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Mendelian inheritance of rust resistance to *Melampsora larici-epitea* in crosses between *Salix sachalinensis* and *S. viminalis*

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Two F_1, two F_2 and two backcross (BC) full-sib families of *Salix sachalinensis* × *S. viminalis* were tested for resistance to two pathotypes of *Melampsora larici-epitea* in leaf-disc inoculation experiments. Two single-pustule isolates, VM and ST, belonging to pathotypes LET1 and LET5, respectively, were used in the tests. Disease was scored based on the number of uredinia, uredinial diameter and inoculum densities. Both F_1 families were completely resistant to both VM and ST. Resistance to VM segregated at a 9:6:1 ratio in the F_2 families and at a 1:2:1 ratio in the BC families, suggesting that two independently segregating genes controlled rust resistance, with resistance dominant over susceptibility. This also indicates incomplete dominance of the resistance alleles over the susceptibility to VM. For ST, the equivalent ratios were 3:1 and 1:1, showing that a single dominant gene was responsible for rust resistance. The broad sense heritabilities were >0·91 for uredinial diameter and 0·1–0·33 for the number of uredinia. There were significant overall correlations between data from inoculations with VM and those from inoculations with ST in the number of uredinia, uredinial diameter and disease scores (Spearman's rank correlation coefficients = 0·31–0·75).

Keywords: incomplete dominance, inheritance, major gene, *Melampsora*, rust resistance, willow

Introduction

In recent years, the rising demand for energy from renewable sources has led to an increased interest in growing biomass crops. Willows (*Salix* spp.) are one of the main biomass crops cultivated in Europe. Biomass willows are grown in short-rotation-coppice (SRC) plantations which are harvested at 2- to 4-year intervals and remain productive for 15–25 years once established. SRC plantations sustain a dense, fast-growing canopy and are often severely attacked by rusts (*Melampsora* spp.). Rust reduces biomass yields by as much as 40% (Parker *et al.*, 1993) and predisposes plants to attacks by secondary pathogens, which often cause death of willow plants. Field experiments (Royle, 1991) suggested that, on susceptible clones, at least five fungicide applications are needed to keep levels of rust low throughout the season. However, routine use of fungicides in SRC plantations is not a viable option because of economic and environmental considerations. Instead, breeding for disease resistance proved to be particularly effective for SRC willow. For example, since biomass willow breeding started in Europe in 1987, levels of leaf rust were reduced considerably and the average yield increased by 50% (Larsson, 2001).

Of several *Melampsora* species occurring on SRC willows, the most widespread and damaging is *M. larici-epitea* (Pei *et al.*, 1996, 1999b). Within *M. larici-epitea*, there is a large variation in pathogenicity to different willows. A number of pathotypes (defined as one or a group of genotypes showing the same virulence/avirulence patterns on a set of host differentials) occurring on biomass willows were identified under f. spp. *larici-epitea typica* (LET) and *larici-retusae* (LR) (Pei *et al.*, 1996, 1999b).

Willow species belonging to section *Vimen* (Skvortsov, 1968; Wang & Fang, 1984) are favoured for biomass production because of their vigour, coppicing ability and growth form. Currently *S. viminalis* is the most important species grown in SRC plantations in Europe. Studies have shown that *S. viminalis* clones are more or less susceptible to LET (Pei *et al.*, 1996, 1999b, 2004a). By contrast, Far Eastern species belonging to section *Vimen*, such as *S. schwerinii* and *S. sachalinensis* (syn. *S. udensis*) are highly resistant to *Melampsora* rusts in Europe (Pei *et al.*, 1996, 2004a). In fact, deployment of the resistance from

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S. schwerinii has been a great success. Biomass clones Tora and Bjorn, which are F_1 hybrids of *S. schwerinii* × *S. viminalis*, have been free of rust over the past two decades. Rust resistance from *S. sachalinensis* is yet to be exploited in breeding.

So far, only limited information is available on how rust resistance is inherited in willow. In intraspecific hybrids of *S. viminalis*, Gullberg & Ryttman (1993) found considerable additive variation in field resistance to rust in Sweden. Fritz *et al.* (1996) tested intra- and interspecific F_1 hybrids of *S. viminalis* and *S. dasyclados* (syn. *S. burjatica*; see Pei *et al.*, 1999a) clones in detached-leaf inoculation experiments. They suggested that inheritance of resistance was additive based on the results that the hybrids between resistant and susceptible parents showed intermediate resistance. Johansson & Alström (2000) assessed the same crosses tested by Fritz *et al.* (1996) for field rust resistance during 1997–9. In 1997 and 1999, interspecific hybrids were more resistant than intraspecific hybrids, suggesting a pattern of hybrid resistance. In 1998, however, rust levels in the interspecific hybrids were intermediate compared to those of the intraspecific hybrids of the two parental species, suggesting an additive pattern of inheritance of rust resistance. In another study (Pei *et al.*, 2001), seven F_1 crosses of willows were tested for rust resistance through leaf-disc inoculation experiments and field disease assessments. The F_1 hybrids were equally or more susceptible than the parents in the crosses *S. disperma* × *S. burjatica*, *S. viminalis* × *S. cinerea* ssp. *oleifolia*, *S. viminalis* × *S. triandra* and *S. viminalis* cv. Bowles Hybrid × *S. viminalis* cv. French Osier. By contrast, the F_1 hybrids were more resistant than the parents in *S. viminalis* × *S. burjatica*, *S. viminalis* × *S. candida* and *S. viminalis* × *S. linearistipularis*. More recently, Rönnberg-Wästljung *et al.* (2008) reported that 8–26% of the variation in rust resistance could be attributed to the quantitative trait loci (QTLs) in nine genomic regions in (*S. schwerinii* × *S. viminalis*) × *S. viminalis* and to the QTLs in seven genomic regions in an F_2 family of *S. viminalis* × *S. dasyclados*. These studies have brought some degree of understanding to the inheritance of rust resistance, but the genetic basis of resistance in *Salix* to *Melampsora* rusts is yet to be resolved.

The aim of this study was to determine the nature of inheritance of rust resistance in *S. sachalinensis* × *S. viminalis* to *M. larici-epitea* using F_1, F_2 and backcross (BC) families in order to assess the potential of *S. sachalinensis* as a source of resistance for willow breeding programmes.

