The genetic architecture of petal number in Cardamine hirsuta

Bjorn Pieper, Marie Monniaux and Angela Hay
Max Planck Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, 50829 Köln, Germany

Summary

- Invariant petal number is a characteristic of most flowers and is generally robust to genetic and environmental variation. We took advantage of the natural variation found in Cardamine hirsuta petal number to investigate the genetic basis of this trait in a case where robustness was lost during evolution.
- We used quantitative trait locus (QTL) analysis to characterize the genetic architecture of petal number.
- Average petal number showed transgressive variation from zero to four petals in five C. hirsuta mapping populations, and this variation was highly heritable. We detected 15 QTL at which allelic variation affected petal number. The effects of these QTL were relatively small in comparison with alleles induced by mutagenesis, suggesting that natural selection may act to maintain petal number within its variable range below four. Petal number showed a temporal trend during plant ageing, as did sepal trichome number, and multi-trait QTL analysis revealed that these age-dependent traits share a common genetic basis.
- Our results demonstrate that petal number is determined by many genes of small effect, some of which are age-dependent, and suggests a mechanism of trait evolution via the release of cryptic variation.

Introduction

Stable merosity (the number of organs within whorls) is one of the major trends in angiosperm floral evolution (Specht & Bartlett, 2009). The majority of core eudicots and monocots have fixed pentameroses and trimerous merosity, respectively, while members of the Brassicaceae are characterized by a fixed cruciform arrangement of four petals. This cruciform flower is typified by the model species Arabidopsis thaliana, which contains four petals, surrounded by four sepals, and internal to these sterile organs are the reproductive organs: stamens and carpels. Cardamine hirsuta is related to A. thaliana and shares a similar four-whorled flower but petal number is unstable, varying from zero to four between individual flowers.

The precise identity, number and placement of floral organs is defined early in flower development and is under strict genetic control (Coen & Meyerowitz, 1991). However, while the genetic basis of floral organ identity is well understood and encapsulated in the predictive ABC model (Bowman et al., 1991), we know less about the genes that control the specific number of different floral organs. The production of four petals in A. thaliana requires genes that control petal identity and the establishment of boundaries that demarcate the position of petal initiation on the floral meristem (Irish, 2008; Huang et al., 2012; Lampugnani et al., 2013). However, this gene activity must also interface with pathways that control the size of the floral meristem, the outgrowth of lateral organs and the patterning of their polarity, and these interactions are not yet captured by predictive models (Eshed et al., 2001; Benkova et al., 2003; Irish, 2008). Two important gene products for petal organogenesis are the trihelix transcription factor encoded by PETAL LOSS (PTL) and the zinc-finger transcriptional repressor encoded by RABBIT EARS (RBE) (Brewer et al., 2004; Takeda et al., 2004). Mutations in these genes cause a reduction in petal number, which is associated with a failure to suppress the growth in the boundary region between sepals (Brewer et al., 2004; Krizek et al., 2006; Lampugnani et al., 2012). RBE promotes activity of the microRNA-regulated CUP-SHAPED COTYLEDON1/2 (CUC1/2) boundary genes by direct repression of MIR164c, while PTL acts in parallel to CUC1/2 to suppress growth between sepals, and this boundary delimitation is required for auxin-induced petal initiation (Huang et al., 2012; Lampugnani et al., 2013).

The analysis of natural genetic variation offers additional insights over induced mutants about which components of a gene regulatory network were targeted by evolution to generate diversity. Natural variation in A. thaliana has proved an important resource to dissect the genetic basis of developmental traits (Michaels & Amasino, 1999; Johanson et al., 2000), however, petal number does not vary among A. thaliana accessions. This reproducibility of petal number reflects its robustness to genetic variation. Phenotypic robustness is a property of many developmental patterning systems but is challenging to address experimentally. Studies in Caenorhabditis show that vulva cell fate patterning is invariant but evolves by accumulating cryptic variation (Felix, 2007; Milloz et al., 2008). In C. hirsuta, the normally invariant trait of petal number expresses phenotypic variation,
providing an opportunity to analyse quantitative genetic variation that may be cryptic in species with robust petal number such as *A. thaliana*. Moreover, this comparative system of two related, genetically tractable species that differ in phenotypic robustness, provides an opportunity to functionally address the contribution of cryptic variation to trait evolution.

Fisher’s polygenic model of inheritance attributes the genetic architecture of quantitative traits to a small number of variants with large effects and a large number of variants with small effects (Fisher, 1918). Major quantitative trait locus (QTL) have been with large effects and a large number of variants with small effects architecture of quantitative traits to a small number of variants of cryptic variation to trait evolution. This provides an opportunity to functionally address the contribution genetically tractable species that differ in phenotypic robustness, genetically tractable species that differ in phenotypic robustness, genetically tractable species that differ in phenotypic robustness, genetically tractable species that differ in phenotypic robustness.

New Phytologist

C. *hirsuta* Ox seeds were mutagenized by agitation with 0.2% ethane methanesulfonate (EMS, Sigma) for 10 h, washed in dH2O, then sown on soil and harvested in pools of five M1 plants. Approximately 1500 M2 plants were subsequently screened to identify the *fp2* mutant. Mutant characterization was performed after backcrossing to Ox twice.

