Fluorescence Cross-Correlation Analyses of the Molecular Interaction between an Aux/IAA Protein, MSG2/IAA19, and Protein–Protein Interaction Domains of Auxin Response Factors of Arabidopsis Expressed in HeLa Cells

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Since auxin may elicit numerous developmental responses by the use of a combination of auxin response factors (ARFs) and their Aux/IAA repressors, it is important to determine the interaction between the two protein families in a quantitative manner. We transiently expressed the C-terminal protein–protein interaction domains (CTDs) of Arabidopsis ARFs, MP/ARF5 and NPH4/ARF7, and MSG2/IAA19, fused to fluorescent proteins in HeLa cells, and determined their molecular interactions with fluorescence cross-correlation spectroscopy (FCCS). Almost complete association was found between MSG2 and MP-CTD and between MSG2 and NPH4-CTD. Approximately 20% association was found for MSG2 homodimers, NPH4-CTD homodimers and MP-CTD/NPH4-CTD heterodimers. Homotypic binding of MP-CTD may be weaker than that of MSG2. MSG2 was localized in cytoplasmic compartments in HeLa cells, whereas it was localized in the nuclei in plant cells. The fact that the heterotypic interaction between MSG2 and ARF-CTDs is stronger than each of the homotypic interactions appears to be the molecular basis for tight control of the transcriptional activity of ARFs by auxin. These results also show that FCCS is useful to examine protein–protein interactions especially for transcriptional regulators.

Keywords: Arabidopsis thaliana — Aux/IAA protein — Auxin response factor — Fluorescence cross-correlation spectroscopy — HeLa cell — Protein–protein interaction.

Abbreviations: ARF, auxin response factor; CTD, C-terminal domain; EGFP, enhanced green fluorescent protein; FCCS, fluorescence cross-correlation spectroscopy; FRET, fluorescence resonance energy transfer; mRFP, monomeric red fluorescent protein; tR₂, mRFP tandem dimer.

Introduction

The transcriptional network facilitated by protein–protein interaction appears to play a more important role in the plant kingdom than in other kingdoms, considering that plants have a greater number of plant-specific transcription factors (Riechmann et al. 2000). We have studied the interactions between plant-specific auxin response factors (ARFs) and their repressive regulators, Aux/IAA proteins (IAAs), in a yeast two-hybrid assay (Tatematsu et al. 2004). ARFs and Aux/IAAs share the C-terminal domain (CTD) through which they interact (Kim et al. 1997, Ulmasov et al. 1997). Auxin has been proposed to elicit numerous developmental and physiological responses by the use of a combination of 23 ARFs and 29 Aux/IAAs in a model plant, Arabidopsis (Leyser 2002). Therefore, it is important to determine which Aux/IAAs can associate with each ARF through its CTD in a quantitative manner.

The yeast two-hybrid assay has often been the method of choice to examine interactions between proteins. However, the yeast two-hybrid assay cannot correctly determine the interaction between transcriptional regulators because it uses transcriptional activation as a measure of the protein–protein interaction. Furthermore, the expression levels of tested proteins must be checked separately with other methods such as Western blotting. Thus, new physical methods to detect protein association in vivo have been eagerly awaited. Fluorescence cross-correlation spectroscopy (FCCS) is an emerging technique that can physically evaluate protein–protein interaction in a quantitative manner (Kettling et al. 1998, Rigler et al. 1998). If fluorescence intensity is measured from a small number of fluorophores in a small detection volume, it fluctuates due to the variations in the number of molecules. In such a condition, correlation of fluorescent intensity is calculated over time. For example, two proteins of interest are fused with different fluorescent proteins: one is fused with enhanced green fluorescent protein (EGFP) and the other is fused with monomeric red fluorescent protein (mRFP). Green and red fluorescence signals are measured simultaneously from molecules in a confocal detection volume, and correlation is calculated as a function of time for EGFP and mRFP, respectively, which is called auto-correlation.

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Correlation is also calculated between EGFP and mRFP (cross-correlation). If the two fused proteins bind together, a high-amplitude cross-correlation signal will be obtained from fluctuating fluorescence from them. If they move independently, a weak cross-correlation signal will be observed. The population of associated molecules can also be estimated from the calculated auto-correlation function and cross-correlation function.

