

An efficient and reproducible *Agrobacterium*-mediated genetic transformation method for the ornamental monocotyledonous plant *Ornithogalum dubium* Houtt.

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Abstract

Ornithogalum is a genus from the *Hyacinthaceae* (*Asparagaceae*) family that comprises about 200 species with remarkable white, yellow, or orange flowers that display exceptional vase life. These properties have made it a popular cut flower and pot plant. Forward genetics approaches may be advantageous to generate novel phenotypes, but the *Agrobacterium*-mediated transformation of plants from this genus remains challenging. Here, a stable and efficient *Agrobacterium*-mediated transformation system was established for *O. dubium*. We found that the timing of transformation with respect to light exposure of the tissue affected transformation rates more than other tested parameters. In the transgenic plants obtained, T-DNA integrations were confirmed by polymerase chain reactions, and positive plants were established in the greenhouse and displayed weak transgene expression. This study exposed an efficient platform for gene function research and germplasm improvement in *O. dubium* plants. The present protocol is now available for the development of novel improved *O. dubium* varieties.

Introduction

Ornithogalum dubium, commonly known as chinchinchee or star-of-Bethlehem, is a bulbous plant that belongs to the *Asparagaceae* family. This genus includes about 200 species, of which *O. thyrsoides*, *O. saundersiae*, and *O. dubium* are the most popular with their remarkable long-lasting white, yellow, and orange flowers. These characteristics have made them popular ornamental cut flowers, whereas other *Ornithogalum* species, including *O. umbellatum*, *O. nutans*, and *O. pyramidale*, are mostly used for gardening (Griesbach et al. 1993; Littlejohn and Blomerus 1997), or as pot plants (Ziv and Lilien-Kipnis 1997). Over the last decades, the market for *O. dubium* has grown and this plant is now sold in the United States, Europe, China, South Africa, and Israel with an ever-increasing demand for novel flower colors and shapes (Niederwieser and Bornman 2004; Tun et al. 2013).

The propagation of *Ornithogalum* species is typically achieved vegetatively using mother bulb propagation. However, in some species, bulbs display a low propagation rate (Naik and Nayak 2005) and are highly sensitive to both viral and bacterial pathogens (Luria et al. 2002), which reduces flower quality and limits their vegetative propagation. Thus, tissue culture methodologies have been successfully developed to propagate high-quality *O. dubium* and *O. thyrsoides* varieties (Hussey 1976; Ziv and Lilien-Kipnis 1997) through direct organogenesis (Hussey 1977; Ascough et al. 2009). In *O. dubium*, micropropagation may be achieved by first producing a suspension culture of callus cells from which shoots and roots will emerge following light exposure (Ziv and Lilien-Kipnis 1997; Luria et al. 2002). However, such cell suspension cultures cannot be maintained for long time periods and lose their regeneration capacity over time (Cohen et al. 2004). The high-throughput *in vitro* propagation of sterile and disease-free plantlets of desired germplasms of *Ornithogalum* thus remains challenging.

Although numerous protocols of micropropagation of ornamental geophytes have been developed, there are still only a few reports on genetic modifications of these crops and even fewer on *Agrobacterium*-mediated transformation (Koetle et al. 2015). This is because, in monocot geophytes, genetic

transformation is not only limited by the failure of *Agrobacterium* to reach the cells that are competent for regeneration, but also by the recalcitrance of many genotypes to callus formation and regeneration (Koetle et al. 2015; Hofmann 2016). Yet, methods using the right biovars or strains, the appropriate co-cultivation conditions, and a competent target tissue have made the *Agrobacterium*-mediated transformation of monocot flower bulbs possible (Koetle et al. 2015). In *O. dubium*, *Agrobacterium*-mediated transformation was achieved but it was not effective enough to routinely generate transgenic plants (van Emmenes et al. 2008). Furthermore, since it was believed that monocotyledonous plants are not susceptible to *Agrobacterium*-mediated transformation (Finer et al. 1992), a particle bombardment method was also developed for *O. dubium*. However, this approach was variable, and some of the transgenic plants were impaired in their growth (Cohen et al. 2004; Lipsky et al. 2014).

The recent developments in molecular biology, especially gene-editing technology, offer rapid and targeted genetic improvement to the ornamental plants industry. This method has been applied to almost all grain crops and many horticultural crops, launching a new era of molecular breeding. However, this approach necessitates an efficient genetic transformation method. To the best of our knowledge, this is why CRISPR/Cas9-mediated genome editing approaches in *O. dubium* have not been applied successfully to date. Here, an efficient *Agrobacterium*-mediated transformation system for *O. dubium* through callus culture was developed and used to generate stable transgenic plants. We found that the timing of *A. tumefaciens* infection with respect to light exposure was crucial for the success of this method, and exposing the cell culture to light prior to inoculation significantly improved the transformation efficiency. In the resulting transgenic plants, the presence of the T-DNA was confirmed by polymerase chain reaction analyses and RFP fluorescence was detected. This study opens the way for genetic manipulations of the ornamental monocot *O. dubium*.

Materials And Methods

Plant material and tissue culture

O. dubium plantlets were grown on the propagation medium (**Supplemental Table S1**). Growth chamber conditions were 24°C ± 2°C, 16/8 h light/dark photoperiod, and light intensity was 110 µmol m⁻² s⁻¹ (van Emmenes et al. 2008). To induce callus formation, leaves of 5 weeks old *O. dubium* were cultivated *in vitro* on an agar-solidified callus induction medium (CIM; **Supplemental Table S1**) for 3–4 weeks (Cohen et al. 2004, 2005). After 3–4 weeks, the calli were grown in liquid CIM (CIM without agar; Cohen et al. 2004), under dark conditions to develop a cell suspension culture. The cultures were transferred to fresh liquid CIM every two weeks. To induce shoot formation, the cultures were transferred to Petri dishes containing CIM with 3% agar under light conditions for 2 weeks. After shoots developed, they were transferred to magenta boxes containing propagation medium (**Supplemental Table S1**). Six weeks later, the proliferating shoots started to develop roots. Following sub-culturing, the plants, whose root system had developed, were transferred to soil. All media and reagents used for tissue culture were purchased from Duchefa Biochemie, Netherlands.

Scanning electron microscopy (SEM)

The regeneration process was monitored by histological observations. Leaf tissues from *in vitro*-grown *O. dubium* plants were regenerated as described above. Samples were harvested at 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 days. The samples were fixed using methanol-ethanol fixation (Talbot and White 2013). Critical point dry (CPD) was conducted according to the manufacturer's directions (K850, QUORUM). Finally, tissue was mounted on a scanning electron microscope (SEM) stub, coated with palladium, and monitored using a versatile benchtop JEOL (JCM-6000) SEM.