Genetic map

The genetic map for the Ox × Wa RILs is described in Hay et al. (2014). For the Ox × GR, Ox × Jpa1 and Ox × Nz populations, only partial genomic coverage could be achieved by genotyping with available markers from the Ox × Wa genetic map. The total coverage of linkage groups was 39.4%, 58.0% and 26.3% for those populations, respectively, when compared with the Ox × Wa map (Hay et al., 2014). Furthermore, 7, 2 and 6 loci were represented by independent markers in these three populations, respectively. Therefore, an integrated genetic map was made from the five individual maps for QTL analysis that consisted of 345 markers and had a total length of 891.7 cM (Supporting Information Fig. S1). All genetic maps were made using the regression mapping algorithm of Joinmap® 4 (Van Ooijen, 2006).

Plant cultivation

Plants were grown 7 × 7 cm pots on a 3 : 1 peat–vermiculite mixture. All experiments were performed under a photoperiod of 16 h : 8 h, light : dark. Experiments with the Ox × Wa and Ox × GR RILs, and the Ox × Jpa1 F2 were conducted in a glasshouse with supplemental light and a temperature of 20°C. The Ox × Az RIL population was analysed in a controlled environment chamber with temperatures of 19°C : 18°C, day : night. The Ox × Nz F2 population was analysed in a controlled environment chamber with temperatures of 22°C : 21°C, day : night. The *fp2* mutant and Ox were grown in a controlled environment chamber with constant temperature of 23°C to analyse petal number and in a glasshouse with supplemental light and a temperature of 20°C to analyse other phenotypes.

Plant phenotyping

Up to 30 of the first flowers to develop on the main inflorescence were consecutively removed from the plant as they opened and the petal number and the total number of trichomes present on the sepals were counted using a dissecting microscope. For the population consisted of 297 plants derived from a cross between Ox and the Nz accession collected from Wellington, New Zealand (Hay et al., 2014). The Ox × Jpa1 F2 population consisted of 418 plants, derived from a cross between Ox and the Jpa1 accession collected from Oumi, Japan (Hay et al., 2014), of which 100 lines belonging to both extremes of the phenotypic distribution were selected for genetic analysis.
Ox × Wa and Ox × Az populations, three replicate lines were analysed per RIL, while for the Ox × GR population a single line was analysed. The average number of petals and sepal trichomes were calculated per plant and used for QTL analysis in the F2 populations and in the Ox × GR RIL population, while for the other populations the mean average per RIL was used. Petal number in fp2 and Ox was scored in up to 15 of the first flowers of 12 plants (n = 171) and 15 plants (n = 225), respectively. Other floral organs in fp2 and Ox were scored in up to 25 of the first flowers of nine plants (n = 193) and seven plants (n = 159), respectively. Rosette leaf number and the average leaflet number in fp2 and Ox were scored in nine and seven plants, respectively. Rosette leaf number was scored as the number of rosette leaves initiated when the first floral buds were visible; leaflet number was scored on all leaves.

Quantitative trait locus analysis

All statistical analyses, including QTL analysis, were done using GenStat 16th edition (VSN International, 2013). Broad sense heritabilities (H²) were calculated according to the implementation in GenStat: 1 – ((non-genetic (within RIL) variance/number of plants per RIL)/genetic (between RILs) variance component), where the variance components are estimated by fitting a mixed model with RIL as a random term. For QTL analysis, genetic predictors were calculated from the molecular marker data and the integrated genetic map with a maximum distance of 2 cM between them. The QTL mapping methodology for multi-trait QTL analysis involved fitting a mixed effects model to a set of traits in which the QTL were fixed effects and the residual genetic and non-genetic variances and covariances were modelled by a random term. The method for single trait analysis is similar but in this case only the residual genetic and nongenetic variance was modelled by the random term. Simple interval mapping scans were followed by several iterations of composite interval mapping. During the latter procedure, cofactors were added and/or removed until no further improvement could be made. The genetic predictors comprising the final set of co-factors were used for fitting the final QTL models in GenStat to estimate the additive allelic effects. The total phenotypic variances explained by the full QTL models were determined as the difference in the residual variance components of the models with no QTL vs the models with all QTL fitted. These differences were then expressed as the percentages of the residual variances from the models with no QTL fitted. In Tables 1 and 2, the phenotypic variances explained by each QTL were estimated as the differences in the total variance explained by the full QTL model and models where each QTL was successively dropped. In SI Tables, we used the QMTESTIMATE procedure in GenStat to estimate the variance explained per QTL. The average number of petals and sepal trichomes per flower were calculated per plant and used for single trait QTL analysis in the F2 populations and in the Ox × GR RILs, while for the other populations the mean average per line was used. Multi-trait QTL analysis was performed on the average petal number and average sepal trichome number, as well as on the averages per bin of five flowers. For the latter procedure, the respective phenotypic data were averaged per five flowers, without overlap, for up to as many as 30 flowers, thus yielding six averages per trait per plant. A multi-trait genome scan was then performed using the set of 12 averages as traits. Following this analysis, moving averages were calculated for each trait per plant per three flowers, with increments of one flower (except for the first of the averages which was an average of only flowers 1 and 2), thus yielding up to 29 trait averages across the flowering shoot. A final multi-trait QTL model with the QTL detected for the nonoverlapping five flower bins was then fitted to the 29 petal and sepal trichome moving averages. Estimating the QTL effects in this way allowed us to determine how they changed over time.

