ENHANCING THE RESISTANCE OF WHEAT TO WATER DEFICIT STRESS THROUGH GENETIC ENGINEERING

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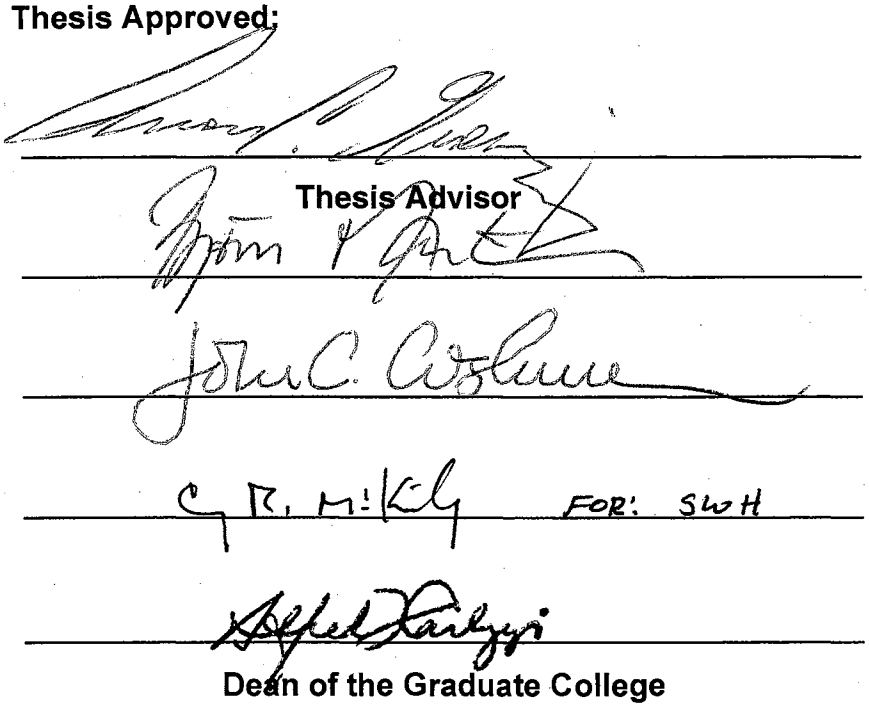

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DEDICATION

I dedicate this work to my brother and best friend Mulu Ambaw Ferede whose whereabouts is unknown since he was kidnapped in Addis Ababa in May 1997 by the security forces of the current government of Ethiopia, which is dominated by the ethnocentric Tigrean People's Liberation Front (TPLF).

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LIST OF SYMBOLS AND ABBREVIATIONS

Ψ_s	Osmotic potential
Ψ_w	Water potential
ABRE	ABA-responsive element
ATCDPK	*Arabidopsis thaliana* CDPK
ATP	Adenine triphosphate
Bp	Base pair
CBF1	C-repeat binding factor 1
CDPK	Calcium-dependent protein kinase
CIM	Callus induction medium
CRT	C-repeat
CSM	Callus selection medium
DHAP	Dihydroacetone phosphate
dNTPs	Deoxynucletide triphosphates
DRE	Dehydration-responsive element
DREB	Dehydration responsive element binding factor
ERD	Early responsive to dehydration
F6P	Fructose-6-phosphate
F16P	Fructose-1,6-bisphosphate
Fwt	Fresh weight
GR	Glutathione reductase
GSH	Reduced form of glutathione
GSSG	Oxidized form of glutathione
GUS	β-glucuronidase
Kb	Kilobase
KDa	Kilodalton
LUC	Luciferase
HPAE	High performance anion exchange
LEA	Late embryogenesis abundant protein
M1P	Mannitol-1-phosphate
MPa	Megapascal
MtlD	Mannitol-1-phosphate dehydrogenase gene
MTLD	Mannitol-1-phosphate dehydrogenase enzyme
-mtlD	Wheat transformed with plasmid pAHC20 alone and does not contain the *mtlD* gene
+mtlD	Wheat transformed with a plasmid containing the *mtlD* gene
NAD	Nicotine adenine dinucleotide (oxidized form)
NADH	Nicotine adenine dinucleotide (reduced form)

NADP	Nicotine adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotine adenine dinucleotide phosphate (reduced form)
OsCDPK	*Oryza sativa* CDPK
QTL	Quantitative trait locus
PAR	Photosynthetically active radiation
3PGA	3-phosphate glyceraldehyde
Pi	Inorganic phosphate
PKAB1	ABA-responsive protein kinase 1
PAD	Pulsed amperometric detector
PAGE	Polyacrylamide gel electrophoresis
PAR	Photosynthetically active radiation
PAT	Phosphinothricin acetyl transferase
PCR	Polymerase chain reaction
PP2C	Protein phosphatase 2C
PEG	Polyethylene glycol
RIM	Root initiation medium
ROS	Reactive oxygen species
Rubisco	Ribulose-1,5-bisphosphate caroxylase/oxygenase
RuBP	Ribulose-1,5-bisphosphate
RWC	Relative water content
SIM	Shoot initiation medium
SOD	Superoxide dismutase
TP	Transit peptide

CHAPTER I

INTRODUCTION

ABSTRACT

Water deficit is the major abiotic factor that limits crop productivity. Water deficit stress is induced by many factors, drought and salinity being major ones. One of the traditional approaches to increase agricultural productivity under conditions of water deficit is breeding for resistant varieties. However, success in breeding resistance has been limited because 1) resistance to water deficit stress is controlled by many genes and their simultaneous selection is difficult, 2) resistance is influenced by the environment more than the allelic compositions making breeding for water-limited areas unreliable, 3) tremendous effort is required to eliminate undesirable genes that are also incorporated, and 4) lack of efficient selection procedures. Genetic engineering offers a novel alternative by selectively transferring resistance genes isolated from other organisms without the need for sexual reproduction. Major targets for engineering resistance to water deficit stress include osmolytes, reactive oxygen species (ROS) scavenging enzymes, late embryogenesis abundant (LEA) proteins, transcription factors and signaling molecules. The osmolyte mannitol accumulates in many plants in response to water deficit stress. Experiments in this dissertation describe the production of transgenic wheat that expressed the *E. coli mtlD* gene for accumulation of mannitol. Transformants were evaluated to determine the effect of mannitol on growth and the resistance of wheat to water stress and salinity.

Resistance of Plants to Water Deficit Stress

Abiotic stress poses a great challenge to our efforts to increase agricultural productivity. Due to stress, crops are unable to express their full genetic potential for yield leading to food deficits in many parts of the world. Water deficit is the major abiotic factor that limits crop productivity (Boyer, 1982; Levitt, 1980; Close, 1996; Bray, 1997). Water deficit stress is induced by many abiotic factors including drought, salinity, and extreme temperatures. About 69% of the United States is affected by water deficit, which causes more yield loss than all biotic factors combined. Drought accounts for 25% of the loss (Kramer and Boyer, 1995). Between 1948 and 1997 about 57% of insurance indemnities paid to farmers for crop losses were due to water deficit stress (NASS, 1999).

Salinity is a serious problem in arid areas where rainfall is limited and in agricultural lands where irrigation is practiced. Worldwide irrigation accounts for 70% of freshwater consumption (UNEP, 2000) and causes salt accumulation in 26% of the cultivated land (Sen and Mohammed, 1994; Szabolcs, 1994). Every year as much as 10 million hectares of irrigated land is abandoned because of excessive salt accumulation (Szabolcs, 1994). Except some plants, known as halophytes that can grow under high salt concentrations, most plants, including agriculturally important crops, are glycophytic and do not grow well under saline conditions. Soil salinity can affect plants in three ways: 1) high concentrations of specific ions (Na and Cl) are toxic to membranes and enzymes and can induce physiological disorders, 2) ionic imbalance can disrupt uptake and distribution of nutritionally relevant ions such as K and Ca, and 3) osmotic depression of the

soil water potential can restrict uptake of water leading to water deficit stress (Glenn *et al.*, 1997).

Because of its central role as a solvent and a reactant in many metabolic reactions, shortage of water has a serious consequence on crop production. When absorption of water lags behind transpiration, water deficit develops that may result in permanent wilting and death by dehydration. Death is usually associated with degradation of cell membranes, protein denaturation and gene mutations (Levitt, 1980). Therefore, in order to survive and maintain some growth during water deficit, plants must develop mechanisms to cope with the stress. Plants respond to water deficit at the morphological, physiological, cellular and metabolic levels. The responses are dependent upon the duration and severity of the stress, genotype, stage of development and cell or organ type (Bray, 1997). Responses to water deficit can be grouped into three basic mechanisms: escape, avoidance and tolerance (Levitt, 1980). Escape from water deficit stress through shorter or better-timed life cycles is the most effective form of adaptation in crop plants. Among cereals, drought stress often has its greatest impact on yield when it occurs during anthesis (Ribaut *et al.*, 1997). Adjustment of flowering time to minimize the chances of drought stress at that stage is an important element in the adaptation of many cereals (Mahalakshmi and Bidinger, 1985).

Avoidance of water deficit stress derives from the ability to maintain high turgor during conditions of stress by either increased water potential or reduced osmotic potential associated with osmotic adjustment. Plants differ greatly in their leaf water potential under particular stress conditions owing to differences in such

characteristics as root growth patterns, hydraulic conductance, glaucousness, movement of leaves to reduce water loss and frequency and responsiveness of stomata. Osmotic adjustment involves accumulation of solutes to lower cellular water potential and thereby increase water uptake and maintain volume and turgor (Bray, 1997). This allows turgor- and volume-dependent processes such as stomatal opening and expansion growth to continue during progressive decline in the availability of water. Varietal differences in this capacity were positively correlated with grain yield of wheat over a range of environments (Morgan, 1983; Morgan *et al.*, 1986).

Plants achieve osmotic adjustment by accumulating osmolytes or inorganic ions. Osmolytes are compatible (non-toxic) organic solutes that can accumulate in the cell without disrupting metabolic functions (Bartels and Nelson, 1994). These include amino acids (e.g. proline), sugar alcohols (e.g. mannitol), soluble sugars (e.g. low molecular weight fructans) and quaternary ammonium compounds (e.g. glycine betaine). Accumulation of osmolytes occurs either through *de novo* synthesis or through a combination of synthesis and catabolism. For example, soluble sugars (such as glucose and fructose) can be released from polymeric forms (starch and fructans) in response to stress. When the stress is relieved the simple sugars can be repolymerized to facilitate rapid reversible osmotic adjustment.

Most frequently, osmolyte accumulation is confined to the small subcellular compartment (the cytosol) with energetically less costly inorganic ions making the major contribution to osmotic adjustment in the vacuole (Hare and

Cress, 1997). Cost benefit analyses of osmotic adjustment with various solutes support this conclusion. Greenway (1973) estimated that osmotic adjustment in 100 mM external NaCl with hexoses would require 20-30% of the total biomass. About 68-101 moles of photons of light is required to synthesize 1 mole of organic solutes, such as sorbitol, mannitol, proline and glycine betaine compared to the 2-4 moles of photons to accumulate 1 mole of KCl or NaCl (Raven, 1985). Furthermore, accumulation of osmolytes in the cytosol minimizes the potential toxicity of inorganic ions. Many inorganic ions adversely affect metabolic processes when present in excess amounts possibly by binding to and altering the properties of substrates, enzymes, membranes and other macromolecules. Many ions also enter the hydration shells of proteins and promote their denaturation. In contrast, osmolytes tend to be electrically neutral at physiological pH and are excluded from the hydration shells of macromolecules (Bray *et al.*, 2000).

Tolerance refers to the ability to sustain less injury when turgor is lost during stress (Bray *et al.*, 2000). Seeds (Black *et al.*, 1999), resurrection plants (Blomstedt *et al.*, 1998; Bockel *et al.*, 1998; Frank *et al.*, 2000) and bryophytes (Wood and Oliver, 1999) are tolerant to dehydration. Seeds can lose more than 90% of their original water content, remain dormant and still germinate when receiving adequate moisture. Resurrection plants can survive severe dehydration down to at least 2% of relative water content of leaves and recover uninjured as soon as they come in contact with water (Ingram and Bartels, 1996). Tolerance involves protection of the cellular machinery by maintaining membrane stability

and repair of damage during dehydration (Bray, 1997). In bryophytes and other lower plants, the components of tolerance are constitutively present and tolerance is thought to be based on cellular repair during rehydration (Oliver, 1991). To the contrary, tolerance in angiosperm seeds and vegetative tissues seems to utilize a protection mechanism that activates the synthesis of specific transcripts and proteins during dehydration (Ingram and Bartels, 1996).

Molecular Basis of Water Deficit Stress Response

Water deficit induces an array of genes whose products can be classified into two major groups: those that directly protect cells against stress-induced damages and those that are involved in signal transduction and regulation of other genes. The first group includes proteins that protect cells from dehydration, such as water channel proteins, LEA proteins, enzymes that synthesize various osmolytes, chaperones, proteases (thiol protease, Clp protease, ubiquitin) and reactive oxygen scavenging enzymes (glutathione S-transferase, soluble epoxide hydrolase, catalase, superoxide dismutase, ascorbate peroxidase). The second group of gene products includes kinases, transcription factors, phospholipase C and 14-3-3 proteins (Shinozaki and Yamaguchi-Shinozaki, 1997).

Despite attempts to elucidate the intricate signaling pathways that participate in alteration of gene expression, little is known of how plants perceive water deficit stress. Davies et al. (1986) suggested the presence of a chemical signal, with the root as a sensing organ. The nature of the proposed signal is unknown, but it is widely accepted to be abscisic acid (ABA) delivered to leaves

via the transpiration stream and causing stomatal closure (Zhang and Davies, 1987). Individual cells perceive water deficit as a loss of turgor or change in cell volume. It is believed that these changes are sensed at the plasmalemma with ABA as a signal. The current working hypothesis for signal transduction involving ABA suggests that the binding of ABA to a receptor on the plasmalemma elicits a complex signaling cascade that induces expression of several genes (Bray, 1997; Bonetta and McCourt, 1998). But receptors of the stress signal are yet to be discovered. ABA levels increase both during embryo development, shortly before the onset of seed desiccation (King, 1976), and in tissues subjected to osmotic stress (Henson, 1984; Squire *et al.*, 1988). Most water stress-inducible genes are also responsive to treatments with exogenous ABA (Thomashow, 1999). Many genes that respond to ABA and water deficit code for late embryogenesis abundant (LEA) proteins (Ingram and Bartels, 1996). LEA proteins are abundant in embryos during the final stages of seed maturation or in immature embryos treated with ABA (Dure 1993a; Ingram and Bartels, 1996).

Not all water deficit stress-inducible genes are responsive to ABA. Many genes are expressed in stressed *Arabidopsis* leaves before the ABA levels rise (Kiyosue *et al.*, 1994). Expression of these *ERD* (early-responsive to dehydration) genes is induced 1 hour after dehydration whereas ABA concentrations do not rise until after 2 hours of dehydration. Addition of ABA does not affect *ERD* expression. Moreover, several ABA-inducible genes are expressed by both drought and cold in ABA-deficient (*aba*) and ABA-insensitive (*abi*) Arabidopsis mutants (Shinozaki and Yamaguchi-Shinozaki, 1996). This

suggests the existence of two pathways for responses to water deficit stress: ABA-dependent and ABA-independent signal transduction pathways (Shinozaki and Yamaguchi-Shinozaki, 1997).

ABA-regulated genes have an ABA-responsive element (ABRE) in their promoter region with a consensus sequence RYACGTGGC (where R refers to a nucleotide with a purine base and Y a nucleotide with a pyrimidine base) containing the ACGT core element (Ingram and Bartels, 1996; Bray, 1997). The ABRE functions as a cis-acting protein-binding DNA element. Nucleotides flanking the ACGT core specify the DNA-protein interactions and subsequent gene activation (Ingram and Bartels, 1996). *ABRE* was first described in wheat (Marcotte *et al.,* 1989). For ABA regulated gene expression, the *ABRE* has been shown to be sufficient, but in some cases a coupling element (CE) is required. Two *ABRE*s in the barley *HVA22* gene can only confer ABA induction in presence of additional sequences from the promoter. The first coupling element (*CE1*) has a consensus sequence TGCCACCGG, whereas the second coupling element (*CE3*) has an ACGCGTGTCCTC sequence (Shen and Ho, 1995).

Based on available evidence so far, four signal transduction pathways can be deduced for activation of water deficit-inducible genes. Two of the pathways are ABA-independent and two are ABA-dependent (Shinozaki and Yamaguchi-Shinozaki, 2000). It is still not clear how ABA activates the ABRE and induces gene expression. As it happens during stomata movement, ABA might initiate signal transduction via secondary messengers, such as Ca^{2+}. The most characterized Ca^{2+}-modulated proteins are calmodulins (CaM) and the Ca^{2+}-

dependent (but CaM-independent) protein kinases (CDPKs). Many plant CDPKs have been isolated and characterized (Sheen, 1996; Harmon *et al.*, 2000). CDPKs are stimulated by the binding of Ca^{2+} to their regulatory domain. The concentration of cytosolic Ca^{2+} is known to rise during water deficit stress. Stress-induced changes in the concentration of cytosolic Ca^{2+} occur due to influx of Ca^{2+} from outside of the cell or release of Ca^{2+} from intracellular pools (Knight *et al.*, 1996; Knight *et al.*, 1998; Kiegle *et al.*, 2000; Knight, 2000). Increased cytosolic Ca^{2+} activates CDPKs and Ca^{2+}/CaM-protein kinases. The kinases in turn activate transcription factors through phosphorylation of specific serine and threonine/tyrosine residues. The phosphorylated transcription factors then bind to the ABRE and initiate expression of stress-inducible genes. The effect of kinases is reversed by protein phosphatases (Sheen, 1996). Expression of two *Arabidopsis* CDPKs (ATCDPK1 and ATCDPK1a) increases in presence of ABA. These CDPKs activate expression of the water deficit stress-inducible HVA1 promoter of barley (Sheen, 1996).

The ABA-independent water stress signal transduction is less understood. The well-characterized *cis*-acting ABA-independent element is the *Arabidopsis* dehydration-responsive element (DRE) identified on the promoter of *rd29A* (variously known as *lti78* or *cor78). Rd29A* is also responsive to low temperature because it contains the ABA-responsive element ABRE (Yamaguchi-Shinozaki and Shinozaki, 1994). The DRE contains a 9-bp consensus sequence, TACCGACAT, and functions in the rapid response (first hour) of *rd29A* to water deficit stress. The ABRE is induced at a later stage of the stress (after about 3

hours). DRE is also found in the promoter regions of other dehydration and cold stress-responsive genes (Kasuga *et al.*, 1999). Another related cis-acting element, named the C-repeat (CRT) or low-temperature-responsive element with a CCGAC motif that forms the core of the DRE sequence, is found in the promoter region of cold-inducible genes, such as the *cor15a* gene of *Arabidopsis* (Baker *et al.*, 1994). Recently Liu *et al.* (1998) described two *trans*-acting factors, DREB1 and DREB2 that bind to the DRE. These elements function in different ways. DREB1A is induced by low temperature whereas DREB2A is responsive to dehydration and salt stress (Liu *et al.*, 1998; Nakashima *et al.*, 2000). DREB1 is identical to the CRT binding protein (CBF) isolated from *Arabidopsis* (Stockinger *et al.*, 1997; Gilmour *et al.*, 1998; Medina *et al.*, 1999).

Breeding for Resistance to Water Deficit Stress

Global population is expected to grow at a rate of 1.1% until 2015 and by 2030 the current population of 6 billion will reach 8.1 billion (FAO, 2000). As a result, demand for food will have to increase to meet population growth. Increase in demand can be met in three ways: increasing yield, increasing the arable land area and increasing cropping intensity. Over the last 30 years more than three-quarters of the increase in food production came from increased yield mainly as a result of the technologies developed during the green revolution, i.e. high yielding varieties, fertilizers, irrigation, and packages for improved cultural practices (Khush, 1999; Miflin, 2000). As a result world wheat production increased from 308 million tons in 1966 to 541 million tons in 1990 (Khush,

1999). Similarly in the next 30 years 69% of the production is expected to come from yield increases, the rest from cropping intensity (12%) and increase in arable land (FAO, 2000). The fact that rainfall in many parts of the world is erratic and unpredictable coupled to the dwindling freshwater resources for irrigation, as well as the increasing environmental problems associated with it, means that new methods must be found to increase productivity in areas prone to shortage of water (Flowers *et al.*, 2000; Miflin, 2000). One way of increasing productivity is to breed crops that are more resistant to stress. However, except for a few varieties with extensive root system (Sloane *et al.*, 1990; Lazar *et al.*, 1997), seedling establishment and early flowering (Kramer and Boyer, 1995), the success in breeding for resistance to water deficit stress has been very limited (Richards, 1996; Yeo, 1998; Flowers *et al.*, 2000).

The slow pace in breeding resistant crops is attributable to four major problems. First, resistance to water deficit stress is a quantitative trait influenced by the action of many genes whose individual effects are very small and difficult to identify in pedigrees (Flowers *et al.*, 2000). Quantitative traits do not fall into discrete categories; instead they show a continuous range of variation. There are no obvious discontinuities in the distribution as might be expected of a classical single gene trait, such as 1:2:1 distribution of genotypes in an F2 (Kearsey, 1998; Yeo, 1998). This means that several regions of the genome must be manipulated at the same time in order to have a significant impact on resistance to water deficit stress. Furthermore, epistatic interactions between genes are common and if the genomic regions involved in the interaction are not incorporated in the

selection scheme, they can bias the selection process. For traits controlled by single, so-called major genes, conventional breeding procedures have been successful because the major genes have a large effect on the phenotype and thus desirable genotypes can be identified through phenotypic evaluation.

Second, unlike the major genes that have a large effect on the phenotype compared to the environment, resistance to water deficit stress is influenced not only by the allelic combination of the genes involved, but also by the effects of the environment. Drought is a very unpredictable climatic phenomenon varying in timing and intensity. Even in the same locality, the severity, timing and duration of drought varies from year to year and cultivars successful in one dry year may fail in another. To make matters worse, drought seldom occurs in isolation; it often interacts with other abiotic and biotic stresses. Thus, breeding for environments where drought is variable and unpredictable makes the target hard to define and hence conventional selection is slow to achieve meaningful results (Cecarelli and Grando, 1996).

Third, introduction of parental materials into breeding programs requires tremendous effort to eliminate undesirable genes that are also introduced. Hybridizing unadapted with adapted parents usually conserves chromosomal segments with large number of unwanted genes. Their linkage with favorable gene combinations may take a long time to separate and therefore considerable time and effort to recover previous yield levels. Many years of breeding in highly bred crops has resulted in combinations of genes that act together in great harmony. Many years may be spent in rebuilding the harmonious combinations

again after introducing new traits from poorly adapted genetic backgrounds (Richards, 1996). Introduction of unadapted parents into breeding programs is therefore a risk that breeders are reluctant to take.

Fourth, lack of efficient selection procedures is another limiting factor for success. Since the ultimate goal of any breeding program is to develop crops with better yield, a relatively higher yield has been considered as a useful selection criterion for resistance to stress. However, selection for high yield under water deficit is inefficient because of low genetic variance of yield components under increased stress resulting in low yield heritability (Ribaut *et al.*, 1996). Therefore, many breeders argue that yield as a selection criterion is less dependable and prefer to devote their time on traits better defined and where selection is known to be effective, such as disease resistance and grain quality. Another reason that makes yield a weaker selection criterion is the probability that a crop performing well under non-stress conditions also performs better under water deficit, even if the relative yield reduction for this crop is large (Ribaut *et al.*, 1997).

