Introgression of crown rust (*Puccinia coronata*) resistance from meadow fescue (*Festuca pratensis*) into Italian ryegrass (*Lolium multiflorum*) and physical mapping of the locus

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Resistance was found in the meadow fescue (*Festuca pratensis*) to crown rust (*Puccinia coronata*), originating from ryegrasses (*Lolium* spp.). A backcrossing programme successfully transferred this resistance into diploid Italian ryegrass (*Lolium multiflorum*) and genomic *in situ* hybridisation (GISH) was used to identify the introgressed fescue chromosome segment. The resistant (R) plants in two BC₃ lines all carried an introgressed segment on a single chromosome, which in one of the lines was confined to the short arm of the chromosome. Susceptible (S) plants either

contained no introgressed chromosome segment or a segment which was physically smaller than the segments in resistant plants. Using GISH the resistance locus could be physically mapped to the midpoint of a short arm. Segregation ratios of the progeny of BC₃ plants, when crossed as R × S and R × R, were in agreement with the hypothesis that the resistance was controlled by a single gene or very closely linked genes. No R plants were produced by crossing S × S plants.

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Introduction

Crown rust (*Puccinia coronata*) is a common disease of ryegrasses, and is probably the most serious fungal disease of perennial ryegrass (*Lolium perenne*) in the UK and western Europe (Potter *et al.*, 1990). It is apparently becoming more widespread in Europe, and while it is normally most prevalent in late summer and autumn, there are many recent reports of the disease appearing earlier (Reheul *et al.*, 2001). It is found in almost every region of the world where perennial ryegrass is grown (Kimbeng, 1999). Many grasses are hosts to crown rust including other agriculturally important species such as meadow fescue (*Festuca pratensis*), as well as other *Festuca* spp (Braverman, 1967, 1986). The fungus exists in different physiological forms and this manifests itself both at the *formae speciales* level – its capacity to infect certain grass species – and as physiological races – specificity to certain genotypes within the species. However, the relationship between different hosts and physiological forms of the fungus is complex (Dinoor *et al.*, 1988). Therefore, within any host species some susceptibility to *P. coronata* from an unrelated species can occur especially under artificial conditions in a glasshouse or growth cabinet. Consequently, within a population of *F. pratensis*, individual plants can differ in their reaction to infection by

P. coronata originating from *Lolium* spp, with some populations having a relatively high proportion of susceptible plants (personal observation). Thus, *F. pratensis* should perhaps be considered as another potential source of resistance parallel to the ones derived from *Lolium* spp rather than as a 'nonhost'.

Meadow fescue (*F. pratensis*) and the two cultivated ryegrass species, Italian ryegrass (*L. multiflorum*) and perennial ryegrass (*L. perenne*), are closely related with a degree of homology between their chromosomes (Jauhar, 1975). On the other hand, the relationship is sufficiently distant that *L. multiflorum* (*Lm*) and *F. pratensis* (*Fp*) chromosomes can be distinguished in hybrid plants using genomic *in situ* hybridisation (GISH) (Thomas *et al.*, 1994). The potential use of *Lolium*/*Festuca* introgression mapping to develop physical and genetic maps, determine the genetic control of important characters and develop new germplasm for plant breeders has been discussed previously (King *et al.*, 1998). Using this technique, it has been possible to identify a segment of chromosome from *F. arundinacea* introgressed into *Lm* and carrying drought tolerance (Humphreys and Paskinskiene, 1996). It should be possible to identify the region of the *Fp* chromosome carrying genes for other traits, such as disease resistance. Wilkins *et al.* (1974) showed that an amphiploid between *Lm* and *F. arundinacea* exhibited the resistance characteristics of both its parents to the corresponding 'nonhost' strain of *P. coronata*. Oertel and Matzk (1999) obtained introgression of crown rust resistance from both *Fp* and *F. arundinacea* into *Lm*. The segregation ratios in the

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progeny of intercrosses of resistant and susceptible BC₃ plants and selfed R plants suggested that the resistance was controlled by two or more dominant genes which were linked in most cases. A possible advantage of using resistance derived from *Festuca* spp is that it might be more stable at high temperature and therefore more effective in warmer regions (Roderick *et al*, 2000).

In this paper we report on the development of introgressed lines between crown rust-susceptible *Lm* and resistant *Fp* and on the use of chromosome painting to identify the region of chromosome carrying resistance.

