Pollen grains as a target for introduction of foreign genes into plants: an assessment

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Abstract Introduction of foreign genes and development of transgenic plants have become an integral part of crop improvement programmes in the last decade. However, most of the present day plant transformation protocols require long periods for development of transgenic plants and need skilled personnel. Development of alternate, simple and rapid transformation protocols for development of transgenic plants can overcome the constraints of *in vitro* culture, regeneration and associated problems. Pollen grains, due to their abundance and ease with which they can be handled are ideal targets for introduction of foreign genes into the germ line. However, progress in introduction of transgenes into pollen grains and their subsequent use in fertilization leading to development of transgenic plants are limited. With the recent progress made in understanding of pollen development along with reports of successful pollen-mediated transformation in important crop plants, it should be possible to extend this simple method of transformation to other crop plants. The review deals with development of pollen grains as a target for introduction of genes with special emphasis on recent developments.

Keywords Pollen grains · Transformation · Transgenic plants

It is essential to develop quick and simple transformation protocols for development of transgenic plants with novel traits so as to be included in modern plant improvement

programmes. The technique of development of transgenic plants should be accessible to conventional plant breeders, so that they can use them as a routine technique in plant improvement programmes. Numerous gene transfer techniques have been developed in the last three decades (Potrykus 1990a, 1991; Christou 1995; Hansen and Wright 1999, Darbani et al. 2008) for introduction of agronomically useful genes into crop plants leading to the large-scale development of transgenic plants and their commercialization (Dunwell 2000; Fernandez-Cornejo 2005). Ever since the first report of successful genetic transformation in plants in 1980's (Herrera Estrella et al. 1983; Horsch et al. 1983) plant biologists are on the lookout for quicker, more efficient and simpler techniques to recover fertile transgenic plants. However, most of the techniques developed based on *Agrobacterium*-mediated and direct DNA transfer techniques require time consuming, labour-intensive and expensive procedures such as prolonged periods of *in vitro* culture, requisite for regenerating protoplasts or explants and use of expensive instruments like particle gun. Most of them lead to production of chimeras and somaclonal variation. Among the alternate methods for development of transgenic plants (Rakoczy-Trojanowska 2002), methods such as the floral dip method (Clough and Bent 1998), pollen transformation (Wang et al. 2001), pistil transformation (Chumakov et al. 2006; Mamontova et al. 2010) and ovary-dip transformation (Yang et al. 2009) have been found to be easier, avoiding the *in vitro* phase and requisite for regeneration. In planta methods such as floral dip method, although demonstrated in a few species such as *Arabidopsis thaliana* (Clough and Bent 1998), is yet to be extended to other species. Pollen transformation, followed by pollination of stigmas with transformed pollen grains and subsequent selection of genetically transformed seeds and plants is a quick and easy alternate method to develop

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transgenic plants, bypassing the requisite for *in vitro* culture. In case of pollen grain transformation, like in other plant cell transformation, either *Agrobacterium tumefaciens* can be used as a vector or DNA can be directly introduced into the target tissue. It has the potential to produce genetically modified plants within a short period, leading to inclusion of the method in crop breeding programmes.

Successful genetic transformation in plants can be achieved through *Agrobacterium* mediated gene transfer (Hansen and Chilton 2000) and by direct DNA transfer methods, which employ particle gun bombardment of the target tissue (Finer et al. 2000) or direct introduction of DNA molecules into protoplasts mediated by polyethylene glycol, electroporation or liposome treatment (Kohler et al. 1987a, b; Hansen and Wright 1999). In *Agrobacterium* mediated transformation, the natural ability of this soil bacterium has been exploited to introduce foreign genes into plants. *Agrobacterium* mediated transformation has the advantage of being a simple protocol with minimum equipment costs coupled with the advantage of having mostly single or low copy inserts (Table 1). Highly efficient vectors (Hansen and Chilton 2000) with extra copies of virulence genes or with mutations which enhance virulence gene expression along with the possibility of introducing large fragments (at least 150 Kb) of DNA into plant genomes (Hamilton et al. 1996) have widened the horizons of *Agrobacterium* mediated gene transfer in plants. However, even after three decades of research, *Agrobacterium*-

mediated gene transfer is still genotype dependent. Frequency of *Agrobacterium* mediated transformation can be enhanced by different techniques such as sonication (Trick and John 1997), vacuum infiltration (Xu et al. 2008) and wounding by aluminium oxide (Kim et al. 2007).

