REVIEW

Problems and possibilities of monocot transformation

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Abstract

Biotechnological improvement of monocots is often hampered by the lack of efficient regeneration systems, requisite wound responses and low cell competence. Despite these limitations, the biolistic and *Agrobacterium* methods have been successfully used to produce several transgenic monocots by adjusting the parameters that govern efficient delivery and integration of transgene(s) into plant genome. It is now possible to transform even difficult monocots using tailor-made gene constructs and promoters, suitable *A. tumefaciens* strains and a proper understanding of the entire process. This success has been reviewed in the present article and a special emphasis was laid on the measures that were taken in overcoming the difficulties that arise due to the differential responses of monocots and dicots. This information is necessary for biotechnological improvement of still newer monocotyledonous plants that have been hitherto difficult to transform.

Additional key words: *Agrobacterium*, microprojectile bombardment, tailor-made gene constructs.

Introduction

Agrobacterium mediated transformation of dicotyledonous plants is well established and a variety of transgenic plants catering to different usages have been produced till date. Monocots on the other hand, are not the natural hosts of *A. tumefaciens* (De Cleene and De Ley 1976). Therefore, until the recent years *Agrobacterium* mediated transformation of monocotyledonous plants was extremely difficult, and reliable transformation methods were absent. Naturally, only the direct delivery methods were used for monocots, and the first transgenic rice, maize and wheat were produced. Progressively, with better understanding of the process of monocot transformation, and availability of superior constructs, strains and vectors, the *Agrobacterium* mediated transformation method gained popularity, and a large number of transgenic monocots were produced. The different problems and the possibilities that were reported to govern successful transformation of monocots are reviewed in the present article.

Advancements in monocot transformation

Direct delivery methods: Different direct delivery methods ranging from polyethylene glycol (PEG) treatment of isolated protoplasts (Potrykus *et al*. 1985) to physical procedures like electroporation (Rhodes *et al*. 1988, D'Halluin *et al.* 1992), microinjection (Neuhaus *et al.* 1987), silicon carbide fiber (Kaeppler *et al.* 1992) and particle bombardment have been used by different workers. These methods are based on the delivery and integration of foreign genes into proliferative and regenerable protoplasts or cells, and their subsequent expression. By the late eighties, the first transgenic maize was produced by electroporation and PEG treatment (Rhodes *et al.* 1988) and the strategies continued to remain the principle methods of monocot transformation

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Abbreviations: FISH - fluorescence *in situ* hybridization; NLS - nuclear localization signal; NSE - nuclear signal E1; PEG - polyethylene glycol; vir - virulence.

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Table 1. Methods employed for genetic transformation of monocots.

Plant	Method	Explant	Reference
Wheat	particle bombardment	embryogenic calli	Vasil *et al.* (1992)
Wheat	particle bombardment	scutellar tissue	Becker *et al.* (1994)
Tall fescue	particle bombardment	embryogenic suspension cells	Spangenberg *et al.* (1995a)
Perennial ryegrass	particle bombardment	embryogenic suspension cells	Spangenberg *et al.* (1995b)
Barley	particle bombardment	green tissue	Cho *et al.* (1998)
Rice	particle bombardment	seed derived explant	Valdez *et al.* (1998)
Oat	particle bombardment	green tissue	Cho *et al.* (1999)
Perennial ryegrass	particle bombardment	embryogenic suspension cells	Dalton *et al.* (1999)
Italian ryegrass	particle bombardment	embryogenic suspension cells	Dalton *et al.* (1999)
Wheat	particle bombardment	green tissue	Kim *et al.* (1999)
Oat	particle bombardment	shoot meristematic culture	Zhang *et al.* (1999)
Barley	particle bombardment	shoot meristematic culture	Zhang *et al.* (1999)
Red fescue	particle bombardment	green tissue	Cho *et al.* (2000)
Tall fescue	particle bombardment	green tissue	Cho *et al.* (2000)
Orchardgrass	particle bombardment	green tissue	Cho *et al.* (2001)
Maize	particle bombardment	type 1 calli	Wright *et al.* (2001)
Blue grama grass	particle bombardment	embryogenic cells	Aguado-Santacruz *et al.* (2002)
Bahiagrass	particle bombardment	embryogenic calli	Smith *et al.* (2002)
Rice	particle bombardment	scutellum derived calli	Martinez Trujillo *et al.* (2003)
Rye	particle bombardment	calli	Popelka *et al.* (2003)
Rice	PEG	protoplasts	Shimamoto *et al.* (1989)
Rice	PEG	protoplasts	Datta *et al.* (1990)
Rice	PEG	protoplasts	Hayashimoto *et al.* (1990)
Rice	electroporation	protoplasts	Zhang *et al.* (1988)
Rice	electroporation	immature zygotic embryos	Christou *et al.* (1991)
Maize	electroporation	immature embryos	Songstad *et al.* (1993)
Rice	electroporation	mature embryos	Xu and Li (1994)
Wheat	electroporation	scutellum	He *et al.* (1994)
Maize	silicon carbide whiskers	embryonic callus	Frame *et al.* (1994)
Maize	silicon carbide whiskers	type 2 calli	Petolino *et al.* (2000)
Rice	*A. tumefaciens*	immature embryos	Chan *et al.* (1993)
Maize	*A. tumefaciens*	immature embryos	Ishida *et al.* (1996)
Wheat	*A. tumefaciens*	immature embryos, embryogenic calli	Cheng *et al.* (1997)
Rice	*A. tumefaciens*	suspension cells	Urushibara *et al.* (2001)
Wheat	*A. tumefaciens*	suspension cells	Weir *et al.* (2001)
Tall fescue	*A. tumefaciens*	embryogenic calli	Dong and Qu (2005)
Wheat	*A. tumefaciens*	immature embryos	Wu *et al.* (2008)
Tall fescue	*A. tumefaciens*	embryogenic calli	Dong *et al.* (2008)
Zoysiagrass	*A. tumefaciens*	organogenic type 3 calli	Toyama *et al.* (unpubl.)
Rice	*A. tumefaciens*	callus	Mahmood *et al.* (2009)
Rice	*A. tumefaciens*	callus	Black and Jung (2010)
Rice	*A. tumefaciens*	callus	Shah and Veluthambi (2010)