Statistical analysis

In order to determine whether flower rank and genotype were independent predictors of the petal number of individual flowers in the mapping populations, petal number was modelled as a function of the fixed terms genotype and flower rank. The model was fitted using the REML directive of GenStat and the significance of the fixed terms was evaluated by the associated Wald tests. The variance explained by flower rank was determined as the difference between the residual variance components of models with (1) only genotype and (2) both genotype and flower rank fitted, as a percentage of the residual variance component of a model with neither of the terms fitted.

Table 1 Characteristics of quantitative trait loci (QTL) detected for average petal number in five Cardamine hirsuta mapping populations

<table>
<thead>
<tr>
<th>QTL</th>
<th>Population</th>
<th>Linkage group</th>
<th>Position (cM)</th>
<th>Add. eff. (petals)</th>
<th>Var. expl. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PN1</td>
<td>Ox × Az</td>
<td>Chr.1</td>
<td>45.88</td>
<td>–0.39</td>
<td>4.4</td>
</tr>
<tr>
<td>PN2</td>
<td>Ox × Wa</td>
<td>Chr.2</td>
<td>6.85</td>
<td>–0.75</td>
<td>13.2</td>
</tr>
<tr>
<td>PN3</td>
<td>Ox × GR</td>
<td>Chr.2</td>
<td>6.85</td>
<td>–0.44</td>
<td>4.4</td>
</tr>
<tr>
<td>PN4</td>
<td>Ox × Az</td>
<td>Chr.3</td>
<td>15.29</td>
<td>0.47</td>
<td>5.2</td>
</tr>
<tr>
<td>PN5</td>
<td>Ox × Jpa1</td>
<td>Chr.4</td>
<td>39.92</td>
<td>0.63</td>
<td>10.1</td>
</tr>
<tr>
<td>PN6</td>
<td>Ox × Wa</td>
<td>Chr.4</td>
<td>80.26</td>
<td>–0.53</td>
<td>7.8</td>
</tr>
<tr>
<td>PN7</td>
<td>Ox × Jpa1</td>
<td>Chr.5</td>
<td>93.89</td>
<td>0.54</td>
<td>6.2</td>
</tr>
<tr>
<td>PN8</td>
<td>Ox × Wa</td>
<td>Chr.5</td>
<td>95.99</td>
<td>0.37</td>
<td>3.6</td>
</tr>
<tr>
<td>PN9</td>
<td>Ox × Jpa1</td>
<td>Chr.6</td>
<td>11.15</td>
<td>–0.44</td>
<td>4.4</td>
</tr>
<tr>
<td>PN10</td>
<td>Ox × Wa</td>
<td>Chr.6</td>
<td>57.49</td>
<td>–1.80</td>
<td>11.9</td>
</tr>
<tr>
<td>PN11</td>
<td>Ox × Jpa1</td>
<td>Chr.7</td>
<td>67.22</td>
<td>–0.51</td>
<td>5.9</td>
</tr>
<tr>
<td>PN12</td>
<td>Ox × Jpa1</td>
<td>Chr.8</td>
<td>72.84</td>
<td>0.55</td>
<td>7.9</td>
</tr>
<tr>
<td>PN13</td>
<td>Ox × GR</td>
<td>Chr.9</td>
<td>100.05</td>
<td>–0.44</td>
<td>5.6</td>
</tr>
<tr>
<td>PN14</td>
<td>Ox × GR</td>
<td>Chr.10</td>
<td>112.39</td>
<td>–0.91</td>
<td>13.9</td>
</tr>
<tr>
<td>PN15</td>
<td>Ox × Jpa1</td>
<td>Chr.11</td>
<td>36.57</td>
<td>–0.44</td>
<td>4.6</td>
</tr>
<tr>
<td>PN16</td>
<td>Ox × GR</td>
<td>Chr.12</td>
<td>116.62</td>
<td>0.53</td>
<td>6.8</td>
</tr>
<tr>
<td>PN17</td>
<td>Ox × GR</td>
<td>Chr.13</td>
<td>118.88</td>
<td>0.92</td>
<td>21.8</td>
</tr>
<tr>
<td>PN18</td>
<td>Ox × GR</td>
<td>Chr.14</td>
<td>1.04</td>
<td>0.65</td>
<td>14.0</td>
</tr>
<tr>
<td>PN19</td>
<td>Ox × GR</td>
<td>Chr.15</td>
<td>8.42</td>
<td>1.84</td>
<td>10.0</td>
</tr>
<tr>
<td>PN20</td>
<td>Ox × GR</td>
<td>Chr.16</td>
<td>69.37</td>
<td>–0.54</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Additive effects (Add. eff.) are given for homozygous allele substitutions with Ox alleles. Var. expl., phenotypic variance explained by the QTL.
Results

To elucidate the genetic architecture of petal number variation, we analysed five independent mapping populations generated from bi-parental crosses with the Ox accession as a recurrent parent. Average petal number in these experimental populations covered a range from 0 to 4, however, flowers with more than four petals were never observed. Transgressive variation was observed in all populations, leading to average petal numbers that exceeded those of the founder accessions, and the broad sense heritability ($H^2$) of this variation was high (0.86–0.90), demonstrating a strong genetic basis for this trait (Fig. 1). We found that petal number varied in response to slight differences in the environmental conditions between separate experiments, suggesting that this trait is determined not only by genetics but also by environment. These effects are most prominent when comparing the average petal number of the recurrent parent Ox between different experiments (Fig. 1). The extensive transgression in these mapping populations indicates that average petal number differences between the founder accessions are not consistent with directional natural selection (Orr, 1998). Furthermore, it shows that there is sufficient standing variation in our sample to generate petal numbers across the possible range of 0–4 through recombination.

