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Chapter 7

Medicago truncatula Functional Genomics – An Invaluable Resource for Studies on Agriculture Sustainability

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Additional information is available at the end of the chapter

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1. Introduction

Legume functional genomics has moved many steps forward in the last two decades thanks to the improvement of genomics technologies and to the efforts of the research community. Tools for functional genomics studies are now available in *Lotus japonicus*, *Medicago truncatula* and soybean. In this chapter we focus on *M.truncatula*, as a model species for forage legumes, on the main achievements obtained due to the reported resources and on the future perspectives for the study of gene function in this species.

2. Why do we need a functional genomics tool for forage legumes?

Legumes are widely grown for grain and forage production, their world economic importance being second only to grasses. Legume species are unique among cultivated plants for their ability to carry out endosymbiotic nitrogen fixation with rhizobial bacteria, a process that takes place in a specialized structure known as nodule. Moreover legumes are able to establish other types of interactions such as arbuscular mycorrhyzal symbiosis with several fungi. For these outstanding biological properties legumes are considered among the most promising species for improving the sustainability of agricultural systems. In fact for farming system to remain productive and to be environmentally and economically sustainable on the long term it is necessary to replenish the reserves of nutrients which are removed or lost from the soil. Nodulating legumes have the potential to provide all nitrogen required for their growth and in this way to influence its balance in the soil and thus its availability for subsequent crops. In addition by reducing the inputs of fertilizers, legumes reduce the risk of nitrogen contamination of water resources. Furthermore, probably due to the wealth of interactions with other organisms, legumes have evolved an intricate network of secondary metabolites

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that, as the recent advances in the knowledge of their nutraceutical properties are proving, can be considered of great importance for livestock welfare and for the quality of their products.

Two legume species, *Medicago truncatula* and *Lotus japonicus*, are being used as model to study legume genetics and genomics. *Medicago truncatula* is closely related to alfalfa, the most important forage legume in the world. It has a small, diploid genome, it is self fertile and amenable to genetic transformation. In the present review we summarize the state of the art of *M. truncatula* genomics with particular emphasis on the available resources for functional genomics studies such as mutant collections.

3. *Medicago truncatula* genome sequencing

Functional genomics is greatly aided by knowledge on genome sequence and transcriptome of the target species. A concerted effort was carried out in *M. truncatula* which made genome data available to the community.

Legumes are the plant family with the greatest amount of genomic data available. Three legume species, *Medicago truncatula*, *Lotus japonicus*, and *Glycine max*, have been sequenced (1). The assembly of *Medicago truncatula* genome is close to completion (2).

M. truncatula sequencing was initially carried out on Bacterial Artificial Chromosome (BAC) libraries following a BAC-by-BAC approach focused on gene-rich BACs. To date the available sequence data consist of three main batches: i) 246Mb of non redundant sequences that could be organized in large scaffolds separated by gaps and anchored to the eight *M.truncatula* physical chromosomes, ii) 17.3Mb of unanchored scaffolds and iii) 104.2Mb of additional unique sequence obtained by next generation sequencing (NGS) with Illumina sequencing. In total, 367.5Mb of *M.truncatula* genome representing 73,5% of the ~500Mb of the predicted genome size and about 94% of the expressed genes is available.

Taken together BAC sequences and non-redundant Illumina assemblies contain 62,388 gene loci with 14,322 gene prediction annotated as transposons. The average *M. truncatula* gene is 2,211 bp in length, contains 4.0 exons and has a coding sequence of 1,001 bp. Genome analysis and comparisons to other sequenced genomes allowed the identification of a 58-Myr-ago whole genome duplication (WGD) that has been associated with the evolution of rhizobial nodulation in *M. truncatula* and its relatives. Some nodulation-specific signalling components might have evolved through duplication and neo-functionalization from more ancient genes involved in host-mycorrhyzal signalling (2).

Another interesting feature is the presence of many amplified and somehow specialized gene families like nine leghaemoglobines, 563 Nodule Cystein-Rich Peptides (NCRs), 764 nucleotide-binding site and leucine-rich repeat (NBS-LRR) genes, genes in the flavonoid pathway such as chalcone synthases (CHS), chalcone reductases, chalcone isomerases. Many gene duplications occurred with the creation of large gene clusters (2).

