TECHNICAL ADVANCE

A Toolbox for Nodule Development Studies in Chickpea: A Hairy-Root Transformation Protocol and an Efficient Laboratory Strain of *Mesorhizobium* sp.

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A *Mesorhizobium* sp. produces root nodules in chickpea. Chickpea and model legume *Medicago truncatula* are members of the inverted repeat-lacking clade (IRLC). The rhizobia, after internalization into the plant cell, are called bacteroids. Nodule-specific cysteine-rich peptides in IRLC legumes guide bacteroids to a terminally differentiated swollen (TDS) form. Bacteroids in chickpea are less TDS than those in *Medicago* spp. Nodule development in chickpea indicates recent evolutionary diversification and merits further study. A hairy-root transformation protocol and an efficient laboratory strain are prerequisites for performing any genetic study on nodulation. We have standardized a protocol for composite plant generation in chickpea with a transformation frequency above 50%, as shown by fluorescent markers. This protocol also works well in different ecotypes of chickpea. Localization of subcellular markers in these transformed roots is similar to the localization observed in transformed *Medicago* roots. When checked inside transformed nodules, peroxisomes were concentrated along the periphery of the nodules, while endoplasmic reticulum and Golgi bodies surrounded the symbiosomes. Different *Mesorhizobium* strains were evaluated for their ability to initiate nodule development and efficiency of nitrogen fixation. Inoculation with different strains resulted in different shapes of TDS bacteroids with variable nitrogen fixation. Our study provides a toolbox to study nodule development in the crop legume chickpea.

Root nodule symbiosis (RNS) is the most successful metabolism-dependent symbiosis on the earth. Leguminous plants get reduced nitrogen directly from RNS at the expense of photosynthate (Werner et al. 2015). The staple crop chickpea (*Cicer arietinum*) (2n = 2x = 16) is the world's second largest cultivated legume. Chickpea seeds are a valuable source of dietary protein in lower socio-economic classes. The predicted genome size of chickpea is approximately 740 Mb. Due to the

symbiotic interaction with a *Mesorhizobium* sp., chickpea can be cultivated in a sustainable way (Jain et al. 2013; Varshney et al. 2013). Research on nodule development has been centered upon model legumes *Medicago truncatula* and *Lotus japonicus*. Today, model legumes are in the forefront of legume biology in terms of both available knowledge and resources. The only disadvantage is that model legumes are not crop species. In spite of being the most important grain legume in tropical and subtropical countries (Jukanti et al. 2012), the literature on chickpea nodule development is scarce. Genome sequencing and the establishment of transcriptomic and proteomic resources have laid the pillars for making chickpea a model among crop legumes (Jain et al. 2013; Pandey et al. 2018; Ramalingam et al. 2015; Varshney et al. 2013). Further, seed-to-seed generation time in chickpea is short, between 85 and 100 days, which makes it even more suitable for future studies (Upadhyaya et al. 2007).

Chickpea belongs to the inverted repeat-lacking clade (IRLC), which diverged from model legume *Medicago* approximately 10 to 20 million years ago (Jain et al. 2013; Varshney et al. 2013). IRLC legumes develop indeterminate nodules, in which a gradient of cells at different stages of development can be seen from the distal to the proximal part of the nodule. A persistent meristem (zone I) is present at the distal end of the nodule. Bacterial endocytosis and colonization take place in the postmeristematic cells of the infection zone (zone II), in which plant membrane-bound bacterial units are formed. These units are called symbiosome (Roth and Stacey 1989). The rhizobia inside the symbiosomes are called bacteroids. The bacteroids divide inside the symbiosome and gradually colonize the whole cell. In the nitrogen-fixation zone (zone III), bacteroid differentiation is terminated, ammonium assimilation genes are repressed, and nitrogen-fixation genes are induced. In the nitrogen-fixation zone, the symbiosome are considered as an ammonium-exporting organelle (Oldroyd 2013; Udvardi and Poole 2013).

Terminally differentiated, enlarged bacteroids with different morphotypes are a typical feature of the IRLC legumes. The major determining factor behind these irreversibly differentiated bacteroids is nodule-specific cysteine-rich (NCR) peptides (Montiel et al. 2016, 2017). The molecular mechanism of NCR peptide-regulated endoreduplication of the symbiont genome has been worked out in model legume *Medicago*. *Medicago* genome encodes more than 700 NCR genes (Mergaert et al. 2003; Young et al. 2011). At least 138 NCR peptides get processed in the endoplasmic reticulum (ER) and targeted toward symbiosomes (Durgo et al. 2015; Wang et al. 2010). NCR

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peptides force endoreduplication of the symbiont genome. As a result, the bacteria (now bacteroids) lose their ability to divide and regrow on culture media, but their size increases up to approximately 10 fold (Mergaert et al. 2006; Sinharoy et al. 2013; Young et al. 2011). The *NCR* gene family is evolved in *Medicago* spp. by a recent local gene duplication (Alunni et al. 2007). *NCR* genes have been identified in several IRLC legumes, including chickpea (Kant et al. 2016; Montiel et al. 2016, 2017). The number of *NCR* genes among different IRLC legumes varies greatly. This results in different morphotypes of bacteroids in different legumes, such as swollen/spherical, elongated, and elongated-branched (Montiel et al. 2017). *Mesorhizobium* TAL620-induced nodules in chickpea express only 63 *NCR* genes, while its bacteroid endoreduplicated up to four fold (Kant et al. 2016; Montiel et al. 2017). Chickpea and *Medicago* NCRs share less than 80% identity. Chickpea NCR peptides have more identity with *Glycyrrhiza uralensis*, *Onobrychis vicifolia*, and *Astragalus canadensis*, while phylogenetically chickpea is closer to *Medicago* (Montiel et al. 2016, 2017). Chickpea swollen or spherical bacteroids are basal to the evolution of *NCR*-guided morphogenesis (Montiel et al. 2017). In contrast to chickpea, *Medicago* has more than 700 *NCR* genes (Young et al. 2011). Bacteroids in *Medicago* nodules are elongated-branched, which depict an advance stage of this trait. Interestingly, this morphogenesis of bacteroids and the genome endoreduplication is thought to determine the efficiency of nitrogen fixation in respective legumes (Oono and Denison 2010). Thus, comparative investigation of nodule development in chickpea and *Medicago* will enhance our knowledge on the evolutionary link between variable nitrogen-fixation efficiencies among different legumes.

