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Contents

ALLAN, E. J., A. H. SCRAGG, and K. PUGH: Cell Suspension Culture of <i>Picrasma guassioides</i> : the Development of a rapidly growing, Shear Resistant Cell Line capable of Quassin Formation	176
AMBERGER-OCHSENBAUER, S. and J. OBENDORFER: Levels of Free Proline in Ornamental Plants: I. Influence of Plant Age, Leaf Age, and Leaf Region in <i>Saintpaulia</i> and <i>Chrysanthemum</i>	758
ANGELOPOULOS, K. and N. A. GAVALAS: Reversible Cold Inactivation of C ₄ -Phosphoenolpyruvate Carboxylase: Factors Affecting Reactivation and Stability	714
ARNOLD, S. VON and I. HAKMAN: Regulation of Somatic Embryo Development in <i>Picea abies</i> by Abscisic Acid (ABA)	164
ARNOZIS, P. A., J. A. NELEMANS, and G. R. FINDENEGG: Phosphoenolpyruvate Carboxylase Activity in Plants Grown with either NO ₃ ⁻ or NH ₄ ⁺ as Inorganic Nitrogen Source	23
AUGSTEN, H., E. KUNZ, and K.-J. APPENROTH: Photophysiology of Turion Germination in <i>Spirodela polyrrhiza</i> (L.) SCHLEIDEN. I. Phytochrome-mediated Responses of Light- and Dark-grown Turions	90
AVANZI, S., P. VERNIERI, N. CECCARELLI, A. M. TAGLIASACCHI, L. M. C. FORINO, R. LORENZI, and R. BONOMO: Developmental Stages and Gibberellin Activity in Ovules of Abscission-affected Fruits of <i>Malus domestica</i> Borkh	10
BASZYŃSKI, T., A. TUKENDORF, M. RUSZKOWSKA, E. SKÓRZYŃSKA, and W. MAKSYMIEC: Characteristics of the Photosynthetic Apparatus of Copper Non-tolerant Spinach Exposed to Excess Copper	708
BAYDOUN, E.-H. and S. C. FRY: [2- ³ H]Mannose Incorporation in Cultured Plant Cells: Investigation of L-Galactose Residues of the Primary Cell Wall	484
BELINGHERI, L., G. PAULY, M. GLEIZES, and A. MARPEAU: Isolation by an Aqueous Two-Polymer Phase System and Identification of Endomembranes from <i>Citrofortunella mitis</i> Fruits for Sesquiterpene Hydrocarbon Synthesis	80
BERGARECHE, C. and E. SIMON: Nitrate Reductase Activity and Nitrate Content under Two Forms and Three Levels of Nitrogen Nutrition in <i>Lolium perenne</i> L.	28
BIRNBERG, P. R., D. A. SOMERS, and M. L. BRENNER: Characterization of Conditioning Factors that Increase Colony Formation from «Black Mexican Sweet Corn» Protoplasts	316
BOLLMARK, M., B. KUBÁT, and L. ELIASSON: Variation in Endogenous Cytokinin Content during Adventitious Root Formation in Pea Cuttings	262
BOTHA, F. C. and J. G. C. SMALL: The Germination Response of the Negatively Photoblastic Seeds of <i>Citrullus lanatus</i> to Light of Different Spectral Compositions	750
CAKMAK, I. and H. MARSCHNER: Increase in Membrane Permeability and Exudation in Roots of Zinc Deficient Plants	356
CAWOOD, M. E., F. C. BOTHA, and J. G. C. SMALL: Properties of the Phosphofructokinase Isoenzymes from Germinating Cucumber Seeds	204
CHANDLER, S. F., E. RAGOLSKY, and T. A. THORPE: Sodium Sulfate Tolerance and Sensitivity in Tissue Cultures and Regenerated Plants of <i>Nicotiana tabacum</i>	155
CHENG, T.-S., J. D. FLOROS, R. L. SHEWFELT, and C. J. CHANG: The Effect of High-Temperature Stress on Ripening of Tomatoes (<i>Lycopersicon esculentum</i>)	459
COSSINS, E., A. R. A. SCOTT, and N. JALEEL: Formate Synthesis in Bundle Sheath Cells of Corn and Sorghum	266
COTTIGNIES, A. and A. JENNANE: Water Content, Water Potential, and Transition from the Non-cycling to the Cycling State in the Pea Cotyledonary Bud	1
CREMERS, H. C. J. CANTER, C. A. WIJFFELMAN, E. PEES, B. G. ROLFE, M. A. DJORDJEVIC, and B. J. J. LUGTENBERG: Host Specific Nodulation of Plants of the Pea Cross-inoculation Group is Influenced by Genes in Fast Growing <i>Rhizobium</i> downstream <i>nodC</i>	398
CROISSANT-SYCH, Y. and M. BOPP: Formation and Degradation of Storage Proteins in the Embryo of <i>Sinapis alba</i>	520
CULLIMORE, J. V. and M. J. BENNETT: Review: The Molecular Biology and Biochemistry of Plant Glutamine Synthetase from Root Nodules of <i>Phaseolus vulgaris</i> L. and other Legumes	387
DABAS, S., K. SWARAJ, and I. S. SHEORAN: Effect of Source Removal on Functioning of the Nitrogen Fixation of Pigeonpea (<i>Cajanus cajan</i> L.) Nodules	690
DALTON, S. J.: Plant Regeneration from Cell Suspension Protoplasts of <i>Festuca arundinacea</i> Schreb. (tall fescue) and <i>Lolium perenne</i> L. (perennial ryegrass)	170
DAMIANI, F., M. PEZZOTTI, and S. ARCIONI: Electric Field Mediated Fusion of Protoplasts of <i>Medicago sativa</i> L. and <i>Medicago arborea</i> L.	474

