Nova Biotechnologica *et* Chimica

Targeting transgene to seed resulted in high rate of morphological abnormalities of *Camelina* transformants

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Abstract

False flax (*Camelina sativa* L.) is currently under-exploited but highly promising oilseed crop. Combining *Camelina's* attractive agronomic traits with its unprecedented ease for genetic engineering makes it an ideal plant chassis for biotechnology applications, in particular synthetic biology strategies. For targeted expression of transgene particularly to seeds requires identification and application of seed specific promoters. In the present study two cultivars of *Camelina,* namely Zuzana and Smilowska, were used for transformation at early flowering stage using the floral dip method. The plants were inoculated with *Agrobacterium* bearing a construct for expression of red fluorescent protein (RFP) under the control of the seed specific cruciferin promoter *CRUC* from *Arabidopsis*. Transgenic seeds and plants were identified on the basis of red fluorescence (RFP) and kanamycin resistance. Relatively high transformation efficiency of 8 % was achieved particularly for the cultivar Zuzana. However, many of regenerants exerted developmental deformations such as lack of shoot apical meristem, deformed or absent cotyledons, etc. Furthermore, the activity of the *CRUC* promoter was still active also in true leaves rendering this promoter as inappropriate for seed targeting of the transgene. Nevertheless, genetic transformation remains a tool for direct modulation of pathways for oil synthesis in oilseed crops.

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Introduction

False flax, *Camelina sativa* (L.), is an ancient crop of the *Brassicaceae* cultivated in Europe as an important oilseed crop for many centuries before it was displaced by higher-yielding crops, such as canola, soybean or sunflower. Like other *Brassicaceae*, *Camelina's* seeds accumulate starch during the early stage of embryo development (Na *et al.* 2018), but later it largely diminishes when seeds mature. It is proposed that the turnover of starch during seed development is functionally linked with cell division (Andriotis *et al.* 2010). *Camelina* seeds store oil and protein as major carbon and nitrogen reserves (Gesch 2014).

The *Camelina* oil yields and fatty acid composition depends on cultivars, soil composition but also environmental conditions (Gesch 2014; Ševčík *et al.* 2018; Obeng *et al.* 2019). The proportion of polyunsaturated fatty acids (PUFAs) in seeds, for example, decreases with temperatures (Obour *et al.* 2017). Since higher content of unsaturated fatty acid in the oil is critical for its uses as jet fuel and biodiesel (Kim *et al.* 2015), the timing

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of *Camelina* planting to ensure flowering and seed filling, periods of adequate soil water supplyand favourable growing temperature are important for *Camelina* production. Further, *Camelina* oil is rich in omega-3 fatty acids but poor in minor lipid components such as tocopherols and phytosterols compared to the other conventional oilseeds (even most fish oils), rendering it suitable for food applications as health-promoting lipids (Budin *et al*. 1995; Belayneh *et al*. 2018).

Although *Camelina* has many attractive agronomic features (e.g. short lifecycle, remarkable adaptation to a wide range of climatic conditions, etc.), its relatively low oil yield (compared to other oilseed plants) represents a real limitation to it using in chemical and/or pharmaceutical industries (Faure and Tepfer 2015). Improving oil yield is therefore a priority for the development of this crop for large-scale use. Desired properties of the oil can be achieved by the use of various transformation techniques. *Camelina* can be successfully transformed using both the floral dip and floral vacuum infiltration methods (Lu and Kang 2008; Liu *et al*. 2012). Recently, two *C. sativa* cultivars were successfully transformed also with *Agrobacterium tumefaciens*, for which the shoot tips with apical meristems appeared the best target tissue (Sitther *et al*. 2018). Fully developed, intact transgenic *Camelina* plants can be obtained within 6 – 8 weeks (Lu and Kang 2008; Sitther *et al*. 2018). Expression of transgenes can result in production of seed oil with attractive physicochemical properties such as reduced viscosity and freezing point, and thus suitable for preparation of biodegradable lubricants, emulsifiers, plasticizers, and second-generation biofuels (Liu *et al*. 2015; Bansal and Durrett 2016; Haslam *et al*. 2016). Recently Zhu *et al*. (2018) suggested that the maize master regulator, *ZmLEC1*, driven by a downstream seed-specific promoter, is a promising target for increasing oil yield in transgenic *Camelina* seeds. Further, *Camelina* plants transformed with *EaDAcT* gene from *Euonymus alatus* synthesize large amounts of *sn*-3-acetyl triacylglycerols (acetyl-TAGs), primarily in the endosperm and mature embryo cells (Durrett *et al*. 2010; Hu *et al*. 2017; Bansal *et al*. 2018; Chhikara *et al*. 2018). Modifying the composition of *Camelina* seed oil opens new possibilities for diversifying oilseed production,

