Gene, phenotype and function: **GLABROUS1** and resistance to herbivory in natural populations of *Arabidopsis lyrata*

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Abstract

The molecular genetic basis of adaptive variation is of fundamental importance for evolutionary dynamics, but is still poorly known. Only in very few cases has the relationship between genetic variation at the molecular level, phenotype and function been established in natural populations. We examined the functional significance and genetic basis of a polymorphism in production of leaf hairs, trichomes, in the perennial herb *Arabidopsis lyrata*. Earlier studies suggested that trichome production is subject to divergent selection. Here we show that the production of trichomes is correlated with reduced damage from insect herbivores in natural populations, and using statistical methods developed for medical genetics we document an association between loss of trichome production and mutations in the regulatory gene **GLABROUS1**. Sequence data suggest that independent mutations in this regulatory gene have provided the basis for parallel evolution of reduced resistance to insect herbivores in different populations of *A. lyrata* and in the closely related *Arabidopsis thaliana*. The results show that candidate genes identified in model organisms provide a valuable starting point for analysis of the genetic basis of phenotypic variation in natural populations.

Keywords: adaptation, *Arabidopsis lyrata*, association analysis, candidate gene, **GLABROUS1**, herbivory

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Introduction

To identify the genetic basis of adaptation represents a major challenge in evolutionary biology, and is of considerable applied interest in breeding and management of genetic resources (Orr & Coyne 1992; Feder & Mitchell-Olds 2003; Wright & Gaut 2005). The current view on the molecular basis of adaptation is based heavily on studies of a few traits in model species (Glazier et al. 2002), and the relationship between gene, phenotype and function has been established in natural populations only in few cases (Bradshaw & Schemske 2003; Clegg & Durbin 2003; Nachman et al. 2003; Colosimo et al. 2005). Thus, there is clearly a need for additional genetic studies of functionally important traits in natural populations.

The link between genotype and phenotype in natural populations can be explored by examining the association between phenotypic variation and molecular variation in candidate genes that have been identified in model organisms. The selection of candidate genes can be based on previous information either on the function of the genes (functional candidates identified in mutational studies), or the expression pattern of the genes (expression candidates identified in expressed sequence tag (EST) libraries and micro-array studies). Genetic association analyses have been used extensively in human epidemiological studies (Cordell & Clayton 2005), but in only a limited number of studies of plant and animal populations (Neale & Savolainen 2004; Gupta et al. 2005).

We combined ecological and molecular genetic approaches to examine the functional significance and genetic basis of a polymorphism in leaf trichome production in the outcrossing perennial herb *Arabidopsis lyrata*. Trichomes are uni- or multicellular hairs that develop on the leaves, sepals, and stems of plants, and may serve...
several functions including protection against herbivores, drought, and UV light radiation (Southwood 1986; Skaltsa et al. 1994; Espigares & Peco 1995). Trichome production may also be costly (Ågren & Schemske 1993; Mauricio 1998), which could explain the considerable variation in trichome density observed among populations of many plant species (Kärkkäinen et al. 2004). In *A. thaliana*, the initiation, number, spacing and shape of leaf trichomes are regulated by many genes (Larkin et al. 1996; Schellmann & Hülskamp 2005), and trichome formation has been used as a genetic and molecular model for studying developmental and cellular mechanisms in plants (Marks 1997; Hülskamp & Schnittger 1998).

Like several other members of the Brassicaceae, *A. lyrata* is polymorphic for trichome production and occurs in a glabrous and a trichome-producing form (Fig. 1). The trichomes of *A. lyrata* are unicellular and nonglandular. Controlled crosses indicate that the polymorphism has a simple Mendelian inheritance with the glabrous allele being recessive to the allele coding for trichome production (Kärkkäinen & Ågren 2002). An earlier study of variation in the frequency of glabrous plants and at isozyme loci in natural populations of *A. lyrata* indicated that differentiation at the locus causing glabrousness was higher than that at neutral marker loci, which is consistent with the hypothesis that trichome production is subject to divergent selection (Kärkkäinen et al. 2004). To determine the functional significance and genetic basis of trichome production in *A. lyrata*, we combined field surveys of natural populations with molecular genetic analyses. We show that (i) glabrous plants are more damaged by insect herbivores than trichome-producing individuals; (ii) the glabrous phenotype is associated with molecular variation in a gene homologous to the regulatory gene *GLABROUS1*; and (iii) sequence variation suggest that independent mutations in *GLABROUS1* have provided the basis for parallel evolution of reduced resistance to insect herbivores in different populations of *A. lyrata*, and in the closely related *Arabidopsis thaliana*.