An efficient hairy-root transformation protocol is an indispensable tool to understand nodule biology, enabling us to study the localization of any protein (fluorescent tag), activities of promoters (β -glucuronidase [*GUS*] fusion), and the effects of overexpression or knockdown of certain genes. We have undertaken an effort to establish hairy-root transformation in chickpea and study nodule development. Unlike *Agrobacterium tumefaciens*, *A. rhizogenes* generates transformed roots from the site of infection. *A. rhizogenes* contains *root locus* (*rol*) genes in the Ri plasmid, which promotes the formation of genetically transformed adventitious hairy roots. *A. rhizogenes* carrying a recombinant Ri plasmid can generate composite plants. These plants contain untransformed shoot and transformed root (Stougaard et al. 1987). When *A. rhizogenes* strain additionally carrying a gene of interest in a binary vector is used for hairy-root transformation, a certain percentage of roots also obtained the binary vector and those roots are called cotransformed roots. Both overexpression and downregulation of a specific gene can be achieved by these cotransformed roots (Limpens et al. 2004; Sinharoy and DasGupta 2009; Sinharoy et al. 2015). Recently, it has been reported that CRISPR/Cas9-mediated gene knockout is also possible in hairy roots (Cai et al. 2015; Ron et al. 2014; Wang et al. 2016). To date, all the successful protocols describing hairy-root transformation in legumes have shown the transformed roots to be biologically similar to untransformed roots, with no difference in nodule development (Boisson-Dernier et al. 2001; Bonaldi et al. 2010; Brijwal and Tamta 2015; Estrada-Navarrete et al. 2006; Habibi et al. 2016; Imanishi et al. 2011; Kereszt et al. 2007; Limpens et al. 2004; Quandt et al. 1993; Sinharoy et al. 2009; Stiller et al. 1997; Stougaard et al. 1987; Thilip et al. 2015; Thwe et al. 2016; Van-de-Velde et al. 2003). *A. rhizogenes*-mediated hairy-root transformation is more efficient and less time-consuming than stable transformation. Thus, it serves as an alternative method for functional validation of genes. Here, we are reporting an efficient protocol for hairy-root transformation in chickpea. We have

developed both in-vitro and ex-vitro protocols. We have used four different *Mesorhizobium* strains to follow nodule initiation and developmental programs and evaluated the nitrogen-fixation efficiency. We have also checked the localization of several sub-cellular markers in transformed chickpea roots and nodules. Additionally, we have shown that different strains of *Mesorhizobium* give rise to mature bacteroids with different shapes. The efficiency of nitrogen fixation is lower when the shape is elliptical instead of spherical.

RESULTS

Hairy-root transformation of chickpea.

We used *Agrobacterium rhizogenes* strains R1000 (Quandt et al. 1993), ARqua1 (Quandt et al. 1993), and MSU440 (Sonti et al. 1995) for hairy-root transformation. All three strains were successful to generate hairy roots (Supplementary Table S1). For detailed characterization, we used a low-virulence strain, *A. rhizogenes* ARqua1 (Quandt et al. 1993). Dicotyledonous plants can be transformed using both in-vitro (tissue culture-based) and ex-vitro (without tissue culture) protocols (Collier et al. 2005; Sinharoy et al. 2015). We attempted both methods. For in-vitro transformation, chickpea seedlings were infected either by cutting 1 to 1.5 cm of the radicle and scraping it in an *A. rhizogenes* lawn culture or using a needle containing *A. rhizogenes* to make a small wound at the hypocotyl region. An overview of the methods is given in Figure 1. In both cases, transformed roots began to emerge from the infected region 14 to 15 days after infection (dai) (Fig. 1, step-Ia). In the case of needle-mediated *A. rhizogenes* infection, the original non-transformed roots were removed from the plant when transformed roots started emerging. These plants were transferred to kanamycin-containing selection medium to increase the efficiency of the transformation. We tried two different concentration of kanamycin (50 and 25 μ g/ml). The plants were maintained in the antibiotic-containing medium for 14 to 15 days for antibiotic selection. All the roots became black and plants died within two weeks when 50 μ g of kanamycin per milliliter was used (20 of 20 plants died). In contrast, the transgenic roots grew normally in the presence of 25 μ g of kanamycin per milliliter. The shoots of composite plants had no difference in morphology with their nontransgenic counterparts (Fig. 1, step IIa). The transformed roots exhibited characteristics of plagiotropic growth. The transformation frequency was determined using either the *Cauliflower mosaic virus 35S* promoter or *Arabidopsis ubiquitin 10* (*AtUBQ10*) promoter-driven expression of the DsRed fluorescent protein (Fig. 1, step IIIa). pCambia2301 vector was used for the determination of *p35S*-driven expression of *gusA* gene. In pCambia2301, the *gusA* gene contains an intron. The glucuronidase activity by the chickpea roots confirmed proper integration of the transformed gene. The *pKGW-Red-Root* vector was used for the determination of *AtUBQ10* driven *DsRed* expression (Table 1). Both in vitro transformation methods either by cutting or injecting showed a similar frequency of cotransformed roots (Table 1). Usually, we obtained a slightly greater frequency of transformed roots after antibiotic selection (Table 1. 53 ± 23 with antibiotic over 49 ± 20 without antibiotic) using the same vector in same chickpea genotype (*BDG256*). Plants generated by in vitro methods were transferred to the substrate (a mixer of 3:1 vermiculite and fireclay balls, leca) 14 to 15 dai (in case of without antibiotic treatment). Nonfluorescent roots were removed from the plants at this point (Materials and Methods section). Nodules were observed in these transformed roots within 10 days postinoculation (dpi) with *Mesorhizobium* (Fig. 1 Step IVa). The cotransformed roots develop healthy and active nodules which can fix nitrogen efficiently (7.6 ± 3 nmole ethylene plant⁻¹ hour⁻¹).

