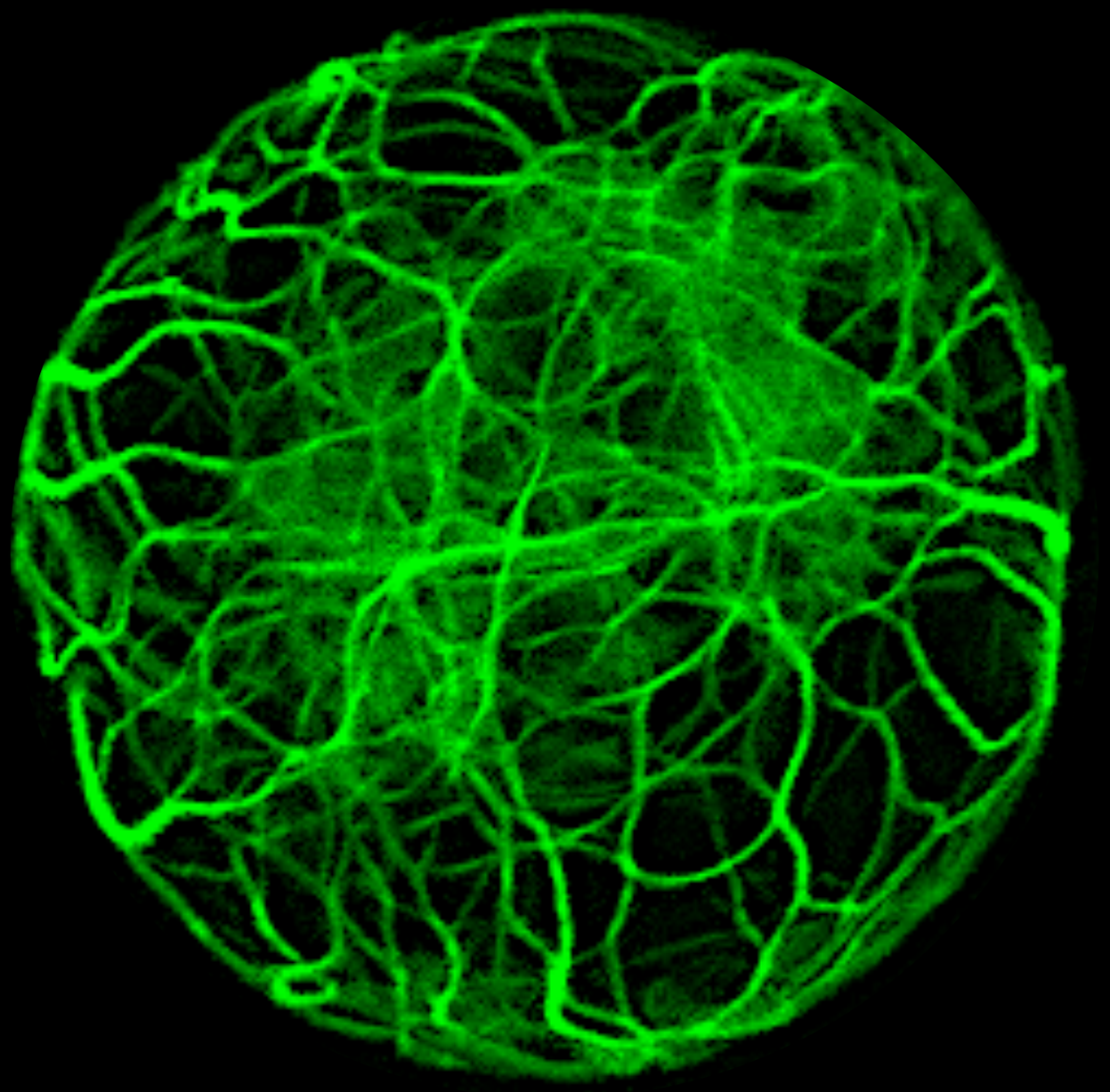
ANALYSIS OF AN ACTIN BINDING GUANINE EXCHANGE FACTOR, GEF8, AND ACTIN DEPOLYMERIZING FACTOR IN *ARABIDOPSIS THALIANA*.

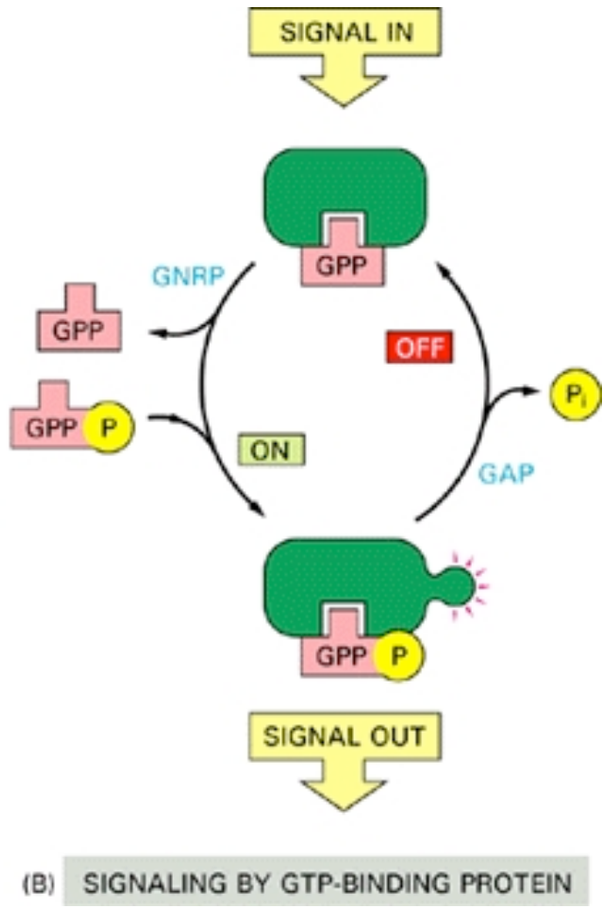


A Thesis presented

By

ALEKSEY CHUDNOVSKIY

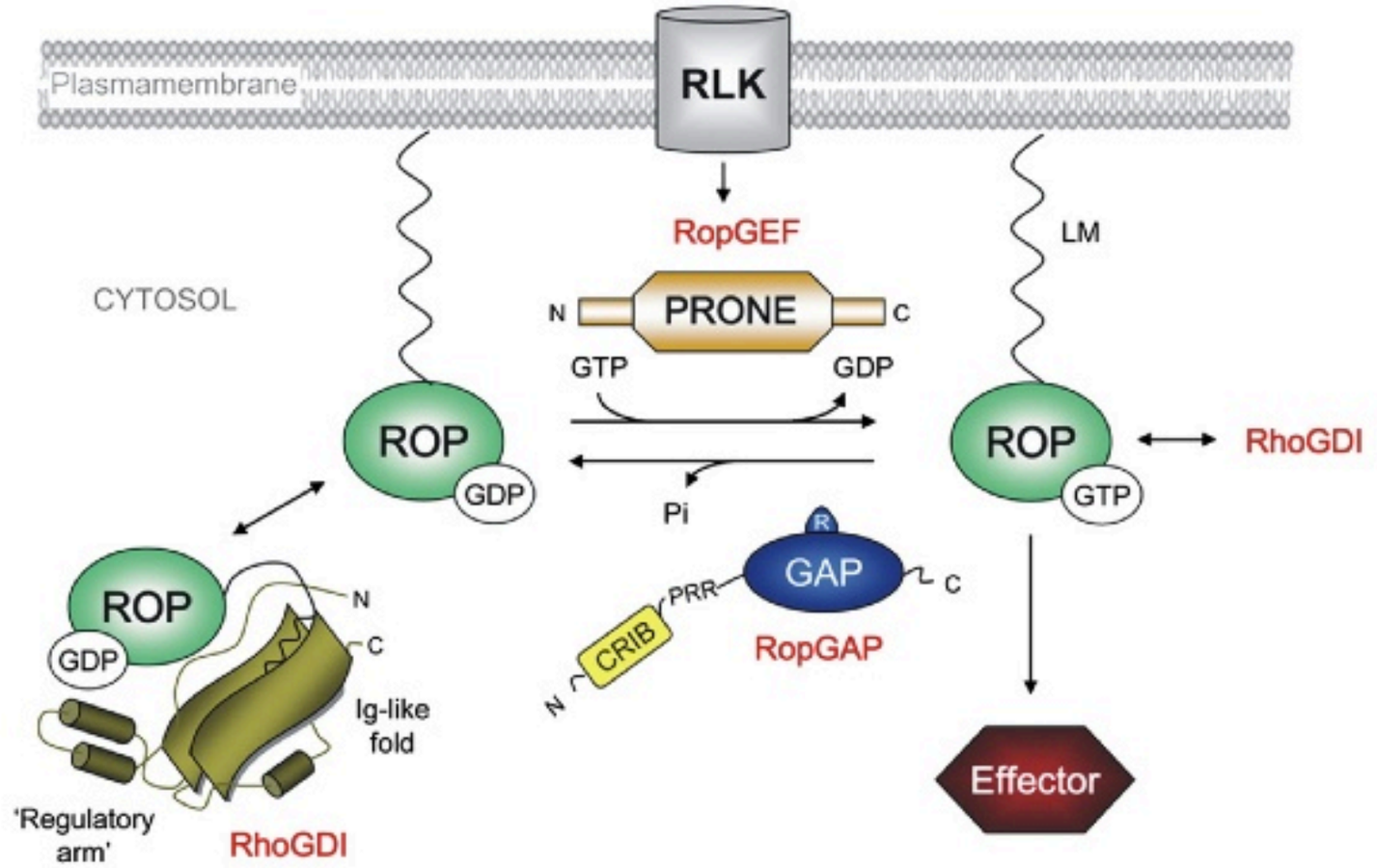
Fig1. Basic Mechanism depicting the Ras cycling between active ON and inactive OFF form