Histological analysis

The regeneration process was monitored by histological observations. Leaf tissues from *in vitro*-grown *O. dubium* plants were regenerated as described above. Samples were harvested at 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 days. The samples were fixed in FAA solution, which consisted of 10% formaldehyde, 5% glacial acetic acid, and 52% absolute ethanol (v/v). Histological analysis was performed as described previously (Singh et al. 2019). Briefly, fixed samples were dehydrated through a graded ethanol series (50, 70, 90, 95, and 100%) at 1 h intervals and embedded in paraffin wax, at 60 °C before sectioning. Serial sections (10µm) were made on a rotatory microtome (Leica RM2245, Leica Biosystems, Nussloch, Germany), and deparaffinized using a histoclear solution, before rehydration with serial ethanol dilutions. The sections were then used for histochemical staining with 1% (w/v) safranin and 0.2% (w/v) fast green to observe the formation of a vascular system and lignin deposition. Microscopic observations and photos were performed under a light microscope (ECLIPSE Ni-E, Nikon), and a Nikon DS-Fi1 digital camera.

Cloning methods

The plasmids designed in this study were assembled using the GoldenBraid cloning system (Sarrion-Perdigones et al. 2013). The putative *UBIQUITIN10* promoters and terminators were previously isolated and cloned into entry vectors and used to generate expression cassettes inside binary vectors (Kumar et al. 2021). Briefly, the *Arabidopsis UBIQUITIN10* gene was used to identify its homologs in tomato (Soly07g064130), potato (Sotub07g026130), sunflower (XM_022178991.1), and maize (XM_008647047.3). The sequences of these genes were retrieved from public databases and their adjacent sequences were identified and used to design specific oligonucleotides. The latter were used for PCR amplification using genomic DNA extracted from the respective plant species, and the resulting amplicons were purified and cloned into entry vectors. The various promoters and terminators were then used to assemble transcription units with specific genes. These transcription units were then combined to produce multipartite assemblies, as described in the GoldenBraid instructions (Supplemental Figure S1).

Determination of the minimum inhibitory concentration (MIC) of kanamycin

Standardization of minimum inhibitory concentration (MIC) of kanamycin (Kan) was conducted on wild-type *O. dubium* calli, in a selection medium containing different Kan concentrations (0, 12, 25, 50, 80, 100,

150 and 200 mg/l). The survival of the WT calli was monitored weekly for 6 weeks and the number of healthy-looking and dark brown-looking calli was determined at each Kan concentration. The Kan sensitivity was expressed as the number of healthy calli / total calli number x100.

Preparation of *A. tumefaciens* bacteria for *O. dubium* transformation

First, a binary vector containing the *RFP* and the *NPT2* genes placed under the control of the *ZmUBIQUITIN10* and *CaMV-35S* promoters was transformed into *A. tumefaciens* (EHA105 and AGL1) using the freeze–thaw method (Höfgen and Willmitzer 1988). *A. tumefaciens* was cultured in Luria-Bertani (LB) broth medium supplemented with 50 mg/L rifampicin (rif) and either kan (100 mg/l) or spectinomycin (spec; 100 mg/l) at 28 °C. Single colonies were picked and grown in liquid LB media at 28°C and 200 rpm. The resulting bacterial cultures were washed once in infiltration buffer consisting of 50 mM MES, 20 mM trisodium orthophosphate, 5 g/l D-glucose, and 200 µM acetosyringone, and further re-suspended in this medium, which was adjusted to a final OD₆₀₀ of different ranges (OD₆₀₀ = 0.5, 0.8, and 1.0).

Agrobacterium -mediated transformation of *O. dubium*

For *A. tumefaciens*-mediated transformation, *O. dubium* callus cell suspension cultures were incubated under light conditions for 0, 5, 10, 15, and 20 days prior to bacterial inoculation. The callus cell aggregates were immersed in the *Agrobacterium* cultures, which were formerly resuspended in the infiltration buffer, for 30 min on a rotary shaker, to ensure full contact with the infection solution. After the explants were removed from the infiltration buffer, the residual bacteria were absorbed with sterile filter papers, and the transformed explants were transferred to Petri dishes containing CIM for four days under dark conditions. Then, the calli pieces were transferred to the pre-selection medium (**Supplemental Table S1**) for seven days under dark conditions. After a week, the calli pieces were transferred onto the selection medium and cultured at 24°C ± 2°C, and 16/8 h light/dark photoperiod with a light intensity of 110 µmol m⁻² s⁻¹. The samples were subcultured every two weeks for ten weeks. Then, to initiate root formation, the shoots were transferred to Petri dishes containing several rooting media (**Supplemental Table S2**) and cultured for 4 weeks. The plantlets were then transferred to magenta boxes containing propagation media (**Supplemental Table S1**) and cultured for 2 to 4 additional weeks. Once rooted plants reached the top of the box, they were taken out, the agar medium was washed away from the roots under running tap water, followed by a wash with sterile distilled water. The plants were then transferred to an autoclaved JIFFY-7® supplemented with ½ MS liquid medium and placed inside sterile magenta boxes. The boxes were covered with thin transparent polybags and kept under a 16/8 h photoperiod at 24°C ± 2°C with 65% relative humidity (RH). One week later, cuts were made in the polybags, and plants were kept until new leaves developed and new roots emerged from the soil block. Next, the rooted plants, with their soil block, were transferred to 1 l pots while still covered with polybags. The polybags were subsequently removed and the plants continued their development in these pots. The number of surviving plantlets was recorded after 70 days and the number of RFP fluorescing plantlets was monitored weekly for 10 weeks. Transformation efficiency was calculated as the ratio of RFP fluorescing plantlets over the total number

of plantlets obtained after transformation x100. All data are expressed as mean $\pm$ standard deviation of triplicate samples.

Genetic characterization of putative transformants

Leaves samples were collected from plants in soil and genomic DNA (gDNA) was extracted from 100 mg leaves of T0 transgenic *O. dubium* plants using the C-TAB method (Doyle 1991). The quantification of gDNA was performed using a nanodrop spectrophotometer (Thermo Fisher Scientific, United States), and 100–200 ng of gDNA was used to perform polymerase chain reaction (PCR; Applied Biosystem by Thermo Fisher Scientific, Waltham, Massachusetts, United States) using the primers that corresponded to the *ACTIN*, *RFP*, and *CaMV-35S* promoter sequences (**Supplemental Table S3**). For genotyping, a 15 μ l PCR reaction mixture containing 7 μ l of 2X ready-to-use green PCR mixture (Promega, Madison, Wisconsin, USA), 1 μ l of each forward and reverse primer from 10 μ M stock, and gDNA was prepared. The PCR program consisted of an initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 kb/min according to the size of the desired amplicon. The final extension was performed at 72°C for 5 min.

Results

Optimized protocol for *O. dubium* micro-propagation

Leaf explants of *O. dubium* were used to initiate callus formation as described previously (Cohen et al. 2005; Lipsky et al. 2014). At first, the leaves were sterilized, excised, and cultured under dark conditions on CIM. Callus initiation started within 2 weeks, and friable calli appeared abundantly after 4 weeks (Fig. 1a, b). Next, the calli were transferred to liquid CIM and cultured under dark conditions. Calli suspension culture was obtained at weeks 8–10 and was kept in the dark for up to four months, by sub-culturing in fresh CIM every two weeks. Next, the cultures were transferred to light, where green shoots developed within a few days (Fig. 1c, d). The calli were then transferred to Petri dishes containing CIM and roots were observed within 4 weeks (Fig. 1e, f). The plantlets were then transferred to a hormone-free medium for an additional 4 weeks, after which the plants were planted in soil.

