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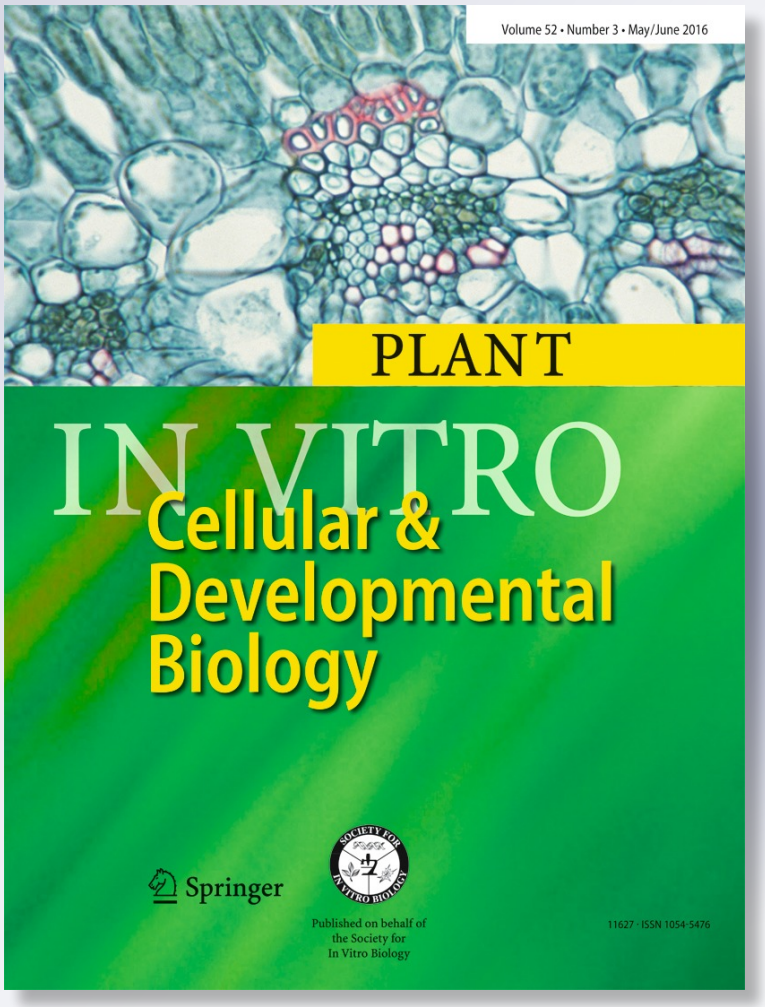

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PLANT TISSUE CULTURE

Tnt1 retrotransposon as an efficient tool for development of an insertional mutant collection of *Lotus japonicus*

Anelia Iantcheva[1] · Miglena Revalska[1] · Grigor Zehirov[2] · Irina Boycheva[1] · Kevin Magne[3] · Mariana Radkova[1] · Pascal Ratet[3] · Valya Vassileva[2]

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Abstract The *Tnt1* retrotransposon of tobacco (*Nicotiana tabacum*) has proven to be a very efficient mutagen for the model legume *Medicago truncatula* ecotype 108 and cultivar Jemalong 2HA and for economically important plants, such as soybean and potato. In this study, the activity of *Tnt1* in the model legume *Lotus japonicus* L. was tested. First, a new regeneration and transformation protocol was developed for *L. japonicus* that represents a new tool for legume mutagenesis and reverse genetics. Using this protocol, the *Tnt1* retrotransposon was introduced into *L. japonicus* by *Agrobacterium tumefaciens*-mediated transformation, and primary transgenic lines, named starter lines, were constructed. *In vitro* regeneration via indirect somatic embryogenesis using starter lines harboring two to eight copies of the transgene resulted in new *Tnt1* transposition events. The *Tnt1* retrotransposon remained inactive during plant growth and in the T_1 progeny, indicating that it is well suited for insertional mutagenesis in *L. japonicus*.

Keywords *Tnt1* retrotransposon · *Lotus japonicus* · Insertional mutagenesis · Starter lines · Regeneration · Embryogenesis

Introduction

Legume plants are a sustainable source of food and feed around the world. They establish beneficial symbiotic interactions with rhizobial bacteria, resulting in special structures, named root nodules. In these structures, rhizobia fix atmospheric nitrogen that plants can use, reducing the need for external fertilizers. Considering the importance of legumes, greater emphasis should be placed on the collection of genetic information for legume species.

Recent advances in "omics" approaches and studies performed on the two model legumes, *Medicago truncatula* Gaertn. (Cook 1999) and *Lotus japonicus* L. (Stougaard 2001), are adding to the understanding of legume biology. Both legume models have relatively small genomes, short life cycles, and can be easily regenerated and transformed. These advantages make it possible to analyze the phenotypic and functional features associated with gene and protein up- and down-regulation in intact plants, as well as the expression patterns of promoters and organelle-specific markers in intact legume cells (Boycheva *et al.* 2015; Revalska *et al.* 2015).

An important goal of legume genomics is knowledge transfer between model and crop legumes, providing means to improve plant breeding efficiency. Information on certain common traits of legumes, including signal transduction events that lead to nodule formation and synthesis of flavonoids and their glycosides, is considerably transferable from models to crops. Knowledge gained from the model legumes on traits of agronomic importance, such as plant architecture, yield components, and stress tolerance, could have a great

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impact for the legumes like alfalfa (*Medicago sativa*) (Zhou *et al*. 2011), pea (*Pisum sativum*), and clover (*Trifolium* sp.). Legume plants and their microsymbionts might even provide a basis for improvement of the performance of other crop species, such as tomato (*Solanum lycopersicum*), sunflower (*Helianthus annuus*), cotton (*Gossypium hirsutum*), corn (*Zea mays*), and rice (*Oryza sativa*) (Dakora 2003).

Because of worldwide efforts, the genomes of *M. truncatula* and *L. japonicus* are now available (Sato *et al*. 2008; Young *et al*. 2011), allowing for comparative genomic analyses. Several technological platforms, such as ethyl methanesulfonate (EMS) mutagenesis combined with targeted induced local lesions in genome (TILLING) techniques (Perry *et al*. 2003), and microarrays of several thousand non-redundant cDNA clones for transcriptomic and proteomic profiling (Wienkoop and Saalbach 2003), have moved forward functional genomic studies in the model legumes. However, there is still a striking incongruity between the level of advancement of these technologies and the efficiency of exploitation of some basic *in vitro* procedures for obtaining transgenic plants and their analysis.