Quantitative trait loci analysis of average petal number was performed in order to discover the loci at which allelic variation affected this trait and to estimate their effects. We detected 15 distinct QTL in total at which allelic variation affected petal number when considering loci identical if $<10$ cM apart (Figs 2, S2, Table 1). Despite partial genomic coverage of some genetic maps, three QTL were detected in the Ox × Nz and Ox × Jpa1 populations, seven QTL in the Ox × Az population and five QTL in each of the other populations. With the exception of the

Table 2 Characteristics of the multi-trait quantitative trait loci (QTL) detected for average petal number and average sepal trichome number in two Cardamine hirsuta mapping populations

<table>
<thead>
<tr>
<th>QTL</th>
<th>Pop</th>
<th>LG</th>
<th>Position (cM)</th>
<th>Add. eff. (petals)</th>
<th>Var. expl. (%)</th>
<th>Add. eff. (trichomes)</th>
<th>Var. expl. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-T1</td>
<td>Ox × Az</td>
<td>Chr.1</td>
<td>44.83</td>
<td>−0.55</td>
<td>60</td>
<td>−0.38</td>
<td>29</td>
</tr>
<tr>
<td>1</td>
<td>Ox × Az</td>
<td>Chr.2</td>
<td>29.16</td>
<td>−0.49</td>
<td>48</td>
<td>0.25</td>
<td>11</td>
</tr>
<tr>
<td>P-T4</td>
<td>Ox × Az</td>
<td>Chr.3</td>
<td>10.51</td>
<td>0.49</td>
<td>40</td>
<td>0.43</td>
<td>3.2</td>
</tr>
<tr>
<td>P-T5</td>
<td>Ox × Az</td>
<td>Chr.4</td>
<td>39.92</td>
<td>0.66</td>
<td>7.6</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>P-T6</td>
<td>Ox × Az</td>
<td>Chr.4</td>
<td>81.98</td>
<td>−0.61</td>
<td>6.6</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>P-T7</td>
<td>Ox × Nz</td>
<td>Chr.4</td>
<td>93.89</td>
<td>0.34</td>
<td>2.3</td>
<td>−0.58</td>
<td>6.8</td>
</tr>
<tr>
<td>P-T8</td>
<td>Ox × Az</td>
<td>Chr.5</td>
<td>20.05</td>
<td>ns</td>
<td>ns</td>
<td>0.55</td>
<td>5.1</td>
</tr>
<tr>
<td>P-T9</td>
<td>Ox × Az</td>
<td>Chr.5</td>
<td>57.49</td>
<td>ns</td>
<td>ns</td>
<td>0.61</td>
<td>4.3</td>
</tr>
<tr>
<td>P-T10</td>
<td>Ox × Nz</td>
<td>Chr.6</td>
<td>68.24</td>
<td>−0.57</td>
<td>8.2</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>P-T11</td>
<td>Ox × Az</td>
<td>Chr.6</td>
<td>94.35</td>
<td>−0.60</td>
<td>7.3</td>
<td>0.43</td>
<td>3.8</td>
</tr>
<tr>
<td>P-T12</td>
<td>Ox × Nz</td>
<td>Chr.6</td>
<td>110.83</td>
<td>−0.90</td>
<td>13.4</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>P-T13</td>
<td>Ox × Az</td>
<td>Chr.7</td>
<td>8.46</td>
<td>ns</td>
<td>ns</td>
<td>−0.17</td>
<td>20.4</td>
</tr>
<tr>
<td>P-T14</td>
<td>Ox × Nz</td>
<td>Chr.7</td>
<td>47.58</td>
<td>ns</td>
<td>ns</td>
<td>0.48</td>
<td>4.6</td>
</tr>
<tr>
<td>P-T15</td>
<td>Ox × Az</td>
<td>Chr.7</td>
<td>79.71</td>
<td>ns</td>
<td>ns</td>
<td>−0.65</td>
<td>5.9</td>
</tr>
<tr>
<td>P-T16</td>
<td>Ox × Nz</td>
<td>Chr.7</td>
<td>99.37</td>
<td>0.31</td>
<td>1.3</td>
<td>−0.62</td>
<td>4.3</td>
</tr>
<tr>
<td>P-T17</td>
<td>Ox × Az</td>
<td>Chr.8</td>
<td>1.04</td>
<td>0.72</td>
<td>11.2</td>
<td>−0.42</td>
<td>3.7</td>
</tr>
<tr>
<td>1</td>
<td>Ox × Az</td>
<td>Chr.8</td>
<td>55.29</td>
<td>ns</td>
<td>0.70</td>
<td>9.3</td>
<td></td>
</tr>
</tbody>
</table>
Ox × Jpa1 population, at least one unique QTL for petal number was always detected. The full additive QTL models explained up to 56.2% (for the Ox × Wa RILs; Table S1) of the total phenotypic variance for average petal number.