The availability of a first draft of the *Medicago truncatula* genome sequence has promoted several initiatives aimed at identifying molecular markers suitable for both evolutionary and genetic mapping studies.

384 inbred lines of *M.truncatula* with a 5x coverage and a subset of 30 with deep coverage (20x) will be resequenced through an Illumina-Solexa sequencing pipeline by the *Medicago* HapMap Project. In the first report on the analysis of sequence data from 26 *M.truncatula* accessions with ~15x average genome coverage, 3,063,923 mapped single nucleotide polymorphisms (SNPs) were described and first estimates of nucleotide diversity (θw =0.0063 and $\theta\pi$ =0.0043 bp−1), population scaled recombination rate and rate of decay of linkage disequilibrium have been published (3). More recently the same material was used to estimate population recombination rates at 1 kb scale and very interestingly in the three chromosomes analysed recombination was higher near centromeric regions in stark contrast to what observed in every non-plant system and in the majority of plants that show a negative gradient of recombination from telomeric to centromeric regions (4).

In parallel, plant phenotyping is ongoing in greenhouse experiments for the *Medicago* lines. The combination of genetic and phenotypic data will be organized in a platform for genome-wide association mapping (GWAS) studies.

4. Functional genomics in *Medicago truncatula*

The discovery of gene function in model species is accomplished by exploiting resources such as mutant collections, using the ability to implement plant genetic transformation and analyzing gene transcription. In *M. truncatula* all the three approaches can be performed.

Several strategies were pursued in *M. truncatula* to produce mutant collections (Tab. 1) and they will be analysed in the following section.

Reference	Mutagenesis technique	Background	Notes
(5)	EMS	Jemalong population 2828	1,500 seeds treated for 24h with 0.2%EMS. 400 M1 plants obtained. 250,000 M2 seeds harvested as a single batch.
(6)	γ-ray	Jemalong line J5	462 M1 plants, screened as M2 families.
(7)	Ethyl-nitrosourea		
(8)	EMS	Jemalong A17	3-7,000 M1 plants in 10-20 lots. M2 seeds bulked from each lot.
(9)	T-DNA	R108-1 (c3)	Test populations with 3 different T-DNAs
(10)	Tnt1	R108-1 (c3)	First test population with Tnt1 (~200 R0 plants)
(11)	FNB	Jemalong A17	80.000 M1, 460.000 M2 http://bioinfo4.noble.org/mutant/
(12;13)	Tnt1	R108-1 (c3)	7,000 Tnt1 mutants, presently extended to 19,000 as reported in http://bioinfo4.noble.org/mutant/

Reference	Mutagenesis technique	Background	Notes
(14)	Tnt1	R108-1 (c3)	~1000 R0
(14)	EMS	Jemalong 2HA	2500 M2 plants
(14)	Activation tagging	R108-1 (c3)	~100 mutant lines
(15)	EMS	Medicago littoralis 'Angel'	Development of new annual medics varieties (i.e. resistant to herbicides)
(16)	Tnt1	Jemalong 2HA	Mutants produced in the frame of the european Grain Legumes Integrated Project (GLIP). The total number of mutants produced by 10 labs all around Europe should be several thousands (~6000). 2000 of them will integrate the Tnt1 collection at Noble.
(17)	FNB	Jemalong A17	31,200 M1 plants, 156,000 M2 plants
(18)	EMS	Jemalong A17	http://195.220.91.17/legumbase/ 2 populations. The first (not using single seed descent, SSD) 500 M1 produced 4500 M2. In the second (using SSD) 4350 M1 and 4350 M2.

Table 1. Mutant collections in *Medicago* spp. **EMS = ethyl methanesulphonate.**

5. Chemical-physical mutagenesis

5.1. Target Induced Local Lesion IN Genomes (TILLING)

Alkylating agents such as ethyl methanesulphonate (EMS) have been used to develop mutant collections of *Medicago truncatula*. EMS induces single base pair C/G to A/T substitution in nucleotides. The mutagenized seeds are germinated and the resulting plants are selfed to produce M1 progenies. The M1 plants are then grown and a TILLING M2 collection is established by growing few seeds from each M1 plant. Total genomic DNA is purified from each M1 plant and pooled. The mutant collections are usually screened with reverse genetics approaches. TILLING involves the identification of mismatches in heteroduplexes formed by single stranded DNA from the wild type and mutant alleles of the target locus. The target sequences are generated by PCR amplification from bulked DNA isolated from single M1 plants using labelled primers appropriate for the detection strategy employed. The amplicons are then heated, causing strand separation, re-annealed in order to form heteroduplexes, cleaved by an endonuclease active on single stranded DNA (i.e. CelI from celery) at the mismatch point and the products separated by electrophoresis. Several EMS mutant collections of *Medicago truncatula* are available. Within the framework of the European Grain Legume Integrated Project two mutant collections were established. The two collections showed the same number of M2 lines that however were obtained from

