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Review

Agrobacterium-mediated transformation of plants: Basic principles and influencing factors

Mohammad Alimohammadi* and Mohammad B. Bagherieh-Najjar

Department of Biology, Faculty of Sciences, Golestan University, Gorgan, Iran.

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Transformation is an important topic in plant biology and transgenic plants have become a major focus in plant research and breeding programs. *Agrobacterium*-mediated transformation as a practical and common method for introducing specific DNA fragments into plant genomes is well established and the number of transgenic plants produced using this method is increasing. Despite the popularity of the method, low efficiency of transformation is a major challenge for scientists. Modification of different genetic and environmental aspects of transformation method may lead to better understanding of the system and result in high efficiency transformation. In this review, we deal with recent genetic findings as well as different environmental factors which potentially influence *Agrobacterium*-mediated transformation.

Key words: *Agrobacterium* transformation, T-DNA integration, transformation efficiency.

INTRODUCTION

Less than 30 years ago, using *Agrobacterium tumefaciens* as an instrument to create transgenic plants was only a prospect for scientists. Today, plant transformation has become an essential tool for plant molecular biologists and creating transgenic plants is a major focus in many plant breeding programs. The first transgenic crop arrived market about 15 years ago, and since then some countries like the United States has commercially approved various transgenic crops. Certain transgenic crop plants are currently grown almost everywhere (e.g. herbicide resistant canola and soya).

A. tumefaciens causes crown gall disease in a wide range of plants, especially members of the rose family. The discovery that the disease has a bacterial nature (Smith and Townsend, 1907) paved the way for other scientists to study the mechanisms used by the bacteria to cause the disease. *A. tumefaciens* can transfer a particular DNA segment named Transfer (T)-DNA of the tumor inducing (Ti) plasmid into the host genome (Binns and Thomashaw, 1988). 95% of the cells in the tumor caused by bacteria are transformed (Deeken et al., 2006). *Agrobacterium* ability to transfer a particular DNA segment into plant genome changed the objectives of researches using *Agrobacterium* for transformation.

*Corresponding author. E-mail: mohamad82@gmail.com.

Recent findings revealed that genes involved in photosynthesis are strongly down regulated in Crown gall disease and that transformation of plant cells with T-DNA of virulent *Agrobacterium* is accompanied by a change from autotrophic to heterotrophic metabolism, where ATP production is mainly powered by glycolysis and fermentation (Deeken et al., 2006).

Virulent strains of *A. tumefaciens* contain a large plasmid (more than 200 kb) that has an important role in tumor induction (Ti Plasmid). The transfer is mediated by proteins encoded by genes in the Ti plasmid virulence region (*vir.* genes) and in the bacterial chromosome. The T-DNA most likely relies on host DNA repair machinery for its conversion into double-stranded T-DNA intermediates and their recognition by proteins such as histone H2A (Mysore et al., 2000; Li et al., 2005) and histone H3 (Anand et al., 2007) for integration into the host chromosome.

Early results of the studies on T-DNA transfer process to plant cells revealed important facts which enabled using this process in plants transformation. Firstly, the tumor formation is a sign of transformation process resulted from integration of T-DNA and expression of T-DNA genes. Secondly, the T-DNA genes are transcribed only in plant cells and not during the transfer process. Thirdly, any foreign DNA placed between the T-DNA borders can be transferred into the plant genome (Rival et al., 1998; Opabode 2002).

