Molecular breeding of *Campanula* for novel flower colour and ethylene insensitivity

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Zusammenfassung

Glockenblumen (*Campanula*) sind bekannte Topfpflanzen mit großem Anteil in ihrem Segment auf dem europäischen Markt. Um den wirtschaftlichen Erfolg zu sichern, muss Ware höchster Qualität vertrieben und der Markt mit neuen, innovativen Sorten bedient werden. Gewünschte Eigenschaften von *Campanula* sind (i) Ethylen-Toleranz und eine dadurch resultierende verlängerte Blütenhaltbarkeit sowie (ii) rote Blütenblätter. Klassische Züchtungsversuche, diese Eigenschaften in kommerziell relevante *Campanula* Sorten einzuführen, schlugen fehl.

In dieser Arbeit sollte sowohl die Ethylen-Sensitivität als auch die Blütenfarbe von *Campanula* mittels genetischer Transformation verändert werden. Dafür wurden zunächst die Regenerations-, Selektions- und Transformationsprotokolle optimiert. Verbesserte Regenerationsraten konnten mit Blattstielen aus Teilstecklingen erzielt werden. Der Vergleich des Neomycin Phosphotransferase/Kanamycin System mit dem Phosphomannose Isomerase/Mannose System identifizierte beide als geeignete Selektionsstrategie. Für die *Agrobacterium*-vermittelte Transformation erwies der Stamm GV3101 die höchsten Transformationsraten. Um die Ethylen-Toleranz zu erhöhen, wurde eine mutierte Version des Ethylenrezeptors ETR1 unter blüten-spezifischer Expression in das *Campanula* Genom integriert. Die Mutation blockiert die Ethylen-Signaltransduktion und Ethylen-vermittelte Seneszenzprozesse. Um die Blütenfarbe von Blau in Rot umzuwandeln, enthielt das Transformationskonstrukt zusätzlich eine palindromische Kopie der Flavonoid 3', 5' Hydroxylase (*F3'5'H*) unter Kontrolle des blüten-spezifischen *pst* Promoters. Dies sollte eine verminderte Expression der Flavonoid 3', 5' Hydroxylase auslösen und somit die Synthese des blauen Pigments Delphinidin verringern.

Test der Ethylen-Sensitivität drei unabhängig generierter transgener Linien bestätigte eine signifikante Verzögerung der Blütenseneszenz der *Atetr1-1* exprimierenden *Campanula* Pflanzen im Vergleich zum Wildtyp. Es konnte aber keine sichtbare Farbänderung festgestellt werden. Expressionsanalysen detektierten keine Veränderung der *F3'5'H* Akkumulation in floralen Geweben der transgenen Linien im Vergleich zum Wildtyp. Dies deutet darauf hindeutet, dass (i) der aus *Arabidopsis* stammende *pst* Promotor in *Campanula* nicht aktiv oder (ii) das ausgewählte RNAi Fragment für die Herabregulation nicht geeignet ist.

Schlüsselwörter: *In Vitro* Kultur, Blütenveränderung, RNAi Genausschaltung, Ethylenesensitivität

Abstract

Campanula also known as bellflower has become a famous potted plant with great ornamental value on the European market. To ensure its economic success, producers have to deliver plants of high quality and supply the market with novel, innovative cultivars. Traits of special interest in *Campanula* are (i) ethylene tolerance resulting in improved flower longevity and (ii) red instead of the naturally occurring blue or white flowers. Classical breeding strategies failed to introduce these traits into commercially relevant *Campanula* species. This study aimed to alter ethylene-sensitivity and flower color of *Campanula* cultivars by genetic transformation. To this end, the regeneration, selection and transformation system was optimized first. Petioles of nodal cuts were the most efficient explants for regeneration. Comparison with the neomycin phosphotransferase/kanamycin system revealed the phosphomannose isomerase/ mannose system as a reliable alternative selection strategy. For the purpose of *Agrobacterium*-mediated transformation, infection with strain GV3101 revealed the highest transformation rates. To enhance ethylene-tolerance, the transformation construct carried a mutated version of the ethylene receptor ETR1 (*etr1-1*) under the control of the flower-specific *fbp1* promoter. The mutation blocks ethylene signal response and ethylene-mediated senescence processes. To change *Campanula* flower color from blue to red, the transformation construct was additionally designed to silence expression of the flavonoid 3', 5' hydroxylase (*F3'5'H*). A palindromic copy of the *F3'5'H* gene under the control of the flower-specific *pst* promoter was supposed to induce RNA interference-mediated silencing of the flavonoid 3', 5' hydroxylase and to inhibit synthesis of the blue pigment delphinidin.

Ethylene sensitivity testing of three independently generated transgenic lines confirmed a significant delay of flower senescence of the *etr1-1* expressing *Campanula* plants compared to wild type plants. However flower color was not visibly changed. Expression analysis revealed that *F3'5'H* was expressed to wild type levels in all floral tissues of the transgenic lines suggesting that either (i) the *Arabidopsis* derived *pst* promoter is not active in *Campanula* or (ii) the chosen RNAi fragment was not suitable to induce gene silencing.

Keywords: *In vitro* tissue culture, flower colour alteration, RNAi gene silencing, ethylene sensitivity

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Dedication

This thesis is dedicated to:

To my husband, Ehsan for his eternal love and endless support and my wonderful daughter, Elena for the passion and joy that she brought to our life.

To my lovely parents and my brothers, Ehsan and Hossein for their pray and encouragement through my study.

To my parents in law, for their all inspiration and kindness.

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Abbreviations

Abs	absorbance
ACC	aminocyclopropane 1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
ain1	*ACC insensitive* 1
ANS	anthocyanidin synthase
AOA	aminooxyacetic acid
AVG	aminoethoxyvinylglycine
BA	6-benzylaminopurine
°C	degree celsius
C4H	cinnamic acid 4-hydroxylase
cDNA	complementray DNA
CHI	chalcone isomerase
CHS	chalcone synthase
CL	4-coumarate Co-A ligase
ctr1	*constitutive triple response* 1
cv.	cultivar
DFR	dihydroflavonol 4-reductase enzyme
DHK	dihydrokaempferol
dsRNA	double-stranded RNA
ein2	*ethylene insensitive* 2
eto1	*ethylene overproduction* 1
etr1-1	*ethylene resistant* 1-1
F2H	Flavanones 2-hydroxylase
F3’5’H	flavonoid 3’, 5’-hydroxylase
F3’H	flavonoid 3’-hydroxylase
FLP	flippase
FLS	flavonol synthase
FNS	flavone synthase
FNSII	flavone synthase II

FRT	FLP recombination target
gusA	*ß-glucuronidase*
HPLC	high-performance liquid chromatography
IFS	2-hydroxyisoflavanone synthase
Km	kaempferol
LPg	leucopelargonidin
MACC	malonyl-ACC
mRNA	messenger RNA
MTA	5'- methylthioadenosine
NAA	1-Naphthaleneacetic acid
npt II	*neomycin phosphotransferase* II
PAL	phenylalanine ammonia lyase
Pg	pelargonidin
PGRs	plant growth regulators
PMI	6-phosphomannose isomerase
PTGS	post transcriptional gene silencing
RISC	RNA-induced silencing complex
RNAi	RNA interference
RT-PCR	reverse transcription polymerase chain reaction
S-AdoMet or SAM	S-adenosyl-methionine
siRNAs	small interfering RNAs
STS	silver thiosulfate
T-DNA	transfer DNA
TDZ	thidiazuron
UV	Ultraviolet
xylA	*xylose isomerase*

Thesis outline

This thesis is a collection of papers, which in detail explains the *in vitro* shoot regeneration protocol and molecular breeding of *Campanula* toward flower colour alteration and improved longevity as main topics.
The dissertation is arranged as follows:

Chapter 1. Introduction, which consists of broad overview of the research and framework.

Chapter 2. Review of the related literature and background information including key findings in tissue culture and micropropagation protocols of *Campanula* species, description of flavonoids biosynthesis pathway and previous attempts in flower colour alteration. In addition, explanation of ethylene biosynthesis pathway and techniques to improve the plants postharvest life are included. Furthermore, this chapter defines characteristics of selection marker genes and use of positive selection system.

Chapter 3. Research paper entitled 'The establishment of effective regeneration and selection systems in *Campanula carpatica* and *Campanula portenschlagiana* cultivars'. This chapter/paper reports the optimal concentrations and combinations of plant growth regulators (PGRs) and the best explant source for micropropagation and shoot regeneration of three *Campanula* cultivars. This chapter also describes the mannose selection technique and presents the optimum concentration of mannose to select the transformed tissues.

Chapter 4. Conference paper and poster entitled 'Effects of cytokinin treatment of nodal cuts and petioles on shoot regeneration of *Campanula portenschlagiana* 'Blue Ocean', which discusses the consequences of hormonal treatments in shoot regeneration. This chapter also compares the effect of various media supplemented with combinations and concentrations of PGRs using for growing nodal cuts in shoot regeneration of harvested petioles.

Chapter 5. Research paper 2 entitled 'Transformation of *Campanula carpatica* to alter flower colour and ethylene sensitivity'. It gives experimental details on gene silencing using the RNA interference (RNAi) technique to manipulate flower colour and improve longevity in *Campanula carpatica* cultivar (cv.) Improved Blue Uniform. This chapter/paper also presents analysis towards differences between non-

transformed and transformed lines in vase life and gene expression of flavonoid hydroxylase enzymes.

Chapter 6. Conclusions on the research and discussion of the key findings considering the previous studies, as well as giving suggestions for future research.

Introduction

1. Introduction

Campanula is a genus of the Campanulaceae family with the common name of bellflower. The genus *Campanula* consists of approximately 400-600 species which are mostly exclusive to the Northern Temperate Zones and the Northern Hemisphere (Kuss et al. 2007). The species contains annual or perennial herbs and grows as potted or bedding plants (Sriskandarajah et al. 2008).

Many of *Campanula* species are cultivated as ornamentals, which raise the demand to generate novel characteristics. Gene manipulation is a sophisticated method to create attractive visual features, such as a new flower colour or shape, increase the product characteristics, and generate plants with improved postharvest quality and longevity.

Even though the lack of efficient regeneration and transformation protocols, limit the application of biotechnological tools, use of this techniques is the most powerful method of generating new characteristics. Efforts have been made towards optimizing shoot regeneration in different *Campanula* species in the *in vitro* condition and various explants, culture media and explants sources have been tested and still some deficiencies are exist due to physiological differences or lack of information. For instance, use of hypocotyls and cotyledons as explants (Sriskandarajah et al. 2001) are not applicable in sterile species such as *C. haylodgensis* (Lewis and Lynch 1989). Shoot regeneration in *C. glomerata* using 6-benzylaminopurine (BA) in media, was observed after 4-6 months, which is a relatively long time. To speed up the shoot regeneration, supplementation of media with thidiazuron (TDZ) was recommended (Joung et al. 2002). In a research on shoot regeneration of four *Campanula* species, high concentrations of TDZ (up to 20 mg l^{-1}) were applied to induce adventitious shoots. Instead a large amount of calli that failed to regenerate shoots was formed (Sriskandarajah et al. 2008). These are some examples of complications that still exist in previous published reports. Furthermore, specific demands of each species and cultivar led us to optimize tissue culture condition as prerequisite of gene transformation as the first step.

Flavonoids, carotenoids and betalains are three classes of pigments, which mostly determine the colour of flowers and fruits. Flavonoids are the most well characterized group of pigments and anthocyanins are coloured group of flavonoids, which mainly contribute wide range of colours to the flowers. To date many colour modification

studies have been performed via engineering of the flavonoids biosynthesis pathway (Tanaka et al. 2010). Different approaches can be applied to manipulate the flavonoids biosynthesis pathway and consequently flower colour alteration including; introduction of the novel sense derivatives of gene to open a new pathway in flavonoids biosynthesis pathway, expression of sense derivatives of appropriate gene to open a blocked pathway or silencing of unwanted gene in the pathway to forward the pathway or open new branches of producing metabolites (Martens et al. 2003).

Along with novel visual characteristics such as the flower colour, improvement of flower longevity is another important target for breeders and could be performed through gene manipulation.

Ethylene gas is a plant growth regulator influencing plant development and responses to adverse environmental conditions. Ethylene has an important role in seed germination, organ elongation, tissue abscission, fruit ripening and flower senescence (Abeles et al. 1992, Gallie 2010). Plants can be roughly categorized into ethylene insensitive or ethylene sensitive plants. The postharvest quality and longevity of the flowers depend on their degree of sensitivity to the ethylene. *Campanula* flowers and buds will wilt within three days when exposed to ethylene and are classified into high ethylene sensitive plants (Sriskandarajah et al. 2007). Chemicals, which block the response of plants to the ethylene by preventing the binding of ethylene to its receptors, act as ethylene inhibitors. While due to disposal concerns and being potentially environment pollutant, application of these chemicals such as silver thiosulfate (STS) are restricted and are not widely used in some countries (Mayers et al. 1997). Therefore, gene manipulation is an effective and promising alternative. Genetic modifications of the genes that encode proteins, which involve in ethylene signaling or response of plants to the ethylene, will alter plant sensitivity and responsiveness to the ethylene and prolong the vase life.

Molecular breeding of plants using *Arabidopsis* mutants such as *etr1-1* that inhibits the binding of ethylene to the ethylene receptors, efficiently represses ethylene signaling in a dominant negative manner and results in reduced ethylene sensitive or ethylene insensitive plants. *Etr1-1* gene was applied in several studies and various plants such as *Dianthus caryophyllus* (Bovy et al. 1999), *Campanula carpatica* (Sriskandarajah et al. 2007), *Kalanchoe blossfeldiana* (Sanikhani et al. 2008),

Solanum lycopersicum (Gallie 2010), and *Petunia hybrida* (Wang et al. 2013) to improve the longevity and shelf life.

The main purpose of the current research was molecular breeding of *Campanula portenschlagiana* cvs. Blue Ocean and Royal, and *Campanula carpatica* cv. Improved Blue Uniform for novel flower colour and improved longevity through gene transformation using *Agrobacterium tumefaciens.*

To modulate flower colour, RNAi gene silencing technique using palindromic copy of *F3'5'H* gene, which is a main enzyme involved in the production of delphinidin and blue colour in flowers, was applied. To improve vase life of flowers the construct harboured *etr1-1* gene, which is a mutant gene from *Arabidopsis thaliana*.

The current report is a successful experiment that used adult plants as the explant source for obtaining transformed independent ethylene insensitive lines. Furthermore, application of non-antibiotic mannose selection system was established for the first time for selection of transformed *Campanula*.

Literature Review

2. Literature review

2-1. Tissue culture and micropropagation

The artificial *in vitro* culture condition is a simulation of natural environments for micropropagation of plants. In recent years, developments of plant genetic engineering have opened new paths for crop improvement and generation of novel characteristic in various plants. The success of plant genetic engineering relies on several factors, which include an efficient tissue culture system. Composition of culture medium, age, position and tissues used as explants, stock plant quality and condition, time of tissue initiation and culture condition prior and after gene transformation affect the success of gene manipulation process and should be optimized for specific species (Wędzony et al. 2014). All of these factors can hinder the efficiency of gene transformation unless they are optimized; therefore various tissue culture systems and target tissues have been improved to reach the high throughput gene transformation system.

2-1-1. Plant growth regulators (PGRs)

Plant hormones (phytohormones) are chemical substances that profoundly influence the growth and differentiation of plant cells, tissues and organs. There are currently five recognized groups of plant hormones including auxins, gibberellins, cytokinins, abscisic acid (ABA) and ethylene (Gaba 2005). Chemical compounds, which have been synthesized by humans, are called PGRs, a short form of plant growth regulators. Auxins and cytokinins are main PGRs used in *in vitro* culture and relative concentrations of auxin and cytokinin has a direct effect on determining the type of organogenesis that occurred in callus (Skoog and Miller 1957). Cytokinins are used as stimulators for shoot multiplication while auxins encourage cell expansion and particularly cell elongation (Mineo 1990).

Despite advances in optimizing plant tissue culture condition, the application of this technique is still hampered by different physiological, developmental problems and regeneration of abnormal tissues occurs (Ruffoni and Savona 2013). The majority of such morphological deformation is caused by excessive application of PGRs during organogenesis, particularly cytokinins in culture media (Ramage and Williams 2004). Abnormalities, such as hyperhyricity (Williams and Taji 1991), proliferation and

regeneration of deformed and stunted shoots (Lincy and Sasikumar 2010) were previously reported as consequences of using excessive cytokinin. Over-supplementation of auxins caused regeneration of abnormal and short roots in *Acacia seyal* Del. (Al-Wasel 2000). In regenerated shoots of *C. carpatica* abnormal leaves formed due to unsuitable hormonal content of culture media (Frello et al. 2002).

TDZ is a growth regulator that is classified in the cytokinin group and known to efficiently stimulate shoot induction and organogenesis in a relatively short time (Schulze 2007). Joung et al. (2002) applied BA in culture media for shoot induction in *C. glomerata* from leaf blades but recommended the application of TDZ instead of BA to increase the shoot regeneration rate as well as shoot induction within a shorter time.

Although TDZ is a hormone that effectively promotes shoot regeneration, some disadvantages have been reported when it is used at high concentrations. In *C. carpatica* regenerated shoot clumps and individual shoots have been transferred to medium containing 5 mg l^{-1} TDZ for further growth. Individual shoots failed to extend and shoot clumps looked like balls of shoots that had a mean height of 2 mm. Medium supplemented with 0.63 mg l^{-1} TDZ or hormone free medium encouraged shoot extension and mean shoot height values were 10.0 and 8.5 mm, respectively (Sriskandarajah et al. 2004).

In the study on shoot regeneration of *Campanula* species, increasing the concentration of TDZ up to 10 mg l^{-1} induced shoot regeneration, whereas higher amounts of TDZ significantly reduced shoot regeneration and promoted rates of callus formation (Sriskandarajah et al. 2008).

Determining the optimal condition including concentration of PGRs, particularly TDZ, is essential for high shoot regeneration rates and avoiding the side effect of over supplementation of hormones in *Campanula* spp.

2-1-2. Explants

There are few research reports that have been focused on *in vitro* propagation of *Campanula*. Organs, media compositions and growth conditions have been tested in different species of *Campanula*.

Leaf blade in *C. glomerata* (Joung et al. 2002), leaf and petiole in *C. punctata* (Sivanesan et al. 2007, Sivanesan et al. 2011), hypocotyls and cotyledons in *C. carpatica* (Sriskandarajah et al. 2001), the first two pairs of leaves harvested from seedlings in *C. carpatica* (Frello et al. 2002), stem segments with axillary or apical bud in *C. isophylla* (Brandt 1992), are some explants which have been used in micropropagation of *Campanula*.

Some of these explants are not applicable in other species due to physiological differences. For instance, in many *Campanula* species, leaves are growing from the crowns and stems will not form until flowering. Therefore, in these species use of stem segments with axillary or apical bud as explant is not possible (Joung et al. 2002). In some species due to low germination rate of seeds in *in vitro* condition and other specific properties concerning seed ecology and physiology such as requirement of moist cold stratification and not available data about length of stratification, using hypocotyls and cotyledons as explants may be inappropriate and cause deficiency in micropropagation (Blionis and Vokou 2005, Stamenković et al. 2012).
Physiology, age, maturation stage and preconditioning of stock plants are some factors that are important that should be considered while harvesting explants (Compton 2011).

2-1-3. Explant source and tissue reinvigoration

Despite desirability of juvenile tissues to harvest explants, in some plant species, the explants should be taken from mature tissues (Bonga and von Aderkas 1992). *C. haylodgensis* is a sterile mutant originating from an interspecific hybrid between *C. carpatica* and *C. cochleariifolia* (Lewis and Lynch 1989), in this species mature tissues would be the explant source. In addition, when germination rate is low as reported in *C. carpatica* (Gargul 2006) explant source would be the mature adult plants. In order to use adult tissues as source of explants, rejuvenation of tissues is necessary. Induction of juvenility is a procedure that can be performed in controlled *in vivo* or *in vitro* environment, leading to forcing adult plants to grow soft and fresh shoots. Grown juvenile tissues can be applied for harvesting explants used in tissue culture for micropropagation (George et al. 2007). Reinvigoration of mature tissues was performed in some species through micrografting (Revilla et al. 1996) or serial

subculturing (Rey et al. 1994) in *in vitro* condition. Mitigation of recalcitrance habit of mature tissues can be achieved by applying PGRs in media (Murthy et al. 1998) or in some species has been made by using hormone-free media (Ewald 1998).

In four *Campanula* species, when greenhouse-grown plants were used as a source of explants, all explants failed to regenerate shoots, thus reinvigoration was performed on greenhouse adult plants using BA and 1-Naphthaleneacetic acid (NAA) or combination of BA, TDZ and NAA. Combination of both BA and TDZ resulted in higher number of adventitious shoots and faster growth while by application of BA shoot number was low and the time taken for shoot primordia formation was about 5 days longer (Sriskandarajah et al. 2008). Similarly, a study by Paunescu (2010) reported the use of adult *C. polymorpha* plants to prepare stem segments for tissue culture; in this experimental example, BA combined with NAA was recommended as the optimal medium for shoot growth from stem segments.