(B) SIGNALING BY GTP-BINDING PROTEIN

Adapted from Alberts Cell

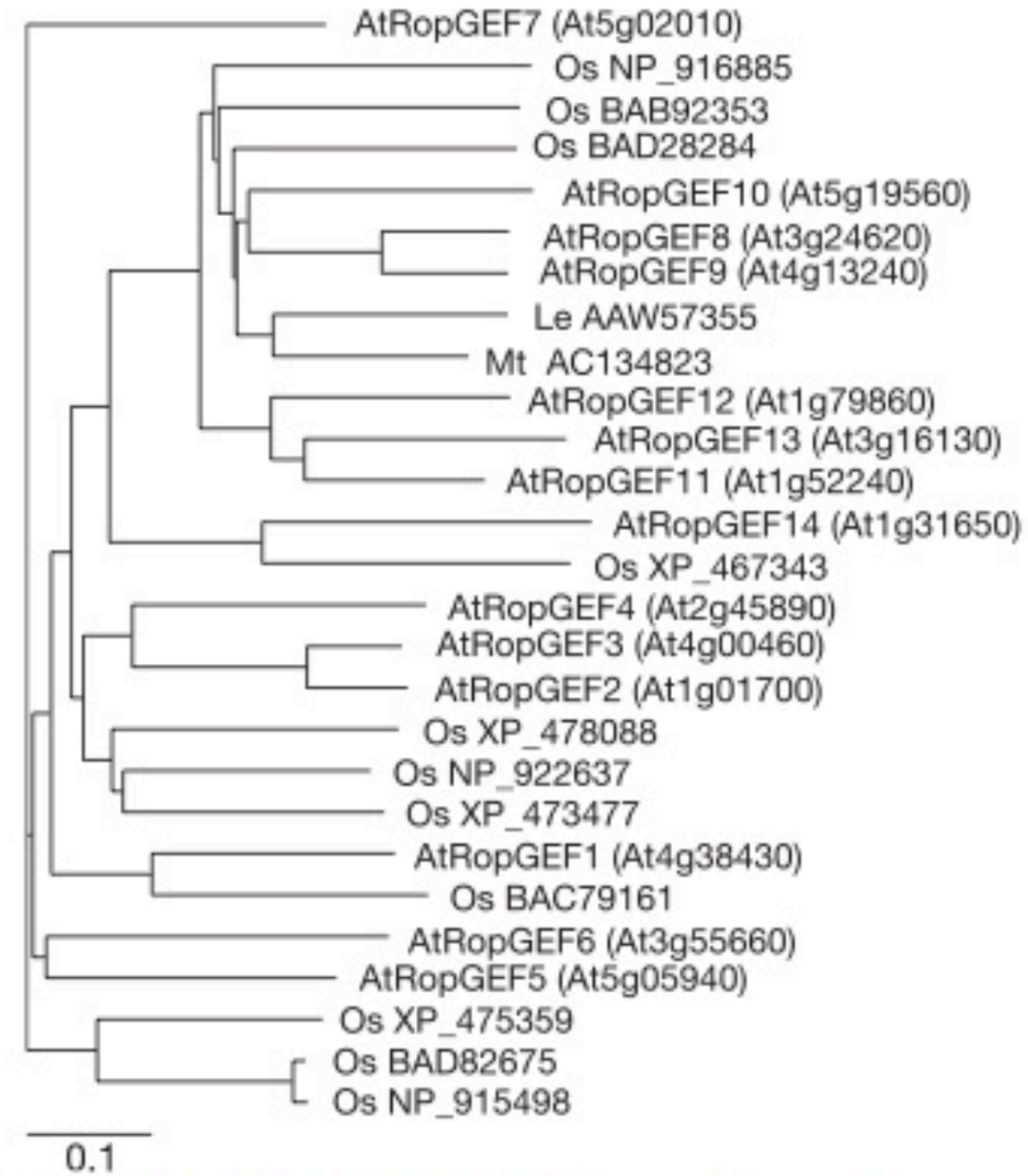
Fig 2 Regulation of Rac activity



Schematic illustration of functionally relevant domains and motifs in the regulators of the ROP cycle. Membrane attached ROPs are activated by RopGEFs via the catalytic PRONE domain promoting GDP/GTP exchange. The GTPase activity of ROPs is stimulated by RopGAPs requiring both a CRIB-motif and a GAP domain with an arginine finger (R) for efficient catalysis (PRR: proline-rich region of unknown function within RopGAPs). RhoGDIs sequester ROPs in the cytosol by hiding the C-terminal lipid-moieties (LM) within their immunoglobulin (Ig)-fold while a regulatory arm binds to prevent GDP dissociation or GTP hydrolysis. Upstream signals can feed into this regulatory cycle via a putative contact between receptor-like kinases (RLK) and RopGEF. Downstream pathways are induced through the interaction of GTP-bound ROP with effectors.

A. Berken, A. Wittinghofer / Plant Physiology and Biochemistry 46 (2008) 380e393

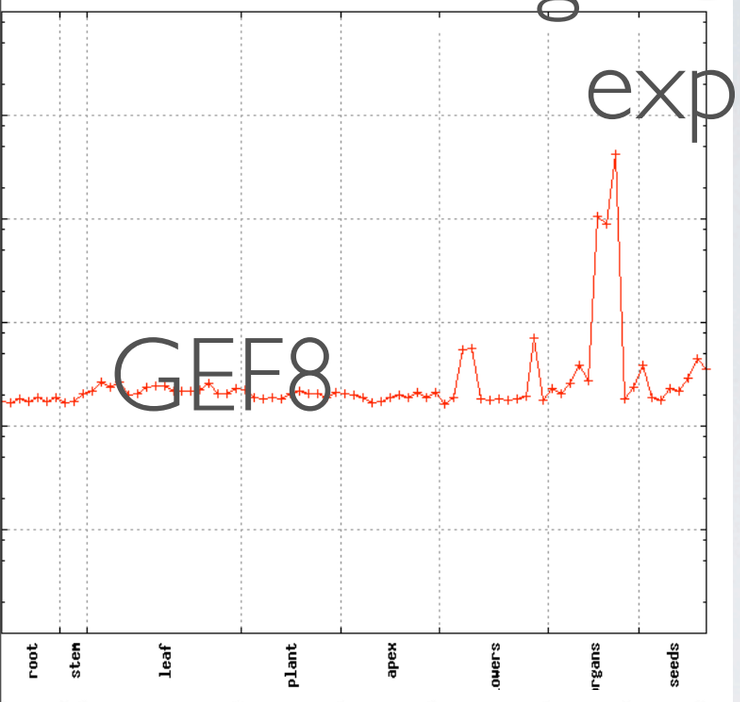
Fig 3 Phylogenetic tree of GEFS in plants

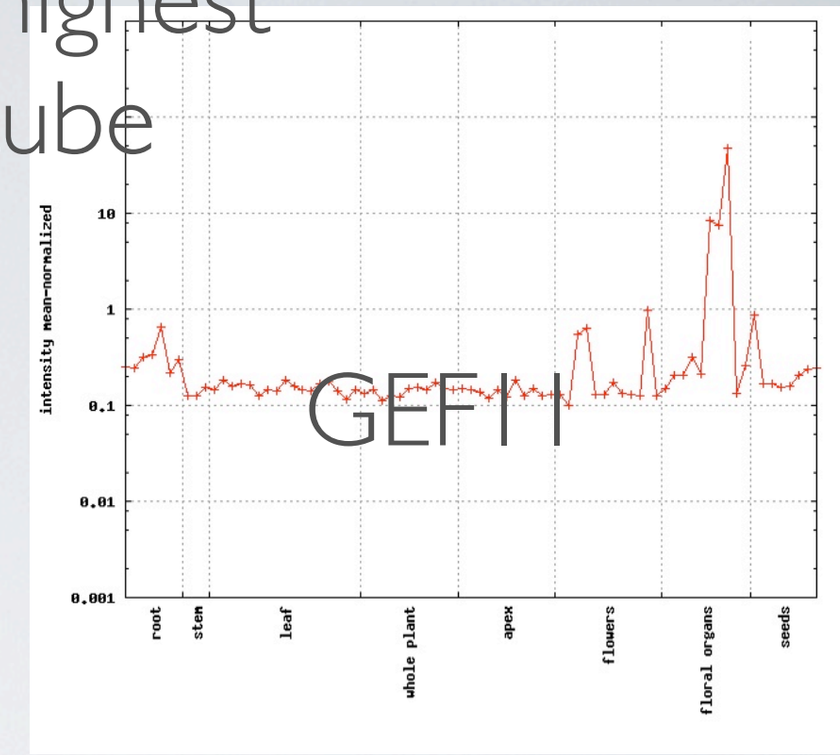


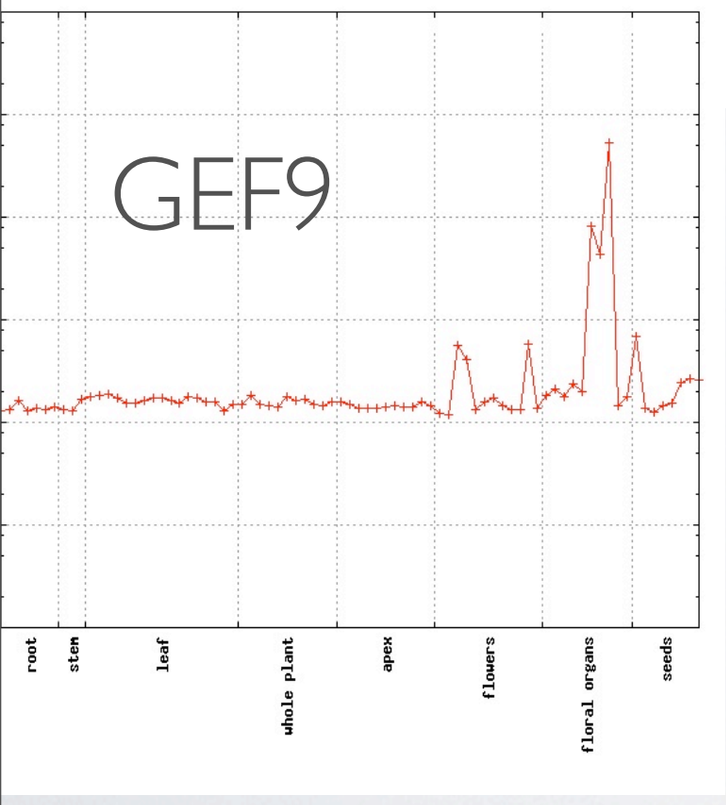
The plant RopGEF family. Phylogenetic tree of sequences obtained from GenBank using BLAST (http://www.ncbi.nlm.nih.gov/ BLAST). Phylogram was constructed with TreeView (http:// taxonomy.zoology.gla.ac.uk/rod/ treeview.html) after alignment with ClustalW (http://www.ebi.ac.uk/clustalw). Arabidopsis thaliana (At) RopGEFs are given with their corresponding loci. The accession numbers for rice (Os: Oryza sativa), the model legume 'barrel medic' (Mt: Medicago truncatula) and tomato (Le: Lycopersicon esculentum) are as indicated

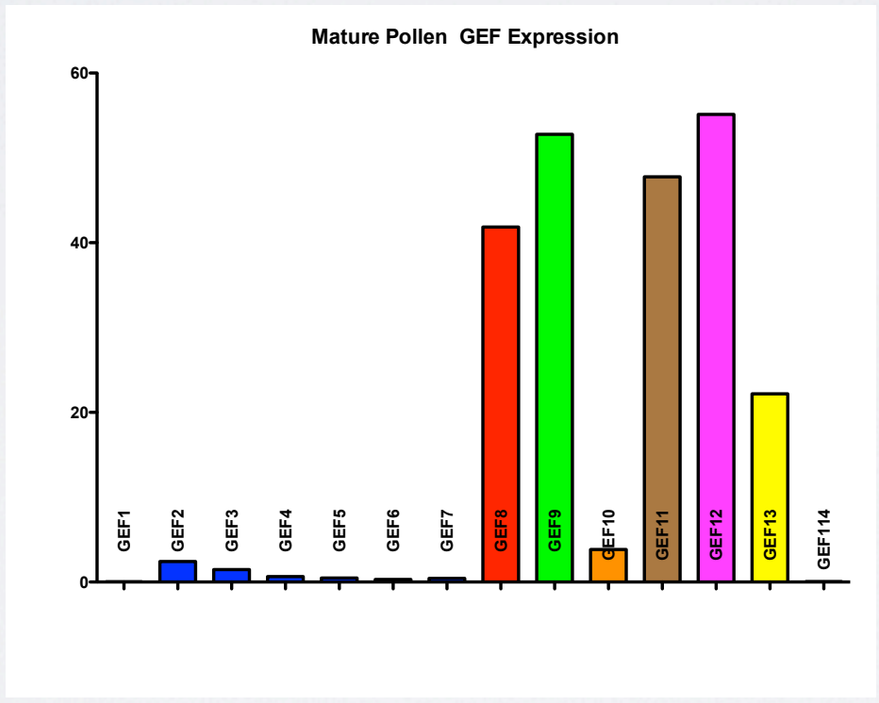
A new family of RhoGEFs activates the Rop molecular switch in plants Antje Berken[1], Christoph Thomas[1] & Alfred Wittinghofer[1]

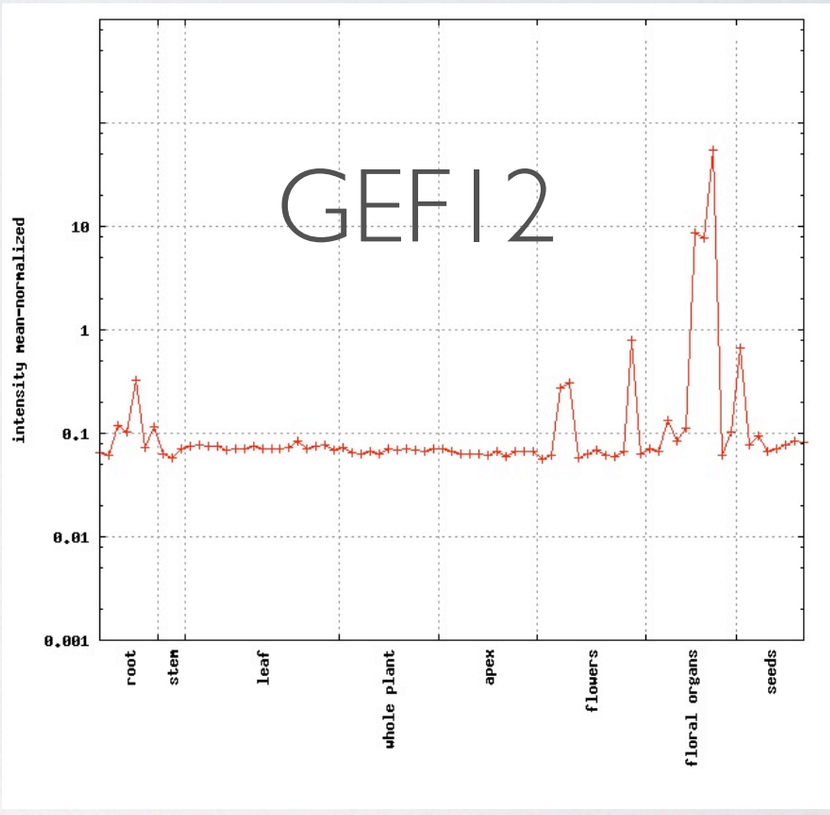
Fig4 GEF8,9,11,12 have the highest expression in the pollen tube







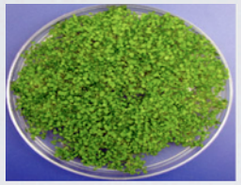




AtGenExpress Visualization Tool (AVT) was used in order to investigate which GEF have the highest expression in the pollen tube

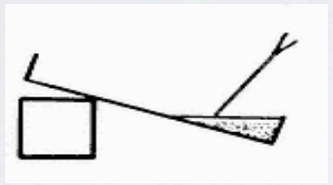
Fig 5 Outline of the experimental procedure for the protoplasts isolation. (see methods section for the details)

✦Cut the 2 week old seedling.

