

IN VIVO LABELLING OF *ANAGALLIS ARVENSIS* L. CELLS WITH GREEN FLUORESCENT PROTEIN

MARCIN ŁUKASZEWICZ¹, DOROTA KWIATKOWSKA²

¹Institute of Microbiology, Wrocław University
Przybyszewskiego 63-77, 54-148 Wrocław
e-mail: lukasz@microb.uni.wroc.pl

²Institute of Botany, Wrocław University
Kanonia 6/8, 50-328 Wrocław, Poland
dorotak@biol.uni.wroc.pl

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ABSTRACT

A few methods only enable to follow the fate of plant cells in vivo. One of the most promising is using the Green Fluorescent Protein (GFP). In our preliminary study we set up the experimental system enabling labelling of *Anagallis arvensis* cells with this marker. We prepared an expression plasmid containing red-shifted *gfp* with optimised translation start site context, under the control of CaMV 35S transcription promoter. The construct was introduced into *A. arvensis* cells by particle bombardment. We developed two methods of material preparation for this transformation: in vitro cultured stem internodes with regenerating adventitious shoots (the earliest stages of regeneration); and shoot tips with temporarily exposed apices. The reflected light fluorescence microscope Olympus with the set of filters U-MNB designed for fluorescein detection enables the observation of GFP fluorescence. Both ordinary epidermal cells and stomata guard cells were transformed. Their fluorescence was observed for up to 14 days. Artefacts (autofluorescence of glandular trichomes and faint green glowing of meristematic tissue) could be overcome by the optimisation of the filter set.

KEY WORDS: *gfp* gene marker, biolistic transformation, cell labelling, *Anagallis arvensis*.

INTRODUCTION

Following individual cell fates during a plant shoot development in real time should deliver significant information on plant morphogenesis. Unfortunately there are only few methods enabling such investigation and each of them has some limitations. A fate of superficial cells of the shoot apex can be followed in vivo by means of sequential replicas made in a dental polymer, which serve as molds for epoxy resin cast preparation. These casts are ready to observe in SEM (Green et al. 1991). Disadvantages of this otherwise very useful method are a relatively long time interval required between successive replicas preparation (24 hrs at least, 48 at best) necessary to keep a meristem alive, and short duration of an individual meristem observation, usually no longer than two weeks. The latter is not an obstacle if chimeras are used for a morphogenetic study. Progeny of meristematic cells representing various genotypes can often be easily recognised in vivo in mature shoot parts (e.g. Hejnowicz 1956; Poethig and Sussex 1985; Tilney-Bassett 1986; Döring et al. 1999). However, in most of chimeras that have been studied by developmental botanists, differences in genotypes of meristematic cells composing stem apices cannot be readily identified in vivo. In case of polyploids, the material requires fixing and sectioning (Satina et al. 1940; Dermen 1945). In albinotic chimeras, the identification of cells with various genotypes

within shoot apical meristems would require still more elaborate microtechniques. Although investigations using the two methods described have already delivered plentiful of information on shoot morphogenesis (Tilney-Bassett 1986; Dawe and Freeling 1991), these methods do not enable either the long-term following of the fate of a chosen meristematic cell progeny, or the study of the molecular mechanisms underlying the ontogenetic development. Thus, setting up new approaches is a great challenge for developmental botanists.

A new potentially powerful method to study plant morphogenesis and gene expression is in vivo labelling of plant cells. The most promising marker is the extensively studied green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Prasher et al. 1992; Reichel et al. 1996). It is now frequently used for specific labelling of cell organelles in vivo (the session Vital Imaging devoted to this kind of research was organised on the recent Botanical Congress in St. Luis). However, the possibility of GFP labelling for the long term observation of cell fate in situ has been only infrequently explored (Łukaszewicz and Kwiatkowska 1998). Wild type GFP emits bright green light ($\lambda_{\text{max}} = 509 \text{ nm}$) when excited with UV ($\lambda_{\text{max}} = 396 \text{ nm}$) or blue light ($\lambda_{\text{max}} = 475 \text{ nm}$, minor peak). This reporter gene has several significant advantages: fluorescence of GFP is an intrinsic property of the protein which is not dependent on exogenous substrates or cofactors (Cody et al. 1993); GFP is non-toxic and does not interfere

