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Molecular Breeding of Forage and Turf

The Proceedings of the
5th International Symposium on the
Molecular Breeding of Forage and Turf

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Preface

Grassland produces feed for livestock, maintains soil fertility, protects and conserves soil and water resources, creates a habitat for wildlife, and provides recreational spaces for sports and leisure while simultaneously maintaining sustainable economic outputs. Turf species similarly contribute considerably to our environment by adding beauty to surroundings, providing a safe playing surface for sports and recreation, and preventing erosion. In addition to food and environment, bio-energy is a global concern related to these species. Renewable biomass energy is increasingly being accepted as a possible alternative to fossil fuels and some forages are promising for energy crops.

Breeding programs in forages have produced improvements in both forage yield and quality. Forage and turf in the future must utilize resources (nutrients and water) more efficiently and must also confer measurable benefits in terms of environmental quality and renewable energy. With a widening range of traits, techniques for more accurate, rapid and non-invasive phenotyping and genotyping become increasingly important. The large amounts of data involved require good bioinfomatics support. Data of various kinds must be integrated from an increasingly wide range of sources such as genetic resources and mapping information for plant populations through to the transcriptome and metabolome of individual tissues. The merging of data from disparate sources and multivariate data-mining across datasets can reveal novel information concerning the biology of complex.

Previous International Symposium on the Molecular Breeding of Forage and Turf (MBFT) Symposia were held in Japan in 1998, Australia in 2000, the USA in 2003 and the UK in 2005. On this occasion the 5th MBFT was held in Sapporo, Japan in 2007. The 5th MBFT was hosted by the Hokkaido University in cooperation with the National Agricultural Research Center for Hokkaido Region and the National Institute of Livestock and Grassland Science in the National Agriculture and Food Research Organization. Attendees included breeders, geneticists, molecular biologists, agronomists and biochemists from 19 countries. The program featured plenary addresses

from leading international speakers, selected oral presentations, volunteered poster presentations, as well as tours of the National Agricultural Research Center for Hokkaido Region, Rakuno Gakuen University and Sapporo Dome.

This book includes papers from the plenary lectures and selected oral presentations of the Conference. A wide variety of themes are included and a collection of authoritative reports provided on the recent progress and understanding of molecular technologies and their application in plant breeding. Almost all relevant areas in molecular breeding of forage and turf, from gene discovery to the development of improved cultivars, are discussed in the proceedings.

The 5th MBFT and the publication of this book, *Molecular Breeding of Forage and Turf*, have been supported by National Agricultural Research Center for Hokkaido Region; National Institute of Livestock and Grassland Science; Sustainability Governance Project, Hokkaido University; Alumni Association, Faculty of Agriculture, Hokkaido University; Japan Grassland Agriculture and Forage Seed Association; Japan Livestock Technology Association; Green Techno Bank; The Akiyama Foundation; The Kajima Foundation; Japan Plant Science Foundation; Novartis Foundation Japan for the Promotion of Science; Life Science Foundation of Japan; Supporting Organization for Research of Agricultural and Life Science (SORALS); The Kao Foundation for Arts and Sciences; The Suginome Memorial Foundation; Sapporo International Communication Plaza Foundation; Hokuren Federation of Agricultural Cooperatives; Snow Brand Seed Co., Ltd.; Toyota Motor Corporation; Monsanto Company; Syngenta Seeds K.K.; Japan Turfgrass II; Nippon Medical & Chemical Instruments Co., Ltd.; Applied Biosystems Japan Ltd.; Nihon SiberHegner Co., Ltd.; Nikon Instech Co., Ltd.; HUB Co., Ltd.; Mutoh Co., Ltd.; Imuno Science Co., Ltd.

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Contents

Molecular Breeding to Improve Forages for Use in Animal and Biofuel Production Systems

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Abstract. Forage cultivars with positive impacts on animal production are currently being released using traditional plant breeding approaches. Molecular breeding is a relatively new term that describes the use of genomic and transgenic biotechnologies in conjunction with traditional breeding. Traits currently under investigation via these biotechnologies include herbicide tolerance, drought tolerance, resistance to disease and insect pests, tolerance to acid, aluminum toxic and/or saline soils, tolerance to cold or freezing injury, expression of plant genes controlling nodulation and nitrogen fixation, increasing nutritional quality via down regulation of lignin genes, flowering control, and reducing pasture bloat via incorporation of genes to express condensed tannins. Molecular breeding approaches are expensive, and in the case of transgenics, controversial, requiring much planning and even partnerships or consortia with others to defray cost, and overcome a "valley of death" for commercialization due to patent and regulatory issues. Trait incorporation via molecular breeding being conducted by the Consortium for Alfalfa Improvement is discussed as an example of this type of research approach. The future of molecular breeding in forage crops is bright, but is tied to funding, and in the case of transgenics, also lies in the hands of regulatory agencies and their ability to establish a fair process to evaluate real versus perceived risks. Finally, the use of forages as cellulosic biofuel crops offers new molecular breeding opportunities, especially for value added traits such as enhanced biomass and fermentation efficiency. The main criteria for any biofuel crops are high yields achieved with low input costs in an environmentally friendly manner. By this definition, many high yielding, currently grown, perennial forages are good candidates as biofuel crops especially if they can be delivered to a biorefinery as cheaply as possible.

