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"A Genetic Analysis of Adh1 Regulation"

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Technical Report of Research Progress from 6-1-91 to 5-31-93

The overall goal of our research is to understand the meaning of the various cis-acting sites responsible for Adh1 expression in the entire maize plant. Latest findings of our mutant analysis has brought attention to the regulation of Adh2 as well, as will be discussed below.

Studies on the TATA box region and analysis of revertants of the Adh1-3F1124 allele.

1. Previously we isolated and characterized a revertant Adh1-3F1124r17 allele, which bears a deletion of 18 nucleotides removing the TATA box and its immediate vicinity. In this revertant the level of Adh1 expression increased from 6 to 60% of wild type in anaerobic roots and scutellum while simultaneously expression in pollen decreased from 100% to 30%. Our most recent findings are that, in pollen, Adh1 transcripts accumulate to wild type levels, while in anaerobic roots transcripts accumulate at 60% levels, a number which agrees with the observed enzymatic activity (in roots). In roots multiple transcription initiation sites, clustered around the wild type site, are utilized. In pollen, transcription initiates at least 25 nucleotides further upstream. These data suggest that the TATA box region influences post-transcriptional processes in the male gametophyte but not in somatic root tissue. Different organs may provide surrogate TATA function in different ways, leading to organ-specific differences. We published this work recently in the EMBO Journal (Kloeckener-Gruissem et al. 1992 see appendix).

Our observations raise further questions which we will address during the next granting period: (1) What is the precise Adh1 transcription initiation site in the revertant pollen? RNase protection and primer extension experiments will show the answer. (2) Which post-transcriptional step is causing the reduced enzyme activity in pollen? We will establish a polysomal profile from male tassel tissue and pollen and use a wild type and a revertant-specific transcription probe to determine whether translation of the 5'-extended revertant Adh1 mRNA can be initiated or whether translational elongation may be effected. (3) Are the revertant-specific elongated transcripts specifically made in gametophytic tissues? We will investigate the transcription pattern in immature ears. We conducted initial in situ experiments which demonstrate that ADH activity is differentially found in the developing immature ear, prior to fertilization. We are currently analyzing Adh1 transcripts from these tissues.

2. We proposed to discover the meaning of the Adh1 TATA box sequences using site-directed mutagenesis and transient expression analysis. We completed the construction of all relevant substitution/deletion promoter constructs. Their effect on transcription was assayed transiently after electroporation into two different tissue culture lines: the Black Mexican Sweet (BMS) cell line which was derived from mesocotyl tissue and a more embryo like cell line, P3377. Although we have not yet been able to assign a single dramatic effect to a particular nucleotide of the TATA box, the following general observations and conclusions can be stated: (i) The two

different cell lines exhibit a differential level of normalized expression when the same mutant construct was tested; indicating differential tissue specific expression of the TATA box region of the promoter in vitro, just as we observed in vivo in revertant r17. (ii) In the embryonic cells every mutant construct directs higher levels of expression when compared to the BMS cells; suggesting that a less differentiated cell type may tolerate the TATA box mutations better than a more differentiated cell. (iii) The largest of all tested deletions (the 18 bp deletion which mimics the r17 deletion), results in 20% level of reporter gene expression as compared to the wild type promoter. This level of expression is not as high as the 60% expression observed in vivo in roots but is closer to the 30% observed in pollen. Again, we interpret these data just as proof of differential gene expression in different tissues in vivo and in vitro and that each tissue requires specific factors which, in combination, allow proper interpretation of the meaning of a cis-acting site. The electroporation experiments also demonstrate that another transient expression system needs to be employed, one which better represents the in vivo situation. For these purposes we proposed to improve the utilization of the particle gun as a mean to deliver DNA into living tissues. We will report progress on the mechanistic aspect of those experiments below; here we describe initial microprojectile bombardment experiments of seedling roots with wild type and the 18 bp deletion constructs. We tested aerobic and anaerobic seedling roots for reporter gene expression and found a drastic decrease in expression when seedlings were bombarded with the deletion mutant construct in comparison to the wild type construct.