Materials and methods

Willow plants and rust isolates

Willow crosses were made using *S. viminalis* and *S. sachalinensis*, both being diploid (2n = 38). *Salix viminalis* occurs naturally across Eurasia, from the British Isles to the Far East, whilst *S. sachalinensis* is native to eastern Siberia, northeastern China, Korea and Japan. Willow shoots carrying flower buds were water-cultivated in heated glasshouses in January/February and crosses were made by hand-brushing female flowers with male pollen. Resulting seeds were harvested during March–April. The seeds were germinated in a mist chamber and cotyledonous seedlings were picked and potted in John Innes Compost No. 3. The plants were grown in pots in glasshouses for several weeks, then transferred to trays (60 × 40 × 10 cm, 24 seedlings in each tray) containing the same compost and placed in an open nursery. The trays were drip-irrigated twice a day.

In all, two F_1, three F_2 and two BC families were tested for rust resistance (Table 1). However, family 1234 was a remake of 1008 and therefore both belonged to the same full-sib family, giving two F_2 families in total. Inoculation experiments were carried out using willow leaves from nursery-grown plants with family 589 in July 1999, with families 761 and 762 in July 2001, with 1008 and 1015 in July 2004 and with 1234 in July 2008. By the time of inoculations, plants of the families 589, 761 and 762 were 110–140 cm in height and those of 1008, 1015 and 1234 were 80–120 cm in height. With family 373, leaves from glasshouse-grown plants (80–100 cm in height) were used for inoculation in May 2002.

Two single-pustule isolates, VM belonging to pathotype LET1 and ST to LET5 (see Pei *et al.*, 1999b), were used for inoculation. VM was derived from *S. viminalis* cv. Mullatin collected at Long Ashton, southwest England, in September 1991, and ST from *S.* × *stipularis* (*S. viminalis* × *S. cinerea*) collected at Long Ashton in September 1995. These isolates were chosen because pathotypes belonging to LET were most prevalent on *S. viminalis* and its hybrids in the UK (Pei *et al.*, 1999b).

Table 1 Crosses between *Salix sachalinensis* and *S. viminalis*, with number of host genotypes tested ( ) and *Melampsora larici-epitea* isolates inoculated [ ]

	Male		
Female	*S. sachalinensis* cv. Sekka	*S. viminalis* cv. Ulv	*S. sach* × *vim* 589-82
S. viminalis cv. Bowles Hybrid	F_1 family 373 (32) [VM, ST]		
S. sachalinensis cv. Kioryu		F_1 family 589 (22) [VM, ST]	
S. sach × *vim* 589-95		BC family 762 (30) [VM, ST]	F_2 family 761 (160) [VM, ST]
S. sach × *vim* 589-79			F_2 family 1008 (154) [VM, ST]
			F_2 family 1234 (147) [VM, ST]
S. sach × *vim* 589-86		BC family 1015 (89) [VM, VMrepeat]	

Salix viminalis cvs Bowles Hybrid and Ulv were susceptible to VM, showing disease scores of 0·15–0·3 (diseases score = square root pustule area/square root inoculum density; see below) in leaf-disc inoculation experiments, but they were resistant to ST, showing disease scores of 0–0·02. By contrast, *S. sachalinensis* cvs Kioryu and Sekka were immune (without symptoms when inoculated) to both of the isolates. All the families, except for BC 1015, were inoculated with the two isolates. Inoculation of 1015 with VM was repeated to validate reproducibility of the inoculation procedure.

Inoculation experiments

Two weeks before inoculation, rust spores were freshly bulked up on detached leaves of *S.* × *stipularis*, which is universally susceptible to LET pathotypes. For testing the F_1, F_2 and BC families, leaf discs 1·1 cm in diameter (95 mm^2 area) were cut from the fifth to 15th leaves from the first unfurled leaf (furled edge less than one-third of total leaf edge) on actively growing willow shoots. Five leaf discs, each from a different leaf, were used as replicates. The discs were placed, abaxial surface uppermost, on blotting paper bridges soaked in tap water in the compartments of 10- × 10-cm-square Petri dishes (each dish contained 5 × 5 = 25 compartments). To measure inoculum densities, 60-mm-diameter round Petri dishes containing 1% water agar, usually one agar plate for every six square Petri dishes, were placed in the spray target area. Before making spore suspensions, rust spores were initially mixed in a drop of distilled water with a hair brush to ensure spores were fully wet before adding more water. Rust spore suspensions were adjusted to 80×10^3 spores mL^{-1} and sprayed evenly on to the spray target area (1 mL per 10- × 10-cm area) using a Humbrol air brush (Humbrol Ltd). After inoculation, the leaf discs were incubated in a growth chamber at 16°C with 16 h/day illumination at an intensity of 80 $\mu E\ m^{-2}s^{-1}$. Inoculum densities (viable spores cm^{-2}) were estimated by counting the number of germinating spores on the water agar in eight fields of view of a light microscope (10 × 10 magnification = 2·4 mm^2 each field) for each Petri dish 24 h after inoculation.

Thirteen days after inoculation, the leaf discs were photographed using a digital camera. Image analysis software SIGMASCAN Pro 5.0 (SPSS Inc.) was used to measure the disease. For each leaf disc, pustule numbers were counted and pustule diameters were measured manually with the TRACE MEASUREMENT MODE function. If there were more than 10 pustules on a leaf disc, only 10 randomly selected pustules were measured to obtain an estimate of average pustule diameter.

Data analysis

Disease scoring

Disease severity was scored for each willow genotype by dividing the average square root of pustule area per leaf disc by the square root of inoculum density in each combination of willow family and rust isolate. This method of disease scoring was proposed previously (Pei & Hunter, 2005) based on the linear relationships between uredinial pustule area and inoculum density in the willow rust *M. larici-epitea* and the poplar rust *M. larici-populina*. A relative disease score (RDS) was calculated for each genotype by dividing its disease score by the highest disease score for that willow family/rust isolate combination. Thus, the most susceptible genotype had a RDS of 1. A relative uredinial diameter (RUD) was also calculated for each genotype by dividing its diameter by the largest diameter for that willow family/rust isolate combination.

Broad-sense heritability (H^2)

Broad-sense heritability (the degree to which individual phenotypes are determined by their genotypes) were calculated as

$$H^2 = \frac{\delta^2{}_G}{\delta^2{}_G + \delta^2{}_E}$$

in which $\delta^2{}_G$ is the variance among genotypes and $\delta^2{}_E$ is the variance within genotypes(i.e. phenotypic variance among replicates of genotypes).