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Introduction

New forage cultivars, developed through plant breeding, have a long history of positively impacting forage and livestock systems. Traditional breeding methods of hybridization and selection have always been, and still continue to be, used. However, forage improvement programs have entered the biotechnology era by the use of molecular biology tools (Brummer et al. 2007). Molecular breeding is therefore a relatively new term that describes the use of genomic and transgenic biotechnologies in conjunction with traditional breeding.

Genomics research received great publicity with the successful completion of the human genome sequencing project. Plant species were next with rice (*Oryza sativa* L.) and *Medicago truncatula* Gaertn., an annual relative of cultivated alfalfa (*Medicago sativa* L.), now being sequenced and used as a reference species for grasses and legumes, respectively. The sequencing data for these reference species, combined with high throughput machinery and data analysis (e.g. bioinformatics), allows more accurate determinations of species relationships and gene expression. From this understanding, new and innovative methods for improving forage crops are evolving.

Transgenics involve the movement of specific and useful genes into the crop of choice and this approach is sometimes referred to as genetic engineering. Scientists using this approach have already shown success in introducing genes which make many important row crops resistant to insects, viruses, and herbicides. The transgenic approach has also been very useful in creating unique plants that allow basic research to be conducted on physiological and biochemical pathways.

Why Molecular Breeding?

An ability to easily manipulate and control genes is fundamental to plant breeding. This is shown historically by the formula P=G+E+GE or Phenotype = Genotype + Environment + Genotype x Environment. Therefore, the genotype or G provides the best estimate for the genes involved in the phenotypic trait or traits being investigated and expressed. However, it is only a general estimate. Molecular tools available through genomics and transgenics offer a powerful ability to move from simply estimating to more accurately measuring G and even ways to manipulate

the actual genes. Combining traditional plant breeding with these molecular tools should assist with making progress in cultivar development.

The Samuel Roberts Noble Foundation's Forage Improvement Division, as probably with most organizations, uses a model of combining traditional breeding approaches with molecular tools to incorporate useful genes (Fig. 1). In this approach, the basic five steps of the cultivar development process, (1) clearly defining objectives, (2) collecting and developing parental germplasm, (3) conducting the actual breeding and selection to produce an experimental cultivar, (4) extensive testing program to prove the worth of this cultivar, and (5) final release and commercialization, proceed as they always have. However, sometimes the traits are very complex to locate and manipulate, or possibly not even contained in a species' primary germplasm. When this happens, biotechnology approaches are an option for trait incorporation and/or validation through more efficient gene discovery, tagging, and even genetic engineering.

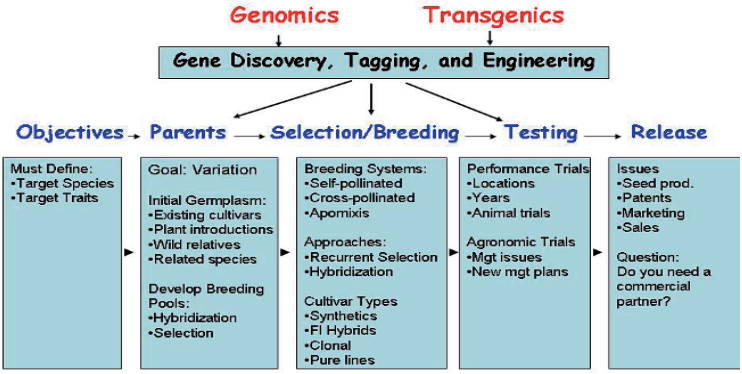

Fig. 1 A cultivar development model demonstrating the traditional steps in the process, and how and where the new transgenic and genomic biotechnologies will likely impact that process

Current Molecular Breeding

Biotechnology research in all forage crops, especially to study and/or incorporate complex traits, is in a time of increased emphasis and success throughout the world. For example, at the International Symposia on Molecular Breeding of Forage and Turf (MBFT) held at Victoria, Australia

in 2000, at Dallas, Texas, in 2003, and at Aberystwyth, Wales in 2005, there were hundreds of scientists in attendance from many countries. Research talks at these meetings are found in the proceedings on many aspects of basic biotechnology in forage grasses and legumes as well as excellent keynote presentations on molecular breeding by Professors Spangenberg, Dixon, and Lübberstedt (Spangenberg 2001; Hopkins et al. 2003; Humphreys 2005), respectively. This current MBFT conference in Sapporo, Japan provides a similar venue.

Some of the research areas traditionally receiving emphasis at MBFT are accurate genomics techniques to more rapidly identify and manipulate important genes (molecular markers and marker assisted selection breeding); tolerance to biotic and abiotic stresses; flowering control; plant-symbiont relations; breeding for animal, human and environmental welfare; transgenics; bioinformatics; population genetics; genomics of the model legume *M. truncatula*; field testing and risk assessment as well as intellectual property rights. These symposia, and many others like them such as The North American Alfalfa Improvement Conference (NAAIC), are proof that research in this area is intense and growing for all forage crops.

