Identification of plant cell wall mutants by means of a forward chemical genetic approach using hydrolases

Sascha Gille^a, Ulrike Hänsel^{b,1}, Mark Ziemann^{b,2}, and Markus Pauly^{a,3}

^aDepartment of Energy Plant Research Laboratory, Michigan State University, East Lansing, MI 48824; and ^bMax Planck-Institute for Molecular Plant Physiology, Am Muehlenberg 1, Golm, Germany

Edited by Maarten J. Chrispeels, University of California at San Diego, La Jolla, CA, and approved July 7, 2009 (received for review May 19, 2009)

A previously undescribed forward chemical genetic screen using hydrolases affecting the extracellular matrix is introduced. The developed screen takes advantage of the power of chemical genetics and combines it with the known substrate specificity of glycosylhydrolases, resulting in the selection of conditional mutants that exhibit structural defects in their extracellular matrix. Identification of the responsible genetic locus in those mutants significantly extends our knowledge of genes involved in the biosynthesis, metabolism, signaling, and functionality of components of the extracellular matrix. The method is exemplified by a screen of mutagenized *Arabidopsis* plants subjected to growth in liquid culture in the presence of a xyloglucanase, an enzyme acting on the major cross-linking glycan found in the extracellular matrix of this plant. Using this hydrolase-based screen, dozens of plant cell wall mutants (*xeg* mutants) were identified, leading to the identification of 23 genetic loci that affect plant cell walls. One of the identified loci is *XEG113*, encoding a family 77 glycosyltransferase (GT77). Detailed analysis of the wall of this mutant indicated that its extensins, structural glycoproteins present in walls, are underarabinosylated. *Xeg-113* plants exhibit more elongated hypocotyls than WT, providing genetic evidence that plant O-glycosylation—more specifically, extensin arabinosylation—is important for cell elongation.

extensin | plant cell wall mutant screen | xyloglucan

The direct contact of cells to the environment is mediated in most organisms by an extracellular matrix. One common aspect of extracellular matrices is that they contain more or less complex sugar moieties in the form of glycoproteins, proteoglycans, and/or polysaccharides. All these glycostructures play vital roles in cell-to-cell and cell-to-environment communication and signaling. An extraordinarily complex example of an extracellular matrix in terms of both structure and function is present in the walls of higher plant cells. Walls provide mechanical support, define the shape of the cell, and play vital roles in signaling, including resistance to pathogens (1). Despite its necessary rigidity, the cell wall is a highly dynamic entity that is metabolically active and plays crucial roles in numerous cell activities such as division, growth, differentiation, and senescence (2). Plants meet this multitude and diversity of functions through complex and dynamic polymer networks. These networks consist mainly of (i) a cellulose/cross-linking glycan network, (ii) a coextensive heterogeneous pectic polysaccharide network, (iii) glycoproteins (to a lesser extent), and (iv) in nongrowing cells, polyphenols such as lignin.

Structural features of plant cell wall polymers and their diversity have been relatively well described (3), yet elucidation of their corresponding functions in the life cycle of a plant is in its infancy. The few insights that we have come from identifying and characterizing mutants with altered wall structures, particularly in the model species *Arabidopsis thaliana* using forward and reverse genetic approaches (4). Successful forward genetic screens include wall sugar compositional analysis (5–7), leading to mutants with altered pectic polysaccharides and the major cross-linking glycan in *Arabidopsis*, xyloglucan (XyG). A screen

for mutants with an irregular xylem phenotype set the foundation for mechanistic insights into cellulose synthesis (8) and more recently, the synthesis of glucuronoarabinoxylan, the major cross-linking glycan in secondary walls (9). The completion of the *Arabidopsis thaliana* genome sequence led to the prediction that about 15% of the plant genes may be involved in the biosynthesis and metabolism of the plant cell wall (10, 11). With the public availability of *Arabidopsis* lines with T-DNA insertions in specific genes (12), reverse genetic approaches have been successful not only in studying the role of the encoded proteins in wall biosynthesis and metabolism, but also in a functional assessment of the concomitant altered wall structures and their effects on plant growth and developmental phenotypes (13–18). However, because little is known about the regulation of wall biosynthesis and metabolism or molecular mechanisms of wall signaling, the selection of candidate genes for a reverse genetic approach has reached its limits.

