## *A. Reorientation of pollen tubes with diffusion experiments*

An easy and straightforward method to modulate pollen tube reorientation consists in placing micropipettes loaded with the molecule of study near the tips of growing pollen tubes. This procedure establishes a diffusion gradient across the tip where growth is located, and pollen tubes have been shown to respond to a variety of chemical agents either by attraction, repulsion, or simply no effect. This is not to imply that the molecule inside the micropipette is in any way an *in vivo* chemoattractant; with this type of test, one can simply infer about intracellular mechanisms. We performed this test in the past as a non invasive method to change growth direction and the results were published in three previous papers (1-3). Among the many compounds tested are $Ca^{2+}$ ionophores, $Ca^{2+}$ channel blockers, inhibitors, and activators of protein kinases. In this work, the same experiment was performed with membrane-permeable cAMP and adenylyl cyclase (AC) antagonists and activators in order to find if cAMP is also involved in the intracellular guidance mechanisms. For these experiments, we used pollen tubes of *Agapanthus umbellatus* in which intensive studies have previously been performed and cell biology techniques mastered, namely pressure microinjection and spatial cAMP imaging.

In all experiments, the micropipette was removed once an effect became visible. This is to prevent possible toxic effects due to excessive concentration of agent (either agonist or antagonist).

Reorientation was defined as a change in the growth axis higher than $5^o$, either to the left or right.

## *B. Caged-probe experiments with UV flash photolysis system*

**1 – Estimate of molecule released by flash photolysis**

Using the lowest aperture in the UV tube and the Plan 60 x dry, the area exposed to UV light is a circle of ~10 µm.

Photoactivation was performed by exposing the pollen tubes to a ~1 msec pulse of UV light (300 mJ in the 330-380 nm band) from an XF-10 flash photolysis system (Hi-Tech Scientific, Salisbury, UK) equipped with a xenon arc lamp (4). The concentration released was estimated with caged-fluorescein as reported previously (1).

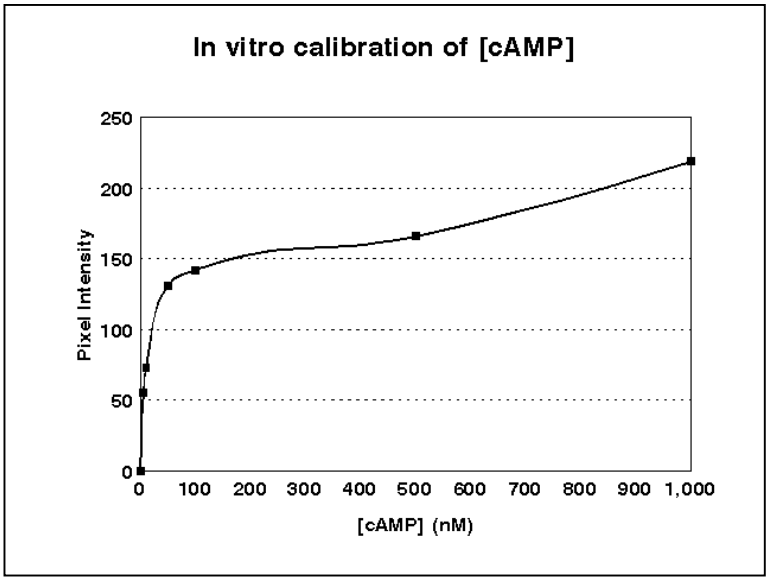
**2 – Release of caged cAMP with flash photolysis system**

- Pollen tubes were exposed to 0.25 mM caged cAMP and left to recover normal growth.
- Reorientation of pollen tubes was defined as a change in the growth axis higher than $5^o$, either to the left or right (5).
- Releasing cAMP in the overall nuclear region (30-50 µm behind the apex) induced similar effects to the sub apical region (see below) but in different proportions: arrested growth (n=6) followed by normal recovery (n=2), slowed growth followed by reorientation (n=12) and no visible effect (n=4). The angle of reorientation induced was always smooth and not abrupt ranging from $25^o$ to $160^o$; the responses were observed 15-25 sec after the release.

- Releasing cAMP in the overall sub apical region (15-30 µm behind the apex) also induced different responses: arrested growth (n=4) followed by normal recovery (n=1), no visible effect (n=3), and slowed growth with random reorientation (n=9). Like the equivalent nuclear release, the angle of reorientation induced was always smooth and not abrupt, ranging from 20$^{o}$ to 85$^{o}$; the responses started to be readily visible 10-20 sec after the release. No tip bursting or arrested growth followed by abnormal recovery was observed.
- Releasing cAMP in the overall tip region (0-15 µm) induced different responses: arrested growth (n=5) followed by normal recovery (n=2), no visible effect (n=4), and slowed growth with random reorientation (n=12). Like the equivalent nuclear release, the angle of reorientation induced was always smooth and not abrupt ranging from 20$^{o}$ to 80$^{o}$; the responses started to be readily visible 5-10 sec after the release. No tip bursting or arrested growth followed by abnormal recovery was observed.
- Release in the left or right part of the tube resulted in different patterns: immediate reorientation towards the side of release (n=14) and no visible effect (n=6). The angle of reorientation induced ranged from 25$^{o}$ to 80$^{o}$.
- As a control for UV exposure, unloaded pollen tubes were exposed to equivalent pulses of UV light, and no effect was observed on growth rates and reorientation.

