Theor Appl Genet (2000) 101:372–378

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Mapping the *jp* (jumbo pollen) gene and QTLs involved in multinucleate microspore formation in diploid alfalfa

Received: 28 January 1999 / Accepted: 16 December 1999

Abstract The objective of this research was to map the jumbo-pollen trait in diploid alfalfa. Homozygous recessive (*jpjp*) plants are characterized by the complete failure of post-meiotic cytokinesis during microsporogenesis resulting in 100% 4n-pollen formation. Three F_1 segregating populations were produced and analyzed for pollen-grain production and the segregation of RFLP markers. The first cross combination did not segregate for the jumbo-pollen trait, but showed a clear segregation for multinucleate (bi-, tri- and tetra-nucleate)-microspore formation. Cytological analysis showed that few plants produced 100% normal (uninucleate) microspores, whereas most of them produced multinucleate microspores at a variable frequency (0–75%). Plants with multinucleate microspores always showed a prevalence of binucleated microspores, even though some plants showed a background presence of tri- and tetra-nucleate microspores. QTL analysis based on ANOVA I and Stepwise Multiple Regression identified three QTLs with a highly significant effect on multinucleate-microspore formation. Two cross combinations, subsequently executed, showed Mendelian segregation for the jumbo-pollen trait and were effective in locating the *jp* gene on linkage group 6 close to the Vg1G1b RFLP locus. Interestingly, this RFLP locus was also linked to one QTL for multinucleate-microspore formation. Genetic models are discussed concerning the presence in linkage group 6 of a cluster of genes involved in multinucleate-microspore formation together with possible relationships between the *jp* gene and the Vg1G1b QTL.

Keywords Alfalfa · Post-meiotic cytokinesis · RFLP · 4n pollen

Communicated by F. Salamini

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Introduction

Genetic linkage maps can be applied to locate chromosome regions carrying genes of agricultural interest (Paterson et al. 1991). The use of molecular markers such as RFLPs (restriction fragment length polymorphisms), RAPDs (random amplified polymorphic DNAs), SSRs (simple sequence repeats) and AFLPs (amplified fragment length polymorphisms) has greatly facilitated the identification of genetic loci controlling qualitative or quantitative traits (Stuber 1992; Dudley 1993; Vos et al. 1995). In species characterized by a ploidy series, such as alfalfa, sexual polyploidization is the main mechanism of polyploid evolution (Harlan and deWet 1975; Ramsey and Schemske 1998). In addition, 2n gametes could be used in germplasm transfer and to maximize heterosis (Bingham 1980). Therefore, molecular markers can be applied to map reproductive mutations conditioning diploid (2n) and/or polyploid (3n and 4n) gamete formation.

Previous genetic analyses carried out in *Solanum* (Mok and Peloquin 1975; Peloquin 1983; Ortiz and Peloquin 1992), *Medicago sativa* ssp. (McCoy 1982; Tavoletti et al. 1991a) and *Trifolium pratense* L. (Parrott and Smith 1986) showed that different cytological mechanisms of 2n gamete formation are controlled by distinct major genes, but that genetic background and environmental conditions influence the frequency of these gametes produced by single genotypes (reviewed by Bretagnolle and Thompson 1995). However, in diploid alfalfa a single recessive gene mutation producing 100% 4n pollen due to the complete lack of post-meiotic cytokinesis in all microspore mother cells has been identified (McCoy and Smith 1983; Pfeiffer and Bingham 1983).

Phenotypic screening for jumbo-pollen formation is relatively easy, because 4n pollen grains are clearly larger than normal pollen grains (McCoy and Smith 1983; Pfeiffer and Bingham 1983; Veronesi et al. 1990). Therefore, the main objective of the present research was to map the jumbo pollen (*jp*) gene in diploid F1 segregating alfalfa populations.

