Making a More Fermentable Plant via Genetic Engineering: a progress report.

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Potential Scale of Energy Crops-derived Fuel Production

Box 1

Conversions

1 exajoule = 10^{18} joules[1]
1 exajoule = 9.48×10^{14} BTU
1 terrajoule = 10^{12} joules
1 terrajoule ~ 0.17 barrels of oil
1 kilojoule ~ 0.2777 watt hours
1 hectare = 2.471 acres
1 bu corn ~ 56 lb[2] (25.4 kg)
1 bu soybean ~ 60 lb
1 bu canola ~ 49 lb
7.7 lbs vegetable oil ~ 1 gallon

[1]Other energy interconversions at http://www.mycomponents.co.uk/energy.htm
[2]Exact value depends on moisture content of seed

Box 2

Biomass energy yield per acre

1 ton of dry *Miscanthus* has 17,252 GJ of heat value [2]
1 acre of *Miscanthus* at 21 dry tons/acre[1] ~ 362,292 GJ
1,021,275 acres of biomass ~ 370 EJ
Terrestrial surface of earth ~32.123×10^9 acres
370 exajoules could be grown on 3.2% of the surface

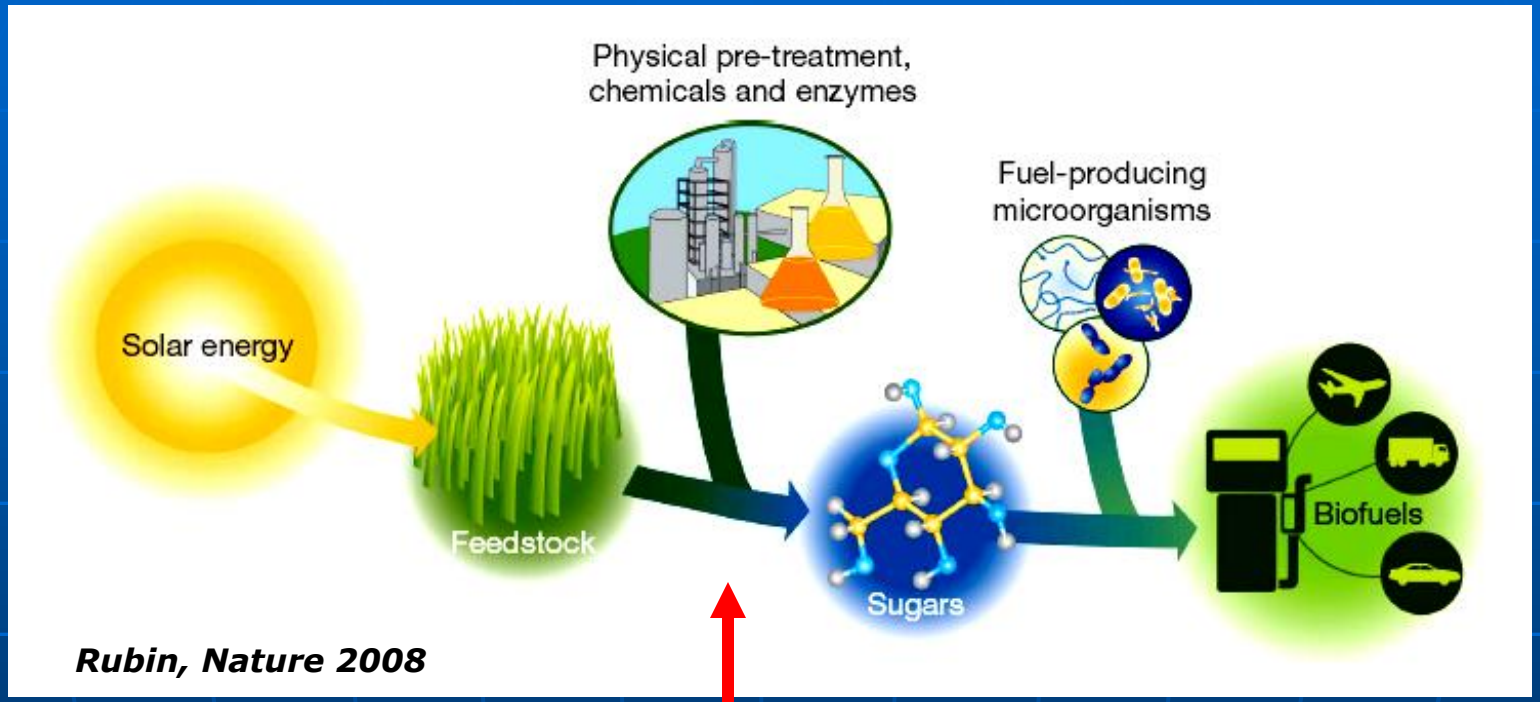
[1]Stephen P. Long, University of Illinois, personal communication.

From Somerville, Curr Biol. 2007

Global energy market: ~370 exajoules/yr. = ~170 M barrels of oil/day

Efficient and sustainable practice for energy crop production as well as cellulosic fermentation will be key ingredients to realizing global "Green Energy" – carbon neutral or carbon negative processes of renewable energy generation.

