Genetic engineering for drought tolerance

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WUEMED training course
June 5-10, 2006
Gene structure (eucaryote)

DNA

Enhancers, silencers, MARS, etc

Promoter

Exon

Intron

Transcription unit

Coding region

3’ end of mRNA

Transcription, capping and polyadenylation

m7G cap

mRNA

mA

Splicing

Translation start (AUG)

Translation end (UGA, UAA, UAG)
Control of eucaryotic gene expression

- Chromatin
- Transcription factor

Mod. from Alberts et al., Molecular Biology of the Cell, 4th edition
Methods of transformation

1. Agrobacterium
   - c/o tissue culture
   - c/o floral dip
2. Particle bombardment (gun)
3. PEG-mediated
4. Electroporation
5. Silicon carbide fibers (Whiskers™)
**Agrobacterium tumefaciens**

Detail of Ti plasmid

Example of binary vector system

Mod from: Griffith et al., Modern Genetic Analysis, Freeman, and Brown, Genomes, Synauer.
Particle Gun
Scheme of particle gun

(a) Rupture disc
(b) Macrocarrer and gold particles
(c) Stopping plate
(d) Plant material
Selection and regeneration
Constructs for genetic engineering: design

- **Gene of interest**
- **Promoter and/or enhancer**
  - constitutive: 35-S, Ubi, actin
  - inducible: By artificial stimuli, Environmentally Endogenously
- **Terminator** e.g.: nos
- **Selection marker for plant cells**
- **Selection marker for bacterial cells**
- **Provide resistance to:**
  - Antibiotic
    - Kanamycin
    - Igromycin
  - Herbicide
    - Bialaphos
    - Bromoxynil
    - Glyphosate
- **Reporter gene**
  - E.g.: gus, gfp (green fluorescent protein), luciferase
Strategies for the genetic engineering of drought tolerance

Gene discovery in the stress response → Genes used for engineering → Construction of transgenes → Genetic engineering

Drought stress → Signal perception → Signal transduction → Transcription factors → Functional proteins

Modification of regulatory proteins:
- Point mutation
- Partial deletion
- Modification + Activation or repression domain

Modification of enzymes, transporters, chaperones, etc.:
- Fused enzyme + Signal peptide

Appropriate promoter:
- Sense
- Antisense
- RNAi

Transgenic plant production:
- Pro gene

Current Opinion in Biotechnology
Synthesis of compatible solutes

- Almost all organisms, ranging from microbes to animals and plants, synthesize compatible solutes in response to osmotic stress.

- Compatible solutes are nontoxic molecules such as amino acids, glycine betaine, sugars, or sugar alcohols which can accumulate at high concentration without interfering with normal metabolism.

- They may have a role in osmotic adjustment, stabilizing proteins and cell structures, scavenging reactive oxygen species.

- Strategies for manipulation:
  
  - require knowledge of biosynthetic pathways
  
  - include up and down regulation of key regulatory enzymes involved in synthesis and degradation, use of feedback inhibition-insensitive forms, ecc.
Synthesis of compatible solutes

<table>
<thead>
<tr>
<th>Classification</th>
<th>Gene name</th>
<th>Transgenic</th>
<th>Origin</th>
<th>Expression</th>
<th>Experiment</th>
<th>Parameters</th>
<th>Year</th>
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<td>Photosynthesis, shoot growth</td>
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</table>
Mannitol is normally synthesized in numerous plant species, but not in wheat.

*MtlD* (from *E.coli*) encodes for mannitol-1-phosphate dehydrogenase that catalyzes the reversible conversion of Fru-6-phosphate to mannitol-1-phosphate. In transgenic plants, mannitol-1-phosphate is converted to mannitol via nonspecific phosphatases.
Tolerance of Mannitol-Accumulating Transgenic Wheat to Water Stress and Salinity


Tilahun Abebe, Arron C. Guenzi, Bjorn Martin, and John C. Cushman

A

Stressed

No Stress  - mtlD  + mtlD

B

Stressed

No Stress  - mtlD  + mtlD

P2-16-1
(+ mtlD)
P2-19-1
(+ mtlD)
P1-13-1
(- mtlD)
Transgenic wheat accumulated mannitol from 0.6 to 2.0 mol g⁻¹ fresh weight in the mature fifth leaf. This amount was inadequate to account for osmotic effects.

Plants that accumulated higher mannitol had severe abnormalities.

- Use of stress-inducible expression systems, which should lack potential detrimental effects on growth.

