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

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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

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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%

2.7. Total methylation of genomic DNA

Plant DNA isolated with DNeasy Plant Kit (Qiagen) was used for the determining of 5-mCytosine content in the plant genome. The assay was performed with use of MethylFlash Methylated DNA 5-mC Quantification Kit (Epigentek, USA), according to the producer's procedure.

2.8. Analysis of methylation pattern of the flax lulcb gene

In order to determine the methylation pattern of the flax *Lulcb* gene a methylation-dependent restriction analysis was performed using methylation dependent restriction enzymes *Msp*I and *Hpa*II. Both of the enzymes recognize the same sequence •••CCGG•••. *Msp*I is an isoschizomer of *Hpa*II. *Msp*I and *Hpa*II cannot cleave when the external C in the sequence •••CCGG••• is methylated,. However, unlike *Hpa*II, *Msp*I can cleave the sequence when the internal C residue is methylated. Plant DNA digested by *Hpa*II or *Msp*I (New England Biolabs, USA) was used as a template in real time PCR. Non-digested DNA was used as a control. Six sites within the *Lulcb* gene body, recognized by the enzymes, were analyzed (Supplementary Table 2). Changes in methylation level in the studied lines were presented as the percent of methylated cytosine residues relatively to wild-type control plants.

2.9. Azacitidine treatment

Flax seeds were first soaked in water for 12 h, followed by 1 mM azacitidine water solution for 12 h. After the treatment the seeds were placed on petri dishes with wet Whatman paper and grown for 7 days in a plant growth chamber. Then the seedlings were collected and total RNA was extracted to obtain the level of *Lulcb* gene transcript with RT-qPCR (Marenkova and Deineko, 2010).

2.10. Abscisic acid treatment

Four-week old wild-type (Linola) flax plants in *in vitro* culture were sprayed with 100 μM ABA solution (in water). Tissue was collected after 6, 12 and 24 h, frozen immediately in liquid nitrogen, and stored at −80 °C before further analyses.

2.11. Assessment of the influence of carotenoids and tocopherols on F. Oxysporum mycelium growth

In order to directly assess the influence of carotenoids and tocopherols on *F. oxysporum* mycelium growth, PDA media supplemented with original standards (Sigma-Aldrich, USA) of lutein, β-carotene, α-tocopherol and γ-tocopherol at 0.5 μM concentration were prepared. Pure solvent, in which the standards were dissolved was used in the control. The PDA media mycelium fragments were grown for 3–5 days at 28 °C in three biological repeats. The mycelia were then photographed and their diameters measured.

3. Results

3.1. Verification of transgenicity of the L flax plants

The transgenicity of the previously described L plants (Boba et al., 2011) was verified and the suppression of the endogenous *Lulcb* confirmed (Fig. 1A and B). Briefly, the L flax lines were generated by introducing *A. thaliana Atlcb* cDNA in sense orientation under the control of a constitutive CaMV35S promoter by means of transgenesis. Based on homology-dependent gene silencing phenomenon, high similarity of the transgenic copy of the *lcb* gene to the endogenous one (79%) led to reduced expression of the latter. However, no explanation of the possible mechanism behind the decrease of the *Lulcb* gene expression was presented, thus we made an attempt to clarify the observation.

3.2. Analysis of methylation pattern of the endogenous lycopene β-cyclase gene in the L plants

There are two isoforms of *lcb* gene in the flax genome with the reciprocal homology of 94.95%. Based on flax genome sequence computer analysis the numbers of CpG islands in both of the isoforms were determined. The analysis showed that five CpG sites were common for the two isoforms at positions −405, +297, +454, +679, +1114. The first isoform had an additional CpG site at position +1395, while the second had additional four sites at positions: −654, −484, −94, +109. The differences in the methylation pattern in *lcb* genes in the L type of flax plants were analyzed using a pair of restriction enzymes sensitive to methylation (*Msp*I and *Hpa*II), which recognized •••CCGG••• site in the nucleotide sequence and RT-PCR technique.

Using methylation sensitive restriction analysis of the *Lulcb* gene we have shown that there are differences between the L lines and the control (Fig. 2). Positions +109 and + 297 are the most discriminatory. In the line L18 non-methylated cytosines at position +109 were by 15% more abundant compared to the control. Simultaneously, in the line L9 non-methylated sequence level was lower by 15%. This correlated with the level of inner cytosine methylation (CCmGG), which in the line L9 was by 16% higher and in the line L18 by 15% lower. At position +297 we observed increased cytosine methylation by 12% in L9 and by 22% in L18. Considering that increased methylation effects in gene inactivation, we suggest that it is the position +297 that plays the key role in causing the epigenetic changes. The remaining sites showed minor differences in the methylation pattern and at the same time indicated that the L18 line, in which the transcript level of *Lulcb* gene is the lowest, was characterized by increased total level of non-methylated cytosines in comparison to the L9 line as well as the control (Supplementary Fig. S1). This is in line with the results obtained after the analysis of the total methylation level of the genome, which was the lowest in the L18 line.

3.3. Total cytosine methylation level in the L plants

In contrast to mammal cells all cytosine nucleotides in the genome sequence can undergo methylation in plants. In the L type flax percent of methylated cytosine nucleotides in the genome was determined. The level of methylated cytosine was lowered in both lines in comparison to the control, by 12% in the L9 and by 23% in the L18 (Fig. 3).

