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KF and EW designed and carried out the experiments, analyzed the data, and wrote the manuscript; AK carried out the experiments and prepared the figures and charts; AZ, KZ, JDA, and AK conducted the microscopy experiments; JK helped in the manuscript preparation

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ORIGINAL RESEARCH PAPER

Ethylene-dependent effects on generative organ abscission of *Lupinus luteus*

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Abstract
The abscission of certain organs from the plant is part of the fulfilment of its developmental programs. The separation process occurs in a specialized abscission zone usually formed at the base of detached organ. The changing level of phytohormones, particularly ethylene, is the element responsible for coordinating anatomical and physiological transformation that accompanies organ abscission. The application of ethylene (ET) on *Lupinus luteus* stimulates flower abortion. However, the treatment with 1-aminocyclopropane-1-carboxylic acid (ACC) – direct ET precursor – does not cause such a strong physiological response. In turn, when applied on the pedicels both ET biosynthesis (2-aminoethoxyvinylglycine; AVG) and action (norbornadiene; NBD) inhibitors reversed the stimulatory effect of ET on generative organ separation. In order to determine ET role in the flower abscission process in *L. luteus*, we identified the sequences coding for synthase (*LlACS*) and oxidase (*LlACO*) of ACC and measured their expression levels. Abscission zone activation is accompanied by a considerable increase both in *LlACS* and *LlACO* cDNAs and also ACC content, which is specifically localized in the dividing cells at the base of the flower being detached. Obtained results suggest that ET is a strong stimulator of flower abortion in *L. luteus*.

Keywords
1-aminocyclopropane-1-carboxylic acid; ethylene; *Lupinus luteus*; phytohormones; organ abscission

Introduction

In yellow lupine, flower formation and development processes are of crucial importance for its productivity, but entail excessive and premature flower abscission. Depending on the level of the inflorescence, between 50 and 90% of all the flowers set are abscised [1,2]. Flower abscission takes place in the abscission zone (AZ), which is located at the base of the pedicel, while this process is related to anatomical and physiological changes coordinated by phytohormones [3,4]. Ethylene (ET) is one of the strongest organ abscission stimulators, as it regulates the function of hydrolytic enzymes (polygalacturonases, peroxidases, esterases, expansins) and, therefore, controls the activation of the cells within separation layer in the AZ [4–6]. Almost every plant tissue is able to produce ET, but in most cases its level is relatively low, only increasing

in certain periods of the plant development, such as seeds germination, stem and root growth, abscission of flowers and leaves, fruit ripening, as well as senescence [7]. Other phytohormones affect ethylene production as well (auxins, jasmonates, abscisic acid), similarly to biotic and abiotic external factors that, in ways differentiated in terms of time and space, regulate the transcriptional activity of genes encoding enzymes involved in their biosynthesis [8–11]. For this process, of key importance are the synthases (ACSs) and oxidases (ACOs) of 1-aminocyclopropane-1-carboxylic acid (ACC) that catalyze the transformation of *S*-adenosyl-methionine (SAM) into ACC and oxidize ACC to ET, respectively [12,13].

In order to study the effect of ET on flower abscission in yellow lupine, we carried out physiological experiments using the solutions of exogenous hormone and the inhibitors of its biosynthesis and action. Moreover, we identified the sequences coding for the synthase and oxidase of ACC (*LlACS*, *LlACO*) and examined their expression patterns in the pedicels of generative organs during their development, and in the pedicels of flowers with an active and inactive abscission zone. We also measured the endogenous ACC content and we determined its cellular localization within the AZ.

Material and methods

Plant material and growth conditions

Plant material, epigonal cultivar Taper of yellow lupine (*Lupinus luteus* L.), was prepared according to Frankowski et al. [14]. The plants were grown in a growth chamber in plastic pots under the conditions as described by Frankowski et al. [14].

Application of plant hormones, inhibitors, and precursors

In order to evaluate the influence of exogenous phytohormones in flower abscission, several treatments were performed. ACC (0.1 mM) or aminoethoxyvinylgycine (AVG; 0.1 mM) solutions in 0.05% Tween 20 were applied by small brushes onto pedicels with an inactive AZ (pedicels of fully developed flowers containing green pedicels). Moreover, gaseous ET or 2,5-norbornadiene (NBD) at concentrations of 100 μL L^{-1} were applied via a syringe with septum into 9-L glass containers containing pots with growing lupins. The control plants were treated with 0.05% Tween 20 solution. At least 15 plants were used in each treatment. After the completion of treatments, the flower abortion rate (%) was determined. All treatments were carried out in three independent biological replications.