The beneficial effect of mannitol might result from protective mechanisms like scavenging of hydroxyl radicals (OH•) and stabilization of macromolecular structures.

Unlike osmotic adjustment, OH• scavenging and other protective functions require only small amounts of mannitol.

The mechanism by which mannitol interacts with OH• remains to be explained.
Proline is the most widely distributed osmolyte; it occurs in plant and in many other organisms. Its accumulation correlates with tolerance to drought and salt stress.

Roles: osmotic adjustment, membranes protection, sink of energy and reducing power, C and N source, OH• scavenger

Synthesis can occurs via two biosyntetic pathways: the ornithine-dependent and the glutamate dependent (predominant under stress conditions)

Constitutive overexpression requires extra energy and building blocks which may hamper the plant growth. Thus, it is desirable to use a stress-inducible promoter to drive the expression of such new functions.
Overexpression of a Δ1-pyrroline-5-carboxylate synthetase gene and analysis of tolerance to water- and salt-stress in transgenic rice

49-bp ABA-responsive element from the barley Hva22 gene

180-bp minimum rice actin1 promoter

Hva22 intron

PC5CS gene from Vigna aconitifolia

Inducible promoter

Selection marker gene
Stress-inducible expression of \( \Delta^1 \)-pyrroline-5-carboxylate synthetase (P5CS) transgene resulted in the overproduction of the P5CS enzyme in transgenic rice plants as well as an increase in proline accumulation.

Increased levels of proline may contribute, at least in part, to an enhanced biomass production, as reflected as higher level of fresh shoot and root weight, of transgenic rice plants under water- or salt-stress conditions.

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**Growth performance of transgenic rice plants in soil under water stress conditions**

<table>
<thead>
<tr>
<th>Transgenic lines</th>
<th>Fresh shoot weight (g per plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.80 ± 0.09 (100)</td>
</tr>
<tr>
<td>N22</td>
<td>1.22 ± 0.08 (153)</td>
</tr>
<tr>
<td>N48</td>
<td>1.12 ± 0.09 (140)</td>
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<tr>
<td>N60</td>
<td>1.41 ± 0.10 (176)</td>
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<tr>
<td>N70</td>
<td>1.90 ± 0.12 (238)</td>
</tr>
<tr>
<td>N71</td>
<td>1.37 ± 0.11 (171)</td>
</tr>
</tbody>
</table>

\( ^a \) Data were collected 28 days after the beginning of initial water stress of 8-week-old \( R_1 \) plants. Four cycles of 6 days of water stress, followed by 1 day of watering were used for the experiment. There were four to ten plants of each line used. At the end of the experiment, the fresh shoot weight of each plant was measured. The values for each plant within a given plant line were averaged and the standard deviation was shown. Numbers in parentheses are the percentages of value in transgenic plants as compared to control plants, which was taken as 100.
Increasing protective proteins

<table>
<thead>
<tr>
<th>Protective proteins</th>
<th>Plant growth</th>
<th>Plant growth, biomass</th>
<th>Shoot growth, RWC, photosynthesis</th>
<th>Shoot growth, RWC, water potential</th>
<th>Shoot growth, survivability</th>
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<tr>
<td>LEA</td>
<td>HVA1</td>
<td>Rice</td>
<td>Barley</td>
<td>Rice Act-1P</td>
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<td>LEA</td>
<td>HVA1</td>
<td>Wheat</td>
<td>Barley</td>
<td>Maize Ubi-1P</td>
<td>Limiting water supply</td>
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<td>Chaperone</td>
<td>BiP</td>
<td>Tobacco</td>
<td>Soybean</td>
<td>CaMV35SP</td>
<td>Water withholding</td>
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<td>Heat shock protein</td>
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<td>HVA1</td>
<td>Rice</td>
<td>Arabidopsis</td>
<td>Rice Act-1P</td>
<td>Water withholding</td>
</tr>
<tr>
<td>LEA</td>
<td>LEA</td>
<td>Chinese cabbage</td>
<td>Canola</td>
<td>CaMV35SP</td>
<td>Water withholding</td>
</tr>
</tbody>
</table>
Late embryogenesis abundant (LEA)

- *Lea* genes encode a diverse group of stress-protection proteins expressed during embryo maturation in all angiosperms.
- Accumulation of LEA proteins during embryogenesis correlates with increased levels of ABA and with acquisition of desiccation tolerance.
- LEA proteins are not normally expressed in vegetative tissues but they are induced by osmotic stress or exogenous application of ABA.
- Evidence derived from expression profiles strongly supports a role for LEA proteins as protective molecules, which enable the cells to survive protoplasmic water depletion.
- From LEA group 1 to group 5. LEA-2 are dehydrins.
Hva1 (LEA3 protein) from barley to rice

Fig. 1. Changes in leaf relative water content in non-transgenic and transgenic lines (30-59 and 30-54) of rice (cv. Nipponbare) under progressive water stress.