with cell growth and function (Chalfie et al. 1994); it enables *in vivo* studies in single cell systems; it is a small protein (238 amino acid residues) which is fluorescent even after the fusion with other proteins; and finally, it is very stable (Sheen et al. 1995) and may be detected directly by fluorescence in SDS-PAGE gels (Baulcombe et al. 1995). Moreover, there are mutants available with different excitation and emission peaks as well as improved photostability and excitation coefficient (Heim et al. 1995). This enables the simultaneous analysis of two reporters (Heim et al. 1994; Zimmerman and Siegert 1998). However, as GFP is a fluorescent protein itself, it does not amplify detected signal like other reporter genes (for example β -glucuronidase or luciferase) exhibiting enzymatic activity. Therefore, it is necessary to optimise transcription, translation, light detection, and transformation methods.

To follow *in vivo* morphogenesis and gene expression at consecutive stages of a meristematic cell ontogeny, meristematic regions have to be exposed *in situ* during the transformation and successive observations. These regions are very vulnerable and are protected by overtopping leaves, which should be temporarily bent outward to expose a shoot apex. A technique of shoot meristem exposure used by Green and collaborators (1991) for the sequential replica preparation seems to be the most promising for this purpose. Another potentially valuable material should be obtained from *in vitro* culture, where growth regulator treatment can lead to cell dedifferentiation. The dedifferentiating cells which progeny forms meristematic centres are not overtopped by leaves and therefore preparations for the transformation are easier.

Our aim was to test the feasibility of the single cell fate observation in the real time *in situ*. To achieve this goal we first constructed an expression plasmid containing *gfp* reporter gene. Then we prepared two kinds of plant material and used it for the biolistic transformation. These were explants of stem internodes cultured *in vitro* on a medium inducing meristematic centre formation from dedifferentiating epidermal cells; and exposed shoot apices, the same as those used by Green and collaborators (1991). We chose *Anagallis arvensis* L. for this study because both the above mentioned methods have already been developed for this particular species (Bajaj and Mäder 1974; Green et al. 1991; Kwiatkowska and Kromer 1999), and because its phyllotaxis and stem elongation patterns pose a number of significant questions on plant morphogenesis (Kwiatkowska 1995, 1997). Finally, we performed primary experiments of biolistic transformation from which we obtained GFP expression which is easy to detect with the epifluorescence microscope with the set of filters designed for fluorescein observation.

MATERIALS AND METHODS

Preparation of constructs used for the biolistic transformation

The 35SGFPRS construct (Fig. 1) was prepared in the pTZ19U plasmid (Genescribe-Z, US Biochemical Corp.). The following sequences were introduced within the Hind III and EcoRI of the pTZ19U: a strong transcription promoter CaMV35S; the *gfp* reporter gene; and the polyadenylation signal from the nopaline synthase (*nos* *ter*) gene. In order to avoid splicing, the sequence of the cryptic intron within the *gfp* gene was modified (Haseloff et al. 1997). The GFP used in the construct is red-shifted, i.e. contains mutation replacing serine 65 to threonine (Heim et al. 1995). This mutation modifies excitation characteristics improving photostability and excitation coefficient. To improve translation initiation

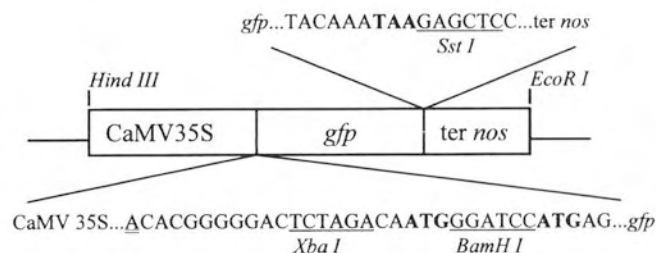


Fig. 1. Schematic representation of the 35SGFP construct. Boxes represent: DNA sequence of the strong transcription promoter from the Cauliflower mosaic virus (CaMV 35S); coding sequence of the green fluorescent protein from the jellyfish *Aequorea victoria* (*gfp*); and transcription terminator from the nopaline synthase gene (*ter nos*). The sequence of the junctions is shown. The double-underlined nucleotide is the transcription start site of the CaMV 35S promoter. Restriction sites are underlined. Translation start and stop codons are in bold. Second ATG is the *gfp* original translation start site.

