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Red Fluorescent Protein DsRed from *Discosoma* sp. as a Reporter Protein in Higher Plants

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ABSTRACT

GFP from Aequorea victoria is a standard genetic marker widely used to visualize cellular events in a noninvasive manner. For simultaneous imaging of different processes, in vivo mutants of GFP with shifted wavelength spectra (e.g., blue fluorescent protein) are conventionally used. The recently reported red fluorescent protein from Discosoma sp., DsRed, represents a new marker that can be used together with GFP variants for multicolor imaging. DsRed is an interesting marker protein for use in plants because of its red-shifted wavelength spectrum that will avoid damaging cells and tissues by excitation light.

In this report, we show that DsRed is an excellent marker in higher plants in spite of the interfering red autofluorescence of chlorophyll, which can be eliminated by using the appropriate filter sets. Transient expression of DsRed1-C1 and a soluble-modified, red-shifted GFP variant has been carried out both individually and jointly in the epidermal cells of three different Nicotiana species and Chenopodium quinoa, which gives rise to dual labeling in plants. For this purpose, a human codon-optimized variant of DsRed has been adopted for expression in plants. Moreover, the DsRed reporter gene was expressed by using a labeled plant viral vector derived from an infectious full-length clone of potato virus X.

INTRODUCTION

Fluorescent proteins are indispensable tools for monitoring cellular events such as gene expression or protein localization in vivo. The GFP from *Aequorea victoria* is a standard reporter protein in several biological systems (6) because of its intrinsic bright, visible fluorescence (22) derived from an internal fluorophore upon excitation with blue light and because no substrates or co-factors are needed (4).

If different biological processes are visualized simultaneously, then marker proteins with distinct spectral properties are required. Because GFP was first reported as an excellent marker for gene expression (4), considerable efforts have been applied to create different wavelength mutations of the wild-type GFP, thus enabling multicolor imaging. The use of a blue-shifted GFP variant (i.e., BFP) was suggested to complement GFP to facilitate two-color assessments of differential gene expression or protein trafficking (11). To date, several GFP variants [e.g., the red-shifted S65T-mutants (6,10,12,15,21)] that show brighter green fluorescence than wild-type GFP, and others such as cyan fluorescent protein (CFP), yellow fluorescent protein (YFP) (19), enhanced cyan fluorescent protein (ECFP), and enhanced yellow fluorescent protein (EYFP), are used in molecular biology applications (3,15).

Recently, the palette of GFP-based fluorescent proteins has been enriched by the isolation of the gene of a red fluorescent protein (DsRed) from the non-bioluminescent coral *Discosoma* sp. (16). The crystal structure of the 28-kDa protein has been reported (23). DsRed differs considerably in its spectral properties from other fluorescent proteins, allowing for investigations that will use DsRed and other GFP variants in combination. The red fluorescent protein has an excitation peak at 558 nm and emits light with a maximum at 583 nm, far beyond the longest wavelength mutants of GFP (16,22). DsRed has been successfully expressed in prokaryotic cells, *Xenopus laevis*, and human embryonic kidney cells (16). In its first technical application, different *Pseudomonas fluorescens* populations were labeled with three GFP variants and DsRed to visualize root colonization of tomato plants by these bacteria simultaneously (3). In addition, mixed *E. coli* cultures labeled with EGFP and DsRed were imaged by confocal, two-photon, and fluorescence lifetime microscopy to evaluate different excitation modes (12). Further, DsRed1-1, a variant optimized for expression in mammalian and bacterial cells, was transiently expressed in *Nicotiana benthamiana* cells (7).

It has been reported that soluble-

modified, red-shifted GFP (smRS-GFP), soluble-modified BFP (smBFP), and BFP can express in plants and act as reporter proteins (6,18). Dual labeling in plant protoplasts has also been shown with YFP and CFP (19). Therefore, there is great interest in using different colored fluorescence markers in intact plant tissues; for example, to enable experiments that focus on “pulse chase” with the same plant virus or studies on complementation of viruses that are labeled with different fluorescent tags (17).