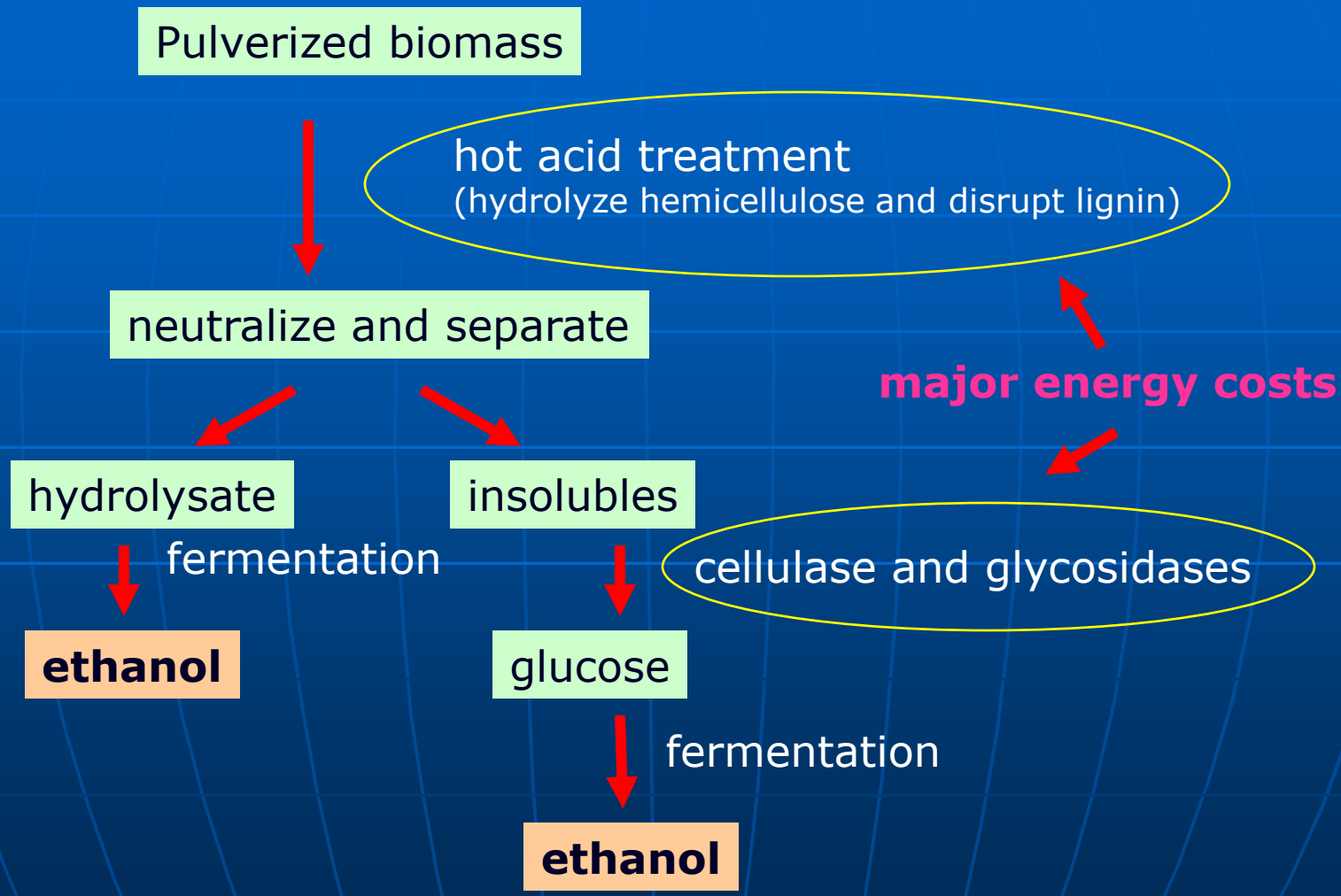
Biomass conversion to "Green Energy"