The use of F_1 populations efficiently avoids segregation distortion (Tavoletti et al. 1996a), which is usually detected when F_2 (Brummer et al. 1993; Kiss et al. 1993) or backcross (Echt et al. 1994) mapping populations are used. However, parental heterozygosity could complicate the genetic analysis because segregating patterns for phenotypic traits are often difficult to forecast in an F_1 population based simply on the phenotypes of parental genotypes. Moreover, it is possible that new traits could segregate in the F_1s because of the high level of heterozygosity of the parents. Therefore, the F_1 itself could give further information about the parental genotypes. Based on the analysis of three F_1 populations, the present research gives mapping information about the *jp* gene location in the alfalfa genome; QTLs for diploid (2n) and polyploid (3n and 4n) male gamete formation have also been identified. Additionally, possible relationships between the *jp* gene and QTLs for partial post-meiotic cytokinesis will be discussed.

Materials and methods

Plant materials

To identify F_1 populations suitable for mapping the *jp* gene, four genotypes (named H20, H25, Wiso6 and W13.2) were chosen (Table 1). In particular, the jumbo-pollen mutant H25 and the normal-pollen producer H20, related to H25, were used. The first cross-combination executed was H25 (100% jumbo pollen, Fig. 1a–d)×Wiso6 (normal pollen, see Fig. 1e). Pollen grains of the parents and of 59 F_1 H25×Wiso6 plants were stained with acetic-carmine and glycerin (1:1) and analyzed using a light microscope. Plants producing 100% jumbo pollen were not observed, whereas segregation for the production of a mixture of normal and large (apparently diploid and polyploid) pollen grains was found (see Fig. 1f–g). Therefore, these plants were subjected to a cytological analysis of microspore formation. To map the *jp* gene, two new cross combinations were produced: H25 (100% jumbo pollen)×W13.2 (normal pollen) and H20 (normal pollen)×W13.2 (normal pollen).

Table 1 Pollen-grain production and pedigrees of the four genotypes used to generate the F_1 mapping populations

H25	Jumbo-pollen producer, selected from the HY6×CADL population (Veronesi et al. 1990)
H20	Normal-pollen producer, selected from the HY6×CADL population (Veronesi et al. 1990)
Wiso6	Normal-pollen producer, selected from the W2x-iso1 population (Bingham 1991)
W13.2	Normal-pollen producer, selected from the PG-F9×Wiso1 population (Tavoletti et al. 1996)

H25×Wiso6: QTL mapping for multinucleate microspore formation

Young buds from each F_1 plant were collected in the summer of 1998 and fixed in FAA (formalin: acetic acid: 70% ethanol, 5:5:90). A stain-clearing technique (Stelly et al. 1984; Tavoletti et al. 1991b) was applied to evaluate microspore formation by observations within intact anthers and a sample of about 1000–3000 microspores per plant was analyzed. Microspores were classified into four types (uni-, bi-, tri- and tetra-nucleate) and the relative frequencies of normal (uni-nucleate) and multinucleate (bi-, tri- and tetra-nucleate) microspores were calculated for each F_1 plant.

DNA extraction, restriction digestion, gel electrophoresis and Southern blotting were carried out following Kidwell and Osborn (1992, 1993), and three restriction enzymes (*Eco*RI, *Hind*III and *Eco*RV) were utilized to identify suitable polymorphisms. Probes detecting RFLP loci distributed across the eight alfalfa linkage groups (Echt et al. 1994, Tavoletti et al. 1996) were chosen, and chemiluminescent detection of RFLP markers was carried out (Gene Images kit, Amersham Biotech). Different RFLP loci detected by a single DNA probe were distinguished by a lower-case letter added after the probe code.