Construction and phenotypic screening of mutant libraries is an effective approach to identify the function of unknown genes (Cigan *et al*. 2005). Different types of mutagenesis, such as T-DNA insertions, chemical mutagens, ionizing radiation, and fast neutron bombardment, have been attempted in legumes; however, with these tools, only a few *M. truncatula* and *L. japonicus* mutant lines have been generated and characterized. Imaizumi *et al*. (2005) demonstrated that T-DNA tagging is possible in *L. japonicus* but it is labor-intensive because of the relatively low transformation efficiency. Similarly, the maize class II transposon *activator* and *dissociation* transposes in the *L. japonicus* genome (Thykjaer *et al*. 1995), but the low efficiency of transposition does not permit its utilization as an efficient tag. To date, only one of the reported symbiotic mutants has been isolated by this transposon tagging strategy (Schauser *et al*. 1999). In contrast, *LORE1*, an endogenous retrotransposon in *L. japonicus*, is mobile in regenerated lines and can be used to establish larger mutagenized populations (Fukai *et al*. 2012). Genome-wide *LORE1* mutagenesis and high-throughput insertion detection in *L. japonicus* have been already reported (Urbanski *et al*. 2012). Currently, around 200, 000 flanking sequence tags (FSTs) have been generated (*Lotus* base http://users-mb.au.dk/pmgrp), allowing the identification of insertions in the majority of the genes of *L. japonicus*.

Retrotranspons are a type of mobile elements (class I) that transpose to new sites in the genome via an RNA intermediate. Their primary transcripts are retro-transcribed into DNA copies by a reverse transcriptase (Wessler 2006), and these copies are inserted back into the genome in multiple places (Kumar and Bennetzen 1999). Retrotransposon-induced mutations are relatively stable because there is no excision during replicative transposition. In addition, some retrotransponsons can be highly mutagenic because of prevalent transposition to gene-rich, low-copy regions, making them suitable for tagging genes in plant species with large genomes.

The tobacco (*Nicotiana tabacum*) retrotransposon *Tnt1* is one of the few elements to achieve a saturation mutagenesis in tobacco and heterologous plant genomes. d'Erfurth *et al*. (2003) first described it during the early steps of *in vitro* transformation and regeneration in the R108 ecotype of *M. truncatula*. Later, *Tnt1* insertional mutagenesis was successfully used for large-scale mutagenesis of *M. truncatula* ecotype R108 (Tadege *et al*. 2008; Cheng *et al*. 2011), during *in vitro* regeneration in *M. truncatula* cv. Jemalong (Iantcheva *et al*. 2009), and in the important crops lettuce (*Lactuca sativa* L.; Mazier *et al*. 2007), soybean (*Glycine max* [L.] Merr.; Cui *et al*. 2013), and potato (*Solanum tuberosum* L.; Duangpan *et al*. 2013).

The high efficiency of *Tnt1* transposition during *in vitro* regeneration of *M. truncatula*, resulting in multiple *Tnt1* insertions in the newly regenerated lines, encouraged evaluating the *Tnt1* tagging as an additional and complementary strategy to the *LORE1* system of mutagenesis in the model legume *L. japonicus*. As a first step, a new protocol for *in vitro* regeneration and transformation via indirect somatic embryogenesis was developed and used to introduce *Tnt1* via *Agrobacterium*-mediated transformation. To reactivate *Tnt1* inserted copies, 'starter lines' containing different numbers of inserted *Tnt1* and having good regeneration capacity were constructed. *Tnt1* transposition frequency was evaluated in the primary regenerated (R_0) lines (starter lines), R_1 lines, and their T_1 progeny. *In vitro* regeneration resulted in new transposition events in the regenerated R_1 lines, and the insertions were stable in T_1 progeny. These findings demonstrate that *Tnt1* can serve as an additional and alternative to the *LORE1* retrotransposon mutagenesis system for the construction of an insertion mutant collection for *L. japonicus*.

Material and Methods

Plant material and growth conditions Seeds of *L. japonicus* ecotype B-129 Gifu, a kind gift of Dr. Hiroshi Kouchi, were scarified with sandpaper and surface sterilized with 70% (*v/v*) ethanol for 30 s, followed by 0.1% (*v/v*) mercury chloride ($HgCl_2$) for 8 min, then rinsed at least three times with sterile distilled water, and germinated on Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) supplemented with 3% sucrose (*w/v*), 0.7% (*w/v*) Phyto agar (Duchefa, Haarlem, the Netherlands). Germinated seedlings were then propagated by cuttings. Explants collected from 30–40-d-old *in vitro* plants were used to initiate callus tissue. *In vitro* plant material was grown in Magenta™ boxes (60 × 60 × 96 mm; Sigma-Aldrich®, St. Louis, MO) in a growth chamber at

24°C, with a 16-h photoperiod and white cool light, tube intensity of 30 μmol m^{-2} s^{-1} (Solar Leading Group Co. Ltd., Jieyang, Guangdong, China).

Composition of plant regeneration and transformation media Callus induction medium (CIM) and embryo induction medium (EIM) were essentially the same as described by Iantcheva *et al*. (2009). CIM was based on B5 (Gamborg *et al*. 1968) solid medium containing 4 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.8 mg L^{-1} kinetin, 1 mg L^{-1} adenine (6-aminopurine), 500 mg L^{-1} casein hydrolysate, 500 mg L^{-1} myo-inositol, 3% sucrose (*w/v*), and 0.7% (*w/v*) Phyto agar (Duchefa). EIM was based on MS medium supplemented with 0.9 mg L^{-1} 6-benzylaminopurine (BAP), 0.3 mg L^{-1} α-naphthaleneacetic acid (NAA), 3% sucrose (*w/v*), and 0.7% (*w/v*) Phyto agar (Duchefa). The newly established embryo development medium (EDM) for *L. japonicus* was based on B5 medium supplemented with 0.2 mg L^{-1} BAP, 3% sucrose (*w/v*), and 0.7% (*w/v*) Phyto agar (Duchefa). Basal MS containing 3% sucrose (*w/v*), 0.7% (*w/v*) Phyto agar (Duchefa), and with no plant growth regulators was used for embryo conversion and rooting (ECR) medium.

The pH of all culture media was adjusted to 5.8 with 1 N NaOH before adding the agar. The media were sterilized by autoclaving at 121°C and 103 kPa for 20 min. All cultivations (with an exception of the callus tissue induced in dark) were carried out in a growth chamber at 24°C, with a 16-h photoperiod and white cool light, tube with intensity of 30 μmol m^{-2} s^{-1} (Solar Leading Group Co. Ltd.).

Regeneration procedure Wounded leaves and petioles of 30–40-d-old *in vitro* plants were transferred onto CIM under light conditions mentioned above or continuous dark. Twenty explants per Petri plate were placed in direct contact with the medium. At day 60, each explant appeared as a ball of friable callus. Embryo induction started after transfer to EIM and lasted about 25–30 d. Development of embryos continued on EDM for two to three passages (each 20 d). Dark green, globular embryos slowly developed and formed cotyledonary leaves after at least two passages (40 d) on this medium. This process continued for 45–60 d. Only embryos with well-developed cotyledonary leaves were transferred to ECR medium for conversion to plantlets. They still looked like a cluster of structures. Subculture (every 25 d) on ECR medium continued until healthy plantlets formed. These plantlets could be separated and maintained on MS basal medium. In total, the optimal period for plant regeneration following this protocol was 150 d.

