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Reducing the tetraploid non-nodulating alfalfa (*Medicago sativa*) MnNC-1008(NN) germ plasm to the diploid level

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Abstract MnNC-1008(NN) (referred to as MN-1008) is a tetraploid alfalfa mutant with two recessive genes (nn_1 and nn_2) conditioning the non-nodulating trait. The tetraploid level (2n=4x=32) of this *Medicago sativa* germ plasm was reduced to the diploid (2n=2x=16) level using the 4x-2x genetic cross originally described as a workable method for the induction of haploidy in alfalfa by T. E. Bingham. In our experiments more than 7000 emasculated flowers of a single non-nodulating MN-1008 mutant alfalfa plant with purple petals were cross-pollinated with pollen from a single, diploid, yellow-flowered alfalfa plant. Mature seeds from these crosses were collected and germinated, after which the plants were subjected to morphological and cytogenetic analyses as well as to DNA fingerprinting. Out of 26 viable progeny, 6 were hybrid plants, 19 proved to be self-mated derivatives of MN-1008, while one descendant turned out to be a diploid (2n=2x=16), purple flowered, non-nodulating plant denoted as *M. sativa* DN-1008. This diploid, non-nodulating alfalfa plant can serve as starting material to facilitate the comprehensive morphological, physiological and genetic analysis (gene mapping and cloning) of nodulation in order to learn more about the biology of the symbiotic root nodule development. To produce diploid, nodulating hybrid F_1 plants, DN-1008 was crossed with a diploid, yellow-flowered *M. sativa* ssp. *quasifalcata* plant. An F_2 population segregating the nn_1 and nn_2 genes in a diploid manner, in which the genetic analysis is more simple than in a tetraploid population, can be established by self-mating of the F_1 plants.

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Introduction

Symbiotic nitrogen fixation between *Rhizobium* bacteria and their host plants is established during a multistep process which is governed by symbiosis-specific genes of both partners. To understand the biological functions of these symbiotic genes they are currently being investigated extensively. Early and late nodulin genes have been isolated on the basis of their differential expression profile in nodules or infected root(hair)s compared to that of uninfected roots (for a review see Schultze et al. 1994). In these cases, however, no evidence was provided for whether or not the identified genes played a significant role in symbiosis. On the other hand, a mutation conditioning non-nodulation or an ineffective phenotype, (i.e. one in which no nodule formation occurs or else nodules are ineffective in symbiotic nitrogen fixation) ensures that the affected genes are responsible for essential functions in symbiosis. Consequently, it is worth using plant mutants impaired in symbiosis to identify, and eventually isolate, symbiotic genes. By this approach not only will new genes be identified but important information can be collected concerning their role in nodule formation and/or function.

Several symbiotic plant mutants have been described to-date (for a review see Caetano-Anollés and Gresshoff 1991). In tetraploid alfalfa (*Medicago sativa*) Peterson and Barnes (1981) described one non-nodulating and four different ineffective traits. The non-nodulating mutant MN-1008 is unable to form nodules following infection with effective *Rhizobium meliloti* and this trait is conditioned by two unlinked recessive genes, nn_1 and nn_2. Further analysis demonstrated that this mutant plant was defective in early plant responses after bacterial infection; that is, neither root hair curling nor cortical cell division occurred (Dudley and Long 1989). In our experiments, the MN-1008 alfalfa mutant did not show root hair deforma-

tion upon treatment with different concentrations of the *R. meliloti* Nod factor, but nodule-like bumps could be induced by adding external 2,4-D (2,4-dichlorophenoxyacetic acid) to the medium (unpublished data). Spontaneous nodule formation was also reported in these mutant plants (Caetano-Anollés et al. 1993). The above results suggested that the tetraploid alfalfa mutant MN-1008 was either impaired in the perception of rhizobial signal molecules or else in the first steps of their subsequent signal transduction pathway. Spontaneously appearing nodules and nodule-like bumps suggest that later steps in the nodule-developing program of the plant are normal. Consequently, it seems likely that this mutant can be an important key to understanding the early events in the signal transduction pathway elicited by Nod-factors leading to nodule development on the root system of the host plant.