With the availability of molecular markers and saturated genomic maps for many crops, methods are being developed to associate quantitative traits with qualitative genetic markers. Interest is growing to utilize these resources to identify and locate quantitative trait loci (QTLs) for resistance to water deficit stress. A QTL is a chromosomal region linked to a marker gene controlling a quantitative trait. QTLs can be single or multiple. The number and distribution of multiple QTLs on chromosomes determines their manipulability (Xu, 1995). QTLs

can be identified by analysis of linkages with molecular markers on an established genomic map and their effect on resistance correlated indirectly by analyzing the segregation of markers. QTL analysis depends on the fact that where such linkage occurs, the marker locus and the QTL will not segregate independently and so differences in those marker genotypes will be associated with the phenotype of the QTL. Once linkage is established, resistance can be evaluated in the presence or absence of stress through marker-assisted selection (MAS), because unlike the phenotype, the DNA of alleles is not affected by the stress (Ribaut and Hisington, 1998). The real advantage of QTL analysis is that resistance can be assessed at the seedling stage without the need for repeated back crossing procedures thus reducing the length of time required to develop genotypes with the desired allelic composition. Besides, multiple selection with markers for different traits can be carried out with very little additional effort in screening since the sampling and DNA preparation usually account for much of the work (Kearsey, 1998).

Several studies have demonstrated the identification of QTLs for resistance to water deficit stress and their localization on specific chromosomal regions. In maize, when drought stress occurs just before and during the flowering period, a delay in silking occurs resulting in an increase in the length of the anthesis-silking interval (ASI). This asynchrony between the male and female flowering results in decreased grain yield (Bolanos and Edmeades, 1993). Selection for grain yield under water deficit conditions is inefficient because of increased environmental variance relative to genetic variance, which decreases

yield heritability. Selection for traits that have relatively high heritability and are correlated to grain yield (such as ASI) has been suggested as an alternative to identify QTLs for resistance to water deficit stress (Bolanos *et al.*, 1993). Using F2 and F3 populations from two maize inbred parental lines differing in their tolerance to drought, Ribaut *et al.* (1996) identified QTLs for ASI. Using 113 polymorphic markers six QTLs were identified on chromosomes 1,2,5,6,8 and 10, which represented a change of 11 days in ASI (47% of the variance).

In wheat differences in osmotic adjustment between genotypes is the result of allelic compositions at a single *or* locus with high response being recessive (Morgan, 1991). Analysis of single chromosome substitution lines of Chinese spring/red Egyptian indicated a location on chromosome 7A. The precise location of the *or* locus was determined by Morgan and Tan (1996) using four RFLP markers (Xpsr119, Xpsr160, Xglk651 and Xglk576) and 14 recombinant inbred lines of a cross between Songlen (resistant) and Codor (susceptible). Linkage analysis using RFLP markers placed the *or* locus on the short arm of chromosome 7A, about 13 cM towards the centromere from the RFLP marker Xpsr119. Rice also has a single QTL locus for osmotic adjustment on chromosome 8. This QTL is homoeologous with the single recessive *or* gene of wheat and accounts for one-third of the variation in osmotic adjustment in rice (Lilley *et al.*, 1997). Specific markers that map the *or* locus of rice chromosome 8 (CDO99, CDO595 and CDO64) also map the wheat chromosome 7 suggesting that the entire region associated with osmotic adjustment in rice has been conserved on chromosome 7 of wheat during evolution (Zhang et al., 1999).

Tuinstra *et al.* (1998) identified three QTLs associated with drought tolerance of sorghum during pre-flowering and post-flowering stages. Ninety-eight near-isogenic sorghum lines (NILs) were derived from a cross between two parental lines contrasting in their response to drought: TX7078 (pre-flowering tolerant; post-flowering susceptible) and B35 (pre-flowering susceptible; post-flowering tolerant). Progenies were analyzed to identify QTLs using three markers: *tM5/75*, *tH19/50* and *t329/132*. QTL marker *tM5/75* was responsible for differences in average yield by more than 1100 kg ha^{-1}. QTL marker *tH19/50* accounted for differences in average yield by more than 700 kg ha^{-1} under drought and non-drought conditions whereas QTL marker *t329/132* caused differences in seed weight by more than 0.25 g per 100 seeds.

QTLs have also been identified for resistance to salt stress. In order to determine the genetic relationship between resistance to salt stress during seed germination and vegetative growth in tomato, Foolad (1999) hybridized a salt-sensitive *Lycopersicon esculentum* (line NC84173) with a salt-resistant *Lycopersicon pimpinellifolium* (line LA722). Seven QTLs were identified for resistance to salt stress during seed germination and 5 QTLs for resistance during vegetative growth. Moreover, QTLs for resistance to salt stress during seed germination were different from those for resistance during vegetative growth (Foolad, 1999).

Despite numerous reports on the identification of QTLs, there is insufficient evidence to suggest the efficiency of MAS in selection for resistance to water deficit stress. To date much of the work has been on the refinement of

statistical designs for improving the accuracy of MAS (Xie and Xu, 1998; Kao *et al.*, 1999).

Engineering for Resistance to Water Deficit Stress

Recent studies with transgenic plants capable of expressing water deficit-related genes have demonstrated the potential of genetic engineering as a novel alternative to breeding for improving resistance to stress. Unlike traditional breeding procedures or marker-assisted selection (MAS), selective introduction of resistance genes into target crops by genetic engineering overcomes species isolation barriers. Therefore, genes cloned from any organism can be introduced to crops to enhance stress resistance without the need for sexual reproduction. By genetic engineering approach it is possible to assemble multiple resistance genes from several initial sources and simultaneously transfer them to a target crop (Bohnert and Shen, 1999). Moreover, genetic engineering is a faster and precise means of achieving improved resistance to water deficit stress (Cushman and Bohnert, 2000), because it avoids the transfer of unwanted chromosomal regions that are also introduced during breeding. Since plants differ in the regulatory control of gene expression and targeting of gene products during stress, installing missing parts of the pathways or establishing a completely new pathway could enhance resistance to water deficit stress. There are many functional targets for engineering stress resistance in plants (Cushman and Bohnert, 2000). Major targets include genes that encode for biosynthesis of osmolytes, reactive oxygen species (ROS) scavenging enzymes, late

embryogenesis abundant (LEA) proteins, transcription factors and signaling molecules.

Engineering for osmolyte accumulation. Amino acids, sugars, sugar alcohols, quaternary ammonium compounds, and tertiary sulfonium compounds are common osmolytes in plants (Hare *et al.*, 1998; Smirnoff, 1998; Trossat *et al.*, 1998; Bohnert and Shen, 1999; McNeil *et al.*, 1999; Nuccio *et al.*, 1999; Sakamoto and Murata, 2000). Accumulation of these molecules during water deficit suggests that they have important roles to play in the survival of plants under stress and thus are excellent targets for improving resistance to water deficit stress through genetic engineering. Osmolytes improve resistance to water deficit stress through at least three mechanisms: 1) osmotic adjustment, 2) reactive oxygen species scavenging, and 3) as a sink for reducing power and storage of carbon and nitrogen.

1. Osmotic adjustment. Osmotic adjustment involves accumulation of solutes to lower cellular solute and water potentials thereby maintaining turgor (Bray, 1997). Most frequently observed levels of osmolyte accumulation are confined exclusively to a small subcellular compartment, such as the cytosol. The energetically less costly electrolytes make the major contributions to osmotic potentials in the vacuole and the apoplast (Hare and Cress, 1997).

2. Reactive oxygen species (ROS) scavenging. ROS, such as singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\bullet$) are very reactive and damage membranes and macromolecules. To counter the

negative effects of these molecules, plants have natural defense strategies (Smirnoff, 1998). *In vitro* and *in vivo* studies have also demonstrated that osmolytes can scavenge ROS. The potential of compatible solutes (such as mannitol, sorbitol and proline) as scavengers of $OH^{\bullet}$ has been demonstrated *in vitro* (Smirnoff, 1989). *In vivo* studies using transgenic tobacco have also demonstrated the $OH^{\bullet}$ scavenging capacity of mannitol (Shen *et al.*, 1997a). Mannitol protects thiol-regulated enzymes (such as phosphoribulokinase) by shielding them from $OH^{\bullet}$ radicals (Shen *et al.*, 1997b).

3. Sink for reducing power and storage of carbon and nitrogen. Since capture of photon energy is insensitive to water deficit stress, plants under stress are exposed to light intensities in excess of those that can be used for carbon assimilation. If the regeneration of $NADP^+$ is limited under conditions of continued photon absorption, redox imbalance is likely to result in photoinhibition and enhanced use of O_2 instead of $NADP^+$ as the electron acceptor in photosynthesis (Hare *et al.*, 1998). Since the biosynthesis of some osmolytes, such as proline, involves utilization of NADPH as a reducing agent, it has been proposed that a stress-induced increase in the transfer of reducing equivalents into these molecules may assist in counteracting photoinhibitory damage under stress conditions by recycling $NADP^+$ (Hare *et al.*, 1998; Kuznetsov and Shevyakova, 1999). Moreover, when the stress is relieved, these molecules can be remobilized and used as a source of carbon and nitrogen (Hare *et al.*, 1998).

Engineering for reactive oxygen species (ROS) scavenging. Reactive oxygen species (ROS) are formed in various metabolic reactions where oxygen is involved. In plants the highly energetic light reactions in photosynthesis and the abundant supply of oxygen makes chloroplasts a rich source of ROS (Allen, 1995). Furthermore mitochondria, endoplasmic reticulum, peroxisomes, and the cell wall are potential sites of ROS production (McKersie and Leshem, 1994; Smirnoff, 1998). Generation of ROS is exacerbated by water deficit stress (Smirnoff, 1993; 1998). ROS damage membranes (through peroxidation), proteins (through amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electrical charge and increased susceptibility to proteolysis) and DNA (through deletions, mutations and other lethal genetic effects associated with base degradation, single strand break and cross-linking to proteins) (McKersie and Leshem, 1994). Prevention of the generation of ROS through genetic engineering is one approach for improving resistance to water deficit stress in plants.

Plants have built in mechanisms to detoxify reactive oxygen species. These include enzymatic and non-enzymatic methods (Bohnert and Sheveleva, 1998; Smirnoff, 1998). Enzymes involved in scavenging ROS are superoxide dismutase (SOD), catalases, peroxidases and glutathione reductase (GR). SOD and catalase/peroxidases work in concert to remove O_2^- and H_2O_2.

$$2O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

$$2H_2O_2 \xrightarrow{\text{catalase}} H_2O + O_2$$

$$H_2O_2 + 2 \text{ ascorbate} \xrightarrow{\text{peroxidase}} 2H_2O + 2 \text{ monodehydroascorbate}$$

GR catalyzes the reduction of GSSG (oxidized form of glutathione) to GSH (reduced form), an important antioxidant in biological reactions (Creisson *et al.*, 1992). There are three isoforms of SOD depending upon their metallic cofactors: FeSOD (chloroplast), MnSOD (mitochondria) and Cu/ZnSOD (cytosol and chloroplast) (Holmberg and Bülow, 1998). Catalase is located in peroxisomes whereas ascorbate peroxidase is a chloroplast enzyme.

The feasibility of enhancing ROS scavenging capacity through genetic engineering has been demonstrated in different plants. The MnSOD isolated from *Nicotiana plumbaginifolia* has been introduced into the mitochondria and chloroplasts of alfalfa (McKersie *et al.*, 1996). Yield and survival of transgenic plants were significantly improved under field conditions. Targeting the *Arabidopsis* FeSOD into the chloroplasts of tobacco protects both the plasmalemma and photosystem II against methyl viologen-induced oxidative damage (van Camp et al., 1996). Introduction of multiple genes that interfere with production of ROS at different steps of the pathway gives greater protection. Tobacco plants transformed with GR and Cu/ZnSOD showed less damage to oxidative stress than the wild type or those transformed with GR or Cu/ZnSOD alone (Aono *et al.*, 1995). Roxas *et al.* (1997) transformed tobacco plants with glutathione S-transferase (GST) and glutathione peroxidase (GPX). Compared to the wild type, plants expressing GST/GPX showed faster growth during chilling and salt stress.

Engineering for accumulation of LEA proteins. Late embryogenesis abundant (LEA) proteins accumulate during the maturation phase of embryos and in vegetative tissues exposed to desiccation, osmotic stress, low temperature and external ABA. LEA proteins have biased amino acid composition (contain high percentage of glycine but lack cysteine and tryptophan), which results in their hydrophilic property (Ingram and Bartels, 1996; Garay-Arroyo *et al.*, 2000). They are localized in the cytoplasm, nucleus and protein storage vacuoles (Dure, 1993a; Martilla *et al.*, 1996). Although the exact function of LEA proteins remains to be elucidated, they are thought to protect desiccating tissues by binding water, sequestering ions and stabilizing protein and membrane structures (Bray, 1994). An enzymatic function for LEA proteins is unlikely because of their wide range of sizes, flexible structure predicted from sequence data and high concentrations in the cell (Campbell and Close, 1997). Regarding their secondary and tertiary structures, several of them seem to exist principally as random coils (which is thought to be responsible for their proposed water-binding property), whereas others exist as an amphiphilic α-helix (Dure, 1993a). Amphiphilic (also synonymously called amphipathic) molecules contain both polar and nonpolar regions.

Several LEA proteins have been identified in different plants (Ingram and Bartels, 1996; Close, 1997) and based on sequence similarity they are divided into at least five major groups (Bray, 1994; Bray *et al.*, 2000). The Group 1 LEA proteins are predicted to have water-binding capacity, the Group 2 and Group 4

LEA proteins maintain protein and membrane structures, and the Group 3 and 5 LEA proteins sequester ions that concentrate during cellular dehydration.

The best-characterized Group 1 LEA is the Em protein of wheat. It is 70% random coil and is expressed in germinating seedlings in response to ABA and in mature leaves during dehydration (Swire-Clark and Marcotte, 1999). It has a 20-mer conserved amino acid sequence. Group 2 LEA proteins (variously known as dehydrins, Rab or the D-11 protein family) have a chaperone-like function to preserve protein folding and assembly during stress. They also function in binding water and being hydrophilic may interact with other proteins and stabilize them at low cytoplasmic water content. Dehydrins have a conserved lysine-rich block, known as the K segment (KIKEKLPG), at their C-terminal and at least once internally. The K segment is predicted to form an amphiphilic α-helix. The consensus 15 amino acid sequences for angiosperm K segment is EEKKGIMDKIKELPG. In addition, a second conserved domain with a phosphorylatable tract of serine residues known as the S segment (SSSSSSS), and an N-terminal consensus called the Y segment ([T/V]DEYGNP) are present (Close, 1997). Group 3 LEA proteins (D-7 family) also have an 11-mer amino acid motif with the consensus sequence TAQAAKEKAGE repeated as many as 13 times (Dure, 1993b). This motif is predicted to form an amphiphilic α-helix. The hydrophobic face is important in forming a homodimer and the outside charged face might be involved in sequestering ions. The cDNA clone MA20005 isolated from wheat belongs to this group (Curry *et al.*, 1991). Group 4 LEA proteins (D-113 family) have a conserved amino acid sequence in their N-terminal, which

forms α-helix. The helix is believed to substitute water in order to preserve membrane structure. They have little conservation in their C-termini although the random coil structure is conserved. Group 5 (D-29 family) also have an 11-mer repeat in which each amino acid in the motif has similar predicted chemical properties to Group 3 LEA. Unlike Group 3, however, a high degree of residue specificity is lacking at each position (Bray, 1994).

Evidence is emerging that LEA-like proteins with hydrophilic amino acids are also widespread in bacteria and fungi. Examples include proteins encoded by *HSP12* and *GRE1* from *Saccharomyces cerevisiae* (Garay-Arroyo and Covarrubias, 1999; Mtwisha *et al.*, 1998) and the GSiB protein from *Bacillus subtilis* (Stacy and Aalen, 1998). Moreover, transcripts of these genes are known to accumulate in response to water deficit stress (Stacy and Aalen, 1998; Garay-Arroyo and Covarrubias, 1999). Garay-Arroyo *et al.* (2000) have suggested the term hydrophilins to encompass LEA proteins and other proteins with strong hydrophilicity index (>1.0) and higher glycine content (>6%) in their amino acid sequence. Using computer algorithms the authors found 5 hydrophilins in *E. coli* 5 and 12 hydrophilins in *S. cerevisiae* and the genes encoding these proteins are induced by water deficit stress (Garay-Arroyo *et al.* 2000).

Because of the number of important protective roles they play, LEA proteins are potential targets for improving resistance to water deficit stress through genetic engineering. Three of these classes of proteins have already been shown to enhance tolerance to water deficit stress in engineered plants and yeast. Rice plants transformed with HVA1 (a group 3 LEA protein from barley)

exhibited constitutive high expression of HVA1 protein ranging from 0.3 to 2.5 % of total protein in the leaf and 0.3% to 1.0% in the root. Over-expression of HVA1 improves drought and salinity tolerance of T_1 progenies as indicated by delayed wilting, dying of old leaves and necrosis of young leaves. On removal of stress, transgenic plants showed better recovery than did the control plants. However, fairly high expression levels (about 0.1% of total soluble protein) were needed before tolerant phenotypes could be observed (Xu *et al.*, 1996).

When the LEA25 protein (a group 4 LEA protein from tomato) was expressed in yeast cells, growth of transformed cells was improved at 1.2M NaCl compared to the control cells (Imai *et al.*, 1996). While controls showed a long lag phase of 40 hours before growth commenced, LEA25-expressing cells showed a shorter lag phase of 10 hours. Moreover, transformants had an increased survival rate after freezing at –20°C. Additional biochemical evidence for the osmoprotective role of LEA proteins comes from a recent study by Swire-Clark and Marcotte (1999). The group 1 LEA, Em protein was overexpressed in yeast cells and the growth performance of transformed cells was evaluated. Enhanced growth was observed in the presence of a variety of osmoticaly active substances (NaCl, KCl and sorbitol) suggesting that Em protein is involved in the mitigation of osmotic stress.

Engineering transcription factors. Expression of regulatory genes that control adaptive responses to water deficit stress is another approach to improve resistance through genetic engineering. This will allow expression of a number of genes at a time when they are needed thus giving more resistance. There are at

least four proposed signal transduction pathways in the induction of water deficit-responsive genes. Two are ABA-dependent and the remaining two are ABA-independent (Shinozaki and Yamaguchi-Shinozaki, 1997). The cis-acting promoter elements in one of the ABA-dependent and one of the ABA-independent pathways have been characterized. The ABA-dependent cis-acting element contains an ABA-responsive element (ABRE) and the ABA-independent element contains the dehydration-responsive element (DRE) (Shinozaki and Yamaguchi-Shinozaki, 1997). A single stress-inducible promoter may contain both ABRE and DRE, as is the case in the *rd29A* promoter of *Arabidopsis*.

Expression of transgenes encoding stress-responsive transcription factors might enable the expression of entire metabolic pathways leading to the accumulation of osmolytes, ROS scavenging molecules and LEA proteins in a developmentally and timely controlled manner (Nuccio *et al.*, 1999). The feasibility of this approach has been shown recently by two independent studies (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Kasuga *et al.*, 1999). A cDNA clone that encodes a DRE-binding factor CBF1 has been isolated from *Arabidopsis thaliana* (Stockinger *et al.*, 1997). CBF1 regulates transcription in response to low temperature and water deficit stress. *Arabidopsis* plants overexpressing CBF1 under the control of the 35S CaMV promoter accumulated CBF1 transcripts at higher concentration than the wild type. CBF1 over-expression induced expression of cold-regulated genes (*Cor6.6, Cor15 and Cor78*) without acclimation to low temperature resulting in increased freezing tolerance.

Two other DRE-binding proteins DREB1 (DREB1A, DREB1B and DREB1C) and DREB2 (DREB2A and DREB2B) have been isolated from *Arabidopsis thaliana* (Liu *et al.*, 1998; Nakashima *et al.*, 2000). Both of these transcription factors activate expression of the *rd29A* gene but under different conditions. Expression of DREB1 is strongly induced by low temperature stress, whereas that of DREB2 is induced by dehydration. Over-expression of DREB1A and DREB2A under the control of the 35S promoter in transgenic *Arabidopsis* induced expression of the target gene *(rd29A)* during freezing and dehydration. However, use of the strong constitutive 35S promoter also resulted in severe growth retardation even under normal growth conditions (Liu *et al.*, 1998). To avoid this problem, Kasuga *et al.* (1999) introduced DREB1A under the control of the stress-inducible *rd29A* promoter. They detected over-expression of six DREB1A-responsive genes (*rd29A, Kin1, Kin2, rd17 and Erd10*), which contain DRE, in both 35S:DREB1A and rd29A:DREB1A transgenic plants. In 35S:DREB1A plants, the proteins were constitutively overproduced under control conditions compared to the *rd29A:*DREB1A plants, which overproduced only in response to stress (Kasuga *et al.*, 1999).

Engineering signaling components. All plants have a genetic makeup for stress responses and these responses are coordinately regulated following the recognition of stress. In this scenario, signal transduction pathway intermediates could be over-expressed to enhance stress response. Post-translational modification of Ser, Thr and Tyr residues by protein kinases and phosphatases is a major transduction route for many signals (Trewavas and Malho, 1997).

Various stresses, including water deficit, are known to influence protein phosphorylation (Sopory and Munshi, 1998). In plants a water deficit stress-related phosphorylation cascade, analogous to HOG pathway for osmotic stress signaling in yeast, is believed to be involved (Nelson *et al.,* 1998). Yupasins *et al.* (1994) found a marked effect of osmotic stress on protein kinase and phosphatase activities in alfalfa seedlings. Stress increased the activities of kinases, whereas phosphatases were inhibited. Anderberg and Walker-Simmons (1992), while studying the effects of exogenous factors on ABA-responsive serine-threonine-type protein kinase (PKABA1) from wheat, found that dehydration, low temperature and salt stress up-regulates PKABA1. In another study, two *Arabidopsis* cDNA clones (ATCDPK1 and ATCDPK2) that code for Ca^{2+}-dependent, calmodulin-independent protein kinases (CDPKs), were rapidly induced by drought and salt stress (Urao *et al.*, 1994). Over-expression of these and other water deficit stress-inducible protein kinases could lead to activation of specific transcription factors leading to improved tolerance.

CDPKs are the most prevalent serine, threonine protein kinases in higher plants. They are induced by drought, cold and ABA. To study their effect on stress signaling, separate chimeric constructs (HVA1:LUC, 35S:ATCDPK1, and 35S:ATCDPK1a) were introduced into maize protoplasts (Sheen, 1996). It was found that constitutive expression of ATCDPK1 and ATCDPK1a increased LUC expression by inducing the HVA1 promoter. HVA1-LUC expression activated by ABA was repressed by the constitutive expression of protein phosphatase 2C (PP2C), which is capable of abolishing kinase-induced responses through

dephosphorylation (Sheen, 1996). Recently Saijo *et al.* (2000) over-expressed a rice CDPK, OsCDPK7, in transgenic rice plants. They found that OsCDPK7 is inducible by salt stress and resistance to stress correlated with the level of OsCDPK7 expression. Over-expression of OsCDPK7 also induced genes responsive to salinity and drought but not genes responsive to cold stress, suggesting that downstream pathways for resistance to salt/drought and cold stress are different. These results suggest that manipulation of protein kinases and phosphatases of the water deficit stress signal transduction pathway can be exploited to improve resistance. At the present, however, our knowledge of the stress signal transduction pathway in plants is incomplete. Further research is needed to dissect the interaction of components of the signal transduction pathway before any major improvement in resistance to stress is achieved through genetic engineering.

Objectives of the Study

Wheat is the leading crop in the world both in terms of the area planted and production. In 1998 about 225 million ha of wheat was planted worldwide with a production of 591 billion metric tons (http://apps.fao.org/cgi-bin/nph-db.pl?subset=agriculture). In the USA, wheat is the second most important cereal next to corn. In the 1999 fiscal year, 25.6 million ha of land was planted and 63.1 billion metric tons of wheat produced. Wheat is the leading crop in Oklahoma with approximately 2.6 million ha planted in 1999 and 4.1 million metric tones harvested with a production value of over $353 million (NASS, 2000). It is the

state's number one agricultural export commodity. Wheat is also used as fall and winter pasture for cattle, a $2.1 billion a year industry in the state. In 1999 Oklahoma ranked second in the United States for winter wheat production and fourth for wheat production regardless of market class (NASS, 2000).

