Supporting Information

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SI Materials and Methods

Protein Expression and Purification. The wild-type *chvE* gene was amplified from plasmid pFH100 (1) by PCR method using the following primers to make the full-length mature *chvE* (residues 25–354) [NSchvE.P1(SapI): ACTGCAGCTCTTCTAACCAGGACAAGGGTTCTGTCG; and NSchvE.P2(BglII): GGAAGATCTTTATTTCAGCTGGTCTTCCTTG] (underlining shows the added restriction site). The resulting fragments were cloned into the SapI/BamHI sites of a pTWIN2 (New England Biolabs) plasmid to produce pJZ1 for overexpression in *Escherichia coli* BL21(DE3). This ORF lacks the periplasmic signal sequence. The coding sequence starting at glutamine 25 was cloned in-frame with an ATG start codon. An Intein affinity tag was fused in-frame at the N terminus to facilitate purification by binding on a chitin resin. *E. coli* BL21(DE3) harboring plasmid pJZ1 was first grown at 37 °C and when the optical density at 600 nm reached 0.6, the culture was transferred to 30 °C with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for induction.

The Intein-tag was cleavaged on column with a pH and temperature shift (pH 7 and 25 °C). The eluted target protein was further purified by size exclusion chromatography (Superdex-75). Then the protein was subject to dialysis against protein stock buffer [10 mM Tris·HCl (pH 7.3), 50 mM NaCl], concentrated with Amicon Ultra devices (molecular weight cutoff, 10,000). Protein concentration was determined by BCA Protein Assay Kit (Pierce). The concentrated protein was aliquoted into 20 μL/vial and taken out when used.

Isothermal Titration Calorimetry. Samples and buffers were filtered and degassed before loading into the cell and syringe. The sugars were dissolved in the dialysis buffer following dialysis of the protein. One titration experiment typically consisted of a preliminary 0.2-μL injection, followed by 19 2-μL injections (5-s injections at 200-s intervals; stirring speed, 1,000 rpm; rotor model, ITC200 from MicroCal; 25 °C). Data from the first 0.2-μL injection were discarded before analysis and data were corrected for the heat of dilution of the sugar into the corresponding buffer without protein in the cell. The data were analyzed by nonlinear least-square fit of a single-site binding model using the software supplied with the instrument.

Crystallization of Galactose and Glucuronic Acid-Bound ChvE. Galactose-bound ChvE was crystallized using the hanging-drop vapor diffusion method by mixing 2 μL of ChvE (18 mg/mL in 10 mM Tris·HCl, 50 mM NaCl, pH 7.3 buffer, 16 °C) with 2 μL of the well buffer of 32% (vol/vol) PEG4000, pH 4.8, 0.2 M $MgCl_2$ (Hampton Research). The glucuronic acid complex was crystallized using protein refolded in the presence of 5 mM glucuronic acid as stock (about 25 mg/mL in 25 mM Mes, pH 5.5, and 50 mM NaCl buffer) against a well buffer of 31% PEG4000, 0.2 M $MgCl_2$ at pH 5.2. Crystals were swiped through the cryoprotectant PEG400 before flash cooling in liquid nitrogen.

Structure Determination of Galactose and Glucuronic Acid-Bound ChvE. The data were indexed and processed with the program HKL2000 (2). Phases for the galactose complex were determined by molecular replacement using the program Phaser (3) with a homologous ribose-binding protein (PDB ID code 1DRK) as the initial search model. Structure refinement was performed using the program Phenix (4) and the model adjustments were carried out with the software coot (5). Alternating cycles of model building and refinement were used to determine the final structure. Water molecules were added at the Fo-Fc electron density peaks with the cutoff level of 3σ. The geometry of the final model was assessed with MolProbity (6). The structure of the glucuronic acid complex was similarly solved by molecular replacement using the galactose complex of ChvE as the starting model.

