Tissue specific response of *Agrobacterium tumefaciens* attachment to *Sorghum bicolor* (L) Moench.

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ABSTRACT

Agrobacterium mediated genetic transformation of plants have advantages over other methods, especially for making single copy transgenic plants with reduced chances of gene silencing and instability. However, monocotyledonous plant species could not utilize the full potential of this system because of possible limitations in *Agrobacterium* interaction with monocot plant cells. *Agrobacterium* attachment as a factor in genetic transformation was studied in the leaf, shoot apex, and leaf derived callus of sorghum (*Sorghum bicolor* (L) Moench). Pre-induction of *Agrobacterium* with acetosyringone was found necessary for *Agrobacterium* attachment to sorghum tissues. All the explants responded positively, with preferential *Agrobacterium* attachment and colonization around the tissues having actively dividing cells. Callus proved to be the best explant for *Agrobacterium* attachment as observed in scanning electron microscopy and transient *GUS* expression. Loss of *Agrobacterium* attachment was observed with an increase in the degree of tissue differentiation. [Physiol. Mol. Biol. Plants 2008; 14(4) : 307-313] E-mail : polumetla@hotmail.com

Key words : Genetic transformation, Acetosyringone, Scanning electron microscopy, Transient gene expression, *GUS* assays, qRT-PCR

Abbreviations : IAA - Indole-3-acetic acid, IBA - Indole-3-butyric acid, BAP - Benzyl amino purine, ADS - Adenine sulphate

INTRODUCTION

Agrobacterium tumefaciens-mediated genetic transformation is the most common approach followed to transfer foreign genes to plant cells. However, many economically important monocotyledon plants including cereals remained inaccessible for genetic manipulation technology for a long time. For these plant species, alternative direct genetic transformation methods such as protoplast uptake of DNA, electroporation and biolistics have been developed (Shillito *et al.*, 1985; Lorz *et al.*, 1985; Fromm *et al.*, 1986; Sanford, 1988; Potrykus, 1991). However, *Agrobacterium*-mediated transformation has remarkable advantages for reducing transgene copy number and lowering the instances of

transgene co-suppression (silencing) and instability of gene expression in the transgenic plants (Matzke *et al.*, 2002). In addition, single-cell transformation by *Agrobacterium* precludes the obtainment of mosaic plants, which are more frequent when direct transformation methods are used.

Earlier the monocots were considered out of the *Agrobacterium* host range. Lippincott and Lippincott (1978) proposed the absence of *Agrobacterium* adherence sites in monocot cell walls. However, recent studies have shown that besides infecting its natural host *Agrobacterium* also transfers DNA to monocotyledons plant species including important cereals like maize (Ishida *et al.*, 1996; Negrotto *et al.*, 2000; Frame *et al.*, 2002), rice (Aldemita and Hodges, 1996; Mohanty *et al.*, 1999; Arockiasamy and Ignacimuthu, 2007) sugarcane (Arencibia *et al.*, 1998; Enriquez-obregon *et al.*, 1998) and sorghum (Zhao *et*

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al., 2000). *Agrobacterium* has also been successfully used for genetic transformation of organisms such as yeast (Piers *et al.*, 1996), fungi (Lee, 2006; Lima *et al.*, 2006; Stenlid, 2006) and human cells (Kunik *et al.*, 2001). Although the transformation efficiency in these species is comparatively less when compared to dicotyledonous plant species. It does suggest that, plant species considered out of *Agrobacterium* host range can be transformed by this bacterium.

As in any other technology for plant transformation, there are multiple factors involved in *Agrobacterium*-mediated transformation that influence the success or failure of the transfer of gene of interest into plants and their subsequent stable integration and expression. These include the wounding response of the plant, attachment and colonization of bacteria, physiological state of explant and DNA repair and replication activity of plant cells (Smith and Hood, 1995). So, most important aspects that require optimization for successful *Agrobacterium* mediated genetic transformation is interaction of the bacterial cells with plant cells that are in a competent physiological state.

In the present study we have investigated the interaction of two strains of *A. tumefaciens* (LBA4404 and EHA105) with three sorghum explants, leaf, split shoot apex and leaf derived callus, each representing cells in different physiological state and having high degree of regeneration potential. The present work was carried out with an aim to extend the methodology of *Agrobacterium* mediated transformation to elite sorghum genotypes.

