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Genetic Transformation of Potato without Antibiotic-Assisted Selection

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Abstract: The genetic engineering of plants often relies on the use of antibiotic or herbicide resistance genes for the initial selection of primary transgenic events. Nevertheless, the commercial release of genetically modified crops containing any marker gene encounters several challenges stemming from the lack of consumer acceptance. The development of strategies enabling the generation of marker-free transgenic plants presents an alternative to address public concerns regarding the safety of biotech crops. This study examined the capabilities of highly regenerative potato cultivars to develop transgenic plants without the presence of selective substances in their media. Internodal segments of in vitro potato plants were inoculated with the *Agrobacterium* strain AGL0 carrying plasmids, which contained the *GFP* or *RFP* gene driven by the *CaMV 35S* promoter to monitor the transformation process by observing in vivo green or red fluorescence. Despite the absence of selective pressure, inoculated explants demonstrated comparable or even higher transient expression compared to experiments based on antibiotic assistant selection. Consequently, under non-selective conditions, non-transgenic, chimeric, and fully fluorescent potato plantlets were concurrently developed. Among the five tested cultivars, the regeneration efficiency of non-chimeric transgenic plants varied from 0.9 ('Chicago') to 2.7 (#12-36-42) plants per 100 detached plantlets. Depending on the regenerative characteristics of potato varieties (early, intermediate, or late), a specific time interval can be determined when a blind collection of transgenic plantlets is more successful, streamlining the transformation procedure. The results indicate that the outlined procedure is simple and reproducible, consistently achieving the transformation efficiency of 7.3–12.0% (per 100 inoculated explants) in potato cultivars without selective pressure. The described transformation procedure holds the potential for obtaining cisgenic or intragenic potato plants with new valuable traits that do not carry marker genes.

Keywords: *Solanum tuberosum* L.; plant regeneration; antibiotic-free; selectable marker gene; *GFP*; *RFP*

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1. Introduction

Genetic engineering has significantly contributed to a fundamental and practical understanding of plant biology and genetics over the past 45 years. Notably, the potato was one of the first plant species to successfully undergo genetic transformation [1]. Since then, numerous publications have detailed the generation of diverse transgenic potato plants carrying a wide array of engineered traits [2–4]. Potato is an important food crop, which is cultivated not only for direct human consumption, but also as animal feed and an industrial crop for the production of alcohol, biofuel, starch, and other products. It stands as the 4th major staple crop globally, following maize, wheat, and rice, and is cultivated in over 150 countries. Faced with various production challenges, breeders are constantly making efforts to develop new varieties adapted to local agricultural needs in order to increase productivity while reducing costs [5]. The breeding of potato is traditionally

achieved through sexual hybridization between related species. However, the limiting factor in potato breeding is the long generation period, which makes it difficult to effectively introduce new traits into commercial varieties using traditional techniques of crossing. For this reason, potato genetic engineering is an important area of research, focusing on improving traits that are not easily modified by conventional breeding [2,3].

The generation of transgenic plants in any species hinges on the efficient selection of transgenic tissues, typically facilitated by various marker genes that confer resistance to selective substances, such as antibiotics or herbicides. However, the presence of antibiotic or herbicide resistance genes in transgenic plants intended for commercial use has raised significant consumer concerns regarding health risks and biosafety. This complicates the regulatory processes overseeing the release of biotech crops [6]. Thus, the development of marker-free transgenic plants stands as one of the options to mitigate perceived risks and reduce public concerns.

In addressing the issue of marker gene presence, various strategies have emerged over the past decades. These include approaches that involve the direct removal of selective sequences from the transformation process or the controlled elimination of marker genes from the plant genome after the marker-assisted selection of transgenic plants is completed. The methods of removal include co-transformation, site-specific recombination, transposition, and the utilization of double right border–binary vectors or multi-auto-transformation vectors [7–10]. In co-transformation, vectors carry two separate T-DNAs: one containing the gene of interest and the other encoding a selection marker gene, both of which are introduced into the plant genome simultaneously. If insertions occur at unlinked genomic loci, they can independently segregate in subsequent generations, yielding marker-free segregants [10]. Similarly, segregation is necessary when transgenic plants are transformed with the double right border twin T-DNA vector, featuring an additional copy of the right border sequence inserted between the marker gene and the gene of interest [11]. Segregation-based strategies are extensively applied for various seed-propagated crops [9,10] but are impractical for vegetatively propagated species, such as potato.

Approaches directly applied to primary transformants, such as site-specific recombination or systems involving transposon-based and multi-auto-transformation vectors, are better suited for potato. Various self-excision site-specific recombination systems, including Cre-lox, FLP-FRT, and R/RS, have already been applied to varying degrees in vegetatively propagated horticulture crops, including apricot [12], tomato [13], blood orange [14], strawberries [15], apples [16–18] and pears [19]. The Cre-lox and R/RS systems have also demonstrated effectiveness in generating marker-free transgenic plants of potato. The heat shock-inducible Cre-lox systems achieved a 71% excision efficiency of the *NPTII* antibiotic resistance marker gene in potato cv. 'Desire' [20]; while the R/RS recombination tool exhibited lower efficacy, producing only 29% marker-free transgenic plants from the regenerated shoots of the same cultivar [21]. The ipt (isopentenyl transferase)-type multi-auto-transformation vector has also been employed to generate marker-free disease-resistant transgenic potato plants [22]. While these approaches have been successful, they do carry certain limitations. These include the need for designing sophisticated constructs, resulting in decreased transformation efficiency; the requirement for additional time for manipulations to re-regenerate marker-free plants after induced excision; the inability to achieve 100% excision efficiency; and unwanted chromosomal rearrangements in the plant genome, as a result of the expression of recombinase systems, leading to genetic and phenotypic changes.

An ideal approach to overcome these limitations involves a marker-free transformation procedure, where a simple expression cassette exclusively encoding the gene of interest is transferred into the plant genome via *Agrobacterium* or gun-mediated transformation. In practice, only a limited number of crops, such as alfalfa [23], peanut [24], tobacco [25] and wheat [26], have been transformed using this approach, with an efficacy ranging from 0.1 to 50%. This straightforward procedure relies on the high regenerative capability of cultivated in vitro tissue to produce plants with a high frequency. Given that potato

is among the highly regenerative species, markerless genetic transformation has been successfully applied to generate late blight-resistant [27,28], virus-resistant [29,30], and oxidative stress-tolerant transgenic potato lines [31]. The primary challenge associated with markerless transformation is the necessity for the molecular screening of several hundreds of regenerated plants to discover a few transgenic events. Since regenerated plants need to be maintained in vitro, until molecular test results are obtained, transformation without selective genes becomes a time-consuming and expensive procedure.

In our current study, we analyzed the possibility of producing transgenic potato plants without using selective substances. We employed an expression construct encoding *GFP* or *RFP* to monitor the transformation process by cultivating internodal explants on both selective and non-selective media. This approach facilitated the analysis of the efficiency of chimeric and transgenic plantlet formation at various time intervals after co-cultivation with *Agrobacterium* and helped to compare transformation efficiencies of potato using antibiotic-free and antibiotic-assisted strategies.

2. Materials and Methods

2.1. Plant Material and General Conditions

The plant materials used in this study consisted of various potato germplasms generously provided by the Doka-Gene Technology Ltd., Russia. These included five commercial cultivars, 'Chicago', 'La Strada', 'Lion Heart', 'Manhattan' and 'Pirol', along with five breeding accessions, specifically #10-9-3, #10-10-10, #12-22-129, #12-36-42 and 'Indigo' (formerly #10-17-10). In vitro stock plants of potato were multiplicated in plastic vessels using phytohormone-free Murashige and Skoog (MS) basal medium supplemented with 3% sucrose, solidified with 7 g L^{-1} agar, and adjusted to a pH 5.8–5.9 before autoclaving. Vessels were maintained in a culture room at 21 ± 2 °C, subjected to a 16 h light/8 h dark cycle under artificial light (40 µmol m^{-2} s^{-1}) provided by OSRAM cool white and fluora fluorescent lamps. In all experiments, internodal segments of 0.5–1 cm in length, excised from three- to four-week-old in vitro plants, were used as explants. Explants were cultured in 100 mm × 20 mm glass Petri dishes containing 25 mL of regeneration medium.