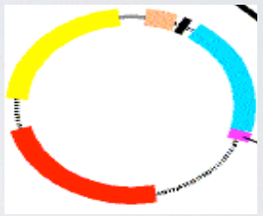


Incubate plants overnight with an enzyme solution in the

Remove the enzyme solution

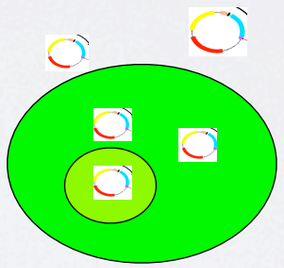


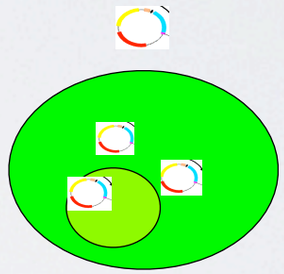
Transform the protoplasts with the construct of interest



Resuspend in K3 sucrose medium

Take the floating intact cells

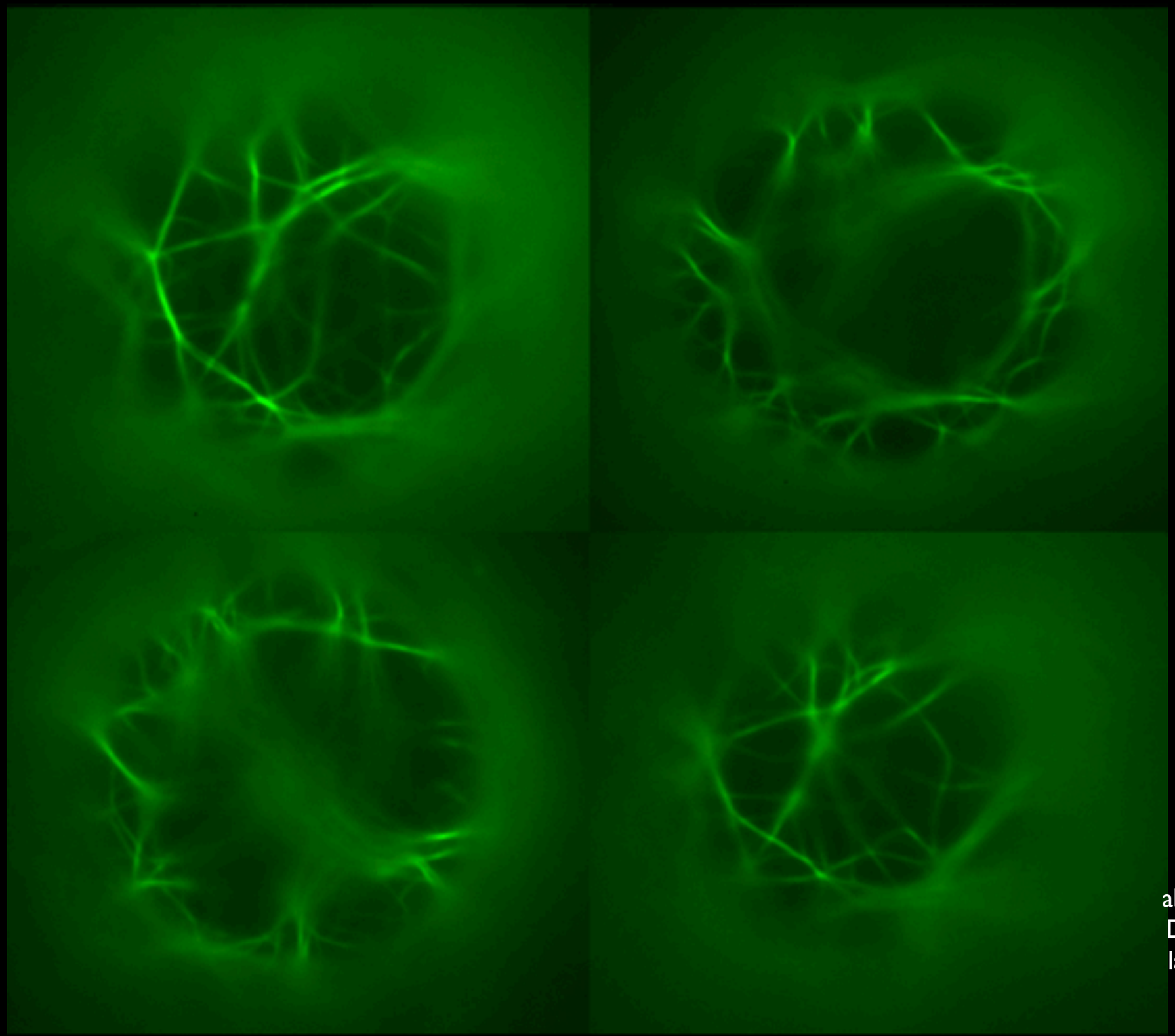




15 hours later assess the expression of the protein

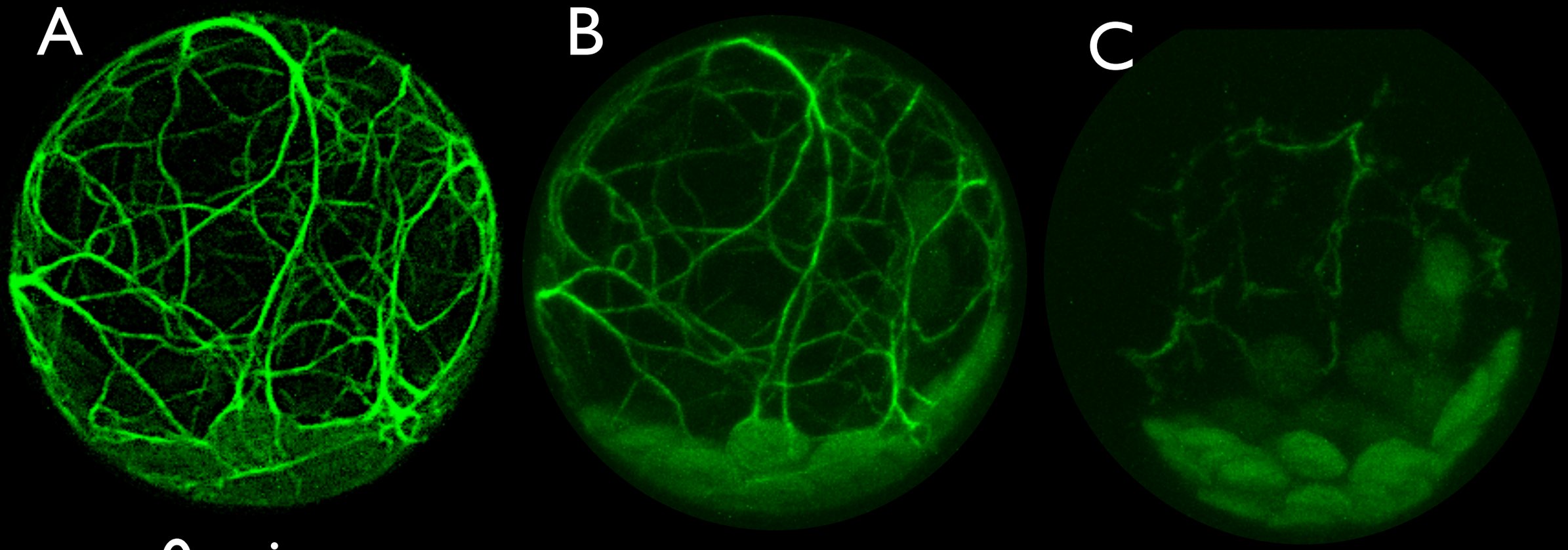
Adapted from
www.escholarship.org/editions/view?docId=ft79...

Fig 6 35S-GFP-GEF8 colocolizes with the prominent cable like structures in the arabidopsis protoplasts



Protoplasts were isolated as described above and transformed with 5ug of plasmid DNA coding 35S-GFP-GEF8. Fifteen hours later the images were acquired using E800 Nikon microscope under 100X magnification

