University of South Bohemia

Faculty of Science

Application of GFP signal gene in the development of the plant transgenesis strategy aimed at increasing the tolerance to biotic stresses

Ph.D. thesis

Marek Hraška

České Budějovice

2007

Acknowledgements:

This is for me quite often one of the most complicated parts of such type of paper works, since so many people encouraged and supported me during my Ph.D. study. Listing of all of these people may lead to the regretful fact, that I can omit to mention some of them. Nevertheless, I would like to express my special thanks to a few people. First of all, my parents for their support and thus the opportunity, to fully concentrate myself on the work on the topics regarding this thesis. Next, special thanks must be given to my supervisors, RNDr. Slavomír Rakouský, CSc., and Doc. Ing. Vladislav Čurn, CSc., for their excellent leadership and help during my study and creation of friendly environment. Finally, I would like to express my thanks to all the colleagues, staff and friends across all laboratories I met within my study. Last, but not least, my thanks also belong to Ms. Marcela Levá, for her technical help with preparation and printing of many my paperwork, I realized during my study, including this thesis.

I hereby declare, that this thesis was prepared by me personally, contain only the results of research I performed in the laboratories within the Faculty of Agriculture and Faculty of Science of the University of South Bohemia in České Budějovice.

Marek Hraška, in Písek, September 02, 2007

Supervisor: **RNDr. Slavomír Rakouský, CSc.**
Fac. of Health and Social Sciences and Fac. of Science,
University of South Bohemia
Consultant/ specialist: **Doc. Ing. Vladislav Čurn, Ph.D.**
Fac. of Agriculture, Biotechnological centre
University of South Bohemia

Annotation

Presented Ph.D. thesis summarizes my research regarding the utility of a vital marker gene coding for green fluorescent protein (GFP) for the research of transgenic plants. The aim of my work was to establish approaches for the use of GFP for the development and production of transgenic plants with novel qualitative traits, perspectively with promoted resistance against fungi and insect. The study of possible use of the GFP as an efficient tool for assessment and selection of transgenic plants, with the aim to qualitatively evaluate and select elite genotypes, was the main topic of my work and novel findings are presented here. Moreover, beside this main topic, the performance of constitutive Ca MV 35S promoter within transgenic plants was also investigated and the original data are also presented within this thesis. Finally preliminary results regarding the possible use of GFP as a selection tool for the flax transformation are included. Presented results contribute to the further development of the approaches utilizing the GFP as a vital marker for the transformation, selection and assessment of transgenic plants. Further, data regarding the performance of CaMV 35S promoter represent another part into the mosaic of a common knowledge regarding the behavior of constitutive promoters.

Content

1. Introduction and background summary of theses topic 1

1. 1 Agriculture and plant breeding on the turn of a new millennium 1

1. 2. Transgenic plants as a part of modern agriculture 2

1. 3. Genetic transformation of plants and development of GMOs 4

1. 3. 1. *Agrobacterium tumefaciens* mediated transfer is relatively easy way for delivery of foreign genes into plant genome 5

1. 3. 2. Particle bombardment is an effective alternative for Agrobacterium- mediated transfer 5

1. 4. Efficient selection of transformed cells/ tissues is a bottleneck of transformation procedure 6

1. 5. Development of transformation protocol is a complex issue and the use of visual markers could be a big benefit 8

1. 6. Green fluorescent protein (GFP) 9

1. 6. 1. Although the GFP possess many beneficial features, it also possess a few weak points 11

2. Aims of presented Ph.D. thesis 13

4. Results 16

4. 1. Protease inhibitors, mode of action and perspectives for plant transgenosis 16

4. 2. Use of a simple semiquantitative method for appraisal of green fluorescent protein gene expression in transgenic tobacco plants 24

4. 3. Green fluorescent protein as a vital marker for non-destructive detection of transformation events in transgenic plants 29

4. 4. Sample topography and position within plant body influence the detection of the intensity of green fluorescent protein (GFP) fluorescence in the leaves of transgenic tobacco plants 46

4. 5. Tracking of the CaMV-35S performance in GFP transgenic tobacco, with a special emphasis on flowers and reproductive organs, confirmed its predominant activity in vascular tissues 57

4. 6. GFP detection in low level signal/ noise ration samples 80

5. Summary 83

6. References 86

7. Summary of publications from the year 2004 where Marek Hraška is listed as a author or co- author 92

1. Introduction and rationale of the thesis topic

1. 1. Agriculture and plant breeding on the turn of new millennium

Current agriculture and plant breeding on the turn of new millennium faces of a new challenges, mainly represented by the need to feed at lest 6 billions of human population worldwide, which still increases. It is estimated that in the year 2025 population of the planet will reach 8.5 billions. Unfortunately, the human population mostly increases in the poorest regions of the world. Together with never ending growth of industry and economy in strong western economies, and nowadays also within many so called "tigers", agriculture has to support further increasing human population, but in parallel from still decreasing area of agricultural land. According to the data presented by the World Bank, average area of cultivated land per human capita in year 1961 was 0.44 ha. In the year 2002 it was 0.26 ha and it is estimated that it will drop to only 0.16 ha per capita in the year 2050 (reviewed by Carliny, Grossi-de-Sá, 2002). Therefore is it expected that agriculture will be able produce more food and also feed, from less area and in parallel in at least the same quality. These challenges expect close integration and cooperation of many scientific disciplines across the biology field (reviewed by Babu et al., 2003).

Although the plant breeding, as an essential prerequisite of successful plant production, recorded in the past decades many successes, it is not possible to meet the above-mentioned requests by conventional approaches only. Conventional methods of plant breeding, represented by controlled crossing of selected individuals and species, followed by the stringent selection of desirable progeny/ progenies, approached nowadays their limits. One of such limits is for the most cases a need of sexual transfer of genetic material, the DNA, reducing the amount of possible desirable traits. Nevertheless these methods still play very important and indispensable role in the production of new varieties and cultivars of crops. Novel methods of recombinant DNA, followed by the transgenesis of plants and also animals, represent a new approach to modification of various desirable plant traits and opens new ways in the crop breeding. They allow for the transfer of desirable traits across various species and moreover classes, which would be never transferred via sexual transfer. This represents a fascinating tool for the production of qualitatively new organisms or organism with features, which could not ever occur after sexual transfer of DNA. Nevertheless, the most desirable, and we can say also the most important trait, the promotion of crop production/ yield is still impossible to reach. The genetic determination of such, very

important trait, is given by many genes of minor influence, which are very complicated to identify and which, moreover, contribute in the different way and intensity to the resulting phenotypic expression of a particular trait, namely production/ yield. Therefore we can expect only a small progress in this area. Nevertheless there are still many other ways to increase the yield in the indirect manner. The genetic determination gives the particular genotype the border of particular trait expression, which could be in the ideal situation reached. In the agricultural practice, this can not be ever reached due to many factors, but this gap, between the plant genetic potential and the real performance on the field gives the plant breeders and molecular biologist a space for genetic manipulations, with the aim to approximate such potential (Lawrence and Koundal, 2002). Among many other, factors like high salinity of soil, drought or on the other side flood, freeze, influence of various plant pests, fungi and viruses are the main factors, which contribute to the particular crop final yield decrease. Therefore, since most of these traits are of qualitative nature, given by one or only a few genes of strong influence, this is an open field for current molecular breeding of plants. Many organisms across various taxons exhibit desired traits, applicable also for the purposes of modern agriculture. It is only a question of time, when such traits will be identified on the molecular level and their regulation will be known and new transgenic plants enriched by such traits will follow soon.

1. 2. Transgenic plants as a part of modern agriculture

Molecular biology and genetics are fast growing disciplines of current science and those experiences a big boom nowadays. Area of genetics modifications of plants is not lag behind and transgenic plants are now part of regular agricultural practice in many advanced, as well as developing countries all over the world. First transgenic plants of tobacco were obtained in the year 1984. Until today transgenic plants of at least 100 plant species were obtained and many others will follow soon (Hraška et al., 2006a). Scope of plant species, which were tested for their susceptibility for genetic modification include various crops of strategic importance, as well as ornamental and medicinal plants. Almost all of them were successfully transformed. Still developed and improved techniques of plant transformations allow for genetic modification of new plants species too. First transgenic crops for commercial agriculture practice (GMOs) were released in 1996 and from this date the area of cultivated transgenic crops still increases. In the year 2000 it reached 44.2 mil ha and in the year 2004 66.7 mil ha worldwide (reviewed by Babu et al., 2003). In 2006 for the first time in

a history their cultivation land exceeded 100 mil. ha (Rakouský and Hraška, 2007). The biggest part of these crops represent transgenic soybean (*Glycine max*), cotton (*Gossypium spp*.), maize (*Zea mays*) and canola (*Brasssica napus*), followed by sunflower (*Helianthus annuus*), potato (*Solanum tuberosum*), cassava (*Manihot esculenta*) and papaya (*Carica papaya*) (ISAA, 2005). Despite such increase of the cultivation area and scientific successes in the development of new transgenic plants enriched by various new traits, transgenic plants also encountered many obstacles. Surprisingly, transgenic analogues of the most important crops, i.e. wheat and rice are still scarcely grown. Monocotyledons are generally less sensitive to the genetic transformation, but since these include the most important crops for living of human population, they represent a big challenge to current science. Another obstacle, maybe more challenging than the scientific background of GMOs is a public concerns, e.g. slowing down wider acceptance of GMOs by consumers, especially in the European countries. GMOs are mostly presented by media as a strange scientific experiment, which can negatively influence human health and ecological equilibrium in the landscape. In addition, many ecological organizations and lobbyist lead various negative campaigns against GMO. Moreover, official opinions and comments of scientific community are not usually presented so frequently so in fact they cannot reach wider public society. But it is not only the public concern and discussion about the GMOs, which leads to the fact that Europe is behind the surrounding world in agricultural biotechnologies. The agriculture in the Europe, and namely in the EU countries, is build up on the different bases than the leading agriculture of overseas countries. While the agriculture in these countries is seen as a industry, with the aim to produce the biggest production/ benefit with minimal incomes, of course in the parallel keeping of all safety issues, agriculture in the European countries is seen also from the cultural and ecological aspects, keeping many traditional and national rules. All above-mentioned facts finally lead to the restrictions of cultivation of GMOs in the European countries and market release of their products, or even products, which in anyway came into contact with such GMOs. Moreover, the agricultural policy implemented within member states of recently established EU in 1962, based on a huge support of the agriculture, lead to the overproduction of many commodities and consequent current market and export obstacles, therefore the benefit of current GMOs (easier and thus cheaper production) is not attractive enough for European farmers. This is a very important fact, since the first generation of GMOs was developed with the aim to make benefits rather for the producers, farmers, than for final consumers in the shop or supermarket. Therefore there was nothing too much attractive and no benefits were seen by consumers buying GMOs. Even so, the European

attitude to GMOs has started to be changed since 2004. Development of the next generation of GMOs should be aimed precisely to the needs of consumers, giving the final consumer not only (maybe) cheaper food, but also more healthy, because e.g., of its favorable spectrum of amino acids and fats and growing without application of pesticides.

1. 3. Genetic transformation of plants and development of GMOs

Successful genetic transformation and obtaining of transgenic plants represents a very complicated process, including many particular processes and requires knowledge or insight into many biological disciplines like e.g., plant physiology and genetics, molecular biology, microbiology and in vitro techniques. Thus the development or transgenic plants needs to fulfill some basic conditions:

- Existence of a suitable target genome
- Precise characterization of gene of interest and its product on a molecular level
- Reliable and efficient system for in vitro cultivation and regeneration of desired organism (acceptor genome), i. e. plant
- The possibility to modify desirable gene by techniques of molecular biology with the aim to increase its efficiency and regulate its expression
- System for identification and selection of transformed cells or tissues
- Characterization of putatively transformed and transformed plants and tracking of the expression on newly inserted gene by molecular methods

Once all these conditions are fulfilled, development and production of transgenic plants, carrying and expressing various desirable genes, is relatively easy and “routine” (Sharma et al., 2000).

Since the first successful genetic transformation of plant, reported in the year 1977 by Chilton et al., who utilized the natural capability of a common soil bacteria *Agrobacteruim tumefaciens* to insert part of its DNA into the genome of higher plants, many other strategies were developed for the genetic transformation of plants till today. Nevertheless, generally two of them are widely used nowadays. The first one, utilizing *A. tumefaciens* as a vector for delivery of desired DNA into the plant genome, also nick named “co-cultivation” and the second one, sometimes also so called “biolistic™” or particle bombardment, is utilizing direct insertion of DNA, coated on metal particles and shot into the plant tissue using various air devices (Klein et al., 1987).

1. 3. 1. Agrobacterium tumefaciens mediated transfer is relatively easy way for delivery of foreign genes into plant genome

Soil bacteria *A. tumefaciens* are capable of inducing plant tumours throughout the integration of a specific part (T-DNA) of its plasmid (Ti-tumour inducing) DNA into the plant genome. Transfer of T-DNA in host genome is a very complex process comprising many biological events and based on cooperation of proteins coded by Ti and chromosomal genes of bacteria (Tinland 1986, Zupan and Zambryski, 1997), affected by various environmental conditions, e.g., temperature (Fullner and Nester, 1986, Dillen et al., 1997). Such genetic modification of plants is relatively easy and cheaper compared to direct transformation mentioned below since it does not require the use of expensive instruments and supplements such as an air-flow gun and metal particles to be coated by plasmid DNA.

1. 3. 2. Particle bombardment is an effective alternative for Agrobacterium- mediated transfer

Although Agrobacterium-mediated transformation is a very efficient way to deliver the endogenous DNA into genomes of many species, there are still some species that are difficult to transform using this approach. The host-range of *Agrobacterium* and the low-regeneration properties of some species, particularly monocots, excludes the general use of this method. An efficient alternative to the method represents the delivery of DNA coated on metal particles via particle bombardment. This was first successfuly applied in transformation of onions and published more than 15 years ago (Klein et al., 1987). Since that time, particle bombardment has become a very efficient tool for transformation of plants where the Agrobacterium- mediated method is less efficient (Finner et al., 1999).

Beside these two main methods, also various other approaches for genetic transformation of plants were reported, like e.g. microinjections of DNA into tissues or cell nuclei, but these were not successful and effective enough to be widely used for the purposes of transformation experiments (Ondřej and Drobník, 2002). *A. tumefaciens* delivery and particle bombardment are nowadays the main methods, which are mostly employed for the plant transformations are being developed and improved. Not all plant species, or better to say particular varieties or genotypes, exhibit the same response to the genetic transformations

using either of these methods. Monocotyledons are generally less susceptible to the *A. tumefaciens* infection than dicotyledons (Birch 1997). This is because naturally *A. tumefaciens* parasite on the roots of dicots. Therefore the monocots are usually transformed by the particle bombardment method, although successful transformations with *A. tumefaciens* were also reported (Repellin et al., 2001). It is not a big surprise that these two so different methods differ also in the transformation efficiency. The *A. tumefaciens* delivery, as a let us say natural method, results in more favorable insertions of delivered DNA, characterized by insertions of one or very low number of inserted DNA, with a low number of subsequent re-arrangements of genetical material. On the other hand, *A. tumefaciens* is capable to transform only some plant species because of some physiological or evolutional barriers and thus many species are recalcitrant to *A. tumefaciens* treatment. This in not an obstacle in the case of particle bombardment method, which is not limited by such evolutional barriers and generally all plant species could be transformed using this approach (Christou 1995, Christou 1997). Nevertheless, the weak points are very low efficiency and insertions of higher number of inserted DNA copies, many times resulting in various re-arrangements in target genome (Snape, 1998). On the other hand low efficiency can be easily circumvented by bombardment of higher amount of target tissue, since once established, such method is relatively easy to use and repeat.

1. 4. Efficient selection of transformed cells/ tissues is a bottleneck of transformation procedure

Methods of genetic transformation of plants are subjected for continuous development and improvement with the aim to increase the range of plant species capable for genetic modifications and increase of their efficiency. Co-cultivation methods are being improved throughout the genetic engineering of new, more virulent bacterial strains, various pretreatment of transformed plant tissues like e.g., sonication, variable length of co-cultivation periods and improvement of subsequent in vitro regeneration/ cultivation and a very important step, the selection of transformed cells or tissues. Regardless which transformation approach is used for particular experiment, only a few cells are transformed by foreign DNA and from this very little number of cells, only few survive this change and are viable and capable of division. Absolute majority of plants cells are not transformed. Thus, there is a big challenge, how ensure preferential growth and division of transformed cell and ideally eliminate the untransformed ones. This is possible only by proper selection.

This can be performed either by negative influence of untransformed cells or by promotion or preferential nutrition/ growth of transformed cell. Irrespective of which approach is chosen, it requires the insertion beside the gene of interest, coding for desirable trait, also some other gene coding for some trait, which can be efficiently employed for either type of selection. The most common is insertion of a gene, coding for enzyme for metabolization of some toxic compound, thus transformed cell are viable on the nutrition medium containing such a toxin, whereas untransformed cell do not posses such enzyme and thus will die or at least their growth and development is suppressed. Such toxin is usually some antibiotic, mostly kanamycin, which is easy to use for the purposes of in vitro techniques. Although these systems are labor-efficient and applicable to a vide range of plant species/ genotypes, they can decrease the number of recovered cells (Stewart, 2001). Furthermore there is a considerable public concern that new transgenes will spread into the environment resulting in wild or feral species containing antibiotic resistance traits, which might disrupt equilibrium of current ecosystems (Custers, 2001), leading to strong public pressure with the aim to exclude these selection systems from the production of transgenic plants. However alternative procedures that are applicable to a wide range of plant species, are not available yet. Nevertheless, recently, the GMO Panel of the European Food Safety Authority (EFSA) issued its opinion regarding the safety and potential dangers of plant selectable antibiotic resistance markers, which resulted in their classification into low-, medium- and high potential risk categories (EFSA, 2004). Based on this classification, antibiotic resistance markers, mostly used in plant biotechnology are mentioned in the first category, i.e. with a low potential risk. In addition, use of toxic compound to eliminate non- transformed cells and tissues from further growth and development, leads to a rapid death and subsequent decay. Such dyeing cells or decaying tissues usually release various toxins, inhibitors and undesirable apoptosis signaling compounds, which can result in possible detrimental effects on the growth and development of regenerating transformed cells (Ebinuma et al., 2001).

Nowadays, novel selection approaches are being developed. These are usually based on the insertion of DNA coding for an enzyme, which allows for metabolisation or modification of some sugar compounds, included into the cultivation medium. These are in a normal way metabolized to some toxic compounds, but with a new added enzyme are metabolized into non-toxic nutrients. Such approach to the selection of transformed cell has been reported, mostly utilizing culture growth on otherwise metabolised mannose sugar (Joersbo et al., 1998, Joersbo 2001). Although many reports about using this method have been published, it has still not become applied to a wider range of plant genotypes for a

routine use in plant transformation. The main limiting factor is the need to determine the optimum level of sugar for each particular genotype in order to determine toxic level of such sugar and subsequently develop a selection system. Other limitations appear to be associated with a taxon/ genotype because, although some transformations of dicots have been reported, mainly transgenic monocots such a maize, wheat, rice and barley have been recovered using this approach (Wright et al., 2001).

Other approaches to the selection of transformed cells and tissues could be simply based on the visual identification and mechanical removal of desired cells. Such are transferred onto a new medium and subsequently cultivated (Ghorbel et al., 1999, Hraška et al., 2006a). Finally, although a wide range of various selections systems have been developed, and are successfully employed in current research and commercial practice, it is evident that the development or use of some general, widely applicable approach is desirable and still subject of intensive research.

1. 5. Development of transformation protocol is a complex issue and the use of visual markers could be a big benefit

It is evident that successful insertion of a new DNA into the plant genome is a very long and complicated process requiring development and subsequent establishment of reliable and reproducible transformation protocol. Such a protocol should lead to the development of most efficient strategy for insertion of genes onto the plant cells (Chilton et al, 1977, Klein et al., 1987), selection and regeneration of putative transgenic cells (Mikki and McHugh, 2004) and subsequent recovery of transgenic plants. For this purposes, generally model or marker genes, which allow for critical appraisal of each particular step within the whole transformation procedure represent a very efficient tool. Therefore, parameters or conditions like e. g., *A. tumefaciens* concentration, length of co-cultivation period, set up of particle bombardment device and subsequent selection and regeneration could be measured and assessed based on the phenotypic performance of such marker (Baranski et al., 2006). Such critical assessment leads to the establishment of the transformations strategies of new plants species or to the improvement of already existing procedures. This results in the more effective, reliable, reproducible and many times also cheaper procedures for development and production of GMOs, by simultaneous reduction of the material, which need to be handled and screened (Hraška et al, 2006a).

Many such markers or marker genes were identified and utilized in the plant research till today. Nevertheless, only two or three of them are widely used in the plant molecular biology or genetic transformation. Genes coding for β-glucuronidase (GUS) (Jefferson et al., 1987), luciferase (LUC) (Ow et al., 1986) and green fluorescent protein (GFP) (Prasher et al., 1992) have become popular tools for monitoring of gene expression in transgenic plants, allowing the study of plant physiology, genetics, molecular and cell biology, plant pathology and many other branches of biology (Hraška et al., 2006a). These markers possess different features and limitations of use, making one particular marker more suitable for some particular purposes than the other one. Nevertheless, within the above mentioned threesome, GFP possess a few unique features, giving the possibility to use this marker not only for the basic research or genetic transformation of plants, but also as marker for quantification or quick estimation of recombinant protein content within transgenic plant (Milwood et al., 2003, Hraška et al., 2005, Hraška et al., 2007) and ecological studies of transgene spread throughout the environment (Halfhill at al., 2003). This unique feature is that GFP does not require any substrate or co-factors for its performance, i.e. green fluorescence (Heim et al., 1994, Misteli and Spector, 1997) and thus allowing for real-time monitoring of its expression in the living organisms, directly in the laboratory, or even in the glasshouse or on the field. This is a strong benefit, compared to very often used GUS marker system, which, otherwise allows for an easy quantification, is based on the destructive assay. So, when summarized, GFP allows for the use as an efficient tool for critical assessment of transformation procedures, study of physiology and genetics, and estimation of recombinant protein content in transgenic plants. All this in real time, on living tissues or at least using small tissue samples.

1. 6. Green fluorescent protein (GFP)

GFP, a nature protein of pacific jellyfish *Aequorea victoria* has been first described in 1992 by Prasher et al. (1992). *A. victoria* contains, among many other proteins, also the aequorin, which emits blue chemiluminiscent light after activation of Ca^{2+} ions. This blue light serves as an excitation signal for the GFP, which result to the green fluorescence. GFP itself is a dimmer of two barrels with the diameter of 30Å and length 40 Å, comprising consisting from eleven β sheets, capped on the top and bottom by α helices. Relative molecular weight is 27 kDa (Yang et al., 1996). The agent responsible for the green fluorescence, chromophore, is enclosed in the geometric centre of the protein, to which is

covalently attached (Shinomura, 1979, Cody et al., 1993). This is formed by posttranslation modification in which is tripeptide Ser65-Tyr66-Gly67 cyclized, followed by later oxidation. Such "wild type" GFP exhibits upon excitation by UV (λ= 360-400 nm) or blue light (λ=440-480) green fluorescence. GFP does not require any additional substrates, cofactors or any other compounds for fluorescence manifestation, since the chromophore formation is either autocatalytic process or requires only ubiquitous cellular components (Heim et al., 1994). GFP possess a very rigid structure with a broad stability range within pH 5-11 and at the temperatures up to 65 °C (Tsien 1998). Moreover, it maintains its fluorescence even in the presence of strong denaturing agents, such as 6 M guanidine HCL, 8 M urea or 1% sodium dodecyl sulfate (Yang et al., 1996).

Due to its favorable properties, GFP rapidly become a very popular tool in many applications within biological research. Till today, it has been introduced into wide spectrum of organisms, including simple bacteria and yeasts, nematodes, insect, fish, plants and mammals (reviewed by Hraška et al., 2006a). The first successful introduction of GFP into the plant was reported in 1995 by Niedz et al. (1995), who transformed the protoplasts of sweet orange (*Citrus sinensis*). This opened new ways in the genetic transformation of plants and transgenic plants of many plants species followed. Nevertheless, very early, scientists encountered complications with the very low expression level and quenching of fluorescence signal in plant cells. Detailed sequence analyses performed by Haseloff et al. (1997) revealed the presence of cryptic intron within the sequence of *gfp* gene. Its presence resulted in abberant splicing between nucleotides 380-463 during the RNA processing and resulted in the loss of the 84 bp region. Therefore a new GFP variant, derived by the altered codon usage, was released. Such a new variant, denoted GFP4, maintains the same spectral characteristics as a "wild type" GFP, but with enhanced fluorescence properties (Haseloff et al., 1997). Subsequently many GFP variants, differing in their spectral characteristics, fluorescence intensity or cell target, were derived (reviewed by Stewart, 2001, Hraška et al., 2006a). GFP has been successfully used for many purposes within plant research, like e. g. the study of expression profiles of promoters (Sheen et al., 1995, Nagatani et al., 1997, Sunilkumar et al., 2002), protein tagging (Chytilova et al., 1999, Shiina et al., 2000) disease tracking (Itaya et al., 1997) and developmental studies (Misteli and Spector, 1997).

Although some concerns about possible toxicity of GFP to plants were raised, these have not been confirmed (Stewart et al., 2001) and GFP did not possess any toxic or detrimental effect on plant growth, development and fertility (reviewed by Hraška et al., 2006a). Moreover GFP has been found non- toxic to rats when these were fed by purified

GFP or by transgenic plants expressing the *gfp* (Richards et al., 2003). Nowadays, the GFP is being mostly employed for various purposes associated with the transformation of plants (reviewed by Stewart, 2001, Hraška et al., 2006a). Anyhow, due to its favorable properties- i. e. monitoring of its expression (fluorescence) in living samples, GFP has been also used for many ecological studies aimed at monitoring of transgene spread throughout the environment (Hudson et al., 2001, Moon et al., 2006) study of competitiveness of GMOs compared to conventional crops (Halfhill et al., 2005) and study of possible transgene transfer from cultivated crop onto its wild relatives (Halfhill et al., 2001). Such studies take very important role in the whole process of the development and mainly placement (release) of GMOs into the commercial practice. Using some excitation source, like e. g. UV hand held lamp, it is relatively easy to identify the GFP tagged GMOs directly on the field or to check the spread of GFP tagged pollen from the field with some GMOs. Besides these applications employing the GFP, which are based on the visual detection of the presence of GFP fluorescence in the studied sample, tissue or whatever, GFP also allows for quantification of its fluorescence in intact plants (Millwood et al., 2003, Hraška et al., 2005) and an estimation of recombinant protein within transgenic plant (Halfhill et al., 2003, Richards et al., 2003).

All applications listed above make the GFP an ideal tool for an assessment of various steps within the procedure of genetics transformation, resulting in the establishment new, or improvement of already existing transformation protocols. Moreover, it allows for the selection of most successful transformation events with the aim to finally obtain elite genotypes, with desired performance of inserted transgene.

1. 6. 1. Although the GFP possess many beneficial features, it also possess a few weak points

Nevertheless GFP is not an ideal marker without any obstacles. Although the use GFP possess many advantages compared to other common marker genes used in plant molecular biology, monitoring of its expression is coupled also with a few weak points. For a monitoring system to be effective, reliable and reproducible, the marker technology should be accurate, without false positives, detectable throughout the whole life cycle of a particular plant, and offer the check of the actual status of genetically linked, i.e. fused, transgene/s of interest (Halfhill et al., 2003).

First, the loss or quenching of the fluorescence intensity in the older tissue, namely leaves, usually occurs (Kamaté et al., 2000, Tamura et al., 2003, Zhou et al., 2004). In addition, the differences in the intensity of GFP fluorescence based on the origin/ position of

studied sample within the whole flower were reported (Halfhill et al 2003, Zhou et al., 2005). Such events were, nevertheless, reported also in the case of GUS marker (Preťová et al., 2001). Various aspects could be found as a cause of such phenomena, but it seems that the presence of some masking or opaque compound in leaf tissue, which could interfere with emitted fluorescence and/ or excitation signal, is most probably the main reason (Hraška et al., 2006a, Hraška et al., 2007). While the content of such compound is changing based on the developmental or physiological stage of particular tissue, also the affected GFP fluorescence is changing. The chlorophyll is mostly mentioned with this fact and it has been reported many times, that different chlorophyll content among different tissues could negatively interfere with the GFP fluorescence (Ponappa et al., 1999, Cho et al., 2002). Moreover, Zhou et al. (2005) reported the reconstruction of once diminished fluorescence in the Medicago leaves after chlorophyll removal.

Next, different levels of detectable green fluorescence were recorded from the different tissue and fluorescence levels tends to higher variability in the older tissues (Halfhill et al., 2001). Also the fluctuations of the fluorescence levels even with one leaf were reported (Hraška et al., 2005). Nevertheless, such phenomena was also reported for other types of marker genes, like e.g. GUS (Preťová et al., 2001) and is probably coupled with different cytoplasmatic density in young and old cell, leading to the “dilution” of GFP within the content of older cells (reviewed by Hraška et al., 2006a), or also different ratio/ level of proteosynthesis and resulting amount of recombinant protein among particular tissues could be another reason (Preťová et al., 2001).

Finally, the cause for differential GFP fluorescence based on the tissue type, location or developmental stage, could be the type of particular promoter, used for each particular experiment. Constitutive promoters such as CaMV 35S of Act1 are mainly used in transformation experiments, and although their constitutive features in transgenic plants were reported (Behfey et al., 1989, Battraw and Hall, 1990), some recent studies revealed some differences in their expression and some developmental and tissue specific features of such promoters (Williamson et al., 1989, Sunilkumar et al., 2002).

When listing the weak points of use of GFP for the transformation and other purposes of plant research, it is worth to mention here also the obstacles associated with the microscopic study of GFP fluorescence in some flower parts or tissues, which can exhibit a certain levels of auto-fluorescence. Such tissues cannot be reliably investigated simply since they do not allow for discrimination of transgenic, *gfp* expressing tissue from the non transgenic or transgenic with low or zero *gfp* expression (Hraška and Rakouský, 2005).

2. Aims of presented Ph.D. thesis

The aims of presented Ph.D. thesis result from the long-term orientation of the laboratory where I realized my work. Our laboratory concentrates its effort on the research and development of new transgenic plants with promoted resistance to locally important fungi and pests. We decided to reach this target by using the insertion of novel genes, coding for various potentially antifungal proteins or proteins with insecticidal properties, derived from insect. Such work is covered by a project across few scientific institutions within the Czech Republic. First such gene coding for serine proteinase inhibitor (PI), was derived from the waxmoth *Galleria mellonella* (Nirmala et al., 2001) and was subsequently modified for its utility for the purposes of genetic transformation of plants. PIs are very promising class of proteins with various antifungal and insecticidal properties and many successful transformation, resulted in the transgenic plant with increased resistance were reported (reviewed by, Schuler et al., 1998, Hraška et al., 2006b). Nevertheless, the use of such new type of genes requires the development and establishment of proper transformation strategy and the approaches for reliable assessment of obtained transgenic plants, namely identification and quantification of recombinant protein content. Due to its properties, proteinase inhibitors are difficult to identify and, moreover, to quantify within the transgenic plant. The biggest obstacle represents the presence of many naturally accruing protein analogues in the plants, especially *Solanaceae*, e.g. tobacco and potato. Usual molecular approaches based on the antigen detection of desired protein were not successful. Therefore we decided to establish a different strategy for molecular characterization (identification and quantification of protein content) and assessment of obtained transgenic plants, based on the fusion of genes coding for PI with even GFP.

The utility of GFP in the genetic transformation of plants and further study and characterization of above mentioned weak points or, let say obstacles, in the use of GFP were the impulses for my study. My work was aimed at the utility of GFP as marker for not only the monitoring of transformation events, but also as an efficient tool for the selection of elite transgenic individuals and possible estimation of a recombinant protein content within selected, desirable individuals. Since the use and benefits of GFP in the assessment of various steps within transformation process were described many times previously, I concentrated my effort on the use of quantification of GFP fluorescence as a tool for indirect estimation of recombinant protein content within transgenic plants, and for the assessment and selection of regenerating and mature transgenic plants. As mentioned above, the intensity of GFP

fluorescence varies among various types of cells and tissues and it seems to be necessary to study and to describe such variability, with the aim to use such approach as a routine tool for assessment of obtained transgenic individuals and to obtain reliable and reproducible data. Since the most common model species in the plant transgenesis is still tobacco (*Nicotiana tabacum*), I decided to study, capture and map the fluorescence patterns within the transgenic tobacco plants, expressing the m*gfp*5-ER marker gene. Since the long-term target of our laboratory is to develop transgenic plants with increased resistance against some important fungi and pests via the insertion of chimaeric GFP constructs fused to genes coding for proteinase inhibitors, the knowledge and definition of GFP fluorescence could ease whole transformation procedure and selection of elite transgenic individuals expressing desirable properties. For this purposes, I decided to use in our laboratory available fluorescent microscope, a collection of images captured by digital color camera or digital CCD camera and to perform the assessment of such images/data by the image analyses software.

More detailed, aims of my Ph. D. thesis were following:

1. Discover, whether it is even possible to perform the assessment and/or selection of regenerating GFP transgenic plants based on a comparison of exhibited fluorescence intensity and to perform relative quantification of GFP fluorescence using digital captures of studied leaf tissues and processing of obtained data with image analyses software.
2. Detailed study and definition of variability of GFP fluorescence patterns within the various leaf tissues, based on the age, developmental stage and vertical position of such tissue within the mature GFP transgenic tobacco plants and further development of previously established methodology and promotion of objectivity of GFP fluorescence evaluations. Beside this, I also tried to study the role of some possible physiological aspects, namely the content of chlorophyll, on the spatial changes in the intensity of GFP fluorescence.
3. Finally, the obtained GFP transgenic tobacco plants gave me a unique opportunity to perform detailed macroscopic studies of the expression profile of the most common promoter, CaMV 35S. Since I have obtained transgenic plants of T_1 and T_2 generations, it gave me the chance to study the stability and uniformity of CaMV 35S performance in particular plant tissues and organs among two different generations of plants. Moreover, although such promoter was exhaustively investigated many times previously, very few information regarding the CaMV 35S performance within floral and generative organs are available. Therefore, I aimed my effort at this stage at

detailed study of CaMV 35S expression profile within two distinct generations of GFP transgenic tobacco plants, with a special emphasis on floral organs.

4. In a view of possible future outputs into agricultural practice, some parts of my work were performed also with flax (*Linum ussitatisimum* L.). Regarding this topic, I tried to utilize the GFP marker as a co-selection tool during the regeneration of putatively transformed flax buds and shoots, with the aim to promote the transformation efficiency of flax, especially in the further experiments regarding the increasing of its resistance to important fungal diseases.

4. Results

4. 1. Protease inhibitors, mode of action and perspectives for plant transgenosis

Hraška, M., Rakouský, S., Čurn, V. (2006) Inhibitory proteas, mechanismy účinků a perspektivy jejich využití v transgenosi rostlin [Protease inhibitors, mode of action and perspectives for plant transgenosis] Chemické listy 100, 501-507

INHIBITORY PROTEAS, MECHANISMY ÚČINKU A PERSPEKTIVY JEJICH VYUŽITÍ V TRANSGENOZI ROSTLIN

MAREK HRAŠKA[a,b], SLAVOMÍR RAKOUSKÝ[a,c] a VLADISLAV ČURN[b]

[a]*Katedra genetiky, Biologická fakulta,* [b]*Biotechnologické centrum, Zemědělské fakulta a* [c]*Zdravotně sociální fakulta, Jihočeská univerzita v Českých Budějovicích, Branišovská 31, 370 05 České Budějovice*
mhraska@seznam.cz

Došlo 11.7.05, přijato 9.12.05.

Klíčová slova: inhibitory proteas, ochrana rostlin, transgenní rostliny

Obsah

1. Úvod
2. Integrovaná ochrana rostlin
3. Transgenní rostliny odolné vůči biotickým stresům
4. Inhibitory proteas
   4.1.1. Inhibitory serinových proteas
   4.1.2. Inhibitory cysteinových proteas
   4.1.3. Inhibitory aspartátových proteas a metaloproteas
   4.2. Mechanismus toxického působení na hmyz
   4.3 Regulace inhibitorů proteas
   4.4. Transgenní rostliny exprimující inhibitory proteas
5. Závěr

1. Úvod

Celosvětová populace se stále rozrůstá, na přelomu tisíciletí dosáhla hranice 6 mld obyvatel a odhady pro rok 2025 mluví již o 8,5 mld. Tento trend klade také stále vyšší nároky na zemědělství, a to nejen co se týče objemu produkce, ale také její kvality. Zemědělství však může vycházet pouze z omezených zdrojů. Podle údajů Světové banky činila průměrná plocha obdělávané zemědělské půdy v roce 1961 0,44 ha na obyvatele planety, v roce 2002 klesla na 0,26 ha a výhled pro rok 2050 hovoří o pouhých 0,15 ha (cit.[1]). Zemědělství a šlechtění tedy stojí před nelehkým úkolem nasytit neustále rostoucí množství lidí ze stále se zmenšujících zdrojů.