The toolbox of biologists has been significantly enhanced by chemical genetic approaches (19). The chemical component of such an approach takes advantage of small organic molecules that interfere with specific biological pathways, resulting in an observable phenotype in organisms (20). The genetic component encompasses the identification of mutants that, when treated with the chemical, display an altered phenotype, usually either hypersensitivity or resistance to the chemical (21). It is then inferred that the mutated gene is either the target of the chemical or a component of the affected biological pathway (22). The major advantage of such chemical genetic approaches is the possibility of modifying the chemical dosage, to address loss-of-function lethality resulting from the tunability of the phenotype. Moreover, genetic redundancy can be overcome because the chemical may target an entire gene family. Using such an approach, important discoveries have been made recently, including the identification of novel components of the auxin signaling pathway (23) and further details of cytoskeleton-cellulose synthesis interactions in plants (24).

In an attempt to identify previously uncharacterized plant cell wall mutants and genes responsible for wall structure and function, we took advantage of the power of chemical genetics by substituting small organic molecules with cell wall-degrading

Author contributions: S.G. and M.P. designed research; S.G., U.H., and M.Z. performed research; S.G., U.H., M.Z., and M.P. analyzed data; and S.G. and M.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

¹Present address: Bayer CropScience AG, Industriepark Hoechst, 65926 Frankfurt/Main, Germany.

²Present address: Environment and Biotechnology Centre, Faculty of Life and Social Sciences, Swinburne University of Technology, P.O. Box 214, John Street, Hawthorn, Victoria 3122, Australia.

³To whom correspondence should be addressed. E-mail: paulymar@msu.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0905434106/DCSupplemental.

Fig. 1. *Arabidopsis thaliana* wild-type (Col-0) plants grown in liquid culture with increasing concentrations of XEG. *A. thaliana* (ecotype Columbia-0) seeds were grown for 14 days in liquid culture containing increasing concentrations of xyloglucanase (XEG). (Scale bar: 1 cm.) Units XEG/mL medium (1 U releases 1 μ mol XyG oligosaccharides from polymeric tamarind XyG per min); zero indicates absence of enzyme in the media.

enzymes. This particular screen involves the germination and growth of mutagenized *Arabidopsis* seeds in liquid culture containing a cell wall hydrolytic enzyme. The presence of this temporal and spatially controllable “agent” alters the plant morphology specifically. Conceptually, cell wall mutants possessing altered structures attacked by the hydrolase should display a different developmental phenotype and can thus easily be selected from the culture. As a proof of concept, we focused on a screen facilitating a xyloglucanase preparation (25). This enzyme specifically cleaves the most abundant cross-linking glycan in nongraminaceous monocots and dicots, XyG (26). It is thought that XyG metabolism plays a major role during cell elongation (27). Applying the xyloglucanase-based screen led to the identification of many mutants and corresponding genes putatively influencing XyG structure and function. An example is a new cell wall mutant affected in a family 77 glycosyltransferase gene, which we will describe in greater detail.

Results

Effect of XEG on Wild-Type *Arabidopsis* Plants and Known XyG Mutants in Liquid Culture. *Arabidopsis* wild-type (WT) seedlings (ecotype Columbia-0) were grown in liquid culture containing a recombinant fungal xyloglucanase (XEG) preparation from seed to 14-day-old seedling stage. We tested various concentrations of the XEG to monitor its effect on plant growth and development (Fig. 1). Already, low concentrations of the XEG preparation in the medium led to shorter hypocotyls, smaller leaves, and shorter roots as well as discolorations on the cotyledons. These observed effects increased in severity with increasing concentrations of XEG. Rudimentary cell wall analysis of these seedlings (monosaccharide composition of noncellulosic wall polysaccharides) showed a XEG dose-dependent decrease in xylose, galactose, and rhamnose with a concomitant increase in arabinose (see [supporting information \(SI\) Fig. S1](#)). Analysis of the XyG present in these plants using oligosaccharide mass profiling (OLIMP) showed a XEG dose-dependent effect on the composition of the XyG structure (see [Fig. S2](#)). To evaluate how deep XEG is able to penetrate into the plant tissue during liquid culture incubation, we labeled sections of stems from these plants with an antibody that recognizes XyG (CCRC-M1) (28) and analyzed them by fluorescence microscopy (see [Fig. S3](#)). Plants grown in the presence of XEG showed an overall decrease of fluorescence in the epidermal and cortical cells, whereas the labeling of the vascular cells remained at a WT level. This suggests that the active hydrolase is indeed able to penetrate deep into the plant tissue during the 14-day period.

