Tolerance of Mannitol-Accumulating Transgenic Wheat to Water Stress and Salinity[1]

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Previous work with model transgenic plants has demonstrated that cellular accumulation of mannitol can alleviate abiotic stress. Here, we show that ectopic expression of the *mtlD* gene for the biosynthesis of mannitol in wheat improves tolerance to water stress and salinity. Wheat (*Triticum aestivum* L. cv Bobwhite) was transformed with the *mtlD* gene of *Escherichia coli*. Tolerance to water stress and salinity was evaluated using calli and T_2 plants transformed with (+mtlD) or without (−mtlD) *mtlD*. Calli were exposed to −1.0 MPa of polyethylene glycol 8,000 or 100 mM NaCl. T_2 plants were stressed by withholding water or by adding 150 mM NaCl to the nutrient medium. Fresh weight of −mtlD calli was reduced by 40% in the presence of polyethylene glycol and 37% under NaCl stress. Growth of +mtlD calli was not affected by stress. In −mtlD plants, fresh weight, dry weight, plant height, and flag leaf length were reduced by 70%, 56%, 40%, and 45% compared with 40%, 8%, 18%, and 29%, respectively, in +mtlD plants. Salt stress reduced shoot fresh weight, dry weight, plant height, and flag leaf length by 77%, 73%, 25%, and 36% in −mtlD plants, respectively, compared with 50%, 30%, 12%, and 20% in +mtlD plants. However, the amount of mannitol accumulated in the callus and mature fifth leaf (1.7–3.7 μmol g^{-1} fresh weight in the callus and 0.6–2.0 μmol g^{-1} fresh weight in the leaf) was too small to protect against stress through osmotic adjustment. We conclude that the improved growth performance of mannitol-accumulating calli and mature leaves was due to other stress-protective functions of mannitol, although this study cannot rule out possible osmotic effects in growing regions of the plant.

Water stress and salinity are major abiotic factors that limit crop productivity in drought-prone areas. One way of increasing productivity in stressful environments is to breed crops that are more tolerant to stress. However, success in breeding for tolerance has been limited because (a) tolerance to stress is controlled by many genes, and their simultaneous selection is difficult (Richards, 1996; Yeo, 1998; Flowers et al., 2000); (b) tremendous effort is required to eliminate undesirable genes that are also incorporated during breeding (Richards, 1996); and (c) there is a lack of efficient selection procedures particularly under field conditions (Ribaut et al., 1997). Genetic engineering offers an alternative approach for developing tolerant crops. Unlike classical breeding, genetic engineering is a faster and more precise means of achieving improved tolerance (Cushman and Bohnert, 2000) because it avoids the transfer of unwanted chromosomal regions. Moreover, through genetic engineering, multiple genes can be assembled and simultaneously introduced to the crop of interest. There are many functional targets for engineering tolerance to water stress and salinity, one of them being accumulation of osmoprotectants (Rathinasabapathi, 2000).

The osmolyte mannitol is normally synthesized in numerous plant species, but not in wheat (*Triticum aestivum*). In celery (*Apium graveolens*), mannitol is synthesized in equal proportion to that of Suc. It also constitutes as much as 50% of the translocated photoassimilate (Loester et al., 1992). Mannitol accumulation increases when plants are exposed to low water potential (Ψ_w; Patonnier et al., 1999), and accumulation is regulated by inhibition of competing pathways and decreased mannitol consumption and catabolism (Pharr et al., 1995; Stoop et al., 1996). In celery, salt stress inhibits Suc synthesis but does not affect the enzymes for mannitol biosynthesis. Moreover, the rate of mannitol use in sink tissues decreases during salt stress mainly because of the suppression of the NAD^+-dependent mannitol dehydrogenase, which oxidizes mannitol to Man (Pharr et al., 1995; Stoop and Pharr, 1996). Studies using transgenic tobacco (*Nicotiana tabacum*) and Arabidopsis also showed improved growth of mannitol-accumulating plants under stress (Tarczynski et al., 1992, 1993; Thomas et al., 1995). However, these studies lack actual measurements of Ψ_w and osmotic potential (Ψ_s) in assessing the role of mannitol in

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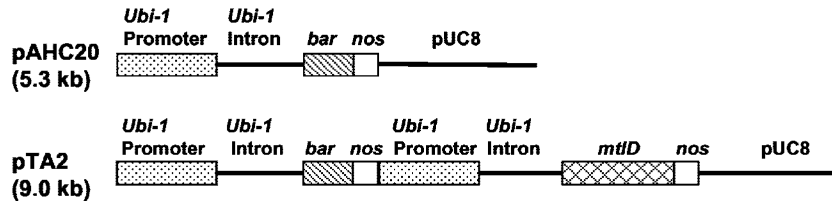

Figure 1. Plasmids used for wheat transformation. Plasmid pAHC20 contains only the selectable marker *bar*. Plasmid pTA2 contains *bar* and the *E. coli mtlD* gene for biosynthesis of mannitol-1-phosphate. Both genes were under the control of the maize (*Zea mays*) *ubi*-1 promoter. Calli and plants transformed with pTA2 were used as mannitol-accumulating lines (+mtlD), and those transformed with pAHC20 served as negative controls (−mtlD).

