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DEDICATED TO PROFESSOR LÁSZLÓ HESZKY ON HIS 70TH BIRTHDAY

FROM CONTENTS

NAKVI National Agricultural Advisory, Educational and Rural Development Institute (NAERDI)

ÖMKi

Ökológiai Mezőgazdasági Kutatóintézet
Research Institute of Organic Agriculture | Forschungsinstitut für biologischen Landbau

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Hungarian Research Institute of Organic Agriculture – ÖMKi

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Cover photo: Selection of mutant plants; Curd of homozygous Or mutant and wild-type cauliflowers (Zsófia Tóth, Zsolt Galli)

DEDICATED TO PLANT SCIENCES: FOR LÁSZLÓ HESZKY'S 70TH BIRTHDAY

ERZSÉBET **KISS**

Szent István University, Faculty of Agricultural and Environmental Sciences, Institute of Genetics and Biotechnology, 2100 Gödöllő, Hungary

"The greatest service which can be rendered any country is to add a useful plant to its culture."

(Thomas Jefferson)

László Heszky member of the Hungarian Academy of Sciences celebrates his 70. birthday this year. He was born on 16th, January, 1945 in Győr. After attending the elementary school in Győr, and taking final examination at the Agricultural High School in Csorna, he enrolled in the Gödöllő University of Agricultural Sciences, where he graduated in 1967. He started working in the Institute of Agrobotany in Tápiószele with academician Andor Jánossy. László Heszky occupied different posts from research assistant to scientific department head.

Figure 1: Academician Heszky in his office for 25 years as the head of the Institute of Genetics and Biotechnology

His first task in Tápiószele was to maintain and investigate the grass species and variety collection of the institute. His talent manifested itself very early, he wrote a book about the timothy grass together with Imre Máté member of HAS (Máté and Heszky 1972), then a few years later he published a monograph on *Festuca* species in the frame of series "Hungarian Cultivated Flora". Beside Andor Jánossy, professors György Mándy, Ádám Boros, Imre Vinczeffy were his masters. He compiled a university doctoral thesis entitled Production of tetraploid forms of *Lolium* and *Festuca* species, which he defended with professor Andor Bálint. He successfully applied *in vitro* culture for rescuing hybrid embryos, and produced *Lolium* x *Festuca* intergeneric hybrids. Following this his interest turned to the development of plant tissue culture methods and their possible applications in plant breeding. He learned the theory and practice of *in vitro* culture at the courses at the Eötvös Loránd University. Between 1978-1980 he was a PhD student under the supervision of Mihály Maróti, the famous professor of plant tissue culture. He was the first to regenerate androgenetic haploid plants from tobacco, rice, and wheat in Hungary and clarified the alternative pathways of ontogeny from somatic and haploid cells (Heszky and Mesch 1976). He summarized his novel results in the thesis entitled „Morphogenesis in anther and callus cultures of different plant species" and became the candidate of science in biology (CSc) in 1979.

He spent a longer period in India (1981-82) and worked in the Ministry of Agriculture (1980-85), where he was involved in organizing agricultural scientific research in Hungary, participating e.g. in the foundation of the Agricultural Biotechnology Centre in Gödöllő.

Based on his results on cell genetic and tissue cultural research he regularly held lectures in the plant breeding courses of the Gödöllő University of Agricultural Sciences, and was awarded with titular professorship in 1984. He was only 40, when Professor Andor Bálint invited him to

Figure 2: The best known books in service of agricultural biotechnology education

become the head of the Department of Plant Breeding at the Gödöllő University of Agricultural Sciences; he had been directing the department, later the Institute of Genetics and Biotechnology of Szent István University for 25 years (Fig. 1). Within a short time after his nomination he established the conditions for the research and education of plant biotechnology and *in vitro* breeding and not only made the colleagues of the department acquainted with these methods, but also successfully involved them in the new research fields. He summarized the results achieved together with the team of his colleagues and PhD students in a thesis entitled „Biotechnology of asexual reproduction in different plant species".

He introduced new subjects into the Hungarian agricultural higher education such as Agricultural biotechnology, Plant biotechnology, Transgenic plants, Plant molecular genetics, Molecular plant breeding, Transgenic plant protection, Genetics, biotechnology and breeding of cultivated plants.

During the almost fifty years of his career László Heszky worked for plant science. In a team of his colleagues he achieved significant novel scientific results manifested in patents ('Noventa' soybean, auxin/cytokinin biotest; Hódosné Kotvics and Heszky 1989, Gyulai et al. 1991); registered new varieties ('Dama' and 'Bioryza' rice, 'Perenne' perennial rye, 'GK Trendi HO' rape seed; Simonné Kiss et al. 1992, Hódosné Kotvics et al. 1998, Falusi et al. 2011). The most important areas of his scientific interest cover plant cell genetics, tissue culture (Binh and Heszky 1990, Gyulai et al. 1992, Kiss et al. 1995), molecular genetics based taxonomy (Galli et al. 2006), gene technology (Kiss et al. 2000, Bittsánszky et al. 2007), functional genomics (Balogh et al. 2005). Cooperating with his colleagues he experimentally proved the organ and ploidy dependent somaclonal variation in plant system, and elaborated a haploid somaclone breeding method. Application of this novel method made it possible to bring about the first plant variety of biotechnological origin in Hungary with Ibolya Simon Kiss (Heszky et al. 1991).

He and the departmental co-workers identified new male-specific molecular markers in the dieocious hemp (Törjék et al. 2002) For prolongation of vase life of cut flower they produced antisense ACC construct and transformed carnation varieties with it (Veres et al. 2005).

His HAS/SIU research group genotyped apple and grape varieties - in collaboration with apple and grape breeders - applying microsatellite based PCR method (Galli et al. 2005, Halász et al. 2005). Microsatellite and markers of other types were used for selecting powdery mildew resistance alleles in grape (Molnár et al. 2007, Katula-Debreceni et al. 2010), predicting berry and curd colour in grape and in cauliflower, respectively (Szőke et al. 2012, Kiss et al. 2014). The same group indentified and characterized genes participating in the maturation of the non climacteric strawberry to better understand the physiology and genetics of fruit ripening (Polgári et al. 2010, Tisza et al 2010).

In recognition of his scientific and public life activity of high level in 1998 he was elected to correspondent member, in 2004 to member of the Hungarian Academy of Science.

In the past 30 years the Institute of Genetics and Biotechnology at SZIU has become the center of plant breeding researches as well as the graduate and postgraduate education based upon biotechnology, gene technology, molecular biology. Under his leadership the Department of Genetics and Plant Breeding, the present Institute of Genetics and Biotechnology initiated specialization in breeding and biotechnology first in Hungary. Between 1992-2007, 119 students graduated from this specialization. László Heszky is a founder member of the PhD School of Plant Science at SZIU and lead it between 2009-2013, in the

Figyelmébe ajánljuk

Biotechnológia

ROVATVEZETŐ: **Dr. Heszky László**

A növekedés és fejlődés géntechnológiai módosításaival foglalkozó VII. fejezet ötödik részében, a virágszínben módosított GM-fajták közül azokat ismertetjük, melyeket részben RNS interferenciával (RNSi) állítottak elő, és köztermesztésben voltak, illetve vannak.

Tanuljunk géntechnológiául (48.)

Növekedésben és fejlődésben módosított GM-fajok és -fajták (VII./5.)

A virág színének géntechnológiai módosítása (lila szegfű, kék rózsa)

Dr. Heszky László
SzIE Mezőgazdaság- és Környezettudományi Kar, Genetika és Biotechnológiai Intézet, Gödöllő

Bevezetés

A növények virágainak színe általában az adott fajra jellemző. A dísznövények között gyakoriak azok a fajok, amiknek különböző virágszínű változatait is előállították. Vannak azonban olyan színek, amiket egy adott fajban sohasem sikerült előállítani. Ilyen például a lila, vagy kék szegfű, rózsa, krizantém és gerbera. A molekuláris genetikai kutatások napjainkban feltárták ennek okait. A rózsában például hiányzik a kék színanyagok bioszintézisében résztvevő egyik enzim génje, emiatt sejtjei képtelenek előállítani a kék színért felelős antocián pigmentet. A géntechnológia eszköztára viszont lehetőséget ad, az evolúció ilyen típusú „hiányosságainak" korrigálására. A virágkertészek „szent Grálja" évszázadokon keresztül a kék rózsa volt. Kék színű rózsát a világon senkinek sem sikerült előállítania. A sikertelen próbálkozások arra sarkallták a virágkertészeket, hogy egyszerűbb és könnyebb megoldást, a festést alkalmazzák, amiről egy, a 12. századi arabul írt mű már említést tesz. A módszer viszonylag egyszerű és napjainkban is használatos. Fehér rózsaszálakat kék festék oldatába kell helyezni és abban órákig tartani. A fehér sziromlevelek néhány óra után elkezdenek kékülni. A virágok kék színe annál sötétebb lesz, minél hosszabb ideig tartjuk a rózsaszálakat a festékben (*1. kép*).

A növényi színanyagok

A virágok színét meghatározó legfontosabb pigment-összetevők a flavonok, a karotinoidok és a betalainok. A ***karotinoidok*** zsíroldékony sárga színt adó vegyületek, melyeket a növény fotoszintetizáló szövetei termelnek. A ***betalainok*** vörös és sárga színű pigmentek, aromás indolszármazékok. A *Caryophyllales* rendbe tartozó fajoknál az antocianinokat helyettesítik. A három csoport közül a növények esetében a legfontosabb színanyagok a flavonok. A ***flavonok*** (flavonoidok) színtelen pigmentek. Különböző komponensei tízféle osztályba sorolhatók. Szintézisük a dihidroflavonból kettős kötés létrehozásával indul, melyet a flavon-szintáz (FLS) katalizál. A flavonoid bioszintézis során keletkeznek az antociánok. Az antociánok, vagy ***antocianinok*** (gör., virág+kék szavakból) a növényvilágban legelterjedtebb színanyagok gyűjtőneve.

Antocianinok bioszintézise

Az antocianinok különböző növényi szervek, szövetek sejtjei vakuólmában találhatók. Ezek legtöbbször a szövetek külső sejtsorai, mint például az epidermisz vagy a külső mezofillum sejtjei. A sejtnedv kémhatásától függően színük savanyú közegben narancsvöröstől pirosig, lúgos közegben kék és kékeszöld között változik. (A pH-nak különös jelentősége lesz a kék rózsa esetében).

Az antocianin bioszintézise során stabil glikozilált formák (antocianidinek) keletkeznek. Kémiai szempontból ezért fenolos glükozidoknak tekinthetők. Szerkezetüket illetően különböző mértékben

1. kép **Festett kék rózsa. A levágott fehérvirágú rózsaszálak virágai, néhány órás kék festékbe való helyezést követően (nyíl), megkékülnek.**
(http://www.123tagged.com/Comments/Most_Used/9/Blue_Roses.html)

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1. 2015

Figure 3: Biotechnology coloumn in Agrofórum edited by László Heszky

frame of which 60 students defended their thesis till 2015, 14 of them under his supervision. He founded Agricultural Biotechnology MSc education at SZIU both in Hungarian and English languages and directed it till 2013, which is unique in Hungary with two accredited specializations: animal and plant biotechnology (Heszky 2007). His textbooks on genetics and books on biotechnology written with co-authors have been used for decades at the universities of agriculture and science (Fig. 2). Beside his 10 books he published 217 scientific articles, more than 500 conference abstracts or popularizing papers.

His creative instinct, educational sense of vocation does not weaken. Nowadays - since 2013 - he has been publishing a series in journal Agrofórum with the title "Let us learn gene technology" offering knowledge not only to the university students but to the inexpert public, as well (Fig. 3). We can deservedly say that Professor Heszky's scientific research and educational activity is inseparable from plant breeding and its foundational sciences. Beside his strives for conserving worth and values, the receptivity for novelty are the driving forces of his lifework.

May his dedication inspire us to be dedicated and determined. May he have a truly happy life. Happy Birthday Professor Heszky!

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Commemorative medal is awarded to Professor László Heszky in recognition of his outstanding work

Prof. Dr. László Heszky is an internationally well-known great authority of plant genetics, plant breeding and plant biotechnology research. He is the founder of *in vitro* plant breeding in Hungary. His results on the field of somatic embryogenesis and *in vitro* androgenesis have been included in university curricula not only in Hungary but in the USA, as well. He and his colleagues have bred the first state certificated plant variety (haploid somaclone breeding, "Dáma" rice variety) which was bred by using biotechnological method. Prof. Dr. László Heszky has been in a close professional relation with the Research Institute of Nyíregyháza of the Agricultural Centre of the University of Debrecen for decades. The plant genetics, plant breeding and plant biotechnological school led by him played an important role in the training of plant breeder generations of the Research Institutes of both Nyíregyháza and Karcag.

Over the years, he worked together with the staff of the research institutes on numerous research projects; these collaborations resulted in joint domestic and international results and publications.
Prof. Heszky participated in a number of events and scientific days of the Research Institute of Nyíregyháza, he held lectures and contributed also to the scientific and research work of our staff and the current breeding and biotechnological research through informal consultations.

In recognition of his activities, the Research Institute of Nyíregyháza honoured him with the Teichmann Award in 2003 and the Westsik Award in 2012.

The Centre of Agricultural Sciences of the University of Debrecen may award a *„Commemorative Medal for the Agricultural Research in Debrecen"* to natural or legal persons who/which significantly facilitated the development of the research activities within the Centre.

On the occasion of the 70th birthday of Prof. László HESZKY, we express our recognition, respect and gratitude through awarding the Commemorative Medal for his active contribution to the development of the research institutions, the agricultural research of Debrecen, the plant breeding and plant biotechnological research and the education of the next scientific generation.

ANTIBIOTIC RESISTANCE IN CHLOROPLASTS: A PRACTICAL APPROACH

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ABSTRACT

Alfalfa *(Medicago sativa* L.*)* is the second most important forage crop in Hungary and the fourth one in the world. Because of its high fibre and nutritional quality, it is used for livestock feeding in various forms as silage, hay or grass. Alfalfa is also an important source of biologically fixed nitrogen and is known for its high biomass production.

Chloroplasts are plant cell organelles with main function of photosynthesis. Their number varies up to 100 per cell and each chloroplast contains 10-100 copies of the prokaryotic type plastid genome. Spectinomycin inhibits protein synthesis on the prokaryotic type 70S plastid ribosomes by binding to the small subunit and induces bleaching in tissue culture. In the majority of the plant species chloroplast shows maternal inheritance, one of the exceptions is the *Medicago* genus, where it is inherited biparentally.

Mutants resistant to spectinomycin were isolated in tissue culture on media containing 30 mg/L spectinomycin. The starting material was young, healthy leaves from greenhouse grown plants of *Medicago sativa* RegenSY line. From leaf tissues we regenerated full plants on selective media. In order to have homoplastomic plants the whole process was repeated with leaves of the regenerated plants two more times on elevated spectinomycin level. After the third regeneration cycle 27 independent green, spectinomycin resistant mutant lines were placed into soil for greenhouse cultivation.

DNA was isolated from the spectinomycin resistant plants and the control wild type plant. The 16S rRNA region of the chloroplast DNA was PCR amplified and sequenced. Comparing the mutant sequences to the original RegenSY sequence we identified different mutations, all were located at a conservative region of the 16s rRNA. In one region (1013-1018) we identified G-C and C-A, C-T and in the other region (1138-1143) A-T, C-G, C-T nucleotide substitutions. After molecular characterization the plants were self fertilized or crossed with wild type plants. The selfed seed progeny was 100% spectinomycin resistant, and the out-crossed progeny showed segregation for the trait. The lines were morphologically characterised and in some lines dry-material and chlorophyll content was measured. Plants were morphologically indistinguishable from the wild type RegenSY line, and neither of the other two parameters showed significant differences.

The mutations can be used as markers in chloroplast inheritance studies, marker associated selection. The mutant plants can also be used for pollen tracing (gene flow) and in plant breeding for selection or identification.

keywords: Medicago sativa L., antibiotic, resistance, chloroplast, maternal, paternal, biparental

INTRODUCTION

Alfalfa is the fourth most important forage crop in the world and the second one in Hungary. The total cultivation area is more than 30 million acres worldwide and 150 thousand acres in Hungary.

Alfalfa has a great role in economy as seed production and future energy-plant too. Recently trifoliate species are coming into view as high biomass producing energy plants for bioethanol production. Seed production is an economically benefitial productivity in Hungary. Most of seeds produced in Hungary (67%) are exported to North and South Europe, and the Hungarian seed production is competitive in the surrounding countries.

Because of it's economical importance alfalfa also carries weight in scientific research. There are many future goals to reach in breeding, like better resistance against pathogens, better stress tolerance, enhanced leaf mass production etc. Every goal is being addressed by traditional breeding and biotechnological techniques. Besides of applied agricultural research alfalfa is also an important model species in basic research. For our research we used *Medicago sativa* cv. RegenSY line, which was selected for its good regeneration ability in tissue cultures.

All green plants have the same, most important subcellular organelle, the choroplast. Chloroplast facilitates the photosynthesys, which is a photochemical reaction, where the Sun's energy is conserved in carbohydrate

molecules (Lopez and Pyke 2005). According to the most popular hypothesis this little cell orgenelle once used to be a free living bacteria, probably a *Cyanobacteria*, wich was eaten by an early Eukaryote. But the digestion process was inhibited somehow and the bacteria was able to live in the host cell. Through several million years, most the genes of this bacteria were transferred and copied to the host cell, transforming the independent bacteria to a fully host dependent cell organelle.