Segregation analysis

The finding that all or some genotypes in the tested families were completely resistant (see Results) indicated that resistance to the tested isolates could be qualitative and was probably controlled by major gene(s). As disease was measured quantitatively, data obtained were more or less continuous (raw data are presented in Figs 1–5). To examine segregation, individuals within a family were assigned to two to five classes according to their RDS or RUD values. For example, in the case of four classes, the individuals having an RDS or RUD value of 0 were assigned to one class and those having values of 0–0·33, >0·33–0·67, >0·67–1 were assigned to the other three respective classes. A chi-squared goodness-of-fit test was conducted with the categorical data under various assumed genetic models, such as that 1, 2 or 3 genes may control rust resistance and these genes may interact with each other.

Correlation between variables

Spearman's rank correlation was tested between the number and the diameter of uredinia on the same leaf disc separately for each family/isolate combination because inoculum densities differed between inoculations. Leaf discs which did not produce uredinia were excluded from the test because they had a value of 0 both for the number and the diameter of uredinia. The same test was also conducted between VM and ST on the number of uredinia, uredinial diameter and disease scores, irrespective of willow families. Families 761, 762, 1008 and 1234 were included in the tests between VM and ST.

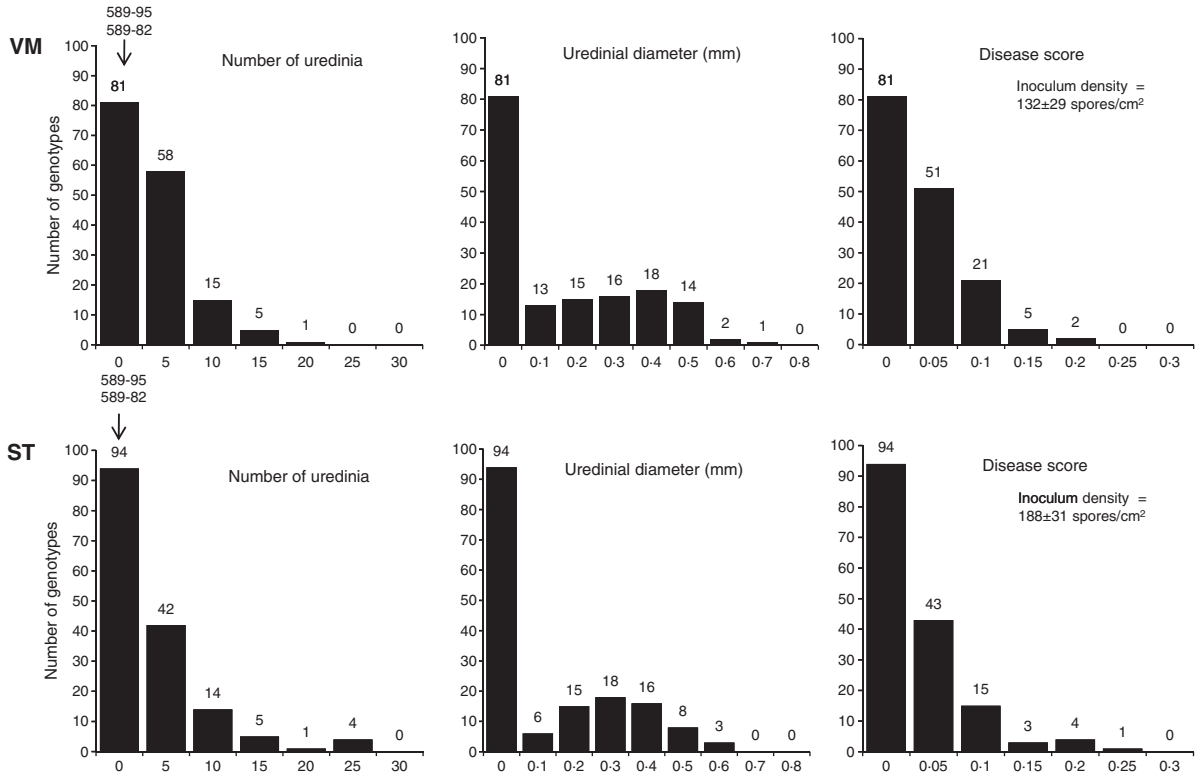

Figure 1 Number of uredinia, uredinial diameter and disease scores in inoculations of the F_2 family 761 with *Melampsora larici-epitea*. Horizontal scales represent the upper value of the range. For example, VM formed 5–10 uredinia on 15 genotypes (upper left). Parental clones 589-95 and 589-82 were completely resistant (no uredinia) in both inoculations.

Results

Inoculum densities were in a range of 80–410 viable spores cm^{-2} (Table 2). All the genotypes in the F_1 families 373 and 589, including those used as parents for other families (589-79, -82,-86 and -85), showed no symptoms or barely recognizable necrotic spots only. The majority of the genotypes in the three F_2 families (761, 1008 and 1234) were highly resistant, having disease scores of 0–0·05 (Figs 1, 2 and 3). The majority of the BC families 762 and 1015 were also scored 0–0·05 (Figs 4 and 5). On the whole, inoculations of the F_2 and BC families with VM resulted in relatively more and larger pustules, hence higher disease scores, than those with ST (Table 2). Disease scores were relatively low in F_2 761 and BC 762, which were more mature (110–140 cm in height) than other families (80–110 cm). In BC 1015, inoculum density and level of disease were slightly higher in the repeated inoculation experiment (Fig. 5, Table 2).

Broad-sense heritabilities were extremely high (H^2 > 0·91) for uredinial diameter and disease scores, but low or moderate (0·1–0·33) for the number of uredinia (Table 3).

In inoculation of F_2 761 with VM, the RDS data differed significantly from the 9:3:3:1 ratio, but the RUD data fitted the same ratio well (P = 0·52; a P value >0·05 indicates that the observed data do not differ significantly from expectations based on the hypothesized segregation ratio) (Table 4). Results from inoculations of F_2 1008 and F_2 1234 with VM did not reject the 9:3:3:1 ratio (P = 0·07 and 0·42, respectively, for RDS and P = 0·31 and 0·15, respectively, for RUD). P values for the combined data for F_2 1008 and F_2 1234 inoculated with VM fell at the margins of rejecting the 9:3:3:1 ratio (P = 0·05 for RDS and 0·06 for RUD). When the two intermediate classes (RDS/RUD = 0–0·33 and RDS/RUD >0·33 – 0·67) were combined, both the RDS and RUD data from the inoculations of F_2 families fitted the 9:6:1 ratio (P = 0·25–0·41).