Molecular cloning of *LlACS* and *LlACO* cDNAs

The floral pedicels of *L. luteus* (1.0 g) were mechanically homogenized and total RNA was isolated according to procedures described in the instructions of NucleoSpin RNA Plant Kit (Macherey-Nagel GmbH & Co. KG, Germany). All designed primers for the studied genes were supplied by Genomed S.A. (Poland). First-strand cDNA was synthesized from 1 μg of total RNA using the Transcriptor High Fidelity cDNA Synthesis Kit (ROCHE Diagnostics GmbH, Germany) following the manufacturer's instruction. In order to identify *LlACO*, we carried out PCR reactions in the T3 Thermocycler (Biometra, Germany). The degenerated primers (Tab. 1) were designed on the basis of conserved fragments of genes encoding *ACOs* in *Lycopersicon esculentum* (GenBank accession No. AJ715790), *Nicotiana tabacum* (GenBank accession No. AB012857), *N. attenuata* (GenBank accession No. AY426756), *N. glutinosa* (GenBank accession No. U62764), *Solanum tuberosum* (GenBank accession No. AY098939), and *Arabidopsis thaliana* (GenBank accession No. AF016100). The sequence of *LlACS* was obtained in PCR reactions by using specific primers listed in Tab. 1, constructed for EST fragments of *ACS* from *Lupinus albus* (EST sequence in the NCBI database of

Tab. 1 Specific primers and probes used in PCR reactions.

Gene name	GeneBank accession No.	Primer sequence 5′–3′	UPL probe No.	Product size (bp)
LlACS	KF573522.1	Specific		
		F: GTGACAAACCCATCCAACCCACT		880
		R: ACACACCAACCATGACCAGGCTAT		
		RACE-PCR		
		F: TGGGTTTACCGGGTTTTCGCGTTGG		870
		R: TTCCTTGAGGACTTCCATGACGCTG		1245
		qRT-PCR		
		F: TTCATTCAAGAAGGCAATGGT	53	71
		R: GGTTTGGGTCAAAAGTCACC		
LlACO	KF573523.1	Degenerate		467
		F: AAGRTTCAAGGAAATGGTGGCAAG		
		R: TCRCCRAGGTTGAYGACAATGGAG		
		RACE-PCR		
		F: TTGCTGTGTGAAAACCTTGGGCTGG		860
		R: ACAGCAAGTCAAGAAGTTGCTCTGCCA		490
		qRT-PCR	53	74
		F: GTGATGAAGGAATTTGCACAAG		
		R: CCAAGGTTTTCACACAGCAA		
LlACT	KP257588	qRT-PCR	165	76
		F: TAATGGTTGGGATGGGTCAG		
		R: TTCAAGGTGAGAATACCCCTCT		

F – forward primer; R – reverse primer.

L. albus: No. AF119413.1). Each PCR reaction mixture, containing 2 µL of obtained cDNA, 2 µL of primers solution (10 µM), 5 µL of 10× TrueStart Hot Start Taq buffer, 4 µL of 25 mM Mg^{2+}, 5 µL of 2 mM dNTP mix, and 1.25 U of TrueStart Hot Start Taq DNA Polymerase (Fermentas, USA), was subjected to the thermal cycling conditions as follows: 95°C for 5 min – one cycle, 95°C for 1 min, 66–58°C for 45 s, 74°C for 45 s – 29 cycles, 74°C for 5 min – one cycle . In order to isolate amplified products from an agarose gel, the GeneMATRIX Agarose Out DNA Purification Kit (EurX, Poland) was used. Subsequently, cDNA fragments were cloned with using the Strata Clone PCR Cloning Kit (Agilent Technologies, USA) and then sequenced by Genomed S.A.