Segregation distortion (departure from a 1:1 segregation ratio) was tested by χ^2. A double pseudo-testcross strategy (Grattapaglia and Sederoff 1994) was applied to study the linkage relationships among RFLP loci. Marker loci segregating for each of the two parents were separately analyzed using MAPMAKER/EXP 3.0 (Lander et al. 1987, Lincoln et al. 1992). The F_2 BACKCROSS option was applied using a maximum recombination frequency of r=0.25, a minimum LOD=3 and the Kosambi mapping function. Linkage groups with no more than five marker loci were ordered using the command COMPARE. For larger groups the TRY and RIPPLE commands were employed. The linkage groups of H25 and Wiso6 were aligned using RFLP loci detected by codominant markers and segregating for both parents, or using previously published alfalfa linkage maps as a reference (Echt et al. 1994, Tavoletti et al. 1996). ANOVA I was carried out to identify putative marker loci with a significant (P<0.01) effect on multinucleate microspore formation. Subsequently, a stepwise multiple regression (forward selection procedure), based on the simultaneous analysis of all RFLP loci segregating in the F_1 H25×Wiso6 population, was applied using P<0.05 as the probability for a variable to enter or exit from the model. The final model, including significant first-order interactions, was subsequently evaluated by a factorial ANOVA. The statistical analysis was conducted using the arcsin-transformed "percentage of multinucleate microspores" as the dependent variable.

Mapping the *jp* gene: analysis of H25×W13.2 and H20×W13.2 F_1 populations

Plants were classified as normal- or jumbo-pollen producers by staining pollen grains of the parents and each F_1 plant with acetic-carmine and glycerin (1:1). A χ^2 test for goodness of fit with 1:1 and 3:1 segregation ratios (normal pollen vs jumbo pollen) was carried out for each segregating population. RFLP segregation and linkage analyses were carried out as described for the H25×Wiso6 population, but only a subset of the DNA probes was utilized. The *jp* gene was mapped using the Mapmaker F_2 BACKCROSS or F_2 INTERCROSS options, when a 1:1 or a 3:1 phenotypic segregation ratio respectively was detected.

Results

H25×Wiso6 cross combination

The large sample of microspores analyzed per plant allowed a precise phenotypic evaluation to be carried out.

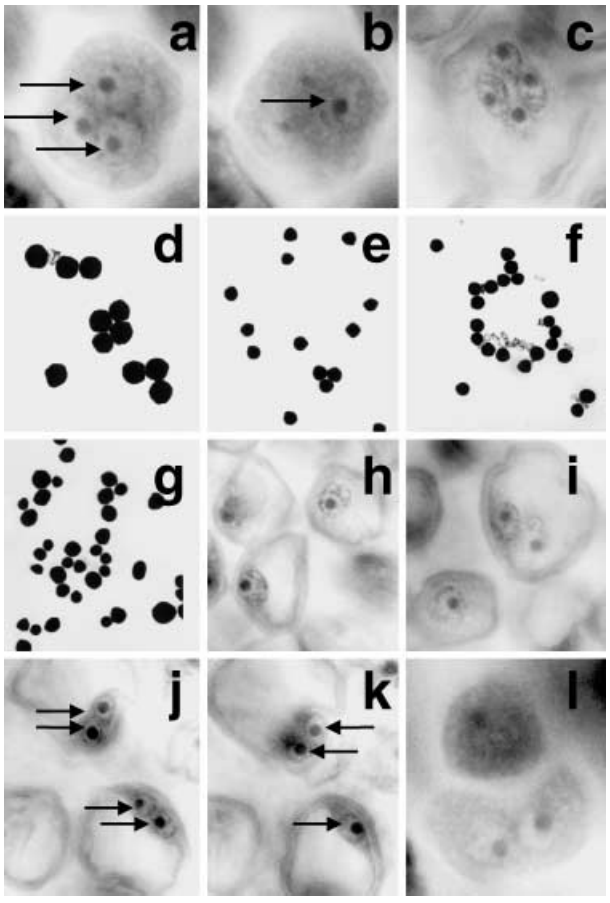

Fig. 1a, b H25 tetranucleate monad (two planes of focus). **c** H25 tetranucleate microspore. **d** H25 jumbo pollen grains. **e** Wiso6 normal pollen grains. **f–l** H25×Wiso6 F_1 plants. **f–g** Plants with a low and high frequency of large pollen grains, respectively. **h** Plant with 100% normal uninucleate microspores. **i** Plant with uni- and bi-nucleate microspores. **j–k** Tri- and tetra-nucleate microspores within the same anther (two planes of focus). **l** Partial post-meiotic cytokinesis

