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Wall-Associated Kinases Are Expressed throughout Plant Development and Are Required for Cell Expansion

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The mechanism by which events in the angiosperm cell wall are communicated to the cytoplasm is not well characterized. A family of five Arabidopsis wall-associated kinases (WAKs) have the potential to provide a physical and signaling continuum between the cell wall and the cytoplasm. The WAKs have an active cytoplasmic protein kinase domain, span the plasma membrane, and contain an N terminus that binds the cell wall. We show here that *WAK*s are expressed at organ junctions, in shoot and root apical meristems, in expanding leaves, and in response to wall disturbances. Leaves expressing an antisense *WAK* gene have reduced WAK protein levels and exhibit a loss of cell expansion. WAKs are covalently bound to pectin in the cell wall, providing evidence that the binding of a structural carbohydrate by a receptor-like kinase may have significance in the control of cell expansion.

INTRODUCTION

In animal, fungal, and algal systems, the physical connection and the communication between the extracellular matrix (ECM) and the cell plays a fundamental role in cell growth and division (Fowler and Quatrano, 1997; Lukashev and Werb, 1998; Tsai, 1998). Similarly, the plant cell wall forms an ECM of carbohydrate and protein that provides structure for individual cells and whole organs. The cell wall must be dynamic as cells divide and elongate, and modulation of its composition and architecture is required during its synthesis and after it has been deposited (Cosgrove, 1997; Reiter, 1998). The wall must therefore be considered in the context of modulating plant development (Kohorn, 2000). Communication between the cytoplasm and the cell wall is necessary and evident because events like cell expansion (Cosgrove, 1997) and pathogen infection (Hammond-Kosack and Jones, 1996) lead to altered biosynthesis and modification of cell wall components and downstream cytoplasmic events such as systemic acquired resistance. How the dynamics and synthesis of the cell wall are coordinated with cytoplasmic events is largely uncharacterized.

Developing cells have walls that are composed of cellulose, hemicellulose, pectin, and proteins. Cellulose is directly secreted by cellulose synthase into the ECM, where it assembles with hemicelluloses and pectins, which are produced in the endomembrane system and secreted by vesicles. The cell wall also includes endoglucanases (Hayashi et al., 1984; Zuo et al., 2000), xyloglucan endotransglycosylases (Fry et al., 1992; Vissenberg et al., 2000), expansins (McQueen-Mason et al., 1992; Cho and Cosgrove, 2000), and a number of other glycosyl transferases that alter carbohydrate linkages and modify secreted cell wall components. Other cell wall proteins, some of which are heavily glycosylated, have been proposed as structural cell wall components or have been implicated in mediating multiple aspects of plant development (reviewed in: Showalter, 1993; Cosgrove, 1997; Kohorn, 2000). These include the families of proline-rich proteins, glycine-rich proteins, hydroxyproline-rich glycoproteins, and arabinogalactan proteins (Majewska-Sawka and Nothnagel, 2000). Different family members show highly regulated and specific patterns of expression. In addition to protein heterogeneity, carbohydrate composition can vary between cell types, and even within one wall of a given cell. For example, pectins can be modified by esterification and detected in distinct regions of the cell wall (McCann et al., 1993; Knox, 1997; Steele et al., 1997). Somehow, the synthesis, secretion, and assembly of wall components is coordinated to produce a functional wall throughout the development of a plant.

There are four classes of proteins that are known to physically link the ECM to the plasma membrane and may facilitate communication between the two compartments. These include cellulose synthases (Pear et al., 1996), a class of arabinogalactan proteins that are reversibly attached to the plasma membrane via a glycosyl phosphatidyl inositol anchor (Oxley and Bacic, 1999; Svetek et al., 1999), a glycolytic enzyme with a transmembrane domain (Nicol et al., 1998), and wall-associated kinases (WAKs; He et al., 1996, 1999). WAKs have the potential not only to link the ECM with the plasma membrane but also to directly

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signal cytoplasmic events. WAKs have an N terminus that is tightly linked to the cell wall, and a transmembrane domain separates the extracellular sequence from a carboxyl cytoplasmic serine/threonine protein kinase. WAKs can only be released from the wall by boiling in 2% SDS and DTT or by treating plant tissue with cell wall–degrading enzymes (He et al., 1996). Immunoelectronmicroscopy confirms that WAKs are associated with the cell wall.

Although the structure of WAKs suggests a role in cell wall–membrane binding and signaling, their true function is unknown. *WAK* expression is required during a response to pathogen because their induction is necessary for plants to survive high levels of salicylic acid (SA; He et al., 1998). Five *WAK* isoforms have been identified that are 40 to 64% identical in their extracellular amino termini and 86% identical in their cytoplasmic kinase domains. Variability in amino acid sequence among the amino termini may indicate distinct interactions between the cell wall components and individual isoforms. At the same time, the amino acid identity among the kinase domains suggests that the WAK isoforms can signal similar cytoplasmic events. Alternately, the presence of multiple *WAK* genes may only provide for a wider variety of tissue-specific, regulated expression.

Here, we describe the cell-specific expression patterns for the five *WAK* genes. *WAK*s are expressed at organ junctions, in shoot and root apical meristems, and in expanding leaves. The patterns overlap, so there is a potential for interactions among these isoforms. Additionally, *WAK* expression responds to wall disturbances, and this pattern is superimposed on the developmental pattern of each isoform. We also show that plants with reduced WAK protein, due to the production of antisense *WAK* RNA, are blocked in leaf cell expansion. Moreover, we find that WAKs are bound to pectin and suggest that this interaction may be important in the regulation of cell expansion.

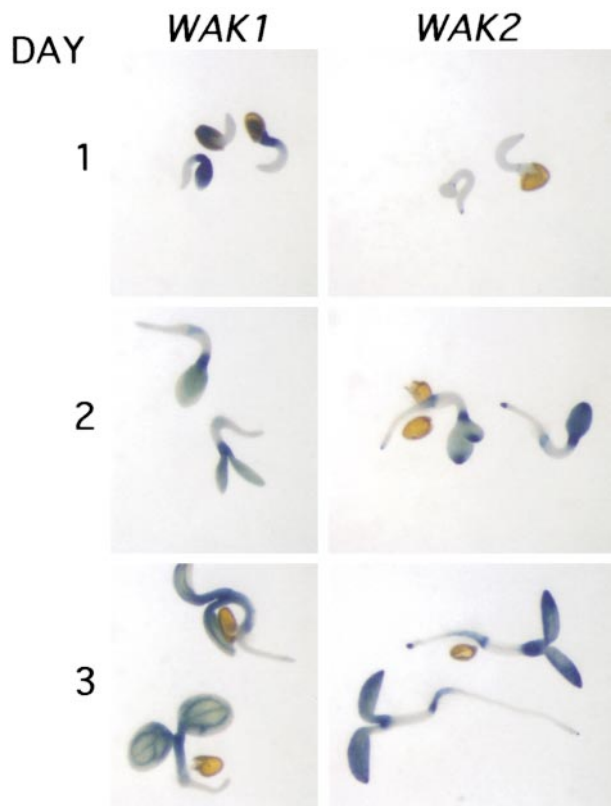

Figure 1. *WAK1*and *WAK2* Promoters Are Differentially Active in Seedlings.

T3 seedlings containing the *WAK1* promoter–GUS transgene (at left) or the *WAK2* promoter–GUS transgene (at right) were grown on plates for 1, 2, and 3 days in the growth chamber and then stained for GUS.

RESULTS

Expression

To examine the cellular expression for individual *WAK*s, we fused the β-glucuronidase (GUS) coding region to each of the *WAK* promoters and transformed them into Arabidopsis Columbia ecotype (Col-0). At least six independently transformed lines, each segregating for a single T-DNA insertion, were examined to characterize each promoter–GUS construct. Previous data obtained by RNA gel blot analysis using gene-specific probes indicated that *WAK1* and *WAK2* were the most abundantly expressed *WAK*s and that they were found predominantly in leaves and stems (He et al., 1999). Therefore, we started our analysis with *WAK1* and *WAK2* to see if there were developmental and cell type differences in their expression. Figure 1 shows representative plants containing the *WAK1* and *WAK2* promoter–GUS fusions in the early stages of development.

The *WAK1* promoter was active in the cotyledons before breaking the seed coat (Figure 1, 1 day), and then the level of GUS decreased as the cotyledons started to open (Figure 1, 2 days). As cotyledons opened, GUS was found in the vasculature (Figure 1, 3 days). Expression was detected at the junction between the cotyledons and the hypocotyl at 1 day and remained at the petiole/stem junction throughout the growth of the vegetative shoot apical meristem (SAM). Longitudinal sections through 7-day-old GUS-stained *WAK1*–GUS seedlings show that the GUS staining at the junction was seen throughout the SAM and leaf primordia (Figure 2, top left) and was not restricted to a particular cell layer or region. Older leaf primordia had prominent GUS staining at their bases and in the vasculature beneath the SAM.