Despite its importance as a leading crop in the world and in the United States, production of wheat remains far below its genetic potential. It is estimated that the genetic potential of wheat is 14.5 metric tons ha^{-1} but the actual yield achieved is only 1.9 metric tons ha^{-1} (13%). The largest proportion (81%) of the loss is caused by abiotic factors (Boyer, 1982; Kramer and Boyer, 1995; Bray *et al.*, 2000). Water deficit is the major abiotic factor that limits crop production (Boyer, 1982) and most of yield loss is attributable to this stress. In Oklahoma, water deficit causes about 60% of yield loss (Arron Guenzi, personal communication). Although there is a wide variation among the response of genotypes (Saulescu *et al.*, 1995; Moustafa *et al.*, 1996), wheat is very susceptible to water deficit stress. Water deficit occurring during the reproductive stage has the most detrimental effect on wheat productivity. Stress during the early vegetative stage also results in decreased tillering that leads to fewer heads per plant and reduced yield.

Wheat breeding programs have made remarkable improvements in productivity through increased harvest index and resistance to numerous diseases and insect pests. However, efforts to increase yield under water deficit conditions have been limited. With the importance of wheat production in the U.S.A. and in Oklahoma's economy in particular, it is essential that available

technologies be explored to improve productivity. As part of a scheme to improve resistance of wheat to water deficit stress, we have transformed wheat with the *mtlD* gene of *E. coli* for accumulation of the osmolyte mannitol. Our objectives were:

1. to determine if mannitol accumulation affects growth of wheat, and
2. to evaluate if mannitol protects wheat from water deficit stress (due to water stress and salinity).

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CHAPTER II

INTEGRATION AND EXPRESSION OF THE *E. COLI* *MTLD* GENE IN TRANSGENIC WHEAT

ABSTRACT

We have transformed wheat (*Triticum aestivum* L. cv. Bobwhite) with the *mtlD* gene of *E. coli* for accumulation of mannitol. *MtlD* encodes for mannitol-1-phosphate dehydrogenase (MTLD), which catalyses the reversible conversion of fructose-6-phosphate (F6P) to mannitol-1-phosphate (M1P). M1P is converted to mannitol via non-specific phosphatases. Mannitol accumulation was targeted either to the cytosol or the plastid. For accumulation in the plastids the transit peptide (TP) sequence of the small subunit of Rubisco was used. A total of 50 plants were recovered with a transformation frequency of 0.4%. Integration and expression of the *mtlD* gene was confirmed by Southern, PCR and enzyme assays. In transformants expressing mannitol in the cytosol, mannitol content reached 0.3 to 2.0 μmoles gfw^{-1} in calli and 0.4 to 1.6 μmoles gfw^{-1} in plants. Although enzyme assays showed an active MTLD, no detectable levels of mannitol accumulated in plastids. Half of the transgenic plants recovered were infertile, dwarfed and had twisted leaves and heads. Abnormal phenotypes correlated with increased mannitol concentration in the flag leaves (>1.0 μmoles gfw^{-1}). Increased mannitol content also reduced the amount of sucrose in source leaves. We hypothesize that stunted growth and infertility of transgenic plants were caused by reduced sucrose supply to apical meristem and developing floral organs.

INTRODUCTION

Wheat is the most important staple crop both in the United States and the world. As a leading crop it can benefit from the introduction of foreign genes that improve grain quality and resistance to biotic and abiotic stresses. However, until the early 1990s, wheat transformation was impossible due to the difficulties in regeneration of plants from tissue culture and the inefficiency of *Agrobacterium* infection. The use of immature embryos as explants (Machii *et al.*, 1998) and the discovery of indirect DNA delivery techniques for plants recalcitrant to *Agrobacterium* infection (Sanford *et al.*, 1987) have now made it possible to transform wheat. The first success in wheat transformation was reported in 1992 using embryogenic cell lines (Vasil, 1992). Since then several published works have reported the production of transgenic wheat using the particle bombardment technique (Weeks *et al.*, 1993; Becker *et al.*, 1994; Altpeter *et al.*, 1996; Takumi and Shimada, 1997). The major limitation with the biolistic technique is that the frequency of transformation is very low, ranging between 0.1 to 2.5% (Altpeter, 1996). Optimizing the tissue culture system can push the upper limit to 8% (Chen *et al.*, 1998), but reproducibility remains problematic mainly due to tissue damage during the bombardment process. To overcome this problem, advances are being made to use aggressive *Agrobacterium* strains to transform wheat (Cheng *et al.*, 1997; Peters *et al.*, 2000). So far the frequency of *Agrobacterium* transformation of wheat is not greater than the conventional biolistic method. One important advantage of *Agrobacterium*-mediated

transformation is the incorporation of single copy transgenes (Cheng *et al.*, 1997), which can reduce silencing.

With refinements in the transformation techniques, attention has now diverted to the manipulation of wheat with economically important genes. The first success in this regard has been the introduction of genes for herbicide resistance as demonstrated in their use as selectable markers in wheat transformation (Vasil, 1992; Weeks et al., 1993). Improvement of grain quality is an area where genetic engineering approaches have potential economic impact. Wheat dough is extensible and strong and can trap gas bubbles during leavening to form light porous structures when processed into food. Dough elasticity is determined primarily by the type and amount of glutenin proteins in the seed. High-molecular-weight glutenin subunit (HMW-GS) genes have been introduced into wheat by different groups in order to improve bread-making quality by modifying the content and type of glutenins (Blechl and Anderson, 1996; Altpeter *et al.*, 1996; Rooke *et al.*, 1999).

Disease and insect resistance is another major trait targeted to improve wheat yield through genetic engineering. Clausen *et al.* (2000) transformed wheat with a cDNA encoding the antifungal protein KP4 from a virus that infects *Ustilago maydis,* the causative agent of stinking smut. The transgene was inherited over several generations and conferred resistance against *Ustilago maydis*. Chen *et al.* (1999) also reported a delay in scab infestation (*Fusarium graminearum*) in transgenic wheat constitutively expressing a rice thaumatin-like protein gene (*tlp*). Wheat seeds accumulating the barley trypsin inhibitor CMe

(BTI-CMe) to 1.1% of total protein significantly impaired survival of Angoumois grain moth (*Sitotroga cerealella;* Altpeter *et al.*, 1999).

In an effort to enhance resistance to water deficit stress we have transformed wheat with the *mtlD* gene of *E. coli* for accumulation of the osmolyte mannitol. Mannitol is the most widely distributed sugar alcohol known to be involved in protection of plants from water deficit stress (Stoop *et al.*, 1996). The *mtlD* gene of *E. coli* (Davis *et al.*, 1988) has been widely used to engineer mannitol accumulation in model organisms and has resulted in improved growth under saline conditions compared to the wild types (Tarczynski *et al.*, 1993; Thomas *et al.*, 1995; Chaturvedi *et al.*, 1997). The open reading frame of the *E. coli mtlD* gene is 1.2 kb in size and encodes for the enzyme mannitol-1-phosphate dehydrogenase (MTLD) with 382 amino acids and a deduced molecular weight of 41 kDa (Novotny *et al.*, 1984; Jiang *et al.*, 1990). *MtlD* is one of the three key components of the mannitol (*mtl*) operon of *E. coli* involved in the catabolism of mannitol. The *mtl* operon consists of an operator-promoter region (*mtlOP*) and two structural genes, *mtlA* and *mtlD*. *MtlA* encodes for the mannitol-specific enzyme II phosphotransferase system (mannitol permease; Jiang *et al.*, 1990). The first step in the metabolism of mannitol is its phosphorylation to mannitol-1-phosphate (M1P). This process is coupled to the transmembrane transport catalyzed by mannitol permease. The cytoplasmic M1P is oxidized to fructose-6-phosphate (F6P) by MTLD in presence of the co-factor NAD^+. MTLD also catalyzes the reverse reaction from F6P to M1P in presence of a reducing agent NADH.

Several stress-responsive genes have been isolated from various organisms. The functions of some of these genes have been demonstrated using model plants (Yoshiba *et al.*, 1995; Pilon-Smiths *et al.*, 1995; Holmström *et al.*, 1996). It is logical to assume that a biological molecule shown to confer resistance in model plants might also function in the same way in crop plants. However, it may also result in certain abnormalities that are detrimental to metabolic processes in crop plants. Recent evidence indicates that certain genes for osmolyte accumulation do result in abnormal growth patterns in transgenic plants (Romero *et al.*, 1997; Sheveleva *et al.*, 2000). Therefore, it is necessary to test how growth and physiological processes in crops respond to ectopic expression of osmoprotectants. This will help us identify appropriate genes for improving crop stress resistance through genetic engineering. Our objective in this study was to determine if expression of mannitol has a negative effect on the growth of wheat.

MATERIALS and METHODS

Gene Constructs

In this study, transcription was driven by the maize ubiquitin-1 (*ubi-1*) promoter. The *ubi-1* promoter is a constitutive promoter, which is also inducible by various environmental stresses (Christensen *et al.*, 1992; Clausen *et al.*, 2000). Plasmids pAHC17 and pAHC20 (Christensen and Quail, 1996) obtained from Dr. Peter Quail (Plant Gene Expression Center, University of California, Albany, CA), plasmid pCAB-MTLD obtained from Dr. John C. Cushman (Department of Biochemistry, University of Nevada, Reno), and plasmid pJIT117 (Guerineau *et al.*, 1988) received from Dr. Phillip Mullineaux (John Inns Center, UK) were used to construct expression cassettes (Fig. 2.1). Plasmid pAHC17 contains the *ubi-1* promoter, 5' untranslated region, *ubi-1* intron, *BamH*I and *Hind*III cloning sites and a nopaline synthase (*nos*) transcription termination sequence. Plasmid pAHC20 contains a selectable marker *bar (*De Block *et al.*, 1987) inserted into the *BamH*I site of pAHC17. Plasmids pCAB-MTLD and pJIT117 contain the *mtlD* gene and a transit peptide (TP) sequence of the pea ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small sub-unit gene, respectively.

Expression cassettes for the synthesis of mannitol in the cytosol. The open reading frame of the *mtlD* gene (1.2 kb) was amplified by PCR from pCAB-MTLD using primers #2512 (5' CGC**GGATCC**ACTATGAAAGCATTAC3') and #2513 (5'CGC**GGATCC**TTATTGCATTGCTTTA3') containing a *BamH*I site (indicated in

boldface). PCR was performed in a 25 µl reaction volume containing 1X Thermopol buffer (pH 8.8), 200 µM of dNTPs (dATP, dGTP, dCTP, dTTP), 0.4 µM of primers, 3 mM $MgSO_4$, 50 ng pCAB-MTLD DNA and 1 U Deep Vent (exo^-) DNA polymerase (New England Biolabs, Inc., Beverly, MA). The mixture was overlaid with mineral oil (Sigma, St. Louis, MO), denatured at 95°C for 1 min and subjected to 25 cycles of denaturation (94°C for 1 min), annealing (55°C for 45 sec) and extension (72°C for 1 min) steps in a PTC-100 thermal cycler (MJ Research, Inc., Waltham, MA). Then a final extension was done for 5 min at 72°C. The PCR amplified fragment was separated in 1.2% agarose gel and purified using the QIAquick gel extraction kit (Qiagen Inc., Chatsworth, CA). The *MtlD* fragment was digested with *BamH*I and ligated into the *BamH*I site of pAHC17, which was *BamH*I digested and dephosphorylated to avoid re-circularization (Sambrook et al., 1989). The resulting plasmid was designated as pTA1 (Fig. 2.2). Since pTA1 lacks a selectable marker it was co-transformed with pAHC20 (Fig. 2.1), which contains the *bar* selectable marker.

To increase the chances of incorporating the *mtlD* gene into the wheat genome, the *ubi-bar-nos* region of pAHC20 was amplified using primers #2879 (5'GGC**AAGCTT**GCTATGACCATGATTACGA3') containing a *Hind*III site (indicated in boldface) and the universal primer 5'GTAAAACGACGGCCAGT3' (designated as #2880). The PCR conditions described for *mtlD* above were used except that annealing was done for 1 min and extension for 3 min. After separation in 1.2% agarose gel and purifying in Qiaquick gel extraction kit, the DNA was digested with *Hind*III and cloned into the *Hind*III site of pTA1 to create

pTA2 (Fig. 2.2). Plasmid pTA2 was independently used for transformation experiments.

Expression cassettes for the synthesis of mannitol in plastids. For accumulation of mannitol in plastids, the open reading frame of *mtlD* was amplified from pCAB-mtlD using primers #4497 (5CGC**CTGCAG**ATACTATG AAAGCATTACA3') containing a *Pst*I site and #2513 (5'CGC**GGATCC**TTATTGC ATTGCTTTA3') containing a *BamH*I site under similar conditions as for pTA1 above. The amplified fragment was digested with *Pst*I and *BamH*I and ligated downstream of the coding region of the transit peptide (TP) sequence of the small subunit of pea Rubisco in pJIT117 (Fig 2.1), which was *Pst*I and *BamH*I digested and dephosphorylated. The resulting plasmid was designated as pTA3 (Fig. 2.2). The TP-*mtlD* fragment from pTA3 was PCR amplified using primers #4460 (5'CGC**GGATCC**AGAAGTGAGAAAAAT3') and #2513 as before, digested with *BamH*I and then cloned into the *BamH*I site of pAHC17 (Fig. 2.1.) to obtain pTA4 (Fig. 2.2). Plasmid pTA4 lacks a selectable marker and was co-transformed with pAHC20. Then the *ubi-bar-nos* sequence from pAHC20 was PCR amplified as in pTA2 and ligated to the *Hind*III site of pTA4 to create pTA5 with a *bar* selectable marker (Fig. 2.2). Plasmid pTA5 was used for transformation independently.

Verification of Correct Orientation and Sequencing of Inserts

All expression cassettes were multiplied in *E. coli* DH5α (Life Technologies, Rockville, MD). Orientation of inserts was verified by restriction

digestion of plasmid DNA prepared using the Qiagen plasmid mini-prep kit (Qiagen, Inc., Chatsworth, CA) and comparing the size of the resulting fragments. For *mtlD*, digestion was performed using *Hind*III and *BstE*II and digested DNA was separated in 1.5% agarose or 5% polyacrylamide gel. Then fragments were evaluated for similarity with the expected 3.16 kb and 2.73 kb fragments for pTA1 and pTA2 or 3.36 kb and 2.73 kb for pTA4 and pTA5 plasmids, respectively. To determine the integrity of inserts, the 5' and 3' ends of inserts were sequenced using the Taq Dideoxy Terminator Cycle Sequencing System (Applied Biosystems, Foster City, CA) available at the DNA/Protein Resources Facility, Department of Biochemistry and Molecular Biology, Oklahoma State University.

Microprojectile Bombardment

Immature kernels were collected 10-12 days post anthesis from wheat plants (*Triticum aestivum* L. cv Bobwhite) grown in the greenhouse or growth chamber depending on time of year. Kernels were surface sterilized in 70% ethanol for 5 min followed by 15 min in 20% (v/v) clorox (1.2%, w/v, sodium hypochlorite). After three washes in sterile distilled water, immature embryos were isolated aseptically from kernels. Forty to 50 embryos (0.5-1.0 mm in length) were placed (scutellum-side up) on callus induction medium (CIM) consisting of MS salts (Murashige and Skoog, 1962); 0.5 mg l^{-1} thiamine-HCl; 2 mg l^{-1} 2,4-D; 150 mg l^{-1} L-asparagine monohydrate, and 20 g l^{-1} sucrose as described by Weeks (1995). The medium was solidified with 2.5 g l^{-1} Phytagel

(Sigma, St. Louis, MO) and the pH adjusted to 5.85 with 1 M KOH prior to autoclaving. Calli cultures were incubated at 21°C under low light (43 $\mu molm^{-2}s^{-1}$ PAR).

After 4-5 days of culture, embryos were placed in a 2 cm diameter circle in the center of a 15x60 mm petri dish containing an osmoticum (0.4 M mannitol) in CIM. After 5 h of osmotic pretreatment, embryos were bombarded with gold particles (1 µm in diameter) coated with appropriate plasmid DNA using the helium-driven PDS-1000/He Biolistic Particle Delivery System (Bio-Rad, Hercules, CA) as described by Weeks (1995). Briefly, plasmid DNA was isolated from overnight *E. coli* DH5α cultures using the Qiagen plasmid purification kit (Qiagen, Chatsworth, CA) according to the manufacturer's instruction. A bombardment mixture was prepared by mixing 25 µl of DNA (1 µg DNA μl^{-1} TE) and 15 mg of ethanol-washed gold particles. To this mixture, 220 µl of sterile water, 250 µl of 2.5 M $CaCl_2$ and 50 µl spermidine (free base) were added in that order while vortexing after each addition. The mixture was shaken with a Tomy mixer (Tomy Tech USA, Inc., Palo Alto, CA) for at least 15 min at 4°C and centrifuged at 10,000xg for 5 min to pellet the DNA-gold particles. The particles were washed in 400 µl ethanol by pipetting up and down. After centrifugation for 5 min the pellet was resuspended in 20 µl ethanol. Five µl of the suspension (6.25 µg DNA) was pipetted on to macrocarrier discs for bombardment. The bombardment procedure was optimized using plasmid pAHC25 (Christensen and Quail, 1996; Fig. 2.1) and assaying for GUS expression. The distance between

the stopping screen and the target was maintained at 6.5 cm and 1,100-psi rupture discs were used.

Selection and Regeneration of Putative Transformants

Selection for bialaphos resistance was initiated 16 h after bombardment. Embryos were subcultured on callus selection medium (CSM) containing CIM supplemented with 1 mg l^{-1} bialaphos (Duchefa Biochemie BV, Haarlem, The Netherlands). Calli were subcultured at two-week intervals onto fresh CSM.

On the fifth transfer, proliferating calli were transferred to a shoot initiation medium (SIM) containing CIM (without 2,4-D) plus 0.5 mg l^{-1} dicamba (Sigma, St. Louis, MO) and 1 mg l^{-1} bialaphos. Calli were kept under fluorescent light (120 µmoles $m^{-2}s^{-1}$ PAR) for 16 h and transferred to fresh SIM every two weeks until shoots develop. Plantlets (>3 cm) were transferred to culture tubes with 15 ml of a root initiation medium (RIM) composed of half-strength CIM (without hormones) and 1.5–3.0 mg l^{-1} bialaphos. When enough root mass developed, plantlets were transferred to pots filled with Metro-mix 366 soil mixture (Scott-Sierra Horticultural Products, Co., Marysville, OH). Pots were covered with plastic bags for few days to prevent desiccation. Plants were grown to maturity in a greenhouse or growth chamber at 22±2/18°C day/night temperatures and a 16 h photoperiod with 220 µmoles $m^{-2}s^{-1}$ PAR.

Analysis of Transgene Integration

DNA isolation. Genomic DNA was isolated using a mini-prep (for PCR) or

large-scale (Southern) procedure. For mini-prep isolation, a modified procedure of Haymes (1996) was followed and DNA was isolated from about 200 mg callus tissue or 3 cm segments of leaf tissue. The leaf tissue was chopped into 2 mm wide pieces using separate razor blades to avoid cross contamination. The samples were placed in sterile 2 ml tubes containing glass beads. Then 250 µl CTAB extraction buffer (containing 100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2% CTAB, and 0.4% ß-mercaptoethanol) was added and homogenized in a Mini-Beadbeater™ (Biospec Products, Bartlesville, OK) set at 5000 rpm for 20 sec. The homogenate was incubated at 65°C for 15 to 30 min. A hole was made in the bottom of the tube using a 21-gauge needle and placed on top of a 5 ml tube and centrifuged for 10 sec at 3000xg. The extract was transferred to a fresh 1.5 ml tube using a sterile Pasteur pipette. Two-hundred µl chloroform:isoamyl alcohol (24:1) was added and centrifuged at 10,000xg for 2 min at 4°C. One volume of isopropanol was added to the supernatant, mixed gently and centrifuged for 10 min at 10,000xg. The DNA pellet was resuspended in 100 µl sterile water containing 1 mg ml^{-1} RNase A. After 1 h incubation at 37°C, 50 µl of 4 M NaCl and 300 µl ethanol were added. The DNA was precipitated at -20°C overnight and pelleted by centrifugation as before. The pellet was washed with 70% ethanol and dried in a speed-vac. The dried samples were redissolved in 20-40 µl of 10 mM Tris-HCl, pH 8.0.

For large-scale isolation, 2 to 4 g of callus or leaf tissue was frozen in liquid nitrogen and ground to a fine powder with pestle and mortar. Powdered tissue was transferred to 15 ml of pre-heated (65°C) extraction buffer (50 mM

Tris-HCl, pH 8.0; 20 mM EDTA, pH 8.0; 0.5M NaCl; 1% SDS) in 50 ml polypropylene tubes. The samples were incubated for 20 min with gentle shaking. Then an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and mixed by gentle inversion of the tube. The mixture was centrifuged at 3,000xg for 10 min at 4°C. The aqueous phase was transferred to a new tube using sterile disposable transfer pipettes and an equal volume of chloroform:isoamyl alcohol (24:1) was added. After gentle mixing the sample was centrifuged for 10 min as before. The aqueous layer was transferred to a new tube and DNA precipitated by adding 0.6 volumes of isopropanol. The DNA was spooled and washed with 1 ml 70% ethanol to remove excess salts. The pellet was resuspended in 500 µl 10 mM TE, pH 8.0 containing 50 µg ml^{-1} RNase A and incubated at 37°C for 1 h. DNA was precipitated with 2 volumes of ethanol and washed with 1 ml 70% ethanol. The DNA was dried in a speed-vac and dissolved in 200-400 µl of 10 mM Tris-HCl, pH 8.0. The concentration and purity of DNA was determined by measuring absorbance at 260 and 280 nm.

Polymerase chain reaction (PCR) analysis. For PCR analysis of transgenic calli and plants, genomic DNA was isolated using the mini-prep procedure of Haymes (1996). PCR was performed in a 20 µl reaction volume using primers specific for *mtlD* (#2512, 5'CGCGGATCCACTATGAAAGCATTAC3' and #3870, 5'GCCAAATGTTTTGAACGATCTGC3') and *bar* (#5606, 5'CATCGAGACAAGC ACGGTCAACTTC3' and #5607, 5'CTCTTGAAGCCCTGTGCCTCCAG3'). The size of amplified fragments is 1.2 kb for *mtlD* and 450 bp for *bar*. The PCR reaction contained 1.5 mM $MgCl_2$, 1X polymerase buffer A, 200 µM of each

dNTP, 0.4 μM of each primer, 0.6 μg DNA, and 1 U Taq DNA polymerase (Promega, Madison, WI). After an initial denaturation of 2 min at 95°C, the reaction was subjected to 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. A final extension was done at 72°C for 5 min. PCR for *bar* was performed in the same way except that annealing was done at 60°C for 30 sec and extension at 72°C for 1 min.

Southern blotting. Genomic DNA was digested with *Pst*I (Life Technologies, Rockville, MD) at 37°C overnight. Five μg of digested DNA was loaded into 0.8% agarose gel and electrophoresed for 3 h in 1X TBE at 80 volts. The DNA was depurinated by incubating the gel in 0.25 M HCl for 15-20 min and neutralized in 0.4 M NaOH. DNA was transferred to HybondN$^+$ nylon filters (Amersham Life Science, Arlington Heights, IL) by downward alkaline (0.4 M NaOH) blotting for 2 h using Turboblotter^{TM} (Schleicher & Schuel, Keene, NH). The filters were briefly rinsed for 20 min in 2X SSC to dissolve any agarose. Then the filters were pre-hybridized overnight in 50 ml of 5X SSPE, 5X Denhardt, 0.5% SDS and denatured salmon sperm DNA (100 μg ml^{-1}) at 65°C. One hundred ng of labeled *mtlD* or *bar* probe (isolated from *Pst*I digested pTA2 plasmids) were mixed in 50 ml fresh pre-hybridization buffer and the filters hybridized at 65°C for 16 h. The probes were labeled with $\alpha[^{32}P]$-dCTP by random priming method using the RediPrime II random prime labeling system and purified using ProbQuant G-50 columns (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) according to the manufacturer's instruction. The filters were sequentially washed in 50 ml each of 2X wash buffer (2X SSPE, 0.1% SDS), 1X wash buffer (1X SSPE, 0.1% SDS)

and 0.5X wash buffer (0.5X SSPE, 0.1% SDS). In each case, washing was performed for 30 min at 65°C. Finally the filters were rinsed in 0.1X SSC, wrapped in Saran wrap and autoradiographed for 1-3 days at -80°C.