3.4. Analysis of transcript levels of genes involved in genome methylation and post-translational histone modification in the L plants

Transcript levels of genes encoding DNA methylase 1 and 3 (*cmt1* and *cmt3*), DNA demethylases (*ros1* and *dme*), as well as those encoding enzymes involved in post-translational histone modification: *ddm1* (Decrease in DNA Methylation) – connected with histone deacetylation and responsible for cytosine methylation in the genome, *gcn5* – histone acetyltransferase, *h3k9me* – histone methyltransferase, *hac1*-histone acetyltransferase were measured (Fig. 4). *Cmt3* gene transcript level was increased in both L lines, 2.5-fold in the L9 and 2-fold in the L18. Transcript levels of *ros1* and *dme* demethylase genes were decreased by 45% in the L9 line. In the case of the L18 the line transcript level of *ros1* gene was lower by 58% and of *dme* by 66% compared to the control. Expression of the genes involved in the post-translational histone modification did not change significantly in the L9 line, while in the L18 line it decreased in all the cases from 30% for *ddm1* gene to 57% for *hac1* gene.

3.5. Analysis of lcb expression level after azacitidine treatment

Our results indicated that the decreased transcription level of the *lcb* gene in the L flax lines may result from changes in their methylation status. Hypermethylation is often connected with gene transcription

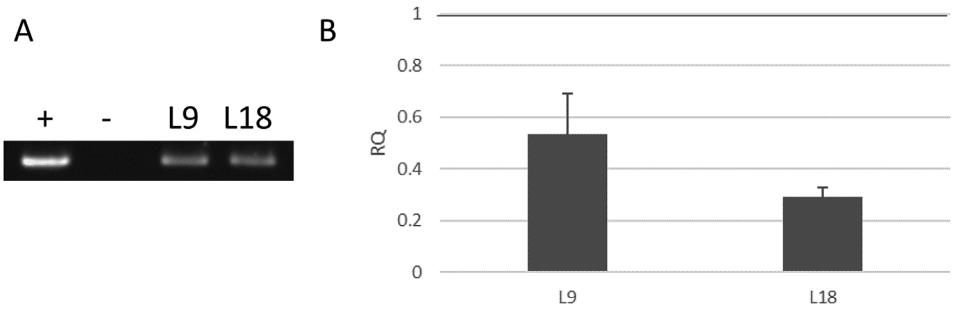

Fig. 1. Verification of the transgene presence and endogenous lycopene β-cyclase (*Lulcb*) gene suppression in L9 and L18 flax plant lines by PCR (A). Transcript levels of the *Lulcb* gene in L flax lines (L9 and L18) presented as RQ in relation to control (wild type; RQ = 1 – marked with black line). Actin gene was used for reference. The results are presented as mean values of three biological repetitions ± SD.

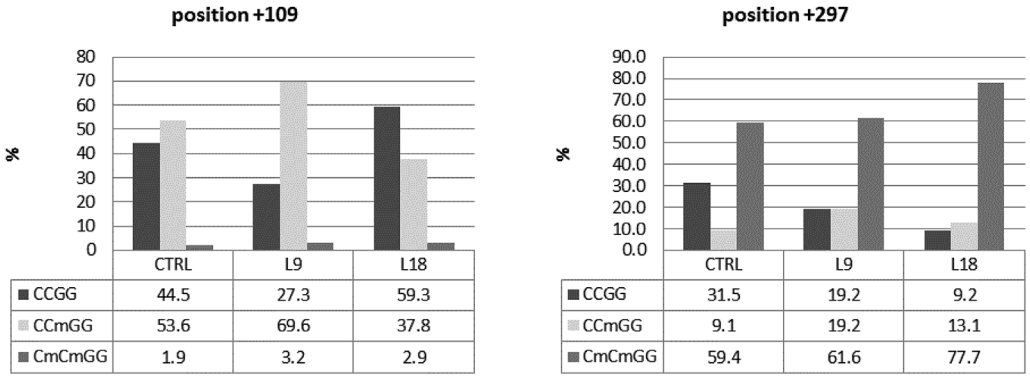

Fig. 2. Pattern of cytosine methylation in •••CCGG••• sequences at positions +109 and + 297 of the *Lulcb* gene in L flax plants (L9 and L18) presented as the percent of the methylated cytosine residues relatively to the control (wild-type – CTRL). The results are presented as mean values ± SD (n = 3).

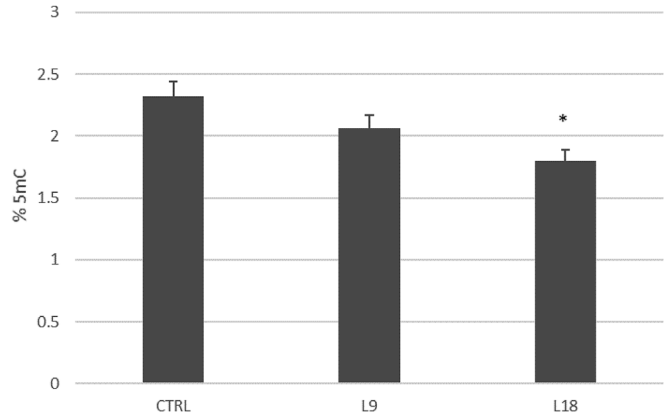

Fig. 3. Percent of total methylation of cytosine (5 mC) in the genome of the L plants with gene repression (L9 and L18) in comparison to the control (wild-type – CTRL). The results are presented as mean values ± SD (n = 3).