2.2. Potato Regeneration Media

All regeneration media were consisted of MS mineral salts and vitamins, supplemented with 30 g L^{-1} sucrose and 100 mg L^{-1} myo-inositol, solidified with 7 g L^{-1} agar, and adjusted to a pH range of 5.8–5.9. Four combinations of phytohormones were used to induce adventitious regeneration, comprising zeatin (Zea) 3 mg L^{-1}, indoleacetic acid (IAA) 2 mg L^{-1}, and gibberellic acid (GA) 1 mg L^{-1}; zeatin-riboside (Zea-R) 3 mg L^{-1}, IAA 2 mg L^{-1}, and GA 1 mg L^{-1}; Zea 3 mg L^{-1}, IAA 0.5 mg L^{-1}; Zea-R 3 mg L^{-1}, and IAA 0.5 mg L^{-1}. The preferred combinations of phytohormones were determined through preliminary experiments.

2.3. Agrobacterium-Mediated Genetic Transformation of Potato with and without Antibiotic Selection

In these experiments *A. tumefaciens* strain AGL0 harboring pBIN-mGFP5-ER binary vector was used [32]. T-DNA of this construct consists of the *NPTII*-selectable marker gene under *NOS* promoter and the modified *GFP* gene driven by *CaMV 35S* promoter along with a leader sequence for expression localization in the endoplasmic reticulum of the cells. Prior to transformation, colonies of bacteria were cultured overnight in liquid LB medium supplemented with 100 mg L^{-1} kanamycin at 28 °C on an orbital shaker. Shortly prior to explant isolation, the agrobacterial suspension was diluted with phytohormone-free MS medium to a final density of OD600 = 0.2. Approximately 50–75 freshly cut internodal segments were immersed in 15 mL of *Agrobacterium* suspension and inoculated in a 100 mL glass for 25–30 min at room temperature. Following this, the explants were placed onto sterilized paper and blotted dry for 2–3 min under laminar flow. After removing the excess of bacteria, the explants were transferred to Petri dishes containing the regeneration medium covered with sterilized filter paper, and maintained for 3 days in the dark at 25 ± 1 °C.

Post-co-cultivation, groups of 10–12 explants were transferred to 100 mm × 20 mm glass Petri dishes containing 25 mL of fresh regeneration medium supplemented with 500 mg L^{-1} of the antibiotic cefotaxime. Dishes were kept in the culture room at 21 ± 2 °C under a 16 h light/8 h dark cycle, with subculturing carried out every 2 weeks. All subsequent media plates contained the same cefotaxime concentration. From the third week after inoculation, plantlets 1.5–3.0 cm in length, regenerated from explants under-non selective conditions, were cut and transferred to culture vessels (ten plantlets per vessel) containing 100 mL of phytohormone–free MS medium supplemented with 150 mg L^{-1} cefotaxime. Once the plantlets grew to 5–10 cm in length, shoot tips with one to two expanded leaves were moved into phytohormone–free MS medium containing 100 mg L^{-1} kanamycin for the identification of transgenic events through rooting. The step-by-step scheme of the non-selective genetic transformation of potato is presented in Figure 1.

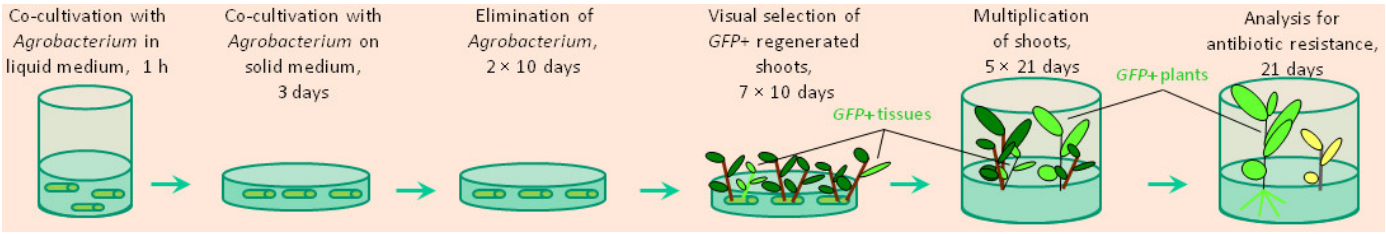

Figure 1. Scheme for plant regeneration of transgenic potato under non-selective conditions.

In parallel, the antibiotic-assisted genetic transformation was carried out. After co-cultivation with *Agrobacterium* in the dark for 3 days, a portion of intermodal segments was transferred to the corresponding regeneration medium supplemented with kanamycin (50 mg L^{-1}). Shoot regeneration and transgenic plant selection were then performed as described [32]. To avoid duplication of transgenic lines, only one kanamycin-resistant shoot was collected from each explant.

As per the aforementioned transformation procedure (Figure 1), two potato cultivars were subjected to transformation using the plasmid pDGB3_5453 (the gift of prof. A.Vainstain, The Hebrew University of Jerusalem), which harbors the *RFP* gene driven by *CaMV 35S* promoter and *NPTII*-selectable marker gene driven by *NOS* promoter. In contrast to previous experiments, each explant that produced a chimeric or transgenic plantlet was carefully labeled. Both the 'mother' explant and the resulting plantlet(s) were tracked throughout the experiment to determine the 'standard' transformation efficiency, calculated as the percentage of inoculated explants that regenerated transgenic plants. Four independent experiments were performed, each involving 50–55 explants for 'Pirol' or 91–100 explants for 'Chicago'. Plantlets displaying any signs of red fluorescence, whether sectoral or full fluorescence, were detached and subsequently cultivated on an antibiotic-free medium. Following at least five sub-cultivations, during which the observation for RFP fluorescence was no longer carried out, all putative transgenic events underwent PCR analysis to confirm the introduction of the *RFP* gene. Concurrently, these events were transferred to a kanamycin-enriched medium to confirm the introduction of the *NPTII* gene from T-DNA through rooting.

2.4. Monitoring of Green and Red Fluorescence

Visual screening for transient and stable T-DNA incorporation was conducted using a ZEISS SteREO Discovery.V12 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with a PentaFluar S 120 vertical illuminator(Leistungselektronik JENA GmbH, Jena, Germany). In the GFP observations, two commercially available filter sets, namely 38 GFP BP (EX BP 470/40, BS FT 495, EM BP 525/50) and 57 GFP BP (EX BP 470/40, BS FT 495, EM LP 550), from Carl Zeiss MicroImaging GmbH (München, Germany) were used. RFP fluorescence was detected using 43 Cy3/Rhod/RFP (EX BP 545/25, BS FT 565, EM BP 606/70) from the same manufacturer. Photographs were taken using the digital

Camera AxioCam MRc 5 with the help of ZEN Pro 2012 software (Carl Zeiss Microscopy GmbH, Jena, Germany).

2.5. Molecular Analysis

To analyze integrated T-DNA copies, Southern hybridization was conducted. For the blot analysis, 30 µg of potato genomic DNA was digested overnight at 37 °C with 60U *HindIII*, which cleaves the T-DNA of pBIN-mGFP5-ER at a single position (5′ end of the *CaMV 35S* promoter). Subsequently, the DNA was separated through gel electrophoresis in 0.9% agarose, and then transferred and immobilized onto a Hybond N+ membrane (GE Healthcare, Amersham Bioscience, Amersham, UK). Membranes were probed with alkaline phosphatase-labeled probes and detected using CDP-Star detection reagent (Amersham CDP-Star Detection reagent, GE Healthcare), as previously described [30]. Hybridizing bands were visualized on X-ray film (Retina XBE blue sensitive, Carestream Health Inc., New York, NY, USA) at room temperature for 24 h. The DNA probe (711 bp) for hybridization was synthesized via PCR using specific primers for the coding region of the GFP gene (forward, 5′-AGTAAAGGAGAAGAACTTTTCACTGGAGTT-3′; reverse, 5′-TTTGTATAGTTCATCCATGCCATGTGT-3′) and labeled with the alkaline phosphatase using the Amersham Gene Images AlkPhos Direct Labelling and Detection System (GE Healthcare, Amersham Bioscience, Amersham, UK).

