OpenIntro Student Project Competition
January 10, 2012

Statistical Identification of Potential CLAVATA2 Interactors by Fluorescence Resonance Energy Transfer Analysis

Sebastian Raschka

Introduction

The overall goal of this study was to identify potential interactors of the CLAVATA2 (CLV2) membrane receptor, which is participating in the stem cell signaling pathway of the model plant *Arabidopsis thaliana.* In order to investigate the physical interaction between those proteins, a fluorescence resonance energy transfer (FRET) analysis was conducted. Data have been collected all by myself during my undergraduate laboratory experiences in August, 2011, at the Department of Developmental Genetics at Heinrich-Heine University Düsseldorf.

The choice of the candidate proteins was based on a systematic screening of the *Arabidopsis thaliana* protein interactome in *Saccharomyces cerevisiae*, which is currently performed by a research group at the *Carnegie Institution for Science* in Stanford, USA [1]. CLAVATA2 (CLV2) is a membrane receptor, which is part of the stem cell regulating CLAVATA pathway in *Arabidopsis* plants [2]. Among others, this plant interactome screening in yeast revealed two potential interactors of CLV2: The stearoyl-ACP desaturase FAB2 [3], and a protein coded by the locus AT5G22540, which has not been described in literature, so far, and therefore will be denoted as UNK1 in this study. However, the Aramemnon *Plant Membrane Protein database* predicts two membrane proteins for UNK1 [4].

Since CLV2 is participating in a stem cell regulating pathway in the shoot apical meristem, and is not associated with other signaling pathways as far as is known, it would be reasonably expected that the expression domain of *CLV2* is restricted to this tissue solely.

But rather interestingly, the expression of *CLV2* has also been observed in many other plant tissues [5]. Hence, if the statistical analysis of FRET data in this study provides significant evidence for physical protein-protein interaction between CLV2 and one of the potential candidates in planta, this could provide a valuable cornerstone for further studies of CLV2 function, leading to a better understanding of the complex stem cell regulation system in plants.

Methods

Biological preparation

In order to investigate the potential protein interaction *in planta*, experiments using fluorescence resonance energy transfer (FRET) were performed as it had been described in a previous study, which successfully revealed the interaction between CLV2 and another membrane protein CORYNE [6].

For the FRET experiments fluorophors with overlapping emission and excitation spectra were fused to the C-termini of each protein. GFP was used as donor molecule, and mCherry (mC) as acceptor molecule, respectively.

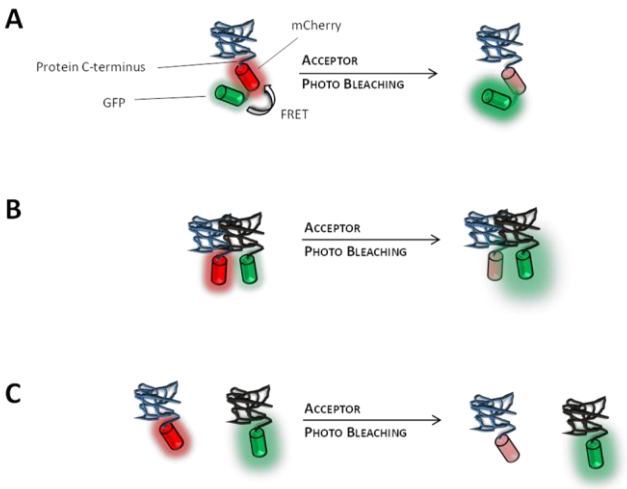


Fig.1. Theoretical scheme of the measurement process between two fusion proteins.
Subframe A shows the positive control where both fluorophors – GFP and mC – are fused to the C-terminus of a protein, in such way that they are in close proximity (< 10 nm) to each other so that FRET can occur. When the mC acceptor molecule is destroyed during photo bleaching the GFP emitted light cannot be quenched anymore, which can be measured by an increased GFP signal strength. In B two proteins are shown, which are physically interacting with each other, so that their conjugated fluorophors are close enough to each other that FRET can occur. Photo bleaching will result in a a quantifiable increase of GFP signal as described in A. Panel C shows two proteins which are not physically interacting with each other, thus FRET does not occur, and the photo bleaching of the acceptor does not lead to an increase of GFP signal.

