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ORIGINAL PAPER

Transfer of anthracnose resistance and pod coiling traits from *Medicago arborea* to *M. sativa* by sexual reproduction

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Abstract Five asymmetric hybrid plants were obtained between *Medicago sativa* ($2n = 4x = 32$) and *Medicago arborea* ($2n = 4x = 32$) through sexual reproduction and the use of a cytoplasmically male sterile *M. sativa* genotype. Over 2,000 pollinations were made to obtain these hybrids. Amplified fragment length polymorphism (AFLP) analysis showed that in the most studied hybrid (WA2273), 4% of the bands unique to the *M. arborea* parent were present, versus 72% for the unique *M. sativa* bands. This suggests that only a single *M. arborea* chromosome or chromosome parts has been transferred. WA2273 had 7% of AFLP bands which were not present in either parent, which is suggestive of chromosome rearrangements as would be expected if only chromosome parts or a single part had been transferred from *M. arborea*. Phenotypic evidence for hybridity was obtained for pod coiling (1.4 coils in WA2273 versus three coils in the *M. sativa* parent and its self and testcross populations, and one coil in *M. arborea*), and *Colletotrichum trifolii* race 2 resistance (transferred from the resistant *M. arborea* parent, as the *M. sativa* parent and the self populations were highly susceptible). The hybrids were self sterile, but were female fertile to a high level when crossed with 4*x*, but not 2*x*, *M. sativa*, indicating they were at or near 4*x*. Both the pod coiling trait and anthracnose resistance segregated in the progeny of testcrosses between WA2273 and *M. sativa*. The work demonstrates that agronomically useful traits can be introgressed into *M. sativa* from *M. arborea* by use of male sterile *M. sativa* and sexual reproduction.

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Introduction

Lucerne or alfalfa (*Medicago sativa*) is the worlds oldest known cultivated forage species, with historical records dating to 1300 BC in Turkey (Hendry 1923). World lucerne area was estimated at 32 m ha, of which 70% was located in the USA, USSR and Argentina collectively (Michaud et al. 1988). To increase the utilisation of lucerne worldwide, there is a need to introgress additional traits, some of those being drought and salinity tolerance, and increased perenniality, all of which are known to exist in *Medicago arborea* (Nenz et al. 1996).

Lucerne is part of the *M. sativa* complex that belongs to the section Falcago, subsection Falcata, which includes 4*x* and 2*x* forms of *M. sativa* ssp. *sativa* (2*x* forms of *M. sativa* are also known as *M. coerulea*), *M. sativa* ssp. *falcata*, and *M. sativa* ssp. *glutinosa* (Lesins and Gillies 1972) among others. The main barrier to gene exchange between representatives of the complex is ploidy, with $2n = 2x = 16$ and $2n = 4x = 32$ being the diploid and autotetraploid karyotypes, respectively. There is considerable genetic diversity within the *M. sativa* complex, with Kidwell et al. (1994) and Musial et al. (2002) demonstrating that *M. sativa* ssp. *falcata*, based on genomic DNA fragment analysis, represents a wide outgroup to *M. sativa* subsp. *sativa*. *M. sativa* ssp. *falcata* has been widely exploited in lucerne breeding in North America for several decades, and introgressing *falcata* into *sativa* through breeding, while increasing winter hardiness of lucerne, results in decreased yields in subtropical

environments due to its high level of winter dormancy (Mackie et al. 2005). For these reasons, transfer of useful traits from *M. arborea*, such as absence of winter dormancy, appears to have merit for breeding of lucernes for warm temperate and subtropical climates.