2-2. Flavonoids and flower colour

Flavonoids are plant's secondary metabolites and consist of different compounds such as anthocyanins, flavones and flavonols. More than 6000 flavonoids have been identified so far and this number is increasing (Lehane and Saliba 2008). Flavonoids have many physiological roles in higher plants including attracting pollinators, protecting tissue against ultraviolet (UV) light (UV-B screening pigments), warding off pathogenic microorganisms (phytoalexins) or pests (antifeedants), fertility and germination of pollen (Forkmann and Martens 2001). Flavonoids also have an antioxidant function and are potentially anticancer agents (Harborne and Williams 2000). One of the most important roles of flavonoids is colouration of flowers, fruits, seeds and leaves. The flavonoids biosynthesis pathway has well characterized and main characteristics of pathway are similar in higher plants (Winkel-Shirley 2001).

Flower colouring is the result of accumulation of a group of flavonoids consisting of anthocyanins, carotenoids or betalains (Nakatsuka et al. 2007). The final colour of flowers is result of the various factors such as anthocyanins structure, pH of vacuoles which contain anthocyanins, co-pigmentation (effects of co-existing compounds such as amino acids or sugars on anthocyanins) and metal iones (Yoshida et al. 2009). Anthocyanidins (aglycones of anthocyanins), are chromophore and precursors of

anthocyanins. Anthocyanins are composed of six main groups of pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin, which are well studied in structure and biosynthesis. Peonidin is the methylated derivative of cyanidin while petunidin and malvidin are delphinidin-based anthocyanins (Fig. 1) (Tanaka and Brugliera 2013).

Biosynthesis of anthocyanins are regulated by two processes of hydroxylation of B-ring and substrate specificity of dihydroflavonol 4-reductase enzyme (DFR) (Forkmann and Ruhnau 1987). In particular, flavonoid 3'-hydroxylase (F3'H), F3'5'H and flavone synthase II (FNSII) are the enzymes mostly involved in controlling flower colouring (Ueyama et al. 2002). F3'H and F3'5'H are two enzymes that determine hydroxylation pattern of B-ring of the dihydroflavonols, which eventually regulate the anthocyanins structure and flower colour (Holton and Tanaka 1994). As increase in the number of hydroxyl groups in B-ring catalyzed by F3'H and F3'5'H shifts the anthocyanins pattern to blue pigment and results in bluer flower colour (Tanaka et al. 1998). FNSII catalyzes biosynthesis of flavones which act as co-pigments to anthocyanins and provide blue and dark colours in flowers (Tanaka 2006). Basically F3'5'H is the key enzyme for expression of blue or purple flower colour and F3'H is the main enzyme to produce red or pink colour in petals or other tissue (Mori et al. 2004).

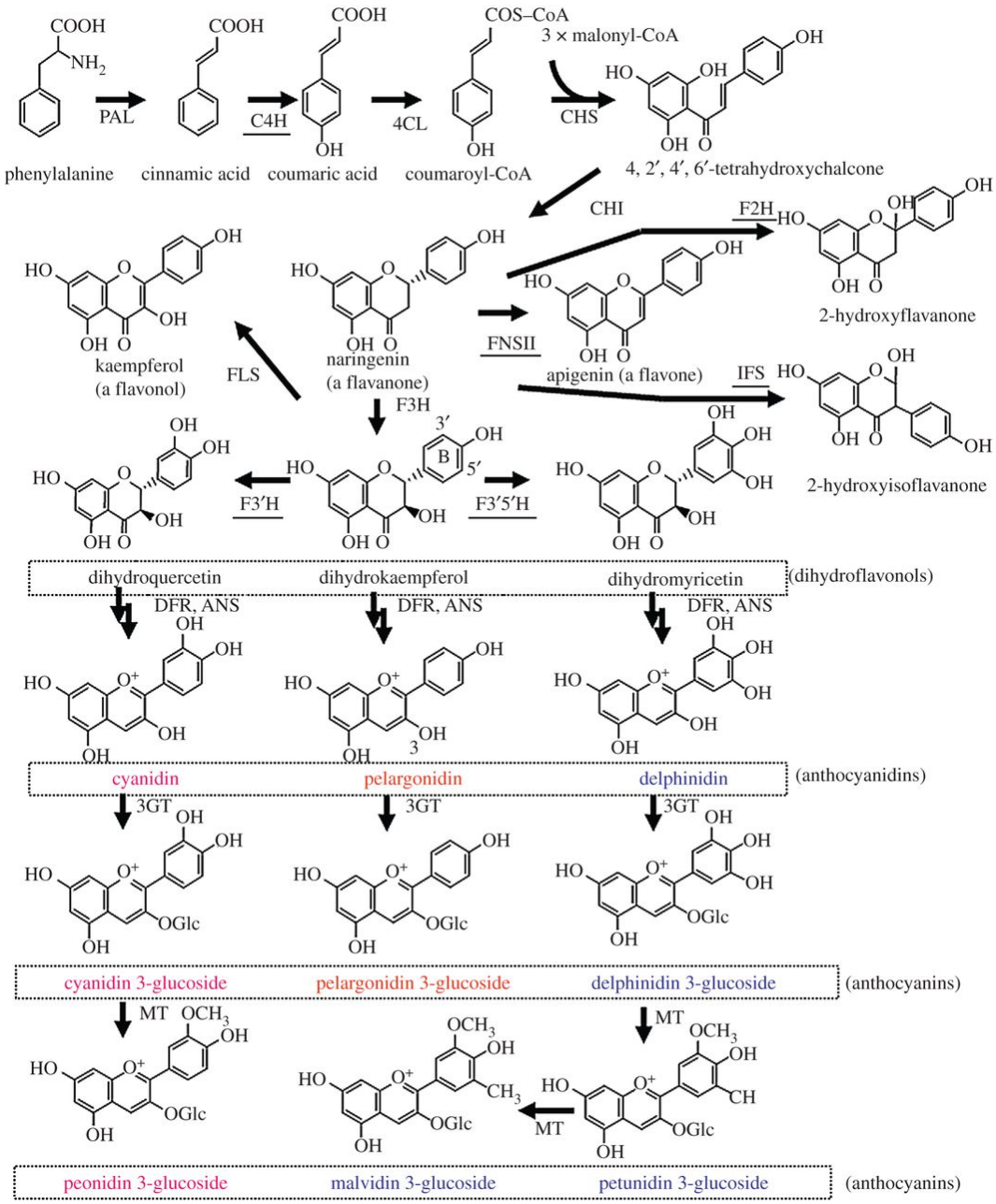

Fig 1. Flavonoids biosynthesis pathway leading the production of pelargonidin cyanidin, peonidin, delphinidin, petunidin and malvidin. Abbreviations include: PAL, phenylalanine ammonia lyase; C4H, cinnamic acid 4-hydroxylase; CL, 4-coumarate Co-A ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; FNS, flavone synthase; F2H; flavanones 2-hydroxylase; IFS, 2-hydroxyisoflavanone synthase; FLS, flavonol synthase; 3GT, UDP-glucose: anthocyanidin 3-O-glucosyltransferase; MT, S-adenosylmethionine: anthocyanin methyltransferase (Tanaka and Brugliera 2013).

2-2-1. Flower colour modulation

Blue/violet flowers contain delphinidin-based anthocyanins, magenta/red flowers have cyanidin-based anthocyanins and orange/brick red flowers contain pelargonidin-based anthocyanins (Yoshida et al. 2009). Due to natural absence of specific flower colour in many ornamentals such as yellow or orange in pelargonium, cyclamen, azaleas and African violets or blue in rose and carnation, breeders are using genetic transformation to generate novel colours in flowers (Debener and Winkelmann 2010).

There are three basic tactics to alter flower colour by means of gene transformation;

1) Introducing the novel sense derivatives of gene to open new pathway for producing a new metabolite.

2) Expression of sense derivatives of suitable gene to open a blocked pathway.

3) Gene silencing and down-regulation of undesirable gene in flavonoids pathway to redirect the pathway or open new branches (Martens et al. 2003).

The latter strategy can be performed in different ways named post-transcriptional gene silencing (PTGS), which is a useful molecular technique for down-regulation of gene expression. This technique can be performed by antisense (van der Krol et al. 1988), sense suppression (co-suppression) (Napoli et al. 1990) and using a hairpin double-stranded RNA (dsRNA) called RNAi (Waterhouse et al. 1998).

Since the discovery of RNAi in nematode *Caenorhabditis elegans*, RNAi has been vastly applied to interfere with the expression of specific endogenous genes and became a powerful tool for functional genomics in eukaryotes including plants (Fire et al. 1998). The RNAi gene suppression mechanism can be generally divided into two steps. The first step includes degradation of dsRNA by Dicer into small interfering RNAs (siRNAs) with 21 to 25 nucleotides long (Fig. 2a), which are homologous with the specific gene that is being suppressed (Bernstein et al. 2001). In the second step, the siRNAs join an RNase complex name RNA-induced silencing complex (RISC), which degrades messenger RNA (mRNA) of gene of interest and blocks the gene expression process (Fig. 2b) (Agrawal et al. 2003).

The frequency of phenotypic changes in transformed plants after gene silencing varies from less than 1% for antisense and up to 40% for co-suppression methods (Tanaka et al. 1998), while using RNAi is a more reliable technique for efficiently down-

regulation of gene expression (Nakamura et al. 2006). Chalcone synthase (CHS) which is the first enzyme in flavonoids biosynthesis pathway has been suppressed using antisense technique in gentian plants with frequency of 18% (Nishihara et al. 2006). Using RNAi gene silencing was more efficient (85%) and stable for down-regulation of CHS in same plant, although the promoter was different (Nakatsuka et al. 2008).

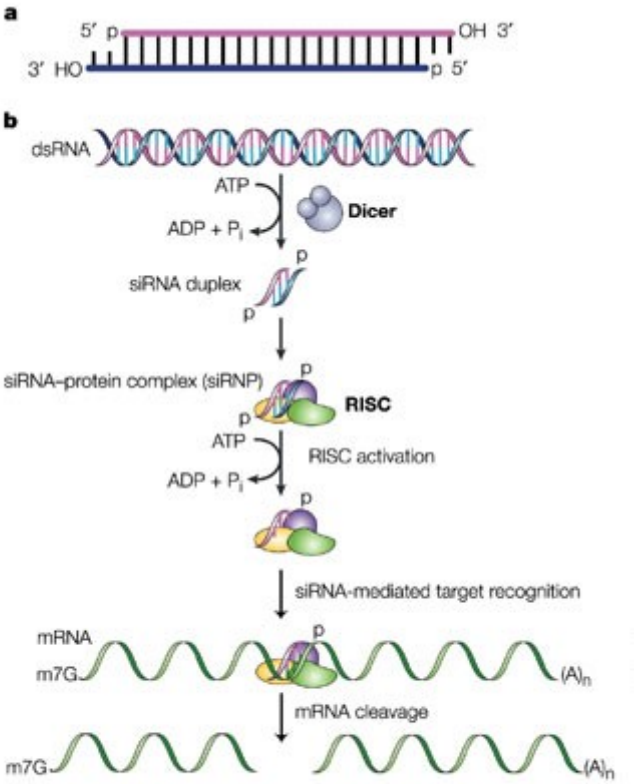

Fig 2. A model for RNAi gene silencing mechanism. a | Short interfering (si)RNAs. b | The siRNA pathway. Long double-stranded (ds)RNA is cleaved by Dicer and into incorporated into the RNA-inducing silencing complex (RISC). Although the uptake of siRNAs by RISC is independent of ATP, the unwinding of the siRNA duplex requires ATP. Once unwound, the single-stranded antisense strand guides RISC to messenger RNA that has a complementary sequence, which results in the endonucleolytic cleavage of the target mRNA. (Dykxhoorn et al. 2003).

RNAi was used in *Torenia hybrida* (Fukusaki et al. 2004) and liliaceous ornamental *Tricyrtis* sp. (Kamiishi et al. 2012) for down-regulation of CHS. Also RNAi has been used to down-regulate the structural genes, which have a function in anthocyanins

biosynthesis pathway to suppress accumulation of specific anthocyanins in tissue and flower colour alteration (Brugliera et al. 2013, Katsumoto et al. 2007, Nakamura et al. 2006, Nakatsuka et al. 2008, Tanaka et al. 2008).

2-2-2. Redirection of anthocyanins pathway toward red

In some plant species such as iris and *Gentiana* spp (gentian) due to accumulation of delphinidin-based anthocyanins and natural lack of pelargonidin-derived anthocyanins, blue or violet colour exhibits in flowers (Tanaka and Brugliera 2013).

DFR substrate specificity and dominant F3'H activity are two main reasons for lack or absence of pelargonidin and consequently nonappearance of red or orange colours in flowers (Tanaka and Brugliera 2013). DFR of many plants such as *Petunia hybrida* (Forkmann and Ruhnau 1987), *Cymbidium hybrida* (Johnson et al. 1999) and African daisy (*Oesteospermum hybrida*) (Seitz et al. 2007) are not able to accept dihydrokaempferol (DHK) and convert it to leucopelargonidin (LPg), which is the precursor of pelargonidin (Pg). But in chrysanthemum (*Dendranthema* × *grandiflora*), substrate specificity of DFR is not the factor for absence of pelargonidin and lack of pelargonidin is due to absence of substrate, results in dominant activity of F3'H (Schwinn et al. 1993).

The first successful metabolic engineering of flavonoids pathway was performed in *Petunia hybrida*. Native petunia DFR is unable to convert DHK to LPg (precursor of pelargonidin) and orange pigment. Therefore there is no orange colour in wild type of *Petunia* species, instead flowers accumulating kaempferol (Km) and DHK (Forkmann and Ruhnau 1987).

Expression of *DFR* coding sequence can contribute the ability of converting DHK to the Pg and exhibition of orange colour in plants. As by introducing *A1* gene from maize into the *Petunia*, Pg-derivatives and orange colour was produced (Meyer et al. 1987). However, neither overexpression of *Gerbera hybrida* nor *Fragaria* × *ananassa*, *DFR* genes which are able to efficiently convert DHK to the LPg, altered flower colour of *Oesteospermum hybrida*. While RT-PCR indicated the successful gene expression of *Gerbera hybrida* DFR in transformed tissues of *Oesteospermum hybrida*, chemical and spectrophotometrical analysis of transformed ray and disk floret did not show any difference in anthocyanins pattern compared to wild type. When same construct harbouring *Gerbera hybrida* DFR altered white flowers of

Petunia hybrida to the brick-red colour, speculated reasons were unavailability of DHK substrate for *Gerbera hybrida* DFR and strong endogenous *F3'5'H* activity in *Oesteospermum hybrida*, also DHK accumulation in *Petunia hybrida* (Seitz et al. 2007).

Because DHK is a common precursor for both anthocyanins and colourless flavonols, DFR and flavonol synthase (FLS) are competing for DHK (Tanaka et al. 1998). Nakatsuka et al. (2007) suggested that both down-regulation of FLS and introducing the *DFR* gene might be required for accumulation of pelargonidin and achievement of red colour.

In *Cyclamen persicum* Mill. which has a natural lack of pelargonidin-based anthocyanins by antisense suppression of endogenous *F3'5'H* gene in naturally purple colour cultivar, various hue of colour from purple to red/pink was attained. High-performance liquid chromatography (HPLC) analysis of transgenic flowers showed a reduction in delphinidin content in comparison with control flowers while cyanidin-based anthocyanins was increased. Total anthocyanins content was decreased up to 80% in some transgenic lines while flavonol levels increased and the quercetin/kaempferol ratio changed (Boase et al. 2010). Reduction of total anthocyanins suggests a low specificity of DFR for pelargonidin or cyanidin precursors. Down-regulation of *F3'5'H* and weak activity of *F3'H* combined with DFR that putatively does not recognize DHK led to limitation in the substrate flow toward pigment production and an increase in the substrate pool for FLS to produce flavonol (Ueyama et al. 2006).

Suppression of *F3'5'H* gene in blue flowers of *Torenia hybrida* resulted in pink flowers with diversion of delphinidin production to the cyanidin pathway. Analysis of anthocyanins content showed an increased amount of cyanidin and peonidin compared with controls and a slight amount of pelargonidin in transformed flowers. However, suppression of *F3'5'H* was not sufficient to generate clear redder flowers (Suzuki et al. 2000). Ueyama et al. (2002) was applied the transgenic line of torenia with pink flowers for further transformation and resulted in co-suppressing of *F3'5'H* gene to generate red flowers. They have considered the low level of *F3'H* expression in wild type and introduce full-length torenia *F3'H* complementary DNA (cDNA) in the sense orientation, driven by a constitutive promoter for overexpression of *F3'H*

gene. One of the eighty regenerated plants showed remarkable redder colour and exhibited an increased amount of cyanidin type anthocyanins in the transformant.

Down-regulation of *F3'5'H* and *F3'H* genes and overexpression of rose or pelargonium DFR gene in a blue or violet torenia cultivar resulted in various pink hue in flowers due to different levels of pelargonidin-based anthocyanins accumulation. Analysis of pelargonidin content in two transformed lines harboured rose or pelargonium DFRs, showed transformed flowers with pelargonium DFR accumulated pelargonidin more efficiently in comparison with transformed flowers which contain rose DFR. Thus it is possible that pelargonium DFR enzyme have a higher affinity for DHK catalysis or are able to more efficiently transcribed and translated in torenia in comparison to rose DFR (Nakamura et al. 2010).

While plant species DFRs have different substrate specificities, choosing the suitable exogenous DFRs is very essential for successful modulation of flower colour toward red colour. The pelargonium DFR gene is a useful molecular tool to engineer pelargonidin-accumulating plants. Also anthocyanins structure of host plant as a gene source is another crucial factor to perform successful redirection of flower colour to the red or orange colour. Furthermore pelargonidin content of flowers should be considered because some ornamental species such as gentian and iris have low pelargonidin content (Nakamura et al. 2010).

2-2-3. Flower colour in *Campanula*

As mentioned earlier, blue/violet petals contain anthocyanins derived from delphinidin, petunidin, or malvidin (delphinidin-based anthocyanins). Most delphinidin glycosides are mauve in colour and presence of co-pigments (flavones and flavonols) or metal ions, and higher vacuolar pH requires the shift to blue colour in petals (Yoshida et al. 2009). Anthocyanin composition of blue-flowering *Campanula isophylla, C. carpatica* and *C. poscharskyana* have been investigated and categorized into four phenotypes. The flowers contained one, two, three or four of the following anthocyanins: bisdeacylplatyconin, violdelphin, monodeacyclampanin and campanin (Brandt et al. 1993).

The flowers of the bellflower, *Campanula medium* L., vary in colour from white through pink to purple. By means of chromatography campanin (delphinidin derivative) and rubrocampanin (pelargonidin derivatives) were identified in

Campanula medium L. (Terahara et al. 1990). Genotypes which contain rubrocampanin as major anthocyanins are pink and flower colour of mutants, which the anthocyanins pathway has blocked after bisdeacylplatyconin, have very light purple colour (Brandt 1990, Terahara et al. 1990).

In violet *Campanula* flowers, four intermediate pelargonidin glycosides designated as Pg 1 (pelargonidin 3,7-diglucoside), Pg 2 (chromatographically indistinguishable from rutinose), Pg 3 (pelargonidin 3-glucoside) and Pg 4 (pelargonidin 7-glucoside) were detected (Asen et al. 1979).

However it is important to note the anthocyanin composition is different in various species and the anthocyanins should be detected in specific plant (Gomez et al. 2009).

2-2-4. Inspection of inhibition the flavonoids hydroxylase enzymes

Detection the presence of pelargonidin in anthocyanin composition or capability of plant to produce pelargonidin is important, specifically when one of the purposes of current work is blocking the *F3'5'H* gene. When anthocyanin content of petals includes pelargonidin, by inhibition the activity of F3'5'H enzyme, pelargonidin will accumulate in the petals and results in red colour. Furthermore, presence of pelargonidin after blocking the activity of F3'5'H and F3'H enzymes indicates that DFR accepts DHK as a substrate to convert to pelargonidin. Also in case of exclusivity of DFR related to dihydroquercetin after inhibition of F3'5'H and F3'H enzymes, different cyanidin derivatives are synthesized and dark red flowers will be produced (Fig. 1). Several chemicals, such as tetcyclacis and 1-aminobenzotriazole have been applied to characterize the anthocyanin biosynthesis pathway in different plants (Menting et al. 1994). Tetcyclacis is the plant growth retardant, which inhibits cytochrome P450 monooxygenases (Rademacher et al. 1987) and has been applied to visualized the colour results in enzyme blocking (Martens et al. 2003).

By application of tetcyclacis, activity of hydroxylase enzymes including F3'5'H and F3'H will be blocked. In case of capability of DFR to catalyze DHK to the pelargonidin, the petal colour will turn to orange/red after tetcyclacis treatments otherwise the petal colour will change nearly to white (Seitz et al. 2007).