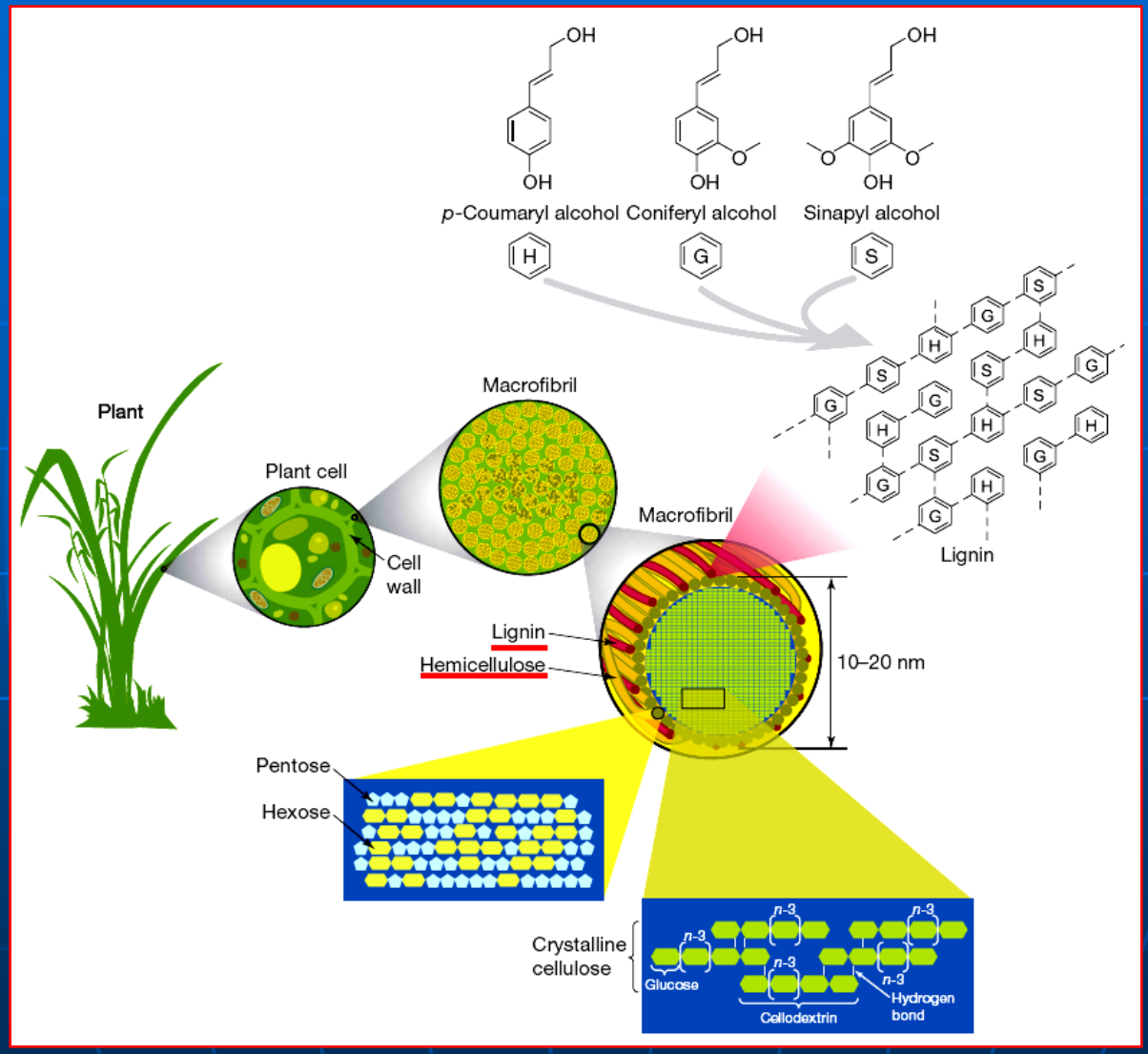
Rubin, Nature 2008

point of major net energy input

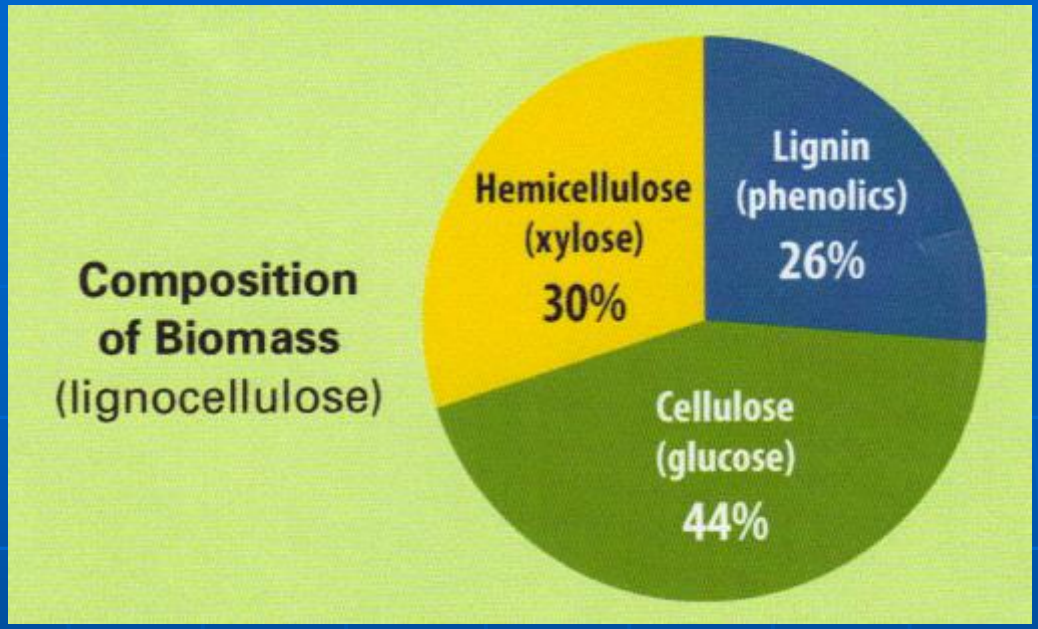
Typical process for cellulosic ethanol production.



Structural Components of Lignocellulose



From Rubin, Nature 2008



Structure of lignocellulose. The main component of lignocellulose is cellulose, a β(1–4)-linked chain of glucose molecules. Hydrogen bonds between different layers of the polysaccharides contribute to the resistance of crystalline cellulose to degradation. Hemicellulose, the second most abundant component of lignocellulose, is composed of various 5- and 6-carbon sugars such as arabinose, galactose, glucose, mannose and xylose. Lignin is composed of three major phenolic components, namely *p*-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S). Lignin is synthesized by polymerization of these components and their ratio within the polymer varies between different plants, wood tissues and cell wall layers. Cellulose, hemicellulose and lignin form structures called microfibrils, which are organized into macrofibrils that mediate structural stability in the plant cell wall.

From Rubin, Nature 2008

Different Types of Hemicellulose are Used by Energy Crops

- Group I : Contains principally xyloglucan as the principal hemicellulose and relatively higher proportion of pectins. Typical of dicotyledonous plants (and some monocots).
- Group II: Contains arabinoxylans and mixed linkage glucan in addition to xyloglucan. Characteristic of monocotyledonous plants such as maize and rice.
- Necessitates different enzymes and treatments to optimize biomass conversion depending on the particular energy crop.

Objective: to create more readily fermentable biomass via genetic engineering of energy crops.

- Endogenous expression of degradative enzymes in energy crop plants to minimize or eliminate the need for their addition - transgenics.
- To facilitate the engineering of energy crops, the suppression of *somaclonal variations* in tissue culture will minimize the time and work for producing desired traits.

Proof-of-concept for first area: facilitating biomass conversion via transgenic approaches.

Enhanced conversion of plant biomass into glucose using transgenic rice-produced endoglucanase for cellulosic ethanol

Hesham Oraby · Balan Venkatesh · Bruce Dale · Rashid Ahmad · Callista Ransom · James Oehmke · Mariam Sticklen

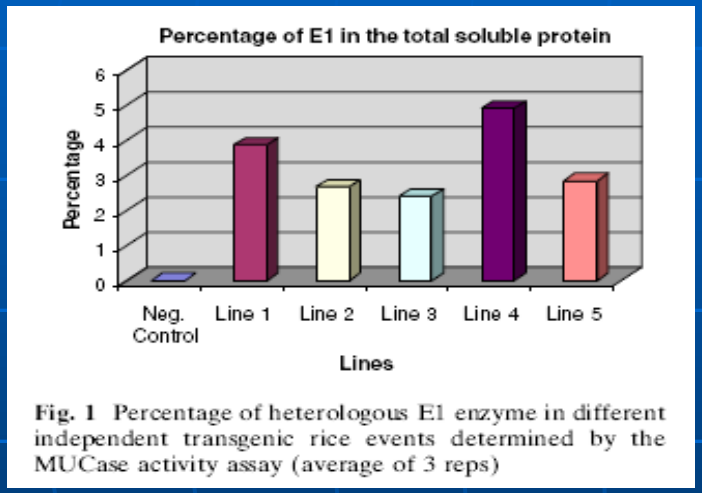


Fig. 1 Percentage of heterologous E1 enzyme in different independent transgenic rice events determined by the MUCase activity assay (average of 3 reps)

Transgenic rice straw extract

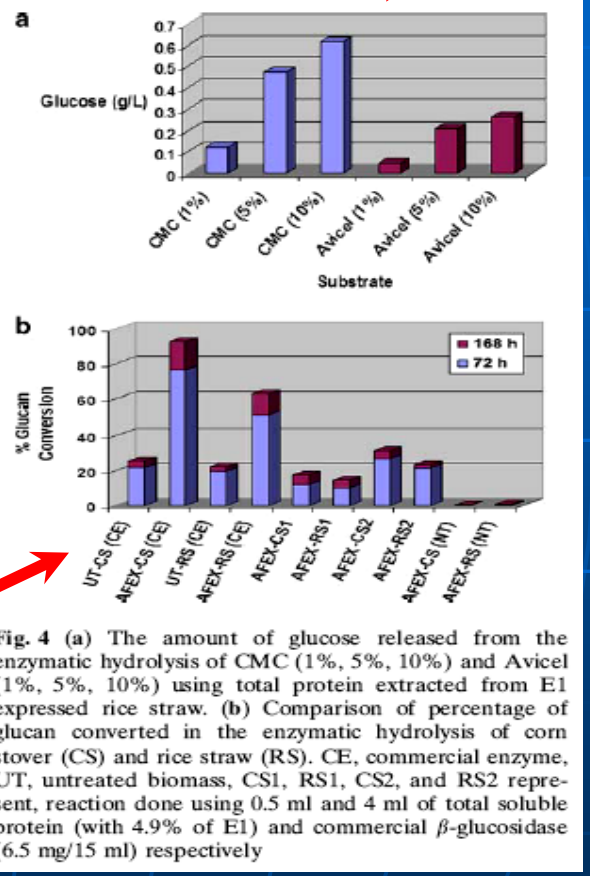


Fig. 4 (a) The amount of glucose released from the enzymatic hydrolysis of CMC (1%, 5%, 10%) and Avicel (1%, 5%, 10%) using total protein extracted from E1 expressed rice straw. (b) Comparison of percentage of glucan converted in the enzymatic hydrolysis of corn stover (CS) and rice straw (RS). CE, commercial enzyme, UT, untreated biomass, CS1, RS1, CS2, and RS2 represent, reaction done using 0.5 ml and 4 ml of total soluble protein (with 4.9% of E1) and commercial β-glucosidase (6.5 mg/15 ml) respectively

