QTL Underlying Self-Fertility in Tetraploid Alfalfa

Joseph G. Robins* and E. Charles Brummer

ABSTRACT
A potential strategy to decrease the levels of self-seed production during the seed increase stages of alfalfa (*Medicago sativa* L.) synthetic cultivar development is selection for decreased self-fertility. The underlying genetics of this trait have not been elucidated and, therefore, a study was designed to identify genetic determinants of alfalfa self-fertility. An F1 mapping population developed by crossing parents representing *Medicago sativa* subsp. *falcata* and *M. sativa* subsp. *sativa* was characterized for self-fertility in a greenhouse during the winter of 1999. Traits representing self-fertility were measured and then mapped to the resulting genetic maps of both parents. Heritability values for each trait were high, suggesting genetic factors are important for their expression. Quantitative trait loci for self-fertility traits were identified on linkage groups (LGs) 2 and 4 from the *falcata* parent and LGs 4 and 8 of the *sativa* parent.

Most alfalfa (*Medicago sativa* L.) cultivars currently in commercial production are synthetic populations formed by intercrossing selected parental plants and then random mating the resulting population for two to four generations to increase seed for sale. During this seed-increase process, various characteristics of the population can change as gene frequencies shift for various reasons. One particularly detrimental cause is self-pollination, which can lead to significant inbreeding depression which reduces vigor and, consequently, yield (Jones and Bingham, 1995). Kehr (1973) identified mean levels of selfed seed production to be ~50%, although in some instances the level approached 70%. Therefore, for commercial applications, keeping the level of self-fertilization as low as possible is desirable. Genotypes with low levels of self-fertility (Campbell and Bauchan, 1990) minimize self-fertilization during the seed-increase phase of synthetic cultivar development, and may also provide a method for developing hybrid alfalfa cultivars (Tysdal and Kiesselbach, 1944; Bauchan et al., 1990).

Self-incompatibility was elucidated by Mather (1943) and refers to mechanisms that prevent the successful fertilization between otherwise fertile genetically related gametes. Self-fertility refers...
to the ability of plants to produce viable seed when self-pollinated. Thus, self-incompatibility and low self-fertility are not the same thing. Although self-fertility encompasses self-incompatibility, they are typically not differentiated in alfalfa because the underlying mechanism of low self-seed set is not usually known or determined (Viands et al., 1988). However, the level of self-incompatibility helps determine the amount of self-fertility. Therefore, the characterization and mapping of the genetic determinants of self-fertility could also signal potential genomic regions involved in self-incompatibility. The genetics of the self-incompatibility system of alfalfa have not been identified (reviewed in Viands et al., 1988). Self-incompatibility levels vary according to genotype (Campbell and Bauchan, 1990), suggesting the existence of genetic variation for this trait. This system is purported to be gametophytic, although additional controls may include nonadditive gene action and cytoplasmic influences (reviewed in Campbell and Bauchan, 1990) and the effects of inbreeding depression, which can result in embryo or zygote failure.

By investigating the genetic control of selfed seed production, we hope to begin to understand and more effectively manipulate self-fertility. The identification of markers associated with self-fertility would allow the development of a marker-assisted selection (MAS) scheme to decrease self-fertility in breeding populations, limiting self-seed production during seed increase. To this end, we mapped quantitative trait loci (QTL) associated with alfalfa self-fertility in a tetraploid segregating alfalfa population by assessing the ability of plants to set self-seed. While not designed to definitively identify the genetic basis of self-incompatibility in alfalfa, this experiment provides a starting point for further investigation of self-incompatibility in this species.

**MATERIALS AND METHODS**

**Plant Material**

Two hundred genotypes from the WISFAL-6 (M. sativa subsp. falkata; Bingham, 1993) × ABI408 (M. sativa subsp. sativa; Forage Genetics Intl., Nampa, ID) cross were grown and clonally propagated in the Iowa State University greenhouses at Ames, IA. These genotypes are the same used in previous field and laboratory studies (Brummer et al., 2000; Robins et al., 2007a, 2007b, 2008).

