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A plastid-localized glycogen synthase kinase 3 modulates stress tolerance and carbohydrate metabolism

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Summary

Glycogen synthase kinase 3 (GSK-3) was originally identified as a regulator of glycogen synthesis in mammals. Like starch in plants, glycogen is a polymer of glucose, and serves as an energy and carbon store. Starch is the main carbohydrate store in plants. Regulation of starch metabolism, in particular in response to environmental cues, is of primary importance for carbon and energy flow in plants but is still obscure. Here, we provide evidence that MsK4, a novel *Medicago sativa* GSK-3-like kinase, connects stress signalling with carbon metabolism. MsK4 was found to be a plastid-localized protein kinase that is associated with starch granules. High-salt stress rapidly induced the *in vivo* kinase activity of MsK4. Metabolic profiling of MsK4 over-expressor lines revealed changes in sugar metabolism, including increased amounts of maltose, the main degradation product of starch in leaves. Plants over-expressing MsK4 showed improved tolerance to salt stress. Moreover, under high-salinity conditions, MsK4-over-expressing plants accumulated significantly more starch and showed modified carbohydrate content compared with wild-type plants. Overall, these data indicate that MsK4 is an important regulator that adjusts carbohydrate metabolism to environmental stress.

Keywords: signal transduction, protein phosphorylation, starch.

Introduction

High soil salinity severely affects agricultural productivity (Boyer, 1982). According to the FAO and UNESCO, 20% of the cultivated land worldwide is impaired by salinity, and the endangered areas are constantly increasing due to global changes and irrigation techniques. Salinity is detrimental to plant growth and is a cause of osmotic stress, ion cytotoxicity and nutritional defects. Plants have evolved various strategies to cope with salinity, including accumulation of osmoprotectants and recovery of ion homeostasis. Furthermore, for growth and development under high-salt conditions, maintenance of metabolic activity is essential (Hasegawa *et al.*, 2000; Munns, 2002; Zhu, 2001).

Genetic and biochemical studies indicate that distinct signal transduction pathways mediate various aspects of high-salt stress in plants (Xiong *et al.*, 2002). Protein kinases constitute important regulators in these circuits. The Ca^{2+}-dependent SOS (salt overly sensitive) pathway regulates ion homeostasis by promoting sodium exclusion from the cytosol (Zhu, 2002; Zhu *et al.*, 1998). A range of MAP kinase signalling pathways is activated by high salinity, and

these appear to mediate hyperosmotic stress conditions generally (Droillard *et al.*, 2000; Hoyos and Zhang, 2000; Ichimura *et al.*, 2000; Kiegerl *et al.*, 2000; Matsuoka *et al.*, 2002; Mikolajczyk *et al.*, 2000; Munnik *et al.*, 1999; Teige *et al.*, 2004). Similarly, members of the sucrose non-fermenting 1-related protein kinase 2 family have been reported to be activated by salt stress and other hyperosmolarity treatments (Bohnert and Sheveleva, 1998; Boudsocq *et al.*, 2004; Mikolajczyk *et al.*, 2000; Umezawa *et al.*, 2004).

Glycogen synthase kinase 3 (GSK-3) was originally identified in mammals as a cytoplasmic serine/threonine protein kinase that regulates metabolism of the animal storage carbohydrate glycogen (Embi *et al.*, 1980; Woodgett and Cohen, 1984). Mammalian GSK-3 phosphorylates and inactivates glycogen synthase, the rate-limiting enzyme of glycogen synthesis. Animal GSK-3 not only controls metabolism but is a multi-functional kinase that acts as a regulator of numerous signalling pathways, including cell fate determination, microtubule function, cell-cycle regulation and apoptosis (Cohen and Frame, 2001; Doble and Woodgett, 2003; Jope and Johnson, 2004).

GSK-3 homologues are found in all eukaryotes. Mammals have two GSK-3 genes, and *Drosophila melanogaster* generates numerous proteins by differential splicing of the SHAGGY gene locus. Plants possess a larger gene family of GSK-3/SHAGGY-like kinases (GSKs) (Jonak and Hirt, 2002). Although only a few functional studies have been performed on plant GSKs, recent evidence indicates that GSKs are involved in diverse important processes including hormone signalling, development and stress responses (Choe *et al.*, 2002; Dornelas *et al.*, 2000; Jonak *et al.*, 2000; Li and Nam, 2002; Perez-Perez *et al.*, 2002; Piao *et al.*, 2001; Vert and Chory, 2006).

Starch is a polymer of glucose and serves as the main carbohydrate store in plants. It is composed of linear amylose and branched amylopectin arranged into granules. In leaves, transitory starch is synthesized in chloroplasts during the day and degraded to supply energy and carbon skeletons for metabolism during dark periods. Reserve starch is synthesized in amyloplasts in storage tissues and is mobilized on demand.

The synthesis of starch involves starch synthases that transfer the glucosyl group of ADP-glucose produced by ADP-glucose pyophosphorylase to the non-reducing end of α-1,4-glucans. Branching points are introduced by branching enzymes. Debranching enzymes are involved in the synthesis of amylopectin and granule initiation (Smith, 2001; Tetlow *et al.*, 2004a).

Hydrolytic starch degradation in cereal endosperm has been studied intensively but appears to differ from starch breakdown in plastids. Increasing evidence indicates that the main route of starch breakdown in leaves proceeds by a β-amylolytic degradation pathway (Lloyd *et al.*, 2005; Smith *et al.*, 2005): Phosphorylation of starch by glucan–water dikinase and phosphoglucan–water dikinase seems to be important for preparing starch for degradation (Kotting *et al.*, 2005; Lorberth *et al.*, 1998; Ritte *et al.*, 2002; Yu *et al.*, 2001). Subsequent release of glucans from starch granules occurs by as yet unknown mechanisms. β-amylase is believed to further process soluble glucans into maltose (Niittyla *et al.*, 2004; Scheidig *et al.*, 2002). Glucose is also formed during hydrolytic starch degradation. Both maltose and glucose are exported to the cytosol by specific transporters (Niittyla *et al.*, 2004; Weber *et al.*, 2000; Weise *et al.*, 2004). It is assumed that maltose is further metabolized by a cytosolic transglucosidase and a glucan phosphorylase to hexose phosphate and sucrose (Chia *et al.*, 2004; Lloyd *et al.*, 2005; Lu and Sharkey, 2004; Smith *et al.*, 2005; Weber, 2004).

The dynamic throughput of plant carbon demands a tight, yet responsive, control of starch metabolism. Despite the fact that starch metabolism has been studied for many years, little is known about the signalling mechanisms that convey intrinsic and environmental stimuli to regulate this important process. Here we have characterized the location and function of MsK4, a novel member of the GSK family in the forage crop *Medicago sativa*. We provide evidence of a starch-associated protein kinase that, when over-expressed, modulates carbohydrate metabolism. Additionally, we identified MsK4 as a novel signalling component in the high-salinity response, suggesting that MsK4 links stress signalling to metabolic adaptation.

Results

MsK4 is a plastid-localized protein kinase

In a search for novel signal transduction components, we isolated the gene encoding MsK4 from the important foliar crop *M. sativa*. Overall, MsK4 shows approximately 50% identity to mammalian GSK-3 and *Drosophila* SHAGGY kinases (Figure S1). The catalytic domains of GSKs are highly conserved between the kingdoms, but the N- and C-terminal regions vary considerably even within a species. MsK4 only shares 60–70% identity with other members of the *Medicago* GSK family. When compared with *Arabidopsis thaliana* GSKs, MsK4 displays highest sequence similarity to AtK-1 (Jonak *et al.*, 1995) over the entire length of the protein (85%), suggesting that MsK4 and AtK-1 might be orthologues.

