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Collection of mutants for functional genomics in the legume *Medicago truncatula*

O. Calderini[1]*, M. Carelli[2], F. Panara[1], E. Biazzi[2], C. Scotti[2], A. Tava[2], A. Porceddu[3] and S. Arcioni[1]

[1]*CNR – Istituto di Genetica Vegetale, Perugia, Italy,* [2]*C.R.A. – Centro per le Produzioni Foraggere e Lattiero-Casearie, Lodi, Italy and* [3]*Dipartimento Di Scienze Agronomiche e Genetica Vegetale Agraria, Università degli Studi di Sassari, Sassari, Italy*

Abstract

We have established mutant collections of the model species *Medicago truncatula* according to current protocols. In particular, we used a transposon (*Tnt1*) tagging method and an ethyl methanesulfonate (EMS) mutagenesis approach (TILLING). The collections were subjected to both forward and reverse genetics screenings, and several mutants were isolated that affect plant traits (e.g. shoot, root developments, flower morphology, etc.) and also biosynthetic pathways of secondary compounds (saponins and tannins). Genes responsible for some of the mutations were cloned and further characterized.

Keywords: *Medicago truncatula*; mutants; Transposon tagging; TILLING

Introduction

Legume species are a major source of protein for both human and animal nutrition. Proteins from legumes are highly sustainable compared with those obtained from other sources as, among other reasons, legume plants have positive impact on soil fertility due to their ability to fix nitrogen via rhizobia symbioses. Functional genomics in model species such as *Medicago truncatula* offers the possibility to speed up the knowledge of the genetic control of traits of agronomic interest and translate such knowledge to crop legumes via different means (Cannon *et al*., 2009). *M. truncatula* was chosen as a model for legumes because it is diploid, with relatively small genome (450–550 Mb), self-fertile, relatively easy to transform, and it has a short generation time. Mutants in *M. truncatula* are currently obtained by three main strategies: (1) chemical mutagenesis mainly based on EMS and the subsequent reverse genetic screening TILLING; (2) deletion mutagenesis based on fast neutron bombardment and γ-rays; (3) insertional mutagenesis based on transposons such as *Tnt1*. Several public resources are being developed both in Europe and in the USA, and some are available for public screening (Tadege *et al*., 2009). In the frame of an Italian functional genomics project 'Post-genomics of forage legumes' (MIUR-FIRB), we developed three complementary mutant collections that can be an integration to the resources already available at the international level (Porceddu *et al*., 2008); we have also produced several hundreds of transposon tagged lines as part of an European collection supported by the EU FP6 'Grain Legumes Integrated Project'.

Materials and methods

Production of the collections

Transposon tagging (Tnt1)

A starter line from the R108-1 genotype harbouring three *Tnt1* insertions was produced via *Agrobacterium* transformation with the construct Tnk23. Transposition was induced *in vitro* according to d'Erfurth *et al.* (2003). Mutant lines were also obtained in the Jemalong background using starter lines as reported in Iantcheva *et al.* (2009).

* Corresponding author. E-mail: ornella.calderini@igv.cnr.it

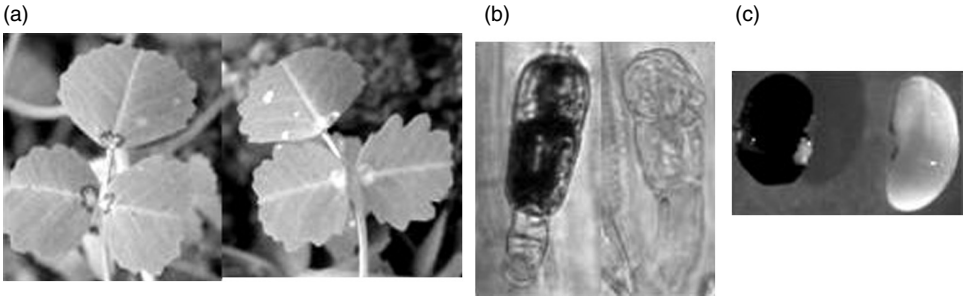

Fig. 1. Phenotypic variation among *Tnt1* lines: (a) lack of anthocyanins in the leaves, (b) no tannin staining in glandular trichomes and (c) seeds.

Activation tagging

Transfer-deoxyribonucleic acid (T-DNA) transformed lines were obtained in the background R108-1 with the pSKI074 vector (Weigel *et al.*, 2000).

TILLING

M. truncatula seeds from cv. Jemalong genotype 2HA10−9−3 were treated with 0.15% EMS to generate a mutant collection. M2 seeds were collected from 2281 M1 individuals together with 65 tester plants from the control treatment (0% EMS). DNA was extracted from almost all the M2 families, and M3 seed was collected; about 1900 plants are present in the final EMS mutant collection (DNA and M3 seed).

