

The two paralogous kiwellin proteins KWL1 and KWL1-b from maize are structurally related and have overlapping functions in plant defense

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Florian Altegoer^{1,*} , Paul Weiland¹, Pietro Ivan Giammarinaro¹, Sven-Andreas Freibert², Lynn Binnebesel¹, Xiaowei Han³, Alexander Lepak¹, Regine Kahmann³, Marcus Lechner¹ , and Gert Bange^{1,*} 

From the ¹SYNMIKRO Research Center and Department of Chemistry and the ²Institute for Cytobiology and Cytopathology, Philipps-University Marburg, Marburg, Germany and the ³Max Planck Institute for Terrestrial Microbiology, Marburg, Germany

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Many plant-pathogenic bacteria and fungi deploy effector proteins that down-regulate plant defense responses and reprogram plant metabolism for colonization and survival *in planta*. Kiwellin (KWL) proteins are a widespread family of plant-defense proteins that target these microbial effectors. The KWL1 protein from maize (corn, *Zea mays*) specifically inhibits the enzymatic activity of the secreted chorismate mutase Cmu1, a virulence-promoting effector of the smut fungus *Ustilago maydis*. In addition to KWL1, 19 additional KWL paralogs have been identified in maize. Here, we investigated the structure and mechanism of the closest KWL1 homolog, KWL1-b (ZEAMA_GRMZM2G305329). We solved the Cmu1–KWL1-b complex to 2.75 Å resolution, revealing a highly symmetric Cmu1–KWL1-b heterotetramer in which each KWL1-b monomer interacts with a monomer of the Cmu1 homodimer. The structure also revealed that the overall architecture of the heterotetramer is highly similar to that of the previously reported Cmu1–KWL1 complex. We found that upon *U. maydis* infection of *Z. mays*, KWL1-b is expressed at significantly lower levels than KWL1 and exhibits differential tissue-specific expression patterns. We also show that KWL1-b inhibits Cmu1 activity similarly to KWL1. We conclude that KWL1 and KWL1-b are part of a redundant defense system that tissue-specifically targets Cmu1. This notion was supported by the observation that both KWL proteins are carbohydrate-binding proteins with distinct and likely tissue-related specificities. Moreover, binding by Cmu1 modulated the carbohydrate-binding properties of both KWLs. These findings indicate that KWL proteins are part of a spatiotemporally coordinated, plant-wide defense response comprising proteins with overlapping activities.

Addressing the food and energy demand of a constantly growing world population has become a major challenge of current generations. Crop yield and quality are directly affected by fungal pathogens, causing large losses in the five most important crops (1). In the case of maize plants (*Zea mays*), pathogens cause an annual loss of ~9% (2). Among these is the smut fungus *Ustilago maydis*, which causes corn smut in maize

leading to the formation of large tumor-like structures on the plant. The infection process is accompanied by the secretion of a plethora of fungal effector proteins that suppress defense responses and modulate the metabolism of the maize plant (3, 4). Expression and activity of effectors is regulated in a spatio-temporal manner and requires universal pathogenicity factors during plant penetration in the early stages, whereas more specific effectors modulate metabolic processes in different tissues in the later stages of infection (5, 6). In infected maize leaves, effector expression is differentially regulated depending on the infected plant tissue (7), which allows addressing the spatio-temporal regulation of the *U. maydis* effectors, as well as the respective plant response (5, 8).

During maize colonization, the secreted chorismate mutase 1 (Cmu1) is one example of a well-characterized and highly abundant effector that confers its activity in the cytoplasm of the host cell (9). Although housekeeping chorismate mutases (CMs) are allosterically regulated by either tryptophan or tyrosine, Cmu1 lacks this allosteric regulation, constantly converts chorismate to prephenate, and thereby prevents production of salicylic acid, an important signal in the immune-response of the plant (9, 10). Cmu1 is a dual-function effector, which when present in the apoplast interacts with the secreted maize protein kiwellin 1 (KWL1). KWL1 has been shown to effectively inhibit Cmu1 activity by blocking the active site of the enzyme (10). Silencing of the KWL1 gene elevated virulence, supporting a function of KWL1 as a plant-defense protein. A more thorough inspection of kiwellin proteins revealed a broad conservation among various monocots and dicots, with 20 kiwellin paralogs being present in the maize genome alone. Kiwellin proteins therefore might be part of the broad defense responses triggered by microbe-associated molecular patterns during pattern-triggered immunity.

Here, we describe the structure and mechanism of another maize kiwellin, named KWL1-b, that inhibits Cmu1 by a mechanism highly reminiscent to that of KWL1. Upon *U. maydis* infection, the induction of KWL1-b is significantly lower than that of KWL1, and both genes show tissue-specific differences in expression. Structural comparison of both KWL proteins with each other and endoglucanase V (EGV) suggest a surface-exposed cleft as possible carbohydrate-binding site located across to the Cmu1-binding site. Biochemical studies reveal that both KWLs can indeed bind carbohydrates, albeit with

This article contains supporting information.

*For correspondence: Florian Altegoer, florian.altegoer@synmikro.uni-marburg.de; Gert Bange, gert.bange@synmikro.uni-marburg.de.

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different preferences. Thus, our study not only clarifies an unforeseen functional feature of the KWLs but also shows how minor structural divergences between two close homologs result in functional diversification during molecular evolution.

Results

KWL1-b interacts with the fungal effector Cmu1

We have recently shown that KWL1 can be efficiently co-immunoprecipitated from lysed maize leaves, which were infected with an *U. maydis* strain expressing a hemagglutinin-tagged Cmu1 (10). Mass spectrometric analysis of the resulting co-elutes reproducibly and unambiguously identified peptides belonging to KWL1, whereas peptides of the 19 remaining KWL proteins were not detected. However, comparison of the KWL1 sequence with those of the others suggested that another so-far uncharacterized KWL (accession code ZEAMA_GRMZM2G305329) is highly conserved within the regions required for interaction with Cmu1 (Fig. S1A). Thus, we hypothesized that this KWL protein might also be able to interact with Cmu1 and termed it KWL1-b.

To challenge this idea, we performed pulldown experiments in lysates of infected maize seedlings with purified glutathione *S*-transferase (GST)-tagged KWL1-b as bait. Subsequent LC-MS unambiguously identified Cmu1, suggesting that KWL1-b also interacted with Cmu1 of *U. maydis*. We could identify eight unique peptides covering 35% of the total Cmu1 sequence (Fig. S1B).

Comparison of the transcript abundance of KWL1-b with that of its close counterpart KWL1 shows that the latter is generally 5- and 10-fold higher expressed in mesophyll-derived tumor cells when compared with bundle sheath-derived tumor cells 4 days postinfection (Fig. S1C) (7). In contrast, KWL1-b showed a more homogenous expression pattern in both cell types despite an overall lower expression (Fig. S1C) (7). These differences in the transcript abundance will probably reflect back to the protein level and might thus explain why KWL1-b has not been identified as an interaction partner of Cmu1 so far. Taken together, these results suggest that maize KWL1-b can interact with the fungal effector Cmu1.