Klasické šlechtění sice zaznamenalo během uplynulých desetiletí řady pokroků, přesto se již v současnosti přiblížilo hranici svých možností, neboť u řady plodin bylo dosaženo hranice biologického výnosu. Naopak nové poznatky genetiky a molekulární biologie nabízejí zcela nové možnosti v tvorbě nových, tzv. geneticky upravených nebo-li transgenních odrůd zemědělských plodin. Přímé šlechtění na výnos je kvůli charakteru komplikovaného genetického založení tohoto znaku v současnosti stále ještě obtížné, ale existuje celá řada možností, jak ovlivňovat znaky a vlastnosti mající na konečný výnos a kvalitu rostlinné produkce významný vliv. Jedná se např. o kvalitativní změny spektra aminokyselin a bílkovin, vlastnosti umožňující jednodušší a levnější pěstování, zvyšování odolnosti rostlin vůči suchu, mrazu či zasolení půd. Významným činitelem je odolnost rostlin vůči biotickým stresům. Ztráty a poškození způsobené různými škůdci, houbovými, bakteriálními a virovými chorobami silně zatěžují ekonomiku rostlinné výroby[1,2]. Ochrana rostlin má kromě přímého vlivu na výši produkce také vliv na rentabilitu pěstování a zdravotní nezávadnost potravin či krmiv.

2. Integrovaná ochrana rostlin

Integrovaná ochrana rostlin představuje moderní trend v boji proti chorobám a škůdcům, jejíž součástí je i tvorba rezistentních odrůd. Možnosti klasického šlechtění jsou však značně omezené, zejména v případech, kdy je rezistence založena kvantitativně, geny rezistence jsou lokalizovány na více lokusech a často ještě nejsou identifikovány. Šlechtitelský pokrok je proto velice zdlouhavý a neodpovídá poměrně rychle se měnícím potřebám pěstitele. Velmi perspektivní alternativu v podobě možnosti záměrného vnesení genů kvalitativního charakteru představují techniky rekombinantní DNA − transgenoze. Z širokého okruhu rostlinných škůdců je hlavním předmětem zájmu hmyz, neboť se jedná o nejpočetnější skupinu škůdců v zemědělství. Transgenoze umožňuje již nyní poměrně vysoce specificky vymezit okruh cílových skupin hmyzu, na něž technologie (genový produkt) přednostně působí, zatímco jiné zůstávají prakticky nedotčeny. Názorným příkladem jsou rostliny s vnesenými geny pro δ (delta)-endotoxin *Bacillus thuringiensis*, které již dosáhly značného rozšíření v zemědělské praxi (např. tzv. *Bt*-kukuřice, *Bt*-bavlník). Navíc využitím vhodných promotorů, vymezujících místo a dobu projevu vloženého genu v rostlině jakož i míru jeho exprese, je potenciální rizikovost modifikovaných plodin dále výrazně snížena. K výčtu pozitiv dané technologie je třeba uvést i snížení zátěže životního prostředí v důsledku omezení či úplné absence postřiků insekticidními přípravky. Hmyz je často také přímým vektorem dalších onemocnění, či jim svým působením otevírá a ulehčuje cestu do rostlinného těla[3]. Nepřímo je tak ovlivněna kvalita rostlinné produkce a potravin z ní vyrobených. Např. v případě *Bt*-kukuřice bylo již nezvratně prokázáno, že její zrna obsahují až o 60 % méně kancerogenních mykotoxinů než běžná kukuřice, u které jsou hmyzem

poškozená zrna napadána houbami[4].

Během evoluce se u rostlin vyvinula řada obranných mechanismů vůči škodlivým organismům. Většina jich je soustředěna do semen a jsou aktivovány konstitutivně nebo indukovaně po napadení či onemocnění. Jedná se převážně o látky bílkovinné povahy. Nejznámější rostlinám vlastní obranné proteiny jsou lektiny, ribosomy inaktivující proteiny, inhibitory proteolytických enzymů, glykosidasy, chitinasy či arcelininy[1]. Rostliny jsou v neustálém kontaktu s okolím a jejich vztahy se škodlivými činiteli se neustále vyvíjejí a mění. Obranné bariéry rostlin jsou časem překonány a je třeba vyvinout si nový způsob ochrany, který je opět posléze překonán.

3. Transgenní rostliny odolné vůči biotickým stresům

Rostliny vytvořené postupy genového inženýrství, označované jako geneticky modifikované vyšší rostliny (GMVR), představují kvalitativně nový příspěvek do mozaiky integrované ochrany rostlin[5]. GMVR mohou efektivně nahradit, a výzkum a praxe potvrzují, že skutečně nahrazují, konvenčně používané agrochemikálie. Nicméně i v této oblasti vyvstávají otázky možného negativního vlivu v důsledku dané genetické modifikace. Konkrétně se jedná o možnost zrychlení selekce rezistentních populací škůdců či ras patogenních hub[6]. K výraznému omezení těchto rizik u hmyzu byly již vyvinuty a do praxe zavedeny účinné strategie využívající tzv. refugií, kdy na polích vedle odolných GMVR plodin musí být současně pěstován i určitý podíl běžných (citlivých) odrůd, které udržují dostatečnou zásobu citlivých forem hmyzu. Ty se pak kříží s ojediněle se vyskytujícími odolnými jedinci z „transgenních“ polí. Jejich potomstvo je obvykle opět náchylné a tak je oddálen nástup rezistentních forem hmyzu[6].

Ochrana plodin vůči biotickým stresům je jedním z hlavních objektů zájmu transgenoze rostlin a v současnosti představuje velmi intenzivně řešenou problematiku. Získat či vytvořit transgenní plodinu však vyžaduje splnění několika základních požadavků jako jsou:

- existence vhodného cílového genomu,
- dostatečná charakterizace kandidátského genu a existence vektoru pro jeho vnesení,
- existence postupů pro kultivace explantátových kultur daného objektu a účinný regenerační systém,
- možnost modifikovat cizí gen a zvyšovat či usměrňovat tím jeho expresi,
- identifikace a selekce transformovaných (transgenních) buněk,
- charakterizace potenciálně transformovaných rostlin na molekulární úrovni.

Pokud jsou tyto požadavky jednou splněny, stává se produkce transgenních plodin nesoucích rozličné nové geny takřka rutinní záležitostí. V minulosti byly vypracovány postupy pro transformaci řady modelových i kulturních rostlin[3]. Zavedení transgenní odrůdy do praxe předchází splnění celé řady dalších náročných kritérií[7].

Do dnešního dne bylo vyvinuto mnoho postupů, jak vpravit cizorodou DNA do rostlinného genomu. Zdaleka nejrozšířenější jsou však dva okruhy postupů, a to přímá transformace nukleovou kyselinou a transgenoze pomocí bakterií *Agrobacterium tumefaciens*. V prvním případě je plasmidová DNA nesoucí požadované geny nanesena na částečky inertního kovu (nejčastěji zlato či wolfram) a pomocí různých vysokotlakých zařízení je „vstřelována“ do cílové tkáně či shluku buněk[8]. Druhý způsob spočívá ve využití přirozeného jevu, kdy bakterie *A. tumefaciens* je sama o sobě schopna vnést část své DNA (tzv. T-DNA – transferred DNA) nesené na plasmidu Ti (tumor inducing) do rostlinného genomu[9] . Metodami molekulární biologie je možno poměrně snadno tuto T-DNA upravovat a vnášet tak do rostliny různé geny.

První transgenní rostliny tabáku byly získány v roce 1984 a od té doby byly získány transgenní rostliny od více než 100 dalších druhů. Z nich nejúspěšnější se dočkaly uvedení do pěstitelské praxe. Pro představu o rozvoji pěstebních ploch GMVR odrůd, první transgenní plodiny začaly být velkoplošně pěstovány v r. 1996, v roce 2000 plocha pěstovaných GMVR plodin dosahovala 44,2 mil ha (cit.[4]) a v r. 2004 již 66,7 mil ha. Odhady pro letošní rok předpokládají až 88 mil ha (zdroj ISAAA 2004, cit.[7]). Co se týče plodin odolných proti hmyzu, největšího rozšíření se dočkaly rostliny exprimující gen pro δ-endotoxin z bakterie *B. thuringiensis*. Osevní plochy těchto *Bt*-plodin celosvětově neustále rostou, nicméně výzkum rezistence neustrnul pouze na tomto jednom úspěšném typu a pokračuje i jinými směry. Mnoho dalších genových produktů ovlivňuje výživu a fyziologii škůdců a jsou tedy potenciálně využitelné pro transgenozi rostlin. Předmětem zájmu jsou jak rostlinám vlastní látky (inhibitory proteas, chitinasy, různé sekundární metabolity či lektiny), tak i látky bakteriálního původu či odvozené od genové výbavy vyšších živočichů. Právě inhibitory proteas různého původu jsou intenzivně zkoumanou skupinou potenciálně insekticidních či antifungálních látek[1].

4. Inhibitory proteas

Proteolytické enzymy katalyzují štěpení molekuly bílkoviny na menší řetězce a posléze až na jednotlivé aminokyseliny. Rozlišují se čtyři hlavní skupiny proteas: serinové proteasy obsahující v aktivním centru aminokyselinu serin, cysteinové obsahující cystein, aspartátové se začleněným zbytkem kyselina asparágové a metaloproteasy obsahující ve svém aktivním centru kovové ionty Zn^{2+}, Ca^{2+} či Mn^{2+} (cit.[1]).

Proteolýza je klíčový proces všech živých organismů, a proto musí být přesně regulována. Nepřekvapí tedy existence přirozeně se vyskytujících inhibitorů proteas (PI) různého původu, které se klasifikují podle cílových enzymů, které inhibují.

V rostlinách zastávají PI různé funkce, např. v zásob-

ních orgánech či při regulaci proteolytické aktivity. Podílejí se také na regulaci mnoha vývojových procesů včetně programované buněčné smrti a v neposlední řadě tvoří významnou složku obranných mechanismů rostlin vůči hmyzu a patogenům. V rostlinách se často vyskytují v překvapivě vysokých koncentracích[10]. Syntetizovány jsou buď konstitutivně nebo jako odpověď na právě vzniklé poškození či probíhající napadení[1].

První náznaky poukazující na možnou roli PI v obraně rostlin se objevily již v roce 1947. Mickel a Standish[11] pozorovali, že pokud jsou larvy rozličného hmyzu udržovány na extraktech sóji, tak ztrácejí schopnost normálního vývoje. Posléze byl prokázán toxický efekt inhibitoru trypsinu sóji na larvy brouka *Tribolium confusum.* Poté byla identifikována celá řada podobných látek, jejichž insekticidní účinky byly prokázány jak v *in vitro* testech na střevních enzymech hmyzu, tak i *in vivo* na živém hmyzu[2].

Z metodického hlediska skýtají geny kódující PI jednu významnou výhodu. Lze je poměrně jednoduše přenášet z jednoho rostlinného (či živočišného) druhu do druhého a docílit jejich exprese v nové rostlině za použití jejích již stávajících regulačních mechanismů, či pod kontrolou souběžně vnesených promotorů. Tímto způsobem Hilder a spol.[12] v roce 1987 transformovali tabák genem pro inhibitor trypsinu z bobovité rostliny vigny a navodili tak jeho zvýšenou odolnost vůči širokému spektru hmyzích škůdců. Doposud nebyl potvrzen negativní účinek nového genového produktu na vyšší organismy. Někteří autoři se dokonce domnívají, že vzhledem ke svému charakteru (jsou bohaté na lysin a cystein) mohou PI zlepšovat nutriční hodnotu rostlin[13].

PI vykazují velmi široké spektrum inhibiční aktivity vůči mnoha organismům, jako např. háďátkům, jsou rovněž schopny potlačit zrání spor a růst mycelia některých houbových patogenů. Všechny tyto vlastnosti představují PI jako vhodnou skupinu látek využitelnou pro tvorbu transgenních odrůd polních plodin. Navíc transformace rostlin geny pro PI není zajímavá pouze z pohledu produkce odolných rostlin jako takových, ale i z pohledu využití rostlin jako „továren“ vyrábějících inhibiční proteiny využitelné i pro jiné účely v ostatních oblastech lidské činnosti[2].

4.1.1. Inhibitory serinových proteas

Role PI serinových proteas jako defenzivní složky ochrany rostlin je již poměrně dlouho známa. Serinové proteasy nejsou ve větším množství využívány v procesech primárního metabolismu a tudíž přítomnost velkého množství inhibitorů právě těchto enzymů vylučuje jejich jakoukoli roli v regulaci vnitřních pochodů rostlin. Inhibitory serinových proteas byly popsány v mnoha rostlinných druzích a rostlinné PI vykazují určitou podobnost. Nejvíce prozkoumanou skupinou jsou inhibitory trypsinu. Poměrně snadná dostupnost trypsinu a snadné měření jeho katalytické aktivity vedly k tomu, že PI serinových proteas se staly předmětem mnohem intenzivnějšího zkoumání než další zástupci PI, nicméně získané poznatky jsou úspěšně aplikovatelné nejen na celou skupinu PI serinových proteas, ale i na ostatní třídy PI. Všechny inhibitory výše zmíněné skupiny jsou kompetitivní inhibitory.

Serinové proteasy byly nalezeny v zažívacím traktu mnohých zástupců hmyzu, zejména řádu motýlů *Lepidoptera*, který zahrnuje celou řadu významných škůdců rostlin. Mnoho těchto trávicích proteolytických enzymů je ovlivňováno právě PI serinových proteas, jejichž optimální pH prostředí 9−11 koresponduje s obvyklým pH střevního traktu řady zástupců *Lepidoptera*. Antinutriční účinek byl demonstrován celou řadou pokusů[2].

4.1.2. Inhibitory cysteinových proteas

Při izolacích střevních proteas z larev hmyzu zavíječe *Callossobruchus macalatus* a mšice *Zabrotes subfaceatus* byla odhalena, mimo jiné, též přítomnost cysteinových proteas. Podobné proteasy byly izolovány také ze střev několika dalších hmyzích druhů. Všechny byly inhibovány jak syntetickými, tak i přirozeně se vyskytujícími PI a jejich příslušnost k dané třídě byla posléze potvrzena řadou testů. Optimální pH cysteinových proteas je v neutrální až mírně kyselé oblasti (pH 5−7).

Pokročilými postupy enzymologie posledních let byla identifikována celá řada inhibitorů proteas, jako např. alpin. PI cysteinových proteas byly popsány v řadě rostlinných druhů, např. u bramboru, vigny, avokáda či papáji. Nejvíce prostudovaný je PI pocházející z rýže, tzv. oryzacystatin.

4.1.3. Inhibitory aspartátových proteas a metaloproteas

Znalosti o této skupině enzymů u hmyzu jsou ve srovnání se dvěma předešlými nesrovnatelně menší. Aspartátové proteasy byly nalezeny spolu s cysteinovými u šesti zástupců skupiny ploštic *Hemiptera*. Nízké pH střevního prostředí zástupců skupin brouků *Coleoptera* a *Hemiptera* představuje mnohem vhodnější podmínky pro aspartátové proteasy než vysoké pH (8−11) střev většiny ostatních druhů. V silně zásaditém prostředí ztrácejí svou aktivitu. U rostlin byly doposud charakterizovány dvě skupiny PI metaloproteas, rodina PI metalo-karboxypeptidas z bramboru a rajčat a skupina cathepsin D PI brambor.

4.2. Mechanismus toxického působení PI na hmyz

Přesný způsob, jakým PI pracují, je stále předmětem intenzivního výzkumu. Získané znalosti o projevech a regulaci inhibitorů pocházejících z rostlin, živočichů, mikroorganismů, ale i třeba z virů, přispěly k vývoji či modifikaci celé řady postupů v medicíně či zemědělství, využívajících právě PI.

Obecně lze konstatovat, že sekrece proteolytických enzymů ve střevě hmyzu je ovlivňována spíše obsahem bílkovin v přijímané potravě, než jejím celkovým množstvím[14]. Sekrece proteas je indukována dvěma odlišnými cestami. Jedná se o přímé působení složek potravy (hlavně bílkovin) na epiteliální buňky střeva hmyzu nebo o hormonální regulaci iniciovanou příjmem potravy. Modelové studie odhalily, že příjem potravy stimuluje syntézu

a sekreci trávicích enzymů z epiteliálních buněk zadní části středního střeva. Enzymy jsou pak uvolněny z membránově asociovaných komplexů a následně odděleny v podobě váčků, které jsou postupně spojovány s cytoskeletem. Peptidasy jsou vyloučeny do ektoperitrofického prostoru v epitelu, ze kterého prostupují transversálně do střevního lumen. Zde pak degradují bílkoviny stravy. PI inhibují proteasovou aktivitu těchto trávicích enzymů a snižují tak množství proteinu, které může být stráveno. Inhibice proteas zároveň vede k nadprodukci trávicích enzymů, což má za následek vyčerpání rezerv sirných aminokyselin. V krajním případě je výsledkem všech těchto pochodů oslabení hmyzu, jeho omezený vývoj a často smrt[2].

Trávicí proteolytické enzymy různých řádů hmyzu většinou náležejí k některé z hlavních skupin proteas. Zástupci skupin *Coleoptera* a *Hemiptera* vykazují přítomnost převážně cysteinových proteas, zatímco příslušníci motýlů *Lepidoptera*, blanokřídlých *Hymenoptera*, „kobylek“ *Orthoptera* a dvoukřídlých *Diptera* využívají spíše serinových enzymů. Účinek PI na hmyz nemusí být vždy inhibice proteolytické aktivity. Nedávné studie prokázaly, že může také dojít ke vzniku zpětné vazby. Cílový hmyz totiž často disponuje dvěma či více odlišnými skupinami trávicích enzymů. Jedny mohou být k inhibitorům citlivé, jiné naopak ne. Výsledkem působení PI na jedince disponujícího takovouto enzymovou výbavou pak může být přednostní produkce PI-rezistentních proteolytických enzymů[15,16].

Způsob, jakým se PI váží na cílové trávicí enzymy, se zdá být pro všechny čtyři skupiny stejný. Inhibitor se naváže na aktivní centrum enzymu a vytvoří komplex o velmi malé disociační konstantě (10^7 až 10^{14} M při neutrálním pH). Tím efektivně blokuje aktivní centrum. Jedná se o kompetitivní inhibici. Střevní proteasy nejsou jedinou skupinou látek ovlivňovaných PI, omezena je aktivita mnoha dalších enzymů, vodní rovnováha, a některé další fyziologické pochody[6].

4.3. Regulace inhibitorů proteas

Inhibitory proteas, které se v rostlinách akumulují jako odpověď na poranění, byly již v minulosti dostatečně charakterizovány. První práce s těmito inhibitory u bramboru odhalily, že iniciační faktor proteas (PIIF- protease inhibitor initiation factor) uvolněný v odpovědi na poranění či poškození rostliny je tou sloučeninou, která uvolňuje sled dějů vedoucích k syntéze PI (cit.[17]).

Dnes se usuzuje na to, že produkce PI je řízena oktadekanovou dráhou, kterou je mimo jiné zprostředkován rozklad kyseliny linolenové na mnoho výsledných produktů, z nichž jedním je kyselina jasmonová (JA). Tyto pochody ve svém důsledku vedou k indukci exprese genů kódujících PI. Zajímavá je také funkční souvislost s poraněním. Za odezvu na poranění rostlin jsou zodpovědné čtyři systemicky působící faktory, signální látky systemin, kyselina abscisová (ABA), hydraulické signály a elektrické signály[18]. Molekuly signálních látek jsou transportovány od místa poranění vodivými pletivy rostliny. První zástupce, systemin, peptid obsahující 18 aminokyselin, byl intenzivně zkoumán u rajčete, v jehož poraněných listech byla silně indukována exprese genů kódujících PI. Oproti tomu, transgenní rostliny, které exprimovaly protismyslovou („antisense“) cDNA prosysteminu, vykazovaly podstatné snížení syntézy PI a následně i snížení odolnosti rostliny vůči hmyzím škůdcům[19]. Je známo, že v odpovědi na poškození hmyzem či patogenem systemin rajčete reguluje expresi asi 20 obranných genů a také aktivuje signální dráhu, během které je kyselina linolenová uvolněna z membránových struktur a konvertována na JA. Povrchový receptor systeminu (160 kDa), indukovaný poraněním, reguluje intracelulární kaskádu zahrnující depolarizaci plazmatické membrány a otevření iontových kanálů. Výsledkem je zvýšený obsah intracelulárního Ca^{2+}, který aktivuje mitogenem aktivovanou fosfokinasu (MAP kinasu) a fosfolipasu A. Tyto rychlé změny ve svém důsledku vedou k uvolnění kyseliny linolenové z intracelulárních membrán, a zřejmě i z plazmatické membrány, a její konverzi na JA, silného aktivátora exprese obranných genů rostliny[20]. Další práce na rajčeti prokázaly, že ke zvýšení hladiny jasmonátu dochází souhrou poranění rostliny, působení systeminu a různých oligosacharidů, vznikajících degradací pektinu, např. působením polygalaktorunasy. Úloha jasmonátu jako agens odpovídajícího na poranění rostliny a zvyšujícího lokální či systemickou expresi PI byla prokázána u mnoha rostlinných druhů[21]. Objev konzervativního motivu v promotoru PI-IIK bramboru, tzv. G-boxu (sekvence CACGTGG), který je indukován JA, tuto myšlenku jen dále podporuje. Další studie na modelových objektech potvrdily významnou roli rostlinných růstových regulátorů, např. ABA, v přenosu signálu poranění. Hladina ABA a paralelně s ní i syntéza PI se zvyšuje v odpovědi na poranění, elektrické signály, tepelné šoky nebo aplikaci systeminu[22]. Přesto se však usuzuje pouze na okrajovou roli ABA v indukci tvorby PI, neboť bylo experimentálně prokázáno, že i velmi vysoké koncentrace ABA (100 mM) indukovaly pouze slabě transkripci mRNA PI (cit.[23]).

Je zcela zřejmé, že dráhy přenášející informace o poranění a obranné dráhy se značně překrývají. Exprese poraněním a JA indukovaných genů může být pozitivně či negativně regulována ethylenem či kyselinou salicylovou (SA). Obě sloučeniny jsou součástí obranné dráhy indukované patogenem. Stimulující efekt JA a tlumící efekt ethylenu byly prokázány ve studiích na modelech *Arabidopsis thaliana*[24] a *Griffonia simplicifolia*[25].

V některých případech jsou rostliny schopny „vzdát“ se jednoho obranného mechanismu ve prospěch jiného. Např. SA a její methylester jsou sloučeniny silně navozující tzv. systémově získanou rezistenci rostlin jako reakci na napadení některé její části patogenem. Nicméně nemusí tomu tak být vždy. V některých případech může SA potlačit obranu rostliny cestou naředění a zeslabení oktadekanové dráhy, zatímco její methylester působí opačně, ve prospěch obranných mechanismů rostliny. V nedávné době byla identifikována celá řada podobně se chovajících drah, zdaleka však nebyly plně charakterizovány. Komponenty

Tabulka I
Některé hmyzu odolné transgenní rostliny exprimující geny pro inhibitory proteas živočišného a rostlinného původu (přehled Schuler a spol., 1998, cit.[6])

PI	Cílový hmyz	Transformované rostliny
Anti-chymotrypsin z *Manduca sexta*	*Homoptera*	bavlník, tabák
Anti-elastasa z *Manduca sexta*	*Homoptera*	vojtěška, bavlník, tabák
α-Antitrypsin (α1 AT)	*Lepidoptera*	brambor
Antitrypsin z *Manduca sexta*	*Homoptera*	bavlník, tabák
Hovězí pankreatický inhibitor trypsinu	*Lepidoptera, Orthoptera*	salát, petúnie, brambor, tabák, jetel plazivý
PI ze sleziny	*Lepidoptera*	brambor
C-II (PI sóji)	*Coleoptera, Lepidoptera*	řepka, topol, brambor, tabák
CMe (inhibitor trypsinu ječmene)	*Lepidoptera*	tabák
CMTI (inhibitor trypsinu dýně)		tabák
CpTI (inhibitor trypsinu luskovin)	*Coleoptera, Lepidoptera*	jabloň, řepka, salát, brambor, rýže, jahodník, slunečnice, sladké brambory, tabák, rajče
MTI-2 (PI hořčice)	*Lepidoptera*	*Arabidopsis*, tabák
OC-1 (PI rýže)	*Coleoptera, Homoptera*	řepka, topol, tabák
PHV (PI soji)	*Lepidoptera*	brambor, tabák
Pot PI-I (PI I bramboru)	*Lepidoptera, Orthoptera*	petunie, tabák
Pot PT-I (PI II bramboru)	*Lepidoptera, Orthoptera*	tabák, salát, rýže, bříza
SKTI (Kunitzův inhibitor trypsinu sóji)	*Lepidoptera*	brambor, tabák
PI I rajčete	*Lepidoptera*	vojtěška, tabák, rajče, lilek
PI II rajčete	*Lepidoptera*	tabák, rajče

těchto drah jsou většinou založené na reverzní fosforylaci, pochodech regulovaných vztahy vápník/kalmodulin a produkci aktivního kyslíku[26].

4.4. Transgenní rostliny exprimující inhibitory proteas

Zjištění, že v některých rostlinách přirozeně se vyskytující PI mohou potlačit činnost trávicích enzymů hmyzu, vedlo k myšlence vnést kódující sekvence PI do genomu zemědělsky významných plodin, či zvýšit expresi v rostlině již přítomných PI. Prvním úspěšným přenosem genu kódujícího PI byla v roce 1987 publikovaná transformace tabáku genem pro inhibitor trypsinu z bobovité rostliny vigny.

Doposud bylo pro transgenozi rostlin použito asi 14 genů kódujících PI. Převážně šlo o inhibitory serinových proteas pocházející z čeledí bobovitých *Fabaceae*, lilkovitých *Solanaceae* a lipnicovitých *Poaceae*. Cíleny byly převážně proti zástupcům hmyzu skupiny *Lepidoptera*, ale také proti některým škůdcům zastupujících řády *Coleoptera* a *Orthoptera*. Předmětem zájmu však nejsou pouze geny pro PI pocházející z rostlin, ale též geny pro PI živočišného původu. Stručný přehled o úspěšně transformovaných rostlinách sekvencemi PI podává tabulka I.

Ačkoliv hlavním objektem zájmu je hlavně hmyz, inhibitory proteas vykazují i aktivitu vůči houbovým patogenům, virům či háďátkům. Inhibitory serinových proteas potlačovaly růst polyfágní houby *Botrytis cinerea*, původce onemocnění luskovin *Fussarium solani* f. sp. *pisi*, či patogena brukvovitých *Alternaria brassicicola*[27]. PI cysteinových proteas pocházející z rýže exprimovaný v transgenním tabáku zvyšoval odolnost vůči potyvirům, viru lepivosti tabáku a Y viru brambor. Proti viru mozaiky tabáku však PI cysteinových proteas nebyl účinný[28]. Inhibitory převážně serinových a cysteinových proteas byly s úspěchem použity pro tvorbu transgenních rostlin odolných vůči některým háďátkům[29,30].

5. Závěr

Přestože byly identifikovány geny kódující celou řadu PI pocházejících z různých organismů a bylo jimi transformováno mnoho druhů rostlin, vývoj tohoto typu transgenních rostlin je stále ještě na svém počátku. Výzkum poukázal na celou řadu limitujících a často i negativních faktorů v současnosti omezujících širší využití transgenních plodin exprimujících PI a dodnes nebyla žádná taková rostlina uvolněna pro komerční praxi.

Významným omezením rychlejšího vývoje této strategie GM rostlin je nesmírná variabilita trávicích proteas hmyzu, kdy je odhadováno, že ve střevě se vyskytuje více než jeden tisíc rozdílných enzymů. Je tedy nemožné ovliv-

nit expresí jednoho PI všechny proteasy hmyzu[2]. Pro navození odolnosti transgenních rostlin tedy bude nezbytné vnášet více genů pro PI, lišících se v mechanismu působení či geny pro další insekticidní látky. Hmyz je navíc schopen kompenzovat inhibici jednoho proteolytického enzymu využitím jiného, který není ovlivněn[31], či jednoduše přejít na produkci enzymů k PI necitlivých[32–36]. V některých případech byl dokonce pozorován opačný efekt exprese PI v transgenní rostlině než snížení jejího poškození. Škodlivý hmyz sice vykazoval inhibici proetolytických enzymů, tuto si však kompenzoval jednoduše tím, že zkonzumoval více rostlinné hmoty[37,38]. Letální účinek PI také není vždy 100%, často dochází pouze ke zpomalení vývoje hmyzu či snížení jeho plodnosti[39].

Z hlediska praktického využití je nezanedbatelnou otázkou také možný nežádoucí vliv rostlin exprimujících PI na necílové organismy. Ať už se jedná o přímou konzumaci rostlinných částí obsahující PI či o potravní vztahy predátorů a jejich potravy, které byly vystaveny účinkům PI, nelze tuto skutečnost přehlížet[40–42]. Rostliny exprimující PI jsou předmětem hodnocení potenciálních rizik stejně jako všechny ostatní transgenní plodiny uvolněné do prostředí nebo u kterých je jejich uvolnění plánováno[43,44]. Potenciální rizika PI pro živočichy a člověka nebyla zatím podrobněji studována vzhledem k tomu, že výzkum se doposud zaměřoval pouze na aspekty spojené s vývojem metodik a studium mechanismů účinku vůči cílovým skupinám organismů.

Efekt a využití transgenních rostlin exprimujících PI nelze doposud srovnávat např. s *Bt*-rostlinami, neboť výzkum v oblasti PI probíhá nesrovnatelně kratší dobu. Dodnes publikované výsledky však poukazují na to, že PI budou v budoucnu jedním ze způsobů efektivní kontroly škodlivých činitelů a najdou si své místo v integrované ochraně rostlin.

Práce vznikla za podpory grantů Ministerstva školství, mládeže a tělovýchovy ČR MŠMT 1PO5ME800 a MSM 60076658-06 a Grantové agentury ČR GA ČR-31/H160.

LITERATURA

1. Carliny C. R., Grossi-de-Sá M. F.: Toxicon *40*, 1515 (2002).
2. Lawrence P. K., Koundal K. R.: Electron. J. Biotechnol. *5*, 93 (2002).
3. Sharma H. C., Sharma K. K., Seetharama N., Ortiz R.: Electron. J. Biotechnol. *3*, 6 (2000).
4. Munkvold G. P., Hellmich R. L, Rice L. G.: Plant Dis. *83*, 130 (1999).
5. Babu R. M., Sajeena A., Seetharaman K., Reddy M. S.: Crop Prot. *22*, 1071 (2003).
6. Schuler T. H., Poppy G. M., Kerry B. K., Denholm I.: Trends Biotechnol. *16*, 168 (1998).
7. ISAAA : http://www.isaaa.org/main.htm, staženo 27. června 2005.
8. Klein T. M., Wolf E. D., Wu R., Stanford J. C.: Nature *327*, 70 (1987).
9. Chilton M.-D., Drummond M. H., Merlo D. J., Sciaky D., Montoya A. L., Gordon M. P., Nester E. W.: Cell *11*, 263 (1977).
10. Murdock L. L., Shade R. E.: J. Agric. Food Chem. *50*, 6605 (2002).
11. Mickel C. E., Standish J.: University of Minnesota Agricultural Experimental Station Technical Bulletin *178*, 1 (1947).
12. Hilder V. A., Gatehouse A. M. R., Sheerman S. E., Barker R. F., Boulter D. A.: Nature *300*, 160 (1987).
13. Ryan C. A., v knize: *Variable Plants and Herbivores in Natural and Managed Systems* (Denno R. F., McClure M. S., ed.), 741–747. Academic Press, New York 1989.
14. Baker J. E., Woo S. M., Mullen M. A.: Ent. Exp. App. *36*, 97 (1984).
15. Jongsma M. A., Bakker P. L., Peters J., Bosch D., Stiekema W. J.: PNAS *92*, 8041 (1995).
16. Michaud D., Nguyen-Quoc B., Vrain T. C., Fong D., Yelle S.: Arch. Insect Biochem. Physiol. *31*, 451 (1996).
17. Melville J. C., Ryan C. A.: Biol. Chem. *247*, 3415 (1973).
18. Malone M., Alarcon J. J.: Planta *196*, 740 (1995).
19. McGurl B., Orozco-Cardenas M., Pearce G., Ryan C. A.: PNAS *91*, 9799 (1994).
20. Ryan C. A.: Biochim. Biophys Acta *1477*, 112 (2000).
21. Wasternack K. F., Parthier B.: Trends Plant Sci. *2*, 302 (1997).
22. Koiwa H., Bressan R. A., Hasegawa P. M.: Trends Plant Sci. *2*, 371 (1997).
23. Birkenmeier G. F., Ryan C. A.: Plant Physiol. *117*, 687 (1998).
24. Epple P., Aprl K., Bohlmann H.: Plant Physiol. *109*, 813 (1995).
25. Zhu-Salzman K., Salzman R. A., Koiwa H., Murdock L. L., Bressan R. A., Hesegawa P. M.: Physiol. Plant. *104*, 365 (1998).
26. León J., Rojo E., Sanchez-Serano J. J.: J. Exp. Botany *52*, 1 (2001).
27. Lorito M., Broadway R. M., Hayes C. K., Woo S. L., Noviello C., Williams D. L., Harman G. E.: Mol. Plant-Microbe Int. *7*, 525 (1994).
28. Gutierez-Campos R., Torres-Acosta J. A., Saucedo-Arias L. J., Gomez-Lim M. A.: Nature Biotech. *17*, 1223 (1999).
29. Urwin P. E., Levesley A., McPherson M. J., Atkinson H. J.: Mol. Breed. *6*, 257 (2000).
30. Urwin P. E., Troth K. M., Zubko E. I., Atkinson H. J.: Mol. Breed. *8*, 95 (2001).
31. Wu Y., Llewellyn D., Mathews A., Dennis E. S.: Mol. Breed. *3*, 371 (1997).
32. Jongsma M. A., Bakker P. L., Stiekema W. J., Bosch D.: Mol. Breed. *1*, 181 (1995).
33. De Leo F., Bonadé-Bottino M. A., Ceci L. R., Gallerani R., Jouanin L.: Plant Physiol. *118*, 997 (1998).
34. Bonadé-Bottino M., Lerin J., Zaccomer B., Jouanin L.: Insect Biochem. Mol. Biol. *29*, 131 (1999).

35. Paulilo L. C. M. S., Lopes A. R., Cristofolleti P. T., Parra J. R. P., Terra E. R., Silvia-Filho M. C.: J. Econ. Entomol. *93*, 892 (2000).
36. Brito L. O., Lopes A. R., Parra J. R. P., Terra W. R. Silva-Filho M. C.: Crop Biochem. Physiol. *128B*, 365 (2001).
37. Cloutier C., Jean C., Fournier M., Yelle S., Michaud D.: Arch. Insect Biochem. Physiol. *44*, 69 (2000).
38. Winterer J., Bergelson J.: Mol. Ecol. *10*, 1069 (2001).
39. De Leo F., Gallerani R.: Insect Biochem. Mol. Biol. *32*, 489 (2002).
40. Girard C., Picard-Nizou A. L., Grallien E., Zaccomer B., Jouanin L., Pham-Delegue M. H.: Transgenic Res. *7*, 239 (1998).
41. Bouchard E., Michaud D., Cloutier C.: Mol. Ecol. *12*, 2429 (2003).
42. Ferry N., Raemaekers R. J. M., Majerus M. E. N., Jouanin L., Port G., Gatehouse J. A., Gatehouse A. M. R.: Mol. Ecol. *12*, 493 (2003).
43. Atkinson H. J., Green J., Cowgill S., Aurora L.: Trends Biotechnol. *19*, 91 (2000).
44. Cowgill S. E., Atkinson, H. J.: Transgenic Res. *12*, 439 (2003).

M. Hraška[a,b], S. Rakouský[a,c] , and V. Čurn[b] *([a]Department of Genetics, Faculty of Biology, [b]Biotechnological Centre, Faculty of Agronomy, [c]Health and Social Faculty, University of South Bohemia, České Budějovice):* **Protease Inhibitors, Mode of Action and Perspectives for Plant Transgenosis**

Contemporary cultivation of field crops utilizes new findings of biotechnologies, specifically recombinant DNA and transgenous techniques to an ever-increasing extent. Transgenic plants enriched in various new genes already became common practice in agriculture of a number of developed but also developing countries. The research in this field intensively grows, also entirely new directions, in addition to already proved gene manipulations, are the subject of interest. The contribution deals with classification and function of some protease inhibitors utilizable in transgenosis of plants. It concentrates also on the aspects associated with possible use of their recombined genes in enhancement of resistance of plants to insect pests and some pathogens. Some examples are given of important transgenic plants which already express genes for most important protease inhibitors.

