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An Arabidopsis cell wall-associated kinase required for invertase activity and cell growth

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Summary

The wall-associated kinases (WAK), a family of five proteins that contain extracellular domains that can be linked to pectin molecules of the cell wall, span the plasma membrane and have a cytoplasmic serine/threonine kinase domain. Previous work has shown that a reduction in WAK protein levels leads to a loss of cell expansion, indicating that these receptor-like proteins have a role in cell shape formation. Here it is shown that a single *wak2* mutation exhibits a dependence on sugars and salts for seedling growth. This mutation also reduces the expression and activity of vacuolar invertase, often a key factor in turgor and expansion. WAKs may thus provide a molecular mechanism linking cell wall sensing (via pectin attachment) to regulation of solute metabolism, which in turn is known to be involved in turgor maintenance in growing cells.

Keywords: cell wall, cell expansion, receptor kinase, plasma membrane, vacuole, sugar metabolism.

Introduction

The shape and size of a cell can be determined by a variety of genetic and environmental factors, and involves interplay between cell turgor and a regulated loosening of the surrounding extracellular matrix (ECM; Cosgrove, 2001). Cellulose of the cell wall is synthesized on the plasma membrane by a family of cellulose synthase rosettes. In contrast, the pectin, hemicellulose and other carbohydrate polymers are synthesized within the Golgi apparatus and then exported to the surface to assemble with the cellulose to form the cell wall (Carpita and McCann, 2000). Little is known of how the synthesis in these two compartments is coordinated. Numerous proteins are also found in the cell wall, and some, such as arabinogalactan proteins (AGPs), are thought to bind the pectin (Nothnagel, 1997), while others may bind the cellulose or hemicellouse (Kohorn, 2000). However, signaling between the ECM and cytoplasm of plant cells remains an elusive area of study and only a few proteins have been identified that have the potential to link these two compartments (Baluska *et al.*, 2003; Kohorn, 2000). These include the wall-associated kinases (WAKs; Wagner and Kohorn, 2001), the proline rich extensin-like receptor kinases (PERKs) (Nakhamchik *et al.*, 2004), the formins (Deeks *et al.*, 2002), the AGPs (Nothnagel, 1997) and COBRA (Schindelman *et al.*, 2001). Only the WAKs and PERKS have the potential to directly signal to the cytoplasm via the protein kinase domain.

WAKs are encoded by five tightly linked and highly similar genes in *Arabidopsis thaliana*, and are expressed in leaves, meristems, and cells undergoing expansion (Wagner and Kohorn, 2001). WAKs are also induced by a variety of agents including pathogens, wounding and stress (He *et al.*, 1996; Sivaguru *et al.*, 2003). A larger family of WAK-like proteins includes members with extracellular epidermal growth factor repeats and a cytoplasmic kinase domain, but the similarity to WAKs is confined to these regions and their function and location in the cell is unknown (Verica and He, 2002; Verica *et al.*, 2003).

Expression of an induceable antisense *WAK2* in Arabidopsis leaf cells led to a 50% reduction in WAK protein levels, with a subsequent loss of cell elongation, and hence dwarf plants (Wagner and Kohorn, 2001). Similar results have been reported where an antisense WAK4 gene was used to reduce total WAK protein levels (Lally *et al.*, 2001). In plants, 68 kDa WAKs have been detected in a Triton X100-insoluble

material that can only be resolved by boiling in SDS and dithiothreitol (DTT). Immunomicroscopy and localization in protoplasts indicate, however, that these receptors indeed traverse the plasma membrane (He *et al.*, 1996). WAK1 has been reported to bind to a glycine-rich protein in the yeast two-hybrid assay, and evidence indicates there is a 95 kDa WAK-cross-reacting protein in a 450 kDa complex present in *Arabidopsis* leaf extracts (Park *et al.*, 2001). However, these results are in contrast to others (He *et al.*, 1999; Wagner and Kohorn, 2001) indicating that WAKs are extremely water- and detergent-insoluble, and thus perhaps cross-linked to cell wall material. The discrepancy remains unresolved.

At least some WAK family members appear to be covalently bound to some form of pectin, as the JIM5 antiserum (Willats *et al.*, 2000) binds to WAK on denaturing polyacrylmide gels (Wagner and Kohorn, 2001). The extracellular domain of WAK1 indeed can bind to pectin *in vitro*, and this requires a calcium-induced caging of the pectin (Decreux and Messiaen, 2005). The WAK-pectin binding is not covalent *in vitro*, indicating that the association is altered in plants.

WAKs have been implicated in the plant's response to pathogens, as their *NPR1*-dependent expression is required during the pathogen response, and expression of the WAK kinase domain can lead to salicylate resistance (He *et al.*, 1998). WAK mRNA expression is also induced by pathogens, Aluminum toxicity, wounding and numerous other stresses (He *et al.*, 1998; Sivaguru *et al.*, 2003; Wagner and Kohorn, 2001).

Here it is shown that a single *wak2* mutation exhibits a dependence on appropriate sugars and salts and reduces vacuolar invertase activity in the roots, thus also limiting this avenue of potential turgor adjustment. WAKs may thus provide a mechanism linking wall expansion sensing (via their attachment to pectin), to solute metabolism, which in turn is known to be involved in systems of turgor maintenance as the cell enlarges.

Results

*The WAK2 insertion mutation (*wak2-1*)*

To determine how WAKs might be involved in cell expansion, insertion mutations in the most abundantly expressed WAK2 were isolated (Syngentia and Salk database of *Agrobacterium* T-DNA insertions, Alonso *et al.*, 2003). An insertion into the coding region of *WAK2* (At1g21270, at amino acid 30, SAIL 286.3 E03.6.1a) was recovered, and backcrossed to wild-type, and the F_1 was allowed to self. The F_2 plants homozygous for the *WAK2* insertion (referred to as *wak2-1*) were identified by Southern blotting with *WAK* gene-specific probes (He *et al.*, 1999, data not shown). RT-PCR using *WAK2*-specific primers demonstrated that the *wak2-1* plant has no detectable *WAK2* mRNA (Figure 1).

