

A. Wenck · C. Pugieux · M. Turner · M. Dunn ·
C. Stacy · A. Tiozzo · E. Dunder · E. van Grinsven ·
R. Khan · M. Sigareva · W. C. Wang · J. Reed ·
P. Drayton · D. Oliver · H. Trafford · G. Legris ·
H. Rushton · S. Tayab · K. Launis · Y.-F. Chang ·
D.-F. Chen · L. Melchers

Reef-coral proteins as visual, non-destructive reporters for plant transformation

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Abstract Recently, five novel fluorescent proteins have been isolated from non-bioluminescent species of reef-coral organisms and have been made available through ClonTech. They are AmCyan, AsRed, DsRed, ZsGreen and ZsYellow. These proteins are valuable as reporters for transformation because they do not require a substrate or external co-factor to emit fluorescence and can be tested in vivo without destruction of the tissue under study. We have evaluated them in a large range of plants, both monocots and dicots, and our results indicate that they are valuable reporting tools for transformation in a wide variety of crops. We report here their successful expression in wheat, maize, barley, rice, banana, onion,

soybean, cotton, tobacco, potato and tomato. Transient expression could be observed as early as 24 h after DNA delivery in some cases, allowing for very clear visualization of individually transformed cells. Stable transgenic events were generated, using mannose, kanamycin or hygromycin selection. Transgenic plants were phenotypically normal, showing a wide range of fluorescence levels, and were fertile. Expression of AmCyan, ZsGreen and AsRed was visible in maize T1 seeds, allowing visual segregation to more than 99% accuracy. The excitation and emission wavelengths of some of these proteins are significantly different; the difference is enough for the simultaneous visualization of cells transformed with more than one of the fluorescent proteins. These proteins will become useful tools for transformation optimization and other studies. The wide variety of plants successfully tested demonstrates that these proteins will potentially find broad use in plant biology.

Keywords AmCyan · AsRed · DsRed · Fluorescent proteins · Plant transformation · Visual markers · ZsGreen · ZsYellow

Abbreviations *BMS*: Black Mexican sweet maize · *CMP*: Cestrum virus promoter · *GUS*: β -Glucuronidase · *LUC*: Luciferase · *nos*: Nopaline synthetase · *pmi*: Phosphomannose isomerase · *PCR*: Polymerase chain reaction

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A. Wenck · M. Dunn · C. Stacy · E. Dunder · E. van Grinsven ·
R. Khan · M. Sigareva · W. C. Wang · J. Reed · K. Launis ·
Y.-F. Chang · L. Melchers
Syngenta Biotechnology Inc.,
3054 Cornwallis Road, Research Triangle Park, North Carolina,
NC 27709, USA

C. Pugieux · M. Turner · A. Tiozzo · P. Drayton · D. Oliver ·
H. Trafford · G. Legris · H. Rushton · S. Tayab · D.-F. Chen
Syngenta Jealott's Hill International Research Centre,
Bracknell, RG42 6EY, UK

Present address:

A. Wenck (✉), BASF Plant Science,
26 Davis Dr, Research Triangle Park, NC 27709, USA
e-mail: wencka@basf.com
Tel.: +1-919-5472024
Fax: +1-919-5472428

Present address:

M. Turner, Danisco AS,
Langebrogade 1, 1001 Copenhagen, Denmark

Present address:

G. Legris, European Development Centre,
Dow AgroSciences,
3 Milton Park, Abingdon, OX14 4RN, UK

Introduction

Marker genes are of great use in transformation technology in order to facilitate development of new transformation techniques. They can be used to monitor gene delivery, selection regimes and the efficiency of eliminating escapes. Frequently used marker genes include regulators of the anthocyanin pathway genes (Goff et al. 1990), β -glucuronidase (*GUS*) (Jefferson 1987) and

Table 1 Spectral properties, references and filter sets for the novel fluorescent proteins (NFPs) utilized

NFP	Excitation maximum (nm)	Emission maximum (nm)	Isolated from	References	Filter set properties		
					Filter set name ^a	Excitation filter	Emission filter
AsRed1 and 2	573	595	<i>Anemonia sulcata</i>	Matz et al. 1999	Chroma 41002c	hq545/30x	hq620/60m
AmCyan1	458	486	<i>Anemonia majano</i>	Lukyanov et al. 2000	Leica CFP	436/20 nm	480/40 nm
ZsYellow1	528	538	<i>Zoanthus sp.</i>	Matz et al. 1999	Leica YFP	510/20 nm	560/40 nm
ZsGreen1	496	506	<i>Zoanthus sp.</i>	Matz et al. 1999	Leica GFP plus	480/40 nm	510 nm
DsRed1 and 2	558	583	<i>Discosoma sp.</i>	Matz et al. 1999; ClonTech 2002	Chroma 41002c	hq545/30x	hq620/60m