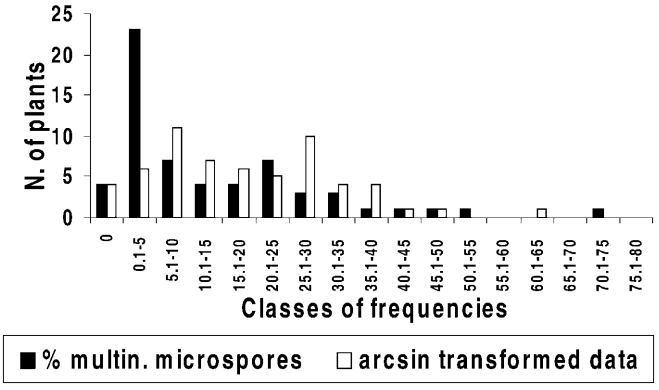

Fig. 2 Frequency distributions of original and arcsin-transformed data

Table 2 ANOVA table of the final multiple-regression model

Sources of variation	*df*	Sum of squares	*F* ratio	*Pr>F*
Vg1A1 (Wiso6)	1	1104	12.66	0.0008
Vg1G1b (Wiso6)	1	1259	14.45	0.0004
Uwg119 (H25)	1	1246	14.30	0.0004
Vg1G1b×Uwg119	1	664	7.62	0.0079
Error	54	4707		
Lack of Fit	3	147	0.55	0.65
Pure error	51	4560		

Fifty five F_1 plants were characterized by a percentage of multinucleate microspores ranging from 0.16% to 75%, and only four plants produced 100% normal microspores (Figs 1h and 2). Plants with multinucleate microspores showed a clear prevalence of binucleate types (Fig. 1i), and 31 of them also produced tri- or tetra-nucleate microspores at a low frequency (between 0.03 and 5%, together with only one plant showing 19%) (Fig. 1j, k). The presence of tri- and tetra-nucleate microspores indicated that abnormal post-meiotic cytokinesis should be effective in these plants (Clement and Stanford 1961, Ramanna 1974, Tavoletti et al. 1991b). The occurrence of binucleated microspores, due to partial post-meiotic cytokinesis following a normal tetraedral arrangement of the four nuclei, was cytologically confirmed in the F_1 H25×Wiso6 population (Fig. 1l).

Twenty four DNA probes (11 Uwg, 12 Vg and 1 Hg DNA clones) detected 18 and 19 RFLP loci segregating for H25 and Wiso6, respectively, which were assigned to linkage groups following Echt et al. (1994) for UWg and Hg RFLP loci and Tavoletti et al. (1996) for Vg RFLP loci (Fig. 3). Segregation distortion was not detected and three marker loci, segregating for H25, and one small group for Wiso6, were classified as unlinked following our previous map (Tavoletti et al. 1996). No segregating loci were available for linkage group 4 of Wiso6 and linkage group 7 of H25. Even though the number of marker loci used in this study was limited, their distribution suggested a good genome coverage.

ANOVA I showed that all group-6 marker loci segregating for Wiso6 and two group-1 marker loci segregating for H25 had significant effects on multinucleate microspore formation (R^2 values and significance levels are shown in Fig. 3). The results of stepwise multiple regression were in agreement with those of ANOVA I. Three RFLP loci, two segregating for Wiso6 (Vg1A1 and Vg1G1b linked in group 6) and one segregating for H25 (UWg119 located in group 1), together with the Vg1G1b×Uwg119 interaction, showed highly significant effects on multinucleate microspore formation (Table 2). Even though the population size was small (59 plants), the Vg1G1b×Uwg119 interaction was included in the model because it clearly improved the lack of fit of the model. Moreover, a multilocus-model R^2 (54%) indicated a good fit given that the Max R^2 value was 56%. Therefore, two QTLs with a significant effect on multinucleate microspore formation were identified in linkage group 6, and another QTL was present on linkage group 1. Our results also suggest that the presence of epistatic interactions between QTLs located on different linkage

