

## A pollen allergen-encoding gene is expressed in wheat ovaries

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### Abstract

To isolate genes specifically expressed at the initiation of plant embryo development we have applied a sensitive subtractive hybridization technique for three isogenic wheat lines of the so-called 'Salmon system' with either zygotic or autonomous embryo development. Here we present a gene sequence showing a high homology to grass pollen allergens of type II/III thought to be expressed in pollen tissue only. Surprisingly, the pollen allergen-like sequence, designated *Tri a* III, is also expressed in gynoceia of the sexual, male fertile wheat line '(aestivum)-Salmon', whereas the two parthenogenetic and male sterile wheat lines '(caudata)-Salmon' and '(kotschy)-Salmon' completely lack any *Tri a* III transcript. Our data suggest a positive correlation between the expression of this clone and the manifestation of male fertility. Northern and *in situ* hybridization analysis revealed that, in addition to its presence in pollen, *Tri a* III is expressed in the parenchymatous tissue of '(aestivum)-Salmon' ovaries exclusively at the day of anthesis. This precise temporal and spatial expression pattern suggests a more general function of the pollen allergen-like sequence *Tri a* III not limited to the exhibition of allergens in pollen grains.

### Introduction

In angiosperms the development of the female gametophyte occurs deeply embedded in carpel derived ovules [26]. Highly complex mechanisms of pollination and fertilization have evolved to ensure the interaction between female and male gametophyte [22, 28]. In addition to the different forms of pollination highly specific interactions of pollen, pollen tube and sperm nucleus with stigma, ovary, and ovule have evolved. On the one hand, the intra-specific gamete fusion is rendered possible by a variety of genetically controlled processes like pollen germination on the stigma, directed pollen tube growth through the transmitting tissue, and double fertilization. On the other hand, in some plant species the intra-individual fertilization is prevented by gametophytically or sporophytically controlled self-incompatibility (for a review, see [8]). Further-

more, the intergenetic fertilization can be prohibited by crossability loci which act gametophytically to inhibit fertilization [18].

Using genetic and molecular approaches a wealth of information has been obtained concerning male gametophyte and pollen development [24]. Much less is known about the female gametophyte development as well as processes connected to the transition from the gametophytic to the sporophytic generation by double fertilization.

Besides the fertilization-dependent induction of embryogenesis several plant species have developed the capability to reproduce by different asexual processes, summarized as apomixis (for recent reviews see [4, 17]). Haploid parthenogenesis is still another pathway of asexual embryo development. Here the egg cell of a reduced embryo sac divides directly without fertilization and forms a haploid embryo [34, 23]. These parthenogenetic processes mimic many of the events of sexual reproduction. Comparative molecular studies of sexually and asexually reproducing lines might there-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Database under the accession number Z50867.

fore provide a suitable experimental system to isolate and characterize genes involved in the regulation of these events.

We have applied the so-called 'Salmon system' of wheat first described by Kihara and Tsunewaki [16] which principally meets the criterion mentioned above. In the sexual wheat line 'Salmon' the short arm of chromosome 1B of the wheat genome is substituted by the short arm of rye chromosome 1R. The transfer of this nucleus into different *Aegilops* cytoplasms (*A. caudata*, *A. kotschy*) results in male-sterile plants with the capability for autonomous embryo development. Without fertilization about 25% of the egg cells start a parthenogenetic development into polyhaploid embryos [34]. Further embryo and seed development depend on endosperm formation upon fertilization of polar nuclei. Based on extensive genetic studies it was concluded that the 1RS chromosome arm carries a gene responsible for parthenogenesis induction (*ptg*), whereas a parthenogenesis-suppressing gene (*spg*) should be located on the 1BS arm [34].

In a first approach to isolate genes specifically expressed at the initiation of embryo development we have screened for gene sequences which are differentially expressed in the three isogenic wheat lines of the improved 'Salmon system' [23] with either zygotic or autonomous embryo development. Several clones have been isolated. The more detailed analysis of one of these clones revealed sequence similarity to pollen allergens. We demonstrate here that a pollen allergen-like gene shows in addition to its expression in pollen a precise temporal expression pattern in the ovary.

## Materials and methods

### Plant material

The 'Salmon' system has been essentially improved by about 20 back-crosses and the generation of doubled haploid lines [23]. The now established system involves three homozygous isogenic lines: '(aestivum)-Salmon' (aS) as standard with normal sexual reproduction and the two alloplasmic, male-sterile lines '(caudata)-Salmon' (cS) and '(kotschy)-Salmon' (kS) exhibiting autonomous embryo development with a frequency of about 90%. All wheat lines mentioned below were grown in pots in the greenhouse with 16 h illumination and a day/night temperature of about 22/12 °C during the generative phase: the parthenogenetic, male-sterile wheat lines cS

and kS, the sexual, male-fertile lines aS, '(aestivum)-Rubin', and '(aestivum)-Hatri', '(aestivum)-Ares' and '(aestivum)-Borenos' as well as the sexual but male-sterile lines '(timopheevi)-Ares' and '(timopheevi)-Borenos', the progenies cS × '(aestivum)-Rubin' (two and three back-crosses: cSR2 and cSR3, respectively) and the segregating cS × '(aestivum)-Chinese Spring' offspring (two back-crosses: cSch<sup>2</sup>). From the two lines cS and kS only the amphidiploid plants ( $2n = 42$ ) have been used for the following analysis. All polyhaploid seedlings were eliminated after determining the ploidy level by flow cytometry from leaf cells (FACStar<sup>Plus</sup> flow cytometer and sorter, Becton & Dickinson).

### RNA isolation and cDNA library construction

Gynoecia from the two parthenogenetic wheat lines '(caudata)-Salmon' and '(kotschy)-Salmon' as well as from the sexual line '(aestivum)-Salmon' were manually dissected only from the area of the spikes corresponding to a developmental stage of 1 to 2 days before anthesis and immediately frozen in liquid nitrogen. About 500 mg of the frozen tissue was ground in 1.5 ml of extraction buffer (1 M Tris-HCl, 5 mM EDTA, 0.1% SDS pH 7.4) and 1.5 ml phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v). After brief centrifugation the aqueous phase was extracted additionally with an equal volume of phenol/chloroform/isoamyl alcohol. Nucleic acids were precipitated with 0.1 vol of 3 M sodium acetate (pH 5.2) and 3 vol of ethanol, immediately centrifuged, and dissolved in 0.6 ml DEPC-treated water. Separation of polysaccharides was achieved by centrifugation of the solution for 10 min at 13 000 rpm. Total RNA was then selectively precipitated with 0.6 ml 4 M LiCl for 16 h on ice. After centrifugation the ribonucleic acids were washed with 2 M LiCl and 70% ethanol, and finally dissolved in water.

Poly(A)<sup>+</sup> mRNA was fractionated from total RNA by adsorption to oligo(dT) cellulose (Pharmacia, type 7) as described [20]. Double-stranded cDNAs were synthesized from cS and aS gynoecium poly(A)<sup>+</sup> mRNAs (5 µg each) using the Amersham kit RPN 1256 Z and an oligo(dT) primer for first strand cDNA synthesis. The average size of ds cDNA was 0.4 to 1.6 kb, the largest was about 4 kb.