Chloroplast have an independent heritability from the host genome. This was first discovered in 1905 by Baur and Correns independently (Baur 1909, Correns 1909 cit. Zhang and Sodmergen 2010). They became aware that some chlorophyll deficiencies were not inherited like the mendelian laws predict it. They discowered the non-mendelian heredability. Chloroplasts are mainly descending from one of the parents in the kingdom of plants. This parent is mainly the mother, so this pattern is called maternal inheritance. In 20% of the angiosperms other patterns exist too. There is paternal inheritance, where the pollen donor's chloroplasts are present in the progeny and there is biparental inheritance, where both chloroplast descend. In alfalfa chloroplasts may descend either way (Masoud et al. 1989).

Many aspects of the chloroplast resembles its bacterial origin. It still has a small genome, called plastome, which is still bacterial like (Levy 1982). The whole cell organanelle functions as a smaller cell, because it has DNA, RNA and protein synthesys. All elements of the synthetic apparatus are bacterial like.

Since Flemming's discovery of the first antibiotic, many antibiotics were discovered and invented. Antibiotics are small molecules produced by microbes against other microbes. They inhibit some important biochemical pathway or structure which are required for normal life functions. Since chloroplasts have bacterial origin, certain antibiotics are effective against them.

Spectinomicyn is an aminoglycoside antibiotic. Aminoglycosides were discowered around the 1940's from different *Streptomyces* spp. bacteria. Most of them have a similar stucture of two aminocyclitol rings, but other functionally similar antibiotics are categorised in this group. These antibiotics bind to the initialising bacterial ribosomes in the cell preventing and stopping protein sinthesys. The key to achieve this is that the antibiotic bonds to the H34 helix of the 16S rRNA interfering with the protein chain elongation (Wax et al. 2008) resulting in terminated bacterial protein synthesis (De Stasio et al. 1989). If we administer spectinomycin to non resistant plants, they bleach out and wither, because their chloroplasts are not functioning properly.

MATERIAL AND METHODS

Tissue culture media

B5h: Gamborg B5 3.16 g/L, supplemented with: L-prolin 0.5 g/L, KNO3 0.5 g/L, MgSO4·7H2O 0.25 g/L, MES 0,64 g/L, suchrose 30 g/L, kinetin 0.1 mg/L, 2.4D 1 mg/L, amino acid complex 30 ml/L, 5.4 g/L Plant agar and 30mg/L Spectinomycin. Amino acid stock solution (adenine 0.016 g/L, serine 3.32 g/L, L-glutamine 26.6 g/L, L-glutatione 3.32 g/L) and spectinomycin stock was filter sterilized.

MMSN: MS media with Nitsch vitamin 4.4 g/L, supplemented with: MES 0.64 g/L, suchrose 30 g/L, 5.4 g/L Plant agar

MS: MS 4.4 g/L, MES 0.64 g/L, suchrose 30 g/L, 5.4 g/L Plant agar

All the base media and supplementary ingredients were produced by Duchefa.

Plant regeneration

Starting materials were young alfalfa (*Medicago sativa* cv. RegenSY) leaves from greenhouse cultivated, vegetatively propagated plants. Leaves were collected from the second and third nodes of the shoot and surface sterilised in a laminar box. The prepared leaves were cut to 3-4 mm slices perpendicular to the main vein and placed on callus inducing B5h media and incubated at 22°C with a 16/8 light cycle. Explants were transferred to fresh media every two weeks, until somatic embryos appeared. Then explants were transferred to MMSN media. From the developing green embryos whole plants were regenerated. For root development and growth the plants were placed on MS media separately.

Mutant selection

All steps were the same as in plant regeneration protocol except, that all media contained 30 mg/L spectinomycin. Explants were kept on the callus induction B5h media for four weeks and then transferred to hormone free media. Only independent mutants were kept.

Enrichment of mutant plastids

In order to produce homoplastomic mutant plants, we repeated the regeneration process with leaves from the spectinomycin resistant alfalfa plants on selective media containing 100 mg/L spectinomycin two more times.

DNA isolation

DNA was isolated with Zenogene Plant DNA isolation kit according to the manufacturer instructions from 100 mg plant material grown in tissue culture or in greenhouse.

DNA concentrations were measured with a NanoDrop™ ND-1000 Spectrophotometer.

PCR and sequencing

All reactions were done in an ICycler™ (Biorad) PCR machine. The *rrn16* gene was amplified with primers A: 5'-GGAGTACGTTCGCAAGAATG-3'; and

B: 5'-TTCCAGTACGGCTACCTTGT-3'. PCR was carried out in 50 µl with the following cycles: initial denaturation: 95°C 5 min; 40 cycles of: denaturation 94°C 30 sec, primer annealing 52°C 30 sec, extension 72°C 45 sec; and final extension 72°C, 10 min. PCRproducts were purified with QIAGEN MinElute PCR Purification Kit by the manufacturer's recommendation. Concentration of the PCR fragment was measured with a NanoDrop™ ND-1000 Spectrophotometer. Purified PCR fragments were sequenced by Biomi Ltd. Sequences were analysed with Chromas Lite (Technelysium Pty Ltd. 2003-2008) and NCBI GeneBank and MultAlin freely accessible browser based programme for the alignments (Corpet 1988).

Restriction digests and gel electrophoresis

We identified the enzymatic cutting sites with NEBcutter (Vincze et al. 2003). Digestions were carried out with HpyF3I and AatII enzyme (Fermentas). Undigested PCR fragments were separated on 1% and digested PCR fragments on 1.5% agarose gel in TBE buffer.

Chlorophyll measurement

Chlorophyll content was measured from 9 leaf blades per plant by the protocol described in Nature Protocols (Ni et al. 2009). The absorption measurements were done with a Jenway 6310 Visible Scanning Spectrophotometer at 645 and 663 nm.

Dry-matter measurement

Leaf blades isolated from three leaves per line were used for the measurement. Weighed samples were homogenized with a Biospec Mini Beadbeater™ laboratory mill, frozen in liquid nitrogen, and then placed into a VirTis® Freezemobile™ 12XL lyophilisator for 20 hours. Samples were weighed again, and then were put into a 70°C drying chamber until mass equation.

Crossing and progeny analysis

Wild type crossing partners were selected from twenty commercial cultivars (Adél, Alexandra, Anna, Hunor, Irisz, Jozsó, Kákai legelő, Kisvárda, Klaudia, KM-Agro, KM-Gyöngy, KM-Norbert, Kőrös, Lily, Maraton, Solar, Szarvasi, Szapkó, Verkó, Viktória). Young apical flowers were pollinated with older basal flowers. Self pollination was prevented by submerging the prepared flower in 51% ethanol, rinsed in water and after drying with tissue paper the stamens were gently rubbed to the prepared younger flowers stigma. The seeds were collected from reciprocal crosses, sowed and grown to full plants. DNA was isolated from the second trifoliate leaf of the F1 progeny plants and chloroplasts was characterised by PCR and restriction digests.

RESULTS

Tissue culture results

First we set up the plant regeneration process, which yielded many regenerated plants. This was only the initial step to verify our method and to prepare for mutant selection. The mutant selection yielded 31 independent mutant lines.

Molecular results

From the isolated DNA we amplified part of the 16S rRNA coding region. The PCR products were uniformly the same size. After purification we sequenced the PCR fragments.

When analysing the nucleotide sequences we could identify two sites, where we found differences. The firs one was at the 1014 and the second was at the 1040 position (Table 1). For the nucleotide changes we could find restriction endonucleases, which digested our PCR products selectively, distinguishing the mutants from the wild type. This way we could identify plants without DNA sequencing.

TABLE 1: All isolated and identified mutant lines

Ms RegenSY 16S rRNA region	Wild-type sequence	All mutants	Substitution	Number of lines	Mutant sequences
1013-1018	CGTCAG	26	G-C	20	CCTCAG
			C-A	4	CGTAAG
			C-T	2	CGTTAG
1138-1143	TGACGT	5	A-T	1	TGTCGT
			C-G	3	TGAGGT
			C-T	1	TGATGT

Mutant characterisation

In the dry-matter and the chlorophyll content of the wild type and selected mutant plants we did not find any significant difference. The chlorophyll measurement was normalised to chlorophyll-a to chlorophyll-b ratio which showed no significant differences.

We also characterised some randomly chosen lines morphologically and compared it to the regenerated wild-type plant. All mutant lines were the same as the wild-type, except four lines. These plants had thicker leaves, longer internodes, less bushy roots and less biomass.

Crossing and progeny analysis

Altogether 600 crossings were done but only 190 yielded seeds (Table 2). In these crosses mutant plants were 101 times mother plants and 89 times father plants. We found many incompatible crossing setups and some which gave plenty of seeds. Some incompatible crossings were repeated many times with different plants from the same type, with the same results. We only accepted those crossing which yielded at least 10 seeds. Those seeds were sowed and planted in Jiffy for further analysis. From the young progeny we took the second leaf and isolated the DNA to identify which parents chloroplasts can be found in them.

TABLE 2: Summary of all alfalfa crosses

	Mutant mother x wild-type pollen	Wild-type mother x mutant pollen	Altogether
All crossings	101	89	190
Number of seeds	2316	2589	4905

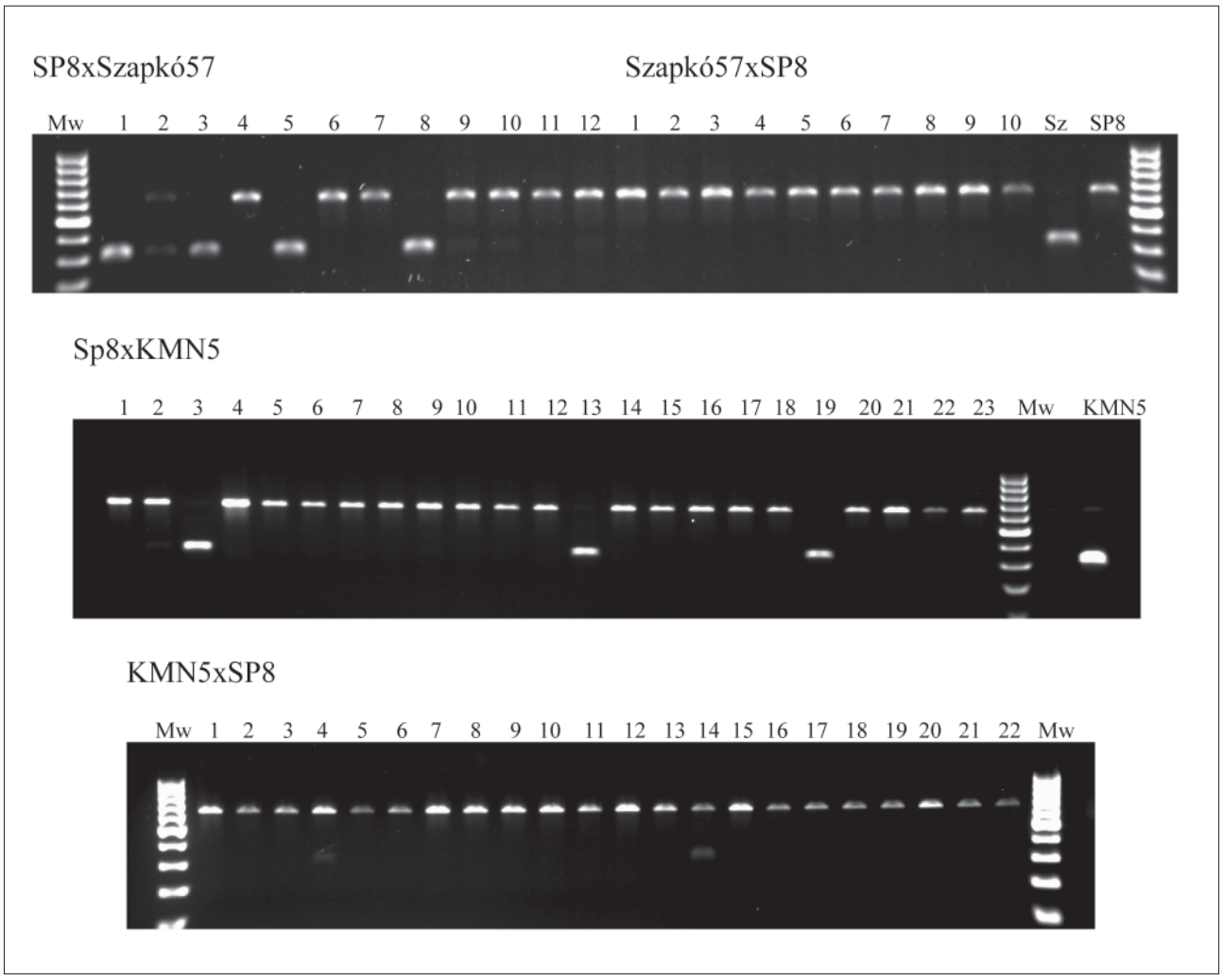

Figure 1: The identification of plastids with restriction digestion (Sz=Szapkó, SP=SP8301, Mw=molecule weight marker)

TABLE 3: Summary of the chosen crosses

	Mutant mother x wild-type pollen	Wild-type mother x mutant pollen	Altogether
All crossings	74	79	153
Number of seeds	787	781	1566
Maternal (M)	443	121	564
Paternal (P)	282	636	918
Biparental (M+P)	62	24	86

Total 46 chosen lines from the crossing of 18 SpecR mutant RegenSY type and 18 commercial type plants were analysed (Table 3).

The first evaluated crossing was the Sp8 x Solar1. Because this was the first experiment, we isolated DNA and identified the chloroplast type in both cotyledons separately, in the unifoliate leaf, and in the first two trifoliate leaves of the F1 progeny. We found that some plants carried only one type of chloroplast, but there were some plants that carried chloroplasts from both parents. This is called heteroplastomy. After a year, the plants were tested again, we found that the heteroplastomy disappeared, and only one type of chloroplast was found in each plant. The Sp8 x Solar1 crossing yielded 8 maternal and 6 paternal chloroplast type plants. The reciprocal crossing yielded 14 paternal and only 1 maternal chloroplast type plants.

The Sp8301 x Szapkó57 and Sp8 x KMN5 crossing had similar results (Fig. 1). We found that the mutant chloroplasts heritability differed in the Sp8301 x KMN5 depending on whether the mutant was the pollen donor (father) or the mother plant. In the first case all progeny carried the SpecR, but in the second we identified progeny with both chloroplast types and heteroplasmic plants, too.

Other evaluated crossings showed the same results (data not shown). When the mutant line was the mother in the crossing setup all variations of chloroplast inheritance could be found in the progeny but the maternal inheritance was more abundant with a 2:1 ratio. However, when the mutant plant was the pollen donor, the ratio shifted to 5:1 in the favour of the paternal chloroplasts.

DISCUSSION

Our mutant isolation procedure yielded 31 independent homoplastomic lines, which were used in further crossing studies. These mutant lines differ from the wild-type plants only in a single nucleotide. The four lines, which showed different phenotype, could be the result of somaclonal variation, which is inherent in tissue culture regeneration. The chloroplasts of the mutant plants were heritable and fully functional. We identified mutant lines with the substitution A to T in the 1140 position, C to G and C to T in the 1141 position of the 16S rRNA gene, G to C in 1014 and C to A, C to T in 1016. The mutations in the SpecR plants were at the same loci, as it was annotated in other species. Back-mutation did not occur in any of these plants after three generations.

Spectinomycin resistant plants were isolated from tobacco (*Nicotiana tabaccum*), carrot (*Daucus carrota*), European night shade (*Eolanum nigrum*) and Fendler's bladderpod (*Physaria fendleri*) so far (Kavanagh et al. 1994; Filipenko et al. 2011a,b; Svab and Maliga 1991). In all these plants the nucleotide substitutions in the plastome were found at the same two loci, as we found in alfalfa. Other aminoglycoside resistant alfalfa is also known from the literature. These mutants were kanamycin resistant plants produced from tissue culture. These plants had developmental defects, like early signs of stress during leaf development. During the apical leaf development the bleached-green leaf-blades became pink from the apex, then slowly turned to normal green. In greenhouse the overall biomass production was 15% lower than the wild-type values. Furthermore the mutant biomass production was 35% less, compared to wild-type, when the plants were bred outdoors (Rosellini et al. 2004). Our mutant lines, except the four somaclonal variants, have no differences comparing to the wild type. This was further supported by chlorophyll and dry-matter measuremenst. Most importantly these plants were able to reproduce by crossing or self pollinating and their offsprings were viable and fertile.

During the evaluation of crossing experiments we found that certain plants are incompatible, and some are shifting the chloroplast inheritance ratio to unexpected values. The biochemical background of these processes has to be researched. These data suggest that there may be several alfalfa genotypes that are incompatible for reproducing and they can be cultured in each others vicinity without the risk of cross pollination.

This mutant isolation method can also be used on some other commercial types to isolate plants with marked plastome in their chloroplasts.

With the mutant plants we can simulate and trace the chloroplast inheritance and we can estimate the ratio of the chloroplast spreading in surrounding alfalfa fields (Dudas et al. 2012). This enables us to precisely evaluate isolation distances in seed production and in transplastomic GM plants. Since alfalfa is an insect pollinated plant different type of bees can carry the pollen to distant plants. Comparing this with recent research data, marked

pollinators can cover a distance of 5 km a day from their homes (Hagler et al. 2011). This data enables us to ask if the current seed production plantings have an appropriate isolating distance? This question can be addressed with traceable chloroplasts and genome markers. Because both of them are independently inherited, a combined usage is recommended.