## *C. Confocal ratio imaging of cAMP in living cells*

The fluorsensor used in this study is based on FRET (fluorescence resonance energy transfer) imaging between the catalytic and regulatory subunits of PKA (labelled respectively with FITC and rhodamine fluorophores; Molecular Probes). It is therefore a very specific method to image the intracellular levels of cAMP in living cells because binding occurs only with this cyclic nucleotide. For this work, an *in vitro* calibration of this dye was used to estimate the intracellular values in living pollen tubes:



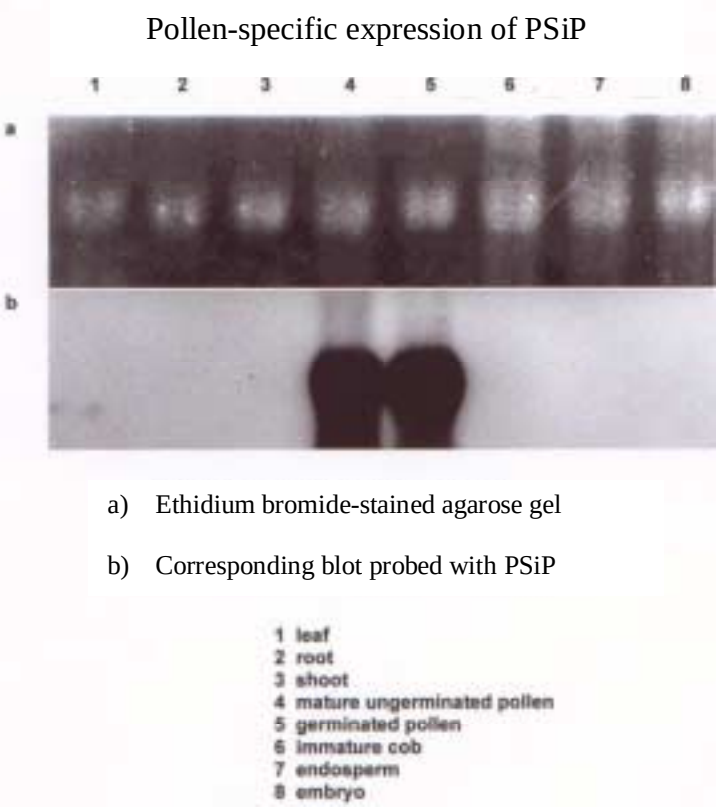
Adams *et al.* (6), who developed this dye, reported that full saturation of the fluorsensor lead to a 31% increase at 530 nm and a 13% decrease at 580 nm (~1.5-fold increase). Our *in vitro* calibration performed with a confocal microscope detecting at 525-555 nm and >600 nm resulted in similar values. Absolute calibration of molecules in cells is, however, recognized to be very difficult, so these numbers should be regarded only as estimates.

Successful pollen tube loading with this 170-kDa dye proved to be extremely difficult and only ~5% of the injected cells recovered growth. The ones that recovered were very sensitive, which defined a limit on the number of tests that one could impose them. Thus, experiments with diffusing chemicals or loading of caged probes could not be achieved.

Images were acquired with a confocal aperture set to optical sections of ~ 8 µm thick and a scan speed of 0.5 sec per frame.

Numerical data presented in Fig. 2 of the manuscript corresponds to single-cell analysis of typical experiments and not to a summary statistics. This is because there is a certain degree of variability, not only on the biological level, but also on the technical one. Even minor changes in the degree of loading, amount of photolyzed biomolecule, area of release (or diffusion from microelectrode), disturbance upon microinjection, and responsiveness of the cell can all play a role in the extent of cellular response.