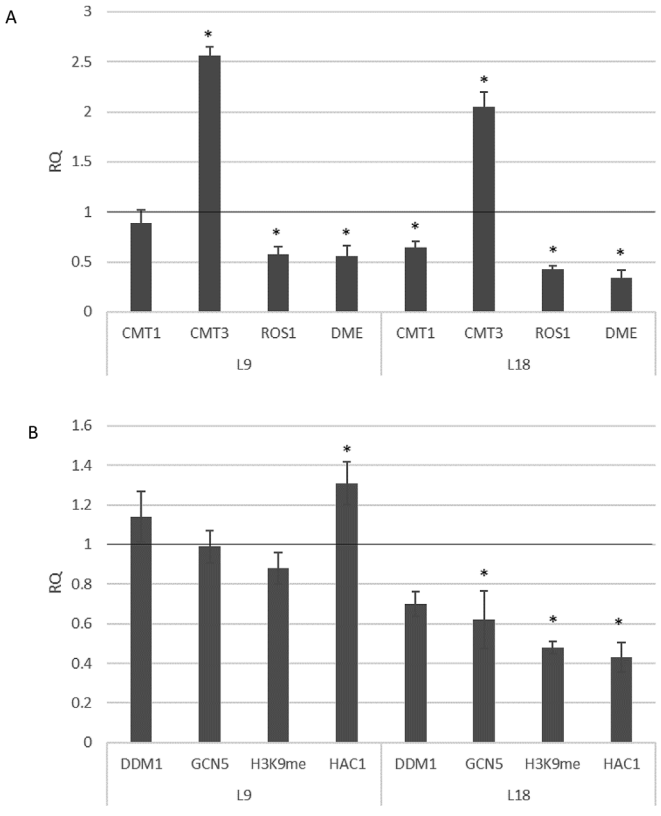

Fig. 4. The transcript level of genes involved in genome methylation (A) and post-translational histone modification (B) in the L flax lines with lycopene β-cyclase gene repression (L9 and L18) presented as RQ relatively to control (wild-type; RQ = 1 – marked with black line). Actin gene was used for reference. The results are presented as mean values ± SD (n = 3).

inactivation, therefore the type L flax plants were treated with 5-azacitidine, a DNA demethylating reagent (Griffin et al., 2016). We observed a restoration of the transcription level of the *Lulcb* gene in the L plants to 92% and 89% of the wild-type control in L9 and L18 lines, respectively (Fig. 5).

3.6. Analysis of transcript levels of genes involved in carotenoid synthesis in the L plants

In order to investigate whether the decrease of the *Lulcb* gene transcript level influenced other carotenoid synthesis gene expressions, their mRNA levels were measured. The expressions of these genes were in most cases decreased (Fig. 6). The exception were carotenoid isomerase (*crtiso*) and lycopene-ε-cyclase (*lce*) in the line L9, where their transcript levels were somewhat increased by 19% and 33%, respectively. A considerable dropdown in the expression was observed in the case of phytoene synthase gene (*psy*), which is the key gene of the

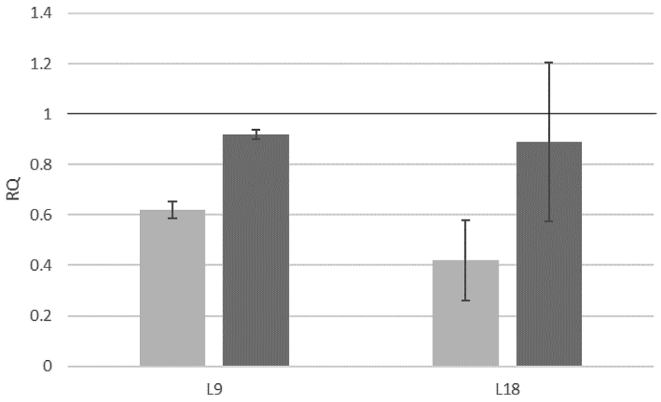

Fig. 5. Transcript level of the *Lulcb* gene in untreated (light grey bars) and azacitidine treated (dark grey bars) L9 and L18 transgenic lines compared to the control (RQ = 1 marked with line). Actin gene was used for reference. The results are presented as mean values ± SD (n = 3).

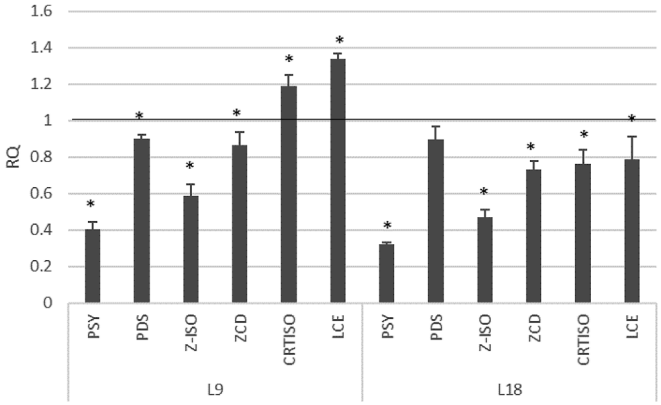

Fig. 6. Level of transcripts of carotenoid pathway genes in the L plants with lycopene β-cyclase gene repression (L9 and L18) presented as RQ relatively to control (wild-type; RQ = 1 marked as with line). Actin gene was used for reference. The results are presented as mean values ± SD (n = 3).

pathway, by 60% in the line L9 and by 68% in the line L18.

