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ALTERNATIVE METHODS OF PLANT TRANSFORMATION – A SHORT REVIEW

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Abstract: Several methods of transformation are currently available for delivering exogenous DNA to plant cells. *Agrobacterium*-mediated transformation, microprojectile bombardment and direct protoplast transformation are routinely used today. However, each of them has certain disadvantages, which led to research into the development of novel alternative systems such as infiltration, electroporation of cells and tissues, electrophoresis of embryos, microinjection, pollen-tube pathway, silicon carbide- and liposome-mediated transformation. The low efficiency of transformation is considered to be the main reason for the limited popularity of the alternative transformation methods, other than infiltration and silicon carbide-mediated transformation, which seem to be the most promising ones for practice.

Key Words: Plant Transformation, Infiltration, Silicon Carbide Fiber, Electroporation, Electrophoresis, Microinjection, Pollen-Tube Pathway, Liposomes

INTRODUCTION

Currently, numerous transformation methods are available. They can be divided into two main groups: indirect and direct ones. The indirect methods of plant transformation are based on the introduction of a plasmid-carrying gene construct into the target cell by means of bacteria – *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. Direct methods do not use bacteria cells as mediators. *Agrobacterium*-mediated transformation is the main method used in the field of biotechnology, where the most often applied direct methods are protoplast transformation or microprojectile bombardment. In the case of *Agrobacterium*-mediated transformation, the efficiency for monocots is still unsatisfactory. However, in recent years, it has become the method of choice for

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this group of plants [31]. The general disadvantages of direct protoplast transformation are problems with plant regeneration (especially in monocotyledonous plants), and a low transient expression of transgenes as compared with organized tissues. The viability of protoplasts and their capability of dividing are strongly reduced by both the electrical field and chemical substances applied to disorganize cell walls. The most distinctive factor limiting the use of the gene gun is the presence of multiple copies of introduced genes, which can lead to various unprofitable effects like their suppressed or changed expression. The high expenses of gene gun accessories should also be taken into consideration. Many methods of plant transformation require the employment of *in vitro* culture, at least during some procedural steps. During *in vitro* regeneration, some somaclonal changes may arise. This fact can make an analysis of transformants difficult, and limits use in further study as well. All the above-mentioned limitations inspired investigators to search for new alternative transformation procedures. Up till now, several such methods have been developed. Among them, the most often listed ones are 1) infiltration, 2) silicon carbide fiber-mediated transformation, 3) electroporation of cells and tissues, 4) electrophoresis of embryos, 5) microinjection, 6) transformation via the pollen-tube pathway and 7) liposome-mediated transformation [34, 35, 40, 43, 47]. The majority of these were thought to be solutions for the effective transformation of recalcitrant species, such as monocots or some legumes, as *Agrobacterium*-mediated transformation was at that time not available for this group of plants.

CHARACTERISTICS OF ALTERNATIVE METHODS OF PLANT TRANSFORMATION

Infiltration

Some transformation procedures do not require *in vitro* culture. In the case of infiltration, the bacterial inoculum is introduced directly into those parts of the plant in which meiotic or mitotic divisions take place intensively. Infiltration has mainly been applied for the transformation of *Arabidopsis thaliana* for the past several last years, and has become the main method of gene delivery for this species. This simple procedure consists of placing plants in the early generative phase of development (the secondary inflorescence is 5-15 cm tall, and the primary one arises from the leaf rosette) upside down in beakers with a solution containing *Agrobacterium tumefaciens* and 5% sucrose in such a way that only the inflorescences are submerged in the inoculum. Beakers carrying plants are placed in vacuum chamber and the vacuum, usually 0.05 bar, is held for several minutes. Seeds are then collected from the infiltrated plants and sown under sterile conditions on selective media. Keeping optimal conditions, it is possible to obtain up to 95% plants which set transgenic seeds at a level of approximately 0.5-4% of all harvested seeds. The vacuum infiltration method for *Arabidopsis* transformation was applied for the first time in 1993 [3]. The optimal conditions leading to a higher than 2% efficiency were established over the next five years.