stress tolerance. Subsequent work suggested that mannitol accounted for only 30% to 40% of Ψ_s changes observed in transgenic tobacco (Karakas et al., 1997). The function of mannitol in stress tolerance has not been evaluated in plants of agronomic importance. Here, we introduced the *mtlD* gene of *Escherichia coli* (Davis et al., 1988) into wheat to evaluate its role in improving tolerance to water stress and salinity. *MtlD* encodes for mannitol-1-phosphate dehydrogenase that catalyzes the reversible conversion of Fru-6-phosphate to mannitol-1-phosphate. In transgenic plants, mannitol-1-phosphate is converted to mannitol via nonspecific phosphatases (Thomas et al., 1995).

RESULTS

Response of Calli to Water and Salt Stress

Recombinant constructs with (pTA2) or without (pAHC20) the mannitol-1-phosphate dehydrogenase (*mtlD*) gene of *E. coli* (Davis et al., 1988) were introduced into wheat calli and were subsequently regenerated into plants (Fig. 1). Calli transformed with pAHC20 and expressing the *bar* gene alone (−mtlD) exhibited a 40% reduction in growth in the presence of −1.0 MPa of polyethylene glycol (PEG) and a 37% growth reduction under 100 mM NaCl stress. Calli transformed with pTA2 expressed *mtlD* (+mtlD), and PEG and NaCl stresses had no effect on their growth (Table I; Fig. 2). PEG and NaCl increased accumulation of soluble carbohydrates in both −mtlD and +mtlD calli (Table II). The +mtlD calli accumulated 81% and 118% more mannitol in the presence of PEG and NaCl, respectively, relative to their unstressed counterparts. Depending on treatment type, mannitol accounted for 8% to 14% of the total soluble carbohydrates in +mtlD calli (Table II). Stress also increased Glc, Fru, and Suc in both types of calli. Accumulation of mannitol in +mtlD calli coincided with reduced Suc content regardless of whether the calli were stressed (Table II). PEG and NaCl reduced the Ψ_w and Ψ_s of both −mtlD and +mtlD calli. However, there were no significant differences in Ψ_w or Ψ_s between the two callus types under either PEG or NaCl stress (Table III). Calli exposed to stress showed osmotic adjustment of −0.34 to −0.29 MPa.

Table I. *Fresh weight of transgenic wheat calli grown under PEG and NaCl stresses*

Stress was applied to 0.25 g of 6-month-old calli (C1–11, −mtlD; and C2–20, +mtlD) by supplementing the Murashige and Skoog medium with −1.0 MPa of PEG or 100 mM NaCl. Measurements were taken 60 d after stress. Data are means of three replications.

Stress Level	Callus Type	Stress Type[a]	
		PEG	NaCl
		g fresh wt	
Unstressed	−mtlD	4.0b	3.8b
	+mtlD	3.6b	4.5b
Stressed	−mtlD	2.4a	2.4a
	+mtlD	3.7b	4.3b
	$LSD_{0.05}$	0.7	0.5

[a] Means followed by the same letter in a column are not significantly different at $P < 0.05$ as determined by Fisher's protected LSD test.

Response to Water Stress and Salinity at the Whole-Plant Level

Water stress reduced growth of both −mtlD and +mtlD plants. However, the effect of stress was more severe on −mtlD than on +mtlD plants (Table IV; Fig. 3). In −mtlD plants, shoot fresh weight was reduced by 70%, dry weight by 56%, plant height by 40%, flag leaf length by 45%, and number of tillers by 75%. In +mtlD plants, fresh weight was reduced by 40%, dry weight by 8%, plant height by 18%, and flag leaf length by 29%. Water stress did not affect the

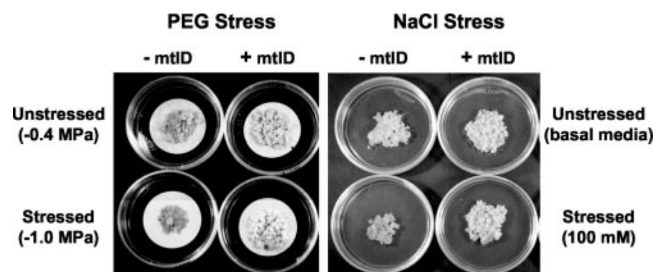

Figure 2. Effect of osmotic stress on the growth of transgenic wheat calli. The mannitol-accumulating callus line C2-20 (+mtlD) and the nonaccumulating line C1-11 (−mtlD) were grown in Murashige and Skoog medium containing PEG 8,000 (−1.0 MPa) or 100 mM NaCl for 60 d.