4. 2. Use of a simple semiquantitative method for appraisal of green fluorescent protein gene expression in transgenic tobacco plants

Hraška, M., Rakouský, S., Kocábek, T. (2005) Use of a simple semiquantitative method for appraisal of green fluorescent protein gene expression in transgenic tobacco plants. Biol. Plant. 49, 313-316

BIOLOGIA PLANTARUM 49 (2): 313-316, 2005

BRIEF COMMUNICATION

Use of a simple semiquantitative method for appraisal of green fluorescent protein gene expression in transgenic tobacco plants

M. HRAŠKA*, S. RAKOUSKÝ*[1] and T. KOCÁBEK

Institute of Plant Molecular Biology, Academy of Sciences of the Czech Republic, and University of South Bohemia, Faculty of Biological Sciences, Branišovská 31, České Budějovice, CZ-37005, Czech Republic*

Abstract

We have applied a simple method for evaluation of *gfp* gene expression in plants using a CCD camera and computerized processing of images. Transgenic tobacco plants were obtained by *Agrobacterium tumefaciens*-mediated transfer of plasmid T-DNA bearing a *m-gfp5-ER* sequence governed by the 35S promoter together with the *nptII* selectable marker gene. Presence of the *gfp* gene in plants was confirmed by a polymerase chain reaction method. Mean brightness values measured using image analysis software showed differences between transgenic and control plants and suggest the possibility of rapid selection of transgenic individuals among regenerants and their progenies.

Additional key words: CCD camera, computerized processing, *gfp* expression, image analysis, *Nicotiana tabacum.*

One of the main obstacles in plant transgenosis is the limited possibility of a simple and efficient selection of transgenic individuals among numerous plants of the first (T_0) generation of regenerants. Although relatively efficient systems (based on co-transfer of selectable or marker gene(s) with the gene of interest) allowing the selection on media containing antibiotics or other substances were developed (for review see Miki and McHugh 2004) those of different reasons are not generally applicable to a wide scale of materials. The aim of this work is to contribute to the development of selection schemes, which either alone or in a combination with selectable marker(s) would be applicable to any higher plant species and enable use of the automated or semi-automated computer driven basic laboratory instruments as well as preliminary evaluation of gene expression.

Many marker genes are used in plant molecular biology in the present time. One of these is the gene coding for green fluorescent protein (GFP) isolated from Pacific jellyfish *Aequorea victoria,* first described by Prasher *et al*. (1992). GFP became a powerful tool in biology for the studying gene expression, protein localization and transport, and many applications in plant research have been reported (Harper *et al*. 1999, Halfhill *et al*. 2001, Miki and McHugh 2004). Mostly, the application of GFP does not require a destructive preparation of studied samples as in the case of β-glucuronidase (GUS) assay and allows investigation of living organisms in real time (Hu and Cheng 1995). Many GFP variants have been created differing in their excitation and emission spectra or in their cell targets (Stewart 2001). In some cases it is suitable to study not only the presence and localization of GFP but also to quantify its activity. We have therefore, performed experiments with a view to detect the activity of *gfp* in transgenic tobacco plants by stereomicroscope and to use the computer-aided quantification of its signal.

Sterile true leaves detached from aseptically cultivated *Nicotiana tabacum*, cv. Petit Havana, SR1 WT plants (Maliga *et al*. 1973) were used for *Agrobacterium* mediated leaf disc transformation according to Horsch

---

Received 14 July 2004, *accepted* 24 November 2004.

Abbreviations: GFP - green fluorescent protein, MB - mean brightness, MS - Murashige and Skoog (1962) culture medium, PCR - polymerase chain reaction.

Acknowledgements: Authors are grateful to Dr. Vincent Lea, Molecular Research Group, NIAB Cambridge, UK for kind proofreading the manuscript. This work was supported by project # QE1123 of the Grant Agency of Czech Ministry of Agriculture.

[1] Corresponding author; fax: (+420) 38 777 2228, e-mail: srak@bf.jcu.cz

et al. (1985). *A. tumefaciens* strain LBA 4404 carrying binary vector pBINm-gfp5-ER (provided by J. Hasseloff, Department of Plant Sciences, University of Cambridge, UK) was used. The vector contains a modified *gfp* gene with endoplasmic reticulum (ER) retention signals mutated to allow visualization at UV (395 nm) or blue light (473 nm). Co-cultivated and control (non transformed) leaf disks were grown under *in vitro* conditions in a growth chamber (16-h photoperiod, 40 - 70 μmol(PAR) m^{-2} s^{-1}, 22 - 25 °C) on a basal Murashige and Skoog (1962; MS) medium supplemented with growth regulators and selective antibiotics (Horsch *et al*. 1985). Regenerated T_0 plants were maintained *in vitro* on a basal MS medium. Putative transformants (14 selected plants) and one control plant were transferred to soil and grown in a glasshouse under optimum temperature 21 - 23 °C. Dried seeds obtained from 12 selfed plants were surface sterilized with 70 % ethanol for 2 min and then for 45 - 65 min in commercial bleach containing 1.6 % m/v sodium hypochlorite and 0.1 % *Tween 20*. Seeds were then rinsed 4 times with sterile distilled water and sown dispersed in 0.125 % agar (*Difco Bacto Agar*, *Difco Laboratories*, Sparks, USA) on MS medium supplemented with 500 mg dm^{-3} kanamycin. Petri dishes (9 cm) with seeds were maintained for 5 weeks in a growth chamber under the same conditions as regenerated plants. Selected T_1 seedlings (11 + 1 control) were transferred to fresh MS without antibiotics. Plants thus obtained were used for further experiments. No visible differences in plant morphology and development between transgenic and control plants were observed either *in vitro* or *in vivo*

To confirm the presence of *gfp* gene in transgenic tobacco plants, DNA was isolated from 2-month-old leaves as described by Edwards *et al.* (1991). The *in vitro* plants used for analyses formed at that time only a ground rosette consisting of 3 - 5 true leaves. The primers *gfp0* (5′-ATG TTG CAT CAC CTT CAC CC) and *gfp1* (5′-AAG CTT ACA GTC TCA AAG ACC AAA G) were used to specifically amplify a region of 570 bp. PCR reactions were performed in 0.02 cm^3 reaction mixture containing buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 200 μM of each dNTP, 0.16 μM of each primer gfp01 and gfp1, 1 U of Taq polymerase (*Top-Bio*, Praha, Czech Republic) and approx. 50 ng DNA. The samples were amplified using 35 cycles (94 °C for 30 s, 52 °C for 30 s, 72 °C for 45 s) and analysed on 2 % agarose gels stained with ethidium bromide in TAE buffer. PCR product corresponding to *gfp* was found in all 11 putative transgenic samples, but not in the control sample (data not shown).

Other leaves detached from the same transgenic and of one control plant were used for further microscopic GFP study. Samples were studied in a normal position with the abaxial side uppermost on a Petri dish in a droplet of distilled water to prevent desiccation and following destruction of tissues. A *Leica MZ 12* stereo dissecting microscope equipped with a fluorescence module consisting of 100 W mercury lamp and GFP excitation and emission filters (*Leica*, Heerbrigg, Switzerland) integrated with CCD camera was used for the study. This set (excitation filter 480/40 nm, dichroic mirror 505 nm LP, barrier filter 510 nm LP), black-white CCD camera *Cohu* (San Diego, USA) permits the visualization and detection of GFP following tissue excitation by blue light. Emitted GFP signal was analysed by *Lucia®4.71* software (*Laboratory Imaging*, Praha, Czech Republic). Leaves were analysed immediately after their separation from plants.

Objective magnification (1.6×) and 1.6 optical zoom were set up on the *Leica MZ 12* stereomicroscope. Two different approaches to obtain data were performed. First, each leaf was scanned from the tip to the base along the midrib. Second, the same leaf was than scanned around its perimeter, excluding the midrib conductive bundles. Mean brightness (MB) values were collected from a defined area (4.2 mm^2) and statistically processed using the Student's *t*-test by means of *Statistica®* software (*StatSoft*, Tulsa, USA). Two to three leaves of each plant were scanned. From each leaf at least 9 measurements were performed. Based on our finding from previous experiments that leaves of different non-transformed (control) plants show very low MB variability (data are not given here) only one plant was used as a control.

Results of the *t*-test showed pronounced differences of MB values between transgenic and non-transgenic (control) plants (Table 1). All transgenic leaves provided visual fluorescence when compared to the control leaves (Fig. 1). Fluorescence outcomes from the area close to the midrib demonstrate considerable variability in MB values between individual measurements. This observation corresponds with the high level of standard deviation (SD) of MB among tested samples. Data collected from the perimeter of each leaf, excluding the midrib part, demonstrate lower levels of SD, except plants 4 and 10. However, differences of SDs in these two cases were not significant. High SD in the medial region of leaves are most probably caused by the influence of the midrib, which exhibits stronger fluorescence than the surrounding tissue. Although leaves of the same physiological age were used, some of them had a stronger midrib than others, which resulted in a higher fluorescence and thus also in higher MB values. This discrepancy was avoided by collecting data from the leaf perimeter, which had lower SD. We suggest that areas away from the midrib should be selected for further use and development of this method.

Data also showed differences in MB values within transgenic leaves from different plants. For example, the fluorescence from leaf 10 was practically invisible and MB was also very low, whereas fluorescence and MB from plant 3 was extremely high. This is probably due to

Table 1. Mean brightness (MB) values of fluorescence signals obtained as summarized outcomes from *Lucia*®*4.71* software following the scanning of transgenic and control leaves. Two to three leaves of each plant were scanned. From each leaf two groups of data were collected (with and without midrib) and at least 9 measurements were performed (C denotes control, * - MB values significantly different at $P \leq 0.01$ from corresponding ones measured on a control plant).

Plant number	1	2	3	4	5	6	7	8	9	10	11	C
MB from the medial part of leaves (including the midrib)												
MB ± SD	134.03 ±26.00*	11.92 ±5.11*	226.55 ±24.29*	13.58 ±4.74*	87.72 ±26.19*	7.18 ±5.05*	143.85 ±57.09*	95.86 ±43.52*	78.14 ±9.36*	1.12 ±0.17	134.98 ±23.79*	0.91 ±0.14
MB from the periphery of leaves (excluding the midrib)												
MB ± SD	137.16 ±15.82*	11.12 ±2.02*	219.38 ±14.60*	13.92 ±3.09*	46.16 ±6.76*	18.64 ±7.75*	86.04 ±14.45*	55.46 ±10.36*	47.73 ±7.69*	1.33 ±0.20*	69.49 ±10.74*	0.47 ±0.11

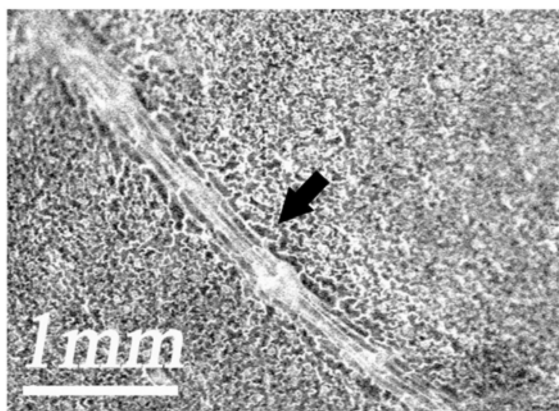

Fig. 1. Example of fluorescence image obtained after the illumination of tobacco leaves with 480/40 nm light using *Leica MZ 12* stereomicroscope equipped with a fluorescence module (objective magnification - 1.6×, optical zoom - 1.6×) and black-white CCD camera *Cohu*. Sample of transgenic plant 1 emits high fluorescence signal (white spots) opposite to the control plant tissue, which is undistinguishable of the low background noise. Region free of the midrib is presented here. *Arrow* indicates the position of conductive bundles on abaxial side of the leaf surface of transgenic plant.

different copy numbers of transgenes inserted in the plant genome and/or their total expression level, which may be influenced by gene silencing (Voinnet and Baulcombe 1997). This idea could be confirmed by Southern blot analyses and quantification of recombinant protein. However, these analyses were not performed, because the aim was to show whether this approach for studying *gfp* expression is possible.

Many other systems for studying fluorescence events are now available. They include the use of various high-sensitivity UV lamps, spectrofluorometers, scanning laser systems and GFP meters (Millwood *et al*. 2003). However, all these instruments, designated for detection and quantification of various fluorescent compounds, are expensive and can be used only for the purposes they were developed for. The detecting set we used here is designated for universal image analyses and represents a new potential utilisation for laboratories equipped with this arrangement and adds value to this set. It was demonstrated here in accordance to other authors (Soukupová and Albrechtová 2003) that results of signal quantification with image analysis may greatly depend on the character and procedure used. If it is done in full respect of conditions and knowledge of plant anatomy it can gain reliable and interesting results. The preliminary results presented in this paper showed, that the detection and quantification of GFP signal from transgenic plant tissue by CCD camera and *Lucia*® *4.71* imaging software is possible and promising.

References

Edwards, K., Johnstone, C., Thompson, C.: A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. - Nucl. Acids Res. **19**: 1349, 1991.

Halfhill, M.D., Richards, H.A., Mabon, S.A., Stewart, C.N., Jr.: Expression of *GFP* and *Bt* transgenes in *Brassica napus* and hybridization with *Brassica rapa*. - Theor. appl. Genet. **130**: 659-667, 2001.

Harper, B.K., Mabon, S.A., Leffel, S.M., Halfhill, M.D., Richards, H.A., Moyer, K.A., Stewart, C.N., Jr.: Green fluorescent protein as a marker for expression of a second gene in transgenic plants. - Nature Biotechnol. **17**: 1125-1129, 1999.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eicholtz, D., Rogers, S.G., Fraley, R.T.: A simple and general method for transferring genes into plants. - Science **227**: 1229-1231, 1985.

Hu, W., Cheng, C.-L.: Expression of *Aequorea* green fluorescent protein in plant cells. - FEBS Lett. **369**: 331-334, 1995.

Maliga, P., Sz.-Breznovits, A., Márton, L.: Streptomycin-resistant plants from callus culture of haploid tobacco. - Nature **244**: 29-30, 1973.

Miki, B., McHugh, S.: Selectable marker genes in transgenic plants: applications, alternatives and biosafety. - J. Biotechnol. **107**: 193-232, 2004.

Millwood, R.J., Halfhill, M.D., Harkins, D., Russotti, R., Stewart, C.N., Jr.: Instrumentation and methodology for quantitative GFP fluorescence in intact transgenic plants. - Biotechniques **24**: 638-643, 2003.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., Cormier, M.J.: Primary structure of the *Aequorea victoria* green-fluorescent protein. - Gene **111**: 229-233, 1992.

Soukupová, J., Albrechtová, J.: Image analysis – tool for quantification of histochemical detections of phenolic compounds, lignin and peroxidases in neeedles of Norway spruce. - Biol. Plant. **46**: 595-601, 2003.

Stewart, C.N., Jr.: The utility of green fluorescent protein in transgenic plants. - Plant Cell Rep. **20**: 376-382, 2001.

Voinnet, O., Baulcombe, D.C.: Systemic signalling in gene silencing. - Nature **389**: 553, 1997.

4. 3. Green fluorescent protein as a vital marker for non-destructive detection of transformation events in transgenic plants

Hraška, M., Rakouský, S., Čurn, V. (2006) Green fluorescent protein as a vital marker for non-destructive detection of transformation events in transgenic plants Plant Cell Tissue Organ Cult. 86, 303-318

Plant Cell Tiss Organ Cult (2006) 86:303–318
DOI 10.1007/s11240-006-9131-1

REVIEW

Green fluorescent protein as a vital marker for non-destructive detection of transformation events in transgenic plants

Marek Hraška · Slavomír Rakouský · Vladislav Čurn

Received: 28 February 2006 / Accepted: 18 June 2006 / Published online: 17 August 2006
© Springer Science+Business Media B.V. 2006

Abstract Transformation of plants is a popular tool for modifying various desirable traits. Marker genes, like those encoding for bacterial *β*-glucuronidase (GUS), firefly luciferase (LUC) or jellyfish green fluorescent protein (GFP) have been shown to be very useful for establishing of efficient transformation protocols. Due to favourable properties such as no need of exogenous substrates and easy visualization, GFP has been found to be superior in to other markers in many cases. However, the use of GFP fluorescence is associated with some obstacles, mostly related to the diminishing of green fluorescence in older tissues, variation in fluorescence levels among different tissues and organs, and occasional interference with other fluorescing compounds in plants. This paper briefly summarizes basic GFP properties and applications, and describes in more detail the contribution of GFP to the establishment, evaluation and improvement of transformation procedures for plants. Moreover, features and possible obstacles associated with monitoring GFP fluorescence are discussed.

M. Hraška · S. Rakouský
Faculty of Biological Sciences, Department of Genetics, University of South Bohemia, Branišovská 31, České Budějovice CZ-370 05, Czech Republic

S. Rakouský (✉)
Faculty of Health and Social Studies, University of South Bohemia, Branišovská 31, České Budějovice CZ-370 05, Czech Republic
e-mail: srak@bf.jcu.cz

V. Čurn · M. Hraška
Faculty of Agriculture, Biotechnological Centre, University of South Bohemia, Studentská 13, České Budějovice CZ-370 05, Czech Republic

Keywords *Agrobacterium tumefaciens* · Green fluorescent protein · Particle bombardment · Plant transformation · Selection

Abbreviations

GFP	Green fluorescent protein
GUS	*β*-Glucuronidase
LUC	Firefly luciferase
RT-PCR	Reverse transcription polymerase chain reaction
gfp	Green fluorescent protein gene
uidA	*β*-Glucuronidase gene

Introduction

Genetic transformation of plants is a promising method not only for improving various agronomic and/or horticultural traits, but also for fundamental studies of plant physiology (Bauchera et al. 1998; Smirnoff and Wheeler 2000), genetics,

molecular and cell biology (Kocábek et al. 1999), pathology (Franchea et al. 1998; Panstruga 2004) and other areas. The development of plants with new qualitative or quantitative traits is the primary objective of plant transgenesis. The successful introduction of new desirable traits usually requires the development of an efficient and reliable transformation protocols. Such protocols should contribute to the development of the most efficient strategy for transferring the genes into plant cells (Chilton et al. 1977; Klein et al. 1987), selection and regeneration of putative transgenic cells (Miki and McHugh 2004) and subsequent recovery of transgenic plant(s). Generally, model genes, which allow for the critical assessment of each step in the procedure, are the most suitable for such fundamental studies. The use of visual markers, which enable direct observation of transformation events, results in a more precise and easier evaluation of various treatments and procedures. They can increase transformation efficiency by reducing the time and amount of material to be handled and screened (Baranski et al. 2006) allowing the most efficient, reliable and reproducible transformation protocol to be established. The ideal marker should possess the following desirable traits. First, it should be readily expressed in plant cells or capable of being engineered for such expression by molecular biology methods. Second, its expression should be easily visualized, and finally, the marker should not be toxic or affect in any way the physiology of living intact plants. Many genes coding for various markers are available now. Markers such as β-glucuronidase (GUS) (Jefferson et al. 1987), luciferase (LUC) (Ow et al. 1986) or β-galactosidase (LacZ) (Helmer et al. 1984) have become very popular tools for monitoring gene expression in transgenic plants. However, these require either destructive assays of the studied sample or the addition of exogenous substrates or some other cofactors for their manifestation. These markers usually do not offer the possibility of determining the exact transgenic status of plants, while also monitoring the transgene expression in real time and in living plants. On the other hand, green fluorescent protein (GFP) marker, in principle, allows for the monitoring of transgene expression from early stages of the transformation procedure though the recovery of living transgenic plants. Moreover, GFP manifestation does not require the addition of any interfering substances like exogenous substrates or enzymes. Thus plants can continue their growth and development, and can be investigated repeatedly at any growth stage (Heim et al. 1995; Chiu et al. 1996). This represents a huge benefit for using GFP as a visual marker during genetic transformation and regeneration of transgenic plants.

Molecular structure, properties and use of GFP

GFP was isolated from the pacific jellyfish *Aequorea victoria* and first described by Prasher et al. (1992). GFP transforms the luminescent blue light emitted by another hydromedusas protein, *aequorin*, into green light. The fluorescing chromophore of GFP is formed by post-translational modification in which a tripeptide Ser65-Tyr66-Gly67 is cyclized and later oxidized. This chromophore is in the geometric centre of the protein to which it is covalently attached (Shinomura 1979; Cody et al. 1993). Eleven β sheets form a barrel structure that is capped with α-helices on the top and bottom of the protein. α-helices also form a scaffold for the centrally placed chromophore. GFP represents a new class of proteins called ‘‘beta can’’. Wild type GFP is a dimer consisting of two monomer units, each consisting of 238 amino acids with a relative molecular weight of 27 kDa. The diameter of the barrels is 30 Å and length is 40 Å (Yang et al. 1996a). This wild type GFP emits light after excitation by UV (λ = 360–400 nm) or blue (λ = 440–480 nm) light with emission spectra at λ = 509 nm and with a minor peak at λ = 540 nm. GFP does not require any endogenous cofactors and substrates or exogenous compounds for fluorescence manifestation, because the formation of the chromophore is either an autocatalytic process or it requires only ubiquitous cellular components (Heim et al. 1994; Misteli and Spector 1997). GFP possesses a rigid structure with a broad stability range in pH 5–11 at temperatures up to 65°C (Tsien 1998). It maintains its fluorescence even in the presence of strong

Table 1 List of various GFP variants that are the most common in plant transformation experiments

GFP variant	Excitation/ emission spectra (nm)	Modification/s	Effect of modification/s	References
Wild type	395*, 475/510	None	None	Chalfie et al. (1994)
mGFP4	395*, 475/509	ACU	Brighter fluorescence in plants	Haseloff and Amos (1995)
mGFP5	395, 473/509	ACU, V163A, I167T, S175G	Better protein folding at 37°C, increased fluorescence, dual excitation peaks-equal amplitude	Haseloff et al. (1997)
sGFP	490/511	Humanized codon usage	Improved expression and fluorescence	Haas et al. (1996)
SGFP S65T	489/511	ACU, S65T, humanized codon usage	Increased detection limits, enhanced brightness, faster chromophore formation, slower photobleaching	Chiu et al. (1996)
EGFP	488/507	ACU, F64L, S65T, Y145F, humanized codon usage	Brighter fluorescence, improvement solubility, more efficient protein folding in bacteria	Yang et al. (1996b), Clontech
mut3GFP	400*, 475/510	ACU, F100S (F99S), M154T (M153T), V164A (V163A)	Putatively improved folding at higher temperatures	Siemering et al. (1996)
smGFP	397*, 480/507	ACU, F99S, M153T, V163A	Improved solubility and brightness	Davis and Viestra (1998)
smRS-GFP	495/510	ACU, S65T, F99S, M153T, V163A	Improved solubility and brightness, red-shifted emission	Davis and Viestra (1998)
GFP	450–490/510	ACU, S65C, S65T	Increased fluorescence	Reichel et al. (1996)
smBFP	385/448	Y66H, M153T	Improved solubility, blue-shifted emission, increase of the fluorescence	Davis and Viestra (1998)
synGFP	395/509	ACU, increasing of CG content up to 48 %, potential polyadenilation sites removed	More efficient translation, increased expression	Rouwendal et al. (1997)
BFP	382/448	ACU, Y66H	Blue-shifted emission, weaker brightness, photobleaching	Reichel et al. (1996)
mYFP	514/527	S65G, S72A, V163A, I167T, S175G, T203Y	Yellow-shifted emission	Haseloff (1999)
mCFP	440/485	Y66W, V163A, S175G	Cyan-shifted emission	Haseloff (1999)

The amino acids mutations are given in a standard format, e.g. S65T, serine to threonine at 65th amino acid; ACU, altered codon usage according to Haseloff et al. (1997); *Major excitation peak in the case of two peaks are available

denaturing agents such as 6 M guanidine HCl, 8 M urea or 1% sodium dodecyl sulphate (Yang et al. 1996a).

Due to its favourable features, GFP rapidly became a popular tool in various applications in biology research. During the last decade, it has been introduced into a wide range of organisms, including bacteria, yeasts (Morschhäuser et al. 1998), nematodes (Chalfie et al. 1994), insects (Wang and Hazelrigg 1994), fish (Kinoshita 2004), mammals (Zolotukhin et al. 1996) and plants (Chiu et al. 1996). Its suitability for plant transformation was first demonstrated by Niedz et al. (1995), who successfully inserted wild type GFP into sweet orange (*Citrus sinensis*) protoplasts. Transformation of other plant species soon followed, but complications with low expression and quenching of fluorescence occurred (Hu and Cheng 1995). Detail sequence analysis performed by Haseloff et al. (1997) revealed the existence of a cryptic intron in the wild type GFP gene sequence. Its presence resulted in aberrant splicing between nucleotides 380–463 during processing in plant cells and finally the loss of the 84-nucleotide region. A new variant, denoted mGFP4, was derived by altered codon usage, maintaining the same spectral characteristics as wild type GFP, but resulting in enhanced protein fluorescence (Haseloff et al. 1997). Subsequently, many other GFP variants have been developed, differing in their spectral characteristics, fluorescence intensity or cell targets, e.g. nucleus, endoplasmic reticulum, plastids (reviewed by Stewart 2001). Different colour GFP variants offer simultaneous tracking and study of various biological events (Baumann et al. 1998; Haseloff 1999). GFP has been used for various purposes in plant research, e.g. for the study of the expression patterns of promoters (Sheen et al. 1995; Nagatani et al. 1997), protein tagging (Chytilova et al. 1999; Shiina et al. 2000), disease tracking (Itaya et al. 1997), developmental studies (Misteli and Spector 1997), expression studies and ecological monitoring of transgene spread (Halfhill et al. 2001). GFP is being increasingly used for various purposes associated with the transformation of plants (Baranski et al. 2006; Yong et al. 2006). Nowadays, many GFP homologues originating from various organisms are available, allowing for broad range of use in biology (Chudakov et al. 2005) (Table 1).

Although some concerns about the possible toxicity of GFP to plants were raised, these have not been confirmed (reviewed by Stewart 2001). GFP did not appear to have any adverse effects on plant growth, development and fertility (Maximova et al. 1998; Ghorbel et al. 1999; Harper et al. 1999; Jordan 2000; Kaeppler et al. 2000; Murray et al. 2004). Moreover GFP has been found to be non-toxic to rats when ingested in purified form or in transgenic plants (Richards et al. 2003a).

GFP as a tool for evaluation of transformation parameters

Although many different approaches to plant transformation are available, most of them involve the insertion of exogenous DNA into plant nucleus via *Agrobacterium*-mediated transfer (Chilton et al. 1977) or particle bombardment (Klein et al. 1987). Transformation methods differ in their suitability for various purposes and plant species (Finer et al. 1999; Repellin et al. 2001), DNA integration patterns (Christou 1995; Birch 1997; Christou 1997) and their efficiency (Snape 1998). It has been shown by many authors that the development of any of transformation procedures may be much faster and more efficient if proper signal gene(s) are used throughout the study (Birch 1997; Baranski et al. 2006).

Compared to other signal genes, GFP has an advantage of wide range of applications covering whole areas of transformation and regeneration procedures. The transformation events, formation of calli followed by the emergence of fluorescing shoots can all be observed sequentially in each step of transformation and during different phases of development by fluorescence microscopy. GFP-expressing cells and tissues can easily be distinguished from untransformed ones, without destroying the studied material (Kamaté et al. 2000). The ratio between fluorescing and non-fluorescing cells, shoots and various organs as a measure of transformation efficiency has been successfully used to improve the various stages and procedures in transformation protocols.

Steps, such as the selection of the most suitable *Agrobacterium* strain for transient and stable expression studies (Galperin et al. 2003; Tang and Newton 2005), determination of the suitable acetosyringone concentration in co-cultivation medium (Jeoung et al. 2002; Tang and Newton 2005; Wang and Ge 2005), or the optimisation of other various pre-cultivation, co-cultivation (Zhou et al. 2004) and post-transformation steps (Eady et al. 2000; Cardoza and Stewart 2003), including e. g. the effect of the antibiotic treatment on explant viability, were critically assessed using the GFP marker (Tang and Newton 2005). Based on differences in GFP fluorescence, the effect of desiccation of co-cultivated explants on efficacy of transformation has also been analysed (Polin et al. 2006). For example, Baranski et al. (2006) successfully employed the GFP fluorescence for critical assessment of the whole transformation procedure of the *Agrobacterium rhizogenes*-mediated transformation of carrot. Based on the green fluorescence intensity they selected the most virulent *Agrobacterium* strain, effective acetosyringone concentration and the most suitable carrot genotype for transformation. Moreover, they were able to assess other parameters, such as the effect of delayed inoculation on the number of adventitious roots production.

In order to achieve higher efficacy of direct transformation, the *gfp* expression has been successfully used as an efficient tool for evaluation and subsequent modification of various parameters and procedures associated with particle bombardment transformation, such as the selection of appropriate tissue to be bombarded (Huber et al. 2002; Tee et al. 2003), modification of gene gun settings (Richards et al. 2001), optimisation of bombardment parameters (Jordan 2000), and evaluation of various promoters (Cho et al. 2002; Tee et al. 2003).

Monitoring the *gfp* expression in primary transformed tissues

At the beginning of the tissue transformation the GFP fluorescence is usually visible in the cuts or other wounded sectors (Zhou et al. 2004), but sometimes it can be confused with a false autofluorescence of wounded tissues (Molinier et al. 2000). For example, high levels of background green fluorescence were observed in both, untransformed (control) and transformed flax hypocotyls. This precluded their use in GFP studies and therefore different plant organs were chosen for this purpose. Moreover, in some cases, transformed tissue could possess so strong autofluorescence, that green fluorescence could not be easily distinguished (Hraška and Rakouský 2005).

Low levels of background fluorescence of various compounds in intact, wounded and untransformed tissues and/or in *Agrobacterium* strains do not usually impede the successful detection of GFP fluorescence and can be restricted by implementation of suitable filter systems (Maximova et al. 1998; Elliott et al. 1999).

A strong GFP fluorescence signal is usually visible within a few hours after co-cultivation, indicating high levels of transient *gfp* expression, which usually decrease within a few days (Elliott et al. 1999; Mercuri et al. 2001; Jeoung et al 2002; Pishak et al. 2003). This has also been reported if other marker genes, (e.g. GUS) were used (Rakouský et al. 1997). Detailed study of *gfp* transient expression in transformed apple leaf explants showed an increase in GFP fluorescence after 9 days of bacterial infection, followed by decrease and stabilization of fluorescence between 11th and 15th day. This was most probably the result of degradation of non-integrated T-DNA or gene silencing of integrated T-DNA. The fluorescence increased at 15 days after transformation, indicating growth of stably transformed cells and transgenic calli formation. Another observed event was the high number of fluorescing cells associated with the cut vascular tissues. This was explained as being due to a higher cell number and density in vascular tissue or due to vascular tissues being more susceptible to *Agrobacterium* infection (Maximova et al. 1998).

The level of GFP fluorescence differs depending on target genotype and tissue, *gfp* variant and the promoter used. For example, if barley immature embryos were transformed with *gfp* gene driven by either rice actin gene (*Act1*) promoter or endosperm-specific hordein promoter, they exhibited stronger transient *gfp* expression

when driven by *Act1* promoter than by the second one. On the other hand, endosperm-specific-hordein-promoter-driven *gfp* possesses more stable expression in T_1 progeny than *Act1* driven *gfp* (Cho et al. 2002).

Following the transformation and subsequent regeneration, only a small number of fluorescing foci stay fluorescent for periods, long enough to indicate stable genetic transformation. This event has been reported for many plant species such as wheat (Jordan 2000), barley (Ahlandsberg et al. 1999; Carlson et al. 2001), oat (Cho et al. 2003), soybean (Ponappa et al. 1999), papaya (Zhu et al. 2004), *Dendrobium* orchid (Tee et al. 2003) and tobacco (Li and Yang 2000). No correlation between the level of transient expression and the subsequent level of stable transformation has been observed (Huber et al. 2002).

Monitoring of the *gfp* expression in transformed tissue can be used to improve the selection efficiency during the subsequent plant regeneration. For example, if the GFP fluorescence was observed during the regeneration of explants cultivated on media supplemented with hygromycin as a selective agent, it resulted in stringent, 4.5% transformation efficiency of red fescue and 82% regenerability, giving an effective transformation frequency 3.7% (Cho et al. 2000). The following example presents quite a different story: monitoring of green fluorescence was used as a tool for critical comparison of the efficacy of two strategies for rhododendron transformation, *Agrobacterium*-mediated and direct transformation. Successful *Agrobacterium*-mediated transformation of *Rhododendron* was previously reported by many authors (Ueno et al. 1996; Pavingerová et al. 1997; Tripepi et al. 1999). Knapp et al. (2001) reported a surprisingly low transformation efficacy (0.2%) after using the particle bombardment of rhododendron leaves as compared with the *Agrobacterium*-mediated transformation efficacy of 5% reported by Ueno et al. (1996). Based on these findings some possible reasons such as the difficulties of penetration of hard and waxy leave cells by gold particles, cell death caused by wounding by gold particles or degradation of naked DNA, were hypothesised and subsequently the transformation protocol was refined (Table 2).

GFP manifestation in regenerating shoots and mature plants

Following the recovery of a new transgenic plant, GFP fluorescence is usually visible in new emerging shoots and young tissues or organs, whereas it declines to give a weak signal in older ones (Kamaté et al. 2000; Tamura et al. 2003; Zhou et al. 2004). On the other hand, the variability in green fluorescence in early transformation stages was reported by some authors (Eady et al. 2000; Taniguchi et al. 2005). During subsequent regeneration the fluorescence normally declines to the extent that it is not visible in older tissues or organs. Weak or no fluorescence has normally been observed in mature leaves (Kamaté et al. 2000; Cho and Widholm 2002; Cui et al. 2003; Zhou et al. 2004; Taniguchi et al. 2005), with the occasional occurrence of small fluorescing regions in some cells (Eady et al. 2000) or organs, e.g. trichomes (Mercuri et al. 2001; Han et al. 2005) or stomatal guard cells (Kim et al. 2004). On the other hand, GFP fluorescence was normally visible in inflorescences, petals, stamens and pistils (Cui et al. 2003; Zhou et al. 2004), roots (Elliot et al. 1999; Zhou et al. 2004), whole flowers, plantlets and seedlings (Kamaté et al. 2000; Zhou et al. 2004), suggesting that the reason for low levels of GFP fluorescence in older leaves is associated with the increasing content of chlorophyll, which possess strong red autofluorescence, or other flourescing compounds.