until 1990. Although other methods of direct DNA transfer gained momentum with time, nearly all genetically engineered monocots were produced through the use of the particle gun technology only. The particle gun bombardment was particularly preferred over other methods (Table 1) because transgene(s) could be directly delivered into a wide range of cellular compartments, cell types and plant species without affecting their regeneration ability. This method also ensured the transfer of exogenous DNA into the nuclear as well as chloroplast genome of several monocotyledonous species (Toriyama *et al.* 1988, Datta *et al.* 1990, Fromm *et al.* 1990, Gordon-Kamm *et al.* 1990, Svab *et al.* 1990, Christou *et al.* 1991, Carrer *et al.* 1993).

Agrobacterium **mediated transformation:** The use of *Agrobacterium* for genetic transformation mostly facilitates stable integration of a single copy of transgene in plant genome with little or no rearrangement. Hence this method is considered to be associated with far fewer problems like transgene instability, gene silencing and/or co-suppression (Koncz *et al.* 1994, Hansen *et al.* 1997). This highly replicative single-cell transformation system is also useful in avoiding mosaicism. Mosaic plants or chimaeras are more frequent when intact organs are transformed by direct methods (Enriquez-Obregon *et al.* 1997, 1998). However, the initial developments in *Agrobacterium* mediated genetic transformation of monocots were rather slow. The first breakthrough came

when some monocot species were shown to be susceptible to *Agrobacterium* infection under natural conditions (De Cleene and De Ley 1976). However, it was not until 1993, *i.e*., ten years after the first dicot crops were transformed (Barton *et al.* 1983) that transgenic rice plants were produced from immature embryos infected with *Agrobacterium* (Chan *et al.* 1993). *Agro*-infection of other cereals and grass species was demonstrated only in the early nineties (Grimsley 1990, Potrykus 1990). With time, many more transgenic monocots were produced by this method.

The early proofs of transformation and T-DNA transfer into monocot cells comprised of tumor-growth on hormone-free medium and opine production from the wound-sites of plant tissues. Based on the presence of nopaline and agrocinopine in the large tumorous outgrowths of *Agro*-infected *Asparagus officinalis* tissues on hormone free medium, Hernalsteens *et al*. (1984) reported stable T-DNA transfer and expression. Since the levels of nopaline and agrocinopine continued to remain persistently constant in the established callus cultures, stable rather than transient expression of opine synthesis genes was considered. Graves and Goldman (1986; 1987) also reported stable transformation of gladiolus cells on the basis of octopine and nopaline synthesizing enzyme activities. However, Christou *et al*. (1986) cautioned that opine production following *Agrobacterium* infection did not always provide a full-proof evidence for T-DNA transfer and integration. This is because the arginine metabolism in un-infected calli and plant tissues can also result in opine production. It was only in 1990, that Prinsen *et al*. provided the first molecular evidence for *onc* gene expression in transformed *Asparagus officinalis* tissues. Later, the presence of T-DNA was also shown in a number of monocotyledonous species.

Suitability of *A. tumefaciens* as a transformation vector for monocots: The suitability of *A. tumefaciens* as a vector for monocotyledon transformation was a highly debated topic until Hooykaas van Slogteren *et al.* (1984) reported transgene expression in *Asparagus*. Seven crucial steps govern *Agrobacterium* infection of plant tissues, *i.e*., cell-cell recognition, signal transduction, transcriptional activation, conjugal DNA metabolism, intercellular transport, nuclear import and T-DNA integration.

Cell-cell recognition: Attachment of *Agrobacterium* to plant cell surface is the first crucial step for tumor initiation (Lippincott and Lippincott 1969, Lippincott *et al.* 1977). Besides plant and bacterial receptors, the products of *Agrobacterium*'s chromosomally encoded genes are required for efficient attachment. Although nothing much is known about monocots, Lippincott and Lippincott (1978) assumed that *Agrobacterium* fails to attach to monocot cells due to the lack of receptor sites. However, when *A. tumefaciens* was found to attach to oat and maize cells in a low frequency, the possibility of attachment to certain monocots but not to others was considered. Receptors for binding (Graves *et al.* 1988) as well as attachment of *Agrobacterium* to different plant species like bamboo cells in suspension (Douglas *et al.* 1985), *Asparagus officinalis* (Draper *et al.* 1983), *Zea mays, Gladiolus* sp. and *Triticum aestivum* (Graves *et al.* 1988) were reported. Although the attachment to dicot or monocot tissues was indistinguishable (Ashby *et al*. 1988), the number of bacterial cells that finally attached were variable. Host factors like age, stage, physiological type of the explants, and also the strain of *A. tumefaciens* used for infection were reported to be the governing factors (Graves *et al.* 1988, Karami *et al.* 2009). While the *A. tumefaciens* strains A66 and T37 attached efficiently to the vascular tissues of wheat, gladiolus and maize (Graves *et al.* 1988), bamboo cell suspension cultures were more susceptible to the strain A723.