Table II. *Mannitol and soluble sugar content of transgenic wheat grown under stress*

Six-month-old calli C1–11 (−mtlD) and C2–20 (+mtlD) were grown in Murashige and Skoog media containing −1.0 MPa of PEG or 100 mM NaCl for 60 d. Wheat plants P1-13-1 (−mtlD) and P2-19-1 (+mtlD) were first grown in soil or hydroponically. Then stress was imposed for 30 d by withholding water from plants grown in soil or by adding 150 mM NaCl to plants grown hydroponically. In plants, carbohydrate content was measured on the fifth leaf. Data are means of three replications.[a]

Growth stage	Stress Level	Callus/Plant Type	Mannitol	Glc	Fru	Suc
				$\mu mol\ g^{-1}$ *fresh wt*		
Callus						
PEG stress	Unstressed	−mtlD	0.0a	5.7a	4.4a	7.7b
		+mtlD	2.1b	5.3a	3.9a	5.1a
	−1.0 MPa	−mtlD	0.0a	9.4b	8.1b	12.0c
		+mtlD	3.8c	8.2ab	6.5b	9.1b
		$LSD_{0.50}$	1.2	3.4	2.0	1.5
NaCl stress	Unstressed	−mtlD	0.0a	8.2a	5.4b	10.8ab
		+mtlD	1.7b	6.6a	4.2a	9.0a
	150 mM	−mtlD	0.0a	14.2b	8.1d	13.6c
		+mtlD	3.7c	12.2b	6.8c	11.3bc
		$LSD_{0.05}$	0.7	3.5	1.1	2.0
Whole plant						
Water stress	Unstressed	−mtlD	0.0a	5.6a	4.9ab	7.1b
		+mtlD	0.6b	4.9a	3.5a	5.3a
	Stressed	−mtlD	0.0a	15.2b	8.7c	19.5c
		+mtlD	1.5c	12.9b	6.7bc	14.6c
		$LSD_{0.05}$	0.16	4.5	3.0	1.7
NaCl stress	Unstressed	−mtlD	0.0a	8.7a	6.9a	9.7ab
		+mtlD	0.9b	7.0a	4.1a	6.3a
	Stressed	−mtlD	0.0a	13.1b	10.1b	25.8c
		+mtlD	2.0c	10.3ab	6.4a	16.7b
		$LSD_{0.05}$	0.51	3.4	2.8	8.8

[a] Means followed by the same letter in a column are not significantly different at $P < 0.05$ as determined by Fisher's protected LSD test.

number of tillers produced in +mtlD plants (Table IV; Fig. 3).

Water stress increased the concentration of soluble carbohydrates in both −mtlD and +mtlD plants (Table II). In +mtlD plants, the concentration of mannitol was increased by 150%. In the absence of water stress, the +mtlD plants had lower Suc content than the −mtlD plants. There was no difference in the concentration of other soluble sugars between the two plants whether the plants were stressed or not.

Under water stress, Ψ_w and Ψ_s were significantly lower in both −mtlD and +mtlD plants (Table III). In −mtlD plants, Ψ_w was reduced to −2.29 MPa compared with −1.43 MPa in +mtlD plants. This difference was not related to changes in Ψ_s because Ψ_s in both types of plants was similar. Decreased Ψ_w also resulted in wilting and leaf rolling of the −mtlD plants in the 2nd and 3rd d of withholding water. In +mtlD plants, these symptoms were delayed until the 4th d. The −mtlD and +mtlD plants showed osmotic adjustment by −0.23 and −0.37 MPa, respectively.

Plants grown hydroponically had twice the shoot fresh weight and dry weight of those grown in soil. Besides, hydroponically grown plants had more tillers than plants grown 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. In −mtlD plants, salt stress reduced shoot fresh weight by 77%, dry weight by 73%, plant height by 25%, flag leaf length by 36%, and number of tillers by 67%. In +mtlD plants, shoot fresh weight was reduced by 50%, dry weight by 30%, plant height by 12%, length of the flag leaf by 20%, and number of tillers by 57% (Table V; Fig. 3). A similar pattern was observed in root growth. In −mtlD plants, salt stress reduced root fresh weight by 80%, dry weight by 82%, and root length by 32%. In +mtlD plants, root fresh weight was reduced only by 50%, dry weight by 55%, and root length by 23% (Table V; Fig. 3).

Exposure to 150 mM NaCl increased soluble carbohydrate content in both −mtlD and +mtlD plants (Table II). Suc showed the largest increase. In −mtlD plants, Suc content rose by 166% and in +mtlD plants by 165%. In +mtlD plants, salt stress increased the mannitol content by 122%. The increase in mannitol content coincided with reduced Suc content in +mtlD plants.

Salt stress significantly reduced Ψ_w and Ψ_s in both types of plants (Table III). The −mtlD plants had lower Ψ_w and Ψ_s than the +mtlD plants. However, there was no difference in Ψ_s at full turgor, and both plant types osmotically adjusted by the same magnitude: −mtlD plants by −0.56 MPa and +mtlD plants by −0.53 MPa. Lower leaves of −mtlD plants showed greater wilting and more chlorotic tips than the +mtlD plants, although the young fifth leaf was tur-

Table III. *Ψ_w and Ψ_s of transgenic wheat*

For calli, water stress was recorded after exposing lines C1–11 (−mtlD) and C2–20 (+mtlD) to −1.0 MPa of PEG and 100 mM NaCl for 60 d. For plants, lines P1-13-1 (−mtlD) and P2-19-1 (+mtlD) were subjected to water stress and 150 mM NaCl for 30 d. In plants, water stress was measured on the fifth leaf. Data are means of three replications.[a]

