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ORIGINAL PAPER

Spectinomycin resistance mutations in the *rrn16* gene are new plastid markers in *Medicago sativa*

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Abstract We report here the isolation of spectinomycin-resistant mutants in cultured cells of *Medicago sativa* line RegenSY-T2. Spectinomycin induces bleaching of cultured alfalfa cells due to inhibition of protein synthesis on the prokaryotic type 70S plastid ribosomes. Spontaneous mutants resistant to spectinomycin bleaching were identified by their ability to form green shoots on plant regeneration medium containing selective spectinomycin concentrations in the range of 25–50 mg/l. Sequencing of the plastid *rrn16* gene revealed that spectinomycin resistance is due to mutations in a conserved stem structure of the 16S rRNA. Resistant plants transferred to the greenhouse developed normally and produced spectinomycin-resistant seed progeny. In light of their absence in soybean, a related leguminous plant, the isolation of spectinomycin-resistant mutants in *M. sativa* was unexpected. The new mutations are useful for the study of plastid inheritance, as demonstrated by detection of predominantly paternal plastid inheritance in the RegenSY-T2 × Szapko57 cross, and can be used as selective markers in plastid transformation vectors to obtain cisgenic plants.

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Introduction

The plastid genome (ptDNA) of higher plants ranges from 120 to 218 kb in size (Chumley et al. 2006; Wakasugi et al. 2001). The ptDNA is highly polyploid and the 1,000–10,000 identical copies in a cell are compartmentalized in up to ~100 plastids per cell (Shaver et al. 2006; Zoschke et al. 2007). Because of the readily obtainable high protein levels, the opportunity to express complex functions from polycistronic mRNAs and natural transgene containment, the plastid genome is an attractive target for biotechnological applications. Plastid transformation involves introduction of the transforming DNA by the biolistic process, incorporation of the marker gene and the gene of interest by two homologous recombination events via the flanking ptDNA sequences, and gradual dilution of the wild-type ptDNA copies in tissue culture on a selective medium. Critical for success is the availability of genetic markers that allow selective enrichment of transformed ptDNA copies (Bock 2001; Koop et al. 2007; Maliga 2004; Maliga and Bock 2011). Plastid transformation is routine only in tobacco (Svab et al. 1990; Svab and Maliga 1993) and is reproducibly used in tomato (Ruf et al. 2001), potato (Valkov et al. 2011), soybean (Dufourmantel et al. 2004), and lettuce (Kanamoto et al. 2006; Lelivelt et al. 2005; Ruhlman et al. 2010). Plastid transformation has also been reported in *Medicago sativa* (Wei et al. 2011), but has not yet been confirmed in other laboratories.

Alfalfa is an important perennial forage crop that is also grown as a means to enhance soil nitrogen. In addition to being an important feed crop, there is interest in developing

M. sativa as a bioreactor for the production of recombinant proteins and industrial enzymes (Austin et al. 1995; Khoudi et al. 1999; Sourrouille et al. 2008). *M. sativa* belongs to the minority of crop species in which plastids are transmitted to the progeny from both parents, with a strong paternal bias (Masoud et al. 1990; Schumann and Hanckok 1989; Smith 1989).

Because we are interested in developing the biotechnological applications of plastid transformation in *M. sativa*, we decided to develop plastid markers. Suitable markers for plastid transformation are resistance to spectinomycin and streptomycin conferred by target site mutations in the plastid 16S ribosomal RNA (Golds et al. 1993; Staub and Maliga 1992, 1993; Svab et al. 1990) or by the expression of an antibiotic inactivating enzyme encoded in a chimeric *aadA* gene (Svab and Maliga 1993). The first report on plastid transformation in *Medicago sativa* also uses *aadA* as the selective marker (Wei et al. 2011). Kanamycin resistance encoded in *neo* (Carrer et al. 1993) or *aphA-6* (Huang et al. 2002) chimeric genes is also a suitable marker to recover transplastomic clones. Kanamycin resistance has been used to transform the nucleus of alfalfa (Deak et al. 1986), but not the plastid genome. However, kanamycin-resistant plastid mutants have been obtained in *Medicago sativa.* The kanamycin resistance mutation in the plastid 16S rRNA had a detrimental effect, causing a chlorotic phenotype in plants and reducing biomass by 85 % (Rosellini et al. 2004). We report here isolation of spectinomycin-resistant mutants in cultured *Medicago sativa* RegenSY-T2 cells conferred by target site mutations in the 16S rRNA. The regenerated plants are free of detrimental effects reported for the kanamycin-resistant mutants. The mutations will be useful for the study of plastid inheritance and as selective markers in cisgenic plastid transformation vectors.