2-3. Ethylene and postharvest quality

Ethylene is a gaseous plant hormone which is involved in regulation of various physiological processes such as growth, seed germination, flower initiation, fruit ripening, senescence and tissue abscission (Johnson and Ecker 1998). Ethylene biosynthesis is strongly regulated by internal signals and environmental factors from biotic and abiotic stresses including pathogen attack, wounding, hypoxia, ozone, chilling, or freezing (Wang et al. 2002). Increased production of ethylene has a direct effect on shortening the postharvest quality of many plants. As senescence and abscission occurs, commercial value of flowers diminishes due to reduction in visual characteristics and decrease in botrytis susceptibility (Owino and Ezura 2008).

Environmental and molecular genetic approaches can be applied to regulate the production or action of ethylene and consequently, growth and development of specific tissue can be controlled (Kumar et al. 2009). To efficiently elongate the flower vase life through anti-ethylene treatments, biosynthesis pathway of ethylene and response of plants to the ethylene should be well characterized.

Plants are considered as being climacteric or non-climacteric depending on the response to the exogenous ethylene or level of endogenous ethylene during the ripening and senescence. Climacteric plants are ethylene sensitive and senescence is accompanied by a temporary increase in ethylene production and respiration. Treatment of non-senescent flowers with ethylene rapidly induces petal senescence in climacteric plants. In non-climacteric flowers, no increases in ethylene production and respiration are apparent during flower senescence and exogenous ethylene has little or no effect on petal senescence. Carnations, Gypsophila and orchids are some climacteric or ethylene sensitive plants while gladiolus, tulip and iris are non-climacteric flowers (Serek et al. 2006).

Woltering and van Doorn (1988) have categorized plants into the family level based on sensitivity to the exogenous ethylene in terms of petal senescence. High sensitivity was found in Campanulaceae, Caryophyllaceae, Geraniaceae, Labiatae, Malvaceae, Orchidaceae, Primulaceae, Ranunculaceae and Rosaceae species; low sensitivity was observed in Asteraceae and Iridacae species and in most of the Amaryllidaceae and

Liliaceae species. Within one plant family, sensitivity of species was generally similar.

2-3-1. Ethylene biosynthesis pathway

Ethylene biosynthesis pathway (Fig. 3) is very active during the senescence of climacteric plants. Methionine is the precursor for ethylene production, which converts to S-adenosyl-methionine (S-AdoMet or SAM) by SAM synthase. SAM inverts to the aminocyclopropane 1-carboxylic acid (ACC) which is an intermediate agent in biosynthesis pathway of ethylene by ACC synthase (ACS) (Yang and Hoffman 1984).

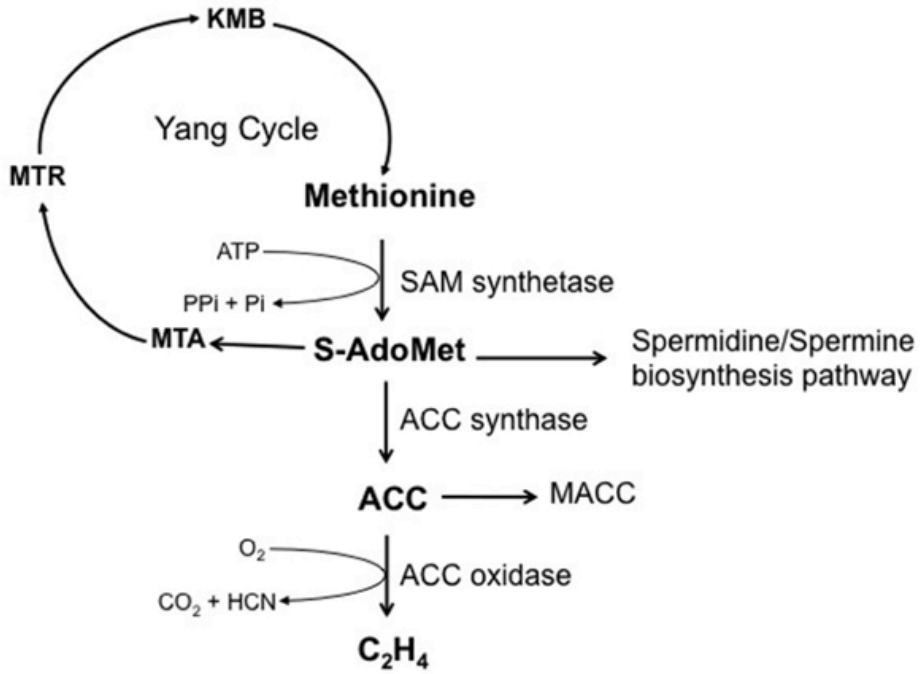

Fig 3. Ethylene biosynthesis pathway, S-AdoMet is catalyzed by SAM synthase from the methionine. ACC is an immediate intermediate product of ethylene, which is catalyzed form SAM by ACC synthase. Beside production of ACC, MTA is produced by ACC synthase (ACS) to use as a recycling source for methionine. Production of malonyl-ACC (MACC) by malonylation of ACC deprives the ACC pool and reduces the ethylene production. In final step of ethylene production, ACC oxidase convert ACC to ethylene (Arc et al. 2013).

In most cases the rate-limiting step of ethylene production is conversion of SAM to the ACC catalyzed by ACS (Wang et al. 2002). However, in some cases activity of ACC oxidase (ACO) an enzyme, which is responsible for conversion of ACC to the ethylene, also plays an important role in the regulation of ethylene production (English et al. 1995, Fernández-Otero et al. 2006, Ruduś et al. 2013, Vriezen et al. 1999, Yamamoto et al. 1995, Zhu et al. 2015).

2-3-2. Ethylene regulations

Different approaches have been applied to regulate the ethylene production in tissues and inhibition of the ethylene action to prolong postharvest quality of ornamental plants.

Serek et al. (2006) have categorized the most common tactics in three groups;

1) Interfering in the production of endogenous ethylene by blocking the components of pathway;

2) Inhibition of ethylene binding to its receptor in the tissues;

3) Blocking the reaction of plant to the binding of ethylene to the receptor.

These three strategies can be performed either by chemical treatments or genetic manipulation.

Blocking the activity of ACS can lower ethylene biosynthesis by decrease in ACC pool and result is delay of senescence. Aminoethoxyvinylglycine (AVG), aminooxyacetic acid (AOA) are chemicals which block the ethylene production by interfering in the activity of ACS (Yang and Hoffman 1984). STS, 1-methylcyclopropene (1-MCP) (Serek et al. 1995), cobalt ion (Co^{2+}) (Yu and Yang 1979) and nickel ion (Ni^{+2}) (Jamali and Rahemi 2011) are other chemicals known as anti ethylene agents and have been vastly used in inhibition of ethylene and prolongation of ornamentals vase life.

Molecular breeding technique is another powerful approach that has been used to inhibit or lower biosynthesis of ethylene. For instance expression of antisense ACO gene into the genome of melon decreased the production of ethylene to less than 1% of the control plants and inhibits the ripening process (Ayub et al. 1996). Same result obtained by double antisense fusion using ACO and ACC genes in tomato and shelf life prolonged over 50 days in transgenic fruits (Xiong et al. 2003). By application of the co-suppression technique and insertion of additional ACS copies of gene in sense orientation to the genome of carnation, ethylene production was decreased and vase life prolonged in transformed flowers (Kepczynski and Kepczynska 2005).

The antisense or over-expression of an antisense approaches also have been applied in other ornamental crops such as begonia, torenia and carnation, to suppress ACC synthase or/and ACC which are active in biosynthesis pathway of ethylene to inhibit or decrease ethylene production (Agarwal et al. 2012).

2-3-3. Ethylene signaling

The key proteins responsible for the reactions to the ethylene and first component of ethylene signaling are ethylene receptors. The ethylene receptors in *Arabidopsis* are classified into two subfamilies of I: ETR1, ERS1 and II: EIN4, ETR2 and ERS2. ETR1 is the first identified ethylene receptor (Chang et al. 1993). The other four members of the *Arabidopsis* receptors are similar in overall structure to ETR1 and greatest level of conservation among all receptors was found in the amino terminal ethylene-binding domains (Chang and Shockey 1999). Downstream from the receptors is the protein kinase, CTR1 that interacts with ETR1 and regulate the ethylene signaling pathway negatively (Ouaked et al. 2003). In presence of ethylene, binding of ethylene to the N-terminal domain that is localized in endoplasmic reticulum inhibits receptor activity and results in inactivation of CTR1. Following the inactivation of CTR1, downstream components of the signaling pathway reveal the repression of signaling and resulting in induction of ethylene responses. But in absence of ethylene, CTR1 is active and represses further signaling (Chen et al. 2002, Klee 2004).

2-3-3-1. Mutations in ethylene signaling pathway

Mutations affecting function of ethylene receptors have been characterized in different species including *Arabidopsis* and tomato (Gallie 2010).

Mutations can be divided into two types; mutants which show the ethylene triple response (alteration of dark grown seedlings in response to the ethylene and involves reduction in elongation, swelling of the hypocotyls and changes in growth direction) and constitutive expression of ethylene-regulated gene even in absence of ethylene. This type of mutants includes constitutive triple response 1 (*ctr1*), ethylene overproduction 1 (*eto1*), *eto2* and *eto3* (Theologis 1998).

Second type, includes ethylene insensitive mutants such as *etr1-1* and ethylene insensitive 2 (ein2), ein3, ein4, ein5, ein6, ein7 and ACC insensitive 1 (ain1) (Theologis 1998). This class of mutants is characterized by constitutive signaling by the receptor, and inability to perform triple response in presence of ethylene, resulting in a dominant negative effect (Bleecker et al. 1988, Chang et al. 1993). *Etr1-1* is one of these mutants that has a Cys-to-Tyr mutation at residue 65 in the N-terminal trans-membrane domain and results in a strong ethylene-insensitive phenotype due to failing the binding of ethylene to the receptor (Chen and Gallie 2010). As *etr1-1*

mutant plants are unable to respond to the ethylene known as ethylene insensitive. Expression of *etr1-1* gene to the genome of different species of plants results in ethylene insensitivity and delays the senescence. To date *etr1-1* gene has been introduced to different plants including tobacco (*Nicotiana tabacum*) (Knoester et al. 1998), carnation (*Dianthus caryophyllus*) (Bovy et al. 1999), petunia (*Petunia hybrida*) (Gubrium et al. 2000), *Campanula carpatica* (Sriskandarajah et al. 2007) and *Kalanchoe blossfeldiana* (Sanikhani et al. 2008).

2-4. Selection markers

In order to efficiently distinguish the transgenic cells or tissues; selectable marker genes are used with the gene of interest. In plant transformation, the neomycin phosphotransferase II (*npt II*) gene is one of the most commonly used selective maker genes (Flavell et al. 1992). The *npt II* gene encodes neomycin phosphotransferase II, which confers resistance on its host cells to a wide range of structurally related aminoglycosides neomycin antibiotics such as kanamycin, neomycin, hygromycin, paromomycin and geneticin (Moazed and Noller 1987). These antibiotics have a generally similar effect on cells and inhibit the growth of plant cells by binding to the 30S ribosomal subunit, thereby inhibiting initiation of translation and consequently protein synthesis (Wilmink and Dons 1993).

As most frequently used marker genes are antibiotic or herbicide resistant, concern about unexpected transfer of these genes by outcrossing into the food product and consequently spread of antibiotic resistance to the human or weeds has been raised (Dale et al. 2002). Furthermore absence of resistance gene in genetically modified products will lower the developing and releasing costs and speed up the publicizing process of these products (Kuiper et al. 2001). Alternative selection markers or removal of selection genes are the main solutions to increase public acceptance of transgenic products.

2-4-1. Positive selectable marker genes

Positive selection system is an approach in which transformed cells use a metabolic ability over untransformed cells to utilize specific agent and offers safe selection method and a harmless alternative for antibiotics or herbicides selection systems. In this method selective agent converts into the compound using positive effects by

expression of specific genes, which transformed cells are harbouring. For instance, *gusA* (ß-glucuronidase) gene from *Escherichia coli* hydrolyzes benzyladenine-glucuronide (Okkels et al. 1997) into active cytokinin and stimulates growing, while development of untransformed cells blocked due to absence of *gusA*.

Expession of xylose isomerase gene (*xylA*) from *Streptomyces rubiginosus* (Wong et al. 1991) or the 6-phosphomannose isomerase (*pmi*) (*manA*) gene of *E. coli* (Miles and Guest 1984) enables transformed cells, the conversion of specific agents into the exerting metabolites, while untransformed cells are starving.

2-4-1-1. Mannose selection system

As many plants are not able to utilize mannose as carbohydrate source, such cells starve and do not grow in media that contain mannose (Malca et al. 1967). In addition, due to deficiency of PMI, which is necessary for conversion of mannose-6-phosphate to fructose-6-phosphate (a precursor for the glycolytic pathway), accumulation of mannose-6-phosphate in cells occurs and causes block in glycolysis and severe growth inhibition (Goldsworthy and Street 1965). In mannose selection system, cells which are transformed with the *pmi* gene that encoding PMI, are able to utilize mannose for ATP production (Sigareva et al. 2004) (Fig. 4).

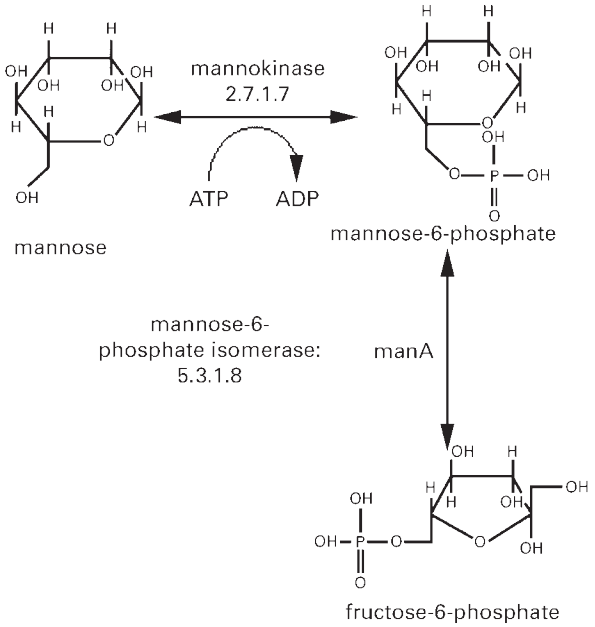

Fig 4. Schematic reaction of mannose conversion to the fructose-6-phosphate catalyzes by phosphomannose isomerase encodes by the *manA* gene (Penna et al. 2002).

Efficient mannose selection has been established successfully in different plants such as rice (*Oryza sativa*) (Lucca et al. 2001), tomato (*Solanum lycopersicum*) (Sigareva et al. 2004), kalanchoe (*Kalanchoe blossfeldiana*) (Ilczuk et al. 2009), orchid (*Oncidium* Gower Ramsey) (Thiruvengadam et al. 2011) and chrysanthemum (*Dendranthema* × *grandiflora*) (Xie et al. 2012).

The Establishment of Effective Regeneration and Selection Systems in *Campanula portenschlagiana* and *carpatica* Cultivars

3. The Establishment of Effective Regeneration and Selection Systems in *Campanula portenschlagiana* and *carpatica* Cultivars

Abstract:

The current study was performed on the following three *Campanula* cultivars: *C. portenschlagiana* cvs. Blue Ocean (BO) and Royal; *C. carpatica* cv. Improved Blue Uniform (IBU). With a focus on avoiding over-supplementation of plant growth regulators (PGRs), the optimal media composition and explant source for shoot regeneration were determined. In addition, the present work selected the optimum *Agrobacterium* strain for gene transformation and compared two different selection systems using mannose and kanamycin in the cultivar IBU. Nodal cuts of mature greenhouse plants, grown in a hormone-free media were the most favorable explant source for harvesting petioles. The best shoot regeneration obtained from the harvested petioles of nodal cuts in the three cultivars were: 85% IBU, 65% BO and 45.8% Royal. After gene transformation of cultivar IBU, 11.42% of the petioles in 100 mg l^{-1} of kanamycin were regenerated shoots while shoot regeneration occurred in 8.33% of petioles cultured in a medium containing 30 g l^{-1} mannose. All the shoots of the selection/regeneration media were examined by GUS assay and indicated GUS activity. According to the results, the non-antibiotic selection system employing mannose was shown to be as effective as the antibiotic (kanamycin) selection method to provide an alternative selection technique.

Keywords**:** explant source, TDZ concentration, gene transformation, mannose, kanamycin

1. Introduction

Several species of *Campanula* genus have been cultivated as ornamental plants, and there is an increasing demand for more attractive and novel characteristics, including new flower shapes and colours. Biotechnological methods and genetic manipulation represent proven functional approaches for producing new characteristics in ornamentals. Furthermore, *in vitro* propagation has been adopted for other purposes, such as the elimination of plant viruses (Cheong et al. 2012) or bacteria (Nadha et al. 2012) during stock plant production. While gene transformation is a useful and effective method to achieve new characteristics, concern has been raised about the possible hazard of employing antibiotic and herbicide selection genes in transformation. Therefore finding of an alternative selection method in transformation is a pressing issue (Hohn et al. 2001). An effective regeneration protocol and selection system for transformed shoots is prerequisite for subsequent successful genetic manipulation. Although approximately 400-600 species exist in the *Campanula* genus (Kuss et al. 2007), only a few species have been the focus of regeneration research (Brandt 1992; Sriskandarajah et al. 2001; Frello et al. 2002; Joung et al. 2002; Sivanesan et al. 2007; Sivanesan et al. 2011) and, to date there has been no study concentrating on the selection method in *Campanula*.

One of the most critical factors affecting shoot regeneration for *in vitro* culture establishment is the explant type and source. Different explant types, such as the stem segment with axillary buds in *C. isophylla* (Brandt 1992), hypocotyls and cotyledons in *C. carpatica* (Sriskandarajah et al. 2004), and leaves and petioles in *C. punctata* (Sivanesan et al. 2011) have been investigated in the *Campanula* species. However, methods found to be effective for one *Campanula* species may not be applicable to other species. For example, although the use of hypocotyls and cotyledons as explants for *C. carpatica* have proven to be successful, this approach failed in *C. velebitica* (Stamenkovic et al. 2012) and *C. poscharskyana and C. portenschlagiana* (Gargul 2006) due to the relatively high contamination and low germination of seeds in *in vitro* conditions. Although juvenile tissue explants are generally favored, mature tissue explants or the explant source are required for certain plants (Bonga and von Aderkas, 1992). Because of the recalcitrant habit of mature plants, it is necessary to determine which strategy is optimal for overcoming this obstacle. Tissue reinvigoration and the mitigation of recalcitrance in some plants can be forced by the

application of PGRs in the culture medium (Murthy et al. 1998) or by the use of a hormone-free medium for some species (Ewald 1998). Employing adult plants as the source of nodal cuts in the *Campanula* species was previously explored (Sriskandarajah et al. 2008, Paunescu 2010). According to the current study's knowledge, although the preparation of adult plants as a source of nodal cuts and explants in the *Campanula* species has been performed by the addition of PGRs to the media, a hormone-free medium has not yet been employed for rejuvenation. The regeneration of abnormal tissue often occurs during *in vitro* culture. Bairu et al. (2006) concluded that PGRs elicit indirect effects on somaclonal variation by increasing the multiplication rate. Other abnormalities, such as shortened internodes and stunted and retarded shoots (Uzelac et al. 2012; Lincy and Sasikumar 2010) and blocked root formations (Arora et al. 2009), were previously demonstrated to be consequences of excessive cytokinin use. Therefore, the manipulation of published *in vitro* culture protocols for the purpose of decreasing hormone application in media is crucial specifically when using a mature explant source is crucial.

TDZ is a hormone that effectively promotes shoot regeneration, some disadvantages have been observed when it is used at high concentrations. Inhibited shoot elongation due to elevated concentrations of TDZ, has been reported in studies investigating *Bambusa edulis* (Lin and Chang 1998). Kadota and Niimi (2003) explored the effects of different cytokinins on shoot regeneration and the *in vitro* hyperhydricity of *Pyrus pyrifolia* and found that explants produced more vitrified shoots during the application of TDZ. Joung et al. (2002) applied benzyl adenine (BA) to induce shoot formation from *C. glomerata* leaf blades while omitting TDZ in the regeneration media; that study based on its findings, recommended the use of TDZ to increase the regeneration rate and induce shoot formation within a shorter time. In the *Campanula* species, an increase in the concentration of TDZ up to 10 mg l^{-1} induced shoot regeneration, whereas higher amounts of TDZ significantly reduced shoot regeneration and promoted increased callus formation. In another work, when greenhouse-grown plants were used as a source of explants, all of the explants failed to regenerate shoots (Sriskandarajah et al. 2008). Frello et al. (2002) found a reduced regenerate rate in *C. carpatica* when cotyledons were utilized via the callus phase. However, a notably impaired transformation by *Agrobacterium tumefaciens* was observed in that study and attributed to deficient shoot regeneration. Thus, for the

high shoot regeneration in *Campanula* spp, it is essential to determine the optimal concentration of PGRs, particularly that of TDZ, as well as the optimal sources of explants.