Fig. 4. Difference in cell membrane leakage among non-transgenic and transgenic lines (30-59 and 30-54) of rice (cv. Nipponbare) 28 days after withholding water. The values above the bars are S.D. of the mean.
### ROS-scavenging proteins

<table>
<thead>
<tr>
<th>Detoxification</th>
<th>MnSOD</th>
<th>Alfalfa</th>
<th><em>N. plumbaginifolia</em></th>
<th>CaMV35SP</th>
<th>Water withholding, field trial</th>
<th>Photosynthesis, electrolyte leakage, yield</th>
<th>1996</th>
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<tbody>
<tr>
<td>Lipid peroxide</td>
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<td>Alfalfa</td>
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<td>Water withholding</td>
<td>Photosynthesis</td>
<td>2000</td>
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<tr>
<td>NAD⁺ breakdown</td>
<td>PARP</td>
<td>Canola</td>
<td>-</td>
<td>CaMV35SP</td>
<td>Water withholding (RNAi)</td>
<td>FW, shoot growth</td>
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Altering ABA concentration

<table>
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<tr>
<th>ABA biosynthesis</th>
<th>AtNCED3</th>
<th>Arabidopsis</th>
<th>Arabidopsis</th>
<th>CaMV35SP</th>
<th>Agrobacterium</th>
<th>Water withholding</th>
<th>Water withholding</th>
<th>Shoot growth</th>
<th>Stomatal conductance, plant growth, survivability, transpiration rate</th>
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<td>Stomata</td>
<td>Chl-NADP-ME</td>
<td>Tobacco</td>
<td>Maize</td>
<td>MAS</td>
<td>Knockout</td>
<td>Hydroponic culture, soil</td>
<td>Shoot growth</td>
<td>2001</td>
<td>2002</td>
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<td>ABA catabolism</td>
<td>CYP707A3</td>
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<td>Water withholding</td>
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<td>2005</td>
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</table>
Drought tolerance by modulating ABA content: ABA synthesis (using *AtNCED3*)

Key enzyme in ABA synthesis. Overexpression of *AtNCED3* increase ABA and drought tolerance. Antisense suppression lead to low ABA and drought sensitivity.
Drought tolerance by modulating ABA content: ABA catabolism (using $CYP707A3$)

- The major ABA catabolic pathway is triggered by cytochrome P450 CYP707A family.
- The expression of CYP707A3 was most highly induced in response to both dehydration and subsequent rehydration.
- They proposed that the pair of $AtNCED3$ and $CYP707A3$ has a pivotal role for ABA biosynthesis and catabolism during dehydration or rehydration.

(Shinozaki and coll, Plant J. 2006)
AVP1 (H+PPase) in *Arabidopsis* and tomato

- AVP1 is a vacuolar H+ pump able to generate pH gradients involved in auxin distribution.
- Modification of Avp1 expression impacts auxin-mediated organogenesis (especially roots!).
Engineering drought tolerance using transcription factors

- Conventional and transcriptome-based analyses have revealed that dozens of transcription factors (TFs) are involved in plant response to drought stress
- Most TFs fall into large gene families like AP2/ERF, bZIP, NAC, MYB, MYC, Cys2His2 zinc-finger and WRKY
- TFs regulates downstream genes which more directly act on drought response
- Two important regulation pathways exist, one ABA-independent (CBF/DREB TFs acting on genes carrying the CRT/DRE – C-repeat/dehydration responsive - elements) and one ABA-dependent (ABF/AREB TFs, acting on genes carrying the ABRE element).
- Genes with both (and other) cis-elements exist!
Transcriptional regulatory networks (cis-acting elements and transcription factors) involved in osmotic and cold-stress responsiveness in *Arabidopsis*

Mod. from: Yamaguchi-Shinozaki and Shinozaki, 2006, Ann Rev Plant Biol
Transcriptional regulatory networks (*cis*-acting elements and transcription factors) involved in osmotic and cold-stress responsiveness in *Arabidopsis*
Engineering drought tolerance using transcription factors (Table 1)