Genetic transformation mediated by *A. tumefaciens*

was first reported in the 1980's (Block et al., 1984). At the beginning of the last decade evidences about transgenic tobacco expressing foreign genes were obtained. Since then, a great progress in understanding the *Agrobacterium* mediated gene transformation of plant cells has been achieved. *A. tumefaciens* naturally infects only dicotyledonous plants. Therefore, genetic manipulation of many important plants remains accessible only by other methods, such as particle bombardment. However, *Agrobacterium*-mediated transformation has advantages over other transformation methods. The transgenic plants obtained from *Agrobacterium*-mediated method are generally fertile and the foreign genes are often transmitted to progeny in a Mendelian manner (Rhodora and Thomas, 1996). *Agrobacterium* mediated gene transfer into monocotyledonous plants was not possible until recently, when reproducible and efficient methodologies were established on rice, banana, corn and wheat (Hiei et al., 1994; Cheng et al., 1998; May et al., 1995; Ishida et al., 1996; Enriquez-Obregon, 1998; Arencibia et al., 1998). Wide host range of *Agro-bacterium*, suggests that in many cases T-DNA integra-tion may remain the limiting step for certain plants. Altering the tissue culture conditions, for example by the use of antioxidants during the transformation can increase the probability of stably transforming cell types that can be regenerated (Obregon, et al., 1999; Olhoft et al., 2001; Frame et al., 2002), but such manipulations may still have limitations.

Different approaches have been employed to identify genes involved in *Agrobacterium* transformation. These approaches can be classified into 4 different groups; genomics approaches to identify plant genes that may be induced or repressed soon after infection by *Agrobacterium*, use of yeast two hybrid systems to identify plant proteins that may interact with virulence proteins, forward genetic screening to identify plant mutants with altered transformation efficiency and reverse genetic screening to test whether particular genes may be involved in transformation. A summary of the results obtained by these approaches are discussed.

GENOMICS APPROACHES

Genes that are induced or repressed during the early stages of *Agrobacterium*-mediated transformation may be used as targets for manipulation of the host to improve the efficiency of transformation. Ditt and his colleagues investigated the response of *Ageratum conyzoides* suspension cell cultures to infection by a non-tumorigenic super virulent *A. tumefaciens* strain (Ditt et al., 2001). Using cDNA amplification fragment length polymorphism (AFLP) to amplify 16,000 fragments, they identified 251 bands that were differentially regulated 48 h after infection. Some of these bands were also induced or repressed 24 h after inoculation. Most of the bands investigated were also differentially regulated following incubation of plant cells with *Escherichia coli*; four genes, including one encoding a nodulin-like protein, responded specifically to *Agrobacterium* infection. The authors believed that this nodulin gene might respond to signals from the bacterium to regulate plant cell division or differentiation. Anand et al. (2007) developed *in planta* and leafdisk assays in *Nicotiana benthamiana* for identifying plant genes involved in *Agrobacterium*-mediated plant transformation using virus-induced gene silencing (VIGS) as a genomics tool. They showed the involvement of a nodulin-like protein and an alpha expansin protein (α-Exp) during *Agrobacterium* infection. Data suggested that α-*Exp* is involved during early events of *Agrobacterium*-mediated transformation but not required for attaching *A. tumefaciens* (Anand et al., 2007).

YEAST TWO-HYBRID SYSTEM

Several *Agrobacterium* virulence proteins interaction with plant proteins includes the processed form of VirB2 (required for transformation), VirD2, the protein that caps the 5′ end of the transferred T-strand, VirE2, the single-stranded DNA binding protein that coats the T-strand, and VirF whose function is still unknown. Ballas and Citovsky (1997) utilized VirD2 as the bait protein in a yeast two-hybrid system to identify an *Arabidopsis thaliana* importin-α (AtKAP) gene which encodes proteins important in the nuclear translocation of many proteins harboring nuclear localization signal (NLS) sequences. They showed that interaction of VirD2 with importin-α AtKAP was NLS dependent both in yeast and *in vitro*. Deng et al. (1998) identified three VirD2 (Including *Arabidopsis* Cyclophilin) and two VirE2 interacting proteins. Cyclosporin A, an inhibitor of cyclophilins, inhibited *Agrobacterium*-mediated transformation of *Arabidopsis* roots.

