Detection of Fluorescence in Transgenic Arabidopsis Lines Expressing Fluorophores in Different Subcellular Localizations

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- Detection of fluorescent proteins in different subcellular locations with NightSHADE
- Correlation of NightSHADE results with fluorescence detected using a fluorescence microscope or Mithras multimode plate reader

Abstract

The usage of fluorophores such as YFP and GFP is a widely distributed and established method in plant research. To monitor fluorescent reporter proteins several techniques based on instruments such as fluorescent microscopes or imaging systems are available. Detection of free or fused fluorescent reporters in different subcellular localizations and with different expression levels can be performed with the NightSHADE LB985 imaging system. The obtained results correlated with data received by detection of fluorescent using the Mithras LB940 multimode plate reader.

Introduction

In plant research fluorescent reporters are widely used to analyze protein localizations, protein trafficking, expression patterns and protein interactions. They function for example as transcriptional reporters under the control of a promoter of interest leading to free fluorophores within the cell, or as translational fusions with the protein of interest. Detection and monitoring of fluorescent reporters are commonly done using wide field or CLS microscopy. Alternatively, fluorescence can be measured using a plate reader or an imaging system with a high performance CCD camera. All these methods offer high sensitivity detection systems, although only the microscope offers a high magnification. The greatest advantage of an imaging system over microscopy is the possibility to monitor reporter gene expression *in planta* in a time and spatial resolution.

To test the performance of our NightSHADE imaging system for detecting fluorescence, a variety of *Arabidopsis* lines expressing different fluorophores at different expression levels, localized to different parts of the cell were used. To compare the NightSHADE to other methods of fluorescence measurements, fluorescence was also detected using a wide field fluorescence microscope and the Mithras LB940 multimode plate reader

Experimental Procedures

NightSHADE Measurements

Plants were grown on MS or Phytoagar plates for 5 days. Fluorescence was measured in seedlings on a glass slide.

Mithras Measurements

Seedlings were transferred into a black 96 well plate containing 100µl H_2O. Seedlings were kept in same order as measured with the NightSHADE. Background values for 100µl H_2O varied between 1870 - 2290 counts/s.

Fluorescence Wide field Microscopy

Microscopy was performed using a Nikon Eclipse 90i Photometrics CoolSNAP ES microscope with FITC filter settings. Images of seedlings were taken at a 100x, 200x or 400x magnification with 500 – 2000 ms detection time. Images were analyzed using MetaMorph Software.

Fluorescence Stereo Microscopy

Stereo microscopy was performed using a Leica MZFLIII microscope with GFP filter settings.

Material

- Mithras LB 940 multimode reader with injectors (Berthold Technologies)
- NightSHADE LB 985 Plant imaging system with IkFlu CCD camera (Berthold Technologies)
- Filter: Ex (15mm) 485nm; Em (25mm) 535nm, Ex (25mm) 475nm; Em (50mm) 520nm
- MikroWin 2000 software, indiGO software

Description of Plant Lines

Line	Fluorophore	Promoter	Subcellular localization	Comments	Reference
YC3.6 - CYT	YFP (cpVenus)/ CFP (ECFP) +NES	UBQ10	Cytoplasm	FRET line to measure Ca^{2+} fluxes	Nagai et al. (2004); Krebs et al. (2011), Plant Biology in Press
YC3.6 - PM	YFP (cpVenus)/ CFP (ECFP)	UBQ10	Plasma-membrane	FRET line to measure Ca^{2+} fluxes	Nagai et al. (2004); Krebs et al. (2011), Plant Biology in Press
YC3.6 - NUC	YFP (cpVenus)/ CFP (ECFP) +NLS	UBQ10	Nucleus	FRET line to measure Ca^{2+} fluxes	Nagai et al. (2004); Krebs et al. (2011), Plant Biology in Press
roGFP - CYT	redox-sensitive GFP		Cytoplasm		Schwarzländer et al. (2008)
roGF -Plastid	redox-sensitive GFP		Chloroplast		Schwarzländer et al. (2008)
roGFP - MIT	redox-sensitive GFP		Mitochondria		Jiang et al. (2006)
HyPer – PER	cpYFP		Peroxisomes	circular permutated YFP inserted into prokaryotic H_2O_2 sensing OxyR protein	Costa et al. (2010)
HyPer - CYT	cpYFP		Cytoplasm	circular permutated YFP inserted into prokaryotic H_2O_2 sensing OxyR protein	Costa et al. (2010)
phyB-GFP	mGFP4	35S	Nucleus		Kircher et al. (2002)
Control					

