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The Induction of Lycopene in Germinating Cottonseed with 2-(4-Methylphenoxy) Triethylamine (MPTA)

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Abstract: Problem statement: Increasing carotenoids content in foods was currently of great interest. The present paper reports strong stimulatory effect of MPTA on the carotenogenesis in cotton, which was used as a model. **Approach:** Cotton seedlings were treated with MPTA vs. water and germinated in the dark. The levels of carotenoids were determined by HPLC and the expression levels of the key genes were determined by real-time PCR. **Results:** In the water treated dark germinating control cotton seedlings only low levels of lutein and β-carotene were detected. Treatment of dark germinating seedlings with substituted triethylamine MPTA resulted in an 88% reduction of lutein and a 100% reduction of β-carotene and nearly 18 fold increases in the total carotenoid production with the increase mainly due to the formation of lycopene and its precursors. Treatment of the germinating cottonseed with MPTA and antibiotics such as actinomycin-D, α-amanitin, cordycepin or cycloheximide reduced the stimulatory effect of MPTA. This indicates that increased biosynthesis of carotenes with MPTA was dependent on gene expression, polyadenylation of the gene transcripts and translation of the mRNA on 80S ribosomes. In addition, the relative transcript levels of phytoene synthase, *psy1* and *psy2* in the MPTA germinating seedlings increased 6.5 and 2.2 fold, respectively, compared to the transcript levels in the H_2O controls. The relative levels of *lyc-β* and *lyc-ε* transcripts in the MPTA treated seed increased 3.7 and 3.6 fold, respectively, compared to the H_2O controls. **Conclusion:** These results support the conclusion that the accumulation of lycopene and lycopene precursors in the MPTA treated germinating cottonseed was dependent on the partial blockage of LYC-β and LYC-ε and the induced expression and translation of the *psy* genes leading to an increased carotenogenesis.

Key words: *Gossypium hirsutum*, biosynthesis, phytoene, Cordycepin, Germinating cottonseed, Lycopene-β Cyclase (LYC-β), lycopene cyclase inhibition, carotenoids, cycloheximide, carotenogenesis, germinating, polyadenylation, quantitative, Triethylamine, MPTA

INTRODUCTION

There is currently great interest in increasing carotenoid content in human foods. In this regard, a number of analogs of triethylamine have been shown to increase the level of lycopene in several plants and microorganisms (Yokoyama *et al*., 1977). Spurgeon and Porter (1983) listed a number of amines including 2-(4-chlorophenylthio) triethylamine (CPTA) that block the later reactions of carotenogenesis at Lycopene Cyclase (LYC) (Fig. 1). This results in the accumulation of lycopene. In germinating lettuce seed under low irradiance, treatment with 2-(4-methylphenoxy) triethylamine (MPTA) severely reduced the levels of β-carotene and xanthophylls by the preferential blockage of LYC-beta (LYC-β)

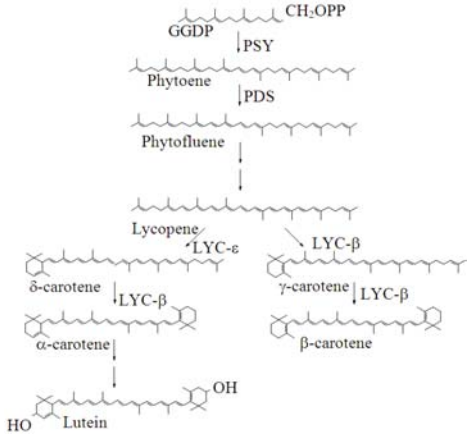

Fig. 1: Biosynthesis of carotenoids

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compared to LYC-epsilon (LYC-ε) and lycopene accumulated at their expenses (Phillip and Young, 2006). The finding that a mutation in the *lyc* gene conferred resistance to MPTA provided strong evidence that LYC is the target site of action of MPTA and related compounds.