Lowering of overall *gfp* expression level during the growth and development of organs may not be the sole reason for diminishing of fluorescence. Some authors studied this event in a more detailed way. For instance, Zhou et al. (2004) also reported high GFP fluorescence in young *Medicago truncatula*, var. A17 leaves and lowering of the fluorescence in older leaves. Based on it they decided to study mRNA levels in leaves of different age. Semi-quantitative RT-PCR showed similar RNA transcript spectra in all samples, indicating that the lack of expression is not the reason. An important fact is that, for many studies *gfp* driven by constitutive promoters such as CaMV 35S or *Act1* were mainly used, and although their constitutive features in transgenic

Table 2 Examples of transformation experiments using various variants of *gfp* gene

Plant species	*gfp* gene variant/s	Promoter/s	Transformed tissue	Selectable marker gene	Transformation method	References
Abies fraseri (Pursh) Poir, *Abies nordmanniana* (Stevens) Spach, *Pinus virginiana* Mill.	*mgfp5*-ER	CaMV 35S	Mature zygotic embryos	*nptII*	At	Tang and Newton (2005)
Aliium cepa L.	*mgfp5*-ER	CaMV 35S	Immature embryos	*nptII*	At	Eady et al. (2000)
Antirrhinum majus L.	*sgfp*(S65T)	CaMV 35S	Hypocotyls	*nptII*	At	Cui et al. (2003)
Arabidopsis thaliana (L.) Heynh.	*mgfp4*	CaMV 35S	Roots	*gfp*	PB	Haseloff et al. (1997)
Artemisia annua L.	*gfp*	CaMV 35S	Leaf explants	*nptII*	At	Han et al. (2005)
Astragalus sinicus L.	*mgfp5*-ER	CaMV 35S	Cotyledons	*nptII*	At	Cho and Widholm (2002)
Avena sativa L.	*sgfp*(S65T)	*Act1*	Shoot meristematic culture	*bar*, *hpt, nptII*	PB	Cho et al. (2003)
Avena sativa L.	*sgfp*	*ubi1*	Calli	*gfp*	PB	Goldman et al. (2003)
Brassica campestris L.	*mgfp5*-ER	CaMV 35S	Cotyledons	*nptII*	At	Malyshenko et al. (2003)
Brassica napus L.	*mgfp5*-ER, *egfp*	CaMV 35S	Hypocotyls	*nptII*	At	Cordoza and Stewart (2003)
Brassica rapa L.	*egfp*	CaMV 35S	Cotyledons	*nptII*	At	Wahlroos et al. (2003)
Cajanus cajan (L.) Millsp.	*mgfp5*-ER	CaMV 35S	Decapitated mature embryo axis	*nptII*	At	Mohan and Krishnamurthy (2003)
Carica papaya L.	*mgfp5*	CaMV 35S	Embryogenic calli	*nptII*	PB	Zhu et al. (2004)
Castanea dentata (Marsh.) Borkh.	*mgfp5*-ER	CaMV 35S	Somatic embryos	*nptII*	At	Polin et al. (2006)
Chamaecyparis obtusa Sieb. et Zucc.	*sgfp*(S65T)	CaMV 35S	Embryogenic tissues	*nptII*	At	Taniguchi at al. (2005)
Citrus aurantium L.	*sgfp*	CaMV 35S	Internodal stems, epicotyls	*nptII, gfp*	At	Ghorbel et al. (1999)
Citrus sinensis L. Osbeck	*egfp*	Double CaMV 35S	Protoplasts	*gfp*	E	Guo et al. (2005)
Cucumis melo L.	*mgfp5*	CaMV 35S	Cotyledons	*nptII*	At	Galperin et al. (2003)
Daucus carota L.	*mgfp5*-ER	CaMV 35S	Root discs	*gfp*	Ar	Baranski et al. (2006)
Dendrobium spp.	*sgfp*(S65T)	*Ubi1*, CaMV 35S, HBT	Tips of inflorescences, various types of calli	*gfp*	PB	Tee et al. (2003)
Festuca arundinacea Schreb.	*mgfp*	CaMV 35S	Embryogenic calli	*hph*	At	Wang and Ge (2005)
Festuca rubra L.	*sgfp*(S65T)	*Act1*	Highly regenerative tissue	*hpt*	PB	Cho et al. (2002)
Glycine max L.	SGFP-TYG, *mgfp4*, *mgfp5*-ER, *smgfp*, *sm*RS-*gfp*	CaMV 35S	Embryogenic tissue	*hpt*	PB	Ponappa et al. (1999)
Helianthus annuus L.	*mgfp*	CaMV 35S	Shoot apices	*nptII*	At	Weber et al. (2003)
Hordeum vulgare L.	*sgfp*(S65T)	*Act1*/ES-hordein	Immature embryos	*bar*	PB	Cho et al. (2002)
Hordeum vulgare L.	*sgfp*(S65T), *pgfp*	*Act1*, CaMV 35S	Microspore culture	*gfp*	PB	Carlson et al. (2001)
Hordeum vulgare L.	*pgfp*	*Act1*	Embryogenic calli	*gfp*	PB	Ahlandsberg et al. (1999)
Ipomoea batatas (L.) Lam.	*mgfp4*	CaMV 34S	Protoplasts, somatic embryos	*gfp*	E, PB	Lawton et al. (2000)
Juglans regia L.	*sgfp*(S65T)	CaMV 35S	Embryos	*nptII*	At	Escobar et al. (2000)

Table 2 continued

Plant species	*gfp* gene variant/s	Promoter/s	Transformed tissue	Selectable marker gene	Transformation method	References
Eustona grandiflorum (Raf.) Shinners, *Osteospermum ecklonis* (DC.) Norlindh	*mgfp4*, *mgfp5*-ER, *sgfp* (S65T/49), *sgfp* (S65T/63), *sgfp* S65C	CaMV 35S, enhanced CaMV 35S	Leaf explants	*nptII*	At	Mercuri et al. (2001)
Malus domestica Borkh.	*sgfp*-TYG	CaMV 35S	Leaf explants	*nptII*	At	Maximova et al. (1998)
Medicago truncatula Gaertn.	*sgfp*	CaMV 35S	Cotyledons, embryogenic axis	*bar*	At	Zhou et al. (2004)
Medicago truncatula Gaertn.	*gfp*	CaMV 35S	Floral organs	*nptII*	At	Kamaté et al. (2000)
Medicago truncatula Gaertn.	*mgfp5*-ER	CaMV 35S	Leaflets form 2–3 week-old plantlets	*nptII*	At	Chabaud et al. (2003)
Melastoma malabathricum L., *Tibouchina semidecandra* Cogn.	*mgfp*	CaMV 35S	Shoot, node explants	*gfp*	At	Yong et al. (2006)
Nemesia strumosa Benth.	*sgfp*(S65T)	CaMV 35S	Stem cuts	*nptII*	At	Cui and Ezura (2003)
Nicotiana tabacum L.	*gfp*	Not reported	Zygote	*gfp*	E	Li and Yang (2000)
Nicotiana tabacum L.	*mgfp5*-ER	CaMV 35S	Leaf discs	*nptII*	At	Chen et al. (2005)
Nicotiana tabacum L.	*s-gfp*	CaMV 35S	Leaf discs	*nptII*	At	Molinier et al. (2000)
Oryza sativa L.	*mgfp4*	CaMV 35S	Immature embryos	*aphIV*, *gfp*	PB	Vain et al. (1998)
Oryza sativa L.	*sgfp*(S65T)	CaMV 35S	Embryogenic calli	*hpt*	At	Sallaud et al. (2003)
Panicum virgatum L.	*sgfp*	*Act1*	Calli	*bar*	PB	Richards et al. (2001)
Pennisetum glaucum L.	*egfp*	CaMV 35S	Embryogenic calli	*bar*	PB	Goldman et al. (2003)
Pyrus communis L.	*gfp*	Not reported	Leaf explants	*nptII*	At	Yancheva et al. (2006)
Prunus persica L.	*sgfp*	CaMV 35S	Embryo sections	*nptII*	At	Pérez-Clemente et al. (2004)
Rhododendron spp.	sm*GFP*	CaMV 35S	Leaves	*nptII*	PB	Knapp et al. (2001)
Rosa hybrida L.	*mgfp5*-ER	CaMV 35S	Embryogenic calli	*nptII*	At	Kim et al. (2004)
Saccharum spp., *Zea mays* L., *Lactuca sativa* L., *Nicotiana tabacum* L.	*sgfp* (S65T), *mgfp5*-ER	CaMV 35S, *Ubi1*	Coleoptyles, calli, cotyledons, leaf explants	*aphA*, *gfp*	At, PB	Elliot et al. (1999)
Sorghum spp.	smRS-*gfp*	CaMV 35S, *Ubi1*, HBT	Embryogenic calli, immature embryos, leaves	Not reported	At, PB	Jeoung et al. (2002)
Spinacia oleracea L.	*smgfp*	CaMV 35S	Cotyledons	*nptII*	At	Zhang and Zeevaart (1999)
Theobroma cacao L.	*egfp*	CaMV 35S	Cotyledons	*nptII*	At	Pishak et al. (2003)
Triticum aestivum L.	S65T*gfp*	CaMV 35S	Immature embryos	*bar*	PB	Huber et al. (2002)
Triticum aestivum L	*sgfp*(S65T)	*Act1*	Embryos	*gfp*	PB	Jordan (2000)
Verbena x hybrida	*sgfp*(S65T)	CaMV 35S	Shoots	*nptII*	At	Tamura et al. (2003)
Vigna angularis (Willd.) Ohwi et Ohashi	*sgfp*(S65T)	CaMV 35S	Epicotyls	*nptII*	At	Yamada et al. (2001)
Vitis vinifera L.	*egfp*	Enhaced CaMV 35S, CsVMV, double CsVMV, ACT2	Somatic embryos	*nptII*	At	Li et al. (2001)
Zea mays L.	*mgfp*	*Ubi1*	Embryogenic calli	*bar*	PB	van der Geest and Petolino (1998)

Table 2 continued

Following abbreviations are used throughout the table:

At, *Agrobacterium tumefaciens*-mediated transfer; Ar, *A. rhizogenes*-mediated transfer; PB, particle bombardment transformation; E, electroporation; *aph*, aminoglykosid phosphotransferase gene; *bar*, phosphinotricin acetyltransferase gene; *hph*, hygromycin phosphotransferase gene; *nptII*, neomycin phosphotransferase gene; *egfp*, engineered *gfp* gene; *mgfp5*-ER, modified *gfp* targeted to the endoplasmatic reticulum; *pgfp*, plant optimized *gfp* gene; *sgfp*, synthetic green fluorescent gene; *sm-gfp*, soluble-modified *gfp* gene

plants have been reported (Benfey et al. 1989; Battraw and Hall 1990), recent studies revealed that some differences in expression can occur (Williamson et al. 1989; Malik et al. 2002; Sunilkumar et al. 2002).

Therefore, some spatial or other fluctuations are possible. Such presumption was confirmed by Zhou et al. (2004), who reported different *gfp* expression patterns in *Medicago truncatula* plants. It is not clear whether an aberrant activity of CaMV 35S promoter is the reason for fluorescence quenching, and/or production of a quenching substance such as protease could also be involved (Zhou et al. 2004). Finally, the expression of *gfp* might be influenced by the positional effect of inserted transgenes or by co-suppression due to the higher transgene copy number (Tamura et al. 2003).

Instrumentation and approaches for GFP visualization, occurrence of interfering factors and diminishing of green fluorescence

The fluorescence properties of GFP allow for detection of gene expression in whole living plants with some simple UV lamp or more precise visualization of various events in living cells using fluorescence microscopy (Haseloff 1999).

Various observation systems are being used to study the GFP fluorescence. These usually consist of an excitation source, detection or observation device and usually appropriate filter sets. Previous investigations of GFP fluorescence mostly utilised high-power microscopes, but recent studies usually found that low-power microscopes and various hand-held UV or blue light sources could be sufficient too (Elliot et al. 1999; Li et al. 2001; Cui et al. 2003). Some instruments, because they exhibit a wide range of broad-wavelengths and wide light-diffusion angles, and therefore possess only limited energy in the wavelengths required for GFP excitation, can be used in situations of high *gfp* expression levels only (Vain et al. 1998). In addition, various confocal laser scanning microscopes are used for more detailed studies, e.g. of the precise sub-cellular GFP localization, allowing for the reconstruction for three-dimensional structures (Haseloff 1999; Belluci et al. 2003;

Pérez-Clemente et al. 2004). The use of the appropriate observation and excitation system is a prerequisite for successful GFP study. For example, Ponappa et al. (1999) reported weaker fluorescent signals after excitation of soybean embryogenic cultures when 50 W mercury lamp was used instead of stronger 100 W source.

Intact plant tissue represents a complicated subject for common fluorescence microscopy. Deep layers of highly refractile walls and aqueous cytosol coupled with the occurrence of various autofluorescence and light scattering compounds also make confocal microscopy a difficult mission. To circumvent these obstacles, fixing and clarification of studied samples in a high refractive index medium (1) or the use of suitable optic set (2) is recommended (Haseloff 1999). Nevertheles, in the case of *Arabidopsis* wholemounts, the first approach was associated with the loss of GFP fluorescence (Haseloff and Amos 1995). It should be noted in this context that direct visualization of GFP fluorescence does not require any fixation, staining or addition of some substrates, and allows for study of various events within the living cells such as cytoplasmatic streaming. Moreover, the presence of various autofluorescent organelles and compounds can be employed as a useful counter staining tool. This can be enhanced by addition of some exogenous substrates (Haseloff 1999).

On the other hand, loss of, or lack of GFP fluorescence is not always associated with the interference of various undesirable signals co-emitted along with the GFP signal. It can also be caused by pigment, which is opaque to exciting UV or blue light and thus negatively affects the effect of exciting light. Mercuri et al. (2001), who detected sufficient levels of GFP protein in transgenic *Limonium* flowers, failed to detect macroscopic green fluorescence due to the presence of various floral pigments. Another cause of the GFP fluorescence quenching in older leaves may be a change in cytoplasmic density of cells. This may explain, why the GFP manifestation is visible better in young cells and organs, than in older ones, especially leaves, since the vacuoles devoid of GFP constitute the largest part of the cell and finally ‘‘dilute’’ the GFP content (Maximova et al. 1998; Molinier et al. 2000; Cho and Widholm 2002). As can be seen from the above discussion, the quenching of GFP signal in mature or older transformed tissues and organs commonly occurs.

However, the most important cause seems to be the chlorophyll red autofluorescence interfering with the GFP green fluorescence, which finally obscures the GFP manifestation, so that it is often only visible in albino tissues lacking the chlorophyll such as roots (Cho et al. 2000; Carlson et al. 2001; Huber et al. 2002). The same observations were published by many other authors (van der Geest and Petolino 1998; Vain et al. 1998; Ponappa et al. 1999; Kaeppler et al. 2000; Jordan 2000; Cho et al. 2002; Zhou et al. 2005), suggesting that the chlorophyll autofluorescence precludes the GFP visualization in tissues with high chlorophyll content. In some cases the GFP fluorescence is visible through the chlorophyll background (Goldman et al. 2003). This can be effectively enhanced by using appropriate filter sets cutting off the undesirable autofluorescence (Ahlandsberg et al. 1999; Jordan 2000; Kamaté et al. 2000; Molinier et al. 2000; Richards et al. 2001; Taniguchi et al. 2005). A brief list of various observation devices coupled with suitable filters is given in Table 3. A different approach was reported by Wahlroos et al. (2003), who used laser-scanning microscopy for study of putative *Brassica rapa* plants, which possess a strong background fluorescence after the illumination with a hand-held long-wave UV lamp to confirm the transgene expression and transgenic status of plants.

Other possible reasons for the poor expression are developmental or cell specific expression of 35S promoter (Ponappa et al. 1999; Zhou et al. 2004), dilution of GFP content in dividing and growing cells (Zhou et al. 2004) or gene silencing (Voinnet and Baulcombe 1997).

Attempts to use GFP as an alternative selection tool in plant transformations

The early visualization and identification of transgenic events using GFP fluorescence allows the regeneration of transgenic cells without any selective (either negative or positive) pressure. GFP fluorescence can serve as a tool for rapid discrimination of transformed and non-

transformed cells, calli and shoots and hence help to eliminate untransformed cells and shoots from further cultivation. Unfortunately, this approach depends on high transformation frequencies, resulting in the development of sufficiently large clusters of cells or organs that can be relatively easily handled. This requires continuous suppression or removal of untransformed cells, followed by sub-culturing of transformed cells. Such approaches have been found to be labour and time consuming (Ghorbel et al. 1999). Elliott et al. (1999) tested the use of visual selection based on GFP fluorescence in comparison with conventional antibiotic selection. They bombarded sugarcane calli and isolated regenerating green fluorescent calli. However, it was difficult to maintain preferential growth of transformed cells, despite the fact that non-fluorescing cells were removed. Furthermore the sectioning of calli was reported to alter the direction rate of growth within individual clusters of cells. After 12 weeks they obtained 2.4 ± 0.9 (SE) green fluorescent calli that reached at least 5 mm in diameter. This was less than average callus formation on geneticin (29.6 ± 1.6). They suggested that the conventional selection is more suitable for routine production of transgenic plants. Quite similar conclusions were reported by Jordan (2000), who cultivated bombarded wheat embryos for the first 4 weeks on a medium without antibiotics, but additional application of antibiotics led to stringent selection of transgenic plants among regenerants. On the other hand, Baranski et al. (2006) were more successful when they screened *A. rhizogenes*-transformed adventitious roots emerged from co-cultivated carrot root discs for GFP fluorescence. Roots positive for green fluorescence were selected for further regeneration and it has been shown that such approach can be an efficient method for the production of transgenic carrot. Although possibilities for selection exclusively based on a screening for GFP fluorescence are limited, due to difficulties in identification of fluorescent tissues and plants among large masses of cells or shoots, some recent reports have confirmed that such an approach is promising for transformation of some objects, and represents a new alternative to current selection schemes (Jordan 2000; Baranski et al. 2006).

Conclusion remarks and further prospects

Green fluorescent protein offers a wide range of applications in plant biology (Leffel et al. 1997; Stewart 2001). Although the study of green fluorescence in plants embodies its own obstacles, it possesses many advantages compared with other marker genes. Monitoring of GFP green fluorescence allows for the rapid non-invasive identification of transformed cells and, therefore, early elimination of non-transformed cells. It has been shown in many cases that GFP fluorescence has been successfully used for the critical evaluation of various transformation parameters resulting in subsequent modifications of transformation protocols. Therefore, plant transformation could be faster and less labour intensive and thus cheaper. Moreover, it may help to identify and therefore to reduce negative events associated with plant transformation (e.g. gene silencing) and to facilitate the successful recovery of transgenic plant tissues, which stably express the gene of interest (El-Shemy et al. 2004). Additionally, various attempts at quantitative or semi-quantitative detection of GFP fluorescence have been reported recently (Millwood et al. 2003; Hraška et al. 2005), allowing for the early identification of homozygotes (Molinier et al. 2000) or estimation of recombinant protein content in transgenic plants (Halfhill et al. 2003; Richards et al. 2003a, b). Such new methods represent an additional asset of GFP use to plant transgenesis.

Acknowledgements The authors are grateful to Prof. Yuri Gleba (Icon Genetics, München, Germany) for critical comments and Dr. Jeremy Sweet (NIAB, Cambridge, UK) for kind proof reading of the manuscript. This work was supported by grants no. 1M06030, 1PO5ME800 and MSM 60076658-06 of the Ministry of Education, Youth and Sport of the Czech Republic. M. H. gratefully acknowledges the support from the Grant Agency of the Czech Republic, no. GA ČR-31/H160.

References

Ahlandsberg S, Sathish P, Sun Ch, Jansson Ch (1999) Green fluorescent protein as a reporter system in the transformation of barley cultivars. Physiol Plant 107:194–200

Bauchera M, Montiesb B, van Montagua M, Boerjana W (1998) Biosynthesis and genetic engineering of lignin. Crit Rev Plant Sci 17:125–197

Baranski R, Klocke E, Schumann G (2006) Green fluorescent protein as an efficient selection marker for *Agrobacterium rhizogenes* mediated carrot transformation. Plant Cell Rep 25:190–197

Battraw MJ, Hall TC (1990) Histochemical analysis of CaMV 35S promoter-β-glucuronidase gene expression in transgenic rice plants. Plant Mol Biol 15:527–538

Baumann CT, Lim CS, Hager GL (1998) Simultaneous visualization of the yellow and green forms of the green fluorescent protein in living cells. J Histochem Cytochem 46:1073–1076

Bellucci M, De Marchis F, Mannucci R, Arcioni S (2003) Jellyfish green fluorescent protein as a useful reporter for transient expression and stable transformation in *Medicago sativa* L. Plant Cell Rep 22:328–337

Benfey PN, Ren L, Chua N (1989) The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J 8:2195–2202

Birch RG (1997) Plant transformation: problems and strategies for practical application. Annu Rev Plant Physiol Plant Mol Biol 48:297–326

Cardoza V, Stewart CN (2003) Increased *Agrobacterium*-mediated transformation and rooting efficiencies in canola (*Brassica napus* L.) from hypocotyl segment explants. Plant Cell Rep 21:599–604

Carlson AR, Letarte J, Chen J, Kasha KJ (2001) Visual screening of microspore-derived transgenic barley (*Hordeum vulgare* L.) with green-fluorescent protein. Plant Cell Rep 20:331–337

Chabaud M, de Carvalho-Niebel F, Barker DG (2003) Efficient transformation of *Medicago truncatula* cv. Jemalong using the hypervirulent *Agrobacterium tumefaciens* strain AGL1. Plant Cell Rep 22:46–51

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994) Green fluorescent protein as a marker for gene expression. Science 263:802–805

Chen S, Li X, Liu X, Xu H, Meng K, Xiao G, Wei X, Wang F, Zhu Z (2005) Green fluorescent protein as a vital elimination marker to easily screen marker-free transgenic progeny derived form plants co-transformed with double T-DNA binary vector system. Plant Cell Rep 23:625–631

Chilton M-D, Drummond MH, Merlo DJ, Sciaky D, Montoya AL, Gordon MP, Nester EW (1977) Stable incorporation of plasmid DNA into higher plant-cells-the molecular-basis of crown gall tumorigenesis. Cell 11:263–271

Chiu W, Niva Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. Curr Biol 6:325–330

Cho H-J, Widholm JM (2002) *Agrobacterium tumefaciens*-mediated transformation of the legume *Astralagus sinicus* using kanamycin resistance selection and green fluorescent protein expression. Plant Cell Tissue Organ Cult 69:251–258

Cho M-J, Ha CD, Lemaux PG (2000) Production of transgenic tall fescue and red fescue plants by particle bombardment of mature seed-derived highly regenerative tissues. Plant Cell Rep 19:1084–1089

Cho M-J, Choi H-W, Jiang W, Ha ChD, Lemaux PG (2002) Endosperm-specific expression of green fluorescent protein driven by the hordein promoter is stably inherited in transgenic barley (*Hordeum vulgare*) plants. Physiol Plant 115:144–154

Cho M-J, Choi HW, Okamoto D, Zhang S (2003) Expression of green fluorescent protein and its inheritance in transgenic oat plants generated from shoot meristematic cultures. Plant Cell Rep 21:467–474

Christou P (1995) Strategies for variety-independent genetic transformation of important cereal, legumes and woody species utilizing particle bombardment. Euphytica 85:13–27

Christou P (1997) Rice transformation: bombardment. Plant Mol Biol 35:197–203

Chudakov DM, Lukyanov S, Lukyanov KA (2005) Fluorescent proteins as a toolkit for *in vivo* imaging. Trends Biotechnol 23:605–613

Chytilova E, Macas J, Galbraith DW (1999) Green fluorescent protein targeted to the nucleus, a transgenic phenotype useful for studies in plant biology. Ann Bot 83:645–654

Cody CW, Prasher DC, Westler WM, Prendergast FG, Ward WW (1993) Chemical structure of the hexapeptide chromophore of *Aeqourea*-green fluorescent protein. Biochemistry 32:1212–1218

Cui M-L, Ezura H (2003) *Agrobacterium*-mediated transformation of *Nemesia strumosa* Benth, a model plant for asymmetric floral development. Plant Sci 165:863–870

Cui M-L, Handa T, Ezura H (2003) An improved protocol for *Agrobacterium*-mediated transformation of *Antirrhinum majus* L. Mol Genet Genomics 270:296–302

Davis SJ, Viestra RD (1998) Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. Plant Mol Biol 36:521–528

Eady CC, Weld RJ, Lister CE (2000) *Agrobacterium tumefaciens*-mediated transformation and transgenic-plant regeneration of onion (*Allium cepa* L.). Plant Cell Rep 19:376–381

Elliott AR, Campbell JA, Dugdale B, Brettell RISL, Grof CP (1999) Green-fluorescent protein facilitates rapid *in vivo* detection of genetically transformed plant cells. Plant Cell Rep 18:707–714

El-Shemy HA, Teraishi M, Khalafalla MM, Katsube-Tanaka T, Utsumi S, Ishimoto M (2004) Isolation of soybean plants with stable transgene expression by visual selection based on green fluorescent protein. Mol Breed 14:227–238

Escobar MA, Park J-I, Polito VS, Leslie Ch A, Uratsu SL, McGranahan GH, Dandekar AM (2000) Using GFP as a scorable marker in walnut somatic embryo transformation. Ann Bot 85:831–835

Finer JJ, Finer KR, Ponappa T (1999) Particle bombardement mediated transformation. Curr Topics Microbiol Immunol 240:59–80

Franchea C, Laplazea L, Duhouxa E, Bogusza D (1998) Actinorhizal symbioses: recent advances in plant molecular and genetic transformation studies. Crit Rev Plant Sci 17:1–28

Galperin M, Patlis L, Ovadia A, Wolf D, Zelcer A, Kenigsbuch D (2003) A melon genotype with superior competence for regeneration and transformation. Plant Breed 122:66–69

Ghorbel R, Juárez J, Navarro L, Pena L (1999) Green fluorescent protein as a screenable marker to increase the efficiency of generating transgenic woody fruit plants. Theor Appl Genet 99:350–358

Goldman JJ, Hanna WW, Fleming G, Ozias-Akins P (2003) Fertile transgenic pearl millet [*Pennisetum glaucum* (L.) R. Br.] plants recovered though microprojectile bombardment and phosphinothricin selection of apical meristem-, inflorescence-, and immature embryo-derived embryogenic tissues. Plant Cell Rep 21:999–1009

Guo W, Duan Y, Olivares-Fuster O, Wu Z, Aris CR, Burns JK, Grosser JW (2005) Protoplast transformation and regeneration of transgenic Valencia sweeet orange plants containing a juice duality-related pectin methylesterase gene. Plant Cell Rep 24:482–486

Haas J, Park E-C, Seed B (1996) Codon usage limitation in the expression of HIV-1 envelope glycoprotein. Curr Biol 6:315–324

Halfhill MD, Millwood RJ, Rufty TW, Weissinger AK, Stewart CN (2003) Spatial and temporal patterns of green fluorescent protein (GFP) fluorescence during leaf canopy development in transgenic oilseed rape, *Brassica napus* L. Plant Cell Rep 22:338–343

Halfhill MD, Richards HA, Mabon SA, Stewart Jr CN (2001) Expression of GFP and Bt transgenes in *Brassica napus* and hybridization with *Brassica rapa* Theor. Appl Genet 130:659–667

Han J, Wang H, Ye H, Liu Y, Li Z, Zhang Y, Zhang Y, Yan F, Li G (2005) High efficiency of genetic transformation and regeneration of *Artemisia annua* L. via *Agrobacterium tumefaciens*-mediated procedure. Plant Sci 168:73–80

Harper BK, Mabon SA, Leffel SM, Halfhill MD, Richards HA, Moyer KA, Stewart Jr CN (1999) Green fluorescent protein as a marker for expression of a second gene in transgenic plants. Nature Biotech 17:1125–1129

Haseloff J (1999) GFP variants for multispectral imaging of living cells. Methods Cell Biol 58:139–151

Haseloff J, Amos B (1995) GFP in plants. Trends Genet 11:328–329

Haseloff J, Siemering KR, Prasher DC, Hodge S (1997) Removal of a cryptic intron and subcellular localisation of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proc Natl Acad Sci USA 94:2122–2127

Heim R, Cubitt AE, Tsien RY (1995) Improved green fluorescence. Nature 373:663–664

Heim R, Prasher DC, Tsien RY (1994) Wavelenght mutations and posttranslational autooxidation of green fluorescent protein. Proc Natl Acad Sci USA 91:12501–12504

Helmer G, Casadaban M, Bevan M, Kayers L, Chilton MD (1984) A new chimeric gene as a marker for plant transformation: the expression of *Escherichia coli* β-galactosidase in sunflover and tobacco cells. Biotechnology (NY) 2:520–527

Hraška M, Rakouský S (2005) GFP detection in low level signal/noise ratio plant samples. J Appl Biomed 3:S19

Hraška M, Rakouský S, Kocábek T (2005) Use of simple semiquantitative method for appraisal of green fluorescent protein gene expression in transgenic tobacco plants. Biol Plant 49:313–316

Hu W, Cheng CL (1995) Expression of *Aequorea* green fluorescent protein in plant cells. FEBS Lett 369:331–334

Huber M, Hahn R, Hess D (2002) High transformation frequencies obtained from a commercial wheat (*Triticum aestivum* L. cv. ''Combi'') by microbombardment of immature embryos followed by GFP screening combined with PPT selection. Mol Breed 10:19–30

Itaya A, Hickman H, Bao Y, Nelson R, Ding B (1997) Cell-to-cell trafficking of cucumber mosaic virus movement protein:green fluorescent protein fusion produced by biolistic gene bombardment in tobacco. Plant J 12:1223–1230

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Jeoung JM, Krishnaveni S, Muthukrishnan S, Trick HN, Liang GH (2002) Optimization of sorghum transformation parameters using genes for green fluorescent protein and β-glucuronidase as visual markers. Hereditas 137:20–28

Jordan MC (2000) Green fluorescent protein as a visual marker for wheat transformation. Plant Cell Rep 19:1069–1075

Kaeppler HF, Menon GK, Skadsen RW, Nuutila AM, Carlson AR (2000) Transgenic oat plants via visual selection of cells expressing green fluorescent protein. Plant Cell Rep. 19:661–666

Kamaté K, Rodriguez-Llorente ID, Scholte M, Durand P, Ratet P, Kondorosi E, Kondorosi A, Trinh TH (2000) Transformation of floral organs with GFP in *Medicago truncatula*. Plant Cell Rep 19:647–653

Kim CK, Chung JD, Park SH, Burrell AM, Kamo KK, Byrne DH (2004) *Agrobacterium tumefaciens*-mediated transformation of *Rosa hybrida* using green fluorescent protein (GFP) gene. Plant Cell Tissue Organ Cult 78:107–111

Kinoshita M (2004) Transgenic medaka with brilliant fluorescence in skeletal muscle under normal light. Fish Sci 70:645–649

Klein TM, Wolf ED, Wu R, Stanford JC (1987) High-velocity microprojectiles for delivering nucleic acids into living cells. Nature 327:70–73

Knapp JE, Kausch AP, Auer C, Brand MH (2001) Transformation of *Rhododendron* through microprojectile bombardment. Plant Cell Rep 20:749–754

Kocábek T, Rakouský S, Ondřej M, Řepková J, Relichová J (1999) Identification and mapping of a T-DNA induced flower mutation in *Arabidopsis thaliana*. Biol Plant 42:349–359

Lawton R, Winfield S, Daniell H, Bhagsari AS, Dhir SK (2000) Expression of green-fluorescent protein gene in sweet potato tissues. Plant Mol Biol Rep 18:139a–139i

Leffel SM, Mabon SA, Stewart Jr CN (1997) Applications of green fluorescent protein in plants. Biotechniques (NY) 23:912–918

Li ST, Yang HY (2000) Gene transfer into isolated and cultured tobacco zygotes by a specially designed device for electroporation. Plant Cell Rep 19:1184–1187

Li Z, Jayasankar S, Gray DJ (2001) Expression of a bifunctional green fluorescent protein (GFP) fusion marker under the control of three constitutive promoters and enhanced derivatives in transgenic grape (*Vitis vinifera*). Plant Sci 160:877–887

Malik K, Wu K, Li X-Q, Martin-Heller T, Hu M, Foster E, Tian L, Wang C, Ward K, Jordan M, Brown D, Gleddie S, Simmonds D, Zheng S, Simmonds J, Miki B (2002) A constitutive gene expression system derived from the *tCUP* cryptic promoter elements. Theor Appl Genet 105:505–514

Malyshenko SI, Tyuĺkina LG, Zvereva SD, Raldugina GN (2003) Transgenic *Brassica campestris* plants expressing the gfp gene. Russ J Plant Physiol 50:276–281

Maximova SN, Dandekar AM, Guiltinan MJ (1998) Investigation of *Agrobacterium*-mediated transformation of apple using green fluorescent protein: high transient expression and low stable transformation suggest that factors other than T-DNA transfer are rate-limiting. Plant Mol Biol 37:549–559

Mercuri A, Sacchetti A, De Benedetti L, Schiva T, Alberti S (2001) Green fluorescent flowers. Plant Sci 161:961–968

Miki B, McHugh S (2004) Selectable marker genes in transgenic plants: applications, alternatives and biosafety. J Biotechnol 107:193–232

Millwood RJ, Halfhill MD, Harkins D, Russotti R, Stewart Jr CN (2003) Instrumentation and methodology for quantitative GFP fluorescence in intact plant organs. Biotechniques (NY) 24:638–643

Misteli T, Spector DL (1997) Applications of the green fluorescent protein in cell biology and biotechnology. Nature Biotech. 15:961–963

Mohan ML, Krishnamurthy KV (2003) Plant regeneration form decapitated mature embryo axis and *Agrobacterium* mediated genetic transformation of pigeonpea. Biol Plant 46:519–527

Molinier J, Himber C, Hahne G (2000) Use of green fluorescent protein for detection of transformed shoots and homozygous offspring. Plant Cell Rep 19:219–223

Morschhäuser J, Michel S, Hacker J (1998) Expression of a chromosomally integrated, single-copy GFP gene in *Candida albicans*, and its use as a reporter of gene regulation. Mol Gen Genet 257:412–420

Murray F, Brettell R, Matthewes P, Bishop D, Jacobsen J (2004) Comparison of *Agrobacterium*-mediated transformation of four barley cultivars using the GFP and GUS reporter genes. Plant Cell Rep 22:397–402

Nagatani N, Takumi S, Tomiyama M, Shimada T, Tamiya E (1997) Semi-real time imaging of the expression a maize polyubiquitin promoter-GFP gene in transgenic rice. Plant Sci 124:49–56

Niedz RP, Sussman MR, Satterlee JS (1995) Green fluorescent protein: an *in vivo* reporter of plant gene expression. Plant Cell Rep 14:403–406

Ow DW, Wood KV, Deluca M, Dewet JR, Helinski DR, Howell SH (1986) Transient and stable expression of firefly luciferase gene in plant cells and transgenic plants. Science 234:856–859

Panstruga R (2004) A golden shot: how ballistic single cell tarnsformation boosts the molecular analyse of cereal-mildew interactions. Mol Plant Pathol 5:141–148

Pavingerová D, Bříza J, Kodýtek K, Niedermeierová H (1997) Transformation of *Rhododendron* spp. using *Agrobacterium tumefaciens* with a GUS-intron chimeric gene. Plant Sci 122:165–171

Pérez-Clemente RS, Pérez-Sanjuán A, García-Férriz L, Beltrán J-P, Canas LA (2004) Transgenic peach plants (*Prunus persica* L.) produced by genetic transformation of embryo sections using the green fluorescent protein (GFP) as an *in vivo* marker. Mol Breed 14:419–427

Pishak S, Young A, Guiltinan MJ (2003) Stable transformation of *Theobroma cacao* L. and influence of matrix attached regions on GFP expression. Plant Cell Rep 21:872–883

Polin LD, Liang H, Rothrock RE, Nishii M, Diehl DL, Newhouse AE, Nairn CJ, Powell WA, Maynard ChA (2006) *Agrobacterium*-mediated transformation of American chestnut (*Castanea dentata* (Marsh.) Borkh.) somatic embryos. Plant Cell Tissue Organ Cult 84:69–79

Ponappa T, Brzozowski AE, Finer JJ (1999) Transient expression and stable transformation of soybean using the jellyfish green fluorescent protein. Plant Cell Rep 19:6–12

Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier MJ (1992) Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene 111:229–233

Rakouský S, Kocábek T, Vincenciová R, Ondřej M (1997/98) Transient ß-glucuronidase activity after infiltration of *Arabidopsis thaliana* by *Agrobacterium tumefaciens*. Biol Plant 40:33–41

Reichel C, Mathur J, Eckes P, Langenkemper K, Koncz C, Shell J, Reiss B, Mass C (1996) Enhanced green fluorescence by the expression of an *Aequorea victoria* green fluorescent protein mutant in mono- and dicotyledonous plant cells. Proc Natl Acad Sci USA 93:5888–5893

Repellin A, Baga M, Jauhar PP, Chibbar RN (2001) Genetic enrichment of cereal crops via alien gene transfer: new challenges. Plant Cell Tissue Organ Cult 64:159–183

Richards HA, Han CT, Hopkins RG, Failla ML, Ward WW, Stewart CN (2003a) Safety assessment of recombinant green fluorescent protein orally administered to weaned rats. J Nutr 133:1909–1912

Richards HA, Halfhill MD, Millwood RJ, Stewart CN (2003b) Quantitative GFP fluorescence as an indicator of recombinant protein synthesis in transgenic plants. Plant Cell Rep 22:117–121

Richards HA, Rudas VA, Sun H, McDaniel JK, Tomaszewski Z, Conger BV (2001) Construction of a GFP-BAR plasmid and its use for switchgrass transformation. Plant Cell Rep 20:48–54

Rouwendal GJA, Mendes O, Wolbert EJH, deBoer AD (1997) Enhanced expression in tobacco of the gene encoding green fluorescent protein by modification of its codon usage. Plant Mol Biol 33:989–999

Sallaud C, Meynard D, van Boxtel J, Gay C, Bes M, Brizard JP, Larmande P, Ortega D, Raynal M, Portefaix M, Ouwerkerk PBF, Rueb S, Delseny M, Guiderdoni E (2003) Highly efficient production and characterization of T-DNA plants for rice (*Oryza sativa* L.) functional genomics. Theor Appl Genet 106:1396–1408

Sheen J, Hwang S, Niwa Y, Kobayashi H, Galbraith DW (1995) Green-fluorescent protein as a new vital marker in plant cells. Plant J 8:777–784

Shiina T, Hayashi K, Ishii N, Morikawa K, Toyoshima Y (2000) Chloroplast tubules visualized in transplastomic plants expressing green fluorescent protein. Plant Cell Physiol 41:367–371

Shinomura O (1979) Structure of the chromophore of *Aeqourea* green fluorescent protein. FEBS Lett 104:220–222

Siemering KR, Golbik R, Sever R, Haselloff (1996) Mutations that supress the thermosensitivity of green fluorescent protein. Curr Biol 6:1653–1663

Smirnoff N, Wheeler GL (2000) Ascorbic acid in plants: biosynthesis and function. Crit Rev Plant Sci 19:267–290

Snape JW (1998) Golden calves or white elephants-biotechnologies for wheat improvement. Euphytica 100:207–217

Stewart CN Jr (2001) The utility of green fluorescent protein in transgenic plants. Plant Cell Rep 20:376–382

Sunilkumar G, Mohr L, Lopata-Finch E, Emani C, Kathore KS (2002) Developmental and tissue-specific expression of a CaMV 35S promoter in cotton as revealed by GFP. Plant Mol Biol 50:463–474

Tamura M, Togami J, Ishiguro K, Nakamura N, Katsumoto Y, Suzuki K, Kusumi T, Tanaka Y (2003) Regeneration of verbena (*Verbena x hybrida*) by *Agrobacterium tumefaciens*. Plant Cell Rep 21:459–466

Tang W, Newton RJ (2005) Transgenic Christmas trees regenerated from *Agrobacterium tumefaciens*-mediated transformation of zygotic embryos using the green fluorescence protein as a reporter. Mol Breed 16:235–246

