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Biotechnological approaches for crop improvement in carnation

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Abstract
Carnations are the world's first genetically engineered commercial flowers. Being a vegetatively propagated crop it has limited availability of gene pool with low chances of flower breeding. This makes it an ideal target for gene transfer technologies that have the potential to hasten the production of new genotypes and broaden the available gene pool. Development of transgenics is the need in the modern era of plant breeding, as they possess the potential to incorporate those characters in crop varieties which are either difficult or impossible through conventional breeding approaches. It is the possible alternative way to varietal development and shortens the duration. Improved morphology, flower colour, resistance to diseases, pests, flower doubleness and fragrance are some of the desired novel traits in carnation where transgenic approaches need to intervene. Genetic modification by means of genetic engineering approaches made remarkable approaches in development of varieties.

Keywords: Biotechnological approaches, improvement

Introduction
Carnation is native to the Mediterranean coastal region, is a member of the family Caryophyllaceae and belongs to the genus Dianthus, which contains more than 300 species. Altered plant and flower morphology, varieties and color combinations, enhanced fragrance and long vase-life are some of the most appealing traits from the consumer's point of view and, as such, the focus of many ornamental breeding programs. Carnation having appeared some 10 years after the first report of success in the genetic manipulation of flower colour through plant transformation [1].

Carnation has been an important target for the breeding of new varieties with novel characteristics. To date, new carnation varieties have been produced mainly via classical breeding, and are propagated vegetatively [2]. New tools for the introduction of foreign genes into plants and the growing knowledge and technology related to gene identification and isolation have enabled the specific alteration of single traits in an otherwise successful cultivar [3]. The application of genetic engineering to cut flowers has become instrumental for carnation.

Applications of Biotechnology in Carnation
Micro Propagation
Used for large-scale plant multiplication of elite superior varieties. Micro propagation is the desired approach for ornamental crops since propagation by cloning are relatively faster and creates exact replicas of the mother plant [4].

Protoplast Culture
Leaf explants of *Dianthus chinensis* L. cultured on Murashige and Skoog's (MS) medium with 6-benzylaminopurine (BAP, 3mg/l)+1-naphthaleneacetic acid (NAA, 0.5 mg/l) or BAP (3mg/l)+NAA (1 mg/l) produced adventitious shoot buds directly on the surface of the leaf explants without formation of intervening callus. Callus formation occurred after the induction of adventitious buds. Leaf explants cultured on BAP (0.5 mg/l) and 2,4-dichlorophenoxyacetic acid (2,4-D, 1 mg/l) supplemented MS medium also produced adventitious shoot buds along with excessive callus formation [5].

Somatic Embryogenesis
Callus has been induced from flower buds, leaf, hypocotyls, internodal sections, shoot-tips

without apex and meristems of various carnation cultivars. MS medium supplemented with 2,4-D was found to be the most suitable medium for callus induction. The balance between auxin and cytokinin is the essential factor for callus induction and regeneration [6].

Karimi *et al.* (2008) [7] conducted an experiment on somatic embryogenesis. Firstly, induce embryogenic calli on petal explants followed by development of primary somatic embryos from the calli. In second step, secondary somatic embryos were obtained and precotyledonary and cotyledonary primary embryos were isolated and transferred onto a series of culture media all containing MS basal salt mixture, and supplemented with different concentrations of 2,4-D, BA, sucrose and mannitol. The highest rate of secondary embryogenesis occurred on mannitol containing media.

In case of carnation, the main objectives to be achieved through transformation methods include different plant forms, colour; specifically the lacking blue, resistance to fusarium wilt and insects and reduced ethylene sensitivity.

Genetic transformation

Lu *et al.* (1991) [8] were the first to describe an *Agrobacterium* mediated carnation transformation procedure. Using stem explants and wild-type *Agrobacterium* strain ICMP8302 containing the binary plasmid pKIWI110, high transformation efficiencies (ca. one kanamycin-resistant transgenic shoot per ten explants) were reported.