Another perennial *Medicago* sp. which may have potential to contribute new genes to the *M. sativa* complex is *M. arborea*. *M. arborea* is a long lived perennial *Medicago* sp., with the most widespread form being $2n = 4x = 32$ (Small and Jomphe 1988). *M. arborea*, and its very close relatives, *M. citrina* and *M. strasseri*, belong to Section Dendrotelis of the genus *Medicago*, which are perennial outbreeding shrubs growing to 4 m tall, and fruits with 0.5–1.5 coils (Small and Jomphe 1988). *M. arborea* is indigenous to the Mediterranean islands and sea coast, has orange-yellow flowers, and is widely used in those regions as a forage (Small and Jomphe 1988). Lefi et al. (2004a, b) have researched water use efficiency in *M. arborea* and *M. citrina*, with both species having a reputation for drought tolerance. Other useful traits of *M. arborea* include absence of winter and summer dormancy (Nenz et al. 1996) and salinity tolerance, all of which have the capacity to increase the adaptation, perenniality and productivity of lucerne. Some accessions of *M. arborea* are also resistant to races 1 and 2 of *Colletotrichum trifolii* (Elgin and Ostazeski 1982), a major disease of lucerne in Australia (Irwin et al. 2001) and the USA (Elgin and Ostazeski 1982). Only three major genes for resistance to *C. trifolii* have been identified in the *M. sativa* complex (Elgin and Ostazeski 1985; Mackie et al. 2007), so introgression of resistance from *M. arborea* to the *M. sativa* complex could be potentially useful in managing anthracnose.

Nenz et al. (1996) reported the generation of somatic hybrids between *M. sativa* and *M. arborea* through symmetrical electrofusion of mesophyll protoplasts of *M. sativa* with callus protoplasts of *M. arborea*. The regenerated somatic hybrids showed considerable genome rearrangement on the basis of restriction fragment length polymorphism (RFLP) and isozyme analysis, but the morphology of the hybrids was generally intermediate between the parents. Flowering was not observed in the hybrids. *M. arborea* does not usually flower until 2 years after sowing, in contrast to *M. sativa* which always flowers in its first year. Bingham (2005) has reported making hybrids between *M. sativa* and *M. arborea* using male sterile lucerne. Glasshouse and field observations on morphological traits unique to both parents were reported. Other previously reported interspecific hybrids involving *M. sativa* are those with *M. rugosa* ($2n = 30$) and *M. scutellata* ($2n = 30$), both of which are annual allopolyploid species (Bena et al. 1998; Mizukami et al. 2006). These hybrids were generated by protoplast electro-fusion, and while the original somatic hybrids were aneuploids ($2n = 31$–59), during vegetative proliferation their chromosome numbers reduced to 32. Chromosomal rearrangements were observed in S_1 offspring of *M. sativa* (+) *M. rugosa* through genomic in situ hybridisation techniques, where small parts of the *M. rugosa* chromosomes were detected among the *M. sativa* chromosomes, and the chromosome number had reduced to $2n = 32$. Hybrids obtained from *M. sativa* + *M. scutellata* lost female fertility and no S_1 seed was obtained.

This paper reports generation of asymmetric *M. sativa* × *M. arborea* hybrids by sexual reproduction, using cytoplasmic male sterile *M. sativa* as the female parent. Introgression of some of the *M. arborea* genome into *M. sativa* has been established, using morphological, anthracnose disease resistance and DNA markers.

Materials and methods

Generation of hybrids between *M. sativa* and *M. arborea* and testcrosses of the hybrids with *M. sativa*

The *M. sativa* and *M. arborea* genotypes used to generate the *M. sativa* × *M. arborea* hybrids are listed in Table 1. A predominantly cytoplasmically male sterile *M. sativa* genotype (WA2071) was used as the female and pollinated by two individuals from the *M. arborea* accession listed in Table 1. A low proportion (<1%) of pollinated flowers produced pods (no chemical treatments were applied), and pods mostly contained only one seed. At least half of the seed which set in macroscopically visible pods later aborted, giving rise to light shrivelled seeds which did not germinate. Viable seed was germinated on moistened filter paper in sterile petri dishes, and transplanted into UC mix (peat/sand, 1:1; Baker 1957) in a naturally illuminated glasshouse at 18–22°C. Six S_1 individuals of WA2071 (to be used later in progeny testing of WA2071) were also generated by tripping its flowers with the end of a spatula. All putative hybrids produced were completely sterile upon selfing, but were female fertile to a high level (at least 50% of a normal lucerne plant) when pollinated using $4x$ plants from the *M. sativa* complex.