Taniguchi T, Kurita M, Ohmiya Y, Kondo T (2005) *Agrobacterium tumefaciens*-mediated transformation of embryogenic tissue and transgenic plant regeneration in *Chamaecyparis obtusa* Sieb. et Zucc. Plant Cell Rep 23:796–802

Tee CS, Marziah M, Tan CS, Abdullah MP (2003) Evaluation of different promoters driving the GFP reporter gene and selected target tissues for particle bombardment of *Dendrobium Sonia* 17. Plant Cell Rep 21:452–458

Tripepi RR, George MW, Sripo T, Johnsen SA, Caplan AB (1999) Infection and transformation of *Rhododendron* by *Agrobacterium tumefaciens* strain B6. HortScience 34:455

Tsien RY (1998) The green fluorescent protein. Annu Rev Biochem 67:509–544

Ueno K, Fukunaga Y, Arusumi K (1996) Genetic transformation of *Rhododendron* by *Agrobacterium tumefaciens*. Plant Cell Rep 16:38–40

Vain P, Worland B, Kohli A, Snape JW, Christou P (1998) The green fluorescent protein (GFP) as a vital screenable marker in rice transformation. Theor Appl Genet 96:164–169

van der Geest AHM, Petolino JF (1998) Expression of modified green fluorescent protein gene in transgenic maize plants and progeny. Plant Cell Rep 17:760–764

Voinnet O, Baulcombe DC (1997) Systemic signalling in gene silencing Nature 389:553

Wahlroos T, Susi P, Tylkina L, Malyshenko S, Zvereva S, Korpela T (2003) *Agrobacterium*-mediated transformation and stable expression of the green fluorescent protein in *Brassica rapa*. Plant Physiol Biochem 41:773–778

Wang S, Hazelrigg T (1994) Implications for *bdc* mRNA localization from the spatial distribution of *exu* protein in *Drosophila* oogenesis. Nature 369:400–403

Wang Z-Y, Ge Y (2005) *Agrobacterium*-mediated high efficiency transformation of tall fescue (*Festuca arundinacea*). J Plant Physiol 162:103–113

Weber S, Friedt W, Landes N, Molinier J, Himber C, Rousselin P, Hahne G, Horn R (2003) Improved *Agrobacterium*-mediated transformation of sunflower (*Helianthus annuus* L.): assessment of macerating enzymes and sonication. Plant Cell Rep 21:475–482

Williamson JD, Hirsch-Wyncott ME, Larkins BA, Gelvin SB (1989) Differential accumulation of a transcript driven by the CaMV 35S promoter in transgenic tobacco. Plant Physiol 90:1570–1576

Yamada T, Teraishi M, Hattori K, Ishimoto M (2001) Transformation of azuki bean by *Agrobacterium tumefaciens*. Plant Cell Tissue Organ Cult 64:47–54

Yancheva SD, Schlizerman LA, Golubowicz S, Ybloviz Z, Perl A, Hanynka U, Flaishman MA (2006) The use of green fluorescent protein (GFP) improves *Agrobacterium*-mediated transformation of ''Spadona'' pear (*Pyrus communis* L.). Plant Cell Rep 25:183–189

Yang F, Moss LG, Phillips Jr GN (1996a) The molecular structure of green fluorescent protein. Nature Biotech 14:1246–1251

Yang T-T, Cheng L, Kain SR (1996b) Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. Nucleic Acids Res 24:4592–4593

Yong WTL, Abdullah JO, Mahmood M (2006) Optimization of *Agrobacterium*-mediated transformation parameters for *Melastomataceae* spp. using green fluorescent protein (GFP) as a reporter. Sci Hortic DOI: 10.1016/j.scienta.2006.03.005

Zhang H-X, Zeevaart JAD (1999) An efficient *Agrobacterium tumefaciens*-mediated transformation and regeneration system for cotyledons of spinach (*Spinacia oleracea* L.). Plant Cell Rep 18:640–645

Zhou X, Carranco R, Vitha S, Hall TC (2005) The dark side of green fluorescent protein. New Phytol 168:313–322

Zhou X, Chandrasekharan MB, Hall TC (2004) High rooting frequency and functional analysis of GUS and GFP expression in transgenic *Medicago truncatula* A17. New Phytol 162:813–822

Zhu YJ, Agbayani R, Moore PH (2004) Green fluorescent protein as a visual marker for papaya (*Carica papaya* L.) transformation. Plant Cell Rep 22:660–667

Zolotukhin S, Potter M, Hauswirth W, Guy J, Muzyczka N (1996) A ''humanized'' green fluorescent protein cDNA adapted for high-level expression in mammalian cells. J Virol 70:4646–4654

4. 4. Sample topography and position within plant body influence the detection of the intensity of green fluorescent protein (GFP) fluorescence in the leaves of transgenic tobacco plants

Hraška, M., Heřmanová , V., Rakouský, S., Čurn, V. (2007) Sample topography and position within plant body influence the detection of the intensity of green fluorescent protein (GFP) fluorescence in the leaves of transgenic tobacco plants Plant Cell Rep. in press

Plant Cell Rep
DOI 10.1007/s00299-007-0431-7

GENETIC TRANSFORMATION AND HYBRIDIZATION

Sample topography and position within plant body influence the detection of the intensity of green fluorescent protein fluorescence in the leaves of transgenic tobacco plants

Marek Hraška · Veronika Heřmanová · Slavomír Rakouský · Vladislav Čurn

Received: 19 February 2007 / Revised: 25 May 2007 / Accepted: 1 August 2007
© Springer-Verlag 2007

Abstract The effect of the type of leaf tissue selected for the study of green fluorescent protein (GFP) fluorescence intensity was investigated here using the T_1 generation of transgenic tobacco expressing the *m-gfp5-ER* gene. The fluorescence of GFP was detected by fluorescence binocular microscope coupled with the CCD camera and quantified by means of image analyses using the Lucia® software. Mean brightness values from various leaf tissues were compared. First, an original data revealing the significant differences in the fluorescence intensity between the abaxial and adaxial surfaces are given. Stronger signal was detected on the abaxial side. Subsequently, the effect of the tissue location within the leaf surface was investigated and higher fluorescence was detected on the samples detached from leaf tips. Finally, the effect of the physiological age of leaves was studied using the in vitro clonally propagated plants. Leaves from the analogous positions within the plant body of three clones were investigated. The decrease in the fluorescence towards the plant top (youngest leaves) was observed in all studied plants. Surprisingly, the variability of the fluorescence within the clones of studied genotype was high enough to conclude, that the fluorescence of each individual is unique and affected by particular genotype and environment. Our study showed that the origin of leaf tissue selected for the GFP quantification is crucial and that the fluctuations in the fluorescence intensity should be taken into account when comparing the GFP fluorescence patterns of different plants. Moreover, the degree of fluorescence variability seems to be individually affected.

Keywords Gene expression · *gfp* · Fluorescence pattern · Image analysis · Leaf position

Communicated by A. Atanassov.

M. Hraška · S. Rakouský (✉)
Faculty of Health and Social Studies,
University of South Bohemia, Jírovcova 24,
370-01 České Budějovice, Czech Republic
e-mail: srak@bf.jcu.cz

M. Hraška · S. Rakouský
Faculty of Biological Sciences, Department of Genetics,
University of South Bohemia, Branišovská 31,
370-05 České Budějovice, Czech Republic

V. Heřmanová · V. Čurn
Biotechnological Centre, Faculty of Agriculture,
University of South Bohemia, Studentská 13,
370-05 České Budějovice, Czech Republic

Introduction

Among its many applications within the field of biology, the *gfp* gene coding for the green fluorescent protein (GFP) has a potential to become a very powerful tool for the monitoring and quantification of the transgene expression in plants (Halfhill et al. 2003). Although its use poses many advantages compared to other common marker genes frequently used in plant biology, monitoring of its expression is coupled with some limitations and obstacles. First, the loss, or the quenching, of the fluorescence signal in older tissues, especially leaves, usually occurs. The presence of some agent, which can mask the GFP fluorescence and/or is opaque to the excitation signal represents an obstacle, which could complicate the monitoring of the fluorescence emitted from particular tissue. Another reason could be the different cytoplasmic density

of cells in young and older leaves, leading to the "dilution" of GFP in older tissues and thus weaker fluorescence (reviewed by Hraška et al. 2006). Moreover, the fluorescence in older leaves tends to display higher variability. Second, the variability of the fluorescence among leaves situated in various positions within the plant body was previously reported (Halfhill et al. 2001, 2003). In addition, there was an indication of the influence of leaf tissue, leaf developmental stage and location within the plant body on the marker gene detection assay (Preťová et al. 2001). Unfortunately, those data were still insufficient for drawing general conclusions since only a few individuals were tested. Moreover, a different marker, the *uidA* gene was used. Another frequently discussed reason of observed fluctuations in transgene expression could be the specific tissue and developmental expression patterns of the promoters used, mostly the constitutive CaMV 35S (Preťová et al. 2001; Halfhill et al. 2003). Although the CaMV 35S is generally considered to be a constitutive promoter, differential expression patterns of transgenes driven by this promoter have been described previously for various plant species (Benfey and Chua 1989; Blumenthal et al. 1999; Rooke et al. 2000; Sunilkumar et al. 2002).

Regardless of the exact cause, some reports indicate, that the expression and/or the detection of marker genes differ in various developmental stages and tissues of plants (Preťová et al. 2001; Halfhill et al. 2003). Therefore, it is evident that the selection of proper tissue for the marker gene quantification is a crucial point for accurate study of GFP fluorescence and can affect the final interpretation of obtained data and their reliability.

The aim of our study was to analyse the GFP performance, especially fluctuations in intensity of its fluorescence, originating from various plant tissues, particularly leaves, by means of image analyses. Our previous study of the GFP fluorescence in leaves using this approach (Hraška et al. 2005) revealed pronounced fluctuations among various tissues within one particular plant or even one leaf. Thus, in this work we concentrated our effort on the deeper mapping of fluorescence intensity variations, with the aim to define the GFP fluorescence patterns within transgenic tobacco tissue and further development of the methodology and objectivity of the GFP fluorescence evaluations. A detailed study of the physiological background of observed events was not of our main concern. Individually obtained T_1 plants as well as clonally propagated T_1 plants of selected genotypes of transgenic tobacco plants were used for the purpose of our studies. The intensity of GFP fluorescence was studied both on the abaxial and adaxial sides of leaves, on the leaf discs detached from various positions within the leaf surface and in leaves of different physiological age.

Materials and methods

Plant material

Transgenic tobacco plants (*Nicotiana tabacum*, cv. Petit Havana, SR1 WT) carrying the *m-gfp5*-ER marker gene were obtained after *Agrobacterium*-mediated leaf discs transformation (Horsch et al. 1985). Transformation and regeneration procedures were described previously (Hraška et al. 2005). Putatively transgenic T_0 shoots regenerated on MS medium supplemented with 500 mg l^{-1} kanamycin-Kn were excised and transferred onto the MS medium of the same composition for rooting stimulation. Best looking, rooting and fresh green explants were transferred into soil in a greenhouse and grown to maturity under optimum temperature 21–23°C. Seeds obtained form selfed T_0 plants were surface sterilised and sown on cultivation medium containing a selective chemical (500 mg l^{-1} Kn) as described previously (Hraška et al. 2005). Selected regenerated transgenic and control T_1 plants were clonally micropropagated in vitro in a growth chamber and after roots emergence transferred to the soil and grown up to maturity in a glasshouse under the same condition as primary regenerants. Randomly selected T_1 transgenic genotypes denoted G1, G2, G3, G4, G5 and G6 and control, non-transgenic plants, were used in the following experiments.

DNA extraction and molecular characterisation of transgenic plants by PCR

Total DNA was extracted from 3 g of fresh tobacco leaves of glasshouse cultivated T_0 and T_1 transgenic and control plants using the CTAB extraction method, with slight modification according to Herskowitz (unpublished report) where an additional centrifugation after the initial heat incubation was included to decrease the presence of the semi-soluble pellet that comes down in the first precipitation. Following this step, chloroform-isoamyl alcohol (IAA) (24:1) was added and the extraction was performed according to the standard CTAB protocol (Doyle and Doyle 1990). Approximately 50 ng of total DNA was used in a PCR amplification performed in a total reaction volume of 20 µl, containing 2× PPP Combi Master Mix (Top-Bio, Czech Republic) and 0.16 µM of each primer. The following primer set was used to specifically amplify a region of 415 bp *gfp5* gene fragment: GFP5-u (5′-ACC CAG ATC ATA TGA AGC GG-3′), GFP5-l (5′-TTG GGA TCT TTC GAA AGG GC-3′) (Fig. 1). PCR amplification was performed on a PTC 100 thermal cycler (MJ Research, USA). Reaction samples were amplified during 32 cycles (94°C for 1 min, 55°C for 30 s, 72°C for 30 s), followed by

a final extension at 72°C for 10 min. PCR products were visualised on 1.5% (w/v) agarose gel stained with ethidium bromide.

Dot-blot hybridization

To confirm the stable transgene integration into the plant genome, the dot-blot hybridization was performed. Approximately 10 μg of DNA were blotted onto positively charged nylon membrane (Hybond N^+, Amersham, Sweden) and probed with ca. 850 bp coding region of *m-gfp5-ER* gene, generated by PCR using the following primers designed according to Eady et al. (2000): 5′-ACG TCT CGA GGA TCC AAG GAG ATA TAA-3′) and GFP5-b-l (5′-ACG TCT CGA GCT CTT AAA GCT CAT CAT G-3′). Probe labelling with alkaline phosphatase, hybridization and detection procedures were conducted according to manufacturer's instructions (AlkPhos Direct Labelling Kit, Amersham, Sweden).

RNA isolation and confirmation of transgene expression by reverse transcription (RT-PCR) analyses

Total RNA was extracted from 100 mg of fresh young leaves ground by mortar and pestle in liquid nitrogen using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions and treated with RNase-free DNase I (Top-Bio, Czech Republic) at final concentration 25 U/100 μl at 37°C to exclude the residual DNA contamination and consequent false positives. DNase was then inactivated by heat treatment at 70°C for 5 min. First strand of cDNA was synthesised using the oligo-dT primers and Sensiscript Reverse Transcription Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. A 2 μl sample of cDNA was used in the PCR amplification, performed as described above and with the same primers.

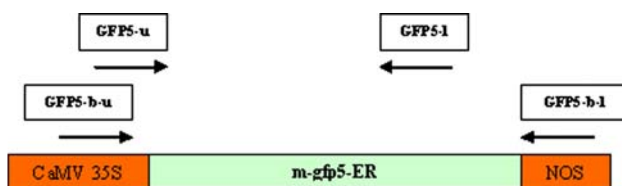

Fig. 1 Schematic diagram of the *m-gfp5-ER* coding region flanked by the CaMV 35S promoter and the nos terminator. Positions of used primers are indicated. The GFP5-u and GFP5-l set of primers yield the 415 bp product and were used for PCR and RT-PCR analyses. Primers GFP5-b-u and GFP5-b-l designed according to Eady et al. (2000) flanked whole *m-gfp-ER* coding region and were used to generate the probe for dot-blot hybridization

Cloning and sequencing of the RT-PCR products

To verify the sequence specifity of polymerase reactions, gel extracted RT-PCR products from T_1 transgenic plants were subcloned into the plasmid vectors using the PCR-Script™ Amp Cloning Kit (Stratagene, La Jolla, CA, USA). The nucleotide sequences were determined by non-radioactive sequencing using the ABI Prism DNA Sequencer. Sequence data were analysed using the Bio-Edit (Ibis Therapeutics, Carlsbad, CA, USA) analyses software.

Protein extraction and native protein PAGE electrophoresis

Approximately 300 mg of tissue from young fully developed leaves from T_1 transgenic tobacco plants were used for protein extraction, performed according to Zhou et al. (2005). Total protein concentrations were determined by the BCA assay using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

Approximately 40 μg of total protein were subjected to native gel electrophoresis, carried out on vertical electrophoresis system (SE 600, Hoefer, USA) according to Laemmli (1973). For protein separation, discontinual electrophoresis system free of SDS, consisting of 10% stacking gel (pH 6.8) and 15% running gel (pH 8.8) was used. The separation was performed at 4°C in Na-borate (pH 8.3) running buffer using constant current of 25 mA . Separated samples were visualised under the fluorescent stereo microscope following the excitation of GFP protein as described previously (Hraška et al. 2005).

Fluorescence microscopy assays

The same observation set and software as described previously in Hraška et al. (2005) was used in all following experiments, allowing the macroscopic survey of studied samples. Intensity of green fluorescence in leaves was evaluated based on the mean brightness value (MB) determinations used as relative units. Fluorescence of each sample (referred as independent measurement) was examined three times to exclude possible discrepancies of measurements. Obtained MB values were statistically evaluated by means of Statistica® ver. 6.0 software (StatSoft, Tulsa, USA), using the Main Effects ANOVA and Multiple Linear Regression modules, with 0.95 confidence interval. The following parameters of GFP fluorescence in transgenic plants and single leaves were investigated:

1. Differences in the green fluorescence intensity measured either on the abaxial or adaxial sides of tobacco leaves were investigated. Samples for fluorescence measurements were randomly taken from eight random positions covering the whole area of fully developed tobacco leaves using a Ø 0.5 cm cork borer. Three leaves from each of four transgenic plants were investigated. MB values taken from measuring points for each leaf surface were summarised as a total average MB value per particular leaf surface and compared reciprocally.
2. Variability of the GFP fluorescence within the leaf surface. From randomly chosen mature transgenic plants (four individuals), at least four leaves taken from different positions were examined. Fluorescence measurements were performed on the samples taken by 0.5 cm cork borer from eight defined positions within the leaf surface as marked on Fig. 2 and MB values for each position were separately recorded. Based on the results from experiments performed in the part 1, fluorescence measurements were done on the abaxial side. Data obtained for each particular position from various leaves within one transgenic plant were expressed as arithmetic averages.
3. Variability in the green fluorescence signals between leaves of different physiological age within the plant. Selected four T_1 plants were clonally propagated in vitro. From each of plants, three leaves denoted A–C (from physiologically oldest-, denoted A, to the youngest-, the second leaf under the inflorescence) were investigated. Based on the outcomes obtained from both previous experiments, all leaves were scanned from abaxial side and from each leaf at least ten independent GFP measurements covering the whole surface were taken. Fluorescence measurements for each line were performed in 1 day as one data set, immediately after the leaf discs were excised. Obtained MB values for each leaf were summarised and expressed as arithmetic averages representing MB value for particular leaf and as such used for subsequent evaluation of spatial fluctuations of GFP fluorescence.

Chlorophyll *a* and *b* extraction and determination

Total leaf chlorophyll was determined spectrophotometrically from leaf extracts in 80% acetone according to Šesták (1971). Leaf disks were taken using the 0.5 cm cork borer from defined positions within the leaf surface as marked on Fig. 2, simultaneously with the samples taken for the GFP fluorescence studies. Disks from one leaf were assessed as one sample and chlorophyll content was expressed in mg gFw^{-1} as an average value obtained from four repeated determinations.

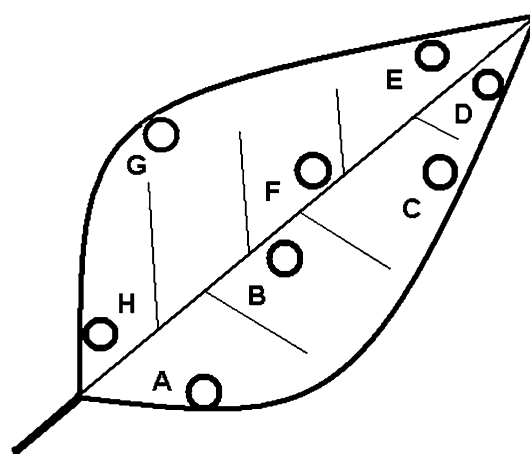

Fig. 2 Schematic drawing of tobacco leaf showing the locations of samples excised with cork borer for the chlorophyll and fluorescence measurements. Eight distinct positions cover the whole leaf area

Results

Molecular characterisation of transgenic tobacco plants carrying the *m-gfp5*-ER gene

Randomly chosen greenhouse T_0 plants were subjected for PCR screening. In these experiments, transformation vector p-Bin 19 was used as a positive control and total DNA extracted from un-transformed plants was used as a negative control. Predicted band with size of 415 bp corresponding to *m-gfp5*-ER gene was present in all 12 T_0 plants and positive control, whereas no PCR product was present in control DNA from non-transformed plants (Fig. 3a), indicating successful transgene integration.

Transmission of the *gfp5* gene to T_1 progeny was verified by means of PCR using the same primers and conditions as for T_0 plants. Randomly selected T_1 plants G1, G2, G3, G4, G5 and G6 were subjected for further analyses (Fig. 3b). The dot-blot hybridization confirmed stable integration of the transgene into tobacco genome, when selected samples and positive control yielded strong hybridization signal (Fig. 3c).

In order to verify the expression of *m-gfp5*-ER gene, cDNA corresponding to selected plants was subjected for PCR amplification and 5 (G2, G3, G4, G5, G6) of the six transgenic plants shown a clear gene specific band at expected size of 415 bp, corresponding to the *gfp5* transcript. No such products were obtained after RT-PCR from control sample and of relevant RNA samples, which

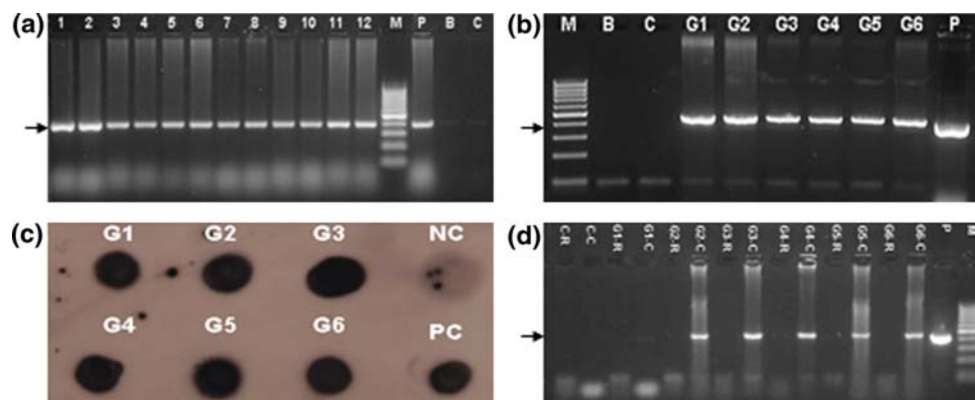

Fig. 3 Molecular analyses of transgenic plants. PCR products analysis of T_0, transgenic plants rooting on the selective MS nutrition medium supplemented with 500 mg l^{-1} Kn **(a)** and of selected T_1 plants derived from selfed T_0 transgenic plants positive for *m-gfp5-ER* gene **(b)**. *Lanes* 1–12 and G1–G6 represent independent transgenic plants of T_0 and T_1 generations, respectively **(b)**. Dot-blot hybridization of DNA from selected T_1 transgenic plants **(c)**. Approximately 10 μg of DNA from each plant were blotted onto negatively charged nylon membrane and probed with the alkaline phosphatase labelled probe. Stable transgene integration was confirmed in all examined samples. *NC* negative control-DNA from untransformed plats, G1–G6 DNA from PCR positive plants, *PC* positive control-plasmid pBINmGFP5-ER. Example of RT-PCR analysis for the *m-gfp5-ER* gene expression in selected PCR positive T_1 transgenic plants **(d)**. *Lanes* G1–G6 represent independent transgenic plants, *C* cDNA samples, *R* corresponding RNA samples subjected simultaneously for PCR to exclude possible DNA contamination of RNA, *C* control samples (untransformed tobacco plants). Legend common for parts **a, b, d**: *lane M* 100 bp molecular weight marker, *lane P* plasmid pBINmGFP5-ER used as a positive control, *B* blank control containing only water, *C* DNA from untransformed tobacco plant

excluded the DNA contamination of RNA. Thus, the PCR product obtained after RT-PCR were solely due to the presence of a transgene transcript (Fig. 3d). The plant denoted G1 showed no RT-PCR product and thus was assumed as a plant with no transgene expression. Sequence analyses confirmed the authenticity of obtained RT-PCR products from T_1 plants (data not shown).

Presence and activity of GFP protein was verified after the total leaf protein gel electrophoresis. Green fluorescing bands with size of 27 kDa corresponding to the commercial GFP standard (Roche Applied Science, Indianapolis, IN, USA) were observed under the fluorescence stereo microscope in all five RT-PCR positive samples. No fluorescing protein bands were observed in the case of control and G1 plant (Fig. 4).

GFP fluorescence microscopy of leaf samples

Intensities of GFP fluorescence significantly differ on both sides of a leaf

A total number of 192 independent measurements were performed on leaves taken from various positions within the transgenic plants G2, G3, G4 and G5. Different intensity of the green fluorescence was evident on the first sight by naked eye, when the green fluorescence observed on the abaxial side was much stronger than from the opposite (adaxial) one. Moreover, the differences in the fluorescence intensity between the abaxial and adaxial sides were revealed during the measurements in all positions within the leaf surfaces and thus also the differences in the MB values

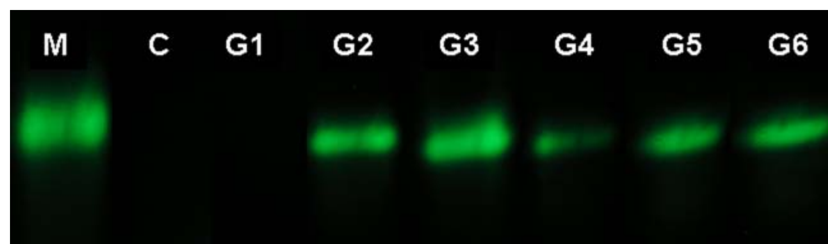

Fig. 4 Detection of the presence of the active GFP protein in crude extracts from leaves of investigated T_1 plants. Approx. 40 μg of total protein were loaded to each well and subjected for native gel electrophoresis. *Lanes* G1–G6, samples from independent transgenic T_1 plants, *M* marker represented by pure, commercially available GFP, *C* extract from the control plant. Note, that none fluorescing band, representing the GFP, is visible in the case of G1 plant. Whole figure is completed from a few partial images because it was not possible to photograph whole gel with the Leica MZ 12 fluorescence microscope

were expected. Statistical analyses of obtained MBs confirmed significant differences ($\alpha = 0.05$) in the fluorescence intensity (MB values) between studied genotypes (data not shown) and between both leaf surfaces (Fig. 5a). Interestingly, data obtained from the adaxial side mostly displayed lower variability in MB value than from the abaxial side, which was confirmed by their lower standard deviation value (adaxial side 23.77, abaxial side 29.54). Nevertheless brighter and stronger fluorescence detected on the abaxial leaf surface was more suitable for our future purposes.

Variability in the GFP fluorescence within a leaf surface

In order to investigate possible fluctuations in the GFP fluorescence within the leaf surface, measurements from eight defined distinct positions, covering its whole area were performed. Leaves detached from four randomly selected tobacco plants were used. A total number of 128 independent measurements were carried out, when four leaves from plants G2, G3, G4 and G5 were investigated. Outcomes from fluorescence measurements showed the differences in the MB values among all eight positions within the leaf surface, indicating also different GFP fluorescence levels among these positions. A general pattern of GFP fluorescence, common to all investigated plants, could be set forth based on our measurements and particular fluorescence patterns of each studied genotypes as shown on Fig. 5b. Generally, the highest GFP fluorescence was observed within areas close to the leaf tips, namely at the positions denoted C, D and E. Nevertheless some variations of the fluorescence intensity among these three positions in each particular genotype were observed. In spite of these fluctuations (Table 1), the general fluorescence profile was common for all studied plants. Surprisingly, high fluctuations in MB values in leaves detached from various plants were observed at positions denoted G. Such fluctuations were most probably caused by their proximity to intensively fluorescing footstalk.

Spatial patterns of GFP fluorescence within transgenic plants

In order to obtain the most reliable data and based on the above described results, we have decided to collect the data from at least eight distinct positions covering the whole leaf

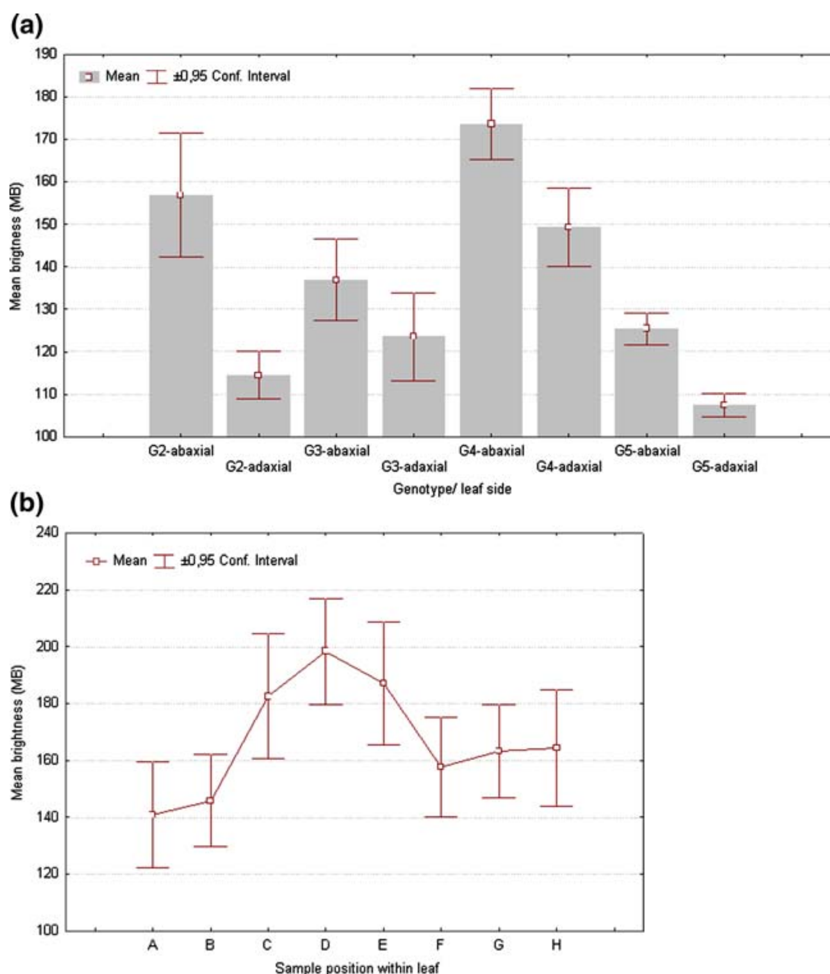

Fig.5 Comparison of the fluorescence intensity between the abaxial and adaxial sides of a leaf **(a)** and among the eight defined positions (A–H) within the leaf surface **(b)**. Weaker GFP fluorescence was detected on the adaxial side of leaves detached from all investigated T_1 plants **(a)**. Each *bar* represents the average MB value obtained from three leaves, eight measurements were performed on each leaf. The increase of the GFP fluorescence intensity on abaxial side of leaf tips was evident in all investigated T_1 plants **(b)**. Average MB values for particular position obtained from all investigated tissue samples are given here. From each plant, at least four leaves were investigated and MB values from defined positions were detected. In both experiments, the fluorescence of control samples expressed as MB value was always below 30

Table 1 The variability of the GFP fluorescence within the leaf surface

Position	Plant				
	G2	G3	G4	G5	Average
A	136.977	108.773	144.877	172.743	140.843
B	135.799	111.902	149.452	186.294	145.862
C	178.946	144.171	167.829	239.161	182.527
D	209.673	159.329	193.38	230.771	198.288
E	177.656	138.000	195.569	236.676	186.975
F	147.092	118.138	165.947	199.281	157.615
G	160.075	127.458	161.223	203.882	163.160
H	149.93	124.471	172.193	210.562	164.289

Each data set represents an average MB value from particular location obtained from four leaves

Positions A–H correspond with those marked on the Fig. 2

surface and to perform all measurements on the abaxial leaf side. From each plant, leaf discs from three leaves were investigated. Moreover, to compare the degree of variability influenced by each individual within one genotype and environment, four randomly selected transgenic genotypes, G3, G4, G5 and G6 were clonally propagated in vitro. Thus, altogether 12 plants were investigated in these series.

Differences in the GFP fluorescence and therefore in the MB values between the oldest and the youngest leaves within each plant were evident in all cases (Fig. 6a). These differences showed a decrease of fluorescence signal in tobacco leaves in the direction towards the plants top. Interestingly, differences in the average MB value between the oldest and the youngest leaf were distinct for each genotype. For example, the difference of medium MB values within the genotype G6 was only 4.829, whereas in the case of genotype G3 such difference was more than three times higher (16.430). Relatively high variability in the GFP fluorescence signal among individual plants of the same genotype was observed, indicating also the strong influence of each individual development/environmental interaction, nevertheless such variability was not so prominent as the variability between studied genotypes (Fig. 6b). Statistical analyses confirmed that differences in the MB values between clones within one genotype were not significant, whereas the differences between studied genotypes were significant. This is an important fact, giving us the chance to discriminate the GFP transformants based on the determined MB values.

Total chlorophyll content has only minor effect on the GFP fluorescence loss in the young leaves

Six plants of genotypes G3 and G6 were investigated with the aim to study a possible masking influence of the chlorophyll content on the GFP fluorescence. Beside these six transgenics, also two clonally propagated control plants were investigated. Data obtained from all plants showed the trend common for both groups, transgenic plants as well as for the control plants: total chlorophyll content increased toward the plant top and was approximately 3–4 times higher in the leaves C (apical, youngest) compared to leaves A (basal, oldest) (Fig. 7). These first observations gave us the reason to speculate about the masking/quenching effect of the chlorophyll on the GFP fluorescence. Nevertheless, following statistical analyses showed a weak negative correlation between these two parameters ($R^2 = -0.36$). Thus, in this context the effect of chlorophyll content on the variability of obtained GFP fluorescence data does not seem to be so strong and the participation of other factors could be hypothesised.

Discussion

Variable intensity in transgene expression/performance in the plants, also those driven by constitutive promoters, is a well known event, reported many times previously (e.g. Preťová et al. 2001; Halfhill et al. 2003) and various reasons were hypothesised. In this study we aimed our effort at the mapping and definition of the differences in the performance of one particular marker, the GFP signal gene, among various leaf tissues of transgenic tobacco, throughout the quantification of its fluorescence. Analyses of fluorescence intensity could be easily used as a powerful toolkit for the definition, measurement and comparison of transformation events among various transgenic plants. Nevertheless, considering the above mentioned GFP fluorescence variability, together with the aim to obtain the most reliable data from a particular experiment or assessment, it is important to define the fluorescence patterns, i.e., intensity detectable from each particular plant tissue. Various commercial or laboratory devices designed for GFP fluorescence measurements are available now (Hraška et al. 2006). Here, we have used a simple equipment consisting of a stereo microscope with the GFP set, allowing the macroscopic examination of leaf tissue, coupled with the CCD camera and image analyses software.

Differences in the GFP fluorescence detected either from abaxial or adaxial sides of leaves were recorded in our study. This could be a very important fact when a quantitative or semi-quantitative approach based on the detection of fluorescence from native samples with the fluorescence microscope is used. Therefore it could be stated, that for the purposes of GFP fluorescence quantification, the detection of the fluorescence from either of the leaf surfaces should be followed uniformly during the whole particular experiment to obtain a representative data set. Differences in the

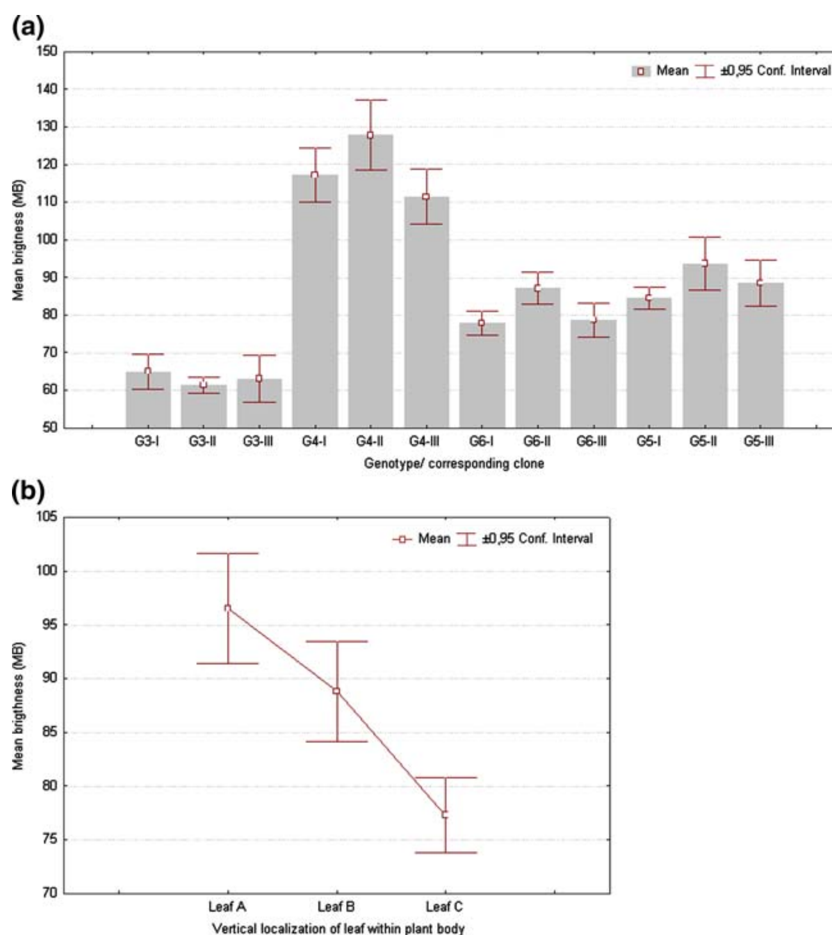

Fig. 6 Results of the GFP fluorescence intensity investigation performed with 12 transgenic plants of four genotypes. The decrease of the fluorescence in the leaves on the plant basement towards the plant top is evident in all investigated genotypes **(a)**. "A" denotes the oldest, lower leaf, "B" denotes the leaf occurring in the middle of plant body and "C" denotes the youngest, upper leaf. The *line* represents an average MB value for all leaf samples detached from particular position. From each leaf, at least ten independent measurements from whole leaf area were performed. The variability of total GFP fluorescence among the individual clones belonging to each genotype was observed **(b)**, nevertheless such variability was not higher then the variability between the studied genotypes. Therefore, each genotype could be distinguished based on the detected fluorescence intensity. Each *bar* represents MB values obtained from all three leaves from one plant. In both experiments, the fluorescence of control samples expressed as MB value was always below 30

chlorophyll and other substances contents and anatomy between these two surfaces could be speculated as a possible factor for such variability. In addition, it has been observed previously that various cell types possess different expression levels of marker genes driven by constitutive promoters, mostly found in the conductive elements which possess much stronger expression as compared to other plant tissues (Hraška et al. 2005).

