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ORIGINAL ARTICLE

Tissue-specific production of limonene in *Camelina sativa* with the Arabidopsis promoters of genes *BANYULS* and *FRUITFULL*

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Abstract

Main conclusion **Arabidopsis promoters of genes *BANYULS* and *FRUITFULL* are transcribed in Camelina. They triggered the transcription of *limonene synthase* and induced higher limonene production in seeds and fruits than *CaMV 35S* promoter.**

Camelina sativa (Camelina) is an oilseed crop of relevance for the production of biofuels and the plant has been target of a recent and intense program of genetic manipulation aimed to increase performance, seed yield and to modify the fatty acid composition of the oil. Here, we have explored the performance of two *Arabidopsis thaliana* (Arabidopsis) promoters in triggering transgene expression in Camelina. The promoters of two genes *BANYULS* ($AtBAN_{pro}$) and *FRUITFULL* ($AtFUL_{pro}$), which are expressed in seed coat and valves of Arabidopsis, respectively, have been chosen to induce the expression of *limonene synthase* (*LS*) from *Citrus limon.* In addition, the constitutive *CaMV 35S* promoter was utilized to overexpress *LS* in Camelina . The results of experiments revealed that $AtBAN_{pro}$ and $AtFUL_{pro}$ are actively transcribed in Camelina where they also retain specificity of expression in seeds and valves as previously observed in Arabidopsis. *LS* induced by $AtBAN_{pro}$ and $AtFUL_{pro}$ leads to higher limonene production in seeds and fruits than when the *CaMV 35S* was used to trigger the expression. In conclusion, the results of experiments indicate that $AtBAN_{pro}$ and $AtFUL_{pro}$ can be successfully utilized to induce the expression of the transgenes of interest in seeds and fruits of Camelina.

Keywords *BANYULS* · *FRUITFULL* · Camelina · Metabolic engineering · Oilseed crop · Limonene

Abbreviations

BAN	BANYULS
FUL	FRUITFULL
LS	Limonene synthase
TT2	Transparent testa 2
TT8	Transparent testa 8
TTG1	Transparent testa glabra 1

Introduction

Arabidopsis thaliana (Arabidopsis) is the best characterized species of the plant kingdom, and function and pattern of expression of many of its genes and promoters are known in deep detail. Therefore, Arabidopsis offers a plethora of promoters that can be potentially used for target manipulation of economically relevant crops (Zhang et al. 2004; Kasuga et al. 2004; Hu and Xiong 2014). *Camelina sativa* L. Cranz (Camelina), a plant of the Brassicaceae family as Arabidopsis, is an oilseed crop that has recently

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been reassessed for the production of biodiesel (Lebedevas et al. 2013; Petrie et al. 2014; Wu and Leung 2011; Biello 2009). Genetically, Camelina has twenty pairs of chromosomes (n=20) organized in an allohexaploid genome (Hutcheon et al. 2010) and shows very high level of conservation with Arabidopsis (Kagale et al. 2014). From an agronomical point of view, the plant is considered low-cost crop management because it requires little input of fertilizers and irrigation, and it has the advantage of being compatible with the existing farm equipment (Putnam et al. 1993; Sawyer 2008.). Hence, the oil from Camelina is designated as low emission biodiesel and it is price competitive with traditional biofuels (Retka-Schill 2008; Sawyer 2008; Bernardo et al. 2003; Ciubota-Rosie et al. 2013; Lebedevas et al. 2013). Recently, an extensive program of genetic improvement has been successfully launched with the aim to make Camelina more competitive as biofuel crop and some of the tangible results recently achieved include fast growing lines with higher yields and increased seed storage of modified fatty acids (Roy Choudhury et al. 2014; Zhang et al. 2012; Nguyen et al. 2014; Horn and Chapman 2012; Horn et al. 2013).

Terpenes are the most diverse group of plant secondary metabolites, which includes natural essential oils used as additives for foods and cosmetics, drugs, rubber and lubricants (Degenhardt et al. 2009). In nature plant terpenes are synthesized by terpene synthases (TPS) that act in the cytosol and/or plastids of the cell, and they can further be modified by oxidases present in mitochondrion and ER (Aharoni et al. 2005). The production of terpenes is often localized in tissues with specialized metabolism and storage capabilities (Lange and Turner 2013). For example, many plants of the Lamiaceae and Solanaceae families, which produce a large number of terpenes, synthesize and store secondary metabolites in glandular trichomes that can be seen as plant phytochemical factories (Lange 2015). These are composed of enlarged epidermal cells often gathered together in multicellular structures and covered with a protective layer of cuticle. Limonene synthase (LS), the TPS enzyme that catalyzes the conversion of geranyl diphosphate (GPP) into the monoterpene limonene has also been shown to localize in glandular trichomes of mint (Turner et al. 1999). After its first isolation from mint leaves, *LS* gene has been cloned from other species and successfully utilized to increase the production of limonene in plants and organisms which naturally produce it in small or null amount (Lucker et al. 2001, 2002, 2004; Ohara et al. 2003, 2010; Augustin et al. 2015; Jongedijk et al. 2014). Recently, limonene has been proposed as additive for biodiesel and fuel propellant used alone or in blends with other terpenes (Tracy et al. 2009; Tomsheck et al. 2010; Peralta-Yahy et al. 2011; Zhang et al. 2011).

To further ameliorate the performance of Camelina we have investigated the use of promoters from Arabidopsis to induce the expression of *LS* in tissues relevant for oil production. To this purpose, the promoters of genes *BANYULS* ($AtBAN_{pro}$) and *FRUITFULL* ($AtFUL_{pro}$) have been chosen because of the expression in seeds and valves, respectively. In addition, the constitutive 35S promoter has also been utilized and its strength in inducing *LS* expression compared to that of $AtBAN_{pro}$ and $AtFUL_{pro}$. Our study shows that $AtBAN_{pro}$ and $AtFUL_{pro}$ trigger limonene production higher than the *CaMV 35S* and retain specificity of expression in seeds and fruits. Therefore, they can be successfully utilized to induce the expression of genes of interest in Camelina.

Materials and methods

Plant material, transformation and selection

Camelina sativa var. calena (Camelina) was grown in Styrofoam pots of 5 cm in diameter (two plants per pot) under standard greenhouse conditions (temperature 23 °C; relative humidity 70 %; photoperiod 16–8 h light–dark; maximum light intensity 500 µmol m^{-2} s^{-1}). Plants were watered once a day with distilled water alternated with commercial all-purpose plant fertilizer (Miracle Gro, The Scotts Company, Marysville, OH) for approximately 50 days. At the beginning of the blooming period, batches of approximately forty plants were transformed twice by vacuum infiltration as described by Lu and Kang (Lu and Kang 2008), with an interval of 7 days between the two transformation events. *Agrobacterium tumefaciens* strain GV3101::mp90(RK) was used for plant transformation. T1 seeds were surface sterilized with 70 % ethanol for 2 min, 35 % bleach for 10 min and thoroughly rinsed with distilled water before being placed on half strength MS salts, pH 5.5 (Caisson Laboratories, UT, USA), solidified with 15 g L^{-1} of agar and containing 80 mg L^{-1} of phosphinothricin (Basta; Goldbio Technologies, St. Louis, MO, USA). Seedlings that survived from the selection were transferred to soil, genotyped and grown to maturity. T2 seeds were also germinated on plates prepared with Basta and only the seedlings that segregated with a ratio of 3:1 (indicative of a single insertion event) were further genotyped and grown to maturity.