Instrument Settings

NightSHADE

Fluorescence measurements were done with 5s exposure time using the GFP filter set 475/520nm. Fluorescence pictures were overlaid with a photo. Data were analysed using indiGO software. To relatively quantify the fluorescence the scale was set to 100-2000 and an automated peak search was performed based on noise detection. This peak search defined the cps and the area of fluorescence emitted by single seedlings. The results of a manual area search, defining parts of the seedlings not emitting fluorescence (e.g. leaves without fluorescence), were added to these values.

Mithras

Fluorescence measurement was done in scanning mode with a 3x3 pattern and a 0.1s counting time. Lamp energy was set to 25.000 and GFP filters 485nm and 535nm were used. Analysis of data was performed using MikroWin2000. For each sample all 9 data points were added together and background was subtracted. Data were correlated to cps measured with NightSHADE for the same single seedling.

Results

Detection of Fluorescence with NightSHADE compared to Fluorescence Microscope

To determine the potential of fluorescence detection of our NightSHADE imaging system 9 plant lines expressing GFP respectively YFP in different cell compartments were examined. To compare the NightSHADE performance to other common detection methods a fluorescence binocular and a wide field fluorescence microscope were used. In no line fluorescence could be detected using a binocular (data not shown), whereas the microscope gave moderate to strong and easy to detect signals as expected (Figure 1). The length of detection time (between 100 and 2000ms) enables a semi-quantitative analysis of the expression strength. YC3.6-NUC showed the strongest signal, followed by YC3.6-PM and YC3.6-CYT whereas roGFP-MI, roGFP-CYT and phy-B GFP emit weak signals. Furthermore roGFP-CYT seems to be a heterozygous line, not all seedlings showed fluorescence under the microscope. NightSHADE measurements were performed with seedlings on glass slides (Figure 2). In general results obtained by NightSHADE correlate very nicely with the results from microscopy. Line YC3.6-NUC and YC3.6-PM show easy detectable signals, whereas YC3.6-CYT, roGFP-PM and HypPER-PER show weaker fluorescence. Weak emitting lines defined by microscopy, roGFP-MI, roGFP-CYT and phy-B GFP, are below NightSHADE detection limit. Sometimes auto fluorescence in roots can be detected, although leaves do not show any fluorescence (Figure 2, control lower row), leading to false positive results. Quantification enables to distinguish between real positive and false positive results (see part 2). Taken together our experiments showed that detection of fluorescence with the NightSHADE is not dependent on localization of the fluorescence protein. Rather the limiting factor is the expression level. All lines with moderate to strong expression defined by microscopy can be detected with the NightSHADE.

Quantitative Analysis of Expression Level using indiGO Software

To relatively quantify measurement data and make them more comparable an automated peak search using indiGO software was performed. No peaks were detected in control 1, showing no autofluorescence, whereas peaks were defined in the second control

showing autofluorescence in roots. To be able to compare cps values of different seedlings, the cps/mm^2 were calculated. Furthermore, for seedlings not showing any fluorescence in leaves the emitted light of a manual defined area, using the manual area function of indiGO, was performed by surrounding the white part of the seedlings and adding the cps/mm^2 to the result of the automated peak search. The results clearly showed, that in most cases seedlings, which were defined as positive by just looking at the false colour picture (Figure 2), also had cps/mm^2 values above the ones determined for seedlings not