Materials and methods

Inoculation procedure and selection of parents

Freshly collected uredospores of an UK isolate of crown rust (*P. coronata*) were collected from infected plants of *L. perenne* and *Lm*. For plant inoculations, the spores were diluted in odourless kerosene (Barretine Ltd, UK) at a concentration of 20 mg/ml and sprayed over the host material using a hand-held atomiser (Humbrol Ltd, UK). The plants were either incubated in a dew simulation chamber at 20°C (Clifford, 1973) or, for more plants, in a 20°C growth chamber fitted with a humidifier which atomised a fine mist of tap water over the plants at intervals of 20 h in the dark. The plants were then transferred to growth chambers at a continuous temperature of 25°C and a 12 h/12 h dark/light period and light intensity of 200 mE m⁻² s⁻². Rust pustules erupted after 9 days on susceptible plants and the expression of resistance was assessed at least twice, after 10 and 14 days. If there was no visible sign of infection (immune) or small chlorotic spots, plants were designated as resistant. Intermediate types had some small pustules along with chlorotic spots, whereas susceptible plants had medium to large pustules. No attempt was made to quantify the amount of infection. Since the F₁ plants had twice as many chromosomes originating from *Lm* as *Fp* (see below), it was considered possible that resistance would not have been fully expressed in the F₁, and therefore no selection was made at this stage. The most resistant plants were selected for further crossing from the BC₁ generation onwards.

Parental material

Five populations of *Fp* from diverse origins were initially screened for resistance to crown rust and two populations contained plants that were resistant. The backcross lines that were eventually selected originated from an ecotype collected in natural grazing pasture at 360 m above sea level in the Moldavia region of Romania (IGER accession Bf 1204), on which there was no apparent host response to infection and was classified as immune. The other, IGER accession Bf 993, was a line bred for a slow senescence character. Susceptible *Lm* parents were obtained from cultivars known to be relatively susceptible to crown rust.

An example of the crossing procedure used to obtain crown rust-resistant BC₃ lines is shown in Figure 1. In this case, triploid F₁ hybrids were produced using a crown rust-susceptible tetraploid genotype of *Lm* (2n = 4x = 28) as the female parent, and a resistant *Fp* as the pollen donor. Tetraploid *Lm* was used as this has been proven to be the most efficient route to obtain

Figure 1 Crossing procedure used to obtain crown rust-resistant introgression line 3/2 between *L. multiflorum* and *F. pratensis*.

introgression (King *et al*, 1998; Thomas *et al*, 1988). For the first backcross generation, the triploid F₁ hybrids (2n = 3x = 21) were used as both the male and female parent, since some were male sterile or had low male fertility. Susceptible *Lm* genotypes of cv. Abercomo (2n = 2x = 14) were selected as the other parent. In the subsequent generations, resistant BC plants were used as the male parent and crossed with a diploid *Lm* genotype.

Cytological assessment and *in situ* hybridisation

Mitotically dividing root cells of selected crown rust-resistant progeny were assessed for their chromosome complement in all generations. Roots were produced from detached vegetative tillers on an aerated culture tank (Morgan, 1976). Excised root tips were kept in ice-cold water either for 16 h (for mitotic cell divisions) or 18 h (for *in situ* hybridisation) and then fixed in ethanol:acetic acid (3:1) for a minimum of 2 h. For chromosome counts, root tips were stained by the Feulgen method and squashed on a microscope slide in 1% acetocarmine. For *in situ* hybridisation, metaphase spreads were treated by the procedure described by Thomas *et al* (1994) and King *et al* (1998). Total genomic *Fp* DNA was used as a probe to detect *Fp* introgressions; this was labelled either with Cy3-dCTP (Amersham Pharmacia Biotech, UK) (red) or dig-11-dUTP (Boehringer Mannheim, UK) and detected by the fluorescein detection procedure (green). The *Fp* DNA was combined with unlabelled *Lolium* DNA in the ratio 1:40. The clone pTa71, which contains the 18S-5.8S-26S rDNA gene cluster, was labelled with dig-11-dUTP and incorporated into the hybridisation mixture to help identify individual chromosomes (Thomas *et al*, 1997); this hybridises to the *Lolium* and *Festuca* rDNA sites. Slides were examined using a Leica DM/RB epifluorescence microscope with filters for DAPI, fluorescein and rhodamine. Images were captured with a Nikon FDX-35 camera on Fujichrome 400 colour slide film or captured directly using a Coolview CCD monochrome camera (Thomas *et al*, 1996). The 35 mm images were subsequently scanned into Adobe Photoshop for digital reproduction.

Results

Both resistant (R) and susceptible (S) triploid F₁ plants were identified. In seven cases, sufficient BC₁ progenies were obtained for crown rust testing. Three of these lines, all originating from resistant F₁ plants, were segregating for resistance (Table 1). In the remaining BC₁ lines, originating from susceptible F₁ plants, only one resistant plant and 13 intermediate types were found.