Microscopic and histological characterization of *O. dubium* shoot development

The developmental processes during micropropagation of *O. dubium* were examined during all stages of the regeneration process by histological and SEM analyses. For this, tissue samples were harvested at regular intervals throughout the regeneration process (Fig. 2a). The SEM observations showed that 5 days after tissue wounding, callus cells started to appear on the leaf cut and completely covered the wound by day 10 (Fig. 2b). The histological analysis revealed that at day 15, callus cells formed small round protuberances (Fig. 2c). Remarkably, between day 20 to day 30, regions of intense cell divisions developed near the surface of these bulges, and we observed a single cell layer that displayed anticlinal cell divisions on the protrusions surrounding (Fig. 2c). At day 30, the explants were transferred to liquid media and further grown under dark conditions. At day 35, the protuberances had increased in size, and

SEM observations revealed the presence of facing leaf structures (Fig. 2b). From day 40, well-defined meristems with lateral leaf structures were observed. At days 50 and 60, the meristems were fully formed and were surrounded by a few lateral leaves (Fig. 2b, c).

Exposure to light prior to *Agrobacterium* inoculation enhances the transformation rate in *O. dubium*

Efficient *Agrobacterium*-mediated transformation of *O. dubium* may greatly facilitate genetic manipulations by CRISPR-cas9. To achieve *O. dubium* transformation, we first determined the minimum inhibitory concentration of Kan as a selection agent for *O. dubium* explants (See methods). The Kan sensitivity was quantified and showed that 80 mg/l kanamycin was sufficient to effectively inhibit cell growth and development (**Supplemental Table S4**).

To monitor the transformation efficiency, a construct was assembled to contain the *RFP* gene fused to the maize *UBIQUITIN10* promoter (*pro-ZmUbiq*) as a fluorescent marker and the *NPT2* gene fused to the CaMV-35S promoter for selection (Fig. 3a). The vector was used to transform *A. tumefaciens* EHA105 and the transformed cells ($OD_{600} = 0.8$) were used for inoculation of *O. dubium* cell suspension cultures. Ten weeks were required for selection with 80 mg/l Kan and 250 mg/l ticarcillin, yielding merely a few shoots and weak RFP fluorescence, suggesting the need to improve this protocol. Since *O. dubium* cell suspension cultures respond to light exposure by initiating differentiation, we determined the optimal timing for *Agrobacterium* inoculation after light exposure. For this, the *O. dubium* cell cultures were transferred to light for 0, 5, 10, 15, and 20 days prior to inoculation with *A. tumefaciens* (Fig. 3b). Following *Agrobacterium* inoculation, the cultures were grown for additional ten weeks on the selection medium. Remarkably, while the transformation of dark-grown cultures yielded two transformants, the cultures that have been exposed to light for 5, 10, 15, and 20 days prior to inoculation yielded 15, 6, 19, and 14 kanamycin-resistant shoots, respectively, among which 4, 6, 6, and 5 shoots (total 21 shoots) displayed weak RFP fluorescence from day 21 (Fig. 4a, b, c). Following selection, the green and healthy plantlets were grown for additional four weeks on the rooting medium without kan, and for four more weeks on the propagation medium without phytohormones. Unexpectedly, this approach did not induce root formation in the transformed plantlets, unlike in the WT cultures. To overcome this issue, several hormone combinations were tested (**Supplemental Table S2**), and it was found that 0.5 mg/l NAA promoted root formation at the highest rate in the transformed plants. After roots were formed, the putatively transgenic plantlets were transferred to the soil (see methods). About two months later, in the greenhouse, the plants reached about 15–20 cm (Fig. 5a) and leaf samples were collected for genomic DNA extraction and RFP fluorescence analysis. Positive plants displayed weak RFP fluorescence (Fig. 5b), and using primers for the *RFP* gene and the CaMV-35S promoter sequences, PCR analysis revealed the presence of the transgenes in 12 plants (Fig. 5c). This result showed the potency of the described protocol in generating transgenic *O. dubium* plants.

To further improve this method, we optimized the concentration of *A. tumefaciens* cells required for the transformation. Cell cultures were exposed to light for 5 days and then inoculated with EHA105, diluted to 0.5, 0.8, and 1.0 OD_{600} . After ten weeks of selection, a larger number of RFP fluorescing plantlets was

obtained using the higher *A. tumefaciens* densities (**Supplemental Figure S2**), suggesting it would be more advantageous to use them for *O. dubium* transformation. In addition, two *A. tumefaciens* strains, EHA105 and AGL1, were compared for their potency to transform *O. dubium*. We found that both strains yielded a similar number of RFP fluorescing plantlets (**Supplemental Figure S3**). According to these results, all additional transformations were performed using the strain EHA105 at a cell density of $OD_{600} = 0.8$.

UBIQUITIN10 putative promoters direct potent gene expression in *O. dubium*

Given the low level of RFP fluorescence obtained using *pro-ZmUbiq*, we attempted to increase transgene expression using different regulatory regions to direct transgene expression. To this end, we tested the potency of *UBIQUITIN10* putative promoters from various plants at directing gene expression in transgenic *O. dubium*, as compared to the widely used CaMV-35S promoter (*pro*-CaMV-35S). The *UBIQUITIN10* putative promoters of *Arabidopsis*, sunflower, maize, tomato, and potato, further referred to as *pro*-AtUBIQ, *pro*-HaUBIQ, *pro*-ZmUBIQ, *pro*-SlUBIQ, and *pro*-StUBIQ, respectively, were already tested in a previous study and were found efficient at directing adjacent gene expression in *Nicotiana* species (Kumar et al. 2021). To determine their potency in *O. dubium*, these promoters were fused to the *RFP* gene, and the resulting cassettes were separately assembled with a *pro*-CaMV-35S::NPT2 cassette (**Supplemental Figure S1**). The resulting constructs were used to transform *O. dubium* cell cultures using the above-mentioned method. After a week of selection, Kan-resistant shoots started to emerge and developed throughout the selection process. From week 5, clear differences in RFP fluorescence intensity were observed (Fig. 6a, c). After ten weeks of selection, the constructs containing the *pro*-AtUBIQ, *pro*-HaUBIQ, *pro*-SlUBIQ, *pro*-ZmUBIQ, *pro*-StUBIQ, and *pro*-CaMV-35S yielded 9, 11, 6, 6, 12 and 3 kan-resistant shoots, and 4, 4, 3, 3, 4, and 2 RFP fluorescent shoots, respectively (Fig. 6b, c). We found the different promoters yielded different levels of RFP fluorescence. While *pro*-ZmUBIQ and *pro*-CaMV-35S induced weak RFP fluorescence, the *pro*-AtUBIQ, *pro*-HaUBIQ, *pro*-SlUBIQ, and *pro*-StUBIQ promoted high RFP fluorescence, which was ~ 3 fold higher than that of the CaMV-35S promoter (Fig. 6c).