Differences in the transgene expression within the leaf surface were indicated previously, e.g. by Preťová et al. (2001). Among various possible reasons, stronger proteosynthesis and thus greater marker gene activity in younger and rapidly developing areas has been hypothesised as one factor (Preťová et al. 2001). Such differences in the transgene manifestation could significantly influence the whole investigation and transgene activity quantification so the choice of measuring point and number of performed measurements are essential factors for an accurate interpretation of obtained data. In our experiments, MB values indicated that the GFP fluorescence differed among various positions within the leaf surface and the strongest fluorescence was detected in the leaf tip area. Interestingly, relatively high fluctuations in MB values in the last positions (G) among various leaves were observed. This was most probably due to the close position of this measuring point to the midrib, which is very wide at the leaf base and thus possesses very strong green fluorescence. Such fluorescence is detectable also in the very close neighbourhood and could affect the measurements performed on the areas in close distance, which was also reported previously (Hraška et al. 2005). Therefore fluctuations in the MB values on the basal parts were most probably due to a

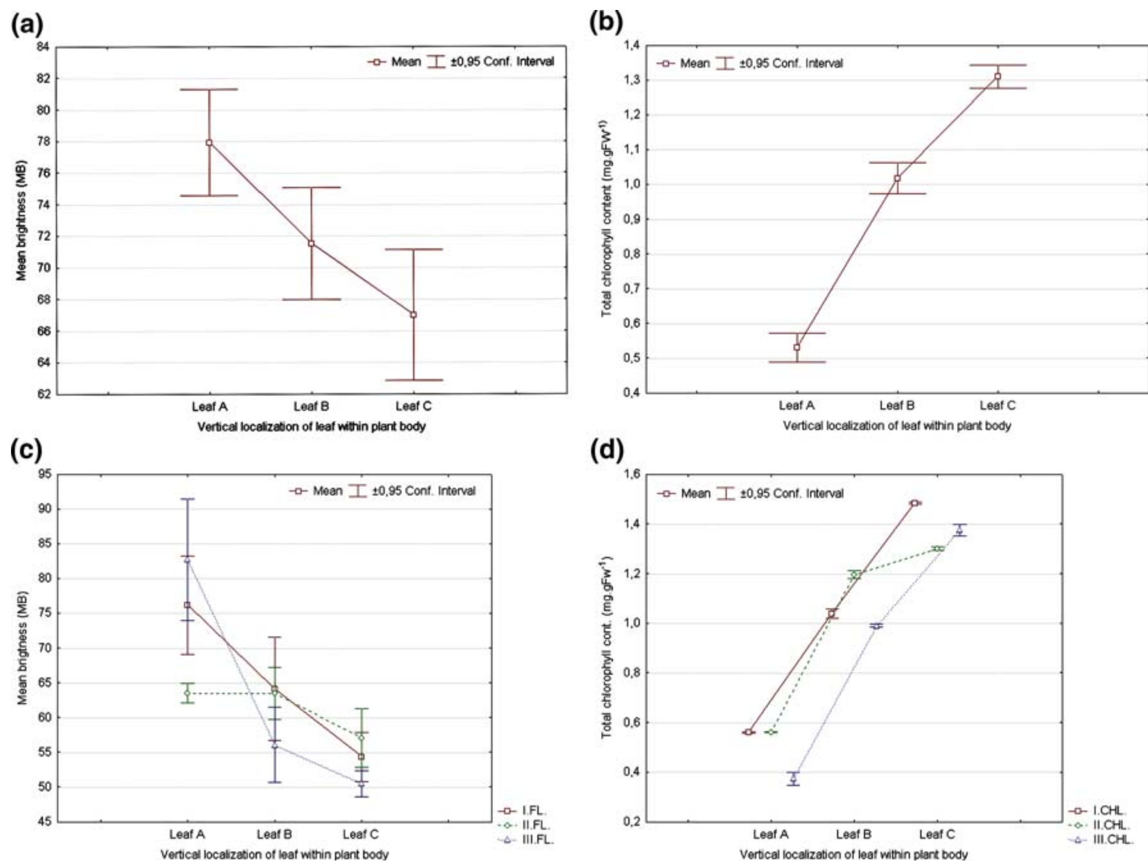

Fig. 7 Analyses of the relationship between the GFP fluorescence intensity and total chlorophyll content within plant body. The decrease of the GFP fluorescence in the leaves towards the plant top and simultaneous increase of the total chlorophyll content is evident. The average GFP fluorescence pattern **(a)** and chlorophyll content **(b)** within plant body for six transgenic plants of genotypes G3 and G6 is shown. Detailed illustration, showing the changes in GFP fluorescence and chlorophyll content for the three clonally propagated plants of the G3 genotype is shown on figures **c** and **d**, respectively. *Arabic numbers* I–III denote the particular plant (clone) of the genotype (**c**, **d**)

limited (small) area in this leaf part, which could be easily influenced by fluorescence originating from a close attendant midrib. Moreover the conductive elements were more developed in the basal part so their fluorescence was more intensive than on the leaf apex. Various aspects, ranging from different activity of constitutive 35S promoter (Sunilkumar et al. 2002) to different cell types and their different physiological properties (Preťová et al. 2001) could be assumed as a possible cause of such GFP fluorescence patterns. Preťová et al. (2001) reported nearly similar patterns of GUS activity within the tobacco leaves. They also found lower GUS activity in the samples taken from basal part than from the apical part. Interestingly, they also reported higher activity in the region of the middle leaf part between the base and the apex (corresponding to region between our positions denoted C and D) compared to the apex. Nevertheless, in our broader scale measurements mostly the highest MB values and therefore GFP fluorescence were detected in parts D and E, so in the leaf apex.

The last investigated topic was the GFP fluorescence pattern within the mature plants. Twelve transgenic plants belonging to four randomly selected genotypes G3–G6 were employed in this part of our experiment. At first, high variability in the GFP fluorescence was revealed among the plant clones within the same genotype, nevertheless this was not higher than the variability between the genotypes. Thus the discrimination of the studied genotypes based on the fluorescence (MB values) was possible. Data obtained from the three leaves from each mature plant showed the decrease of the fluorescence towards the plant top therefore to youngest leaves. This is in contrast with the findings reported by Halfhill et al. (2003), who detected the highest GFP fluorescence in the youngest oilseed rape leaves. The highest intensity of the fluorescence was retained by the youngest leaves during whole oilseed rape life cycle. Moreover, the decline in the fluorescence intensity towards the plant base and thus in the older leaves was reported. Nevertheless, the decline of the GFP fluorescence intensity in older leaves reported by Halfhill et al. (2003) seems to

be often also for some other species except the oilseed rape. An expression pattern of the GUS marker similar to that reported by Halfhill et al. (2003) was also reported by Preťová et al. (2001) in the case of *uidA* marker expressing tobacco, but they investigated only two transgenic plants. Loss of GFP fluorescence with the increasing leaf age was also reported by Zhou et al. (2005) for *Medicago* and rice plants, whereas they did not observe such event in the case of *Arabidopsis*.

Although we were interested mainly in the mapping and definition of patterns of the GFP fluorescence intensity among different leaf tissues, some authors concerned also on the study of possible physiological background of such variability. One of the first reasons could be the different GFP content within the total soluble protein. Halfhill et al. (2003) reported positive correlation between the fluorescence quenching and level of soluble protein content per unit of a wet mass in leaves of all ages of oilseed rape. On the other hand, the GFP quantification within the total extractable protein from transgenic tobacco as found by western blotting, reported by Harper and Stewart (2000) showed relatively the same GFP content within the whole plant body. Moreover, they assumed that the observed GFP expression patterns would be comparable with other GFP transgenic lines in other situations. Another important factor seems to be the presence of some masking or quenching agent(s), namely chlorophyll. It has been reported many times that chlorophyll content could negatively interfere with the GFP fluorescence (Ponappa et al. 1999; Cho et al. 2002). Moreover, Zhou et al. (2005) reported reconstruction of once diminished fluorescence in the *Medicago* leaves after the chlorophyll removal. Our observation confirmed some role of the chlorophyll content in the decrease of GFP fluorescence intensity, nevertheless the negative correlation was not strong enough to conclude the dominant role of the chlorophyll only. Therefore, it seems that the GFP fluorescence intensity may be affected by synergic incidence/action of several factors, including various physiological aspects, particular plant species, maybe the genotype and even environment.

In the study presented here, we have defined the fluorescence patterns of GFP, a common marker in plant biology, in the leaves of transgenic tobacco plants, using a simple method of image analyses. Various variables in the fluorescence were identified based on the leaf tissue type selected for the investigation. Based on our results, it is evident that the GFP manifestation differs in various leaf tissues and in leaves of different physiological age, though the exact physiological reasons are still debatable. This fact strengthens the necessity to perform comparative studies of GFP fluorescence/promoter activity using the same methodology for all plants and tissues of the comparable physiological age or developmental stage. Moreover, the influence of each individual plant within cloned genotypes was revealed. It is evident, that the origin/position of investigated leaf tissue could affect the interpretation of data obtained based on the detection of GFP fluorescence. Moreover, when evaluating the fluorescence profile of a group of plants, the individual variability, most probably affected by the environment, should be taken into account and such studies should be provided with wider numbers of individuals, clones or populations.

Acknowledgments The authors gratefully acknowledge the financial support received from the Ministry of Education, Youth and Sport of the Czech Republic (grants 1M06030, 1PO5ME800 and MSM 60076658–06). M. H. and V. H. received the support from grant GA ČR 31/H160 provided by the Grant Agency of the Czech Republic and both contributed to this work equally. The authors thank Dr. Jan Bárta (Department Plant Production, Faculty of Agriculture, University of South Bohemia) for his valuable advice and guidance in the part of this work concerning the proteins. Whole manuscript was kindly revised by Mentewab Ayalew.

References

Benfey PN, Chua N-H (1989) Regulated genes in transgenic plants. Science 244:174–181. doi:10.1126/science.244.4901.174

Blumenthal A, Kuznetzova L, Edelbaum O, Raskin V, Levy M, Sela I (1999) Measurement of green fluorescence protein in plants: quantification, correlation to expression, rapid screening and differential gene expression. Plant Sci 142:93–99. doi:10.1016/S0168-9452(98)00249-0

Cho M-J, Choi H-W, Jiang W, Ha ChD, Lemaux PG (2002) Endosperm-specific expression of green fluorescent protein driven by the hordein promoter is stably inherited in transgenic barley (*Hordeum vulgare*) plants. Physiol Plant 115:144–154. doi:10.1034/j.1399-3054.2002.1150117.x

Doyle JJ, Doyle JL (1990) A rapid total DNA preparation procedure for fresh plant tissue. Focus 12:13–15

Eady CC, Weld RJ, Lister CE (2000) *Agrobacterium tumefaciens*-mediated transformation and transgenic-plant regeneration of onion (*Allium cepa* L.). Plant Cell Rep 19:376–381. doi:10.1007/s002990050743

Halfhill MD, Millwood RJ, Rufty TW, Weissinger AK, Stewart CN (2003) Spatial and temporal patterns of green fluorescent protein (GFP) in transgenic oilseed rape, *Brassica napus* L. Plant Cell Rep 22:338–343. doi:10.1007/s00299-003-0696-4

Halfhill MD, Richards HA, Mabon SA, Stewart CN Jr (2001) Expression of GFP and Bt transgenes in *Brassica napus* and hybridization with *Brassica rapa*. Theor Appl Genet 103:659–667. doi:10.1007/s001220100613

Harper BK, Stewart CN Jr (2000) Patterns of green fluorescenct protein expression in transgenic plants. Plant Mol Biol Rep 18:141a–141i

Horsch RB, Fry JE, Hoffmann NL, Eicholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231

Hraška M, Rakouský S, Kocábek T (2005) Use of simple semiquantitative method for appraisal of green fluorescent protein gene expression in transgenic tobacco plants. Biol Plant 49:313–316. doi:10.1007/s10535-005-3316-z

Hraška M, Rakouský S, Čurn V (2006) Green fluorescent protein as a vital marker for non-destructive detection of transformation

events in transgenic plants. Plant Cell Tissue Organ Cult 86:303–318. doi:10.1007/s11240-006-9131-1

Laemmli UK (1973) Cleavage of structural proteins during assembly of head bacterophage-T4. Nature 227: 680–685. doi:10.1038/227680a0

Ponappa T, Brzozowski AE, Finer JJ (1999) Transient expression and stable transformation of soybean using the jellyfish green fluorescent protein. Plant Cell Rep 19:6–12. doi:10.1007/s002990050702

Prešová A, Obert B, Wetzstein HY (2001) Leaf developmental stage and tissue location affect the detection of β-glucuronidase in transgenic tobacco plants. Biotech Lett 23:555–558. doi:10.1023/A:1010311524048

Rooke L, Byrne D, Salgueiro S (2000) Marker gene expression driven by the maize ubiquitin promoter in transgenic wheat. Ann Appl Biol 136:167–172. doi:10.1111/j.1744-7348.2000.tb00022.x

Sunilkumar G, Mohr L-A, Lopata-Finch E, Emani Ch, Rathore KS (2002) Developmental and tissue specific expression of CaMV 35S promoter in cotton as revealed by GFP. Plant Mol Biol 50:463–474. doi:10.1023/A:1019832123444

Šesták Z (1971) Determination of chlorophyll *a* and *b*. In: Šesták Z, Čatský J, Jarvis PG (eds) Plant photosynthetis production. Manual and methods. Dr. W. Jung N. V. Publ., The hague 1971, pp 672–701

Zhou X, Carranco R., Vitha S, Hall TC (2005) The dark side of green fluorescent protein. New Phytol 168:313–322. doi:10.1111/j.1469-8137.2005.01489.x

4. 5. Tracking of the CaMV-35S performance in GFP transgenic tobacco, with a special emphasis on flowers and reproductive organs, confirmed its predominant activity in vascular tissues

Hraška, M., Rakouský, S., Čurn, V.: Tracking of the CaMV-35S promoter performance in GFP transgenic tobacco, with a special emphasis on flowers and reproductive organs, confirmed its predominant activity in vascular tissues. Submitted to Plant Cell Tissue and Organ Culture 8/2007

Tracking of the CaMV-35S performance in GFP transgenic tobacco, with a special emphasis on flowers and reproductive organs, confirmed its predominant activity in vascular tissues

Marek Hraška [1, 2], Slavomír Rakouský [1, 2, *], and Vladislav Čurn [3]

[1] Faculty of Health and Social Studies, University of South Bohemia, Jírovcova 24, 370 01 České Budějovice, [2] Faculty of Biological Sciences, Department of Genetics, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, and [3] Biotechnological Centr, Faculty of Agriculture, University of South Bohemia, Studentská 13, 370 05 České Budějovice, Czech Republic.

* Corresponding author:
Slavomír Rakouský
Phone/fax: +420 38 777 5537
E-mail: srak@bf.jcu.cz

Abstract

Constitutive promoters are the most common promoters used to drive the expression of various genes in monocots and dicots. Therefore, it is of intense interest to ascertain their expression patterns in various plant species, organs and during their ontogenic development. In this study, the activity of the CaMV 35S promoter in transgenic tobacco plants was assessed. In contrast to other studies, performed rather on the primary

transformants (T_0 generation), here, individuals of T_1 and T_2 generations were used. The expression profiles of the CaMV 35S promoter were tracked within various plant organs and tissues using the GFP marker. Special attention was given to floral tissues for which the original data regarding the CaMV 35S expression were obtained. As expected, distinct developmental and organ/ tissue specific expression patterns in a plant body were observed. CaMV 35S activity was detected in most of the plant tissues and during different developmental stages. The GFP signal was not visible in dry seeds only, but it became clearly apparent within 24-48 hours after sowing onto the medium, what, among other things, enables the discrimination of transgenic and non-transgenic seeds/ seedlings. Afterwards, the most pronounced GFP fluorescence intensity was usually visible in various vascular tissues of both, T_1 and T_2 plants, indicating the high promoter activity. A stable manifestation of the promoter was retained in the next T_2 generation without any evident changes or losses of activity, showing the expression stability of the CaMV 35S.

Key words: CaMV 35S promoter, expression pattern, fluorescence, green fluorescent protein marker, transgenic tobacco (*Nicotiana tabacum*)

Introduction

Strategies aimed at modifying the productivity and/or quality traits of plants require a stable and controlled transgene expression driven by a proper promoter. Besides the tissue specific, inducible and other promoters, the constitutive promoters are the most commonly used for driving the transgene expression in plants. No matter which type of promoter is chosen for a particular experiment, it is desirable to monitor its expression in various tissues and during plant development. The Cauliflower mosaic virus promoter CaMV 35S is the most widely used promoter for effective transformation of both monocots and dicots. Although it is generally considered to be a constitutive promoter (Odell et al. 1985), some reports suggest that it is not always expressed in all tissue types (Williamson et al. 1989, Yang and Christou, 1990, Malik et al. 2002, Sunilkumar et al. 2002).Detailed information regarding the activity of the CaMV 35S promoter in transgenic plants is still unsatisfactory and limited to only a few species. Moreover, most studies concerning this topic used the reporter gene *uidA* coding for β-glucuronidase (Jefferson et

al. 1987) enabling to follow the histochemical localization of the enzyme product in plant tissues (Benfey et al. 1989, Battraw and Hall, 1990, Yang and Christou, 1990). Although the *uidA* is a widely used marker in plant transgenesis, GUS histochemical staining for visualization of *uidA* expression suffers from some limitations. Firstly, diffusion of the enzyme or reaction product into the surrounding, transgene non-expressing tissue, can occur (Yang and Christou, 1990, Mascarenhas and Hamilton, 1992). This could lead to an inadequate interpretation of the results obtained. Secondly, the irregular penetration of the substrate throughout different tissue types and a stronger expression in younger cells with dense cytoplasm can influence the final data interpretation (Preťová et al. 2001). Thirdly, the histochemical staining usually requires fixed, i. e. dead tissues. Thus, the histochemical GUS assay is complicated, relatively time consuming and destructive to the studied samples. Therefore, the use of an alternative marker with different qualitative and quantitative traits is highly desirable. In 1992 Prasher et al. (1992) presented the detailed structure of a novel fluorescent maker gene coding for the green fluorescent protein (GFP), originating from the pacific jellyfish Aquorea victoria. The GFP itself possesses many unique features allowing its use for various purposes in research (Stewart, 2001; Hraška et al. 2006). Upon excitation with UV or blue light, wild type GFP emits green fluorescence, which is relatively easily visualized with appropriate observation devices. The use of GFP as a marker of gene expression in plants possesses many advantages compared with GUS and, moreover, it permits monitoring of various events in real time in living plants (Hraška et al. 2006). Nowadays, many variants differing in their spectral and other characteristics are available to allow the study of various events within living plants (Tsien 1998; Stewart, 2001).

Previous studies of the expression patterns of constitutive promoters mostly utilised the *uidA* marker gene and the histochemical localization of its expression in mature seeds, vegetative parts and some floral organs (Benfey and Chua, 1989, Benfey et al. 1990a, Terada and Shimamoto, 1990). Nevertheless, the need for a more comprehensive study describing CaMV 35S activity within various plant tissues and organs and during various developmental stages was evident. In addition, the use of some novel marker gene, with qualitatively different features, allows for a different approach in such type of studies and could lead to new insights on the performance of promoters. The first such study concerning CaMV 35 S expression patterns in transgenic cotton was presented in 2002 by Sunilkumar et al. (2002), who utilised the GFP-based marker in primary transformants of T_0 generation.

The objective of the present study was a detailed investigation of the CaMV 35S promoter activity in transgenic tobacco (Nicotiana tabacum L.) plants, a common model species of plant transgenesis, using the GFP marker. To our knowledge, CaMV 35S promoter manifestation in tobacco tissues and organs was not previously studied so exhaustively, especially using a GFP marker. This paper together with work presented by Sunilkumar et al. (2002), these are only two studies concerned solely on this topic. Nevertheless, in contrast to Sunilkumar et al. (2002), who employed primary transformants, stably transformed plants of the T_1 generation carrying the m*gfp*5-ER gene (Haseloff et al. 1997) and its progeny (T_2 generation) were examined in the study presented here, with the aim to also track promoter activity in the next generations of plants. In addition to this comprehensive investigation of promoter activity in main plant organs, a detailed study was carried out on different floral tissues and reproductive organs, since the information regarding the CaMV 35S performance in such tissues is still insufficient.

Plant material and methods

Transformation and regeneration of transgenic plants

Transgenic tobacco (Nicotiana tabacum L.) plants were obtained after co-cultivation of leaf discs with *Agrobacterium tumefaciens* – a disarmed helper strain LBA 4404 (Ooms et al. 1982), carrying a binary vector pBINmGFP5-ER (kindly provided by J. Haseloff, MRC, Cambridge, UK) according to Horsch et al. (1985). The plasmid pBINmGFP5-ER contains the nos driven nptII gene as a plant selection marker and a CaMV 35S driven m*gfp*5-ER gene, targeted to the endoplasmic reticulum. Putatively transgenic shoots were regenerated on a basal MS medium (Murashige and Skoog 1962), supplemented by 500 mg L^{-1} kanamycin (Kn). Regenerated rooting plants were transferred to soil and grown to maturity under controlled conditions in a greenhouse. Seeds obtained from selfed T_0 plants were harvested and, following the dormant period, the seeds were surface sterilised with sodium hypochlorite and then sown in vitro on the MS medium of the same composition as described above. Then, the T_1 seedlings obtained were transferred to the same greenhouse as primary T_0 regenerants and grown to maturity. The mature

transgenic plants thus obtained were assumed to have integrated T-DNA in a stable way and thus no fluctuations of transgene expression or other changes associated with the transformation process and in vitro regeneration were expected. In addition, selected T_1 transgenic plants were selfed and the T_2 plants obtained were investigated too. Four randomly selected independent T_1 transgenic plants and four T_2 plants obtained from each T_1 individual plant were investigated, giving the total number of twenty plants employed in this research.

Molecular characterization of transgenic plants

DNA extraction and PCR detection of the presence of the m*gfp*5-ER gene in T_0, T_1 and T_2 plants were performed as previously described (Hraška et al. 2005), but with the different primers described below. A detailed study of transgene expression in various organs in different developmental stages, detached from T_1 plants, was performed on an mRNA level by means of reverse transcriptase PCR (RT-PCR). Total RNA was extracted from the following plant parts and organs using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions: mature seeds, germinating 7 days old seedlings, immature seeds of the T_2 generation, roots, stems, leaf blades, isolated conductive elements, sepals, senescent sepals, petals, anthers, stamens, stigmas and styles, ovaries, immature inflorescences of three different developmental stages, pollen and immature capsules. Residual DNA was removed from RNA samples by RNase-free DNase digestion (25 U/ 100 µl at 37 ºC for 10 min, inactivation at 70 ºC for 5 min) and first strand cDNA was synthesised using oligo- dT primers and the Revert Aid First Strand cDNA Kit (Fermentas, Lithuania) according to the manufacturers instructions. A 2 µl sample of obtained cDNA was used in a PCR amplification of m*gfp*5-ER region with primers GFP5-u (5′-ACC CAG ATC ATA TGA AGC GG- 3′) and GFP5-l (5′-TTG GGA TCT TTC GAA AGG GC- 3′). A PCR reaction was performed in a total volume of 20 µl reaction mixture, containing 2× PPP Combi Master Mix (150 mM Tris-HCl, 40 mM $(NH_4)_2SO_4$, 0.02% Tween 20, 5mM $MgCl_2$, 400 µM of each dNTP, 1U of Taq polymerase, 38 mM monoclonal anti-Taq) (Top-Bio, Czech Rep.) and 0.2 pmol/ ul of each primer. The samples were amplified using 32 cycles (94 ºC for 1 min, 55 ºC for 30 s, 72 ºC for 30 s) and the reaction products were analysed on 1.5% (w/v) agarose gel stained with ethidium bromide. DNA and RNA from T_2 plants were isolated and analysed in the same way, but the RNA was isolated only from young leaves. Ten T_2 plants from each selected T_1 plant progeny

were analysed by PCR for the presence of the transgene and four plants selected for further microscopic studies were analysed for the transgene expression using the RT-PCR with the same primers and reaction conditions mentioned above.

Visualization of green fluorescent protein

Fresh tissues and organs were dissected from T_1, T_2 transgenic and control tobacco seedlings and plants at various developmental stages and investigated directly on Petri dishes. All hand made cuts were prepared from fresh tissues and covered by a droplet of distilled water to avoid rapid desiccation of objects before subsequent microscopic studies. The GFP fluorescence was visualized with a Leica MZ 12 stereo dissecting microscope coupled with an excitation source and filter set permitting the GFP visualization- excitation filter 480/ 40 nm, dichroic mirror 505 nm and emission filter 510 nm LP. Photographs were taken with an Olympus digital camera C5050 Zoom. Fluorescence images of samples studied from the transgenic and control plants were taken simultaneously and when possible also bright field images were taken.. Because some tissues from untransformed plants lack any visible fluorescence, such objects were not photographed under the excitation light.

Results

Molecular characterization of transgenic plants

The presence of the transgene in regenerated T_0 plants grown on the selection medium containing 500 mg L^{-1} Kn was confirmed by PCR reaction (data not shown). Also the presence of the transgene in all T_1 and T_2 was verified by PCR (examples given on Figs. 1a, 1b), when the expected band sized 415 bp was amplified in all tested samples. Moreover, m*gfp*5-ER expression in T_1 plants was confirmed on the mRNA level by RT-PCR. The particular band sized 415 bp corresponding to a specific region of the m*gfp*5-ER transcript was present in all studied samples from the main floral parts (Fig. 1c). Controls supplied with only the total RNA samples without a reverse transcription reaction did not yield any product, indicating that there was no DNA contamination of RNA samples and

thus the PCR products obtained after RT-PCR were solely due to the presence of a transgene transcript. Therefore, the expression of the transgene was confirmed in all tissues of T_1 transgenic plants investigated subsequently by fluorescence microscopy. The transgene expression was also confirmed in all studied T_2 samples (Fig. 1d).

GFP expression patterns during seed germination

The first developmental stages studied in our work were seeds taken from four selfed independent T_0 transgenic tobacco plants. Very low levels of GFP fluorescence were detected in the mature seeds immediately after the end of a dormant period when sown on an agar nutrition medium. Nevertheless, RT-PCR revealed the transgene expression, indicating that some low levels of *gfp* transcript are present in seeds (Fig.1c). Clear bright green fluorescence was observed after the next 24 h. Such fluorescence was strong enough to distinguish between the fluorescent, putative transgenic and dark, most probably non-transgenic seeds (Fig. 2A). Following the germination, differences in the fluorescence intensity between putatively transgenic and non-transgenic seeds were more evident, especially when the root tip started to emerge from the seed coat (Figs. 2B, 2BB). The non-transgenic mature and germinating seeds did not show any green fluorescence and images captured under the same magnification and exposure times showed only faint contours of samples studied (Fig. 2BB). The observations confirmed that the green fluorescence in transgenic seeds is most probably due to the presence and activity of GFP. Following the emergence of plantlets, strong green fluorescence was observed in the growing roots, especially in the root tips and hairs and in the bases of the newly emerging hypocotyls (Fig. 2C). Surprisingly, high levels of green autofluorescence were observed also in the growing roots of the control seedlings (data not shown). Thus this green fluorescence could not be explained solely as a result of GFP and promoter activity. After the complete development of the hypocotyls and cotyledons, the green fluorescence was evident throughout the whole transgenic plantlet aerial part; not as strong as in the root section, but still intense enough to permit visual discrimination between putative transgenic and non-transgenic individuals. Strong GFP fluorescence and thus CaMV 35S activity was detected in stomatal guard cells on the abaxial surface of the developed cotyledons, visible as small bright green spots on the cotyledon surface (Fig. 2D), which was also easily distinguished later during the development of true leaves, as well as in mature leaves. Again, the non-transformed controls exhibited no visible GFP fluorescence (Figs. 2E, 2EE). Finally, at the

end of this period (emergence of the first true leaves) the GFP fluorescence was evident in whole plantlets, with observed fluctuations when the strongest GFP activity was detected in the stomatal guard cells.

GFP expression in leaves, stems and roots

Various levels of green fluorescence were observed within the main plant organs and tissues. In leaf blades, GFP green fluorescence was apparent throughout the whole leaf surface, but younger leaves from transgenic plants exhibited more intense green fluorescence than the older ones (data not shown). This loss in fluorescence indicated a possible decrease of CaMV 35S promoter activity in older cells or some changes in the cell physiology. Very strong GFP fluorescence was detected in vascular tissues and all conductive elements of various sizes, and was evident throughout the whole leaf area. Using the stereomicroscope equipped with the GFP adaptor even very thin vascular elements were easily distinguished within the leaf blade (Figs. 2F and 2G). The fluorescence was strongest in the midrib area. Beside the central midrib, also other vascular elements were visible in the leaf blade and exhibited fluorescence stronger than the surrounding mesophyll and epidermal cells. The stomatal guard cells, visible as a glowing green spots and tips of trichomes exhibited much stronger fluorescence than the neighbouring cells (Figs.2GG). No such fluorescence was visible in the control stomatal guard cells, and only faint autofluorescence was recorded on the trichomes from control samples. Strong CaMV 35S activity in the central conductive elements was also evident in the first, second and third transversal cuts measured from the footstalk throughout the leaves (Figs. 2I and 2II). Hand-made longitudinal cuts throughout the separate midrib (footstalk) showed the CaMV 35S activity inside this tissue – seefigure 2H. Strong fluorescence was visible in phloem cells while epidermal and xyleme cells lacked the fluorescence (Figs. 2I. 2II). Longitudinal cuts revealed strong GFP activity in the central sieve elements (Fig 2H).

As in the case of leaves, fluctuations in GFP activity within the stem body were observed, but such differences were not as obvious. A relatively constant green signal was visible over the surface of a stem detached from the transgenic plants (Fig. 2J). As shown on a longitudinal cut of the stem in figure 2K, the fluorescence was nearly stable within the whole stem body and only phloem cells possessed stronger GFP fluorescence. This fluorescence pattern was constant among stem cuts taken from various positions within the

plant body. A different situation was observed when transversal cuts were prepared. These observations revealed that epidermal cells and the cortex layer possessed very faint green fluorescence, with some visible brighter spots on the surface only, which were identified as trichomes (Fig. 2M). In the direction from the surface towards the stem centre, the fluorescence intensity culminated at first in the phloem cells, which possessed very strong green fluorescence. But more faint GFP fluorescence and thus the promoter activity was observed in xyleme cells and the central pith parenchyma (Fig. 2M). Dark areas lacking any fluorescence in the central stem part were identified as intercellular spaces. Changes were also observed in the xyleme constitution during stem ageing, when a compact cell mass without any intercellular spaces was observed in the younger parts and growing intercellulars in the older ones (Figs. 2M to 2R).

The fluorescence patterns in the roots of seedlings in very early stages have already been mentioned above. Following the roots' emergence, the meristematic area of the root tip was clearly distinguished, indicating the high level of CaMV 35 S promoter activity in root cells (Fig. 2S). Moreover, single cells of root hairs emerging from the main root were also visible. Strong green fluorescence was observed over the entire length of the mature root (3SS). Longitudinal and transversal cuts of physiologically mature roots are captured on images 2T and 2TT, respectively. Nevertheless, all these findings were subsequently found confusing and controversial since surprisingly high levels of autofluorescence were also observed within root tissues excised from control plants, where very similar fluorescence patterns were revealed. Such autofluorescence was uniform along the whole organ, in all of its parts (data not shown). This autofluorescence possessed by some genetically unmodified tissues is one of the few weak features in the GFP visual marker system. In such cases, additional investigation of control and transgenic samples could be required for their clear-cut discrimination. An investigation of the root parts subsequently performed under the transmission fluorescence microscope also gave unsatisfactory results. It was possible, rarely, to distinguish the control samples, exhibiting yellowish autofluorescence, from the brightly green transgenic samples, but a detailed comparative study of GFP fluorescence in roots is still not fully reliable (data not shown).

GFP expression patterns in floral organs

Buds and flowers detached from transgenic tobacco plants were investigated very extensively as the information about CaMV 35S activity in plant reproductive organs is

still insufficient. GFP fluorescence was observed along the whole surface of flowers detached from transgenic plants (Figs. 3C, 3CC). For comparison, fluorescent images of flowers from control plants are given on Figs. 3D and 3DD. From the beginning of flower bud development, the GFP fluorescence was visible over the whole surface of the enclosing sepals and frequently occuring trichomes (Fig. 3E). During the flower opening, a little more intensive green fluorescence was visible at the edge of the still unopened petals. Mature sepals and petals exhibited clear GFP fluorescence, constant throughout the organ surface. As shown in figure 3C, the green fluorescence of petals was much stronger compared to sepals and true leaves (Fig. 2F). Very strong GFP activity was visible in vascular tissues throughout the whole petal (Fig. 3EE).

The reproductive organs were well visible under excitation light inside the whole flower (Fig. 3CC). The fluorescence was seen in anthers and stamens (Figs. 3G, 3GG, 3H), the, stigma, style and ovary (Figs. 3HH, 3I). The GFP signal was strong in stamen filaments, styles and ovary tissues, whereas fluorescence in anthers was weaker. Stamen filaments consisting of massive conductive elements covered by a thin indistinct cortex exhibited very intensive green fluorescence and their conductive elements were easily visible also within the anther body (Fig. 3H). Compared with the male organs, the stigma exhibited weaker GFP activity (3HH).

Detailed investigations of GFP fluorescence patterns in developing inflorescences were performed on sections from transgenic plants at various stages of flowering. Petals in an early developmental stage could be easily distinguished from sepals. Strong GFP activity was visible in the ovary (Fig. 3J) and was pronounced throughout the whole inflorescence development and later flower maturity (Figs. 3JJ and 3K). Following further inflorescence growth, sepals lacked the visible GFP fluorescence whereas it was evident in petals, stamen filaments, anthers and styles (Figs. 3J to 3K). Patterns of GFP fluorescence observed among the floral organs are similar to those seen in the earlier stage described above: bright green fluorescence was visible in petals, the basal part of a pistil (ovary), stamen filaments and anthers, in contrast to a weak fluorescence possessed by sepals and stigma. Intensively fluorescing filaments, continuing as conductive elements inside the anthers were again evident, indicating that the strong CaMV 35S promoter activity is characteristic for this tissue type (Figs. 3L and 3M). Strong GFP activity was observed in the ovary and surrounding integuments and in stamens (Fig. 4A). Petals showed bright green fluorescence, while sepals exhibited a weaker signal (Fig. 4A). Pollen from both the

transgenic and control plants showed very weak fluorescence, making it impossible to differentiate transgenic and non-transgenic samples (Figs. 4G, 4GG).

GFP activity in capsules and seeds

Capsules detached from transgenic tobacco plants possessed faint fluorescence when observed under the fluorescence microscope, but such intensity was still sufficient for an easy discrimination from untransformed ones, which were completely dark (4B, data from untransformed capsule are not shown). Capsule coats lacked any intesive green fluorescence, whereas high levels of GFP activity were seen in the placenta, immature seeds and central conductive elements, as revealed by transversal cuts (Fig. 4BB). Longitudinal cuts showed intensively fluorescing central vascular tissue and immature seeds under the thin medial membrane (Fig. 4C). Interestingly, low levels of autoflorescence occurring mainly in immature seeds were observed in capsules excised from untransformed plants. Nevertheless, such fluorescence was faint when compared with organs detached from transformed plants (Fig.4D). Immature seeds dissected from green capsules showed intensive green fluorescence (Fig. 4E), but during the seed maturation this signal decreased and it finally diminished in fully mature, dormant seeds (Fig. 4EE).