Fig. 2. *A. thaliana* wild-type (WT) and mutant seedlings after growth in liquid culture containing XEG. (A) Wild-type (Col-0), (B) *mur1*, and (C and D) examples for *Arabidopsis* mutants identified in the XEG liquid culture screen. (Scale bar: 3 mm.)

In summary, it is evident that growth of *Arabidopsis* seedlings in XEG has a dose-dependent effect on seedling growth, morphology, cell wall, and XyG composition. Hence, XEG, as a representative glycosylhydrolase, displays the properties of a chemical agent as defined in chemical genetics (21). Conceptually, these conditions, growth of seedlings in liquid culture containing XEG, could represent a unique approach for a forward genetic screen to identify new XyG mutants altered not only in their structure but also in their functionality. Mutants would be identified by changes in their visual seedling phenotypes when specifically grown in the presence of XEG (21). To ascertain the feasibility of such an approach, known and well-studied *Arabidopsis* XyG mutants such as *mur1* (6), *mur2*, and *mur3* (7) were subjected to growth in XEG and their morphological phenotype compared with that of WT seedlings grown under the same conditions. Indeed, all 3 mutants displayed distinct altered morphological phenotypes (Fig. 2 and [Fig. S4](#)). The *mur1* mutant exhibited chlorotic cotyledons and smaller leaves (Fig. 2B), whereas *mur2* and *mur3* showed a brown discoloration of the cotyledons ([Fig. S4 B and C](#)), when compared with WT (Fig. 2A). Hence, all 3 XyG mutants could have been easily selected as XyG mutants using this chemical genetic approach facilitating the glycosylhydrolase.

XEG-Based Liquid Culture Screen of EMS and T-DNA Insertion Mutants. Based on these findings, a 2-staged XEG liquid culture mutant screen was developed to identify novel conditional XyG mutants

Fig. 3. Principle of the 2-staged XEG-based liquid culture forward genetics screen. In the first stage of the screen, mutagenized *Arabidopsis* seeds were subjected to XEG in liquid culture. After 14 days, the morphology of the seedlings was assessed. Seedlings exhibiting a phenotype different from WT were selected and placed on soil for seed production. The resulting seed population was split and subjected to growth in liquid medium either with or without XEG to select only mutants with an XEG-dependent phenotype.

in a population of randomly chemical and T-DNA activation-tagged mutagenized *Arabidopsis thaliana* seed populations (Fig. 3). In the first phase of the screen, pools of mutagenized seeds (≈ 300 per flask) were grown in liquid culture containing XEG. After 14 days, the seedlings were visually inspected and plants exhibiting morphological differences compared with the WT seedlings were transferred from the liquid medium to solid MS agar plates followed by transfer to soil for the plants to recover and produce seeds. In total, $\approx 25,000$ T-DNA activation-tagged and 16,000 EMS-mutagenized *Arabidopsis* seeds were screened. During the first phase, $\approx 1,700$ T-DNA and 460 EMS mutants were selected as displaying distinct changes in plant morphology. Among the observed alterations were mutant seedlings that were not obviously affected by XEG displaying larger leaves (Fig. 2C), plants where XEG had even more detrimental effects on overall seedling development (Fig. 2D), plants with necrotic lesions on stems and leaves (Fig. S4F and I), black spots on leaves (Fig. S4I), brown discoloration of cotyledons (Fig. S4F), chlorotic cotyledons (Fig. 2D), elongated hypocotyls and/or leaves (Fig. S4G and H), and crippled roots and leaves (Fig. S4E and F). Of the selected T-DNA mutants, only 775 plants (46%), and of the EMS mutants, only 260 plants (56%) fully recovered and produced seeds. These seeds were subjected to the second stage of the screen, in which the seeds of individual mutants were split. One portion was subjected again to the XEG liquid culture conditions to confirm the phenotype results of the first stage. If the morphological alteration was not reproducible, the mutant was dismissed. Another portion of the mutant seeds was grown in liquid culture without the enzyme so that we could identify mutant phenotypes specifically arising from the presence of XEG, and thus exclude general developmental mutants. To date, of 137 tested T-DNA mutants, 50 passed the second-stage selection criteria (36%); of 64 tested EMS mutants, 23 (40%) passed. These remaining mutants were termed *xeg* mutants based on the enzyme used in the screen.