Specific biotech traits currently under investigation and reported at the current and past MBFT symposia include herbicide tolerance, drought tolerance, resistance to disease and insect pests, tolerance to acid, aluminum toxic and/or saline soils, tolerance to cold or freezing injury, expression of plant genes controlling nodulation and nitrogen fixation, increasing nutritional quality via down regulation of lignin genes, flowering control, and reducing pasture bloat via incorporation of genes to express condensed tannins.

These traits are therefore ones that breeders have made little progress for improvement through conventional breeding. Another aspect is the high potential impact for farmers if these traits can be incorporated into cultivars. This type of impact would justify the use of biotechnologies even when considered against the issues surrounding their use.

Considerations and Issues

There are several issues to consider when deciding to use biotechnologies especially in cultivar development programs. The first is cost. Compared to the traditional model for cultivar development, everything is more costly with molecular breeding. That one has to recover these costs through the

sale of seed, a notoriously low margin product, provides less incentive for using molecular breeding by many commercial seed companies. Second, one must have freedom to operate for all enabling technologies and patents involved in the process; especially for using transgenics. This again is a cost issue, but can become a legal issue if all the proper patents and licenses are not put in place. Third, the regulatory costs for transgenic traits are problematic and rising. For example, applications for de-regulation of the Roundup Ready (RR) gene were submitted in the USA for a turf and a forage crop, creeping bentgrass (*Agrostis palustris* Hud.) and alfalfa. Although the RR gene is a 1990s technology that is currently found in millions of acres of corn (*Zea mays* L.) and soybean (*Glycine max* L.), crops also fed to livestock, only alfalfa was de-regulated. However, RR alfalfa has been re-regulated and still not being sold (Tietz 2007), and RR creeping bentgrass has yet to be de-regulated (it has also been in the application process longer than any crop to date). This slow progress is not encouraging for production of transgenic cross-pollinated forage or turf crops. These delays are due, in part, to initial estimations that pollen can flow for extreme distances and into related weedy species potentially causing herbicide resistant weeds to develop. Whatever the reasons, these delays further adds to the regulatory costs, and if these two applications are not finally successful, it could set a negative precedent for the future of transgenic, cross-pollinated, perennial forages. Additionally, these problems with transgenic development have created further negative public perception for use of other biotech developed traits and methods.

All molecular breeding approaches are therefore expensive, and in the case of transgenics, controversial, creating for many a “valley of death” for the commercialization. Although there is no problem for conducting basic molecular biology research by creating unique plants to study, there may need to be new models created and used to overcome these inherent problems.

Models for Using Transgenics

Due to the cost and controversy of using transgenics, it is usually the option of last resort. However, although transgenic biotechnologies provide very powerful and useful alternatives to not having the trait altogether, the main question is this: Is the trait of such value and impact that it will justify a transformation approach? If the answer to this question is yes, then a good model of how to do this is the Consortium for Alfalfa Improvement (CAI).

The CAI is composed of researchers from Noble Foundation, the U.S. Dairy Forage Research Center (USDFRC) in Madison, WI, and Forage Genetics International (FGI), a commercial alfalfa research and seed company. Therefore, these three organizations have complementary strengths coming together to improve important characteristics in alfalfa. The main steps for using transgenics that are all covered by at least one of the CAI partners include (1) investigating and obtaining the requisite biotech pieces (including freedom to operate for patents on genes and enabling technologies), (2) trait development including introgression into commercially viable cultivars, proof of concept studies, and animal testing, and (3) commercialization including regulatory trials.

The first initiative by the CAI focused on improving protein utilization and cell wall digestibility via lignin reduction, and the second was expression of condensed tannins to reduce pasture bloat and improve protein utilization in ruminant animals. Therefore, the consortium's overall goal is to re-design alfalfa as the major forage source. This would be of such impact as to justify use of any biotechnologies. It also brings to bear additional resources to leverage with those existing for each organization as a separate entity. The CAI is therefore a good model of what may need to be done to justify the costs and reduce risks when using transgenics.

Other Options

On its face, transgenics simply create unique variation not found in the primary germplasm. However, it is the cost and controversy of using transgenics that cause most of its problems. Therefore, are there other, less controversial and costly approaches to creating unique variation?

Stebbins (1950) wrote that three main driving forces in the evolution of higher plants were inter-specific hybridization, mutation with Mendelian segregation, and polyploidy. For example, crop plants such as wheat (*Triticum aesativum* L.) evolved with inter-specific hybridization and polyploidy; while in alfalfa, polyploidy underpinned its development as an autotetraploid. Gene mutations that control traits such as yield, maturity, seed size, flower color, disease resistance, etc. have always been recognized in the primary germplasm of all crop plants. These same driving forces therefore underpin the basic approaches used by most plant breeders in the modern era with hybridization and selection for the natural mutations being the most used.