Discussion

We presented here a comprehensive study describing the performance of the CaMV 35S promoter in various tissues during the tobacco plant development, using the GFP as an efficient tool for the visualization of promoter activity. GFP transgenic plants of T_1 and T_2 generations were employed in our study. GFP fluorescence and thus the promoter activity was tracked from early seed germination (T_1 generation) throughout the whole plant life cycle, to the production of a new generation of seeds and plants (T_2). Special emphasis was given to the study of reproductive and floral organs, since the knowledge of the CaMV 35S performance in these tissues is still unsatisfactory. Transgene expression in various plant tissues was confirmed by RT-PCR and organs and tissues excised from non-transgenic (control) plants were investigated too.

The CaMV 35S expression was studied here beginning with the mature seeds of selfed T_0 transgenic plants (a new T_1 generation). In agreement with the results of previous studies (Benfey and Chua 1989; Benfey et al. 1990a), no visible GFP fluorescence was observed. This is different from Arabidopsis, where, for example, the red fluorescent- (RFP) or yellow fluorescent protein (YFP) can be detected also in dry seeds and used as a selectable marker instead of antibiotic resistance. This picture changed when tobacco seeds were sown on a selectable nutrition medium. As early as 24-48h later the bright green GFP fluorescence could clearly be seen. The absence of fluorescence in mature dried seeds was most probably due to very low water content and thus an overall low level of vital processes within the seeds. Nevertheless, the RT-PCR revealed a low degree of the *gfp* expression and therefore some level of GFP could be assumed. But such amount was probably too low to be revealed by fluorescence microscopy. Sunilkumar et al. (2002) considered that the truly constitutive promoter should possess expression beginning from the very early plant developmental stages; thus they examined the cotton zygote. They failed to detect any fluorescence during the globular, torpedo and heart stage embryos and concluded that the promoter was either not expressed or the expression was too low to detect. Custers et al. (1999) reported the first detectable transgene activity in the mid-cotyledon stage of Brassica napus transformed with a CaMV 35S driven *uidA* gene. However, no further data or photographs of histochemical staining were provided. We found that, following the seed embryo development, an intensive green fluorescence was detected in the emerging roots, hypocotyls and cotyledon bases with a very pronounced fluorescence in various meristem cells. During the next seedling growth, the GFP activity spread throughout the vascular system and stomatal guard cells of fully developed cotyledons. In contrast to the findings reported by Sunilkumar et al. (2002), constant green fluorescence with higher activity in the root tip was observed along the whole radicle, but, as mentioned above, these finding are not fully reliable since relatively high levels of autofluorescence were revealed also in radicles and roots from untransformed plants. Thus we cannot say that such green fluorescence was due only to the GFP performance. Nevertheless, simultaneously performed studies of developing seeds originating from untransformed plants showed no visible fluorescence among the main tissues except the radicles/ roots.

Following the plant’s development, GFP activity was apparent over the whole leaf surface, in the blade as well as in the petiole (footstalk); nevertheless the highest levels of GFP activity were observed in the vascular tissues of all sizes and types. Very intense GFP

fluorescence visible in the petioles and midribs was detected also in our previous investigations (Hraška et al. 2005). In addition, also easily visible were stomatal guard cells and trichomes, which however exhibited weaker fluorescence. Weaker CaMV 35S activity in trichomes was reported previously by Yang and Christou (1990) and Benfey et al. (1990b) in soybean and tobacco, respectively. Moreover, Yang and Christou (1990) reported higher levels of promoter activity in stomatal guard cells on the basis of histochemical GUS staining. Generally, the same expression patterns within mature genuine leaves as we observed, were reported also by Rooke et al. (2000) who worked with the ubi1 driven *uidA* gene constructs, and Sunilkumar et al. (2002), with the CaMV 35S driven *gfp* gene.

Our results suggest that the CaMV 35S promoter is active in all cell types of true tobacco leaves, with some variability detected among leaves of different ages. The decline of GFP fluorescence in older leaves was reported previously by many authors and various reasons are hypothesised (reviewed by Hraška et al. 2006). Here we recorded sufficient fluorescence in older (mature) leaves, indicating the CaMV 35S expression. Rooke et al. (2000) also reported stronger GUS staining in younger tissues than in the older ubi1 *uidA* transgenic wheat lines, indicating the possibly stronger activity of constitutive promoters in younger cells. The CaMV 35S promoter is presumed to be more active in younger cells and tissues which are in addition usually characterized by stronger level of proteosynthesis (Preťová et al. 2001).

Fluorescence photographs of mature stems confirmed the consistent GFP signal indicating the CaMV 35S activity along the whole stem surface, regardless of physiological age. Stronger fluorescence was observed again in vascular tissues and/ or phloem, whereas lack of fluorescence was evident in epidermal cells, cortex and xyleme cells. Sunilkumar et al. (2002) reported the appearance of GFP fluorescence also in the epidermis, cortex tissue and pith region of transgenic cotton. Expression of the CaMV 35S promoter in the stem in tobacco and soybean was described previously (Benfey et al. 1990b, Yang and Christou, 1990). Mature, fully developed roots of transgenic plants possessed strong GFP fluorescence visible in most parts throughout the whole organ. Nevertheless, as mentioned above, relatively strong green autofluorescence observed also in the roots detached from untransformed plants makes these findings somewhat uncertain. It seems that, due to the presence of some autofluorescing compound in the tobacco roots, the GFP is not a reliable tool for the assessment and tracking of the expression of promoters in roots and should be replaced in this particular case by some another marker.

For example, strong GUS staining associated with the main root, secondary roots and root hairs, with intensive staining in the root tip of transgenic wheat was also reported for the CaMV 35S promoter Rooke et al. (2000). Moreover, they observed the diminishing of GUS activity in older roots, restricted only to the vascular tissue. Benfey et al. (1989) reported much higher CaMV 35S driven *uidA* activity in a root cap.

The study of CaMV 35S promoter activity among different organs within inflorescence during various developmental stages was a main concern in our work. The first detailed study focused on this point was reported by Sunilkumar et al. (2002). In our report, whole fully developed inflorescences generally exhibited bright green fluorescence, showing the brightest fluorescence in regions of vascular tissues. In addition, the GFP activity was observed in all main floral organs. Rooke et al. (2002) also reported GUS activity in all developed flower parts of ubi1 *uidA* transformed wheat. Moreover, they observed stronger GUS staining in young carpels compared to older ones, where GUS activity was restricted in lodicules, stigma and the style. Using longitudinal and transversal cuts we revealed various degrees of GFP fluorescence occurring among different organs and throughout inflorescence development. Constant CaMV 35S activity was observed in ovaries, stamens, anthers and vascular elements, whereas lack of fluorescence was apparent in stigma. An intense GFP signal was evident in various vascular tissues within floral organs as well as in the immature capsule and therefore the predominant activity of the CaMV 35S promoter in such tissue type was confirmed. Anyhow, a strong marker gene expression in vascular tissues was apparent within the whole plant body, thus indicating that the strong CaMV 35S promoter activity is associated with cells acting in the transport of solutes over the plant body. This finding is supported by many previously presented reports describing generally the same expression pattern of constitutive promoters (Rooke at al., 2001, Sunilkumar et al. 2002,). Cornejo et al. (1993) suggested that this higher expression could be due to a higher level of cell division within some organs. Preťová et al. (2001) noticed that CaMV 35S activity is usually stronger in tissues with higher cell density and proteosynthesis.

The aim of the study presented here was to investigate the activity of the CaMV 35S promoter throughout the whole tobacco life cycle using the GFP fluorescence as a tracking tool. A special emphasis was given to flowers and reproductive organs and original data regarding these organs are presented. To our knowledge, this is the first detailed report about the behaviour of CaMV 35S in such types of organs, and moreover performed on two subsequent generations of autogamized transgenic plants. As we

expected, different promoter activity in all main plant organs and tissues within the plant body, and during various developmental stages of the tobacco life cycle was detected. Predominant CaMV 35S performance in vascular tissues was confirmed GFP has been found to be an efficient tool for the evaluation of promoter activity in most tissues and organs; nevertheless some limitations of its use, caused by strong autofluorescence of some tissues, have been encountered. This should be taken into account when interpreting such data. CaMV 35S is generally considered to be a constitutive promoter, nevertheless nowadays some authors conclude that it is no longer adequate to describe it as “constitutive” solely based on the expression in all plants organs without taking into account criteria such as different metabolic activity and corresponding levels of transcription and thus also translation (Preťová et al. 2001). The information presented in this paper adds another useful part into the mosaic of our knowledge about the behaviour of the CaMV 35S promoter in different plant organs and tissues during plant development and stability of its expression pattern in the next generations of plants.

Acknowledgements

The authors are grateful to Stanislav Vitha (Microscopy and Imaging Center Texas A&M University) for his valuable critical comments and revisions. The authors would like also gratefully acknowledge the financial support received from Ministry of Education, Youth and Sport of the Czech Republic (grants 1M06030, 1PO5ME800 and MSM 60076658-06). M. H. received the support from grant GA ČR 31/H160 provided by the Grant Agency of the Czech Republic. Linguistic revision was kindly performed by John McAvoy, Journal of Applied Biomedicine.

References

Battraw MJ, Hall TC (1990) Histochemical analysis of CaMV 35S promoter- β-glucuronidase gene expression in transgenic rice plants. Plant Mol Biol 15:527–538. DOI 10.1007/BF00017828

Benfey PN, Chua N-H (1989) Regulated genes in transgenic plants. Science 244:174–181

Benfey PN, Ren L, Chua N-H (1990a) Tissue specific expression from CaMV 35S enhancer subdomains in early stages of plant development. EMBO J 9:1677–1684

Benfey PN, Ren L, Chua N-H (1990b) Combinatorial and synergistic properties of CaMV 35S enhancer subdomains. EMBO J 9:1685–1696

Cornejo MJ, Luth D, Balkenship KM, Andreson OD, Blechl AE (1993) Activity of a maize ubiquitin promoter in transgenic rice. Plant Mol Biol 23:567–581. DOI 10.1007/BF00019304

Custers JBM, Snepvangers SCHJ, Jansen HJ, Zhang L, van Lookeren C (1999) The 35S-CaMV promoter is silent during early embryogenesis but is activated during non-embryogenic sporophytic development in microspore culture. Protoplasma 208:257–264. DOI 10.1007/BF01279097

Haseloff J, Siemering KR, Prasher DC, Hodge S (1997) Removal of a cryptic intron and subcellular localisation of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly. PNAS USA 94:2122–2127. DOI 10.1073/pnas.94.6.2122

Horsch RB, Fry JE, Hoffmann NL, Eicholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231

Hraška M, Rakouský S, Kocábek T (2005) Use of simple semiquantitative method for appraisal of green fluorescent protein gene expression in transgenic tobacco plants. Biol Plant 49:313–316. DOI 10.1007/s10535-005-3316-z

Hraška M, Rakouský S, Čurn V (2006) Green fluorescent protein as a vital marker for non-destructive detection of transformation events in transgenic plants. Plant Cell Tiss Organ Cult 86:303–318. DOI 10.1007/s11240-006-9131-1

Jefferson RA, Ravanagh TA, Bevan MW (1987) GUS fusion: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Malik K, Wu K, Li X-Q., Martin-Heller T, Hu M, Foster E, Tian L, Wang C, Ward K, Jordan M, Brown D, Gleddie S, Simmonds D, Zheng S, Simmonds J, Miki B (2002) A

constitutive gene expression system derived from the tCUP cryptic promoter elements. Theor Appl Genet 105:505–514. DOI 10.1007/s00122-002-0926-0

Mascarenhas JP, Hamilton DA (1992) Artifacts in the localization of GUS activity in the anthers of petunia transformed with a CaMV 35S-GUS construct. Plant J 2:405–408. DOI 10.1111/j.1365-313X.1992.00405.x

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497. DOI 10.1111/j.1399-3054.1962.tb08052.x

Odell JT, Nagy F, Chua N-H (1985) Identification of DNA sequence required for activity of cauliflower mosaic virus 35S promoter. Nature 313:810–812

Ooms G, Hooykaas PJJ, van Veen RJM, van Beelen P, Regensburg-Tuink TJG, Schilperoot RA (1982) Octopine Ti-plasmid deletion mutants of *Agrobacterium tumefaciens* with emphasis on right side of T-region. Plasmid 7:15–29

Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier MJ (1992) Primary structure of the Aequorea victoria green-fluorescent protein. Gene 111:229–233

Preťová A, Obert B, Wetzstein HY (2001) Leaf developmental stage and tissue location affect the detection of β-glucuronidase in transgenic tobacco plants. Biotech Lett 23: 555–558. DOI 10.1023/A:1010311524048

Rooke L, Byrne D, Salgueiro S (2000) Marker gene expression driven by the maize ubiquitin promoter in transgenic wheat. Ann Appl Biol 136:167–172. DOI 10.1111/j.1744-7348.2000.tb00022.x

Stewart CNJr (2001) The utility of green fluorescent protein in transgenic plants. Plant Cell Rep 20:376–382. DOI 10.1007/s002990100346

Sunilkumar G, Mohr L-A, Lopata-Finch E, Emani Ch, Rathore KS (2002) Developmental and tissue specific expression of CaMV 35S promoter in cotton as revealed by GFP. Plant Mol Biol 50:463–474. DOI 10.1023/A:1019832123444

Terada R, Shimamoto K (1990) Expression of CaMV 35S-GUS gene in transgenic rice plants. Mol Gen Genet 220:389–392

Tsien RY (1998) The green fluorescent protein. Ann Rev Biochem 67:509–544. DOI 10.1146/annurev.biochem.67.1.509

Williamson JD, Hirsch-Wyncott ME, Larkins BA, Gelvin SB (1989) Differential accumulation of a transcript driven by the CaMV 35S promoter in transgenic tobacco. Plant Physiol 90:1570–1576

Yang N-S, Christou P (1990) Cell type specific expression of a CaMV 35S-GUS gene in transgenic soybean plants. Develop Genet 11:289–293

Figures and figure legends:

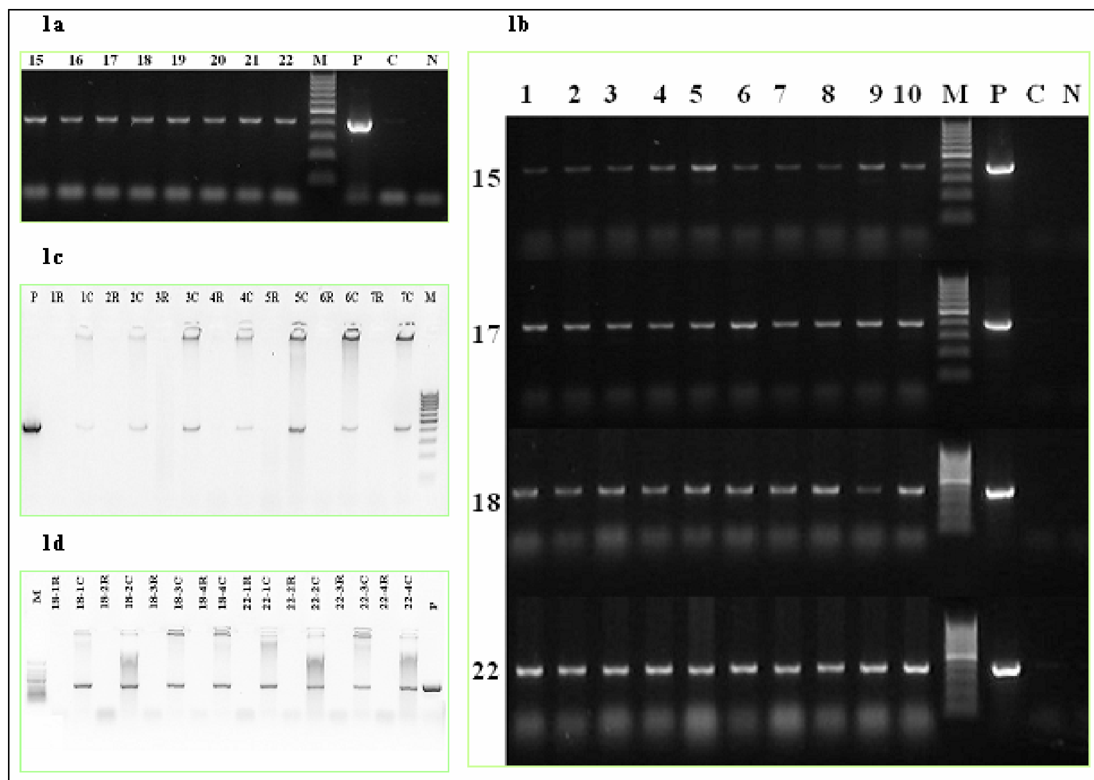

Figure 1a. Example of PCR products analyses of T_1 plants. Primers GFP5-u and GFP5-l were used in PCR. Eight independent plants of T_1 generation grown on the MS selection medium containing 500 mg L^{-1} kanamycin were subjected for PCR detection. Among these individuals, four plants were randomly chosen for microscopic studies of Ca MV35S expression. 15–22 = independent T_1 individuals; M = 100 bp molecular weight marker; P = positive control (pBINmGFP5-ER plasmid), 415 bp; C = negative control (DNA from untransformed plants); N = negative control without DNA template (water only).

Figure 1b. Example of PCR products analyses of T_2 plants. Primers GFP5-u and GFP5-l were used in PCR. Ten T_2 seedlings from each of four individuals (previously selected plants denoted 15, 17, 18, 22) subjected for microscopic studies were cultured on the selection medium containing 500 mg L^{-1} kanamycin and subjected for PCR detection.

Among these ten PCR positive seedlings, four individuals belonging to each of four T_1 plants were randomly chosen for further microscopic studies and RT-PCR detections. 1–10 = independent T_2 individuals; M = 100 bp molecular weight marker; P = positive control (pBINmGFP5-ER plasmid), 415 bp; C = negative control (DNA from untransformed plants); N = negative control without DNA template (water only).

Figure 1c. RT-PCR products analyses of T_1 plants examined for the m-*gfp*5-ER gene expression in selected plant tissues. A representative image of seven samples is given here. P = positive control (pBINmGFP5-ER plasmid), 415 bp; 1 = seeds; 2 = leaf blade; 3 = conductive elements; 4 = sepal; 5 = petal; 6 = anthers; 7 = stamen filaments; M = 100 bp molecular weight marker; C = cDNA samples; R = corresponding RNA samples subjected simultaneously for PCR to exclude the possible DNA contamination of RNA.

Figure 1d. An example of RT-PCR products analysis of T_2 plants for the expression of m-*gfp*5-ER gene. Progeny (four plants, 1–4) from two selected T_1 plants denoted 18 and 22 was subjected for analysis. M = 100 bp molecular weight marker; C = cDNA samples; R = corresponding RNA samples subjected simultaneously for PCR to exclude the possible DNA contamination of RNA; P = positive control (pBINmGFP5-ER plasmid), 415 bp.

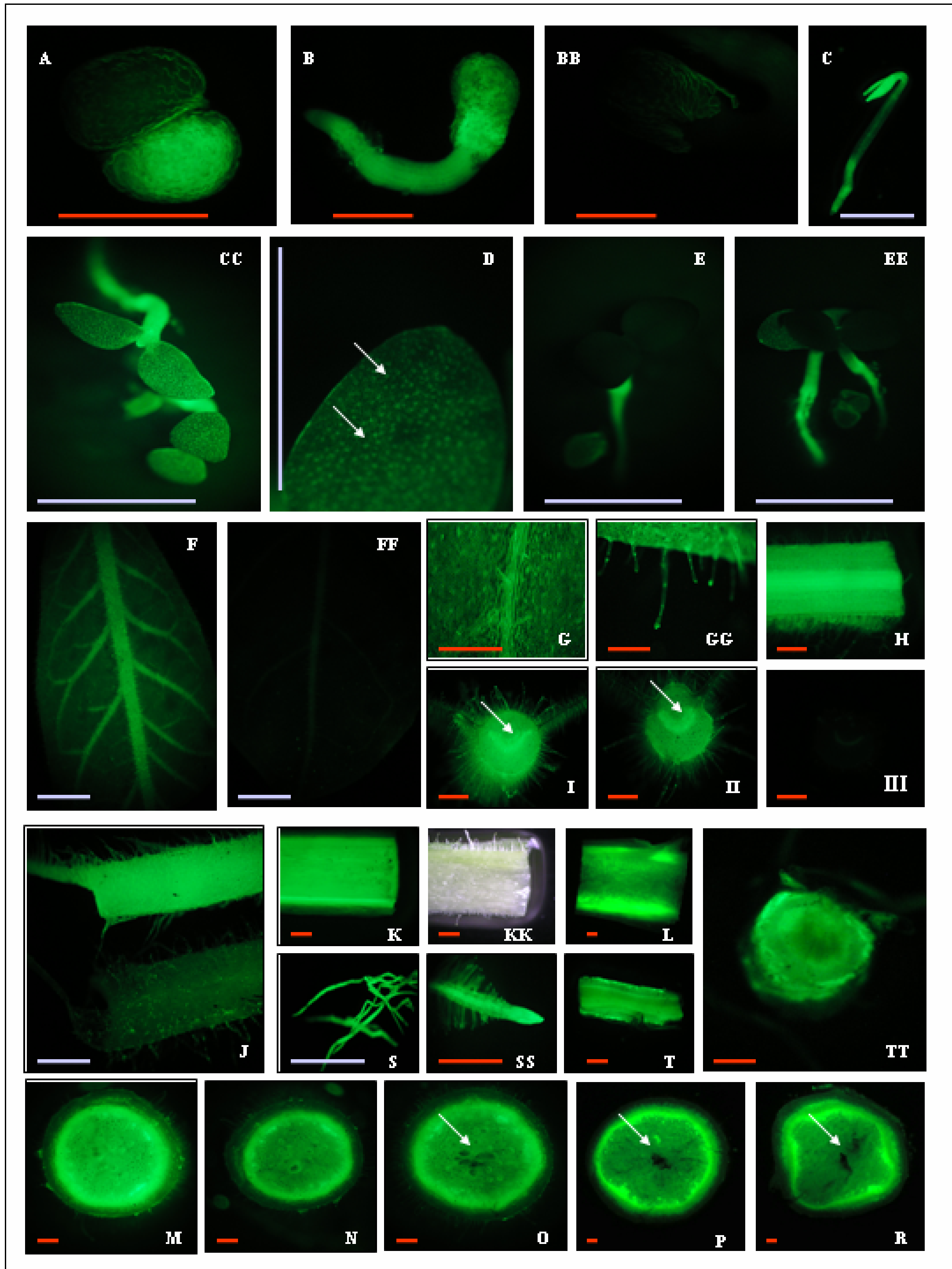

Figure 2. Expression patterns of CaMV 35S promoter driven GFP synthesis, revealed by its fluorescence after light excitation, in seeds, leaves, stems and roots detached from transgenic tobacco plants carrying the m-*gfp*5-ER gene and control (unmodified) plants. (A) represents the fluorescence image of two T_1 seeds, one transgenic, showing brightly green fluorescence and thus expressing the *gfp* (right), whereas the second lacks any fluorescence (left) and thus not carrying and/or expressing the *gfp* gene. On parts B and BB

is a comparison of the fluorescence of putatively transgenic seed three days after sowing on MS medium and non-transgenic control seed respectively. The non-transgenic seed lacks any fluorescence and only faint contours of a seed coat are visible. C and CC are fluorescence images of seedlings with developing, and fully developed cotyledons, respectively. Small bright green spots, indicating high CaMV 35S promoter activity, representing the stomatal guard cells are clearly visible within the cotyledon adaxial surface (CC). A detail of the stomatal guard cells is presented in more detail on the image D (arrow indicates stomatal guard cells). Note, that the seedling on the figure (C) was cultivated in the dark to achieve the hypocotyl elongation and thus enable easier study of GFP expression in this part. E and EE are fluorescence images of untransformed, control seedlings (E) and seedlings of transgenics segregating for the presence of *gfp* gene (EE). An untransformed (dark) seedling is visible on the clearly fluorescing background of the transformed one (EE). F and FF are fluorescence images of the abaxial side of fully developed true leaves detached from transgenic plants and control plants, respectively. Note, that only faint contours of vascular tissue could be recognized within the dark surface of untransformed leaf. A high level of GFP fluorescence (promoter activity) is visible in the conductive elements. Trichomes and stomatal guard cells (small, bright green spots) are visible under higher magnifigation as well (G). A detailed fluorescence image of trichomes merging from the leaf surface is given in figure GG. I and II are fluorescence images of transversal cuts done throughout the leave blade in its first third, close to the footstalk (I) and the second third (II), further from the footstalk (arrows indicate central conductive elements). III is an image of the same tissue type as on the I, but detached from untransformed plants. H represents a longitudinal cut throughout the central midrib (footstalk). J, K represent fluorescence images of young stem surface and its longitudinal cut, respectively. A bright green fluorescing transgenic stem is easily visible when compared with the faint, weakly visible stem detached from the control plant. KK is a representative light image of longitudinal cut throughout the stem in a droplet of distilled water. O is a fluorescence image of cut throughout the stem form basal plant part. Images M to R are fluorescence images of transversal cuts throughout the stem of different age M – the youngest (apical)-, R – the oldest (basal) part, arrows indicate intracellular areas lacking the GFP fluorescence. S and SS are fluorescence images of mature roots, (S) of root hairs and a root tip SS. Simultaneously, longitudinal and transversal cuts of a mature root are given on images T and TT, respectively. Blue and red bars represent 1 cm and 1mm, respectively.

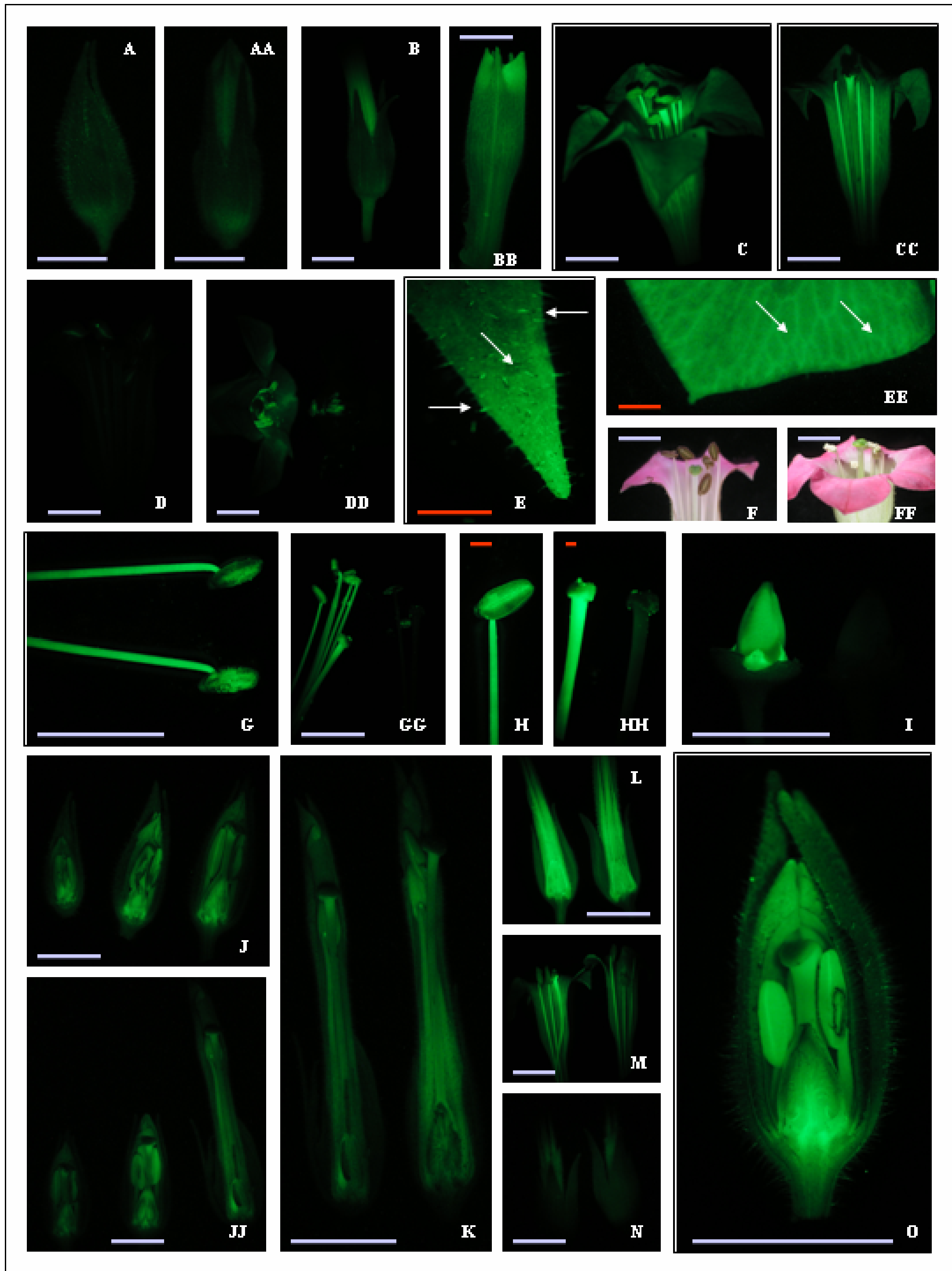

Figure 3. Expression patterns of CaMV 35S promoter driven GFP synthesis in flowers and generative organs of transgenic tobacco in comparison to control (unmodified) plant. Sections A and AA are fluorescence images of growing inflorescence at early stages of young buds. Just faint fluorescence signal could be detected within the base of the inflorescence indicating a low promoter activity. B and BB are fluorescence images of

basal- (B) and upper (BB) part of nearly fully developed flower before the opening. C is a fluorescence image of fully opened flower detached from transgenic plant. Image CC reveals the top parts of reproductive organs after partial removal of petals. D is an image of mature flower with removed petals, detached from control (untrasformed) plant. DD represents an image showing the apical parts of opened transgenic (left) and untrasformed (right) flowers placed against each other. E is a fluorescence image of a sepal part; note that trichomes (arrows) are easily visible within the sepal surface. EE shows a fluorescence image of a petal surface under high magnification. Note, that intensely fluorescing conductive elements (arrows) are clearly visible within the petal surface. F and FF are illustrative light images of apical parts of opened flowers prepared for investigations. G to I are fluorescence images of reproductive organs detached from the same transgenic tobacco plant. Note, that on figures GG, HH and I representative fluorescence images of transgenic (left) and control (right) stigmas, anthers (GG), individual stigmas (HH) and ovaries (I) are presented. J, JJ and K are fluorescence images of longitudinal cuts throughout the inflorescence during various developmental stages. Petals partially covering anthers are visible and moreover carpels can be distinguished in a pistil. Note, that the ovary exhibits the most intense GFP fluorescence, as compared to other organs, from the early developmental stage of a flower bud. Longitudinal cuts throughout the opened flower are captured on images L (basal parts) and M (apical parts). N is and fluorescence image of basal flower parts showing the fluorescence pattern in sepal. O gives a detail fluorescence image of developing inflorescence. Note, that strong fluorescence (and thus assumed also CaMV 35S activity) is visible in the ovary, anthers and petals. Also trichomes on the sepal surface are easily visible. Blue and red bars represent 1 cm and 1mm, respectively.

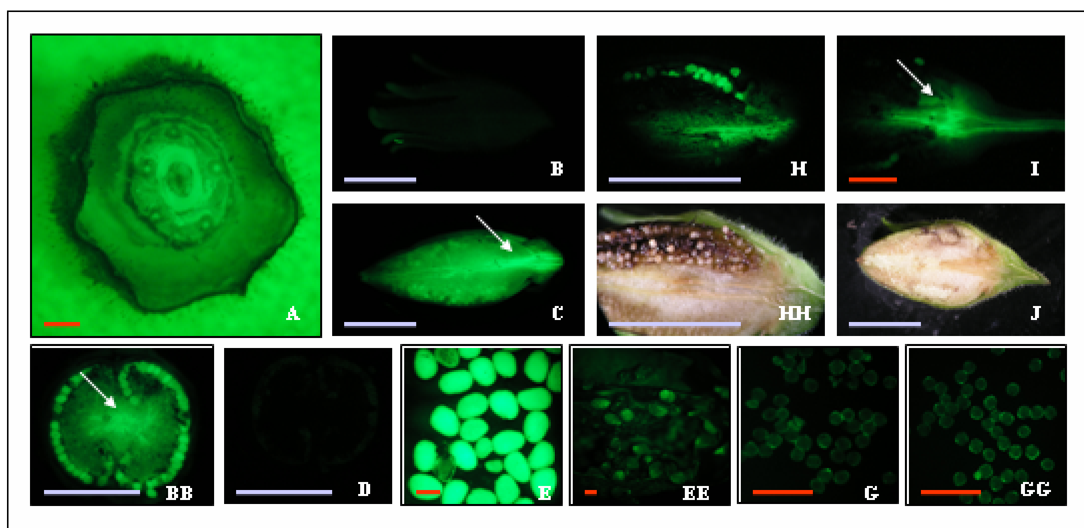

Fig. 4. Expression patterns of CaMV 35S promoter driven GFP synthesis in flower, capsules, seeds and pollen from transgenic and control (unmodified) plants. Part A shows the fluorescence image of transversal cut throughout the base of a fully developed flower. The surrounding light green signal comes from the synthetic foam used to fix the sample in the desired position. B is a fluorescence image of surface of immature transgenic capsule. Note, that capsule possess only faint green fluorescence. BB and C are fluorescence images of longitudinal and transversal cuts throughout the immature capsule. Arrows indicate strong GFP fluorescence and thus CaMV 35S activity within the vascular tissues. D represents the fluorescence image of a transversal cut throughout the immature capsule, detached from untransformed plant. E and EE are fluorescence images of immature and nearly mature seeds from transgenic plants. G and GG are images of pollen originating from control and transgenic plants, respectively (pollen of transgenic plants shows a slightly more intense fluorescence due to GFP protein presence). H is a detailed fluorescence image of longitudinal cut with revealed immature seeds. HH gives a corresponding light image. I is a detailed image of highly fluoresent vascular tissue in basal part (arrow), indicating high CaMV 35S activity. J is an illustrative light image corresponding to image C. Blue and red bars represent 1 cm and 1mm, respectively.

4. 6. GFP DETECTION IN LOW LEVEL SIGNAL/ NOISE RATIO PLANT SAMPLES

Hraška, M., Rakouský, S., (2005): GFP detection in low level signal/noise ratio plant samples. J. Appl. Biomed. 3 (Suppl. 1): S19. (Abstr. Conf. Cells VI., České Budějovice, Czech Republic, October 24-26, 2005). ISSN 1214-0287

GFP DETECTION IN LOW LEVEL SIGNAL/NOICE RATIO PLANT SAMPLES

Hraška M. (1,3), Rakouský S.. (1,2)

[1]Faculty of Biological Sciences (e-mail: mhraska@seznam.cz), [2]Faculty of Health and Social Studies, [3]Biotech. Centre, Agricultural Faculty, University of South Bohemia, 370 05 České Budějovice, Czech Republic.

Green fluorescent protein (GFP) offers a wide range of applications in plant biology (Stewart, 2001). GFP represents an efficient tool for the detection/selection of transgenics and moreover for the improvement of transformation, selection and regeneration protocols for the recovery of stable transgenic plants. GFP has been found to be superior to other marker genes used in plant biology such as GUS or LUC owing to many advantages it displays. Early identification of transgenic cells is crucial point for efficient recovery of transgenic plants and the application of GFP can reduce the amount of material to be handled and analysed through culture and regeneration. GFP green fluorescence allows rapid non-invasive identification of transformed cells and therefore early elimination of non-transformed cells, silencing events or developing chimeras. Moreover, various approaches for quantification of GFP fluorescence have been recently reported with the aim to quantify gene expression and to identify early homozygots (Millwood *et al.* 2003; Hraška *et al.* 2005).

On the other hand problems associated with a low signal/low signal-noise ratio could be met quite often. Low levels of background fluorescence of various compounds present in intact, wounded and untransformed tissues and/or in *Agrobacterium* strains do not usually impede the successful GFP fluorescence detection and can be restricted by implementation of suitable filter systems. Choose of the right detection device is an important factor. Another way to circumvent such complications represents the use of proper tissue or plant part(s) because the intensity of visible fluorescence is affected by

numerous factors among which chlorophyll content and presence of other flouresencing compounds play an important role.

Supported by grants from the Grant Agency of the Czech Republic (GA ČR-31/H160) and from the Ministry of Education of the Czech Republic (1PO5ME800).

References:

Hraška M., Rakouský S., Kocábek T.: Biol. Plant. 49:313-316, 2005.

Millwood R.J., Halfhill M.D., Harkins D., Russotti R., Stewart C.N.Jr.: BioTechniques 4:638-643, 2003.

Stewart C.N.Jr.: Plant Cell Rep. 20:376-382, 2001.