Hsu *et al*. (1972) demonstrated that cycloheximide inhibited the accumulation of lycopene in CPTA treated *Blakespora trispora,* while Camara (1984) showed that cycloheximide prevented the enhancement of Phytoene Synthase (PSY) by CPTA in *Capsicum annum*. Benedict *et al*. (1985) demonstrated that treatment of lemon flavedo with MPTA increased lycopene biosynthesis, but α-amanitin, cordycepin, anisomycin or cycloheximide significantly reduced the MPTA induction of lycopene. Increases in lycopene required the blockage of LYC and gene expression, polyadenylation of gene transcripts and translation of the mRNA on 80S ribosomes. Al-Babli *et al*. (1999) demonstrated that application of CPTA to narcissus flowers increased the mRNA levels of *psy*, phytoene desaturase (*pds*), *lyc* genes and protein levels of PSY, PDS and LYC. Lycopene accumulation caused by the CPTA inhibition of LYC was accompanied by reduction in β-carotene derivatives suggesting carotenogenesis may undergo end-product regulation.

In this study we assess the action of MPTA in inducing the accumulation of lycopene and lycopene precursors. We used cotton cotyledonary leaves as a model to more fully characterize the induction of carotenogenesis by MPTA.

MATERIALS AND METHODS

General: MPTA was synthesized by the procedure of Schuetz and Baldwin (1958). Cottonseed from the glandless *Gossypium hirsutum* Acala line were soaked in H_2O for 6 h and their seed coats removed. The dehulled seeds were imbibed for an additional 3 h in either H_2O, 7.2×10^{-4} M MPTA, or 7.2×10^{-4} M MPTA and antibiotics and germinated in the dark at 32°C for 72 h.

Carotenoid extraction and HPLC analysis: Cotyledons of the three day old treated cottonseed were rinsed with water and ground in liquid nitrogen to a fine powder. The powder was further ground in 80% acetone for an additional 5 min transferred to a centrifuge tube and centrifuged at 10,000 g for 10 min. The pellet was extracted once more with 80% acetone followed by two times with 100% acetone to remove the carotenoids. The acetone extracts were combined and the acetone removed in vacuo. The carotenoids were dissolved in 20 mL methanol and 4 mL 60% (w/v) KOH was added for saponification. The flasks were flushed with nitrogen and stored overnight in the dark. The resulting solutions were extracted with dichloromethane 4 times. The combined organic extracts were dried with anhydrous sodium sulfate and the resulting solution concentrated *in vacuo* and brought to a final volume of 1.71 mL g^{-1} fresh weight of seeds for use in HPLC analysis. For UV-Vis analysis, an aliquot of the above solution was dried in vacuo and the residue dissolved in hexane in a final volume of 17.1 mL g^{-1} fresh weight of seeds. The absorption spectra of the hexane fractions were obtained from 250-550 nm on a Hewlett Packard 8453 UV-Visible spectrometer.

An Agilent Technologies 1200 Series HPLC instrument was used for the analysis. Carotenoids were separated by reverse-phase HPLC using an Agilent Zorbax Eclipse XDB-C18 (4.6×250 mm, 5 μm) column maintained at 30°C with an isocratic elution of 70% ACN, 20% dichloromethane and 10% MeOH at a flow rate of 1.25 mL^{-1} min. The DAD signals were collected at 286, 348, 400 and 473 nm to detect all the carotenoid compounds. The carotenoids were identified by their characteristic absorption spectra and their typical retention time.

Total RNA extraction and real time PCR analysis: Total RNA was extracted from the cotyledons of the three day old MPTA treated and H_2O control cotton seedlings as previously described (Liu *et al*., 2002). Total RNA was treated with DNAase (Roache Applied Science, Indianapolis, IN, USA) to remove residual DNA. The first strand cDNAs for each sample were generated using random hexamers and Multiscript reverse transcriptase (Applied Biosystems, Foster City, CA, USA) using 1μg total RNA in 100μL of reaction. The relative transcript levels of the *psy* and *lyc* genes were determined in the MPTA treated sample relative to the H_2O control samples using Step One Real-Time PCR systems (Applied Biosystems, Foster City, CA, USA). The *G. hirsutum* EST database was searched with a number of *psy* genes from plant species (accession numbers: AY056287, X68017, AJ308385, AJ304825, X60441, M84744 and X78814) as query. Alignment of the resulting sequences gave two major groups of *psy* genes designated as *psy*1 and *psy2*. The *G. hirsutum* database was also searched with a number of *lyc* genes from other plant species (accession numbers: AF117256, AY091396, AF254793, X86452, X98796, AY206862, AY079371, AY533827 and EU533951). Alignment of the resulting sequences gave one group of *lyc-β* genes and one group of *lyc-ε* genes. Gene specific primers were designed based on the