One putative hybrid, WA2273, was also used as the female and test crossed to an anthracnose resistant $4x$ lucerne genotype (WA2702) with purple flowers from the cv Pac L901. Fourteen testcross progeny were grown to maturity, and then flower colour, pod shape and coiling were assessed on each plant. WA2273 was also test crossed (to determine if anthracnose resistance is transferred) with a *C. trifolii* race 2 susceptible *M. sativa* $4x$ genotype (WA3021) from Oman 2 (highly winter active line collected at Sur, Oman) growing in a naturally illuminated glasshouse running at 25°C over the period October to November, which in Brisbane, Australia is late spring with 13 h of daylight.

The testcross progeny (WA2273 × WA3021) were assessed for reaction to *C. trifolii* race 2 as seedlings.

Morphological observations

Flower colour was recorded for all plants listed in Table 1 and the 14 testcross progeny (WA2273 × WA2702), using the notation of Barnes (1972). Determinations were done both in young unfaded flowers, and in older flowers which had sufficient time to fade, allowing detection of yellow pigmentation, if present. All flower colour determinations were made on plants growing in a glasshouse in Brisbane, Australia in the September to November period of 2007. The number of coils per seed pod, diameter of pod, and whether the pods were flat or crinkled was also determined on 15 pods for the parents, F_1, and 14 testcross (WA2273 × WA2702) individuals at the time of pod maturity.

Anthracnose reaction

Disease response reactions of the parents (WA2071 and WA3187), the six S_1 plants of WA2071, and the hybrid WA2273 were determined to *C. trifolii* race 2 (isolate UQ4504) as mature plants using spray inoculation methods outlined in Mackie et al. (2007). The highly susceptible *M. sativa* 4*x* clone D was included in the inoculations, as a susceptible check (Mackie et al. 2007). In all cases, 5–7-day-old succulent regrowth after cutting back was inoculated. Plants were inoculated on two separate occasions, following cutting back. The reactions to the race 2 (isolate UQ4504) inoculations of the testcross progeny (WA2273 × WA3021) were assessed on 3–4-week-old seedlings, as described in Mackie and Irwin (1998). Also assessed were seedlings from the cross WA2071 × WA3021, which were included as controls. One hundred and twenty-two seedlings of each of the above populations were assessed.

Somatic chromosome counts and breeding behaviour

The somatic chromosome number was estimated for the *M. sativa* parent (WA2071) and the *M. arborea* parent (WA3187) of WA2273, (the putative hybrid from the above genotypes), and for WA2273 itself. Procedures were those described in Bingham (1968), except root tips were taken from clonal material of the above individuals, not seedling radicals. With the exception of the *M. arborea* individuals, all genotypes shown in Table 1 were used as females and pollinated with pollen from known $2n = 4x = 32$ genotypes (WA2702 and WA3021) as previously described, and from a known $2n = 2x = 16$ genotype (WA2291), selected from CADL (Bingham and McCoy 1979), respectively. The ease of producing pods containing viable seed was used as further evidence of ploidy level.

Molecular characterisation

Amplified fragment length polymorphism (AFLP) markers were used to determine if genetic transfer of all or part of the *M. arborea* genome to *M. sativa* had been achieved. AFLP analysis was performed on the parents and hybrids listed in Table 1. Leaf material (less than 4 weeks regrowth) for DNA isolation and marker analysis was harvested from glasshouse grown individuals described in Table 1. The methods for DNA extraction and AFLP marker analysis were described in Musial et al. (2007). Briefly, genomic restriction fragments produced by *Eco*RI/*Mse*I digestion were detected by PCR amplification using radiolabelled *Eco*RI primers. A total of 12 selective *Eco*RI/*Mse*I primer combinations were used to analyse the parents and putative *M. sativa* × *M. arborea* hybrids. Amplified products were run on a 6% denaturing polyacrylimide gel, the gel was dried onto Whatman 3MM Chr paper and exposed to Kodak X-Omat film. The film was developed,