KWL1-b inhibits the chorismate activity of the fungal effector Cmu1

To study the consequences of the Cmu1-KWL1-b interaction, we next performed *in vitro* pulldown assays employing GST-tagged KWL1-b as bait. Cmu1 and the maize-housekeeping CMs *ZmCm1*, *ZmCm2*, and *ZmCm3* served as prey. All these proteins were recombinantly produced in *Escherichia coli* BL21(DE3) and purified by two-stepped protocol consisting of nickel-ion affinity purification followed by size-exclusion chromatography (SEC). This experiment confirmed that GST-KWL1-b interacts with Cmu1 while not interacting with any of the three maize CMs (Fig. 1A). Analytical SEC employing KWL1-b and Cmu1 further confirmed the interaction and provided an apparent molecular mass of ~100 kDa, which is best explained by a 2:2 stoichiometry of KWL1-b and Cmu1 in a heterotetrameric complex (Fig. 1B). Isothermal calorimetry (ITC) suggested a dissociation constant of $1.09 \pm 0.11 \mu\text{M}$ for the Cmu1-KWL1-b interaction (Fig. 1C). Analysis of the CM

activity at a substrate concentration reflecting the maximal velocity of Cmu1 showed an ~6-fold decrease in the presence of KWL1-b (Fig. 1D). Taken together, these data show that KWL1-b inhibits the CM activity of the fungal effector Cmu1 in a heterotetrameric complex.

Crystal structure of the Cmu1-KWL1-b heterotetramer

Next, we determined the crystal structure of the Cmu1-KWL1-b complex to a resolution of 2.75 Å by molecular replacement with the structures of Cmu1 and KWL1 (PDB code 6FPG) as search models (Table 1). The overall structure shows a highly symmetric Cmu1-KWL1-b heterotetramer in which each KWL1-b monomer interacts with a monomer of the Cmu1 homodimer (Fig. 2A). Closer comparison of the Cmu1-KWL1-b structure (*this study*) with that of the Cmu1-KWL1 complex (10) revealed a nearly identical overall architecture with an RMSD of 0.5 Å in Cα atoms (Fig. 2A). The interface between KWL1-b and Cmu1 covers a surface area of ~1200 Å², which compares well to that in the Cmu1-KWL1 complex (10). The interaction between the two proteins is established through a combination of polar and nonpolar interactions and involves three loops (L1-L3) and the N-terminal β1/2 domain in KWL1-b (Fig. 2B). A closer inspection of the four interfaces revealed that although the overall sequence conservation is high, some of the residues interacting with Cmu1 at KWL1-b deviate from KWL1 (Fig. 2, C and E).

Within the β1/2 domain at KWL1-b, polar contacts are formed by the backbone amide and carboxyl groups of Tyr⁴² and Asn²⁰¹ in Cmu1 (Fig. 2, C and E). The same residue in Cmu1 is contacted by the carboxyl group of Ser⁴⁵ within KWL1. Although residue Pro⁴⁴ at KWL1-b forms a backbone contact with Trp¹⁹⁷ in Cmu1, Gln⁴⁹ in KWL1 interacts with Glu²⁹ in Cmu1 (Fig. 2C). In addition to these differences, Arg⁶⁹ from β2 within KWL1 establishes a salt bridge with Glu³² (Cmu1), but this contact is completely absent in KWL1-b (Fig. 2C). Conversely, the strong interaction of Arg⁴⁶ (Arg⁵¹ at KWL1) with several negatively charged residues of Cmu1 is conserved among KWL1 and KWL1-b (Fig. 2, C-E). Within the L1 region, the residues mediating the interaction between KWLs and Cmu1 are again different (Fig. 2E and Fig. S2A). An interesting observation is that Gly⁹³ within KWL1-b contacts Asn²⁵⁰ within the second Cmu1 monomer (Cmu1'; Fig. S2B). This leads to a large deviation within L1. Furthermore, the charged patch within L3 that protrudes into the active site of Cmu1 is conserved among the two proteins with respect to the "clamping" aspartate and glutamate (Fig. 2, D and E) but differs in the residue in between being an aspartate in KWL1-b but a lysine in KWL1 (Fig. 2D). In Cmu1, important residues within the active site (Arg⁴³ and Gln²²⁹) are tethered by both KWL1 and KWL1-b (Fig. 2D). Despite their redundant function as potent Cmu1 inhibitors, both KWLs show slight variations in the way they interact with Cmu1.

KWL1-b and KWL1 differ in a surface cleft distal to their Cmu1-binding site

We next wondered whether other relevant differences between KWL1 and KWL1-b might exist. Overall, both KWL proteins consist of nine β-strands (β1 to β9) with β3 to β9