M1 populations with different size. Genetic analysis of the two collections allowed to define that the number of meristematic cells that contribute to seed (germ-line) in *Medicago truncatula* is 3. The number of mutations detected in the two EMS populations was 1 every 485 kb. A pilot reverse genetic experiment with 56 target genes revealed an efficiency of 13 independent alleles per exon screened, 67% of which were missense and 5% nonsense mutations. An Italian functional genomics initiative produced a small collection of TILLING mutants with about 2500 M2 lines and a reported efficiency of about 4 independent alleles for target sequence. Catalogue of mutant phenotypes were developed and services for reverse screening with target sequence are available (http://inra.fr/legumbase). A list of *M.truncatula* mutants is reported in Table 2.

Reference	Mutagenesis technique	New mutants	Phenotype	Gene/Mutant line
(5)	EMS	1	Nod+Fix-	TE7=Mt*sym*1
(6)	γ-ray	2	Nod-, Myc-	TR25, TR26
(6)	γ-ray	4	Nod±, Myc+	TR34, TR79, TR89, TRV9
(6)	γ-ray	9	Nod+Fix-, Myc+	TR3, TR9, TR13, TR36, TR62, TR69, TR74, TR183, TRV15
(6)	γ-ray	3	Nod++Nts, Myc+	TR122, TRV3, TRV8
(8)	EMS	1	EIN, Nod++	*sickle = skl1*
(8)	EMS	1	Nod-	C71 = *Domi*
(19)	γ-ray	1	Nod-	TRV25
(20)	EMS	3	developmental	*mtapetala (tap), palmyra (plm), speckle (spk)*
(21)	EMS	5	Nod-	B85, B129, C54, P1, Y6 Individuation of 4 complementation groups (DMI1, DMI2, DMI3, NSP): *dmi1-1* = C71 = *domi* *dmi1-2* = B129 *dmi1-3* = Y6 *dmi2-1* = TR25 *dmi2-2* = TR26 *dmi2-3* = P1 *dmi3-1* = TRV25 *nsp1-1* = B85 *nsp1-2* = C54
(22)	EMS	7	Calcium oxalate defective	*cod1, cod2, cod3, cod4, cod5, cod6, cod7*
(23)	EMS	1	Nod-, root hair deformation	*hcl = B56*
(24)	EMS	1	Blocked in the formation of nodule primordial	*pdl1*

Reference	Mutagenesis technique	New mutants	Phenotype	Gene/Mutant line
(24)	EMS	1	Blocked in the formation and/or maintenance of epidermal cell infection	*lin1* (first citation)
(21)	EMS	3	Nod-, root hair deformations	*hcl-1* = B56 *hcl-2* = W1 *hcl-3* = AF3
(25)	EMS	6	Oxalate crystal morphology defective	*cmd1, cmd2, cmd3, cmd4, cmd5, cmd6*
(26)	Fast Neutron	2	Nod-, cortical cell division	*nsp2-1* *nsp2-2*
(27)	EMS	1	Nod-, does not respond to Nod Factors by induction of root hair deformation	*nfp* = C31
(28)	EMS	1	Nod++	*Sunn*
(29)	EMS	1	Blocked in the formation and/or maintenance of epidermal cell infection	*lin1* (first description)
(30)	EMS	1	Numerous infections and polyphenolics	*Nip*
(31)	EMS	1	Nod-, defective in lateral root development	*Latd*
(32)	γ-ray	2	Nod+,Fix-,Myc+	*Mtsym20* = TRV43, TRV54 *Mtsym21* = TRV49
(32)	γ-ray	1	Nod-/+,Myc+	*Mtsym15* = TRV48;
(32)	γ-ray	1	Nod-,Myc-/+	*Mtsym16* = TRV58
(33)	Fast Neutron	6	Fix-	*dnf1-1* = 1D-1; *dnf1-2* = 4A-17; *dnf2* = 1B-5; *dnf3* = 2C-2; *dnf4* = 2E-1; *dnf5* = 2F-16; *dnf6* = 2H-8; *dnf7* = 4D-5
(34)	Fast Neutron	1	Nod-	*bit1*
(35)	Tnt1	1	Single leaflet	*sgl1*
(36)	Fast Neutron	1	Increased nodule number	*Efd*
(37)	EMS	1	Impaired in nodule primordium invasion	*Api*
(38)	EMS	1	Aberrant root hair curling and infection thread formation	*Rpg*
(39)	EMS	1	Myc++, Nod-/+	B9