Table 1 Phenotypic observations of *M. sativa* × *M. arborea* F_1 hybrids, and hybrid pedigree

F_1 hybrid clone identification	Flower colour		Pod coiling and shape		Parents
	Unfaded	Faded	Number of coils	Shape	
WA2266	Light purple (1.3)	Light blue (2.4)	1–2	Crinkled	WA2071[a] × WA3186[b]
WA2268	Light purple (1.3)	Light blue (2.4)	1–2	Crinkled	WA2071[a] × WA3186[b]
WA2271	Pink/maroon (2.2)	Pink/maroon (2.2)	3	Non crinkled	WA2071[a] × WA3186[b]
WA2272	Pink/maroon (2.2)	Light blue (2.4)	3	Non crinkled	WA2071 × WA3187[b]
WA2273	Pink/maroon (2.2)	Light blue (2.4)	1–2	Crinkled	WA2071 × WA3187[b]

[a] Genotype WA2071 is a *M. sativa* cytoplasmic male sterile, derived from a male sterile genotype MB (Magnum × Blazer) and obtained from E T Bingham, University of Wisconsin—Madison (Bingham 2005). WA2071 has very faintly variegated light blue flowers upon fading (2.4), [refer to Barnes (1972) for detailed descriptions of flower colours], moderately dark purple when newly opened (1.2), and produces non crinkled pods with three coils

[b] WA3186 and WA3187 are different individuals from the accession SA 30528 = CPI 131489, *Medicago arborea* ex Spain, and produce orange flowers (4.4) and flat pods with one coil

and then manually scored for the presence or absence of bands. Reproducibility was established by repeat testing of the parents and WA2273 as described above.

Results

Phenotypic and molecular characterisation of the parents and putative F_1 plants and their testcross progeny with *M. sativa*

Root tip chromosome number was determined for the *M. sativa* (WA2071) and *M. arborea* (WA3186 and WA3187) parents, and a somatic chromosome number of or near 32 chromosomes was established, indicating they were at or near tetraploid. Only <0.5% of pollinated flowers produced viable seed and over 2,000 pollinations were made to produce the material listed in Table 1. Because WA2071 was faintly variegated (light blue, 2.4) upon fading indicating presence of some yellow pigment, flower colour was not the only phenotypic indicator of introgression of *M. arborea* used. Of the six S_1 individuals of WA2071 obtained, four were light blue (2.4) on fading and two were moderately dark purple (1.2), indicating WA2071 was segregating at one of the Y loci that conditions yellow pigment (Barnes 1972). Three (WA2271, 2272 and 2273) of the putative hybrid F_1 individuals (Table 1) had pink/maroon (2.2) unfaded flowers, which remained that colour in WA2271, the other two fading to light blue. WA2266 and WA2268 were light purple (1.3) when young, fading to light blue (2.4). The pink/maroon colour is observed infrequently (Barnes 1972), but it was predominant in this family, indicating possible introgression of genes conditioning flower colour from *M. arborea*.

Evidence for introgression of *M. arborea* was observed with pod morphology and coiling (Table 1; Fig. 1). WA2071 had typical *M. sativa* pods which were non-crinkled and with three coils, as did all of the six S_1 individuals examined from it, while the *M. arborea* parent had broad and flat single coiled pods (Fig. 1). Three of the putative hybrids (WA2266, 2268 and 2273) produced crinkled pods with 1–2 coils (Table 1), and the remaining two individuals produced three coiled non crinkled pods similar to the maternal parent. For 15 pods assessed for WA2273, the mean number of coils was 1.4 with a standard deviation of 0.4, versus 3.0 ± 0.3 for WA2071.