Fluorescence resonance energy transfer or the Förster resonance energy transfer (FRET) technique is another fluorescent spectroscopic method for studying protein–protein interactions in live cells, in which the efficiency of energy transfer from a donor fluorophore bound to one protein to an acceptor fluorophore on the other protein is determined. It has been used more often than FCCS (Miyawaki 2003), and is completely different from FCCS which is based on thermal fluctuation of molecules. A disadvantage of FRET is that FRET efficiency greatly depends on the distance and angle between the donor and acceptor fluorophores. The efficiency is poor if the distance between the two fluorophores is greater than a few nanometers. Furthermore, the emission spectrum of the donor fluorophor should overlap with the excitation spectrum of the acceptor fluorophore. In contrast, any combinations of two spectrally distinct fluorescent probes are utilized for FCCS analysis irrespective of their relative geometry because FCCS is based on only the coincidence of the two fluorescences. In principle, therefore, FCCS is free from the limitations of FRET (Takagi et al. 2004). The two methods also differ from each other with respect to data acquisition: most in vivo FRET experiments are based on the ratio image of fluorescence intensity from sequentially acquired images collected by a laser scanning microscope or a charge-coupled device (CCD) camera. On the other hand, standard FCCS equipment does not provide simultaneous measurements at many points in cells, and thus does not provide an image for visualizing the subcellular localization of fluorophores.

Here, we have used FCCS to investigate the interaction between MSG2/IAA19 (Tatematsu et al. 2004) and MP/ARF5- or NPH4/ARF7-CTDs (Hardtke and Berleth 1998, Harper et al. 2000, Hamann et al. 2002), which were transiently expressed as fusion proteins with the fluorescent proteins in HeLa cells. Through this study, we also show that the FCCS method can be successfully applied to the analysis and measurement of plant protein interactions, broadening its use from the few in vitro and in vivo model systems reported so far (Bacia et al. 2002, Kim et al. 2004, Saito et al. 2004, Baudendistel et al. 2005).

Results and Discussion

We first estimated maximum cross-correlation by measuring the cross-correlation of EGFP fused to the mRFP tandem dimer (tR2) expressed in HeLa cells (Figs. 1, 2b). EGFP and tR2 separately expressed in one cell were also examined for minimum cross-correlation (Fig. 2a). Determination with these cell lines resulted in a 74.8±10.7% cross ratio \( \frac{G_c(0)}{G_r(0)}-1 \) for the maximum cross-correlation and a 23.3±5.5% cross ratio for the minimum cross-correlation (Fig. 3). When MSG2 fused to EGFP was expressed with MP-CTD fused to tR2, 73.0±13.9% cross ratio was observed (Fig. 2c). Essentially the same interaction was obtained for a reverse combination of fusion proteins, EGFP–MP-CTD and tR2–MSG2 (Fig. 3). These values coincided with the maximum value of the cross ratio, indicating complete association between MSG2 and MP-CTD. Essentially the same results were obtained for the interaction between MSG2 and NPH4-CTD (Fig. 2d). On the other hand, the cross ratio between EGFP–MSG2 and tR2–MSG2 was 33.7±6.2% (Fig. 2e), which was significantly higher than the minimum value of the cross ratio \( P=0.0054 \) by Student’s t-test. It was also higher than that for interaction between two fluorescent proteins, only one of which was fused with MSG2 (one-sided free fluorescent protein

![Fig. 1](https://academic.oup.com/pcp/article-abstract/47/8/1095/2329665) Schematic drawing of fusion proteins expressed in this study. Black boxes represent domains III and IV of the C-terminal domain (CTD) which is conserved in ARFs and Aux/IAAs. Numbers below the CTDs indicate amino acid numbers of the N- and C-termini of CTDs.
controls; \( P < 0.026 \). If we assume that the maximum and minimum cross ratios correspond to 100 and 0% association, respectively, about 20% of MSG2 molecules are judged to form a homodimer. Values for the homotypic interaction of MP- and NPH4-CTD and those for the heterotypic interaction between MP-CTD and NPH4-CTD (Fig. 2f) were all similar in magnitude to that between MSG2 monomers, which were significantly larger than the minimum cross ratio (\( P < 0.017 \) for all combinations). The homotypic interaction between NPH4-CTD and the heterotypic interaction between MP- and NPH4-CTD were also significantly higher than that of the one-sided free fluorescent protein controls (\( P < 0.022 \)), except for the interaction between EGFP-MP-CTD and free tR2 (\( P > 0.20 \)). On the other hand, the homodimeric interaction between MP-CTD molecules was as weak as that of the one-sided free fluorescent protein controls (\( P > 0.137 \)), suggesting that homotypic interaction of MP-CTD was negligible.

