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Sixième congrès international de biologie moléculaire des plantes

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ISPMB

Du 18 au 24 juin 2000
Quebec

Compte-rendu

Pascal Montoro
CIRAD-CP
Programme Hevea

Valérie Pujade-Renaud
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Résumé
Le sixième congrès international de biologie moléculaire des plantes, organisé par l'ISPMB (Société Iinternationale de Biologie Moléculaire des Plantes) s'est déroulé du 18 au 24 juin 2000 à Québec. Les travaux exposés montrent une nette progression dans les domaines fondamentaux comme dans les biotechnologies. L'utilisation des mutants et des micro-collections de cDNAs sont largement utilisés pour la caractérisation moléculaire fine de nombreux mécanismes. Les outils d'analyse à haut débit du génome, du transcriptome ou du protéome sont de plus en plus utilisés. Parmi les thématiques les plus fréquemment abordées lors de ce congès, l'étude des mécanismes de défense contre les stress biotiques et abiotiques tient une place importante. La transformation génétique est très largement utilisée, comme outil d'analyse de l'expression des gènes, comme stratégie d'amélioration, ou pour la production de molécules à haute valeur ajoutée.
Concernant l'hévéa, outre les 3 posters présentés par le CIRAD, un poster et une communication ont été présentés par le RRIM (Rubber Research Institut of Malaysia).

Mots-clés : congress, plant molecular biology, genome, biotechnology, rubber tree

Sommaire

Sixth Congress of the International Society for Plant Molecular Biology (ISPMB)

Quebec – Canada
June 18 – 24, 2000

1. Presentation of the congress

This international congress takes place every 3 years with the objective to highlight the advances, but also to focus on the future orientations, in plant molecular biology.

This year in Quebec, there were 2,200 delegates from 54 countries with an important participation of USA and Canada.

Plenary sessions, symposia, workshops and poster sessions have been conducted on the following topics :

Plant metabolic pathways : regulation and re-routing
Signal transduction
Plant genomics and bioinformatics
Molecular development of Plants
Membranes : receptors and transporters
Flux control and Metabolite partitioning
Molecular systematics
Molecular evolution
Protists and Cryptogams
Mechanisms of disease resistance
Plant biotechnology and molecular farming
Secondary metabolism
Plant cell cycle and development
Environmental interactions
Photosynthesis and photoregulation
Commercialization and Regulatory affairs

2. General idea about the congress

This 6th edition of the ISPMB International Congress in Plant Molecular Biology was marked by the explosion of works relating to genomics and transcriptomics. The use of high rate technics of gene expression analysis (microarrays) becomes more democratic and even indispensable. It is often coupled to approaches using extensive cDNA sequencing. It allows some fundamental studies of the broad mechanismes involved in specific physiological events. It has often been presented in works aiming to characterize the mechanisms of plant defense against biotic or abiotic stresses.

The new concept of proteome analysis is also under development. A still limited number of laboratories have access to that technology (Dr Rossignol's team in Montpellier is one of these).

During the last congress in 1997, a lot of studies concerning transgene silencing had been presented. The progresses in that field allowed to discover that intrusive DNA is recognized by host surveillance systems. More work will be devotted to this topic in the futur.

With regards to genetic transformation, only a few posters have been presented on the technical aspects, other more specialized congress beeing devoted to this, or because the procedure is now well mastered for a high number of species. However, this technique was widely used as a tool to determine gene function by transgenic test, for crop improvement, or for molecular farming;

The knowledge of the mechanisms ruling *Agrobacterium tumefaciens*-mediated transformation is still not complete, in particular the step of the T-DNA integration into the plant. A very interesting lecture was given by Barbara Hohn on this topic.

Finally, the use of Matrix Associated Regions in plant transformation vectors, first reported in 1994 by T. Hall, then by J-P Nap in 1995 and well developed in the last congress in 1997, seems now to be often (although not generally) used in tranformation vectors. These MAR sequences allow to reduce the variability of transgene expression They proved efficient to fight silencing observed after several generations but may however affect temporal expression. These studies have participated to the development of knowledge concerning the influence of chromosomal integration domains on gene expression.

3. Sessions attended

3.1. Plenary sessions

Monday 19 June

1. *Cell cycle*. The ins and outs of the plant cell cycle. Dirk Inze.
2. *Chromatine structure*. DNA methylation, chromatine and epigenetic variations. Eric Richards.
3. *The plant cytoskeleton*. Distribution and functions of microtubules and actin filaments during cell division. Ann Cleary.
4. *Genetic determination of plant morphology*. Linking leaf and meristem in *Arabidopsis*. Kathy Barton.

Tuesday 20 June

1. *Functional genomics*. Plant EST microarrays: cautionary tales. Shauna Somerville.
2. *Genome evolution*. Grass genome structure and evolution. Jeff Bennetzen.
3. *Gene silencing*. Gene silencing in plants: antiviral defense with application in functional genomics. David Baulcombe.
4. *Subcellular targeting, post-translational processing*. Subcellular targeting and post-translational processing. Loic Faye.

Thursday 22 June

1. *Plant-pathogen interactions*. Mechanisms of specific disease resistance. Jeff Dangl.
2. *Abiotic stress*. Molecular responses and tolerance to osmotic stress: gene expression and signal transduction. Kazuo Shinozaki.
3. *Chloroplast molecular biology*. Molecular and genetic analysis of a retrograde signaling pathway from the plastid of the nucleus. Joanne Chory.

4. *Plant hormones.* The AXR1-TIR1 pathway and auxin response. Mark Estelle.

Friday 23 June

1. *Metabolic engineering.* Metabolic engineering : are some enzymes more equal than others? Mark Stitt.
2. *Secondary metabolism.* Biotechnology exploitation of medicinal plant lipid biosynthesis. Tony Kutchan.
3. *Molecular biology of plant lipid biosynthesis.* Molecular biology of plant lipid biosynthesis. John Shanklin.
4. *Molecular farming.* New century, new technology and new products. Elizabeth Hood.

3.2 Concurrent sessions

Pascal Montoro

Monday 19 June

Session: Transformation and recombinant gene expression

1. Transgenic choloroplasts are a unique system for expression of recombinant proteins in higher plants. Pal Maliga.
2. Plant genes involved in Agrobacterium –mediated transformation. Stanton Gelvin.
3. Transgene expression : phenotypic cost vs. benefits. Swapan Datta.
4. Analysis and utilization of phaseolus vulgaris arcelin-5 gene, which directs high seed-specific gene expression. Geert Angenon.
5. Is histone phophorylation involved in phas promotor activation. Guofu L.
6. Gene amplification: a system for enhanced expression of introduced genes in transgenic plants. Nikolai Borisjuk.

Session: Plant genomics

7. Identification of defense-related genes from rice by c-DNA arrays. Shiping Wang.

Tuesday 20 June

Metabolic engineering / industrial products

1. Creating novel biosynthesis pathway through artificial metyabolic sinks. Vincentzo de Luca.
2. Synthesis of novel biopolymers in plants. Yves Poirier.

Embryogenesis and seed development

3. Genetic analysis of embryogenesis in Arabidopsis. Martine Devic.

Transcription factors

4. Methylation and transcriptional repression in carrot somatic cell culture. Letizia Pitto.

Wednesday 21 June

Organellar targeting

1. Transport to the vacuole. Natasha Raikhel.
2. Chloroplast protein import. Jurgen Soll.

Proteomics and genomics

3. Proteomics to test protein variation in the cyanobacterium Synechocystis. Ronney Wiklund.
4. A protein fragment complementation strategy to study prtotein-protein interactions in plant cells. Rajagopal Subramaniam.
5. Gateway cloning system. Gary Temple.
6. Functional genomics for sucrose accumulation gene discovery in sugarcane. Lynne McIntyre.

7. Proteomics in Arabidopsis : an overview form the construction of resources to functional approaches. Michel Rossignol .

Thursday 22 June

DNA integration mechanisms

1. Integration of T-DNA into the plant DNA. Barbara Hohn.
2. Hyper-recombinogenic plants. Avraham Levy.

Tree biotechnology

3. Cloning and analysis of a rubber biosynthesis stimulator protein in *Hevea brasiliensis*. Keng-See Chow.
4. Gene expression associated with shifts in carbon and nitrogen allocation in populus. Janice Cooke.
5. Cellulose metabolism by cellulases in poplar. Takahisa Hayashi.
6. Molecular dissection of phase change in Eucalyptus. Angela Collins.
7. Genomics of heartwood formation. Kyung-hwan Han.

Friday 23 June

Abiotic/Environmental stresses

1. Expression of spinach ascorbate peroxidase isoenzymes. in response to several environmental stresses. Takihiro Ishikawa.
2. Regulation of novel stress-induced genes in pea plants. Helena Savenstand.

Valerie Pujade-Renaud

Monday 19 June

Session: mRNA stability/Interdiction of Gene Expression

1. Application of genetic and genomic approaches to the study of mRNA decay in *Arabidopsis*. Pamela Green.
2. RNA directed promoter methylation and transcriptional gene silencing. Florian Mette.
3. Autoregulation of cystathionine gamma-synthase mRNA stability in response to methionine application. Yukako Chiba.
4. Inhibition of chlorophyll biosynthesis by virus-induced gene silencing (VIGS). Kirsi Lehto.
5. The INTIR approach: efficient gene silencing in transgenic plants by in vitro prepared interrupted invertd repeats. Jan-Peter Nap.
6. Post-transcriptional and transcriptional gene silencing are induced by cauliflower mosaic virus infection: two faces of the same coin? Nadia S. Al-Kaff.
7. Evolution and epigenetic control of MuDR, the regulator of Mu transposons. George N Rudenko.

Tuesday 20 June

Metabolic engineering / industrial products

1. Creating novel biosynthesis pathway through artificial metyabolic sinks. Vincentzo de Luca.
2. Synthesis of novel biopolymers in plants. Yves Poirier.
3. Enhanced nitrogen efficiency by tissue specific expression of an aminotransferase gene. Allen G Good.
4. Starch encapsulation technology as a vehicle for producing recombinant proteins in plants. Hanping Guan.

Wednesday 21 June

Insect resistance

1. Signal interactions in induced resistance to pathogens and insect herbivores. Richard Bostock.
2. Genetic manipulation of fatty acid hydroperoxide metabolism in potato. Jose-Sanchez Serrano.

Organelle Molecular Biology

3. Conter-acting oxidizing and reducing activities control translation of psbA mRNA. Avihai Danon.

Plant lipid metabolism

4. Characterization of an *Euphorbia lagascae* epoxide hydrolase gene which is induced bearly during germination. Johan Edqvist.

Thursday 22 June

DNA integration mechanisms

1. Integration of T-DNA into the plant DNA. Barbara Hohn.
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Tree biotechnology

3. Cloning and analysis of a rubber biosynthesis stimulator protein in Hevea brasiliensis. Keng-See Chow.
4. Gene expression associated with shifts in carbon and nitrogen allocation in populus. Janice Cooke.
5. Cellulose metabolism by cellulases in poplar. Takahisa Hayashi.
6. Molecular dissection of phase change in Eucalyptus. Angela Collins.
7. Genomics of heartwood formation. Kyung-hwan Han.

Friday 23 June

Molecular farming

1. Protein expression systems based in rice alpha-amylase promoter and signal peptide sequences. Su-May Yu.
2. Protective anti-Herpes antibodies for reproductive health. Kristin Brigg.

Abiotic/Environmental stresses

3. Expression of spinach ascorbate peroxidase isoenzymes in response to several environmental stresses. Takihiro Ishikawa.
4. Regulation of novel stress-induced genes in pea plants. Helena Savenstand.

Gene mapping and genomic synteny

5. Construction of rice artificial chromosome. Nori Kurata.

4. Workshop attended

Plant molecular biology. Applications in developing countries

1. The context. Malcolm Elliott
2. The crops. Swapan Datta and Kiran Sharma
3. Food security. Indra Vasil
4. The regions/countries. Luis Herrera-Estrella and Qifa Zhang
5. New horizons. Maurice Moloney
6. The multinationals point of vue. Barbara Mazure

The main ideas arising from this worshop are:

- The urgent necessity for an increase in food productions (a 60 to 100 % increase is necessary to feed the earth population by the year 2050 !)

- Also a demand for an increase in quality and protection of the environment
- The anti GMO protest is generated and entertained by mass media from developped countries whereas the developping countries (according to representatives from Mexico, China, India, Philippines...) reaffirm their demand for GMOs.
- Importance to train young scientists fromdeveloping countries

5. Posters

About 1400 posters were presented.
All abstracts are available on http://www.ISPMB-2000.org/frameset3.html with the help of an abstract search tool.

Posters were classified under 30 different categories:

	Categories	*Nbre of posters*
S1	Plant Genomics	100
S2	Rhizobium and Non-Pathological Plant Microbe Interactions	22
S3	Transformation and Recombinant Gene Expression	127
S4	Plant Cell Wall and Extracellular Matrix	27
S5	Leaf and Shoot Development	33
S6	Plant Secondary Metabolism	52
S7	mRNA Stability/Interdiction of Gene Expression	41
S8	Bioinformatics	9
S9	Signal Transduction	95
S10	Metabolic Engineering/Industrial Products	21
S11	Embryogenesis and Seed Development	33
S12	Lower Plants and Prokaryotes	9
S13	Plant Secretory Pathway	20
S14	Transcription Factors	46
S15	Proteomics & Genomics II	5
S16	Insect Resistance	38
S17	Organellar Targeting	12
S18	Root Developmental Biology	14
S19	Organelle Molecular Biology	57
S20	Plant Lipid Metabolism	26
S21	Evolution of Terrestrial Plants	23
S22	Plant-Pathogen Interactions	108
S23	DNA Integration Mechanisms	27
S24	Photomorphogenesis	22
S25	Fertilization / Plant Reproduction	38
S26	Tree Biotechnology	29
S27	Plant Carbohydrate Metabolism	47
S28	Molecular Farming	33
S29	Gene Mapping and Genomic Synteny	8
S30	Plant Viruses	49
S31	Abiotic/Environmental Stresses	124
S32	Plant Cytoskeleton	10

S33	Hybrid Technology/Control of Fertility	9
S34	Plant Cell Cycle	30
S35	Hormonal Regulation	42
S36	Late Abstracts and Biotechnology Related Topics	9

The number of posters per section may give an idea of the importance devoted by the international community to each research field. In particular, it highlights the very important interest for plant defense mechanisms against biotic and abiotic stresses, as well as the important use of genetic transformation, both as a research tool and as a method for plant improvement. It also demonstrates the development of the genomic tools.

A list of abstracts of interest is given in Annexe. They were selected among the following poster categories:

Transformation and Recombinant Gene Expression,
Metabolic Engineering/Industrial Products,
Embryogenesis and Seed Development,
Plant-Pathogen Interactions,
DNA Integration Mechanisms,
Tree Biotechnology,
Plant Carbohydrate Metabolism,
Molecular Farming and Hormonal Regulation.

6. Conference and posters about rubber tree

One conference given by Dr Keng-See Chow (Rubber Research Institut of Malaysia) and 4 posters (see Annexe) were concerning rubber tree.

CLONING AND ANALYSIS OF A RUBBER BIOSYNTHESIS STIMULATOR PROTEIN IN *Hevea brasiliensis*. (Conference and poster S26-2)
Keng-See Chow, Faridah Yusof, Wan Halizah, Wan Ishak, Latifah Abdullah, Hoong-Yeet Yeang.
Rubber Research Institut of Malaysia

ACCUMULATION OF A POLYPEPTIDE IDENTIFIED AS THE LATEX ALLERGEN HEV B 3 IN THE LATEX CYTOSOL OF RUBBER TREES DISPLAYING THE TAPPING PANEL DRYNESS SYNDROME (Poster S 01-69)
Valerie Pujade-Renaud[1], Regis Lacotte[1], Uncheera Sookmark[2], Phyao Romruensukharom[3], Cinda Naiyanetr[2], Jarunya Narangajavana[2], Herve Chrestin[4].
[1]CIRAD; [2]Mahidol University, Thailand; [3]RRIT, Thailand; [4]IRD

CLONING OF POTENTIALLY ETHYLENE-INDUCIBLE AND/OR LATICIFER-SPECIFIC PROMOTORS FROM HEVEA BRASILIENSIS (Poster S 26-21)
Valerie Pujade-Renaud[1], Pascal Montoro[1], Panida Konsawadworakul[2], Phyao Romruensukharom[3], Jarunya Narangajavana[2], Herve Chrestin[4].
[1]CIRAD; [2]Mahidol University, Thailand; [3]RRIT, Thailand; [4]IRD

DIFFERENTIAL DISPLAY AS A TOOL FOR SCREENING SEQUENCES RELATED WITH EARLY SOMATIC EMBRYOGENESIS IN RUBBER TREE (Poster S 11-5)
Erika Charbit, Thierry Legavre, Ludovic Lardet, Marc-Philippe Carron
CIRAD Biotrop

7. Main discussions with scientists

P Montoro et V Pujade-Renaud: Discussion with Dr Keng-See Chow, from the Rubber Research Institute of Malaysia. Dr Chow is involved in molecular biology projects. We introduced ourselves to her and we talked about our respective activities. She mentionned that one of her collegue is also working presently on the hevein promoter. Each molecular biologist in RRIM works in tandem with a biochemist.

V Pujade-Renaud: discussion with Pierre Maraccini (Nestlé, France) about wether or not make a pattent on the hevein promoter. We arrived to the conclusion that it would be difficult and not interesting financially. He advised us to rather release our promoter sequence in the public databank, in order to protect ourselves from a possible pattent coming from the RRIM.

V Pujade-Renaud: discussion with Dr Han, formerly at Kumho Life and Environmental Science Laboratory, Korea. Dr Han was the scientist who conducted the work on ESTs from rubber tree latex (annonced in a publication). He now moved to the Michigan State University (USA) were he works on wood species, in the Department of Forestry. He intends to developp a bigger programm on rubber tree ESTs. Dr Han said that nobody from korea is continuing the work on the rubber tree ESTs. We may however contact him directly in USA to obtain some ESTs of interest for our programm, or for collaboration on the EST approach.

P Montoro: Meeting with Dr Armand Seguin (Canadian Forest Service) and Dr Gilles Pilate (Inra-Orleans) about the genetic transformation of poplar and conifers. Their project on modification of lignine content in poplar will be ended soon. Dr Seguin has other projects on the introduction of Bt genes in spruce and on the characterization of tree-pathogens interaction in poplar. Dr Pilate has also conducted research on differential gene expression analysis in poplar tension wood and he plans to develop molecular genetic approach on it. Besides, a scheduled visit of Dr Seguin's laboratory has been cancelled because of a personal problem.

The 7th edition of the ISPMB International Congress of Plant Molecular Biology will take place in Barcelona in 2003

ANNEXES

ANNEXE 1

S1 PLANT GENOMICS

S1 – 32
DIFFERENTIALLY EXPRESSED SEQUENCE TAGS IN SUNFLOWER

Ruth A. Heinz, Diego Lijavetzki, Norma Paniego, Maritza Lopez Bilbao, Paula Fernandez, Horacio, E. Hopp
1- Instituto de Biotecnologia, I.N.T.A., Castelar, Buenos Aires, Argentina

Sunflower (Helianthus annuus L.) is one of the major annual crops grown for edible oil in the world and particularly in Argentina with a considerable increase in the production area and exportation volume in the last years. Despite its economic importance relatively scarce genomic studies have been accomplished. In this project, the development of expressed sequence tags (ESTs) obtained from different cDNA libraries is explored as one of the genomic tools used for cultivar identification, genomic analysis and plant breeding as well as for the discovery of new genes and metabolic pathways involved in crop quality and productivity. Differential and equalized cDNA libraries were constructed from different sunflower organs including leaves and developing flower. A PCR-selected cDNA substraction technique was used to produce differential and/or enriched libraries and the final amplified cDNA product was cloned in a PCR cloning vector. This strategy was selected in order to increase the probability of detection of low abundance transcripts and simultaneous enrichment in sequences expressed in a specific organ/tissue under defined environmental conditions. About 2000 clones have been obtained from the leaf substracted cDNA library from which 300 randomly selected clones are in the process of being sequenced. Sequence data and similarity searches against known sequences in database will be presented and discussed. We expect to obtain, analyze and map at least 1,200 ESTs randomly selected from four distinct sunflower differential cDNA libraries.
Simultaneously, other genomic tools, including single sequence repeats (SSRs) and resistance gene analogs (RGAs) are being generated and mapped. The final goal of this project is to develop a genetic map for cultivated sunflower for most of the developed markers (SSRs, ESTs and RGAs) as the standpoint of understanding the genome organization, and as a platform for map-based cloning in combination with the construction of a BAC library.

S1 - 34
THE ROLE OF ETHYLENE IN NON-HOST AND ACQUIRED RESISTANCE IN TOBACCO

Jan Hoekstra (1), John, F. Bol (1), Huub, J.M. Linthorst (1)
1- Institute of Molecular Plant Sciences, Leiden University, Leiden, The Netherlands

The gaseous hormone ethylene plays an important role in plant development and defence. Recently, tobacco plants were transformed with the dominant mutant etr1-1 ethylene receptor gene from Arabidopsis thaliana. The resulting transgenic plants (Tetr) are insensitive to ethylene. Tetr plants are sensitive to infection with normally non pathogenic soil borne organisms, especially some Pythium species (Oomycetes). After infection with Tobacco Mosaic Virus (TMV), the induction of the ethylene regulated basic PR genes is sharply reduced in Tetr plants when compared to wild type plants. To test if there is a correlation between these two phenomena, the cDNAs corresponding to the anti Oomycete basic PR1 and PR5 were cloned behind the 35S promoter in an Agrobacterium transformation vector. Single gene constructs and a construct containing both basic PR cDNAs were transformed to Tetr line 18. Constitutive expression of the transgenes in primary transformants was confirmed by Northern blot. Several primary transformants of all three constructs showed spontaneous disease symptoms as was previously observed with Tetr plants, indicating that the overexpression of basic PR1 and or basic PR5 gene transcripts does not prevent infection of non-host fungi in ethylene insensitive tobacco.
Tetr plants also have a reduced capability to induce systemic acquired resistance (SAR). To study this in more detail chimaeric grafts were made between Samsun NN tobacco and Tetr18 plants. TMV infection of Tetr18 rootstock leaves was not able to induce a significant SAR response in Samsun NN scion tissue. This shows that ethylene-sensitivity is essential for the generation of the mobile signal that leads to SAR. In addition, the reverse grafts showed that a SAR response can be generated in Tetr scion tissue when grafted onto wildtype rootstock, suggesting that ethylene sensitivity is not needed for the perception of the SAR signal in the systemic tissue.

S1 - 40
A NOVEL STRESS-INDUCIBLE PEROXIDASE PROMOTER FROM SUSPENSION CULTURES OF SWEET POTATO

Sang-Soo Kwak (1), Kee-Yeun Kim (2), Suk-Yoon Kwon (1), Haeng-Soon Lee (1), Yun-Kang Hur (2)
1- Plant Cell Biotechnology Laboratory, Korea Research Institute of Bioscience and Biotechnology, Yusong, Taejon 305-333, Korea
2- Department of Biology, Chungnam National University, Yusong, Taejon 305-763, Korea

We have established an efficient production system of peroxidase (POD) in suspension cultures of sweet potato (Ipomoea batatas) and characterized four POD cDNAs (three anionic cDNAs swpa1, swpa2, swpa3 and one neutral cDNA swpn1)isolated from suspension cultures of sweet potato in terms of environmental stresses. Four genes were strongly expressed in cultured cells compared to intact plant tissues, and induced by various stresses such as chilling, wounding and ozone in leaves. Particularly, swpa2 was not expressed in intact plant tissues, but strongly induced by various stresses. We have isolated an swpa2 genomic clone (SWPA2) containing about 1824 bp of 5'-upstream sequence, two intron (743 bp and 96bp), and the coding sequence. The SWPA2 promoter contains several cis-element sequences bearing a high degree of similarity with GCN-4, AP-1, HSTF, SP-1 and G-box which are involved in oxidative stress. Promoter activity was analyzed with different 5'deletion mutants of the SWPA2 promoter-GUS fusion genes by the transient system using tobacco protoplasts. The transient expression studies revealed that SWPA2 promoter showed about 30 times higher expression than CaMV 35S promoter. A gel mobility shift assay indicated that the HSTE motif might bind the nucleus proteins of sweet potato in vitro. The functional analysis and possible applications of the promoter using transgenic tobacco plants and cells will be discussed.

S1 - 41
GENOMIC ANALYSIS OF EMBRYOGENESIS IN POTATO THROUGH VIRTUAL SUBTRACTION : AN EST TARGETED PROJECT

Marie Lagac□, Corine Zotti, Sier-Ching Chantha, Daniel P. Matton
1- Institut de recherche en biologie v□g□tale, Universit□ de Montr□al, 4101 Sherbrooke est, Montr□al, Qc, Canada, H1X 2B2

To understand the molecular basis underlying plant sexual reproduction from pollination to embryogenesis, we propose to undertake a genomics approach to analyze and isolate new regulatory genes differentially expressed during the early stages of embryogenesis. The main approach currently used to study embryogenesis at the molecular level is based on the production of mutants, either by chemical mutagenesis followed by map-based cloning or DNA tagging. Although these are very successful methods, they are also time-consuming and there seems to be a limitation in the number of genes that can be targeted by mutation. This suggests that mutagenesis approaches will uncover only a fraction of the genes expressed during fertilization and embryogenesis. The virtual subtraction strategy ensure that an enriched population of cDNAs representing rare transcripts will be target by this EST project, the assumption being that genes with regulatory function are generally very weakly expressed. In a preliminary screen by virtual subtraction of 7872 cDNAs from a Solanum chacoense pistil and ovule libraries, 718 lowly expressed cDNAs were sequenced and about 20% were found by identity search to have potential regulatory functions, thus confirming that our pool is enriched. The sequenced cDNAs include genes involved in signal transduction (Protein kinases, Histidine kinase, Protein phosphatases, G proteins, G-protein coupled receptor, WD40 proteins), transcriptional regulation (MADS-box gene, HD-Zip, b-Zip, Myb-like factors, AP2, ANT, PHAN, Zinc finger factors, Lim-domain factors, etc), chromatin remodeling (Histone deacetylase and acetyltransferase, SWI/SNF) and RNA processing (Splicing factors, RNA binding protein, PABP). Another 40% had no functional identites (although some were also found in EST sequences from other species). Roughly 40 % had similarities to know gene product. Global results from the virtual subtraction will be presented.

S1 - 43
TRANSGENIC CUCUMBER PLANTS OVEREXPRESSING SUPEROXIDE DISMUTASE

Haeng-Soon Lee (1), Jae-Whune Kim (1), Yu-Jeong Jeong (1), Suk-Yoon Kwon (1), Sang-Soo Kwak (1)
1- Plant Cell Biotechnology Lab., Korea Research Institute of Bioscience & Biotechnology, Yusong, Taejon, Korea

Superoxide dismutase (SOD) plays an important role in cellular defense against oxidative stress in plants. Transgenic plants overexpressing SOD have been developed to reduce the oxidative damage in plants. In addition, SOD may be very useful in the field of medicinal, food and cosmetic industries. In this report, to develop the fruits of cucumber (Cucumis sativus L.) producing high yields of SOD as a plant bioreactor, the SOD cDNA was introduced into cucumber. For this we designed a new vector system, pGPTV-Bar harboring ascorbate oxidase promoter expressing dominantly in cucumber fruits, mSOD1 isolated from cultured cells of cassava, and bar gene as a selectable marker. An mSOD1 cDNA was highly expressed in cultured cells, and differently responded to various stresses including. The excised hypocotyl segments bearing cotyledons of cucumber seedlings were cocultivated with A. tumefaciens carrying the vector. The Bastar-resistant shoots were selected on the selection medium containing MS basal salts, 2 mg/l BA, 300 mg/l claforan, 2 mg/l Bastar. After 6 weeks of cultures on the selection medium, the shoots were transferred to MS containing 1 mg/l IAA, 300 mg/l claforan, 2 mg/l Bastar to induce roots. PCR analysis using the primers for bar revealed that several plantlets were transformed. The putative transgenic cucumber plants are growing in the green house and will be analyzed. The cucumber fruits overexpressing SOD will be useful for cosmetic and medicinal materials.