We subjected the selected T-DNA insertion mutants to TAIL-PCR to identify the location of the T-DNA insertion in the genome. To date, the location of T-DNAs in 23 *xeg* mutants in or close to coding regions of the *Arabidopsis* genome has been identified. In addition to 6 genes encoding proteins of unknown functions, genes encoding proteins involved in the circadian cycle, sugar conversion pathways, proteins with kinase activities, transferases, hydrolases, and DNA-binding proteins were tagged (Table S1).

***xeg113*: Identification and Characterization of a New Cell Wall Mutant.** One of the T-DNA mutants identified by the XEG liquid culture screen was *xeg113*, hereafter termed *xeg113-1*. When subjected

Fig. 4. Phenotypes of *A. thaliana* WT, *xeg113-2*, *xeg113-3*, and *113comp*. (A) Phenotype after growth in liquid culture with XEG. (Scale bar: 3 mm.) (B) Seven-day-old etiolated seedlings. (Scale bar: 5 mm.) (C) Length of 7-day-old etiolated seedlings, $n = 10$. (D) Time course of hypocotyl growth of etiolated seedlings, $n = 10$.

to growth in the presence of XEG, *xeg113-1* displays an elongated hypocotyl and longer petioles of the rosette leaves (Fig. S4G) compared with WT grown under the same conditions (Fig. S4A). However, when the mutant is grown in liquid culture without the enzyme, no alteration in plant morphology compared with WT can be observed (Fig. S5D). The mutant thus passed our 2-stage screen criteria. TAIL-PCR of isolated genomic DNA of *xeg113-1* revealed a T-DNA insertion 228 bp upstream of the start codon of At2g35610 (Fig. S5A). This locus encodes a protein of unknown function with a length of 644 aa and a calculated molecular weight of 73.2 kDa; it is classified as a family 77 glycosyltransferase (GT77) protein (www.cazy.org/). The expressed protein is predicted to contain a single transmembrane domain within the first 50 aa in the N terminus (ARAMEMNON database) (29) and conserved domain known as reticulon (RTN; PF02453 and IPR003388), suggesting a subcellular localization in the secretory pathway, likely in the Golgi apparatus, where the protein was localized in a proteomic study by Dunkley et al. (30).

To confirm the link between the discovered genetic disturbance and the observed XEG-dependent phenotype, additional T-DNA lines with insertions in the At2g35610 locus were acquired. Indeed, both lines, SALK_066991 (*xeg113-2*) and SALK_058092 (*xeg113-3*), displayed an elongated hypocotyl and long petioles, again only when grown in the presence of XEG (Fig. 4A). Also, genetic complementation was achieved by cloning the genomic At2g35610 locus, including a 2-kbp promoter sequence upstream of the start codon, into a binary vector and transformation into *xeg113-2*. This line was termed *113comp*. When *113comp* was grown in liquid culture with or without XEG, only a WT-like phenotype in both culture conditions was observed (Fig. 4A and Fig. S5D). Taken together, these findings confirm that a loss of transcript of this GT77 gene is responsible for the observed phenotype.

