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MPDover May 22, 2000
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DOE FINAL REPORT

Transcription Factors in Xylem Development

Award # DE-FG02-92ER20085

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The questions asked in this research were:

1. Do the two pine Myb proteins previously identified as candidate transcription factors bind to DNA and activate transcription?
2. In what cell types are these Myb proteins expressed?
3. Are these proteins localized to the nucleus?
4. Do other proteins in pine xylem interact with these Myb proteins?
5. Does altered expression of these genes have an impact on xylogenesis, specifically the expression of monolignol biosynthetic genes?

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JUN 13 2000
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1. Analysis of Myb function

Susan Rodzik, Malcolm Campbell (University of Oxford)

Plant Myb transcription factors have been shown to bind to three different A+C rich sequences termed AC elements. AC elements are found in the promoters of every sequenced monolignol biosynthetic gene. Experiments to test the DNA-binding and transcriptional-activation functions of pine Mybs 126 and 413 were done in yeast using various combinations of the three AC elements fused to the reporter gene LacZ, which encodes beta-galactosidase. Expression of either pine Myb126 or pine Myb413 gave greater production of beta-galactosidase than that seen in negative controls with neither Myb expressed. Similar experiments were done in tobacco mesophyll protoplasts, using parts of the bean PAL2 promoter fused to the uidA gene, which encodes GUS. The pine Myb constructs were expressed under the control of the CaMV35S promoter. Mutation of different AC elements in the bean PAL2 promoter showed that both pine Mybs will bind the ACII sequence, but that the presence of ACI or ACIII elements with the ACII element increases GUS expression. Electrophoretic mobility shift assays (EMSAs) and binding site selection assays were also done, showing that the pine Mybs have slightly different binding specificities for the different AC elements. These experiments showed that pine xylem Mybs were able to bind to DNA and activate transcription in two different systems, yeast and tobacco suspension-cultured cells.

2 and 3. Analysis of expression

Malcolm Campbell

Ribonuclease protection assays (RPAs) were performed to examine pine Myb transcript expression in different pine tissues and organs. RNA samples from needles, shoot tips, roots, two stages of differentiating xylem (stage 1, primary cell wall only; stage 2, secondary wall present), megagametophytes and phloem were used, with the two different pine Myb clones and a ribosomal protein coding sequence as probes. Ribosomal protein transcript was detected in all tissues, but Myb126 and Myb413 transcripts were detected only in stage 1 differentiating xylem (primary cell wall only). This experiment suggested that Myb126 and Myb413 are certainly expressed at a much higher level in

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stage 1 differentiating xylem than in any other tissue or organ tested, although we cannot completely rule out low levels of expression in other tissues. Antibodies were elicited in chickens using Myb126 and Myb413 proteins expressed in bacteria, but the affinity and avidity of the resulting antibodies were insufficient to allow immunolocalization of Myb proteins in differentiating xylem. As an alternative means of detecting subcellular localization, onion epidermis was bombarded with chimeric genes composed of the pine Myb coding sequences fused to the GUS reporter enzyme coding sequence. Staining of bombarded cells showed GUS activity exclusively in the nuclei. This experiment showed that the pine Myb proteins contain nuclear localization signals and are targeted to the nucleus.

4. Interactions of other proteins with Myb126 and Myb413

Alison Morse, Ross Whetten

Post-translational modification of Myb proteins is known to play a role in modulating transcriptional activation. We tested whether pine Myb transcription factors are substrates for, and therefore regulated by, a mitogen-activated protein kinase (MAPK) during xylem differentiation. Basic helix-loop-helix transcription factors (bHLH) have also been shown to interact with Myb proteins in plants, and are known to be regulated by MAPK cascades in animal systems. We therefore sought to clone MAPKs and bHLH factors expressed in pine xylem and test them for interactions with each other and with the Myb proteins. Two MAPKs were cloned from a cDNA expression library made of differentiating pine xylem. One of these, called pine MAPK-1, has been expressed in bacteria. This recombinant MAPK-1 protein autophosphorylates on tyrosine residues, consistent with the activity of similar proteins from animals, and phosphorylates myelin basic protein on threonine and serine residues, again consistent with mammalian MAPKs.

We also cloned a bHLH factor from differentiating pine xylem, using a partial clone obtained from an EST project as a probe. The cDNAs isolated using this probe fell into three classes, which differed only in the presence or absence of short peptides within the body of the protein. This seemed consistent with a hypothesis that a single gene gives rise to multiple, closely-related proteins through differential splicing of a primary transcript. Further investigations into the genome organization of the gene encoding bHLH factor used both Southern blot analysis of pine genomic DNA and PCR analysis of megagametophytes from a single parent tree, and both techniques confirmed the hypothesis that a single gene encodes this family of related bHLH proteins. RT (reverse transcriptase)-PCR analysis of mRNA pools shows that three alternative splicing patterns can be detected, with minor differences in the levels of the three different versions of the protein. All three versions of the protein were expressed in bacteria and tested for DNA-binding activity. The longest and the shortest protein both bind the canonical bHLH recognition sequence CANNTG (the "E-box" first identified in studies of mammalian bHLH factors) and the "G-box" sequence CACGTG, while the intermediate protein fails to bind either sequence. The potential physiological relevance of these differences in DNA-binding activity has yet to be determined. Antibodies have been raised in rabbits against the full-length bHLH1c protein (the shortest version), and against the 100-amino acid peptide present in the longest version and absent in the shortest (the "intron peptide"). The anti-bHLH1c antiserum detects three bands on Western blots of

