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Antisense perturbation of protein function in living pollen tubes

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Abstract During the past few years pollen tubes grown in vitro became a popular model system for cell biology studies of signal transduction in plant cells. Here we report a simple and fairly inexpensive way of studying protein function by transiently perturbing expression of the target gene in living pollen tubes. The ability of antisense oligodeoxynucleotides (ODNs) to bind to complementary mRNA sequences was used to selectively inhibit gene expression and thus assess the putative function of specific proteins in tip growth. The delivery of ODNs to growing pollen tubes was accomplished with the help of a liposomal formulation, originally developed for transfection assays in animal cells. The limitations and potentialities of this technique are discussed.

Keywords Antisense · *Agapanthus umbellatus* · Cytofectin · Oligodeoxynucleotides · Pollen tube

Introduction

Pollen tubes are polarised growing cells capable of orienting themselves inside female tissue to fertilise the ovule. In their intracellular signalling cascade, the key role of cytosolic free calcium as a second messenger has been reported (Malhó et al. 1995). The multitude of extracellular stimuli that evoke a Ca^{2+} response is impressive, and downstream events are likely to involve a myriad of target molecules. Among these are calmodulin (Moutinho et al. 1998a), Ca^{2+}-dependent protein kinases (Moutinho et al. 1998b), Ins (1, 4, 5) P3 (Malhó 1998) and Rho GTPases (Lin et al. 1996). The identification of these target molecules is often complex and requires various types of cellular and molecular approaches. In this work we used a simple and rapid method to assess the putative role of key proteins in tip growing mechanisms. This consists of loading growing pollen tubes with antisense oligodeoxynucleotides (ODNs) directed against specific mRNAs.

Antisense-mediated inhibition of gene expression has become a valuable experimental tool in many fields of research. Antisense ODNs are being evaluated in clinical trials as novel therapeutical agents, and various models have been studied intensively, both in vivo and in vitro, to further improve their properties. Antisense ODNs are able to interfere with genetic information at various levels: transcription, mRNA stability in the cytoplasm and translation.

Two important factors that determine the effectiveness of antisense ODNs in vivo are susceptibility to degradation and intracellular uptake. Negatively charged molecules, like ODNs, possess very little or no ability to diffuse across cell membranes. Various strategies have been employed to improve the internalisation of ODN through the plasma membrane, the most significant being covalent bonding to carriers and encapsulation in liposomes. Cationic lipids are effective not only in enhancing ODN uptake but also in preventing extra- and intracellular degradation. In the cytoplasm, the major route of degradation is the rapid hydrolysis of phosphodiester bonds by exo- and endonucleases. Phosphorothioates (or S-oligos) are a variant of normal DNA in which one of the non-bridging oxygens is replaced by a sulphur. Apparently, this modification renders them more resistant to a variety of nucleases, while retaining the parental construct´s properties of high water solubility and specificity in forming duplexes with specific target mRNAs.

The use of transfection agents increases the specificity of the antisense effect by permitting the use of ODNs at lower concentrations. Recently, a novel transfection agent was reported which showed improved levels of transformation efficiency and low toxicity in a variety of animal cells (Lewis et al. 1996). In this work a similar

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strategy was applied to living pollen tubes, as a means to assess a particular protein function in growth and reorientation.

Material and methods

Designing the antisense oligodeoxynucleotides

Antisense ODNs were designed on the basis of available sequence data relative to the proteins under study. In this work we report the results obtained by using two different approaches. Firstly, using PCR cloning and RACE techniques, cDNAs encoding a putative signalling protein were cloned from pollen of *Agapanthus umbellatus*, and the conserved regions used to design antisense probes. Special attention was devoted to avoiding sequences with significant secondary structure, as the mRNA target regions are less accessible for hybridisation. Secondly, an available pollen cDNA library (non-homologous) was screened for clones that encode proteins with a putative role in tip growth. The candidate sequences were submitted to database searches and alignment algorithms that allowed us to map the consensus regions among various members of the gene family and identify conserved motifs for antisense binding. In the in vitro assays different oligonucleotide sequences, from 16 to 21 bp, were tested. Controls included the correspondent sense sequences and non-specific sequences from synthetic plasmids. Custom-made oligodeoxynucleotides (HPLC purified) were obtained from Sigma-Genosys with two different types of backbones, native phosphodiester and with phosphorothioate modifications in both the 5´ and 3' terminus (three or four bases modified in each end), indicated in lowercase: AS1 (5'-tagtGATGGGAAcagc-3'); AS2 (5'-tgagCATGCAGGtctt-3'); AS3 (5'-gagGGCGTCCACGTCGCCgat-3'); AS4 (5'-gtcGATGAGGAAGGCctt-3'); and AS5 (5'-ctcCTCCTTCTCGGTCTCgtc-3').

