Metabolic engineering of plants for osmotic stress resistance
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Genes encoding critical steps in the synthesis of osmoprotectant compounds are now being expressed in transgenic plants. These plants generally accumulate low levels of osmoprotectants and have increased stress tolerance. The next priority is therefore to engineer greater osmoprotectant synthesis without detriment to the rest of metabolism. This will require manipulation of multiple genes, guided by thorough analysis of metabolite fluxes and pool sizes.

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Abbreviations
GlyBet glycine betaine
MCA metabolic control analysis

Introduction
Improving crop resistance to osmotic stresses is a long-standing goal of agricultural biotechnology [1,2•]. Drought, salinity and freeze-induced dehydration constitute direct osmotic stresses; chilling and hypoxia can indirectly cause osmotic stress via effects on water uptake and loss. Soil salinity alone affects some 340 million hectares of cultivated land [2•]. To withstand osmotic stresses, certain plants have evolved a high capacity to synthesize and accumulate non-toxic solutes (osmoprotectants or compatible solutes), predominantly in the cytoplasm, as part of an overall mechanism to raise osmotic pressure and thereby maintain both turgor and the driving gradient for water uptake [3]. Many microorganisms also produce osmoprotectants [4]. Figure 1 shows the structures of some common osmoprotectant compounds; they fall into three groups — amino acids (e.g. proline), onium compounds (e.g. glycine betaine, dimethylsulfoniopropionate), and polyols/sugars (e.g. mannitol, D-ononitol, trehalose). Being non-toxic, osmoprotectants can accumulate to osmotically significant levels without disrupting metabolism; some of them can also protect enzymes and membranes against damage from high salt concentrations [4] and others (especially polyols) protect against reactive oxygen species [5].

The accumulation of osmoprotectants has been a target for plant genetic engineering for more than 15 years [6], and work is now in progress on all the compounds in Figure 1. In several cases, introduction of a single foreign gene into a transgenic plant has led to modest accumulation of an osmoprotectant and, apparently in consequence, a small increase in stress tolerance. The physiological and agricultural implications of these experiments have been thoroughly reviewed [2•,5,7–9,10•]. We will focus here on the metabolic implications of the genetic engineering work, and on what needs to be done to drive more flux towards osmoprotectant synthesis, with emphasis on glycine betaine, polyols and trehalose.

Overview of progress in engineering osmoprotectant synthesis
Table 1 catalogs the osmoprotectant work published to date, carried out with tobacco, Arabidopsis, rice and potato. The table illustrates several important points. First, many of the transgenes are of a non-plant origin, which reflects

Figure 1

Structures of various osmoprotectants found in plants.
the lack of investment in plant biochemistry. The plant gene pool should not be overlooked, as plants have evolved unique genes to synthesize osmoprotectants [11], and plant genes can possess particularly useful characteristics. For example, in transgenic tobacco, choline monooxygenase (CMO) is stabilized by salt-stress via a post-transcriptional mechanism, which leads to higher CMO activity when it is most needed [12••]. Second, most of the transgenes have been expressed from a constitutive promoter. Tissue-specific and inducible promoters eventually will be necessary, and could be developed from genes known to be induced when and where high osmoprotectant synthesis is required [13,14]. Third, most of the plants described express a single transgene and so represent only the first step in the engineering process, which is by nature iterative [1]. Finally, very few of the transgenic plants produced to date have been thoroughly analyzed at the metabolic level. This is critical because most of them accumulate only small amounts of the desired osmoprotectant, and further progress requires that the metabolic constraints be identified.

What are these constraints likely to be? First, certain metabolic networks can be rigid in that they have evolved to maintain metabolite flux distributions that are optimal for growth, and oppose any flux redistribution following expression of a transgene [15]. Second, the engineered pathway may divert flux away from primary metabolism and so create undesirable side effects [16•]. Finally, a foreign metabolite may be degraded, limiting its accumulation [17••]. We will now use published results to illustrate these points.

**Glycine betaine synthesis**

Glycine betaine (GlyBet) is synthesized in the chloroplast in two steps from choline (Figure 2a) and can accumulate to high levels (>20 mM on a tissue water basis, Table 1) in plants such as spinach and sugar beet under osmotic stress.