for example towards the accumulation of bioactive compounds (e.g. terpenes) used in food additives, cosmetics, drugs, and for other bio-based products. Transformation of *Camelina per se* does not appear as demanding, although failure of the applied method has also been reported (Lu and Kang 2008). Moreover, targeted expression of transgene particularly to seeds can be problematic. Model organisms are being widely screened for identifying promoters that are tissue- or developmental stage-specific to maximize the effect of transgene expression without affecting other tissues during growth and development (Jeong *et al*. 2014; Polóniová *et al*. 2015). The development of promoter systems expressed specifically in seeds or in particular constituents or tissues/compartments of seeds is indispensable not only for the mentioned reasons, but also to control the nourishment of the embryo, dispersal to a new location, and dormancy under unfavourable conditions (Jeong *et al*. 2014). *Camelina* has previously been transformed with seed-specific phaseolin promoter (Lu and Kang 2008).

In this work, we tested the suitability of the cruciferin (*CRUC*) promoter to drive transgene expression in *Camelina* seeds. This strong promoter from *Arabidopsis thaliana* is embryo-specific with activity that starts between mid-globular and early heart embryo stages (Becerra *et al*. 2006), but is inactive in other (vegetative) tissues like leaves or flowers (Mietkiewska *et al*. 2000). We show that the transformation of *Camelina* can be hampered, while potential reasons are discussed. We used two *Camelina* cultivars Zuzana and Smilowska, which are promising for breeding in Slovakia.

Experimental

Bacteria

The bacterial strain of *Agrobacterium tumefaciens* strain LBA4404 was used, carrying a binary vector pEV1 with the *RFP* reporter and selectable *nptII* genes (Moravčíková *et al*. 2008). The former gene was under the control of the cruciferin promoter sequence (*CRUC*), while the latter one by the *NOS* promoter. Bacteria were cultivated on LB medium, (10 g.L^{-1} peptone, 6 g.L^{-1} NaCl, 6 g.L^{-1} yeast
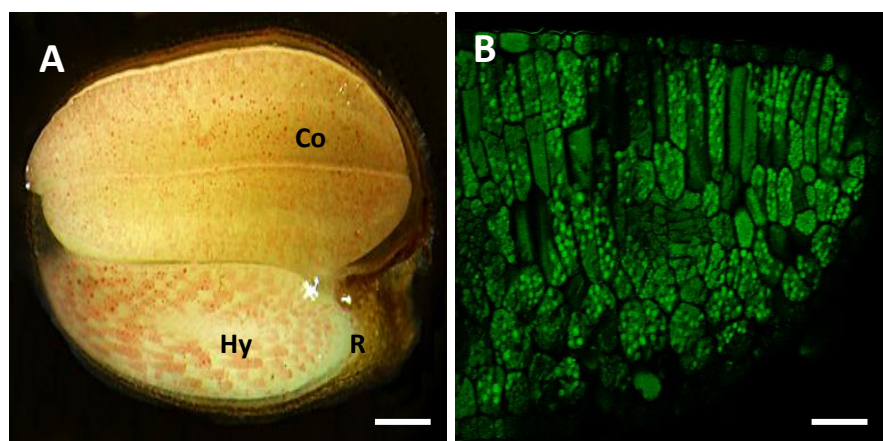
Fig. 1. Longitudinal sections of *Camelina* seeds observed by stereomicroscope (M 165 FC Leica, Germany) (**A**) and confocal laser microscopy fluorescence image (FV1200 IX83, Olympus, Japan) of cotyledons showing oil bodies stained with BODIPY TM 493/503 according to Martinčová *et al.* (2019) (**B**). Co −cotyledons; Hy − hypocotyl; R − radicle. The bar represents A = 500 µm; B = 50 µm.

extract; pH 5.8). Bacterial suspension (300 µL) and appropriate antibiotics (100 mg.L^{-1} of kanamycin and 25 mg.L^{-1} of rifampicin) were added into a liquid LB medium and cultivated for overnight on a shaker (SHAKER DOS-20L; 220 rpm) in the dark at 28 °C. The *Agrobacterium* cells were separated from medium by centrifugation at 2,500 rpm for 2 min and the pellet was suspended in the infiltration medium (OD_{600} ~ 1.0), consisting of half-strength MS salts and 0.025 % (v/v) Silwet L-77.