Materials and methods

Plant tissue culture media

The B5h embryo induction medium is based on the Gamborg B5 Medium (Brown and Atanassov 1985). The B5h medium is prepared using 3.2 g of Gamborg B5 medium including vitamins (Duchefa Biochemie B.V., Haarlem, The Netherlands, Catalog No. G0210), 30 g sucrose, 30 ml amino acid stock solution, 1 mg 2,4D, 0.1 mg kinetin, and 5.6 g plant agar (Duchefa Biochemie B.V., Haarlem, The Netherlands, Catalog No. E1001) in 1 l medium adjusted to pH 5.7. One liter of the B5h amino acid stock solution contains L-glutamine, 26.6 g; serine, 3.32 g; adenine, 16 mg; L-glutathione, 332 mg. The amino acid stock solution is filter sterilized and stored at 4 °C.

The MMSN plant regeneration medium contains 4.43 g Murashige and Skoog salts (Duchefa Biochemie B.V., Haarlem, The Netherlands, Catalog No. MO221), 1 ml of the 1,000× Nitsch and Nitsch vitamin stock (Duchefa Biochemie B.V., Haarlem, The Netherlands, Catalog No. N0410), 30 g sucrose, 100 mg myo-inositol, and 5.6 g plant agar in 1 l medium (pH 5.7).

The 1/2 MS medium contains 2.21 g Murashige and Skoog salts containing Gamborg B5 vitamins (Duchefa Biochemie B.V., Haarlem, The Netherlands, Catalog No. MO231), 15 g sucrose, and 5.6 g plant agar in 1 l medium adjusted to pH 5.7.

Selection of spectinomycin resistant mutants

Mutant selection was carried out in embryogenic callus derived from *Medicago sativa* line RegenSY-T2 leaves. RegenSY-T2 is a single, vegetatively maintained clone derived from the RegenSY genetic line (Bingham et al. 1975). To initiate the cultures, fully expanded leaves of greenhouse-grown plants were cut from node 2–7 and immediately submerged in a beaker with cool tap water. The leaves were sterilized in 70 % ethanol (5 s) and 2 % commercial bleach containing 0.05 % Tween 20 (5 min). The bleach was removed by rinsing the leaves five times in sterile water. The leaves were blotted on sterile filter paper; the leaflets of the trifoliate leaves were cut into pieces (0.5 cm × 0.5 cm) and placed abaxial side up on a selective B5h medium containing different concentrations of spectinomycin dihydrochloride pentahydrate (S9007, Sigma, St. Louis, MO). The cultures were incubated at 24 °C at 16/8 h light/dark cycles and the explants were transferred to fresh medium every 2 weeks. After 1 month the leaf callus was transferred to selective MMSN plant regeneration medium where green, spectinomycin-resistant embryos appeared in 12–14 weeks after culture initiation. Shoots were obtained from well-developed green embryos after separation from the callus and individually transferring them to a Petri dish containing selective MMSN medium. The shoots regenerated roots on the same selective MMSN medium. Each mutant derived from a different leaf.