The transgenic status of plants, which were discovered after the initial *RFP* monitoring, was validated using PCR with the following primers: *RFP* forward 5′-CATCACCGAGTTCA TGCGCTTCAAG-3′, *RFP* reverse 5′-GGCTTCTTGGCCATGTAGATGGACT-3′; the size of the amplified fragment was 537 bp. PCR conditions included preheating to 95 °C for 2 min, followed by 35 cycles with denaturation at 95 °C for 20 s, annealing at 61 °C for 20 s, elongation at 72 °C for 45 s, and ending with min at 72 °C. The PCR products were analyzed in 0.8% (*w*/*v*) agarose gel stained with ethidium bromide.

2.6. Statistical Analysis

The data presented are the mean ± SD. Significant differences were determined via t-test with $p < 0.05$ as a threshold. Within experiments, data were analyzed by Statistica10 software (©StatSoft Inc., Tulsa, OK, USA) using analysis of variance (ANOVA), followed by Tukey's HSD (Duncan's) test.

3. Results

3.1. Efficiency of Adventitious Plant Regeneration of Potato Cultivars

This experiment describes the regenerative capacity of potato cultivars in producing multiple shoots using zeatin-enriched regeneration media. Each analyzed cultivar was tested on four phytohormonal combinations, with the most favorable combination for adventitious shoot regeneration being determined (manuscript in preparation). Summarized results are presented in Table 1. All ten cultivars demonstrated the ability to regenerate plantlets from cultured internodal segments. However, the percentage of explants producing shoots varied from 75.4 ('Indigo') to 100% (#12-22-129, 'Pirol' and #10-9-3). Eight cultivars exhibited an efficiency of plantlet regeneration higher than 93%. The number of regenerated shoots per explant significantly varied depending the genotype. Particularly, 'Indigo' (2.4 plantlets per explant) and 'Manhattan' (3.5 plantlets per explant) displayed the lowest ability, while 'Chicago' and #12-22-129 produced an average of more than 15 shoots per initial explant (Table 1).

The experiment revealed a noticeable difference among cultivars in their timing abilities for generating plantlets. Nodal explants of #12-22-129 and 'Chicago' promptly responded to phytohormonal induction, initiating the formation of adventitious shoots during the second week following culture initiation. By the end of the third week, they notably produced visible plantlets ranging from 0.5 to 2.0 cm in length (Figure 2). Furthermore, the explants of #12-22-129 and 'Chicago' were characterized by the emergence of shoots at both nodal excision sites, ultimately yielding a higher number of shoots compared

to other cultivars. In contrast, the explants of 'Pirol', 'Indigo', 'Lion Heart', and #10-9-3 demonstrated a significant delay in plantlet formation, as shoots of a comparable size only emerged at the 6th week following culture initiation (Figure 2). Meanwhile, 'La Strada', 'Manhattan', #10-10-10, and #12-36-42 exhibited an intermediate timing pattern (Figure 2), alongside a varying number of regenerated adventitious shoots per explant (Table 1).

Table 1. The efficiency of plant regeneration from internodal segments of potato cultivars.

Cultivar	Combination of Phytohormones *	Regeneration Type	Efficiency of Plantlet Regeneration (%)	Number of Plantlets per Explant
Chicago	3 Zea + 2 IAA+ 1 GA	early/medium	98.3 ± 3.3	15.2 ± 3.9
Indigo	3 Zea-R + 0.5 IAA	late	75.4 ± 2.8	2.4 ± 0.3
Lion Heart	3 Zea + 2 IAA+ 1 GA	late	93.1 ± 6.3	6.3 ± 0.4
La Strada	3 Zea-R + 2 IAA+ 1 GA	medium	97.8 ± 3.8	12.3 ± 2.9
Manhattan	3 Zea + 0.5 IAA	medium	76.2 ± 10.6	3.5 ± 1.9
Pirol	3 Zea + 0.5 IAA	late	100 ± 0.0	8.2 ± 2.0
#10-9-3	3 Zea + 2 IAA+ 1 GA	late	100 ± 0.0	6.6 ± 1.8
#10-10-10	3 Zea + 0.5 IAA	medium	93.6 ± 6.3	4.8 ± 0.6
#12-22-129	3 Zea + 0.5 IAA	early	100 ± 0.0	15.0 ± 1.6
#12-36-42	3 Zea + 0.5 IAA	medium/late	95.0 ± 4.4	6.4 ± 2.1

* All regeneration media consisted of MS mineral salts and vitamins, supplemented with 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol, and solidified with 7 g L^{-1} agar. Various combinations of phytohormones were used to induce adventitious regeneration, including 3 mg L^{-1} of zeatin (Zea) or zeatin-riboside (Zea-R), 2 or 0.5 mg L^{-1} of indoleacetic acid (IAA), and 1 mg L^{-1} of gibberellic acid (GA).

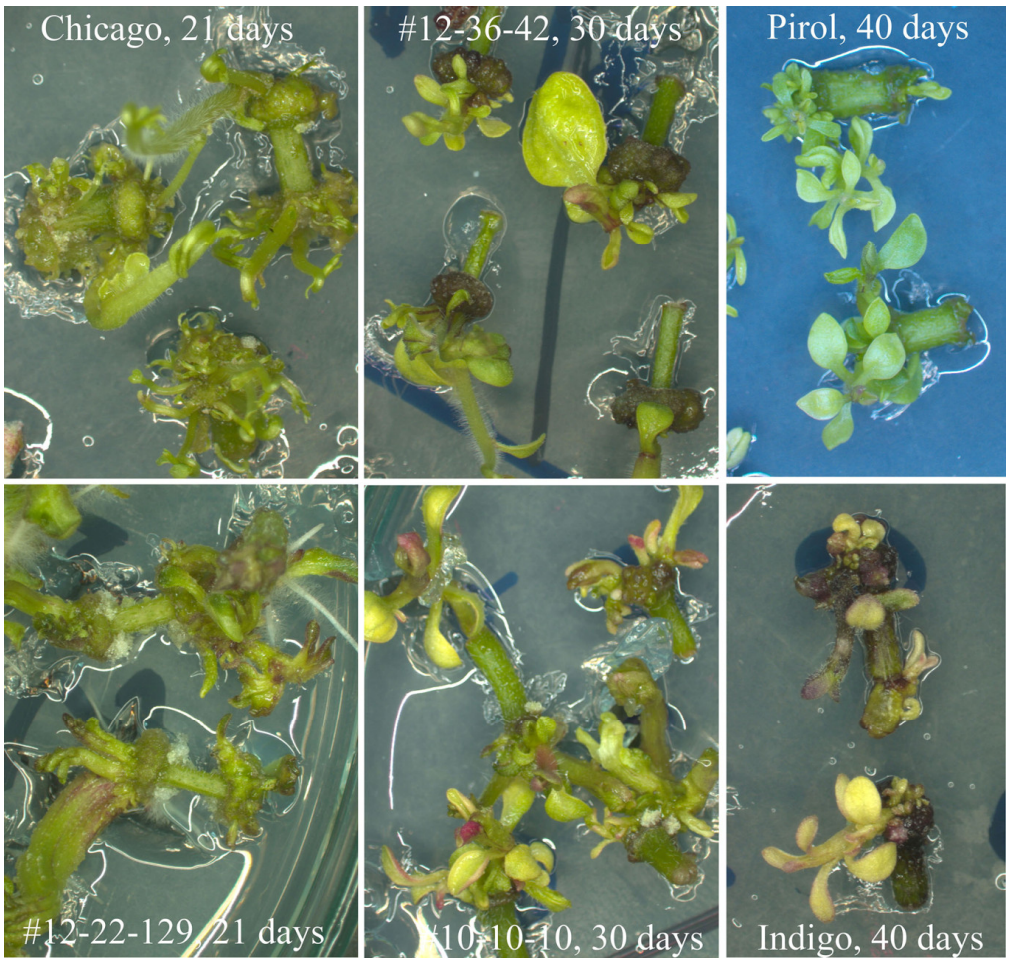

Figure 2. Plantlets regeneration from in vitro cultured internodal segments of various potato cultivars.