Libraries were prepared from 270 ng double-stranded cDNA by ligation of *Eco*RI/blunt-ended adaptors (Amersham) to the cDNA fragments, size fractionation with a GeneClean kit (Bio 101, removal of fragments smaller than 200 bp), and cloning of the

adapted cDNA into the *Eco*RI site of the phage vector  $\lambda$ ZapII (Stratagene). The cDNA library specific for genes expressed in gynoecea of '(aestivum)-Salmon' was used to screen for full length cDNA clones via plaque hybridization.

#### Subtractive hybridization

The strategy to enrich cDNA fragments specific for *cS* and *aS* gynoeceum gene sequences was basically the same as presented by Wang and Brown [37] with one important modification: by applying two different linker primers for each pool of cDNA fragments to be subtracted from each other there is no need anymore to digest PCR-amplified cDNAs before photobiotinylation to inhibit amplification of residual driver in later PCR experiments [6].

To gain multiple DNA fragments from each cDNA in the range of 200 to 800 bp, half of the amount of cDNA fragments were completely digested with *Alu*I and *Alu*I/*Rsa*I separately for 4 h at 37 °C with 10 U restriction enzyme per 10  $\mu$ l. The cDNA fragments from *cS* and *aS* thus treated were then ligated to different double-stranded phosphorylated linker primers: LP1 (25-mer dTAG TCC GAA TTC AAG CAA GAG CACA and 21-mer dCTC TTG CTT GAA TTC GGA CTA) for *cS* cDNA fragments and LP2 (25-mer dAGC CAT TCT AGA CGT GTA ACT GATA and 21-mer dAGT TAC ACG TCT AGA ATG GCT) for *aS* cDNA fragments, respectively. The oligonucleotides were synthesized on an Applied Biosystems apparatus and were purified by polyacrylamide electrophoresis. Corresponding oligonucleotides were phosphorylated and annealed leading to duplex linker primers with one blunt end and one 4 base 3' protruding end. 7 nucleotides from the blunt end LP1 includes an *Eco*RI restriction site, LP2 a *Xba*I site, respectively. To purify ligated cDNAs from excess linker primer half of each reaction mixture was size fractionated on a 1.4% low-melting agarose gel.

Linker-ligated *Alu*I and *Alu*I/*Rsa*I cDNA fragments from *cS* and *aS* in the size range of 200 to 2000 bp were combined and stored in the low-melting agarose at -20 °C. *cS* and *aS* cDNA fragments were amplified directly from the agarose by PCR through 25 cycles in a 100  $\mu$ l reaction containing 1  $\mu$ l cDNA agarose solution (30–40 pg DNA), 0.2 mM dNTPs, 1.5 mM MgCl<sub>2</sub>, 3 U *Taq* polymerase (Promega M1861) and 1  $\mu$ M of the appropriate 21-mer oligonucleotide specified above using a thermocycler (Mastercycler, Eppendorf) under the following conditions: denaturation at 94 °C for

1 min, annealing at 50 °C for 1 min, and elongation at 72 °C for 2 min with 25 s of autoextension per cycle.

After six cycles of subtractive hybridization the enriched cDNA fragment pools *P6* and *S6* were finally amplified with 25 cycles of PCR. Reaction products were cleaved with *Eco*RI or *Xba*I, respectively, and ligated to dephosphorylated pBluescribe vector pBS (Stratagene) for transformation into competent *Escherichia coli* DH5 $\alpha$  cells.

#### Southern and cDNA library analysis

DNA was isolated from leaf tissue (wheat, barley, tobacco, and *Arabidopsis*) essentially as described by Thompson *et al.* [33]. The genomic DNA (15  $\mu$ g) was digested for 16 h with 150 units of different restriction endonucleases and additionally with 20 units fresh enzyme for another 2 h. Electrophoresis was performed through a 0.8% agarose gel in a running buffer of TPE (90 mM Tris-phosphate, 2 mM EDTA pH 8.0). After depurination in 250 mM HCl the DNA was transferred onto nylon membrane (Hybond N+, Amersham) under alkaline conditions (0.4 M NaOH) and hybridized with [<sup>32</sup>P]dCTP-labelled SA7X1 or *Tri a* III. The membranes were prehybridized and hybridized at 65, 60 or 55 °C in 250 mM NaCl 1.25 mM EDTA 125 mM sodium phosphate, 7% SDS, 1% BSA, and 10% PEG in the presence of 200  $\mu$ g/ml of denatured sonicated calf thymus DNA. The hybridized membranes were washed two times at hybridization temperature in 2 $\times$  SSC/0.1% SDS (1 $\times$  SSC is 0.15 M NaCl, 0.015 M sodium citrate), two times in 1 $\times$  SSC/0.1% SDS, and finally two times in 0.1 $\times$  SSC/0.1% SDS, and exposed to Kodak X-Omat AR films.

For cDNA library screening 3  $\times$  10<sup>5</sup> plaque lifts were taken onto Hybond N membranes (Amersham) as described by the manufacturer. The membranes were prehybridized (2 h at 65 °C) in 2 $\times$  SSPE (1 $\times$  SSPE is 0.18 M NaCl, 0.01 M sodium phosphate, 0.001 M EDTA pH 7.5) 1 $\times$  Denhardt's solution (1 $\times$  Denhardt's solution is 0.02% Ficoll, 0.02% BSA, 0.02% PVP 6000), and 0.1% SDS and hybridized (16 h at 65 °C) in 5 $\times$  SSPE, 5 $\times$  Denhardt's solution, 0.5% SDS, and 25  $\mu$ g/ml of denatured sonicated calf thymus DNA. The membranes were washed under stringent conditions two times at 65 °C with 2 $\times$  SSPE/0.1% SDS and two times with 1 $\times$  SSPE/0.1% SDS. The cDNA probe SA7X1 generated by the subtractive hybridization was labelled with the Mega Primed DNA Labelling system (Amersham) to screen the *aS* library for full-length cDNA clones. Only one positive clone could

be isolated not containing any poly(A) tail. Sequence information of the 3' end of cDNA clones was achieved by applying the 3'RACE kit (Rapid Amplification of cDNA Ends) according to the manufacturer (Gibco-BRL).

#### Northern analysis

Total RNA (10 µg) or poly(A)<sup>+</sup> mRNA (1.5 µg) was dissolved in 25 µl of 25 mM N-morpholinopropanesulfonic acid (MOPS, pH 7.0), 5 mM sodium acetate, 15% formaldehyde, 50% formamide, denatured (15 min at 55 °C) and electrophoresed through a 1% agarose gel containing 17% formaldehyde in 25 mM MOPS pH 7.0. The RNA was transferred onto Hybond N+ by capillary blotting in 20× SSC overnight, and fixed with UV light (320 nm) for 12 s. Prehybridization and hybridization was at 65 °C as described for Southern analysis. Post-hybridization washing was usually performed at 65 °C twice in 2× SSC/0.1% SDS and once in 1× SSC/0.1% SDS.

#### DNA sequencing

The DNA sequence was determined using an A.L.F DNA Sequencer (Pharmacia LKB) and a fluorescence labelled Autoread Sequencing kit (Pharmacia). Homology searches against GenBank, SwissProt, and EMBL databases were performed using the software packages PC/Gene (IntelliGenetics), HIBIO DNASIS, and HIBIO PROSIS (Hitachi Software Eng.).