Direct DNA transfer using particle bombardment (biolistics) (Eapen 2003a), although known to be genotype independent, can also result in a complex pattern of transgene integration with multiple copy inserts. Use of mesoporous silica nanoparticles to deliver DNA into plants (Torney et al. 2007) is another breakthrough technology to introduce DNA directly into plant cells. Protoplast transformation, which needs great finesse, employs direct DNA uptake using polyethylene glycol treatment, electroporation (Kohler et al. 1987a, b) or liposomes (Deshayes et al. 1985; Shillito 1999). Other methods of gene transfer include use of silicon carbide whiskers (Kaepper et al. 1990), microinjection (Crossway et al. 1986), sonication assisted transformation (Trick and John 1997), electroporation (Matthews et al. 1990) and laser treatment mediated gene transfer (Badr et al. 2005) (Table 1).

In planta transformation methods which include floral dip method (Clough and Bent 1998), pipetting *Agrobacterium* into developing spikelets (Hess et al. 1990), pollen tube mediated gene transfer (Zhou et al. 1983), pistil transformation (Chumakov et al. 2006) and direct pollen transformation (Wang et al. 2007, 2008) have been successful only in a few species.

Table 1 Pollen mediated genetic transformation in plants

Plant	Method	Result	Reference
<i>Zea mays</i> <i>Lycopersicon esculentum</i>	Incubation of pollen with genomic DNA	No transgenic plant after pollination	Sanford et al. 1985
<i>Nicotiana tabacum</i>	Electrotransformation	Transgenic plants	Smith et al. 1994
<i>Nicotiana tabacum</i>	Exine detached pollen + Electroporation	Gus expression	Shi et al. 1996
<i>Medicago sativa</i>	Microprojectile bombardment	Presence of gus A gene	Ramaiah and Skinner 1997
<i>Nicotiana tabacum</i>	Unicellular pollen grain treated biolistically, matured <i>in vitro</i> and used for pollination	Kanamycine resistant transgenic plant	Touraev et al. 1997
<i>Petunia hybrida</i>	Incubation of pollen with <i>Agrobacterium</i> under vacuum infiltration and pollination	Transgenic plants	Tjokrukusumo et al. 2000
<i>Zea mays</i>	Ultrasonication of pollen grains and plasmid DNA	Transgenic plants	Wang et al. 2001
<i>Nicotiana tabacum</i>	Particle gun bombardment of pollen	Transgenic plants	Aziz and Machray 2003
<i>Gossypium hirsutum</i> L	Pollen + <i>Agrobacterium</i> + Vacuum infiltration	Transgenic plants with <i>acs A</i> and <i>acs B</i> genes	Li et al. 2004
<i>Lilium longiflorum</i>	Pollen wounding by aluminium oxide + <i>Agrobacterium</i>	Gus A expression	Kim et al. 2007
<i>Sorghum bicolor</i>	Pollen + plasmid DNA subjected to sonication	Transgenic plants developed by pollination	Wang et al. 2007
<i>Brassica juncea</i>	Sonication assisted Plasmid DNA incubation	Transgenic plants developed by pollination and fertilization	Wang et al. 2008
<i>Nicotiana tabacum</i>	Male gametophyte using biolistics	GFP expression	Akhond and Machray 2009

Tissue culture prerequisite

In the majority of the protocols using *Agrobacterium*-mediated, particle gun bombardment-mediated and other direct DNA transformation methods, a tissue culture period is an essential requirement for developing transgenic plants. Plants can be regenerated by *de novo* shoot differentiation, somatic embryogenesis and by axillary bud multiplication after the introduction of the gene (Eapen 2003b). The process of *in vitro* culture is labour intensive, time consuming and needs skilled personnel. Besides, development of somaclonal variation (Larkin and Scowcroft 1981) may lead to unwanted variants among transgenics. Also, it is essential to have a reproducible regeneration system for successful development of transgenic plants.

Developing tissue culture-independent genetic transformation system has been a dream for plant biotechnologists, since this would avoid constraints such as genotype specificity and transgenic plants would be produced inexpensively and rapidly. Hence, alternate systems for introduction of genes are highly desired.

Pollen grains—a target for gene transfer

Formation of gametes is a key step in the life-cycle of sexually reproducing plants (Kagi and Grob-Hardt 2007). The whole concept has centered on the assumption that at fertilization, the egg cell accepts the genome of the sperm cell and consequently introduction of foreign gene into the pollen grain prior to fertilization will give rise to transgenic zygote and plants. The treated pollen grains can be used for pollinating the stigma of male sterile or emasculated flowers, develop seeds and transgenic plants selected on medium supplemented with selectable marker chemicals.

