# Transformation of leguminous plants to study symbiotic interactions

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ABSTRACT Legume plants are important in agriculture because they represent an important source of protein for human and animal consumption. This high protein content results from their capacity to use atmospheric nitrogen for their nutrition as a consequence of their symbiotic interaction with rhizobia. Understanding this interaction at the molecular level is a prerequisite for its better use in agriculture and for the long term objective of its transfer to other crops. *Agrobacterium*-mediated transformation is a tool of choice for studying this interaction and for unraveling the function of the different genes discovered through classical genetic approaches. However, legume plants are often recalcitrant to regeneration and transformation. This paper describes the technology developments (regeneration, transformation, insertion mutagenesis) related to *Agrobacterium* transformations that were established in the legume plants, as well as different examples of the technology developments or gene discoveries resulting from these studies.

KEY WORDS: *legume plant, agrobacterium, rhizobium, transformation, symbiosis*

## Legumes and their importance

Amongst the mineral nutrients required by plants, nitrogen is the most important but its availability is unfortunately low in most soils. The intensive use of industrial nitrogen fertilizers in modern agriculture in the last 50 years has allowed the development of very productive crops, but their intensive use also resulted in eutrophication of continental waters including some areas in Europe. There is thus a strong need to reduce application of chemical nitrogen fertilizers and improve alternative nitrogen inputs (Graham and Vance 2003; Ferguson *et al.,* 2010) in agriculture. A key contribution of legumes to sustainable agriculture and nitrogen cycle is their ability to fix atmospheric nitrogen in most agricultural ecosystems. They form specialized organs, the root nodules, in association with specific soil bacteria called rhizobia (including the genera *Azorhizobium*, *Allorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*). In these organs the bacteria differentiate into a form called the bacteroids and catalyze the reduction of atmospheric nitrogen gas ($N_2$) into ammonia using the nitrogenase enzyme complex. This process is commonly referred as "symbiotic nitrogen fixation". Annually the legume-rhizobia symbiosis produces around 200 million tons of nitrogen (Graham and Vance 2003) to the host plant thus reducing the need for nitrogen fertilizers. Members of the family *Leguminosae* are major crops used for human food, animal feed, and vegetable oil. They represent the third largest group of angiosperms and are the second largest group of food and feed crops grown worldwide. These plants are cultivated on 12–15% of available arable land and are responsible for more than 25% of the world's primary crop production. Around 250 million tons of grain legumes are produced annually world-wide. The ability of legumes to enter into symbiosis with nitrogen-fixing rhizobia provides them with a unique advantage compared to other plant species. For this reason legumes are called "green manure" and they are used in common farming practice as an intercrop or to rotate crop species, using legumes such as clover or alfalfa in the rotation. This rotation is a process that dramatically improves the organic content of the soil and for this reason improvement of the agricultural capacities of the legumes is always a goal for researchers in addition to the hope that one day this symbiotic capacity could be transferred to other non-leguminous crops (Charpentier and Oldroyd 2010).

## The legume-rhizobia symbiotic interaction

The first step of the symbiotic interaction starts with the exudation of flavonoids (phenolics compounds) into the rhizosphere by the

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*Abbreviations used in this paper:* AM, arbuscular mycorrhiza; N2, nitrogen gas; NF, Nod factor; TDZ, thidiazuron; T-DNA, agrobacterium tranfered DNA.

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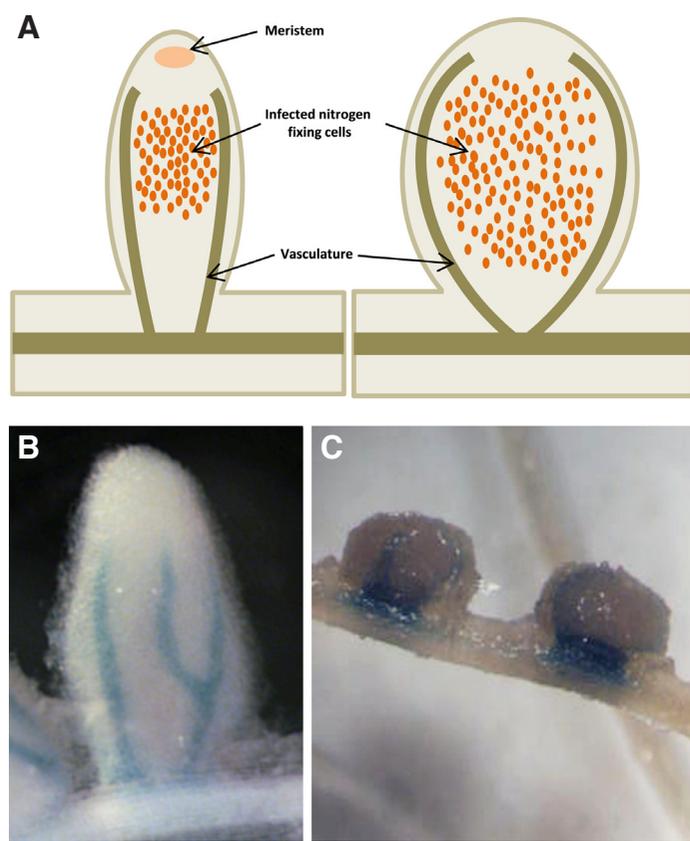
Fig. 1. **Indeterminate and determinated nodules. (A)** *Schematic representation of the indeterminate (left) and determinated (right) nodules. The meristem position is represented by an orange oval in apical position. The infected cells in which nitrogen fixation takes place are represented by orange dots.* **(B)** *An indeterminated nodule expressing a nodule vascular tissue specific GUS fusion.* **(C)** *A determinated nodule expressing a cyclin F box promoter-GUS fusion at the base of the nodule and in the vascular tissues.*