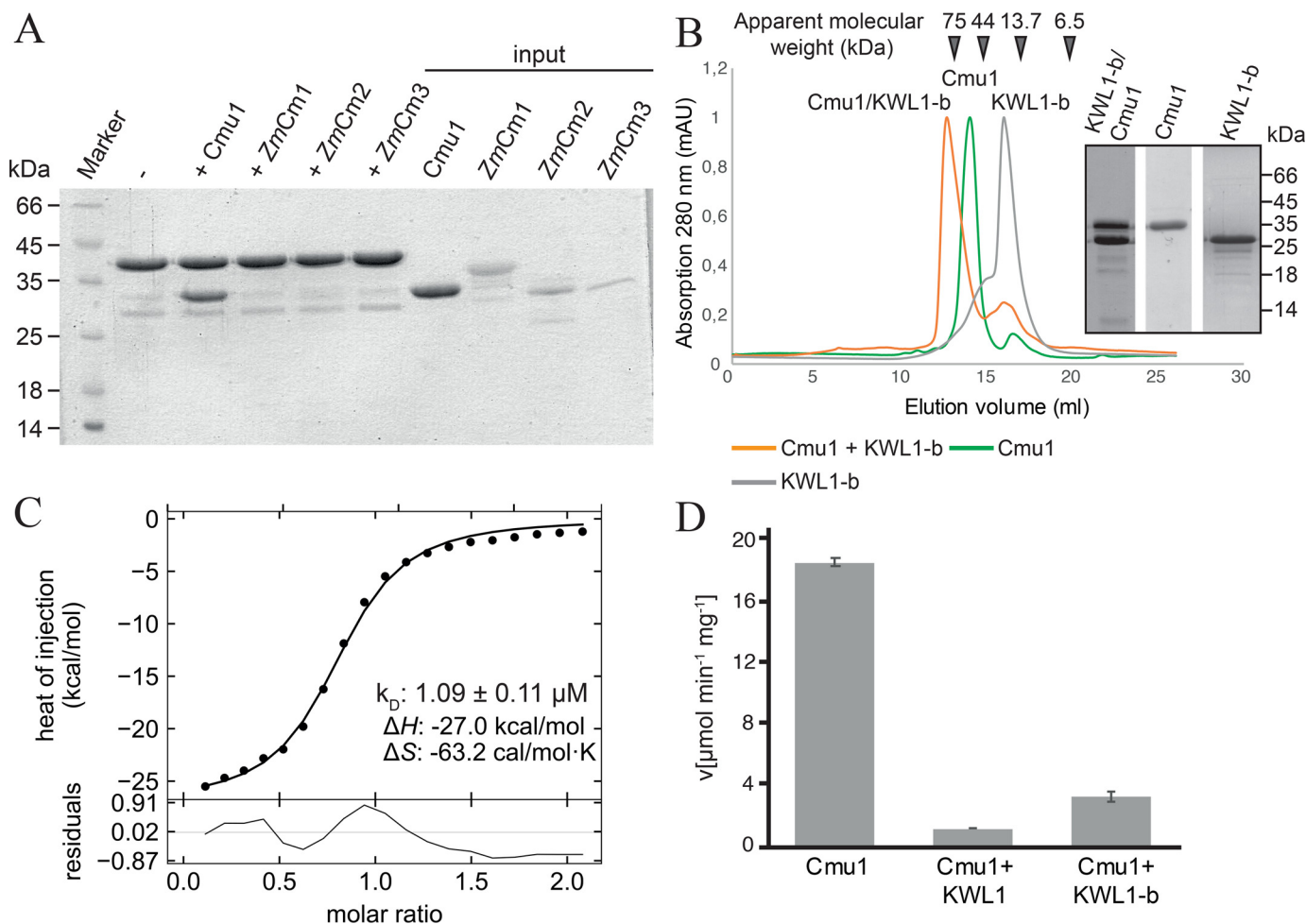


Figure 1. KWL1-b binds to Cmu1 and inhibits its activity. A, GST interaction assay employing a GST-tagged KWL1-b, UmCmu1, and the three housekeeping chorismate mutases ZmCM1, ZmCM2, and ZmCM3. B, size-exclusion chromatograms of Cmu1 (green), KWL1-b (gray), and the Cmu1–KWL1-b complex (red). The inset shows a Coomassie-stained SDS-PAGE of the peak fractions. C, ITC of Cmu1 and KWL1-b yielding a K_D of $1.1 \pm 0.1 \mu\text{M}$. KWL1-b was added to the sample cell and titrated with Cmu1. D, KWL1-b inhibits the CM activity of Cmu1. CM activity was determined with the chorismate activity assay. Error bars represent the S.D. of three technical replicates.

forming the β -barrel and a short α -helical segment connecting the strands $\beta 8$ and $\beta 9$ (Fig. 3A). In the case of KWL1-b, $\beta 6/7$ forms one large strand that closes the barrel. Five disulfide bridges formed through 10 highly conserved cysteine residues stabilize both KWL proteins (Fig. S3A). Consequently, the α -backbones of both KWL proteins align well with an RMSD of 0.5 (Fig. 2A).

However, analysis of surface topology and electrostatic potential revealed pronounced differences between KWL1-b and its counterpart KWL1 (Fig. S3B). Both KWL proteins significantly differ in a surface cleft that localizes on the opposite side of their Cmu1-binding site. In both KWL proteins, this cleft is formed by the β -sheets 3, 4, 6, and 7 and L3 between $\beta 6$ and $\beta 7$ that aligns to the $\beta 1/2$ domain (Fig. 3B). Although the overall volume of the cavity is comparable between KWL1-b and KWL1 with ~ 2400 and $\sim 2900 \text{ \AA}^3$, respectively, they show pronounced differences at the amino acid level (Fig. 3D and Fig. S3C). More precisely, the negatively charged residues toward Cmu1 (Asp¹⁴⁹, Glu¹⁵⁰, and Asp¹⁵² at KWL1), as well as Glu¹⁶⁴ and Asp¹⁷³, “clamping” the cleft are conserved between KWL1 and KWL1-b, whereas the residue “guarding” entrance to the cleft exhibits opposing charge (Arg¹⁶⁸, KWL1 and Asp¹⁶³,

KWL1-b). Taken together, despite a high sequence conservation and similar binding of Cmu1, both KWL proteins display differences in a surface cleft located across to the Cmu1-binding site in both proteins.

KWL1-b and KWL1 differ in their carbohydrate-binding properties

Our structural analysis identified pronounced clefts at the surfaces of KWL1 and KWL1-b, which differed in their calculated electrostatic properties (Fig. S3B). DALI search for structural relatives of the KWL proteins identified barwins, expansins, and cerato-platanins (10, 11) but also revealed similarity to several glycoside hydrolases (GH) such as EGV from *Humicola insolens* (PDB code 4ENG), which cleaves the β -1,4-linkage of cellulose as a primary component of plant cell walls (12, 13). KWL1-b and KWL1 superimpose well with EGV with RMSD values of 1.26 and $2.66 \text{ \AA}^2$ over 188 and 184 atoms, respectively. Closer structural comparison showed that the cleft conserved between the two KWL proteins can also be found on the surface of EGV, albeit smaller (Fig. 4A). The central β -barrel that forms one side of the cleft is present in both EGV and kiwellin proteins, but the opposite side of the cleft is

Table 1

Data collection and refinement statistics for the Cmu1/KWL1-b complex

The values in parentheses are for the outer shell.

Cmu1/KWL1-b	
Data collection	
Space group	$P2_1$
Cell dimensions	
a, b, c (Å)	57.74, 124.58, 98.21
α, β, γ (°)	90, 96.216, 90
Resolution (Å)	48.82–2.7 (2.796–2.7)
R_{merge}	0.0896 (0.8243)
$I/\sigma I$	7.91 (1.12)
Completeness (%)	98.46 (99.73)
Redundancy	3.0 (3.1)
Refinement	
Resolution (Å)	47.99–2.75 (2.85–2.75)
No. reflections	35,373 (3546)
$R_{\text{work}}/R_{\text{free}}$	0.22/0.26
No. atoms	
Protein	6594
Ligand/ion	0
Water	19
B-factors	
Protein	73.82
Ligand/ion	0
Water	64.16
Ramachandran statistics	
Favored (%)	94.66
Allowed (%)	4.99
Outlier (%)	0.36
RMSDs	
Bond lengths (Å)	0.006
Bond angles (°)	0.98

tightly closed at EGV, whereas $\beta 1/2$ and L3 generate a larger surface groove within the KWLs. In the GH enzyme EGV, the cleft is required for binding of the cellohexaose sugar substrate (Fig. 4B).

