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Genetic analysis and associated SRAP markers for flowering traits of chrysanthemum (*Chrysanthemum morifolium*)

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Abstract The inheritance of two flowering traits of chrysanthemum, initial blooming time and the duration of flowering, was investigated using segregation within an F_1 population derived from a cross between the autumn-flowering 'Yuhualuoying' and the summer-flowering 'Aoyunhanxiao' cultivars. The analysis, based on a single segregating generation and the major gene plus polygene mixed inheritance model, showed that the inheritance of both traits was compatible with the presence of two pairs of major genes displaying additivity–dominance–epistasis, with additivity predominating. As the heritability of both pairs of major genes was high (initial blooming time ~65%, duration of flowering ~72%), it should be possible to select for both traits in early breeding generations. A marker-trait association analysis based on sequence-related amplified polymorphism (SRAP) genotyping uncovered 10 (initial blooming time) and 12 (duration of flowering) markers significantly associated with phenotype, cumulatively explaining, respectively, 46 and 54% of the variation. Some potentially useful markers were identified.

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Introduction

Chrysanthemum, *Chrysanthemum morifolium* (Ramat.) Kitam, is one of the most popular ornamentals in the world. Its diversity with respect to growth habit and both inflorescence form and colour have ensured its economic importance. The demand for chrysanthemum production, encompassing cut-flower, garden, potted plants and ground-cover types, is increasing worldwide. The flowering traits of a variety determine its adaptation and productivity in a given agro-ecological zone (Hao et al. 2008a). In China, most cultivars flower in autumn, with only a few flowering in early summer. Therefore, protected cultivation has been adopted for year-round production of chrysanthemum, which is rather costly and laborious. Understanding the inheritance pattern of a target trait is necessary for its improvement. Most genetic studies in chrysanthemum have targeted the ornamental traits of inflorescence such as colour (Li et al. 2005) and floret form (Chen et al. 1991, 2003; Zhang et al. 2008, 2010a), as well as vegetative traits such as plant type (Jiang et al. 2003; Zhang et al. 2010a; Zhao et al. 2009a). Flowering is photoperiod, temperature and vernalization dependent, so its genetic control is expected to be complex. Flowering

time is a quantitative trait in kiwifruit (Zhu et al. 2002), snapdragon (Martin and Stimart 2003, 2005), *Vernonia galamensis* (Baye and Becker 2005) and cotton (Anbessa et al. 2006; Hao et al. 2008a). Little is known regarding the genetic control of flowering in chrysanthemum, which has hindered the improvement of this trait to some extent.

Historically, quantitative traits have been analyzed by calculating the total genetic effect based on population means and genetic variance (Cheng et al. 2006; Rumpunen and Kviklys 2003), since a quantitative trait is generally believed to be controlled by many polygenes. The effects of genes controlling quantitative traits can vary in magnitude, with some showing strong genetic effects (major genes) and some showing only weak ones (minor genes) (Chen et al. 2008; Oyant et al. 2008; Thumma et al. 2010). Therefore, the inheritance of a quantitative trait may be determined by a few major genes as well as by many polygenes. Based on this idea, Gai et al. (2003) have developed the concept of a major gene plus polygene mixed inheritance model (mixed model) to analyze quantitative traits in plants. This requires a single segregating generation and several jointly segregating families (Wang and Gai 1997; Zhang et al. 2000), and has been successfully applied to the genetic analysis of a number of agronomically important quantitative traits (Chen et al. 2009; Guo et al. 2006; Hao et al. 2008b; Zeng and Pu 2006; Zhang et al. 2006). Quantitative trait locus (QTL) analysis has been attempted in the ornamental species lily (Abe et al. 2002), rose (Dugo et al. 2005; Oyant et al. 2008) and carnation (Yagi et al. 2006), and genetic linkage maps of chrysanthemum have recently been constructed (Zhang et al. 2010b). Among the major methods of QTL analysis, marker-based variance analysis has become the preferred means where only limited genetic linkage map information is available—such as in the tree species poplar (Huang et al. 2004a, b; Su et al. 2000), *Taxus yunnanensis* (Su et al. 2009) and *Camellia sinensis* (Yao et al. 2010).