Random Mutagenesis of ChvE^{K262}. To obtain all possible residues at site ChvE^{K262}, we conducted random mutagenesis at this site. To do this, random primer 3 (AGCAGTACTCCACCATCTTCNNNGACACCCGCGAACTCGCC) (NNN indicates random nucleotides at codon K262) was used to do PCR with primer 4 (GGGGTACCCCTTATTATTTTTCGAACTGCGGGTGGCTCCAAGCGCTTTTCAGCTGGTCTTCCT) (primer 4 is located in the 3′-terminus of *chvE* and underlining shows the added KpnI site) using pGN102 (1) as template. Primer 1 (CATCCGTTTCCACGGTGTG) (located upstream of *chvE*) was used with primer 2 (GAAGATGGTGGAGTACTGCT) (partly complementary to primer 4) to do PCR using pGN102 as template. The PCR products from the two PCRs were mixed and used as template to do another PCR with primer 1 and primer 4 as primers. The final PCR product was digested with NcoI and KpnI and cloned into pBBR1MCS-5 (7) and electroporated into *Agrobacterium tumefaciens* DC1. The colonies were randomly picked up and used to isolate plasmids for sequencing to get all of the desired mutants.

Western Blot Analysis of the Expression of *chvE* Mutants. For Western blot, the plasmid pJZ16 (8) harboring constitutively expressed *araD1* was transformed into the strains used above in *vir* gene induction assays as an inner control. After growth in AB induction (ABI) medium with 0.25% glycerol for 24 h, the bacteria were collected and adjusted to an OD_{600} of 20 in sample buffer (9). After boiling for 10 min, 10-μL samples were subjected to SDS/PAGE and analyzed using anti-ChvE rabbit antibody (10). Subsequently, the membrane was stripped and used to check AraD1 level with RGS-His mouse monoclonal antibody (Qiagen).

Bacterial Growth Assays. The growth assay of *A. tumefaciens* strains was described previously (8). Glucuronic acid (Sigma) was used at 3 mM.

Scheme S1. Proton linkage in the glucuronic acid binding of ChvE:

$$ChvE \bullet BDP \bullet H \xleftrightarrow{K_B} ChvE + BDP \bullet H \xleftrightarrow{K_H} ChvE + BDP^- + H^+$$

Scheme S1 for proton binding linked to binding of glucuronic acid (BDP) to ChvE.

For a binding reaction in which association of a ligand is coupled to uptake or release of a proton as shown in *Scheme S1*, the equilibrium dissociation constants can be expressed, respectively, as follows:

$$K_B = \frac{[\mathrm{BDP} \bullet H] \cdot [ChvE]}{[ChvE \bullet BDP \bullet H]}$$

$$K_H = \frac{[\mathrm{BDP}^-] \cdot [H^+]}{[\mathrm{BDP} \bullet H]},$$

where [ChvE], [BDP•H], [ChvE•BDP•H], [BDP^-], [H^+], and [BDP•H] are the concentrations of free ChvE, proton-bound glucuronic acid, protein complex, free BDP, proton, proton-bound BDP, respectively, K_B is the ligand dissociation constant when the ionizable group in the ligand is protonated, K_H is the proton dissociation constant of the ionizable in the free form.

The observed ligand binding constant K_{obs} is written as follows:

$$K_{\text{obs}} = \frac{([\text{BDP}^-] + [\text{BDP} \bullet H])\cdot[ChvE]}{[ChvE \bullet BDP \bullet H]} = \frac{\left(\frac{K_H}{[\text{H}^+]} + 1\right)[\text{BDP} \bullet H]\cdot[ChvE]}{[ChvE \bullet BDP \bullet H]} = \left(\frac{K_H}{[\text{H}^+]} + 1\right)K_B = K_B\left(1 + 10^{\log\left(\frac{K_H}{[\text{H}^+]}\right)}\right) = K_B\left(1 + 10^{(pH - pK_H)}\right)$$

$$\log(K_{\text{obs}}) = \log(K_B) + \log\left(1 + 10^{(pH - pK_H)}\right). \quad \textbf{[S1]}$$

Eq. **S1** shows how the observed disassociation constants depend on K_B and K_H as pH changes. By nonlinear least-squares regression analysis, K_B was fitted by fixing pK_H as 2.9. The fitted K_B is 79 nM with $R^2 = 0.94$. The fitted plot is shown in Fig. 1.

