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Ploidy of embryogenic *Medicago sativa* subsp. *falcata* germplasms

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Abstract – The germplasms *Medicago sativa* L. subsp. *falcata* WY-RF1 (Reg. no. GP-263, PI 564263) and WY-RF2 (Reg. no. GP-264, PI 564262), maintained at the Wyoming Agricultural Experimental Station, were reported to have improved regeneration capacity, to be embryogenic and are registered as diploid. We show that, in fact, germplasm WY-RF1 is diploid but WY-RF2 is tetraploid. The latter is more vigorous, but the two have close flowering times. The mean 2C nuclear DNA values from flow cytometry using ethidium bromide, verified upon many seeds including those from descendants, were: 1.88 pg for RF1 (1C = 907 million base pairs) and 3.41 pg for RF2 (1C = 1645 million base pairs). Chromosome numbers in root meristem metaphases were, respectively, $2n = 16$ and 32 . This distinction is capital for biotechnological and genetic studies. (© Inra/Elsevier, Paris.)

genome-size / ploidy / flow cytometry / embryogenesis / *Medicago sativa* L. subsp. *falcata*

Résumé – Ploidie de lignées embryogènes de *Medicago sativa* subsp. *falcata*. À la station expérimentale d'agriculture du Wyoming (États-Unis), deux lignées de *Medicago sativa* L. subsp. *falcata* WY-RF1 (Reg. no. GP-263, PI 564263) et WY-RF2 (Reg. no. GP-264, PI 564262) ont été sélectionnées pour leurs capacités de régénération et d'embryogenèse in vitro. Ces deux lignées sont définies comme étant diploïdes. Nous montrons, entre autres, que si la lignée WY-RF1 est bien diploïde par contre la lignée WY-RF2 est tétraploïde. Le temps nécessaire pour obtenir la floraison est proche pour les deux lignées mais la tétraploïde est plus vigoureuse. La valeur moyenne de la teneur en ADN, déterminée par cytométrie en flux avec le bromure d'éthidium, est de 1,88 pg pour WY-RF1 (1C = 907 millions de paires de bases) et 3,41 pg pour WY-RF2 (1C = 1645 millions de paires de bases). Le nombre de chromosomes, dans les pointes racinaires, est de 16 pour WY-RF1 et de 32 pour WY-RF2. Ces lignées sont utilisées dans des expériences génétiques et de transformations, ce qui rend capital la connaissance de leur niveau de ploïdie et de leur teneur en ADN. (© Inra/Elsevier, Paris.)

taille du génome / ploïdie / cytométrie en flux / embryogenèse / *Medicago sativa* L. subsp. *falcata*

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1. INTRODUCTION

Medicago sativa L. subsp. *sativa* (alfalfa) can be readily regenerated from tissue, apparently owing to its tetraploidy [2], and somatic embryogenesis is obtained [8]. The diploid *Medicago* species unfortunately are much more recalcitrant to regeneration, although major progress has been made [6, 10, 11, 14–16]. The agronomic tetraploid cultivars of subsp. *sativa* are composite, often including part of the genome of *Medicago sativa* subsp. *falcata*. Germplasms of these contributing subspecies with high regeneration capacity are useful, notably if they are diploid. They may be essentially allogamous, but with a certain frequency of autogamy. Here we report on two germplasms of *Medicago sativa* subsp. *falcata* with high regeneration capacities [10], one of which we show to be tetraploid in contrast to its registration as a diploid.

2. MATERIALS AND METHODS

Seeds of *Medicago sativa* L. subsp. *falcata* WY-RF1 (Reg. no. GP-263, PI 564263) and WY-RF2 (Reg. no. GP-264, PI 564262), both registered as diploid, were kindly provided by R.W. Goose at the Wyoming Agricultural Experimental Station, as duplicate dispatches to A. Kondorosi and to E. Kondorosi. WY-RF1 and WY-RF2 were developed by Goose and Li by three cycles of recurrent phenotypic selections from accessions PI 251830 (Austria) and PI 260993 (Russia), so improving from initial somatic embryogenesis frequencies of 46 and 39 % to 100 and 93 %, respectively. Their culture in vitro was only used as an assay and was never the source of germplasm.

Our plants were sown in a glasshouse with 16 h natural light complemented by incandescent and fluorescent sources for 15 W·m⁻² and 22 °C constant temperature, 70 % relative humidity, using vermiculite and nutrient solution [3], conducive to good pollen viability [4]. Field plants were also grown at Gif-sur-Yvette.

2.1. Light microscopy

Root tips of 2 cm were taken from germinated seeds, transferred to saturated α -bromonaphthalene [1] for 60

min at room temperature to accumulate metaphases. After rinsing with distilled water, root-tips were fixed in absolute ethanol/acetic acid, 3:1 (v/v) for 24–48 h, then stored at –20°C. Later, fixed roots were abundantly rinsed with water and the digestion of the meristem was carried out using an enzyme mixture (0.25 % pectolyase Y23, 3 % cellulase Onozuka R-10, 0.4 M sorbitol, pH 5.5) at 37 °C. After 60 min maceration, the root-tips were transferred to cold 45 % acetic acid for 15 min. Single root-tips were taken with a 20 μ L pipette onto slides before gentle squashing in one drop of 45 % acetic acid. A Diaplan phase contrast optic was used with PanF 50ASA Ilford film.