Constructs for plant transformation

The full length cDNA sequence of limonene synthase (LS_{cp}) from *Citrus lemon* L. Burm f. (lemon) (GenBank AF514287, isolate C62) and its shorter cDNA fragment lacking the first 153 nucleotides encoding the plastid transit

peptide (LS_{ct}) were optimized for expression in Camelina and synthesized at GeneScript (Piscataway, NJ, USA). Flanking *attL* Gateway sequences were also included to allow for LR recombination with binary destination vectors. *LS* from lemon was chosen because it has been previously shown to promote limonene production in heterologous organisms (Lücker et al. 2004; Jongedijk et al. 2014). pB7FWG2 (Karimi et al. 2002), which bears the constitutive *CaMV 35S* promoter and GFP reporter was used to express LS_{ct} and LS_{cp} in whole plant. For the expression in seed coat, 324 nucleotides from the promoter of gene At1g61720 (BAN_{pro}) that have been previously shown to be sufficient to guide the transcription of *BAN* gene (pBAN6; Debeaujon et al., 2003) were fused upstream to LS_{ct} and LS_{cp} sequences. For the expression in valves, 682 nucleotides (between positions −2952 and −2271) of the core promoter of gene At5g60910 (FUL_{pro}) previously shown to positively regulate the expression in valves (Nguyen 2008) were used. BAN_{pro} and FUL_{pro} promoters were cloned into pBGWFS7 (Karimi et al. 2002) which bears β-glucuronidase (GUS) as a reporter gene. A schematic representation of all the constructs used in the experiment is given in Fig. 1b. BAN_{pro}, FUL_{pro} and LS_{ct}, LS_{cp} optimized sequences are reported in the supplementary material.

Transgenic lines were selected as previously described and genotyped with the following sets of primers: FUL_{pro}:LS_{ct}, DYX799F and DYX726R; FUL_{pro}:LS_{cp}, DYX733F and DYX802R; BAN_{pro}:LS_{ct} and BAN_{pro}:LS_{cp}, DYX799F and DYX660R; BAN_{pro}:LS_{ct}, DYX690F and DYX802R; BAN_{pro}:LS_{cp}, DYX686F and DYX802R; *CaMV 35S*:LS_{ct} and *CaMV 35S*:LS_{cp}, DYX646F and DYX647R (Table S1).

Transgene expression

Total RNA was extracted with Trizol Plus RNA purification kit starting from 0.1 g of fresh tissue and 100 ng of total RNA were used to synthesize cDNA with SuperScript Vilo cDNA synthesis kit (Life Technologies, USA). qRT-PCR reactions were performed in a StepOne Plus qRT-PCR machine (Applied Biosystem, USA) with 2.5 ng of cDNA as template and the following sets of primers: BAN_{pro}:LS_{ct}, DYX903F and DYX904R; BAN_{pro}:LS_{cp}, DYX905F and DYX906R; FUL_{pro}:LS_{ct}, DYX907F and DYX908R; FUL_{pro}:LS_{cp}, DYX909F and DYX910R; *CaMV 35S*:LS_{ct} and *CaMV 35S*:LS_{cp}, DYX875F and DYX876R; DYX961F and DYX962R, *CsaTT8*; DYX963F and DYX964R, *CsaTT2*; DYX965F and DYX966R, *CsaTTG1*. Primers DYX815F and DYX816RF which amplify *CsaACT* gene (Hutcheon et al. 2010) were used for the normalization across samples. PCR cycles were set as it follows: stage I, 50 °C per 2 min; stage II, 95 °C per 10 min; stage III, 95 °C per 15 s, 60 °C per 1 min (repeated 40 times). Denaturation curves were run at the end of each PCR cycle to verify the specificity of amplificates.

GUS Staining

Siliques were harvested from FUL_{pro}:LS_{ct} and FUL_{pro}:LS_{cp} transgenic and wild-type plants, and immediately immerged in the GUS staining solution (100 mM sodium phosphate buffer, 10 mM EDTA pH 5.0, 0.1 % Triton-X, 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6 3H_2O$, 1 mg·mL^{-1} X-Gluc. Siliques from BAN_{pro}:LS_{ct} and BAN_{pro}:LS_{cp} lines were carefully opened with a razor blade and only the seeds contained inside were used in the assay. Seeds and siliques

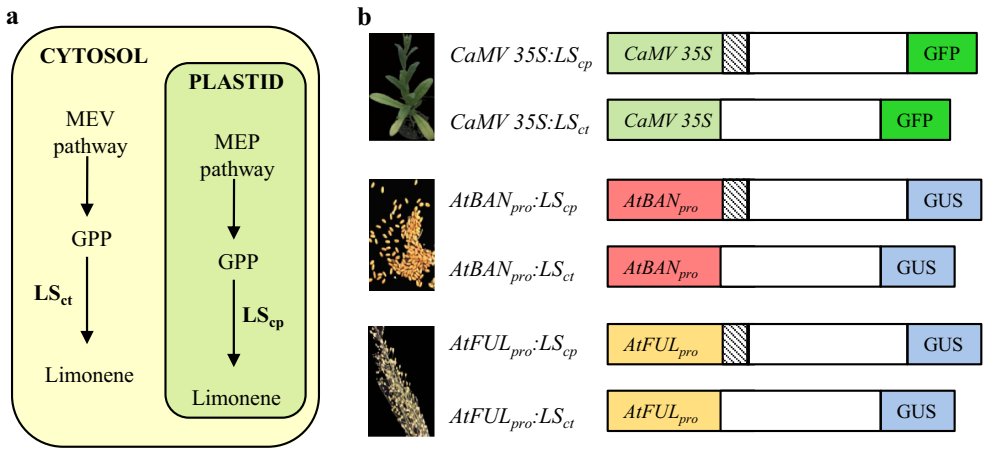

Fig. 1 Schematic representation of the site of activity of limonene synthase (*LS*) along MEV and MEP pathways and constructs used in the experiment. **a** Site of activity of LS along MEV cytosolic (LS_{ct}) and MEP plastidial (LS_{cp}) pathways. **b** Constructs used for *LS* expression in whole plant (*CaMV 35S*:LS_{ct} and *CaMV 35S*:LS_{cp}), seed coat ($AtBAN_{pro}$:LS_{ct} and $AtBAN_{pro}$:LS_{cp}) and valves ($AtFUL_{pro}$:LS_{ct} and $AtFUL_{pro}$:LS_{cp}). *Dashed filled boxes* indicate the transit peptide. GFP and GUS reporters are shown in *green* and *blue*, respectively

still submerged in the staining solution were vacuum infiltrated at −80 psi for 10 min and then placed in the dark at 37 °C for 2 days to allow the blue color to develop. Chlorophylls and natural pigments were removed by thoroughly rinsing the tissues with 70 % ethanol until they appeared white. Images were visualized with a Leica MZ FL III microscope connected to an Olympus DP71 color camera.

