# Transfer of Disease Resistance from Diploid to Tetraploid Alfalfa by Unreduced Female Gametes

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**ABSTRACT**

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Diploid (2n = 2x = 16) *Medicago* spp. have been described as sources of resistance to diseases of cultivated tetraploid (2n = 4x = 32) alfalfa (*M. sativa*). The objective of this research was to transfer sexually to the 4x level germ plasm selected and bred at the 2x level for resistance to Phytophthora root rot, caused by *Phytophthora megasperma* f. sp. *medicaginis*. Direct 2x-4x crosses using a susceptible 4x male parent transferred resistance to *P. m.* f. sp. *medicaginis* in 2x *M. falcata* to the 4x level. One *M. falcata* plant, MfR-28, produced unreduced (2n) female gametes at a high frequency (13 4x plants per 100 flowers pollinated) and allowed for efficient sexual transfer of 2x *M. falcata* germ plasm to the 4x level. Direct 2x-4x crosses were also successful in achieving tetraploidization of 2x plants resistant to *P. m.* f. sp. *medicaginis* and bred by crossing resistant *M. falcata* plants with resistant diploids derived from the 4x plant M193 by interploid bridge crosses. Two derived diploid by *M. falcata* hybrids produced 2n female gametes at a frequency equal to or greater than that observed for MfR-28, thus facilitating transfer of resistance to *P. m.* f. sp. *medicaginis* from 2x to 4x alfalfa. Our results demonstrate that direct interploid crosses can efficiently transfer disease resistance from the 2x to 4x level and that genes conditioning resistance to *P. m.* f. sp. *medicaginis* selected at the 2x level are expressed in 4x alfalfa. Interploid crosses also allow for combination of different disease resistances during tetraploidization.

The great genetic variability that exists in diploid (2n = 2x = 16) *Medicago* species has not been extensively used in breeding cultivated tetraploid (2n = 4x = 32) alfalfa, *M. sativa* L. (1). Diploid *Medicago* species have been described as sources of resistance to diseases of 4x alfalfa (6,8,11,18). Germ plasm selected at the 2x level must be tetraploidized for the improvement of cultivated 4x alfalfa. Colchicine has been used to double the chromosome number of alfalfa (5,16). Problems with the use of colchicine include the time-consuming process of treatment, chimeras, identification of tetraploid shoots, and the low fertility often observed in the tetraploidized plant. In contrast, sexual tetraploidization takes advantage of unreduced (2n) gametes, which occur at low frequencies in alfalfa populations (17,20). Hybrid 4x progeny can be quickly identified by genetic markers and/or pollen fertility with known 4x plants (2,15).

We have transferred resistance to Phytophthora root rot, caused by *Phytophthora megasperma* Drechs. f. sp. *medicaginis* Kuan & Erwin, from the 4x alfalfa plant M193 to the 2x level by interploid bridge crosses (12). The relationships between genes conditioning resistance to *P. m.* f. sp. *medicaginis* from the derived diploids of the 4x plant M193 and 2x *M. falcata* L. were studied at the 2x level, taking advantage of disomic inheritance ratios. Diploid plants with independently inherited genes conditioning resistance to *P. m.* f. sp. *medicaginis* from 2x and 4x alfalfa were identified (12). The technique of breeding cultivated 4x alfalfa at the 2x level represents analytic breeding of an autopolyploid, as proposed by Chase (7), and is an efficient method to combine and determine the relationships between resistance genes from 2x and 4x alfalfa (12). Havey et al (12) have discussed the advantages and disadvantages of breeding 4x alfalfa at the 2x level. The final step of analytic breeding of alfalfa for resistance to *P. m.* f. sp. *medicaginis* is the transfer of 2x resistant germ plasm to the 4x level. The objective of this study was to determine if 2x-4x crosses can efficiently transfer to the 4x level germ plasm selected and bred at the 2x level for resistance to *P. m.* f. sp. *medicaginis* and to assess the usefulness of derived diploids as female parents in 2x-4x crosses in order to increase the chances of successful sexual tetraploidization of 2x alfalfa germ plasm.