When MSG2–GFP was transiently expressed in onion epidermal cells by the use of particle bombardment, it was exclusively found in the nucleus (Fig. 4a). However, EGFP–MSG2 was seen diffusively in the cytoplasm as well as in the nucleus when expressed in HeLa cells (Fig. 4c). Although tR2–MSG2 was also present in both compartments, it was excluded from the nucleus more readily than EGFP–MSG2 (Fig. 4d), probably due to its larger molecular size (72.5 kDa) than EGFP–MSG2 (48.4 kDa). EGFP–MSG2 was found almost exclusively in the cytoplasm when co-expressed with tR2–NPH4-CTD (65.4 kDa; Fig. 4h), which was found in both the cytoplasm and nucleus when expressed alone (Fig. 4g). These results

![Fig. 2 Cross-correlation analyses. Typical auto- and cross-correlation curves of EGFP and tR2 (a), EGFP–tR2 (b), EGFP–MSG2 and tR2–MP-CTD (c), EGFP–MSG2 and tR2–NPH4-CTD (d), EGFP–MSG2 and tR2–MSG2 (e), and EGFP–NPH4-CTD and tR2–MP-CTD (f) expressed in HeLa cells. The fluorescence intensities in the red and green channels are shown in the upper graph of each panel in red and blue, respectively. Auto-correlation curves for red and green channels, and a cross-correlation curve between the two colors are depicted in the lower graph of each panel in red, blue and black, respectively. Intensity of fluorescence was measured at cross hairs in each inset with a 10-μm scale bar.](https://academic.oup.com/pcp/article-abstract/47/8/1095/2329665)
strongly suggest that EGFP–MSG2 interacted with tR2–NPH4-CTD so strongly that the resultant complexes were efficiently excluded from the nucleus because of their large molecular size. The subcellular localization of EGFP–MSG2 was not affected by the presence of either tR2–MSG2 (Fig. 4i) or tR2 (Fig. 4j), suggesting that there was no significant interaction between the MSG2 monomer molecules. Essentially the same results were obtained between EGFP–MSG2 and tR2–MP-CTD (data not shown).

In this study, we were able to determine the protein–protein interaction between MSG2 and MP- or NPH4-CTDs in a quantitative manner with FCCS. Almost complete association was observed between MSG2 and ARF-CTDs, while ~20% association was found between MSG2 monomers. Homodimeric interaction between NPH4-CTD monomers and heterodimeric interaction between MP- and NPH4-CTDs are also similar in strength to homodimeric interaction between MSG2 molecules. The interaction between MP-CTD monomers may be more subtle (Fig. 3). These results imply that Aux/IAA and ARF proteins primarily exist as a heterodimeric form between the two protein families. When one of them cannot find its partner, it is often present in a monomeric form; homodimeric species and heterodimeric species between ARFs are found on fewer occasions. This conclusion is consistent with the observed subcellular localization of EGFP–MSG2 in HeLa cells, which is affected by the expression of tR2–NPH4-CTD, but not by tR2–MSG2 (Fig. 4). The strong interaction between Aux/IAA and ARF suggests that transcriptional regulation conducted by ARFs is readily modified by the change in Aux/IAA level, which is under the direct control of the auxin F-box receptors (Dharmasiri et al. 2005, Kepinski and Leyser 2005).

The molecular interaction between Aux/IAA proteins and ARF-CTDs has been investigated with the yeast two-hybrid system (Kim et al. 1997, Ulmasov et al. 1997, Ouellet et al. 2001, Hamann et al. 2002, Hardtke et al. 2004, Tatemetatsu et al. 2004, Fukaki et al. 2005, Weijers et al. 2005, Weijers et al. 2006) and immunoprecipitation (Tatematsu et al. 2004, Weijers et al. 2006). Homotypic interaction of IAA1, AXR3/IAA17 or its CTD was observed with size exclusion chromatography (Kim et al. 1997) or electrophoretic (Ouellet et al. 2001) and cross-linking methods (Kim et al. 1997). Stronger interaction between MSG2 and MP- or NPH4-CTD than each homodimeric interaction was reported by the use of the yeast two-hybrid assay (Tatematsu et al. 2004). Similar results were obtained between BDL/IAA12 and MP- or NPH4-CTD (Hardtke et al. 2004, Weijers et al. 2005, Weijers et al. 2006) and between AXR3 and ARF1 (Ouellet et al. 2001). When maximum interaction was evaluated as the activity of the reporter gene in the presence of the GAL4 transcription factor in the yeast two-hybrid system, the interactions between MSG2 and MP- or NPH4-CTD were ~30% of the maximum interaction in our previous study (Tatematsu et al. 2004). A slightly stronger interaction was detected between BDL and MP- or NPH4-CTD (Hardtke et al. 2004). Furthermore, only 0.9% of the
Although these results cannot be directly compared with those of the present FCCS experiments, they appear to be in sharp contrast to almost complete association between MSG2 and ARF-CTDs and ~20% association between MSG2 in the FCCS study. The difference probably arises from the repressive nature of Aux/IAA proteins in transcription, which has been well characterized in plant cells (Tiwari et al. 2004). This clearly illustrates the limitation of the yeast system and the marked advantage of the physical FCCS method to evaluate protein–protein interaction between transcription factors quantitatively.