Our future goal is to map the two recessive genes conditioning the non-nodulation phenotype on the genetic map of alfalfa (Kiss et al. 1993). However, mapping this trait in a tetraploid segregating population is especially complex since it is determined by two recessive genes. The expected incidence rate of a plant with the synchronous presence of the nulliplex alleles of two unlinked loci in a tetraploid F_2 segregating population is 1:1296, while it is 1:16 in a diploid F_2 population. Therefore it is more reasonable to perform a diploid cross to produce the segregating population for mapping the nn_1 and nn_2 genes. To reduce the ploidy of MN-1008 to a diploid level, namely to isolate 2x haploids of this tetraploid alfalfa mutant, the 4x–2x cross method described by Bingham (1969, 1971) was applied.

In the present paper we describe the generation of a diploid non-nodulating derivative of the tetraploid MN-1008 mutant and its successful cross with the diploid yellow-flowered *M. sativa* ssp. *quasifalcata* to generate F_1 progeny.

Materials and methods

Plant materials and treatments

The tetraploid (2n=4x=32) non-nodulating *M. sativa* mutant MN-1008 was kindly provided by Prof. D. Barnes, University of Minnesota, MN, USA (Peterson and Barnes 1981). It is a purple-flowered plant and was used as the female parent in the 4x-2x cross. The nodulation-competent, diploid (2n=2x=16), yellow-flowered *M. sativa* ssp. *quasifalcata* k93 (abbreviated as Mqk93, Kiss et al. 1993) was the pollinator plant.

Since the purple-flowered tetraploid MN-1008 plant is self fertile, emasculation of the flowers prior to foreign pollination had to be performed. Emasculation was carried out by clipping the standard petal followed by tripping the flowers by gently squeezing them at the base. The whole raceme was immersed in 51% ethyl alcohol for 3–4 s then washed in water for a few s. After becoming dry the stigma was covered with pollen from the male parent. The efficiency of the alcoholic treatment was checked in control experiments. The self-pollination frequency of MN-1008 flowers after emasculation proved to be about 0.5%.

The nodule-forming ability of the plants was determined in a symbiotic plant test. Plants were infected with *R. meliloti* in the absence of fixed nitrogen as described previously (Kondorosi et al. 1977).

Cytogenetic analysis

For cytogenetic analyses shoot apices from well growing plants were cut down, incubated in 2% glucose solution at 2°C for 24 h then fixed in Carnoy solution for 2–3 h. Staining was carried out in 4% carmine acetic acid for 2–3 days. For softening, the plant material was transferred into hot 45% acetic acid. Squash preparations were made in 45% acetic acid and were analyzed under a phase-contrast microscope. The chromosome numbers were counted in at least 40 metaphase cells from several shoot apices per plant.

DNA manipulation, PCR analysis

Total DNA was isolated from young shoots as described by Kiss et al. (1993). PCR amplification was carried out in 25 µl 1×*Taq* polymerase (Promega) buffer in the presence of 1.5 mM $MgCl_2$, 1 pmol/µl of ENOD12 gene-specific primers (Allison et al. 1993), 1 U *Taq* polymerase enzyme (Promega), 200 µM of each dNTP and 1 ng/µl template DNA and overlayed with 20 µl of mineral oil (Sigma). The reactions were run under the following conditions: 94°C for 30 s, 60°C for 1 m, 72°C for 1 m over 35 cycles and the reaction was completed with a 4-min step at 72°C. The amplified products were separated in 2.0% agarose gel and stained with ethidium bromide.

Results and discussion

The 4x-2x cross

To generate diploid derivatives of the tetraploid alfalfa mutant MN-1008 carrying the two recessive genes nn_1 and nn_2 the 4x-2x cross method of Bingham (1969, 1971) was applied. According to these earlier studies 4x–2x crosses provided 2x haploids (2n=2x=16) from several different tetraploid cultivated alfalfa (2n=4x=32). In these cases the diploid male parent did not contribute germ plasm; its role was simply to induce the unfertilized egg (n=2x) to develop into a 2x haploid plant (2n=2x), as was indicated by the inheritance of a recessive marker of the tetraploid seed parent (Bingham 1969). Later Bingham and Gillies (1971) demonstrated that 90% of the 2x haploids of several different tetraploid alfalfa had an acceptable level of fertility. They were crossable with wild diploid *M. falcata* or *M. sativa* and the F_1 progenies were essentially normal in male and female fertility.

