4

平成１０年度
地球環境産業技術開発推進事業
国際セミナー事業　　資料
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「２１世紀の植物バイオテクノロジー」ワークショップ報告書

平成11年3月

新エネルギー・産業技術総合開発機構（NEDO）
財団法人　地球環境産業技術研究機構（RITE）

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緒　言

地球温暖化や砂漠化の防止と急増しつつある世界人口に見合う食糧の確保は、２１世紀の人類の存亡を考慮するうえで非常に重要である。いま、これらの諸問題に有効に立ち向かう科学を模索するとき、植物機能の２面性、すなわち地上部のエネルギーや産業への有効利用と地下系への環境CO_2の埋蔵は特筆すべきものである。この卓越した植物の２面性を、すでに砂漠化が進行し、人口が急増して近い将来に極度のエネルギー不足が予想される諸国で生かす科学技術は、植物バイオテクノロジーである。

各種の自然環境に成育する植物は，自然環境で一般にかつ頻繁に，日中の高温，夜間の低温，日中の乾燥，根系への塩の作用など複合環境ストレス下で成育している。このため，植物は本来持っている能力の２０%程度しか発揮できていない。地球上には広大な砂漠地域や準砂漠地域が存在するが，ここでは上記のような厳しい環境ストレスから植物は限定的にしか成育できない。しかし，もしもこのような地域で植物が生育できれば，二酸化炭素の固定可能な陸地面積を大きく拡大できるのみならず，成育する植物の有効利用の観点からは，食糧問題や植物による物質合成などの更なる付加価値が期待できる。　細胞生育環境には、温度、光、水等数々の環境因子が存在し、これらに応答機能を持った生物創成のための研究開発が進められている。すなわち、現在、細胞への１遺伝子導入による形質転換により環境ストレスの緩和を目指す研究が世界的に数多く試みられており、米国を中心に１～２の遺伝子の導入により改良された生物が近年数多く出現している。しかし、これらの生物は、シンプルな環境ストレスには耐性を有するものの、通常の自然環境下において見られる複合環境に強い応答性を有するものとはなっておらず、基本的にはある程度管理された条件下での生育が前提となっている。エネルギー収支の観点からは従前の方法と比較し優位な改善効果を見いだせていない。

そこで複合的環境化下でも生育可能な生物の開発が求められている。これを達成する第一の方法が、複合環境応答型生物の創成を可能とする、多重遺伝子導入技術である。

前述のとおり、生物への１～２つの遺伝子の導入はこれまで多くの先例があり、すでに実用製品（インターフェロン、害虫耐性植物等）が多数出現しているが、複数（３個以上）の遺伝子を一つの細胞に発現調節が可能な形で同時に導入する技術はいまだ確立していない。その導入技術は基盤技術として、動・植・微生物バイオテクノロジー分野において２１世紀の世界の中心に据えられる技術と考えられる。

本ワークショップは，こうした観点から２１世紀の植物バイオテクノロジーを論じたものである。

平成１１年３月

財団法人　地球環境産業技術研究機構

目　次

福崎　英一郎（大阪大学助教授）
林　　誠（大阪大学助手）
佐藤　文彦（京都大学教授）
福澤　秀哉（京都大学助教授）
大和　勝幸（京都大学助手）
村田　紀夫（基礎生物学研究所教授）
小林　裕和（静岡県立大学助教授）
丹羽　康夫（静岡県立大学助手）
重岡　　成（近畿大学教授）
石川　孝博（島根大学助教授）
林　　秀則（愛媛大学教授）
柴田　大介（㈱三井業際主任研究員）

４．概要

プログラムは山田奈良先端科学技術大学院大学長（写真１）のワークショップ開催の所見に始まり，２１世紀の植物バイオテクノロジーの概観について学会および官庁の立場から，新名奈良先端科学技術大学院大学教授（写真２）および堅尾通産省生物化学産業課長（写真３）によりそれぞれ論じられた。

個々のトピックスについての講演および討議が引き続き２日にわたって行われた（写真４）。主な内容は，植物の各組織で特異的に，また日中，夜間，乾燥，塩，高温，低温で遺伝子をＯＮにするためのスイッチ遺伝子の取得に対する，ジェノミックアレー法の有用性について，細胞核あるいは葉緑体への超長鎖ＤＮＡ導入技術の現状，複合ストレス耐性遺伝子，生産性向上，高付加価値等有用遺伝子等に関する現状の知見と将来像について，複合ストレス耐性遺伝子，生産性向上，高付加価値等有用遺伝子が導入された形質転換の複合ストレス耐性能力や生理機能の生物学的な評価について等である。

２１世紀の植物バイオテクノロジーを考える上で，基礎研究の重要性も議論された。たとえば，乾燥、塩ストレス、そして低温ストレスには共通の要素が含まれるが、またそれぞれに特有のストレス因子も存在し，これらの環境ストレスは多様な生化学経路と発達過程に影響を及ぼすため、これらのストレスに対する植物の耐性あるいは抵抗性は、付加的そして共同的に機能できる多様な遺伝的特性によることは明らかであることから、耐性機

１．組織

（１）主催　　財団法人　地球環境産業技術研究機構（RITE）

奈良先端科学技術大学院大学（NAIST）

（２）共催　　新エネルギー・産業技術総合開発機構（NEDO）

２．スケジュール

（１）期日　　平成１０年１２月２日（水）、３日（木）

（２）会場　　虎ノ門パストラル

東京都港区虎ノ門

（３）プログラム（詳細はAbstractsのプログラムを参照）

１２月２日

開会の辞　　奈良先端科学技術大学院大学学長　山田教授

Overview from Academia　　奈良先端科学技術大学院大学　新名教授

Overview from MITI　　通商産業省　生物化学産業課　堅尾課長

講演　　１２件

１２月３日

講演　　８件

General Discussion

閉会の辞　　財団法人　地球環境産業技術研究機構　山口専務理事

３．出席者

国内外合わせておよそ６０名が参加した。

招聘研究者

海外：Hans J. Bohnert（Professor, University of Arizona, USA）

Douglas A. Gage（Assistant Professor, Michigan State University, USA）

Paul Mike Hasegawa（Professor, Purdure University, USA）

Ray A. Bressan（Professor, Purdure University, USA）

国内：室岡　義勝（大阪大学教授）

小林　昭雄（大阪大学教授）

構の基礎的理解が必須であることが参加者の共通認識とされた。

１日目の討議に引き続き懇親会が行われたが，この席においても２１世紀の植物バイオテクノロジーについて，ワークショップの講演者間のみならず，企業等からの参加者をも含めた白熱した議論が行われた（写真５）。

２日目は上記の個々のトピックスについての講演および討議が１日目の討議に引き続き行われた後，山口地球環境産業技術研究機構専務理事（写真６）のワークショップ閉会の所見で終了した。

本ワークショップにおいて２１世紀の植物バイオテクノロジーと２０世紀のそれの対比がかなり鮮明になったといえる。２０世紀の植物バイオテクノロジーでは個々の研究室が興味を持つ過程の解析と証明のために形質転換植物を作製してきたのに対し，２１世紀の植物バイオテクノロジーでは，環境ストレス耐性の向上、貯蔵種子の改良、二酸化炭素固定の増大へ向けた光合成特性のエンジニアリング、そして発達の改変という多くの異なる特性の積み重ねが初めて試みられるであろう。

NAIST/RITE Workshop

Plant Biotechnology in the 21st Century

Toranomon Pastoral, Tokyo

December 2 (Wed)-3 (Thu), 1998

NAIST/RITE Workshop

Plant Biotechnology in the 21st Century

Toranomon Pastoral, Tokyo
2 and 3 December, 1998

Program

December 2

8:30-9:00	Registration	
9:00-9:10	Opening Remarks	Y. Yamada (NAIST)
9:10-9:20	Overview from Academia	A. Shinmyo (NAIST)
9:20-9:30	Overview from MITI	K. Katao (MITI)
9:30-10:00	Isolation of Vavious Plant Gene Promoters by "Genomic" DNA Array and Efficient Transfer of Large DNA Fragments into Plant Genomes	D. Shibata (Mitsui Plant Biotech.)
10:00-10:30	Gene Identification for Improved Crop Performance by Linkage between Stress Tolerance at Tissue Level and Whole Plant Fitness	T. Kohchi (NAIST)
10:30-10:50	Coffee break	
10:50-11:20	Development of Polycistronic Expression Vectors for Higher Plants	K. Hiratsuka (NAIST)
11:20-11:50	Regulation of Expression of Transgenes in Plants	A. Shinmyo (NAIST)
11:50-13:00	Lunch	
13:00-13:30	Development and Establishment of Chloroplast Transformation Technology for Transferring Multiple Genes into Chloroplast Genome of Higher Plants	K. Tomizawa (RITE)
13:30-14:00	Engineering of Transcription of Chloroplast Genes in Higher Plants: Increase of Photosynthetic Productivity and Potential Production of Industrial Materials in Chloroplasts	H. Kobayashi (Univ. Shizuoka)
14:00-14:20	Coffee break	
14:20-14:50	Production Technology for Environmental Compatibility: Technology Development for Renerwable Industrial Materials Production by Plants Adapted to Stressful Environments	H. Bohnert (Univ. Arizona)

14:50-15:20 Creation of Plants with Tolerance to Oxidative Damage Imposed by Environmental Stresses
S. Shigeoka (Kinki Univ.)

15:20-15:50 Production Technology for Environmental Compatibility: Technology Development for Renerwable Industrial Materials Production by Plants Adapted to Stressful Environments
D. Gage (MSU)

15:50-16:20 Production Technology for Environmental Compatibility: Technology Development for Renerwable Industrial Materials Production by Plants Adapted to Stressful Environments
R. Bressan (Purdue Univ.)

16:20-16:50 New Factors Which Alleviate High Intensity-Light and Low-Temperature Stress
N. Murata (NIBB)

16:50-17:20 Protein Sedretion System into the Rhyzosphere
T. Hashimoto (NAIST)

18:00- Reception

December 3

9:00-9:30 Functional Identification of Stress Tolerance Genes by Transposon Tagged Sites (TTS) and Microarray Technology
K. Shimamoto (NAIST)

9:30-10:00 Molecular Analyses of Gravitropism in *Arabidopsis*
M. Tasaka (NAIST)

10:00-10:30 Production Technology for Environmental Compatibility: Technology Development for Renerwable Industrial Materials Production by Plants Adapted to Stressful Environments
M. Hasegawa (Purdue Univ.)

10:30-10:50 Coffee break

10:50-11:20 Over Production of Amino Acids in Soybean Seeds Which Are Acid and Salt Tolerants
Y. Murooka (Osaka Univ.)

11:20-11:50 Oil Production as Raw Materials in Genetically Engineered Plants – Gene Transfer of Fatty Acid Production from Bryophytes to Oil Seed Plants –
K. Ohyama (Kyoto Univ.)

11:50-13:00 Lunch

13:00-13:30 Material Production in Plants Using the Transcriptional Activator-Target

	Regulons System	
		H. Sano (NAIST)
13:30-14:00	Molecular and Cellular Engineering of Useful Secondary Metabolite Production in Higher Plants	
		F. Sato (Kyoto Univ.)
14:00-14:30	Development of Plant Potency for Hydrocarbon Production	
		A. Kobayashi (Osaka Univ.)
14:30-15:00	General Discussion	H. Sano (NAIST)
15:00-15:10	Closing Remarks	T. Yamaguchi (RITE)
15:10-17:00	Closed Meeting	

NAIST/RITE Workshop "Plant Biotechnology in the 21st Century"

Good morning, ladies and gentlemen.