To estimate whether the clefts of both KWL proteins would allow carbohydrate binding, we superposed EGV bound to a cellohexaose in the -4 to -2 side (precleavage sides; Fig. 4B). The superposition clearly suggested that cellohexaose binding to either KWL1-b and KWL1 should generally be possible from a structural point of view (Fig. 4C). Therefore, we analyzed the carbohydrate-binding ability of KWL1 and KWL1-b by microscale thermophoresis (MST). Because of decreased solubility of higher-order carbohydrates, we employed glucose, xylose, galactose, arabinose, and mannose. Although KWL1 showed specific binding to galactose with a dissociation constant (K_d) of $41 \pm 8 \mu\text{M}$, KWL1-b preferred mannose and xylose over the other sugars with K_d values of 29 ± 10 and $31 \pm 14 \mu\text{M}$, respectively (Table 2 and Figs. S4B, D, and E). In addition to monosaccharides, we further examined the binding of mannotriose and xylotriose to KWL1-b. To our surprise, the K_d of mannotriose was comparable with mannose and xylose with $45 \pm 7 \mu\text{M}$, whereas binding of xylotriose could not be observed (Table 2 and Fig. S6). Thus, our results suggest that KWL1 and KWL1-b are capable of sugar binding but differ in their specificity.

Next, we wondered whether Cmu1 binding to KWL1 or KWL1-b would impact their ability to interact with sugars. Thus, we analyzed the ability of the Cmu1–KWL1-b and Cmu1–KWL1 complexes to interact with glucose, xylose, galactose, arabinose, and mannose. To our surprise, Cmu1 binding to KWL1 and KWL1-b abolished the interaction with galactose and xylose, respectively. However, Cmu1-bound

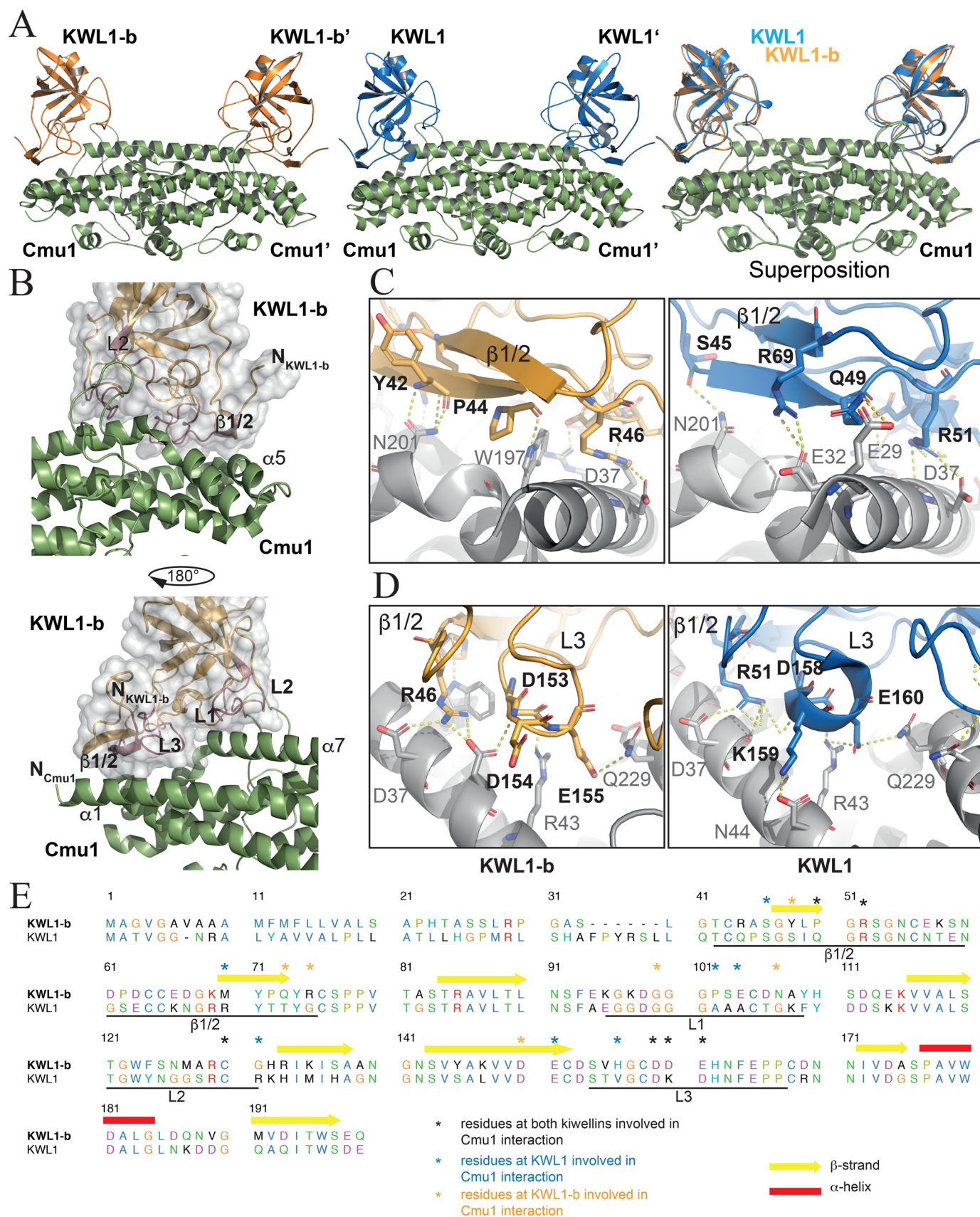
KWL1-b could still interact with mannose and mannotriose (Table 2, and Figs. S4, D and E, and S5A). From our results, we conclude that Cmu1 binding modulates the sugar-binding abilities of KWL1 and KWL1-b.

In a next step, we aimed at a more thorough investigation of residues at KWL1 and KWL1-b potentially involved in carbohydrate binding. Based on EGV co-crystallized with cellohexaose, we varied three acidic amino acids in close vicinity to the cellohexaose and conserved among KWL1 and KWL1-b. At KWL1, Asp¹⁴⁹, Glu¹⁶⁴, and Asp¹⁷³ were varied to alanine, corresponding to Asp¹⁴⁴, Glu¹⁵⁹, and Asp¹⁶⁸ at KWL1-b (Fig. 4, C and D). All variants at KWL1 significantly decreased the affinity of galactose toward KWL1 by ~ 10 -fold (Table 2 and Fig. S5B). Varying Asp¹⁴⁴ and Glu¹⁵⁹ to alanine at KWL1-b completely abolished binding of both mannose and mannotriose, whereas exchanging the central Asp¹⁶⁸ to alanine did not affect the binding (Table 2 and Fig. S5, C and D). Similarly, xylose could still bind to the D168A variant of KWL1-b, although the interaction was weakened ($168 \pm 28 \mu\text{M}$). A residual binding of xylose was also observed toward KWL1-b_{D144A} but considered not trustworthy because of bad fitting and a high standard deviation (Fig. S5E). We conclude that conserved acidic residues at KWL1 and KWL1-b contribute differently to carbohydrate binding, providing a potential explanation for a difference in substrate specificity by the two proteins.