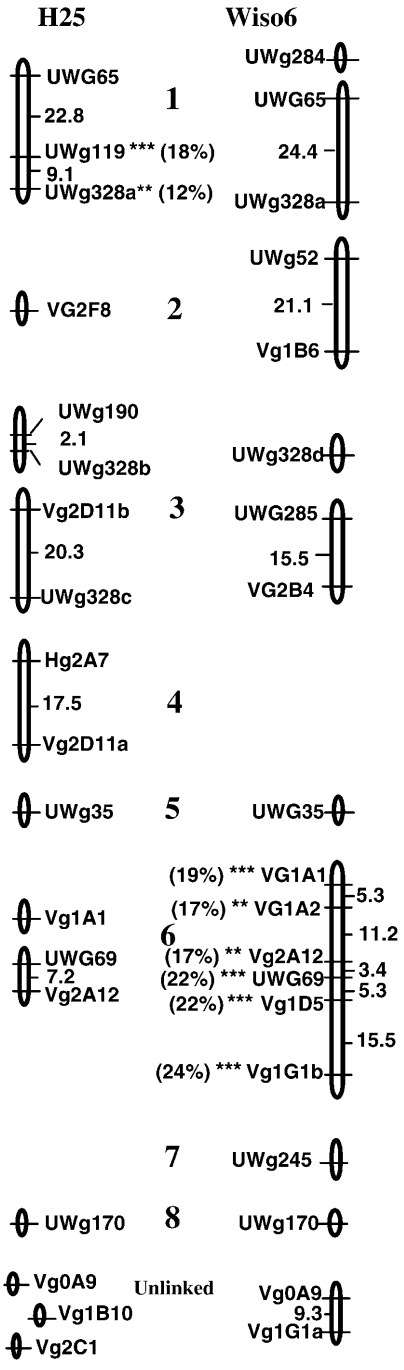

Fig. 3 RFLP linkage maps of H25 and Wiso6 showing ANOVA I results (***=significant at $P<0.001$; **=significant at $P<0.01$; R^2 values are shown in parenthesis)

groups should be verified by using a larger segregating population.

H25×W13.2 cross combination

Analysis of pollen grains, carried out on 85 H25×W13.2 plants, showed a Mendelian 1:1 phenotypic segregation ratio (Fig. 4, $\chi^2_{1\ df}=0.10$, $0.7<P<0.9$). Assuming that H25 is homozygous recessive *jpjp*, this segregation ratio suggested that W13.2 was heterozygous *Jpjp*. To map the *jp* gene, 53 randomly chosen F_1 plants were analyzed for the segregation of RFLP markers, and loci on groups 1 and 6 were the first to be screened. The *jp* gene mapped in the W13.2 linkage group-6 and cosegregated with the Vg1G1b locus (Fig. 4), which previously showed a significant effect on multinucleate microspore formation. The Vg1G1b RFLP locus segregating for W13.2 was detected by a dominant marker and normal phenotypes were associated with the presence of the segregating RFLP band, indicating that the genotype of H25 was *jpjp* and that of W13.2 was *Jpjp*.