In the forage crop bermudagrass (*Cynodon dactylon* L.), however, hybridization, including inter-specific hybridization, was successful in producing vegetatively propagated, clonal F_1 cultivars that are currently planted on millions of hectares in the southeastern USA, and many other areas in the sub-tropics (Burton and Hanna 1995). In the case of the bermudagrass hybrid, "Coastal", it was unique enough to be used as a parent to produce other hybrids. So, as this example indicates, inter-specific hybridization provides unique plants, that if their propagation methods are worked out, can become cultivars themselves or used as parents to produce other unique plants. There are now several forage species, such as the clovers (*Trifolium* spp.), where the phylogenetic relationships among species are being examined through molecular markers (Ellison et al. 2006). These species are therefore good candidates for an inter-specific hybridization approach due to an improved ability to predict the success of each potential cross. It is also a good example of how a genomics based approach can be successfully employed in inter-specific hybridization.

Other avenues to create unique variation in plants that are possibly less controversial than transgenics include somatic hybridization and selection via somaclonal variation. One problem with producing inter-specific hybrids is that reproductive barriers prevent embryo or endosperm development. Therefore, somatic hybridization, or fusion of protoplasts under tissue culture conditions, offers a method to overcome these barriers and create unique inter-specific, and possibly inter-generic, hybrids (Arcioni et al. 1997). Likewise, when growing any cells in tissue culture, stable genetic changes are common, leading to unique cell to cell variation called somaclonal variation. Therefore, somaclonal variation offers another, and safer, form of mutation breeding (Evans 1989). If the tissue culture media also contains a specific stress or toxin, then the cells are simultaneously selected for ability to grow in these conditions. Further selection is then practiced among the regenerated plants for the desired changes. Its best application to conventional breeding occurs when the best available germplasm is used to begin the process.

So, does using inter-specific hybridization, or even somaclonal variation and somatic hybridization, create variation as useful to breeders as transgenics? This is a legitimate question because these approaches are generally less expensive, and surely less controversial, than transgenics.

Future of Molecular Breeding

As stated above, there is no problem for using biotechnologies to create unique plants in order to conduct basic plant molecular biology research. This is important work that will not only continue, but increase in scope. However, the irony is not whether basic biotechnology research is increasing in forages, because it is, but whether useful biotechnology traits can be delivered directly to the farmer in an improved cultivar.

The future of molecular breeding for cultivar development in forage crops is also tied to funding, and in the case of transgenics, lies in the hands of regulatory agencies and their ability to establish a fair process to evaluate real versus perceived risks. Consortia of various partners like those described for the CAI will also be important to bring the fruits of these new technologies to researchers and farmers alike. However, it is hoped that more funding will be available to help the regulatory agencies in assessing the question of real versus perceived risks. At the end of the day, these agencies will need to make decisions on what are the real risks, establish a rigorous regulatory process to assess these risks, oversee the regulatory process in a fair manner, and make a decision! We can all then move forward based strictly on the value of the traits to the well-being of the environment, the farmer, agriculture, and all citizens.

The use of forages as cellulosic biofuel crops also now offers new molecular breeding opportunities, especially for value added traits such as enhanced biomass and fermentation efficiency. The main criteria for any biofuel crops are high yields achieved with low input costs in an environmentally friendly manner. By this definition, several high yielding, perennial forages are good candidates as biofuel crops. However, the initial requirement of low cost of the delivered feedstock may be the greatest hurdle for breeders and growers to overcome for most forages.

In addition to their direct use as cellulosic feedstock, the evolving biofuel industry has created other opportunities for forage and pasture crops, such as an increased need for high value forage finishing systems created by expensive feed grain prices due ironically to the current use of corn grain as a main ethanol producing feedstock.

Biofuels

Biofuels include ethanol, biodiesel, and other hydrocarbons achieved either through a fermentation or gasification process using plant biomass as a "feedstock". However, this current discussion will concentrate mainly on forages for use as cellulosic feedstock to produce ethanol.

Cellulosic ethanol is ethanol produced from cellulosic material (e.g. all plant parts especially stems, leaves, seedheads, etc.). Cellulosic feedstocks are generally comprised of three components: cellulose (~44%), hemicellulose (~30%) and lignin (~26%). The cellulose and hemicellulose provide a rich supply of carbohydrates that are ultimately used to produce ethanol. Sources of cellulosic material include grasses, wood and wood residue, and crop residues such as corn stover and wheat straw. However, ethanol produced from any feedstock, corn grain, perennial grasses, wheat straw, etc. is all chemically identical.

The technology to create cellulosic ethanol is becoming closer to reality. Many companies world-wide are in the later stages of development and entering the early stages of commercial scale-up into ethanol plants (also called biorefineries). Though most of the pieces are in place, the key is to continue to make ethanol production more cost-effective and economically competitive.

A biorefinery produces fuel-grade ethanol, and that ethanol is then blended in a percentage with gasoline to make a finished motor fuel. Commonly, we hear about E10 (10% ethanol/90% gasoline) and E85 (85% ethanol/15% gasoline). It is unlikely most vehicles will run on pure ethanol anytime soon.

At this time, there are not many service stations selling fuel grade ethanol at the pump. This is one of the national issues concerning its use and adoption especially in the USA where there are over 150,000 outlets – gas stations and convenience stores – and fewer than 1,000 sell ethanol.