In comparison, the *WAK2* promoter was first active at the root tip and at the cotyledon/hypocotyl junction (Figure 1, 1 day). GUS stained throughout the SAM (Figure 3, top left), similar to the *WAK1* promoter. As the cotyledons started to

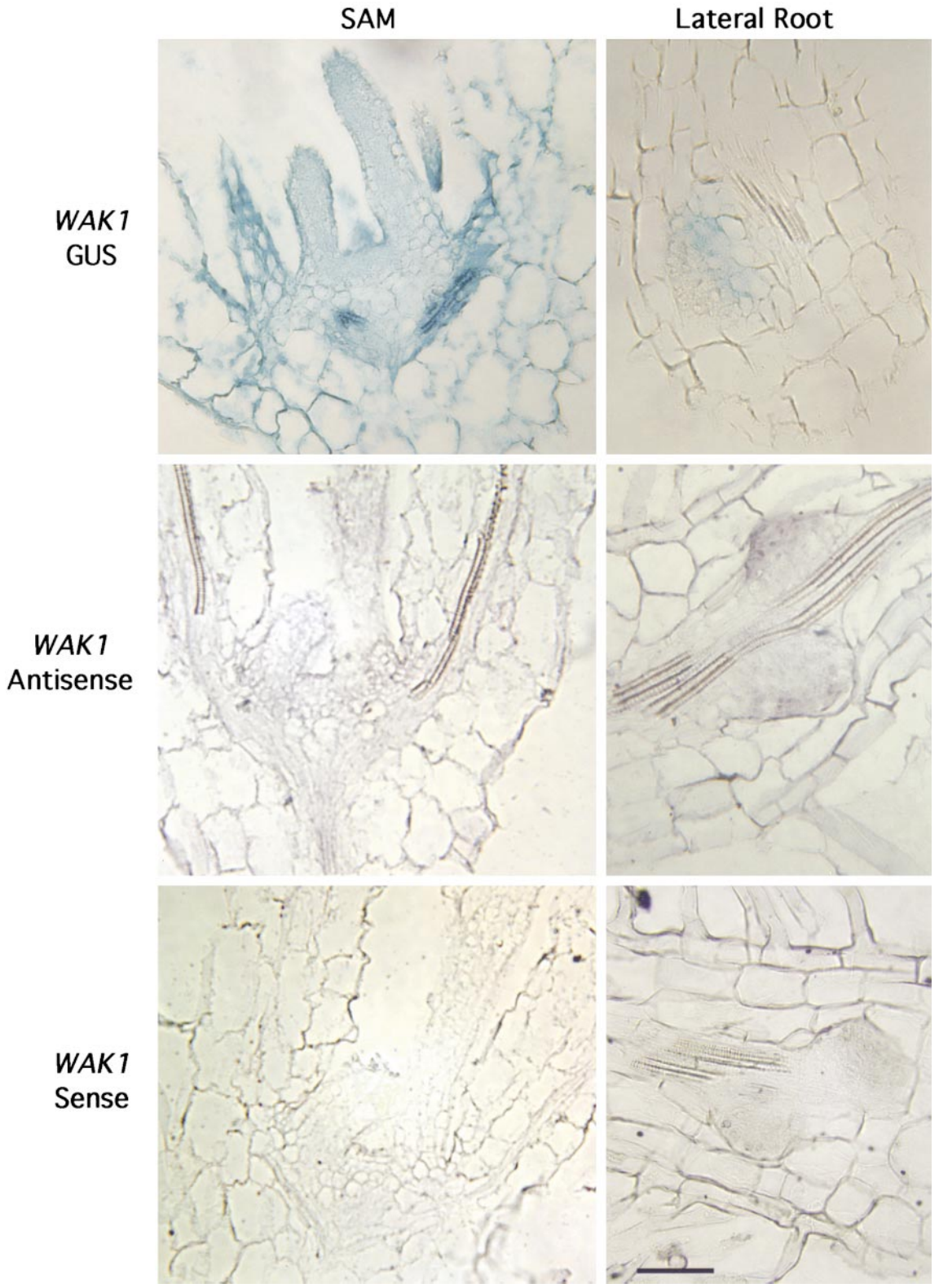

Figure 2. *WAK1* Is Expressed in Root and Shoot Meristems.

Sections through 7-day-old seedlings containing the *WAK1* promoter–GUS transgene and stained for GUS (top) were compared with similar sections that were probed with the antisense (middle) and sense (bottom) *WAK1* transcript. Bar = 50 μm.

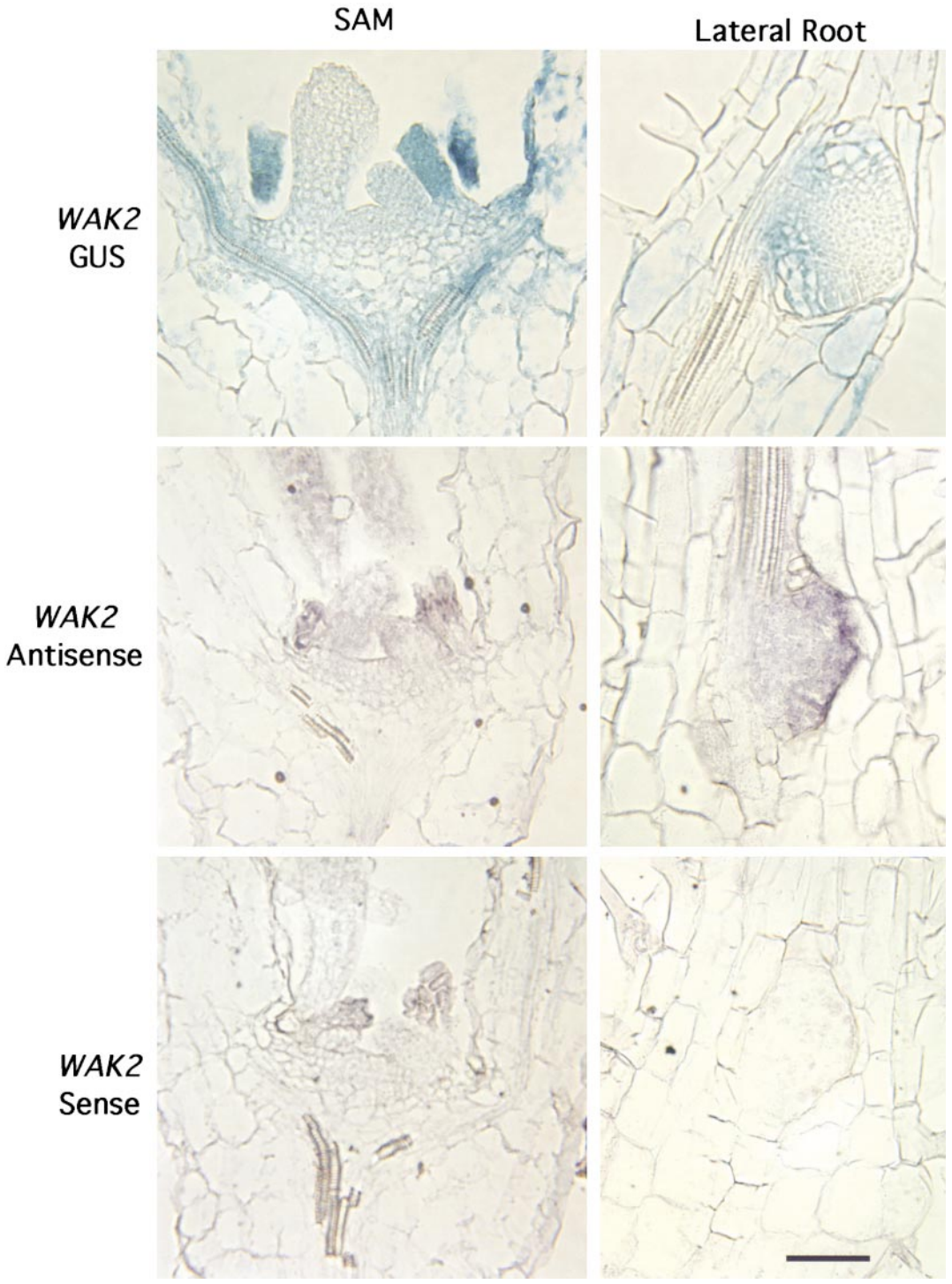

Figure 3. *WAK2* Is Expressed in Root and Shoot Meristems.

Sections through 7-day-old seedlings containing the *WAK2* promoter–GUS transgene and stained for GUS (top) were compared with similar sections that were probed with the antisense (middle) and sense (bottom) *WAK2* transcript. Bar = 50 μm.

expand and open, GUS was visible at the cotyledon tip and then throughout the cotyledon, although the margins were darker than the interior (Figure 1, 2 to 3 days). The root/hypocotyl region of both *WAK1* and *WAK2* promoter–GUS seedlings showed GUS staining after 2 to 3 days (Figure 1, 2 to 3 days). This region is where lateral roots will first emerge (Figures 2 and 3, top right).