Pictures and measurements were taken with a Zeiss 510 Meta Confocal Microscope using a 488 nm Argon-Laser for the excitation of the GFP donor fluorophors, and a 561 nm diode for exciting and bleaching the mC acceptor fluorophors, respectively.

The FRET measurements via acceptor photo bleaching were done in leaf epidermis cells of *Nicotiana benthamiana* plants.

GFP-mC-fusion proteins of UNK1 and FAB2 were used as positive controls to quantify the intra-molecular energy transfer between GFP and mC in close proximity (Fig.1). As negative controls GFP-fusion proteins were bleached in absence of mC-acceptor molecules, in order to quantify the GFP signal fluctuation within the bleaching step, which can be regarded as "FRET-background".

The FRET efficiency in percent was calculated using this formula:

$$\frac{X_2 - X_1}{X_2} \times 100$$

Where

X_1: GFP intensity before acceptor photo bleaching
X_2: GFP intensity after acceptor photo bleaching

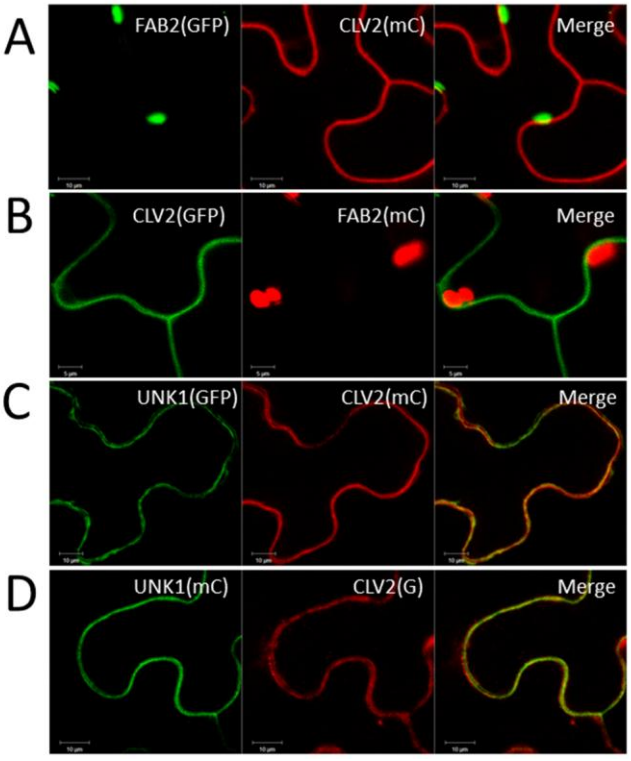


Fig. 2. Localization studies of UNK1 and FAB2 fusion proteins via fluorescence microscopy in leaf epidermis cells of *Nicotiana benthamiana*. The left panel shows the filtered signal from the GFP donor molecule, and the middle panel shows the filtered signal from the mC acceptor molecule, respectively. The right panel (Merge) shows an overlay of both signals.

Statistical Analysis

For the collected data, statistical analyses were done using the Minitab 16 statistical software. The analyses involve graphical investigation including histograms, boxplots, normal probability plots, and numerical testing such as two-sample *t* test.

Results

During localization studies of the fusion proteins in *Nicotiana benthamiana* leaf epidermis cells FAB2 showed localization in chloroplast only (Fig 2A and 2B). Because of this physical separation between CLV2 and FAB2 fusion proteins FRET measurements between these proteins where not performed.

But instead, FRET experiments between FAB2 fusion proteins were done. It was reported in previous studies that FAB2 forms homodimers in chloroplasts [7] [8] [9]. Thus, FAB2 homodimeric complexes should serve as a control for the feasibility of the experimental procedures of this study.

In contrast to CLV2, UNK1 also does not seem to be located exclusively at the plasmamembrane, indicated by its fuzzy fluorescence signal (Fig 2C and 2D). However, in contempt of this non-obvious membrane localization a FRET measurement between UNK1 and CLV2 was performed.

Normality tests

In order to check whether the data is normally distributed, histograms and normal probability plots were made for all measured FRET data (Fig. 3 and 4).