Ex-vitro composite plant generation is a rapid, efficient, simple, and low-cost method for producing composite plants without tissue culture (Collier et al. 2005; Sinharoy et al. 2015). The steps for ex vitro transformation are shown on the right-hand side of Figure 1. For ex vitro transformation, 25-30 days old apical stem tissue of young branches were cut and dipped in the *A. rhizogenes* culture and transferred to the substrate (Fig. 1 Step Ib). Further, the plants were maintained in a humid chamber for 30 days to promote root production in presence of full nitrogen fertilization regime. Both transformed and adventitious root production were observed by 20 dai. The transformed roots were screened based on dsRed fluorescence (Fig. 1 step IIb). At this point, the substrate containing plants were washed thoroughly with distilled water, and transferred to a new pot and maintained under nitrogen free condition. After 2-3 days the plants were inoculated with *Mesorhizobium* culture for root nodule development, and nodules were noticed within 10 dpi (Fig. 1 step IIIb). Ex vitro transformation

protocol showed slightly lower transformation efficiency compared with any of the in vitro transformation protocols (Table 1).

We have tested this protocol in different chickpea ecotypes. A list of different cultivars of chickpea from different origin, for which we successfully tested our transformation protocol, has been given in Table 1. Both in vitro and ex vitro methods had comparable transformation efficiency. While we obtained greater transformation efficiency in *BDG256*, and 'local' variety (above 50%), *ICC4958* and *ICC8261* had a lower efficiency. In summary, using our protocols hairy roots can be generated in several different ecotypes of chickpea.

Expression of different subcellular markers in transformed hairy roots and nodules of chickpea.

To test the activity of transgenes transformed following our protocol we employed already known markers for their

Fig. 1. *Agrobacterium rhizogenes* induced hairy roots in chickpea. For in-vitro transformation, germinated seedlings were infected with *Agrobacterium rhizogenes* and were plated on Fahraeus medium. At 14 to 15 days after infection, hairy roots were seen and the composite plants were transferred in selective antibiotics (step Ia). After 14 to 15 days, the plants were transferred to growing substrate (step IIa). Alternatively, after 14 to 15 dai, plants were directly transferred to growing substrate. Transformed roots were screened under the red channel of the stereomicroscope (step IIIa). Composite plants were inoculated with *Mesorhizobium ciceri* CC1192 after 22 to 23 days (step IVa). For ex vitro transformation, the wounded portion was inoculated with *Agrobacterium rhizogenes*. At 30 days after infection, hairy roots were seen (step Ib) and transformed roots were screened under the red channel of the stereomicroscope (step IIb). The composite plants were inoculated with *M. ciceri* CC1192 for nodulation (step IIIb). The transformed root pictures were taken under the stereomicroscope either under bright field or under the red channel.

subcellular localization in chickpea root and nodules. These markers were already published for either *Arabidopsis thaliana* (*At*) (model plant), or *Medicago truncatula* (*Mt*) (model legume) (Nelson et al. 2007; Ivanov and Harrison 2014). We expressed some of these markers in hairy roots and nodules of chickpea (Supplementary Fig. S1). A construct created by fusing the signal peptide of *AtWAK2* (*Arabidopsis thaliana* wall-associated kinase 2) at the N-terminus of a *mCherry* sequence which also contained endoplasmic reticulum (ER) retention signal HDEL at the C-terminus, was used as ER marker. This *mCherry* was under *AtUBQ10* promoter (He et al. 1999). An extensive characteristic tubular and sheet-like ER network was seen throughout the cytoplasm of chickpea root epidermal cells. As expected, ER network around the nucleus was more uniform. The mitochondrial marker was created by adding the first 29 amino acids of *Saccharomyces cerevisiae* cytochrome c oxidase IV with a *mCherry* sequence under *pAtUBQ10* (Kohler et al. 1997). Typical small and round mitochondria were present in high copy numbers in every cell in chickpea hairy root epidermis. The mitochondria were generally clustered in the periphery of the cells, and around the nucleus. A similar result was obtained earlier in *Medicago* (Ivanov and Harrison 2014). We have used *pAtUBQ10* driven *mCherry* fused with Lifeact, a 17 amino acid peptide which binds filamentous actin as the actin filament marker (Riedl et al. 2008). In root epidermal cells, we noticed extensive filamentous actin networks. The marker for peroxisome was created by fusing SKL signal peptide of *AtPTS1* to the C-terminal of *mCherry* (Reumann 2004). Innumerable peroxisomes were seen in the cells of chickpea root epidermis. Unlike mitochondria, peroxisomes were scattered all around the cells. To monitor the localization of apoplasts, we used the *pAtUBQ10*-driven expression of *Medicago* blue copper protein's (*MtBCP1*) 23 amino acid signal peptide (*BCPsp*) fused to *mCherry* (Pumplin and Harrison 2009). *MtBCP1* signal peptide drives the routing of *mCherry* to apoplasts, as was also seen in *Medicago* before (Ivanov and Harrison 2014). From the *MtBCP1sp-mCherry* localization, it is clear that the epidermal cells are connected through apoplastic connection. Additionally, we used the *p35S* promoter-driven expression of a plasma membrane aquaporin (*PIP2a*) fused to cyan fluorescent protein (CFP) (Cutler et al. 2000). We noticed the uniform labeling of CFP along the surface of the cell. A marker created by fusing plasmodesmata-located protein 1 gene (*PDLPI*) with *mCherry* at the N terminus under *pAtUBQ10* was used to visualize the cytoplasmic connections

between the cells (Thompson and Wolniak 2008). A cross-section of chickpea root showed the symplastic connections between the cortical cells. The Golgi body marker was created by fusing the N-terminal 49 amino acids of *GmMAN1* to the N terminus of *mCherry* (Saint-Jore-Dupas et al. 2006). The Golgi apparatus were scattered throughout the cytoplasm in the chickpea root epidermal cell. To check the hormone responsiveness in these transformed roots, we used a construct created by fusing a nuclear localization signal (*NLS*) at the C-terminus of GFP under auxin-responsive synthetic *DR5* promoter (*DR5-GFP-NLS*) (Suzaki et al. 2013). We noticed high GFP expression around the proliferating lateral root primordial cells, but GFP fluorescence was not observed in the mature root cortex. In summary, by using eight different subcellular markers under both *35S* and the *AtUBQ10* promoter and the *DR5-GFP* construct, we confirmed the normal physiological functioning of these transformed roots.