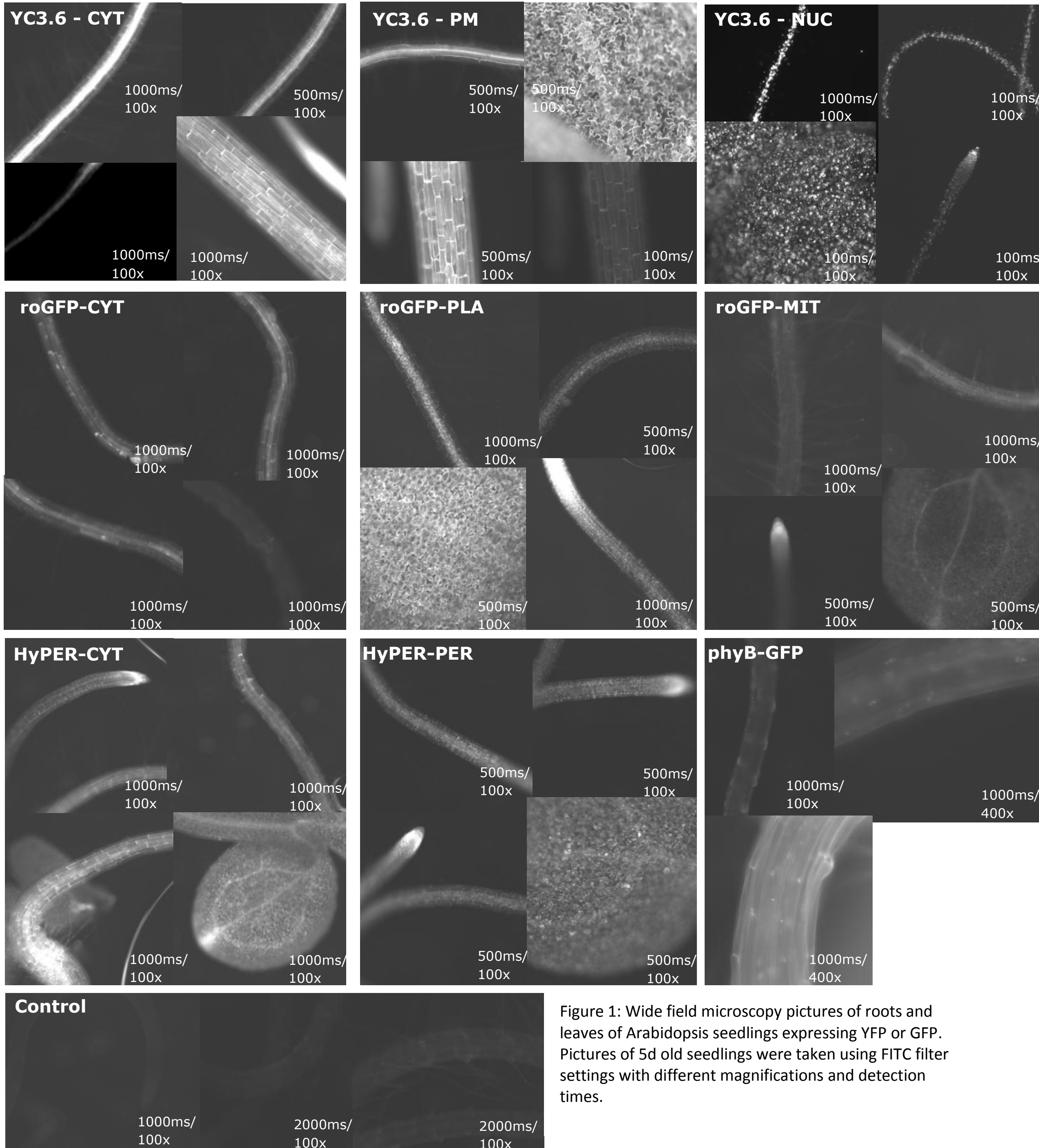

Figure 1: Wide field microscopy pictures of roots and leaves of Arabidopsis seedlings expressing YFP or GFP. Pictures of 5d old seedlings were taken using FITC filter settings with different magnifications and detection times.