In my capacity as supervisor of this project, I am pleased to welcome you to the opening of this NAIST/RITE Workshop, "Plant Biotechnology in the 21st Century."

Throughout the 19th and 20th centuries, basic physics and the applied science of engineering have made remarkable advances that have raised our standards of living to levels inconceivable in the past. Today, we can fly non-stop from Tokyo to New York. We have seen men walk on the moon and a robotic vehicle negotiate the surface of Mars. Using a computer, at home or work, we can send mail between almost any two points on Earth in minutes.

These are only a few examples of the astounding developments of recent years. Looking ahead to the 21st century, we anticipate the use of computers equipped with artificial intelligence. Nonetheless, we must squarely face the reality that the achievements of the physical and chemical sciences have led to the mass consumption of energy, seriously disrupting the closed cycle of our planet's ecosystem.

During the past two centuries, the science of biology has progressed from observations of natural phenomena to the study of genetics and thence to the powerful scientific field now known as biochemistry, all of which have deepened our understanding of life science. Biochemical investigation has given us the DNA double helix and the central principles of heredity. With identification of the chemical components of the gene in the 1960s, a new science emerged--molecular biology. Its development marked the beginning of our understanding of the chemistry of heredity, the most important phenomenon of life. Molecular biology has generated the applied science of biotechnology, which now has many practical applications. Today, a general expectation is that plant biotechnology will be a "savior"that resolves many of the critical environmental problems of the 20th century, problems that seriously threaten the concept of sustainable development and the very survival of humanity. As scientists we place great confidence in plant

biotechnology helping us to overcome the grave threats to the global ecosystem.

In October, I hosted an international symposium in Nara that examined the place of plant biotechnology in eliminating some of these problems facing humanity by providing solutions to the environmental and food issues. Many of the achievements of biotechnology were presented and the challenges we face outlined. As you well know, one such achievement is the successful transduction of foreign species into plants, an impossibility with conventional breeding technologies. As the world-famous researchers in our distinguished audience here today know, transduction has dramatically increased plant resistance to salinity, drought, intense light, and extremes of temperature. It is also common knowledge that the developed nations are investing heavily in biotechnology in order to lead in the production of plants in the coming century.

Most researchers in this field hope to obtain a single effect by single-gene transduction, but I, as a plant scientist, wish to raise an important question: Is our current approach to research the best way to solve the problems that threaten human survival in the 21st century? I believe that the answer is "no." Although the narrow development of this type of technology may achieve the short-sighted goal of making a fortune, it does not answer our pressing need to conserve the environment and secure sufficient food supplies in the future. The Nara symposium identified this issue as one requiring urgent attention.

Last year,RITE and NAIST, co-operated in convening a workshop in which we discussed the future directions of plant biotechnology. At that workshop, attended by a number of the distinguished plant scientists present at this workshop, we concluded that future research should be directed at creating new plant species resistant to compound environmental stresses through multiple-gene transduction. This would revive our dying global ecosystem and degraded breeding environment, enabling them to function effectively as absorbers of carbon dioxide and providers of food sources. Multiple-gene transduction will enable us to explore ways of developing plants that can grow and thrive amid compound envionmental stresses that include salinity, drought, intense light, extremes of temperature, and harmful insects. We speculate that this technology also will activate the plant's metabolism so that solar energy will be converted into large amounts of various useful compounds.

In response to this change in direction, Japan's Ministry of International Trade and Industry recently announced a 5-year research project starting in 1999 that will focus on the use of multiple-gene transduction to develop plants that are resistant to compound environmental stresses and capable of producing useful materials. As the world's first such initiative, this innovative project points the way that biotechnology industry will take in the 21st century, and many successes are anticipated.

RITE and NAIST, after evaluating the outcome of last year's workshop, decided to organize today's workshop to help determine the path of plant biotechnology in the coming century. I am confident that our discussions here will be successful in envisioning a new role for plant biotechnology that provides comprehensive solutions to food and environmental issues and will light the path of plant biotechnology in the 21st century.

In conclusion, I thank you very much for coming and wish you all a most rewarding and productive workshop.

NAIST/RITE共同ﾜｰｸｼｮｯﾌﾟ
「２１世紀における植物のバイオテクノロジー」

奈良先端科学技術大学院大学
学　長　　山　田　康　之

東京　虎ノ門パストラルにて

会場の皆様おはようございます。

NAIST/RITE共同ワークショップ「２１世紀における植物のバイオテクノロジー」に、御参集下さいましてありがとうございます。このプロジェクトの顧問として、大変喜ばしく思います。

１９世紀から２０世紀にかけて、基礎物理学及び応用工学の分野の飛躍的進歩により、我々の生活水準は過去からは想像もつかないほどになりました。今日では、東京とニューヨーク間をノンストップで飛行できますし、また月面を歩く人や火星の表面を探索するロボットまで見ることができます。さらに家や職場でコンピューターを使えば、地球上のほとんどの地点に瞬時にメールを送ることも可能です。

これらの先端技術は近年のめざましい発展のほんの数例です。２１世紀の将来を考えてみますと、我々は人工知能を備えたコンピューターを使うことになると予測されます。しかしながらまた一方では、物理学や化学の進歩が多大な資源の消費、閉鎖系地球環境システムの深刻な崩壊を招いているという現実にも正面から向き合わなければなりません。

過去２世紀の間、生物学は自然現象の観察から遺伝学へ、そして今日生化学として知られている有用な科学分野へ進歩してきました。それらは生命科学のより深い理解を促しました。ことに生化学の研究は、我々にDNA２重らせん構造の知見や遺伝法則の基本原理をもたらしました。1960年代は、遺伝子の化学成分の同定を通して新しい科学、

すなわち分子生物学への幕開けとなりました。その発展は最も重要な生命現象である遺伝の化学的実体の理解を促すことになりました。分子生物学はバイオテクノロジーという応用科学を生じ、現在において多くの実務的な利用に役立っています。今日植物バイオテクノロジーは２０世紀に起こった多くの危機的環境問題、つまり絶え間ない進歩と人類の存続という概念を脅かしている問題を解決する『救世主』になるものとして世間から期待されています。我々研究者は、植物バイオテクノロジーが地球生態系の崩壊という脅威に打ち勝つための手助けになってくれるものと信じております。

私は今年１０月奈良において、植物バイオテクノロジーが環境や食料問題に対する解決策を提示することにより人類が直面している様々な問題に打ち勝つことができるかどうかを議論するために、国際植物シンポジウムを開催致しました。そこでは多くのバイオテクノロジーの成果が発表され、また我々が直面している課題も提示されました。皆様よく御存知のように、バイオテクノロジーの成功例のひとつとして、従来の育種技術では到底不可能と思われていた、植物への外来遺伝子導入の技術があります。ここにお集まりのこの分野では世界的に有名な科学者に方々はよくご存じのこととは存じますが、遺伝子導入技術は植物を塩害、干ばつ、強光、激しい気温の変化に著しい耐性を付与してきました。また、共通の認識として、先進国においては次世紀の植物の生産性の向上を目指してバイオテクノロジーへの投資に重きをおいていることはよくご存じのことと思います。

この分野の多くの研究者は単一遺伝子を導入することにより一つの成果を得るために研究しています。しかし私は一植物科学者として、ここに重要な問題を提起したいと思います。それは私たちが現在進めているこのような研究が、２１世紀に人類が生き残るための多様な問題の解決へ向かう最善の道であろうかということです。私はその答は『否』だと思います。この種の技術のこ手先の改良は、短期間の目的を達成するかもしれませんが、しかし我々の逼迫する要求つまり環境保全や将来の安定的な食糧供給に対する答にはならないと思います。奈良国際シンポジウムはこの緊急の問題を議論しようとしたものです。

昨年、RITEとNAISTが共同で植物バイオテクノロジーの未来ついてワークショップを行いました。そこでは多くの優れた植物科学者にご出席頂き、今後の研究は多重遺伝子導入を通して複合環境ストレスに耐性を持った新しい植物種を創成する方向に進むべきだということで意見は一致しました。つまりは、この方向こそが末期的地球生態系や病弊した植生を蘇生させ、環境CO_2の吸収源と食糧供給源として効果的に機能させる道なのです。多重遺伝子導入技術は、塩害、干ばつ、強光、気温の急激な変化や害虫などの複合的ストレス下でも育ち生長する新種植物を作り出すことを可能にするでしょう。さらには、この技術が植物代謝の活性化を促し、その結果我々は太陽エネルギーが大量の多様な有効化合物に変換するようになると考えています。

この研究の方向性の変更に対応して、日本の通商産業省は1999年より５年間の研究プロジェクトを開始することを発表しました。それは植物を複合環境ストレスに耐性にしたり、植物に有用な物質を作らせるための多重遺伝子導入技術に焦点を合わせたものです。この世界にさきがけた先導的なプロジェクトは、２１世紀のバイオテクノロジー業界が目指す方向を指し示したものであり、多くの成果をもたらすものとして期待されます。

RITEとNAISTは昨年のワークショップの成果を踏え、次世紀の植物バイオテクノロジーの進路を決める手助けとなるべく本日のワークショップを開催することを決定致しました。私は、我々のディスカッションにおいて食糧や環境問題の多面的な解決への植物バイオテクノロジーの新しい役割を提起し、また２１世紀における植物バイオテクノロジーの方向を指し示すことができると確信しております。

締め括りと致しまして、このワークショップに御参集下さいましたことに深く感謝の意を述べますとともに、本ワークショップが多くの成果をおさめますことを切に願うものであります。

Isolation of various plant gene promoters by "genomic" DNA array and efficient transfer of large DNA fragments into plant genomes

Daisuke Shibata (Mitsui Plant Biotech. Res. Inst.)

We contribute a major objective of the Shinmyo-Yokota project, "Multiple gene transfer to plants", by our genomic DNA array technology and by using our TAC vector that has been developed for Agrobacterium-mediated transformation of large DNA fragments. We will also contribute to develop a new method for connecting large DNA fragments in a vector.

1) Promoter isolation by "genomic" DNA array

To drive useful genes under suitable promoters, we will isolate various plant promoters from Arabidopsis thaliana using our ÉÑgenomicÉÜ DNA array. We have developed a novel DNA array in collaboration with Kazusa DNA Res. Inst. (Chiba, Japan). In the genome sequencing project of Kazusa DNA Res. Inst., more than one hundred of P1 and TAC clones were sequenced by a shotgun-based strategy. The sequence data obtained were confirmed with the sequences of "bridge clones" of 2-4 kbp. We selected and assigned a total of 1,859 bridge clones with minimum overlapping, which corresponds to 4,660,772 bp on 47 P1 and 12 TAC clones used for the sequencing project. The regions covered are calculated as approximately 1/5 of chromosome 5 or 1/20 of the whole genome. The genomic DNA fragments of the bridge clones were amplified by PCR with a set of universal primer pairs outside of the cloning site. The amplified fragments were spotted on nylon membrane filters. We call this filter "genomic" DNA array.

We will isolate poly(A)-RNA from leaves that are grown under various conditions such as continuous light, dark, cold, and drought, and then synthesize cDNA from the RNAs. The genomic DNA array will be hybridized with the labeled cDNA. Genes that show strong expression under certain conditions will be identified on the array. The promoter region of these genes will be used for driving useful genes.