Based on current estimates, cellulosic feedstocks are far better than grain in producing ethanol. Cellulosic feedstocks are estimated to produce approximately five times more energy that corn grain. Further, cellulosic feedstocks are intended to have a broader range of adaptability to poorer soils, which would allow them to be grown in regions that cannot support large-scale grain production. The cellulosic feedstocks being considered are crop residues, perennial crops such as grasses and trees, animal manures,

and even municipal waste. For the perennial grasses, the main ones being investigated are switchgrass (*Panicum virgatum* L.), giant miscanthus (*Miscanthus* × *giganteus* Greef & Deuter ex Hodkinson & Renvoize), and giant reed (*Arundo donax* L.).

Switchgrass as a Biofuel Crop

Although any high yielding perennial forage will suffice, switchgrass is being investigated as one of the main perennial biomass species for cellulosic ethanol production in the USA. This is because it is a perennial grass native to the prairies of North America that was also identified by the United States Department of Energy (DOE) as a primary target for development as a dedicated energy crop because of its potential for high fuel yields, drought tolerance, and ability to grow well on marginal cropland without heavy fertilizing or intensive management (McLauglin and Kszos 2005).

The initial DOE program to evaluate and develop switchgrass as a bioenergy crop was recently reviewed and demonstrated that switchgrass has potential as an alternative to corn for ethanol production and as a supplement for coal in electricity generation (McLaughlin and Kszos 2005). The program identified the best varieties and management practices to optimize productivity, while concurrently developing a research base for long-term improvement through breeding and sustainable production in conventional agro-ecosystems. Gains through plant breeding were found for switchgrass yield to exceed that of corn. Significant carbon sequestration was projected for soils under switchgrass that should improve both soil productivity and nutrient cycling. Co-firing switchgrass with coal will also reduce greenhouse gas production. Finally, collaborative research with industry included fuel production and handling in power production, herbicide testing and licensing, release of new cultivars, and genetic modifications for chemical co-product enhancement.

More research will need to be conducted on crops like switchgrass that incorporate biotechnologies. In the USA, the DOE-USDA biomass genomics research program announced projects with switchgrass as one of the main species (USDA, DOE News Release; URL: http://genomicsgtl.energy.gov/research/DOEUSDA/), that along with a new bioenergy center concentrating on improving switchgrass recalcitrance (DOE Bioenergy Research Center Fact Sheets; URL: http://www.science.doe.gov/News_Information/News_Room/2007/Bioenergy_Research_Centers/DOE%20BRC%20fact%20sheet%20

final%206-26-07.pdf), are important developments. The future is therefore bright for switchgrass as a dedicated energy crop with millions of hectares projected to be planted in order to meet DOE goals.

Other Forages as Biofuel Feedstock

Again, the main criteria for any biofuel crops are high biomass yields achieved with low input costs in an environmentally friendly manner. It is also important that these crops have alternate uses besides feedstock for biorefineres such as forage for livestock. This is why switchgrass is a very good choice. By this definition, the traditional, high yielding forages like bermudagrass, tall fescue (*Festuca arundinacea* Schreb.), ryegrasses (*Lolium* spp.), red clover (*Trifolium pratense* L.), white clover (*Trifolium repens* L.), and alfalfa are also good candidates. However, the requirement of low cost of the delivered feedstock, possibly as low as $50USD per US ton, is the greatest hurdle for growers of these crops to overcome.

For high value crops like alfalfa to be used, the harvested product needs to be divided into components, such as leaves and stems, and using the leaves to produce high value meal and the stems for sale to a biorefinery. If co-products such as pharmaceuticals are simultaneously extracted from the leaf material, this allows the economics of using alfalfa as a biofuel crop to work even better.

It is possible that each specific geographic region will have its own cropping system(s) based on several adaptive crops to supply a local biorefinerery. Co-cropping alfalfa or tall fescue with switchgrass to achieve an off-season supply of biomass, or inter-cropping switchgrass with alfalfa or clovers to supply nitrogen into the production system are good examples of how this could work.

Summary and Conclusions

Molecular breeding is important and will be used extensively in future forage research efforts. The overall participation and depth of research in this area as presented at this and past MBFT meetings supports this fact. Transgenics will have a big role to play in future forage cultivar development efforts, but other approaches such as inter-specific hybridization and somatic hybridization need to be re-examined for potential use.

Molecular breeding also needs to develop from a platform of good conventional breeding and include supporting agronomic research and partnering with commercial industry where appropriate.

Future problems for molecular breeding in forages include high development costs, poor breeding histories and the polyploid nature of the main species, accurate phenotyping for most of the genomics based approaches, and freedom to operate, regulatory, and public perception issues for transgenics. To overcome these problems, development and regulatory costs will need to be funded by government grants and organizational consortia. The regulatory agencies will likewise need to establish a fair system that separates real from perceived risk.