consensus sequences of the aligned G. *hirsutum* EST sequences using Primer Express version 3.0 (Applied Biosystems, Foster City, CA). The gene specific forward and reverse primers were as follows: *psy1*, 5'-GCTTGTTAAATGAAGCATATGATCGT-3' and 5'-TCAGCAAAGTTCCCAAATAAAAGG-3'; *psy2*, 5'-GGATTTAGGAACAAGAACTGAAACTGA-3'and 5'- CACATACTTCACCACACCGATCA-3'; *lyc-β*, 5'-AGCAGGACTTTCAGTGTGTTCAAT-3' and 5'-AGCTTCAAATTCATCAACCCAAA-3'; *lyc-ε*, 5'-GCCTGACAAGGGACATTCTCA- 3' and 5'-CTTTCCTCTGCCGATCTCATTC-3'. 18S rRNA forward and reverse primers (5'-CGTCCCTGCCCTTTGTACAC-3' and 5'-ACACTTCACCGGACCATTCAA-3') were used for RNA normalization. Reactions that lacked reverse transcriptase were included to check the possible amplification of genomic DNA contamination for each RNA sample. Samples were run in triplicate on each plate. The quantitative PCR was repeated on at least two sets of independent RNA samples. Real-time PCR was performed in a 20 µL reaction mixture containing 7.2 µL dd H_2O, 10 µL Fast SYBR green Master Mix (Applied Biosystems, Foster City, CA), 200 nM each forward and reverse primers and 2 µL template cDNA (10ng μL^{-1}). The PCR conditions were 20 sec pre-denaturation at 95°C, 40 cycles of 3 sec at 95°C and 30 sec at 60°C, followed by steps for dissociation curve generation (15 sec at 95, 1 min at 60°C and 15 sec at 95°C). The step one software version 2.0 (Applied Biosystems) was used for data collection and analysis. Dissociation curves for each amplicon were carefully examined to confirm the specificity of the primer pair used. The amplicons were also cloned and their sequences verified. The threshold cycles (CT value) of the target genes and 18S rRNA in MPTA and water treated samples were obtained after quantitative real-time PCR. Relative transcript levels for MPTA treated samples were compared to the water treated samples using the comparative CT method.

RESULTS AND DISCUSSION

The chloromethane extract of the MPTA treated and H_2O controls of cotyledonary leaves 72 h after germination in the dark were analyzed by HPLC. Phytoene, phytofluene and lycopene are the main pigments present in the MPTA treated seedlings. In contrast, the H_2O control samples show the presence of lutein and β-carotene. The levels of these carotenoids are shown in Table 1. In the H_2O controls, there were 8.4 µg g^{-1} fr. wt. lutein and 1.6 µg g^{-1} fr. wt. β-carotene.

Table1: Increase of carotenoids in dark germinated cotton cotyledonary leaves from seed treated with 7.2×10^{-4} M MPTA or water and carotenoids in non-germinated cottonseed

Carotenoid	MPTA (µg gm^{-1} fr. wt.)	H_2O (µg gm^{-1} fr. wt.)	Cottonseed[a] (µg gm^{-1} fr. wt.)
Phytoene	74.9±4.8	ND[b]	ND
Phytofluene	4.8±0.8	ND	ND
Lycopene	96.7±18.7	ND	ND
β-Carotene	ND	1.6±0.2	ND
Lutein	1.0±0.7	8.4±0.7	0.5±0.1
Total carotenoids	177.4±25.0	10.0±0.9	0.5±0.1

[a]: Non-germinated cottonseed; [b]:Not detected

These represent de novo synthesis of carotenoids during dark germination as the non-germinated seed contained only trace amount of lutein (0.5 µg g^{-1} fr. wt). However, in the 7.2×10^{-4} M MPTA treated seedlings the level of lutein was reduced by 88% and β-carotene was not observed; rather high levels of phytoene, phytofluene and lycopene were observed (74.9 µg g^{-1} fr. wt., 4.8 µg g^{-1} fr. wt. and 96.7 µg g^{-1} fr. wt. lycopene, respectfully). Overall, the 7.2×10^{-4} M MPTA treatment resulted in a 17.7 fold increase in carotenoids biosynthesis in the cotton cotyledonary leaves. This contrasts with the 2-fold increase in carotenoids in the narcissus flowers treated with 1 mm CPTA (Al-Babli *et al*., 1999) and the 10% increase in carotenoids in germinating seed of radish and lettuce treated with 10 and 20 µm MPTA (Phillip and Young, 2006). These differences may be due to different responses to substituted tertiary amines in the different plant materials and treatment with different concentrations of CPTA and MPTA. What seems clear is that the large increase in carotene biosynthesis in cotyledons from germinating cottonseed treated with MPTA may be a response in a plant material that initially has a low level of carotenoid biosynthesis.