Interestingly, etiolated seedlings of both *xeg113-2* and *xeg113-3* grown on agar plates exhibited higher growth rates

Table 1. Monosaccharide composition and hydroxyproline content of total AIR ($\mu\text{g mg}^{-1}$ AIR) and Ba(OH)₂ extract after sequential cell wall extraction ($\mu\text{g mg}^{-1}$ dry weight); for analyses of other extracts see Table S2

	Line	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose	Hyp
Total AIR	WT	20.19 $\pm$ 4.08	4.17 $\pm$ 0.66	30.69 $\pm$ 2.72	25.54 $\pm$ 2.34	6.44 $\pm$ 1.11	41.93 $\pm$ 4.49	8.48 $\pm$ 2.50	4.98 $\pm$ 0.43
	<i>xeg113-2</i>	19.09 $\pm$ 0.84	4.28 $\pm$ 0.12	25.27* $\pm$ 1.04	27.86 $\pm$ 1.45	8.79* $\pm$ 0.53	44.47 $\pm$ 1.48	13.71* $\pm$ 1.67	4.89 $\pm$ 0.39
	<i>xeg113-3</i>	19.39 $\pm$ 0.87	3.94 $\pm$ 0.26	26.80* $\pm$ 1.24	25.35 $\pm$ 1.17	8.02* $\pm$ 0.43	43.72 $\pm$ 0.92	12.86* $\pm$ 1.61	5.03 $\pm$ 0.43
	<i>113comp</i>	22.81 $\pm$ 1.89	3.62 $\pm$ 0.16	32.84 $\pm$ 0.96	27.87 $\pm$ 1.65	5.52 $\pm$ 0.35	41.89 $\pm$ 2.04	12.43* $\pm$ 1.79	5.39 $\pm$ 0.65
Ba(OH) ₂ extract	WT	0.09 $\pm$ 0.01	N.D.	4.54 $\pm$ 0.80	1.19 $\pm$ 0.21	0.51 $\pm$ 0.11	0.99 $\pm$ 0.21	2.57 $\pm$ 0.45	1.60 $\pm$ 0.14
	<i>xeg113-2</i>	0.10 $\pm$ 0.01	N.D.	3.06* $\pm$ 0.72	0.96 $\pm$ 0.13	0.45 $\pm$ 0.09	0.88 $\pm$ 0.24	2.40 $\pm$ 0.40	1.48 $\pm$ 0.15
	<i>xeg113-3</i>	0.09 $\pm$ 0.02	N.D.	3.02* $\pm$ 0.79	0.94 $\pm$ 0.19	0.48 $\pm$ 0.11	0.90 $\pm$ 0.28	2.34 $\pm$ 0.45	1.61 $\pm$ 0.22
	<i>113comp</i>	0.08 $\pm$ 0.01	N.D.	4.41 $\pm$ 0.97	1.14 $\pm$ 0.25	0.53 $\pm$ 0.08	0.98 $\pm$ 0.20	2.63 $\pm$ 0.71	1.70 $\pm$ 0.34

N.D., not detected; Hyp, hydroxyproline.

*Significantly different ($P \leq 0.01$) when compared with WT ($n = 8$) $\pm$ SD.

leading to longer hypocotyls when compared with WT (Fig. 4 B–D). The rescued line, *113comp*, did not display any significant difference in hypocotyl length (Fig. 4 B–D). Moreover, when grown on soil in light, both knockout mutants had a larger rosette leaf diameter and displayed earlier inflorescence bolting compared with WT and *113comp* (Fig. S6). No other change in plant morphology was observed.