3.7. Analysis of transcript levels of carotenoid oxidative cleavage and abscisic acid synthesis genes in the L plants

Oxidative cleavage of carotenoids is connected with the synthesis of volatile compounds and abscisic acid. Apocarotenoids play an important role during pathogen infection (Sun et al., 2008; Adie et al., 2007), hence transcript levels of the genes involved in their synthesis were determined in the L plants (Fig. 7). Transcript levels of oxidative cleavage oxigenases leading to abscisic acid (*nced3*, *nced6*) and other apocarotenoids (*ccd1*, *ccd7*, *ccd8*) and of other genes involved in ABA synthesis (SDR reductase (*aba2*) and abscisic aldehyde oxidase (*aao*)) were investigated. Among the investigated dioxygenase genes, a decrease in the transcript levels of *ccd7* and *ccd8*, by 30% and 90%, respectively, was observed in the L9 line. Simultaneously, a slight increase in *nced3* (by 10%) and a substantial decrease in *nced6* transcript level was observed. Transcript levels of genes directly connected with ABA synthesis (*aba2* and *aao*) were decreased by 50% and 30%, respectively. In the L18 line *ccd7* and *aba2* gene transcript levels were elevated (1.4- and 2.1-fold, respectively). The remaining gene transcript levels were decreased and the changes were most prevalent in the case of *ccd8* and *nced6* genes.

3.8. Abscisic acid content in the L plants

The content of ABA was measured in the L plants. Despite the

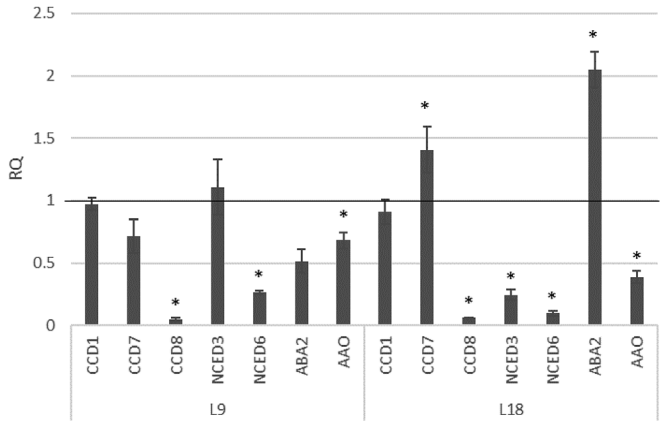

Fig. 7. Transcript level of genes involved in carotenoid catabolism and ABA synthesis in the L plants with lycopene β-cyclase gene repression (L9 and L18) presented as RQ relatively to control (wild-type; RQ = 1 marked with line). Actin gene was used for reference. The results are presented as mean values ± SD (n = 3).

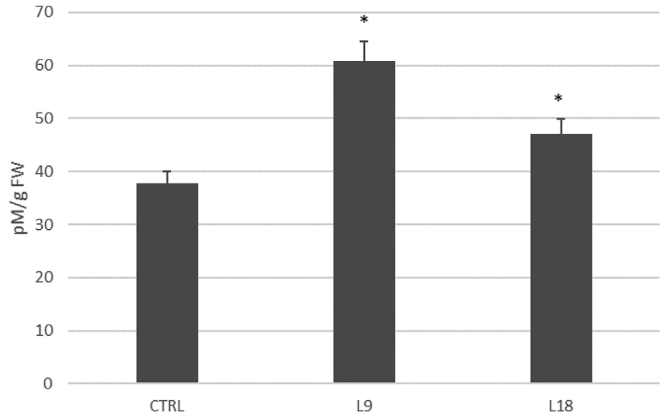

Fig. 8. Abscisic acid content in the L plants with lycopene β-cyclase gene repression (L9 and L18). Changes in ABA content are presented in comparison to the control (CTRL). The results are main values of three independent repetitions (n = 3) ± SD.

decreased transcript levels of the genes involved in ABA synthesis, the content of this compound was increased in the studied plants, by 62% in the L9 and 27% in the L18 (Fig. 8).

3.9. Analysis of transcript levels of genes involved in abscisic acid degradation in the -L plants

In order to investigate the elevated content of the abscisic acid observed despite the decrease in the transcript level of the genes involved in ABA biosynthesis, the levels of transcript of three ABA hydroxylases were measured. It turned out that in all cases a significant decrease by over 99% was observed (Fig. 9).

3.10. Analysis of transcript level of chitinase gene in the L plants

We have investigated whether the decreased susceptibility of the L plants to *Fusarium* infection (Boba et al., 2011) is connected with the induction of chitinase (Pathogenesis-Related proteins (PR proteins) chitinase). The protein belongs to a group of enzymes hydrolyzing fungal cell wall components. In both of the L lines a significant increase in the transcript level of chitinase gene was noticed: 11-fold in the L9 and 9-fold in the L18 (Fig. 10).

