Genetic engineering to improve β-carotene content in pepper

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ABSTRACT

A laboratory study was conducted to develop a broadly applicable *in vitro* regeneration and transformation methods for increasing β-carotene content in pepper fruits. Therefore, two different pepper genotypes were analyzed with regard to their efficiency for regeneration and genetic transformation *in vitro*. Cotyledons were used *in vitro* from young seedlings as target tissue for regeneration and transformation of pepper. Over expression of *β-Lcy* gene via Agrobacterium-mediated transformation was used. The presence of the transgene was assayed on leaf DNA via PCR and biochemical analysis using HPLC. Results strongly showed that there were significant differences between pepper genotypes. The transgenic pepper plants showed a significant increase in fruit β-carotene content reaching 7 to 10 folds according to the genotype. The fruits from these plants displayed different colour phenotypes, from orange to orange-red. The difference in the fruit colour phenotypes reflects the beta-carotene ratio, which ranges from 4.5 mg/100g dw in the wild plants to 45.0 in the transgenic plants of genotype Balady during maturity stage. Concerning to the genotype Topepo rosso, the β-carotene content was increased from 5.2 mg/100g dw in the wild plants to 36.39 mg/100g dw in the transgenic plants during mature stage.

Key words: pepper, *in vitro* regeneration, genetic transformation, *β-Lcy*, carotenoids.

Introduction

Pepper (*Capsicum annuum* L.) fruits are one of the most important vegetables in the human diet in Egypt and many other regions around the world (Slavin and Liloyd, 2012; El Nagar and Mekawi, 2015; Tomlekova et al., 2017). Increasing population, inadequate food and nutrition, hunger, malnourishment of vitamins, micronutrients and macronutrients are the biggest challenges to address most of the nations across the world. Vitamin A deficiency predominant in developing countries among children and women which leads to more than 600, 000 deaths each year globally among children less than 5 year of age (Gomathi et al., 2017). Inadequate availability of these important vitamins resulted in many health and physical disorders in human beings (West, 2003; Mason et al., 2005; WHO, 2009). Dietary intake of carotenoids can mitigate against these risks (Von Lintig, 2010). Pepper fruits can often be a major dietary source of carotenoids, especially β-carotene (precursors of Vitamin A) (O'Neill et al., 2001; Chaiter et al., 2007; Biehler et al., 2011). There is considerable natural variation in β-carotene concentrations in pepper fruit (Wall et al., 2001; Ha et al., 2007; Rodriguez-Uribe et al., 2012, El Nagar and Mekawi, 2015). Thus it becomes pertinent to study these variations in different genotypes during maturity to select the best for health benefits and to improve it using genetic transformation methods (Marín *et al.*, 2004; Matsufuji *et al.*, 2007; El Nagar and Mekawi, 2015).

Metabolic engineering is one of the possible approaches to improve the levels of vitamins in plants (Giuliano, 2017). Already cloning of the carotenoid pathway gene has been demonstrated in the endosperm of rice, a major staple food in areas at high risk of vitamin A deficiency (Ye et al., 2000). The dose reachable with this engineered rice (2 mg beta-carotene/kg dry rice endosperm) which is insufficient to reach the Recommended Dietary Allowance to prevent severe vitamin A deficiency. The US Recommended Dietary Allowance is 1mg/day retinol equivalents (approximately 6 mg beta-carotene equivalents). Therefore, further engineering efforts in edible plants are required to provide optimal vitamin A supplementation from diversified sources.

Although, significant progress has been made in the field of *Capsicum* improvement using conventional breeding techniques, it still suffers from some limitations for the introduction of new alleles. Genetic transformation holds great potential to overcome constraints like limited gene pool and species barrier posed by conventional breeding methods. As compared to other solanaceous crops which have been used as model plant systems, *Capsicum* has always been less responsive towards regeneration and transformation (Christopher and Rajam, 1997; Manoharan et al., 1998; Wolf et al., 2001, Chen, et al., 2003; Lee et al., 2009; Kothari et al., 2010; El Nagar 2012; Verma et al., 2013). Genetic engineering of Capsicum, with regard to enhanced carotenoid production, will be an area of commercial importance (Giuliano, 2017).