DAVIES, H. V., H. A. ROSS, and R. THOMPSON: The Influence of Nitrogen Supply on the Development of Nitrate Reductase Activity in Leaves of Potato (<i>Solanum tuberosum</i> L.)	540
DELVALLÉE, I. and C. DUMAS: Anther Development in <i>Zea mays</i> : Changes in Protein, Peroxydase, and Esterase Patterns	210
DEVINE, T. E. : Role of the Nodulation Restrictive Allele <i>Rj4</i> in Soybean Evolution	453
DRAGET, K. I., S. MYHRE, G. SKJÅK-BRÆK, and K. ØSTGAARD: Regeneration, Cultivation and Differentiation of Plant Protoplasts Immobilized in Ca-alginate Beads	552
DÜBALL, S. and A. WILD: Investigation on the Nitrogen Metabolism of Spruce Needles in Relation to the Occurrence of Novel Forest Decline	491
ELENA, E. B. and H. D. GINZO: Effect of Auxin Levels on Shoot Formation with Different Embryo Tissues from a Cultivar and a Commercial Hybrid of Wheat (<i>Triticum aestivum</i> L.)	600
FELICOLI, R., A. ROMAGNOLI, A. GIACHI, S. BERNINI, and E. BALESTRERI: The Use of a Native Enzyme Protein as Substrate for Plant Proteinase Assay	34
FELIPE, M. R. DE, M. M. LUCAS, and J. M. POZUELO: Cytochemical Study of Catalase and Peroxidase in the Mesophyll of <i>Lolium rigidum</i> Plants Treated with Isoproturon	67
FERRARI, L., A. ALPI, and E. BALESTRERI: Characterization of an Endopeptidase from Alfalfa (<i>Medicago sativa</i> L.) Leaves	74
FLORES, A., A. GRAU, F. LAURICH, and K. DÖRFFLING: Effect of New Terpenoid Analogues of Abscisic Acid on Chilling and Freezing Resistance	362
GARBERS, C., R. MECKBACH, R. B. MELLOR, and D. WERNER: Protease (Thermolysin) Inhibition Activity in the Peribacteroid Space of <i>Glycine max</i> Root Nodules	442
GIMMLER, H.: Erythrosine B – a Specific Inhibitor of Plasmalemma ATPase in Intact Microalgae?	545
GEUNS, JAN M. C.: Synergism between IAA and Cortisol in the Adventitious Root Formation in Mung Bean Cuttings	370
GIVAN, C. V., S. TSUTAKAWA, J. M. HODGSON, N. DAVID, and D. D. RANDALL: Glyoxylate Reductase Activity in Pea Leaf Protoplasts Nucleotide Specificity and Subcellular Location	593
GLAND, A., R. LICHTER, and H.-G. SCHWEIGER: Genetic and Exogenous Factors Affecting Embryogenesis in Isolated Microspore Cultures of <i>Brassica napus</i> L.	613
GÖTTFERT, M., J. WEBER, and H. HENNECKE: Induction of a <i>nodA-lacZ</i> Fusion in <i>Bradyrhizobium japonicum</i> by an Isoflavone	394
GOMES FILHO, E. and L. SODEK: Effect of Salinity on Ribonuclease Activity of <i>Vigna unguiculata</i> Cotyledons during Germination	307
GORHAM, J., O. S. TOMAR, and R. G. WYN JONES: Salinity-Induced Changes in the Chemical Composition of <i>Leucaena leucocephala</i> and <i>Sesbania bispinosa</i>	678
GRESSHOFF, P. M., A. KROTZKY, A. MATHEWS, D. A. DAY, K. A. SCHULLER, J. OLSSON, A. C. DELVES, and B. J. CARROLL: Suppression of the Symbiotic Supernodulation Symptoms of Soybean	417
GUIÑAZÚ, M., G. ABDALA, and R. TIZIO: Effect of Free and Conjugated Gibberellins on Roots of Potato Cuttings Treated with CCC[(2-chloroethyl) trimethylammonium chloride] in Relation to Tuber Formation	725
HAHNE, G. and H. LÖRZ: Release of Phytotoxic Factors from Plant Cell Walls During Protoplast Isolation	345
HASCHKE, H.-P., S. GRÖTSCH, and U. LÜTTGE: Proton Transporting Enzymes at the Tonoplast of Leaf Cells of the CAM Plant <i>Kalanchoe daigremontiana</i> III. Regulation of the ATPase	604
HAVAUX, M., M. ERNEZ, and R. LANNOYE: Tolerance of Poplar (<i>Populus</i> sp.) to Environmental Stresses II. Photosynthetic Characteristics of Poplar Clones Grown at Low and High Light Intensities	664
HAVEL, L. and F. J. NOVÁK: Regulation of Somatic Embryogenesis and Organogenesis in <i>Allium carinatum</i> L.	373
HOLLOWAY, P. J. and M. J. DALLING: Modulation of the Light-Stimulated Loss of Polypeptides from Isolated Wheat Thylakoids <i>in vitro</i>	273
HOWITT, S. M., D. A. DAY, K. F. SCOTT, and P. M. GRESSHOFF: Mutants of <i>Bradyrhizobium (Parasponia)</i> sp. ANU 289 Affected in Assimilatory Nitrate Reduction also Show Lowered Symbiotic Effectiveness	5
IMESON, H. C., N. JALEEL, and E. A. COSSINS: One-Carbon Substituted Folylpolylglutamates of Isolated Bundle Sheath Cells	465
JACKSON, J. A. and P. J. DALE: Callus Induction, Plant Regeneration and an Assessment of Cytological Variation in Regenerated Plants of <i>Lolium multiflorum</i> L.	351
JACKSON, J. A. and R. F. LYNDON: Cytokinin Habituation in Juvenile and Flowering Tobacco	575
JAMES, D. J., A. J. PASSEY, and E. RUGINI: Factors Affecting High Frequency Plant Regeneration from Apple Leaf Tissues Cultured <i>in vitro</i>	148
JESCHKE, W. D. and O. WOLF: Effect of NaCl Salinity on Growth, Development, Ion Distribution, and Ion Translocation in Castor Bean (<i>Ricinus communis</i> L.)	45

JUARRANZ, A., M. J. HAZEN, and J. C. STOCKERT: Effect of Copper Phthalocyanine Derivatives on Sister Chromatid Exchanges in BrdU-substituted Chromosomes of <i>Allium cepa</i>	557
KARTHA, K. K., L. C. FOWKE, N. L. LEUNG, K. L. CASWELL, and I. HAKMAN: Induction of Somatic Embryos and Plantlets from Cryopreserved Cell Cultures of White Spruce (<i>Picea glauca</i>)	529
KELLER, FELIX: A Large-scale Isolation of Vacuoles from Protoplasts of Mature Carrot Taproots	199
KĘPCZYŃSKI, J. and J. STANISLAW KNYPL: Inhibition of Germination by Growth Retardants AMO-1618 and Ancymidol, and its Reversal by Gibberellin A ₃ in <i>Amaranthus caudatus</i> L. Seeds	584
KERBAUY, G. B., W. R. MONTEIRO, J. E. KRAUS, and K. G. HELL: Some Physiological and Structural Aspects of Cytokinin-Autonomy in the Callus of Tobacco (<i>Nicotiana tabacum</i> L.)	218
KERSCHER, F. and G. FRANZ: Isolation and Some Properties of an UDP-Glucose: 2-Hydroxyflavanone-6(or 8)-C-Glucosyltransferase from <i>Fagopyrum esculentum</i> M. Cotyledons	110
KLECZKOWSKI, L. A. and C. V. GIVAN: Serine Formation in Leaves by Mechanisms other than the Glycolate Pathway	641
KLOTZ, F. and W. J. HORST: Genotypic Differences in Aluminium Tolerance of Soybean (<i>Glycine max</i> L.) as Affected by Ammonium and Nitrate- Nitrogen Nutrition	702
KRISHNA, T. G. and D. R. MURRAY: Effects of Cycloheximide and Actinomycin D on Glycosidase Activities in the Cotyledons of Legume Seeds Following Imbibition	745
LARCHER, W., C. LÜTZ, M. NAGELE, and M. BODNER: Photosynthetic Functioning and Ultrastructure of Chloroplasts in Stem Tissues of <i>Fagus sylvatica</i>	731
LASTRA, O., A. CHUECA, M. LACHICA, and J. LÓPEZ GORGÉ: Root Uptake and Partition of Copper, Iron, Manganese, and Zinc in <i>Pinus radiata</i> Seedlings Grown under Different Copper Supplies	16
LEHMANN, H. and P. VLASOV: Plant Growth and Stress – The Enzymic Hydrolysis of Abscisic Acid Conjugate	98
LEÓN, J., L. C. ROMERO, and F. GALVÁN: Intracellular Levels and Regulation of O-Acetyl-L-Serine Sulfhydrylase Activity in <i>Chlamydomonas reinhardtii</i>	618
LINDSTRÖM, K. and P. LIPSANEN: Properties of <i>Rhizobium galegae</i> and its Symbiosis with <i>Galega</i> sp.	456
LITZ, RICHARD E.: Somatic Embryogenesis from Cultured Leaf Explants of the Tropical Tree <i>Euphoria longan</i> Stend.	190
LOYOLA-VARGAS, V. M., M. EUGENIA ROMÁN, J. QUIROZ, C. OROPEZA, M. L. ROBERT, and K. N. SCORER: Nitrogen Metabolism in <i>Canavalia ensiformis</i> (L.) DC. I. Arginase and Urease Ontogeny	284
LOYOLA-VARGAS, V. M., A. YÁÑEZ, J. CALDERA, C. OROPEZA, M. L. ROBERT, J. QUIROZ, and K. N. SCORER: Nitrogen Metabolism in <i>Canavalia ensiformis</i> (L.) DC. II. Changing Activities of Nitrogen-Assimilating Enzymes during Growth	289
MANGA, V. A. and R. SHARMA: Sustainment of Uninterrupted Increase of β -amylase Activity in Mustard (<i>Sinapis alba</i> L.) Cotyledons during Light/Dark Cycles	116
MARCO, E. and M. I. ORÚS: Variation in Growth and Metabolism with Phosphorus Nutrition in two Cyanobacteria	339
MARTIN, C. E., M. HIGLEY, and W. WANG: CAM-Cycling and CAM-Idling in <i>Drosanthemum hispidum</i> (L.) Schwant. (Mesembryanthemaceae)	303
MENON, M. K. C. and E. HARTMANN: Secretion of Protein-bound Hydroxyproline from Moss Callus Cells	569
MILLS, D. and T. K. HODGES: Characterization of Plasma Membrane ATPase from Roots of <i>Atriplex nummularia</i>	513
MONTEIRO, A. M., A. CROZIER, and G. SANDBERG: Endogenous Hormones, Germination and Early Seedling Growth of <i>Dalbergia dolichopetala</i>	762
MOROHASHI, Y. and H. MATSUSHIMA: Effect of the Embryonic Axis on the Development of Glyoxysomes, Plastids and Mitochondria in Cotyledons of Germinated Cucumber Seeds	279
NESSLER, C. L.: Comparative Analysis of the Major Latex Proteins of Opium Poppy	588
NICHOLSON, R. I. D. and J. VAN STADEN: Cytokinins and Mango Flower Malformation. I. Tentative Identification of the Complement in Healthy and Malformed Inflorescences	720
O'GARA, F., K. BIRKENHEAD, B. NOONAN, and S. S. MANIAN: Dicarboxylic Acid Utilisation and Regulation of Nitrogen Fixation in <i>Rhizobium</i> species	439
PALACIOS, J. M., A. LEYVA, and T. RUIZ-ARGÜESO: Generation and Characterization of Hydrogenase Deficient Mutants of <i>Rhizobium leguminosarum</i>	412
PEÑA, J., P. APARICIO-TEJO, and M. SANCHEZ-DIAZ: Dormancy Mechanism and the Effect of Scarification in the Germination of <i>Halimium halimifolium</i> Seeds	54
PEDERSEN, T. J. and S. C. MINOCHA: Effects of n-Sodium Butyrate on Cell Division in Jerusalem Artichoke (<i>Helianthus tuberosus</i> L.) Tuber Explants Cultured <i>in vitro</i>	623
PICQUOT, P. and A.-M. LAMBERT: Tubulin from Monocotyledon Endosperm Cells: Particular Biochemical and Immunological Properties	561