H20×W13.2 cross combination

Pollen production was tested on 112 plants and a 3:1 phenotypic segregation ratio was detected (Fig. 5a, $\chi^2_{1\ df}=0.15, 0.5<P<0.7$) suggesting that both parents were heterozygous *Jpjp*. Segregation for RFLP markers detected by the Vg1G1 probe was tested using 52 randomly chosen plants of the H20×W13.2 F_1 population. Each parent showed two RFLP dominant markers segregating in the F_1 population (Fig. 5c). For W13.2, *Eco*RV gave a better banding pattern than *Eco*RI (Fig. 5c) to follow the segregation of the Vg1G1b marker linked to the *jp* gene. To identify Vg1G1b between the two RFLP markers segregating for H20, a model based on independent segregation between a dominant marker, segregating for H20, and the jumbo-pollen trait segregating 3:1 was developed and tested by χ^2 (Fig. 5b). For each Vg1G1 RFLP marker, the overall χ^2 with 3 *df* was subdivided into three χ^2 each with 1 *df* in order to test segregation for the jumbo-pollen trait, segregation for the RFLP marker, and independent assortment between the RFLP locus and the *jp* gene. Based on these results, the RFLP marker corresponding to the Vg1G1b locus was identified (Fig. 5b, c). The jumbo-pollen phenotype was associated with the absence of the Vg1G1b RFLP band for both parents. Therefore, the linkage phase between Vg1G1b and the *jp* gene was determined and an estimated two-point distance of 2.4 cM between Vg1G1b and the *jp* gene was obtained by using F_2 INTERCROSS Mapmaker option and the Kosambi mapping function (Fig. 5c).

Discussion

Mapping the *jp* gene

Two F_1 mapping populations (H25×W13.2 and H20×W13.2) independently indicated that the *jp* gene maps in alfalfa linkage group 6 close to the Vg1G1b RFLP locus. Our results also identified well-defined diploid genotypes, such as H25, H20 and W13.2, which could now be efficiently used to generate large segregating populations in order to obtain better estimates of the recombination frequency between Vg1G1b and the *jp*

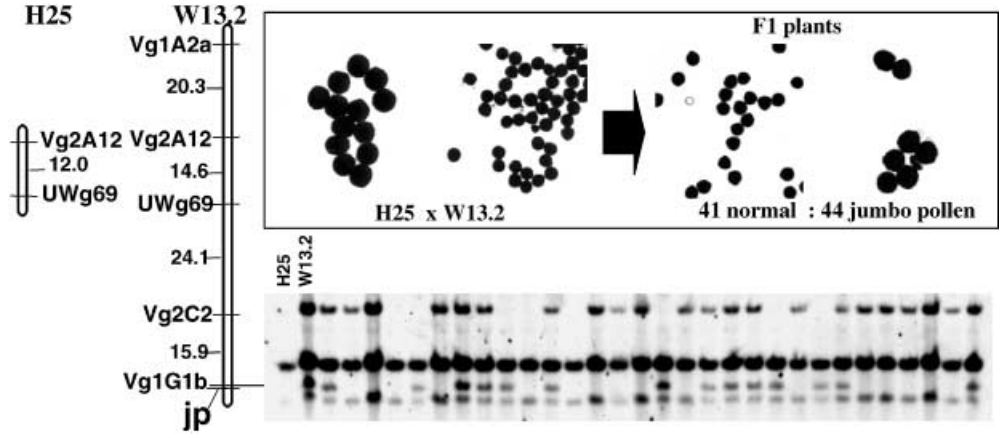

Fig. 4 Mendelian segregation (1:1) of the jumbo-pollen trait in the H25×W13.2 F_1 population, the map location of the *jp* gene and segregation of the W13.2 RFLP marker corresponding to the Vg1G1b locus

