The role of explant type and selective agent application in the initial transformation rate of *Lens culinaris* Medik.

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Abstract. *Lens culinaris* Medik. (lentil) is an agronomically important leguminous species, but its genome modification is rarely used for obtaining new varieties, probably due to a low efficiency of transformation protocols. Development of universal, genotype-independent protocols for obtaining transgenic plants usually relies, among other factors, on the possibility of obtaining a substantial number of transgenic cells *in vitro*. This study aimed to adapt a previously developed *Agrobacterium*-mediated transformation protocol, used for a related legume, for the production of transgenic callus tissue in *L. culinaris*. We used two different markers of transgenic tissue, beta-glucuronidase and green fluorescent protein, to find an optimal type of explant for obtaining transgenic tissue in lentil. We also evaluated the impact of hygromycin, a common selective agent, on the amount of transgenic tissue in developing transformed explants of *L. culinaris*. According to our results, the transformation protocol commonly used for *Medicago truncatula* Gaertn. leaf explants can also be applied for obtaining transgenic calli from *L. culinaris* shoot apices. Explants from shoot apices demonstrated higher initial transformation rate in comparison with explants from roots, stems and leaves. Moreover, explants of different types, which were cultivated on medium without hygromycin, developed significantly fewer calli expressing reporter genes than those grown on hygromycin-containing medium, confirming that hygromycin may be used as an effective selection agent for lentil. During our analysis, we noticed GUS-like staining in calli which didn't contain plasmids for *GUS* gene expression. This can be explained with so-called intrinsic GUS-like activity, which was described in previous research. These data can be used for further development of effective and universal *L. culinaris* transformation and genome editing protocols.

Key words: lentil; hygromycin; GUS; GFP; transformation; *Lens culinaris*

Роль типа экспланта и применения селективного агента в первичной эффективности трансформации *Lens culinaris* Medik.

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Аннотация. *Lens culinaris* Medik. (чечевица) – важная сельскохозяйственная культура из семейства бобовых, но модификация ее генома редко используется для создания новых сортов. Вероятно, это связано с низкой эффективностью существующих протоколов трансформации. Как правило, разработка универсальных, независимых от генотипа протоколов получения трансгенных растений зависит, среди прочего, от возможности образования существенного количества трансгенных клеток *in vitro*. В настоящем исследовании мы предприняли попытку адаптировать разработанный ранее для другого бобового протокол агробактериальной трансформации для получения трансгенной каллусной ткани у *L. culinaris*. Чтобы оценить эффективность трансформации, мы выбрали две независимые репортерные системы: бета-глюкуронидазу и зеленый флуоресцентный белок. С помощью этих систем мы нашли оптимальный тип экспланта для создания каллусной ткани чечевицы, экспрессирующей рекомбинантную ДНК. Мы также оценили влияние гигромицина, одного из распространенных селективных агентов, на количество

трансгенной ткани в развивающихся трансформированных эксплантах *L. culinaris*. Согласно нашим результатам, протокол трансформации, который обычно применяется для эксплантов листьев *Medicago truncatula* Gaertn., может быть использован и для получения трансгенных каллусов из верхушек побегов *L. culinaris*. Экспланты из апексов побегов продемонстрировали более высокую первичную эффективность трансформации по сравнению с эксплантами из корней, стеблей и листьев. Кроме того, экспланты разных типов, культивируемые на среде без гигромицина, образовывали значительно меньше каллусов, экспрессирующих репортерные гены, по сравнению с эксплантами, выращиваемыми на среде с гигромицином. Это подтверждает возможность использования гигромицина в качестве эффективного селективного агента для чечевицы. В ходе нашего анализа мы также обнаружили GUS-подобное окрашивание в каллусах, не содержащих плазмид для экспрессии гена *GUS*, что можно объяснить так называемой внутренней GUS-подобной активностью, которая была описана в предыдущих исследованиях. Эти данные могут быть полезны для дальнейшей разработки эффективных и универсальных протоколов трансформации и редактирования генома *L. culinaris*.

Ключевые слова: чечевица; гигромицин; GUS; GFP; трансформация; *Lens culinaris*

Introduction

Lentils (*Lens culinaris* Medik.) are one of the oldest domesticated crops (Erskine et al., 2009), the cultivation history of which dates back over 8,000 years (Liber et al., 2021). Lentil production has doubled since the beginning of the 21st century (Kaale et al., 2023), coinciding with a rising societal interest in plant-based meat alternatives and growing trends towards more sustainable food production (Runte et al., 2024). Being a leguminous plant, *L. culinaris* is used both as a nitrogen-fixing crop improving the soil fertility and as a source of protein for human nutrition. The level of carbohydrates, including starch and dietary fiber, is also quite high in lentils, making them a valuable crop among other legumes. Furthermore, biochemical properties, specific for different lentil cultivars, provide a wide range of lentil applications in food production (Kaale et al., 2023).