PIRO, G., S. PEROTTO, P. BONFANTE-FASOLO, and G. DALESSANDRO: Metabolism of D-[U- ¹⁴ C]glucosamine in Seedlings of <i>Calluna vulgaris</i> (L.) Hull	695
PIWOŃWARCZYK, W.: A New Method of Measurement of Changes in Turgor Pressure in the Mesophyll Cells of Leaves	653
POJOVA, L. P., T. D. TSONEV, and S. G. VAKLINOVA: Changes in Some Photosynthetic and Photorespiratory Properties in Barley Leaves after Treatment with Jasmonic Acid	257
POJOVIC, R., R. CARPENTIER, and L. MORIN: Determination of Fluorescence Inductions in a PSII Submembrane Fraction Affected by Additives	754
POJOVIC, R., R. M. LEBLANC, and M. BEAUREGARD: Photoacoustic Studies of Bundle Sheath Cell Photosynthesis in <i>Zea mays</i>	94
POSTMA, J. G., E. JACOBSEN, and W. J. FEENSTRA: Three Pea Mutants with an Altered Nodulation Studied by Genetic Analysis and Grafting	424
RAĐOJEVIĆ, L.: Plant Regeneration of <i>Aesculus hippocastanum</i> L. (Horse Chestnut) through Somatic Embryogenesis	322
RAMAGOPAL, SUBBANAIIDU: Sodium Chloride Effects on Dedifferentiation and Protein Synthesis in Root Meristem Cultures of Two Contrasting Barley Genotypes	245
RAÏET, P., K. PAWLOWSKI, H. MEYER Z. A., J. SCHELL, and F. J. DE BRUIJN: Regulation of Nitrogen Fixation (<i>nif</i>) Genes of <i>Azorhizobium caulinodans</i> ORS571 in Culture and <i>in planta</i>	405
REKOSLAVSKAYA, N. I., T. A. MARKOVA, and K. Z. GAMBURG: Appearance of N-malonyl-D-tryptophan in Excised Leaves during Wilting. 1. The Content of Tryptophan and N-malonyl-D-tryptophan as Affected by Water Deficit	86
REVILLA, M. E., L. MARTIN, G. NICOLAS, M. E. LEGAZ, and N. VILLALOBOS: Effects of High Temperature on the Variation and Transport of Endogenous Cytokinins during the Germination of Chick-pea Seeds	223
RUDNICKI, K., R. RZEPECKI, and J. SZOPA: Rearrangement of the Nuclease-Inhibitor Complex Components within Cells of White Bush Treated with Phytohormone	658
SANTA MARÍA, G. E. and D. H. COGLIATTI: Bidirectional Zn-Fluxes and Compartmentation in Wheat Seedling Roots	312
SAUTER, J. J.: Temperature-induced Changes in Starch and Sugars in the Stem of <i>Populus × canadensis</i> «robusta»	608
SAXENA, P. K. and J. KING: Recovery of Prototrophic Clones Following Introduction of Isolated <i>Brassica</i> Nuclei into the Protoplasts of a <i>Datura</i> auxotroph	140
SCHNYDER, H., U. EHSES, J. BESTAJOVSKY, R. MEHRHOFF, and W. KÜHBAUCH: Fructan in Wheat Kernels during Growth and Compartmentation in the Endosperm and Pericarp	333
SCRAGG, A. H., E. J. ALLAN, and P. MORRIS: Investigation into the Problems of Initiation and Maintenance of <i>Cinchona ledgeriana</i> Suspension Cultures	184
SHANTHARAM, S., K. S. ENGWALL, and A. G. ATHERLY: Symbiotic Phenotypes of Soybean Root Nodules Associated with Deletions and Rearrangements in the Symbiotic Plasmid of <i>R. fredii</i> USDA191	431
SHARMA, S. and N. CHANDRA: Organogenesis and Plantlet formation <i>in vitro</i> in <i>Dalbergia sissoo</i> Roxb.	145
SHEA, E. M., A. SKARIA, and N. C. CARPITA: Growth of Carrot Cell Suspension Cultures in Medium Containing Amino Acid Conjugates of Indoleacetic Acid	298
SHEORAN, I. S., A. KAUR, and R. SINGH: Nitrogen Fixation and Carbon Metabolism in Nodules of Pigeonpea (<i>Cajanus cajan</i> L.) Under Drought Stress	480
SILBERBUSH, M., A. GOLAN-GOLDHIRSH, Y. HEIMER, J. BEN-ASHER, and S. H. LIPS: Response of Peanuts (<i>Arachis hypogaea</i> L.) grown in Saline Nutrient Solution to Potassium Nitrate	229
SOLL, J. and I. FISCHER: Analysis of Chloroplast Envelope Membranes Using Photoaffinity Label	631
SPRUYT, E., J.-P. VERBELEN, and J. A. DE GREEF: Ultradian and Circannual Rhythmicity in Germination of <i>Phaseolus</i> Seeds	234
STELZER, R., J. KUO, and H.-W. KOYRO: Substitution of Na ⁺ by K ⁺ in Tissues and Root Vacuoles of Barley (<i>Hordeum vulgare</i> L. cv. Aramir)	671
STUART, D. A. and J. NELSEN: Isolation and Characterization of Alfalfa 7S and 11S Seed Storage Protein	129
STUART, D. A., J. NELSEN, and J. W. NICHOL: Expression of 7S and 11S Alfalfa Seed Storage Proteins in Somatic Embryos	134
TA, T. C., F. D. H. MACDOWALL, and M. A. FARIS: Assimilation and Partitioning of Labeled Nitrogen from ¹⁵ N ₂ and ¹⁵ NO ₃ ⁻ by Alfalfa (<i>Medicago sativa</i> L.)	239
TAKIO, S. and S. HINO: Nitrate Reductase from Suspension Cultured Cells of <i>Marchantia polymorpha</i> L.	470
TAKIO, S., S. TAKAMI, and S. HINO: Photosynthetic Ability of Dark-grown <i>Marchantia paleacea</i> Cells in Suspension Culture	195
THALOUARN, P., V. PHILOUZE, and S. RENAUDIN: Nitrogen Metabolism Key Enzyme Activities in a Scrophulariaceae Holoparasite <i>Lathraea clandestina</i> L.	63