In situ hybridization using DIG-labeled *WAK1* and *WAK2* cDNAs as probes was used to confirm the GUS staining detected in seedlings (Figures 2 and 3). Compared with the sense controls, the *WAK1* was clearly expressed in the SAM, and this mirrors the pattern seen in GUS sections (Figure 2). *WAK1* was not restricted to any region or layer in the SAM. *WAK1* was also found in the lateral root meristem by DIG labeling. Lateral root sections probed with *WAK2* antisense resemble the *WAK1* pattern (Figure 3). *WAK2* was detected clearly with antisense probes in the SAM and leaf primordia. However, the hybridization signal was weak, and the expected expression in the vasculature underneath the SAM was difficult to detect. In summary, at early stages in seed germination, the *WAK1* and *WAK2* promoters showed distinct yet overlapping patterns of expression. Their expression overlaped at the shoot and root apical meristems and throughout the cotyledon.

The *WAK1* and *WAK 2* expression patterns were also studied in older tissues, and the results are shown in Figure 4. For the *WAK1* promoter fusion, leaf primordia stained, but the emerging leaf had very little GUS. GUS staining was then seen in the vasculature and increasingly throughout the leaf, such that older leaves had high levels of GUS expression in the blade and petiole (Figure 4A). This pattern was true for all rosette leaves. The *WAK2* promoter was active in young leaves at the leaf tip, hydothodes, and at the leaf margin. GUS accumulated throughout the blade and petiole as the leaf expanded (Figure 4B). *WAK2*–GUS plants showed some vasculature staining, but not as much as did *WAK1*–GUS plants. Thus, *WAK1* and *WAK2* were expressed in both juvenile and adult leaves, and the promoters remained active as leaves continued to expand.

In contrast to rosette leaves, there was very little expression of *WAK1* and *WAK2* in cauline leaves (Figures 4D and 4E) except for faint vascular *WAK1*–GUS staining. There was some expression of the *WAK1* and *WAK2* promoters in the inflorescence stem, flowers, and siliques. In the inflorescence stem, GUS staining for *WAK1* and *WAK2* was only seen at nodes and at the base of cauline leaves (Figures 4D and 4E). RNA gel blot analysis of RNA from inflorescense stem tissue divided into nodes and internodes confirmed the GUS data (data not shown). There were some subtle differences in flower staining between *WAK1* and *WAK2* (Figures 4G and 4H). For the *WAK1* promoter fusion, GUS was present at the sepal tips early in development. This staining was clearly visible by stage 7 (Smyth et al., 1990), but sometimes the staining was seen in sepals that did not yet enclose the flower bud (Figure 5A). Staining at the base of the flower coincided with the time that the sepals opened. All lines examined showed variablity in the timing and amount of ovary expression (data not shown). The *WAK1* promoter was frequently active at the stigmata surface before the flower opened (Figure 4G). In contrast, the *WAK2* promoter was active at the flower base between stages 6 and 9 (Figure 4H), earlier than *WAK1*. This is the period in which the ovary and stamens mature. *WAK2*–GUS staining at sepal tips occured later than *WAK1*, around stage 9 (a time of rapid flower expansion). Sometimes the stigma was stained in open flowers. The base and often the tip of the siliques stained for GUS in both *WAK1* and *WAK2* promoters (Figure 4F; data not shown). In green siliques that were cut open, GUS was detected thoughout the ovules and the ovary wall in *WAK1* promoter–GUS plants (Figure 4I).

GUS staining was detected faintly in longitudinal sections of GUS-stained *WAK1* and *WAK2* floral apical meristems. In some cases, staining of the flower primordia was limited to the L1 layer (Figure 5). The staining of the inflorescence SAM was reduced compared with the expression seen in the vegetative SAM. Faint blue spots could be seen in *WAK1*–GUS developing sepals and stamens. Floral buds (stages 1 through 5) showed faint *WAK2* promoter activity in the rib meristem and in the pith and vasculature of the young inflorescence stem (Figure 5B). Unfortunately, the low-level GUS patterns seen in flower buds, flowers, and siliques could not be confirmed by in situ hybridization because no signal was detected above background.

The *WAK1* and *WAK2* promoters also responded to environmental cues. *WAK1* and *WAK2* mRNA has previously been shown to be induced by pathogen-related events (He et al., 1998, 1999). We tested the *WAK1* and *WAK2* promoter–GUS fusions for similar induction by growing seedlings in the absence or presence of 100 μM 2,6-dichloroisonicotinic acid (INA) (Figure 6A). INA is a commercial analog of SA, a compound that is necessary for systemic acquired resistance. In the presence of INA, GUS staining was increased in the hypocotyl and cotyledons and decreased at the hypocotyl/petiole junction. The results are shown for *WAK1*, but the *WAK2* promoter was similarly induced, although the induction in cotyledons was difficult to detect above the strong developmentally regulated expression. In the process of testing leaves for GUS expression, we observed wound-inducible expression for *WAK1* and *WAK2*. The wound response was most visible in leaves that had ceased expansion since the background, noninduced GUS staining had abated (Figure 6B). Younger rosette leaves, removed at the same time, also showed wound induction (Figure 6B) over and above a high level of developmental expression. Thus, the *WAK1* and *WAK2* promoters are not active in leaves that have stopped expanding unless the leaves are environmentally stressed. The wound response was very rapid, because leaves that were wounded and immediately put into GUS substrate containing 20 μM cycloheximide still showed the wound response. The *WAK2* wound induction has been confirmed by RNA gel blot (data not shown). It is likely that the developmental pattern of

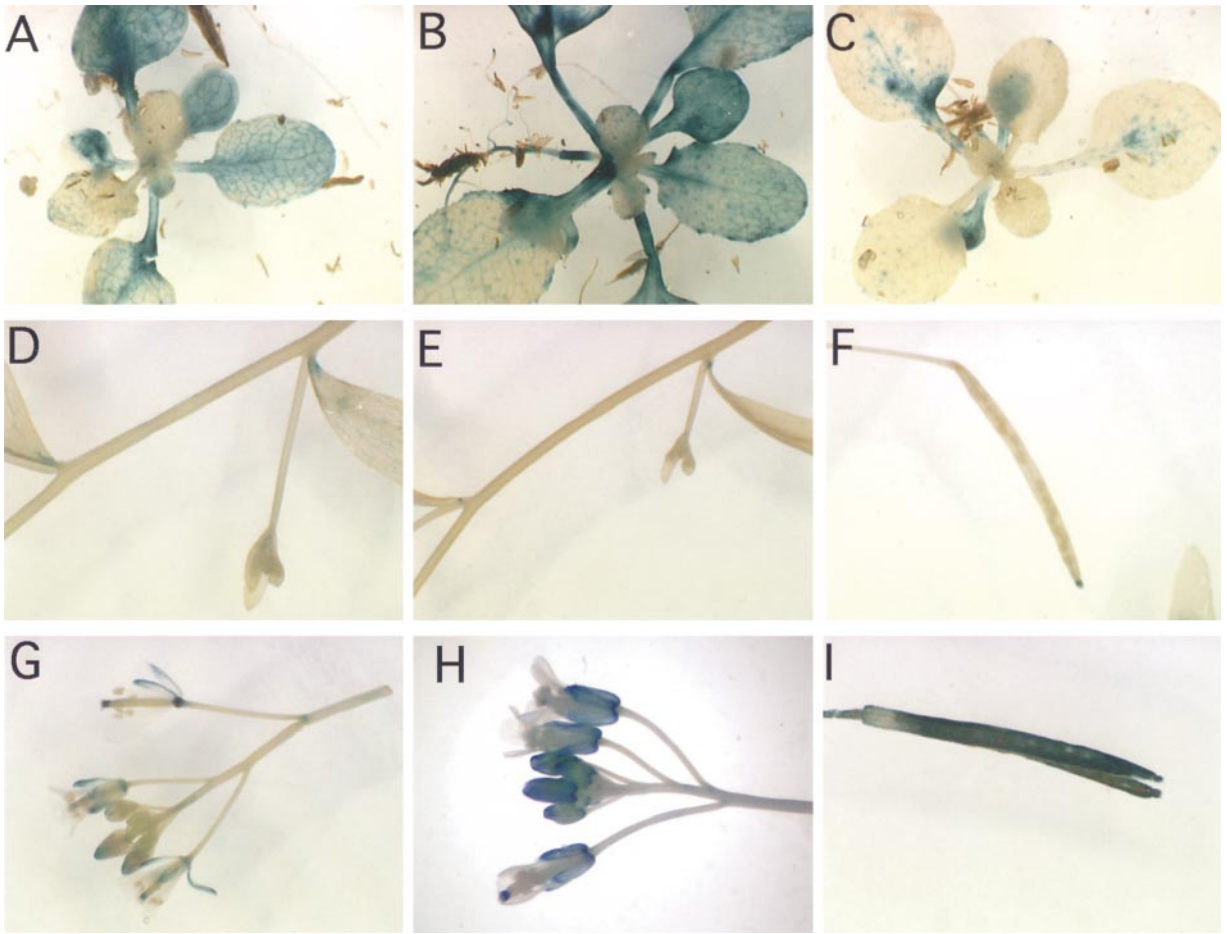

Figure 4. The *WAK1*, *WAK2*, and *WAK3* Promoters Are Active in Adult-Stage Plants.