The overall wall structure of *xeg113* was characterized. Analyses of the monosaccharide composition of the noncellulosic polymers of etiolated seedlings revealed an up to 18% decrease in arabinose and a concomitant increase in mannose and glucose in *xeg113-2* and *xeg113-3*, when compared with WT and *xeg113comp* (Table 1), suggesting a change in the composition of an arabinosylated matrix component, such as pectic polysaccharides, arabinogalactan proteins (AGP), or the glycoprotein extensin. For a more detailed analysis, we sequentially extracted whole etiolated seedlings from WT, *xeg113-2*, and *xeg113-3* using specific enzymes and salt solutions; the resulting extracts were subsequently analyzed for their monosaccharide composition (Table 1 and Table S2). No consistent significant changes in the monosaccharide composition between the *2xeg113* knockout lines and WT were observed in the AGP/soluble components, the pectic fraction, and the EGII (cross-linking glycans and noncrystalline cellulose) (Table S2). The remaining residue was treated with Ba(OH)₂ at high temperature to release cross-linked glycosylated extensins from the wall. In this extensin-enriched fraction, a decrease of up to 33% in arabinose was observed (Table 1), suggesting an underarabinosylation of extensins in *xeg113*. The remaining material was hydrolyzed under strong acidic conditions and high temperature to solubilize all remaining cell wall components, including crystalline cellulose.

No significant changes in the monosaccharide composition of this residue were observed (Table S2).

We determined the abundance of hydroxyproline (Hyp), a prominent component of the glycoprotein extensin (Table 1 and Table S2). The highest amount of Hyp was detected in the Ba(OH)₂ fraction (Table 1), confirming that the largest amount of extensin is released with this treatment. No significant changes in the Hyp content released from the cell wall were observed. For the Ba(OH)₂ fraction, the molar ratios between Hyp and arabinose in WT and both mutants were calculated. For the WT, this ratio was calculated to be 1:3.2 (Hyp:Ara), for *113comp*, 1:2.97. For the knockout lines this ratio was lower (*xeg113-2*, 1:2.3; *xeg113-3*, 1:2.2). Taken together, these data show that *xeg113* exhibits a significant underarabinosylation of the extensins in the wall.

The Ba(OH)₂ fraction was also subjected to glycosidic linkage analysis (Table 2). Here, both mutants showed a decrease of up to 38% in 2-linked arabinofuranose (2-Araf) and up to 80% in 3-linked arabinofuranose (3-Araf), 2 of the major constituents of extensins. Terminal-linked arabinofuranose (t-Araf) and terminal-linked galactopyranose (t-Galp) remained at WT levels. All other detected glycosyl residues showed no significant changes when compared with WT (Table 2), providing strong evidence for an altered extensin arabinosylation in *xeg113* (see model Fig. S7).

Discussion

A forward chemical genetic approach facilitating glycosylhydrolases was developed to identify previously uncharacterized conditional plant cell wall mutants and thus genes responsible for wall structure and function. When growing an Arabidopsis plant

Table 2. Linkage analysis of Ba(OH)₂ extracted extensins (percent of total peak area)

Glycosidic linkage	WT	<i>xeg113-2</i>	<i>xeg113-3</i>	<i>113comp</i>
t-Araf	14.16 $\pm$ 1.01	16.33 $\pm$ 2.25	18.44 $\pm$ 1.63	13.64 $\pm$ 1.45
t-Xylp	4.91 $\pm$ 0.37	5.21 $\pm$ 0.73	6.49* $\pm$ 0.75	4.49 $\pm$ 0.54
2-Araf	22.06 $\pm$ 1.83	13.50* $\pm$ 1.57	16.56* $\pm$ 1.18	22.77 $\pm$ 2.20
t-Glcp	4.45 $\pm$ 1.39	6.75 $\pm$ 2.30	4.95 $\pm$ 0.89	4.72 $\pm$ 1.80
3-Araf	8.31 $\pm$ 0.59	1.58* $\pm$ 0.19	1.65* $\pm$ 0.31	8.99 $\pm$ 1.21
t-Galp	3.76 $\pm$ 0.52	4.73 $\pm$ 0.94	5.87 $\pm$ 0.72	4.69 $\pm$ 1.09
5-Araf	4.45 $\pm$ 0.23	8.52* $\pm$ 1.04	6.31 $\pm$ 1.06	5.18 $\pm$ 0.37
4-Xylp	5.07 $\pm$ 0.33	6.12 $\pm$ 0.39	6.19 $\pm$ 0.91	5.19 $\pm$ 0.78
3-Glcp	3.58 $\pm$ 1.53	4.47 $\pm$ 2.01	2.67 $\pm$ 0.65	2.71 $\pm$ 0.47
4-Glcp	21.57 $\pm$ 5.06	23.91 $\pm$ 2.54	21.42 $\pm$ 1.25	20.20 $\pm$ 3.81
2,5-Araf	0.69 $\pm$ 0.37	0.61 $\pm$ 0.18	0.68 $\pm$ 0.10	0.79 $\pm$ 0.08
6-Galp	1.10 $\pm$ 0.09	1.54 $\pm$ 0.26	1.75* $\pm$ 0.13	1.25 $\pm$ 0.20
4,6-Glcp	3.76 $\pm$ 2.47	3.76 $\pm$ 1.22	3.62 $\pm$ 0.81	2.81 $\pm$ 1.02
3,6-Glcp	2.08 $\pm$ 0.21	2.92 $\pm$ 0.52	3.33* $\pm$ 0.37	2.52 $\pm$ 0.57