^a CFP AmCyan fluorescent protein, YFP ZsYellow fluorescent protein, GFP green fluorescent protein

luciferase (*LUC*) (Schrott 1995). All of these markers have their use, but they also have some drawbacks. Anthocyanin expression works well for monocot crops but is not useful in dicots. In addition, there is phytoactivity associated with the constitutive expression of this pathway that makes recovery of plants difficult (Wu et al. 1998). This marker is, however, one of the most sensitive markers available since, as a transcription factor, even low levels of expression can be observed (McCormac et al. 1998). The *GUS* gene is also extremely useful for monitoring not only gene delivery, but also stable transformation. Nevertheless, in order to visualize *GUS* activity, the tissue, or a sub-sample, must be sacrificed. In addition, background *GUS*-like activity may be present in plant tissues (Hansch et al. 1995). The *LUC* gene has also been used as a reporter for monitoring the transformation process. It has proven particularly useful in identifying the transition from transient to stable expression in wheat and rice (Lonsdale et al. 1998; Baruah-Wolff et al. 1999), but visualization at cellular level was not possible even when a costly, purpose-built luminometer (Berthold LUMAT LB950) was used.

A new visual marker that has shown promise in plants is green fluorescent protein from *Aequorea victoria*. This marker has been used in several monocot and dicot plants (Reichel et al. 1996; Haseloff et al. 1997). In addition, versions exist that emit not only green light but also blue, yellow and cyan (Cubitt et al. 1995). However, all of these versions have significant overlaps in excitation and emission wavelengths making them difficult, if not impossible, to discriminate when expressed together within the same cell.

Recently, a new set of fluorescent proteins was isolated from non-bioluminescent species of reef coral. These proteins have been made available through Clontech [Palo Alto, Calif. (Clontech 2002)] and include the following:

- AmCyan1 from *Anemonia majano* (Matz et al. 1999);
- ZsGreen1 and ZsYellow1 from *Zoanthus sp.* (Matz et al. 1999);
- DsRed1 and DsRed2 from *Discosoma sp.* (Matz et al. 1999); and
- AsRed1 and AsRed2 from *Anemonia sulcata* (Lukyanov et al. 2000).

The fluorescence spectrum details of these proteins are given in Table 1. DsRed has been shown to function in tobacco as a marker for both transient expression and stable transformation (Jach et al. 2001).

In this report, we tested the use of AmCyan, ZsGreen, DsRed, AsRed and ZsYellow in a variety of different monocot and dicot species for both transient and stable expression studies. Stable transgenic events were generated for most of these constructs. Transgenic plants were phenotypically normal and fertile. Expression was visible in T1 seed and could be used to segregate transgenic from non-transgenic plants visually.

Materials and methods

Vector construction

AmCyan1, ZsGreen1 and AsRed1 coding sequence were cloned in front of the 346-bp version of the cestrum virus promoter (CMP) into binary vector pNOV2822 which contains the phosphomannose isomerase gene (*pmi*) under control of the 400-bp version of CMP (Stavolone et al., submitted). The use of CMP allows for expression of both the visual marker and selectable marker in monocot and dicot tissues. Binary vectors for ZsGreen1, AsRed1 and AmCyan1 are pNOV2145, pNOV2146 and pNOV2147 respectively. The binary vector pNOV2145 was created using the following steps: ZsGreen1 was excised from pZsGreen-N1 (Clontech) as a *NotI* fragment [all enzymes were obtained from New England Biolabs (Beverly, Mass.)]. Subsequent cloning steps created the intermediate vector, pBSC11250, which places ZsGreen1 between the CMP promoter and *nos* (nopaline synthetase) terminator for plant expression. This expression fragment was then subcloned into a plant binary vector containing the *pmi* gene for *Agrobacterium*-mediated transformation and mannose based selection (Reed et al. 2001). pNOV2146 was created as above except that the initial vector was pAsRed1-N1 (Clontech). The intermediate vector created during cloning (used for transient expression in onion) was pBSC11249. The AmCyan1 gene was isolated from pAmCyan1-N1 (Clontech) using the polymerase chain reaction (PCR) with the addition of a *SacI* site at the 3' end. The intermediate vector containing the expression fragment created was pBSC11251. Further cloning into the binary vector was as above. Vectors for DsRed1 were created using the following steps: the DsRed1 coding sequence was amplified from pDsRed1-N1 (Clontech) by PCR with the addition of an *NcoI* site at the 5' end and *SfiI* site at the 3' end. The resulting fragment was subcloned between the double enhanced 35S promoter of the cauliflower mosaic virus or maize ubiquitin 1 modified promoter and the *nos* terminator from *Agrobacterium tumefaciens* to create vectors for use in biolistic transformation, which were pJH0166 and pCeP1001 for dicot and monocot expression respectively. Further vectors for biolistic