Vegetatively, all of the five putative hybrids resembled the *M. sativa* parent, with the exception that they appeared to produce more primary shoots, and more shoots from auxiliary buds on the primary shoots than the *M. sativa* parent or its selfs, a trait observed by Nenz et al. (1996) for their *M. sativa* × *M. arborea* hybrids. Fourteen testcross plants (WA2273 × WA2702) were grown to maturity, and

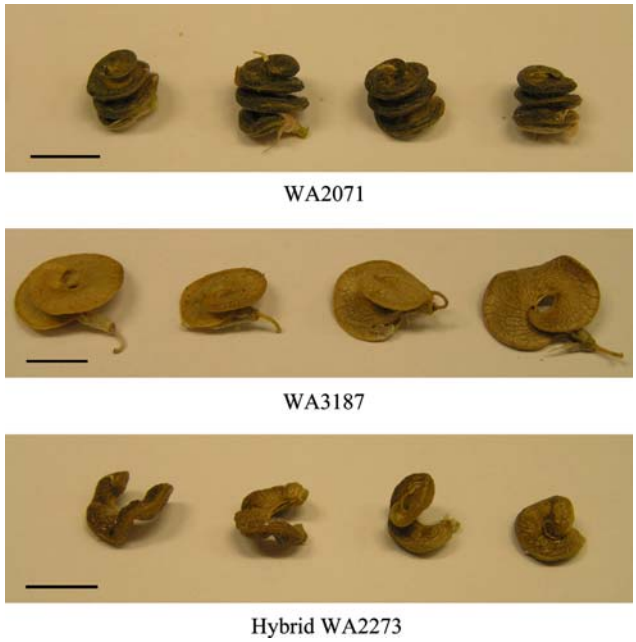

Fig. 1 Pod coiling in hybrid WA2273 and parents. WA2071 is the *M. sativa* cytoplasmic male sterile female parent, and WA3187 is the *M. arborea* pollen donor. *Scale bars* indicate 5 mm

they showed segregation for flower colour, pod shape and pod coiling. One plant resembled WA2273, with 1.5 coils per pod and exhibited a crinkled pod. Five plants had three coils per pod, and the pods resembled those of *M. sativa*. The remaining eight plants had 2–2.5 coils per pod, with no crinkling. Flowers from 11 of the testcross plants were variegated (2.4) and three were purple (1.3).

The *M. sativa* parent (WA2071), its six S_1 progeny and the susceptible check clone D were all highly susceptible to spray inoculation with *C. trifolii* race 2 as mature plants, each reacting to give a disease score of 4 (coalescing sporulating lesions). The *M. arborea* parent (WA3187), as with >20% of the plants tested in its accession (SA 30528) (authors, unpublished data), was resistant, producing necrotic flecks (disease score = 2, Mackie and Irwin 1998). Only hybrid WA2273 showed higher levels of resistance than the *M. sativa* parent WA2071, giving a disease score of $2^+/3^-$ [mostly narrow necrotic flecks (2^+) and occasionally producing narrow and weakly sporulating lesions(3^-)] after spray inoculation. The seedling testcross progeny (WA2273 × WA3021) segregated for 34.4% resistant plants (Table 2), demonstrating the heritability of the resistance derived from *M. arborea* in WA2273. In the testcross of WA2071 with the highly susceptible WA3021, 2.4% of seedlings gave a resistant reaction, and these plants could have been disease escapes, or else one of either parent may contain QTLs which condition low levels of resistance, as previously reported by Irwin et al. (2006) for a *M. sativa* genotype.

Table 2 Reaction to *Colletotrichm trifolii* race 2 of seedlings from the testcrosses (WA2273 × WA3021) and (WA2071 × WA3021)

Testcross	Number of seedlings in each class					% Resistant (1 + 2)
	1	2	3	4	5	
WA2273[a] × WA3021[b]	2	40	57	23	0	34.4
WA2071[c] × WA3021	0	3	43	76	0	2.4
LSD ($P \leq 0.01$)						7.2

Seedling reaction was classified on a scale where 1 = highly resistant to 5 = highly susceptible. A reaction of 1 or 2 was considered resistant, and 3, 4 or 5 were considered susceptible

