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Molecular biology of male gametogenesis

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Abstract

Pollen development has been studied at a molecular level in several systems that are amenable to genetic or transgenic analysis. We have characterized several tomato genes that are expressed late in pollen development. Our goals in this research were 1) to determine the cis- and trans-acting factors that mediate pollen expression, and 2) to determine the functions of the proteins encoded by these genes. We currently favor the hypothesis that pollen-specific gene expression is mediated in a combinatorial manner. Antisense experiments have indicated an important role for the LAT52 protein during pollen hydration.

Introduction

The development of a functional male gametophyte (pollen) requires tightly controlled gene expression in both the gametophytic and sporophytic tissues of the anther; this system therefore offers the opportunity to study several aspects of gene regulation, including tissue-specific gene expression, translational control, and cell-cell interactions. Pollen development, as well as plant reproductive biology as a whole, was extensively reviewed in the October 1993 special issue of *Plant Cell*.

To identify pollen-specific genes, a cDNA library prepared from mature anthers of tomato (containing mature pollen) was subjected to differential screening with probes prepared from seedling and from mature anther (McCormick et al., 1987). Twenty cDNAs were selected, and cross-hybridizations within the set of 20 showed that most were unique. Subsequent analysis with Northern blots showed that most of these cDNAs were expressed solely in the male gametophyte, although occasional isolates also showed weak expression in petals or in roots. We selected several of these cDNAs for further analysis (originally termed 9651, 9652, 9656, 9568 and 9659, and later renamed LAT51, etc., for *late anther tomato*). These genes were shown by Southern analysis to be single-copy genes

in the tomato genome, and were mapped to chromosomal position (Ursin et al., 1989; Wing et al., 1989; Twell et al., 1989b). LAT51 maps near the S-locus on chromosome 1, LAT56 and LAT59 are linked, and map near the invertase gene on chromosome 3, LAT58 is unlinked to LAT51, but also maps to chromosome 1, and LAT52 maps near the RFLP marker TG122 on chromosome 10.

Sequence analysis of these tomato cDNAs revealed tantalizing similarities with several already characterized proteins. The LAT51 gene shows sequence similarity with ascorbate oxidase (McCormick et al., 1991a). The pollen-expressed *Brassica* Bp10 gene (Albani et al., 1992) and the tobacco NTP303 gene (Weterings et al., 1992), also show sequence similarity to ascorbate oxidase. It is likely that the LAT51 and NTP303 genes are homologs, in that the sequence similarity is high, and even extends into the 5' untranslated region. The *Brassica* gene Bp10 is likely to be a related family member, rather than a true homolog, since its sequence similarity to the tomato and tobacco genes is only about 63%, and it has introns that are not found in the tomato/tobacco genes. We do not think that any of these pollen-expressed genes are really ascorbate oxidases, since ascorbate oxidase is a copper-binding protein, and none of the pollen proteins conserve the amino acids required for copper binding.