2) Large DNA transfer to plant genome

To transfer multiple useful genes into plant genomes by Agrobacterium-mediated transformation, we will use our transformation-competent artificial chromosome (TAC) system. We designed a TAC vector, pYLTAC7, to meet the following requirements: (i) efficient cloning of large DNA fragments, (ii) stable maintenance of inserted fragments in both E. coli and A. tumefaciens, and (iii) competence of transferring inserted DNA into plant genomes via Agrobacterium. The vector has the P1 bacteriophage replicon and the pRiA4 replicon of the Ri plasmid, that render the copy number of the plasmid single in *E. coli* and *Agrobacterium*, respectively. Therefore, large DNA fragments are maitained in *E. coli* and *A. tumefaciens* without deletion. The disadvantage of the low yield of the single-copy plasmid for DNA preparation is overcome by amplifying the plasmid by releasing the suppresser of the P1 lytic replicon with IPTG. As HindIII cohesive ends show a high ligation efficiency, we created a unique HindIII site in the vector. The HindIII site was inserted
between the sacB gene and its promoter. As the gene functions to convert sucrose

to lavan that is toxic to the *E. coli* growth, *E. coli* clones carrying inserts are selected on sucrose-containing agar plates. Two I-SceI sites flanking the cloning sites and the Pnos sequence were engineered in the vector. As statistically the 18 bp recognition site for I-SceI occurs once in 6.9 x 1010 bp, in most cases, entire transgenes with the Pnos/HPT sequence tag are cut out from genomes, allowing us to check sizes of transgenes by Southern analysis using a HPT probe. We demonstrated that entire genomic DNA fragments of 40-80kb were integrated into plant genome without obvious deletions. We also showed that a 45kb fragment introduced into the *Arabidosis* genome was faithfully inherited to the T3 generation. The multiple genes will be integrated into the TAC vector and transfered to model plants by Agrobacterium-mediated transfromation.

Gene identification for improved crop performance by linkage between stress tolerance at tissue level and whole plant fitness

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Background

To improve plant performance under stress condition, it is necessary to consider the underlying physiological mechanism that control plant response to biotic or environmental stress. Many scientists in plant breeding have believed that the potential for success in improving plants by genetic engineering is higher in molecular level by introducing specific genes whose products are tightly linked to primary response or metabolism under stress condition ("bottom up" approach). On the other hand, the potential has been considered to be lower in whole plant level since it had been difficult to regulate plant development by genetic engineering. Recently our knowledge to plant development is accumulated from molecular genetics. Now biotechnology in plants needs to increase greatly in sophistication by linkage between stress tolerance at tissue level and whole-plant fitness.

One of the most powerful source to find a clue to the linkage would be in plant genomics. International efforts to genome analysis are undergoing in many plant species, such as in Arabidopsis and rice. Functional genomics, including transposon/T-DNA tagging and systematic expression profile analysis, is one of our choice to find genes for biological processes of interest.

In my laboratory, we have been studying plant development, especially in plant plasticity in meristem function using cDNA-based functional genomics. Here we show our proposal to the project to improve whole plant performance by linking stress tolerance with developmental regulation.

Strategy

We have made equalized cDNA libraries that contain equal representation of all expressed genes; the cDNA clones whose gene messages are expressed at low level are enriched, and the population for abundant messages are reduced in the equalized libraries. One of the libraries in our stocks was prepared from shoot apical tissue from Arabidopsis. Using this library, we will examine gene expression profile at apical tissue under stress conditions. Also we will check gene expression pattern in mutants that show abnormal response to stress. After expression analyses, we will focus in particular on regulatory genes for plant body plans that are influenced under stress conditions and in mutants. Once identified such genes, their functions and ability to be used in genetic engineering will be carefully evaluated in transgenic plants.

1. Preparation of DNA chips for equalized cDNAs from shoot apical tissues, using microarray technology (-2000).
2. ESTs for equalized cDNAs from shoot apical tissues (-2000).
3. Systematic examination of gene expression profile at shoot apices under biotic and stress conditions and in mutants (-2000).
4. Identification and isolation of "regulatory" genes whose expression were influenced by stress (-2001).
5. Characterization of identified genes by molecular analysis (-2002).
6. Evaluation of identified genes in transgenic model crop in the project (-2003).

Our original materials available to the project

1. Equalized cDNA libraries prepared from shoot apex of Arabidopsis.
2. Regular cDNA libraries from shoot apical tissue.
3. Genes and promoters for apex-specific genes.
4. Individual genes studied in my laboratory in NAIST (Genes for receptor kinase, zinc finger, ring finger, xyloglucan endotransferase, enzymes for tetrapyrrole biosynthesis, etc.)

Research Proposal

Kazuyuki Hiratsuka
Laboratory of Plant Molecular and Cellular Biology
Graduate School of Biological Sciences
NAIST

Development of Polycistronic Expression Vectors for Higher Plants

BACK GROUND

Standard methods used to introduce multiple genes into plant cells require transformation with T-DNA based plasmid vectors that contain multiple promoter-cDNA-terminator cassettes (Figure 1). These cassettes are introduced by way of Agrobacterium mediated transformation or by direct gene transfer. Although these strategies are widely used, it is known that the presence of repeated sequences often causes instability of introduced DNA sequences. In addition, construction of these relatively large plasmid DNA is tedious and time consuming.

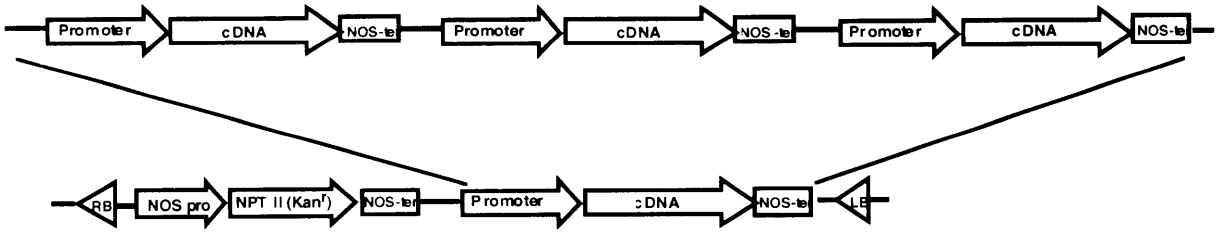

Figure 1. Schematic diagram of the plasmid vector that contain multiple gene cassettes.

For translation of most eukaryotic mRNAs, ribosomes scan the mRNA from the 5' end until an initiation codon is reached. In case of some viruses, ribosomes begin translation at internal sites (internal ribosome entry site: IRES) in the mRNA. The plasmid vectors containing the IRES of the picornaviruses that permits the translation of two open reading frames from one mRNA have been developed in animal system (Figure 2).

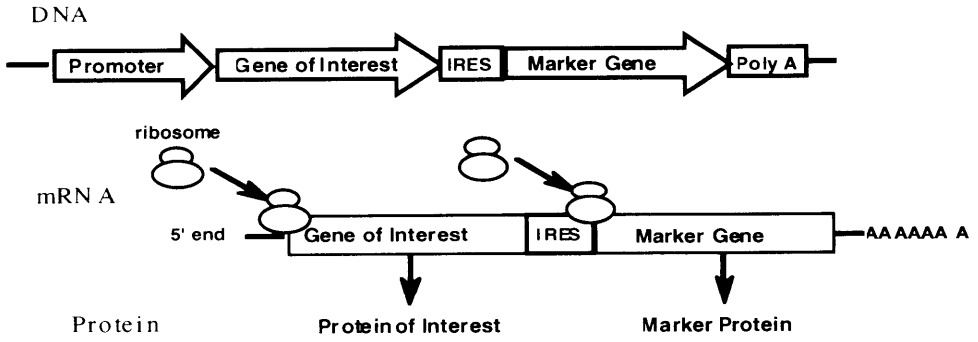

Figure 2. IRES mediated bicistronic gene expression.

In order to simplify the strategy for multigene transformation and for the efficient expression of introduced genes, we propose the application of IRES based expression vector for higher plants. In the proposed research we will focus on developing the polycistronic gene vector system for multigene transfer and expression in higher plants.

EXPERIMENTAL DESIGN AND METHODS

A schematic diagram of the experimental design is shown in Figure 3. We have chosen a cultured tobacco cell line (BY-2) for the pilot studies because such cells are uniform, grow rapidly, easy to transform, are convenient for initial experiments for gene expression studies. For the monitoring the gene expression levels, we will use firefly luciferase(LUC), *Renilla* luciferase(Rluc), β-glucuronidase(GUS) and green fluorescent protein(GFP). A bicistronic vector with IRES sequence from phytopicornavirus that contains LUC and Rluc will initially be tested. We will try transient assay by microprojectile bombardment for the evaluation of IRES function with bicistronic vector. In vitro translation system will also be used for further study. After confirming the IRES function we will try polycistronic version. Finally, studies of transgenic plants transformed with polycistronic vectors will be carried out.

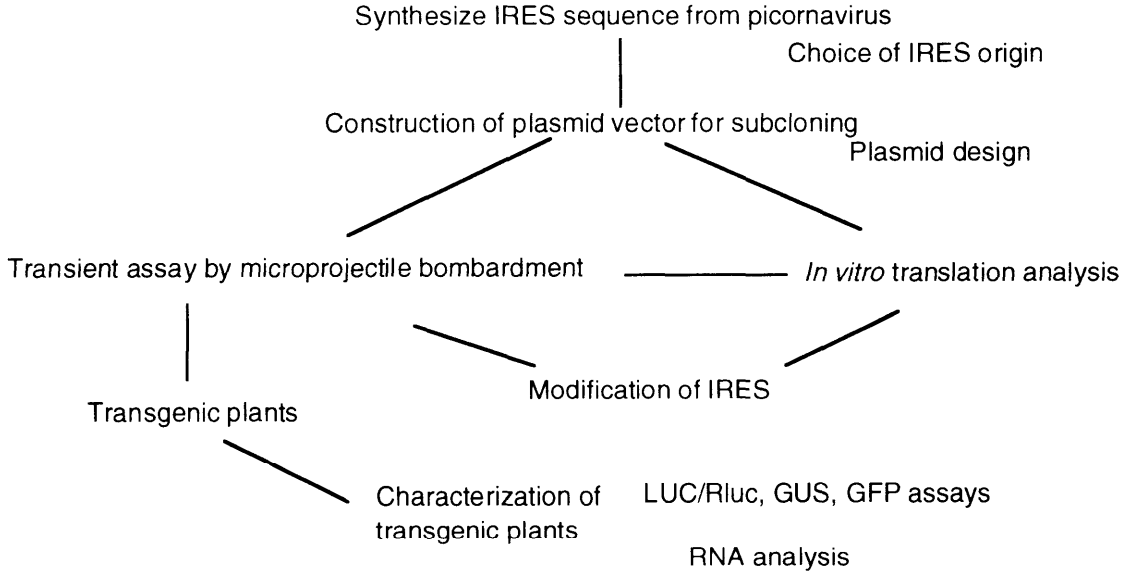

Figure 3. Schematic diagram of the experimental design.

Genes and Techniques

Genes and Plasmids

Genes:

LIM genes: 18 cDNAs that are specifically expressed during microsporogenesis of *Lilium longiflorum*.

Plasmid vectors:

Firefly luciferase vectors: 221-LUC+, 121-LUC

Renilla luciferase vector: 221-Rluc

GFP vector: 221-EGFP-c1

Techniques

Transient assay system by microprojectile bombardment.

High efficiency transformation of BY-2 cells.

Hand held gene gun(Helios).

In vivo luciferase assay system.

Regulation of Expression of Transgenes in Plants

Atsuhiko Shinmyo (Nara Institute of Science and Technology)

CaMV35S promoter is a strong and constitutive promoter, and has been often used to express transgenes in plants. However, future transgenic plants introduced multi-genes have to be controlled the expression of each gene in suitable cell, tissue or organ and at required stage of plant development. We are collecting specific, inducible and/or strong promoters and *cis*-elements.