A BD SMART RACE cDNA Amplification Kit (Clontech-Takara Bio Europe, France) was used to prepare amplification of complementary DNA ends (RACE)-ready cDNA . Specific 5′-RACE and 3′-RACE primers for *LlACS* and *LlACO* were designed with public version of the software Fast PCR (http://primerdigital.com/fastpcr.html) and all the reactions were performed using the Advantage 2 PCR Enzyme System (Clontech-Takara Bio Europe, France). Purified RACE products were cloned by Strata Clone PCR Cloning Kit and transformed using StrataCloneSoloPack Competent Cells (Agilent Technologies, USA). Plasmid DNA containing the cloned RACE products was isolated with the GeneMATRIX PLASMID MINIPREP DNA Purification Kit (EurX, Poland). Each DNA product was sequenced by Genomed S.A.,

whereas full-length sequences of examined genes were deposited in the GenBank database. Bioinformatic analysis was performed using ClustalW (http://www.genome.jp/tools/clustalw/), BLAST 2.2.25 (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and ExPASY (http://www.expasy.org) applications.

LlACS and *LlACO* expression analyses in pedicels

LlACS and *LlACO* expression analyses were performed for pedicels collected in the subsequent developmental stages (photograph in Fig. 1) and in naturally active (NA) or artificially activated (AC) AZ described by Frankowski et al. [15]. The control, green pedicels, which showed no symptoms of senescence were excised from fully opened flowers from sixth stage of their development (inactive AZ, IN).

Real-time quantitative PCR analyses of the *LlACS* and *LlACO*

Each amplification reaction mixture for *LlACS* and *LlACO* expression was prepared with using a LightCycler TaqMan Master Kit (ROCHE Diagnostics GmbH, Germany) and carried out on the LightCycler 2.0 Carousel-Based System instrument (ROCHE Diagnostics GmbH, Germany). The cDNA templates for transcriptional activity analysis were prepared similarly as for genes molecular cloning. Specific primers for examined genes (*LlACS*, GenBank accession No. KF573522.1 and *LlACO*, GenBank accession No. KF573523.1) and gene specific hydrolysis probe (UPL) were designed by Universal Probe Library Assay Design Center (https://qpcr.probefinder.com/organism.jsp) (Tab. 1). Expression was normalized to the housekeeping gene *LlACT* (GenBank accession No. KP257588.1). Transcripts were amplified under following qPCR conditions: one cycle at 95°C for 10 min, 45 cycles at 95°C for 10 s, 58°C for 30 s, 72°C for 1 s, one cycle of cooling at 40°C for 30 s.

The qPCR mixtures consisted of 1 µL of first strand cDNA, 0.4 µL of the gene specific primers solution (10 µM), 0.2 µL of the UPL probe, and 4 µL of TaqMan Master Mix, containing a reaction buffer, dNTP mix, and DNA Polymerase (ROCHE Diagnostics GmbH, Germany). Serial dilutions of cDNA were used to generate a standard curve for relative quantification. The data were analyzed and presented with LightCycler Real-Time PCR Systems software (ROCHE Diagnostics GmbH, Germany), MS Office Excel (Microsoft), and SigmaPlot 2001 v.5.0. qPCR reactions were carried out in triplicate for each RNA template. Data are presented as mean ± standard error (*SE*).