LAT 52 - like Genes

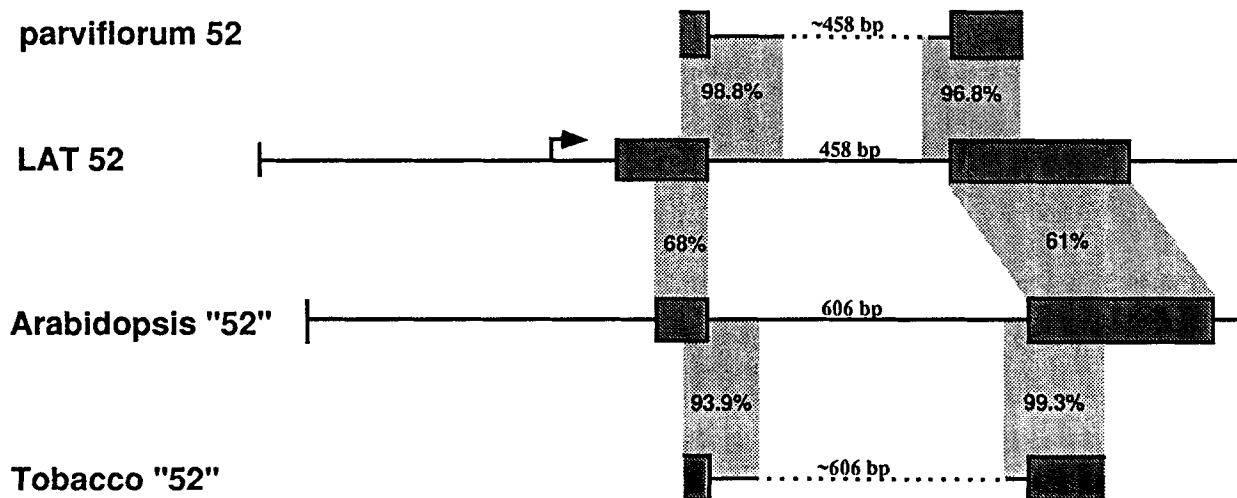


Fig. 1. Schematic representation of the LAT52 gene, showing the start of transcription (arrow), the two exons (filled boxes) and the intron (thin line between the two exons). The *parviflorum* 52 sequence is from a PCR product obtained using primers that span the LAT52 intron. Sequence identities are noted. The *Arabidopsis* '52' sequence (genomic clone) and the tobacco '52' sequence (PCR product) conserved the intron position of the LAT52 gene. The region 5' to the first exon in *Arabidopsis* '52' may contain an intron.

The LAT52 gene shows some sequence similarity to several pollen-expressed genes that have been isolated from other plants, including the Zm13 gene of maize (Hanson et al., 1989), the PS1 gene of rice (Zou et al., 1993), and the major allergen (Ole e I) gene from olive trees (Villalba et al., 1993). The amino acid sequences of all of these proteins are only about 32% identical to each other, so we believe that they are not homologs, but merely distantly related members of a large family of pollen-expressed genes. All of these genes also show distant similarity to the Kunitz-type proteinase inhibitors of legume seeds. However, the similarities between the pollen proteins and the inhibitor proteins do not include the region of the proteinase inhibitor that is known to contain the active site; therefore, it is unlikely that these pollen proteins are proteinase inhibitors. An alignment of these pollen proteins is shown in Muschiatti et al. (1994). We have used degenerate primers in attempts to clone the homologs of the LAT52 gene from wild species of tomato, from tobacco and from *Arabidopsis*. The primers were designed to span the intron in LAT52, and were chosen based

on regions where the amino acids in the maize Zm13 gene and the LAT52 gene were identical. The results of these analyses are shown in Fig. 1. Sequence from a *Lycopersicon parviflorum* PCR product indicates that we have likely identified a true homolog of LAT52 in this species. However, the products we obtained from *Arabidopsis* and tobacco show that though they are quite similar to each other, they are rather different from the tomato LAT52 sequence. The intron position is conserved in all 4 sequences shown in Fig. 1.

The intron position is also conserved in the rice gene, but the maize gene does not have an intron, and a genomic clone of the olive allergen gene is not available. Because tomato and tobacco are related (both *Solanaceae*), if this tobacco gene was a LAT52 homolog, it is unlikely that it would be more similar to a *Arabidopsis* gene sequence than to a tomato sequence. We therefore view this finding as evidence for another group of proteins that shows similarity to the LAT52 superfamily, and we predict that members of this sub-group will also be found in tomato and in *L. parviflorum*.

We used an antisense approach to determine if the LAT52 protein played an important role during pollen development. The results of these experiments (Muschietti et al., 1994) indicate that the antisense pollen tubes are abnormal – the end result is that the pollen tubes cannot grow the length of the style and deliver the sperm cells to the ovary. We found that the pollen that is deficient in LAT52 protein has a water hydration/retention problem, and hence cannot build up the hydrostatic pressure required for germinating a proper pollen tube. It is still unclear why the lack of LAT52 protein causes an abnormality in water retention. If LAT52 protein directly contributed to the solute capacity of the pollen grain (by binding water molecules itself) one might expect it to be a abundant protein in pollen, but it is not. Perhaps the LAT52 protein somehow regulates water channels or solute capacity indirectly. Because we do not believe that the olive, maize and rice genes are true homologs of LAT52, these antisense results cannot address the possible functions of these family members, and would require antisense experiments to target specifically the function of the Zm13 gene, for example.