(A) to **(C)** Young rosette plants grown in soil for 14 days. **(A)** *WAK1* promoter–GUS; **(B)** *WAK2* promoter–GUS; and **(C)** *WAK3* promoter–GUS.
(D) and **(E)** GUS staining at the node of the inflorescense stem. **(D)** *WAK1* promoter–GUS; **(E)** *WAK2* promoter–GUS.
(F) Silique with GUS staining at the tip. *WAK1* promoter–GUS.
(G) and **(H)** Flower clusters. **(G)** *WAK1* promoter–GUS; **(H)** *WAK2* promoter–GUS.
(I) Silique taken from the plant shown in **(F)**. *WAK1* promoter–GUS.

WAK expression is not caused by, and is distinct from, wound induction. Applying pressure or bending tissues did not induce any *WAK* expression, and all plants were intact when placed in GUS substrate to ensure that the GUS patterns were not caused by wounding.

Results with the *WAK3* promoter–GUS fusion revealed a pattern reminiscent of microlesions or "microbursts" (Figure 4C; Alvarez et al., 1998). *WAK3* was previously detected by RNA gel blot analysis in leaves and stems, but to a lesser extent than *WAK1* or *WAK2* (He et al., 1999). Using GUS as a marker, the *WAK3* promoter was indeed shown to be active in cotyledons, leaves, and stems, but only in large and small spots of GUS staining (Figure 4C). Some spots were the size of a single cell, and the spots were frequently seen along the vasculature as opposed to the tissue within. The spots of GUS appeared to coalesce as the leaves matured. The number and intensity of the spots increased when the plants were grown on media containing 100 μM INA (data not shown). To determine if these spots were related to reactive oxygen species, leaves and seedlings were treated with 2 μM hydrogen peroxide for 1 hr or 2.5 μM diphenylene iodinium (an NADPH oxidase inhibitor) for 2 hr, and then the tissues were put into the GUS substrate and stained. These treatments did not change the appearance or number of spots (data not shown), so the underlying mechanism behind the spot pattern is not clear. *WAK3*–GUS expression was rarely seen in the infloresence (data not shown). The *WAK1* and *WAK2* promoter–GUS plants did show a spotty

pattern on top of their normal developmental pattern (e.g., Figures 4A and 4B), so there is a potential overlap of *WAK3* expression with that of *WAK1* and *WAK2* at the cellular level, particularly in older expanding leaves.

The promoter–GUS fusions for *WAK4* and *WAK5* indicated that they are not widely expressed. *WAK4* was previously detected by RNA gel blot to be silique specific (He et al., 1999), and the *WAK4* promoter fused to GUS was not sufficient to drive GUS expression in siliques. In the case of *WAK5*, expression was barely detectable by RNA gel blot in leaves and stems (He et al., 1999), and the *WAK5* promoter was active only in the roots of seedlings that were at least 5 days old. The GUS staining was weak, occurred in spots (similar to *WAK3*), and the staining was more evident in seedlings grown on plates than on soil (data not shown). Reverse transcription–polymerase chain reaction (RT-PCR) failed to detect a product for *WAK5* in RNA either from roots or from the leaves and stem (combined) of 7-day-old seedlings (data not shown).

In summary, the different *WAK* genes showed distinct patterns of expression with a great deal of overlap. *WAK1* was expressed earlier than *WAK2* in cotyledons and sepal tips. *WAK2* was expressed at the margins of leaves, whereas *WAK1* was found more often in the vasculature. *WAK3* was expressed in a spotty pattern in the rosette plant, and *WAK1* and *WAK2* shared this pattern to a lesser extent. This analysis did not find significant expression of *WAK*s in the elongation zone of roots, the inflorescence stem, cauline leaves, and flower organs other than the base, sepals, and ovaries. *WAK1* and *WAK2* were expressed in shoot and root apical meristems at organ junctions in response to wall disturbances, and their expression in leaves and sepals correlates with these organs' expansion.

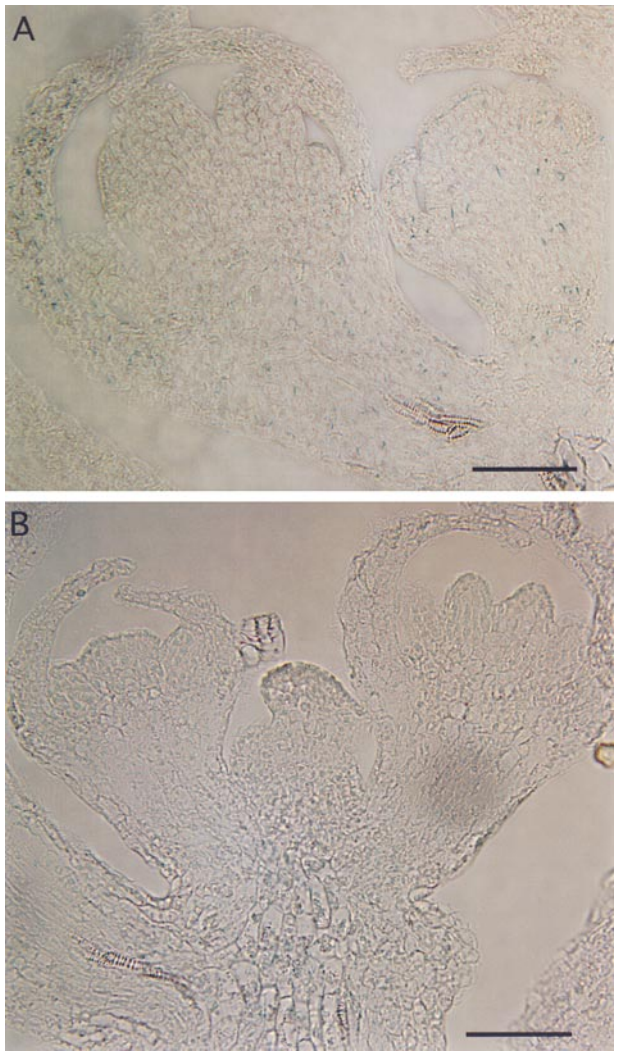

Figure 5. Floral Organ Activity of the *WAK1* and *WAK2* Promoters.

(A) A representative section of *WAK1*–GUS floral apical meristem showing promoter activity in the L1 layer of young flower buds and single spots at the floral base and in the developing sepal.
(B) A representative section of a *WAK2*–GUS floral apical meristem showing promoter activty in the L1 layer of developing floral organs, the rib meristem, and the floral base.
Bars = 50 μm.

WAKs Are Involved in Leaf Cell Expansion

WAK1 and *WAK2* were increasingly expressed in leaves as the leaves expanded. To determine if WAKs function in leaf expansion, the constitutive 35S promoter was first used to drive antisense expression in transgenic plants, but this contruct was presumably lethal because no transformed plants were obtained. Therefore, the dexamethesone (Dex)-inducible promoter (McNellis et al., 1998) was fused to the *WAK2* cDNA in the antisense direction and transformed into Arabidopsis (Col-0). Because the construct contains the conserved kinase domain, it was expected that the expression levels of all the WAK isoforms would be reduced, not just those of WAK2. Additionally, sequences specific for *WAK1* and *WAK2* from the N terminus were fused to the Dex promoter in the antisense direction. The empty vector was also transformed into Arabidopsis to control for phenotypes resulting from the constitutively expressed transcription factor that is required for the inducible system. Dex-inducible phenotypes have been reported for transgenic lines containing just the empty vector, and these phenotypes include seedling lethality, stunting in germinating seedlings, leaf chlorosis, and epinasty (Kang et al., 1999). Because of these limitations, the analysis of Dex-inducible phenotypes was limited to rosette-staged plants.