1. He F, et al. (2009) Molecular basis of ChvE function in sugar binding, sugar utilization, and virulence in *Agrobacterium tumefaciens*. *J Bacteriol* 191(18):5802–5813.
2. Otwinowski Z, Minor W (1997) *Processing of X-Ray Diffraction Data Collected in Oscillation Mode* (Academic, New York), pp 307–326.
3. McCoy AJ, Grosse-Kunstleve RW, Storoni LC, Read RJ (2005) Likelihood-enhanced fast translation functions. *Acta Crystallogr D Biol Crystallogr* 61(Pt 4):458–464.
4. Afonine PV, Grosse-Kunstleve RW, Adams PD (2005) The Phenix refinement framework. *CCP4 Newsletter* 42.
5. Emsley P, Cowtan K (2004) Coot: Model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 60(Pt 12 Pt 1):2126–2132.
6. Chen VB, et al. (2010) MolProbity: All-atom structure validation for macromolecular crystallography. *Acta Crystallogr D Biol Crystallogr* 66(Pt 1):12–21.
7. Kovach ME, et al. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166(1):175–176.
8. Zhao J, Binns AN (2011) Characterization of the *mmsAB-araD1* (*gguABC*) genes of *Agrobacterium tumefaciens*. *J Bacteriol* 193(23):6586–6596.
9. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227(5259):680–685.
10. Shimoda N, Toyoda-Yamamoto A, Aoki S, Machida Y (1993) Genetic evidence for an interaction between the VirA sensor protein and the ChvE sugar-binding protein of *Agrobacterium*. *J Biol Chem* 268(35):26552–26558.