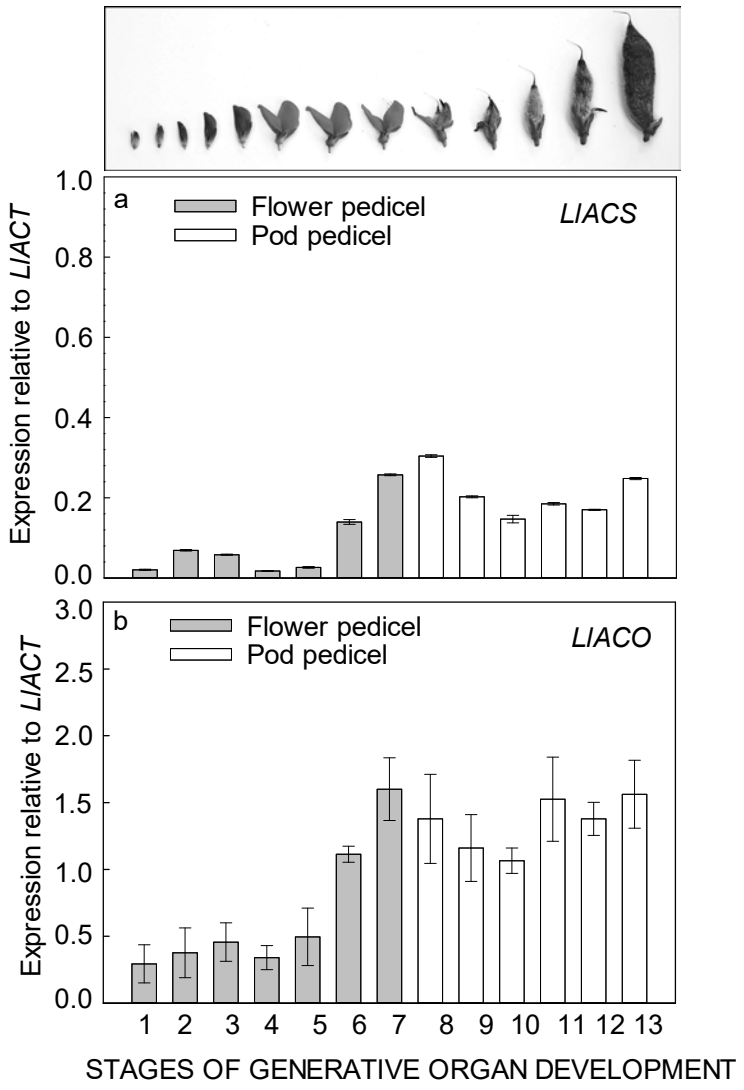

Fig. 1 Expression pattern (related to *LlACT*) of ethylene biosynthesis genes *LlACS* (**a**) and *LlACO* (**b**) in the pedicels during generative organ development. *SE* is marked at the bars.

Microscopy sample preparation and immunofluorescence experiments

Pedicels to microscopy analysis were prepared according to Frankowski et al. [15]. For

immunofluorescence studies, the sections were incubated overnight at 4°C in primary antibody solution prepared by dilution the primary antibody (anti-ACC) 1:50 in 1% bovine serum albumin (BSA) prepared in 1× PBS (pH 7.2). A DyLight Alexa 488 conjugated IgG diluted 1:250 in PBS buffer for 2 h at 37°C served as the secondary antibody. The samples were observed in a Leica DMI4000B inverted microscope using the filter combination: BP365, FT395, and LP397.

Determination of ACC levels

The tissue fragments (0.5 g) containing AZ were homogenized in liquid N_2, extracted twice in 5% sulphosalicylic acid (1.5 mL) for 24 h at 21°C and centrifuged. 500 ng of d_2ACC internal standard was added before first extraction. All subsequent procedures of ACC level isolation and determination were carried out according to the modified procedure of Kęsy et al. [16]. Fractions from the HPLC, containing phthalimido-ACC derivative, collected at 12.8 ±0.5 min, were evaporated to dryness, methylated with diazomethane, dissolved in 50 µL of methanol and analyzed by GC/MS-SIM following to conditions described by Kęsy et al. [16].

Results

Ethylene effectively stimulates flower abscission in *L. luteus*

Lupinus luteus grown under phytotron conditions formed 15 flowers per plant on average, of which 40% were aborted (Tab. 2). Under the effect of exogenous ACC and ethylene, the number of flowers abscised grew to 49% and 95%, respectively. In turn, the treatment with inhibitors of ET biosynthesis (AVG) or action (NBD) reversed the stimulatory effect of ET on generative organ abortion in such a manner that 15% and 5% of the flowers, respectively, were abscised (Tab. 2).

Tab. 2 The effect of ET, AVG, NBD, and ACC treatment on flower abortion rate in *L. luteus*.

	Control	ET	NBD	ACC	AVG
Flower abortion rate (%)	40	95	5	49	15

Pedicels were treated with ethylene (ET) precursor ACC (1-aminocyclopropane-1-carboxylic acid; 0.1 mM) or ET biosynthesis inhibitor AVG (aminoethoxyvinylgycine; 0.1 mM) solutions in 0.05% Tween 20. ET or its perception inhibitor NBD (2,5-norbornadiene) at concentrations of 100 µL L^{-1} were put via a syringe into the glass containers. The control plants were treated with 0.05% Tween 20 solution. At least 15 plants were used in each treatment. Upon completion of the treatments, the flower abortion rate was determined. All treatment experiments were designed in three independent biological replications.