**Materials and methods**

**Study species**

The self-incompatible perennial herb *Arabidopsis lyrata* ssp. petraea (Brassicaceae; syn. *Arabis petraea* L., syn. *Cardaminopsis petraea* [L.] Hiit.) is a close relative of *Arabidopsis thaliana* (Price et al. 1994; Koch et al. 1999). It has a disjunct distribution in Europe (Jalas & Suominen 1994). In Sweden, it occurs in an area along the coast of the Gulf of Bothnia (Hultén 1971). The 5–20 cm long inflorescence is produced from a leaf rosette. The rosette leaves are typically 1–5 cm long and about 0.5 cm wide. Trichome-producing plants may form trichomes on leaves and stems. In this study, we adhere to a very strict definition of glabrousness: only plants without a single trichome were classified as glabrous.

**Field study**

To examine the functional significance of trichome production, we quantified leaf damage caused by insect herbivores in five *A. lyrata* populations located on the east coast of Sweden (for the exact location, see Kärkkäinen et al. 2004). Four study-populations were polymorphic for trichome production and one was monomorphic (glabrous). The polymorphic populations included Stubbsand [P2 in Kärkkäinen et al. 2004; number of established plants in the population, N = 800, frequency of glabrous plants, Fr(gl) = 0.375], Storstensudden [P6; N = 1 400, Fr(gl) = 0.63], Skommarskaten [P4; N = 1 200, Fr(gl) = 0.54], and Svartlandsudden [P7; N = 1 500, Fr(gl) = 0.35]. The Storsanden population was monomorphic for the glabrous morph [P9; N = 30 000, Fr(gl) = 0.99; Kärkkäinen et al. 2004].

At the time of fruit maturation, we recorded the size and magnitude of leaf damage of between 69 and 104...
flower-producing plants in each population. The rosette diameter was measured to the nearest 0.5 cm, and the proportion of leaf area removed by insect herbivores was estimated by eye. Plants were sampled in transects laid across the populations; in polymorphic populations, a roughly equal number of glabrous and trichome-producing plants were scored. Data from the polymorphic populations were used to examine the effects of population and trichome production (glabrous vs. trichome-producing) on rosette diameter and on proportion leaf area removed with two-way analysis of variance (ANOVA) using the software JMP 5.0.1. Proportion leaf area removed was arcsine square-root transformed prior to analysis. Rosette diameter varied among polymorphic populations (two-way ANOVA, $F_{3,275} = 7.1$, $P = 0.0001$), but did not differ between glabrous and trichome-producing plants ($F_{1,275} = 2.1$, $P = 0.15$; no statistically significant population $\times$ trichome-production interaction, $P = 0.22$). Any effect of trichome production on damage was thus not likely to be confounded by differences in rosette size.

Genotypic assays

In the genetic analysis, we included three populations: the polymorphic Storstensudden ($N = 51$ plants) and Stubbsand ($N = 55$) populations, and the monomorphic glabrous Storsanden ($N = 33$) population. Storstensudden plants were grown in the greenhouse under standard conditions, whereas leaf material from the other two populations was collected in the field. For each sampled individual, we recorded whether it had produced any trichomes, and counted the number of glabrous and trichome-producing plants from each population; in polymorphic populations, a roughly equal sequence variation in the coding region of GL1.