The amino acid sequences of the LAT56 and LAT59 proteins show about 67% similarity. The LAT56 and LAT59 genes are members of a large super-family of reproductive-tissue-expressed proteins that all share some distant similarity to the pectate lyases of plant pathogenic bacteria. The pollen proteins include the major ragweed allergens (i.e. *Amb a* I, Rafnar et al., 1991), a gene from maize (Zm58, Turcich et al., 1993), a tobacco gene (Tp10) that is most likely a homolog of the LAT56 gene (Rogers et al., 1992), a tobacco gene that is most likely the homolog of the LAT59 gene (Kulikauskas and McCormick, unpublished), and an *Arabidopsis* family member (Kulikauskas and McCormick, unpublished). There is at least one report of a family member (9612) that is expressed in the pistil (Budelier et al., 1990). Although we have speculated in the past (Wing et al., 1989; McCormick, 1991) that it seems reasonable that pollen and pistils would require pectate lyase activities for wall dissolution and pollen tube growth, we have not been able to demonstrate pectate lyase activity for the LAT56 and LAT59 proteins, nor to our knowledge has anyone else done so for the other family members. Thus the biological function of these proteins remains obscure. We have shown that the LAT56 and LAT59 proteins traverse the secretory pathway when expressed in a baculovirus system, and thus may be secreted to the pollen wall, consistent with the sequence similarity to ragweed allergens,

which have been localized on the pollen surface. A PCR based approach to identify homologs in other plant species outside the *Solanaceae* usually results instead in the isolation of additional family members, rather than bona-fide homologs, as we also noted when searching for homologs of LAT52.

Translational control

During male gametophyte development, several transcripts that are first detected in the late phase of microsporogenesis (after microspore mitosis) are not translated until later times during pollen development, and the protein products of such mRNAs are presumed to be required during pollen germination or pollen tube growth (for reviews see Mascarenhas, 1990, 1993). However, since many enzymes are translated in pollen before germination, this raises the question of how translation of a specific set of mRNAs are discriminated against, when some mRNAs are actively translated. The 5' untranslated regions (5' UTR) of transcripts often are implicated in translational control and in determining the translatability of mRNAs (for review, see Kozak, 1992). Mascarenhas (1993) notes that the mRNA for Zm13 is transcribed 10 days before the protein is first apparent. Although the Zm13 gene is not believed to be a homolog of LAT52 (see discussion above), it is interesting to note that in his 1993 review, Mascarenhas mentions (unpublished) that there is a cis-acting element in the 5' untranslated leader of the Zm13 gene which is responsible for translational repression.

In the process of testing whether there were elements in the 5' UTR of the LAT52 promoter that contributed to pollen specificity, we made constructs composed of the LAT52 promoter, with and without the LAT52 leader, fused to the reporter gene in transient assays. The leader-less construct was not expressed in non-pollen tissues, indicating that the leader does not contribute to specificity by repressing expression in other tissues. We also saw no repressive effect on gene expression in pollen in the presence of the leader, and in fact Twell et al. (1994) reports that the LAT52 leader can act to enhance translation, although his experiments compared the LAT52 leader to a synthetic leader, while our experiments compared the presence and absence of the leader.