In two BC₂ lines, obtained by crossing crown rust-resistant plants of BC₁ line 1/3 with a susceptible diploid *Lm* plant, clearly different segregation ratios were recorded. The ratio of R:S plants in BC₂ line 2/1 was significantly different from an expected 1:1 ratio ($P < 0.05$), with susceptible types predominating (Table 1). Susceptible plants also predominated in line 2/2, but the ratio did not differ statistically from 1:1.

The amount of *Fp* introgression was analysed using GISH in a sample of crown rust-resistant plants, which were also selected for an *Lm* growth habit. The chromosomes of two of the BC₂ plants analysed are shown in Figure 2. Plant 3 in line 2/2 (2/2/3) had two introgressed *Fp* segments, one of which was on an *Lm* chromosome with an rDNA site that was identified morphologically as being on chromosome 2 (Thomas, 1981). However, this chromosome is now known to correspond to the Poaceae linkage group/chromosome 3 (Armstead *et al*, 2002; Jones *et al*, 2002) and this numbering has now been adopted. A second, larger *Fp* segment was on a smaller submedian chromosome, where it had replaced the short arm, centromere and part of the long arm (Figure 2a). Plant 44 in line 2/1 (2/1/44) had one *Fp* segment (Figure 2b). As this chromosome

Figure 2 Fluorescent *in situ* hybridisation of BC₂ and BC₃ introgression plants. (a, b) GISH with total genomic *Fp* DNA (Cy3 – red) and pTa71 rDNA (fluorescein – green); (a) BC₂ genotype 2/2/3 part cell with introgressed *Fp* segments on the target chromosome (plain line indicating centromere) and chromosome 3 (plain line indicating centromere and arrow indicating NOR); (b) BC₂ genotype 2/1/44 part cell with introgressed *Fp* segment on the target chromosome; (c) the target chromosome from five BC₃ R genotypes, line 3/2 with introgressions identified by GISH with *Fp* DNA that was labelled with either Cy3 (red) or fluorescein (green); (d) chromosome 3 from BC₃ S genotype, line 3/2 with introgression labelled green (plain line indicating centromere and arrow indicating NOR); (e) the target chromosome from five BC₃ R genotypes, line 3/1 with variable size introgressions with *Fp* DNA labelled with Cy3; (f) the target chromosome from three BC₃ S genotypes, line 3/1 with *Fp* DNA labelled with either fluorescein or Cy3. The plain line in (c, e, f) indicate the centromere position. The bar in (a) represents 10 µm; all images are the same magnification, but chromosome condensation may vary.

Table 1 Segregation ratios for crown rust resistance in BC₁, BC₂, and BC₃ progenies of crosses between *Festuca pratensis* and *Lolium multiflorum*^a

BC line	Rust reaction type			Expected ratio	χ^2 -values
	R	I ^a	S		
BC ₁					
Derived from resistant F ₁					
1/1 (Bf 993/3)	6	1	10	—	—
1/2 (Bf 993/3)	10	1	27	—	—
1/3 (Bf 1204/23)	13	4	22	—	—
Derived from susceptible F ₁					
1/4 (Bf 993/3)	1	1	31	—	—
1/5 (Bf 1204/20)	0	5	39	—	—
1/6 (Bf 1204/5)	0	5	22	—	—
1/7 (Bf 1204/5)	0	2	38	—	—
BC ₂					
2/1	6		47	1:1	31.72***
2/2 ^b	37	2	55	1:1	2.72 NS
BC ₃					
3/1	31		51	1:1	4.88 *
3/2	41		48	1:1	0.55 NS

IGER accession no./genotype no. of *F. pratensis* parent in parenthesis after BC₁ code. R=resistant, I=intermediate and S=susceptible types.

^aIntermediate types grouped with resistant class for analysis.

^bDerived from a cross where the BC₁ parent was used as the female parent.

* and *** denote significance at $P = 0.05$ and 0.001 , respectively. NS = no significant difference.

cannot be identified by its morphology, it will be referred to in this paper as the 'target chromosome'. One of the introgressed segments found in 2/2/3 was on a chromosome resembling the target chromosome in 2/1/44 and for now it is assumed to be the same chromosome.

In the BC₃ generation lines 3/1 and 3/2, which were derived from plants 2/1/44 and 2/2/3, respectively (Table 1), gave a very clearcut segregation of R:S plants, with almost all of the resistant plants showing immunity. Both lines were also derived from the same *Fp* parent and F₁. As with the previous generation, one of the lines (3/1) had significantly more susceptible plants than the expected 1:1 ratio.