The amphipathic N-terminal region of MsK4 was predicted by the PSORT algorithm to function as a chloroplast import sequence with a certainty of 0.95. To test whether MsK4 is located in plastids, processing and import of MsK4 protein into intact chloroplasts were analysed. Firstly, we investigated whether MsK4 is processed by the stromal processing peptidase. Incubation of radiolabelled *in vitro*-translated protein with lysed or intact chloroplasts led to the appearance of a smaller fragment (Figure 1a), indicating that MsK4 can be processed. Consistent with the predicated putative

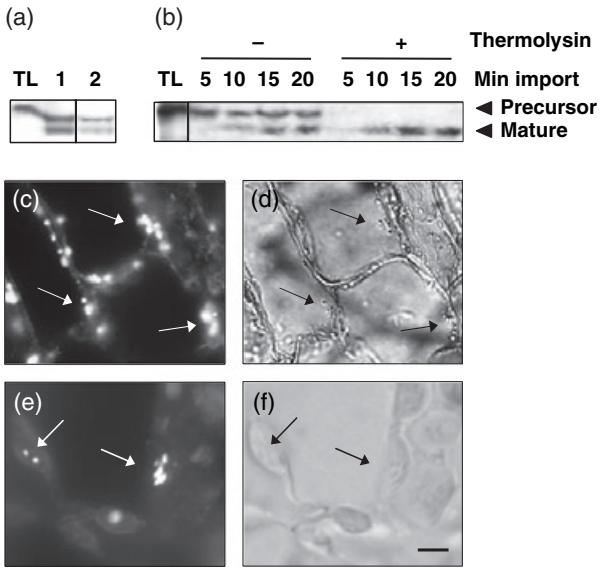

Figure 1. Import and subcellular localization of MsK4 to plastids.
(a) Radiolabelled *in vitro*-translated MsK4 (TL) was incubated with lysed (lane 1) or intact chloroplasts (lane 2).
(b) *In vitro*-translated MsK4 (TL) was incubated with intact chloroplasts for the indicated periods of time without or with subsequent thermolysin treatment.
Indirect immunofluorescence with MsK4-antibody (c,e) and corresponding bright-field images (d,f) of root epidermis and cortex (c,d) and leaf mesophyll cells (e,f). Bar = 3 μm for (c) and (d); bar = 10 μm for (e) and (f).

cleavage site (at position 29), the mature protein is about 3 kDa smaller than the precursor. To confirm the translocation of MsK4 into chloroplasts, the kinetics of MsK4 protein import were analysed. As shown in Figure 1(b), MsK4 was processed and imported in a time-dependent manner. In contrast to the precursor, the processed protein was resistant to digestion by the protease thermolysin, indicating that MsK4 was indeed internalized into intact chloroplasts.

To study the localization and function of the MsK4 protein kinase *in vivo*, an antibody was produced against the C-terminal peptide sequence of MsK4 (Figure S2). We investigated the subcellular location of MsK4 protein in roots, leaves and cultured cells by immunofluorescence microscopy using the anti-MsK4 antibody. In roots, MsK4 was found in amyloplasts, plastids specialized for starch storage, within epidermis and cortex cells (Figure 1c,d). MsK4 was also found in starch-containing plastids of leaf mesophyll cells (Figure 1e,f) and in amyloplasts of suspension-cultured cells (data not shown). In controls, no labelling was detected when the anti-MsK4 antibody was omitted or when anti-MsK4 antibody was pre-incubated with MsK4 peptide (data not shown). Taken together, these results indicate that MsK4 is a plastid-localized protein kinase.

Association of MsK4 with starch granules

Immunofluorescence microscopy suggested that MsK4 might be associated with starch granules within different types of plastids. To investigate MsK4 localization in more detail, immunogold electron microscopy of root cells was performed. MsK4 localized directly to starch granules in plastids of various developmental stages, including young, developing and mature amyloplasts (Figure 2a–c, respectively). Statistic evaluation of the immunogold labelling clearly showed that the majority of MsK4 signal was localized to amyloplasts (Table 1). Analysis of the spatial distribution of gold within amyloplasts showed that about 70% of the gold label was associated with starch granules. Preincubation of the antibody with the C-terminal MsK4 peptide and omission of the anti-MsK4 antibody abolished the MsK4 signal from the starch granules (Figure 2d,e, respectively).

Furthermore, protein gel blot analysis of purified starch granules with the anti-MsK4 antibody detected mature MsK4 as a starch-associated protein, while both the likely precursor and the mature MsK4 protein were present in total protein extracts (Figure 2f). Washing of starch granules with SDS or treatment with thermolysin abolished the MsK4 signal on the immunoblots, indicating that, as expected for a signal transduction component, MsK4 is associated with, but not an integral part of, the starch granules.

To exclude the possibility that the anti-MsK4 antibody cross-reacted non-specifically with components of starch granules, a *Medicago truncatula* suspension culture over-expressing myc-tagged MsK4 was generated. Starch-associated proteins were purified from control cultures and those over-expressing MsK4-myc. Subsequent protein gel blot analysis with anti-myc antibody showed that MsK4-myc was also associated with starch granules (Figure 2g). Taken together, these data indicate that MsK4 is a plastid-localized protein that is associated with starch granules.

Rapid induction of MsK4 kinase activity by hyperosmotic stress

As plant GSKs have been implicated in the response to environmental stress (Jonak and Hirt, 2002), we tested the *in vivo* MsK4 kinase activity in plants grown under standard growth conditions or after exposure to environmental stress. Immunokinase assays with the MsK4-specific antibody showed very little MsK4 activity in roots grown under normal conditions. However, treatment of roots with increasing amounts of NaCl induced MsK4 kinase activity (Figure 3a). High levels of NaCl impose sodium toxicity and osmotic stress. To distinguish between these two phenomena, MsK4 activity was also assayed after KCl and sorbitol treatment. MsK4 activity was similarly induced at high concentrations of NaCl, KCl and sorbitol, indicating that the MsK4 kinase activity is enhanced by hyperosmotic conditions.

Induction of MsK4 activity by NaCl is very rapid and was detected within 2 min after addition of 250 mM NaCl (Figure 3b), showing that induction of MsK4 activity is one of the