In inoculation of BC 762 with VM, the RDS data differed significantly from the 1:1:1:1 ratio, but the RUD data did not reject the same ratio (P = 0·18). When the two intermediate classes were combined, the data from the inoculation of BC 762 with VM fitted the 1:2:1 ratio (P = 0·46 for RDS and P = 0·43 for RUD). The results from BC 1015 and its repeat fitted either the 1:1:1:1 ratio (P = 0·11–0·59; two intermediate classes separate) or the 1:2:1 ratio (P = 0·36–0·51; two intermediate classes combined).

In inoculations with ST, data from the F_2 families 1008 and 1234 fitted remarkably well a 3:1 ratio (P = 0·65–0·93). However, data from the inoculation of the F_2 family 761 with ST did not fit the 3:1 ratio (P = 0·04). On

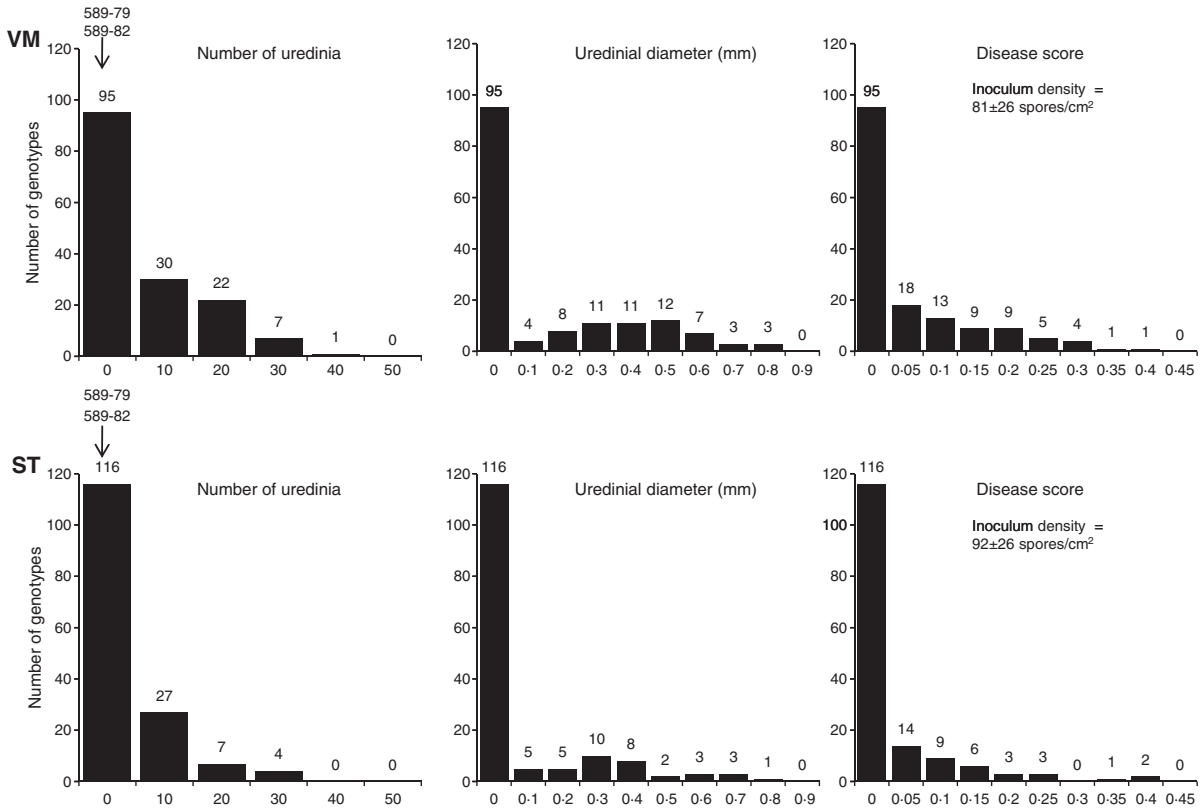

Figure 2 Number of uredinia, uredinial diameter and disease scores in inoculations of the F_2 family 1008 with *Melampsora larici-epitea*.

the other hand, results from the inoculation of BC 762 with ST did not reject the 1:1 ratio of segregation (*P* value = 0·15).

Spearman's rank correlation coefficients between the number of uredinia and the diameter of uredinia on the same leaf discs were relatively low in F_2 761 and BC 762 (0·22–0·47) (Table 4) but high in BC 1015 (0·7–0·79). Correlations between the two variables were significant in all combinations of willow family and rust isolate.

Spearman's rank correlation coefficients between VM and ST for the number of uredinia, uredinial diameter and disease scores were 0·73–0·75 including all genotypes, 0·31–0·43 excluding those which scored 0 against both isolates, and 0·5–0·54 excluding the genotypes which scored 0 against one or both isolates (Table 5). There were significant correlations in these parameters between the data from the two isolates (Table 6).

Between the data from the repeated inoculations of 1015 with VM, Spearman's rank correlation coefficients were in a range of 0·88–0·96 for number of uredinia, uredinial diameter and disease scores.

Discussion

In this study, the inheritance of rust resistance in willow was examined using two rust pathotypes of *M. larici-epitea* and six full-sib families of *S. sachalinensis* × *S. viminalis*. The use of the leaf-disc inoculation procedure allowed a large number of samples to be tested and the resulting disease measured precisely. In previous studies (Pei *et al.*, 1996, 2001), disease was scored using a 0–4 ranking system. A similar ranking system was also used to score the size of uredinia in studies of *Melampsora* on poplars (*Populus* spp.) (Lefèvre, 1998; Dowkiw *et al.*, 2003; Dowkiw & Bastien, 2004; Jorge *et al.*, 2005). With these ranking systems, estimation of pustule size is subject to the assessor's judgment and, therefore, considerable experience is required to achieve consistency. Also, in *Melampsora* rusts on willow and poplar, inoculum densities can greatly influence the number of pustules produced (Pei *et al.*, 2002, 2004b; Pei & Hunter, 2005). In the present study, direct measurement of uredinia provided objective and precise data and the weighting of the differences in inoculum density made it easier to compare the disease data between inoculations. The repeated inoculations of 1015 with VM produced almost identical results (Spearman's correlation coefficients = 0·88–0·96), indicating that the results obtained using the present inoculation procedure were robust. Also, between the families 1008 and 1234 (remake of 1008), the results obtained were very similar.