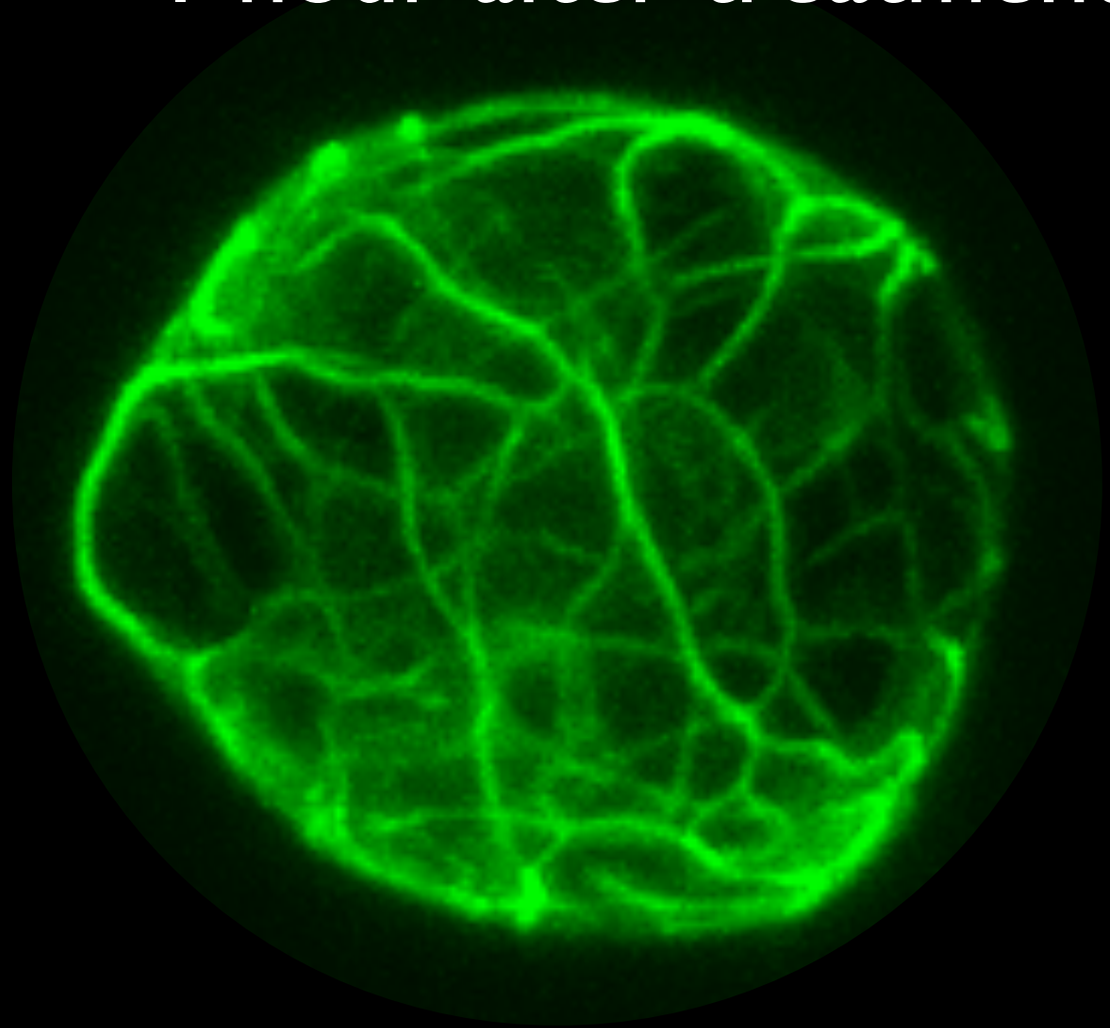
Fig 7 250 nM Latranculin treatment



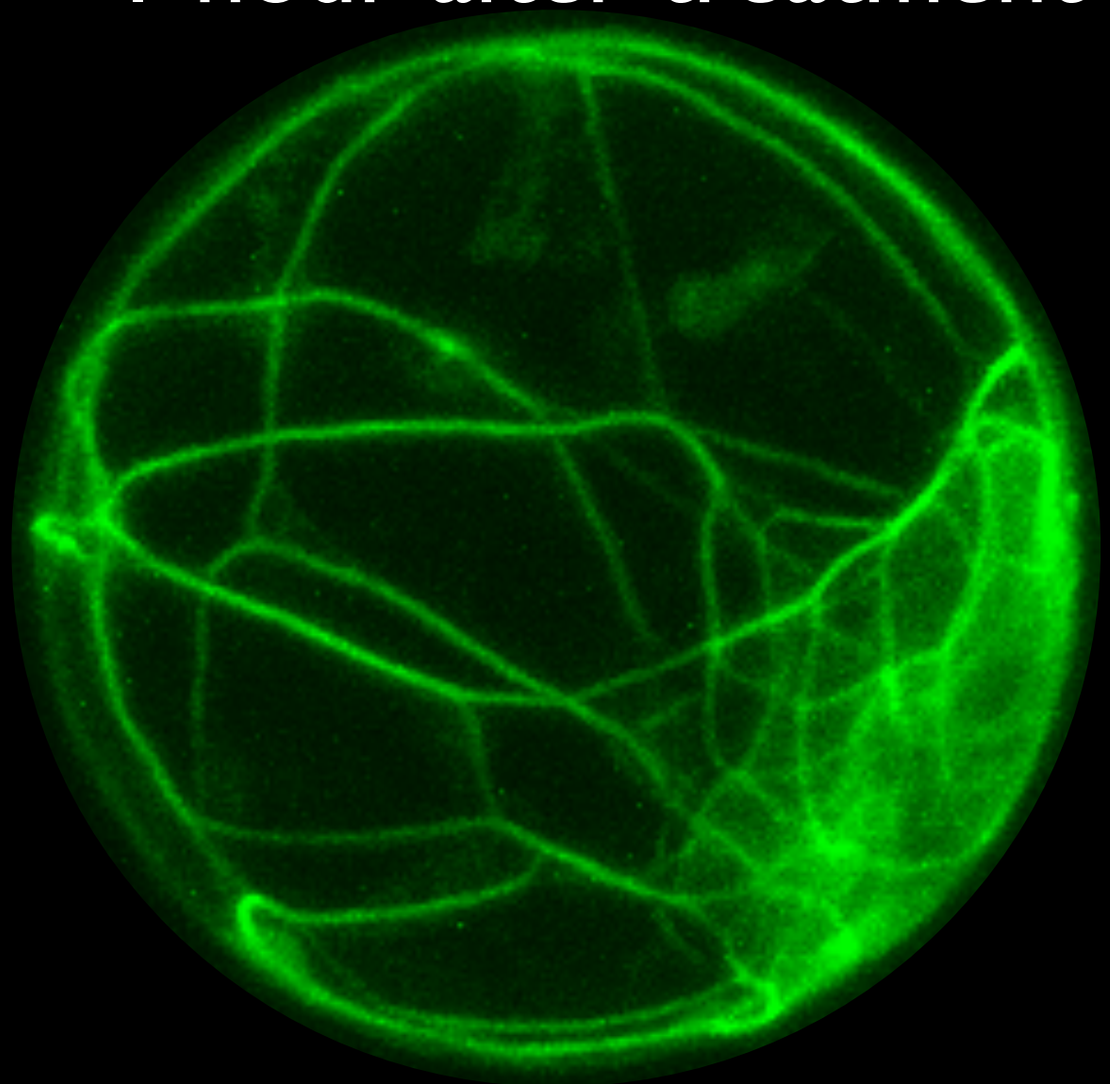
Arabidopsis protoplasts were treated with 250nM concentration of Latranculin and Z stacks of the same cell were taken at 0, 2 and 10 min after the treatment using Confocal Zeiss microscope. Images show the 3D reconstruction of the Z Stacks.

Fig 8 Oryzalin 10um treatment

1 hour after treatment



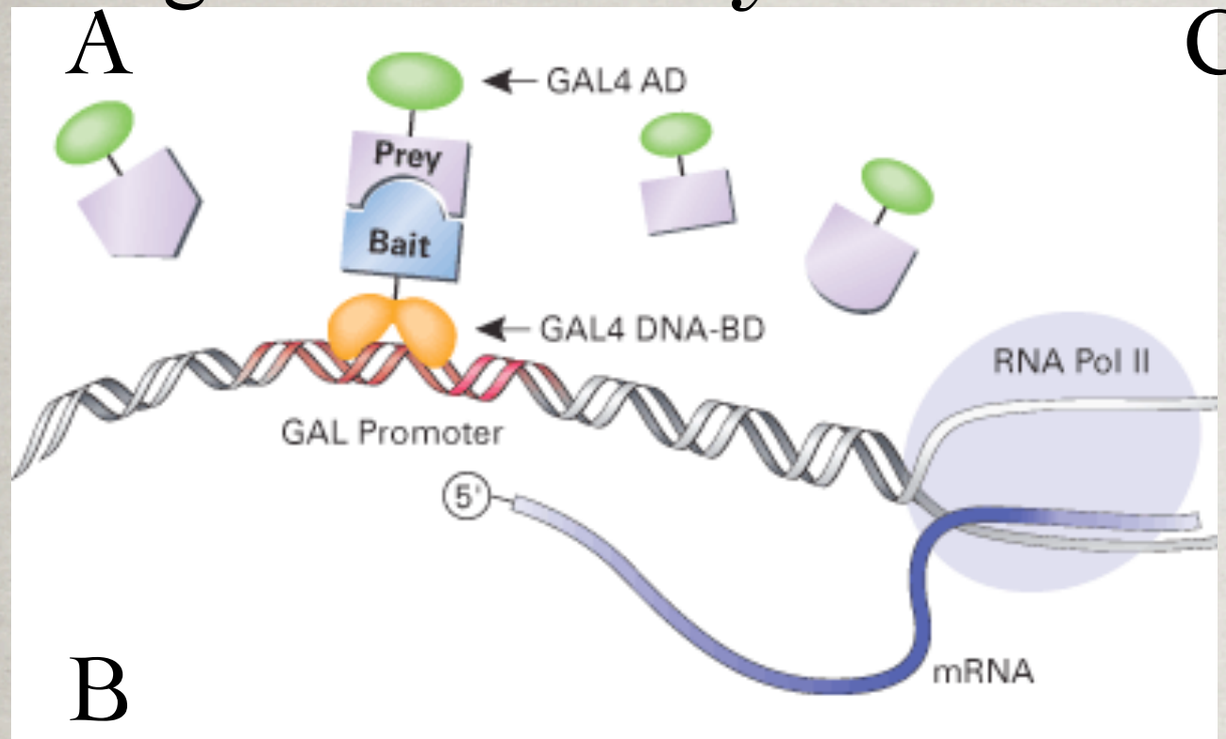
1 hour after treatment



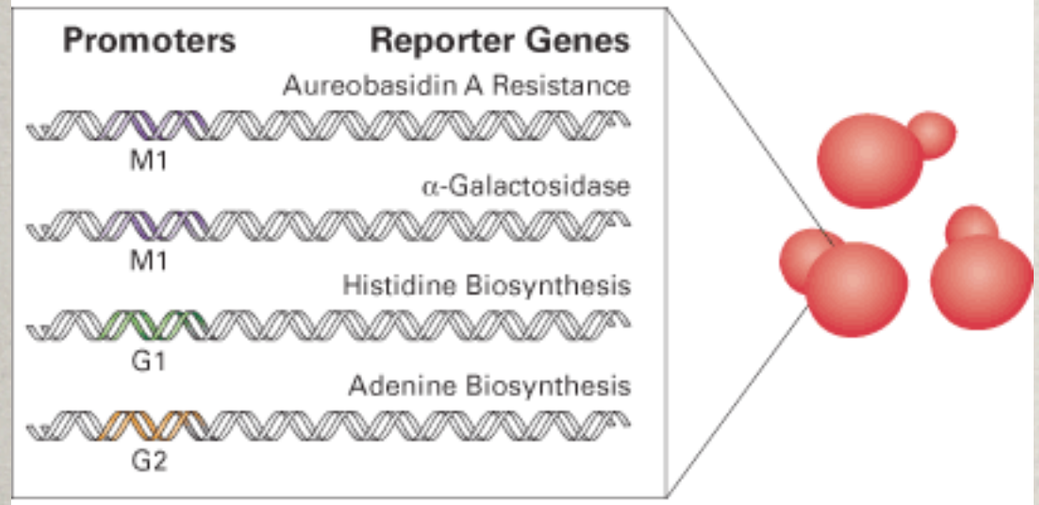
Protoplasts were treated for 1hour with oryzalin and Z stacks were acquired using Zeiss confocal micorscope. Images show the 3 D reconstitution of 2 different cells after !hour of oryzalin treatment.

Fig 9 Yeast 2 Hybrid

A



B



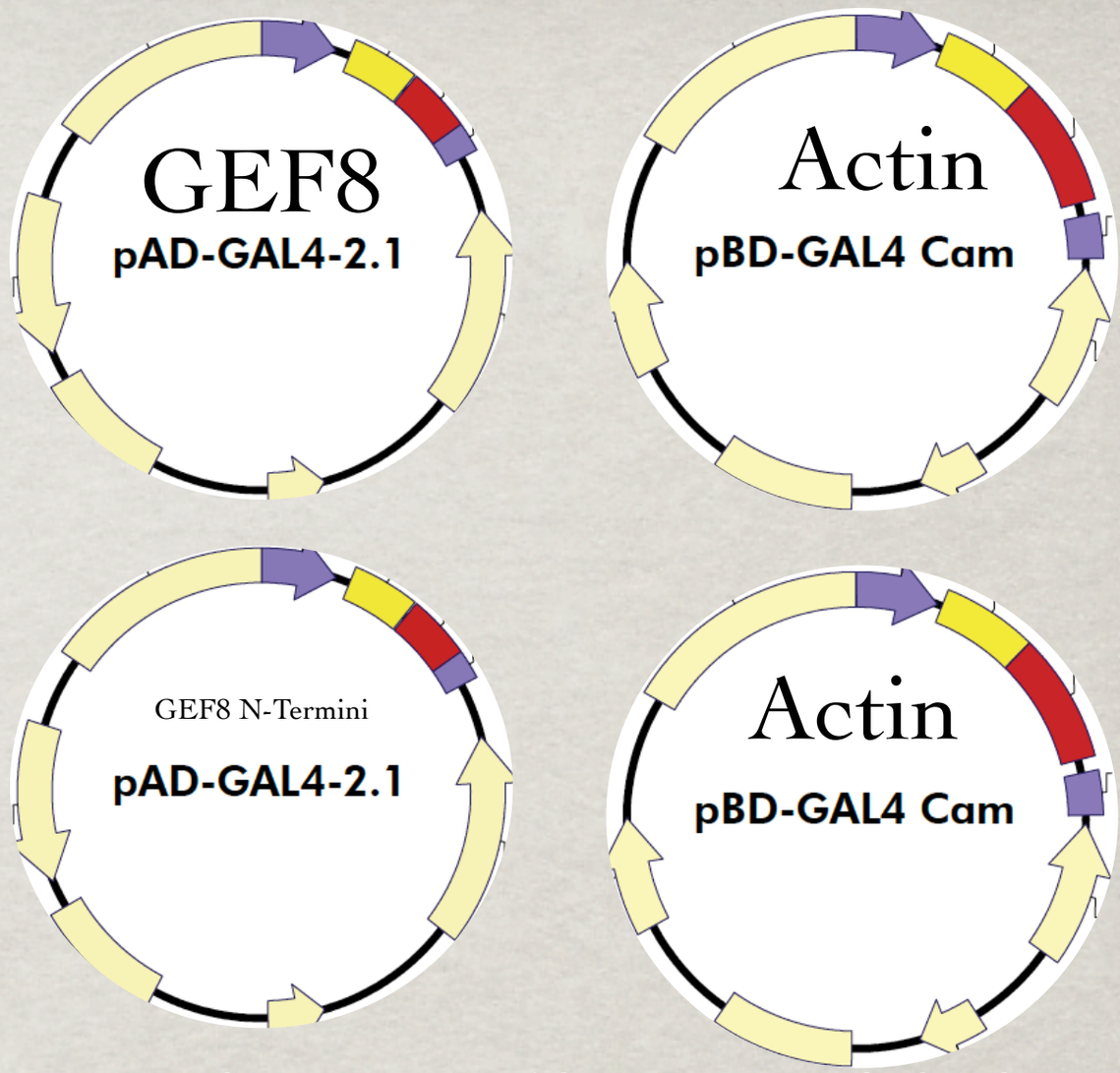
A-The yeast two-hybrid principle. A bait protein interacts with the GAL4 recognition sequence (or promoter) upstream of a reporter gene. Transcription of the reporter is activated when a prey protein containing the GAL4 transcriptional activation domain interacts with the bait.