1) Promoters active in log phase of tobacco cultured cells

Isolation of strong promoters in tobacco cultured cells (*Nicotiana tabacum* L. cv. BY2) was tried. cDNA clones, in which the mRNA level is high in log phase cells and the copy number in the genome is low, were isolated. These genes showed high homology with F1-ATPase (mitochondria type, ATPD), elongation factor 1-α, and a gene with an unknown function of *A. thaliana* (clone 27), respectively. Promoter of *ATPD* and clone 27 genes was constitutively expressed the *GUS* reporter gene and clone 27 promoter showed 5-6 times the activity of the CaMV35S in BY2 cells. This promoter is expected to be active in growing tissues, such as meristem and root tip in whole plant.

2) Promoters active in stationary growing phase of BY2 cells

Three cDNA clones, which are expressed in the stationary growth phase of BY2 cells, were isolated by a differential screening. These clones showed high sequence homologies to alcohol dehydrogenase (ADH), pectin esterase (PES) and extensin. Promoter of *ADH* and *PED* genes expressed the GUS reporter genes at the stationary phase and the maximum GUS activity obtained by both promoters was several tens times of that by CaMV35S. In transgenic tobacco plant the *GUS* was specifically expressed in root under the control of *ADH* promoter. Effector(s) controlling de-repression of *ADH* and *PED* genes is under study.

3) Construction of inducible promoters

A 925 bp promoter fragment of a heat-shock protein gene (*HSP18.2*) of *Arabidopsis thaliana* showed clear heat-shock response of expression of the *GUS* reporter in BY2 cells. *GUS* transcript was detected 15 min after shift of the incubation temperature from 25℃ to 37℃, and reached maximum level at 2 h and then disappeared at 4 h. The maximum GUS activity observed at 4 h was 1000-fold greater than that before temperature shift. Two cDNAs encoding heat-shock factors were obtained from BY2 cells.

Since exposure of plants at 37℃ for a long period will damage total metabolic activities of plants, expression of a positive transcription factor by *HSP18.2* promoter for a few hours might be possible application. Vector 1 contains a hybrid DNA of the coding region of the DNA binding domain of yeast *Gal4* gene and the transcription activation domain of*VP16* under

control of the *HSP18.2* promoter. Vector 2 contains binding sequence of Gal4 protein upstream of CaMV35S promoter/GUS fused gene. Expression of the GUS gene by heat-shock in BY2 cells transformed with vectors 1 and 2 will be studied.

4) Effect of coding region of plant gene on post-transcriptional regulation

We have constructed 2 types of fused genes; (1) The above *ADH* promoter was directly ligated to *GUS* structure gene, and (2) In construct 1, the DNA fragment coding for N-terminal 7 amino acid residues of ADH was placed at the 5' end of the GUS. In BY2 cells with construct 2, GUS activity was higher more than 10 times than those with the construct 1, in spite of a similar level of *GUS* transcript in both cells. This result suggests that the 5' coding region of *ADH* has effect on translation efficiency or stabilities of GUS mRNA and/or GUS protein. We will analyze details of this phenomenon to establish an efficient expression system of transgenes in plants.

5) Effect of insulator on stable gene expression

In general, variation of expression level of a foreign gene in transgenic plants is believed to be dependent on a position of insertion of transgene in chromosome (position effect). A 0.6 kbp DNA fragment containing insulator, which is known to shut off the effect of enhancer and located upstream of arylsulfatase (Ars) gene of sea urchin, was ligated to a minimum promoter of CaMV35S/GUS gene and introduced into chromosome DNA of BY2 cells. GUS activity was measured in independent 50 transformants. About 20% of transformants without insulator were not detected GUS activity, but all transformants with insulator showed a significant GUS activity, suggesting that the insulator might reduce the position effect.

6) Future projects

Screening of organ-specific, stress-specific and/or inducible promoters is our main research project. Differential screening and micro-tip array technology will be used.

Materials and genes in the laboratory

1) Genes for synthesis of a compatible solute, ectoine, which was found in salt-torelant bacteria, *Halomonas elongata.*
2) Genes for cell cycle regulation in tobacco: cdc2, cyclin A, B, and D, Rb homolog, E2F homolog, and protein phosphatase
3) Transformation system of chloroplasts of *Chlamydomonas reinhardtii* and strong promoters active in chloroplast.
4) Peroxidase (prx) genes in horseradish. *prxC1a* gene has effect on growth stimulation of plants and was applied in popular. *prxC2* gene is expressed by wounding. Two *cis*-elements and transcription factors were identified. Ascorbate oxidase gene of cucumber is specifically expressed in fruit and expressed by wounding in leaf.

Theme: Development and establishment of chloroplast transformation technology for the transferring multiple genes into chloroplast genome of higher plants

Proposer: Tomizawa, Ken-Ichi
Research Institute of Innovative Technology for the Earth (RITE)

1. Outline of project

The aim of the project is to establish chloroplast transformation technology for the transferring multiple genes into chloroplast genome of higher plants. In summary, the theme of the research in this project is classified into 2 parts; (1) the establishment of chloroplast transformation technology using model plants and (2) the application of the established technology towards the practical use.

The former part will create more general and sophisticated techniques of chloroplast transformation for multi-gene transfer/expression to the model plants, tobacco and Arabidopusis. In the second part, newly developed techniques will be applied and modified to such model crops as sweet potato and others. In addition, we are aiming at creation of a novel model plant with the fixation ability of nitrogen in the atmosphere. This novel plant will provide us the reality of the extensive cultivation of the plants.

2 Current accumulation of knowledge concerning the relevant theme and the subject of technical development

Compared to plant nuclear transformation technique, the progress of the chloroplast transformation technology is about 10 years behind. In early 1990s, a leading research for chloroplast transformation of a higher plant was done by Paul Maliga's group in US using tobacco as an experimental material. After that the technique has been tried and established by more than 10 groups including our lab in the world using such tobacco, Arabidopusis and so on as experimental materials.

From 1980s, the set of genes involved in nitrogen fixation has been revealed by molecular biological and biogenitical analysis with mutants. In the case of nitrogen-fixation bacteria, Kllebsiells pneumoniae, 18 genes related on nitrogen fixation have been found within 23 kbase successively.

3. Project plan

During the project period from 1999 to 2004, the theme of our proposal is classified into 2 parts; (1) the establishment of chloroplast transformation technology

using model plants and (2) the application of the established technology towards the practical use.

(1) The establishment of chloroplast transformation technology using model plants

Although the chloroplast transformation technique is used several laboratories, the technique is not so general and requires some skills even when we carry a single gene transfer. Thus more general technology should be established in a single gene transfer as well as a multiple gene transfer method by using tobacco and Arabidopusis as model host plants. To facilitate the basic study, attempts will be made A) to search for suitable marker genes for chloroplast transformation, B) to develop in vitro transcription-translation system to obtain the information whether the constructed gene that will be introduced is expressed satisfactory, and C) to develop cytological monitoring system for gene transfer-amplify process. In parallel, D) we will also develop gene transfer/expression technique for multi-structural genes with a suitable promoter and terminator.

A) Search for suitable marker genes

Several genes code for resistance against anti-biotics are frequently used to facilitate screening of the transformants. However, for chloroplast transformation, few number of those genes are available. Therefore, early period in this project, it will be done to search for several genes useful for this purpose.

B) Development of in vitro transcription/translation system

In chloroplast transformation, it takes at least a half year to obtain the final transformant. At a moment, we have no way to judge whether the construct gene that will be introduced is expressed sufficiently or not unless the transformant obtained. This newly developed system will allow to check the possibility of the expression within a week. The basic concepts and techniques have been reported using tobacco chloroplast, so that modification of the techniques adapted for our purpose will be done for tobacco and Arabidopusis in this proposal.

C) Development of cytological monitoring system for gene transfer-amplify process

One of the difficulty of the chloroplast transformation in higher plants is thought to be due to the lack of monitoring system for gene transfer-amplify process. The system we will develop is based on in situ PCR technique. The newly developed system allow to observe the behavior of introduced DNA within a cell and to monitor the amplification process of the DNA. This system will become a basic tool to establish chloroplast transformation method for practical useful crops.

D) Development of gene transfer-expression technique for multi-genes

There have accumulated no evidence on multi-gene introduction to chloroplast genome. To access this matter, we should obtain at least two basic information: One is to determine both minimum and maximum sizes of chromosome that could behave as chloroplast genome. The other is to know whether consecutive multi-structural genes between a suitable promoter and terminator could be expressed satisfactory. Considering the construction of the multi-gene transfer to chloroplast genome system, to obtain these basic information is faced on. Thus we are going to elucidate those problems within the first 3 years in this project, and we will establish the multi-gene transfer to chloroplast genome system until the end of the project.

(2) The application of the established technology towards the practical use

Although the proposal of (1) section has importance and a lot of benefits for basic science, it seems to have trivial aspect in terms of the feature of this project. Considering the disposition of the project, we propose to carry out the (2) section that is the utilization and application of the basic technology obtained by the (1) section. This section can be divided into 2 parts: One is the application of newly developed techniques to such model crops as a sweet potato. The other is the creation of a novel model plant as tobacco having nitrogen fixation ability.

E) Development of sweet potato chloroplast transformation system

When chloroplast transformation system will be constructed, we have to consider the construction of suitable vector, the effective delivery system, reliable regeneration process and sufficient verification method. In our proposal, this part will be done step by step, parallel to the progress of section (1) but with incorporation of the results obtained by section (1). We will achieve those problems within the first 3 years in this project, and we will establish the multi-gene transfer to chloroplast genome system until the end of the project.

F) The creation of a novel model plant having nitrogen fixation ability

For extensive cultivation of higher plants including crops and trees, the supply of nitrogen is a big problem which cannot be avoided. Concerning this problem, we have started for the analysis of genes involved in the nitrogen fixation in sea cyanobacterium. The analysis has revealed that 14 different genes among 18 genes related on the nitrogen fixation are required for the addition of nitrogen fixation ability to a higher plant. In this proposal, we will try introduction of these 14 genes into chloroplast genome in tobacco to add the fixation ability of nitrogen in the atmosphere.

(3) Proposed schedule of each research project

Section	program	99	00	01	02	03	04
(1)	A						
	B						
	C						
	D						
(2)	E						
	F						

4. Expected effectiveness

The project proposed here aims to establish the technology by which provides the basic knowledge of chloroplast transformation, and the application of the provided knowledge will be done by ourselves. In most of cases, the obtained techniques are quite new and patentable technology. In addition, if the novel plant with nitrogen fixation ability will come true, it will picture not only the reality of the extensive cultivation of the plants but provide one worthy step for food crisis we have faced on.

For practical use of transgenic plants, the effects on environment of gene scattering due to pollen scattering should be considered. However, the gene transfer to chloroplast we proposed here is no obstacles for securing the safety since the chloroplast genome transmits with maternal inheritance manner.

Engineering of Transcription of Chloroplast Genes in Higher Plants: Increase of Photosynthetic Productivity and Potential Production of Industrial Materials in Chloroplasts

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The chloroplast is an organelle where photosynthesis occurs to assimilate CO_2 by solar energy. Enhancement of this ability by genetic engineering is invaluable for energy and carbon skeleton supply for potential production of industrial materials in plants. Since photosynthesis is the most sensitive to environmental stresses, tolerance ability of plants to them may be gained by engineering of the photosynthesis machinery. Genes for photosynthesis are encoded in both the nuclear and chloroplast genomes, but genetic manipulation technology for the chloroplast genome has less been advanced. Furthermore, to the chloroplast attention has recently been paid as an intracellular compartment where foreign gene products let be accumulated, and maternal inheritance of recombinant genes in the chloroplast genome may result in higher security to prevent recombinant plants from distributing transgenes to other species through pollens. In these aspects, I intend to develop the technology to manipulate chloroplast gene expression.