Reference	Mutagenesis technique	New mutants	Phenotype	Gene/Mutant line
(40)	Fast neutron	1	Leaf dissection	*palm1*
(41)	T-DNA	1	Compact roots	*cra1* (not tagged)
(42)	Tnt1	various	Leaf epidermal morphology	Various
(43)	Tnt1	1	Lack of lignin in the interfascicular region	*nst1*
(44)	Tnt1	1	Secondary cell wall thickening in pith	*mtstp1*
(45)	Fast neutron	1	Compund leaf development	*fcl1*
(46)	Fast neutron	1	Root determined noudulation	*rdn1*
(47;48)	Fast neutron	1	Myc-, Nod-	*Vpy*
(49)	Activation tagging	1	Lack of hemolytic saponins	*Lha*
(50)(1)	Tnt1	1	Stay green	*MtSGR*
(51)	Tnt1	1	Smooth leaf margin	*slm1*
(52)	Tnt1	1	Reduced leaf blade expansion	*Stf*
(53)	Tnt1	1	Inhibition of rust germ tube differentiation	*irg1*

Table 2. *Medicago truncatula* mutants. Nodulation phenotypes: Nod++ = hypernodulator, Nod+ = wild type nodulator, Nod± = reduced nodulation, Nod- = lack of nodules, Nod-/+ = late nodulation. Nitrogen fixation phenotypes: Fix+ = wild type, Fix- = no fixation. Mychorrhization phenotypes: Myc+ = wild type, Myc- = absent or reduced mychorrhizas, Myc-/+ = mix of normal colonization and events of formation only of appressoria with no intercellular hyphae developing from them, Myc++ = hyper responsive to mychorrhization. Nts = nitrate tolerant nodulation. EIN = ethylene insensitive.

5.2. "Delete a gene" collections

Irradiation of plant seeds to appropriate dose of fast neutrons and γ-rays results in deletion of DNA fragments of variable lengths with an average modest reduction of seed viability.

Large mutant collections by seed irradiation have been created for *Medicago truncatula* functional genomics studies. Although the first experiments were based on γ-ray irradiation of the Jemalong J5 seeds (6) the main body of the collection was obtained by Fast Neutron Bombardment (FNB) of the genotype A17. Globally the two larger collections, stored at the John Innes Center and at the Noble Foundation, consist of about 616,000 M2 FNB families.

Both reverse and forward genetic approaches have been successfully applied to study mutants from these collections.

Reverse screening of FNB populations have been carried out by the DeTILLING strategy described by Rogers C *et al.* (17). This strategy allows detection of mutants by PCR on bulks of DNAs of FNB mutants. The wild type target amplification is avoided by a strategy that combines restriction enzyme digestion of the template and the use of poison primers. With this strategy a mutant recovery rate of 29% has been obtained from a population of 156.000 M2 plants (4 genes out of 14 screened).

Nevertheless deletion size can hamper reverse genetics screening (Chen R., personal communication) leaving forward genetics as the main choice in case of FNB populations. However map-based cloning required to discover the mutation of interest is helped by strategies such as transcriptional cloning, originally devised by Mitra R M *et al.* (54), which has allowed the identification of FNB induced mutations (see Table 3). This approach relies on the identification of mutated genes through detailed genome-wide transcriptomic analyses. Also genome-wide analyses of FNB mutant are expected to benefit of the recent development of a *Medicago truncatula* genome-wide tiling array by Nimblegen. A list of *Medicago truncatula* FNB mutants characterized by forward genetics approaches is reported in Table 2.