#### In situ hybridizations

Ovaries from the three wheat lines under study were dissected from the area of the spike corresponding to a developmental stage of 1 day before anthesis and immersed in ethanol/chloroform/acetic acid (6:3:1, v/v/v) for 1 h under slight vacuum at 0 °C followed by another 3 h incubation at room temperature. After washing with absolute ethanol the tissue was kept in 100% ethanol for 24 h. Subsequently, the fixed pistils were rinsed in 70% ethanol and transferred to a mixture of ethanol/glycerol/water (1:1:1, v/v/v) in which they were stored until embedding. After dehydration in graded ethanol series the tissue was embedded in Paraplast Plus (Sherwood Medical). Sections of 12 µm were cut with a steel knife (Microtom, Microm HM 400 S), transferred to poly-L-lysine-coated slides (Sigma), and dried overnight at 45 °C. The slides were cleared with xylene, blocked with 1% of acetylated bovine

|                                                                         |     |
|-------------------------------------------------------------------------|-----|
| GGA TCA ACC GAG AAG ATG GCC TCC GGC TCC AAG ATG CTC ACG GCG GCG CTG     | 54  |
| G S T E K M A S A S R <u>M L T V A V L</u>                              |     |
| GCG GCG CTG TTC GCG GCG GCG ATG GCG GCG AAA GTC AAG TTG ACG CTG CAG AAG | 108 |
| A A L F A G A M A                                                       |     |
| GCC TCG GAC AAA AAG AAG CTG GCG CTG AAG ATC GAC TAC ACA AAG CCA AAC GAC | 162 |
| G S D K K K L A L K T D Y T R P N D                                     |     |
| AAC CTG TCA GAG GTG GAG CTC CCG CAG TAC GGC TCA GAG GAG TGG CAG CCC TTG | 216 |
| S L S R V E L R Q Y G S E E W P L                                       |     |
| ACC AAG AAG GGC GAC GTG TGG GAG GTC TCG TCC AAG CCG CTG GTT GGC CCC     | 270 |
| T K K K G D V W R V S C S K P L V G P                                   |     |
| TTC AAC TTC GCG TTC TTG TCC AAG AAT GGC ATG AAG AAA GTC TTC GAC GAG GTC | 324 |
| F N F R P L S K N G M K K V F D R V                                     |     |
| TTC TCC ACC GAT TTC AAG ATC GGC AAA ACC TAC GAA CCG GAA TAT TGA TCC ACA | 378 |
| F S T D F K I G K P Y K P E Y *                                         |     |
| CGG TCC AAG TTT CAG TTG ATC AAG ATC GTC GTA ATT AAT TAA TCC CCA TGT ATG | 432 |
| AGT GCG GTT GGA AAT ATG CCG CTG CAT GAG TGG ATA GTG AAT ATT TTT TTA TGC | 486 |
| ACA TAC ATG CAT ATG GTA GGA GAA GCA ACG ACG GAC ATG TTT GCT CTC CCA TGC | 540 |
| ATG CAT CAA CTG TCT TTT TTT GTT TTT GGA TTA TCT TTG GAT TAG TTT GAA TTG | 594 |
| CTT ACC ACA TAT GTC ATG TGG T                                           | 616 |

Figure 1. cDNA and predicted amino acid for *Tri a* III. The deduced amino acid sequence represented by the single-letter code is shown below the DNA sequence and begins at the first potential in-frame initiation codon. The open reading frame continues for 118 amino acids and ends with the TGA stop codon denoted by an asterisk. The putative transmembrane segment is underlined. The location of the primary cDNA clone SA7X1 is indicated by bold letters.

serum albumin (Sigma, in 10 mM Tris-HCl pH 8.0), treated with 5 µg/ml proteinase K, and acetylated to block non-specific charge interactions.

*In situ* hybridization was performed according to Harris and Wilkinson [13] at 45 °C for 16 h in a formamide system using either <sup>35</sup>S-labelled cRNA [9] or <sup>33</sup>P-labelled DNA probes.

## Results

#### Isolation of differentially expressed cDNA fragments

To identify genes specifically expressed in '(caudata)-Salmon' (cS) or '(aestivum)-Salmon' (aS) plants poly(A)<sup>+</sup> mRNA was isolated from gynoecia 1 day before anthesis and reverse transcribed into double-stranded cDNA. Using an improved subtractive hybridization technique [6] several clones specific for either the sexual aS line or for the parthenogenetic line cS have been isolated.

Here we describe a detailed analysis of one of these cloned cDNA fragments, designated SA7X1, which is expressed in the gynoecia exclusively of the sexually reproducing wheat line aS. A corresponding transcript of about 800 bp is missing in the two parthenogenetic lines (Fig. 5).

This 436 bp gene sequence was first cloned directly from the subtractive hybridization procedure. Since it most likely represented only a partial sequence we

|                  |                                                           |     |
|------------------|-----------------------------------------------------------|-----|
| <i>Tri a</i> III | MA---SASRMLTVAVLAALFAGAM-AVKVKLTIVQKGSDDKKLALKEDYTRFGDGL  | 51  |
| <i>Lol p</i> III | -----TKVDLTVEKGSDAKTLVLNKKYTRPGDIL                        | 29  |
| <i>Lol p</i> II  | -----AAPVEFTVEKGSDEKNLALSLKYNKEGDSM                       | 30  |
| <i>Phl p</i> II  | MSMASSSSSSLLAMAVLAALFAGAWCPKVTFVEKGSNEKHLAVVKKY--EGDLM    | 54  |
|                  | * * * * *                                                 |     |
| <i>Tri a</i> III | SEVELRQDSSEEWQPLTKGD-VWEVSCSKPLVGPFFNFRFLSKNGMKKVFDEVFST  | 106 |
| <i>Lol p</i> III | AEVELRHGSEEWEPMTKGN-LWEVKSAPLGTGPMNFRFLSKGGMKNVDFEVIET    | 84  |
| <i>Lol p</i> II  | AEVELKEHGSNEWLALKKNGDGVWEIKSDKPLKGPFFNFRFVSEKGMKNVDFDVVHA | 86  |
| <i>Phl p</i> II  | AEVELRHGSDIEWVAMTNGEGGVWTFDSEEPLOGPFFNFRFLSEKGMKNVDFDVVBE | 110 |
|                  | * * * * *                                                 |     |
| <i>Tri a</i> III | DFKIGKTYEPEY-                                             | 118 |
| <i>Lol p</i> III | AFTVGKTYTPEYN                                             | 97  |
| <i>Lol p</i> II  | DFKVGTTYKPE--                                             | 97  |
| <i>Phl p</i> II  | KYTIGATYAPEE-                                             | 122 |
|                  | * * * * *                                                 |     |

Figure 2. Comparison of the *Tri a* III deduced amino acid sequence with other type II/III pollen allergens. Region of homology of the deduced *Tri a* III amino acid sequence to *Lol p* III and *Lol p* II from *Lolium perenne* and *Phl p* II from *Phleum pratense*. The character to show that a position in the alignment is perfectly conserved is an asterisk below the sequence. Conservative substitutions of amino acids are indicated by dots below the sequence. The potential transmembrane segment is underlined. Potential sites for glycosylation (48 N) and tyrosine sulfation (59 Y) are indicated by a circle. In shaded boxes the conserved B-cell and T-cell epitopes are shown. The sequence alignments were performed using the CLUSTAL program [14].

have screened a conventional cDNA library specific for the aS line and isolated an overlapping longer cDNA fragment. The sequence is shown in Fig. 1. It contains an open reading frame (ORF) of 356 bases from the first methionine at position 16 terminated with a TGA stop codon at position 370. This ORF encodes a polypeptide of 118 amino acids with a calculated molecular mass of 13 282 Da. The proposed translation initiation site and its flanking region (nucleotides 12–20) share 67% identity with the consensus plant initiation sequence AAC AAT GGC [19]. The translation initiation site was confirmed by analysis of the genomic *Tri a* III sequence (data not shown).