Conversion of cellulose to glucan in non-transgenic corn stover and rice straw is dependent on added cellulase

from Transgenic Research, 2007

E1 = catalytic domain of *Acidothermus cellulolyticus* endo-1,4-β–glucanase

Biomass Feedstocks and Degraders With Ongoing or Completed Genome Projects Provide Available Gene Sets

Feedstocks : prospects to customize cell walls

Populus trichocarpa (poplar) *
Chlamydomonas reinhardtii *
Glycine max (soya bean)
Manihot esculenta (cassava)
Sorghum bicolor
Eucalyptus globulus
Brachypodium distachyon
Zea mays (maize)
Elaeis guineensis (oil palm)
Panicum virgatum (switchgrass)
Setaria italica (foxtail millet)

*** : completed genomes**

Degraders : tool box for genetic engineering

Acidothermus cellulolyticus 11B *
Bacillus pumilis SAFR-032 *
Caldicellulosiruptor saccharolyticus DSM 8903 *
Clostridium phytofermentans ISDg *
Clostridium thermocellum ATCC 27405 *
Cytophaga hutchinsonii ATCC 33406 *
Flavobacterium johnsoniae UW101 *
Rubrobacter xylanophilus DSM9941 *
Saccharophagus degradans *
Thermobifida fusca strain YX *
Clostridium cellulolyticum H10
Elusimicrobium minutum Pei191
Nectria haematococca/Fusarium solani
Phanerochaete chrysosporium K-3
Postia placenta
Sagittula stellata E-37
Trichoderma reesei/Hypocrea jecorina
Cellulomonas flavigena DSM 20109
Cellvibrio japonicus Ueda107
Fibrobacter succinogenes subsp. *succinogenes* S85
Ruminococcus albus
Teredinibacter turnerae T7902
Termite hindgut community *
Poplar biomass degrading community
Asian longhorned beetle (*Anoplophora glabripennis*) gut community
Bovine rumen community transcriptome

From Rubin, Nature 2008

Expression of cell wall degradation enzymes in energy crop plants: immediate goals.

- Overexpression of target enzymes in stable transgenic plants to degrade hemicellulose and disrupt lignin.
- Demonstration of enzyme activities in plant cell extracts and compare rates of biomass conversion rate between wild-type and transgenic plants.

Laccase and Lignin Peroxidase are key enzymes in lignin biodegradation.

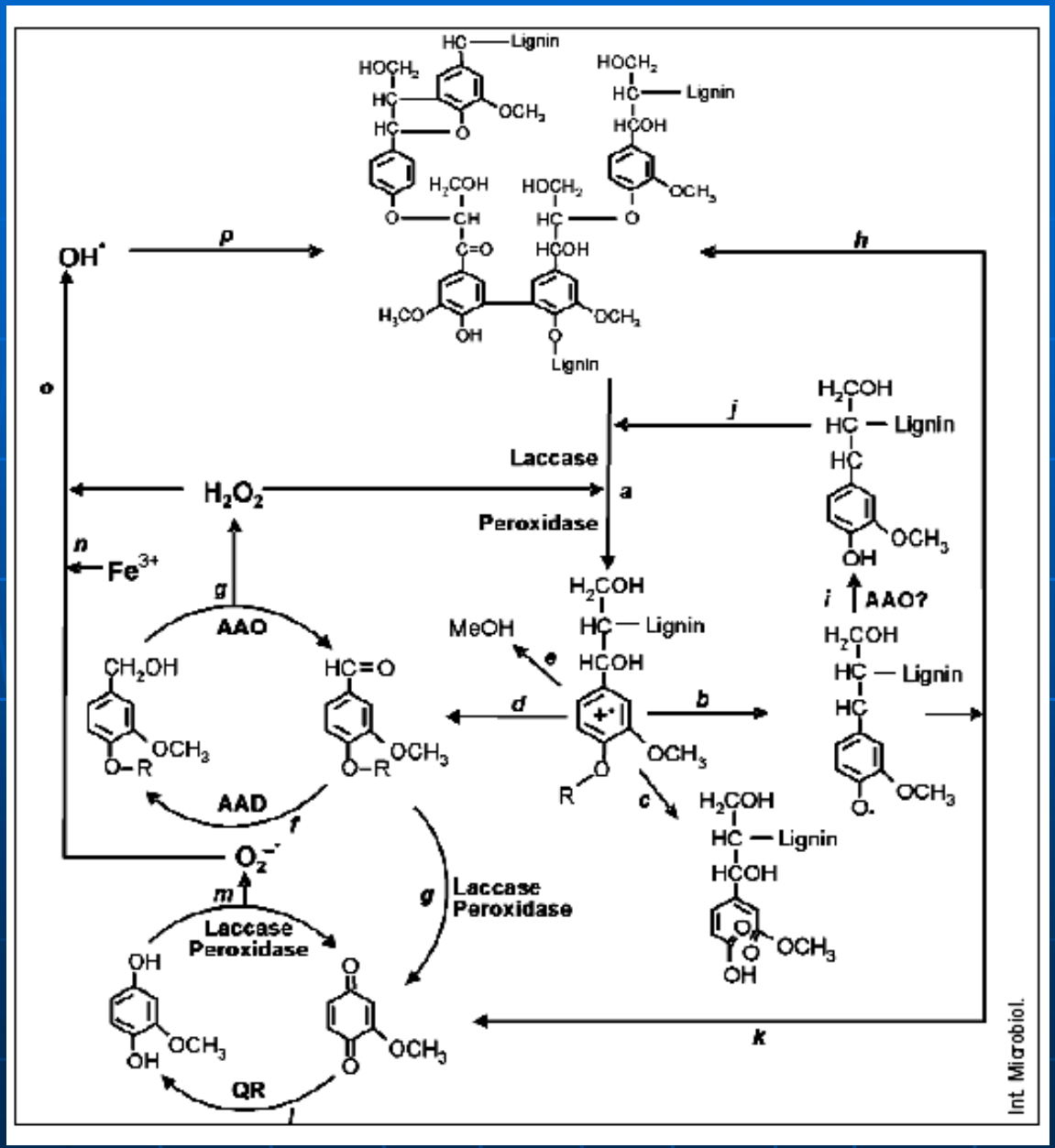


Fig. 4. A scheme for lignin biodegradation including enzymatic reactions and oxygen activation, (for explanation see text). Updated from Gutiérrez and Martínez [22].

Xylanase is a class of enzymes that degrade linear polysaccharide beta-1,4-xylan into xylose, thus breaking down hemicellulose. *It is especially relevant for group II cell walls (monocots) since it is able to degrade arabinoxylan while cellulases cannot*. For this purpose, xylanases are present in fungi for degrading plant biomass into carbon source.