Agrobacterium tumefaciens strain A 281 was the most virulent in *in vitro* stems of carnation and regenerated transformed plants from leaf and petal bases. However, transformation was most successful using *in vitro* leaf bases co cultivated with *Agrobacterium tumefaciens* EHA101 containing the binary vectors [9].

Flower Colour

Colour is an important objective for the breeder as it has the potential to create novelty, which is an important marketing trait. Carnations are available in a wide range of shades, e.g. white, pink, red, orange and yellow. Flecked, mottled and edged petal colours are also available.

Introduction of novel colours in carnation was based upon the gene encoding F3′5′-hydroxylase, which was isolated from petunia [10]. Transgenic violet carnations have been successfully developed by the introduction of a F3′5′H gene together with a petunia DFR gene into a DFR deficient white carnation Zuker *et al.* (2002) [3] 85 observed the colour change from orange-red to white through introduction of the antisense of flavonoid biosynthetic gene, flavanone 3-hydroxylase (F_3H). Along with the colour modification, transgenic plants emitted higher levels of methyl benzoate and so were more fragrant than control plants. Anti-sense technology can be used to create white varieties from colored parents by suppression of chalcone synthase gene(s), providing petal pigmentation is due to anthocyanin accumulation, and not to carotenoids. Chalcone synthase is the first key enzyme on the flavonoid pigment biosynthesis pathway and inhibition of its synthesis completely suppresses anthocyanin formation [11].

Anti-sense could therefore be used to produce white cultivars from elite, pigmented, parent lines. Insertion of genes encoding the enzyme dihydroflavonol reductase may alter the rate of accumulation of anthocyanins, or change the ratio of type of anthocyanin, thereby altering colour shade. Using conventional breeding, blue carnations would be impossible to achieve but their introduction is likely to lead to a new, wide range of novel colour varieties.

Post-harvest quality

Many cut flower crops deteriorate rapidly after harvest due to production of ethylene. Attempts made in carnation to lower the ethylene level leads to increase in vase life. The senescence and ripening process is characterized by a climacteric increase in the production of ethylene, leading to the induction of genes involved in programmed cell death and ripening.

Carnation is highly sensitive to ethylene and during senescence, autocatalytic production of ethylene leads to deterioration of petals [12]. Two major contributing genes are identified, *viz*. DC-ACS1 (encoding ACC synthase) and DC-ACO1 (encoding ACC oxidase) to control of senescence in carnation flowers by regulating ethylene production, [13, 14]. By regulating ethylene-induced senescence can enhance post-harvest longevity of carnation, as transgenic carnation with reduced ethylene production will lead to the complete elimination of costly and harmful chemicals used to lengthen the vase life [15].

Antisense ACO gene was inserted in carnation varieties Scania, White sim leading to control the production of ethylene levels and increased vase life.

Ethylene biosynthesis is reported that ACC synthase (ACS) catalyzes the conversion of S-adenosylmethionine to 1-aminocyclopropane-1- carboxylic acid (ACC) [16], while ACC oxidase (ACO) catalyzes the subsequent step, the conversion of ACC to ethylene [17]. An ACO e DNA isolated from carnation cv. Scania was used to produce transgenic carnation plants containing an antisense ACO gene.

Genetic Modification

Genetic modification (GM) has been used for the development of varieties of numerous important food species. Genetic modification answers these constraints and provides a way for variety improvement [18]. Biotechnology also shortens the duration of variety development in an industry where phenotypic novelty, such as flower color, is an attractive marketing factor [19].

Several traits of ornamental plants have already been modified including flower color, fragrance, flower shape, plant architecture, flowering time, postharvest life and resistance for both biotic and abiotic stresses. Currently, at least 50 ornamental plants can now be transformed. [20]. Transgenic ornamentals have been produced by several different techniques, the most common techniques being *Agrobacterium*-mediated transformation and particle bombardment Ornamental plant traits are classified according to their value in the market chain. There are traits with more value to the grower than to the consumer. These are traits related to ease of production and shipping such as disease resistance and shelf life. Meanwhile, other traits have more value to the consumer such as novel colors, dwarfed plants, modified growth, improved fragrance, flower shapes and flower sizes [21].