At last, the transformed plantlets that showed RFP fluorescence were transferred to soil. After about 2 months, the plants reached 15–20 cm (Fig. 7a) and displayed weak RFP fluorescence (Fig. 7b). Leaf samples were collected for genomic DNA extraction and PCR analysis. Using primers for the *RFP* gene and the CaMV-35S promoter sequences, the presence of the transgenes was detected in 3 plants for *pro*-AtUBIQ, 2 for *pro*-HaUBIQ, 1 for *pro*-SlUBIQ, 1 for *pro*-ZmUBIQ, 1 for *pro*-StUBIQ and 0 for *pro*-CaMV-35S (Fig. 7c). Overall, the results suggested *UBIQUITIN10* promoters may be used as an efficient tool to direct potent gene expression during the first weeks following transformation, compared to the commonly used CaMV-35S promoter.

Discussion

Ornithogalum dubium is an important commercial crop for the cut flowers and potted plants market. Extensive cross-species hybridization was performed to expand the range of naturally occurring flower

colors and embryo rescue was used to recover viable hybrids (Niederwieser et al. 1990; Griesbach et al. 1993). Moreover, tissue culture procedures and bioreactors were developed for high throughput production while maintaining genetic integrity (Hvoslef-Eide and Preil 2005). Genetic improvement in *O. dubium* may be achieved by means of gene editing to develop novel traits suitable for the ornamental plants industry. However, gene editing most often relies on genetic transformation, and in monocotyledonous ornamental plants, this is limited by the failure of *Agrobacterium* to reach competent cells on one hand, and on the other by the lack of efficient regeneration systems for these plants (Sood et al. 2011; Koetle et al. 2015). Adjusting the parameters that govern efficient infection has facilitated the successful development of *Agrobacterium*-based transformation methods of flower bulbs nearly 40 ago in *Narcissus papyraceus* (Hooykaas-Van Slogteren et al. 1984). Since then, successful protocols for numerous monocotyledonous ornamentals have been developed, and the genus *Lilium* has been the subject of the largest number of reports, starting 30 years ago (Cohen and Meredith 1992). In *O. dubium*, an *Agrobacterium*-mediated stable transformation was developed (van Emmenes et al. 2008) but it was not efficient enough to routinely generate transformants. Successful *O. dubium* transformation was also obtained by the use of particle bombardment of cell clusters (Lipsky et al. 2014, 2016), but this approach did not yield high efficiencies and was not reproducible.

In this study, we first characterized the morphological changes that occur during *O. dubium* regeneration in the *in vitro* system. The histological analysis revealed that exposure to CIM caused the formation of ball-shaped callus aggregates that later exhibited an organized single-cell layer in their surroundings. Following light exposure, we found that these structures further developed into functional meristems and that roots developed subsequently to form functional organisms that could be transferred to soil. Similar results were recently obtained using IAA and TDZ (Petti 2020), suggesting *O. dubium* plants are highly reactive to *in vitro* hormone treatments and thus greatly suitable for tissue culture manipulations. Interestingly, our observations suggest a period of darkness followed by irradiance causes shoot development in *O. dubium*, although, in sugar cane (Garcia et al. 2007), lily (Mori et al. 2005), and sorghum (Pola et al. 2007), this treatment induces embryo formation.

Despite advances in *Ornithogalum* tissue culture methods, the genetic transformation of these species remains challenging. To achieve it, we used *A. tumefaciens* to infect callus cell aggregates, but this approach did not yield transformants in a robust manner, as reported previously (van Emmenes et al. 2008). Since, in this system, shoots develop following light exposure, we determined the optimal timing for *A. tumefaciens* infection with respect to irradiance. We found that light exposure for at least 5 days significantly increased the transformation efficiency. This suggests differentiating tissues may be more amenable to *A. tumefaciens* transformation than undifferentiated callus cells while retaining their regenerative capacities. This parameter was most critical as compared to the other parameters tested here. Besides light exposure, two *A. tumefaciens* strains, EHA105 and AGL1, were tested at different OD₆₀₀ (0.5 to 1), and achieved similar transformation efficiencies. This may be explained by the genetic similarity of these *A. tumefaciens* strains, which both harbor the C58 chromosomal backbone. The C58 *A. tumefaciens* strain has long been used to transform *Lilium* (Cohen and Meredith 1992; Hoshi et al. 2004),

Agapanthus (Suzuki et al. 2001), and *Gladiolus* (Kamo 1997), and was found more infectious than other strains in *Lilium* transformation assays (Langeveld et al. 1995).

Unexpectedly, unlike their wt counterparts, transgenic *O. dubium* did not root spontaneously, even though Kan was not used in the rooting assays. While a shorter selection period may improve tissue response, it may also lead to the emergence of false positives. Thus, to overcome rooting recalcitrance, we used 0.5 mg/l NAA, which promoted abundant root development and facilitated plant establishment in the soil. In addition, while the presence of the transgene was detected in the transformed plants after their establishment in soil, RFP fluorescence was weak, suggesting transgene silencing, which has long been recognized as a major obstacle to transgenic technologies (Baulcombe 1996). This may also be particularly cumbersome because it may limit transgene expression in gene editing assays. In an attempt to improve transgene expression, we used different *UBIQUITIN10* promoters (Kumar et al. 2021). We observed that these promoters directed *RFP* expression at various levels merely during the first weeks of growth in tissue culture. However, in mature plants, RFP fluorescence was very weak and difficult to monitor, which may explain the occurrence of false positives. Thus, to achieve gene editing in *O. dubium*, it may be needed to isolate rare transformation events with limited silencing effect, or use silencing suppressors, such as P19 (Silhavy et al. 2002) or *SGS3*-silencing (Kumar et al. 2022).

Recent developments in plant gene-editing technology now allow rapid and targeted plant breeding. While this is well advanced in food crops, gene editing in bulbous ornamentals remains marginal. This study presents a robust protocol for the stable transformation of *O. dubium* plants and opens the way for its genetic manipulation and the development of new varieties.

Conclusion

This study focused on establishing a genetic transformation of *O. dubium* testing various transformation factors. Key transformation conditions, including *A. tumefaciens* strains, light exposure, kan concentrations, genetic promoters, and infection conditions were optimized. The resulting protocol facilitated the reproducible transformation of *O. dubium* and transgenic plants were readily obtained. While *UBIQUITIN10* promoters directed relatively strong RFP fluorescence during the first weeks of growth, in mature plants the transgene was silenced, suggesting the need for silencing suppressors. This study exposes a potent protocol for *O. dubium* transformation and paves the way for genetic manipulations in this species to yield new varieties.

Declarations

Authors' contributions

SB conceived and designed the research. PKT, DA, MK, and NZ conducted the experiments. SB and IY supervised the overall work and critically analyzed all the results. PKT, DA, IY, and SB wrote the manuscript. All the authors read and approved the final manuscript.

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Data availability The data underlying this article are available in the article and in its online supplemental material

Competing interest The authors declare they have no competing interest.