Adapted from Clontech Yeast 2 hybrid systems manual

B-Matchmaker Gold reporter genes. Yeast strain Y2HGold expresses 4 genes from 3 separate GAL4-responsive promoters in response to protein-protein interactions.

Adapted from Clontech Yeast 2 hybrid systems manual

C



C- Schematic representation of the vector constructs used in the study.

Adapted from Stratagene HybriZAP® 2.1 Two-Hybrid System

Table 1

Construct	Construct	Growth on-Leu,-Trp	Growth on -Leu.-Trp,-His
GEF8	Actin	+	+
GEF8	pBD	+	-
pAD	actin	+	-
N-termini GEF8	actin	+	+
N-terminiGEF8	pBD	+	-

Table 1 shows different constructs transformed into the Yeast. +indicates the growth on different selection plates. - indicates no growth (See text for the details).

Fig 10 CLUSTALW alignment of pollen specific GEFs

```
gi|240254246|ref|NP_175634.5|      MLEGKAMVEDSDMPVKMQMQAMAFASQALDLFDVFDCKSIAGHIKKEFDE 50  GEF11
gi|15220004|ref|NP_178104.1|       ------MVRAS----------------------------------EQEQE 10  GEF12
gi|15230137|ref|NP_189105.1|       ------MVAAL----------------------------------ERGLS 10  GEF8
gi|15235630|ref|NP_193060.1|       ------MVPSL----------------------------------ERGIS 10  GEF9
                                         **                                    :.  .

gi|240254246|ref|NP_175634.5|      RYGSGWQCVALLLLNYTTLIMGMPKLREEVDFNDNMNENSASRHVKSWSS 100
gi|15220004|ref|NP_178104.1|       TYRS-------RLFNFK--------------WRNNDN-NSATRHNKSLSV 38
gi|15230137|ref|NP_189105.1|       ASKS---------FNFK-----------------RMFDSSSTKQQQSQTI 34
gi|15235630|ref|NP_193060.1|       ITSS---------FNLD-----------------RMFDSSPGKEQQ---- 30
                                      *         :*                   .   .*. :. :

gi|240254246|ref|NP_175634.5|      DCAMRMDGSDNLDDDDNDMMMFRSQPGKCGSVDRPSLPIGGVTPNRNDKL 150
gi|15220004|ref|NP_178104.1|       ETGLDEAATG--SHDAEPLTIIHPSQG-------PPLSR---SAADEAVL 76
gi|15230137|ref|NP_189105.1|       VV-------ENGDSHIVESNTPESQNS-------DSFVESPVESSLPMIS 70
gi|15235630|ref|NP_193060.1|       ------------QPHLAETTMPESQTQ-------DSLGGSPVETSRPMTS 61

gi|240254246|ref|NP_175634.5|      PRVSSSDSMEALIILQAAMEQMKEKFSKLLLGEDMSGGGKGVSSALALSN 200
gi|15220004|ref|NP_178104.1|       AALAASQARERQ--LLADMEQMKERFSKLLLGEDNSGGGKGVSSALALSN 124
gi|15230137|ref|NP_189105.1|       PLTRPGKRSERQ---QADMEMMKDRFAKLLLGEDMSGGGKGVSSALALSN 117
gi|15235630|ref|NP_193060.1|       RLIS--RRQDKQ---QSETEMMKDRFTKLLLGEDMSGGGKGVSSALALSN 106
                                         :      :  * **::*:******* ***************

gi|240254246|ref|NP_175634.5|      AITNLAASAFGEQRRLEPMAVDRKTRWRREIGWLISVADYIVEFAPTQQT 250
gi|15220004|ref|NP_178104.1|       AITNLAASVFGEQRRLEPMPAERRARWRKEIDWLLSVTDYVVEFAPSQQK 174
gi|15230137|ref|NP_189105.1|       AITNLAASIFGEQTKLQPMPQDRQARWKKEIDWLLSVTDHIVEFVPSQQT 167
gi|15235630|ref|NP_193060.1|       AITNLAASIFGEQTKLQPMAPDRRARWKKEIDWLLSVTDHIVEFVPSQQI 156
                                   ******** **** :*:**. :*::**::**.**:**:*::***.*:**

gi|240254246|ref|NP_175634.5|      NKDGTSMEVMSTRQRTDLLCNIPALKKLDAMLLDCLDKFKDQDEFYYVKK 300
gi|15220004|ref|NP_178104.1|       NKDGTNMEIMTTRQRTDLHMNIPALKKLDAMLIDCLENFKDQSEFSYISK 224
gi|15230137|ref|NP_189105.1|       SKDGVCTEIMVTRQRGDLLMNIPALRKLDAMLIDTLDNFRGHNEFWYVSR 217
gi|15235630|ref|NP_193060.1|       SKEGVCTEIMVTRQRGDLLMNIPALRKLDAMLIDTLDNFRGHNEFWYVSR 206
                                   .*:*.  *:* **** **  *****:******:* *::*:.:.** *:.:
```

Alignment of the amino-acid sequnces of the full lengths GEF8,9,11,12 using the ClustalW2 program * indicates the identical residues.: indicates consreved residues.
. indicates non conserved residues.

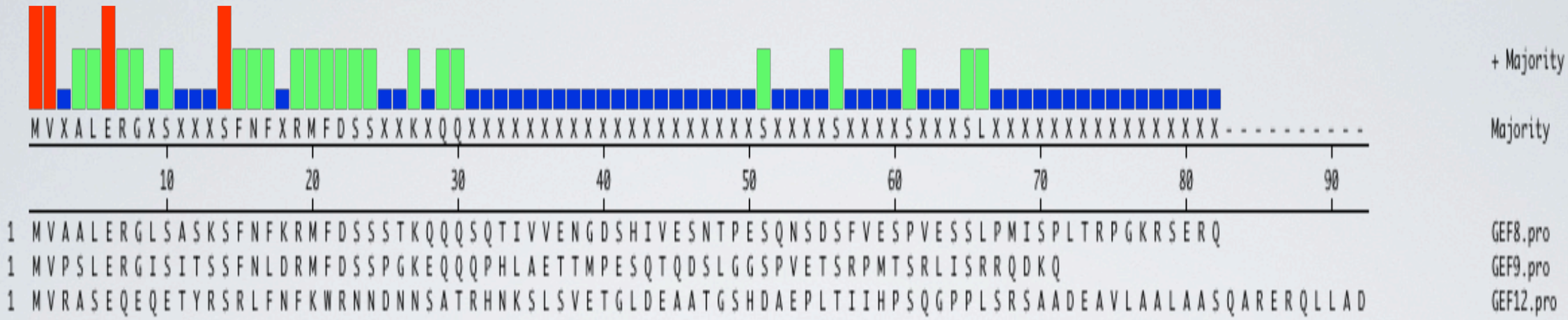
Larkin M.A., Blackshields G., Brown N.P., Chenna R., McGettigan P.A., McWilliam H.*, Valentin F.*, Wallace I.M., Wilm A., Lopez R.*, Thompson J.D., Gibson T.J. and Higgins D.G. (2007)
ClustalW and ClustalX version 2.
Bioinformatics 2007 23(21): 2947-2948.
abstract full-text PDF

Fig 10 continued

```
gi|240254246|ref|NP_175634.5|      DSPDSCETRND---EKWWLPAVKVPPNGLSEISRRFLQSQKECVNQVLKA 347
gi|15220004|ref|NP_178104.1|       DSPDLDGKRND---EKWWIPTVKVPPDGLSEASRRFLQYQKDCVNQVLKA 271
gi|15230137|ref|NP_189105.1|       DSEEGQQARNDRTNDKWWLPPVKVPPGGLSEPSRRMLYFQKDSVTQVQKA 267
gi|15235630|ref|NP_193060.1|       DSEEGKQARNERTKDKWWLPPVKVPPNGLSESARRMLHFQKDSVSQVQKA 256
                                   ** :    **:   :***:*.*****.**** :**:*  **:.*.** **

gi|240254246|ref|NP_175634.5|      AMAINAQVLSEMEIPESYLESLPKNGRASLGDVIYRMITVEMFDADQFLI 397
gi|15220004|ref|NP_178104.1|       AMAINAQVLFEMEIPESYIDSLPKNGRASLGDQMYKNITVDFFDPDQFLS 321
gi|15230137|ref|NP_189105.1|       AMAINAQVLSEMEIPESYIDSLPKNGRASLGDSIYKSITEEWFDPEQFLA 317
gi|15235630|ref|NP_193060.1|       AMAINAQVLSEMAIPDSYIESLPKNGRVSLGDSLYKSITEEWFDPEQFLS 306
                                   ********* ** **:**::*******.**** :*: ** : **.:***

gi|240254246|ref|NP_175634.5|      EMDLSSEHKILDLKNRIEASIVIWKRKMVQKDTKS--PWGSTVSIEKREQ 445
gi|15220004|ref|NP_178104.1|       SMDMSSEHKIVDLKNRIEASIIIWKRKMVYKDNKSSAPWASGVSLEKREV 371
gi|15230137|ref|NP_189105.1|       MLDMSTEHKVLDLKNRIEASVVIWKRKLHTKDTKS--SWGSAVSLEKREL 365
gi|15235630|ref|NP_193060.1|       TLDLSTEHKVLDVKNRIEASIVIWKRKLHLKDNKS--SWGSAVSLEKREL 354
                                    :*:*:***::*:*******::*****:  **.**  .*.* **:****

gi|240254246|ref|NP_175634.5|      FEERAETILLLLKQGFPGISQSSLDISKIQFNRDVGLAILESYSRVLESL 495
gi|15220004|ref|NP_178104.1|       FEERAETILLILKQRYPGISQSSLDISKIQFNEDVGQAVLESYSRILESL 421
gi|15230137|ref|NP_189105.1|       FEERAETILVLLKQKFPGLPQSSLDISKIQFNKDVGQAVLESYSRILESL 415
gi|15235630|ref|NP_193060.1|       FEERAETILVLLKQKFPGLPQSSLDISKIQYNKDVGHAVLESYSRILESL 404
                                   *********::*** :**:.**********:*.*** *:******:****
gi|240254246|ref|NP_175634.5|      AHTVMSRIEDVLYADQLTQEPTNNAPSKNRYSLKENEKLREERLSFTEDM 545
gi|15220004|ref|NP_178104.1|       AYTVLSRIDDVLEADR--------AGNKRNTPLEAEEETLVGSMTLSDFM 463
gi|15230137|ref|NP_189105.1|       AYTVMSRIEDVLYTDTLALKQTLLAEETSDGG-RTTETDSESAGSSN-SG 463
gi|15235630|ref|NP_193060.1|       GYTEMSRIDDVLYADSLARKQ-CTGEETSDGGKIATETDSASAGSSNYSG 453
                                   .:* :***:*** :*         . ..        *       : .

gi|240254246|ref|NP_175634.5|      ASGTLSDVMQWGNKNNEMKKESFFGDREKPLLSKVTGIMTNNKKSSYLDN 595
gi|15220004|ref|NP_178104.1|       G----WDFDQAANAELESKKD----LPDDPLIKEKLSVVTT-KKTSYLET 504
gi|15230137|ref|NP_189105.1|       EEAEKHDPHS---KTLLDFMGWNDNSSKG-GDKPTKSPNLTPKKLSYLEK 509
gi|15235630|ref|NP_193060.1|       EEIEKLESQNSSKTTLLDFIGWSDNSSKGQSEKPPKSPRMTPKKLSYLEK 503
                                         :  .                .    .   .   . ** ***:.

gi|240254246|ref|NP_175634.5|      LG---AMRSPTARYS 607
gi|15220004|ref|NP_178104.1|       LG---GVKSPTARH- 515
gi|15230137|ref|NP_189105.1|       LENLNGFRSPKDRH- 523
gi|15235630|ref|NP_193060.1|       LENLNGFRSPKDRH- 517
                                   *    ..:**. *:
```

Fig 11

N-terminal alignment of pollen specific GEFs



CLUSTAL W. Multiple sequence alignment of Arabidopsis GEF8,9 and 12. The alignment shows the N-terminal of the GEFS right before the PRONE domain.