CONCLUSIONS

We succeded to create several spectinomycin resistant chloroplast mutant alfalfa plants that would be ideal for on-field application to determine and test different seed production and GM safety isolation distances. These non-GM plants only contain a mutation which can be found in the nature too, but in a very low percentage.

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SUPPLEMENTARY – TABLE 4: Total listing of the seed yielding crosses. M – Maternal, P – paternal, biP – biparental.

Crosses						Reciprocal crosses					
Parent plants		Genotype of F1 progenies				Parent plants		Genotype of F1 progenies			
Mother	Pollen	M	P	biP	Σ	Mother	Pollen	M	P	biP	Σ
Adél 120	RY6		18		18	**RY6**	Adél 120	15	1		16
Adél 125	RY1	2	8		10	**RY1**	Adél 125	7	5	3	15
Alex 96	RY13	2	11	1	14	**RY13**	Alex 96	8	5	1	14
Hunor 152	RY13	1	34	1	36	**RY13**	Hunor 152	11	11	3	25
Írisz 18	RY5		20		20	**RY17**	Írisz 18	10	8		18
Írisz 224	RY8	1	11	1	13	**RY8**	Írisz 224	7	4	2	13
Jozsó 10	RY5, RY12, RY15, RY17	7	13	1	21	**RY5, RY12, RY15, RY17**	Jozsó 10	12	11	5	28
Jozsó 31	RY15, RY17	5	7		12	**RY5, RY15, RY17**	Jozsó 31	8	2	1	11
Kákai legelő 76	RY17	1	11	1	13	**RY17**	Kákai legelő 76	9	7	1	17
Kisvárda 26	RY1, RY3,RY5, RY15,RY17	4	26	1	31	**RY1, RY3, RY17**	Kisvárda 26	16	12	1	29
Kisvárda 270	RY4		10	1	11	**RY4**	Kisvárda 26	14	3		17
Klaudia 166	RY17	6	11	2	19	**RY17**	Klaudia 166		30	2	32
Klaudia 179	RY7	6	11		17	**RY7**	Klaudia 179	6	12	4	22
KMA 14	RY17		11		11	**RY9, RY17**	KMA 14	5	3	5	13
KMA22	RY11	5	14		19	**RY11**	KMA22	4	7		11
KMG 191	RY7	2	10		12	**RY7**	KMG 191	4	3	3	10
KMG201	RY13, RY16	8	8		16	**RY16**	KMG201	7	2	2	11
KMN 5	RY15, RY17	4	34		38	**RY17**	KMN 5	22	5	2	29
KMN 20	RY11, RY17		40	1	41	**RY11, RY17**	KMN 20	13	21	5	39
KMN 23	RY15,R Y17		35		35	**RY15**	KMN 23	7	6		13
Körös 217	RY4, RY18	7	4	2	13	**RY4, RY18**	Körös 217	7	2	1	10
Maraton 349	RY8, RY17	3	13		16	**RY8, RY17**	Maraton 349	6	3	1	10
Solar 1	RY1, RY3, RY9	6	34	2	42	**RY3, RY9**	Solar 1	24	14	6	44
Solar 2	RY6,RY17	3	15		18	**RY6, RY17**	Solar 2	10	15	3	28
Solar 36	RY17		15		15	**RY17**	Solar 36	7	6		13
Solar 43	RY9, RY10, RY17	2	41		43	**RY9, RY10, RY17**	Solar 43	18	16	2	36
Solar 44	RY6	1	11	1	13	**RY5**	Solar 44	10	4		14
Solar 51	RY17		14		14	**RY17**	Solar51	4	6	1	11
Solar 52	RY17		15		15	**RY17**	Solar52	8	7	3	18
Solar 61	RY9, RY17	3	25	1	29	**RY9, RY17**	Solar 61	10	11	3	24
Solar 63	RY5, RY9	3	21		24	**RY5, RY9**	Solar 63	12	9	3	24
Solar 64	RY7, RY17	6	4	2	12	**RY7**	Solar 64	5	5	5	15
Solar 65	RY14		12		12	**RY8, RY14**	Solar 65		10	2	12
Solar 71	RY7	1	11	1	13	**RY7**	Solar71	3	9	3	15
Szapkó 8	RY15, RY17		16		16	**RY15, RY18**	Szapkó 8	7	8		15
Szapkó 57	RY1, RY15, RY18	8	40	1	49	**RY1, RY15, RY18**	Szapkó 57	26	22	9	57
Szarvasi 316	RY3	11	16	3	26	**RY3**	Szarvasi 316	14	7	2	23
Szarvasi 327	RY10, RY17	5	14	2	21	**RY10, RY17**	Szarvasi 327	11	7	1	19
Verkó 305	RY11		11		11	**RY11**	Verkó 305		4		4
Verkó 313	RY18		8		8	**RY15, RY18**	Verkó 313	22	5	1	28
Viktória 63	RY9		2	2		**RY9**	Viktória 63	6	2		8

RECENT RESULTS AND ROOTS OF IN VITRO ANDROGENESIS RESEARCH IN CEREAL BREEDING

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In memoriam Prof. Ibolya Kiss Simon

ABSTRACT

In plant biotechnology, the research of *in vitro* androgenesis has been initiated more than 40 years ago. In Hungary, Prof. László Heszky played an important part in creating the fundaments of *in vitro* androgenesis research. His first scientific result was achieved in anther culture of tobacco. In our country, his result founded and launched the haploid research in crop plants in the 1970s. This work was continued with cereal species in his career. In breeding, the first biotech-derived variety was the 'Dáma' rice variety, registered in 1992. These results were followed by some other rice and wheat varieties from other institutions. Recently, the well-developed and optimized DH plant production can be applied in different wheat and rice breeding programmes. Androgenesis offers a quick tool to accelerate the breeding process and produce pure lines for breeding goals, molecular examinations and to study genetic background of biotic and abiotic stress resistance and services.

keywords: haploid, doubled haploid, wheat, rice, breeding

INTRODUCTION

Scientific roots of in vitro haploid research in Hungary

The first *in vitro* haploid plant induction was reported by Guha and Maheswary in Nature (Guha and Maheswary 1964). This publication initiated a real scientific revolution in plant genetics and breeding including thousands of the papers dealing with in *vitro* haploid plant induction dominantly in crop plants. In Hungary, a young scientist (L. Heszky), freshly graduated from the Agricultural University in Gödöll launched a very consequent haploid induction research in a tiny laboratory of the Hungarian Gene Bank Institute in Tápiószele. His research was motivated by the founder of the Hungarian tissue culture research, Prof. Mihály Maróti, Loránd Eötvös University, Budapest.

In Hungary, the first haploid plantlet was produced from anther culture (AC) of tobacco in 1973 (Heszky 1973). This procedure offered an excellent opportunity for geneticists and breeders to produce homozygous lines, in relatively big quantity, in one plant generation. So many efforts were made to improve the doubled haploid (DH) plant production methods (anther and microspore culture) in Hungary and around the word, too.

The first AC–derived rice plant was published in 1968 by Niizeki and Oono (1968). It was repeated in Hungary relatively quickly in 1975 (Heszky and Pauk 1975). It was followed by haploid induction of common wheat (Heszky and Mesch 1976), too. After the first haploid induction improvements, the breeding applications also started and the first Hungarian DH variety in rice, namely Dáma, was registered in 1992 (Heszky et al. 1996).

In the current study, we shortly summarise the results of breeding application of *in vitro* androgenesis in winter wheat and rice in Cereal Research Non-profit Ltd.

MATERIALS AND METHODS

Plant materials and growing conditions

Six winter wheat ('6621', '6656', '6670', '6680', '6715' and '6724') crossing combinations were used in AC experiments. In rice, a standard variety (Nucleoryza) and two crossing combinations (Miara/No.3, Kalaris/Timis) were used for the successful tissue culture experiments. The donor plants were grown under nursery condition. The nurseries were managed according to a standard protocol for Hungarian small grain cereals (wheat and rice).

Figure 1: Winter wheat DH lines in the wheat nursery of CR Ltd. showing the homogenous nature of DH method produced wheat strains

Pre-treatment of donor tillers, isolation of anthers and AC experiments

The donor tillers were collected when the microspores were in early and mid-uni-nucleate stages. The harvested donor tillers were cold pre-treated (2-4°C in wheat, 8-10°C in rice) for one-two weeks in Erlenmeyer flasks containing tap water. Sterilization and other tissue culture (induction, regeneration, acclimatization) steps were carried out according to the protocol of Lantos et al. (2013) in wheat and Pauk et al. (2009) in rice. The regenerated and well-rooted green plantlets were transplanted into greenhouse. Fertile seed propagations and selections were carried out under wheat and rice nursery conditions and breeding protocols.

Statistical analysis

In our experiment, each treatment was repeated at least six times. The collected data - number of embryo-like structures (ELS), green plantlets and albino plantlets- were analysed by one-way ANOVA (Microsoft Excel 2002 statistical software).

RESULTS

In vitro androgenesis research in wheat

The genotype effect on ELS and the green/albino plantlet production are the characteristics that influence the applicability of the DH plant production in the routine breeding process. Six winter wheat segregated crossing combinations were used to demonstrate the efficiency of AC and the effect of genotype. The androgenesis was induced in each genotype and microspore-derived ELS were obtained in the Petri dishes. The genotype influenced the number of ELS/Petri dish. The number of ELS ranged 3.5-54.688 ELS/Petri dish depending on genotype (Table 1).

The microspore derived ELS produced green and albino plantlets. The number of regenerated plantlets were also influenced by genotype (Table 1). The number of green plantlets ranged from 0.5 to 13.125 green plantlets/ Petri dish. The number of albinos was limited among the regenerated plantlets. The number of green plantlets were more than three times higher than the number of albinos. After the acclimatization period, the spontaneous DH plants were fertile and produced seeds. The DH lines

can be integrated into different winter wheat breeding programmes (Fig. 1).

TABLE 1: The effect of genotype in AC of wheat. The different letters of the alphabet marked the significantly (p=0.005) different values in each column.

Genotype	ELS	green plantlets	albino plantlets
'6621'	17.063 c	0.500 b	4.125 a
'6656'	54.688 a	13.125 a	0.125 b
'6670'	3.500 c	1.563 b	0.000 b
'6680'	14.313 c	1.375 b	0.563 b
'6715'	16.800 c	1.133 b	1.000 b
'6724'	35.000 b	3.750 b	0.063 b
Mean	23.560	3.574	0.979
LSD	14.84	3.78	1.46

Combining traditional and in vitro methods in rice breeding

During a long time in collaboration with Ibolya Kiss Simon, rice breeder in Szarvas, different cell and tissue culture methods were carried out in pre-breeding phase. The segregated generation of Miara/No.3 mutant single cross combination was used for an *in vitro* haploid induction. Because of the high percentage (76) of the plantlets was haploid, the plantlets were colchicine treated. From the colchicine treated haploids, DHs were obtained. From the regenerants six DH lines were passed through a serious nursery selection and multi-location tests. After the 3 years in the official plant variety testing, the best line was released as a new variety under the name 'Risabell'. The main characteristics of 'Risabell' are resistance to blast disease, high cooking quality which comes from its long grain character (long B).

Our deperment spent a lot of time with creating protoplast plant system in rice. From rice 'Nucleoryza' variety we developed a long-term embryogenic haploid cell suspension. To control the regeneration capacity of the suspension, haploid regenerants were obtained. The most vigorous haploid regenerants were colchicine treated to produce fertile lines. Altogether 20 DH lines were obtained from this experiment. These fertile lines were tested for agronomic parameters and after the first nursery selection only nine lines were selected for further testing. In the four replicated experiments, three lines had an outstanding

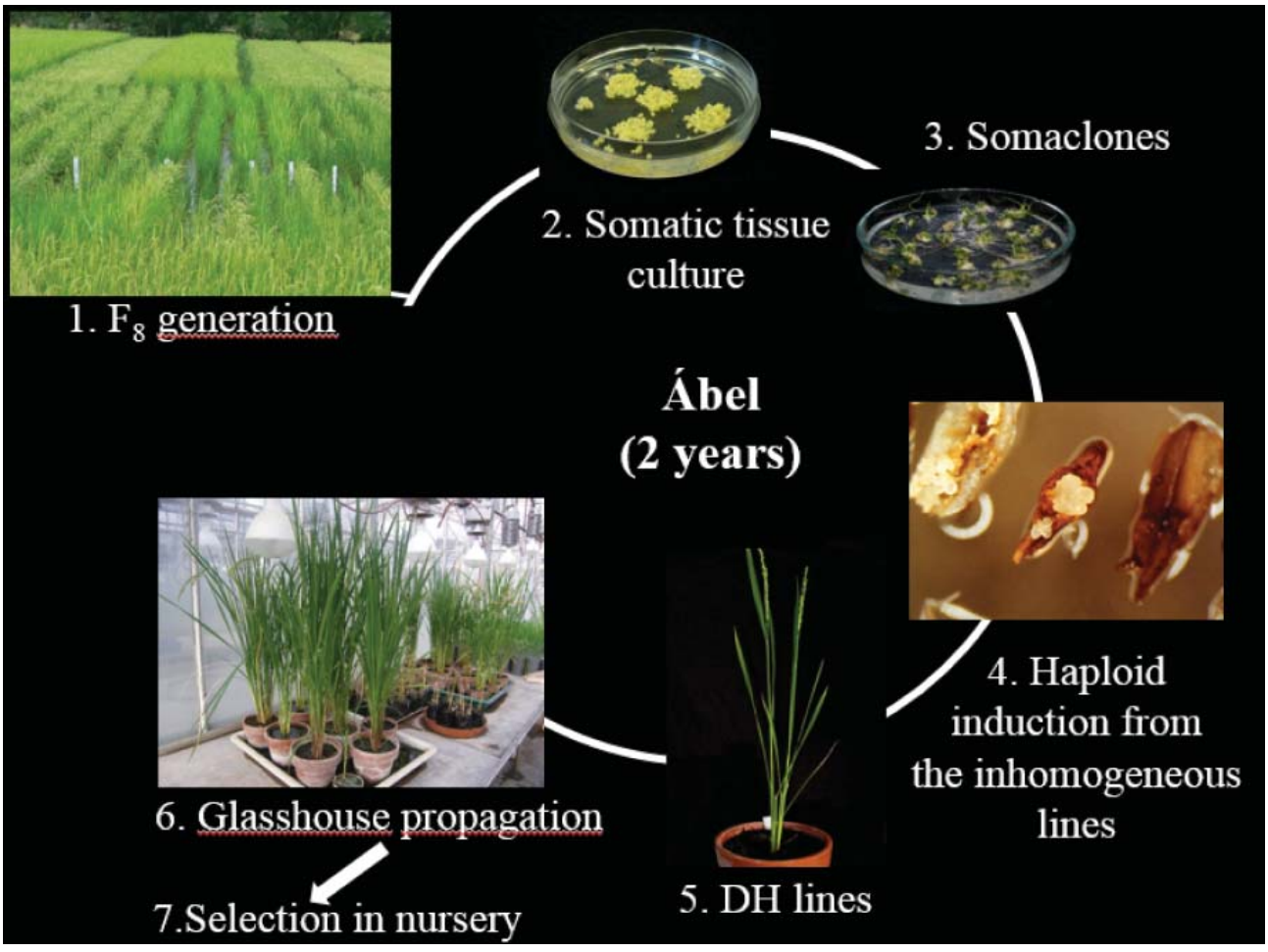

Figure 2: Schematic breeding story of 'Ábel', extra early rice variety

grain profile index compared to the variety 'Nucleoryza'. The best one was selected for the official plant variety testing to be registered. After 3 years of positive results, this line was released as a new variety under the name 'Janka'. The main characteristics of this variety are earliness, vigorous seedling growth, drought tolerance and good grain quality (long A).

The advanced lines (F_8) of Kalaris/Timis single-cross combination were used for the induction of somaclonal variants. In the greenhouse significant differences were noted in the earliness and fertility in the somatic tissue-derived lines. Because these lines were relatively inhomogeneous, the best ones were subjected to haploid induction to produce homogenous lines. From the DHs, 31 DH lines were tested for abiotic stress (seedling cold stress) tolerance. In the selection, the earliness and early stage cold tolerance were in the focus. The best one had extra earliness and it was applied for the official plant variety testing. Figure 2 summaries the biotech life story of this induction and selection process. The genotype 'Ábel' was released as a new variety in 2005.

DISCUSSION

In vitro androgenesis in winter wheat

The development of DH plant production methods are in the focus of modern crop breeding and applied researches. In small grain cereals, the AC is one of the most important DH plant production methods. Some critical reviews reported that the high ratio of albinos and genotype dependency limited the practical application of AC in cereals (Jauhar et al. 2009; Islam and Tuteja 2012; Niu et al. 2014).

In our experiments (wheat), the number of albinos was significantly lower than the number of green plantlets. The phenomenon of albinism did not mitigate the green plantlets production. The genotype influenced the number of ELS, green and albino plantlets. These results are in harmony in our earlier published data (Lantos et al. 2013, 2014). The AC offers an opportunity for breeders to produce homozygous lines in one generation.