Floral dip method of Camelina sativa *transformation and selection*

Two *C. sativa* cultivars Zuzana (Czech Republic) and Smilowska (Poland) were transformed *via* floral dip infiltration using the above-mentioned strain of *Agrobacterium tumefaciens*.

One hundred *C. sativa* seeds planted in 15 × 35 cm pot filled with soil were cultivated in a growth chamber under controlled environmental conditions (16/8 h long-day photoperiod with the light intensity 100 µmol.m^{-2}s^{-1} and stable temperature 24 °C). Plants at early flowering stage were dipped into *Agrobacterium* cell suspension for 15 seconds and covered with plastic dark film and placed in the dark for 24 h. These T$_0$ plants were then moved to the growth chamber and cultivated (16/8 h long-day photoperiod; 100 µmol.m^{-2}.s^{-1} with stable temperature 24 °C) until mature seeds were harvested.

One hundred seeds from T$_0$ plants were sterilized with 4.7 % (w/v) sodium hypochlorite solution for 15 min and then washed three to five times with sterile distilled H$_2$O. Washed seeds were sown on Petri dishes with a half-strength MS medium (pH 5.8) supplemented with 10 g.L^{-1} sucrose, 7 g.L^{-1} agar and 150 mg.L^{-1} kanamycin. The antibiotics concentration to be applied was determined based on preliminary screening of its (obviously negative) effect on non-transgenic germinating seedlings after 7 days growth. The seeds were cultivated in a growth chamber with a 16/8 h light/dark cycle for germination and seedling growth. Transgenic plants were examined for red fluorescence under a stereo-microscope M 165 FC (Leica, Germany) after 7 – 14 d cultivation.

Results and Discussion

Like other *Brassicaceae* plants, *Camelina's* seeds accumulate non-polar lipids, presence of which was detected in many oil bodies, primarily in cotyledons (Fig. 1). Oil content and properties render this species extremely attractive for food, but relatively cheaper agronomic costs and the possibility to modify the properties of its oil makes it promising for the biofuel industry as well. Genetic transformation approach has been successful in achievement of the desirable *Camelina* traits (for review see Aznar-Moreno and Durrett 2017), while specific promoters are important for seed-targeted modification to occur. We transformed two *Camelina* cultivars Zuzana and Smilowska with *Agrobacteria* using floral dip method. Seeds were collected from 100 plants. A total of 100 seeds from each cultivar were let to germinate on media with kanamycin and the number of resistant individuals was scored.

Table 1. Overview of the *Camelina* transformation.

Camelina cultivar	Seeds of transformed plants			
	Total[a]	RFP[b]	Regenerants[c]	TE [%][d]
Zuzana	100	11	8	8
Smilowska	100	6	2	2

[a] Total number of seeds from T_0 plants screened;
[b] Number of seeds expressing red fluorescence;
[c] Number of potential transgenic regenerants identified on the basis of kanamycin resistance;
[d] Transformation efficiency (TE) expressed as a number of kanamycin resistant regenerants obtained as percentage of the seeds used.

The data showed that two cultivars markedly differ in transformation efficiency; efficiency of 8 % was achieved for the *cv*. Zuzana but only 2 % for the *cv*. Smilowska (Table 1). The former value is much higher than the transformation efficiency of 0.8 % obtained using the same transformation method by Liu *et al*. (2012), and also than the efficiency of 1.3 % obtained using vacuum infiltration by Lu and Kang (2008). Noteworthy, the later authors failed to obtain transformed *C. sativa* (*cv*. Celine) plants using floral dip technique. Previously,