Isolation of *LlACS* and *LlACO* cDNAs

The full-length *LlACS* (GenBank accession No. KF573522) and *LlACO* (GenBank accession No. KF573523) cDNAs were obtained by the traditional PCR (Fig. S1a, Fig. S2a) and 5′–3′-RACE PCR (Fig. S1b,c, Fig. S2b,c) methods, and are composed of 1859 bp and 1235 bp, respectively. The predicted amino acid LlACS (486 aa) includes seven conserved regions among ACC synthases (Fig. S1d). One of the domains contains the active site – lysine residue, which binds to pyridoxal 5′-phosphate and *S*-adenosyl-L-methionine [17]. Moreover, the LlACS sequence contains 11 amino acids characteristic for ACSs and various aminotransferases (Fig. S1d) [18,19]. The full-length of *LlACO* cDNA encoded 317 amino acids (Fig. S2d). The predicted LlACO sequence and ACC oxidases from other plant species were compared and it was found that the LlACO protein also included conserved motifs: a cofactor and a cosubstrate binding [Hsp177-X-Asp179-X(54)-Hsp234 and Arg244-X-Ser246, respectively] [20] (Fig. S2d). Moreover, the C-terminus of LlACO protein contained the Lys and Arg residues essential for enzymatic activity (Positions 294–301).

Multiple amino acid sequences alignment demonstrated that the predicted LlACS is closely related to the ACS from *Vigna radiata* (86%; GenBank accession No. AAD41083), *Medicago truncatula* (83%; GenBank accession No. XP003611535), and

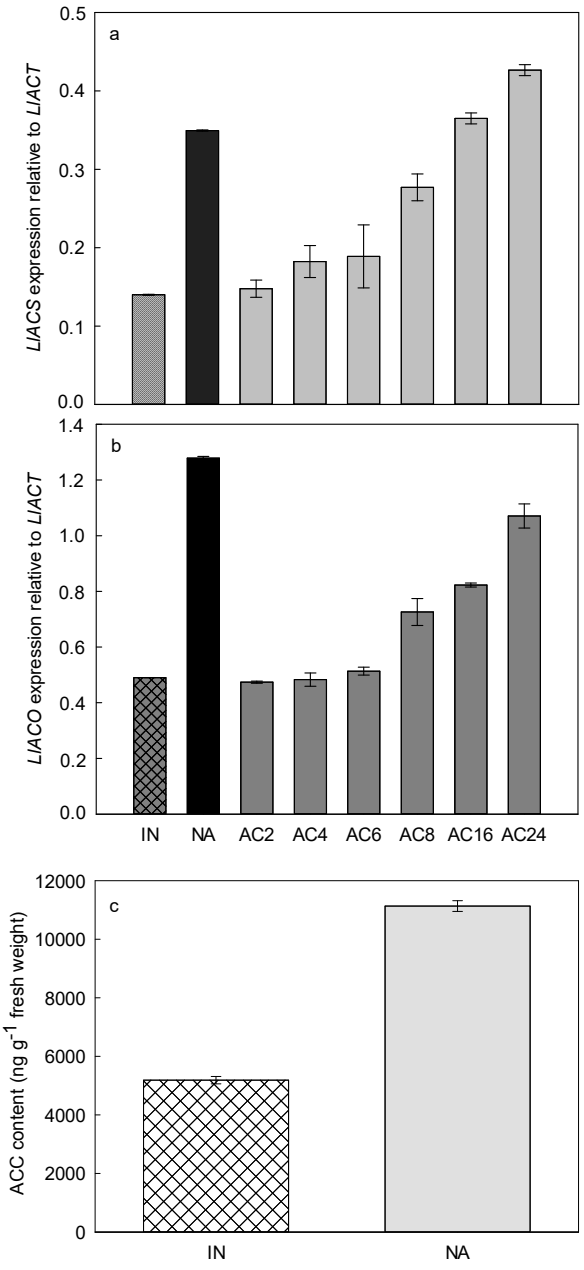

Fig. 2 Expression analysis (related to *LlACT*) of the *LlACS* and *LlACO* ethylene biosynthesis genes, and the ACC level in the AZ. The *LlACS* (**a**) and *LlACO* (**b**) transcriptional activity was measured in flower pedicels with an inactive (IN) abscission zone (AZ), the AZ from naturally abscised (NA) flowers, and the abscission zone activated (AC) by flower removal after 2, 4, 6, 8, 16, and 24 h. **c** The level of ACC in the pedicels with the IN or NA abscission zone. *SE* is marked at the bars.