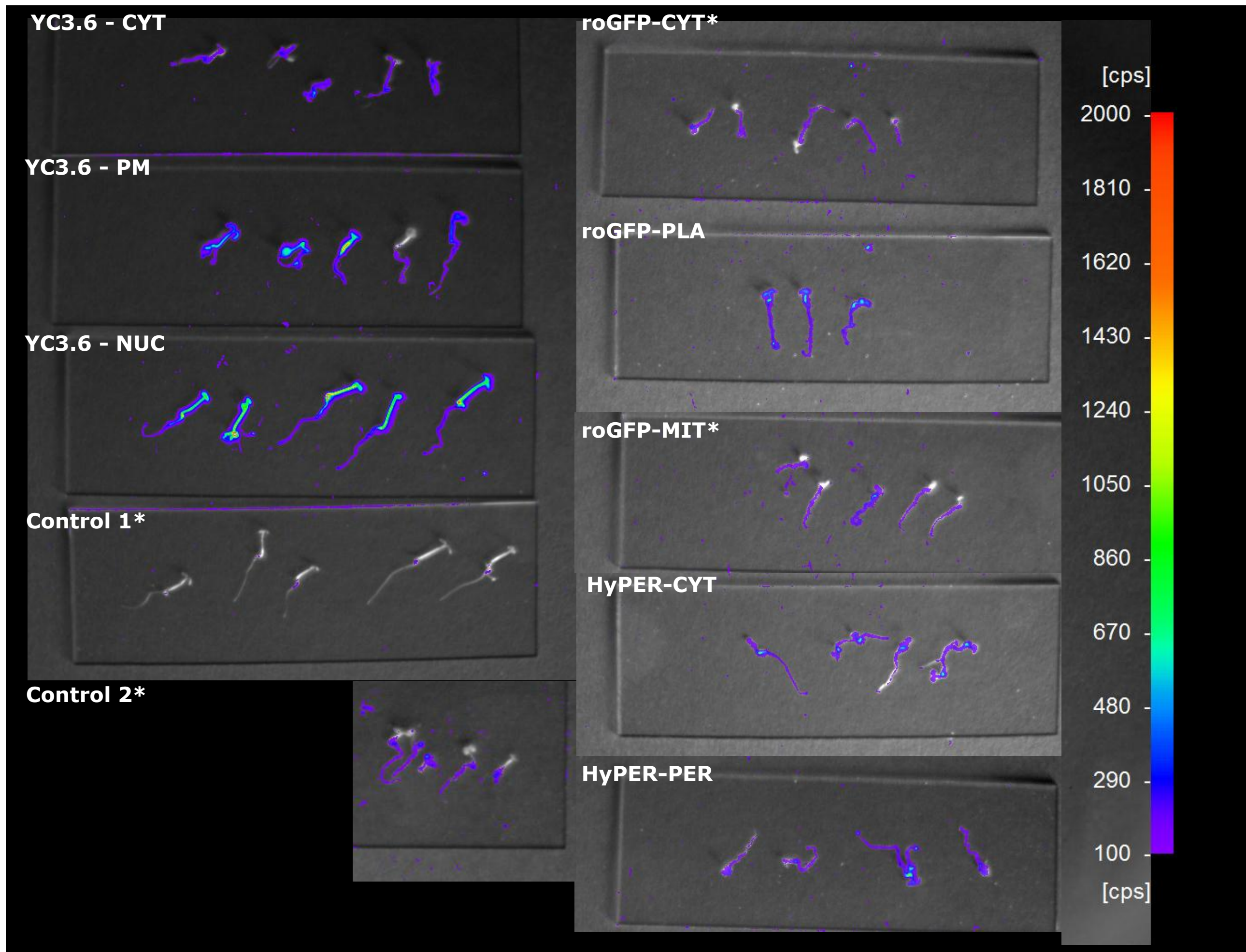

Figure 2: Fluorescent Measurement with NightSHADE. Transgenic Arabidopsis lines expression GFP respectively YFP with different localizations were tested for their emission of fluorescence using the NightSHADE LB985 imaging system. * mark lines with no clear fluorescence detectable with the NighSHADE system.

expressing fluorophores (Table 1 + 2). Wildtype seedlings emission was between 210 and 410 cps/mm^2 (Table 1). All seedlings showing values above 450 were considered to be positive ones.