Analysis of Transgene Expression

β-Glucuronidase (GUS) assay. To optimize the transformation procedure, embryos bombarded with pAHC25 were assayed for GUS expression 48 h after bombardment (Jefferson, 1987). Embryos were incubated overnight in darkness at 37°C in a buffer consisting of 1.0 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc), 0.1 M sodium phosphate buffer (pH 7.0), 10 mM EDTA, 0.5 mM $K_4Fe(CN)_6$, 0.5 mM $K_3Fe(CN)_6$ and 0.1% triton X-100. The tissue was cleared in 70% ethanol and blue spots counted under a microscope.

Phosphinothricin acetyl transferase (PAT) assay. The phosphinothricin acetyltransferase (PAT) activity of the *bar* gene was assayed in crude protein extracts of calli and leaves according to a modified protocol of Spencer *et al.* (1990). One hundred mg of tissue was ground in liquid nitrogen with a pestle and mortar and transferred to 1.5 ml tubes. Then 100 µl of ice-cold extraction buffer was added and homogenized. The extraction buffer contained 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.1 mg l^{-1} PMSF, 0.01 mg l^{-1} leupeptin, 0.3 mg l^{-1} DTT and 2% (w/v) polyvinylpyrolidone (40K). The homogenate was centrifuged at 14,000xg for 10 min and the supernatant transferred to fresh tubes. Total protein content was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). In a final 25 µl reaction volume 20 µg protein, 1 µl of 60 mCi $mmol^{-1}$

[^{14}C]acetyl CoA (Amersham Life Science, Arlington, IL) and 4 µl of 1 mM phosphinothricin (PPT; Sigma, St. Louis, MO) were added and the mixture incubated at 37°C for 1 h. Six µl of the reaction mixture was spotted onto cellulose thin layer chromatography plates (Whatman International Ltd, Maidstone, England). The plates were developed in a 3:2 ratio (v/v) of 1-propanol and NH_4OH (28% NH_3) and exposed to X-ray films. Transgenic Caucasian blue stem plants containing the *bar* gene served as a positive control.

Mannitol-1-phosphate dehydrogenase (MTLD) assay. Mannitol-1-phosphate dehydrogenase (MTLD) was assayed using native polyacrylamide gel electrophoresis (PAGE). One to 2 g of leaf and callus tissue were ground in liquid nitrogen with a pestle and mortar. The ground tissue was transferred to 15 ml tubes containing 1.5 volumes of the extraction buffer (described above for PAT assay). The homogenate was centrifuged at 5,000xg for 5 min at 4°C and the aqueous phase transferred to a fresh tube. Samples were then separated using nondenaturing polyacrylamide gel electrophoresis (PAGE) as described by Bollag and Edelstein (1991). Briefly, 1 mm thick gels consisting of 10% separating and 5% stacking gel were prepared in a Bio-Rad Miniprotean II apparatus (Bio-Rad, Richmond, CA). For two separating gels 3.33 ml of 30% acrylamide/bisacrylamide, 2.5 ml of 1.5 M Tris-HCl (pH 8.0), 50 µl of 10% ammonium persulfate (APS), 5 µl TEMED, and 4.1 ml water were carefully mixed so as not to create air bubbles. The stacking gels were composed of 0.67 ml of 30% acrylamide/bisacrylamide, 1 ml of 0.5 M Tris-HCl (pH 6.8), 30 µl of 10% APS, 5 µl TEMED, and 2.3 ml water for two gels. The gels were pre-run in 0.375

M Tris-HCl, pH 8.3 for 2 h. Prior to sample loading the buffer was replaced with a running buffer containing 25 mM Tris and 190 mM glycine, pH 8.5. Two hundred μg protein samples were mixed with 5X sample loading buffer (4.6 mM Tris, pH 8.0; 200 mM EDTA, pH 8.0; 0.125%, w/v bromophenol and 40%, v/v glycerol) and electrophoresed for 4 h at 80 volts. The gels were carefully removed from the casting plates and incubated for 5 min in a staining buffer containing 100 mM Tris-HCl, pH 8.0; 1 mM β-NAD^+; 0.075 mM phenazinemethosulfate, and 0.5 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). After 5 min, 1 mM M1P was added and incubated further for 15 min or until dark blue bands appeared (Novotny *et al.*, 1984). The staining solution was removed and gels washed in water and fixed in 50% ethanol. Protein extracted from *E. coli* DH5α served as a positive control.

Extraction and determination of carbohydrates. To determine the concentration of mannitol and soluble carbohydrates, the method developed by Adams *et al.* (1993) was followed. One hundred to 300 mg callus and leaf tissue was quickly frozen in liquid nitrogen. The frozen tissue was ground to a fine powder with mortar and pestle and transferred to 2 ml tubes. Two volumes (200-600 μl) of ice-cold ethanol/chloroform/water (12:5:3) was added and mixed by vortexing. Two volumes of ice-cold water were added, vortexed and centrifuged (14,000xg) for 2 min. The upper ethanol/water phase was transferred to a new tube. The pellet was re-extracted once in 2 volumes of water at 60°C for at least 30 min. This second extract was combined to the first and dried in a speed-vac. The pellet was resuspended in 300 μl water and passed through a C_{18} solid-

phase extraction column (Alltech Associates, Inc., Deerfield, IL) to remove hydrophobic substances. The columns were washed with 700 ml purified water (using 0.2 μm cellulose acetate filters). Lactose was used as an internal standard to correct for losses during extraction.

Carbohydrates were separated using a high-performance anion exchange chromatography (HPAE) system coupled to a pulsed amperometric detector (PAD). Fifty μl samples were injected into a 200 μl sample loop connected to a 9x250 mm Carbopac PA1 column (Dionex, Sunnyvale, CA) maintained at room temperature. Samples were separated isocratically at a flow rate of 2.0 ml min^{-1} with degassed 150 mM NaOH as a mobile phase. Peak areas and retention times were determined using an integrator. Authentic carbohydrates (inositol, sorbitol, mannitol, glucose, fructose and sucrose) purchased from Sigma (St. Louis, MO) were used as standards.

Growth Measurements

To determine the effect of mannitol accumulation on growth plant height, length of the flag leaf and number of tillers were recorded in T_0 plants at anthesis. Height of plants was measured from the crown. Length of the flag leaves was measured from the ligule. Also number of seeds $plant^{-1}$ was counted in matured plants.

RESULTS

Bombardment, Selection and Regeneration of Transformants

Fig. 2.3 shows the steps in the regeneration of putative transgenic wheat plants. Plasmid pAHC25 (Christensen and Quail, 1996), which contains the GUS gene, was used to optimize the bombardment procedure and to check the DNA/gold coating protocol. During bombardment experiments, a tube containing a coating mixture with pAHC25 was run alongside the other plasmids. Forty-eight h after bombardment, calli were assayed for GUS expression (Fig. 2.3e).

Selection of calli was initiated 16 h after bombardment in callus selection medium (CSM) that contained 1 mg l^{-1} bialaphos. Calli were maintained in CSM for 4 transfers of two weeks each. At the end of the fourth transfer period, calli were screened visually for any sign of increase in size. At this stage transgenic calli were very distinct from non-transgenic ones in their appearance and size. Transgenic calli were friable, light yellowish in color and bigger in size. Non-transgenic calli were watery in appearance and had reduced growth or died altogether (Fig. 2.3f). At the end of the fourth transfer period, putative transgenic calli identified by their vigorous growth on CSM were transferred to a shoot regeneration medium (SIM) containing 1 mg l^{-1} bialaphos. Calli were maintained in SIM until shoots were developed with transfers to fresh media at two-week intervals (Fig. 2.3g). Shoot regeneration took about 1.5 months (Table 2.1).

When shoots were 3 cm, they were transferred to tubes with a rooting medium (RIM) supplemented with 1.5-3.0 mg l^{-1} bialaphos. Shoots were kept in

RIM until they had well developed roots. This selection was enough to eliminate non-transformed shoots that escaped the shoot regeneration step. Non-transgenic shoots became necrotic and died (Fig. 2.3h). Most plantlets were able to develop enough roots in 5 weeks and were transferred to soil. It took ca. 141 days from embryo excision to transfer of putative transgenic plants to soil and ca. 67 days from soil transfer to maturity (Table 2.1).

Table 2.2 shows summary of results for the transformation experiments. A total of 57 bialaphos-resistant calli were obtained. Except a few calli that did not produce shoots, most calli regenerated at least one plant and 50 plants were recovered with a transformation frequency of 0.4%. However, a significant portion (50%) of these plants were infertile.

PCR and Southern Analysis of Transgene Integration

PCR and Southern analysis were performed on calli and T_0 plants to confirm integration of *bar* and *mtlD* genes. Both analyses showed that *bar* and *mtlD* were incorporated into the wheat genome (Figs. 2. 4 and 2.5). Copy number reconstructions, indicated that the number of copies for both genes varied from 5 to more than 10 copies per haploid wheat genome. PCR and Southern analyses also demonstrated inheritance of *bar* and *mtlD* into the T_2 generation (Figs. 2.6 and 2.7). However, *mtlD* was not detected in all plants. All fertile plants recovered from co-transformation experiments using pTA1 or pTA4 (Table 2.2) were positive for *bar*, but did not contain *mtlD* (data not shown).

Transgene Expression in Transformants

Expression of the *bar* gene. Expression of the *bar* gene was determined by assaying for its phosphinothricin acetyl transferase (PAT) activity in calli and plants. PAT activity in calli was determined in tissues maintained in shoot regeneration medium (SIM). In T_0 and T_2 plants, activity was determined using portions of the flag leaf. PAT activity was detected in calli as well as T_0 and T_2 plants suggesting that the *bar* gene was active at the tissue and whole plant level and was inherited into the T_2 progeny (Figs. 2.8 and 2.9).

Analysis of *mtlD* expression. Expression of the *mtlD* gene in transgenic wheat was evaluated using an enzyme assay specific for mannitol-1-phosphate dehydrogenase (MTLD) and by determining the mannitol content in calli, T_0 and T_2 plants. For transformants that expressed MTLD in plastids, chloroplasts were isolated from leaf protoplasts using Percoll density gradient centrifugation (Power and Davey, 1990). Assays using nondenaturing PAGE showed that MTLD that corresponds to the activity in *E. coli* was functional in calli or plants transformed with *mtlD*, but not in wild type Bobwhite or those transformed with pAHC20 alone (Fig.2.10). HPAE profiles for carbohydrates from *mtlD* positive calli and plants showed typical distribution of soluble carbohydrates found in *mtlD* negative plants, but also contained one additional peak with a retention time of about 3.4 min. This peak corresponded to the peak of authentic mannitol (Fig. 2.11). The amount of mannitol in calli and plants that were positive for *mtlD* was variable. In calli it ranged from 0.3 to 2.0 μmoles gfw^{-1} (data not shown). Table 2.4 shows the

concentration of mannitol and soluble carbohydrates in the flag leaf of selected T_0 plants that expressed mannitol in the cytosol. Mannitol content in these plants varied from 0.4 to 1.6 μmoles gfw^{-1}. Unlike the observation in other similar studies (Thomas *et al.*, 1995), increased accumulation of mannitol resulted in reduced concentration of soluble carbohydrates. Glucose content varied from 3.37 to 5.05 μmoles gfw^{-1}, fructose from 3.82 to 4.83 μmoles gfw^{-1} and sucrose from 1.94 to 3.07 μmoles gfw^{-1}.

Data for calli and plants transformed with pTA5 and expected to accumulate mannitol in the plastids were unclear. As shown in Figs. 2.5 and 2.7 the *mtlD* gene was integrated into the genome and the MTLD enzyme was functional (Fig. 2.10), but did not result in accumulation of detectable levels of mannitol. This discrepancy could be explained if the substrate F6P is limiting and the pathway leading to sucrose synthesis is favored over mannitol synthesis. However, this possibility seems less likely, at least in calli, where there was plenty of sucrose in the medium. Because of the lack of mannitol accumulation in plastids, calli and plants transformed with pTA5 were excluded from water deficit stress studies (Chapter 3).

Effect of Mannitol Accumulation on Growth

The effect of mannitol accumulation on growth was evaluated using T_0 plants. Many T_0 plants were infertile, stunted in growth, and had twisted leaves and heads (Tables 2.2 and 2.3; Fig. 2.12). These phenotypes were not observed in tissue culture-derived wild type Bobwhite plants. Therefore, the effects are

likely due to transgene activity. However, all the dwarf T_0 plants were infertile and it was impossible to evaluate their progeny for these abnormalities. Moreover, seed set was negatively correlated with mannitol content (Table 2.4). Thus, infertile plants had higher mannitol content when compared to fertile plants.

DISCUSSION

Experiments described here were designed to produce transgenic wheat plants that accumulate mannitol and to evaluate if mannitol had a negative effect on growth. These transgenic plants were evaluated to determine if mannitol enhances resistance to water and salt stress as well (Chapter 3). Integration and expression of the *mtlD* gene was verified using PCR, Southern, enzyme assays and quantification of mannitol content using HPAE-PAD (Figs 2.5, 2.7, 2.10, 2.11 and Table 2.4). Determination of the enzyme activity of mannitol-1-phosphate dehydrogenase (MTLD) in plants transformed with *mtlD* is the first to be reported (Fig. 2.10). Accumulation of mannitol in transgenic tobacco, *Arabidopsis* and yeast expressing the *E. coli mtlD* gene has been reported previously (Tarczynski *et al.*, 1993; Thomas *et al.*, 1995; Chaturvedi *et al.*, 1997), but *mtlD* expression was determined indirectly from measurements of mannitol content.

Accumulation of mannitol in plants transformed with *mtlD* is a two-step process. The conversion of F6P to M1P is catalyzed by MTLD. An endogenous enzyme is required to remove the phosphate group from M1P for synthesis of mannitol to occur. Like tobacco and *Arabidopsis*, wheat does not naturally accumulate mannitol and is unlikely to have a specific enzyme to catalyze this step. Accumulation of mannitol in transgenic wheat indicates that nonspecific enzymes were involved in this process. Tarczynski *et al.* (1993) and Thomas *et al.* (1995) proposed that nonspecific phosphatases could hydrolyze M1P to mannitol, but no direct evidence has been reported so far. To investigate if nonspecific phosphatases in wheat can hydrolyze M1P, a simple enzyme assay

for acid and alkaline phosphatases was performed. The standard procedure for detecting acid and alkaline phosphatase activity utilizes p-nitrophenylphosphate (pNPP) as a substrate under acidic and alkaline pH, respectively. In our case the assay was performed by substituting M1P for pNPP. Mannitol produced from the hydrolysis of M1P was determined by HPAE-PAD. Fig 2.13 shows that both phosphatases can effectively hydrolyze M1P resulting in a peak that corresponds to the elution time for authentic mannitol. The mannitol peak was not detectable in the control reaction without a protein extract. Because of the negatively charged phosphate group, which strongly binds to the positively charged particles of the CarboPac PA1 column, M1P was not eluted for 40 min under the conditions used to analyze mannitol and other carbohydrates (data not shown). Based on this information the pathway for the conversion of F6P to mannitol in transgenic wheat can be depicted as shown in Fig 2.14.

Transformation of wheat with *mtlD* gene has resulted in severe abnormalities in regenerated plants. About 50% of the T_0 plants were infertile (Table 2.2). The infertile plants were dwarf and had twisted heads and leaves (Fig. 2.12). Abnormal phenotypes are common in tissue culture-derived plants, especially with lengthy tissue cultures that allow mutations to accumulate and greater chances of abnormal phenotypes to develop. Regeneration in our study was very slow (106 days) compared to that reported by Altpeter *et al.* (1996) and Chen *et al.* (1998) who obtained regenerated plants in 60-70 days following culture initiation using the same wheat cultivar. Composition of the media used in our study was different from these studies and could have resulted in longer

regeneration time (Table 2.1) and limited infertility is expected. However, the regeneration time found in our study was similar to that of Weeks *et al.* (1993), who used the same cultivar and media except that in our case root initiation took 35 days compared to the 14 days obtained by these authors. Weeks *et al.* (1993) also obtained infertile plants, but much fewer than in our study. The similarity in regeneration time between our study and that of Weeks *et al.* (1993) suggests that the exceptionally greater infertility and abnormal growth patterns of transgenic plants in our study is unlikely to be explained by differences in the duration of the regeneration time. Furthermore, because of its polyploid nature wheat is particularly tolerant to chromosomal aberrations occurring in tissue culture. Combined, these results suggest that infertility and other pleiotropic effects obtained in transgenic plants were caused by accumulation of mannitol. As shown in Table 2.4 deleterious pleiotropic effects were correlated with increasing mannitol content. Those plants accumulating higher levels of mannitol were severely affected.

Reduced growth and other pleiotropic effects have also been reported in related studies on transgenic plants engineered for ectopic osmolyte accumulation. Karakas *et al.* (1997) reported slower growth of tobacco plants transformed with *mtlD*. Romero *et al.* (1997) found abnormalities including stunted growth, reduced glucose and sucrose content, loss of apical dominance and infertility in 40% of transgenic tobacco plants accumulating up to 0.17 mg gfw^{-1} trehalose. These phenotypes were inherited in the T_2 generation. A recent study by Sheveleva *et al.* (2000) found that tobacco plants derived from a cross

between a line expressing MTLD in chloroplasts and a line expressing myo-inositol-O-methyltransferase (IMT1) in the cytosol had infertility problems. Hybrids expressing both enzymes accumulated mannitol and D-ononitol in amounts comparable to those expressing only single genes and had normal phenotypes until the flowering stage. In contrast to the wild type, hybrid plants expressing both MTLD and IMT1 had curled and smaller upper leaves, elongated stems during flowering and incomplete floral development leading to abortion. The concentration of mannitol and D-ononitol increased in youngest leaves close to the vegetative meristem and in flowers, whereas that of soluble sugars and potassium was very low. The authors ascribed this phenomenon to decreased sink demand leading to reduced sugar sensing and reduced carbon import into the reproductive organs.

We did not measure mannitol content in individual leaves of T_0 plants due to sample limitation. However, we found that the amount of mannitol measured in the flag leaves was different between the fertile and infertile lines. Fertile lines either did not accumulate mannitol at all or the amount accumulated was very small (Table 2.4). Contrary to the results of Sheveleva *et al.* (2000) in tobacco, we did not obtain fertile plants with high mannitol content that were normal in height or taller than wild type plants. All plants with high mannitol content were infertile, dwarfed, and had twisted leaves and heads. Furthermore, increased mannitol content resulted in the reduction of soluble carbohydrates, mainly sucrose. This observation suggests that in infertile and dwarf plants the amount of sucrose to be translocated out of the source leaves into the growing sink

tissue (apical meristem and developing flowers) was very small resulting in metabolic disturbance that ultimately resulted in infertile plants with stunted growth and other abnormal phenotypes.

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Table 2.1. Time required for wheat transformation.

Steps in procedure	Time (days)
Embryo excision and culture	4
↓	
Osmotic treatment and bombardment	2
↓	
Callus selection	60
↓	
Shoot regeneration	40
↓	
Root initiation	35
↓	
Acclimation in soil	7
↓	
Maturity	60
Total	208 days (7 months)

Table 2.2. Summary of transformation results. The numbers given correspond to calli or plants that thrived in media containing bialaphos and were positive for bar as determined by PCR, Southern or PAT assay.

Plasmid	Embryos bombarded	Transgenic calli obtained		Transgenic plants recovered				
		Number	Designation*	Designation†	Fertile plants (%)	Infertile plants (%)	Lost to insects	Total (freq)
pTA1	3,413	15	C1-1 to C1-15	P1-1-1 to P1-15-1	4 (45.5%)	5 (27.3%)	2	11 (0.3%)
pTA2	5,083	20	C2-1 to C2-20	P2-1-1 to P2-20-2	7 (30.4%)	13 (56.5%)	2	23 (0.5%)
pTA4	2,412	9	C4-1 to C4-6	P4-1-1 to P4-6-1	3 (50.0%)	2 (33.3%)	1	6 (0.3%)
pTA5	2,849	13	C5-1 to C5-10	P5-1-1 to P5-10-2	4 (50.0%)	5 (50.0%)	2	10 (0.4%)
Total	13,757	57			18 (36.0%)	25 (50.0%)	7 (14.0%)	50 (0.4%)

* C1, C2, C4 and C5, are calli transformed with plasmids pTA1, pTA2, pTA4 and pTA5, respectively. Numbers following hyphens indicate the serial number for calli transformed with respective plasmids.

†P1, P2, P4, and P5 represent plants transformed with plasmids pTA1, pTA2, pTA4 and pTA5, respectively. Numbers following the first hyphen correspond to the callus serial number and numbers that follow designate the plant number regenerated from the same callus line.

Table 2.3. Plant height (cm), flag leaf length (cm), number of tillers and seed setting ability of selected T_0 and wild type Bobwhite plants derived from tissue culture. Data are means ±SE of 3 to 6 plants.

Plasmid	Fertile plants				Infertile plants		
	Plant height	Flag leaf	No. tillers	Seeds plant^{-1}	Plant height	Flag leaf	No. tillers
None (Wild type)	62.0±2.8	31.0±0.9	2.5±0.3	31.0±2.0	N/A	N/A	N/A
pTA1	55.3±2.4	30.8±0.5	2.5±0.3	32.0±3.0	22.5±2.7	13.0±1.1	2.3±0.3
pTA2	54.3±3.0	29.0±1.5	3.0±0.4	13.0±3.0	17.2±1.9	11.3±1.3	2.5±0.2
pTA4	50.3±3.8	29.0±0.6	3.0±0.0	33.0±5.0	21.0±4.0	11.5±1.5	2.5±0.5
pTA5	56.5±1.8	28.8±1.1	3.5±0.5	33.0±4.0	25.5±3.1	13.8±0.5	2.8±0.3

Table 2.4. Mannitol and carbohydrate content (μmoles gfw^{-1}) of selected T_0 plants. Codes for plants are as shown in Table 2.2. Data are means for flag leaves from two main tillers of the same plant.

Carbohydrate	Fertile plants			Infertile plants	
	-mtlD	P2-15-1	P2-19-1	P1-15-1	P2-16-1
Inositol	0.75	1.07	0.79	0.77	0.82
Mannitol	0.00	0.42	0.72	1.51	1.64
Glucose	5.11	4.38	5.05	3.65	3.37
Fructose	5.50	4.83	4.52	3.82	3.98
Sucrose	4.50	3.07	3.04	2.20	1.94

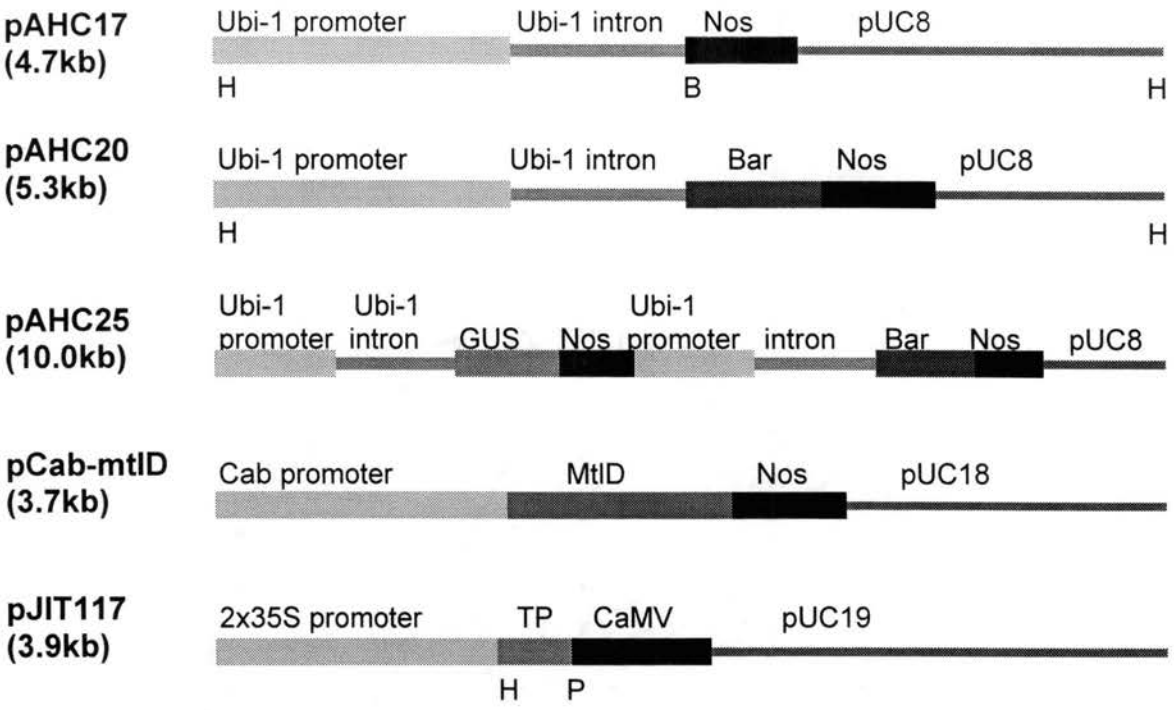


Fig. 2.1. Plasmids used for constructing expression cassettes (pAHC17, pCab-mtlD and pJIT117), for co-transformation (pAHC20) and for optimizing the transformation procedure (pAHC25). B, *BamHI*; H, *HindIII*; P, *PstI*. Fragments not drawn to scale.