To isolate the GL1 coding region from A. lyrata, we used A. thaliana GenBank sequences to design the primers (GenBank Accession nos M79448 and AB006078). The primers (5'-GAATGAGAAATGGAAGAGAGGAAA-3' and 5'-CTAGAGCGACTTATCTCACC-3') were used to PCR-amplify the exons 1–3 from GL1. The PCRs were carried out in a reaction volume of 25 µl by using a PCR-optimization kit (FailSafe PCR System, Epicentre) and Premix E2X as the buffer. The buffer includes MgCl$_2$ and an appropriate dNTP-mix. To the buffer, we added 10 pmol of each primer, 1–10 ng genomic DNA and 2 U of polymerase mix provided by the kit. The reactions were performed with the following cycling conditions: 5 min denaturation at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 50–52 °C depending on the quality of the template and 2 min at 72 °C. To detect sequence polymorphisms in GL1, we cloned the gene fragment into pGEM-T easy vector (Promega) for five glabrous and five trichome-producing plants from each polymorphic population. The exon areas were sequenced (LI-COR 4200 DNA analyser) from three to five clones per individual to detect both alleles and eliminate PCR errors. No variation was found in the first or second exon but several polymorphic sites were detected in the third exon of GL1, and thus this region was screened from the remaining samples by direct sequencing of the purified PCR product (Montage PCR Filter Unit, Millipore; sequencing primer 5'-GAATCATAAACATCTACTTATAGG-3') and run on Beckmann Coulter capillary electrophoresis. The cef8000 software was used to detect heterozygotes in the samples (SNP variation). The occurrence of indels in the third exon of GL1 was confirmed by fragment analysis, done by nested PCR for the diluted coding region amplification (with one fluorescently labelled primer 5'- CGCTACATTGTCATGCAGCGC-3' and an unlabelled primer 5'-GCAAAATTCTACCATCGAGTGA-3') and run on LiCor 4200 DNA analyser to detect fragment size variation. PCR conditions were as described for the genomic PCR with FailSafe PCR kit with annealing temperature of 54 °C and 25 cycles of amplification. BIOEDIT sequence alignment editor program (Hall 1999) and cef8000 software were used to align the DNA sequences.

Association of genotypes and phenotypes

We used the software FSTAT (Goudet 1995; http://www2.unil.ch/izea/softwares/fstat.html) to estimate linkage disequilibrium between all pairs of polymorphic loci in the GL1 gene and microsatellite markers in the three
study populations, and to estimate population differentiation. To study associations between DNA polymorphisms in the GL1 gene and trichome production, we followed methods proposed by Pritchard et al. (2000a, b). To avoid spurious associations caused by population substructuring, we first analysed variation at microsatellite loci and assessed the number of subpopulations in the sample. The Pritchard et al. (2000a) method uses genotypic correlations among unlinked markers to reveal the structure of populations from which samples have been taken and the ancestry of the sampled individuals. Individuals are assigned to subpopulations based on associations among unlinked markers and a Markov chain Monte Carlo method is used to estimate the number of subpopulations. Based on variation at 12 microsatellite loci, the Pritchard et al. (2000a) method identified three clusters corresponding to the three populations sampled ($F_{ST} = 0.176$; 95% CI 0.077–0.296), but no differentiation was detected at microsatellite loci between glabrous and trichome-producing plants within populations. The inferred population structure was taken into account when estimating associations between glabrousness and individual mutations in GL1 using the case-control approach of Pritchard et al. (2000b). Associations between phenotype (glabrous vs. trichome-producing) and variation at each locus (microsatellite loci, three variable sites detected in GL1) were tested within the identified subpopulations, which should minimize the risk of spurious associations due to hidden population structure.

Furthermore, to assess the relative importance of different mutations for initiation of trichome production, we used the program POLYPhen (Ramensky et al. 2002), which estimates the possible effects of mutations based on conservation of similar sequences found in GenBank and on the nature of amino acid change.

Different variable sites in GL1 can explain the appearance of glabrous plants in other A. lyrata populations and in closely related species. To assess whether the glabrous phenotype has a common, monophyletic origin, we sequenced the third exon of GL1 in five glabrous plants from a Norwegian A. lyrata population at Spiterstulen (61°41′N, 8°25′E, 1100 m a.s.l.). We combined these data with data from the three Swedish populations and previously published sequence information from a Russian and a German population (Hauser et al. 2001) to produce a minimum evolution tree with the software MEGA (http://evolgen.biol.metro-u.ac.jp/MEGA/default.html). This analysis included 13 sequences from six A. lyrata populations, three in Sweden (Storsanden (P9) GenBank Accession numbers DQ336628–DQ336629, Storstensudden (P6) DQ336619–DQ336622, and Stubban (P2) DQ336623–DQ336626), one in Norway (NO, DQ336627), one in Russia (Hauser et al. 2001) (RU; AF263721), and one in Germany (Hauser et al. 2001) (GE; AF263720), and one sequence from A. thaliana (A. thal.; AB006078).