Bacterial strain and plasmid For plant transformation, *Agrobacterium tumefaciens* EHA105 (Hood *et al*. 1993) was used, carrying the plasmid p1381XC::Tnt1, which is a pCambia1381XC (http://www.cambia.org) vector with the autonomous *Tnt1* retrotransposon and a chimeric *hptII* gene encoding plant resistance to hygromycin. *A. tumefaciens* EHA105 (pCambia1381XC::Tnt1) was maintained on agar-solidified (1.5%; *w/v*) YEB nutrient medium with 100 mg L^{-1} rifampicin and 100 L^{-1} kanamycin for p1381XC::Tnt1 plasmid selection. Prior to transformation, the cultures were incubated in YEB for 24 h under constant agitation (250 rpm) at 28°C. Bacterial suspensions at an optical density of OD_{600} = 0.3 were used for inoculation.

Transformation procedure Leaf and petiole explants detached from *in vitro* plants were wounded with a scalpel blade and cultured on CIM for 48 h in dark at 24°C before inoculation with bacterial suspension. Explants were inoculated with bacterial suspension for 1 h and co-cultivated for 2 d on solid CIM without selective antibiotics. Then, the explants were transferred to solid CIM medium for callus induction with hygromycin (5.5 mg L^{-1}; HygroGoldTM, Invivogen, San Diego, CA) for selection of the transformed cells and carbenicillin (400 mg L^{-1}; Duchefa) to remove bacterial infection. The selective medium was refreshed every 20 d. Embryogenic calluses were then transferred to EIM and EDM as described above. Hygromycin selection was omitted during the embryo development stage and continued at the same concentration on medium for embryo conversion and rooting. Carbenicillin was reduced to 200 mg L^{-1} in EIM and EDM and removed from ECR medium. All regeneration and transformation experiments were conducted three times with three replicates.

PCR analysis Genomic DNA was extracted from transgenic plants using the DNeasy Plant Mini Kit (Qiagen, Germantown), according to the manufacturer's instructions. PCR amplification to detect the presence of the *hptII* gene in the genome of the transgenic plants was performed in a reaction containing 1× PCR amplification buffer, 200 μM of each dNTP, 0.5 μM each of forward and reverse primers, 1 U Taq DNA polymerase, and Milli-Q H_2O to a final volume of 20 μL. The following amplification conditions were used: [94°C 3 min (1×); 94°C 30 s, 55°C 45 s,72°C 45 s (35×); 72°C 5 min (1×)]. PCR products were evaluated by electrophoresis through 1.0% (*w/v*) agarose gels. Gene-specific primers for the *hptII* sequence that were used to amplify a 550-bp product were forward 5′-CCATTCGGACC GCAAGGAATC-3′ and reverse 5′-TCGGCGAGTAC TTCTACACAG-3′.

Inverse PCR Inverse PCR (IPCR) allows amplification of the flanking sequences of *Tnt1* copies inserted into the genome (Ratet *et al*. 2010). Genomic DNA was isolated using the same kit as mentioned above, then digested, self-ligated in a 200-μL reaction volume, and the borders were amplified through a nested (two-step) PCR. Genomic DNA (500 ng) of all tested

lines was digested in 50-μL reaction volumes for at least 2 h at 37°C (or overnight at 16°C). The digestion was performed with enzymes *EcoR*I and *Mfe*I in 1× Tango buffer (Thermo Fisher Scientific, Waltham, MA) and heat inactivated at 65°C for 20 min. Fifty microliter of the digestion reaction were used for ligation (T4 DNA ligase 5U μL^{-1}; Thermo Fisher Scientific) in 200 μL reaction overnight at 16°C. Two nested PCR reactions were performed with primers specific for the borders of *Tnt*1. IPCR1 [94°C 2 min (1×); 94°C 20 s, 72°C 3 min (30×); 72°C 5 min (1×)] was performed using LTR5 (5′ GCCAAAGCTTCACCCTCTAAAGCCT3′) and LTR3 (5′ AGTTGCTCCTCTCGGGGTCGTGGTT3′) primers. IPCR2 [94°C 2 min (1×); 94°C 20 s, 60°C 20 s, 72°C 3 min (30×); 72°C 5 min (1×)] was performed using LTR6 5′ GCTACCAACCAAACCAAGTCAA3′ and LTR4 (5′ TACCGTATCTCGGTGCTACA3′). PCR reactions were done using the Takara Ex Taq™ DNA polymerase enzyme (Lonza Rockland Inc., Rockland, ME). PCR products were analyzed on 1.5% agarose gels.

FST characterization Cloning and sequencing of the FSTs were performed as described by Ratet *et al.* (2010).

Materials and reagents All chemicals and cell culture reagents were purchased from Sigma-Aldrich® (Munich, Germany) unless otherwise specified.

Results

Development of Tnt1 efficient regeneration and transformation protocols for *L. japonicus* During regeneration, the presence of a high 2,4-D concentration in the CIM resulted in total dedifferentiation of the plant explants and production of soft and friable callus tissue (Fig. 1*A*, *B*). The use of either light or dark conditions led to the production of massive callus tissue which differed only in color (yellow under dark and greenish under light conditions; Fig. 1*A*, *B*). Approximately 85% of the initial explants (16 of 20 plated) responded positively to the inductive signal (Table 1). Combination of BAP and NAA in the EIM resulted in the formation of embryogenic zones, resembling green spots, which further developed to two to five globular embryos per each callus tissue (Fig. 1*C*, *D*) for a period of 30 d. In the newly established medium for embryo development (ED), the auxin NAA was removed and only BAP triggered further development of globular embryos. Globular embryos slowly developed to torpedos, which formed cotyledonary embryos with emerging cotyledonary leaves after at least two passages (40 d) on this medium. This process continued around 45–60 d. The conversion rate of the globular embryos to torpedo and cotyledonary stage embryos was 50% (27 cotyledonary embryos per 20 explants; Fig. 1*E*–*G*). Only embryos with well-developed cotyledonary