The chemotactic movement of *Agrobacterium* towards the *vir* inducers was also a much debated topic for quite some time. Both monocots and dicots were shown to exude chemoattractants that were equally potent for the *A. tumefaciens* strain, C58C. While the importance of Ti plasmid was reported by Ashby *et al*. (1987) and Shaw *et al*. (1988), plasmid independent attachment was also considered (Mooney and Goodwin 1991). Different evidence indicated that bacterial recognition of susceptible hosts, chemotaxis and subsequent attachment were not the limiting steps in *Agrobacterium* mediated transformation of monocots. Tumor initiation in monocots was probably blocked at a much later point. The effect of monocot genome on the T-DNA transfer was also not overruled (Douglas *et al.* 1985).

Signal transduction and transcriptional activation: After attachment, the *vir* gene induction, signal transduction and transcriptional activation pathways govern the *Agrobacterium* infection of both dicots and monocots, and are largely similar (Usami *et al*. 1988). An intact *vir*A locus is an absolute requirement for these steps (Grimsley *et al.* 1989) and mutations result in variable responses (Shen *et al*. 1993). Thus, while mutated *vir*A and *vir*G failed to transform rice roots, mutated *vir*B eliminated the transient expression in immature embryos of maize. The mutated *vir*C1 or *vir*C2 on the other hand, reduced the infection of *Zea mays* drastically. It was also noticed that *vir*A and not *vir*B locus was necessary for widening the host range of *Agrobacterium*.

Depending upon the number of copies present in the monocot genome, *vir*A and *vir*G genes performed multifunctional roles (Shaw *et al.* 1988). While low number of copies of *vir*A gene induced chemotaxis in monocots, higher number of copies was required for the induction of the entire *vir* locus (Ashby *et al.* 1988). Copy number also changed the *vir* gene expression in response to pH, temperature, radiation, *etc*. (Alt-Morbe *et al.* 1989, Turk *et al.* 1991). Thus, the pH dependent induction of *vir*G was partially relieved when the copies of *vir*A and *vir*G increased in number (Turk *et al*. 1991). Multiple copies of *vir*G in rice tissue also enhanced the transgene expression by several fold (Vain *et al*. 2004).

Intercellular transport and nuclear import: As in dicots, the processes involved in cellular transport and nuclear import govern the transfer of T-DNA into the monocot genome (Wei *et al.* 2000). That the nuclear import step is not limiting in *Agrobacterium*-monocot interactions was obvious from the findings of Sheng and Citovsky (1996). Their earlier studies with gus:VirE2 and gus:VirD2 fusions had clearly shown the accumulation of both VirE2 and VirD2 proteins in the nuclei of maize and tobacco leaves (Citovsky *et al.* 1994). However, the level of accumulation was different in the two plants. While the accumulation of both VirD2 and VirE2 was quantitatively similar in tobacco nuclei, the nuclear import of VirD2 was more than VirE2 in maize. Actually, the nuclear localization signal (NSL) of VirE2 and VirD2 proteins regulate the import of T-complex into the dicot and monocot nuclei depending on the developmental stage of the plant/explants (Binns and Thomashow 1988, Citovsky *et al.* 1994). However, in case of tobacco protoplasts, both the nuclear signals of VirE2 (NSE 1 and NSE 2) functioned independently, whereas, only NSE 1 was independently active in maize leaves.

T-DNA integration: According to Binns and Thomashow (1988) and Narasimhulu *et al.* (1996), major blocks prevent the normal integration of T-DNA into the genome of maize and other monocots. However, studies on transgenic *Asparagus* tissues revealed that the mechanism of T-DNA integration in monocots is similar to that in dicots (Bytebier *et al.* 1987). Molecular analysis of another monocot, *i.e.*, transformed *Dioscorea* also confirmed this observation and described the structure of T-DNA during its integration into a monocot genome. As in several dicots, two full length copies of wild type T-DNA and an additional copy of truncated T-DNA with different integration sites formed nopaline producing crown gall tumors (Schafer *et al.* 1987). As generally observed in dicots, different transgene integration patterns led to variable expression in the segregated progenies of maize and rice (Gould *et al*. 1991 and Hiei *et al*. 1994). During the early twenties, studies mainly focused on the transgene integration patterns in rice (Kim *et al*. 2003). Thus, in molecular analysis of the junctions of T-DNA borders and plant DNA in 20 transgenic lines from three rice cultivars, single non-rearranged inserts were observed in two lines only (Azhakanandam *et al*. 2000). While Yin and Wang (2000) reported truncated T-DNA inserts in 14 % of the rice transformants, Dong *et al*. (2001) reported rearranged, truncated and variable copies of transgenes in 18 lines. Co-localization of a single copy of transgene with a satellite DNA at the distal end of the metaphase chromosome was also observed in transgenic *Allium cepa* analyzed with tyramide-FISH (Khrustaleva and Kik 2001). Despite these studies, the actual mechanism of transgene integration into monocot genome is still not clear.