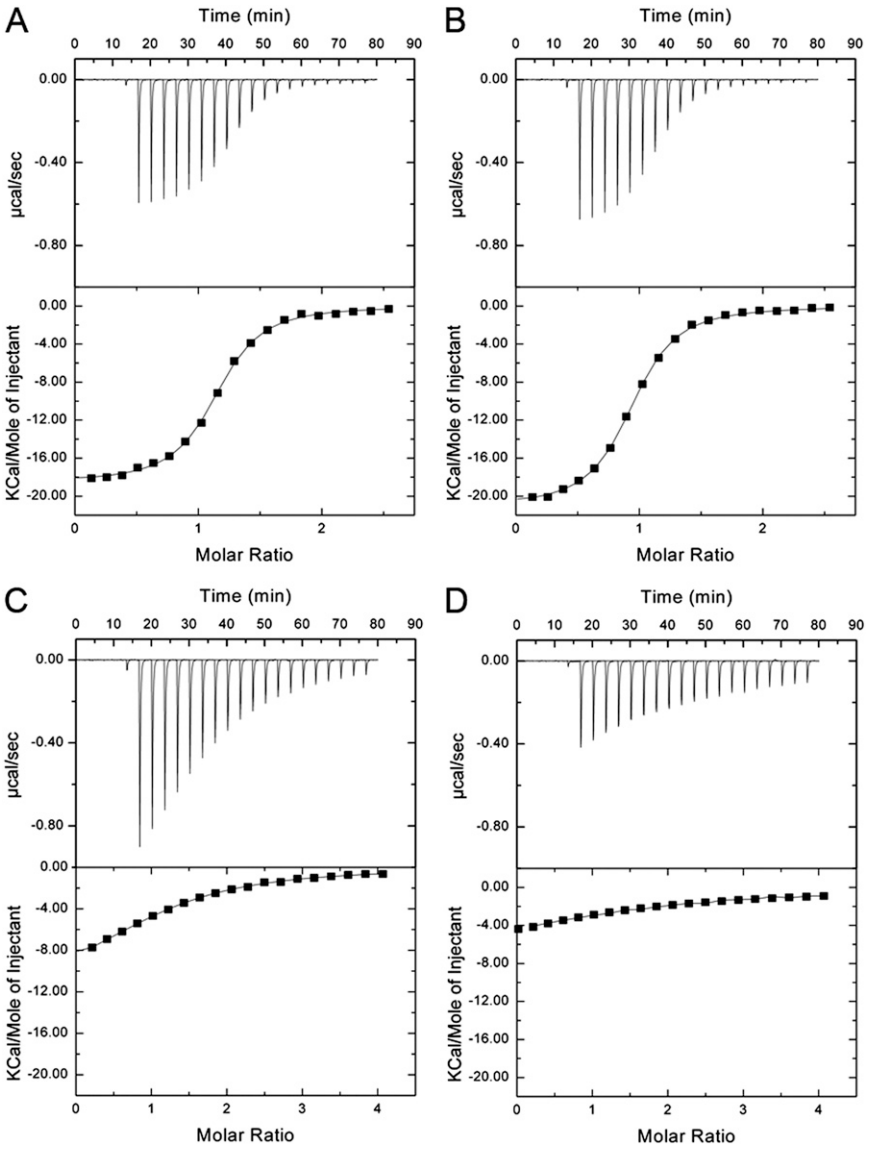

Fig. S1. A calorimetric titration profile and integrated injection heats for the titration of different ligands to ChvE at buffer condition 25 mM Mes, 50 mM NaCl, pH 5.5, (*A*) 250 µM arabinose to 20 µM ChvE, (*B*) 250 µM glucose to 20 µM ChvE, (*C*) 1 mM glucuronic acid to 50 µM ChvE, and (*D*) 1 mM galacturonic acid to 50 µM ChvE. The fitted data are shown in Table S2.

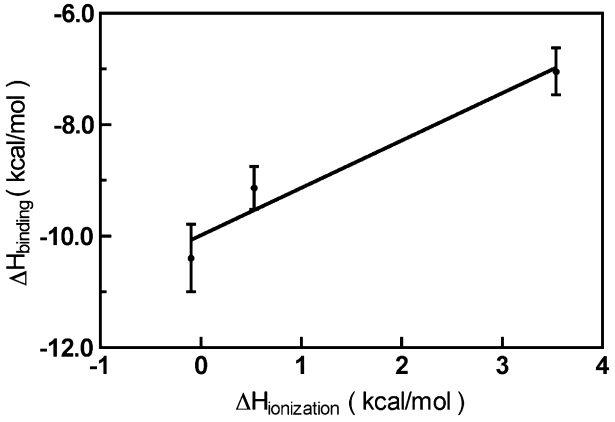

Fig. S2. Enthalpy change vs. buffer ionization enthalpy at pH 5.0 for three different buffers (25 mM Mes, 50 mM NaCl; 25 mM NaAc, 50 mM NaCl, and 25 mM Na-citrate, and 50 mM NaCl).

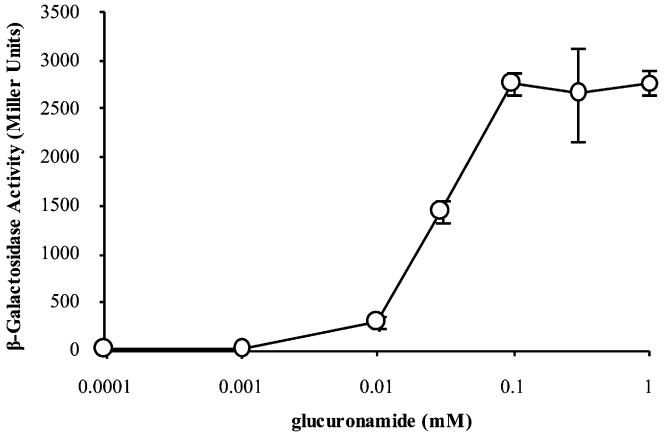

Fig. S3. *vir* gene expression in response to different glucuronamide concentrations. Wild-type strain A348 carrying pSW209Ω was grown in AB induction medium for 24 h with 10 μM AS and the indicated concentration of glucuronamide. β-Galactosidase activity was determined and reported in Miller units. Samples were assayed in triplicate, and results are plotted as means with SDs.