Based on the results, five potato cultivars were selected for the non-selective transformation experiment. We used cultivars exhibiting varied timing of plantlet emergence (ranging from early to late), which demonstrated a higher number of regenerating shoots and a higher percentage of explant producing plantlets. These cultivars included #12-22-129 (an early type of regeneration), 'Chicago' (early/intermediate), 'La Strada' (intermediate), #12-36-42 (intermediate/late), and 'Pirol' (late).

3.2. Agrobacterium-Mediated Genetic Transformation of Potato without Antibiotic–Assisted Selection

3.2.1. Efficiency of Transient Expression under Selective- and Non-Selective Cultivation

In this experiment, five cultivars were transformed with the pBIN-mGFP5-ER vector containing the sequence of green fluorescent protein *GFP*, allowing the easy monitoring of the generation of transgenic events from single cells to plantlets. To gain initial insights into the potential of potato cultivars to produce transgenic shoots without selective pressure, transient GFP expression was monitored over four weeks following co-cultivation of nodal segments with agrobacteria. The impact of the absence or presence of selective pressure was investigated by culturing explants of five genotypes in parallel, both on a medium containing no selective compound and on a medium supplemented with kanamycin. A significant genotype-dependent effect was observed (Figure 3). Cultivars with an earlier regeneration type, such as 'Chicago' and #12-22-129 (Figure 3C,D), showed earlier transient expression in cells of the explant cuts, particularly during the first two weeks of culture, compared to 'La Strada' (Figure 3A), 'Pirol' (Figure 3B) and #12-36-42 (Figure S1). Overall, nearly all explants (>90%) of 'Chicago' and #12-22-129 showcased single cells or cell conglomerates expressing GFP throughout the observation period (25th day). The percentage of GFP-positive explants in other cultivars was comparatively lower: 84–89% in #12-36-42, 80–86% in 'La Strada', and 58–75% in 'Pirol'.

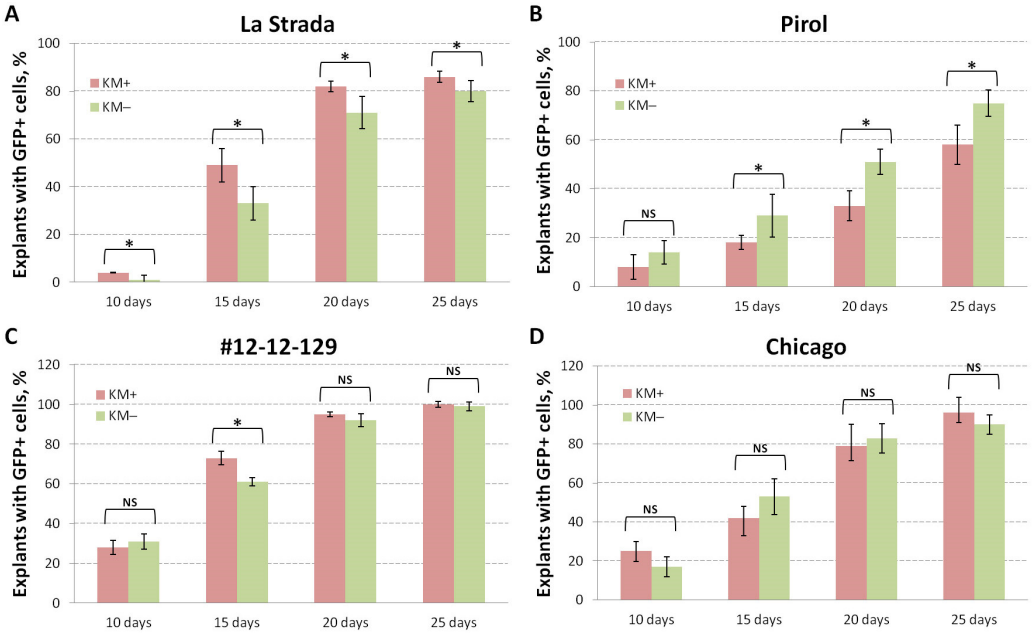

Figure 3. The percentage of explants displaying GFP expression in cells of potato cultivars, including 'La Strada' (**A**), 'Pirol' (**B**), # 12-12-129 (**C**), and 'Chicago' (**D**) under selective (Km+) and non-selective (KM−) cultivation. "*" indicate statistically significant differences calculated according to *t*-test ($p \leq 0.05$), NS—non-significant.

The presence of the antibiotic in the medium noticeably affected the transient expression in two cultivars. The percentage of 'La Strada' explants exhibiting GFP expression was higher in the presence of kanamycin throughout the observation period, especially during the first two weeks following inoculation (Figure 3A). In contrast, the cultivation

of nodal explants of 'Pirol' without an antibiotic proved beneficial, as a higher portion of explants displayed GFP expression (Figure 3B). However, there was no clear effect of selective pressure in the other three cultivars, as an equivalent number of explants exhibited cell fluorescence throughout the observation period, or the difference was not statistically proved (Figure 3C,D).

3.2.2. Efficiency of Regeneration of GFP-Positive Transgenic Plants under Non-Selective and Selective Cultivation

In the absence of antibiotic in the regeneration medium, the emergence of non-transgenic, transgenic, and chimeric buds was observed on the cut ends of explants during the initial subcultures (Figure 4A,B). These buds easily progressed into shoots, leading to the development of both chimeric and transgenic individuals among the predominant non-transgenic plantlets, as confirmed by GFP observation. Chimeric plantlets exhibited sectored *GFP*-expressing tissues (Figure 4C), while completely transgenic plants demonstrated clear green fluorescence throughout the entire plantlet, inclusive of stem, petioles, and leaves (Figure 4D,E). Typically, only a single *GFP*-expressing plantlet was selected from a single explant during observation, albeit occasionally two independent *GFP* positive plantlets were observed simultaneously among the cluster of non-transgenic shoots (Figure 4F).