<table>
<thead>
<tr>
<th>Classification</th>
<th>Gene name</th>
<th>Transgenic</th>
<th>Origin</th>
<th>Expression</th>
<th>Experiment</th>
<th>Parameters</th>
<th>Year</th>
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Engineering drought tolerance using transcription factors (Table 2)

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<td>Arabidopsis</td>
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<td>Survivability, electrolyte leakage</td>
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<td>Water withholding</td>
<td>Survivability</td>
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</table>
• *CBF3/DREB1A* and *ABF3* are two *Arabidopsis* TFs related to the ABA-independent and ABA-dependent pathways, respectively

• *CBF3* enhances tolerance to cold and other abiotic stresses and the expression of target genes (*cor* genes, *rd29a*, etc) involved in cold responses

• *ABF3* enhances tolerance to drought and expression of target genes (LEA genes *rd29b* and *rab18*; protein phosphatase, ecc) involved in drought responses

Aim

To test the expression of *Arabidopsis CBF3* and *ABF3* under constitutive promoter in rice
Arabidopsis CBF3/DREB1A and ABF3 in Transgenic Rice Increased Tolerance to Abiotic Stress without Stunting Growth

- Matrix attachment regions from chicken lysozyme gene
- Maize ubiquitin1 promoter and its first 5’ UTR (leader) intron
- CBF3 (or ABF3) coding sequences from Arabidopsis
- 3’ UTR from potato proteinase inhibitor II
- Selection marker gene

- Agrobacterium-mediated transformation
Arabidopsis CBF3/DREB1A and ABF3 in Transgenic Rice Increased Tolerance to Abiotic Stress without Stunting Growth\textsuperscript{1[w]}

![Graph showing fresh and dry weight growth over time](image)

![Images of plant growth at various time points](image)

\textbf{A}

- 0 day
- 2 day
- 4 day
- +1 day
- +3 day
- +5 day

\textbf{B}

- +5 day
- +7 day
Arabidopsis CBF3/DREB1A and ABF3 in Transgenic Rice Increased Tolerance to Abiotic Stress without Stunting Growth

Figure 4. Changes in chlorophyll fluorescence during drought, high-salinity, and low-temperature stresses. Three independent T₄ homozygous lines for Ubi1:CBF3, Ubi1:ABF3, and NC seedlings grown in the greenhouse for 14 d were subjected to various stress conditions as described: for drought stress, the seedlings were air-dried for 2 h at 28°C and for high-salinity stress seedlings were exposed to 400 mM NaCl for 2 h at 28°C. For low-temperature stress, they were exposed to 4°C for 6 h. All of the experiments were carried out under continuous light of 150 μmol m⁻² s⁻¹. Each data point represents the mean ± se of triplicate experiments (n = 6).
Arabidopsis CBF3/DREB1A and ABF3 in Transgenic Rice Increased Tolerance to Abiotic Stress without Stunting Growth

RNA from Ubi1:CBF3, Ubi1:ABF3 and control, 14-days seedling

- Ubi1:CBF3 activated 12 genes
- Ubi1:ABF3 activated 7 genes
- 3 genes (Hsp70, PP2Ca and a receptor kinase gene) were activated by both
- The 12 CBF3-activate genes had one ore more DRE elements while the 7 ABF3-activated genes had one ore more ABRE element in their promoters

58,417 known or predicted ORFs
2 slides
70-mer oligos
Arabidopsis CBF3/DREB1A and ABF3 in Transgenic Rice Increased Tolerance to Abiotic Stress without Stunting Growth\textsuperscript{1[w]}

- \textit{CBF3} over-expression improved tolerance to drought and salinity and only marginally to cold
  - The low effect on cold correlates with the low number of regulated genes in rice (overexpression of \textit{CBF3}: 38 genes activated in Arabidopsis vs 12 genes in rice)
  - This could be due to i) lack of appropriated target genes and/or ii) Arabidopsis \textit{CBF3} does not efficiently recognize rice promoters
- \textit{ABF3} over-expression improved tolerance to drought only
- Lack of negative or pleiotropic effects is probably due to low number of genes which are altered by Ubi:CBF3 and Ubi:ABF3
Genetic engineering of maize using the rice transcription factor OsMyb4 for adaptation to abiotic stresses

Natoli, Tuberosa, Coraggio et al., in preparation

- OsMyb4 is expressed in rice following cold stress and is able to activate the expression of downstream genes enhancing cold stress tolerance in Arabidopsis
- Constitutive expression of OsMyb4 in Arabidopsis resulted in improved cold tolerance and variable dwarf phenotypes
- Aim:
  to test the effect on cold and drought stresses of regulated and ectopic expression of OsMyb4 in maize
Genetic engineering of maize using the rice transcription factor OsMyb4 for adaptation to abiotic stresses

Natoli, Tuberosa, Coraggio et al., in preparation

• T4 plants homozygous for transgenic events were identified using PCR.
• Drought treatment:

  OsMyb4 +/- and the isogenic -/- were tested. At 3-leaf stage, water was withheld until 60% RWC (-/-) was reached (+/+ reached ca. 80% RWC).
No regenerants were obtained when OsMyb4 was driven by a constitutive (Ubi) promoter.