Disease Resistance

Sarcotoxin gene of Sarcofaga peregrine inserted in carnation to develop genetically modified carnation crop resistance against *Burkholderia caryophylli* in Carnation.

The RAPD technique was used to identify genetic markers useful in the development of a diagnostic method for *E oxysporum* f. sp. *dianthi*, the causal agent of the carnation wilt disease. A total of 18 different strains isolated from different locations around the world, as well as 17 *F. oxysporum* isolates corresponding to f. sp. other than dianthi (non-

dianthi) were amplified using 15 primers. No direct correlation was observed between the RAPD pattern and the race of an isolate [22].

Flower Doubleness

In ornamentals polyploid individuals (those with multiple sets of chromosomes) are widely used for their improved characters such as larger flowers and thicker petals. Polyploid individuals can either spontaneously appear in nature or be induced by *in vitro* chromosome doubling.

Scovel *et al*. (1998) [23] Flower doubleness is an important breeding characteristic in carnation. Flowers of standard and spray varieties, which constitute the largest market share, are usually of the double and semi-double type respectively. These flower types are not easily distinguishable due to phenotypic overlaps caused by environmental conditions. Using random decamer primers, RAPD marker which is tightly linked to this recessive allele was identified. The RAPD marker was cloned and used to generate a RFLP marker. This RFLP marker discriminated with 100% accuracy between the semi-double and double-flower phenotypes in carnations of both Mediterranean and American groups.

Genetic Engineering of Blue Carnation

The GM ornamental plants that are on the market have colour modified flowers, and both have been developed by Florigene Pty. Ltd. /Suntory Ltd. The product range comprises eight varieties of transgenic *Dianthus caryophyllus*. The colour modification is the result of manipulation of the anthocyanin biosynthetic pathway [24].

In nature, *Dianthus caryophyllus* do not contain delphinidin-derived anthocyanins, due to absence of flavonoid 3′5′-hydroxylase. There are three anthocyanin aglycones, cyanidin, pelargonidin and delphinidin. The former two lead to red-shaded anthocyanins, while delphinidin is a pre-requisite to form blue anthocyanins.

Carnation and all other *Dianthus* species lack the enzyme 3'5'-hydroxylase necessary to produce delphinidin. This gene has now been isolated from *Petunia* by Florigene and the expectation is that delphinidin-producing carnations will have the capacity to produce blue flowers. Expression of petunia F3'5'H (under the control of a promoter region from the snapdragon CHS gene) and petunia DFR (under the control of a constitutive promoter) genes in one such DFR mutant resulted in exclusive accumulation of delphinidin derivatives and significant colour change toward blue [25].

Petunia has a cytochrome *b5* that specifically transfers electrons to F3'5'H by which petunia can efficiently synthesize 3',5'-hydroxylated flavonoids [26]. Expression of a petunia F3'5'H (*Hf1*) gene along with a petunia cytochrome *b5* gene in a carnation cultivar producing cyanidin derivatives resulted in efficient production of delphinidin based anthocyanins and subsequent change in petal colour [27, 28].

To date, the “Moon” series from Suntory Limited and Florigene Pty Ltd are the only GM ornamental products commercialized on a significant scale [29]. The Moon series carnations, containing various flower colors, have been commercially available in Australia, European Union, Japan and USA since the late 1990s while Colombia approved them in the early 2000s. Genetically Modified *Dianthus caryophyllus* products were first marketed in Australia in 1997.

The carnation “Moon” series namely: (please refer Fig. 1)

1. Moon shadow - blue (1996)
2. Moon dust - blue (1996)
3. Moon shade - purple (2001)
4. Moon lite - lavender (2001)
5. Moon vista - dark purple (2001)
6. Moon aqua - mauve (2001)
7. Moon pearl - lavender (2010)
8. Moonique - purple (2010)
9. Moon berry - light purple (2010)
10. Moon velvet - dark purple (2010)
11. Moon burst - bicolour lavender (2017)
12. Moon strike - purple (2017)
13. Moon tea (dark purple)

Table 1: Summary of studies conducted on genetic transformation of carnations (Zuker *et al*. 2001) [2].