host plant roots. The specific flavonoid composition of the exudate determines the rhizobia species attracted. With the exception of rhizobium species NGR 234, which is able to colonize about 112 species, rhizobia are generally able to establish symbiosis with only few legumes. This attraction of specific rhizobia to the roots of host legume leads to the activation of bacterial *nod* genes and production and secretion of specific lipochito-oligosaccharides called Nod Factors (NFs) (Ferguson *et al.*, 2010; Oldroyd *et al.*, 2011) that are necessary for the bacterial invasion and the nodule organogenesis. The NFs are composed of an oligosaccharide backbone of *N*-acetyl-D-glucosamine units with a fatty acyl group attached to the non-reducing sugar. Specific modifications of this backbone happen in different rhizobium strains that define the specificity required for the plant bacterial recognition. In the rhizosphere, rhizobia attach to root-hair tips, initiate root hair deformation and curling and penetrate the root hair via a structure called the infection thread. This structure allows the symbiotic bacteria to reach the cortical cells of host plant. Increasing concentration of NF leads to mitotic activation of cortical cells in the root and starts the development of the nodule primordium. The infection thread grows towards the newly induced dividing cells and bacteria are released into an infection droplet in the host cell cytoplasm,

forming an organelle like structure called the symbiosome. In the symbiosome, bacteria differentiate into bacteroids. In fully developed mature nodules, atmospheric $N_2$ is reduced into ammonia by bacteroids and subsequently assimilated by the plant (Ferguson *et al.*, 2010; Oldroyd *et al.*, 2011).

Legume nodules are of the determinate or indeterminate type (Fig. 1A). There are three main differences between these two nodule types: the site of initial internal cell division, the lifetime of meristematic cells and the shape of the mature nodules. Indeterminate nodules are characterized with initial anticlinal division of inner cortex and periclinal divisions in endodermis and pericycle, leading to the formation of nodule primordia. Their persistent meristem divides continuously giving rise to new nodule cells and thus results in nodules with elongated shape. Legume species like *Medicago sativa* (alfalfa), *Trifolium repens* (clover), *Pisum sativum* (pea) and the model legume *Medicago truncatula* form indeterminate nodules. Determinate nodules are characterized by their round shape and lacks persistent meristem that results in the absence of developmental gradient. The initial cell division occurs sub-epidermally in the outer cortex. Legumes that form determinate nodules include *Glycine max* (soybean), *Phaseolus vulgaris* (common bean), and *Lotus japonicus*. *M. truncatula* and *L. japonicus* are used as model legumes to study indeterminate and determinate types of nodules, respectively (Handberg and Stougaard, 1992; Stiller *et al.*, 1997).

In this review we discuss about various protocols available for legume regeneration and transformation. In addition, we will also describe how transformation technology has helped in deciphering some of the molecular events that allow legume plants to establish symbiosis with rhizobia.

## Legume regeneration and transformation

The main component of most system biology approaches is a high-throughput regeneration/transformation system to decipher gene function. Transformation offers strategies for overexpressing or suppressing endogenous genes as well as expressing various gene fusions thus understanding their biological role during plant development. Large scale accumulation of data from "omics" platform in legumes necessitates the development of transformation protocols for rapid *in vitro* and *in vivo* investigation of functions and subcellular localization of the target genes. In addition to understanding gene function legume transformation also provides possibilities for crop improvement. For example, transgenic soybean is being widely cultivated in North and South America (Yamada *et al.*, 2012).