It is known that the age of leaves of plants and growing conditions can influence levels of rust disease. In poplars, Sharma (1980) showed that older leaves were more resistant to *M. larici-populina* than younger leaves. Experience with willow rusts in the past 20 years

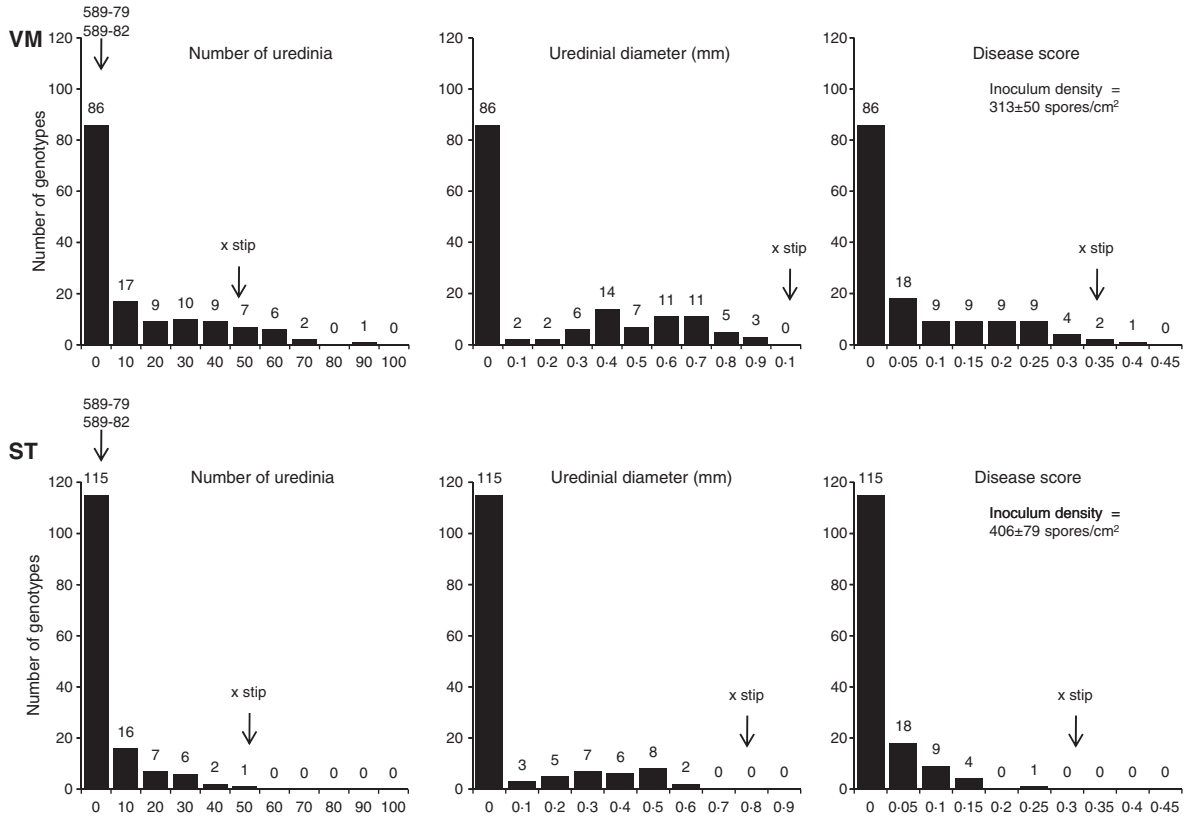

Figure 3 Number of uredinia, uredinial diameter and disease scores in inoculations of the F_2 family 1234 (remake of 1008) with *Melampsora larici-epitea*.

(Pei *et al.*, 1996; Pei & Ruiz, unpublished data) suggests that older leaves, leaves from older plants and plants grown under unfavourable conditions are less susceptible to rust. In the present study, disease levels were relatively low in the families F_2 761 and BC 762 (Table 2; Figs 1 and 4). Maximum disease scores against VM were 0·16 in both 761 and 762, but 0·24–0·39 in other families, in which the plants were younger than in 761 and 762. Introduction of the RDS and the RUD made the data derived from F_2 761 and BC 762 somewhat more comparable to those from other families. However, this relative approach needs to be adopted with caution because, in cases where all individuals in a family are more or less resistant because of genetic factors, RDS and RUD values would exaggerate disease severity. In such cases, a highly susceptible clone can be included in inoculation experiments as a reference clone. On the whole, it is important to standardize the experimental conditions, including the maturity and growth stage of plant material, as rigorously as possible in inoculation experiments.

In the past two decades, extensive research has been carried out to determine the genetics of rust resistance in poplars, which are closely related to willows and serve as hosts to many species of *Melampsora*. Studies have shown that, in North America, *P. trichocarpa* possesses at least two major genes for resistance, to *M. medusae* and *M.* × *columbiana*, and *P. deltoides* possesses at least two major genes for resistance to some pathotypes of *M.* × *columbiana* (Newcombe, 2005). In Europe, Lefèvre *et al.* (1994) reported that inheritance of qualitative resistance to *M. larici-populina* in *P. deltoides* × *P. trichocarpa* is relatively simple: one or two genes control resistance to a given set of isolates of the pathogen, and some of these genes appear to be organized in a cluster (Lefèvre, 1998). Also, statistical associations have been observed between qualitative and quantitative resistances to *M. larici-populina* (Dowkiw *et al.*, 2003; Dowkiw & Bastien, 2004). In these studies, mapping populations have been used for detection of QTLs for poplar rust resistance (Cervera *et al.*, 1996; Newcombe *et al.*, 1996; Lefèvre, 1998; Dowkiw & Bastien, 2004; Jorge *et al.*, 2005).

In comparison with research on rust resistance in poplars, very limited work has been done on the genetics of rust resistance in willows. Previously, Gullberg & Ryttman (1993), Fritz *et al.* (1996) and Johansson & Alström (2000) examined hybrids within and between *S. viminalis* and *S. dasyclados*. Although 40 or more families were studied, the small sample sizes (8–16 genotypes in each family), the fact that only F_1 hybrids were examined and the differences in ploidy level (2n = 38 in *S. viminalis* and 2n = 76 in some *S. dasyclados* clones) made it difficult to draw conclusions on the inheritance of