In our experiment the non-nodulating, tetraploid (2n=4x=32), purple-flowered MN-1008 mutant was used as the female parent, and the nodulating, diploid (2n=2x=16), yellow-flowered Mqk93 served as the pollinator (Kiss et al. 1993). As a result of this cross the following categories of the progeny were expected: (1) hybrid tetraploids and triploids coming from cross-pollination; (2) self-pollinated tetraploids (since MN-1008 is self fertile); and (3) the required diploids containing the reduced genome of the tetraploid female parent (Table 1). The yellow-flowered, nodulation-competent pollinator plant was selected to make the identification of the hybrid descendants easier. In order to reduce the number of self-pollinated derivatives, alcoholic treatment was applied to the flowers of MN-1008 to get rid of fertile pollen. The cross pollinations carried out on approximately 7000 emas-

Table 1 Expected progeny categories of the tetraploid MN-1008 – diploid Mqk93 cross

Progeny categories	Symbiotic phenotype	Flower		Leaf morphology	No. of plants
		Colour	Size		
1. Triploid, tetraploid hybrid plants	Nodulating	Inter-mediate	Normal	Normal	6
2. Self-pollinated tetraploids	Non-nodulating	Purple	Normal	Normal	19
3. Diploid plants (2× haploids of the tetraploid parent)	Non-nodulating	Purple	Small	Small leaves, no serrations	1

Table 2 Cytogenetic analysis of representative individuals of the progeny categories

Plant		Number of cells examined[1]	Number of				Ploidy level of the plant
			Diploid 2n=16	Triploid 2n=24	Tetraploid 2n=32	Aneuploid cells	
MN-1008		51 (2)	–	–	39	12	Tetraploid
Mqk93		43 (3)	43	–	–	–	Diploid
Progeny							
Categ.	Names						
1	Dj13	64 (4)	–	58	–	6	Triploid
1	Dj4	54 (10)	–	25	4	25	Mixoploid
2	Dj2	40 (5)	–	–	35	5	Tetraploid
2	Dj17	51 (4)	–	–	27	24	Mixoploid
2	P93/18	65 (7)	–	–	45	20	Tetraploid
2	P24/15	40 (2)	–	–	31	9	Tetraploid
3	Dj5[2]	42 (6)	42	–	–	–	Diploid

[1] The number of shoot apices the cells originated from is in brackets
[2] This diploid plant was designated later as DN-1008

culated flowers have produced 72 mature seeds. Out of these 72 seeds, 22 did not germinate, 24 seeds germinated but perished at an early plantlet stage, and 26 viable plants were obtained. Their morphological features and nodulation ability, coupled with a cytogenetic analysis, were used to classify each of them into one of the progeny categories shown in Table 1 and to pick out the desired 2x haploid derivative(s).

Symbiotic and morphological characterization of the progeny

The symbiotic behaviour of the progeny was determined in a plant nodulation test. Out of the 26 viable plants 6 nodulated normally in the presence of effective *R. meliloti* bacteria, while the remaining 20 plants were not able to form symbiotic nodules on the roots (Table 1). The nodulating phenotype is one of the characteristic features of the hybrid progeny of this cross (1st category, Table 1) since the male parent was normal in nodulation. The hybrid nature of these plants was also substantiated by the greenish intermediate colour of their flowers and by DNA fingerprinting using the PCR-based RAPD method (Williams et al. 1990) (data not shown).

The non-nodulating phenotype and the purple flowers of the other 20 descendants indicated that their genetic material was exclusively of maternal origin. The majority of plants (19 out of 20 plants, 2nd category) had a normal tetraploid appearance in respect of their growth and leaf morphology. One non-nodulating plant (called Dj5), however, displayed an overall morphology distinct from both the tetraploid parent and the progeny (3rd category). This plant was reduced in growth and had smaller flowers and leaves. The leaves also had an altered shape without leaflet serrations. These morphological characters coincided with the features of the 2x haploid derivatives of tetraploid alfalfa plants described by Bingham and Binek (1969), and indicated that this plant could be a diploid progeny of the tetraploid MN-1008 mutant.