When we tested whether there were any elements in the 5' UTR of the LAT59 gene which might contribute to pollen specificity, we discovered that the LAT59

leader had a repressive effect on gene expression. The LAT59 gene has a relatively long 5' untranslated region of 235 base pairs. These experiments compared the level of reporter gene activity (luciferase) in constructs that fused the LAT59 promoter and 5' UTR to constructs with the same LAT59 promoter but only 10 of the 5' most base pairs of the 235 bp 5' UTR. Both constructs were bombarded into tobacco pollen grains. The results showed a 20-fold increase in LUC activity when the 5' UTR was deleted, and a similar trend (a 10-fold increase in reporter activity when the leader is deleted) is seen in transgenic plants transformed with these constructs. Further experiments using the transient expression bombardment assay indicated a progressive decline in gene activity as the 5' UTR was added back to the constructs. There are two predicted stem-loop structures in the 5' UTR of the LAT59 gene, one of which is quite close to the 5' end of the UTR. Such secondary structures are known to interfere with translation initiation (Kozak, 1992). The LAT59 homolog in tobacco conserves the 5'-most stem-loop structure; this evolutionary conservation suggests that this leader plays an important role in regulating the expression of the LAT59 gene and its homolog in tobacco. The LAT59 5' UTR is also able to repress expression in tissues other than pollen. Since the LAT59 promoter is inactive except in pollen, this was demonstrated by bombarding tobacco cell cultures with constructs driven by the CaMV35S promoter, with the LAT59 5' UTR inserted between the promoter and the reporter gene. Transient expression results indicate a 20-fold reduction in gene expression when the 5' UTR is present in the construct.

We now need to address whether the repression acts at the post-transcriptional or translational level (that is, does the LAT59 5' UTR contain a transcriptional inhibitor or in some way decrease message stability, or does it inhibit translation). Secondly, we are interested to learn whether there is a biological reason that LAT59 expression is so tightly regulated, especially in light of the fact that the LAT59 protein is a member of a large super-family – are only some members regulated in this way, and why?

Transcriptional control

We have characterized the minimal promoter elements that are required for pollen expression of the LAT52, LAT56 and LAT59 genes, by analyzing promoter-reporter gene constructs in both a transient assay sys-

tem (Twell et al., 1989a) and in transgenic tomato plants (Twell et al., 1991). In this work, we identified two elements (the so-called 52/56 box and the 56/59 box) that were shared by two of the three promoters. However, sequence comparisons revealed no regions that were shared between all three promoters, nor have any other universally shared sequence elements been found in other pollen specific promoters (see McCormick, 1991, for review). Since removal of the 52/56 or 56/59 boxes had no effect on the specificity of expression, it remained to be answered which cis-acting elements controlled the specificity of expression of these genes. In order to address this question, we performed additional linker scan mutagenesis experiments in the minimal promoters of the LAT52 and LAT59 genes. These constructs were assayed in both the transient bombardment assay and in stably transformed tomato plants. In each promoter, we identified 30 base-pair regions that are important for pollen expression, because they caused a decrease in the level of expression of the reporter gene. We have named these elements PP52 and PP59 (for proximal promoter) boxes. The transgenic plant analyses performed to date confirm the importance of these PP boxes for quantitative expression in pollen. Our results also suggest that these elements do not contribute to pollen specificity by acting as repressors, in that reporter gene expression is not seen in tissues other than pollen when these PP regions are mutagenized. The recent report of Bate and Twell (1994) that the -41 to +100 GUS construct (which includes the 5' UTR) was sufficient to give blue pollen indicates that there may be yet another redundant element present within the 5' untranslated leader that can mediate pollen expression, but further promoter dissection will be required to determine the precise regions involved.

We have indirect evidence that the PP52 box (at least) does interact with protein factors to mediate gene expression. This was shown by an *in vivo* competition assay after particle bombardment with constructs that contained multimers of the wild type PP52 box, or a mutated version of the PP52 box, on the same DNA molecule as the LAT52 promoter-reporter gene construct. The wild-type PP52 multimer was able to compete for putative trans-acting factors (and hence lowered reporter gene expression), whereas the mutated multimer of the PP52 box could not. To prove that the PP boxes interact with transcription factors, we will have to isolate the proteins that bind to the PP boxes. It is difficult to obtain large amounts of tomato pollen, so classical approaches such as gel retardations to sup-