*Significantly different ($P \leq 0.01$) when compared with WT ($n = 4$) $\pm$ SD.

rides per min), not further purified, was added to the cultures. Seedlings were grown for 14 days before visual analysis.

Thermal Asymmetric Interlaced (TAIL) PCR for Mapping T-DNA Insertion Flanking Sequences. The location of the T-DNA insertion in *xeg* mutants from the activation-tagged mutant populations was determined by thermal asymmetric interlaced (TAIL) PCR. We adapted and modified the method previously described by Liu et al. (42) (see *SI Materials and Methods*).

Preparation and Analysis of Cell Wall Material. Sterilized *A. thaliana* seeds were grown for 7 days in the dark on MS medium containing 1% agar. Etiolated seedlings were harvested, pooled, and frozen in liquid nitrogen. The alcohol-insoluble residue (AIR) of the etiolated seedlings was prepared by the adapted method previously described by Fry (43) and Lerouxel et al. (13). The tissue was ground to a fine powder using a beat mill (Retsch). The powder was extracted with 1 mL 70% aqueous ethanol. The supernatant was removed by centrifugation for 10 min at 14,000 rpm. A 1:1 (v:v) chloroform/methanol mixture (1 mL) was added. The supernatant was again removed by centrifugation and the resulting residue air-dried.

Freeze-dried WT and mutant plant material was sequentially extracted using specific polysaccharide-degrading enzymes and salt solutions. In brief, 7-day-old etiolated whole seedlings were freeze-dried, ground to a fine powder, and aliquoted into 3–5 mg dry weight. After the extraction of water-soluble components, pectic polysaccharides, cross-linking glycans, and

partially cellulose (for details, see *SI Materials and Methods*), the remaining pellet was resolubilized in 1 mL of saturated barium hydroxide solution (≈ 0.22 M) and incubated for 6 h at 105 °C (44). The supernatant containing solubilized extensins was separated by centrifugation, neutralized with 1 M sulfuric acid, and the resulting precipitated salts removed by centrifugation.

The monosaccharide composition of 1 mg total cell wall material and sequentially extracted cell wall components was determined by hydrolyzing cell wall material in trifluoroacetic acid (TFA) followed by alditol acetate derivatization and GC-MS analysis as described (45). Glycosidic linkage analysis on the $\text{Ba}(\text{OH})_2$ fraction was performed by GC-MS analysis of partially methylated alditol acetates as described by Ciucanu and Kerek (46) with modifications by Ciucanu (47).

The hydroxyproline (Hyp) content of sequentially extracted cell wall components was determined by the colorimetric assay described by Kivirikko and Liesmaa (48) after the extracts were hydrolyzed in 6N hydrochloric acid for 18 h at 110 °C.

ACKNOWLEDGMENTS. We thank Kirk Schnorr (Novozymes) for the generous gift of the XEG enzyme; Michael Hahn (Complex Carbohydrate Center, University of Georgia) for providing the CCRC-M1 antibody, Marcia Kieliszewski for protocol discussions of extensin extractions, and Karen Bird for editing. This work was funded by the Max-Planck Society and by the Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences, Office of Science, US Department of Energy (Award No. DE-FG02-91ER20021).

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