Supplemental information The online version contains supplementary material available at

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Figures

Figure 1

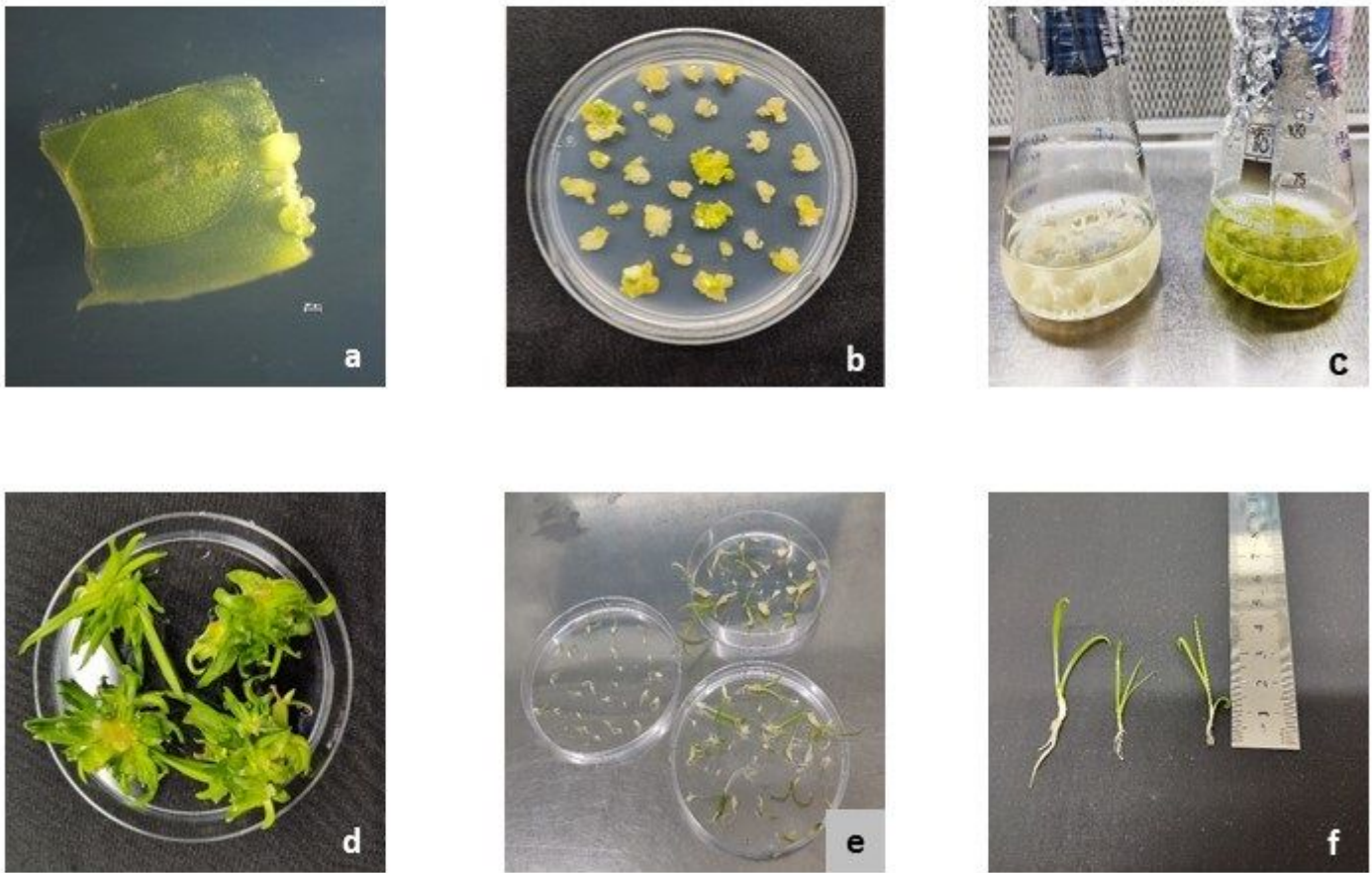


Figure 1

Regeneration procedure used for *O. dubium*

a Callus formation observed on leaf explants that were cultured on CIM for two weeks

b Callus proliferation on CIM after 4 weeks

c Callus cells proliferation in suspension medium grown in dark (left) and light (right) conditions for 6 weeks

d Elongated shoot clusters isolated after 10 weeks

e Bulbs growth after 12 weeks

f Rooted shoots after 14 weeks

Figure 2

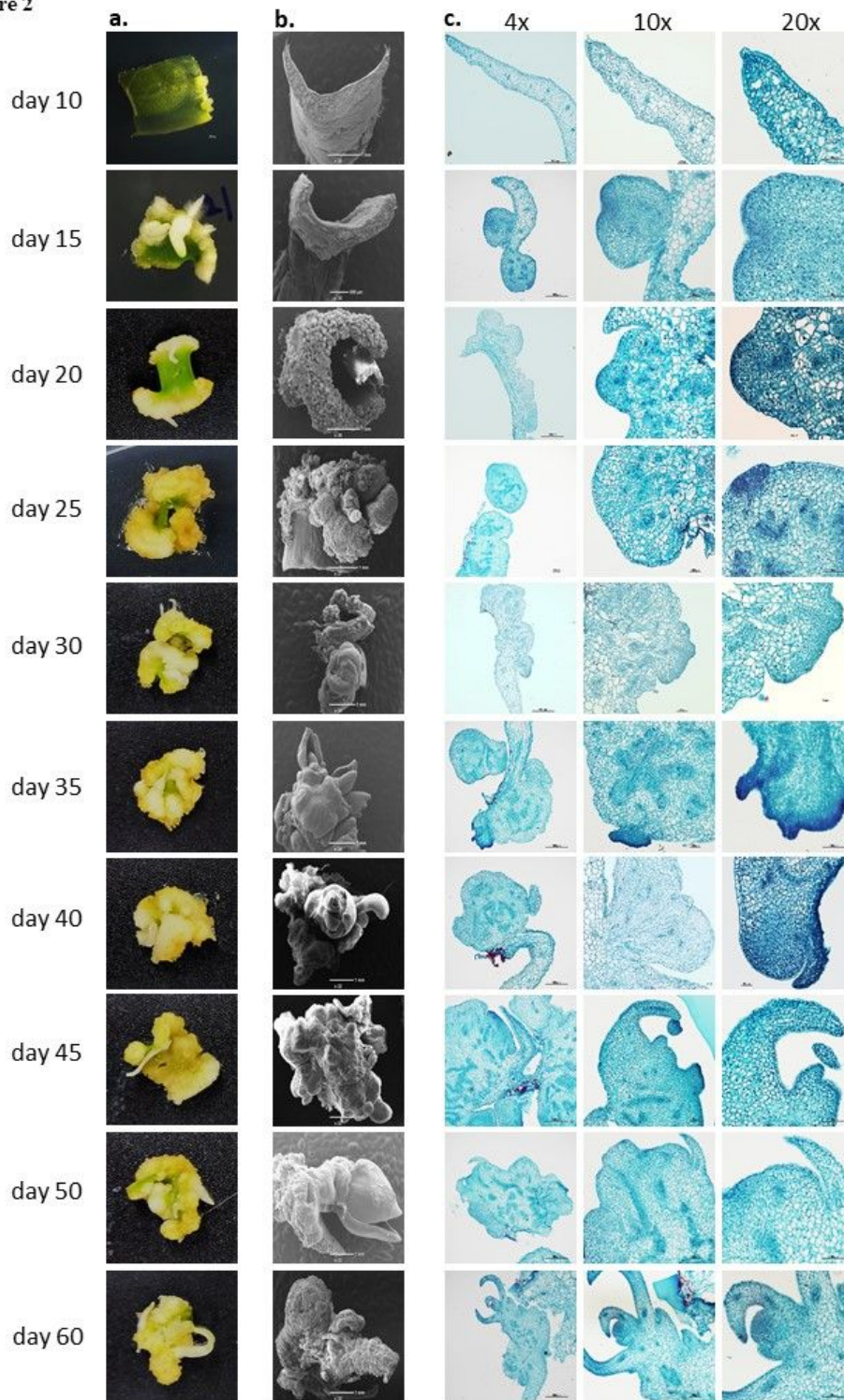


Figure 2

Histological characterization of *O. dubium* regeneration

a Bright light, **b** SEM observations, and **c** histological analysis of the *O. dubium* explants observed at the indicated days during the regeneration process described in Figure 1. Transverse explants' sections were stained with safranin and fast green.