Seven independent lines containing the inducible *WAK2* antisense gene were studied. Whereas two lines appeared wild type upon Dex treatment, five lines showed an inducible phenotype when sprayed with 20 μM Dex. Five to 7 days after spraying, rosette leaves of these five lines were

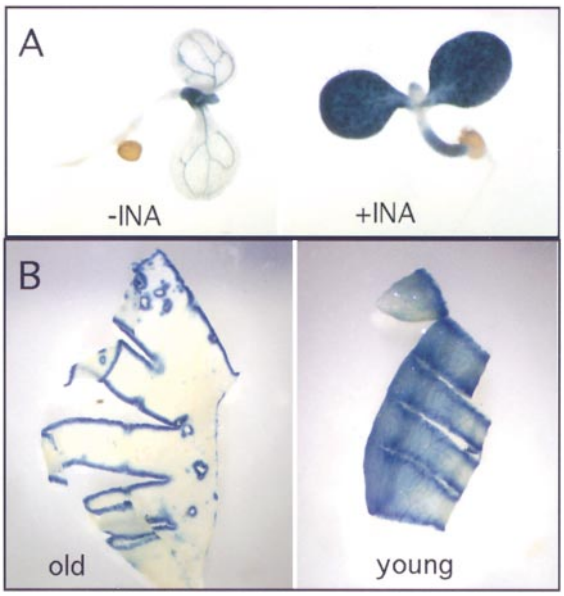

Figure 6. *WAK1* and *WAK2* Expression Is Induced by Environmental Signals.

(A) GUS-stained 5-day-old *WAK1*–GUS seedlings grown on media without (−) INA or (+) on media containing 100 μM INA.
(B) Old and young rosette leaves taken from a fully flowering *WAK2*–GUS plant and cut several times with scissors before immediately staining for GUS.

noticeably small compared with mock-sprayed plants (Figure 7A), and this phenotype was maintained as the plants aged through seed set. This small rosette phenotype was not seen in >10 independent lines containing the empty vector that had been sprayed with Dex. In contrast, induced *WAK1*- and *WAK2*-specific antisense plants (five independent lines each) did not show any phenotypes different from the empty vector lines (data not shown). Additional phenotypes were observed in the induced *WAK2* antisense plants. Occasionally, the Dex-sprayed *WAK2* antisense plants flowered earlier than the control plants, but this phenotype was variable (e.g., Figure 7A). Older leaves of the inducible antisense plants showed some chlorosis, necrosis, and leaf epinasty, but these phenotypes are sometimes seen in empty vector control plants.

To determine if small leaves from induced plants had reduced WAK protein, we isolated whole-cell extracts from leaves 3 days after spraying and analyzed them by SDS-PAGE and protein gel blotting. The experiment was performed with multiple lines and repeated three times with similar results. This serum was raised to the WAK kinase domain, which is 86% identical between the five WAK isoforms and, as expected, reacted with all WAKs expressed in *Escherichia coli* (data not shown; He et al., 1996, 1998). Protein gel blotting with the WAK antibody showed that *WAK2* antisense–expressing lines had, on average, a 0.5-fold reduction in total WAK protein compared with mock-treated leaves and empty vector controls (Figure 7B). Because leaves used in this analysis were not noticeibly different in chlorophyll content, a protein gel blot with a Cytochrome f antibody was used to normalize total protein amount.

Scanning electron microscopy of the abaxial leaf surface was used to determine if the reduced leaf size of the induced antisense plants was due to reduced cell division, reduced cell expansion, or some combination of the two. The same stage leaf, leaf 3, was taken from Dex-treated and untreated *WAK2* antisense plants 7 days after spraying, so that the tissues analyzed were at a similar stage of development before spraying. The leaves were photographed and their areas were calculated. These same leaves were then fixed and prepared for scanning electron microscopy analysis, and representative micrographs are shown in Figure 8A. The treated leaves had more and noticibly smaller cells than did the control leaves in both the petiole and the blade. For each leaf, five different scanning electron microscopy images along the leaf blade were captured. The average number of cells per 0.09-mm^2 area photographed is presented in Figure 8B. The Dex-treated leaves have more cells than do mock-treated leaves, and this difference is significant (analysis of variance: $P = 0.015$). When these cell numbers were put into the context of the total abaxial surface area (Figure 8C), the total number of cells was not significantly different ($P = 0.44$) in the induced antisense plants compared with the controls. Therefore, reduction of WAK protein levels leads to a loss of cell expansion and not of cell division.

WAKs Are Bound to Pectin

WAKs may facilitate cell expansion through their physical link to the cell wall. To determine what cell wall components bind WAKs, we tested several cell wall–degrading enzymes to see if they could release WAK from the cell wall. Out of hemicellulase, xyloglucanase, cellulase, and pectinase treatments, only the pectinase digestion was effective. A purified preparation of tomato pectinase (courtesy of D. Dellapenna, Michigan State University) was incubated with ground leaves and compared with the release by SDS/DTT extraction. The release of WAK from the cell was assayed by centrifugation at 6000*g* and running the supernatant on a denaturing polyacrylamide gel. WAKs were detected on a protein gel blot using serum directed to the kinase domain conserved between the five isoforms, and the results are shown in Figure 9A. WAK serum detected a single protein band of ~68 kD in SDS/DTT samples (lane SDS/DTT), as previously reported (He et al., 1996). After pectinase treatment, WAKs also appeared in an ~67-kD band that accumulated with increasing time of digestion. The 80-min digestion showed decreasing WAK levels, perhaps due to protein degradation. Protein gel blotting of the pectinase

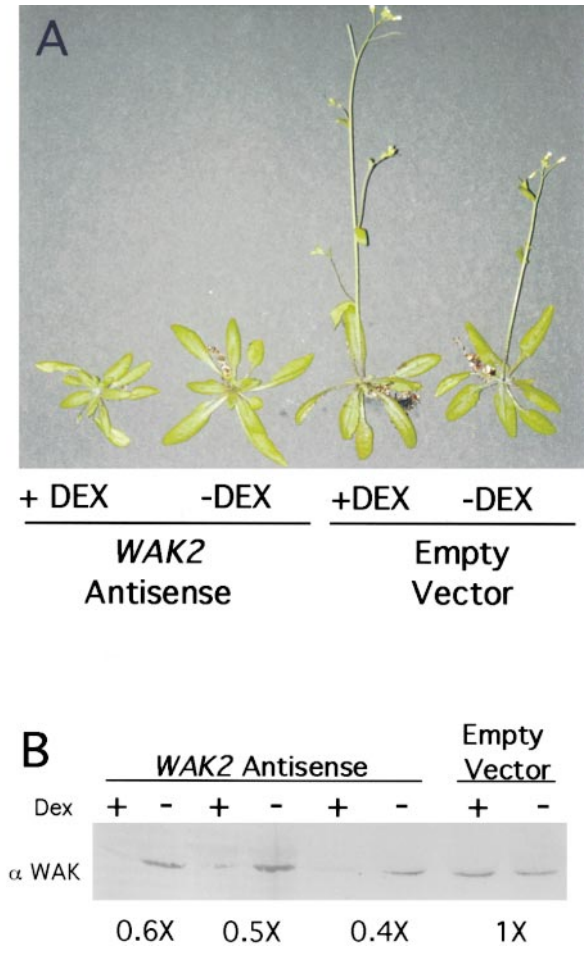

Figure 7. Reduction in WAK Protein Coincides with Reduced Leaf Size.

(A) Representative plants containing the inducible *WAK2* antisense gene or an empty vector 7 days after spraying with (+) or without (−) 20 μM Dex.

(B) Protein gel blot using the WAK antibody against total protein isolated from induced (+) and uninduced (−) *WAK2* antisense and empty vector plants 3 days after spraying. The fold reduction for each treated leaf is shown below the blot. Cytochrome f antiserum was used to normalize protein levels (data not shown).

preparation in the absence of plant tissue did not detect any proteins (lane np).

Because pectinase releases WAKs, it is possible that WAKs are bound to pectin. Duplicate samples of pectinase-treated leaves (as shown in Figure 9A) were protein gel blotted and probed with JIM5, an anti-pectin serum that recognizes homogalacturonan, and the results are shown in Figure 9B. JIM5 detects a 68-kD band from the SDS/DTT-extracted sample and a 67-kD band in the pectinase-released sample. These bands are the same size as those recognized by the WAK antibody. Protein gel blots with JIM7, a serum that binds methyl-esterified pectin, were identical to those of JIM5. Both JIM5 and JIM7 reacted more strongly with pectinase-released WAK than with the SDS/DTT-released band, indicating that the pectinase-released form may have more pectin bound to it.

DISCUSSION

Cell walls are dynamic structures whose synthesis, assembly, and modifications are complex and must somehow be coordinated with the cell interior. The structure and location of WAKs in the cell suggest that they could structurally link the cell wall with the plasma membrane and facilitate communication between the two compartments. In this report, we show that *WAK*s are expressed in a variety of cell types and that their expression is required for leaf cell expansion. WAKs are covalently linked to pectin, providing evidence that the binding of a structural carbohydrate may have significance for the control of cell expansion.