S1 - 44
BIOCHEMICAL PROPERTY COMPARISON OF BRASSICA CPRXII WITH 2CYS-PRX ISOLATED FROM THE SAME PLANT

Sang Yeol Lee, Yeon Ok Choi, Kyun Oh Lee, Bae Gyo Jung , Yong Hun Chi , Ji Yeon Lee, Ho Hee Jang, Moo Je Cho
1- School of Applied Life Sciences, Gyeongsang National University, Chinju, 660-701, Korea

A cDNA encoding a newly identified isotype of peroxiredoxin (Prx) was isolated from a chinese cabbage flower bud cDNA library and designated CPrxII. The predicted amino acid sequence of CPrxII has no conserved cysteine, peptide domain, or signal sequence present in most of the 2Cys-Prx subfamily members. Database searches using the predicted CPrxII amino acid sequence revealed no substantial homology to other proteins with the exception of the yeast type II Prx with which CPrxII shares 27.8% sequence identity. However, the CPrxII shows no immuno cross-reactivity to antiserum of the yeast type II Prx, and vice versa. Southern analysis using the cDNA insert of CPrxII revealed that it consists of a small multigene family in chinese cabbage genome. The recombinant protein of CPrxII expressed in E. coli migrates as a dimer in a nonreducing SDS-polyacrylamide gel and as a monomer in a reducing condition. Recombinant CPrxII was able to protect glutamine synthetase from inactivation in a metal catalyzed oxidation system and to reduce H2O2 with electrons provided by thioredoxin. This specific antioxidant activity of CPrxII was about 6 fold higher than that of 2Cys-Prx of the same plant. In contrast to 2Cys-Prx, which is predominantly expressed in leaf tissue of cabbage seedlings, CPrxII is highly expressed in root tissue as revealed by Northern and Western blot analyses.
[This is supported by BK21

S1 - 45
ANALYSIS OF THE GENE EXPRESSION PROFILE OF ARABIDOPSIS LEAF BY SAGE METHOD

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Serial analysis of gene expression (SAGE), one of the powerful expression analysis methods, allows the quantitative and simultaneous analysis of large number of transcripts. It is highly efficient, able to detect low abundance genes, very accurate, and extremely sensitive. In this study SAGE technology was utilized for the systematic analysis of transcripts expressed in leaf cells of Arabidopsis. Several steps were modified to improve the efficiency of the cDNA tag library construction. Sequence analysis of several thousands tags revealed a gene expression pattern characteristic of leaf cells of Arabidopsis. As expected, a large part of abundant tags matches with that are widely expressed in many cell types, notably genes encoding ribosomal proteins, proteins associated with protein synthesis and the cytoskeleton. Among abundant transcripts, chlorophyll a/b binding protein and photosystem subunit proteins for photosynthesis represents the match with transcripts that are fairly specific for green leaf cell.

S1 - 46
MAP-BASED CLONING OF A SOYBEAN CYST NEMATODE RESISTANCE GENE

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Soybean cyst nematode (SCN) is the most serious pest of soybeans in the US. The most desirable control method is the use of soybean cultivars having SCN resistance genes. The SCN resistance gene, Rhg4, is located near the I locus controlling seed coat color. From published sequence of the I locus, we developed a PCR marker. In addition, the molecular marker, pBLT65, is close to Rhg4. Both markers were used to screen a 'Williams 82' (susceptible) Bacterial Artificial Chromosome (BAC) library, identifying one 150kb BAC. SSR markers and a subclone library were developed from this BAC. Subclones from the BAC were sequenced: 1) to identify genes in this region, and 2) to develop additional markers to use in finding the same region in resistant genotypes. These markers identified 87 BACs from a PI 437.654 (resistant) BAC library. Restriction fragment analysis using FPC (fingerprinted contigs) software at several stringency levels assigned BACs to contigs. RFLP markers pBLT65 and the I locus were assigned by FPC at all stringency levels to separate contigs. However, connection of contigs containing these markers was confirmed by PCR primers developed from BAC-end sequencing. Because these primers did not amplify a product from the Williams 82 BAC, and because the physical distance between pBLT65 and the I locus marker is greater in PI 437.654 than in Williams 82, we are investigating the possibility that there may be an insertion in PI 437.654 relative to Williams 82. This is of interest, because at least four resistance genes have been found in insertions in plant genomes. We are focusing our efforts on two BACs that connect the contigs containing pBLT65 and the I locus marker. We are taking two approaches: 1) complementation tests through transformation, and 2) sequencing and assembly of subclones of the two BACs.

S1 - 52
FUNCTIONAL GENOMICS FOR SUCROSE ACCUMULATION GENE DISCOVERY IN SUGARCANE.

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Commercial varieties of sugarcane accumulate up to 50-60% of the dry weight of stem tissues as sucrose. The accumulation of sucrose occurs in a developmentally programmed tissue-specific manner with a sharp transition from active growth and elongation to sucrose storage functions occurring over a few internodes. We are interested in identifying and characterising the genes that determine the development and function of the sucrose accumulating tissues. Eventually, this may facilitate the manipulation of sugarcane and other plants for enhanced sugar concentration. Our aim is to develop a substantial set of annotated ESTs from stem tissues, followed by further analysis using bioinformatics and expression arrays to identify genes with potentially useful functions or expression patterns. These genes will then be studied in more detail either as molecular markers or as transgenes in cell culture or transgenic plants.
A dataset of 4000 ESTs has been obtained from two complete cDNA libraries, one derived from immature stems (1092 sequences) and one from near-mature stems (2908 sequences) of the sugarcane cultivar Q117. For the immature and mature libraries respectively, 56.5% and 44.4% of sequences were homologous to genes of known function, 19.2% and 23.7% were homologous to anonymous genes and 24.3% and 31.7% had no known homologue.
A total of 8761 ESTs have now been sequenced from the near-mature stem library and the database is currently being annotated and analysed for homologies. Expression profiles will be performed on a non-redundant set of clones and attempts made to correlate expression with the onset of sucrose accumulation and with genotypic variation. ESTs of interest are currently being mapped in both sugarcane and sorghum populations segregating for level of sucrose accumulation and other traits of interest. Microsatellites from the EST collection are also being investigated.

S1 - 69
ACCUMULATION OF A POLYPEPTIDE IDENTIFIED AS THE LATEX ALLERGEN HEV B 3 IN THE LATEX CYTOSOL OF RUBBER TREES DISPLAYING THE TAPPING PANEL DRYNESS SYNDROME

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The Tapping Panel Dryness (TPD) syndrome of rubber tree is characterized by the partial or ultimately complete stoppage of latex flow upon tapping. A 22 kDa polypeptide (PDF22) was found to accumulate in the latex cytosol of TPD trees. A cDNA encoding PDF22 was cloned. Sequence analysis identified PDF22 as the latex allergenic protein Hev b 3, which presents some sequence homology both with the Rubber Elongation Factor (REF) and with PvSRP, a stress-related protein from Phaseolus vulgaris. PDF22 gene expression was strongly increased by tapping, although not by wounding alone (tapping without loss of latex), nor by ethylene or ABA. No significant difference in gene expression was observed between healthy and TPD trees, indicating that the TPD-related accumulation of the PDF22 polypeptide was not due to transcriptional regulation mechanisms. Studies on latex protein compartmentation demonstrated that the PDF22 protein, normally bound to the rubber particle surface, could be released in the cytosol in vitro upon hypotonic lysis of the latex organelles. It is suggested that such a decompartmentation process may be responsible for the accumulation of PDF22 in the latex cytosol of TPD trees in vivo.

S1 - 77

CONSTRUCTION OF FULL-LENGTH ARABIDOPSIS cDNA LIBRARIES BY BIOTINYLATED CAP TRAPPER AND MONITORING GENE EXPRESSION PATTERN UNDER DEHYDRATION AND COLD STRESS USING FULL-LENGTH cDNA MICROARRAY

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Full-length cDNAs are essential for functional analysis of plant genes. We constructed full-length Arabidopsis cDNA libraries from plants in different conditions, such as dehydration-treated plants and cold-treated plants, and plants at various developmental stages from germination to mature seeds. We used thermoactivated reverse transcriptase and chemical introduction of a biotin group into the diol residue of the CAP structure of eukaryotic mRNA, followed by RNase I treatment, to select full-length cDNA1). Until now, we have done single-pass sequencing of 13,109 cDNA clones from the 5' end. These clones were classified into 6,345 independent genes. We also prepared a Arabidopsis full-length cDNA microarray using ca. 1300 full-length cDNAs including drought-inducible genes. The cDNA microarray was used to identify drought- and cold- inducible genes, and target genes of DREB1A, a transcription factor that controls stress-inducible gene expression. The rd29A, cor15a, kin1, kin2, rd17, and erd10 genes were also identified as DREB1A target genes using the cDNA microarray, which confirmed the previous report. Also, 6 genes, such as a homolog of wheat putative cold acclimation protein, were identified as novel drought- and cold-inducible genes, and DREB1A target genes. These results show that our full-length cDNA microarray is a useful material to analyze expression pattern of Arabidopsis genes under dehydration and cold stress and to identify target genes of stress-related transcription factors.
1) Seki, M., Carninci, P., Nishiyama, Y., Hayashizaki, Y., and Shinozaki, K.(1998) Plant J. 15: 707-720.

S1 - 80

PLANT EST MICROARRAYS: CAUTIONARY TALES

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With the eminent completion of the sequencing of the Arabidopsis genome, plant biologists are faced with the twin challenges of determining functions for all ~25,000 genes of this plant and of utilizing this information to enhance understanding of basic plant processes. Many new technologies that will be required to meet these challenges. The DNA microarray technology is one of the first genomic-scale technologies to be developed. With this technology, mRNA abundance can be monitored for thousands of genes simultaneously, thus providing a genome-wide view of plant responses to developmental transitions or environmental treatments. DNA microarrays are being produced for a number of plant species, including Arabidopsis. I will outline results from our initial experiments to characterize powdery mildew resistance in Arabidopsis using 2,400-element microarrays enriched for genes implicated in plant-pathogen interactions. In addition, I will describe the Arabidopsis Functional Genomics Consortium (http://afgc.stanford.edu) project to produce large scale 11,500-element Arabidopsis microarrays.

S1-91

GENE EXPRESSION PROFILING IN ARABIDOPSIS USING DNA MICROARRAYS

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The microarray facility at MSU is part of the Arabidopsis Functional Genomics Consortium (AFGC), a collaboration recently funded by National Science Foundation. Together with Shauna Sommerville at Carnegie Institute and Mike Cherry at Stanford University our goal is to make DNA microarray technology available to the academic community. This will be accomplished through the establishment of a service facility and by providing the data generated to the community through the WWW. In the first year of our project we have printed and tested a microarray containing 11,000 non-redundant Arabidopsis ESTs selected from the 37,000 ESTs available in the public domain. The DNA fragments spotted on the microarray have been amplified from the collection of EST clones sequenced by Tom Newman. By hybridizing these arrays with probes corresponding to mRNA extracted from different tissues global gene expression patterns can be investigated. To this end, the labeling and hybridization techniques have been optimized. The microarray facility has started its service at the beginning of this year. In the first of three rounds of applications we have received 44 proposals from scientists of 6 different countries. Most experiments compare mutant to wild type or treated to untreated plants. Two experiments use Brassica plant material rather then Arabidopsis. The proposal titles and our services can be viewed on the website http://afgc.stanford.edu/. Our project will determine the gene expression patterns in different tissues and organs or from Arabidopsis plants subjected to different stimuli. We will also examine the expression of plant specific genes of unknown function under a set of standardized conditions.

S1 - 93

THE ARABIDOPSIS GERANYLGERANYLTRANSFERASE-I AND FARNESYLTRANSFERASE ARE STRUCTURALLY AND FUNCTIONALLY DISTINCT.

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Gernaylgeranyltransferase-I (GGTase-I) and farnesyltransferase (FTase) are heterodimeric enzymes that share a common alpha subunit, have distinct beta subunits and modify proteins with key functions in signaling cascades. Both GGTase-I and FTase recognize a C-terminal

CaaX-box recognition motif in which C designates the prenylated cysteine, a are usually aliphatic amino acids, and X is almost exclusively a leucine residue when a protein is prenylated by GGTase-I, and can be one of several residues if prenylated by FTase. Despite its regulatory importance, cloning of a plant GGT-IB gene and characterization of GGTase-I activity in plants were not reported. The AtGGT-IB gene from Arabidopsis, was cloned using the tomato LeFTA as bait in a yeast two hybrid screen. Sequence comparison showed that the AtGGT-IB protein displays higher homology to its mammalian than to its yeast homologue. Recombinant AtGGTase-I was purified from baculovirus infected insect cells. Kinetic studies, using the purified AtGGTase-I, geranylgeranyldiphosphate , and derivatives of the Petunia calmodulin CaM53 as protein substrates, showed that proteins containing a domain of basic residues proximal to the CaaX box were prenylated with higher Vmax values. The affinity, however, is determined by the amino acid composition of the CaaX box and remained the same in the absence of the polybasic domain. Moreover, prenylation by GGTase-I was inhibited at high concentrations of a GST-CaaX box fusion protein indicating an inefficient release of the prenylated protein from the enzyme in the absence of the polybasic domain. RT-PCR and protein blot analysis showed that AtGGT-IB is ubiquitously expressed throughout the plant, and that expression AtGGT-IB remains unaltered in era1-2 plants in which the AtFTB gene is deleted. Also, a GFP-CaM53 fusion protein that was transiently expressed in era1-2 leaves was localized in the plasma membrane indicating prenylation of the fusion protein by GGTase-I.

S1 – 95

RICE cDNA MICROARRAY SYSTEM: TOWARDS FUNCTIONAL GENOMICS OF RICE.

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cDNA microarray have become the preferred method for genome-wide transcriptional monitoring. RGP (Rice Genome Research Program) determined about 8800 partial cDNA sequences representing individual genes in rice genome. This effort allowed us to survey the gene expression on a large scale. In order to use these cDNAs as probes for expression monitoring, a Microarray Project in Rice has been started by MAFF since April 1999.
As the first step towards genomic-scale expression analysis of rice, we have constructed microarray containing 1265 rice cDNAs spotted with a high-speed robotic arrayer from Amasham-Pharmacia (MTAP). Among the spotted clones, 495 sequences correspond to unidentified genes in rice. We have made two kinds of cDNA array by spotting PCR products; Full insert array; Gene-specific array with PCR products using specific primers designed from 3'untranslated regions of the cDNAs (The gene-specific PCR products have also been using for EST mapping of rice genome by RGP).
To analyze the gene expression patterns in different tissues and between plants under normal growth condition and plants under environmentally-stressed condition, we isolated mRNAs from leaf, root, flower, callus, germinating seeds, and germinating seeds exposed to high temperature (40C), as target RNAs for microarray hybridization experiments.
As the results, we found that the gene-specific arrays could detect weaker signals but much more specific signals than the full insert arrays. In addition to tissue-specific genes, several genes with unknown functions also reproducibly showed the difference in expression level between different mRNA samples. These results suggest that the microarray system has the high efficiency and reproducibility in quantitating gene expression levels in various samples and will be a powerful tool in functional genomics, that is, in characterizing functions of novel genes and understanding genetical regulation of physiological and developmental processes in rice.

S3 Transformation and Recombinant Gene Expression

S3 - 4

AGROBACTERIUM TRANSFORMATION OF INDICA RICE: FACTORS AFFECTING TRANSIENT AND STABLE TRANSFORMATION

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Morphologically normal, fertile transgenic rice plants were obtained by co-cultivating mature seed scutellum-derived embryogenic calli of the cvs. BR-26 and Binni with Agrobacterium tumefaciens strain LBA4404 carrying the super binary vector pTOK233 [1]. Inclusion of acetosyringone (100µM) in the co-cultivation medium was essential for efficient transformation. The optimum temperature for co-cultivation was 25-28oC; selection with hygromycin B (50 mg l-1) was essential for stable transformation. SAAT (Sonication Assisted Agrobacterium-mediated Transformation) treatment of embryogenic calli and suspension cells increased transient _-glucuronidase (GUS) activity 2-6 fold. Stable integration and expression of the gus, neomycin phosphotransferase (nptII) and hygromycin phosphotransferase (hpt) genes in T0 transformants were confirmed by GUS histochemical and fluorometric assays, NPTII ELISA and Southern analysis. Fluorometry and ELISA revealed that GUS activity and NPTII protein varied between transformed T0 plants. Southern blot analysis confirmed integration of 1-2 copies of T-DNA into most of the T0 plants. Interestingly, in some transformants, the copy number of the nptII gene, present near the right T-DNA border, was less than that of the hpt gene, near the left border of the T-DNA. Transgene expression levels were not correlated with integration copy number in T0 plants. Mendelian segregation of transgenes was observed in the T1 generation.

1. M. Al-Forkan, K. Azhakanandam, M.R. Davey, J.B. Power and K.C. Lowe (1999). J. Exp. Bot. 50 (supp.), Abst. No. P2.45, p21.

S3 - 8

MOLECULAR ANALYSIS OF MUTATIONS ASSOCIATED WITH REGENERATION FROM PROTOPLASTS AND AGROBACTERIUM-MEDIATED TRANSFORMATION

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We have used a reversion assay to study (1) genomic DNA instability during regeneration from protoplasts and (2) the occurrence of mutations in a transgene during an Agrobacterium-mediated transformation procedure. The assay is based on constructs containing versions of the kanamycin resistance gene, nptII, that are translationally inactive. Activation of these genes requires a specific mutation, such as a deletion or a basepair substitution. These mutations can be selected for on kanamycin containing medium and can subsequently be analysed at the molecular level.
In vitro culture of plant cells and tissues is an environment that promotes genomic instability, a phenomenon referred to as somaclonal variation. Somaclonal variation comprises many types of genetic and epigenetic modifications, however little is known about the relative importance of the different forms of variability and about factors affecting their appearance. Our reversion assay allowed us to conclude that basepair substitutions and small deletions (1 bp to 2 kbp) occur with frequencies of several hundred per 109 bp during regeneration of tobacco protoplasts, and hence constitute a significant portion of the total somaclonal variability. Molecular analysis of those mutations shows that they probably result from reduced fidelity of DNA replication or mismatch repair, on the one hand and, and from illegitimate recombination activities, on the other hand.
Similarly, the occurrence of basepair substitutions and small deletions in internal T-DNA sequences during an Agrobacterium-mediated transformation procedure was measured. Such mutations are estimated to arise with a frequency of 10-4 to 10-5 per basepair, and would therefore only affect a minority of transgenic plants. This mutation frequency is, however, orders of magnitude higher than spontaneous mutation rates. The mutations are likely a consequence of lesions in the single-stranded T-DNA transfer intermediate or from the error-prone conversion of the T-strand to a double stranded form.

S3 - 12
TRANSGENE EXPRESSION IN WHEAT: INVESTIGATION AND IMPROVMENT

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Wheat transformation protocol by particle bombardment has been worked out and optimised allowing the regeneration of large number of independent transgenic plants. Achieving consistent and high expression level of transgenes in genetically modified crops is one of the main goals in plant biotechnology. As an attempt to improve the expression levels in transgenic plants, we are studying on "the behaviour of transgene expression". Constructs containing the Luciferase coding region (reporter gene) flanked by chicken lysozyme MAR elements, loxP recognition sites and carrying different maize introns inserted at different positions within the coding region were evaluated. In parallel, to increase expression levels, introns located at three different positions of the coding region of the Luciferase coding region were transiently tested in maize BMS cells. Although all four introns tested were correctly spliced, only one of them, located at one particular position, was able to enhance transgene expression. The Cre/loxP system showed activity in wheat embryos and at present, transgenic lines carrying the Luciferase reporter cassette flanked by loxP recognition sites, are being crossed with a wheat line containing an active site specific Cre recombinase. This recombinase will be use to reduce copy number of the transgene cassette and assess the correlation between copy number and transgene expression at known chromosomal location. The mapping of independent transgenic lines is being performed using FISH method and by crosses to 21 wheat monosomic lines. These methods will allow us to clarify the relation between gene positioning and expression levels. Crossings with a wheat winter variety (Riband) will determine if the genetic background plays any significant role in transgene expression.

S3 - 13
TRANSGENIC WHEAT (TRITICUM AESTIVUM L.) FOR IMPROVED PHOSPHATE BIOAVAILABILITY

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In cereal grains, up to 85% of the phosphate is bound in phytic acid (phytate, myo-inositol 1,2,3,4,5,6-hexakisphosphate). At germination, the phosphorus is liberated due to activity of the hydrolytic enzyme 3- and 6-phytase (myo-inositol hexakisphosphate 3- and 6-phosphohydrolase). In the dry seeds and in the digestive tract of non-ruminants animals, there is little or no phytase activity. In order to improve the nutritional value of the wheat grain, the Aspergillus niger phytase encoding gene phyA has been expressed in transgenic wheat, either under control of the constitutive maize ubiquitin-1 (Ubi-1) or the wheat seed specific high molecular weight glutenin subunit (HMW-GS) 1DX5 promoter. To ensure transport of the heterologous protein into the lumen of the endoplasmic reticulum, a 72 bp sequence for a barley alfa-amylase signal peptide was fused upstream to phyA. Ubi-1 controlled expression led to strong accumulation of heterologous protein in leaves and seeds while the HMW-GS 1DX5 promoter resulted in Aspergillus phytase accumulation exclusively in the endosperm. Purification and N-terminal sequencing of heterologous protein indicates that the signal peptide allowed processing of the heterologous protein via the default secretory pathway. An up to 3-fold increase in phytase activity was observed in flour made from transgenic seeds. The effect of hetrologous phytase accumulation on seed myo-inositol phosphate composition was examined in a time study. Metal dye detection HPLC revealed that after 50 min of incubation, heterologous phytase had caused a 60-70% reduction of the phytate level and a complete elimination of myo-inositols with a lower degree of phosphorylation (penta-, tetra-, tri- di- and monophosphate). In comparison, wild type seeds showed only limited (ca. 10%) reduction of the phytate level accompanied by an increase in the level of the less phosphorylated myo-inositols.

S3 - 34
DEVELOPMENT OF EFFICIENT REGENERATION AND TRANSFORMATION SYSTEMS IN EGYPTIAN MAIZE LINES.

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A number of elite Egyptian maize lines (Zea mays L.) were screened for callus induction capability on two different culture media using immature embryos as explants. Mainly compact type I callus was produced. Modification in both callus induction media resulted in the formation of embryogenic calli and the improvement of regenerability.
To overcome the genotype restriction of callus induction, another system based on the formation of multiple shoot meristems was adopted. Although this system was reported as being non-genotype specific, variation in the ability and rate of shoot multiplication was observed among the lines surveyed. Line Gz 643 was identified as the best line revealing the highest number of multiple shoots, type II callus and regeneration frequency.
Transformation of both explants was performed with the particle delivery system using the GUS and Bar genes in a single plasmid (pAB-6) or by co-transformation with two plasmids (pAct1-F and pTW-a). Different transformation parameters were used, i.e., osmotic treatment, acceleration pressure and number of shots. For immature embryos, osmotic treatment along with the use of acceleration pressure 1300 psi and two shots gave the best results as expressed by the number of blue spots in the GUS assay. The use of acceleration pressure 1550 psi and four shots was the best for transformation of the shoot clumps. Stable transformation was confirmed in R0 transformed plants by means of histochemical GUS assay and herbicide application. Moreover, PCR and Southern blot analysis proved the integration of the full-length genes in some of the transgenics.

S3 - 38
CONTROL OF GENETIC TRANSFORMATION OF TOBACCO BY LIGHT

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We demonstrate that genetic transformation of tobacco by Agrobacterium tumefaciens is inhibited by light, and that transformation efficiency can be increased by a period of darkness. The effect of light on the process of genetic transformation of tobacco (Nicotiana tabacum cv Samsun NN) by Agrobacterium tumefaciens strain EHA105 was studied using conventional methods, and selection on 250 mg/l kanamycin. When leaf explants from greenhouse-grown plants are incubated continuously in darkness (except for transfers and other treatments) a large number of independent shoots (13-15) are recovered from each plate, compared to the 2-5 independent shoots per plate recovered following treatment in a 16h photoperiod of white fluorescent light. A large proportion of the shoots regenerated in darkness failed to root (40-60%) subsequently in light on kanamycin-containing medium, as compared to 20-30% of shoots derived from light-grown cultures. However, 7-10 shoots per plate still root from dark treatments, compared to 2-3.5 shoots per plate from light treatments. The population of rooted plants was screened for expression of the GUS-intron gene and for the NPTII gene by PCR. Rooted non-transgenic escapees are always found among dark-recovered plants at this stage, but rarely from light-grown explants. Despite the reduction in efficiency due to shoots not rooting, and the production of non-transformed escapees, the transformation rate of tobacco is increased 2- to 5-fold by incubation in darkness, instead of the usual white fluorescent photoperiod. We have identified that the maximal sensitivity to light is the period of 2 days of co-cultivation with Agrobacterium tumefaciens. The shorter dark treatment also reduces the problem of rooted non-transgenic escapees to a level proportional to that of the illuminated control. We are now examining the photobiology of the inhibition of tobacco transformation, to establish which photoreceptor(s) control the inhibition of the transformation.

S3 - 39
PLANT GENES INVOLVED IN AGROBACTERIUM-MEDIATED TRANSFORMATION

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We have screened Arabidopsis T-DNA tagged mutants for mutants that are resistant to Agrobacterium transformation (rat mutants). rat mutants constitute approximately 0.7% of the approximately 9000 mutants screened to date. These mutants are characterized by an extremely low transformation rate as assayed by crown gall tumorigenesis, transformation to phosphinothricin-resistance, and transformation to yield stable GUS expression. Most mutants are recalcitrant to transformation to express GUS activity transiently. However, at least 5 mutants can be transformed to yield high levels of transient GUS expression. This latter class of mutants is likely blocked at the T-DNA integration step. In most mutants, the T-DNA co-segregated with the resistance phenotype. Almost all mutants investigated appeared dominant or semi-dominant. However, other data suggest that these mutants are actually haplo-insufficient. We cloned T-DNA/plant DNA junctions from nine of the rat mutants and identified corresponding cDNA and genomic clones for many of them. In all cases investigated, the T-DNA did not integrate into the structural gene, but rather integrated into the 5Ô or 3Ô untranslated regions of the gene. Rat1 encodes an arabinogalactan protein (AGP). Using beta-glucosyl Yariv reagent, we showed that AGPs are necessary for Agrobacterium transformation. Rat3 likely encodes a cell wall protein. Both the rat1 and rat3 mutants are deficient in binding Agrobacterium to Arabidopsis roots, and are hyper-susceptible to infection by Pseudomonas syringae pv. tomato. Rat4 encodes a cellulose synthase-like protein. The rat5 and rat17 mutants are deficient in T-DNA integration. Rat5 encodes a histone H2A protein, and is one of a 6-member histone H2A gene family. Rat17 encodes a myb-like transcription factor. The rat17 mutant is allelic to the caprice (cpc) mutant, and shows altered root hair development. Although all rat mutants identified so far are resistant to root transformation, they all can be transformed efficiently by vacuum infiltration of flowers.

S3 - 44
TRANSFORMATION AND REGENERATION OF LOBLOLLY PINE: USING SHOOT APEX INOCULATION WITH AGROBACTERIUM

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Loblolly pine (Pinus taeda L.) is the most important commercial tree species in the US. This and other pine species can be transformed using particle bombardment and Agrobacterium; however, regeneration of loblolly tissue is difficult and recovery of transgenic plants of this species has been rare. A genotype-independent, shoot-based transformation method using Agrobacterium was used with P. taeda to facilitate

recovery of plants and to allow transformation of elite germplasm. Shoots isolated from 4-6 week old seedlings, and adventitious shoots, were inoculated with either A. tumefaciens EHA101 (pGUS3), or EHA105 (pSSLa.3), subjected to kanamycin selection and regenerated. Shoots that survived selection exhibited expression of the transferred uidA gene that was typical of either the CaMV35S(pGUS3), or larch RbcS(pSSLa.3) promoter used. Recovered plants were screened by PCR for the transferred uidA and nptII genes, as well as for picA and virG of Agrobacterium. Southern analyses confirmed genomic integration of the transferred uidA and nptII genes, and absence of picA and virG. Shoot-based transformation and regeneration methods offer a genotype-independent approach to pine transformation that is not possible with callus-based methods, or by somatic embryogenesis, and can be used with seedlings identified through marker-aided selection. Overall recovery of intact rooted plants of P. taeda following selection was low (0.9%), but recovery of P. eldarica and P. radiata plants was more efficient (10-20%). The addition of improved Agrobacterium virulence, shoot multiplication steps and effective rooting protocols would improve the recovery of transgenic plants of P. taeda and other pines from inoculated shoots

S3 - 49

INFLUENCE OF EXPLANT AGE AND GENOTYPE ON RATES OF TRANSIENT TRANSFORMATION IN BRASSICA OLERACEA.