For the application of fluorescent proteins in plants, long-wavelength mutants are required to avoid the damage of plant tissues by the excitation light (17). For this reason and because of the limited applications of GFP variants for dual monitoring in plants, the new reporter protein DsRed with its red-shifted excitation and emission

spectra is a suitable companion for GFPs. However, the red autofluorescence spectrum of chlorophyll ranges from 660 to 800 nm (20) and might be a limiting factor for using DsRed in plants. Thus, it is necessary to eliminate the far-red autofluorescence of chlorophyll by using the appropriate filter sets to visualize DsRed fluorescence.

MATERIALS AND METHODS

DsRed Constructs

A human codon-optimized variant of DsRed (Living Colors® DsRed1-C1; BD Biosciences Clontech, Palo Alto, CA, USA) was used in all experiments. The marker gene was PCR amplified for subsequent cloning with two primers. The sense primer, 5'-GCAT-CGATTAGGAGATATAACAATGGT-

GCG-3', was used to generate a *Clal* site (underlined), a ribo- somal binding site (bold), and a plant-optimized surrounding region (underlined and Reference 14) of the start codon to the 5' end of the marker. The antisense primer, 5'-GCTCTAGACTCGACCAGGAACA-GGTGGTGGCGGC-3', was used to add *XbaI* and *XhoI* sites (underlined) at the 3' end of the gene.

For transient expression of the modified DsRed1-C1, the marker gene was digested (*Clal/XbaI*) and cloned into a modified pT7T3 19U plasmid (Amersham Biosciences, Freiburg, Germany) containing an enhanced 35S cauliflower mosaic virus promoter (CaMV35S) (13) and a CaMV termination signal. The nucleotide sequence at the 35S promoter (bold) and the transcription start site is 5'-**GAGAGGCGCGCCG**ATC-CAAGCTTATCGATTAGGAGATATA-ACA**ATG**-3', where the transcript is

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given in capital letters, and the initiation codon of DsRed is underlined. The resulting construct is referred to as pe35AscIoptRed. A similar plasmid was constructed without an optimized start codon surrounding region, 5'-GAGAGGCGCGCCGGATCCACCGGTCCGCCACCATG-3', named pe35AscIRed.

For dual labeling, smRS-GFP (6) was used in conjunction with pe35AscIoptRed. The smRS-GFP expression vector, CD3-328smRS-GFP (GenBank® accession no. U70497), is based on pUC118. The marker gene contains the S65T substitution for a highly fluorescent signal, the corrected splice site (9), and additional mutations F99S, M153T, and V163A to increase the solubility of the native protein (5).

The DsRed1-C1-labeled PVX vector is based on pPVX201, which is an infectious, full-length clone of the viral RNA genome of the plant virus, potato

virus X (PVX) (2). After PCR amplification, the optimized DsRed1-C1-fragment (described earlier) was digested with *ClaI/XhoI*, cloned into the pPVX201 vector, which was digested with *ClaI* and *SalI*, giving pPVX201-optRed.

Particle Bombardment

For transient marker expression, 1 µg column-purified plasmid DNA (Qiagen, Hilden, Germany) pe35AscIoptRed and CD3-328smRS-GFP was inoculated onto fully expanded detached leaves of *N. benthamiana*, *N. tabacum* 'Xanthi-nc', and *N. occidentalis* by microprojectile bombardment using a particle inflow gun (PIG), according to a previously described method (8). Air pressure was at 4 bar, and the vacuum was at -800 mbar. The inoculated leaves were stored for 48 h in the dark at room temperature using petri dishes with

moistened filter papers (595 1/2 Folded Filters; Schleicher & Schuell GmbH, Einbeck, Germany) to avoid drying the leaves. The co-bombardment of both plasmids was done using a mixture of 0.5 µg each plasmid DNA attached to tungsten particles using 1 M Ca(NO₃)₂, pH 10.0. *N. benthamiana* plants were inoculated with plasmid DNA of pPVX201-optRed by PIG as described earlier, except with the pressure of 3 bar to prevent damaging the plants.