To further investigate the genetic architecture of petal number, we examined the allelic effects of the detected QTL. Both negative and positive allelic effects on petal number were found in all mapping populations, which is consistent with the observed transgressive variation. The strongest effects were observed in the Ox × Jpa1 F2 population with the largest effect being 2.12 petals for QTL PN4 (Table 1). However, selection of plants from the extremes of the phenotypic distribution has likely led to an overestimation of these effects (see the Materials and Methods section). PN4 was also detected in the Ox × Az RIL population with an allelic effect of 0.63 petals, and another two QTL detected in the Ox × Jpa1 F2, PN8 and PN14, also had considerably weaker effects in the other populations where these were detected. The strongest effects observed in the unbiased populations was 0.92 and −0.91 petals for homozygous substitutions with Ox alleles in the Ox × Wa RIL population at PN14 and the Ox × Nz F2 population at PN11 respectively. The absolute magnitude of all other QTL effects ranged between 0.37 and 0.75 petals for homozygous substitutions. We found that mutant alleles induced by ethyl methanesulfonate (EMS) in the Ox accession can have much stronger effects on petal number than the natural alleles detected in our mapping populations. For example, a recessive allele of the four petals 2 (fp2) mutant increased the average petal number by 2.0 over the wild-type value when homozygous (Fig. 3a,b). We observed no pleiotropic effects of the fp2 mutant on the number of other floral organs, the number of rosette leaves produced before flowering, or the number of leaflets per leaf, suggesting that fp2 specifically affects petal number (Fig. 3c–e). This result shows that single mutations can produce large phenotypic effects on petal number but such large-effect alleles are not found in natural accessions of C. hirsuta.

To understand petal number in the context of variation found in other floral organs in C. hirsuta, we compared the location of QTL identified here with those previously identified for stamen

![Figure 2](image-url)
number in the Ox × Wa RILs (Hay et al., 2014). We found that the major QTL influencing petal and stamen number did not co-localize, indicating that each trait has a distinct genetic basis. We next considered how petal number varied within individual plants. Following the phase transition from vegetative to reproductive development in C. hirsuta, the shoot apical meristem remains indeterminate, yet produces floral meristems on its flank rather than leaf primordia. Modelling petal numbers on individual flowers as a function of genotype and flower rank (i.e. the number in sequence when counting from the first flower to develop) revealed that the rank of the flower was a highly significant source of variation (P < 0.001). Although most variance for petal number per flower was explained by genotype in the populations, up to 16% of the remaining variance was accounted for by flower rank. This observation indicated that the variance around the mean petal number per plant was not random but rather, there existed a strong relationship between the rank of the flower and the number of petals it produced. Closer inspection revealed a typical pattern whereby petal number on the first flowers to open was relatively high, and would then decrease over time, to eventually rise again towards the last flowers that a plant produced (Fig. 4a). To gain insight into this temporal pattern, we sought to identify another quantitative floral trait that is temporally regulated to compare its dynamics with that of petal number. Trichome distribution is a classical marker of developmental phase transitions in A. thaliana (Telfer et al., 1997). The distribution of trichomes during flowering is temporally regulated by microRNA156-targeted SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL) genes in A. thaliana (Shikata et al., 2009; Yu et al., 2010), which increase in expression due to a decline in miR-156 levels as the plant ages (Wang et al., 2009). We quantified trichome distribution on the abaxial surface of sepals in C. hirsuta and found that the temporal change in sepal trichome number closely resembled that of petal number (Fig. 4a,b). Based on this observation that the two traits co-varied during plant ageing, we hypothesized that they may share a common genetic basis.

To investigate whether the genetic basis of petal number variation, a derived trait in C. hirsuta, shared common components with sepal trichome density; a conserved trait between C. hirsuta and A. thaliana, we performed multi-trait QTL analysis of both traits. We quantified petal number and sepal trichome number in the Ox × Nz F2 and the Ox × Az RIL populations and observed transgression beyond the trait values of the founder accessions in both populations (Fig. 4c, d). This variation in sepal trichome number was highly heritable with an $H^2$ of 0.74 in the RIL population, where broad sense heritability could be determined, indicating that trichome number has a strong genetic basis. We detected 12 QTL in the Ox × Az RIL population using multi-trait QTL analysis of average petal number and trichome number, and five different QTL in the Ox × Nz F2 population (Figs 4e, S3, Table 2). Five of the QTL detected in the Ox × Az population had significant effects on both petal number and trichome number (P-T1, 4, 11, 17 and the QTL on chromosome 2 between P-T2 and P-T3) while two QTL affected only petal number (P-T5, 6) and five QTL affected only trichome number.

![Fig. 3](image)

Fig. 3 The non-pleiotropic four petals2 mutant increases petal number in Cardamine hirsuta. (a) Representative flower showing the model petal number found in fp2 (left) and representative flowers of all petal numbers found in wild type Ox (right). (b) Barchart showing average petal number in wild type Ox and fp2 genotypes grown at 23°C, n = 225 and 171 flowers, respectively. (c–e) Barcharts showing average floral organ numbers (c, n = 159 and 193 flowers, respectively), average rosette leaf number (d, n = 7 and 9 plants, respectively), and average leaflet number (e, n = 7 and 9 plants, respectively) in wild type Ox (light grey) and fp2 (dark grey) genotypes. Error bars show ± SE of the mean.
Six of the QTL with effects on petal number had been detected in our previous analysis (PN1, 3, 4, 5, 10, and 14; Fig. 2; Table 1). We found that the weakest effect QTL detected in our previous analysis (PN12; Fig. 2; Table 1) was no longer significant but we detected a novel QTL effect on petal number on chromosome 2 at 29.16 cM, accompanied by a significant effect on trichome number (Fig. 4e between P-T2 and P-T3, Table 2). We detected two QTL with effects on both petal number and trichome number in the Ox9Nz F2 population (P-T7, P-T16) that had both been detected before as QTL affecting petal number (PN6, 13; Fig. 2, Table 1), and another three QTL had effects on only petal number (P-T10, 12) or trichome number (P-T14). All three QTL that we had previously detected in this population (PN6, 9 and 11; Fig. 2, Table 1) were also found using multi-trait QTL analysis. In summary, 7 of 11 QTL that affected petal number also had significant effects on trichome number, showing that indeed both traits are partly under the control of the same loci, either pleiotropically or due to closely linked QTL. However, these loci did not consistently affect both