Gene transformation is a crucial technique to generate novel characteristics in plants. *Agrobacterium*-mediated transformation is one the most applicable tools for gene transformation. Stable transformation is a lengthy procedure and involves organ regeneration, while transient transformation is a suitable alternative to analyse particular targets in the gene transformation process (Wroblewski et al. 2005). For most plants, transient transformation performs by the infiltration of bacteria suspensions into the plant cells (Manavella et al. 2009). To determine a clear result from transient transformation, the expression of a reporter gene, such as β-glucuronidase (GUS) (Jefferson et al. 2009) or green fluorescent protein (GFP) (Chiu et al. 1996) will be assayed. In the current study, transient transformation is applied to explore the most penetrative strain of *Agrobacterium* in *Campanula*.

After transformation, only a few cells are transformed and must be selected. Selection methods are based on the ability of transformed cells to metabolize a substance, a conversion which is not possible for non-transformed cells (positive selection) (Penna et al. 2002); or the resistance of transformed cells to antibiotics or herbicides despite the non-transformed tissue (negative selection). Most plants do not have the ability to convert mannose to fructose-6-phosphate and to utilize it as a source of energy. The *manA* gene, which encodes the phosphomannose isomerase (PMI) transfers the ability of methaboliting mannose to the transformed tissue; therefore in a medium containing mannose, the newly transformed tissue can survive and the non-transformed tissue will starve and die. The mannose selection system has been investigated in different plants, such as *Torenia fournieri* (Li et al. 2007), *Kalanchoe blossfeldiana* (Ilczuk et al. 2009), cucumber (He et al. 2006) and sugarcane (Jain et al. 2007). To date, this study is the first one to clarify the possibility of using mannose selection in *Campanula*.

In the present work, different explant sources and a variety of concentrations and combinations of PGRs are investigated with the goal of improving shoot regeneration in three *Campanula* species. Also, different *Agrobacterium* strains and two methods of selection were compared. The main objective of the current investigation was to

improve the shoot regeneration capacity in greenhouse-grown *Campanula* plants by using a minimal concentration of PGRs; this was to prevent negative effects and to demonstrate the capacity of the non-antibiotic selection method when replacing the antibiotic selection system in cv. IBU.

2. Materials and methods

Plant materials

The adult plants of two *Campanula* species, *C. portenschlagiana* cvs. Blue Ocean and Royal and *C. carpatica* cv. Improved Blue Uniform, were grown in an experimental greenhouse whose temperature was maintained at 18-22˚C under 337 μmol m^{-2} s^{-1} light. Short-day conditions (8 h light) were simulated to keep the plants in a vegetative stage for the collection of nodal cuts (Fig. 3a).

Nodal cut shoot growth and regeneration of petioles

Media optimization for the shoot growth of greenhouse-grown nodal cuts of three cultivars

Fresh shoots were harvested from all three *Campanula* cultivars that were grown under the present study's greenhouse conditions. After the removal of the leaf blades, the shoots were washed under running tap water for 15 minutes and then sterilized for 20 minutes in 1.5% (v/v) sodium hypochlorite solution supplemented with a drop of Tween-20. Next, the nodal cuts were washed three times in sterilized distilled water. Each explant was prepared with an 8-10 mm inter-node and one or two nodes (Fig. 3b). All of the media were based on MS macro and microelements (Murashige and Skoog, 1962) and were supplemented with 10 g l^{-1} glucose (AppliChem GmbH, Darmstadt, Germany), vitamin B_5 and 15 g l^{-1} sucrose (Duchefa, Haarlem, the Netherlands). The media were gelled using 3.5 g l^{-1} plant agar (Duchefa) and 1.25 g l^{-1} gelrite (Duchefa), and the pH was adjusted to 5.7-5.8 prior to autoclaving. Two PGR-containing media, 0.5 mg l^{-1} BA and 10 mg l^{-1} TDZ, both supplemented with 0.25 mg l^{-1} naphthaleneacetic acid (NAA) (Duchefa, Haarlem, the Netherlands), and one hormone-free medium were compared for the cultivation of nodal cuts.

In each treatment condition, 6 replicates of 4 explants each were tested. Nodal cuts were grown in the dark at 24˚C (Fig. 3c). The data were collected after 2 weeks.

Optimization of the explant source for shoot regeneration

Explant sources and cytokinin (TDZ) levels of three Campanula cultivars

Two different petiole sources were compared in terms of the shoot regeneration capacity of all investigated cultivars. The first type of explant was comprised of petioles harvested directly from plants cultured *in vitro* under 16 hours of 32-48 μmol m^{-2} s^{-1} light and 24 ± 1˚C. The plants cultured *in vitro* (Fig. 3g) were maintained for approximately 3 months in tissue culture conditions. The second type of explant was comprised of petioles that were prepared from two-week-old shoots grown *in vitro* (darkness, 24 ± 1˚C) and derived from the nodal cuts of greenhouse-grown plants. The MS medium was supplemented with 1.0, 2.0 or 3.0 mg l^{-1} TDZ. All of the media contained 0.1 mg l^{-1} NAA. Five replicates, each consisting of 4 explants, were investigated. The petioles were maintained in the dark at 24 ± 1˚C to regenerate shoot, and the data were collected after 7 weeks of cultivation.

Explant sources and auxin (NAA) concentrations in C. portenschlagiana cv. Royal

To determine the optimal explant sources, two types of petiole sources of cv. Royal were assessed for shoot regeneration capacity. The first type of petiole was harvested from the shoots of nodal cuts from greenhouse-grown plants raised in the dark in a hormone-free medium. The second type of petiole was harvested from three-month-old plantlets grown *in vitro* and cultivated in a hormone-free medium. Three different auxin concentrations (NAA) were tested. A combination of two cytokinins, 5.0 mg l^{-1} TDZ and 5.0 mg l^{-1} BA, supplemented with three concentrations of NAA (0.5, 1.0 or 2.0 mg l^{-1}), was used to promote shoot regeneration. In each treatment, 6 replicates of 4 explants each were examined.

Gene transformation

Transient transformation

The *Agrobacterium* strains AGL1, GV3101 and EHA105 harbouring the *GUSi* reporter gene (for easy detection of transformed shoots) were inoculated with a LB medium containing a spectinomycin antibiotic and grown overnight at 28˚C up to OD_{600} = 0.6. An infection buffer was prepared by using 10 mM MES plus 10 mM $MgCl_2$ and the pH was adjusted to 5.6 before autoclaving. Bacteria cells were

collected by centrifuging and re-suspended in an infection buffer. To increase the infectious ability of the bacteria 40µL mL^{-1} of acetosyringone was added to the prepared *Agrobacterium* inoculums. For each strain, 10 flower buds of *C. carpatica* cv. IBU were sprayed with prepared inoculums in the greenhouse with a pressure pump sprayer. In addition, 10 flower buds were sprayed with an infection buffer liquid as a control. The sprayed flowers were collected four days after spraying and submerged in a GUS assay substrate for vacuum infiltration. Afterwards, the flowers were transferred to a temperature of 37˚C for overnight. To remove the colour of the petals, so as to make the blue colour of the GUS product visible, the flowers were submerged in 70% ethanol for a few days. The best penetrative strain for stable transformation was chosen according to the presence of the colour blue in the petal's tissues.

Optimum kanamycin concentration

To investigate what is the optimal kanamycin concentration for the selection of transformed shoots a pre transformation experiment was performed. Harvested petioles of nodal cuts (prepared as described) were transformed with the *Agrobacterium tumefaciens* strain GV3101 harbouring the *GUSi* reporter gene. Transformed petioles were then placed for three days in a medium containing no selection as a pre-culture for bacteria incubation. After those three days the petioles were transferred to the same MS medium supplemented with 3 mg l^{-1} TDZ and 0.1 mg l^{-1} NAA and containing 0, 50 or 100 mg l^{-1} kanamycin. The transformed petioles were kept in the dark at 24 ± 1˚C in order to regenerate shoots. Six weeks after transformation, the shoot regeneration was evaluated to choose the kanamycin concentration; the regenerated shoots were then tested by the GUS assay to confirm the successful transformation. In each treatment, 5 replicates of 5 explants each were tested.

Best mannose level for selection

Different concentrations of mannose/glucose: 0/20, 5/15, 10/10, 15/5, 20/0, 25/0, 30/0 g l^{-1} were tested to determine the sensitivity of the explants to the mannose. Nodal cut petioles of cv. IBU were prepared according to the described method and cultivated in different media containing combinations of mannose/glucose and 5 mg l^{-1} TDZ and

0.1 mg l^{-1} NAA. Six weeks after cultivation, shoot regeneration was evaluated in each medium. In each treatment, 5 replicates of 4 explants each were investigated.

Transformation using kanamycin or mannose

To compare the two methods for the selection of transgenic tissue, nodal cut petioles, which had been grown in the dark, were prepared in a MS hormone-free medium. Used for transformation, the *Agrobacterium tumefaciens* strain GV3101 harbours the plasmid p9Ndo containing *nptII* gene (for kanamycin resistance) or plasmid pNOV2819 contained *pmi* gene (for mannose catalyzing). Three days after gene transformation, the petioles were transferred to a media containing selection. The same process using no bacteria was performed as a transformation control. Depending on the plasmids applied for gene transformation, 100 mg l^{-1} kanamycin or 30 g l^{-1} mannose was used for selection. For each treatment, 9 replicates of 4 explants were examined. Six weeks after cultivation, the regenerated shoots were counted and GUS test was performed.

Statistical analysis

The data were subjected to ANOVA by the use of the MSTATC software (Michigan State University, version 1.42). The mean values were separated according to the least significant difference test (LSD) at an alpha level of 5% ($P \leq 0.05$).

3. Results

Optimization of media for nodal cut growth and shoot regeneration of petioles

The effect of hormone treatments on the shoot growth of nodal cuts harvested from greenhouse-grown plants of three Campanula cultivars

The experiment was designed to clarify the effects of growth regulators on the growth of axillary shoots derived from nodal cuts. As Fig. 1 shows, supplementation of the medium with 10 mg l^{-1} of TDZ induced a 95.8% growth of axillary shoots from cv. BO nodal cuts. In media with 0.5 mg l^{-1} BA, 79.1% of the nodal cuts produced secondary shoots, while this figure was 91.6% for the cuts derived from axillary shoots in a hormone-free medium. The lack of significant differences among the tested media indicated that a hormone-free medium is just as effective as the media containing PGRs. In cv. Royal (Fig. 1), no significant differences were observed between the number of shoots from the nodal cuts that were cultivated and

regenerated in a hormone-free medium and the nodal cuts cultured in a medium with 10 mg l^{-1} TDZ. In a 0.5 mg l^{-1} BA medium, only 38.9% of the nodal cuts produced shoots. In cv. IBU (Fig. 1), the growth of nodal cuts from media containing TDZ or BA was 100%, whereas in the hormone-free medium there was only a 72.1% shoot growth.

The effects of explant source on shoot regeneration

The effects of explant sources and TDZ levels on the shoot regeneration of three cultivars

The present experiment's aim was to compare the effects of two explant sources and three concentrations of TDZ on the shoot regeneration of three cultivars of *Campanula*. In cv. BO, the nodal cuts from greenhouse-grown plants represented a more favorable source for petioles compared to petioles from plants that were cultivated under *in vitro* conditions (Fig. 2).

In cvs. BO and Royal, no shoot regeneration was observed from the petioles of tissue culture plants that were grown in a medium containing 1 mg l^{-1} TDZ. On the other hand, cv. IBU 35% of the petioles regenerated shoots in such medium (Fig. 2). In cv. Royal (Fig. 2), no shoot regeneration occurred in any of the three TDZ concentrations from petioles harvested from plants grown *in vitro*.

The effects of explant sources and auxin (NAA) concentrations on the shoot regeneration (%) of C. portenschlagiana cv. Royal

Two types of explant sources (petioles harvested from the nodal cuts of greenhouse-plants and the petioles of plants grown in tissue culture) and three concentrations of NAA were compared by the current study. The results identified an optimal explant source and NAA concentration in a medium to achieve an optimal regeneration in cv. Royal. The use of petioles harvested from the nodal cuts of greenhouse-grown plants significantly improved the shoot regeneration. In contrast, petioles harvested from plants grown in tissue culture resulted in poorer shoot regeneration when compared to that of nodal cuts from plants cultivated in all three media.

The percent of shoot regeneration exhibited significant differences at α=0.05 in two types of petioles cultivated in media containing 5.0 mg l^{-1} TDZ, 5.0 mg l^{-1} BA and 2 mg l^{-1} NAA. Increasing the amounts of NAA efficiently augmented the shoot

regeneration, and using 2.0 mg l^{-1} NAA in combination with 5.0 mg l^{-1} TDZ and 5.0 mg l^{-1} BA boosted the regeneration of shoots from petioles to 45.8% (Fig. 3d, e and f). There was no significant difference between the two types of petioles in media containing 0.5 mg l^{-1} NAA and 1.0 mg l^{-1} NAA (Fig. 4). The current study's cv. Royal shoot regeneration results confirmed the advantage of using petioles harvested from nodal cuts over petioles from plants grown in tissue culture.

Gene transformation

Transient transformation

The petals of cv. IBU were sprayed by inoculums of three strains of AGL1, GV3101 and EHA105 harbouring the *GUSi* construct. After GUS assay and the removal of the petal colour by ethanol, the GUS product was visible in 8 flowers, which were sprayed by the GV3101 strain (Fig. 5). In flowers that treated with AGL 1 just 1 flower exhibited the GUS signal while no signal was detected in flowers, which were sprayed with EHA105. Therefore, GV3101 was selected as the best strain of *Agrobacterium* for the transformation of cv. IBU.

Using antibiotic selection system (kanamycin)

To clarify the proper kanamycin concentration, an experiment was designed using 0, 50 or 100 mg l^{-1} kanamycin in the selection media after transformation. Shoot regeneration of the transformed petioles of cv. IBU was measured after six weeks (Fig. 6). As expected, in media supplemented with 50 mg l^{-1} kanamycin (8.0%) or 100 mg l^{-1} kanamycin (10.66%), shoot regeneration was reduced and there was a significant difference in comparison with the shoot regeneration of explants in the control medium (51.32%).

Mannose as a carbon source and its effect on shoot regeneration

To examine the response of *Campanula* cv. IBU and find the border of explant sensitivity, an experiment was designed. A mixture of mannose and glucose was examined in different concentrations (Table 1). In a medium containing 5/15 g l^{-1} mannose/glucose, 61.11% of explants regenerated shoots while in medium supplemented with 15/5 g l^{-1} mannose/glucose shoot regeneration dramatically decreased and a 15.78%, shoot regeneration was observed. In media contained 20/0, 25/0 and 30/0 g l^{-1} mannose/glucose shoot regeneration was suppressed and no shoots were regenerated.

Positive and negative selection systems in gene transformation of C. carpatica cv. IBU

In this experiment, 100 mg l^{-1} kanamycin or 30 g l^{-1} mannose was applied in regeneration media for selection of transformed shoots during regeneration. Also, a medium containing no selection agent was used as a control for regeneration condition. Shoot regeneration (%) in a medium with 100 mg l^{-1} kanamycin was 11.42% and, in a medium supplemented with 30 g l^{-1} mannose 8.33% of the explants were regenerated shoots. In addition, 69.44% of non-transformed petioles acting as a control in a medium having no selection agent were regenerated shoots (Table 2).

4. Discussions

To establish nodal cuts, a variety of cytokinin and auxin combinations have previously been studied for *Mecardonia tenella* (Alderete et al. 2006) and *Capparis spinosa* (Musallam et al. 2011). The current study's demonstrated that in cvs. Royal and BO, the use of PGRs did not enhance nodal cut growth in comparison with the hormone-free medium. Furthermore, the side effects of utilizing media supplemented with high PGRs concentration, reported in previous studies by Sriskandarajah (personal communication) and Meng et al. (2004) by use of hormone-free media in current study were avoided. Sriskandarajah et al. (2008) witnessed some side effects following the use of high concentrations of TDZ (> 10 mg l^{-1}), such as calluses that fail to regenerate shoots. Meng et al. (2004) previously reported that the pretreatment of cultures with 1.0 μM TDZ (0.2 mg l^{-1}) significantly increased the frequency of abnormalities.

For *C. polymorpha*, combinations of BA and kinetin with a variety of auxins have been assessed for their effects on nodal segment growth (Paunescu, 2010). According to the current study's experimental results, media supplemented with PGRs induced the growth of axillary shoots, although a hormone-free medium was equally effective for cvs. BO and Royal. The use of a hormone-free medium during the rejuvenation of nodal cuts was previously recommended by Ewald (1998). A hormone-free medium is strongly recommended due to its efficiency in axillary shoot growth; and its reduction if explant exposure to PGRs and the chance of abnormal shoot regeneration.

Generally, after the rejuvenation of mature tissues, invigorated explant sources have been favored for harvesting explants compared to explants from *in vitro* tissue

cultured plants. This preference is attributed to several underlying factors that reduce the regeneration rates of explants harvested from plants grown in tissue culture. Bhojwani and Razdan (1996) have previously found that a long-term culture can results in the loss of totipotency in cells remaining recalcitrant, as they have lost their ability to respond to factors that induce regeneration in tissue culture. In all three cultivars, a rising shoot regeneration trend was observed in petioles harvested from nodal cuts cultivated in progressively increasing concentrations of TDZ. The same results were obtained with *Cassia sophera*; by increasing the TDZ concentration up to 2.5 μM, the shoot regeneration frequency was increased (Parveen and shahzad 2010).

In the current work's nodal cut culture, the cuts were cultivated in media for a relatively short time for axillary shoot growth and, after two weeks, petioles were harvested from the newly grown shoots. Hence, the newly grown plant tissue was fresh, of good quality and capable of regeneration. In addition, nodal cuts that were used as a source of explants were cultivated in the dark for shoot growth. The advantage of employing etiolated tissues as sources of explants for regeneration was confirmed by several experiments (Li et al, 2009). Joung et al. (2002) highlighted the contamination problems encountered with *C. glomerata* while using material derived from greenhouse-grown plants. In this study, infected shoots were eliminated during the establishment of nodal cuts and healthy grown shoots were chosen for the harvesting of petioles. Therefore, the contamination of explants did not affect shoot regeneration (%), which was calculated from the percent of regenerated explants from all the examined explants in the current study. These advantages can explain the favorability of using nodal cuts.

For gene transformation, GV3101 is one of the most commonly used strain for gene transformation as for transient transformation of rose petals, was selected as best strain (Yasmin and Debener, 2010). To select transformed cells different selection system can be applied. Kanamycin is a well-known antibiotic, which used for selection of transformed tissues. Decreases of shoot regeneration and basically increscent of concentration of antibiotics causes full or partial blocking of regeneration (Chauvin, et al, 1999). Using low kanamycin concentrations leads to the regeneration of too many escape shoots, which are not transformed (Zhang et al, 2001). Although there was no significant difference in the shoot regeneration of explants cultivated in media containing 50 and 100 mg l^{-1} kanamycin, was chosen by

the present work the use of 100 mg l^{-1} kanamycin due to inhibition of non-transformed shoot regeneration.

Mannose has been applied in different plants as a non-antibiotic selection agent (Li et al, 2009). In the selection of shoots in rice plants, almost the same concentration of mannose in comparison of current work was used and no escape was observed in the regenerated shoots (Lucca et al. 2001). In the current experiment, all surviving shoots in the both media that were supplemented with kanamycin and mannose tested by a GUS assay and every regenerated shoot showed GUS activity. Moreover, the regenerated shoots of the control were tested by the GUS assay and, as expected, no GUS signal was detected. These findings showed the absence of endogenous PMI in wild type plants and confirmed that *Campanula* is not able to utilize mannose as a carbon source. Therefore PMI/mannose selection is a suitable candidate for the non-antibiotic selection system *in Campanula*. He et al (2006) showed that by increasing the mannose concentration in cucumber after transformation, escapes in regenerated shoots are reduced. Therefore, in order to reduce non-transformed shoots, the present work chose 30 g l^{-1} mannose was chosen for the *Campanula* selection. It was shown that the mannose concentration required for selection in *Campanula* is much higher than that in sugar beets (Joersbo et al, 1998) and in cucumbers (He et al, 2006). According to the current study's results the GUS signal was observed in all regenerated shoots; therefore we strongly suggest the application of mannose for the selection of transformed *Campanul*a shoots to be an environmentally safe and reliable selection method. Reed et al. (2001) investigated the environmental safety of mannose and unable to find any adverse effect on the transformed plants, which had been selected by the mannose system. This method is a safe alternative for antibiotic or herbicide selection and considerably applicable to the gene transformation of *Campanula*.