Despite many features, which make lentils a valuable crop, its production and usage are related with diverse obstacles, including undesirable traits common for most legumes and also drawbacks which are specific for this culture. The features which require alleviation in lentil, include high content of different anti-nutrients, such as phytate, as well as low levels of some amino acids (Joshi et al., 2017), pod shattering (Cao et al., 2024), etc.

Advancements in next-generation sequencing led to the discovery of many genetic markers and genes related to different desirable and undesirable traits in lentils (Kumar et al., 2023). These data can be widely used for selection and breeding of lentil cultivars with specific useful features. However, currently this information may rarely be used for genome modification of lentils, because, similarly to many legumes, the transformation and regeneration effectiveness of this species is quite low. There are only a few studies on *L. culinaris* transformation, and, to our knowledge, all of them report on low effectiveness of obtaining transgenic lentil plants (Gulati et al., 2002; Celikkol Akcay et al., 2009; Khatib et al., 2011; Chopra et al., 2012; Das et al., 2012, 2019; Polowick, Yan, 2023), which doesn't exceed 7 percents (i. e. 7 transgenic plants per 100 explants) for individual cultivars. Moreover, these protocols are developed for specific lentil varieties and, therefore, their effectiveness may depend greatly on genotype transformed.

The transformation effectiveness depends both on the success of foreign DNA introduction into plant cells and on further fate of the transformed cell, i. e. its ability to give rise to a new plant. Consequently, these parameters are influenced by many factors accompanying transformation, including the method of transformation, explant type, cultivation medium and the method of transformant selection. A significant part of plant transformation protocols includes the stage of obtaining transgenic callus. At this stage, modified cells proliferate and some of them acquire the capacity for regeneration. Therefore, the efficiency of transgenic callus formation plays an important role in overall transformation efficiency. Moreover, maximization of transgenic tissue yield for a plant species facilitates the screening for morphogenic regulators, i. e. genes that are able to induce plant regeneration from transgenic tissue and can be used for effective cultivar-independent transformation (Bakulin et al., 2025).

In the current study, we made an attempt to find an optimal type of explant for obtaining transgenic callus tissue in lentil. We also evaluated the impact of hygromycin, a common selective agent, on the amount of transgenic tissue in developing transformed explants of *L. culinaris*. We built our transformation protocol on the highly effective transformation system used for *Medicago truncatula*, a well-studied model leguminous plant (Cosson et al., 2006). This system includes the formation of a significant amount of transgenic callus tissue. Moreover, as a model legume, *M. truncatula* provides an efficient platform for identifying key morphogenic regulators (Yakovleva et al., 2024), which can subsequently be validated in *L. culinaris*. The optimized protocol presented in this study provides a foundation for such validation experiments.

Materials and methods

Plant material and bacterial strains. *L. culinaris* Ekaterininskaya zelenosemyannaya cultivar (k-2942) seeds from the collection of the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR) were used in the study. For cloning, the *Escherichia coli* DH10B strain was used. For *Agrobacterium*-mediated transformation, the *Rhizobium radiobacter* (*Agrobacterium tumefaciens*) AGL1 strain was used. *E. coli* and *A. tumefaciens* cultivation and transformation were performed as described in (Potsenkovskaia et al., 2022).

Plasmids used in the study. For *L. culinaris* transformation, two plasmids were used: pMDC32_GUS (Tvorogova et al., 2019) with the beta-glucuronidase (GUS) gene and pMDC32_eGFPer obtained in this study. To construct the pMDC32_eGFPer plasmid, the *eGFPer* gene, encoding the enhanced green fluorescent protein (GFP) with endoplasmic re-

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ticulum localization signal, was amplified from the pB7WG2D vector (Karimi et al., 2002) with primers containing attB1 and attB2 sites (forward primer: GGGGACAAGTTTGT ACAAAAAAGCAGGCTTCATGGTGAAGACTAATC TTTTTCTC; reverse primer: GGGGACCACTTTGTACA AGAAAGCTGGGTCTTACAGCTCGTCCTTCTTG) and cloned into the pDONR207 entry vector (Invitrogen) and then to the pMDC32 vector (Curtis, Grossniklaus, 2003) using Gateway technology (Invitrogen).