Using this system, mapping populations can be produced to study the genetic background of agronomic parameters, biotic and abiotic stress tolerance (Cuthbert et al. 2008; Zhang et al. 2008; Szabó-Hevér et al. 2014).

In vitro androgenesis in rice

In rice, traditional breeding steps and in *vitro* haploid induction- and somatic tissue culture improvements were combined and used for breeding goals to release modern rice varieties for Hungarian producers. The summarised rice breeding improvements had been generated here in the past 20-22 years. These results confirmed the applicability of the biotech-method induced lines in rice breeding. The biotech-method generated genotypes in breeding published earlier in rice (Heszky et al. 1996; Khush and Virmani 1996; Thomas et al. 2003; Lapitan et al. 2004) and in other crops like barley, rapeseed, wheat etc. (Cistué et al. 2004; Forster and Thomas 2005; Tanács et al. 1997) are in harmony with our published results. Our results confirmed – in compliance with the previously cited papers – the usefulness of the biotechnology methods in rice improvement.

CONCLUSIONS

After more decades of androgenesis research in CR Ltd., Szeged, this research area produced more routinely applicable methods. Recently AC offers opportunity for (i) reducing breeding process of new varieties and (ii) produce mapping populations. The new tissue culture methods combined with traditional breeding processes may result in new released genotypes and varieties with good agronomic values.

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CYTOKININS AND PHOTOSYTHETIC APPARATUS OF LEAVES ON *IN VITRO* AXILLARY SHOOTS OF APPLE CV. FREEDOM

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ABSTRACT

Operability of photosynthetic apparatus in *in vitro* shoots is of great importance because it may influence the survival of plantlets when transferring to *ex vitro* conditions. Effect of different cytokinins (BA, benzyl-adenine; TOP, *meta*-topolin; Kin, kinetin; and BA+TOP, combined application of benzyl-adenine and *meta*-topolin) were studied on chlorophyll fluorescence (in dark and light adapted leaf samples) and chlorophyll content in leaves of 3-week-old apple shoots cv. Freedom. Chlorophyll fluorescence (maximum quantum yield (F_v/F_m) and maximum efficiency of the photochemical process (F_v/F_0)) was found to be the highest when BA and TOP were applied in combination in the medium; and they were the lowest at TOP-supply. The highest quantum yield (Y(II)) of photosystem II was measured when Kin and BA+TOP were used as a cytokinin source in the medium, respectively. According to our results the cytokinin-types and its applied concentration affected the chlorophyll a and chlorophyll b content of the leaves. Using TOP in the medium resulted in the highest chlorophyll a content. TOP used at low concentrations (0.5 and 2.0 µM) and BA at 12.0 µM resulted in the highest chlorophyll b content in the leaves. We could not find any correlation between the chlorophyll fluorescence parameters and the chlorophyll content, which shows that measuring the chlorophyll content itself cannot characterize the capacity of the photosynthetic apparatus. Strong genotype effect was proven both in chlorophyll fluorescence and chlorophyll content of the leaves when comparing our recent results to earlier results with cv. Royal Gala.

keywords: Freedom, aromatic cytokinins, chlorophyll fluorescence, chlorophyll content, *in vitro* axillary shoots

INTRODUCTION

Recently the micropropagation is used worldwide for the production of homogeneous and healthy (pathogen-free) plantlets of various fruit and ornamental species, among them also in apple. Apple micropropagation includes four steps, such as *in vitro* culture establishment from *ex vitro* (glass-house or field-grown) plants, *in vitro* shoot multiplication by axillary or adventitious shoot induction and development, rooting of *in vitro* shoots and acclimatization of *in vitro* plantlets (rooted shoots) (Dobránszki and Teixeira da Silva 2010).

Micropropagation includes artificial conditions (media components and environmental factors) which influence both the morpho-physiology and photosynthetic apparatus of shoots and plantlets (Yue et al. 1992; Desjardins 1995; Triques et al. 1997; Valero-Aracama et al. 2006). The success of micropropagation depends not only on the quantity of plantlets produced within a period but also on their quality because morphological and physiological state of *in vitro* shoots and plantlets affect their survival potential when transferred to *ex vitro* conditions during the acclimatization. Development and capacity of the photosynthetic apparatus of *in vitro* shoots and plantlets are of great importance in the quality and the utilization of micropropagated plant materials (Yue et al. 1992; Hazarika 2006; Fila et al. 2006; Ziv and Chen 2008; Dobránszki and Teixeira da Silva 2010). Chlorophyll fluorescence measurement is a non-invasive and rapid tool for characterization of the function of the photosynthetic apparatus (Roháček 2002; Baker 2008).

Cytokinins (CKs) are the main plant growth regulators in *in vitro* shoot growth and development therefore they are indispensable media components in the shoot multiplication phase of micropropagation (George and Debergh 2008; Van Staden et al. 2008; Dobránszki and Teixeira da Silva 2010; Magyar-Tábori et al. 2010; Dobránszki 2014). CKs were proven to regulate the photosynthesis *in vivo* (Reeves and Emery 2007; Haisel et al. 2008; Rivero et al.

2009). Morphological and physiological development and disorders of shoots in plant tissue culture (PTC) are proven to be affected by CKs applied in the medium (Genkov et al. 1997; Xie et al. 2004; Dobránszki et al. 2005; Magyar-Tábori et al. 2010; Aremu et al. 2012; Dobránszki and Mendler-Drienyovszki 2014). In our earlier experiments we described that both the chlorophyll content and chlorophyll fluorescence of leaves in *in vitro* apple shoots were affected by CK-supply of the medium when using apple scion cultivar Royal Gala as a model cultivar (Dobránszki and Mendler-Drienyovszki 2014). However, different genotypes (cultivars, hybrids, clones) of a species replies very variously to the *in vitro* environment and media components, and the growth and development in PTC are highly genotype-dependent (Fig. 1). This is especially observed in apple PTC (Dobránszki and Teixeira da Silva 2010; Magyar-Tábori et al. 2010).

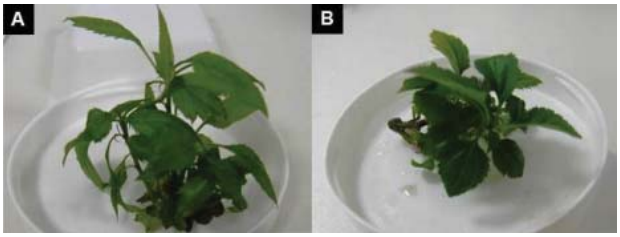

Figure 1: Genotype-dependent growth and development of apple in vitro shoots. Axillary shoot cultures of different apple scion cultivars (A: Royal Gala, B: Freedom) on medium with the same citokinin (1.0 mg l^{-1} benzyl-adenine riboside) content.

In present work we studied the effect of different CK-supply of the medium on the chlorophyll content and the capacity of photosynthetic apparatus in *in vitro* apple leaves in the apple scion cv. Freedom. Shoot development and growth of apple cv. Freedom in PTC were proven to be very different from that of cv. Royal Gala (Dobránszki and Teixeira da Silva 2010; Magyar-Tábori et al. 2010).

MATERIALS AND METHODS

Plant materials and treatments

Five of 4-week-old *in vitro* shoots of apple (*Malus* X *domestica* Borkh.) scion cv. Freedom were placed vertically in Killner jars (400 ml in volume) on 40 ml of MS (Murashige and Skoog, 1962) proliferation medium (Dobránszki and Mendler-Drienyovszki 2014) contained various CK supply. Different types of CKs were applied, as single CK source of the media, such as benzyl-adenine (BA), its hydroxylated derivate, *meta*-topolin (3-hydroxy-benzyladenine, TOP) and kinetin (Kin), moreover dual CK effect was studied using BA and TOP, where the level of BA was constant of 2.2 µM and the concentrations of TOP were different. CKs were applied at different concentrations in the media (Figs. 1, 2). Before measurements the cultures were grown for 3 weeks at 22 °C, 16 h photoperiod (cool-white fluorescent lamps, 400-700 nm, PPF of 57 µmol m^{-2} s^{-1}).

Measurement and evaluation of chlorophyll fluorescence and chlorophyll content of leaves

Chlorophyll fluorescence was measured in the second apical leaves of *in vitro* plantlets as described in details previously (Dobránszki and Mendler-Drienyovszki 2014) by using OS5p Modulated Fluorometer (Opti-Sciences, USA). Chlorophyll fluorescence was measured both in dark-adapted leaves using F_v/F_m test to determine the maximum quantum yield (F_v/F_m) and the maximum efficiency of the photochemical process (F_v/F_0) in photosystem II (PSII) and in light-adapted leaves using Yield protocol ($Y(II)=(F_{ms}-F_s)/F_{ms}$) to determine the actual quantum yield of PSII under the actual lighting condition.

In the dark adapted leaves the variable fluorescence ($F_v=F_m-F_0$) and the values of F_v/F_m were calculated by the software of the Fluorometer, and the F_v/F_0 were counted from the measured parameters. In light adapted leaves the actual quantum yield (Y(II)) of PSII was counted by the software of the Fluorometer.

Chlorophyll a (chl a), chlorophyll b (chl b) and total chlorophyll (chl a + chl b) contents of the leaves collected from the apical two, fully developed leaves were measured using spectrophotometric method described by Felföldy (1987). Chlorophyll contents were then calculated for µg per 1 g fresh weight (FW) of the leaves.

Data collection and analysis

Three independent shoots were used for measurements from each CK treatment and measurements were repeated three-times.

Data were analysed statistically using SPSS for Windows (SPSS®, version 21.0) by one- and two-way-ANOVA followed by Duncan's test for all pairwise comparisons at $p \leq 0.05$. Pearson correlation coefficient was determined and its significance (at $p \leq 0.05$ and at $p \leq 0.01$) was tested to study the relationship between the chlorophyll fluorescence and chlorophyll content.

RESULTS AND DISCUSSION

Chlorophyll fluorescence

Analysis of variance proved that CK-type affected the maximum quantum yield (F_v/F_m), maximum efficiency of the photochemical process (F_v/F_0) and actual quantum yield of PSII, as well, significantly. However, ANOVA did not confirm significant effect of CK concentration.

Values of F_v/F_m varied between 0.709 and 0.790, which indicates that the photosynthetic apparatus in the leaves of the 3-week-old *in vitro* plantlets of cv. Freedom was well-developed and operable (Triques et al. 1997). Both the maximum quantum yield (F_v/F_m) and maximum efficiency of the photochemical process (F_v/F_0) of PSII were the highest at

BA+TOP supply; this effect was statistically significant at all concentrations, except for 6.0 µM (Fig 2). The lowest values of F_v/F_m and F_v/F_0 were measured when TOP was applied as single source of CK in the medium. The effect of CK-supply was unlike as detected in the cv. Royal Gala (Dobránszki and Mendler-Drienyovszki 2014), where application of BA resulted in the highest F_v/F_m and F_v/F_0 at 0.5 µM, but at higher concentration there were no significant differences between the effects of BA and BA+TOP.

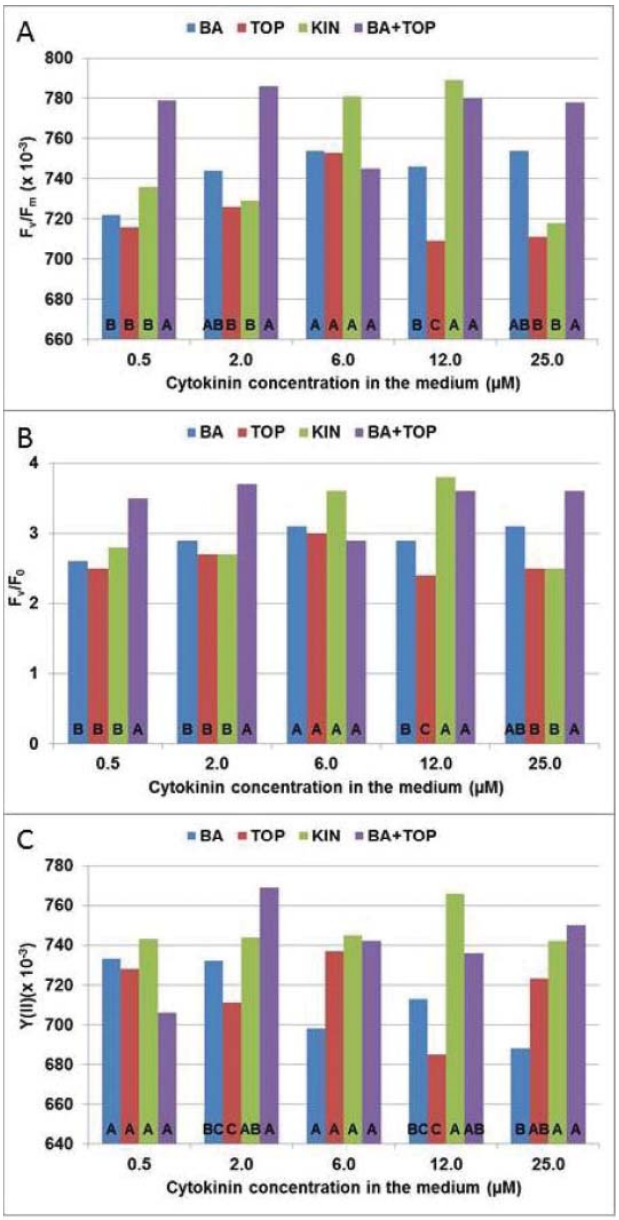

Figure 2: Chlorophyll fluorescence of the second apical leaves on in vitro axillary apple shoots of cv. Freedom. A: maximum quantum yield (F_v/F_m), B: maximum efficiency of the photochemical process (F_v/F_0) and C: actual quantum yield of PSII under the actual lighting condition, at different cytokinin-supplies. (BA: benzyl-adenine, TOP: meta-topolin, KIN: kinetin, BA+TOP: 2.2 µM BA and different concentrations of TOP). Different capitals in the columns indicate significantly different homogeneous groups between different CK-supply within the applied concentrations according to the Duncan's test ($p \leq 0.05$).

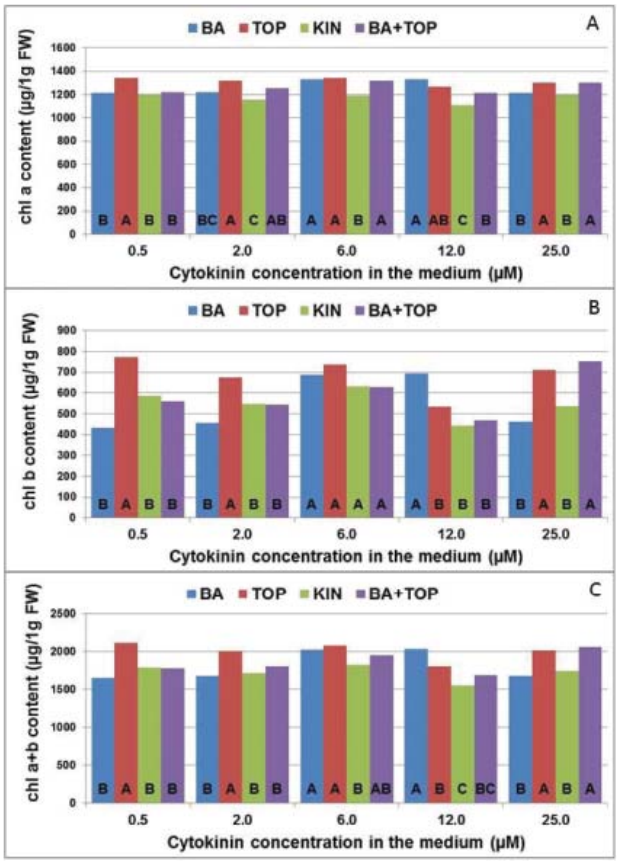

Figure 3: Chlorophyll content of the two apical leaves on in vitro axillary apple shoots of cv. Freedom. A: chlorophyll a, B: chlorophyll b, C: chlorophyll a + chlorophyll b (µg/1g fresh weight of leaves) (BA: benzyl-adenine, TOP: meta-topolin, KIN: kinetin, BA+TOP: 2.2 µM BA and different concentrations of TOP). Different capitals in the columns indicate significantly different homogeneous groups between different CK-supply within the applied concentrations according to the Duncan's test ($p \leq 0.05$).

Quantum yield of PSII measured in light-adapted leaves of cv. Freedom under the actual lighting condition (Y(II)) tended to be the highest when Kin and BA+TOP were applied, respectively, at higher than 0.5 µM. Such effect of BA+TOP was not detected in cv. Royal Gala (Dobránszki and Mendler-Drienyovszki 2014).

Comparing our earlier and present results with different cultivars (Royal Gala and Freedom), a very strong genotype-dependence can be confirmed in chlorophyll fluorescence of *in vitro* apple shoots grown on media with various CK contents.