efficient transformation of two *C. sativa* cultivars (Pl650159 and Pl650161) has been described by Sitther *et al*. (2018), while the authors concluded that shoot tips with apical meristems were the best target tissues for *Agrobacterium*-mediated transformation of *C. sativa* plants *in vitro*. As easily scorable reporter gene we used the red fluorescing *DsRFP*, which has previously been used to transform *Camelina* under control of *CaMV* promoter (Lu and Kang 2008), and had shown to remain active in desiccated mature seeds with no obstruct excitation or emission of the fluorescence by the light-colored seed coat. We could not detect fluorescence in any intact *Camelina* seed at all, probably due to week transgene expression. In contrast, strong fluorescence was typical for germinating embryos (Fig. 2 and 3). However, not all red-fluorescing embryos survived on selection media in later developmental stage, pointing on insufficient tolerance to antibiotics (e.g. due to chimeric nature), or incomplete transgene (Boszorádová *et al*. 2019). The phenomenon of chimerism, for example, frequently occurs
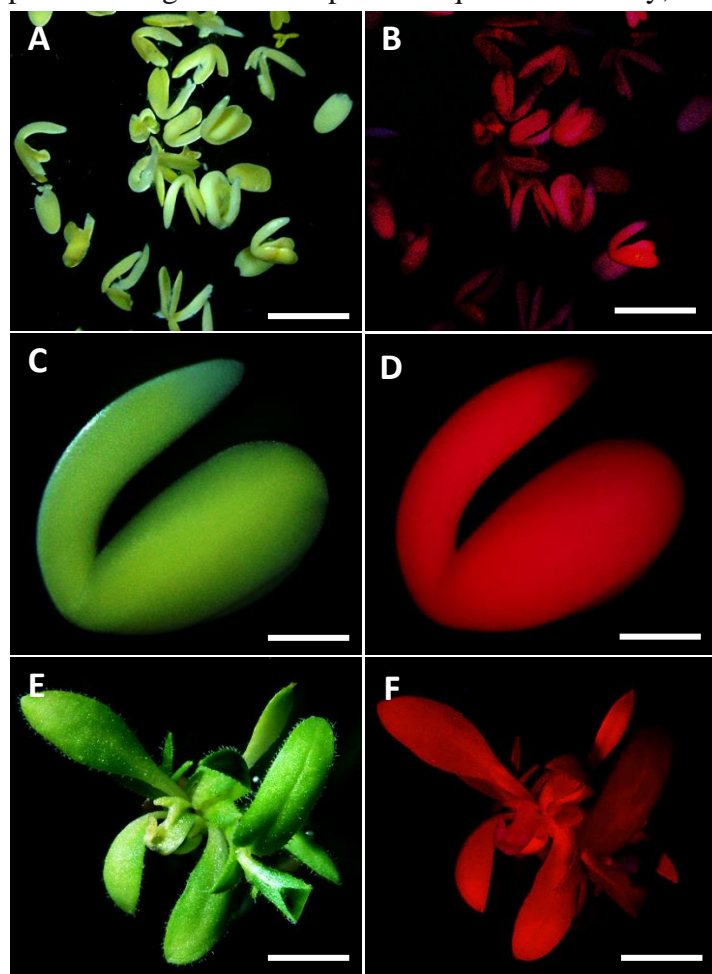
Fig. 2. Selection of RFP⁺ *C. sativa* embryos after 7 days of cultivation (**A** – **D**) and regeneration of RFP⁺ potential transgenic plants (**E** – **F**) after 14 days of cultivation. The bar represents **A**, **B** = 1 cm; **C**, **D** = 250 μm; **E**, **F** = 1 cm.
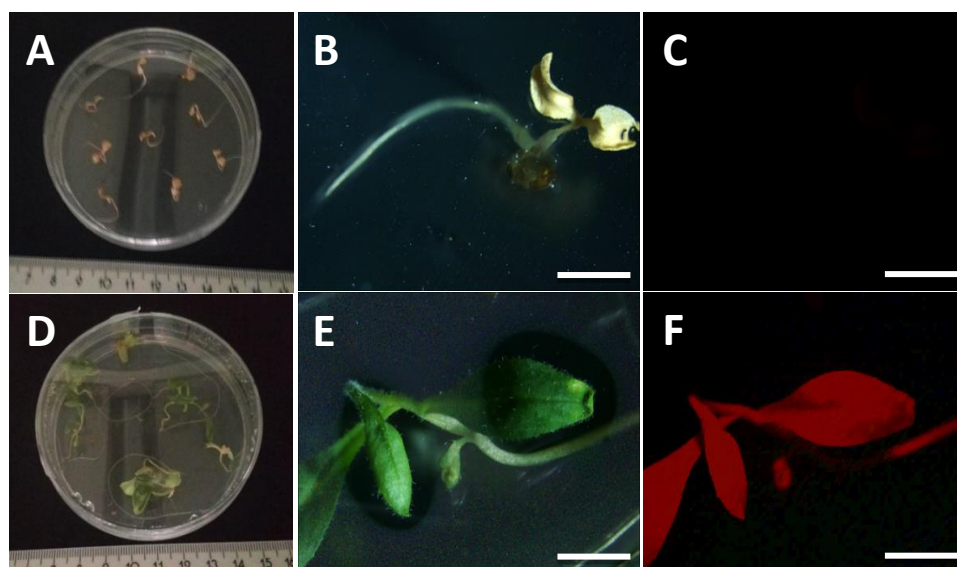
Fig. 3. Generation of transgenic *Camelina sativa* plants. Seeds of transformed plants were plated on media with 150 mg.L^{-1} kanamycin (**A**, **D**) and their transgenic nature was identified on the basis of red fluorescence (RFP$^+$) and resistance to antibiotics. Non-transgenic *Camelina* seedlings (**B**) had white cotyledons and showed very low or no RFP activity (**C**). Red fluorescence indicated transgene expression in transgenic *Camelina* plants regenerated on MS media with kanamycin (**F**). The bars at **A** – **F** = 1 cm.

