Plant Physiology and Biochemistry 127 (2018) 143–151

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

# Expression of heterologous lycopene β-cyclase gene in flax can cause silencing of its endogenous counterpart by changes in gene-body methylation and in ABA homeostasis mechanism

Aleksandra Boba[a,*,1], Kamil Kostyn[a,b,**,1], Marta Preisner[a,b], Wioleta Wojtasik[a], Jan Szopa[a,b], Anna Kulma[a]

[a] Faculty of Biotechnology, University of Wroclaw, Przybyszewskiego 63/77, 51-148 Wroclaw, Poland
[b] Department of Genetics, Plant Breeding and Seed Production, Faculty of Life Sciences and Technology, Wroclaw University of Environmental and Plant Sciences, Plac Grunwaldzki 24A, 53-363 Wroclaw, Poland

ARTICLE INFO

*Keywords:*
Flax
Co-suppression
Epigenetics
DNA methylation
Abscisic acid regulation
Pathogen resistance

ABSTRACT

Previously we described flax plants with expression of *Arabidopsis* lycopene β-cyclase (*lcb*) gene in which decreased expression of the endogenous *lcb* and increased resistance to fungal pathogen was observed. We suggested that co-suppression was responsible for the change. In this study we investigated the molecular basis of the observed effect in detail. We found that methylation changes in the *Lulcb* gene body might be responsible for repression of the gene. Treatment with azacitidine (DNA methylation inhibitor) confirmed the results. Moreover, we studied how the manipulation of carotenoid biosynthesis pathway increased ABA level in these plants. We suggest that elevated ABA levels may be responsible for the increased resistance of the flax plants to pathogen infection through activation of chitinase (PR gene).

## 1. Introduction

Recently, a carotenoid biosynthesis pathway modification in flax via introduction of lycopene β-cyclase gene from *Arabidopsis thaliana* (*Atlcb*) under the control of the constitutive promoter CaMV35S was described. The presence of *Atlcb* gene, which is homologous with the corresponding flax gene in 74%, led to the down-regulation of the endogenous gene by 50% on average. The obtained L plants demonstrated lower contents of β-carotene and lutein, but higher resistance against *Fusarium* infection (Boba et al., 2011). Although the knowledge on the machinery of co-suppression phenomena has been widened in the last few years, the exact mechanism of the endogenous *Lulcb* gene silencing in the L lines of flax remained unknown.

Co-suppression was first observed in an attempt of overexpressing chalcone synthase (*chs*) gene in petunia, when some of the obtained transformants unexpectedly showed white flowers instead of colored ones, which was the result of the endogenous gene suppression (Stam et al., 1997). Co-suppression, that is silencing of an endogenous gene caused by exogenous gene copy may refer to silencing at the transcriptional (TGS) or post-transcriptional (PTGS) level. Gene silencing on transcriptional level results from reduced RNA synthesis efficiency or total transcription inhibition. TGS may be influenced by the transgene insertion site in a chromosome, homologous DNA sequence interaction or DNA methylation. This epigenetic silencing may persist over many cell divisions or plant generations (Vaucheret et al., 2001). The mechanism of PTGS leads to degradation of specific, homologous RNA sequences in cytoplasm. In result the number of transgene RNA copies and of all homologous RNAs decreases. PTGS has been well recognized mainly due to studies on transgenic plant resistance to viruses, where the viral RNA degradation was dependent on the homology of the transgene and virus sequences (Lindbo and Dougherty, 1992). TGS and PTGS are mechanistically and possibly also functionally related as they are associated with some of the same events, including DNA methylation. It is assumed that TGS is connected with changes in the promoter sequence methylation, while PTGS with changes in transcribed sequence methylation (Paszkowski and Whitham, 2001; Marenkova and Deineko, 2010).

In plants methylation of cytosines occurs at CG, CNG and CNN context (where N is A, C, or T) and is catalyzed by cytosine methyltransferases yielding 5-methylcytosine (m5C). Regulation of gene

* Corresponding author.
** Corresponding author. Faculty of Biotechnology, University of Wroclaw, Przybyszewskiego 63/77, 51-148 Wroclaw, Poland.
*E-mail addresses:* aleksandra.boba@uwr.edu.pl (A. Boba), kamil.kostyn@upr.edu.pl (K. Kostyn), marta.preisner@upr.edu.pl (M. Preisner), wioleta.wojtasik@uwr.edu.pl (W. Wojtasik), szopa@ibmb.uni.wroc.pl (J. Szopa), anna.kulma@uwr.edu.pl (A. Kulma).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.plaphy.2018.03.023
Received 9 January 2018; Received in revised form 20 March 2018; Accepted 20 March 2018
Available online 21 March 2018
0981-9428/ 

transcription might be associated with maintenance and *de novo* methylation of DNA. CG methylation is maintained by methyltransferase 1 (*met1*) and is controlled by Variant In Methylation 1 (*vim1*) and the chromatin remodeler *ddm1* (Decrease in DNA Methylation 1). CNG methylation is maintained by Chromomethylase 3 (*cmt3*) and Histone Methyltransferase (*hmt*), Kryptonite (*kyp*), and is controlled by *cmt3* and *drm2* (Domains Rearranged Methyltransferase 2). CNN methylation is maintained by the RdDM (RNA-dependent DNA methylation) pathway, and *de novo* methylation of DNA in all of these sequence contexts is generally established by *drm2*. Biochemical and cytological evidence from mammalian systems suggests that DNA methylation status is recognized by proteins that bind to the methylated DNA or by the methyltransferase itself, and translated to the corresponding chromatin structure using histone deacetylase (*hdac*) complexes that influence chromatin organization (Robertson et al., 2000). To shed some light on the source of changes in the endogenous *Lulcb* gene expression observed in the L flax lines we performed additional, detailed studies of these plants, including analysis of methylation profile of the *Lulcb* gene and determination of transcription levels of genes involved in epigenetic changes.