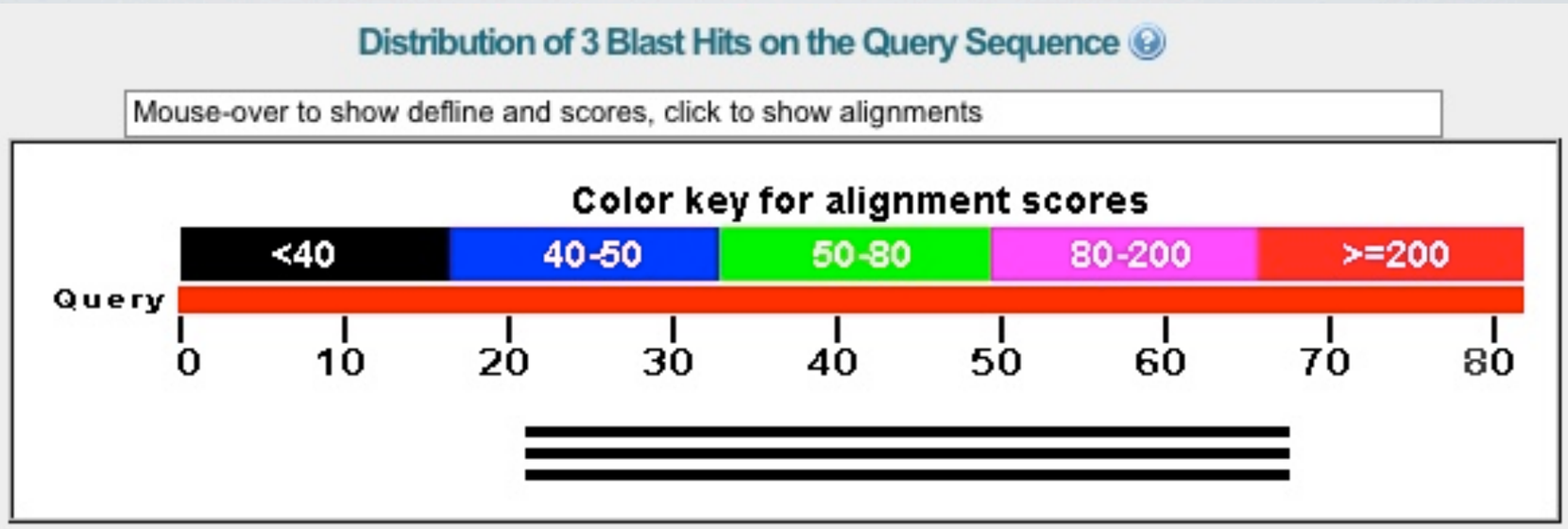
Non Conserved residues

Identical in two sequences

Identical in all three sequences

Fig 12

gi|15230137|ref|NP_189105.1| ROPGEF8; Rho...



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Links
EDN63569.1	actin filament binding protein [Saccharomyces cerevisiae YJM789]	26.2	26.2	56%	3.9	
EEU04836.1	Abp140p [Saccharomyces cerevisiae JAY291]	25.8	25.8	56%	6.0	
NP_014882.2	Abp140p [Saccharomyces cerevisiae] >sp|Q08641.3|AB140_YEAST RecName: Full=Uncharacteriz	25.4	25.4	56%	6.5	UG

Alignment of the N-termini of GEF8 against Saccharomyces cerevisiae genome using Genbank BLAST Search. http://www.ncbi.nlm.nih.gov/ BLAST

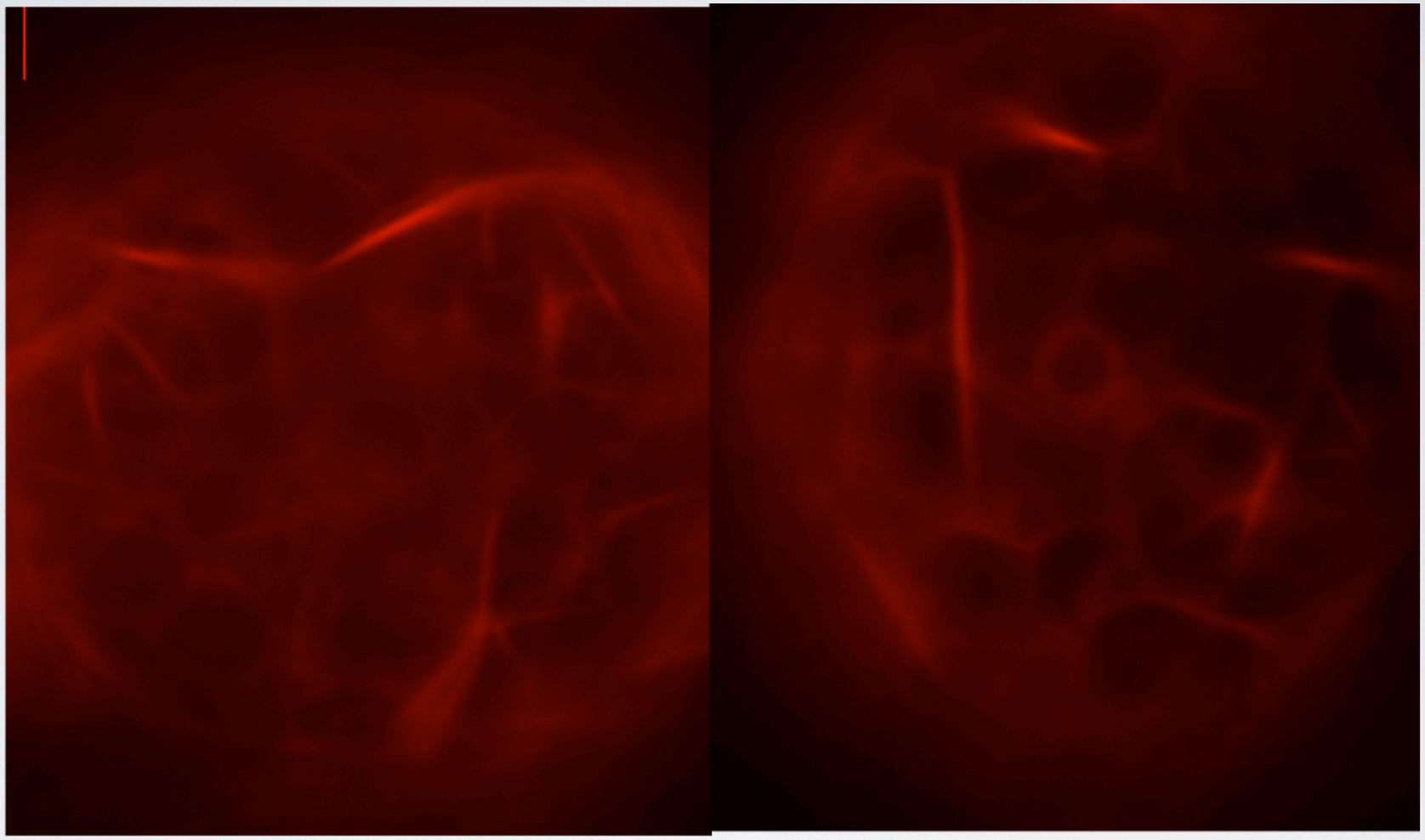
Blast search of the N-terminal sequence of the GEF8 against Saccharomyces Cerevisiae. Showing the sequences producing the most significant alignments.

```
>gb|EDN63569.1|  actin filament binding protein [Saccharomyces cerevisiae YJM789]
Length=628

 Score = 26.2 bits (56),  Expect = 4.2, Method: Compositional matrix adjust.
 Identities = 16/46 (34%), Positives = 23/46 (50%), Gaps = 0/46 (0%)

Query  22  DSSSTKQQQSQTIVVENGDSHIVESNTPESQNSDSFVESPVESSLP  67
           D +    QQ  T V +  D +    N PE+ NS    E P+E++LP
Sbjct  35  DETKELHQQESTAVPQEVDVNEEFENEPETTNSSRTAEKPLETNLP  80


>gb|EEU04836.1|  Abp140p [Saccharomyces cerevisiae JAY291]
Length=598

 Score = 25.8 bits (55),  Expect = 6.2, Method: Compositional matrix adjust.
 Identities = 16/46 (34%), Positives = 23/46 (50%), Gaps = 0/46 (0%)

Query  22  DSSSTKQQQSQTIVVENGDSHIVESNTPESQNSDSFVESPVESSLP  67
           D +    QQ  T V +  D +    N PE+ NS    E P+E++LP
Sbjct  35  DETKELHQQESTAVPQEVDVNEEFENEPETINSSRTAEKPLETNLP  80


>ref|NP_014882.2|  Abp140p [Saccharomyces cerevisiae]
 sp|Q08641.3|AB140_YEAST  RecName: Full=Uncharacterized methyltransferase ABP140; AltName:
Full=140 kDa actin-binding protein
 tpg|DAA11008.1|  TPA: Abp140p [Saccharomyces cerevisiae]
Length=628

 GENE ID: 854414 ABP140 | Abp140p [Saccharomyces cerevisiae]
(Over 10 PubMed links)

 Score = 25.4 bits (54),  Expect = 6.8, Method: Compositional matrix adjust.
 Identities = 16/46 (34%), Positives = 23/46 (50%), Gaps = 0/46 (0%)

Query  22  DSSSTKQQQSQTIVVENGDSHIVESNTPESQNSDSFVESPVESSLP  67
           D +    QQ  T V +  D +    N PE+ NS    E P+E++LP
Sbjct  35  DETKELHQQESTAVPQEVDVNEEFENEPETINSSRTAEKPLETNLP  80
```

Fig 13 Restriction map of GEF 8 showing BGL 2 site.

Schematic representation of GEF8 sequence showing the unique Bgl2 restriction site right before the PRONE domain.

Fig 14 35S-RFP-Fimbrin Actin Binding Domain ABD



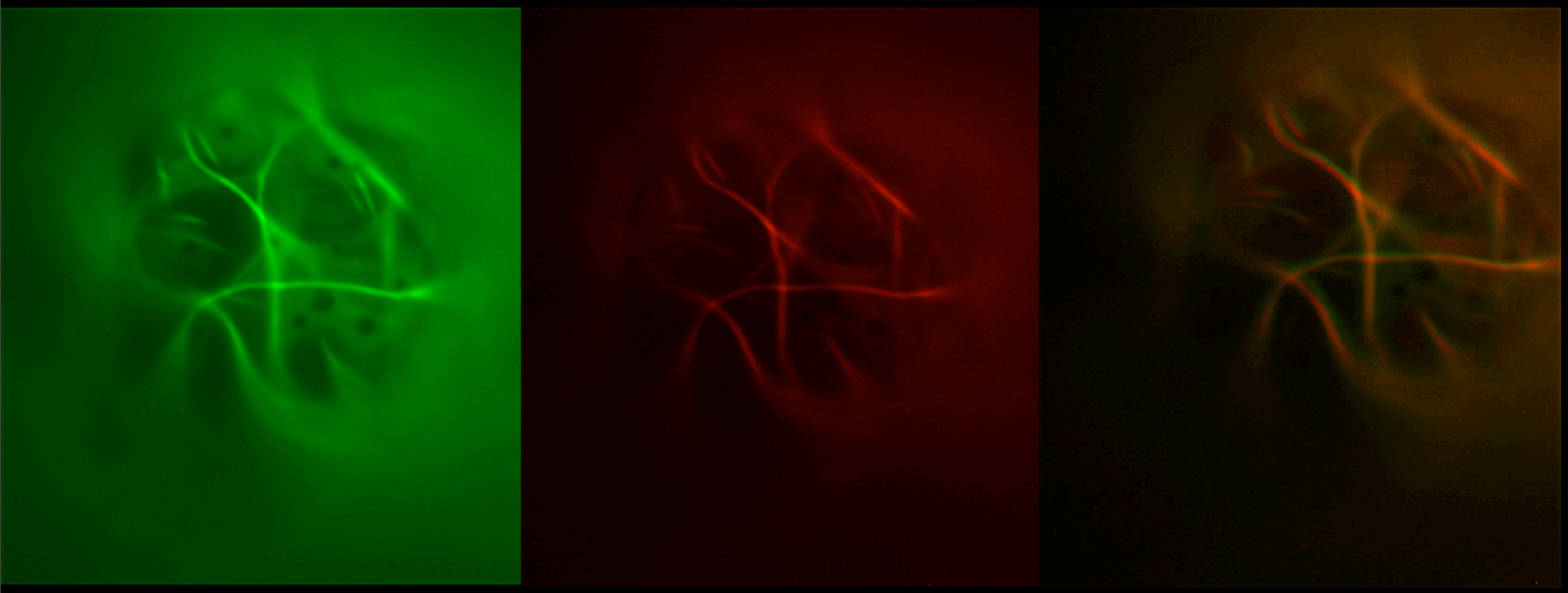
Arabidopsis protoplasts were transformed with the construct encoding 35S-RFP-Fimbrin 16 hour later the images were acquired using E-800 Nikon microscope. The picture shows 2 respresentative cells.