and enable easy modifications of the translation start site context, the second ATG was introduced upstream the *gfp* within the XbaI and BamHI. This was achieved by PCR amplification using GFP5' (GCTCTAGAACAATGGGATCCATGAG-TAAAGGAGAAGAAC) and GFP3' (CTGGAGCTCT-TATTTGTATAGTTCATCC) primers. PCR-amplified fragment was electrophoresed on a 2% agarose gel, purified with Qiaex DNA Gel Extraction (DIAGEN GmbH, Hilden, Germany) and cloned within BamHI-SacI of the SK⁺ bluescript plasmid. After sequencing on double-stranded DNA by the dideoxy termination method (Sanger et al. 1977) using the T7 polymerase (Pharmacia), *gfp* was cloned to the 35SGUS (Michelet et al. 1994) within XbaI and SacI, thus replacing *gus* reporter gene.

In vitro cultures

Explants of stem internodes, 8-12 mm long, excised from *A. arvensis* shoots grown *in vitro* on the modified Murashige and Skoog (1962) medium (MS), were passaged on MS supplemented with 0.2 mg dm⁻³ 6-benzylaminopurine (BA). This growth medium was earlier found to effectively stimulate the formation of meristematic centres, which develop into adventitious shoots (Kwiatkowska and Kromer 1999).

The growth medium contained 8 g dm⁻³ of agar and 30 g dm⁻³ of sucrose; pH was adjusted to 5.8. Cultures were kept in OSRAM Fluora lamps, with 90 μ m⁻² s⁻¹ PAR (Experiment No 1 – 24 h light per day; Experiment No 2 – 16 h). Temperature and humidity were not controlled.

For the transformation and the microscopic observation stem internodes were placed in Petri dishes, 6 cm diameter, half-filled with the growth medium (Fig. 2).

A method of shoot apices exposure

Shoot apical meristems of *A. arvensis* were temporary exposed for the time of transformation and successive microscopic observations with the method Green and collaborators (1991) designed for the preparation of sequential *in vivo* replicas of the meristem surface. Six shoots, c. 5 cm long, cut off from potted plants in the generative phase of development, were placed in a scaffolding, which enabled to unbend leaves overtopping the shoot apex (see Fig. 3 for further explanations). These shoots together with their scaffoldings were placed in vessels filled with water, and handled as such both for the transformation and microscopic observation. A

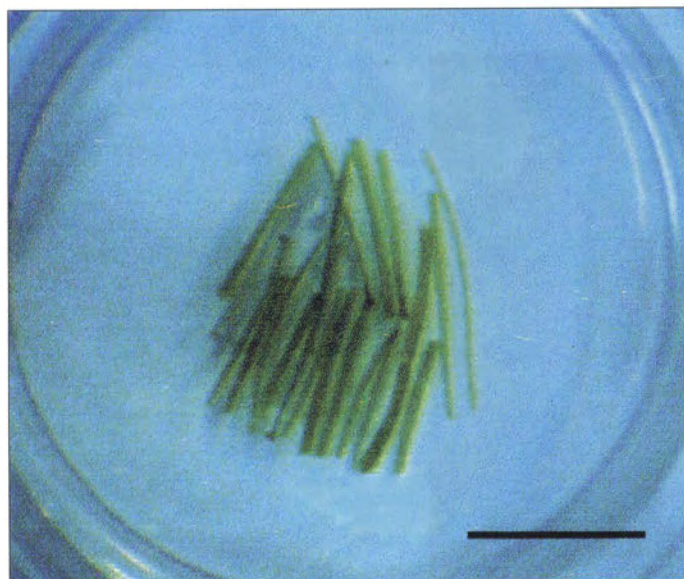


Fig. 2. Top view of a Petri dish with stem internode explants of *A. arvensis* placed on the solid medium containing 0.2 mg dm^{-3} BA, like those used for biolistic transformation in experiments 1 and 2. Bar = 15 mm.

drop of distilled water was placed on the shoot tip immediately after bombardment or microscopic observation. In the meantime, shoot tips were covered with a Petri dish bottom with wet filter paper attached. The shoots were kept in the same growth conditions as in vitro cultures, with 16 hrs of light per day (experiment 3).

DNA preparation and biolistic transformation

Plasmid DNA was amplified in *Escherichia coli* JM109 strain growing in the LB medium and purified on QIAGEN affinity columns (QIAGEN GmbH, Hilden, Germany). Biolistic transformation was performed using PDS-1000/He System (Bio-Rad) essentially as that described by Łukaszewicz and collaborators (1998).