6. Insertional mutagenesis with DNA mobile elements

6.1. Tnt1

T-DNA tagging has been the strategy of choice for many mutant collections in *Arabidopsis* and it has allowed fundamental discoveries in gene functions and advances in both basic and applied plant research (55). Unfortunately only *Arabidopsis* can be transformed easily by the floral-dip method which allows the generation of large numbers of mutants in a cost-effective manner. Up to now transformation for the other plant species including *M. truncatula* can only be achieved by tissue culture-based protocols requiring great efforts to produce the number of mutants that would allow a significant genome coverage. An interesting strategy has been recently published in the legume *Lotus japonicus* based on the endogenous retrotransposon LORE1 (56;57). LORE1, originally activated via tissue culture, retained its activity for some regenerated plants in the subsequent generations. Based on such discovered germline activity, tagged M1 mutant collections were produced by seed propagation from activated starter lines (M0) (57;58).

In *M.truncatula* large scale collections of mutants have been constructed using the tobacco Tnt1 retrotransposon. d'Erfurth and colleagues have demonstrated that in the *Medicago truncatula* R108 genotype, this element has the ability to transpose during the early steps of in vitro regeneration (10) with a high rate of insertion in transcribed genomic regions. Sequence analyses of insertion sites has showed the virtual absence of insertion site preference. The average amount of new insertions per regenerated line was calculated in the order of ~25. Based on these data it was shown that a collection of 14-16.000 Tnt1 lines will store tagging events for about 90% of *M.truncatula* genes (13). Such an ambitious objective has been pursued by working on two *Medicago truncatula* lines.

The collection maintained at the Noble Foundation (http://bioinfo4.noble.org/mutant/) which includes also the first mutants generated by CNRS in France, is based on the genotype R108-c3. Another collection of about 1000 lines from the same R-108 line was produced by CNR-IGV in Italy.

In the framework of the GLIP project 8000 Tnt1 mutants were produced from the Jemalong 2HA (2HA3-9-10-3) line. The GLIP collection is maintained by the various labs that participated to the project and a subset of plants were merged with the collection at the Noble Foundation.

Iantcheva and colleagues reported that Tnt1 transposition efficiency in Jemalong 2HA has a lower efficiency with only 10-15 new insertions per line and a variable percentage of regenerated plants without transposition (16). The adoption of 2HA line for mutagenesis instead of R108, was motivated by the highest DNA homology to the line used for genome sequencing (Jemalong A17), and for the presence of active and characterized endogenous retroelements (59).

Tnt1 mutant collections have been screened with both forward and reverse genetic approaches. Forward approaches have been based on cloning of host sequence flanking the insertion sites and subsequent identification of events linked to the studied mutation. Based on the duplicated Tnt1 long terminal repeats (LTR) sequences several molecular approaches including thermal asymmetric interlaced (TAIL)-PCR, Inverse-PCR have been used to recover the host sequences flanking the insertion sites (60). Segregation analysis of each cloned insertion site can then be used to select the event linked to the mutation. In alternative the insertion sites associated with the mutations can be selected by segregation analysis prior to host sequence cloning by employing a sequence specific amplification polymorphism (S-SAP) based protocol.

Confirmation of the identity of the mutation can be obtained by means of complementation tests based on the reintroduction of the wild type gene sequence in the mutated background. In alternative one could obtain independent alleles of the target gene and compare their similarity to the original mutant phenotype. This can be done using TILLING and Tnt1 mutant populations as demonstrated by many publications that report successful recovery of alleles by reverse screening (61) and Table 3. The power of the Tnt1 mutagenesis approach is also witnessed by the prevalence of publications reporting successful gene cloning based on such strategy compared to the others since 2008 (Table 2 and 3).