The transgenic hairy roots also showed efficient nodule development (Fig. 1). After the bacterial endocytosis in the postmitotic cells, bacteroids multiply and rapidly fill up the entire cell. The peribacteroid membrane is a plant-derived membrane. Hence, the division of bacteroids accompanies a huge expansion of the elements of the host cell membrane. Several parallel evidences suggest that membrane trafficking toward symbiosomes increases significantly during this phase (Gavrin et al. 2017). Symbiosomes in *Medicago* spp. have a unique mosaic identity with plasma membrane syntaxin SYP132 and endomembrane marker (regulatory small GTPases of the Rab family) Rab7 (Limpens et al. 2009). This suggests that, under certain circumstances, established markers may behave differently inside the infected nodule cells. To demonstrate the utility of this protocol, we used three subcellular markers (peroxisome, ER, and Golgi) to check their localization inside nodule cells. Peroxisomes are organelles that play key roles in plant cell metabolism (Hu et al. 2012). The central infected nodule tissue is surrounded by three layers of uninfected peripheral tissues, namely, from inside to outside, the nodule parenchyma, endodermis, and cortex (Xiao et al. 2014). We noticed the presence of a higher number of peroxisomes in the nodule cortex around the vascular tissue (Fig. 2A). The number of peroxisomes was low in nodule parenchyma and endodermis (Fig. 2A), but the density of peroxisomes was more or less similar in all infected zones (Fig. 2B and C). The Golgi apparatus (*cis*-Golgi) were noticed everywhere inside the infected cells surrounding the symbiosomes (Fig. 2D). We failed

Table 1. *Agrobacterium rhizogenes* ARqual-mediated transformation of different chickpea ecotypes with binary vector *pKGW-Red-Root* and *pCAMBIA2301*

Genotype	Region	Method of transformation (fraction of transformed plants) ^a			DsRed- or GUS-positive hairy roots per cotransformed plant ^b (%)		
		In vitro			In vitro		
		Cut	Inject	Ex vitro	Cut	Inject	Ex vitro
ICC4958 ^{1c}	India	16/20	ND	ND	26 ± 9	ND	ND
ICC17258 ^{1c}	India	20/20	ND	ND	40 ± 17	ND	ND
ICC1885 ^{1c}	India	18/20	ND	ND	37 ± 16	ND	ND
ICC8261 ^{2c}	Turkey	9/20	ND	ND	27 ± 7	ND	ND
Local Variety ^{1c}	India	35/35	31/35	34/35	50 ± 21*	51 ± 21*	40 ± 17
BDG265 ^{1c}	India	19/20	17/20	14/20	49 ± 20	40 ± 16	38 ± 16
BDG256 ^{1c}	India	21/25	ND	ND	53 ± 23*	ND	ND
BDG256 ^{1d}	India	10/10	ND	ND	40 ± 17	ND	ND

^a Fraction of cotransformed composite plants were determined based on either DsRed fluorescence or β -glucuronidase positive hairy roots. ND = not determined.

^b Four weeks after infection, the fraction of cotransformed roots were determined. Asterisks (*) indicate values that show the percentage after antibiotic selection.

^c Numbers of cotransformed hairy roots were counted based on DsRed fluorescence. A superscript 1 following the genotype name indicates Desi and a superscript 2 indicates Kabuli variety.

^d Numbers of cotransformed hairy roots were counted based on β -glucuronidase (GUS) positive hairy roots per plant.

to detect any Golgi apparatus surrounding the infection thread (Fig. 2D). ER also shows a similar localization pattern to the Golgi, close to the symbiosomes (Fig. 2E). Additionally, we were able to detect ER around the infection thread (Fig. 2F).

Evaluating the symbiotic potential of different *Mesorhizobium* strains.

An efficient laboratory strain is a prerequisite to study nodule development. We examined several different *M. ciceri* strains, originating from different sources, to evaluate their potential for healthy nodule development and N₂ fixation efficiency. *M. ciceri* CC1192 is an Australian isolate used as a commercial inoculant, and, recently, the complete genome sequence has been published (Haskett et al. 2016). *Mesorhizobium ciceri* IC-59 was isolated by ICRISAT (International Crops Research Institute for the Semi-Arid Tropics, Hyderabad, India) (Esfahani et al. 2014; Rupela and Sudarshana 1990) and is being used for different field trials (Esfahani et al. 2014). *M. ciceri* TAL620 was used for studies in the past (Chandra and Pareek 1987) and recently has been used for the transcriptome sequencing of chickpea root nodules (Kant et al. 2016). Our own isolation effort from the experimental garden of the National Institute of Plant Genome Research (NIPGR), New Delhi, India, has resulted in the culturing of NIPGR isolate 7 or NI-7. Chickpea plants with healthy pink nodules were collected from the experimental garden. The pink nodules were surface-sterilized, and rhizobia were isolated from the nodules (details below). The 16S rDNA sequence of the NI-7 strain has been submitted to the National Center for Biotechnology Information (NCBI) database (MH517450). Interestingly, NI-7