3.11. Analysis of chitinase gene transcript level after abscisic acid treatment

In silico analysis revealed the presence of cis-regulatory elements

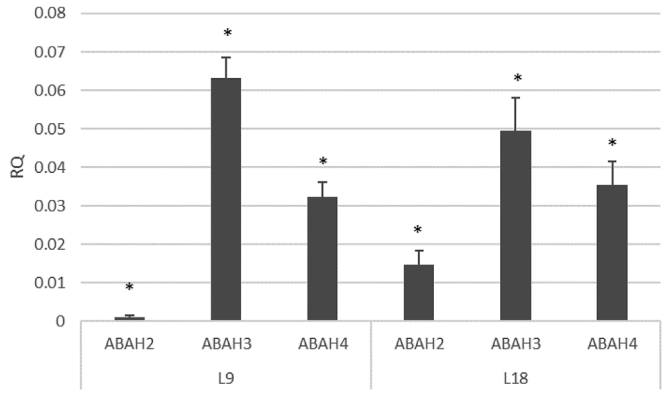

Fig. 9. The transcript level of genes involved in ABA catabolism in the L plants with lycopene β-cyclase gene repression (L9 and L18) presented as RQ relatively to control (wild-type; RQ = 1). Actin gene was used for reference. The results are presented as mean values ± SD (n = 3).

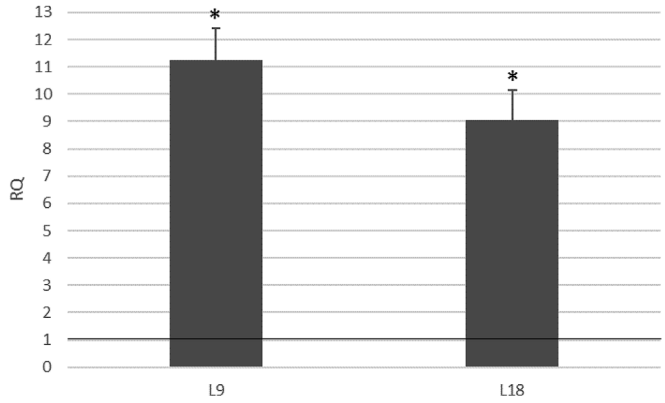

Fig. 10. The transcript level of chitinase gene in the L plants with lycopene β-cyclase gene repression (L9 and L18) presented as RQ relatively to control (wild-type; RQ = 1 marked with line). Actin gene was used for reference. The results are presented as mean values ± SD (n = 3).

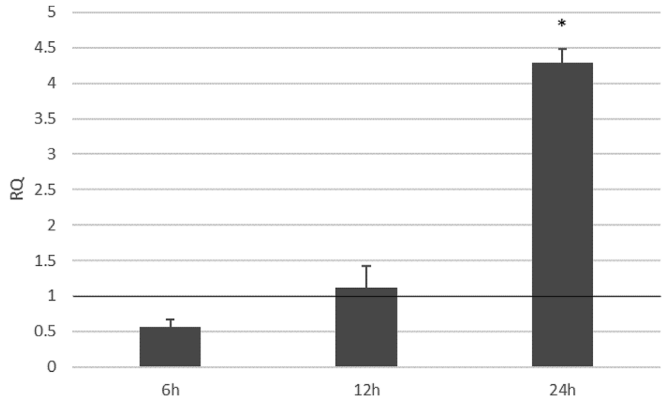

Fig. 11. Transcript level of chitinase gene in the non-transgenic plants treated with 100 μM abscisic acid presented as RQ compared to the non-treated control (RQ = 1 marked with line) measured at 6 h, 12 h and 24 h after the treatment. Actin gene was used for reference. The results are presented as mean values ± SD (n = 3).

sensitive to ABA in chitinase gene promoter: ABREOSRAB21, ABRELATERD1. In order to verify whether the elevated transcription of this gene is related to higher ABA contents in the L plants, non-transgenic flax plants were treated with 100 μM abscisic acid and transcript level of chitinase gene was measured. A 4-fold increase was noted at 24 h after ABA treatment (Fig. 11).

4. Discussion

Recently described transgenic plants with decreased level of endogenous lycopene β-cyclase (*Lulcb*) gene transcript (L lines) were generated through heterologous expression of *lcb* gene from *A. thaliana*. The observed inactivation of the *Lulcb* gene was ascribed to the co-suppression phenomena, however, no detailed explanation was presented. Although transgene-induced silencing in some plants appears to involve post-transcriptional level (post-transcriptional gene silencing, or PTGS), in others silencing occurs by gene-specific methylation (transcriptional gene silencing, or TGS) (Rajeevkumar et al., 2015). The study of DNA methylation in higher plants has focused mainly on the modification of stably integrated transgenes and relatively little is known about the methylation status of endogenous plant genes. The majority of DNA methylation in plants concerns the addition of a methyl group to position 5 of a cytosine residue lying within the sequence consensus CG, CNN, or CNG (Gruenbaum et al., 1981). It has been proposed that methylation of symmetrical sequences constitutes a signal for a maintenance methylase that adds methyl group to C residues in a newly synthesized strand, if the opposite strand carries a m5C residue in the complementary sequence. However, non-symmetrical methylation leading to silencing of endogenous genes has been also reported in plants (McGinnis et al., 2006). RNA-mediated epigenetic modifications are among the factors possibly involved in this process (Miki and Shimamoto, 2008).