Using VirE2 as the bait protein identified two interacting proteins from *Arabidopsis*, VIP1 and VIP2 was identified (Tzfira et al., 2001). They suggested that VIP1 might be involved in nuclear targeting of the T-complex because antisense inhibition of VIP1 expression resulted in a deficiency in nuclear targeting of VirE2. VirE3 is transferred from *Agrobacterium* to the plant cell and then imported into its nucleus via the karyopherin α –dependent pathway. VirE3 interacts with VirE2. The VirE2 nuclear import in turn is mediated by a plant protein, VIP1. Data indicate that VirE3 can mimic this VIP1 function, acting as an 'adapter' molecule between VirE2 and karyopherin α (Lacroix and Tzfira, 2005). *A. thaliana* VIP2 with a NOT domain that is conserved in both plants and animals is identified (Anand et al., 2007). Evidences suggest that VIP2 interaction with VIP1 is required for nuclear import and integration of T-DNA into the genome. Double mutation of VirF and VirE3 leads to strongly diminished tumor formation on tobacco, tomato and sunflower. VirE3 interacts *in vitro* with importin- α and

VirE3-GFP fusion protein is localized in the nucleus (Garcia-Rodriguez et al., 2006).

FORWARD GENETIC SCREENING

In order to find plant genes involved in *Agrobacterium*-mediated transformation, Gelvin and his co-workers tried to identify *Arabidopsis* T-DNA insertion mutants (rat mutants) that are resistant to *Agrobacterium* transformation (Nam et al., 1999). More than 70 mutants were identified. Many of these genes are involved in the transformation process. Mutants, including the rat5 (a histone H2A mutant) are probably involved in T-DNA integration (Nam et al., 1999). Other experiments indicate that histone H2A-1 gene (HTA1) expression and *Agrobacterium*-mediated transformation are highly correlated.

Transgenic *Arabidopsis* plants containing additional genomic (Mysore et al., 2000) or cDNA (Gelvin, 2003) H2A-1 copies are 2-6 fold more transformation competent, compared to plants containing the normal histone HTA1 gene. Over expression of the histone H2A1 gene complements the rat5 mutant and increase the transformation efficiency of *Arabidopsis* ecotypes (Gelvin, 2003). Finally, over expression of the RAT5 histone H2A-1 gene in various rat mutants (other than the rat5 mutant) also restores transformation competency (Gelvin, 2003). Expression of the RAT5 gene is therefore epistatic over the rat phenotype of other rat mutants and thus may sensitize plant cells to *Agrobacte-rium* mediated transformation.

RNA encoded by *HTA* genes accumulates to differing levels in roots and whole plants; HTA1 transcripts accumulate to levels up to 1000 fold lower than the transcripts of other HTA genes (Yi et al., 2006). Over expression of all tested HTA cDNAs restored transformation competence to the rat5 mutant. However, only the HTA1 gene could phenotypically complement rat5 mutant plants when expressed from their native promoters. Data suggest that, with respect to *Agrobacterium*-mediated transformation, all tested histone H2A proteins are functionally redundant. However, this functional redundancy is not normally evidenced because of different expression patterns of the *HTA* genes (Yi et al., 2006).

REVERSE GENETIC SCREENING

Scientists identified plant genes encoding proteins that interact with *Agrobacterium* virulence proteins (Bachrati and Hickson, 2003). This suggests that these genes play a role in plant transformation. One way to test the role of a particular gene in transformation would be to mutate that gene and then assay the plant for transformation susceptibility. At pre-sent site directed mutagenesis is not an efficient method for use in plants. An alternative reverse genetic approach is to identify mutant plants containing transposons or T- DNA insertions in genes of interest. Several PCR-based strategies have been described in order to identify such knockout mutants in *Arabidopsis* (Feldmann, 1991; Frey et al., 1998; Krysan et al., 1999). Later, a reverse genetic strategy introduced to produce crown gall-resistant plants (Escobar et al. 2001). In this method, transgenic *Arabidopsis* plants expressing double-stranded RNA constructions were generated which target the T-DNA-encoded auxin and cytokinin biosynthetic ontogenes. These genes are highly homologous among *Agrobacterium* strains. Many of transgenic plants expressing these RNAi constructions were highly resis-tant to crown gall disease.