## *D. PSiP*




a) Ethidium bromide-stained agarose gel

b) Corresponding blot probed with PSiP

## *E. Sequence alignment of PSiP*

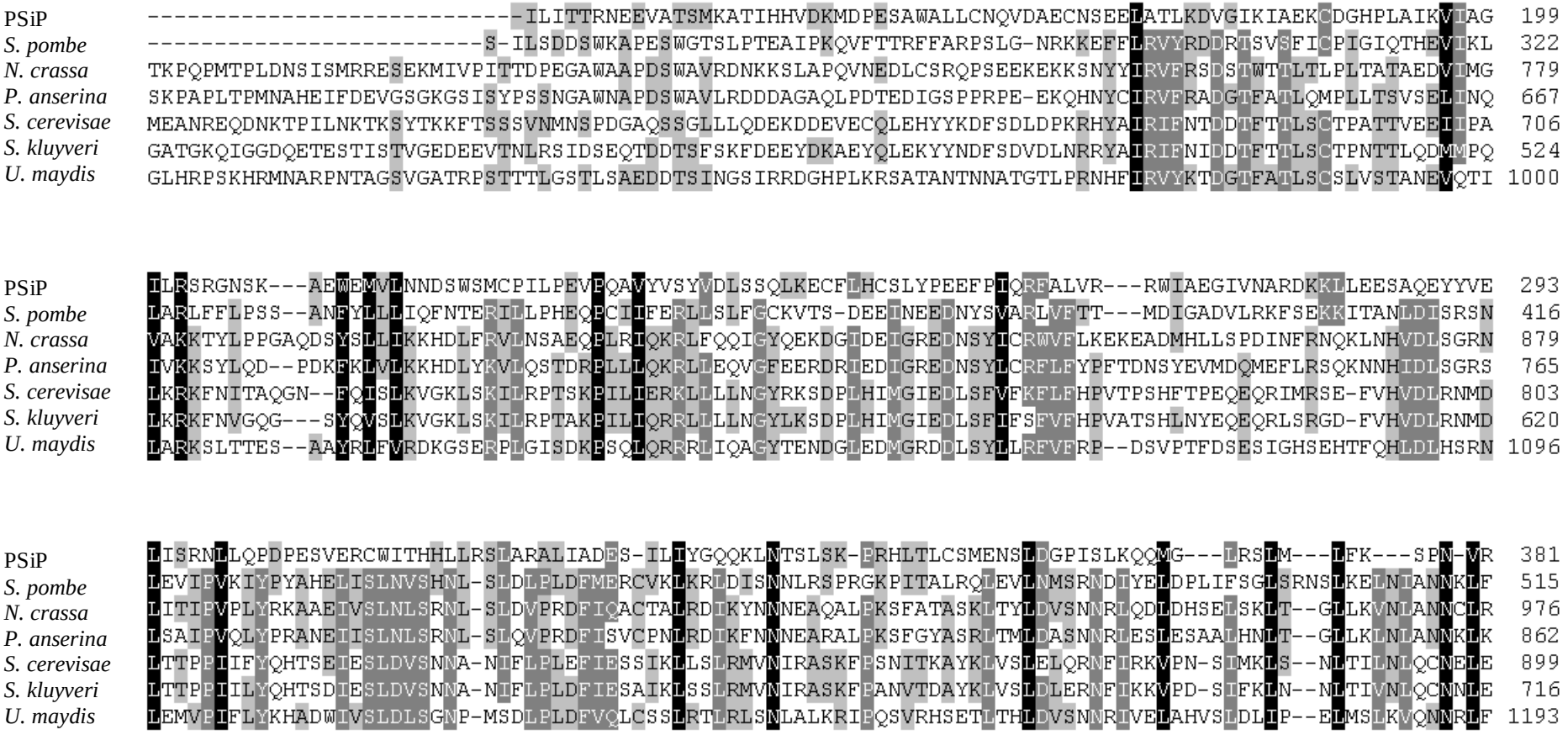
**(1)** Leucine-rich motifs identified in the middle region of PSiP. Numbers on the left are amino acid coordinates, and blanks indicate the placement of gaps that align the sequence to reflect the periodic structure.

