BIOTECHNOLOGY AND MOLECULAR-BASED METHODS FOR GENETIC IMPROVEMENT OF TULIPS

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Abstract

Although the conventional methods of improvement have changed significantly throughout the last fifty years, additional tools and novel approaches are needed in order to fasten the process of creation new and highly valuable tulip varieties.

The genetic base of tulip production can be preserved and widen by an integration of biotechnology tools in conventional breeding. Micropropagation *in vitro* may produce very fast large numbers of vigorous plants with high quality and free of endogenous pathogens. The *in vitro* rescue of embryos resulted from interspecific crosses between more or less distant species, chromosome doubling, somaclonal variation, transformation, and marker-aided selection and breeding are just a few of the examples of the applications of biotechnology in tulip improvement. This review provides an overview of the opportunities presented by the integration of plant biotechnology into the tulip improvement efforts.

Keywords: Tulipa sp., tissue culture, ploidy manipulation, embryo rescue, micropropagation, embryogenesis, organogenesis, somaclonal variation, transformation, molecular markers, genetic improvement

1. INTRODUCTION

Tulip is ranked third among the top ten flowers sold worldwide (Podwyszynska and Sochacki, 2010), being extremely popular for landscaping, but also as garden plants and cut flowers. Due to the constant or even increasing interest for this flower species, continuous and consistent efforts have been made to develop improved breeding methods, including biotechnology and molecular methods. Despite all appearances, creating of new tulip varieties is not an easy task. Most often, intra- and interspecific crosses are made in order to generate new combinations of specific growth characteristics, colors, flower longevity and resistance against diseases. The great majority of tulip varieties created along the centuries have derived from only two species, *Tulipa gesneriana* and *T*. suaveolens (Van Tuyl and Van Creij, 2006). Because of the differences in ploidy level and fertilization barriers which hamper breeding, a few other wild species from more than 150 belonging to the Tulipa genus have contributed to gene pool of the tulip world assortment. As estimated by van Scheepen (1996), from more than 3000 varieties registered, the T. gesneriana and so called Darwin hybrids (originating from interspecific crossing of T. gesneriana and T. fosteriana) consist of more than 1100 cultivars. Over the last decades, valuable cultivars derived from intraspecific crosses of T. fosteriana, T. greigii and T. kaufmanniana have also been released and introduced into commercial cultivation. Mutagenesis has been also successfully employed to generate desirable and valuable changes in flower traits (Fig. 1). Many varieties remarkable for their beauty, such as Estella Rijnveld (Fig. 2) revealed that mutagenesis (mainly X radiation) is a powerful tool for the improvement of tulips.

Despite their limitations, conventional methods of breeding are still essential in any strategy for creating new varieties of tulips. Significant progress was achieved in identifying pre- and post-fertilization barriers (Kho and Baer, 1971; Van Tuyl and Lim K.B., 2003), which now can be overcome for obtaining viable hybrids. Also, ploidy manipulations can be applied in order to avoid the infertility of hybrid progenies as result of differences in ploidy level (Van Tuyl et al., 2002). As reported by Kroon (1975), Sen (1977), Sayama et al. (1982), Kroon and Jongerius (1986), Straathof

and Eikelboom (1997), Masoud et al. (2002), and Okazaki et al. (2005), the tulip assortment consists mainly of diploids with 2n = 2x = 24 (Fig. 3), but include also several dozens of triploid varieties (2n = 3x = 36) and a few tetraploid varieties (2n = 4x = 48). It was estimated that about 5% of Darwin hybrids (which are remarkable for their plant vigor and large flower, as well as for their good bulb yield) are triploid (Van Tuyl and Van Creij, 2006).