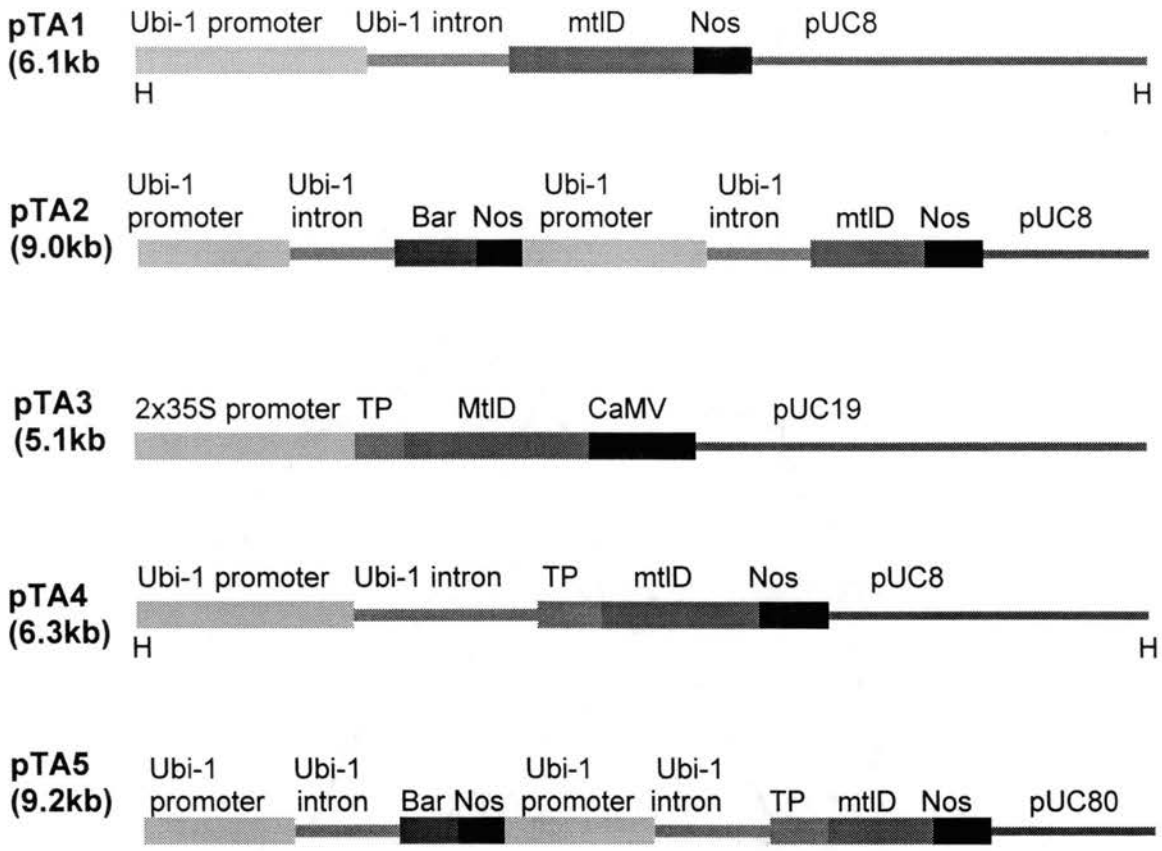
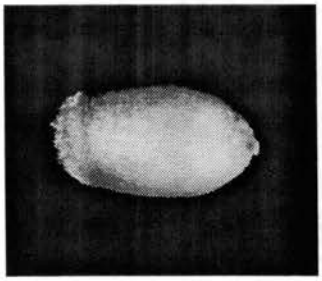


Fig. 2.2. Gene constructs prepared for wheat transformation. Plasmids pTA1 and pTA2 were used for accumulation of mannitol in the cytosol. Plasmids pTA4 and pTA5 were used for accumulation in plastids. Plasmid pTA3 was used to amplify the TP-mtlD fragment in order to create pTA4 and pTA5. H, *HindIII*. Fragments not drawn to scale.

a



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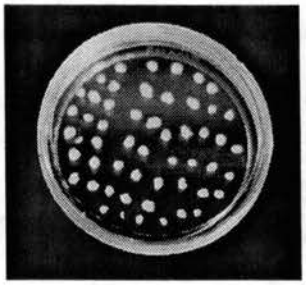

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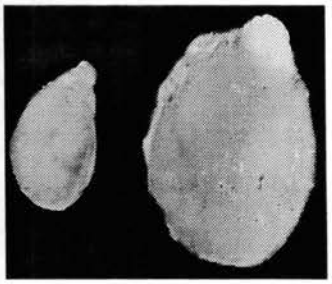

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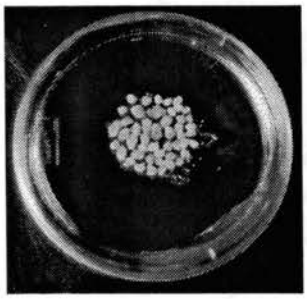

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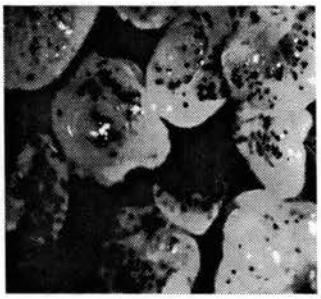

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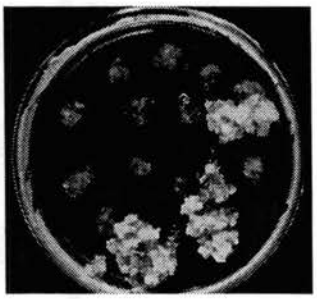

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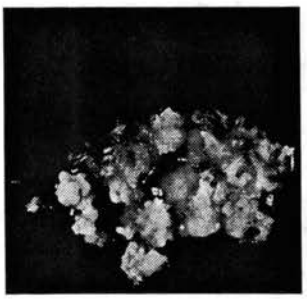

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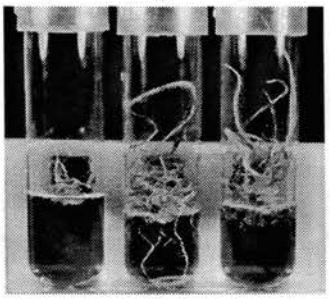

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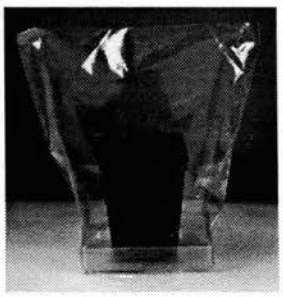

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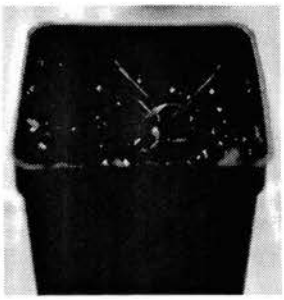

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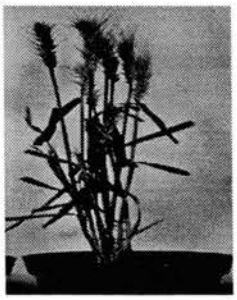

Fig. 2.3. Wheat tissue culture and regeneration of transformants. a) immature wheat kernel used for excision of embryo; b) immature embryos plated on CIM after excision; c) immature embryos after excision (i) and after 4 days on CIM (ii); d) embryos arranged in a circle on CIM supplemented with 0.4 M mannitol for osmotic pretreatment; e) transient GUS expression in embryos bombarded with pAHC25; f) proliferation of transgenic calli in CIM supplemented with 1 mg l^{-1} bialaphos at the end of the fourth transfer; g) regeneration of shoots from transgenic callus on SIM supplemented with 1 mg l^{-1} bialaphos; h) regeneration of roots from shoots, (i) nontransgenic plant, (ii) and (iii) transgenic plants; i) a transgenic plant shortly after transfer to soil covered with a polyethylene bag to prevent dehydration; j) a transgenic plant 10 days after transfer to soil; k) a mature transgenic plant

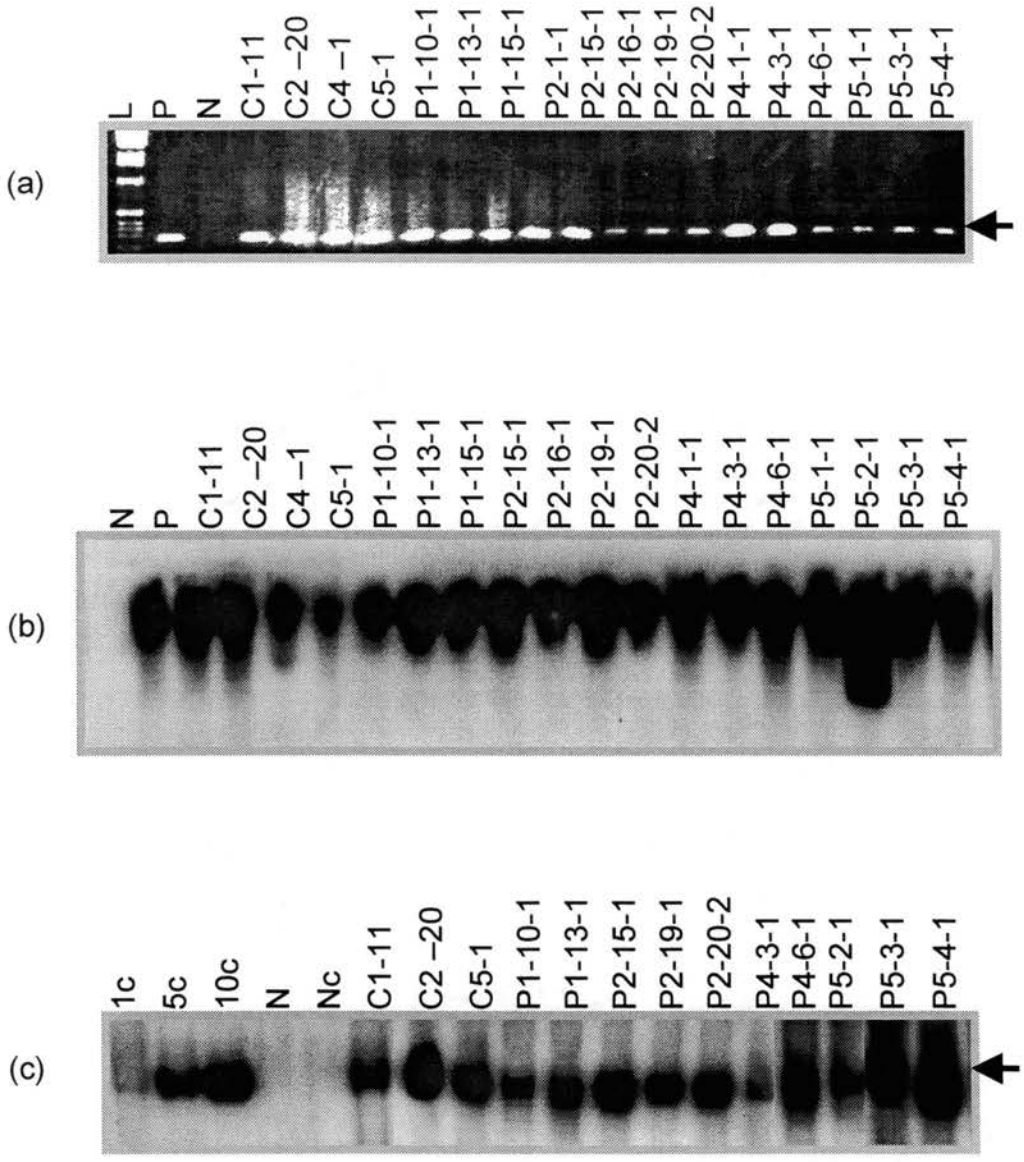

Fig. 2.4. Analysis of integration of the *bar* gene into the wheat genome using T_0 transformants. (a) PCR of the 450 bp *bar* fragment; (b) undigested genomic DNA hybridized with 0.6 kb ^{32}P-labeled *bar* probe; (c) DNA digested with *Pst*I and hybridized with ^{32}P-labeled *bar* probe. Positive band is indicated by an arrow. L, 1 kb DNA ladder; N, negative control (wild type plant); P, positive control (pTA2 plasmid); C1, C2, C4 and C5 are calli transformed with plasmids pTA1, pTA2, pTA4 and pTA5 and P1, P2, P4 and P5 are plants regenerated from the respective calli; 1c, 5c and 10c represent 1, 5 and 10 copies of the transgene, respectively. Designations of transgenic calli and plants are as shown in Table 2.2.

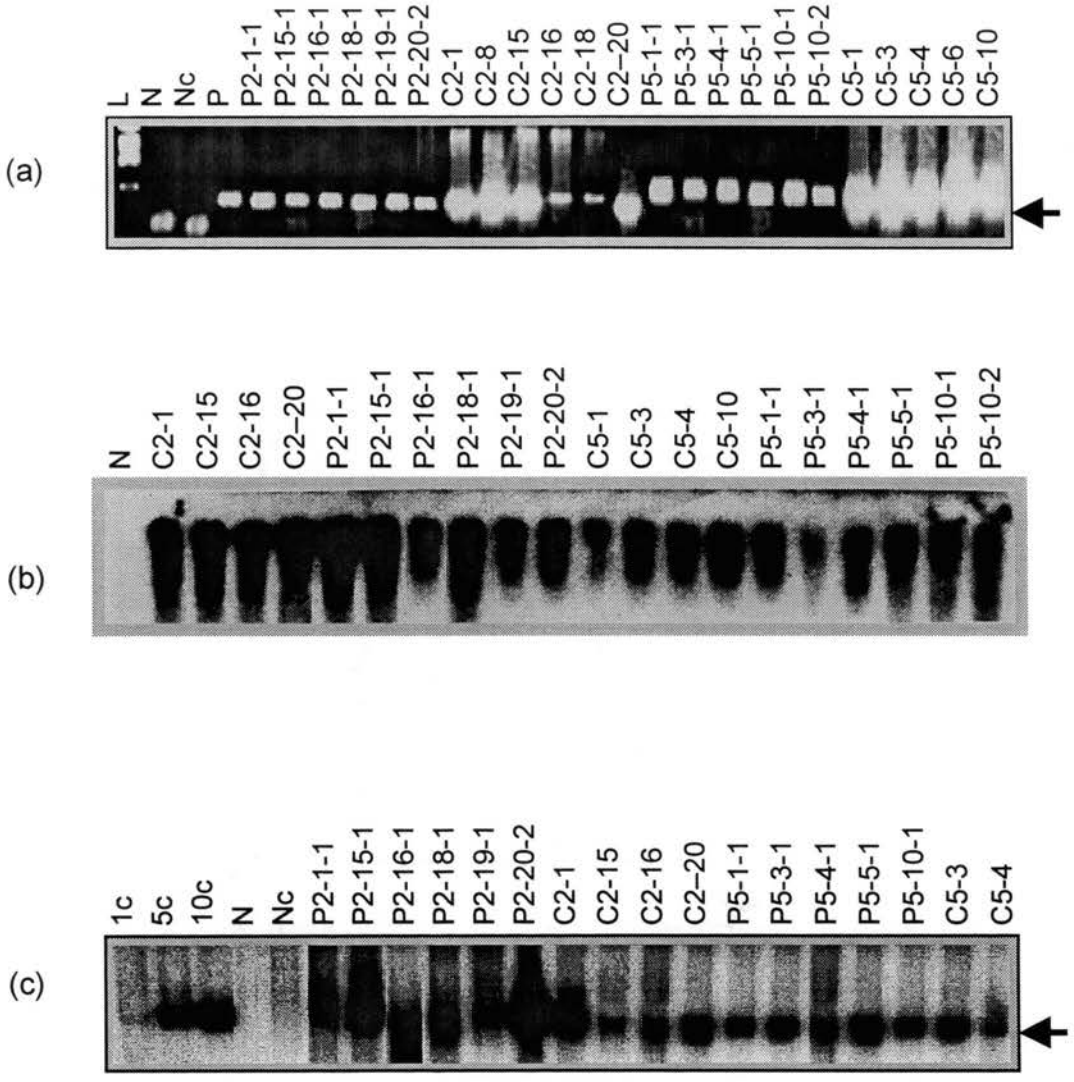

Fig. 2. 5. Integration of the *mtlD* gene in calli and T_0 plants transformed with plasmids pTA2 and pTA5. (a) PCR of the 1.2 kb *mtlD* gene; (b) genomic DNA hybridized with ^{32}P-labeled *mtlD* probe, and (c) DNA digested with *Pst*I and hybridized with ^{32}P-labeled *mtlD* probe. The expected 1.2 kb fragment is indicated by an arrow. L, 1 kb DNA ladder; N, negative plant (wild type plant); Nc, negative callus (wild type); P, positive control (pTA2 plasmid); 1c, 5c and 10c represent 1, 5 and copies of the transgene, respectively. Designation of calli and plants is as shown in Table 2.2.

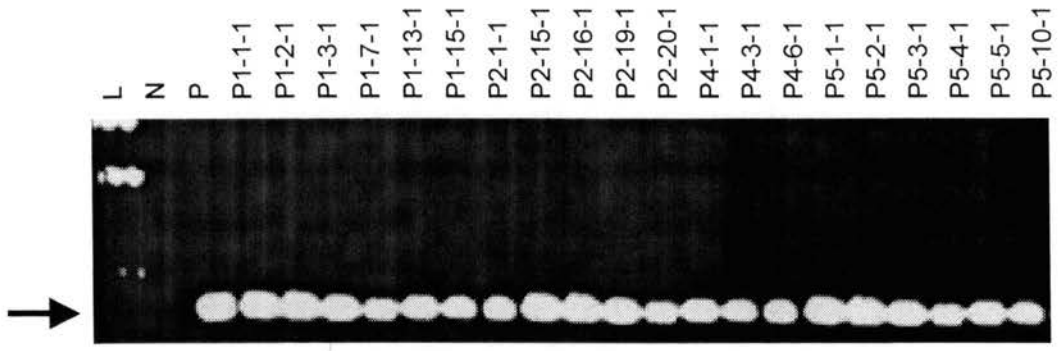

Fig. 2.6. PCR analysis of the *bar* gene in T_2 progenies. L, 1kb ladder; N, negative control (wild type plant); P, positive control (pTA2 plasmid); P1, P2, P4, P5 represent plants transformed with plasmids pTA1, pTA2, pTA4 and pTA5 respectively. Arrow indicates the positive band. Designation of transgenic lines is as shown in Table 2.2.

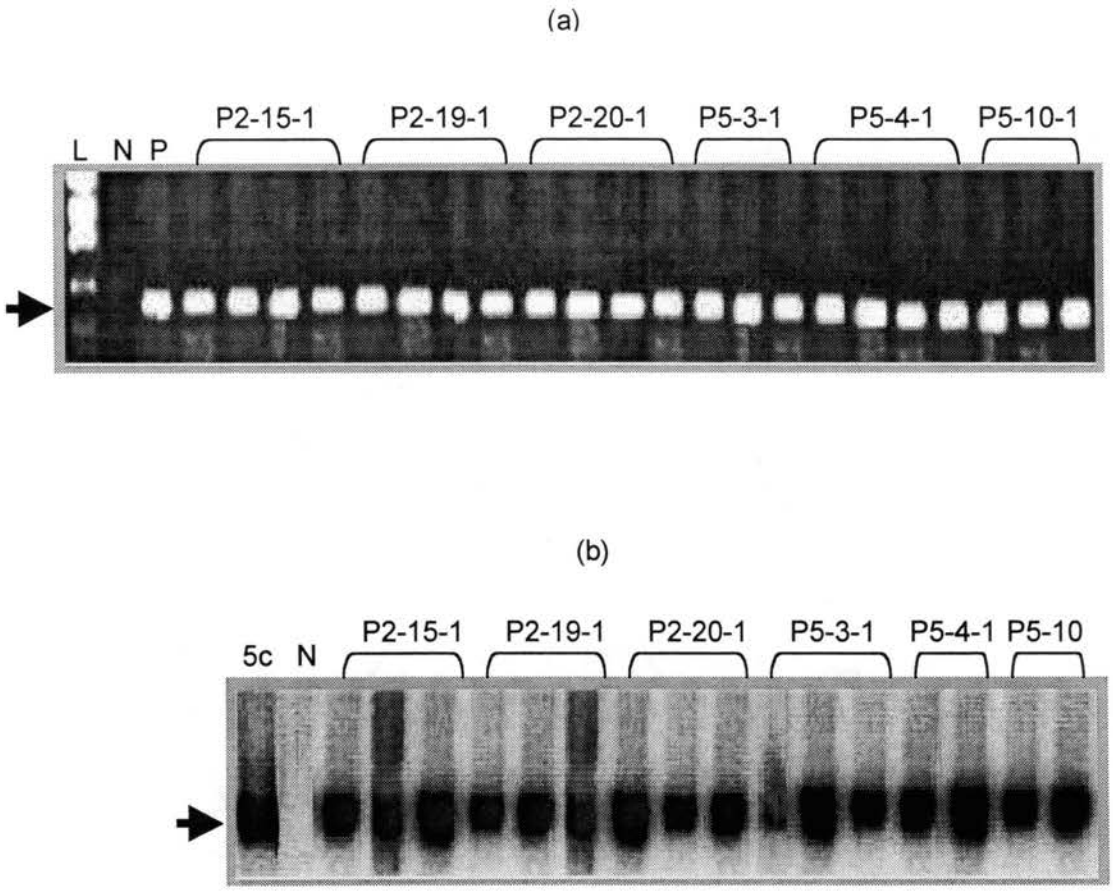

Fig. 2.7. Inheritance of *mtlD* intoT_2 progenies. Individual T_2 plants from lines Plants transformed with plasmids pTA2 and pTA5 were used for PCR (a) and Southern analysis (b). For Southern, genomic DNA was digested with *Pst*I and probed with ^{32}P-labeled *mtlD* probe. The positive 1.2 kb band is shown by an arrow. L, 1 kb DNA ladder; N, negative control (wild type plant); P, positive control (pTA2 plasmid); 5c, represents 5 copies of the transgene per haploid genome. Designation of transgenic lines is as shown in Table 2.2.