Growth Stage	Stress Level	Callus/Plant Type	Ψ_w	Ψ_s	
				Fresh Tissue	Fully Turgid Tissue
				MPa	
Callus					
PEG stress	Unstressed	−mtlD	−0.44a	−0.57a	−0.42a
		+mtlD	−0.43a	−0.60a	−0.48a
	−1.0 MPa	−mtlD	−1.03b	−1.23b	−0.71b
		+mtlD	−1.04b	−1.31b	−0.82b
		$LSD_{0.05}$	0.20	0.28	0.18
NaCl stress	Unstressed	−mtlD	−0.48a	−0.63a	−0.50a
		+mtlD	−0.43a	−0.65a	−0.47a
	150 mM	−mtlD	−0.84b	−1.20b	−0.80b
		+mtlD	−0.82b	−1.17b	−0.76b
		$LSD_{0.05}$	0.17	0.31	0.22
Whole plant					
Water stress	Unstressed	−mtlD	−1.02a	−1.67a	−1.18a
		+mtlD	−0.97a	−1.66a	−1.16a
	Stressed	−mtlD	−2.29b	−2.22b	−1.41b
		+mtlD	−1.43c	−2.20b	−1.53b
		$LSD_{0.05}$	0.25	0.30	0.21
NaCl stress	Unstressed	−mtlD	−0.89a	−1.19a	−1.14a
		+mtlD	−0.84a	−1.21a	−1.10a
	150 mM	−mtlD	−2.00b	−2.71b	−1.70b
		+mtlD	−1.74c	−2.14c	−1.63b
		$LSD_{0.05}$	0.23	0.50	0.17

[a] Means followed by the same letter in a column are not significantly different at $P < 0.05$ as determined by Fisher's protected LSD test.

gid, as revealed by the difference between Ψ_w and Ψ_s (Table III).

DISCUSSION

Our results demonstrate that mannitol improves growth of transgenic wheat under water stress and salinity both at the callus and whole-plant level (Tables I, IV, and V; Figs. 2 and 3). These findings are in agreement with earlier studies that used the same *mtlD* gene in tobacco (Tarczynski et al., 1992, 1993; Karakas et al., 1997; Shen et al., 1997a) and Arabidopsis (Thomas et al., 1995). The amount of mannitol accumulated in transgenic wheat was in the low end of the range reported for tobacco and Arabidopsis. In tobacco, mannitol accumulated to between 1 and 7 μmol g^{-1} fresh weight (Tarczynski et al., 1992, 1993; Shen et al., 1997a). Transgenic Arabidopsis accumulated between 0.05 and 12 μmol g^{-1} fresh weight mannitol (Thomas et al., 1995). In our experiment, depending on the severity of stress, wheat accumulated 1.7 to 3.7 μmol g^{-1} fresh weight in the callus and 0.6 to 2.0 μmol g^{-1} fresh weight in the mature fifth leaf. Earlier studies concluded that the amount of mannitol accumulated was inadequate to account for osmotic effects (Tarczynski et al., 1992, 1993; Thomas et al., 1995). However, no direct measurement of plant water status was made. In a subsequent

Table IV. *Shoot fresh weight, dry weight, plant height, and number of tillers of transgenic wheat plants exposed to water stress*

T_2 plants (P1-13-1, −mtlD; and P2-19-1, +mtlD) were grown in soil and exposed to stress by withholding water. Measurements were taken after 30 d of the imposition of stress. Data are means of three replications.[a]

Stress Level	Plant Type	Shoot weight		Plant Height	Flag Leaf Length	No. of Tillers
		Fresh Wt	Dry Wt			
		g		*cm*		
Unstressed	−mtlD	11.7c	2.5b	47.0c	28.3b	4.0b
	+mtlD	11.9c	2.6b	45.0c	26.3b	3.0b
Stressed	−mtlD	3.5a	1.1a	28.0a	15.6a	1.0a
	+mtlD	7.1b	2.4b	36.7b	18.7a	3.0b
	$LSD_{0.05}$	2.9	0.8	7.3	7.0	1.6

[a] Means followed by the same letter in a column are not significantly different at $P < 0.05$ as determined by Fisher's protected LSD test.

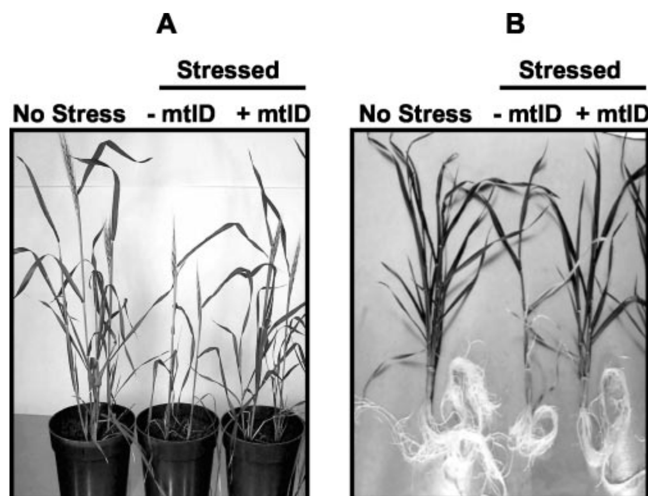

Figure 3. Effect of water stress and salinity on the growth of +mtlD and −mtlD plants. The mannitol-accumulating transgenic wheat line P2-19-1 (+mtlD) and the nonaccumulating P1-13-1 (−mtlD) were stressed by withholding water (A) or by supplementing the nutrient solution with 150 mM NaCl (B) for 30 d. Pictures were taken 30 d after the imposition of water stress and 20 d after NaCl stress. In the absence of stress, −mtlD and +mtlD plants were similar in size; thus, for unstressed controls, only the −mtlD plants are shown.