Three Fungal Genes are Chosen for Overexpression Studies

1. Laccase 1 from *Trametes versicolor* 52J (wood rot fungus)
2. Lignin peroxidase H8 from *Phanerochaete chrysosporium* *
3. Xylanase 2 from *Trichoderma reesei* *

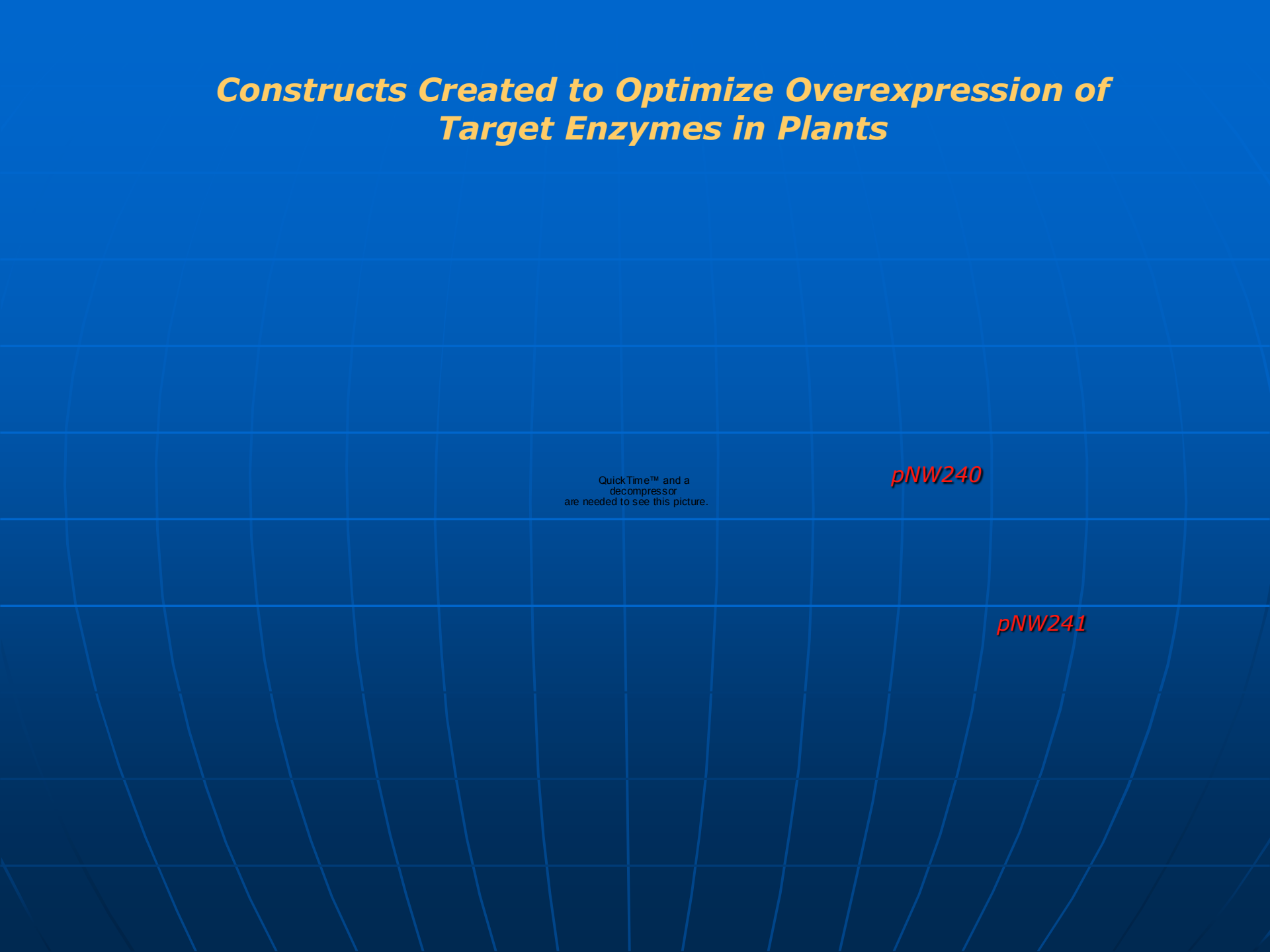
** Draft sequence of cell wall degrader completed*

Strategies for Overexpression of Cellulose Degradation Enzymes *in planta*

1. Design and synthesize codon-optimized genes: using known codon-usage of rice and Arabidopsis where whole genome annotation have been performed, synthetic versions of laccase 1, lignin peroxidase H8 and xylanase 2 were made and cloned.

2. Sequestration of translated proteins in multiple subcellular compartments. Secretion signal peptide (SP) at the 5' end of the fungal genes were substituted with the dual targeting transit peptide (DuTP) sequence from the Arabidopsis histidyl-tRNA synthase (*AtHRS1*) gene. This should target the linked peptides to the mitochondria and plastids. A peroxisome targeting tripeptide sequence (SKL) is inserted at the 3' ends of the the synthetic genes as well to affect triple targeting of the desired gene product.

Constructs Created to Optimize Overexpression of Target Enzymes in Plants



pNW240

pNW241

Localization of GFP fusion proteins in guard cells of transgenic Arabidopsis.

** Needs to co-express peroxisome-specific mCherry marker together with triple-targeting construct to verify peroxisome targeting.*

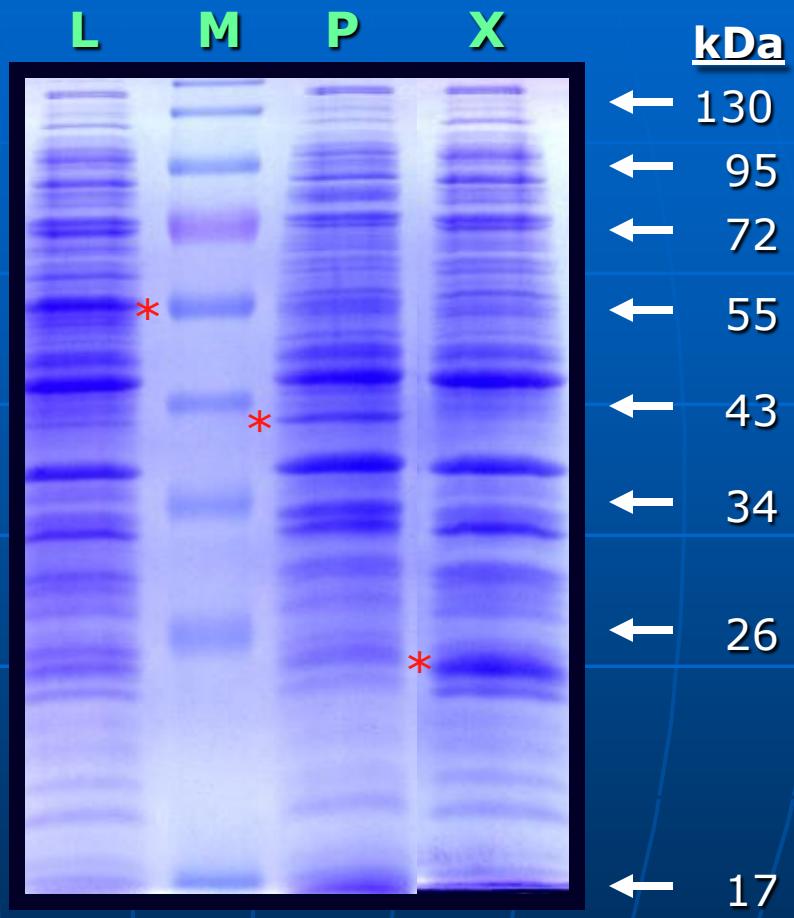
Expression of Recombinant Synthetic Genes using pET23a