![Table 1](https://example.com/table1.png)

<table>
<thead>
<tr>
<th>Osmoprotectant</th>
<th>Gene constructs</th>
<th>Gene expression</th>
<th>Observed phenotype*</th>
<th>Product level†</th>
<th>Enzyme activity‡</th>
<th>Metabolic measurements§</th>
</tr>
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<tr>
<td>Proline</td>
<td>Mothbean P5CS</td>
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<td>180</td>
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<td>√</td>
<td>16</td>
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<td></td>
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<tr>
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<td>√</td>
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<tr>
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<td>√</td>
<td>8–16</td>
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<tr>
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<td>0.7–1.3</td>
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<td>D-Ononitol</td>
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<td>atsA</td>
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</table>

*A tick under ‘stress resistance’ indicates that the transgenic plant displayed more resistance than controls. A tick under side-effects indicates that transgenic plants had a growth defect. †This column reports the level of osmoprotectant synthesis in osmotically-stressed transgenic plants as a percentage of the level found in a representative plant that naturally accumulates the osmoprotectant in similar conditions. These levels (given in mM in tissue water) are: proline 36mM [35]; polyols 50mM [44]; trehalose 50mM [17••]; GlyBet 24mM [45]. ‡Indicates that the enzyme activity of the transgene product was assayed and localized to a subcellular compartment. §Indicates measurements were made of the endogenous levels of the osmoprotectant’s precursor or of any pertinent intermediate, or whether flux through the pathway was measured with isotopic tracer methods.
Several groups have reported engineering GlyBet synthesis by introducing choline-oxidizing genes from *E. coli* [18], *Arthrobacter* spp. [19,20], and spinach [12••]. GlyBet levels in the transgenic plants represented just a few percent of those found in plants that naturally accumulate it. Two groups have shown that supplying choline in axenic cultured plants enhanced GlyBet synthesis, indicating a constraint in choline synthesis ([12••], Huang et al. abstract in *Plant Physiol* 1997, 114S:120). Detailed analysis of choline metabolism in tobacco demonstrates that it is embedded in a rigid network in which choline is directed almost exclusively to phosphatidyl-choline synthesis, making it difficult to divert choline to GlyBet [12••]. Also, in tobacco *de novo* choline synthesis is probably constrained at the step catalyzed by phosphoethanolamine-N-methyltransferase [12••]. In this context, a comparison of the demand for choline moieties between a plant that lacks GlyBet and a plant that synthesizes it is instructive. Figure 3 makes clear that GlyBet synthesis represents a huge demand on choline synthesis, requiring more than 90% of the choline moieties. This suggests that the choline synthesis pathway is far more active in GlyBet accumulators. Increasing the choline supply in tobacco will require additional engineering steps. One possibility is to increase choline synthesis via up-regulation of phosphoethanolamine-N-methyltransferase activity.

**Figure 2**

Metabolic pathways associated with GlyBet, polyol and trehalose synthesis. (a) GlyBet synthesis (modified from [12••]) and (b) polyol and trehalose synthesis. The osmoprotectants are given in bold. Abbreviations are CDP-, cytidyldiphospho-; EA, ethanolamine; MME, monomethylEA; DME, dimethylEA; Bet ald, betaine aldehyde; cmo, choline monooxygenase; nsdh, endogenous non-specific aldehyde dehydrogenase activity; cdh, Cho dehydrogenase; codA, Cho oxidase; peamt, P-EA-N-methyltransferase; mtldh, mannitol dehydrogenase; s6pdh, sorbitol dehydrogenase; imt1, inositol methyltransferase; tps1, trehalose-6-phosphate synthase; tpp, trehalose-6-phosphate phosphatase. Reactions with an asterisk (*) are non-specific phosphatase activity.
Polypol synthesis

In some plants polypol synthesis is upregulated in response to osmotic stress, leading to significant polypol accumulation (up to 50 mM on a tissue water basis, Table 1). Polypols are derived from sugar phosphates by reduction and dephosphorylation (Figure 2b). Mannitol, sorbitol and D-ononitol synthesis have been introduced into transgenic plants. Two reports are of particular interest from a metabolic standpoint; one illustrates a competitive or antagonistic effect between transgene activity and host-plant metabolism, and the other a synergistic effect. First, analysis of sorbitol levels in transgenic tobacco showed that high sorbitol accumulation was associated with growth defects and necrosis. This was attributed to myo-inositol depletion, although sorbitol toxicity and cytosolic Pi depletion due to sorbitol-6-P build-up were not ruled out [16•]. Second, in tobacco, the myo-inositol pool expands as the plants are salt- or drought-stressed, and in plants engineered to synthesize D-ononitol from myo-inositol, the increased precursor supply makes D-ononitol production stress inducible [21••].