All biofuel industries will be local with their own cropping systems, but high yielding forage crops will play a large role as feedstocks for this emerging industry. Initial context for the biofuels industry is for cheaply produced feedstock and this is why switchgrass is being touted as an initial dedicated crop. The future context is unclear, but should involve value-added feedstocks. The main traits to be improved are increased biomass yield, reduced input costs, and reduced chemical recalcitrance. Molecular breeding is therefore poised to make positive impacts in the biofuel feedstock development area.

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DREB Regulons in Abiotic-Stress-Responsive Gene Expression in Plants

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Abstract. Plant growth and productivity is affected by various abiotic stresses such as drought, high salinity, and low temperature. Expression of a variety of genes is induced by these stresses in various plants. In the signal transduction network from perception of stress signals to stress-responsive gene expression, various transcription factors and *cis*-acting elements in the stress-responsive gene expression function for plant adaptation to environmental stresses. The dehydration-responsive element (DRE)/C-repeat (CRT) *cis*-acting element is involved in osmotic- and cold-stress-inducible gene expression. Transcription factors that bind to the DRE/CRT were isolated and named DREB1/CBF and DREB2. DREB1/CBF regulon is involved in cold-stress-responsive gene expression, whereas, DREB2 is involved in osmotic-stress-responsive gene expression. Recently, we highlight transcriptional regulation of gene expression in response to drought and cold stresses, with particular emphasis on the role of DREB regulon in stress-responsive gene expression.

Function of Drought Stress-Inducible Genes

Drought, high salinity, and freezing are environmental conditions that cause adverse effects on the growth of plants and the productivity of crops. Plants

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respond and adapt to these stresses to survive under stress conditions at the molecular and cellular levels as well as at the physiological and biochemical levels. Expression of a variety of genes is induced by these abiotic stresses (Thomashow 1999; Shinozaki and Yamaguchi-Shinozaki 2000; Bray et al. 2000; Zhu 2002; Yamaguchi-Shinozaki and Shinozaki 2006). Transcriptome analysis using microarray technology has proven to be very useful for the discovery of many stress-inducible genes involved in stress response and tolerance (Shinozaki et al. 2003). Numerous genes that are induced by various abiotic stresses have been identified using various microarray systems (Seki et al. 2002; Fowler and Thomashow 2002; Kreps et al. 2002; Maruyama et al. 2004; Vogel et al. 2005).

Genes induced during stress conditions are thought to function not only in protecting cells from stress by the production of important metabolic proteins but also in the regulation of genes for signal transduction in the stress response. Thus, these gene products are classified into two groups (Seki et al. 2002; Fowler and Thomashow 2002; Kreps et al. 2002). The first group includes proteins that probably function in stress tolerance, such as chaperones, LEA (late embryogenesis abundant) proteins, osmotin, antifreeze proteins, mRNA binding proteins, key enzymes for osmolyte biosynthesis such as proline, water channel proteins, sugar and proline transporters, detoxification enzymes, enzymes for fatty acid metabolism, proteinase inhibitors, ferritin and lipid-transfer proteins. Some of these stress-inducible genes that encode proteins such as key enzymes for osmolyte biosynthesis, LEA proteins and detoxification enzymes have been overexpressed in transgenic plants and have been found to produce stress-tolerant phenotypes in the transgenic plants (Holmberg and Bulow 1998; Cushman and Bohner 2000). These results indicate that the gene products of the stress-inducible genes really function in stress tolerance.

The second group contained protein factors involved in further regulation of signal transduction and gene expression that probably function in stress response. They included various transcription factors suggesting that various transcriptional regulatory mechanisms function in the drought-, cold- or high-salinity-stress signal transduction pathways (Seki et al. 2003). The others were protein kinases, protein phosphatases, enzymes involved in phospholipids metabolism, and other signaling molecules such as calmodulin-binding protein and 14-3-3 proteins. At present the function of most of these genes are not fully understood. It is important to elucidate the role of these regulatory proteins for further understanding of plant responses to abiotic stress.

DREB Regulons in *Arabidopsis*

The promoter of a drought-, high-salinity- and cold-inducible gene, RD29A/COR78/LTI78, in *Arabidopsis* contains a major cis-acting element, the dehydration-responsive element (DRE)/C-repeat (CRT), that is involved in stress-inducible gene expression and its consensus was G/ACCGAC. DRE functions in one of the ABA-independent pathways in response to drought, high-salinity and cold stresses (Shinozaki and Yamaguchi-Shinozaki 2000). cDNAs encoding DRE binding proteins, *DREB1/CBF*, and *DREB2*, have been isolated by using yeast one-hybrid screening (Stockinger et al. 1997; Liu et al. 1998). These proteins contained the conserved DNA-binding domain found in the ERF and AP2 proteins. These proteins specifically bind to the DRE sequence and activate the expression of genes driven by the DRE sequence.