MATERIALS AND METHODS

Plant material and *Agrobacterium* strains

Three different explants of *Sorghum bicolor* var. 296 B were used for the *Agrobacterium* interaction studies. These included (i) shoot apex, which was excised from 24–36 hrs old *in vitro* raised seedlings. The shoot apex was split longitudinally into two portions and separated so as to expose the meristematic corpus region, (ii) Leaf sections from seven days old seedling that have leaves at expansion stage and mature leaves (iii) Callus derived from leaf base at different stages of shoot meristem differentiation. To test the suitability of different strains of *Agrobacterium*, two strains namely LBA4404 and EHA105 harboring pCambia1304 vector were used for transformation.

Agrobacterium culture and explant infection

Freshly streaked *Agrobacterium* was grown for a period 24 to 28 hrs in YEM medium at 28°C. Acetosyringone was added to the *Agrobacterium* culture medium one hour prior to harvesting the cells. The bacteria were collected by centrifugation at 4000 rpm for 3 min in 1.5 ml Eppendorf tubes. The supernatants were carefully decanted and the pellets were suspended in 20 ml of without sucrose Murashige and Skoog (1962) medium. The final OD₆₀₀ of *Agrobacterium* culture was maintained at 0.6 and explants were infected for 15 min., blotted dry and cultured on co-cultivation medium (MS medium, 1.5% sucrose, 1.5% maltose, 2 mg/l 2,4-D, 100 µM acetosyringone) for 24hrs.

Preparation of explant for scanning electron microscopy

The explants incubated with *Agrobacterium* for 24 hrs were washed three times by vortexing in phosphate buffer saline (8 mM NaHPO₄, 2 mM NaH₂PO₄ and 150 mM NaCl, pH 7.4). Samples were fixed in 0.1 M phosphate buffer containing 3.5% glutaraldehyde overnight, followed by washing twice in 0.1 M sodium phosphate buffer pH (7.2) and dehydration through ascending series (5–70%) of cold acetone. Subsequently, the samples were subjected to critical point drying (CPD) and mounted, on the aluminum stubs, sputter coated with gold-palladium and viewed on Leo VP-45 Scanning Electron Microscope.

RT-PCR

Total RNA was isolated using RNeasy plant mini kit (Qiagen) and quantified using Nanodrop spectrophotometer. Semi quantitative analysis of *GUS* gene expression was carried by RT-PCR using gene specific primers: *GUS* forward: 5'-GGAAGTGATGGAGCATCAGGGCGGC-3', *GUS* reverse: 5'-CAGCCCGGCTAACGTATCCACGCCG-3', in 25 nanogram of RNA template. Each RT-PCR reaction was conducted in a final volume of 50 µL. Rice *β-tubulin* (forward primer 5'-GGAGTCACATGCTGCCTAAGGTT-3' and reverse primer 5'-ATCATCTGCTCATCAACCTCCT-3') was used as an internal control in qRT-PCR. The master mix (Onestep RT PCR kit, Qiagen) contained all components required for RT-PCR except for the template RNA, which was added separately. RT-PCR amplification was initiated by placing the PCR tubes in the thermal cycler when the temperature reached 50 °C. Following the reverse transcription at 50 °C for 30 min, PCR was initiated by heating at 95 °C for 15 min to activate the Hot-Start *Taq* Polymerase. The reaction mixture was

cycled 40 times at 94 °C for 30 s (denaturation), 55 °C for 30 sec (annealing), and 72 °C for 60 sec (extension), followed by final extension at 72 °C for 7 min.

GUS assay

Twenty five independent explants infected with *Agrobacterium* harboring pCAMBIA1304 vector were analyzed for *in situ* GUS expression according to Jefferson *et al.* (1987). Plant materials were stained with 2 mM X-Gluc, 100 mM Tris-HCl (pH 7.0), 50 mM NaCl, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide and 0.1% (v/v) Triton X-100 at 37 °C overnight. After staining, the tissues were incubated in 70% ethanol to clear chlorophyll and were subsequently fixed in 70% ethanol.

RESULTS

Agrobacterium attachment to plant cells can be affected by plant tissue age, cell type, cell cycle stage, and other physiological parameters (Graves *et al.*, 1988). In the present study three different explants *viz.* leaf, shoot apex and callus were used. The shoot apex obtained from young seedling was split longitudinally to expose the meristematic corpus region for bacterial attachment.

Effect of acetosyringone

The scanning electron microscopic (SEM) observation found negligible number of *Agrobacterium* cells attached to the sorghum tissues. However, with the

increase in the concentration of the acetosyringone in the *Agrobacterium* culture medium, there was an increase in the attachment frequency. Acetosyringone at a concentration of 100 µM gave maximum response in terms of number of attached *Agrobacterium*; increasing the concentration above it did not enhance the bacterium attachment to the sorghum leaf derived callus significantly (Fig. 1A-D).