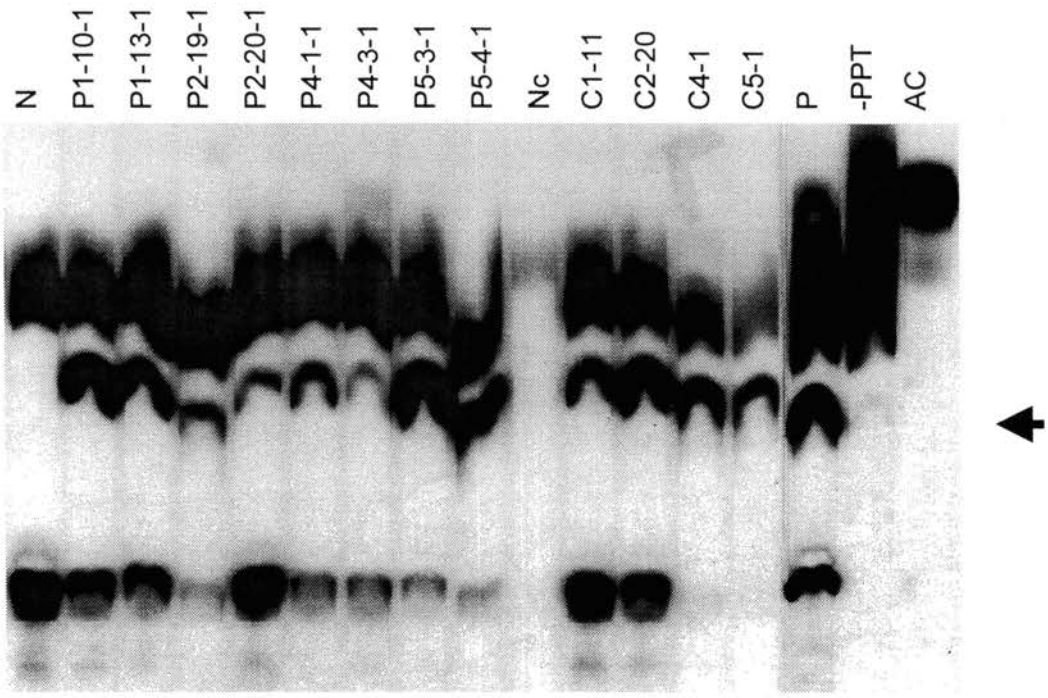

Fig. 2.8. PAT activity in transgenic calli and T_0 plants. C1, C2, C4 and C5 represent calli transformed with pTA1, pTA2, pTA4 and pTA5 plasmids and P1, P2, P4 and P5 are plants regenerated from the respective calli. The positive band is indicated by an arrow N, negative control (wild type plant); Nc, negative callus (wild type); P, positive control (Caucasean bluestem transformed with the gene); -PPT, reaction without phosphinothricin; AC, [^{14}C]acetyl-CoA

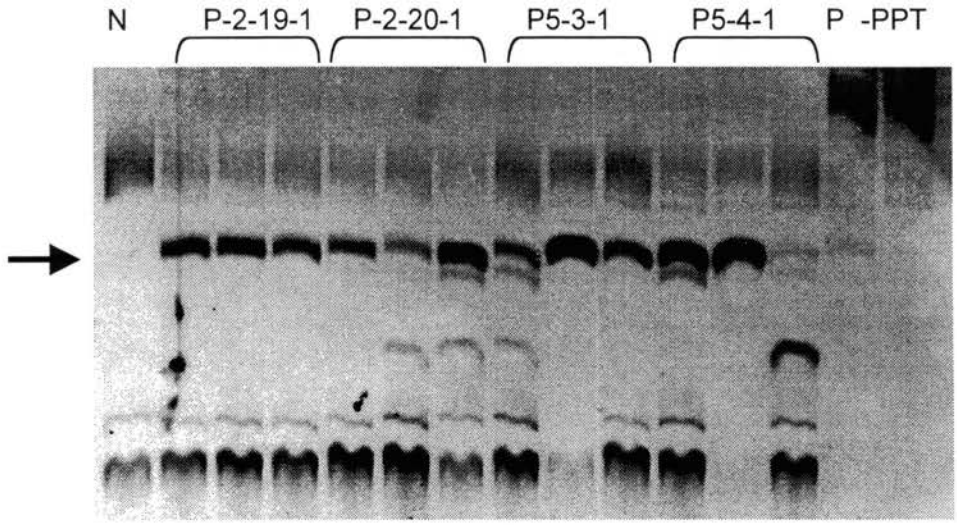

Fig. 2.9. PAT activity in T_2 plants. Individual T_2 plants from lines transformed with pTA2 and pTA5 plasmids were used for the assay. The positive band is indicated by an arrow. N, negative control (wild type plant); P, positive control (Caucasean bluestem transformed with the *bar* gene); -PPT, reaction without phosphinothricin. Designation of transgenic lines is as shown in Table 2.2.

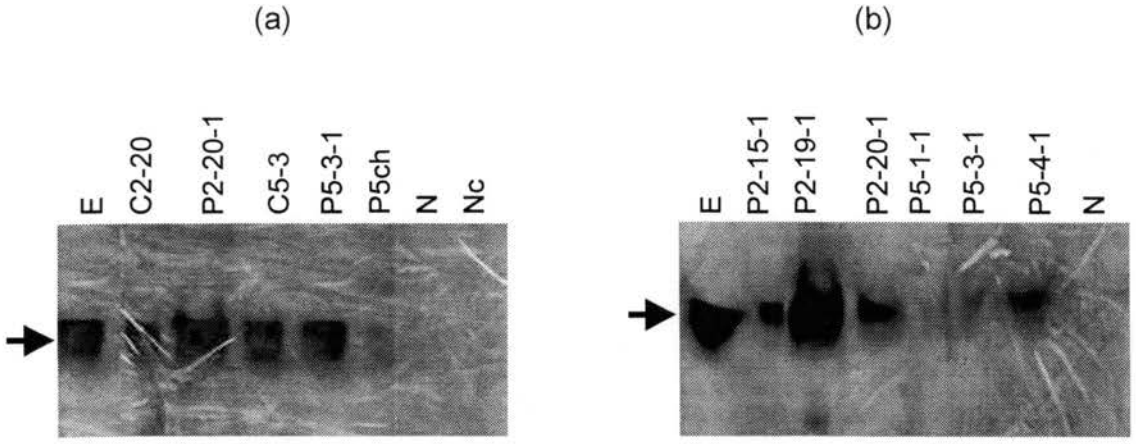

Fig. 2.10. MTLD activity in T_0 (a) and T_2 (b) transformants. E, positive control (*E. coli*); C2, callus transformed with pTA2; P2, plants transformed with pTA2; C5, callus transformed with pTA5; P5, plants transformed with pTA5; P5Ch, chloroplast extract from pTA5-transformed plants; Nc, negative control callus (wild type); N, negative control plant (wild type).

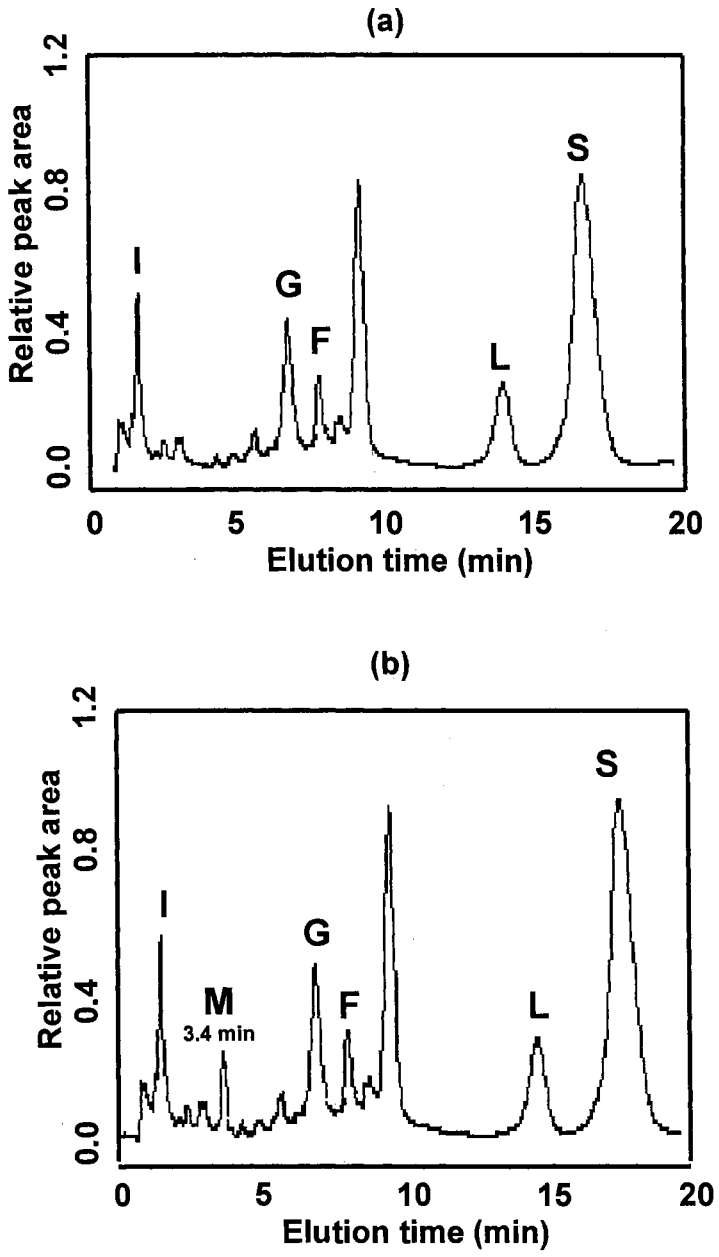

Fig. 2.11. HPAE-PAD separation of mannitol and soluble carbohydrates in transgenic wheat. (a) carbohydrates from line P1-13-1 (transformed with pAHC20 alone) and (b) from line P2-19-1(transformed with pTA2). I, inositol; M, mannitol; G, glucose; F, fructose; L, lactose (internal standard); S, sucrose. Note that the mannitol peak is present in P2-19-1 but not in P1-13-1.

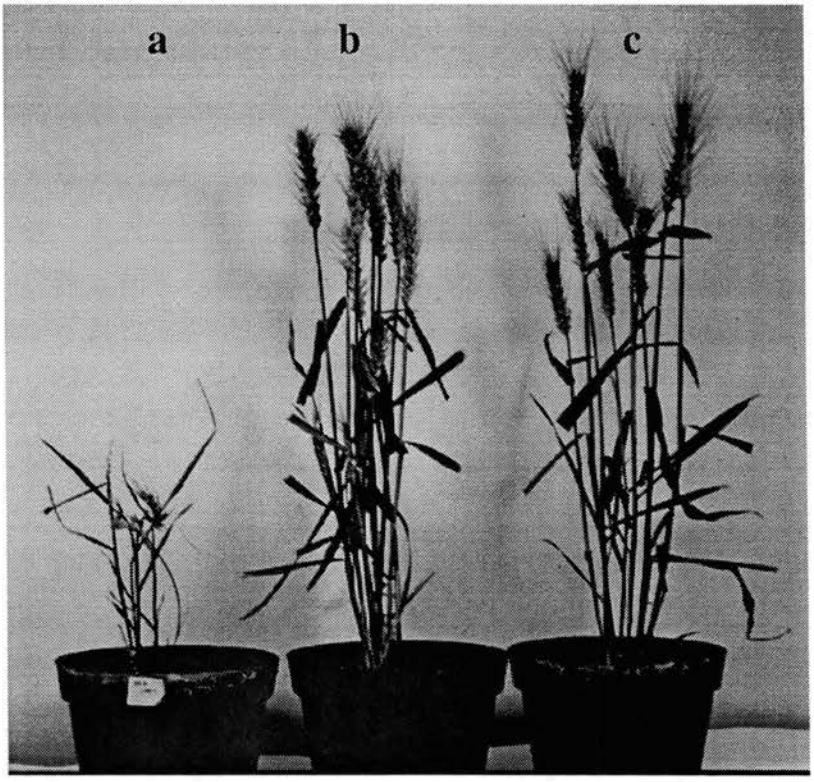

Fig. 2.12. Phenotypes observed in T_0 plants. Lines P2-16-1 (a) and P2-19-1 (b) were transformed with plasmid pTA2 for accumulation of mannitol in the cytosol. Line P1-13-1 (c) was transformed with pAHC20 alone and did not accumulate mannitol. Most plants that expressed *mtlD* were stunted in growth, infertile, and had twisted leaves and heads as shown in (a)

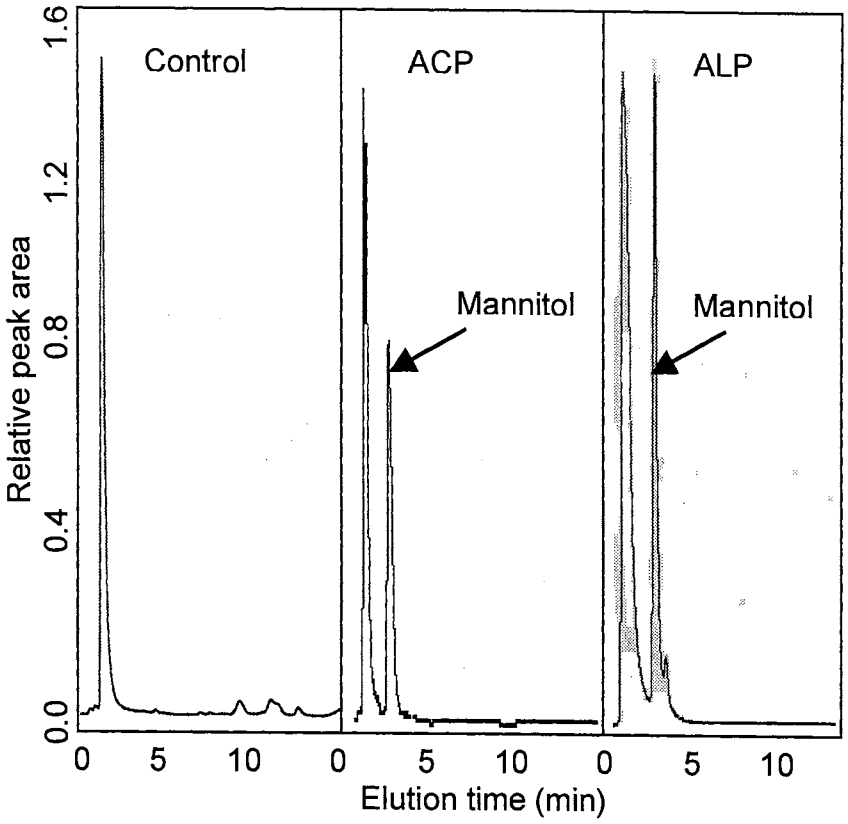

Fig. 2.13. Assay for acid (ACP) and alkaline (ALP) phosphatases in wheat. Five mM of M1P was incubated with 20 μg total leaf protein at 30 °C for 1 h at pH 5.0 (ACP) or 8.5 (ALP). Mannitol produced from the hydrolysis of M1P was detected by HPAE-PAD. A control reaction without protein was included for each phosphatase assay. Chromatograms in both controls were identical hence only the control chromatogram for ACP is presented.

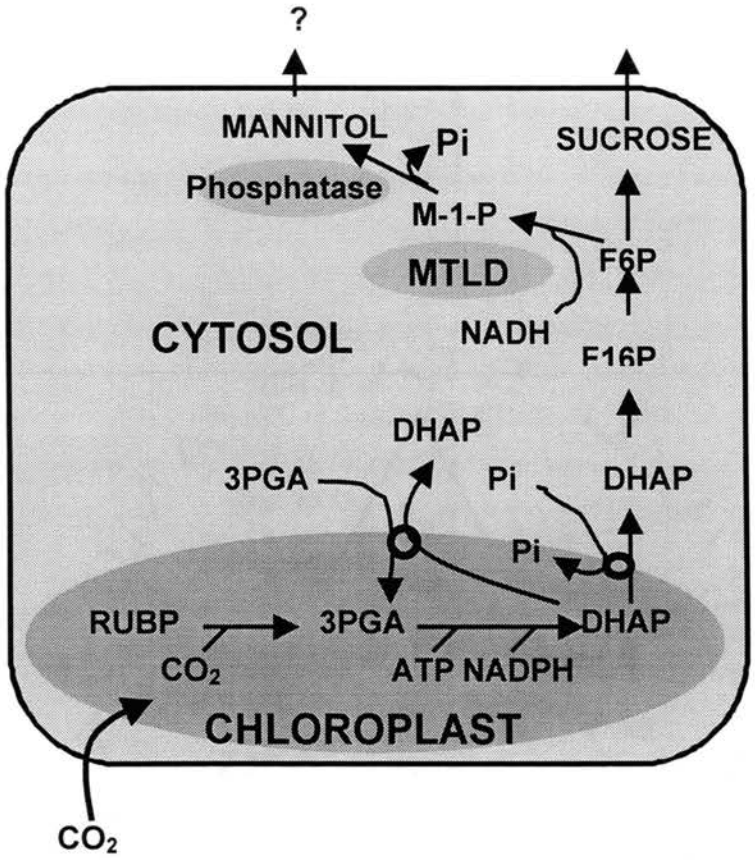

Fig. 2.14. Pathway for synthesis of mannitol in transgenic wheat.

CHAPTER III

RESISTANCE OF MANNITOL ACCUMULATING TRANSGENIC WHEAT TO WATER DEFICIT STRESS

ABSTRACT

We have evaluated the performance of transgenic wheat expressing the mannitol-1-phosphate dehydrogenase (*mtlD*) gene for accumulation of the osmolyte mannitol (+mtlD) to water and salt stress. Wheat transformed with the *bar* selectable marker served as a negative control (-mtlD). Response to stress was evaluated at the tissue and whole plant level using calli and T_2 plants that accumulated 2.0 and 0.7 μmoles gfw^{-1} mannitol respectively. Calli were exposed to -1.0 MPa PEG and 100 mM NaCl for 60 days. Water stress at the whole plant level was imposed by watering plants with 1/3 (50-75 ml) of the unstressed level (150-225 ml) for 30 days at 4-day intervals. Salt stress was induced by growing plants in nutrient solution containing 150 mM NaCl for 30 days. Fresh weight of -mtlD calli was reduced by 45% and 37% in the presence of PEG and NaCl, but there was no significant effect on +mtlD calli. Similarly, fresh weight, dry weight and plant height were reduced by 70%, 56% and 40%, respectively in -mtlD plants under water stress compared to 40%, 8%, and 18% in +mtlD plants. Salt stress reduced shoot fresh weight, dry weight and length of the flag leaf by 78%, 72% and 36% in -mtlD plants, respectively compared to 50%, 30% and 5% in +mtlD plants. Mannitol content increased by 79% and 118% in PEG and NaCl-stressed +mtlD calli. In plants, mannitol increased by 138% and 129% in water and salt-stressed plants, respectively. However, the amount of mannitol accumulated was too small to have a major effect on osmotic adjustment. It is concluded that the improved performance of +mtlD transformants under stress could be due to other stress protective functions of mannitol.

INTRODUCTION

The sugar alcohol mannitol is synthesized in several plant species. In celery, a naturally mannitol accumulating plant, mannitol is synthesized in equal proportion to that of sucrose. It also constitutes as much as 50% of the translocated photoassimilate (Loescher *et al.*, 1992). Mannitol accumulation increases when plants are exposed to low water potential (Patonnier *et al.*, 1999) and accumulation is regulated by inhibition of competing pathways and decreased mannitol consumption and catabolism (Stoop *et al.*, 1996). In celery, salt stress inhibits sucrose synthesis, but does not affect the enzymes for mannitol biosynthesis. Moreover, the rate of mannitol utilization in sink tissues decreases during salt stress mainly because of the suppression of the NAD^+-dependent mannitol dehydrogenase (MTD), which oxidizes mannitol to mannose (Stoop and Pharr, 1993; Pharr *et al.*, 1995).

Mannitol improves resistance to limited water availability through osmotic adjustment, reactive oxygen species (ROS) scavenging, and serves as a store of carbon. In osmotic adjustment the tissue osmotic potential (Ψ_s) decreases as a result of accumulation of solutes, which in turn lowers the water potential (Ψ_w) of the cell below the surrounding environment thus allowing flow of water towards the cells (Bray *et al.*, 2000). Recent studies using transgenic tobacco and *Arabidopsis* showed that accumulation of mannitol enhances growth during water deficit stress. However, the amount of mannitol accumulated was considered insufficient for osmotic adjustment to account for the observed resistance (Tarczynski *et al.*, 1993; Thomas *et al.*, 1995; Karakas *et al.*, 1997). Subsequent

studies have shown that the improved growth is due to the ROS scavenging ability of mannitol (Smirnoff and Cumbes, 1989; Shen *et al.*, 1997a).

Mannitol also serves as a sink for reducing power and storage of carbon. Since the primary capture of photon energy is insensitive to water deficit stress, plants under stress are exposed to light intensities in excess of those that can be used for carbon assimilation. If the regeneration of $NADP^+$ is limited under conditions of continued photon absorption, redox imbalance results in photoinhibition and enhanced use of O_2 instead of $NADP^+$ as the electron acceptor in photosynthesis (Hare *et al.*, 1998). In plants, such as celery, mannitol synthesis involves utilization of NADPH as a reducing agent. As a result it has been proposed that a stress-induced increase in the transfer of reducing equivalents into mannitol may assist in counteracting photoinhibitory damages during stress by recycling $NADP^+$. When the stress is relieved, mannitol is mobilized and serves as a source of carbon (Stoop *et al.*, 1996). The massive NADP-NADPH turnover in the cytosol during the reduction of mannose-6-phosphate to mannitol-1-phosphate and the additional cytosolic sink for photosynthetically fixed CO_2 provided by mannitol synthesis in addition to sucrose, increases the outlets for carbon exported from the chloroplast and ultimately from the leaf. This gives celery (a C_3 plant) an exceptionally high photosynthetic rate that is comparable to C_4 plants. Furthermore, mannitol metabolism in plants results in more efficient carbon use, especially in sink tissues. This may result from energy production in the initial steps of mannitol

catabolism that generates NADH thus giving a higher net ATP yield than the catabolism of an equal amount of sucrose (Stoop *et al.*, 1996).

Here we report the response of mannitol accumulating transgenic wheat to water and salt stress conditions. Our objective was to determine if mannitol improves resistance to water stress and salinity at the tissue (callus) and whole plant level using T_2 progenies.

MATERIALS and METHODS

Plant Materials

Transgenic calli and plants expressing the *E. coli mtlD* gene were developed as described in Chapter 2. Callus line C1-11 and plant line P1-13-1 transformed with pAHC20 alone (hereafter referred to as -mtlD) served as negative controls. Callus line C2-20 and plant line P2-19-1 that expressed mannitol in the cytosol (hereafter referred to as +mtlD) were used as positive controls to determine if mannitol enhances stress resistance in wheat. Two criteria were taken into consideration to choose calli and plant materials for this experiment. First, to avoid variations due to differences in their physiological state, calli of same age (6 month-old) were used. Second, to be able to observe real differences in the response of +mtlD and -mtlD materials it was necessary to select +mtlD lines with high mannitol content. Thus for experiments involving calli, line C2-20 was selected. This line was transformed with pTA2 and accumulated about 2 μmoles gfw^{-1} mannitol in the cytosol (Chapter 2). Due to the high rate of sterility in T_0 plants that expressed high levels of mannitol, it was not possible to obtain enough seeds from plants with high mannitol. It was therefore necessary to conduct the experiment using T_2 plants. Plant line P2-19-1, which was transformed with pTA2 and accumulated moderate levels of mannitol in the cytosol (0.70 μmoles gfw^{-1}) was selected (Chapter 2).

Growth Conditions

The calli used in this experiment were initially maintained in shoot induction medium (SIM) to regenerate shoots. After one month on SIM (4 months after culture initiation) portions of each callus line were transferred to callus induction medium (CIM) supplemented with 1 mg l^{-1} bialaphos to increase callus proliferation. After 2 months in CIM (6 months since culture initiation), calli were exposed to different levels of water and salt stress as described below.