Methylation sensitive restriction analysis of methylation pattern in the *Lulcb* gene indicated differences between the L lines and the control. Our conclusion is therefore, that the increased methylation of certain sites in the *lcb* gene sequence led to its suppressed expression. Why the sites +109 and + 297 are relevant in the *lcb* gene expression decrease requires further research. It must be noted, however, that only cytosine methylation in CCGG context was investigated. Since all cytosines may be methylated in plants, and moreover adenine nucleotide may also undergo methylation, additional analyses using different methods are needed (Vanyushin and Ashapkin, 2011). Total methylation level in the L plants was lowered, though such phenomena might have occurred during callus stage of plant agrotransformation process (Stroud et al., 2013). Elevation of the methylation level in a gene is usually connected with its repression (Cao and Jacobsen, 2002) and the participation of DNA (cytosine-5)-methyltransferase (cmt3) in RNA-dependent methylation of genomic DNA in plants is well recognized (Gohlke et al., 2013). Transcript level of the *cmt3* DNA methyltransferase gene was significantly elevated in the L plant lines and this correlated with higher content of abscisic acid. The promoter sequence of the *cmt3* gene contains cis-regulatory element responsive to ABA (ABRELATERD1). In addition, exogenous application of ABA caused increased *cmt3* gene expression (Supplementary Fig. S2). Although ABA-regulated genetic processes are well known, recent findings reveal that epigenetic processes are an integral part of ABA-regulated processes. The basic unit of eukaryotic chromosome is the nucleosome, consisting of a protein complex (H2A, H2B, H3 and H4) wrapped around by 146 bp DNA and the linker histone H1 associated with the linker DNA (8–114 bp) (Chinnusamy et al., 2008). Nucleosome structure depends on the histone variants and post-translational modifications in the N-terminal tails of core histones. Treatments of *Arabidopsis* with ABA or salt also triggered various fluctuations in the level of histone modification, namely, an enrichment of the H3K9K14ac and H3K4me3, normally associated with gene induction and a decrease of the H3K9me2 seen as having a repressive role on gene expression regulation (Chen et al., 2010). Although the obtained data on transcript levels of genes associated with histone modifications differed for both of the L lines, the dropdown in *h3k9me* gene expression was consistently decreased in both cases. The influence of ABA on DNA methylation changes may utilize the non-coding regulatory small RNAs (~20–24 nt long; miRNA, siRNA). RNA-dependent RNA polymerases (rdr) play a crucial role in siRNA and miRNA biogenesis. Promoter of *rdr6* gene contains an ABA-

responsive DNA cis-element and exogenous ABA application enhanced the *rdr6* transcript level in rice cells. Moreover, ABA silences the isocitrate lyase (*icl*) gene through the RDR6-generated siRNA(*icl*) mediated post-transcriptional gene silencing mechanism (Yang et al., 2008). *Arabidopsis* RDR2 is involved in transcriptional gene silencing (Chan et al., 2004). Non-coding RNAs can also induce epigenetic changes through chromatin or DNA methylation (Bond and Finnegan, 2007). Some ABA-induced small RNAs and *rdr*s can play a role in epigenetic regulation of genetic expression. ABA has been shown to up-regulate miR402 in *Arabidopsis*. The predicted target of ABA-up-regulated miR402 is At4g34060, which encodes a DNA glycosylase domain protein dml3 (DEMETER-LIKE PROTEIN 3) similar to ros1 (Sunkar and Zhu, 2004). Later it was shown that the expression of *dml3* mRNA was down-regulated in miR402-overexpressing transgenic plants (Kim et al., 2010). Transcription levels of *dme* and *ros1* genes, which are responsible for DNA demethylation, were significantly decreased in both the L lines. ABA-induced miRNAs might reduce the transcript levels of *dme* and *ros1* genes, which in turn can result in increases in methylation levels at certain loci and thus, repression of gene expression.