shows the highest similarity with *Mesorhizobium tianshanense*. *M. tianshanense* nodulating *Cicer arietinum* and *Cicer canariense* have already been reported (Armas-Capote et al. 2014; Rivas et al. 2007). We generated nodules by inoculation of all four above-mentioned *Mesorhizobium* strains (Supplementary Fig. S2). All four strains were able to initiate nodules by 6 dpi (bump formation). Numerous small white round-shaped nodules were observed by 10 dpi. At 15 dpi, nodules were pink (due to the expression of leghemoglobin) in CC1192, IC59, and NI-7 but remained white in TAL620. When followed further, all four nodules become pink by 21 dpi. By 28 and 35 dpi, CC1192, IC59, and NI-7 nodules showed a similar developmental fate by becoming elongated and cylindrical, while TAL620 nodules were comparably smaller and pale pink. Senescence zones, a typical feature of indeterminate nodules, started appearing by 35 dpi in most of the nodules. In TAL620 nodules, the senescence zone was visible even from 28 dpi. This suggests that TAL620 is a comparably inefficient strain for chickpea nodule development. We performed acetylene reduction assays (ARA) at 21 and 35 dpi, to determine the nitrogen-fixation efficiency of these strains. CC1192 showed the highest ARA, followed by NI-7, IC59, and TAL620 at both timepoints (Fig. 3A). Both NI-7 and IC59 show a significantly low ARA when compared with CC1192. But TAL620 showed a significantly low ARA compared with CC1192, NI-7, and IC59 at both timepoints. Further, we determined the biomass of the full plants at 35 dpi, to understand how nodule development by different *Mesorhizobium* strains affects overall plant growth. Based on biomass, TAL620-inoculated plants showed significant growth retardation and IC59-inoculated plants showed slight

Fig. 2. Localization of different subcellular protein markers in transgenic chickpea nodules. **A to F,** Transformed nodules were hand-sectioned and were stained with SYTO13 (DNA, green). **A to C,** mCherry-SKL showing localization in the peroxisome whole nodule (**A**), invasion zone (**B**), and nitrogen-fixation zone (**C**). **D,** MAN49-mCherry localization in Golgi bodies in the nitrogen-fixation zone. **E and F,** mCherry-HDEL localization in the endoplasmic reticulum in the nitrogen-fixation zone of transgenic chickpea nodules induced by *Mesorhizobium ciceri* CC1192. N = nucleus and IT = infection thread. Scale bars in **A** = 50 μm and **B to F** = 5 μm.

growth retardation compared with CC1192 inoculated plants (Supplementary Fig. S3). Taken together, our data show that CC1192 is the most efficient strain for chickpea, followed by NI-7. Further, we have used SYTO dyes to stain the nitrogen-fixation zone of these chickpea nodules at 21 dpi. Interestingly, we found small spherical symbiosomes inside CC1192-, IC59-, and NI-7-generated nodules (Fig. 3B to D), whereas in the case of TAL620 the symbiosomes were small and elliptical (Fig. 3E). Our result shows that elliptical symbiosomes are associated with low ARA. Acidification of symbiosome space is crucial for functional nitrogen fixation. LysoTracker DND189 has been successfully used in *Medicago* spp. for in-planta detection of peribacteroid space (PBS, also called symbiosome space) acidification (Pierre et al. 2013). We then asked whether there is any correlation between the PBS acidity and ARA by the above-mentioned strains. We used pH-sensitive dyes DND189 to stain the most efficient (CC1192) and the least efficient (TAL620) *Mesorhizobium*-induced nodules. DND189 is a weak base linked with a fluorophore. It gets protonated in acidic organelles and is retained in the membranes of those organelles. The biggest advantage of DND189 over most of the lysotrackers is that it is almost nonfluorescent except when inside acidic compartments (Lopez et al. 2005). DND189 starts fluorescing at a pH ≤ 5.2 and reaches an optimal emission between pH 4 and 5. We used DND189 in combination with SYTO82/PI (propidium iodide) to check the acidity

of chickpea symbiosome space. In CC1192, DND189 was accumulated in the PBS and we observed the clear spherical shape of the acidic zone (PBS) (Fig. 4A and B). The bacterial nucleic acid showed clear red staining when it was counter-stained with SYTO82 (Fig. 4B). There was some acidity in the PBS of the symbiosomes made by strain TAL620, but we rarely obtained a clear demarcation of PBS boundary when stained with DND189 (Fig. 4C). To test the viability of bacteria inside TAL620-induced nodules, we used PI staining (Haag et al. 2011). Live bacteria with intact cytoplasmic membranes would be stained by membrane-permeable SYTO dye, while dead bacteria with degraded cytoplasmic membranes would be stained by membrane-impermeable PI. When TAL620-induced nodules were stained with PI, the nucleic acid in most of the samples did not take up the stain. Interestingly, the few instances where sporadic symbiosomes took up the stain, they were spherical (Fig. 4D). The frequency of occurrence of such zones inside the nodules is scarce, whereas SYTO dye shows live bacteroids inside TAL620-induced nodules (Fig. 3D). Hence, from our observation, it appears that DND189 is accumulated less specifically in the PBS of TAL620-induced symbiosomes, indicative of less acidity.

DISCUSSION

Unlike model legumes, nodule development studies in chickpea are limited. Nodule development in chickpea and

Fig. 3. Characterization of chickpea nodules induced by different *Mesorhizobium* strains. **A**, Acetylene reduction activity with *M. ciceri* CC1192, *M. ciceri* IC59, *Mesorhizobium* sp. strain NI-7, and *M. ciceri* TAL620 induced nodules at 21 and 35 days postinoculation (dpi). Data presented are the means $\pm$ standard error of mean per plant from four independent experiments (with five plants from each experiment). Data with different letters reveal significant differences as measured by a *t* test ($P \leq 0.05$). **B** to **E**, Chickpea nodules (21 dpi) were hand-sliced and were stained with SYTO82. *M. ciceri* CC1192 (**B**), *M. ciceri* IC59 (**C**), *Mesorhizobium* sp. strain NI-7 (**D**), and *M. ciceri* TAL620 (**E**) induced nodules in *BDG256* chickpea ecotype. IT = infection thread. Scale bars in **A** to **D** = 5 μ m.