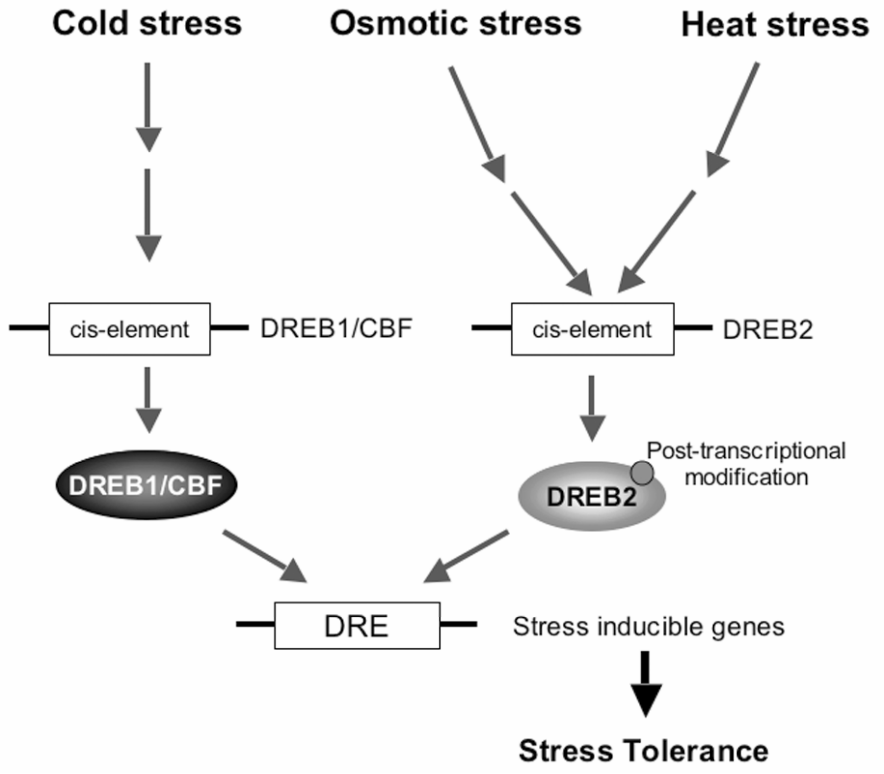

Fig. 1 A model of the induction of abiotic-stress-inducible genes that have the DRE cis-element in their promoters. Two different type DRE-binding proteins, DREB1/CBF and DREB2, distinguish different signal transduction pathways. DREB1/CBF-type transcription factors function in response to cold, and DREB2-type transcription factors function in drought and heat stresses

In *Arabidopsis*, three DREB1/CBF proteins are encoded by genes that lie in tandem on chromosome 4 in the order of *DREB1B/CBF1*, *DREB1A/CBF3* and *DREB1C/CBF2* (Gilmour et al. 1998; Liu et al. 1998). *Arabidopsis* also contains two DREB2 proteins, DREB2A and DREB2B (Liu et al. 1998). Expression of the *DREB1/CBF* genes is induced by cold, but not by dehydration and high-salinity stresses (Liu et al. 1998; Shinwari et al. 1998). By contrast, expression of the *DREB2* genes is induced by dehydration and high-salinity stresses but not by cold stress (Fig. 1; Liu et al. 1998; Nakashima et al. 2000). Later, Sakuma et al. (2002) reported three novel *DREB1/CBF*-related genes and six novel *DREB2*-related genes that were not expressed at high levels under various stress conditions. However, one of the *CBF/DREB1* genes, *CBF4/DREB1D* is induced by osmotic stress, suggesting the existence of crosstalk between the CBF/DREB1 and the DREB2 pathways (Haake et al. 2002).

DREB1/CBFs, Major Transcription Factors in Cold-Responsive Gene Expression

Transgenic *Arabidopsis* plants overexpressing *CBF1*/*DREB1B* under control of the cauliflower mosaic virus (CaMV) 35S promoter showed strong tolerance to freezing stress (Jaglo-Ottosen et al. 1998). Overexpression of the *DREB1A*/*CBF3* under the control of the CaMV 35S promoter also increased the tolerance to drought, high-salinity, and freezing stresses (Liu et al. 1998; Kasuga et al. 1999; Gilmour et al. 2000). Six genes have been identified as the target stress-inducible genes of DREB1A using RNA gel blot analysis (Kasuga et al. 1999). By using microarray analyses, more than 40 target genes of DREB1/CBF have been identified (Seki et al. 2001; Fowler and Thomashow 2002; Maruyama et al. 2004; Vogel et al. 2005). Most of these target genes contained the DRE or DRE-related core motifs in their promoter regions (Maruyama et al. 2004). These gene products are transcription factors, phospholipase C, RNA-binding protein, sugar transport protein, desaturase, carbohydrate metabolism-related proteins, LEA proteins, KIN (cold-inducible) proteins, osmoprotectant biosynthesis-protein, protease inhibitors, and so on. Many of them were proteins known to function against stress and were probably responsible for the stress tolerance of the transgenic plants. However, overexpression of the DREB1A protein also severely retarded growth under normal growth conditions. Use of the stress-inducible *rd29A* promoter instead of the constitutive 35S CaMV promoter for the overexpression of DREB1A minimizes negative effects on plant growth (Kasuga et al. 1999).