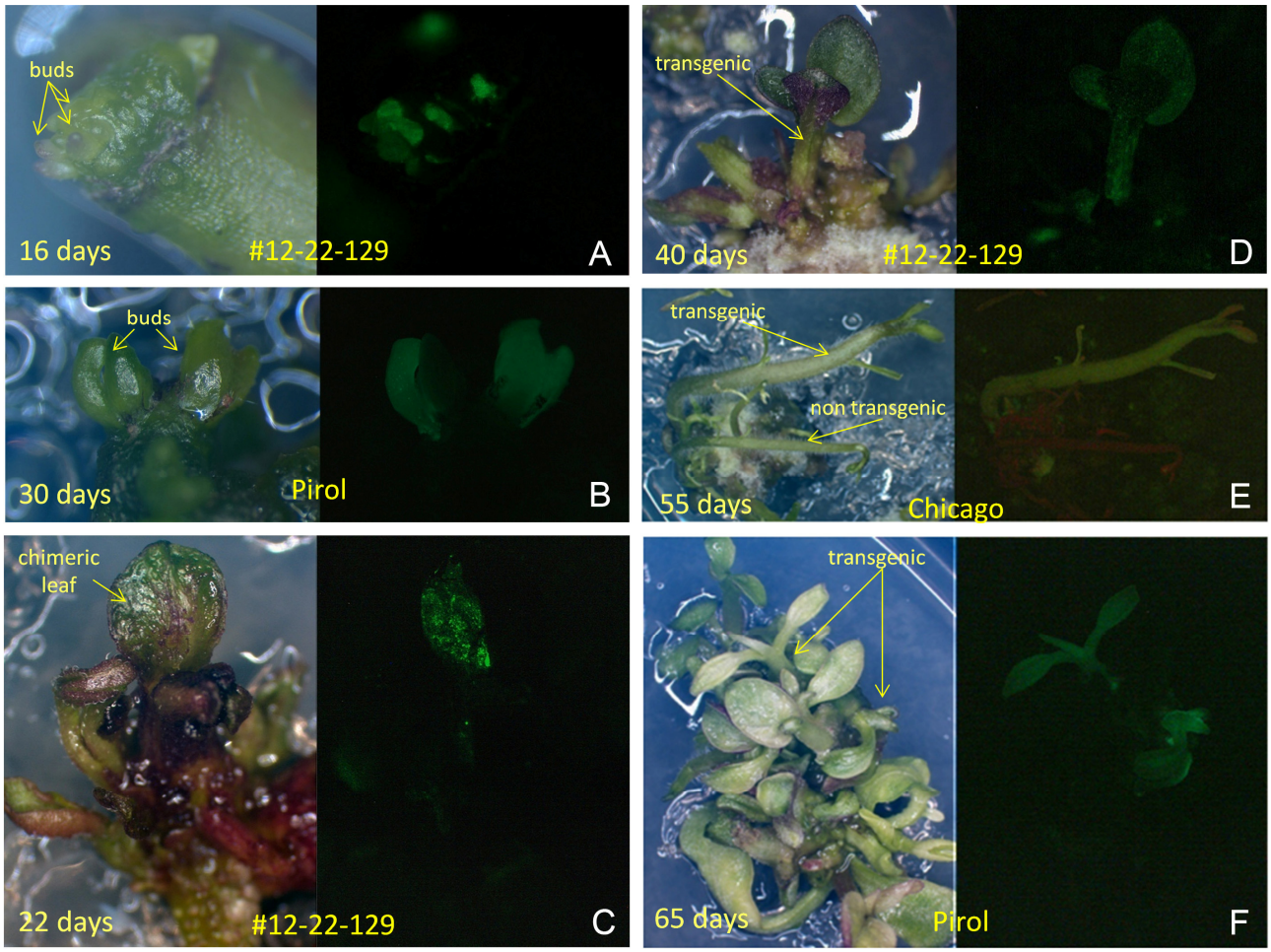

Figure 4. Regeneration of transgenic potato plants under non selective conditions. (**A**,**B**) Formation of *GFP*-expressing buds on the cut edge of explants; (**C**) development of chimeric plantlets with the *GFP* positive sector on expanding leaf; (**D**,**E**) regeneration of single transgenic plantlets of #12-22-129 and 'Chicago' with a visible green fluorescence throughout all tissues; (**F**) two independent transgenic shoots generated from the internodal explant of 'Pirol' among the non-transgenic plantlets.

Each chimeric and fully transgenic plantlet was regularly separated from the cultivated explants and independently proliferated on the antibiotic-free medium. At this stage, it became challenging to monitor the in vitro plant growth with GFP observation due to technical limitations (maintaining aseptic conditions became difficult with increased sizes) and physical issues (GFP fluorescence diminished in mature tissue and was obscured by chlorophyll fluorescence and tissue autofluorescence). As the T-DNA of the pBIN-mGFP5-ER vector also contains the *NPTII* gene, after several rounds of sub-culturing without selective pressure, all the putative transgenic plants were subsequently tested by rooting in the medium supplemented with a lethal dose of kanamycin (100 mg L^{-1}). The presence of the selective antibiotic prevented the growth and rooting of non-transgenic plants (Figure S2). The rooting was used with the aim to clarify the introduction of *NPTII* gene after subculturing and proliferation without the addition of a selective compound. The results of two independent experiments, each involving 50–75 explants for each cultivar, are summarized in Table 2.

Table 2. The efficiency of antibiotic-free genetic transformation of five potato cultivars.

Cultivar	Regeneration Type	Number of Explants with *GFP*+ Buds *	Number of Shoots Regenerated (90 Days) *	Number of *GFP*+ Shoots Detached from Explants *	Number of Stable Transgenic Events (*GFP*+) *	Percentage of *GFP*+ Stable Events per 100 Regenerated Shoots (%)	Transformation Efficiency with Antibiotic-Assisted Selection ** (%)
#12-22-129	early	36	1519	66	32	2.1 ± 0.4	100
Chicago	early/intermediate	16	1554	22	15	0.9 ± 0.1	98
La Strada	intermediate	30	1434	40	29	2.0 ± 0.5	47
#12-36-42	intermediate/late	39	964	50	25	2.6 ± 0.4	68
Pirol	late	29	916	33	16	1.8 ± 0.7	44

* Average number per 100 inoculated explants, ** kanamycin-assisted transformation frequency defined as the percentage of initially inoculated explants which produced *GFP*-positive events.

In our experiment, we employed a 'blind' approach, where *GFP*-positive plantlets (both chimeric and non-chimeric) were not specifically tracked in relation to the mother explant. For this reason, the frequency of transformation was defined as the total number of detached plantlets at the end of experiment. The efficiency of transformation varied among the analyzed cultivars, ranging from 0.9% ('Chiacago') to 2.6% (#12-36-42). According to the rooting analysis, a significant number of initially collected chimeric / non-chimeric shoots developed into non-transgenic plants during the subsequent antibiotic-free subculture. Specifically, from half (Pirol, #12-22-129, and #12-36-42) to one third ('Chicago' and 'La Strada') of the plantlets, which initially exhibited *GFP* expression in tissues at the point of detachment, subsequently became non-transgenic (Table 2).

It is noteworthy that the number of explants that initially produced GFP-positive transgenic buds exceeded the resulting number of transgenic shoots (Table 2). Considering the significant portion of chimeric escapes, it is plausible that some of the generated stable transformants are duplicates, potentially stemming from the same transgenic cell aggregates of the explant, or some explants may produce several independent transgenic events. To address this, we carried out a molecular characterization of several transgenic events through Southern blot analysis. For instance, the analysis of 13 out of 15 transgenic 'Chicago' plants using a *GFP* probe revealed the disparate pattern of T-DNA insertions (Figure 5). Similarly, the analysis of insertion patterns in transgenic 'Pirol' plants, which we initially suspected to be duplicates due to their origin from the same explants, confirmed their independent origin (Figure 5).

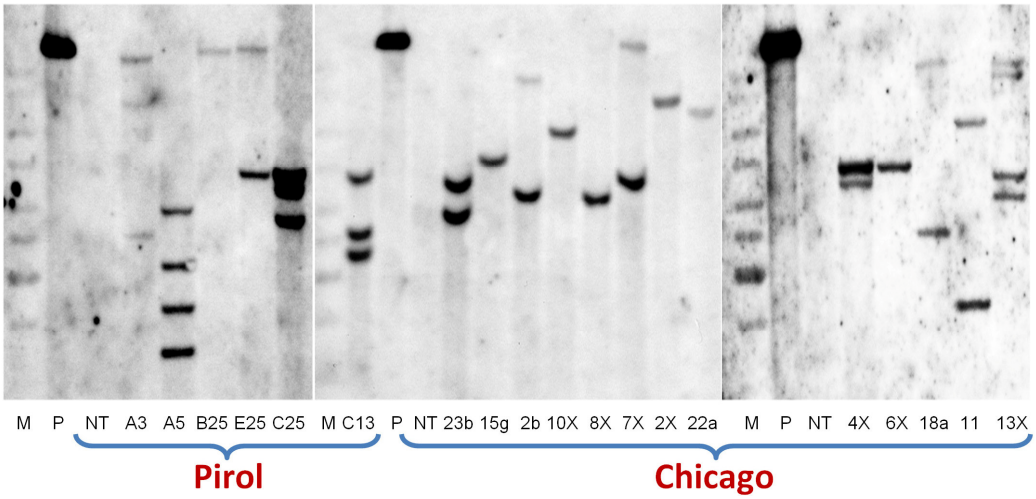

Figure 5. Example of the Southern blot analysis for GFP insertions in selected potato lines of 'Pirol' and 'Chicago' transformed with the binary vector pBIN-mGFP5-ER. Genomic and plasmid control DNA was digested with *HindIII* and membranes were probed using the 711 bp *GFP* probe; lane M, molecular marker; lane P, plasmid pBIN-mGFP5-ER; lane NT, non-transgenic potato plant.

In a parallel experiment, we also performed kanamycin-assisted genetic transformation of the same cultivars to compare the efficiency of transgenic plant production. Monitoring the transformation process revealed the significant suppression of plant regeneration in cultured explants, alongside the emphasized formation of aggregates of transgenic cells and callus, which subsequently produced single and multiple shoots with distinct *GFP* expression (Figure S3). When measuring the efficiency of genetic transformation in antibiotic-assisted selection, defined as the percentage of initially inoculated explants that produced transgenic shoots, our analysis indicated markedly higher transformation abilities in potato cultivars under selective pressure compared to the non-selective approach (Table 2). Contrary to the non-selective approach, we observed a substantial genotypic effect. While two cultivars, #12-22-129 and 'Chicago', showed 100% and 98% efficiency of genetic transformation, respectively, 'Pirol' and 'La Strada' yielded transgenic events at half the efficiency, specifically 44% and 47%, respectively.