As described previously, the L type flax plants showed increased resistance to pathogen infection (Boba et al., 2011). It is suggested that 1%–10% of the plant genome is regulated by ABA, of which 25%–50% of the genes regulated by this hormone are also associated with response to drought and salinity (Seo and Koshiba, 2002). For a long time it was thought that the role of abscisic acid in response to pathogenic infection consists mainly in blocking pathogen access to plants. Closing the stomata and affecting the decomposition and callose synthesis in the cell wall, ABA blocks the entrance to many pathogens, which has a special role in the first phase of infection. Since this mechanism in many cases is sufficient to block the spread of the infection, ABA inhibits the development of the plant response via salicylic acid signaling, and blocks the formation of reactive oxygen species (ROS), which is intended to prevent unnecessary activation of “costly” defense mechanisms (Ton et al., 2009; Mauch-Mani and Mauch, 2005). ABA can function as a positive or a negative regulator of plant response depending on the plant–pathogen interaction analyzed. ABA (signaling or synthesis) mutants in tomato and *Arabidopsis* (*abi1-1, abi2-1, aba1-6, aba2-12, aao3-2,* and *pyr1pyl1pyl2pyl4*) were shown to overexpress defensive-signaling pathways, leading to enhanced resistance to different pathogens, such as *B. cinerea*, *P. syringae*, and *F. oxysporum*. During incompatible interactions between nonpathogenic strains of *Phytophthora sojae* and soybean, ABA decreases resistance. In case of *P. cucumerina* infection *Arabidopsis* plants impaired in ABA biosynthesis, such as *aba1-6*, or in ABA signaling, like the quadruple *pyr/pyl* mutant (*pyr1pyl1pyl2pyl4*), showed enhanced resistance to the fungus, while the *hab1-1abi1-2abi2-2* mutant impaired in three phosphatases that negatively regulate ABA signaling are more susceptible to this fungus. Interactions of ABA with the major hormones involved in the plant defense response (SA, JA, and ET) have been verified by exogenous hormone treatment. For instance, almost 65% of the up-regulated genes and 30% of the down-regulated genes in the *aba1-6* mutant were found to be up- or down-regulated by either ET, JA, or SA treatment. In addition, ABA plays a direct role in regulating R (resistance) protein activity. But abscisic acid can also positively regulate the resistance to some pathogens, such as *A. brassicicola*, *R. solanacearum*, and *P. irregulare*, as ABA-deficient and -insensitive mutants (*abi1- 1*, *abi2-1*, *abi4-1*, *aba1-6*, *aba2-12*, *aao3-2*, and *npq2-1*) were found to be more susceptible than wild-type plants to these pathogens. In *Arabidopsis*, ABA has been shown to be required for JA biosynthesis, which is essential for resistance to *P. irregulare*. However, ABA signaling and ABA-dependent callose formation are crucial for ABA-induced resistance against *P. cucumerina* and *A. brassicicola* in *Arabidopsis*. The ABA-deficient mutant *aba1-5*, the ABA insensitive mutant *abi4-1* and the callose-deficient mutant *pmr4-1* could not induce ABA resistance (Chen & Yu, 2014; Sanchez-Vallet et al., 2012; Denancé et al., 2013). ABA is a key hormone in *Arabidopsis* response to *R. solanacearum* infection, as 40% of the genes up-regulated during the development of wilting symptoms were related to ABA, including those encoding proteins for ABA biosynthesis (e.g. nced3). Numerous examples suggest that the role of abscisic acid in response to infection varies according to the analyzed interaction. There are no data indicating the function of this hormone in response to *F. oxysporum* f. sp. *lini* (Fol) infection in flax.

Reactive oxygen species (ROS) are produced by a plant being infected during the first reaction (O'Brien et al., 2012). The same ROS must be swiftly neutralized by the plant to prevent oxidation of cellular components, including nucleic acids, proteins, chlorophyll, and lipids. To cope with oxidative stress, plants have evolved two general functionally interlocked protective mechanisms, enzymatic and non-enzymatic detoxification. The ROS is especially dangerous to the photosynthetic systems, thus the plant requires an efficient system of quenching the free radicals preventing them from decoupling photosynthesis, with carotenoids and tocopherols being among them (Sakuragi et al., 2006). As in the L flax lines decreased levels of carotenoids were observed ((Boba et al., 2011), Supplementary Fig. S3), it was proposed that the substrates for carotenoid synthesis were rerouted to another branch of the terpenoid pathway – tocopherol synthesis. In fact, elevated levels of α- and β-tocopherols were noted for both the L flax lines. It can be assessed that stress-tolerant plants usually display increased tocopherol levels, but the most sensitive ones show tocopherol loss under stress, which leads to oxidative damage and cell destruction (Munné-Bosch, 2005). Tocopherol synthesis is regulated in plant responses to environmental stress, and stress sensitive hormones such as jasmonic acid, salicylic acid and abscisic acid appear to play a role. ABA-specific motif has been identified in the promoter region of the *hpd* gene (which catalyzes p-hydroxyphenylpyruvate conversion to homogentisate), which indicates that tocopherol biosynthesis may be stimulated by this compound (Munné-Bosch, 2005). However, it must be considered that a net activity of various antioxidants rather than solely tocopherols is necessary to fight plant pathogen infection, especially that when we investigated the direct influence of tocopherols on *Fusarium* growth by adding the compounds to the fungal growth medium, no anti-fungal activity of these compounds was observed (Supplementary Fig. S4).

In search of the origin of elevated ABA levels in the L lines we investigated the transcription levels of the genes leading to its biosynthesis. Abscisic acid origins from the carotenoid biosynthesis pathway. β-carotene is converted to xanthophylls, which undergo transformation to abscisic acid in a multistep reaction involving aba1, aba2, nceds and aao. The levels of β-carotene in the L flax plants as well as of other carotenoids were decreased. It corresponded with the significant decrease in the transcription of *psy* gene coding for phytoene synthase, the key-enzyme in carotenoid production (Liu et al., 2015). In general, down-regulation of the genes involved in the carotenoid biosynthesis was observed in both of the L flax lines. Despite decreased levels in ABA precursors and in the expression of the most of the genes involved in ABA synthesis, the content of this hormone was elevated in the L flax lines. Regulation of ABA level in plants is based on a dynamic balance between its synthesis and metabolism. In fact, in addition to ABA synthesis, catabolism is a major mechanism for regulating ABA levels (Finkelstein, 2013). This observation was confirmed by our study in flax plants. We investigated key genes involved in ABA catabolism and found out that their transcript levels were significantly decreased compared to the non-transgenic control. Hydroxylation of ABA is predominantly involved in homeostatic regulation of ABA, thus down-regulation of genes encoding the hydroxylases might be a natural response to decreased synthesis of the hormone (Endo et al., 2014). Reactive oxygen species were proposed among agents involved in the regulation of this homeostasis. Exogenous H_2O_2 and NADPH oxidase inhibitor increased ABA catabolism by enhancing the expression of *cyp707a* genes, which encode ABA 8′-hydroxylases in dormant *Arabidopsis* seeds and non-dormant barley seeds (Ishibashi et al., 2017). ROS molecules can be generated by NADPH oxidases, which were proposed

