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The Utility of GFP in Genetic Engineering of Horticultural Plants

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Summary

Reporter proteins play a significant role in developing and optimizing transformation protocols for plant species and they show a unique activity to visualize gene expression and protein localization. Chemical based selection for plant transformation is associated with a number of problems that might be avoided through visual selection. In recent years, the reporter protein GFP (Green Fluorescent Protein) has become very effective and highly valuable for use in biotechnology, cell biology, and biochemistry. It is a unique tool for monitoring gene expression, protein localization, detection of the gene flows and protein dynamics in both prokaryotic and eukaryotic living cells because it requires neither exogenous substrates nor cofactors for its activity. We discussed the use of jellyfish green fluorescent protein (GFP) as a reporter in horticultural plants.

Zusammenfassung

Der Nutzen von GFP für die gartenbauliche Züchtung. Reporterproteine spielen eine wichtige Rolle in der Entwicklung und Optimierung der Transformationsprotokolle für Pflanzenspezies und sie besitzen eine einzigartige Aktivität um Genexpression und Proteinlokalisation zu zeigen. Auf Chemikalien basierende Selektion bei Pflanzentransformation ist mit Problemen verbunden, die mit visueller Selektion vermieden werden können. In den letzten Jahren wurde das neue Reporterprotein GFP (Grün fluoreszierendes Protein) sehr wertvoll für den Gebrauch in der Biotechnologie, Zellbiologie und Biochemie. Es ist ein einzigartiges Werkzeug, um die Genexpression und Protein-Lokalisation sowie den Nachweis von Genfluss und Protein-Dynamik in lebenden Zellen von Prokaryonten und Eukaryonten zu überwachen, denn es braucht weder exogen Substrate noch Faktoren für seine Aktivität. Wir haben den Einsatz von grün fluoreszierendem Protein (GFP) aus Quallen als Reporter im Gartenbau diskutiert.

Key words. reporter protein – Green fluorescent protein – horticultural plants – vegetables – fruit – ornamentals

Introduction

Reporter proteins play a significant role in both developing and optimising transformation protocols for plant species and visualising gene expression and protein localization. They are also useful for monitoring cellular events that are difficult or impossible to observe. GUS (ß-glucuronidase), the *uid A* gene product (JEFFERSON et al. 1987), and LUC (luciferase), encoded by the *luc* gene, are widely used reporter proteins in plants. These systems were helpful in many studies but each of them has some limitations. The histochemical GUS assay is destructive for the tissue, therefore not suitable for direct visual selection of transformed plants (SANTI et al. 2003). Luciferase can be monitored *in vivo* but it requires an exogenous substrate and emits light only at very low intensity (OW et al. 1986). In recent years, a novel reporter protein, GFP (Green Fluorescent Protein) has become very effective and highly valuable for use in biotechnology, cell biology, and biochemistry. It is a unique tool for monitoring gene expression, protein localization, detection of the gene flows and the protein dynamics in both prokaryotic and eukaryotic living cells (OLIVARES-FUSTER et al. 2002) because it requires neither exogenous substrates nor cofactors for its activity. GFP is a 27kDa, small, barrel-shaped protein surrounding a fluorescent chromophore that emits bright green fluorescence (λ_{max} =509 nm) upon excitation with ultraviolet (λ_{max} =395 nm) or blue (λ_{max} =475 nm) light encoded by the *gfp* gene isolated from the crystal jellyfish *Aequorea victoria* (ORMO et al. 1996; ELLIOTT et al. 1999; HARPER and STEWART 2000).