Fig 15 GEF8 co-localizes with fimbrin ABD

35S-GFP-GEF8

35S-RFP-Fimbrin ABD

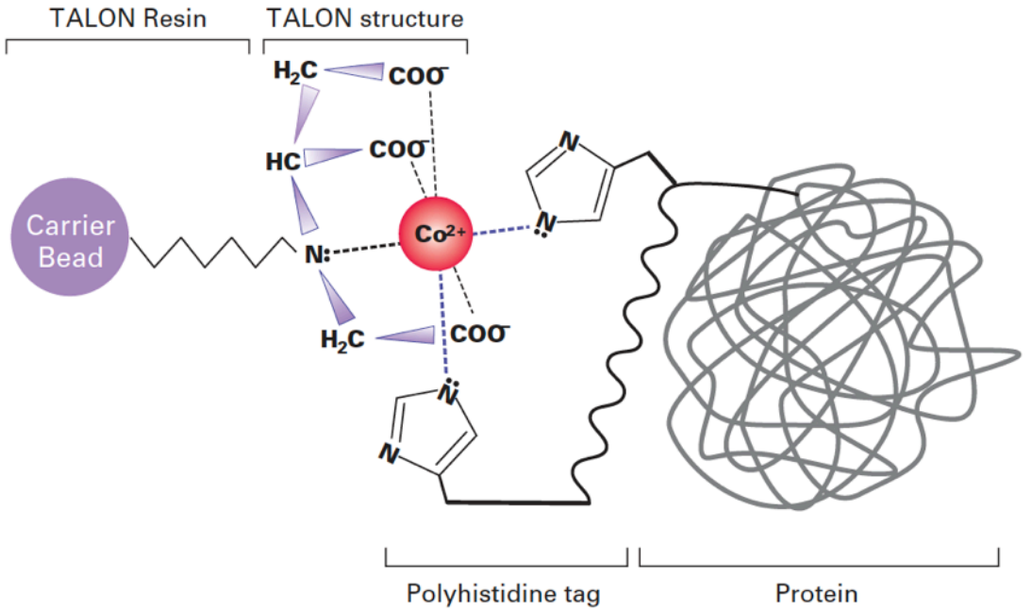
merge



Arabidopsis protoplasts were cotransformed with 35s-GFP-GEF8 and 35s-RFP-Fimbrin ABD. 16 hours later the images were acquired using E800.

Fig 15

A

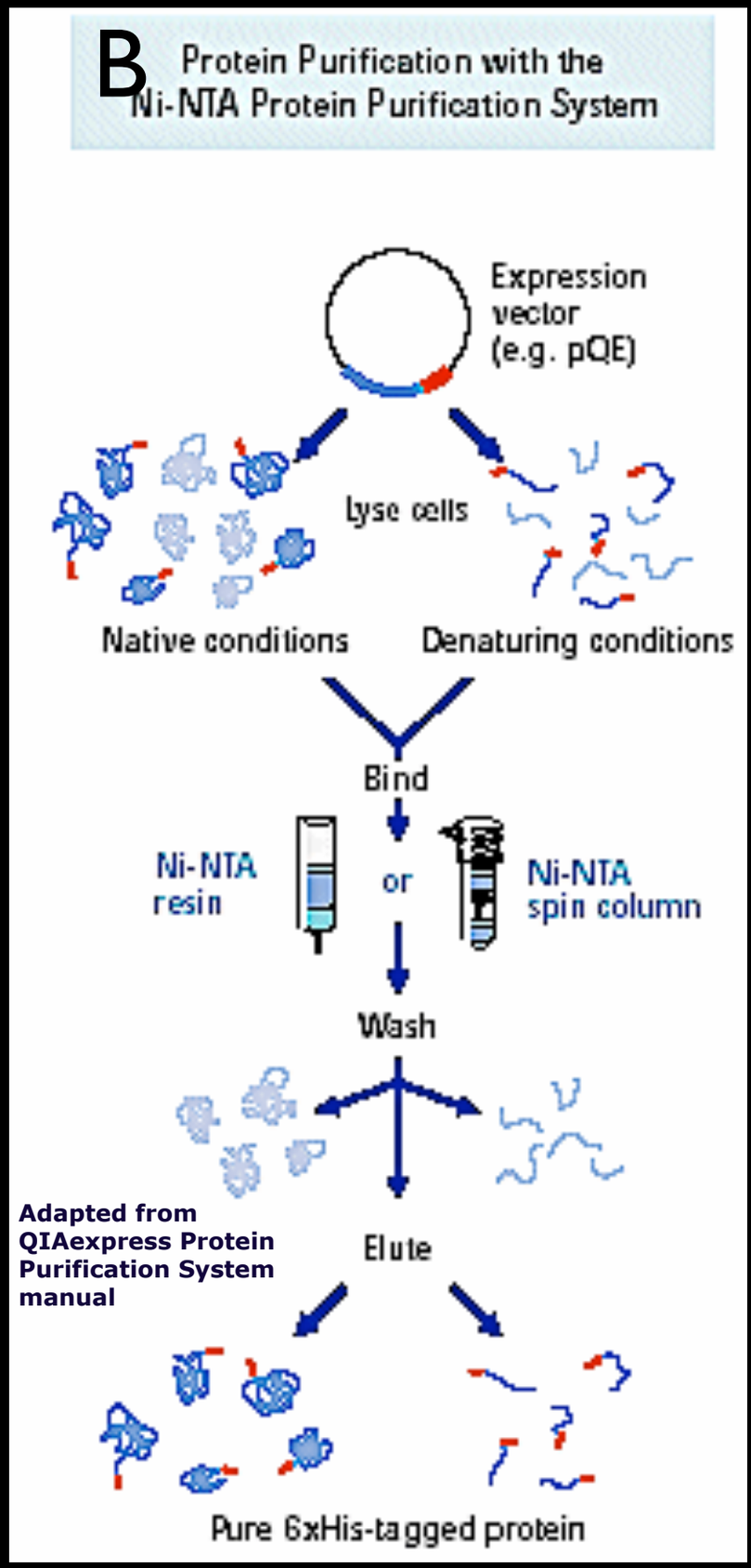


Adapted from ClontechMetal Affinity Resins User Manual

C

A-Schematic representation of the interaction between PolyHis protein and Talon resin. B-Outline of the experimental procedure for the PolyHis protein purification. C-Western blot showing the elution fraction of the N-terminal polyhis tagged GEF8.

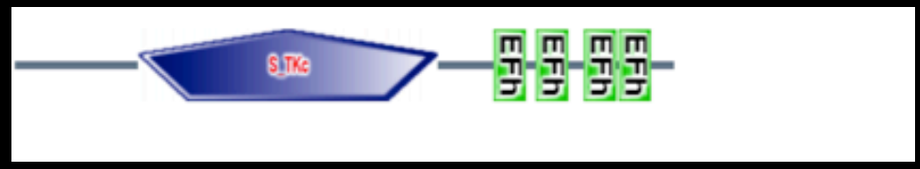
B



Adapted from QIAexpress Protein Purification System manual

Fig 16

A



B

3141 pollen	2	46402	400	
315 pedicel	3	62	21	

CDPK16

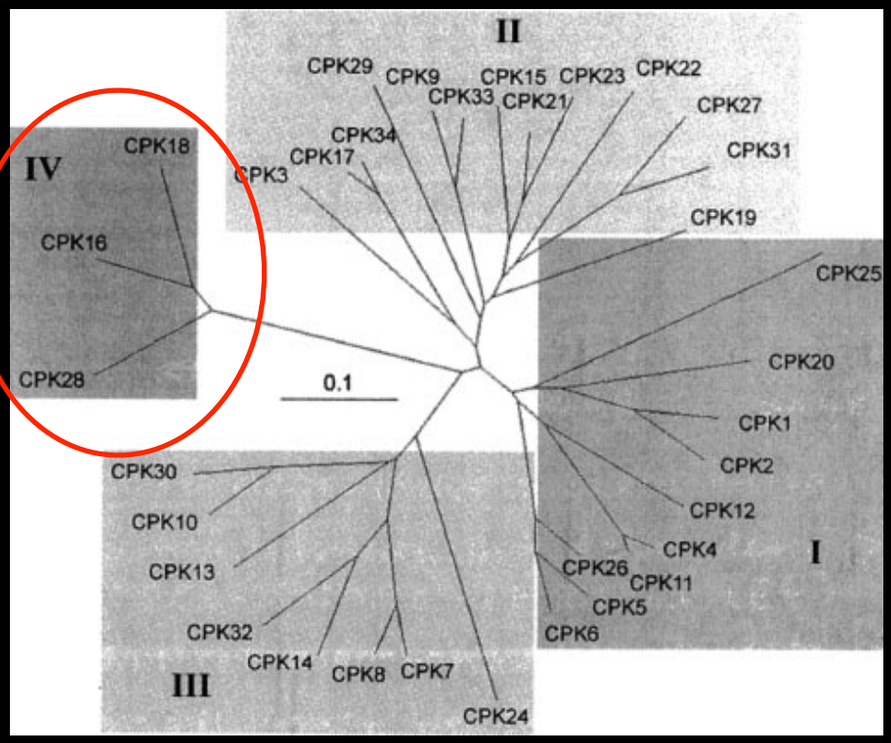
313 sepal	6	5	1	
314 stamen	15	149	41	
3141 pollen	2	299	127	
315 pedicel	3	5	1	

CDPK 18

313 sepal	6	1169	25	
314 stamen	15	225	49	
3141 pollen	2	103	92	
315 pedicel	3	842	47	

CDPK 28

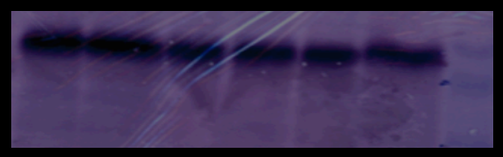
C



A- schematic representation of the CDPK16 structure constructed using the Simple Modular Architecture Research Tool (SMART). B- microarray data from AT Gene Express showing the relative expression of Group IV CDPK. C- Phylogenetic tree of the Arabidopsis CDPK family. (adapted from **Shu-Hua Cheng et. al.)**

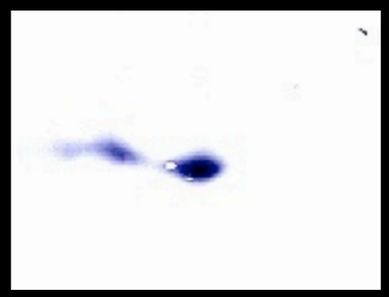
Fig 17 ADF expression in Arabidopsis protoplasts.