Figure 1. Flower color mutants in Tulipa gesneriana



Figure 2. Estella Rijnveld tulip variety – mutant derived from the Red Champion variety



Figure 3. Metaphase chromosomes in Tulipa sp. (Masoud et al., 2002)

Breeding of tulips and creating new varieties benefited from advances in manipulating the ploidy, mainly from the use of 2n (unreduced) pollen grains or artificially induced tetraploid plants in controlled crosses with selected diploid cultivars. The method based on the treatment of tulip plants with N₂O at one week after pollination (Zeilinga and Schouten, 1968) have been proven to be very efficient in inducing formation of tetraploids, and therefore highly important within the strategies to create new varieties. The fertility of obtained polyploids can be subsequently improved by reciprocal crossings. This method allowed obtention of highly fertile tetraploid forms of T. gesneriana, T. fosteriana, and T. kaufmanniana, which were released in 1989 and 1991, becoming available for the Dutch companies (Straathof and Eikelboom, 1997). More recently, Okazaki et al. (2005) and Barba-Gonzales et al. (2006) proposed the method of inducing unreduced pollen formation in tulips by treating the bulbs with N2O for 24-48 hours, which was proven to be efficient in generating triploid progenies, despite the fact that very few polyplois were observed in some cross combinations, even those involving pollen with a relatively high proportion of giant grains. However, the long juvenile period remains a major drawback in breeding tulip by hybridization. As emphasized by Wilmink et al. (1995), from seed to a flowering bulb takes 4 to 6 years, and then it takes about 10 years to evaluate the value of a promising hybrid.

2. BIOTECHNOLOGY METHODS FOR GENETIC IMPROVEMENT OF TULIPS

The process of creating and introducing a new tulip variety takes about 25-30 years (Podwyszynska and Marasek, 2003), not only because the time interval from planting o the flowering of the hybrids is long, but also because the period for bulbs propagation until the level of commercial scale is very long (Juodkaite et al., 2008). This is the main explanation for the low rate of replacing varieties within the tulip assortment, as compared to that of lilies or gladiolus. In the last decades, biotechnology methods contributed significantly to the shortening of breeding cycles. Similarly to the other flower species (and, generally, to the other horticultural species), the first application of the biotechnology in tulips was the *in vitro* clonal propagation. Micropropagation may produce very fast large numbers of vigorous plants with high quality and free of endogenous pathogens. It can be achieved by inducing formation of adventitious shoots (organogenesis) from the bulb scales or by somatic embryogenesis. During the last three decades, optimal conditions for the in vitro culture of tulip genotypes and factors influencing the efficiency of plant micropropagation and shoot regeneration have been studied extensively (Riviere and Müller, 1976; Riviere and Müller, 1979; Wright and Alderson, 1980; Wright, 1981; Alderson et al., 1983; Gabryszewska and Saniewski, 1983; Le Nard et al., 1987; Le Nard, 1989; Baker et al., 1990; Taeb and Alderson, 1990; Hulscher et al., 1992; Le Nard and Chanteloube, 1992; Koster, 1993; Wilmink et al., 1995; Podwyszynska et al., 1997; Van Rossum et al., 1997; Van Rossum et al., 1998; Podwyszynska and Rojek., 2000; Podwyszynska, 2001; Podwyszynska and Marasek, 2003; Podwyszynska and Ross, 2003; Ghaffoor et al., 2004; Podwyszynska, 2004; Kalyoncu et al., 2006; Minas, 2007; Podwyszynska and Sochacki, 2010). Not surprisingly, the first attempts of in vitro cultures of tulips have been carried out not in The Netherlands (the kingdom of tulips), but in Japan, this country having also a long and impressive tradition in growing and breeding of Tulipa gesneriana varieties. Actually, Japan is ranked second among the world producers of tulip bulbs (before France, USA, and Poland). The studies carried out by Nishiuchi (1973, 1979, 1980, 1983, 1986, 1990), Nishiuchi and Myodo (1976), and Nishiuchi and Koster (1988) contributed by their results to the understanding of the physiological factors which influence the *in vitro* culture of tulips. They allowed establishment of the influence of some growth regulators on either in vitro plant development and multiplication from bulb scales, or formation of adventitious shoots (by organogenesis) from such type of explants. Alderson et al. (1983) developed an efficient method for regeneration of adventitious shoots from immature flower stems formed from dry bulbs. It was reported that by using this method, the formation of bulblets from such shoots can be induced by the cultivation at 20°C for 14

to 18 weeks, followed by the transfer to 4° C for 8 weeks and subsequent incubation at the temperature of 20°C.