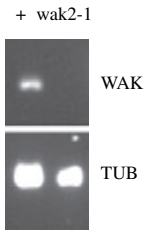

Figure 1. *wak2-1* is a null allele.
Equal amounts of total RNA were used as a template for semi-quantitative RT-PCR using *WAK2* or tubulin-specific primers (TUB), and the products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Expression of the adjacent WAK1 and distal WAK4 are not affected in the *wak2-1* mutant plant (data not shown). The *wak2-1* mutant showed no difference in growth on soil in 150 μM photons m^{-2} sec^{-1} of light, nor when grown on full-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 2% sucrose. In the absence of sucrose, the *wak2-1* plants sometimes, but not always, arrested at the seedling stage. Those seedlings that did escape arrest grew more slowly than wild-type. This phenotype was most pronounced on 1/6 strength MS media that lacked sucrose (Figure 2). The arrested or slowed seedlings recovered normal growth when transferred to sucrose plates. Use of 60 mM sorbitol also allowed *wak2-1* to grow normally on 1/6 MS.

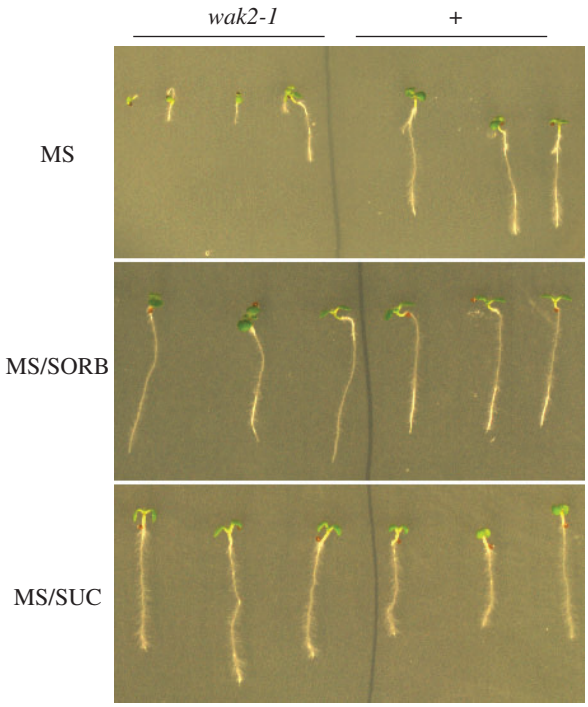

Figure 2. Sucrose requirement for growth of *wak2-1*.
Seedlings of wild-type (+) or *wak2-1* were plated on MS media of 1/6 strength supplemented with 60 mM sorbitol (SORB) or sucrose (SUC). Plants were grown for 8 days in 150 μm m^{-2} sec^{-1} light (14 h, 22°C).

The wak2-1 *mutant requires sugar and salts*

To determine why a reduction in the strength of MS would affect growth in the mutant, plants were plated on minimal media (pond water, PW; Shabala and Lew, 2002), and the results are shown in Figure 3. On PW without sucrose, most *wak2-1* seedlings arrested or slowed growth (Figure 3, PW). As there was variation in both wild-type and mutant seedling growth, the root and hypocotyl length of 49 *wak2-1* were compared to those of 49 wild-type seedlings grown on PW. The *wak2-1* seedlings were significantly ($P < 0.001$) shorter than wild-type, with the roots being affected more than the hypocotyls (*wak2-1* roots/hypocotyls 9.4 ± 6.6/1 ± 0.4 mm; wild-type roots/hypocotyls 18.5 ± 7/1.3 ± 0.4 mm). Arrest of *wak2-1* on PW was rescued by the addition of 60 mM sucrose, fructose or glucose, but not sorbitol or mannitol (Figure 3). Representative seedlings are shown in Figure 3, with additional measurements of root length from wild-type and *wak2-1* seedlings grown on these sugars as follows (the type of sugar in the media is noted, followed by the genotype, the average root length, and the *P* value of a *t* test): sucrose; wild-type (25.67 ± 9.22 mm), *wak2-1* (26.70 ± 5.81), $P > 0.1$; mannitol; wild-type (12.80 ± 4.19 mm), *wak2-1* (5.42 ± 2.19), $P < 0.01$; sorbitol; wild-type (12.70 ± 2.97 mm), *wak2-1* (5.61 ± 2.58), $P < 0.01$; glucose; wild-type (24.21 ± 4.3 mm), *wak2-1* (21.40 ± 6.71), $P > 0.1$; fructose; wild-type (20.2 ± 2.73), *wak2-1* (17.38 ± 2,31 mm), $P > 0.1$; no sugar; wild-type (13.4 ± 5.01 mm), *wak2-1* (7.24 ± 6.23), $P < 0.01$. Sorbitol and mannitol can be metabolized, but less efficiently than are glucose, sucrose and fructose. A comparison of results from Figures 2 and 3 indicates that *wak2-1* is arrested or slowed due to conditions contributed by both salts and metabolized sugars that influence the whole plant and cellular concentrations. These effects relate to long-term growth, rather than short-term external osmotic changes.

