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FINAL TECHNICAL REPORT

"Regulation of Cell Division in Higher Plants"
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We have no objection from a patent standpoint to the publication or dissemination of this material.

MPDowscale
Office of Intellectual Property Counsel
Mar 24, 2000
San Francisco, California

Objectives of 1993 Renewal Application

Research proposed in our 1993 renewal application to the Department of Energy was divided into two parts. First, we set out to expand our macromolecular tool kit for studying plant cell division. Second, we proposed experiments in which the roles played by plant cell cycle regulators were to be cast in the light of the emerging yeast and animal cell paradigm for molecular control of the mitotic cycle.

The first objectives were accomplished to a very satisfactory degree (see below). With regard to the second part of the project, we were driven to change our objectives for two reasons. First, the families of cell cycle control genes that we cloned encoded such closely related members that the prospects for success at raising distinguishing antisera against each were sufficiently dubious as to be impractical. Epitope tagging is not feasible in *Pisum sativum*, our experimental system, as this species is not realistically transformable. Therefore, differentiating the roles of diverse cyclins and cyclin-dependent kinases was problematic.

Secondly, our procedure (Prewett and Jacobs, 1995) for generating mitotically synchronized pea root meristems for biochemical studies was far too labor intensive for the proposed experiments. Indeed, investigating the biochemistry of mitotic mechanisms in intact plants is prohibitively constrained by the practical limitations imposed by the growth habits and developmental strategies of the organisms themselves. There simply is no mitotically synchronizable meristematic system in intact plants in which discovery biochemistry can be performed on a practical scale. We are limited to correlative studies in which meristem extracts are assayed for the presence or state of proteins identified by other means. We therefore shifted our objectives to identifying connections between the conserved proteins of the cell cycle engine and factors that interface it with plant physiology and development. In this, we have obtained some very exciting results.

The following manuscripts are either published and attached or actively in preparation, to be forwarded before the panel meets. Some results described are not explicitly included in the manuscript under the reference for which they are presented below because, for these findings, we have yet to complete a story in which they can be incorporated. Yet they merit mention and the location in which they appear below was considered most appropriate.

Renaudin, J.-P., J. Doonan, D. Freeman, J. Hashimoto, H. Hirt, D. Inze, T. Jacobs, H. Kouchi, P. Rouze, M. Sauter, A. Saviour, D. Sorrell, V. Sundaresan and J. Murray (1996) Plant cyclins: a unified nomenclature for plant A-, B- and D-type cyclins based on sequence organisation. *Plant Mol. Biol.* 32:1003-1018.

Passage of every eukaryotic cell through the mitotic cycle is regulated by CDKs (cyclin dependent kinases) whose activity is controlled primarily at the post-translational level by phosphorylation and by positive and negative regulatory subunits, cyclins and CDK inhibitors, respectively. We probed a pea cDNA library with a PCR clone (isolated during the previous

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cycle of DOE support) encoding part of a pea mitotic cyclin, and obtained three cyclin clones, *CycPs1*, encoding a mitotic, B-type cyclin, and *CycPs2* and *CycPs3*, both encoding S phase, A-type cyclins. These cyclin clones were among those analyzed in the Renaudin *et al.* survey.

We also probed our pea cDNA library with PCR product encoding part of a pea CDK (Feiler and Jacobs, 1990; Feiler and Jacobs, 1991) and obtained three clones. CDKPS1 and CDKPS2 are 87% identical at the amino acid level. CDKPS1 is 79% identical to the CDC28 gene product of budding yeast, the archetype CDK. Both *CdkPs1* and *CdkPs2* rescue temperature sensitive *cdc28* budding yeast strains. *CdkPs3* encodes a putative 57 kDa protein resembling a human CDK that phosphorylates the retinoblastoma tumor suppressor protein (Grana *et al.*, 1994). An alfalfa clone encoding a highly homologous product was recently reported (Magyar *et al.*, 1997).

Pomerening, J., P. Wakeley, T. Duncan and T. Jacobs (1997) Protein synthesis and the cell cycle: Interaction between a cyclin-dependent kinase and translation apparatus. (submitted to Plant Cell)

We began this study by assuming that at least some proteins that participate in regulatory interactions with CDKs must bind to them. Far-western library screening (Kaelin *et al.*, 1992) was used to identify pea cDNAs encoding proteins that bind to CDKPS1. That is, we probed our pea cDNA expression library with radioactively labeled CDKPS1. 25 isolates survived a second screen and two were selected for further study.

One selected CDKPS1-binding protein was a homolog of CYC07, a protein encoded by the eponymous clone previously selected for its expression during S-phase in cultured plant cells (Ito *et al.*, 1991). CYC07 has since been identified as S3a, one of the 33 polypeptides that make up the 40S subunits of eukaryotic ribosomes. We will pursue the CDKPS1-S3a connection under alternative support.