3.2.3. Impact of the Regeneration Type on the Identification of GFP-Positive Plants under Non-Selective Pressure

In the present study, we found that the genotype-specific timing of plantlet regeneration significantly influenced the identification of the *GFP*-positive plants. In Figure 5, we have combined the number of detached plantlets with the occurrence of explants that formed GFP+ buds, along with the number of detached chimeric/non-chimeric plantlets throughout the entire regeneration stage. The results for two contrasting genotypes are presented. Considering the regeneration experiments, #12-22-129, characterized as the 'early' regenerative cultivar, produced shoots much faster than the 'late' regenerating cultivar 'Pirol'. By the 50th day post agrobacterial inoculation, almost 100 plantlets were detached from Pirol' explants, while with #12-22-129, over half of all regenerated explants (nearly 1000 shoots) had already been collected. Similarly, the first *GFP*-expressing buds were observed in #12-22-129 explants within the second week of cultivation, whereas in 'Pirol' they emerged 10–15 days later. After 45 days of cultivation, no new explants with *GFP*-positive buds were found on #12-22-129 explants, whereas in 'Pirol', the number of explants with such buds continued to appear until the 60th day of the experiment (Figure 5).

The dynamics of detaching *GFP*-expressing plantlets revealed 'empty' periods of culture, when most picked plantlets were non-transgenic (Figure 6). Consequently, the majority of *GFP*-positive shoots (approximately 75%) of the early regenerative cultivar #12-22-129 were discovered between 30 and 60 days of cultivation. In contrast, about

three-quarters of putative transgenic plantlets of the 'late' cv. 'Pirol' were generated by explants between 50 and 80 days of the experiment (Figure 6). The earlier and the later periods of plant regeneration were ineffective in collecting *GFP*-expressing plantlets for cultivars with an intermediate type of regeneration. For instance, the majority of putative transgenic events (over 80%) of 'Chicago' were collected between 30 and 60 days of culture (Figure S4). In 'La Strada' and #12-36-42, the formation of *GFP*-positive plantlets was more extensive and consistent between 40 to 80 days, although a half of the putative transgenic plants were detached between 60 and 80 days of culture (Figure S4).

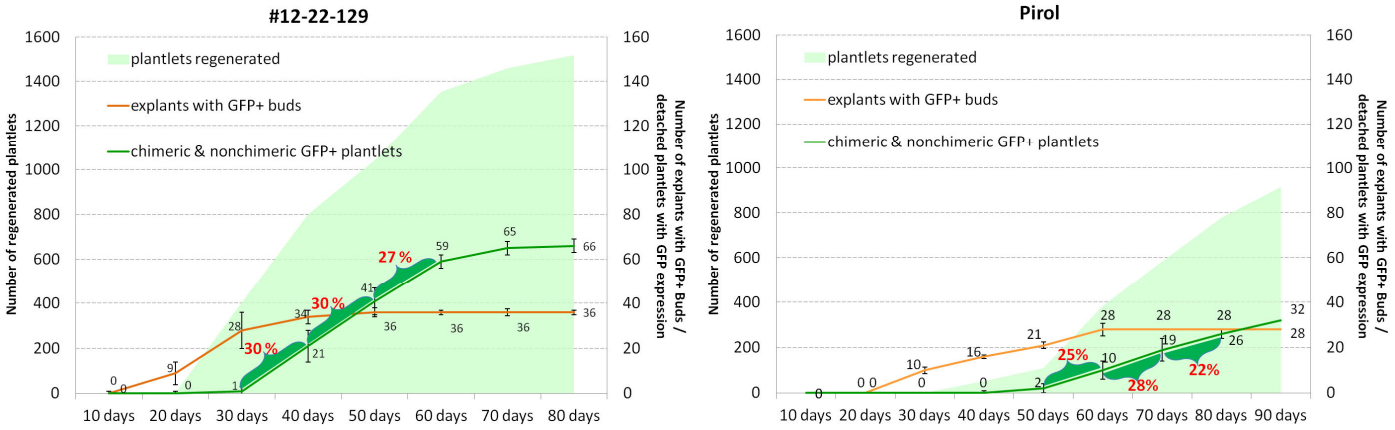

Figure 6. Genotype-dependent timeline for formation of *GFP* expressed buds and plantlets of #12-22-129 ('early' type of regeneration) and 'Pirol' ('late' type of regeneration) under non-selective conditions in comparison with the regenerated shoots.

3.2.4. Efficiency of Antibiotic-Free Genetic Transformation of Two Potato Cultivars Using RFP Monitoring

To confirm the stability and repeatability of antibiotic-free transformation, we conducted an experiment employing a modified in vivo monitoring strategy. In this experiment, each explant and resulting transgenic plantlets were meticulously monitored throughout the experiment to assess the effectiveness of the 'standard' transformation. The plasmid encoding the *RFP* gene driven by the *CaMV 35S* promoter was used to monitor the transformation process. Two potato cultivars with different regeneration characteristics, 'Chicago' and 'Pirol', were subjected to transformation.

Similarly to the previous experiments, the early regenerating cv. 'Chicago' produced chimeric and transgenic plantlets by the end of the 4th week after co-cultivation (Figure 7A,B). Conversely, on explants of the 'late' cv. 'Pirol' fluorescent buds primarily appeared during the same cultivation period (Figure 7C). As a result, the majority of *RFP*-expressing plantlets (Figure 7D) of 'Pirol' were developed between 50 and 80 days of the experiment (Figure 7D), two-three weeks later than in 'Chicago' (Figure 7B).

The use of the *RFP* gene in this study facilitated in vivo observation of transgenic tissue development, as it yielded a brighter signal and exhibited a lower level of autofluorescence compared to experiments involving *GFP* monitoring. On average, the formation of *RFP*-expressing buds was observed on 13–20% of inoculated explants (Table 3). However, the overall number of plantlets exhibiting chimeric or complete red fluorescence exceeded the number of explants with fluorescent buds. An increased number of plantlets resulted from the regeneration of two to three *RFP*-expressing plantlets from some explants, either due to the formation of morphogenic transgenic clusters (Figure 7E,F) or as a result of the independent development of plantlets on both cuts of the internodal explant.