Why are monocots more difficult to transform?

The reasons that have been propounded till date for the difficulties encountered in *Agrobacterium* mediated transformation of monocots include chemotaxis, attachment, Ti plasmid mediated T-DNA transfer and integration, wound response and differences in cellular structures (Fig. 1*A*,*B*).

Anatomical differences: The basic anatomy has been implicated to govern the monocot response to *Agrobacterium* infection. It is believed that T-DNA fails to target the specific meristematic cells that are competent to dedifferentiate in monocots. Adding to the problem, the monocot cells unlike the dicots lose the ability to dedifferentiate at a very early stage of development (Graves *et al*. 1988).

Differences in cell wall chemistry between dicots and monocots, especially, the members of *Poaceae* are thought to govern the success of *Agrobacterium* infection. While the dicot cell wall is composed of β-linked glucose residues with interlocking chains of β-D-xyloglucans, the glucuronoarabinoxylans and linear chains of β-*D*-xylose characterize the interlocking polysaccharides in grasses (Carpita 1996). Instead of hydroxyproline-rich extensions that accumulate in the dicot cell walls, threonine-rich proteins with sequences reminiscent of extension are observed during cellular differentiation in *Poaceae* (Kieliszewski *et al.* 1990, Xing *et al.* 2009).

Meristematic cell types have been reported to affect the attachment as well as *vir* gene inducing steps in monocots (Hernalsteens *et al*. 1984, Grimsley *et al*. 1988, Raineri *et al*. 1990, Gould *et al*. 1991, Chan *et al*. 1992, 1993, Delbreil *et al*. 1993). It is also believed that these cells fail to exude the *vir* gene inducing compounds in monocots.

Wound response: Wounds are the portals of bacterial attachment and transformation-facilitating-processes (Braun 1952, Baron and Zambryski 1995). Besides exuding compounds like phenolics, flavonoids and sugars, these sites transduce and regulate multiple signals for the induction of *vir* genes (Messens *et al.* 1990). Despite successful *vir* gene induction in its absence (Brencic *et al*. 2005), wound response is considered to be a major factor governing *Agrobacterium* mediated transformation of monocots. Extremely weak wound response (Hiei *et al*. 1997) and absence or low levels of *vir*-inducing exudates probably hamper *Agrobacterium* mediated transformation of monocots (Hooykaas 1989). Moreover, wounding in monocots is not always followed by extensive cell divisions. Rather the wounded monocot tissues differentiate into a lignified or sclerified ring of hardened cells that quickly seal the plant's wound-site from invading *Agrobacterium* (Kahl 1982, Mahalakshmi