Since it has more advantages than the other two reporter genes, GFP is preferred in plant cells instead of ß-glucuronidase and luciferase. It allows direct imaging of the fluorescent gene product in living cells without lethal histochemical staining procedures and since it is autocatalytic, the fluorescent chromophore requires no supplementary components (CHALFIE et al. 1994). GFP fluorophore requires a series of posttranslational intramolecular reactions, involving cyclisation and autoxidation of amino acids Ser65-Tyr66-Gly67 (HASELOFF and AMOS 1995). Further modifications of the GFP were done. Recently, the performance of *Aequorea victoria* GFP in plant cells has been greatly improved over the native form by the introduction of key amino acid substitutions that enhance the formation and

function of the fluorophore or by the alteration of the gene sequences for improved expression (STEWART 2001). Since the processing of gfp mRNA may vary depending on the species and visualization of the protein may be complicated by background fluorescence in certain tissues, the selection of the appropriate version of this reporter gene is critical (PONAPPA et al. 1999). For this reason, several GFP mutants have been expressed either in monocotyledonous (PONAPPA et al. 1999; JORDAN 2000) or dicotyledonous (SHEEN et al. 1995; HASELOFF et al. 1997; PONAPPA et al. 1999) species. Nevertheless, the fluorescence performance of GFP variants has seldom been compared in the same species except for simple comparisons of mutant versus wild-type GFP (STEWART 2001). ELLIOTT et al. 1999 reported that sGFP-S65T had a brighter fluorescence in sugarcane than mGFP5-ER, while PONAPPA et al. (1999) found in soybean that of five different GFP versions tested, smRS-GFP was the easiest to detect based on the brightness and number of GFP spots. GFP is also used as a fluorescent tag to observe the behavior of proteins in plant cells. Virtually any protein of interest which can be fluorescence-labelled with GFP have been used to explore targeting to a variety of subcellular locations, including the endoplasmic reticulum (HASELOFF et al. 1997), mitochondria and chloroplasts (KÖHLER et al. 1997). In recent years, the use of the green fluorescent protein as a visual marker in several plant species has been reported. In this article, the use of the GFP in genetic engineering of horticultural plants is reviewed.

GFP Utility in Vegetable Crops

A lot of studies have been done on several vegetable crops related with GFP (Table 1) and the most important ones are discussed in this review. There are a few studies on legumes. In one of these studies a system has been developed by using green fluorescent protein (GFP) and soybean glutamine synthetase (GS), to examine the function of genes that are vital for legume plants. Consequently, green fluorescence was detected in infected broad bean, kidney bean, and soybean plants. Early flowering and tolerance to herbicide glufosinate were observed in plants that express glutamine synthetase (GS) and GFP (MASUTA et al. 2000). A further study using GFP on *Leguminosae* was done by PACIOS-BRAS and his colleagues (2003). They transformed GFP and GUS (ß-glucuronidase) reporter genes under control of GH3 (soybean auxin-responsive promotor) of *Lotus japonicus* lines and observed auxin distribution in transformed *L. japonicus* lines in order to determine if it belongs to the determinate nodulating legume group. They found that *L. japonicus* belonges to the nodulating group. Thus *L. japonicus* is different from white clover, which forms intermediate nodules.

There are more legume studies, which evaluate GFP with the goal of improving and developing transformation and regeneration strategies for soybean. For this purpose, several GFP modifications (smRS-gfp, smgfp (DAVIS and VIERSTRA 1998) mgfp4-ER, mgfp5-ER (HASELOFF et al. 1997)) were introduced into embryogenic soybean cultures by particle bombardment. Green fluorescence was detected in soybean cultures by using all modifications; however, soluble modified red shifted GFP (*smRS-gfp*) (DAVIS and VIERSTRA 1998) was detected easier than the other modifications. (PONAPPA et al. 1999).

GFP was also used as a reporter gene to monitor plastid gene expression. HIBBERD et al. (1998) demonstrated transient gene expression of GFP in chloroplasts and non-photosynthetic plastids in higher plants. With this goal, *gfp* gene has been introduced in several plants by particle bombardment and GFP has been detected in amyloplasts of potato tubers and in chromoplasts of marigold petals, carrot roots, and pepper fruits 4 days after bombardment. Therefore, GFP should be an appropriate reporter both in photosynthetic and non-photosynthetic plastids.