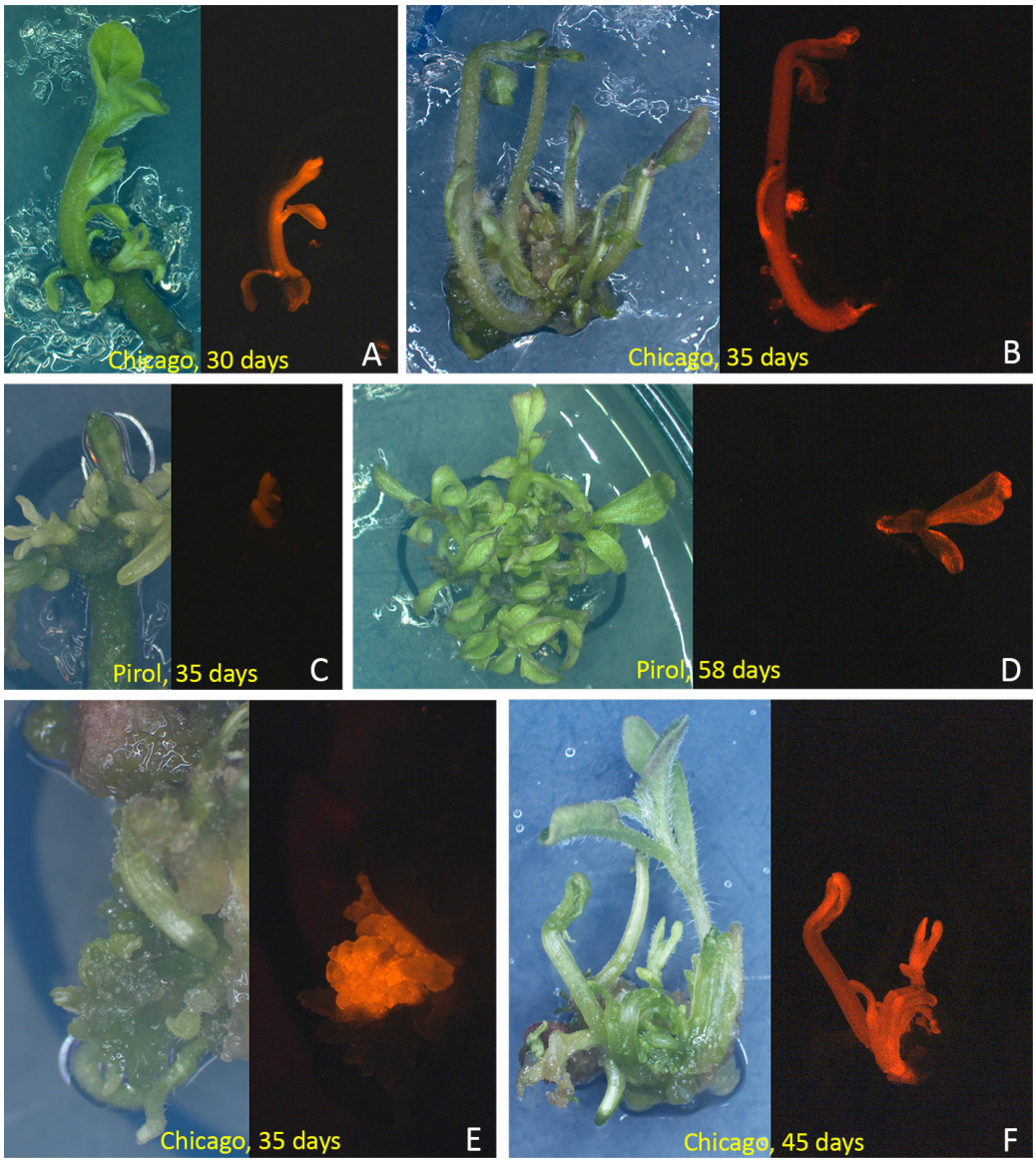

Figure 7. Regeneration of transgenic plants from internodal explants of cvs. 'Chicago' and 'Pirol' under non-selective conditions visualized by red fluorescence. (**A**) Formation of the chimeric plantlet with sectoral *RFP* expression; (**B**) development of the single transgenic plantlet of 'Chicago' with a bright red fluorescence throughout all tissues after 35 days of inoculation; (**C**) formation of RFP-expressing buds on the cut edge of 'Pirol' nodal explant; (**D**) identification of the single transgenic plantlet of 'Pirol' among a bunch of non-transgenic plantlets using RFP observation; (**E**) formation of the morphogenic cluster with a visible red fluorescence at the cut edge of explant; and (**F**) regeneration of several *RFP*-expressing plantlets with various degree of development from the transgenic morphogenic cluster using antibiotic-free transformation.

Following at least five rounds of the sub-culturing of fully fluorescent plantlets and chimeras on an antibiotic-free medium, the total DNA from putative transgenic lines was extracted to confirm the presence of the introduced expression cassette and to explore chimerism. The PCR analysis revealed that 43–74% of 'Chicago' plants and 40–58% of 'Pirol' plants retained their transgenic status, demonstrating the presence of a 537 bp fragment of the *RFP* gene (Figure S5). Similarly, these same plants showed resistance to kanamycin in a parallel rooting test, confirming the introduction of *NPTII* gene. Additionally, tissues of both positive and negative events were assessed for the *RFP* signal, revealing plants with either a bright red fluorescence over the entire plant or plants lacking any sights of

RFP expression. Interestingly, no chimeras were found, as the majority of initial chimeras (>85–95%) transitioned to non transgenic plants, with transgenic sectors being eliminated during in vitro cutting and proliferation.

The efficiency of transgenic plant regeneration remained consistent with the previous experiment. On average, the number of fully transgenic plants per 100 regenerated plantlets was 0.80 ± 0.18 in 'Chicago' and 1.29 ± 0.12 in 'Pirol' (Table 3). Simultaneously, the transformation efficiency, calculated as the percentage of initially inoculated explants producing at least one transgenic plant, was 11.1 ± 1.8% in 'Chicago' and 9.5 ± 2.0% in 'Pirol' (Table 3).

Table 3. The efficiency of antibiotic-free genetic transformation of cvs. Chicago and Pirol with vector.

Cultivar	Experiment	Number of Explants			Number of Plantlets			Transformation Efficiency, %	
		Inoculated	with RFP+ Buds *	with Stable RFP+ Plants	Regenerated (90 Days)	Collected from Explants *	Stable Transgenic Events **	per 100 Regenerated Plantlets	per 100 Inoculated Explant
Chicago	I	95	15	11	1617	24	13	0.80	11.6
	II	91	12	8	1481	21	9	0.61	8.8
	III	100	14	11	1583	19	14	0.91	11.0
	IV	100	16	13	1750	22	16	0.88	13.0
							Average	0.80 ± 0.18	11.1 ± 1.8
Pirol	I	57	9	5	589	14	7	1.19	8.8
	II	55	9	4	523	12	7	1.34	7.3
	III	50	8	5	501	11	6	1.20	10.0
	IV	50	10	6	416	15	6	1.44	12.0
Total/Average							Average	1.29 ± 0.12	9.5 ± 2.0

* Chimeric and fully fluorescent plantlets at the time of collection; ** PCR-positive plants (*RFP*+).

4. Discussion

The large number of scientific reports published since the late 1980s and worldwide commercial releases of GM varieties confirm that potato is a relevant crop for improvement through biotechnology [2–5]. The successful genetic transformation of potato depends on the efficient plant regeneration protocol and the optimized method of recognizing transgenic tissues. Since it is assumed that the likelihood of obtaining transgenic events is higher when more cells are able to differentiate into shoots, we have preliminarily screened ten potato cultivars for the regeneration potential to choose better ones for antibiotic-free transformations. As a rule, a two-stage regeneration protocol is used in potato, when the stage of regeneration of shoots is preceded by the stage of callus induction [4]. In most cases, cytokinins are of paramount importance to initiate morphogenesis in potato. Although BAP, kinetin, and TDZ are known to stimulate regeneration in potato, currently zeatin is the most popular cytokinin for generation of transgenic potato plants. Most transformation protocols include the supplementation of 1–5 mg L^{-1} of zeatin in combination with a low auxin level into the regeneration medium.

Several zeatin-containing variants of regeneration medium combined with different auxins were used in the present study. Experiments also included twin variants, when zeatin was replaced with zeatin ribozide. The latter was reported to be superior to zeatin in terms of regeneration frequency and even allowed the one-step transformation of potato [33]. Tested varieties positively reacted to the various plant phytohormone combinations, although the two variants were more effective in practice. The combination of 3 mg L^{-1} Zea and 0.5 mg IAA L^{-1} was the best for five cultivars, while the combination of the same level of zeatin with a significantly higher concentration of IAA (2 mg L^{-1}) and the additional incorporation of gibberellic acid (1 mg L^{-1}) to support the better outgrowth of morphogenic buds was essential for successful regeneration in 'Chicago', 'Lion Heart', and #10-9-3. The replacement with the more expensive zeatin ribozide generally had no

significant effect in listed above varieties as they were already able to produce plants with 95–100% efficiency using zeatin. Nonetheless, zeatin ribozide was more successful in two cultivars ('Indigo', 'La Strada') and this observation supports the early notion that its application is a really good alternative for particular varieties to achieve increased efficiency of regeneration [33].

