Expression Effects of CHI and CHS Genes and Colchicine Treatment in Yellow Flowered Cyclamen: a Review

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Abstract

Nowadays, the field of entirely artificial hybrids raises ethical problems in the animal world and to a lesser amount in plants. Throughout the years, yellow *Cyclamen* has been particularly important for both breeders and passionate growers as being a peculiar color for this species. The possibility to artificially induce hybrids between species that can never normally cross it's now achievable. This paper describes the possibility of obtaining high ornamental yellow flowered cyclamen, through chromosome doubling. The pollen and seed sterility can be overcome by doubling the chromosomes. In this sense, there are two full sets from each parent, resulting in a fertile hybrid, by introducing the *in vitro* culture into colchicine supplemented medium.

Keywords: colchicine, *Cyclamen persicum*, pigments, polyploidy

Introduction

The *Cyclamen* name has its origin due to its spherical shape tuber or the spiral shape of the peduncle following pollination, shape that in Greek means *kyklos*. Romans called it *Cyclaminis*, and later around year 1700, the french botanist Tournefort named it *Cyclamen* as a genus (Preda, 1971).

In antiquity, *Cyclamen* was recognized for its therapeutic qualities, due to the presence of cyclamin (Fig. 1). This bitter substance with purgative powers is used as a basic remedy in homeopathy against grief and depression being well known for its medicinal properties since antiquity (Fournier, 1999).

The *Cyclamen* genus consists of 24 species, belongs to the *Primulaceae* family (Grey-Wilson, 2015) and is distributed in and near the

Mediterranean region. Some of them are frost resilient, while others can bloom from late summer until late spring in a palette of colors from white to red. Throughout the years, yellow *Cyclamen* has been particularly important for both breeders and passionate growers.

This paper depicts the possibility of obtaining high ornamental yellow flowered cyclamen, through chromosome doubling.

Colchicine is a toxic natural product extracted from the bulb-like corms of *Colchicum autumnale*, that is used in producing polyploid strains (Planchais *et al.*, 2000) (Fig. 1). Colchicine is also used for inducing polyploidy in plant cells during cellular division by inhibiting chromosome segregation during meiosis. Polyploids are organisms with two or more sets of chromosomes (Sattler *et al.*, 2015).

Figure 1. Chemical compound of colchicine (left) and cyclamin (right)
(source: <https://pubchem.ncbi>)

Figure 2. Hybrid vigour in polyploids as a result of increased gene dosage
(source: original)

Seed sterility poses a barrier in the breeding and propagation of cyclamen. In many plants, sterility caused by the lack of affinity between different genomes can be overcome by chromosome doubling (Fig. 2).

Numerous theoretical works predict that polyploids may exhibit less inbreeding depression

than diploids, because of the presence of multiple gene copies (Lande and Schamske, 1985). Inbreeding depression in cyclamen was also reported by Wellensiek (1959).

Most genotypes show inbreeding depression, although the degree varies with genotype. Also, cross compatibility between diploid and tetraploid

cultivars was very low in the research conducted by Takamura and Miyajima (1996a).

Key enzyme genes responsible for yellow coloration/pigmentation

The major pigments produced in flowers comprise flavonoids, carotenoids, betalains and chlorophylls. Amid flavonoids, flavonol glycosides are colorless to pale compounds while anthocyanins range from red to purple, both types being broadly distributed in multiple organs, particularly in cyclamen. In flowers the presence of flavonol glycosides as co-pigments changes anthocyanins, resulting in a broad assortment of colors (Nakayama *et al.*, 2012, Takamura and Sugimura, 2008).

Anthocyanidin structures and flavonoid biosynthetic pathways responsible for color are well-known and effectively all genes that encode the biosynthesis enzymes have been isolated in previous studies (Fig. 3).

Chalcone synthase (CHS), a polyketide synthase, is the first committed enzyme in the pathway, and it catalyzes the synthesis of THC (from one molecule of 4-coumaroyl CoA and three molecules of malonyl CoA) (Fig. 4). THC is rapidly and stereospecifically isomerized to the colorless (2S)-naringenin by chalcone isomerase (CHI).