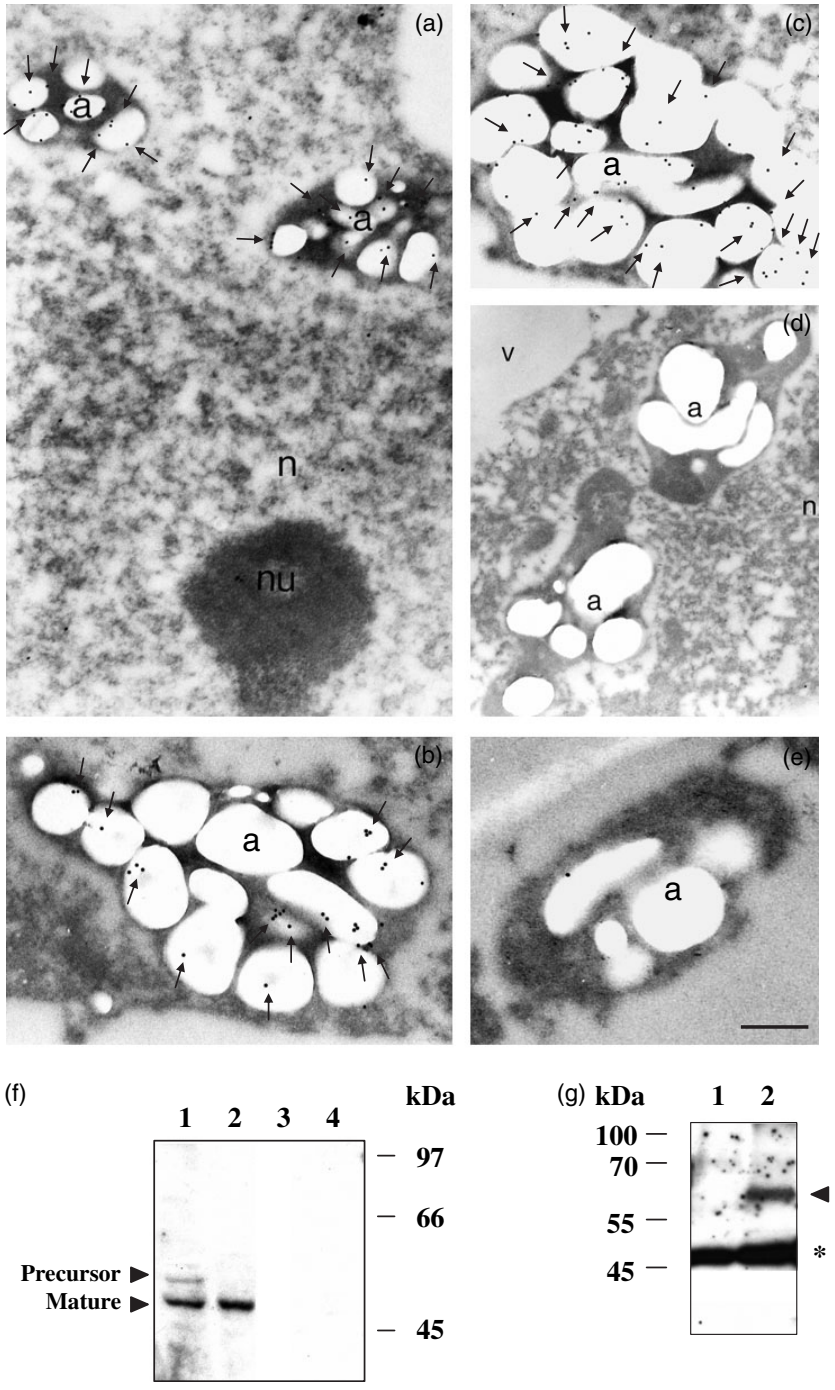

Figure 2. MsK4 is associated with starch granules.
(a–e) Electron micrographs showing immunogold localization of MsK4 in *Medicago* root cap cells. a, amyloplast; n, nucleus; nu, nucleolus; v, vacuole. Bars = 140 nm for (a), (b) and (d), 190 nm for (c) and 110 nm for (e). Arrows indicate gold label.
(a) Overview of immunogold labelling with anti-MsK4 antibody on a section with young amyloplasts.
(b) Developing and (c) mature amyloplasts.
(d) Pre-incubation of anti-MsK4 antibody with MsK4 peptide.
(e) Omission of anti-MsK4 antibody.
(f) Protein gel blot analysis with anti-MsK4 antibody of total protein extract from cultured cells (lane 1), starch-associated proteins (lane 2), protein extracts of starch granules washed with 1% SDS (lane 3) and starch granules treated with thermolysin (lane 4).
(g) Protein gel blot analysis with anti-myc antibody of starch-associated proteins from control *Medicago truncatula* suspension culture (lane 1) and MsK4-myc transformed suspension culture (lane 2). Arrowhead indicates MsK4-myc protein; asterisk indicates constitutive, non-specific labelling.

earliest salt-stress responses detectable in plants. Although MsK4 kinase activity peaked at 10 min, it remained at elevated levels during the 60 min experimental period. To investigate whether the activity of MsK4 can also be stimulated by other abiotic stresses, roots were challenged by cold and drought and assayed for MsK4 kinase activity (Figure 3b). Neither of these conditions induced MsK4 activity, suggesting a specific involvement of MsK4 in the hyperosmotic stress response.

Over-expression of MsK4 enhances salt-stress tolerance

The rapid induction of MsK4 activity by hyperosmotic conditions suggested that this GSK-based pathway might be involved in mediating the salt-stress response in plants. In order to gain insight into the function of MsK4, we generated transgenic *A. thaliana* plants expressing myc-tagged MsK4 under the control of the CaMV 35S promoter. Among the transformants, we obtained four independent single-copy

Table 1 Quantification of immunogold labelling density. Values represent the mean number (±SD; $n = 30$) of gold particles per μm^2 after immunogold labelling with MsK4 antibody and/or in corresponding controls within different subcellular compartments including amyloplasts, cytosol, nucleus, vacuole, mitochondria and cell wall. Note the clear and statistically significant localization of MsK4 to amyloplasts. Control 1, immunodepletion of MsK4 antibody with MsK4 peptide; control 2, omission of anti-MsK4 antibody (primary antibody)

	MsK4 antibody	Control 1	Control 2
Amyloplasts	33.1 ± 3.8	3.8 ± 0.5	1.2 ± 0.1
Cytosol	4.3 ± 1.7	3.5 ± 0.6	0.6 ± 0.2
Nucleus	4.9 ± 2.1	1.4 ± 0.4	0.8 ± 0.1
Vacuole	3.7 ± 1.3	1.2 ± 0.3	0.4 ± 0.1
Mitochondria	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
Cell wall	0.6 ± 0.2	0.4 ± 0.1	0.6 ± 0.2

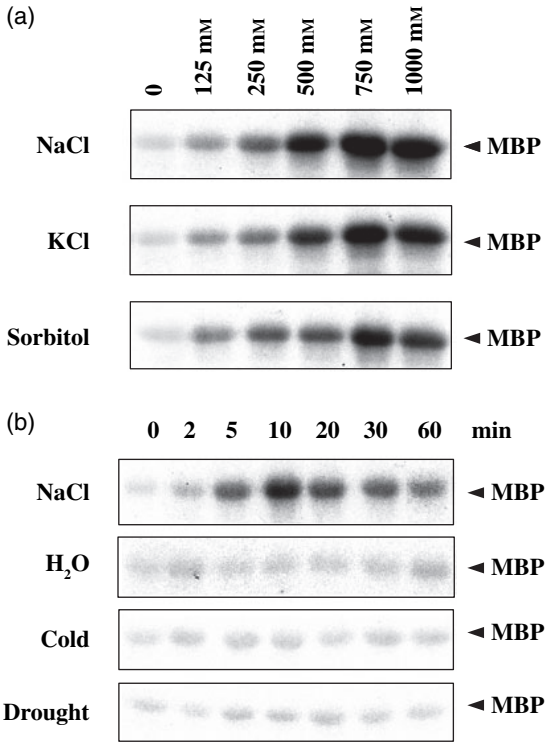

Figure 3. Induction of MsK4 kinase activity by hyperosmotic stress.
(a) Alfalfa roots were exposed for 10 min to 0, 125, 250, 500, 750 and 1000 mM NaCl, KCl or sorbitol.
(b) Kinetics of MsK4 activation by NaCl in alfalfa. Roots were exposed to 250 mM NaCl, water, cold (4°C) or drought for the indicated period of time. Root protein extracts were immunoprecipitated with the anti-MsK4 antibody. Kinase reactions for the immunoprecipitated proteins were performed using myelin basic protein as a substrate. Three independent experiments yielded comparable results.

insertion lines (lines 2, 4, 8 and 9). These lines expressed the MsK4-myc protein (Figure 4a), and MsK4-myc kinase activity could be enhanced by high-salt conditions (Figure 4b).

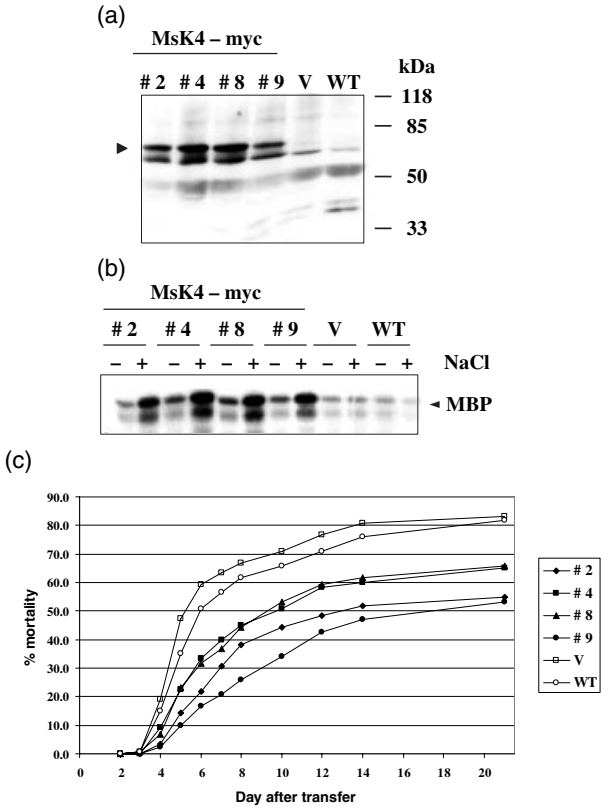

Figure 4. Over-expression of MsK4 enhances salt-stress tolerance.
(a) Protein gel blot analysis with anti-myc antibody of total protein extracts from MsK4-myc-over-expressing lines (lines 2, 4, 8 and 9), vector control (V) and wild-type (WT) plants. The arrowhead indicates the MsK4-myc protein.
(b) Immunokinase assays of MsK4-myc-over-expressing lines (lines 2, 4, 8 and 9), vector control (V) and wild-type (WT) plants. Protein extracts of plants without treatment (–) or after treatment with 250 mM NaCl for 10 min (+) were immunoprecipitated with anti-myc antibody. Subsequently, MsK4-myc kinase activities were determined using myelin basic protein as substrate.
(c) High-salinity stress tolerance test. Eight-day-old seedlings were transferred to 1/2-strength MS plates supplemented with 200 mM NaCl. Four hundred seedlings were used for each line. The mortality rate was determined over a period of 3 weeks. Lines 2, 4, 8 and 9 are distinct MsK4-myc-over-expressing lines; V, vector control; WT, wild-type. Representative data from one of three independent experiments are shown.