Legume *in vitro* regeneration which has long been an obligatory step for transgenic production is still a challenge for plant scientists, thus making many legumes recalcitrant to efficient *Agrobacterium tumefaciens*-mediated plant transformation. However, the extensive use of the model legume plants like *M. truncatula* and *L. japonicus* for molecular studies has favoured the development of efficient regeneration and *Agrobacterium*-mediated transformation protocols for these two plant species. *Agrobacterium*-mediated transformation protocols rely on the availability of highly totipotent cells (targets for the transformation events), specific genotypes and proper selection of transformed tissue followed by 4 to 6 months of tissue culture.

*In vitro* regeneration of legumes is based on direct organogenesis, indirect organogenesis or somatic embryogenesis (Fig. 2) starting with different plant explants. Successful regeneration is

accomplished by determination of species-specific parameters like explant source, plant genotype and media components. The somatic embryogenesis approach has been favoured because each event of regeneration is supposed to be derived from one cell and chromosomal rearrangements are less frequent (Arcioni *et al.*, 1989). In the legume crops like alfalfa and red clover (*Trifolium pratense*) different dominant genes are thought to be involved in the control of somatic embryogenesis (McLean and Nowak, 1988; Reisch and Bingam, 1980). Dramatic improvement of embryogenic capacity has been observed after several cycles of recurrent selection mainly in the *Medicago* genus. Using this approach, lines with high embryogenic capacity were selected in *M. sativa* Rengelander (Atanassov and Brown, 1984), *M. sativa* Regen–SY (Samac *et al.*, 2004), *M. varia* A2 (Deak *et al.*, 1986), *M. sativa* R4 (Barbulova *et al.*, 2002), *M. falcata* 47/1/150 (Denchev *et al.*, 1991), *M. truncatula* cv. R108-1 (Hoffman *et al.*, 1997), *M. truncatula* cv. Jemalong 2HA (Nolan *et al.*, 1989) and *M. truncatula* cv.Jemalong M9-10a (Araujo *et al.*, 2004). It has been shown in *Medicago* (Rakocevic *et al.*, 2009) and in *Lotus* (Madsen *et al.*, 2005; Fukai *et al.*, 2008; Fukai *et al.*, 2012) that *in vitro* regeneration activates endogenous retroelements. We can therefore postulate that these recurrent selections have resulted in chromatin modification (epigenetic modifications) and/or insertion of new copies of endogenous elements that are deregulating genes that act as negative regulators of embryogenesis (Srinivasan *et al.*, 2007). These highly embryogenic lines can then be considered as mutant lines.

For *M. truncatula*, direct regeneration as well as somatic embryogenesis protocols are well described (Cosson *et al.*, 2006; Wright *et al.*, 2006; Crane *et al.*, 2006, Iantcheva *et al.*, 2001, Chabaud *et al.*, 1996; Zhou *et al.*, 2004). It should be noted that each protocol was developed for a specific line and may not be applicable to the whole species. For *L. japonicus cv.* Gifu, regeneration and stable *Agrobacterium*-mediated transformation protocols, mainly based on the use of hypocotyl explants, were develop by Handberg and Stougaard (1992) and improved by Stiller *et al.* (1997) who also established an *A. rhizogenes* hairy root transformation method.

Somatic embryogenesis in legumes is affected by exogenously supplied auxin, which plays important role in somatic embryo induction and development. In most legumes the auxin analog 2,4D (2,4-dichlorophenoxyacetic acid) induces high frequency of embryo production, but large numbers of these embryos are morphologically abnormal and unable to convert to plantlets (Garg *et al.*, 1996). In pea and soybean, embryos with normal morphology are obtained using the auxin NAA (1-naphthaleneacetic acid; Lazerri *et al.*, 1987; Ozcan *et al.,* 1993). Induction of somatic embryogenesis by cytokinin alone is rare but it is required for induction of somatic embryos from intact seedlings of *Phaseolus* species (Malik and Saxena, 1992a). In contrast, its presence strongly inhibited direct somatic embryo induction in pea and soybean (Kysley and Jacobsen, 1990; Lazzeri *et al.*, 1987). The highly embryogenic effect of cytokinin is observed for indirect somatic embryogenesis after an extensive callus induction step notably in the genera *Medicago* and *Trifolium*. The synthetic plant growth regulator thidiazuron (TDZ) with both auxin and cytokinin activity possess remarkable regeneration ability in some leguminous plants like bean (Malik and Saxena, 1992), peanut (*Arachis hypogeal*; Murthy *et al.*, 1995) and some diploid *Medicago* species (Iantcheva *et al.*, 1999). Interestingly, in peanut TDZ induced somatic embryogenesis is genotype independent (Saxena *et al.*, 1992). Somatic embryo maturation is a crucial step during somatic embryogenesis and it has been shown that cytokinin alone or in combination with an auxin is important for this somatic embryo maturation in chickpea (*Cicer arietinium*; Kumar *et al.,* 1995) and *Vigna aconitifolia* (Kumar *et al.*, 1988).