To ensure that the *wak2-1* phenotype was due to the insertion into the *WAK2* locus, and not to some linked mutation, *wak2-1* plants were transformed with a wild-type *WAK2* cDNA with a hemagglutinin (HA) epitope inserted in-frame C-terminal to the transmembrane domain (at amino acid 361), and under the control of the CaMV 35S promoter. The F_1 plants were selected for co-transformation of the kanamycin resistance marker on the pBI1.4t vector (Mindrinos *et al.*, 1994). Figure 4(a) shows that transformants (*wak2-1*:35SWAK2HA) and wild-type (+) did not arrest on PW, while *wak2-1* plants did. The root length of 28 rescued *wak2-1* seedlings and 28 wild-type seedlings grown on PW did not differ ($P = 0.6$). To ensure that the *WAK2HA* gene was expressed, whole-seedling extracts were made from a rescued transformant (Figure 4b, lane 2) and from two transformants that were not rescued on PW (Figure 4b, lanes 1, 3), and equal amounts of protein were run on a Western blot and probed with an anti-HA antiserum. The results show that only the lines expressing significant levels of WAK2HA were rescued.

While growth of the *wak2-1* plants on soil in light was normal, it was expected that if the plants were grown in the dark, carbon supplies would become limiting, and more markedly affect growth of the *wak2-1* mutant. Figure 5 shows that *wak2-1* plants did indeed have less hypocotyl extension than the wild-type when grown in the dark for 3 weeks on soil.

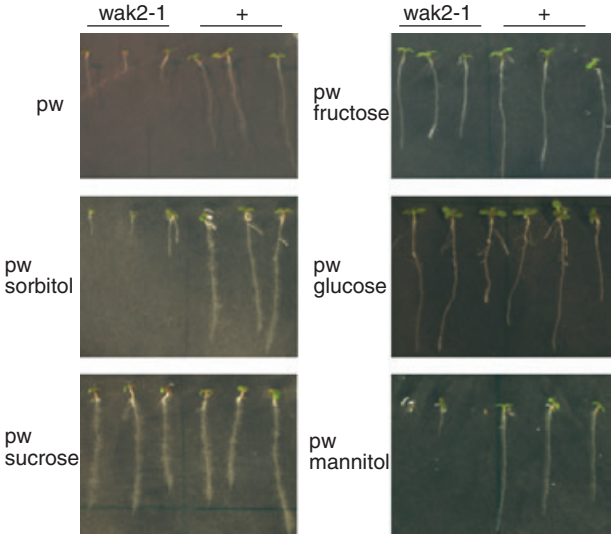

Figure 3. *wak2-1* rescue by metabolized and signal active sugars.
Mutant (*wak2-1*) and wild-type (+) seedlings were grown in 150 μm m^{-2} sec^{-1} light for 8 days on pond water (pw, minimal medium) supplemented with 60 mM sugar, as indicated.

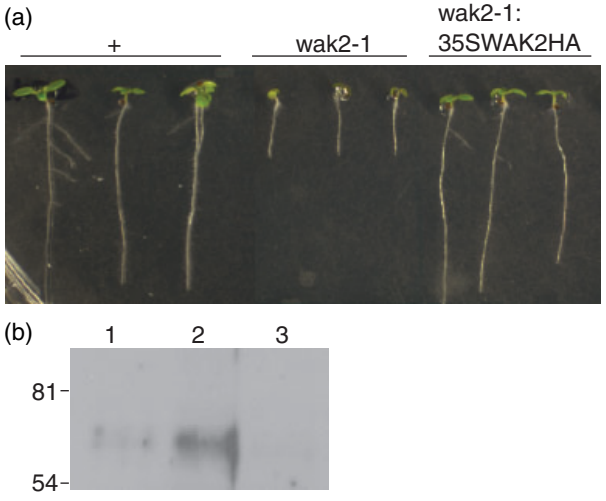

Figure 4. Complementation of *wak2-1*.
(a) Complementation of *wak2-1* by wild-type *WAK2*. Wild-type (+), *wak2-1* or F_2 *wak2-1* transformed with 35S:WAK2HA were grown for 8 days on pond water in 150 μm m^{-2} sec^{-1} light.
(b) HA Western blot of leaf extracts of *wak2-1* transformed with 35S:WAK2HA. Plants 1 and 3 are transformed but not rescued, while plant 2 is transformed and rescued on pond water. Numbers on the left indicate molecular weight $\times 10^{-3}$.

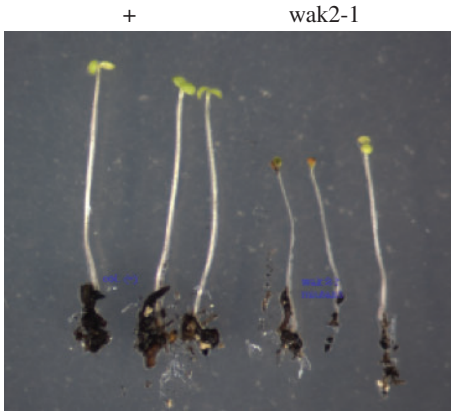

Figure 5. *wak2-1* mutation affects growth in the dark on soil (Metromix). The mutant or wild-type (+) lines were planted in soil and grown for 3 weeks in the dark.

These results parallel those of Figures 2 and 3 in suggesting that root and seedling arrest of *wak2-1* may be especially sensitive to limitation of endogenous carbon supplies.

wak2-1 *affects the rate of cell elongation in roots*

To determine whether the cells of *wak2-1* roots were changed in elongation rates or size, an anatomical analysis was performed. Root tips from 8-day-old wild-type and *wak2-1* seedlings were incubated with the lipophilic dye FM4-64 that labels plasma membranes and endocytotic vesicles (Ueda *et al.*, 2004; Vida and Emr, 1995), and a single longitudinal optical section though the center of the roots was visualized by confocal scanning microscopy (Zeiss 510 Meta; Zeiss, Thornwood, NJ, USA). Open Lab digital software (Improvision, Lexington, MA, USA) was used to quantify the length of cells within a continuous column that originated just distal to the quiescent zone near the root tip, and enlarged toward the hypocotyl (Figure 6). Some of the images used are shown to the right of Figure 6. The sizes of cells in two columns (c1 and c2) of wild-type (Col) show the same increase as those in two columns of cells in the *wak2-1* line (wk2 c1 and wak2 c2). Although the measurements of each column may not begin at exactly the same point relative to the meristem, overall progression of cell sizes within the zone of elongation was similar. This, together with the marked reduction in overall root length of *wak2-1* mutants, implies that the rate of cell elongation plays a greater role than cell size in the *wak2-1* phenotype.

