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TISSUE CULTURE AND QUANTIFICATION OF INDIVIDUAL-LEVEL RESISTANCE TO ANTHER-SMUT DISEASE IN *SILENE VULGARIS*

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Resistance to systemic diseases is often difficult to measure quantitatively because individuals can be scored only as either diseased or healthy. A technique is described for cloning individuals of *Silene vulgaris* plants using the tissue culture of seedling meristems. Inoculation of such clonally replicated genotypes within two families shows that segregation of resistance to anther-smut disease caused by the systemic plant pathogen *Microbotryum violaceum* is highly bimodal. This suggests that resistance to this disease in *S. vulgaris* may have a relatively simple genetic basis.

Keywords: callus, flowering time, gynodioecy, male sterility.

Introduction

Anther-smut disease, caused by the fungal pathogen *Microbotryum violaceum*, has become a widely used model for studying disease in natural populations (Alexander et al. 1996; Antonovics et al. 2002; Hood et al. 2003). *Microbotryum violaceum* is a species complex that infects many members of the Caryophyllaceae (Thrall et al. 1993; LeGac et al. 2007). The pathogen is systemic and sterilizing, and the pollen in diseased plants is replaced by spore-filled anthers. Insect pollinators serve as vectors of the disease (Antonovics and Alexander 1992).

Substantial genetic variation in disease resistance occurs in the natural populations of several hosts. For example, Alexander (1989) used vegetative cloning of adult *Silene latifolia* plants to show large differences in resistance among genotypes from one population, and field experiments using progeny from crosses among these genotypes confirmed that resistance was highly heritable (Alexander et al. 1993). Antonovics et al. (2002) showed differences in disease resistance among families of *Silene vulgaris* sampled as seeds by a common female parent in a natural population where the disease had recently undergone a host shift from *S. latifolia* (Hood et al. 2003). However, although it has been established that ecological, morphological, and physiological factors contribute to variation in field resistance (Biere and Antonovics 1996; Giles et al. 2006), neither the resistance pathway nor the genes responsible have been identified.

Cloning of individuals of *S. vulgaris* using vegetative rosette cuttings is slow, requiring several rounds of cloning to obtain a sufficient numbers (M. Olson, personal communication), and therefore previous studies on this species have used family-level measures of resistance (Antonovics et al. 2002). To obtain quantitative measures of resistance at the individual level, we undertook this study with the goal of cloning individuals of *S. vulgaris* by callus tissue culture. We derived protocols from those used for cloning horticultural members of the same family, especially carnations (*Dianthus caryophyllus*) (Watad et al. 1996; Thakur et al. 2002). We then applied this protocol to individuals from two full-sib host families derived from crosses between parents that differed in their resistance. We asked whether the variation among individuals was continuous or there were discrete patterns of segregation within each family with regard to levels of resistance to anther-smut disease.

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Material and Methods

Study Organisms

The host plant *Silene vulgaris* (bladder campion), of the carnation, or pink, family (Caryophyllaceae), is a short-lived perennial that grows from a taproot. It is native to Europe and was introduced into the eastern United States, where it has well-established populations in fields and roadsides. *Silene vulgaris* plants produce flowers that are either hermaphroditic or male sterile (Taylor et al. 2001). In Europe, high-elevation populations of *S. vulgaris* (above ca. 2000 m) are a known host for two divergent lineages of *Microbotryum violaceum* (Le Gac et al. 2007). However, in North America, the only documented occurrence of anther-smut disease on *S. vulgaris* is a recent host shift of the fungus from *Silene latifolia* to *S. vulgaris* in a mown pasture in Broadway, Rockingham County, Virginia (Antonovics et al. 2002; Hood et al. 2003). *Silene vulgaris* used in this study was derived from plants growing at this North American site.

The experimental plants came from two full-sib families (identified as families 3 and 9) differing in their resistance to *Microbotryum violaceum*. These full-sib families were obtained as follows, as part of another study. First, seeds were collected at the field site by common female parent (i.e., half-sib families) and tested for their family-level resistance by seedling inoculation using standard methods described by Antonovics et al. (2002). Inoculum came from diseased

S. vulgaris plants collected at the same field site, where the pathogen is derived from a lineage of the fungus endemic to the original host, *S. latifolia* (Hood et al. 2003). Full-sib family 3 was generated by a cross between two adults derived from two relatively resistant field-collected families. Full-sib family 9 was generated by a cross between two adults derived from two relatively susceptible field-collected families.