Discussion

KWL1 and KWL1-b are structurally and functionally divergent

In a recent study, we could demonstrate that the KWL1 protein might be part of the pattern-triggered immunity because it is secreted from plant cells into the apoplastic space after pathogen attack to counteract *U. maydis* (10, 14). The secreted chorismate mutase (Cmu1) from *U. maydis* was shown to target KWL1 from *Z. mays* (10). We now show that a second KWL protein, termed KWL1-b, specifically interacts with Cmu1 of *U. maydis*. Our structural and biochemical analysis shows that KWL1-b employs an identical mechanism as its counterpart KWL1 to inhibit the CM activity of Cmu1, although KWL1-b employs other amino acids than KWL1. More precisely, only four residues tethering charged residues in the active site of Cmu1 are identical between KWL1 and KWL1-b, although they overall architecture of both complexes is almost identical. This observation might be the reason for the differences in the interaction strengths between KWL1 and KWL1-b for Cmu1 (*i.e.* 80 nM and 1 μM , respectively), accompanied by a slightly less efficient inhibition of Cmu1 by KWL1-b. The reason for the apparent functional redundancy of KWL1 and KWL1-b can at this point only be answered at the speculative level. One idea could be that KWL1-b might more efficiently counteract the activity of closely related Cmu1 proteins of another maize-specific pathogen or represents an earlier, less-efficient version of KWL1. Another possibility could be considered from transcriptome data sets that revealed *U. maydis* cell type-specific gene expression upon *Z. mays* infection (7). During plant infection, hyphae of *U. maydis* first proliferate within the mesophyll of *Z. mays* leaves and then colonize bundle sheath cells, allowing entry in the veins and more efficient spread (7, 15) (Fig. 5A).



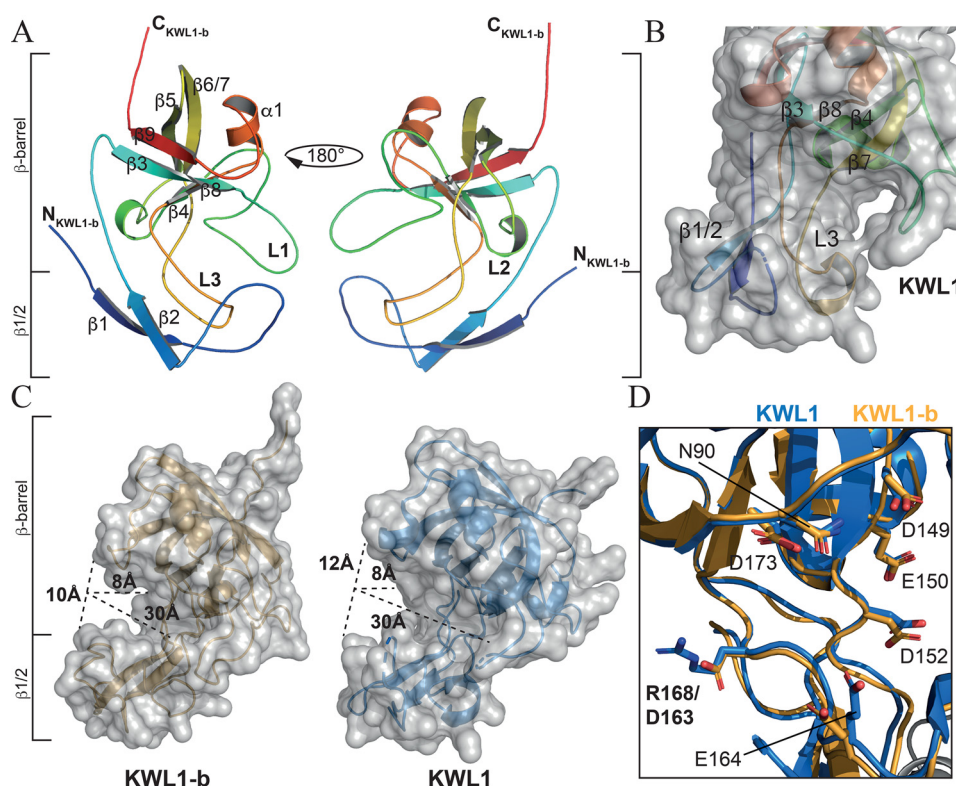


Figure 3. KWL1-b and KWL1 differ in a pronounced surface cleft distal to their Cmu1-binding sites. A, KWL1-b consists of a central β -barrel and two short antiparallel β -strands at the N terminus termed $\beta 1/2$. The loops L1–L3 together with the $\beta 1/2$ domain confer interaction with Cmu1. The structure has been colored in rainbow colors from the N terminus (blue) to the C terminus (red). B and C, both KWLS have a pronounced cleft on the rear of the protein distal of the Cmu1-binding site that is formed between $\beta 1/2$ –L3 and the β -strands 3, 4, 7, and 8. D, residues within the cleft are conserved between KWL1 and KWL1-b with the exception of Arg¹⁶⁸ (KWL1) and Asp¹⁶³ (KWL1-b).

The analysis revealed a high abundance of transcripts corresponding to *KWL1*, but *KWL1-b* was only poorly resolved. Interestingly, *KWL1* was significantly enriched in mesophyll cells compared with bundle sheath cells, indicating a role in the early defense response toward *U. maydis* colonization. Despite the overall low transcript abundance, *KWL1-b* showed similar transcript abundance in both tissues. Therefore, KWL1-b could also just play a role during the later stages of infection.

KWL1 and *KWL1-b* have different carbohydrate-binding properties

Our structural comparison between KWL1 and KWL1-b identified a pronounced surface cleft, which both proteins share with the structurally related glycoside hydrolase EGV. Indeed, our biochemical analysis shows that KWL1 and KWL1-b specifically recognize the monosaccharides galactose and mannose/xylose, respectively. EGV proteins have several adjacent carbohydrate-binding sites within their active site that allow oligosaccharide binding and subsequent cleavage

between the -1 and $+1$ sites (Fig. 4B) (13). Cellulases of this type belong to the GH family and cleave the β -1,4-linkage of cellulose, which is the primary component of plant cell walls. In these enzymes, the catalytic activity is mediated by two aspartic acids and a stabilizing tyrosine on either side of an open groove in the surface of the protein (13, 16, 17). However, only one aspartate of three catalytically relevant residues is conserved between EGV and the two KWLS (Fig. S6). From our structural analysis and biochemical evidence, we conclude that the two studied KWL proteins are carbohydrate-binding proteins, which might have lost their glycoside hydrolase activity. Similar observations were made for the structurally related family of the cerato-platanins, which also exhibit carbohydrate-binding properties while lacking glucosidase activity (18, 19). This idea is furthermore supported by the fact that KWL proteins were initially isolated from cell wall extracts of kiwi fruit (20, 21).