Reference	Mutant	Gene	Approach
(62)	*dmi2*	NORK	Physical mapping
(63)	*dmi1*	AY497771, possible membrane receptor	Physical mapping
(64)	*dmi3*	Ca_2 and Calmodulin dependent protein kinase	Physical mapping/Transcriptional based cloning

Reference	Mutant	Gene	Approach
(65)	*nsp2*	GRAS Transcriptional regulator	Physical mapping
(66)	*nsp1*	GRAS Transcriptional regulator	Physical mapping
(46)	*sunn*	CLV1-like LRR receptor kinase	Physical mapping and gene homology
(67)	*mtpim*	MADS-box	Reverse screening on Tnt1 collection
(68)	*mtpt4*	Phosphate transporter	RNAi and TILLING (reverse)
(34)	*bit1*	ERF transcription factor required for nodulation (ERN)	Transcriptional based cloning
(35)	*sgl1*	MtUNI (transcription factor)	Tnt1 forward
(36)	*efd*	Ethilene responsive factor required for nodule differentiation	Fast neutron reverse
(38)	*rpg*	Putative long coiled-coil protein	Map based cloning
(28)	*sickle*	MtEIN2, ethylene signaling gene	Map based cloning and gene homology
(69)	*lin*	E3 ubiquitin ligase containing a U-box and WD40 repeat domains	Positional cloning
(70)	*srlk*	LRR kinase	TILLING reverse and RNAi
(71)	*mate1*	MATE	Tnt1 reverse
(72)	*ugt78g1*	Glucosyl transferase	Tnt1 reverse
(73)	*mtapetala*	*MtPI*, MADS Box transcription factor	RNAi and mutation segregation analisys
(40)	*palm1*	Cys(2)His(2)zinc finger transcription factor	Fast neutron forward and Tnt1 reverse
(74)		*MtSYMREM1*, remorin	Tnt1 reverse
(44)	*dnf1*	Signal peptidase complex subunit	Fast neutron microarray based cloning
(43)	*nst1*	NAC transcription factor	Forward screening and Tnt1 flanking region cloning
(44)	*mtstp1*	WRKY transcription factor	Forward screening and Tnt1 flanking region cloning
(75)	*ccr1, ccr2*	Cinnamoyl CoA Reductase	Tnt1 reverse
(76)	*ugt73f3*	Glucosyl transferase	Tnt1 reverse
(45)	*fcl1*	Class M KNOX	Fast neutron forward, map based cloning and Tnt1 reverse
(77)	*rdn1*	Uncharacterized plant family	Mapping

Reference	Mutant	Gene	Approach
(78)	*fta, ftc*	MtFTa, MtFTc, protein ligands	Tnt1 reverse
(48)	*vpy*	Vapyrin	Microarray based cloning, Tnt1 reverse
(49)	*lha*	CYP716A12, Cytochrome P450	Flanking sequence tagging and TILLING
(50)	*MtSGR*	Stay green gene	Tnt1 forward and flanking sequence cloning
(51)	*slm1*	Auxin efflux carrier protein	Tnt1 forward and flanking sequence cloning
(52)	*stf*	Stenofolia, WUSCHEL-like homeobox transcription factor	Tnt1 forward and flanking sequence cloning
(71)	*mate2*	MATE	Tnt1 reverse
(53)	*irg1*	Cys(2)His(2) zinc finger transcription factor	Tnt1 forward and flanking sequence cloning
(79)	*mtpar*	MYB transcription factor	Tnt1 reverse

Table 3. *Medicago* genes characterized using mutants.

7. RNAi and VIGS

Reverse genetics studies in *Medicago truncatula* did not only take advantage of the many mutant populations available but also of techniques based on post-transcriptional gene silencing (PTGS). In this case plants are transformed with a construct that will produce double-stranded RNAs that will guide sequence-specific mRNA degradation of the target gene. The phenotype of the transformed plants can gradually vary from wild type to knock-out thus many transformants are needed to obtain the desired effect. Mild effects can be beneficial in case of essential genes whose complete loss-of-function may cause lethal phenotypes. RNAi in *M.truncatula* has been extensively used to study gene function but it has not been a matter of a functional genomics approach as for *Arabidopsis* and the AGRIKOLA collection (80). Nevertheless many gene functions have been characterized exploiting RNAi. A list of gene function and *Medicago truncatula* physiology studies that used RNAi approaches is reported in Table 4.