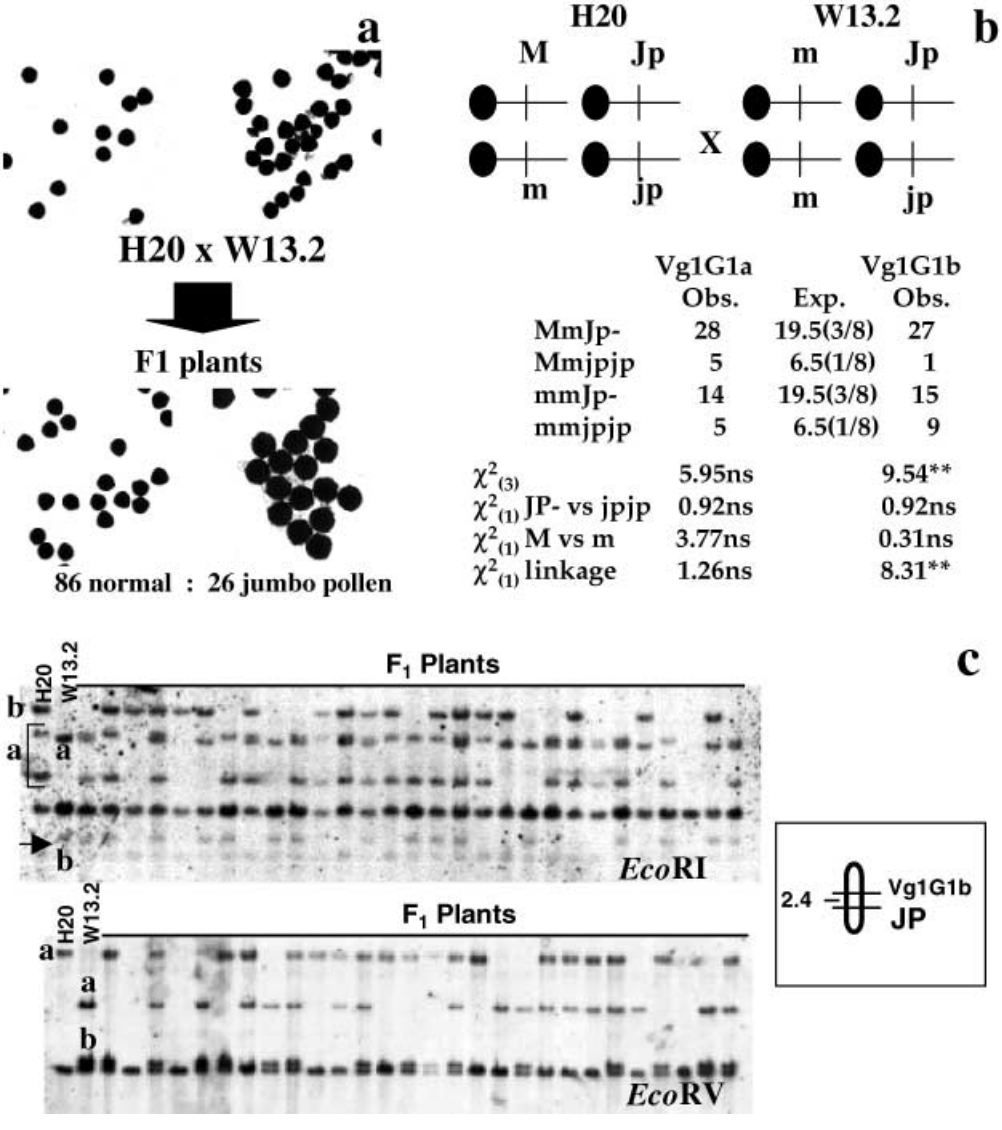

Fig. 5 **a** Mendelian segregation (3:1) of the jumbo-pollen trait in the H20×W13.2 F_1 population. **b** Genetic model and χ^2 analysis to identify the Vg1G1b locus segregating for H20. **c** RFLP markers corresponding to the Vg1G1a and b loci (*Eco*RI and *Eco*RV restriction digests) segregating for both parents and the two-point distance between Vg1G1b and the *jp* gene

gene. They could also be good testers to screen for the presence of *jp* mutant alleles within diploid alfalfa populations. Since the *jp* mutant allele was always linked in coupling with the absence of the RFLP band, a saturated linkage map for this chromosome region is under development, by using both RFLP and AFLP molecular markers, in order to verify if the jumbo-pollen trait is due to a deletion. Eventually, cloning of the *jp* gene will be attempted because this gene seems to be specifically expressed during post-meiotic cytokinesis and could there-

fore be the source of a promoter specific for this stage of microsporogenesis.