Results and discussion

Tnt1 *tagging*

We have produced approximately 1000 R0 lines in the R108 background and 1000 lines in the Jemalong background; all the lines are stored as legumes/seeds. In a small scale experiment, 96 flanking sequence tags (FSTs) were recovered from 13 R108 mutant plants. BLAST analysis of the FSTs showed that at least 47% of insertions are inside genes, 27% are in sequenced but not yet annotated *M. truncatula* BAC clones and 25% do not show similarity with any sequence in the database. This preliminary molecular analysis of the *Tnt1* insertion sites confirms the data from Tadege *et al.* (2008); in fact in a larger scale experiment, these authors reported an overall FSTs match with *M. truncatula* sequences in the database of 78.6, 60.2% of which having high homology with a range of known genes. The rate of transposition in R108 was as high as reported in the literature (~80%). Differently in the Jemalong background, we noticed a lower transposition efficiency (60%), which was also reported in Iantcheva *et al.* (2009).

Tnt1 R0 lines were visually screened during multiplication, and several mutants were identified concerning leaf, flower and root morphology. A forward screening was conducted on the content of condensed tannins in the leaves, which was also extended to 2000 lines from the collection at the Samuel Roberts Noble Foundation (Tadege *et al.*, 2008). Even in this case, several mutants were identified, namely two main phenotypic classes

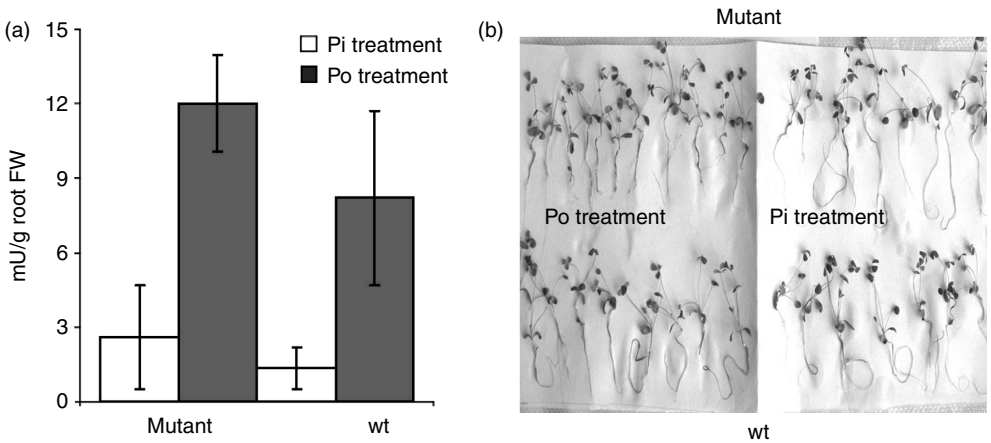

Fig. 2. Phytase activity in the mutant MtPHY1598: (a) enzymatic assay (five root bulks of ten plants/treatment); (b) histochemical assay on intact roots. FW, fresh weight; Pi, inorganic phosphorous; Po, organic phosphorous; wt, wild type.

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could be detected: plants lacking both anthocyanins and tannins and plants lacking anthocyanins but producing tannins (Fig. 1). For several of the mutants mentioned, it was possible to isolate a candidate gene based on FST recovery and segregation analysis.

Activation tagging

A small population (128) of activation tagged lines was produced in R108 as an initial test of transformation. The population was screened for the presence of haemolytic saponins in the leaves, and a loss-of-function mutant was identified. The corresponding gene was cloned and characterized (Carelli *et al.*, unpublished).

TILLING

The TILLING collection is based on the initial treatment of *M. truncatula* seeds from cv. Jemalong genotype 2HA10–9–3 with 0.15% EMS. The M2 generation (1658 families, represented by 1–5 plants/family for a total of 2560 plants) and 34 tester families were grown in a cold greenhouse and phenotypically screened for identifying mutants. DNA was extracted from almost all the M2 families, and M3 seed has been collected; about 1900 plants are present in the final EMS mutant collection (DNA and M3 seed). TILLING analysis was performed in collaboration with the Genomic Platform of the Parco Tecnologico Padano (Lodi, Italy); the estimated rate of mutation was one mutation/Kbp/400 plants similar to the collection generated by the R. Cook laboratory as reported by Anè *et al.* (2008). It is worth of mention that two alleles were recovered for the candidate gene related to the absence of haemolytic saponin phenotype; the mentioned mutants lack haemolytic saponins in the leaves (Carelli *et al.*, unpublished). Two more genes were analysed by TILLING: the extracellular phytase MtPHY1 (Xiao *et al.*, 2005) and the trypsin inhibitor MsTI (Balestrazzi *et al.*, 2004). Interesting results were obtained with an MtPHY mutant (MtPHY1598) that showed an enhanced phytase activity when grown on organic phosphorus (Fig. 2).

The collections reported in the present paper have proved to be efficient in isolating mutants related to relevant agricultural traits and in cloning the underlying genes. The genetic information achieved can be easily translated to alfalfa, a closely related crop to *M. truncatula* and one of the major forage crop worldwide and possibly used in breeding programmes.

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