Three series of biolistic transformation were performed. In experiments 1 and 2 explants of stem internodes, 8-12 mm

long (four Petri dishes, each containing c. 20 explants in both experiments), were used (Fig. 2). In experiment 1 they were bombarded 9 days after the passage to a medium stimulating the regeneration; in experiment 2 – after 2 days. In experiment 3 four shoot tips with exposed apices (Fig. 3) were bombarded. Two shots were performed for every specimen. In experiment 1, $1 \mu\text{m}$ tungsten particles were used, whereas in the other two experiments gold particles (Fig. 4), $1.1 \mu\text{m}$ in diameter, were used instead of tungsten. We found that coating gold particles with DNA is more reproducible than tungsten particles (data not shown).

GFP fluorescence

GFP fluorescence was observed in a light microscope Olympus BX50 with a reflected light fluorescence attachment BX-FLA. We used the excitation cube U-MNB (designed for fluorochromes like FITC, acridine orange, auramine), which contains: exciter filter BP470-490 nm; a barrier filter BA515 transmitting light longer than 515 nm; and a dichroic mirror DM500 transmitting wavelengths longer than 500 nm.

RESULTS

An average number of adventitious shoots regenerating per internode explant after two weeks of in vitro culture equaled 12. The majority of these shoots did not exhibit teratologic development like fasciations or abnormal leaf shapes.

Reflected light fluorescence microscope Olympus BX50 with the filter cube U-MNB allowed the observation of GFP fluorescence. This equipment, however, did not enable to quantify the fluorescence intensity, although our microscopic observation revealed that green light intensity was variable depending on transformed cell. It should be also noted that GFP fluorescence might be confused with autofluorescence of glandular trichomes (Fig. 5). The green-yellowish glowing of meristematic tissue (Fig. 6) caused other technical problem.

In the first experiment (Tab. 1A) where eighty stem segments were bombarded, the total of 17 ordinary epidermal cells showed GFP fluorescence 72 hrs after the bombardment.

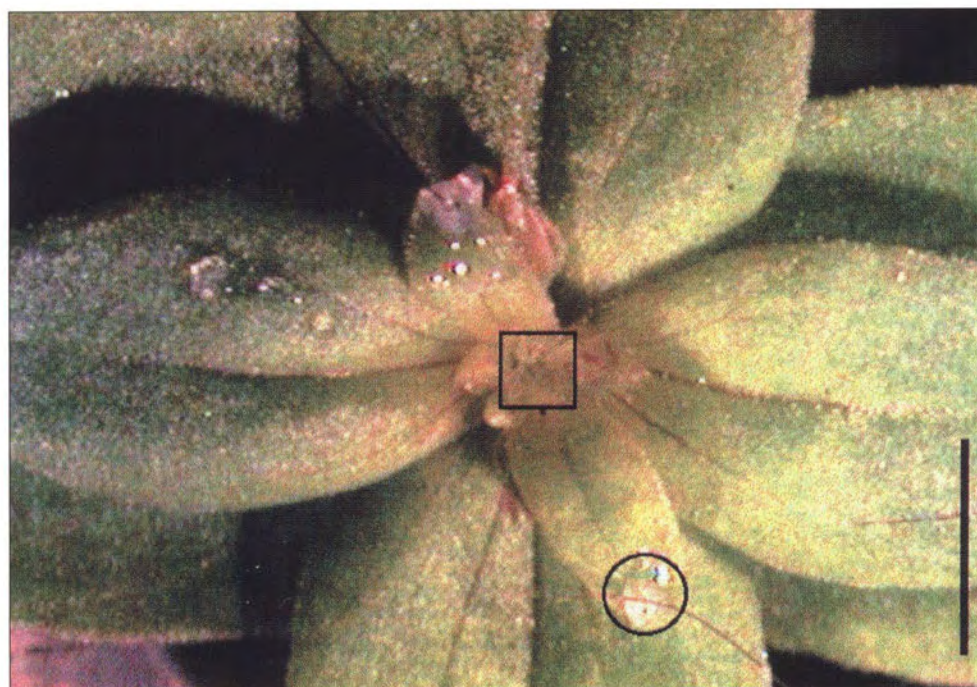


Fig. 3. A shoot tip of *A. arvensis* with the apical meristem exposed using the technique developed by Green and collaborators (1991) (exp. 3). Microsurgical threads are glued with an epoxy gel to leaf tips (circled) at one end, and to the scaffolding at the other end. Pulling the threads enables temporary unbending of leaves which overtop the shoot apex region (in square). Bar = 5 mm.