Figure 3

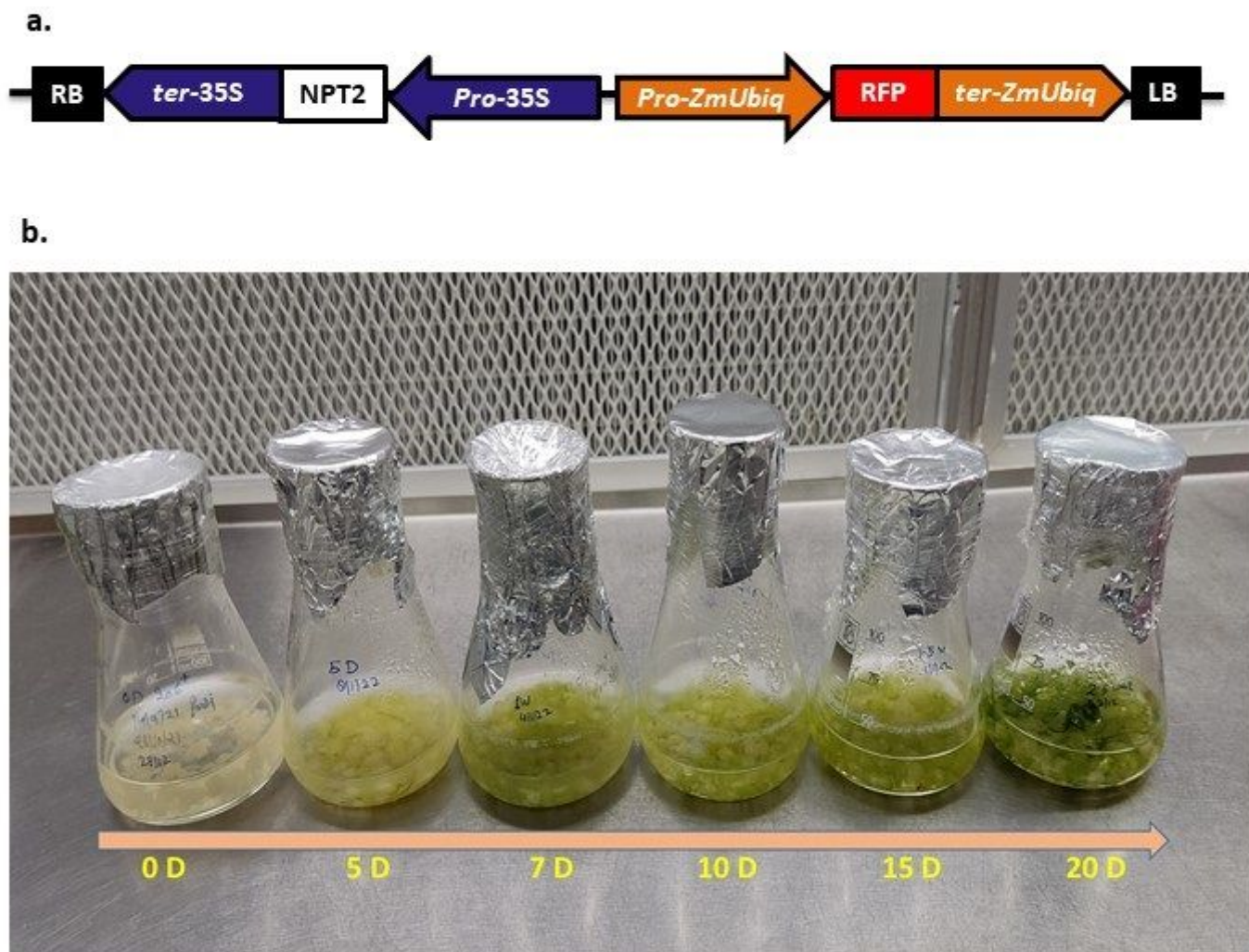


Figure 3

a Schematic representation of the construct used for *O. dubium* transformation

b Light exposure treatments of *O. dubium* callus cells culture promotes differentiation