Medicago spp. follow a similar course. Hence, the knowledge on *Medicago* nodule development holds true for many aspects of nodule development in chickpea. Nonetheless, differentiation of bacteroids in both systems shows major dissimilarity. Bacteroid differentiation takes place in the invasion zone of both *Medicago* and chickpea nodules. Bacteroids inside *Medicago* nodules endoreduplicate up to 24-fold, whereas a maximum of fourfold endoreduplication is observed in chickpea (Montiel et al. 2017). Certain *NCR* genes (*dnf4-NCR211* and *dnf7-NCR169*) are indispensable for nitrogen fixation in *Medicago* spp. (Horvath et al. 2015; Kim et al. 2015). Recently, it has been shown that two *NCR*, nitrogen-fixation specificity genes (*NFS1* and 2) also function as negative regulators of symbiont persistence (Wang et al. 2017; Yang et al. 2017). Such a specific role of a few *NCR* peptides amid overall redundancy among the members of this family suggests complex regulation behind the evolution of terminal differentiation of bacteroids. Also, the presence of a much lower number of *NCR* genes and the absence of the symbiotically essential *NCRs* in the chickpea genome suggest major differences in the molecular regulation of bacteroid differentiation (Montiel et al. 2017).

The recalcitrance of chickpea toward transformation and its low regeneration efficiency is a bottleneck at this moment (Indurker et al. 2007; Varshney et al. 2010). For root biologists, hairy-root transformation is an easy alternative to stable lines. The cotransformed roots can be used for overexpression, knockdown, or CRISPR/Cas9-mediated deletion of a gene of interest (Limpens et al. 2009; Ron et al. 2014). To date, most of the literature on RNS in model legumes uses hairy-root transformation. Two preliminary reports, by Altinkut et al. (1997) and Khawar and Ozcan, (2004), have previously described hairy-root transformation using chickpea. Those reports are preliminary without cotransformation efficiency calculation and, also, nodule development by the cotransformed roots had not been evaluated. Here, we are reporting an efficient transformation protocol, showing 26 to 53% cotransformed root generation and the normal physiological functioning of the cotransformed roots, by using eight different subcellular markers and auxin-responsive *DR5-GFP* construct. Further, the nodule development and symbiotic nitrogen fixation by the cotransformed roots had been evaluated (Figs. 1 and 2). The cotransformation efficiency in the BDG256 ecotype is 49 ± 20 without antibiotic whereas, with antibiotic, it is 53 ± 23 (Table 1). We only observed a mild increase in the transformation frequency when antibiotic selection is used. Hence, the antibiotic selection step can be omitted during composite plant generation in chickpea. The transformation efficiency of the model legume ranges around 60 to 70%. On the other hand, the transformation efficiency of the large-seeded grain legumes soybean, *Vigna* spp., and pea varies greatly between 20 and 80% (Sinha et al. 2015). According to our protocol, the transformation efficiency of chickpea varies greatly between ecotypes, ranging between 26 and 53% (Table 1). This is comparable to other large-seeded grain legumes. These transformed roots develop healthy and active nodules (Fig. 1) that can fix nitrogen efficiently. We have tested our protocol using nine different constructs carrying different transgenes, and all of them showing an expected pattern and subcellular localization (Fig. 2).

Our data show that peroxisomes are enriched around the vascular bundle in the nodule cortex (Fig. 2). Peroxisomes play a major role in ureide transport inside the determinant nodules of soybean (Hanks et al. 1981). The role of peroxisomes in amide transporting nodules of temperate legumes is not clear. Scavenging of toxic H_2O_2 around the nodule cortex could be one possibility. Presence of active peroxisomes has been reported also in the meristem and the invasion zones of pea

nodules (Borucki 2007). On the contrary, we have noticed a higher number of peroxisomes in the nodule cortex (Fig. 2A). Discrimination between active and inactive peroxisomes was beyond the limits of the present study. Our observation demands further studies on the significance of peroxisome accumulation around the nodule cortex of temperate legumes like chickpea. In *Medicago* spp., the ER apparatus is assembled within the epidermal cells around an area where the future infection thread would penetrate, even before the actual penetration (Genre et al. 2005). The localization of ER around the infection threads in chickpea nodules indicates similar organization (Fig. 2F). ER-mediated trafficking is important during endocytosis of bacteroids. Normally, proteins of the exocytosis pathway go from the ER to the membrane through the Golgi bodies (Gavrin et al. 2017; Ivanov et al. 2012). Surprisingly, we did not find Golgi bodies around the infection threads. The significance of selective concentration of ERs in combination with the absence of Golgi bodies around the infection threads is not clear.

The efficiency of nitrogen fixation differs significantly, depending on bacterial strains. A systematic effort to evaluate the behavior of different *Mesorhizobium* strains during chickpea nodule development has not been given to date. Our study highlights a striking difference of symbiotic performances between different *Mesorhizobium* strains (Fig. 3). Early induction of senescence in TAL620-induced chickpea nodules (Fig. 4D) raises the question whether there are certain *NCR* genes in the chickpea genome that cause a toxic effect in the TAL620-induced nodule. It is worth mentioning that a toxic effect of *NCRs* has already been reported in *Medicago* spp. (Wang et al. 2017; Yang et al. 2017). We detected sporadic spherical-shaped

Fig. 4. Characterization of the acidity of the peribacteroid space of chickpea nodules. At 21 days postinoculation, chickpea nodules were hand-sliced and stained with DND189. **A**, Nodules induced by *Mesorhizobium ciceri* CC1192 and **B**, counterstained with SYTO82 (DNA, a live cell). **C**, Nodules induced by *Mesorhizobium ciceri* TAL620 and **D**, counterstained with propidium iodide (DNA of a dead cell). Scale bars in **A** to **C** = 5 μ m and in **D** = 10 μ m.

dead bacteroids inside TAL620-induced nodules (Fig. 4D). This, together with the late onset of pink coloration and early senescence of the above-mentioned nodules indicate a delay in bacteroid differentiation and reduced survival time. A total of 63 NCRs express in chickpea BDG256 nodules induced by TAL620, as shown by transcriptomic experiments (Kant et al. 2016; Montiel et al. 2017). This suggests that combinations of at least 63 NCRs are required for the development of this uniquely-shaped symbiosome. Our study highlights the importance of further transcriptomic study on chickpea nodules containing normal spherical-shaped symbiosomes. A comparative transcriptomic study between nodules containing spherical and elliptical symbiosomes will highlight the NCRs behind the transition between these two unique shapes. It is also very important to screen diverse chickpea ecotypes inoculated by both TAL620 and CC1192/NI-7 to see whether different hosts can generate different morphotypes of bacteroids.