L : *pET23a-Lcc1*
~55 kDa

M: EZ protein markers

P: *pET23a-Lph8*
~40 kDa

X: *pET23a-Xyn2*
~24 kDa



** Enzyme assays with bacterial extracts will be performed and synthetic genes will now be subcloned into plant expression vectors to test for expression in planta by transient and stable transformation.*

Tissue Culture Induces Transposable Element Activities

Retrotransposons of rice involved in mutations induced by tissue culture

(retroelements/transposable elements/stress/insertion mutations)

HIROHIKO HIROCHIKA*†, KAZUHIKO SUGIMOTO*, YOSHIAKI OTSUKI‡, HIDEHITO TSUGAWA‡, AND MARI KANDA§

Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 7783–7788, July 1996
Genetics

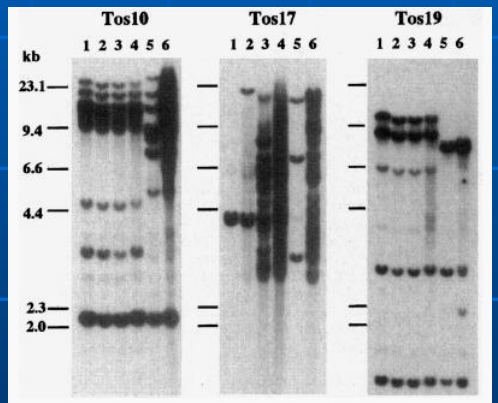


FIG. 2. An increase in the copy number of *Tos10*, *Tos17*, and *Tos19* during tissue culture. DNAs of leaves or cultured cells were digested with *Xba*I and analyzed by Southern blot hybridization using ^{32}P-labeled *Tos10*, *Tos17*, and *Tos19* probes. Lanes 1 and 5, leaves of Nipponbare and C5924 varieties, respectively; lanes 2–4, cultured cells derived from Nipponbare cultured for 3, 12, and 24 months, respectively; lane 6, the Oc cell line derived from C5924.

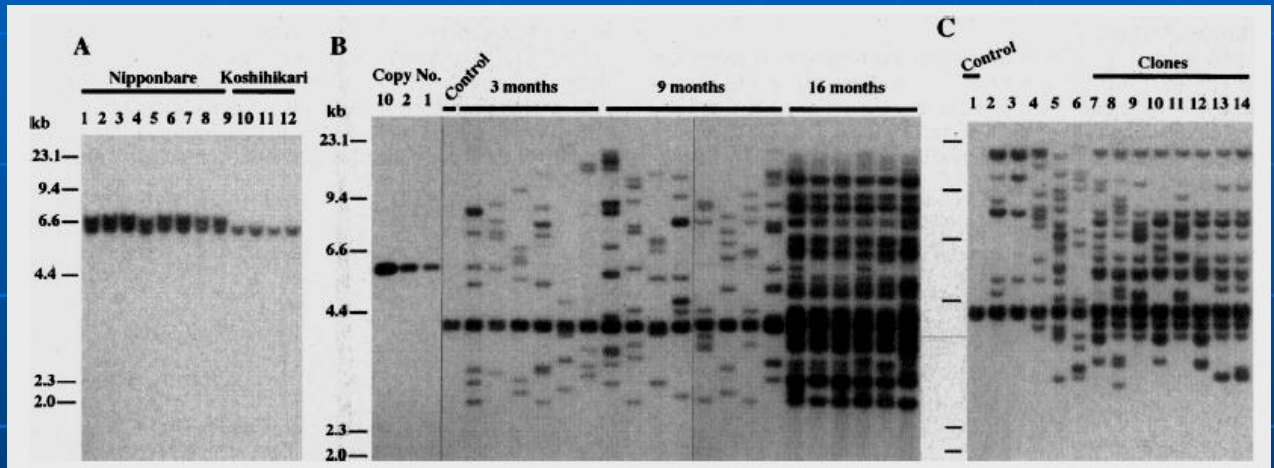


FIG. 3. An increase in the copy number of *Tos17* in plants regenerated from culture and transgenic plants. DNAs were prepared from leaves and analyzed by Southern blot hybridization after digestion with *Hin*dIII (*A*) or *Xba*I (*B* and *C*). (*A*) Lanes 1–8 and 9–12: normally propagated plants of Nipponbare and Koshihikari varieties, respectively. (*B*) Plants regenerated from tissue cultures of Nipponbare. Plants were regenerated from 3-, 9-, and 16-month-old cultures. Left three lanes: cloned *Tos17*-1 digested with *Xba*I as a copy number control. (*C*) Lane 1, the control Nipponbare plant; lanes 2–14, transgenic plants (lanes 7–14, clones derived from a single transformed cell).

Rice retrotransposon number increases as a function of time in tissue culture - activation of transposable elements is likely an important factor for somaclonal variations.

The Plant Cell, Vol. 12, 357–368, March 2000, www.plantcell.org © 2000 American Society of Plant Physiologists

Silencing of Retrotransposons in Arabidopsis and Reactivation by the *ddm1* Mutation

Hirohiko Hirochika,[1] Hiroyuki Okamoto, and Tetsuji Kakutani

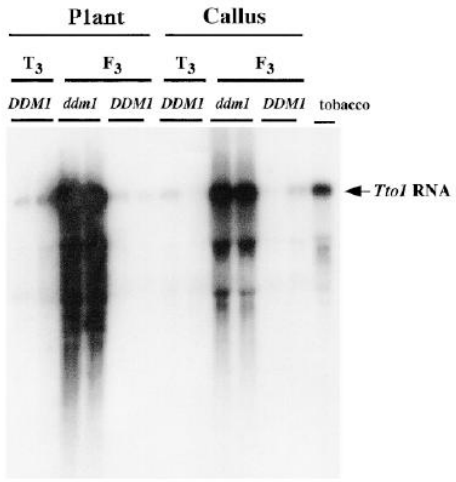


Figure 6. Analysis of *Tto1* RNA in the Wild Type and *ddm1* Mutant.