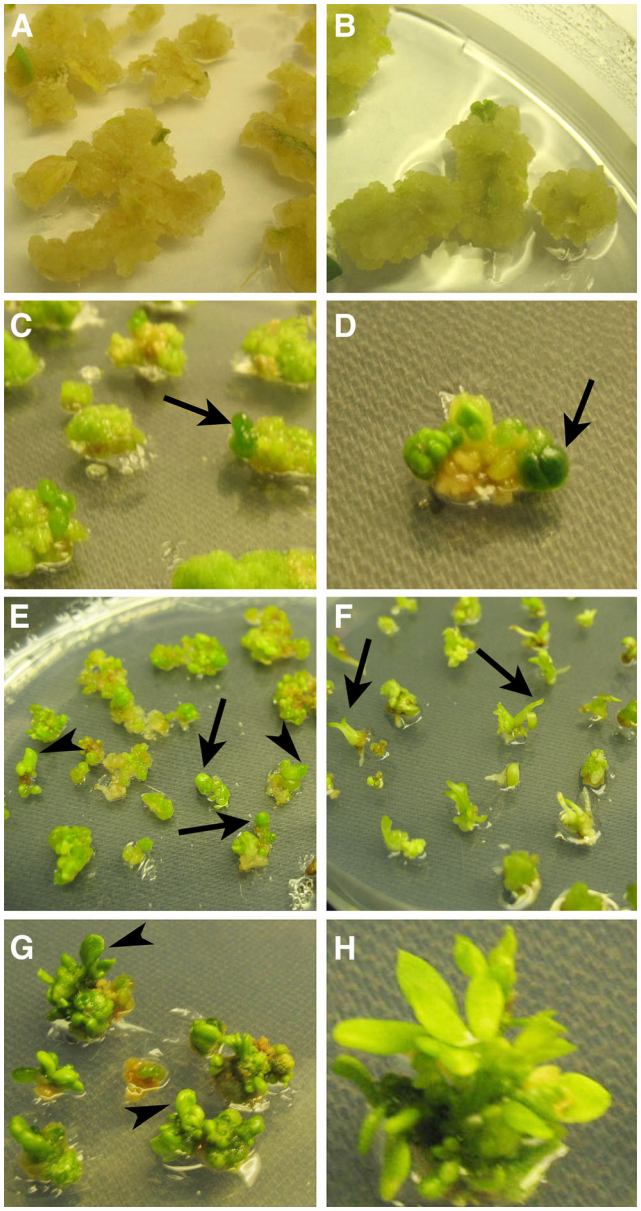

Figure 1 Basic steps in regeneration of *Lotus japonicus* from leaf and petiole explants. (*A*) callus tissue initiated in dark conditions, (*B*) callus tissue initiated in light conditions, (*C*) green embryogenic zones on EI medium, (*D*) cluster of globular embryos on medium for ED, (*E*) globular and torpedo embryos (*arrows*, globular; *arrowhead*, torpedo), (*F*) cotyledonary embryos with emerging cotyledonary leaves, (*G*) cluster of cotyledonary embryos, and (*H*) healthy plantlets.

leaves either alone or grouped into clusters were transferred to ECR medium for conversion to plantlets. From each 20 initial explants, 14 healthy plantlets were regenerated (Fig. 1*H* and Table 1). The overall regeneration efficiency of the established protocol was 70%.

Pretreatment of explants on CIM for 48 h before *Agrobacterium* infection, using bacterial suspension at $OD_{600} = 0.3$, and inoculation for 1 h were found to be optimal conditions for genetic transformation of leaf and petiole explants, preserving their strength and ability for regeneration

Table 1 Efficiency of the regeneration and transformation procedures for *L. japonicus*

Regeneration efficiency				
Reacting explants (%)/number of explants inducing callus	Globular embryos per callus/total globular embryos		Cotyledonary embryo (%)/total cotyledonary embryos	Number of embryos converted to plantlets
85.33 ± 6.02/15.75 ± 1.45	2–5/53.68 ± 6.53		49.66 ± 3.05/26.83 ± 1.91	14.33 ± 3.21
Transformation efficiency				
Transformed explants	Independent transgenic lines	*hptII*-positive lines	Starter lines with one or two bands	Starter lines with three to eight bands
200	57	49	42	9

Data represent mean ± SD

(data not shown). Selective pressure was kept during the first two regeneration stages—callus and embryo induction, but was removed during embryo development. This led to the successful regeneration of 57 healthy and vigorous, putatively independent transgenic plants, starting from 200 transformed explants. PCR analysis confirmed incorporation of the *hptII* gene in 86% of the regenerated transgenic lines (Fig. 2*A*). The observed 14% rate of escapes (Table 1) could be related to the selection on medium containing 5.5 mg L^{-1} hygromycin, which did not completely eliminate initial callus induction in control explants but helped identify transformed tissues that grew faster. The transformation step of the protocol resulted in 25% efficiency. In total, 51 starter lines with 1 to 8 copies of *Tnt1* were generated.

Defining the Tnt1 copy number in starter lines of *L. japonicas* The *Tnt1* copy number for 57 primary transformants (starter lines; St) was analyzed. Most of the transgenic lines (39) showed one band of approximately 600 bp (Fig. 2*B*). This fragment corresponds to the flanking sequence of the *Tnt1* retrotransposon in the pCambia1381XC:Tnt1 T-DNA that was integrated into the transgenic plants. This further confirmed the transgenic nature of the plants. The fragment of less than 200 bp in St4 and St5 lines (lanes 4 and 5; Fig. 2*B*) and St6 and St8 (Fig. 3) corresponded to the two borders of the element plus a few additional base pair and probably represent free (extra-chromosomal) copies of the element present in the tissue of the transgenic plants. The free copies indicate expression and reverse transcription of the retrotransposon in these lines (Fig. S1). In the line St4, three to four additional fragments were detected that corresponded to new insertion sites of the element, indicating that *Tnt1* was active in this line during the transformation/regeneration process.

The results presented in Fig. 3 indicate that some of the regenerated (R_0) lines (St7, St8, and St9) did not contain the 600-bp fragment, as expected for the T-DNA construct. However, these lines were PCR-positive for *Tnt1* FST. For example, St9 contains multiple FSTs that could represent multiple insertion sites of the *Tnt1* element. Among the other lines, some showed only the T-DNA copy (St1) or both the T-DNA copy plus putative extra-chromosomal copies [lines St3, St6, St11, St12, and St13 (Fig. 3) and lines St3, St12, and R6 (Fig. S1)]. This was another indication that transposition had occurred during the transformation process. The characteristics that indicate the presence of T-DNA, the number of *Tnt1* inserts, fertility, and marker segregation in the progeny of the R_0 and R_1 lines, are given in Table 2.