DRE has been shown to function in gene expression in response to stress in tobacco plants, which suggests the existence of similar regulatory systems in tobacco and other crop plants (Yamaguchi-Shinozaki and Shinozaki 1994). The DRE-related motifs have been reported in the promoter region of cold-inducible *Brassica napus* and wheat genes (Jiang et al. 1996; Ouellet et al. 1998). Additionally, the changes that occur in the *Arabidopsis* metabolome in response to cold were examined and the role of the CBF/DREB1 cold response pathway were assessed (Cook et al. 2004). On the other hand overexpression of the *Arabidopsis DREB1/CBF* genes in transgenic *B. napus* or tobacco plants induced expression of orthologs of *Arabidopsis* DREB1/CBF-targeted genes and increased the freezing tolerance of transgenic plants (Jaglo et al. 2001; Kasuga et al. 2004). These observations suggest that the DREB1/CBF regulon can be used to improve the tolerance of various kinds of agriculturally important crop plants to drought, high-salinity and freezing stresses by gene transfer.

Interestingly, Zhang et al. (2004) reported that tomato, a chilling sensitive plant, encodes three *DREB1/CBF* homologs, *LeCBF1-3*, that are present in a tandem array in the genome. Only the tomato *LeCBF1* gene was found to be cold-inducible. Constitutive overexpression of *LeCBF1* in transgenic *Arabidopsis* plants induced expression of DREB1/CBF-targeted genes and increased freezing tolerance. These results clearly indicated that *LeCBF1* encodes a functional homolog of the *Arabidopsis* DREB1/CBF proteins. Overexpression of *Arabidopsis CBF1/DREB1B* in tomato has been shown to increase the chilling and drought tolerance of transgenic tomato plants (Hsieh et al. 2002a,b). However, constitutive overexpression of either *LeCBF1* or *Arabidopsis DREB1A* in transgenic tomato plants did not increase freezing tolerance (Zhang et al. 2004). microarray analysis only identified four genes that were induced 2.5-fold or more in the *LeCBF1* or *DREB1A* overexpressing plants. Three out of the four identified genes were putative members of the tomato DREB1/CBF regulon as they were also upregulated in response to low temperature and they concluded that an intact CBF/DREB1 cold response pathway is present in tomato but the tomato CBF/DREB1 regulon differs from that of *Arabidopsis* and appears to be considerably smaller and less diverse in function.

In rice, four CBF/DREB1 homologues and one DREB2 homologous genes, *OsDREB1A*, *OsDREB1B*, *OsDREB1C* and *OsDREB1D*, and *OsDREB2A*, respectively have been isolated (Dubouzet et al. 2003). Overexpression of OsDREB1A in transgenic *Arabidopsis* resulted in improved high-salinity and freezing stress tolerance. A DREB1/CBF-type transcription factor, ZmDREB1A was also identified in maize (Qin et al. 2004). The ZmDREB1A

was shown to be involved in cold-responsive gene expression, and the overexpression of this gene in *Arabidopsis* resulted in improved stress tolerance to drought and freezing. These results indicate that similar regulatory systems are conserved in monocots as well as dicots. Pellegrineschi et al. (2004) showed that overexpression of DREB1A/CBF3 driven by the stress-inducible rd29A promoter in transgenic wheat improved drought stress tolerance. Oh et al. (2005) reported that constitutive overexpression of DREB1A using the 35S promoter in transgenic rice resulted in increased stress tolerance to drought and high salinity. Similarly, Ito et al. (2006) also developed transgenic rice plants that constitutively expressed *DREB1A* or *OsDREB1A* genes. In this work, these factors in transgenic rice elevated tolerance to drought, high salinity, and low-temperature. These observations suggest that the DREB regulon can be used to improve the tolerance of various kinds of agriculturally important crop plants to drought, high-salinity and freezing stresses by gene transfer.

DREB2, Major Transcription Factors in Osmotic-Responsive Gene Expression

The DREB2A protein has a conserved ERF/AP2 DNA-binding domain and recognizes the DRE sequence like DREB1A (Liu et al. 1998). Among the eight DREB2-type proteins, DREB2A and DREB2B are thought to be major transcription factors that function under drought and high-salinity stress conditions (Nakashima et al. 2000; Sakuma et al. 2002). However, overexpression of DREB2A in transgenic plants neither caused growth retardation nor improved stress tolerance, suggesting that the DREB2A protein requires post-translational modification such as phosphorylation for its activation (Liu et al. 1998), Nevertheless, the activation mechanism of the DREB2A protein has not yet been elucidated. Domain analysis of DREB2A using *Arabidopsis* protoplasts revealed that a negative regulatory domain exists in the central region of DREB2A and deletion of this region transforms DREB2A to a constitutive active form. Overexpression of the constitutive active form of DREB2A (DREB2A-CA) resulted in growth retardation in transgenic *Arabidopsis* plants. These transgenic plants revealed significant tolerance to drought stress but only slight tolerance to freezing. Microarray analyses of the transgenic plants revealed that DREB2A regulates expression of many drought-inducible genes. However, some genes downstream of DREB2A are not downstream of DREB1A, which also recognizes DRE but functions in cold-stress-responsive gene expression (Sakuma et al. 2006a). The genes downstream of DREB2A play an important