(P-T8, 9, 13, 15 and the QTL on chromosome 8 between P-T18 and P-T19). Six of the QTL with effects on petal number had been detected in our previous analysis (PN1, 3, 4, 5, 10, and 14; Fig. 2; Table 1). We found that the weakest effect QTL detected in our previous analysis (PN12; Fig. 2; Table 1) was no longer significant but we detected a novel QTL effect on petal number on chromosome 2 at 29.16 cM, accompanied by a significant effect on trichome number (Fig. 4e between P-T2 and P-T3, Table 2). We detected two QTL with effects on both petal number and trichome number in the Ox × Nz F2 population (P-T7, P-T16) that had both been detected before as QTL affecting petal number (PN6, 13; Fig. 2, Table 1), and another three QTL had effects on only petal number (P-T10, 12) or trichome number (P-T14). All three QTL that we had previously detected in this population (PN6, 9 and 11; Fig. 2, Table 1) were also found using multi-trait QTL analysis. In summary, 7 of 11 QTL that affected petal number also had significant effects on trichome number, showing that indeed both traits are partly under the control of the same loci, either pleiotropically or due to closely linked QTL. However, these loci did not consistently affect both

Fig. 4 Petal and sepal trichome number share a common genetic basis in Cardamine hirsuta. (a) Moving averages of petal number (open circles) and sepal trichome number (closed circles) show a similar age-dependent change when plotted against flower position. Organ counts were averaged in a window of three flowers that was shifted by one flower except the first average, which was calculated for flowers 1 and 2. Dashed and continuous lines show nonlinear models fitted to the petal and trichome numbers, respectively, to illustrate the similarity in their relation with flower number. (b) Scanning electron micrograph of a C. hirsuta inflorescence; varying numbers of sepal trichomes are indicated by arrowheads on representative flowers with 1, 2 and 3 trichomes; bar, 0.5 mm. (c, d) Frequency distributions of average sepal trichome number in the Ox × Nz F2 (c), and the Ox × Az recombinant inbred lines (RILs) (d). Mean average sepal trichome number for the founder accessions of each population is indicated by triangles (Ox: blue, Az or Nz: red) and the standard error of the mean by the black line crossing it. (e) Summary of multi-trait quantitative trait locus (QTL) analysis of petal and sepal trichome numbers averaged over all flowers and in consecutive bins of five flowers (up to as many as 30 flowers). Detected QTL are shown as arrows on the integrated genetic map to either the left (Ox × Nz F2) or the right (Ox × Az RILs) of the eight linkage groups according to the mapping population in which they were detected. The black marker inside each arrow is placed at the position where the detected QTL effect was most significant and the length of each arrow is scaled to a 2-log(p) interval around that position. Arrows are terminated with a black mark where the most significant position or the 2-log(p) interval could not be accurately determined due to incomplete map coverage. Arrows point up or down according to whether the Ox allele increases or decreases petal number. The two traits are shown in different colours: petal number (red) and sepal trichome number (blue), and the phenotyping method is indicated by colour shade: averaging all flowers (bright) and averaging five flower bins (pale). The number of traits analysed with multi-trait QTL analysis of the binned averages totalled to 10 and 12 for the Ox × Nz F2 and the Ox × Az RILs, respectively. The results are summarized with a single arrow if a significant effect was detected for at least one bin. The direction was always the same when effects were detected in multiple bins.
traits in the same way: at five QTL (P-T7, 11, 16, 17 and the QTL on chromosome 2 between P-T2 and P-T3) the allelic effects for petal number and trichome number were of opposite sign while at the remaining two QTL (P-T1, 4) they were of the same sign. Therefore, averaging petal number and trichome number per plant may obscure the age-dependent effects of these alleles on each trait.