The sequence-related amplified polymorphism (SRAP) technique amplifies open reading frames. It has been used to understand genetic diversity (Budak et al. 2004; Hao et al. 2008b), construct genetic (Li and Quiros 2001) and transcriptome (Li et al. 2003) maps, and for gene tagging (Li et al. 2003; Han et al. 2008; Pan et al. 2003). Recently, we have reported the use of SRAP markers in chrysanthemum (Zhang et al. 2009, 2010b). Here, we describe a genetic analysis of initial blooming time and the duration of flowering in chrysanthemum, based on SRAP genotyping. We believe that these data will contribute to an improved understanding of the inheritance of these two flowering traits, and perhaps lay the foundations for future gene cloning and marker-assisted breeding in chrysanthemum.

Materials and method

Materials

The female mapping parent 'Yuhualuoying' flowers in autumn, while the male 'Aoyunhanxiao' flowers in summer. Both varieties were bred by the Chrysanthemum Germplasm Resource Preserving Centre, Nanjing Agricultural University, China. Artificial hybridization was used to make the 'Yuhualuoying' × 'Aoyunhanxiao' cross to generate a population of 142 F_1 progeny (Liu et al. 2010; Sun et al. 2010).

Design of field experiments and investigation of flowering traits

The field experiments were carried out in the nursery of the Chrysanthemum Germplasm Resource Preserving Centre in 2008 and 2009. The 142 F_1 seedlings, along with both parental lines, were vegetatively propagated in April of both 2008 and 2009, and a month later, rooted cuttings were transplanted to a nursery field in a randomised block design with three replications. The size of the plot was 1.0 m × 60 m and the plant spacing was 35 cm × 35 cm. Field management followed standard commercial practices. Initial blooming time and the duration of flowering were measured in both years. The former was defined as the number of days between transplanting to the date on which approximately 50% of the total flower buds were half-opened and fully pigmented. The latter was defined as the number of days between initial blooming and the date on which approximately 10% of the total flowers appeared wilted.

Genomic DNA isolation and SRAP marker analysis

Total genomic DNA was extracted from young unexpanded leaves using CTAB procedure (Murray and Thompson 1980). A set of 30 SRAP primers (10 forward and 20 reverse) was designed according to Li and Quiros (2001), producing 200 primer combinations as a means of finding polymorphism between the parental cultivars. Informative primer combinations were used to genotype the mapping population. All PCR and electrophoretic procedures followed Zhang et al. (2010b).

Heterosis and genetic analyses

Heterosis was measured both by mid-parent heterosis (H_m) and the ratio of mid-parent heterosis (RH_m) (Li and Wu 1997). H_m was defined as the difference between F_m (the mean trait value of the F_1 progeny) and MPV (the mean trait value of the two parental lines). RH_m was given by $(F_m - MPV)/MPV \times 100\%$. Statistical analyses of the flowering traits and one-sample *t*-tests were performed using SPSS v11.5 software. Since both phenotypic and genetic data were consistent over the 2 years (not shown), the 2 year mean was used for trait values. A single segregating generation and the mixed inheritance model analysis method described by Gai et al. (2003) were used. Briefly, suppose under the modifications of polygene and environment, the effects of major gene in segregation generation display an independent normal distribution, then the whole segregation generation could be seen as a mixed distribution made up by many independent normal distributions. The single generation segregation analysis may involve the two genetic model of null or one pair of major genes (A model) and two pairs of major genes (B model) in 11 kinds with special reference to major gene. Statistical deduction can be regarded as an estimation of probability distribution of observed value according to the principle of entropy maximization suggested by Akaike (1977). Therefore, comparison of the goodness-of-fit of the practical frequency distributions with the standard curves can approximate the inheritance model of a quantitative trait. The most-fitting genetic model was chosen according to Akaike's Information Criterion (AIC). The smallest AIC value is the most-fitting genetic model. Akaike (1977) suggested that the hypothesis maximizing the expected entropy should be selected as the most fitting model. For this purpose, based on good-of-fit and parsimony, the hypothesis that leads to the smallest AIC will be chosen. A calculated software was provided by Dr. Gai, National Centre for Soybean Improvement, State Key Laboratory of Crop Genetics and Germplasm Enhancement of Nanjing Agricultural University. Genetic parameters were estimated with the method as detailed in Wang and Gai (1997) and Gai et al. (2003).