3. A third experimental approach to the understanding of the meaning of the TATA box sequences focuses on trans-acting protein factors that recognize and bind the TATA box as an early step in transcription. We proposed to isolate the gene(s) encoding the transcription factor TFIID, which was shown in yeast and mammalian cells to bind the TATA box and to play a role in initiation of transcription. Substantial progress was made towards isolation and characterization of the maize Tbp genes. In contrast to animals and yeast, the maize genome carries two unlinked Tbp loci as determined by southern blot analysis and cDNA cloning. The genes were localized, using RFLP mapping, to chromosome arms 1L and 5S. The cDNA clone for locus one (Tbp-1) was identified based on sequence homology to primers obtained from animal TFIID. A fragment was obtained in a PCR reaction which was used to screen a maize seedling cDNA library. resulting in the isolation of a full length clone representing Tbp-1 Using the Tbp-1 cDNA, a second cDNA class, representing the transcripts of Tbp-2 was isolated. We studied the transcript expression pattern of the two Tbp genes with the primary question why plants (note that Arabidopsis also carries two Tbp loci as reported by the Chua laboratory) carry two loci, whereas animals contain only a single Tbp gene. We hypothesized that perhaps these loci are differentially expressed with respect to gametophytic vs somatic tissue or their expression may be differentially regulated in a developmental fashion in the plant. We know

now that both maize Tbp genes are actively transcribed and that they are differentially expressed only with respect to somatic tissue. Experimental complications, primarily based on the fact that both genes are very similar in sequence composition and exhibit only one short region of non-homology to use for loci-specific probing, have hindered the precise identification of loci-specific transcripts.

We successfully expressed both maize Tbp genes in vitro, and the isolation of antibodies is in progress. We soon will combine the TATA box mutant construct experiments with the TFIID studies. The over expressed TBP proteins and their antibodies will be used in DNA-binding assays to analyze the requirements of the sequences within and surrounding the TATA box promoter region for the Adh1 gene expression.

Studies on cis-acting sequences required for root-specific Adh1 expression.

We proposed to analyze a revertant Adh1-3F1124r53, which exhibits a novel tissue-specific effect on Adh1 expression: 50% activity in the scutellum and pollen and no detectable activity in aerobic and anaerobic roots. We postulated that this pattern fits a knock-out of a root-specific box, rather than an anaerobic-specific box which exerts its effects in the roots.

During our attempts to obtain a promoter fragment using the PCR reaction we discovered that homology to one (or both) of the primers was disturbed by the mutation. We performed detailed Southern Blot analyzes which focused on short restriction fragments encompassing the 1 kbp BamHI-HindIII promoter region and concluded that this mutation is not a simple deletion or insertion but rather a combination of both or an inversion. It became clear that precise nucleotide information is needed of the r53 promoter region which extends the initial limits set by the primers as described in our proposal. Therefore we cloned the 11 kbp genomic BamHI fragment which contains the entire r53 locus, including up- and downstream sequences, into bacteriophage lambda. DNA sequencing of the 1kbp promoter region shows three types of changes: First, a deletion which removed 30 bp including the TATA box, second, a duplication of parts of the untranslated leader and the first exon which became inserted as an inversion just proximal to the TATA deletion and third, an inversion of the anaerobic regulatory region (ARE) just proximal to the deleted TATA region. Such complex DNA structural changes were most likely generated during the excision event of transposon Mu3. These findings might aid in the understanding of Mutator activity, which is the focus of another research project in our laboratory.