In the narcissus flowers treated with CPTA (Al-Babli *et al*., 1999), the level of lycopene accounted for most of the increase in carotenoids with minor reductions in violaxanthin, neoxanthin and antheraxanthin. CPTA did not change the level of lutein but did substantially increase the level of abscisic acid. There was no accumulation of lycopene precursors in the treated narcissus flowers and the reduction of the β-xanthophylls would not account for the amount of lycopene accumulated. In the MPTA treated radish seedlings (Phillip and Young, 2006) at low light intensity, the formations of β-carotene and violaxanthin were the most reduced, while the level of lutein and neoxanthin were the least affected. In the lettuce seedlings treated with MPTA there was an 18-fold reduction in β-carotene and neoxanthin with a 4-fold

reduction in the levels of violaxanthin and lutein and a substantial increase in lactucaxanthin. In the radish seedlings treated with 10 μm MPTA the loss in the levels of β-carotene, lutein, neoxanthin and violaxanthin would nearly account for the accumulation of lycopene. In lettuce seedlings treated with 10 μm MPTA the reduction in the levels of β-carotene, lutein, neoxanthin and violaxanthin would account for the amount of accumulated lycopene but at 20 μm MPTA the formation of lycopene would exceed the losses in these carotenoids. In the MPTA treated cotton seedlings there was an 88% decrease in lutein concentration compared to the H_2O controls and β-carotene was not observed. These losses are presumably due to the partial blockages of LYC-β and LYC-ε by MPTA. These blockages agree with the finding of Pecker *et al*. (1996) that LYC-β is the target site for MPTA and by Philip and Young *et al*. (2006) that MPTA preferentially blocks LYC-β compared to LYC-ε. However, in the cotton seedlings the partial blockage of LYC-β and LYC-ε with subsequent losses in β-carotene and lutein would not alone account for the increases in lycopene and lycopene precursors accumulated in the treated seedlings.

MPTA treated cottonseed were also treated with various antibiotics including actinomycin D, an inhibitor of RNA synthesis (Martindale, 1979), α-amanitin, an inhibitor of RNA polymerase II (Meinecke and Meinecke-Tillmann, 1993), cordycepin, a polyadenylation inhibitor (Muller *et al*., 1977) and anisomycin and cycloheximide, inhibitors of protein synthesis on 80S ribosomes (Grollman, 1967; Stockein and Piepersberg, 1980) (Table 2). At the highest concentrations, these antibiotics inhibited the MPTA induced formation of lycopene by 73-100%. These observations support the concept that the enhancement of lycopene formation by MPTA in the cotton seedlings is dependent on mRNA synthesis, polyadenylation of the primary gene transcripts and protein synthesis on 80S ribosomes.

To investigate this further, the relative transcript levels of *psy* and *lyc* genes were determined in the germinating seedlings by qPCR. The *G. hirsutum* EST database was searched and compared to a number of *psy* genes and *lyc* genes from plant species. Alignment of the resulting sequences gave two groups of *psy* genes (designated as *psy1* and *psy2*) and *lyc-β* and *lyc-ε* genes in cotton. Primer pairs were designed with the consensus sequences and the sequences of the amplified products from cDNA of Acala cultivar of *G. hirsutum* were verified through sequencing. The relative levels of *psy1, psy2, lyc-β* and *lyc-ε* were determined using 18S rRNA as the reference gene.

