

A method to quantify amounts of mRNA coding for Lol p 1 in pollen from *Lolium perenne*

Britt Berggren^{1*}, Mari Källersjö² & Cenk Suphioglu³

¹Palynological Laboratory, Swedish Museum of Natural History, S-104 05 Stockholm, Sweden; ²Molecular Systematic Laboratory, Swedish Museum of Natural History, S-104 05 Stockholm, Sweden; ³Department of Allergy and Clinical Immunology, Monash Medical School, Alfred Hospital, Prahran 3181, Australia
(*author for correspondence, e-mail: britt-berggren@swipnet.se)

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Abstract

A method for quantification of mRNA coding for the allergen Lol p 1 is described. Quantitative RT-PCR is performed using a mutant RNA sequence as an internal standard, slightly longer than corresponding wild-type RNA. The mutant, which contains a unique *Hind* III restriction site, has been synthesised by splice overlap extension PCR of corresponding wild-type cDNA. Purification has been effected by ligation into pCR-Script SK(+) and introduction into *E. Coli* XL1-MFR. The mutant is obtained by *in vitro* transcription of purified and *Bam*H I-cut plasmid.

RT-PCR is performed in one-tube procedures where RTase and polymerase are both added at the same time, thus minimising the danger of contamination. After digestion with *Hind* III the product is analysed by polyacrylamide gel electrophoresis in an automated sequencer, AlfExpress. A special software (Fragment manger) allows direct estimation of the areas under respective peaks. A linear correlation between area and DNA amount exists over more than 2-log range. A preliminary “titration” to estimate best relation between standard and unknown sample is recommended. Samples comprising 30 pollen grains can be successfully analysed. Standard deviations of about 10% can be obtained in triplicate samples. For increased reliability at least five samples of each batch of pollen should be analysed. The quality of the pollen material should be checked by light microscopy.

1. Introduction

The number of people allergic to pollen has increased greatly during recent years. The reason why environmental pollution has enhanced the allergenicity (Scheiner and Kraft, 1995) has not yet been explained. However, interaction between the major ryegrass pollen allergen Lol p 1 and diesel exhaust carbon particles (DECP) has recently been reported (Suphioglu, 1998). The composition or quantities of the allergens in airborne pollen grains might have been effected. Some kind of synergism is also possible. A third explanation is that environmental pollutants have brought about an increased expression of IgE binding compounds. Investigation of how pollen allergens are influenced by

environment demands a thorough knowledge of their function.

Two main functions of pollen allergens have been suggested. Firstly: a role connected with pollen germination on the stigma, and secondly: as a protection against pathogens and perhaps other kinds of stress. One or several of the allergens might be so-called stress proteins. There are many examples of proteins and peptides that are synthesized by pollen grains and other plant cells in response to various kinds of stress. Most studied are heat shock proteins (e.g. Frova et al., 1991) and metallothioneins and phytochelatin (peptides) that protect against heavy metals.

It is worth noting that the mRNA that encodes one of the major birch pollen allergenes (Bet v 1) is tran-

scribed relatively late during pollen development. It has been proposed that this allergen is a disease resistance protein (Breiteneder et al., 1989), i.e. acting as a stress protein. The theory is founded on homology between the gene encoding Bet v 1 and a family of pathogenesis-related proteins, encoded by genes found in pea, bean, parsley, and potato (Scheiner and Kraft, 1995). These authors also refer to data indicating that environmental pollution, especially car-exhaust fumes, might lead to enhanced expression of Bet v 1 in birch pollen and other tissues of birch.

Two other birch pollen allergens have been isolated and described. One of them, Bet v 2, that is a minor allergen, belongs to the group of profilins (Valenta et al., 1991). The complex between profilin and actin is dissociated during rehydration of the pollen grains (Grote et al., 1993), for instance in connection with pollen germination. Leaching of profilin should thus promote formation of the cytoskeleton and facilitate pollen tube growth.

Another allergen, Bet v 3 is a calcium binding protein (Seiberler et al., 1994) with an ability to effect many functions in the mature pollen grain. The importance of calcium during pollen germination is well known.