Confocal laser scanning microscopy

Confocal laser scanning microscopy was conducted with a Zeiss LSM 710 confocal microscope on lines transformed with constructs carrying the GFP reporter. Images were taken with 10X or 20X objectives, and with wavelengths of 488 nm/492–570 nm excitation/emission.

Chlorophyll content and seed traits

Chlorophylls *a* and *b* were extracted from four foliar disks of 7 mm in diameter with N, N-dimethylformamide (1:20, w:v) for 48 h at 4 °C in the dark. Absorbance of chlorophylls *a* (664 nm) and *b* (647 nm) were measured in a Helios-γ spectrophotometer (Thermo scientific, USA) and the chlorophyll content calculated accordingly with the formula developed by Moran (1982). Seed traits were analyzed with the software SmartGrain (Tanabata et al. 2012).

GC–MS analysis of volatiles

Plant tissues spiked with 100 ng of borneol as internal standard were ground to a fine powder with liquid nitrogen, transferred into a cool glass vial to which hexane was added in the amount of 1:5, weight/volume. The vials were sealed with a silicone rubber cap and the volatiles extracted overnight at 4 °C on a shaker. The supernatant was separated from the cellular debris by centrifugation at 5000 rpm at 4 °C for 15 min and further concentrated to 0.5 mL under a stream of nitrogen gas while continuously kept on ice as previously described (Lee and Chappell 2008). 1 or 2 µL per sample were injected in a 6890 N gas chromatography system equipped with 5975C Inert XL MSD detector (Agilent Technologies, Santa Clara, CA, USA) set in splitless mode. Separation of volatiles was performed with a HP-5MS capillary column (30 m x 0.25 mm Agilent Technologies, Santa Clara, CA, USA) with the following method adapted from (Lucker et al. 2004): oven initial temperature 40 °C for 2 min; ramp to 125 °C at 5 °C per minute; post run at 320 °C for 3 min. Pure helium was used as a carrier gas at a rate of 1.2 mL per minute at the nominal pressure of 16 psi. Mass spectrometry was performed with a transfer line temperature of 150 °C, source temperature of 230 °C, quadrupole temperature of 150 °C, ionization potential of 1459 eV, and scan range of 40–600 atomic mass units. When specifically indicated in the text volatiles were collected with the static method with the use of 100-µm polydimethylsiloxane-coated SPME fiber (Supelco, USA). For this, plant tissues were enclosed into a 10 mL clear glass vial sealed with PTFE/rubber septum aluminum cap. Head space collection was performed for 30 min at room temperature and soon after the analytes were injected in the GC–MS via thermal desorption. Analytical standards of (−)-borneol, (+)-limonene and 1,8-cineole were purchased from Sigma and used for the comparison with the samples.

Results

Characterization of transgenic Camelina lines

T1 transgenic lines generated via vacuum infiltration were selected on Petri dishes supplemented with the herbicide basta (Fig. S1). The plants that survived from the selection were transferred to soil, genotyped and grown to maturity.

Leaf samples harvested from T1 *CaMV 35S:LS*$_{ct}$ and *CaMV 35S:LS*$_{cp}$ plants were utilized to extract RNA with which the level of transgene expression was measured. Figure 2a shows the abundance of *LS* transcripts in the best performing lines that were further propagated to T2 and T3 generation.

On the same plants the emission of limonene was assessed. Volatiles were collected for 30 mins from individual leaves enclosed in glass vials sealed at the bore with aluminum foil. Figure 2b shows the typical chromatograms of transgenic and wild-type plants in the region of limonene. Higher production of limonene was measured in *CaMV 35S:LS*$_{cp}$ lines with *LS* expression targeted to the chloroplast. Transgenic lines also emitted 1,8-cineole (Fig. 2b, peak 2), which was not detected in wild-type plants. Retention times and spectra of limonene and 1,8-cineole were confirmed by comparison with authentic analytical standards. Sites of subcellular LS localization were visualized with confocal scanning microscope through the visualization of the GFP signal on tissues from 15 days old seedling grown in plates. Figure 3 (left panels) shows a portion of the stem from *CaMV 35S:LS*$_{cp}$ T2 plant in which the florescence emitted from the GFP reporter is colored in green. The overlapping between the GFP signal and the fluorescence emitted from the chlorophyll is an indication that *LS* is expressed in the chloroplast. In plants transformed with the cytosolic form of *LS* cDNA, the GFP signal was better visualized in the root tips, which showed diffused fluorescence to the whole cytoplasm (Fig. 3, panels on the right). Because the GFP reporter is fused in