#### Sequence homology of the SA7X1 clone with genes coding for pollen allergens

Database searches revealed considerable sequence homology with genes coding for grass pollen allergens of type II/III (Fig. 2). Although the allergen function was not directly proven the cloned gene was preliminary designated *Tri a* III in accordance with the IUIS (International Union of Immunological Societies) nomenclature [21] for pollen allergens. In the overlapping region the *Tri a* III sequence shows 57.7% (68%), 69.1% (75.3%) and 55.1% (67%) identical (functionally conserved) amino acid positions with the pollen allergens *Lol p* II and *Lol p* III from *Lolium perenne* [2, 3, 30] and with *Phl p* II from *Phleum pratense* [11], respectively. Significant sequence similarity (63.9%) was also found with a partial amino acid sequence deduced from an incomplete cDNA clone thought to

code for a type II allergen, *Dac g* II, from *Dactylis glomerata* [27]. In contrast to the sequences of *Lol p* II, *Lol p* III and *Phl p* II, the amino acid sequence of *Tri a* III contains one potential glycosylation site at position 48 and one potential tyrosine sulfation site at position 59 (Fig. 2). Moreover, the *Tri a* III sequence but not the other mentioned pollen allergen sequences can be classified as an integral membrane protein with one predicted transmembrane segment. No homologies to known leader peptide sequences could be found which is also the case for other signal-anchor membrane proteins [15].

#### Southern analysis and chromosomal localization

Southern analysis using genomic wheat DNA and the *Tri a* III cDNA fragment as probe revealed the existence of a small gene family (Fig. 3a). 3'RACE experiments which were performed to obtain the missing 3'-end sequence information of the cDNA clone *Tri a* III revealed different 3'-untranslated regions also emphasising that in wheat several genes encode nearly identical *Tri a* III proteins. In addition, cross-hybridizing sequences could be detected in the genomes of barley and tobacco (Fig. 3b). Only under low stringency conditions weak hybridization signals were found when genomic *Arabidopsis thaliana* DNA was probed with the fragment (data not shown).

In an attempt to localize the gene on wheat chromosomes we applied genomic DNA from nullitetrasonic lines of *Triticum aestivum* (kindly provided by Dr Andreas Börner, IPK Gatersleben) for Southern

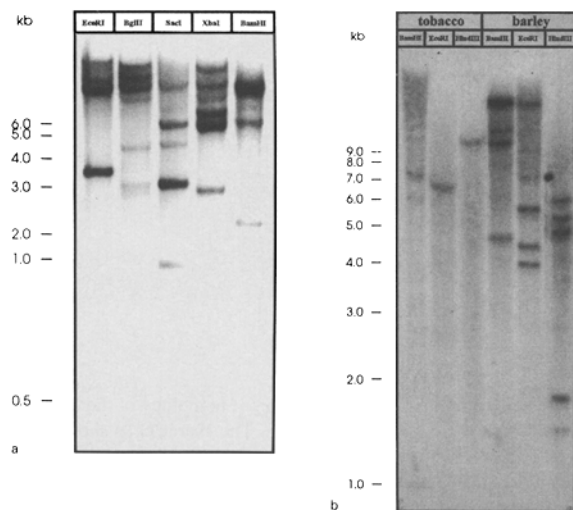


**Figure 3.** Southern blot analysis of genomic DNA from wheat, tobacco, and barley. A. 15  $\mu$ g of genomic wheat DNA was digested with *EcoRI*, *BglII*, *SacI*, *XbaI*, and *BamHI*, fractionated on a 0.8% agarose gel and transferred onto nylon membrane. The membrane was hybridized either with the  $^{32}$ P-labelled fragment SA7X1 or with the full-length clone *Tri a III* at 65 °C. Washing was carried out at the same temperature under stringent conditions (1 $\times$  SSC/0.1% SDS). B. 15  $\mu$ g of genomic DNA from tobacco and barley were digested with *BamHI*, *EcoRI*, and *HindIII*. Gel fractionation, immobilisation, and hybridization were carried out as described above.

analysis. Missing hybridization signals in N5AT5B, N4BT4A and also in N4DT4A indicated the location of the *Tri a III* gene on the long arms of chromosomes 5A, 4B and 4D (to be reported elsewhere) corresponding to the proposed evolutionary translocation between chromosome arms 4AL and 5AL [25].

#### Organ specificity of *Tri a III* gene expression

The sequence of *Tri a III* shows homology to proteins found to be specifically expressed in pollen. Surprisingly, we have isolated the *Tri a III* fragment as being differentially expressed in the gynoecia of the wheat lines under study. To determine the tissue specificity of *Tri a III* expression in the three alloplasmic wheat lines the accumulation of the transcript in various tissues has been investigated. The RNA blots in Figs. 4 and 5 show that the *Tri a III* mRNA is expressed in ovules and in anthers (containing immature pollen) one day before anthesis of '*aestivum*'-Salmon' and only in anthers (1 dbA) of '*kotschyii*'-Salmon' whereas no hybridization signal was obtained with RNA from leaves, roots or pollen-free anther sacs. Therefore, the expression of

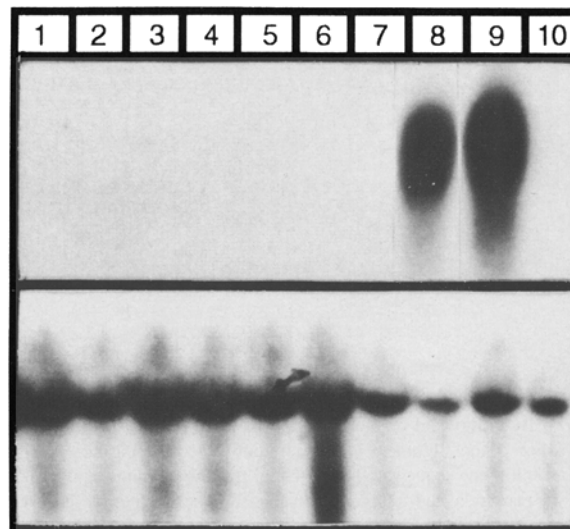


**Figure 4.** Organ specificity of *Tri a III* expression. A (top). Total RNA from different tissues of the three wheat lines was hybridized with the  $^{32}$ P-labelled cDNA fragment SA7X1: lanes 1–3, 20  $\mu$ g of total RNA of leaf tissue from cS, kS and aS, respectively; lanes 4–6, 20  $\mu$ g of total RNA of root tissue from cS, kS and aS, respectively; lane 7, 8  $\mu$ g of total RNA of empty anther sacs 1 day after anthesis from aS; lane 8, 8  $\mu$ g of total RNA of anthers from kS containing pollen grains 1 to 2 days before anthesis; lane 9, 8  $\mu$ g of total RNA of anthers from aS containing pollen grains 1 to 2 days before anthesis; lane 10, 8  $\mu$ g of total RNA of mature pollen grains from aS 1 day after anthesis. B (bottom). Northern blot analysis of the same membrane using an ATP/ADP translocator cDNA fragment as probe to standardize loading.