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Previously we established optimum bombardment conditions for cotyledon explants of B. oleracea cv. Marathon (Puddephat et al 1999). Using these conditions the influence of explant age and plant genotype on rates of transient transformation was investigated using the Bio-Rad Helium-driven PDS-1000.Transient transformation events were followed using the gus reporter gene system and visualised by histochemical staining of cells. Rates of transient transformation were compared in cotyledon discs excised from seedlings (cv Marathon) aged between 8-15 days. Explants from younger donor seedlings gave significantly ($p<0.05$) higher rates of transformation than those excised from older material. Rates of transient transformation were greatest in explants excised from 8-days-old seedlings. A linear decline in rates of transient transformation was observed in explants taken from seedlings aged between 8 and 12 days old, which reached a plateau in explants excised from seedlings aged 12 - 15 days old. Rates of transient transformation were compared in six different brassica genotypes, representative of the crop morphotypes cabbage, broccoli, cauliflower and Brussels sprout. Rates of transient transformation were found to vary in different genotypes but these differences were not significant. Common protocols for Agrobacterium-mediated transformation of Brassica oleracea have not been developed to date due to pronounced genotype x bacterial strain interactions. The results of this study support the proposition that direct gene transfer methods are less genotype specific than Agrobacterium-mediated transformation.
Reference:
Puddephat IJ, Thompson N, Robinson HT, Sandhu P and Henderson J (1999): Biolostic transformation of broccoli (Brassica oleracea var. italica) for transient expression of the bÐglucuronidase gene. Journal of Horticultural Science and Technology 74: 714 Ð 720.

S3 - 51

SIMULTANEOUS DOWN-REGULATION OF TWO RELATED ENZYMES IN EARLY STEPS OF THE POLYAMINE BIOSYNTHETIC PATHWAY IN TRANSGENIC RICE BY A SINGLE ANTISENSE mRNA

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Transgenic rice cell lines engineered with the heterologous oat arginine decarboxylase cDNA in an antisense orientation exhibited significant ($P < 0.05$) down-regulation of enzyme activity for both endogenous arginine and ornithine decarboxylase enzymes compared to wild type and hpt-transformed controls. Changes in enzyme activity translated to a direct decrease in the level of putrescine ($P < 0.001$) and spermidine ($P < 0.01$) but not spermine ($P > 0.01$) in the majority of cell lines we analyzed. Consistent with previous results, we confirmed that cell lines with low levels of polyamines exhibited normal morphogenic responses. In vegetative tissue at the whole plant level no significant variation ($P > 0.05$) in polyamine levels was observed. However, we measured significant reductions ($P < 0.001$) in putrescine levels in seeds derived from three out of five plants we analyzed. We report that a single molecular species can down-regulate simultaneously two alternative enzymes in the polyamine pathway resulting in significant reduction in end product accumulation in seeds of transgenic plants.

S3 - 52

ANTIMICROBIAL GENE, Asa-AMP FROM GARLIC AND ITS EXPRESSION IN TRANSGENIC RICE

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Non-specific lipid transfer proteins (ns LTPs) are one of the most abundant protein in plants. The plant LTPs have some common properties briefly that LTPs can bind and transfer phospholipids between membranes in vitro. Moreover certain LTPs are reported to have antimicrobial activity and to be responsive to abiotic stresses such as cold, drought and salt.
In trying to screening useful disease-resistance gene, an antimicrobial protein gene (Asa-AMP) was cloned from garlic (Allium sativum). Determination of the DNA sequence indicated a 396-nucleotide open reading frame which encodes a 132 amino acids including 27 signal peptide on N-terminus and 12 propeptide on C-terminus. It has high homology, 88% in amino acid sequence, with Ace-AMPs [Plant Physiol. 109:445-455], which suggest very similar protein structure. They are likely to be counter parts in each plant which are closely related in Allium species. Both AMPs belong to plant lipid transfer protein family based on the sequence homology. But unlikely other known LTPs, both AMPs have additional propeptide in C-terminal. So these proteins might go into secretory pathway and be sorted to vacuole. But the exact sorting system remains to be solved. Ace-AMP1 was known to have broad resistant spectrum to phytopathogenic fungi in vitro, which implicate similar activity for Asa-AMP from high sequence homology. However the confirmation of antifungal activity of both AMPs in vivo needs transformation.
In this study we transformed rice with the gene using Agrobacterium. To transfer Asa-AMP into rice, plasmid vector was constructed with MAR sequence of chicken lysozyme gene locus. Transgenic plants were selected by herbicide, phospinothricin, resistance. Genomic Southern blot and Northern analysis shows its integration into rice genome and expression. MAR sequence appeared to affect the

integration and expression pattern by lowering its copy number and copy number dependent expression. Some transgenic plants showed fertility and produced seeds. Disease resistance is supposed to be tested in next generation.

S3 - 56
GENETIC TRANSFORMATION OF MAJOR WINE GRAPE CULTIVARS OF VITIS VINIFERA

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As part of a significant effort to develop genetic engineering strategies for the improvement of grapevine, we have developed an Agrobacterium-mediated transformation system for a number of important grapevine cultivars used in wine production. Embryogenic callus was initiated from anther filaments from nine cultivars and a clear genotype-media interaction was observed for both initiation and proliferation. Transgenic plants were obtained for the seven cultivars: Cabernet Sauvignon, Shiraz, Chardonnay, Riesling, Sauvignon Blanc, Chenin Blanc and Muscat Gordo Blanco. The transformation system allowed the recovery of germinated transgenic embryos 10-12 weeks after Agrobacterium inoculation and plants within 18 weeks. Examination of the expression patterns of the green fluorescent protein gene from the CAMVS promoter in leaf tissue of transgenic plants showed that for up to 35% of plants the pattern was not uniform suggesting silencing or lack of activation of the gene in some cells. The successful transformation of a genetically diverse group of wine grape cultivars indicates that the transformation system may have general application to an even wider range of Vitis vinifera cultivars.

S3 - 57
THE EFFECT OF MATRIX ATTACHMENT REGIONS ON TRANSGENE EXPRESSION LEVELS AND STABILITY IN RICE

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The transformation of plants is an important tool for both molecular genetic studies and crop improvement strategies. Particle gun bombardment is the most widely used technique for transforming cereals, but despite its efficiency, often produces complex integration patterns and transgene rearrangements. This can be associated with variability in transgene expression levels and stability. Flanking trangenes by matrix attachment regions (MARs) has been shown to stabilise expression in tobacco and rice primary transformants (T0) and their progeny (T1), possibly by reducing the effects of gene silencing.
In this work, homozygous rice lines containing a CaMV 35S - gusA expression unit, either unflanked or flanked by tobacco Rb7 MARs or yeast ARS1 MARs, were obtained from transformed rice plants produced previously (Vain et al., 1999, Plant Journal 18: 233-242). Transgene expression levels and stability in these plants have been monitored in 27 independently transformed lines, up to the T5 generation. The presence of flanking MARs appears to stabilise transgene expression levels when compared to lines with no MARs, across several generations.

S3 - 61
GENETIC TRANSFORMATION OF VITIS VINIFERA L. GRAPEVINES WITH GENES ENCODING CHITINASES AND ANTIMICROBIAL PEPTIDES.

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Vitis vinifera grapes, highly prized for fine wine production, are plagued in many growing regions all over the world by the fungal diseases powdery mildew, downy mildew, and Botrytis bunch rot. Resistance of grapevines to fungal pathogens would reduce yield losses and cut the number of fungicidal applications. Using genetic engineering methods, elite grapevine cultivars may be enhanced for disease resistance while retaining other beneficial characters of the genotype. Our laboratory has developed a biolistic transformation system for grapevines that employs highly regenerable embryogenic suspension cultures (Kikkert et al. 1996, Plant Cell Rep. 15:311-316). Using the biolistic system, transgenic plants of V. vinifera L. 'Merlot' and 'Chardonnay' which express a chitinase gene from the biocontrol fungus Trichoderma harzianum were produced. A total of 124 Merlot and 93 Chardonnay putatively transformed plants were regenerated and evaluated for expression of chitinase using a fluorometric assay and Western blot analysis. About 43% of the Merlot and 60% of the Chardonnay regenerates had 10- to 100-fold higher chitinase activity than non-transformed control plants. Transformation was further confirmed by PCR and Southern blot analysis. Several of the Chardonnay lines appeared more tolerant to Botrytis (B. cinerea) infection in detached leaf assays, and to powdery mildew (Uncinula necator) in preliminary field tests. Of the 20 transgenic Merlot lines tested, none were more tolerant to powdery mildew in either greenhouse tests or early field trials. Transformation experiments currently underway are aimed 1) to improve selection of transformants by comparison of paromomycin and kanamycin at different concentrations, and 2) to introduce antimicrobial peptides of the magainin family into grapevines, alone or in combination with genes encoding chitinolytic enzymes. Gene pyramiding may provide for broader spectrum disease resistance and reduce the chance that pathogenic organisms will overcome the resistance.

S3 - 66
TRANSFORMATION OF TOBACCO CELL SUSPENSION CULTURE VBI-0 VIA AGROBACTERIUM

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Tobacco cell suspension culture VBI-0 exhibits a specific filamentous phenotype represented by chains of unseparable cells. Therefore, a simple cocultivation with Agrobacterium does not lead to single-cell-derived transgenic line. In order to obtain transgenic lines expressing

various genes involved in hormonal regulation of plant development, we have optimized methods of Agrobacterium-mediated protoplast transformation and subsequent regeneration.
Plant transformation vectors derived from pBIN19 have been used, carrying genes abp1 (auxin binding protein) from tobacco, chax1 from cherry tree (cherry tree cDNA homologue of aux1 gene from Arabidopsis, coding for auxin uptake carrier) and ipt (izopentenyl transferase) oncogene from Agrobacterium tumefaciens, all driven by CaMV35S promoters.
Physiological characteristics of VBI-0 strain will be discussed in context with optimal conditions for transformation and regeneration.

S3 - 67
ENGINEERING RESISTANCE IN ELITE INDICA RICES OF TAMIL NADU TO MAJOR DISEASES

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Putative transgenic lines of an indica rice cultivar, Pusa Basmati1 were evolved by an Agrobacterium-mediated transformation protocol using suspension cells or scutellum-derived calli as explants. A rice chitinase gene, chi11 (Courtesy: S. Muthukrishnan, Kansas State University, Manhattan, USA) which was known to confer resistance against Rhizoctonia solani, the rice sheath blight pathogen was cloned into an Agrobacterium binary vector, pCAMBIA1301 which was subsequently mobilized into EHA105. Another construct (pJRB3) harbouring a CaMV 35S promoter-chi11-NOS terminator cassette mobilized into LBA4404 was also used in the present study. Putative transgenic rice lines of Pusa Basmati1 expressing chi11 were evolved and expression of chi11 gene confirmed by Western blot analysis. Elite indica rice varieties of local importance, ASD16, ADT38, IR50 or White Ponni failed to produce any stable transformants expressing chi11.
Xa21 gene (Courtesy: Pamela C. Ronald, University of California, Davis, USA), the rice chitinase gene (chi11) and thaumatin-like protein (TLP; a gift of S. Muthukrishnan, Kansas State University, Manhattan, USA) gene were used as candidate genes against these diseases and elite indica cultivars namely, White Ponni, ASD16, IR50 and ADT38 were transformed using these genes in separate Biolistic experiments. Several transgenic rice lines evolved in the present study were analyzed for transgene expression. Polymerase chain reaction/Southern hybridization analyses for detecting incorporation of transgenes, chi11, gusA and hph proved positive. Molecular analyses of other transgenes in putative transgenic lines are in progress. Testing reaction of these lines for resistance/susceptibility to the pathogens is underway.

S3 - 73
USING MATRIX ATTACHMENT REGIONS TO IMPROVE HOMOLOGY-DEPENDENT VIRUS RESISTANCE

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Homology-dependent virus resistance is a type of transgenic resistance in which a plant is transformed with a viral gene. Although this may provide high resistance to a plant virus, this resistance can be unstable in later generations, possibly due to transcriptional silencing of the transgene. We are working to determine if the placement of matrix attachment regions (MARs) around the viral construct will help stabilize resistance. MARs are DNA elements that can increase gene expression and stabilize expression in later generations when placed around a transgene. We have transformed tobacco with tomato spotted wilt virus (TSWV) nucleocapsid gene constructs with or without flanking MARs. In the R1 generation, the MAR population has a higher percentage of highly TSWV resistant lines than the nonMAR population. When lines with two transgene copies are compared, the majority of MAR lines show no symptoms of TSWV, while the majority of nonMAR lines show local lesions and later recover from TSWV. Therefore, MARs appear to provide a higher level of resistance when lines with two copies are compared. The copy number of lines with high resistance (no TSWV symptoms) has also been examined. The majority of highly resistant MAR lines have only two copies, while the majority of highly resistant nonMAR lines have three or more copies. MARs appear to provide a high level of resistance with fewer copies than lines without MARs. A 1999 field trial in Tifton, GA showed that MAR and nonMAR lines that have high resistance in greenhouse tests also have high resistance to TSWV under field conditions. The MAR and nonMAR populations will be tested in the R4 generation to determine if the presence of MARs prevents loss of TSWV resistance in later generations.

S3 - 75
ENHANCED COMPETENCE OF SOMATIC EMBRYOGENESIS BY THE EXPRESSION OF A SMALL SUBUNIT CDNA IN AN ANTI-SENSE ORIENTATION IN SWEET POTATO

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ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.27) plays a key role to regulate starch biosynthesis in higher plants. It catalyzes the synthesis of ADP-glucose and PPi from glucose-1-phosphate and ATP. ADP-glucose is then used as glucosyl donor in the biosynthesis of starch. A small subunit AGPase cDNA from sweet potato (sTL1) was introduced into embryogenic cells of sweet potato in an anti-sense orientation by Agrobacterium-mediated genetic transformation. Transformed cells regenerated into whole plants. Leaf explants from transgenic plants and from control plants were cultured on MS medium supplemented with 1Êmg/L 2,4-dichlorophenoxyacetic acid.
After six weeks of culture, leaf explants from transformed plants gave rise to embryogenic calluses and somatic embryos at a frequency of up to 95% whereas those from control plants did not produce. The frequency of embryogenic calluses and somatic embryos from leaf explants was proportional to the mRNA level of the sTL1 in explants. It is suggested that probably elevated internal osmolarity of glucose in leaf explants causes enhanced competence of somatic embryogenesis.

S3 - 81
CHROMATIN ELEMENTS AND TRANSGENE EXPRESSION IN TRANSGENIC TOBACCO CELLS

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We have tested the effect of SCS elements on transgene expression in tobacco cells using a variety of transformation methods. The Drosophila insulator elements SCS and SCS' elevate transgene expression 5-17 fold in tobacco cells transformed by microprojectile bombardment but not in cells transformed by Agrobacterium. In bombarded cells there was also a 3 fold increase in the percentage of transformants expressing detectable amounts of luciferase. We have also analyzed the interaction of the RB7 MAR with six different promoters in tobacco cell cultures. When transgenes are flanked by the RB7 MAR, the number of non-expressing transformants recovered was reduced. The constitutive promoters CaMV 35S, NOS and OCS all showed significant increases in gene expression with MAR-flanked transgenes. However, the RB7 MAR did not cause expression from either the pea ferredoxin promoter, normally not expressed in NT1, or a soybean heat shock promoter in the absence of induction. However, expression form an induced heat shock promoter was increased relative to control, unflanked genes.

S3 - 85
MODULATION OF POLYAMINE SYNTHESIS IN TRANSGENIC MUSTARD PLANTS EXPRESSING ANTISENSE SPERMIDINE SYNTHASE RNA

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The enzyme spermidine synthase (SPDS, EC 2.5.1.16) is responsible for the synthesis of spermidine (SPD) that, together with putrescine (PUT) and spermine (SPM), is the major polyamine (PA) in plants. As part of effort to elucidate the regulatory mechanism of PAs on plant growth and development, the main objective in this study was to investigate the feasibility of PA modulation by downregulation of SPDS expression in transgenic mustard plants expressing antisense SPDS RNA. We have previously cloned a SPDS cDNA (MSPDS1) from mustard. The cDNA was inserted in an antisense orientation into the binary vector pBI121 between the CaMV 35S promoter and the GUS coding sequence. The vector was mobilized into Agrobacterium tumefaciens LBA4404, which was used for mustard transformation. Several putative transformed green shoots regenerated from explants grown on culture medium containing 20 mg/l kanamycin were selected and verified by Southern analysis. Three transgenic plants (TR1, TR2 and TR3) carrying the chimeric gene consisting of antisense SPDS cDNA were selected for further study. Tissue analysis for the PA content showed that the levels of both conjugated and free PUT in all transgenic plants, except free PUT in TR3, were significantly higher than those in the control tissue. In TR1, synthesis of free SPD appeared to be inhibited, whereas the amount of conjugated SPD in TR2 and TR3 was higher. With respect to SPM, the conjugated form in all transgenic plants was higher but the free form was lower. Apart from the PA content, our preliminary results also showed that transgenic tissues grown in culture formed roots earlier and more abundant than the control counterpart, suggesting that PAs may be involved in rhizogenesis.

S3 - 86
A SYSTEM FOR THE HIGH-LEVEL EXPRESSION OF RECOMBINANT PROTEINS IN PLANTS

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Bean Yellow Dwarf Virus is a monopartite geminivirus that can infect dicotyledonous plants. We have developed a high-level expression system that utilizes elements of the replication machinery of this ssDNA virus. The replication initiator protein allows high-copy replication of replicons that contain plant expression cassettes for a gene of interest flanked by cis-acting elements of the virus. Using this system we have obtained up to 30 fold increase in expression levels.

S3 - 93
CLONING AND EXPRESSION OF HUMAN CALCITONIN GENE IN TRANSGENIC POTATO PLANTS

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Synthetic hCT monomeric (hCTm) and tetrameric(hCTt) genes were cloned under the control of the 35S cauliflower mosaic virus (CaMV) promoter linked to the tobacco etch virus (TEV) 5í-nontranslated leader sequence acting as a translational enhancer. The resulting constructs were cloned into the binary vector Bin19 and potato minituber discs were then transformed using an Agrobacterium strain. Transformants were selected by their resistance to kanamycin and Northern dot-blot and RT-PCR were applied for monitoring of transcription. Translation of the hCTm and hCTt mRNAs was studied by immunobloting and radioimmunoassay and molecular size of the recombinant proteins was determined by polyacrylamide-SDS gel electrophoresis. The estimated average yield of recombinant hCT in the transgenic potato plants was about 0.02% of the total soluble protein.

S3 - 94
CHEMICAL INDUCIBLE SYSTEM TO ACTIVATE OR INACTIVATE PLANT GENE EXPRESSION

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An inducible system to activate or inactivate plant gene expression has many potential applications in basic understanding of gene function, in manipulating complex developmental pathways, and in plant biotechnology. Rohm and Haas company is developing an inducible system with potential for field application using spruce budworm ecdysone receptor (EcR) and non-steroidal ecdysone agonists. Chimeric transcription factors were made using different DNA binding and activation domains and EcR ligand binding domain. Reporter gene or gene of interest was cloned behind a DNA element where chimeric EcR transcription factor can bind. Such transcription factor does not activate or inhibit transcription in the absence of a ligand. Addition of Rohm and Haas ligand, which has an exceptional health and environmental safety profiles, activates or inhibits the transcription of the gene(s) of interest. We used our system in transient assays with tobacco protoplasts and in tobacco and Arabidopsis plants. Results show that Rohm and Haas GSTM system based on EcR is very effective and can be used to express high levels of protein. Data from transient and transgenic experiments will be presented.

S3 - 96
EXPRESSION OF A GENE ENCODING A SYNTHETIC ANTIMICROBIAL PEPTIDE CONFERS FUNGAL RESISTANCE IN VITRO AND IN PLANTA IN TRANSGENIC PLANTS

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Plants produce, either preformed or in response to microbial invasion, cysteine-rich antimicrobial peptides similar to animals and insects. These peptides have been shown to be effective against a wide range of microbes due to their lytic properties or interference with synthesis of cell wall constituents. Compared to naturally occurring antimicrobial peptides, synthetic analogs of natural antimicrobial peptides offer more target specificity, increased efficacy at lower concentrations, and reduced degradation by plant proteases. We transformed tobacco and cotton with a gene encoding a synthetic, linear antimicrobial peptide, D4E1, which has a broad-spectrum activity against several bacterial and fungal phytopathogens and is fairly resistant to degradation by fungal and host proteases. Crude protein extracts from leaf tissue of transgenic tobacco plants expressing D4E1 significantly reduced the number of fungal colonies arising from germinating conidia of Aspergillus flavus and Verticillium dahliae by upto 75% and 99%, respectively, compared to extracts from plants transformed with pBI 121. Compared to negative controls, tobacco plants expressing the D4E1 gene showed greater levels of disease resistance in planta to the fungal pathogen, Colletotrichum destructivum, which causes anthracnose. A parallel experiment with immature cottonseeds from transformed R0 cotton plants showed a similar inhibition in vitro of germinated conidia of A. flavus andV. dahliae.
Remarque: essai preliminaire sur culture cellulaire resistant au champignon

S3 - 103
GROWTH MODULATION OF TRANSGENIC POTATO PLANTS BY CBD

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Cellulose binding domain (CBD) derived from the cellulolytic bacterium Clostridium cellulovorans was found to modulate the growth of different plant tissues in vitro in a concentration dependent manner. Transgenic potato plants expressing the CBD under the control of the CaMV 35S or the Arabidopsis thaliana endo-1,4-b-glucanase (CEL1) promoter were employed to further explore the effect of this protein on plant development. Binary expression vectors containing the respective promoters, a signal peptide to direct the expressed protein to the cell wall, and the CBD gene were inserted into potato plants using an Agrobacterium mediated transformation. More than 30 independent transgenic plants were identified displaying various copy number. The different level of expression was verified by Western blot analyses. Growth analyses of whole plants grown under greenhouse conditions were performed on R1 and R2 generations. These measurements included plant height, node and shoot number, leaf weight, leaf area, tuber weight and distribution of tuber size. In addition, tuberÕs starch and sugar content as well as level of compression were used as parameters to characterize their quality. Several transgenic lines exhibited a significant higher elongation rate of the main stem and longer nodes as compared with the control line. This effect was often associated with higher leaf area and biomass production. The results are discussed in terms of the mode(s) by which the CBD exerts its influence over the growth rate of specific tissues and the development of the whole plant.

S3 - 104
TARGETED EXPRESSION OF ANTIFUNGAL PROTEIN GENES IN BARLEY

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Fungal diseases continue to cause heavy losses in barley and wheat crops. Aided by climatic conditions and the adoption of no-till agriculture, Fusarium graminearum, and its associated mycotoxins, has emerged as a major threat to food, feed and beverage products. We are trying to produce Fusarium-resistant barley by redirecting the expression of a native thionin gene so that it is expressed in the path of Fusarium invasion. For this purpose, we are developing tissue-specific gene promoters and subcellular targeting strategies. The goal is to express thionin specifically in the extracellular space of the lemma/palea and pericarp. The differential display method was used to produce clones for genes expressed in either the lemma/palea or in the pericarp but not in vegetative leaves. These were used as probes to locate BAC clones (A. Kleinhofs, Washington State U.), from which regions upstream from coding sequences were isolated. These promoter-containing sequences were fused to a sgfp reporter gene in pAHC17 and pAHC25 derivatives and used in transient expression assays with

the appropriate barley tissues. The promoter from an unknown gene, designated D5, was used to drive sgfp expression in the lemma/palea of young spike tissue; virtually no expression occurred in leaves. Stable transformation to test this promoter in intact barley is underway. For subcellular targeting, thionin was fused to targeting signals, cloned into pAHC25, and used in particle bombardment experiments to achieve transient expression in etiolated barley coleoptiles. The signals were designed to compete with the (as yet undefined) vacuolar and protein body targeting signal(s) in the native protein. Secretion of GUS activity has been achieved in cells that were judged to be intact by having no secreted malate dehydrogenase activity.

S3 - 107
PRODUCTION OF HUMAN GM-CSF USING TRANSGENIC PLANT CELL CULTURE

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We tested if a disease therapeutic cytokine, human GM-CSF (hGM-CSF), could be produced through plant cell suspension culture as a biologically active form. Initially, we cloned hGM-CSF gene from PHA and hIL-2 stimulated PBMC. The gene was sub-cloned into the plant expression vector, pMY27, and Ti-plasmid mediated transformation of tobacco leaves was conducted by using Agrobacterium tumefaciens harboring the hGM-CSF gene. Cell suspension culture was established from the leaf-derived calli of transgenic tobacco plant and integration of the introduced hGM-CSF gene into plant chromosome was confirmed through genomic PCR by using specific primers. In addition, Northern blot analysis indicated the expression of the introduced hGM-CSF gene in cultured plant cells. Biological activity of the hGM-CSF from plant cell suspension culture was confirmed by measuring the proliferation of the hGM-CSF dependent TF-1 cells. (Travel expenses for this presentation has been supported by BK21 program from Korean Ministry of Education)

S3 - 113
THE GREEN FLUORESCENT PROTEIN AS A VITAL SCREENABLE MARKER FOR TRANSFORMATION OF THE C3-C4 INTERMEDIATE SPECIES MORICANDIA ARVENSIS (BRASSICACEAE).

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A modified form of the green fluorescent protein (GFP) from the jellyfish Aequorea victoria was used to develop an efficient and rapid transformation system for the C3-C4 photosynthetic intermediate species Moricandia arvensis (Brassicaceae) via Agrobacterium-mediated transformation of stem internodes and leaf explants. The cauliflower mosaic virus (CaMV) 35S promoter::mgfp4-ER gene construct (Haseloff et al., 1997, Proc. Natl. Acad. Sci. USA 94, 2122-2127) produced bright green fluorescence in sectors of developing calli and in emerging shoots. Visual screening of transformed tissue, combined with antibiotic selection, enabled early identification of transformed plants in a non-destructive manner and contributed to improvements in the transformation technology. A wide range of GFP fluorescence intensities was observed in leaves, roots, flowers and flower buds of primary transformed plant lines. In many plant lines, GFP fluorescence was clearly visible by eye, using a hand-held long-wavelength UV lamp. The mgfp4-ER expression levels in about 100 independent M. arvensis plant lines are currently being characterized. The high efficiency and reliability of the regeneration and transformation procedure was also confirmed by stable transformation of M. arvensis with a CaMV 35S promoter-driven _-glucuronidase-intron construct. Further, the results show that this transformation technique can be utilized in promoter studies aimed at the further understanding of the C3-C4 intermediate photosynthetic phenotype.

S3 - 116
DIFFERENCES IN SPATIAL AND TEMPORAL REGULATION OF TRANSGENE PROMOTER ACTIVITY CONTRIBUTE TO THE POSITION EFFECT OF TRANSGENES

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Quantitative differences in transgene expression between individual transformed plants are generally ascribed to different integration sites of the transgene and is referred to as 'position effect'. With the firefly luciferase (luc) reporter system we characterised the origin of these position induced differences in transgene expression by monitoring both spatial and temporal aspects of transgene promoter activity in planta. The activity of three different promoters (Cauliflower Mosaic Virus (CaMV) 35S, modified CaMV 35S and the promoter of an Arabidopsis thaliana Lipid Transfer Protein gene) was shown to vary not only among independent transformants, but also between leaves on the same plant and within a leaf. We confirmed that the differences in local LUC activity in a leaf correlates with differences in local luc mRNA steady state levels. Expression analysis of selected endogenous plant genes indicated that some (endo)genes show a variegated expression that coincides with the LUC variegation. This observation implies that this variegation is caused by differences in transcriptional activity that also affects some plant genes. By imaging the LUC activity in the same leaves over a 50-day period, we show that individual transformants show different types of temporal regulation. Both the spatial and the temporal type of luc transgene expression are inherited to the next generation. Over prolonged periods of time many of the variations in (trans)gene promoter activity within a tissue may be cancelled out at the level of the final gene product, due to the shifts in spatial expression and depending on the stability of the gene-product. Our results show that transgene promoter activity can be very dynamic and that the differences in transgene product steady-state levels in leaves is the accumulated result of differences in spatial and temporal regulation of transgene promoter activity in independent transgenic lines.

S3 - 117
STRATEGIES TO OPTIMIZE CRE-MEDIATED TARGETED INTEGRATION OF AGROBACTERIUM T-DNA IN PLANTS.