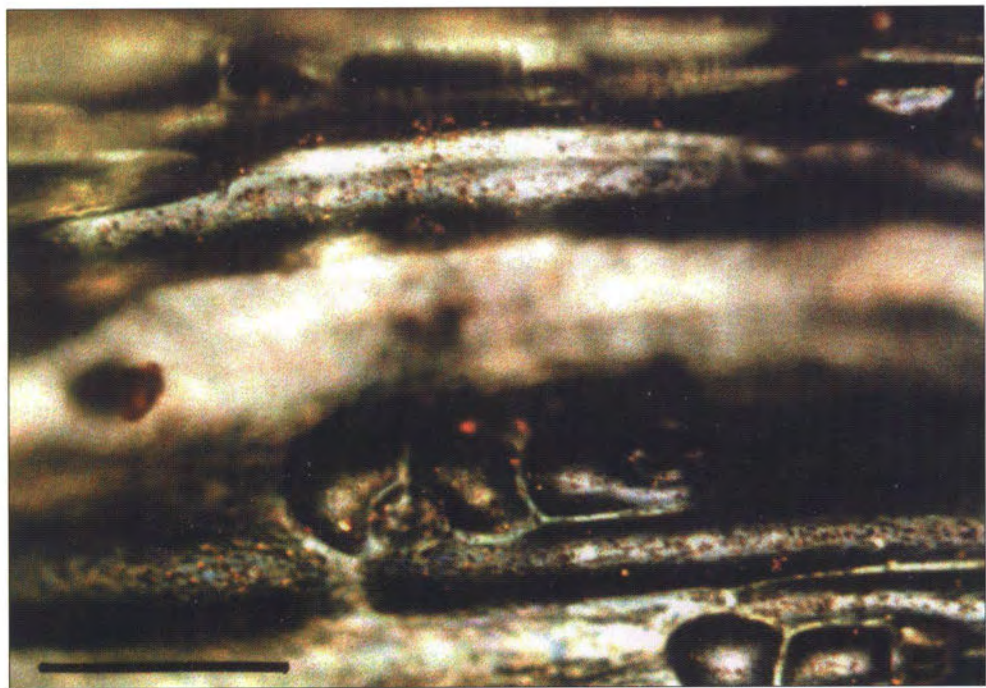


Fig. 4. Gold particles on the surface of an internode explant observed immediately after the biolistic transformation (exp. 2). Bar = 50 μ m.

In 11 of these cells the fluorescence ceased within six days; in two – it lasted for ten days; and in one – for 14 days after which the cell died. A strong fluorescence occurred even in this plasmolysed dying cell, which suggests that the protein is extremely resistant to degradation in cells of *A. arvensis*. None of transformed cells participated in formation of adventitious shoots.

Similar results were obtained from the second experiment. As a result of this transformation, however, apart from ordinary epidermal cells (Fig. 7), a large number of stomata guard cells were transformed (Tab. 1B, Fig. 8). Like in the previous experiment, in the majority of cells the fluorescence ceased after one week.

The third series of bombardment, i.e. of exposed shoot apices, resulted in the GFP fluorescence in a number of stomata guard cells and in a single ordinary epidermal cell (Tab. 1C). Fluorescence of all these cells ceased within seven days.

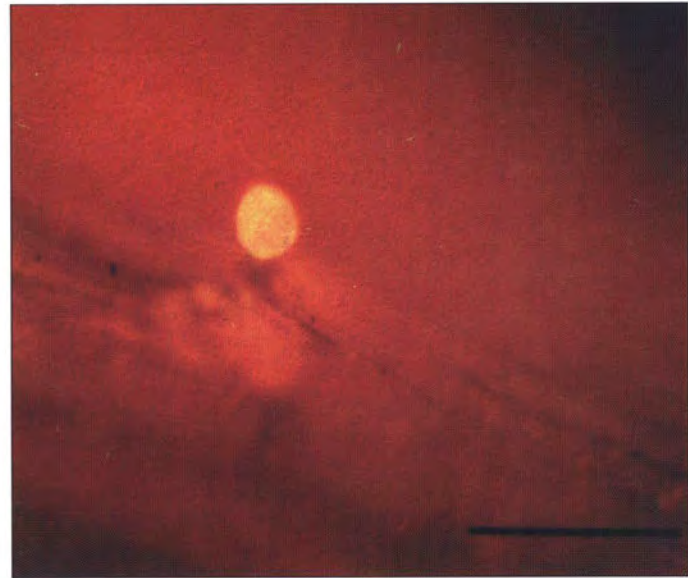


Fig. 5. Autofluorescence of a glandular trichome (exp. 1; three days after the transformation). Bar = 50 μ m.