In a mixed inheritance model, the phenotypic value (p) can be expressed as the sum of the population mean (m), major gene effects (g), polygene effects (c) and the environmental effect (e), i.e. $p = m + g + c + e$, where g is dependent on major gene effects, and both c and e are normally distributed. The heritability of each major gene h^2_{mg} was given by $\sigma^2_{mg}/\sigma^2_p \times 100\%$. Because this study was undertaken using a single segregating generation, neither the polygene nor the environmental variances could be estimated.

Marker-trait association analysis

Marker profiles were scored by assigning '0' for the absence and '1' for the presence of each polymorphic fragment. Only reproducible and well-defined bands were scored. Each marker was identified by the primer pair used and a suffix was attached to the designation, where multiple fragments were identified from a single primer combination. According to the marker-based method of Singh et al. (1991), for each marker, the segregating progeny were assigned into groups, formed by those scored as '0' and those as '1', and a one-way variance analysis was then performed for each marker in turn to detect any SRAP loci linked to the trait.

Results

Trait distribution and heterotic performance

Initial blooming time and the duration of flowering segregated widely, with coefficients of variation of >9 and >21%, respectively. The skewness and kurtosis values (Table 1) and the frequency distributions (Fig. 1) indicated that both initial blooming

Table 1 Phenotypic and heterosis performance for initial blooming time and the duration of flowering in the F_1 population derived from the cross 'Yuhualuoying' (P_1) × 'Aoyunhanxiao' (P_2)

Trait	Parents			F_1 segregating population		
	P_1	P_2	MPV	Minimum	Maximum	Range
Initial blooming time	156	95	126	9	29	20
Flowering duration	19	12	16	79	167	80

F_1 segregating population

Mean	SD	CV	Skewness	Kurtosis	H_m	RH_m (%)
141.29	13.39	9.47	−1.11	3.69	15.79*	12.58
16.42	3.55	21.65	1.30	2.52	0.92*	5.91

The difference between H_m and MPV was analysed by a one-sample *t*-test

* Indicates a significant difference at $P < 0.01$

time and the duration of flowering in the F_1 population fitted a normal distribution. There were highly significant differences between MPV and H_m (Table 1). The RH_m for initial blooming time was >12% and that for the duration of flowering ~6%, indicating a substantial level of heterosis for both traits. Transgressive segregation was present for both traits (Fig. 1), but transgressive heterosis was not observed (Table 1).

Goodness-of-fit tests

The goodness-of-fit of the 11 genetic models with the observed phenotypic data is reported in Table 2. Following the assumption that the model with the smallest AIC value is the best model, several models with the smallest AIC values were chosen for each trait. For initial blooming time, models B-1 and B-6 had the lowest AIC values, while for the duration of flowering the optimal models were A-1, A-4 and B-1. These models were therefore taken forward for further goodness-of-fit tests. Three tests for goodness-of-fit were then applied: equal distribution (U_1^2, U_2^2 and U_3^2), Smirnov ($_nW^2$) and Kolmogorov test (D_n) (Table 3). According to Wang and Gai (1997), if the test statistics of all the models tested show significant differences, the model with the least significant statistic should be chosen, but if there are no significant differences, then the model with the smallest AIC should be preferred. On this basis, B-1 was selected for both traits.