Detection of Fluorescence

The marker fluorescence was detected with an Axiophot® microscope (Carl Zeiss, Oberkochen, Germany) fitted with filter sets (AHF Analysentechnik AG, Tübingen, Germany) consisting of the following bandpass and narrow-band filters. The smRS-GFP was visualized by illumination using an

excitation filter of 470/40 nm, a dichromatic beam-splitting mirror at 495 nm, and an emission filter of 525/50 nm (termed AHF_{smRS-GFP}). For the detection of DsRed fluorescence, the filter characteristics were 565/30, 585, and 620/60 nm respectively (AHF_{DsRed}). Two additional filter sets (Carl Zeiss) were used to visualize autofluorescence of chlorophyll and marker fluorescence—a UV filter (365/11, 395, and 395 nm) and a conventional rhodamine filter (546/12, 580, and 590 nm). Photographs were taken with FarbWelt Zoom 800/37° film (Eastman Kodak GmbH, Stuttgart, Germany).

RESULTS AND DISCUSSION

Because no optimized DsRed exists for expression in plants, we chose a variant with a human-optimized codon usage as a compromise. An smRS-GFP that was optimized for applications in plants (6) was used to compare two different fluorescent signals and to exam-

ine whether both marker genes are expressed within the same cell.

To adopt DsRed1-C1 to plant conditions, we made two modifications to the marker gene. The start codon was optimized for gene expression in plants (14) by introducing AACA just in front of the start codon. To facilitate a proper binding of the DsRed1-C1 mRNA at the plant ribosomes, a Shine-Dalgarno motif (AGGA) was generated nine nucleotides upstream of the start codon. After the specific modification of DsRed1-C1 and the subsequent introduction of a strong enhanced CaMV-35S promoter (13), a fluorescence signal was detectable 48 h after inoculation with pe35AscIoptRed by microprojectile bombardment. No fluo-

rescence was obtained from the non-modified DsRed1-C1 construct, pe35AscIRed (data not shown).

Single epidermal cells of the model plants *N. benthamiana*, *N. tabacum* 'Xanthi-nc', and *N. occidentalis* emitted a bright red fluorescence, demonstrating that the DsRed1-C1 gene is successfully expressed from pe35AscIoptRed. With its corresponding filter set AHF_{DsRed}, DsRed1-C1 fluorescence was visualized and localized in the cytoplasm and the nucleus (Figure 1, A2 and C2). We also found that *Chenopodium quinoa* cells were able to express DsRed1-C1, but the fluorescence signal was weak (data not shown). This might be due to different pH values in the cells of *C. quinoa* and

Figure 1. Expression of the marker genes DsRed1-C1 and smRS-GFP in epidermal *N. benthamiana* cells. The first row (A) shows images of the transient expression of the DsRed1-C1 marker gene in a single cell using the expression vector pe35AscIoptRed (magnification: 200×). The second row (B) shows the transient expression of smRS-GFP in single cells after inoculation with CD3-328smRS-GFP and the third row (C) demonstrates the transient co-expression of DsRed1-C1 and smRS-GFP within the same cell (magnification in B and C: 200×). Pictures are taken using fluorescence microscopy with four different filter sets: AHF_{smRS-GFP}, AHF_{DsRed}, a conventional rhodamine filter, and a conventional UV filter (panels labeled 1–4, respectively). The different expression vectors were introduced into leaves, respectively, by microprojectile bombardment. Fluorescence was observed at 48 h after inoculation.

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tobacco plants because it is known that the functionality of fluorescent proteins in plants is dependent on the pH in cells (E. Balazs, personal communication). As expected, the smRS-GFP fluorescence was visualized with AHF_{smRS-GFP} in *N. benthamiana* and *N. tabacum* Xanthi-nc cells also within 48 h, and the localization of the fluorescence was similar to that of DsRed1-C1 fluorescence (Figure 1, B1 and C1). Moreover, the successful co-expression of both markers in one cell was demonstrated (Figure 1, C1–C4). Both marker genes caused comparable strong fluorescent signals in single and co-expression, but no quantification of the quantum yield was done.