The histograms of FAB2 PK and FAB2 NK are unimodal, and concluding from their shape it seems as if the data could be presumably normally distributed (Fig. 3A and 3B). However, the histogram of FAB2 PK FRET efficiency data shows slightly skewed to the right, and FAB2 NK data has some aberration to the left. From the normal probability plots it is clearly evident that FAB2 NK has an outlier to the very left and FAB2 PK has two outliers to the very right, which are supposed to be responsible for the skewness (based on the 1.5 × IQR rule). These data points could be due to measurement errors, and therefore were excluded from further analyses.

In the case of the FRET efficiency data measured between FAB2(G) and FAB2(mC) the histogram is unimodal, but shows a small second peak to its right side (Fig 3C). As it can be seen from the normal probability plot outliers to the right might explain this observation. But basically, as it can be seen in the normal probability plots, the data points are most widely on a linear line, indicating that the data is close to normal. Thus, with a combined sample size of 58 measurements, two-sample *t* procedures are supposed to be robust enough for hypothesis testing in this particular case.

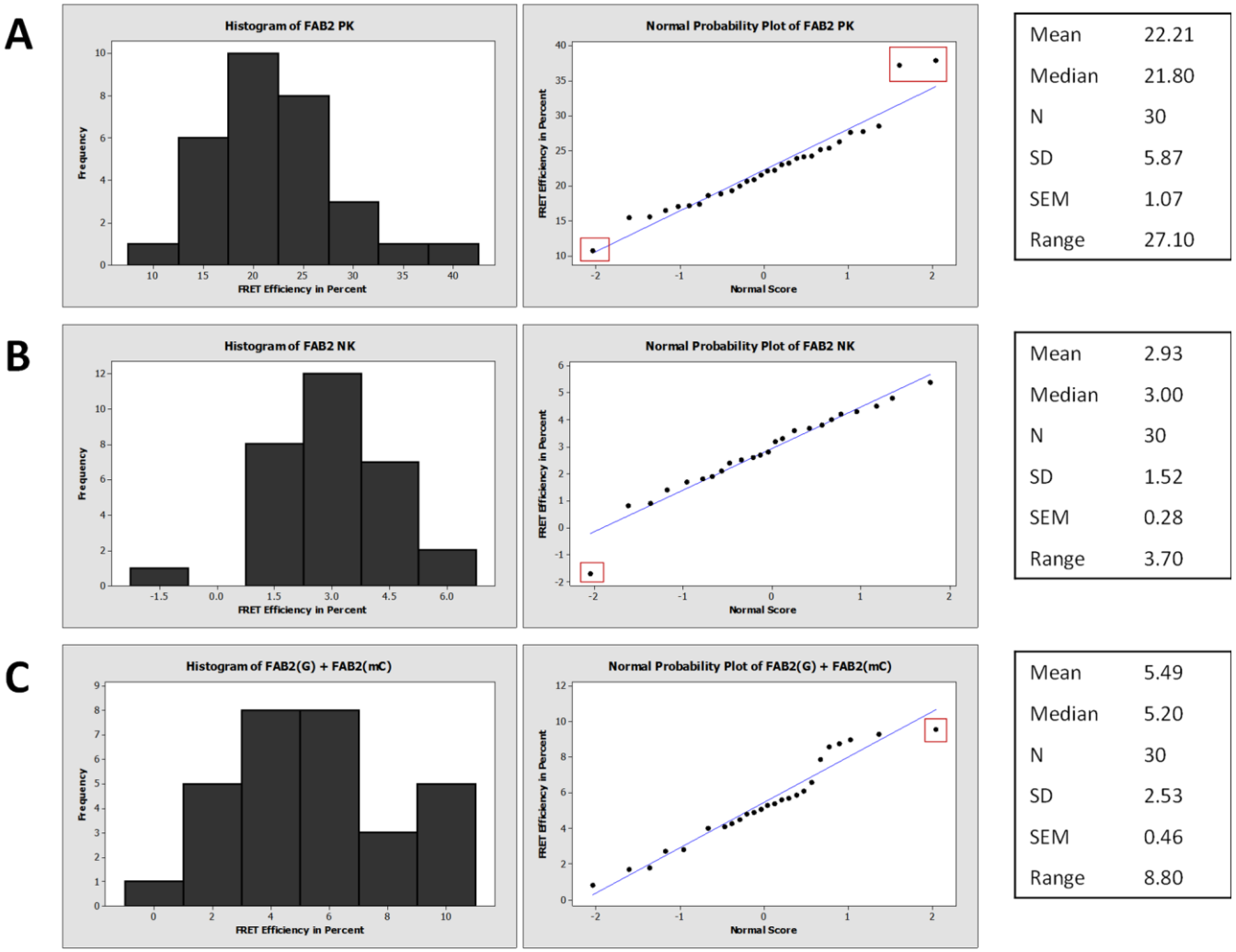


Fig. 3. Histograms, normal probability plots, and descriptive statistics of FAB2 PK (3A), FAB2 NK (B), and FAB2(G)+FAB2(mC) (C). Red boxes in the Normal quantile plots encircle obvious outliers, which were removed for further analyses.