In contrast to the birch pollen allergens, enzymatic activity of some grass pollen allergens has also been suggested. Dumas et al. (1984) proposed that some of them could have a role in recognition between pollen grains and the stigma of the pistil. Bufer et al. (1995) have recently shown that Phl p 5b from timothy grass has RNase function and suggested that it might be involved in defence against pathogens. Many other key roles in plant development have been ascribed to ribonucleases, e.g. differentiation, reaction to light, water stress (Newbigin et al., 1993).

cDNA cloning and sequencing is becoming a very useful tool in the investigation of pollen allergens. The allergens (Lol p 1, Lol p 2, Lol p 3, Lol p 5) from *Lolium perenne* have been studied in this respect by two groups of scientists, i.e. Perez et al. (1990) and Knox's group (Knox and Suphioglu, 1996). Complete mRNA sequences have been published.

In the current study, we intend to investigate how transcription of the mRNAs which code for major allergens is affected by heavy metal ions in the cultivation substrate. This transcription takes place late during the development of the microspore into a mature pollen grain. It is important to avoid differences caused by the genetic diversity of the plant mater-

ial. Kwaasi et al. (1994) have found great differences between different cultivars of *Phoenix dactylifera* in allergens and antigens in their pollen material. A comparison between members of one clone of plants is needed in order to eliminate these differences and to be able to evaluate the effect of pollutants. For practical reasons we have decided not to work with trees (birch pollen) but with some herbaceous plants.

The aim of this study is to develop a method to quantify the amount of Lol p 1 mRNA, coding for one of the major allergens in *Lolium perenne*. The purpose is then to use this method to find out if/how different concentrations of heavy metals, such as lead and cadmium, added to the cultivation media, affect the amount of allergen in *Lolium perenne* pollen.

The introduction of PCR has facilitated qualitative examination of nucleic acids. PCR is a sensitive method for detection of a target DNA. Demands to quantify small amounts of nucleic acids (e.g. specific mRNA) cannot be met by conventional methods, such as Northern blot hybridization, dot blot hybridization etc., when a limited number of cells should be analyzed. Reverse transcription (RT) followed by PCR offers a possibility to quantify the amount of a specific mRNA sequence. Tube-to-tube variations can, however, influence reaction rate and effect the yield of PCR products. This has led to development of competitive PCR. Quantitative PCR analysis is, however, more complicated than qualitative PCR analysis. In this method an internal standard is co-amplified together with the sample. Generally a series of replicates must be processed. Thus, a range of standard concentrations is simultaneously co-amplified together with a constant amount of wild-type template (Gilliland et al., 1990; Becker-André and Hahlbrock, 1989). These multiple PCR reactions are then followed by electrophoretic separation of the products and analysis. This is a very cumbersome method if many samples shall be analysed. Development of a calibration curve simplifies the analysis. It is possible to perform analysis on a simple set of samples, each containing a known amount of standard.

Construction of the internal standard can be performed in several ways. Besides the claim that it should be amplified by the same primer pair as the selected wild type fragment, it should also be possible to distinguish the PCR products of the wild type fragment and of the standard. A length difference should be an easy way to separate them. The importance of the length of the mutant as compared to the wild-type fragment has been much discussed. According

to Arnold et al. (1992) and Bouaboula et al. (1992) the size difference between the two amplicons has no effect on the amplification efficiency, in contrast to what others have reported. A general opinion has however been that the wild-type and the mutant targets should be of about the same length. The only difference between the target sequences is the introduction of a unique restriction site in the mutant. This, however poses another problem, i.e. the possibility of formation of heteroduplex between mutated and non-mutated sequences, especially if the PCR reaction has been carried to the plateau phase. In one method the problem is avoided by always limiting the PCR reaction to the exponential phase. In another method the PCR product is diluted several times before a final cycle of PCR reaction, and this is claimed to give no heteroduplex formation (Becker-André and Hahlbrock, 1989).

A further possibility is to optimize formation of the heteroduplex. After a high number of PCR cycles, the amplification reaction products can be heat denatured and allowed to cool down slowly. This promotes stabilization of heteroduplexes based on random re-annealing of mutated and non-mutated sequences (de Kant et al., 1994). Only the double stranded DNA molecules that consist of two mutant strands can be cut by the restriction enzyme.

2. Material and methods

2.1. Pollen material

Lolium perenne was chosen for this study because complete mRNA sequences and/or amino acid sequences for the major allergens (i.e. Lol p 1, Lol p 2, Lol p 3, and Lol p 5) have been published. Pollen material was obtained from Allergon.