DISCUSSION

The aim of this work was to test two experimental systems of labelling *in vivo* plant cells for the observation *in situ*. We prepared a construct containing *gfp* as reporter gene, which expression in *A. arvensis* cells was easily detected by fluorescence microscope. This suggests that additional translation initiation start site, making the GFP protein three aminoacids (namely methionine, glycine, serine) longer, did not abolish the fluorescent properties of the protein. It is not surprising

TABLE 1. Numbers of cells exhibiting GFP fluorescence after the biolistic transformation. A. Experiment 1; B. Exp. 2; C. Exp. 3.

A.

Cell type	Number of days after bombardment						
	3	5	6	9	10	14	17
Ordinary epidermal cells	17	9	6	3	2	1	0
Stomata guard cells	0	0	0	0	0	0	0

B.

Cell type	Number of days after bombardment			
	2	6	9	12
Ordinary epidermal cells	10	1	0	0
Stomata guard cells	16	4	2	0

C.

Cell type	Number of days after bombardment	
	2	7
Ordinary epidermal cells	1	0
Stomata guard cells	10	0

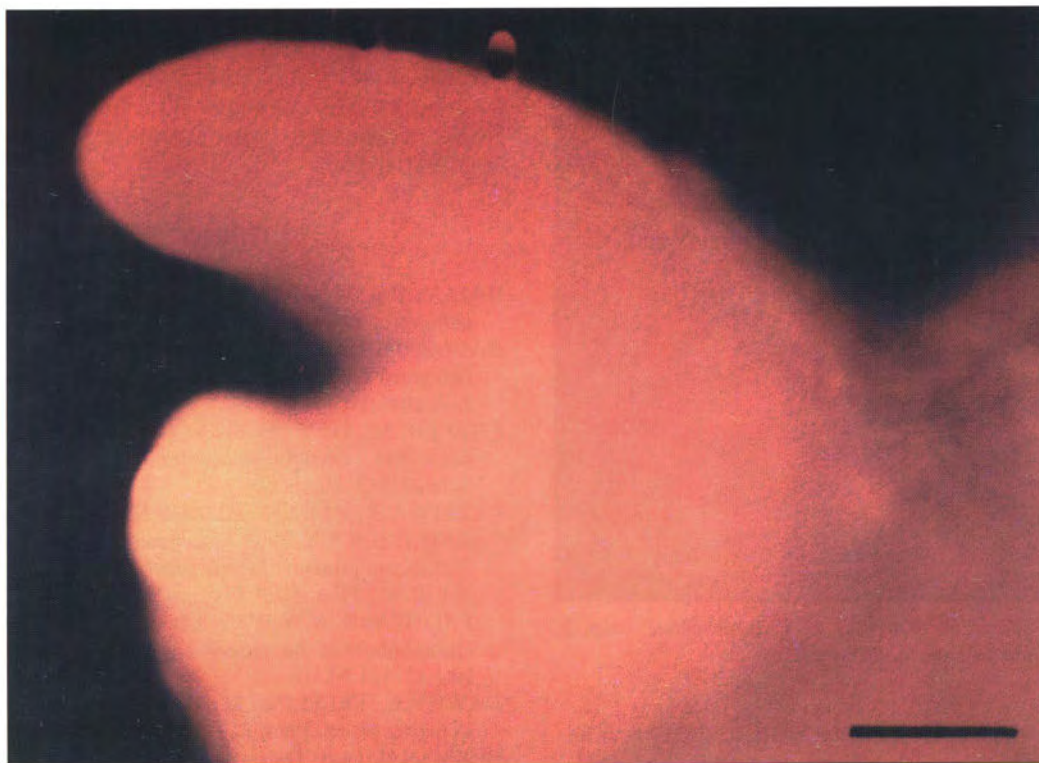


Fig. 6. Yellow-greenish glowing of a young adventitious shoot, regenerating from the internode epidermis. The two youngest leaf primordia are visible (exp. 1; ten days after the transformation). Bar = 50 μm.

since several other N and C end terminal fusion proteins have already been shown to be fluorescent (Cubitt et al. 1995). It is also possible that the GFP protein is expressed from its own translation start site. This, however, is less probable as the nucleotide context predicts this AUG as a weak translation initiation start site (Joshi et al. 1997).