Table 2 ClonTech fluorescent proteins tested in various crops for stable or transient expression. The T-region for the binary vectors used or the expression cassettes used in the biolistic approaches include the reporter gene and plant selectable marker gene

Construct	Reporter gene			Plant selectable marker			Construct type	Assay type	Crop tested
	Promoter ^a	NFP gene	Terminator ^b	Promoter ^c	Selectable marker ^d	Terminator ^b			
pBSC11249	CMP	AsRed1	nos	–	–	–	pUC based	Transient	Onion
pBSC11250	CMP	ZsGreen1	nos	–	–	–	pUC based	Transient	Onion
pBSC11251	CMP	AmCyan1	nos	–	–	–	pUC based	Transient	Onion
pBSC11579	CMP	DsRed2	nos	–	–	–	pUC based	Transient	Onion
pBSC11580	CMP	ZsYellow1	nos	–	–	–	pUC based	Transient	Onion
pJH0166	CaMV d35S	DsRed1	nos	–	–	–	pUC based	Transient	Potato, banana
pCeP1001	ZmUbi-1	DsRed1	nos	–	–	–	pUC based	Transient	Onion and rice suspension cells
pCeP1001	ZmUbi-1	DsRed1	nos	ZmUbi-1	PMI	nos	pUC based	Stable (co-transformation)	Maize Black Mexican sweet cells, wheat
pNOV2145	CMP	ZsGreen1	nos	CMPS	PMI	nos	Binary	Stable and transient	Maize, rice, tomato, soybean
pNOV2146	CMP	AsRed1	nos	CMPS	PMI	nos	Binary	Stable and transient	Maize, rice, tomato
pNOV2147	CMP	AmCyan1	nos	CMPS	PMI	nos	Binary	Stable and transient	Maize, rice, wheat, tomato, soybean, barley
pBSC11135	CMP	AsRed1	nos	AtUbq3+i	Hyg	nos	Binary	Stable	Cotton
pBSC11136	CMP	ZsGreen1	nos	AtUbq3+i	Hyg	nos	Binary	Stable	Cotton
pBSC11137	CMP	AmCyan1	nos	AtUbq3+i	Hyg	nos	Binary	Stable	Cotton
2004	CaMV d35S	DsRed1	nos	nos	nptII	nos	Binary	Stable	Tobacco

^a *CMP* Cestrum virus promoter (346-bp version), *CaMV d35S* double enhanced 35S promoter of cauliflower mosaic virus, *ZmUbi-1* maize ubiquitin 1 modified promoter

^b *nos* Nopaline synthetase

^c *ZmUbi-1* Maize ubiquitin 1 modified promoter, *CMPS* cestrum virus promoter (400-bp version), *AtUbq3+i* *Arabidopsis thaliana* ubiquitin 3 promoter with intron, *nos* nopaline synthetase

^d *PMI* Phosphomannose isomerase, *Hyg* hygromycin, *nptII* neomycin phosphotransferase II

transformation for transient results in onion were pBSC11579, pBSC11580, pBSC11249, pBSC11250 and pBSC11251. Vectors pBSC11579 and pBSC11580 were created using PCR amplification of the DsRed2 gene from pDsRed2-N1 (Clontech) and ZsYellow1 from pZsYellow1-N1 (Clontech) respectively, and cloned as described above for pBSC11251. Vectors for cotton transformation and hygromycin selection were pBSC11135, pBSC11136, and pBSC11137. Vector pBSC11135 was created from the AsRed1 intermediate vector pBSC11249 (above) by first cloning the AsRed expression fragment into a binary vector containing a hygromycin-resistance cassette for selection of stable transgenic events. ZsGreen1 and AmCyan1 were created in the same manner as for AsRed1. The construct used for tobacco transformation was 2004. This was created by cloning the DsRed1 cassette from pJH0166 above into a binary vector containing the neomycin phosphotransferase gene driven by the 5' *nos* promoter and with the *nos* terminator for kanamycin selection. A summary of all of the vectors utilized in this report is given in Table 2.