***Agrobacterium*-mediated transformation.** For explants preparation, seeds of *L. culinaris* were incubated in concentrated sulfuric acid for 7 minutes, rinsed with tap water 7 times, then incubated in 5–7 % sodium hypochlorite solution (the "Belizna" bleach) for 8 minutes and rinsed with sterile water 10 times in a laminar hood. After sterilization, seeds were put on modified Fahraeus medium (Fåhraeus, 1957; Tvorogova et al., 2019) and left to germinate for 12 days at 21–24 °C, 16 h (light)/8 h (dark) photoperiod. After that, germinated seedlings were used to prepare explants. Root explants (about 10–15 mm length) were obtained from root tips, shoot apex explants (about 10–15 mm length) were taken from the main and/or lateral shoots. Stem explants (about 3–8 mm length) were obtained from stem fragments between the first and the second leaves of the seedlings. For leaf explants, one or two first true leaves were taken. Seedling growth was asynchronous, and the explant size depended on the seedling development stage.

For *Agrobacterium*-mediated transformation itself, a modified protocol of Cosson et al. (2006) for *M. truncatula* Gaertn. was used. Agrobacterial strain carrying either pMDC32_GUS or pMDC32_eGFPer plasmids was recovered from glycerol stock (500 μl of 50 % glycerol mixed with 500 μl of overnight stationary-phase culture) stored at −80 °C and cultured on solid YEP medium (Table S1)[1] containing kanamycin (50 mg/l) and rifampicin (40 mg/l). Kanamycin resistance was provided by the pMDC32_GUS or pMDC32_eGFPer plasmid, whereas rifampicin resistance was characteristic of the AGL1 strain. On such medium, bacteria were cultivated at 30 °C for 1–2 days. Then, a small amount of bacteria from the Petri dish was sown to the liquid YEP with the same concentrations of antibiotics, in which they were grown overnight in a thermoshaker at 30 °C and 200 rpm. On the next day, 2 ml of bacterial culture were transferred to 50 ml of AB-MES medium (Wu et al., 2014, Table S1) with the addition of 40 mg/l (200 μM) acetosyringone, kanamycin (50 mg/l) and rifampicin (40 mg/l). Bacteria were cultivated in this medium at 30 °C and 200 rpm for about 3 hours. Then, they were centrifuged (4,000g, 15 minutes, room temperature) and resuspended in liquid ABM-SH medium (Table S1) with 4 mg/l (18 μM) 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l (2.22 μM) 6-benzylaminopurine (BAP), and 40 mg/l (200 μM) acetosyringone to the final OD600 0.1. ABM-SH represented a mixture of 0.5X AB-MES components and 0.5X modified liquid PCI-4 medium (Hoffmann et al., 1997) components, similarly to the infiltration medium from the AGROBEST protocol for *Arabidopsis thaliana* (L.) Heynh. (Wu et al., 2014).

[1] Supplementary Table S1 and Figures S1, S2 are available at:
https://vavilovj-icg.ru/download/pict-2026-30/appx17.pdf

Prepared explants were incubated in *Agrobacterium* suspension in ABM-SH medium for 15 minutes with slow gentle agitation. After the inoculation stage, explants were blotted on sterile filter paper, then put on solid ABM-SH medium (Table S1) and cultivated in the dark at 21–24 °C for about 36 hours. After cocultivation, explants were put on solid PCI-4 medium (Hoffmann et al., 1997, Table S1) for callus formation with 4 mg/l (18 μM) 2,4-D, 0.5 mg/l (2.22 μM) BAP, and with the addition of cefotaxime (250 mg/l), to eliminate agrobacteria. In some experiments, hygromycin (10 mg/l), necessary for the selection of transgenic plant cells, was added to the medium. On this medium, explants were cultivated for 44 days, with transfer on the fresh medium every 14–16 days. At the end of cultivation, explants were either stained with X-Gluc and analysed with stereomicroscope to detect *GUS* expression, or analysed with the fluorescent stereomicroscope to detect GFP fluorescence.