QTLs for multinucleate microspore formation

The large map distance between Vg1A1 and Vg1G1b, the significance levels concerning all Wiso6 marker loci, and the average multinucleate microspore frequencies of recombinant haplotypes (data not shown) suggest the presence of two QTLs instead of one in group 6. These QTLs were detected by RFLP markers segregating from the normal parent, indicating that Wiso6 is heterozygous for alternative alleles with significantly different effects on multinucleate microspore formation. Conner et al. (1998) discussed in detail the limitations of QTL mapping based on the double pseudo-testcross approach, which does not allow genetic models based on additive and dominant QTL effects to be developed. However, in our experiment, Wiso6 produced only normal pollen and so alleles promoting multinucleate microspore formation at the Vg1G1b and Vg1A1 loci should be recessive. Therefore, it could be hypothesized that Wiso6 was heterozygous and H25 was homozygous recessive at these QTL loci. However, as previously described, H25 is also homozygous recessive at the *jp* locus. This finding suggests that the *jp* gene has an epistatic effect (recessive epistasis) on genes for multinucleate microspore formation because *jpjp* plants always produce 100% jumbo pollen independently from their genotypes at QTLs for partial cytokinesis. This hypothesis also suggests that, in a biochemical pathway which eventually leads to post-meiotic cytokinesis, the *jp* mutation occurs upstream of mutations involved in partial post-meiotic cytokinesis. A third QTL was found on group 1 by the Uwg119 RFLP marker segregating for H25, the jumbo-pollen parent. Therefore, for this QTL, H25 appeared to be heterozygous and Wiso6 was homozygous. In this case, the H25 allele which increases the frequency of multinucleate micospores should be dominant or codominant to the wild-type allele, and the effect of the H25 genotype at this locus seems to be masked by homozygosity for the *jp* allele.

Since one QTL for multinucleate microspore formation was located close to Vg1G1b, the same RFLP locus linked to the *jp* gene, the presence of a multiple allelic series at the *jp* locus, instead of a separate QTL, could also be hypothesized: Wiso6 could be heteroygous $Jpjp_w$, where jp_w, a "weak" allele determining partial post-meiotic cytokinesis, is recessive to the wild-type *Jp* allele but dominant to the *jp* allele carried by H25. Under this hypothesis, the F_1 H25(*jpjp*)×Wiso6($Jpjp_w$) should include 50% *Jpjp* and 50% $jp_w\,jp$ plants. As shown in Fig. 2, 27 plants produced a very low frequency (<5%) of multinucleate microspores, and the 27:32 segregation ratio fits with the expected 1:1 (*Jpjp*: jp_wjp) ratio. The low frequency of multinucleate microspores of *Jpjp* plants could be due to the effects of QTLs linked to Vg1A1 and UWg119. However, it is interesting to note that single recessive gene mutations determining the complete lack of post-meiotic cytokinesis have also been isolated and studied in soybean (Brim and Young 1971) and more recently in *Arabidopsis thaliana* (Hulskmp et al. 1997; Spielman et al. 1997). Both in soybean (Palmer et al. 1978) and *Arabidopsis*, independent mutations at this gene always resulted in 100% tetranucleate microspore production. These results suggest that the *jp* and partial-cytokinesis mutations in alfalfa involve different genes. However, further research will be necessary to fully discriminate between linkage or a multiple allelic series at the *jp* locus in alfalfa.

Acknowledgements The authors thank Prof. E.T. Bingham and T.C. Osborn, Department of Agronomy, University of Wis., Madison, WI, USA, and Prof. Wayne Parrott, University of Georgia, Athens, USA, for critical reading of the manuscript and suggestions. This research was funded by the Italian "Ministero per l'Università e la Ricerca Scientifica", Project "Characterization of mutations concerning sporogenesis and gametogenesis in *Medicago* spp.."

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