ODN delivery to growing pollen tubes

Pollen of *Agapanthus umbellatus* was germinated in 50 µl of liquid medium, modified from Brewbacker and Kwack (1963), containing 2.5% sucrose, 0.01% H_3BO_3, 0.02% $MgCl_2$, 0.02% $CaCl_2$ and 0.02% KCl, pH 6.0 as described previously (Malhó and Trewavas 1996). BK medium was supplemented with ODN and transfection agent according to the different assays. Germination was performed in the dark, at 20–23°C under humid conditions. Cytofectin GS 3815 vesicles (Glen Research, Sterling, Va.) is 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 lipid suspension stock (2 $mg.ml^{-1}$) was stored at 4° C, and the complex ODN/cytofectin prepared freshly for each delivery assay. Several parameters were assessed during growth in germination medium supplemented with a concentration range of cytofectin: 1, 5, 10, 15, 20, 25 and 50 $\mu g.ml^{-1}$. After evaluating the efficiency and toxicity of cytofectin in growing pollen tubes, a concentration of 15 $\mu g.ml^{-1}$ was used in the experiments.

The ODN/cytofectin complexes were prepared as described by Lewis et al. (1996). Briefly, the stock oligo DNA and cytofectin were incubated for 15 min at room temperature. This formulation was then diluted with germination medium to achieve a final concentration of 30 µM ODN and 15 $\mu g.ml^{-1}$ cytofectin. Pollen grains were germinated in liquid medium with the supplements correspondent to each assay, and observations were recorded every 10 min. All results shown were individually repeated a minimum of six times.

Imaging

Images were collected with a Nikon Microphot microscope, using a PlanApo 20× objective, and recorded on Kodak ektachrome film, or captured with a V-scan cooled charged-coupled device (CCD) camera (Photonic Science) attached to an Olympus IX-50 inverted microscope, and using an Olympus LCP PlanFl 40× objective. Length, diameter and growth rate of the pollen tubes were calculated for 20 pollen tubes per assay using Image-Pro Plus 4.0 software (Media Cybernetics).

Molecular control of RNA hybridisation with ODN probes

Total RNA was isolated from germinated pollen by a phenol/SDS standard procedure. For northern blots, total RNA was electrophoresed in a 1% denaturing formaldehyde gel and capillary transferred to nylon positive membranes (Roche Biochemicals). cDNA probes were PCR-labelled, using DIG-11-dUTP (Roche Biochemicals) in the reaction mixture, and ODNs were 3'end-labelled with DIG-11-ddUTP (Roche), following manufacturer instructions. Dot plots were prepared using 5 µg of total RNA and probed with DIG-labelled ODNs. Detection was performed with CSPD substrate (Roche) and chemiluminescence signals recorded on Kodak Biomax film.

Results and discussion

Growth conditions

Pollen tubes from angiosperms can be germinated in vitro provided some essential nutrients are included in the growth medium. These include ions like calcium, potassium, magnesium and boron, along with a carbohydrate source like sucrose. The optimisation of growth media is outside the scope of this report and should be ameliorated for each particular species. Under optimised conditions, in vitro pollen tubes can grow at very high rates (*Agapanthus umbellatus* standard in-vitro rate is ~15 $\mu m\ min^{-1}$), which implies a fast metabolism and high protein turnover.

Contrary to what happens with the majority of animal cell types, the incubation and observation periods of in vitro germinating pollen tubes are normally restricted to less than 24 h. This is probably caused by a shortage of nutrients that in vivo are supplied by the female tissue, e.g. arabinogalactans (Wu et al. 2000). Thus, in order to avoid observations at periods where erratic behaviour and cellular death is common, we restricted our experiments to incubation periods of 8 h maximum.