OsMyb4 expression under the control of an inducible (cold and ABA responsive element) promoter conferred drought and cold tolerance in maize.

Absence of negative pleiotropic effects on phenotype when plants were grown in well-watered and mild drought-stress conditions.
# Engineering drought tolerance using signaling factors

<table>
<thead>
<tr>
<th>Classification</th>
<th>Gene name</th>
<th>Transgenic</th>
<th>Origin</th>
<th>Expression</th>
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<th>Parameters</th>
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<td>Farnesyl-transferase</td>
<td>ERA1</td>
<td>Arabidopsis, canola</td>
<td>Arabidopsis</td>
<td>CaMV35SP/ RD29AP (antisense)</td>
<td>Water withholding, field test</td>
<td>Survivability, water loss, seed yield, oil content</td>
<td>2005</td>
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</table>
The *ERA1* mutation in Arabidopsis increases sensitivity to ABA (tight closure of guard cells with reduced wilting under drought stress) but has severe pleiotropic phenotypes.

*ERA1* encodes farnesyltransferase b-subunity (Farnesilation is a type of regulative protein modification where a lipid chain -15 C- is attached to the aa chain).

A negative regulator of guard cell sensitivity to ABA signalling must be farnesylated to modulate ABA response.

Aim: to place under control such regulative function in order to enhance a positive response to drought in *Arabidopsis* and *Brassica napus*.
Molecular tailoring of farnesyltransferase for plant drought tolerance and yield protection

Wang et al., 2005, Plant J 43, 416-424

ABA signal

Farnesyltransferase (AtFTB)

Rd29:anti-AtFTB

negative regulator (unknown)

Open stomata

Close stomata
Molecular tailoring of farnesylation for plant drought tolerance and yield protection

Wang et al., 2005, Plant J 43, 416-424
Engineering of stomata

It would be beneficial to agriculture for crop plants to show wide stomatal opening for CO2 intake when water is available, but to close stomata during drought periods, thereby slowing desiccation and damage. However, conventional high-yield breeding approaches may have contributed to selection of crop plants with reduced stomatal ABA responsiveness, because genes controlling guard cell signalling are also expressed in other tissues and control other yield parameters.

(Schroeder et al, Guard cell abscisic acid signalling and engineering drought hardiness in plants, Nature, 2001)

**Aim:** to optimize the balance between stomatal CO2 influx and water efflux in order to provide the best drought tolerance

**Strategy:** Genetic engineering to control guard cell movements

**Possible avenues:**

- To improve guard cell sensitivity to both increase and decrease of water availability
- To modify guard cell sensitivity in order to meet crops’ and environments’ specificities
AtMYB60 is a R2R3-MYB gene specifically expressed in guard cell and down regulated upon drought stress.

A null mutation (atmyb60-1) showed a constitutive reduction of stomata opening and decreased wilting under drought stress.

The atmyb60-1 mutation results in guard-cell-specific defects without affecting other developmental and physiological processes.

- **AtMYB60** is a R2R3-MYB gene specifically expressed in guard cell and down regulated upon drought stress.
- A null mutation (atmyb60-1) showed a constitutive reduction of stomata opening and decreased wilting under drought stress.
- The atmyb60-1 mutation results in guard-cell-specific defects without affecting other developmental and physiological processes.

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**A Guard-Cell-Specific MYB Transcription Factor Regulates Stomatal Movements and Plant Drought Tolerance**

**A**

- **AtMYB60**
- **TSB1**

**B**

- **Wild type**
- **atmyb60-1**

**C**

- **atmyb60-1**
- **Wild-type**
• High concentration of ions increases turgor pressure of guard cells and increased stomatal pore size

• Ions accumulated in guard cells during stomatal opening include K, Cl and malate

• In transgenic plants expressing a NADP malic enzyme, malate concentration was decreased

• Transgenic plants had decreased stomatal conductance and gained more fresh mass per unit water consumed while they were similar to wild type in their growth rate and development
Relevant references

Reviews


Research papers