We show that several residues within the potential binding cleft at KWL1 and KWL1-b contribute differently to carbohy-

Figure 2. KWL1-b forms a heterotetrameric complex with Cmu1. A, KWL1-b and KWL1 form highly similar complexes with the fungal effector Cmu1. Left panel, crystal structure of two KWL1-b proteins (orange) bound to the Cmu1 homodimer (green; this study). Middle panel, crystal structure of two KWL1 proteins (blue) bound to the Cmu1 homodimer (green; PDB code 6FPG). Right panel, superimposition of both complexes shown in the left and middle panels. B, close-up of the interaction between Cmu1 (green) and KWL1-b (orange; gray surface). The interacting loops at KWL1-b are highlighted in red. C and D, detailed view on residues involved in the interaction with Cmu1 at KWL1-b and KWL1 within the $\beta 1/2$ domain and L3, respectively. KWL1-b is colored in orange, KWL1 is colored in blue, whereas Cmu1 is colored in gray across all images. Dotted lines represent polar interactions. E, sequence alignment of KWL1 and KWL1-b with secondary structures according to the KWL1-b structure. β -Strands and α -helices are indicated in yellow and red, respectively. Residues involved in the interaction are marked with an asterisk and colored according to whether they are involved in interactions between KWL1 and KWL1-b to Cmu1, respectively.

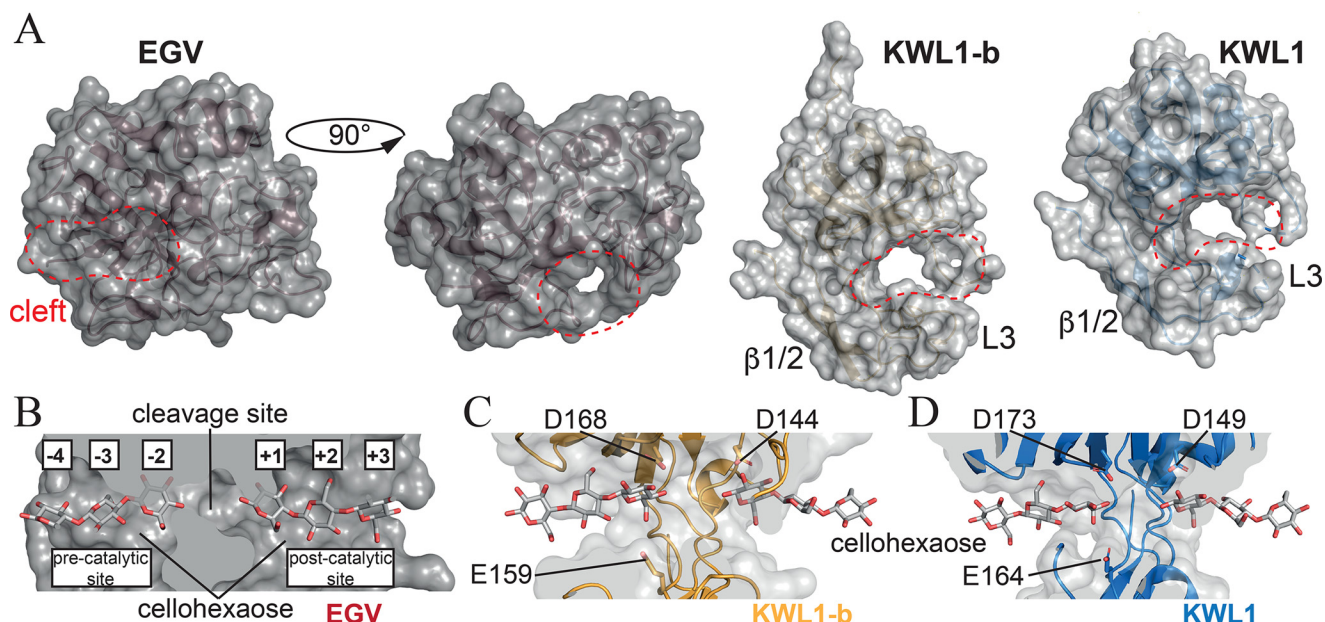


Figure 4. KWL1 and KWL1-b share structural homology to endoglucanases. *A*, side-by-side view of EGV (PDB code 4ENG), KWL1-b, and KWL1 in surface representation. The cleft is highlighted with a dashed line. EGV is colored in dark red, KWL1-b is colored in orange, and KWL1 is colored in blue. *B*, EGV bound to cellohexaose. The different carbohydrate-binding sites are highlighted. *C* and *D*, superposition of EGV-bound to cellohexaose and KWL1-b/KWL1 shows that cellohexaose *per se* fits into the cleft at KWL1-b and KWL1. Residues in the cleft varied to alanine are highlighted.

Table 2

Carbohydrate binding of KWL1 and KWL1-b

The table summarizes the dissociation constants (K_d) of KWL1, KWL1-b, and variants in the absence and presence of Cmu1 for various carbohydrates, as determined by MST. All experiments have been performed in three technical replicates. The values are given in μM . The standard deviation is given on the right side of the table. N.D., no fitting possible; [em], not measured. Original data can be found in Figs. S4 and S5.

	Galactose	Glucose	Mannose	Mannotriose	Xylose	Xylotriose	Arabinose
KWL1	41 ± 8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
KWL1 _{D149A}	463 ± 183	[em]	[em]	[em]	[em]	[em]	[em]
KWL1 _{E164A}	422 ± 272	[em]	[em]	[em]	[em]	[em]	[em]
KWL1 _{D173A}	192 ± 79	[em]	[em]	[em]	[em]	[em]	[em]
Cmu1/KWL1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
KWL1-b	N.D.	N.D.	29 ± 10	49 ± 13	31 ± 14	N.D.	N.D.
KWL1-b _{D144A}	[em]	[em]	N.D.	N.D.	80 ± 54 ^a	[em]	[em]
KWL1-b _{E159A}	[em]	[em]	N.D.	N.D.	N.D.	[em]	[em]
KWL1-b _{D168A}	[em]	[em]	31 ± 5	43 ± 18	135 ± 28	[em]	[em]
Cmu1/KWL1-b	N.D.	N.D.	47 ± 4	28.2 ± 4	N.D.	N.D.	N.D.

^a Bad fitting.

drate binding. Although mutation of three acidic residues at KWL1 reduces the affinity for galactose by 10-fold, variation of two of their conserved counterparts at KWL1-b abolish mannose and mannotriose binding (Fig. 4, C and D, and Fig. S5, B–D). However, an exchange of Asp¹⁶⁸ to alanine affects the binding of neither mannose nor mannotriose. Moreover, this variation impacts xylose binding only moderately. We therefore conclude that carbohydrate binding to KWL1-b occurs slightly different than to KWL1 (Fig. 5C).