Experiments have also shown that the timeline of plantlet regeneration from internodal explants is an important aspect to pay attention to. We observed significant differences between genotypes in the speed of adventitious shoot formation. Some varieties, such as 'Chicago' and #12-22-129, started to generate plantlets much earlier than the others. In contrast, the 'late' cultivars such as Pirol', #10-9-3, 'Indigo', and 'Lion Heart' begin to develop plantlets when the early regenerative cultivars had already produced a maximum of shoots. This genotype-specific property could be used when potato cultivars were subjected to marker less transformation. Our results indicate that early regenerative cultivars produced the most transgenic plants (~75%) between 30 and 60 days after inoculation with *Agrobacterium*, while the late regenerative cultivars preferably generate transgenic events during the 50–80 days of cultivation. The cultivars with an intermediate regeneration timeline generate most transgenic plants between 40 and 70 days after co-cultivation with *Agrobacterium*. This could be considered as a means to reduce the number of tested plantlets, as a bunch of manipulations, such as DNA extraction, PCR analysis, and prolonged in vitro cultivation of each regenerated plant, are required to detect transgenics. Our data are in agreement with those of Jo et al. 2014 [27], who under non-selective conditions and also observed differences between three potato cultivars in the number of PCR-positive plants over the number of analyzed regenerated shoots. Unlike the present study, shoots were collected only at three-week intervals, and only a part of generated shoots were analyzed; however, the timeline-dependent effect was clearly observed, as a higher portion of PCR positive plants of 'Potae9' and 'Bintje' was found between 31–50 and 31–70 days, correspondently, while explants of 'Atlantic' produced a significant portion of transgenic plants during the 50–90 days [27].

There is general agreement that regeneration without selective pressure leads to a problem of chimerism, when the shoots are composed of both transgenic and non-transgenic cells. Through the in vivo monitoring of *GFP* or *RFP* fluorescence, we found that a substantial portion of plants (from a third to a half) displaying visible fluorescent signals represent chimeras of varying degrees (Figures 4C and 7A). Even though chimeric plants were more prevalent during the first period of regeneration, they continued to appear during the later stages of transformation. Our findings suggest that sequential cultivation of chimera shoot tips helps overcome the problem of chimerism. Five cycles of multiplications enable the stabilization of putative transgenic plants. During this period, chimeras containing the transgenic meristem turn into completely transgenic plants, while the majority of chimeras (approximately 80–95%) exhibiting sectoral fluorescence transform into non transgenic plants.

An additional consideration is the possible transgenic chimerism of regenerated potato plants. Direct regeneration may result in transgenic plants composed of cells with diverse T-DNA introduction events. Analysis of transgene insertions indicated a low number of transgene copies in the resulting transgenic potato plants, with mostly one to three independent insertions identified (Figure 5). Observed patterns of integrations align with the prevalent type of transgene insertions reported by various authors when producing transgenic potato plants with the aid of selectable marker genes [20,26,34–37]. This finding prompts us to propose that chimerism issues initially observed in plants collected without selective pressure could be effectively addressed through the application of standard tissue culture methods, such as tip sub-culturing. This practice yields transgenic potato plants that consistently demonstrate the presence of transgenes, confirmed by molecular analysis (PCR or southern-blot) or the expression of introduced genes (red or green fluorescence of tissues, rooting on medium supplemented with kanamycin). In our prior study [29], where marker-free intragenic plants of 'Pirol' were regenerated using the same procedure, the

transgenic status remained stable over five years of in vitro propagation and was regularly confirmed by qRT-PCR and virus resistance.

The Southern blot analysis corroborated that the majority of the regenerated plants represent independent events, even if they originate from the same explant. Our tracking of explants producing transgenic plantlets showed the possibility of duplicating the same transgenic event in the case of morphogenic transgenic cluster formation (Figure 7E). Continuous in vivo monitoring for *RFP* fluorescence, however, indicated that the duplication was rarely observed, giving the low rate of callus formation in our study. It is plausible that the direct induction of buds at various sites of the explant (Figure 4F) could augment the generation of independent events. This notion is supported by the fact that most of the cultivars used for antibiotic-free transformation exhibited the ability to regenerate shoots on both sides of the internodal explant (Figure 2), thereby increasing the likelihood of the independent production of transgenic events from the same explant.

Our results are consistent with previous studies, underscoring that antibiotic-free transformation is less genotype-dependent compared to antibiotic-assisted transformation [25,27]. In the present study, antibiotic-assisted transformation across five cultivars varied from 44 to 100% (Table 2), whereas the efficiency of antibiotic-free transformation was within a narrow range of 0.8 and 2.8% (per 100 plants). A similar trend was previously observed following the *Agrobacterium*-mediated transformation of 'Atlantic', 'Potae9', and 'Bintje' potato cultivars [27], demonstrating high variation in the efficiency of kanamycin-assisted transformation (ranging from 10 to 71%) and low variation in markerless transformation efficiency (0.6–2.4 plants per 100 tested plants). An average marker-free transformation efficiency of 2.2% was reported for cv. 'Superior' [30], where leaf explants were used instead of intermodal segments for plantlet regeneration. In another report, the highly transformable cv. 'Karnico' achieved a slightly higher efficiency, reaching 4.5% of markerless transformation, while the cultivars with lower marker-assisted transformation abilities showed a lower percentage of PCR-positive shoot recovery (0.6–2.4%) [26]. There was no such correlation in our study. For instance, 'Chicago', the cultivar with remarkably high efficiency of antibiotic-assisted selection (100%), demonstrated the lowest antibiotic-free transformation (0.8–0.9%) calculated per 100 detached plantlets. By contrast, cultivars 'Pirol' and 'La Strada', with a lower kanamycin-assisted transformation efficiency of 44–47%, produced more transgenic plants without selective pressure (1.8–2.0 per 100 plants) than 'Chicago' (Table 2). It is pertinent to note that the marker-free transformation, calculated relative to the total number of plants tested, may not be directly comparable for the same cultivar, as the efficiency of antibiotic-assisted transformation is typically defined as the percentage of explants producing transgenic plants. Consequently, the ability to generate more or fewer plantlets from the same number of cultured explants can influence the difference in marker-free transformation efficiency per 100 tested plants in a specific cultivar. This was further affirmed in our study of the 'standard' transformation efficiency by tracking all inoculated explants/transgenic plantlets using in vivo *RFP* fluorescence. The outcomes indicated that the previously mentioned cultivars, 'Pirol' and Chicago', which differ in antibiotic–assisted transformation, displayed nearly equivalent efficiency of antibiotic free transformation, defined as the percentage of initial explants producing transgenic plants (Table 3). We found that approximately every tenth explant of both cultivars is capable of producing a transgenic plant, although the efficiency in individual experiments could vary from 7.3 to 13%.

Despite the simplicity of marker-less transformation, the chances of recovering transgenic plants are usually low [22–24,27–29], as there is the supposition that without the application of selective substances the fraction of *Agrobacterium*-treated cells that may become transgenic is limited. In our study, the number of explants displayed that *GFP* fluorescence until 30 days after agrobacterial inoculation of four potato cultivars was surprisingly equal or even higher than without the selective pressure. Only cultivar 'La Strada' displayed a better transient expression when the kanamycin-assisted selection was used; however, the efficiency of stable genetic transformation was only 47%, lower than in other

genotypes. These results, on the one hand, indicate that a high level of transient expression does not necessarily lead to high efficiency of transformation in potato, while the presence of explants with high transient expression, regardless of selective pressure, makes the generation of fully transgenic plants in the absence of marker-assisted selection realistic.

Although the ability to generate transgenic elite cultivars of potato without using selectable marker genes is not as effective as using a marker-assisted selection, we believe it will facilitate the potential commercial release of new biotech potato varieties produced by cisgenic and intragenic approaches of genetic transformation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10030222/s1, Figure S1: The percentage of explants displaying the GFP expression in cells of potato cv. #12-36-42 under selective- (Km+) and non-selective (KM−) cultivation. Figure S2: Identification of transgenic events by in vitro rooting of putative transgenic plants (cv. #12-36-42) on the medium, supplemented with a lethal dose of kanamycin (100 mg L^{-1}). Figure S3: Regeneration of transgenic potato plants under kanamycin-assisted conditions. Figure S4: Genotype-dependent timeline for formation of GFP-expressed buds and plantlets of 'Chicago' ('early/intermediate' type of regeneration), 'La Strada' ('intermediate' type of regeneration) and #12-36-42 (intermediate/late type of regeneration) under non-selective conditions in comparison with the regenerated shoots. Figure S5: Putative transgenic plants of potato cv. 'Pirol' produced within experiments of antibiotic-free transformation were analyzed for the presence of *RFP* gene by PCR amplification.

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