We observed the fluorescence of two types of cells: ordinary epidermal cells and stomata guard cells. Unfortunately we did not succeed in transforming a meristematic cell, for which several possible reasons can be suggested. Firstly, the area covered by meristematic or potentially meristematic cells

is only a small portion of the total area under bombardment. In case of internode explants this could be overcome by the performance of a higher number of shots per explant. The possible way to increase the transformation probability in meristems of exposed shoot apices, is to concentrate bombarding particles within the smaller area, e.g. by inserting a micropipette tip between the stopping screen and the target. Other reasons for which no meristematic cells were transformed can be their much smaller size and possibly also different surface properties (thinner cell walls and small vacuoles in comparison with mature epidermal cells). They are

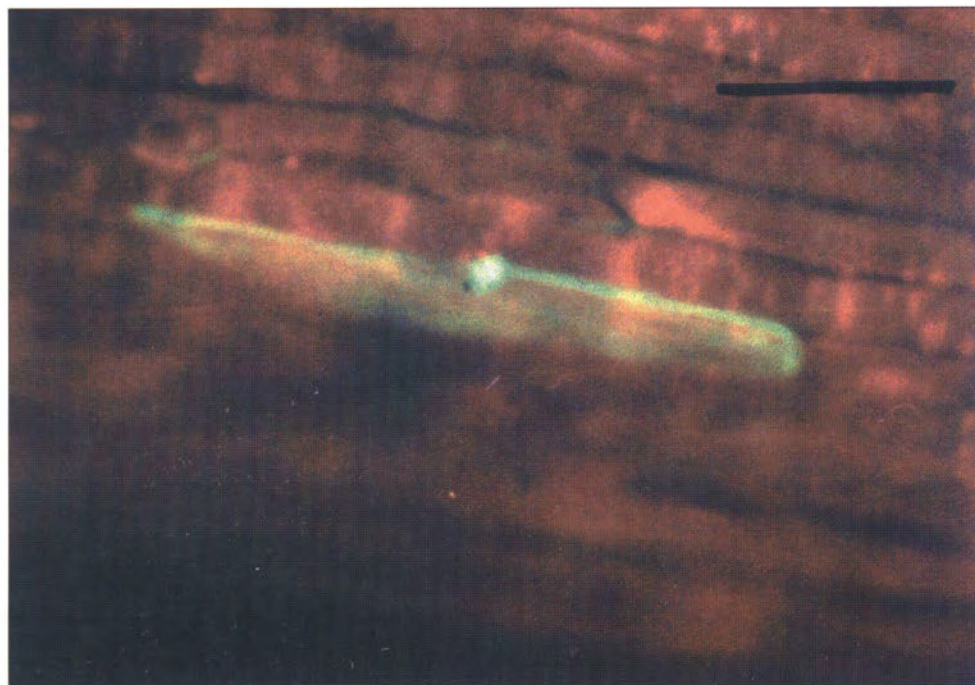


Fig. 7. Transformed ordinary epidermal cell (exp. 2, three days after the transformation). A gold particle, dark spot near the cell nucleus, is visible. Bar = 50 μm.