The second CDKPS1-interacting protein identified was a translation elongation factor, eEF1Bb (see Scientific Background, below, for discussion of the eEF1 complex). We verified that labeled CDKPS1 binds to western-blotted, recombinant eEF1Bb and vice versa, whether CDKPS1 was fused to GST or 6-His. We also showed that recombinant glutathione agarose-bound GST-CDKPS1 precipitates *in vitro*-translated eEF1Bb out of the translation reaction mixture whereas glutathione agarose-GST alone does not.

We raised murine antisera against recombinant pea eEF1Bb. If eEF1Bb is bound to one or more CDKs *in vivo*, then the latter's signature histone H1 kinase (H1K) activity should be detectable in eEF1Bb immunoprecipitates. We recovered substantial H1K activity in eEF1Bb immune precipitates from whole cell pea protein extracts and negligible activity in control precipitations. Thus it appears that eEF1Bb binds to active CDK complexes.

Neither recombinant nor immunoprecipitated pea eEF1Bb were phosphorylated *in vitro* by affinity-purified pea CDKs. However, purified wheat germ eEF1B complexes (provided by Karen Browning, University of Texas) were efficiently phosphorylated on the a, b, and g subunits by pea CDKs. Antisera raised against recombinant pea eEF1Bb recognize the structurally related eEF1Ba and eEF1Bb polypeptides from wheat germ.

Wakeley, P. and T. Jacobs (1997) The retinoblastoma-associated protein RbAp48 interacts with the *no apical meristem* gene product of *Arabidopsis thaliana* (in preparation)

At the end of the G1 phase of the mitotic cycle, cells decide whether to divide again, arrest, or die. Plant cells display this decision point, as well as another in the G2 phase (Van't Hof, 1985). Central to the G1 decision process in vertebrate cells is Rb, the retinoblastoma tumor suppressor protein. During G1, hypophosphorylated Rb sequesters factors that are needed for the cell to enter S phase. Phosphorylation of Rb by specific G1 CDKs frees these proteins to promote the G1-S transition (Hinds, 1995).

RbAp48 is a human protein originally identified solely on the basis of its capacity to bind to Rb (Qian et al., 1993). RbAp48 has more recently been found to "escort" histones, first to the enzymes that acetylate them, then to chromatin assembly factors, and finally to the nucleus for chromatin maturation and deacetylation (ref. (Roth and Allis, 1996) and see Scientific Background, below).

We identified an RbAp48 homolog within the *Arabidopsis* EST collection, verified its sequence, cloned it into a yeast two-hybrid prey vector (Fields and Song, 1989), and demonstrated that it interacts strongly with human Rb in a two-hybrid assay. We then asked, with which proteins does the *Arabidopsis* RbAp48 associate *in vivo*? We moved the RbAp48 clone into a bait vector and screened an *Arabidopsis* library in a prey vector via the yeast two-hybrid system. One interacting clone encoded a homolog of RbAp46, a protein closely related to RbAp48 (Qian and Lee, 1995). Another interacting clone, *AtTAF1*, was an *Arabidopsis* homolog of the *Petunia nam* (*no apical meristem*) gene (Souer et al., 1996). Transposon mutants of *Petunia nam* result in seedlings lacking shoot apical meristems (see Scientific Background for further discussion).

RbAp48 carries seven "WD" repeats that may promote protein-protein interactions (Neer et al., 1994). *AtTAF1* has two versions of this motif in reverse ("DW" repeats). Deletion and site-directed mutagenesis of *AtTAF1* delineated portions of its encoded protein required for interaction with RbAp48 in a yeast two-hybrid assay. Interaction is abolished by deletion of the N-terminal-most DW repeat or substitution of this motif's N-terminal tryptophan (W) with leucine.

We generated transgenic *Arabidopsis* in which the CaMV35S promoter drives RbAp48, in sense and antisense orientations. CaMV35S-RbAp48(sense) seedlings germinate and grow faster than wild-type and ultimately produce earlier and more inflorescence bolts than wild-type. Antisense RbAp48 plants show no abnormal phenotype.

We raised murine antisera against recombinant *Arabidopsis* RbAp48 and used these to localize the protein in *Arabidopsis* cells. RbAp48 is concentrated in the nuclei of some cells, whereas it is either not present or thinly dispersed throughout others.

Duerr, B., M. Gawienowski, T. Ropp, T. Jacobs (1993) MsERK1: A mitogen-activated protein kinase from an flowering plant. *Plant Cell* 5:87-96.

