Supplementary material for Wiedenmann *et al*. (2000) *Proc. Natl. Acad. Sci. USA* **97** (26), 14091–14096. (10.1073/pnas.011235398)

Relationship of Green Fluorescent Protein (GFP) Homologes

The phylogenetic relationship of asFP499, asCP562, GFP (1), and homologes from anthozoan species (2) was examined. The study was based on an multiple alignment using the CLUSTAL method (3) of the residues 1–135 as this region exists in all proteins. The pigments derived from the same species (*A. sulcata*, *Zoanthus* sp.) appear as direct neighbors (Fig. 1). A comparable relationship was found for the green and the orange fluorescent proteins from *Discosoma striata* and *Discosoma* sp. "red." In contrast, the pigments of the two *Actinaria* spp. are only distant relatives. Therefore, it seems likely that the two forms of *Discosoma* sp. represent two color morphs of the same species or that they are closely related. The phylogenetic tree shows no direct connection between proteins with similar spectral properties. For this reason, it seems likely that the different colors of fluorescence have evolved independently from a distant ancestor protein.

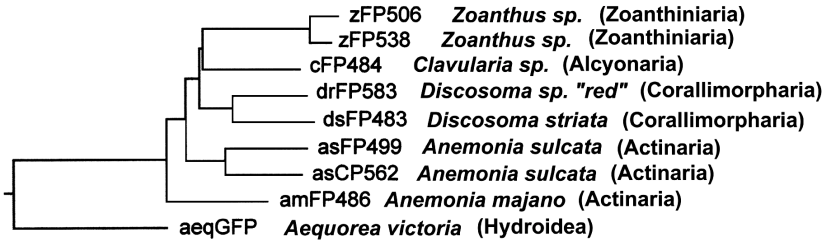

Fig. 1. Relationship of asFP499, asCP562, GFP, and homologes from *Anthozoan* species. The phylogenetic tree is based on a multiple alignment of the residues 1–135 of the protein pigments of these species. The relative length of each pair of branches represents the distance between sequence pairs as determined by the neighbor-joining method built into CLUSTAL (3, 4).

Application of asFP499 as *In Vivo* Marker

The fluorescent protein asFP499 cloned from *A. sulcata* can potentially be applied as marker proteins. The use of the proteins is patent pending (5). The GFP asFP499 has spectral properties differing from all known GFP variants and homologues. The applicability as reporter gene in eukaryotic systems analog to GFP from *A. victoria* is demonstrated by the transfection of *Drosophila melanogaster* cells (SL2) and protoplasts of *Nicotiana tabaccum*

and *Arabidopsis thaliana* (Figs. 2, 3*a*, and 4). Compared to the fluorescence of chlorophyll, asFP499 expressed in tobacco protoplasts appears to be more fluorescent as the optimized variant for plant expression of *Aequorea*-GFP (smGFP) (ref. 6; Fig. 3*b*). The comparison of smGFP and asFP499 expressed in chlorophyll depleted *A. thaliana* indicates about three to four times brighter fluorescence of asFP499, when the first is excited at its minor absorbance peak (Fig. 4). The bright fluorescence of asFP499 and the functional expression in prokaryotic, plant, and insect cells suggests that it will have a general utility for biological studies in a wide range of organisms.