The L flax lines were previously characterized by increased resistance to *Fusarium* infection with simultaneous decrease in carotenoids (β-carotene, lutein) and increase in tocopherols. The elevated resistance was connected with increased antioxidative potential of metabolites extracted from the L plants compared to the control. Redirection of substrates to different branches of the terpenoid pathway and/or their activation was proposed as a mechanism behind the observed changes. To further elucidate the suggested way of action we performed a complex analysis of transcript levels of genes involved in the studied pathway, including not only the metabolite synthesis genes, but also the genes involved in their processing/catabolism. We found increased abscisic acid (ABA) contents in the L flax plants and we suggest that this may be the reason for the elevated resistance of these plants to pathogen infection, as ABA is a well-known messenger in plants responses to such stress (Raghavendra et al., 2010). To our knowledge this is the first report on the flexibility of the terpenoid pathway in flax in response to pathogen infection.

## 2. Materials and methods

### 2.1. Plant material

Flax seeds (*Linum usitatissimum* cv. Linola) were obtained from the Flax and Hemp Collection of the Institute of Natural Fibres and Medicinal Plants, Poland. Flax lines with reduced expression of lycopene beta cyclase gene obtained via agrotransformation with a construct containing *A. thaliana lcb* cDNA sequence under 35S promoter (lines L9 and L18) as described previously (Boba et al., 2011) were maintained in tissue culture in Murashige and Skoog medium (Sigma-Aldrich) supplemented with 1% sucrose and solidified with 0.9% agar, under a 16 h light (21 °C), 8 h darkness (16 °C) regime. Shoot tips were grown for four weeks to about 10 cm height before experiments.

### 2.2. Identification of gene sequences

The cDNA sequences of flax genes were identified on the basis of homology alignments with the known gene sequences from other plant species. The sequences were amplified by means of PCR using cDNA template transcribed from mRNA isolated from 14 day-old flax seedlings, with primers designed for the most homologous regions. The amplified reaction products were analyzed via electrophoresis and following gel extraction (Qiaquick Gel/PCR Purification Kit, Qiagen), they were cloned with a TOPO TA Cloning Kit (Invitrogen) and sequenced (Genomed SA). The obtained DNA sequences were compared with the flax genome sequence (*Linum usitatissimum* cv. Bethune) and aligned with corresponding genes from other plants in the GenBank database (http://www.ncbi.nlm.nih.gov/blast/).

### 2.3. Gene transcript level analysis

Total RNA was isolated with TRIzol reagent (Life Technologies) according to the manufacturer's protocol. Its quantity was determined spectrophotometrically at $\lambda = 260$ nm. The RNA was treated with DNase I and transcribed to cDNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was determined with real time PCR (RT-PCR) technique using a DyNAmo SYBR Green qPCR Kit (Thermo Scientific, USA) on the StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) in triplicates. Primers used in the reaction were designed using LightCycler® Probe Design v2 software (Roche, Switzerland) and their sequences are presented in Supplementary Table 1. Primer annealing temperature was 57 °C. Melting curve was applied for primer annealing verification. Changes in the transcript levels of the investigated genes were expressed as relative quantification (RQ) relatively to control (RQ = 1). Actin gene was used for reference.

### 2.4. Isolation of terpenoids

Plant green tissue was ground in liquid nitrogen and lyophilized. 50 mg aliquots were extracted with 1 ml of methanol in an ultrasonic bath for 15 min and then centrifuged at 16000 rpm, 4 °C for 5 min. The supernatants were collected and the pellet was extracted in the same manner two more times, but acetone and then petroleum ether were used instead of methanol. The combined supernatants were then dried under nitrogen flow and then re-suspended in 1 ml of acetonitrile:methanol (1v:1v) mixture and submitted to further analysis.

### 2.5. UPLC analysis of terpenoids

Ultra performance liquid chromatography (UPLC) was used to analyze the terpenoids isolated from flax plants. Acquity BEH C18 2.1 × 100 mm, 1.7 µm column was employed. The mobile phase was passed through the column at a flow rate of 0.5 ml/min and consisted of the following solvents: A – acetonitrile:methanol:water (5v:3v:2v) and B – acetonitrile:methanol (1v:1v) according to the conditions depicted in Table 1. The column was kept at 25 °C. A photodiode array (PDA) was used to detect absorption between 210 and 500 nm. The identities of components were determined based on their retention times, and UV spectra comparison to authentic standards (Sigma-Aldrich, USA).

### 2.6. Abscisic acid isolation and quantification

200 mg of plant green tissue ground in liquid nitrogen were extracted with 500 µl of 200 µg/ml of sodium diethyldithiocarbamate in 90% methanol. The mixture was moved to glass tubes and placed at 4 °C overnight. The next day the samples were centrifuged at 8000 rpm, 4 °C, for 10 min and the supernatants were moved to ice-cooled eppendorfs and then evaporated in vacuum at 4 °C. The resulted pellet was re-suspended in 400 µl of buffer consisted of 10% methanol, 50 mM Tris pH 8.0, 1 mM $MgCl_2$, 150 mM NaCl. The quantities of abscisic acid were determined with ABA Test Kit (Agdia, USA) according to the producer's protocol.

**Table 1**
Conditions of UPLC solvent flow.

| TIME | 0 min | 1 min | 5 min | 10 min | 11 min |
|---|---|---|---|---|---|
| Solvent A | 100% | 100% | 0% | 0% | 100% |
| Solvent B | 0% | 0% | 100% | 100% | 0% |