THIEL, G., J. LYNCH, and A. LÄUCHLI: Short-term Effects of Salinity Stress on the Turgor and Elongation of Growing Barley Leaves	38
VASIL, V., R. J. FERL, and I. K. VASIL: Somatic hybridization in the Gramineae: <i>Triticum monococcum</i> L. (Einkorn) + <i>Pennisetum americanum</i> (L.) K. Schum. (Pearl Millet)	160
VENEKAMP, J. H. and J. T. M. KOOT: The sources of free proline and asparagine in field bean plants, <i>Vicia faba</i> L., during and after a short period of water withholding	102
VICENTE, C. and E. PÉREZ-URRIA: Tolbutamide, a Urea Derivative, Impedes Phenolic Accumulation in the Lichen <i>Evernia prunastri</i>	580
WELANDER, M.: Plant Regeneration from Leaf and Stem Segments of Shoots Raised <i>in vitro</i> from Mature Apple Trees	738
WIEL, C. VAN DE, J.-P. NAP, A. VAN LAMMEREN, and T. BISSELING: Histological Evidence that a Defence Response of the Host Plant Interferes with Nodulin Gene Expression in <i>Vicia sativa</i> Root Nodules Induced by an <i>Agrobacterium</i> Transconjugant ..	446
WURTELE, E. S., G. L. KELLER, B. J. NIKOLAU, and T. H. ULRICH: Quantitation of Starch and ADP-Glucose Pyrophosphorylase in non-Embryogenic Cells and Embryogenic Cell Clusters from Carrot Suspension Cultures	683
YONEYAMA, T.: Natural Abundance of ¹⁵ N in Root Nodules of Pea and Broad Bean	59
ZAPATA, F. J. and E. S. ELLA: Specific Gravity of the Grain – a Factor to Consider in Rice Tissue Culture	294
ZIEGLER, R., A. BLAHETA, N. GUHA, and B. SCHÖNEGGE: Enzymatic Formation of Pheophorbide and Pyropheophorbide during Chlorophyll Degradation in a Mutant of <i>Chlorella fusca</i> SHIHIRA et KRAUS	327
Short Communications	
AKIYAMA, T., S. YAMAMOTO, and H. HORI: Membrane-bound α -Mannosidase Activity toward Man ₉ GlcNAc Oligosaccharide in Wheat Germ	505
ALAZARD, D. and E. DUHOUX: Diversity of Stem Nodulation Sites in <i>Aeschynomene</i> spp.	123
BELL, P. R.: Control Points in the Generation Cycle of Ferns	378
BLANKE, M. M. and A. LEYHE: Stomatal and Cuticular Transpiration of the Cap and Berry of Grape	250
CHEN, H. and B. G. ROLFE: <i>Rhizobium</i> Infection of <i>Leucaena leucocephala</i> via the Formation of Infection Threads in Curled Root Hairs	379
COPPENS, L., D. VERCRUYSE-DEWITTE, and E. GILLIS: Influence of Germination Delay on Callus Induction from Shoot Meristems of Barley (<i>Hordeum vulgare</i> L.) Embryos	383
DREIER, S. I. and G. H. N. TOWERS: Activity of Ecdysterone in Selected Plant Growth Bioassays	509
GUPTA, H. S. and D. N. DE: Acridine Orange-induced Vacuolar Uptake of Cytoplasmic Organelles in Plant Cells: An Ultrastructural Study	254
JAIN, A. and K. R. SHIVANNA: Storage of Pollen Grains in Organic Solvents: Effects of Solvents on Pollen Viability and Membrane Integrity	499
JÖRGENSEN, J.: Embryogenesis in <i>Quercus petraea</i> and <i>Fagus sylvatica</i>	638
MOLERO, M. L. and M. J. HAZEN: Photodynamic Effect of Acridine Orange, Eosin Y and Orcein in a Plant System <i>in vivo</i> Measured by the Sister Chromatid exchanges test	636
NORMAN, E. G. and B. COLMAN: Evidence for an Incomplete Glycolate Pathway in Cyanobacteria	766
RENGEL, Z. and H. A. KORDAN: Effects of N, P, and K Deficiencies on Light-dependent Anthocyanin Formation in <i>Zea mays</i> L. Seedlings	126
STADEN, J. VAN and A. D. BAYLEY: A Comparison of Two Extraction Procedures For The Recovery of Cytokinin Nucleotides ...	502

Authors Index

- ABDALA, G. 725
 AKIYAMA, T. 505
 ALAZARD, D. 123
 ALLAN, E. J. 176, 184
 ALPI, A. 74
 AMBERGER-OCHSENBAUER, S. 758
 ANGELOPOULOS, K. 714
 APARICIO-TEJO, P. 54
 APPENROTH, K.-J. 90
 ARCIONI, S. 474
 ARNOLD, S. VON 164
 ARNOZIS, P. A. 23
 ATHERLY, A. G. 431
 AUGSTEN, H. 90
 AVANZI, S. 10

 BALESTRERI, E. 34, 74
 BASZYŃSKI, T. 708
 BAYDOUN, E.-H. 484
 BAYLEY, A. D. 502
 BEAUREGARD, M. 94
 BELINGHERI, L. 80
 BELL, P. R. 378
 BEN-ASHER, J. 229
 BENNETT, M. J. 387
 BERGARECHE, C. 28
 BERNINI, S. 34
 BESTAJOVSKY, J. 333
 BLAHETA, A. 327
 BLANKE, M. M. 250
 BIRKENHEAD, K. 439
 BIRNBERG, P. R. 316
 BISSELING, T. 446
 BODNER, M. 731
 BOLLMARK, M. 262
 BONFANFTE-FASOLO, P. 695
 BONOMO, R. 10
 BOPP, M. 520
 BOTHA, F. C. 204, 750
 BRENNER, M. L. 316
 BRUIJN, F. J. DE 405

 ÇAKMAK, I. 356
 CALDERA, J. 289
 CANTER, C. J. 398
 CARPENTIER, R. 754
 CARPITA, N. C. 298
 CARROLL, B. J. 417
 CASWELL, K. L. 529
 CAWOOD, M. 204
 CECCARELLI, N. 10
 CHANDLER, S. F. 155
 CHANDRA, N. 145
 CHANG, C. J. 459
 CHEN, H. 379
 CHENG, T.-S. 459
 CHUECA, A. 16
 COGLIATTI, D. H. 312
 COLMAN, B. 766
 COPPENS, L. 383
 COSSINS, E. A. 266, 465
 COTTIGNIES, A. 1
 CREMERS, H. 398

 CROISSANT-SYCH, Y. 520
 CROZIER, A. 762
 CULLIMORE, J. V. 387

 DABAS, S. 690
 DALE, P. J. 351
 DALESSANDRO, G. 695
 DALLING, M. J. 273
 DALTON, S. J. 170
 DAMIANI, F. 474
 DAVID, N. 593
 DAVIES, H. V. 540
 DAY, D. A. 5, 417
 DE, D. N. 254
 DELVALLÉE, I. 210
 DELVES, A. C. 417
 DEVINE, T. E. 453
 DJORDJEVIC, M. A. 398
 DÖRFFLING, K. 362
 DRAGET, K. I. 552
 DREIER, S. I. 509
 DÜBALL, S. 491
 DUHOUX, E. 123
 DUMAS, C. 210