**Results**

**Trichome production and herbivory**

Trichome production was associated with reduced leaf damage caused by insect herbivores (Fig. 2a). Glabrous plants were consistently more damaged by insect herbivores than trichome-producing plants in polymorphic populations (two-way ANOVA, $F_{1,275} = 13.6, P = 0.0003$; no statistically significant population $×$ trichome–production interaction, $P = 0.37$), and the level of leaf damage varied among populations ($F_{3,275} = 6.9, P = 0.0002$; Fig. 2a).

**Genetic basis of glabrousness**

We detected five variable sites in the third exon of GL1: two synonymous substitutions, one nonsynonymous substitution causing the amino acid alanine to position 95 to change into aspartate (hereafter called A95D), a 3-bp deletion causing a serine deletion at position 148, and a 7-bp insertion causing an altered amino acid sequence after position 215 in the C-terminus compared to the GL1 sequence of trichome-producing Arabidopsis thaliana (Fig. 3).
The large monomorphic glabrous population P9 Storsanden (about 30,000 plants) had ample variation in microsatellite loci, but harboured very few plants with leaf trichomes and the frequency of mutations altering the amino acid sequence of GL1 was also very high compared to the polymorphic populations (Table 1; Fig. 2b).

Glabrousness was associated with both the nonsynonymous substitution and the two indels in the third exon of GL1 (Table 1), and strong linkage disequilibrium was detected between these three sites in the two polymorphic populations. Multilocus analysis of microsatellite loci revealed significant genetic differences among the three study populations, and indicated no recent population admixture or clustering of the trichome-producing and glabrous individuals (Fig. 4). An association analysis, which controlled for population structure following Pritchard et al. (2000b), demonstrated that glabrousness was associated with both the nonsynonymous substitution and the two indels in the third exon of GL1 (Table 1). The three mutations are closely located in GL1 and were in strong, statistically significant linkage disequilibrium in the two polymorphic populations ($P < 0.0005$). In contrast, no significant linkage disequilibrium was found between any of the 12 microsatellite loci examined or between microsatellites and mutations in GL1 in any population after Bonferroni correction. Taken together, the results suggest that glabrousness is caused by one or more of the detected mutations or some other closely linked mutation in the regulatory region of GL1.

The results of the present study and comparisons with sequence data from other populations indicate that the A95D substitution is more likely to cause glabrousness than the two indels observed in the study populations. The A95D is located in the R3 Myb-repeat (Fig. 3). This is one of the most conserved residues (94%) found in the Myb region (Stracke et al. 2001), and on the basis of sequence conservation estimated by the program PolyPhen (Ramensky et al. 2002), the substitution A95D is likely to disrupt function. Of the 52 plants that were homozygous for the A95D mutation, only three produced any trichomes at all. The latter
formed very few trichomes, and the trichomes were located on the leaf margin (Table 1), which is consistent with observations of GL1 mutants in A. thaliana (Oppenheimer et al. 1991). Of the five plants homozygous for the 3-bp deletion, two were not homozygous for the A95D mutation, and these two plants had produced 71 and 222 trichomes on the three leaves examined (two plants in the Stubbsand population; Table 1). About one-third of the plants homozygous for the 7-bp insertion were trichome-producing (25 of 79; Table 1). Moreover, the 3-bp and 7-bp indels documented in the present study have previously been detected in a trichome-producing plant from a German A. lyrata population (Hauser et al. 2001; Fig. 3). In conclusion, these observations suggest that the two indels do not affect the functionality of GL1.

<table>
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*, all individuals had ≤ 5 trichomes and the trichomes were located on the leaf margin.