Age-dependent variation in petal and sepal trichome number appeared as uncertainty around the average trait values in our analysis above, while this may actually be under genetic control. Considering the relationship between the age of the shoot and the numbers of petals and sepal trichomes produced by flowers, and the similarity in temporal patterning of both traits, we investigated whether and how QTL affected these traits in an age-dependent manner. To do this, we performed multi-trait QTL analysis on petal number and trichome number averaged in nonoverlapping bins of five flowers (i.e. 12 averages in the Ox × Az population and 10 in the Ox × Nz population). Across both populations, 19 QTL were detected with significant effects on at least one bin for either petal number or trichome number (Figs 4e, S4, Tables S2–S4). These QTL included the same loci as we found with multi-trait analysis of petal number and trichome number averaged over the entire inflorescence, with a few exceptions. For example, a QTL was detected for petal number and trichome number averaged across all flowers in the Ox × Az population on chromosome 2 at 29.16 cM (between P-T2 and P-T3), whereas a QTL for petal number and trichome number in binned flowers (P-T2) was detected at 1.23 cM instead (Fig. 4e, Table S2). Allelic effects of the age-dependent QTL (P-T2) were of the same sign for petal number and trichome number while those of the QTL for average petal number and trichome number were of the opposite sign (Fig. 4e, Table S3). In another example from the same population, two separate QTL were detected on chromosome 8 at 36.55 and 69.37 cM: one for trichome number (P-T18), and one for petal number and trichome number (P-T19), in binned flowers (Fig. 4e, Table S2), whereas a single QTL for average trichome number was found between them at 55.29 cM. These differences are probably caused by closely linked QTL in each case, which hindered the accurate determination of the QTL position on chromosome 2, while the single QTL effect on average trichome number on chromosome 8 was resolved into two distinct loci (Fig. 4e, Table S2). Interestingly, we detected 13 of the 15 QTL that we previously detected for average petal number across all five populations, as QTL for petal number and/or trichome number using only two populations (Ox × Az RIL and Ox × Nz F2; Table S2). The increased power of multi-trait analysis and the increased accuracy of phenotyping by binning flowers rather than averaging across all flowers, resulted in the detection of significant effects on both petal number and trichome number at 11 out of the 19 QTL, lending further support to the notion that the two traits are under common genetic control. These results also showed that some QTL indeed acted in an age-dependent manner as the significance or strength of their allelic effects changed over consecutive bins (Tables S3, S4).

To accurately determine the age-dependent effects of the 19 detected QTL, we fit the final multi-trait QTL model of petal number and trichome number in binned flowers, to trait values averaged in a sliding window of three flowers to give an overlapping moving average (Fig. 5a, Tables S5, S6). This analysis revealed that the effect of different QTL could be either consistent, or increasing, or decreasing in strength and significance during ageing of the shoot (Fig. 5b–e). We found both correlated as well as anti-correlated QTL effects on petal number and trichome number but the direction of effects never changed significantly for any single QTL (Fig. 5). Those QTL that mostly affected either petal number or trichome number tended to have consistent effects during ageing of the shoot (P-T5, 6, 8, 9, 13, 15, 18). In other cases, where QTL had anti-correlated effects on average petal number and trichome number; this detailed analysis revealed correlated changes in the effects during specific periods of development. For example, at P-T11 the Ox allele increased trichome number for moving averages in flowers 1–17, after which it was no longer significant, while it was not significant for petal number until flower 11, after which it reduced petal number with increasing strength (Fig. 5c). The correlated change in the effect of the Ox allele leading to a reduction in both petal number and trichome number can be observed from approximately flower 10 onwards (Fig. 5c). In this light, P-T11 acts similarly to P-T1 although here, the Ox allele reduced both petal number and trichome number from flower 1 onwards, and later the magnitude of these effects reduced in concert (Fig. 5d). We found similar age-dependent effects for P-T2 yet with a negative correlation in the change of both traits (Fig. 5e). Here, the Ox allele reduced trichome number until flower 15 with no significant effect on petal number, and it reduced petal number between flowers 16 and 24 with no significant effect on trichome number (Fig. 5e). We also observed age-dependent effects that were more temporally restricted for trichome number (P-T10 and 12) or petal number (P-T3, 19). In fact, we found that P-T8 and 9 had effects on petal number in a very restricted window spanning four flowers (two adjacent moving averages) that were not detectable in bins of five flowers (Fig. 4e). Taken together, these results suggest that allelic variation in general age-dependent mechanisms, trait-specific integrators of ageing signals, as well as genes specific to petal or sepal trichome number underlie these QTL.

**Discussion**

Variable petal number is derived in *C. hirsuta* from a stable petal number of four that characterizes the Brassicaceae. We found that petal number varied from zero to four within five *C. hirsuta* mapping populations and that this variation was highly heritable. We identified at least 15 loci with effects on average petal number that diverged between the six *C. hirsuta* accessions analysed here. Understanding this genetic architecture can provide insight into the genetic basis and evolutionary forces responsible for petal number variation in *C. hirsuta*. A polygenic architecture of many small to moderate effect QTL, affecting the trait in both positive and negative directions, likely contributes to maintaining *C. hirsuta* petal number within its variable range below four. In contrast to this, traits governed by large effect mutations with
low pleiotropy can respond to directional selection more readily, and under this type of architecture, the average petal number could readily revert back to four petals in *C. hirsuta*. We analysed an EMS mutant in *C. hirsuta* that increased the average petal number by two petals over the wild-type Ox value, without apparent pleiotropic effects. This phenotype of the *fp2* mutant demonstrates that *C. hirsuta* petal development could, in principle, accommodate natural allelic effects of this magnitude that would increase the average petal number and reduce phenotypic variability without incurring obvious pleiotropic effects on

![Figure 5](image_url)