to mediate ABA-induced ROS generation (Kwak et al., 2003). Hence, H_2O_2 may be viewed as a mediator of positive feedback regulation of ABA synthesis (Xiong and Zhu, 2003). H_2O_2 is not only responsible for ABA level regulation but also plays an important role in ABA signaling. Moreover, recent studies have demonstrated the interplay between ABA and other plant hormones such as auxins, cytokinins, gibberellins, ethylene, and BRs in a number of physiological processes (Zhou et al., 2014; He et al., 2012; Chen et al., 2014; Zheng et al., 2015). With few exceptions, abscisic acid has been considered a negative regulator of disease resistance due to the interference of ABA with biotic stress signaling that is regulated by salicylic acid, jasmonic acid and ethylene (Mauch-Mani and Mauch, 2005). It is evident that ABA can positively prevent pathogen infection by functioning in PAMP-induced stomatal closure (Lim et al., 2015; Zeng and He, 2010). Upon the recognition of a successful invasion, the host plant would activate post-invasive resistance responses, such as rapid callose deposition and generation of ROS, during which ABA is involved. However, compared with its positive roles in stomatal innate immunity, the role of ABA in post-invasive resistance responses is controversial and seems to vary among different plant-pathogen interactions (Chen & Yu, 2014). In some plant–fungal–oomycetes interactions, ABA can exert a positive role by stimulating callose deposition or by modulating the priming of callose deposition (Ton and Mauch-Mani, 2004; Oide et al., 2013). Moreover, ABA is a stimulant of other pathogenesis related proteins like chitinase (Yu et al., 1998), also in flax, where after treatment with ABA we showed elevated chitinase gene transcript level as well as callose synthase (*pmr4*) gene expression (data not shown). In fact, ABRE motifs can be found in promoters of genes encoding chitinases and callose synthase. Chitinases act in at least two different ways: directly, by degrading the cell walls of the pathogen, and indirectly, by promoting the release of cell wall-derived materials that can act as elicitors of defense reactions, stimulating the production of other PR proteins and low molecular weight antifungal compounds, such as phytoalexins (Wojtasik et al., 2014). Experimental evidence suggests that ABA is a component of the signaling pathway activating plant defense against some, but not all the necrotrophic pathogens (Richa et al., 2016).

To conclude, in the type L flax plants the decreased expression of the endogenous *Lulcb* gene is connected with epigenetic effects, changed DNA methylation in coding region within CCGG motifs. This may be connected with increased expression of DNA methylase *cmt3* gene and repressed demethylase genes (*ros1* and *dme*). The *Lulcb* gene silencing is strictly dependent on its methylation pattern, however, further experiments, like *LuLCB* gene sequencing after bisulfite treatment, need to be planned to answer the questions such like which methylated motifs are responsible for silencing of the gene? Is this change stable through generations or the pattern is constantly re-created *de novo* due to the transgene presence? We have also shown flexibility of the terpenoid pathway. Decreased reservoir of substrates for abscisic acid synthesis caused by silencing flax *Lulcb* gene expression led to inhibition of ABA catabolism. The cellular ABA level fluctuates in response to physiological and environmental conditions, and these concentration changes determine ABA function in plant physiology and development. Plants continuously adjust the ABA level to meet physiological needs, but how ABA homeostasis occurs is not fully understood (Dong et al., 2014; Merilo et al., 2015). The interplay between ABA synthesis and catabolism may be one of the methods of regulating/maintaining this hormone concentration (Ji et al., 2011). The increased resistance to *Fusarium* of the L flax lines might result from the increased ABA levels leading to increase in PR gene expression. Further studies on understanding the regulation mechanisms of ABA level in flax plants are necessary.

Authors' contributions

AB performed all RT-PCR analyses, determined methylation levels, participated in writing the manuscript; KK performed azacitidine treatments, determined metabolite contents, wrote the manuscript and calculated statistics; MP performed ABA treatment; WW performed infection tests; JS participated in designing the study and helped in discussion writing; AK participated in writing the manuscript and study designing, was the author of the general concept.

Competing interests

The authors have no competing interests to declare.

Acknowledgements

This study was supported by grants No. 2013/11/B/NZ9/00150 and 2014/15/B/NZ9/00470 from the National Science Centre (NCN, Poland) and Wroclaw Centre of Biotechnology, The Leading National Research Centre (KNOW) programme for the years 2014–2018.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2018.03.023.

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