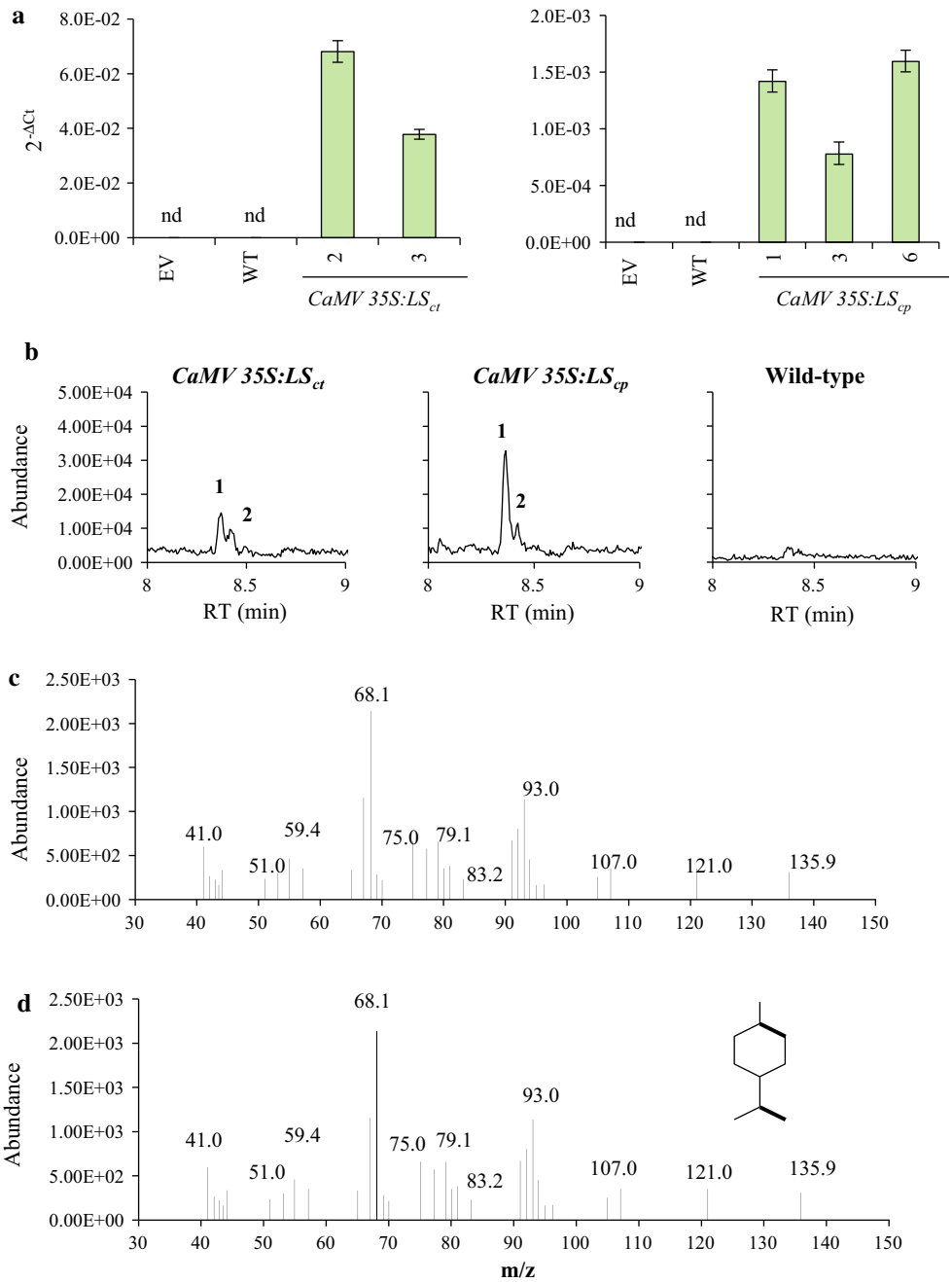

Fig. 2 Characterization of *CaMV 35S:LS*$_{ct}$ and *CaMV 35S:LS*$_{cp}$ T1 lines. **a** Level of *LS* mRNA measured in leaves of transgenic and wild-type plants. Data are presented as 2^{-DCT} and are calculated from three independent biological replicates per genotype. *CsaACT* has been used for the normalization across the samples. *Error bars* represent the standard deviation. **b** GC–MS chromatograms of the headspace collected from leaves of T1 Camelina lines transformed with *CaMV 35S:LS*$_{ct}$ (*left*), *CaMV 35S:LS*$_{cp}$ (*middle*), and wild-type plants (*right*). The collection of volatiles was performed for 30 mins on single leaves enclosed in a 10 mL glass vial sealed at the bore with aluminum foil. **c** Mass spectrum of compound in peak number 1 and (**d**) authentic limonene standard. Numbered peaks are: 1, limonene; 2, 1,8-cineole

frame in C-terminal to *LS*$_{cp}$ and *LS*$_{ct}$ the detection of the GFP signal is also an indication that both *LS* and GFP genes are correctly transcribed and translated into mature proteins.

AtBAN$_{pro}$*:LS* and *AtFUL*$_{pro}$*:LS* T1 lines were also selected on plates prepared with basta, transferred to soil and genotyped (Fig. S1). As soon as *BAN*$_{pro}$*:LS*$_{cp}$ and *BAN*$_{pro}$*:LS*$_{ct}$ T1 lines started blooming, volatiles produced in the inflorescences were collected with the SPME fiber and analyzed. The analysis revealed that all genotypes released limonene, but the production in transgenic lines was three times higher than that of the wild-type (Fig. 4a). In addition to limonene production, LS expression was measured in T2 seeds of *AtBAN*$_{pro}$*:LS* and T1 valves of *AtFUL*$_{pro}$*:LS* lines via qRT-PCR. All lines aside from those originally transformed with the empty vector and wild-type plants showed positive *LS* expression (Fig. 4b, c).

Because in these plants LS protein is fused in frame with the reporter GUS, the X-glu staining method was utilized to confirm the expression in target tissues. Figure 5 shows representative photographs in which a typical blue color developed in seeds and valves of transgenic lines, which

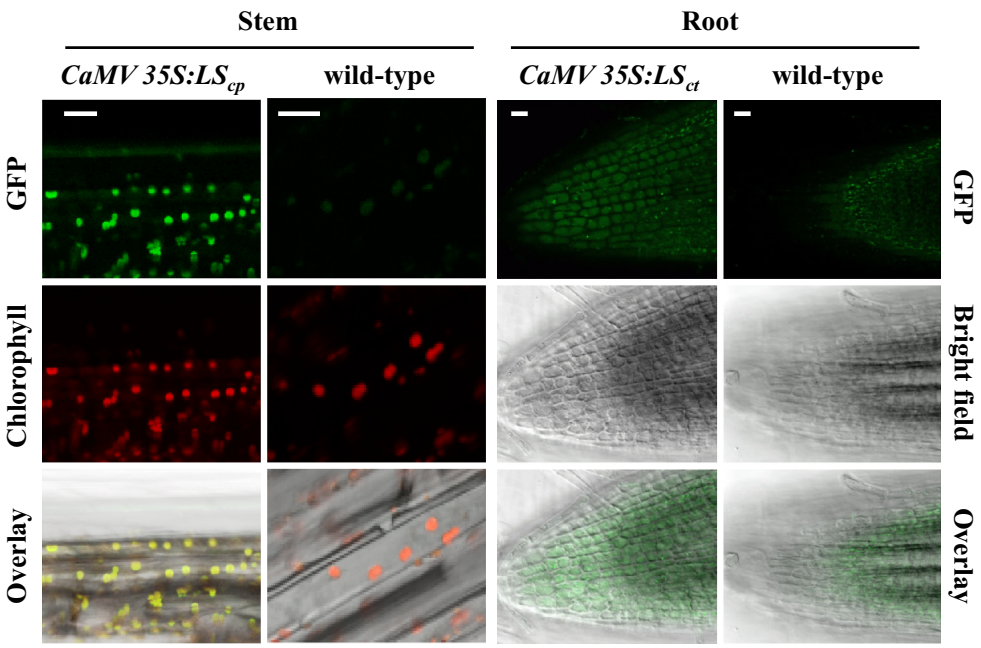

Fig. 3 Visualization of the GFP fluorescence in transgenic Camelina. Subcellular localization of limonene synthase in transgenic T2 Camelina. Comparison between stems from Camelina lines transformed with *CaMV 35S:LS$_{cp}$* constructs and wild-type (*left panels*), and root from *CaMV 35S:LS$_{ct}$* and wild-type plants (*right panels*). GFP and chlorophylls emission are colored in *green* and *red*, respectively. Overlay was performed in bright field. *Bars* represent 25 μm

was not detected in wild-type plants. The best performing lines were further propagate to T3 generation and LS expression newly measured to verify that unwanted silencing effects did not occur (Fig. S2).