Estimation of genetic parameters

Genetic parameters for initial blooming time and the duration of flowering were estimated using model B-1 (Table 4). For initial blooming time, the additive effect values of the two pairs of major genes were, respectively, 11.6 and 11.1, and their dominance effects 11.0 and 10.7. Thus, both the additive and

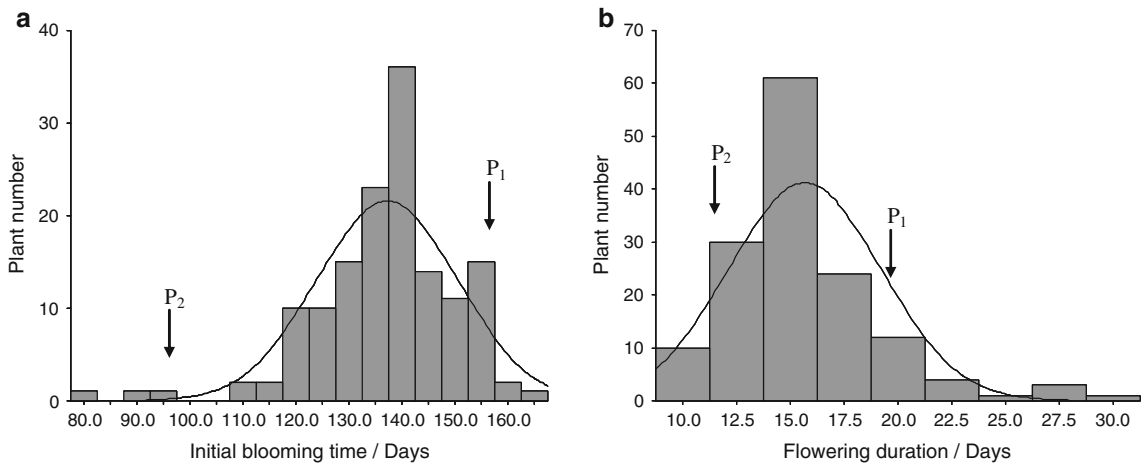

Fig. 1 Frequency distributions for initial blooming time and the duration of flowering in an F_1 population derived from the cross between 'Yuhualuoying' (P_1) × 'Aoyunhanxiao' (P_2)

Table 2 Model parameters for the analysis of segregation of initial blooming time and the duration of flowering in the F_1 population derived from the cross 'Yuhualuoying' × 'Aoyunhanxiao'

Model	Code	Initial blooming time		Flowering duration	
		MLV	AIC	MLV	AIC
A-0	0MG	−567.35	1138.69	−390.71	785.42
A-1	1MG-AD	−566.11	1140.21	−383.69	775.37
A-2	1MG-A	−567.35	1140.69	−390.71	787.42
A-3	1MG-EAD	−566.11	1140.21	−390.71	789.42
A-4	1MG-AEND	−567.35	1142.69	−383.69	775.37
B-1	2MG-ADI	−556.68	1133.35	−372.32	764.64
B-2	2MG-AD	−565.28	1142.56	−383.08	778.16
B-3	2MG-A	−567.35	1142.70	−390.71	789.42
B-4	2MG-EA	−567.35	1140.70	−390.71	787.42
B-5	2MG-AED	−565.28	1138.56	−390.71	789.42
B-6	2MG-EEAD	−565.28	1136.56	−390.71	787.42

Thus, model B-5 (2MG-AED) implies that the additive effect equals the dominance effect of two pairs of major genes

MLV maximum likelihood-value, *AIC* Akaike's information criterion, *MG* major gene model, *A* additive effect, *D* dominance effect, *I* interaction (epistasis), *N* negative, *E* equal

dominance effects of the first pair of major genes were larger than those of the second pair. For the duration of flowering, the additive effect effects of the two pairs of major genes were 2.9 and 2.8, and their dominance effects were −2.8 and −0.5; once again, the additive and dominant effects of the first pair of major genes were larger than those of the second pair. The additive effects of the two pairs of major genes for initial blooming time and duration of flowering were larger that their dominance effects, indicating that the additive effects predominated. However, the size of the dominance effects of the two pairs of major genes was similar to that of the additive effects, suggesting that the two pairs of major genes showed over-dominance for both traits. The additivity × additivity, additivity × dominance, dominance × additivity and dominance × dominance interaction effects were all substantial (Table 4), indicating a significant level of epistasis between the two pairs of major genes. The heritability of the major genes for initial blooming time and duration of flowering were ∼65 and 72%, respectively (Table 4).