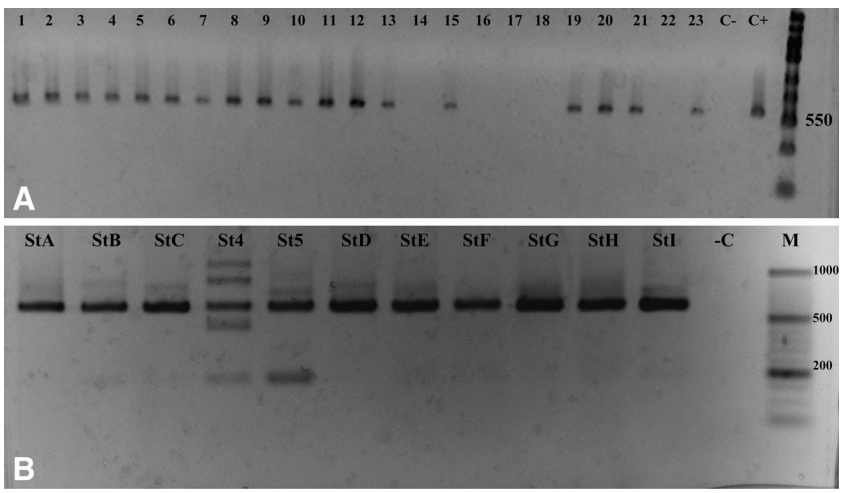

Figure 2 Representative PCR analyses confirming the presence of transgenes. (*A*) PCR analysis for the presence of *hptII* gene in the genome of *Tnt1* R_0 transgenic lines. *Lanes 1–23*, tested plants; *lane 24*, negative control (C−); *lane 25*, positive control (C+); *lane 26*, DNA marker. (*B*) Inverse PCR analysis of *Tnt1* starter lines (StA-I) with the most common observed profile. *Lanes 1–11*, *Tnt1* positive lines; *lanes 4 and 5*, St *lines 4 and 5* with five and three *Tnt1* copies, respectively; *lane 12* (−C), negative control; *lane 13* (M), DNA marker.

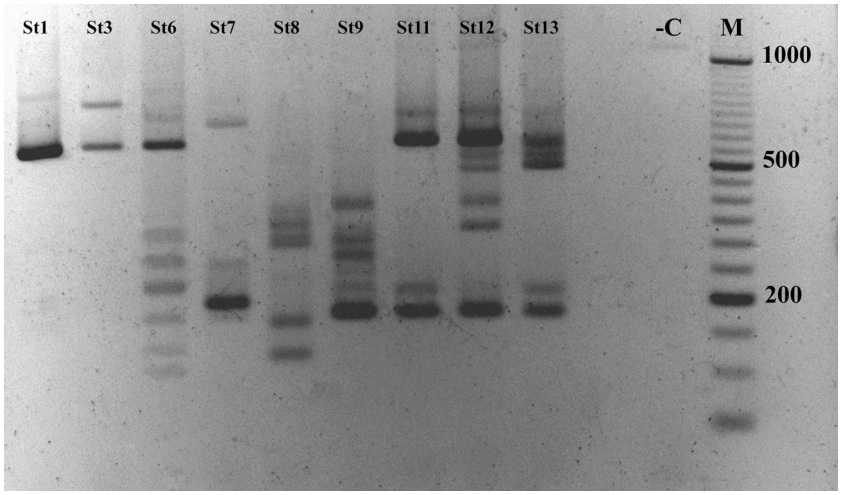

Figure 3 IPCR of *Tnt1* starter lines. Number of *Tnt1* copies: *lanes 1 and 2*, St1 with one copy; St3 with two copies; *lane 3*, St6 with eight copies; *lane 4*, St7 with three copies; *lane 5*, St8 with four copies; *lane 6*, St9 with five copies; *lane 7*, St11 with four copies; *lane 8*, St12 with seven copies; *lane 9*, St13 with five copies; *lane 11*, negative control; *lane 12*, DNA marker.

Tnt1 transposition was reactivated via indirect somatic embryogenesis To investigate whether tissue culture-induced activation of *Tnt1* is applicable for *L. japonicus*, *in vitro* regeneration was tested to determine if it could reactivate *Tnt1* transposition. All starter lines had conserved the ability to regenerate plantlets via indirect embryogenesis (data not shown). The regeneration protocol described in this work was established particularly for *Tnt1* reactivation in *L. japonicus*. For the *Tnt1* reactivation experiments, St3, St6, and St11 were selected (Fig. 3 and Table 2) and their regenerants and T_1 progeny were analyzed. The transposition characteristics of the randomly selected regenerants R_{12}, R_{14}, and R_{18} (derived from St11, with four *Tnt1* inserts), and the regenerants R_{13}, R_{15}, and R_{16} (derived from St3, with two inserts) and R_6 (derived from St6, with eight *Tnt1* inserts), are presented in Table 2. The regenerated lines R_{12} and R_{14} contained three and four new *Tnt1* inserts when compared to St11. In the regenerated line R_{13}, two new inserts were detected, and the line R_{15} four new transposition events were detected. The new transposition events, resulting from *Tnt1* reactivation in the *L. japonicus* ecotype B-129 Gifu background, varied from 1 to 4, significantly less than reported for *M. truncatula* cv. Jemalong, where 8 to 12 new transpositions occurred in tissue culture experiments using a starter line with 8 copies.

Tnt1 inserts were heritable in T_1 progeny Vigorous *in vitro* plants from starter and regenerated lines were transferred to

Table 2 *Tnt1* transposition characteristics in different regenerated lines and their progeny

Line number	Parental line	Regeneration level	T-DNA	Extra T-DNA copies	Free copies	PCR for Hyg resistance	Fertility	T1 progeny/ segregation ratio
StA	Gifu wt	R0	+	0	−	+	+	+/3:1
StB	Gifu wt	R0	+	0	−	+	+	+/3:1
StC	Gifu wt	R0	+	0	−	+	+	+/3:1
St3	Gifu wt	R0	+	1	+	+	+	+/3:1
St4	Gifu wt	R0	+	4	+	+	+	+/3:1
St6	Gifu wt	R0	+	7	+	+	+	+/1:1
St8	Gifu wt	R0	−	4	+	−	+	+
St9	Gifu wt	R0	−	5	+	−	+	+
St11	Gifu wt	R0	+	3	+	+	+	+/3:1
St13	Gifu wt	R0	+	4	+	+	+	+/1:1
R13	St3	R1	+	3	+	+	+	+/3:1
R15	St3	R1	+	5	+	+	+	+/3:1
R16	St3	R1	+	1	+	+	+	+
R6	St6	R1	+	10	+	+	−	−
R12	St11	R1	+	6	+	+	+	+/3:1
R14	St11	R1	+	7	+	+	+	+/3:1
R18	St11	R1	+	4	+	+	+	+/3:1

greenhouse in order to obtain their offspring. The plants had regular flowering time and pod production. The only exceptions were for some of the regenerants (line R_6) deriving from St6 that were sterile. Self-fertilized T_1 progeny were obtained from all other starter and regenerated lines with new transposition events.

Transgenic T_1 seeds from several starter lines and seeds of regenerants were grown on selective ECR medium with 5.5 mg L^{-1} hygromicin. All tested lines segregated in the ratio 3:1 with an exception of T_1 progeny of lines St6 and St13, which segregated in a 1:1 ratio. This deviation could be explained by the small population of analyzed seeds (only 12) or as a result of chromosomal rearrangements.