As result of the present study, improved protocol was established for the successful regeneration of three *Campanula* cultivars via reduction of the PGRs application. In cv. IBU, 85% shoot regeneration was observed when using just 3.0 mg l^{-1} TDZ, that is, just a 30% concentration as recommended by Sriskandarajah et al. (2008). Improved regeneration was also achieved in cvs. Royal and BO by reducing the concentrations of TDZ. Relatively high regeneration rates were obtained in short time periods, which can benefit transformation studies in *Campanula*. Furthermore, no regeneration of deformed or abnormal shoots was observed. The *Agrobacterium*

tumefaciens strain GV3101 was chosen through transient transformation as the best strain in *Campanula carpatica* cv. IBU. A non-antibiotic selection system employing the *pmi* gene for the mannose selection system was clarified to be as effective as kanamycin in selection of transformed shoots and was successfully established for selection after gene transformation. However, in previous attempts of using mature plants as an explant source, no shoot regenerated after gene transformation, mostly transformed callus was obtained (Sriskandarajah et al. 2008). For further studies, PCR and southern blot investigations of regenerated shoots are recommended.

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Table 1 Shoot regeneration of *C. carpatica* cv. IBU in different concentrations of glucose and mannose.

Carbohydrate (g l^{-1})		Shoot regeneration (%)
Glucose	Mannose	
20	0	80.00
15	5	61.11
10	10	57.89
5	15	15.78
0	20	0
0	25	0
0	30	0

Table 2 Summary of transformation results.

Selection agent	No. of tested explants	No. of regenerated explants	Shoot regeneration (%)	No. of survived shoots	GUS positive shoots
Kanamycin (100 mg l^{1})	35	4	11.42	4	4
Mannose (30 g l^{-1})	36	3	8.33	2	2
Control (0)	36	25	69.44	25	0

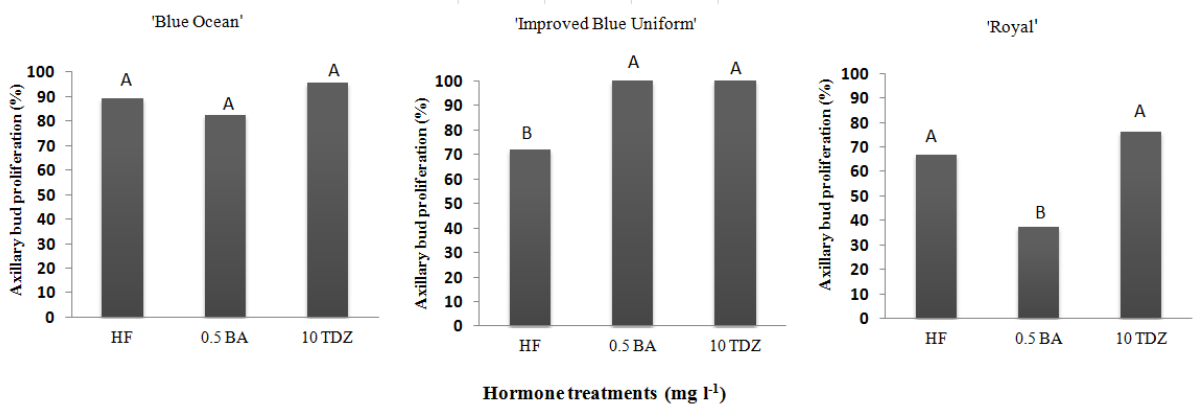

Fig 1 The effect of different media supplemented with growth regulators (mg l^{-1}) and hormone-free (HF) medium on the growth of axillary shoots from nodal cuts in three cultivars of *Campanula*. The columns marked with same letter within the same chart are not significantly different at $\alpha = 0.05$.

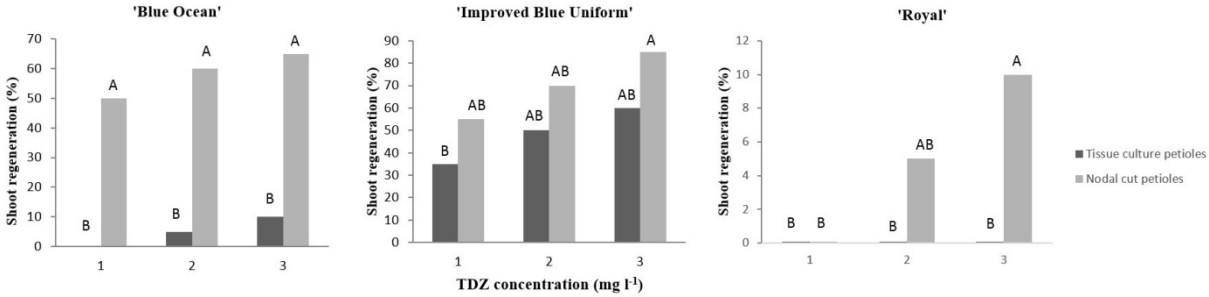

Fig 2 A comparison of the shoot regeneration of petioles from cvs. BO, IBU and Royal harvested from plants grown in tissue culture and from nodal cut petioles in media containing three different PGR combinations. Columns marked with same letter within the same chart are not different significantly at $\alpha = 0.05$.

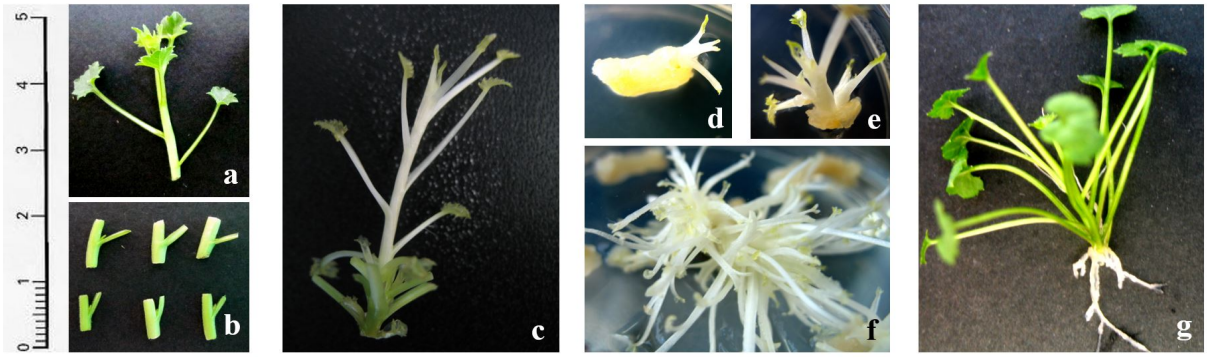

Fig 3 Greenhouse-grown shoots of *C. portenschlagiana* cv. Royal (**a**), nodal cuts of *C. portenschlagiana* cv. BO (**b**), dark-grown nodal cuts of *C. carpatica* cv. IBU after two weeks (**c**), petiole-regenerated shoot in *C. portenschlagiana* cv. Royal after 6 weeks (**d**), shoot regeneration of *C. portenschlagiana* cv. Royal after 7 weeks (**e**), shoot regeneration of *C. portenschlagiana* cv. Royal after 8 weeks (**f**), and an *in vitro* plantlet of *C. portenschlagiana* cv. Royal for the harvesting of petioles as an experimental explant source (**g**).

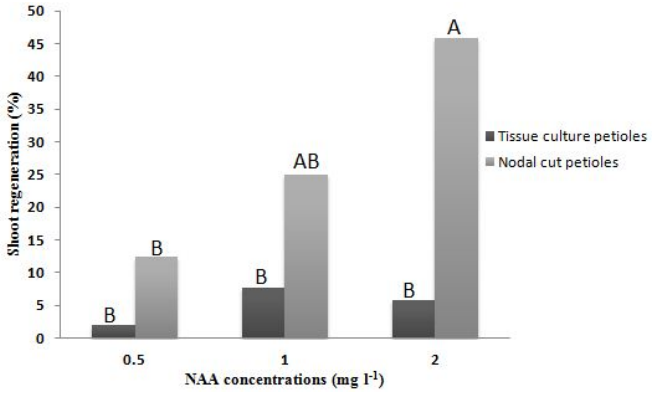

Fig 4 Comparison of effects of two different petiole sources (petioles harvested from nodal cuts and petioles harvested from plants grown *in vitro*) and three NAA concentrations on shoot regeneration in cv. Royal. All of the media were supplemented with 5.0 mg l^{-1} TDZ and 5.0 mg l^{-1} BA. The columns marked with same letter are not significantly different at $\alpha = 0.05$.

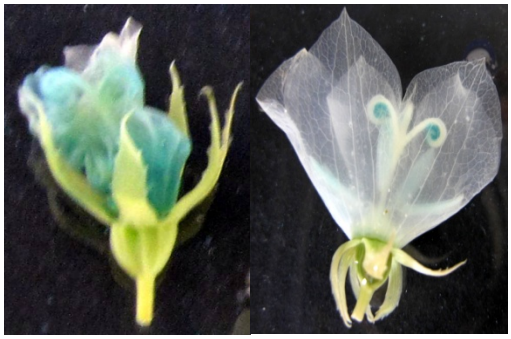

Fig 5 Transient transformation with GV3101 strain (left), control (right).

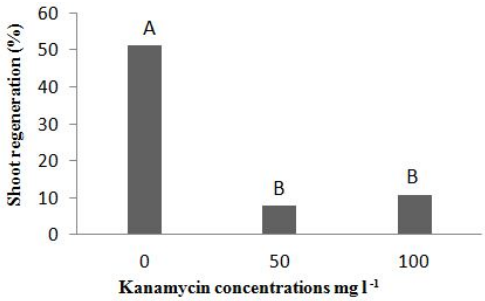

Fig 6 Shoot regeneration of cv. IBU in different kanamycin concentrations after transformation.

Effects of Cytokinin Treatment of Nodal Cuts and Petioles on Shoot Regeneration of *Campanula portenschlagiana* 'Blue Ocean'

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4. Effects of Cytokinin Treatment of Nodal Cuts and Petioles on Shoot Regeneration of *Campanula portenschlagiana* 'Blue Ocean'

Z. Ghayoor Karimiani, H. Mibus and M. Serek

Keywords: Campanula, BA, cytokinins, shoot regeneration, TDZ

Abstract

Several species of *Campanula* genus have been cultivated as ornamental plants and there is still growing demand for more attractive characteristics via breeding including genetic modification. While competence and condition of transformed tissue have a direct effect on success of gene transformation, obtaining efficient protocol with balanced hormonal content, which induce healthy shoot regeneration, is necessary. To determine the optimum type and concentration of cytokinins that induce shoot regeneration in 'Blue Ocean', an experiment in two phases (I and II) was designed. In the initial phase (I), nodal cuts were harvested from mature greenhouse-grown plants and cultured on Murashige and Skoog (MS) media containing various concentrations of benzyladenine (BA) or thidiazuron (TDZ). In the second phase (II), petioles were harvested from the newly grown shoots, derived from the nodal cuts and were cultivated on MS media supplemented with different concentrations of BA or TDZ for shoot regeneration. Results revealed that best rate of axillary node growth were exhibited by the nodal cuts, which cultivated in medium containing 0.5 mg/LTDZ (in phase I). The most favorable shoot regeneration rate was obtained from petioles cultivated in 1.0 mg/L TDZ (in phase II). According the results of this experiment, insignificant positive effects of hormone treatments in phases I was observed in improving the shoot regeneration efficiency in phase II,

consequently, the final hormonal content of the media elicited a direct influence on the rate of shoot regeneration.

1. INTRODUCTION

Genus *Campanula* consists of more than 500 species, which include annual or perennial plants (Contandriopoulos, 1984). *Campanula* species are cultivated as potted or bedding plants, ornamental garden plants and more recently cut flowers (Scariot et al., 2008). There is a growing demand to generate novel characteristics in ornamental plants to increase the commercial value. Genetic modifications techniques are precise methods to improve the quality of ornamental plants and prerequisite of gene manipulation studies is the optimized tissue culture protocol that provides relatively high regeneration rate (Wędzony et al., 2014). Although *Campanula* genus contains various species, which are cultivated as ornamental plants, only few studies have been focused on shoot regeneration in *in vitro* condition. Some of these reports still have deficiencies or the described media composition or explant is not applicable in other species and need to be manipulated for specific species.

Type of explants and age of explant source are important factors, which involve in efficiency of tissue culture and determination the media composition. The optimized medium recommended for adventitious shoot induction of explants harvested from seedlings in *C. carpatica* (Sriskandarajah et al., 2001) was not the best medium for shoot regeneration of explants harvested from mature plants (Sriskandarajah et al., 2008).

Although organogenesis potential of mature plant materials is lower than juvenile tissues (Basto et al., 2012), in some circumstances use of adult stock plants as explant source is inevitable. In sterile species such as *C. haylodgensis* (Lewis and Lynch, 1989) or when seed contamination is high or germination rate is low as reported in *C. portenschlagiana* and *C. poscharskyana* (Gargul, 2006), mature plants should be used as explant source. To use adult plants as explant source tissue rejuvenation should be performed via serial subculture of shoot tips (Bonga and von Aderkas, 1992) or micrografting (Revilla et al., 1996).

For tissue invigoration, plant growth regulators (PGRs) are usually applied. Excessive supplementation of PGRs, particularly cytokinins reported as the main reason of tissue deformation and abnormal morphological developments (Naik et al.,

1999). Stem fasciation was increased in *Kalanchoe blossfeldiana* by rising the concentration of PGRs supplementation in media specially cytokinins (Varga et al., 1988). In *Campanula carpatica* increasing the concentration of TDZ up to 10 mg/L was efficiently triggered the shoot induction in calli but higher TDZ level enhanced more callus formation and blocked the shoot regeneration (Sriskandarajah et al., 2008). In addition application of inappropriate hormone type may delay or hinder shoot regeneration and media composition has a direct influence on shoot regeneration. In *Campanula glomerata*, N^6-(2-isopentenyl) adenosine (2iP) and BA was used for shoot regeneration, but best results obtained after 4 months of culture, which is a relatively long time; To promote shoot within the shorter time application of TDZ was recommended (Joung et al., 2002).

Purpose of current study is to optimize media composition for shoot regeneration of *C. portenschlagiana* 'Blue Ocean' using adult plants as explant source considering to avoid over-supplementation of PGRs and reduce the explants exposure time to the PGRs. In the present work, influences of type and concentrations of cytokinins, used for pre-treatments of nodal cuts, were investigated on shoot regeneration of harvested explants.

2. MATERIALS AND METHODS

Plant materials

Adult plants of *C. portenschlagiana* 'Blue Ocean' were grown in an experimental greenhouse. Plants were maintained at 18˚ -22˚C under 337 μ mol m^{-2} s^{-1} light. Short-day conditions (8 h light) provided to keep the plants in a vegetative stage for the collection of nodal cuts.

Nodal cuts preparation

Fresh shoots of adult plants grown in above mentioned conditions in greenhouse were harvested. Leaf blades were removed and branches were sterilized and disinfected as follows; explants washed for 15 minutes in running tap water and sterilized in 1.5% (v/v) sodium hypochlorite solution supplemented with a drop of Tween-20 for 20 minutes. Then, rinsed three times in sterilized distilled water and were cut to prepare 8-10 mm nodal cuts with one or two nodes. The media were based on MS macro and microelements (Murashige and Skoog, 1962) supplemented

with 10 g/L glucose (AppliChem GmbH, Darmstadt, Germany), 112 mg/L B_5 vitamin and 15 g/L sucrose (Duchefa, Haarlem, The Netherlands). The media were silicified using 3.5 g/L plant agar (Duchefa) and 1.25 g/L gelrite (Duchefa), and the pH was adjusted to 5.7-5.8 prior to autoclaving.

Media compositions

To investigate the optimal type and concentration of cytokinins, the two experimental phases (I and II) were designed (Fig. 1).

1. Phase I. In the first phase (I), nodal cuts were harvested from adult greenhouse-grown plants, disinfected as described above, and cultured on MS media containing different concentrations of BA (0.5, 1.0 or 2.0 mg/L) or TDZ (0.5, 1.0 or 2.0 mg/L). All media were supplemented with 0.25 mg/L 1-naphtaleneacetic acid (NAA). The nodal cuts were grown in the dark at 24˚C for two weeks.

2. Phase II. The petioles of newly grown shoots derived from the nodal cuts were harvested and placed on MS media supplemented with 0.5, 1.0 or 2.0 mg/L BA, or 1.0 mg/L TDZ for shoot regeneration. All media contained 0.25 mg/L NAA.

In this experiment, 24 different combinations and concentrations of PGRs have been assessed. Each hormonal treatment (Fig. 1) included three replicates of 4 explants. Regenerated shoots from the petiole cultures were evaluated after 7 weeks to determine the optimal hormonal combination that promoted the shoot regeneration of 'Blue Ocean'.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using MSTATC software (Michigan State University, Lansing, Michigan, USA, version 1.42), and the mean values were compared according to the least significance difference test (LSD) at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

The effects of hormonal pre-treatments of nodal cuts on the shoot regeneration of *C. portenschlagiana* 'Blue Ocean'

To clarify the possible effects of the hormonal content applied for growing nodal cuts on shoot regeneration, we designed an experiment that assessed 24

different combinations and concentrations of PGRs in two phases on the 'Blue Ocean'. Results showed the optimal rate of shoot regeneration was exhibited by the nodal cuts that were cultivated in medium containing 0.5 mg/L TDZ and 0.25 mg/L NAA (in phase I). The petioles of these nodal cuts were harvested and then cultivated in medium supplemented with 1.0 mg/L TDZ and 0.25 mg/L NAA (in phase II), exhibited best shoot regeneration rate of 66.67% (Table 1). Shoot regeneration rate of the petioles harvested from same medium (in phase I), cultured in other media composition (in phase II) contained various BA concentrations was significantly lower than those cultured in 1.0 mg/L TDZ and 0.25 mg/L NAA. When nodal cuts were grown on medium containing 0.5 mg/L BA and 0.25 mg/L NAA (in phase I), the most favorable shoot regeneration rate (50.0%) was obtained from petioles cultivated in 1.0 mg/L TDZ and 0.25 mg/L NAA (in phase II) and shoot regeneration in other three media compositions containing BA were significantly lower. According to these results, final hormonal content of the media in phase II has direct influence to induce efficient shoot regeneration. The underlying reason might be the short period (two weeks) of hormone exposure in the nodal cut growth media (in phase I), which is insufficient for hormone accumulation in tissues.

Also we reached the conclusion that not only PGRs content of media in phase I, did not stimulate shoot regeneration of petioles in phase II (especially in all concentrations of BA), but also application of TDZ, higher than 0.5 mg/L reduced shoot regeneration frequency. Reduction of shoot regeneration rate (%) in consequence of increasing the TDZ concentration of media was observed on explants of bamboo (Singh et al., 2001) and lentil (Khawar et al., 2004). The threshold of preventive concentration of TDZ is varying by genus and species, for instance study on *Petunia hybrida* shows same results when exceeding 2 mg/L TDZ (Thirukkumaran et al., 2009). When application of BA or TDZ in growing nodal cuts, do not promote shoot regeneration in higher efficiency, time of explants exposure to the PGRs can be reduced by utilization of hormone-free medium in phase I to cultivate nodal cuts.

Obtained results demonstrated that TDZ induce shoot regeneration of petioles, more efficient compared to the BA (Table 1). While the ability of TDZ to stimulate shoot regeneration has been well documented in different plants, avoiding the disadvantages over-supplementation of TDZ by reducing the concentration (Huetteman and Preece, 1993) or decreasing the explants exposure time to TDZ is

essential (Naz et al., 2012). In the current work, best results of shoot regeneration obtained when 1.0 mg/L TDZ was applied in regeneration medium. The applied TDZ concentration was only 10 % of the amount that previously described in a study by Sriskandarajah et al. (2008). In mentioned study, four *Campnuala* species have been investigated and 10 mg/L TDZ was selected as optimal concentration for shoot regeneration. In described study relatively high concentration of PGRs, particularly cytokinins have been applied for rejuvenation of tissues and promotion of shoots, but the obtained shoot regeneration rate was very low (Sriskandarajah et al., 2008).

In the present work, the possibility of decreasing the exposure time of explants to the hormone and reducing PGRs concentration of regeneration media along with obtaining efficient shoot regeneration have been investigated. We reached the conclusion that hormone-free media can be applied to cultivate the nodal explants, hence reduce the hormone exposure time. Also application of lower TDZ concentration compared to previous study (Sriskandarajah et al., 2008) results in better shoot regeneration efficiency. Along with lower costs for commercial operation by reduction in hormonal usage in media; regeneration of deformed or abnormal plants in consequence of over-supplementation of PGRs can be prevented.

4. ACKNOWLEDGEMENT

Thanks to Danish growers, Gartneriet Thoruplund A/S, Gartneriet Tvillingegaarden A/S and Gartneriet Toftly A/S (Odense, Denmark) for kindly providing research funds.

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Table

Table 1**.** The interactions of hormonal content in the media used for nodal cuts (phase I) and the PGR content of the regeneration media in phase II on the shoot regeneration rate (%) of 'Blue Ocean' 7 weeks after culture. Hormonal content of treatments are mg/L in medium. All of the media in both phases were supplemented with 0.25 mg/L NAA.