Chlorophyll content of leaves

Chlorophyll content (chl a, chl b and chl a+b) of the two apical leaves of cv. Freedom was significantly affected both by the type and the concentration of CKs ($p \leq 0.05$) (Fig. 3). The highest chl a was measured when shoots grown on medium supplemented with TOP and it was the lowest after grown on medium with Kin in tendency at any concentrations, but the differences were proven to be significant only at 0.5, 2.0

TABLE 1: Correlation analysis between the parameters of the chlorophyll fluorescence and that of the chlorophyll contents. Test of two-tailed significance of Pearson correlation coefficients (Pearson's rho) at $p < 0.05$ (*) and $p < 0.01$ (**)

	chl b	chl a+b	chl a/b	F_v/F_m	F_v/F_0	Y(II)
chl a	0.790**	0.914**	-0.602**	-0.289	-0.299	-0.344
chl b	-	0.971**	-0.952**	-0.154	-0.152	-0.071
chl a+b	-	-	-0.866**	-0.215	-0.218	-0.182
chl a/b	-	-	-	0.117	0.115	-0.057
F_v/F_m	-	-	-	-	0.994**	0.391
F_v/F_0	-	-	-	-	-	0.425

and 25.0 µM TOP and at 6.0-12.0 µM Kin, respectively. At low concentrations (0.5 and 2.0 µM) the TOP, at 12.0 µM the BA while at the highest concentration (25.0 µM) the TOP and the BA+TOP resulted in the highest chl b content. However, the most effective cytokinin regarding both chl a and chl b content was the BA in cv. Royal Gala (Dobránszki and Mendler-Drienyovszki 2014), clearly indicating the different responses of the genotypes of the same species.

Statistical analysis did not prove any correlation between the chlorophyll content and chlorophyll fluorescence parameters in cv. Freedom (Table 1). This result indicates that measurement of chlorophyll content in *in vitro* leaves cannot give exact information about the functionability of the photosynthetic apparatus. Similarly no correlation could be proven between the chlorophyll content and F_v/F_m of cv. Royal Gala (data not presented here). These results are in contradiction with the finding of Sáez et al. (2012), who found chlorophyll content to be a good indicator of photosynthetic capacity of *Castanea sativa*.

CONCLUSIONS

Performance of the photosynthetic apparatus of *in vitro* leaves was modified by the CK supply in our model experiments with apple cv. Royal Gala (Dobránszki and Mendler-Drienyovszki 2014). Results with cv. Freedom presented here confirmed our earlier finding. However, they simultaneously indicated also the genotype-dependence of the CK-effect since the effect of CKs on the quantity (chlorophyll content) and quality parameters (chlorophyll fluorescence) of leaves differed in cv. Freedom from those of in cv. Royal Gala.

Results showed that the type of CKs used in the medium affected significantly the chlorophyll fluorescence of the leaves in *in vitro* apple cv. Freedom. Chlorophyll fluorescence, mainly F_v/F_m is a good indicator of the functionability of photosynthetic system and therefore a good measure of the developmental state of chloroplasts, too. As it was proven under *in vivo* conditions (Kualeva 2002), functional chloroplasts are prerequisite of normal leaf response to CKs. CKs applied in the medium of shoot cultures are of importance in regulation of growth and development and they have post-effect also for the subsequent developmental processes, i.e. for the next cycle of axillary or adventitious shoot development or for rooting ability (Magyar-Tábori et al. 2010). In present experiments with apple cultivar Freedom both the dark and light chlorophyll fluorescence was the highest when dual CK-supply, BA+TOP, was applied. As described earlier (Magyar-Tábori et al. 2010), dual application of BA and TOP was proven to be the most efficient CK pre-treatment before adventitious shoot regeneration in cv. Freedom.

Both the type and the concentration of applied CKs affected significantly the chlorophyll content of the leaves in cv. Freedom; both chl a and chl b were the highest after application of TOP at low concentrations (0.5-2.0 µM) and the application of Kin resulted in the lowest chlorophyll content at all concentrations.

In cv. Royal Gala (data not presented here) no correlation was detected between the chlorophyll content and maximum quantum yield or photochemical efficiency (F_v/F_m, F_v/F_0) however there was a weak but significant correlation between the chlorophyll content and actual quantum yield (Y(II)). Unlike in cv. Freedom (Table 1) no correlation could be detected between chlorophyll content and chlorophyll fluorescence parameters (F_v/F_m, F_v/F_0, Y(II)) indicating that no conclusion can be drawn from the chlorophyll content for the developmental state and functionability of the photosynthetic apparatus in *in vitro* leaves of cv. Freedom.

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DEVELOPMENT AND USE OF MOLECULAR MARKERS FOR TWO GAIN-OF-FUNCTION COLOUR GENE MUTATIONS IN CAULIFLOWER

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ABSTRACT

Breeding of cauliflowers with enhanced level of health-promoting secondary metabolites such as β-carotenes and anthocyanins can provide an additional vegetable component in human diet. Through more than 30 year work by many breeders and scientists, the orange and purple cauliflowers are now commercially available and the genetic background of these natural mutations is known. Surprisingly, both of these striking mutations were caused by transposable genetic elements which usually ruin the function of the affected genes. However, in these cases the mutants gained a high level accumulation of carotenoids and anthocyanins, respectively.

We have developed PCR based molecular markers for these mutations in this study for earlier and faster selection of coloured cauliflower mutants in practice. These markers made it possible to differentiate not only the purple and orange coloured genotypes from any other cauliflowers, but also to distinguish their homozygous allelic forms from the heterozygous ones regardless of the developmental stage of the plants. Introgression of these mutations into related *Brassica* crops and species and following their inheritance is also possible with the developed markers. Here the first step is presented to enhance the β-carotene levels in kohlrabi *via* introduction of the mutant *Or* gene found in cauliflower. These results may pave the road to a new approach for increasing β-carotenes and anthocyanins also in other food crops, in this way improving their nutritional and agronomic value.

keywords: β-carotene, anthocyanin, retro-transposon, DNA transposon, coloured cauliflower, Or gene, Pr gene, nutrition enhancement

INTODUCTION

Many plant secondary metabolites such as anthocyanins and carotenoids are highly beneficial for human since they provide important natural nutrients and antioxidants in our diets (Hou 2003, Zhou et al. 2008). Coloured vegetables and fruits are in major; focus now as functional foods due to their potent nutritional and health-promoting effects due to mainly their high levels of plant pigments. Among them, orange and purple cauliflowers are really attractive vegetables (Fig. 1) and already commercially available worldwide from some seed companies, like Syngenta. The purple and orange colouration in the cauliflower curds is the consequence of anthocyanin and β-carotene accumulation, respectively. Surprisingly, the development of both colour variants belong to the group of rather rarely happening gain-of-function natural gene mutations caused by transposable elements.

Transposable genetic elements are representing a major fraction of eukaryotic genomes (Gaut and Ross-Ibarra 2008) and induce structural and epigenetic reorganization in their host genome during their movement (Michalak 2009; Parisod et al. 2014). DNA fragments of different sizes can insert into new chromosomal locations and often make duplicate copies of themselves in the process (Feschotte et al. 2002). They were discovered by Barbara McClintock as the genetic agents that are responsible for the sectors of altered pigmentation on mutant kernels of corn (McClintock 1951).

Transposable elements can be divided into two classes by their transposition intermediate (Finnegan 1989). In case of 'class I' or **retrotransposons**, the transposition mechanism is commonly called 'copy-and-paste' because they are moving to new chromosomal locations *via* an RNA intermediate, while the transposition of 'class II' or **DNA**

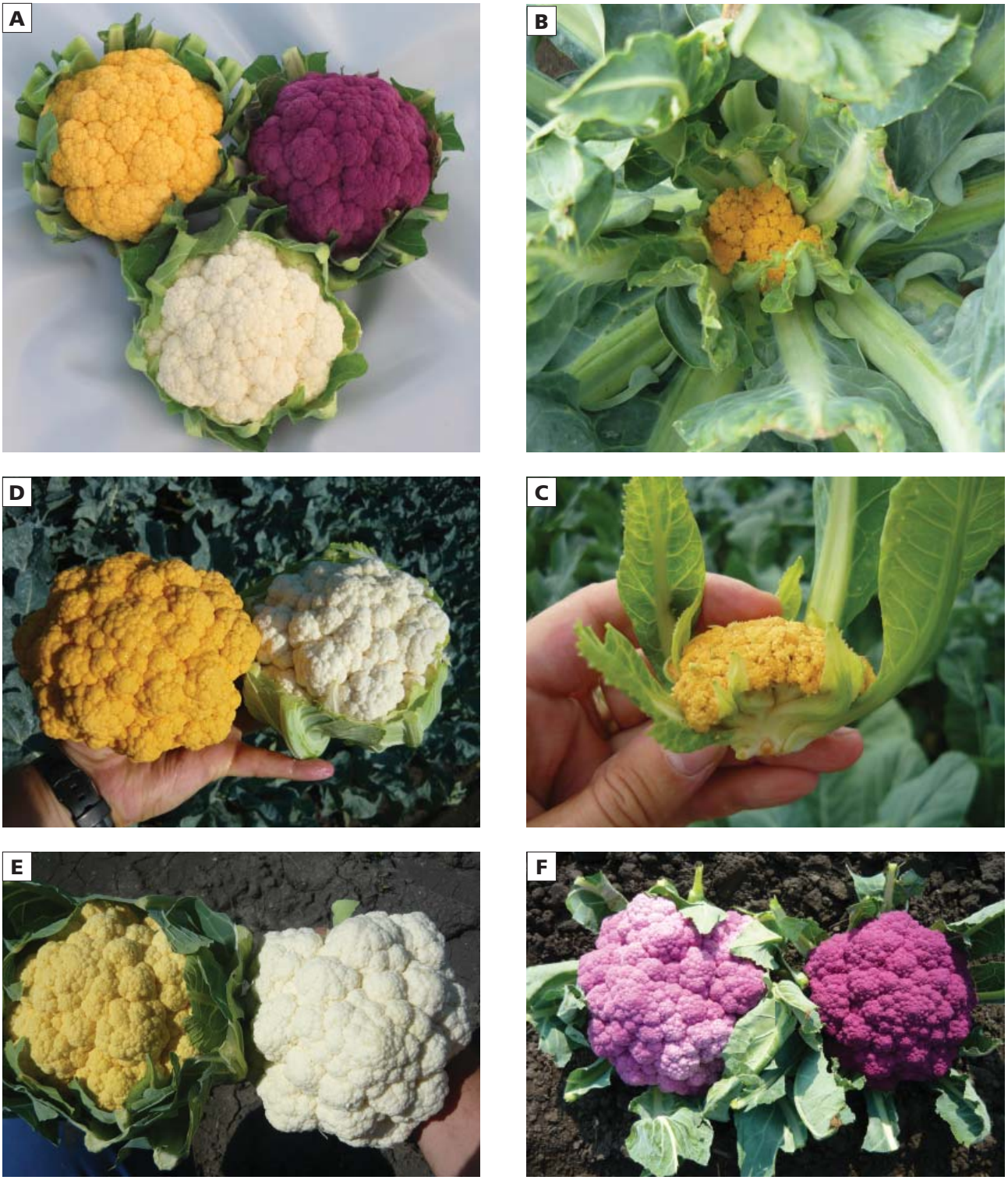

Figure 1: (A) Curd of orange, purple and white cauliflowers. (B) Phenotype of the homozygous *Or* mutant in the field shows dwarf plant habit. (C) The normal curd size of homozygous *Or* mutant is 4-5 cm in diameter. (D) Curd of homozygous *Or* mutant (left) and wild-type (right) cauliflowers. The linkage with dwarfism was broken, the homozygous mutant shows normal growth and curd size. (E) Curd of heterozygous *Or* mutant (left) and wild-type (right) plants. (F) Curd of heterozygous (left) and homozygous (right) *Pr* mutants grown in the field.

transposons occurs with DNA movement ('cut-and-paste'). Retrotransposons can be also separated into two major subclasses that mainly differ in their structure. Elements of 'subclass I' are bounded by two long terminal repeats (LTRs) and are termed **LTR retrotransposon**s; elements of 'subclass II' do not possess LTRs and are therefore termed **non-LTR retrotransposon**s.

The orange and purple colour mutations in cauliflower

are nice examples of the movement and effect of transposable elements caused by a LTR retrotransposon and a DNA transposon, respectively.

The spontaneous mutation of a cauliflower gene, designated as (*Or*) for *Orange* gene (Crisp et al. 1975) confers carotenoid (mainly β-carotene) accumulation in the normally unpigmented curd (Fig. 1). The mutation imparts also visible orange colouration to the shoot meristems, the pith of the stem and the vasculature at the base of the petioles (Li et al. 2001). β-carotene is the primary dietary source of provitamin A and offer protection against the incidence of certain diseases, such as cancer, cardiovascular disorder, and age-related eye deterioration (Mayne 1996; Giovannucci 1999). The curd tissues of *Or* homozygous plants accumulate approximately 800 µg/100g fresh weight of β-carotene, a level several hundred fold higher than that detected in comparable wild-type tissues (Li et al. 2001).

The *Or* gene is a single-copy sequence in the cauliflower genome. The wild-type gene (*or*) contains eight exons and seven introns. The mutation of this gene was caused by a *copia*-like LTR retrotransposon insertion (4.7 kb) in the third exon. This retrotransposon insertion turns the white colour of curd tissue into orange as a result of β-carotene accumulation. Genetic and cellular analysis suggests that the *Or* gene is not functioning directly in the biosynthesis, but controlling the carotenoid accumulation by inducing the differentiation of proplastids and/or other non-coloured plastids into chromoplasts. This provides a new metabolic sink for carotenoid accumulation in the specific tissues (Lu et al. 2006).

The cauliflower purple mutation was also found to be controlled by a single, semidominant gene designated as (*Pr*) for *Purple* gene. The wild type gene (*pr*) encodes a R2R3 MYB transcription factor which has been shown to play an important role for colour difference in plant species (Chiu et al. 2010). It was proven that the activation of R2R3 MYB transcription factor confers anthocyanin production in a number of anthocyanin-accumulating mutants like in *Arabidopsis* (Borevitz et al. 2000), tomato (Mathews et al. 2003) and red-fleshed apple (Espley et al. 2007). In contrast, the loss of function of a R2R3 MYB causes colour loss in the normal anthocyanin-accumulating tissues in grapes (Walker et al. 2007). The purple cauliflower mutant appears to display a different mechanism in activating gene expression.

A Harbinger DNA transposon insertion into the promoter region of the *Pr* gene in cauliflower is responsible for the increased transcription of the gene which in turn up-regulates anthocyanin expression, finally resulting in purple phenotype (Chiu et al. 2010). The purple curded cauliflower mutants are also exhibit tissue-specific anthocyanin accumulation in the leaves, flower buds, siliques and seeds. In the curds of the purple cauliflower the anthocyanin (mainly cyanidin 3-(coumaryl-caffeyl) glucoside-5-(malonyl)glucoside) content is about 375 mg/100 g (Chiu et al. 2010), similarly as it was found in blueberries (Gao and Mazza 1994). The wild type (white) cauliflower curd contains undetectable amount of anthocyanins. The transposon insertion in the *Pr* allele introduced additional E-box cis-acting elements which provide more binding sites for bHLH transcription factors and up-regulate *Pr* expression (Chiu et al. 2010).

It is getting more and more important to develop anthocyanin-rich foods to meet the increasing demand for health-promoting components in our diet. These kinds of foods offer protection against cardiovascular disorders (Lazze et al. 2006), certain cancer (Wang et al. 2012) and some other chronic diseases (Hou 2003; Lila 2004). The health promoting roles are most frequently associated with the high antioxidant activity of anthocyanins.

The aim of this study was to develop molecular markers for these colour mutations in cauliflower which can be applied in breeding for early and easier selection of the mutants, to differentiate their allelic forms and to confirm the phenotypic observations with objective data. It can be also used for the transfer and selection of orange and purple colour mutations in related *Brassica* crops (cabbage, broccoli, kohlrabi, Brussels sprouts etc.) to increase their nutrient and antioxidant levels in the future. Some of these conversions are already in progress what can pave the road to a new strategy for enhancing these healthy elements to the required level in other food crops, as well.

MATERIAL AND METHODS

Young leaf samples of different white-, orange-, purple- and green curded cauliflowers were collected at the Trial Station of Syngenta Kft. in Ócsa, Hungary. Samples were kept at -70°C until DNA isolation. Genomic DNA was extracted using a DNeasy® Plant Mini Kit (Qiagen) from approximately 100 mg leaf samples. DNA concentration was determined by a NanoDrop spectrophotometer in each sample and then was adjusted to 10 ng/µl for using as template for polymerase chain reactions (PCR). PCR amplifications were carried out by a Perkin Elmer 9700 thermocycler or by BioRad iCycler. Primer sequences used were as follows (Kiss et al. 2014) for the orange mutation (Fig. 2):

P1 (forward primer): 5'–TTTGTTATGCCCATGAGAGTTG –3',

P3 (reverse primer): 5'–AAGGGAGGGAGTATATGCCAAT –3',

P2 (retrotransposon specific reverse primer): 5'–TTCATTAGGGAATGTGTTGAGC –3'

Primer sequences used were as follows (Bedzsó et al. 2012) for the purple mutation (Fig. 3):

F1 (forward primer): 5'–GCCTCTGGTGCGTGAAGTTGCT–3',

R (reverse primer): 5'–CGGGCAAAGGGGAATGACGTGC–3',

F2 (transposon specific forward primer): 5'–ACCCGTGACAGAAGAATGCTCCA –3'.