Regeneration/transformation procedures based on repetitive somatic embryogenesis (a process of formation of secondary somatic embryos on primary ones) have been described for several legume species including alfalfa, soybean, peanuts and diploid medics (Lupotto 1983; Finer and Nagasawa, 1988; Parott and Bailey, 1993; Iantcheva *et al.*, 1999). Repetitive embryogenesis was explored for *Agrobacterium*-mediated transformation of *M. sativa* (Nincovic *et al.*, 1995) and *M. truncatula* (Iantcheva *et al.*, 2005). In soybean, regeneration and transformation protocols are established either on somatic embryogenesis using immature embryos (Parott at al. 1989) or cotyledons (Hinchee *et al.*, 1988) or organogenesis from cotyledonary nodes or geminating seeds (Olholt *et al.*, 2003). In chickpea, different regeneration/transformation protocols have been developed using leaf or stem (Srinivasan and Sharma 1991; or cotyledons and cotyledonary nodes (Bhattacharjee *et al.*, 2010)
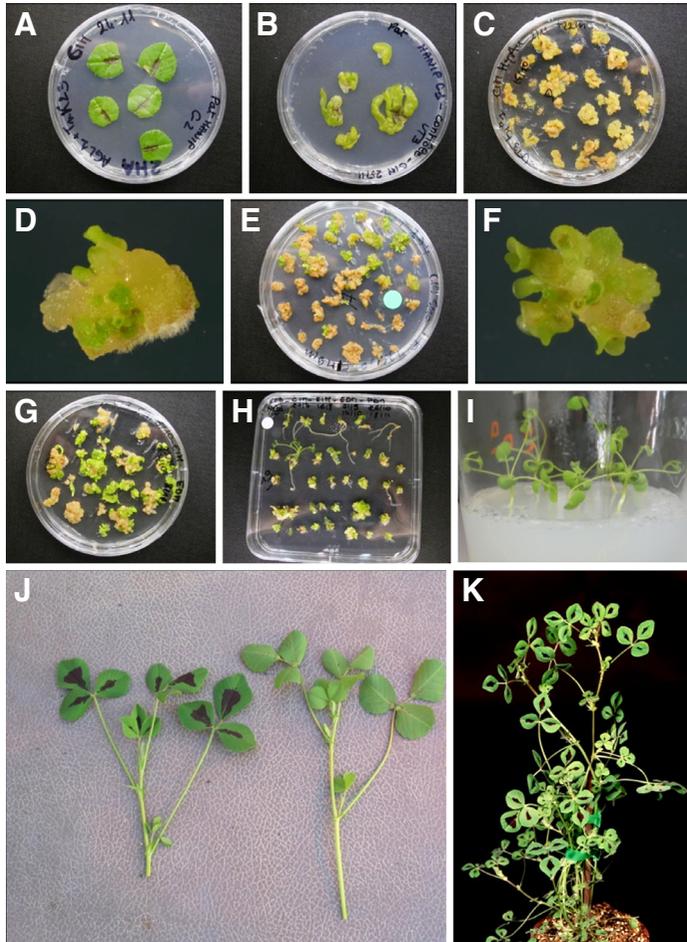


**Fig. 2. Regeneration of *Medicago* plants using *in vitro* somatic embryogenesis.** *Leaf explants **(A)** are placed on callus inducing medium **(B)** in order to induce embryogenesis **(C)**. Embryos **(D-F)** are differentiating on hormone free medium in plantlets **(G)** that are placed on rooting medium **(H)** and allow to develop in true plantlets **(I)**. These are later adapted and transferred in the green house for further development **(J)** and seed production **(K)**. In panel J the plant in the left is* M. truncatula *ssp* truncatula Jemalong *and the plant in the right is* M. truncatula *ssp* tricycla *R108.*

or mature embryogenic axes (Mehrotra *et al.*, 2011). In pea, segments of embryogenic axis (Polowick *et al.*, 2000), cotyledonary nodes (Bean *et al.*, 1997), and immature cotyledons (Grant *et al.*, 2003) have been used for transformation. The above protocols are generally genotype dependent, less efficient and lead to production of chimeric plants.

In addition to the peculiarity of the *in vitro* regeneration of legume, another bottle neck to generate transgenic legume plants is the choice of the *Agrobacterium* strain used for transformation. Some strains used currently for *Brassicaceae* or *Solanaceae* species (for example strain LBA4404) are less efficient in transforming legume cells. Legume transformation has been improved by the use of hyper virulent strains like EHA105, EHA101 (Hood *et al.*, 1993), KYRT1 (Grant *et al.*, 2003; Ko *et al.*, 2003) and eventually by adapting protocols to reduce the biotic stress created by the infection. Use of acetosyringone (improved virulence gene activation) and/or stress protectants like cystein and dithiothreitol, offers improved *Agrobacterium*-mediated plant transformation in soybean (Olhoft *et al.*, 2003, Paz *et al.*, 2004), maize (Vega *et al.*, 2008) and cell suspension of *M. truncatula* (Iantcheva *et al.*, unpublished data). Finally the optimization of selection-marker used during the transformation procedure is also important and should be carefully defined for each plant species (Cosson *et al.*, 2006).