Expression

The cellular expression of the five *WAK* isoforms was characterized using promoter–GUS fusions, in situ hybridization, and RNA gel blot analysis to further distinguish between the WAK isoforms and to determine if any of the expression patterns overlap. *WAK1*, *WAK2*, and *WAK3* show distinct patterns of expression with many opportunities for overlap. *WAK1* and *WAK2* expression are both detected at organ junctions, in shoot and root apical meristems, and in expanding leaves and sepals. However, the timing of expression differs between *WAK1* and *WAK2*. *WAK1* is found in cotyledons before they emerge from the seed coat and at the tip of sepals before *WAK2* expression is found. In contrast, *WAK2* is expressed earlier than *WAK1* at the base of flowers. Also, *WAK1* and *WAK2* are expressed in different locations in the same organ. *WAK2* expression is stronger than *WAK1* at cotyledon and leaf margins and at the base of organs, whereas *WAK1* is expressed strongly in cotyledon and leaf vasculature.

The expression of *WAK* genes is both environmentally and developmentally regulated. *WAK1* and *WAK2* genes are INA and wound inducible, but there is variation in the degree to which the environmentally induced expression overlaps with the developmental pattern. Some tissues that normally do not express *WAK*, such as fully expanded rosette leaves and cauline leaves, show wound-inducible *WAK* expression, but the inflorescence stem does not. The developmental GUS patterns are likely not due to wounding because entire plants or organ pieces were placed in GUS substrate, and bending or applying light pressure did not induce *WAK* expression. When induced purposefully, wound-induced expression was immediately evident.

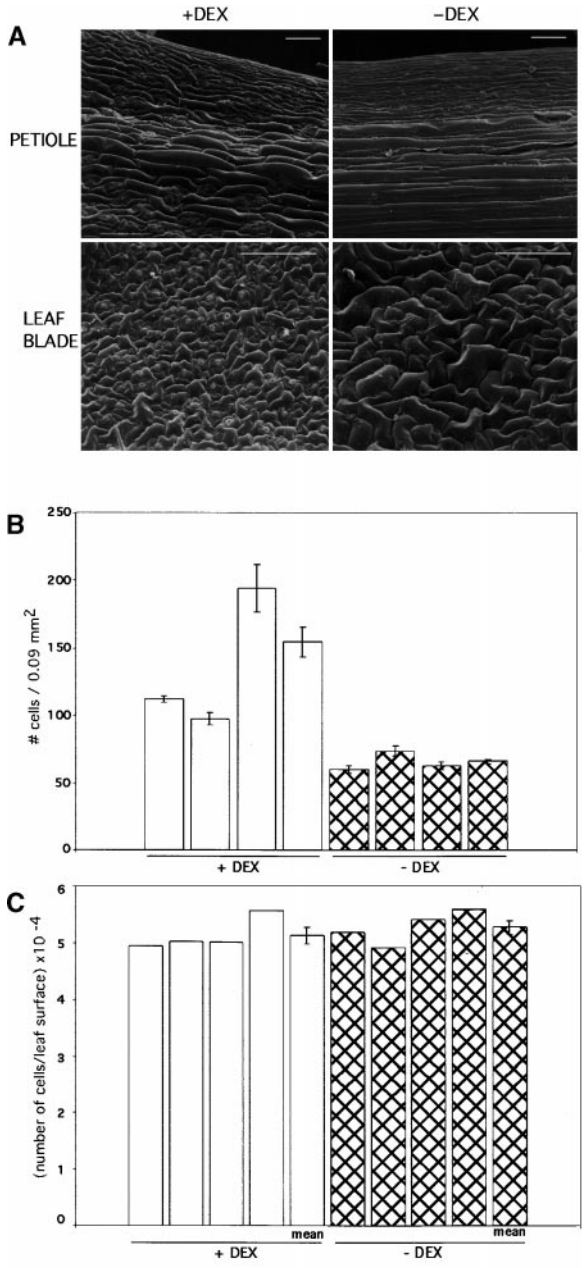

Figure 8. Reduced Cell Expansion in Induced *WAK2* Antisense Leaves.

(A) Representative scanning electron micrographs of the abaxial leaf blade and petiole showing that the epidermal cells are small and that there are more cells per given area in the induced leaves than in uninduced antisense leaves. Bars = 100 μm.

(B) Dex-treated *WAK2* antisense leaves have more pavement cells per area than do controls. Each bar represents a single plant and shows the average number of cells in five fields from an individual leaf.

(C) The total number of pavement cells per leaf surface is indistinguishable between Dex-treated and untreated *WAK2* antisense leaves. Bars represent the estimated number of pavement cells per leaf (see Methods). The mean for either the Dex-sprayed or control leaves is shown with standard error to demonstrate that they are not significantly different.

The expression pattern for *WAK3* is reminiscent of "microbursts" or "microlesions" because it is expressed in variable spots in cotyledons, leaves, and stems, mostly on or near veins. Treating seedlings with hydrogen peroxide or diphenylene iodonium did not alter *WAK3* promoter activity, as might be expected of a function related to the production of reactive oxygen species. On the other hand, INA does increase the size and number of *WAK3*–GUS spots, indicating some relation to the pathogen response. The spotty pattern in leaves is seen to a far lesser extent for *WAK1* and *WAK2*.

The GUS data for *WAK1* and *WAK2* seedlings agrees with in situ hybridization results, indicating that the necessary promoter regions and correct staining conditions were present. Six independent lines were examined for each construct, and the patterns reported here were observed in all lines, although GUS staining intensity was variable between lines. The variation in staining intensity was probably the result of position effects. At a general level, the results from the *WAK1-3* promoter–GUS fusions agree with the previous RNA gel blot analysis of organs and INA induction. There was difficulty in detecting the cell-specific pattern of expression in flower organs by in situ hybridization, so these patterns remain to be confirmed. The *WAK3* mRNA was not detected by in situ hybridization because of the inability to predict the location of *WAK3* spots and the difficulty of finding a section with *WAK3* expression.

The patterns described for *WAK1* and *WAK2* show some overlap with GUS patterns described for other genes. Promoter–GUS fusions for the metallothionein gene *LSC54* show staining at the junction of the cotyledon petiole and hypocotyl, in hydathodes, and in the stigma, as well as pathogen and wounding induction (Butt et al., 1998), which is strongly reminiscent of *WAK2* and slightly of *WAK1*. The basic leucine zipper transcription factor *TGA6* promoter–GUS fusion shows activity in hydathodes and the root tip, like *WAK2* (Xiang et al., 1997), and the *TGA6* root staining appears in random spots, like *WAK1* and *WAK2*. The promoter for a legume peroxidase is expressed in cotyledons, at cotyledon and leaf nodes, in the stigma, and in patches in leaves, the latter similar to *WAK3* (Curtis et al., 1997). This gene is also induced by wounding, pathogen induction, and methyl jasmonate. The expression pattern of *WAK2* in seedlings is also remarkably similar to places where naphthylalamic acid, an auxin transport inhibitor, is degraded, namely, in the root tip, base of cotyledons, and at the root/hypocotyl transition zone, (Murphy and Taiz, 1999), indicating the presence of an amidase at these locations. The functional significance of this amidase-to-auxin transport and signaling is unknown. Taken together, gene induction at cotyledon

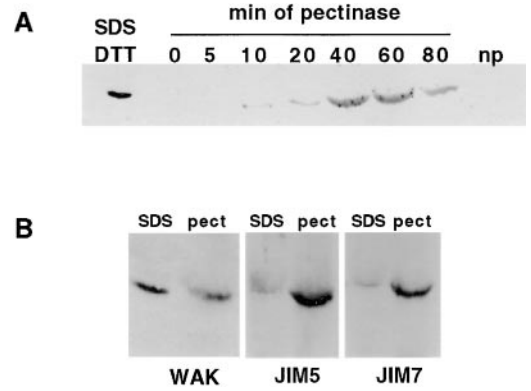

Figure 9. WAKs Are Bound to Pectin and Are Phosporylated.

(A) Protein was extracted by boiling in SDS/DTT or by incubation in pectinase for the indicated time (above each lane), followed by centrifugation at 6000*g*. The supernatant was analyzed by SDS-PAGE and protein gel blotting with WAK serum. np, 80-min reaction containing no plant tissue.

(B) Same as **(A)** except that pectinase treatment was for 30 min. Extracted protein was reacted with antiserum, as indicated below each panel. pect, pectinase; SDS, SDS/DTT.

and leaf nodes, hydathodes, and the stigma appears to be correlated with some form of stress and perhaps auxin regulation. Thus, WAKs may be induced by similar events. WAKs are clearly inducible by wounding and pathogen, and this is overlayed on a normal developmental program. It remains to be determined if the *WAK* that is expressed during development serves the same function as that induced by wall disturbance.