Sequencing of plastid *rrn16* genes

Total leaf DNA was isolated by the DNeasy Plant Mini Kit (Qiagen, Valencia, CA 91355, Cat. No. 69104) following the manufacturer's instructions. The *rrn16–rps7* region of the RegenSY-T2 ptDNA was sequenced with primer pairs no. 1 to no. 7 listed in Table 1. The DNA sequence was deposited in GenBank under accession no. JX174425. The region containing the *rrn16* spectinomycin resistance mutations was amplified and sequenced with primer pair

no. 8 and the sequences were deposited in GanBank (Table 1). The *rrn16* genes can be viewed as a PopSet under accession numbers: wild-type RegenSY-T2, JX185400; MsRSY-SP4251, JX185401; MsRSY-SP5501, JX185402; MsRSY-SP7171, JX185403; MsRSY-SP8301, JX185404; and MsRSY-SP8401, JX185405.

PCR assay to test for spectinomycin resistance mutation in SP8301 *rrn16* gene

The *rrn16* genes were amplified with Primer pair 8 (Table 1), digested with the *Aat*II restriction endonuclease and the fragments were separated in 1 % agarose gel. The C to G (1141) mutation (Table 3) eliminates the *Aat*II site present in the wild-type *rrn16* gene, yielding a 634-nt mutant fragment cleaved to 330- and 304-nt fragments in the wild-type amplicons.

Results

Selection of spectinomycin-resistant mutants

Combining the alfalfa plant regeneration protocol with selection for spectinomycin resistance led to the isolation of the spectinomycin-resistant mutants (Fig. 1). Plant regeneration from alfalfa leaves is obtained by first inducing embryogenic callus on B5h medium and then transferring the callus to MMSN plant regeneration medium. Callus formed on the B5h medium is initially white and then forms green embryos. These embryos, when transferred to hormone-free MMSN medium with the callus, continue to grow. The embryos will form shoots in 3–4 weeks and regenerate into plants on MMSN medium, if separated from the callus when they are 2–3 mm in size. Spectinomycin, when incorporated in the B5h and MMSN media, allows white embryogenic callus to form but inhibits greening and shoot regeneration (Fig. 2a). Spectinomycin-resistant mutants were isolated by their ability to form green embryos and shoots in the presence of spectinomycin (Fig. 2b). The spectinomycin-resistant shoots appeared at 10–12 weeks after initiating the leaf cultures.

To determine the antibiotic concentration suitable for mutant selection, plant regeneration from leaf explants was carried out in the presence of 25–500 mg/l spectinomycin. Mutants were recovered on media containing 25–50 mg/l spectinomycin. The mutant frequency in the initial screen was about 1 per 300 explants (7/2,340) (Table 2). After the initial screen on different concentrations of spectinomycin, mutant selection was carried out on a medium containing 30 mg/l spectinomycin. The mutant frequency on the larger scale screen in the presence of 30 mg/l spectinomycin was 1 in 270 explants (11/3,000). The regenerated plants were chimeric and had resistant and sensitive sectors when grown in the presence of spectinomycin. This is because

Table 1 Sequencing primers

Primer pairs	Primer name	Target sequence
1	*rrn*16-25700	5′ tatcaacttgttccgaccta 3′
	*rrn*16-26584	5′ ccgtgcaatgctgtagctaa 3′
2	*rrn*16S-901	5′ acatgctccaccgcttgtgc 3′
	*rrn*16S-205	5′ cgcgtctgattagctagttg 3′
3	*rrn*16S-68	5′ caccgccttggtaagctatt 3′
	*rrn*16S-666	5′ ttgacgtggtggaagtcatc 3′
4	*rrn*16S-557	5′ cacaagcctcttatccattc 3′
	*rrn*16S-1041	5′ tccatctcatcttgccttag 3′
5	*rrn*16S-819	5′ attgtcaactgctcctatcc 3′
	*rrn*16S-1459	5′ ttagtgatctcggttcagtg 3′
6	*rps*7-28292	5′ tcctcttgcctagtattcag 3′
	*rps*7-29125	5′ cttactgccactctacagaa 3′
7	*rps*7-29064	5′ aattgaacgaggagccgtat 3′
	*rps*7-29001	5′ ctgccattctatgagtctct 3′
8	*rrn*16S-837F	5′ ggagtacgttcgcaagaatg 3′
	*rrn*16S-1469R	5′ ttccagtacggctaccttgt 3′