We also show that the presence of Cmu1 inhibits the binding of galactose and xylose to KWL1 and KWL1-b, respectively, although it does not affect the binding of mannose and mannotriose to KWL1-b. Combining these results with our mutational studies, we predict that the galactose- and xylose-binding sites of KWL1 and KWL1-b overlap at least partially with their Cmu1-binding sites, although this cannot be true for the binding of mannose and mannotriose to KWL1-b (Fig. 5, B and C). A superposition of the Cmu1–KWL1 (or Cmu1–KWL1-b) complex and cellohexaose-bound EGV showed that Cmu1-binding generates a sterical

clash with the cellohexaose within the postcatalytic pocket (Fig. 5C). The galactose- and xylose-binding sites possibly overlap with this region of KWL1 and KWL1-b, respectively, preventing carbohydrate binding when Cmu1 is present. We also predict that mannose/mannotriose preferentially binds to a position at KWL1-b equivalent to the precatalytic site at EGV, because its binding affinity remains largely unaffected by Cmu1 binding (Fig. 5C).

In all likelihood, both KWLs bind oligosaccharides rather than the tested monosaccharides. Thus, future research needs to identify the cognate oligosaccharides to which KWL1 and KWL1-b bind. It could well be that these oligosaccharides will be found in the context of the cell wall. At this point, we cannot exclude that KWL proteins also address oligosaccharides of the fungal cell wall. However, our data suggest that Cmu1 and sugar binding might be mutually exclusive. Thus, we favor the idea that KWL proteins reside tethered to specific oligosaccharides of the plant cell wall and release upon binding to the fungal effector Cmu1 to inhibit its activity. The carbohydrate specificity of KWL proteins might be due to the tissue-specific proper-

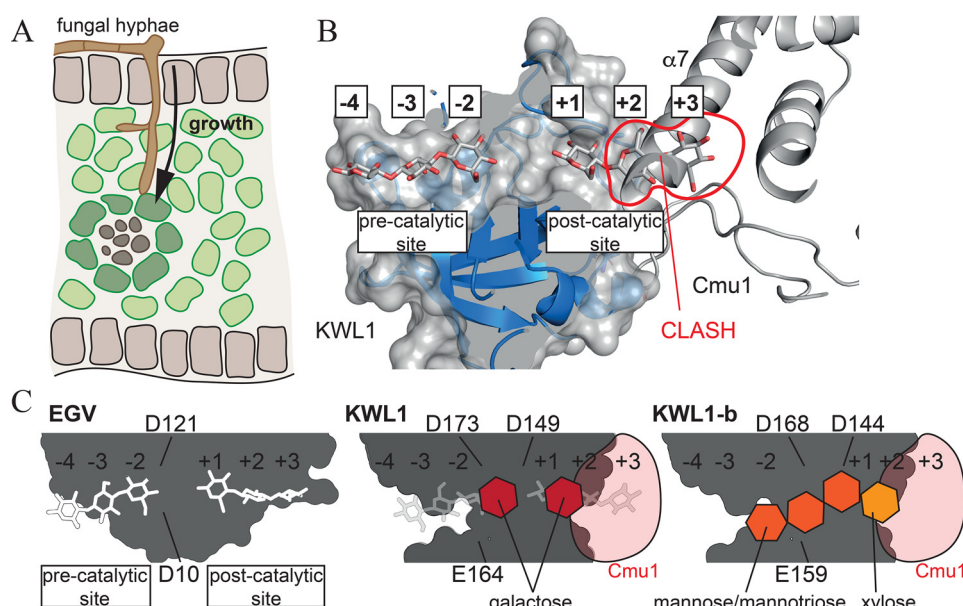


Figure 5. Functional and structural diversity of two redundant KWL proteins. A, illustration of the early infection stages of maize leaves by *U. maydis*. Fungal hyphae is in brown; epidermal layer is in light brown; mesophyll cells are in light green; bundle sheath cells are in dark green; and veins are in dark brown. B, the superimposition of Cmu1–KWL1-b (this study), Cmu1–KWL1 (PDB code 6FPG), and cellohexaose-bound EGV (PDB code 4ENG) shows that Cmu1 binding to KWL1 could interfere with oligosaccharide binding in the postcatalytic pocket. C, schematic representation of monosaccharide-binding sites in EGV (left panel) and their putative equivalents in KWL1 (middle panel) and KWL1-b (right panel). –4, –3, –2, +1, +2, and +3 refer to the monosaccharide-binding sites at the pre- and postcatalytic sites, respectively. The Cmu1-binding area is indicated in red. The positions of the catalytic aspartates and their equivalents in the KWLs are indicated. Potential carbohydrate-binding sites at KWL1 and KWL1-b are indicated with hexagonal models.

ties of cell walls *per se* or cell wall remodeling during fungal infection.

Experimental procedures

Molecular cloning

The gene encoding KWL1-b was amplified from genomic DNA of *Z. mays* EGB without the signal peptide (KWL1-b^{31–193}) and cloned into pEMGB1 (22) and pGAT3 (Novagen) vectors yielding in pEMGB1-KWL1-b and pGAT3-KWL1-b, respectively. Single amino acid variations were obtained using a modified quick-change protocol. Briefly, the entire backbone of pEMGB1-*Zm*KWL1-b and pET28a-*Zm*KWL1 was amplified with the respective primers in a single step, digested with BsaI-HFv2 (NEB), and ligated to obtain the final plasmids (Tables S1 and S2). Plasmids encoding Cmu1, KWL1, and the three CMs from *Z. mays* were obtained from an earlier study (10).

Protein production and purification

Protein production and purification was performed as described earlier (23). Briefly, the proteins were produced in *E. coli* SHuffle T7 (NEB) and BL21(DE3) (Novagen). *E. coli* SHuffle T7 were transformed with plasmids encoding GB1-KWL1-b. The cultures were grown in lysogeny broth medium in the presence of ampicillin (100 µg/ml) at 30 °C under constant shaking to an A_{600} level of 0.5, the temperature was shifted to 20 °C, and the cultures were induced with 0.5 mM isopropyl β -D-thiogalactopyranoside. The cultures were harvested by centrifugation after 20 h. In the case of Cmu1 and KWL1, *E. coli* BL21(DE3) were transformed with the respective plasmids. Protein production was performed in autoinductive lysogeny broth medium containing 1% (w/v) of lactose and incubated at 30 °C under constant shaking for 20 h.