It is therefore, imperative to study the changes in the content of Beta carotene and total carotenoids as influenced by genetic transformation in pepper. Therefore, the aim of the present work was to introduce the Arabidopsis lycopene beta-cyclase (*β-Lcy)* gene via Agrobacterium-mediated transformation to produce high β-carotene concentrations in the fruits of two pepper genotypes.

Materials and Methods

The present experiment was conducted in the Biotechnology Laboratory, Research Park, and in the Vegetable farm, Faculty of Agriculture, Benha University, Egypt to evaluate the carotenoids content in fruits obtained from *in vitro* regeneration and transformation of two pepper genotypes.

Plant material:

Mature seeds of two pepper genotypes were used to raise seedlings for the present study. Seeds of the local genotype Balady, and Topepo rosso, were obtained from the Preservation Germplasm Laboratory of the Department of Horticulture, Faculty of Agriculture, Benha University, Egypt. The three genotypes had red fruits at ripening stage.

***In vitro* plant regeneration and genetic**

Transformation system

I*n vitro* regeneration experiments of pepper were performed as described previously (El Nagar and Mekawi, 2015). Explants of cotyledon of two pepper genotypes were taken from aseptic plants 10 days old after *in vitro* germination of the seed. Cotyledons divided into 1-cm pieces and cultured horizontally on the MS medium (Murashige and Skoog, 1962) supplemented with 0.57 µM IAA, 22.81 µM Zeatin and 10.0 µM $AgNO_3$ for 2 days. *Agrobacterium tumefaciens* strain GV3101 with the binary plasmid pBI121 was used for transformation. It contains a selectable marker gene nptII encoding the enzyme neomycin phosphotransferase conferring kanamycin resistance (Pridmore, 1987) and the *β-Lcy* gene under the control of the pepper Pds promoter. The constructs were introduced into explants of cotyledon of two pepper genotypes using previously published techniques (El Nagar, 2012). The explants of cotyledons were incubated in the Agrobacterium suspension in a small petri plate for an additional period of 30 min. They were then blotted dry on a sterilized Whatman filter paper and cocultured in petri plates on MS medium with 0.57 µM IAA, 22.81 µM Zeatin and 10.0 µM $AgNO_3$ for two days in the dark. Following co-culture the explants were washed several washed times in liquid MS medium with gentle shaking until no opaque suspension was seen. The infected explants were finally dried with a sterile Whatman filter paper and placed on the shoot induction media (MS medium supplemented with 0.28 µM IAA and 13.86µM Zeatin). The infected explants were then placed in the growth room for regeneration under 16/8 hours light/dark cycle at 25 ± 1ºC. To eliminate non transformed tissues, the regenerating explants were sub-cultured on a fresh regeneration medium initially with 35mg/l kanamycin after two weeks. The concentration of selection antibiotic was increased with each subculture at two weeks intervals up to 100mg/l kanamycin. During each subculture the dead and deep brown tissues were discarded and green shoots and shoot buds were sub- cultured on elongation medium (MS media contained 0.27 µM NAA and 9.12 µM Zeatin) containing the next higher concentration of kanamycin. Those cultures were transferred to fresh medium every second week. For rooting and adaptation, shoots with a length of at least 2 cm were transferred to root induction medium (½ MS-medium).

Acclimatization and field transfer

Regenerated putative transgenic shoots with well-developed leaves and roots were transferred to pots containing soil with high relative humidity and maintained in the growth room under 12 h light photoperiod at 25ºC. Plantlets were watered twice a week with water. Then, plantlets were transferred into greenhouse. Plants grew to maturity, producing flowers and fruits. Seed viability from ripening fruits was tested in soil in the greenhouse and on germination medium *in vitro*.

All peppers received similar water and fertilizer treatments based on the recommendation of Egyptian Ministry of Agriculture. Fruits of two pepper genotypes were harvested at the same time but at three successive maturity stages viz. green (fruits showed characteristic green colour), intermediate (50% of the fruit showed transition from green to red) and finally at full maturity stage (bright red colour). Immediately after harvest, the fruits were placed in polyethylene bags and were then stored at -20 °C until analyzed. Quantitative analysis was carried out for total carotenoids and β-carotene contents.