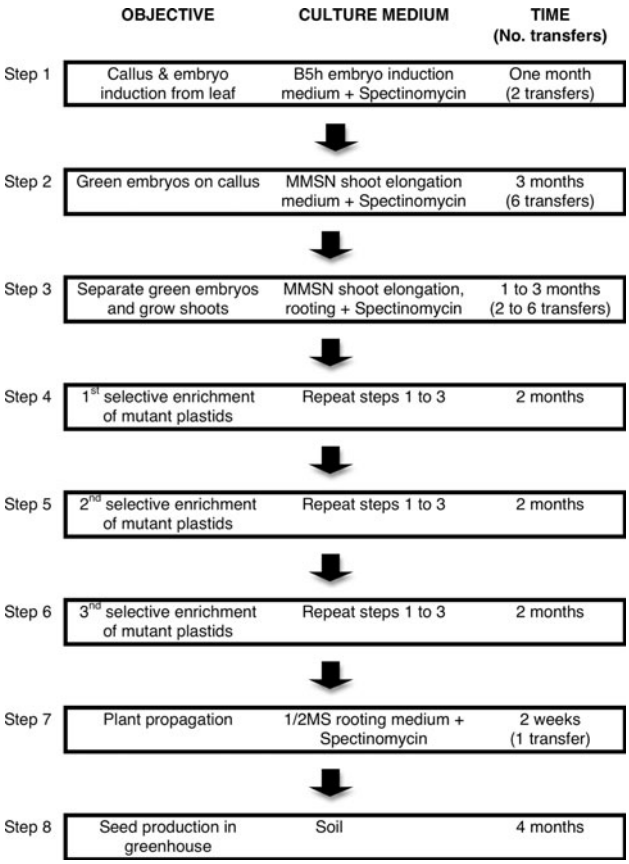

Fig. 1 An overview of the protocol for the isolation of spectinomycin-resistant mutants in *Medicago sativa* RegenSY-T2 cultures

the mutation that confers spectinomycin resistance is likely to occur in only a single ptDNA copy. Genetically stable plant lines were obtained through a gradual process of diluting the non-mutant wild-type ptDNA copies during growth on a selective medium and regenerating new plants from the resistant sectors on a selective medium. Genetically stable lines were obtained after three cycles of plant regeneration on a selective medium containing 50 mg/l spectinomycin. The spectinomycin resistance phenotype of the regenerated plants was judged by formation of green callus on selective spectinomycin medium (Fig. 2a). Repeating steps 1–3 in Fig. 1 with the spectinomycin-resistant shoots took significantly shorter time than the original screen, 2 months instead of 4–6 months, because the mutant plastids in the regenerated shoots were already present in significant numbers.