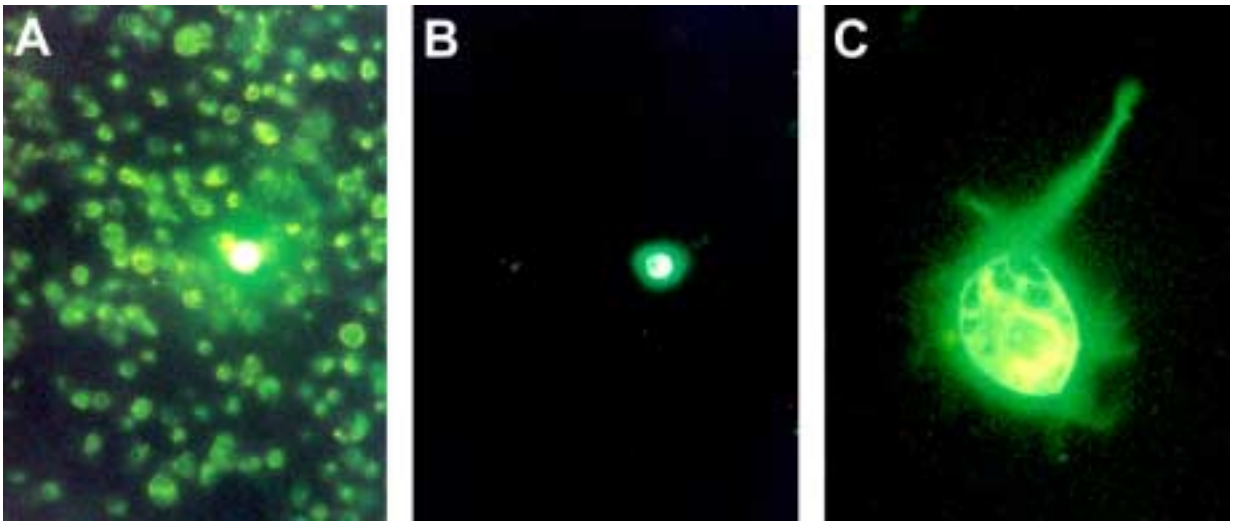

Fig. 2. Expression of asFP499 in *Drosophila* cells (SL2). Pictures were taken with the Axioplan filter set 487901 (Zeiss; excitation wavelength, 450–490 nm; FT510; LP530). (*A*) A transfected cell among nonrecombinant cells illuminated with additional white light (magnification, ×400). Nonrecombinant cells are not fluorescent but appear also greenish because of the filter set used. (*B*) The same sample without additional white light. (*C*) A single cell expressing asFP499 (magnification, ×630).

Experimental Procedures

Transfection of *Drosophila* Cells. *Drosophila melanogaster* cells (Schneider's line 2/SL2) (7) were transfected with the original plasmid (pBK-CMV phagemid vector; Stratagene) containing the sequence coding for asFP499. Transfection was performed using Effectene Transfection Reagent (Qiagen, Heidelberg, Germany). After a 48-h incubation period at 25°C following the experiment, cells were screened for functional expression of asFP499. Photographs were taken using Fuji Color Sensia films.

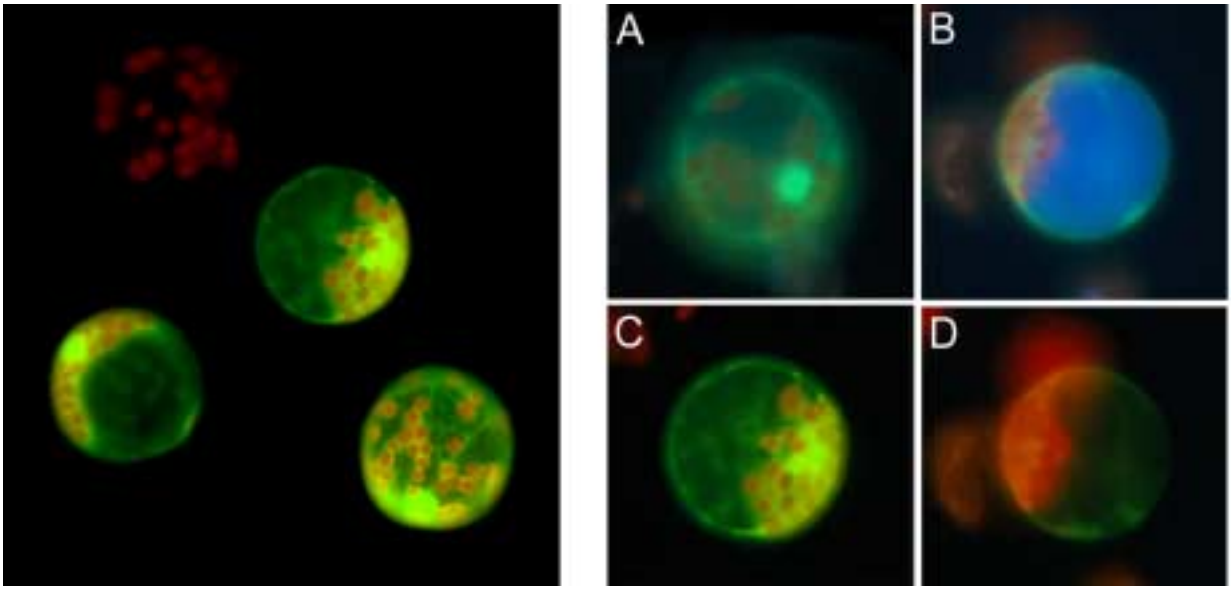

Fig. 3. (*a*) Green fluorescence of asFP499 expressed in protoplasts of *N. tabaccum* (excition wavelengths, 450–490 nm; filter, FT510, LP520; Zeiss; magnification, ×630). The untransfected protoplast in the upper part of the figure shows only red chlorophyll fluorescence. (*b*) Comparison of green fluorescence produced by asFP499 and smGFP expressed in protoplasts of *N. tabaccum* (magnification, ×630). Protoplasts transfected with asFP499 (*A*) and smGFP (*B*) excited with UV light (365 nm) (filter, FT395, LP520; Zeiss). Large parts of the cell in *B* show blue autofluorescence. Fluorescence of asFP499 (*C*) and smGFP (*D*) excited with 450–490 nm (filter, FT510, LP520; Zeiss). Compared to the intesity of the red fluorescence of chlorophyll, the green fluorescence of asFP499 appears to be brighter as of smGFP. The protein asFP499 seems to be localized both in cytoplasm and nucleus (*A*).