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## MATERIALS AND METHODS

The 2x *M. falcata* plants MfR-17, MfR-22, MfR-25, MfR-28, and MfR-36, all resistant to *P. m.* f. sp. *medicaginis* (11), were crossed as the female parent, without emasculation, with the 4x susceptible *M. sativa* plant M269 (14). Progeny were grown in the greenhouse, and those with variegated flower color were crossed as the male parent with the 4x self-incompatible plant M194 (14) or the 4x male-sterile plant 6-4. Chromosomes in root tips were counted, as previously described (12), to confirm tetraploidy in five plants that demonstrated male fertility in crosses with the known 4x plants. Each plant was cloned by stem cuttings, and resistance to *P. m.* f. sp. *medicaginis* was assessed using the mature root assay (13).

The *P. m.* f. sp. *medicaginis*-resistant 2x plants $F_1$R-1170, $F_1$R-1171, $F_1$R-1175, $F_1$R-1176, $F_1$R-1179, $F_1$R-1194, $F_1$R-1195, $F_1$R-1196, $F_1$R-1204, $F_1$R-1205, $F_1$R-1208, and $F_1$R-1216 were generated by crossing *P. m.* f. sp. *medicaginis*-resistant derived diploid DDR-850 or DDR-1051 as the female parent with *P. m.* f. sp. *medicaginis*-resistant *M. falcata* plants (12). Each 2x derived diploid by *M. falcata* hybrid was crossed in the greenhouse as the female parent, without emasculation, with the 4x plant Arc 9. Arc 9 was resistant (9) and the 2x plants were susceptible to anthracnose, caused by *Colletotrichum trifolii* Bain. Hybrid progeny were identified by resistance to *P. m.* f. sp. *medicaginis* and *C. trifolii*. Resistance to *P. m.* f. sp. *medicaginis* was evaluated using the mature root assay (13). Resistance to *C. trifolii* was assessed using a detached stem assay (10). Stem cuttings were taken from the regrowth of recently cut plants, dipped in rooting hormone, placed in unsterilized silica sand, covered with plastic, and allowed to grow in a growth chamber (24 C, 12-hr days at approximately 40 $\mu E/m^2/sec$) for 10–14 days. A single droplet of a suspension of race 1 *C. trifolii* conidia ($10^6$/ml) was placed in the stem with a 25-gauge sterile hypodermic needle on a 1-ml plastic syringe. Control plants were inoculated with sterile water. After inoculation, the plants were returned to the growth chamber, and 10–14 days later a susceptible phenotype showed a water-soaked rotting of the stem. A resistant phenotype had only a necrotic fleck where the needle had been inserted. Tetraploid plants resistant to *P. m.* f. sp. *medicaginis* and anthracnose were identified by their fertility as a male parent in crosses with M194 or 6-4. Chromosomes in root-tip cells of seven plants that demonstrated fertility in the testcrosses were counted to confirm tetraploidy.

## RESULTS AND DISCUSSION

Resistance to *P. m.* f. sp. *medicaginis*

**Table 1.** Yield of tetraploid plants from five diploid *Medicago falcata* plants crossed as females with the tetraploid *M. sativa* plant M269

| Diploid *M. falcata* parent | Number of flowers pollinated | Number of tetraploid progeny[a] | Yield of tetraploid progeny per 100 flowers pollinated |
|---|---|---|---|
| MfR-17 | 152 | 0 | 0.0 |
| MfR-22 | 250 | 0 | 0.0 |
| MfR-25 | 420 | 3 | 0.7 |
| MfR-28 | 492 | 64 | 13.0 |
| MfR-36 | 220 | 1 | 0.5 |