MsK4 over-expressor plants showed normal growth behaviour under standard growth conditions. However, when plants were exposed to salt stress, MsK4-myc over-expressor lines showed a significantly higher survival rate in comparison with control lines (Figure 4c). High-salinity tolerance was evaluated by transferring MsK4-over-expressing lines 2, 4, 8 and 9 as well as wild-type and vector control plants from normal growth conditions to medium supplemented with 200 mM NaCl and scoring for the mortality rate. As shown in Figure 4(c), under these conditions, wild-type and vector control plants died more rapidly than MsK4-over-expressing plants. Even after 3 weeks, MsK4-over-expressing plants could withstand the severe high-salinity stress significantly better than wild-type and the vector control

line. As a control, plants over-expressing either a kinase-dead version of MsK4 or the related MsK1 were tested for their survival rate under high-salt conditions but did not show any change in tolerance to high salinity compared to wild-type (data not shown). MsK4 over-expressor plants also flowered under high-salt conditions whereas wild-type plants failed to do so (data not shown). These data indicate that over-expression of MsK4 enhances resistance to high-salinity stress.

MsK4-over-expressing plants show altered metabolite levels

The specific localization of MsK4 prompted us to study the impact of MsK4 on metabolism. To gain a comprehensive view on the metabolite status of MsK4 over-expressors in comparison to wild-type plants, we analysed the metabolic profiles by GC-MS (Fiehn *et al.*, 2000). As plant metabolism is highly dynamic, metabolite levels were determined in the middle of the light period (noon) and at the end of the light period (evening). Metabolites were identified by retention time indices and specific mass spectra. About 80 known and several as yet unidentified metabolites were monitored (Kopka *et al.*, 2005; Schauer *et al.*, 2005). Comparison of the metabolic profiles of MsK4 over-expressor lines 2 and 9 with those of wild-type plants revealed quantitative differences in the abundance of several carbohydrates and compounds of the nitrogen metabolism at both time points (Table 2). Levels of myo-inositol, galactinol, raffinose and melibiose were elevated in MsK4 over-expressors in comparison to wild-type, suggesting an alteration of the raffinose family oligosaccharide pathway (Figure 5). MsK4 over-expressors displayed a 1.4-fold increase in the glucose to fructose ratio (Table 3) and 50% higher maltose levels compared to wild-type plants (Figure 5). Additionally, the levels of some other sugars (arabinose, mannose and xylose) were significantly elevated at noon. The amount of galactonic acid was also significantly increased in MsK4-over-expressing plants. Galactonic acid can serve as a precursor for ascorbate biosynthesis, but ascorbate levels were not found to be elevated in the MsK4 over-expressors. Higher amounts of amine-containing metabolites such as uric acid, putrescine and 4-aminobutyric acid were detected. Several amino acids (valine, tyrosine, threonine, tryptophan, phenylalanine, lysine and asparagine) showed elevated levels. Interestingly, the levels of compounds of the TCA cycle such as malate, succinate and fumarate were comparable in wild-type and MsK4-over-expressing plants. The observed metabolic changes might be caused by enhanced carbohydrate and nitrogen metabolism in MsK4 over-expressors. The overall differences in the metabolite composition between wild-type and MsK4 over-expressor lines were confirmed in independent experiments (Figure S3).

Starch contents of MsK4-over-expressing plants

The association of MsK4 with starch granules and the observed modulation of carbohydrate metabolism in MsK4-over-expressing plants led us to investigate the starch levels. Firstly, we studied the diurnal fluctuation in starch content (Figure 6a). Starch levels were high at the end of the day, decreased during the night and increased again during the subsequent light period. No significant changes in starch levels were observed between MsK4 over-expressor lines and wild-type plants. However, when plants were exposed to high-salt stress by watering with 100 mM NaCl for 4 weeks, MsK4-over-expressing plants accumulated four- to fivefold more starch than wild-type plants (Figure 6b), suggesting an influence of MsK4 on starch metabolism during high-salt stress conditions.

MsK4-over-expressing plants show an altered carbohydrate metabolism after salt treatment

MsK4-over-expressing plants were more tolerant to high-salt stress and displayed higher starch contents when grown for longer periods under high-salinity conditions. These observations prompted us to investigate the impact of MsK4 on the metabolism of plants exposed to high salinity. As MsK4 activity is very rapidly induced by high-salt stress, we analysed the metabolite contents at the onset of stress. The metabolite profile of MsK4 over-expressor and wild-type plants was analysed 6 and 24 h after treatment with 150 mM NaCl in comparison with unstressed plants. Raffinose and galactinol levels were not enhanced significantly during this short period of salt stress in either wild-type or MsK4 over-expressors (data not shown). However, in wild-type plants, starch contents decreased by approximately 20% at the 6 h time point, and further declined by 40% 24 h after salt treatment (Figure 7). Similarly, maltose, glucose and glucose-6-phosphate (G6P) levels were reduced. At both time points, MsK4 over-expressors consistently displayed higher levels of starch, maltose, glucose and G6P compared to wild-type, suggesting a regulatory role of MsK4 on carbohydrate metabolism during high-salinity stress.

Discussion

Protein kinases are major components of intracellular signal transduction. They mediate various extracellular signals to different intracellular targets, enabling plant cells to rapidly acclimatize to prevailing environmental conditions. Adaptive responses that allow survival and growth under saline stress conditions include metabolic adjustment, in particular the maintenance of carbon supply, a process that is still poorly understood at the molecular level. In this work, we identified MsK4 as a plastid-localized protein kinase that is associated with starch granules. High-salinity conditions

Table 2 Metabolite composition of MsK4 over-expressors The metabolite contents of rosette leaves of 4-week-old wild-type and MsK4-over-expressing plants of lines 2 and 9 was determined by GC-MS analysis. Plants were grown under a short-day light regime (8 h light/16 h dark), and harvested at noon (after 4 h light) and in the evening (after 8 h light, end of light period). Pools of ten individual leaf rosettes were analysed four times using GC-MS. Compounds were identified by the quantitative mass tag (tag mass), the retention index (tag RI) and the reverse match value (reverse match) of the mass spectra comparison. Values are fold changes compared with the corresponding wild-type controls. Bold letters indicate significant changes for these metabolites (*t*-test; *P* value < 0.05)