All histograms of UNK1 data are virtually unimodal, though the histograms of UNK1 PK and UNK1(G)+CLV2(mC) are skewed to the left.

As it can be seen from the normal probability plot the skewnesses have their origin in outliers in x- and y-position. Further, the normal probability plots also show that the data are normal to a large extend, and here, too, the combined sample sizes (N > 52) are sufficient for the usage of *t* testing procedures on these data. To visualize the spread of the data, boxplot diagrams were drawn after the outliers described above were removed (Fig. 5).

The boxplot of UNK1(G)+CLV2(mC) is in stark contrast to the other boxplots, salient through showing a large variability of data. Both sample data, UNK1(G)+CLV2(mC) and FAB2(G)+FAB2(mC), are also noticeably skewed. Obviously FAB2 data are less spread than UNK1 data.

Further, it should also be mentioned that no suspected outlier can be detected in any boxplot diagram, which falls more than 1.5 times the interquartile range below the first, or above the third quartile.

Hypotheses

The question of this study was to figure out if two membrane proteins are physically interacting with each other. A significant FRET signal (i.e. a significant increase of the donor fluorescence after acceptor photo bleaching) between the tested proteins would imply that these proteins are in close proximity to each other and therefore might be physical interactors.

To that effect one-sided two-sample *t* significance tests were conducted to test the null hypothesis (H_0) that “the percentage FRET efficiency of the potential interactors does not differ from the negative control” against an alternative hypothesis (H_a) that “the percentage FRET efficiency of the potential interactors is greater than the negative control”. Hypothesis testing was done at a significance level of $\alpha = 0.05$.