Name	Seedling No	manual area search		
		overall cps	area	cps/mm^2
Control 1	1	6278	28.43	220.8
	2	3314	13.88	238.8
	3	4307	18.6	231.6
	4	6286	27.56	228.1
	5	5839	27.13	215.2
Control 2	1	7919	23.22	341.0
	2	2922	7.13	409.8
	4	5870	14.5	404.8
	5	4431	11.27	393.2

Table 1: Background emission of *Arabidopsis* wildtype plants

3. Correlation of Mithras and NightSHADE results

Fluorescence in the same seedlings as used in the NightSHADE experiment was also measured with the multimode reader Mithras LB 940. To ensure that the whole seedling was taken into account the measurement was performed using the scanning mode. The medium value for buffer without seedlings was subtracted as background noise. Wildtype plants had cps values ranging between -53 (after background subtraction) and 1150. All seedlings showing a cps value >1200 are considered to be positive for fluorescence. The results reflect very nicely the ones determined by imaging e.g. emission of seedling #4 of line YC3.6-PM is below the threshold. Furthermore in roGFP-MIT only seedling #3

has a cps value above background (Table 3). To correlate the Mithras measurements with NightSHADE

Name	Seedling #	detectable with NightSHADE	Cps/mm^2
YC3.6 - CYT	1*	yes	302
	2	yes	491
	3	yes	467
	4	yes	505
YC3.6 - PM	1	yes	731
	2	yes	808
	3	yes	566
	4	no	292
YC3.6 - NUC	1	yes	469
	2	yes	505
	3	yes	534
	4	yes	505
roGFP - CYT	1	not definable	417
	2	no	408
	3	no	359
	4	not definable	338
roGFP -PLA	1	yes	453
	2	yes	454
	3	yes	465
roGFP - MIT	1	no	413
	2	no	351
	3*	yes	423
	4	no	345

Table 2: Quantification of light emission of different *Arabidopsis* lines expressing fluorophores. Most values correlate nicely with results seen in figure 2.(* marks lines were figure 2 indicates a different result than cps values)

the cps values defined by the Mithras were compared to the overall cps determined by imaging. Because the plate reader values do reflect the whole emission of one seedling it is important to use the overall cps for the NightSHADE measurements to make both experiments comparable. The results were plotted on a scatter plot (Figure 3). These plots show a nice correlation between these two different ways to detect fluorescence emission.

Conclusions

The NightSHADE imaging system and the Mithras multimode reader both offer highly sensitive systems feasible for detecting and quantifying fluorescence in plants. Fluorophores localized to cytoplasm, nucleus, plasma membrane, mitochondria, peroxides and chloroplasts could be detected showing that detection is not dependent on localization. Rather than that the detection limit is defined first by expression strength. The low expression of phyB-GFP prevented its detection using the NightSHADE or the Mithras system (data not shown), although fluorescence could be seen with a wide field microscope. Second, if seedlings are too small quantification of fluorescence is difficult, due to low overall cps values for a single seedling. In general one can say, if a line shows moderate to strong fluorescence in wide field microscopy, fluorescence can be detected and quantified either using the NightSHADE imaging system or the Mithras plate reader. Results of both systems strongly correlate as can be seen by plotting them, making the quantification of cps highly trustable. Taken together imaging systems and plate readers both offer alternatives for microscopy to monitor fluorescence in transgenic plants. Although resolution is not as high compared to the microscope there are other advantages. Both systems allow a much higher throughput than microscopy. Furthermore imaging enables researchers to monitor fluorescence over the time without disturbing plants and keeping environmental conditions stable.

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Name	Seedling #	detectable with NightSHADE	Mithras [cps]	NightSHADE overall cps
Control no autofluorescence	1	no	1115	6278
	2	no	615	3314
	3	no	-53	4307
	4	no	-2016	6286
Control autofluorescence	1	no	958	7919
	2	no	375	2922
	4	no	423	4555
	5	no	1102	4431
YC3.6 - CYT	1	yes	4855	3329
	2	yes	10703	3311
	3	yes	4569	6187
	4	yes	13251	2663
YC3.6 - PM	1	yes	21124	23029
	2	yes	18752	30826
	3	yes	13607	14448
	4	no	318	6792
YC3.6 - NUC	1	yes	18778	16514
	2	yes	48946	34689
	3	yes	18336	23488
	4	yes	23768	20604
roGFP - CYT	1	n.d.	185	3357
	2	no	459	2654
	3	no	685	3754
	4	n.d	936	3210
roGF - PLA	1	yes	3235	9969
	2	yes	2639	8425
	3	yes	2222	8244
roGFP - MIT	1	no	173	5110
	2	no	555	4743
	3	yes	1448	8283
	4	no	244	3211