IPCR was utilized to confirm that the transposition profile of some of the starter lines and showed that their progeny did not change during plant growth in a greenhouse. The transposition profiles of T_1 regenerants R_{14} and R_{12} and starter lines St3, St11, St8, and St13 are shown in Fig. 4. There were no new transposition events during plant growth and in the obtained progeny, suggesting that the *Tnt1* element is not active during normal plant life cycle.

Sequencing of FSTs To confirm the nature of the FSTs deduced by IPCR, FSTs were cloned and sequenced for lines R_6, T_1 St3, and T_1 St12 (Fig. S1). Six fragments from each line were sequenced. Ten fragments corresponding to free copies of the *Tnt1* element with only a few base pairs between the two borders were obtained. Similarly, four fragments corresponding to the *Tnt1* junction with the T-DNA vector were recovered. From line T_1 St3, four sequences were obtained that represented the same FST fragment and which showed *Tnt1* insertion in a putative gene. This sequence (493 bp) is not available in the *Lotus* genome present in public databases. It corresponds to a putative gene coding for a cytochrome P450, highly homologous (90% amino acid identity) to the cytochrome P450 (XP_006596023.1) gene from *Glycine max*. The sequence of this IPCR fragment shows the 5-bp duplication (TTTTC) characteristic of *Tnt1* transposition. This result clearly shows that *Tnt1* was active in this line and inserted into a gene.

Discussion

Building large mutant collections for different plant species contributes to identification of mutated genes and an understanding of gene function. During the last two decades, different tools have been developed for efficient generation of mutant collections. Insertional mutagenesis is one of the most powerful tools to generate mutants and understand gene function in plants. T-DNA has been successfully applied as a mutagen in *Arabidopsis thaliana* (Alonso *et al*. 2003), but for plants with larger genomes, like model legumes, it is not practical to use this approach.

The use of transposable elements is a good alternative strategy for generation of large insertional mutant collections and has become a powerful tool for gene tagging in model legumes and crops (Mathieu *et al*. 2009; Cheng *et al*. 2011; Cui *et al*. 2013; Grandbastein 2015). Activity of *Tnt1* and *Tto1* retrotransposons has been detected in interspecific hybrids of tuber-bearing species of *Solanum*, and represents a source of genetic variability, important for adaptation of potato species (Paz *et al*. 2015).

In the last decade, *Tnt1* has been successfully used as a valuable tool for building *M. truncatula* mutant collections and applied in forward and reverse genetic studies (Tadege *et al*. 2008; Zhou *et al*. 2011; Bourcy *et al*. 2013; Veerappan *et al*. 2016). Phenotypic screening of the *Tnt1* mutant lines is an efficient way to explore functions of new genes or new regulatory pathways. Analysis of morphological characteristics of *M. truncatula* mutants generated by *Tnt1* retrotra nsposon insertions has shown that leaf phenotypic characteristics are influenced by the *Tnt1* insertions and display visually recognizable phenotypes (Vassileva *et al*. 2010).

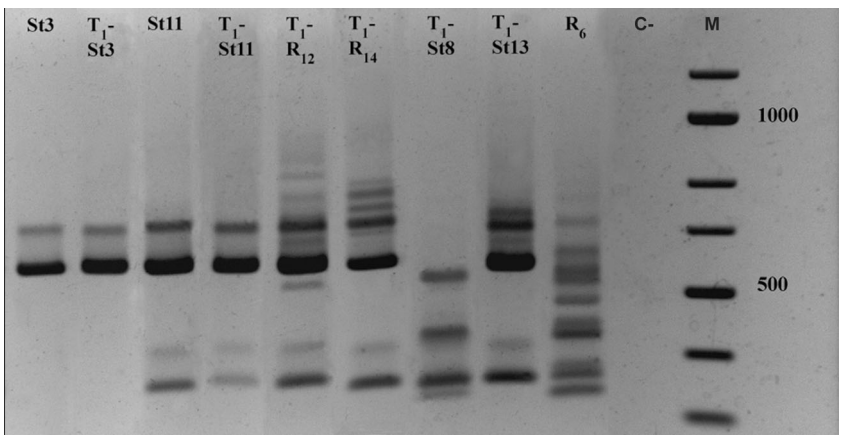

Figure 4 IPCR of selected starter lines, regenerated lines and T_1 progeny: *lane 1*, St3 in greenhouse; *lane 2*, T_1 of St3; *lane 3*, St11 in greenhouse; *lane 4*, T_1 of St11; *lane 5*, T_1 of R_{12}; *lane 6*, T_1 of R_{14}; *lane 7*, T_1 of St8; *lane 8*, T_1 of St13; *lane 9*, R6 regenerant of St6; *lane 10*, negative control (C−); *lane 11*, DNA marker.

In this paper, *Tnt1* insertional mutagenesis has been shown capable of producing collections of insertional mutants in *L. japonicus*. Application of *Tnt1* mutagenesis in the second model legume *L. japonicus* could provide additional tagged insertion mutant populations and serve as an alternative of the existing *LORE1* strategy. The constructed *Tnt1* mutant lines will further enrich the legume reverse genetic resources. These mutants may be a valuable resource for functional and comparative genomic studies in other legumes.

The obtained regeneration/transformation protocol was mainly developed to enable efficient *Tnt1* incorporation and transposition into the *L. japonicus* genome. The current study confirmed the importance of the synthetic auxin 2,4-D and the callus induction step for successful *Tnt1* transposition, which was explored during the construction of *Tnt1* mutant collection for *M. truncatula* cv Jemalong. Under these optimized conditions, the frequency of transposition of the *Tnt1* retrotransposon in the *M. truncatula* genome was 80–85% (Iantcheva *et al*. 2009). The present study showed that the initiation of soft and friable callus in the presence of a high concentration of 2,4-D (4 mg L^{-1}) was an important prerequisite for complete dedifferentiation of starting explants (leaf and petiole) of the model legume *L. japonicus*. Other works related to regeneration and genetic transformation of *L. japonicus* confirm the importance of the plant hormones NAA and BAP and the hypocotyl as a starting explant or a combination of auxin and cytokinins for dedifferentiation and production of poor callus from root explants (Lohar *et al*. 2001; Lombari *et al*. 2003). The published protocols point to the induction of compact callus tissue followed by organogenesis and shoot regeneration.

The described regeneration and transformation protocols via indirect somatic embryogenesis had relatively high transformation efficiency (25%) and allowed producing 51 independent *Tnt1* starter lines. The number of *Tnt1* copies in these starter lines (1–8) was much smaller than reported for *M. truncatula* (up to 30), for soybean (4–19), and for potato (1–20) (d'Erfurth *et al*. 2003; Cui *et al*. 2013; Duangpan *et al*. 2013). Three of the starter lines were PCR positive for *Tnt1* FST, suggesting that *Tnt1* inserted into the *L. japonicus* genome independently from T-DNA. This result is similar to previous reports for *M. truncatula* and potato (d'Erfurth *et al*. 2003; Duangpan *et al*. 2013).