GFP was also used to demonstrate phosphinothricin herbicide resistance in onion plants. EADY et al. 2003 produced phosphinothricin resistant onion plants by *Agrobacterium*-mediated transformation. The inheritance of both GFP expression and tolerance to phosphinothricin in a Mendelian segregetion has been reported. GFP has also been transferred to melon, sweet potato, and spinach plants. GALPERIN et al. (2003) described an efficient transformation protocol by *Agrobacterium*-mediated delivery of GFP reporter gene into cotyledon explants of a newly characterized melon breeding line "BU-21/3". GFP was also used as a reporter gene in sweet potato by LAWTON et al. (2000). Transient and stable GFP gene expression in leaf and petiole tissues of sweet potato was monitored after delivering the *gfp* gene by electroporation and particle bombardment. Moreover, the *gfp* gene was used as a reporter gene for the transformation of spinach. ZHANG and ZEEVAART (1999) reported an efficient *Agrobacterium*-mediated transformation method by using *smgfp* (DAVIS and VIERSTRA 1998) and the expression of GFP was also observed in progeny plants.

GFP Utility in Fruits

The use of GFP has been reported in several *Citrus* species, grapevine, apple, plum, peach, and banana plants (Table 1). Wild type GFP visualization in plant cells and its utility in plant transformation and expression was monitored firstly in sweet orange (*Citrus sinensis*) protoplasts by NEIDZ et al. (1995). Another published study in *Citrus sinensis* was done by OLIVARES-FUSTER et al. (2002). They firstly demonstrated the potential of green fluorescent protein as a somatic hybridisation marker. The results showed the utility of GFP to improve or establish somatic hybridisation protocol in other plant systems (OLIVARES-FUSTER et al. 2002). There are more studies demonstrating GFP reporter gene in *Citrus* plants. Plasmid DNA encoding both mgfp5-ER (HASELOFF et al. 1997) and Egfp and the plasmid DNA of a potential canker resistance gene (pC822) from the xa21 gene family of rice were transferred into Sweet Orange protoplasts via PEG by GUO et al. (2004). In another study, the expression of three different gfp mutants (egfp (YANG et al. 1996), gfpc3 (CRAMERI et al. 1996) and mgfp5-ER (HASELOFF et al. 1997) was studied in *Citrus* sp. by KAYIM et al. (2004) by using *Agrobacterium*-mediated transformation. Although green fluorescent protein expression was ob-

Table 1. The usage of GFP in vegetables, fruits, and ornamentals.

Species	Gene(s)	Methods	References
Vegetables			
Soybean (*Glycine max*)	GFP modification (smRS-gfp, mgfpER, mgfp5-ER, smgfp)	particle bombardment	PONAPPA et al. 1999
Broadbean, kidney bean (*Vicia faba*), soybean (*Glycine max*)	GFP, GS (soybean glutamine synthetase)	particle bombardment	MASUTA et al. 2000
Lotus (*Lotus japonicus)*	GFP, GUS	particle bombardment	PACIOS-BRAS et al. 2003
Potato (*Solanum tuberosum*)	GFP in amyloplast	particle bombardment	HIBBERD et al. 1998
Carrot (*Daucus carota*)	GFP in chromoplast	particle bombardment	HIBBERD et al. 1998
Pepper (*Capsicum annum*)	GFP in chromoplast	particle bombardment	HIBBERD et al. 1998
Onion (*Allium cepa*)	GFP, herbicide phosphinothricin	*Agrobacterium*	EADY et al. 2003
Spinach (*Spinacia oleracea*)	GFP	*Agrobacterium*	ZHANG and ZEE-VAART 1999
Melon (*Cucumis melo*)	GFP, GUS	*Agrobacterium*	GALPERIN et al. 2003
Sweet potato (*Ipomoea batatas*)	GFP	electroporation and particle bombardment	LAWTON et al. 2000
Fruits			
Sweet orange (*Citrus sinensis*	GFP	particle bombardment	NIEDZ et al. 1995
Sweet orange (*Citrus sinensis*)	gfp mutants (Egfp, gfpC3, mgfp5-ER	*Agrobacterium*	HIBBERD et al. 1998
Sweet orange (*Citrus sinensis*)	GFP	particle bombardment	OLIVARES-FUSTER et al. 2002
Sweet orange (*Citrus sinensis*)	mgfp5-ER, Egfp, canker resistance gene (pC822)	PEG	Guo et al. 2004
Grapevines (*Vitis* spp.)	EGFP/NPT II	*Agrobacterium*	LI et al. 2002
Apple (*Malus* sp.)	SGFP	*Agrobacterium*	MAXIMOVA et al. 1998
Plum (*Prunus domestica*)	GUS, GFP	*Agrobacterium*	GOLIS et al. 2002
Peach (*Prunus persica*)	GUS, GFP	*Agrobacterium*	GOLIS et al. 2002
Banana (*Musa* spp.)	uidA (ß-glucuroni-dase), SGFP	micro-particle bombardment	DUGDALE et al. 1998
Ornamentals			
Orchid (*Dendrobium* 'Sonia 17')	SGFP	Particle bombardment	TEE et al. 2003
Daisy *(Osteospermum ecklonis)*	GFPS65C	*Agrobacterium*	MERCURI et al. 2001
Lisianthus *(Eustoma grandiflorum)*	GFPS65C	*Agrobacterium*	MERCURI et al. 2001