Pollen grain is an ideal target for transformation, since they occur in large numbers, can be easily isolated from anthers and transgenes can be introduced directly in the form of naked DNA or through mediation of *Agrobacterium*. The mature pollen grain harbours a vegetative cell and a generative cell, the latter giving rise to two male gametes. Sporogenous initial cells also called as pollen mother cells, undergo meiosis and a mitosis resulting in a tetrad of microspores. Each tetrad is enclosed in a thick callose wall and individual microspores are released from the tetrad by the action of callase—an enzyme produced by the surrounding tapetal cells (McCormick 2004; Edlund et al. 2004). The microspore enlarges and undergoes an asymmetric division resulting in a large vegetative cell and a small generative cell. The generative cell is engulfed in the cytoplasm of the vegetative cell. The generative cell undergoes mitosis (second pollen mitosis) to form two sperm cells (Fig. 1). The timing of second mitosis varies in

different plant species and in some cases, as in case of gramineae and crucifers, it takes place when the microspores are inside the anthers, while in others, it may occur during pollen tube growth on stigma.

The pollen grains are contained inside cell walls and at maturity, they are covered with two different cell walls—an inner intine made up of cellulose and an outer multilayered exine, composed of a chemically resistant polymer sporopollenin and interrupted by apertures. Besides, a pollen coat made up of lipids, proteins, pigments and aromatic compounds fill the sculptured cavities of pollen exine.

Pollen grain transformation

Desired foreign gene should be introduced into the pollen grain and they can subsequently be used for pollination and fertilization. At fertilization, the egg cell fuses with one of the sperm cells of the pollen grain and zygote is produced (Fig. 1). Hence, it is appropriate to integrate transgenes into the particular generative cell of the pollen, which will fuse with the egg cell and subsequently give rise to the zygote. Often, it is difficult to introduce genes into mature pollen grains with conventional methods of transformation because of the thick cell walls of pollen grains. Some of the initial experiments on treatment of pollen with exogenous DNA did not yield transgenic plants after pollination and fertilization (Sanford et al. 1985; Booy et al. 1989). Particle gun bombardment of pollen grains (Stoger et al. 1992; van der Leede-Plegt et al. 1995; Touraev et al. 1997, electroporation [Li et al. 2004] and vacuum infiltration (Xu et al. 2008) can help in introduction of transgenes into pollen grains. For earlier work in pollen or pollen tube mediated transformation, reviews by Alwen et al. (1990) and Roeckel et al. (1992) can be referred. Presence of thick cell walls of pollen grains coupled with the release of strong nucleases by germinating pollen grains have been hindrances for successful entry and integration of transgenes. Matousek and Tupy (1983) and Roeckel et al. (1988) have reported the release of active DNA nucleases in the pollen during germination, which may hinder pollen and pollen tube transformation.

Co-cultivation of pollen grains with *Agrobacterium* (Hess and Dressler 1989) and application of *Agrobacterium* prior to or after pollination (Luo and Wu 1989; Ohta 1986) have been reported to give rise to transgenic zygotes and plants. However, these results could not be reproduced in all laboratories and contradictory evidence has slowed down further research in this area (Stoger et al. 1992; Langridge et al. 1992; Shou et al. 2002). Langridge et al. (1992) showed that transformation of cereals by pollen pathway was difficult and they suggested that transformation observed in earlier studies was probably an artifact. However, since the successful reports on pollen-mediated

gene transfer using ultrasonication leading to transgenic plant development in some of the crop plants like *Zea mays* (Wang et al. 2001), *Sorghum bicolor* (Wang et al. 2007) and *Brassica juncea* (Wang et al. 2008), there is renewed interest in pollen-mediated transformation.

Pollen transformation is conducted on the basis of the assumption that DNA uptake by pollen will lead to integration of transgene into the germline with subsequent transmission to the progeny. Mature pollen grain contains a vegetative cell and two sperm cells. Since only one of the