Homologous T_2 plants used in the stress experiments were derived from immature embryos isolated from kernels 25 days after anthesis of the corresponding T_1 plants. Kernels were surface sterilized in 70% ethanol for 5 min followed by 15 min in 20% clorox. After 3 washes with sterile water, embryos were isolated and plated on root initiation medium (RIM) without hormones containing 3 mg l^{-1} bialaphos. RIM consisted of half-strength MS salts (Murashige and Skoog, 1962), 10 g l^{-1} sucrose, 0.25 mg l^{-1} thiamine-HCl, 75 mg l^{-1} L-asparagine monohydrate and solidified with 2.5 g l^{-1} Phytagel (Sigma, St. Louis, MO) as described by Weeks (1995). Embryos were allowed to germinate in 100 ml of RIM in Magenta jars for two weeks. Those that grew under bialaphos selection were transferred to a small plastic box lined with paper towel and moistened with nutrient solution as described below, covered with Saran wrap and maintained under low light. The seedlings were allowed to acclimate to ambient conditions by slowly opening the plastic wrap over a 2-day period. Then for water stress 3 uniform seedlings were planted in 15x12 cm pots filled with equal amounts (350 g) of Metro-mix 366 soil mixture (Scott-Sierra Horticultural

Products, Co., Marysville, OH). The weights of empty pots were recorded before filling with soil in order to adjust the total weight to uniformity during the start of the stress experiment. To minimize variations in moisture content, soil from one bag was mixed well before use. After planting, the pots were watered with 200 ml nutrient solution modified from Bingham and Stevensen (1993) and kept at room temperature under low light for 4 days. The nutrient solution consisted of 2 mM KNO_3, 2 mM $Ca(NO_3)_2.4H_2O$, 0.75 mM $MgSO_4.7H_2O$, 0.5 mM $NH_4H_2PO_4$, 50 µM NaFeEDTA, 5 µM $MnSO_4.H_2O$, 0.5 µM $CuSO_4.5H_2O$, 0.5 µM $ZnSO_4.7H_2O$, 25 µM H_3BO_3, 0.1 µM $CoCl_2.6H_2O$, 0.2 µM $Na_2MoO_4.2H_2O$, 50 µM NaCl, and 0.5 µM $Na_2SiO_3.5H_2O$. For the salt stress experiment, seedlings were transferred to support tubes (made out of 1.5 ml centrifuge tubes) in lids of rectangular plastic containers filled with 3.5 l aerated nutrient solution. The seedlings were kept at room temperature under low light for 4 days. On the fifth day plants were transferred to a growth chamber with 200 µmole $m^{-2}s^{-1}$ PAR, 23°C/17°C day/night temperatures, 70% relative humidity and 16 h photoperiod. Plants in soil were watered with 150 ml nutrient solution every 4 days for 3 weeks. For plants in hydroponics, the nutrient solution was changed every 4 days.

Imposition of Water and Salt Stress in Calli

Calli were subjected to osmotic stress using polyethylene glycol 8,000 (PEG) and NaCl. To determine the concentrations of PEG and NaCl at which noticeable changes in the growth of -mtlD and +mtlD calli occur, a pilot experiment was conducted using PEG that lowers Ψ_w of the medium to -0.40

(unstressed), -0.70 and -1.0 MPa. For NaCl, unstressed (no supplemental NaCl), 100 and 150 mM were tested for 30 days. It was found that -1.0 MPa PEG and 100 mM NaCl reduced the size of -mtlD calli compared to +mtlD calli and these levels of PEG and NaCl were selected for the actual experiment.

For PEG treatment, a tissue culture system using filter paper-polyurethane support (Fig 3.1) saturated with liquid CIM (without Phytagel) was used since PEG inhibits gelling agents from solidifying in plant tissue culture media (Weeks, 1991). Whatman No.1 filter papers (4.25 cm diameter) were placed on top of circular 10x80 mm polyurethane foam in 25x100 mm petri dishes. The foam and filter paper support system was saturated with 45 ml of CIM with 19.3 mM PEG 8,000 to lower Ψ_w to -1.0 MPa. Unstressed calli were grown in CIM without PEG. Then 0.25 g calli were placed on the filter paper-foam support. For salt stress, 0.25 g calli were grown at 21°C in solidified CIM with (stressed) or without (unstressed) 100 mM NaCl. Use of 19.3 mM PEG and 100 mM NaCl lowered the Ψ_w of the medium to -1.0 MPa. In both stress types a completely randomized design with three replications per treatment level was used. Calli were transferred to fresh media every two weeks for 60 days.

Imposition of Water and Salt Stress in Plants

Water stress and salt stress were imposed by withholding water from plants grown in soil and addition of NaCl to nutrient solution in plants grown in hydroponics. To determine the level of water stress and NaCl concentration at which visible reduction in growth was observed, a preliminary experiment was

conducted with 4-week old wild type Bobwhite plants. For water stress 50, 100, and 150 ml water was applied to plants in 13x15 cm pots every 3 days. For salt stress plants were grown in nutrient solution containing 100, 150 and 200 mM NaCl. With 50 ml water and 150 mM NaCl there were clear signs of reduced growth, wilting of older leaves, rolling of younger leaves and necrosis of leaf tips (for NaCl). These levels were selected for the actual stress experiments.

After 3 weeks in soil and hydroponics, -mtlD and +mtlD plants grown as above were randomly assigned to two water levels and two salt levels, respectively. For water stress, plants were thinned to 2 uniform plants per pot. Then, the weight of pots was adjusted to the original weight with water. The unstressed plants received 150 ml and stressed plants were watered with 50 ml of water at 4-day intervals. Each treatment was replicated three times for each plant type. Pot positions were randomized daily to minimize differences that may result due to conditions in the growth chamber.

For salt stress, two uniform plants each for -mtlD and +mtlD grown in hydroponics were assigned to stressed and unstressed groups. Plants in the unstressed group were grown without supplemental NaCl. The stress group was exposed to 150 mM NaCl. To avoid sudden shock and death of plants, the concentration of NaCl was slowly raised by an increment of 30 mM per day over a 5-day period. The solution was continuously aerated and changed every 4 days for two weeks. Then as plants became larger the solution was changed every three days for two additional weeks. A randomized complete block design with containers as blocks was used. Each treatment was replicated three times.

Growth Measurements

Growth of calli was determined by measuring fresh weight. After the 60-day stress period calli were blotted dry with paper towels and fresh weights measured. For plants, at the end of the stress period water and salt-stressed plants were harvested to measure fresh weight, dry weight, height of the main shoot, length of the fully expanded flag leaf and root length (salt stress only). Dry weight was detrmined after oven drying plants at 70°C for 48 hours. Height of the main shoot and length of the flag leaf and the roots were measured using a ruler. Length of the flag leaf was measured from the ligule and that of the root from the crown.

Measurement of Water Relations

For plants, Ψ_w, Ψ_s and relative water content (RWC) were measured for the fifth leaf. For Ψ_w and Ψ_s measurements the region between 3 and 5 cm from the ligule was used. The rest of the leaf excluding 3 cm of the tip was cut into 1 to 1.5 cm segments for RWC determination. Ψ_w was measured using a Wescor HP-115 automatic water potential measurement system (Wescor Inc, Logan, UT) and leaf cutter psychrometer (Merrill Specialty Equipments, Logan, UT). Six-millimeter diameter discs cut with a cork borer were immediately transferred to the leaf cutter thermocouple psychrometer sample chambers. Measurements were taken after 2 h equilibration of psychrometers in a water bath maintained at 30°C. Sodium chloride solutions (0, 200, 400, 600, 800 and 1000 mmol kg^{-1}) were used to calibrate each thermocouple psychrometer. The Ψ_w of samples was

estimated from the resulting calibration plots. For calli about 0.1 g of tissue was gently blotted-dry with paper towel until no visible wetting. Then tissue was placed in the sample chamber and Ψ_w measured.

Ψ_s of leaf and callus tissue were measured using a Vapro^{TM} vapor pressure osmometer (Wescor Inc, Logan, UT). About 1.5 cm long leaf segment or 0.1 g callus was placed in 0.5 ml centrifuge tubes and frozen in liquid N_2. Samples were thawed at room temperature and centrifuged to collect the sap. Ten µl of the sap was loaded into the sample chamber and Ψ_s determined. The osmometer was calibrated using 290 and 1000mOsm NaCl solutions.

RWC of leaves was calculated according to the equation

$$RWC = \frac{FWT-DWT}{TWT-DWT} \times 100\%$$

Where, FWT is leaf fresh weight, TWT is turgid weight of leaf after floating for 2 h on distilled water, and DWT is dry weight taken after drying turgid leaves for 48 hours at 70°C.

Determination of Osmotic Adjustment

If calli or plants osmotically adjusted during stress, it is expected that they show reduction in the Ψ_s at full turgor. If they do not adjust, Ψ_s at full turgor will remain unaffected. Then osmotic adjustment between the -mtlD and +mtlD was compared. If +mtlD calli or plants accumulated osmotically significant quantities of mannitol (assuming no change in the concentration of the other low molecular

weight carbohydrates), it is expected that +mtlD calli or plants will have lower Ψ_s than the -mtlD calli or plants.

Osmotic adjustment of calli and plants was determined as the difference between the Ψ_s at full turgor (after rehydration in distilled water) of stressed tissue from the Ψ_s at full turgor of unstressed tissue. To determine Ψ_s at full turgor, six-millimeter diameter leaf discs and 0.1 g callus tissue were floated on distilled water in petri dishes for 2 h. The petri dishes were wrapped with parafilm to create 100% humidity. After 2 h, leaf discs and calli were blotted dry, transferred to 0.5 ml centrifuge tubes and frozen in liquid N_2. Ψ_s was measured using the Vapro™ vapor pressure osmometer as described above.

Determination of Mannitol and Carbohydrate Content

The concentration of mannitol and carbohydrates in calli and plants was determined as described by Adams *et al.* (1993). Briefly, samples were harvested and quickly frozen in liquid nitrogen. The frozen tissue was ground to a fine powder with mortar and pestle and 100-200 mg tissue was transferred to 2 ml tubes. Two volumes (200-400 µl) of ice-cold ethanol/chloroform/H_2O (12:5:3) was added and mixed by vortexing. Then an equal volume of water added, vortexed again and centrifuge at 10,000xg for 5 min. The upper ethanol/water phase was transferred to a new tube and the pellet was re-extracted twice with water at 60°C for 30 min each. The extracts were dried in a speed-vac and the pellet was resuspended in 300 µl H_2O and passed through a C_{18} solid-phase extraction column (Alltech Associates, Inc., IL) to remove hydrophobic

substances. The column was eluted under vacuum using a vacuum manifold attached to a vacuum source or by spinning at 200xg for 2 min. The columns were washed with 400 ml purified water (using 0.2 μm cellulose acetate filters). Lactose was used as an internal standard to correct for losses during extraction.

Carbohydrates were separated using a high-performance anion exchange chromatography (HPAE) system coupled to a pulsed amperometric detector (PAD). Fifty μl samples were injected into a 200 μl sample loop connected to a 9x250 mm Carbopac PA1 column (Dionex, Sunnyvale, CA) maintained at room temperature. Samples were separated isocratically in 150 mM degassed NaOH at a flow rate of 2.0 ml min^{-1}. Peak areas were quantified and retention times determined using an integrator. Purified carbohydrates (inositol, mannitol, glucose, fructose, lactose and sucrose) purchased from Sigma (St. Louis, Mo) were used as standards.

RESULTS

Response to Water and Salt Stress at the Tissue Level

Growth. The effect of water and salt stress on the growth performance of calli was examined after two months of exposure to -1.0 MPa PEG 8,000 and 100 mM NaCl in the medium. Calli transformed with *mtlD* and, which accumulated mannitol in the cytosol (+mtlD) were less affected by stress than calli transformed with pAHC20 alone (-mtlD). Growth of -mtlD calli was reduced by 44.6% and 36.8% under PEG and NaCl stress, respectively, but there was no significant change in the growth of +mtlD calli (Table 3.1, Figs 3.2 and 3.3).

Mannitol and carbohydrate content. PEG and NaCl stresses increased accumulation of carbohydrates in both -mtlD and +mtlD calli (Tables 3.2 and 3.3). Inositol, glucose, fructose and sucrose levels increased in both types of calli. In +mtlD calli the mannitol content increased by 79% and 118% during PEG and NaCl stress, respectively. There was no detectable mannitol in the -mtlD calli. Depending on treatment type mannitol accounted for 7.1 to 12.5% of total soluble carbohydrates in +mtlD calli. Accumulation of mannitol in PEG- but not NaCl-stressed calli resulted in reduced sucrose content of the +mtlD calli.

Water relations of calli. There was a significant reduction in the Ψ_w and Ψ_s of both -mtlD and +mtlD calli under PEG and NaCl stresses. However, there was no difference in the Ψ_w or Ψ_s of the two callus types (Tables 3.4 and 3.5). Osmotic adjustment calculated as the difference between the Ψ_s of stressed and

unstressed calli at full turgor indicated that -mtlD and +mtlD calli had adjusted by 0.29 MPa and 0.34 MPa, respectively during PEG stress. Under NaCl stress, the -mtlD and +mtlD calli adjusted by 0.30 MPa and 0.34 MPa, respectively.

Response to Water Stress at the Whole Plant Level

Growth. Under well-watered condition there was no difference in the growth performance of -mtlD and +mtlD plants. Water stress reduced the dry weight, fresh weight and length of the fifth leaf of both -mtlD and +mtlD plants. The impact of stress was more severe on the -mtlD than on the +mtlD plants (Table 3.6 and Fig 3.4). Fresh weight, dry weight, and plant height were reduced by 70%, 56% and 41%, respectively in -mtlD plants compared to the 40%, 8% and 18% in +mtlD plants. The -mtlD plants also had fewer tillers than the +mtlD plants during stress.

Mannitol and carbohydrate content. Like in the callus experiment water stress increased the concentration of soluble carbohydrates in -mtlD and +mtlD plants (Table 3.7). Mannitol concentration in stressed +mtlD plants increased by 138%. Depending on plant type other carbohydrates also increased between 37.5% (inositol) and 190% (fructose). Although the amount of mannitol present in +mtlD plants was small and was not expected to have significant effect on the amount of other carbohydrates, the concentration of sucrose was smaller in +mtlD than in -mtlD plants. There was no difference in the concentration of inositol, glucose and fructose between -mtlD and +mtlD plants under stress and unstressed conditions.

Water relations. Data on the water relations of unstressed and stressed plants are given in Table 3.8. There was no difference in the RWC, Ψ_w and Ψ_s of -mtlD and +mtlD plants under well-watered condition. Under stress condition these parameters were significantly low in both plants. Also there were clear differences in the RWC and Ψ_w of -mtlD and +mtlD plants. In -mtlD plants RWC and Ψ_w were reduced to 79.3% and -2.29 MPa, respectively. In +mtlD plants RWC and ψ_w were reduced to 88.5% and -1.43 MPa. However, these differences were not related to changes in Ψ_s, since Ψ_s in both plants was similar. Differences in RWC and Ψ_w also resulted in different wilting responses. The -mtlD plants showed reversible wilting and leaf rolling in the second and third day after watering but +mtlD plants did not show these symptoms until the fourth day. Values of Ψ_w and Ψ_s in stressed -mtlD plants are consistent with the visually observed wilting (Table 3.8). The -mtlD and +mtlD plants osmotically adjusted by 0.23 and 0.37 MPa, respectively.

Response to Salt Stress at the Whole Plant Level

Growth. Plants in hydroponics had twice the shoot fresh weight and dry weight as compared to plants in soil. Besides, plants in hydroponics had more tillers than those in soil. Apart from these differences +mtlD plants grew better in 150 mM NaCl than -mtlD plants, as did +mtlD plants in the water stress experiment. Salt stress reduced shoot fresh weight, dry weight and flag leaf length by 78%, 72% and 36% in -mtlD plants, respectively compared to 50%, 30% and 5% in +mtlD plants (Table 3.9, Fig 3.5).

A similar response was observed in root growth. Salt stress reduced root fresh weight and dry weight in both -mtlD and +mtlD plants. However, fresh weight and dry weight in -mtlD plants were reduced by 80% and 81%, respectively compared to only 62% and 51% in +mtlD plants (Table 3.9).

Mannitol and carbohydrate content. The total soluble carbohydrate content increased 94% and 90% in -mtlD and +mtlD plants, respectively during exposure to 150 mM NaCl (Table 3.10). Inositol, glucose, fructose and sucrose increased during salt stress regardless of plant type. Sucrose showed the largest increase. Mannitol content increased by 129% in +mtlD plants. As was the case for calli and plants in PEG and water stress, +mtlD plants used for the salt stress experiment had lower sucrose content than the -mtlD plants

Water relations. There was no difference in the RWC, Ψ_w and Ψ_s between -mtlD and +mtlD plants under unstressed conditions (Table 3.11). Salt stress, however, significantly reduced these parameters in both plants. Furthermore, there were clear differences in the RWC of the two plants. RWC of -mtlD plants was 10 percentage points less than that of +mtlD plants. The -mtlD plants also had lower Ψ_w and Ψ_s than the +mtlD plants. Interestingly, however, there was no difference in Ψ_s at full turgor and both plants had osmotically adjusted by the same amount (0.56 and 0.53 MPa). Moreover, -mtlD plants showed wilting of lower leaves (although turgor was greater, Table 3.11) and chlorotic tips, which were rare in +mtlD plants. Taken together these results suggest that although the -mtlD plants

osmotically adjusted by an equal amount as their +mtlD counterparts, osmotic adjustment alone was not able to overcome the damage caused by stress.

DISCUSSION

In an effort to improve the resistance of wheat to water deficit stress, we previously introduced the *mtlD* gene of *E. coli* into wheat (Chapter 2). Here we evaluated the response of the transgenic wheat to water deficit stress at the tissue (callus) and whole plant level. Our results demonstrate that *mtlD* increased the growth performance of transgenic wheat under limited water availability and salinity conditions both at the tissue level and the whole plant level (Tables 3.1, 3.6, 3.9; Figs 3.2 to 3.5). These observations are in agreement with other studies that used the same *mtlD* gene in model plants. Tobacco plants expressing mannitol were shown to grow better under 250 mM NaCl conditions (Tarczynski *et al.*, 1993) and transgenic *Arabidopsis* seeds germinated at 250 mM NaCl, whereas the wild type seeds ceased to germinate at 100 mM NaCl (Thomas *et al.*, 1995).

It has been proposed that mannitol enhances resistance to water deficit stress primarily through osmotic adjustment (Loescher *et al.*, 1992). Osmotic adjustment involves net cellular accumulation of solutes in response to falling Ψ_w in the environment. Net accumulation of solutes lowers Ψ_s, which in turn attracts water into cells and maintains turgor. Turgor is a prerequisite for various metabolic processes enabling osmotically adjusted plants to maintain growth under stress (Kramer and Boyer, 1995). Our data show that there was no difference in the Ψ_s of -mtlD and +mtlD transformants at the tissue and whole plant level and that both osmotically adjusted to water deficit stress (Tables 3.4, 3.5, 3.8, 3.11). As shown in Tables 3.2, 3.3, 3.7 and 3.10 the amount of mannitol

accumulated in response to water deficit stress was very small both in calli (3.66 μmoles gfw^{-1} during PEG and 3.82 μmoles gfw^{-1} during NaCl stress) and plants (1.45 μmoles gfw^{-1} during water stress and 2.02 μmoles gfw^{-1} during 150 mM NaCl) and that compared to the other carbohydrates its effect on osmotic adjustment may not be relevant.

The contribution of mannitol and other carbohydrates to Ψ_s can be estimated by the van't Hoff's equation assuming no dissociation. Van't Hoff's equation is given by, Ψ_s = cRT, where, Ψ_s is osmotic potential, *c* is the concentration of solutes in mol l^{-1}, R is 0.00821 l MPa mol^{-1} K^{-1} and T is the temperature in °K. Assuming 85% and 95% water content of the stressed and unstressed calli, 3.66 μmoles gfw^{-1} (PEG-stressed) and 3.88 μmoles gfw^{-1} (NaCl-stressed) of mannitol would contribute 0.0093 and 0.009 MPa, respectively. The osmotic contribution is very low on a whole cell basis. It becomes higher, yet not dramatic, if we assume all the mannitol to be localized in the cytoplasm. If we assume that the cytoplasm represents 5% of the total water content of the tissue, mannitol could contribute 0.166 MPa and 0.160 MPa to the Ψ_s of calli exposed to PEG and NaCl, respectively. This represents 20% and 21% of the Ψ_s at full turgor or 47% and 55% of the osmotic adjustment. Similarly, at 88% and 90% of water content 1.45 μmoles gfw^{-1} mannitol in water-stressed plants and 2.02 μmoles gfw^{-1} in salt-stressed plants would contribute 0.0035 MPa and 0.0049 MPa to Ψ_s, respectively. With the same assumptions made above the contribution of mannitol to Ψ_s in the cytoplasm could be 0.066 MPa and 0.093 MPa, respectively. This represents 4.7% and 5.7% of total Ψ_s of water stressed

and salt stressed plants at full turgor, respectively or 18% of the Ψ_s in both cases. This is rather insignificant relative to the total osmotic adjustment. Besides, there was no significant difference in the osmotic adjustment of the -mtlD and +mtlD wheat either at the tissue or whole plant level suggesting that the beneficial effect of mannitol resulted from something other than osmotic adjustment.

In related studies modest increases in the level of mannitol (Tarkzynski *et al.*, 1993; Thomas *et al.*, 1995; Karakas *et al.*, 1997), fructan (Pilon-Smit *et al.*, 1995) and trehalose (Holmström *et al.*, 1996) resulted in improved performance of transgenic plants, but were considered insufficient for osmotic adjustment. Tobacco, a salt sensitive plant expressing the *mtlD* gene accumulated up to 4 µmole gfw^{-1} mannitol and had improved growth (Tarczynski *et al.*, 1993). Seeds of *Arabidopsis* plants accumulating between 0.05 and 12 µmole gfw^{-1} mannitol germinated in the presence of 250 mM NaCl (Thomas *et al.*, 1995). Furthermore, transgenic tobacco plants which accumulated twice as much proline as the unstressed (9 µmol gfw^{-1}) displayed improved growth when exposed to drought and salinity, but did not undergo osmotic adjustment upon drying of the soil (Kavi Kishore *et al.*, 1995). Osmotic adjustment was not observed in transgenic *Arabidopsis* plants, which accumulated 1.0 µmol gfw^{-1} glycine betaine (Hayashi *et al.*, 1997). The growth advantage of transgenic plants under stress without a significant change in osmotic adjustment suggests a role for mannitol and other osmolytes independent of their osmotic effect.

How can a small mannitol content, which is not sufficient for osmotic adjustment (particularly in plants), enhance the resistance of transgenic wheat to

stress? It is known that mannitol is an active scavenger of reactive oxygen species (ROS) and plays a key role in the maintenance of macromolecular structure. Unlike osmotic adjustment these protective functions require very small amounts of mannitol (Nelson *et al.*, 1998) and it is very likely that the improved performance of transgenic wheat may be due to ROS scavenging and other functions of mannitol. ROS, such as singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\bullet$) are formed in various metabolic reactions where oxygen is involved (Asada, 1992). ROS are very reactive to biological molecules and can cause lipid peroxidation, breakdown of macromolecules and mutation of nucleic acids (McKersie and Leshem, 1994; Smirnoff, 1998). Oxidative stress is a common problem in plants during water deficit stress (Smirnoff, 1993 and 1998; Wise, 1995). Correlations between the reduction in the level of toxic oxygen species and an increase in the concentration of polyols during stress were demonstrated *in vitro* (Elstner, 1982). *In vitro* studies by Smirnoff and Cumbes (1989) showed that mannitol is an active scavenger of ROS, particularly $OH^\bullet$. Transgenic experiments have also demonstrated that mannitol is involved in $OH^\bullet$ scavenging. Targeting mannitol into the chloroplast of tobacco significantly reduced the buildup of $OH^\bullet$ (Shen *et al.*, 1997a). In a subsequent study, Shen et al. (1997b) showed that mannitol protects thiol-regulated enzymes (such as phosphofructokinase) and other biological molecules such as thioredoxin, ferrodoxin and glutathione from attacks by $OH^\bullet$ radicals. The mechanism by which mannitol interacts with $OH^\bullet$ remains to be explained.