Reference	Silenced gene	Phenotype
(75)	Lyk3	Marked reduction of nodulation when inoculated with Sm 2011ΔNodFE-GFP
(75)	Lyk4	Effect on infection thread morphology
(81)	CDPK1	Reduced root hair and root cell lengths. Diminution of both rhizobial and mycorrhizal symbiotic colonization.
(82)	DMI2	Reduction of organelle-like symbiosomes in nodules

Reference	Silenced gene	Phenotype
(83)	NFP	Nod-
(76)	MtHAP2-1	Alteration of nodule development
(84)	MtCPK3	Increased average nodule number
(85)	MtCRE1	Cytokinin-insensitive roots, increate number of lateral roots, strong reduction in nodulation.
(86)	MtPIN2, MtPIN3, MtPIN4	Reduced number of nodules
(87)	CHS	Reduced levels of flavonoids and subsequent inability to nodulate
(88)	PR10-1 (pathogenesis related)	Reduced colonization by the root pathogen A.euteiches.
(89)	HMGR1	Dramatic decrease in nodulation.
(90)	IPD3	No obvious phenotype observed
(63)	MtPT4	Premature death of mycorrhizal arbuscules.
(91)	MtSNF4b	Reduced seed longevity, alteration in non reducing sugar content.
(92)	ENOD40-1, ENOD40-2	Reduced nodule number and altered symbiosome development.
(93)	MtFNSII-1, MtFNSII-2	Reduced nodulation
(94)	MtCDD1	Alteration of the Arbuscular Mycorrhizal – mediated accumulation of apocarotenoids
(95)	MtDXS2	Reduction of AM-induced apocarotenoid accumulation.
(78)	MtSERF1	Strong inhibition of somatic embryogenesis
(73)	MtPI, MtNGL9	Altered flower development
(96)	MtWUS	Strong inhibition of somatic embryogenesis
(65)	Srlk	Transgenic root growth less inibited by salt stress.
(97)	FLOT2, FLOT4	Reduced nodulation and root development.
(98)	MtMSBP1	Aberrant mycorrhizal phenotype with thik and septated appressoria, decrease number of arbuscules and distorted arbuscule morphology.
(99)	MtCDC16	Decreased number of lateral roots and increased number of nodules. Reduced sensivity to auxin.
(100)	NPR1	Acceleration of root hair curling at the beginning of symbiosis estabilishment
(101)	MtSNARP2	Aberrant early senescent nodules where differentiated bacteroids degenerate rapidly.
(74)	MtSYMREM1	Reduced nodulation and abnormal nodule development

Reference	Silenced gene	Phenotype
(102)	MtN5	Reduced nodulation
(103)	Vapyrin	Impaired passage across epidermis by AM fungi. Abolition of arbuscule formation.
(104)	MtAOC1	No nodulation phenotype observed
(105)	γECS	Lower homoglutathione content. Lower biological nitrogen fixation associated with a reduction in the expression of the leghemoglobin and thioredoxin S1 genes. Reduction in nodule size.
(106)	MtSAP1	Lower level of storage globulin proteins, vicilin and legumin in seeds and germination deficiency.
(107)	MtNR1, MtNR2	Reduced nitrate or nitrite reductase activity and NO level.
(108)	MtNoa/Rif1	Decrease in NO production in roots but not in nodules. Reduction of nodule number and nitrogen fixation capacity.
(109)	MtROPGEF2	Effect on cytosolic Ca2+ gradient and subcellular structure of root hairs. Reduced root hair growth.
(110)	MtROP9	Reduced growth , no ROS generation after microbial infection. Promoted mycorrhizal and A.euteiches early hyphal root colonization. Impaired rhizobial colonization.
(111)	MtNAC969	Improved growth under salt stress.

Table 4. Use of RNAi approaches in *Medicago truncatula*.

Virus-induced gene silencing (VIGS) is a PTGS technique that can be used transiently by scrubbing leaves or introducing the viral vector in the plant by agro-infiltration. VIGS is being used for large scale forward genetics screening by inoculation of cDNA library and subsequent identification of the gene involved in the process of interest (112). Viral vectors working on *Medicago truncatula* have been recently described. Grønlund *et al.* used successfully a Pea Early Browning Virus (PEBV) based vector for both transient expression of reporter genes and for silencing of the Phytoene Desaturase (PDS) gene that causes a bleaching phenotype (113). Várallyay and colleagues constructed two VIGS vectors based on the Sunnhemp Mosaic Virus (SHMV) that can systemically infect *M.truncatula* without causing severe symptoms and reported a successful silencing of the Chlorata 42 gene (114). Large scale screenings based on VIGS analysis have not been reported for *M. truncatula* as far.