Production of limonene in seeds and siliques of transgenic Camelina

T2 transgenic lines selected based upon positive expression of transgene, visualization of GFP/GUS and emission of limonene were set to seed and limonene content measured in tissues (leaves, seeds and valves) of third generation transgenics. Seeds and siliques from three to five independent homozygous plants per genotype were pooled together, homogenized with liquid nitrogen and the volatiles extracted with hexane and analyzed via GC–MS. All transgenic lines showed higher limonene content than the wild-type and the lines transformed with the empty vector (Fig. 6). Average limonene content was 7.1 ± 0.2 ng g^{-1} FW in seeds of and *CaMV 35S:LS$_{ct}$* and *CaMV 35S:LS$_{cp}$*, 20.3 ± 0.3 and 38.6 ± 0.3 ng g^{-1} FW in seeds and siliques of plants with *LS* expression induced by *AtBAN$_{pro}$* and *AtFUL$_{pro}$*, respectively. Limonene content measured in siliques and seeds of wild-type lines ranged between 4.22 ± 0.3 and 4.47 ± 0.4 ng g^{-1} fresh weight (FW), which is in line with the small amount of limonene previously measured in the oil (Krist et al. 2006).

Fitness of T3 seeds and plants

T3 lines transformed with *LS$_{cp}$* and *LS$_{ct}$* triggered by the constitutive 35S promoter expressed transgenic *LS* and produced limonene also in leaves (Fig. 7a, b). Average limonene content measured in leaves of transgenic plants was 17.2 ± 7.4 ng g^{-1} FW and negligible amounts were also detected in wild-type. Transgenic lines were visually monitored during their life cycle and carefully examined for the development of undesirable traits possible due to detrimental effects associated with the constitutive production of limonene. Particular attention was given to *CaMV 35S:LS$_{cp}$* lines that express *LS* in the chloroplast. To assess whether any detrimental impairment to the photosynthetic apparatus may have occurred, the concentration of chlorophyll *a* and *b* was measured and compared to wild-type. As shown in Fig. 7c no differences were detected.

Quantitative seeds traits, such as seed shape and size were measured in batches of transgenic and wild-type seeds. Length to width ratio (LWR) showed the lowest degree of variability among all lines and no significant differences were reported (Fig. 8, black bars). Similarly it appeared for the seed area (Fig. 8, white bars). Seeds germination has been assessed on plates and did not reveal any impairment.

Discussion

Arabidopsis *BAN* and *FUL* promoters are actively transcribed in Camelina

This study assessed whether Arabidopsis *AtBAN$_{pro}$* and *AtFUL$_{pro}$* promoters are suitable to induce tissues specific expression of *LS* in Camelina and accumulation of limonene in seeds and fruits. For this purpose six constructs

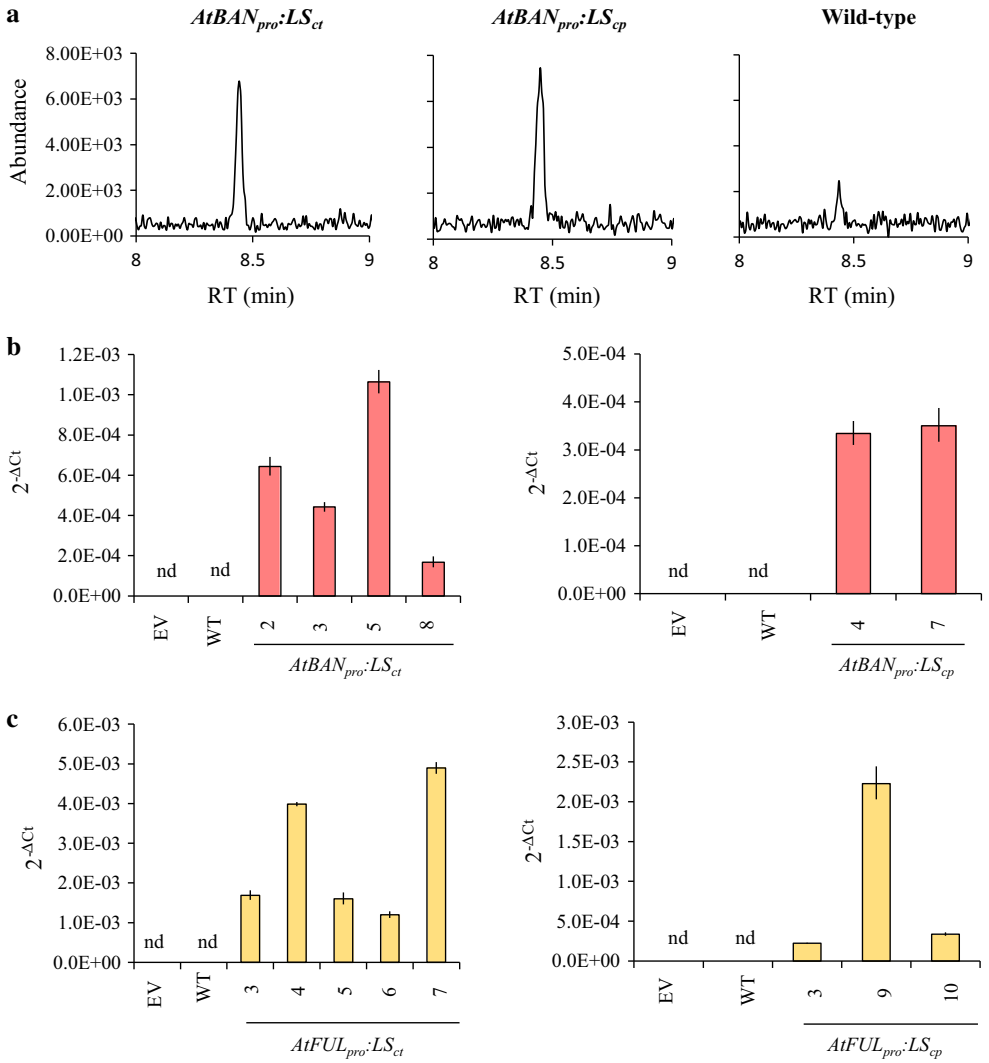

Fig. 4 Limonene emission and LS expression in seeds and siliques. **a** Chromatograms of the headspace collected from inflorescences of T1 Camelina lines transformed with AtBANpro:LSct (*left*), AtBANpro:LScp (*middle*), and wild-type (*right*) plants. Volatiles were collected for 30 mins from a single inflorescence enclosed into a 10 mL glass vial sealed at the bore with aluminum foil. **b**, **c** Level of LS expression in T2 AtBAN:LSct and AtBAN:LScp seeds and T1 AtFUL:LSct and AtFUL:LScp siliques. CsaACT was used as endogenous control for the normalization across samples. Data are presented as $2^{-\Delta Ct}$ and are calculated from three independent biological replicates per genotype. *Error bars* represent the standard deviation. *WT* wild-type; *EV* empty vector

were prepared in which *LS* cDNA was alternatively guided by $AtBAN_{pro}$, $AtFUL_{pro}$ or the constitutive *CaMV 35S* and targeted to the cytosol and plastids. The results of this experiment indicate that all promoters can trigger *LS* expression in Camelina (Figs. 2a, 4, Fig. S2) and boost the production of limonene in flowers, seeds and/or leaves of transgenic plants (Figs. 2, 4, 6, 7).