2.2. Extraction of RNA

Total RNA was extracted from *Lolium perenne* pollen and purified with the aid of a kit, RNeasy™ (Qiagen Ltd., UK). This involves grinding the pollen grains in a microfuge tube immersed in liquid nitrogen, adding buffer, followed by ethanol, and purification in RNeasy™ spin column. After washing with washing buffer, the total RNA was eluted from the spin columns with 2 × 20 ml RNase free water.

Table 1. Primers

A = 5'-CGCGAAGAGCACCTGGTA
B = 5'-AGGTGTCGGCCTTCCAGC
C = 5'-GGTACTTGCACA/AGCTTTTGACCCGCCTGAACTG
D = 5'-AGGCGGGTCAA/AGCTTGTGCAAGTACCCGGACG
Cy5-labelled primer A
The <i>Hin</i> d III cleavage site in primers C and D is underlined

2.3. Reverse transcription

For the first of these steps we used a "First strand cDNA synthesis kit" (Pharmacia Biotech Inc., Sweden) and a random hexadeoxynucleotide primer.

2.4. PCR

Several more or less differing sequences of the Lol p 1 mRNA, maybe representing some isoallergens, were obtained in the GenBank. A set of primers was developed that could work with all these possible sequences. The primers must be sequence-specific and not amplify unwanted products that might disturb later work. Furthermore, they should not complement within themselves or to each other. We used a program, "Amplify" (Bill Engels[®], 1992), that can be used to simulate PCR reactions, before we tested the primers in practical experiments. Primers A and B (Table 1) meet all these criteria.

We generally used a kit, *fmoI*[®] DNA Sequencing System (Promega Corp., USA). The reaction mixture contained 1x buffer, 4 µl dNTP (10 mM), 1 µl each of primers A and B (10 mM), 4 µl MgCl₂ (25 mM), 1 µl cDNA (corresponding to 0.15–0.2 µg total RNA), and 2.5 units AmpliTaq in a volume of 50 µl. The cycling was preceded by denaturation 45 sec. at 95 °C, followed by a 30 cycles program; denaturation 30 sec. at 94 °C, annealing 45 sec. at 60 °C, and extension 2 min at 72 °C. The program was concluded by a final extension for 8 min at 72 °C and soak at 4 °C.

2.5. Preparation of standard DNA (mutant)

A mutant with *Hind* III restriction site close to the middle of the amplified fragment was synthesised (Figure 1). For this purpose another set of primers was developed (primers C and D in Table 1). They contain a *Hind* III restriction site and are partly complementary to each other, so that the two smaller fragments obtained could be combined by PCR (Higuchi, 1990).

These smaller fragments ought to be blunt ended, i.e. without 3' overhang. For this reason we used another DNA polymerase (Deep Vent DNA polymerase from New English Biolabs). The reaction mixture contained 1x buffer (10 mM KCl, 10 mM (NH₄)₂ SO₄, 20 mM Tris-HCl – pH8.8, 2 mM Mg SO₄, 0.1% Triton X-100), 4 μ l dNTP (10 mM), 4 μ l each of the primer pair in question (10 mM), 1 μ l cDNA (corresponding to 0.15–0.2 μ g total RNA), and 0.5 units of Deep Vent DNA polymerase in a volume of 50 μ l. The conditions for the PCR reactions were; 45 sec. at 95 °C, followed by a 30 cycles program; denaturation 30 sec. at 94 °C, annealing 45 sec. at 60 °C, and extension 2 min at 72 °C, followed by a final extension for 8 min 72 °C and soak at 4 °C. The two fragments were purified with the aid of a DNA purification kit (Qiagen Ltd., UK). Equal amount of the two fragments were finally combined into the desired mutant by the same PCR procedure using Deep Vent DNA polymerase and the primer pair A and B. The mutant that we finally obtained was purified by ligation into plasmids and cloned.

2.6. Purification of mutant (ligation into plasmids)

The resulting mutant, which had been prepared with blunt ends, was ligated into a plasmid (pCR-Script SK(+) vector, Stratagene Ltd., UK) and cloned into *E. coli* XL1-Blue Script before *in vitro* transcription. The bacteria were stored at –70 °C, and when needed grown in SOC medium.