[a] *M. sativa* × *M. arborea* hybrid. The *M. arborea* parent (WA3187) is resistant (disease score = 2) to race 2 of *C. trifolii*

[b] Oman 2 genotype highly susceptible (disease score = 4) to race 2 *C. trifolii*

[c] Male sterile *M. sativa* parent of WA2273, and it is highly susceptible (disease score = 4) to race 2 of *C. trifolii*

Table 3 AFLP marker analysis of parental and putative hybrid material of *M. sativa* and *M. arborea*

	Monomorphic in both parents and hybrid	Monomorphic in both parents but absent from hybrid	Unique sativa[a]	Unique arborea[b]	Unique hybrid[c]	Total
M. arborea WA3186	193	18	–	571	–	782
M. sativa WA2071			545	–	–	756
Hybrid WA2266	193	–	494	8	23	718
M. arborea WA3186	190	21	–	571	–	782
M. sativa WA2071			545	–	–	756
Hybrid WA2268	190	–	483	15	17	705
M. arborea WA3186	200	11	–	571	–	782
M. sativa WA2071			545	–	–	756
Hybrid WA2271	200	–	498	15	23	736
M. arborea WA3187	201	13	–	555	–	769
M. sativa WA2071			542	–	–	756
Hybrid WA 2272	201	–	480	7	19	707
M. arborea WA3187	202	12	–	555	–	769
M. sativa WA2071			542	–	–	756
Hybrid WA2273	202	–	484	20	51	757

Markers were scored for band presence/absence; marker presence was categorised for each hybrid, *M. arborea* pollen donor and the male sterile *M. sativa* female parent WA2071

[a] AFLP markers present in the *M. sativa* female parent (WA2071) only

[b] AFLP markers present in the *M. arborea* pollen donor only

[c] AFLP markers present in the individual hybrid but not either parent

AFLP analysis of more than 700 scored fragments that were derived from 12 primer combinations confirmed the asymmetric genomic composition of the hybrids (Table 3). WA2273 was the hybrid which contained the largest number of unique *M. arborea* alleles scored, 20 out of 555, or 3.6%. This genotype also showed the highest proportion (6.7%) of bands which were unique to the hybrid and not present in either parent, suggesting possible chromosomal rearrangement(s) contributing to new alleles (Table 3). Each of the remaining hybrids showed the presence of at least some (1.3–2.6%) alleles unique to the *M. arborea* parent, as well as alleles unique to the hybrid (2.4–3.2%). This analysis was repeated for WA2273 and both parents, and the reproducibility of the scored bands was confirmed.

The five hybrid genotypes were self sterile, producing little or no pollen, but were fertile to a high level when used as females and pollinated with 4*x* but not 2*x* *M. sativa* (authors, unpublished data). All flowers pollinated generally produced a pod, and pods contained at least half of the number of seeds as would be obtained with a lucerne parent. Chromosome counts on root tips indicated each hybrid was tetraploid, or near tetraploid ($2n = 4x = 30$–32), with WA2273 appearing to be deficient in up to two chromosomes.

Discussion

There have been several previous attempts made to generate hybrids between *M. sativa* and Medicago spp. outside the *M. sativa* complex using sexual reproductive processes (Fridriksson and Bolton 1963; Lesins and Lesins 1979). In the work of Fridriksson and Bolton (1963), pollination of *M. sativa* stigmas from highly self incompatible clones with a range of annual and perennial Medicago spp., including *M. arborea*, failed to produce mature embryos. However, the early stages of embryonic growth were initiated with some of the species, including *M. arborea*. Nenz et al. (1996) reported interspecific hybrid plants following symmetrical electrofusion of mesophyl protoplasts of *M. sativa* with callus protoplasts of *M. arborea*. These plants failed to flower, precluding further genetic studies, and although they also showed extensive genomic rearrangements as evidenced by isozyme and RFLP analysis, their hybridity was established with about half of the species specific bands of the two parent species present in them. This paper reports the generation of asymmetric hybrids between *M. sativa* and *M. arborea* by sexual reproduction.