An alternative method for production of transgenic material uses the *A. rhizogenes*-mediated transformation, as the production of transgenic legume plants is not always necessary when studying symbiosis. This is a rapid method to produce hairy roots on composite plants (transformed roots are induced on a non-transformed plant). Induced transgenic hairy root system can be nodulated by symbiotic bacteria or can establish symbiosis with mycorrhizal fungi. Hairy roots possess hormone-independent growth, are much more branched, and contain numerous root hairs. The capacity to produce chimeric transgenic plants is an important tool for scientists involved in root, nitrogen fixing and endomycorrhizal symbiotic studies. *A. rhizogenes*-mediated transformation in legumes was first described for *Lotus corniculatus* (Jensen *et al.*, 1986). Such transgenic roots can be nodulated directly and this technology has now been reproduced in other (model) legumes such as *M. truncatula* (Boisson-Dernier *et al.*, 2001; Gourion *et al.*, 2012), *V. aconitifolia* (Lee *et al.*, 1993), *L. japonicus* (Stiller *et al.*, 1997), red clover (Diaz *et al.*, 2000), bean (Estrada-Navarrete *et al.*, 2006), *Aeschinomene indica* (Bonaldi *et al.*, 2010) and peanut (Sinharoy *et al.*, 2009).

In the past 15 years, the efforts of the legume scientific community concentrated on studying host genes involved in the legume-rhizobium symbiosis. As indicated above, two model species, *M. truncatula* and *L. japonicus* are popularly used for these studies. Subsequently, a large number of genes involved in NF perception and symbiotic signal transduction, bacterial infection, nodule organogenesis, and regulation of nitrogen fixation have been identified in these two species. It should be noted that the arbuscular mycorrhizal (AM) symbiosis can also be studied in these model leguminous plants and has benefited from the knowledge developed for the rhizobia symbiosis. However, the benefit of the transformation methods to study AM symbiosis will not be discussed here. This review emphasizes the role of the transformation approach as a tool for understanding the functions of key regulatory and signaling related genes, as well as the role of phytohormones and other signal molecules involved in different stages of the symbiosis between legume plants and rhizobia.

## Insertion mutagenesis

One important outcome of establishing regeneration/transformation protocols in model legumes was the possibility to develop insertion mutagenesis in these plants. Insertion mutagenesis has

TABLE 1

**A REPRESENTATIVE (NON EXHAUSTIVE) LIST OF SYMBIOTIC GENES STUDIED USING *AGROBACTERIUM*-MEDIATED STABLE OR HAIRY ROOT TRANSFORMATION IN *M. TRUNCATULA***

| Experiment | Gene | Gene function | References |
|---|---|---|---|
| ***Agrobacterium tumefaciens*-stable transformation** | | | |
| Gene fusion (GUS) | Enod12-early nodulin gene | Early nodule development | Chabaud et al. (1996) |
| Gene fusion (GUS) | DNF2-GUS | Bacteroid maintenance | Bourcy et al., (2013) |
| Gene fusion (GUS) | NOOT-GUS | Nodule identity | Couzigou et al., (2013) |
| Overexpression | *P5CS*-(D-pyrroline-5-carboxylate synthetase) | Enhanced nodule activity during the condition of osmotic stress | Verdoy et al. (2006) |
| Ectopic expression of dominant negative form of the *AT* SINAT5DN | *SINA* (Seven in Absentia Proteins ) | Affects plant growth and nodulation | Den Herder et al. (2008) |
| Genomic DMI2 construct fused to a dual affinity tag | *DMI2* (does not make infection) | Biochemical characterization of symbiotic receptor kinase | Reily et al (2012) |
| ***Agrobacterium rhizogenes*-hairy root transformation** | | | |
| Complementation | *MtLyk3* (HCL) | NF entry receptor | Smit et al. (2007) |
| Gene fusion (GUS) | *MtIRE* | Unknown | Pislariu et al., (2007) |
| RNAi | *MtLyk3* (HCL) | NF entry receptor | Limpens et al., (2003) |
| RNAi | *NFP* (nod factor perception) | NPF Nod factor perception | Arrighi et al. (2006) |
| RNAi | *DMI1*(does not make infection) | DMI1 (calcium spiking) | Ane et al 2004 |
| Complementation | *NSP1*, *NSP2* Nodulation Signaling Pathway | Efficient nodulation | Kalo et al., (2005); Smit et al., (2005) |
| Complementation; RNAi | *EFD* ethylene response factor required for nodule differentiation | Nodule initiation and development | Vernie et al. (2008) |
| RNAi | *CHS* chalcone synthase | Nodule initiation and regulation of auxin transport | Wasson et al (2006) |
| RNAi | *MtCRE1* Cytokinin Response1 | Control of nodulation | Gonzalez-Rizzo et al. (2006) |
| RNAi | *LAX* auxin influx carrier | Early steps of nodule development | Billy et al. (2001) |
| RNAi | *PIN* auxin efflux carrier | Nodule development | Huo et al. (2006). |
| | *CDC16* gene / *CELL DIVISION CYCLE16* / | Root and nodule development | Kuppusamy et al. et al. (2009) |