Pisum sativum (83%; GenBank accession No. AAD04198) (Fig. S1e). In turn, a comparison of LlACO with ACOs from other plant species showed that LlACO has 86% sequence identity to ACOs from *Vigna radiata* (GenBank accession No. CAJ56064) and *Glycine max* (GenBank accession No. NP001241899), and is also closely related (85%) to ACOs from *Trifolium repens* (GenBank accession No. AAD28196), *Medicago sativa* (GenBank accession No. ABF61805), and *Carica papaya* (GenBank accession No. AAK57516) (Fig. S2d).

Activation of the AZ is correlated with changing *LlACS* and *LlACO* expression and the ACC level

The transcriptional activity of both *LlACS* and *LlACO* was the highest in the pedicels of fully developed flowers and young pods (Fig. 1a,b). In the pedicels from naturally abscised flowers, a significant accumulation of both *LlACS* and *LlACO* transcripts was observed (Fig. 2a,b). Additionally, artificial AZ activation (flower removal) caused gradually increase of the the mRNA level of both the genes in comparison to control plants (an inactive AZ) (Fig. 2a,b). The transcriptional activities of the studied genes were also correlated with the level of ET precursor (Fig. 2c), which was specifically localized within the dividing AZ cells (Fig. 3c–f).

Discussion

The mechanism of organ abscission has not been completely clarified. The separation of some plant organs is preplanned in its developmental programs. This phenomenon is first of all connected with plant reproduction, protection mechanisms, and getting rid of organs which have lost their function. The abscission process takes place in the specialized abscission zone, which forms at a place and time characteristic of particular plant species [3]. As we previously showed, the floral abscission zone in *L. luteus* is located at the base of the pedicel. In this region, cells are round, contain dense cytoplasm, and are smaller than adjacent cells below and above AZ [15].

In general, a change in the transcriptional activity of genes coding for transcription factors and cell wall hydrolyzing enzymes leads to the AZ cells activation and, as a consequence of that, to maturation in the distal part of the separation layer. The changing level of phytohormones is a factor responsible for coordinating the anatomical and physiological changes accompanying organ abscission [9].

Due to the pivotal role of ethylene in the senescence and abscission of different plant organs, we studied the effect of this hormone and of its precursor (ACC) on flower abortion in *L. luteus*. As our physiological studies suggest, ethylene and ACC increased the number of abscised flowers by 95% and 49%, respectively (Tab. 2). By contrast, the application of ethylene biosynthesis or action inhibitors (AVG or NBD) significantly decreased the stimulatory effect of ethylene on flower abscission (Tab. 2). Similar results relating to the effect of ethylene and its inhibitors on the organ AZ were obtained for other plant species, including *A. thaliana*, apple, citrus, and *Pelargonium* [21–25].