Tissue Culture

Seed germination. To obtain meristems for tissue culture, seeds were surface sterilized using a bleach, alcohol, and surfactant solution (Hood and Antonovics 2000). Seeds were germinated in 15×150-mm petri dishes, on one-tenth strength Murashige and Skoog Basal Salt Mixture with sucrose and agar (MS; product no. M9274, Sigma-Aldrich; Murashige and Skoog 1962) supplemented to 1% agar, and incubated at 25°C. After 9 d, once the seedlings reached the cotyledon stage, 15 seedlings from each of the two families were randomly selected for cloning.

Shoot induction. The apical meristem was either left whole or cut into two or three pieces (explants) with a sterile surgical blade. Explants were cultured on the shoot induction medium (Watad et al. 1996), consisting of 42.4 g MS, 1 mg thidiazuron (TDZ), and 1 mg 1-naphthaleneacetic acid (NAA) per liter, pH = 5.8. The explants all formed callus, and after 3–4 wk, multiple shoots appeared. The explants were then divided into one to eight subclones with at least one shoot per subclone and transferred to new shoot induction medium. After 11–12 wk, numerous shoots were produced per subclone (see "Results"). Genotypes with 35 or more total shoots were further subdivided into callus tissues with at least one shoot each and were used for root induction.

Root induction. The rooting medium used was from Thakur et al. (2002) and consisted of 42.4 g MS, 0.5 mg NAA, and 2 mg kinetin per liter, pH = 5.8. The kinetin was added after autoclaving. After 6 wk on rooting medium, the cultures produced roots and were transplanted into a soil mix (13.2 L topsoil, 6.6 L peat, 11 L turkey grit gravel, 19.8 L perlite, 26.4 L pine and bark nuggets, 319 g dolomitic lime, 142 g hydrated lime, and 191 g super phosphate) in 3.81-cm Conetainers (Stuewe and Sons, Corvallis, OR). At transplanting, the roots of the plants were dusted with the rooting hormone Rootone (GardenTech, Lexington, KY). Plants were kept on a mist bench for ca. 1 wk before they were placed in the greenhouse.

Unsuccessful protocols. Cotyledons (cut into squares with the abaxial surface on the medium) and hypocotyl segments (cut transversely) did not form callus in the shoot induction medium. The shoot induction medium of Kantia and Kathari (2002), consisting of MS, 3 mg/L 6-benzylaminopurine (BAP), and 1 mg/L NAA, produced fewer shoots than when TDZ was used (see "Shoot induction" above). Apical meristem explants transferred to MS with 0.5 mg/L NAA and 2 mg/L kinetin produced few calli. When BAP was used as a replacement for kinetin, no roots were formed.

Inoculation

After 12 wk growth in the greenhouse (March 2005), plants were inoculated with a mixture of A1 and A2 haploid mating types of *M. violaceum* derived from one meiotic tetrad of the pathogen from the same field site as the host parents. Inoculum concentration was 650 each of A1 and A2 haploid cells per microliter of water, and inoculum was applied by misting the shoots until runoff. Because we wanted to test for family differences and maximize the numbers of plants inoculated within each family, all plants within a family were inoculated, rather than some also being left as "controls." No vertical transmission via seeds has been observed in this disease system. Immediately after inoculation, the plants were placed inside clear plastic bags to maintain a high humidity and to promote infection. The plants were left in the lab at room temperature for 7 d, after which the bags were removed and plants were transferred to the greenhouse. As the plants flowered, the date of flowering was noted, and they were scored for disease (the presence of *M. violaceum* spores in the anthers of the flowers) once or twice a month for a period of 5 mo (until the end of July 2005). Another dose of inoculum was applied in August 2005 using teliospores collected from flowers of plants from the same experiment that had become diseased as a result of the inoculation. Plants were inoculated by watering the aboveground plant tissues with a concentrated spore suspension until runoff. The concentration was not measured precisely, but the solution of spores had a light purple tinge. Data were collected at 3-mo intervals until May 2006. Plants were cut back after each data collection to stimulate production of additional branches, which were scored for presence of disease. The sex of the flowers (male sterile or hermaphrodite) was noted in September 2005, January 2006, and May 2006.