2.7. In vitro transcription

Plasmids were isolated and purified with the aid of Qiagen kit (Qiagen Ltd., UK) and linearized with the aid of *Bam* H I (40 μ l plasmid and 5 μ l enzyme were incubated for 3 hours at 37 °C in a total volume of 50 μ l) and used for preparation of RNA standard.

Mutant RNA was prepared with the aid of a kit, Riboprobe[®]MAX (Promega Ltd., UK). The reaction mixture consisted of 1x buffer, 10 μ l 100 mM DTT, 20 u RNasin (ribonuclease inhibitor), 20 μ l NTP (2.5 mM each of ATP, GTP, CTP and UTP), 40 μ l DNA, and 2 μ l T3 RNA polymerase, in 100 μ l. Incubation at 37 °C was performed for 120 min. Target DNA was removed by incubation with RNase free DNase for 15 min at 37 °C and the specimen was further purified with RNeasy Mini Kit. The purity and concentration of the preparation was controlled by spectrometry, and the absence of DNA was verified by

PCR reaction. The product was diluted to a concentration of ca 10 μ g/ml, and stored at –70 °C in small aliquots.

2.8. Reverse transcription of PCR in one step

Handling of tubes, pipetting etc. always involves some danger of contamination, and we have also tested methods to perform RT and PCR in one tube and adding all necessary reagents at one time.

1. The PCR Access kit (Promega Ltd., UK) offered such a possibility. The reaction mixture contained 1x buffer, 1 μ l dNTP (10 mM of each dNTP), 5 μ l each of primers Cy5-A and B (10 mM), 2 μ l (25 mM MgSO₄), 1 μ l AMV Reverse Transcriptase (5 μ / μ l), 1 μ l Tfl DNA Polymerase (5 μ / μ l), and RNA in a volume of 50 μ l. The amplification program was; cDNA synthesis at 48° for 45 min, AMV RTase inactivation and RNA/cDNA/primer denaturation at 95° for 2 min, followed by second strand synthesis and PCR; 45 cycles; denaturation 30 sec. at 94 °C, annealing 1 min at 60 °C, extension at 72° for 1 min, followed by a final extension for 8 min at 72 °C and soak at 4 °C.
2. Ready-To-Go RT-PCR Beads (Pharmacia Biotech Inc., Sweden), was another possibility. Ready-To-Go RT-PCR Beads contain Taq DNA polymerase, 10 mM Tris-buffer, 1.5 mM MgCl₂, 200 mM of each NTP, Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase, RNA guard Ribonuclease Inhibitor, and BSA. In this case the bead was first dissolved in DECP treated water in an amount to give a final reaction volume of 50 μ l. 5 μ l each of primers Cy-5-A and B (10 mM) and 10 μ RNA was added. The amplification program was; cDNA synthesis at 42° for 3 min, M-MuLV RTase inactivation and RNA/cDNA/primer denaturation at 95° for 5 min, followed by second strand synthesis and PCR; 45 cycles; denaturation 1 min at 90 °C, annealing 1 min at 60 °C, extension 1 min at 72° C, followed by a final extension for 8 min at 72 °C and soak at 4 °C.

2.9. Quantification

The mutant DNA was transcribed *in vitro* and the resulting RNA was used as a standard. RNA extracts from *Lolium perenne* pollen were mixed with known amounts of mutant RNA immediately before the extract was purified with the RNeasy kit. The product

was subjected to RT and PCR amplification using the primer pair Cy5-A and B, cut with *Hind* III, and analysed with an AlfExpress equipment (Pharmacia LKB Biotechnology, Sweden). The fragments were separated in a polyacrylamide gel using an AlfExpress automated sequencer. The amounts of fluorescent compounds were detected by a laser. The relative amount of Lol p 1 mRNA in the pollen samples was finally calculated using a standard line, obtained by plotting the logarithm of the ratio between amount of wild-type and amount of standard against the logarithm of the known amount of standard.