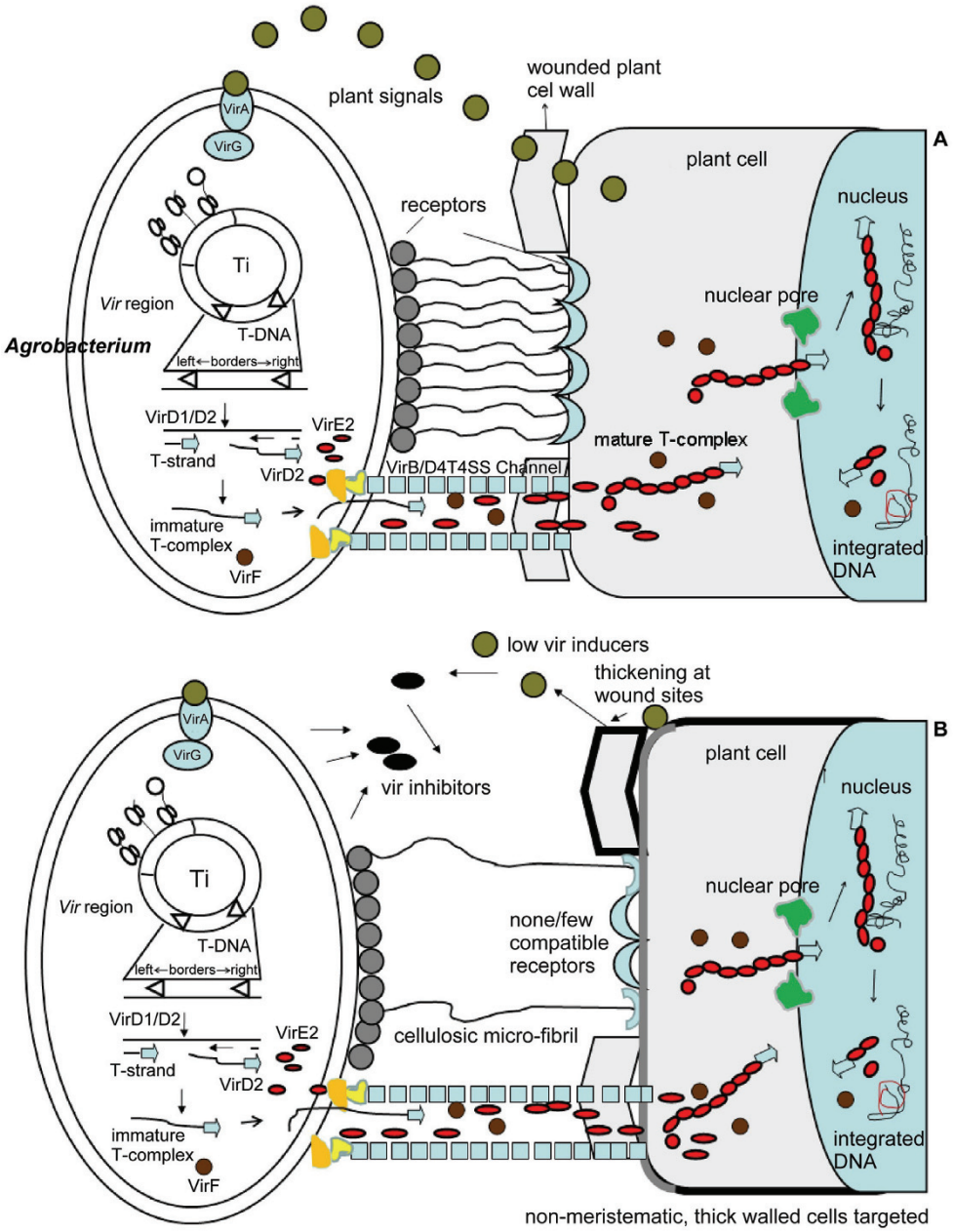

Fig. 1. *A* - Schematic diagram showing *Agrobacterium* infection of a dicot cell adapted from Sheng and Citovsky (1996). Optimal amounts of inducers secreted from wound sites promote chemotaxis, T-DNA transfer and integration followed by extensive cell division of transformed cells/sectors. Generally rapidly dividing healthy meristematic cells are targeted thereby making the transformation process a successful event. *B* - Schematic diagram showing blocks in *Agrobacterium* infection of a monocot cell. Very few or no inducers along with inhibitors are secreted from wound sites thereby, inhibiting chemotaxis, T-DNA transfer and integration. Sclerification of wound sites prevent further cell division and cordons off the transformed sector. Ability to de-differentiate is lost very early, hence the transformed cells do not multiply.

and Khurana 1997). This rapid differentiation of wound sites in monocots leaves only a few cells marginally competent for either plant regeneration or transformation or both. The actual number of cells receiving the T-DNA is also critically low (Graves *et al.* 1988). In contrast, wounding in dicots converts potentially competent cells to actually competent ones, and a sector of competent cells is generally created (Binns 1990).

The *vir* gene induction: Earlier it was believed that monocotyledons, in particular grasses, did not produce *vir* inducing compounds (Usami *et al*. (1987) or if they did, the levels were extremely insufficient for *vir* induction (Smith and Hood 1995). However, it was later shown that monocots do produce *vir* inducing compounds (Usami

et al. 1988, Messens *et al.* 1990, Wang and Fang 1998), and mixtures, rather than individual compounds had stronger activity (Xu *et al.* 1989). Although more than 40 phenolic as well as non-phenolic compounds like galacturonic, glucuronic and arabonic acids have been identified, only acetosyringone emerged as an essential component of transformation of rice (Hiei *et al.* 1994), wheat, barley (Guo *et al.* 1998) and maize (Ishida *et al.* 1996), *etc*. Monocot inducer molecules differ widely from the dicots and also amongst the monocot species. For example, the *vir* inducer from *Triticum aestivum* is a hydrophilic high molecular mass compound (Usami *et al.* 1988), whereas, that from *Triticum monococcum* suspension cells is a low molecular mass ethyl ferulate, a compound more potent than acetosyringone (Messens *et al.* 1990). Since both inducers and inhibitors of *vir* genes are present in monocots, it is important to remove or suppress the inhibitors while inducing a strong expression of the *vir* genes by the use of proper compounds. Mismatched receptor protein is another important factor responsible for the incompatibility of *Agrobacterium* and monocots and the importance of *vir*A for maize transformation was demonstrated (Raineri *et al*. 1993). Moreover, while several monocots exhibit specific preferences for some Ti plasmids only, dicots do not do so (Grimsley *et al*. 1986). Even certain active onco-genesis genes like the *onc* gene 1 were found to impose a lethal effect in the tumor tissue of some monocots (Prinsen *et al*. 1990).

Growth regulators: Although phytohormones mediate autotrophic growth in dicot tumors, for quite some time, monocots were considered incapable of responding to either auxins and/or cytokinins in the culture medium. Despite the presence of PGRs, *A. tumefaciens* infected monocot tissues were generally un-amenable to de-differentiation, and tumors would not form. All attempts to culture callus from stem and leaf sections also met with minimal success.

Also certain plant growth regulators or secondary metabolites have been reported to inhibit the process of *vir* gene induction. A heat labile, bacteriostatic compound from maize (Sahi *et al*. 1990), indole-3-acetic acid (Liu and Nester 2006) and 2-hydroxy-4,7-dimethoxy-benzoxazin (MDIBOA) from the roots of maize seedlings (Zhang *et al.* 2000, Maresh *et al*. 2006, Karami *et al.* 2009) are examples of such plant chemicals.

Methylation: Transgene inactivation due to methylation of T-DNA sequences (Matzke *et al.* 1989, Matzke and Matzke 1991) is supposedly much higher in monocots (Prinsen *et al.* 1990). This probably accounts for the resistance of monocots to *Agrobacterium* mediated transformation. Transgene integration into methylated sites within the genome or promoter region of the transgene(s) and also varying levels of methylation are responsible for reduced transformation efficiency in some monocots (Bussingler *et al.* 1983, Klein *et al*. 1990).