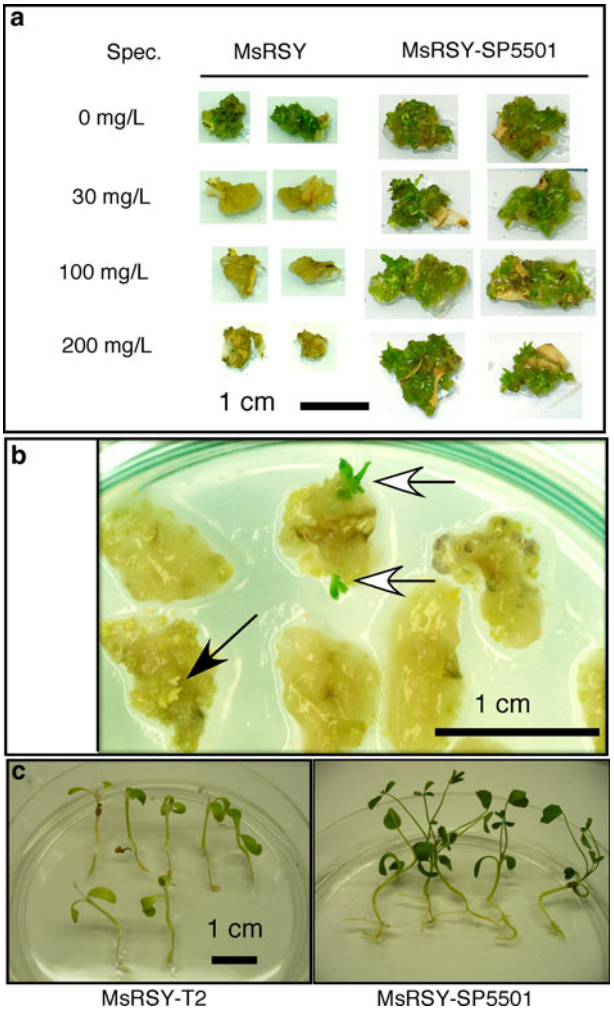

Fig. 2 Selection of spectinomycin-resistant mutants in embryogenic *Medicago sativa* RegenSY-T2 callus. **a** Spectinomycin inhibits greening and shoot formation in wild-type RegenSY-T2 callus on selective MMSN medium, but not in the mutant MsRSY-SP5501 culture. Note that the mutant is resistant up to 200 mg/l spectinomycin. **b** Spectinomycin-resistant clone appearing on MMSN medium containing 25 mg/l spectinomycin. Note green resistant (*open arrowhead*) and white sensitive embryos (*black arrow*) on the calli. **c** Inheritance of spectinomycin resistance in the seed progeny. Wild-type MsRSY-T2 and spectinomycin-resistant MsRSY-SP5501 seed was germinated on ½ MS medium containing 100 mg/l spectinomycin. The sensitive MsRSY-T2 primary leaf is bleached and further shoot development is inhibited while the resistant MsRY-SP5501 progeny developed resistant green primary and one or two trifoliate leaves. The seedlings are 4 weeks old

The shoots were then rooted and the plants were transferred to the greenhouse to obtain seed. The regenerated plants had a normal phenotype indistinguishable from wild-type plants, exhibiting no obvious deleterious phenotypes caused by the mutations. So far seed was obtained from 17 of the mutant lines (all lines in Table 3, except SP4252). Three spectinomycin-resistant lines yielded plants with abnormal morphology and were not studied.

Expression of spectinomycin resistance in the seed progeny

To confirm seed transmission of spectinomycin resistance, seed was germinated on ½ MS medium containing 100 mg/l spectinomycin (Fig. 2c). Wild-type MsRY-T2 seed germinated and the cotyledons were green. However, the primary leaf was bleached and further development of the shoot system was inhibited by spectinomycin. In contrast, within the same time period (4 weeks), the resistant seeds developed a green primary leaf and a shoot system with several green trifoliate leaves. Heritable transmission of spectinomycin resistance has been confirmed in seventeen independently isolated lines in the selfed seed progeny (all lines in Table 3, except SP4252).

Localization of mutations conferring spectinomycin resistance in the plastid 16S rRNA

Spectinomycin inhibits protein synthesis on the prokaryotic type 70S plastid ribosomes by direct interaction with the 16S rRNA. Spontaneous mutations conferring spectinomycin resistance most commonly arise by mutations in the 16S rRNA preventing spectinomycin binding (Svab and Maliga 1991). Therefore, we PCR amplified and sequenced

Table 2 Frequency of spectinomycin-resistant mutants in *M. sativa* RegenSY-T2 leaf culture

Spectinomycin (mg/l)	0	25	30	40	50	60	75	100	200	500
No. of explants	200	200	400	400	200	340	200	200	200	200
No. of mutants	n/a	2	1	1	3	0	0	0	0	0

Table 3 Mutations in the plastid 16S rRNA in the spectinomycin-resistant *M. sativa* RegenSY-T2 mutants