Total RNA was prepared from whole plants or calli of T_3 plants (T_3; lines 121 and 122) and F_3 *ddm1*/*ddm1* (*ddm1*; lines 119 and 120) and *DDM1*/*DDM1* (*DDM1*; lines 123 and 124) families. As a control, total RNA from tobacco BY2 cells (Nagata et al., 1981) was analyzed. Twenty micrograms of total RNA was loaded on the gel.

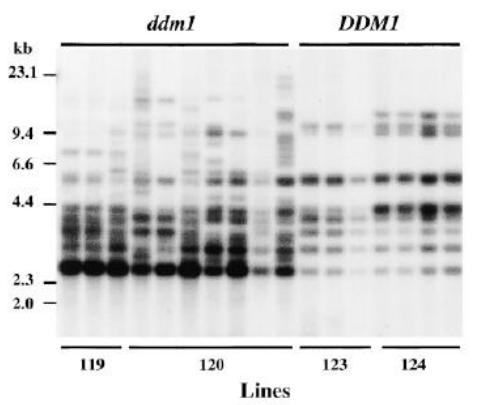


Figure 7. Reactivation of *Tto1* Transposition by the *ddm1* Mutation in Calli.

Calli were induced from F_3 *ddm1*/*ddm1* (*ddm1*; lines 119 and 120) and *DDM1*/*DDM1* (*DDM1*; lines 123 and 124) families and cultured for 3 months. Induced calli were smashed into pieces, and each piece was cultured separately for one more month. DNA from each callus was digested with EcoRV and analyzed by DNA gel blotting with the *Tto1 gag* probe. DNA length markers are shown at left in kilobases.

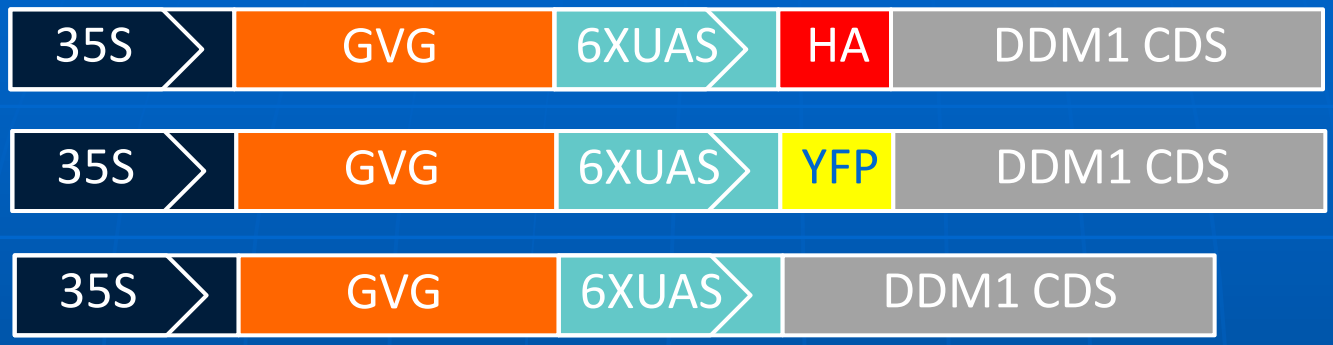
Tobacco retrotransposon *Tto1* is silenced in Arabidopsis via a DDM1-dependent pathway in plants or callus tissue.

Hypothesis: DDM1 (Decrease in DNA Methylation 1), which encodes a conserved SWI2/SNF-like chromatin remodeling factor, may be a critical silencing component that can be used to control somaclonal variation.

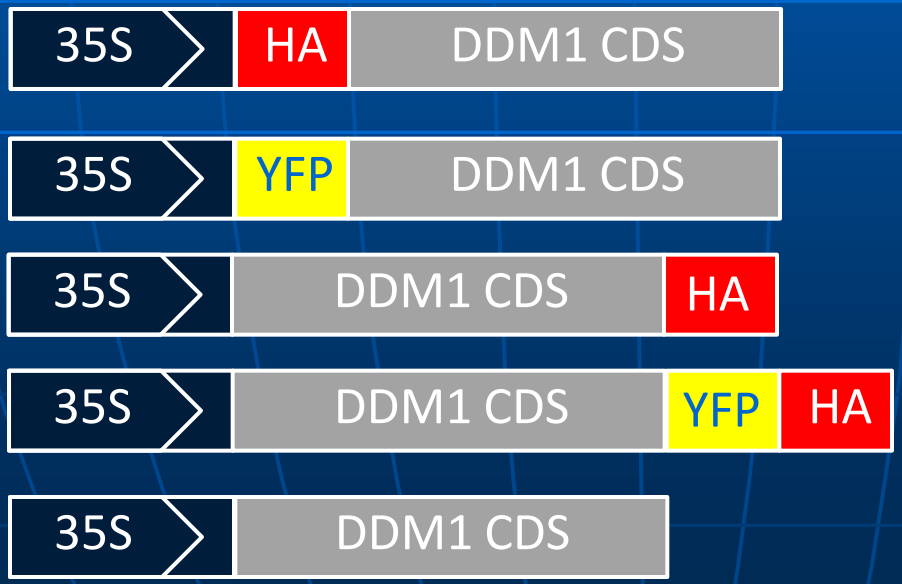
Objective: to ectopically express DDM1 in callus tissues during plant transformation in order to suppress activation of transposable elements, thereby minimizing somaclonal variations.