sperm cells fuse with the egg cell giving rise to the zygote and the second sperm cell fuses with the central cell to produce the endosperm (Berger et al. 2008), the introduction of DNA into the sperm cell, destined to fuse with the egg cell is important in determining the development of transgenic seed, while DNA transferred to vegetative cell and to the second sperm cell involved in triple fusion to produce endosperm will not produce transgenic plants, with subsequent transmission of the trait to the progeny. Hence, it will be ideal to introduce the transgene into the microspore when it is uninucleate, so that the transgene is passed on to both the generative cells. Touraev et al. (1997) pioneered *in vitro* culture of unicellular microspores of tobacco followed by introduction of transgene and subsequent *in vitro* maturation of microspores, which were finally used for pollination, fertilization and development of transgenic plants. However, frequency of transformation was low in their experiments. Aziz and Machray (2003) optimized tobacco microspore transformation by biolistics to achieve very high transformation frequency in the progeny. Stable transformants with low copy number were produced using this procedure. The technique of *in vitro* maturation of unicellular microspores after introduction of transgene has to be standardized in other important crops, so as to use this technique as a routine protocol for transformation. Exine-detached microspores of tobacco were shown to have enhanced expression of uid A gene (Shi et al. 1996). However, maturation of these microspores has to be standardized before further use in pollination and fertilization.

Improving pollen grain-mediated gene transfer

Electroporation of pollen grains (Matthews et al. 1990; Abdul-Baki et al. 1990) have been used to introduce DNA into pollen grains and development of transgenic plants containing genes encoding B-glucuronidase and chloramphenicol acetyl transferase has been demonstrated (Smith et al. 1994). Transgenic plants have also been obtained by pollinating pollen grains after vacuum infiltration with *Agrobacterium tumefaciens* in *Petunia hybrida* (Tjokrukusumo et al. 2000). Ultrasonication was used to introduce plasmid DNA into maize pollen grains, which were subsequently used for pollination of flowers and transgenic maize plants developed (Wang et al. 2001) (Fig. 1). Sonication-assisted introduction of aroAM1 gene using glyphosate as a selectable agent in microspores was successfully used to develop transgenic plants in *Brassica juncea* (Wang et al. 2008). In their experimental protocol, 0.1 g of fresh pollen grains were collected in the morning during anthesis and dispersed in 15 ml of 0.3 M sucrose solution. The solution was treated with sonication in presence of 1 mg/ml plasmid DNA. They used a sonication intensity of 300 w and four sonications,

each lasting 10 s at intervals of 5 s, followed by pollination of pollen on stigma of emasculated or male sterile flowers (Fig. 1). Sonication is believed to act by cavitation phenomena. The hydrostatic pressure imposed on the pollen membrane may help in exchange of solutes from the medium to the cytoplasm (Muorten and Janne 1990). Sonication was shown to enhance *Agrobacterium* mediated transformation in somatic tissues of different plants (Trick and John 1997) and may also help in the entry of T-DNA into pollen grains. DNA can also be directly introduced into pollen by incubation and subjecting them to sonication (Wang et al. 2007, 2008). Pollen transformation, if standardized will become a routine technique in conventional plant breeding. After fertilization and seed development, selection of transgenics can be done by addition of a strong dominant selectable marker chemical in the seed germination medium. In conventional plant breeding, hybridization of plants is a normal procedure and the technique of pollen transformation and subsequent *in situ* pollination to produce seeds can become a routine and rapid plant breeding technique to develop new cultivars with alien genes. Table 1 shows a few selected examples of pollen-mediated transformation in plants. Some advantages and disadvantages of pollen mediated transformation and other transformation methods are shown in Tables 2 and 3 respectively.

Conclusions and future prospects

Angiosperm pollen grains are the products of meiotic division and harbor vegetative and generative cells. Generative cell produces two sperm cells, one of which fuses with the egg cell to produce the zygote and the second sperm cell fuses with central cell to produce the endosperm.

Table 2 Advantages and disadvantages of pollen mediated transformation

Advantages	
1	Fast and regeneration independent method
2	Not prone to chimerism
3	Avoids somaclonal variation
4	Genotype independent
5	Cheap method
6	No need for skilled workers
7	No need of <i>in vitro</i> conditions
8	Can be routinely employed
9	Availability of large number of pollen grains
10	Can be used by plant breeders
Disadvantages	
1	Not demonstrated in many species
2	Genes have to be introduced into pollen at correct stage
3	Frequency of transformation is low