The *Tnt1* retrotransposon could be a useful tool for building a collection of *L. japonicus* mutant lines only if *Tnt1* insertional activity remains inactive during plant growth and if new transpositions do not occur in subsequent generations. The first evidence of *in vitro* regeneration of transgenic lines containing the *Tnt1* retrotranspososn was shown to be sufficient to activate *Tnt1* transposition in *M. truncatula* ecotype R108 and for cultivar Jemalong (d'Erfurth *et al*. 2003; Iantcheva *et al*. 2009). *In vitro* regeneration-induced activation of *Tnt1* has been reported also for other plant species (Courtial *et al*. 2001; Mazier *et al*. 2007; Cui *et al*. 2013; Duangpan *et al*. 2013). This approach is very powerful for a large-scale mutagenesis since *in vitro* regeneration is faster and more efficient compared to genetic transformation. The regeneration protocol for *L. japonicus* developed in this study induced the transposition activity of *Tnt1* and the insertion of the element in different locations in the *Lotus* genome and showed activation of *Tnt1* via *in vitro* regeneration. Activation of different tobacco retrotransposons was shown to be specifically modulated by the elicitor cryptogein (Anca *et al*. 2014) and enhanced by osmotic pretreatment of the starting explants (Iantcheva *et al*. 2009).

The number of new transpositions observed in this study (two to four) is comparable to the efficiency of the endogenous retrotransposon *LORE1* (Fukai *et al*. 2012), where the average number of the new insertions per plant was 2.6. Despite the low transposition frequency, tissue culture-induced activation of *Tnt1* after further optimization and selection of lines devoid of background mutations could offer a promising basis for the construction of large mutant populations of *L. japonicus*.

Conclusion

The present study showed incorporation of the retrotransposon into *L. japonicus* ecotype B-129 Gifu and that starter lines with different copy numbers of *Tnt1* were obtained. *In vitro* regeneration from starter lines containing from two to eight copies of *Tnt1* resulted in new transposition events, increasing the number of insertions per line. The inserted *Tnt1* copies remained inactive during normal plant life cycle and were stably inherited in the progeny. These findings can serve as a methodological basis for the construction of a large insertional mutant collection for *L. japonicus*. The efficient transformation and regeneration protocols developed for this study can be used by researchers producing transgenic legume lines.

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References

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Hom E, Karnes M, Mulholland C, Ndubaku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003)

Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science 301:653–657

Anca IA, Fromentin J, Bui QT, Mhiri C, Grandbastien MA, Plas FS (2014) Different tobacco retrotransposons are specifically modulated by the elicitor cryptogein and reactive oxygen species. J Plant Physiol 171:1533–1540

Bourcy M, Brocard L, Pislariu CI, Cosson V, Mergaert P, Tadege M, Mysore KS, Udvardi MK, Courion B, Ratet P (2013) *Medicago truncatula* DNF2 is a PI-PLC-XD-containing protein required for bacteroid persistence and prevention of nodule early senescence and defense-like reactions. New Phytol 197:1250–1261

Boycheva I, Vassileva V, Revalska M, Zehirov G, Iantcheva A (2015) Cyclin-like F-box protein plays a role in growth and development of the three model species *Medicago truncatula*, *Lotus japonicus* and *Arabidopsis thaliana*. Res Rep Biol 6:117–130

Cheng X, Wen J, Tadege M, Ratet P, Mysore KS (2011) Reverse genetics in *Medicago truncatula* using *Tnt1* insertion mutants. Methods Mol Biol 678:179–190

Cigan AM, Unger-Wallace E, Haug-Collet K (2005) Transcriptional gene silencing as a tool for uncovering gene function in maize. Plant J 43: 929–940

Cook DR (1999) *Medicago truncatula*—a model in the making. Curr Opin Plant Biol 2:301–304

Courtial B, Feuerbach F, Eberhard S, Rohmer L, Chiapello H, Camilleri C, Lucas H (2001) *Tnt1* transposition events are induced by *in vitro* transformation of *Arabidopsis thaliana*, and transposed copies integrate into genes. Mol Genet Genomics 265:32–42

Cui Y, Barampuram S, Stacey MG, Hancock CN, Findley S, Mathieu M, Zhang Z, Parrott WA, Stacey G (2013) *Tnt1* retrotransposon mutagenesis: a tool for soybean functional genomics. Plant Physiol 161: 36–47

Dakora FD (2003) Defining new roles for plant and rhizobial molecules in sole and mixed plant cultures involving symbiotic legumes. New Phytol 158:39–49

Duangpan S, Zhang W, Wu Y, Jansky SH, Jiang J (2013) Insertional mutagenesis using *Tnt1* retrotransposon in potato. Plant Physiol 163:21–29

d'Erfurth I, Cosson V, Eschstruth A, Lucas H, Kondorosi A, Ratet P (2003) Efficient transposition of the *Tnt1* tobacco retrotransposon in the model legume *Medicago truncatula*. Plant J 34:95–106

Fukai E, Soyano T, Umehara Y, Nakayama S, Hirakawa H, Tabata S, Sato S, Hayashi M (2012) Establishment of a *Lotus japonicus* gene tagging population using the exon-targeting endogenous retrotransposon *LORE1*. Plant J 69:720–730

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Grandbastein MA (2015) LTR retrotransposons, handy hitchhikers of plant regulation and stress response. Biochim Biophys Acta 1849: 403–416

Hood EE, Gelvin SB, Melcshers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res 2:208–218

Iantcheva A, Chabaud M, Cosson V, Barascud M, Schutz B, Primard-Brisset C, Durand P, Barker DG, Vlahova M, Ratet P (2009) Osmotic shock improves *Tnt1* transposition frequency in *Medicago truncatula* cv Jemalong during *in vitro* regeneration. Plant Cell Rep 28:1563–1572

Imaizumi R, Sato S, Kameya N, Nakamura I, Nakamura Y, Tabata S, Ayabe S, Aoki T (2005) Activation tagging approach in a model legume, *Lotus japonicus*. J Plant Res 118:391–399

Kumar A, Bennetzen JL (1999) Plant retrotransposons. Annu Rev Genet 33:479–532

Lohar DP, Schullee K, Buras DM, Gresshoff PM, Stiller J (2001) Transformation of *Lotus japonicus* using the herbicide resistance bar gene as a selectable marker. J Exp Bot 52:1697–1702

Lombari P, Ercolano E, Alaoui HE, Chiurazzi M (2003) A new transformation-regeneration procedure in the model legume *Lotus japonicus*: root explants as a source of large numbers of cells susceptible to *Agrobacterium*-mediated transformation. Plant Cell Rep 21:771–777