3. Results and discussion

Competitive RT-PCR appeared to be the best method to perform a quantitative analysis of Lol p 1 mRNA in *Lolium perenne* pollen. A standard (mutant) RNA slightly different from the wild type RNA sequence has been synthesised. Differences in length can be obtained via an insertion or deletion in the original RNA sequence. Differences between wild type and standard can also be obtained by inclusion of a unique restriction site (Becker-André and Hahlbrock, 1989; Gilliland et al., 1990). We decided to make the mutant somewhat longer than the wild-type target by a splice overlap extension PCR, thus minimizing the danger of heteroduplex formation between wildtype and mutant. With a complete knowledge of all restriction sites in the wild type we chose to construct a mutant containing a unique *Hind* III restriction site (Figure 1). The primers C and D (Table 1) were developed such as to introduce this restriction site near the middle of the sequence. This mutant had almost the same length as the wild type fragment (655 bp as compared to 648 bp) and cutting with *Hind* III gave a fragment 356 bp long. The identity of the mutant has been verified by minigel electrophoresis, PCR amplification and DNA sequencing in an AlfExpress automated sequencer and shown to have the expected DNA sequence. Ligation of the product into a plasmid and cloning into *E. Coli* XL1-BlueScript also involved a purification of the mutant. Colonies were tested by plasmid isolation, purification, and PCR reaction. A colony that contained the expected mutant was selected for further amplification.

When needed standard RNA was prepared by linearization with *Bam*H I, followed by *in vitro* transcription of the purified and *Bam*H I cut plasmid. The purity of the produced RNA was controlled spec-

trophotometrically, by running an agarose gel, and by performing PCR reaction. No detectable product was obtained after PCR reaction using the primers developed for the quantitative analysis. The spectrometric analysis gave ratios A_{260}/A_{280} between 1.87 and 2.02, mean value 1.93.

It has been recommended (Zimmermann and Manhalter, 1996) that the standard should be added before the extraction, but we found this impossible from a practical point of view. The procedure we developed involved grinding the pollen material in a tube immersed in liquid nitrogen, and then adding extraction buffer immediately followed by the mutant. By this procedure we assume to have a safeguard against varying extraction efficiency. Elution from the RNeasy spin column in two steps is a means to increase the extraction yield. RNA extraction worked well using the Qiagen RNeasy kit, that is claimed to give an efficient extraction and a product free from DNA. The total RNA eluted with RNase free water was analysed in the same way as *in vitro* prepared RNA. The absence of DNA was verified by spectrophotometry and PCR reaction.

A final step to consider is the detection and analysis of the PCR products. If the two fragments are of very different length they could simply be separated by conventional gel electrophoresis. Quantification by a video image analysis system after visualization by ethidium bromide has been described (Zachar et al., 1993). It appeared, however, much simpler to use a fluorescence labeled primer. The label could then be detected and analysed in an automated laser fluorescent DNA sequencer (AlfExpress, cf. Porcher et al., 1992). A computer program (Fragment Manager, Pharmacia LKB Biotechnology) allows direct calculation of the relative amounts of the PCR products. Since we had introduced a unique *Hind* III restriction site in the standard, we could after digestion with *Hind* III obtain one longer fragment (the wild-type) and one shorter fragment (the standard). Originally we took great care to purify the products by treatment with Qiagen tubes but we soon found that polyacrylamide gel electrophoresis in the Alf apparatus gave as good separation without this preliminary purification. A 300 bp molecular weight standard was added in some samples to ascertain that the analysed peaks appeared in the right positions (Figure 2).

The reliability of the analysis was first checked by submitting different dilutions (1/10, 1/20, 1/40, 1/100, 1/200, 1/250, and 1/625) of Cy5-marked DNA product (corresponding to 1 μ g/ml RNA) to polyacrylamide