Table 3 Advantages and disadvantages of different methods of transformation

No.	Method	Advantage	Disadvantage
1	<i>Agrobacterium</i> -mediated transformation of protoplasts	No chimeras	Protoplast regeneration is essential. Tedious procedure
2	<i>Agrobacterium</i> -mediated transformation of explants	Easy	Chimeras, somaclonal variation, <i>in vitro</i> culture necessary
3	Floral dip method with <i>Agrobacterium</i>	Easy, No need of tissue culture	Demonstrated only in a few species
4	Pistil transformation	Easy, No tissue culture	Demonstrated only in a few species
5	Particle gun bombardment using explants	Genotype independent	Costly, somaclonal variation, chimeras, complex DNA integration pattern
6	Seedling transformation	Easy, No tissue culture	Not demonstrated in all species
7	Microinfection into protoplasts	No chimeras	Tedious, Time consuming, requirement for skilled personnel
8	Macroinfection		Tedious, Time consuming, requirement for skilled personnel, Chimeras
9	Liposome mediated DNA transfer into protoplasts	No chimeras	Tedious, requirement for regeneration from protoplasts, chimeras, somaclonal variation
10	PEG mediated DNA transfer to protoplasts	No chimeras	Tedious, requirement for regeneration from protoplasts, chimeras, somaclonal variation
11	Electroporation of protoplasts	No chimeras	Tedious, requirement for regeneration from protoplasts, chimeras, somaclonal variation
12	Laser-mediated DNA transfer		Needs highly skilled personnel, Not demonstrated in different plants, costly
13	Ca- phosphate for DNA transfer to protoplasts	No chimeras	Tedious, protoplast regeneration needed
14	Silicon fibre mediated	Easy	Toxic to cells
15	Electrophoretic DNA transfer		Not demonstrated in different plants

Our current knowledge of pollen development, although has expanded in the last decade (McCormick 2004; Russell et al. 2010) is still very fragmentary and discrete. In the last decade, more and more emphasis has been placed on model systems such as *Arabidopsis* to understand the development of pollen grain and events involved in fertilization. Availability of *Arabidopsis* genome sequencing has dramatically accelerated genetic research on pollen biology and has helped in designing new experimental strategies. Borges et al. (2008) developed a method to isolate *Arabidopsis* sperm cells by fluorescence-activated cell sorting, allowing Gene Chip analysis of their transcriptome at a genome wide level. They identified several candidate genes involved in sperm cell development. If the foreign DNA can be specifically targeted to sperm cells using new experimental strategies, frequency of pollen transformation can be enhanced, which will help in developing transgenic plants at a higher frequency. However, the translation of *Arabidopsis* genomics and proteomics for effective utilization in pollen transformation is yet to be achieved.

If high frequency pollen transformation can be developed, it will be an alternate and easy method for developing transgenic plants. Hence pollen grains are ideal targets for introduction of foreign genes. Both *Agrobacterium* and direct DNA transfer methods can be

employed for pollen transformation. To improve particle gun bombardment mediated pollen transformation efficiency, magnetic particles can be tried (Kakuta 2003). Since mature pollen grains contain three cells, of which only one of them is involved in fertilization to produce the zygote, it is perhaps ideal to introduce the transgene at the unicellular stage of the microspore and later allow their maturation with suitable techniques.

Since the mature pollen grains are covered by plasma membrane, intine and exine, for successful introduction of transgenes, the DNA has to cross all the three barriers and also withstand the chemicals present in the pollen coat. The observation that lasers can create perforations in plant cells (Wehner et al. 2007; Schinkel et al. 2008) can perhaps be tested out for laser-mediated transgene introduction into pollen grains. Similarly, whisker supersonic method (Khalafalla et al. 2006), use of silicon-carbide whisker mediated method (Matsushita et al. 1999) and aluminium borate whisker-mediated method (Mizuno et al. 2004, 2005), which are tested out in somatic tissues for transgenic plant production can also be tried out for introduction of genes into pollen grains. Use of mesoporous silica nanoparticles to deliver DNA and chemicals into plants (Torney et al. 2007) can also be tested for pollen transformation. The recent reports on use of ultrasonication to introduce

transgenes into *B. juncea* (Wang et al. 2008) and Sorghum bicolor (Wang et al. 2007) has renewed the interest for transferring genes into pollen grains.

Minimization or elimination of tissue culture and regeneration steps and developing transgenic seeds directly by using treated pollen grains for fertilization will bring down the cost and plant breeders can use the technique for developing novel genotypes with ease. However, methods have to be standardized to obtain reproducible pollen transformation. It can perhaps be extended to crops such as maize, rice and pearl millet where male sterile lines are available thus avoiding the tedious task of emasculation.

Two decades back, transformation of ‘cereals’ was considered very difficult (Potrykus 1990b), but today it is a routine technique. Although scientists were pondering over pollen transformation for a long time without success, it can possibly become a successful technique in future if right efforts are made. The inheritance and stability of transgene using pollen transformation also need to be demonstrated. However, like any new procedure, pollen transformation should be reproducible for the benefit to reach the plant transgenic community and finally the end users.

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