To further correlate the expression pattern seen for *WAK1* and *WAK2*, the PLACE database was searched for potential *cis* regulatory elements in the *WAK1* and *WAK2* promoters (Higo et al., 1999). The two promoters contained similar kinds of motifs but in different amounts and order. Although none of these have been directly confirmed for *WAK*s, sequence motifs associated with SA, auxin, gibberellin, abscisic acid, and light induction were identified. There were also motifs involved in sugar repression, circadian rythym, protein storage in seeds, and phenylpropanoid and flavanoid metabolism. Thus, there are both stress- and growth-related *cis* factor motifs present in the *WAK1* and *WAK2* promoters, and this correlates with their expression pattern.

Earlier immunofluoresence micrographs showed WAK protein present in all vegetative cells. More detailed analysis of the family finds expression throughout the nonflowering rosette plant, in which *WAK*s are expressed in the meristems and increasingly in the leaves and rosette stem over time. In contrast, there is very little to no detectable *WAK* expression detected in the elongation zone of roots, flowering SAM, inflorescence stem, cauline leaves, and some flower organs such as petals and stamens. Thus, whereas WAKs are required for full leaf cell expansion in the rosette, it appears that alternative, perhaps related proteins are involved in other tissues' expansion.

WAK1-3 expression patterns overlap in several instances. Providing that the proteins are localized to the same regions of the cell, this overlap indicates that there is a potential for heterodimer formation between WAK isoforms. This could be significant in building general models for receptor kinase function in plants as well as models for WAK function. The common paradigm for receptor kinases holds that ligand binding is coupled to receptor dimerization or further clustering, and this causes the receptors to activate and become phosphorylated (Heldin, 1995). However, this model is still undergoing revision because recent evidence indicates that transforming necrosis factor receptors interact before ligand binding (Chan et al., 2000). It is unknown if plant receptor kinases function as dimers or if ligand binding causes their association and activation, although some clearly function as large complexes of related proteins (Kohorn, 1999; Trotochaud et al., 1999).

Cell Expansion

Our results show that WAKs are required for leaf cell expansion. Plants containing a *WAK2* antisense construct under the control of the dexamethasone promoter have small rosette leaves when induced. This phenotype is specific to plants expressing full-length *WAK2* antisense RNA capable of hybridizing to all *WAK* messages, and it has never been observed in lines containing the empty vector or gene-specific *WAK1* and *WAK2* fragments in the antisense direction. The amount of WAK protein in induced *WAK2* antisense leaves was reduced compared with the controls. Scanning electron microscopy analysis of cell size revealed that the induced antisense plants have more cells per area than controls. When the reduced total area of the leaf in induced antisense plants is taken into account, the approximate total number of cells in induced and uninduced leaves is the same. Thus, WAKs are required for leaf cell expansion and not cell division.

Plant growth and cell expansion are influenced by a variety of plant hormones, such as auxins, gibberellins, ethylene, and brassinosteroids. Mutations in gibberellin (Sun and Kamiya, 1994) and brassinosteroid (Fujioka et al., 1997) synthesis result in dwarf plants, and exogenous application of auxin (Jones, 1998) and ethylene (Bleecker et al., 1988) can alter cell elongation. Analysis of signal transduction pathways for these hormones has identified many pathway components as well as alterations in downstream gene expression (reviewed in Walker and Estelle, 1998; Chang and Shockey, 1999; Schumacher and Chory, 2000; Silverstone and Sun, 2000). However, with the exception of auxin-induced acid growth (reviewed in Rayle and Cleland, 1992) and the induction of expansin expression (Caderas et al., 2000; Catala et al., 2000), physical changes in the cell wall have yet to be

directly linked to the hormones. Because WAKs are also required for full leaf expansion, it will be useful to determine if the WAK signal transduction pathway overlaps or interacts with those pathways affected by hormones.

Additional phenotypes for the *WAK2* antisense plant, such as leaf epinasty and chlorosis, cannot be determined to be WAK related using this inducible system because these phenotypes are seen in control plants as well. Also, constitutive expression of the inducible transcription factor can sometimes cause seedling lethality, so to avoid this complication, this analysis was limited to plants at least 2 weeks old. Examination of WAK function in seedlings will require alternative inducible promoters. The fact that the induced *WAK2* antisense lines showed a leaf cell expansion phenotype but induced antisense constructs containing *WAK1*- and *WAK2*-specific sequences did not suggests that there is functional redundancy between WAKs or limitations with induced antisense expression. Although there are times and places in development at which only one WAK isoform is expressed at cotyledon emergence or in the primary root tip, these places cannot be examined for a phenotype using the Dex system. The Dex system was used for these studies because constitutive antisense expression of *WAK*s was lethal; no transformants of *WAK* antisense under the control of the 35S promoter were obtained. We presume that the lethality is due to a requirement of WAK expression during early times of plant growth. Because antisense *WAK* does not abolish WAK protein levels, it is likely that there is sufficient WAK remaining in the *WAK2* antisense lines to permit survival. The PR1 promoter was also used for inducible expression (He et al., 1998), but this promoter did not allow for a straightforward analysis of development because *WAK*s were discovered to be pathogen inducible.

Pectin Association

To determine what components of the cell wall interact with WAK, we tested different cell wall–degrading enzymes for their ability to liberate WAK from the cell wall into a low-speed supernatant. Only pectinase treatments released WAKs from the wall, and the WAK that was released was still attached to pectin. Given that pectins form a malleable matrix that can undergo modifications that alter their structural properties, it is tempting to speculate that the binding of WAKs to pectin has some role in modulating cell expansion.

WAK can be extracted from leaf tissue by detergent that solubilizes membranes and by enzymatic digestion of the cell wall with pectinase. It is not known if the pectinase-released population is the same as that released by detergent. However, both fractions are likely to originate from the plasma membrane because WAK is only detected on the surface of the cell (He et al., 1996). Whereas the enzymatic release does not require detergent to solubilize the membrane, it is likely that WAKs carry with them the membrane; indeed, most cell wall preparations have an abundant plasma membrane component. The 67-kD pectinase-released WAK protein has a homogalacturonan epitope still bound. Serum raised to a variety of different pectins also identify the 67-kD pectinase-released WAK, and this includes (1-4)-β-galactan, (1-5)-α-*L*-arabinan, deesterified homogalacturonan, and homogalacturonan (data not shown). This attachment of WAK to pectin is likely to be covalent because it survives boiling in SDS and DTT and denaturing acrylamide gels, but the nature of the bond is unknown. The length of the pectin chain cannot be determined by this analysis, and because the various side chains can alternate within one chain, it is not possible to predict if WAKs are bound to the pectin backbone or to side chains. The migration of the pectinase-released WAK is slightly faster than that of WAK released by SDS/DTT, and this may be due to the increased levels of pectin detected by the JIM serum. However, the true effect of bound carbohydrates on migration cannot be estimated until their identities are known. Two-dimensional gel analysis using isoelectric focusing and denaturing second dimensions fails to detect the 68- and 67-kD species. Attempts to immunoprecipitate with either JIM or WAK serum have also not been successful.

The WAK 67- and 68-kD species appear to be major pectin binding proteins in these leaf extracts. Pectin-specific serums have been used with dot blots or artificially linked carbohydrate–protein complexes to detect material, but they do not detect bands in polyacrylamide gels (Willats and Knox, 1996; Knox, 1997). However, these studies did not use pectinase or cellulase in the tissue preparation, and thus, the identification of a major pectin binding protein that is reported here would have been missed. It remains likely, however, that there is a variety of other proteins that are bound to pectin and not detected by this analysis because they stay with large molecular complexes only detected by dot blots and immunofluorescence (Domingo et al., 1999). Although there may be a population that cannot enter denaturing acrylamide gels, the data suggest that WAKs are major protein components of the ECM that bind pectin. These results do not distinguish between the WAK isoforms, so isoform-specific antibodies are necessary to determine if only certain types of WAK are released by pectinase. Future work may also determine if there is a temporal relationship between the detergent- and pectinase-released fractions that is significant to the role of WAKs in communication between the cell wall and cytoplasm.

WAKs and Development

Some have proposed that the controlled modulation of tension, provided by the epidermis, is necessary for proper organ initiation and development. When tension in the plant has been altered experimentally by applying external force (Hernandez and Green, 1993; Lynch and Lintilhac, 1997) or altering expansin levels (Fleming et al., 1997; Cho and Cosgrove, 2000), cell division, organ initiation, and organ

fate are affected, and abnormal development results. *WAK*s are expressed in the meristem and, because they are linked to pectin, one might speculate that they function there as tension sensors. However, this is unlikely because the induced antisense plants show no meristem phenotypes, such as changes in organ number or identity. This does not rule out a role for WAKs as tension sensors in leaf expansion, but this remains to be tested. In addition to its importance at the whole-plant level, tension is also necessary for single-cell expansion. Cell walls require a minimum amount of wall stress to extend (reviewed in Cosgrove, 1997). It is possible that in expanding leaves, WAKs provide or sense part of the tension necessary for expansion via their connection to pectin and the plasma membrane. Alternately, WAKs may simply provide a sensing function related to the state or architecture of pectin or other unidentified ligands.