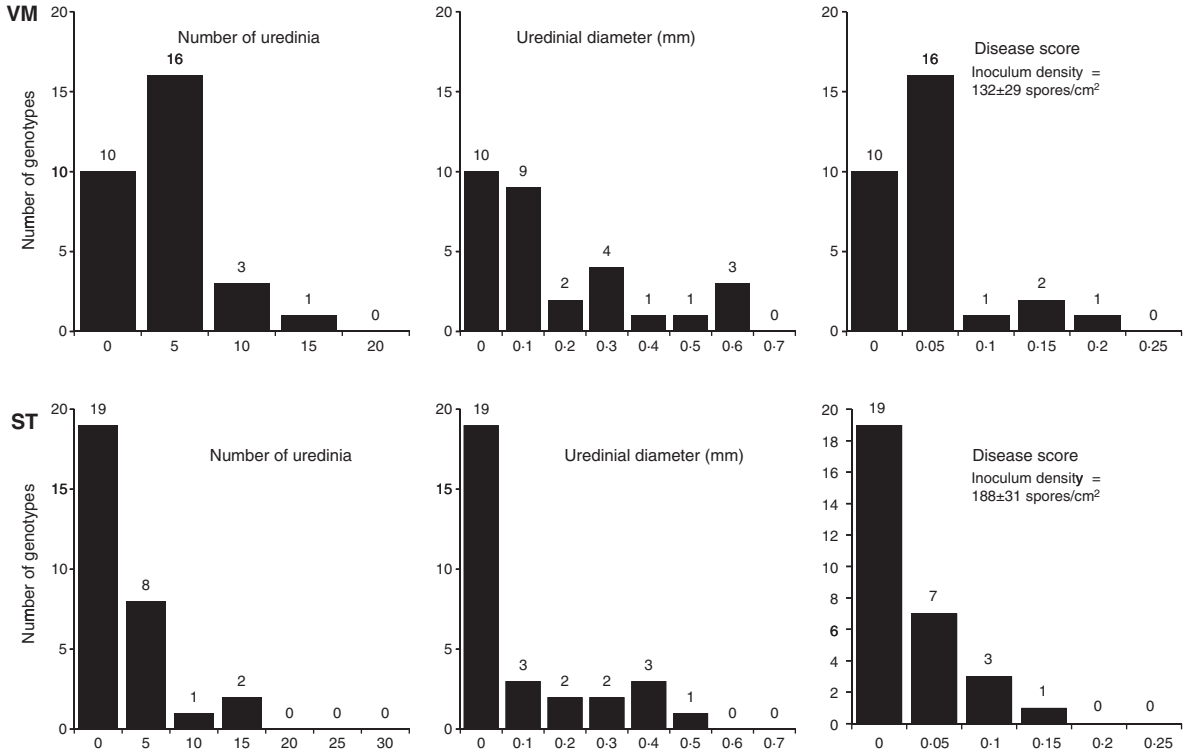

Figure 4 Number of uredinia, uredinial diameter and disease scores in inoculations of the BC family 762 with *Melampsora larici-epitea*.

rust resistance. Also, their data were mainly derived from assessments of rust severity in the field. Under field conditions, many factors, such as weather, soil and water conditions and field pathotype composition, can influence rust severity. For example, Johansson & Alström (2000) found that, compared with the intraspecific hybrids they studied, interspecific hybrids were more resistant in 1997 and 1999, but not in 1998. The two families studied by Rönnberg-Wästljung *et al.* (2008) (*S. schwerinii* × *S. viminalis*) × *S. viminalis* and an F_2 of *S. viminalis* × *S. dasyclados*, contained only 73 and 85 genotypes, respectively. The authors pointed out that there were indications of the involvement of a few genes or gene clusters with a large impact, but a larger population was needed to facilitate further study. Compared with previous work, the present study was much more focused. For example, of the six full-sib families tested, five were derived from a male clone of *S. sachalinensis* and a female clone of *S. viminalis*. This ensured that the resistance examined had the same genetic background and facilitated investigation of the same resistance genes from different angles, i.e. using F_1, F_2 and BC. Also, a reasonable number of genotypes (22–89 for F_1/BC and 147–160 for F_2 families) were tested in each inoculation experiment to obtain robust results.

The present study provides, for the first time, conclusive evidence that rust resistance is inherited in a classic Mendelian fashion in a willow cross. The dominant nature of the gene(s) for rust resistance was evident in the early stages of this study as all F_1 genotypes were completely resistant. This also suggested that at least one resistance gene was homozygous in the resistant parent *S. sachalinensis*. In inoculations with VM, the goodness of fit tests with RDS and RUD data rejected neither a segregation ratio of 9:6:1 for the F_2 families nor a ratio of 1:2:1 for the BC families (P = 0·25–0·51). This means that, in this host-pathogen system, two independently segregating genes conditioned rust resistance, with resistance dominant over susceptibility. It also suggests that the dominant resistance alleles were homozygous at both loci in the resistant parent cv. Kioryu but were absent in the susceptible parent cv. Ulv. For convenience of explanation, the two genes in Kioryu resistant to VM can be denoted as R1R1 and R2R2, and those in Ulv susceptible to VM as r1r1 and r2r2. The present results show that all the F_1 genotypes were completely resistant, being heterozygous at both loci, i. e. R1r1R2r2. In the F_2 families, the R1_R2_, R1_r2r2, r1r1R2_ and r1r1r2r2 genotypes segregated at 9:3:3:1 ratio. Of these, R1_R2_ were completely resistant and r1r1r2r2 highly susceptible, whilst R1_r2r2 and r1r1R2_ were intermediate. Thus, the ratio of resistant : intermediate : highly susceptible phenotypes would be 9:6:1. The involvement of the two independent genes for rust resistance was confirmed by the data from the backcrosses, in which R1r1R2r2, R1_r2r2, r1r1R2_ and r1r1r2r2 genotypes segregated at a 1:1:1:1 ratio (1 resistant: 2 intermediate : 1 highly susceptible phenotypes). The results also showed that the two independent