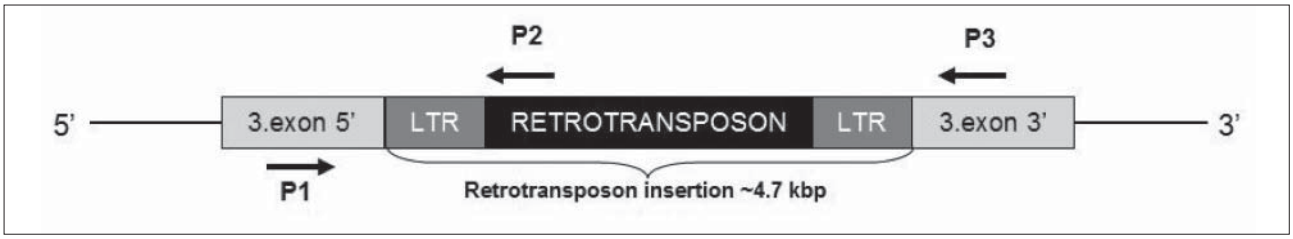

Figure 2: Schematic diagram of the third exon of the mutant *Or* gene. Horizontal arrows represent the designed primers and show their orientation. The insertion site of retrotransposon is flanked by the primers P1 and P3 while P2 is a retrotransposon specific primer.

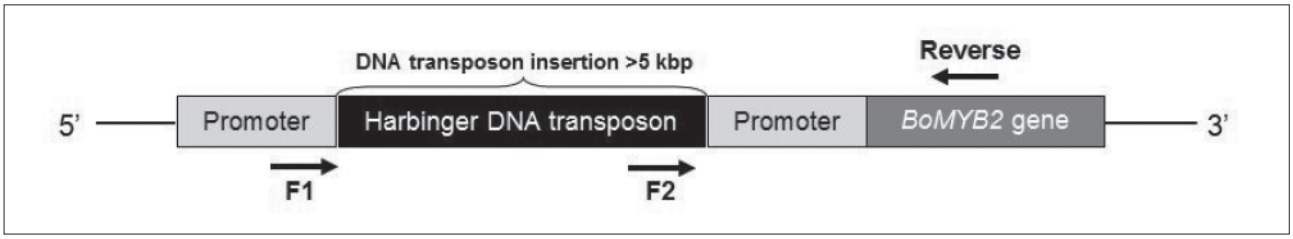

Figure 3: Schematic diagram of the promoter region of the mutant BoMYB2 gene. Horizontal arrows represent the designed primers and show their orientation. The insertion site of transposon is flanked by the primers F1 and Reverse while F2 is a transposon specific primer.

The reactions were performed in a final volume of 10 µl, the reaction mixture contained the following components: 25 ng template DNA, 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCL pH: 8.3, 1.1 mM $MgCl_2$ 0.01% gelatin), plus 0.9 mM $MgCl_2$ (Fermentas, Biocenter Kft., Szeged), 0.3 µM of used primer(s), 0.2 mM each of dNTPs (Fermentas, Biocenter Kft., Szeged), and 0.6 unit of WestTeam Taq-DNA polymerase (West Team Biotech Kft., Pécs). The cycling profile consisted of an initial denaturation step of 2 min followed by 40 cycles of 30 s at 94°C, 30 s at 57-62°C (depending on the melting points of the used primer(s)), and 90 s at 72°C. The amplification process was finished with 5 min at 72°C. Products were tested by running on 1.2% agarose gel (80V) necessarily diluted with TE1 buffer, painted by EtBr. Pictures were taken by fixed digital camera above UV transilluminator on 313 nm.

RESULTS

Development of molecular markers for the orange mutation

The published genomic nucleotide sequence of the *Or* mutant allele (Lu et al. 2006) was used to design two primers (one forward /P1/ and one reverse /P3/) for the flanking regions of the retrotransposon insertion site and one retrotransposon specific /P2/ reverse primer (Fig. 2).

At first, the designed primers were tested on DNA isolated from 'normal' white curded cauliflower samples alone or in different combinations (Fig. 4). As we expected, the primers alone did not amplify any well-defined specific fragments during the reactions, the results look like RAPD patterns. We did not expect specific fragment also with P1+P2 combination since the genome of white cauliflower does not contain the retrotransposon insertion in the 3rd exon of the *Or* gene. Only the P1+P3 and P1+P2+P3 primer combinations were able to amplify the awaited 605 bp long fragment from the 3rd exon of *Or* gene itself. Of course, in the latter case the P2 primer is not necessary for this, since its priming site is missing in white curded cauliflowers. These results prove that the designed primers work in white cauliflowers, as expected.

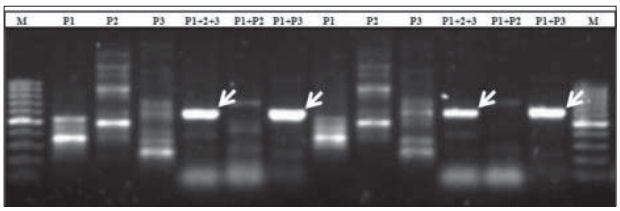

Figure 4: Testing of different primer combinations designed for the 3rd exon of Or gene (P1+P3) and for the retrotransposon (P2) on white curded cauliflowers. M: GeneRuler 100 bp DNA ladder: 100 bp-1031 bp. Arrows are indicating the expected 605 bp long fragment.

As a next step, it was tested whether the designed primers are suitable to reveal the mutant *Or* allele in orange curded cauliflowers, or not. The combination of P1+P2 primers was used for this test because theoretically this combination

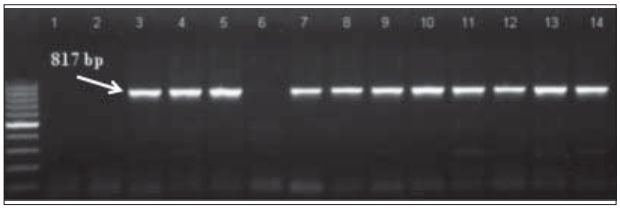

Figure 5: PCR results using the combination of P1+P2 primers on samples of different white and orange curded cauliflower genotypes. M: GeneRuler 100 bp DNA ladder: 100 bp-1031 bp. Template DNA was isolated from white cauliflowers (lane 1,2,6), and different orange cauliflower (lane 3-5 and 7-14) genotypes. The arrow shows the awaited 807 bp long fragments amplified in orange cauliflowers.

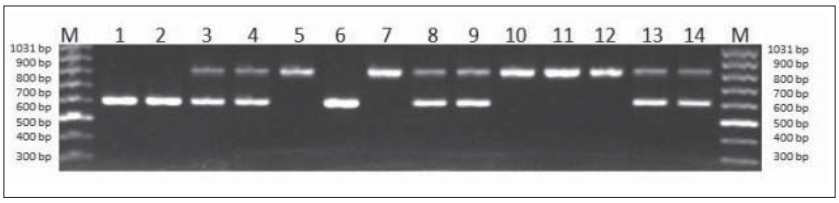

Figure 6: Results of the PCR using all (P1+P2+P3) primers in one reaction. M: GeneRuler 100 bp DNA ladder: 100 bp-1031 bp. Template DNA was isolated from white cauliflowers (lane 1,2,6), and different orange cauliflower (lane 3-5 and 7-14) genotypes.

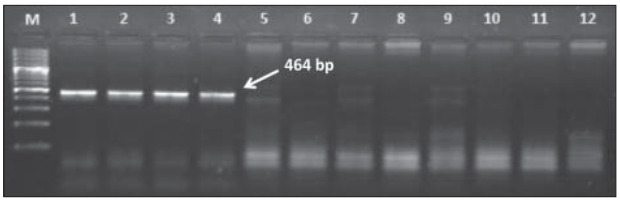

Figure 7: Results of the PCRs using F2+R primer combination on different white and coloured cauliflower genotypes. M: GeneRuler 100 bp DNA ladder: 100 bp-1031 bp. Template DNA was isolated from different purple- (lane 1-4), green- (lane 5), Romanesco- (lane 6), orange- (lane 7-9), and white (lane 10-12) genotypes. The arrow shows the expected 464 bp long fragments amplified in purple cauliflowers.

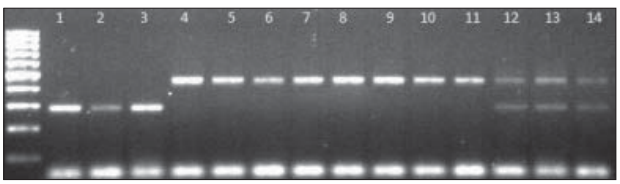

Figure 8: Results of the PCR using all (F1+F2+R) primers in one reaction. M: GeneRuler 100 bp DNA ladder: 100 bp-1031 bp. Template DNA was isolated from: white cauliflowers (lane 1-3), homozygous purple genotypes (lane 4-11), heterozygous purple genotypes (lane 12-14).

amplifies a fragment of 817 bp length only in DNA isolated from orange cauliflowers where the retrotransposon was inserted into the 3rd exon of *Or* gene (Fig. 5). The reaction did not amplify any product from DNAs isolated from the white curded cauliflowers. Hereby, the white and orange cauliflowers can be easily distinguished genotypically by using this primer combination as dominant molecular marker.

To make this marker co-dominant, we need to add also the designed P3 primer to the reactions (Fig. 6).

Since the reaction mixture contained all of the three primers, it made it possible to differentiate the orange coloured genotypes from the white ones (theoretically from any other coloured materials) and also to distinguish the homozygous mutants from the heterozygous ones within the orange genotypes. If there is no mutation in the gene (so in case of white and other coloured /purple, green/ cauliflowers) we get only a 605 bp long single product (lanes 1,2,6 in Fig. 6). In the homozygous mutant plants we get another single product which is 817 bp in length, since the retrotransposon specific primer is working as a reverse primer (lanes 5,7,10,11,12 in Fig. 6). In this case, the 605 bp long product is not amplifying since its length would be more than 5.5 kbp thanks to the inserted retrotransposon which is too long for a normal reaction. In the heterozygous plants we get both products (lanes 3,4,8,9,13,14 in Fig. 6).

Development of molecular markers for the purple mutation

The published genomic nucleotide sequence of the purple mutant allele (Chiu et al. 2010) was used to plan two primers (one forward /F1/ and one reverse /R/) for the flanking regions of the insertion site in the promoter region of BoMYB2 gene and one transposon specific /F2/ forward primer (Fig. 3).

Similarly to the previously shown results with the 'orange' marker, if we just want to differentiate the purple genotypes from any other coloured genotypes (white, orange, green), the F2+R primer combination can be used as a dominant marker. In this case, we get a single 464 bp product only in purple genotypes independently of their allelic forms (homo- or heterozygous) since the forward primer is transposon specific. We do not get product in any other coloured materials (Fig. 7).

Using all three designed primers in one reaction makes it possible to differentiate the purple coloured genotypes from any other coloured materials and also to distinguish the homozygous mutants from the heterozygous ones within the purple genotypes (Fig. 8). The marker became co-dominant.

If there is no mutation in the promoter region of BoMYB2 gene (so in the case of white and other coloured /orange, green/ cauliflowers and probably in many plant species) we get only a 292 bp long single product (lanes 1-3 in Fig. 8). In the homozygous mutant plants (deep purple colour) an other single 464 bp product amplified, since the transposon specific primer is functioning as a forward primer (lanes 4-11 in Fig. 8). In this case, the 292 bp long product is not produced in a normal reaction since its length would be too large due to the transposon insertion. (The precise length of this transposon is still unknown, but in *Arabidopsis* the average size of Harbinger transposons is around 5300 bp.) In the heterozygous plants we get both products (lanes 12-14 in Fig. 8).

An example of application of the developed co-dominant molecular marker for introgression of orange mutation into kohlrabi background

Crosses were made in order to introduce the semi-dominant orange mutant allele discovered in cauliflower into other related *Brassica* crops, such as kohlrabi. Inbreeding of some selected heterozygous hybrids originated from homozygous orange cauliflower x kohlrabi crosses resulted in segregating populations for many traits, including the mutant *Or* allele, as well.

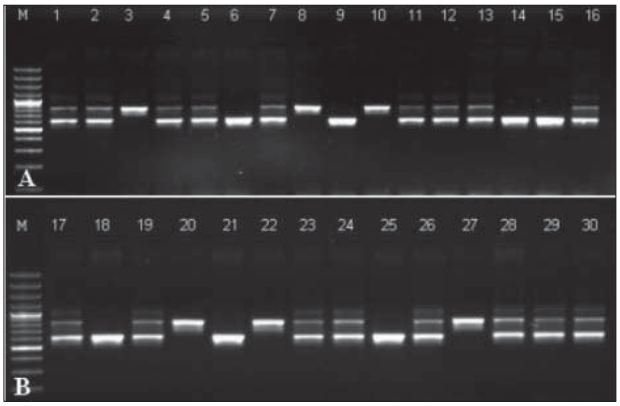

Figure 9 A/B: PCR results of an F_2 segregating population using all (F1+F2+R) primers. M: GeneRuler 100 bp DNA ladder: 100 bp-1031 bp. Lane 1-30: template DNA was isolated from different F_2 individuals coming from a cross of orange cauliflower and kohlrabi.

It has become possible to easily reveal the mutant *Or* allele in the F_2 progeny and to distinguish the homozygous mutants from the heterozygous ones applying the developed molecular marker (Fig. 9). Normal Mendelian inheritance of *Or* gene was observed in the segregating population (Table 1) despite the relatively low number of individuals: 6/30 plants proved to contain the wild type gene in homozygous form (*or/or*), so these were lost the orange phenotype. 17/30 were heterozygous for this mutation (*Or/or*) while 7/30 were homozygous (*Or/Or*). The *Or* mutation conferred orange discolouration in the hetero- and homozygous plants mainly in the middle and close to the outer periphery of the bulb. Similarly to cauliflowers the colour is more intense in homozygous than in heterozygous form.

The genotypic data presented here absolutely agreed with the phenotypic data judged in the field at harvest maturity (not shown data). (In practice, 1-9 scale was used for the colour evaluation, giving the number 2-3-4-5 for the putative heterozygous orange plants while the number 6-7-8-9 was given for the presumed homozygous plants depending on the visible hue of the colour.)

Although the orange colour is not uniform in the bulbs of these plants and still many back-crosses are needed to restore the kohlrabi background, this result verifies the capability of the developed molecular markers to use in breeding and also the possibility to enrich the carotenoid and/or anthocyanin content employing this approach in other crops, as well.

DISCUSSION AND CONCLUSIONS

The spontaneous, single-locus *Or* and *Pr* gene mutations in cauliflower confers high levels of β-carotene or anthocyanin accumulation in the curds where the accumulation of these secondary metabolites normally repressed (Fig. 1). In this study, we have successfully developed molecular markers for these natural mutations which can be used in breeding practice for easier selection of the mutants and to differentiate their allelic forms independently from their phenological phase.

The *Pr* gene in the purple cauliflower mutants represents a novel mutation of a transcription factor gene (*BoMYB2*) that controls the expression of other anthocyanin regulators in controlling anthocyanin accumulation. It seems to be a very important transcription factor probably across all *Brassica* genomes, because the constitutive anthocyanin production is also associated with an increased expression of a gene in red cabbage which shares 99.6% nucleotide sequence identity with *BoMYB2* in cauliflower (Yuan et al. 2009). The Harbinger DNA transposon insertion in the promoter region of *Pr* gene introduced additional E-box cis-acting elements (Chiu et al. 2010), which likely provide more binding sites for bHLH transcription factors to activate its expression, as suggested also in a study of *mPing* DNA transposon in rice (Naito et al. 2009).

Carotenoids are synthetized by all chlorophyll-containing photosynthetic organisms, some bacteria and many species of fungi but not produced in animals and humans. These are indispensable in providing precursors for vitamin A synthesis. Its deficiency, which affects millions of people in many parts of the world, remains one of the most noticeable nutritional problems. Current advances to enhance carotenoid accumulation in food crops have been mainly focused on modification of the genes encoding the metabolic enzymes in the pathway (Taylor and Ramsay 2005, Botella-Pavia and Rodriguez-Concepcion 2006). Golden Rice is one of the best known examples of this strategy (Potrykus 2001). In the improved generation of Golden Rice, the level of total carotenoids in the endosperm reached the 37 µg/g that provides adequate quantity

TABLE 1: Comparison of the observed and expected segregation ratios of Or gene in an F_2 population (30 individuals) derived from a homozygous orange cauliflower and kohlrabi cross.

Phenotype	Genotype	Observed segregation	Expected (statistical) segregation	Expected phenotypic ratio	Expected genotypic ratio
White	or/or	6	7.5	1	1
Orange	Or/or	17	15	3	2
Orange	Or/Or	7	7.5		1
		$\Sigma = 30$	$\Sigma = 30$		

of vitamin A in an average daily consumption of rice (Paine et al. 2005). Although the genetic modification of biosynthetic genes can be effective, it has been proved to be difficult to reach the desired level of carotenoids in transgenic plants in many cases (Fraser and Bramley 2004) or even leads to unexpected phenotypic changes (Fray et al. 1995). This makes essential to search for novel approaches to accumulate carotenoids in plants. The *Or* gene in cauliflower reveals that changing chromoplast formation to provide new metabolic sink for carotenoid accumulation is an alternative strategy to increase their content in plants.

The transformation with *Or* gene isolated from cauliflower under the control of a tuber-specific promoter has already provided evidence that this mutant gene works across species to enhance carotenoid accumulation (Lopez et al. 2008). The total levels of carotenoid increase were over six fold comparing to untransformed controls. Surprisingly, the cold storage of the transgenic tubers further increased the carotenoids to a level of ten fold over controls. This effect had been also detected by us in the case of stored orange cauliflowers and the colouration became more intense (unpublished data).