Molecular Analysis:

For the examination of the Arabidopsis lycopene beta-cyclase (*β-Lcy)* in putative transgenic pepper plants, DNA was isolated from young leaves as described by (El Nagar, 2012). The PCR reaction was typically carried out in a 25 µl reaction volume with the following constituents: 10-50 ng template DNA, 5 pmole forward primer (5´TCATCAACAGTTTTTTACAAAAGAAATG-3´), 5 pmole reverse primer (5´AACACACTACATTACATTATTGATAACA-3´), 0.2 mM dNTPs, 5 µl of 5x Taq buffer, 2 U Taq-polymerase and H_2O up to 25 µl. The amplification conditions were done in a PCR thermocycler using 94º C for 3 min; 10 cycles at 94º C for 30 s, 60º C (-

0.5º C per cycle) for 30 s, 72º C for 45 s; 25 cycles at 94º C for 30 s, 72º C for 45 s. The program was terminated by a final extension step at 72º C for 10 min. The amplification products were analyzed by electrophoresis on 1% agarose gels and visualized with ethidium bromide using standard procedures (Sambrook et al., 1989).

Biochemical analysis

For biochemical analysis, samples from three different fruits from each transgenic and wild plant, harvested from different ripening stages, were subjected to HPLC analysis of carotenoids.

The extraction of carotenoids was carried out according to the method described by Mınguez-Mosquera and Hornero-Mendez (1993). A known weight dry samples were milled in a coffee grinder and 2 g of obtained powder sample was extracted with acetone in mortar and pestle. Extractions were repeated until the complete exhaustion of colour (usually 4–5 extractions were enough). All extractions were pooled in a separating funnel and shaken with diethyl ether. A sufficient quantity of 10% NaCl was added at the end to facilitate separation of the two phases. Aqueous phase was discarded. The lipophilic phase was washed with 100 ml of an anhydrous Na2SO4 (2%) solution to remove all the remaining water. It was saponified with the addition of 40 ml of 10% KOH in methanol and shaken vigorously before being left in a dark place for 1 h. After addition of water, the pigments were subsequently extracted with diethyl ether, evaporated in a rotary evaporator and then made up to 25 ml with acetone. One milliliter aliquot of this solution was centrifuged at 12,000 rpm and stored at -20 °C until analysed. Losses occurring during the process were monitored with the use of all-transb-apo-80-carotenal as internal standard. All analysis was carried out in triplicate. Because of no availability of standards for different carotenoids, total carotenoids were estimated by taking the absorbance of extracts at 450 nm (Ranganna, 1986). However, the separation and quantification of β-carotene was carried out by the method of Chávez-Mendoza (2013) using C18-type column (Hypersil ODS C18) of 4.6 mm × 15 cm. Chromatographic analyses were carried out using a young lin HPLC, series YL-9100, equipped with a quaternary pump, an autosampler (YL9150), a degasser, and a YL-9160 spectrophotometric detector (Photo Diode Array detector–PDA), which was set at 455 nm. The solvent system consisted of acetonitrile/THF/H2O (85:12.5:2.5). The analyses were made at 24 °C. The final results were expressed as mg/100 g dry weight *Capsicum* tissue.

Experimental design and Statistical analysis

Experiments were arranged in a completely randomized block design with 3 replications. Data were estimated as the mean and its standard error of the different traits. The calculations were done using Microsoft Excel 2010 program.