The WAK isoforms have distinct amino termini and could bind to different cell wall components. However, their cytoplasmic kinases are 86% identical. This suggests that their binding to different ECM components can lead to the activation of similar pathways in the cell and thus coordination of extracellular events. Genetic analysis and the identification of WAK-specific ligands and substrates will help to determine the roles WAKs play in plant function and development.

METHODS

Plant Growth Conditions

Arabidopsis thaliana ecotype Columbia (Col-0) were grown at 22°C under a cycle of 16 hr of light and 8 hr of darkness. Plants were grown either on Metromix 200 or on plates containing 1 × MS salts and vitamins (Murashige and Skoog, 1962) supplemented with 2% sucrose, 0.5 g/L Mes, 0.8% Bacto agar, and 50 μg/mL ampicillin, pH 5.7. Seeds were vernalized at 4°C for at least 48 hr on growth media.

Generation of Promoter Constructs

Promoter regions were initially identified as sequences that lay between the tandemly repeated coding regions of the five *WAK*s (He et al., 1999). All promoter fragments included the 5′ untranslated regions. The *WAK1* promoter was amplified with primers 5′-CGTGTCGACCGGGTATCAGCTGGGTAGTAG-3′ (W3-363F Sal) and 5′-CTGCACCTCCATGGTCTACCTCTCTTCTCTCTTTATTTC-3′ (W3NcoI R) using long distance polymerase chain reaction (PCR) with genomic DNA template (He et al., 1999). The SalI/NcoI fragment was subcloned into pUC β-glucuronidase (GUS) (Dewdney, 1993). The EcoRI (the promoter contains an EcoRI site) fragment containing 3 kb of *WAK1* promoter fused to GUS was then inserted into a modified pBI1.4t vector (the HindIII/SalI fragment containing the 35S promoter was removed; Mindrinos et al., 1994). The *WAK2* promoter was cloned in a similar way. PCR with primers 5′-GAACTGTATGCTGCAGATAATC-3′ and 5′-CCTGTACCTCCATGGTCTCTCTTTCTTTCTCTCTCAC-3′ (W2P NcoI R) generated a PstI/NcoI fragment that was cloned into the pUCGUS vector. A HindIII/PstI fragment of the *WAK2* promoter was added, and the HindIII/EcoRI fragment containing 2.7 kb of the *WAK2* promoter and GUS was inserted into pBI1.4t. PCR with primers 5′-GTCAAGGGCGCAACTTCGCGA-3′ (W5-100F) and 5′-CTGGAACTCCATGGCCTACCTTTCTCTTCTTTCTCTCTA-3′ (W3P-NcoI R) was used to generate the *WAK3* promoter. A 3.5-kb HindIII/NcoI fragment containing the entire promoter plus some WAK5 coding sequence was then cloned into the pUCGUS vector. The *WAK3* promoter plus GUS was then subcloned into pBI1.4t as a HindIII/EcoRI fragment. The primers used to amplify the *WAK5* promoter were 5′-GCAATCCTGAATACGTTGAATGG-3′ (6.4R-1700) and 5′-TTATCTAGATCCATGGTCTCCCTTTCCCTCTCTTTATTTTTC-3′ (W5P NcoI XbaI). This fragment was blunt cloned into pBluescript II. A 2.3-kb NcoI/XbaI fragment (which did not contain the *WAK5* start codon) was cloned into pUCGUS. The 1.2-kb NcoI fragment containing the start codon was added to the above construct. An EcoRI fragment containing 2.5 kb of the *WAK5* promoter and GUS was added to the modified pBI1.4t vector.

Generation of WAK Antisense Constructs

PCR was used to generate a SpeI/XhoI fragment, and the fragment containing the entire *WAK2* coding region was then ligated in an antisense orientation into the dexamethesone (Dex)-inducible pTA7002 vector. The primers were W2Sp, 5′-GGTTAAAAGAGACTAGTGAGAGAGAAAGAAAG-3′, and W2Xh, 5′-CTAAACAAACATGTAGAGCTCTAGTTATTAGTAC-3′. Amino acids 91 to 204 for *WAK1* and 88 to 200 for *WAK2* (this is the least conserved region among the WAK isoforms) were initially cloned into the pGEX-2TK vector to express these peptides in *Escherichia coli* and to generate antiserum against them. These pGEX constructs were then used as templates to generate Spe1/XhoI PCR fragments for antisense cloning into pTA7002. The primers against the pGEX-2TK polylinker used for PCR were Spe1, 5′-GGATCTCGTCGTACTAGTGCATCTGTTGGATCC-3′, and Xho1, 5′-GATCGTCAGTCCTCGACGCACGATGAATTC-3′.

Generation of Transgenic Plants

All of the constructs used for these experiments were confirmed by sequencing before the plasmids were transformed into *Agrobacterium tumefaciens* (strain GV3101) by electroporation. The promoter–GUS constructs were transformed into Col-0 by the dipping procedure (Clough and Bent, 1998), and transgenic plants were selected on plates containing 50 μg/mL kanamycin. These transformants were transplanted to soil and allowed to self. The resulting T2 seedlings were further screened for lines segregating for a single insertion. The T2 and T3 seedlings were analyzed for GUS staining, and the results presented here are based on six independent lines per construct. The Dex-inducible constructs were transformed into plants in the same way except that transgenic plants were selected on plates containing 20 μg/mL hygromycin. For induction experiments, plants growing on soil were sprayed or dabbed with a solution containing 20 μM Dex (dissolved in ethanol) and 0.01% Tween 20.

GUS Staining Conditions

Plants and plant organs were stained for GUS according to Silverstone et al. (1997). Blue color could be seen within 4 hr, but staining was

permitted to continue for 14 hr before clearing the tissues of chlorophyll or dehydrating and embedding them if necessary. Dissection of siliques was required for reliable GUS staining (Silverstone et al., 1997).

Histochemistry

GUS-stained tissues were dehydrated through an ethanol series, embedded in Steedman's wax (Vitha et al., 1997), and cut into 8-μm sections. Tissue for in situ hybridization was fixed and embedded in Paraplast Plus, according to Jackson (1991). Eight-micron sections were made with a rotary microtome.

Scanning Electron Microscopy Analysis

Leaves for scanning electron microscopy analysis were prepared as described by McConnell and Barton (1995). Leaf areas were calculated from light micrographs using NIH image software (http://rsb.info.nih.gov/nih-image). Cells were counted on scanning electron micrographs of five separate 0.09-mm^2 areas for each leaf. The total number of cells per leaf was calculated by multiplying the mean number of cells per 0.09 mm^2 by the actual area of the leaf/0.09 mm^2.

In Situ Hybridization

The *WAK1* cDNA was cloned into pGEM4Z (Promega) such that the SP6 RNA polymerase generated the sense strand and the T7 RNA polymerase generated the antisense probe. The templates for sense and antisense *WAK2* probes were made in two separate PCR reactions, and T7 polymerase was used for the synthesis of both probes. The primer used to make the *WAK2* sense probe template was 5′-CCAAGCTTCTAATACGACTCACTATAGGGCACAACCATCCTACTGTGCAT-3′. The primer used to make the antisense probe template was 5′-CCAAGCTTCTAATACGACTCACTATAGGGCCATCTAAGTATTCAGGCCT-3′. Both primers contained the T7 binding site and promoter (Kain et al., 1991). The reverse primers were the same as given above but without the T7 binding site and promoter. The PCR generated a 500-bp fragment from the N terminus of *WAK2*. Probes were made with the Digoxigenin (DIG) RNA labeling kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's directions. The *WAK1* and *WAK2* probes were hydrolized for 30 and 15 min, respectively. The nonradioactive in situ protocol detailed in Dr. Kathy Barton's Web site (www.wisc.edu/genetics/CATG/barton/protocols.html) was followed for the pretreatment and post-treatment of slides and detection of the DIG label. Hybridization conditions were overnight at 50°C, according to the procedure given by McKhann and Hirsch (1993).

WAK Analysis

The release of WAK by SDS-DTT extraction has been previously described (He et al., 1996). For the pectinase treatments, rosette leaves were gound in 5 mM MES, 1 mM $CaCl_2$, 2 μg/mL leupeptin, 10 μg/mL pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 0.1 μg/μL purified tomato polygalacturonase 2 (courtesy of D. DellaPenna, Michigan State University). Samples were incubated for 30 min (or as indicated) at room temperature and then centrifuged at 6000*g* for 10 min. The supernatants were then analyzed by SDS-PAGE and protein gel blotting, as previously described (He et al., 1996). The JIM5 and JIM7 antibodies are described by Knox et al. (1990) and were supplied by M. McCann (John Innis Institute). The Cytochrome f antibody used to normalize protein amount was initially described by Smith and Kohorn (1994).

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