GUS staining. For GUS staining, X-Gluc substrate (Sisco Research Laboratories Pvt. Ltd.) was used. Plant tissues were collected in NT buffer (100 mM Tris-HCl, 50 mM NaCl) and then transferred to X-Gluc buffer (100 mM Tris-HCl, 50 mM NaCl, 0.2 mM $K_3[Fe(CN)_6]$, 2.5 mM X-Gluc), where they were incubated at 37 °C overnight. After staining, tissues were transferred to 70 % ethanol. Photos of single explants were taken with a ZEISS SteREO Discovery.V12 stereomicroscope. Photos of Petri dishes with explants were taken with the Vilber Fusion-FX6.Edge imaging system. For microscopic images, pieces of callus tissue were squashed and examined under a Leica DM500 microscope.

Statistical analysis and software. For evaluation of explant type and hygromycin presence impact, 20–28 explants per group were used. Statistical significance of differences was evaluated with Fisher's exact test with Bonferroni adjustment for multiple comparisons, if necessary. Statistical analysis of results and diagram drawing were performed in the R studio using the vcd (Meyer et al., 2006, 2024) and dplyr (Wickham et al., 2025) packages. Final figures were created using the Inkscape software.

Results

Evaluation of transgenic tissue development from different explant types using the GFP marker

We decided to build our transformation protocol on the transformation system used for *M. truncatula* (Cosson et al., 2006), in which transgenic plants are developed from transformed leaf explants through somatic embryogenesis. To find the optimal variant of explant type for transformation, we performed *Agrobacterium*-mediated transformation of root, stem, leaf and shoot apex explants with plasmid pMDC32_eGFPer for *eGFPer* overexpression. After cocultivation, explants were transferred to the callus-inducing medium with or without hygromycin. The concentrations of plant growth regulators and cefotaxime for agrobacteria elimination were identical to the ones used for *M. truncatula*, but the concentration of hygromycin was lower (10 mg/l instead of 25 mg/l used for *M. truncatula*), because 10 mg/l concentration is successfully used for other legumes to obtain transgenic tissue (Olhoft et
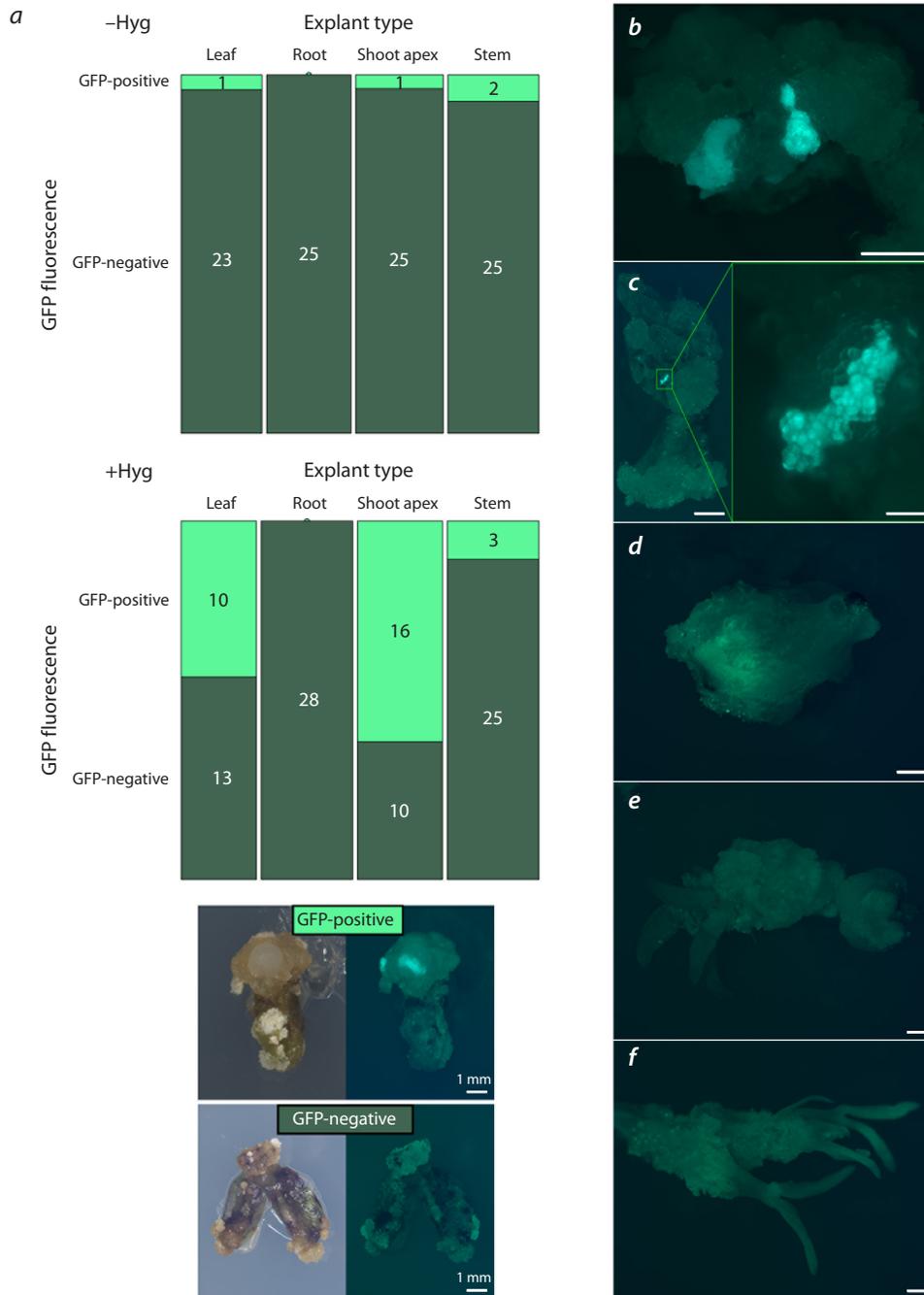
Fig. 1. *a*, Mosaic plots demonstrating the number of calli with GFP fluorescence (GFP-positive) and without GFP fluorescence (GFP-negative) developed from different types of explants, on the medium without hygromycin (above) or on the medium with addition of 10 mg/l hygromycin (below). In a legend below, the photos of explants were taken in white light (left) and fluorescent (right) light. *b–d*, Calli with GFP fluorescence. *e*, *f*, Calli without detectable GFP fluorescence, obtained after explant transformation with the pMDC32_GFP (*e*) or pMDC32_GUS (*f*) plasmids.