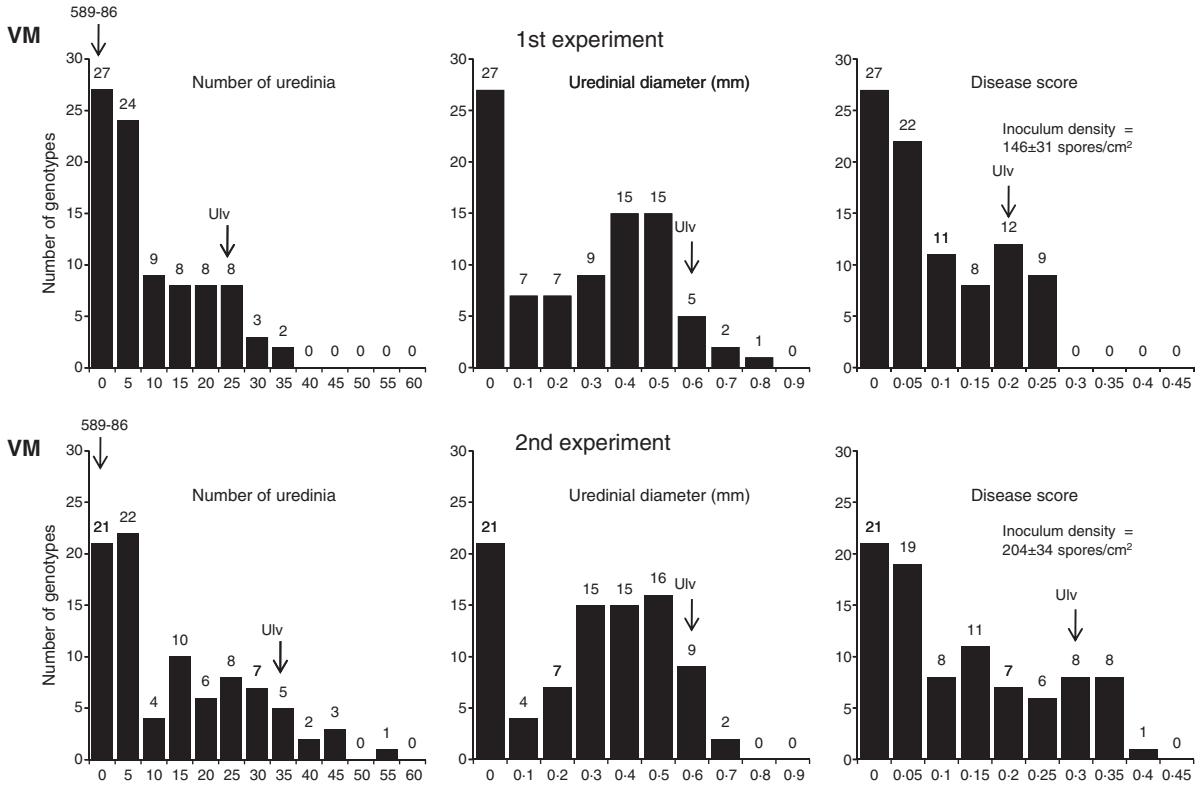

Figure 5 Number of uredinia, uredinial diameter and disease scores in inoculations of the BC family 1015 with *Melampsora larici-epitea*. The second experiment, which had a higher inoculum density, was a repeat of the first.

Table 2 Average number of uredinia, uredinial diameter (mm) and disease scores in inoculation experiments of the crosses between *Salix sachalinensis* and *S. viminalis* with *Melampsora larici-epitea*

	VM				ST			
Family	Inoculum density	Number of uredinia	Uredinial diameter	Disease scores	Inoculum density	Number of uredinia	Uredinial diameter	Disease scores
373	245 ± 62[a]	0	0	0	218 ± 61	0	0	0
589	235 ± 75	0	0	0	312 ± 65	0	0	0
761	132 ± 29	1·8	0·13	0·022	188 ± 31	2·2	0·11	0·022
762	108 ± 14	1·5	0·14	0·025	131 ± 15	1·3	0·08	0·014
1008	81 ± 26	3·6	0·14	0·044	131 ± 15	2	0·08	0·026
1015	146 ± 31	7·6	0·24	0·071				
	208 ± 34	12·2	0·27	0·112				
1234	313 ± 50	11	0·2	0·055	406 ± 79	2·8	0·071	0·013

[a]Standard error of mean (P = 0·05).

Table 3 Broad-sense heritabilities in inoculations of the crosses between *Salix sachalinensis* and *S. viminalis* with *Melampsora larici-epitea*

	VM			ST		
Family	Uredinial number	Uredinial diameter	Disease score	Uredinial number	Uredinial diameter	Disease score
761	0·32	0·92	0·99	0·24	0·91	0·98
1008	0·20	0·95	0·97	0·24	0·97	0·98
1234	0·13	0·97	0·98	0·1	0·94	0·98
762	0·33	0·94	0·99	0·31	0·93	0·99
1015	0·26	0·96	0·98			
	0·20	0·97	0·98			

Table 4 Segregation and Chi-squared values for relative disease scores (RDS) and relative uredinial diameter (RUD) in the F_2 and backcross families of *Salix sachalinensis* × *S. viminalis* inoculated with *Melampsora larici-epitea*

Family	RDS (RUD)				Sum	χ^2	*P* value
Inoculations with VM							
F_2							
	0	0–0·33	>0·33–0·67	>0·67–1			
761	81 (81)	53 (32)	20 (35)	6 (12)	160	23·47 (2·27)	0 (0·52)
1008	95 (95)	34 (20)	18 (29)	6 (9)	153	7·22 (3·6)	0·07 (0·31)
1234	86 (86)	30 (18)	20 (30)	11 (13)	147	2·78 (5·25)	0·42 (0·15)
1008 + 1234	181 (181)	64 (38)	38 (59)	17 (22)	300	8·04 (7·51)	0·05 (0·06)
Expected ratio	9	3	3	1			
	0	0–0·67	>0·67–1				
761	81 (81)	73 (67)	6 (12)		160	2·28 (2·78)	0·32 (0·25)
1008	95 (95)	52 (49)	6 (9)		153	2·02 (2·29)	0·36 (0·33)
1234	86 (86)	50 (48)	11 (13)		147	1·8 (1·98)	0·41 (0·37)
1008 + 1234	181 (181)	102 (97)	17 (22)		300	2·27 (2·49)	0·32 (0·29)
Expected ratio	9	6	1				
BC							
	0	0–0·33	>0·33–0·67	>0·67–1			
762	10 (10)	16 (11)	2 (5)	2 (4)	30	18·53 (4·93)	0 (0·18)
1015	27 (27)	26 (16)	18 (29)	18 (17)	89	3·27 (6·06)	0·35 (0·11)
1015Repeat	21 (21)	30 (18)	20 (27)	18 (23)	89	3·81 (1·92)	0·28 (0·59)
Expected ratio	1	1	1	1			
	0	0–0·75	>0·75–1				
762	10 (10)	18 (16)	2 (4)		30	1·55 (1·68)	0·46 (0·43)
1015	27 (27)	44 (45)	18 (17)		89	2·06 (2·04)	0·36 (0·36)
1015Repeat	21 (21)	50 (45)	18 (23)		89	1·3 (1·37)	0·41 (0·51)
Expected ratio	1	2	1				
Inoculation with ST							
	0		0–1				
F_2							
761	94		66		160	22·53	0·04
1008	116		38		154	0·01	0·93
1234	115		31		147	0·82	0·65
1008 + 1234	231		70		301	0·022	0·88
Expected ratio	3		1				
BC							
762	19		11		30	2·133	0·15
Expected ratio	1		1				