Five R and five S plants were analysed by GISH from each line. In 3/2, all the five R plants had the introgressed segment on the target chromosome and, given the resolution of GISH, the segments appeared not to have been reduced by further recombination (Figure 2c). Two of the R plants also had the *Fp* segment on chromosome 3. None of the S plants had the target chromosome introgression, but three of the five plants had the chromosome 3 introgression (Figure 2d). The five R plants in line 3/1 had an introgressed segment on the chromosome, and this varied in size indicating that further recombination had occurred (compare Figure 2b, e). Three of the five S plants also had an *Fp* segment on the

Table 2 Segregation ratios in progenies of pair crosses between crown rust-resistant and -susceptible BC₃ plants of crosses between *Festuca pratensis* and *Lolium multiflorum*^a

Cross	Rust reaction			Expected ratio	χ^2 -values
	R	I ^a	S		
S × S					
3/1/5 × 3/2/25	0	0	59	—	—
3/2/27 × 3/1/5	0	0	66	—	—
R × R					
3/1/30 × 3/2/49	44	0	15	3:1	0.005 NS
3/2/49 × 3/1/3	50	3	21		0.45 NS
R × S					
3/2/18(S) × 3/1/44(R)	72	0	57	1:1	1.74 NS
3/1/25(R) × 3/2/36(S)	62	0	51		1.07 NS

R = resistant, I = intermediate and S = susceptible types.

^aIntermediate types grouped with resistant class for analysis.

NS = no significant difference.

short arm of the target chromosome, again reduced in size from the original (compare Figure 2b, f).

Intercrosses were also made between R and S plants from the two BC₃ lines (Table 2). No R plants were produced in lines from crosses between S plants. The segregation ratios for R × R and R × S lines did not differ from expected ratios, assuming a single gene or very closely linked genes on the *Fp* segment.

Discussion

Crown rust resistance has been transferred from the original *Fp* parent into *Lm*. A sample of resistant BC₃ plants from two crosses all contained an *Fp* chromosome segment, while susceptible plants had either a smaller segment or no segment. The ratios of R:S plants from intercrosses within a sample of BC₃ plants demonstrated that they were segregating for a single dominant gene or a group of closely linked genes. This supports the assumption that the introgression in 3/1 and 3/2 is the same segment and therefore the same chromosome. The resistance gene(s) is probably therefore located on this introgressed *Fp* chromosome segment. In 3/2, the second introgressed segment on chromosome 3 segregated independently of rust resistance with two of the five R plants and three of the five S plants having the segment.

The segment on the target chromosome has undergone further recombination in 2/1/44, as in the progeny (3/1) the segment has been reduced with a range of segment sizes found in both R and S genotypes. The physical position of the crown rust resistance locus can be roughly estimated – the *Fp* segments in the S plants appeared similar in size to the smaller of the segments in the R plants (compare Figure 2e, f). The resistance locus must therefore be close to the recombination point and maps physically to the midpoint of the short arm. The resolution of GISH on such contracted mitotic chromosomes means that the gene can only be located to regions of the chromosome arm, that is, in this case, the midpoint. The same *Fp* segment, though larger, in 2/2/3 appears not to have recombined as the segment is either present in the R genotype or absent in the S genotypes in population 3/2 with no variation in size.

Apart from confirming the presence of the resistance locus in the chromosome arm, it does not provide further information on its position.

Disturbed segregation ratios in introgression lines with two marker loci in crosses between BC₁ heterozygous plants (*Lm/Fp*) and *Lm* were reported by Humphreys and Thorogood (1993). They found that when the BC₁ was used as the male parent, there was a reduced transmission of *Fp*-derived alleles. In our study, there was a higher proportion of crown rust-susceptible plants in one of the BC₂ and BC₃ families, indicating a link between deleterious alleles and crown rust resistance. However, the normal segregation ratios in the BC₃ intercrosses indicate that the linked deleterious genes have been lost.

Wilkins (1975) found that in the *Lm* cultivar RvP, crown rust resistance was under the control of a single dominant gene and additional minor genes. Despite selecting susceptible *Lm* plants for crossing at each generation, a major concern from the outset was the possibility of accumulating minor genes. By selecting the most resistant plants, that is, giving no disease symptoms (immune), it is possible that this was eliminated since it is unlikely that enough minor genes were accumulated in one generation to give this level of resistance. The intercrosses between susceptible BC₃ plants produced only susceptible progeny, an indication that these at least were not carrying any effective genes for resistance.

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