Mathieu M, Winters EK, Kong F, Wan J, Wang S, Eckert H, Luth D, Paz M, Donovan C, Zhang Z, Somers D, Wang K, Nguyen H, Shoemaker RC, Stacey G, Clemente T (2009) Establishment of a soybean (*Glycine* max Merr. L) transposon-based mutagenesis repository. Planta 229:279–289

Mazier M, Botton E, Flamain F, Bouchet JP, Courtial B, Chupeau M-C, Chupeau Y, Maisonneuve B, Lucas H (2007) Successful gene tagging in lettuce using the *Tnt1* retrotransposon from tobacco. Plant Physiol 144:18–31

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Paz RC, Rendina Gonzales AP, Ferrer MS, Masuelli RW (2015) Short-term hybridisation activates *Tnt1* and *Tto1* Copia retrotransposons in wild type tuber-bearing *Solanum* species. Plant Biol 17:860–869

Perry JA, Wang TL, Welham TJ, Gardner S, Pike JM, Yoshida S, Parniske M (2003) A TILLING reverse genetics tool and web-accessible collection of mutants of the legume *Lotus japonicus*. Plant Physiol 131:866–871

Ratet P, Wen J, Cosson V, Tadege M, Mysore KS (2010) *Tnt1* induced mutations in *Medicago*: characterisation and applications. In: Meksem K, Kahl G (eds) Handbook of plant mutation screening (mining of natural and induced alleles). Wiley-VCH Verlag Gmbh & Co. KGaA, Weinheim, Germany, pp 83–99

Revalska M, Zehirov G, Vassileva V, Iantcheva A (2015) Is the auxin influx carrier *LAX3* essential for plant growth and development in the model plants *Medicago truncatula*, *Lotus japonicus* and *Arabidopsis thaliana*? Biotechnol Biotechnol Equip 29:786–797

Sato S, Nakamura Y, Kaneko T, Asamizu E, Kato T, Nakao M, Sasamoto S, Watanabe A, Ono A, Kawashima K, Fujishiro T, Katoh M, Kohara M, Kishida Y, Minami C, Nakayama S, Nakazaki N, Shimizu Y, Shinpo S, Takahashi C, Wada T, Yamada M, Ohmido N, Hayashi M, Fukui K, Baba T, Nakamichi T, Mori H, Tabata S (2008) Genome structure of the legume, *Lotus japonicus*. DNA Res 15:227–239

Schauser L, Roussis A, Stiller J, Stougaard J (1999) A plant regulator controlling development of symbiotic root nodules. Nature 402: 191–195

Stougaard J (2001) Genetics and genomics of root symbiosis. Curr Opin Plant Biol 4:328–335

Tadege M, Wen J, He J, Tu H, Kwak Y, Eschstruth A, Cayrel A, Endre G, Zhao PX, Chabaud M, Ratet P, Mysore KS (2008) Large-scale insertional mutagenesis using the *Tnt1* retrotransposon in the model legume *Medicago truncatula*. Plant J 54:335–347

Thykjaer T, Stller J, Handberg K, Jones J, Stougaard J (1995) The maize transposable element AC is mobile in the *Lotus japonicus*. Plant Mol Biol 27:981–993

Urbanski DF, Małolepszy A, Stougaard J, Stig Uggerhøj Andersen SU (2012) Genome-wide *LORE1* retrotransposon mutagenesis and high-throughput insertion detection in *Lotus japonicus*. Plant J 69: 731–741

Vassileva V, Zehirov G, Ugrinova M, Iantcheva A (2010) Variable leaf epidermal morphology in *Tnt1* insertional mutants of the model legume *Medicago truncatula*. Biotechnol Biotechnol Equip 24: 2060–2065

Veerappan V, Jani M, Kadel K, Troiani T, Gale R, Mayes T, Shulaev E, Wen J, Mysore KS, Azadand RK, Dickstein R (2016) Rapid identification of causative insertions underlying *Medicago truncatula Tnt1* mutants defective in symbiotic nitrogen fixation from a forward genetic screen by whole genome sequencing. BMC Genomics. doi:10.1186/s12864-016-2452-5

Wessler SR (2006) Transposable elements and the evolution of eukaryotic genomes. Proc Natl Acad Sci U S A 103:17600–17601

Wienkoop S, Saalbach G (2003) Proteome analysis. Novel proteins identified at the peribacteroid membrane from *Lotus japonicus* root nodules. Plant Physiol 131:1–11

Young ND, Debelle F, Oldroyd GED, Geurts R, Cannon SB, Udvardi MK, Benedito VA, Mayer KFX, Gouzy J, Schoof H, Van de Peer Y, Proost S, Cook DR, Meyers BC, Spannagl M, Cheung F, Mita SD, Krishnakumar V, Gundlach H, Zhou S, Mudge J, Bharti AK, Murray JD, Naoumkina MA, Rosen B, Silverstein KAT, Tang H, Rombauts S, Zhao PX, Zhou P, Barbe V, Bardou P, Bechner M, Bellec A, Berger A, Bergeś H, Bidwell S, Bisseling T, Choisne N, Couloux A, Denny R, Deshpande S, Dai X, Doyle JJ, Dudez A, Farmer AD, Fouteau S, Franken C, Gibelin C, Gish J, Goldstein S, González AJ, Green PJ, Halla A, Hartog M, Hua A, Humphray SJ, Jeong D, Jing Y, Jőcker A, Kenton SM, Kim D, Klee K, Lai H, Lang C, Lin S, Macmil SL, Magdelenat G, Matthews L, McCorrison J, Monaghan EL, Mun J, Najar FZ, Nicholson C, Noirot C, O'Bleness M, Paule CR, Poulain J, Prion F, Qin B, Qu C, Retzel EF, Riddle C, Sallet E, Samain S, Samson N, Sanders I, Saurat O, Scarpelli C, Schiex T, Segurens B, Severin AJ, Sherrier DJ, Shi R, Sims S, Singer SR, Sinharoy S, Sterck L, Viollet A, Wang B, Wang K, Wang M, Wang X, Warfsmann J, Weissenbach J, White DD, White JD, Wiley GB, Wincker P, Xing Y, Yang L, Yao Z, Ying F, Zhai J, Zhou L, Zuber A, Dénarie J, Dixon RA, May GD, Schwartz DC, Rogers J, Quétier F, Town CD, Roe BA (2011) The *Medicago* genome provides insight into the evolution of rhizobial symbioses. Nature 480:520–524

Zhou C, Han L, Pislariu C, Nakashima J, Fu C, Jiang Q, Quan L, Blancaflor EB, Tang Y, Bouton JH, Udvardi M, Xia G, Wang ZW (2011) From model to crop: functional analysis of a STAY-GREEN gene in the model legume *Medicago truncatula* and effective use of the gene for alfalfa improvement. Plant Physiol 157:1483–1496