differentiating pine xylem, consistent with the hypothesis that all three versions of the bHLH protein are present *in vivo*, while the anti-"intron peptide" antiserum detects the larger two proteins. Both the pine Myb and bHLH transcription factors have been tested for their ability to serve as substrates for recombinant pine MAPK-1. *In vitro* phosphorylation assays using recombinant proteins revealed that MAPK-1 appears to prefer Myb 413 as a substrate over Myb 126. No phosphorylation of bHLH recombinant proteins was detected. This indicates that the recombinant MAPK-1 protein is capable of discriminating between potential phospho-acceptor sites. Phosphoamino acid analysis and mutational analysis were performed to characterize MAPK phosphorylation sites in Myb 413. The phosphoamino acid analysis indicated that recombinant pine MAPK-1 phosphorylates recombinant Myb 413 on serine residue(s).

MAPKs are proline-directed serine/threonine kinases, and will therefore phosphorylate target proteins only on S or T residues immediately followed by a P residue. The predicted amino acid sequence of Myb 413 reveals four candidate SP phosphorylation sites, three in and around the DNA-binding domain and one in the C-terminal domain of the protein. It was thought unlikely that the DNA-binding domain was phosphorylated, because EMSAs (electrophoretic mobility shift assays) do not detect an effect of phosphorylation on DNA binding. The C-terminal SP site in the activation domain was therefore considered the most likely site for phosphorylation of Myb413 by MAPK-1. In order to test this hypothesis, site-directed mutagenesis was used to mutate the C-terminal SP in Myb413 to an EP dipeptide (glutamate followed by a proline), a change predicted to have a phosphomimic effect on Myb413 function. When used as a substrate in *in vitro* phosphorylation assays, this Myb mutant, Myb 413SE, was found to be only minimally phosphorylated when compared to wild type Myb 413. Antibodies raised against recombinant pine MAPK-1 have been used to immunoprecipitate the endogenous kinase and determine its activity in protein extracts made from differentiating xylem. The results from these experiments indicate that recombinant MAPK-1 and MAPK immunoprecipitated from differentiating xylem both recognize similar substrates and imply a role for MAPKs in regulating gene transcription in response to developmental signals. Myb413 could interact with MAPK-1 *in vivo*, although we have not yet demonstrated this interaction. We have yet to test for direct interactions between the Myb proteins and the bHLH proteins *in vivo*. Both these experiments will depend on the availability of higher quality antisera against the pine Mybs, which would allow immunoprecipitation experiments and co-immunolocalization experiments to test the hypotheses of *in vivo* interactions.

Another facet of analysis of expression was to test the involvement of the Fos and Jun transcription factors in the regulation of xylem differentiation, using biochemical and genetic approaches in pine and model organisms. These two proteins bind DNA as dimers of different types, either Fos-Jun heterodimers or Jun-Jun homodimers. Active dimers are known as AP-1 transcription factors and have been shown in animals to regulate genes involved in the control of growth and differentiation. Phosphorylation of the AP-1 transcription factor by MAPK cascades in response to extracellular signals, results in changes in AP-1 function in animal cells. A number of sequences similar to the mammalian AP-1 binding site have been identified in the pine PAL promoter. *In vitro* EMSAs indicate that these PAL sequences are recognized by proteins in pine xylem

extracts, indicating that the pine PAL gene in differentiating xylem may be regulated by a transcription factor similar to AP-1.

Purification of the AP-1 DNA-binding activity from differentiating pine xylem is in progress. Fractions eluted from a DNA affinity column containing AP-1 DNA-binding activity were further purified on an affinity column comprised of AP-1 binding site oligomers. The eluate from the affinity column contains AP-1 DNA-binding activity, as determined by EMSA, and contains Jun-like proteins as determined by Western blot analysis with antisera against the mammalian Jun protein. The next goal is to separate the protein(s) in this fraction by 2-D electrophoresis and determine the identity of the proteins by N-terminal amino acid sequencing. Random cDNA sequencing of clones derived from differentiating pine xylem has identified a putative Jun-interacting protein based on predicted protein sequence homology. This cDNA is currently being cloned into an expression vector for protein production in *E. coli* and will be tested for AP-1 DNA-binding activity and for protein-protein interactions.

5. Analysis of physiological function

Transgenic plant experiments have not been carried out as proposed, due to technical difficulties in producing transgenic conifers. The proposal relied heavily on analysis of Myb protein functions in transgenic model plants, primarily *Arabidopsis*, but comparison of results using the bean PAL2 promoter in pine and tobacco convinced us that this was perhaps unwise. In transgenic tobacco, the promoter of the bean PAL2 gene is more strongly expressed in differentiating xylem, while the promoter of the bean PAL3 gene is expressed more strongly in flower petals and other non-vascular tissues. Transient expression experiments in pine xylem, using microprojectile bombardment to introduce bean PAL2-GUS and bean PAL3-GUS fusion genes, showed that in differentiating pine xylem, the bean PAL3 promoter is more strongly expressed than the PAL2 promoter. This indicates clearly that tobacco and pine regulate these two promoters in different ways, and cast serious doubts in our minds as to the utility of analyzing pine promoter or transcription factor function in a model angiosperm system. We have instead focused our attention on isolation and characterization of proteins likely to interact with Myb proteins in differentiating pine xylem (see section 4, above).