Transfection of Tobacco and *Arabidopsis thaliana.* The insert containing the coding sequence for asFP499 was cut off from the original plasmid by digestion with Bam*HI* and Kpn*I* (Boehringer Mannheim). The insert was separated from the plasmid on an agarose gel and purifided using Agarose Gel DNA Extraction Kit from Boehringer Mannheim and subcloned into the high-copy pUC119 plasmid containing a CaMV 35S promoter for plant expression. Protoplasts of *N. tabaccum* and *A. thaliana* were transfected according to the polyethylene glycol-mediated transfection protocol provided by Koop *et al.* (8). After incubating the samples in the dark for 24 to 48 h at room temperature, transient transgene protoplasts were analyzed under a fluorescence microscope (Axioplan; Zeiss). Exposure time series were documented with a digital camera (Polaroid).

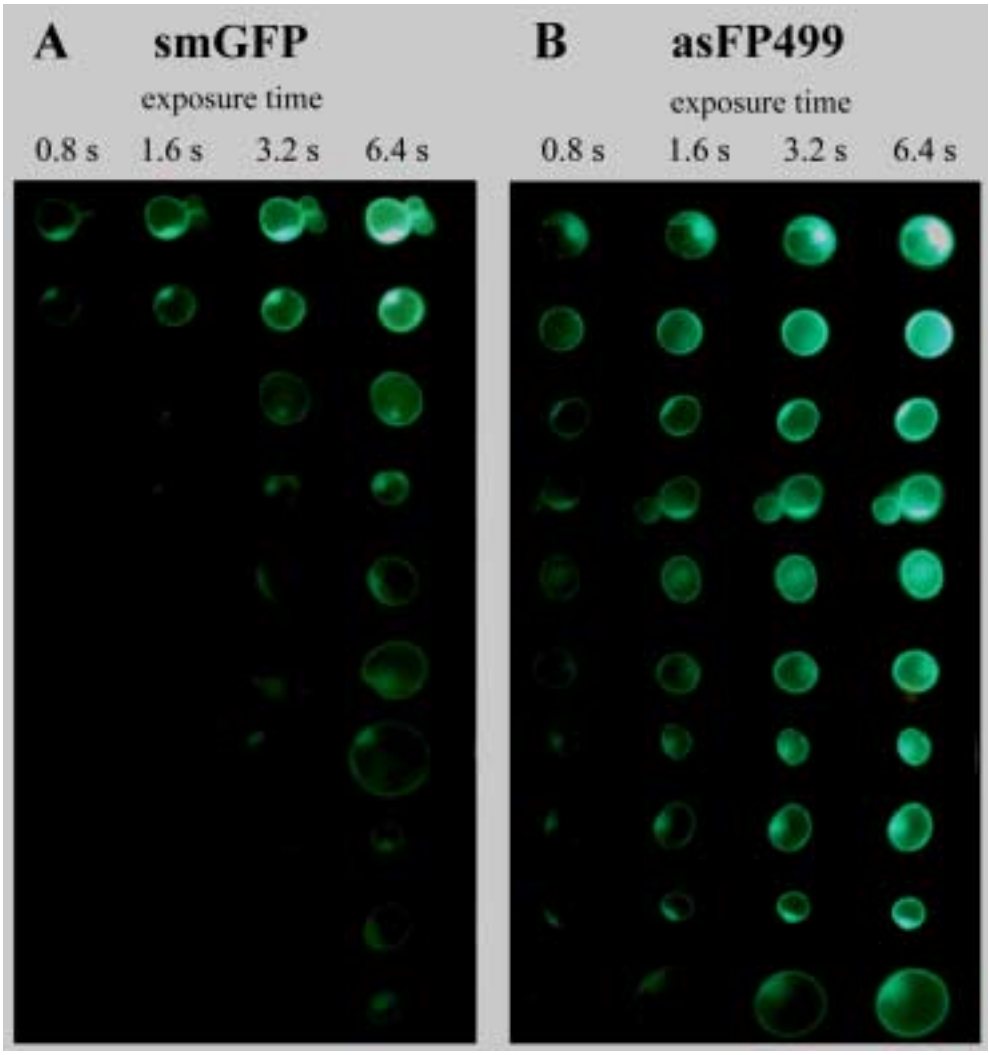

Fig. 4. Comparison of chlorophyll-depleted protoplasts of *A. thaliana* transfected with smGFP (*A*) and asFP499 (*B*). Protoplasts were excited with 450–490 nm (filter, FT510, LP520; Zeiss; magnification, ×630). From each protoplast preparation, 10 positive cells being observed first were documented in exposure time series. The fluorescence of asFP499 appears to be about three to four times brighter as compared to smGFP.

References

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