Cytogenetic characterization of the progeny

To further characterize the nature of the progeny of the 4x-2x cross, cytogenetic analyses were carried out on the parents and representative individuals of the progeny categories to reveal their ploidy level (Fig. 1, Table 2). Two out of the six hybrid plants were cytogenetically analysed. One of them, Dj13, was a triploid (2n=3x=24, Fig. 1C),

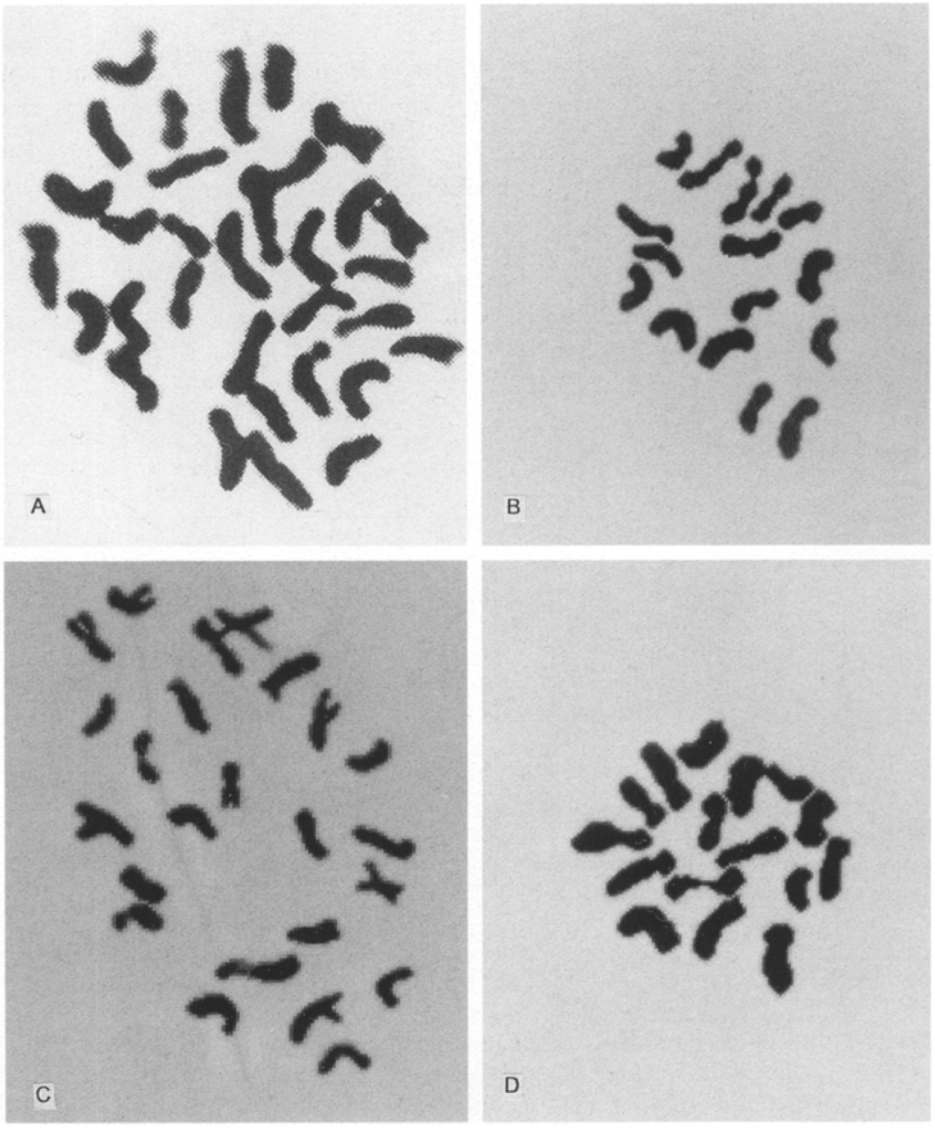

Fig. 1A–D Cytogenetic analysis of tetraploid MN-1008: 2n=4x=32 (**A**) diploid Mqk93: 2n=2x=16 (**B**), triploid progeny Dj13: 2n=3x=24 (**C**), and a diploid derivative of the tetraploid *MN-1008 – DN-1008*: 2n=2x=16 (**D**)

while the other, Dj4, contained tetraploid (7%), triploid (46%) and also aneuploid (46%) cells. The chromosome number of cells reaching meiosis mostly stabilized at the triploid level in this mixoploid plant. The self-mating efficiency (seed production after self pollination) of the hybrid plants was below 1%. These phenomena indicate the instability of the chromosomes in these hybrid plants.