Results

Tissue culture. All explants from the 30 seedlings produced callus tissue, and all but three regenerated shoots (table 1). In general, where two explants were generated by splitting the meristem, only one of the explants produced shoots (five out of seven instances); in one case, both explants produced shoots, and in another case, neither produced shoots. There was no difference between the two families in shoot production (and correspondingly in the number of callus subcultures), except that two genotypes of family 9 failed to produce many shoots and did not survive subculturing. The number of shoots produced per callus by the two families was also nearly identical (table 1). All cultures produced roots successfully. The roots appeared to grow out of the calluses, but dissection of the calluses and microscopic examination showed that the roots were coming directly from the shoots. There was no difference in transplant success between the families.

Disease resistance. The percentage of cloned individuals within each genotype that became diseased was either very low (0%–5%) or intermediate (ca. 40%–60%) (table 2). Family 3, derived from resistant parents, had six of eight genotypes that were highly resistant, while family 9, derived from susceptible parents, had two of six individuals highly resistant. The difference between families 3 and 9 was not significant (Fisher's exact test, $P < 0.28$).

Flowering time and male sterility. Most clones within genotypes could be scored as either hermaphrodite or male sterile, although there was some variation in expression of the

Table 1

Outcome of Tissue Culture of Genotypes of *Silene vulgaris* Sampled from Two Families

Family and genotype	Explants	Explants producing shoots	Subclones per explant	Shoots per subclone	Transplant success	Number of shoots per transplant
9.1	2	1	5	14.0	.83	3.14
9.2	1	1	3	12.3	.94	3.70
9.3	1	1	4	13.3	.97	3.18
9.4	1	1	4	21.3	.83	3.69
9.5	1	1	1	8.0		
9.6	1	1	4	21.3	.80	3.71
9.7	1	1	1	0		
9.8	1	0				
9.9	1	1	2	0		
9.10	1	0				
9.11	1	1	2	4.0		
9.12	1	1	5	15.0	.91	3.72
9.13	1	1	2	12.5		
9.14	2	1	1	20.0		
9.15	2	0				
Family 9 average			2.833	11.798	.881	3.522
3.1	1	1	1	15.0		
3.2	1	1	3	12.7	.83	3.72
3.3	1	1	1	5.0		
3.4	1	1	8	11.6	.91	3.88
3.5	1	1	5	8.6	.86	2.83
3.6	1	1	3	20.0	.91	3.56
3.7	1	1	3	4.0		
3.8	1	1	2	4.0		
3.9	1	1	8	10.6	.97	3.41
3.10	1	1	1	3.0		
3.11	1	1	2	17.5	.86	3.73
3.12	2	1	1	6.0		
3.13	2	1	2	22.5	.89	3.00
3.14	2	1	1	20.0		
3.15	2	2	3	16.5	.88	2.87
Family 3 average			2.933	11.801	.889	3.376

Note. All explants from the meristems produced callus tissue. Blank cells indicate that subcloning was not possible because no shoots were produced or that transplanting was not carried out because of the small number of total shoots.

trait (table 2). When plants had both types of flowers, usually some branches were hermaphroditic and others were male sterile. There was no correlation among genotypes between male sterility and resistance (Fisher's exact test, $P = 0.99$). The two families differed significantly in time to first flowering (ANOVA: $F = 25.8$, df = 1, 12, $P = 0.003$), with family 3 flowering 25.1 d earlier than family 9 (table 2). Within family 9, genotypes differed significantly in flowering time (analysis on first flowering by 28, 63, and 103 d: $\chi^2 = 30.9$, df = 10, $P = 0.006$), while within family 3, the difference approached significance ($\chi^2 = 22.3$, df = 14, $P = 0.073$).