$AtBAN_{pro}$ was chosen because it is known to be expressed in seeds. In fact, *AtBAN* codifies for the anthocyanidin reductase enzyme that catalyzes the conversion of the colorful anthocyanidin into flavan-3-ols, the building block units for the biosynthesis of condensed tannins (Xie et al. 2003; Xie et al. 2004). Condensed tannins accumulate in the seed coat and function to protect the embryo from pathogens and UV light exposure (Bharti and Khurana 1997). Previous studies have shown that Arabidopsis $AtBAN_{pro}$ triggers the expression of the GUS reporter in seed coat of *Brassica napus* (Nesi et al. 2009) and our study presented here shows that $AtBAN_{pro}$ is also effective in inducing *LS* expression in the seed coat of Camelina (Figs. 4, 5, 6). In Arabidopsis three transcription factors regulate *AtBAN* expression in the seed coat and these are TRANSPARENT TESTA GLABRA 1 (AtTTG1), TRANSPARENT TESTA 2 (AtTT2) and AtTT8 (Debeaujon et al. 2003; Nesi et al. 2000; Nesi et al. 2001). AtTT2 (a MYB transcription factor) and AtTT8 (a bHLH transcription factor) physically bind $AtBAN_{pro}$ in the upstream region between nucleotides −111 and −75,

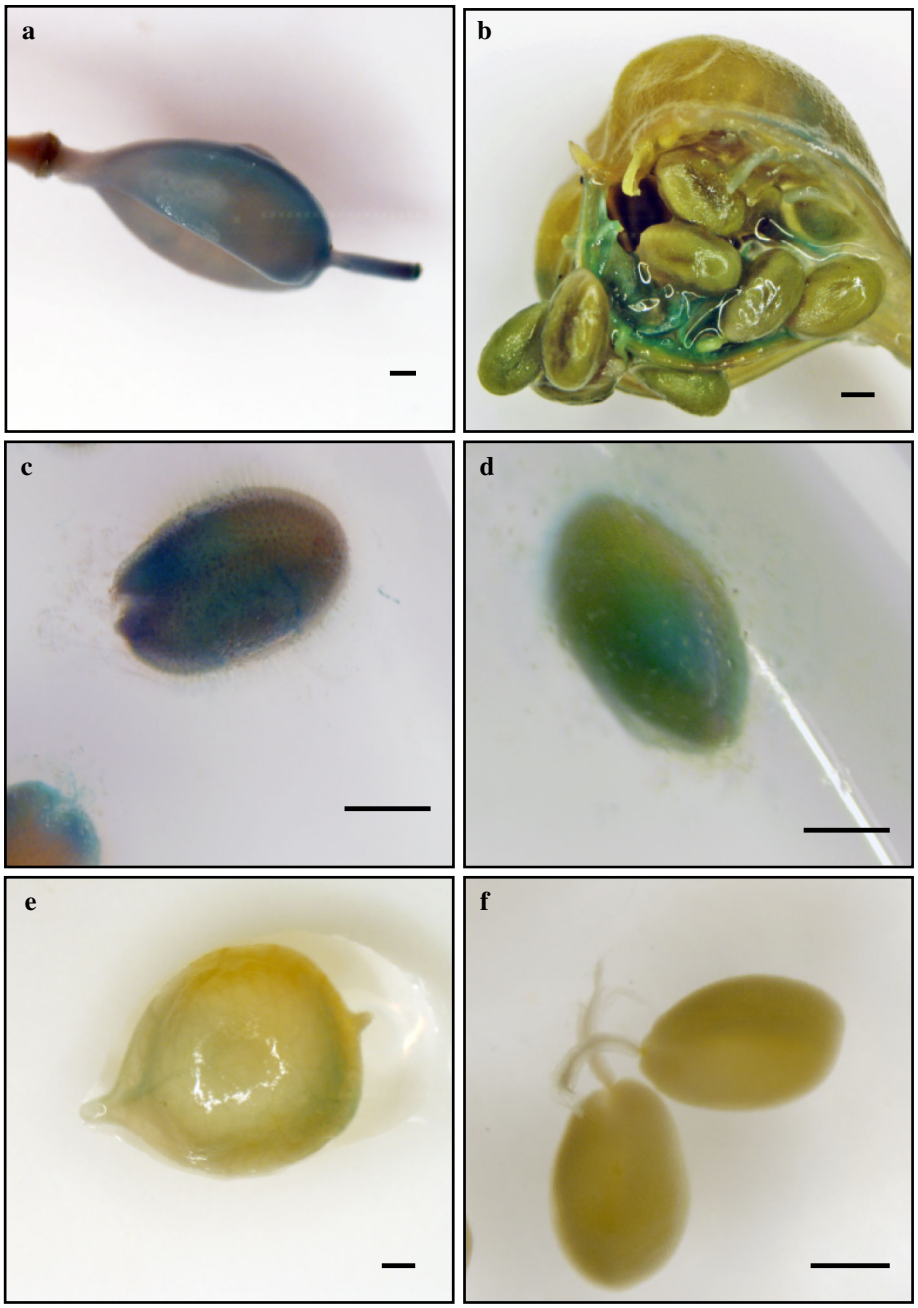

Fig. 5 Histochemical visualization of GUS activity in Camelina. GUS staining of representative siliques and seeds harvested from **a**, **b** $AtFUL_{pro}$:LS_{ct} and $AtFUL_{pro}$:LS_{cp}; **c**, **d** $AtBAN_{pro}$:LS_{ct} and $AtBAN_{pro}$:LS_{cp}; **e**, **f** wild-type plants. *Bars* correspond to 1 mm

which contains MYB and G-box binding sites (Debeaujon et al. 2003). AtTTG1 (a WD-repeat protein) does not directly interact with the sequence of $AtBAN_{pro}$, but stabilizes the AtTT8 and AtTT2 multi-protein complex so to enhance *AtBAN* transcription (Baudry et al. 2004; Baudry et al. 2006). Because transcriptional regulation from $AtBAN_{pro}$ in Camelina can only be initiated when proteins with similar function to AtTT1, AtTT2 and AtTT8 are present and active, our experiment together with the previous observations (Nesi et al. 2009) supports the conclusion that the protein complex that regulates *BAN* expression is conserved among different species of the Brassicaceae family. Indeed, genes homologs to *AtTT2*, *AtTT8* and *AtTTG1* have been identified in Camelina, which all show conserved amino acid sequence between the two species (Table S3), and pattern of tissue expression similar to what previously described in the literature for Arabidopsis (Fig. S3a). Moreover, the sequence upstream of Csa16g053480, which codifies for one of the three *CsaBAN* homologs (*Camelina sativa* is allohexaploid; therefore, three homologs per each Arabidopsis gene are present in the genome) is surprisingly highly conserved between the two species. In fact, bHLH and MYB *cis* regulatory elements that in Arabidopsis recruit AtTT2 and