Alteration of SPS activity in wak2-1

If indeed sugar metabolism and carbohydrate source–sink relations are altered in the *wak2-1* seedlings, then the ectopic expression of metabolic enzymes that alter sucrose levels in the leaf should also affect the growth of *wak2-1*.

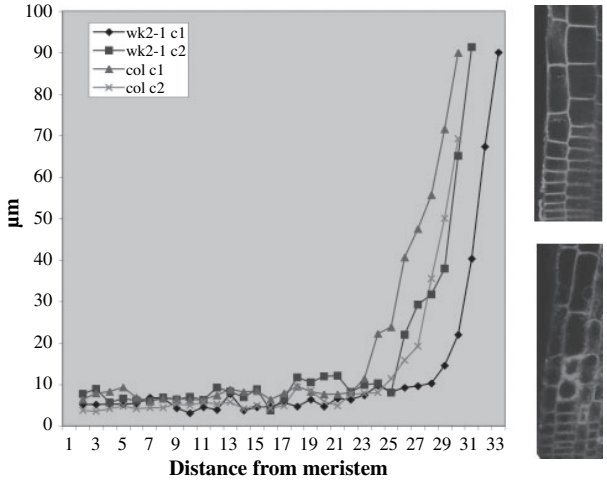

Figure 6. Cell length with distance from the root meristem.
Roots were stained with FM4-64, visualized by confocal microscopy, and cell length measured using Open Lab software. Two columns of cells (c1 and c2, images shown on right) in both wild-type (Col, top) and *wak2-1* roots (bottom) were measured. The *x*-axis indicates the cell number (numerical position) relative to the meristem. The *y*-axis shows the length in µm of cells within a column.

Sucrose phosphate synthase (SPS) converts UDP-glucose and fructose-6-phosphate to sucrose phosphate, which in turn can be converted to sucrose and used as the primary transported sugar from the leaves to the roots in Arabidopsis (Huber and Huber, 1996). SPS is primarily expressed in the leaves and not roots. To determine whether perhaps SPS activity was altered in *wak2-1*, SPS activity in *wak2-1* and wild-type were compared in leaf and root extracts from plants grown on PW. No differences were detected (Table 1).

SPS is regulated by phosphorylation, but when a maize isoform is expressed in Arabidopsis, it has constitutive

Table 1 Enzyme activity for leaves and roots of *wak2-1* as a percentage of wild-type

Enzyme	Organ	% of wild-type	*P*-value
CW invertase	Leaves	123 ± 13	0.046
	Roots	88 ± 12	0.107
Vacuolar invertase	Leaves	84 ± 9	0.043
	Roots	62 ± 5	0.003*
Hexokinase	Leaves	91.7 ± 26	0.31
	Roots	93.0 ± 12	0.22
SUSY	Leaves	115 ± 21	0.172
	Roots	123 ± 33	0.179
SPS	Leaves		
	V_{max}	97 ± 6	0.204
	Limit	101 ± 4	0.332
	Roots		
	V_{max}	96 ± 18	0.366
	Limit	85 ± 17	0.135

Total cell extracts were desalted and adjusted to equal protein concentrations. *Significant difference. CW, cell wall; SUSY, sucrose synthase; SPS, sucrose phosphate synthase.

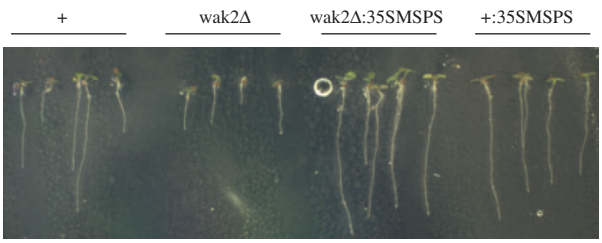

Figure 7. Expression of maize SPS (MSPS) promotes growth of *wak2-1* on pond water.
Wild-type (+) and F_2 *wak2-1* transformed with 35S:MSPS in pBI1.4t were grown on pond water (PW) in 150 μm photons m^{-2} sec^{-1} of light (14 h) for 8 days.

activity and can alter carbon flux (Lunn and MacRae 2003). The maize SPS (MSPS; Genbank accession no. M97550) under the control of the CaMV 35S promoter was therefore transformed into both *wak2-1* and wild-type. The F_2 were tested for their ability to grow on PW, and the results are shown in Figure 7. The *wak2-1* MSPS transformants are not arrested on PW, and grow as well as wild-type transformed with the same construct. Thus manipulation of internal sugar metabolism can also rescue the *wak2-1* phenotype.

Root vacuolar invertase is altered in wak2-1

The dependence of *wak2-1* on sugars suggested that there could be an alteration in some aspect of sugar metabolism, and there are several key enzymes that may be affected. Sucrose that is either added exogenously or transported to the roots from the leaves can be used by sucrose synthase to provide carbon for cellulose synthesis (Ruan *et al.*, 2003). A reduction in cellulose synthase can result in altered root morphology and radial swelling (Arioli *et al.*, 1998). Neither of these are observed in the *wak2-1* mutant, and stress tests (Cosgrove, 2001) of wild-type and mutant seedlings indicate no difference in cell wall strength (data not shown). In addition, sucrose synthase activity is not altered in the mutant relative to wild-type (Table 1).