Factors affecting monocot transformation

The success of transformation either by *Agrobacterium* or biolistic depends to a great extent on the age and physiological status of the explant (Birch 1997). The few competent cells that finally receive the transgene should have the ability to quickly recover from the shock imposed by the transformation method, and also proliferate and regenerate into complete plants. However, the recovery of fertile plants from transformed monocot explants is extremely difficult. Therefore, for quite some time, almost all work on monocot transformation focused mainly on the optimization of factors governing plant regeneration (Chen *et al.* 1988, Toriyama *et al.* 1988, Zhang and Wu 1988, Zhang *et al.* 1988, Klein *et al.* 1989, Datta *et al.* 1990, Fromm *et al.* 1990, Gordon-Kamm *et al.* 1990, Christou *et al.* 1991, Potrykus 1991, Cao *et al.* 1992, Li *et al.* 1993, Hiei *et al.* 1994, Rancé *et al.* 1994, Tian *et al.* 1994, Xu and Li 1994, Zhang 1995, Sivamani *et al.* 1996, Zhang *et al.* 1996, Aulinger *et al.* 2003, Shahzad *et al*. 2009).

Rapidly dividing meristematic tissues of maize, wheat and other cereals were found to be more susceptible to *Agrobacterium* infection (Hernalsteens *et al.* 1984, Graves and Goldman 1986, Woolston *et al.* 1988, Chen and Dale 1992, Li *et al.* 1992, Vijaychandra *et al.* 1995). In general, highly embryogenic genotypes were preferred, yet, explants rather than genotype was important for agroinfection of maize (Boulton *et al*. 1989). Despite a high cell division index, some tissues/cells had poor regeneration and transformation potential (Park *et al.* 1996, Hiei *et al.* 1997). Even these could be transformed through extensive optimization experiments (Chan *et al.* 1993, Hiei *et al.* 1994, Aldemita and Hodges 1996). For example, the frequency of tissue recovery was improved by osmotic treatment as it suppressed *Agrobacterium* overgrowth on explant surfaces. Species-dependent osmotic pre-treatment or medium with sugars and other agents also increased the cell competency of rice and maize explants quite effectively (Hiei *et al.* 1994, Ye *et al.* 2000, Lucca *et al.* 2001, Zhao *et al.* 2001, Frame *et al.* 2002). Although immature embryos of wheat failed to respond to such treatments (Uze *et al.* 1997, 2000, Cheng *et al.* 2003), pre-culturing of immature embryos and embryogenic calli improved the transformation efficiency in several other plants (Dong *et al.* 1996, Rashid *et al.* 1996, Cheng *et al.* 1997). Even the replacement of solid with liquid media, and 'pre and/or post transformation' desiccation of explants improved the transformation efficiency of sugarcane, wheat, rice and maize by several folds (Hiei *et al.* 1994, 1997, Arencibia *et al.* 1998, Urushibara *et al.* 2001, Cheng *et al.* 2003). The strain of *Agrobacterium* and its ability to produce opines was another important factor governing monocot

transformation (Hooykaas van Slogteren *et al.* 1984, Chibbar *et al.* 1993). Thus, strains like octopine producing LBA1010 and 1023, nopaline producing LBA2318 and 2347, and the opine non-producing, avirulent strain LBA288 that did not produce opines were tested. Based on their monosaccharide binding proteins or *Chv*E factors (Heath *et al.* 1997), *vir* gene induction potential and ability to utilize opines (Raineri *et al.* 1993, Shen *et al.* 1993, Hansen *et al.* 1994), the nopaline strain, C58C1 was found to be far more superior than the octopine strain LBA4404 in their infectivity. Different monocotyledonous species were also transformed successfully with compatible strains, helper plasmid derivatives and super virulent strain A281 harboring the pTiBo542 plasmid (Jin *et al.* 1987, Ritchie *et al.* 1990, Chan *et al.* 1993, Shen *et al.* 1993). While Jin *et al.* (1987) and Komari (1989) improved the virulence of some strains by extra copies of *vir*B, *vir*C and *vir*G, Cheng *et al.* (1997) and Tingay *et al.* (1997) showed that super virulent strains were not indispensible for monocot transformations.

Till date, significant improvements in transformation efficiency have been brought about by extensive optimization of procedures. For example, *A. tumefaciens* attachment was facilitated by eliminating the protective inhibitory substances and/or waxy cuticle present on explant surfaces (Kumar *et al.* 2004) by chemical agents and surfactants such as *Tween 20*, *Silwet L77* and *Pluronic acid F68* (Cheng *et al.* 1997). Use of an optimal *Agrobacterium* density can also facilitate attachment of finite number of bacteria to probable receptor proteins on the explant surface (Hiei *et al.* 1994). However, the requisite population density varies from plant to plant. Thus, while a density of 1.0×10^{10} colony-forming units (cfu) cm^{-3} was required for rice, and 0.5×10^{10} cfu cm^{-3} for wheat suspension cells, a range of cell densities (1.0×10^{6} and 1.0×10^{10} cfu cm^{-3}) were optimal for other plants (Hiei *et al.* 1994, 1997). Any changes in these optimized densities resulted in a decrease in both transient and stable transformations. A density higher than 1×10^{10} cfu cm^{-3} damaged plant cells, lowered plant cell recovery and reduced stable transformations (Cheng *et al.* 1997, Zhao *et al.* 2000, 2001). However, a short inoculation time was recommended when a higher density of *A. tumefaciens* was absolutely necessary for recalcitrant plants or explants (Kumria *et al.* 2001).