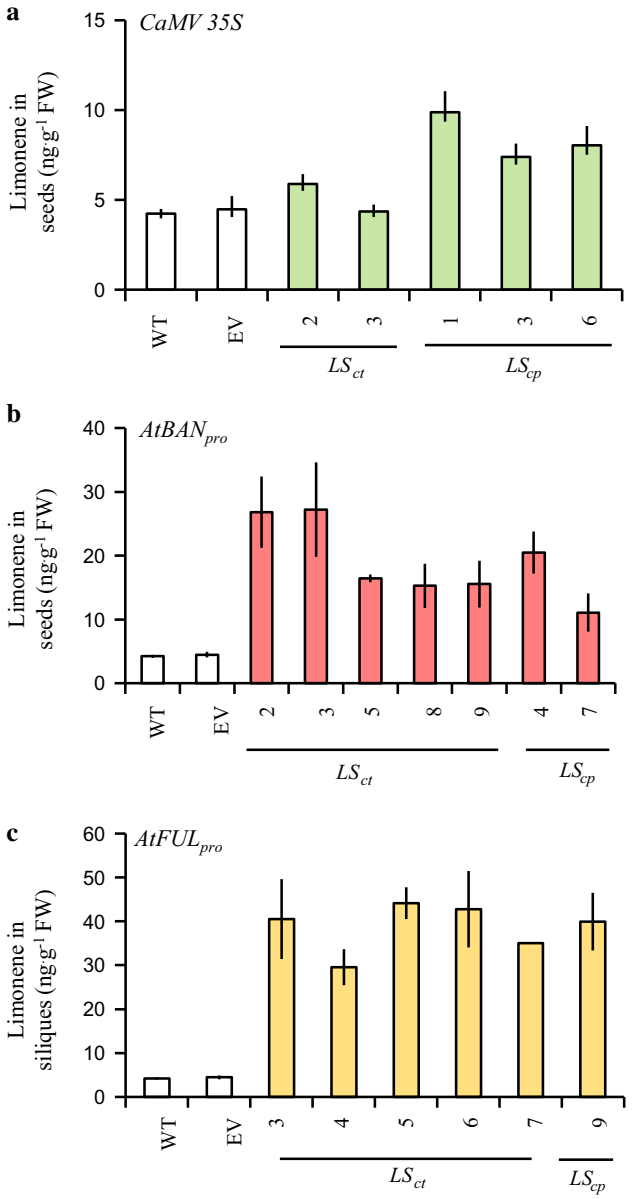

Fig. 6 Limonene content in T3 seeds and siliques of transgenic Camelina. Limonene content in **a** seeds of *CaMV 35S:LS*$_{ct}$ and *CaMV 35S:LS*$_{cp}$, **b** *AtBAN*$_{pro}$*:LS*$_{ct}$ and *AtBAN*$_{pro}$*:LS*$_{cp}$ **c** *AtFUL*$_{pro}$*:LS*$_{ct}$ and *AtFUL*$_{pro}$*:LS*$_{cp}$ lines. *WT* wild-type; *EV* empty vector

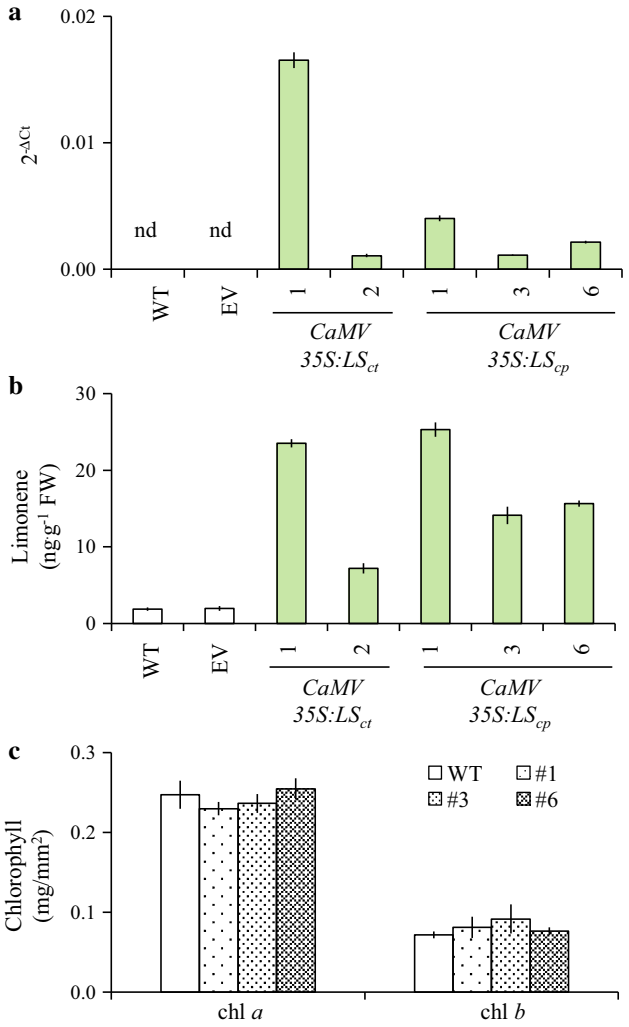

Fig. 7 Characterization of *CaMV 35S:LS*$_{ct}$ and *CaMV 35S:LS*$_{cp}$ Camelina lines of third generation transgenics. **a** Level of *LS* transcript. The expression of *CsaACT* was used as endogenous control for the normalization across samples. Data are presented as 2^{-DCt} and are calculated from three independent biological replicates per genotype. *Error bars* represent the standard deviation. **b** Limonene content in leaves of T3 *CaMV 35S:LS*$_{ct}$ and *CaMV 35S:LS*$_{ct}$ lines. **c** Chlorophyll *a* and *b* contents in three independent CaMV 35S*:LS*$_{ct}$ lines. *WT* wild-type; *EV* empty vector

AtTT8 transcription factors are also present in the upstream sequence of Csa16g053480 and syntenic to Arabidopsis (Fig. S3b).