*Tri a III* is strongly related to those tissues of the wheat plant which are involved in fertilization processes.

#### Temporal and spatial expression pattern of *Tri a III* in wheat gynoecia

To analyse the temporal expression pattern of the *Tri a III* gene we have probed RNA isolated from developing gynoecia of all three lines at eight different stages: three days after meiosis, three days before anthesis, one day before anthesis, anthesis, 20 h after pollination, three days after pollination, three days after anthesis, and five days after anthesis. As shown in Fig. 5, the gene is exclusively expressed in aS gynoecia one day before but mainly at the day of anthesis. Already 20 h after pollination (zygote starts the first division) no accumulation of the *Tri a III* transcript is detectable anymore. This remarkably precise temporal regulation was reproduced in a second independent experiment.

To analyse the spatial expression pattern within the gynoecium we performed *in situ* hybridization

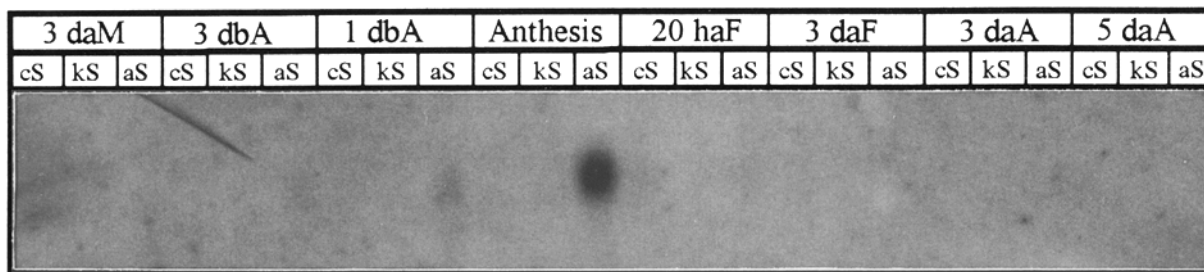


Figure 5. Expression pattern of *Tri a III* at different stages of wheat gynoecia development. Northern blot analysis of total RNA (20  $\mu$ g per lane) from gynoecia of different developmental stages of the three wheat lines cS, kS and aS using SA7X1 cDNA as a probe: 3 days after meiosis (3 daM), 3 days before anthesis (3 dbA), 1 days before anthesis (1 dbA), anthesis, 20 h after pollination (20 haP), 3 days after pollination (3 daP), 3 days after anthesis (3 daA), 5 days after anthesis (5 daA). Specific activity of the labelled probe =  $1.6 \times 10^9$  dpm/ $\mu$ g. Film exposure time 5 days.

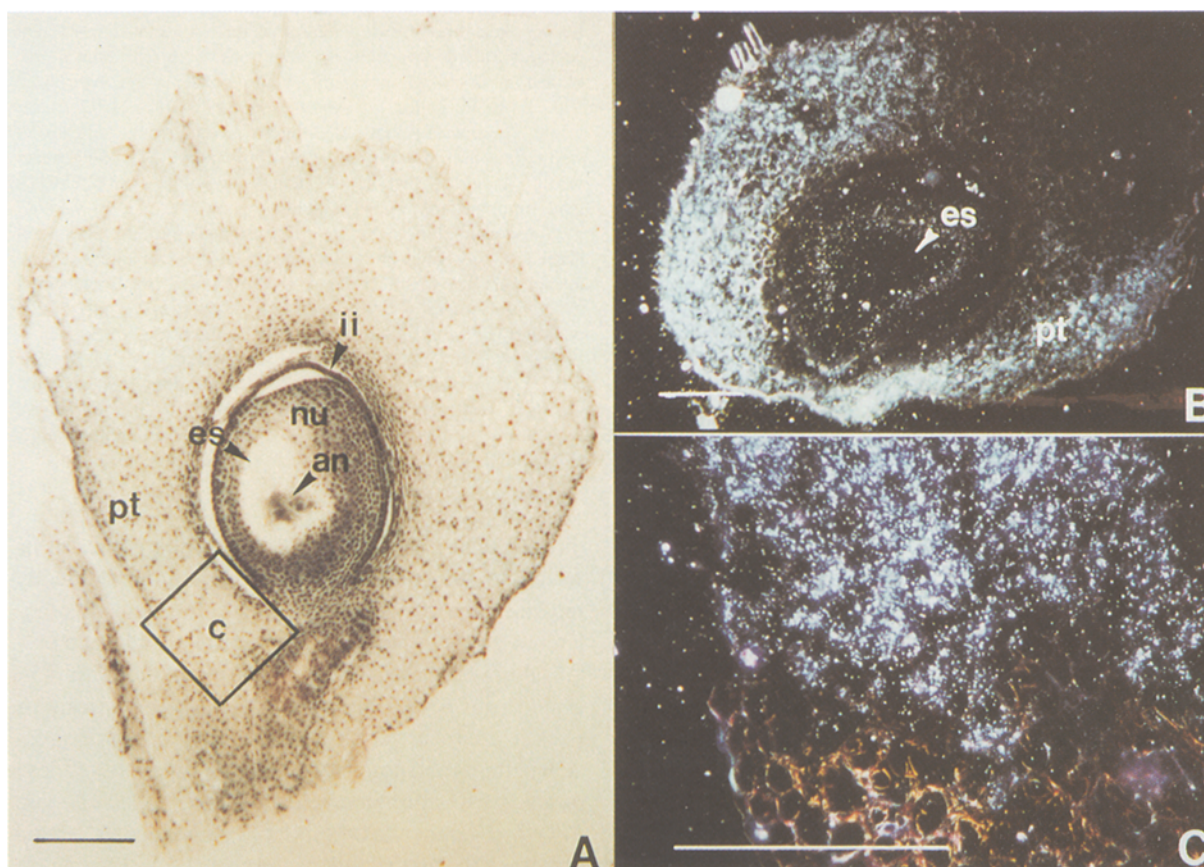


Figure 6. Spatial expression pattern of *Tri a III*. The cDNA fragment SA7X1 was labelled with [ $\alpha^{32}$ P]dATP and *in situ* hybridized to gynoecia sections from all three wheat lines '*caudata*'-Salmon', '*kotschy*'-Salmon', and '*aestivum*'-Salmon'. Photomicrographs of aS gynoecium sections are shown in bright field (segment A) and dark field (segments B and C). A. Sagittal-longitudinal section of a wheat gynoecium. B. The dark-field photomicrograph shows the expression pattern of the aS-specific *Tri a III* mRNA in the parenchymatous tissue of the ovary from the sexual wheat line '*aestivum*'-Salmon'. No cross-hybridization could be detected with sections from the parthenogenetic lines cS and kS (data not shown). C. Expression pattern of *Tri a III* in aS ovary tissue close to the myrophylar entrance of the embryo sac as shown in the boxed region of segment A (diagonal section). Structural and vascular transport tissues are free of any hybridization signal. Abbreviations used: an, antipodals; es, embryo sac; ii, inner integument; nu, nucellus tissue; pt, parenchymatous tissue. Bar in section A: 200  $\mu$ m,  $\times 57$ . Bar in section B: 200  $\mu$ m,  $\times 57$ . Bar in section C: 100  $\mu$ m,  $\times 350$ .

on ovary tissue microdissected 1 day before anthesis. Gene-specific transcripts could be found only in the parenchymatous tissue of aS gynoecia reaching from close to the base of the style along the outer integument to the micropylar entrance (Fig. 6). The specificity of the detection was controlled by the use of sense and antisense *in vitro* transcripts as probe as well as by RNase treatment of the sections. Control sections of gynoecia from the cS and kS line did not give any signal.