Sucrose can also be converted to glucose and fructose by invertase within the vacuole and cell wall (Koch, 2004; Winter and Huber, 2000). Vacuolar invertase can contribute prominently to the internal turgor of the cell in a number of instances (Koch, 2004), so extracts from roots and leaves were used to determine whether invertase activity was changed in *wak2-1* samples relative to those of wild-type. The most marked difference was a highly significant reduction ($P < 0.01$) in the vacuolar invertase activity of *wak2-1* roots to $62 \pm 5\%$ of that in wild-type (Table 1). A similar extent of change was not observed for other tissues (leaves) or invertases (cell wall activities in either leaves or roots). The response of the root vacuolar invertase could be key, however, as reduced production of glucose and fructose in the root vacuole could limit contributions from this means of turgor maintenance and lead to the observed arrest of growth in *wak2-1* plants.

Sugar metabolism has a direct influence on gene expression (Koch, 1996), often mediated by hexokinase (Moore *et al.*, 2003; Rolland *et al.*, 2002). Table 1 indicates that no changes were detected in hexokinase activity in the mutant relative to the wild-type. The possibility remains that some aspects of WAK function may alter signals through hexokinases, but this apparently does not involve gross changes in enzyme capacity. An altered localization of hexokinase in the *wak2-1* mutant also has not been determined.

As the vacuolar invertase activity is lower in the *wak2-1* roots, it was of interest to determine whether this regulation extended to the transcriptional level. The *Arabidopsis* genome contains two genes for vacuolar invertase, and six for the cell wall isoform. Quantitative real-time PCR using primers specific for each of the two vacuolar invertase mRNAs showed that the most abundant transcript, *AtvacINV1*, is reduced by 60% in mutant roots but not in shoots (Figure 8). This mRNA typically predominates by many-fold over the other vacuolar invertase in roots (*AtvacINV2*), and changes in the *AtvacINV1* transcript abundance here are consistent with those of enzyme activities (Table 1).

Effects of the *wak2-1* mutation on the six cell wall invertase mRNAs were generally negligible (not shown), although some reduction was evident in the highly variable levels of *AtcwINV6* transcript in roots. This *AtcwINV6* mRNA, however, may not encode a classical invertase, as studies of its recombinant protein show cleavage of fructan and trisaccharide substrates rather than sucrose (De Coninck *et al.*, 2005). Any response of this transcript to WAK2 might thus involve a non-invertase contribution. The lack of significant changes in other cell wall invertase mRNAs is consistent with minimal detectable differences in cell wall invertase enzyme activity (Table 1).

Lastly, total soluble carbohydrate analysis by HPLC was performed (Chia *et al.*, 2000). While no difference was detected between mutant and wild-type (data not shown), it was not possible to determine the relative location of different pools within the roots or cells.

BRI–WAK chimeric fusion

If indeed WAK regulation can affect osmotic content of cells, then activation of WAK in a protoplast might be expected to increase its capacity to withstand shrinkage. As the activating ligand of WAK is unknown and protoplasts lack cell walls to which WAKs bind, the extracellular domain of the brassinosteroid receptor BRI1 (Li and Chory, 1997) was used to replace the entire extracellular domain of WAK2. A similar replacement has been effective in activating the XAR21 kinase (He *et al.*, 2000). The BRI–WAK chimeric fusion protein expressed under the constitutive 35S promoter was cloned into a plasmid also containing

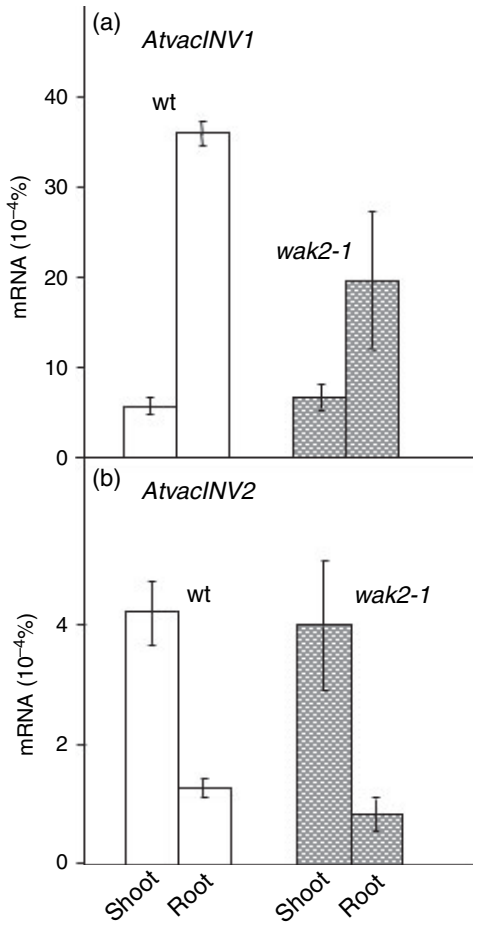

Figure 8. The *wak2-1* mutation reduces mRNA levels of the *AtvacINV1* vacuolar invertase in *Arabidopsis* roots.
Levels of *AtvacINV1* mRNA (a) *AtvacINV2* mRNA (b), and six cell wall invertases (not shown) were each determined by absolute, quantitative real-time PCR using 200 ng total RNA from each sample. Gene-specific values were normalized relative to 18S rRNA and expressed as percentages of total mRNA (note differences in scale for *y*-axes). Error bars indicate the SD of values from replicate experiments. The effects of the *wak2-1* mutation on the six cell wall invertases were negligible (not shown), although some reduction was evident in the highly variable levels of an *AtcwINV6* transcript in roots.