been instrumental in the development of molecular tools necessary for the understanding of plant physiology and development because it allows efficient gene disruption (KO mutations) and gene tagging that facilitate isolation of the mutated gene. In both *L. japonicus* and *M. truncatula*, the T-DNA tagging strategy developed in *Arabidopsis thaliana* could not be applied because of the absence of high throughput transformation protocols (Brocard *et al.*, 2006) necessary for the production of large insertion mutant collections. On the contrary, this was possible using the *Tnt1* and *MERE* retroelements in *M. truncatula* (d'Erfurth *et al.*, 2003; Tadege *et al.*, 2008; Revalska *et al.*, 2011; Rakocevic *et al.*, 2009) and *Lore1* retroelement in *L. japonicus* (Madsen *et al.*, 2005; Fukai *et al.*, 2012). The *Tnt1* tobacco retroelement was introduced in *M. truncatula* using a T-DNA based approach but it was shown that its autonomous transposition is induced only during *in vitro* somatic embryogenesis. The *MERE1* endogenous retroelement was first characterized as a somaclonal mutation in a transgenic line obtained after T-DNA transformation. Similarly to *Tnt1*, *MERE1* transposes during the process of *in vitro* somatic embryogenesis. The *Lore1* element in *L. japonicus* was characterized as an element responsible for somaclonal variations in the progeny of plants, obtained through *in vitro* culture regeneration. Transposition occurs at high frequency in genes of these plants. The retroelement-mediated insertion mutant collections in these two plants have now allowed the characterization of numerous symbiotic mutants in both the plant species (http://medicago-mutant.noble.org/mutant/, http://www.kazusa.or.jp/lotus, see also Table 1 and Table 2) and continue to contribute to the understanding of the symbiotic process in legume plants.

## Model plants

### Medicago truncatula, *a model for indeterminate nodule formation*

In *M. truncatula*, the model plant to study indeterminate nodules, the different transformation technologies have facilitated the identification of the signaling pathway necessary for symbiotic interactions. Both stable transformation and hairy root transformation systems have been used in *M. truncatula*. The hairy root system was used mainly for complementation or gene knockdown. In addition, promoter-GUS fusions or fusions to other reporter genes were used in these studies. Only few of the research investigations related to endosymbiosis were done using stable transgenic plants. This is because obtaining stable transformants through *Agrobacterium*-mediated transformation is labour-intensive, expensive and time consuming. There are two pioneer studies of Chabaud *et al.* (1996) and Trinh *et al.* (1998) on transformation of *M. truncatula* and *M. sativa* with transcriptional reporter constructs. These stable transgenic plants have the advantage that they can be reproduced, propagated and crossed to mutant genotypes and used for different studies.

Some examples of the results obtained, using transgenic technology, to understand the *M. truncatula-Sinorhizobium* symbiosis based on stable and hairy roots transformation methods are summarized below in Table 1. A transgenic nodule expressing the GUS reporter gene in vascular tissues is schown Fig. 1B.

### Lotus japonicas, *a model for determinate nodule formation*

*L. japonicus* a member of the genera *Loteae* is used as a model legume to study determinate type of nodulation (Handberg and Stougaard, 1992). Similar to what has been achieved in *M. truncatula*, the different transformation technologies developed for *L. japonicus* have facilitated the identification of various components of the Nod factor signaling pathway necessary for the interaction. Some examples using the transgenic technology to understand the *L. japonicus-Mesorhizobium loti* symbiosis are outlined in Table 2. A transgenic Lotus nodule expressing a cyclin-like F box-GUS gene fusion is schown Fig. 1C.