Uptake of ODNs

Whenever the objective is to introduce exogenous molecules inside living cells, the plant researcher faces an additional ordeal, the plant cell wall. When it comes to cell wall structure, pollen tubes represent a unique case since the growing tip is surrounded by a loose matrix of hemicellulose and pectins (Mascarenhas 1975, 1993). This is likely to expose the plasma membrane to diffusing substances from the outside and to facilitate internalisation of substances from the medium, possibly by endocytotic mechanisms (O'Driscoll et al. 1993).

The effectiveness of ODNs depends directly on the concentration of molecules that are taken up by the cell.

The uptake of ODNs by pollen tubes was first demonstrated by Estruch et al. (1994). In this work 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. However, high extracellular concentration of ODNs (even when encapsulated) may induce toxicity. Thus, the exact concentrations of cytofectin and ODNs used should be optimised for each species being tested. Of particular importance is the concentration of lipid suspension, since the behaviour of pollen tubes is strongly dependent on the oil composition of the growth media (Wolters-Arts et al. 1998). For *A. umbellatus*, cytofectin concentrations above 20 µg ml^{-1} have proved to be cytotoxic, leading to abnormal tip morphology. A concentration of 15 µg ml^{-1} did not affect growth rate, tip morphology or polarity maintenance and was thus chosen for the antisense experiments.

Intracellular degradation of ODNs

The cytoplasm is an exonuclease-rich environment, and the lifetime of the ODNs was shown to be dependent 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, compared to approximately 35 h for phosphorothioate ODNs (Glen Research). But for an ODN to show increased 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). In this work, and for the same sequences, both phosphodiester and phosphorothioate-capped oligos were tested. The observations showed that the latter are indeed more effective, with visible phenotypic changes occurring sooner than was registered for its normal phosphodiester counterpart.

Hybridisation of ODNs with pollen mRNA

One of the methods used to design the antisense ODNs was based on information gathered after screening a non-homologous pollen cDNA library. When this strategy is used, hybridisation of the non-homologous probes to the mRNA of the species under study must be determined. In this work we tested the hybridisation of *A. umbellatus* pollen mRNA by northern and dot blotting. The DIG-labelled cDNA containing the coding region of the signalling protein selected from the pollen library was shown to hybridise to a 2.0-kb transcript (Fig. 1a). The antisense probes were also labelled with DIG and hybridised to *A. umbellatus* pollen RNA, demonstrating the presence of an mRNA containing the target motifs (Fig. 1b).

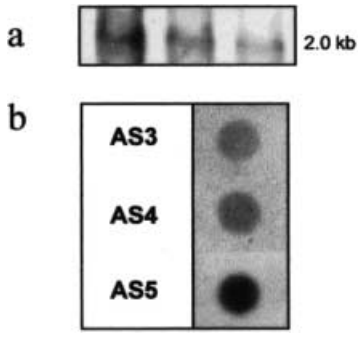

Fig. 1 Northern and dot blot analyses of *Agapanthus umbellatus* pollen RNA probed with antisense ODNs. **a** The DIG-labelled cDNA (coding region of a putative signalling protein) hybridised to a transcript of 2 kb. **b** 3'end-DIG-labelled antisense AS3, AS4 and AS5 ODNs show positive hybridisation in total RNA dots

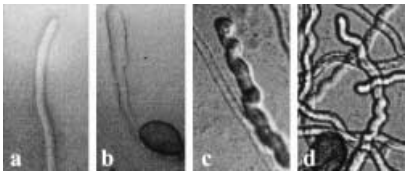

Fig. 2 Effect of antisense ODNs in pollen tubes germinated in vitro. **a** Control pollen tube germinated in medium supplemented with 15 µg ml^{-1} cytofectin. **b** Cells treated either with AS1 or AS2 show a pronounced increase in tube diameter after 1 h. **c–d** Effect of AS5 probe in growing pollen tubes after 3 h germination. Clear perturbation of tip growth leads to abnormal morphology. AS3, AS4 and AS5 have similar effects on the growth patterns

Controls of antisense assays included experiments with the correspondent sense ODNs, as well as antisense oligos targeted for plasmid sequences (M13 and universal reverse primers). Antisense ODNs without cytofectin encapsulation failed to evoke detectable responses.