to a different extent in all systems where a recombination event has to take place in multicellular structures, e.g. during development from calli (Moravčíková *et al.* 2008).

Surprisingly, red fluorescence of surviving transformants was observed also later during development and growth, especially in the regenerants of the ana (Fig. 2 and 3). This suggests that the *CRUC* promoter does not stop activation of the *rfp* as expected. Ectopic expression of this promoter has been reported previously in rapeseed (Boszorádová *et al.* 2019) and tobacco (Moravčíková *et al.* 2008), but shifted activation of a heterologous promoter in time has also been observed by others (Luo *et al.* 2007; Polóniová *et al.* 2015). Given the critical requirement of transgene expression solely in the seeds of *Camelina*, the *CRUC* appears inappropriate for use in oil biotechnology.

The transgenic (in each case still RFP positive) seedlings have green expanded cotyledons and green true leaves. In contrast, control and non-transgenic seedlings had white cotyledons and they were unable to produce true leaves in the given (cultivation) conditions (Fig. 3). The surprising result of our transformation efforts was ~30 % of aberrant phenotypes seedlings (Fig. 3 and 4).

The observed phenotypic and developmental abnormalities included reduced or partially disrupted shoot apical meristems, and deformed or absent cotyledons (Fig. 4). These defects likely hamper the completion of embryo development and/or seed germination. According to Schwartz *et al.* (1994), defects in *Arabidopsis* embryo development appear in the globular stage of embryogenesis, while the abnormalities in the suspensor function were detected soon after at the heart stage. Altered development of the embryo in mutant seeds leads indirectly to the proliferation of suspensor cells and expression of properties characteristic of the embryo proper. In this context is also important that storage protein and lipid bodies, which normally accumulate only in the *Arabidopsis* embryo, were present in both structures (Schwartz *et al.* 1994). This is consistent with the work recently published by Zhu *et al.* (2018), who achieved more than 20 % increased oil content in the transgenic *Camelina* seeds, but they did not observe any adverse effects on growth and development. Furthermore, the changes in the composition of oil do not affect the germination of *Camelina* seeds, and therefore these oils can be used by plants as an energy source during the embryo development, seed germination, in regulation processes of seedlings growth and development, and even during the cultivation in the field (Liu *et al.* 2015).

Previously, tissue-specific expression of aspartate aminotransferase in *Brassica napus* produced transformants with aberrant phenotype, such as abnormal leaf morphology and photosynthesis as well as stunted growth (Wu *et al.* 2002; Shu *et al.* 2002; Ahlert *et al.* 2003; McAllister *et al.* 2016). Ahlert *et al.* (2003) speculated that the position of transgene insertion may have played a key role in the phenomenon.