The initial phenotypic characterization of the r53 mutant as described above and in the proposal was extended to monitor ADH1 expression in other organs, tissues and cells. We obtained preliminary evidence that shows novel ADH1 activity in young leaves, where enzyme activity in wild type plants can not be detected. How this ectopic expression is correlated with the above described DNA rearrangements in the promoter and untranslated leader will be approached within the next year. We will spend continuous

effort on the analysis of this revertant allele in the whole plant, at all developmental stages and under various environmental conditions. As did revertant r17, this revertant offers new surprises about sequence requirements and their consequences on Adh1 expression.

Studies on cis-acting sequences involved in pollen specific expression.

In our proposal we suggested to analyze the involvement of the untranslated leader region in pollen expression. We obtained DNA sequence information from PCR amplified genomic DNA from many of the germinal revertants of the unstable Adh1-Fm335 mutant which suggests that very small changes in the DNA sequence can result in dramatic changes in enzyme activity. Analysis of mRNA of the germinal revertants indicates that the activity levels are reflected at the level of transcript accumulation. For more details, see Dawe et al.s manuscript in the appendix.

Refinement of the use of the particle gun

The availability of the particle gun as a mean to deliver DNA constructs, whose transient expression can be measured in living tissues and cells, is essential to our studies. We are continuously working to improve the frequency of successful DNA delivery. We tested some of the obvious technical aspects and decided to conduct our experiments according to the best results which are listed below. One major breakthrough in the ease of measuring bombardment success came with the availability of a plasmid pC17 which contains the maize color gene C1/ B-Peru driven by a 35S promoter (provided by Michael Fromm). We discovered that expression of this gene within 24 hours after bombardment of seedlings carrying the genotypes of our Adh1-3F, Adh1-3F1124 and Adh1-3F1124r53 alleles results in red colored epidermal and sub-epidermal cells, reflecting frequencies of success. Using this color reaction as a measuring unit we found consistently fewer spots in seedling roots versus seedling shoots. We find best results if every material is assaulted with two shots, we determined the optimal distance between the position of the macroprojectile and the living material and the use of two metal screens of different mesh sizes placed between the projectile and the target material. It should be noted that "best" and "optimal" reflects the momentary conditions and we continue with improvements.

Screening for more, different mutants that affect Adh1 expression differentially.

1. Search for mutants deficient in the expression of trans-acting proteins.

A. We chose the transposon Mutator system as the mutagen. Mu-active plants (in which transposon activity was monitored by an unstable color gene "bronze") which carried a normal, active Adh1-1F allele, were grown to maturity. Alive , ADH1 positive pollen grains were collected and treated with allyl alcohol vapors, which kill ADH positive pollen. Ideally, only ADH inactive pollen can survive. Treated pollen grains were crossed to tester ears, carrying an active Adh1 allele which product migrates at a distinguishable position in an electrical field. The scutellum of M1 kernels from this cross were subjected to ADH analysis. Some numbers may demonstrate the powerful selection of this procedure: We treated approximately 5×10^7 pollen grains; from crossing to tester ears we obtained about 100 kernels of which 10 were lacking an active ADH1 enzyme in the scutellum. We anticipate several scenarios of events leading to the mutant phenotype: (i) The mutation occurred within the Adh1 locus. (ii) The mutation occurred at an unlinked site. These represent the class of mutants that is of highest interest to us since they might identify a trans-acting factor which is either generally or differentially required for Adh1 expression. We grew a few M1 kernels which showed no ADH1 deficiency in the scutellum and assayed ADH activity in pollen. In two cases, enzyme activity segregated, suggesting that these two new mutants carry a deficiency that affects ADH expression in trans and in an organ-specific manner. Further genetic and molecular analysis of these new, and other potential mutants will allow us to identify factors that control Adh1 expression in trans.