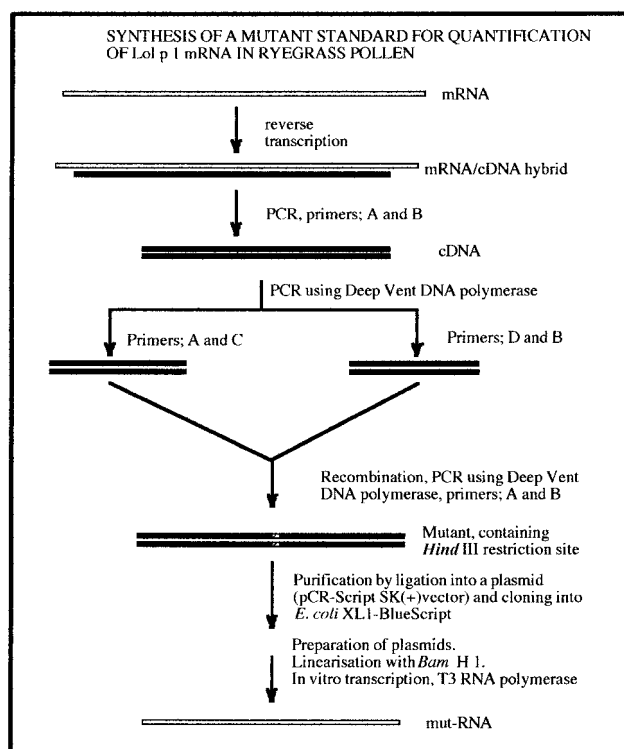


Figure 1. A schematic outline of the procedure for preparation of mutant.

gel electrophoresis and plotting the logarithm of the observed values against the known dilution. Known amounts of the 300 bp-sizer were added to check for variations between different lanes in the electropherogram. A linear correlation between the logarithms over more than a 2-log range of standard-cDNA was apparent (Figure 3). The slope of the line was as close to 1 as could be expected, and the reliability was good ($R^2 = 0.987$, confidence limits ± 0.134).

The calibration line (Figure 4) was plotted as the logarithm of the ratio between amount of wild-type and amount of standard against the logarithm of the known amount of standard. A linear relation with high reliability ($R^2 = 0.976$) was obtained for a (wild/standard) ratio between at least 0.1 and 10, and for amounts of standard between 2×10^{-1} and 2×10^{-4} pg. In tentative experiments we found that each pollen grain contains a rather high copy number of Lol p 1 mRNA, and thus it is possible to use relatively small samples in each analysis. Samples consisting of 30 pollen grains proved to give well reproducible results. It might be possible to use still smaller samples, but as the quality of individual pollen grains can vary some-

what (Figure 5), smaller samples increases the danger of great variation between duplicate samples.

A schematic outline of the quantification method is shown in Figure 6. The primers used for cDNA synthesis and PCR defined a fragment of 655 and 648 bp length, respectively. Digestion with *Hind* III selectively cuts the mutant DNA fragments into two fragments, 356 bp and ca. 300 bp in length. Only the 356 bp sequence is Cy5-marked.

Tentative experiments with pollen grains collected from *Lolium perenne* plants growing outside our laboratory showed a great variation between individual plants (Table 2). We have obtained good correlation between triplicate samples. The standard deviation between triplicate samples was between 2 and 20%, quite comparable with what has been reported by others (Zimmermann and Mannhalter, 1996), mostly dependent on the total yield of product. We found it highly recommendable to start each analysis by making a 'titration' using a few different amounts of standard to find the best relation between wild-type and standard. For increased safety at least five samples of each pollen batch should be analysed.

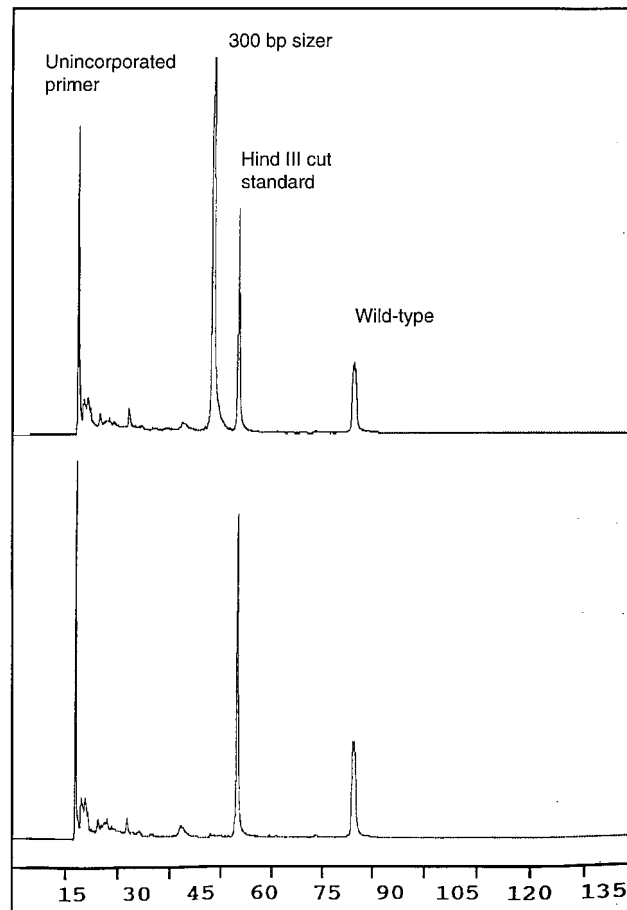


Figure 2. Alf gel electrophoresis of samples, one of them (A) with an added molecular weight sizer.