Variation in genetic basis of glabrousness

A minimum-evolution tree based on sequence variation in GL1 suggested that the single nucleotide polymorphism causing A95D has evolved more recently than the two indels (Fig. 5), and that different mutations in GL1 may be responsible for glabrousness in other populations. Whereas glabrous individuals in the Norwegian Arabidopsis lyrata population carried the same mutations as glabrous plants in the Swedish populations, glabrous plants from a population in Russian Karelia carried a 4-bp insertion at the beginning of the third exon (Hauser et al. 2001). This 4-bp insertion has not been observed in any trichome-producing plant (Fig. 5), and causes a frameshift and subsequently a stop-codon truncating most of the polypeptide.

Discussion

Association analysis can be a powerful method in evolutionary studies

This study has shown that trichome production is correlated with reduced damage from insect herbivores and that glabrousness in natural populations of the perennial herb Arabidopsis lyrata is associated with molecular variation in a gene homologous to the candidate gene GLABROUS1 described in A. thaliana. Association analysis is widely used in human genetics. However, until now it has been used only in a few studies of plants and animals other than humans, and most of these studies concerned domesticated species and traits subject to artificial selection (Glazier et al. 2002). This study demonstrates that genetic association methods can be used to reveal the genetic basis of phenotypic variation also in natural populations of plants.

The genetic basis of trichome formation is well understood in A. thaliana, and this provided necessary background information for the present study. GL1 is a regulatory gene that belongs to the Myb gene family and codes for an R2R3-type transcription factor (Stracke et al. 2001). The gene family is diverse with over 125 members characterized in A. thaliana and has various important regulatory functions in plants (Stracke et al. 2001). In the coding region of GL1, we detected three mutations altering the amino acid sequence. The three mutations were in strong linkage disequilibrium in the study populations, but the phenotypes
of the few plants that showed recombination in the third exon of GL1, and sequence variation available from other populations, suggested that glabrousness is more likely to be caused by the A95D than the two indels documented. The A95D substitution is situated in the conserved Myb-domain, and causes the neutral amino acid alanine to change into the negatively charged aspartate. The GL1 protein interacts with other proteins in forming the trichome-promoting complex needed for trichome initiation (Szymanski et al. 2000) and the A95D substitution may affect binding properties. Sequence conservation of alanine 95 was also confirmed by the program polyphen (Ramensky et al. 2002) indicating that changes in this residue would be deleterious.

The association between the A95D substitution and glabrousness was very strong, but did not completely follow the pattern expected for a recessive mutation blocking trichome production. A few plants homozygous for this mutation produced a few trichomes on leaf margins. This is consistent with observations made on GL1 mutants in A. thaliana (Oppenheimer et al. 1991), and may be explained by the action of one or several other genes. Recently Kirik et al. (2005) characterized a Myb gene in A. thaliana, MYB23, which regulates the formation of trichomes on leaf margins. Moreover, a few plants heterozygous for the A95D mutation were glabrous rather than trichome-producing. Trichome production is not necessarily expressed in early leaves (Kärkkäinen et al. 2004), and it is possible that some plants were scored before they had initiated trichome production. Alternatively, glabrousness in these plants may be due to variation in some other gene. Trichome formation can be affected by several cues and control mechanisms. In A. thaliana, mutational studies indicate that, in addition to GL1, TRANSPARENT TESTA GLABRA (TTG) may also cause glabrousness (Koornneef 1981; Herman & Marks 1989; Larkin et al. 2003). Moreover, allele–specific interactions causing glabrous phenotype have been documented between TTG and GL1 in A. thaliana (Larkin et al. 1999), and mutations in other genes have also been shown to inhibit trichome formation (Sawa 2002). Thus, similar to other seemingly simple traits (Scriver & Waters 1999; Clegg & Durbin 2003), trichome production may be influenced by several genes, epistatic interactions and environment. However, it is noteworthy that in the present study, we did not observe any of the pleiotropic effects of mutations in TTG documented in A. thaliana (pale colour of the seeds, lack of seedcoat mucilage and anthocyanin production; Koornneef 1981).