To evaluate whether the SEM observations of positive interaction between *Agrobacterium* and sorghum tissues are productive for T-DNA delivery as well, transient *GUS* expression was studied. The *GUS* assay of *Agrobacterium* infected callus shows that with the increase in concentration of acetosyringone in *Agrobacterium* culture medium, there was an increase in size and intensity of patches having *GUS* expression (Fig. 1E). The effect of acetosyringone on successful *Agrobacterium*-sorghum tissue interaction was further quantified by qRT-PCR for *GUS* transcript and a direct relationship between the increases in *GUS* transcripts with the increasing concentrations of acetosyringone was observed (Fig. 2). These observations point out that induced *Agrobacterium* cells not only attach themselves to sorghum plant cells but also deliver the T-DNA to the host cell nucleus and this interaction requires pre-induction of *Agrobacterium* with acetosyringone. Since pre-induction of *Agrobacterium* with 100 µM acetosyringone gives maximum response further studies were carried out with pre-induced *Agrobacterium*.

Fig. 1. A-D. Scanning electron microscopy of undifferentiated leaf derived sorghum callus infected with *Agrobacterium*, induced with increasing concentrations of acetosyringone 0, 20, 50 and 100 µM. E. transient GUS assay of sorghum callus treated in same way as A-D.

Fig. 2. Quantitative RT PCR for *GUS* and β -tubulin (internal control) transcripts present in undifferentiated callus infected with *Agrobacterium* induced with different concentrations of acetosyringone 0, 20, 50, and 100 µM.

Effect of tissue age

Callus

The leaf-derived callus responded positively and formed large number of aggregates of pre-induced *Agrobacterium* over the surface of young callus, having actively dividing cells (Fig. 1D). However, with the increase in tissue differentiation and meristem formation over the callus surface, *Agrobacterium* attachment gradually becomes restricted to apical regions of meristems (Fig. 3A-C). These observations point out that sorghum cells at younger stage are more competent to *Agrobacterium* mediated transformation and this competence is lost with the increase in tissue differentiation with age. To re-evaluate these observations callus at different stage of differentiation was used for *Agrobacterium* mediated transformation, followed by transient GUS expression analysis. These results show that in young undifferentiated callus *Agrobacterium* attachment to host plant cell occurs at the whole surface (Fig. 1E), while the callus that has meristems differentiated over its surface shows GUS activity which gradually becomes limited only to apical regions of the newly formed meristems (Fig. 3D-F). It proves that with an increase in the degree of differentiation competence of sorghum cells for *Agrobacterium* mediated transformation is lost.

Leaf

Sorghum leaves at various stages of development interacted differently with *Agrobacterium*. Leaf surface at younger age showed massive *Agrobacterium*

Fig. 3. A-C. Scanning electron microscopy of callus at different stages of differentiation and infected with acetosyringone (100 μ M) induced *Agrobacterium*. D-E. Transient GUS assay of callus treated in same way as A-C.

Fig. 4. Scanning electron microscopy of leaf infected with acetosyringone (100 μ M) induced *Agrobacterium*. A. Leaf surface at cell expansion stage. B. Mature leaf surface.

attachment at the region of cell-cell junction (Fig. 4A). However, evenly distributed individual cells of *Agrobacterium* were found attached on the surface of mature leaf (Fig. 4B). This indicated that with the increase in cell age *Agrobacterium* attachment sites are gradually lost and mature sorghum leaf surface has limited number of *Agrobacterium* attachment sites. Transient GUS expression analysis further confirmed that intact leaf surface does not support productive *Agrobacterium* interaction.

Quantitative *Agrobacterium* attachment at cell-cell junctions of young leaf surface was comparable to young undifferentiated callus, which supported good attachment and colonization. However, contrary to the callus surface it was restricted to regions of cell-cell junction over leaf surface.

Shoot Apex

The split shoot apex also interacted positively with the pre-induced *Agrobacterium*. The number of *Agrobacterium* cells attached to split shoot was considerably less than that found on the young leaf surface. On the split shoot apex only a limited number of *Agrobacterium* aggregates could be observed around the cell situated in the meristematic region (Fig. 5B). Another tissue that supports *Agrobacterium* attachment was vascular tissue (Fig. 5C.)