Co-culturing of the target explant and the infecting *Agrobacterium* under *in vitro* conditions is termed as 'co-cultivation'. Since the induction of *vir* genes leading to signal transduction, T-DNA delivery and integration occur during this step, it requires extensive optimization with respect to duration, temperature, irradiance, medium composition and pH. In general, 2 - 3 d of co-cultivation was required for successful transformation of most members of *Gramineae* (Hiei *et al.* 1994, Dong *et al.* 1996, Ishida *et al.* 1996, Rashid *et al.* 1996, Cheng *et al.* 1997). Even periods as long as 5 - 7 d increased the transformation efficiency of *Lilium usitatissimum* and *Agapanthus* explants (Dong and McHughen 1991, Suzuki *et al.* 2001). The optimal co-culture temperatures for most monocots ranged between 23 to 25 °C (Rashid *et al.* 1996, Arencibia *et al.* 1998, Enriquez-Obregon *et al.* 1998, Hashizume *et al.* 1999, Salas *et al.* 2001). Particularly, 22 °C resulted in highest transient β-glucuronidase (GUS) expression (64 % of the total callus) in garlic when 18, 20, 22 and 24 °C were tested (Kondo *et al*. 2000). A number of crops, particularly rice (Dong *et al.* 1996, Enriquez-Obregon *et al.* 1999, Mohanty *et al.* 1999, Lucca *et al.* 2001), maize (Ishida *et al.* 1996), zoysiagrass (Yaxin *et al.* 2006), *etc*. were also successfully transformed by optimization of parameters like medium strength, composition, sugars, plant growth regulators, and *vir* inducing chemicals. Reduction in the salt strength of co-culture and inoculation media was also found to improve the transformation efficiency of wheat (Cheng *et al.* 1997) and maize significantly (Armstrong and Rout 2001, Zhang *et al.* 2003).

Calcium-induced plant defence and resistance to pathogenic microorganisms (Dierk 1998) probably accounted for significantly high *gus* activity in explants of zoysiagrass and rice co-cultivated on $CaCl_2$ free medium (Toyama *et al*. unpublished). However, even in the presence of $CaCl_2$, high transformation efficiency was reported in barley (Kumlehn *et al.* 2006). Sugars, particularly *D*-glucose, *D*-mannose, *D*-galactose, *D*-talose, D-xylose or *L*-arabinose were reported to enhance the acetosyringone-dependent-expression of *vir* genes (Shimoda *et al.* 1990). Even glycinebetaine enhanced the *vir* gene induction by acetosyringone at low pH (Vernade *et al.* 1988). Addition of silver nitrate or thiol compounds to the agar solidified co-cultivation medium also facilitated higher stable transformation in maize and cotyledonary-node cells, respectively (Armstrong and Rout 2001, Olhoft and Somers 2001, Zhao *et al.* 2001, Olhoft *et al.* 2003). In addition to these compounds, Bytebier *et al.* (1987), Domisse *et al.* (1990), Raineri *et al.* (1990), Gould *et al.* (1991), Hiei *et al.* (1994), Philipp *et al*. (1995), Ishida *et al.* (1996) and Zakharchenko *et al.* (1999) reported the use of different phenolic and non-phenolic inducers, inhibitors of *vir* gene repressors and dicot plant extracts for successful transformation of different monocots. For example, potato suspension culture filtrate was a requirement for the transformation of rice (Chan *et al.* 1993). Since most inducers facilitate T-DNA transfer at a very early stage of co-cultivation, their addition to the infecting cultures and/or co-cultivation medium improved the transformation of cereals (Hiei *et al.* 1994, Vijaychandra *et al.* 1995, Aldemita and Hodges 1996, Ishida *et al.* 1996, Cheng *et al.* 1997).

Special care is also required to prevent the loss of transformants due to necrosis and bacterial over-growth in *Agrobacterium* mediated transformations. Thus elimination of residual *Agrobacterium* is important for higher transformant recovery and increased transformation efficiency (Zhao *et al.* 2000, 2001, Cheng *et al.*

2003, Zhang *et al.* 2003). Either a gentle rinsing of explants with fresh inoculation medium (Zhao *et al.* 2001) and/or use of antibiotics such as cefotaxime, carbenicillin, tricarcillin, timentin, *etc*., for suppressing or eliminating the residual *Agrobacterium* enhanced the transformation efficiency of several monocots (Cheng *et al.* 1996, Naureby *et al.* 1997, Bottinger *et al.* 2001). However, the detrimental effects of high concentrations of antibiotics like cefotaxime on explants and reduction in the transformation frequency by several folds was investigated by Ishida *et al.* (1996). While silver nitrate can suppress *Agrobacterium* growth and enhance stable transformation when present in the co-culture medium (Armstrong and Rout 2001, Zhao *et al*. 2001), oxidative-burst reducing antinecrotic mixtures (Enriquez-Obregon *et al.* 1999) impart tolerance to plant tissues against oxidative stresses (Cassells and Curry 2001).