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Integration of transgenes in the plant genome occurs at random positions by illegitimate recombination. This causes variation in transgene expression level between independent transformants (position effects) by the difference in chromosomal context. Targeted integration could

avoid this and therefore would be desirable for the introduction and acceptance of genetically modified crops. Since introduction of transgenes via homologous recombination is highly inefficient in plants, a strategy for the site-specific integration of transgenes using Cre recombinase was developed. Cre can mediate recombination between two DNA recognition sequences (lox) and allows to target a lox-containing plasmid to a lox site, previously introduced in the genome. In mammalian cells it was shown that targeted integration mediated by Cre-lox resulted in reproducible transgene expression levels. Since a frequently used method for the introduction of transgenes in plants makes use of Agrobacterium, we analyzed the possibility to target T-DNA to a specific location. Earlier we showed that Agrobacterium T-DNA could be directed to such a target lox sequence in the genome of Arabidopsis. The efficiency was sufficiently high (2% of random integration), but the method used to prevent the reversible excision event (by preventing cre expression after the integration event) was not completely tight. Thus, we aimed to introduce mutant lox sites, selected for their inability to recombine with wildtype loxP sites. By using a recombinase-mediated cassette exchange strategy (RMCE) we attempted to optimize the site-specific integration of T-DNA. In our hands the mutant lox sites (lox511 as well as lox5171) are not as recombination deficient as reported in the presence of Cre enzyme. Furthermore, we analyzed whether it would be possible to reduce the number of random integration events occurring simultaneously with site-specific integration events. Here we report about the recent results we obtained and discuss the relevance for use in targeted integration strategies.

S3 - 119

TRANSGENIC PLANTS FOR THE DELIVERY OF VACCINES: EXPRESSION OF HUMAN PAPILLOMAVIRUS TYPE 11 (HPV-11) L1 CAPSID PROTEIN IN TOMATOES

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Molecular farming, the production of recombinant proteins in plants, is coming of age. In addition to the production of therapeutic proteins, plants can be engineered as production and delivery systems for vaccines. Expression of subunit vaccine proteins in transgenic plants offers many potential advantages including convenience of oral delivery of edible plant material and no need for expensive cell culture.
For the present study we have chosen the human papillomavirus type 11 as a vaccine candidate. Infection with certain human papillomaviruses (HPV) can cause invasive cervical carcinoma and other anogenital malignancies; thus an effective vaccine may reduce the incidence of these diseases. Virus-like particles (VLPs) formed by self-assembly of HPV L1 major capsid proteins are good vaccine candidates. We have expressed native HPV-11 L1 protein in tomato plants, and have also generated a synthetic plant-optimized version of the HPV-11 L1 gene in order to increase expression levels. In this report we compare expression levels of native and synthetic HPV-11 L1 genes, and evaluate the efficiency of VLP formation in plants.

S3 - 120

THE CAUSES OF TRANSIENT EXPRESSION IN TOBACCO

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Since the first descriptions of transient expression in plant cells, assumptions about the underlying processes have gone unchallenged. We sought to test the causes of loss of transient expression in one transformation system. The transient nature of T-DNA expression was studied using a gfp reporter gene transferred to Nicotiana plumbaginifolia suspension cells from Agrobacterium tumefaciens. Individual GFP-expressing protoplasts were isolated after 4 days co-cultivation. The protoplasts were cultured without selection and 4 weeks later the surviving protoplasts were again screened for GFP expression. 50% of proto-calli initially expressing GFP had lost detectable GFP activity during the first 4 weeks of culture. 64% of protoplasts died within the first 4 weeks of culture. Multiple T-DNA copies of the gfp gene were detected in 10 of 17 cell lines lacking visible GFP activity. The remaining 7 cell lines contained no gfp sequences. Our results confirmed that transiently expressed T-DNAs can be lost during growth of somatic cells and demonstrated that transiently expressing cells frequently integrate multiple T-DNAs that become silenced. In cells competant for DNA uptake, cell death and gene silencing were more important barriers to the recovery of stably expressing transformants than lack of T-DNA integration.

S6 Plant Secondary Metabilism

S6 - 1

UNRAVELING METABOLIC PATHWAYS AND GENE FUNCTION IN STRAWBERRY USING CDNA MICROARRAYS

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Strawberry fruit development and maturation is characterized by activation of genes of key metabolic pathways leading to fruit softening, expansion, water uptake, pigmentation, enhanced sweetness and flavor. Some of the changes are due to newly formed mRNA. Microarray technology provides a powerful tool for following temporal changes in mRNA levels. Using high-speed robotics, 1700 cDNAs from a strawberry fruit cDNA library were spotted in high density on glass microscope slides. The arrays were used for parallel hybridization of fluorecently labeled mRNA populations. Gene expression during strawberry fruit development and in seed (achene) compared to the receptacle was analyzed. The results support previous biochemical and physiological evidence concerning strawberry fruit development. In addition genes pointing to the activity of certain metabolic pathways not known to be involved in strawberry fruit maturation were discovered. Specific genes were further characterized and subsequent proof of function obtained. One such gene termed SAAT was shown to encode an alcohol acyltransferase, which plays a major role in flavor biogenesis in ripening fruit. The enzyme is responsible for the formation of volatile esters in fruits, which are quantitatively, and qualitatively the most important compounds providing fruity notes. Biochemical evidence that the SAAT encoded enzyme is involved in the formation of fruity esters is provided by the characterization of the recombinant enzyme produced

in E.coli. The results validate the capability of the system to provide quantitative and accurate chronological data, to link gene to function, which will no doubt aid future plant functional genomics research.

S10 Metabolic Engineering/Industrial Products

S10 - 5

MAIZE AS A PRODUCTION AND DELIVERY SYSTEM OF ORAL VACCINES: THE EXPRESSION OF SYNTHETIC E. COLI HEAT LABILE TOXIN B SUB-UNIT (LT-B) IN MAIZE.

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Production of specific antigens from bacterial and viral pathogens in the edible tissue of transgenic plants has given rise to the development of oral vaccines. The antigens make effective vaccines when they are expressed in a form in which they retain their native immunogenic properties. Since the concept of plant-based vaccines was introduced by Mason et al, 1992 (PNAS 89:11745), there has been a surge in research towards production of antigenic/immunogenic proteins in crops such as potato, alfalfa and Arabidopsis. These plant species are relatively easy to transform via Agrobacterium tumefaciens. Advances in maize transformation have allowed us to investigate the possibility of using this crop as a vaccine production and delivery system. We report progress on the development of a maize-based oral vaccine production system. Maize is a major feed component for livestock, does not require extensive cooking (which might denature the antigens) in the feed making process, as do legumes, and is widely grown. Our system is based on the expression of a synthetic version of the E. coli heat labile toxin B sub-unit (sLT-B) in maize grain. LT-B has been shown to be an excellent oral adjuvant, which stimulates immune responses against co-fed antigens, enhancing their value as vaccines. The expression of the sLT-B gene with a plant biased codon composition in maize at high level lays the foundation for the development of a variety vaccines against pathogens that invade or colonize epithelial surfaces in humans and livestock.

S10 - 6

MODIFICATION OF THE PHASEOLUS VULGARIS L. SEED PROTEIN ARCELIN 5 TO INCREASE THE METHIONINE CONTENT OF PHASEOLUS BEANS

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Plant seeds represent the major protein source in human and animal diets. However, the amino acid composition of plant seed proteins is unbalanced with reference to the needs of humans as well as domesticated monogastric animals. The primary limiting essential amino acids in legume seed storage proteins are methionine and cysteine. Gene engineering offers a direct method for manipulating the amino acid composition of seed storage proteins. Different strategies can be used to balance the methionine content. One approach is to modify a gene coding for a major native seed protein by insertions or substitutions with methionine codons and to transform the target plant species with this modified gene. We are using this strategy to increase the methionine content of seeds by modification of the Phaseolus vulgaris L. seed protein arcelin 5. Arcelin 5 is a suitable protein for this purpose because it is a very abundant protein, the gene encoding it as well as the protein itself are well characterised and the crystallographic structure has been determined allowing to predict the influence of mutations by computer modelling. To increase the methionine concentration of Phaseolus beans to that of the FAO reference protein a modified arcelin 5 gene with at least ten additional methionine codons is needed. In a first step, we designed four arcelin 5 genes with three to four substitutions with methionine codons and two genes with a methionine-rich insertion. Mutations resulting in a stable methionine-enhanced protein were combined in a second step in order to obtain modified arcelin 5 genes with at least ten methionine codons. Currently, we are testing the stability of these modified proteins in Phaseolus seeds. These modified arcelin 5 genes can be used to improve not only Phaseolus beans but also other legumes which are used for human and animal nutrition.

S10 - 7

CREATING NOVEL BIOSYNTHETIC PATHWAYS THROUGH ARTIFICIAL METABOLIC SINKS

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Metabolic engineering of complex pathways requires knowledge about the genes involved in biosynthesis, their promoters, and the regulatory mechanisms that control metabolic flux into the pathway. Genetic engineering can be used to create artificial metabolic sinks for substrates in order to probe the ability of a pathway to accommodate the production of a novel end product. We have used the substrate specificity of tryptophan decarboxylase (TDC) for Trp and tyrosine decarboxylase (TYDC) for Tyr to modify the in vivo pools of these amino acids in transgenic, canola, potato and tobacco. The creation of these sinks for Trp and/or Tyr also drastically affected the levels of phenylalanine, as well as those of the non-aromatic amino acids methionine, valine, and leucine. Transgenic plants that produced high levels of Tryptamine and/or tyramine, were modified in their ability to accumulate characteristic secondary metabolites, as a result of the redirected and increased flux imposed by the presence of these decarboxylases. The results of these studies will be used to demonstrate how metabolism can be redirected and how novel biosynthetic pathways can be constructed based on the ability of transgenic plants to accommodate the biosynthesis of a particular end product.

S10 - 9
ENHANCED NITROGEN EFFICIENCY BY TISSUE SPECIFIC EXPRESSION OF AN AMINOTRANSFERASE GENE

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Nitrogen is the most important factor limiting crop growth worldwide and all field crops have a fundamental dependence on nitrogenous fertilizers. To address this requirement, transgenic canola plants (Brassica napus) that overexpress alanine aminotransferase (AlaAT) were engineered by introducing an AlaAT cDNA driven by a tissue specific, osmotic stress promoter. These plants show an increased growth rate under low nitrogen conditions and under hydroponic conditions where the AlaAT gene has been switched on using an osmotic compound. Both the AlaAT gene and the tissue specific promoter were necessary to achieve this result. The increased growth rate is observed as an increase in fresh weight, dry weight, leaf area and stem diameter. Changes in specific metabolites were monitored between wild type and transgenic lines and significant differences were found in several key metabolites in the transgenics. These results and the potential for modifying nutrient uptake and metabolism will be discussed.

S10 - 10
PRODUCTION OF A HIGH AMYLOSE POTATO

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Starch is the major storage carbohydrate in plants. Starch consist of two different molecules, amylopectin (75-80%) and amylose (20-25%). Individually the two molecules are beneficial for different industrial applications. Both amylopectin and amylose are polymers of glucose residues where amylopectin is a branched molecule and amylose is a mainly linear molecule. Branching of the amylopectin molecule in Solanum tuberosum is catalysed by starch branching enzymes. Until today two branching enzymes have been found in S. tuberosum (SBEI and SBEII). cDNAs corresponding to the two branching enzyme genes (S. tuberosum) were isolated and fragments inserted in antisense orientations downstream of the granule bound starch synthase (gbss) promoter (S. tuberosum) and then transformed to potato. Approximately 0.5% of the transgenic clones yielded a total inhibition of both branching enzyme I and II activities. Field tested transgenic clones had an increase of amylose to at least 70 % of the total starch.

S10 - 11
INCREASED LEVELS OF CYTOSOLIC GLUTAMINE SYNTHETASE ACTIVITY IN TRANSGENIC LOTUS JAPONICUS PLANTS IS ACCOMPANIED BY ENHANCEMENT IN GROWTH, PROTEIN CONTENT AND PLANT BIOMASS.

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Higher plants assimilate nitrogen in the form of ammonia through the concerted activity of glutamine synthetase (GS) and glutamate synthase (GOGAT), in which glutamine and glutamate are synthesized from 2-oxoglutarate. Diferent GS isoforms have been identified in higher plants. The GS enzyme is either located in the cytoplasm (GS1) or in the chloroplast (GS2). Since GS activity plays a central role in utilizing carbon skeletons to produce nitrogen compounds, a question that is important to address is whether modulating GS activity levels would affect N and C metabolism in plants. Towards accomplishing this goal we have introduced a cDNA clone for GS1 from alfalfa behind the constitutive CaMV 35S promoter into Lotus japonicus, a model legume. The transformants showed increased GS activity and a corresponding increase in GS1 polypeptide level in all the organs tested. Native gel electrophoresis of protein extracts also showed the presence of a unique GS isoenzyme. The GS1 polypeptide was immunolocalized in the mesophyll cells of the leaves and in the vasculature of the stems and roots in the transformants. The transformants showed increased growth, chlorophyll content, total amino acid and protein content and an increase in the level of asparagine/aspartate. An increase in GS1 polypeptide level in the leaves and the stem of the transformants was accompanied by a decrease in the level of GS2 polypeptide but not in the level of GS2 transcript suggesting that the regulation of GS2 in these plants is at a post-transcriptional level. Furthermore, the transformants showed increased expression of many of the genes involved in carbon assimilation, suggesting some kind of crosstalk between the N and C assimilatory pathways. We will present models to explain the basis for increased growth resulting from increased GS1 activity in the mesophyll cells and the root and stem vasculature.

S10 - 12
SYNTHESIS OF NOVEL BIOPOLYMERS IN PLANTS

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Polyhydroxyalkanoates (PHAs) are bacterial polyesters having a wide range of thermoplastic and elastomeric properties. At present, the main limitation to the industrial use of these polymers as commodity plastics is the high cost of bacterial fermentation. Synthesis of PHAs in transgenic plants could thus be used as an alternative to fermentation. Synthesis of polyhydroxybutyrate (PHB) in the plastids was shown to lead to polymer accumulation to over 14% dry weight in leaves and 8% in seeds. Unfortunately, PHB is a somewhat stiff and brittle plastic. There is thus considerable interest in engineering plant metabolic pathways for the synthesis of PHA copolymers with better physical properties, such as medium-chain-length PHAs (MCL-PHAs), a group of polymers having properties ranging from flexible plastics to elastomers and rubbers. MCL-PHAs are synthesized in bacteria using intermediates of the beta-oxidation of alkanoic acids. We have recently shown that Arabidopsis expressing the PHA synthase in the peroxisomes accumulated MCL-PHAs containing saturated and unsaturated 3-hydroxyalkanoic acids ranging from 6 to 16 carbons. A wide range of monomers derived from fatty acid degradation could be included in the plant PHA, such as branched-chain hydroxyalkanoic acids. Furthermore, the nature and quantity of PHA produced in leaves or seeds could be modulated through the co-expression of a peroxisomal PHA synthase along with transgenes involved in the synthesis of unusual fatty acids, such as medium-chain thioesterases, or by blocking triacylglyceride synthesis. Yet, the amount of MCL-PHA synthesized is still low and further engineering is required to increase the amount to commercial levels. In addition to its application in biopolymer

synthesis, PHA synthesized from beta-oxidation intermediates can be utilized as a novel tool to study various aspects of fatty acid degradation, including the pathway of degradation of unusual fatty acids.

S11 Embryogenesis and Seed Development

S11 - 5

DIFFERENTIAL DISPLAY AS A TOOL FOR SCREENING SEQUENCES RELATED WITH EARLY SOMATIC EMBRYOGENESIS IN RUBBER TREE.

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Somatic embryogenesis could be a powerful tool for rubber tree (Hevea brasiliensis Mull.Arg.) propagation and improvement. The CIRAD has developped a process to maintain embryogenic callus lines and produce somatic embryos. In our system, we suspect that the embryogenic capacity has an early determination. Many genes specifically expressed during embryogenesis have already been identified in a variety of plant models. However, most of them have late patterns of expression (Late Embryogenesis Accumulation). Previous findings have shown that embryogenic induction and the early steps of embryogenesis are controlled by only a few genes. Apart from a limited number of genes with very specific and abundant expression, the regulation of embryogenesis seems to take place essentially via post transcriptional control. Then, the study and comparison of the mRNA pattern could be an efficient strategy to find genes involved in early somatic embryogenesis control.
In order to get more information on the underlying mechanisms of somatic embryo induction, we wished to complete the histological knowledge of our material by a molecular study of gene expression. Our study has been focused on the early steps of embryogenesis. Among many maintained lines, some have an embryogenic potential (regenerant lines), some other do not give any embryos (non-embryogenic lines), or do not allow the complete embryo germination into plantlets to take place (embryogenic, but non-regenerant lines).
The protocol of Differential Display was adapted to rubber tree calli. A very high quality of mRNA was necessary to perform the reverse transcription and PCR in order to get significant and reproducible amplification patterns. In the first experiments, the Differential Display technique allowed us to look for sequences that were differentially expressed during the course of embryogenic induction (14 days), on an embryogenic and regenerant line chosen as the reference (clone PB260, line LC/06/95). As most of the differences have been observed between D0 and D14 of induction, we shall now be focusing more specifically on those two steps of the process, using the SSH (Substractive Suppressive Hybridization) technique. Afterwards, the informations collected will be used to compare the embryogenic lines with the non-embryogenic and non regenerant ones.
To date, a hundred of potentially differently expressed sequences have been obtained, and some of them have been cloned and sequenced. One of these sequences shows significant similarities with a Drosophila gene involved in the early steps of embryogenesis, and more precisely in the zygote polarisation. The complete study of the differential bands and the validation steps will be presented in the poster. Apart from their fundamental interest, these sequences will also be useful for an early selection of the callus lines with the best embryogenic potential.

S11 - 7

GENETIC ANALYSIS OF EMBRYOGENESIS IN ARABIDOPSIS

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Many genes are expressed in a highly co-ordinate manner to ensure that the zygote develops into an organised multicellular structure capable of producing a viable seed. It is therefore not surprising that the genetic analysis of Arabidopsis embryogenesis has led to the characterization of a wide class of mutants, which has been assigned by David Meinke as emb, for embryo-defective (1). Many emb mutants are likely to be altered in basic housekeeping functions whose esssential roles become apparent during early stages of embryo development. Some of the EMB genes might be involved more specifically in the establishment of the body pattern of the plant. In order to gain further insight as to the molecular basis of this particular class of mutants whose morphological defects are poorly understood, we have undertaken a systematic study of emb mutants of the INRA Versailles collection of T-DNA transformed Arabidopsis (2). The screening of 6,000 T1 lines has identified 160 emb lines. Their study revealed 3 main sensitive points in embryo development : the first division of the embryo, the formation of the protoderm and the globular-heart transition. The phenotypic description, the study of the expression of marker genes in the emb seeds and the primary molecular characterization of the corresponding EMB genes will be presented for these 3 groups. Furthermore, additional data on the emb 506 mutant (3) will be presented.
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(2) Bechtold et al. (1993) C.R. Acad. Sci. Paris 316, 1194-1199.
(3) Albert et al. (1999) Plant J. 17, 169-179.

S 11 - 10

THE GENES DIFFERENTIALLY EXPRESSED DURING SOMATIC EMBRYOGENESIS IN CUCUMBER (CUCUMIS SATIVUS L.) SUSPENSION CULTURE

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Somatic embryogenesis of plants can be a useful system for studying changes in gene expression. In competent cucumber cell suspension cultures the embryogenesis can be induced by removal of 2,4-D from the media. In order to clone cDNA fragments represent-inggenes differentially transcribed in this system, the differential display technique was used. The cucumber cell suspension culture was induced towards somatic embryogenesis and the tissue samples were collected in 7 time points during first two weeks after induc-tion. The differential display analyses were done in two series: first covered early changesin gene expression (0 to 24 h.) and second covered subsequent processes (1 to 14 days after induction). We identified 43 and 77

candidate bands respectively. The cloned cDNAs were screened with probes specific to tissues before and after the induction of somatic embryogenesis. We confirmed that 16 clones were repressed and 10 clones were induced after start of somatic embryogenesis. The candidate clones were sequenced. Three induced clones showed high sequence similarity to xyloglucan endotransglycosylase genes. The expression of xyloglucan endotransglycosylase genes during somatic embryo-genesis can be explained by high activity of the plant cell wall in morphogenesis.

S 11 - 11

MODIFICATION OF GENE EXPRESSION INDUCED BY 5-AZACYTIDINE DURING CAR-ROT SOMATIC EMBRYOGENESIS

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In mammals the correct methylation pattern is critical for normal embryonic development and plays important roles in the regulation of gene expression, X-chromosome inactiva-tion, genomic imprinting, chromatin modification and silencing. Several experimental data showed that plants with hypomethylated genome, despite suffering significant develop-mental abnormalities, are viable; however the consequence of demethylating treatments on plant somatic embryogenesis seems to be more dramatic. Previously we demonstrated that if hypomethylation is induced with drug such as 5-azacytidine (5-aza), ethionine or ethoxy-carbonyl-pyrimidine, carrot somatic embryogenesis progression is immediately blocked. Hyper- or hypo-methylation have no effect on undifferentiated carrot callus cell growth. In this study, to acquire a better knowledge about the inhibition of in vitro differen-tiation induced by hypomethylating drugs, we added 5-aza at different stages of carrot somatic embryogenesis. At every stage tested so far, the presence of the drug always caused a developmental arrest that turned out to be reversible after the 5-aza removal from the medium.
Contrary to this pattern, the addition of 5-aza at the 4-5th day of differentiation determined an irreversible block of somatic embryo development. This developmental stage repre-sents for carrot somatic embryos the switch point from globular embryos (radial symmetry) to heart-shape embryos (bilateral symmetry). The two mRNA populations from 5-aza blocked and control embryos were compared by mRNA differential display analysis. The drug treatment leads to minor changes in the expression of individual genes. Bands of dif-ferentially expressed genes, confirmed as such by reverse Northern analysis, were cloned and sequenced. A classical Northern experiment also confirmed for one of them the differ-ential expression, being null in the control sample. All the products so far analysed show various degree of homology with existing EST, cDNA or genomic sequences. The best match correspond to a zinc finger protein and its role in embryogenesis is discussed.

S 11 - 14

ANTIBIOTICS INCREASE SOMATIC EMBRYOGENESIS FROM LEAVES OF RECALCI-TRANT CLONES OF CAMELLIA JAPONICA

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Camellia japonica L., commonly known as "camellia", is an evergreen ornamental plant from Theaceae family. Several protocols were described for the production of somatic embryos from embryonic (Barciela and Vieitez, 1993; Kato, 1982) and non-embryonic tis-sues (Pedroso and Pais, 1993; Pedroso e Pais, 1995; Vieitez et al, 1991). Early results showed, however, that in vitro embryo production decreased with the number of subcul-tures, subculture interval and in vitro shoot-culture age. Direct embryo formation decreased drastically for clones established in vitro for more than 2 years (Pedroso and Pais, 1993). Antibiotics have been described to increase somatic embryogenesis (Nakano and Mii, 1993) and leaf regeneration efficiency (Yepes and Aldwinckle, 1994). Cefotaxime and carbenicillin were tested to determine their effect on somatic embryogenesis. Entire camellia leaves from a 5-year-in vitro established clone were used as explants. Carbenicillin (100mg/L) and cefotaxime (200-300mg/L) added together to culture medium enhanced embryogenic response, whereas when added separately they did not show sig-nificant increase. Cefotaxime (300 mg/L) showed some phytotoxic effect when added alone to the culture medium. Regeneration was achieved on medium with 200 mg/L car-benicillin and on medium with 100mg/L carbenicillin and 150 mg/L cefotaxime. The possi-bility that antibiotics might act on the stress that induces somatic embryogenesis in recalci-trant clones of Camellia is discussed.

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S 11 - 25

POSSIBLE INVOLVEMENT OF AN ACIDIC CHITINASE DURING DIRECT SOMATIC EMBRYOGENESIS IN COFFEA ARABICA L

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Chitinase has been proposed to be involved in somatic embryogenesis in carrot. A chiti-nase secreted by a wild type line was able to rescue a temperature-sensitive mutant line (ts11) in which embryos are arrested at globular stage at the non-permisive temperature of 32oC. A role in restoring the endomembranal system and protoderm of mutant line was suggested. In this work, we surveyed the gene expression pattern during the development of direct somatic embryogenesis in coffee by a differential display approach. A gene frag-ment, showing differential expression, was cloned and sequenced. It showed a high homology with several acidic chitinases. No induction of its expression was observed by mechanical damage or pathogenesis and its expression was highly reduced when embryos passed from globular to germinated stage. Determination of activity in polyacril-amide gels after isoelectrofocusing revealed that two forms of the enzyme have a pattern that resemble that of the expression of the gene. This two forms are not activated by mechanical damage or pathogenesis.
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S22 Plant-Pathogen Interactions

S22 - 70
EXPRESSION OF GENES INVOLVED IN THE RESISTANCE OF SOYBEAN TO THE SOYBEAN CYST NEMATODE

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The soybean cyst nematode (SCN), Heterodera glycines, is the major pest of soybean worldwide and is responsible for damage estimated at $1.5 million each year in the U.S. We are identifying genes involved in the resistance response of soybean to SCN using array techniques. Over 1000 ESTs were sequenced from a cDNA library constructed from cv. Peking mRNA two days after invasion of roots by SCN race 3. Numerous genes were identified by BLAST searches, including genes involved in secondary metabolism, signaling, and the defense response. Several genes involved in phenylpropanoid synthesis were found including phenylalanine ammonia lyase, chalcone synthase, cinnamyl alcohol dehydrogenase, 4-coumarate coA ligase, and others. Numerous peroxidases, protein kinases and transcription factors also were identified. Insert sizes range from ca. 400 bp to over 3.2 kb. Some clones were added to the database from differential display experiments that identified SCN-induced soybean genes. A set of 96 clones also was added from a cDNA library made from cotyledons to serve as non-induced genes in the array. Inserts of several clones are completely sequenced and contain complete reading frames: these are from genes that were available in this or other USDA laboratories. The database containing clone information is searchable by clone address, identity and size. The database can be viewed by collaborators at http://bldg6.arsusda.gov/benlab. A subset of the array is being hybridized to cDNA made from mRNA representing genes expressed in resistant and susceptible soybean inoculated and not inoculated with SCN to identify genes greatly induced in response to SCN.

S 22 - 74
A PLANT ESTERASE INDUCES DISEASE RESITANCE IN PLANTS

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Carboxylesterases are enzymes that catalyze the hydrolysis of compounds containing an ester bond. In plant-microbe interactions, an esterase gene from the hypersensitive reac-tion, and a lipase gene that is an essential component of R gene-mediated disease resis-tance and a lipase-like gene for salicylic acid signaling have been recently isolated.
However, physiological role of the enzymes in plant defense mechanisms is still unclear. In addition, many of phytopathogenic fungi secrete cutinases as esterases that break the ester linkages between cutin molecules of plant cuticles to invade plant. Another role of the cutinases is hypothesized to induce disease resistance in plant by generating cutin monomers from plant cuticles. We report here the cloning and characterization of an esterase gene of pepper (Capsicum annuum) that is accumulated to high levels only in the incompatible interaction with anthracnose fungus Colletotrichum gloeosporioides, but not in the compatible interaction. Exogenous application of the pepper esterase elicit H2O2 that is parameter for plant defense as well as defense-related genes, and inhibits the appressorium formation that is prerequisite of the fungus to infect the host during infection process. Finally the applications protect pepper fruits against fungal infection. Here we show that a pepper esterase induces disease resistance in peppers.

S23 DNA Integration Mechanisms

S 23 – 7
NTEGRATION OF T-DNA INTO PLANT DNA

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Instrumental for the efficiency of T-DNA transfer from Agrobacterium tumefaciens to plants and the precision with which the bacterial DNA is integrated are virulence proteins which are carried over to the plant cell, along with the T-DNA. Specific virulence proteins aid in overcoming the barrier of the nuclear membrane, other functions are important for integra-tion. The elements on the plant side which contribute to successful movement of T-DNA through membranes to the plant chromatin are slowly being identified. For the in-vitro analysis of T-DNA integration an assay using artificial T-DNA-VirD2 com-plex and an oligonucleotide derived model target DNA was developed. It was demonstrat-ed that VirD2, covalently attached to the 5' terminus of a single-stranded oligonucleotide, could not ligate the model T-DNA to the model target. However, the addition of plant nuclear extracts allowed a low level of ligation, which could be substantially increased using T4 ligase or Arabidopsis thaliana ligase I. Thereby ATP served as source for AMP, on which the reaction depended. As intermediate in the ligation a phosphotriester consist-ing of the model T-DNA, VirD2 (with a tyrosine linkage) and AMP could be detected. As final step this intermediate reacted with the substrate DNA and ligase, yielding an integrat-ed version of the T-DNA, free VirD2 and AMP. Involvement of other plant components is anticipated in in-vivo T-DNA integration.