Overall, our studies with different bacterial strains along with the efficient hairy-root transformation protocol will render the community of nodule biologists a new platform on which genetic experiments of chickpea could easily be performed. Our results presented both in-vitro and ex-vitro methods of chickpea hairy-root transformation, which is more comprehensive than previous publications (Aggarwal et al. 2018; Altinkut et al. 1997; Khawar and Ozcan 2004). Moreover, we have presented enough intriguing features of nodule development in chickpea, which merits further attention in the future.

MATERIALS AND METHODS

Plant material and growth condition.

Seeds of chickpea (*Cicer arietinum*) varieties ICC4958 (genome sequenced [Jain et al. 2013] and drought-tolerant genotype), BDG256 (used for nodule transcriptomics by Kant et al. [2016], also known as BG 256), ICC17258 (germplasm accession number ICCV96029, early maturing cultivar), ICC1885 (close relative of ICC1882, used by Varshney et al. [2013] for single nucleotide polymorphism determination), ICC8261 (drought-tolerant genotype used by Varshney et al. [2013]), and local varieties (Desi variety, bought from local market) were surface-sterilized by 30% commercial bleach solution, containing active sodium hypochlorite 1.2%, for 3 min. The seeds were thoroughly washed with sterile water. They were placed on plates containing sterile wet filter paper for germination. The plates were kept in 20 to 22°C temperatures in the dark. After 2 to 3 days, the germinated seedlings were placed in pots with growing substrate (a mixture of 3:1 vermiculite and fire clay balls, leca). The pots were placed in a growth chamber under 200 $\mu\text{E m}^{-2} \text{s}^{-1}$ with a 16-h light and 8-h dark cycle, at 22°C and 40 to 60% relative humidity. The Desi chickpea variety is characterized by small, brown seeds and is widely cultivated in India (ICC4958, BDG256, ICC17258, and ICC1885) and Kabuli is characterized by large, beige seeds consumed as an international commodity (ICC8261).

Bacterial strains and growth condition.

The *Agrobacterium rhizogenes* R1000, ARqua1, and MSU440 strains were grown at 28°C in solidified Luria Bertani (LB) medium with appropriate antibiotics. The *Agrobacterium* strains were transformed with the vectors (pCMU-ERr, pCMU-APr, pCMU-ACTLr, pCMU-MITr, pCMU-PERr, pCMU-GAr, pCMU-PDESr, pm-ck-CFP-CD3-1001, pDR5-GFP-NLS, pCAMBIA2301 and pKGW Red root) (Ivanov and Harrison 2014; Karimi et al. 2002; Nelson et al. 2007; Suzaki et al. 2013) by the freeze-thaw method (Hofgen and Willmitzer 1988). For hairy-root transformation, *Agrobacterium* strains were grown for 48 h at 30°C on an LB agar plate with suitable antibiotics to

produce a lawn in the plate. *M. ciceri* IC59, *M. ciceri* TAL620, *M. ciceri* CC1192, and *Mesorhizobium* sp. strain NI-7 were cultivated at 28°C in yeast mannitol broth.

Hairy-root transformation.

Preliminary reports on hairy-root transformation in chickpea (Altinkut et al. 1997; Khawar and Ozcan 2004) using cut radicles encouraged us to initiate our attempt using the same explant. For the in-vitro method of transformation, seeds were surface-sterilized and germinated. For cut-mediated transformation, when seedlings had a radicle length of approximately 40 mm, the radicles were sectioned 10 to 15 mm from the root tip with a sterile scalpel. The freshly cut root surface of the radicles was coated with *A. rhizogenes* culture by rubbing it on a bacterium lawn containing appropriate vectors. For inject mode of transformation, the germinated seedlings were pierced by a needle (radius 0.45 mm) containing *A. rhizogenes* culture in the tip of the needle. The piercing was done in epicotyl region. After infection by either method, infected seedlings were transferred to a plate containing solidified Fahraeus medium (Boisson-Dernier et al. 2001) without antibiotics. The lower parts of the plates were covered with aluminum foil. The plates were placed longitudinally, allowing the root to grow gravitropically, at 22°C under a 16-h light and 8-h dark cycle. After 7 days, hairy root production was noticed. When the transformed roots started emerging from the injection site, nontransformed main roots were removed by cutting with a sterile scalpel, after 14 to 15 dai. Transformed roots were screened under either the Leica stereo fluorescence microscope M205FA equipped with a Leica DFC310FX digital camera (Leica Microsystems) or stereo fluorescence microscope Nikon AZ100 equipped with Nikon digital camera (Nikon digital sight DS-Ri1) (Nikon). For antibiotic selection, after 14 to 15 days, the composite plants were transferred to a solidified Fahraeus medium with suitable antibiotics in culture tubes (25 × 150 mm) and were maintained for 2 weeks. Further, these antibiotic-selected composite plants were transferred to the growing substrate. Alternatively, the composite plants with transformed roots were directly transferred to growing substrate without antibiotic selection after 2 weeks of infection with *A. rhizogenes*. The growing substrate was pretreated with full-nitrogen B and D medium (Broughton and Dilworth 1971). The plants were bottom-watered four times per week with sterile water. After 10 days of transfer to substrate, the plants were treated with full-nitrogen B and D medium. After 20 days from the initial planting in the growing substrate, composite plants were watered (in the pot) with excess distilled water; further, the composite plants were transferred to a new pot and tray for removal of nitrogen. For 2 to 3 days, the plants were maintained in this nitrogen-free condition and were then inoculated with *Mesorhizobium* strains for nodulation.