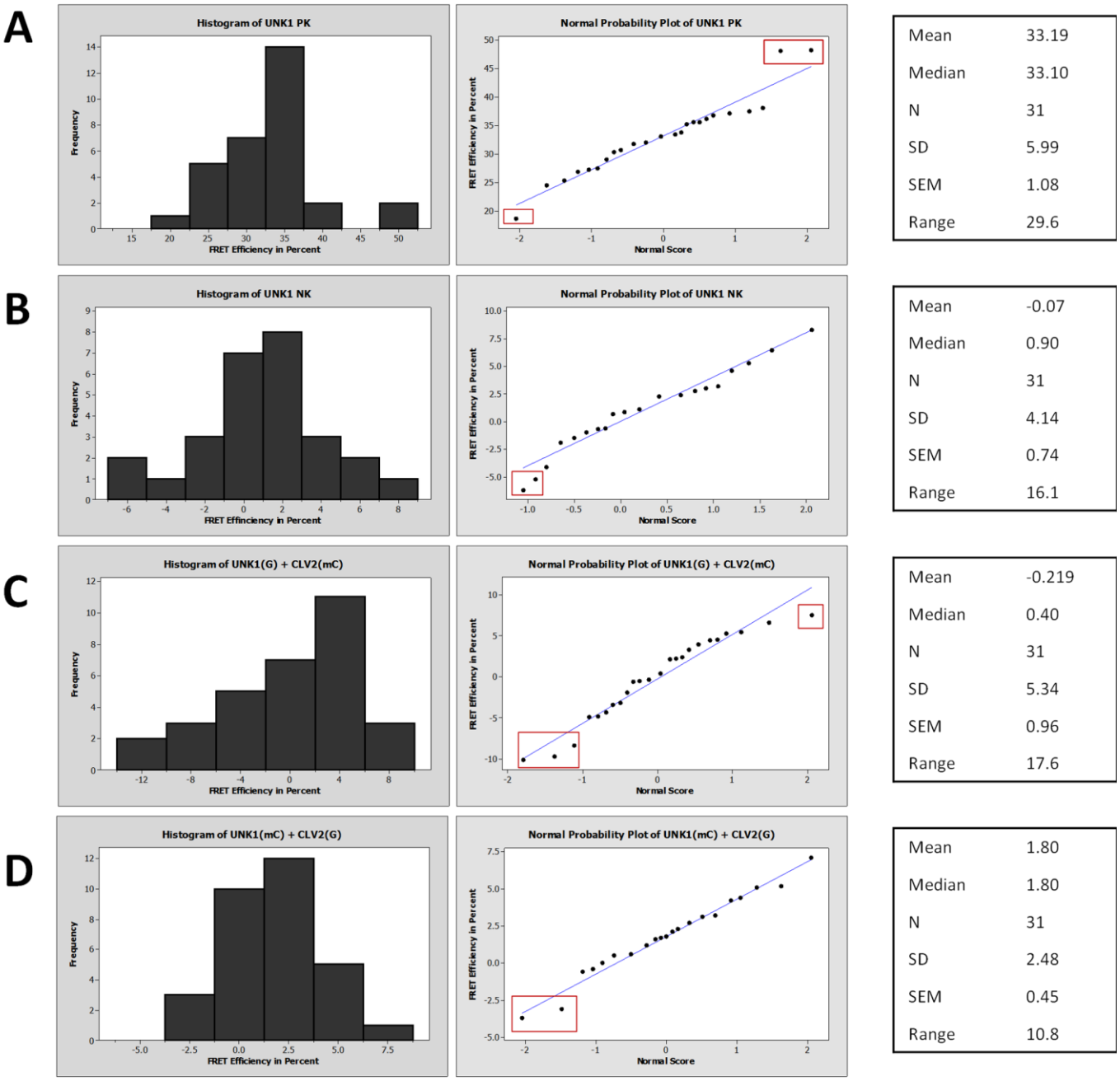


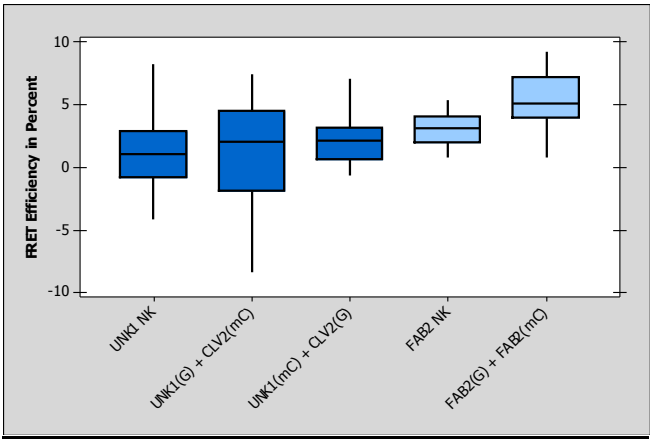
Fig. 4. Histograms, normal probability plots plots, and descriptive statistics of UNK1 PK (A)UNK1 NK (B), UNK1(G)+CLV2(mC) (C), and UNK1(mC)+CLV2(G) (D). Red boxes in the Normal quantile plots encircle obvious outliers, which were removed for further analyses.

One-sided two-sample t significance tests

The *p*-value of the one sided two-sample *t* test between FAB2(G)+FAB2(mC) and FAB2 NK was smaller than 0.001, which is clearly below the significance level of 0.05, and therefore provides strong evidence for rejecting the null hypothesis in favor of the alternative hypothesis (Tab.1). However, the *p*-values of *t* significance tests of UNK 1 data (0.631 and 0.107) are larger than 0.05 and thus the null hypothesis cannot be rejected in this case.

Power of the two-sample t test

In order to quantify the probability of making a false-negative error, i.e. failing to reject the null hypothesis if it is false, a power test was done for the FAB2 one-sided two-sample *t* significance test. The power to detect a difference of 2.259 between FAB2(G)+FAB2(mC) and FAB2 NK, as it has been observed in this study, was 93% (N = 25, assumed equal standard deviations = 2.5). The power curves (Fig. 6) also show that a sample size of 20 could also be sufficient to detect a similar difference between the