				Fold increase			
				Noon		Evening	
Tag mass	Tag RI	Reverse match	Compound	MsK4 line 2	MsK4 line 9	MsK4 line 2	MsK4 line 9
198	1568.50	837	2-Ketoglutaric acid	0.94	2.57	0.92	0.78
166	1525.70	887	4-Aminobutyric acid	**1.69**	1.59	**2.75**	**1.97**
189	1652.70	791	Arabinose	**1.91**	**2.62**	1.27	1.78
332	1937.30	967	Ascorbic acid	1.32	0.68	**0.51**	**0.64**
103	1665.75	735	Asparagine	1.27	**1.59**	1.24	**1.81**
232	1509.85	971	Aspartic acid	0.84	1.20	1.07	0.93
174	1424.35	914	β-Alanine	1.31	3.75	1.11	**1.33**
147	1739.90	912	*cis*-aconitic acid	1.87	1.46	0.81	0.75
338	2055.40	665	*cis*-sinapic acid	1.26	**1.51**	1.23	1.31
273	1803.35	973	Citric acid	**0.72**	**0.66**	0.88	0.85
157	1839.40	974	Dehydroascorbic acid	1.40	**2.42**	1.69	**2.15**
205	1493.50	930	Erythritol	1.27	**1.68**	1.06	1.50
220	1529.15	919	Erythronic acid	0.93	1.32	1.30	**1.59**
100	1262.00	945	Ethanolamine	**1.72**	1.30	**1.48**	1.30
117	1853.30	939	Fructose	**0.69**	**0.54**	1.37	0.90
315	2292.35	855	Fructose-6-phosphate	0.98	1.04	0.62	0.56
245	1347.20	989	Fumaric acid	1.09	**0.78**	1.07	**0.89**
204	2970.60	952	Galactinol	**2.47**	**2.78**	**2.63**	**2.50**
292	1982.40	934	Galactonic acid	**1.36**	**1.43**	**1.33**	**1.43**
217	1873.60	894	Galactono-lactone	0.93	2.72	1.34	1.87
160	1897.10	679	Galactose	0.50	0.83	1.09	0.80
305	2000.00	679	Gluconic acid	2.56	2.24	2.14	**2.56**
160	1879.60	965	Glucose	0.93	1.00	**1.57**	1.19
299	2307.80	860	Glucose-6-phosphate	0.89	0.86	0.74	0.60
246	1614.30	970	Glutamic acid	0.58	1.01	0.89	0.97
227	1993.60	892	Glutamine	0.69	1.08	**0.58**	0.98
205	1321.70	980	Glyceric acid	**1.30**	**1.62**	0.92	0.96
293	1262.00	953	Glycerol	0.95	1.03	**0.83**	0.92
174	1303.90	965	Glycine	0.88	0.95	0.98	1.07
177	1067.65	921	Hydroxyproline	**1.20**	1.38	0.92	1.01
117	1051.50	965	Lactic acid	2.04	1.43	0.71	0.98
204	2675.25	940	Lactose	0.91	1.21	0.92	1.59
86	1157.70	825	Leucine	1.62	1.30	0.57	0.65
317	1911.00	827	Lysine	1.59	2.53	1.08	2.17
147	1300.40	931	Maleic acid	0.72	0.77	1.35	1.10
147	1477.40	974	Malic acid	0.96	0.84	0.89	0.89
361	2744.70	782	Maltose	**1.61**	**1.41**	**1.72**	**1.45**
160	1868.45	772	Mannose	**1.36**	**2.04**	1.11	1.22
217	3019.10	869	Melibiose	**1.50**	1.42	**1.84**	**1.81**
128	1514.35	770	Methionine	0.75	1.44	0.98	0.77
305	2081.00	980	Myo-inositol	**1.20**	**1.35**	**1.23**	**1.42**
318	2411.70	674	Myo-inositol-phosphate	1.08	1.45	0.77	0.50
132	1389.90	744	*O*-acetyl-serine	1.06	1.38	0.28	0.41
192	1628.55	851	Phenylalanine	1.27	**1.59**	**1.24**	1.81
147	1264.15	835	Phosphoric acid	1.00	1.00	**0.87**	1.03
142	1297.30	942	Proline	1.16	1.47	0.89	1.11
174	1736.10	952	Putrescine	**2.19**	**2.62**	1.44	**1.78**
156	1519.60	982	Pyroglutamic acid	0.99	1.23	1.15	1.05
174	1043.10	822	Pyruvic acid	1.02	**1.40**	1.19	**0.86**
255	1840.00	731	Quinic acid	1.30	**1.42**	0.69	0.89
217	3356.30	868	Raffinose	1.38	**2.29**	1.47	**2.46**

Table 2 Continued

				Fold increase			
				Noon		Evening	
Tag mass	Tag RI	Reverse match	Compound	MsK4 line 2	MsK4 line 9	MsK4 line 2	MsK4 line 9
267	1506.80	825	Salicylic acid	1.08	1.89	1.00	1.15
116	1253.60	966	Serine	1.21	0.79	0.95	**1.60**
204	1792.80	898	Shikimic acid	**1.20**	**1.39**	1.09	**1.35**
147	1853.80	939	Sorbose	**0.72**	**0.61**	1.21	1.36
174	2250.00	945	Spermidine	0.75	**0.30**	1.00	0.86
129	1310.00	977	Succinic acid	1.10	**0.85**	1.12	**1.12**
271	2626.95	959	Sucrose	1.05	0.88	1.05	1.07
335	1851.50	903	Tagatose	**0.74**	**0.68**	1.50	1.82
147	1546.30	962	Threonic acid	1.40	2.29	0.29	0.39
147	1374.20	947	Threonic acid lactone	1.02	2.25	**1.52**	**1.55**
117	1291.25	976	Threonine	**1.46**	1.29	1.23	**1.90**
159	1940.70	889	*trans-p*-coumaric acid	**1.84**	**4.36**	1.36	1.38
338	2243.70	913	*trans*-sinapic acid	**1.26**	**1.37**	1.12	**1.11**
243	2727.00	919	Trehalose	1.12	1.58	**1.47**	1.82
202	2213.75	682	Tryptophan	1.47	**2.53**	1.32	1.14
220	1931.50	855	Tyrosine	0.80	**3.12**	1.50	**3.18**
441	2090.10	982	Uric acid	**2.32**	**2.66**	**7.00**	**2.50**
156	1207.50	936	Valine	1.90	1.62	2.16	1.05
217	1643.50	857	Xylose	**1.49**	**1.79**	1.30	1.45

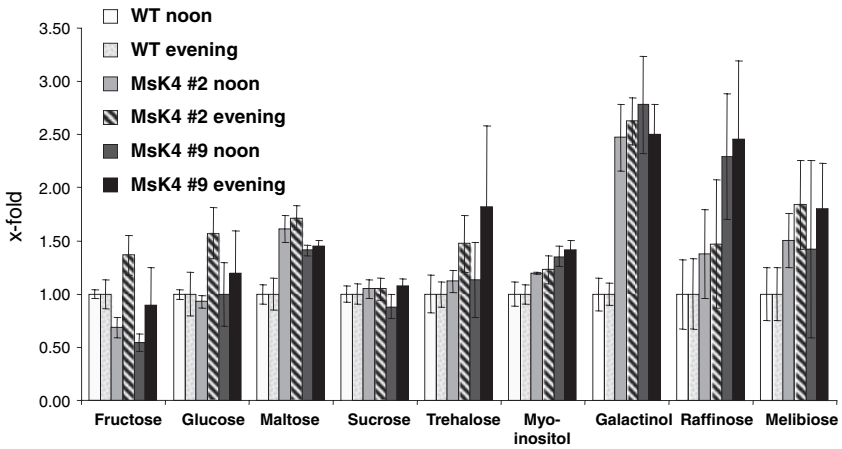

Figure 5. Modulation of sugar levels in MsK4-over-expressing plants.
GC-TOF-MS-based metabolic profiling was performed on 4-week-old plants harvested at noon (4 h after the start of the light period) and at the end of the photoperiod (after 8 h light). Changes in metabolite levels were determined from four replicate pools of ten individual leaf rosettes each. Independent MsK4-over-expressing lines 2 and 9 were compared to wild-type. Changes in levels of selected sugars are indicated as fold increases compared with the average for the wild-type reference sample. Error bars indicate SD.

induced MsK4 protein kinase activity in both *Medicago sativa* (alfalfa) and Arabidopsis. Distinct signalling pathways appear to transduce various features of high-salt stress and to drive specific physiological responses. The rapid induction of MsK4 activity by elevated levels of NaCl, KCl and sorbitol indicates that this GSK pathway is involved in mediating the osmotic aspect of the salt-stress response in plants. MsK4-over-expressing transgenic plants corroborate this, showing enhanced salt tolerance.

Interestingly, metabolic profiling of MsK4-over-expressing plants under normal growth conditions revealed elevated levels of the osmoprotectants galactinol and raffinose. The capacity to accumulate these metabolites is associated with stress tolerance in many species (Yancey, 2005). In *A. thaliana* and *M. sativa*, galactinol and raffinose accumulate in response to high salinity, drought and cold stress (Castonguay and Nadeau, 1998; Cook *et al.*, 2004; Kaplan *et al.*, 2004; Taji *et al.*, 2002). The shift to an increased glucose to fructose ratio in MsK4 over-expressor plants might suggest that the elevated contents of galactinol and raffinose arise from modified carbohydrate metabolism. An attractive hypothesis is that over-expression of MsK4 resulted in basal MsK4 activity even without stress, putatively mimicking mild stress conditions. This pre-conditioning to stress might constitute a vital advantage at the onset of stress and account for the observed stress tolerance of these plants.