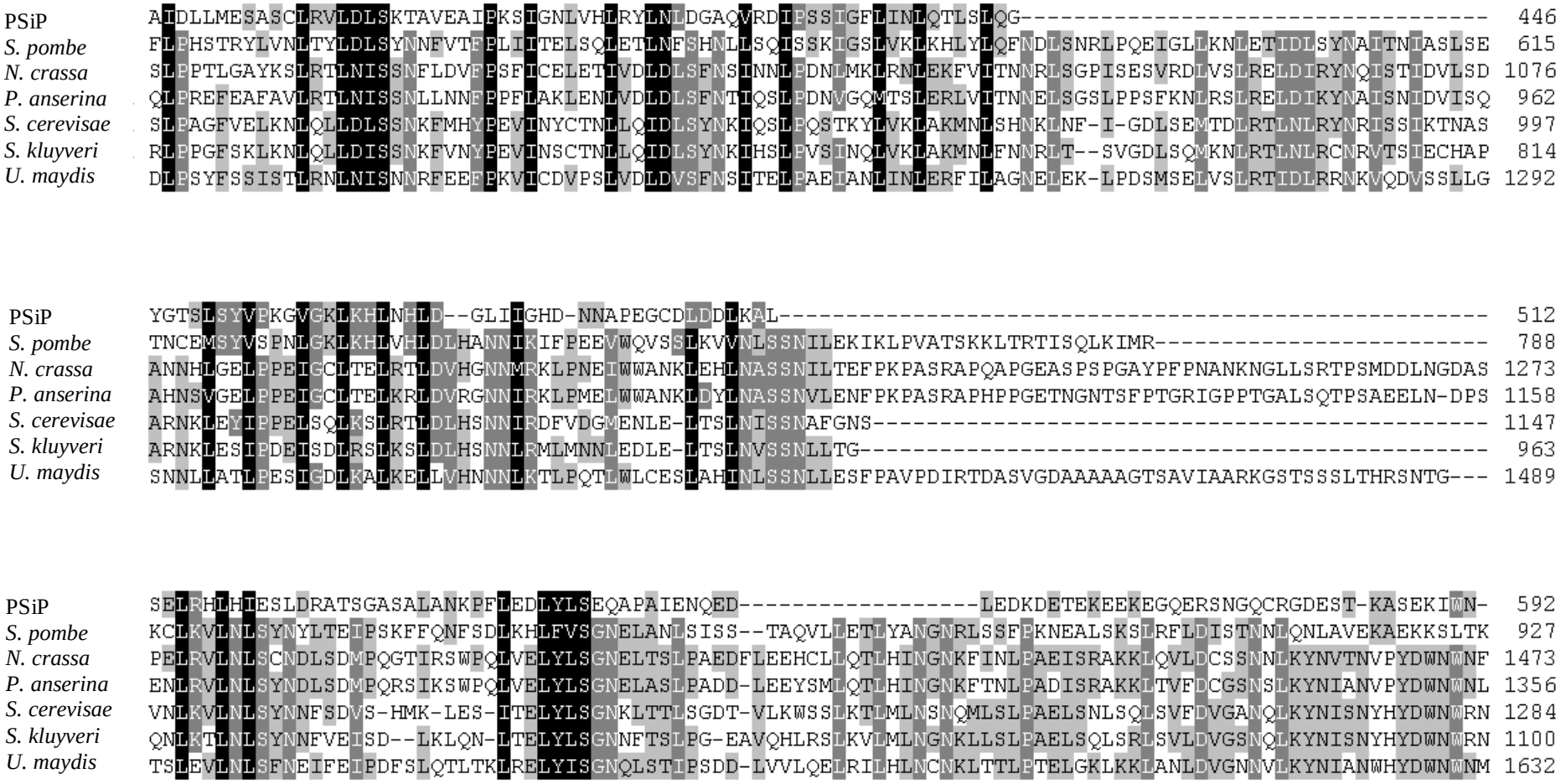
```
360 PISLKQQMGLRSL-ML
407 PKSIGNLVHLRYL-NL
430 PSSIGFLINLQTL-SL
454 PRSIRALLELRCL-CL
477 PKGVGKLKHLNHLDGL
501 PEGCD-LDDLKALSEL
644 PS-LGLLSQLQSLQI-
683 PK-LEVLK-LRNMKKL
711 PC-LKSLQIQWC----
722 PK-LKALPEGLKHVAL
754 PK-LSDELHLKDNKVL
825 PRWLELL--IQKCKGL
```

**(2)** Sequence alignment of PSiP with six fungal ACs that produced significant hits on a BLAST search. The relative similarities are "expect values" (E value) taken directly from a BLAST search of the PSiP vs. SwissProt database. These values estimate the statistical significance of the match by specifying the number of matches expected to occur by chance.

| | ***Similarity (E value)*** |
|---|---|
| *Schizosaccharomyces pombe* | *8.6 x $10^{-2}$* |
| *Neurospora crassa* | 1 x $10^{-6}$ |
| *Podospora anserina* | 9 x $10^{-4}$ |
| *Saccharomyces cerevisae* | 5 x $10^{-2}$ |
| *Saccharomyces kluyveri* | 4.3 x $10^{-1}$ |
| *Ustilago maydis* | 1 x $10^{-4}$ |