Table 3: Detection of fluorescence in different Arabidopsis lines expressing fluorophores using the Mithras LB940 plate reader. Values correlate nicely with results seen in figure 2

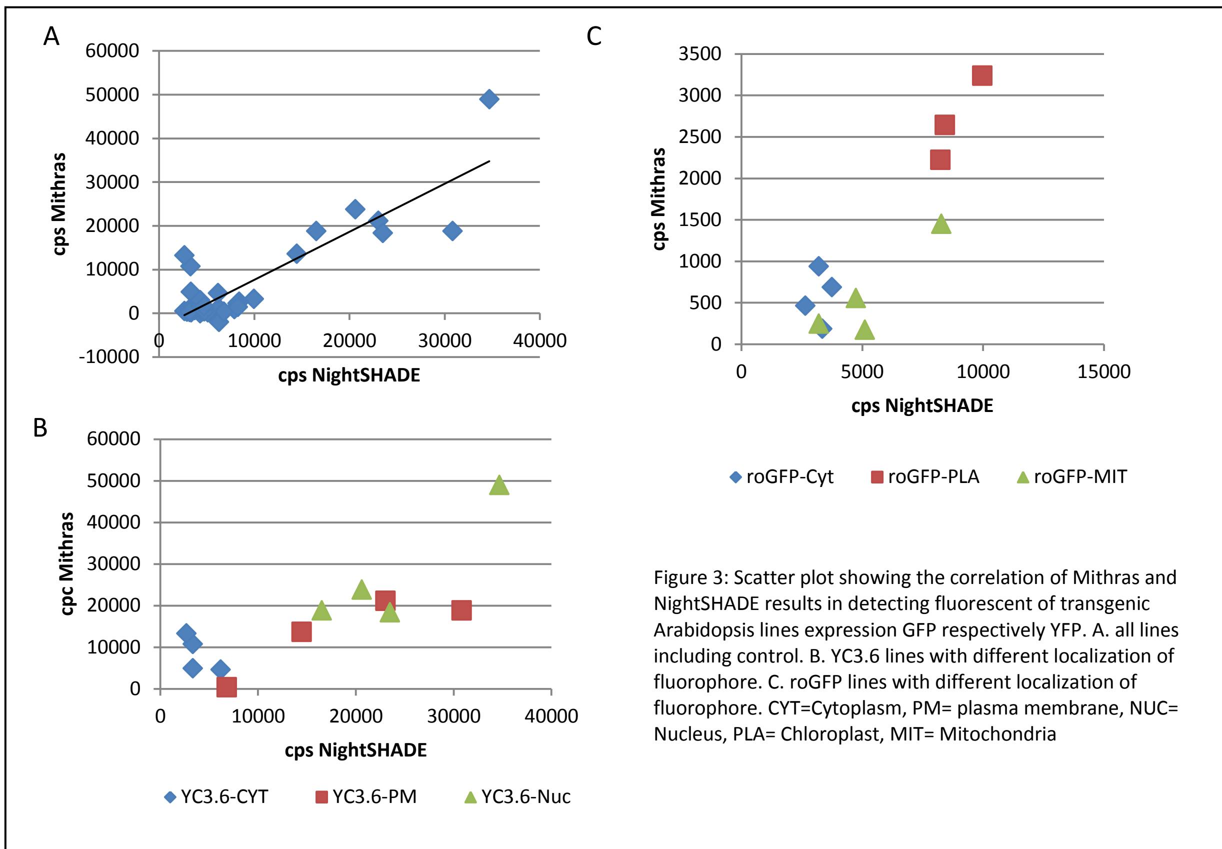

Figure 3: Scatter plot showing the correlation of Mithras and NightSHADE results in detecting fluorescent of transgenic Arabidopsis lines expression GFP respectively YFP. A. all lines including control. B. YC3.6 lines with different localization of fluorophore. C. roGFP lines with different localization of fluorophore. CYT=Cytoplasm, PM= plasma membrane, NUC= Nucleus, PLA= Chloroplast, MIT= Mitochondria