 EHSSES, U. 333
 ELENA, E. B. 600
 ELIASSON, L. 262
 ELLA, E. S. 294
 ENGWALL, K. S. 431
 ERNEZ, M. 664

 FARIS, M. A. 239
 FEENSTRA, W. J. 424
 FERL, R. J. 160
 FELICOLI, R. 34
 FELIPE, M. R. DE 67
 FERRARI, L. 74
 FINDENEGG, G. R. 23
 FISCHER, I. 631
 FLORES, A. 362
 FLOROS, J. D. 459
 FORINO, L. M. C. 10
 FOWKE, L. C. 529
 FRANZ, G. 110
 FRY, S. C. 484

 GALVÁN, F. 618
 GAMBURG, K. Z. 86
 GARBERS, C. 442
 GAVALAS, N. A. 714
 GEUNS, J. M. C. 370
 GIACHI, A. 34
 GILLIS, E. 383
 GIMMLER, H. 545
 GINZO, H. D. 600
 GIVAN, C. V. 593, 641
 GLAND, A. 613
 GLEIZES, M. 80
 GÖTTERT, M. 394
 GOLAN-GOLDHIRSH, A. 229
 GOMES FILHO, E. 307
 GORHAM, J. 678

 GRAU, A. 362
 GREEF, J. A. DE 234
 GRESSHOFF, P. M. 5, 417
 GRÖTSCH, S. 604
 GUHA, N. 327
 GUIÑAZÚ, M. 725
 GUPTA, H. S. 254

 HAHNE, G. 345
 HAKMAN, I. 164, 529
 HASCHKE, H.-P. 604
 HARTMANN, E. 569
 HAVAUX, M. 664
 HAVEL, L. 373
 HAZEN, M. J. 557, 636
 HEIMER, Y. 229
 HELL, K. G. 218
 HENNECKE, H. 394
 HIGLEY, M. 303
 HINO, S. 195, 470
 HODGES, T. K. 513
 HODGSON, J. M. 593
 HOLLOWAY, P. J. 273
 HORI, H. 505
 HORST, W. J. 702
 HOWITT, S. M. 5

 IMESON, H. C. 465

 JACKSON, J. A. 351, 575
 JACOBSEN, E. 424
 JAIN, A. 499
 JALEEL, N. 266
 JAMES, D. J. 148
 JENNANE, A. 1
 JESCHKE, W. D. 45
 JÖRGENSEN, J. 638
 JUARRANZ, A. 557

 KARTHA, K. K. 529
 KAUR, A. 480
 KELLER, F. 199
 KELLER, G. L. 683
 KEPczyński, J. 584
 KERBAUY, G. B. 218
 KERSCHER, F. 110
 KING, J. 140
 KLECZOWSKI, L. A. 641
 KLOTZ, F. 702
 KNYPL, J. S. 584
 KOOT, J. T. M. 102
 KORDAN, H. A. 126
 KOYRO, H.-W. 671
 KRAUS, J. E. 218
 KRISHNA, T. G. 745
 KROTZKY, A. 417
 KUBÁT, B. 262
 KÜHBAUCH, W. 333
 KUNZ, E. 90
 KUO, J. 671

 LACHICA, M. 16
 LÄUCHLI, A. 38

 LAMBERT, A.-M. 561
 LAMMEREN, A. VAN 446
 LANNOYE, R. 664
 LARCHER, W. 731
 LASTRA, O. 16
 LAURICH, F. 362
 LEBLANC, R. M. 94
 LEGAZ, M. E. 223
 LEHMANN, H. 98
 LEÓN, J. 618
 LEUNG, N. L. 529
 LEYHE, A. 250
 LEYKA, A. 412
 LICHTER, R. 613
 LINDSTRÖM, K. 456
 LIPS, S. H. 229
 LIPSANEN, P. 456
 LITZ, R. E. 190
 LÖRZ, H. 345
 LÓPEZ-GORGÉ, J. 16
 LOYOLA, R. 10
 LOYOLA-VARGAS, V. M. 284, 289
 LUCAS, M. M. 67
 LUGTENBERG, B. J. J. 398
 LÜTTGE, U. 604
 LÜTZ, W. 731
 LYNCH, J. 38
 LYNDON, R. F. 575

 MACDOWALL, F. D. H. 239
 MAKSYMIEC, W. 708
 MANGA, V. A. 116
 MANIAN, S. S. 439
 MARCO, E. 339
 MARKOVA, T. A. 86
 MARPEAU, A. 80
 MARSCHNER, H. 356
 MARTIN, C. E. 303
 MARTIN, L. 223
 MATHEWS, A. 417
 MATSUSHIMA, H. 279
 MECKBACH, R. 442
 MEHRHOFF, R. 333
 MELLOR, R. B. 442
 MENON, M. K. C. 569
 MEYER z. A., H. 405
 MILLS, D. 513
 MINOCHA, S. C. 623
 MOLERO, M. L. 636
 MONTEIRO, A. M. 762
 MONTEIRO, W. R. 218
 MORIN, L. 754
 MOROHASHI, Y. 279
 MORRIS, P. 184
 MURRAY, D. R. 745
 MYHRE, S. 552

 NAGELE, M. 731
 NAP, J.-P. 446
 NELEMANS, J. A. 23
 NELSEN, J. 129, 134
 NESSLER, C. L. 588
 NICHOL, J. W. 134

- NICOLAS, G. 223
 NIKOLAU, B. J. 683
 NICHOLSON, R. I. D. 720
 NOONAN, B. 439
 NORMAN, E. G. 766
 NOVÁK, F. J. 373
- OBENDORFER, J. 758
 O'GARA, F. 439
 OLSSON, J. 417
 OROPEZA, C. 284, 289
 ORÚS, M. I. 339
 ØSTGAARD, K. 552
- PALACIOS, J. M. 412
 PASSEY, A. J. 148
 PAULY, G. 80
 PAWLOWSKI, K. 405
 PEDERSEN, T. J. 623
 PEES, E. 398
 PEÑA, J. 54
 PÉREZ-URRÍA, E. 580
 PEROTTO, S. 695
 PEZZOTTI, M. 474
 PHILOUZE, V. 63
 PICQUOT, P. 561
 PIRO, G. 695
 PIWOWARCZYK, W. 653
 POPOVA, L. P. 257
 POPOVIC, R. 94, 754
 POSTMA, J. G. 424
 POZUELO, J. M. 67
 PUGH, K. 176
- QUIROZ, J. 284, 289
- RADOJEVIĆ, L. 322
 RAGOLSKY, E. 155
 RAMAGOPAL, S. 245
 RANDALL, D. D. 593
 RATET, P. 405
 REKOSLAVSKAYA, N. I. 86
 RENAUDIN, S. 63
 RENGEL, Z. 126
 REVILLA, M. E. 223
 ROBERT, M. L. 284, 289
 ROLFE, B. G. 379, 398
 ROMAGNOLI, A. 34
 ROMÁN, M. E. 284
 ROMERO, L. C. 618
 ROSS, H. A. 540
 RUGINI, E. 148
 RUIZ-ARGÜESO, T. 412
 RUDNICKI, K. 658
 RUSZKOWSKA, M. 708
 RZEPECKI, R. 658
- SANCHEZ-DIAZ, M. 54
 SANDBERG, G. 762
 SANTA MARÍA, G. E. 312
 SAUTER, J. J. 608
 SAXENA, P. K. 140
 SCHELL, J. 405
 SCHNYDER, H. 333
 SCHÖNEGGE, B. 327
 SCHULLER, K. A. 417
 SCHWEIGER, H.-G. 613
 SCORER, K. N. 284, 289
 SCOTT, K. F. 5
 SCOTT, R. A. 266
 SCRAGG, A. H. 176, 184
- SHANTHARAM, S. 431
 SHARMA, R. 116
 SHARMA, S. 145
 SHEA, E. M. 298
 SHEORAN, I. S. 480, 690
 SHEWFELT, R. L. 459
 SHIVANNA, K. R. 499
 SILBERBUSH, M. 229
 SIMON, E. 28
 SINGH, R. 480
 SKARIA, A. 298
 SKJÅK-BRÆK, G. 552
 SKÓRZYŃSKA, E. 708
 SMALL, J. G. C. 204, 750
 SODEK, L. 307
 SOLL, J. 631
 SOMERS, D. A. 316
 SPRUYT, E. 234
 STADEN, J. VAN 502, 720
 STELZER, R. 671
 STOCKERT, J. C. 557
 STUART, D. A. 129, 134
 SWARAJ, K. 690
 SZOPA, J. 658
- TA, T. C. 239
 TAGLIASACCHI, A. M. 10
 TAKAMI, S. 195
 TAKIO, S. 195, 470
 THALOUARN, P. 63
 THIEL, G. 38
 THOMPSON, R. 540
 THORPE, T. A. 155
 TIZIO, R. 725
 TOMAR, O. S. 678
- TOWERS, G. H. N. 509
 TSONEV, T. P. 257
 TSUTAKAWA, S. 593
 TUKENDORF, A. 708
- ULRICH, T. H. 683
- VAKLINOVA, S. G. 257
 VASIL, I. K. 160
 VASIL, V. 160
 VENEKAMP, J. H. 102
 VERBELEN, J. P. 234
 VERCRUYSE-DEWITTE, D. 383
 VERNIERI, N. 10
 VICENTE, C. 580
 VILLALOBOS, N. 223
 VLASOV, P. 98
- WANG, W. 303
 WEBER, J. 394
 WELANDER, M. 738
 WERNER, D. 442
 WIEL, C. VAN DE 446
 WIJFFELMAN, C. A. 398
 WILD, A. 491
 WOLF, O. 45
 WURTELE, E. S. 683
 WYN JONES, R. G. 678
- YAMAMOTO, S. 505
 YÁÑEZ, A. 289
 YONEYAMA, T. 59
- ZAPATA, F. J. 294
 ZIEGLER, R. 327