S 23 – 11
HYPER-RECOMBINOGENIC PLANTS

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Homologous recombination (HR) is involved in the generation of genetic diversity, in DNA repair, and in exogenous DNA integration. In plants, DNA integration usually occurs through non-homologous recombination and HR is not efficient. The low rates of HR enable to

maintain the stability of the repeat-rich plant genome, but on the other hand it impedes sophisticated genomic manipulations such as gene targeting. In order to identify the genes that control homologous recombination in plants, and to develop gene targeting, we are using the following approaches: (i) screening for hyper-recombinogenic mutants; (ii) screening for radiation-resistant mutants; (iii) ectopic expression of bacterial genes with known functions. One Hyper-recombinogenic tobacco mutant, "Hyrec", was found. The mutation is dominant and is inherited in a Mendelian manner. In Hyrec, somatic recombi-nation between homologous chromosomes is increased by three orders of magnitude. Recombination between extrachromosomal substrates is increased by 6-9 fold and recom-bination between tandem genomic repeats (direct or inverted) is not affected. Hyrec shows significant resistance to gamma-irradiation but not to UV. Three additional mutants were generated using gain-of-function mutagenesis and selection for resistance to gamma irradiation. These mutants are being tested for HR efficiency. Finally, we showed that plants expressing the bacterial resolvase RuvC show stimulated rates of HR. All the recombinogenic plants are being tested for gene targeting efficiency.

S 23 – 16
THE USE OF THE CRE-LOX SYSTEM FOR THE EVALUATION OF CHROMATIN BOUNDARIES IN PLANTS

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Matrix-associated regions (MARs) are a structural feature of plant chromosomes and belong to the general class of chromatin boundary elements. They are supposed to create topological units of transcriptional regulation. In transgenic plants, flanking of transgenes with (some) MARs results in significant reduction in position effects and enhances overall stability and predictability of transgene expression. Currently, MAR assays in plants require the comparison(s) of different constructs in relatively large populations of plants. The inter-pretation of MAR action may therefore be confounded with other parameters, such as dif-ferent integration sites within the populations, or differences in the relative frequency of occurrence of complex integrations. For easier MAR evaluation, we aim to generate popu-lations of plants with and without MAR elements on a given chromosomal position with the help of the Cre-lox recombination system. To excise two MARs independently, we use a pair of the wild-type lox site, loxP, and two different pairs of mutated lox sites, the single spacer mutant lox511 and the double spacer mutant lox2272, both of which should recom-bine exclusively with the identical lox site. Binary plasmids used for evaluation carry the T-DNA configuration lox1>-MAR-lox1>-GUS-NPTII-lox2>-MAR-lox2>. Such plasmids were combined in E. coli with a plasmid supplying Cre recombinase and excision of MAR ele-ments was analysed by restriction analysis of plasmid DNA without any biased selection for excision events. In unexpectedly high percentages (up to 80%), the GUS and NPTII genes were excised as well. This was shown to be due only in part to promiscuity of the lox sites used, suggesting that the presence of multiple heterologous lox sites can interfere with proper excision events. The results of analyses of similar plasmids in tobacco proto-plasts upon transient and stable expression assays will be presented.

S 23 – 25
RAD50 OF ARABIDOPSIS

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The Rad50 protein is involved in the cellular response to DNA-double strand breaks, including the detection of damage, activation of cell-cycle checkpoints and DSB repair via recombination. It is also essential for meiosis in yeast, is involved in telomere maintenance and is essential for cellular viability in mice.
We present here the isolation and preliminary characterisation of the Arabidopsis thaliana RAD50 homologue and of a rad50 mutant plant. The gene has 27 exons and Southern analysis and YAC-based PCR mapping show that there is a single locus for this gene in the Arabidopsis genome located on chromosome II. Northern analysis shows a single 4.3 Kb mRNA species in all plant tissues tested, which is strongly enriched in flowers and other tissues with many dividing cells. The predicted protein length (1316aa) is conserved with repect to the other known Rad50 homologues (human, mouse and Caenorabditis elegans), whilst the amino acid sequence conservation is largely limited to the amino- and carboxy-terminal regions of the predicted protein. The central part of the protein shows conservation of the predicted coiled-coil con-formation also found in the other known Rad50 proteins. The mutant plant is sterile and cultured mutant cells are strongly sensitive to the radiomimetic agent MMS. Arabidopsis RAD50 is thus involved in both meiosis and the repair of DNA double-strand breaks in Arabidopsis. Furthermore we show that, as is the case in yeast, Rad50 protein is involved in the maintenance of telomeric repeats in Arabidopsis.

S 23 - 26
ISOLATION AND CHARACTERIZATION OF A LARGE Ac/Ds MEDIATED INVERSION IN ARABIDOPSIS THALIANA

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We have identified a new allele of rooty (rty), a mutation affecting auxin homeostasis, in Ds-mutagenized Arabidopsis. The molecular characterization of rty-5 has revealed the presence of a large, Ac/Ds mediated inversion. Genetic analysis demonstrates the perfect co-segregation of a Ds element with rty-5. However, the characterization of Arabidopsis genomic DNA, recovered from each end of the Ds element, failed to reveal the presence of a structural gene. Database searches utilizing the recovered sequences demonstrate that each flanking sequence displays identity to a distinct chromosome II BAC. The avail-ability of the complete chromosome II sequence has allowed the determination of the distance separating these two BACs. In the wild-type chromosome II configuration, a physi-cal distance of 1.2 Mb, or a genetic distance of 4.0 cM, separates the BACs. This result, coupled with other analyses, is suggestive of an inversion. By assuming that the Ds ele-ment is positioned at one breakpoint of the inversion, PCR was used to isolate the second, predicted breakpoint. This result confirms the existence of a large inversion in rty-5 lines. Further analyses of the inversion indicate that rty-5 is contained within the inverted chro-mosome II sequence, 4.0 kb away from the second breakpoint and 1.2 Mb away from the Ds element. Sequence analysis of rty-5 demonstrates the presence of a small lesion in the coding sequence. This lesion is presumably the basis of the rty-5 mutation and repre-

sents an event independent of the observed inversion. The failure to genetically separate rty-5 and the Ds element is most likely the result of suppressed recombination in the inverted region. A model depicting the role of Ac/Ds in the formation of the inversion will be presented.

S 23 - 27
IN VITRO INTEGRATION OF T-DNA INTO THE PLANT GENOME

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Agrobacterium tumefaciens, a gram-negative soil bacterium, transfers DNA causing crown gall disease on dicotyledonous plants rending it the system of choice for plant transgene-sis. The transferred DNA (T-DNA) that resides on a large Ti (tumor inducing) plasmid is processed within the bacterium and is exported as a protein-T-DNA complex to the plant cell where it is integrated into the genome. An in vitro T-DNA ligation/integration assay has been designed to investigate the mechanism of T-DNA integration and the factors involved in this process. The bacterium derived VirD2 protein that is covalently attached to T-DNA was not able to perform in vitro ligation reaction. These results suggest that plant factors are necessary for T-DNA integration. Indeed, in vitro ligation/integration reaction occurred when plant nuclear extracts were added. Prokaryotic DNA ligases could substitute for plant extracts in this reaction. T4 DNA ligase mediated ligation of the VirD2 bound oligonu-cleotide to the target sequence was slower and less efficient than when the oligonu-cleotide was free. ATP was found to be source of AMP moiety for ligation mediated by T4 DNA ligase or plant enzymes. A reaction intermediate consisting of T-DNA, AMP and VirD2, in a phosphotriester linkage, was detected. VirD2 and AMP were released from T-DNA only at the last step of ligation. These findings do not, however, exclude the involve-ment of other plant enzymes in T-DNA integration.

S26 Tree Biotechnology

S 26 - 2
CLONING AND ANALYSIS OF A RUBBER BIOSYNTHESIS STIMULATOR PROTEIN IN *Hevea brasiliensis*

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The pathway of rubber biosynthesis in latex of the Hevea brasiliensis tree begins from the glycolytic processing of the photosynthate, sucrose. Subsequent steps lead to the forma-tion of high molecular weight rubber molecules (polyisoprene) via the intermediate, isopen-tenyl diphosphate, IDP. The final steps of rubber biosynthesis occur on the surface of rub-ber particles where IDP units are assembled into short-chain initiator molecules before fur-ther polymerization into long-chain polyisoprene. Although enzymes catalyzing the major steps of IDP polymerization are known, ancillary proteins have also been reported. Two rubber particle located proteins which play a role in rubber polymerization are the 14 kDa rubber elongation factor, REF, and the 24 kDa small rubber particle protein, SRPP. Our study focuses on a 13 kDa cytosolic protein named as the rubber biosynthesis stimulator, RBS. Native RBS was shown to enhance the incorporation of 14C-labeled IDP in vitro in the presence of washed rubber particles. Amino acid sequencing of RBS revealed that it is similar to the eukaryotic initiation factor 5A, eIF-5A. Using a tobacco eIF-5A cDNA as a probe, we isolated seventeen Hevea homologs. Complete sequencing of these cDNAs showed that they share more than 90% DNA identity and can be classified into seven dif-ferent protein isoforms. This indicated a large family of Hevea eIF-5A genes but also raised the question of whether one or more protein isoforms produce the stimulatory effect. Candidate clones of six isoforms (the seventh was not full-length) were selected for recombinant protein expression. Subsequent biosynthesis assays in vitro showed that in the presence of washed rubber particles, all six protein isoforms stimulated the incorpora-tion of 14C-labeled IDP ranging from 15-120% of the negative control. Lire Yusof et al. (1998) J.Rubb.Res 1 (1) 48-66

S 26 – 5
GENE EXPRESSION ASSOCIATED WITH SHIFTS IN CARBON AND NITROGEN ALLO-CATION IN POPULUS

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Nitrogen is an important determinant of plant growth and architecture. Under N-replete conditions, plants preferentially allocate C and N resources to foliage, stems and branch-es, whereas under N-limited conditions, an increased proportion of C and N resources are allocated to roots. As part of our ongoing efforts to identify factors that mediate C and N resource allocation in Populus trichocarpa x deltoides, we have used differential display to identify cDNAs whose expression patterns are altered in response to limiting or luxuriant levels of N fertilization. These cDNAs represent a diverse array of biochemical and cellu-lar processes that are modulated in response to N availability prior to or concomitant with visible changes in growth, and thus are useful "sentinels" for monitoring global responses to changing N status. Analysis of a subset of these N-responsive cDNAs demonstrated that N-induced shifts in resource allocation to shoots and roots are correlated with changes in the expression of cDNAs associated with C assimilation and partitioning. These findings suggest that C/N interactions may be important in determining the relative distribution of plant resources to roots and shoots. Experiments in which girdling was used to manipulate C:N ratios in plants indicate that the expression patterns of several of the N-responsive cDNAs are altered by shifts in C/N balance. The expression of some of these cDNAs can also be up- or downregulated by incubating excised tissues on MS media supplemented with sugars or nitrogen metabolites. We are currently characterizing N-responsive cDNAs that encode putative modulators of sink strength, as well as cDNAs that are similar to regulatory factors, with the goal of determining the role that these gene products play in allocating C and N resources amongst plant parts.

S 26 – 6
DETECTION OF QUANTITATIVE TRAIT LOCI INVOLVED IN KEY AGRONOMIC TRAITS IN CACAO

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Cacao (Theobroma cacao L.) is a tropical, diploid (2n=20) outbreeding tree. This species is one of the major cash crops for a number of developing countries. Cacao production is affected by the low yield of cacao trees, and the use of low genetic potential material in regard to diseases such as black pod caused by Phytophthora sp. The aim of this study was to investigate the integration of molecular markers to detect, quantify and identify the genes involved in yield and disease resistance. A core genetic map of the ten cacao haploid chromosomes representing 768 cM was defined by using mainly RFLP markers. Ten yield QTL were detected over a period of 15 years on an F1 cross between Catongo, a highly homozygous clone, and Pound 12, a highly heterozygous cacao. Some of these QTL were frequently detected over 15 years of production, while others were specific for a given year. Total yield variance, on a yearly basis, ranged from 0 to 56%. Two major QTL explained each 20% of the total variance were detected. Phytophthora resistance was assessed on the same F1 cross and on a backcross popula-tion Catongo X (Catongo X Pound 12). Six different QTL were detected in the two popula-tions. One QTL was found in both populations and appeared to be a major component of disease resistance. Some major QTL found in these two studies showed overlapping with other traits suggest-ing a possible pleiotropy. The use of candidate genes and genomic synteny could facilitate the identification of the genes involved in these traits of interest for cacao breeding.

S 26 – 11
DNA METHYLATION IS INVOLVED IN SOMACLONAL VARIATION IN OIL PALM

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Clonal propagation of oil palm (Elaeis guineensis Jacq.) through somatic embryogenesis has led to the production of several million clonal plantlets to date. The occurrence of somaclonal variants (ca 5%) among populations of somatic embryo-derived oil palms (Elaeis guineensis Jacq.) currently hampers the scaling-up of clonal plant production. Variant "mantled" palms show a feminisation of male flower parts and can result in com-plete sterility in the most severe cases. Preliminary studies of ploidy level and RAPD/AFLP polymorphism have not revealed any genomic changes that could be linked with the vari-ant phenotype, thus confirming the hypothesis of an epigenetic origin for the abnormality. In order to investigate the relationship between the "mantled" somaclonal variant pheno-type and possible alterations in genomic DNA methylation rate, two complementary approaches have been used. HPLC quantification of relative amounts of 5-methyl-deoxy-cytidine has shown that global methylation in leaf DNA of abnormal regenerants is 0.5 to 2.5% lower than in their normal counterparts (20.8% versus 22%, respectively on aver-age). Genomic DNA from nodular compact calli (NCC) and fast growing calli (FGC), which yield respectively 5% and 100% "mantled" plantlets was also compared: in this case, an even more marked difference (4.5%) was observed (23.2% for NCC against 18.7% for FGC). An alternative method, the SssI-Methylase Accepting Assay (SssI-MAA), based on the enzymatic saturation of CG sites with methyl groups, gave compatible results. This work demonstrates that a correlation exists between DNA hypomethylation and the "man-tled" somaclonal variation in oil palm. We are now carrying out RFLP studies using isoschizomeric restriction enzyme pairs which show differential sensitivity to dC methylation (eg MspI and HpaII). Similarly an MSAP approach is being pursued with the aim of identifying polymorphic AFLP markers which characterise changes in DNA methylation patterns. Since active chromatin may show a reduced methylation rate as compared with its inactive counterpart, we are now investigating whether methylation rates differ between the active chromatin fractions of DNA extracted from normal and variant regenerants.

S 26 - 17
1) Oil palm 2) Tissue culture 3) Somaclonal variation 4) methylation-sensitive amplified polymorphism (MASP)

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The occurrence of somaclonal variants (ca. 5%) among populations of somatic embryo-derived oil palms (Elaeis guineensis Jacq.) currently hampers the scaling-up of clonal plant production. These variants display the "mantled" fruit phenotype, where the stamen pri-mordia have been converted into carpel-like tissues leading to abnormal fruit development. The severity of this phenotype shows heterogeneity between different clonal lines, between palms of the same clonal lines and even between different flowers of the same individual and can also show differing degrees of reversion. These observations lead to the hypothesis that the underlying cause responsible for the mantled phenotype might be epigenetic in nature. Here we report the investigation of possible alterations of genomic DNA methylation between the mother plant used as tissue source (ortet) and regenerated plants (ramets) by performing AFLPs using different methylation sensitive enzymes for the preparation of AFLP templates. Polymorphisms between ortet and ramet were highest using the enzyme combination HpaII/EcoRI (2%) and were detected as the appearance of additional bands in the ramets, suggesting that a reduction in methylation had occurred during tissue culture. Conversion of 10 polymorphic fragments into probes for Southern blot analysis confirmed that these polymorphisms were not due to partial digestion of the AFLP templates and indicated that the majority were found in single copy sequences. Although this method is an effective way of detecting variation in tissue culture-derived plants, no polymorphism could be detected which was specific for the mantled phenotype, indicating that different approaches will be required to identify the causal basis of the man-tled fruit abnormality.

S 26 – 18
USE OF DIFFERENTIAL GENE EXPRESSION ANALYSIS TO STUDY SOMACLONAL VARIATION IN OIL PALM SHOOT APICES

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Micropropagation of oil palm (Elaeis guineensis Jacq.) by somatic embryogenesis has been found to result in the induction of a flowering abnormality, known as mantled, which involves the homeotic transformation of stamens into carpelloid structures. Several key features of the mantled abnormality reveal that it is epigenetic in nature. In particular, it has been observed that reversion to a normal floral phenotype may occur over a period of years following the onset of flowering. In addition, although the mantled abnormality is strongly transmitted through tissue culture, only a weak non-Mendelian transmission of the mantled character is observed via seeds. The fact that the abnormal phenotype is unlikely to have resulted from a classical genetic mutation is borne out by ploidy, RAPD and AFLP studies previously carried out in our lab. Given that the mantled character is epigenetic in nature, we have used an experimental approach based on differential display analysis as a means of identifying genes which are differentially expressed in a mantled-related fash-ion. The data thus obtained will be used to study the molecular mechanisms which give rise to the mantled abnormality. Furthermore, we hope that the approach used will provide us with early markers of the flowering abnormality for the screening of regenerants. Differential display analysis has been performed on oil palm shoot apices harvested from in vitro-derived leafy shoots. Differential expression markers were cloned and their expres-sion patterns rechecked by Northern hybridisation on a range of materials of different genotypes. Subsequently, markers representing mRNAs which accumulate in a consistent-ly mantled-related fashion were used as probes to obtain full length cDNA clones. The results obtained to date will be discussed.

S 26 – 20
DIFFERENTIAL GENE EXPRESSION IN POPLAR TENSION WOOD

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Tension wood is formed in poplar at the upper side of bended stems in response to a gravitational stimulus. Although important for tree architecture, a high percentage of tension wood is a major problem for most wood industrial utilizations. Tension wood exhibits strik-ing differences compared to regular wood: 1) presence of gelatinous fibre cells, called G-fibres, containing high amount of cellulose; 2) modification in lignin content and composi-tion and 3) important changes in cell ultrastructure and wood mechanical properties. Using an AFLP-based technique for RNA fingerprinting, we identified more than 100 transcript-derived fragments (TDFs) from gene sequences specifically regulated during tension wood formation in poplar. AFLP analyses have been carried out on poly (A)+ RNA purified from xylem tissues harvested on poplar stems bended for 2 months. About 80% of the differen-tially amplified TDFs appeared to be specifically or mainly amplified from tension wood compared to opposite or normal wood samples. Presently, 40 of these TDFs have been cloned and sequenced. Some of the corresponding genes appear to be involved in the biosynthesis of cellulose, hemicellulose or lignin, whereas others seem to participate to xylem differentiation. A number of sequences correspond to ESTs with no assigned func-tion and a few other TDF sequences does not have any hit in the different databases. RT-PCR, northern and reverse northern will be carried out to follow the expression pattern of these sequences.

S 26 – 21
CLONING OF POTENTIALLY ETHYLENE-INDUCIBLE AND/OR LATICIFER-SPECIFIC PROMOTORS FROM HEVEA BRASILIENSIS

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Cloning of ethylene-inducible and/or laticifer-specific promotors from rubber tree was untertaken with the objective to optimize transgene expression in genetically engineered rubber tree. Glutamine synthetase (GS) and hevein gene promotors were targetted, based on the fact that (1) gs gene overexpression was observed after ethylene treatment in latex, and (2) the hevein protein has been found in laticifers only. Several genomic clones were obtained. Partial sequencing revealed that hevein and GS are encoded by at least 2 and 3 genes respectively (hv1, hv2 and gs1, gs2, gs3). Unfortunately, the clone corresponding to gs1 was truncated and lacked the promotor region. Specific probes were designed from the 3' non coding region of gs1 and gs2. However, it was not possible to distinguish gs2 from gs3, nor hv1 from hv2, as these genes were highly homologous, including in their non coding regions. Gene expression analysis revealed that : (1) both gs1 and gs2/gs3 were responsive to eth-ylene in latex, with gs1 apparently strictly induced and gs2/gs3 overexpressed; (2) hv gene expression in latex was very strong but not significantly responsive to ethylene; (3) gs1 and gs2/gs3 were differentially expressed in tissues from in vitro culture at various stages of development ; (4) both gs and hv genes were highly expressed in undifferentiated tissue; (5) hv gene expression increased with embryo development, according probably to the laticifer differentiation stage. Subcloning of hv1, hv2, gs2 and gs3 promotor regions in a vector for transformation, in fusion with the gus reporter gene, was untertaken. Preliminary results of functional analy-sis are presented : the gs3 promotor-gus construct was introduced into rubber tree callus tissue by particle gun bombardment. Transient GUS activity was detected, which demon-strated functionality of the isolated gs3 promotor. Inducibility by ethylene will then be ana-lyzed.

S 26 - 22
MOLECULAR GENETIC DIAGNOSTICS OF SOMACLONAL VARIATION IN MICRO-PROPAGATED REE SPECIES

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Micropropagation of diverse groups of elit plant species has nowbecome a multibillion dollar commercial industry being practiced all over the world. For the long-term benefits of the technique, the micropropagated plants mus tretain their genetic fidelity so as the advantages in the selective use ofelite genotypes are maintained. Since organised meristems have long beenpresumed to give rise to genetically identical plants, most of the commercial micropropagation protocols are based either upon enhanced axillary branching culture or somatic embryogenesis. Notwithstanding this consideration, however, there are now several convincing reports of the occurrence of somaclonal

variation even in the organised meristem- derived plants. We have critically analysed the genetic fidelity in enhanced axillary branching-derived plants of 5 Clones (L34, G3, G48, S7C15, and D121) of Populus deltoides (PD), and Eucalyptus tereticornis (ET) and E.camaldulensis (EC) vis-a-vis their respective mother plant(s) using many molecular DNA markers (RFLPs of nuclear, mitochondrial and chloro-plast genomes; RAPD and ISSR fingerprinting). Total DNA of 175 hardened micropropa-gated plants of above three species were digested with 8 restriction endonucleases and hybridized to 13 nuclear, mitochondrial and synthetic oligonucleotide probes to produce 102 bands. Hybridization patterns indicated variation in few micropropagated plants of 2 (L34 and G3) clones of PD and ET. The variation observed in the latter species was moderate.Further, 12 arbitrary 10-mer and 6 ISSR primers produced 133 monomorphic frag-ments in Eucalyptus species, while in PD a total of 13 and 7 polymorphic bands across 6 and 5 plants in clones L34 and G3 , respectively were produced. We observed modertae (ET) to drastic (PD : L34, G3) variation at DNA sequence level among organised meris-tem- derved plants, which were otherwise observed to be monomorphic at the cytogeneti-cal, cytochemical and biochemical level(s). Molecular DNA markers have enabled a fast and unambiguous screening of microproprgated plants at an early stage of in vitro multipli-cation.

S 26 - 23
INSECT RESISTANCE OF TRANGENIC SPRUCE EXPRESSING A SYNTHETIC BACILLUS THURINGIENSIS GENE

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A synthetic version of the, cryIA(b) gene from Bacillus thuringiensis (Bt) was introduced into two genotype of white spruce (Picea glauca) by microprojectile bombardment. A plas-mid carrying the cryIA(b) gene, driven by either the 35S or ubiquitin constitutive promoters, was co-transferred with a plasmid containing the gus-nptII fusion gene as a screenable/selection marker. Transgenic lines were obtained by selection on kanamycin and identified by GUS histochemical staining. Molecular analysis showed co-integration of the Bt gene as well as the selective marker in over 90% of the transgenic lines tested. A range in expression levels of the cryIA(b) gene were obtained, with ubiquitin promoter appearing stronger that the 35S promoter. CryIA(b) proteins were detected in both embry-onal tissues and needles of plants. Insect feeding trials showed that expression levels of the cryIA(b) gene in many of the lines were lethal to the development of the larvae of the spruce budworm.

S 26 - 24
14-3-3 GENE FAMILY IN HYBRID POPLAR AND ITS POSSIBLE IMPLICATION IN TREE DEFENSE AGAINST PATHOGENS

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In ongoing investigations of the role of the signal transduction pathway in tree-pathogen interactions, we have isolated several 14-3-3 fragments which are members of a gene family. We obtained four clones from hybrid poplar. Sequence comparisons show high homology between the fragments, and, in some cases, higher than 75% sequence similari-ty with previously published sequences. Treatments of trees with chitosan, jasmonates, or cold, or by wounding of leaves, caused either an increase or a slight down-regulation in 14-3-3 mRNA. Since jasmonates and chitosan are signal transducers of defense reac-tions in plants, our results suggest a possible role of 14-3-3 in pathogen defense response in deciduous trees. This role remains to be tested. In addition, pal, a stress-related gene also involved in plant defense against pathogens, was used to verify its induction following treatment of trees with the previously cited elicitors. This is the first time 14-3-3s have been reported in a tree species. It shows that high conservation of 14-3-3 genes exists within a high range of organisms. As well, it suggests a high conservation in the signal transduction pathway and in the elicitor response.

S 26 - 25
ASSESSING THE INCIDENCE OF DNA RELEASE INTO THE SOIL FROM GENETICALLY MODIFIED POPLARS

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Genetic engineering offers the opportunity to generate transgenic trees with useful new traits conferred by genes obtained from a variety of organisms. However, the development of resistance by insects and diseases to foreign genes in plants could have an important environmental impact when transgenic plants are established in the field. Also, the DNA released into the soil by decaying leaves and roots from the transgenic trees may become available for incorporation by soil microbes, and this problem is exacerbated if the trans-genic plants are perennials. The objectives of this study were to establish methods for investigating the persistence of recombinant plant marker genes in degrading transgenic poplar leaf material. We studied the stability of the DNA encoding the neomycin phospho-transferase II resistance marker, used in tree genetic engineering. DNA persistence in the environment was determined by placing transgenic poplar leaves in permeable bags that were located on top of weeds, on top of the soil and below the soil, and left under natural conditions on the site of a field trial for up to 12 months. Our study represents the first quantitative analysis of tree DNA stability under field conditions and indicates that large fragments of the recombinant plant genomic DNA did not persist in the field for more than 3 months. Under experimental conditions where transgenic poplar leaves were mixed with soil, recombinant DNA was initially degraded at a high rate.

S 26 – 26
FLORAL REGULATORY GENES AS TOOLS FOR CONTROL OF REPRODUCTION IN POPLARS

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The long juvenile period of trees, and their propensity for extensive gene flow via pollen and seed, pose major constraints to breeding and biotechnology. Although poplar (genus Populus, including cottonwoods and aspens) is highly suitable for genetic transformation because of its ease of transformation and vegetative propagation, ecological and regulato-ry concerns about transgene spread are likely to pose a strong barrier to commercial use of transgenic trees. Our laboratory's primary focus has therefore been on the isolation of genes that can be used to impart male and female sterility as a means of transgene con-tainment. To be useful as a biosafety measure, sterility must be highly reliable, predictable in the seedling phase, and have no deleterious effects on tree growth. Data from a multiple-year field experiment with

transgenic poplars demonstrated that heterologous floral promoter::cytotoxin transgenes can have cryptic, deleterious effects on growth. Native genes from poplar, and alternative kinds of sterility transgenes, are therefore being pur-sued. We have studied poplar homologs to LEAFY, AGAMOUS, APETALA1, and APETA-LA3, and will analyze their value for deriving various kinds of sterility transgenes, including dominant negative mutants. To develop means for precocious flowering, we have experimented with the poplar and Arabidopsis LEAFY genes in transgenic poplars, and begun to isolate a number of poplar homologs to the diverse array of genes that influence flowering time in Arabidopsis. The poplar LEAFY gene is less effective at induction of early flowering in poplar than is the Arabidopsis LEAFY gene, phenotypic effects vary with tree age, and effects on flowering and vegetative growth vary widely among poplar genotypes. For effective control of flow-ering in trees, it appears that genes that act to regulate maturation state, rather than genes like LEAFY that control floral meristem development, will be required.