1. Clarification of Critical Steps for Enhanced Gene Expression

What is the most critical step in determining levels of gene products in chloroplasts? There were arguments about the steps: gene dosage, transcription, RNA stability, translation, and protein turnover. We have revealed that transcriptional regulation is the most crucial in tissue-specific expression of chloroplast genes in *Arabidopsis thaliana* (1). Therefore, we will focus on the transcriptional regulation. However, we must further clarify what steps are more responsible for levels of chloroplast gene products depending on ways of culture, plant species, and growth stages.

2. Improvement of Endogenous RNA Polymerase

We have found and characterized three species of σ factors of bacterial-type RNA polymerase responsible for transcription of genes for photosynthesis in chloroplasts of *A. thaliana* (2). The enhanced and ectopic expression of genes for σ factors (*SIGs*) under the control of CaMV 35S promoter elevated transcription of chloroplast photosynthesis genes such as *rbcL*, *psbA*, *psbD*, *etc.*, and resulted in rapid growth of seedlings and early flowering. We will precisely analyze what happens when expression of σ factors is manipulated.

3. Employment of Foreign RNA Polymerase

Bacteriophage T7 RNA polymerase system is worth being considered for higher expression of foreign genes. We have introduced the gene for T7 RNA polymerase fused with a chloroplast transit peptide into *A. thaliana*. In our transient expression assay by particle bombardment with reporter genes for β-glucuronidase (GUS) and green fluorescent protein (GFP) (3), the combination of T7 RNA polymerase and its recognizing promoter exhibited the highest transcriptional activity among combinations of endogenous RNA polymerase and promoters of *psbA*, 16S rDNA, and *tac*. The *A. thaliana* line harboring T7 RNA polymerase in chloroplasts will further be evaluated in production of foreign gene products in chloroplasts.

4. Development of Chloroplast Genome Transformation System

There is an example of success in stable transformation of the chloroplasts genome with tobacco in higher plants. However, it is very difficult to transform the chloroplast genome in other higher plants, although we made trial to do it with *A. thaliana* over these 6 years. In addition to particle bombardment, we will try employment of polyethylene glycol (PEG) which is used as another strategy for chloroplast transformation of tobacco.

5. Designing of Gene Constructs

We must consider the following points for efficient expression of foreign genes in chloroplasts: codon usage, possible RNA editing, possible RNA splicing, structures of promoters and its upstream and downstream regions responsible for transcriptional enhancement, stem-loop (hairpin) structures behind the 3'-end of ORFs, 5'- and 3'-untranslated regions (UTRs) for RNA stability, and stem-loop structures closely ahead of the Shine-Dalgarno (SD) sequence for enhanced translation. Each point will be carefully evaluated for efficient gene expression in chloroplasts in higher plants. Genes for the super-Rubisco associated with enhanced carboxylation activity made in collaboration with Akiho Yokota, as well as mutated *psbA* encoding D1 protein species coffering tolerance to irradiance on a cyanobacterium (4) will be tried to be ectopically expressed in higher plants by stable chloroplast transformation.

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Materials and Methodologies to Be Shared

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Original Materials Capable of Being Sheared

1. A λ library of *A. thaliana* total DNA [3 X 10^5 clones, 10^8 pfu/ml; leaf DNA partially digested with *Mbo*I and cloned into λ Fix (Stratagene)].
2. Clones of promoters of *rbcL* and *atpB/E* (ref. 1, in the abstract) and of cDNAs for three σ factors (*SIG1*, *SIG2*, and *SIG3*) (ref. 2) from *A. thaliana*.
3. A gene for engineered GFP, sGFP(S65T) (ref. 3, http://sfns.u-shizuoka-ken.ac.jp/pctech/gfp/).

Methodologies Experienced or Developed at My Laboratory and Ready to be Taught

1. Transient expression assays for both nuclear and chloroplast genes with reporter genes in *A. thaliana* and tobacco (refs. 2 and 3).
2. Stable transformation of the nuclear genome of *A. thaliana* and tobacco.
3. Genetical analysis of the nuclear genome of *A. thaliana*.
4. *In vitro* random mutagenesis (ref. 4).

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Production Technology for Environmental Compatibility

Technology Development for Renewable Industrial Materials Production by Plants Adapted to Stressful Environments

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Our group will contribute to the MITI-Japan/US project the expertise and knowledge from the ongoing program: "Functional Genomics of Plant Stress Tolerance", and a new interdisciplinary program on "Metabolic Engineering of One-Carbon Metabolism". Within our group, individual members pursue different, independently funded projects, which are coordinated to our core research objectives. The group consists of Drs. Andrew Hanson (U. Florida), Doug Gage (Michigan State U.), Ray Bressan and Mike Hasegawa (both Purdue U.), and Jian-Kang Zhu and Hans Bohnert (both U. Arizona). Arizona will be the lead institution for the MITI project.

By far the most severe reduction of plant productivity and possible crop yield is due to environmental factors, drought, high salinity and adverse temperature, both high and low. Abiotic stress is responsible for more than two third of yield reduction compared with pathogen infestations and insect damage. The mechanisms important for plant protection are gradually emerging; we can now point to a limited number of crucial biochemical reactions, which lead to improved tolerance of adverse environmental factors. The MITI project will combine engineering of stress tolerance traits with plant alteration and improvement of traits leading to industrial and agronomical production of materials in a multidisciplinary program the success of which depends on the coordination of efforts in diverse areas.

The four areas in which we have proven expertise and documented credentials are **(1)** gene discovery, **(2)** plant metabolic engineering,

including the production of transgenic plants, (**3**) analytical biochemistry and (**4**) metabolic modeling. Finally, each of us have exemplary records in training and education.

(1) Gene discovery and suitable promoters for plant engineering.

R. Bressan, *Purdue Univ., USA*
M. Hasegawa, *Purdue Univ. USA*
H. Bohnert, *Univ. Arizona, USA*
J.-K. Zhu, *Univ. Arizona, USA*

One aspect important for stress-tolerance engineering that is not sufficiently known concerns the control elements for pathway induction and maintenance. First, we do not have the promoters that lead to suitable expression. Second, we lack knowledge about how strongly, when and where – subcellular, and cell- and tissue-specific – these mechanisms should be expressed to promote tolerance.

Several members of our group (Bressan, Hasegawa, Zhu, Bohnert) are working on a "stress tolerance genome analysis" project. Relevant to this project is work that will define the core-set of stress-induced transcripts in three higher plants and a salt-tolerant alga. By sequencing approximately 10,000 transcripts from each species we will be able to distinguish the set of common stress-relevant genes from the set that is unique to each species.

For the MITI project we propose to analyze stress-induced promoters for a sub-set of these genes. In addition to induction by stress, we will analyze tissue- and cell-specificity and developmental regulation of these promoters. The goal will be studying, and preparing for utilization in transgenic plants, a set of promoters covering the entire life cycle and several stress responses of target species.

Because we understand that the target species for the MITI project are not yet decided, we will justify here the strategy for promoter detection and analysis. From our experience, most promoters from one species will function very similarly in a different species, although the relative strength of expression might vary. In our experience, promoters from grass species (monocots) retain cell specificity faithfully after transfer into dicot species, unless the promoters are derived from C4-type genes. The majority of promoters that we will find will therefore be suitable for use in various crops.

Another data set argues for using promoters from foreign species, rather than isolating the control elements from the target crop species. In more than 20 transgenic experiments, the use of a foreign promoter resulted

in excellent expression without problems of gene silencing which may be encountered when a resident promoter is duplicated.

With these promoters, the expression of transferred genes can be controlled, but additional protein domains will also become available through our work, by which the proteins can be delivered to the sub-cellular compartment in which they should function. Protein domains, e.g., for nuclear, cytosolic, chloroplast, vacuolar or membrane attachment, will be engineered to provide multi-purpose cassettes.

The techniques to be used are established in our laboratories, including the necessary models for testing promoters and domain fusions, and the use of reporter genes that indicate in life tissues and cells the expression characteristics of promoters in a tissue context and the validity of sub-cellular targeting domains.

(2) Metabolic Engineering.

D. Gage, *Michigan State Univ., USA*
H. Bohnert, *Univ. Arizona, USA*
A. Hanson, *Univ. Florida, USA*

Three members of the group (Hanson, Gage and Bohnert) have successfully developed a coordinated, multidisciplinary group approach for a new US research initiative for plant metabolic engineering. The main focus of this effort is to understand how primary one-carbon metabolism can be altered in response to the addition or subtraction of metabolic sinks for methyl groups in plants. This involves expertise in gene expression, transformation, biochemistry, analytical chemistry and metabolic modeling.

It is now becoming clear that the transfer of a single gene or suites of genes to introduce novel metabolic pathways into target organisms is only the first step in effective metabolic engineering of these pathways. For example, the introduction of the two genes for the biosynthetic enzymes in the higher plant glycine betaine pathway does not produce significantly elevated glycine betaine levels in the transgenic plants. Even though the biosynthetic enzymes were found to be properly targeted to the appropriate cell compartment, primary metabolic pathways involving methyl group metabolism were not flexible enough to respond to the new metabolic demands. The techniques of both molecular biology and analytical biochemistry have been equally important in helping us to reach this level of understanding, but there is clearly more to be done. In order to engineer new pathways, we will need to understand how primary metabolism has been altered (or is regulated) in the organisms that naturally contain the metabolic pathways of interest. We will also need to learn how primary pathways respond in these organisms to alterations in metabolic flux (e.g., using antisense technology or gene knockouts). This knowledge will help us to design strategies for overcoming the factors limiting the successful

engineering of metabolism. Simple genetic transformations for a discrete pathway in target organisms alone will not accomplish our objectives in metabolic engineering.

The expression of new biosynthetic or regulatory genes will alter primary metabolism and gene expression in unpredictable ways. Therefore, it is necessary to first understand how the transgenics are metabolically perturbed. This requires both the measurement and modeling of metabolite flux. It is also important to determine how the expression of other genes is modified. Gene array technology can be used to examine gene expression in transgenics. With the knowledge gained from many different levels of analysis (gene, gene product and metabolite), repeated rounds of genetic engineering may ultimately be necessary to produce the desired result. We are convinced that the multidisciplinary approach will be essential for making metabolic engineering a practical reality.

The development of transgenic plants tolerant of environmental stresses, as proposed in the MITI project, will clearly require more than the introduction of newly discovered genes. Linking the MITI project to the US metabolic engineering group will allow a valuable exchange of ideas and strategies that will be mutually beneficial. Inter-laboratory training of graduate students and postdocs is a key component in our projects. The expansion of the training effort by bringing young Japanese investigators to our labs will be an obvious means to help accomplish the MITI project objectives.

(3) Training.

B. Hanson, *U. Florida, USA*
D. Gage, *Michigan State Univ., USA*
R. Bressan, *Purdue Univ., USA*
M. Hasegawa, *Purdue Univ. USA*
J.-K. Zhu, *Univ. Arizona, USA*
H. Bohnert, *Univ. Arizona, USA*

Training of young Japanese investigators in the multi-disciplinary approaches represented within the group can be our primary contribution to the MITI effort.

Large interdisciplinary, multi-component projects succeed only when they are well coordinated and when the major players manage to generate synergy among themselves and with all participants, including postdoctoral fellows, graduate students, and technicians. We must generate the conviction among all our people that the whole is more than simply a numerical addition of person-power and projects; that this is a common project. For the project to be successful, close interactions will be important. Such

interactions can best be assured by the exchange of people. We suggest that people are exchanged and that young Japanese colleagues, postdoctoral Fellows, join our laboratories for extended time periods. For people from our labs to work in Japan, if funds can be made available, sub-projects for later years of the project should be discussed.