Cytogenetic studies were carried out on five non-nodulating blue-flowered plants. Three of them, Dj2, P93/18 and P24/15, proved to be tetraploids (2n=4x=32). Dj17 contains aneuploid cells in quite a high ratio (47%), but the cells attaining meiotic division were stabilized at the tetraploid level. We concluded that these plants originated from self-pollination. Subsequent chromosome counting confirmed the diploid character of Dj5 which exhibited distinguishing morphological features; every cell examined (42) contained 16 chromosomes (2n=2x=16, Fig. 1D). Referring to its ploidy level (diploid) and to its origin from the tetraploid non-nodulating MN-1008 mutant, this plant was designated hereafter as DN-1008.

Generating a hybrid F_1 plant for producing a diploid population segregating the nn_1 and nn_2 genes

Using the 4x-2x cross method described by Bingham (1969, 1971) the diploid derivative of the tetraploid non-nodulating MN-1008 plant could be isolated. Since DN-1008 contains the 2x haploid genome of MN-1008 it carries nn_1 and nn_2 recessive alleles as is supported by its non-nodulating phenotype. This diploid non-nodulating *Medicago* enables us to establish segregating population for the non-nodulation trait in order to map the nn_1 and nn_2 genes on the eight linkage groups of alfalfa. For this purpose the yellow-flowered Mqk93 has been crossed with the purple-flowered DN-1008 mutant. Mqk93was selected since this

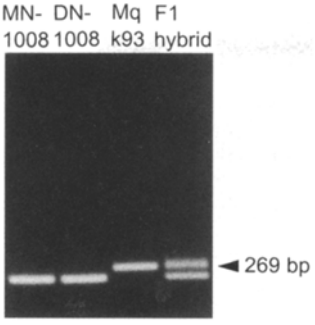

Fig. 2 PCR amplification products of the tetraploid MN-1008 plant; the diploid DN-1008 and Mqk93 parents, and their hybrid using ENOD12-specific primers. The exact size of the Mqk93 fragment was determined earlier (Csanádi et al. 1994)

plant served as the female parent of the segregation population used for the construction of the basic genetic map of alfalfa (Kiss et al. 1993). When DN-1008 was used as a pollinator no hybrid offspring appeared on the female parent Mqk93. Since all descendant plants were yellow-flowered and the PCR-based molecular markers tested were all inherited from the maternal parent (data not shown), we concluded that progenies of this cross originated only from the self-pollination of Mqk93. Consequently DN-1008 is male-sterile, which is in accord with earlier results obtained with 2x haploids of tetraploid alfalfa plants by Bingham and Gillies (1971). On the other hand, in the reverse cross DN-1008 proved to be fertile when used as the female parent. Following fertilization the stigmas of DN-1008 with pollen of Mqk93, seed formation occurred on the diploid non-nodulating plant. Though seed production of this cross was low (about 1%) up to now seven seeds have been collected and three of them have germinated. DNA was isolated from them at the seedling stage and both DNA fingerprinting and gene-specific PCR amplification experiments were carried out to confirm their hybrid nature. The inheritance of the polymorphic ENOD12 alleles amplified with ENOD12-specific primers demonstrated clearly the hybrid nature of an F_1 plant of this cross (Fig. 2). DN-1008 has homozygous ENOD12 alleles derived from its tetraploid MN-1008 progenitor giving one amplified fragment; Mqk93 carries ENOD12 alleles producing a PCR-amplified fragment with a higher mobility; while the ENOD12 amplification of the heterozygote F_1 hybrid plant results in two fragments originating from the two parents. The diploid, nodulation-competent F_1 hybrid plants are currently under intensive physiological and genetic investigation.

Conclusions

The above study demonstrates that the 4x-2x cross method described originally by Bingham (1969) is a feasible way to convert valuable tetraploid alfalfa mutants to the diploid level. By this means the complex tetraploid segregation pattern can be reduced to the diploid level and the trait in question can be more easily genetically analyzed. Haploidy is especially useful if the phenotype of interest is conditioned by two recessive mutations, as in case of the MN-1008 alfalfa mutant. The 2x haploid derivative DN-1008 provides a facility for further genetic studies on the nn_1 and nn_2 genes conditioning the non-nodulating phenotype. The production of more F_1 hybrid plants from the DN-1008 – *M. sativa* ssp. *quasifalcata* cross is continuing and the best self-pollinating hybrid plant will be selected for generating a segregating F_2 population. We plan to map and eventually clone the genes nn_1 and nn_2 using the map-based cloning strategy after establishing a population segregating for the non-nodulating phenotype.

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