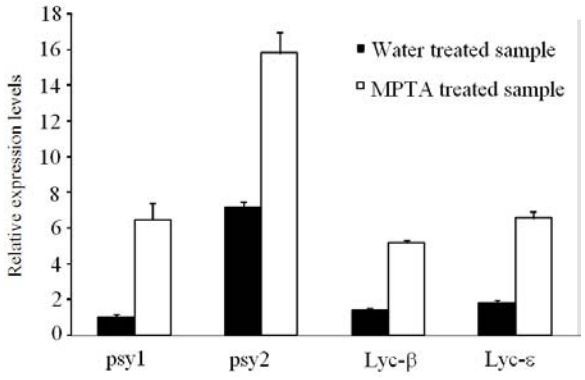

Fig. 2: Real-time PCR analysis showing relative expression levels of genes involved in carotenoid biosynthesis in cotton seedling cotyledons from seed treated with H_2O or 7.2×10^{-4}M MPTA (error bars represents means±SD)

Table 2: Effect of antibiotics on the MPTA induction of lycopene in cotton cotyledon seedlings germinated in the dark

Antibiotic[a]	Lycopene (μg)/ cotyledon pair	Inhibition of lycopene (%)
H_2O Control	9.3	00.0
Actinomycin D (10 μg mL^{-1})	6.1	34.0
Actinomycin D (100 μg mL^{-1})	None	100.0
α-Amanitin (1 μg mL^{-1})	4.1	55.8
α-Amanitin (10 μg mL^{-1})	None	100.0
Cordycepin (10 μg mL^{-1})	6.2	33.1
Cordycepin (100 μg mL^{-1})	2.5	73.1
Cycloheximide (1 μg mL^{-1})	2.4	74.1
Cycloheximide (10 μg mL^{-1})	None	100.0
Anisomycin (1 μg mL^{-1})	3.4	63.3
Anisomycin (10 μg mL^{-1})	None	100.0

[a]: Antibiotics were added together with 7.2×10^{-4}M MPTA

The results (Fig. 2) show the effect of MPTA on the relative transcript levels of *psy* and *lyc* genes in the germinating cottonseed. The MPTA treatment increases the gene transcripts of *psy1* and *psy2* by 6.5 and 2.1 fold compared to the transcripts levels of these *psy* genes in the H_2O controls. The increase in the expression of the *psy* genes by MPTA agrees with the actinomycin-D data in Table 2 that MPTA increases gene expression in cotton seedlings and in agreement with the earlier work of Al-Babli *et al*. (1999) that CPTA increases *psy* mRNA in narcissus flowers. Thus, the increase in the expression and translation of *psy* is a major contributor to the MPTA induction of carotenogenesis in the cotton seedlings. The data in Fig. 2 also show that MPTA increases the transcript levels of *lyc-β* and *lyc-ε* genes by 3.7 and 3.6 fold, respectfully, compared to the transcript levels in the H_2O controls. The reduction of β-carotene and lutein in the MPTA treated seedlings compared to the H_2O

controls indicates that the concentration of MPTA used in these experiments is sufficient to partially block any increase in the LYC produced from the higher concentrations of *lyc* gene transcripts in the MPTA treated seedlings.

CONCLUSION

These results demonstrate that treating germinating cottonseed with MPTA partially blocks LYC-β and LYC-ε resulting in a complete inhibition in β-carotene biosynthesis and a substantial reduction in lutein. The stimulation of carotenogenesis and the partial blockage of LYC-β and LYC-ε results in the accumulation of phytoene, phytofluene and lycopene. The reduction in β-carotene and lutein was not sufficient to account for the accumulation of lycopene and lycopene precursors as would be suggested from the work of Phillip and Young (2006).

Inhibition of the ability of MPTA to increase lycopene in the germinating cottonseed with actinomycin D, α-amanitin, cordycepin or cycloheximide indicate that MPTA action is dependent on gene transcription, polyadenylation of the gene transcripts and translation of the mRNA on 80S ribosomes. MPTA treatment of the germinating seedlings significantly increased the relative expression of *psy1, psy2, lyc-β* and *lyc-ε* genes compared to that in the H_2O controls. The concentration of the MPTA used in these experiments was adequate to partially block any formation of LYC-β and LYC-ε resulting from the increase in the *lyc-β* and *lyc-ε* transcripts. The small pool size of β-carotene in the H_2O controls (10^{-6}M g^{-1} fr. wt.) may not be sufficient to function in a feed-back regulation. Therefore complete reduction of β-carotene in the MPTA treated germinating cottonseed would probably have a minimum impact on gene expression of *psy1, psy2, lyc-β* and *lyc-ε* and the majority of the induced expression of the *psy* and *lyc* genes may result from the action of MPTA with gene transcription factors. The results in this study indicate that the accumulation of lycopene and lycopene precursors in the MPTA treated germinating cottonseed results from the partial blockage of LYC-β and LYC-ε and the increased expression of the *psy* genes leading to an overall increase in carotenogenesis.

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