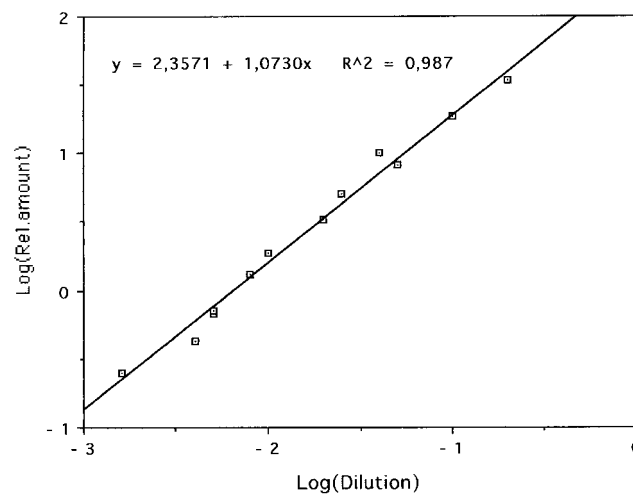


Figure 3. Relation between observed amount of DNA (peak area after gel electrophoresis) and sample dilution. Log-log-diagram.

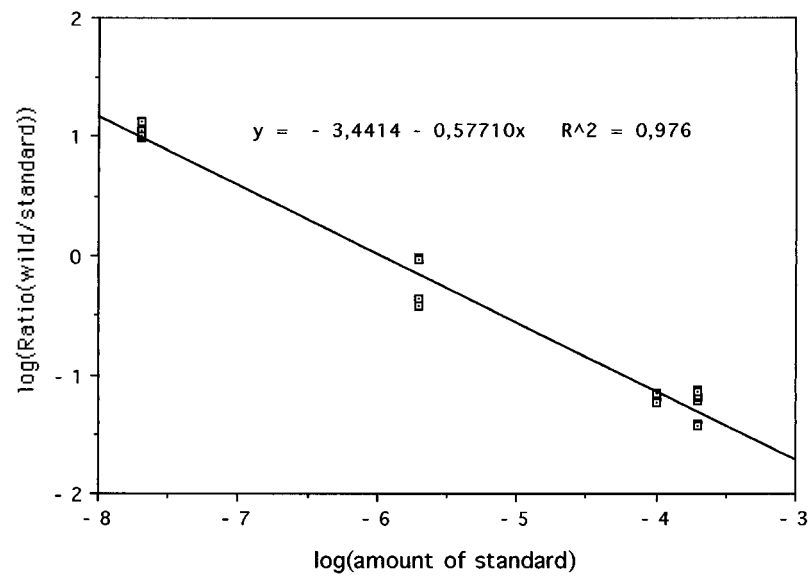


Figure 4. Calibration line for the relation between amount of standard RNA (2×10^{-1} to 2×10^{-4} pg) and the ratio between peak areas corresponding to wild-type and standard. Log-log-diagram.