The genome of our hybrids was clearly biased towards *M. sativa* with 72% of the sativa specific AFLP bands present, versus 4% of the arborea specific bands present in the most studied hybrid. In this same hybrid (WA2273), 7% of the AFLP bands were unique to the hybrid, indicating genome rearrangement creating new AFLP markers. WA2273 was also resistant to spray inoculation with *C. trifolii* race 2, which was transmissible to its testcross progeny, and this level of resistance can only have come from the resistant *M. arborea* parent, or as a result of genome rearrangement due to transfer of *M. arborea* chromosomal material, as the *M. sativa* parent WA2071 and its self and testcross progenies were highly susceptible.

Anthracnose is a major disease of lucerne worldwide, and only three major loci for resistance to *C. trifolii* races in *M. sativa* have been identified, and two of these are closely linked (Mackie et al. 2007; Elgin and Ostazeski 1985). Mackie et al. (2007) reported that in 4*x* *M. sativa* major genes for resistance to anthracnose are located on chromosome 8 (races 1 and 4) and chromosome 4 (race 2). QTLs (quantitative trait loci) were also identified in this work which explained smaller amounts of the phenotypic variation for reaction to anthracnose. Whether the resistance in the *M. arborea* parent we have used is conditioned by the same genes previously identified in *M. sativa* remains to be determined. In a testcross of WA2273 with a highly susceptible genotype (WA3021), 34% of the population were resistant. This result is similar to that found for inheritance of resistance to race 2 in lucerne, where the resistance was incompletely recessive, and 37% of the population were resistant in a testcross of resistant × susceptible genotypes (Mackie et al. 2007). The above work has demonstrated the capacity to transfer agronomically useful genes from *M. arborea* to *M. sativa* through normal sexual reproduction.

McCoy and Smith (1986) reported interspecific diploid hybrids were obtained by crossing diploid *M. sativa* with a range of diploid perennial Medicago spp., including *M. papillosa*, and ovule–embryo culture was necessary for recovery of hybrids with equal numbers of genomes of the two species. Only tetraploidised hybrids were fertile in *M. sativa* × *M. papillosa*, and these showed disomic segregations, indicating little or no exchange between homoeologus chromosomes (McCoy and Quarisa 1989). Mizukami et al. (2006) used electro-fusion of protoplasts of *M. rugosa* and *M. scutellata*, both annual species, with 4*x* *M. sativa* to produce somatic hybrids. These hybrids were very unstable, and during vegetative proliferation their chromosome numbers quickly reduced to 32, from a potential of 64 in the *M. scutellata* hybrids. Genomic in situ hybridisation showed that small fragments of the annual Medicago genome had integrated into the *M. sativa* genome, in the somatic hybrids with both annual species, and in the S_1 progeny of *M. sativa* + *M. rugosa*. In our studies, the asymmetric hybrids produced by sexual reproduction appear to have undergone a similar phenomenon to that reported above, where only a relatively small proportion of the *M. arborea* genome has introgressed with that of *M. sativa*. The proportion of *M. arborea* bands observed in hybrid WA2273 (3.6%) could indicate transfer of a whole chromosome, or introgression of several smaller parts, as theoretically each *M. sativa* chromosome constitutes an average of 1/32 or 3.1% of the genome. It is possible that further introgression of *M. arborea*, not detected by the AFLP analysis, may have occurred as one quarter of the markers in this hybrid (202/757) were present in both parents. Whether the introgressed *M. arborea* genome represents a chromosome, an arm, or several small pieces of chromosome, remains unresolved. The hybrids and their progeny, as with those of *M. sativa* + *M. rugosa*, as reported by Mizukami et al. (2006), are more similar to *M. sativa*, as would be expected with such unequal genome representation.