Data	N	Min.	Q1	Med.	Q3	Max.
UNK1 NK	25	-4.10	-0.85	1.10	2.90	8.30
UNK1(G) + CLV2(mC)	27	-8.40	-1.90	2.10	4.50	7.50
UNK1(mC)+ CLV2(G)	28	-0.60	0.60	2.20	3.20	7.10
FAB2 NK	29	0.80	2.00	3.20	4.10	5.40
FAB2(G) + FAB2(mC)	29	0.80	4.00	5.10	7.30	9.30

Fig. 5. Boxplot diagrams of FAB2 and UNK1 data. Outliers mentioned in the text and described in Fig. 3 and 4 were excluded from this diagram. N = sample size, Min. = minimum, Q1 = first quartile, Med. = median, Q3 = third quartile, Max. = maximum.

means with a power greater than 80% (for assumed standard deviations of 2.5). This could be taken into account for following experiments toward saving time and resources. As it can be derived from the diagram a sample size of at least 40 would be required in order to get a power close to 100% for the two-sample significance test under these conditions.

Discussion

FAB2 forms homodimers in chloroplasts

In case of the FAB2 FRET analysis the null hypothesis could be successfully rejected ($p < 0.001$), which confirms the assumption of previous studies that FAB2 forms homodimers in the chloroplast [7] [8] [9]. Although a FRET analysis between FAB2 and CLV2 was not conducted by reason of the separation of the proteins in different subcellular compartiments, a significant FRET signal between FAB2 proteins might prove this technique to be feasible for investigating protein-protein interactions in plant cells.

No interaction between UNK1 and CLV2

The FRET analysis of UNK1 did not provide sufficient evidence to reject the null hypothesis at the specified significance level ($\alpha = 0.05$). In favor of the alternative hypothesis it can be concluded that UNK1 does not interact with CLV2 at the plasma membrane. This outcome is not unexpected due to the fact that UNK1 did not show unequivocal co-localization with CLV2 at the plasma membrane.

Reasons why UNK1 did not localize at the plasmamembrane, despite the prediction of two transmembrane domains, might be that the fusion-proteins were not folded properly or additional factors like additional interactors were missing. In addition, the over-expression in this experiment may have resulted in an incorrect targeting of UNK1 fusion-proteins. These assumptions could be tested by rescue experiments with UNK1-mutant *Arabidopsis* lines in further experiments.

Tab. 1. One-sided *t* significance tests. St.Dev = standard deviation, SEM = standard error of the mean, H_0 = null hypothesis, H_A = alternative hypothesis, DF = degrees of freedom.

Data	Mean	Difference	St.Dev	SEM	H_0	H_A	p-Value	DF
FAB2(G)+FAB2(mC) VS FAB2 NK	5.35 3.09	2.259	2.45 1.27	0.45 0.24	equal	greater than	< 0.001	41
UNK1(G)+CLV2 (mC) VS UNK1 NK	1.17 1.50	-0.329	4.17 2.83	0.80 0.57	equal	greater than	0.631	46
UNK1(mC)+CLV2(G) VS UNK1 NK	2.34 1.50	0.847	1.91 2.83	0.36 0.57	equal	greater than	0.107	41