Looking out for all people in a project has become something that not only all successful companies do these days, federal research agencies in the US place much emphasis on this aspect of a project. Our combined laboratories have in fact become models for such interactive, multidisciplinary groups. Taken together, we have trained well in excess of 100 postdoctoral fellows and graduate students and even more undergraduate students have worked in our labs.

Rigorous experimentation and intelligence we expect from all people in our laboratories. What most students must learn, however, is to share experiences, to discuss problems, to open up to others, talk about their work in a non-defensive attitude, develop a competitive spirit in fairness to others, and to learn how to quickly and creatively rethink and alter experimental approaches that have failed. This is what we can teach and such trained colleagues will assure success to the project. We can of course also train young colleagues to sharpen their skills in English scientific writing and in making oral presentations.

Creation of plants with tolerance to oxidative damage imposed by environmental stresses

Shigeru Shigeoka (Kinki University, Nara 631-8505, Japan)

Environmental stress is the major limiting factor in plant productivity. Plant cells are prone to oxidative stress because they both produce and consume oxygen. Much of the injury to plants caused by stress exposure is associated with oxidative damage at the cellular level. The destruction of a regulated balance between the generation and scavenging systems of active oxygen species (AOS) causes the inactivation of enzymes and damage to important cellular components. In plant tissues the chloroplasts are potentially the most powerful source of oxidants and sites within the cell most at risk from photooxidative damage. Many stresses cause stomatal closure and the limitation of transpiration, which inhibit the photosynthetic capacity. As soon as the CO_2 concentration decreases in chloroplasts, there is a lower availability of NADP to accept electrons from PSI, thus initiating O_2 reduction with the concomitant generation of O_2^- and H_2O_2. Higher plants have developed defense systems, nonenzymatic antioxidants and antioxidant enzymes against photooxidative damage; the chloroplasts contain the ascorbate (AsA)-glutathione (water-water) cycle including SOD, ascorbate peroxidase (APX) isozymes and the regenerating system of AsA.

Physiological and genetic evidence clearly indicates that the scavenging systems of AOS of plants are an important component of the stress protective mechanism. The ability to manipulate the levels of specific enzymes in these systems using gene transfer technology raises hopes that this approach can be used to improve the stress tolerance of economically important plants. Recently, we generated transgenic tobacco plants which expressed the catalase (*kat E*) from *E. coli* in chloroplasts and showed increased tolerance to photooxidative damage caused by drought at high light intensity, suggesting the possibility that this plant can grow even in a semidesert environment. Furthermore, we have cross-fertilized the transgenic tobacco plant expressing *kat E* catalase in chloroplasts with the transgenic plant expressing mannitol 1-phosphate dehydrogenase in cytosol. The transgenic plants showed increased tolerance to photooxidative stress and salt stress.

Our attainable goal is to increase the oxidative stress tolerance of crop plants, which is being accomplished through the following research objectives.

1) Search for effector genes and command genes involved in oxidative stress

From the data accumulated thus far, attention should be directed forward the compartment-specific location of the AOS-scavenging enzymes and cell-specific, tissue-specific, and developmental stage-specific expression of the genes. It is well known that two complementary ways are necessary to approach the engineering of crop stress resistance: first by finding and inserting new effector genes from stress-resistant plants and other species, and secondly, by modifying the command genes that determine the perception and transduction of the stress signal.

With respect to the affnity for H_2O_2 and the enzyme stability under the oxidative stress conditions, catalase from prokaryotic cells is quite superior to APX as a stress-resistant enzyme. More noteworthy is that the transformed *E. coli* cells expressing selenium-independent glutathione peroxidase (GPX) or APX from halotolerant *Chlamydomonas* sp. W80 exhibit tolerances to salt stress and oxidative stress.

Manipulation of the regulatory processes controlling the expression of enzymes involved in the antioxidant systems may provide an additional means of stress tolerance improvement. Accordingly, it is necessary to clarify what *cis*-acting signals and *trans*-acting factors are involved in the regulation of gene transcription in response to oxidative stress. In addition, we will focus on the two-component signal transduction system in higher plants, because the two component system in photosynthetic bacteria has recently been shown to integrate the control of photosynthesis, carbon dioxide assimilation, and nitrogen fixation.

2) The generation of transgenic plants with enhanced level of antioxidant

Tobacco leaves suffering from drought at high light intensity lost their chloroplastic APX activities as well as their photosynthetic activity, indicating that APX is much more strongly inactivated by oxidative stress than phosphoribulokinase (PRK), which is believed to be one of the enzymes most sensitive to H_2O_2. Miyake and Asada have demonstrated that the inactivation of spinach chloroplastic APX isozymes in an AsA-depleted medium is caused by the instability of Compound I to H_2O_2 when AsA is not available for Compound I. Under the stress conditions, the level of AsA in the chloroplasts of tobacco plants significantly decreased, suggesting that the complete inactivation of chloroplastic APXs is associated with the decrease in the level of total AsA. It seems likely that under the oxidative stress condition, the excess generation of AOS causes the depletion of AsA in the vicinity of tAPX, thus resulting in the inactivation of tAPX. Based on the data accumulated thus far, it is clear that a high level of endogenous AsA is essential to effectively maintain the antioxidant system that protects plants from oxidative damage due to biotic and abiotic stresses. L-Galactono-γ-lactone dehydrogenase (GLDase), an enzyme that catalyzes the final step in the biosynthesis of AsA, is a rate-limiting factor. Accordingly, the isolation of the GLDase cDNA may allow us to possibly engineer crops containing stably increased levels of AsA, thus increasing their nutritional value and stress tolerance.

3) Combination of genes and attempt of multigene transfer

Studies on transformed plants expressing increased activities of enzymes in the stress defense system indicate that it is possible to confer a degree of tolerance to stress by this means. Attempts to increase stress tolerance by simply increasing the activity of one enzyme have not always been successful. Accordingly, an attempt of multigene transfer for the simultaneous increase in several components of the defense systems against many stresses seems to be necessary to obtain a substantial increase in stress tolerance. We have found that the algal photosynthesis, that is, the CO_2 fixation, shows tolerance to a high level of H_2O_2, which is due to the nonsusceptibility of the thiol-modulated enzymes, that is, FBPase, GAPDH, PRK, and SBPase, of the Calvin cycle to H_2O_2. We are trying to combine our genes including a new gene involved in oxidative stress and attempt multigene transfer.

1. cDNAs encoding stromal, thylakoid, microbody, and cytosolic APX isozymes from spinach and tobacco, and cDNAs encoding selenium-independent glutathione peroxidase and APX from halotolerant *Chlamydomonas* sp. W80
2. *Cpx* encoding catalase-peroxidase from *Synechococcus* 7942), *Apx II* encoding spinach chloroplastic APX isozymes, *kat E* encoding catalase from *E. coli* , and genes encoding thiol-modulated enzymes from *S.* 7942 and *Synechocysitis* 6803

New factors which alleviate high intensity-light and low-temperature stress.

Norio Murata
National Institute for Basic Biology

High-intensity light and low temperature are important environmental factors that limit productivity of plants. These factors interact with each other to induce damages to the photosynthetic machinery; e.g., photoinhibition of the photosystem II complex is apparently enhanced at low-temperature.

The molecular mechanism of photoinhibition has been intensively studied, and it is established that photoinhibition is caused by the light-induced inactivation to the photosystem II complex with damage to and degradation of the D1 protein (a component involved in the photochemical reaction center in the photosystem II complex). In intact cells or plants, however, the photosystem II complex restores its activity by synthesis *de novo* of the D1 protein. This recovery process is depressed at low temperature. This explains that the photoinhibition is enhanced at low temperature.

We have previously demonstrated that photoinhibition is alleviated by acceleration of the recovery of the photosystem II complex by several factors: (1) Desaturation of fatty acids in membrane lipids; (2) the presence of glycinebetaine; (3) treatment with abscisic acid; (4) removal of salt stress; (5) elimination of active oxygen. Therefore, it can be concluded that the genetic engineering of the recovery process should enhance the tolerance of the photosynthetic machinery to high intensity-light and low-temperature stress.

Based on these consideration, our proposal of the research plan is as follows; (1) Identification of reactions which limit the overall recovery process of the photosystem II complex from photoinhibition under various stress conditions; (2) Cloning of genes responsible for these reactions; (3) Genetic modification of these genes in order to improve the stress tolerance by acceleration of the recovery from photoinhibition.

List of materials in hand

1. Genes for fatty acid desaturases
2. Specific antibodies against fatty acid desaturases

Protein Secretion System into the Rhizoshpere

Takashi Hashimoto (Nara Institute of Science and Technology)

1. Objective

Design and demonstrate a strategy which delivers recombinant proteins into the rhizosphere to improve the performance of transgenic plants and the root environment

2. Background

Soil and sand sheaths usually cling tightly to the many field-grown plants, and are thought to be formed by the binding of soil particles in mucilage originating from the root. Mucilage, soil particles, sloughed-off root cap cells, and some soil bacteria together comprise the rhizosphere. Despite its potential importance in determining root growth and therefore crop productivity, the rhizosphere has not been the subject of biotechnological improvement. In this project, a genetic engineering approach will be taken to secrete recombinant proteins into root mucilage which would spread into the rhizospere.

We previously cloned a cDNA (*zmGRP4*) encoding a member of the maize glycine-rich proteins. Detailed expression studies showed that *zmGRP4* is strongly expressed in lateral root cap and weakly in root epidermis, and that a modified, insoluble form of zmGRP4 accumulates in the root mucilage. A genomic clone of *zmGRP4* will be used to design the protein secretion system.

3. Strategy

1) Molecular cloning of genomic clones of *zmGRP4* from maize
2) Design a series of plant transformation vectors which express:
   - *zmGRP4* promoter::GFP for accumulation in lateral root cap
   - *zmGRP4* promoter::zmGRP4-GFP for secretion into the rhizosphere (local distribution)
   - *zmGRP4* promoter::zmGRP4 transit peptide-GFP for secretion into the rhizosphere (wide distribution)
3) Generate transgenic plants (rice, tobacco, and Arabidopsis)
4) Examine distribution of the GFPs in the root and the rhizosphere

4. Applications

1) protection of roots against damages caused by bacteria, fungi, and insects
2) improved nutrition uptake
3) phytoremediation

5. Available genes/cDNAs

- zmGRP4 encoding a glycine-rich protein accumulating in the root mucilage
- D1/MP10 encoding novel maize membrane-proteins expressed specifically in the outermost cells of root cap
- spermidine synthase from *Arabidopthis thaliana, Atropa belladonna*, and *Hyoscyamus niger*
- PMT, TR-I, TR-II, H6H, and A622, encoding enzymes involved in nicotine/tropane alkaloid biosynthesis from several solanaceous plants

Functional identification of stress tolerance genes by transposon tagged sites (TTS) and microarray technology

Ko Shimamoto, Laboratory of Plant Molecular Genetics, Nara Institute of Science and Technology

Background

Numerous biotic and abiotic stresses are responsible for huge loss of yields of agricultural products worldwide. To help solve this problem genes that are functionally important in stress tolerance need to be systematically isolated from various plant species. A traditional approach has been to identify genes whose expression are induced in plants under various stress conditions. Transgenic experiments are later performed to study their gene functions. Although this approach has been successful, numerous genes which have functional roles in stress tolrance in plants have been uncovered yet. The proposed research is to systematically identify such genes by combining the transposn-tagged lines and the microarray technology. The method could be called TTS-MA (Transposon-Tagged Site-MicroArray).