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Analysis of Chloroplast Envelope Membranes Using Photoaffinity Label

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Summary

Direct photoaffinity labeling technics were used in order to get more information on the possible function of envelope membrane polypeptides. Using [α - 32 P] ATP the major labeled proteins had a molecular weight of 75 and 56 kDa. Of the labeled polypeptides, a 58 kDa and a 26 kDa protein were found in the inner membrane while labeled proteins of 75, 64, 56, 52 and 42 kDa were confined to the outer membrane. UTP specifically labeled proteins at 23, 31 and 50 kDa while CTP gave rise to a photoaffinity marked protein with an apparent molecular weight of 90 kDa in the inner membrane. The only cross-reacted polypeptide by CTP in the outer envelope had a size of 48 kDa on SDS-PAGE. The major precursor in galactolipid synthesis, UDP-galactose, was also used as a photoaffinity probe. This substance specifically labeled proteins at 31, 33, 35, 38 and 50 kDa.

Key words: Chloroplast envelope, nucleoside triphosphates, photoaffinity label, *Pisum sativum* L., UDP-galactose.

Introduction

The plastid envelope is a two membrane barrier surrounding the organelle. While the internal membrane system in plastids undergoes dramatical changes during the development of the organelle, the envelope is seen as a conservative structure throughout plastid development. Very little is known regarding the molecular changes in the envelope during development. The envelope membranes have important functions during plastid biogenesis as well as mediating metabolite flow between the organelle and the surrounding cell. The envelope is the sole site of many biosynthetic pathways essential for chloroplast biogenesis; e.g. galactolipids, which make up more than 80% of the chloroplast lipids are made in this membrane. Further biosynthetic capacities in lipid metabolism include the synthesis of prenylquinones phosphatidylglycerol, sulfolipids and long-chain acyl-CoA. Other known properties of the envelope include such polypeptides as a protein kinase, ATPase, ATP-translocator and specific metabolite translocators (Douce et al., 1984).

Although a large number of biochemical functions have been shown to reside in the envelope membranes, very few proteins have been identified, isolated and characterized. The

quantity of purified envelope membranes available as starting material is very small and the purification of active enzymes from envelopes is hampered by the high lipid content of the membranes; the outer envelope contains about 75% lipids on a dry weight basis. Direct photoaffinity labeling technics are a powerful and sensitive tool to assign biochemical functions to polypeptides. We used [α - 32 P] ATP, CTP and UTP as well as [14 C] labeled UDP galactose as direct photoaffinity probes. The results show specific protein labeling for each of the substances used and this should enable us to approach the purification of envelope membrane polypeptides with greater ease.

Material and Methods

[α - 32 P] NTP's (400 Ci/mmol) were from Amersham Buchler, Braunschweig. [14 C] UDP-galactose (250 mCi/mmol) was from New England Nuclear. All other chemicals were commercially available, of reagent grade and used without further purification.

Isolation of Chloroplasts and Chloroplast Envelopes

Intact, purified chloroplasts were isolated from two week old pea plants (*Pisum sativum* L., var. Golf) in low ionic strength buffer

(Nakatani and Barber 1977) and further purified on silica sol gradients (Mourioux and Douce 1981). Mixed envelope membranes were isolated on sucrose gradients after hypotonic lysis of chloroplasts (Douce et al., 1984; Cline et al., 1981). Separation of inner and outer envelope membranes was achieved by hypertonic treatment of chloroplasts and rupturing of shrunken chloroplasts by a Dounce homogenizer. Membranes were separated on a sucrose gradient (Keegstra and Youssif 1986).

UV Photoaffinity Labeling Experiments

Photoaffinity labeling of chloroplast envelope membranes was performed essentially according to (Yue and Schimmel, 1977). Labeling was performed in 24 μ l aliquots, containing 250 nM α -nucleoside triphosphates, 25 μ g envelope protein, 10 mM Tricine-KOH (pH 7.9), 4 mM $MgCl_2$, at 4 °C for 15 min. The mixture was placed in an Eppendorf tube and irradiated at a distance of about 3.5 cm with a Camag TL-900 UV lamp at 254 nm. UV treated membranes were centrifuged for 5 min at 15,000g and washed twice with 10 mM Tricine-KOH (pH 7.9), 4 mM $MgCl_2$ and 0.5 mM of the respective unlabeled NTP's. Samples were further analyzed by SDS-polyacrylamide gel electrophoresis with a 7.5–15% linear polyacrylamide gradient gel (Laemmli, 1970). Following electrophoresis, gels were stained with Coomassie Brilliant blue, destained, dried and autoradiographed over night at -80 °C using an intensifying screen (Agfa-Gevaert MR 800).

Results and Discussion

Initial photoaffinity labeling experiments using 8-azido-adenosine [γ - ^{32}P]ATP and chloroplast envelope membranes showed a specific labeling pattern. Though experiments were done at 4 °C, labeling of a number of proteins was due to ^{32}P -transfer from the γ -position of 8-azido-adenosine [γ - ^{32}P]ATP by the outer envelope bound protein kinase (Soll and Buchanan, 1983), as detected when assays were done in complete darkness. Control experiments revealed that the envelope protein kinase had still about 50% of its activity at 4 °C and about 15% at -10 °C (assay in the presence of 40% glycerol, Soll, 1985). Furthermore it was not possible to eliminate labeling in the excess of nonradioactive ATP, thus showing that 8-azidoadenosine-ATP cross-reacted unspecifically in this complex assay system.