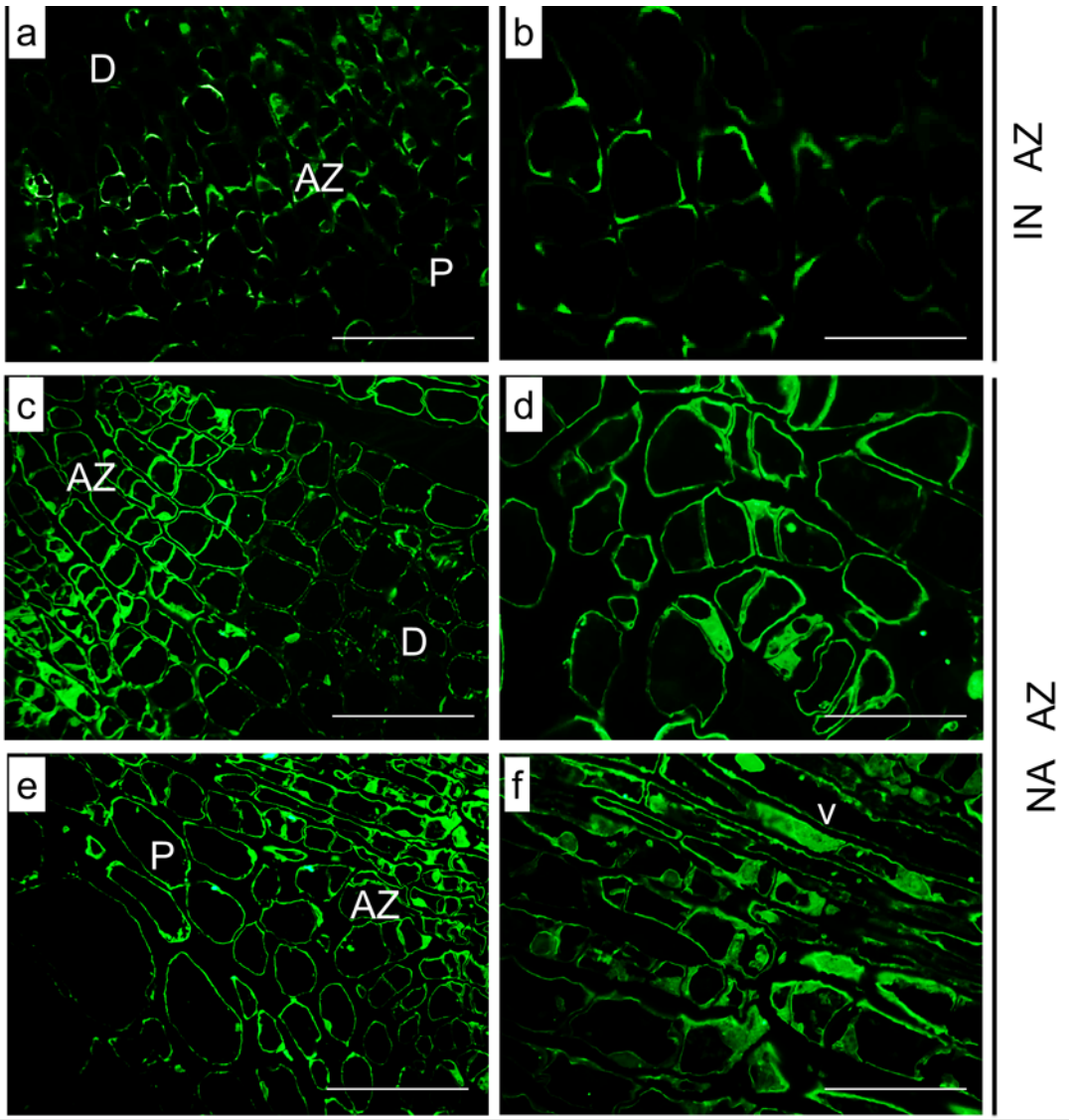

Fig. 3 Immunolocalization of ACC (anti-ACC Ab) in the pedicels with IN (**a,b**) or NA abscission zone (**c–f**). The symbols are: ACC – 1-aminocyclopropane-1-carboxylic acid; P – proximal zone; AZ – abscission zone; D – distal zone; v – vascular bundle. Scale bars: **a,c,e** 400 µm; **b,d,f** 100 µm.

Ethylene is commonly considered as the effector of separation processes induced by various stress conditions [26]. Both internal conditions and environmental factors may control the ethylene production process by affecting the transcriptional activity of the gene coding for ACC synthases and/or oxidases [27,28]. Considering that, in this study we identified those genes in yellow lupine (*LlACS*, *LlACO*) (Fig. S1, Fig. S2). The alignment of LlACS/LlACO deduced amino acid sequences and ACSs/ACOs from other species, revealed that both LlACS and LlACO contain all of the characteristic motifs found in their homologous (Fig. S1d, Fig. S2d). In the predicted LlACS, there were seven evolutionarily conserved regions found in all ACC synthases (Fig. S1d) [29–32], and conserved amino acid residues that are important for enzymatic activity [18,19], e.g., the Lys residue (V region) contained in the active center and involved in cofactor (PLP) and substrate (SAM) binding [17], as well as the Glu residue (I region) responsible for substrate specificity [33]. The Ser residue, located at Position 460 of the LlACS protein, is a part of the characteristic tripeptide R/K-L/V-S and may, similarly to ACC synthases from other plant species, be the place of phosphorylation [34]. In turn, identified LlACO contained motifs that are characteristic for ACC oxidases: the Fe(II) binding pocket containing three conserved amino acid residues (H, D, H) [20]. The sequence also contained residues of Lys and Arg that are important for oxidase enzymatic activity. Therefore, it appears that LlACS and LlACO encode for functional enzymatic proteins.