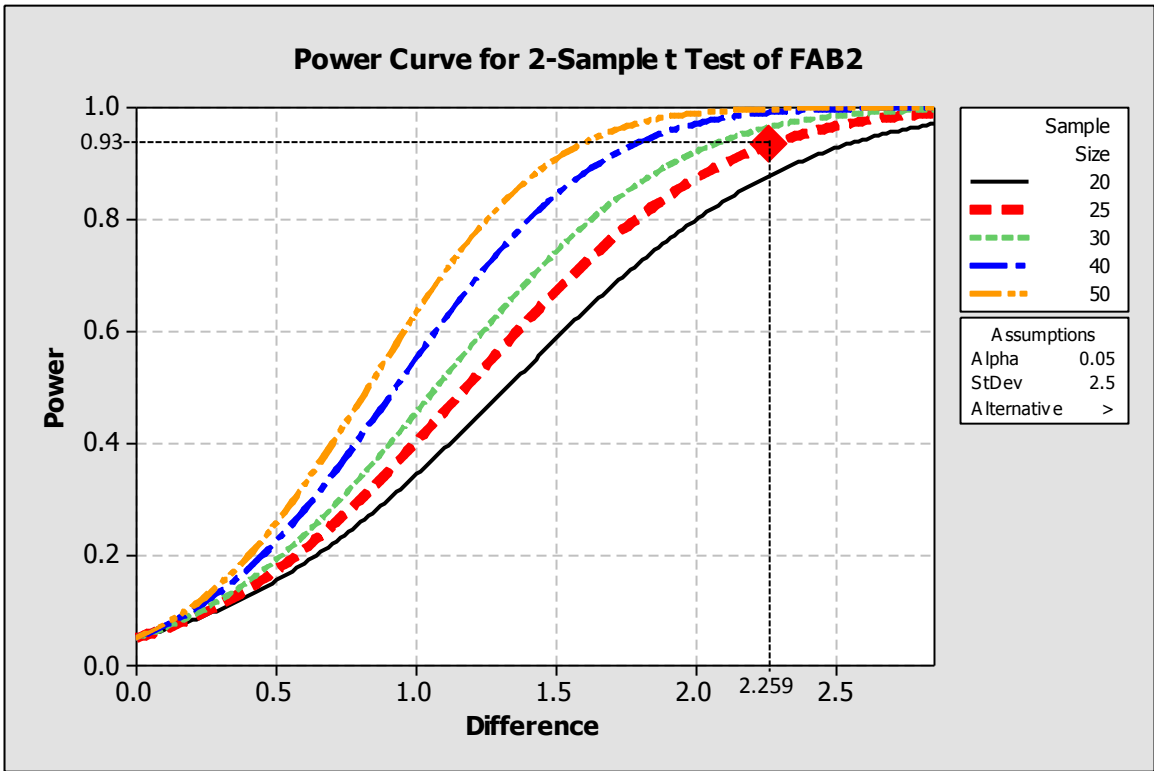


Fig. 6. Power curves for the two-sample *t* tests of FAB2 data shown for different sample sizes. For the calculation of these power curves equal standard deviations of FAB2 NK and FAB2(G)+FAB2(mC) were assumed at a significance level of α = 0.05. The red diamond denotes the power (93%) to detect the difference (2.259) between the means of tested FAB2 data in this study. Alpha = significance level alpha, StDev = standard deviaton, Alternative = alternative hypothesis.

Yeast as heterologous system

The FRET analysis *in planta* produced opposing results to the protein-protein interaction studies of plant proteins in *Saccharomyces cerevisiae*. The heterologous yeast system may be suitable for high-throughput screenings of the plant interactome, but on the other hand yeasts have different subcellular cell structures in comparison to plants. Because yeasts are lacking chloroplasts the observed interaction between CLV2 and FAB2 might have been due to random co-localization of these proteins in yeast cells, leading to a false positive error.

Because only little is known about UNK1 the disagreement between yeast studies and the FRET analysis in plant cells is less obvious. Assuming that no false-negative error in not-rejecting the null hypothesis was made, a possible explanation for the observed interaction between UNK1 and CLV2 in yeast might also be a false-positive error.

Outlook

Since all FRET data stem from one single experiment, at least one second experiment should be done to validate the consistency of the data.

More sophisticated tests beyond an introductory statistics level can be conducted to test the population standard deviations for equality, which could justify the utilization of the more powerful pooled two-sample *t* procedures.

Further experiments using compartiment-specific subcellular markers could provide unambiguous clues for the subcellular localization of UNK1 fusion-proteins. Fusion proteins with fluorophors on the N-termini could be constructed as alternative testing objects.

It is also important to mention that these FRET experiments in *Nicotiana benthamiana* are not to be regarded as ultimate results, but costlier experiments under the endogenous promotor of *Arabidopsis plants* would provide more certainty about the outcomes. Once, as the required fusion proteins are constructed, also alternative approaches like the pixel-specific fluorescence lifetime imaging microscopy (FLIM) [10] could be conducted, if devices are available.

Cited Literature

1. Lalonde, S. *et al.* A Membrane Protein/Signaling Protein Interaction Network for Arabidopsis Version AMPv2. *Front Physiol* 1, (2010).
2. Kayes, J.M. & Clark, S.E. CLAVATA2, a regulator of meristem and organ development in Arabidopsis. Development 125, 3843-3851 (1998).

3. Lightner, J., Wu, J. & Browse, J. A Mutant of Arabidopsis with Increased Levels of Stearic Acid. Plant Physiology 106, 1443 -1451 (1994).