Obvious objective is to introduce these mutations found in cauliflower into crossable related *Brassica* crops and species to increase carotenoid content by conventional breeding methods. It is especially important in these years of intense opposition against gene technology. The first steps are presented in this study proving that the *Or* gene works also in a kohlrabi background and the developed marker is suitable to identify the presence of *Or* gene and to distinguish the homozygous mutants from the heterozygous ones. This differentiation is not always obvious phenotypically because of the phenological phase and/or environmental factors.

Indeed, the mutation of the *Or* gene leads not only to high level of β-carotene accumulation in the cauliflower curd, but in homozygous form also causes other phenotypic changes like dwarf plant habit, smaller curds, enhanced petiole elongation (Fig.1: B-C). In the latter case it has been already proven that the cauliflower *Or* gene controls petiole elongation by suppressing the expression of the eukaryotic release factor 1 (eRF1) genes (Zhou et al. 2011). More breeding related effects are the undesired smaller curds and stunted growth of *Or* homozygous plants. In heterozygous form of this mutation, the plant and its curd has normal size and exhibits a less intense orange colour than in homozygous form (Fig.1: E). Although the explanation for the dwarf plant habit is still unknown, it seems it is not due to the over production of phytoene synthase which diverts the necessary intermediate away from gibberellin acid biosynthetic pathway which was found in tomato plants transformed with a copy of phytoene synthase cDNA controlled by CaMV 35S promoter (Fray et al. 1995). The phytoene accumulated at comparable levels and no major differences in carotenogenic gene expression were observed either between the wild type and *Or* cauliflower calli (Li et al. 2006). The tight linkage with dwarfism had been already broken (Fig.1: D) by breeders from Daehnfeldt and Syngenta companies (not published data), so it was not caused by pleiotropic effect assumed in the scientific literatures, though the genetic background is still unknown. Nevertheless, deep orange coloured cauliflower genotypes showing normal plant growth are already available for breeding and to exploit commercially in the future.

Enriching carotenoid and anthocyanin levels in major crops is expected to have a broad and significant impact on human nutrition and health in the future. The demonstration of use of the unique *Or* and *Pr* genes to increase carotenoid and cyanidin content in *Brassica* crops may furnish a potent new genetic tool for nutritional improvement of plants.

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DNA DEMETHYLATION-INDUCED GENE REACTIVATION IN GM POPLAR

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ABSTRACT

DNA de-methylating agent DHAC (5,6-dihydro-5'-azacytidine hydrochloride) was applied (10^{-4} M for 7 days) for gene reactivation of wild type (WT) and 35S(CaMV)-*gsh*I GM (genetically modified) transgenic poplar (*Populus x canescens*; plant family Salicaceae) clones (6*lgl* and *11ggs*) by using aseptic leaf discs cultures. Gene expression levels were determined by RT-qPCR (reverse transcriptase quantitative PCR) measuring the mRNA levels of the prokaryotic *gsh*I-mRNA (γ-glutamylcysteine synthetase) cloned from *E. coli,* and two endogenous poplar genes of *gsh*1-mRNA and *gst*-mRNA (the allergenic glutathione S-transferase). For internal control, the constitutively expressed housekeeping poplar genes α-*tubulin* and *actin* were used, and the $2^{-\Delta\Delta Ct}$ method was applied for data analysis. After DHAC treatment the expression levels of 35S(CaMV)-*gsh*I transgene showed a double (1.8-fold) increment. The endogenous poplar gene *gsh*1 increased by 19.7-fold in the WT, and by 8.7-fold in the GM *6lgl* clone. The endogenous *gst* gene showed a 4.9-fold (in WT) and a 2.9-fold (in GM) increment, respectively. Sequence analysis of known plant DNA methylating enzymes were analyzed *in silico* to distinguish the three main classes of plant DNA methylases (METase): the METs (maintenance methyltranferases), CMTs (chromomethylases) and DRMs (*de novo* domains rearranged DNA methylases). A *de novo* root and rare shoot development was also observed on DHAC-treated poplar leaf discs incubated for long term (21 days) treatment. The DHAC-treated WT poplars with increased gene expressions of *gsh*1 and *gst* genes might provide novel plant resources for application for detoxification and soil remediation and acceptable to the general public concerned about GMOs.

keywords: *Populus* x *canescens*, DNA demethylation, DHAC, RT-qPCR

INTRODUCTION

Poplars (*Populus* ssp) are capable of removing and degrading toxic substances from the polluted soils through phytoremediation due to the extensive root system, high water uptake capacity, rapid growth, and large biomass production [1]. This phytoremediation capacity of *Populus x canescens* has been significantly increased by genetic transformation with the prokaryotic *gsh*I gene, which encodes for γ-glutamylcysteine synthetase (γ-ECS, EC 6.3.2.2). Gene *gsh*I was cloned from *E. coli* (NCBI, X03954). The transformed poplar clones showed higher contents of both GSH and its precursor of γ-L-glutamyl-

Figure 1: Clones of Populus x canescens (WT) and transgenic (GM) 35S(CaMV)-gshI (6lgl) tree grown in glass houses (left), micropropagated in aseptic shoot cultures (middle), and the sources of explants (petioles, nodal segments, and leaf discs) prepared for the experiments (right) [in 1].

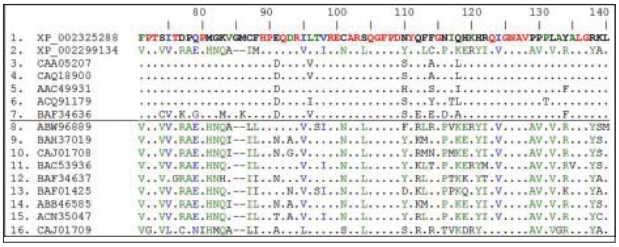

Figure 2: Partial amino acid (70-140 aa) sequence alignments of enzymes METs (DNA-METHYLTRANSFERASES, 1-7) and CMTs (CHROMOMETHYLASES, 8-16) aligned by Multalin server, (http://multalin.toulouse.inra.fr/multalin/) prior to blasting to XP_002325288 (*Populus trichocarpa*) by NCBI server (http://www.ncbi.nlm.nih.gov/). Consensus aa (*dots*) and aa-changes (color letters) are indicated. NCBI Gene Bank Accession numbers are: (1) XP_002325288 (MET, *Populus trichocarpa*, 1549 aa). (2) XP_002299134 (MET, *Populus trichocarpa*, 973 aa). (3) CAA05207 (DNA cytosine-5-MET, *Solanum lycopersicum*, 1559 aa). (4) CAQ18900 (DNA cytosine-5-MET, *Nicotiana sylvestris*, 1558 aa). (5) AAC49931 (DNA cytosine-5-MET, *Pisum sativum*, 1554 aa). (6) ACQ91179 (MET1-type, *Fragaria x ananassa*, 1557 aa). (7) BAF34636 (MET1b, *Brassica rapa*, 1519 aa). (8) ABW96889 (CMT-type MET, *Elaeis guineensis*, 925 aa). (9) BAH37019 (CMT, OsMET2a, *Oryza sativa* Japonica, 907 aa). (10) CAJ01708 (CMT1, *Hordeum vulgare*, 735 aa). (11) BAC53936 (CMT-like, *Nicotiana tabacum*, 741 aa). (12) BAF34637 (CMT, *Brassica rapa*, 805 aa). (13) BAF01425 (CMT, *Arabidopsis thaliana*, 839 aa). (14) ABB46585 (MET2a, *Oryza sativa* Japonica, 371 aa). (15) ACN35047 (CMT, *Zea mays*, 329 aa). (16) CAJ01709 (CMT2, *Hordeum vulgare*, 187 aa).

L-cysteine (γ-EC) than the WT, which led to an improved detoxification capacity against various environmental pollutants [1-4]. Both *gsh* and *gst* genes play central role in plant detoxifications.

Transgenic poplar clone studied here (6*lgl,* and *11ggs*) has been maintained in aseptic shoot cultures for about a decade without 35S(CaMV)-*gsh*I transgene elimination [in 1]. However, transgenes have been exposed to gene silencing either in the region of the constitutive 35S(CaMV) promoter, or in the coding regions. By the application of a DNA de-methylating agent DHAC in the study presented, the natural gene silencing process was reversed, and self genes and transgene were aimed to be reactivated.

MATERIALS AND METHODS

Clones of the genetically transformed (INRA 717-1-B4) lines (6*lgl* and *11ggs*) overexpressing 35S(CaMV)-*gsh*I gene (γ-glutamylcysteine synthetase; EC 6.3.2.2; cloned from *Escherichia coli*; (1.557 bp; NCBI X03954) was used in the control of WT (*Populus x canescens* = *P. tremula* x *P. alba*; 2n = 4x = 38; 4.5 to 5.5 x 10^8 bp). Shoot culture of nodal segments were micropropagated (Fig. 1) and maintained *in vitro* [1].

RT-qPCR. Relative gene expression levels of 35S(CaMV)-*gsh*I transgene (*E. coli*) and the endogenous poplar gene *gsh*1 (γ-glutamylcysteine synthetase; EC 6.3.2.2) and *gst* (glutathione S-transferase; EC 2.5.1.18) were analyzed in the control of constitutively expressed housekeeping poplar gene α-*tubulin* and *actin* [2, 3]. Total RNA was extracted from 0.05 g leaf disc tissues using the Absolutely RNA Miniprep Kit (# 400800, Stratagene, USA) following the manufacture's protocol. Three individual leaf discs were analyzed in duplicate measurements (n = 6) in each case. The quality and quantity of extracted RNA samples (2 µl) were measured by NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA) [2].

First strand cDNAs: Reverse transcription of first strand cDNA was synthesized on the *mRNA* templates by RT (reverse transcriptase of Moloney Murine Leukemia Virus: M-MuLV) with primer oligo$(dT)_{18}$ (0.5 µg) following the manufacturer's protocol (# K1622; Fermentas). First strand cDNAs (2.5 µl) were directly applied in RT-qPCR (25 µl) and probed by gene specific primers (400 nM) [2].

Primers used were as follows: for transgenic 35S(CaMV)-*gsh*I, the 5'-AGGTCAGGACATCGAACTGG-3' and 5'-GATGCACCAAACAGATAAGG-3' were used, which amplified a fragment of 273 bp of the incorporated transgene (from 667 bp to 939 bpt). Primers used for endogenous *gsh*1 (5'-AGTTCCGAGGCTGACATGAT-3' and 5'-CAGCACGGTTGTTGTCAGTA-3'), endogenous *gst* (5'-GCACAAGAAAGAGCC(A/G)TTCC-3' and 5'-AGCTCCCAGTTCAGCTTTGA-3'), α-tubulin (poplar) (5'-TAACCGCCTTGTTTCTCAGG-3' and 5'-CCTGGGGTATGGAACCAAGT-3'), and actin (poplar) (5'-AATGGTACCGGAATGGTCAA-3' and 5'-CCCAACATACGCATCCTTTT-3') were applied according to [1-5]. Kits of DyNAmo HS SybrGreenI qPCR, and Finnzymes RT-qPCR kit (# F-410L) was used. The reactions were performed by Rotor Gene 6000 cycler (Corbett Research, Australia) in forty cycles (95 °C / 20 sec, 60 °C / 20 sec, 72 °C / 20 sec) prior to a hold at 95 °C for 10 min, and a final hold at 4 °C [2, 3].

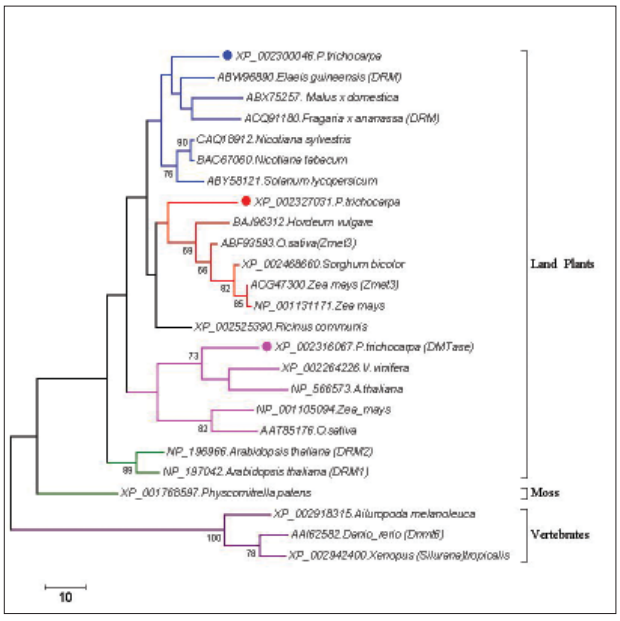

Figure 3: Protein phylogram (Maximum Likelihood) of the enzymes (consensus 654 aa) of DNA methylases (METases), METs (DNA-methyltransferases; orthologous to *Dnmt* in vertebrates), CMTs (chromomethylases) and DRMs (*de novo* domains rearranged DNA methylases) of plant and moss species compared to Vertebrates (edited by MEGA5; http://www.megasoftware.net/). Main clades, relative genetic distance (scale, 10 aa substitutions per site), branch informations of high bootstrap values (x1000 replicates), *Populus trichocarpa* (●), and the NCBI accession numbers are indicated.

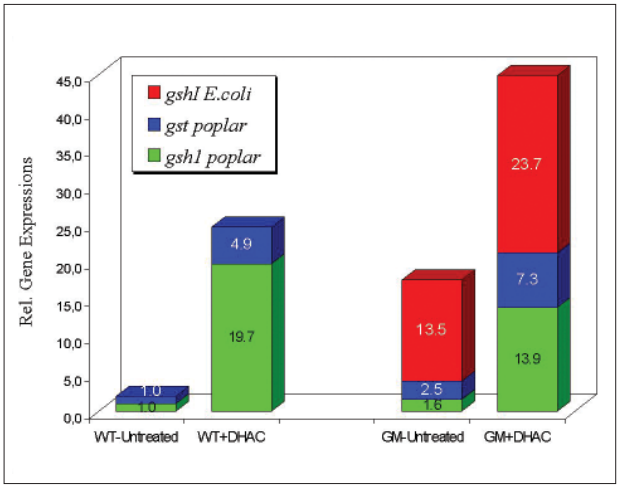

Figure 4: Cumulative gene expression levels (RT-qPCR) of *gsh*1-mRNA and *gst*-mRNA of WT (wild type); and 35S(CaMV)-*gsh*I-mRNA (cloned from *E. coli*) of transgenic (GM) poplar (*Populus x canescens*) clone *6Igl* exposed to DHAC (10^{-4} M, 7 days).

Ct values (threshold cycle): The threshold of fluorescence value (*dR*) of the amplified PCR products was determined manually above the background of fluorescence signals. Standard curve correlating Ct values to log amount of DNA were plotted at high R^2-ratio (0.976 to 0.987) [4].

Lypoxigenase (LOX) activity. Cell-free extracts of leaf tissues of GM and WT clones were prepared before and after the DHAC treatment and the enzymatic activities of lipoxygenase (LOX; EC 1.13.11.12) were detrmined at pH range 5.0 – 9.5 according to [1].

Multiple sequence alignments were analyzed *in silico* by programs of BioEdit Sequence Alignment Editor (North Carolina State University, USA; http://www.ctu.edu.vn/~dvxe/Bioinformatic/Software/BioEdit), MULTALIN (http://www-archbac.u-psud.fr/genomics/multalin), CLUSTALW (http://bioweb.pasteur.fr/seqanal/interfaces/clustalw.html), and FastPCR (www.biocenter.helsinki.fi/bi/Programs/). For BLAST (*Basic Local Alignment Search Tool*) analysis the NCBI (*National Center for Biotechnology and Information;* http://www.ncbi.nlm.nih.gov/) server was used. Molecular dendrograms were edited by using the MEGA5 program (http://www.megasoftware.net/) [5].

RESULTS AND DISCUSSION

Plant Biotechnology

The Biological Research Centre (Szeged, Hungary) can be considered as the *Genius Loci* (Smil, 2001) of the current plant biotechnology since methodologies of plant cell line selections for chloroplast mutants (Maliga et al. 1973; Garab et al. 1974), cell fusion (Dudits et al. 1976), genetic transformation (Márton et al. 1979; Koncz et al. 1984), bacterial nitrogen fixation (Kiss et al. 1980), and artificial chromosomes (Hadlaczky 2001) were either fundamentally developed or highly improved there. Following all these pioneering works the research and teaching of plant biotechnology has also started in 1986 at the St István University, Instutute of Genetics and Biotechnology with series of significant results in the past decades [in 4].

The first stable higher plant mutant, the antibiotic (*i.e.* streptomycin, SR) resistant (*i.e.* mutant) tobacco (SR1) was selected (Maliga et al. 1973) *in vitro*, followed by the selection (Maliga et al. 1975) and identification of SR1A15 (Sváb and Maliga 1986) the first double mutant of higher plants, the albino (chloroplast) and SR-resistant tobacco (Maliga et al. 1975; Páy and Smith 1988). Later, as the early forms of gene transfer, protoplast cell fusion plants (*i.e.* cybrids) were developed in several laboratories (Kao and Michayluk 1974; Melchers and Labib 1974; Power et al. 1976; Dudits et al. 1977; Medgyesi et al. 1985) [in 4].

Alternatives to the conventional haploid genome transfer (*i.e.* pollination), the technologies of gene transfer resulting in stable transgenic crops (*i.e.* GM - genetically modified, or GMO - genetically modified organism) were developed in four labs at the same time in 1983: GM *Nicotiana plumbaginifolia* (resistant to the antibiotic kanamycin) (Bevan et al. 1983), tobacco lines resistant to kanamycin and methotrexate (a drug used to treat cancer and rheumatoid arthritis) (Herrera-Estrella et al. 1983), GM petunia resistant to kanamycin (Fraley et al. 1983), and GM sunflower transformed by phaseolin gene isolated from bean (Murai et al. 1983) [in 4].