	Phase I					
Phase II	0.5 BA	1.0 BA	2.0 BA	0.5 TDZ	1.0 TDZ	2.0 TDZ
0.5 BA	8.33 d[1]	11 cd	8.33 d	33 bcd	8.33 d	16.67 cd
1.0 BA	8.33 d	11 cd	33 bcd	11 cd	8.33 d	58.33 ab
2.0 BA	25 cd	11 cd	25 cd	22 cd	16.67 cd	30.77 bcd
1.0 TDZ	50 abc	22 cd	8.33 d	66.67 a	16.67 cd	25 cd

[1]The values marked with same letter are not significantly different at P ≤ 0.05.

Figure

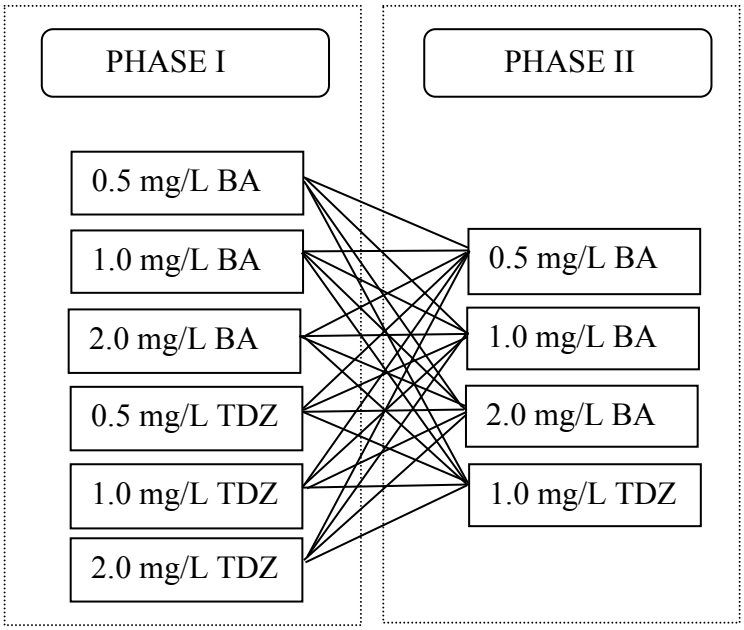

Fig. 1. Combinations of PGR treatments. All media supplemented with 0.25 mg/L NAA.

Transformation of *Campanula carpatica* to alter flower colour and ethylene sensitivity

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Transformation of *Campanula carpatica* to alter flower colour and ethylene sensitivity

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SUMMARY
This study is the first report of gene manipulation to alter flower colour in the genus *Campanula*. The experiment was designed using *Campanula carpatica* 'Improved Blue Uniform' (IBU) with two main purposes: to generate a red flower colour by down-regulation of delphinidin production through RNAi silencing of the flavonoid 3', 5' hydroxylase (*F3'5'H*) gene, and to improve vase-life by inducing insensitivity to ethylene using the *ethylene-resistant 1-1* (*etr1-1*) gene. Three independent transgenic lines were obtained after genetic transformation via *Agrobacterium tumefaciens* strain GV3101. Southern blot analysis was performed to determine the integrated T-DNA copy number, and reverse transcription-polymerase chain reactions (RT-PCR) were performed to evaluate expression of the *F3'5'H* and flavonoid 3' hydroxylase (*F3'H*) genes. An ethylene sensitivity test was conducted to evaluate the tolerance of the transformed plants to exposure to ethylene. Southern blot analysis revealed the integration of two or three T-DNA copies in each of the three transformed lines. The flower colour of the transformed lines was not visually altered, and expression of the *F3'5'H* and *F3'H* genes in all three transformed and in non-transformed lines was confirmed by RT-PCR. Although all flowers on non-transformed *Campanula* plants were senescent 6 d after exposure to ethylene, 63.3% of the flowers on transgenic line 11-213 and 86.4% of the flowers on transgenic line 21-1 survived. The ethylene sensitivity test showed that transformed plants exposed to ethylene showed a significant delay in senescence compared to non-transformed plants.

Flower colour and vase-life are important characteristics for ornamental plants and have significant effects on their market success. The introduction of new visual characteristics always increases opportunities for the floral industry. *Campanula* spp. are commercially important ornamentals that are cultivated mainly as potted or bedding plants.

Flavonoids, carotenoids, and betalains are the pigments responsible for flower colour (Grotewold, 2006). Anthocyanins (a class of coloured flavonoids) consist of delphinidin (Dp; blue), pelargonidin (Pg; orange), and cyanidin (Cy; red; Tanaka *et al*., 2005). In many ornamentals, a natural lack of a specific flower colour such as yellow or orange in cyclamen, or blue in rose, has encouraged breeders to use genetic transformation to introduce or to silence genes to create novel flower colours (Debener and Winkelmann, 2010). Flower colour can be modified by inducing the synthesis of non-native pigments in the flower or by suppressing the synthesis of endogenous pigments (Nishihara and Nakatsuka, 2011).

Two enzymes, flavonoid 3',5' hydroxylase (F3'5'H) and flavonoid 3' hydroxylase (F3'H), directly affect the synthesis of different flavonoids (Forkmann and Martens, 2001) and produce blue or purple colours and red or pink colours in petals, respectively (Mori *et al*., 2004). Blocking the expression of the *F3'5'H* or *F3'H* genes is an effective and genetically stable way to modulate flower colour. By suppressing expression of the *F3'5'H* gene in *Cyclamen persicum* (Boase *et al*., 2010) and in *Osteospermum hybrida* (Seitz *et al*., 2007), the concentrations of Dp-derived pigments decreased, and a different hue of red was produced in the transgenic lines. Double-stranded RNA (dsRNA)-mediated gene silencing, termed RNA interference (RNAi), is a reliable method that can be used to down-regulate the flavonoid biosynthesis pathway. RNAi was used to down-regulate the chalcone synthase gene (*CHS*), which is the first enzyme in the flavonoid biosynthesis pathway in *Torenia hybrida* (Fukusaki *et al*., 2004).

The effect of inhibition of an enzyme can be visualised by *in vivo* infiltration of a specific enzyme inhibitor. Tetcyclacis (BASF, Limburgerhof, Germany) is a plant growth retardant that inhibits the cytochrome P450-dependent enzymes involved in gibberellic acid (GA) biosynthesis, herbicide detoxification, and sterol biosynthesis (Menting *et al*., 1994). Tetcyclacis strongly inhibits enzymes of the flavonoid hydroxylase class (Stich *et al*., 1988). Due to the non-specific action of this inhibitor, the hydroxylase activities of both enzymes (F3'5'H and F3'H) were blocked. Tetcyclacis has been used in certain ornamentals such as *O. hybrida* (Seitz *et al*., 2007) and *Anagallis monelli* (Martens *et al*., 2003), to determine the effects of an inhibitor of hydroxylase enzyme activities on petal colour. In addition, the flower colour achieved after tetcyclacis treatment can indicate whether or not the endogenous dihydroflavonol 4-

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reductase (DFR) accepts dihydrokaempferol (DHK) to produce pelargonidin (orange-red colour; Boase *et al.*, 2010).

Although *Campanula* spp. are important ornamentals, few investigations have focussed on the flavonoid biosynthetic pathways in this genus. The anthocyanin composition of *Campanula* cultivars has been studied, and the production of Dp and Dp-derived anthocyanins such as violdelphin has been verified (Brandt *et al.*, 1993). While cDNA of the *F3'5'H* gene of *Campanula* has been transformed into several plants such as tobacco (Okinaka *et al.*, 2003) and chrysanthemum (Brugliera *et al.*, 2013) to produce blue petals, no studies have reported the molecular breeding of the flavonoid synthesis pathways in *Campanula* spp. In this study, RNAi was used to try to silence the *F3'5'H* gene to block the production of Dp. By blocking this gene, which is responsible for producing blue pigments, we expected to reduce the dominant blue colour (Dp) of *C. carpatica* 'Improved Blue Uniform' (IBU) flowers and cause the appearance of different red hues (Cy or Pg) in transformed flowers.

In addition to flower colour, vase-life and post-harvest quality are also commercially important characteristics of ornamentals. Ethylene plays a key role in plant development and floral senescence (Lütken *et al.*, 2012). Post-harvest treatments with chemicals can improve the quality and longevity of cut flowers; however, unfortunately, some of these compounds are not environmentally safe. Silver thiosulphate (STS), which is the most effective chemical treatment for blocking ethylene binding sites, has been restricted in several countries due to its toxicity (Mibus *et al.*, 2009). Genetic modification to achieve ethylene insensitivity is a long-term and environmentally safe solution for improving floral longevity in ethylene-sensitive plants (Arora, 2005). Chang *et al.* (1993) identified a dominant mutant gene called *ethylene-resistant 1-1* (*etr1-1*) that blocked ethylene action, resulting in the insensitivity of *Arabidopsis thaliana* seedlings to ethylene. Several research groups have used the *etr1-1* gene to generate ethylene-insensitive transgenic plants with an improved post-harvest flower vase-life (Sanikhani *et al.*, 2008; Sriskandarajah *et al.*, 2007). Members of the family Campanulaceae have been classed as highly ethylene-sensitive (Serek *et al.*, 2006). The flowers and buds of *C. carpatica* senesce within only 3 d of exposure to ethylene; however, introduction of the *etr1-1* gene significantly enhanced the tolerance of flowers to exogenous ethylene (Sriskandarajah *et al.*, 2007).

This study is the first report of an attmpt to modify flower colour in *Campanula* spp. The strategy was to generate red flowers by blocking the production of Dp through RNAi gene silencing using a palindromic copy of the endogenous *F3'5'H* gene. Tetcyclacis was applied to clarify the possible redirection of substrates via other enzymes upstream of the inhibited F3'5'H and F3'H enzymes and to determine whether *Campanula* DFR was able to accept DHK to produce leucopelargonidin (LPg), the precursor of Pg-based anthocyanins (orange-red colour). Furthermore, the levels of expression of both the *F3'5'H* and *F3'H* genes were studied in wild-type and transformed plants. In addition, we introduced the *etr1-1* gene of *A. thaliana* using *Agrobacterium tumefaciens*-mediated transformation to induce ethylene insensitivity into flowers and buds.

MATERIALS AND METHODS

Plant material

Adult *C. carpatica* 'IBU' plants were grown in an experimental greenhouse under a light intensity of 337 µmol m^{-2} s^{-1}. The plants were grown under short-day conditions (8 h) at 18° – 22°C to maintain the vegetative stage. Long-day conditions (16 h; 17° – 19°C) were used to induce flowering.

Tetcyclacis treatment

Different concentrations (0, 5, 30, 50, or 100 µM) of tetcyclacis solution (100 ml) were sprayed onto the green, closed flower buds of control plants in a growth chamber. The different concentrations of tetcyclacis were prepared by dissolving tetcyclacis in 100% (v/v) acetone. For each treatment, five plants were sprayed, and five flower samples from each replication were collected. The flowers were evaluated by visual inspection, and their anthocyanins were extracted 3 d after spraying using the method of Hosokawa *et al.* (1996) with some modifications. The absorbance of the extract at 525 nm A_{525} had a direct linear correlation with anthocyanin concentration (Wang *et al.*, 2013). A_{525} was measured using a Smartspec 3000 spectrophotometer (Bio-Rad, Richmond, CA, USA) with three replications for each treatment, and the anthocyanin concentrations in each extract were compared.

The experiment was arranged in a completely randomised factorial block design using five concentrations of tetcyclacis in five replications. The results were analysed using two-way ANOVA and Duncan's multiple range test at $P \leq 0.05$ with MSTAT-C software (Michigan State University, Lansing, MI, USA).

Plant transformation

Greenhouse-grown nodal explants of *C. carpatica* 'IBU' were harvested and disinfected as described by Sriskandarajah *et al.* (2008). The medium used for growing the nodal explants was based on full-strength MS macroelements and microelements (Murashige and Skoog, 1962) supplemented with 10 g l^{-1} glucose (AppliChem GmbH, Darmstadt, Germany), 112 mg l^{-1} vitamin B_5, and 15 g l^{-1} sucrose. The medium was solidified using 3.5 g l^{-1} plant agar and 1.25 g l^{-1} Gelrite (Duchefa, Haarlem, The Netherlands). The pH was adjusted to 5.7 – 5.8 with 0.1 M HCl or 0.1 M NaOH before autoclaving at 121°C and 1.05 kg cm^{-2} for 20 min.

Petioles were harvested from the nodal explants after 2 weeks of culture in the dark and used as explants for transformation.

Agrobacterium tumefaciens strain GV3101, harbouring the binary vector p9Ndo (pLH9000; GenBank Accession No. AF458478; DNA Cloning Service, Hamburg, Germany) which contained a palindromic copy of the *F3'5'H* gene (540 bp; D14590.1) from the splice site of the gene, under control of the *pst* promoter (a petal- and stamen-specific promoter from a MADS box gene of *A. thaliana*; AF198055; Krizek and Meyerowitz, 1996), was used to transform the petioles. The plasmid also harboured the *etr1-1* gene under

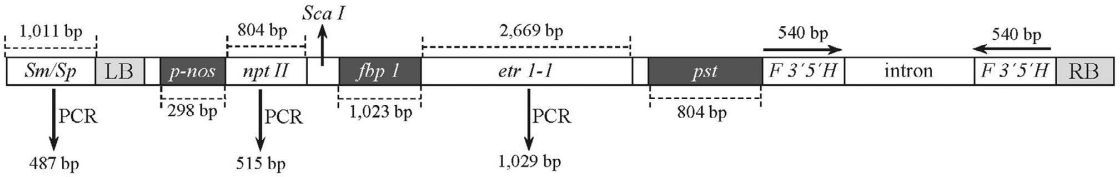

FIG. 1
Schematic diagram of the transformation cassette in the binary vector p9Ndo. The vector carried the antibiotic resistance genes (*Sm/Sp* and *npt II*), the *etr1-1* gene controlled by the *fbp1* promoter, and a palindromic copy of the *F3'5'H* gene (*F3'5'H*-RNAi) driven by the *pst* promoter. The cleavage site of *Sca* I and the three positions of sequences amplified by PCR are indicated. Numbers refer to the size of each segment or amplicon in bp.

control of the flower-specific *fbp1* promoter (EF192606) to generate ethylene insensitivity in transformed lines (Figure 1). The backbone of the binary plant transformation vector p9Ndo contained the bacterial spectinomycin/streptomycin resistance (*Sp/Sm*) gene, a marker to select for transformed *A. tumefaciens* in culture. The T-DNA also harboured the neomycin phosphotransferase II gene (*npt II*) for kanamycin resistance as a marker to select for regenerated transformed shoots. Transformation was performed according to Sriskandarajah *et al.* (2008).

Transformed petioles were transferred to full-strength MS medium supplemented with 5.0 mg l^{-1} thidiazuron (TDZ; Duchefa), 0.1 mg l^{-1} 1-naphthaleneacetic acid (NAA; Duchefa), 100 mg l^{-1} kanamycin, and 500 mg l^{-1} cefotaxime. The petioles were then maintained in the dark at 24° ± 1°C to regenerate shoots.

A total of 16 independent transformation experiments, each using 20 Petri dishes containing four petiole explants (20 × 4) as replicates for each transformation event, were performed. The rate of regeneration was evaluated 6 weeks after each transformation event. Rooted transformed plantlets were transferred to a greenhouse for acclimatisation at 17° – 19°C under 16 h long-day conditions to promote flowering.

Southern blot analysis

Seven months after transformation, genomic DNAs (gDNAs) were extracted from a leaf blade on each putatively transformed shoot using the Edwards *et al.* (1991) method, modified by Dorokhov and Klocke (1997). PCR was performed using specific primers for the *etr1-1* gene (Sanikhani *et al.*, 2008) for an expected amplicon size of 1,029 bp to verify the presence of T-DNA in the transformed shoots. In addition, specific primers for the *Sp/Sm* gene (AF458478) located in the binary vector p9Ndo outside the T-DNA region (forward 5'-TATCCAGCTAAGCGCGAACT-3'; reverse 5'-ATTTGCCGACTACCTTGGTG-3') were designed to yield an amplicon of 487 bp. These primers were used to screen for *A. tumefaciens*-free transformed shoots to exclude false positives by PCR.

Southern blot analysis was performed on each gDNA sample from each transformed plant that had been confirmed by PCR according to Sriskandarajah *et al.* (2008). The gDNA was digested using 30 Units of *Sca* I (Fermentas, Leon-Rot, Germany) at 37°C overnight. Specific primers for the *F3'5'H* gene (forward 5'-GGATCCTTGCATGCTCAACT-3'; reverse 5'-GCGACGGACCTGAATTCTAA-3') were designed to prepare the 562-bp *F3'5'H* intron probe.

Evaluation of F3'5'H *and* F3'H *gene expression*

To detect *F3'5'H* gene expression in transformed and wild-type *C. carpatica* plantlets, reverse transcription-polymerase chain reaction (RT-PCR) was performed. F3'H is the key enzyme that produces dihydroquercetin (DHQ), which is the precursor of Cy (red colour) in petals of *Campanula*. Therefore, expression of the *F3'H* gene was also monitored in transformed and non-transformed plants.

For RT-PCR, total RNA was extracted from 50 ± 3 mg of petals from transgenic or control *C. carpatica* 'IBU' plants according to Sanikhani *et al.* (2008). Flower development was sub-divided into three stages: (i) green flower bud (GB); (ii) pale violet bud (VB); and (iii) fully-coloured purple flowers (PF). Total RNA was extracted from the petals of both transformed and control plants at each developmental stage using the InviTrap Spin Plant RNA Mini Kit (STRATEC Molecular, Berlin, Germany). The extracted RNA was reverse-transcribed to cDNA, and RT-PCR was performed. Specific primers to detect *F3'5'H* (D14590.1) gene expression (forward 5'-TAGCCCCTGCACATATCCTC-3'; reverse 5'-TGAAGTGGACGGATTTCACA-3') and *F3'H* (AB221082.1) gene expression (forward 5'-TTCTACCG GGTGGTGAAAAG-3'; reverse 5'-AGCCCATAGC CTTCTTCCAT-3') were used. Each PCR reaction was incubated in a thermocycler (Biometra, Goettingen, Germany) for 2 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C. The same PCR amplification conditions were used for both pairs of primers. The expected amplicon sizes for *F3'5'H* and *F3'H* were 206 bp and 211 bp, respectively.

Sensitivity to ethylene

Two transgenic lines (11-213 and 21-1) and control plants of *C. carpatica* were grown in a controlled climate chamber for flower induction. All un-opened flowers were removed at the start of the experiment, and the number of open flowers were counted. The experiment was performed as a split-plot design with air quality (ethylene-free or 2 µl l^{-1} ethylene) as the main factor and plant line (non-transformed control, 11-213, or 21-1) as the secondary factor. In total, 48 flowering plants were placed in eight air-tight glass chambers. The air in four chambers contained 2 µl l^{-1} ethylene, whereas the air in the other four chambers did not. Each chamber contained two pots each of 11-213, 21-1, and control plants. The vase-life of the flowers was recorded as the percentage of open flowers after 3, 6, and 9 d. Comparisons between the three lines, plus or minus ethylene, were performed using two-way ANOVA and

Duncan's multiple range test at $P \leq 0.05$ in MSTAT-C software (Michigan State University, Lansing, MI, USA).

RESULTS AND DISCUSSION

Tetcyclacis treatments

The tetcyclacis treatments were performed to determine the effect of a lack of hydroxylase enzyme activity on flower colour and to clarify whether *Campanula* DFR was able to accept DHK or DHQ to produce Pg or Cy precursors, respectively.

In *C. carpatica* 'IBU' plants exposed to increasing concentrations of tetcyclacis, the intensity of the petal colour was visibly reduced and, following the application of 100 µM tetcyclacis, the petals turned white. This trend was confirmed spectrophotometrically by measuring the A_{525} of anthocyanin extracts (Figure 2). *In vivo* inhibition of F3'5'H and F3'H in *Anagallis monelli* resulted in the production of white flowers due to the accumulation of kaempferol (Km; Martens *et al.*, 2003). Similar results were obtained by applying tetcyclacis to *O. hybrida* (Seitz *et al.*, 2007). Orange-red flowers were expected after tetcyclacis treatment if DFR was able to accept DHK to produce Pg-based anthocyanins, and white flowers were expected if DFR could not use DHK as a substrate. After inhibition of the hydroxylase enzyme activities in *C. carpatica* 'IBU', the production of Dp and Dp-derived anthocyanins was blocked due to the inability of *Campanula* DFR to use DHK and white flowers were obtained.

Transformation and Southern blot analysis

The regeneration percentage of shoots of *C. carpatica* 'IBU' after transformation ranged from 3.75 – 18.75% in each of the 16 independent transformation experiments each using 80 petiole explants.