In this study it has been observed that acetosyringone is most critical for the sorghum-*Agrobacterium* interaction leading to bacterial attachment and T-DNA delivery to the host cell nucleus. The degree of attachment and colonization however differ in different tissues i.e. leaf epidermis shows good attachment but did not support bacterial colonization. Split shoot apex shows bacterial attachment with limited colonization. Callus with actively dividing cells was the best explant that supported maximum attachment and colonization of *Agrobacterium*.

Fig. 5. Scanning electron microscopy of different regions of split shoot apex infected with acetosyringone (100 μ M) induced *Agrobacterium*. A. Cortex region B. Meristematic region, C. Vascular bundle region.

Use of different strains

A. tumefaciens strains differ in their ability to transfer T-DNA to plant cells as the strains exhibit different host specificity. The majority of identified strains have a wide host range. The sorghum plant cells did not show any preference to the two strains EHA105 and LBA4404 used. Both of the strains of bacterium formed aggregates on the callus cells and attached to all the tissues. Tissues that are in an active state of cell division showed maximum colonization.

DISCUSSION

Sorghum is the fifth most important cereal crop in the world. It is largely grown on marginal soils with residual moisture where other major cereals cannot be grown due to inadequate water. Sorghum is chiefly grown in low input conditions and development of host plant resistance to biotic and abiotic stresses is much pursued objective. Introduction of resistance through conventional breeding is limited due to the lack of resistance sources. There are a few reports on genetic transformation of sorghum but the transformation frequency remains low (Zhao *et al.*, 2000). Attachment of *Agrobacterium* to host plant cells is the first and most important step in the genetic transformation process. This step was studied with an aim to facilitate genetic transformation of elite sorghum genotypes.

Pre-induction of *Agrobacterium* with acetosyringone was found a prerequisite for host recognition and binding. Monocots are not known to secrete the phenolic compounds such as acetosyringone or even if they do so, the level is not enough for induction of *Agrobacterium* virulence gene. For a successful T-DNA delivery, *Agrobacterium* must bind itself to the host cell wall that is achieved by a group of genes located within the bacterial chromosome (Matthysse *et al.*, 2000;

Gelvin 2003). The precise mechanism for attachment is still unknown. However, it is proposed that these bacteria anchor at the site of injury, by the production of cellulose fibrils (Matthysse *et al.*, 1981). The fibrils attach to the cell surface of the plant host and facilitate the clustering of other bacteria on the cell surface. It is believed that this clustering may help the successful transfer of T-DNA. Mutagenesis studies show that non-attaching mutants loss the tumor-inducing capacity (Douglas *et al.*, 1982; Cangelosi *et al.*, 1987; Bradley *et al.*, 1997).

Although reports on involvement of plant genes in *Agrobacterium* mediated transformation are very limited (Fullner *et al.*, 1996; Gelvin, 2000; Tzvi *et al.*, 2002). RAT4 mutants in *Arabidopsis* are resistant to *Agrobacterium* mediated genetic transformation. Cellulose synthase-like genes such as 'CSLA9' are involved in determination of sugar composition of cell wall and ability of roots to bind *Agrobacterium* (Zhu *et al.*, 2003).

In the present study it has been observed that sorghum tissues interact positively with *Agrobacterium*, but as the cell matures their competence to *Agrobacterium* mediated transformation is lost. Three sorghum explants, leaf derived callus, leaf and split apex responded differentially for *Agrobacterium* attachment and T-DNA delivery. The possible reason of observed differential response of *Agrobacterium* attachment to different sorghum tissues may be due to change in cell wall composition of cells that are at different physiological and developmental stages. As the plant cell gets mature, its cell wall composition changes in such a way that *Agrobacterium* binding sites are lost gradually. Mature cells of intact leaf epidermis having limited number *Agrobacterium* attachment sites have evenly distributed *Agrobacterium* while undifferentiated callus and split shoot apex having young cells with plenty of *Agrobacterium* attachment sites favored development of *Agrobacterium* aggregates.

The scanning electron microscopic observations demonstrated the presence of bacteria individually bound or massively attached to the surface of different sorghum tissues. It can be concluded that at least during the two early steps of *Agrobacterium* attachment and gene delivery *A. tumefaciens* is compatible with sorghum, indicating its potential for genetic transformation. The present study shows that undifferentiated callus and split shoot apex have maximum number of cells in competent stage for *Agrobacterium* mediated transformation. It is concluded that regeneration from

shoot apical meristem, more preferably from callus is essential to develop transgenic plants of sorghum.

ACKNOWLEDGMENTS

The scanning electron microscope work was carried out at SAIF (DST), Department of Anatomy, AIIMS, New Delhi- 110029.

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