study, Karakas et al. (1997) estimated that in salt-stressed transgenic tobacco mannitol contributes only 3×10^{-3} to 4×10^{-3} MPa to osmotic adjustment.

Mannitol has been proposed to enhance tolerance to water deficit stress primarily through osmotic adjustment (Loester et al., 1992). Our data show that there was no difference in the Ψ_s of −mtlD and +mtlD transformants at the callus level or in mature fifth leaves and that both adjusted osmotically to an equal extent when exposed to water and osmotic stresses (Table III). The amount of mannitol accumulated in response to stress was small (Table II), and its effect on osmotic adjustment was less than that of other carbohydrates. We estimated the contribution of mannitol to osmotic adjustment using the van't Hoff's equation, $\Psi_s = -cRT$, where c is the solute concentration in mol L^{-1}, R is a constant (8.2×10^{-3} L MPa mol^{-1} K^{-1}) and T is temperature in Kelvins (Table VI). Accordingly, at 85% relative water content (stressed), 3.8 μmol g^{-1} fresh weight of mannitol in PEG-stressed calli and 3.7 μmol g^{-1} fresh weight in NaCl-stressed calli would contribute only -1.1×10^{-2} MPa to Ψ_s of fresh calli. At 95% relative water content (unstressed), the contribution of mannitol to Ψ_s of turgid calli would be -9.9×10^{-3} (PEG stress) and -9.5×10^{-3} MPa (NaCl stress). This represents only 1.2% of the Ψ_s at full turgor or 2% to 3% of the osmotic adjustment of stressed calli. The fate of mannitol in transgenic plants is unknown. Because wheat does not naturally synthesize mannitol, it may not have the mechanism to transport or metabolize this sugar alcohol, and mannitol is most likely stored in the cytosol as a dead-end product. Assuming that mannitol is accumulated in the cytosol and that the cytosol represents 5% of the total water content of fully turgid tissue, mannitol would contribute -2.0×10^{-1} and -1.9×10^{-1} MPa to Ψ_s of calli exposed to PEG and NaCl, respectively (Table VI). If Ψ_s and osmotic adjustment of the cytosol are similar to the values measured on the tissue basis, this will represent 25% of the Ψ_s at full turgor of PEG- and NaCl-stressed calli.

Similarly, at 88% relative water content (stressed), mannitol levels of 1.5 μmol g^{-1} fresh weight in water-stressed plants and 2.0 μmol g^{-1} fresh weight in salt-stressed plants would contribute -4.9×10^{-3} MPa and -6.3×10^{-3} MPa to Ψ_s of the fresh leaf, respectively. At 96% relative water content (unstressed), the contribution of mannitol to Ψ_s of turgid leaves would be -4.3×10^{-3} (water stress) and -5.6×10^{-3} MPa (NaCl stress). This represents 0.4% and 0.3% of the Ψ_s at full turgor of water- and NaCl-stressed leaves, respectively. With the same assumptions made above, the contribution of mannitol to Ψ_s in the cytosol of water- and salt-stressed plants would be -8.6×10^{-2} and -1.1×10^{-1} MPa at full turgor, respectively (Table VI). This corresponds to 5.6% and 6.7% of the Ψ_s of leaves at full turgor. This is rather insignificant relative to the total osmotic adjustment. Besides, there was no significant difference in the osmotic adjustment between the −mtlD and +mtlD wheat either at the callus or whole-plant

Table V. *Shoot weight, root weight, plant height, length of the flag leaf, length of root, and number of tillers of transgenic wheat plants exposed to NaCl*

T_2 plants (P1-13-1, −mtlD; and P2-19-1, +mtlD) were grown hydroponically, and stress was imposed by raising the salt concentration of the nutrient medium to 150 mM NaCl. Measurements were taken after 30 d of stress. Data are means of three replications.[a]

Stress Level	Plant Type	Shoot					Root		
		Fresh Wt	Dry Wt	Plant Height	Flag Leaf Length	No. of Tillers	Fresh Wt	Dry Wt	Length
		g		*cm*			*g*		*cm*
Unstressed	−mtlD	28.3c	4.8c	50.0b	34.7c	6.0c	17.8c	1.1c	63b
	+mtlD	24.2c	3.7c	51.0b	33.7c	7.0d	16.9c	1.1c	61b
Stressed	−mtlD	6.4a	1.3a	37.7a	22.7a	2.0a	3.5a	0.2a	43a
	+mtlD	12.2b	2.6b	45.0b	27.0b	3.0b	8.5b	0.5b	47a
	$LSD_{0.05}$	5.7	1.1	6.6	2.3	0.5	4.2	0.2	12.1

[a] Means followed by the same letter in a column are not significantly different at $P < 0.05$ as determined by Fisher's protected LSD test.