These particular metabolites are involved in diverse biotic and abiotic functions as well as plant structure, chemical defenses against pathogens, root nodulation, UV protection, pollen development and flower color.

The advances in molecular analysis of CHS have shown that it is universally present in plants, including early land plants and *Charophyceae*, the class of green algae (Schroder, 1997). Firstly reported in parsley (Reimold *et al.* 1983), CHS has been mostly isolated, including in ornamental plants as petunia (Morgret *et al.*, 2005), phalaenopsis (Han *et al.*, 2006) and herbaceous peony (Zhao *et al.*, 2012b). Their protein sequences are greatly conserved among various plants with ~80–90% homologies (Beerhues and Wiermann, 1988). CHS plays an important role in the accumulation and synthesis of anthocyanins, which results in flower color alteration. In transgenic petunia the expression of *Freesia hybrida* CHS1 results in flower color modification from white to pink (Sun *et al.*, 2015), and in transgenic tobacco the expression of CHS

of *Malus crabapple* exhibits higher anthocyanin accumulations and deeper red petal colors compared to the control untransformed lines (Tai *et al.*, 2014).

Additionally, CHS expression is often regulated by different developmental stages and tissue specificity and, with a wide-ranging sensitivity to environmental stimuli. For example, CHS of safflower is responsive to salinity stress, salicylic acid treatment and wounding, respectively (Dehghan *et al.*, 2014). In case of CHS in *Dryopteris fragrans*, UV light and temperature can affect its expression (Sun *et al.*, 2014).

CHI (chalcone isomerase) encodes the second key enzyme gene in plants anthocyanin biosynthesis, which catalyzes the isomerization of chalcone. Chalcone is modified by CHI to form flavanone. This enzyme is essential in the metabolic branch pathways of flavone, flavonol, proanthocyanidin, and anthocyanin synthesis. Therefore, CHI is expressed and its expression level affects flavonoid metabolism, ultimately affecting flower color development. For example, a decrease of CHI expression in the petals of carnations, asters, cyclamen and tobacco leads to the accumulation of chalcone resulting into modifying the pigmentation to yellow (Nishihara *et al.*, 2005).

Regarding cyclamen, “Golden Boy” is a natural mutant of ‘Pure White’, which most likely arose by the CHI gene loss-of-function. This yellow flowered cyclamen was developed by induction of the Ch2G through a CHI gene deficiency (Miyajima *et al.*, 1991).

Therefore, “Golden Boy” is thought to have acquired the fourth branch for disposing of chalcone surplus accumulated as the result of inactivation of CHI gene. Furthermore, because ‘Golden Boy’ accumulates Ch2G in flowers as well as leaves, petioles and cotyledons, these organs are yellow-green (Takamura *et al.*, 1993; 1995).

Flowers of ‘Golden Boy’ accumulate chalcone 2-O-glucoside (Ch2G) into their pale yellow flowers as their major flower pigment, and also quercetin and kaempferol in trace amounts, suggesting that the biosynthetic pathway from chalcone to a flavonoid is inactivated (Tab. 1) (Miyajima *et al.*, 1991; Sugimura *et al.*, 1997).

Other enzymes, such as F3'H (flavonoid 3'-hydroxylase) and F3'5'H (flavonoid 3',5'-hydroxylase) are necessary for cyanidin and delphinidin

Figure 3. The biosynthetic pathway leading to the biosynthesis of anthocyanidins

Enzymes and flavonoid classes are indicated by abbreviated and bold letters, respectively. The actual color of compounds depends on various factors as described in the text. CHS - chalcone synthase; THC2'GT - UDPglucose:tetrahydroxychalcone 2'GT; CHI - chalcone isomerase; THC4'GT - UDP-glucose:tetrahydroxychalcone 4'GT; AS - aureusidin synthase; F3H - flavanone 3-hydroxylase; F3'H - flavonoid 3'-hydroxylase; F3'5'H - flavonoid 3',5'-hydroxylase; DFR - dihydroflavonol 4-reductase; ANS - anthocyanidin synthase.