Importantly, the hybrids we have generated by sexual reproduction have excellent female fertility (authors, unpublished data) when crossed with 4*x* *M. sativa*, unlike those from previous attempts utilising protoplast fusion (Nenz et al. 1996; Kaimori et al. 1998; Mizukami et al. 2006). Another cultivated autotetraploid which has a very large number of related wild and cultivated species at different ploidy levels is the potato, *Solanum tuberosum* ($2n = 4x = 48$), and sexual polyploidisation through $2n$ gametes has played a major role in their evolution (Camadro et al. 2004). As well as $2n$ gametes, the need for a balance of chromosome sets between maternal tissue, embryo and endosperm, has been established (Boyes and Thompson

1937; Valentine 1956). In generation of the asymmetric hybrids, we have reported between *M. sativa* and *M. arborea*, 2*n* gametes and endosperm development are two factors which appear to have influenced the outcome. A possible mechanism is that a 2*n* egg arising from second division restitution (Pfeiffer and Bingham 1983) from the male sterile *M. sativa* female parent has been fertilised with a normal 2*x* gamete of *M. arborea*. During subsequent cell divisions, the *M. arborea* chromosomes have rapidly been lost due to differences in the timing of mitotic events between the two species, except for a chromosome or chromosome parts, the latter integrating into the *M. sativa* genome. This explanation is consistent with the DNA marker analysis of the hybrids, and their breeding behaviour. Also, the endosperm of the male sterile female parent used, WA2071, and its maternal parent MB appear to be able to tolerate an unbalanced genome dosage (Johnston and Hanneman 1980; Bingham 2005). This is consistent with earlier work of Fridriksson and Bolton (1963) who reported post-fertilisation barriers were the main reason for abortion of embryos of *M. sativa* × *M. arborea* crosses. Ovule–embryo culture, as reported for *Medicago* spp by McCoy and Smith (1986), may provide a means of obtaining *M. sativa* × *M. arborea* hybrids with balanced genomes.

The 7% of AFLP fragments unique to the hybrid WA2273 is indicative of genome rearrangement in the hybrid. Song et al. (1995) and Gaeta et al. (2007), working with synthesised *Brassica* hybrids with polyploid genomes, found that in early generations after polyploidisation, most genome changes resulted in loss and/or gain of parental restriction fragments, and the appearance of novel fragments. These changes were attributed to chromosome rearrangements, transpositions, deletions and other processes including epigenetic changes. Exchanges among homoeologous chromosomes were found to be a major mechanism creating novel allele combinations and phenotypic variation in newly formed *B. napus* polyploids (Gaeta et al. 2007). Integration of *M. arborea* chromosomal material into the *M. sativa* genome would be one cause for the novel AFLP fragments found in the hybrid WA2273. The mechanisms involved in generation of the asymmetric hybrids will require further research.

This work has demonstrated the potential to transfer agronomically useful genes from *M. arborea* to *M. sativa* by the use of male sterile lucerne as the female parent and sexual reproduction. While the genomes in the hybrids we generated were unbalanced in favour of the *M. sativa*, the hybrids showed good female fertility and the *M. arborea* traits of a single coiled pod and *C. trifolii* race 2 resistance segregated in the testcross with *M. sativa*. Hybrids have also been generated in earlier work at the University of Wisconsin–Madison (Bingham 2005; Bingham et al. 2006) using MB as the female parent; MB being the female parent of WA2071. In the Wisconsin hybrids, a range of additional phenotypic traits have been transferred from *M. arborea*, including orange flower colour, flat single coiled pods and large seeds (refer to http://www.medicago-reports.org/). Importantly for future breeding work, this indicates that while these hybrids are still more like *M. sativa* and are highly female fertile with 4*x M. sativa*, different parts of the *M. arborea* genome have been transferred to that reported in this paper. There thus appears to be the potential to transfer mitotically stable parts of the *M. arborea* genome to *M. sativa* by sexual reproduction, without having to use protoplast fusion. Further work is needed to determine whether agronomically important traits such as greater perenniality, absence of winter dormancy, drought hardiness and others, which would improve lucerne productivity and utilisation (Irwin et al. 2001), can be transferred from *M. arborea* to *M. sativa* through sexual reproduction.

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