#### Correlation with male sterility

The results of northern and *in situ* hybridization experiments showing that the *Tri a* III transcript was only found in the ovarian tissue of the sexual and male-fertile line aS and not in the parthenogenetic, male-sterile lines cS and kS suggested a possible inverse correlation between parthenogenesis and/or male sterility of the wheat lines under study and the expression of the pollen allergen-like sequence. To distinguish between these possibilities, messenger RNA of several male-fertile or male-sterile wheat lines has been probed with the *Tri a* III cDNA. As shown in Fig. 7, the expression of *Tri a* III is strongly reduced in gynoecia from the male-sterile wheat lines with one exception. The hybridization signal of the labelled cDNA probe with mRNA from ovaries of cSCH<sub>2</sub> is as prominent as the one with aS mRNA. Detailed analysis of the cSCH<sub>2</sub> offspring revealed that this segregating second back-cross generation did indeed regain male-fertile plants.

As a control mRNAs from '(aestivum)-Salmon' gynoecia isolated from emasculated '(aestivum)-Salmon' plants (aSe: emasculated 1 dbA, dissected at anthesis) has been applied in this northern analysis, too. This tissue also shows a distinct hybridization signal. A contamination of styles and stigmata with pollen grains during the dissection procedure leading to an artificial detection of the *Tri a* III transcript in the female tissue can therefore be excluded.

#### Discussion

##### Homology of *Tri a* III to pollen allergens

Grass pollen allergy is a major human health problem world-wide. Several allergens from *Lolium perenne*, *Phleum pratense* and other plant pollen have been isolated and characterized (for a review see [5]). Most of

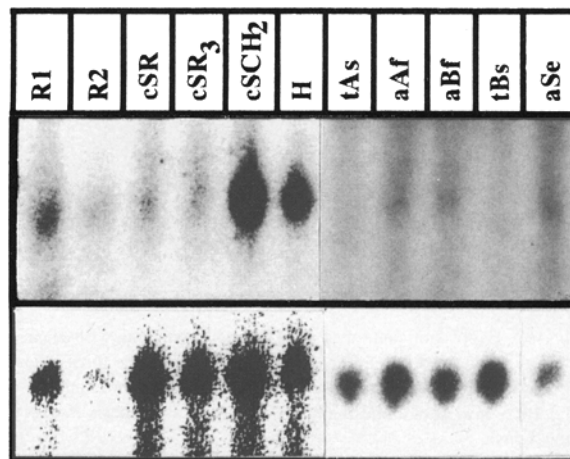


Figure 7. Expression of *Tri a* III in various male-sterile and male-fertile wheat lines. A (top). 2 µg of mRNA isolated from gynoecia of various wheat lines at anthesis were fractionated and blotted as described in experimental procedures and hybridized with the radiolabelled full length clone *Tri a* III: R1, R2: male-fertile '(aestivum)-Rubin' (2 different preparations); cSR: male-sterile '(caudata)-Salmon' × '(aestivum)-Rubin' (F1); cSR3: male-sterile '(caudata)-Salmon' × '(aestivum)-Rubin' (F3); cSCH<sub>2</sub>: male-fertile/male-sterile segregating progeny of '(caudata)-Salmon' × '(aestivum)-Chinese Spring' (F2); H: male-fertile '(aestivum)-Hatri'. 15 µg of total RNA from gynoecia at anthesis were loaded per lane for the following wheat lines: tAs: male-sterile '(timopheevi)-Ares'; aAf: male-fertile '(aestivum)-Ares'; aBf: male-fertile '(aestivum)-Borenos'; tBs: male-sterile '(timopheevi)-Borenos'; aSe: '(aestivum)-Salmon' (emasculated 1 dbA). Specific activity of the labelled probe =  $1.6 \times 10^9$  dpm/µg. Film exposure time was 10 days for the first 6 lanes and 5 days for the last 5 lanes. B (bottom). Northern blot analysis of the same membranes using a β-tubulin cDNA fragment as probe to standardize loading.

these proteins exist in multiple, isoallergenic forms which are variants that appear to be immunologically indistinguishable forming a quite large and inhomogenous group of pollen allergens. Most studies on pollen allergens have focused mainly on their immunological properties and possible clinical applications [12]. In contrast, very little is known about the expression patterns and potential biological functions of these proteins. For example, the major birch pollen allergen *Bet v* I was shown to be similar to pathogenesis-related proteins [7], and the pollen allergens *Amb a* I from ragweed and *Cry j* I from Japanese cedar were suggested to be pectate lyases [35, 32]. However, the function of the structurally completely different type II/III grass pollen allergens is unknown.

In this paper we demonstrate for the first time that a grass pollen allergen-like sequence thought to be specific for pollen tissue [10] is also expressed in wheat ovaries. Since a high degree of sequence homology was

found with *Lol p* II, *Phl p* II, and *Lol p* III we designated the wheat cDNA clone *Tri a* III. The presence of the *Tri a* III messenger also in the female tissue strongly supports a more general cellular function of this protein not limited to the expression in the male gametophyte.

#### *Correlation of Tri a III expression with male sterility*

Interestingly, the pollen allergen-like gene *Tri a* III was isolated as a cDNA fragment specifically enriched from gynoecia of a sexual, male-fertile wheat line when compared to gynoecia of an isogenic, parthenogenetic, and male-sterile wheat line via subtractive hybridization. Northern blot analysis (Fig. 5) showed that the two parthenogenetic, male-sterile lines cS and kS under study completely lack any *Tri a* III transcript in ovary tissue. It was therefore tempting to assume that in cS and kS the inhibition of the gynoecia specific *Tri a* III expression is connected to the induction of parthenogenesis. However, the transfer of the 'Salmon' wheat nucleus into the cytoplasm of *Aegilops caudata* or *A. kotschy* (lines cS and kS) not only leads to parthenogenesis induction but also to male sterility. In both alloplasmic lines pollen development is affected at different stages. In the cS line development of pollen is already aborted at an early stage whereas in the kS line pollen development nearly reaches maturity but leads to impotent pollen grains. Moreover, kS anther lobes are not able to release the pollen [23]. The expression of *Tri a* III in nearly mature pollen grains (aS and kS lines) (Fig. 4) is followed by the expression of the gene in the gynoecium only if the pollen really reaches maturity and fertility (aS line only). This argument is reinforced by the precise temporal expression of *Tri a* III in aS ovaries (Fig. 5) suggesting a 'cross-talk' of anthers and gynoecia after pollen maturation has been achieved.

A reciprocal example of gene expression in pollen and ovule tissue associated with male sterility has recently been demonstrated by Abad *et al.* [1]. We therefore propose that the expression of *Tri a* III in the parenchymatous tissue of ovaries from sexual, male-fertile wheat lines at anthesis is caused by an anther signal to prepare the gynoecium for the reception of the pollen tube. Such signal would not be emitted from the male tissue in the male-sterile lines cS and kS. The missing or at least strongly reduced expression of *Tri a* III in ovaries of other male sterile wheat lines seems to support this hypothesis (Fig. 7). In Fig. 8 our observations are summarized: both female and male gamete development as well as the expression of the pollen

allergen-like *Tri a* III sequence seem to be influenced by the cytoplasm. However, a linkage of parthenogenesis and *Tri a* III expression is unlikely.