Cultivar	Explant	Vector/method	Selection marker	References
White Sim Red Sim Crowley Sim	Stem segments	AGLO/pKIWIl05 AGLO/pCGP407	Kanamycin	Lu *et al.* (1991) [8]
CPRO 89100 CPRO 89127 CPRO 89117 CPRO 89132	Ex vitro leaves	AGLO/pCGN7001	Kanamycin	van Altvorst *et al.* (1995) [30]
White Sim	Stem segments	Microprojectile bombardment	Bialaphos	Zuker *et al.* (1998) [32]
White Sim Manon Nathalie	*In vitro* leaves	EHA101/pWTTI084 EHA101/pSLJ1911	Chlorsulfuron Kanamycin	Firoozabady *et al.* (1995) [31]
CPRO 89100	Petals	AGLO/pCGN7001 AGLO/pGUSint	Kanamycin	van Altvorst *et al.* (1996) [30]
White Sim Eilat Desio	Stem segments	Microprojectile bombardment AGLO/pCGN7001	Kanamycin	Zuker *et al.* (1998) [32]

Table 2: Achievements of gene expression in carnation [18].

Gene	Result
F3′-5′h gene	overexpression produces blue flowers in combination with a silenced *dfr* gene in Carnation (Petunia)
Sarcotoxin gene from Sarcofaga Peregrina	Resistance against *Burkholderia caryophylli* in Carnation
ACO-coding genes	Increased vase life in carnation

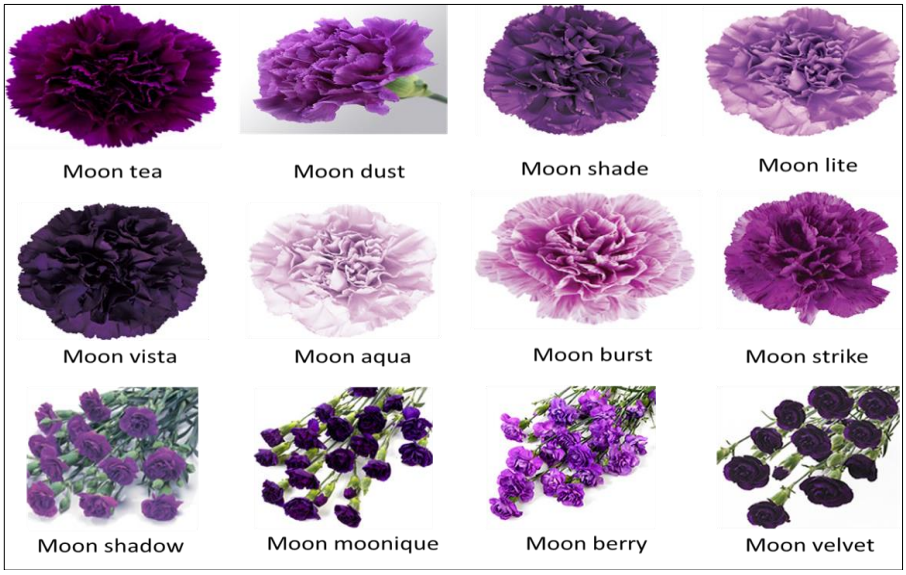

Fig 1: MOON series of carnation released by Florigene Pty ltd

Conclusion

It could be concluded that by using different biotechnological approaches we can improve the flower morphology, color, resistance to biotic, abiotic stresses, flower doubleness, fragrance and long vase life. It showed that by down-regulating the action of genes responsible for ethylene production through transformation, longevity of cut carnations can be enhanced. Down-regulation of the F3'H and F3'5'H genes in combination with over expression of a correctly identified DFR gene should generate gentian flowers producing pelargonidin based pigments. Moon series of carnation which has been commercialized only in a few countries despite the novel blue color. There is a need to widen the scope of efficient genetic transformation systems and transgenic technology to improve novel varieties among flower crops and ornamentals as such.

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