We did not observe increased levels of galactinol and raffinose in MsK4 over-expressors compared with wild-type soon (6 and 24 h) after the application of high-salt stress. At

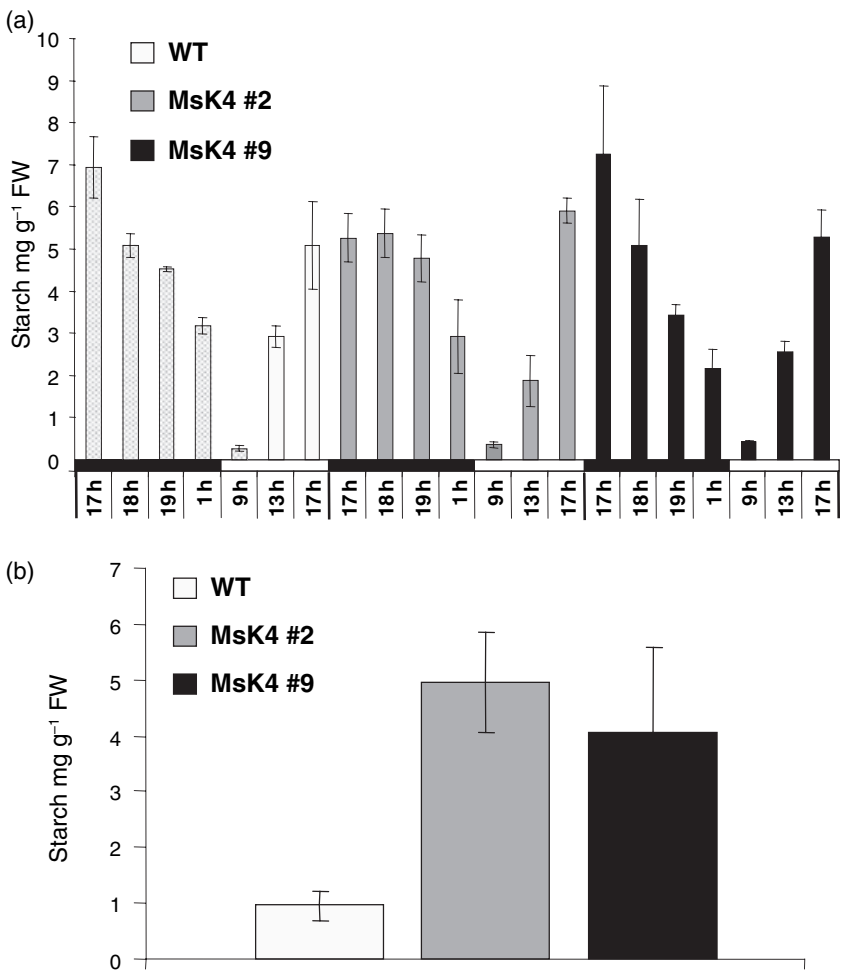

Figure 6. Steady-state starch content.
(a) Diurnal starch levels of 4-week-old soil-grown wild-type and MsK4-over-expressing plants.
(b) Starch content at the end of the light period for wild-type and MsK4 over-expressors exposed to high-salt stress. Four-week-old soil grown plants were watered with 100 mM NaCl for 4 weeks.
Starch content was determined in pools of (a) six and (b) ten plants in three replicates. Plants were grown under short-day conditions (8 h light). FW, fresh weight.

Table 3 Glucose to fructose ratio of MsK4 over-expressors. The relative glucose and fructose contents of 4-week-old MsK4-over-expressing plants were calculated from the data presented in Table 2. The average glucose to fructose ratio for MsK4-over-expressing plants was 1.42. Bold letters indicate significant changes for these metabolites (*t*-test; *P* value < 0.05)

	Noon			Evening		
	WT	MsK4 line 2	MsK4 line 9	WT	MsK4 line 2	MsK4 line 9
Glucose (fold change)	1.00	0.93	1.00	1.00	**1.57**	1.19
Fructose (fold change)	1.00	**0.69**	**0.54**	1.00	1.37	0.90

first sight, this finding may appear unanticipated but is consistent with previous observations that galactinol and raffinose accumulate only after several days of high salinity (Taji *et al.*, 2002).

Piao *et al.* (2001) have reported that over-expression of AtGSK1 enhanced NaCl tolerance in *A. thaliana*. AtGSK1 is an Arabidopsis GSK that shows only 66% protein sequence identity with MsK4 and belongs to a distinct class of plant GSKs (Jonak and Hirt, 2002). Based on sequence similarity analysis, AtK-1 might be the Arabidopsis orthologue of MsK4. In line with this, computer-based algorithms indicate a plastidial localization for AtK-1 but a cytoplasmatic localization for AtGSK1. The available data indicate that MsK4 and AtGSK1 are regulated differently. AtGSK1 mRNA could be induced by the plant hormone abscisic acid (ABA) and NaCl but not by KCl (Piao *et al.*, 1999). No change in MsK4 transcript levels were observed in salt-stressed roots (data not shown). In addition, MsK4 protein kinase was activated by NaCl, KCl and sorbitol but not by ABA (data not shown). The dissimilarity in the responsiveness to various stimuli and most importantly the apparently different subcellular localization of MsK4 and AtGSK1 indicate that these two

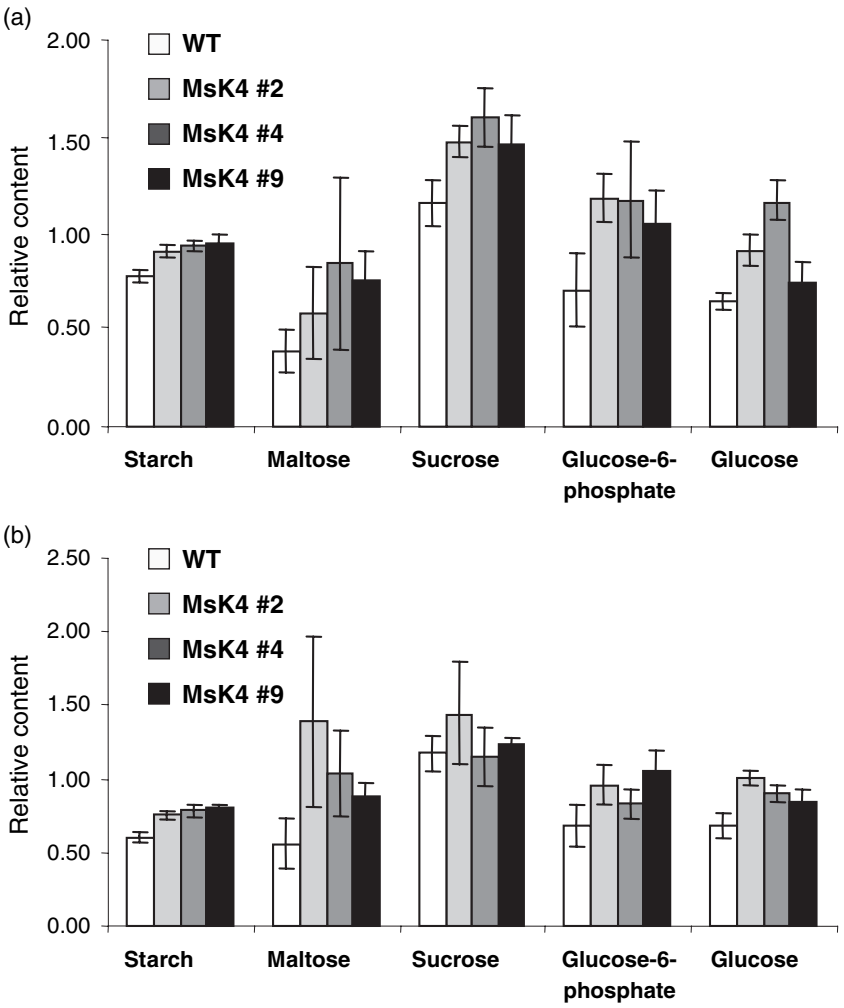

Figure 7. Early response of starch-related carbohydrates to high-salt stress.
Relative starch and sugar contents of 4-week-old MsK4 over-expressor plants after (a) 6 h and (b) 24 h treatment with 150 mM NaCl. Pools of ten plants were harvested, and the metabolite and starch contents were analysed. The relative metabolite levels are shown as fold increases normalized to unstressed wild-type plants.

different GSKs might be involved in distinct aspects of the high-salinity response.