Hulscher et al. (1992) developed a method for the micropropagation of tulips from stem and axillary bud explants cultivated on media containing α -naftylacetic acid (NAA), N⁶-[2-isopentenyl-adenine (2iP) and 6-benzylaminopurine (BAP) or zeatine. The regenerated shoots are able to form bulbs after cold treatment on a medium supplemented with 70 g/l sucrose and lacking growth regulators. Kuijpers and Langens-Gerrits (1997) have reported the regeneration of tulip shoots on a culture medium containing 5µM zeatine and 5µM α -naftylacetic acid, pointing out the significant increase of the rate of meristems formation on stem explants when silver thyosulfate and, especially, paclobutrazol and methyl jasmonate were used. Recently, Podwyszynska and Sochacki (2010) have described a new method of tulip micropropagation, based on cyclic multiplication of shoots at intervals of 2-3 years, using thidiazuron (TDZ) instead of the currently used cytokinins (N⁶-[2izopentenyl-adenine (2iP) and 6-benzylaminopurine). Fragments of flower stems are used as initial explants, and the adventitious shoots regenerated on medium containing TDZ are sub-cultivated at every 8 weeks. Formation of bulblets by the shoots resulted by in vitro multiplication is induced by the cold treatment, followed by cultivation on a sucrose rich medium, at the temperature of 20°C (Fig. 4 and Fig. 5). In order to avoid the risk of mutations (somaclonal variation), it was recommended that the number of multiplication subcultures should not exceed 5-10. This method make possible the production of 500-2000 de microbulblets from a single healthy plant.



Figure 4. Adventitious shoot regeneration from in vitro cultured bulb scales of tulips (left); tulip bulblets growing in the in vitro cultures (right).

As shown by the results obtained from numerous studies, the regeneration ability is dependent on cultivar (genotype) and growth regulators. For instance, a study carried out by Podwyszynska and Marasek (2003) showed clearly different regeneration potential of different tulip cultivars. Thus, the percentage of explants forming leaf-like structures ranged from 80% in 'Blue Parrot' and 'Prominence' to below 30% in 'Apeldoorn' and 'Mirjoran'.



Figure 5. Stages of tulip micropropagation (after Podwyszynska and Marasek, 2003): a) direct regeneration of leaflike structures similar to somatic embryos on flower stalk explants after 10 weeks of culture on initiation medium; b) subsequent development of leaf-like structures, 8 weeks after transfer onto multiplication medium; c) stabilized shoot cultures during multiplication stage); d) in vitro formation of bulbs on sucrose rich medium, under exposure to the light and temperature of 23°C, at 8 weeks after the end of treatment for inducing bulb formation (chilling at 4°C for 12 weeks).



Figure 6. Somatic embryogenesis in tulip: (a) embryogenic callus grown with 25 μM Picloram + 0.5 μM BA (bar = 1 mm); (b) embryogenic callus grown with 25 μM zeatin + 0.5 μM BA (bar = 1 mm); (c) non-embryogenic cells of colorless callus (bar = 25 μm); (d) isolated embryogenic cells (bar = 90 μm); (e) somatic embryos at globular stage (10 μM Picloram + 0.5 μM BA) (bar = 2 mm); (f) cross-section of the shoot and nodular callus formation (bar = 290 μm); (g) conversion of embryos into plants (5 μM BA + 0.5 μM NAA) (bar = 2 mm); (h) longitudinal section of the somatic embryo (bar = 2 mm); (from Bach and Ptak, 2005).

Also, the major influence of growth regulators has been revealed by the results of almost all studies on in vitro tulip regeneration. One of the most important finding was that thidiazuron, a cytokinin-like compound, greatly increase the regeneration potential up to 70-100% (Podwyszynska and Marasek, 2003), being far more efficient than cytokinins such as benzylaminopurine or kinetin.