Figure 4

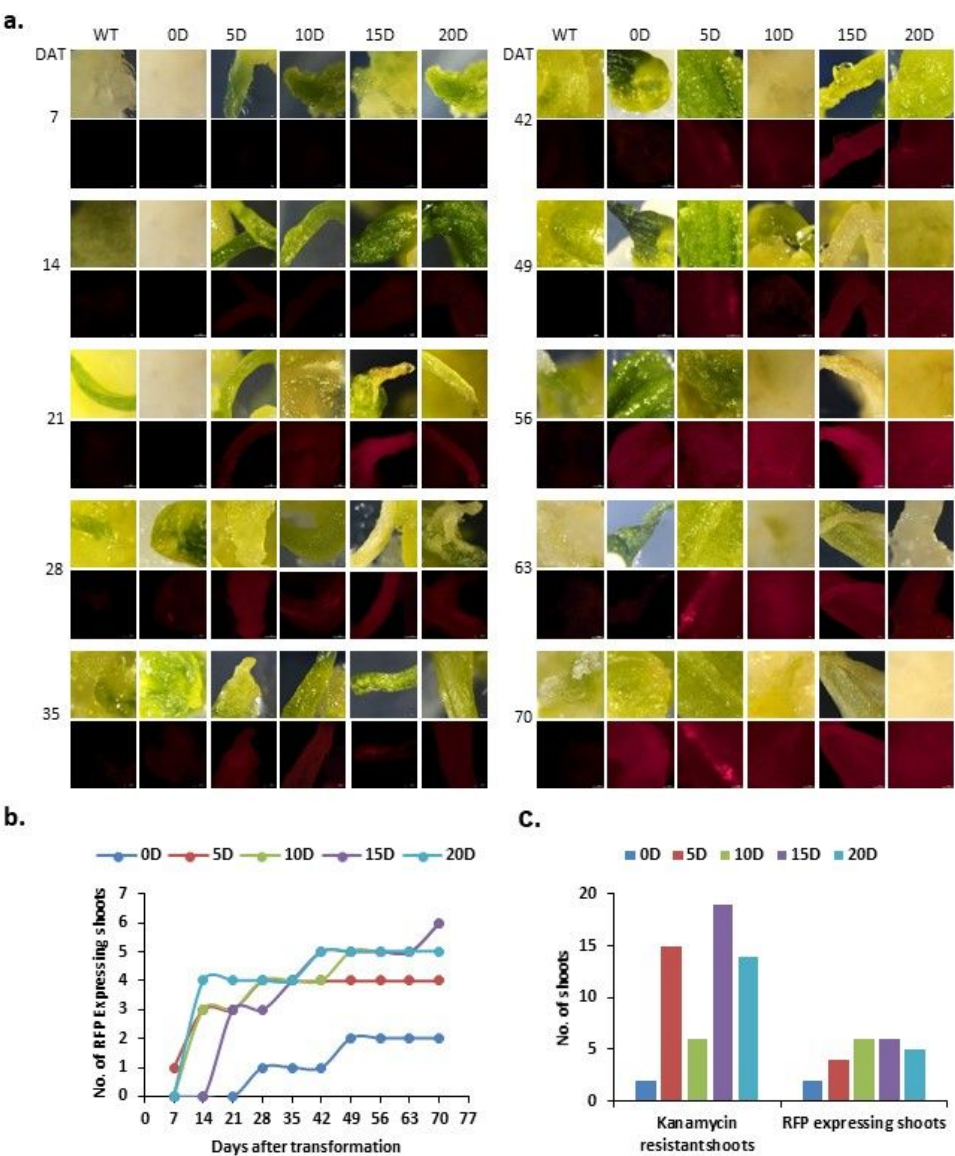


Figure 4

Observations of shoot development following *O. dubium* transformation

a RFP fluorescence observed during the regeneration process of *O. dubium* plants transformed using the construct depicted in Figure 3 and pretreated with 0, 5, 10, 15 and 20 days of light exposure

b Number of RFP fluorescent shoots detected using various periods of light exposure

cNumber of Kan-resistant and RFP fluorescent shoots detected after ten weeks, using various periods of light exposure

Figure 5

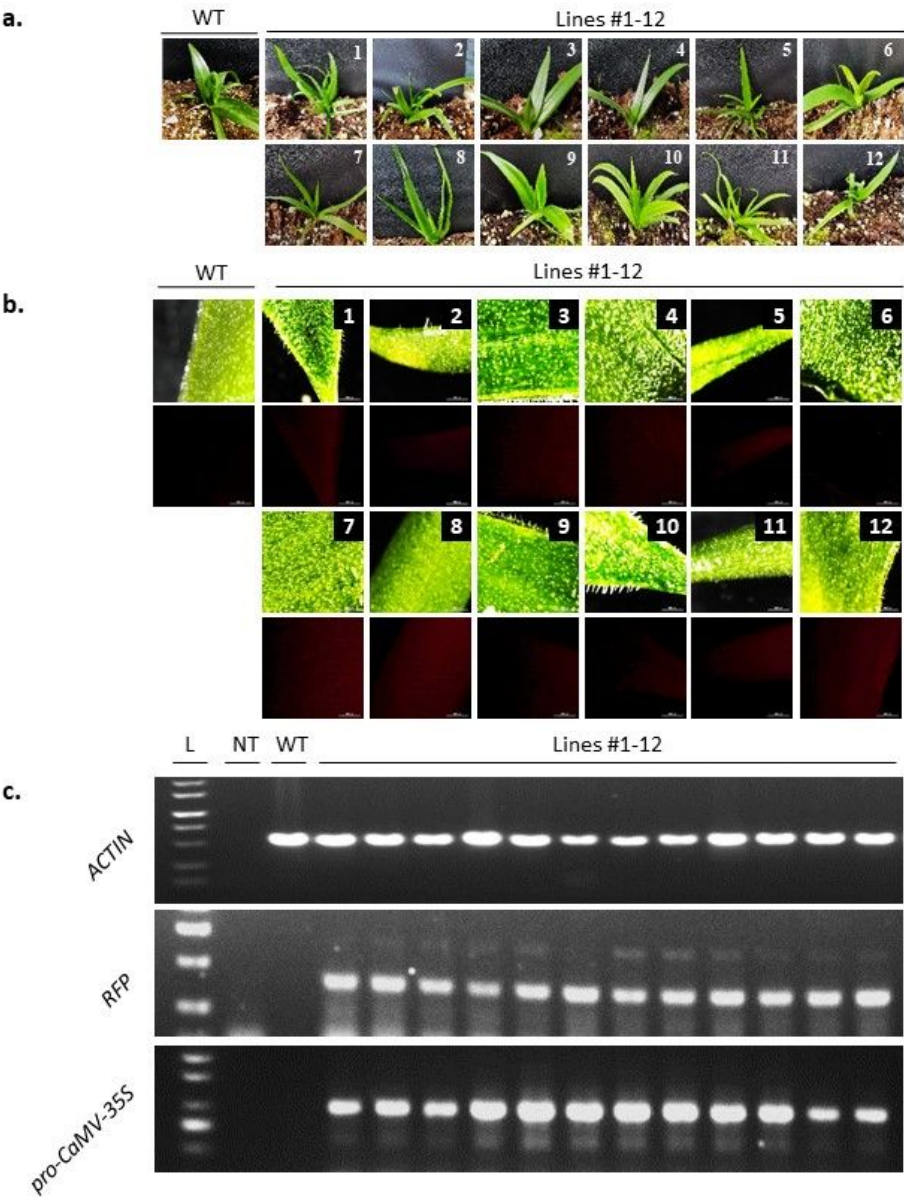


Figure 5

Characterization of transgenic *O. dubium* plants transformed with reporter genes

a Observations of the *O. dubium* plants obtained following transformation using the construct depicted in Figure 3a, and growing in the greenhouse 14 weeks after transformation.

b RFP fluorescence detected in the plants depicted in Figure 5a

c PCR analysis of the transgenic plants depicted in Figure 5a, using primers specific for *ACTIN*, *RFP* and the CaMV-35S promoter. L: 1 kb ladder; NT: Non-template; WT: wild type.