port the presence of DNA-protein interactions are also difficult to achieve. Furthermore, the LAT promoters are only expressed in the vegetative cell nucleus of the two-celled tomato pollen grain (Twell, 1992). For these reasons, we are now using an alternative approach to identify trans-acting factors that interact with one or more of the LAT promoter elements. This scheme is based on genetic selection in yeast, and was used to clone the cDNA encoding the olfactory binding protein (Wang and Reed, 1993). In our case, it involves fusing the LAT promoter elements upstream of the Gal4 minimal promoter, which drives the His3 gene. Separately, a cDNA library from tomato is cloned in a *trp+* centromeric plasmid, in a fusion with the DNA sequence encoding the Gal4 activator domain. The cDNA library is then transfected into yeast cells containing the LAT promoter element construct. When a protein encoded by one of the tomato cDNAs binds to the LAT gene promoter sequence, it brings the Gal4 activator domain in contact with the Gal4 minimal promoter, and allows transcription of the His gene. Therefore, His⁺ colonies are obtained, and the cDNA plasmid can be rescued from these cells and sequenced. This scheme should allow us to isolate genes that encode proteins that interact directly with the LAT promoter elements, and in theory should not depend on the cooperative presence of multiple trans-acting factors, as may be the case in the endogenous system.

To date, we have tested two constructs and one library. We fused trimers of the 52PP and 59PP 30-base pair elements upstream of the Gal4 minimal promoter. These constructs are not activated to give His⁺ cells when present in yeast, which shows that there are no endogenous yeast transcription factors that can 'turn on' these constructs. A cDNA library prepared from anthers at the green petal (GP) stage of flower development has thus far yielded no positives. Three reasons for the lack of the success can be considered. Firstly, it is possible that the cDNA library was prepared from a stage of pollen development that was too early, (i.e. the mRNA for the transcription factor that binds to these cis-elements was not yet present, or very abundant), although we know that the LAT52 and LAT59 promoters are active around the GP stage of development (Twell et al., 1990, 1991), so the transcription factors should be present. Secondly, it is possible that the mRNA for these putative transcription factors is present, but because the library was prepared from anthers, and not isolated microspores, there is a large dilution factor with mRNAs from the sporophytic tissue, which are not expressing the transcription factor.

Thirdly, the scheme may not work for other technical reasons. To address the first two possibilities, we are currently using a cDNA library that was prepared from mature pollen. From our results to date, we favor the idea that pollen specificity is mediated in a combinatorial manner, with protein factors (that may not be pollen-specific themselves) acting in a pollen-specific manner. Once we have obtained protein factors that bind to the PP elements, we can begin to test whether a combinatorial model for pollen gene expression will prove true.

The LAT52 and LAT59-reporter gene constructs are also expressed in a pollen-specific manner in *Ara-bidopsis* (Twell et al., 1990). A few years ago we set out a mutagenesis screen for trans-acting factor mutants. The scheme involved mutagenizing homozygous LAT52-GUS and LAT59-GUS plants, and screening the M1 plants for those that showed only 50% blue pollen, with the supposition that the white (non-GUS staining) pollen would harbor a mutation in a trans-acting factor, thus disabling the ability to express the GUS gene in 50% of the pollen. Although we obtained several putative mutants in the primary screen, none bred true into subsequent generations. In retrospect, if the LAT promoters are composed of multiple, redundant elements, any of which can mediate some level of pollen expression in the absence of others, this scheme was doomed to failure, since mutation of only one trans-acting factor would have no phenotype. Using a similar mutation screen with the promoter of the chalcone synthase gene fused to GUS, Jackson et al. (1994) identified candidates for mutations in trans-acting factors that interacted with the CHS promoter, in that the lines had reduced CHS-GUS expression. However, several of those tested proved to be due to methylation of the transgene, since the level of the endogenous CHS was not altered. We did not examine the molecular basis for the 50% white pollen in our putative mutants, so we do not know whether methylation differences contributed to their identification.

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