Considering these results, it was decided to use [α - ^{32}P]ATP as the direct affinity reagent. Nucleoside triphosphates, and not only the azido compounds are susceptible to UV-radiation (Yue and Schimmel, 1977) and can be used directly as affinity label (Fig. 1). Time course studies revealed that photolysis of the nucleoside triphosphates was slower than for the corresponding azido compounds. Samples were irradiated for 15 min instead of 5 min for the corresponding azido compounds to yield maximal cross-linking (not shown). The outer envelope membrane bound protein kinase activity was not altered by prolonged UV-radiation at 254 nm (60 min). When [α - ^{32}P]ATP photoaffinity labeled membranes were analyzed by SDS-PAGE and autoradiography, envelope proteins with an apparent molecular weight of 75 kDa and 56 kDa were labeled to a significant extent (Fig. 1, 2, 3). The appearance of labeled polypeptides is completely dependent on radiation at 254 nm; no labeling occurs in dark controls (Fig. 1). These results also demon-

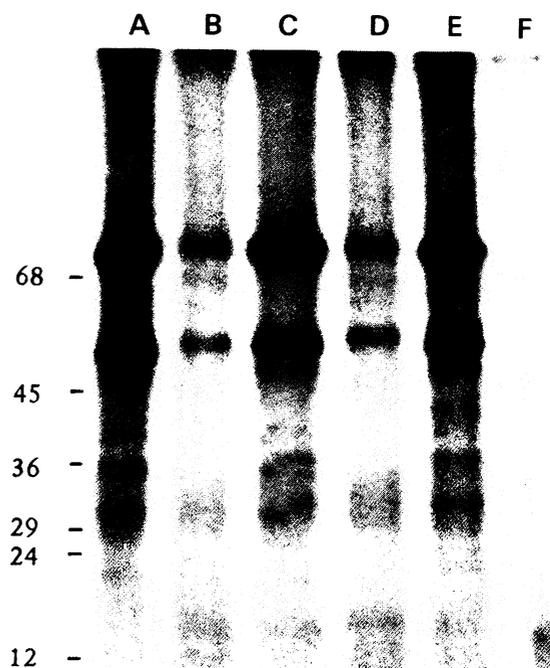


Fig. 1: Photoaffinity labeling of pea chloroplast envelope membranes by [α - ^{32}P]ATP. Membranes were analyzed by SDS-PAGE on 7.5–15% polyacrylamide gels. Autoradiograph of samples irradiated for 15 min at 254 nm and 250 nM [α - ^{32}P]ATP is shown. A, no further additions; B, + 100 μ M ATP; C, + 100 μ M d-ATP; D, + 100 μ M ADP; E, + 100 μ M AMP; F, dark control. Numbers indicate molecular weight standards in kDa.

strate that no transfer of $^{32}P_i$ occurs from [α - ^{32}P]ATP to give a phosphoester bond as was the case for [γ - ^{32}P]azido-ATP due to the protein kinase.

Experiments in the presence of a 400 fold excess of nonradioactive ATP or ADP showed that labeling almost completely disappeared and only proteins of 75 and 56 kDa showed minor incorporation. Other substrates like d-ATP, AMP, adenosine, NADP and NADPH did not compete with [α - ^{32}P]ATP and labeling was as in control experiments (Fig. 2).

Preincubation of the membranes with ATP prior to photolysis did not alter the labeling pattern. In this case the envelope membranes were pelleted and washed once after a 5 min treatment with 100 μ M ATP (not shown). Envelope membranes solubilized in 0.6% Brij W1 and 0.3% N,N-dimethyldodecylamin-N-oxid prior to UV treatment showed the same affinity labeling pattern except for an additional band at about 105 kDa (not shown). We used very low concentrations of ATP (250 nM) in our experiments showing that the labeled proteins had a high affinity for ATP. Increasing the ATP concentration up to 1 μ M did not change the pattern of labeling (Fig. 2).

Recently methods have become available to separate inner and outer envelope membranes (Cline et al., 1981; Douce et al., 1984). When we compared the photoaffinity labeling profile of mixed envelope membranes to that of separated inner and outer envelope membranes it was possible to

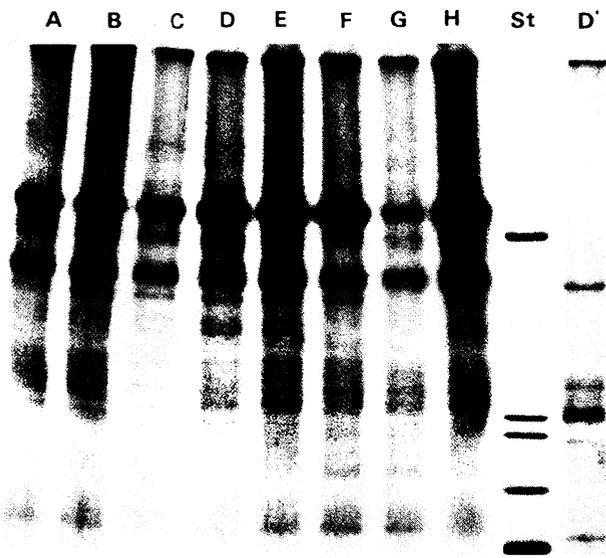


Fig. 2: Autoradiograph of the effect of various ATP concentrations, adenosine, NADP and NADPH on photoaffinity labeling of envelope proteins. Products were analyzed by SDS-PAGE. Conditions as in Fig. 1. A, B, C, 0.5, 0.75, 1.0 μM ATP respectively; D, + 100 μM NADPH; E, + 100 μM NADP; F, + 100 μM adenosine; G, + 100 μM ATP; H, no further additions; St, molecular weight standards in kDa, bovine serum albumin 68, ovalbumin 45, glycerinaldehyd-3-phosphate dehydrogenase 36, carbonic anhydrase 29, trypsinogen 24, cytochrome c 12; D', Coomassie Brilliant blue stained polypeptide pattern of the autoradiogram shown in lane D.

ascribe each labeled polypeptide to either the outer or the inner membrane (Fig. 3, lanes F–G and Table 1). UV photolysis of [α - ^{32}P]ATP labels many more proteins in the outer membrane than in the inner envelope membrane. The major band visible on the autoradiogram could be aligned with a Coomassie Brilliant blue stained polypeptide of 75 kDa molecular weight. This protein is predominantly confined to the outer chloroplast envelope. The second major labeled protein at 56 kDa could not be aligned to a Coomassie Brilliant blue stainable protein, but it was clearly distinguishable from the large subunit of ribulose-1,5-bisphosphate carboxylase, which has an apparent molecular weight of 54 kDa on SDS-PAGE (compare Fig. 3).

ATP dependent protein phosphorylation of envelope membrane polypeptides due to protein kinase activity has been reported recently (Soll and Buchanan, 1983; Soll, 1985). As shown in Fig. 3 the labeling regimes by [γ - ^{32}P]ATP (protein kinase) and [α - ^{32}P]ATP (photoaffinity label) showed no significant homology. ^{32}P is preferentially incorporated into proteins of 86 and 23 kDa by the membrane bound protein kinase.

One of the major roles of the chloroplast envelope is its function in lipid biosynthesis; these reactions also require nucleoside triphosphates, e.g. the synthesis of CDP-diacylglycerol en route to phosphatidylglycerol from phosphatidic acid requires CTP. We tested CTP and UTP in the direct photoaffinity labeling approach with separated inner and outer envelope membranes (Fig. 4). The labeling profile is

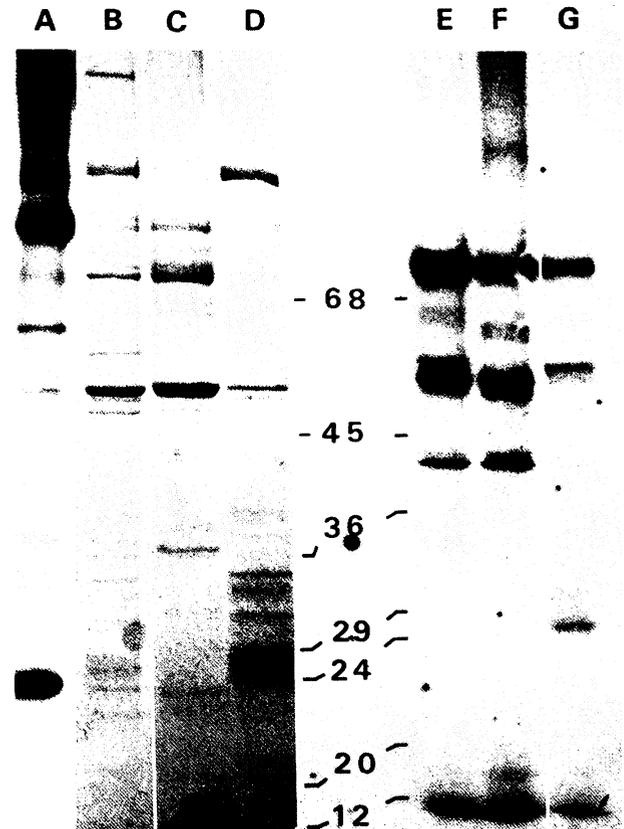


Fig. 3: Separation and labeling of inner and outer chloroplast envelope. A, autoradiograph of [γ - ^{32}P]ATP labeling of mixed envelope membranes. B, C, D, Coomassie Brilliant blue stain of mixed envelope membranes, outer envelope and inner envelope, respectively. E, F, G, autoradiograph of [α - ^{32}P]ATP photoaffinity labeling of mixed, outer and inner envelope membranes, respectively.