**Experimental Design**

Clonal ramets of the 200 F1 genotypes and their parents were grown in a greenhouse during winter 1998–1999. A randomized complete block design consisting of three blocks was used with one clonal ramet of each genotype per block. Plants were grown for approximately 5 mo before beginning the experiment. Ambient light was supplemented with high-intensity greenhouse lights to obtain a 16-h daylength. The temperature was maintained at 25°C throughout the length of the experiment. Insects, predominantly flower thrips (Frankliniella occidentalis Pergande), were controlled by periodic applications of Avid (a.i. abamectin 17 g L−1 [Syngenta, Greensboro, NC]). All plants were self-pollinated by tripping fully opened florets with toothpicks. We tripped florets on each plant weekly for approximately 1 mo, with the objective to trip at least 100 florets on each plant during that time period. During the second month, seeds were allowed to ripen and were then harvested. Data collected from each plant included the total number of florets tripped, the number of seedpods produced, and the number of seeds produced. From these data, we derived three traits representing self-fertility (Sleper and Poehlman, 2006): (i) the number of seedpods produced per floret tripped (PF) = total seedpods produced/number of florets tripped; (ii) the number of seeds produced per floret tripped (SF) = total seeds produced/number of florets tripped; and (iii) the number of seeds produced per seedpod (SP) = total seeds produced/total seedpods produced.

**Statistical Analysis and QTL Mapping**

The MIXED procedure (Littell et al., 1996; SAS Institute, 2006) was used to calculate means for each of the measured traits and corresponding least significant differences among genotypes. Genotypes were analyzed as fixed effects. Broad-sense heritabilities, using a random model (Holland et al., 2003), and genotypic correlations (Holland, 2006) were calculated using the MIXED and IML procedures of SAS. Due to skewness toward lower values, all analyses were based on natural logarithm-transformed data. A small constant (C = 0.001) was added to values that were equal to 0 to allow logarithm transformation (Steel et al., 1997). Values used in the results and discussion were reverse-transformed for ease of interpretation. Unless otherwise stated, statistical significance was assessed at the 5% probability level.

Linkage mapping procedures are described in Robins et al. (2007b, 2008). As discussed in Robins et al. (2008), the map was re-created using the updated TetraploidMap software (Hackett et al., 2007). The effects of this remapping were discussed in the Robins et al. (2008) study. Briefly, the mapping order is changed somewhat when compared with the Robins et al. (2007b) study due to use of a different algorithm and the exclusion of markers segregating at a ratio higher than 5:1 (presence:absence). This update was justified because it allowed the use of the interval mapping portion of the program, which was unavailable for the Robins et al. (2007a, 2007b) studies when QTL identification was limited to single-marker analysis. The theory underlying interval mapping in an autotetraploid is complex and is thoroughly described elsewhere (Cao et al., 2005; Doerge and Craig, 2000; Hackett et al., 2001).

Restriction fragment length polymorphism and simple sequence repeat (SSR) marker alleles are identified by the marker name (see Robins et al. [2007b] for probe and primer sources) followed by a letter indicating the parental genome that contributed the allele (“a” from WISFAL-6, “b” from ABI408, or “c” from both parents) and a distinct number for each individual allele. For example, the SSR allele aw690847a2 is the second largest allele contributed by the WISFAL-6 parent and amplified by the aw690847 primer. The location of putative QTL was explored using the interval mapping procedure of TetraploidMap. Although many of the loci contained information for multiple alleles, many did not and behaved as dominant rather than codominant markers. Thus, epistatic interactions were not explored using the marker data. Putative
QTL locations were identified based on logarithm of the odds (LOD) values larger than the 5% (or 10%, as noted) threshold determined through 1000 permutations.

**RESULTS**

For all but 15 of the F1 genotypes, the mean number of florets tripped on each plant was >100. Only three genotypes had <50 florets tripped. For each of the three self-fertility traits, there were significant differences among genotypes and the F1 population exhibited a wide range of values including high and low transgressive segregants (Table 1). The mean values of individual F1 genotypes ranged from 0 to 1.0 PF, 0 to 1.4 SF, and 0.2 to 2.3 SP. Broad-sense heritabilities on a genotype-mean basis were at least 0.70 for each trait (Table 1).