The new improved protocol involved an optimal sucrose concentration and a supplement of bacteria inoculum by the surfactant, Silwet L-19 [8]. Currently, the protocol developed by Clough and Bent is the most often used for *Arabidopsis* transformation [7, 45]. A modified version of this protocol involves transformation without applying vacuum infiltration. In this simple procedure, the plants are immersed or sprayed with a bacterial suspension several days after the first leaf has been removed. Chung and co-workers [7] compared the classical and modified methods of transformation and concluded that spraying plants with a bacterial suspension gave the best result (2.41% vs. 1.76% for vacuum infiltration and 2.09% for immersion). The authors suggested that by omitting the vacuum, infiltration could become a useful method for the transformation of other plant species which are larger in size than *Arabidopsis*. However, the attempts to employ infiltration for other species, e.g. Chinese cabbage [27] have not succeeded so far.

Silicon carbide-mediated transformation (SCMT)

SCMT is one of the least complicated methods of plant transformation. Silicon carbide fibers are simply added to a suspension containing plant tissue (cell clusters, immature embryos, callus) and plasmid DNA, and then mixed in a vortex, or in other laboratory apparatus such as commercial shakers, blenders etc. DNA-coated fibers penetrate the cell wall thanks to the presence of small holes created in collisions between the plant cells and fibers [21, 22, 44]. The most often used fibers in this procedure are single crystals of silica organic minerals like silicon carbide, which have an elongated shape, a length of 10-80 μm , and a diameter of 0.6 μm , and which show a high resistance to expandability.

The efficiency of SCMT depends on the fiber size, the parameters of vortexing, the shape of the vessels used, the plant material and the characteristics of the plant cells, especially the thickness of the cell wall.

The main advantages of this easy and quick procedure are the low expenses and usefulness for various plant materials. Among the main disadvantages are a low transformation efficiency, damage to cells negatively influencing their further regeneration capability, and the necessity of obeying extraordinarily rigorous precaution protocols during lab work, as breathing the fibers in, especially asbestos ones, can lead to serious sicknesses [40].

There are several known examples of deriving transgenic forms – cell colonies or plants – in maize [5, 15, 22, 36, 44], rice [32], wheat [4, 39], tobacco [21], *Lolium multiflorum*, *Lolium perenne*, *Festuca arundinacea*, and *Agrostis stolonifera* [10] by SCMT. Kaeppeler *et al.* [22] transformed a cell suspension of the Black Mexican Sweet (BMS) variety of maize with the plasmid carrying genes *bar* and *uidA*. The authors obtained approximately 3.4% transgenic cell lines expressing both transgenes from a 300 ml of packed cell volume, which means that the integration of transgenes occurred in one per one million cells. The efficiency was significantly lower than that described earlier by the same

team or other authors (among others Klein and co-workers [23]) when microbombardment was applied. One of the reasons for such a low efficiency could be the notable reduction of cell viability, up to 29%, caused by damages during vortexing with silicon carbide fibers.

The first fertile transgenics were produced for maize in 1994 [15]. Three hundred and eleven transgenic plants were derived from 22 independent transgenic cell lines, and eight of those turned out to be stable transformants. However, the efficiency was significantly lower (5-10 fold) than that obtained earlier for gene gun-mediated transformation in the same lab. A similar observation – the efficiency being much lower in comparison with microbombardment – was reported by Petolino *et al.* [36]. The authors also considered the serious damage to transformed tissue by silicon carbide fibers to be the main reason for the unsatisfactory results.

The above-mentioned reports clearly indicate a low efficiency of silicon carbide fiber-mediated transformation as the main limitation for its practice. However, SCMT is an easy, fast and inexpensive procedure. Therefore, it could be an attractive alternative method of plant transformation in particular situations, e.g. when a gene gun is not available and *Agrobacterium*-mediated transformation is difficult or not possible (as in the case of numerous monocots). Moreover, the SCMT system of using commercial paint shakers, which has recently been reported for maize [5], seems to be very promising for commercial large-scale transformation.