*For further information see the alignments (7) below:*


PSiP
S. pombe
N. crassa
P. anserina
S. cerevisae
S. kluyveri
U. maydis


PSiP
S. pombe
N. crassa
P. anserina
S. cerevisae
S. kluyveri
U. maydis

**BLASTP 2.0.14 [Jun-29-2000]**

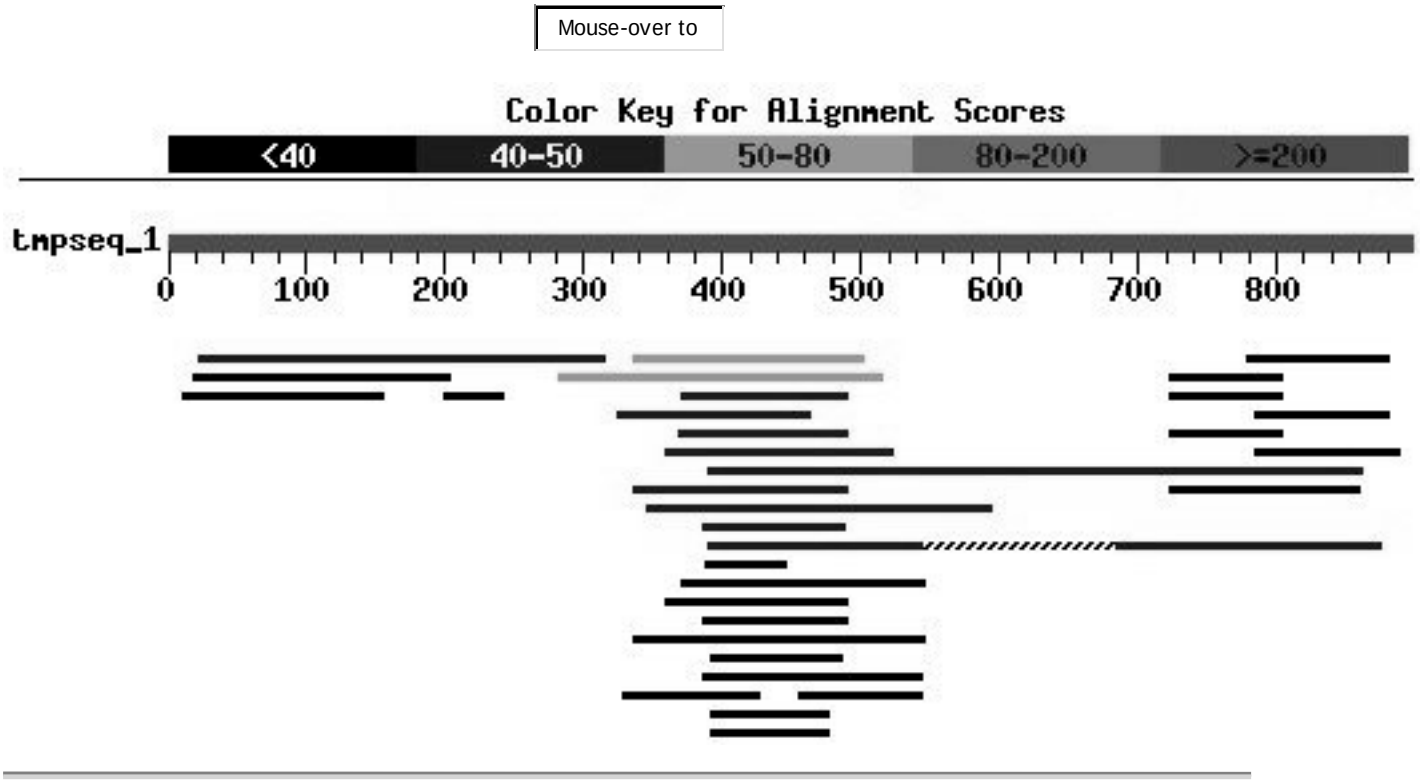
RID: 965383887-22116-15582

**Query=** pac
(897 letters)

**Database:** Non-redundant SwissProt sequences
88,237 sequences; 31,799,033 total letters

Taxonomy reports

## Distribution of 54 Blast Hits on the Query Sequence



| Sequences producing significant alignments: | | Score (bits) | E Value |
|---|---|---|---|
| sp\|Q01631\|CYAA_NEUCR | ADENYLATE CYCLASE (ATP PYROPHOSPHATE-L... | 54 | 1e-06 |
| sp\|P34268\|FLIH_CAEEL | FLIGHTLESS-I PROTEIN HOMOLOG | 50 | 2e-05 |
| sp\|P49606\|CYAA_USTMA | ADENYLATE CYCLASE (ATP PYROPHOSPHATE-L... | 48 | 1e-04 |
| sp\|Q09564\|YR71_CAEEL | HYPOTHETICAL 118.2 KD PROTEIN F43C1.1 ... | 46 | 2e-04 |
| sp\|Q01513\|CYAA_PODAN | ADENYLATE CYCLASE (ATP PYROPHOSPHATE-L... | 45 | 9e-04 |
| sp\|Q15404\|RSU1_HUMAN | RAS SUPPRESSOR PROTEIN 1 (RSU-1) (RSP... | 41 | 0.008 |
| sp\|O14727\|APAF_HUMAN | APOPTOTIC PROTEASE ACTIVATING FACTOR ... | 41 | 0.008 |
| sp\|P23799\|ESA8_TRYBB | PUTATIVE ADENYLATE CYCLASE REGULATORY ... | 41 | 0.008 |
| sp\|O08742\|GPV_MOUSE | PLATELET GLYCOPROTEIN V PRECURSOR (GPV... | 41 | 0.010 |
| sp\|P47735\|RLK5_ARATH | RECEPTOR-LIKE PROTEIN KINASE 5 PRECURSOR | 41 | 0.010 |
| sp\|P31384\|CCR4_YEAST | GLUCOSE-REPRESSIBLE ALCOHOL DEHYDROGEN... | 41 | 0.013 |
| sp\|P26337\|ESA8_TRYEQ | PUTATIVE ADENYLATE CYCLASE REGULATORY ... | 41 | 0.013 |
| sp\|Q14392\|GARP_HUMAN | GARP PROTEIN PRECURSOR (GARPIN) | 39 | 0.039 |
| sp\|P08678\|CYAA_YEAST | ADENYLATE CYCLASE (ATP PYROPHOSPHATE-... | 39 | 0.052 |
| sp\|Q01730\|RSU1_MOUSE | RAS SUPPRESSOR PROTEIN 1 (RSU-1) (RSP... | 38 | 0.068 |
| sp\|P14605\|CYAA_SCHPO | ADENYLATE CYCLASE (ATP PYROPHOSPHATE-L... | 38 | 0.089 |
| sp\|O08770\|GPV_RAT | PLATELET GLYCOPROTEIN V PRECURSOR (GPV) ... | 36 | 0.45 |
| sp\|P23466\|CYAA_SACKL | ADENYLATE CYCLASE (ATP PYROPHOSPHATE-L... | 36 | 0.45 |
| sp\|P02750\|A2GL_HUMAN | LEUCINE-RICH ALPHA-2-GLYCOPROTEIN (LRG) | 35 | 0.77 |
| sp\|P12175\|MATK_ORYSA | PROBABLE INTRON MATURASE | 34 | 1.3 |
| sp\|Q01129\|PGS2_RAT | BONE PROTEOGLYCAN II PRECURSOR (PG-S2) (... | 34 | 1.7 |
| sp\|P25941\|AFSR_STRCO | REGULATORY PROTEIN AFSR | 33 | 2.3 |
| sp\|P28654\|PGS2_MOUSE | BONE PROTEOGLYCAN II PRECURSOR (PG-S2... | 33 | 2.3 |
| sp\|P48190\|MATK_MAIZE | PROBABLE INTRON MATURASE | 33 | 2.3 |
| sp\|P17778\|YOPM_YERPE | OUTER MEMBRANE PROTEIN YOPM | 33 | 3.0 |