Moreover, to achieve self-fertilization the development of the spatially separated female and male organs has to be synchronized. This synchronisation event needs a 'cross-talk' between the two sexual organs. That some aspects of female and male meiosis are under common genetic control has been reported [31]. Thus, the presence of a pollen-specific gene product in the ovary as well as in anthers only shortly before and at anthesis (Figs. 4 and 5) could be another example of such a 'cross-talk' and synchronized gene expression.

#### *Tri a III might be involved in the guidance of pollen tube growth*

In addition, there are genetic data available suggesting that gynoecial factors may determine the pattern of pollen tube behaviour such as direction and rates of pollen tube growth [29]. As proficiently examined by Vishnyakova and Willemse [36] the pistil transmitting tract for pollen tube pathway in wheat includes all stigmatic hairs, the transmitting tissue of stylodia and style and the passage between outer integument and ovarian epidermis in the ovarian cavity. After passing through the transmitting tissue, pollen tubes will enter this ovarian cavity and grow in a space between the ovary and the ovule walls towards the micropyle. However, all those pollen tubes which do not follow the major pathway after reaching the base of the style but grow between the cells of the outer integument will immediately arrest in their growth [36]. Interestingly, the spatial expression pattern of *Tri a* III borders exactly on the outer integument, the outer barrier of the pollen tube tract, reaching from close to the base of the style where a functional control of pollen tube growth would be expected down to the micropylar entrance. Therefore, the *Tri a* III gene product present in this parenchymatous tissue might be involved in the guidance of pollen tube growth along the transmitting tract by keeping it away from the surrounding ovarian tissue. Since the parthenogenetic as well as the other male sterile wheat lines analysed can still be fertilized by foreign pollen at anthesis the missing *Tri a* III gene product is obviously not an essential component involved in the complex network of pollen tube guidance.

The manifestation of its function will require the subcellular localization of the *Tri a* III protein and the generation of transgenic plants with a reduced expression level of *Tri a* III.

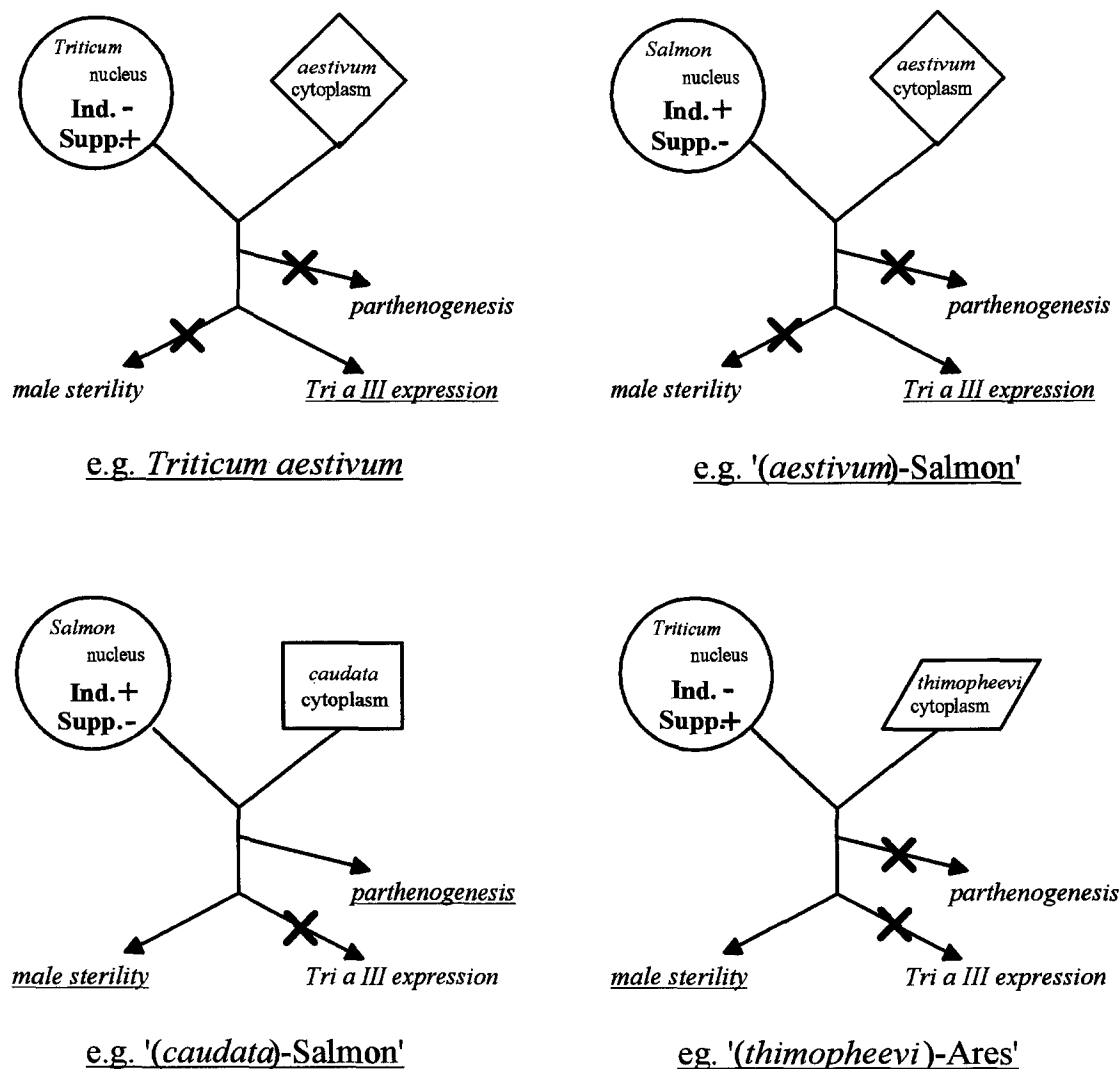


Figure 8. Correlation of *Tri a III* expression with male sterility and parthenogenesis. This figure summarises the observed effects of different cytoplasm on the wheat nuclear genome with or without the 1BL/1RS translocation (Ind. +/Supp. -; Ind. -/Supp. +, respectively) regarding the display of male sterility, parthenogenesis and the expression of the *Tri a III* gene sequence. In all wheat lines analysed the pollen allergen-like sequence is only expressed in male fertile, non-parthenogenetic plants. A correlation of *Tri a III* expression with parthenogenesis is unlikely. Abbreviations used: Ind. -, inducer of parthenogenesis not present on chromosome 1B; Ind. +, inducer of parthenogenesis present on chromosome 1B (through translocation of rye 1RS); Supp. -, suppressor of parthenogenesis not present on chromosome 1B (through translocation of rye 1RS); Supp. +, suppressor of parthenogenesis present on chromosome 1B.

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## References

1. Abad AR, Mehrtens BJ, Mackenzie SA: Specific expression in reproductive tissues and fate of a mitochondrial sterility-associated protein in cytoplasmic male-sterile bean. *Plant Cell* 7: 271-285 (1995).
2. Ansari AA, Shenbagamurthi P, Marsh DG: Complete amino acid sequence of a *Lolium perenne* (perennial rye grass) pollen allergen, *Lol p II*. *J Biol Chem* 264: 11181-11185 (1989).