We have generated ca. 18000 rice plants carrying active *Ac/Ds* transposons, isolated DNA and harvested seeds from all the plants. Based on our previous studies on sequencing TTS it has been shown that *Ac/Ds* preferentially transposes into coding sequences with high frequencies. Hence these rice population are a potential source of mutants for any biological processes of rice. Microarray technology is a convenient method to analyze transcription of numerous genes at once.

Proposed Experiments

We will isolate 3000 TTS in the rice population carrying active *Ac/Ds* by the TAIL-PCR method. Each TTS will be sequenced and placed in microarray. To evaluate possible functions of these TTS, RNAs will be isolated from rice plants that are i) infected with avirulent pathogens and ii) treated with low temperature. RNAs will be isloted at different stages of stress treatments. These RNAs will be hybridized with TTS and their expression levels at different stages will be measured. When a TTS shows a response to a stress, seeds derived from a plant from which a TTS is isolated will be identified. Then we will find identify those plants in which a gene is interrupted by *Ac/Ds*. Finally the phenotype of mutants lacking the gene under the stress conditions will be examined to determine the role of the TTS in stress tolerance.

Expected Outcomes of This Proposal

i) The partial sequence of 3000 rice genes. ii) Identification of many genes that have functional roles in stress tolerance. iii) Identification of 1000 knockout mutants of rice.

Molecular analyses of gravitropism in Arabidopsis

Masao Tasaka and Takashi Hashimoto; Nara Institute of Science and Technology

Shoots and roots of higher plants exhibit negative and positive gravitropism, respectively. Gravitropism includes many important and essential plant physiological processes such as perception of an environmental stimulus, signal formation in the gravity perceptive cell, intra- and inter-cellular signal transduction, growth control by plant hormones, inducing asymmetric cell elongation between the upper and lower sides of the responding organs.

To analyze the molecular mechanisms of gravitropism, many gravitropic mutants have been isolated from *Arabidopsis thaliana*. Our characterization of these mutants demonstrates that the molecular mechanisms of gravitropic responses in roots, hypocotyls and inflorescence stems are genetically different[1]. We have identified some of these mutated genes. The cytological and molecular biological investigations of two mutants of scr/sgr1 and shr/sgr7, which are agravitropic in both inflorescence stems and hypocotyls, clearly indicates that endodermis is the site of gravity perception in shoots. The *SCR/SGR1* gene encodes a possible transcription factor from a new bZIP family[2]. *agr* is agravitropic only in roots. The AGR/EIR1 protein is related to the apical-basal polar transport of auxin in roots, emphasizing the importance of this transport for root gravitropism[3]. *solitary-root (slr)* is agravitropic in both roots and hypocotyls, and is specifically resistant to exogenous auxin. The *SLR* gene appears to encode a member of the *AUX/IAA* family of short-lived nuclear proteins[4].

In this study, we want to continue to isolate more gravitropic mutants and combine the sequence data with physiological, cytological and molecular biological data from these mutants. These analyses should provide both an outline of gravitropism and the detailed scenario of each step at a molecular level. The analysis of gravitropism should advance many other fundamental fields of plant biology and the outcome of understanding might be the design of many useful plants including quickly getting up crops and horizontally growing vegetables and flowering plants, which provide new ideas for agriculture and gardening.

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Available mutants (*Arabidopsis thaliana*)
sgr1/scr, sgr2, sgr3, sgr4/zig, sgr5, sgr6, sgr7/shr, rhg, slr, agr

Available genes
SGR1/SCR, SLR, AGR/EIR1

Over production of amino acids in soybean seeds which are acid and salt tolerants

by Yoshikatsu Murooka

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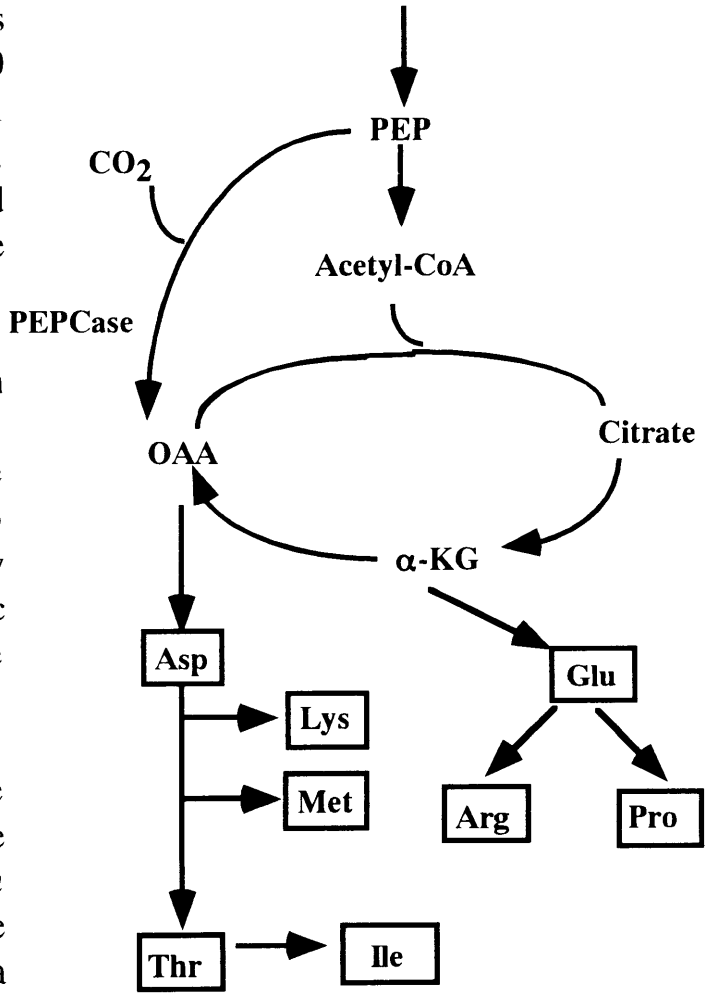
Some of the ten essential amino acids, which are required in an animal diet, are deficient in many grains. Soybean meal, which is rich in lysine and other essential amino acids, is used to supplement corn-based animal feeds which is relatively poor in amino acid content. For example, about 200,000 tons of lysine are produced annually by fermentation, mostly for use as an animal feed additive. The cost of crystalline lysine is currently, about $ 1.20/lb (¥ 300 /kg). An increase in the essential amino acid content of corn, soybean, or other animal feed sources would reduce the need to supplement the seed with crystalline amino acid.



Transgenic soybean seeds with increased amino acids

An approach to raising the amino acid content of seed is to increase its productivity by amplification of the biosynthetic genes which are insensitive to the feedback inhibition.

Example 1. Lysine

Lysine-feedback-insensitive dihydrodipicolinic acid synthase encoded by the *Corynebacterium dapA* gene and aspartokinase by the *E. coli lusC* gene were linked to a chloroplast transit peptide (*ctp*) and expressed from a seed-specific promoter (phaseolin) in transgenic canola and soybean seeds. *Agrobacteium*-mediated transformants of canola and soybean were self-pollinated and mature seeds were harvested and analyzed. The transgenic seed accumulated several hundred hold amount of free lysine and increased total seed lysine content by as much as 5-fold

(S. C. Falco et al., Biotechnol., 13:677-582, 1995).

A soybean meal with double the normal lysine content is worth an additional $3.6/100lbs (¥1000/100kg) over commodity soybean meal. They applied this technology to corn and achieved a 3-fold increase in corn seed lysin content.

Example 2. Tryptophan

Transgenic soybean that contains the feedback-insensitive anthranilate synthase gene increased 400 times higher amounts of free tryptophane (Cho, personal communi.). The crystalline tryptophane is about ¥1000/kg?

The genes may increase the production of other amino acids.

Methionine: methionine- & threonine- insensitive- threonine dehydrogenase

Threonine: Threonine-insensitive-aspartokinase & threonine dehydrogenase

Glutamic acid: Glutamate dehydrogenase

Aspartic acid: Phosphoenolpyruvate carboxylase, aspartate aminotransferase

Development of symbiotic engineering

The symbiotic system between plant and bacterium, such as legume and rhizobia, will be useful for a gene expression in plant. Since genes from microorganisms can be expressed in bacteroides in legume nodules. We have isolated several acid tolerant *Bradyrhizobium* strains which infect and form nodules on soybean roots.

The techniques should be broken through for overproduction of useful products in soybean seeds are as follows:

1) Selection of acid tolerant soybean:
   Isolate it in Southeast Asian countries.
2) Over expression system in bacteroid:
   Use the *nif* gene promoter or other strong promoter which can express in bacteroid.
3) Translocation of the product produced in bacteroid to the seeds:
   Use the seed-specific promoter, link to a chloroplast transit peptide?
4) Efficient extraction and purification of materials from seeds.
   Fermentation: cultivation of cane-- cane molasses-- fermentation-- purification
   Transgenic plant: cultivation of soybean-- purification
5) Development of salt tolerant transgenic soybean and *Bradyrhizobium*:
   Use the ectoine synthesizing genes, betaine-glycine synthesizing genes.

These technologies are applicable to other leguminous plants and also crops to accumulate useful materials (vitamines B1, C, B12 and E, vactines, PHB etc.) in seeds.

The following genes cloned in my laboratory can be transferd for this project

Y. Murooka

atsA, arylsulfatase from *Klebsiella aerogenes*
atsB, arylsulfatase positive regulator
moaR, positive regulator of monoamine regulon from *K. aerogenes*
moaEF, tyramine metabolic enzymes
maoCA, monoamine oxidase from *K. aerogenes* and *E. coli*
pulA, pullulanase from *K. aerogenes*
galK, galactokinase from *E. coli*
choA, cholesterol oxidase from *Streptomyces*
ksd, ketosteroid dehydrogenase from *Arthrobacter*
ksi, ketosteroid isomerase from *Arthrobacter*
hMTII, human metallothionein II
mMTII, monkey metallothionein II
ectABC, ectoine synthesis genes from *Halobacterium elongata*
hem, several hem genes
nif, seveal nitrogen-fixation genes from *Astragalus sinicus*
nod, several nodulation genes from *Astragalus sinicus*
ENODs, several noduline genes from *Astragalus sinicus*

Host vector system developed in my laboratory
CI_{857}-P_RP_L expression vectors for *E. coli*
Klebsiella host-vector system
Erwinia host-vctor system
Xanthomonas host-vector system
Streptomyces host-vector system
Lactobacills host-vector system

Oil production as raw materials in genetically engineered plants – Gene transfer of fatty acid production from bryophytes to oil seed plants –

Kanji Ohyama (Laboratory of Plant Molecular Biology, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University)

We are facing two major problems in coming 21 century to coexistence of livings, especially prosperity of human beings. The first one is environmental problems such as global warming by CO_2 increase in the atmosphere, and expansion of desert area in major continents. The second one is food shortage in especially developing countries by increasing population and less and less cultivated lands. We, as plant scientists, have the great responsibility to dissolve these problems toward the survival and welfare of people in the world because plants are so important livings that have a key role in the dissolution of problems mentioned above.

Now we think over more details of these problems and how to dissolve it. In order to dissolve these problems we first try to find out the causes of the problems described above. The former problem is caused by to much energy consumption of fossil fuel which can not be recycled in the short period of time. However we can not stop it in the present time unless it can be replaced by another sources of clean energy. In the long term, therefore, we can contribute with energy supply from plant resources which are products of photosynthesis in plants. That is, improvement of the photosynthetic activity is an important key which gives some ideas for dissolution of the first problem by direct energy supply as fuel, and also photosynthesis itself directly stimulates CO_2 reduction in the atmosphere. We also can overcome the expansion of desert area by the creation of drought-resistant plants.