In direct transformations also, a wide range of parameters affect the transformation efficiency. Of these, the pre- and post-transformation treatments play a significant role. Thus, plant recovery and transformation efficiency of embryogenic maize cultures in suspension increased by four fold when treated with 0.2 M each of sorbitol and mannitol for 4 h before and 16 - 20 h after bombardment (Vain *et al*. 1993). Pre and post bombardment osmotic treatment with 0.25 M mannitol in the culture media also increased the transient GUS activity in scutellar calli of wheat by 3- to 4-fold (Perl *et al.* 1992), and the transformation efficiency of rice by 8-fold (Fauquet *et al.* personal communication). An effective 12-h pre-bombardment osmotic treatment of rice callus was also reported (Martinez-Trujillo *et al*. 2003). Plasmolysis or partial drying of tissues by osmotic treatment generally maintains the pressure potential of wounded cells by reducing or preventing cell damage and/or leakage of protoplasm (Finer and McMullen 1991). Transformation efficiency can be also increased by repeated bombardments and optimized microparticle velocity. While two bombardments improved the transient expression in some species (Lonsdale *et al.* 1990), greater tissue damage and reduced expression were observed in others (Kartha *et al.* 1989). The transformation efficiency improved in some monocots when explants were given 4 - 6 d of osmotic treatment prior to bombardment with 700 - 900 kPa of helium pressures and finally 2 - 4 d on antibiotic free culture (O'Kennedy *et al.* 2001). In recent years, Pinghua and Rukai (2004) improved the transformation efficiency of sugarcane with suitable rupture pressure and target distance. Depending upon genotype and species, size and concentration of gold particles are other important factors that govern the transformation efficiency of monocots. Microprojectile size of 0.8 - 1.2 µm was preferred by Klein *et al*. (1988) and Birch and Franks (1991). However, microprojectile size was not important for the transformation of maize coleoptiles (Reggiardo *et al*. 1991). An increase in concentration beyond a specific limit resulted in increased cell damages in embryonic axes of bean and maize cell suspensions and a concomitant decrease in transient gene expression (Klein *et al.* 1988, Aragao *et al.* 1993). Microprojectile agglutination is another problem encountered in monocot transformation. Generally, high concentrations of DNA used for coating the microprojectiles lead to their agglutination and subsequent reduction in transformation efficiencies. Klein *et al.* (1988) and Oard (1991) suggested the use of 2 µg(DNA) mg^{-1}(tungsten) for optimal transient gene expression in maize suspension cultures.

Irrespective of the method of transformation, use of suitable reporter genes, monocot specific promoters (Franks and Birch 1991) and modification of selectable marker genes by insertion of introns into coding regions (Wang *et al*. 1997) are some approaches that have improved the transformation efficiencies of different plants. Modification within *hpt* gene elevated the expression but reduced the copy number in rice and barley cultivars (Simpson and Filipowics 1996, Upadhyaya *et al.* 2000, Wang *et al.* 2001). On the other hand, specific promoters like actin, ubiquitin and α-amylase improved the transformation of different plant species and/or tissue types. In this regard, the effect of the first intron *Ubi1* and/or the promoters and first exon of the maize ubiquitin gene on transgene expression in transformed wheat, maize, *Panicum maximum, Pennisetum glaucum, P. purpureum and Saccharum officinarum* was studied (Taylor and Vasil 1991). GUS expression was significantly enhanced by strong monocot promoters like *Emu* (Last *et al.* 1991) or the maize alcohol dehydrogenase intron 1 inserted between the *35S* promoter and reporter genes (Callis *et al.* 1987, Franks and Birch 1991). Bower and Birch (1992) further suggested the possibility of using weaker promoters, once the transformation and selection conditions were optimized. Constructs with nopaline synthase (*nos*) terminator linked to promoter fusions have also been used extensively (Chibbar *et al.* 1993). Some other popular promoters include those of maize alcohol dehydrogenase (*Adhl*) and the rice actin (*Actl*) genes with their respective first introns, CaMV*35S* or the enhanced *35S* promoters with the maize *Adhl* intron 1 or the maize shrunken locus (*shl*) intron 1. Of these, the rice *Actl* promoter with its first intron yielded the highest expression in barley cells, followed by the *E35S* promoter with *shl* intron 1. However, Reggiardo *et al*. (1991) and Abumhadi *et al*. (2005) reported maximum gene expression in maize cells by the fusion of the maize *Adhl* promoter with its first intron as compared to the use of intact CaMV*35S* promoter alone. A slightly higher gene expression with the intact maize *Adhl* promoter as compared to *Adhl* and CaMV*35S* promoters broken by the *Adhl* intron1was also reported by Bekkaoui *et al.* (1990) and Luehrsen and Walbot (1991). Since the level of gene expression driven by a promoter cannot be generalized, Assem *et al*. (2002) suggested that each plant species should be tested with a set of promoters in the presence and/or absence of introns. Besides promoters, combining features like introns and overdrive sequences in different gene

constructs, and plasmid vectors continue to be important for monocot transformation. In this regard, the isolation, and characterization of novel transcription factors from monocot(s) may be particularly, useful (Gao *et al.* 2009). In recent years, further improvements were achieved through the incorporation of matrix attachment regions (MAR) also called scaffolding attachment regions (SCAR) in the gene construct (Oh *et al.* 2005).

Conclusions

Despite the absence of efficient regeneration systems, requisite wound response and low competence for transformation, it is now possible to transform almost all monocots based on the current knowledge of their response to transformation methods and adjustment of the parameters that govern these responses. In the present review alone, more than forty different transgenic monocots have been listed, and of these, almost half have been produced by the *Agrobacterium* method.

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