## Crop plants

### Alfalafa (Medicago sativa)

Alfalfa is one of the major forage crops in the world and also the first legume species regenerated and transformed *in vitro* (Deak

TABLE 2

**A REPRESENTATIVE (NON EXHAUSTIVE) LIST OF SYMBIOTIC GENES STUDIED USING *AGROBACTERIUM*-MEDIATED STABLE OR HAIRY ROOT TRANSFORMATION IN *L. JAPONICUS***

| Experiment | Gene | Gene function | References |
|---|---|---|---|
| ***Agrobacterium tumefaciens*-stable transformation** | | | |
| pGH3:GUS:GFP | Soybean auxin responsive promoter. | Auxin distribution during the process of nodule development | Pacios-Bras et al. (2003) |
| Complementation | HAR1 | Control of the nodule number | Nishimura et al., (2002a) |
| Complementation | ASTRAY | Control of the nodule number | Nishimura et al., (2002b) |
| Complementation | IGN1 | Bacteroid persistence | Kumagai et al., (2007) |
| Overexpression | NahG salicylate hydroxylase | Reduction of SA levels in respect to control of nodule | Stacey et al. (2006) |
| ***Agrobacterium rhizogenes*-hairy root transformation** | | | |
| Complementation | NFR1; NFR5 nod factor recognition | Initiation of the symbiotic interaction | Radutoiu et al. (2003); Madsen et al., (2003) |
| Complementation | CASTOR POLLUX | Ion channels essential for perinuclear calcium spiking | Miwa et al., (2006) |
| Complementation | SEN1 | Nitrogen fixation | Hakoyama et al. (2011) |
| Complementation | NUP133 symbiotic nucleoporin | Rhizobial colonization | Kanamori et al. (2006) |
| Complementation | NUP85 symbiotic nucleoporin | Rhizobial colonization | Saito et al. (2007) |
| Complementation | CCaMK CYCLOPS | Initiation of symbiotic interaction | Kistner et al, (2005) |
| Complementation | NIN | Initiation of nodule organogenesis | Schauser et al, (1999) |
| Gene function | Cytokinin receptor | NF signaling | Tirichine et al., (2006) |
| Gene function | Ca2+/Calmoduline dependant kinase | NF signaling | Tirichine et al., (2007) |
| Complementation | NSP1; NSP2 | Formation of infection threads | Heckmann et al, (2006) |

*et al.*, 1986; Pichon *et al.*, 1992). The cultivated alfalfa is peren-
nial, tetraploid and allogamous and possesses a relatively large
genome, making it not suitable for genetic analysis. In contrary,
*M. truncatula* that is closely related to alfalfa has a relatively small
sized diploid genome making it suitable for genetic/genomic studies
(see above). However, some research related to the role of legume
lectins during symbiosis was done in alfalfa. One of the proposed
functions of legume lectins is to determine the specificity of the
rhizobia-legume symbiosis (Hirsh 1999). For this study, Brill *et al.*
(2004) obtained transgenic alfalfa plants that expressed the alfalfa
lectin *MsLEC1* and *MsLEC2* in sense and antisense orientations.
*MsLEC1*-antisense (*LEC1AS*) plants were stunted, exhibited
hypernodulation phenotype, and developed not only abnormally
large nodules but also numerous small nodules, both of which se-
nesced prematurely. *MsLEC2*-antisense plants were intermediate
in growth and nodule numbers compared with *LEC1AS*. To confirm
lectin function, seed lectin gene (*PSL*) from pea was expressed in
white clover hairy roots. Transformed "hairy" roots were nodulated
by the pea specific symbiont *Rhizobium leguminosarum* bv. *viciae*
(Díaz *et al.*, 2000) thus demonstrating the role of the lectins in the
specificity of symbiotic interaction.

### Pea (Pisum sativum)

Pea has been a model organism in genetic research for more
than a century. Intensive genetic and phenotypic analyses using
pea symbiotic mutant collections showed that more than 40 genes
are involved in the symbiotic process (Tsyganov *et al.*, 2002).
However, pea is an orphan species among legumes in respect to
root symbioses (nodulation and mycorrhiza-formation) molecular
studies. Pea has a larger genome, relatively long life cycle and
larger size thus making it unattractive to do genetic research when
compared to other model legumes. In addition, the pea genome
sequence is not yet publically available and there are no efficient
regeneration/transformation-systems developed. For this reason
only few laboratories have published symbiotic studies using
transgenic pea plants (Krejci *et al.*, 2007; DeMason *et al.*, 2013).
There is only one report related to symbiotic studies in pea where
*Agrobacterium*-mediated transformation has been used to study
*ENOD12A:GUS* gene construct (Schneider *et al.,* 1999). In addition,
there is one report showing successful formation of hairy roots on
composite pea plant, for complementation of a nodulation mutant
(Hohnjec *et al.*, 2003). Clemow *et al.* (2011) recently established
a protocol for the rapid development of transformed hairy roots on
pea composite plants using *A. rhizogenes*. The authors showed that
the transformed roots of two non-nodulating pea *sym10* mutants
complemented with the wild-type gene. This protocol should now
facilitate the studies done with various pea symbiotic mutants.