3. Ansari AA, Shenbagamurthi P, Marsh DG: Complete primary structure of a *Lolium perenne* (perennial rye grass) pollen allergen, *Lol p* III: Comparison with known *Lol p* I and II sequences. *Biochemistry* 28: 8665–8670 (1989).
4. Asker SE, Jerling L: Apomixis in Plants. CRC Press, Boca Raton/Ann Arbor/London/Tokyo (1992).
5. Avjioglu A, Hough T, Singh M, Knox RB: Pollen allergens. In: Willams EG, Clarke AE, Knox RB (eds) Genetic Control of Self-Incompatibility and Reproductive Development in Flowering Plants, pp. 336–359. Kluwer Academic Publishers, Dordrecht, Netherlands (1994).
6. Balzer H-J, Bäumlein H: An improved gene expression screen. *Nucl Acids Res* 22: 2853–2854 (1994).
7. Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, Scheiner O, Breitenbach M: The gene coding for the major birch pollen allergen *Bet v* I is highly homologous to a pea disease resistance response gene. *EMBO J* 8: 1935–1938 (1989).
8. Clark AG, Kao T-H: Self-incompatibility: theoretical concepts and evolution. In: Willams EG, Clarke AE, Knox RB (eds) Genetic Control of Self-Incompatibility and Reproductive Development in Flowering Plants, pp. 220–242. Kluwer Academic Publishers, Dordrecht, Netherlands (1994).
9. Cox KH, Goldberg RB: Analysis of plant gene expression. In: Shaw CH (ed) Plant Molecular Biology: A Practical Approach, pp. 25–35. IRL Press, Washington, DC (1988).
10. Davies SP, Singh MB, Knox RB: Identification and *in situ* localization of pollen-specific genes. In: Russel SD, Dumas C (eds) Sexual Reproduction in Flowering Plants. *Int Rev Cytol* 140: 19–34 (1992).
11. Dolecek C, Vrtala S, Laffer S, Steinberger P, Kraft D, Scheiner O, Valenta R: Molecular characterization of *Phl p* II, a major timothy grass (*Phleum pratense*) pollen allergens. *FEBS Lett* 335: 299–304 (1993).
12. Ferreira FD, Hoffmann-Sommergruber K, Breiteneder H, Pettenburger K, Ebner C, Sommergruber W, Steiner R, Bohle B, Sperr WR, Valent P, Kungl AJ, Breitenbach M, Kraft D, Scheiner O: Purification and characterization of recombinant *Bet v* I, the major birch pollen allergen. Immunological equivalence to natural *Bet v* I. *J Biol Chem* 268: 19574–19580 (1993).
13. Harris N, Wilkinson DG: *In situ* Hybridization: Application to Developmental Biology and Medicine. Cambridge University Press, Cambridge (1990).
14. Higgins DG, Sharp PM: Clustal: a package for performing multiple sequence alignments on a microcomputer. *Gene* 73: 237–244 (1988).
15. High S: Protein translocation at the membrane of the endoplasmic reticulum. *Prog Biophys Mol Biol* 63: 233–250 (1995).
16. Kihara H, Tsunewaki K: Use of an alien cytoplasm as a new method of producing haploids. *Jpn J Genet* 37: 310–313 (1962).
17. Koltunow AM: Apomixis-other pathways for reproductive development in angiosperms. In: Willams EG, Clarke AE, Knox RB (eds) Genetic Control of Self-Incompatibility and Reproductive Development in Flowering Plants, pp. 486–512. Kluwer Academic Publishers, Dordrecht, Netherlands (1994).
18. Lein A: Die genetische Grundlage der Kreuzbarkeit zwischen Weizen und Roggen. *Z Indukt Abstamm Vererbungsl* 81: 28–61 (1943).
19. Lütcke HA, Chow KC, Mickel FS, Moss KA, Kern HF, Scheele GA: Selection of AUG initiation codons differs in plants and animals. *EMBO J* 6: 43–48 (1987).
20. Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1982).
21. Marsh DG, Goodfriend L, King TP, Lowenstein H, Platts-Mills TAE: Allergen nomenclature. *J Allergy Immunol* 80: 639–645 (1987).
22. Mascarenhas JP: Molecular mechanisms of pollen tube growth and differentiation. *Plant Cell* 5: 1303–1314 (1993).
23. Matzk F, Meyer H-M, Bäumlein H, Balzer H-J, Schubert I: A novel approach to the analysis of the initiation of embryo development in Gramineae. *Sexual Plant Reprod* 8: 266–272 (1995).
24. McCormick S: Male gametophyte development. *Plant Cell* 5: 1265–1275 (1993).
25. Naranjo T, Roca A, Goicoechea PG, Giraldez R: Arm homoeology of wheat and rye chromosomes. *Genome* 29: 873–882 (1987).
26. Reiser L, Fischer RL: The ovule and the embryo sac. *Plant Cell* 5: 1291–1301 (1993).
27. Roberts AM, Van Ree R, Cardy SM, Bevan LJ, Walker MR: Recombinant pollen allergens from *Dactylis glomerata*: preliminary evidence that human IgE cross-reactivity between *Dac g* II and *Lol p* I/II is increased following grass pollen immunotherapy. *Immunology* 76: 389–396 (1992).
28. Russell SC: The egg cell: development and role in fertilization and early embryogenesis. *Plant Cell* 5: 1349–1359 (1993).
29. Sanders LC, Lord AM: A dynamic role for the stylar matrix in pollen tube extension. In: Russel SD, Dumas C (eds) Sexual Reproduction in Flowering Plants. *Int Review of Cytol* 140: 297–318. Academic Press, New York (1992).
30. Sidoli A, Tamborini E, Giuntini I, Levi S, Volonte G, Paini C, De Lalla C, Siccardi AG, Baralle FE, Galliani S, Arosio P: Cloning, expression, and immunological characterization of recombinant *Lolium perenne* allergen *Lol p* II. *J Biol Chem* 268: 21819–21825 (1993).
31. Staiger CJ, Cande WZ: Microfilament distribution in maize meiotic mutants correlates with microtubule organization. *Plant Cell* 3: 637–644 (1991).
32. Taniguchi Y, Ono A, Sawatani M, Nanba M, Kohno K, Usui H, Kurimoto M, Matuhasi T: *Cry j* I, a major pollen allergen of Japanese cedar pollen, has pectate lyase enzyme activity. *Allergy* 50: 90–93 (1995).
33. Thompson RD, Bartels D, Harberd NP, Flavell RB: Characterization of the multigene family coding for HMW glutenin subunits in wheat using cDNA clones. *Theor Appl Genet* 67: 87–96 (1983).
34. Tsunewaki K, Mukai Y: Wheat haploids through the Salmon method. In: Bajaj YPS (ed) Wheat: Biotechnology in Agriculture and Forestry, vol 13, pp. 460–478. Springer-Verlag, Berlin/Heidelberg (1990).
35. Turcich MP, Hamilton DA, Mascarenhas JP: Isolation and characterization of pollen-specific maize genes with sequence homology to ragweed allergens and pectate lyases. *Plant Mol Biol* 23: 1061–1065 (1993).
36. Vishnyakova MA, Willems MTM: Pollen-pistil interaction in wheat. *Acta Bot Neerl* 43: 51–64 (1994).
37. Wang Z, Brown D: Gene expression screen. *Proc Natl Acad Sci USA* 88: 11505–11509 (1991).