SRAP marker analysis

Of the 200 SRAP primer pairs screened against DNA of the two parental cultivars and 10 of the F_1 progeny, 62 were applied to the whole F_1 population, leading to the identification of 346 polymorphic fragments (5.6 per primer pair). The parental and a sample of 45 progeny profiles amplified with the Me13Em4 primer pair are shown in Fig. 2. The SRAP markers significantly associated with the flowering traits are listed in Table 5. Ten SRAP markers were significantly associated with initial blooming time and 12 with the duration of flowering. Both had a high cumulative contribution ratio of 45.54% for initial blooming time and 53.48% for duration of flowering. The contribution of any single marker to the phenotypic variance ranged from 3.0 to 10.1%.

Discussion

Heterotic performance for two flowering traits

Heterosis is common in chrysanthemum. Chen et al. (2003) claimed that hybrid vigor of flower diameter,

Table 3 Test for goodness-of-fit of the selected genetic models

Trait	Model	U_1^2	U_2^2	U_3^2	${}_nW^2$	D_n
Initial blooming time	B-1	0.01 (0.94)	0.00 (0.97)	0.02 (0.88)	0.10	0.07
	B-6	0.13 (0.72)	0.02 (0.89)	3.79 (0.05)	0.23	0.10
Flowering duration	A-1	0.02 (0.90)	0.20 (0.65)	1.66 (0.20)	0.20	0.09
	A-4	0.02 (0.89)	0.20 (0.65)	1.66 (0.20)	0.20	0.09
	B-1	0.00 (0.98)	0.00 (0.97)	0.00 (0.96)	0.17	0.09

U_1^2, U_2^2, U_3^2 refer to uniformity tests, ${}_nW^2$ the Smirnov test statistic, D_n the Kolmogorov test statistic. The numbers in parentheses refer to probabilities

Table 4 Estimation of genetic parameters for flowering traits of chrysanthemum using the optimal genetic model

Genetic parameters	Initial blooming time	Duration of flowering	Genetic parameters	Initial blooming time	Duration of flowering
m	131.46	18.17	j_{ab}	−10.73	−0.47
d_a	11.60	2.85	j_{ba}	−10.84	−2.79
d_b	11.09	2.80	l	−10.71	0.47
h_a	10.97	−2.82	σ_p^2	179.22	12.64
h_b	10.73	−0.47	σ_{mg}^2	116.55	9.06
i	−10.90	2.80	h_{mg}^2 (%)	65.04	71.70

m population mean, d major gene additive effect, h major gene dominant effect, d_a the additive effect of the first pair of major genes, d_b the additive effect of the second pair of major genes, h_a the dominance effect of the first pair of major genes, h_b the dominance effect of the second pair of major genes, i additivity × additivity interaction effect, j_{ab} additivity × dominance interaction effect, j_{ba} dominance × additivity interaction effect, l dominance × dominance interaction effect, σ_p^2 phenotypic variance, σ_{mg}^2 major gene variance, h_{mg}^2 major gene heritability