The presence of MsK4 in starch-containing plastids is highly remarkable as some enzymes important in starch metabolism, including starch synthases and glucan–water dikinase, are bound to starch (Mikkelsen and Blennow, 2005; Ritte *et al.*, 2000; Rongine De Fekete *et al.*, 1960). Consistent with the association of MsK4 with starch granules, MsK4 over-expressors display increased maltose levels during the day. Maltose constitutes the main degradation product of starch by the β-amylolytic pathway in plastids, and is exported to the cytosol by the maltose transporter MEX1 (Niittyla *et al.*, 2004; Weise *et al.*, 2004) where it is used to meet various metabolic demands. The increased glucose to fructose ratio observed in MsK4 over-expressors points to a modulation at the heart of carbohydrate metabolism that might stem from increased starch turnover.

Quantification of starch levels in leaves did not show any apparent effect of MsK4 over-expression in plants grown under standard growth conditions. This result suggests that MsK4 does not influence starch levels during normal light–dark cycles but could be responsible for mediating other signals to starch. Consistent with this notion, MsK4 activity is induced by high salinity, and plants with elevated MsK4 levels have significantly more starch under salt-stress conditions than wild-type plants. Starch metabolism is highly sensitive to changes in the environment, and its accurate regulation is crucial for adaptation of carbon and energy flow during stress conditions (Geigenberger *et al.*, 1997; Gilbert *et al.*, 1997; Kaplan and Guy, 2004, 2005; Oparka and Wright, 1988; Todaka *et al.*, 2000; Yano *et al.*, 2005; Zenner and Stitt, 1991).

Salinity stress leads to changes in carbohydrate metabolism (Arbona *et al.*, 2005; Balibrea *et al.*, 1997; Fougere *et al.*, 1991; Gilbert *et al.*, 1997; Jouve *et al.*, 2004; Kawasaki *et al.*, 2001; Morsy *et al.*, 2006; Ndimba *et al.*, 2005; Seki *et al.*, 2002). Consistently, we found that high-salt conditions induce a rapid alteration of carbohydrate levels. After irrigation of plants with NaCl, starch contents as well as the levels of several soluble sugars decreased significantly, indicating an impairment of the central carbohydrate metabolism. MsK4-over-expressing plants had higher levels of

starch, maltose, glucose and G6P, suggesting enhanced carbohydrate metabolism during high-salinity stress compared with wild-type plants (Figure 8). Given the better stress tolerance of MsK4 over-expressors, maintenance of the central carbohydrate metabolism at a reasonable level, as observed in MsK4-over-expressing plants, might contribute to the improved fitness under stress conditions. This hypothesis is compatible with the observation that acclimatization of the halotolerant green algae *Dunaliella salina* to high-salt conditions includes the upregulation of key enzymes in carbohydrate metabolism (Liska *et al.*, 2004). Moreover, it is worth noting that plants with lowered plastidic G6P dehydrogenase activity not only show elevated levels of G6P and starch, but are also more tolerant to oxidative stress (Debnam *et al.*, 2004).

Fluxes of carbon into and out of starch are extremely complex and must be highly controlled. However, the signal transduction pathways that regulate starch metabolism are still obscure. Evidence is emerging that protein phosphorylation plays an important role in the regulation of starch-associated carbon metabolism. Phosphorylation of starch metabolic enzymes has been shown to control their activity and complex formation (Tetlow *et al.*, 2004b). MsK4 can bind to some isoforms of soluble and granule-bound starch synthase *in vitro* (C. Jonak, unpubl. results) suggesting that enzymes involved in starch metabolism might be direct targets of MsK4 action. Moreover, a starch-associated protein phosphatase has been identified as involved in the regulation of starch metabolism (Kerk *et al.*, 2006; Niittyla *et al.*, 2006; Sokolov *et al.*, 2006). SEX4/DSP4 is a dual-specificity phosphatase similar to laforin that binds to starch and glycogen and regulates glycogen metabolism in animals (Fernandez-Sanchez *et al.*, 2003). Interestingly, laforin was shown to interact with and dephosphorylate GSK-3

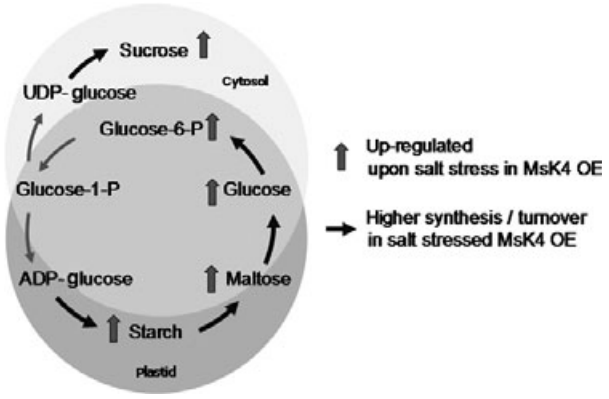

Figure 8. Schematic presentation of changes in carbohydrate metabolism in plants over-expressing MsK4 upon high-salt stress.
A simplified metabolic pathway from starch to sucrose is shown. Black arrows indicate a higher synthesis or turnover of the respective metabolite; grey arrows show increased levels of metabolites. Grey shading indicates the subcellular localization (light grey, cytoplasm; dark grey, plastid; medium grey, cytoplasm and plastid).

(Lohi *et al.*, 2005). SEX4/DSP4 phosphatase activity has been shown to be regulated by the redox state (Sokolov *et al.*, 2006). Unfavourable environmental stress conditions alter the redox balance within the cells. This raises the interesting possibility that SEX4/DSP4 phosphatase activity may be regulated in response to environmental stress, and that there might be a functional interconnection between MsK4/AtK-1 and SEX4/DSP4.

Overall, our data confirm and extend observations indicating that starch metabolism is tightly regulated by protein phosphorylation, showing that MsK4 is a starch granule-associated protein kinase, the activity of which is induced by high salinity. The discovery that over-expression of MsK4 modulates carbohydrate metabolism and stress tolerance opens new perspectives as to how metabolic carbon flux can be regulated in response to environmental stress.

Experimental procedures

Plant material, plant growth and stress treatments

Medicago sativa cv. Europa was grown under a 16 h light/8 h dark cycle at 22°C. Stress treatments are described in the text.

For starch and metabolite analysis, soil-grown *A. thaliana* Col-0 plants were cultivated under an 8 h light/16 h dark cycle at 22°C, 60% humidity and a light intensity of 150 μE m^{-2} sec^{-1}. For long-term salt-stress treatments, plants were watered with 100 mM NaCl for 4 weeks. For short-term salt-stress treatments, pots with plants were incubated in 150 mM NaCl for 1 h to ensure thorough soaking of the soil with the NaCl solution.

Isolation of MsK4 cDNA

Primers corresponding to two highly conserved regions of *MsK1*, *MsK2* and *MsK3* were used to isolate new members of the *GSK-3* gene family from alfalfa by PCR (Pay *et al.*, 1993). Sequence analysis revealed a new type of alfalfa *GSK*, denoted *MsK4*, which was used to screen an alfalfa cDNA library. The nucleotide sequence of the *MsK4* gene has been deposited in the GenBank database under the accession number AF432225.

Chloroplast processing and import assay

For the processing assays, *Pisum sativum* (pea) chloroplasts corresponding to 100 μg chlorophyll were pelleted, and resuspended in 100 μl 10 mM HEPES/KOH, pH 7.6. Subsequently, 20 μl of *in vitro*-translated protein (T3/T7-coupled *in vitro* translation kit; Amersham Life Sciences; http://www5.amershambiosciences.com/) was added. Import assays were performed as described previously (Qbadou *et al.*, 2003; Schleiff *et al.*, 2001).

Antibody production and specificity tests

The MsK4-specific antibody was produced in rabbits against a synthetic peptide corresponding to the C-terminus of the *MsK4* cDNA (KQNLFMALHT). The polyclonal antibody was affinity-purified on a CNBr–Sepharose column charged with the C-terminal MsK4 peptide.