Electroporation of intact plant cells and tissues

The electroporation of plant cells and tissues is very similar in its principles to the electroporation of protoplasts. The main difference lies in the use of other plant material, such as pollen, microspores, leaf fragments, embryos, callus, seeds or buds. For transformation, both plasmid DNA and *Agrobacterium* inoculum can be applied. The first attempts to adopt methods employed in protoplasts for organized plant tissues were reported in the early nineties, and their main idea was to check the transient expression of transgenes under different organo- or tissue-specific promoters. Efficient protocols for the electroporation of cell suspensions have been worked out for many species, e.g. tobacco [1], rice [12], and wheat [46]. Experiments on obtaining transgenic plants also started in the early nineties. So far, the best results have been obtained for maize. D'Halluin *et al.* [14] transformed immature embryos and embryogenic callus type I, which were briefly digested in a solution of pectolytic enzymes, followed by transfer into electroporating cuvettes. The efficiency of electroporation was relatively high: 90 transgenic plants were regenerated from 1440 embryos (6.25%) and 31 plants from 55 callus clusters (54.6%), which is fully comparable with the best results obtained for this species after microbombardment. Similar results were obtained for this species by other authors, with 445 transgenic cell lines selected from 24 ml PCV [26]. Authors calculated that the integration of transgenes took place approximately in one per 10,000 cells (1 ml PCV \approx 200,000 cells). Finally, 6 transgenic plants were

regenerated. Such an efficiency is similar to those characteristic for microbombardment. A much lower efficiency – 3 transgenic plants from 1080 immature embryos (0.28%), was stated in the case of wheat electroporation [41]. The postpulse addition of ascorbic acid or another ascorbate could significantly increase the transformation efficiency without any negative influence on cell viability, as shown for a maize BMS cell suspension [37].

Although electroporation seems to be an extremely simple and effective method, for at least some species, it has not yet been widely used for plant transformation.

Electrophoresis

At the end of the eighties a method employing electrophoresis was developed for the transformation of immature embryos, especially for the embryos of monocotyledonous plants [40]. It was proposed as an alternative method of transformation to expensive and not always efficient microprojectile bombardment [2, 17]. Transfected embryos are placed between the tips of two pipettes connected to electrodes. The pipette connected to the anode is filled in its narrow part with agar (or agarose) followed by an electrophoresis buffer containing EDTA. The pipette connected to the negative electrode contains agar mixed with DNA and an electrophoresis buffer. This pipette is in contact with the apical meristem of the embryo, whereas the second one is located near its basal apical part. Switching on current causes a slow flowing of DNA from cathode to the anode through the embryo (from the apical meristem to its base part). The efficiency of electrophoresis-mediated transformation depends on numerous factors, mainly on the parameters of the electrical field, the duration of electrophoresis, the contents of the electrophoresis buffer, and the physico-chemical properties of the embryo tissue. A voltage of 25 mV and an amperage of 0.5 mA for 15 minutes are the most often used parameters for electrophoresis [2, 17, 40]. Electrophoresis has a rather inconsiderable importance in plant transformation in spite of its simplicity and relatively low cost. The main reason is the poor viability of the treated embryos. Although the first attempts of Ahokas [2] resulted in the derivation of plants from embryos of barley, none of them expressed the *uidA* gene carried by the plasmid taken for transformation. Up till now, the only transgenic plants were obtained for *Calthe orchid* L. [17].