Trehalose synthesis

The non-reducing disaccharide trehalose is believed to enable desiccation resistant organisms to survive dehydration stress [17••]; it is synthesized from glucose-6-phosphate and uridine-diphosphoglucose (Figure 2b). In plants engineered to synthesize trehalose, only very small amounts accumulate (Table 1). In two reports trehalose-6-P-synthase (tps) alone was introduced, leaving dephosphorylation of trehalose-6-P to an endogenous non-specific phosphatase activity. In one report both tps and trehalose-6-P phosphatase (tpp) were inserted, but even with both genes, a dramatic increase in trehalose did not occur due to its degradation by trehalase [17••]. This was demonstrated by the use of a trehalase inhibitor, which increased trehalose accumulation and identified a constraint on trehalose synthesis in plants that do not naturally accumulate it [17••].

Metabolic engineering requires a guided, iterative approach

As noted above, Table 1 highlights the relative lack of attention to metabolic analysis of transgenic plants, in particular in vivo estimates of pathway fluxes and intermediate pool sizes made using isotopic tracer methods. Such data can be incorporated into metabolic models, which are very helpful tools to describe and predict the behavior of the target pathway (Figure 4). Models have a long track record of application to the metabolic engineering of microorganisms [22••], and to metabolic pathway characterization in plants [23]. Model development begins with a metabolic map as shown for GlyBet synthesis (Figure 2a) or polypol synthesis (Figure 2b). The input data consist of intermediate and end-product pool size measurements, and flux-rates calculated from timed in vivo isotope labeling data. The modeling program is used to fit the data to the described pathway, which is done interactively by varying flux rates and pool sizes (see [23]). Once developed, the model can be tested experimentally to assess its accuracy. The most important feature of models is their potential to predict the impact of a modification (e.g. inserting a transgene to upregulate a step in the pathway) on pathway flux. This can guide the engineering process by simulating the outcome of various experimental strategies. For instance, a model for choline metabolism in tobacco has been developed to simulate strategies for engineering GlyBet synthesis. It can be accessed at the world wide web site (http://www.hort.purdue.edu/cfpesp/models/models.htm) for constructing interactive metabolic models.

A related, but distinct, approach to understanding and predicting metabolic flux is metabolic control analysis (MCA). MCA is basically concerned with the steady state of a metabolic pathway, in which the enzyme activities and metabolite pool sizes remain constant. MCA shows that control of pathway flux is typically shared among all the enzyme activities in the pathway, in sharp contrast with the traditional concept of a single ‘rate-limiting’ step [24]. Each enzyme’s contribution to control of flux can vary and MCA defines this behavior as it relates to an individual step, or the entire pathway. The analysis can be used in simulation experiments similar to those outlined above. MCA has contributed both to microbial metabolic engineering [22••], and to basic understanding of the control of metabolism [25••]. For example, a recent report, using MCA to evaluate transgenic plants, clearly demonstrates that the control of pathway flux is shared among the component reactions within the pathway and is not limited to an individual step [26••].
Advancing technology to aid the engineering process

Two useful tools to help diagnose problems in transgenic plants are DNA micro-array technology and in vivo NMR spectroscopy. Micro-array technology was developed to evaluate the expression of many genes at once. The analytical power of this technology has been demonstrated in yeast [27••], and is being applied to plants [28]. It provides high-resolution gene expression data which can be used to identify (unanticipated) changes in expression that follow the insertion of a transgene.

In vivo NMR spectroscopy can be used to detect and quantitate several metabolites at once. In some cases the compartmentation of a metabolite can be distinguished [29••]. Although rarely as sensitive as standard in vivo biochemical or mass spectral methods, in vivo NMR techniques can be used to make otherwise impossible measurements — for instance, high resolution two-dimensional 31P-NMR spectroscopy was recently used to directly measure metabolic flux rates [30*].