Discussion

This study shows that it is possible to use tissue culture to clone *Silene vulgaris* and that clonal progeny produced in this way provide a useful means of quantifying disease resistance at the level of the individual genotype, as well as other traits such as flowering time and male sterility. Our results also show that there are large and bimodal differences in the resistance among individuals within two full-sib families, suggesting relatively simple genetic control of this important disease-related trait in *S. vulgaris*. Moreover, although the relationship was not significant, genotypes from parents that were from resistant families tended to produce more-resistant genotypes. The number of genotypes investigated per family was too small to provide power for testing alternative Mendelian hypotheses of inheritance, but our results are consistent with resistance being determined by one or a few major genes. The initial inoculum consisted of just one genotype of *Microbotryum violaceum*, and therefore it was not possible to assess whether the resistance reflected a gene-for-gene system (Keen 1990; Meyer et al. 2001). Single-locus control of resistance is well established in many host-pathogen systems, including resistance of cereal crops to smut fungi in the Ustilaginales (Holton et al. 1968). However, there have been very few studies on the genetic basis of resistance by dicotyledonous hosts to smut fungi in the Microbotryales. Evidence of single-locus control of resistance to *Microbotryum* was claimed by Goldschmidt (1928), but the data he presented do not clearly support this conclusion. Nevertheless, the demonstration of genetic variation in resistance in *S. vulgaris* is consistent

Table 2

Disease Susceptibility, Flowering Date, and Male Sterility of Genotypes of *Silene vulgaris* Cloned by Tissue Culture

Family and genotype	% disease	Flowering date	Number scored[a]	% male sterile	Number scored[a]
9.1	4.5	71.7	22	5.0	20
9.2	46.9	61.0	32	2.6	19
9.3	0	48.1	28	8.9	28
9.4	60.7	50.4	28	9.5	21
9.6	40.0	58.1	25	4.3	23
9.12	56.7	77.4	30	86.4	22
Family 9 average	34.80	61.12		19.45	
3.2	0	47.8	24	2.1	24
3.4	0	44.1	28	96.4	28
3.5	0	29.1	26	22.0	25
3.6	0	36.3	26	1.9	26
3.9	3.0	35.3	33	92.4	33
3.11	0	36.5	30	98.3	30
3.13	50.0	30.4	28	97.9	24
3.15	38.5	28.7	26	96.0	25
Family 3 average	11.48	36.02		63.38	

[a] Number of clonal individuals scored for each character.

with prior studies carried out at the family level using genotypes from the same site (Antonovics et al. 2002).

The results from *S. vulgaris* are in contrast to those from *Silene latifolia*, where variation in resistance appears to be more continuous. Alexander (1989) and Alexander et al. (1993) propagated genotypes from one population by rosette division and found that although there were highly susceptible and highly resistant genotypes present, the variation was continuous between these extremes. Subsequent studies using experimental crosses and testing the progeny at a family level showed that the resistance was highly heritable (Alexander and Antonovics 1995). In this study, despite two successive inoculations, the maximum infection rate of susceptible genotypes was only 60%, indicating the additional presence of substantial "background" resistance. The 60% maximum infection rate is comparable to the rate found in a prior study using inoculation of seedlings of *S. vulgaris* (Antonovics et al. 2002) and suggests that genetic analysis using progeny testing alone would be very difficult. The methods outlined here therefore provide a useful avenue to investigate inheritance patterns of resistance to *M. violaceum*.

The tissue culture and inoculation protocols described here are likely to be applicable to many other hosts in the Caryophyllaceae because the propagation methods were derived from work on carnations, *Dianthus caryophyllus*, and were adapted easily to *S. vulgaris*. However, our success at cloning plants was limited to the use of callus derived from meristematic tissue. Thus, we failed to obtain shoot regeneration from cotyledon and hypocotyl tissues, and dissection of the seedling apex almost always produced only one explant with shoots; whether the technique is successful for meristems other than those from seedlings remains to be determined. Although we did not investigate this explicitly, we think that the duration of shoot production could be reduced substantially from the 11–12 wk used in this study and that subcloning could be carried out more frequently to produce larger numbers of clonal progeny.

The cloned genotypes differed not only in their disease resistance but also in flowering time and in another binary trait, namely, male sterility. However, there were no correlations between these traits and resistance, which is not surprising, given the small sample size and low power to detect such correlations. Male sterility in *S. vulgaris* has been shown to be determined by the interaction of nuclear and cytoplasmic factors (Taylor et al. 2001). Our results show that although there is some variation in sex expression within cloned individuals, in general, cloned genotypes could be scored as either male sterile or hermaphrodite.

This study has confirmed that it is relatively easy to develop methods for cloning genotypes in the Caryophyllaceae using quite general tissue culture techniques. This has the potential to open up important avenues to examine the inheritance of binary traits, such as the genetics of disease resistance and male sterility, that up to now have proved difficult to study using family-based samples and progeny testing.

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