Table 1. Flower pigments detected in *Cyclamen persicum* cultivars, wild *C. purpurascens* and their interspecific hybrids (adapted from Ishizaka, 2018)

Plant materials	Flower pigments detected ¹						
	Kae	Que	Ch2'G(ISPP)	Mv3G	Mv3,5dG	Pn3G	Pn3nH
<i>C. persicum</i> "Strauss"	2	2	-	4	-	3	3
<i>C. persicum</i> "Pure white"	2	2	-	-	-	-	-
<i>C. persicum</i> "Golden Boy"	2	2	2	-	-	-	-
<i>C. purpurascens</i>	2	2	-	-	2	-	-
GBCP (Golden Boy x <i>C. purpurascens</i>)	2	2	-	-	2	-	-

Note: ¹Kae - Kaempferol glycosides; Que - Quercetin glycosides; Ch2'G - Chalcone 2'-O-glucoside (ISPP, isosalipurposide); Mv3G - Malvidin 3-glucoside, Mv3,5dG - Malvidin 3,5-diglucoside, Pn3G - Peonidin 3-glucoside, Pn3nH - peonidin 3-neohesperidoside; ²Pigment detected in slip and eye; ³Pigment detected in slip; ⁴Pigment detected in eye; - Not detected.

production, respectively. They are the key enzymes that determine the structure of anthocyanins and consequently their color (Tanaka *et al.*, 2008).

Accumulation of isosalipurposide (Ch2G), which is a glycosylated form of 4,2',4',6' - tetrahydroxychalcone has been previously attributed to mutations in the CFI (chalcone flavanone isomerase) gene in other experiments too. The petals of the diploid yellow-flowered cyclamen are paler than those of the yellow-flowered *Dianthus caryophyllus* and *Callistephus chinensis*, although they also contain chalcone (Kuhn *et al.*, 1978; Forkmann and Kuhn, 1979; Forkmann and Dangelmayr, 1980; Miyajima *et al.*, 1991).

In addition to the structural genes in the anthocyanin biosynthetic pathway, transcription factors also play important roles in flower color development through regulating the temporal and spatial expression of structural genes (Xie *et al.*, 2006). There are three main types of transcription factors that affect flower color, MYB, bHLH and WD40 (Ramsay and Glover, 2005). These transcription factors activate or suppress the transcription and expression of target genes through binding to specific DNA sequences and affect protein-protein interactions. MYB TFs affect flower color by regulating petal cell morphology (Noda *et al.*, 1994; Baumann *et al.*, 2007; Di *et al.*, 2009).

As mentioned above, physical factors that affect flower color are temperature, light and water content. For example, darker flowers result by exposure to low temperatures due to increased anthocyanin content in plants, such as plantain

(Stiles *et al.*, 2007). High temperatures lead to lighter flower colors due to reduced anthocyanin content, in plants such as *Rosa hybrida* cv. Jaguar (Dela *et al.*, 2003), *Chrysanthemum morifolium* Ramat. (Nozaki *et al.*, 2006) and *Lilium* spp. (Lai *et al.*, 2011). These are a consequence of the suppressed expression of genes involved in anthocyanin biosynthesis, such as *CHS*, *F3H* and *DFR*. Therefore, the anthocyanin biosynthesis rate is reduced at high temperatures affecting the anthocyanin concentrations (Lai *et al.*, 2011).

In addition to the three physical factors discussed above, pollinators (Adriana *et al.*, 2011), gamma rays (Dwivedi *et al.*, 2000; Bala and Singh, 2013) and ion beam irradiation (Hase *et al.*, 2010; Masayoshi *et al.*, 2012; Kameari *et al.*, 2012) are known to affect flower pigmentation in ornamental plants.

Colchicine induced tetraploids in different cultivars

The most significant features in *Cyclamen* are represented by flower and foliage characteristics. Beside multiple flower colors, petal shape and edge are of interest.

For a better understanding of the major characteristics in *C. persicum* cv. *Neo Golden girl*, it must be noted that the flowers consist of a pink "eye", which is the region of the petal base and a pale yellow "slip", which is the petal region excluding the eye (Fig. 4).

For the pollination process, the method used is relatively simple: the selected plants, *Golden boy* and *Neo Golden girl* varieties, were crossed through

Figure 4. Flower characteristics of *Cyclamen persicum* cultivar Neo Golden girl.
(source: www.engel.net)

the traditional method, transferring pollen from one flower to another (Grey-Wilson, 2015).