Figure 6

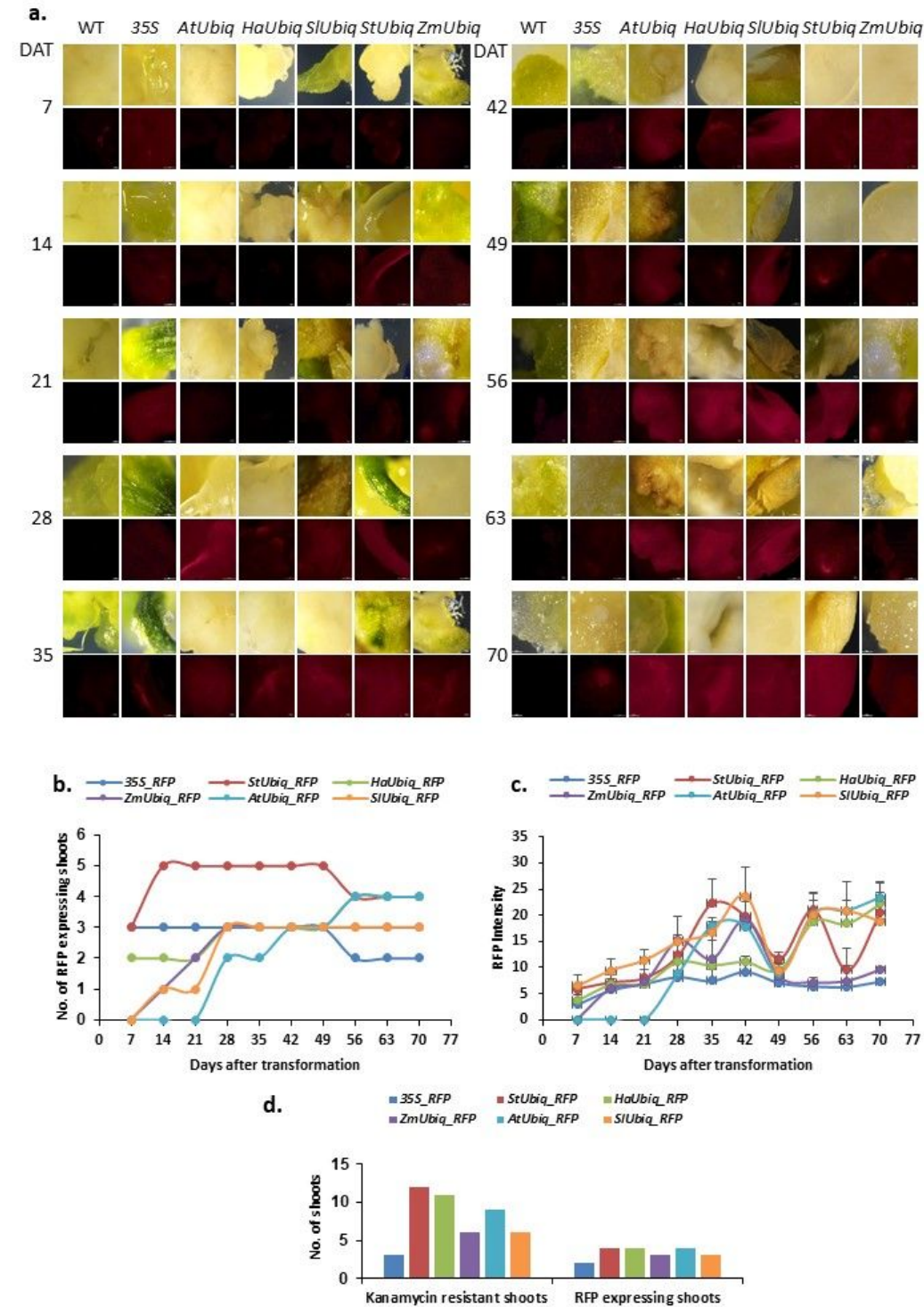


Figure 6

UBIQUITIN10 promoters direct various levels of transgene expression in transgenic *O. dubium* plants

a RFP fluorescence observed during the regeneration process of *O. dubium* plants transformed using the constructs depicted in Supplemental Figure S1

- b** Number of RFP fluorescent shoots detected using the different constructs
- c** Intensities of RFP fluorescence observed in developing plantlets following transformation with the different constructs
- d** Number of Kan-resistant and RFP fluorescent shoots detected ten weeks after transformation using the different constructs

Figure 7

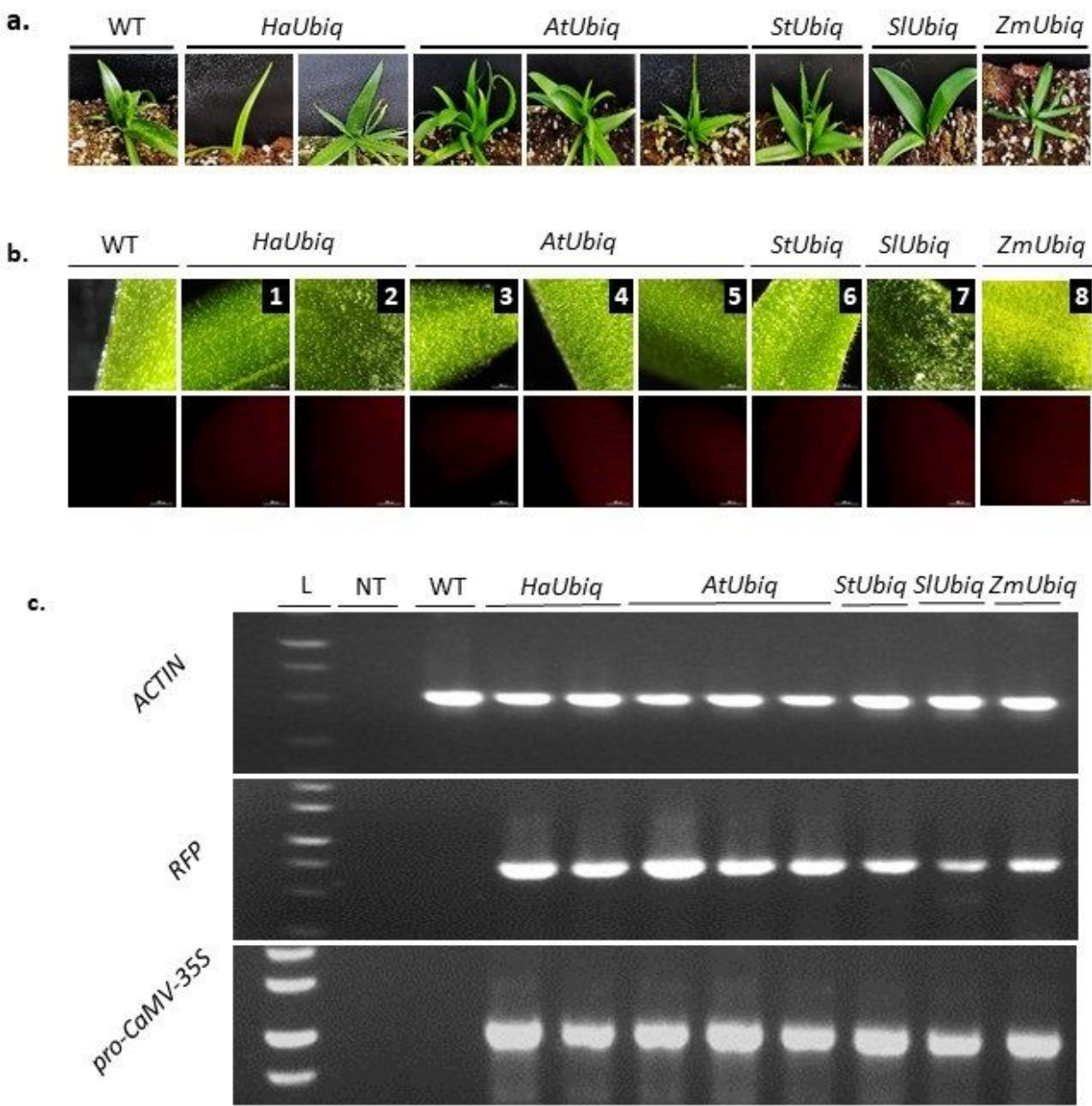


Figure 7

Characterization of transgenic *O. dubium* plants transformed with reporter genes directed with various *UBIQUITIN10* promoters

a Pictures of the *O. dubium* plants obtained following transformation using the construct depicted in Supplemental Figure 1, and growing in the greenhouse 14 weeks after transformation.

bRFP fluorescence detected in the plants depicted in Figure 7a

cPCR analysis of the transgenic plants depicted in Figure 7a, using primers specific for *ACTIN*, *RFP* and the CaMV-35S promoter. L: 1kb ladder; NT: Non-template; WT: wild type.

Supplementary Files

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