35S:green fluorescent protein (GFP) as a marker for transformation; hence only those cells expressing GFP should express BRI–WAK, and those not expressing GFP served as an internal standard. This plasmid was transformed into Arabidopsis protoplasts, and these were treated with 2 μm brassinolide (BL). The size of transformed (T) and non-transformed (NT) cells was monitored using light microscopy and Open Lab software, and their numbers, as well as those of each that died (Td or NTd) were tabulated. Incubation in media with BL (see Experimental procedures) caused 20% shrinkage of all cells every 24 h (Table 2). Notably, 54% of non-transformed cells (no GFP) shrunk and died within 48 h, while only 15% of the BRI–WAK transformed cells died (Table 3). Death was indicated by a loss of chlorophyll fluorescence and by the collapse of the plasma membrane. Cells expressing BRI–WAK with an inhibitory alanine substitution for a lysine at the catalytic site of the WAK protein kinase (K432A, Anderson *et al.*, 2001) behaved just as non-transformed cells did; *approximately* 50% of both cell types die within 48 h (Table 3). There was also no difference between transformed and non-transformed cells in cultures not treated with BL (Table 3). RT-PCR of RNA isolated from a population containing BRI–WAK transformed and non-transformed cells shows that both the BRI–WAK fusion and the BRI–WAK K432A fusion are expressed. As only approximately 20% of the cells are transformed, any changes in gene expression or cellular osmolytes would probably be masked by untransformed cells. Nevertheless, the results shown here are consistent with a WAK-mediated role in processes aiding the maintenance of cellular osmotic status that reduced cell death via excessive shrinkage in these experiments.

Discussion

An insertion mutation in *WAK2* results in a dependence on a combination of metabolized sugars and salts for seedling growth. These mutant plants show reduced vacuolar invertase activity, and a corresponding decrease in steady-state levels of mRNAs specific for the most prominent of the vacuolar invertases. Thus we propose that WAK2 either directly or indirectly regulates the transcription and activity of vacuolar invertase (*AtvacINV1*) as one constituent of a mechanism modulating solute concentrations. As changes in the concentration of solutes are known to be involved in turgor regulation (Koch, 2004), this provides a possible mechanism for WAK to regulate cell expansion. Cells with insufficient vacuolar invertase activity would be compromised in one avenue of turgor maintenance, thus leaving them vulnerable to insufficient turgor during expansion, and hence slow or arrested growth.

As a cell expands, there is increased need for a compensatory adjustment in turgor, and invertase activity

Table 2 Shrinkage of protoplasts

Construct	% T (24 h)	% Td (24 h)	% T (48 h)	% NT (24 h)	% NTd (24 h)	% NT (48 h)
BR1–WAK + BL	84.6 ± 8.5	83.6 ± 9.8	85.7 ± 11.6	82.3 ± 9.8	81.8 ± 14.8	81.7 ± 3.3
BR1–WAK K432A + BL	73.1 ± 6	67.8 ± 5.1	70.1 ± 9	73.2 ± 7.7	73.2 ± 5.8	81.2 ± 7.1

%(N)T, percentage of area relative to previous 24 h of (non)-transformed cells; Td or NTd, cells that died within 24 h; BL, brassinolide.

Table 3 Brassinolide (BL) causes the BRI–WAK fusion to protect protoplasts from death

Construct	% T broken	% NT broken	*P*-value
BR1–WAK + BL	8 ± 6.7	41 ± 9.5	0.008
BR1–WAK K432A + BL	49 ± 5	48 ± 4	0.93
BR1–WAK, no BL	52 ± 3.7	50 ± 6.1	0.36

%(N)T broken, percentage of (non)-transformed cells broken after 48 h.

can provide a critical source of organic solutes (one sucrose yielding two hexoses). WAKs may sense cell wall expansion by their attachment to pectin, and provide a mechanism for transducing these signals to systems regulating solute changes. This in turn can influence the maintenance of turgor as the cell enlarges. It is of interest that WAK is bound to pectin as this is thought to be the more flexible component of the cell wall (Willats *et al.*, 2001). In the model proposed here, it would be expected that the cell turgor be similar between the mutant and wild-type, as growth arrest in the mutant would compensate for osmotic deficiencies during growth; arrest or slowed growth would occur before turgor could drop below a critical level. Indeed, estimates of root turgor by plasmolysis studies indicate no differences between wild-type and mutant (data not shown).

Invertase activity does not appear to be markedly altered in *wak2-1* leaves, and this may be due to several factors. WAK2 is abundant in leaves, and thus would be expected to be active and influence leaf invertases. However, leaves contain higher levels and different pools of sugars, and these may override either a WAK2 role in metabolic regulation and/or its regulation of vacuolar invertase. Thus the root may be more sensitive to alterations of sugar metabolism, and to the absence of WAK2. This view is consistent with the observation that *wak2-1* is rescued by supply of sucrose (i) from the media, (ii) from leaves via enhanced photosynthesis on soil, or (iii) by ectopic SPS expression. Thus it is likely that the effect of reduced invertase activity is overcome by elevated supplies of sucrose that could overcome the lower rates of glucose and fructose production. In addition, invertase expression and activity are known to be regulated by the abundance of sucrose, and also a variety of other environmental stimuli (Koch, 1996, 2004), such that invertase activity in the *wak2-1* mutant may be modulated through this mechanism. As the location of sugars in different pools and organelles of the cell cannot be easily measured, this model has yet to be tested, but it is consistent with results presented here. Invertase may be activated by a WAK2-independent pathway in the leaf, thereby masking a *wak2-1* leaf phenotype. The lack of a visible leaf phenotype may also be due to the presence of overlapping functions of the WAK gene family that are less effective in the root, yet this remains to be tested.