On the other hand, the interaction between MSG2 monomers appears to be near the limit of detection for FCCS, although it would be detected easily by the yeast two-hybrid assay if it is not a transcriptional repressor. This seems to represent a weakness of FCCS. Recently, the interaction between GFP-fused BDL and HA (hemagglutinin epitope)-tagged MP was examined in planta with immunoprecipitation. When protein extracts were immunoprecipitated with anti-HA antibody, almost all the BDL was recovered in precipitates, indicating that BDL primarily exists as a heterodimer with MP in flower buds (Weijers et al. 2006). This is consistent with our observation that MSG2 binds to ARFs more strongly than it binds to itself.

Two oncoproteins, Fos and Jun, are transcription factors of the basic region leucine zipper (bZIP) type. Since they act as a heterodimer, the thermodynamics of their dimerization has been intensively studied. A dissociation constant of ~50 nM was reported for their peptides that consisted of the basic DNA-binding region and leucine zipper motif (Kohler and Schepartz 2001). Baudendistel et al. (2005) also investigated the interaction between the whole Fos and Jun proteins in HeLa cells with FCCS. If we assume that the maximum and minimum cross ratios correspond to 100 and 0% association, respectively, again, ~60% of the oncoproteins are estimated to form a heterodimer in HeLa cells. The maximum cross-correlation observed in the present study (~80%) is much higher than that (45%) reported by Baudendistel et al. (2005). This seems to be due to a few improvements in our experiments: narrowing the confocal detection volume for the green channel, and brightening fluorescence in the red channel by the use of tR2 instead of mRFP in their work. Expressing plant proteins in human cells may also be important because plant proteins artificially expressed in HeLa cells may interact with each other freely without interference by endogenous ARFs, Aux/IAAs or other interacting plant proteins. However, this could also be a drawback of our FCCS analyses since the protein interaction was observed in a non-native environment.

In this connection, it is interesting to note that the subcellular localization of MSG2 in HeLa cells (Fig. 4)
is totally different from the consistent nuclear localization of Aux/IAAs in plant cells reported previously (Fig. 4a; Abel et al. 1994, Abel and Theologis 1995, Ouellet et al. 2001, Fukaki et al. 2002, Hamann et al. 2002, Weijers et al. 2006). Aux/IAA proteins contain conserved nuclear localization signals (Abel et al. 1994, Abel and Theologis 1995, Ouellet et al. 2001). The results suggest that HeLa cells may lack efficient molecular machineries to recognize the nuclear localization signal of plant-specific Aux/IAAs or to transport them into the nucleus.

In conclusion, we have shown that FCCS is a useful physical technique to determine molecular interaction quantitatively, which complements the use of conventional biological or immunochemical approaches. Using FCCS, we show that MSG2 mostly exists as a heterodimer with NPH4- or MP-CTD in HeLa cell cytoplasm. The strong association between MSG2 and ARFs may make it possible for auxin to control the transcriptional activity of ARFs tightly through changes in the MSG2 level.

Materials and Methods

The nucleotide sequence for MSG2, MP-, or NPH4-CTD was amplified by PCR from cDNA using a pair of oligonucleotide primers: 5′-GTCGACATGGAGAAGGACTCGG-3′ and 5′-GATCGACACCTGGACGAAACCAGCTCGG-3′ for MSG2; 5′-GATCGACATGGAGAAGGACTCGG-3′ and 5′-GATCGACACCTGGACGAAACCAGCTCGG-3′ for MP-CTD; and 5′-GTCGACATGGAGAAGGACTCGG-3′ and 5′-GATCGACACCTGGACGAAACCAGCTCGG-3′ for NPH4-CTD. All the forward and reverse primers contained SalI and SmaI sites respectively, at their 5′ ends. The PCR product was subcloned into pT7-blue (Novagen, San Diego, CA, USA) digested with EcoRV, and its sequence was confirmed by sequencing. After digestion with SalI and SmaI, the inserted DNA was cloned downstream of EGFP in pEGFP-C1 (Clontech, Mountain View, CA, USA) or rT2 in the rT2-replaced pEGFP-C1 (Saito et al. 2004).