Southern blot analysis using a DIG-labelled 562-bp *F3'5'H* intron probe confirmed three independent lines of transformed 'IBU' (Figure 3). Two copies of the T-DNA were integrated into the genome in two transgenic lines (11-213 and 21-1) and, in one line (16 B), three copies of the T-DNA were integrated into the genome. The insertion of multiple T-DNA copies into the genome

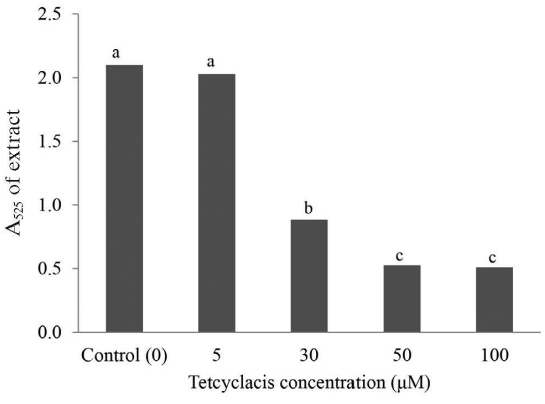

FIG. 2
Absorbance at 525 nm of anthocyanin extracts obtained from petals of *C. carpatica* pre-treated with tetcyclacis at 0, 5, 30, 50, or 100 µM. Columns with the same lower-case letter are not significantly different at $P \leq 0.05$.

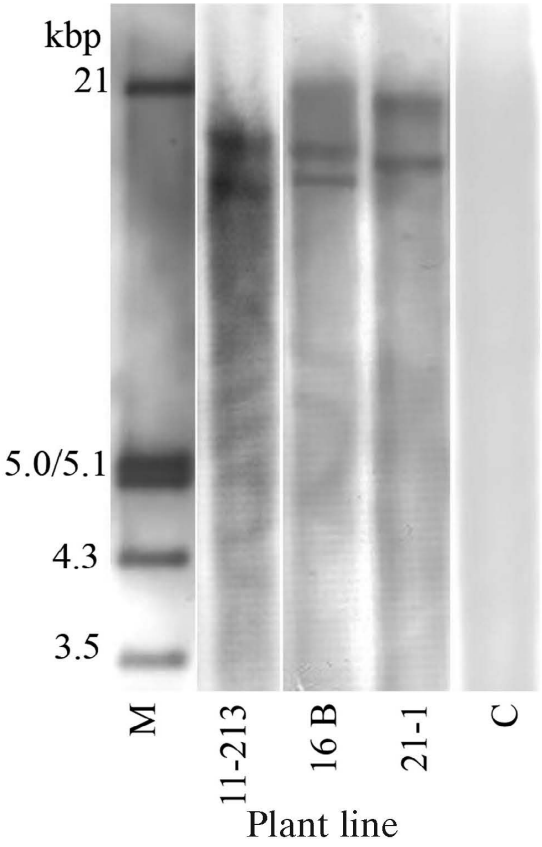

FIG. 3
Southern blot hybridisation of genomic DNA cut with *Sca* I from three putative T0 lines of *Campanula carpatica* (11-213, 16 B and 21-1). Lane C, non-transformed control plants. Lane M, size marker (1 kbp ladder). The DNAs were probed with a DIG-labelled 562-bp PCR-amplicon specific for the *F3'5'H* transgene.

can alter interactions between the T-DNA and the host genome as well as the organisation of the genome in specific regions (Jorgensen *et al.*, 1987). In addition, truncations, rearrangements, or repetition of the introduced T-DNA may occur, which can affect the expression of foreign genes (Deroles and Gardner, 1988). The level of gene silencing can therefore fluctuate from complete knock-out, to partial silencing, to no silencing, depending on the copy number and position(s) of the T-DNA in each transgenic line (Travella *et al.*, 2006). Kerschen *et al.* (2004) compared the effectiveness of gene silencing between multi-copy and single-copy RNAi lines and demonstrated that target gene transcript concentrations were not significantly reduced in multi-copy lines. Since multiple copies of introduced transgenic DNA can interact to co-suppress each other (Assaad *et al.*, 1993), single-copy T-DNA lines are preferred to achieve optimal results.

Evaluation of F3'5'H *and* F3'H *gene expression*

Because the flower colour of the transformed plants was not visually altered, RT-PCR was used to verify expression of the *F3'5'H* gene in petals. Figure 4 shows similar expression of the *F3'5'H* gene in petals of non-

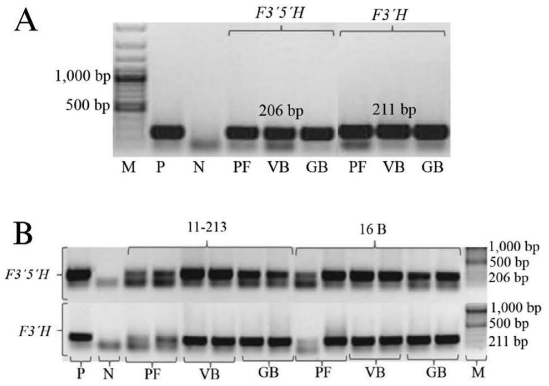

FIG. 4
Panel A, expression of the *F3'5'H* gene (amplicon size 206 bp) and the *F3'H* gene (amplicon size 211 bp) in fully-coloured purple flowers (PF), pale violet buds (VB), and green buds (GB) from non-transgenic *C. carpatica* 'IBU' plants. M, size marker (100 bp ladder); P, binary plasmid (p9Ndo used for transformation); N, negative control. Panel B, expression of the *F3'5'H* and *F3'H* genes in PF, VB, and GB of transgenic lines 11-213 and 16 B (two replications for each cDNA sample). M, size marker (100 bp ladder); P, binary vector plasmid (p9Ndo used for transformation); N, negative control.

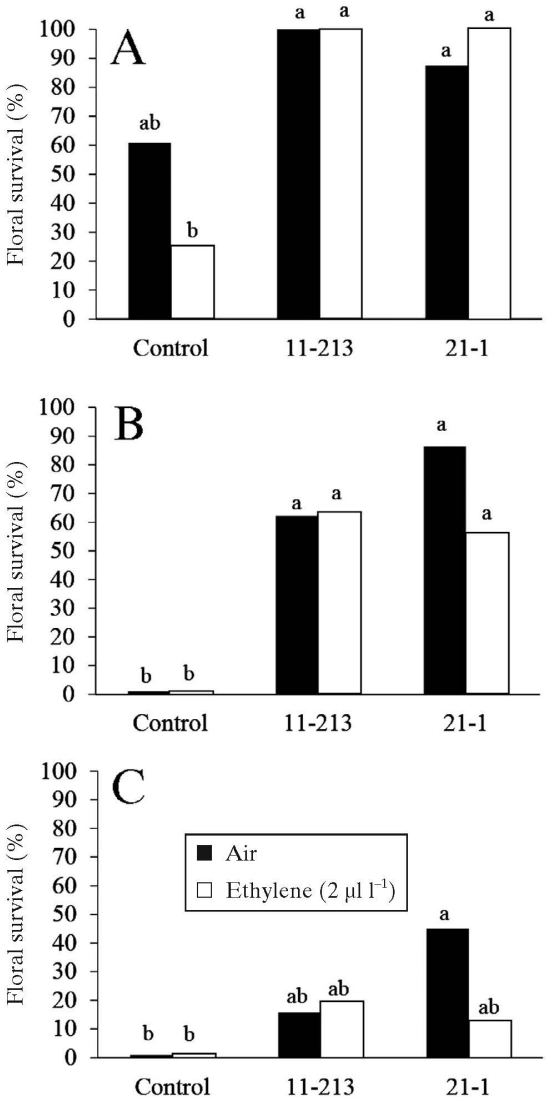

FIG. 5
Rates of flower survival (%) on day-3 (Panel A), day-6 (Panel B), and day-9 (Panel C) in two transgenic lines (11-213 and 21-1) of *C. carpatica* 'IBU' and in control (non-transformed) flowers exposed to air or to 2 µl l^{-1} ethylene. Columns with the same lower-case letters in each Panel are not significantly different at $P \leq 0.05$.

transformed (Figure 4A) and transformed (Figure 4B) plants at all three stages of flower development. Expression of the *F3'H* gene was also monitored in both non-transformed (Figure 4A) and two lines of transformed plants (Figure 4B). *F3'5'H* gene expression was detected and Dp was produced at all three stages of flower development in both transgenic lines (11-213 and 16 B); thus, the dominant blue colour was maintained in the petals of transformed plants. Several possibilities have been proposed as reasons for unsuccessful alterations of flower colour in different plants. After silencing the *F3'5'H* gene in *Torenia hybrida*, the blue colour occasionally reappeared on petals in most of the selected lines due to release of gene suppression (Nakamura *et al.*, 2010). It was not possible to predict the T-DNA copy number after genetic transformation (Tenea and Cucu, 2006); however, genetic transformation via *Agrobacterium* usually results in the integration of a large number of T-DNA copies into the genome (Jorgensen *et al.*, 1987). Thus, the failure to silence *F3'5'H* gene expression after transformation with *F3'5'H*-RNAi could result from the integration of multiple copies of T-DNA.

Sensitivity to ethylene

The ethylene sensitivities of the two transgenic lines of *Campanula* (11-213 and 21-1) were compared to that of wild-type plants. On day-3, a significant difference was observed between the number of flowers that senesced in non-transformed and transformed plants exposed to ethylene ($P \leq 0.05$; Figure 5A). On day-6, all flowers on non-transformed plants had senesced under both the +ethylene and –ethylene conditions (Figure 5B), while, on day-9, 19.4% of 11-213 flowers and 12.8% of 21-1 flowers still survived under the +ethylene conditions (Figure 5C). The numbers of open flowers on both transformed lines (11-213 and 21-1) was significantly higher than the numbers of open flowers on control plants, even 6 d after the start of the experiment.

The results of this experiment demonstrate increased resistance of *etr1-1*-transformed plants to ethylene compared to control, non-transformed plants. To date, the *etr1-1* gene has been introduced into a wide range of plants including *Dianthus caryophyllus* (Bovy *et al.*, 1999), *C. carpatica* (Sriskandarajah *et al.*, 2007), and *Petunia hybrida*, to confer an ethylene-insensitive phenotype (Wilkinson *et al.*, 1997).

Although the integration of a single copy of a foreign transgene into each transformant is usually preferable, the performance of the *etr1-1* gene generating ethylene-resistant plants appeared to be independent of copy number (Sanikhani *et al.*, 2008, Sriskandarajah *et al.*, 2007).

CONCLUSIONS

This report describes an efficient transformation and regeneration protocol using greenhouse-grown, adult *Campanula* spp. plants as a source of petiole explants. We achieved a reasonable frequency of shoot regeneration (18.8%) after transformation and produced three independently transformed lines. Southern blot analysis confirmed the integration of two (11-213 and 21-1) or three (16 B) T-DNA copies per genome in the transformed lines. The RNAi construct designed to target the *F3'5'H* gene failed to silence the gene. The white flower colour observed after tetcyclacis treatment indicated that *Campanula* DFR was not able to use DHK to produce Pg-based anthocyanins. Thus, after efficient *F3'5'H* gene silencing, white flowers were expected. To induce *Campanula* to produce red flowers, a suitable DFR that was capable of converting DHK to Pg and a silenced *F3'5'H* gene would be required. Because different plants have different DFR isoenzymes, the ability of the enzyme to accept DHK as a substrate should be determined prior to the expression of exogenous DFR. In addition, over-expression of the *F3'H* gene in *Campanula* should enhance the accumulation of Cy derivatives to produce a red colour.

Although the functionality of the *etr1-1* gene has been reported previously in *Campanula*, it has never been co-expressed with another flower-specific promoter in the same transformed plant. In the current study, the *etr1-1* gene reduced ethylene sensitivity and delayed senescence during flowering.

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Discussion

6. Discussion

The current research was conducted for two main purposes: to alter the flavonoid biosynthesis pathway via gene silencing of the *F3'5'H* gene and to improve the postharvest quality of flowers by expression of the *etr1-1* gene from *Arabidopsis* in *Campanula*. Since an ideal tissue culture condition is a basic requirement for gene transformation, the shoot regeneration of three *Campanula* cultivars was optimized: *C. carpatica* cv. Improved Blue Uniform and *C. portenschlagiana* cvs. Blue Ocean and Royal. Mature greenhouse-grown plants were applied as an explants source and medium compositions for growing nodal cuts and shoot regeneration were adjusted.

Prior to the gene transformation, the best kanamycin concentration for selecting transformed tissues and the most penetrative *Agrobacterium tumefaciens* strain were selected. In addition, the flower buds of non-transformed plants were treated with different concentrations of tetcyclacis for *in vivo* inspection of flavonoid hydroxylase enzymes blocking. After the gene transformation and shoot regeneration, the following were performed: PCR for detection of transformed shoots, RT-PCR for evaluation of *F3'5'H* and *F3'H* gene expression, and southern blot for confirming the integration and copy number of the T-DNA to the genome.

6-1. Micropropagation and *in vitro* tissue culture

6-1-1. Optimal explant source for shoot regeneration

The explant source and developmental stage of the starting materials directly influence shoot regeneration frequency (Volkaert et al. 1990). Comparisons have been made on the effects of different explant sources on various plants and recommendations given regarding various sources for explant harvesting. For instance, in many plants, juvenile tissues were a superior explant source for harvesting explants (Basto et al. 2012, Ismail et al. 2012). However, for *Quercus robur* L., no difference was observed in the multiplication rate of explants taken from juvenile or mature tissues (Vieitez et al. 1985). In current study, the effects of two different explant sources on shoot regeneration were compared. The first type of starting materials was three-month-old tissue-cultured plant, which cultivated in *in vitro* conditions ins under 32-48 μmol m^{-2} s^{-1} light and at 24 ± 1˚C. The second type

of explant source was greenhouse-grown mature plant, which were maintained in conditions of 18-22˚C under 337 μmol m^{-2} s^{-1} light during a short day (8 hours light) so as to conserve the plants in a vegetative stage.

The petioles of three *Campanula* cultivars namely, Blue Ocean, Improved Blue Uniform and Royal, were harvested from two different sources and cultivated in 1, 2 or 3 mg l^{-1} TDZ. All media was supplemented with 0.1 mg l^{-1} NAA. In all three cultivars, the shoot regeneration frequency of petioles harvested from greenhouse-grown plants was higher than that of shoot regeneration (%) obtained from petioles of tissue-cultured plants **(Fig. 2, Chapter 3)**. In all three medium compositions applied for cvs. Royal and Blue Ocean, the shoot regeneration of the two compared explant sources showed significant differences at $\alpha = 0.05$. In cv. Royal, none of the petioles harvested from the tissue-cultured plants were regenerated shoots **(Fig. 2, Chapter 3)**.

In addition, the effects of different concentrations of NAA (0.5, 1.0 and 2.0 mg l^{-1}) were tested in the shoot regeneration of petioles harvested from the two sources in cv. Royal. The results showed that newly grown nodal cuts were a better explant source when compared to three-month-old tissue-cultured plants **(Fig. 4, Chapter 3)**.

Sriskandarajah et al. (2008) reported a better shoot regeneration capacity in nodal cuts harvested from greenhouse-grown materials in comparison with prolonged *in vitro* cultivated tissues that had been applied in two cactus cultivars (Sriskandarajah and Serek 2004) and apple (Sriskandarajah et al. 1982). Generally, for harvesting explants, rejuvenated explant sources have been preferred to *in vitro* tissue-cultured plants due to specific *in vitro* conditions that may cause a reduction in the regeneration of *in vitro*-cultured tissues. There are several reasons for a reduction in the regeneration of tissues harvested from *in vitro*-cultured plants. For instance, long-term *in vitro* cultivation causes a reduction in the shoot regeneration of tissue-cultured petioles due to a loss of cell totipotency (Bhojwani and Razdan 1996). The hyperhydricity of tissues is another abnormality that reduces the multiplication rate of plants grown in a tissue culture and so affects shoot regeneration (Mitić et al. 2012).

6-1-2. Optimum medium composition for growing the nodal cuts of greenhouse-grown plants

Because adult plants are recalcitrant and less competence to *in vitro* culture conditions and regeneration, rejuvenation process of mature tissues to harvest explants is

recommended (Cervera et al. 2008). The mitigation of recalcitrance can be encouraged by the application of PGRs in the culture medium (Murthy et al. 1998) or by the use of a hormone-free medium (Ewald 1998). In *in vitro* conditions, the invigoration procedure has been performed through serial sub-culturing or micro-grafting (Fernández-Lorenzo and Fernández-López 2005). The current study explored the optimum hormonal content of a medium that was employed for the reinvigoration of mature tissues has been studied in three *Campanula* cultivars. Three different medium compositions (0.5 mg l^{-1} BA and 10 mg l^{-1} TDZ, both supplemented with 0.25 mg l^{-1} NAA and one hormone-free medium) were compared for the cultivation of nodal cuts. The results indicated a lack of significant differences at an alpha level of 5% among all three media in cv. Blue Ocean. Furthermore, in cv. Royal, no significant difference was observed in the axillary bud proliferation (%) of nodal cuts cultured in hormone-free media and media supplemented with 10 mg l^{-1} TDZ **(Fig. 1, Chapter 3)**. The obtained results imply that a hormone-free medium is just as efficient as a medium supplemented with PGRs. For shoot induction of the axillary buds of *Acacia auricaliformis*, the application of relatively higher concentrations of BA and kinetin (2.0 mg l^{-1} BA and 0.1 mg l^{-1} kinetin) enhanced shoot regeneration. However, the regeneration of defoliated and brown leaves was observed, along with the induction of callus instead of shoots (Ismail et al. 2012). Despite previous studies that employed PGRs for the reinvigoration of adult tissues in different *Campanula* species (Paunescu 2010, Sriskandarajah et al. 2008), the current report is the first experiment that successfully utilized and recommends a hormone-free medium for the rejuvenation of *Campanula*. Even though the growth induced by PGRs was more favorable in cv. Improved Blue Uniform than that induced by a hormone-free medium, the advantages of a hormone-free medium led the present study to employ it for the further cultivation of nodal cuts.

6-1-3. Effect of hormonal pre-treatments of nodal cuts on shoot regeneration of harvested petioles

To discover if pre-treatments of nodal cut influence shoot regeneration frequency, a two-phase (I and II) experiment was designed employing different cytokinin types and concentrations **(Fig. 1, Chapter 4)**. The nodal cuts of greenhouse-grown Blue Ocean plants were tested in this experiment. MS media consisting different concentrations of BA (0.5, 1.0 or 2.0 mg l^{-1}) or TDZ (0.5, 1.0 or 2.0 mg l^{-1}) and 0.25

mg l^{-1} NAA were applied in Phase I to grow the nodal cuts. In Phase II, the petioles of newly grown nodal explant were collected and cultured in MS media containing 0.5, 1.0 or 2.0 mg l^{-1} BA or 1.0 mg l^{-1} TDZ and 0.25 mg l^{-1} NAA. Shoot regeneration was evaluated seven weeks after Phase II cultivation. According to the results, it was concluded that the PGRs that employed in media in Phase I, did not stimulate petiole shoot regeneration in Phase II (especially in all concentrations of BA). Also, a TDZ concentration higher than 0.5 mg l^{-1} decreased shoot regeneration frequency **(Table 1, Chapter 4)**. The same result was obtained from the shoot regeneration of *Liquidambar formosana* L. when higher TDZ concentrations (> 2.27 μM) induced explants to form callus instead of shoot clusters (Xu et al. 2007). Jitsopakula et al. (2013) showed that concentrations of BA higher than 3.0 mg l^{-1} and TDZ higher than 1.0 mg l^{-1} reduced the shoot regeneration of *Vanda coerulea*.

Since PGRs used for growing nodal cuts in Phase I, did not positively influence shoot regeneration frequency in Phase II, the application of a hormone-free medium for growing nodal cuts is recommended. Along with the outcome of the current study's previously mentioned experiments, these results confirmed the advantage of utilizing a hormone-free medium to reinvigorate the *Campanula* nodal explants **(Chapter 3)**.

6-2. Establishment of a positive selection system in *Campanula*

Within the transformation of a foreign gene, the selection marker gene is transferred to tissues so as enable the selection of transformed tissues or cells out of the non-transformed ones (Penna et al. 2002). There are two main selection methods: the traditional system which uses a specific compound, such as antibiotic or herbicide, to kill non-transformed cells (negative selection) and a second method based on the ability of transformed tissues to metabolize a substrate, in which non-transformed cells are disabled or cannot efficiently convert a substrate (positive selection) (Wenck and Hansen 2004).

6-2-1. Optimal kanamycin concentration for selection

Kanamycin is an aminoglycoside antibiotic that has been widely used as selection agent for the growth inhibition of a non-transformed cell in the case when transformed tissues utilizing the *npt II* gene can detoxify this antibiotic out of a growth medium (Zhang et al. 2001). Until now, kanamycin, in various concentrations has been applied as a selective agent in different *Campanula* species. In *C. glomerata*,

50 mg l^{-1} kanamycin was added to a selection medium (Joung et al. 2001); however, in another experiment performed by Sriskandarajah et al. (2004), the application of 100 mg l^{-1} kanamycin was suggested as an optimal concentration for the selection of transformed shoots in *C. carpatica*. Relatively low selection pressure results in poor selection and high frequency regeneration of escapes, while high pressure inhibits shoot regeneration and causes tissue necrosis (Hinchee et al. 1994). Because the sensitivity of different plants and regeneration systems to kanamycin concentrations is varies among species and even cultivars, the current work designed an experiment to determine the proper concentration **(Fig. 6, Chapter 3)**.