16S rRNA stem sequence	Mutation	Mutant lines
5′-CGTCAG-3′ (1013–1018)	G to C (1014)	MsRSY-SP24151
		MsRSY-SP2911
		MsRSY-SP3531
		MsRSY-SP5501
		MsRSY-SP5502
		MsRSY-SP5503
		MsRSY-SP7141
		MsRSY-SP7151
		MsRSY-SP9601
		MsRSY-SP10401
		MsRSY-SP15131
		MsRSY-SP15232
		MsRSY-SP18291
	C to A (1016)	MsRSY-SP4251
		MsRSY-SP4252
5′-TGACGTC-3′ (1138–1144)	A to T (1140)	MsRSY-SP8401
	C to G (1141)	MsRSY-SP8301
	C to T (1141)	MsRSY-SP7171

Nucleotide position is given in 16S rRNA sequence

The *rrn16* gene sequences have been deposited in GanBank as follows: wild-type RegenSY-T2, JX185400; MsRSY-SP4251, JX185401; MsRSY-SP5501, JX185402; MsRSY-SP7171, JX185403; MsRSY-SP8301, JX185404; and MsRSY-SP8401, JX185405

the region of the plastid *rrn16* gene known to be involved in spectinomycin binding. Spectinomycin resistance in each of the lines could be attributed to nucleotide exchanges known to confer spectinomycin resistance in other species (Table 3). Mutations in the lines affected a stem structure formed by base pairing in the 16S rRNA. Some of the mutations mapped to the 5′-CGTCAG-3′ region (nucleotides 1013–1018). The G to C point mutation at nucleotide position 1014 in the 16S rRNA was the most frequent; 13 out of the 18 lines carried this exchange. This creates a new *Dde*I restriction site. The C to A (1016) mutation was less frequent, represented by two lines only, MsRSY-SP4251 and MsRSY-SP4252. The second region (5′-TGACGTC-3′, 1138–1144) where spectinomycin resistance mutations clustered contains an *Aat*II restriction site (GACGTC). The A to T (1140), C to G (1141) and C to T (1141) mutations are represented by a single example each and eliminate the *Aat*II restriction site, a convenient marker for screening PCR amplified ptDNA fragments.

Interestingly, spectinomycin resistance mutations in *M. sativa* were biased towards the 1013–1018 region. In *Nicotiana sylvestris* we found the opposite bias: each of the seven spontaneous spectinomycin resistance mutations affects the *Aat*II site (1138–1144 region), indicating species-specific differences in the likelihood of nucleotide exchanges conferring spectinomycin resistance (Thyssen et al. 2012).

Testing biparental plastid inheritance using the SP8301 SNP

The A to T mutation in the SP8301 mutant eliminates an *Aat*II restriction site, providing a convenient marker to distinguish the SP8301 ptDNA from all wild-type (spectinomycin sensitive) ptDNA. To confirm the utility of the spectinomycin resistance mutations for ptDNA genetic analyses, we obtained seed progeny from reciprocal crosses between the RegenSY-T2 SP8301 mutant and the Szapko57 lines. To identify the plastid types in the F1 progeny, total cellular DNA was PCR amplified, digested with the *Aat*II restriction endonuclease, and separated in an agarose gel (Fig. 3). When the RegenSY-T2 parent was the mother, we found seven paternal, four maternal, and one biparental (mixed) ptDNA in 12 F1 plants in total. When the Szapko57 line was the mother, only paternal ptDNA could be detected.

Discussion

We report here isolation of spontaneous spectinomycin-resistant mutants in *Medicago sativa* conferred by mutations in the 16S rRNA. The mutant plants are very similar

Fig. 3 Plastid inheritance in the RegenSY-T2 SP8301 and Szapko57 F1 progeny. Shown are *Aat*II-digested PCR fragments derived from the cross with RegenSY-T2 SP8301 (SP) as maternal (*lanes 1–12*) and paternal (*lanes 13–22*) parent. *Aat*II-digested parental RegenSY-T2 SP8301 (SP) and Szapko57 (Sz) amplicons are also shown. Mw is the 100-bp Fermentas Molecular Weight marker. Note that the 634-bp mutant fragment does not contain an *Aat*II site, whereas the wild-type amplicon is cleaved to 330- and 304-bp fragments, and that the 330- and 304-bp fragments do not separate in the gel