Size bar is 1 mm for *b–f* and 100 μm for the inset in *c*.

al., 2003; Li et al., 2023; Tisseyre et al., 2024). After 46 days of cultivation on this callus-inducing medium, calli were developed from all types of explants both on the medium with hygromycin and hygromycin-free medium (Figs. S1, S2). We analysed GFP fluorescence to evaluate the effectiveness of transformation.

To avoid false positive results, we also analysed the green fluorescence in the explants transformed with pMDC32_GUS construction, which didn't contain the *GFP* gene. Bright GFP fluorescence in separate tissue areas (Fig. 1*b*, *c*), as well as less bright diffuse fluorescence (Fig. 1*d*), were easily distinguishable from autofluorescence (Fig. 1*e*, *f*), which was observed in control pMDC32_GUS explants. It is worth noting that some fluorescent areas with weak signal, as well as autofluorescent areas, can only be seen with high magnification (60× and more). Therefore, for robust comparative analysis, it was

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crucial to evaluate the presence of fluorescence for all explants without changing magnification and other visualization settings on the stereomicroscope.

According to our results, on the medium supplemented with 10 mg/l hygromycin, the type of explant had an impact on the frequency of fluorescent calli (Fisher's exact test, p-value 4.359e–08). The highest frequency of transformed calli with GFP fluorescence was detected for explants from shoot apices (Fig. 1a). Leaf explants also demonstrated a rather high frequency of transformation, whereas only 3 out of 28 stem explants developed fluorescent calli. Calli from the root explants didn't demonstrate any transgenic fluorescent tissue at all (Fig. 1a). Overall, shoot apex explants formed significantly more fluorescent calli in comparison with explants from roots or stems (Fisher's exact test with Bonferroni adjustment for multiple comparisons, p-values 1.51e–06 and 8.46e–04, respectively), whereas differences between explants from shoot apices and leaves were not statistically significant.

At the same time, explants of all types, cultivated on medium without hygromycin, developed significantly fewer fluorescent calli than those grown on hygromycin-containing medium (Fisher's exact test, p-value 2.09e–06), confirming that hygromycin may be used as an effective selection agent for lentil (Fig. 1a).