The first field trial of GM cotton was carried out in 1990, followed by the first FDA-approved (Unites States Food and Drug Administration) transgenic food of Flavr-Savr tomato in 1994 (Bruening and Lyons 2000). A series of further GM crops were released in 1995, such as the canola oil seed rape (*Brassica napus*) with modified oil compositions (Calgene), Bt (*Bacillus thuringiensis*) corn (Ciba-Geigy) resistant to the herbicide bromoxynil (Calgene), Bt cotton (Monsanto), GM soybeans resistant to herbicide glyphosate (Monsanto); virus-resistant squash (Asgrow), and delayed ripening tomatoes (DNAP, Zeneca/Peto and Monsanto) (Conner et al. 2003). Later, a series of woody plants were also bred by genetic transformation (Arisi et al. 1997; Noctor et al. 1998; Bittsánszky et al. 2005; Gyulai et al. 2012, 2014) [in 4].

Here we present a case study of the upregulation of the 35S(CaMV)-*gsh*I transgene in poplar (*Populus x canescens*) compared with endogenous poplar gene expressions of *gsh*1 and *gst*.

DNA de/methylation

DNA de/methylation is a natural enzymatic process of TGS (Transcriptional Gene Silencing) catalyzed by DNA methyltransferase enzymes, which results in the meiotically heritable methylation pattern (*i.e.* inprints). DNA methylation

is not universal, as in the insect fruit fly *Drosophyla* has not been detected.

In *Arabidopsis*, there are at least three classes of DNA methyltransferases (METases), which catalyze asymmetric DNA methylation. These are METs (maintenance methyltranferases), CMTs (chromomethylase3) and DRMs (de novo domains rearranged dna methylases) [1]. MET1 genes are similar in sequences and homologues in functions to mammalian DNMT1. CMT3 is specific to the plant kingdom and contains a chromo domain. CMTs transfer a methyl group (CH_3) from S-adenosyl methionine (AdoMet-dependent methyltransferases) mainly to the position of cytosine-C_5 (EC 2.1.1.73), cytosine-N_4 (E.C. 2.1.1.13); and adenine-N6 by adenine DNA methyltransferases (E.C. 2.1.2.72). The first eukaryotic adenine DNA methyltransferase was isolated from plants (wheat) and were found mainly responsible for the methylation of mitochondrial DNA [in 1]. The protein sequence analyses revealed an extreme molecular diversity of DNA methylases with indications for the possibility of site specific (single gene directed) DNA demethylation (Fig. 2, 3).

The DRM class of genes includes DRM1 (624 amino acid - aa) and DRM2 (626 aa) (syn. DNA-METase) (both EC 2.1.1.37) and contain catalytic domains which shows sequence similarity to mammalian *de novo* DNMT3 [1]. The conversion of 5-methylcytosine to 5-hydroxymethylcytosine was discovered recently in mammalian DNA [in 1]. In *Arabidopsis,* the same enzyme (DRM2) can methylate both cytosine and adenine nucleotides [in 1].

A process of RdDM (siRNA and micro-RNA directed DNA methylation) might also occur in eukaryotes which was also observed first in plants [in 1].

Triggering of DNA methylation

Gene expression *vs.* TGS can be triggered *in vitro* by up/down (syn.: re/activation, hypo/hyper/de-methylation) regulation of genes of DNA methylase [1-5]. For induced gene up-regulation, MTase-inhibitors such as the structurally modified cytosine analogues *zebularine*, *5-azacytidine* (5-azaC), *5-aza-2'-deoxycytidine* (5-azadC) and *DHAC* have been shown to be highly effective [1]. Alternatively, the drug *3-aminobenzamide* has been used for gene down-regulation in a series of genes. Gene reactivation through the application of thymidine analogues can also occur in demethylation-independent gene up-regulations [in 3]. These exogenously applied DNA de-methylating agents act via covalent complex formation when present either in the cytosol or when incorporated into DNA as DNA base analogues.

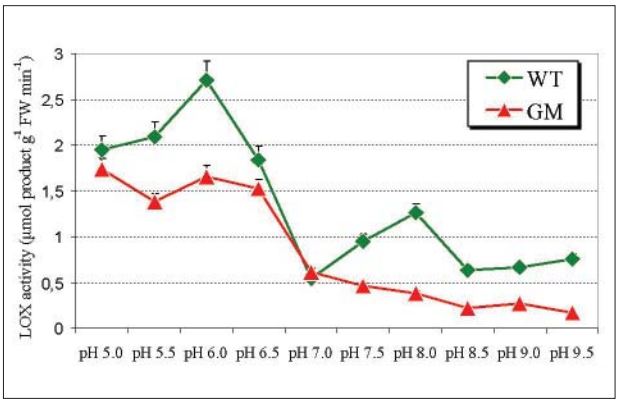

Figure 5: Lipoxygenase (LOX) activity of DHAC (10^{-4} M, 7 days) treated WT and transgenic (GM) 35S(CaMV)-*gsh*I poplar (*Populus* x *canescens*) clone *6lgl* at the range of pH 5.0-9.5 [in 1].

The study presented here aimed to upregulate simultaneously both the prokaryotic tansgene *gsh*I and the endogenous eukaryotic poplar gene *gsh*1 and *gst* in clones of WT and 35S(CaMV)-*gsh*I (*6lgl* and *11ggs*) after DHAC-treatment (10^{-4} M, for 7 days).

Reverse transcription (RT) followed by qPCR analysis was found to be an exceptionally sensitive method for gene expression analyses compared to RNA-DNA hybridizations (*i.e.* Northern blots) in both cases of absolute and relative quantification [3]. In the study presented, relative quantification was used as it is more relevant than absolute quantification used to compare expression levels of different treatments.

The results showed, that gene expression of transgene 35S(CaMV)-*gsh*I increased from a high rel. expression level (13.5 rel. unit) to 23.7 (rel. unit) with about a two-fold increment compared to the endogenosus *gsh*1 gene expression which increased from a lower level (1.3 rel. unit) to 13.9 (rel. unit) with an 8.7-fold increment after DHAC-treatment (Fig. 4).

Gene expression of the endogenous poplar gene *gsh*1 of the WT clone showed also high responsiveness to DHAC-treatment with an extremely high expression (19.8-fold increase). This result indicates a difference in DNA methylating capacity between transgenes and proper poplar self genes as a type of cosuppression [1]. The endogenous poplar *gst* gene also showed DHAC-inducibility with a 4.9-fold (WT) and a 2.9-fold increase (from 2.5 rel. unit to 7.3 in the GM-clone).

Increased levels of *gsh*1-*mRNA* (syn.: γ-ECS-*mRNA*) have also been reported in *Brassica juncea*, *Brassica napus*, *Arabidopsis thaliana* exposed to cadmium stress [in 1]. The moss *Physcomitrella patens* also showed a high level of γ-ECS overexpression (5.7 to 7.9-fold increase) in response to 10 µM Cd^{2+} [in 1].

For functional analysis, enzyme activities of lipoxygenase (LOX, EC 1.13.11.34), which catalyzes the conversion of arachidonic acid to 5-HPETE were measured. The LOX activity was higher in the DHAC-treated WT clones than in the transgenic clone *6lgl* at all pH levels (Fig. 5). The curves with two pH optima between the range of pH 5.0-9.5 indicated that two LOX izozymes are present in poplar. The promoter region of *lox* gene was found to be downregulated by DNA methylation in human U937

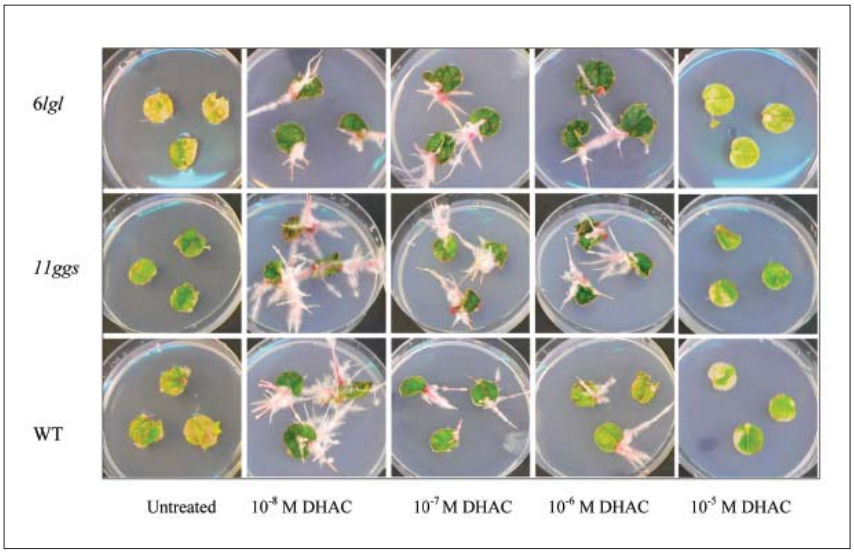

Figure 6: Root initiation capacity of DHAC (10^{-8} M to 10^{-6} M) on leaf discs of untransformed (WT) and 35S-*gsh*I-transformed poplar (*Populus* × *canescens*) clones (*6lgl* and *11ggs*) incubated in long-term (21 days) aseptic cultures of agar media [in 5].

and HL-60TB cells [in 1], however in our experiments LOX activity measured at pH 6 and pH 8 in DHAC (10^{-4}M and 10^{-5} M) treated poplar did not show concentration dependent increase (detailed elsewhere).

A *de novo* root and rare shoot development was also observed on DHAC-treated poplar leaf discs incubated for long term (21 days) DHAC treatment. This result indicates a multi-target action of DHAC, especially at the auxin-related root initiating genes (Fig. 6). Plant morphogenetic capacity of DNA de-methylating agents has also been reported. Flower development of *Arabidopsis* and shoot induction of *Petunia* were initiated by DNA de-methylating agents [in 5]. Early flower bud development (vernalization, *i.e.* 'remembering winter') in *Arabidopsis* was found to be coupled with low levels of DNA methylation. Low level of methyl-cytosine were also found associated with organogenetic capability of sugarbeet (*Beta vulgaris* L. *altissima*). Contrary to the induction of morphogenesis, increased DNA methylation levels occurred during bud dormancy [in 1, 5]. Methylation also plays a key role in the chromosome re/modelling as it turned out that the Pc-G (Polycomb Group) protein complexes, encoded by *pcg-g* genes (polycomb group genes), regulate plant flowering. These genes are orthologue to PRC2 (Polycomb Repressive Complex 2) in animals, and functions as histone methyltransferases [in 5].

CONCLUSIONS

In conclusion, since DNA methylation patterns are inherited in the vegetative clones (*i.e.* epigenetic memory), the DHAC-treated WT poplars regenerated from leaf discs and with increased gene expression levels of endogenous genes *gsh*1 and *gst* might provide novel plant resources for the application to air and soil detoxification and remediation, and prove acceptable to the general public concerned about GMOs and the recently changing global environment.

ACKNOWLEDGMENTS

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PROGRAMME

18-19 March, 2015

Day 1	Section	Time	Theme	Presenter
Springtime harvesting	***Production, ensilage and conservation of forages***	9:00-10:00	**Harvesting:** Current situations and actualities of rye harvesting.	Ferenc Iván
		10:00-11:00	**Harvesting :** 1. Springtime harvesting of winter crop mixtures in the aspect of cost-efficiency of milk production. 2. Actualities of sawing springtime-mixtures (possibilities to fit the pea or vetch in crop rotation).	Dr. Szilvia Orosz
	Demonstration	11:00-12:00	Precise feeding solutions 'in time - on farm' moisture evaluation. Demonstration of a digital moisture measurement method (Digi-Star).	Harm Franssen
		12:00-13:00	**Crop production:** producing soybean and pea.	Helga Bosnyák-Egri
	Presentation of experts	14:00-15:00	The field practice of harvesting good quality forages.	Ferenc Filátz
	Mycotoxin-prevention on the field	15:00-16:00	Pest control of the corn in the aspect of the breeder.	István Hevesi (pig breeder)
		16:00-17:00	Up-to-date technologies (soil injection and stable treatment).	Péter Vajda
	Dairy nutrition	17:00-18:00	The role of carbohydrates in the dairy nutrition.	Dr. László Dégen
Day 2	***Breeding and health***	9:00-10:00	Presentation organized by the Hungarian Holstein Association.	International presenter
		10:00-11:00	Presentation organized by the Hungarian Holstein Association.	Hungarian presenter
		11:00-12:00	Actualities in Bluetongue situation in Hungary.	Dr. Tamás Abonyi
		12:00-13:00	Reproduction management in a herd of 2500 dairy cows.	Dr. Levente Kátai

03-04 June, 2015

Day 3	Section	Time	Theme	Presenter
Summertime harvesting	***Production, ensilage and conservation of forages***	9:00-10:00	**Crop production:** Actualities in rye and winter crop mixtures (making arrangements for sowing).	Dr. Richárd Hoffmann
		10:00-11:00	**Crop production:** Intensive grass management in dry/hot conditions.	Prof. Chris Dannhauser
		11:00-12:00	**Crop production:** Joint growing of legumes with cereals in dry/hot climate.	Prof. Chris Dannhauser
		12:00-13:00	**Harvesting-conservation:** Technological aspects of harvesting the silo maize Ensiling of silo maize.	Dr. Szilvia Orosz
Feeding in summertime	***Presentation of experts***	14:00-15:00	The feeding practice of high milking dairy cows in summer. (Komárom Mg Ltd., Csém)	Ferenc Mészáros
	Dairy nutrition	15.00-16.30	Recent research development of dairy cow's nutrition management under heat stress.	Dr. Jan van Eys
		16.30-17.30	Problem solving of low milk fat production in Dairy cow.	Dr. László Dégen
Day 4	***Breeding and health***	9:00-10:00	Presentation organized by the Hungarian Holstein Association.	International presenter
		10:00-11:00	Presentation organized by the Hungarian Holstein Association.	Hungarian presenter
		11:00-12:00	Cattle hoof trimming.	Dr. János Lehoczky
		12:00-13:00	The effect of the heat stress on reproduction, based on PAG results.	Dr. Attila Monostori

07-08 October, 2015.

Day 5	Section	Time	Theme	Presenter
	Production, ensilage and conservation of forages	9:00-10:00	**Crop production:** Actualities in winter grains and roughages.	Dr. Richárd Hoffmann
		10:00-11:00	**Conservation:** Aerobe stability.	Prof. Limin Kung
		11:00-12:00	**Conservation:** The fermentation and aerobe stability of maize silage. Homo- and heterofermentative bacteria, as the silage additive. Silo wall protection in the USA.	Prof. Limin Kung
		12:00-13:00	**Conservation:** Partial results of maize silage of 2015.	Dr. Szilvia Orosz
	Presentation of experts	14:00-15:00	The long term practical consequences of the most important 90 days around calving (close up, parturition and involution) in large dairy herd.	Dr. Petra Bérdi Dr. Levente Kátai
	Dairy nutrition	15:00-16:00	Up-to-date nutrition management of high milking dairy herds in the USA (focusing on cost-efficiency).	Corwin Holtz
		16:00-17:00	Up-to-date nutrition management of high milking dairy herds in the USA (focusing on reproductive performance).	Corwin Holtz
		17:00-18:00	The nutrition management background of retained placenta.	Dr. László Dégen
Day 6	***Breeding and health***	9:00-10:00	Presentation organized by the Hungarian Holstein Association.	International presenter
		10:00-11:00	Presentation organized by the Hungarian Holstein Association.	Hungarian presenter
		11:00-12:00	Elimination of *Staphylococcus aureus* in the practice.	Dr. János Tibold Dr. Péter Kovács
		12:00-13:00	Introduction of the milk based ketosis monitoring system in Hungary.	Dr. Attila Monostori

25-26 November, 2015

Day 7	Section	Time	Theme	Presenter
	TMR-technology	9:00-10:00	Recent updates of the nutrition content of TMRs, based on Hungarian (ÁT Ltd.) monitoring results.	Dr. Szilvia Orosz
		10:00-11:00	Physical structure of TMRs (inhomogenity, sorting, supply of effective fiber, DMI and fiber intake and milk production).	Dr. Szilvia Orosz
		11:00-12:00	Technical background of feedout and feeding management (management of silo surface, scaling order, mixing time and the TMR wagons).	Tibor Barkóczi
		12:00-13:00	Technical background of preparing dairy feeds.	Tibor Barkóczi
	Presentation of experts	14:00-15:00	Calf rearing on Vajhát Farm (Hódmezőgazda Ltd.).	Péter Toldi
	Dairy nutrition	15.00-16.30	Nutrition management and the consequences for the dairy cow's longevity and performance.	Dr. Jan van Eys
		16.30-17.30	The nutrition management background of ketosis.	Dr. László Dégen
Day 8	***Breeding and health***	9:00-10:00	Presentation organized by the Hungarian Holstein Association.	International presenter
		10:00-11:00	Presentation organized by the Hungarian Holstein Association.	Hungarian presenter
		11:00-12:00	Downer cow syndrome.	Dr. Tamás Varga
		12:00-13:00	The Hungarian animal health administration update in the diary sector.	Dr. Lajos Bognár

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