Table VI. *Contribution of mannitol to osmotic potential in transgenic wheat (+mtlD)*

Osmotic potentials were calculated from mannitol contents in Table II using van't Hoff's equation, $\Psi_s = -cRT$, where c is solute concentration in mol L^{-1}, R is a constant (8.2×10^{-3} L MPa mol^{-1} K^{-1}), and T is temperature in K (at room temperature T = 298 K).

Growth Stage	Stress Type	Stress Level	Mannitol Content		Ψ_s[c]		Contribution to Cytosolic Ψ_s[f]
					Fresh Tissue[d]	Fully Turgid Tissue[e]	
			μmol g^{-1} fresh wt[a]	*Mol L^{-1}*[b]	*MPa*		
Calli	PEG	Unstressed	2.1	2.5×10^{-3}	-6.1×10^{-3}	-5.8×10^{-3}	-1.1×10^{-1}
		−1.0 MPa	3.8	4.8×10^{-3}	-1.1×10^{-2}	-9.9×10^{-3}	-2.0×10^{-1}
	NaCl	Unstressed	1.7	2.0×10^{-3}	-4.8×10^{-3}	-4.6×10^{-3}	-9.2×10^{-2}
		150 mM	3.7	4.6×10^{-3}	-1.1×10^{-2}	-9.5×10^{-3}	-1.9×10^{-1}
Whole plant	Water	Unstressed	0.6	0.8×10^{-3}	-2.0×10^{-3}	-1.9×10^{-3}	-3.8×10^{-2}
		Stressed	1.5	2.0×10^{-3}	-4.9×10^{-3}	-4.3×10^{-3}	-8.6×10^{-2}
	NaCl	Unstressed	0.9	1.1×10^{-3}	-2.7×10^{-3}	-2.6×10^{-3}	-5.2×10^{-2}
		150 mM	2.0	2.6×10^{-3}	-6.3×10^{-3}	-5.6×10^{-3}	-1.1×10^{-1}

[a] Mannitol content for stressed tissues are from Table II. [b] Mol L^{-1} = [(μmol mannitol g^{-1}fresh weight × 100/water content per fresh weight)/1,000]. Water accounted for 85% and 80% of the fresh weight of unstressed and stressed calli, respectively. For plants in water stress experiment, 76% (unstressed leaf) and 73% (stressed leaf) of fresh weight was water. For plants in salt stress, 80% (unstressed leaf) and 78% (stressed leaf) of fresh weight was water. [c] Relative water content (a) Unstressed calli: fresh calli, 95%; fully turgid calli, 98%; stressed calli: fresh calli; 85%; fully turgid calli; 95%. (b) Unstressed fifth leaf: fresh leaf, 95%, fully turgid leaf, 97%; stressed fifth leaf: fresh leaf, 88%; fully turgid leaf, 96%. [d] Ψ_s of fresh tissue under stress = $-cRT$. [e] Ψ_s of (a) fully turgid calli: unstressed calli, $-cRT \times (95/100)$; stressed calli, $-cRT \times (85/100)$; and (b) fully turgid leaf: unstressed leaf, $-cRT \times (96/100)$; stressed leaf, $-cRT \times (88/100)$. [f] Osmotic contribution of mannitol to the cytosol (assuming all the mannitol is in the cytosol, which accounts for 5% of cell volume) = Ψ_s of turgid tissue × (100/5).

level (Table III), suggesting that the beneficial effect of mannitol resulted from protective mechanisms other than osmotic adjustment. Although very unlikely, the possibility that mannitol may have an osmotic effect in growing regions of +mtlD wheat plants remains to be determined. As mentioned above, previous studies in tobacco and Arabidopsis have shown accumulation of two to three times higher levels of mannitol than we found in wheat, yet they were not considered meaningful in terms of osmotic adjustment (Tarczynski et al., 1992, 1993; Thomas et al., 1995; Karakas et al., 1997; Shen et al., 1997a). Studies with other osmolytes also showed that marginal accumulation of fructan, Pro, and trehalose improves growth of transgenic tobacco under stress without being involved in osmotic adjustment (Kavi Kishor et al., 1995; Pilon-Smits et al., 1995; Holmström et al., 1996).