B. We chose the alkylating agent Ethyl methanesulfonate (EMS) to mutagenize pollen. EMS is known to cause point mutation but last year reports from Sue Wesslers group suggests that EMS, when exposed to maize pollen, causes small deletions. Wild type Adh1 pollen was treated, crossed to Adh1 wild type ears and resulting kernels were grown to maturity, pollen samples were analyzed for segregating Adh1 mutant activity, and plants were self pollinated and outcrossed. In addition to ADH1 deficient plants, we were able to score other abnormal pollen phenotypes that were either male sterile mutants or showed segregation of pollen size, which potential effect on function can be analyzed, but is clearly not the focus of our work. Of 3000 tested plants, 0.5% showed an ADH1 deficiency. We found one of these mutants to act in cis, while the others are still under investigation.

2. Search for additional mutants derived from a partial revertant of Adh1-3F1124.

Revertant r16 was described in our proposal. It specifies 30% of wild type activity in the scutellum and roots but shows no alteration in pollen expression levels (=100% of wild type). The transposon Mu3 did not excise and is still sitting in the Adh1 promoter. In situ ADH activity in aleurone cells indicates that this allele, like the mutant 1124 is also unstable. We will take advantage of this instability and by varying selection conditions we will screen for either up or down mutation. In preparation for this screen, we are

currently constructing a maize line that carries a morphological marker linked to the Adh1-3F1124r16 allele. The marker we chose is the knotted phenotype, caused by a dominant mutation, Kn1-0, which is located one map unit apart from Adh1. Homozygous r16 plants were crossed to a Knotted plant which carries an Adh1-1S allele. Heterozygous kernels were self pollinated and will be analyzed for recombination events leading to the Adh1-r16 allele linked to Knotted.

Functional analysis of a non glycolytic anaerobic protein.

We continued work on one of the anaerobically inducible genes, previously denoted as ANP31. Thorough DNA sequence analysis of a full-length cDNA clone led us to rename the gene as ANP27 This cDNA hybridizes to a polyadenylated message of approximately 1.1 kb whose steady-state levels are induced 2-3 fold in hypoxic seedling roots and shoots. We cannot detect transcripts for ANP27 in aerobic root tissue, nor in cold- or heat-treated seedlings; however we did find abundant levels of ANP27 message in developing embryos The cDNA contains an open reading frame of 765 nucleotides, encoding a polypeptide of 255 amino acids with a predicted molecular mass of 27.5 kDal. The protein is prolin (10%) and arginine (10%) rich; yet it does not show obvious sequence homology to known proteins rich in these two amino acids. We constructed appropriate plasmids to obtain in vitro expressed ANP27 polypeptides which were injected into rabbits and ANP27 specific antibodies were obtained. With these molecules in hand we will be able to analyze the gene's expression pattern and elucidate more of its (hopefully regulatory) functional aspects. Identification of the cellular localization of the encoded protein will allow a better understanding of the potential regulatory role of ANP27 non glycolytic anaerobic protein in hypoxic roots and in noninduced developing embryos.

Expression of a new ADH activity in hypoxic roots.
During the course of analyzing the mutant Adh1-3F1124r53, we stumbled across an unexpected pattern of ADH expression, which is encoded by the Adh2 locus. This hypoxically inducible activity is expressed primarily in epidermal cells, in contrast to ADH1, which is expressed in every cell, but most staining is found in the central part of the root, the steele. Surprisingly, not every epidermal cell is expressing this ADH; a variegation pattern of cellular expression is observed in cross sections of roots. Along the longitudinal axis of the roots, we also find a characteristic local expression pattern: enzyme activity is concentrated to the region of cell division and early differentiation. Our immediate future experiments will test an involvement with the cell cycle by the use of drugs that interfere with cell division (i.e. aphidocolin) as well its response to other stimuli known to induce (or suppress) ADH1 activity.

Publications resulting from work supported by this grant:

Kloeckener-Gruissem B, Vogel JM and M Freeling (1992) The TATA box promoter region of maize Adh1 affects its organ-specific expression. EMBO J. **11**, 1, 157-166.

Dawe RK, Lachmansingh AR and M Freeling (1992) Transposon-mediated mutations in the untranslated leader of maize Adh1 that increase and decrease pollen-specific gene expression. The Plant Cell, inpress.

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