to the wild type, and do not have any readily detectable, deleterious phenotype. This is in contrast to the plastid-encoded 16S rRNA kanamycin resistance mutations in *Medicago sativa* that were associated with a chlorotic phenotype during early development and a severe (~85 %) reduction in biomass (Rosellini et al. 2004). Spontaneous spectinomycin-resistant mutants could be readily obtained at the frequency of about one mutant in ~300 explants. The ready identification of spectinomycin-resistant mutants in *Medicago sativa* is in contrast to the absence of spectinomycin-resistant mutants in soybean (*Glycine max*), a related leguminous plant (Dufourmantel et al. 2004). Spontaneous spectinomycin-resistant mutants were also readily obtained in the solanaceous species *Nicotiana tabacum* (Fromm et al. 1987; Svab and Maliga 1991), *Nicotiana sylvestris* (Thyssen et al. 2012) and *Solanum nigrum* (Kavanagh et al. 1994), and two species in the Brassicaceae family, *Lesquerella fendleri* (Skarjinskaia et al. 2003) and *Arabidopsis thaliana* (Azhagiri and Maliga 2007). Spectinomycin resistance in each case was the result of a point mutation within the same conserved stem structure preventing spectinomycin binding and thereby preventing inhibition of plastid protein synthesis on the organelle's prokaryotic type 70S ribosomes.

The spectinomycin-resistant mutants were isolated in embryogenic leaf cultures following a protocol that is normally used to recover transplastomic clones. Recovery of spontaneous spectinomycin-resistant mutants in plastid transformation experiments is very common, because the protocol for the recovery of spontaneous spectinomycin-resistant mutants is identical with the protocol used for the recovery of transplastomic clones (Svab et al. 1990; Svab and Maliga 1993). Therefore, we expect to achieve plastid transformation in *Medicago sativa* using the tissue culture selection protocol reported here. The mutants described here will also be useful to study plastid inheritance. The plastid genome in ~80 % of the higher plant species, including the important agronomic crops such as wheat, rice, maize, and tobacco and the model plant *Arabidopsis thaliana* is transmitted to the progeny by the maternal parent only (Azhagiri and Maliga 2007; Hagemann 2002; Mogensen 1996; Reboud and Zeyl 1994). *M. sativa* belongs to the minority (~20 %) of higher plant species, in which plastids are transmitted biparentally (Schumann and Hanckok 1989; Smith 1989; Smith et al. 1986). Spectinomycin resistance was a useful marker to study plastid inheritance in *Nicotiana tabacum* (Ruf et al. 2007; Svab and Maliga 2007) and *Arabidopsis thaliana* (Azhagiri and Maliga 2007). Therefore, we expect to gain new insights into the biparental mode of plastid inheritance in crosses with the new alfalfa mutants, in which the spectinomycin resistance mutations will serve as SNPs. The five *rrn16* plastid mutations described here will be suitable to distinguish six plastid types, including the wild-type ptDNA. We have shown the utility of the spectinomycin resistance mutation by testing biparental plastid inheritance in reciprocal crosses between the RegenSY-T2 SP8301 mutant and the Szapko57 lines. We have found strong paternal bias in plastid transmission, in line with earlier reports on plastid inheritance in *M. sativa* (Masoud et al. 1990; Schumann and Hanckok 1989; Smith 1989).

In addition, we expect to incorporate the new mutations in cisgenic plastid transformation vectors, which will be used to obtain cisgenic crop plants that have been genetically modified with one or more genes isolated from a crossable donor plant. An example for a cisgenic plant is a cisplastomic tobacco plant, the plastid genome of which was transformed with a fatty acid desaturase gene using only plant sequences (Craig et al. 2008). Although selection of engineered plastids by *rrn16* vectors is likely to be less efficient than selection for the *aadA* transgene (Svab and Maliga 1993), reduced regulatory hurdles in a changing regulatory environment for cisgenic plants, as compared with their transgenic counterparts, might make the investment in time worthwhile (Schouten et al. 2006; Waltz 2011).

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