Effect of ODNs in pollen tube growth and morphology

During the first hour of germination antisense-treated cells behaved similarly to controls (Fig. 2a). Pollen hydration occurred after approximately 5 min and pollen tubes emerged after approximately 20 min germination. Pollen tubes grew at a standard rate of 12 µm min^{-1} and tube diameter was about 10 µm. After the first hour signs of perturbation started to be observed when using AS1 and AS2 (for sequences see Material and methods). Pollen tubes treated with either AS1 or AS2 showed a lower growth rate (Fig. 3) and had a much wider diameter at the growing tip (Fig. 2d). Three hours after germination the perturbations were clear and disseminated. Treatment with AS3, AS4 and AS5 took longer to achieve a visible effect, obtained 3 h after germination (Fig. 2b,c). The observations showed that tip morphology was clearly altered, rendering growth significantly slower and suggesting a fundamental role for this protein in the guidance mechanisms underlying tip growth.

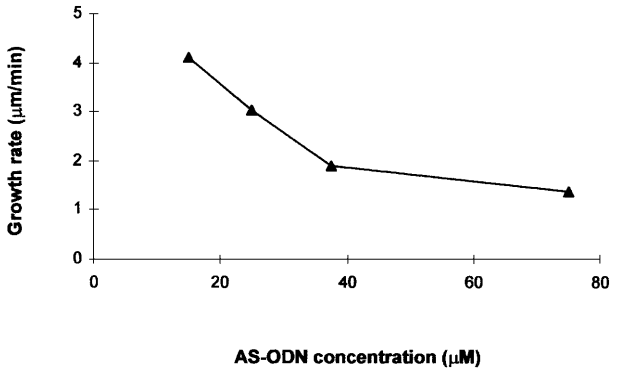

Fig. 3 Dose-response curve of pollen tube growth rate with AS-ODN concentration. Control pollen tubes, germinated in medium supplemented with 15 μg ml^{-1} cytofectin grow at a standard rate of 12 μm min^{-1}. Pollen tubes treated with AS2 show a decrease in growth rate, which is inversely correlated with the AS-ODN concentration. Data are mean ± SD from 20 pollen tubes

Advantages and drawbacks

The use of antisense ODNs presents several advantages but also considerable limitations that must be taken into account. The main limitation of this technique is the loading of the molecules into cells. Even with a liposomal formulation, only small-sized ODNs could be loaded into pollen tubes. The uptake of the ODNs is likely to occur through diffusion across cell membranes, and it is possible that the technique works only in cells where this membrane is made accessible by the high turn-over of cell wall components, such as in the tips of pollen tubes and fungal hyphae.

An advantage of antisense ODNs, when compared to knockout experiments, is that it is possible to test different concentrations and obtain a gradient on the intensity of responsive phenotypes. Figure 3 shows a dose-response curve using different ODN concentrations. It clearly indicates that pollen tube growth is perturbed in a dose-dependent manner by the AS2 ODN. These experiments were performed using an extracellular concentration of cytofectin that did not affect growth rate, tip morphology or polarity. However, it is possible that different ODNs may exhibit different cytotoxic effects. The purity of commercially acquired ODNs is variable and may significantly affect tip growth. It is therefore imperative that appropriate controls are performed before tracing a dose-response curve.

The time delay between the incubation with antisense ODNs and the appearance of the first perturbations in tip growth can also be used as an indication of the turnover of a particular protein during tip growth. In the first stages of germination, protein synthesis is not essential, as shown by incubation with actinomycin D (Mascarenhas 1966). However, in the later stages, de novo synthesis of both protein and RNA occur (Mascarenhas 1975). These observations are consistent with our results where the ODNs had no effect on initial tube germination and tube growth.

With the growing availability of database information, namely the near completion of the *Arabidopsis* genome, the antisense strategy can be very useful in preliminary tests to determine the effect of a protein for tip growth. The usefulness of this method is greater when the species under study is not a "model" system for molecular studies (e.g. *Arabidopsis*), but has considerable advantages in cell biology experiments (e.g. *Liliaceae*). Gene families are frequently characterised by consensus motifs and so this method is a possible alternative when the complete homologous sequence has not been identified in the species under study. In this work, the strategy proved adequate for designing antisense ODNs. Furthermore, it is a rapid and inexpensive way to assess the putative function of proteins in tip growth and can thus be an alternative for difficult and time-consuming knockout experiments.

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