger overall flower size, and thus, is strongly associated with the attractiveness of the flower for ornamental purposes. Consequently, the underlying molecular mechanisms of the trait have attracted significant research interest. Studies of flower development have assigned the MADS-box ABC class genes as key controllers in specifying floral organ type and, hence, in determining the development of the double flower trait (Coen and Meyerowitz, 1991; Bowman et al., 1991; Weigel and Meyerowitz, 1994). The A and B class genes act together to specify petal identity, and over-expression of either class of gene can lead to increased petal number (Sun et al., 2014; Jing et al., 2014). The C class of genes, determining the formation of stamen and carpel floral organs, plays a dual role in the development of the double flower trait. Loss of expression of the C class gene *AGAMOUS* (*AG*) in *Arabidopsis* results in the conversion of stamens to petals and carpels to sepals, so resembling the 'double flower' feature with an excess of petals (Bowman et al., 1989). Similar phenotypes have been described in the mutants of *AG* homologous genes in *Petunia hybrida* (Heijmans et al., 2012), *Ipomoea nil* (Nitasaka, 2003), *Thalictrum thalictroides* (Galimba et al., 2012) and *Prunus lannesiana* (Liu et al., 2013). Furthermore, determinacy in floral meristems of single flowers relates to a fixed number of whorls whereas, double flowers are indeterminate, exhibiting extra floral whorls and increased floral organ number. It has been reported that floral determinacy is determined by a negative feedback loop created by *AG* and *WUSCHEL* (*WUS*) genes (Lenhard et al., 2001; Lohmann et al., 2001), and this regulatory mechanism thereby underlies the formation of double flowers (Roeder and Yanofsky, 2001). Thus, the cohort of genes affecting *AG* or *WUS* will influence formation of the double flower phenotype; such genes include *APETALA2* (*AP2*) (Zhao et al., 2007), *PERIANTHIA* (*PAN*) (Das et al., 2009), *ULTRAPETALA* (*ULT*) (Fletcher, 2001) and *CLAVATA* (*CLV*) (Clark et al., 1993, 1995; Kayes and Clark, 1998). Additionally, the genes involved in the organogenesis of individual floral organs have been reported to influence petal number in the whorl, e.g. *PETAL LOSS* (*PTL*) and *CUP-SHAPED COTYLEDON (CUC)* (Brewer et al., 2004; Lampugnani et al., 2012; Baker et al., 2005). Thus, different studies have ascribed the formation of double flowers to diverse molecular aspects of floral development and, therefore, further studies are needed to resolve these discrepancies.

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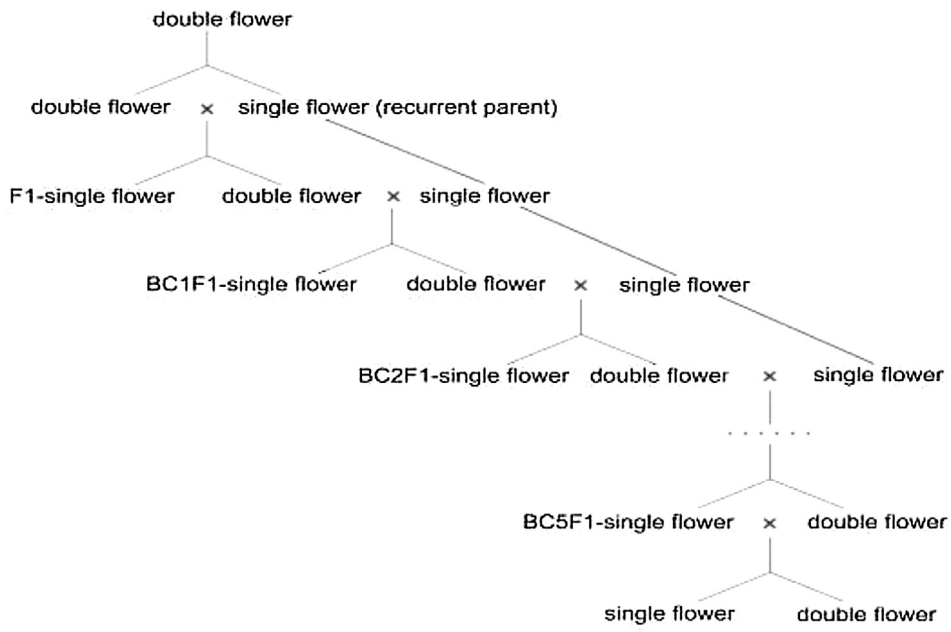

Fig. 1. Construction of a near-isogenic population line in petunia.