Evaluation of transgenic tissue development from different explant types using GUS marker

We performed a similar experiment with transformation of different explant types using GUS, another transgenic tissue marker. Explants were transformed with the pMDC32_GUS plasmid (Tvorogova et al., 2019) using the protocol mentioned above. After 46 days of cultivation (starting from the day of transformation), GUS activity was evaluated in explants. Usage of GUS allowed us to detect single stained cells (at about 80× magnification on stereomicroscope), which we were not able to visualize with the GFP marker. The results of transgenic tissue detection were similar to those obtained using the GFP marker. On the media with hygromycin, the highest transformation efficiency was shown for shoot apex explants, followed by leaf explants. Both root and stem explants demonstrated a low transformation rate (Fig. 2). In total, shoot apex explants formed significantly more stained calli in comparison with explants from leaves, roots or stems (Fisher's exact test with Bonferroni adjustment for multiple comparisons,
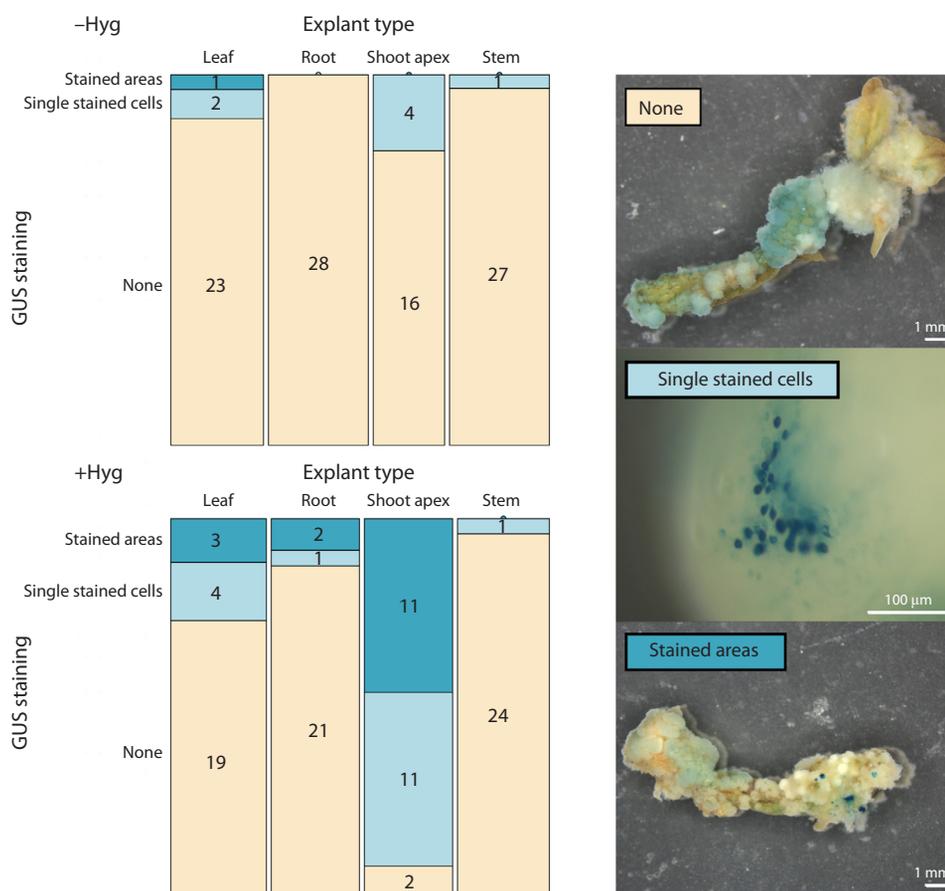