Six weeks after transformation, the frequency of shoot regeneration in media containing 50 and 100 mg l^{-1} kanamycin did not show a significant difference at an alpha level of 5% ($P \leq 0.05$), **(Fig. 6, Chapter 3)**. Because the utilization of 100 mg l^{-1} kanamycin in a selection medium did not decrease the shoot regeneration frequency, when compared to the 50 mg l^{-1}, it was concluded that 100 mg l^{-1} should be applied for selecting transformed shoots. In this way, the poor selection and regeneration of too many escape shoots were avoided. In *Arabidopsis thaliana*, different kanamycin concentrations were assessed and no significant differences were observed in the shoot regeneration frequency of transformed explants cultured in 35, 50 and 75 mg l^{-1} kanamycin. However, increasing the kanamycin concentration up to 100 mg l^{-1} dramatically reduced the shoot regeneration. Hence, 75 mg l^{-1} kanamycin was selected as an optimal concentration (Dehestani et al. 2009).

6-2-3. Evaluation of the sensitivity of *Campanula* to mannose and the optimal concentration for successful selection

Mannose is a selective agent that has been successfully applied for the positive selection of transgenic tissues in various plants (Degenhardt et al. 2006, Ilczuk et al. 2009, Sigareva et al. 2004). However, it has not yet been assessed in the *Campanula* species. Since each plant has a specific degree of sensitivity to selective agents, different concentrations of mannose were assessed in different plants in order to establish a mannose selection system. For instance, non-transformed *lotus corniculatus* L. (Guo et al. 2015) is relatively less sensitive than sugar beet (Joersbo et al. 1998) or maize (Negrotto et al. 2000) to mannose toxicity. To determine the proper mannose concentration for selection and clarify the incapacity of wild type *Campanula* to utilize mannose as an energy source, an experiment was performed on

cv. Improved Blue Uniform **(Table 1, Chapter 3)**. The results indicated 15.78% of explants, regenerated shoots in a medium supplemented with 15 g l^{-1} mannose, but that an application of mannose in higher concentrations caused inhibition of growth and failure to regenerate shoots **(Table 1, Chapter 3)**. These findings showed the inhibitory effects of mannose on *Campanula* cells and indicated the absence of the endogenous PMI gene in wild type *Campanula*. Just as a relatively low mannose concentration can cause regeneration of too many escapes (He et al. 2006), the application of a higher selection pressure can also significantly reduce the regeneration of non-transformed plants (Wallbraun et al. 2009). Therefore, it was decided that the selection medium should be supplemented with 30 g l^{-1} mannose **(Table 2, Chapter 3)**.

6-2-4. Comparison of selection systems (positive (mannose) and negative (kanamycin)) to distinguish transformed tissues

Briza et al. (2010) compared the effectiveness of mannose and kanamycin when selecting transformed shoots of *Lactuca sativa* L. In their study, mannose was also shown to be an efficient non-antibiotic selection agent by equal competence with the traditional antibiotic selection method.

In *C. carpatica* cv. Improved Blue Uniform, both selection systems (antibiotic and non-antibiotic) were compared in the gene transformation. The regenerated shoots were examined by GUS assay and all regenerated shoots in both media showed the GUS signal **(Table 2, Chapter 3)**. The transformation frequency obtained in *Campanula* by mannose selection (8.33%) was lower than that by kanamycin (11.42%) **(Table 2, Chapter 3)**. Although similar results were observed in the transformation frequency of Chinese cabbage, when compared with the application of kanamycin and mannose as selective agents, the practicality of mannose selection was declared (Min et al. 2007). Transformation frequency fluctuates by differences in genotypes (Guo et al. 2015), selection pressure, and the level of PMI gene expression. Gui et al. (2014) found that there is a positive correlation between the protein expression level and the optimum selection pressure.

Although, in some plants, a transformation frequency based on kanamycin was higher compared to that of mannose selection, biosafety concerns were avoided by the use of mannose instead of antibiotics. Privalle et al. (2000) assessed the biosafety of the PMI

gene in regard to toxicity and allergenicity and found no adverse effects on mammalian. Mannose has been reported to be a safe and efficient alternative selection method for traditional antibiotic systems in the selection of *Campanula* **(Chapter 3)**.

6-3. Tetcyclacis treatments on *Campanula* flowers

6-3-1. *In vivo* blocking assessment of flavonoid hydroxylase enzymes

The activity of hydroxylase enzymes can be detected by the application of chemical compounds, which inhibit or enhance these enzymes (Menting et al. 1994). Tetcyclacis is a typical cytochrome P-450 inhibitor (Stich et al. 1988) which has been applied to characterize hydroxylase enzymes in different flowers (Boase et al. 2010, Martens and Forkmann 1998).

Furthermore, tetcyclacis can be applied to visualize and predict the outcome of hydroxylase enzymes blockage and to determine if endogenous DFR is able to accept DHK and produce pelargonidin (orange-red colour) (Boase et al. 2010). In the current study, different tetcyclacis concentrations were sprayed on the flower buds of three *Campanula* cultivars and the anthocyanin content of the petals were extracted three days after treatments. The absorbance (Abs) of extracts was measured at 525 nm when estimating the anthocyanins content **(Chapter 5)**. **Fig. 2, chapter 5** presents the spectrophotometer measurment of 525 nm for the Abs of anthocyanin extractions of cv. Improved Blue Uniform. Also, **Fig. 1, Appendix** represents the absorbance of anthocyanin, extracted from cv. Blue Ocean and **Fig. 2, Appendix** shows the Abs of the Royal flowers, anthocyanins extractions three days after tetcyclacis treatments. The results demonstrated that, by increasing the tetcyclacis concentration, the flavonoid content of petals decreased and, in 100 μM tetcyclacis, the colour of the flowers turned white **(Fig. 3, Appendix)**. Obtaining a white flower colour after tetcyclacis treatments by the inhibition of the F3'5'H and F3'H enzymes suggests the absence of other responsible enzymes that may produce a flower colour in *Campanula* and an incapability of DFR to accept DHK as a substrate. Martens et al. (2003) and Seitz et al. (2007) also have came to the same conclusion after tetcyclacis treatments on *Anagallis monelli* and *Osteospermum hybrida* respectively. In addition, by obtaining white flowers in *Anagallis monelli* when hydroxylase enzymes were

inhibited by the use of tetcyclacis, a high substrate specificity of DFR concluded (Martens et al. 2003). Martens et al. (2003) mentioned the accumulation of 4'-hydroxylated flavonoids, such as pelargonidin, as a consequence of hydroxylase enzyme inhibition. The result of white flowers after F3'5'H and F3'H enzyme inhibition shows that *Campanula* DFR does not accept DHK and is not able to convert DHK to a pelargonidin precursor (LPg). Thus, pelargonidin is not naturally produced in the flavonoid pathway of *Campanula*. Hence, the efficient inhibition of *F3'5'H* gene leads to achieve the white flower colour in *Campanula* **(Chapter 5)**.

6-4. Assessments on transformed plants

The down-regulation of a specific endogenous gene for flower colour modification is an efficient method to alter the flavonoid pathway and this has been employed in several ornamentals (Boase et al. 2010, Nakamura et al. 2010). In the present work, gene silencing was performed using a palindromic copy of the *F3'5'H* gene under the control of the *pst* promoter, petal- and stamen-specific, from the MADS box gene of *Arabidopsis* **(Fig. 1, Chapter 5)**.

6-4-1. PCR and southern blot analysis

Putative transformed plants were tested using PCR and the positive lines were transferred to a greenhouse to grow and flower. As the flower colour of the transformed plants did not alter visually, the integration of T-DNA into the genome and the copy number of T-DNA, in each independent transformed line, were analysed by the southern blot. Southern blot analysis confirmed three independent transformed lines with the integration of two (11-213 and 21-1) or three (16 B) copies of T-DNA **(Fig. 3, Chapter 5)**.

The number of the T-DNA copy number can directly affect the stability of transgene expression (Jorgensen et al. 1996) and the introduction of a single copy transgene is preferable when compared to the multiple copy number integration of T-DNA, which causes gene expression variability or susceptibility to the silencing (De Paepe et al. 2012). The effectiveness of transgene-induced RNAi on gene silencing has been compared in different single and multiple copies lines of transgenic *Arabidopsis*. The results found that single copy transgenic lines showed a maximum gene knockdown,

unless some of the multiple copy T-DNA lines indicated an intermediate RNAi effect on gene expression. However, the reduction effect on gene expression is not reliable due to progressive loss over generations and it may show reversibility and somatic diversity in the transformed tissues (Kerschen et al. 2004).

6-4-2. Evaluating the expression of *F3'5'H* and *F3'H* genes

After gene transformation, the expression of *F3'5'H* and *F3'H* genes was evaluated for three different stages of flower developments in transgenic plants. The results show the expression of both genes in the transformed plants' three stages of flower development **(Fig. 4, Chapter 5)**. In the current work, RNAi construct aiming to silence the *F3'5'H* gene failed to down-regulate the gene **(Chapter 5)**.

The functionality of RNAi gene silencing can vary from a completely silenced gene to a partial gene down-regulation and an un-silenced gene, depending on the position of the inserted T-DNA and the copy number (Travella et al. 2006). Furthermore, Travella et al. (2006) referred the variations in the efficiency of gene silencing to the differences that exist in some phenotypes. They demonstrated that some phenotypes are less sensitive to the amount of protein expected to be down-regulated by gene silencing and that even the reduced level of gene expression is still sufficient for their proper function.

6-4-3. To generate ethylene insensitive plants

Gene transformation was performed to generate improved postharvest quality plants as well as flower colour alterations **(Chapter 5)**. Therefore, transformed plants were assessed to identify sensitivity to ethylene. To produce ethylene-insensitive plants, the plasmid was constructed to harbour the *etr1-1* gene (an ethylene receptor mutant from *Arabidopsis*) under the control of the *fbp1* flower-specific promoter.

In many gene manipulation studies, the 35S promoter has been used to drive the expression of *etr1-1* for the generation of ethylene insensitive transgenic plants (Clark et al. 1999a, Jones 2008). The 35S promoter is a general constitutive promoter that regulates gene expression in dicot plants. Because the ethylene-signaling pathway is involved in many plant developmental processes, several undesirable physiological problems were reported from the use of the 35S promoter in transformed plants (Clark et al. 1999b). Gubrium et al. (2000) hypothesized that the constitutive inhibition of plant response to ethylene via the expression of *etr1-1* driven by the 35S promoter

affects several processes in all developmental stages, such as seed germination, fruit ripening, flower senescence and adventitious root formation. Therefore, depending on the product the application of tissue (flower)-specific promoters to drive the *etr1-1* gene has been suggested. For instance, by the application of a flower specific promoter, the alteration of organs other than a flower can be prevented and a strong phenotype with a longer vase life and better horticultural performance will be generated.

The introducing the *etr1-1* gene using a flower-specific promoter *fbp1*, into the genome of different ornamentals, such as *Kalanchoe blossfeldiana* and *Campanula carpatica* decreased the ethylene sensitivity of flowers. In addition, the side effects of the 35S promoter were not observed (Sanikhani et al. 2008, Sriskandarajah et al. 2007). The expression of the *etr1-1* transgene in various plants resulted in ethylene insensitive lines (Clark et al. 1999b, Wilkinson et al. 1997, Gallie 2010, Kuniyoshi 2013) and functionality of *etr1-1* gene has been well characterized.

The present work monitored the flower longevity of two transgenic lines (21-1 and 11-213) containing two T-DNA copies and wild-type plants in two different air qualities (ethylene-free air or 2 μ l^{-1} ethylene). Observed was a significant delay in the flower senescence of the transformed lines when compared to the non-transformed plants **(Fig. 5, Chapter 5)**.

Although the integration of a single copy transgene by gene transformation is usually preferable, integration of several T-DNA copies or truncated transgenes often occurs (Tzfira et al. 2004). However, in the current study, there were no single copy transgenes obtained, the function of the *etr1-1* gene was not affected by the copy number and the transformed plants were ethylene insensitive **(Chapter 5)**. The absence of a correlation between the transgenes' copy number and the functionality of the *etr1-1* gene in generating ethylene insensitive plants had been previously reported by (Sriskandarajah et al. 2007), when the same level of resistance to the ethylene was observed in single and double T-DNA copies lines. Sanikhani et al. (2008) came to the same conclusion and showed that resistance to ethylene is independent of the T-DNA copy number.

7. Outlook

Despite the vast production of *Campanula* plants, a limited numbers of studies have concentrated on the *Campanula* flower colour. To date, there has been no investigation reporting on flower colour manipulation of the *Campanula* species. Furthermore, information about the enzymatic activity of *Campanula* hydroxylase enzymes, DFR and CHS enzymes is very limited. Although, no single copy T-DNA line was achieved in the current study, further experiments that generate single copy transformed plants can be helpful in finding the correlation between the T-DNA copy number and RNAi expression in *Campanula*.

The results of the tetcyclacis treatments demonstrated that after efficient *F3'5'H* gene silencing, white flowers could be expected, when DFR is not capable of accepting DHK and so converts it to pelargonidin. Therefore, to generate a red-orange colour hue in this plant, it is necessary to express an exogenous DFR, such as *Gerbera hybrida*, so as able to efficiently convert DHK to pelargonidin or over-express the *F3'H* gene.

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8. References

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Appendix

9. Appendix

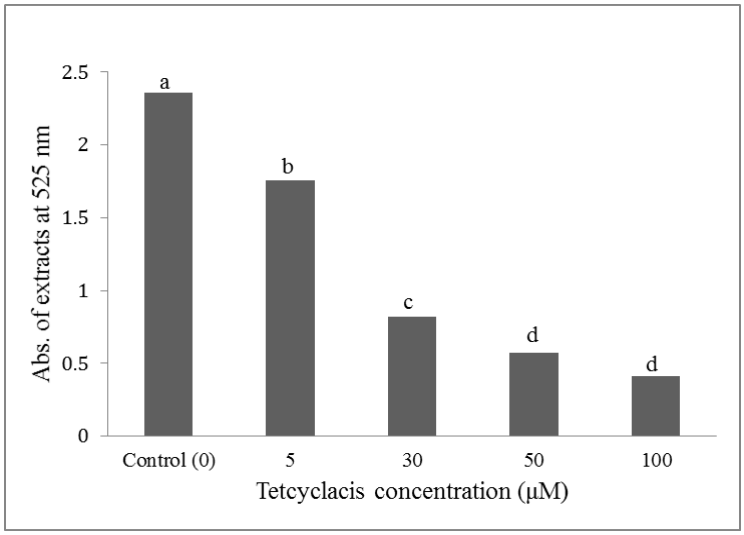

Fig 1. Flavonoids content of cv. Blue Ocean, 3 days after tetcyclacis treatments. Flower buds were sprayed with 0, 5, 30, 50 and 100 µM tetcyclacis from left to right respectively. Column marked with same letter within the same chart are not different significantly at α=0.05.

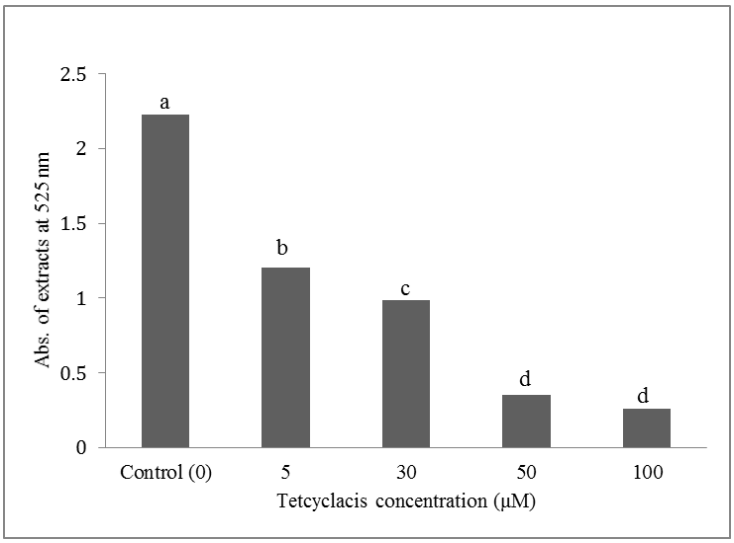

Fig 2. Flavonoids content of cv. Royal, 3 days after tetcyclacis treatments. Flower buds were sprayed with 0, 5, 30, 50 and 100 µM tetcyclacis from left to right respectively. Column marked with same letter within the same chart are not different significantly at α=0.05.

Fig 3. *Campanula* cv. Royal flowers after tetcyclacis treatments. Flower buds were sprayed with 0, 5, 30, 50 and 100 µM tetcyclacis from left to right respectively.

Curriculum Vitae

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Ausbildung (education)

[2009-2015]	**Doktorandin, Leibniz Universität Hannover** **Abteilung Zierpflanzenbau** **Herrenhäuser Str. 2** **D 30419 Hannover, Germany**
[2005-2007]	**MSc Agricultural Biotechnology** **Ferdowsi University of Mashhad** **Mashhad, Iran**
[2000-2005]	**BSc Horticultural Engineering** **Ferdowsi University of Mashhad** **Mashhad, Iran**

Computerkenntnisse (Computer skills)

Statistical analysis (SAS, R)	„sehr gute Kenntnisse“
Bioinformatics (MEGA, R)	„sehr gute Kenntnisse“
Microsoft Office (Word, Excel ...)	“sehr gute Kenntnisse“

Fremdsprachenkenntnisse (foreign language skills)

Persian	"Muttersprache"
English	"Mündlich: Sehr gut, schriftlich: Sehr gut"
German	"Mündlich: Basic , schriftlich: Basic"

Theses:

- **M.Sc Thesis** (2007) Chromosome doubling of *Gerbera jamesonii* via tissue culture. Under supervision of Prof. A. Bagheri and co-supervision of Prof. M. J. Kermani
- **PhD Thesis** (2015) Molecular breeding of *Campanula* for novel flower colour and ethylene insensitivity. Under supervision of Prof. Dr. Margrethe Serek.

Publications

1. E. Davarynejad**,** G.H. Davarynajad, A. Tehranifar and **Z. Ghayoor**, 2005. New experiment on Year round Production of Saffron in hydroponic system, Abstract in the Proceedings of the 4th Iranian Horticultural Science Congress, Mashhad, 8th-10th November, p. 404.

2. **Z. Ghayoor** and G. H. Davarynejad, 2006. Effect of oryzalin on chromosome doubling and plant morphology of some species of *Gerbera jamesonii*, Abstracts of the 27th International Horticultural Congress & Exhibition, Seoul, Korea, 13th-19th August. Book of Abstracts, p. 346.

3. E. Davarynejad, A. Tehranifar, **Z. Ghayoor**, and G. H. Davarynejad, 2008. Effect of different pre-harvest conditions on the post-harvest keeping quality of cut Gerbera. Europe-Asia Symposium on Quality Management in Postharvest Systems-Eurasia, Bangkok, Thailand, 3rd-6th December 2007. Acta Horticulturae 804: 205-208.

4. **Z. Ghayoor**, A. Bagheri, M. J. Kermani and G. H. Davarynejad, 2009. Investigating the *in vitro* growth inhibition of oryzalin treated *Gerbera jamesonii*. 6th International Symposium on In Vitro Culture and Horticultural Breeding, Brisbane, Australia, 24th-28th August 2008. Acta Horticulturae 829: 309-312.

5. **Z. Ghayoor**, A. Bagheri, M. J. Kermani, G. H. Davarynejad, 2011. Effects of TDZ and kinetin on regeneration and proliferation of *Gerbera jamesonii*, Journal of Horticultural Science, 24(2): 170-174.

6. **Z. Ghayoor,** A. Bagheri, M. J. Kermani and G. H. Davarynejad, 2009. Effect of oryzalin on chromosome doubling and *in vitro* growth of *Gerbera jamesonii.* 23rd EUCARPIA SYMPOSIUM - Section Ornamentals "Colourful Breeding and Genetics", Leiden, Netherlands, 31st August-4th September Book of Abstracts, p. 114.

7. **Z. Ghayoor**, H. Mibus, M. Serek, 201X. The effects of cytokinin treatment of nodal cuts and petioles on the shoot regeneration of *Campanula portenschlagiana* cv. Blue Ocean. 6th International Symposium on Production and Establishment of Micropropagated Plants, Sanremo, Italy, 19th -24th April 2015. Acta Horticulture (in press).

8. **Z. Ghayoor**, M. Serek, H. Mibus, 2015. Transformation of *Campanula carpatica* to alter flower colour and ethylene sensitivity. Journal of Horticultural Science & Biotechnology, 90(5): 518-524.

9. **Z. Ghayoor** et al, The establishment of effective regeneration systems in *Campanula portenschlagiana* and *carpatica* cultivars**.** (in preparation for submission).