2.2. Flow cytometry

Nuclei were isolated mechanically, similar to [5]. One leaflet was chopped with a razor-blade in 600 μ L cold buffer [9] supplemented with 5 mM sodium metabisulfite with about 1 cm² piece of leaf of the internal standard, *Petunia hybrida* PxPC6 (2n = 2x = 14; 2C = 2.85 pg DNA) [12]. This was filtered through 48 μ m nylon, treated with 5 U/mL final Ribonuclease A (Boehringer no. 109169 DNase-free) and stained with 50 μ g/mL ethidium bromide for 15 min, prior to flow cytometry (EPICS V, Coultronics France, Margency) with the laser at 514 nm and taking the emission above 590 nm. The ratio of fluorescence intensity of the 2C peaks was used to calculate the 2C value of *Medicago*, taking a linear relationship. Data were treated by analysis of variance followed by the F-test.

3. RESULTS

For glasshouse plants with 2–3 trifoliate leaves, the 2C nuclear DNA means $\pm$ SE from flow cytometry with ethidium bromide were: 1.88 $\pm$ 0.022 pg (degrees of freedom, df = 11) for RF1 and 3.41 $\pm$ 0.023 pg (df = 8) for RF2. The F-test indicated that the variance was the same in the two populations, RF1 and RF2. These mean values have a confidence interval of about 2 %. However, with field samples, exceptions occurred during the 3 year programme even on clonal material when our estimates became dependent upon the day of analysis: at its extreme, 7 % systematic variation was observed

under particular seasonal conditions and growth stages.

Self-pollination seeds were harvested from these glasshouse plants and resown. In this second generation, sibling plants from RF1 gave 2C values (each the mean of six measurements) of 1.86, 1.88, 1.90 and 1.92 pg. Likewise, for the second generation of RF2, one lot of siblings gave 2C values of 3.53, 3.58 and 3.66 pg; a second lot of siblings gave 3.42, 3.43 and 3.46; a third lot 3.41, 3.43 and 3.44 pg; a fourth lot 3.39, 3.41 and 3.46 pg. In more than 100 independent measurements of plants grown under glasshouse or field conditions, no exception has been found to the clustering of RF1 and RF2.

Chromosome numbers on six individuals of each germplasm were consistent: $2n = 16$ for RF1 and $2n = 32$ for RF2 (figure 1).

Under glasshouse conditions, RF1 and RF2 have their flowering dates close: flower emergence began at 46–52 days and 50–56 days, respectively. The generation time was 4 months. In the field, RF1 began spring growth 1 week later but the genotypes had similar flowering dates (mid- till late-June at Gif-sur-Yvette according to the year). RF2 was clearly more vigorous: in the field, the average surface of an adult leaf was about double that of RF1; in the glasshouse, it was 20 % larger.

4. DISCUSSION

Here the 2C DNA values, the chromosome counts, and the phenotype show that *M. sativa* subsp. *falcata* RF1 is diploid ($2n = 16$ with 1.88 pg DNA) and RF2 is tetraploid ($2n = 32$ with 3.41 pg DNA). These genotypes originate from Austria (RF1) and Russia (RF2).

In an earlier study [5] the closely related diploid Russian ecotype of *M. sativa* subsp. *quasifalcata* had 2C DNA of 1.72 pg; RF2 has double this value. Other values concerning *M. sativa* subsp. *falcata*, deduced by Blondon et al. [5] from Essad [7], were 1.49 and 1.97 pg for diploid ecotypes and 2.82 pg for a tetraploid. There is clearly intraspecific variation of both genome size and ploidy level. As the

Figure 1. Metaphase spreads of α -bromonaphthalene treated root tissues from *Medicago sativa* subsp. *falcata*. (upper) WY-RF1; (lower) WY-RF2. Phase contrast; magnification $\times 1\,800$.

present study concerns germplasm derived from different sources, the fact that the tetraploid genome is not exactly twice that of the diploid is not surprising. Over 100 plants have been analysed, covering two generations, without finding any exception to these systematic diploid and tetraploid groupings. This is not surprising: we recall that these germplasms are not the product of in vitro manipulation, so somaclonal variation should not be present.

The 1C value for RF1 was 0.94 pg, equivalent to 907 million base pairs. The mean genome size of RF2, 1645 million base pairs, was 181 % that of RF1.

Given the intraspecific variation of the *Medicago* genomes which exhibit much plasticity, given their frequent use for genetic manipulation and their agronomic significance, we propose that flow cytometry should be more routinely applied as a quality control [12]. One demonstration of this practice comes from research into somatic embryogenesis in rose cultures [13]. This is all the more necessary when culture conditions risk to introduce somaclonal variation, such as during in vitro manipulations.

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