In co-expression, smRS-GFP and DsRed1-C1 fluorescence can be clearly distinguished because the single expression of the markers showed that AHF_{DsRed} only detects DsRed1-C1 fluorescence but no fluorescence of smRS-GFP and vice versa (Figure 1, A1, A2, B1, and B2). To demonstrate that AHF_{DsRed} is reliably eliminating the far-red fluorescence of chlorophyll, additional pictures of DsRed1-C1 (and

smRS-GFP) single and co-expressing cells were taken with a rhodamine and a UV filter set. A rhodamine filter is able to detect DsRed1-C1 fluorescence, but the signal is weak because of interfering chlorophyll autofluorescence, whereas no smRS-GFP fluorescence was detectable with this filter (Figure 1, A3 and B3). If a UV filter was used, then only smRS-GFP but no DsRed1-C1 fluorescence (Figure 1, A4 and B4) was visible. Similar to the rhodamine filter, strong autofluorescence of the surrounding cells interfered with the marker signal when the UV filter was used. The use of both nonoptimized filter sets (rhodamine and UV) also demonstrates that the marker genes DsRed1-C1 and smRS-GFP work parallel in one cell when co-expressed (Figure 1, C3 and C4).

When *N. benthamiana* plants were inoculated with pPVX201-optRed, systemic infections occurred seven days after inoculation. The DsRed1-C1 fluorescence signal was detected in systemically infected *N. benthamiana* tissues with AHF_{DsRed} (Figure 2) but not with AHF_{smRS-GFP}. Areas with a change from fluorescent to nonfluorescent cells were clearly detected, indicating the border of the virus spread in the tissues. The marker insertion in PVX201-optRed was stable even if the labeled virus was mechanically passaged onto new plants. Four direct passages were carried out, and one passage was done after four weeks using plant sap from plants that were first inoculated. In all passages, the same fluorescence characteristics were observed.

The results show that expression of DsRed1-C1 in plant cells is evident because of the bright and distinguishable red fluorescence of DsRed-expressing cells (Figures 1 and 2) and can be used in conjunction with an enhanced GFP variant. Moreover, autofluorescence of the plant tissues was suppressed by AHF_{DsRed}, allowing the visualization of DsRed1-C1 despite chlorophyll autofluorescence. However, the red fluorescence of DsRed1-C1 was reliably distinguishable from the surrounding cells and from smRS-GFP fluorescence.

Surprisingly, DsRed1-C1 showed a very strong fluorescence signal in plants. In contrast, DsRed has been reported to possess only 24% of the rela-

Figure 2. Systemic spread of PVX in *N. benthamiana* leaf tissues revealed by the DsRed1-C1-expressing PVX-vector PVX201-optRed. Plants were inoculated by microprojectile bombardment. Marker fluorescence was observed in systemically infected leaves seven days after inoculation under a fluorescence microscope with a specific DsRed filter set (magnification: 100×).

tive brightness of wild-type GFP (17). The relative fluorescence of smRS-GFP, used as a reference, is about 168% when compared with a soluble-modified variant of GFP (smGFP) (6), which shares the same chromophore with the wild-type GFP. Our results correspond with recent observations that DsRed has a higher quantum yield than reported previously (1). Further, the strong oligomerization and slow maturation of the protein are considered to be major drawbacks of DsRed (1). However, these observations relate to DsRed expressed in bacterial cells. Thus, we believe that the expression of this marker follows other requirements in plants. It is possible that bright DsRed fluorescence is caused by more efficient maturing and/or folding of the protein in plant cells than in other organisms.

In addition to the strong fluorescence and its use for double labeling with smRS-GFP, the red-shifted wavelength spectra of DsRed1-C1 makes this protein interesting for applications in plants particularly because the longer excitation wavelength avoids the risk of damaging plant tissues (17). Therefore, DsRed1-C1 might be a better companion for red- or yellow-shifted GFPs than BFP, which was proposed for the simultaneous imaging of different labeled viruses in plants (17). DsRed is an ideal marker protein for imaging on confocal laser microscopes because its excitation maximum matches with the wavelengths of commonly used green lasers.

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