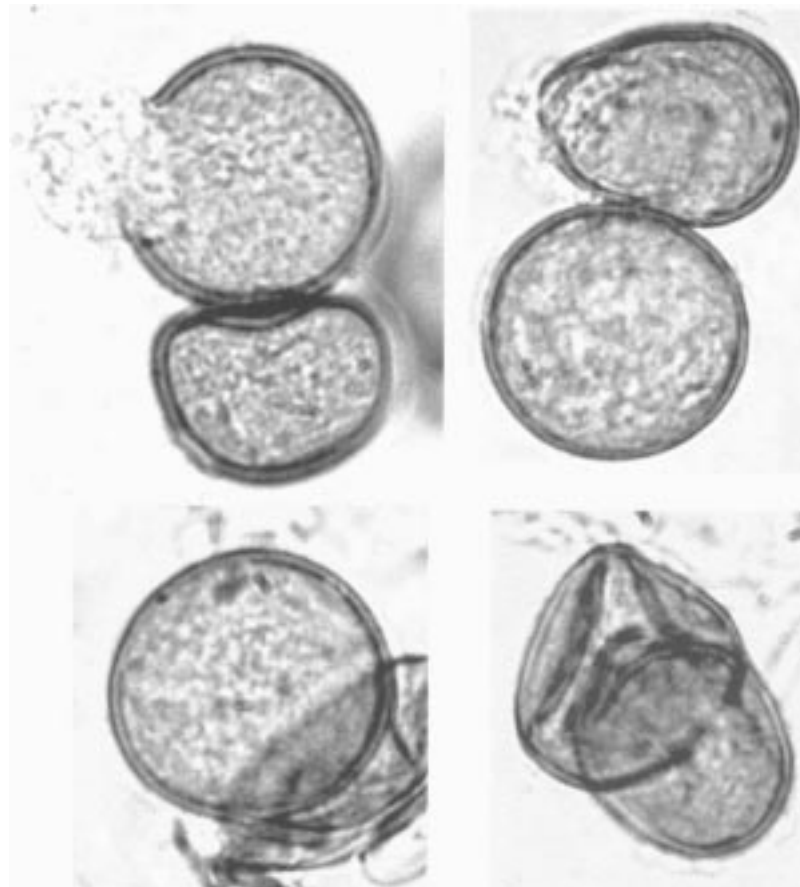


Figure 5. Quality of some pollen samples. Light microscopic picture.

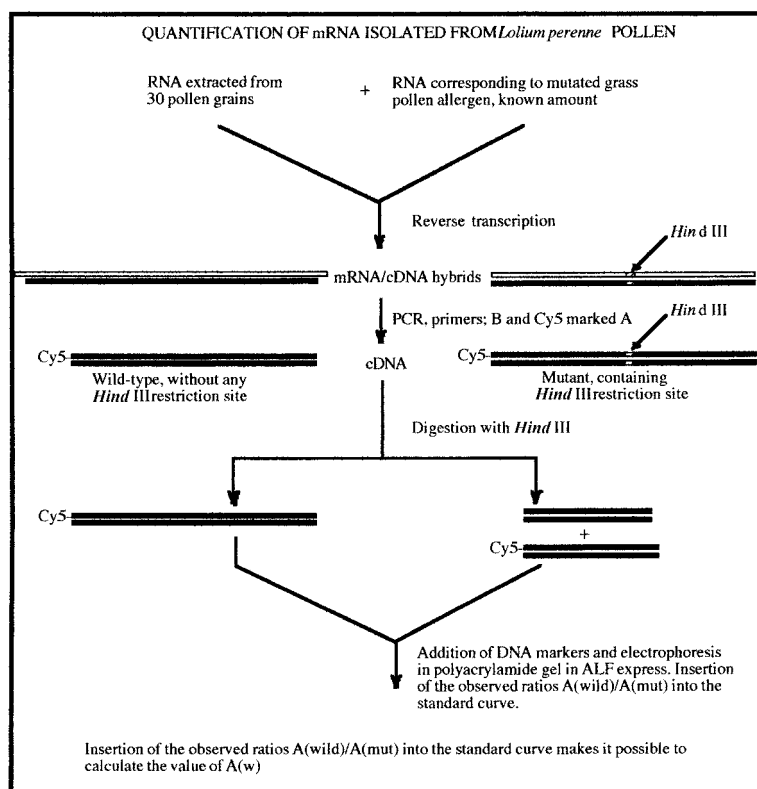


Figure 6. A schematic outline of the competitive analysis procedure.

Table 2. Relative amount of Lol p 1 mRNA obtained from some *Lolium perenne* plants growing close together outside our laboratory

Sample No.	Rel. amount	Stdev	Stdev, %	Note
B1	0,9142	0,034	3,7	
E4	1,328	0,024	1,8	
G1	0,0752	0,0028	3,7	
G2	0,1308	0,025	19,1	*
H	0,0653	0,0022	3,4	
I2	0,4149	0,0708	17,1	*
J2	0,1707	0,009	5,3	
K1	0,2317	0,0597	25,8	
K2	0,0731	0,0143	19,6	*

* = Ratio(wild/standard) was too high.

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