| sp\|Q28888\|PGS2_RABIT | BONE PROTEOGLYCAN II PRECURSOR (PG-S2)... | 32 | 5.1 |
| sp\|P17158\|MATK_HORVU | PROBABLE INTRON MATURASE | 32 | 5.1 |
| sp\|Q63912\|OMGP_MOUSE | OLIGODENDROCYTE-MYELIN GLYCOPROTEIN P... | 32 | 6.7 |
| sp\|O83110\|CLPB_TREPA | CLPB PROTEIN | 32 | 6.7 |
| sp\|Q66431\|RRPL_DUGBV | RNA-DIRECTED RNA POLYMERASE (L PROTEIN) | 31 | 8.7 |
| sp\|Q02763\|TIE2_HUMAN | ANGIOPOIETIN 1 RECEPTOR PRECURSOR (TY... | 31 | 8.7 |
| sp\|P23515\|OMGP_HUMAN | OLIGODENDROCYTE-MYELIN GLYCOPROTEIN PR... | 31 | 8.7 |
| sp\|P06695\|TRA5_PSEAE | TRANSPOSASE FOR TRANSPOSON TN501 | 31 | 8.7 |

## *F. Antisense Oligodeoxynucleotides (ODNs) from PSiP perturb pollen tube growth*

Antisense ODNs are able to interfere with genetic information at various levels: transcription, mRNA stability in the cytoplasm, and translation. The uptake of ODNs by pollen tubes and inhibition of gene expression was first demonstrated by Estruch *et al.* (8). In this work, the ODNs were added to the growth medium or combined with calcium phosphate to increase uptake, which potentiated cytotoxic effects. Covalent bonding to carriers and encapsulation in liposomes can ameliorate these problems. Cytofectin GS 3815 vesicles (Glen Research, Sterling, VA) are a formulation of two equivalents of a cationic lipid (dimyristylamidoglycyl-N-α-isopropoxycarbonyl-arginine dihydrochloride) with one equivalent of the zwitterion DOPE (L-α-dioleoylphosphatidylethanolamine), which works within a wide range of lipid/DNA complexes. The cytotoxicity of cytofectin was assayed by germinating pollen under various concentrations, ensuring that, at 15 $\mu g.ml^{-1}$, cells behave similarly to controls in growth medium alone.

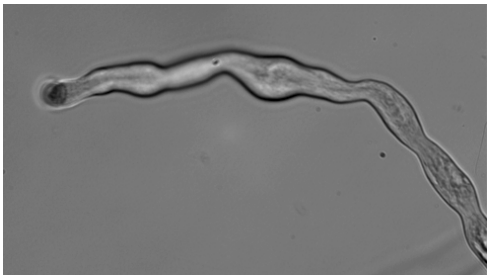
The cytoplasm is an exonuclease-rich environment, and the lifetime of the ODNs depends on the type of backbone linkages. It is generally accepted that common phosphodiester ODNs are rapidly degraded in most cells, with a typical half-life of 20 min, against ~35 h for phosphorothioate ODNs (Glen Research). But for an ODN to increase its resistance, it does not need to have the S modification throughout the molecule. In fact, extensively thiolated molecules tend to be sticky, giving rise to spurious antisense effects from non specific bindings. Therefore, modification of only a few bases at the terminus also blocks nuclease degradation, significantly increasing the half-life of ODNs in cell culture (Genosys, The Woodlands, TX). To augment their resistance to nuclease attack, the ODNs used in our work were designed with a terminal capping of phosphorothioate modifications.