Plant transformation

Standard procedures were utilized for plant transformation. Species transformed using *Agrobacterium*-mediated transformation with modifications of previously described procedures were as follows:

- Tobacco leaf disk (*Nicotiana tabacum* cv. Samsun NN) (Rosahl et al. 1987);
- Maize immature embryos (*Zea mays* L.) (Negrotto et al. 2000);
- Cotton petiole (*Gossypium hirsutum* L.) (Chen and Zhang 2000);
- Rice callus (*Oryza sativa* L.) (Hiei et al. 1994);
- Soybean (*Glycine max* L.) (R. Khan, unpublished);

- Tomato cotyledon and hypocotyls (*Lycopersicon esculentum* L.) (M. Sigareva, in preparation);
- Wheat immature embryos (*Triticum aestivum* L.) (W. Wang, unpublished); and
- Barley immature embryos (*Hordeum vulgare* L.). (Tingay et al. 1997).

Mannose was used for selection of transformed events (Negrotto et al. 2000; review by Reed et al. 2001; Wright et al. 2001), except for tobacco, in which kanamycin selection was used (Rosahl et al. 1987) and cotton in which hygromycin selection was used (Chen and Zhang 2000).

Biolistic transformations of onion (*Allium cepa* L.) bulb epidermal cells, wheat immature embryos, maize BMS callus (black Mexican sweet) and potato epidermal cells (*Solanum tuberosum* L.) were performed essentially as described by Wright et al. (2001), except that intact plasmid was used instead of isolated fragments. Banana (*Musa acuminata*) fruit peel was transformed but using a Helios gene gun (Martiniuk et al. 2002). Mannose was used for selection of transformed wheat events (Wright et al. 2001).

Plant analysis

Fluorescent protein expression was monitored using a light microscope in some cases (such as for high expression of AsRed and AmCyan) or with a Leica MZ FLIII scope equipped with the proper filter sets (Table 1). Transient expression was observed from 1 to 7 days following DNA delivery or co-culture. Tissues coming through selection were also monitored for expression of the fluorescent proteins. In some cases expression was also followed into the leaf, pollen and seeds. Plants coming through regeneration were further characterized using TaqMan copy number analysis as

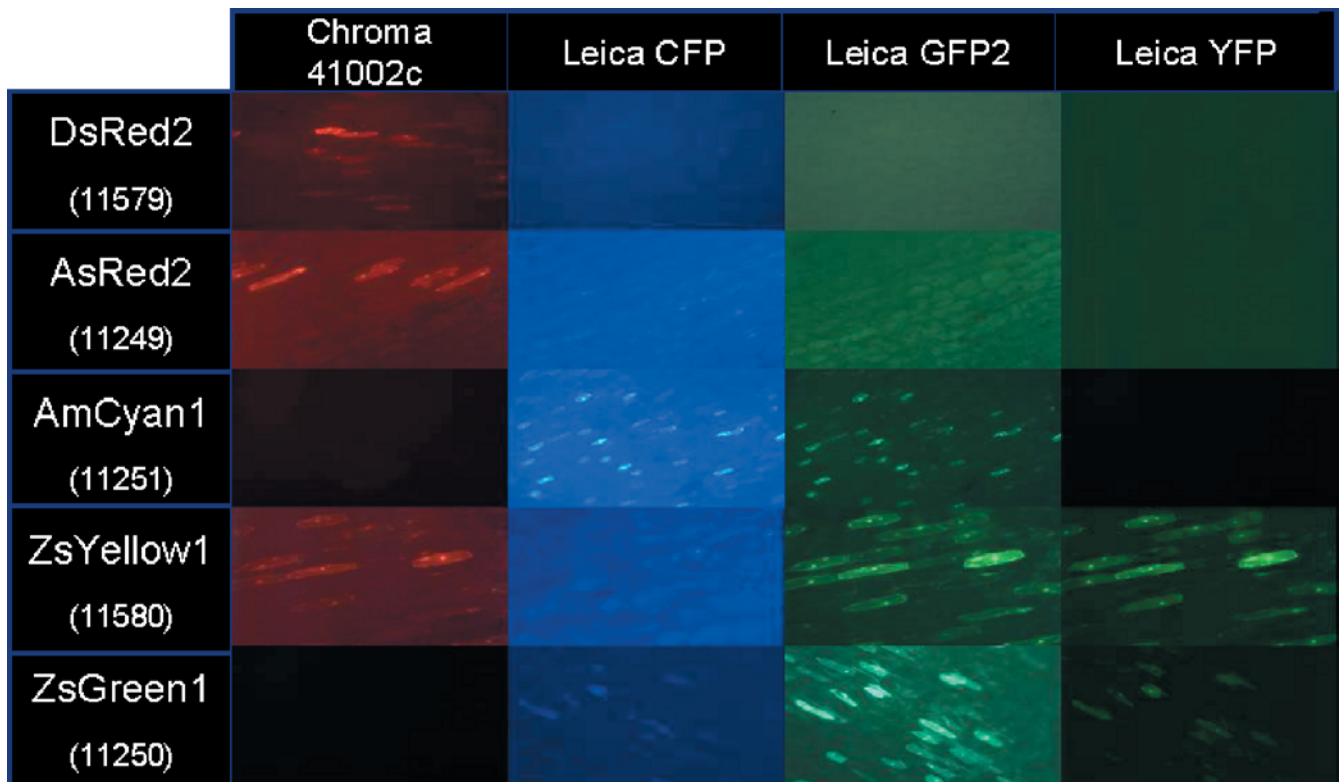
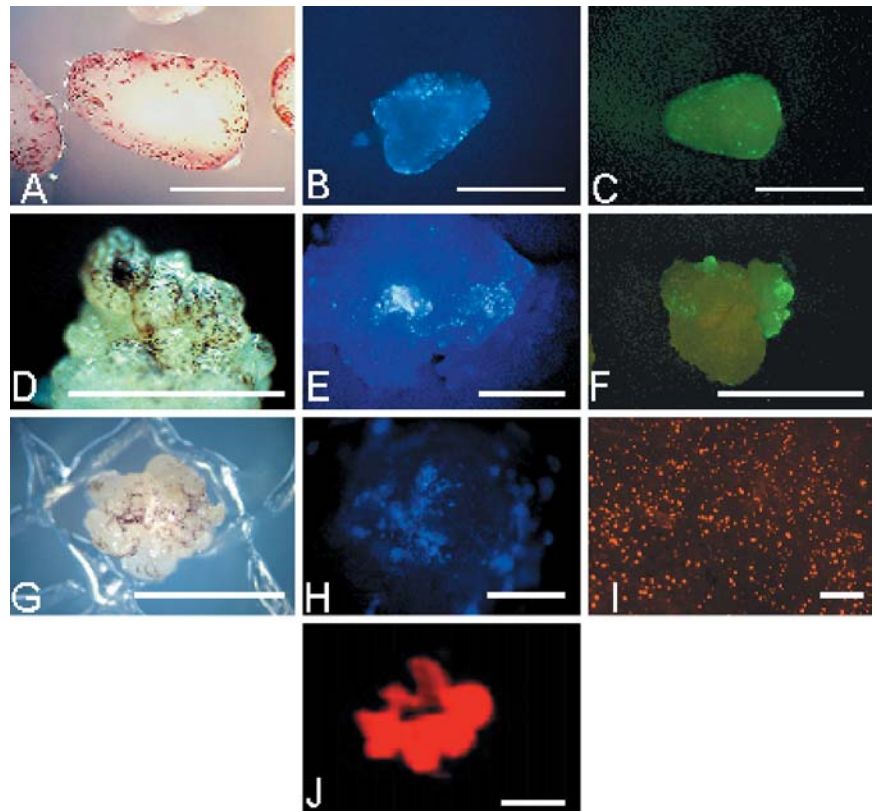