Fig. 2. Mosaic plots demonstrating the number of stained and non-stained calli developed from different types of explants after transformation with pMDC32_GUS plasmid, on the medium without hygromycin (above) or on the medium with addition of 10 mg/l hygromycin (below). The legend on the right demonstrates calli belonging to different categories relative to GUS staining: none – no detectable GUS staining apart from the light-blue color characteristic of endogenous GUS-like activity; single stained cells – small spots of GUS staining visible only with high (about 80×) magnification, stained areas – patches of GUS staining visible with naked eye or with low magnification.
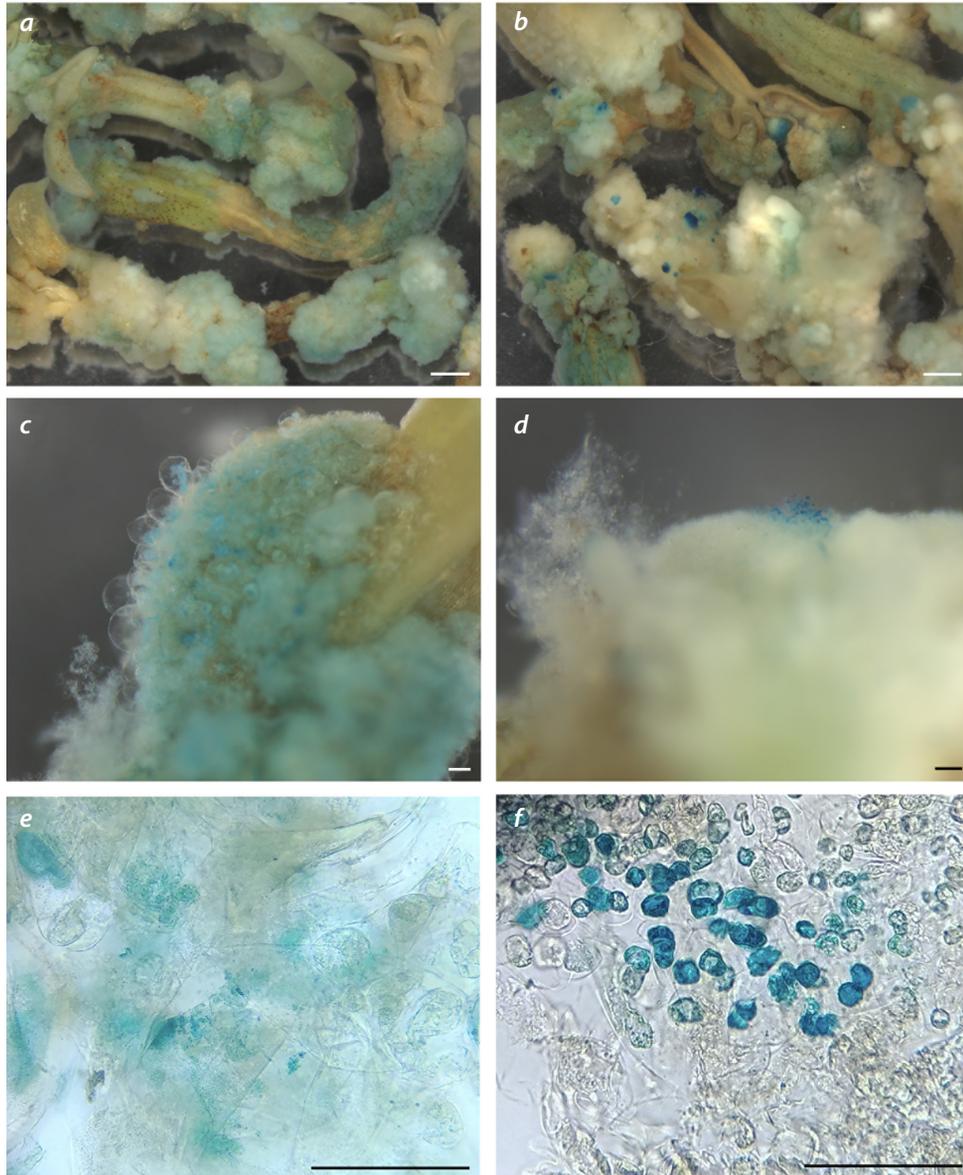
Fig. 3. GUS staining of control calli obtained from explants transformed with the pMDC32_eGFPer plasmid (*a*, *c*, *e*) or calli obtained from explants transformed with the pMDC32_GUS plasmid (*b*, *d*, *f*).

Size bar is 1 mm for *a*, *b* and 100 μm for *c*–*f*.

p-values 6.243734e–05, 2.191140e–07 and 7.429123e–10, respectively). In contrast, all types of explants grown on the hygromycin-free media mostly didn't develop detectable transgenic tissues. The difference was statistically significant when compared to explants cultivated on hygromycin-containing medium (Fisher's exact test, *p*-value 5.842e–12), confirming our results obtained for the GFP marker (Fig. 1).

Evaluation of GUS false positive staining

During the experiment with GUS activity analysis described above, we also performed GUS staining of explants transformed with the pMDC32_eGFPer construction, which didn't carry the *GUS* gene. Unexpectedly, these control explants also demonstrated blue staining, although the staining pattern differed from the one provided by true *GUS* expression (Hu et al., 1990). In false positives, the blue coloration was more diffuse and didn't have distinct borders compared with true GUS staining (Fig. 3).

Microscopic analysis also revealed differences between false positive and true GUS staining. *GUS*-expressing cells had deep blue coloring (Fig. 3*f*), which was not the case for false positive GUS-like staining (Fig. 3*e*). Therefore, the GUS marker can be used to detect transgenic tissue in lentil, but the staining of negative controls is necessary.