Previously published work reported that antisense WAK expression in the leaves, where there was a 50% reduction in total WAK protein levels, resulted in smaller leaf cells and hence dwarf plants. Antisense WAK affected total WAK protein and thus at least expression of WAK1–WAK5, and not just WAK2, and this alone may account for the lack of a leaf phenotype in *wak2-1* plants. In addition, the induction of WAK antisense in roots was not possible (Wagner and Kohorn, 2001), and hence it is not known what effect this has on root cells. Nevertheless, the loss of leaf cell expansion in the WAK antisense plants is consistent with the reduction in root cell expansion in the *wak2-1* mutant. While the root cells themselves are not shorter than wild-type, the roots are. As roots grow from their tips, a reduced rate of expansion could be manifested by an overall shorter root, with cells in the elongation zone having an apparent size progression similar to those of a longer root, and this is indeed what is observed. In leaves, a reduced or arrested cell expansion results in smaller cells.

If indeed WAK can indirectly regulate invertase, then ectopic expression of WAK2 in protoplasts might be expected to increase cellular solute levels.. The results of the BRI–WAK protoplast expression are consistent with this model. BRI–WAK, but not the BRI–WAK K432A mutant kinase, increased the survival of protoplasts. Protoplasts are active in both sucrose metabolism and transport and some photosynthesis, but the actual sugar flux and concentrations of metabolites in these protoplasts is not known. It is clear that expression and activation of WAK causes these shrinking protoplasts to survive longer, consistent with a net accumulation of solutes within them. It was expected that BRI–WAK expression would slow the rate of shrinkage, but this was not detected. It is possible that the activation of invertase under these conditions is only effective below a certain osmotic potential, and thus only those cells reaching this threshold are affected. Cell that go beyond this threshold lyse. Future studies with native WAK protein and invertase promoters may help to clarify this possibility. In this protoplast system, only 20% of the protoplasts are transformed, so it is unlikely that changes in activity, mRNA, or solute composition can be detected against the larger background of untransformed material. Although such measurements will require the establishment of a stably transformed line, the present data clearly show that WAK2 activation confers a survival advantage to shrinking protoplasts.

This work provides a direct link between a protein bound to the plant cell wall and obvious alterations in metabolism that are critical to plant function. Indeed, causal relationships between sugar metabolism and programs for structure and development have been suggested in the literature (Koch, 2004; Roitsch and Gonzalez, 2004; Rolland *et al.*, 2002;

Sherson *et al.*, 2003). The results presented here indicate that there is also a signaling path potentially linking input from the cell wall to adjustments in endogenous sugar metabolism. Further investigations of this system may help to decipher how cell shape and plant form are related to the function of internal metabolic enzymes.

Experimental procedures

Plant materials

Arabidopsis thaliana (ecotype Columbia) wild-type and T-DNA insertion mutant lines were grown at 22°C under 14 h light condition (150 μM photons m^{-2} sec^{-1}) either on soil (Metromix; Scotts-Sierra Hort Products, Marysville, OH, USA) or agar plates.

DNA constructs and transformation

CaMV35S:WAK2HA. PCR was performed with the following primers using the full-length *WAK2* cDNA in pZL1 (clone 230O3; ABRC, The Ohio State University, Columbus, OH, USA) as a template. The forward primer was 5′-cgcctgcaggtaccggtccggaattcccggg-3′, annealing to the vector arm; the reverse primer was 5′-gaatttttgtcggagctcagcgtagtctgggacgtcgtatgggtatggttcttccggtgc-3′. The product encodes the first half of WAK2 cDNA (amino acids 1–360) followed by the HA epitope (YPYDVPDYA). The product was digested with *Eco*RI and *Sac*I and cloned into pBI1.4t (Mindrinos *et al.*, 1994) together with the *Sac*I–*Bam*HI fragment of *WAK2* cDNA to make 35S:WAK2HA. *wak2-1* plants were transformed with the construct by the *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998), and transformants were selected on kanamycin-containing medium.

35S:MSPS. A *Bam*HI-*Eco*RI fragment of p35SMSPS (C.H. Foyer, Institute of Arable Crops Research, Rothamstead, UK, unpublished data), which contains the full-length maize SPS cDNA (Genbank accession no. M97550), was transferred to pET-24a (Novagen, Madison, WI, USA). A 3.5 kb *Bam*HI–*Sac*I fragment containing the full-length cDNA was transferred to pBI1.4t, and the resulting construct was used to transform wild-type and *wak2-1* plants.

BRI–WAK fusion

The BRI extracellular domain and transmembrane domain were amplified to generate a 5′ *Sal*I 3′ *Spe*I site using the forward primer 5′gtcgacaaactcttgagaaatgaagacttttta, and reverse primer 5′cgtggcggatactagttccgcaaacg. The WAK kinase domain was amplified to generate a 5′ *Spe*I site (present in WAK coding) and a 3′ *Bam*HI site using forward primer 5′gctgtctacaactagtacagaaaattaagcacc, and reverse primer 5′gtcagagcattaataggatcctcaacgccag. These were cloned, along with a *Kpn*I–SaI fragment containing the 35S promoter and a *Bam*HI–*Hin*dIII nos terminator from pBIN19, into the *Kpn*I–*Hin*dIII sites of pCambia1302.

Protoplasts were prepared from Arabidopsis leaves and transformed as described by Sheen (http://genetics.mgh.harvard.edu/sheenweb/jen-archive.html). Transformed protoplasts were incubated at room temperature in medium containing 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl and 2 mM MES-KOH (pH 5.7) supplemented or not with 2 μM brassinolide. Cell size was measured using Open Lab software.