Results and discussion

***In vitro* regeneration and genetic transformation of pepper**

According to previous research (El Nagar and Mekawi, 2015) screening of different pepper genotypes for their carotenoids content in fruits in various ripening stages, cvs Balady and Topepo rosso were chosen for improving β carotene content in current study. Differences were observed between genotypes after ten days of culture on germinating agar medium. The germination rate of genotype Topepo rosso and Balady were 75.0% and 65.0%, respectively (Table 1). Explants of cotyledons of two pepper genotypes under study regenerated shoots on shoot induction medium. The *Agrobacterium tumefaciens* strain GV3101 used in this study has *β-Lcy* gene and *nptII* gene and this gene confers kanamycin resistance to the transformed cells. Therefore, selection of the transformants was carried out using various concentrations of kanamycin. It was observed that co-cultivated explants of cotyledon, even in the presence of lower concentrations of kanamycin in regeneration medium, failed to regenerate and consequently died. This observation was similar to the results obtained by Prematilake et al., 2002; Sarker et al., 2009. Therefore, selection pressure was not applied immediately after co-cultivation. Co-cultivated explants were first allowed to regenerate in regeneration media without any selective agents. After one week the infected cotyledon explants showing very small shoots and shoot buds were subjected to selection pressure. All control shoots died in the selection medium with 100mg/l kanamycin. In this investigation, a lower concentration of kanamycin (35 mg/l) was applied in the initial selection medium and selection pressure was increased gradually in subsequent subcultures. For selection, 100mg/l kanamycin was reported to be suitable in obtaining transformed pepper shoot. Genotype Balady regenerated shoots at highest rates of 35.0% while inoculated explants with Agrobacterium suspension regenerated 4% shoots. Genotype Topepo rosso formed 44% shoots while inoculated explants with Agrobacterium suspension regenerated 6% shoots (Table 1). Explants of two pepper genotypes initiated multiple shoots. Genotype Balady developed the highest number of shoots per explant of cotyledon with a mean value of 4.8 and 3.1 shoots for control and inoculated explants with Agrobacterium, respectively. The percentage of shoot formation was observed for genotype Topepo rosso 2.9 and 2.3 shoots per explant for control and inoculated explants with Agrobacterium, respectively (Table 1). Putative transgenic shoots from genotype Topepo rosso were the best of shoot elongation (6%)

as well as rooted shoots (100%) following by putative transgenic shoots of genotype Balady (3%) and 100% on rooting medium (Table 1). Successfully rooted plants generally grew well under greenhouse conditions. Using our optimized procedure, we repeatedly regenerated mature transgenic pepper plants from two different *Capsicum* genotypes using Agrobacterium mediated transformation (Table 1). These plants showed no significant alteration of growth habit or leaf colour phenotype, and provided normal fruit set. Among the overexpression transformants, many showed an altered fruit colour phenotype, varying from the red of the parental Balady and to bright orange of Topepo rosso. The low efficiency of plant regeneration and transformation by pepper cultures has been widely reported (Steinitz *et al*., 1999; Li et al., 2003; Sharma et al., 2006; El Nagar 2012; Verma et al., 2013; Orlinska and Nowaczyk, 2015).

Table 1. Comparison of the regeneration and transformation frequencies of two pepper genotypes during different stages of *in vitro* cultivation.

Genotypes	Germination of seed (%)	Induced shoots (%)	Mean ± SE of shoots per explant	Elongated shoots (%)	Rooted shoots (%)	Mature plants (%)	Transgenic plants (%)
Balady (wild type)	65	35	4.8±0.3	58	90	88	-
Balady (T0)	65	4	3.1±0.2	3	100	-	3
Topepo rosso (wild type)	75	44	2.9±0.2	25	55	53	-
Topepo rosso (T0)	75	6	2.3±0.2	6	100	-	6

Detection of *β-Lcy* Expression Patterns by PCR:

To modify *β-Lcy* gene expression in pepper fruits, the overexpression of Arabidopsis *β-Lcy* gene under the control of the pepper *Pds* promoter was used. The construct was introduced into pepper (cvs. Balady and Topepo rosso) via Agrobacterium-mediated transformation and primary transformants were brought to maturity in the greenhouse. The presence of the transgene was assayed on leaf DNA via PCR. Only PCR-positive euploid plants were subjected to further studies. For the present study three and six T0 overexpressed transgenic pepper plants of Balady and Topepo rosso were obtained respectively (Table 1). *β-Lcy* expression was assayed, by PCR (Figure 1 a and b). The presence of an easily identifiable marker linked to an agronomically desirable gene would facilitate efficient selection in pepper which consider one of the recalcitrant crop for genetic transformation (Verma et al., 2013).