For ex-vitro transformation, plants were grown in pots in 22°C in the growth room. A slanted cut (5 to 6 cm) was made in the apical stem sections of 25- to 30-day-old plants. The freshly cut stem region was rubbed on a bacterial culture plate (containing 10 mM morpholineethane sulfonic acid, pH 5.6, and 20 μM acetosyringone) of *Agrobacterium* strains with the appropriate vector. Infected stems were transferred into 4-cm long pots with growing substrate. The pots were placed on a tray containing sufficient full-nitrogen B and D medium to overcome water stress. A humid chamber was created around the tray with clear wrap and the tray was placed in a growth chamber under a 16-h light and 8-h dark cycle at 22°C. Plants were regularly checked for maintaining water and humidity. After 15 days, roots were seen, and, after 30 days, the growing substrate containing plants were washed thoroughly with distilled water and were transferred to a new pot to create

nitrogen-depleting condition. After maintaining this nitrogen-free condition for 2 to 3 days, the plants were inoculated with *Mesorhizobium* for nodulation.

GUS staining of chickpea root.

Histological assay for checking *GUS* gene expression by GUS activity was performed according to Jefferson et al. (1987). Briefly, hairy roots were vacuum-infiltrated with 0.1 M sodium phosphate buffer containing 0.5 M EDTA, 0.1% Triton X-100, 100 mM potassium ferricyanide (III), 1 mg of X-Gluc per milliliter and were then incubated for 10 h at 37°C. Expression of the *GUS* gene in hairy roots was visually assayed and the percentage of transformed roots was calculated.

Nodulation assay.

The growing substrate was vigorously washed for removal of nitrogen prior to nodulation and was tested for ammoniacal nitrogen and nitrate nitrogen with a soil testing kit (Himedia). The inoculum was prepared from different *Mesorhizobium* cultures of optical density at 600 nm (OD₆₀₀) of 0.7 to 0.9 in yeast mannitol medium. The bacterial culture was harvested and was diluted in nitrogen-free B and D media. Each pot was inoculated with 50 ml of diluted 0.03- to 0.05-OD₆₀₀ bacterial culture in B and D medium.

ARA.

ARA was performed as described previously (Oke and Long 1999). Briefly, chickpea BDG256 was grown on growing substrate and was inoculated separately with the four *Mesorhizobium* strains (*M. ciceri* IC59, *M. ciceri* TAL620, *M. ciceri* CC1192, and *Mesorhizobium* sp. NI-7). At 21 and 35 dpi, plants were harvested for ARA. This experiment was repeated four times. For each experiment, five plants were used for ARA for each timepoint. For each plant, the entire root system was placed in a test tube and sealed with suba-seal septa (Sigma-Aldrich) and 1 ml of pure acetylene gas was injected through the suba-seal septa. The tubes were kept in the dark at room temperature for 16 h. Ethylene production was measured by gas chromatography (Shimadzu GC-2010 equipped with HP-PLOT 'S' Al₂O₃ 50 m, 0.53 mm column [Agilent Technologies]). The mean and standard error of mean were calculated between twenty ARA measurement data (four biological replicates and five plants from each experiment). Plants from these experiments were also used for fresh weight determination. A total of 18 plants (three biological replicates and six plants from each experiment) were taken, after 35 dpi, for fresh weight. To determine the acetylene reduction activity of the transformed nodules, 10 pCambia2301 (*p35S*) vector-transformed plants were used and were inoculated with *M. ciceri* CC1192.

Confocal microscopy.

For confocal microscopy, the plants were harvested and the sample was hand-sectioned. The tissues were stained with a 5-μM solution of SYTO13 (Invitrogen) and SYTO82 (Invitrogen) and were kept for 15 min in dark. For staining with DND189 (Invitrogen), the tissue was incubated with 100 μM DND189 for 15 min. For determination of the status of the bacteroids, the DND189-stained tissue was incubated with a 1 mg/ml solution of PI for 10 min. Then the samples were washed and mounted with water. The confocal microscopy was performed either on an Olympus Model IX81 or a Leica TCS SP5 confocal microscope, using an excitation wavelength and emission bandpass of 488 nm and 500 to 530 nm for SYTO13, 541 nm and 550 to 580 nm for SYTO82, 448 and 500 to 530 nm for DND189, 587 nm 600 to 630 nm for mCherry, 535 nm and 610 to 640 nm for PI.

Isolation of *Mesorhizobium* strains from chickpea nodules.

The plants were collected from the NIPGR experimental field. Healthy-looking pink nodule-containing plants were selected. Roots were washed thoroughly to remove soil. About 10 pink nodules were collected by cutting the nodules from the root about 0.5 cm on each side. The nodules were placed in sterile water with 0.12 g of glass beads and were vortexed for 30 to 60 s in repetitive cycles to remove adhering soil particles. It was treated with 30% commercial bleach solution (Hindustan Unilever Limited) containing 1.2% active sodium hypochlorite for 3 min and was washed with sterile water five or six times. After surface sterilization, the nodules were rolled over solidified yeast mannitol medium and were incubated for 2 to 3 days at 28°C (control). Immediately, the same nodules were squashed in sterile water. It was serially diluted with sterile water and was plated in solidified yeast mannitol medium. If the control plate did not show growth, then only the corresponding experimental plates were processed for further characterization. The experimental plates were placed in 28°C for 5 to 6 days. The growth was checked regularly. The bacterial colony in the experimental plates were subcultured to purify the bacterial culture and were further used for the nodule developmental experiment.

Cloning of 16S rDNA.

NI-7 16S rDNA was amplified by forward 5'-TAACACAT GCAAGTCGAACG-3' and reverse 5'-ACGGGCGGTGTGTAC-3' primers, using PrimeSTAR Max DNA polymerase (Takara, Clontech). It was then cloned in a pJET 1.2/blunt vector (CloneJET PCR cloning kit; Thermo Fisher). Sanger sequencing was done with sequencing primers provided by the CloneJET PCR cloning kit (forward 5'-CGACTCACTATAGGAGAGCGGC-3' and reverse 5'-AAGAACATCGATTTTCCATGGCAG-3'). The sequence was assembled and processed with Geneious v9.1.8. The 16S rDNA sequence of the NI-7 strain has been submitted to NCBI (MH517450).

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