Table 5 Spearman's rank correlation coefficients between the number and diameter of uredinia on the same leaf disc (leaf discs without uredinia were excluded) in inoculations of the crosses between *Salix sachalinensis* and *S. viminalis* with *Melampsora larici-epitea*

	VM			ST		
Family	Correlation coefficient	Degrees of freedom	*P* value	Correlation coefficient	Degrees of freedom	*P* value
761	0·31	275	<0·001	0·22	241	<0·001
762	0·47	49	<0·001	0·38	36	0·006
1008	0·50	198	<0·001	0·655	146	<0·001
1015	0·79	294	<0·001			
	0·70	254	<0·001			
1234	0·76	276	<0·001	0·57	124	<0·001

Table 6 Spearman's rank correlation coefficients between inoculations with VM and that with ST of *Melampsora larici-epitea*

Parameter	Coefficient	Degrees of freedom	*P* value
Including all genotypes			
Number of uredinia	0·73	488	<0·001
Diameter	0·75		<0·001
Disease score	0·75		<0·001
Excluding genotypes scoring 0 against both isolates			
Number of uredinia	0·31	231	<0·001
Diameter	0·43		<0·001
Disease score	0·41		<0·001
Excluding genotypes scoring 0 against one or both isolates			
Number of uredinia	0·5	130	<0·001
Diameter	0·54		<0·001
Disease score	0·5		<0·001

alleles controlling resistance to VM exhibited incomplete dominance over the respective susceptible alleles. Had the two resistance alleles been completely dominant over susceptibility, the segregation ratio of resistant and susceptible genotypes would have been 15:1 in the F_2 families and 3:1 in the BC families. This means that, if resistance were completely dominant over susceptibility at both loci, R1_R2_, R1_r2r2 and r1r1R2_ genotypes would be completely resistant, and only r1r1r2r2 susceptible to VM.

Although the results revealed incomplete or partial dominance of the alleles R1 and R2 in resistance to VM, the relative effect of these alleles on the extent of resistance to VM is not yet clear. If either R1 or R2 is more effective than the other, R1_r2r2 is more resistant than r1r1R2_ or *vice versa*. Hence, the segregation ratio would be 9:3:3:1 instead of 9:6:1 in the F_2 families and 1:1:1:1 in the BC families instead of 1:2:1. It is interesting to note that both the RDS and RUD data from F_2 families 1008 and 1234 fitted the 9:3:3:1 ratio and that from BC 1015 fitted the 1:1:1:1 ratio. In F_2 761 and BC 762, although the RDS data did not conform to 9:3:3:1 and 1:1:1:1 ratios, respectively, their RUD data did.

In inoculations with ST, data from the F_2 families 1008 and 1234 (both derived from the same parents) fitted well the 3:1 ratio (*P* values 0·65–0·93), indicating that a single locus conditioned rust resistance, with resistance dominant over susceptibility. Data from inoculation of F_2 family 761 with ST did not fit the 3:1 ratio ($P = 0{\cdot}04$). A possible explanation could be that ST may be more sensitive to maturity of leaves or plants. Assuming that a single gene is responsible for rust resistance, BC families inoculated with ST should segregate in a 1:1 ratio. In this study, the results from the inoculation of BC 762 with ST did not reject the 1:1 segregation ($P = 0{\cdot}15$). This suggests a single gene is acting here too, but further work is needed to confirm this.

In the present study, the values of the broad-sense heritability (H^2) for uredinial diameter (>0·91) were much higher than that for the number of uredinia (0·1–0·33). Therefore, uredinial diameter is a more reliable heritable trait than the number of uredinia. Rönnberg-Wästljung *et al.* (2008) found that the broad-sense heritability of uredinial diameter was in a range of 0·76–0·85. Dowkiw *et al.* (2003) showed that uredinial size is the resistance component in the poplar–*M. larici-populina* system that best predicts the effects under field conditions. Jorge *et al.* (2005) also reported $H^2 > 0{\cdot}89$ for uredinial diameter in inoculations of F_1 families of poplars with several isolates of *M. larici-populina*.

Results from the present study showed significant correlations in the number of uredinia, uredinial diameter and disease scores between inoculations with VM and those with ST (Spearman's rank correlation coefficients = 0·31–0·75). This suggests that there is an association among the genes controlling resistance to VM and the gene(s) controlling resistance to ST. With SRC willows, linkage maps have been constructed in Sweden (Rönnberg-Wästljung *et al.*, 2008) and in the UK (Hanley *et al.*, 2002, 2006). The UK mapping population K8, derived from two *S. viminalis* × (*S. viminalis* × *S. schwerinii*) hybrid sibs, contains a large number of genotypes (947 in total) (Hanley, 2003). Future studies involving the UK mapping population, VM and ST may provide an insight into how the genes for resistance to VM and to ST are organized in the willow genome.

Breeding for disease resistance is a high priority in many crop systems. *Salix* is one of the largest genera of woody plants in the northern hemisphere, comprising 300–500 species according to different authorities. Studies involving inoculation tests and field disease assessments have shown that there are abundant sources of resistance in *Salix* against rust (Pei *et al.*, 1996, 2004a). Also, willows are dioecious (male or female), hybridize with relative ease, and those used for SRC usually reach sexual maturity in 2 years. Furthermore, unlike grains, fruits and vegetables, in which flavour, colour and shape may have prime importance, interspecific hybrids are readily accepted for biomass production. These attributes make breeding for resistance a favourable option to combat rust disease in SRC willow.

The present results clearly demonstrated that major genes are involved in the inheritance of rust resistance in *S. sachalinensis* × *S. viminalis* and indicated that *S. sachalinensis* may be a useful source of resistance for future breeding programmes. Like their hosts, *Melampsora* rusts on *Salix* are highly diverse. The results from a preliminary study (Pei *et al.*, 2001) indicated that the mechanisms involved in the inheritance of rust resistance may vary in different willow crosses. Judging from the immense diversity both in the host and in the pathogen, genetic control of rust resistance in the *Salix–Melampsora* system may be complex. Further work involving a wide range of willow crosses may help to clarify the genetic basis of resistance in different *Salix* spp. to *Melampsora* rusts and help inform breeding programmes aimed at improving rust resistance in SRC willow.

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