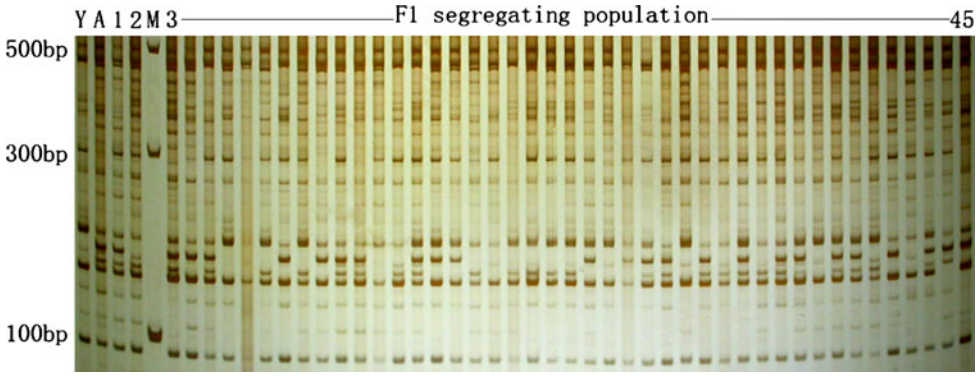

Fig. 2 Amplification profiles of chrysanthemum 'Aoyunhanxiao', 'Yuhualuoying' and their F_1 progeny with SRAP primer combination Me13Em4. *M* DNA marker, *Y* chrysanthemum 'Yuhualuoying', *A* chrysanthemum 'Aoyunhanxiao', *1–45* 45 individuals of F_1 progeny, sequence of Me13 is 5′-TGAG TCCAAACCGGTAA-3′ and that of Em4 is 5′-GACTGCG TACGAATTTGA-3′

number of ray and tubular florets increased significantly in F_1 progeny of chrysanthemum of small inflorescences, and the increasing extend in F_1 population became slow with increase in flower diameter, number of ray and tubular florets of parental lines. The heterosis mainly originated from the heterogeneity between parental lines. In the current study, we have analysed a cross between an autumn- and a summer-flowering chrysanthemum. In the progeny population, both initial blooming time and the duration of flowering varied considerably. Heterosis was present for both traits, with evidence for over-dominance. Therefore, chrysanthemum breeding should greatly benefit from the heterosis available in wide crosses.

The genetics of flowering traits with reference to major genes

Chrysanthemum is largely self-incompatible and suffers from inbreeding depression (Anderson and Ascher 2000; Li and Chen 2007; Xu et al. 2009). This makes it practically difficult to obtain advanced generations of inbred lines of the sort used for genetic research in self-compatible species. A number of tree species are similarly heterozygous, and in these the F_1 generation is generally used as a pseudo-F_2 population for genetic studies (Grattapaglia and Sederoff 1994; Marron and Ceulemans 2006; Song et al. 2005; Wang et al. 2009). Such a pseudo-F_2 population was also widely applied in horticultural crops of

Table 5 The SRAP markers significantly associated with flowering traits in the F_1 population derived from the cross 'Yuhualuoying' × 'Aoyunhanxiao', as detected by one-way ANOVA method

Traits	Marker name	Sum of squares between groups	Sum of squares within groups	*F* value	*P* value	Ratio of contribution (%)
Initial blooming time	*Me11Em19-2*	796.02	21425.73	5.02	0.027	3.58
	Me13Em1-1	1026.57	21195.18	3.25	0.043	4.62
	Me13Em4-8	1014.75	21207.00	3.21	0.044	4.57
	Me14Em10-1	1163.24	21058.51	3.70	0.027	5.24
	Me15Em14-1	1693.22	20528.54	5.53	0.005	7.60
	Me18Em5-1	845.79	21375.96	5.34	0.022	3.81
	Me19Em5-5	685.08	21536.67	4.29	0.040	3.08
	Me20Em1-1	1902.59	20319.16	12.64	0.001	8.56
	Me20Em7-4	994.86	21226.89	6.33	0.013	4.48
	Me20Em15-1	1894.30	20327.45	12.58	0.001	8.52
Total						45.54
Flowering duration	*Me13Em4-8*	170.75	1512.17	7.57	0.001	10.14
	Me13Em15-1	79.33	1603.59	6.68	0.011	4.71
	Me14Em16-4	75.10	1607.82	3.13	0.047	4.46
	Me18Em1-3	69.79	1613.13	5.84	0.017	4.14
	Me18Em9-3	72.78	1610.14	6.10	0.015	4.33
	Me18Em11-2	53.57	1629.35	4.44	0.037	3.18
	Me18Em14-6	130.43	1552.49	11.34	0.001	7.75
	Me19Em10-2	58.74	1624.18	4.88	0.029	3.49
	Me19Em14-2	89.07	1593.85	7.54	0.007	5.29
	Me19Em17-1	50.03	1632.89	4.14	0.044	2.97
	Me20Em1-1	50.87	1632.05	4.21	0.042	3.02
	Me20Em15-1	68.20	1614.72	5.70	0.018	4.05
Total						53.48