GFP DETECTION IN LOW LEVEL SIGNAL/NOISE RATIO PLANT SAMPLES

Marek Hraška [1,3], *Slavomír Rakouský* [1,2]

[1]Faculty of Biological Sciences (e-mail: mhraska@seznam.cz), [2]Faculty of Health and Social Studies,
[3]Biotechnological Centre, Agricultural Faculty, University of South Bohemia, 370 05 České Budějovice, Czech Republic

Introduction

Many heritable markers are being used in the present plant molecular biology for various purposes. One of such popular markers is a green fluorescent protein (GFP) isolated from pacific jelly fish *Aequorea victoria* and first described by Prasher *et al.* (1992). GFP transforms chemiluminescent blue light emitted by another hydromedusan protein, aequorin, into green light. The agent responsible for fluorescing is the chromophore, which is formed by post-translational modification in which a tripeptide Ser65-Tyr66-Gly67 is cyclized and later oxidized. This chromophore is in the geometric centre of protein to which it is covalently attached. Eleven β sheets form a barrel structure which is capped with α helices on the top and bottom of the protein. α helices also form a scaffold for the central placed chromophore. GFP represents a new class of proteins called "beta can". "Wild type" GFP is a dimer consisting of two monomer units, each consisting of 238 amino acids with a relative weight of 27 kDa. The diameter of the barrels is 30 Å and length is 40 Å (Fig. 1). This wild type GFP emits light after excitation by UV (λ~360–400 nm) or blue (λ~440–480 nm) light with emission spectra at λ=509 nm and with a minor peak at λ=540 nm (Yang *et al.*, 1996). GFP does not require any endogenous cofactors, substrates or other compounds for fluorescent manifestation. Due to its favourable features GFP become a popular tool for various applications in plant biology. Because the detection of fluorescence is a non-invasive process which does not require the destruction of studied sample, the GFP can be successfully employed in the co-selection (or single-step selection) of transgenic plant cells, usually based on the use of various compounds added to the cultivation medium *in vitro*. Such an alternative selection is based on the localization of fluorescing, which means transgenic cells, followed by their isolation and later transfers to another cultivation medium. The fluorescence is mostly studied using various observation sets, usually consisting of some suitable excitation source and confocal microscope or stereomicroscope. Because many other compounds such as chlorophyll possess some levels of fluorescence too, the use of various filter sets is necessary to restrict undesirable background autofluorescence and to allow easier detection of GFP fluorescence. Despite the use of the filters, the study of fluorescence is still complicated even, influenced e.g. by the variant of *gfp* gene, intensity of transcription and used target tissue. For some plant species the choice of appropriate target tissue seems to be an important event influencing successful detection of the fluorescence signal. Although nowadays many plant species were subjected to genetic transformation, still many species and varieties are recalcitrant. Flax (*Linum usitatissimum* L.) belongs to a group of species, usually forming high numbers of escapes, that means cells and shoots without inserted genetic information nevertheless regenerating on a selection medium. Due to it, such selection process is time consuming and little efficient. Addition of another marker gene such as GFP could promote the transformation efficiency of flax experiments. The aim of this study was to compare the effect of some variables of the transformation protocol, especially the suitability of two different sources of regenerating tissues, hypocotyls and cotyledons for GFP based selection.

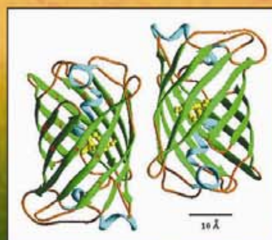

Fig. 1.: Schematic diagram of the GFP molecule. Eleven β sheets representing the structure of barrels are designated green, α helices forming the caps on the top and the bottom of barrel are designated blue. Chromophore placed in the centre of barrels and hold by a scaffold formed by α helices is marked yellow. (Yang *et al.*, 1996)

Results

Total of 96 and 24 hypocotyl explants of cv. Biltstar and cv. Venica, respectively were subjected to *Agrobacterium*-mediated transformation. Altogether 278 regenerated buds of cv. Biltstar and 55 buds of cv. Venica were obtained in the first experiment. Usually, each explant gave rise to 1–3 buds. Studies of the GFP fluorescence were performed beginning from the 2nd day after the co-cultivation and repeated after 2 days, later in 4 days periods. Although the filter set for restriction of noise signals was used, all hypocotyl explants exhibited strong background green fluorescence (Fig. 2a). The same fluorescence level possessed also control, non-transformed explants, indicating that such fluorescence did not originate from the GFP manifestation (Fig. 2b). Identification of transformed and green fluorescing cells in early stages of regeneration thus was not possible. Later, following the regeneration, some of proliferating buds and shoots exhibited green fluorescence signal, which was slightly stronger and brighter than the fluorescence found early in bacteria-treated hypocotyls, but still hardly distinguishable (Fig. 2c). In the second experiment, 43 cotyledons and 126 hypocotyls of cv. Venica, and 28 cotyledons and 55 hypocotyls of cv. Biltstar were subjected to *Agrobacterium*-mediated transformation. Because media promoting callogenesis were used exclusively, no regenerating buds or shoots were found. The same background fluorescence level was observed in the hypocotyls segments as in the first experiment. Proliferating calluses did not possess any distinguishable fluorescence signal originating from hypocotyls of both, transformed and control explants in early stages of cultivation. On the other hand, distinct bright spots in the callus mass proliferating from the cotyledonary explants were visible after 6 days following the co-cultivation (Fig. 3a). No such fluorescence was visible in the control cotyledons (Fig. 3b). Following the cell number outgrowth, the green clusters of fluorescing cells become more evident, but no such cells or fluorescing clusters of cells occurred on some other bacteria-treated cotyledons. Simultaneously, no green fluorescence was detected in control cotyledons, indicating that the fluorescence detected in transformed ones originated from GFP manifestation. Following the futher growth of calluses, the fluorescing sectors become relatively smaller when compared to total size of calluses, indicating, that they were overgrown by non-transformed, escapes cells. Nevertheless the use of flax cotyledons seems to be more suitable for the detection of GFP fluorescence than hypocotyls and promise more stringent and efficient selection of hard-to-transform plant species showing high levels of escapes. Simultaneously, it could help also to reveal the negatively interferring factor(s) obstructing the GFP visualisation in some plant tissues.

Material and methods

Flax (*Linum usitatissimum* L.) plants of the two varieties (cvs. Venica, Biltstar, obtained from AGRITEC, Research, Breeding & Services, Ltd., Šumperk, Czech Republic) were subjected to the *Agrobacterium*-mediated transformation. *A. tumefaciens* strain LBA 4404, carrying binary vector pBINm-*gfp5*-ER (kind gift from Dr. J. Hasseloff, Department of Plant Sciences, Univ. Cambridge, UK) was used for our experiments. 7–10 days old flax seedlings hypocotyls and cotyledons were subjected to transformantion according to Rakouský *et al.* (1999) Two sets of experiments were performed, in the first one only hypocotyls were used for transformation and in the second variant also cotyledons were included. The co-cultivation was performed *in vitro* on basal MS (Murashige, Skoog, 1962) medium without antibiotics, supplemented with 1mg.l^{-1} BAP and 0,02 mg. l^{-1} NAA, to promote callogenesis, in the growth chamber under controlled conditions (16-h photoperiod, 40–70 μmol (PAR) m^{-2} s^{-1}, 22–25 °C). After three days control (non-transformed) and bacteria-treated "transformed" segments were transferred on the MS medium with 50 mg. l^{-1} kanamycin (to enable transformants selection), 500 mg. l^{-1} Timentin, 300 mg. l^{-1} Claforan (for bacteria elimination), 0,1 μM BAP and 0,005 μM NAA for the regeneration of shoots (first experiment). For the second experiment, callus initiation MS medium of the same phytohormone composition (1 mg. l^{-1} BAP and 0,02 mg. l^{-1} NAA) was used throughout the whole period of time, i.e. without any change in the growth regulators level after the subcultivation. Such medium was designated for further promotion of the calogenesis. Explants were maintained under the same conditions as mentioned above. The detections of the GFP fluorescence were performed regularly in two, and later in four days periods. Detection device consisting of *Leica MZ 12* stereo dissecting microscope equipped with a fluorescence module with 100 W mercury lamp and GFP excitation and emission filters (*Leica*, Heerbrigg, Switzerland) was used for study of GFP fluorescence. Various magnifications were used during this study based on the nature of studied sample.

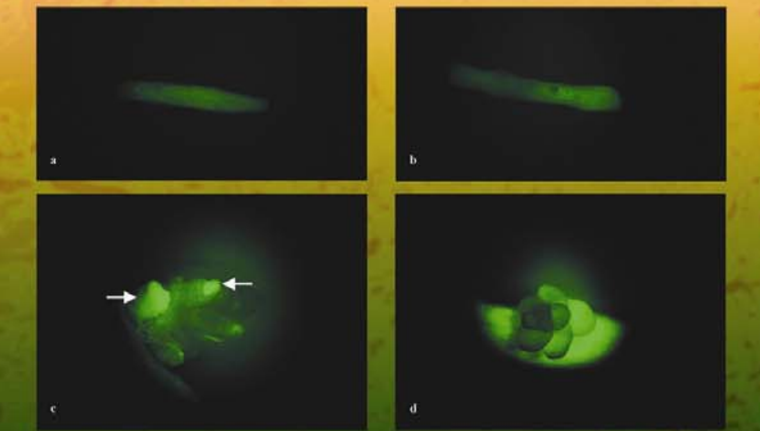

Fig. 2.: Visual comparison of fluorescence signal of transformed and control flax hypocotyls. Transformed (a) and control (b) explants exhibit the same level of background autofluorescence, which makes visual differentiation nearly impracticable. Moreover, in both cases the total level of fluorescence is very faint. Following the regeneration (35–50 days), some buds emerging from the transformed tissue (c) possess a brighter fluorescence and generate localized green fluorescent foci (white arrows), indicating possible transgenic fluorescence, while buds emerging from control hypocotyls (d) possess no such fluorescence signal.

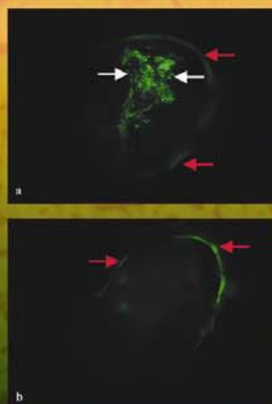

Fig. 3.: Visual comparison of fluorescence signal of putatively transformed and control flax cotyledons. Arrows (white) indicate green fluorescing foci of *gfp* expressing cells. GFP fluorescence in the putative transgenic calluses proliferating on a cotyledon wounded with forceps is evident (a). Faint noise signal (autofluorescence) in surrounding green belt (red arrows) represents the slowly decaying tissue on the margin of the cotyledon. In the non-transformed cotyledon (b) no GFP green fluorescence is visible on the surface of the cotyledon except the same faint green belt of decaying tissue (red arrows).

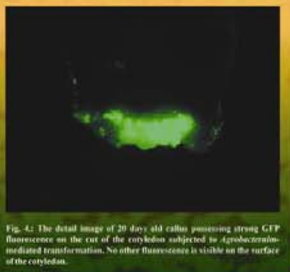

Fig. 4.: The detail image of 20 days old callus possessing strong GFP fluorescence on the cut of the cotyledon subjected to *Agrobacterium*-mediated transformation. No other fluorescence is visible on the surface of the cotyledon.

Discussion

Of the total 333 buds and shoots arising from *Agrobacterium*-treated hypocotyls of Biltstar and Venica varieties no GFP fluorescence was visible in early stages of regeneration due to relatively high levels of the background fluorescence. In later stages of plant regeneration only minor fluorescent foci were visible, but such fluorescence was faint and insufficient for a successful selection. Non-transformed explants expressed the same background fluorescence. Same results of observations and conclusions were confirmed in the second set of experiments, suggesting that flax hypocotyls are not suitable material for efficient selection of transformants based on the GFP manifestation. Obstacles associated with low GFP fluorescence signals of flax hypocotyls were recognised also by other author (Jordan, unpublished). On the other hand, in our study we have shown that distinct GFP foci occurred in the calluses formed on transformed cotyledons and were easily distinguishable from the surrounding tissue. No such fluorescence was visible in the control cotyledons. Nevertheless, following the calluses proliferation, green foci tend to slowly disappear, most probably by intensive growth of untransformed cells, causing overlaying of transformed ones. It is speculated that this could be circumvented by additional changes, e.g. proper timing of the next subcultivation (use of different cultivation media). Nevertheless, this was not the main aim of this study, which was to reveal the role of external factors, such as nature (origin) of studied material on the level of the detected GFP fluorescence. This study was designed to elucidate in a more detail way the role of choice of the target explant for the *Agrobacterium*-mediated transformation and subsequent GFP based selection of flax.

Acknowledgements

This work was supported by grants from the Grant Agency of the Czech Republic (GA ČR-31/H160) and from the Ministry of Education of the Czech Republic (1PO5ME800).

References

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497, 1962.
Prasher, D., C., Eckenrode, V., K., Ward, W., W., Prendergast, F., G., Cormier, M., J.: Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene 111: 229–233, 1992.
Rakouský, S., Tejklová, E., Wiesner, I., Wiesnerová, D., Kocábek, T., Ondřej, M.: Hygromycin B - an alternative in flax transformant selection. Biol. Plant. 42(3): 361–369, 1999.
Yang, F., Moss, L., G., Philips, G., N., Jr.: The molecular structure of green fluorescent protein. Nature Biotech. 14: 1246–1251, 1996.

5. Summary

Results of my thesis presented above contribute to the establishment of an efficient strategy for genetic transformation of plants using the *gfp* linked genes, which could be utilized in the following research/ work aimed at development of transgenic plants with an increased resistance against fungal pathogenes and pests based on expression of specific genes coding for proteinase inhibitors (PIs). PIs as a part of GMOs development represent a relatively novel strategy (as compared to e.g., commercially available Bt plants) for increasing of plant resistance to diseases and pests, and as such are being intensively investigated now. Chemical properties, mode of action and history of the use of PIs in the research of transgenic plants were summarized in a recent review article published in Czech in journal Chemické Listy (Hraška et al., 2006b).

Nevertheless, the core part of my study represents the development of an efficient and reliable system for plant transgenesis just with PIs, but with utility of GFP marker. Properties and summary of its use for the purposes of genetic transformation, together with the list of plant species, which were recently successfully transformed with the *gfp* marker were presented in a recent review article published in the Plant Cell Tissue and Organ Culture journal (Hraška et al., 2006a).

For a monitoring system based on the use of GFP to be effective, reliable and reproducible, it is important to investigate and define its properties and performance in the real situation, i.e. glasshouse or field. For such monitoring of GFP fluorescence and quantitative assessment of obtained transgenic plants, we decided to use an image analysis of fluorescence images of transgenic tissues from various plants. In the first part, I concentrated my effort on the initial investigation, whether our preliminary hypothesis, that such approach can be used for a relative quantification of marker gene expression, is realistic and accomplishable. Obtained results, presented in the Biologia Plantarum (Hraška et al., 2005) showed that such approach could be successfully used for the quantification assessment of regenerated tobacco transgenic plants. Nevertheless, during this research we revealed the existence of slight differences in GFP fluorescence patterns, observed among the samples detached from one transgenic plant and even among various cells and tissues within one leaf. Since such variables were reported also by some other authors as a relatively common event, this fact together with our aim to develop the most reliable system based on the GFP marker we decided to investigate such variability in a more detailed way.

Therefore, the subsequent research was aimed at the study and definition of GFP fluorescence patterns detectable on tissue samples detached from the different spatial locations within the plant and also within one particular leaf. Obtained results, also presented in the journal Plant Cell Reports (Hraška et al., 2007), showed that the proper and consistent tissue sampling is crucial for obtaining reliable and reproducible data. GFP fluorescence patterns, recorded during our research, significantly differed within one leaf and also among the leaves at different positions on a stem, i.e. depending on a physiological age within one plant. Moreover, we also recorded variability in the GFP fluorescence among the clonally propagated individuals, indicating the possible role of environment. Nevertheless, such differences were not significantly higher than the differences recorded among different transgenic plants, thus confirming our preliminary assumption, that the quantification of GFP fluorescence using the image analysis can be used for an assessment of transgenic plants. The last, but not least outcome was the difference in a quantity of detectable GFP fluorescence from different leaf surfaces. This is an important fact, to investigate the leaf samples subjected for GFP fluorescence assessment/ quantification in the same way and on the same surface, consistently throughout the whole particular experiment. Beside this investigation and mapping of GFP fluorescence patterns, I tried also to discover possible physiological background for such previously mentioned disturbances. Since the chlorophyll content is mostly mentioned as a possible cause, which could affect the GFP fluorescence I decided to investigate the correlation between the total chlorophyll content and GFP fluorescence among the leaves of different physiological age. Obtained results were a bit equivocal, since some influence of chlorophyll content on the decrease of GFP fluorescence intensity was recorded, nevertheless the obtained negative correlation was not so strong enough to see the chlorophyll as the only cause. Therefore, some other aspects could play their role in this event.

Since the cause for a different GFP fluorescence among various tissues need not always be associated with the plant physiology, but rather with the regulation sequences used for the particular research, I decided to investigate also the expression pattern of CaMV 35S promoter within the plant and results of research. Although this promoter is usually reported as a constitutive, some reports indicated, that it could be also developmentally and tissue specifically regulated. The CaMV 35S driven GFP gave me an excellent opportunity to track the performance of this promoter during the whole life cycle of plants, from the seed stage, throughout the growth and development of mature plant, till the production of a new progeny. Moreover, special emphasis was given on the floral and

reproducible organs, since the information regarding the CaMV 35S expression within those tissues was still unsatisfactory. Obtained results indicate some degree of tissue specific regulation and confirmed the preferential performance/ expression of this promoter in various vascular tissues. Such strong expression within vascular tissue was observed also in the floral and reproducible organs. These are the original data regarding the CaMV 35S expression in such organs. Nevertheless, this study also confirmed the expression of this promoter nearly in all main plant organs and tissues. Moreover, all these investigations were performed on two distinct generations of transgenic plants, T_1 and T_2, (as compared to the most of other studies done on primary transformants T_0 only) and the same expression profile of the CaMV 35S promoter was identified in the plants belonging to both generations, what also indicated that the transgene did not underwent to a gene silencing. Beside other tissues and plant organs, an intensive expression of CaMV 35S driven GFP was always recorded in various vascular tissues. Thus, our results confirmed high expression stability of this promoter across distinct generations of transgenic plants. On the other hand, in a spite of these positive results, this research revealed also a weak point in the use of GFP as a vital marker for the promoter tracking purposes. We have found that some tissues posses a relatively high level of green auto-fluorescence, which could be easily misinterpreted as the GFP fluorescence. This was revealed by simultaneous investigation of analogous types of tissue detached from control, non-transgenic plants. Such auto-fluorescence was recorded in roots and pollen exclusively.

In my introductory studies performed with flax some preliminary results were already obtained. Using the co-cultivation method transgenic flax plants bearing either *SPI-2* or *gfp* gene were obtained. First experiments aimed on the utility of GFP as a selection tool revealed high levels of background auto-fluorescence within flax hypocotyls. Nevertheless such auto-fluorescence was not recorded in the case of hypocotyls and thus such first attempts to use the GFP for an early evaluation and co-selection of flax transgenics seem to be very promising. Outcomes were presented in the form of poster on the conference Cells VI held in 2005 in České Budějovice.

Results of the research reported in the above presented Ph.D. thesis can significantly contribute to further development and improvement of the methodology utilizing the GFP as an efficient tool for monitoring of gene expression within transgenic plants. Such results could be used also to advanced research studies regarding the transgenic plants. The results regarding the performance of the CaMV 35S promoter added another useful information to the mosaic of our knowledge of the promoter expression within transgenic plants.

6. References

Babu, R, M., Sajeena, A, Seetharaman, K, Reddy, M, S (2003) Advances in genetically engineered (transgenic) plants in pest management-an over view. Crop. Prot. 22: 1071-1086

Baranski R, Klocke E, Schumann G (2006) Green fluorescent protein as an efficient selection marker for *Agrobacterium rhizogenes* mediated carrot transformation. Plant Cell Rep. 25: 190-197

Battraw MJ, Hall TC (1990) Histochemical analysis of CaMV 35S promoter- β-glucuronidase gene expression in transgenic rice plants. Plant Mol Biol 15:527–538.

Benfey PN, Ren L, Chua N-H (1990) Tissue specific expression from CaMV 35S enhancer subdomains in early stages of plant development. EMBO J 9:1677–1684

Birch RG (1997) Plant transformation: problems and strategies for practical application, Annu. Rev. Plant Physiol. Plant Mol. Biol. 48 297-326

Carliny, CR., Grossi-de-Sá, MF (2002) Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides. Toxicon 40: 1515-1539

Chilton, M-D, Drummond, MH, Merlo, DJ, Sciaky, D, Montoya, AL,.Gordon, MP, Nester, EW (1977) Stable incorporation of plazmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis, Cell 11 263-271

Cho M-J, Choi H-W, Jiang W, Ha ChD, Lemaux PG (2002) Endosperm-specific expression of green fluorescent protein driven by the hordein promoter is stably inherited in transgenic barley (*Hordeum vulgare*) plants. Physiol. Plant. 115: 144-154

Christou, P (1995) Strategies for variety-independent genetic transformation of important cereal, legumes and woody species utilizing particle bombardment, Euphytica 85
13-27

Christou, P (1997) Rice transformation: bombardment, Plant Mol. Biol. 35 197-
203.

Chytilova E, Macas J, Galbraith DW (1999) Green fluorescent protein targeted to the nucleus, a transgenic phenotype useful for studies in plant biology. Ann. Bot. 83: 645-654

Cody CW, Prasher DC, Westler, WM, Prendergast F G, Ward W W (1993) Chemical structure of the hexapeptide chromophore of *Aeqourea*-green fluorescent protein.
Biochemistry 32: 1212-1218

Custers, R (ed.), Safety of genetically engineered crops, VIB publication, Flanders Interuniversity Institute for Biotechnology, 159 pp. 2001.

Dillen W, De Clercq J, Kapila J, Zambre M, Van Montagu M, Angenon, G (1997) The effect of temperature on *Agrobacterium tumefaciens*-mediated gene transfer to plants. Plant J. 12: 1459-1463

EFSA (2004) Opinion of the Scientific Panel on Genetically Modified Organisms on the use of antibiotic resistance genes as marker genes in genetically modified plants EFSA J. 48 1-18

Ebinuma H, Sugita K, Matsunaga E, Endo S, Yamada K, Komanine A (2001) Systems fro the removal of a selection marker and their combination with a positive marker, Plant Cell Rep. 20: 383-392

Fullner KJ, Nester EW (1996) Temperature affects the T-DNA transfer machinery of *Agrobacterium tumefaciens.* J. Bacteriol. 6: 1498-1504

Finer JJ, Finer KR, Ponappa T (1999) Particle bombardment mediated transformation, Plant Biochtechnol. Curr. Topics Microbiol. Immunol. 240, 59-80

Ghorbel R, Juárez J, Navarro L, Peña L (1999) Green fluorescent protein as a screenable marker to increase the efficiency of generating transgenic woody fruit plants, Theor. Appl. Genet. 99 350-358

Halfhill MD, Richards HA, Mabin SA, Stewart CN Jr (2001) Expression of GFP and Bt transgenes in *Brassica napus* and hybridization with *Brassica rapa*. Theor. Appl. Genet. 103: 659-667.

Halfhill MD, Millwood RJ, Rufty TW, Weissinger AK, Stewart CN (2003) Spatial and temporal patterns of green fluorescent protein (GFP) in transgenic oilseed rape, *Brassica napus* L. Plant Cell Rep. 22: 338-343

Halfhill MD, Sutherland JP, Moon HS, Poppy GM, Warwick SI, Weissinger AK, Rufty TW, Raymer PL, C. Neal Stewart Jr (2005) Growth, productivity, and competitiveness of introgressed weedy Brassica rapa hybrids selected for the presence of Bt cry1Ac and gfp transgenes. Molecular Ecology. 14:3177-3189

Haseloff J, Siemering KR, Prasher DC, Hodge S (1997) Removal of a cryptic intron and subcellular localisation of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proc. Natl. Acad. Sci. U.S.A. 94: 2122-2127

Heim R, Prasher DC, Tsien RY (1994) Wavelenght mutations and posttranslational autooxidation of green fluorescent protein. Proc. Natl. Acad. Sci. U.S.A. 91: 12501-12504

Hraška M, Rakouský S (2005) GFP detection in low level signal/noise ratio plant samples. J. Appl. Biomed. 3:S19

Hraška M, Rakouský S, Kocábek T (2005) Use of simple semiquantitative method for appraisal of green fluorescent protein gene expression in transgenic tobacco plants. Biol. Plant. 49: 313-316

Hraška M, Rakouský S, Čurn V (2006a) Green fluorescent protein as a vital marker for non-destructive detection of transformation events in transgenic plants Plant Cell Tissue Organ Cult. 86, 303-318

Hraška M, Rakouský S, Čurn V (2006b) Inhibitory proteas, mechanismy účinků a perspektivy jejich využití v transgenosi rostlin [Protease inhibitors, mode of action and perspectives for plant transgenosis] Chemické listy 100, 501-507

Hraška M, Heřmanová V, Rakouský S, Čurn V (2007): Sample topography and position within plant body influence the detection of the intensity of green fluorescent protein fluorescence in the leaves of transgenic tobacco plants. Plant Cell Rep. – in press, DOI 10.1007/s00299-007-0431-7

Hudson LC, Chamberlain D, Stewart CN Jr (2001) GFP-tagged pollen to monitor gene flow of transgenic plants. Molecular Ecology Notes 1:321-324

ISAAA: http://www.isaaa.org/main.htm, downloaded on June 27. 2005

Itaya A, Hickman H, Bao Y, Nelson R , Ding B (1997) Cell-to-cell trafficking of cucumber mosaic virus movement protein: green fluorescent protein fusion produced by biolistic gene bombardment in tobacco. Plant J. 12: 1223-1230

Jefferson RA, Ravanagh TA, Bevan MW (1987) GUS fusion: *ß*-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901-3907

Joersbo M, Donaldson I, Kreiber J, Petersen SG, Brunstedt J, Okkels (1998) FT Analysis of mannose selection used for transformation of sugar beet, Mol. Breed 4 111-117

Joersbo M (2001) Advances in the selection of transgenic plants using non-antibiotic marker genes, Physiol. Plant, 111 269-272

Kamaté K, Rodriguez-Llorente ID, Scholte M, Durand P, Ratet P, Kondorosi E, Kondorosi A, Trinh TH (2000) Transformation of floral organs with GFP in *Medicago truncatula*. Plant Cell Rep. 19: 647-653

Klein TM, Wolf ED, Wu R, Stanford JC (1987) High-velocity microprojectiles for delivering nucleic acids into living cells, Nature 327 70-73

Lawrence PK, Koundal KR (2002) Plant proteinase inhibitors in control of phytophagous insect. EJB 5: 93-109

Miki B, McHugh S (2004) Selectable marker genes in transgenic plants: applications, alternatives and biosafety. J. Biotechnol. 107: 193-232

Millwood RJ, Halfhill MD, Harkins D, Russotti R, Stewart CN Jr. (2003) Instrumentation and methodology for quantitative GFP fluorescence in intact transgenic plants. Biotechniques
(N.Y.) 24: 638-643

Misteli T, Spector DL (1997) Applications of the green fluorescent protein in cell biology and biotechnology. Nature Biotech. 15: 961-963

Moon HS, Halfhill MD, Hudson LC, Millwood R.J, Stewart CN Jr. (2006) Expression of green fluorescent protein in pollen of oilseed rape (*Brassica napus* L.) and its utility for assessing pollen movement in the field. Biotechnology Journal 1:1147-1152

Nagatani N, Takumi S, Tomiyama M, Shimada T, Tamiya E (1997) Semi-real time imaging of the expression a maize polyubiquitin promoter-GFP gene in transgenic rice, Plant Sci. 124: 49-56

Niedz RP, Sussman MR, Satterlee JS (1995) Green fluorescent protein: an *in vivo* reporter of plant gene expression. Plant Cell Rep. 14: 403-406

Nirmala X, Kodrík D, Žurovec M, Sehnal F (2001) Insect silk contains both a Kunitz-type and a unique Kazal-type proteinase inhibitor. Eur. J. Biochem. 268, 2064-2073

Ondřej M, Drobník J Trasnsgenoze rostlin. Academia, Praha, 1 vydání, 316 s, 2002

Ow DW, Wood KV, Deluca M, Dewet JR, Helinski DR, Howell SH (1986) Transient and stable expression of firefly luciferase gene in plant cells and transgenic plants. Science 234: 856-859

Ponappa T, Brzozowski AE, Finer JJ (1999) Transient expression and stable transformation
of soybean using the jellyfish green fluorescent protein. Plant Cell Rep. 19: 6-12

Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier MJ (1992) Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene 111: 229-233

Preťová A, Obert B, Wetzstein HY (2001) Leaf developmental stage and tissue location affect the detection of β-glucuronidase in transgenic tobacco plants. Biotech Lett 23:555–558

Rakouský, S., Hraška, M. (2007): Transgenní plodiny – realita a perspektivy. In: Geneticky modifikované organismy v agroekosystému a jeho okolí. Sborník ze semináře pořádaného Ministerstvem zemědělství ČR a Českou zemědělskou univerzitou v Praze, 17.

květen, 2007, Ministerstvo zemědělství ČR ve spolupráci s Českou zemědělskou univerzitou v Praze, Praha 2007. str. 18−23. ISBN 978-80-7084-588-2

Repellin A, Baga M, Jauhar PP, Chibbar RN (2001) Genetic enrichment of cereal crops via alien gene transfer: New challenges, Plant Cell Tiss. Org. Cult. 64 159-183

Richards HA, Halfhill MD, Milwood RJ, Stewart CN (2003) Quantitative GFP fluorescence as an indicator of recombinant protein synthesis in transgenic plants. Plant. Cell. Rep. 22: 117-121

Schuler TH, Poppy GM, Kerry BK, Denholm I (1998) Insect-resistant transgenic plants. TIBTECH 16: 168-175

Sheen J, Hwang S, Niwa Y, Kobayashi H, Galbraith DW (1995) Green-fluorescent protein

as a new vital marker in plant cells. Plant J. 8: 777-784

Shinomura O (1979) Structure of the chromophore of *Aeqourea* green fluorescent protein. FEBS Lett. 104: 220-222

Snape JW (1998) Golden calves or white elephants-biotechnologies for wheat improvement, Euphytica 100 207-217

Stewart CN Jr (2001) The utility of green fluorescent protein in transgenic plants. Plant Cell Rep. 20: 376-382

Sunilkumar G, Mohr L, Lopata-Finch E, Emani C, Kathore KS (2002) Developmental and tissue-specific expression of a CaMV 35S promoter in cotton as revealed by GFP. Plant Mol Biol 50: 463-474

Tamura M, Togami J, Ishiguro K, Nakamura N, Katsumoto Y, Suzuki K, Kusumi T, Tanaka Y (2003) Regeneration of verbena (*Verbena x hybrida*) by *Agrobacterium tumefaciens*. Plant Cell Rep. 21: 459-466

Tinland B (1996) The integration of T-DNA into plant genomes. Trends Plant Sci., Rev. 178-184

Tsien RY (1998) The green fluorescent protein. Annu. Rev. Biochem 67: 509-544

Wright M, Dawson J, Dunder E, Suttie J, Reed J, Kramer C, Chang Y, Novitzky R, Wang H, Artim-Moore L (2001) Efficient bilolistic transformation of maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) using the phosphomannose isomerase gene, *pmi*, as the selectable marker, Plant Cell Rep. 20 429-436

Williamson JD, Hirsch-Wyncott ME, Larkins BA, Gelvin SB (1989) Differential accumulation of a transcript driven by the CaMV 35S promoter in transgenic tobacco. Plant Physiol 90:1570–1576

Yang F, Moss LG, Phillips GN Jr. (1996) The molecular structure of green fluorescent protein. Nature Biotech. 14: 1246-1251

Zhou X, Chandrasekharan MB, Hall TC (2004) High rooting frequency and functional analysis of GUS and GFP expression in transgenic *Medicago truncatula* A17. New Phytol. 162: 813-822

Zhou X, Carranco R., Vitha S, Hall TC (2005) The dark side of green fluorescent protein. New Phytol 168:313–322

Zupan, J, Zambryski P (1997) The *Agrobacterium* DNA transfer complex. Critical Rev. Plant Sci. 16: 279-295

7. Summary of publication from the year 2004 where Marek Hraška is listed as a author or co- author

Publication in the international journals, listed in the CC or similar databases (Medline, MBASE etc.):

1. Hraška, M., Heřmanová, V., Rakouský, S., Čurn, V. (2007): Sample topography and position within plant body influence the detection of the intensity of green fluorescent protein fluorescence in the leaves of transgenic tobacco plants. Plant Cell Rep. – in press, DOI 10.1007/s00299-007-0431-7

2. Hraška, M., Rakouský, S., Čurn, V. (2006) Green fluorescent protein as a vital marker for non-destructive detection of transformation events in transgenic plants Plant Cell Tissue Organ Cult. 86: 303−318.

3. Hraška, M., Rakouský, S., Čurn, V. (2006) Inhibitory proteas, mechanismy účinků a perspektivy jejich využití v transgenosi rostlin [Protease inhibitors, mode of action and perspectives for plant transgenosis] Chemické listy 100: 501−507.

4. Hraška, M., Rakouský, S., Kocábek, T. (2005): Use of a simple semiquantitative method for appraisal of green fluorescent protein gene expression in transgenic tobacco plants. Biol. Plant. 49(2): 313−316.

Other publications and abstracts:

1. Hraška, M., Heřmanová, V., Rakouský, S., Čurn, V. (2007): Topography factors influencing the quantification of GFP fluorescence in leaves of transgenic tobacco as revealed by image analysis. In: Libiaková, G., Gajdošová, A. (eds): Plant Biotechnology: Impact on High Quality Plant Production. Book of Abstracts 7th Int. Symp. Recent Advances in Plant Biotechnology, Stará Lesná, Slovakia, June 10-16, 2007, Institute of Plant Genetics and Biotechnology SAS, Nitra. Pp. 87−98. ISBN 978-80-89088-51-5

2. Hraška, M., Rakouský, S., Čurn, V. (2007): Tracking of the CaMV35S expression pattern in the flowers and reproductive organs of GFP transgenic tobacco revealed its high activity in vascular tissues. In: Libiaková, G., Gajdošová, A. (eds): Plant Biotechnology: Impact on High Quality Plant Production. Book of Abstr. 7th Int. Symp. Recent Advances in Plant Biotechnology, Stará Lesná, Slovakia, June 10-16, 2007, Institute of Plant Genetics and Biotechnology SAS, Nitra. Pp. 103−104. ISBN 978-80-89088-51-5

3. Rakouský, S., Hraška, M. (2007): Transgenní plodiny – realita a perspektivy. In: Geneticky modifikované organismy v agroekosystému a jeho okolí [Transgenic plants- reality and perspectives.In: Genetically modified organisms in agro-ecosystem and its surroundings]. Book of Abstracts, Seminar organized by Ministry of Agriculture of Czech Republic and Czech University of Life Sciences in Prague, May 17, 2007, Ministry of Agriculture of Czech Republic in cooperation with Czech University of Life Sciences in Prague, Prague, 2007, pp 18−23. ISBN 978-80-7084-588-2

4. Rakouský, S., Hraška, M., Žaludová, J., Riegelová, L. (2006): Current results of long-term studies on biosafety of GM winter oilseed rape cultivation in the Czech Republic. In: Abstr. 23rd World Congr. Czechoslovak Soc. Arts and Sciences, České Budějovice, June 25-July 2, 2006, p. 117. ISBN 80-903600-5-X

5. Rakouský, S., Hraška, M., Bříza, J., Psota, B. (2005): Long-term monitoring of GM winter oilseed rape volunteers and the implications for Co-existence. In: Messéan, A. (ed.): Proc. Second Int. Conference on Co-existence between GM and non-GM based Agricultural Supply Chains. November 14-15, 2005, Montpellier, France. Agropolis Production, Montpellier 2005. Pp. 89−91. ISBN 87-984996-5-3

6. Hraška, M., Rakouský, S., (2005): GFP detection in low level signal/noise ratio plant samples. J. Appl. Biomed. 3 (Suppl. 1): S19. (Abstr. Conf. Cells VI., České Budějovice, Czech Republic, October 24-26, 2005). ISSN 1214-0287

7. Rakouský, S., Tejklová, E., Hraška, M., (2005): Úloha mastných kyselin v prevenci a léčbě závažných onemocnění. In: Adámková, V., Zimmelová, P. (eds.): Výživa –

nedílná součást léčby závažných chorob. Sborník příspěvků a abstraktů z mezinárodní konference. 22.9.2005 České Budějovice. Jihočeská univerzita v Českých Budějovicích, Zdravotně sociální fakulta, České Budějovice. str. 13−19. ISBN 80-7040-820-0

Submitted for publication:

1. Hraška, M., Rakouský, S., Čurn, V.: Tracking of the CaMV-35S promoter performance in GFP transgenic tobacco, with a special emphasis on flowers and reproductive organs, confirmed its predominant activity in vascular tissues.
   Submitted to Plant Cell Tissue and Organ Culture 8/2007