Pollen tubes treated with antisense ODNs from PSiP were found to develop an abnormal growth approximately 3 h after germination. The perturbation on ODN-treated cells results in growth over different focusing planes, unlike control cells that grow often in the same plane. Therefore, images of ODN treated cells have a much lower "quality" when it comes to definition. To overcome this problem, the images presented in the manuscript were acquired at low magnification (higher depth of field) and digitally zoomed thus their low resolution. An example of images acquired at higher magnification is given below.

As part of the control experiments, some cells were incubated with 20 µM dibutyryl cAMP together with cytofectin and the antisense ODNs. Under these circumstances, abnormal tip morphology was abolished, suggesting that bypassing the AC activity is enough to maintain normal growth.

A different set of controls consisted in the addition of the dibutyryl cAMP only after the ODN effect became clearly visible (3 h and 30 min after germination). Approximately 65% of the cells showing abnormal tip growth recovered a normal tip morphology within 5-15 min (Fig. 6H).

In a set of parallel experiments, we replaced the ODN incubation by 0.5 mM dideoxyadenosine, an AC antagonist. The rationale behind this test is that, if PSiP has AC activity, an AC antagonist should produce the same effect as the antisense oligos. Using such concentration of dideoxyadenosine, we found this to be the case, as shown in the figure below:



## *G. cAMP and $Ca^{2+}$*

The relationship of cAMP with $[Ca^{2+}]_c$ is a recurrent theme in plant cell signaling. A soluble 84-kDa putative AC, detected by biochemical methods in alfafa roots, was shown to be activated by $Mg^{2+}$, $Ca^{2+}$ and calmodulin (9). The gating of some of the plasma membrane $K^+$ channels of carrot cells was shown to be controlled by cAMP and the inward $K^+$ current induced by the nucleotide elicited $Ca^{2+}$ influx into the cells, possibly through the activation of voltage-dependent $Ca^{2+}$ channels (10). In tobacco protoplasts, the addition of cAMP resulted in elevation of $[Ca^{2+}]_c$ (11). A similar situation was observed in pollen (12), and it was suggested that the $Ca^{2+}$ response is likely to be part of a feedback mechanism, with the activation of cAMP downstream targets inducing further $Ca^{2+}$ influx or release.

In animal cells, the study of AC has fostered the importance of G proteins in many signaling processes, but current studies suggest that regulation by $[Ca^{2+}]_c$ may be as important (13). An assessment of known ACs reveals that no two forms are regulated in precisely the same manner, and if $[Ca^{2+}]_c$ can activate AC through calmodulin (14), it can also inhibit other isoforms of the enzyme (15). This inhibitory role of $[Ca^{2+}]_c$ is also patent in the regulation of phosphodiesterase, the enzyme that degrades cAMP and is known to be $Ca^{2+}$/calmodulin-activated. Cyclic nucleotide phosphodiesterases are a large multigene family that play a pivotal role in cAMP signal termination and in establishing compartmentalized responses (16). Chiatante *et al.* (17) found a multifunctional cyclic nucleotide phosphodiesterase in pea root tissues capable of hydrolyzing cAMP, but the molecular databases are still devoid of significant homologues to the well known animal counterparts.

Changes in intracellular cAMP and activities of AC and phosphodiesterase have also been reported to occur during meiosis in lily microsporocytes (18).

A major advantage of $Ca^{2+}$ sensitivity in the cAMP pathway would be to make it possible that cAMP levels oscillate in harmony with $[Ca^{2+}]_c$ oscillations (13). Pollen tubes are known to exhibit $[Ca^{2+}]_c$ oscillations (19) but technical limitations (adequate temporal resolution prevented by photobleaching) did not allow us to investigate whether cAMP also oscillates in pollen tubes. Zaccolo *et al.* (20) have recently produced a fluorescent indicator that, using the appropriate mutants of GFP fused to PKA sub units, can generate cAMP-sensitive fluorescence inside living cells, without the need of demanding microinjection techniques. Application of these technologies to plant cells is thus in demand.

## *H. Disease-resistance response and cyclic nucleotide-gated (CNG) cation channel*

Several components involved in signaling resistance reactions have recently been identified and characterized (21), but although many resistance genes have been isolated, receptor function of the corresponding proteins remains to be demonstrated (22).

*Arabidopsis* AtCNGC2 encodes a CNG ion channel that can allow passage of $Ca^{2+}$, $K^+$, and other cations (23). A mutation in this gene was identified as belonging to the *dnd* (defense, no death) class of mutants, which have a reduced ability to produce the hypersensitive response to avirulent *Pseudomonas syringae* (24). $Ca^{2+}$ influx is a critical early step in defense activation processes, and some of these CNG ion channels also carry a calmodulin-binding domain within their amino terminus and can be regulated by intracellular $Ca^{2+}$ levels.