self-incompatibility such as artichoke (Lanteri et al. 2006; Portis et al. 2009) and garlic (Ma et al. 2006). In chrysanthemum, flower diameter, central flower diameter, and ray and tubular floret number are all predominantly controlled by additive genes (Ghimiray et al. 2005; Pal and George 2002; Sirohi and Behera 2000; Zhang et al. 2008, 2010a), but little is known of the inheritance of flowering traits. Although flowering time depends on planting date, photoperiod, temperature and other climatic factors (Chardon et al. 2004), genes also play an important part (Anbessa et al. 2006; Cai et al. 2008; Godoy and Palomo 1999; Hao et al. 2008a). The outcome of the present genetic analysis was that both flowering traits were compatible with the action of two pairs of major genes displaying additivity–dominance–epistasis. Their contribution to the phenotypic variation was substantial and their heritabilities were as high as 0.88. Thus, it should be relatively easy to select for these two traits in the early generations of a breeding programme. The additive effect of these major genes predominated, although both dominance and epistasis were detected. Theoretically, both dominance and epistasis in heterosis could be adopted in a breeding programme. Although we were unable to estimate the contribution of polygene and environmental variation, as is possible using several jointly segregating families (Chen et al. 2009; Zeng and Pu 2006), the detection of major genes for initial blooming time and duration of flowering of chrysanthemum is a useful start to identifying molecular markers associated with these traits.

SRAP markers associated with flowering traits of chrysanthemum

Genetic linkage maps, molecular markers and QTL analysis have been attempted in a number of ornamental crops (Abe et al. 2002; Dugo et al. 2005; Dunemann et al. 1999; Remay et al. 2009; Yagi et al. 2006). In chrysanthemum, in contrast, there has been less progress to date in this direction (Zhang et al. 2010b; Zhao et al. 2009a, b). Here, we have identified SRAP markers associated with initial blooming time and the duration of flowering were identified by the marker-based variance method based on 2-year phenotypic data in an F_1 (pseudo-F_2) population. That the contribution of each individual marker explained 3–10% suggested that the genetic effects of quantitative trait genes controlling flowering traits of chrysanthemum varied in magnitude, which further confirmed the detection of major genes for the flowering traits described above. Moreover, some significant differences at the 0.05 or 0.01 level were detected between every two significantly associated markers by double-factor variance analysis for initial blooming time and duration of flowering, respectively (data not shown), which also provides some molecular evidence for the above-mentioned interaction of the two pairs of major genes.

Compared to marker-trait analyses performed in other crops (Huang et al. 2004a; Li and Zheng 1998; Su et al. 2009), the contribution of a single marker locus to the phenotypic variation of flowering traits studied here is relatively low. However, we believe that some useful SRAP markers of flowering behaviour QTL have been detected in this study. For example, the locus *Me20Em15-1* explained ~8.5% of the variation in initial blooming time and approximately 4.1% in the duration of flowering, as did *Me13Em4-8* and *Me20Em1-1*. As SRAP marker amplifies open reading frames, therefore, the associated genetic markers with relatively high contributions to the phenotypic variation of flowering traits identified here may be useful in target gene cloning.

The marker-based variance method is limited, especially as it has difficulty in determining whether the marker loci are associated with one or more QTL, and cannot easily define intra-chromosomal QTL location with any precision. It also tends to underestimate the size of the genetic effect, and suffers from a high false positive rate in the detection of genetic markers or QTL associated with a trait (Huang et al. 2004b; Tanksley et al. 1982). The outcome of marker-based variance, however, is generally quite consistent with map-based methods in terms of approximate QTL location (Huang et al. 2004b; Li et al. 1999; Su et al. 2000). Thus, the SRAP marker analysis will help to understand the inheritance pattern of flowering traits of chrysanthemum. Even so, further research using map-based QTL analysis is needed to accurately map horticulturally important traits like the flowering behaviour of chrysanthemum.

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