**Fig. 5** Age-dependent effects of petal and sepal trichome number quantitative trait loci (QTL) in *Cardamine hirsuta*. (a) Allelic effects of QTL detected in the Ox × Az recombinant inbred lines (RILs) (pink background) and the Ox × Nz F$_2$ (green background) for petal and sepal trichome number in consecutive bins of five flowers (Fig. 4) on moving averages of these traits. Effects are shown plotted to the right of the linkage groups of the integrated genetic map. The position of each QTL is indicated by a red dot and QTL effects are plotted in the adjacent bar graphs. Significant effects on petal number are shown in red and on sepal trichome number in blue. Nonsignificant effects are shown in grey. Axis labels and numbering are omitted but are identical to those in (b). Note that the bi-modal distribution of QTL effects on trichome number for P-T7, T4 and T6 probably reflects the lack of phenotypic variation in flowers with no sepal trichomes that are produced at the mid-point of this age progression. (b–e) Detailed view of the allelic effects of four QTL.
development. Therefore, our results suggest that species-specific developmental constraints do not prevent C. hirsuta from attaining a high average petal number, but rather that a polygenic architecture contributes to maintaining the variable petal number in C. hirsuta.

In Brassicaceae species with a stable petal number of four, such as A. thaliana, this phenotype is robust to natural genetic variation. In contrast to this, petal number varied from zero to four in C. hirsuta and we detected at least 15 QTL at which natural allelic variation affected petal number. Additionally, the average petal number varied between isogenic individuals of the Ox accession in response to environmental variation that was unintentionally introduced between different experiments. Thus, evolutionary change has produced a nonrobust phenotype in C. hirsuta where petal number is shifted outside of the buffered zone that produces four petals in other crucifers (Monniaux et al., 2015). Although petal number varied between zero and four in C. hirsuta flowers, it never increased beyond four. This indicates that phenotypic variation in C. hirsuta petal number is one-sided with respect to the buffered trait value of four petals that exists in most crucifers. Two-sided phenotypic variation is good evidence for a loss of developmental robustness as this can more readily produce a wider phenotypic distribution with a similar mean (Felix & Barkoulas, 2012). Therefore, the evolutionary transition from invariant to variable petal number that occurred in the lineage leading to C. hirsuta may not reflect a complete loss of robustness in this developmental patterning system, but rather a shift outside of its buffered zone.

Petal number variation is a character that defines C. hirsuta and manifests at different scales: from between genetically distinct accessions to between the different flowers produced on a single plant. By quantifying phenotypic variation at each of these scales we identified a significant contribution of plant ageing to petal number variation. Moreover, we found that petal number shared a common genetic basis with a temporally correlated trait: sepal trichome number, but not with lateral stamen number, even though each lateral stamen and its two adjacent petals are thought to share developmental commonalities such as pre-patterning though each lateral stamen and its two adjacent petals are thought to share developmental commonalities such as pre-patterning though each lateral stamen and its two adjacent petals are thought to share developmental commonalities such as pre-patterning.
flowers with petals (chasmogamous) and obligatory self-pollinating flowers with reduced or absent petals (cleistogamous) (Culley & Klooster, 2007). As a selfing species, C. hirsuta seems to efficiently self-pollinate under standard glasshouse conditions irrespective of petal number variation, but these conditions hardly reflect the real world. Therefore, it will be interesting to address whether variable petal number contributes to the life history strategy of C. hirsuta as an invasive, pioneer weed with a cosmopolitan distribution (Hay et al., 2014).

Acknowledgements

We thank M. Tsiantis, and members of his group (A. Tattersall, M. Cartolano, J. Lamb and S. Langer) for generating and making available the C. hirsuta RIL populations and providing sequence and molecular marker information. We thank M. Tsiantis for critical comments on the manuscript, M. Bonsall for helpful discussions and S. McKim for a scanning electron micrograph. This work was supported by Biotechnology and Biological Sciences Research Council grant BB/H10133X/1 to A.H. and A.H. was supported by the Max Planck Society W2 Fellowship and M.M. by a European Molecular Biology Organisation MM Programme and a Royal Society University Research Fellowship and M.M. by a European Molecular Biology Organisation Long Term Fellowship.

References


Van Ooijen JW 2006. Joinmap® 4, software for the calculation of genetic linkage maps in experimental populations. Wageningen, the Netherlands: Kyazma B.V.


Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 An integrated genetic map for the five experimental *C. hirsuta* populations analysed.

Fig. S2 Genome-wide QTL mapping scans for average petal number in five *C. hirsuta* populations.

Fig. S3 Genome-wide multi-trait QTL mapping scans for average petal number and average trichome number in two *C. hirsuta* populations.

Fig. S4 Genome-wide multi-trait QTL mapping scans for petal number and trichome number averaged in bins of five flowers in two *C. hirsuta* populations.

Table S1 Phenotypic variances explained for average petal number by the full QTL models in five mapping populations

Table S2 Positions of multi-trait QTL detected for petal number and sepal trichome number averaged in nonoverlapping bins of five flowers

Table S3 Effects of multi-trait QTL detected for petal number and sepal trichome number averaged in nonoverlapping bins of five flowers in the Ox × Az RIL population

Table S4 Effects of multi-trait QTL detected for petal number and sepal trichome number averaged in nonoverlapping bins of five flowers in the Ox × Nz F2 population

Table S5 Effects of multi-trait QTL detected for petal number and sepal trichome number averaged in nonoverlapping bins of five flowers in the Ox × Az RIL population on moving averages

Table S6 Effects of multi-trait QTL detected for petal number and sepal trichome number averaged in nonoverlapping bins of five flowers in the Ox × Nz F2 population on moving averages

Please note: Wiley Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.