Quantitative real-time PCR

Total RNA was extracted using RNeasy Plant Mini Kits (Qiagen, Valencia, CA, USA), and treated with DNase (DNA-*free* kit, Ambion, Austin, TX, USA). Gene-specific mRNA levels were determined by absolute, quantitative real-time (Q-RT) PCR for each of the eight acid invertases in *Arabidopsis*, using 200 ng of RNA in 25 μl reactions with *Taq*-Man One-Step RT-PCR Master Mix Reagents (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems GeneAmp 5700 sequence detection system. Primers and Taq-Man probes were designed using Applied Biosystems Primer Express software (Version 2.0) as described by Huang (2006). The fluorescent FAM reporter and TAMRA quencher were bonded to the 5′ and 3′ ends of the probes, respectively. Amplicon lengths were 66–78 bp. To avoid amplification of trace contamination by genomic DNA, most gene primers and probes spanned two exons. In addition, controls without reverse transcriptase were included to test for any detectable amplification of DNA. RT-PCR was conducted at 48°C for 30 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. RNA standards were synthesized from cDNAs, with standard curves being linear from 1.2×10^{-10} to 2.4×10^{-14} g. The *Ct* threshold value was determined as 0.075. Values were normalized relative to 18S ribosomal RNA (TaqMan® Ribosomal RNA Control Reagents, Applied Biosystems).

Enzyme assays

Hexokinase. Tissues were homogenized in a chilled mortar in buffer (4–6 ml g^{-1} fresh weight) containing 50 mM HEPES-KOH (pH 7.5), 1 mM $MgCl_2$, 1 mM EDTA, 10 mM KCl, 2.5 mM DTT, and protease inhibitors (Complete Mini; Roche, Indianapolis, IN, USA). The homogenate was centrifuged at 12 000 ***g*** for 5 min and the supernatant was desalted on Sephadex G-25 equilibrated with 50 mM Hepes-NaOH (pH 7.5), 1 mM EDTA and 1 mM DTT. Protein concentration of desalted extact was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with BSA as a standard.

Hexokinase activity was assayed with 100 μl of desalted extract in 1 ml reactions containing 30 mM HEPES-NaOH (pH 7.5), 1 mM $MgCl_2$, 0.5 mM EDTA, 10 mM KCl, 1 mM NAD, 1 mM ATP and 2 units of NAD-dependent Glc-6-P dehydrogenase (from *Leuconostoc*, Sigma, St Louis, MO, USA). The reaction was initiated with 2 mM Glc, incubated for 1 h at 37°C, and the absorbance at 340 nm (A_{340}) was measured. A blank without ATP was included to subtract the background.

Sucrose phosphate synthase. Tissue homogenate was prepared as described for hexokinase using extraction buffer containing 50 mM HEPES-KOH (pH 7.5), 15 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, 0.1% v/v Triton X-100 and protease inhibitors, and the centrifuged supernatant was desalted into extraction buffer minus Triton X-100 and protease inhibitors.

SPS activity was assayed with 50 μl of desalted extract in 70 μl reactions containing HEPES-KOH (pH 7.5), 15 mM $MgCl_2$, 10 (2.5) mM fructose-6-phosphate (Fru-6-P), 40 (10) mM glucose-6-phosphate (Glu-6-P), 10 mM UDP-glucose (UDP-Glc) and 0 (10) mM Pi, for non-limiting (and limiting) substrate conditions. Reactions were initiated by the addition of extracts and incubated at 37°C for 7 min. They were stopped by the addition of 70 μl 5 M KOH and boiling for 10 min. Blanks without Glc-6-P and Fru-6-P were included to subtract the background. Sucrose was determined by the anthrone method as described previously (Weber *et al.*, 1996).

Sucrose synthase. Tissue homogenate was prepared as described for hexokinase using extraction buffer containing 50 mM HEPES-KOH (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 0.1% Triton X-100, 10% (v/v) glycerol and protease inhibitors (Complete Mini), and the centrifuged supernatant was desalted into 25 mM HEPES-KOH (pH 7.0), 0.5 mM DTT and 0.5 mM EDTA.

Sucrose synthase activity was determined in the catabolic direction (UDP-dependent formation of UDP-Glc). The assay was carried out with 170 μl of desalted extract in 200 μl reactions containing 25 mM HEPES-KOH (pH 7.0), 100 mM sucrose and 4 mM UDP. A blank without UDP was included to subtract the background. Reactions were started with sucrose and incubated for 1 h at 37°C. After boiling for 5 min, 1 ml of 300 mM glycine-NaOH (pH 8.9), 5 mM $MgCl_2$, 2 mM NAD and 0.0025 units of UDP-Glc dehydrogenase (from bovine liver, Sigma) were added, and the reaction mixture was incubated for 30 min at 37°C before measurement of A_{340}.

Invertases. Cell wall and vacuolar invertase activities were measured in the leaves and roots of 2-week-old seedlings grown on PW as described previously (Link *et al.*, 2004) except that the enrichment of vacuolar invertase was not carried out.

Immunoblotting

Leaves were ground in 2X sample loading buffer (100 mM Tris-HCl, pH 6.8, 100 mM DTT, 8% SDS, 0.05% bromophenol blue, 20% glycerol) in microtubes using plastic pestles, boiled for 5 min, and centrifuged at 10 000 ***g*** for 3 min. The supernatants were fractionated by SDS–PAGE on 10% gel. Proteins were electroblotted to nitrocellulose membranes in 50 mM Tris, 380 mM glycine, 0.1% SDS and 20% methanol. Blots were blocked in 5% w/v non-fat dry milk in Tris-buffered saline supplemented with 0.3% v/v Tween-20. Anti-HA antiserum (HA-Probe, Y-11; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-SPS antiserum was used at 1:2500 dilution. Blots were further incubated with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (Molecular Probes, Eugene, OR, USA) and signal was detected by chemiluminescence.

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