Although this method may result in commercially less valuable plants, this can be overcome by introducing the *in vitro* culture to colchicine supplemented medium.

After the treatment with colchicine, the plants were cross pollinated. In the research conducted by Ishizaka and Uematsu (1994 and 1995a), they affirmed that the method inducing the largest number of fertile plants was with 50 mg/l colchicine treatment and a duration of 5-10 days.

Tetraploid yellow flowers were successfully induced by colchicine treatment in the study conducted by Takamura *et al.*, 1996c (Tab. 2). A two days treatment proved to be the most effective in inducing yellow flowered plants with polyploidy derivatives. This resulted in a higher accumulation of pigments in petals, increased petal size and pollen diameter and viability.

In recent studies, *in vitro* colchicine treatment of placenta-attached ovules derived from crosses of *C. persicum* "Golden Boy" (all $2n = 2x = 48$, AA) $\times$ *C. purpurascens* ($2n = 2x = 34$, BB) (GBCP) has produced allotetraploids ($2n = 4x = 82$, AABB)

by embryo rescue, due to chromosome doubling (Ishizaka and Uematsu, 1995b, Kameari *et al.*, 2010). The allotetraploids produce viable pollen grains through frequent formation of 41 bivalent chromosomes in the pollen mother cells by pairing homologous chromosomes within the A and B genome, and produce fertile seeds by self-pollination (Ishizaka, 1997).

Other allotetraploids (AABB) produced by chromosome doubling of allodiploids of diploid *C. persicum* 'Golden Boy' (AA) $\times$ *C. purpurascens* (BB), known as "GBCP", have not yet been developed into commercial cultivars, but GBCP is a useful breeding material for creating yellow flowered cyclamen by mutation breeding (Kameari *et al.* 2010).

C. persicum "Golden Boy" (AA), lacking anthocyanins, has pale yellow flowers with chalcone 2'-O-glucoside (ISPP) as a major pigment. Inactivation of the CHI gene by insertion of an unknown sequence leads to a deficiency of chalcone isomerase. This results in an excess of chalcone, which is later converted to chalcone 2'-O-glucoside by glycosylation and ultimately accumulated in the vacuole (Miyajima *et al.* 1991).

Table 2. Polyploidization using colchicine in Cyclamen plants

Species	Ex-plant	Concentration/ Duration	Method of colchicine application	PL and CC	PL determination	Characteristics	References
D Yellow girl	T	0, 100, 500 mg/l, 7-days treatment	<i>in vitro</i> , on liquid 1/3 MS	U	CC	Anatomical (guard cell length↑, guard cell width↑, pollen diameter↑, pollen viability↑) Morphological (petal size↑, flower pigmentation↑)	Takamura Tet al. (1996c)
<i>C. persicum</i> "Sonja"	EC	0.5%, 10 and 15 days	<i>in vitro</i> , MS without plant hormones	2n=48	CC	Anatomical (guard cell length↑, guard cell width↑, pollen diameter↑, pollen viability↑) Morphological (petal size↑, flower size↔, auricle↓, peduncle arched, heart-shaped leaf)	Ishizaka H, Uematsu J (1994)
<i>C. hederifolium</i>	EC	0.5%, 10 and 15 days	<i>in vitro</i> , MS without plant hormones	2n=34	CC	Morphological (petal size↑, peduncle coiled, ivy leaf)	Ishizaka H, Uematsu J (1994)
H <i>C. persicum</i> Sonja x <i>C. hederifolium</i>	EC	0.5% and 0.5%, 10 and 15 days	solid colchicine medium	2n=41	CC	Morphological (flower size↓, peduncle coiled, ivy leaf)	Ishizaka H, Uematsu J (1994)
A1 <i>C. persicum</i> "Sonja" x <i>C. hederifolium</i>	HO	0.5%, 10 and 15 days	<i>in vitro</i> , MS without plant hormones	2n=82	CC	Anatomical (guard cell length↑, guard cell width↑) Morphological (flower size→, peduncle coiled, ivy leaf)	Ishizaka H, Uematsu J (1994)
A2 <i>C. persicum</i> "Sonja" x <i>C. hederifolium</i>	MH	0.5%, 10 and 15 days	<i>in vitro</i> , MS without plant hormones	2n=82	CC	Anatomical (guard cell length↑, guard cell width↑) Morphological (flower size↔, peduncle length↑, auricle↓, peduncle coiled, heart-shaped leaf)	Ishizaka H, Uematsu J (1994)
D Kage Yellow	S	100 and 500 mg/l, 1, 2 and 4 days	<i>in vivo</i>			Seed germination↓ by colchicine concentration ↑	Takamura and Miyajima (1996b)
D Kage Yellow	T	0, 20, 100 or 500 mg/l	<i>in vitro</i> , 1/3 MS 1.0 p,M n6-BA, 3% sucrose and 0.3% gellan gum	U	FCM= flow cytometry	Anatomical (guard cell length↔, guard cell width↔, pollen diameter↔, pollen viability↑); Morphological (dose↑ =plant regeneration↔; dose↓= polyploidy↓, flower size↔, petal pigmentation↓)	Takamura and Miyajima (1996b)
TP Kage Yellow	T	0, 20, 100 or 500 mg/l	<i>in vitro</i> , 1/3 MS 1.0 p,M n6-BA, 3% sucrose and 0.3% gellan gum	U	FCM= flow cytometry	Anatomical (guard cell length↑, guard cell width↑, pollen diameter↑, pollen viability↔); Morphological (high dose↑=plant regeneration↔; dose↓= polyploidy↓; flower size↑; petal pigmentation↑)	Takamura and Miyajima (1996b)