[a] Progeny had variegated flower color and set seed as male parents in crosses with known tetraploid plants. Chromosome counts in five plants confirmed tetraploidy. Fifty-three of the 68 progeny were resistant to *Phytophthora megasperma* f. sp. *medicaginis.*

**Table 2.** Yield of tetraploid plants resistant to *Phytophthora megasperma* f. sp. *medicaginis* and *Colletotrichum trifolii* from crosses of derived diploid by *Medicago falcata* hybrids with Arc 9

| Diploid parent | Number of flowers pollinated | Number of resistant 4x plants[a] | Yield of resistant 4x plants per 100 flowers pollinated |
|---|---|---|---|
| $F_1$R-1170 | 127 | 17 | 13.4 |
| $F_1$R-1171 | 120 | 6 | 5.0 |
| $F_1$R-1175 | 72 | 7 | 9.7 |
| $F_1$R-1176 | 198 | 3 | 1.5 |
| $F_1$R-1179 | 207 | 1 | 0.5 |
| $F_1$R-1194 | 145 | 2 | 1.4 |
| $F_1$R-1195 | 183 | 3 | 1.6 |
| $F_1$R-1196 | 62 | 15 | 24.2 |
| $F_1$R-1204 | 146 | 2 | 1.4 |
| $F_1$R-1205 | 126 | 1 | 0.8 |
| $F_1$R-1208 | 100 | 2 | 2.0 |
| $F_1$R-1216 | 155 | 1 | 0.6 |
| Total | 1,641 | 60 | 3.7 |

[a] Ten plants were testcrossed as male parent to known 4x plants and all set seed. Tetraploidy was confirmed by chromosome counts in seven plants.

was successfully transferred to the 4x level by 2x-4x crosses for three of the five *M. falcata* plants; 68 progeny were generated with variegated flower color and pollen fertility in crosses with the 4x testers (Table 1). Fifty-three of the 68 progeny were resistant to *P. m.* f. sp. *medicaginis*. MfR-28 produced 2n female gametes at a greater frequency than observed for the other *M. falcata* plants (Table 1). This was a fortunate discovery because MfR-28 made possible the efficient transfer of resistance to *P. m.* f. sp. *medicaginis* from 2x *M. falcata* to the 4x level by 2x-4x crosses.

Crosses of 2x derived diploid by *M. falcata* hybrids with 4x Arc 9 generated 60 progeny resistant to *P. m.* f. sp. *medicaginis* and *C. trifolii* (Table 2). Ten of the doubly resistant plants were crossed with M194 and all demonstrated fertility. Chromosome counts in root-tip cells of seven of the plants confirmed tetraploidy.

Although large differences exist between individual plants, the frequency of 4x progeny from all 2x-4x crosses using the derived diploids as seed parents (3.7% in Table 2) was greater than that previously reported (2,20). Veronesi et al (20) observed a slightly lower frequency of 4x progeny (3.2%) from 2x-4x crosses using CADL, which is a population of derived diploids selected for fertility and maintained at the 2x level (4). The frequency of 4x progeny observed in our 2x derived diploid by 4x crosses (Table 2) is a minimum because 4x progeny susceptible to *P. m.* f. sp. *medicaginis* and/or *C. trifolii* were discarded. Derived diploids $F_1$R-1170 and $F_1$R-1196 (Table 2) produced 2n female gametes at a frequency equal to or greater than MfR-28 (Table 1). Crosses of each of the 12 derived diploids with Arc 9 generated at least one 4x progeny (Table 2). The chances of successful sexual transfer of 2x alfalfa germ plasm to the 4x level may be increased by using derived diploids as seed parents in 2x-4x crosses, due to higher frequencies of 2n gametes in diploids derived from tetraploids than in naturally occurring diploids (Tables 1 and 2) (3,19). Sexual tetraploidization also allows one to combine simply inherited traits, such as resistances to *P. m.* f. sp. *medicaginis* and *C. trifolii*, during the tetraploidization step.

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