Fig. 1 Detection of Clontech fluorescent proteins following bombardment of onion epidermis

Fig. 2A–J Transient expression of reef-coral proteins in various crop systems. **A** Maize immature embryo—anthocyanin expression ($bar=1$ mm). **B** Maize immature embryo—AmCyan expression ($bar=1$ mm). **C** Maize immature embryo—ZsGreen expression ($bar=1$ mm). **D** Rice callus—anthocyanin expression ($bar=0.5$ cm). **E** Rice callus—AmCyan expression ($bar=1$ mm). **F** Rice callus—ZsGreen expression ($bar=0.5$ cm). **G** Wheat callus—anthocyanin expression ($bar=0.5$ cm). **H** Wheat callus—AmCyan expression ($bar=1$ mm). **I** Banana fruit peel—DsRed expression ($bar=1$ mm). **J** Potato epidermal single cell—DsRed expression ($bar=20$ μ m)



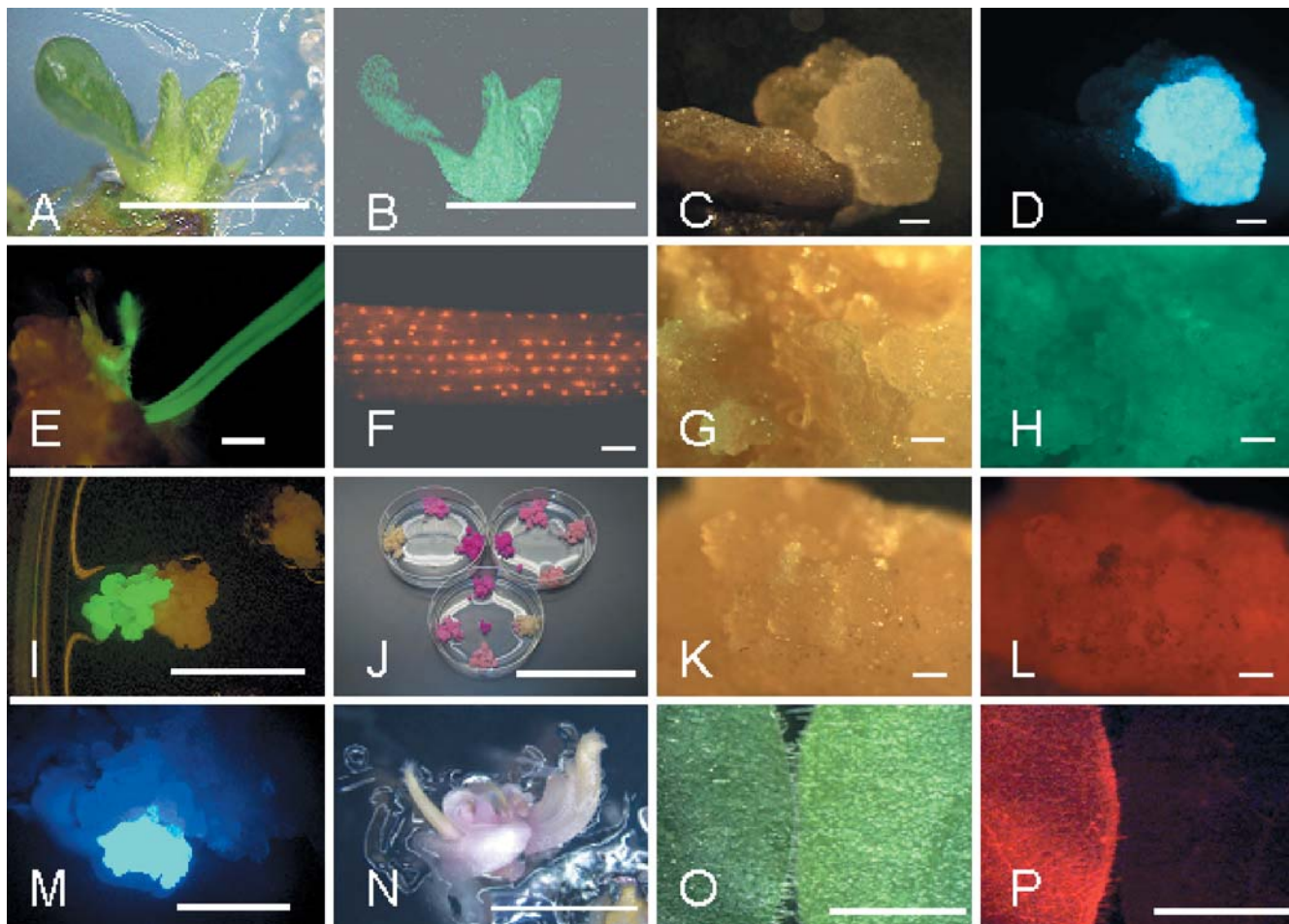


Fig. 3A–P Expression of the various fluorescent proteins in mannose, kanamycin or hygromycin selected transformants. **A** Soybean under white light ($\text{bar}=1\text{ cm}$). **B** Soybean under fluorescent light [ZsGreen ($\text{bar}=1\text{ cm}$)]. **C** Cotton callus under white light ($\text{bar}=1\text{ cm}$). **D** Cotton callus under fluorescent light [AmCyan ($\text{bar}=1\text{ cm}$)]. **E** Barley shoot under fluorescent light [AmCyan, but green filter set ($\text{bar}=2\text{ mm}$)]. **F** Wheat leaf under fluorescent light [DsRed ($\text{bar}=2\text{ mm}$)]. **G** Cotton callus under white light ($\text{bar}=1\text{ mm}$). **H** Cotton callus under fluorescent light [ZsGreen

($\text{bar}=1\text{ mm}$)]. **I** Rice callus under fluorescent light [ZsGreen ($\text{bar}=0.5\text{ cm}$)]. **J** Rice callus under white light [AsRed ($\text{bar}=10\text{ cm}$)]. **K** Cotton callus under white light ($\text{bar}=1\text{ mm}$). **L** Cotton callus under fluorescent light [AsRed ($\text{bar}=1\text{ mm}$)]. **M** Maize callus under fluorescent light [AmCyan ($\text{bar}=0.5\text{ cm}$)]. **N** Maize callus under white light [AsRed ($\text{bar}=1\text{ cm}$)]. **O** White light—DsRed transformed tobacco on left, untransformed on right ($\text{bar}=1\text{ cm}$). **P** Fluorescent light—DsRed transformed tobacco on left, untransformed on right ($\text{bar}=1\text{ cm}$)

previously described (Ingham et al. 2001). TaqMan analysis was also used to test for the presence of the T-DNA in seed visually scored as being positive or negative based on fluorescence.