Plant regeneration by organogenesis and somatic embryogenesis

Several types of explants (flower stalk, flower stem, ovary, isolated microspores) have been investigated for their potential to regenerate plants *in vitro* by either organogenesis or somatic embryogenesis. The results reported several authors (Alderson and Taeb, 1990; van den Bulk et al., 1994; Famelaer et al., 1996; Gude and Dijkema, 1997; Bach and Ptak, 2001; Podwyszynska and Marasek, 2003; Bach and Ptak, 2005; Ptak and Bach, 2007) have shown that the regeneration capacity is influenced by many factors, among the most important being the genotype (van den Bulk et al., 1994; Podwyszynska and Marasek, 2003), the basal culture medium (Podwyszynska and Marasek, 2003), the growth regulators (Podwyszynska and Marasek, 2003; Ptak and Bach, 2007), the type of explants, the physical culture conditions (Bach and Ptak, 2001; Bach and Ptak, 2005), the developmental stage and physiological state of the initial explants (van den Bulk et al., 1994). Both cytokinins and auxins are necessary to induce organogenesis or somatic embryogenesis in tulip somatic tissue explants (Fig. 6). For instance, the formation of somatic embryos in ovary tissue cultures was induced in the presence of 5 μ mol BA and 0.5 μ mol NAA (Bach and Ptak, 2001).

In experiments carried out with tulip flower stalk explants of six cultivars, excised from cooled and subsequently forced bulbs, Podwyszynska and Marasek (2003) were successfully in inducing plant regeneration by somatic embryogenesis with a frequency ranging (depending on the cultivar and medium composition) from 14.3% to 100%, and with a rate of leaf-like structures formed per explant ranging from 13.7 to 22.8. Subsequently, adventitious shoots developed from this primary regenerants, and formed clusters.

An important advance resulted from the studies carried out by Bach and Ptak (2005), who reported that a high frequency of adventitious bulb formation took place on stolons developing from embryos converted into plantlets in the liquid medium lacking growth regulators and containing 6% of sucrose. Both the number of bulblets per explants and individual fresh weight significantly increased in liquid medium as compared to control.

Embryo rescue

In vitro culture for the rescue of embryos resulting from interspecific crosses is another important application of biotechnology in tulips. This is actually the only way to overcome the post-fertilization barriers, and to avoid the abortion of embryos formed as result of such crosses. Therefore, considerable efforts have been made towards the establishment of optimum culture conditions and most favorable composition of culture media for inducing high rates of embryo rescue (Van Tuyl et al., 1990; Custers et al., 1992; Custers et al., 1995; Van Creij et al., 1999; Van Creij et al., 2000; Kalyoncu et al., 2006).

Embryo-rescue was found to be successful in the interspecific cross *T. gesneriana* x *T. kaufmanniana*, which is hampered by embryo breakdown. Custers et al. (1995) reported that optimal embryo-rescue was achieved in cultures initiated seven weeks after pollination. Compared to embryo culture, ovule culture allowed a higher efficiency of embryo rescue at earlier developmental stage (four weeks after pollination). Also, more embryos could be rescued at each culture date. A very important finding was that ovary-slice culture, started at various dates after pollination, allows obtention of comparable or even better results than that of direct ovule culture.

Polyploidization

Due to their vigorous growth, very attractive flower colour and large flower size, triploid tulips are gaining more and more popularity and, therefore, are in great demand (Kroon and Van Eijk, 1977; Okazaki, 2005; Marasek et al., 2006). Consequently, there is a great demand for tulip polyploids as initial breeding material.

Since the 90', methods for *in vivo* polyploidization were gradually replaced by the techniques of *in vitro* polyploidization, which were reported to contribute significantly to the efficiency of creating tetraploid varieties of tulips (Eikelboom et al., 1991; Van Tuyl et al., 2002). Although the *in vivo* chromosome doubling induced by treatment with polyploidization agents is still largely used within the breeding programs (especially for obtaining triploid varieties), doubling of chromosomes by the

in vitro culture offer some important advantages, including that the process is more easily to be programed and measured. Moreover, in the in vitro tissue and organ culture, can be induced the spontaneous doubling of chromosomes by explants wounding and forcing the production of abnormal mitotic divisions, generating of endopolyploidy. Regeneration of shoots from the cells situated in the areas of explant's wounding and selection of the tetraploid shoots by using flowcytometry, allows formation of the stock of parental plants necessary for crosses with the different cultivars of diploid tulips, as a stage in obtention and subsequent selection of triploid hybrids with valuable traits. Two decades ago, Van Tuyl et al. (1992) reported that tetraploid tulip cultivars have been produced after treating in vitro grown tulip bulbs with oryzalin or colchicine. A major achievement resulted from the studies of Chauvin et al. (2005), who developed a method for obtaining tetraploid clones from diploid varieties of tulip by using a system for plant regeneration from stem discs cultivated in vitro on medium containing oryzalin. It was reported that this method allowed the production of tetraploid clones in all the tulip varieties tested. Although the tetraploidy induction rate was low in some cultivars, this was sufficient for initiating crossing experiments between diploid and tetraploid genitors for obtaining triploid offspring. An important finding was that the regeneration efficiency is dependent on genotype, this explaining the significantly higher regeneration ability of 'Lucky Strike' and 'Gander' varieties, compared to that of 'Lustige Witwe' and 'Don Quichotte' varieties.