In genetic studies of developmental pathways, molecular marker technologies have been proved to be effective and practical in identifying numerous linkage markers for interested traits in multiple ornamental species including, *Phalaenopsis* (Gawenda et al., 2012), *Rosa* (Crespel et al., 2002; Spiller et al., 2011), *Tagetes erecta* (He et al., 2009) and *Dianthus caryophyllus* (Scovel et al., 1998; Yagi et al., 2012). *Petunia* is not only widely grown as a horticultural plant, but it is also increasingly employed as a model plant in molecular research studies due to its experimental tractability (Angenent et al., 1995; Colombo et al., 1995; Kapoor et al., 2002; Vandenbussche et al., 2003). The molecular marker techniques have provided a value resource for studies of petunia. High-density genetic maps have been constructed via restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR) and cleaved amplified polymorphic sequence (CAPS) (Strommer et al., 2000, 2002; Bossolini et al., 2011) to provide the framework for map-based gene cloning and references for sequence assembly of the petunia genome. Combining with the technique of quantitative trait locus (QTL) analysis and genetic maps, a large number of QTLs for key aspects of the pollination process (Stuurman et al., 2004; Klahre et al., 2011) and for several crop physiological traits (Vallejo et al., 2015) have been identified. However, few studies to date have referred linkage analysis to the single-flower versus double-flower trait in petunia.

Double-flowered petunia plants possess higher ornamental and, thus, commercial value and so tend to comprise the most popular varieties. Thus, understanding the genetics of doubleness in petunia is important to breeding programs. As in many plant species, petunia contains two *AG* homologs, i.e. *PETUNIA MADS BOX GENE3* (*PMADS3*) and *FLORAL BINDING PROTEIN6* (*FBP6*), that act redundantly in floral organ identity and meristem development (Heijmans et al., 2012), thereby contributing to double flower formation. In the *Pmads3-RNAi fbp6-1* mutant, the stamens are converted to petals, and the carpels are completely absent with the organelles converted to extra sepals and petals. Thus, this interference RNA mutant resembles a strong *ag* phenotype, but shows key differences from a naturally occurring double flower form. Thus, it is necessary to take a natural double flower genotype as the research subject in order to develop linked markers for marker-assisted selection breeding and map-based gene cloning.

In this project, we constructed a near-isogenic line (NIL) of single and double flower petunia and used sequence-related amplified polymorphism (SRAP) and SSR techniques, combined with bulked segregant analysis (BSA), to develop molecular markers linked to the double-flower (*df*) allele in *Petunia hybrida*. Because the detection of SRAP marker was time-consuming, we converted the amplified fragment into the sequence characterized amplified region (SCAR) marker, which was stable and convenient for individual analysis.

2. Materials and methods

2.1. NIL (near-isogenic line) population construction

The original material was the first generation progeny of the self-cross of a double fertile petunia individual, resulting in segregation for individual plants possessing either single (fertile) flowers (*sf*) or double (female-sterile) flowers (*df*). The *sf* and *df* plants were crossed to obtain the F1 generation. Subsequently, self-cross of *sf* flowers was conducted to maintain the single flower type as the recurrent parent, and *df* flowers producing fertile pollen were used as the donor (male) parent to conduct back-crosses for 5 generations, as shown in Fig. 1. As the segregating phenotype exhibited stable inheritance, sister hybridization between *sf* and *df* flowers was carried out to maintain the distinct characteristics, until ultimately resulting in the NIL population, which comprised 1065 plants, 478 single flower plants and 587 double flower plants.

2.2. DNA extraction

The modified cetyltrimethyl ammonium bromide method (Doyle and Doyle, 1990) was used to extract genomic DNA from fresh leaves of 1065 flowering plants of the NIL population of

Table 1
The primers used in the conversion of the SRAP marker into SCAR marker.

Forward primers (5′-3′)	Reverse primers (5′-3′)
F1: GTCCTAAACTTGGCTCTTTCCTGT	R1: GATCTTGACGTGCTAAGAATTCCA
F2: AAACGTCTAATTCTGATGACCTCTA	R2: ATTGCTTGGCTTTGTATACTTCTTC