For competition assays, the MsK4 antibody was pre-incubated with an excess of MsK4 peptide before the antibody was added to the filters. Immunoprecipitation of *in vitro*-translated protein kinases (T3/T7-coupled *in vitro* translation kit) was performed as described for immunokinase assays.

Indirect immunofluorescence microscopy

Alfalfa root apices and pieces of leaves were fixed either with 3.7% formaldehyde in stabilizing buffer (SB; 25 mM PIPES buffer, 2.5 mM $MgSO_4$, 2.5 mM EGTA, pH 7.0) or with 3% formaldehyde and 0.5% glutaraldehyde in 50 mM phosphate buffer, pH 7.0, respectively. After washing in SB (for roots) and phosphate buffer (for leaves) and in PBS (Sigma; http://www.sigmaaldrich.com/) (100 mM, pH 7.2), samples were dehydrated in a graded ethanol series. Tissue was embedded in Steedman's wax. Sections 7 μm thick were mounted on slides coated with glycerol albumin (Serva; http:www.serva.de) or Biobond (BioCell; http:www.biocell.com). Sections were de-waxed in absolute ethanol, passed through a graded ethanol series, and rinsed in SB and PBS containing 5% BSA. Subsequently, sections were incubated with affinity-purified anti-MsK4 antibody in PBS containing 1% BSA. After four washes with PBS containing 1% BSA, sections were incubated with FITC-conjugated goat anti-rabbit IgGs (F-9887; Sigma; http://www.sigmaaldrich.com/). After rinsing in PBS, the sections were treated with 0.01% toluidine blue in PBS for 10 min prior to mounting in anti-fade mountant: 100 mg *p*-phenylenediamine dissolved in 10 ml 0.01 m PBS, pH 8,0. As negative controls, the primary antibody was omitted or was pre-incubated with the C-terminal MsK4 peptide. Immunofluorescence was examined with Axioplan 2 and Axiorent microscopes (Zeiss; http://www.zeiss.com/).

Immunogold cytochemistry and statistic evaluation

Apical segments of alfalfa root tips were fixed, washed and dehydrated as described above before embedding in LR White resin (hard grade; BioCell). Ultra-thin sections of root cap cells (ultramicrotome OM U3; Reichert; http://www.reichert.com) were collected on Formvar-coated Ni grids (British Biocell International, Cardiff, UK; http://www.bb-international.com). Residual aldehydes on these sections were blocked with 0.05 M glycine in PBS, and non-specific binding of proteins was avoided by applying 5% BSA and 5% normal goat serum. Grids were washed with washing mixture (WM; 1% BSA and 0.1% fish gelatine in PBS) and incubated with affinity-purified anti-MsK4 antibody. For negative controls, the primary antibody was omitted or the anti-MsK4 antibody was pre-incubated with the C-terminal MsK4 peptide. Sections were washed five times with WM and incubated with goat anti-rabbit IgGs conjugated with 10 nm gold particles (BioCell). Further washing with WM and PBS was followed by post-fixation with 3% glutaraldehyde, washing with PBS and deionized water, and staining with 2% uranyl acetate. Immunogold-labelled sections were examined with a Zeiss EM 10A at 60 kV.

The quantity and distribution of MsK4 immunogold labelling within different subcellular compartments, including amyloplasts, cytosol, nucleus, cell wall and vacuole, were analysed by the point-counting method (Rivett *et al.*, 1992) on 30 randomly selected electron micrographs of each compartment.

Isolation of starch granules

Isolation and purification of starch granules, detergent extraction and proteinase digestion were performed as described previously (Mu-Forster *et al.*, 1996).

Western blot analysis

Western blotting of 40 μg protein extract with affinity-purified MsK4 antibody and anti-myc antibody (Santa Cruz Biotechnology; http://www.scbt.com) was performed as described previously (Jonak *et al.*, 2000). Alkaline phosphatase-conjugated goat anti-rabbit (Santa Cruz Biotechnology) and anti-mouse IgG (Sigma) were used as secondary antibodies, and the reaction was visualized by chemoluminescence (CDP-Star; Amersham Life Sciences).

Immunokinase assays

Roots of hydroponically grown 4-day-old alfalfa plants were exposed to 0, 125, 250, 500, 750 and 1000 mM NaCl, KCl or sorbitol for the indicated times before shock-freezing in liquid nitrogen. Immunokinase assays from root extracts containing 100 μg of total protein were performed with MsK4 antibody as described previously (Jonak *et al.*, 2000). Arabidopsis plants over-expressing MsK4-myc were grown on 1/2-strength Murashige and Skoog (MS) medium. Twelve-day-old plants were either treated with 250 mM NaCl or with water for 10 min. Immunokinase assays with anti-myc antibody were performed using 100 μg of protein extract.

Generation of transgenic plant material

A myc-epitope-tagged version of MsK4 was cloned into the binary plant expression vector pGWR6 under the control of the 35S promoter. pGWR6 is a derivate of pGreenII0029 (Hellens *et al.*, 2000) carrying the translational leader of the tobacco etch virus for efficient translation. pGWR6-MsK4-myc and pGWR6 were transformed into *A. thaliana* Col-0 using the floral dipping method (Clough and Bent, 1998).

Medicago truncatula var. Jemalong was stably transformed with pGWR6-MsK4-myc by *Agrobacterium rhizogenes*-mediated root transformation as described previously (Boisson-Dernier *et al.*, 2001). After selection of stably transformed roots on Fahraeus medium (Boisson-Dernier *et al.*, 2001) containing 20 mg l^{-1} kanamycin, transgenic roots were cut and placed on modified White's medium (Bécard and Fortin, 1988) complemented with 500 μg ml^{-1} carbenicillin to remove residual *A. rhizogenes*. Subsequently, a 35S::MsK4-myc transgenic root cell culture was established.

Analysis of starch content and metabolite profiling

Ten rosettes of four-week-old Arabidopsis plants were pooled and frozen in liquid nitrogen, and 60 mg of ground plant material was extracted using 300 μl pre-cooled methanol. Internal standards for quantitative and retention time index calibration were added as described previously (Wagner *et al.*, 2003). Then, 300 μl chloroform were added to the extracts and mixed. After centrifugation, 160 μl of the methanolic supernatant were lyophilized and stored until metabolite analysis at −80°C. The solid fraction of the extracts was used for determination of starch content. For quantification of starch content, the pellet was washed twice with 80% ethanol. Subsequently, the pellet was incubated with 460 μl 0.2 M KOH at 85°C for 1 h, and the pH was adjusted with 140 μl 1 M acetic acid. The supernatant (100 μl) was incubated overnight with 1 U α-amylase (Fluka; http://www.fluka.org), 1 U β-amylase (Sigma) or 1 U 01a0myloglucosidase (Fluka). Free glucose was quantified using a glucose Trinder kit (Sigma).

For metabolite analysis, samples were prepared as described previously (Fiehn *et al.*, 2000; Kaplan *et al.*, 2004). The derivatized extracts were analysed by GC-TOF-MS (Wagner *et al.*, 2003), and

the resulting chromatograms were processed by CHROMATOF software (Leco; http://www.leco.de) for peak identification using a customized mass spectral and retention time index library of approximately 1000 non-redundant entries, which currently covers 360 identified metabolic components of plant, microbial and animal origin (Kopka *et al.*, 2005; Schauer *et al.*, 2005). The identity of all automatic annotated metabolites was confirmed manually by comparing mass spectra and retention indices. The signal intensities of mass tags that were unique at a certain retention index for a single metabolite were extracted from GC-TOF-MS files using the CHROMATOF software and used for relative quantification. The intensities were normalized to the exact sample amount and corrected for volume losses through sample preparation and analysis using the internal standard ribitol.

Bioinformatic and statistical data analyses

Statistical screening for significant metabolic changes among monitored metabolites was performed using Microsoft Excel. The correspondence analysis was performed using TIGR MEV freeware (TIGR MEV 3.0) (http://www.tm4.org/mev.html).

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Sequence alignment of MsK4 with AtK-1, MsK1 and GSK-3β.

Figure S2. Specificity of the anti-MsK4 antibody.

Figure S3. Metabolite profiling of two distinct MsK4 over-expressor lines and wild-type.

This material is available as part of the online article from http://www.blackwell-synergy.com

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