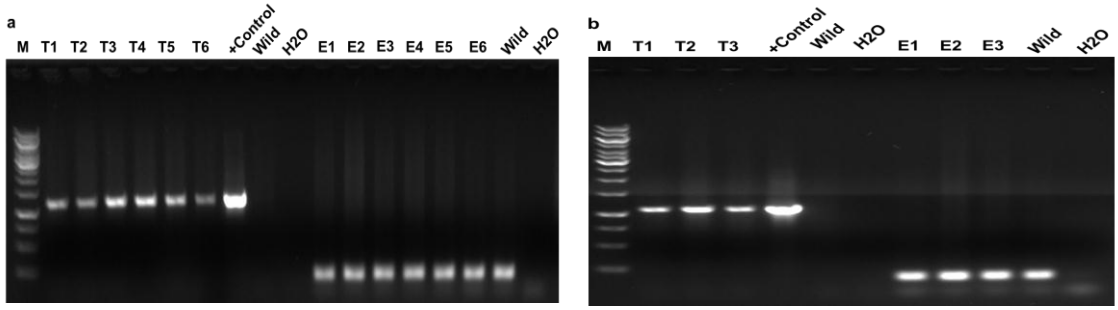

Figure 1 (a-b): Molecular characterization of putative transgenic plantlets of two pepper genotypes.

(a) PCR analysis showing amplification of (0.75kb) *npt*-II specific fragment in regenerated transgenic plantlets of pepper genotype Topepo rosso. Lane M: DNA Marker (GeneRuler^{TM}1Kb DNA ladder); T1-T6: independent lineages of putative transgenic plantlets; Lane + control: positive control (pBI121plasmid DNA); Lane wild: negative control from genomic pepper DNA (untransformed pepper) ; Lane H_2O: negative control (H_2O).; Lanes E1- E6: positive control with specific primers for genomic pepper DNA.

(b)) PCR analysis showing amplification of (0.75kb) *npt*-II specific fragment in regenerated transgenic plantlets of pepper genotype Balady. Lane M: DNA Marker (GeneRuler^{TM} 1Kb DNA ladder); T1-T3: independent lineages of putative transgenic plantlets; Lane + control: positive control (pBI121plasmid DNA); Lane wild: negative control from genomic pepper DNA (untransformed pepper) ; Lane H_2O: negative control (H_2O).; Lanes E1- E3: positive control with specific primers for genomic pepper DNA.

Total carotenoids and β-carotene:

Since 1999, over 90 papers have been published on carotenoid metabolic engineering in plants. The

main enzymatic steps engineered and the relative crops were reported in the publication of Giuliano, 2017. The different strategies used have been the subject of several reviews (Giuliano et al., 2008, Farre et al., 2011 and Giuliano, 2014).

Results in figure 2 showed that total carotenoid content (dry weight) of wild pepper plants ranged from 8 mg/100 g in Balady to 45 mg/100 g in Topepo rosso at the green stage. There was sharp increase in carotenoid content with maturity and at the red stage. Topepo rosso showed the highest content of 134 mg/100 g while Balady had the least (13 mg/100) (Figure 2). Thus with advancing maturity a maximum of 3 fold variation was observed in genotype Topepo rosso (Figure 2). Concerning to total carotenoid content (dry weight) of transgenic pepper plants, fruits of Topepo rosso genotype had 315 and 938 mg/100 g at the green and red stages, respectively (Figure 2). Fruits of transgenic plants of Balady genotype showed 80 mg/100 g at green stage while at ripening stage increased to 130 mg/100 g dw (Figure 2).

β-carotene content (dry weight) ranged from a lowest of 0.50 mg /100 g in Balady to 1.65 mg/100 g in Topepo rosso at the green stage (Figure 2). At the red stage it ranged from 4.50 mg/100 g in Balady to 5.29 mg/100g in Topepo rosso. β-carotene content increased with advancing maturity. Significant differences in β-carotene content with respect to different maturity stages were observed in genotypes, Topepo rosso, and Balady (Figure 2). Dramatic increase (13 fold) in β-carotene content was observed in Balady, during maturity (Figure 2).