tained in transgenic callus and shoots transformed with all three mutants, the transformants containing *Egfp* and *gfpC3* turned into brownish colour and died. They reported that most of the stable transgenic shoots survived when transformation was performed with mgfp5-ER gene.

LI et al. (2002) used EGFP/NPT II fusion marker to monitor transgene expression and developed Pierce's disease (PD)-resistant grapevines using transgenic technology. GFP expression patterns in various plant tissues and organs were monitored microscopically. In another study, factors that affect *Agrobacterium*-mediated transformation of grapevine were determined using the green fluorescent protein reporter gene as a non-destructive marker (LI et al. 2001).

For a successful transformation in apple, a synthetic green fluorescent protein gene (SGFP) (CHIU et al. 1996) was transferred into apple leaf explants using *Agrobacterium tumefaciens*. Although GFP expression was detected within 48 h, expression level decreased 11 days after agrobacterial infection (MAXIMOVA et al. 1998).

The utility of GUS and GFP reporter genes was compared between plum hypocotyl slices and different explants of peach. Since *gfp* gene provides not only a more rapid and non-destructive process of transformation but also an early selection of transgenic cells, it is considered as the most effective reporter gene (GOLIS et al. 2002). To understand the replication and the control of gene function in BBTV (Banana Bunchy Top Virus), *uidA* (ß-glucuronidase) and SGFP were used as reporter genes. BBTV promoters fused to the GUS and GFP genes were transiently introduced into banana embryogenic cells via micro-particle bombardment. (DUGDALE et al. 1998).

GFP Utility in Ornamentals

Green Fluorescent Protein as a flower fluorescent was used by some scientists (Table 1). MERCURI et al. 2001 demonstrated the generation of green-fluorescent flowers and thus they selected *Eustoma grandiflorum* and *Os-*

teospermum ecklonis for *gfp* transformation. They reported that the transformed *Eustoma* flowers fluoresced brightly upon illumination with UV light and GFP-transgenic *Osteospermum* flowers also appeared distinctly green upon UV illumination. TEE et al. (2003) did another study about gfp transformation to an ornamental plant. They compared five different promoters, which promote synthetic green fluorescent protein gene (CHIU et al. 1996) for the genetic transformation of *Dendrobium* cultivar Sonia 17S and they observed that the highest GFP transient expression rate was obtained by CaMV 35S promoter, whereas HBT and Ubi1 promoters showed a relatively lower expression rate in all the target tissues tested.

Conclusions

This review shows that GFP was widely used as a reporter gene alone or with other genes in horticultural plants. GFP has revolutionized experimental analysis of the dynamic molecular organization of cells. GFP has proven to be a tremendously versatile biological tool that works into nearly all aspects of biology. The use of this gene in biotechnology, cell biology, and biochemistry will highly contribute to successful further studies. In conclusion, the reporter gene discussed in this paper is recommended strongly for the future transformation studies.

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