C

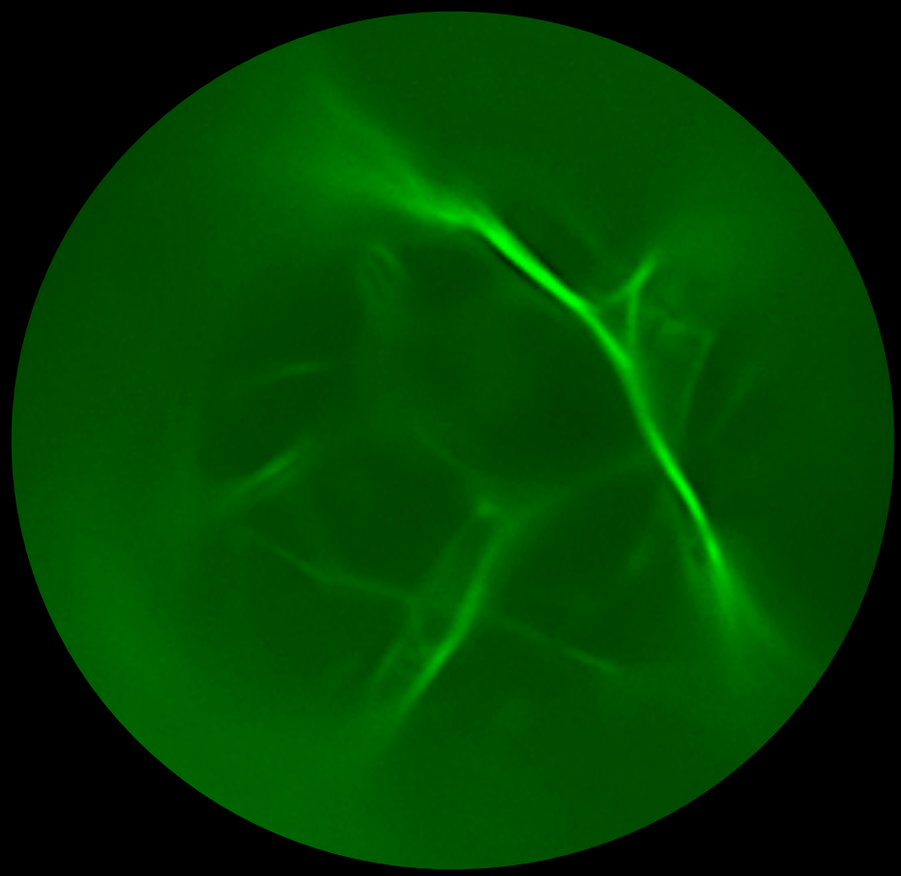


D

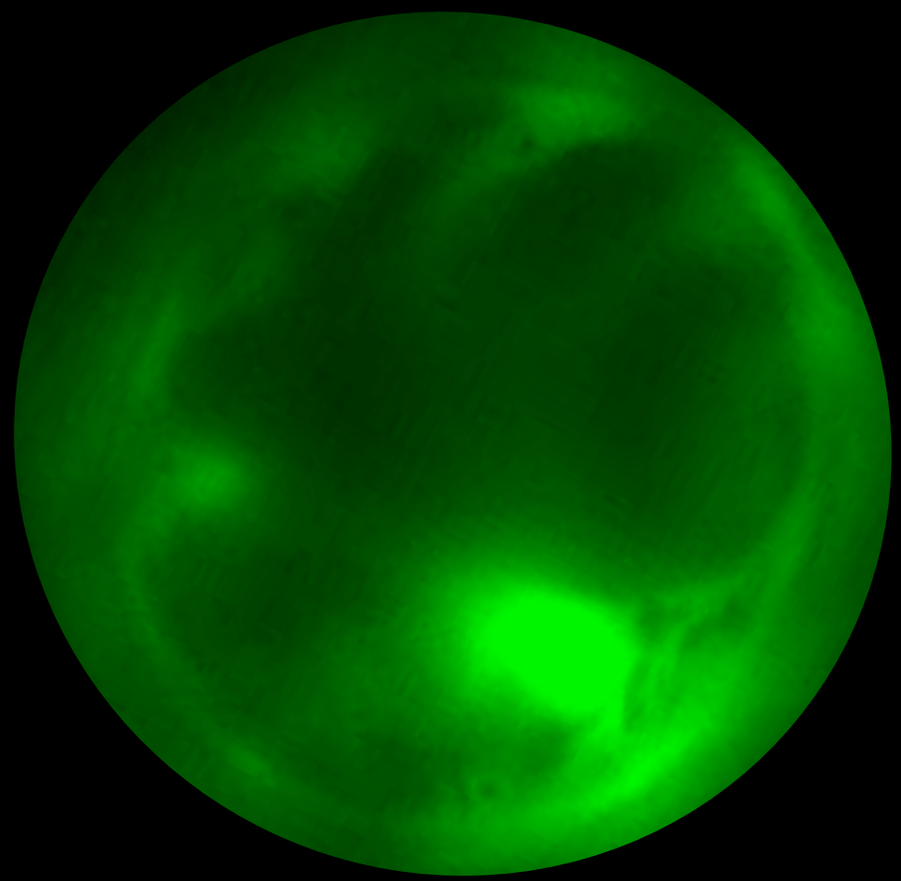
PH4 PH7



A



B

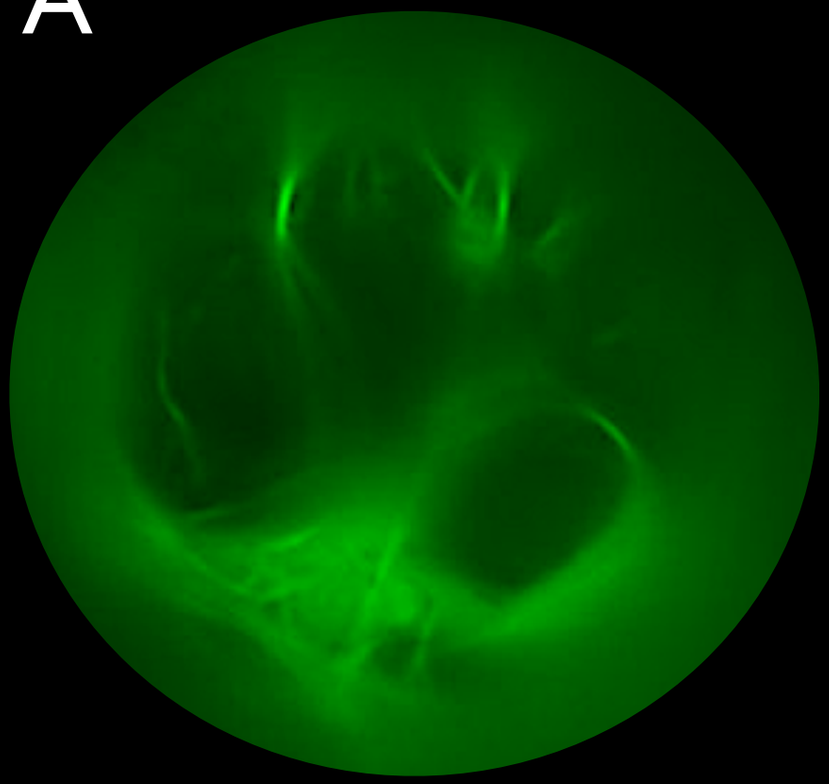


A- plasmid DNA encoding the 35S-GFP-ADF was transformed in to the Arabidopsis protoplasts 16 hours later the images were taken using Nikon E800 microscpe. B 35S-HA-ADF S6D mutant was transformed in to the protoplasts 16 hours later the images were taken. C- The total protein was extracted from the protoplasts transformed with 35S-HA-ADF, resolved on the 15% protein gel, transfered on the PVDF membrane and developed with Anti-HA antibody.
D-2D gel showing the different forms of ADF present in the Arabidopsis protoplasts. The protein was extracted using 2D gel extraction kit (Invitrogen) according to the manufacture instructions.

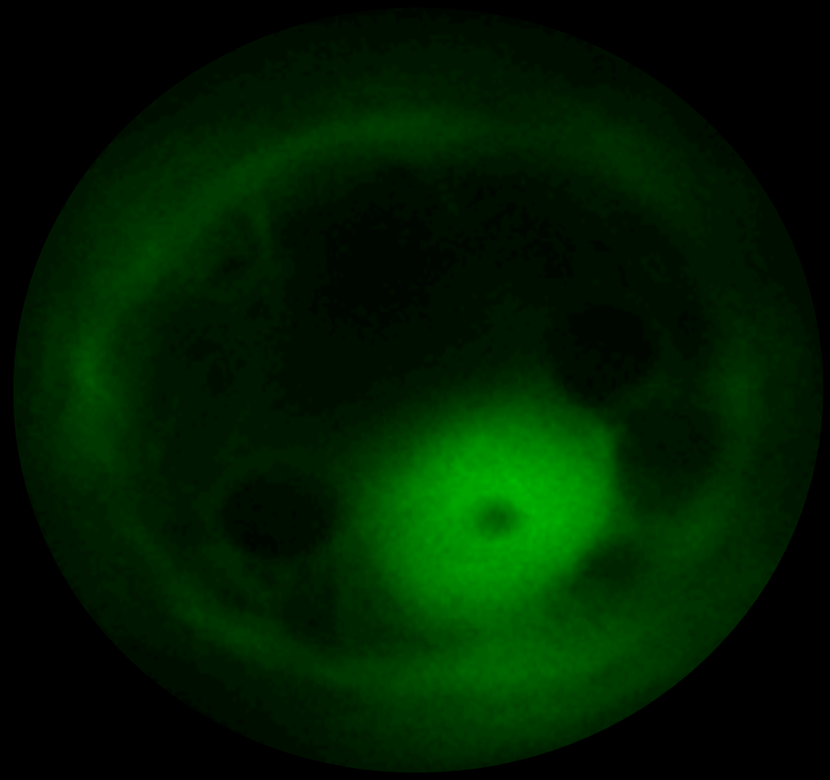
Fig 18

ADF+ CDPK16

A



B



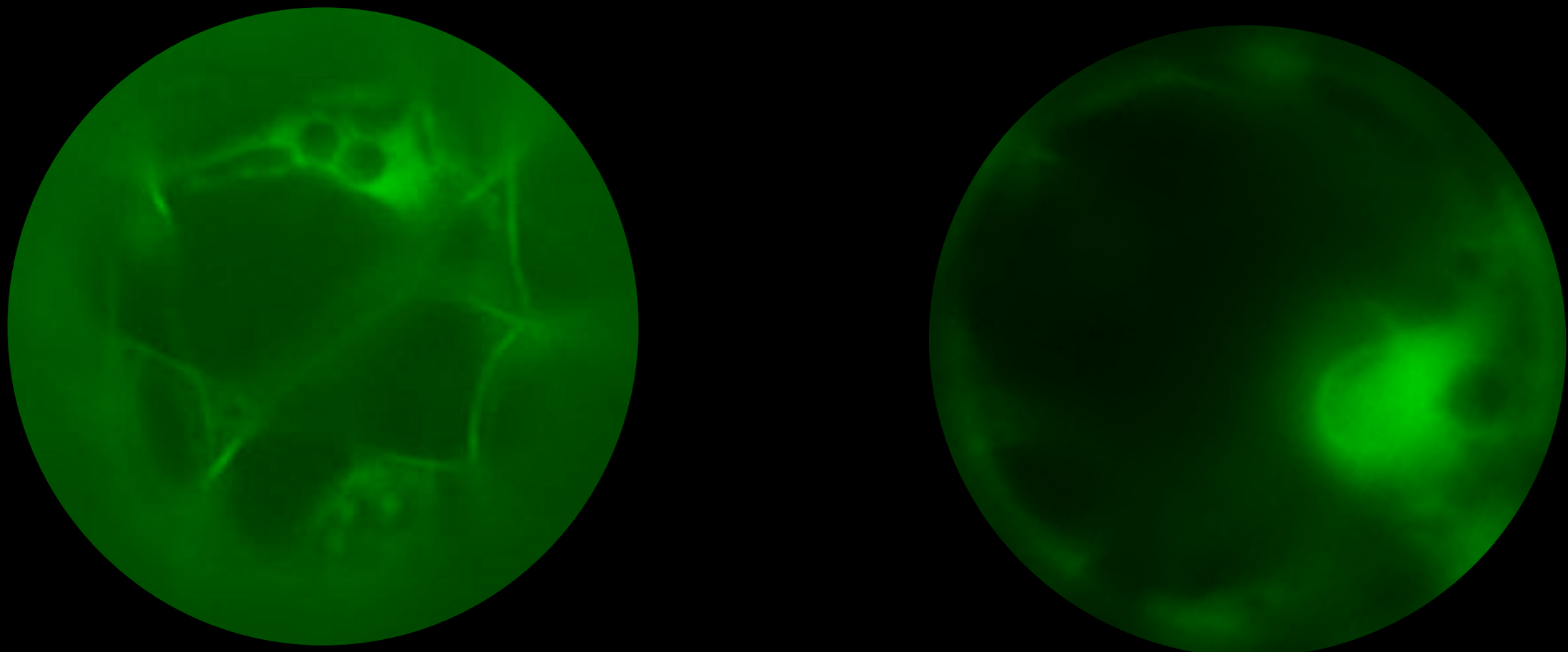
A - Arabidopsis protoplasts were transformed with 35S-GFP-ADF alone
B-Arabidopsis protoplasts cotransformed with 35S-GFP-ADF and 35S-HA-CDPK16 Representative images were taken 16 hours later at 100X magnification.

Fig 19

A

B

ADF+Ric4



A- Protoplasts transfromed with 35S-GFP-ADF alone
B-Protoplasts cotransformed with 35S-GFP-ADF and 35S-HA-Ric4