Somaclonal variation

Somaclones regenerated from tissue culture can be an interesting material for tulip breeding. Podwyszynska (2005), and Podwyszynska et al. (2006, 2010) reported that all the plants from 'Blue Parrot' cultivar regenerated *in vitro* from four-year-old cultures had modified flower color (red-purple instead of purple-violet), when they used a new protocol for tulip micropropagation, based on cyclic multiplication of adventitious shoots in the presence of thidiazuron. Also, among the juvenile plants derived from shoot cultures which had been propagated *in vitro* for four years or longer occurred variants with variegated leaves or altered morphology. RAPD and ISSR analyses revealed that the phenotypic variation shown by regenerants is the consequence of some genetic changes. Thus was demonstrated that *in vitro* plant regeneration in long term cultures gives chances of selecting somaclonal variants with modified characteristics.

Transformation

Considerable enrichments of the cultivated tulip with foreign genetic material has been accomplished using interspecific hybridization (van Eijk et al., 1991; Wilmink et al., 1995), but further transfer of germplasm from interesting related species into the cultivated varieties is hampered by strong crossing barriers.

Gene transfer to the tulip was obtained both by particle bombardment and Agrobacterium mediated transformation. Using a Particle Delivery System, transient expression of the reporter gene for β -glucuronidase was demonstrated in flowers stem explants of 14 cultivars and *Tulipa* species (Wilmink et al., 1992). However, successful tulip transformation requires efficient regeneration and selection systems Chauvin et al. (1997) have analyzed *in vitro* development of tulip floral scape segments in the presence of kanamycin, hygromycin and phosphinithricin. Unlike kanamycin, to which the tulip proved to se insensitive even at high concentrations, hygromycin was effective in controlling the regeneration of plantlets. The most effective selective agent was phosphinithricin, which totally inhibited regeneration for tulip even at low medium concentration.

3. MOLECULAR-BASED METHODS FOR GENETIC IMPROVEMENT OF TULIPS

In almost all cases, the F1 hybrids of distant related species are highly sterile as a consequence of the disturbed chromosome division during meiosis, leading to the formation of gametes with unbalanced chromosome constitution. The most widely used method of restoring fertility in interspecific hybrids is that of doubling the chromosome number in the offspring which should lead to formation of homologous chromosome pairs and, therefore, to normal meiosis. However, this

approach has a great drawback, arising from the preferential pairing of chromosomes between the constituent genomes of the hybrid and, consequently, reduced possibility for homoeologous chromosome pairing and crossing-over. Since homoeologous recombination is a crucial prerequisite for introgression of specific desirable traits into a cultivar, chromosome doubling of the F1 hybrids is not a suitable method. Rather, backcrossing would be the appropriate method.

Backcrossing is often useful, resulting in recombination between chromosomes and thus leading to introgression of desired traits into the recipient parent (Marasek and Okazaki, 2008; Hanzi, 2009). As shown in Fig. 7 and Fig. 8, this can be studied by either fluorescence *in situ* hybridization (FISH) or genomic *in situ* hybridization (GISH). Also, based on the recent achievements in understanding the genetics behind traits that are of interest, molecular markers are generated and used for mapping. Linkage studies in progenies or association mapping in collections of cultivars (varieties) can now identify molecular markers or QTLs linked to specific traits.