No differences were present for any of the traits between the mean performance of the ABI408 (0.12 PF, 0.13 SF, and 1.1 SP) and the overall F1 population mean (0.12 PF, 0.13 SF, and 1.0 SP). However, WISFAL-6 (0.55 PF, 0.91 SF, and 1.6 SP) exhibited higher mean values than either ABI408 or the overall F1 population mean for PF and SF and a higher mean value than the F1 population mean for SP (Table 1). Genotypic correlations were 0.99 between PF and SF, 0.77 between PF and SP, and 0.86 between SF and SP.

Interval mapping identified the location of nine putative QTL on WISFAL-6 linkage groups (LGs) 2 and 4 and ABI408 LGs 4 and 8 (Table 2; Fig. 1). Quantitative trait loci for PF localized on WISFAL-6 LGs 2 and 4 and ABI408 LG 8. Quantitative trait loci for SF localized on WISFAL-6 LGs 2 and 4 and ABI408 LGs 4 and 8. Quantitative trait loci for SP localized on WISFAL-6 LGs 2 and 4. Each QTL was significant at the 5% threshold, with the exception of the SP QTL on WISFAL-6 LG 2 that was significant at the 10% threshold (Table 2). Typically, LOD peaks occurred for each of the three traits in the same general region of the LG (Fig. 1). Although, on WISFAL-6 LG 4, the peak associated with PF was 20 cM removed from the PF and SF peaks. The percent variation explained by individual QTL ranged from 9 to 24% and LOD values ranged from 3.3 to 6.1 (Table 2).

**DISCUSSION**

The wide range of values exhibited for PF, SF, and SP and the high broad-sense heritability estimates suggest high levels of genetic variation for each trait in this population. This result was consistent with previous estimates of genetic variation for important agronomic traits in this population (Robins et al., 2007a, 2007b, 2008). The higher self-seed set of the WISFAL-6 parent may indicate that its self-incompatibility system is less effective than that of the ABI408 parent, although the F1 population values were closer to the

### Table 1. Mean self-fertility values and broad-sense heritability estimates ($H^2$) in alfalfa for the parents (ABI408 and WISFAL-6) and F1 population measured following self-fertilization in a greenhouse at Ames, IA, during winter 1998–1999. Self-fertility traits include pods floret$^{-1}$ (PF), seeds floret$^{-1}$ (SF), and seeds pod$^{-1}$ (SP).

<table>
<thead>
<tr>
<th>Trait</th>
<th>PF</th>
<th>SF</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 population mean</td>
<td>0.1</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>WISFAL-6 mean</td>
<td>0.6</td>
<td>0.9</td>
<td>1.6</td>
</tr>
<tr>
<td>ABI408 mean</td>
<td>0.1</td>
<td>0.1</td>
<td>1.1</td>
</tr>
<tr>
<td>F1 population range</td>
<td>0–1.0</td>
<td>0–1.4</td>
<td>0.2–2.3</td>
</tr>
<tr>
<td>LSD$_{0.05}$</td>
<td>0.3</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>$H^2$ ± SE</td>
<td>0.77 ± 0.03</td>
<td>0.82 ± 0.03</td>
<td>0.70 ± 0.04</td>
</tr>
</tbody>
</table>

**Table 2. Characterization of quantitative trait loci (QTL) putatively associated with alfalfa self-fertility as identified by interval mapping, including the linkage group (LG) to which each QTL belongs, the position on the LG of the maximum logarithm of the odds (LOD) value (5% LOD threshold values as determined by 1000 permutations are in parentheses), the percentage of the phenotypic variation explained (exp.) by the QTL, and the phenotypic values of F1 genotypic classes carrying specific homologous chromosomes at the QTL location.**