Note: A1,2=Amphidiploids; BA=benzyladenin; CC=chromosome counting; D=diploid; EC=embryogenic callus; FCM=flow cytometry; H=hybrid; HO=hybrid ovules; MH=mature hybrids; MS=Murashige and Skoog medium (Murashige and Skoog, 1962); PL=ploidy level; T=tuber; TP=tetraploid; U=chromosome number was not counted; S=seeds; ↑=higher; ↔=intermediate; ↓=lower

Other chemical factors besides colchicine that affect plant color are environmental pH and plant hormones, such as gibberellins. The later has been found to induce expression of *CHS*, *CHI*, *DFR* genes by its anther production and transportation to petals (Weiss *et al.*, 1995). Also, transgenic methods prove to be effective strategies for the regulation of flower colors. Boase *et al.* (2010) suppressed the *F3'5'H* gene in cyclamen via antisense inhibition. This led to a reduced delphinidin content and higher cyanidin content, resulting in petal color shifting from purple to red to pink. RNA interference technology controlled flower color in blue gentian (Nakatsuka *et al.*, 2010).

Induction of polyploidy proves to be challenging due to the incompatibility of multiple crosses between species different from *Cyclamen*, mainly caused by the incompatible mechanism in pollination biology and different cytology. With all these, come the inheritance of yellow pigments and correlation between the “slip” and “eye”, which are controlled by different genes (Takamura *et al.*, 2000).

The most well-known consequence of polyploidy in plants is the increase in cell size, caused by the larger number of gene copies (Sattler, 2016). The natural origin of polyploidy stands in the rare incidence of somatic mutations (mitotic doubling of chromosome number), with the development and fusion of unreduced gametes in diploids. This leads to the formation of tetraploids.

Concluding remarks

This paper emphasizes that the possibility to induce yellow cyclamen is attainable with overcoming the aspect of cross incompatibility by chromosome doubling, for obtaining flowers with high ornamental value. Flower color induction in ornamental plants is a consequence of multiple factors. The color of colchicine treated plants can be predicted, along with its viability and sterility, and expression of related compounds in the original and hybrid plants. Anatomically speaking, colchicine induced high guard cell length and width, and pollen viability. Morphologically speaking, colchicine led to the formation of larger plants, high width and length of leaves and increased pigmentation in petals.

Therefore, overcoming the aspect of cross incompatibility by colchicine treatment, along

with gene manipulation, proves to be an efficient method when it comes to breeding new varieties. Colchicine treatment leads to higher accumulation of interest compounds in petals and vegetative organs, whereas gene manipulation acts as a targeted method by regulating petal cell morphology.

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