Regarding to the transgenic pepper plants, six of genotype Topepo rosso and three of genotype Balady overexpression transformants showed high levels of expression of the At *β-Lcy* transgene (Figure 2). All six transgenic plants of genotype Topepo rosso showed increased β-carotene content reaching seven fold while all three transformants of genotype Balady had β-carotene content, reaching ten fold (Figure 2). These transgenic plants displayed different fruit colour phenotypes from 'orange –red' of genotype Balady to 'light red' of genotype Topepo rosso. The difference in the fruit colour phenotypes reflects the beta-carotene ratio, which ranges from 4.5 mg/100g dw in the wild plants to 45.0 in the transgenic plants of genotype Balady during maturity stage. Concerning to the genotype Topepo rosso, the β-carotene content was increased from 5.2 mg/100g dw in the wild plants to 36.39 mg/100g dw in the transgenic plants during mature stage (Figure 2).

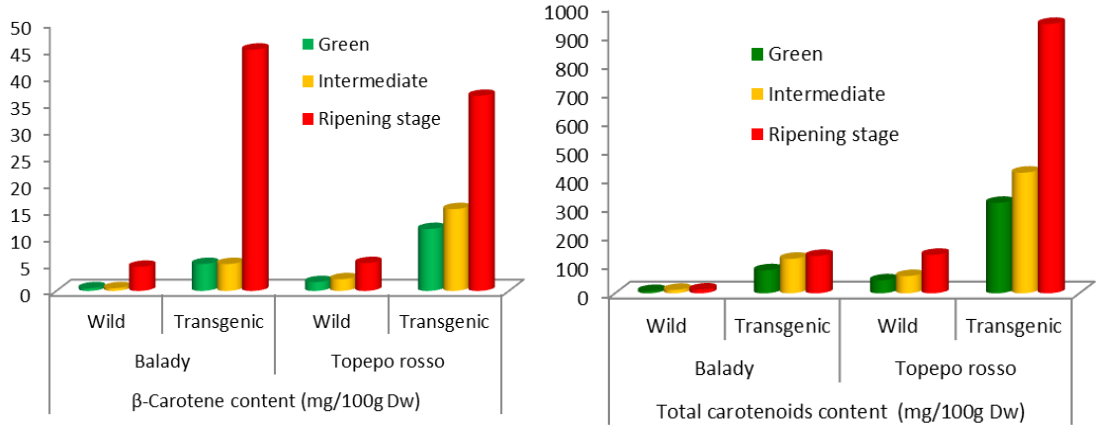

Figure 2: β-carotene and total carotenoids content in wild and transgenic pepper fruits during different maturity stages.

Over-expression of the *β-Lcy* in pepper fruit under the control of the Pds promoter results in the accumulation of beta-carotene. This result, along with that of Romer et al. (2000), paves the way for increasing the pro-vitamin A content of tomato fruits without the long breeding efforts. It also opens the possibility for the metabolic engineering of compounds further downstream in the carotenoid pathway, for which 'natural' accumulating mutants or genotypes are not available. An elegant example of this type of metabolic engineering is provided by the overexpression of the Hematococcus pluvialis ketolase in tobacco under the control of the Pds promoter (Mann et al., 2000). Carotenogenic enzymes have been proposed to be present in multi-enzyme aggregates (Cunningham and Gantt, 1998). Therefore, the alteration of the levels of one enzyme may affect the activity of other enzymes in the complex. This hypothesis may explain the unexpected increase in beta-carotene levels found in crtl overexpressiors of rice (Ye et al., 2000) or tomato (Roemer et al., 2000). A more detailed discussion of the possible regulatory phenomena occurring in crt/ transformants can be found in Giuliano et al., 2000.

Conclusion

The present study demonstrates a simple and promising protocol for *in vitro* plantlet regeneration and transformation of *C. annuum* L. from cotyledon explants to increase β-carotene content (precursor of vitamin A). Transgenic pepper plants increased β-carotene content in fruits reaching 7 to 10 folds according to the genotype. Genetically modified plants have the potential to solve many of the world's hunger and malnutrition problems, especially in developing countries, where vitamin A deficiency poses serious public health concerns and is responsible for various diseases and mortality.

Acknowledgement:

We would like to thank Scientific Research Fund-Benha University, Egypt for providing financial support to the project. A special thank goes to Prof. Dr. G. Giuliano, Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), Casaccia Research Center, Roma, Italy for providing the At *β-Lcy* gene .

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