Positive expression of *LS* transgene and increased limonene content were also measured when *AtFUL*$_{pro}$ was employed to trigger LS expression (Figs. 4, 5, 6). In Arabidopsis *AtFUL* participates in the proper regulation of fruit development. Indeed, the gene was initially identified through a screening of mutants with short siliques full of seeds, after which the causative gene was named (Gu et al. 1998). This happens because the valves of the mutant fail to properly elongate, so that the siliques are shorter and the seeds inside tightly compressed (Gu et al. 1998; Mandel and Yanofsky 1995). From a functional dissection study of *AtFUL*$_{pro}$ it emerged that the region between nucleotides −2952 and −2271 is necessary and sufficient to guide *FUL* expression in valves (Nguyen 2008) and the results presented in the current study show that this region is also functional in fruits of Camelina. For this to happen, transcriptional regulatory mechanisms that regulate *FUL* expression must be conserved in the two species. In Camalina three genes were identified for their homology with *AtFUL,* namely Csa18g034280, Csa11g094760 and

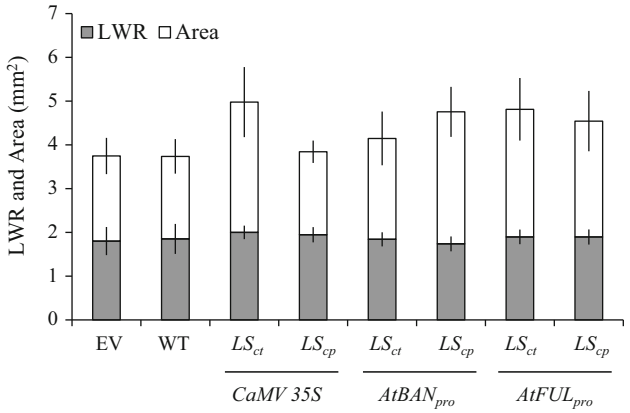

Fig. 8 Phenotypes of seeds from transgenic Camelina. Length to width ratio (LWR) and area of T3 seeds harvested from wild-type and transgenic plants. *Bars* represent the average value from 77 to 238 seeds per genotype. *Error bars* show the standard error

Csa02g067860, and their respective proteins all show very high level of identity with the protein codified by At5g60910 (Table S4). However, the mechanisms that control the transcriptional regulation of these genes are currently unknown. In Arabidopsis *AtFUL* expression is regulated by *Squamosa Promoter binding protein Like 3* (*SPL3*), *SPL4* and *SPL5* (Yamaguchi et al. 2009; Wang et al. 2009), which in their turn are regulated by *miR156* (Wang et al. 2009; Fornara and Coupland 2009). Whether microRNA also regulates *AtFUL* expression in Camelina is a possibility, although too speculative to be inferred. In fact, *FUL* genes from *Betula pendula* (birch tree) and *Camellia japonica* (camellia) show high degree of homology with the Arabidopsis gene, but while the pattern of expression is conserved between Arabidopsis and the birch tree, this is different in camellia. (Elo et al. 2001; Lännenpää et al. 2005; Sun et al. 2014). Nevertheless, the results described in the current experiment are in favor of a common mechanism of regulation.

The promoter of choice impacts limonene production in Camelina

The distribution of the average limonene content measured in seeds and siliques of transgenic Camelina illustrates that the promoter chosen to initiate *LS* transcription largely affects the amount of limonene produced (Fig. 9). Thereafter, transgenic lines can be classified as low (*CaMV 35S:LS*$_{cyt}$ and *CaMV 35S:LS*$_{pl}$, green bars), intermediate (*AtBAN*$_{pro}$*:LS*$_{cyt}$ and *AtBAN*$_{pro}$*:LS*$_{pl}$, red bars) and high (*AtFUL*$_{pro}$*:LS*$_{cyt}$ and *AtFUL*$_{pro}$*:LS*$_{pl}$, yellow bars) producers of limonene. *AtFUL* and *AtBAN* are tuned to regulate vital processes of fruit development and seed dispersal; therefore, it is reasonable to speculate that they are more efficiently expressed in seeds and valves than the constitutive *CaMV 35S* promoter. It is documented that tissue and organ of transgene expression influence the production of secondary metabolites (Lücker et al. 2001; Gutensohn et al. 2014; Gutensohn et al. 2013); therefore, tissue-specific promoters are often exploited to boost the production of metabolites of interest, as for example to increase food aromas (Davidovich-Rikanati et al. 2007; Lewinsohn et al. 2001) or to explore non-conventional biosynthetic pathways (Gutensohn et al. 2014).

Glandular trichomes have been shown to be elective sites for the synthesis of terpenes because of their peculiar metabolic resources and storage capacity (Lange and Turner 2013). Indeed, trichomes-specific promoters represent a great potential for the genetic engineering of secondary metabolites and have been successfully utilized for target engineering of trichomes in tobacco and cotton (Tissier 2012b). In Camelina trichomes are present on the upper lamina and leaf margins of young leaves (Sigareva and Earle 1999), but in mature leaves, trichome density considerably decreases because of leaf expansion. Target modification of Camelina trichomes may be marginally relevant for industrial applications related to biofuel

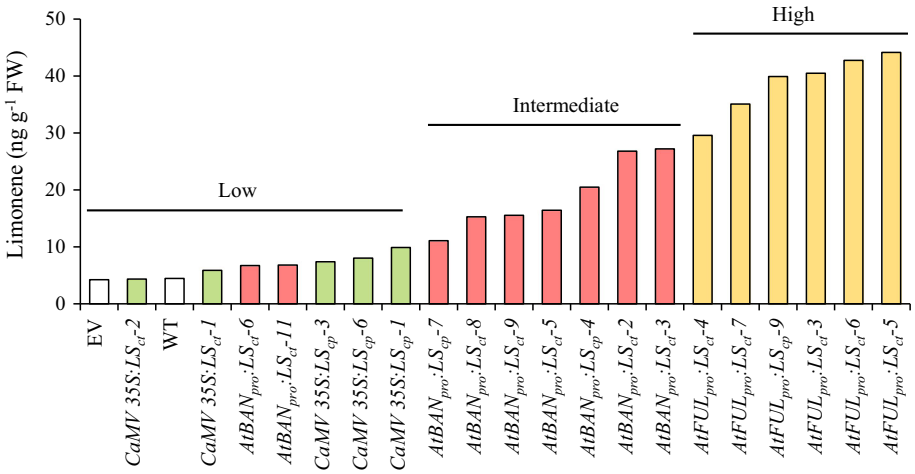

Fig. 9 Distribution of the average limonene content in seeds and siliques of second generation transgenics. *Bars* represent the following genotypes: *green*, *CaMV 35S:LS*$_{ct}$ and *CaMV 35S:LS*$_{cp}$; *red*, *AtBAN*$_{pro}$*:LS*$_{ct}$ and *AtBAN*$_{pro}$*:LS*$_{cp}$; *yellow*, *AtFUL*$_{pro}$*:LS*$_{ct}$ and *AtFUL*$_{pro}$*:LS*$_{cp}$. *White bars* represent wild-type (*WT*) and empty vector (*EV*) lines

production; however, it would bring insights into substrate availability and pathways to the synthesis of secondary metabolites (Tissier 2012a). Since these questions have not been yet investigated in Camelina, it is uncertain to infer whether substrate availability or the capacity of each individual tissue to hamper limonene volatility or both, are causative of the differences observed in our experiment. However, independently from the biological mechanism underlying the process, our results clearly show that Arabidopsis promoters of genes *BAN* and *FUL* are actively transcribed in Camelina, in which they also retain specificity of tissues expression as seen in Arabidopsis. Therefore, they can be successfully utilized to boost the expression of transgenes of interest in transgenic plants.

Author contribution statement DYX developed this project. MB and DYX designed experiments, analyzed data and wrote the paper. MB performed the experiments.

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