As in many other horticultural species, DNA markers became extremely useful tools, applied for either controlling the genetic uniformity of *in vitro* micropropagated tulip plants, or detecting somaclonal variation in micropropagated tulips or plantlets regenerated from somatic tissues. RAPD (random amplified polymorphic DNA) and ISSR (inter-simple sequence repeat) markers have been proven efficient in detecting somaclonal variation among the micropropagated plants of the 'Blue Parrot' cultivar (Podwyszynska et al., 2006). While no phenotypic off-types were noted in the progeny-line derived from a two-year-old culture and polymorphism on DNA level was not detected, genetic changes resulting in phenotypic variation (a changed color of flowers, from purple-violet to red purple) were revealed by the RAPD and ISSR analyses within the juvenile plants derived from 4- to 7-year-old cultures. A proof of the reliability of this molecular markers is the fact that they easily distinguished distantly related cultivars of tulips (such as 'Blue Parrot', 'Prominence', and 'Giewont'), but within the family of sports both RAPD and ISSR did not reveal polymorphism.

Recently, Podwyszynska et al. (2010) reported that ISSR technique confirmed that the variants with major phenotypic changes (highly malformed leaves and/or flowers), which occurred among the tissue culture-derived plants of several tulip cultivars, resulted from genetic changes.

DNA markers were recently recommended as efficient molecular tools for the identification of tulip cultivars. Bondrea et al. (2007) have been successful in using AFLP (amplified fragments length polymorphism) as molecular tool for distinguishing the various genotypes of *Tulipa*. It was emphasized that AFLP can be tailored by varying the number of selective nucleotides added to core primers and can allow accurate amplification even in complex template mixtures generated from plant species with very large genomes, such as tulip. As shown by the results obtained with several cultivars and hybrid progenies, reproducible AFLP patterns can be obtained with only seven selective nucleotides.



Figure 7. Discrimination of chromosomes originating from Tulipa gesneriana (green fluorescence) and T. fosteriana (red fluorescence) in the genomes of (a) 'Judith Leyster' (2n = 4x = 48), (b) 'Purissima' (2n = 2x = 24), (c) 'Hakuum' (2n = 2x = 24), (d) 'Hatsuzakura' (2n = 2x = 24), (e) 'Kikomachi' (2n = 2x = 24), (f) 'Momotaro' (2n = 2x = 24), (g) 'Tonbou' (2n = 2x = 24) şi (h) 'Kouki'(2n = 3x = 36). Recombinant chromosomes are indicated by letters F for T. fosteriana and G for T. gesneriana. Chromosomes are counterstained with DAPI (blue). Each bar represent 10 μm (Marasek and Okazaki, 2008).



Figure 8. GISH results from different genotypes of hybrids. T. gesneriana (G) genome was labeled by biotin-16dUTP (red), and T. fosteriana (F) genome was labeled by dig-11-dUTP (green). Recombinant chromosomes are defined as F/G and G/F indicating a T. fosteriana centromere with T. gesneriana chromosome segment(s) and a T. gesneriana centromere with T. fosteriana chromosome segment(s), respectively. Arrows indicate types of genome or recombination (after Hanzi, 2009).

4. CONCLUSIONS

The above review emphasizes that the use of recent advances in biotechnology and molecular tools can lead to significant improvements in tulip. The recent integration of advances in biotechnology, genomic research, and molecular marker applications with conventional plant breeding practices has created the foundation for molecular breeding of tulips.

Plant tissue culture became an essential component of the present-day techniques in tulips. Micropropagation allows the rapidly multiplying of elite plants bred through conventional plant breeding methods or obtained via somaclonal variation. The ovule culture and embryo rescue procedures allow novel crosses and offer new possibilities for the introduction of desirable genes into tulip cultivars. The doubling of chromosomes and the use of 2n gametes was proven to be suitable and efficient to overcome F1 sterility and enhance introgression of characters.

Since the introgression of one or a few genes into a current elite cultivar via backcrossing is a common breeding practice, methods for marker-assisted backcrosing were developed for the introgression of transgenic traits and reduction of linkage drag, where molecular markers can be used in genome scans to select those individuals that contain both the transgene(s) and the greatest proportion of favorable alleles from the recurrent parent genome. Therefore, the application of molecular genomic and cytogenetic techniques such as FISH, GISH and MAS can be of great help for fastening interspecific hybridization programs and shortening the duration of creating new varieties.

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