4. Schwacke, R. et al. ARAMEMNON, a Novel Database for Arabidopsis Integral Membrane Proteins. Plant Physiology 131, 16 -26 (2003).

5. Jeong, S., Trotochaud, A.E. & Clark, S.E. The Arabidopsis CLAVATA2 Gene Encodes a Receptor-like Protein Required for the Stability of the CLAVATA1 Receptor-like Kinase. The Plant Cell Online 11, 1925 -1934 (1999).

6. Bleckmann, A., Weidtkamp-Peters, S., Seidel, C.A.M. & Simon, R. Stem Cell Signaling in Arabidopsis Requires CRN to Localize CLV2 to the Plasma Membrane. Plant Physiology 152, 166 -176 (2010).

7. McKeon, T. & Stumpf, P. Purification and characterization of the stearoyl-acyl carrier protein desaturase and the acyl-acyl carrier protein thioesterase from maturing seeds of safflower. J. Biol. Chem. 257, 12141-12147 (1982).

8. Fox, B.G., Shanklin, J., Somerville, C. & Münck, E. Stearoyl-acyl carrier protein delta 9 desaturase from Ricinus communis is a diiron-oxo protein. Proceedings of the National Academy of Sciences 90, 2486 -2490 (1993).

9. Ohlrogge, J. & Browse, J. Lipid biosynthesis. Plant Cell 7, 957-970 (1995).

10. Bastiaens, P.I. & Squire, A. Fluorescence lifetime imaging microscopy: spatial resolution of biochemical processes in the cell. Trends Cell Biol. 9, 48-52 (1999).

Appendix

Tab. 2. Calculated FRET efficiencies of all collected data.

Sample #	FAB2 PK	FAB2 NK	FAFAB2(G) + FAB2(mC)	UNK1 PK	UNK1 NK	UNK1(G) + CLV2(mC)	UNK1(mC) + CLV2(G)
1	16.5	2.7	8.6	31.7	0.9	-8.4	2.7
2	25.4	4.5	9.3	33.1	-1.9	-10.1	0.5
3	17.1	3.7	2.8	37.1	-1.0	-0.4	-3.1
4	15.6	3.6	9.3	38.1	1.1	5.4	0.6
5	10.7	1.4	4.8	35.2	-6.3	0.4	5.1
6	22.2	2.1	4.0	48.2	2.4	3.9	3.2
7	23.2	5.4	2.8	32.0	-4.1	-4.3	5.2
8	17.0	4.2	4.5	29.0	2.3	2.1	2.7
9	15.4	4.8	9.0	48.1	4.6	7.5	1.7
10	23.0	1.7	6.1	36.8	8.3	-0.5	1.2
11	23.9	2.5	1.7	18.6	-7.7	6.6	7.1
12	27.7	1.8	0.8	26.8	6.5	6.6	2.1
13	28.5	2.8	5.9	33.1	0.7	5.3	4.2
14	24.2	4.3	1.8	33.8	2.3	-1.9	-3.7
15	18.8	-1.7	7.9	27.4	-6.2	3.3	3.1
16	37.8	0.9	8.8	35.6	-0.6	-4.8	1.2
17	19.3	3.8	5.6	35.5	-5.2	4.4	0.6
18	25.1	2.6	2.7	30.3	3.2	2.4	-0.4
19	18.6	2.4	5.7	36.2	5.3	-0.6	4.4
20	21.5	4.3	5.4	33.4	-0.7	-3.2	1.8
21	20.9	2.5	4.0	30.7	-1.5	-9.7	3.2
22	18.8	1.9	4.0	37.5	2.8	-4.9	2.3
23	17.4	1.7	5.1	24.5	2.3	2.2	0.0
24	20.0	3.2	9.3	25.3	-7.8	-3.4	-0.6
25	20.6	3.3	4.3	27.2	3.0	4.5	1.6
26	26.2	3.6	4.9	31.7	0.9	-8.4	2.7
27	27.6	5.4	9.6	33.1	-1.9	-10.1	0.5
28	37.2	3.7	4.1	37.1	-1.0	-0.4	-3.1
29	22.1	4.0	6.6	31.7	1.1	5.4	0.6
30	24.1	0.8	5.3	33.1	-6.3	0.4	5.1
31	-	-	-	37.1	2.4	3.9	3.2