For expression in onion (Allium cepa) epidermal cells, the nucleotide sequence for MSG2 was amplified as described above with the exception of the forward primer, 5′-CACCATGGAGAAGGACTCGG-3′. The PCR product was subcloned into pENTR-D-TOPO (Invitrogen, Carlsbad, CA, USA). The GFP gene was amplified by PCR using forward and reverse primers containing SmaI and SphI sites, respectively, at their 5′ ends. The PCR product was inserted between the SmaI and SphI sites at the 3′ end of the MSG2 sequence. The DNA fragment, MSG2::GFP, was inserted downstream of the cauliflower mosaic virus 35S promoter of the gateway binary vector pH35GS (Kubo et al. 2005), using LR clonase (Invitrogen). pBI121-GFP was used for free GFP (Sugikawa et al. 2005). These plasmids were introduced into onion epidermal cells by particle bombardment (PDS-1000/He, Bio-Rad, Hercules, CA, USA) using 1.0 µm gold particles at 1,100 p.s.i. After incubation in MS medium (Murashige and Skoog 1962) for 24 h, the onion cells were examined with a confocal laser scanning microscope (LSM410; Zeiss, Oberkochen, Germany).

HeLa cells were grown as described elsewhere (Saito et al. 2004). Transfection was carried out on LAB-TEK chambered coverslips with eight wells (Nalge Nunc, Rochester, NY, USA), using FuGENE 6 (Roche, Basel, Switzerland). During FCCS measurements, HeLa cells were maintained in Opti-MEM I reduced serum medium (Invitrogen). FCCS measurements were carried out with a ConfoCor2 (Zeiss) (Saito et al. 2004), which consisted of a CW Ar+ laser and a He–Ne laser, a water immersion objective (C-Apochromat, 40 ×, 1.2NA; Zeiss) and two channels of avalanche photodiodes (SPCM-200-PQ; EG&G, Gaithersberg, MD, USA). EGFP and rT2 were excited at 488 and 543 nm, respectively. The confocal pinhole diameter was adjusted to 40 and 78 µm for the 488 and 543 nm laser lines, respectively. The emission signals were split by a dichroic mirror (570 nm beam splitter) and detected at 505–530 nm by the green channel for EGFP and at >610 nm by the red channel for rT2.

Fluorescence data were processed in essentially the same manner as described by Saito et al. (2004). In brief, the fluorescence auto-correlation functions of the red and green channels, Gg(t) and Gr(t), are calculated by

\[ G_g(t) = \frac{I_1(t)I_1(t+\tau)}{\langle I_1(t)\rangle \langle I_1(t) \rangle}, \]

where \( t \) represents the time delay, \( I_1 \) is the fluorescence intensity in the green (\( x = g \)) or red channel (\( x = r \)), and \( \langle \cdot \rangle \) denotes the time average (for 15 s in this study). The cross-correlation function, \( G_c(t) \), is given by

\[ G_c(t) = \frac{I_1(t)I_r(t+\tau)}{\langle I_1(t)\rangle \langle I_r(t) \rangle}. \]

The average number of fluorescent molecules, \( N \), in the confocal detection volume was calculated by resolving the observed \( g(t) \) into a two-component model. Then, the average numbers of red fluorescent molecules (\( N_r \)), green fluorescent molecules (\( N_g \)), and molecules that emit both red and green lights (\( N_{rg} \)) were calculated by

\[ N_r = 1/(G_r(0) - 1), \quad N_g = 1/(G_g(0) - 1), \quad N_{rg} = (G_r(0) - 1)[(G_r(0) - 1)(G_g(0) - 1)]. \]

We usually carried out FCCS measurements in the condition that \( N_r < N_g \), but that their molar ratio was near one. Therefore, the ratio of the associated molecules to the sum of the associated and the monomeric molecules corresponded to \( N_{rg}/N_g \), which was given by the cross ratio, \([G_r(0) - 1]/[G_g(0) - 1]\).

Fluorescence images of live cells were obtained with an inverted confocal laser scanning microscope LSM510 (Zeiss) (Saito et al. 2004), using the same laser lines described above. Emission signals were detected at 505–550 nm for EGFP and at >610 nm for rT2 by sequential scanning.

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