The later problem is caused by drastic increase of world population and also by no increasing cultivated lands. On the other hand, the ordinary plant breeding system is also reaching to the maximum level of production of crops. Therefore it is only possible to improve plant production in quantity as well as in quality of their products by so called plant genetic engineering. We have so far succeeded to some extent this approach by manipulation of disease resistant-, herbicide-resistant genes from the other organisms. However, we again realize that plant growth is basically results or products of photosynthesis using solar energy. Therefore it is reasonable to overcome food shortage by improving of plant growth with the higher photosynthesis activity, and at the same time by acquiring of good quality of plant products using gene transfer techniques.

We, therefore, choose oil synthesis system in plants as mass production that can be utilized as the raw materials in the industrial scale. Plant lipids are usually produced in the chloroplast compartments by photosynthesis, and transferred and accumulated in the seeds as oil body. We use bryophytes as experimental plants because they are well-known to produce lipids such as fatty acids, sterol, and their related compounds.

We have already some tools such as DNA recombinant techniques and plant cell culture system. We have constructed genomic libraries, and cDNA libraries from a liverwort Marchantia polymorpha. We have the complete genetic information of liverwort chloroplast and mitochondrial genomes.

We here propose the cloning of genes for elongation of fatty acids (C18 to C20; C20 to C22) that can be used as good quality of food materials and for sterol biosynthesis the products of which can be used as raw materials of medical compounds. We analyze the gene function and regulation of the expression. Finally we apply these methodology to crop plants such as rapeseed, soybean, sesame plants, resulting in high quality of oil as well as in the large scale production of oil that can be utilzed as raw materials for the industries in the replacement of the substances from fossil oil.

Experimental plan

Years	Subjects
1999	Cloning of genes for fatty acid elongation and sterol biosynthesis in bryophytes
2000	Characterization of their gene expression and regulation
2001 – 2003	Gene transfer to oil seed plants and analysis of transgenic plants and their products

Materials production in plants using the transcriptional activator-target regulons system

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In order to create agromonically and industorially useful plants, introduction of multiple genes into a single plant is one of the indispensable strategies. To date, two methods have been developed: one is to deposit genes in a plant by crossing parental plants that contain one gene; the other is to introduce tandemly organized genes that contain own promoter for each gene. However, both require tedious work and time consuming, and also expression efficiency of introduced genes is currently not clear. To overcome these disadvantages, we propose here other possibilities in condition that intensive basic studies are available. The first is to construct an operon system, by which one promoter drives successive multiple genes. The second is to create a regulon system, by which single transcription factor drives related multiple genes. In this proposal, we mainly describe the second option.

Up to now two research groups, one in Michigan State University and the other in RIKEN, have identified a transcriptional activator-target regulons system in *Arabidopsis thaliana*. The CBF1 (DREB) is the transcriptional activator which plays a role both in low-temperature- and drought-signal transduction pathways, and recognizes and binds to a C-repeat (or DRE, drought responsive element) sequence. This C-repeat (DRE) sequence was found in the promoters of CBF1 (DREB)-target genes such as *rd29A* and *cor15A* genes. The finding indicates that multiple genes with *cis*-element described above are switched on by a master gene, *CBF1* (*DREB*) expression.

We have identified the similar gene, wizz that encodes a zinc-finger type transcription factor. Wizz is induced within 10 minutes after mechanical

wounding, and recognizes particular nucleotide sequences that are found in several wound- and pathogen-induced genes. The other system we have is the genes involved in sulfur metabolic pathways, which respond coordinately to sulfur and/or nitrogen deficiency. We identified several key genes, of which expression simultaneously activated by sulfur, suggesting the presence of a common transcriptional control system. We propose here to utilize these genes as a model system to construct an appropriate regulons system in higher plants.

Our project:

1. To characterize wizz system which is composed of a transcriptional activator, wizz and its downstream regulons.

2. To identify the common transcriptional activator for sulfur assimilation pathways.

3. To establish a sweetpotato transformation system and to isolate tuber-specific promoters.

4. To produce valuable materials using a DREB-*rd29A* promoter system or a novel system that we will develope.

Techniques we are able to provide:

FDD cloning.

Clones we are able to provide:

N. tabacum (wound-induced genes, MAP kinase genes), *A. thaliana* (genes which participate in sulfur metabolism), wheat (light- and cytokinin-induced protein kinase gene), rice (genes encoding a small GTP-binding protein, low-temperature-induced genes), maize (low-temperature-induced genes, CDPK gene)

Molecular and Cellular Engineering of Useful Secondary Metabolite Production in Higher Plants

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Higher plants are important natural resources for chemicals. Although plants are renewable resources, they are becoming more difficult to obtain sufficient amounts due to the increased demand and drastic reductions in plant availability. While cell culture of plants provide promising alternatives, still their productivity is limited. Here, we propose the new strategy to produce novel useful metabolites more efficiently in plants by genetic engineering.

1. Introduction of whole biosynthetic pathway;
With the development of large-size (-100kb) gene transfer, multi-gene transfer is now available. So far, we have isolated several cDNAs involved in the biosynthesis of secondary metabolite, i.e., isoquinoline alkaloids. Isoquinoline alkaloids show an unusually rich variety of structural types and are of pharmaceutical value. However, plants which produce secondary metabolites are usually slow growing.

Thus, our first proposal is to transfer whole biosynthetic pathway into rapid growing annual plants using isolated genes (see list of isolated genes) under the control of the inducible or constitutive promoter.

2. Development of the technology to trim the metabolic branch

We also propose to develop the new strategy to regulate the biosynthetic pathway. One of the problem to use plants as the raw material for chemicals, is that plant cells usually synthesize structurally related chemicals with desired one. Therefore, downstream processing is very important for the commercialization. One of our project is to trim the side branch of biosynthetic pathway. We have examined the antisense-, cosuppression-, and novel RNA-mediated gene switch technology. We believe the development of these technology to trim the certain metabolic step would be very useful for the general application of metabolic engineering.

3. Development of the high accumulator plants

Another problem is the accumulation of metabolites. Usually limited species of plant cells can accumulated the secondary metabolites. We speculate that metabolite transporter would be the rate-limiting step for the accumulation. Our investigation indicated that ABC transporter would be involved in the accumulation of metabolites (at least, in the case of berberine). Then, we have been trying to isolate the candidate of the transporter. Isolated gene would be useful to enhance the accumulation of metabolites and also to excrete the metabolites by the modification of the localization of the transporter.

4. Isolation of master gene and simultaneous activation of whole pathway;

During the course of characterization of induction of biosynthetic enzymes, we found that whole metabolic pathway has been induced simultaneously. Therefore, we expect that master-switch gene also exists in other secondary metabolism except anthocyanin biosynthesis. To isolate such master gene, we have started to isolate the gene which activates the whole biosynthetic pathway by enhancer-tagging method. Because the accumulation of alkaloid is rather easily detected by the cell squash method, we are conducting to establish more than thousand transformants for further screening.

We believe that the integrated research proposed above would be very useful and essential to establish new methodology and to develop novel metabolite producing/accumulating plants.

List of genetic materials in hand

(Laboratory of Molecular and Cellular Biology,
Division of Applied Life Sciences, Graduate School of Agriculture,
Kyoto University, Nov., 12, 1998)

cDNAs

Secondary metabolism

berberine biosynthesis;
from Coptis; norcoclaurine-6-O-methyltransferase, 3'-hydroxy-N-methylcoclaurine-4'-O-methyltransferase, putative coclaurine-3'-hydroxylase, ABC transporter which we expect to be involved in the metabolite transport, berberine-bridge forming enzyme.

Primary metabolism

from tobacco; PEPCase, triosephosphate isomerase, chloroplast DNA-binding protein (CND41), PS2-oxygen evolving complex 23kD protein and more than 60 unique EST fragments (ESTs tentatively identified by the sequence homology were not included)
from cucumber, spinach;PS2-oxygen evolving complex 23kD protein
from yeast; invertase, hexokinase

stress-response

from tobacco; PR-5d (Osmotin-like protein), EREBP (Ethylene-responsive element-binding protein)

Promoter (genomic clones)

chloroplast-DNA-binding protein (*CND41*) from *Nicotiana sylvestris*; high expression in heterotrophically cultured cells and young leaf

OLP from *Nicotiana sylvestris*: ethylene inducible, constitutive expression in root and cultured cells

EREBP2, 3, 4 from *Nicotiana sylvestris*; ethylene inducible, constitutive expression in root and cultured cells

PEPCase from *Nicotiana sylvestris*; constitutive expression and induction by phosphate depletion

Development of plant potency for hydrocarbon production

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1. Introduction

Plant materials are the most abundant biomass resource on the earth, and they have been used for foods, building materials, fuels, and so on. Although the future energy problem has been rushed with misgivings about exhaustion of fossil fuels and environmental pollution, any promising candidates in terms of safe, cleanness and inexpensiveness have not been suggested.

From the view point of environmental protection, we may propose an approach for utilization of the plant potency for production of hydrocarbons. Our tentative research aims are elucidation of biosynthetic pathway of hydrocarbon in plant, enhancement of hydrocarbon production and application of plant hydrocarbons.

2. Production of plant hydrocarbons

Waxes and terpenes are the one of the most abundant natural products of plants, and they have been used as fuels and paint additives. Moreover, terpenes are also very useful for cosmetics, flavors, medicines and so on. Among the terpenes plant polyisoprenes are the main component of rubber and gutta percha products. The latex of the rubber trees mainly contains *cis*-polyisoprene and that of gutta percha trees *trans*-polyisoprene. Such conformational difference markedly affects physical and chemical properties of the polymers. Plant origin *trans*-polyisoprene has unique properties such as resistance to strong acids, potentials for insulator and thermoplastics. Due to such properties it used to be a raw material for golf balls, durable submarine cables, artificial legs and point channel filling

for dentists. Gutta percha is produced by the latex of gutta percha trees limitedly distributed in the tropical area. We are interested in other plants which contain *trans*-polyisoprene, and *Eucommia ulmoides* tree was found to contain this polymer. This plant is very unique in the rareness that the plant has no other species in the same family. This plant can grow in the subtropical and temperate zones and it is often found as wood fossil. This plant is very important as a genetic resource.

Besides polyisoprene the tree contains monoterpenes. So, we are interested in the switching mechanism of the biosynthetic pathway of such terpenes since their contents are markedly different in the seasons. The fundamental study of the biosynthesis enables us to breed a new variety of *E ulmoides* with a high content of gutta percha.

3. Strategy

The main purpose of this project is the creation of a transgenic *E ulmoides* plant which produces larger amount of gutta purcha.

We will aim at the goal with following tactics: 1) Reinforcement of gutta purcha biosynthetic pathway; 2) Repression of the most abundant byproduct biosyntheses; 3) Introduction of cold-resistance to expand habitation of the plants. First, elucidation of biosynthetic pathway of gutta percha in *E. ulmoides* will be carried out. Isopentenylpyrophosphate (IPP), a key intermediate for terpene production, had been thought to be synthesized *via* mevalonate. However, Rohmer*et al.* found a novel biosynthetic pathway for IPP *via* 1-deoxyxylurose-5-phosphate synthesized from glyceraldehyde-3-phosphate and pyruvate. We would like to confirm whether IPP is biosynthesized *via* mevalonate or Rohmer's pathways. Second, the biosynthetic pathway of geniposide, a main byproduct, which is one of the most abundant terpenes among the terpene fraction will be elucidated and the key enzyme genes will be obtained. Third, transformation of *E. ulmoides* will be performed to breed potential varieties, and novel application of gutta percha will be also considered.