The association between glabrousness and mutations in GL1 could not be explained by population structure. Stratification or admixture of populations can produce spurious associations between genotypes and phenotypes (Long & Langley 1999; Cardon & Palmer 2003). However, there was no evidence of hidden population structure in the study populations of A. lyrata, and glabrous and trichome-producing plants did not differ at microsatellite loci. Cryptic relatedness may also influence tests of association, especially in founder populations or populations with extensive inbreeding (Voight & Pritchard 2005). However, surveys of variation at putatively neutral marker loci indicate that rates of inbreeding are very low in Swedish populations of A. lyrata (van Treuren et al. 1997) and have not revealed any clear signs of rapid and recent population growth (Kärkkäinen et al. 2004).

**Genetic basis of functional variation**

Comparative data suggest that glabrousness is caused by different mutations in GL1 in other populations of A. lyrata.
and in the closely related *A. thaliana*. Glabrous plants in Swedish and Norwegian populations of *A. lyrata* carried different mutations in GL1 than glabrous plants in a Russian population. However in both cases, mutations likely to be deleterious to the function of the gene were found in a highly conserved area of the third exon: a nonsynonymous substitution in the Swedish and Norwegian populations and a 4-bp insertion causing an early stop in the Russian population (Fig. 3). Two other mutations in GL1 have been described from glabrous accessions of *A. thaliana*: a single-base-pair deletion in the second exon resulting in an early stop codon (Mir-0, Br-0) and a large deletion of the gene (Hauser et al. 2001). Thus, although the number of genes identified in the developmental pathway of trichome development is high, different mutations in the same gene can apparently explain the occurrence of glabrous plants in both species.

It is not clear how commonly orthologous genes contribute to trait variation in different plant or animal lineages (Hoekstra & Price 2004; Wood et al. 2005), but several examples have been identified during recent years. Independent mutations in the gene *Melanocortin-1-receptor* (*MC1R*) cause melanism and colour polymorphism of several bird and mammal species (Nachman et al. 2003; Mundy et al. 2004). Similarly, independent mutations of the same gene have been found to cause albinism in different cavefish populations (Protas et al. 2006). In plants, different loss-of-function mutations in *FRIGIDA* were found to affect flowering time in different *A. thaliana* accessions (Johanson et al. 2000; Le Corre 2005).

The intensity and direction of selection on trichome production is likely to vary spatially and temporally. We documented a correlation between trichome production and reduced damage from insect herbivores, which should give trichome-producing plants an advantage in environments where the risk of herbivory is high. These findings are consistent with previous studies showing that damage caused by insect herbivores is negatively related to trichome density in other members of the Brassicaceae (Ågren & Schemske 1993; Mauricio 1998; Handley et al. 2005). The diamond-back moth, *Plutella xylostella*, was the most frequently observed herbivore on *A. lyrata* in the year of the field survey, and the differential damage may be related to its feeding preferences. Experimental work has shown that *P. xylostella* larvae preferentially feed on *A. lyrata* individuals with few or no leaf trichomes (R. Handley, P. Huttunen and J. Ågren, unpublished data). Moreover, field studies indicate that the relative fitness of the glabrous morph increases when the intensity of herbivory is experimentally reduced (G. Lee and J. Ågren, unpublished data).

The Fisherian view that adaptation is a function of many genes of small effect (Fisher 1930) has recently been challenged on theoretical grounds (Orr 1998), and by empirical studies on model organisms and natural populations (Bradshaw & Schemske 2003; Remington & Purugganan 2003). Here we have presented strong evidence that functionally important variation in trichome production is due to a major mutation in the regulatory gene GL1 in natural populations of *A. lyrata*. Our results are consistent with those suggesting that individual genes with large effects can be important in trait evolution (Remington & Purugganan 2003). Further studies are needed to determine how often orthologous genes have contributed to parallel evolutionary changes in multiple lineages as in the case of GL1 in *A. lyrata* and *A. thaliana*. We suggest that traits with a relatively simple inheritance will continue to be highly valuable in research programs exploring the complex process of adaptation, and that candidate genes identified in model organisms provide an obvious starting point in the search for the genetic basis of phenotypic variation.

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