S27 Plant Carbohydrate Metabolism

S27 - 5

TARGETING THE FUNCTION OF THE DIFFERENTIALLY EXPRESSED GENES IN THE SINK-SOURCE TRANSITION LEAVES

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In our effort to understand the molecular mechanisms controlling leaf ontogeny, we initiated a molecular approach to identify and isolate the developmentally regulated genes in the sink and source leaves of tobacco. Ten differentially expressed gene fragments (S/S1-S/S10) were identified by differential display analysis. mRNA expression profiles of these gene fragments were determined by RNA blotting analysis, in which gene expression patterns could be categorized into five types; ubiquitous expression (S/S1), sink tissue-specific expression (S/S4, S/S5, S/S6,S/S9 and S/S10), sink tissue-enhanced expression (S/S7 and S/S8), source tissue-specific expression(S/S3)and source tissue-enhanced expression(S/S2). Sequence analyses and database searches showed that three gene fragments (S/S1, S/S5 and S/S6) were novel. Seven gene fragments had varying percentages of similarity to known proteins whose function in sink or source transition leaves is unclear. To explore the function of these genes in the leaf development, we introduced these gene fragments (3'-terminal sequence of genes)into a plant viral vector(Potato virus X expression vector) in sense or antisense orientation. To induce post transcriptional gene silencing of the homologous host gene after inoculation of Nicotiana benthamiana, we have determined that symptoms of systemic infection appeared at 7 days post inoculation (dpi) and the silencing initially occurred at 14 dpi with the altered phenotype in the young emerging leaves. At 21 dpi, virus symptoms were decreased in the emerging leaves and the silencing phenotype appeared in a veincentric pattern. The molecular and genetic analyses of silencing phenotypes of different genes are in progress.

S27 - 8

ANTISENSE REPRESSION OF FRUCTOSE-6-PHOSPHATE, 2-KINASE/FRUCTOSE-2, 6-BISPHOSPHATASE IN ARABIDOPSIS THALIANA ALTERS THE PHOTOSYNTHETIC CARBON PARTITIONING IN GREEN TISSUE.

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Fructose-2,6-bisphosphate (Fru-2,6-P2) is an important metabolite involved in the regulation of carbohydrate metabolism in plants. The bi-functional enzyme fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase (F2KP) is responsible for the synthesis and degradation of Fru-2,6-P2. The regulatory role of Fru-2,6-P2 was studied by the use of transgenic Arabidopsis plants with reduced expression of F2KP. A relationship between the enzymatic activity of fructose-6-phosphate,2-kinase and the level of Fru-2,6-P2 was observed in the transgenic plant lines. The partitioning of carbon was studied by incorporation of 14CO2 into photosynthetic products. Plant lines with less than 10% Fru-2,6-P2 compared to wild type exhibited an altered 14C-sucrose to 14C-starch ratio during the photoperiod (1.2-1.5 for plants with low levels of Fru-2,6-P2 compared to 0.7 for wild type). Plant lines with intermediate levels of Fru-2,6-P2 had an unaltered 14C-sucrose to 14C-starch ratio compared to wild type. The diurnal content of sugars and phosphorylated intermediates determined in the transgenic plants, indicated that the level of Fru-2,6-P2 has an effect on the partitioning of carbohydrates in the photoperiod. In addition, the photosynthetic capacity of Fru-2,6-P2 low plants was compared to the wild type.

S27 - 12

REGULATION OF SOURCE-SINK RELATIONS BY BRASSINOSTEROIDS

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Brassinosteroids (BRs) induce various growth responses when applied exogenously to plant tissues and the analysis of biosynthetic mutants revealed an essential role for plant growth and development. Only few BR regulated genes have been identified so far and the corresponding gene products are assumed to be involved in cell elongation. The present study shows that BR growth responses are linked to the regulation of carbohydrate metabolism by the induction of the mRNA for the key enzyme of an apoplastic phloem unloading pathway. Addition of BRs to autotrophic tomato suspension culture cells specifically elevates the activity of cell wall bound invertase, whereas the intracellular invertase activities were not affected. This enhanced enzyme activity was shown to correlate with the induction of the mRNA of extracellular invertase Lin6, whereas the mRNA levels of the other three extracellular invertase isoenzymes were not affected. The induction level induced by different BRs correlates with their growth promoting activity. The physiological significance of this regulation is further supported by the low concentrations and short incubation times required to induce Lin6 mRNA. This regulatory mechanism results in an elevated uptake of sucrose via the hexose monomers and thus an increased supply of carbohydrates to the BR treated cells. Experiments with tomato seedlings showed that the localized BR dependent growth response of the hypocotyl elongation zone was accompanied by a specific induction of Lin6 mRNA that is restricted to the corresponding tissues. This study demonstrates a role of BRs in tissue specific source/sink regulation.

S27 - 17
NEW ASPECTS ON SUCROSE TRANSPORT IN HIGHER PLANTS

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In leaves, sucrose uptake kinetics are composed of several components with different kinetic parameters. SUT1 serves as a high affinity transporter essential for phloem loading and long distance transport in solanaceous species as demonstrated by transgenic plants. The presence of SUT1 protein was shown to be restricted to the plasma membrane of sieve elements, whereas SUT1 mRNA was localized in companion cells as well. The presence of SUT1 mRNA in the phloem sap and the ability of CmPP16 to traffic this mRNA through plasmodesmata was demonstrated by other groups (Xoconostle-C‡zares, et al., 1999; Ruiz-Medrano et al., 1999). Phloem mobility of the sucrose transporter mRNAs and their proteins has been analysed in heterografted plants. We identified new sucrose transporter-like proteins from tomato. Their expression pattern overlaps that of SUT1. We immunolocalized the transporters on the light, as well as on the electron microscopic level in several solanaceous species and analyzed their expression pattern by RNase protection assays and by Western Blots. Functional analyses will be described.

Xoconostle-C‡zares, B., Xiang, Y., Ruiz-Medrano, R., Wang, H. L., Monzer, J., Yoo, B. C., McFarland, K. C., Franceschi, V. R., and Lucas, W. J. (1999). Plant paralog to viral movement protein that potentiates transport of mRNA into the phloem. Science 283, 94-98.
Ruiz-Medrano, R., Xoconostle-C‡zares, B., Lucas, W.J. (1999) Phloem long-distance transport of CmNACP mRNA: implications for supracellular regulation in plants. Development 126, 4405-4419.

S27 - 19
MANIPULATION OF SIGNALLING PATHWAYS INVOLVED IN CARBON PARTITIONING IN POTATO AND WHEAT.

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SNF1 (Sucrose-Non-Fermenting) related protein kinases (SNRK1's) have been isolated from a number of higher plants including barley (Halford et al., 1992) and potato (Man et al., 1997), and recently a homologue to the barley gene has been cloned from wheat (Laurie & Halford, unpublished data). We are studying the involvement of these genes in carbon partitioning in wheat and potato using a transgenic approach. Antisense constructs have been introduced into wheat and potato and plants are currently being analysed. In addition to stable transformation experiments, a system of transient expression of promoter-reporter constructs in isolated wheat endosperm is being developed, to look at the interaction of the kinase with the alpha-amylase promoter from wheat. The involvement of different sugars in signalling of gene activity is being examined in wheat by incubation of cut ears in nutrient medium, and in potato using culture of tissue discs. Kinase activity is then being monitored by the SAMS peptide assay. A yeast two-hybrid system has been used to identify further components of the signal transduction pathway and an Apetala 2-type transcription factor has been isolated from barley that interacts with SNRK1. Attempts are underway to clone this in wheat and potato, and fully characterise its function. 5'-truncated sequence from wheat is currently being cloned into suitable constructs for use in transient expression studies, and full length sequence will be expressed in E.coli for further study of the protein.

Refs; Halford, N.G., Vicente-Carbajosa, J., Sabelli, P.A., Shewry, P.R., Hannappel, U., Kreis.M. (1992) Molecular analyses of a barley multigene family homologous to the yeast protein kinase gene SNF1. Plant J., 2:791-797.
Man, A.L., Purcell, P.C., Hannappel, U., Halford. N.G. (1997) Potato SNF-1 related protein kinase: molecular cloning, expression analysis and peptide kinase activity assay. Plant Mol. Biol. 34:31-43

S27 - 30
CLONING AND CHARACTERIZATION OF GENES REGULATING WATER STRESS TOLERANCE IN RICE

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Drought is a major constraint to rice production in rainfed ecosystems. Thickness, length and penetration ability of roots are associated with drought tolerance in rice. Incorporation of root traits into breeding program has been difficult due to the laborious nature of measuring root characters. While root-specific genes of unknown function have been cloned from rice, genes governing root-pattern and drought adaptation have not been isolated. Identifying genes regulating root growth under water stress could be used to develop rice cultivars better adapted to drought prone environments. Based on the genetic variation in root morphology and anatomy under normal and water stress conditions, root penetration ability under wax-petrolatum system, seminal root elongation under rapid screening test, germination and early seedling growth under reduced water potentials and drought recovery ability, we have identified land races, Nootripathu and Norungan with desirable root system traits as potential sources of genes influencing water stress tolerance in rice. The cultivar, IR 20 with shallow rooting habit is used as susceptible check. The differential display RT-PCR of RNA isolated from the irrigated and drought subjected roots of Nootripathu and subsequent electrophoretic analysis of the DD-RT-PCR products revealed a number of differentially expressed sequences. Thirty putative differentially expressed sequences were eluted from the gel and reamplified. Northern hybridization analysis of irrigated and drought subjected root RNAs of Nootripathu and IR20, using the putative differentially expressed sequences as probes are performed. The results will be discussed in terms of understanding the molecular basis of root growth under stress and their utility as molecular markers for drought resistance breeding in rice.

S28 Molecular Farming

S28 - 1
EVALUATION OF PLANT OILBODIES AS A VACCINE DELIVERY SYSTEM

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Developments in genetic engineering and immunology are providing major opportunities for novel approaches in vaccine design. Oral vaccines are being developed by producing antigens within the edible portion of genetically engineered plants. However, there are many potential problems and limitations associated with oral vaccines and its delivery, including immune tolerance and allergic reactions. Edible vaccines are also not amenable to other forms of delivery since purifying antigens from plant crude extracts is often difficult and expensive.
A plant expression system has been developed based on targeting recombinant proteins to oilbodies. Oilbodies are subcellular organelles found in seeds that function to store oil during periods of dormancy. They have a diameter 0.5-1.0 mM and are composed of triacylglycerol surrounded by a half unit phospholipid membrane. Embedded in this membrane are proteins known as oleosins. Oleosins prevent oilbodies from coalescing within the cytosol. Recombinant proteins can be targeted to the surface of oilbodies by engineering a translational fusion between an oleosin gene and a foreign sequence. Since oilbodies are less dense than water, they can be purified from other plant constituents by flotation centrifugation. The physical properties of oilbodies have prompted our interest in using them as an antigen presentation vehicle.
The objective of this study was to assess whether transgenic oilbodies can be used as a vaccine delivery system. Since producing transgenic plants is a lengthy and labor intensive process, we have developed a model oilbody system that emulates transgenic oilbodies. The model system involves chemically biotinylating oilbodies and then adding a protein complex consisting of streptavidin and an enzymatically biotinylated antigen. This model system has allowed us to address questions dealing with the use of oilbodies as a presentation vehicle. Mice immunized with model oilbodies displaying a bacterial antigen from Neisseria meningitidis produce a specific immune response towards the antigen.

S28 - 2
PRODUCTION OF A CANINE ORAL PAPILLOMAVIRUS VACCINE IN TOBACCO

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We are testing the feasibility of using tobacco to produce a vaccine for canine oral papillomavirus (COPV), the causative agent of a common disease in dogs, oral papillomatosis. While most cases are benign, some progress to malignant tumors. Recombinant versions (produced in insect cells) of virus like particles (VLPs) formed from self-assembly of the major viral capsid protein (L1) have been shown to be highly immunogenic. Because preparation of the vaccine using the insect production system is inefficient and laborious, it is not scaleable to the commercial level.
Tobacco produces sufficient biomass to allow cost-effective recovery of both native and recombinant proteins. The value derived from high yields of ribulose-1,5- bisphosphate carboxylase (RUBISCO), a protein with numerous applications, should be sufficient to offset crop production cost.
We produced stable transformed tobacco cell cultures with a cassette containing the COPV L1 encoding sequence fused to the 35S promoter. These cultures are producing L1 RNA. We have also transformed tobacco plants with a similar cassette and these too are producing L1 message. We are currently working to determine the level of L1 protein made in both the tobacco cell cultures and plants. Use of the 35S promoter, a pseudo-constitutive promoter, is expected to result in production of the recombinant protein in most of the harvestable biomass of the plant, greatly facilitating protein recovery and enhancing yield. Based on studies performed in insect cell cultures, we expect L1 extracted from tobacco to produce immunogenic virus like particles after separation from RUBISCO. Papillomaviruses are associated with several types of human cancers. We hope to use a similar approach to produce vaccines for these diseases.

S28 - 4
TOWARDS THE DEVELOPMENT OF A HERBAL VACCINE TO HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 (HIV-1): EXPRESSION OF ENV AND TAT ANTIGENS IN PLANTS.

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WHO and UNAIDS have estimated that, by the end of 1999, 33.6 millions people would be living with HIV/AIDS worldwide, with the hardest-hit region concentrated in Sub-Saharan Africa. An edible vaccine (costÐeffective and safe) able to induce mucosal and systemic immune responses would be particularly indicated for HIV-1, which is mainly transmitted via mucosal surfaces. Plants and plant viruses have been recently utilised to express a variety of viral and bacterial antigens successfully applied as experimental immunogens either by parenteral inoculation or oral administration.
In this study, highly conserved immunogenic epitopes/proteins of HIV-1 have been expressed through the viral vector PVX (potato virus X). To this end, two different strategies have been used. The "epitope presentation" has been used to express an epitope from the envelope glycoprotein gp 41, which is recognised by a neutralising human monoclonal antibody (mAb 2F5). This strategy is based on the fusion of the 2F5 epitope to the coat protein (CP) of PVX, which is used as a carrier of this peptide (PVX-2F5). The "gene insertion" has been used to express the regulatory Tat protein as a single entire ORF (PVX-tat). Upon systemic infection of N.benthamiana plants, the correct expression of foreign sequences has been verified by RT-PCR, Western blot and ELISA. Our results have shown that the modified CP (PVX-2F5) and the accessory ORF (PVX-tat) did not interfere with correct assembly of virions, which moved both locally and systemically. The PVX-2F5

chimaeric virus has been purified, and used to immunise mice through intranasal and intraperitoneal routes. We have also verified that the expression of Tat protein in infected tissues is sufficient to use the crude leaf extracts directly as experimental immunogens. Ongoing studies will evaluate if the immune response elicited by Tat protein expressed in plant tissues is comparable or better to that induced by E. coli-derived Tat protein.

S28 – 6
PROTECTIVE ANTI-HERPES ANTIBODIES FOR REPRODUCTIVE HEALTH

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Herpes is an incurable sexually transmitted disease (STD) caused by the herpes simplex virus (HSV). In the United States, 22% of people 12 years and older are infected with HSV-2. This year in the United States, 15 million people will contract a STD; approximately 1 million of these will be new HSV-2 infections. At present, there is no effective way for a sexually active person to prevent HSV infections. HSV can also cause potentially fatal infections in the immunocompromised and in infants infected during delivery. We are developing antibody products that will prevent infection by HSV and an antibody product that will be used to treat infected neonates. The human sequences encoding anti-HSV antibodies as an IgG,IgA, dIgA, and sIgA were introduced into rice cells. Transgenic cells were selected and regenerated into plants. The majority of rice lines assayed produced assembled antibody. The rice-produced antibodies were able to neutralize HSV-2 in standard in vitro neutralization assays. The rice-produced antibodies were also able to prevent vaginal HSV-2 infection in mice. For large-scale consumer health applications, plants may offer the only method for producing protective antibodies at costs low enough to be used for over-the-counter products and in developing world markets.

S28 - 7
EXPRESSION OF HUMANIZED ANTIBODY IN TRANSGENIC TOBACCO CHLOROPLASTS

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Dental caries (cavities) is probably the most prevalent disease of humankind. Colonization of teeth by Streptococcus mutans is the single most important risk factor in the development of dental caries. A topical monoclonal antibody therapy to prevent adherence of S. mutans to teeth has recently been developed. The incidence of cariogenic bacteria (in humans and animals) and dental caries (in animals) was dramatically reduced for periods of up to two years after the cessation of the antibody therapy. The annual requirement for this antibody in the US alone may eventually exceed 1 metric ton. The high copy number of the chloroplast genome (~10,000 copies per cell) makes the hyper-expression of foreign genes in chloroplasts possible. Chloroplasts also process a number of eukaryotic proteins, including proper folding and formation of disulfide bonds. Coordinated expression of multi-subunit proteins may be facilitated by the ability of chloroplasts to translate polycistronic messages. The compartmentalization provided by chloroplasts also facilitates purification of foreign proteins. Environmental concerns (out-cross, toxicity of pollen to insects etc) and other undesirable consequences (position effect, gene silencing, pleiotropic effects etc.) of nuclear expression could also be overcome.
Therefore, this antibody was expressed via the chloroplast genome to achieve higher levels of expression and proper folding. The integration of antibody genes into the chloroplast genome was confirmed by PCR and Southern blot analysis. The expression of both heavy and light chains was confirmed by western blot analysis under reducing conditions. The expression of fully assembled antibody was confirmed by western blot analysis under non-reducing conditions. This is the first report of successful assembly of a multi-subunit eukaryotic (human) proteins in transgenic chloroplasts. Production of monoclonal antibodies at agriculture level should reduce their cost and create new applications of monoclonal antibodies.

S28 - 9
EXPRESSION OF HUMAN INSULIN IN TRANSGENIC TOBACCO CHLOROPLASTS

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Attempts have been made to synthesize high value pharmaceutical proteins in nuclear transgenic plants. But, levels of expression have been low. Hyper-expression of foreign proteins (up to 30% of total cellular protein) has been accomplished via chloroplast genetic engineering with expression of 10,000 copies of foreign genes per cell. Chloroplasts are ideal for this purpose because of their ability to process eukaryotic proteins, including folding and formation of disulfide bridges and compartmentalization of foreign proteins facilitating their purification. Tobacco is also an ideal choice for this purpose because of its large biomass, ease of scale-up, genetic manipulation and an impending need to explore alternate uses for this hazardous crop.
The first objective of this project is to use a thermally responsive biopolymer as a fusion protein to enable hyper-expression of insulin and accomplish rapid single step purification of fusion peptides utilizing the inverse temperature transition properties of this polymer. Also, feeding a small dose of insulin conjugated with the non-toxic b subunit moiety of the cholera toxin (CTB) suppressed beta cell destruction and clinical diabetes in adult non-obese diabetic (NOD) mice. Therefore, the second objective of this project is to develop insulin-CTB fusion protein in chloroplasts of nicotine free edible tobacco (LAMD 605) for oral delivery to NOD mice. Chloroplast vectors were constructed for expression of proinsulin as a fusion protein either with the polymer or CTB. Expression of the biopolymer-proinsulin fusion protein in E. coli was confirmed by western blots. Proinsulin fusion with polymer did not alter the inverse temperature transition property of the biopolymer. After expression of the fusion protein was confirmed in E. coli, tobacco plants were transformed by particle bombardment. Integration of the biopolymer-proinsulin fusion gene into the chloroplast genome was confirmed by PCR. Among the transgenic plants tested, 22 out of 23 showed integration of foreign genes into the chloroplast genome. Characterization of transgenic expression of proinsulin CTB or polymer fusion proteins in chloroplasts will be presented.

S28 - 10

DEVELOPMENT OF NEW PROPHYLACTIC AND THERAPEUTIC VACCINES AGAINST CERVICALCANCER USING PLANT-DERIVED ANTIGENS OF THE HUMAN PAPILLOMA VIRUS 16.

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Plants are currently being used as a cost-effective and safe heterologous system for the expression of functional biomolecules. Plant-derived recombinant antigens of animal viruses or bacteria have already been successfully used as experimental vaccines, administered either by parenteral inoculation or by oral administration. Two basic strategies can be used to obtain recombinant proteins in plants, the generation of transgenic plants or the use of plant viruses as vectors for transient expression. Both strategies have some limitations, and have been compared in the present research. The human papilloma virus (HPV16) is involved in the pathogenesis of cervical cancer. In an attempt to develop a preventive vaccine against HPV, the gene encoding the HPV16 L1 coat protein (which has been shown to self-assemble into virus-like particles in eukaryotic cells) was cloned into PVX-derived epichromosomal expression vectors under the duplicated coat protein promoter of PVX. When infective RNA transcripts or DNA plasmids were used to infect N. benthamiana plants, no recombinant L1 were detected. Most probably, the adjunctive HPV16 coat protein gene interfered with the PVX replication process, which indicates that the production of this antigen might only be possible through stable transformation. Conversely, when a PVX-derived vector was used to express the original HPV16 E7 gene, high amounts of soluble E7 protein were detected. E7 is an oncoprotein highly expressed in human cells during malignancy, and presumably a 'Tumor Associated Antigen' believed to be a good candidate to develop a therapeutic vaccine. Ongoing experiments consist in administering subcutaneously crude E7-expressing plant extracts to C57black/6 mice, in order to raise a cell-mediated immune response.

S28 - 11

COST ANALYSIS FOR GMP PRODUCTION OF RECOMBINANT PROTEINS IN AN OILBODY BASED SYSTEM

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Traditionally, recombinant protein production has been carried out using microbial fermentation or cell culture systems. More recently, recombinant proteins have been produced in the milk of transgenic animals. Many of these methods of production have been amenable to scale-up to produce commercial quantities of proteins, demonstrating various degrees of success and cost. With advances in plant transformation and expression, plant-based systems are now being considered as alternatives to these more traditional methods of recombinant protein production. The potential advantages of plant-based systems include higher capacity and lower costs of production. The question that arises is, do these represent any significant advantages in production requirements to result in a competitive economic advantage and cost savings to the consumer? Both fermentation and animal based systems fall under strict production guidelines known as Good Manufacturing Practices (GMP). These provide minimum acceptable limits for the processes of extraction, purification, quality control, supply continuity and reproducibility for biopharmaceutical products to be accepted for release and use by humans. For biopharmaceutical protein production the rigorous GMP guidelines will also apply to plant production systems so the cost savings, if any, will need to be determined. The ultimate goal is to develop GMP guidelines that ensure the products produced from transgenic plants are as safe and effective as biologicals produced by any other method. In order to produce GMP quality products, traditional systems and some plant-based systems will require several chromatographic steps, which on a commercial scale are extremely costly. At SemBioSys Genetics Inc. we have developed a proprietary oilbody-based production system in oilseeds plants which will significantly reduce the need for many of these costly chromatographic steps to produce ultra-pure protein products. We will describe this system and present a cost model to confirm the advantages of our plant-based production system of recombinant proteins for the pharmaceutical, industrial, food and cosmetic industries.

S28 - 17

A NOVEL METHOD FOR CLEAVAGE OF RECOMBINANT FUSION PROTEINS

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Oleosin technology involves expression of foreign peptides as oleosin fusions that are specifically targeted to the surface of plant seed oil bodies. This system allows for rapid and simple separation of the desired peptide from endogenous plant proteins through flotation centrifugation of the oil bodies. Subsequent downstream purification requires enzymatic or chemical cleavage of the oleosin fusion in order to recover the desired protein in its native form. Very few proteolytic enzymes provide for the specificity and efficiency that is required for commercial production. Yield losses of recombinant protein higher than 90% due to inefficient cleavage are not atypical. In addition, the currently widely used proteolytic enzymes, such as blood clotting factor Xa, thrombin or enterokinase are very expensive.
Autocatalytic maturation of inactive protease precursors (zymogens) such as pepsin and chymosin involves an activation step, elicited by low pH, followed by a processing step in which the amino terminal propeptide is cleaved. Both intramolecular and intermolecular cleavages are involved in the processing step that results in the formation of the mature protease. We have used the protease maturation system as a method for cleaving recombinant fusion proteins. DNA encoding the prosequence for the aspartic protease chymosin, was fused upstream of two heterologous genes encoding either hirudin or carp growth hormone and expressed in a bacterial host. We show that the resulting fusion peptides are cleaved at the C-terminus of the pro sequence by active chymosin . This novel use of a protease pro sequence in a heterologous system provides possibilities for accurate, efficient and cost effective cleavage of commercially produced fusion peptides.

S28 - 18
STRATEGIES USED TO EXPRESS HEPATITIS A VIRUS CAPSID IN TRANSGENIC PLANTS

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Transgenic plants expressing recombinant pharmaceuticals proteins for therapeutic applications are increasingly used due to the low cost of production of molecules from vegetal cultures.
Hepatitis A is an infectious and endemic disease that is caused by a virus of the same name (VHA). The virus is present in most countries and the main mode of transmission is by the fecal-oral route, from one person to another and through the ingestion of contaminated water or food. The virus has been isolated from excreta, blood and seminal liquid. A single RNA strand of 7,480 kb is the viral genome component that codifies for a large polyprotein which has a post-translational processing to form structural and non-structural proteins. The evidence supports the existence of immunodominant antigenic regions, which depend on the final conformation. Recombinant polypeptides and synthetic peptides expressed in different heterologous systems have shown poor antigenic activity; while, the synthetic capsid or assembled pentamers show increased antigenic properties. Here, we show our strategy to obtain the synthetic capsid of HAV in transgenic plants using the virus's ORF and the coexpression of the P1-2A and P3 regions under 35S CaMV promoter.

S28 – 20
HUMAN INTERLEUKIN-10 PRODUCED IN TRANSGENIC PLANTS IS CORRECTLY ASSEMBLED AND BIOLOGICALLY ACTIVE

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Plant biotechnology is transforming agriculture, adding new traits to crop plants at a fast pace. For some time, emphasis has been placed on input traits that allow better yield and benefit the producer more than the consumer. Recently, more attention has been directed toward using plants as bioreactors for the production of value-added proteins at a lower cost than presently available. Such proteins include vaccines, antibodies, therapeutic proteins, industrial enzymes and nutraceuticals. Human interleukin-10 (hIL-10) is an anti-inflammatory cytokine that has been reported to play a therapeutic role in numerous autoimmune disorders such as inflammatory bowel disease (IBD), psoriasis, thyroiditis, allergic airway inflammation and encephalomyelitis. To explore the effectiveness of oral administration of IL-10 in treating IBD, sufficient low-cost IL-10 is needed. We have transformed low-nicotine tobacco plants with the hIL10 cDNA using Agrobacterium. The hIL10 gene was under the control of either a viral or a plant-derived constitutive promoter, and was targeted to different cellular compartments. Plant recombinant hIL10 was produced in both greenhouse and field trials. Western analysis has clearly demonstrated that the hIL-10 homodimer is properly assembled by plants, and murine cell assays have shown that it is biologically active. We are in the process of conducting IL-10 feeding trials on a mouse model for IBD.

S28 - 25
A PLANT-BASED VACCINE AGAINST PORCINE PARVOVIRUS

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Porcine parvovirus (PPV) is a single-stranded DNA virus that affects swine and is widespread in all pig-producing regions of the world. PPV causes fetal death and mummification, reproductive failure in sows, diarrhea in young piglets, and a delayed return to oestrus. Certain strains also cause skin lesions. The viral capsid is comprised of VP1 and VP2 proteins. When VP2 is expressed in a baculovirus system these proteins self-assemble to form a virus-like particle, and have been shown to elicit protective immunity in vaccinated pigs. Even with vaccination, this disease causes significant mortality in swine and, therefore, better vaccines are required. We have expressed the VP2 protein from PPV in transgenic tobacco plants. For this, the VP2 gene (Kresse strain) was introduced into the binary vector pCamTer X downsteam of the duplicated 35S cauliflower mosaic virus promoter and the alfalfa mosaic virus leader sequence, and upstream of the nos terminator. This binary vector was used for the Agrobacterium-mediated transformation of tobacco. The resulting transgenic plants were confirmed to have the VP2 gene by PCR. Soluble protein was extracted from transgenic plants and analysed by Western blotting. Two proteins of approximately 60 kDa and 30 kDa were detected using VP2-specific antiserum, but only the 60 kDa protein was visible on a stained gel. These transgenic plants will be used to develop an economical, easily administered oral vaccine against PPV.