<table>
<thead>
<tr>
<th>Linkage group</th>
<th>QTL position</th>
<th>LOD value</th>
<th>Variation exp.</th>
<th>F1 population mean</th>
<th>Q12†</th>
<th>Q13</th>
<th>Q14</th>
<th>Q23</th>
<th>Q24</th>
<th>Q34</th>
</tr>
</thead>
<tbody>
<tr>
<td>LG2-WISFAL-6</td>
<td>48</td>
<td>4.1 (4.0)</td>
<td>14</td>
<td>0.1</td>
<td>0.10</td>
<td>0.23</td>
<td>0.18</td>
<td>0.09</td>
<td>0.17</td>
<td>0.08</td>
</tr>
<tr>
<td>LG4-WISFAL-6</td>
<td>26</td>
<td>3.7 (3.0)</td>
<td>11</td>
<td>0.14</td>
<td>0.14</td>
<td>0.13</td>
<td>0.22</td>
<td>0.07</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>LG8-ABI408</td>
<td>52</td>
<td>3.8 (2.9)</td>
<td>10</td>
<td>0.11</td>
<td>0.10</td>
<td>0.09</td>
<td>0.18</td>
<td>0.06</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>LG2-WISFAL-6</td>
<td>48</td>
<td>4.3 (4.0)</td>
<td>16</td>
<td>0.1</td>
<td>0.10</td>
<td>0.29</td>
<td>0.19</td>
<td>0.09</td>
<td>0.21</td>
<td>0.09</td>
</tr>
<tr>
<td>LG4-WISFAL-6</td>
<td>26</td>
<td>4.0 (3.0)</td>
<td>12</td>
<td>0.19</td>
<td>0.15</td>
<td>0.14</td>
<td>0.26</td>
<td>0.07</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>LG4-ABI408</td>
<td>34</td>
<td>3.3 (2.8)</td>
<td>24</td>
<td>0.17</td>
<td>0.17</td>
<td>0.17</td>
<td>0.03</td>
<td>0.17</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>LG8-ABI408</td>
<td>52</td>
<td>3.7 (3.0)</td>
<td>9</td>
<td>0.14</td>
<td>0.12</td>
<td>0.09</td>
<td>0.20</td>
<td>0.06</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>LG2-WISFAL-6</td>
<td>66</td>
<td>3.8 (3.9)**</td>
<td>10</td>
<td>1.0</td>
<td>0.87</td>
<td>1.19</td>
<td>1.00</td>
<td>1.11</td>
<td>1.09</td>
<td>0.94</td>
</tr>
<tr>
<td>LG4-WISFAL-6</td>
<td>46</td>
<td>6.1 (3.5)</td>
<td>16</td>
<td>1.0</td>
<td>0.87</td>
<td>1.19</td>
<td>1.00</td>
<td>1.11</td>
<td>1.09</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**Significant at the 10% LOD cutoff level.**
†Values indicate the persistence percentages self-seed production (PF, SF, SP) of F1 genotypes that inherited the corresponding homologues of the respective LGs, i.e., Q12 indicates the mean persistence self-seed production values of genotypes inheriting homologues 1 and 2 of the corresponding LG.
Figure 1. Graphical representation of linkage groups (LG) associated with self-fertility quantitative trait loci (QTL) in alfalfa, specifically (A) WISFAL-6 LG 2 and (B) LG 4, and (C) ABI408 LG 4 and (D) LG 8 with corresponding diagram of logarithm of the odds (LOD) values representing potential QTL positions of the QTL underlying seedpods floret tripped^{-1} (PF), seeds floret tripped^{-1} (SF), and seedpods^{-1} (SP). Putative QTL are significant based on a minimum 5% LOD value determined through 1000 permutations (Table 2), with the exception of the SP QTL on WISFAL-6 LG 2 that is significant based on a 10% LOD value. Although QTL were not identified for PF or SP on ABI408 LG 4 or SP on ABI408 LG 8, LOD traces are included for these traits on the LGs to show the correspondence of LOD peaks with the other traits.
ABI408 parent than the WISFAL-6 parent. The reasons for this are not completely clear, but may be associated to the action (additive/dominant) of the underlying genetic determinants or the complexities of autotetraploid meiosis and inheritance, that is, the allele(s) from WISFAL-6 are present in only copy and inherited at a low rate in the population. The identification of transgressive segregants is also consistent with the previous studies of other traits in this population (Robins et al., 2007a, 2007b, 2008).

Previous studies identified a strong genetic component to self-fertility in alfalfa based on the results of selection (Busbice, 1968; Campbell and Bauchan, 1990) and on estimates of heritability (Campbell and He, 1997). Our study provides further evidence that manipulation of these traits through
selection would be successful. Additionally, based on the high genotypic correlation estimates between PF, SF, and SP, these traits likely share common genetic determinants.