How could a small amount of mannitol enhance the tolerance of transgenic wheat to water stress and salinity? Besides its function in osmotic adjustment, mannitol improves tolerance to stress through scavenging of hydroxyl radicals ($OH^{\bullet}$) and stabilization of macromolecular structures (Smirnoff and Cumbes, 1989; Crowe et al., 1992; Shen et al., 1997a, 1997b). Reactive oxygen species in general react aggressively with biological molecules and can cause lipid peroxidation, breakdown of macromolecules and damage to nucleic acids (Smirnoff, 1998). Oxidative stress is common in plants during water stress (Smirnoff, 1993, 1998). The importance of mannitol as a scavenger of the hydroxyl radical ($OH^{\bullet}$) has been demonstrated in vitro (Smirnoff and Cumbes, 1989) and in vivo using transgenic tobacco (Shen et al., 1997a). In tobacco, mannitol protects the thiol-regulated enzyme phosphoribulokinase, thioredoxin, ferredoxin, and glutathione from $OH^{\bullet}$ (Shen et al., 1997b). The mechanism by which mannitol interacts with $OH^{\bullet}$ remains to be explained. Stabilization of macromolecular structure involves formation of hydrogen bonds. Under limited water availability, osmolytes can form hydrogen bonds with macromolecules and thus prevent formation of intramolecular H-bonds that would otherwise irreversibly change their three-dimensional structure (Crowe et al., 1992). Unlike osmotic adjustment, $OH^{\bullet}$ scavenging and other protective functions require only small amounts of mannitol, and it is likely that the improved performance of transgenic wheat observed in this study was the result of $OH^{\bullet}$ scavenging and/or improved stability of macromolecular structures.

We have shown that similar to previous studies in model plants, the improved performance of mannitol-accumulating wheat under stress was not likely to involve osmotic adjustment in calli and mature leaves. For osmotic adjustment to be important, a higher concentration of mannitol is needed. Theoretically, increased accumulation of mannitol can be achieved by diverting more carbon to mannitol biosynthesis. However, accumulation of too much mannitol may have adverse effects. First, diverting carbon that is normally destined to Suc synthesis will reduce or deplete the Suc pool with a deleterious effect on growth especially in plants where Suc is the major carbohydrate translocated. Second, even though mannitol is a compatible solute, the target plant may not tolerate high levels of mannitol. The appropriateness of the term compatible solute for

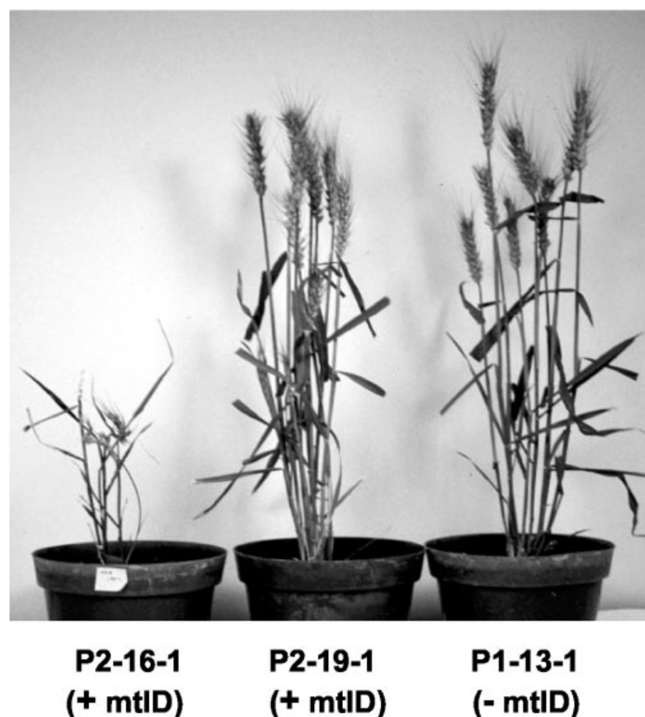

Figure 4. Phenotypes observed in transgenic wheat plants. Lines P2-16-1 and P2-19-1 were transformed with plasmid pTA2 for accumulation of mannitol in the cytosol (+mtlD). Line P1-13-1 was transformed with pAHC20 (−mtlD) and did not accumulate mannitol. Most +mtlD plants were short and sterile and had twisted leaves and heads similar to P2-16-1. In addition, the sterile plants had high mannitol (more than 1.5 μmol g^{-1} fresh weight) and low Suc content (less than 2 μmol g^{-1} fresh weight). In the fertile +mtlD plants, mannitol content ranged from 0.4 to 0.7 μmol g^{-1} fresh weight.

osmolytes is questionable because marginal accumulations can induce pleiotropic effects (Hare et al., 1998). The plant line P2-19-1 (+mtlD) used in our study accumulated only 0.7 μmol g^{-1} fresh weight mannitol in the flag leaf under unstressed conditions. This was the highest amount of mannitol accumulated without causing any noticeable side effects in transgenic wheat. Plants that accumulated higher mannitol had severe abnormalities including sterility, stunted growth, twisted heads, and curled leaves (Fig. 4). For instance, the sterile plant line P2-16-1 (Fig. 4) accumulated 1.6 μmol g^{-1} fresh weight mannitol in the flag leaf. The Suc content in this line was exceptionally low; only 1.9 μmol g^{-1} fresh weight compared with 3.5 μmol g^{-1} fresh weight in the fertile +mtlD line P2-19-1 and 4.5 μmol g^{-1} fresh weight in the −mtlD line P1-13-1. In a related study, transgenic tobacco plants expressing *mtlD* were 20% to 25% smaller in size and had reduced Suc compared with the wild type (Karakas et al., 1997). Sheveleva et al. (2000) found that tobacco plants expressing *mtlD* and *IMT1* (*myo*-inositol-*O*-methyltransferase) had abnormal flower development and reduced sugar content. Exogenous application of Gly betaine to a non-accumulating plant was found to destabilize membranes and to inhibit protein synthesis and osmotic-induced accumulation of Pro (Gibon et al., 1997). Stress-induced accumulation of Pro also results in reduced growth (Hare and Cress, 1998). These results point to the need to carefully optimize the use of existing osmoprotectant-based mechanisms and to explore the development of alternative engineering strategies, such as the use of stress-inducible expression systems for stress tolerance determinants, which lack potential detrimental effects on growth.