### Soybean (Glycine max)

Soybean is one of the most important legume crops in the world.
Genetically modified (transgenic) soybean plants have been pro-
duced mainly to improve their field performance and to understand
gene function. For this, *Agrobacterium*-mediated or biolistic (par-
ticle bombardment) methods have been used to transform shoot
meristems, cotyledonary nodes or cultured embryogenic tissues
(Finner *et al.*, 1991; Hinchee *et al.*, 1988). These transforma-
tion techniques are labor-intensive and inefficient for large scale
production. For studies related to root biology and symbiotic and
pathogenic interactions, an efficient *A. rhizogenes* transformation

method was developed by Kereszt *et al.* (2007). Indrasumunar *et
al.* (2011) described two highly related lipo-oligochitin LysM-type
receptor kinase gene products (GmNFR1α and GmNFR1β) as puta-
tive Nod factor receptor components in soybean. Transgenic hairy
roots of non-nodulating mutant lines overexpressing *GmNFR1a*
but not *GmNFR1b* form nodules with *Bradyrhizobium japonicum,*
demonstrating the functionality of *GmNFR1a*. Other host genes
referred as *Rj* genes have been identified as controlling nodulation
traits upon inoculation with compatible *Bradyrhizobium* (Hayashi
*et al.*, 2012). In addition Radwan *et al.* (2012) have used RNA
interference (RNAi) approach to silence *SGF14c* and *SGF14l*
gene expression in soybean roots using *A. rhizogenes*-mediated
root transformation. Silencing of these genes encoding 14-3-3
proteins resulted in reduced numbers of mature nodules and large
numbers of arrested nodule primordia following *B. japonicum* in-
oculation. This work suggests a critical role of 14-3-3 proteins in
early stages of nodule development. Recently it was shown that
soybean ureide transporters play a critical role in nodule develop-
ment, nodule functioning and in nitrogen export out the symbiotic
organ. For this, Collier and Tegeder (2012) demonstrated, using
*A. rhizogenes* transformation combined with an RNAi approach,
the function of two soybean proteins, GmUPS1-1 and GmUPS1-2
in allantoin and allantoic acid transport out of the nodule. The
repression of *GmUPS1-1* and *GmUPS1-2* in nodules resulted in
an accumulation of ureides and decreased nitrogen partitioning
between roots and shoots.

### Common bean (Phaseolus vulgaris) and cowpea (Vigna un-guiculata)

Consortia focusing on common bean (www.phaseolus.net) and
cowpea (http://www.entm.purdue.edu/ngica/) have allowed making
progresses in these two plants. For common bean and cowpea,
genetic transformation protocols are not well established. Although
progress has been made in establishing a transformation protocol
for *Phaseolus acutifolius* (Zambre *et al.*, 2005), the developed
protocol is not routinely used. By using *A. rhizogenes*-mediated
hairy root transformation, Estrada-Navarrete *et al.,*2006) reported
the successful production of composite plants for the otherwise
transformation recalcitrant bean species, *P. vulgaris*, *P. coccineus*,
*P. lunatus* and *P. acutifolius*.

## Conclusions

Leguminous plants are recognized worldwide because of their
environmental and agricultural benefits. Recent progresses in
molecular biology, genomics, transcriptomics, proteomics, metabo-
lomics and bioinformatics of model legumes, *M. truncatula* and *L.
japonicus* have provided new opportunities for deciphering gene
function in legumes. The genome sequences or draft genomes
of *M. truncatula, L. japonicus, G. max, P. vulgaris* and *C. arietum*
are now available (www.phytozome.net; http://cicar.comparative-
legumes.org). These legume plants share considerable genetic
synteny with other crop legumes and this has allowed the suc-
cessful translation of knowledge from these well characterized
legumes to other agriculturally important legumes that are not
well characterized. A long-lasting research idea is to transfer the
legume genes required to establish symbiosis with nitrogen-fixing
rhizobia to non-legume crop species (Charpentier and Oldroyd,
2010). Such genetic modifications will require transfer of multiple

genes or pathways. Recently, Untergasser *et al.,*2012) developed a vector able to transfer eight genes in one-step by *Agrobacterium*-mediated transformation. Genes essential for *M. truncatula* to establish a symbiosis with rhizobia were transferred to four non-leguminous species; strawberry, poplar, tomato and tobacco and all the transgenes were shown to be expressed in the root tissue of these non-legumes. Long term efforts of scientists will then continue to focus on the establishment of successful symbiotic interactions of rhizobia with non-legume crops.

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