Due to the impossibility of determining gaseous ethylene production in flower and pod pedicels in *L. luteus*, we studied the transcriptional activity of the identified sequences encoding the genes of its biosynthesis. We found that the level of *LlACS* and *LlACO* mRNAs kept changing across the subsequent developmental stages and was the highest in the pedicels of fully developed flowers and young pods (Stage 7–8) (Fig. 1). That elevated expression of both the genes in Stage 7–8 may be related to abscission zone activation and/or be a consequence of the existence of a positive feedback loop between ethylene produced by ageing components of the perianth and the genes of ACC synthases and oxidases. We claim so after [25], who in their research on *Pelargonium* proved an increased transcriptional activity of the *GACS2* and *GACO2* genes involved in the abscission of some components of the perianth. Additionally, prior to petal abscission in *Rubus idaeus*, *Digitalis*, banana, rose, and *Pelargonium*, ethylene production significantly increased [35–40].

The level of *LlACS* and *LlACO* expression as well as the content of the ethylene precursor, ACC, was over two times larger in the pedicels of flowers aborted naturally in comparison with the pedicels with an inactive abscission zone (Fig. 2a–c). Furthermore, ACC was localized especially within the dividing cells of the AZ and in the vascular bundles of the pedicel (Fig. 3e–f). The results obtained suggest that a substantial portion of the ACC pool present in the AZ originates from de novo synthesis, although it is not excluded that the particle may also be transported from other parts from the plant. The accumulation of studied genes transcripts related to the process of natural generative organ abscission was also confirmed by research in which the AZ was activated artificially by removal of flowers (Fig. 2a,b). The increasing expression of the genes coding for ET biosynthesis enzymes, the increase in the ACC content, as well as studies of ACC immunolocalization in the abscission zone, all suggest that ethylene is directly involved in the functioning of the flower abscission zone in *L. luteus*. This hypothesis was also confirmed by the results of research performed in other plant species, in which the factors activating abscission of particular organs stimulate the expression of both ACC synthases and oxidases, e.g., in apple (*MdACO3*, *MdACO4*, *MdACO1*) or tomato (*SlACO5*, *LACS2*) [41–44]. Moreover, it was found that immediately before flower abscission in *Ecballium elaterum* and *S. lycopersicum* [45,46], the ethylene production rate increases. Not all of the results obtained so far in respect of ET role in regulating flower abscission are explicit. Analyses of *A. thaliana* (*etr1-1*, *ein2*) and *S. lycopersicum* (*Nr*) mutants show that ET determines the organ abortion time [3]. There are also such mutants as *Sletr1-2* (*S. lycopersicum*) and *dab* (*A. thaliana*), which are characterized by normal sensitivity to ET and that do not abort organs. On this basis, the existence of two pathways leading to plant organ separation was proposed: one ET-dependent and one ET-independent.

The research results presented in this paper indicate that in *L. luteus*, there is an ethylene-dependent pathway controlling flower abscission. The transcriptional activity of this phytohormone biosynthesis genes, as well as the changes in the ET precursor content, unambiguously indicate that ET plays a substantial role both in the formation and functioning of the AZ in this species. Therefore, ET is one of the factors responsible for earlier abortion of generative organs in *L. luteus* and, as a result of that, for the decreased yielding.

Supplementary material

The following supplementary material for this article is available at http://pbsociety.org.pl/journals/index.php/asbp/rt/suppFiles/asbp.3540/0:

Fig. S1 Molecular cloning of *LlACS* cDNA.

Fig. S2 Molecular cloning of *LlACO* cDNA.

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