S28 - 26
PLANT TRANSFORMATION FOR THE PRODUCTION OF AN ORAL VACCINE AGAINST INFLUENZA VIRUS

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Oral vaccines have considerable advantages over parenteral delivered vaccines including ease of administration and improved safety and have been highlighted as a priority by the World Health Organization ChildrenÕs Vaccine Initiative. The production of transgenic plants which provide an oral vaccine against several disease organisms has now been well-established. We are developing an edible oral vaccine to influenza virus A/Sichuan/2/87 using the highly immunogenic haemagglutinin (HA) protein. The target plants for transformation are tomato and lettuce. Two approaches will be taken in the engineering of the plants with vaccine genes, (1) Agrobacterium tumefaciens-mediated transformation, resulting in stable nuclear transformation and (2) Biolistic transformation of chloroplasts, which should lead to high levels of protein expression. To test whether the HA gene constructs are suitable for chloroplast transformation, the HA gene was expressed in a prokaryotic protein expression system. Full length HA genes are not expressed in the prokaryotic system, however, deletion of the amino-terminal transit peptide and the carboxy-terminal membrane anchoring domain restores protein expression. Therefore, a modified HA gene lacking the transit peptide and membrane anchoring domain, but including a SEKDEL motif is being cloned under the control of the CaMV

35S promoter into the vector, pROK2, for nuclear transformation. Another approach we are using to boost vaccine efficacy is to include genes for an adjuvant, the cytokine interleukin 12 (IL-12) which can be administered in the same foodstuff as the vaccine. IL-12 is a heterodimeric protein, with subunits of 35 kDa and 40 kDa. Protein expression data for the IL-12 subunit genes and progress in plant transformation will be reported.

S28 - 27
CEREAL GRAINS AS PRODUCTION FACTORIES FOR VALUABLE PHARMACEUTICAL ANTIBODIES

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The potential of antibodies for human health demands large amounts of diverse recombinant antibodies at reduced cost with no risks for the patient for applications in diagnosis and therapy. The use of plants to produce recombinant antibodies has distinct advantages over microbial or animal production systems.
Our work demonstrates the use of transgenic wheat and rice as bioreactors to produce macromolecules of high therapeutic and diagnostic value. We successfully expressed a therapeutic single chain Fv antibody (scFvT84.66) against carcinoembryonic antigen (CEA), a well characterized tumor-associated marker antigen. ScFv constructs were engineered for recombinant antibody targeting to the plant cell apoplast and ER. Up to 30 ug g-1 of functional recombinant antibody was detected in the leaves and seeds of wheat and rice. We confirmed that transgenic dry seeds could be stored for at least five months at room temperature, without significant loss of the amount or activity of scFvT84.66. In addition, we are also working with a secretory immunoglobulin A with specificity to the oral pathogen Streptococcus mutans. The use of edible plants such as rice, maize and wheat as production vehicles for this molecule may eliminate the need for purification of the antibody prior to treatment, thus providing a cost-effective option for the passive treatment of diseases of the oral cavity.
Our findings provide a foundation for exploiting alternative uses of cereal crops for production of antibody molecules that have already shown efficacy in clinical applications.

S29 Gene Mapping and Genomic syntheny

S29 - 3
PROFILING OF GENE EXPRESSION IN SHOOT ORGANOGENIC CULTURES OF LOBLOLLY PINE USING DD-PCR AND cDNA CLONE ARRAYS

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In plant tissue culture, organogenesis is a process of differentiation by which plant organs are formed de novo or from pre-existing structures. Plant growth and development is inherently complex. In the de novo shoot organogenesis, precursor cells, triggered by plant growth regulators, will develop competence for cell division, differentiation and subsequently formation of shoot buds. These developmental events require changes in the expression level of numerous selected genes which result in different structural and morphological characteristics of organs. In this report we describe utilization of newly developing molecular techniques, a combination of differential display and a large number of cDNA clone arrays to study gene expression differences between organogenic and non-organogenic tissues. These methods enable us to monitor the profile of genes differentially expressed in different tissues, at a specific developmental stage. Several genes that may have biological relevance in the process of de novo shoot organogenesis have been identified through this approach. Correlation of the altered expression patterns of these genes with the developmental process may provide new insight into the regulation of morphogenesis and in vitro plant regeneration.

S31 Abiotic/environmental stresses

S31 - 1
TRANSGENIC TOMATO PLANTS EXPRESSING A LYCOPERSICON CHILENSE DROUGHT- INDUCED GENE DEMONSTRATE ENHANCED TOLERANCE TO WATER STRESS

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During our previous studies, we isolated a drought- and ABA- induced gene, pLC30-15, from the drought tolerant wild tomato, Lycopersicon chilense (Chen et al., 1993. Plant Physiol. 103:301). The protein encoded by pLC30-15 shares common characteristics with several drought- and ABA- induced proteins isolated from higher plants. However, it is unique due to a high content of glutamic acid and three lysine-rich K boxes in its primary structure. Transcripts of pLC30-15 increases to a high level upon dehydration and returns to control level once drought stressed plants are rewatered. pLC30-15 was introduced to cultivated tomato through Agrobacterium mediated gene transfer technique, using the CAMV 35S promoter. Transgenic plants with different levels of expression of pLC30-15 were self pollinated. Kanamycin resistant R1 plants along with R0 and control plants were evaluated for their level of tolerance to drought stress. A positive correlation was found between the level of expression of pLC30-15 gene, leaf water potential and the greenhouse performance of transgenic plants. Indeed, plants with a higher level of pLC30-15 transcripts were able to maintain a higher leaf water potential and a higher percentage of healthy, non wilted leaves. Overall our results suggest that pLC30-15 plays a positive role in drought tolerance. The potential function of this gene will be discussed.

S31 – 3
ANTIOXIDANT ENZYME ACTIVITIES ACCOMPANYING THE INDUCTION OF SALT TOLERANCE IN MANGROVE AVICENNIA MARINA

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Despite extensive research into salt tolerance in plants, little is known about the salt-defence related mechanisms. We are utilizing a halophyte, A. marina to elucidate and understand the biochemical functions associated with salt tolerance in halophytes. Treatments of A. marina with NaCl and seawater enhanced the activities of a number of antioxidant enzymes, most notably SOD, ascorbate peroxidase and glutathione reductase. In contrary, antioxidant enzyme activities decreased in cucumber (a glycophyte) following salt treatment. This indicates that salt treatment directly or indirectly causes oxidative stress in plants. Therefore it may be expected that amplified antioxidative enzyme activities in halophytes, as shown for A. marina may enhance resistance to salinity.

S31 - 5
OXIDATIVE STRESS AND CROP PLANTS: POSSIBLE STRATEGIES FOR IMPROVING TOLERANCE

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Plants can be exposed to an array of environmental stress conditions such as heat, drought, cold, air-pollution, herbicides, heavy metals and ionising radiations. Almost all of these adverse conditions result in enhanced production of active oxygen species that can cause severe damage to nucleic acids, cellular membranes and organelle, and consequently affecting plant growth and productivity. To provide temporary protection against the oxidative stress in plants, in addition to a variety of protective compounds, there are a number of antioxidant enzymes that facilitate the dismutation of active oxygen species. In recent years, genetically engineered plants with continuous over-production of an antioxidant enzyme have been produced that are more tolerant against one or other kind of environmental stress. However, a timely burst in expression, rather than a continuous over-production, of multiple antioxidant enzymes involved would be a better option. To understand the regulation of these antioxidant enzyme genes, under a variety of environmental stresses, a detailed study on their expression was undertaken in wheat and rice seedlings. Tolerant genotypes seem to have higher levels of superoxide dismutase transcripts than in sensitive genotypes. Besides the information on specific role of these antioxidant enzymes, specific inducer(s), to over-express these genes in non-transgenic plants, have been identified. Induction response was found to be more pronounced for plastidic-superoxide dismutase gene(s) than for the cytosolic-enzymes. Additionally, promoter sequences from wheat and rice, with potential application in inducible over-expression of stress related proteins, have been isolated. Strategies in coordinate induction of a battery of stress tolerant genes, to provide increased protection against a range of abiotic stresses, will be presented.

S31 - 14
CHARACTERIZATION OF A ROOT OXALATE OXIDASE (GERMIN-LIKE) INDUCED BY ALUMINUM EXPOSURE IN WHEAT

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Previously, we reported that oxalate oxidase (Oxo) mRNA expression increases in wheat (Triticum aestivum) root tips in response to toxic Al concentrations (Hamel et al 1998, Planta 205:531-538). This paper reports an increase in oxalate oxidase protein and activity in response to Al in tolerant and sensitive cultivars. The monomer protein accumulation was detected by an antibody directed against an antigenic region of WAR13.2 and the enzyme activity was detected using a colorimetric assay on nitrocellulose or by histochemical staining in situ. The protein and activity were generally apparent 24h after Al exposure. Higher Al concentrations elevated both Oxo protein accumulation and activity and was thus associated with Al toxicity. Since we have detected a high Oxo activity outside the cell by oxalate -dependent production of H2O2 and an important increase in root thickness following Al exposure, we propose that this source of H2O2 provides the necessary substrate to allow lateral cell wall expansion. This activity may also reduce cell wall porosity and raise the net negative charge thus reducing the rate of Al entry in the cell at low to medium Al toxic concentrations. Al does not induce these responses by amplifying root sensitivity to pathogens since axenic cultures are inducing Oxo activity at the same Al concentrations. At high Al concentrations, we have found, by Evans blue staining, that an increase in Oxo activity is correlated with cell death in wheat roots. This high H2O2 production thus results in symptoms that are similar to a hypersensitive response and supports our previous hypothesis that root tip cells possess a general defense response triggered by Al which may act as an abiotic elicitor.

S31 - 18
DROUGHT TOLERANCE CONFERRED BY THE YEAST TREHALOSE-6-PHOSPHATE SYNTHASE GENE ENGINEERED VIA THE CHLOROPLAST GENOME

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Water stress due to drought, salinity or freezing is a major limiting factor in plant growth and development. Trehalose is a non-reducing disaccharide of glucose and its synthesis is mediated by the trehalose-6-phosphate (T6P) synthase and trehalose-6-phosphate phosphatase complex in Saccharomyces cerevisiae. Because of its accumulation under various stress conditions such as freezing, heat, salt or drought, there is general consensus that trehalose protects against damages imposed by these stresses. Trehalose is also known to accumulate in anhydrobiotic organisms that survive complete dehydration, the resurrection plant and some desiccation tolerant angiosperms. This study has been undertaken to compare chloroplast and nuclear expression of TPS1 in transgenic plants to confer drought tolerance.
Yeast trehalose phosphate synthase (TPS1) gene was introduced into the tobacco chloroplast and nuclear genomes to confer drought tolerance. Northern blot analysis of transgenic tobacco showed that the chloroplast transformant expressed 16,966-fold more TPS1

transcript than that of the highly expressing nuclear transgenic plant. Although both the chloroplast and nuclear transgenic plants showed significant TPS1 enzyme activity, no significant trehalose accumulation was observed in F1 nuclear transgenic plants whereas chloroplast transgenic plants showed 15-fold higher accumulation of trehalose than the highly expressing nuclear transgenic plant. This may be due to the lack or lower trehalase activity in chloroplasts. Nuclear transgenic plants (F0) that showed significant amounts of trehalose accumulation showed stunted phenotype, sterility and other pleiotropic effects whereas chloroplast transgenic plants (F1/F2) showed normal growth and no pleiotropic effects. Chloroplast transgenic plants also showed a high degree of drought tolerance as evidenced by growth of transgenic plants in 6% polyethylene glycol whereas respective control nuclear transgenic plants died. In order to prevent escape of drought tolerance trait to weeds and associated pleiotropic traits to related crops, it is desirable to genetically engineer crop plants for drought tolerance via the chloroplast genome instead of the nuclear genome.

S31 - 19

A CRITICAL ROLE OF ETHYLENE IN HYDROGEN PEROXIDE RELEASE DURING PROGRAMMED CELL DEATH IN TOMATO SUSPENSION CELLS

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Programmed cell death (PCD) involves a sequence of events in which a cell directs its own death. PCD is essential for normal plant development (xylogenesis, reproduction, senescence) and survival (hypersensitive response). The most common form of PCD in animals, apoptosis, is accompanied by characteristic morphological changes and executed by a class of specific proteases, designated caspases. A tomato suspension culture was employed to study pathways involved in plant PCD. Treatment of the cells with the well known animal apoptosis inducer camptothecin, a topo-isomerase I inhibitor, induces cell death in tomato suspension cells. This chemical-induced cell death is accompanied with characteristic morphological changes and by internucleosomal DNA fragmentation. These observations indicate that camptothecin induces apoptotic cell death in tomato suspension cells.
To elucidate the different processes involved in apoptotic cell death, the effect of a range of specific inhibitors on camptothecin-induced cell death was tested. Our results show the involvement of a calcium influx, release of H2O2 and activation of caspase-like proteases, eventually leading to cell death. Ethylene, although in itself not capable of inducing cell death in tomato cells, was shown to play an essential role in apoptosis as it greatly stimulated camptothecin-induced cell death. Conversely, inhibitors of ethylene biosynthesis or action completely diminished cell death. Ethylene was found to exert its effect at the level of the oxidative burst as it greatly stimulated camptothecin induced H2O2 release. A hypothetical model of apoptotic cell death regulation in plants will be presented.

S31 - 24

ISOLATION AND IDENTIFICATION OF OSMOTIC STRESS INDUCED mRNAs FROM OAK (QUERCUS SPP.)

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The adaptation of individuals and populations to the environment is not well understood due to the lack of knowledge on genes involved in these processes. Therefore there is a need to develop DNA polymorphisms representing genes involved in adaptive processes; such non-neutral markers would indicate the differentiation of populations on the basis of selective and adaptive traits. The two main central European oak species, Quercus robur and Quercus petraea are genetically closely related, however, Q. petraea tends to grow predominantly on slightly drier soils covering lower altitude slopes and ridges while Q. robur populates temporary waterlogged, heavy low land soils.
In our study, we intended to provide tools for analyzing the adaptation of these two species to the different microclimatic conditions. Several types of physiological functions may be responsible for the adaptation of oaks to these microclimatic conditions, one of those could be genes involved in the osmotic regulation of the cells. Therefore the identification of such genes was the goal of the present study, using oak suspension culture cell line for cloning mRNAs induced under osmotic stress.
Messenger RNA populations of cells growing in normal as well as high osmotic media were compared by a PCR based subtraction hybridization procedure (CLONTECH). Eventually we could identify 34 osmotic stress modulated clones. Clarifying the putative identity of 22 of them by BLAST search allowed us to group some of them as i/ glycolysis and Krebs cycle (4 clones), ii/ ribosome structure and protein translation (6 clones), and iii/ lipid metabolism (2 clones) related clones. Two additional isolates contained repetitive elements, micro and minisatellite regions respectively, however the putative identity of these clones is still obscure. Three of the clones: the putative Fiddlehead protein (FDH), the Betaine aldehyde dehydrogenase (BADH) and the putative lipid transfer protein (LTP) were fully sequenced.

S31 - 25

ENHANCED ACCUMULATION OF BiP IMPACTS PLANT DEVELOPMENT AND CONFERS TOLERANCE TO ABIOTIC STRESSES 1

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The binding protein BiP is an important component of both the constitutive endoplasmic reticulum (ER) function and the ER-stress response of cells. Despite extensive studies in cultured cells, a protective function of BiP against stress has not yet been demonstrated in whole multicellular organisms. Here, we have obtained transgenic tobacco plants (Nicotiana tabacum L. cv. Havana) constitutively expressing elevated levels of BiP to analyze the protective role of this ER lumenal stress protein at the whole plant level. Under greenhouse conditions, the transgenic plants exhibited increased growth rate that led to a significant reduction in their time to flowering, as compared to wild type plants. The developmental differences in the transgenic plants suggest that the basal level of BiP may limit the ER function in plants under normal, non-stressed conditions. Furthermore, elevated levels of BiP in transgenic lines conferred resistance to the glycosylation inhibitor tunicamycin during germination and tolerance to water deficit during plant growth. Under progressive drought, the leaf BiP levels correlated with the maintenance of the shoot cellular turgor. Collectively, these results demonstrate that overexpression of BiP in plants alters growth dynamics under normal conditions and confers tolerance to abiotic stresses.

S31 - 40

CHANGES IN GENE EXPRESSION DURING PROGRAMMED CELL DEATH IN TOMATO CELL SUSPENSIONS

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Programmed cell death (PCD) is a process aimed at eliminating unnecessary or harmful cells during growth and development of multicellular organisms. In plants, PCD plays an essential role in, for example, xylogenesis, the hypersensitive response (HR), aerenchyma formation, senescence, and embryogenesis. To identify genes involved in plant PCD, changes in gene expression were studied in a model system of suspension-cultured tomato cells undergoing PCD. In this system, cell death is triggered by treatment with camptothecin, an inhibitor of topoisomerase I. Cell death was accompanied by internucleosomal DNA degradation, indicating that the cell death process shares similarities with apoptosis in animals. Tomato homologues of DAD1 and HSR203, two genes that have been implicated in PCD elsewhere, were isolated. Furthermore, a differential display approach was used to identify novel genes that show changes in expression levels during camptothecin-induced PCD. This resulted in isolation of two up-regulated and five down-regulated cDNA clones. Addition of the calcium channel blocker lanthanum chloride prevented camptothecin-induced cell death. The effect of lanthanum chloride on camptothecin-induced gene expression was studied to discriminate between putative cell death genes and general defence genes. The various (predicted) gene products are compared to sequences present in public databases and their possible roles in plant PCD are discussed.

S31 - 42

EXPRESSION OF SPINACH ASCORBATE PEROXIDASE ISOENZYMES IN RESPONSE TO SEVERAL ENVIRONMENTAL STRESSES

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Ascorbate peroxidase (APX) isoenzymes play a key role for scavenging excess amount of hydrogen peroxide generated in cellular organelles and locate in chloroplasts (stromal and thylakoid-membrane), microbody, and cytosol.
We studied the response of each APX isoenzyme in spinach leaves to stress conditions imposed by high-light intensity, drought, salinity, and paraquat. The steady state transcriptipt level of cytosolic APX remarkably increased in response to high-light intensity and paraquat treatment, but not in response to the other stress treatments.
The transcript levels of the chloroplastic (stromal and thylakoid membrane-bound) and microbody-bound APX isoenzymes were not changed in response to all of the stress treatments.
To explore the responses of APX isoenzymes to photooxidative stress, the transcript and protein levels and activities of each isoenzyme were studied during high-light intensity followed by its recovery. The cytosolic APX activity increased in parallel with its transcript abundance during high-light intensity, while the protein level was not altered. The other isoenzymes showed no significant changes in their transcripts and protein levels and activities, except for the gradual decrease in the activities of chloroplastic isoenzymes.
These data indicate that the expression of cytosolic APX is definitely responsible for the photooxidative stress caused by high-light intensity to protect each organelle and minimize the tissue injury. In contrast, the constitutive expressions of the chloroplastic and microbody-bound APX isoenzymes are due to detoxify immediately hydrogen peroxide generated in each organelle under normal and stress conditions.
Plant Physiol. (2000) in press.

S31 - 44

NONRADIACTIVE DETECTION OF mRNAs DIFFERENTIALLY EXPRESSED DURING COLD ACCLIMATION IN ALFALFA (Medicago sativa L.) BY COUPLING OF DIFFERENTIAL DISPLAY AND AFLP APPROACHES.

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We modified the differential display of 3Ō-end restriction fragments of mRNA technique by combining it with the amplified fragment length polymorphism (AFLP) approach and silver staining. The procedure, named DD-AFLP (Ivashuta et.al 1999, Mol.Biotech.,12: 137-141) utilizes advantages of AFLP approach resulting in highly reproducible fingerprints. Furthermore, DD-AFLP fingerprint represents mainly 3Ō-end of mRNAs, including a non-coding terminal exon, that is almost always unique to the transcript. Thus, procedure might identify rare transcripts which belong to a gene family and share conserved coding exons with others members of family. Introducing of asymmetric PCR pre-amplification step allows to clear visualization of generated PCR products on silver-stained PAGE and facilitates subcloning. We applied DD-AFLP to identify genes differentially expressed during cold acclimation in alfalfa. Sequencing and searching against GenBank revealed the transcripts with strong, limited and no homology (by aa sequence similarity) to the known proteins. Preliminary analysis indicates that transcripts represent mRNAs in all frequency classes (superprevalent, moderately prevalent and complex). Details on some differentially expressed mRNAs will be presented.

S31 - 49

DIFFERENTIAL EXPRESSION OF PETUNIA ZINC-FINGER GENES IN RESPONSE TO ENVIRONMENTAL STRESSES AND PLANT HORMONES

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In plants, dynamic modulation of gene expression has been known to occur in response to environmental stresses. Such modulations are supposed to be mediated by transcription factors under the control of several signal transduction pathways. We have previously isolated more than 30 genes for Cys2/His2-type zinc-finger (ZF) proteins (EPF family) encoding one, two, three and four ZF motifs from petunia. One of them (ZPT2-2) was found to respond to wounding, low-temperature, desiccation and UV-B (1). This finding prompted us to examine the stress responses of other members of the EPF family. A systematic survey revealed that nine out of fourteen two-fingered ZF genes were inducible by wounding, desiccation, salt and/or low-temperature treatments, however, neither of three- and four-fingered ZF gene responded

to these stresses. Upon wounding, ZPT2-2, 2-3, 2-4, 2-12 and 2-13 genes showed rapid and transient induction. These genes also responded to desiccation and low-temperature but with differing profiles of transcript accumulation. Of these, ZPT2-3, 2-12 and 2-13 responded to salt treatment as well, while ZPT2-2 and 2-4 did not. ZPT2-3, 2-12 and 2-13 were induced by both of jasmonic acid (JA) and hydrogen peroxide, while ZPT2-2 was induced only by JA. These results suggest that ZPT2-3, 2-12 and 2-13 are components of the same signaling pathway(s) and ZPT2-2 belong to a different pathway. ZPT2-5, 2-9 and 2-14 showed slow response to wounding but no response to either low-temperature or desiccation. ZPT2-1 responded only to low-temperature. Taken together, EPF family ZF genes seem to be involved in the transcriptional regulation under the control of diverse stress-responsive signaling pathways.

1. van der Krol, A. R. et al. (1999) Plant Physiol 121(4), 1153-1162.

S31 - 52

USE OF MICROARRAYS TO CLONE LOW OXYGEN RESPONSIVE GENES

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Waterlogging, causing low oxygen stress in plant roots, is a major threat in many crop plants. Plants respond to low oxygen stress by switching their metabolism from an oxidative to a fermentative pathway. However, fermentation results in an 18-fold reduction of energy per metabolised carbohydrate molecule. This causes severe damage to the plants, resulting in decreased crop yield, when subjected to low oxygen stress for a longer period of time. In the current project, we aim to improve the low oxygen response in plants, in order to increase plant survival during waterlogging. One of our approaches is to clone the key genes involved in the anaerobic response. Therefore, we aim to clone all the genes that show a changed expression profile in Arabidopsis thaliana roots upon low oxygen stress. DNA microarray slides were prepared containing cDNA clones from low oxygen stressed roots. The slides were hybridised with labelled cDNA derived from roots harvested at different time points during low oxygen treatment. The results show a stress-induced increase, or decrease, of expression of several genes. Blast searches revealed that some of these genes are known anaerobically induced genes, whereas most of them are new genes with known or unknown functions.

S31 - 61

ANALYSIS OF STRESS-RESPONSIVE REGIONS OF A CHITINASE GENE PROMOTER OF WINTER WHEAT

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Freezing injury and snow mold infection are major environmental stresses resulting in low yields of winter crops in northern regions. In order to improve the ability of plants to tolerate various abiotic and biotic stresses in winter, an understanding of the mechanisms underlying plant responses to these stresses is essential. However, few hypotheses on the interactions among host plant, low temperature and snow mold pathogens have been presented. It has been reported that plant pathogenesis-related proteins (PR-proteins) may play certain roles in freezing tolerance and snow mold resistance in winter wheat, which shows a cross-adaptation ability in winter crops. This study focused on the analysis of promoter responses of a wheat chitinase gene and the identification of responsive regions associated with two different kinds of stress. The 5'-flanking region of Chi1 (Kawakami et al, PCP supple, 2000) was cloned by Inverse PCR from a highly snow mold-resistant winter wheat cultivar, PI 173438. GFP(S65T) and GUS reporter gene fusion systems were developed for a promoter specificity assay with winter wheat leaf. Analysis of the expression of the fusion gene with Chi1 promoter deletions revealed that the region between -438 to -319 contributed to the increased expression following 50 000 pmol/L exogenous ABA, low temperature (0-3 C) and 200 mmol/L mannitol treatments, suggesting that a cross-net exists in signal transduction cascades of Chi1 responsive to exo-ABA, low temperature, and mannitol-induced osmotic stress. In addition, the region between -851 to -439 responded to 250 mmol/L salicylic acid and elicitors derived from snow mold, Typhula ishikariensis and Microdochium nivale. The results suggest that both abiotic and biotic environmental factors up-regulate the expression of Chi1 gene, which, in turn, maybe protect the plant from these stresses.

S31 - 86

SUGAR METABOLISM AND PATHOGENESIS-RELATED PROTEINS DURING THE LUPIN RESPONSE TO DROUGHT

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We observed that L. albus strategy to cope with water deficit (WD) leads to a combination of stress avoidance and tolerance mechanisms that entail distinct behaviours in what concerns leaves and stems. Plant survival appeared to be dependent on nutrient mobilisation from leaves and development of a defence/resistance/adaptation mechanism in stems. At an initial stage, WD caused a general alteration in sucrose metabolism in the leaves and, later on, stem changes in several proteins related to stress (induction of thaumatin-like protein and increase in chitinase levels and peroxidase activity). It has already been suggested that the induction of pathogenesis-related genes occurs through a sugar dependent pathway (FEBS Lett. 1996, 397, 239-44). When rewatered, plants fully recovered. So, these results suggest a sequential and compartmentalised response to WD, dependent on drought severity. The early alterations in sugar metabolism of the leaves may have contributed to amplify the stress signal(s), allowing the plant to survive.

S31 - 89

cDNA CLONING AND DIFFERENTIAL GENE EXPRESSION OF TWO CATALASES IN PEACH (Prunus persica L.)

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Many environmental and abiotic stress exert at least part of their effect causing oxidative damage. Consequently, the antioxidant defence system of plants has a crucial role in protecting plants against stress. A primary enzymatic antioxidant defence is provided by catalase which catalyses the decomposition of H2O2. Multiple catalase encoded by more than one gene have been found in plants, but in woody species no catalase genes have been so far isolated. We report the cloning and the expression analysis of two catalase cDNAs in peach. The catalase

multigene family in peach include three genes encoding individual subunits that associate to form at least six isoenzymes that are resolved by non denaturing gel electrophoresis. The individual isoenzymes showed distinct patterns of spatial (organ specific) expression. Two distinct catalase cDNA, CAT-1 and CAT-2 were isolated by mean of RT-PCR from leaf and petiole respectively. Both the genes were sequenced, and their deduced amino acid sequences were used to determine the similarity with other plant catalase. A high level of homology (87%) was shared by the two catalase isolated. The CAT-1 sequence shares an homology of 84% with cotton CAT-1 and 83% of the Class III catalase of Nicotiana plumbaginifolia. Both peach catalases displayed different developmental expression patterns. CAT-1 mRNA predominated in leaves and the peak in its abundance occurred in the mature fully expanded leaf. CAT-2 is highly expressed in buds, stem and micropropagated shoots, whilst a low expression was observed in leaves. The implications of these findings with respect to the role and developmental regulation of the catalases in peach will be discussed.

S31 - 92
COMPARATIVE ANALYSES OF DROUGHT RESPONSES IN PLANTS USING MICROARRAY TECHNOLOGY

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Water deficit stress or drought is the major limiting factor for plant growth. Plants respond to drought at multiple levels, one of these being alterations in gene transcription profiles. Identification of drought-induced and drought-repressed genes has been an ongoing effort in our lab for many years. We have recently applied the technology of microarray screening of cDNA libraries to both identify drought-responsive genes and to monitor global alterations in patterns of gene expression during drought. Microarrays were printed on nylon membranes and probed with cDNAs labeled with biotin or digoxigenin. Hybridization was detected colorimetrically. cDNAs from six different libraries are under investigation: Capsicum chinense: leaf and placenta, Phaseolus acutifolius: leaf and root, Trifolium purpureum: leaf and Dactylis glomerata: leaf. All of these libraries except for the fruit were made from drought-stressed plants. The log ratio was calculated as the ln (hybridization signal from cDNA probe of droughted sample/hybridization signal from cDNA probe of control sample). Genes with log ratios greater than 1.6 are considered drought induced and less than -1.6 are considered drought-repressed. A list of the genes, their function based on sequence annotation, and their drought responsiveness will be presented. Quantitative northern analyses were also performed on selected genes. A comparison of the microarray results with the northern results will be presented. The two methods produce similar rank orders for responsiveness, however the absolute values of the log ratios are different. This work was supported in part by New Mexico Agricultural Experiment Station, USDA CSREES, IALC, and NIH grant S06 GM07667-21.