Li et al. (2005) used reverse genetics to dissect VIP1 functionally and demonstrate its involvement in the stable genetic transformation of *Arabidopsis* plants by *Agrobacterium*. Their findings indicate that the ability of VIP1 to interact with the VirE2 protein component of the T-complex and localization to the cell nucleus is sufficient for transient genetic transformation, whereas its ability to form homomultimers and interact with the host cell H2A histone *in planta* is required for tumorgenesis and, by implication, stable genetic transformation.

The ectopic expression of *Arabidopsis* RecQl4A in yeast RecQ deficient cells suppressed the enhanced rate of homologous recombination (HR). Furthermore, inactivation of RecQl4A in *Arabidopsis* leads to 7.5 to 20 fold increase in the frequency of HR suggesting that under standard growth conditions RecQl4A acts as a suppressor of homologous recombination (Bagherieh-Najjar et al., 2005). Therefore, it is highly possible that we can modify transformation efficiency in *Arabidopsis* by modification of the RecQl4A gene. This suggested an application for RecQl4A in developing new tools for gene targeting in plants. Our recent preliminary data reveal that RecQl4A indeed modulates T-DNA transformation efficiency in plants (Unpublished Data).

FACTORS WHICH INFLUENCE *AGROBACTERIUM*-MEDIATED TRANSFORMATION

The transfer of T-DNA and its integration into the plant genome is influenced by several factors. These include plant genotype, explants, vector-plasmids, bacteria strain, culture media composition, tissue damage, suppression and elimination of *A. tumefaciens* infection after co-cultivation. Recently, some other factors have found importance in influencing the efficiency of *Agrobacterium*-mediated genetic transformation of crops (Opabode, 2006; Alt-morbe et al., 1989; Bidney et al., 1992; Hoekema et al., 1993; Hiei et al., 1994; Komari et al., 1996; Nauerby et al., 1997; Klee, 2000). Here we briefly discuss some of these factors and their effects on transformation efficiency

Osmotic treatment of explants

In-vitro manipulation of explants is necessary to enhance

competency of plant cells to T-DNA delivery, and plant cell recovery after infection. Osmotic enhancement of *Agrobacterium*-mediated transformation largely depends upon species. Enriched co-culture medium with 200 mM sucrose and 200 mM glucose was used in rice and maize transformation (Hiei et al., 1997; Zhao et al., 2001; Frame et al., 2002). Plasmolysis with 292 mM sucrose improved T-DNA delivery into pre-cultured immature rice embryos (Uze et al., 1997). This treatment was extensively used to produce large numbers of transgenic plants in various projects (Ye et al., 2000; Lucca et al., 2001). Osmotic treatment was not effective with pre-cultured immature embryos of wheat and also did not have a beneficial effect on T-DNA delivery in wheat (Uze et al., 2000; Cheng et al., 2004). Nevertheless, in various wheat species significant differences in transient expression of synthetic green fluorescent protein (GFP) mediated by *Agrobacterium* were found in regard to osmotic treatment with 0.4 M mannitol (Carsono et al. 2007). Induction of embryogenic calli from sweet potato treated with different time periods of mannitol before being mixed with bacteria resulted in transformation improvement after 60 min. mannitol treatment (Xing et al., 2007). The result suggests that osmotic treatment period has a clear impact on gene transformation efficiency.

Desiccation of explants

Desiccation of explants before or after *Agrobacterium* infection is a significant factor that enhances transformation of crop species. Air drying sugarcane suspension cells for 15-60 min under laminar flow conditions prior to bacteria inoculation slightly improved T-DNA delivery and subsequently increased transformation efficiency (Arencibia et al., 1998). Similarly, air-drying calli derived from rice suspension cultures for 10-15 min increased the transformation efficiency 10 fold or more, as compared to the control plants (Urushibara et al., 2001). Desiccation of precultured immature embryos, suspension culture cells, embryonic calluses of wheat, and embryogenic calluses of maize greatly enhanced T-DNA delivery and plant tissue recovery after co-culture, which leads to a more stable transformation frequency (Cheng et al., 2003). This treatment was not only effective in monocot species, but also improved T-DNA delivery in recalcitrant dicot species such as soybean suspension cells (Cheng and Fry, 2000). It is unclear, however, what factors were affected by air drying of plants, but it is possible that plasmolysis of the cells or tissue wounding might be important.