## *I. PSiP vs.* Nicotiana tabacum AC

Ichikawa *et al.* (25) reported the identification of an AC enzyme in a paper that was later retracted. The putative AC enzyme identified in *Nicotiana tabacum* (accession no. AF026389) is a 406-aa soluble peptide, with blocks of leucine-rich repeats and low-level similarity to the AC of *Schizosaccharomyces pombe*.

In the *Arabidopsis thaliana*, database there is a 388-aa homolog to this sequence (26), with 48% identity and 68% structural conservation (Fig. 7).

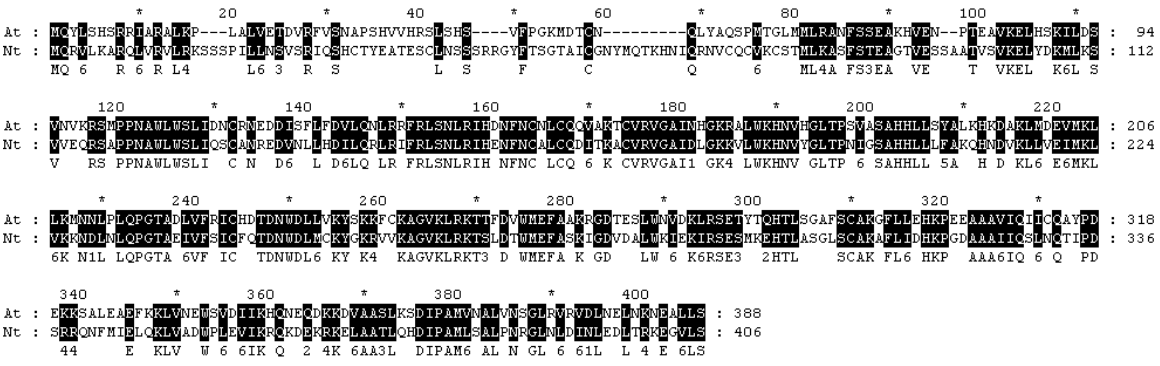


Fig. 7. Alignment between *Nicotiana tabacum* putative AC and an *Arabidopsis thaliana* homolog. The sequences are registered under the GenBank accession nos. AF026389 and BAB02340, respectively. The alignment was performed with ClustalW and edited with GeneDoc software.

PSiP and *N. tabacum* AC sequences share some short conserved motifs, but it is difficult to attribute them any functional significance (Fig. 8). Both sequences putatively translate into a soluble protein and show similarity to fungal AC enzymes, e.g. *Schizosaccharomyces pombe*, especially in the repeats of leucines, a motif known to be involved in protein-protein interactions in signaling pathways.

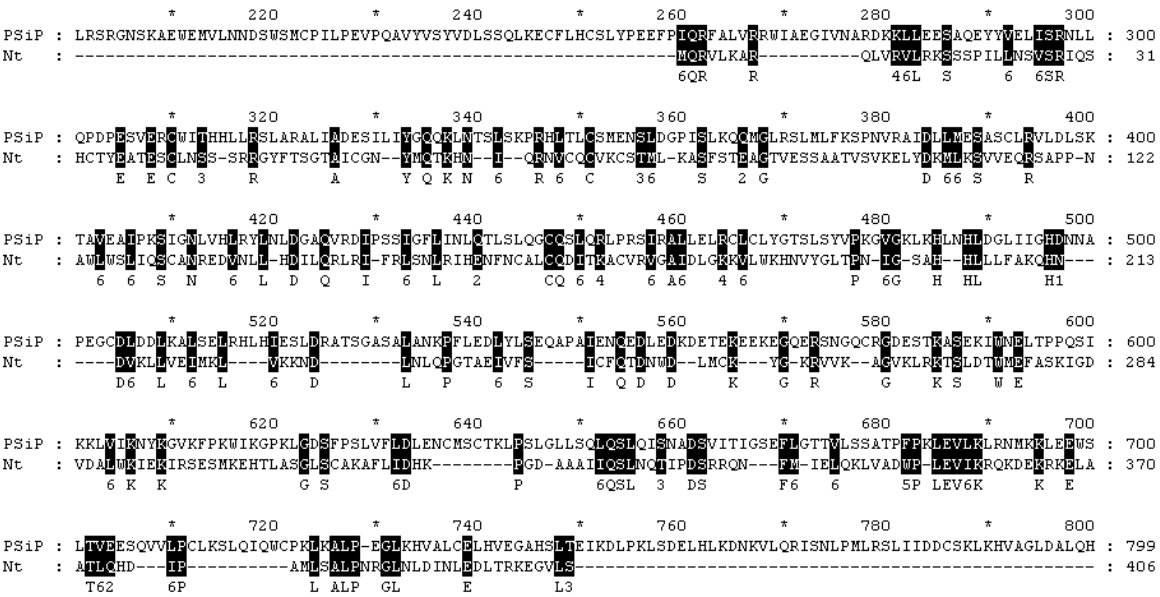


Fig. 8. Alignment between *N. tabacum* putative AC and PSiP. The alignment was performed with ClustalW and edited with GeneDoc software.

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