Regulon engineering – manipulating entire pathways using regulatory genes

Expression of regulatory genes that control several steps in a pathway or in related pathways (i.e. a regulon [31••]), may provide an alternative to introducing pathway genes one at a time. Such an approach was used to manipulate secondary metabolite production in maize cells [32]. The basic principle is to manipulate the expression of a regulatory gene (e.g. a specific transcription factor), which in turn alters the expression of the entire regulon. Over-expression of CBF1, a transcription factor that controls the expression of cold-responsive genes in Arabidopsis, demonstrated the feasibility of this approach in a plant stress context [31••]. Other distinct transcription factors that may function in a similar way to CBF1 have also been described [33]. An Arabidopsis regulatory locus (esk1) that governs genes involved in both the synthesis and breakdown of proline has been identified [34•], and is, therefore, an attractive candidate for regulon engineering. It is important to note, however, that ectopic expression of regulatory genes can only modify a plant’s natural capabilities; it cannot confer new ones (e.g. the ability to synthesize a novel osmoprotectant).

Conclusions

Engineering metabolic change is not a trivial endeavor. It will typically require iterative rounds of transformation guided by thorough analysis of each transgenic generation. Interactive metabolic modeling promises to streamline this process by helping predict which step(s) will require alteration through additional rounds of engineering. Emerging
technology including DNA micro-arrays and _in vivo_ NMR could also speed and simplify the analysis of genetically engineered plants. Only when a biochemical trait, such as osmoprotectant biosynthesis, is successfully engineered can the physiological consequences of that trait be assessed. Metabolic engineering research will teach us not only how to engineer biochemical change but also much about the metabolic pathways themselves.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

* of special interest
** of outstanding interest


This report highlights a downside in metabolic engineering. It shows that extensive redirection of metabolic flux to sorbitol depletes the myo-inositol pool, and that this correlates with necrosis and growth defects. Clearly, transgene expression created an imbalance in primary metabolism; this could perhaps be corrected through additional rounds of engineering.


The analytical experiments in this paper show that an endogenous trehalase activity is one factor limiting trehalose synthesis in transgenic plants. Specific inhibition of trehalase increased trehalose accumulation. It also reports the expression of trehalose-6-P synthase both alone and with trehalose-6-P phosphatase (tpp), and that the presence of tpp has a small positive effect on trehalose accumulation.


This was the first report on the expression of a foreign choline oxidizing gene in chloroplasts. The transformed plants synthesized GlyBet and were slightly more tolerant to salt and cold stress.


This paper reports the production of _D_-ononitol in transgenic plants. The myo-inositol pool size increased with stress, which led to greater D-ononitol production and osmotic stress resistance. It illustrates how metabolic engineering can lead to insight into endogenous metabolic characteristics.


A historical discussion of mathematical modeling and its application to metabolic problems. It highlights the importance of modeling complex metabolic networks which, in turn, facilitate the engineering process.


This book provides a thorough and highly readable introduction to metabolic control analysis (MCA). It bridges the gap between traditional enzymology and MCA, and demonstrates how MCA overcomes some of the descriptive limitations of traditional enzymology.


An instructive demonstration of the use of MCA. Phosphofructokinase has been traditionally thought of as a ‘rate-limiting’ enzyme in glycolysis. However, overexpressing it in transgenic plants does not result in an increase in glycolytic flux. The authors use MCA to show that flux control is shared by the many steps between fructose-6-P and the Krebs cycle.

DNA microarray technology enables the expression of many genes to be measured at once. This paper is the first to report bringing this analytical procedure to the genomic scale, in yeast. The experiments clearly demonstrate how alterations in growth, mutations and genetic manipulations can affect the expression of many genes.


A good example of how in vivo NMR spectroscopy can complement classical biochemical analysis. $^{13}$C- and $^{31}$P-NMR were used to monitor the uptake and metabolism of homoserine. The experiments demonstrate how metabolite pool size data and enzymatic rate data can be derived from NMR spectra. An experiment to determine the distribution of phosphohomoserine between the cytosol and the vacuole is also described.


This paper describes how in vivo NMR spectroscopy can be used to measure the flux of phosphate from substrates to products. The methods to monitor four enzymatic reactions in central metabolism are described and used to show how each reaction responds to changes in external stimuli.


This is the first report to demonstrate 'regulon engineering' in a plant stress context. CBF1 had been shown to interact with a number of cold-responsive genes during the 'cold-acclimation' process, which leads to an increase in freezing tolerance. The experiments show that overexpressing CBF1 induces the acclimation effect and enhances freezing tolerance.


This paper describes the identification and characterization of an Arabidopsis mutant that is defective in a key regulatory gene. It is a new locus, distinct from other cold-acclimation pathways. The data show that, in proline metabolism, this gene can simultaneously upregulate synthesis genes and downregulate degradative genes, implying that it is near the top of a signaling cascade.