Antinecrotic treatments

Application of antinecrotic mixture for pre-induction was shown to be important for reducing oxidative burst. Obregon (1998) developed an efficient transformation system by treatment of meristigmatic spindle sections of sugarcane and rice (Obregon et al., 1999), with a medium containing 15 mgl^{-1} (0.09 µM) ascorbic acid, 40 mgl^{-1}(0.33 µM) cysteine, and 2 mgl^{-1}(0.01 µM) silver nitrate. Inclusion of cysteine in the co-culture medium led to an improvement in stable transformation frequency in maize. T-DNA transfer into cotyledon node cells and genomic integration was increased through the inclusion of thiol compounds in the solid co-cultivation medium, resulting in an increased production of transgenic plants (Olhoft et al., 2003). Other findings show that silver nitrate significantly suppresses *Agrobacterium* growth during co-culture without compromising T-DNA delivery and subsequent T-DNA integration. The suppressed *Agrobacterium* growth on the target explants could facilitate plant cell recovery and result in increased efficiency of transformation (Cheng et al., 2003).

Temperature

The effect of temperature during co-culture on T-DNA delivery was first studied in dicotyledonous species. A temperature of 22°C was found to be optimal for T-DNA delivery in tobacco leaves (Dillen et al., 1997). However, in another report, co-culture at 25°C led to the highest number of transformed plants of tobacco, even though 19°C was optimal for T-DNA delivery (Salas et al., 2001). In monocots, co-culture temperature for most of the crops ranged from 24 to 25°C, and in some cases, 28°C was used for co-culture (Rashid et al., 1996; Arencibia et al., 1998; Enriquez-Obregon et al., 1998; Hashizume et al., 1999). The optimum co-culture temperature for *Brassica juncea* was 25°C while higher temperatures resulted in a very low number of transgenic plants (Zhang et al., 2006). Similarly transformation efficiency in sweet potato increased from 22 to 28ºC but at 30ºC it was strongly reduced (González et al., 2008); in line with previous work suggesting that T-DNA transfer machinery works more efficiently under temperatures below 28ºC (Ditt et al., 2005). Higher transformation frequency was observed in maize immature embryo transformation from 20 to 23°C when using a standard binary vector (Frame et al., 2002). Therefore, it seems optimal temperature for stable transformation should be evaluated with each specific explants and *Agrobacterium* strain involved.

Surfactants

Addition of surfactants such as Silwet L77 (0.01-0.075% v/v) and pluronic acid F68 (0.01-0.2% w/v) in inoculation medium was shown to enhance T-DNA delivery in immature embryos of wheat (Cheng et al., 1997). This may be resulted by elimination of factors that inhibit *Agrobacterium* attachment. The addition of F68 (0.03%) to the

inoculation medium dramatically increases transient GUS expression by up to 100 fold in sorghum (Henrique et al., 2004). The surfactant Silwet L77 was also shown to be useful to the success of the floral dip method of *A. thaliana* transformation (Ye et al., 1999; Bechold et al., 2000; Desfeux et al., 2000). Notably, high levels of Silwet L77 caused notable flower mortality in some experiments (Clough et al., 1998). In most experiments, L77 is used at 0.03%, but even at those level deformities in the pistils of flowers used for crosses is occasionally noted in the first few days after inoculation (Desfeux et al., 2000). . Wu et al. (2003) reported that concentrations of Silwet L77 up to 0.04% had positive effects on T-DNA delivery, while higher than 0.05% reduced survival and callus formation.