Microinjection

Transformation *via* microinjection is based on introducing DNA into the nucleus or cytoplasm by means of a glass microcapillary-injection pipette [9, 29]. This operation requires a micromanipulator. During the introduction of DNA into the nucleus, cells are immobilized with a holding pipette and gentle suction. Both pipettes contain mineral oil, which works as a cylinder. Microinjection is mainly used for the transformation of large animal cells. Its importance for plant transformation is rather limited due to the characteristics of plant cell walls, which contain a thick layer of lignins and cellulose. The plant cell wall is a barrier for glass microtools. The microinjection of protoplast could theoretically

resolve this limitation, but it carries with it the danger of releasing of hydrolases and other toxic compounds from the vacuole to the cytoplasm, which can cause rapid death of the protoplast. Although it is possible to remove vacuoles before microinjection without any consequences for protoplast viability, their loss significantly decreases the capability for division and plant regeneration [25]. The microinjection of protoplasts requires different methods of immobilization – instead of using a sucking capillary, protoplasts are attached to glass by coating them by poly-L-lisine or agarose. None of these solutions has proved useful, as poly-L-lisine can be toxic for some species and agarose (even a very thin layer) reduces visibility in the area of manipulation.

Currently, microinjection is widely used for the transformation of large animal cells e.g. frog egg cells or the cells of mammalian embryos, whereas it has not been developed into a routine transformation method for plants. The procedure is very slow and requires an expensive micromanipulator. However, one of the unquestionable improvements of microinjection is that it allows the introduction not only of DNA plasmids but also of whole chromosomes into plant cells [16, 20]. Moreover, it has become a very powerful tool for studying the cellular functions of plant cells and plastid physiology, e.g. in tobacco and *Vicia faba* [24]. Transgenic plants were only recovered in several studies involving such species as tobacco [38], petunia [16], rape [33], and barley [18], and usually at very low frequency.

The pollen-tube pathway method

Foreign DNA can be applied to cut styles shortly after pollination. The DNA reaches the ovule by flowing down the pollen tube. This procedure, the so-called pollen-tube pathway (PTP), was applied for the first time for the transformation of rice [28]. The authors obtained transgenic plants at remarkably high frequency. Afterward PTP was used for other species e.g. wheat [30], soybean [19], *Petunia hybrida* [42], and watermelon [6].

A bacterial inoculum or plasmid DNA can also be injected into inflorescences with pollen mother cells in the premeiotic stage without removing the stigma. In that case, it is expected that foreign DNA will be integrated with the gamete genome. Such an approach has been employed for rye [12]. Pollen collected from inflorescences injected with a suspension of genetically engineered *A. tumefaciens* strain was predestined for the pollination of the emasculated spikes of the maternal plant. In the next generation, two transgenic plants were derived from 3023 seeds. Although the result of the described experiment could be considered as positive, the final transformation efficiency was about 10-fold lower than that approximately reached for this species via microprojectile bombardment.

Liposomes

The idea of a method of direct plant transformation elaborated in the middle eighties was to introduce DNA into the cell by means of liposomes. Liposomes

are microscopic spherical vesicles that form when phospholipids are hydrated. They can be loaded with a great variety of molecules, including DNA. In the case of protoplasts, the transfection (lipofection) occurs thanks to membrane fusion and endocytosis. When pollen grains are transformed, liposomes are delivered inside through pores. Liposome-mediated transformation is far from routine, in spite of the low expense and equipment requirement. A probable reason is its laboriousness and low efficiency. Only several reports on the integration of genes introduced by means of liposomes followed by transgenic plant regeneration for tobacco [13] and wheat [47] have been published thusfar.

CONCLUSION

As mentioned above, none of the alternative transformation methods found a wide employment in the laboratory. A low efficiency of transformation in almost all of these procedures, a consequence of the decreased viability of cells, is one the most often listed limitations of their application. However, it seems that at least two of the described methods, infiltration and silicon carbide fiber-mediated transformation, can gain greater interest. Both of them are characterized by simplicity, reduced costs and a low equipment requirement. The infiltration is observed to become the main transformation method for *Arabidopsis*, whereas SCMT is for maize. With optimizing studies these experimental procedures might soon be available for a broad spectrum of plant species.

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