Genomic regions containing QTL for at least one of the traits were identified on WISFAL–6 LGs 2 and 4 and ABI408 LGs 4 and 8. The PF and SP QTL on WISFAL–6 LGs 2 and 4 and ABI408 LG 8 appeared to colocalize, suggesting that a single gene may control both traits. On LG 2 the QTL for each trait all fell within the same LOD–1 confidence interval (Lander and Botstein, 1989), although the SP QTL on WISFAL–6 LG 4 did not appear to colocalize the PF and SF QTL. Thus, while there was an indication of common genetic determinants for each trait based on the genotypic correlations and colocalization of QTL, there was not sufficient evidence to clearly determine control by the same gene(s). Distinct genomic regions controlling the traits were identified in each parent, except for the SF QTL on WISFAL–6 LG 4, but given that these QTL collectively only explained a portion of the genetic variation and that our genetic maps on each homologous chromosome are not completely saturated, our results do not conclusively demonstrate that different loci are important in each parent.

Interval mapping enabled us to identify the effect of individual homologous chromosomes on the trait values (Table 2). In general, a single homolog did not have a large effect by itself, but rather specific combinations of homologs were necessary to produce a given phenotype. For example, SF decreased when homolog 3 of ABI408 LG 4 was inherited with either homolog 2 or 4; homolog 3 plus homolog 1 did not show that depression (Table 2). The complicated nature of these results is at least partially due to the complexities of mapping in autotetraploid plants, and they indicate the difficulty in applying mapping results to a MAS program.

In each of the previous mapping studies using this population (Robins et al., 2007a, 2007b, 2008) phenotypic associations were identified on these four linkage groups. However, with the exception of persistence QTL on ABI408 LG 2 (Robins et al., 2008), other regions of the alfalfa genome were considered more important. In particular, LG 7 was identified as an important area of the genome in each of the previous studies, and in other Medicago genomic studies (Julier et al., 2007). There was no indication of LG 7 being instrumental in promoting self-fertility in this study. Previous molecular marker studies of self-seed set in alfalfa focused on the effect of genetic diversity on the selection of clones for self-incompatibility (Campbell 2000). This is the first study attempting to localize the genetic determinants of self-seed set in the alfalfa genome by characterizing self-fertility. Self-fertility suggests the absence of a self-incompatibility locus, and the lack of self-seed suggests that a self-incompatibility locus is present. Likely mechanisms underlying the identified QTL for self-fertility include self-incompatibility and/or ovule abortion (reviewed in Viands et al., 1988). Plants with high levels of self-fertility exhibit lower self-incompatibility and less postfertilization failure than do plants with low self-fertility. Because of the likely involvement of self-incompatibility locus (or loci), we have identified several candidate intervals for controlling self-incompatibility.

There are several limitations that preclude the results of this study from being applied to an applied alfalfa breeding for decreased self-fertility. The inclusion of only one greenhouse season is a limitation of the study. Environmental effects, including temperature, humidity, pollinator activity, etc., play an important role in determining self-fertility levels (Kehr, 1973; Viands et al., 1988; Campbell and Bauchan, 1990). More definitive QTL identification will require multiple-environment evaluation. Further, these results are only reflective of this biparental cross (M. sativa subsp. falcata × M. sativa subsp. sativa). More detailed evaluation across broader genetic backgrounds, including use of population-based strategies (Skøt et al., 2005), are necessary for more definitive QTL identification. Finally, identified QTL intervals are quite large in some cases. Improved map resolution, with higher density, and improved allele dosage identification would result in more precise results. Despite these limitations, this study provided important findings on potential genomic loci controlling alfalfa self-fertility and a basis for further investigation of the underlying genetics of self-fertility and self-incompatibility.

Acknowledgments

This research was supported by USDA-NRI (97-35300-4573), USDA-IAFAS (00-52100-9611), and Hatch Regional Research Project NE-1010 (all to E.C.B.) and an Iowa State University Plant Sciences Institute Fellowship (to J.G.R.).

References