Results and discussion

Transient expression was readily observed at 1–7 days following DNA delivery or co-culture in multiple crops. Examples of transient expression for the five fluorescent proteins following bombardment into onion bulb epidermis are shown in Fig. 1. As can be observed with the various fluorescence filter sets, it is possible to differentiate combinations of these proteins visually within the same cell, such as either of the red versions with green or cyan. Transient expression in several different crops was observed and a subset is shown in Fig. 2. For maize, rice and wheat, transient expression of anthocyanin is given

for reference. Transient expression could be detected in 100% of the maize embryos co-cultured with either the fluorescent protein construct or the construct containing the regulators of anthocyanin expression. The exact number of scutellum cells fluorescing was not counted for comparison with the number of cells that showed anthocyanin accumulation. However, as can be seen in Fig. 2, there appeared to be an overall greater number of cells with anthocyanin accumulation compared to the number expressing visible fluorescence (compare Fig. 2A with B and C). The same reduction in overall number could also be observed in rice (compare Fig. 2D with E and F) and wheat (compare Fig. 2G with H). The regulators of anthocyanin expression are transcription factors, so even very low levels of T-DNA expression can be visualized (Goff et al. 1990). However, there must be sufficient accumulation of the fluorescent protein marker

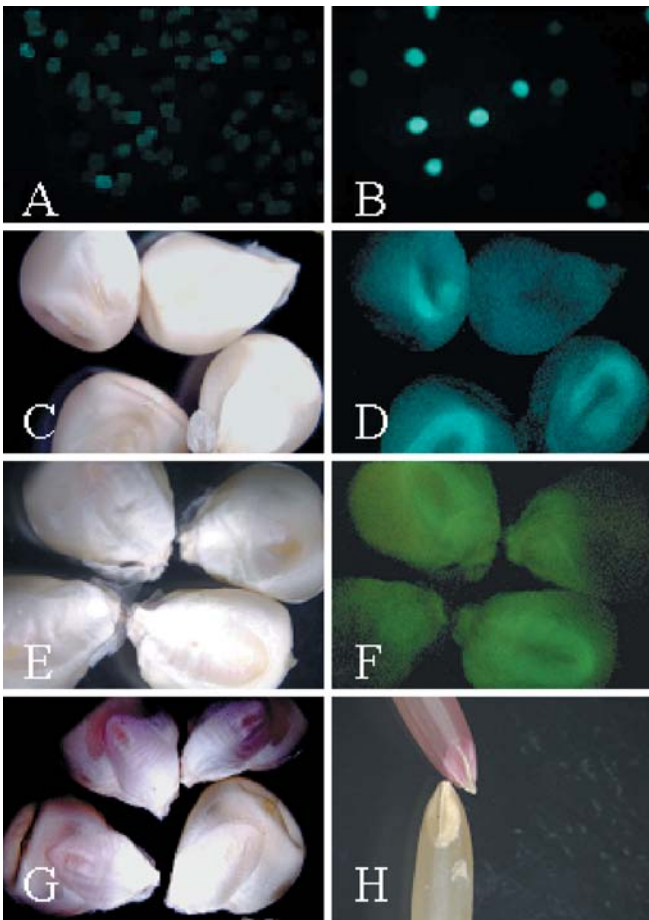


Fig. 4A–H Transmission of fluorescent reef-coral proteins to progeny. **A** Pollen from untransformed maize under fluorescent light. **B** Pollen from transformed maize under fluorescent light (AmCyan). **C** Maize T1 seed under white light. **D** Maize T1 seed under fluorescent light (AmCyan). **E** Maize T1 seed under white light. **F** Maize T1 seed under fluorescent light (ZsGreen). **G** Maize T1 seed under white light (AsRed). **H** Rice T1 seed under white light (AsRed)

in order to visualize expression. Low-level expression may not be observed. Expression could be localized to single cells (Fig. 2J) making these markers potentially useful for promoter studies.

Mannose, hygromycin or kanamycin were used as a selection marker in order to generate stable events expressing the fluorescent proteins. Stable transformants were recovered from maize (both embryogenic callus and BMS cell cultures), rice, barley, wheat, soybean, tomato, cotton, tobacco and potato (hairy root cultures). Examples are shown in Fig. 3. Transformed callus sectors were readily observed for all of the fluorescent proteins tested. The growth rate was not measured, but callus growth was according to normal expectations and the efficiency of transformation was similar for constructs with the same promoter-*pmi* combination. For maize, average efficiencies for this promoter with mannose selection were 28% compared to 20%, 34% and 16% in single experiments with ZsGreen, AsRed and AmCyan, respectively.