MATERIALS AND METHODS

Gene Constructs and Biolistic Transformation

The open reading frame of *mtlD* (1.2 kb) was amplified by PCR from pCab-mtlD (a gift from Dr. Hans Bohnert) and ligated to the *Bam*HI site of pAHC17 (Christensen and Quail, 1996). To the *Hin*dIII site of the resulting plasmid, the *ubi-bar-nos* region of pAHC20 (Christensen and Quail, 1996) was ligated to create pTA2 (Fig. 1). Both *mtlD* and the selectable marker *bar* in pTA2 were under the control of the maize (*Zea mays*) *ubi-1* promoter (Christensen et al., 1992). For accumulation of mannitol in the cytosol, wheat (*Triticum aestivum* L. cv Bobwhite) was transformed with pTA2 using the He-driven PDS 1000 and regenerated as described by Weeks (1995). As a negative control, wheat was also transformed with pAHC20.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

Plant Materials and Growth Conditions

Twenty T_0 plants transformed with pTA2 (+mtlD) were obtained. Thirteen of these plants were sterile, were stunted in growth, and had twisted leaves and heads. In the remaining seven plants, mannitol concentration ranged from 0.4 to 0.7 μmol g^{-1} fresh weight. Mannitol concentration in calli ranged from 0.3 to 2.0 μmol g^{-1} fresh weight (data not shown). To be able to observe differences in the response of pTA2-transformed (+mtlD) and pAHC20-transformed (−mtlD) lines, a transformation event that resulted in progeny with a high mannitol content and phenotypically identical to the control for plant traits under non-stress conditions was selected for analyses of growth, water relations, and soluble carbohydrates in response to water or salinity stresses. The event represented by callus line C2-20 and plant line P2-19-1 was transformed with pTA2 (+mtlD) and accumulated 2.0 and 0.7 μmol g^{-1} fresh weight of mannitol, respectively; it was selected for characterization. Moreover, for experiments involving calli, tissues of the same age (6 months) were used to avoid variations due to differences in physiological states. Accordingly, the event represented by callus line C1-11 and plant line P1-13-1 transformed with pAHC20 (−mtlD) served as negative controls. Stress at the tissue level was imposed on 0.25 g of calli grown on Murashige and Skoog maintenance media (Weeks, 1995) containing PEG 8,000 (Ψ_s = −1.0 MPa) or 100 mM NaCl for 60 d. Calli were transferred to fresh media every 2 weeks.

Stress at the whole-plant level was imposed on T_2 plants derived from immature embryos of +mtlD and −mtlD T_1 kernels. Seedlings were first screened for the *bar* gene on media containing 3 mg L^{-1} bialaphos. After 2 weeks, healthy seedlings were transferred to 15- × 12-cm pots filled with 350 g of soil or to troughs filled with 3.5 L of aerated nutrient solution. Plants were maintained in a growth chamber at 200 μmol m^{-2} s^{-1} photosynthetically active radiation, 23°C/17°C day/night temperature, 70% relative humidity, and 16-h photoperiod. After 3 weeks, three uniform plants were randomly assigned to stress. Stress was imposed by watering plants with 50 mL of water at 3- to 4-d intervals compared with 150 mL for unstressed controls. For salt stress, plants were exposed to 150 mM NaCl by

raising the NaCl concentration of the nutrient solution 30 mm per day over a 5-d period. The solution was changed every 3 to 4 d.

Measurement of Growth and Water Relations

Growth was measured at the end of the stress period. For calli, fresh weight was measured. For plants, fresh weight, dry weight, plant height, length of the flag leaf, and number of tillers were recorded. In addition, for salt-stressed plants, fresh weight, dry weight, and length of the root were measured. Ψ_w and Ψ_s were determined for calli and the fifth leaf. Ψ_w was measured with leaf cutter psychrometers (Merrill Specialty Equipment, Logan, UT) connected to an automatic Ψ_w measurement system (HP-115, Wescor, Logan, UT). Ψ_s was measured using a Vapro vapor pressure osmometer (Wescor). Osmotic adjustment was determined as the difference between Ψ_s at full turgor (after rehydration in distilled water for 2 h) between stressed and unstressed tissues.

Determination of Carbohydrate Content

Mannitol and other soluble carbohydrates were extracted from calli and leaves as described before (Adams et al., 1993) and separated using a high-performance anion-exchange chromatography system coupled to a pulsed amperometric detector. Fifty-microliter samples were injected into a 9- × 250-mm Carbopac PA1 column (Dionex, Sunnyvale, CA), and carbohydrates 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 were determined using an integrator.

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