8. Perspectives

Functional genomics of forage legumes started with the aim of determining the molecular and genetic bases of nitrogen fixation and since the beginning mutant collections have been thoroughly screened also for mycorrhyzal symbiosis. These aspects are still being

investigated and we expect that many more results will be published in the next years. A better understanding of nitrogen fixation and symbiosis is fundamental for the development of a sustainable agriculture aiming at a reduction of inputs and at maintaining soil fertility. Nitrogen (N) is one of the crucial nutrients for all organisms including plants. The doubling of world food production in the past four decades was contributed by a sevenfold increase of N fertilization (115). The anthropogenic N which is mostly lost to air, water and land affects climate, the chemistry of the atmosphere, and the composition and function of terrestrial and aquatic ecosystems (116). Improving the ability of plants to exploit environmental nitrogen would decrease N fertilization and its negative consequences; therefore a deep understanding of legume symbiosis with nitrogen fixing bacteria could help the long term goal of transferring the associative ability of legume species to non-symbiotic crops of agronomic relevance. As a consequence functional genomics of nodulation will have an impact on reduction of intensive agriculture practices with benefits for the preservation of environment and quality of human activities.

Another positive role for legumes in an environmental perspective is addressed by species such as *Lotus spp.* that have strong adaptive characteristics making them good candidates for restoration and phytoremediation of degraded environments (117). This happens in the Flooding Pampa (Argentina) where the presence of proteinaceous forages was re-established by the introduction of *L. tenuis*, being the other legume species reduced by the harsh environmental condition.

Pastures and feedstuff including forage legumes have a higher quality compared to those based only on grasses and provide an important input of protein in animal nutrition. More recently public and scientific debate has reassessed forage legumes importance for the quality of livestock nutrition and welfare has having relevant consequences on the quality of final products (meat, milk etc.) and ultimately on human health. This happened because of the occurrence of bovine spongiform encephalopathy (BSE) related to the traditional use of offal in animal feed lots as a source of protein.

Functional genomics in *M.truncatula* proved useful in the study and comprehension of many aspects of plant development and plant secondary metabolism that could not be discovered in earlier models such as *Arabidopsis.* The availability of genomics tools in an increasing number of species has the effect of widening the possibility of new discoveries in the field of plant biology. Worth mentioning the recent advances in understanding compound leaf development and zygomorphic flower ontogeny based on the analysis of several mutants in *M.truncatula* .

Living organisms, and among them plants, can be considered as an abundant and diverse set of biofactories with the ability to synthesize an enormous variety of chemical compounds. Legumes contain chemicals that can prove useful for their anti-oxidant, anti-viral, anti-microbial, anti-diabetic, anti-allergenic and anti-inflammatory properties (118) . These properties are related to secondary molecules such as flavonoids and saponins.

Modest levels of protoanthocyanidins (PAs) in forages reduce the occurrence of bloat and at the same time promote increased dietary protein nitrogen utilization in ruminant animals (119). The lack of PAs in the leaves of the major forage legume such as alfalfa has prompted

studies for the understanding of the molecular and cellular biology of PA polymerization, transport, and storage helped by the functional genomics tools available for *M.truncatula*. Recent positive achievements were obtained by biotechnological strategies based on the overexpression of MYB transcription factors that induced PAs accumulation in both alfalfa and clover leaves (79).

In addition to well-known beneficial properties of flavonoids (cit) recent evidence suggests that flavonoids themselves, particularly fractions rich in PAs, can significantly reduce cognitive deterioration in animal model systems (120-122), and may more generally promote improvements in memory acquisition, consolidation, storage, and retrieval under nondegenerative conditions.

In Chinese medicine one of the oldest herbal medicine was obtained by the roots of the legume plant licorice (*Glychyrriza glabra*).containing the triterpenoid saponin glychyrrizin exhibiting a wide range of pharmacological activities. Cytochrome P450 monooxygenases were proved to be responsible for synthesis of glychyrrizin via oxidative steps based on biochemical experiments (123).

In forage legumes saponins can be toxic to monogastric animals and reduce forage palatability for ruminants. Mutant analysis in *M.truncatula* has unveiled the genetic control of key biosynthetic steps for saponins related to oxidation and glycosilation (49;124), opening possibilities of biotechnological manipulation of saponins in alfalfa.

Both human and animal nutritional science are bound to profit from plant genetic analysis and nutritional genomics, opening possibilities to more personalized approaches to medicine and improvement of the quality of life.

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