By using the proper filter sets, it was possible to visualize expression of the fluorescent proteins in the stable transformed callus and plants generated. Examples of several of these are given in Fig. 3. ZsGreen expression in soybean is shown in Fig. 3B compared to normal light (Fig. 3A) and in rice (Fig. 3I) and cotton (Fig. 3H). AmCyan expression in cotton is shown in Fig. 3D and also in maize (Fig. 3M). This protein fluoresces green if the filter set for green fluorescent protein (Table 1) is used, barley being shown as an example (Fig. 3E). AmCyan expression was also visible as a slight green/yellow glow in rice callus selected on mannose even without the use of fluorescence optics (not shown). In maize and rice callus tissue transformed with AsRed, red color was readily observed without the filter set [Fig. 3J and N (and other images not shown)]. For cotton, the fluorescent light was required to visualize expression (Fig. 3L). Given the results of Jach et al. (2001) and the results presented here with tobacco (Fig. 3O and P), it can be seen that red fluorescence, emitted by DsRed, can easily be observed in green tissue, which is an improvement over the green fluorescent protein from *Aequorea*. This result is expected since the wavelength utilized for DsRed excitation is different from that required for chlorophyll. Background fluorescence was not a problem with any of the proteins in the plants and tissues analyzed.

T1 seed was produced for maize, rice, wheat, soybean, tomato and tobacco. Fluorescent protein expression was visible in pollen (maize) and seed. Examples from maize and rice are shown in Fig. 4. Fluorescence was also visible in seed of tobacco, wheat and soybean (not shown). It was possible to segregate T1 seed visually based on the fluorescence phenotype (Fig. 4).

Two independent maize events with ten seeds visually scored as positive and ten as negative for each of the fluorescent proteins (AmCyan1, ZsGreen1 and AsRed1) were planted in the greenhouse (total of 120 T1 plants). TaqMan analysis was used to confirm visual segregation. Correlation between visual segregation and TaqMan analysis was shown to be greater than 99%. In only one case in the 120 resulting T1 plants was the visual scoring not confirmed by the TaqMan analysis. In this case one kernel scored as negative was positive by TaqMan analysis (data not shown). The high level of correlation allows for these proteins to be used as tools for high-confidence, rapid segregation of transgenic and non-transgenic progeny.

In addition to the maize tested above, T1 wheat and tobacco plants were grown. DsRed1 fluorescence could be detected in leaf in both wheat and tobacco seedlings (Fig. 3F, P). The plant growth was normal compared to untransformed plant controls. No negative effects were observed in any of the species tested. The five fluorescent proteins tested were expressed and could be detected visually in the crop species transformed (Table 2).

In the future it will be possible to stack these proteins within the same plant cell as predicted by transient results from onion. An example of stacking is shown in Fig. 5. In this example, a culture of rice expressing AmCyan was

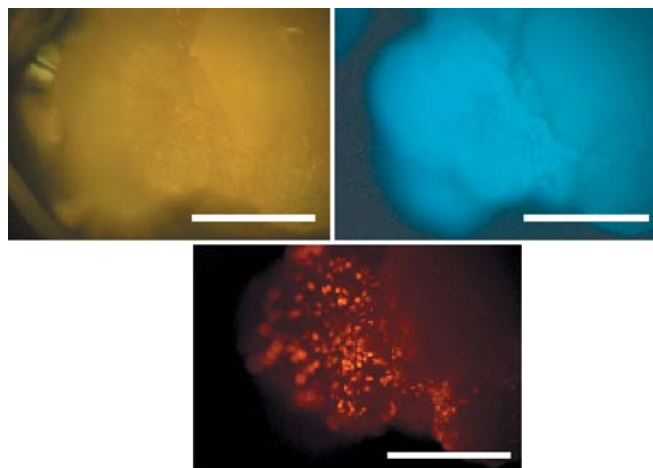


Fig. 5 Retransformation of an AmCyan expressing rice callus line with AsRed. *Top left* white light; *top right* fluorescent light (filter set for AmCyan fluorescent protein), *bottom center* fluorescent light (Chroma filter set). Bar=1 mm

retransformed with *Agrobacterium* containing the AsRed construct. Transient expression of the AsRed was readily visible in the stable AmCyan line. Several pairs can be constructed from the existing proteins for use in either retransformation experiments or experiments in which two different fluorescent reporters are required. In addition to the proteins available, new fluorescent proteins are still being discovered that will allow for even greater flexibility (Dove et al. 2001). Not only will these proteins find use in transformation studies, but they will be useful for other fields such as research into virally induced gene silencing studies where fluorescent markers have become an integral part of the work (Niwa et al. 1999; Johansen and Carrington 2001; Klahre et al. 2002)

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