Table 1: Localization of photoaffinity labeled polypeptides of pea chloroplast envelope membranes by various [α - ^{32}P] nucleoside triphosphates and [γ - ^{32}P] ATP. Data comprises results from Fig. 1–4; numbers indicate approximate molecular weight in kDa, as determined by one dimensional SDS–PAGE. Numbers in () indicate labeled polypeptides, whose presence is probably due to cross-contaminations.

	mixed envelope	outer envelope	inner envelope
[γ - ^{32}P] ATP	86, 64, 52, 38, 34.5, 23	–	–
[α - ^{32}P] ATP	75, 64, 58, 56, 52, 42, 37, 35, 30, 21	75, 64, 56, 52, 42	(75), 58, 30, 26
[α - ^{32}P] CTP	–	48	90, (48)
[α - ^{32}P] UTP	–	(70), (50), (23)	86, 70, 50, 40, 33, 31, 23

distinct from each other and from ATP. In the inner envelope UV₂₅₄ photolysis of UTP labeled three protein bands at an apparent molecular weight of 23, 31 and 50 kDa. Less prominent are bands at 33, 40, 70, 86 kDa. CTP also labels

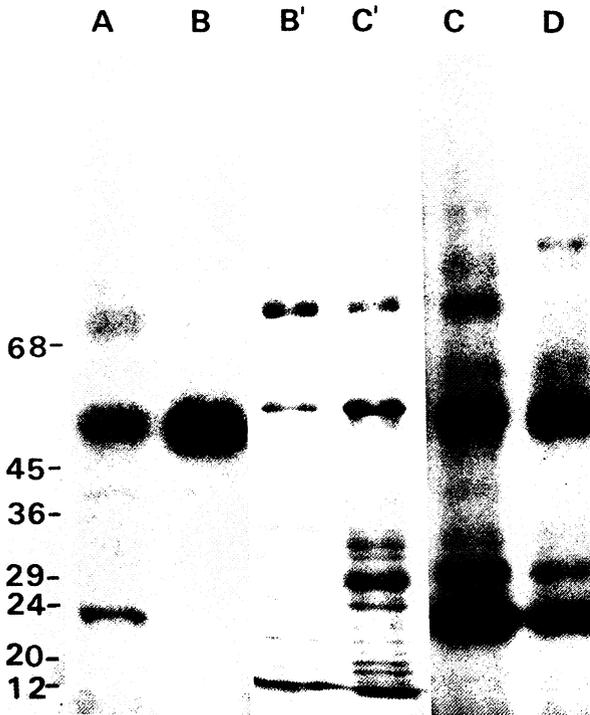


Fig. 4: Inner and outer chloroplast envelope membranes were photoaffinity labeled by [α - 32 P]CTP and [α - 32 P]UTP. Outer membranes labeled by UTP (A) or CTP (B), Inner envelope labeled by UTP (C) or CTP (D). B', C', Coomassie Brilliant blue stain of outer envelope and inner envelope respectively. B', C' correspond to the autoradiograph shown in B and C.

proteins at 23 and 31 kDa like UTP but to a much lesser extent. Specifically marked are only two proteins in the inner membrane by CTP, namely at 90 and 48 kDa. The outer envelope does not show a specific labeling profile using [α - 32 P]UTP, since most of it is probably due to contamination of the outer membrane by the inner envelope. This cross-contamination is generally between 5–10% and thus can account for the labeling intensity seen (Fig. 4). In contrast, CTP almost exclusively labeled a protein at 48 kDa, thus showing that the CTP label in the inner membrane at 48 kDa is also most likely due to cross-contamination of the inner membrane by outer envelope (Table 1). CTP and UTP label two proteins of very similar molecular weight at 50 and 48 kDa, but they can clearly be distinguished by their size on SDS-Page and more important by their confinement to one of the two membrane (Fig. 4; Table 1). Both labeled proteins are clearly different from the large subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (compare Fig. 4).

Galactolipids are the major structural lipids of all chloroplast membranes. UDP-galactose and diacylglycerol give rise to monogalactosyldiacylglycerol which can subsequently be further galactosylated by UDP-galactose to yield digalactosyldiacylglycerol. They are solely synthesized at the envelope (Douce et al., 1984) and have to be transported to the thylakoids. The purification of enzymes active in galactolipid synthesis is not yet achieved. The versatility of direct photoaffinity labeling and its specificity was again demon-

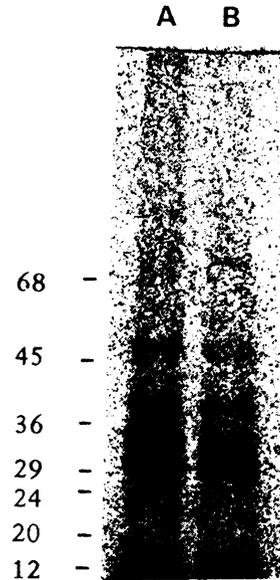


Fig. 5: [U - 14 C]UDP galactose ($0.5 \mu\text{M}$) was used as photoaffinity marker for pea chloroplast envelopes (A) and for spinach chloroplast envelopes (B). Fig. 4 shows a fluorogram of a 7.5–15% SDS-PAGE which was exposed for 3 weeks (Bonner and Laskey, 1974).

strated by using [U - 14 C]UDP-galactose as a probe. UDP-galactose is used only in a very limited number of enzymatic reactions in the envelope membrane. Hence it gave rise to a limited and distinct set of labeled membrane proteins at 31, 33, 35, 38 and 50 kDa in envelope membranes from pea and spinach chloroplasts (Fig. 5). This labeling pattern is very different from those described for the nucleoside triphosphates ATP, CTP and UTP except that the 31 and 50 kDa proteins being labeled by UTP and UDP-galactose might be identical.

Conclusions

We have shown that direct photoaffinity labeling using nucleoside triphosphates as probes gave rise to specific and unique labeling of a very limited number of envelope polypeptides. Direct photoaffinity labeling gave reliable and specific data in this complex assay system containing multiple components, lipids, pigments and polypeptides. It is of much greater use in this complex membrane system than photoaffinity labeling substrates containing bulky or highly reactive photolysable groups like azido residues. Each nucleosidetriphosphate gave rise to distinct labeled proteins in the inner and outer membrane. In our attempts to assign biochemical function to certain proteins and to purify functionally active enzymes from the envelope membranes, the above results can be taken as a starting point at which proteins to look at under given circumstances. Nucleotides have a multifunctional role in cellular metabolism. They serve not only as co-substrates in an enzymatic reaction but also have a regulatory function, in which in most cases they do not bind to the active site but to a regulatory site. Our experiments

were not designed to resolve this question or to check for changes in certain enzyme activities; this has to await purification of the proteins in question. Photoaffinity labeling of an envelope protein has been used to identify an inner envelope bound NTP'ase after partial purification (McCarty and Selman, 1986). This NTP'ase has an apparent molecular weight of 37 kDa. A protein of similar molecular weight is labeled in our *in situ* experiments (Fig. 1).

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