Sugars and sugar alcohols are also known to prevent the damaging effects of water deficit stress through the maintenance of macromolecular structure and glass formation (Crowe *et al.*, 1987; Koster, 1991). Sugar alcohols can form hydrogen bonds with macromolecules and thus may prevent formation of intramolecular H-bonds that would otherwise irreversibly change the 3D structure. They also form a strong water structure and maintain the equivalent of complete hydration of macromolecules even with a reduced number of available water molecules (Stoop *et al.*, 1996; Bohnert and Jensen, 1996). For membrane lipids the hydrophobic-hydrophilic orientation is maintained during limited water availability (McKersie and Leshem, 1994). Formation of an intracellular glass is common in seeds and resurrection plants. During desiccation cellular solutes can become concentrated with an accompanying increase in viscosity. When the viscosity reaches the point where diffusion of water ceases, the solution assumes the properties of a plastic solid and at this state the solution is called a glass (Koster, 1991). Glasses inhibit chemical reactions requiring diffusion, fill space and prevent cellular collapse (Burke, 1986). The involvement of mannitol, and for that matter other soluble carbohydrates, in glass formation in our wheat plants is unlikely because wheat is not a resurrection plant and that reported glass formation requires higher concentrations than observed in our study.

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Table 3.1. Fresh weight of transgenic wheat calli after 60 days of growth in -1.0MPa PEG and 100 mM NaCl. Callus line C1-11 (-mtlD) and C2-20 (+mtlD) were used in the study. Data are means±SE of three replications.

Callus type	Treatment Level	PEG treatment*	NaCl treatment*
-mtlD	Unstressed	3.97±0.20^{b}	3.83±0.21^{b}
	Stressed	2.40±0.27^{a}	2.43±0.06^{a}
+mtlD	Unstressed	3.59±0.26^{b}	4.45±0.20^{b}
	Stressed	3.74±0.08^{b}	4.27±0.08^{b}

*Means followed by the same letter in a column are not significantly different at $p< 0.05$

Table 3.2. Mannitol and carbohydrate content (μmoles gfw^{-1}) of C1-11 (-mtlD) and C2-20 (+mtlD) calli after 60 days of growth in -1.0 MPa PEG. Data are means±SE of three replications.

Treatment	Callus type	Inositol	Carbohydrate*				
			Mannitol	Glucose	Fructose	Sucrose	Total
Unstressed (-0.45 MPa)	-mtlD	1.28±0.26[a]	0.00±0.00[a]	5.65±1.47[a]	4.35±0.09[a]	7.69±0.79[b]	18.97[a]
	+mtlD	1.50±0.54[a]	2.12±0.18[b]	5.25±0.64[a]	3.85±1.04[a]	5.05±0.91[a]	17.77[a]
-1.0 MPa	-mtlD	2.14±0.34[b]	0.00±0.00[a]	9.40±1.45[b]	8.06±0.51[b]	12.01±0.60[c]	31.61[b]
	+mtlD	2.65±0.71[b]	3.80±0.64[c]	8.16±0.35[b]	6.51±0.78[c]	9.07±1.20[b]	30.19[b]

*Means followed by the same letter in a column are not significantly different at $p<0.05$.

Table 3.3. Mannitol and carbohydrate content (μmoles gfw^{-1}) of transgenic callus lines C1-11 (-mtlD) and C2-20 (+mtlD) after 60 days exposure to 100 mM NaCl. Data are means±SE of three replications.

Treatment	Callus type	Inositol	Carbohydrate*				
			Mannitol	Glucose	Fructose	Sucrose	Total
Unstressed	-mtlD	1.89±0.54[a]	0.00±0.00[a]	8.19±0.77[a]	5.35±0.22[b]	10.78±2.39[ab]	26.21[a]
	+mtlD	1.59±0.46[a]	1.68±0.20[b]	6.61±1.00[a]	4.23±0.33[a]	9.59±0.41[a]	23.70[a]
100 mM	-mtlD	2.65±0.71[b]	0.00±0.00[a]	14.2±2.86[b]	8.13±0.34[c]	13.58±1.0[b]	38.56[b]
	+mtlD	2.29±0.16[ab]	3.67±0.0.46[c]	12.19±1.62[b]	6.80±0.69[b]	11.26±0.84[b]	36.21[b]

*Means followed by the same letter in a column are not significantly different at $p<0.05$.

Table 3.4. Water potential and osmotic potential (MPa) of transgenic wheat callus lines C1-11 (-mtlD) and C2-20 (+mtlD) exposed to -1.0 MPa PEG for 60 days. Data are means±SE of three replications.

Stress level	Callus type	Ψ_w*	Ψ_s*	
			Non-rehydrated callus	Rehydrated callus
Unstressed (-0.45 MPa)	-mtlD	-0.44±0.02[a]	-0.57±0.05[a]	-0.42±0.07[a]
	+mtlD	-0.43±0.04[a]	-0.60±0.03[a]	-0.48±0.05[a]
-1.0 MPa	-mtlD	-1.03±0.04[b]	-1.23±0.08[b]	-0.71±0.15[b]
	+mtlD	-1.04±0.05[b]	-1.31±0.14[b]	-0.82±0.05[b]

*Means followed by the same letter in a column are not significantly different at $p<0.05$.

Table 3.5. Water potential and osmotic potential (MPa) of transgenic wheat callus lines C1-11 (-mtlD) and C2-20 (+mtlD) exposed to 100 mM NaCl for 60 days. Data are means±SE of three measurements.

Stress level	Callus type	Ψ_w*	Ψ_s*	
			Non-rehydrated callus	Rehydrated callus
Unstressed	-mtlD	-0.48±0.03[a]	-0.63±0.03[a]	-0.50±0.02[a]
	+mtlD	-0.43±0.02[a]	-0.65±0.05[a]	-0.47±0.09[a]
100 mM	-mtlD	-0.84±0.03[b]	-1.20±0.15[b]	-0.80±0.03[b]
	+mtlD	-0.82±0.04[b]	-1.17±0.10[b]	-0.76±0.10[b]

*Means followed by the same letter in a column are not significantly different at $p<0.05$.

Table 3.6. Shoot fresh weight, dry weight, plant height, length of the flag leaf and number of tillers of transgenic wheat lines P1-13-1 (-mtlD) and P2-19-1 (+mtlD) after 30 days of imposition of water stress. Data are means±SE of three replications.

Stress level	Plant type	Shoot weight (g)*		Plant height (cm)*	Length of flag leaf (cm)*	No. of tillers*
		Fresh	Dry			
Unstressed	-mtlD	11.7±0.6^{c}	2.49±0.22^{b}	47.0±3.8^{c}	28.3±1.5^{b}	4.0±0.0^{c}
	+mtlD	11.9±1.6^{c}	2.61±0.36^{b}	45.0±3.1^{c}	25.3±2.9^{b}	3.0±1.0^{bc}
-1.0 MPa	-mtlD	3.48±0.59^{a}	1.09±0.15^{a}	28.0±1.2^{a}	15.67±0.9^{a}	1.0±0.0^{a}
	+mtlD	7.09±0.28^{b}	2.40±0.02^{b}	36.7±1.2^{b}	18.7±2.9^{a}	3.0±0.0^{b}

*Means followed by the same letter in a column are not significantly different at $p<0.05$.

Table 3.7. Mannitol and carbohydrate content (μmole gfw^{-1}) of transgenic wheat lines P1-13-1 (-mtlD) and P2-19-1 (+mtlD) after 30 days of water stress. Data are means±SE of three replications.

Plant type	Stress level	Carbohydrate*					
		Inositol	Mannitol	Glucose	Fructose	Sucrose	Total
-mtlD	Unstressed	0.48±0.32^{a}	0.00±0.00^{a}	5.60±0.06^{a}	4.89±0.22^{a}	7.14±0.19^{b}	18.11
	Stressed	0.66±0.05^{a}	0.00±0.00^{a}	15.17±3.42^{b}	8.68±0.66^{b}	19.53±5.38^{c}	44.04
+mtlD	Unstressed	0.39±0.02^{a}	0.61±0.11^{b}	4.88±1.79^{a}	3.54±0.39^{a}	5.27±0.08^{a}	14.69
	Stressed	0.72±0.37^{a}	1.45±0.02^{c}	12.90±4.61^{b}	6.71±1.70^{ab}	14.62±0.94^{c}	36.40

*Means followed by the same letter in a column are not significantly different at $p<0.05$.

Table 3.8. Relative water content (RWC), water potential and osmotic potential (before and after rehydration) of the fifth leaf of transgenic wheat lines P1-13-1 (-mtlD) and P2-19-1 (+mtlD) 30 days after the imposition of water stress. Data are means±SE of three replications.

Stress level	Plant type	RWC (%)	ψ_w*	ψ_s*	
				Non-rehydrated leaf	Rehydrated leaf
Unsressed	-mtlD	95.2±0.8[a]	-1.02±0.06[a]	-1.67±0.07[a]	-1.18±0.05[a]
	+mtlD	96.2±1.4[a]	-0.97±0.02[a]	-1.66±0.07[a]	-1.16±0.08[a]
Stressed	-mtlD	79.3±0.5[b]	-2.29±0.14[b]	-2.22±0.31[b]	-1.41±0.16[b]
	+mtlD	88.5±1.7[c]	-1.43±0.01[c]	-2.20±0.09[b]	-1.53±0.09[b]

*Means followed by the same letter in a column are not significantly different at $p<0.05$.

Table 3.9. Shoot weight, root weight, plant height, length of the flag leaf, length of root, and number of tillers of transgenic wheat lines P1-13-1 (-mtlD) and P2-19-1 (+mtlD) exposed to 150 mM NaCl for 30 days. Data are means±SE of three replications.

Stress Level	Plant type	Shoot*					Root*		
		Fresh weight (g)	Dry weight (g)	Height (cm)	Flag leaf length (cm)	No. of tillers	Fresh weight (g)	Dry weight (g)	Length (cm)
Unstressed	-mtlD	28.26±2.66^{c}	4.77±0.60^{c}	50.0±2.9^{c}	34.7±1.86^{c}	6.0±1.0^{a}	17.84±1.09^{a}	1.11±0.05^{a}	63±4.04^{a}
	+mtlD	24.15±2.98^{c}	3.74±0.45^{c}	51.0±2.1^{c}	33.7±1.67^{c}	7.0±2.o^{a}	16.90±2.08^{a}	1.05±0.11^{a}	61±3.79^{a}
150 mM	-mtlD	6.40±1.37^{a}	1.35±0.29^{a}	37.7±1.8^{a}	23.7±0.67^{a}	2.0±1.o^{b}	3.50±0.88^{b}	0.22±0.03^{b}	43±1.15^{b}
	+mtlD	12.19±1.77^{b}	2.61±0.38^{b}	43.0±3.0^{b}	27.0±0.58^{b}	3.0±1.o^{c}	6.50±0.76^{c}	0.51±0.06^{c}	47±4.98^{b}

*Means followed by the same letter in a column are not significantly different at $p<0.05$.

Table 3.10. Mannitol and carbohydrate content (μmole gfw^{-1}) of transgenic wheat lines P1-13-1 (-mtlD) and P2-19-1 (+mtlD) after 30 days of growth in 150 mM NaCl. Data are means±SE of three replications.

Plant type	Stress level	Carbohydrate*					
		Inositol	Mannitol	Glucose	Fructose	Sucrose	Total
-mtlD	Unstressed	0.61±0.15[a]	0.00±0.00[a]	8.65±1.36[a]	6.85±2.85[a]	9.70±1.64[b]	25.81[a]
	Stressed	0.89±0.17[a]	0.00±0.00[a]	13.10±2.60[b]	10.13±3.37[b]	25.82±2.58[c]	49.94[c]
+mtlD	Unstressed	0.81±0.16[a]	0.88±0.13[b]	7.04±0.31[a]	4.09±0.49[a]	6.27±0.99[a]	19.09[a]
	Stressed	0.93±0.09[a]	2.02±0.35[c]	10.26±2.53[b]	6.39±2.33[b]	16.73±6.38[b]	36.33[b]

*Means followed by the same letter in a column are not significantly different at $p<0.05$.

Table 3.11. Relative water content (RWC), water potential and osmotic potential (before and after rehydration) of the fifth leaf of transgenic wheat lines P1-13-1 (-mtlD) and P2-19-1 (+mtlD) after 30 days of exposure to 150 mM NaCl. Data are means±SE of three independent measurements.

Stress level	Plant type	RWC (%)	ψ_w*	ψ_s*	
				Non-rehydrated leaf	Rehydrated leaf
Unstressed	-mtlD	97.8±0.6[a]	-0.89±0.09[a]	-1.19±0.02[a]	-1.14±0.02[a]
	+mtlD	96.9±1.0[a]	-0.84±0.04[a]	-1.21±0.04[a]	-1.10±0.04[a]
150 mM	-mtlD	80.7±1.1[b]	-2.00±0.13[b]	-2.71±0.28[b]	-1.70±0.09[b]
	+mtlD	90.3±1.3[c]	-1.74±0.10[c]	-2.14±0.12[c]	-1.63±0.04[b]

*Means followed by the same letter in a column are not significantly different at $p<0.05$.

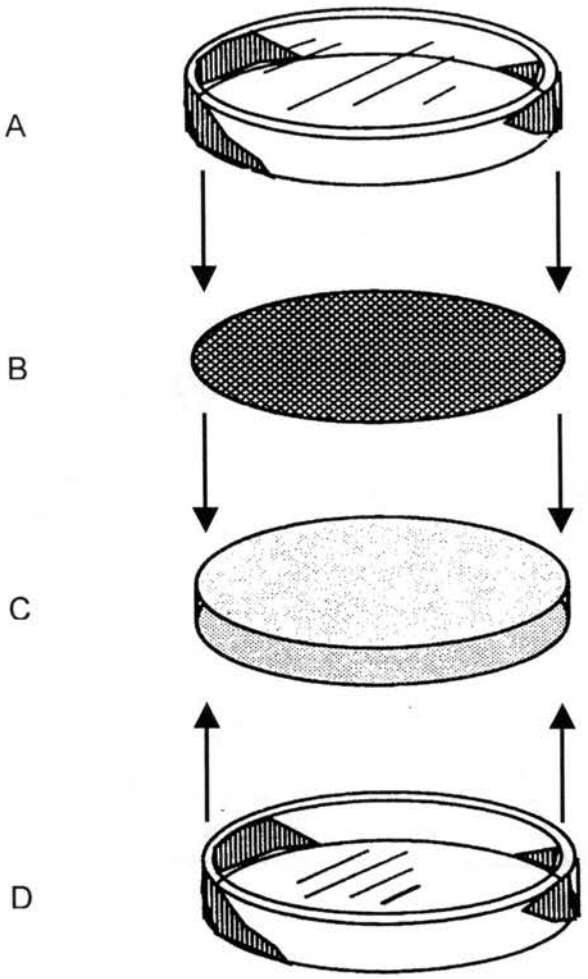

Fig. 3.1. Composition of the filter paper-foam support system used in the PEG stress experiment of calli. (A) petri dish lid; (B) filter paper; (C) polyurethan foam, and (D) petri dish bottom (Adapted from Weeks, 1991).

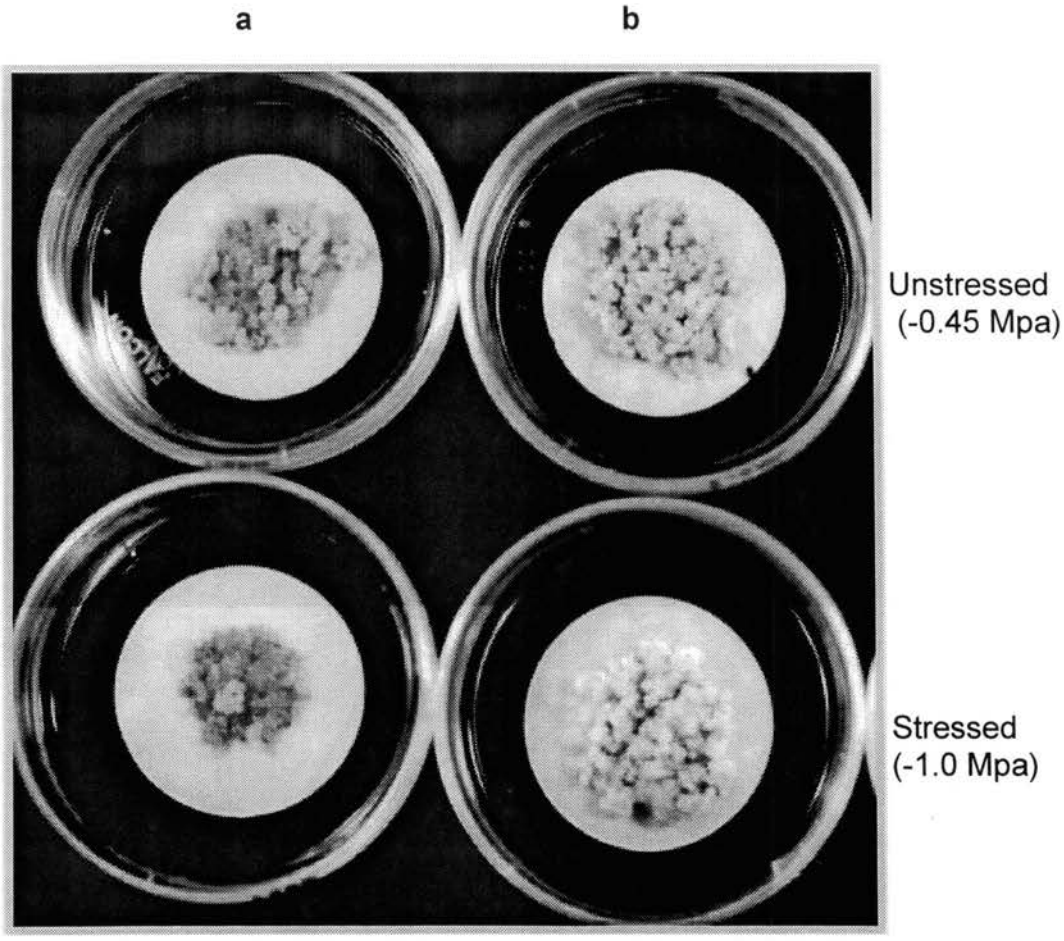

Fig. 3.2. Transgenic wheat callus lines C1-11 (-mtlD) and C2-20 (+mtlD) after 60 days of growth in -1.0MPa PEG. (a) C1-11 calli and (b) C2-20 calli.

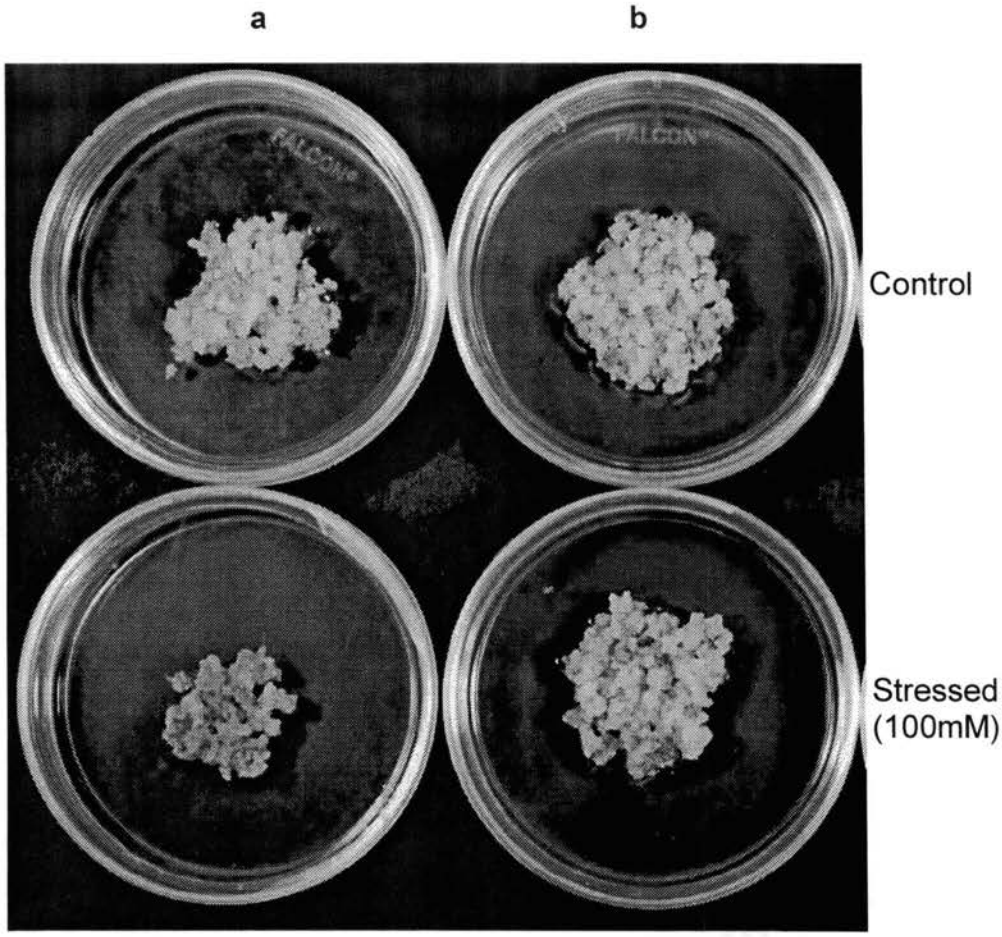

Fig. 3.3. Transgenic wheat callus lines C1-11 (-mtlD) and C2-20 (+mtlD) 60 days after imposition of 100mM NaCl. (a) C1-11 calli and (b) C2-20 calli.

Fig. 3.4. Transgenic wheat lines P1-13-1 (-mtlD) and P2-19-1 (+mtlD) 30 days after the imposition of water stress. Left, unstressed control; middle, stressed P1-13-1 plant; right, stressed P2-19-1 plant.

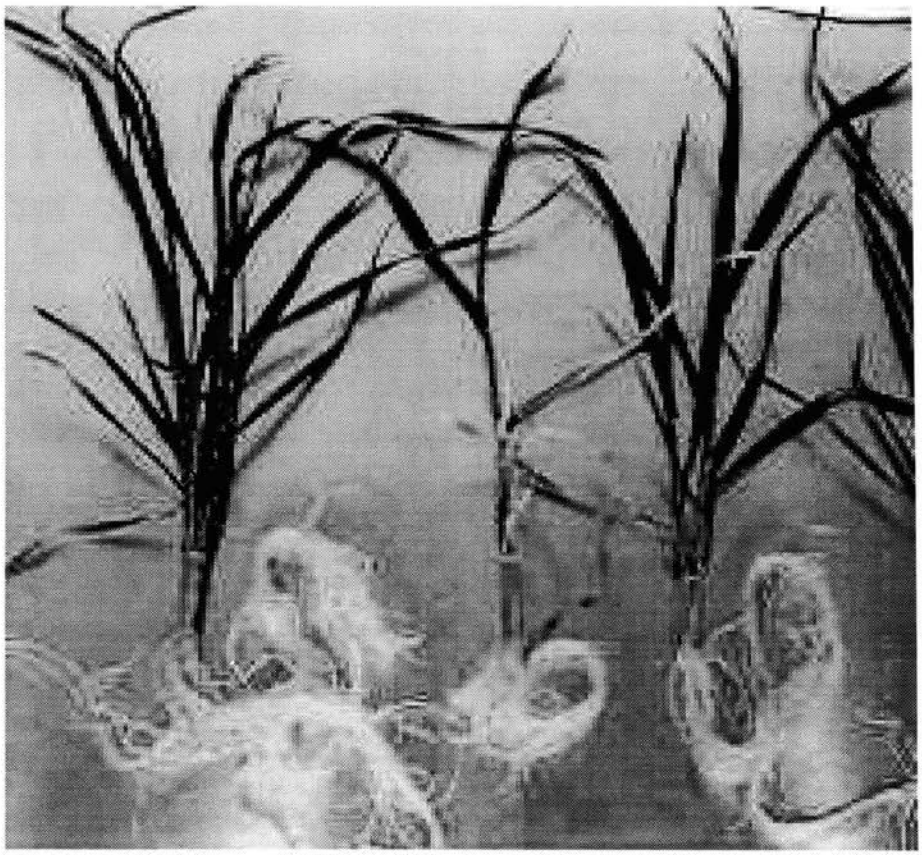

Fig. 3.5. Transgenic wheat lines P1-13-1 (-mtlD) and P2-19-1 (+mtlD) after 20 days of growth in 150 mM NaCl. Left, unstressed control; middle, stressed P1-13-1 plant; right, stressed P2-19-1 plant.

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