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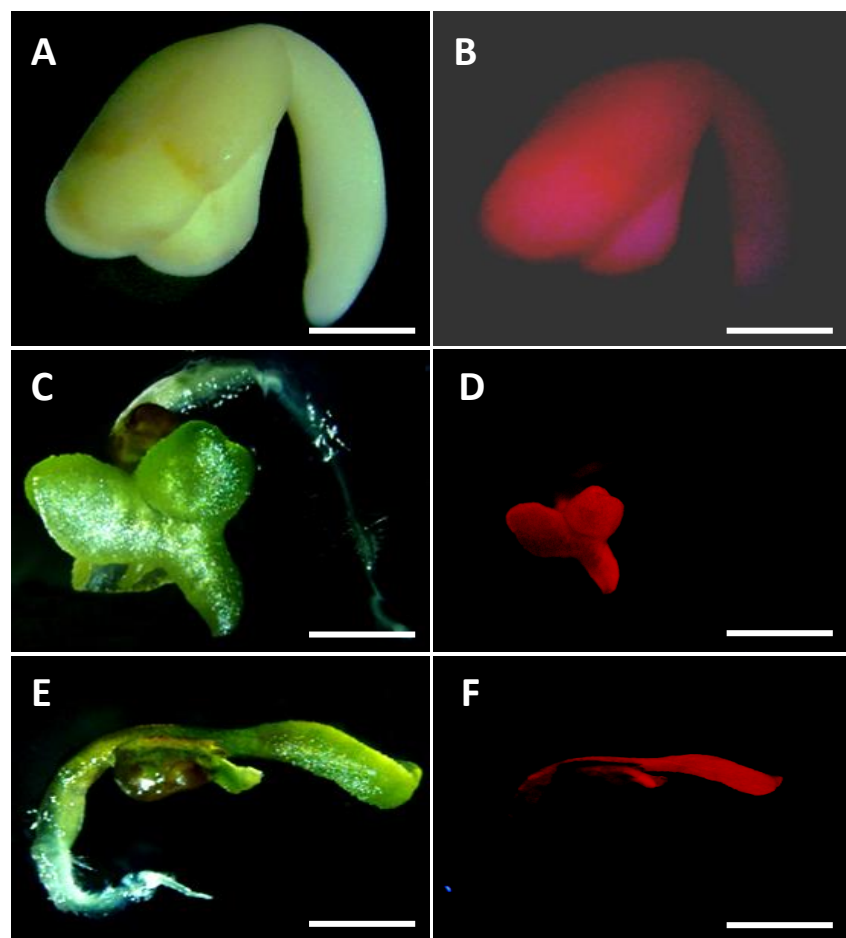
Fig. 4. Red fluorescence in large number of embryos with developmental deformations in development, such as deformed (**A** – **D**) or absent cotyledons (**E** – **F**). The bar represents **A**, **B** = 250 μm; **C** – **F** = 1 cm.

Since aberrant transformants are undesired side-products of many experiments, they are rarely discussed in detail and their reasons are not further explored. There are many quite well described reasons for changes at DNA level (ranging from point mutations and methylation differences to transposon induction, gene amplification, chromosomal aberrations and ploidy level changes; Kaeppler *et al*. 2000; Jain 2001; Bregitzer *et al*. 2002; Boszorádová *et al*. 2011, 2014). Numerous aspects of plant transformation are associated with the stress of the exposed tissues. For example, the use of antibiotics – especially at higher concentrations like in our study – may induce epigenetic and/or genetic changes of the plant genome (Bardini *et al*. 2003; Madlung and Comai 2004). The *Agrobacterium* infection itself, and also insertions of small fragments of transgenic DNA can also be a potential source of genome-wide mutations. As these would usually be missed by Southern blot and even by polymorphism analysis techniques, the numbers of genome-wide

mutations in plants transformed by *Agrobacterium* (and possibly also by particle bombardment) are probably underestimated (Makarevitch *et al*. 2003). Importantly, the presence of strong transgene promoters, including the commonly used cauliflower mosaic virus (*CaMV*) promoter and also the *CRUC* promoter used in this study, may also result in mis-expression (especially over-expression) of endogenous genes (Moravčíková *et al*. 2008; Boszorádová *et al*. 2019) at a distance of up to 12 kbp (Ichikawa *et al*. 2003). Transcriptional read-through and improper mRNA processing (protein translation/folding) can also occur when the *NOS* terminator is used (Rang *et al*. 2005).

Conclusions

Since the current plant transformation methods are mutagenic, efficient production of safe transgenic plants for biotechnology would require more data on the frequency and molecular basis of

transformation-induced mutations (Wilson *et al.* 2006). Those presently available describe aberrant *Arabidopsis* plants created by *Agrobacterium*-mediated transformation, while those from other species (including important crop plants) describe at most a few transgenic individuals with incompletely analysed genome changes. In our system, both strong (ectopically active) promoter and terminator combined with high antibiotics concentrations could be responsible for aberrant regenerants. Though formation of aberrant plants has not been reported as consequence of neither RFP production not *CRUC* promoter activity, for *Camelina* the *CRUC* did not prove to be promising for seed-directed genome modification.

Acknowledgement

Conflict of Interest

The authors declare that they have no conflict of interest.

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