Discussion

Transformation is a powerful tool for obtaining new varieties of different plant species (Garcia Ruiz et al., 2018; Noack et al., 2024), which is why improving its effectiveness for lentil is important. Nevertheless, current *L. culinaris* transforma-

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tion protocols have rather low efficiency, probably due to the low regeneration rate. One of the possible solutions for this problem is the usage of morphogenic regulators, i. e. genes that can be added to transformation vectors and that are able to induce proliferation of transgenic cells and plant regeneration from transgenic tissue. Still, the search for such morphogenic regulators, suitable for lentils, can be hampered with low initial transformation rate, i. e. low effectiveness of transgenic callus formation. Therefore, the development of effective protocols for maximizing the transgenic tissue amount can be very useful for further optimization of obtaining transformed and edited plants.

The protocols for obtaining transgenic lentil plants usually involve cotyledons and/or cotyledonary nodes, parts of embryos or embryo axes (Gulati et al., 2002; Cellikol Akcay et al., 2009; Khatib et al., 2011; Das et al., 2012, 2019; Polowick, Yan, 2023) or whole seeds (Chopra et al., 2012). In our study, we tried to perform *Agrobacterium*-mediated transformation using different types of explants obtained from 12-day old seedlings. According to our results, the transformation protocol, developed for *M. truncatula* leaf explants, can also be used for obtaining transgenic tissue from *L. culinaris* explants. Still, although the leaf explants are used in this protocol for *M. truncatula*, a different type of explant, shoot apex, gave better results for *L. culinaris*. It can possibly be related to a relatively high proportion of stem cells and, in general, proliferating cells in the shoot apex in comparison with other explant types. It would be interesting to evaluate the efficiency of usage of seedling shoot apices for *M. truncatula* transformation. The usage of hygromycin at the concentration 10 mg/l allowed us to obtain a significant amount of transgenic tissue from shoot apex explants. Nevertheless, the optimal hygromycin concentration has to be defined in the future.

There are also several studies aiming to obtain transgenic tissues in lentil from diverse explant types, including different parts of germinated seedlings (Warkentin, McHughen, 1992), cotyledonary nodes (Mahmoudian et al., 2002) and embryo axes (Lurquin et al., 1998), although in most cases, no selection agent and no 2,4-D-mediated callus induction was applied, in contrast to our research. As far as we know, the current study is also the first in which optimization of lentil transformation was performed with the usage of two different transgenic tissue markers.

In a study by Warkentin and McHughen (1992) *Agrobacterium*-mediated transformation of lentil shoot apex explants yielded a high percentage of explants, which contained transgenic tissue, without selection for transgenic cells at early stages of cultivation. In our study, cultivation on the medium without a selection agent was not effective, whereas hygromycin application made it possible to obtain a significant amount of transgenic tissue. The discrepancy between our results and results obtained previously may be due to differences in genotypes, *in vitro* cultivation and transformation conditions.

In experiments on evaluation of *in vitro* regeneration capacity in lentil, explants from shoot tips were shown to give callus on the medium with 2,4-D, as well as regenerating shoots on other types of media, with the addition of BAP, IAA or NAA (Polanco et al., 1988). Shoot regeneration from shoot apex

explants was also observed after *Agrobacterium*-mediated transformation, although shoots regenerated during kanamycin selection were non-transgenic (Warkentin, McHughen, 1992). Therefore, the development of a protocol for producing transgenic lentil plants from shoot tip explants may be feasible upon identification of suitable selection and regeneration conditions. Based on our data, hygromycin selection appears to be a promising approach.

During our analysis, we noticed the GUS-like staining in calli that didn't contain plasmids for *GUS* gene expression. This can be explained with so-called intrinsic GUS-like activity, which was described in previous research (Hu et al., 1990). Indeed, beta-glucuronidase (GUS) genes or proteins were described in several species, including *Scutellaria baicalensis* (Zuo et al., 2025) and *Zea mays* (Muhitch, 1998), whereas endogenous GUS activity was detected in *Brassica napus* (Abdollahi et al., 2011), *Oryza sativa*, *A. thaliana*, *Nicotiana tabacum* and other plants (Sudan et al., 2006). Still, in other studies involving GUS staining of lentil explants, no false positive GUS staining was detected on control explants, which may be due to different staining buffer composition or different tissue features (Warkentin, McHughen, 1992; Lurquin et al., 1998; Mahmoudian et al., 2002).

Conclusion

Our results show that *Agrobacterium*-mediated transformation of lentil shoot apices according to the protocol developed for *M. truncatula*, can be successfully used for obtaining transgenic callus tissues. These data can be used for further development of effective and universal lentil transformation and editing protocols.

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