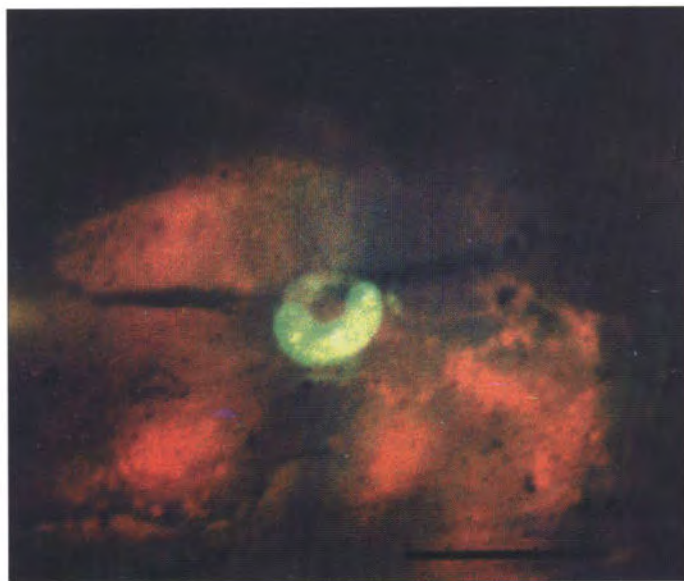


Fig. 8. The stomata guard cell exhibiting GFP fluorescence. (exp. 2, nine days after the transformation). Bar = 50 μ m.

also more fragile and could possibly be destroyed by bombarding particles. This might have caused necroses of meristematic cells which we observed after the third series of bombardment. Thus it could be a good idea to use smaller particle size and optimise other transformation conditions. Nevertheless, using exposed shoot apices as an experimental system has a number of significant advantages in comparison with the internode explants. Most importantly, there is no need of performing all manipulations in the sterile conditions, which is especially troubling during microscopic observation. Also, the meristematic centres formed on internode explants are very soon covered by growing leaves making the observation of fluorescent cells impossible. Exposing apices of in vitro grown shoots would be much more difficult than of potted plants, both because of their very small size and of the requirement of sterile conditions. At the same time, in potted plant shoots, successively formed leaves can be attached to threads and the scaffolding with virtually no limitation. Also in case of potted plants, one works with genetically uniform and normally growing shoots, whereas adventitious shoots are often teratologic (Kwiatkowska, Kromer 1999) and can possibly exhibit somaclonal variation.

The encountered artefacts, i.e. the autofluorescence of glandular trichomes and faint green glowing of meristematic tissues, make the screening of material for transformed cells difficult in our microscope. These could be improved by optimisation of the filter cube characteristics. Exchanging the barrier filter BA 515 nm into BA 510 nm should result in the increase of GFP fluorescence, since the former filter cuts off the peak of GFP emission located at 510 nm wavelength. Also we would probably get rid of the yellowish fluorescence of glandular trichomes and autofluorescence of chlorophyll in subepidermal cells, if a filter removing the light with wavelengths longer than 575 nm was added.

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ZNAKOWANIE PRZYŻYCIOWE KOMÓREK *ANAGALLIS ARVENSIS* L. BIAŁKIEM GFP (GREEN FLUORESCENT PROTEIN)

STRESZCZENIE

Przyżyciową obserwację komórek roślinnych umożliwiają tylko nieliczne metody badawcze. Jedną z najbardziej obiecujących jest znakowanie komórek białkiem GFP (Green Fluorescent Protein). We wstępnych badaniach, które prezentujemy w niniejszej pracy, zajmowaliśmy się opracowaniem systemu doświadczalnego umożliwiającego znakowanie GFP komórek *Anagallis arvensis*. Przygotowaliśmy plazmid do ekspresji białka GFP (red-shifted) ze zoptymalizowanym kontekstem translacyjnym, pod kontrolą silnego promotora transkrypcji. Konstrukcję ten został wprowadzony do komórek *A. arvensis* na drodze transformacji biolistycznej. Opracowaliśmy dwie metody przygotowania materiału do transformacji: eksplantaty międzywęzli pędów hodowanych w kulturach in vitro na pożywce stymulującej tworzenie pędów przybyszowych oraz szczytowe części pędów z merystemami wierzchołkowymi odsłanianymi na czas transformacji i obserwacji mikroskopowych. Obserwacja fluorescencji GFP jest możliwa z wykorzystaniem mikroskopu fluorescencyjnego Olympus z zestawem filtrów U-MNB przeznaczonym do obserwacji fluoresceiny. Transformacji uległy komórki właściwe epidermy oraz komórki szparkowe. Fluorescencję poszczególnych komórek obserwowaliśmy przez okres do 14 dni. Artefaktów, takich jak autofluorescencja włosków gruczołowych i zielonawa poświata tkanki merystematycznej utrudniających obserwację, będzie można uniknąć w przyszłości przez zmianę parametrów zestawu filtrów.

SŁOWA KLUCZOWE: marker genowy *gfp*, transformacja biolistyczna, znakowanie komórek, *Anagallis arvensis*.