S31 - 101
LEVEL OF THE ACTIVE OXYGEN SPECIES ARE CONTROLLED BY ASCORBIC ACID AND ANTHOCYANIN IN ARABIDOPSIS

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It has been supposed that stabilization of the level of the active oxygen species would be important for the survival of the orgasms. Flavonoid has been considered as a strong radical scavenging contents in Arabidopsis. Accumulation of the flavonoid was observed in the many oxidative stress responses. The radical scavenging activity was measured by chemiluminescence method. According for the accumulation of the flavonoid, the increase of the radical scavenging activity was detected. However, in ordinary conditions, plants did not synthesize and accumulate flavonoid. It suggest that some other substances act as radical scavengers. It is possible to distinguish radical species by ESR (electron spin resonance) method. In order to clarify the defense system for oxidative stress of the plants, the radical scavenging activity were measured in wild type and anthocyanin mutant (tt3, tt4, tt5, ttg) of Arabidopsis thaliana by ESR method. In the ordinary condition, the three week-old plant contains about 50-100 SOD unit/g radical scavenges activity in the whole body. The fact that the anthocyanin mutant has almost the same radical scavenging activity indicates that some substances other than anthocyanin might scavenge the active oxygen species in the ordinary conditions. Assignment of the ESR pattern indicates that the main content of the radical scavenging activity was caused by an accumulation of the ascorbic acid. It was also confirmed by the in vitro treatment of ascorbic acid oxidase. Almost all of the radical scavenging activity was disappeared by treatment with the ascorbic acid specific oxidase. These results indicate that there are two radical scavenging activities, such as anthocyanin and ascorbic acid. We have observed the contribution of anthocyanin and ascorbic acid to the radical scavenging activity is different among ecotypes. In the Ler, level of ascorbic acid accumulates twice high as that of Col, and induction of anthocyanin was 1/5 of the Col. These results indicate that the radical scavenging substances are differentially utilized among ecotypes.

S31 - 112
REGULATION OF GENE EXPRESSION AND OZONE SENSITIVITY BY ETHYLENE, JASMONATES AND SALICYLIC ACID IN ARABIDOPSIS

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Ozone is an air pollutant, which induces oxyradical production and signaling processes inside the cells in much the same way as in the hypersensitive pathogen response. Signaling molecules that are known to be involved include ethylene, jasmonates and salicylic acid. These signaling pathways induce expression of various defense and stress-related genes. Ethylene production enhances ozone-induced lesion formation, and functional ethylene signaling is required in this process. In an opposite manner, jasmonates are believed to protect plants from ozone damage through containment of the ozone-induced lesions. Salicylic acid seems to have a dual role in damage formation since salicylates are involved in both stress protection and potentiation of oxidative burst and cell death.
We have isolated four ozone-sensitive Arabidopsis mutants that display various degrees of lesion formation in response to a 250-ppb ozone treatment. Defense responses are not defective in these mutants as determined by superoxidase dismutase activity, ascorbate concentrations and expression of defense-related genes. A cDNA array of 92 genes revealed in fact upregulation of some of the

pathogenesis-related proteins in three of the mutant lines (ml2-40, ml2-11, ml2-3). Mutant line 2-40 had additional changes in salicylate-related gene expression, and rcd1 (for radical-induced cell death) and ml2-11 in jasmonate-related gene expression.
Ozone sensitivity of known Arabidopsis signaling mutants was also tested. Compared to Columbia wild type, ethylene-insensitive etr1-1 and ein2 were more tolerant to ozone, jasmonate-resistant jar1 more sensitive and salicylate-deficient transgenic NahG line and salicylate-insensitive npr1 mutant were similar to the wild type. Expression of 144 genes was studied in a cDNA macroarray in order to characterize the role of ethylene, jasmonates and salicylic acid in the processes of defense and cell death during oxidative stress. These results will be discussed in relation to ethylene emission and the quantity of salicylates and jasmonates in these mutants.

S31 - 116
FUNCTIONAL ANALYSIS OF THE GLUTATHIONE TRANSFERASE FAMILY OF ARABIDOPSIS

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Glutathione-transferases (GSTs) are ubiquitous enzymes that catalyze the conjugation of the tripeptide glutathione to diverse hydrophobic compounds. GSTs are known to play roles in normal cellular metabolism as wellas in detoxification systems activated by a multitude of stress conditions. In plants, GSTs have been studied intensively in relation to their detoxification potential towards herbicides. However, little is known about their roles in normal plant metabolism. The goal of our project is to learn more about the endogenous functions of individual GSTs in Arabidopsis thaliana. Thirty-eight GSTs have been identified in Arabidopsis. This complex enzyme family shows extreme sequence divergence with less than 20% amino acid identity between the most divergent members. m-RNA expression profiling of a set of 12 different GSTs in plants exposed to different stress conditions such as herbicide or phytohormone treatments, wounding or pathogen attack indicated that the GST family is differentially regulated in a stress dependent manner. This suggests that individual GSTs might have specific roles in protection from diverse stresses. Twelve GST cDNAs were functionally expressed in E. coli and tested for enzymatic activity with 6 diagnostic substrates. The deduced substrate profiles were dramatically different. Surprisingly, there was no correlation between sequence similarity and substrate preference. Our results indicate that the apparent broad substrate range of GSTs measured in crude plant extracts is not so much the property of individual enzymes but a reflection of the diversity of GSTs present in plants. It seems as if this diversity is needed to counteract the many consequences of unfavourable conditions. Plant GSTs are likely to have well defined metabolic functions. The knowledge of the individual expression profiles and the preferred substrates allows us to hypothesize in which metabolic events individual GSTs might be involved. These hypotheses will be tested by manipulating GST expression in transgenic plants.

S31 - 117
STRUCTURAL AND FUNCTIONAL STUDIES OF BspA: A HEAT SHOCK AND STRESS-RESPONSIVE PROTEIN IN ASPEN (Populus tremula)

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In a previous work, we identified a novel 66 kDa (later re-designated as 116 kDa) boiling-stable protein, BspA, which accumulated in aspen (Populus tremula L.) in response to various stresses and following abscisic acid application. bspA cDNA was isolated by immuno-screening an expression library and sequenced. The bspA cDNA was found to encode a 12.4 kDa hydrophilic polypeptide, as verified also by its overexpression in E. coli. Thus, the identified BspA has a higher molecular mass than the protein encoded by bspA. Northern blot analysis revealed that BspA is encoded from a small mRNA (about 0.6 kb) which is constitutively expressed in aspen, but accumulation of bspA mRNA is stimulated considerably by drought stress, salinity stress and elevated temperatures. The 12.4 kDa protein was later detected in aspen plants. Protease digestion pattern, amino acids analysis, and N-terminal sequences of large and small species of BspA revealed that BspA is a homo-oligomeric protein. Gel filtration chromatography, size exclusion native-PAGE, TEM and MALDI-TOF-MS studies indicate that BspA is composed of 6-9 subunites, the structure of which is similar to that of sHsps. The potential protection by BspA of heat-inactivated citrate synthase and horseradish peroxidase was studied, and it was demonstrated that BspA possesses chaperone activity, which can function in enzyme stabilization. The expression and possible function of BspA in transgenic plants will be discussed.

S31 - 121
THE EXPRESSION MECHANISM OF CHLOROPLASTIC ASCORBATE PEROXIDASE IN HIGHER PLANTS

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We have shown that stromal and thylakoid-bound ascorbate peroxidase (APX) isoenzymes of spinach chloroplasts arise from a common pre-mRNA by alternative splicing in the 3Ō-terminal of the isoenzymes. Four mRNA variants, one form (tAPX-I) for thylakoid-bound APX (tAPX) and three forms (sAPX-I, -II, and -III) for stromal APX (sAPX), were identified.
The sAPX-I and sAPX-III mRNAs were generated through excision of intron 11 and encoded the previously identified sAPX protein. Interestingly, the sAPX-II mRNA was generated by the insertion of intron 11 between exons 11 and 12. The similar observation also has been found in tobacco, pumpkin, and Mesembryanthemum crystalinum.
The use of this insertional sequence was in frame with the coding sequence and would lead to the production of a novel isoenzyme which contains the C-terminus in which a 7-amino acid sequence replaces the last amino acid of the previously identified sAPX. The recombinant novel enzyme expressed in E. coli showed the same enzymatic properties except for the molecular mass as the recombinant sAPX from the previously identified sAPX-I mRNA, suggesting that the protein translated from the sAPX-II mRNA is functional as a soluble APX in vivo.
The S1 nuclease protection analysis showed that the expression levels of mRNA variants for sAPX and tAPX isoenzymes are in nearly equal quantities in the spinach leaves throughout the growth under normal conditions. The present data demonstrated that the expression of chloroplastic APX isoenzymes is regulated by a differential splicing efficiency, which is dependent on the 3'-terminal processing of the ApxII.
We are progressing toward clarifying cis-acting signals and trans-acting factors which regulate the 3Ō-end processing including splicing to produce mature mRNA variants.
Biochem. J. (1997) 328, 795-800; Biochem. J. (1999) 338, 41-48.

S31 - 122
CLONING OF SALT-STRESS RESPONSIVE GENES FROM BARLEY LEAVES BY DIFFERENTIAL DISPLAY

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High salinity in soil is a limiting factor of plant growth, development and agricultural productivity. For this reason plant salt tolerance has been widely studied so far, although molecular mechanisms underlying response to high salinity are not fully clear. Cloning of salt-induced genes is essential to understand plant salt tolerance mechanism. We isolated salt-induced cDNA clones from leaves of salt-stressed barley (Hordeum vulgare L. cv. Harunanijyo) by differential display method. Totally 480 RAPD primers were used to screen out. As a result of sequence analysis, some cDNAs showed homology with stress related genes such as pyrroline-5-carboxylate synthetase, sugar transporter, and plasma membrane H+-ATPase, etc. Some other cDNAs had similarities to genes whose relations to salt tolerance are not clear. On the other hand, a large number of isolated cDNAs are functionally unknown. Isolation of full-length cDNAs and their functional analysis are now in progress.

S31 - 123
STRESS RESPONSE OF ARABIDOPSIS THALIANA DURING LEAF SENESCENCE

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The death of organisms is one of the fundamental steps in their development. It is the prerequisite to guarantee the genetic variability of species and is absolutely necessary for evolution. The final step of development that leads to the death of an organism is the phenomenon of senescence. Senescence is not a chaotic breakdown but an orderly loss of normal cell functions which is under the control of the nucleus. Free radicals are thought to play an essential role in senescence especially those derived from oxygen. Increased levels of activated oxygen in senescing tissues occur either through an enhanced production of activated species or a decline of the various defence mechanisms that normally afford protection against oxidative injury. Both seems to be realized in senescing tissues. The total activity of the H2O2 scavenging enzymes catalase (EC 1.11.1.6.) as well as the separated activities of the different isoforms of leaves (CAT2 and CAT3) decline during senescence of the rosette leaves of Arabidopsis thaliana in parallel or even before the loss of chlorophyll can be measured. In young plants, the activity of CAT3 is increased approximately 3-fold by oxidative stress which can be induced by paraquat treatment. This substrate induction via paraquat is no longer realized in plant cells with the beginning of leaf senescence. In contrast, the response to other stress conditions like water logging or wounding, which also lead to the induction of CAT3 enzyme activity, is maintained. Other stress responses like heat stress response, which induces the massive expression of heat-shock proteins, are also maintained all over leaf development. This indicates that the response to oxidative stress seems to be actively suppressed during senescence and that this phenomenon is highly restricted to oxidative stress.

S35 Hormonal Regulation

S35 - 2
ETHYLENE INVOLVMENT DURING TENSION WOOD FORMATION

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Tension wood is formed on the upper side of leaning stems or branches in hardwood. The function is to restore the stem or branch to its natural position. Tension wood differs from normal wood anatomically, physically and chemically. In particular, tension wood is associated with increased growth rate and formation of fibers rich in cellulose. The molecular controls regulating tension wood are unknown but physiological studies have implicated a role for ethylene.
Ethylene is produced by the Yang cycle where S-adenosyl methionine is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase and ACC is converted to ethylene by ACC oxidase.
To gain an understanding of the role of ethylene in wood formation, and specifically during tension wood formation, we have used both molecular and chemical approaches.
A full-length cDNA of ACC oxidase was identified from an EST library made from cambial-region tissues of hybrid aspen (Populus tremula x P. tremuloides). Northern blot analysis revealed expression primarily in the developing xylem in straight trees (non-inductive conditions). During tension wood formation, the ACC oxidase expression drastically increased in the developing xylem on the tension wood side. In contrast, the levels of ACC, as measured by mass spectrometry, revealed a low level of ACC on the tension wood side, but an increase on the lower, non-tension wood side.
The significance of these results as they relate to wood formation and the molecular regulation of tension wood development is discussed.

ANNEXE 2

Cloning of potentially ethylene-inducible and/or laticifer-specific promoters from *Hevea brasiliensis*

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Cloning of ethylene-inducible and/or laticifer-specific promoters from rubber tree was undertaken with the objective to optimize transgene expression in genetically engineered rubber tree.

	Ethylene-inducible promoter	Laticifer-specific promoter
Objective	To induce/over-express transgene expression in situations of intense exploitation where ethephon is used as a stimulant of rubber production.	To limit transgene expression to latex cells exclusively (limited disturbance for the whole tree, limited risk in case of accidental dissemination)
Targeted promoter	GLUTAMINE SYNTHETASE *(gs)*	HEVEINE *(hev)*
Why	*gs* genes are highly and early over-expressed in response to ethylene treatment (1)	The hevein protein is exclusively located in the latex cells

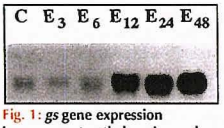

Fig. 1: ***gs* gene expression in response to ethylene in newly opened trees (first tapping).**
C, untreated control trees; E_3-E_{48}, trees treated with ethephon respectively 3, 6, 12, 24 and 48 h before the first tapping.

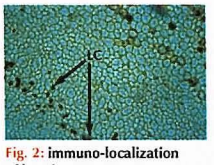

Fig. 2: **immuno-localization of hevein.**
Transversal section of a young stem incubated with polyclonal antibodies directed against hevein. LC : latex cells.

1. Cloning and molecular characterization

Strategy

A genomic library (genotype RRIM600) was screened with cDNA probes coding for glutamine synthetase (1) or hevein (2).

Results

Glutamine synthetase: Three different clones bearing GS genes (*gs1*, *gs2* and *gs3*) were obtained. The clone carrying *gs1* corresponded to the cDNA used to screen the library. However, it was truncated and lacked the promoter region. The other two genes were highly homologous to each other in their ORF as well as non coding regions but differed in their upstream region. They differed significantly from *gs1*. No ethylene-related upstream element could be identified from the sequence.

Hevein: Three classes of genes were obtained: 2 classes of genes encoding hevein and a third class encoding pseudo-hevein (3). Class I and II hevein genes were highly homologous but differed in their upstream regions. The promoter region of pseudo-hevein and class II hevein genes had significant sequence homology.

Table 1: **Cloned promoter region: available size upstream the start codon.**

	Genomic clone	Subclone (in pCambia vector)
gs1	- (truncated clone)	-
gs2	1.4 kb	1 kb
gs3	3.9 kb	0.9 kb
hev1 (class I hevein)	320 pb	320 pd
hev4 (class II hevein)	1.9 kb	1.9 kb
hev7 (pseudo-hevein)	(>2.4 kb)	-

2. Northern blot analysis of Glutamine Synthetase and Hevein gene expression

Probes used

Specific probes were designed from the 3' non coding regions of *gs1* and *gs2*. As *gs2* and *gs3* 3' non coding regions were highly homologous (99%), it was not possible to discriminate *gs2* from *gs3*. For the same reason, no specific probes could be designed to discriminate *hev1* from *hev4*. Hevein: total cDNA

Plant material analyzed (4, 5):

L	latex from non stimulated trees
SL	latex from ethephon-stimulated trees (48 h)
TobL	tobacco leaves
T	tegument
TC	tegument-derived callus (day 25)
ECC	embryogenic compact callus (day 50)
FC	proliferating friable callus
EFC	embryogenic friable callus
E	embryo
DE	developed embryo
ME	mature embryo
G	very young germination
R	root from germination
S	stem from germination
Lf	leaf from germination

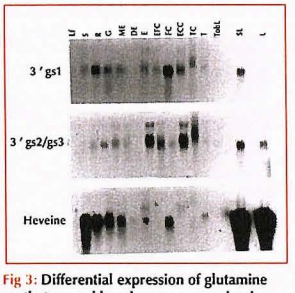

Fig 3: **Differential expression of glutamine synthetase and hevein gene expression in response to ethylene and in various tissues from *in vitro* culture.**

Results

Differential expression in response to ethylene treatment, in latex

- Both *gs1* and *gs2/gs3* respond to ethylene treatment. However, *gs1* expression appears to be *de novo* induced whereas *gs2* and(or) *gs3* are over-expressed, with a significant endogenous level in control trees.
- The hevein genes display a very high level of expression, with no additional over-expression in response to ethylene.

Differential expression during development

Gs and *hev* gene expression was tested in various tissues at different stages of the somatic embryogenesis process (4, 5).

The *gs1* gene was preferentially expressed in proliferating friable callus (maintained callus line) compared to other types of callus. In later developmental stages (embryos and plantlets), only roots displayed a significant expression level for *gs1*. On the contrary, *gs2/gs3* were expressed preferentially in embryogenic callus as well as in young embryos.

Hev genes were expressed in proliferating friable callus, excluding any other type of callus, and in later developmental stages (nature embryo and plantlets).

3. Functional analysis, preliminary result

Transient expression of the *gs3* promoter after microprojectile bombardment-mediated transformation of rubber tree callus.

The *gs3* promoter fused to the *gus* reporter gene in a transformation vector (pCambia-P*gs3-gus* construct) was introduced *via* microprojectile bombardment into rubber tree embryogenic compact callus J13 (i.e. sub-cultured from maintenance medium onto embryogenesis expression medium for 13 days).

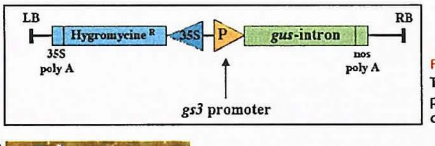

Fig. 4: **T-DNA region of the pCambia-P*gs3-gus* construct.**

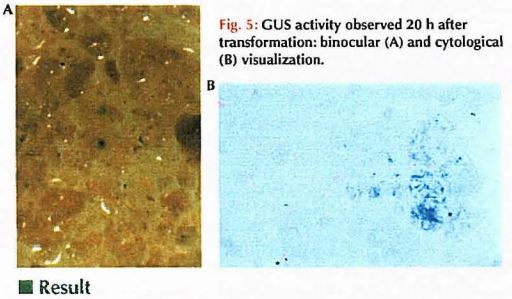

Fig. 5: **GUS activity observed 20 h after transformation: binocular (A) and cytological (B) visualization.**

Result

The isolated 900 bp *gs3* promoter is functional, which validates the cloning strategy.

Appropriately chosen callus transformed by microprojectile bombardment can be successfully used for transient expression analysis of the promoters.

Conclusions/Perspectives

❑ Glutamine synthetase and hevein genes were cloned in order to isolate ethylene-inducible and laticifer-specific promoters respectively. GS and hevein appeared to be encoded by a small multigene family (respectively *gs1*, *gs2* and *gs3* for glutamine synthetase, *hev1* and *hev4* for hevein, *hev7* for pseudo-hevein). The *gs* genes (*gs1* and *gs2/gs3*) were differentially expressed in response to ethylene-treatment or during the somatic embryogenesis process. All three *gs* promoters are interesting in terms of responsiveness to ethylene, although the *gs1* promoter would probably allow a higher level of over-expression. Concerning the hevein promoters, a very strong activity is expected in the latex cells, considering the very high level of expression in this tissue.

❑ Preliminary functional analysis (transient expression) should be possible for all promoters in appropriately chosen callus tissue, as confirmed for the *gs3* promoter after microprojectile bombardment-mediated transformation.

Moreover, an *Agrobacterium*-mediated transformation system is currently being set up (6). High rates of gene transfer have already been obtained and developing transformed callus can be maintained for several weeks. Therefore, such system is already available for stable analysis of the *gs* promoters activity in response to ethylene, using exogenous ethylene and/or inhibitors of ethylene biosynthesis. However, regeneration of at least mature embryos, will be necessary for detailed analysis of the hevein promoters in terms of tissue specificity.

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Accumulation of Polypeptides Identified as the Latex Allergens Hev b 3 and Hev b 1 in the Latex Cytosol of Rubber Trees Displaying the Tapping Panel Dryness Syndrome.

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The tapping panel dryness (TPD) syndrome of rubber tree is characterized by the decrease and ultimately complete stoppage of latex flow, as a consequence of in situ latex coagulation. The process of latex coagulation involves bursting of part of the vacuolyzosomes (lutoïds) and subsequent interaction of intravacuolar components with the rubber particles, leading to their coagulation by charge neutralization (1) or by a mechanism involving a lectin-like protein (2). Two types of TPD have to be distinguished (3): the irreversible necrotic TPD affecting the whole bark tissue, and a reversible form of TPD affecting the latex cells only and induced by over-exploitation (overtapping or over-stimulation, i.e. excessive use of ethephon as a stimulant of latex production). TPD-associated variations of the protein pattern have ben described (4).

By looking for molecular markers of TPD, our aim was first to get a better understanding of the syndrome and eventually obtain tools for early diagnosis of TPD in rubber plantation as well as in selection programs.

Identification of PDF (Pannel dryness factors) polypeptides

Protein screening by 2D protein gel electrophoresis revealed the accumulation of 2 major polypeptides of 22 and 15 kDa respectively in the cytosol of overstimulation-induced TPD trees (Fig. 1) as well as necroctic TPD trees (not shown) compared to healthy trees. They were excised out for molecular characterization (Fig.2) and identified as the latex allergens Hev b 3 (or SRPP, i. e. Small Rubber Particle Protein) and Hev b 1 (or REF, i.e Rubber Elongation Factor) respectively (Table 1). They share significant sequence homology with each other (5), are both located on the rubber particle membrane (6), and were both shown to be involved in rubber biosynthesis, *in vitro* (5, 7).

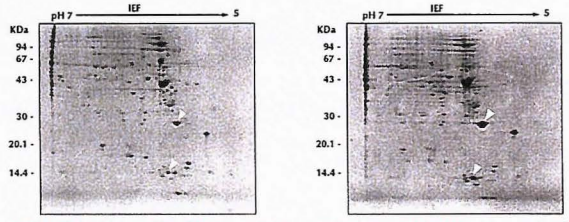

Fig. 1: 2-D SDS-PAGE profile of latex cytosolic proteins from healthy (A) and overstimulation-induced TPD trees (B). IEF: pH 5 to 7; SDS-PAGE: 12.5% acrylamide; silver staining.

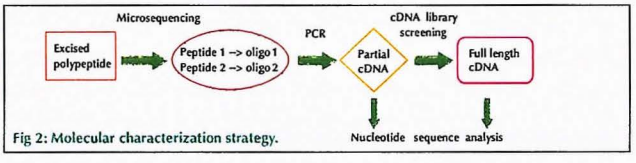

Fig 2: Molecular characterization strategy.

Name	Molecular weight	pHi	Cloned cDNA	Identification	Reference
PDF22	22 kDa	5.5	Full length	Hev b 3, SRPP	(5, 8)
PDF15	15 kDa	5.8	Partial	Hev b 1, REF	(9, 10)

Table 1: result of the PDF polypeptides molecular characterization.

Gene expression analysis

Northern blot analysis was performed on total RNA from latex. The probes used were specific to PDF22 (upper lane) and PDF15 (lower lane) respectively. They did not cross-hybridize.

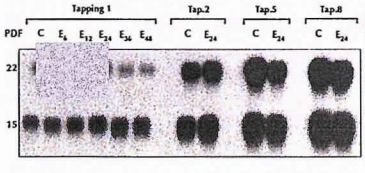

Fig. 3: Effect of tapping and ethylene treatment on PDF22 and PDF15 gene expression.
C: untreated control trees. E6-E48: trees treated with 2.5% ethephon respectively 6, 12, 24, 36 or 48 hours before the first tapping. All trees were virgin (previously untapped). After opening, they were tapped every 2 days.
Both PDF22 and PDF15 are highly over-expressed by tapping but not by ethylene treatment *per se*

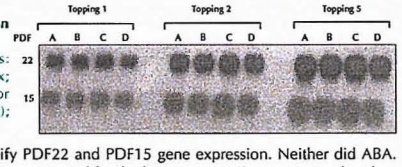

Fig. 4: Effect of wounding without loss of latex on PDF22 and PDF15 gene expression.
Virgin trees were submitted to various stresses: A, no stress; B, superficial cut without loss of latex; C, nails were stuck into the wood every 2 cm for 2 days (then removed just prior tapping); D, treatment with ABA.

Wounding without loss of latex did not modify PDF22 and PDF15 gene expression. Neither did ABA. The tapping-induced over-expression should be accounted for the latex regeneration process rather than wounding.

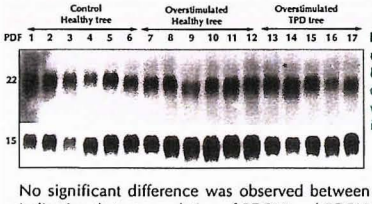

Fig 5: PDF22 and PDF15 gene expression in the latex of healthy and overstimulated trees.
Lane 1-6: untreated healthy control trees. Lane 7-12: overstimulated trees (2.5% ethephon every 2 weeks) without symptoms of TPD, Lane 13-17: overstimulation-induced TPD trees displaying symptoms of TPD.

No significant difference was observed between healthy and overstimulation-induced TPD trees, indicating that accumulation of PDF22 and PDF15 polypeptides in the latex cytosol of TPD trees was not due to transcriptional regulation mechanisms.

Compartmentation analysis

Latex can be separated into 3 main compartments: the rubber particles, the cytosol and an organelle fraction composed mainly of vacuo-lysosomes called lutoids. PDF22 (Hev b 3) and PDF15 (Hev b 1) are normally located on the rubber particles (6). We demonstrate here that organelles osmotic lysis can induce the release of PDF22 and PDF15 from the rubber particles into the cysosol, in vitro.

Procedure: Latex from healthy trees was divided into 2 batches. One was diluted with mannitol-containing buffer (100 mM mannitol final) in order to maintain the integrity of the organelles, the other with mannitol-deprived buffer, which caused bursting of the lutoids. The different compartments were separated by centrifugation and protein content analyzed by SDS-PAGE.

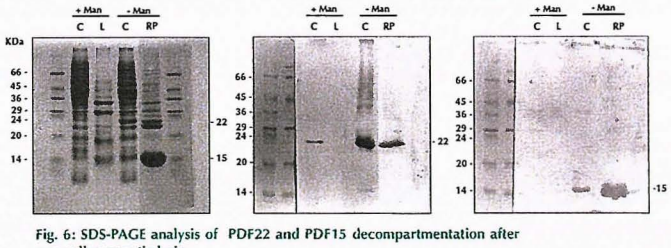

Fig. 6: SDS-PAGE analysis of PDF22 and PDF15 decompartmentation after organelles osmotic lysis.
Coomassie blue staining (panel A). Western blot using polyclonal antibodies directed against PDF22 (panel B) or PDF15 (panel C). C, cytosol; O, organelles; RP, rubber particles proteins extracted by SDS and triton. Sample were in slightly hypertonic (+ mannitol) or hypotonic (no mannitol) buffer.

Conclusion

❑ Two major polypeptides (PDF22 and PDF15), identified as the latex allergen Hev b 3 and Hev b 1 respectively, were found to accumulate in the cytosol of TPD trees. This was not due to transcriptional regulation but rather an indirect consequence of the organelles (lutoids) bursting. The increased membrane fragility and therefore higher lutoids "bursting index" in TPD trees has been documented (11). We suggest that Hev b 3 and Hev b 1 in TPD trees may be released from the rubber particles under the action of some vacuolar hydrolytic enzymes. It is therefore likely that the rubber biosynthesis process is affected in TPD trees.

❑ PDF22 and PDF15 present striking similarities with each other in terms of sequence homology and function (5, 7) but also, as demonstrated here, in terms of gene expression patterns.

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