Constructs for Testing DDM1 Functions in Arabidopsis and Tobacco

Dexamethasone-inducible Expression Constructs

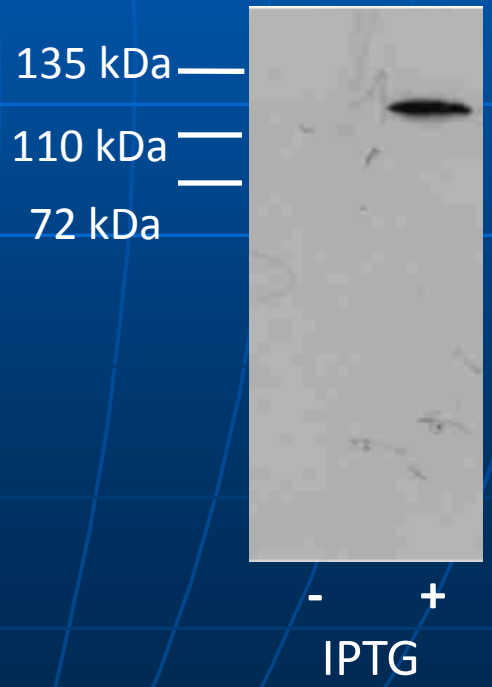


Constitutive Over-expression Constructs



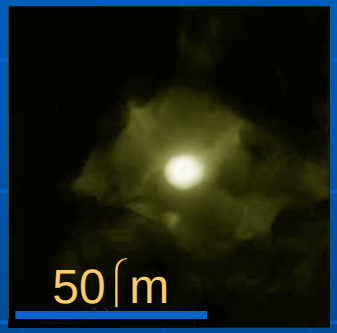
T7::HA-DDM1

E. coli Expression

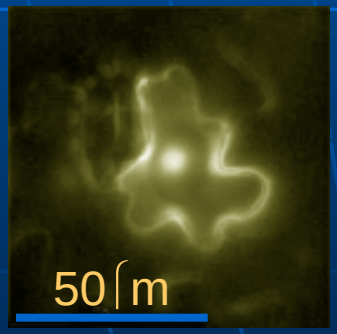


Detection and Localization of HA-tagged DDM1 in Tobacco Cells

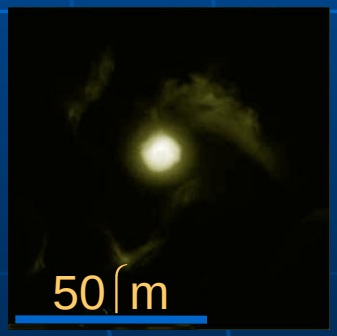
Tobacco Transient Expression 35S::YFP-DDM1



YFP-NLS



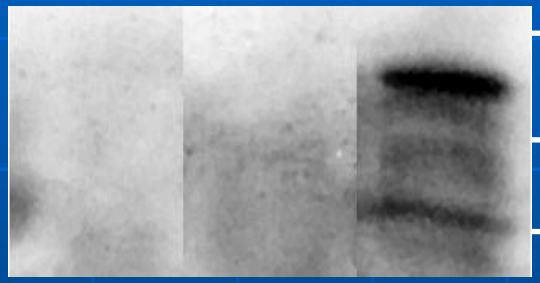
YFP

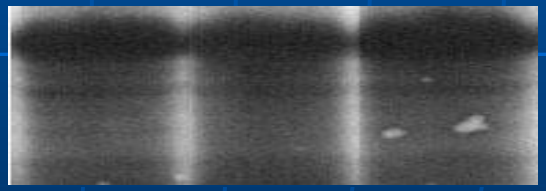


NYFP-DDM1

Tobacco Transient Expression 35S::HA-DDM1

Anti-HA

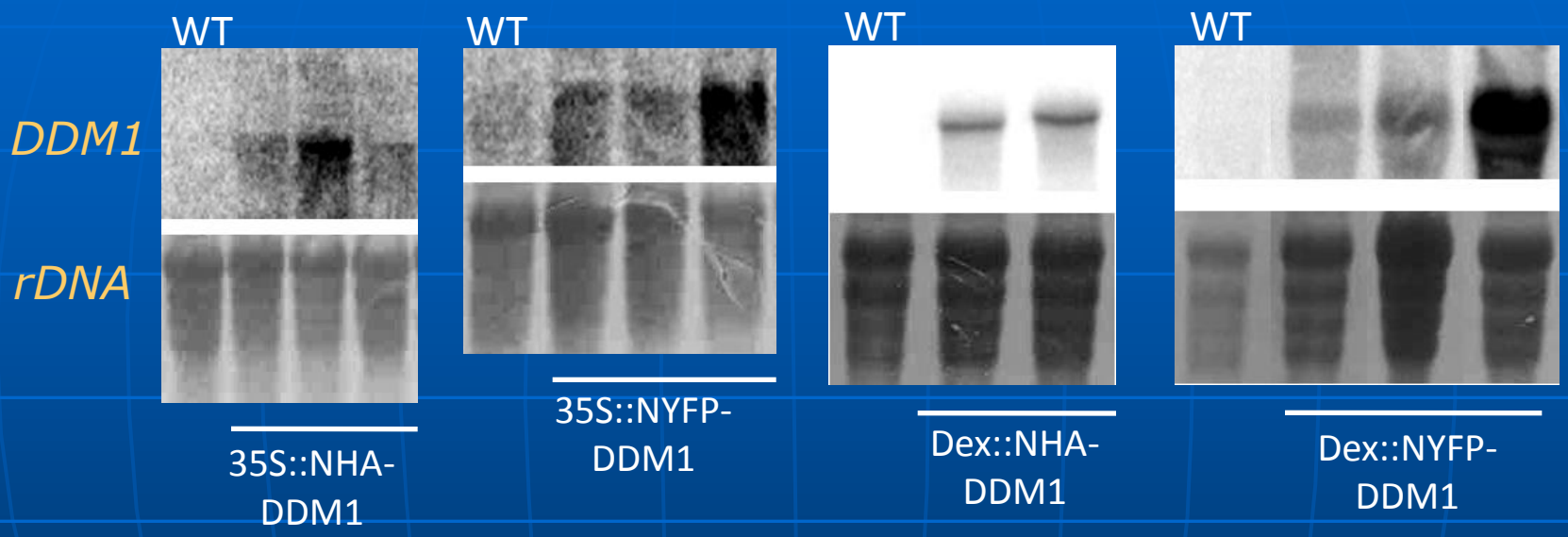




Ponceau S Staining

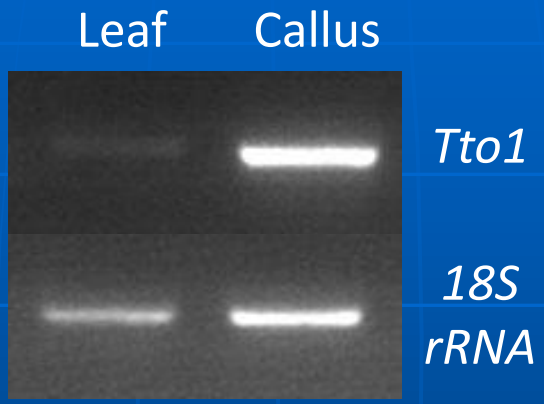
Mock | Vector Backbone | 35S::HA-DDM1

Verification That DDM1 can be Expressed in Transgenic Arabidopsis



DEX: Gene Expression driven by the Dexamethasone-inducible system.
Arabidopsis rosette leaves are soaked in 10 μM Dexamethasone for 24 hrs to induce expression.

Activation of Retrotransposon Expression as a Functional Assay for DDM1 Transgenes in Plant Cells



* Transgenic tobacco calli will be examined to determine if overexpression of AtDDM1 can suppress the expression of the tobacco retrotransposon *Tto1*. Similar strategy will be used to examine stable and transient expression of the target gene in Arabidopsis with calli-induced retrotransposon expression.

Acknowledgement

Lam lab.

Naohide (Peter) Watanabe - synGene design and expression

Chongyuan Luo - DDM1 and somaclonal variations

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Helaine Carrer (ESALQ) - sugarcane vectors and methods

Glaucia Souza (USP) - sugarcane promoters and genes

Marcio de Castro Silva Filho (ESALQ) - subcellular targeting

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