After cell lysis by a microfluidizer (M110-L, Microfluidics), cell debris was removed by high-speed centrifugation, and the proteins were purified by nickel-ion affinity purification (FF-HisTrap columns; GE Healthcare) and SEC as described recently (23). The SEC buffer consisted of 20 mM HEPES-Na, pH 7.5, 200 mM NaCl, and 20 mM KCl. In the case of GB1-KWL1-b, elution fractions were collected after SEC, pooled, and incubated with 0.2 mg of TEV protease (NEB) in the presence of 1 mM DTT at ambient temperature for 10 h. Proteolytic cleavage was analyzed by SDS-PAGE, and the TEV and cleaved KWL1-b were retrieved by reverse nickel-ion affinity purification. Protein containing fractions were pooled and concentrated in Amicon Ultra-10K centrifugal filters. The Cmu1–KWL1-b complex was reconstituted by mixing both proteins in a molar 1:2 ratio and subjecting the mixture to another SEC. Fractions containing the Cmu1-KWL1-b complex were pooled and concentrated for crystallization experiments.

Crystallization and structure determination

Crystallization was performed by the sitting-drop method at 20 °C in 0.5-µl drops consisting of equal parts of protein and precipitation solutions. Cmu1–KWL1-b crystallized at 161 µM concentration within 24 h days in 0.1 M HEPES, pH 7.0, and 8% (w/v) PEG 8000. Prior to data collection, the crystals were flash-frozen in liquid nitrogen employing a cryo-solution that consisted of mother liquor supplemented with 30% glycerol. The data were collected under cryogenic conditions at the European Synchrotron Radiation Facility at Beamline ID30A-1 (MAS-SIF-1) (24).

The data were integrated and scaled with XDS (25) and merged with XSCALE (25). Structures were determined by molecular replacement with PHASER (26), manually built in

COOT (27), and refined with PHENIX (28). The structure of the Cmu1–KWL1-b complex was determined by molecular replacement using the crystal structures of KWL1 and Cmu1 of the Cmu1–KWL1 complex (PDB code 6FPG) as search models. The figures were prepared with PyMOL (29) and Chimera (30).

GST-binding assays

GST interaction assays were performed with SEC buffer + 0.05% Tween (20 mM HEPES, pH 7.5, 200 mM NaCl, 20 mM KCl, 20, 0.05% Tween) at 4 °C using Mobicol “classic” spin columns (MoBiTec). A total amount of 4 nmol of SEC-purified GST-tagged protein was immobilized on 25 μ l of GSH Sepharose (GE Healthcare) and incubated on a turning wheel for 5 min. Two equivalents of putative interaction partner proteins were added to the beads and incubated for 20 min on a turning wheel. After removal of residual protein by centrifugation (4 °C, 4000 rpm, 1 min), the column was washed three times with SEC buffer + 0.05% Tween. The proteins were eluted with 80 μ l of GSH elution buffer (20 mM HEPES, pH 8.0, 200 mM NaCl, 20 mM KCl, 0.05% Tween, 20 mM GSH) and analyzed by Coomassie-stained SDS-PAGE.

Isothermal titration calorimetry

Prior to the measurement, the Cmu1 and KWL1-b protein solutions were dialyzed against the identical buffer, which consisted of 20 mM HEPES-Na, pH 7.5, 200 mM NaCl, 20 mM KCl. Titration was carried out at a temperature of 25 °C with a MicroCal ITC200 (Malvern Panalytical Ltd.). 280 μ l of KWL1-b at 25 μ M were placed in the sample cell, and the syringe was fully loaded with 25 μ M of Cmu1. A first injection of 0.3 μ l was followed by 19 injections of 2 μ l to generate the thermogram representing the interaction. The data were processed with the MicroCal PEAQ-ITC analysis software (Malvern Panalytical Ltd.).

CM activity assay

Analysis of the CM activity of Cmu1 was carried out as described earlier (16). Briefly, the assay monitored the disappearance of chorismate at a wavelength of 274 nm (A_{274} ; the extinction coefficient of chorismate is $\epsilon_{274\text{ nm}} = 2630\text{ M}^{-1}\text{ cm}^{-1}$). The A_{274} was measured using a TECAN Infinite 200 PRO plate reader (Tecan Trading AG). Standard assays were performed at 30 °C in 250 μ l of reaction buffer composed of 40 mM Tris-HCl, pH 7.0, and 100 mM NaCl. In a standard reaction, chorismate mutase activity was measured with 100 ng of Cmu1 protein (*i.e.* 13 nM concentration) and 0.5 mM chorismate (Sigma).

Measurements of binding constants by MST

MST experiments were performed in a buffer containing 20 mM HEPES, pH 7.5, 20 mM KCl, and 200 mM NaCl using a Monolith NT.115 with red LED power set to 50% and IR laser power set to 75% (31). KWL1, KWL1-b, Cmu1–KWL1, Cmu1–KWL1-b, and all mutant proteins (50 μ M) were each labeled according to the supplier's instructions (dye NT 647, NanoTemper Technologies). Subsequently, 500 nM of each protein was titrated with increasing amounts of galactose, glucose,

mannose, xylose, or arabinose starting from 6 mM each. At least three independent MST experiments were recorded at 680 nm and processed by NanoTemper analysis 1.2.009 and Origin8G.

RNA sequencing data analysis

RNA sequencing data for runs SRR6202430–SRR6202441 (NCBI BioProject [PRJNA415355](#)) were downloaded from the Short Read Archive (7, 32). They comprise three replicates each for infected cells, as well as mock-treated maize mesophyll and bundle sheath tumor cells (*Z. mays* infected with *U. maydis* strain SG200, 4 days postinfection). Quality trimming and adapter removal were performed using Trim Galore, a wrapper tool around Cutadapt (33). A Phred score threshold of 20 was used. Processed reads with at least 20 bp were mapped to the *Z. mays* transcriptome B73 RefGen v4 (GCF_000005005.2) using Segemehl (34) with an *e*-value threshold of 1. Here we identified transcripts XM_008646524.3 and XM_008665441.3 as *KWL1* (ZEAMA_GRMZM2G073114) and *KWL1-b* (ZEAMA_GRMZM2G305329), respectively (best reciprocal blast hit). The RPKM was calculated based on all reads that uniquely mapped to these transcripts (read counts/(transcript length/1000 $\times$ mapped reads/1,000,000)). Significance was evaluated using a one-sided Wilcoxon rank-sum test in R.

Data availability

The coordinates and structure factors have been deposited in the PDB under accession code [6TI2](#). The authors declare that all other data supporting the findings of this study are available within the article and its [supporting information](#).

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Abbreviations—The abbreviations used are: CM, chorismate mutase; RMSD, root-mean-square deviation; MST, microscale thermophoresis; PDB, Protein Data Bank; EGV, endoglucanase V; GST, glutathione *S*-transferase; SEC, size-exclusion chromatography; ITC, isothermal calorimetry; GH, glycoside hydrolase.

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