Inoculation and co-culture medium

Medium component, sugar, plant growth regulators, and some chemicals are also some important factors that affect transformation frequency. The modified N6 medium (Chu et al., 1995) containing 2,4-dichlorophenoxyacetic acid (2,4-D) and casamino acids was shown to be suitable for co-culture in rice. MS (Murashige and Skoog, 1962) or a modified MS-based medium was shown to be suitable for inoculation and co-culture in several reports of rice transformation (Dong et al., 1996; Enriquez-Obregon et al., 1999; Mohanty et al., 1999; Luca et al., 2001). Transformation of maize immature embryo is reported using LS-based medium (Linsmaier and Skoog, 1965). More recently, the use of L-cysteine in combination with modified medium salts has improved *Agrobacterium*-mediated transformation of three maize inbred lines (Frame et al., 2006). Use of low salt media during the *Agrobacterium* infection stage of transformation represents an additional strategy to improve T-DNA transfer in canola (Fry et al., 1987) and wheat (Cheng et al., 1997). This treatment was used to regenerate stable transformed wheat plants from embryogenic callus with a super binary vector (Khanna and Daggard, 2003). Medium with reduced salts also enhanced T-DNA delivery in maize (Armstrong and Rout, 2001), and half-strength MS salts in both inoculation and co-culture media have been used in maize transformation (Zhang et al., 2003). The use of 1/10 MS salts tended to increase the percentage of embryos but reduced embryo survival and callus formation after co-cultivation in sorghum (Henrique et al., 2004).

Antibiotics

Antibiotics such as cefotaxime, carbenecillin and timentin are regularly used in *Agrobacterium*-mediated transformation of crops and co-culture medium to eliminate *Agrobacterium* (Cheng et al., 1996; Bottinger et al., 2001; Sunikumar and Rathore, 2001). Although cefotaxime works in *Agrobacterium*-mediated transformation of rice and maize, it is found that at concentrations above 250 mgl^{-1} have detrimental effects on maize callus formation (Ishida et al., 1996). Callus formation was greatly reduced in maize when cefotaxime (50 or 250 mgl^{-1}) was added in the callus induction medium, and transformation ratio was reduced 3 fold compared to that with carbenicillin (100 mgl^{-1}). Carbenicillin has been the antibiotic of choice in reports of *Agrobacterium*-mediated transformation of wand maize (Cheng et al., 1997, 2003; Zhang et al., 2003). However 100 mg^{-l} kanamycin was economical and improved the transformation efficiency in white spruce (Le et al., 2001) and increased positive transformed shoots during subculture on kanamycin containing medium in peanut and pigeon pea (Sharma and Anjaiah, 2000; Thu et al., 2003). Kanamycin selection on the regeneration step was efficient in sweet potato with concentration of 50 mgL^{-1} (González et al., 2008). After co-cultivation in sorghum the explants were allowed to grow for 6 to 12 days in a medium without hygromycin but with cefotaxime to kill *Agrobacterium*. This period without selection seemed to help the explants to recover from infection and apparently did not affect embryo selection with hygromycin (Henrique et al., 2004).

Conclusion

Identification of the genes involved in the transformation process result in improvement of transformation methods. Genomics approaches, yeast two-hybrid systems, forward and reverse genetics are four major approaches employed to identify these genes. After finding target genes, influencing factors in transfer of T-DNA and its integration into the plant genome should be considered. Among these factors are osmotic treatment of explants, desiccation, antinecrotic treatments, temperature, surfactants, inoculation, co-culture medium and antibiotics employment. Recent findings revealed indisputable role of these factors in *Agrobacterium* mediated transformation system and discuss the possibility of modifying these factors in order to result in high efficiency transformation of target genes into plant genomes. Hopefully, specific studies around these subjects especially on economically important crops will lead to producing transgenic plants with higher growth rate in adverse environmental conditions, against potential pathogen organisms and with top quality products.

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