Despite the fact that this paper became the basis of an NSF-funded project in the laboratory, it started as part of our DOE-supported cell division work. We were probing an alfalfa cDNA library with a PCR clone of a pea CDK, and recovered the first MAP kinase cDNA to be cloned from a plant source. We over-expressed the alfalfa cDNA in *E. coli* and showed that recombinant alfalfa protein, MsERK1, was antigenically closely related to animal MAP kinases.

By site-directed mutagenesis and probing with anti-phosphotyrosine antibodies, we showed that the recombinant protein autophosphorylated *in vitro* at the same tyrosine residue at which animal MAP kinases do. We raised an anti-peptide antibody against a carboxy-terminal epitope of MsERK1, and used it to detect a protein of the appropriate size (44 kDa) in extracts from alfalfa roots and shoots. Finally, we showed that this novel plant enzyme could phosphorylate myelin basic protein, the standard substrate used for MAP kinase assays in animal systems. A sequel to this report, describing the auxin and fusicoccin induction of MsERK1 activity in alfalfa roots, is being submitted for publication.

Prewett, M., T. Duncan, H. Feiler and T. Jacobs (1997) The molecular basis for mitotic activity along the pea seedling root axis. (*in revision*)

What is the biochemical basis of the mitotic profile displayed by cell lineages along the developmental gradient of the angiosperm root? We began by surveying diverse plant organs with respect to levels of cyclin mRNA and CDK mRNA, protein, and enzymatic activity. We went on to show that the activity profile of affinity-purified CDKs correlates closely with mitotic activity and steady state concentration of a mitotic cyclin mRNA (but not CDK mRNA or protein) per cell along the pea root axis.

Although these and other published data (Doerner et al., 1996) suggest that cyclin mRNA levels may generate the mitotic gradient in seedling roots, both positive and negative factors are likely involved. We investigated the role of negative factors by asking whether proteins present in the more basipetal, less mitotic, end of the root apex could inhibit CDK activity purified from the meristem itself. Affinity matrix-bound CDKs from the meristem were incubated in protein extracts from successively basipetal root segments, washed, and assayed for residual CDK activity. This experiment revealed a very strong positive correlation between strength of CDK inhibitory activity and distance from the root tip. The inhibitor could be a kinase, a phosphatase, and/or a CDK inhibitor (Parker et al., 1992; Poon and Hunter, 1995; Sherr and Roberts, 1995; Harper and Elledge, 1996). Owing to the difficulties in performing the necessary bucket biochemistry on pea seedling roots required to pursue this phenomenon beyond pharmacology, we have set it aside while we devise a more lucrative strategy for approaching it.

Prewett, B., and T. Jacobs (1995) Chemically induced mitotic synchrony in root apical meristems. In *Methods in Cell Biology*, Vol 49, eds. D. W. Galbraith, H. J. Bohnert, D. P. Bourque, pp. 355-365. Academic Press, San Diego.

In this technical paper, we describe a protocol for inducing mitotic synchrony in the root apical meristems of hydroponically grown plants. Included is our protocol for isolating pea root nuclei and preparing them for flow cytometry. The methods were modified from those supplied to us by Jack Van't Hof (Brookhaven)(Van't Hof, 1966), and rely on the reversible DNA replication inhibitor, 5-aminouracil, a thymidine analog (Prensky and Smith, 1965; Díez et al., 1976). The method is simple, inexpensive, and highly reproducible, but rather labor intensive if enough root tips are to be prepared for biochemical studies.

Jacobs T. (1995) Cell cycle control. *Ann Rev Plant Physiol Plant Mol Bio* 46:317-339.

The purpose of this review was to provide interested plant biologists with a useful introduction to the current model of eukaryotic cell cycle control, along with a snapshot of the state-of-the-art in plant systems. Special emphasis was placed on relatively obscure studies in yeast and animal cells that relate to methods in vogue in plant systems (e.g., RNA level analyses of cell cycle regulators). I also provided a catalog of cloned plant CDKs and cyclins (circa 1994), and highlighted issues regarding the application of the yeast/animal cell paradigm of cell cycle control to plant systems.

Jacobs T. (1997) Why do plant cells divide? *Plant Cell* 9:1021-29

In this essay, I made no attempt to review comprehensively the plant cell cycle/cell division field. Rather, I used two case studies as starting points from which to explore the particular role that cell division may play within the developmental strategies of higher plants. Whereas in animals cell division supplies modular building blocks for organogenesis and tissue maintenance, plants may use mitosis merely to subdivide spaces created as organs expand. This distinction has profound consequences for any hypotheses one might formulate about biochemical links between the cell cycle engine and plant (vs. animal) development and physiology. The two studies discussed were Peter Doerner's report of faster seedling root growth in *Arabidopsis* ectopically expressing a mitotic cyclin (Doerner et al., 1996) and Hans Kende's long term study of gibberellin-induced elongation and cell division in deepwater rice (Sauter and Kende, 1992; Sauter et al., 1995).

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