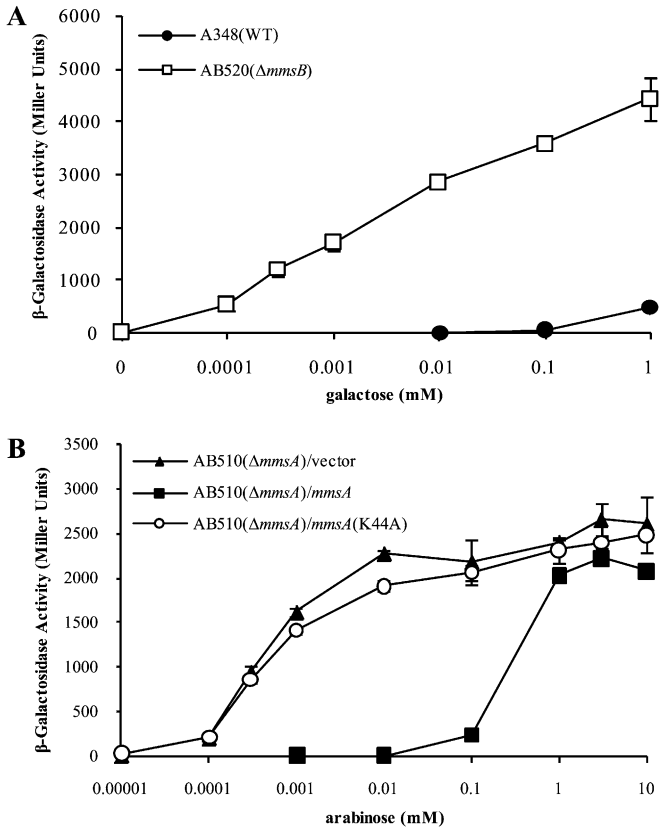

Fig. S4. (*A*) Effects of *mmsB* deletion on galactose dose–response for *vir* induction. *mmsB* deletion strain AB520 carrying pSW209Ω and wild-type strain A348 carrying pSW209Ω were grown for 24 h in AB induction medium with 10 μM AS and indicated concentration of galactose. β-Galactosidase activity was determined and reported in Miller units. (*B*) Effects of *mmsA* site-directed mutagenesis on arabinose dose–response for *vir* induction. *mmsA* deletion strain AB510 carrying pSW209Ω and 6His-tagged wild-type *mmsA* (pJZ20), 6His-tagged *mmsA*(K44A) mutant (pJZ21), or vector control (pBBR1MCS-5) were grown for 24 h in AB induction medium with 10 μM AS and indicated concentration of arabinose. β-Galactosidase activity was determined and reported in Miller units. Samples were assayed in triplicate, and results are plotted as means with SDs.

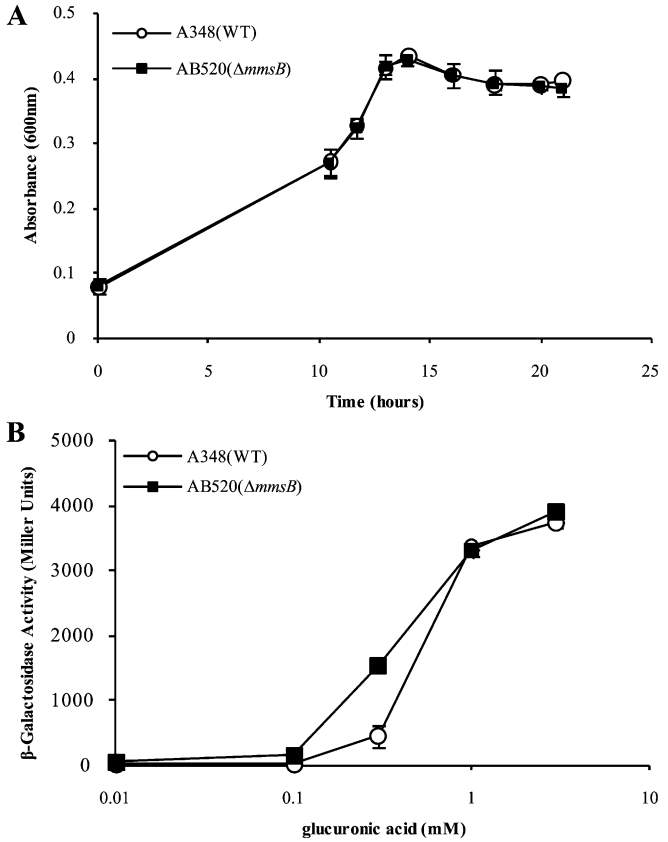

Fig. S5. (*A*) Growth of *A. tumefaciens* wild-type strain (A348) and *mmsB* mutant (AB520) in minimal medium containing 3 mM glucuronic acid. Bacteria were grown at 25 °C in AB minimal medium with 10 mM glucuronic acid as the sole carbon source. At intervals, the optical density at 600 nm of the cultures was determined. Data shown are the averages of triplicate values. SDs are indicated. (*B*) *vir* gene expression in response to different glucuronic acid concentrations. Wild-type strain A348 or *mmsB* deletion strain AB520 carrying pSW209Ω was grown in AB induction medium for 24 h with 10 μM AS, and the indicated concentration of glucuronic acid. β-Galactosidase activity was determined and reported in Miller units. Samples were assayed in triplicate, and results are plotted as means with SDs.

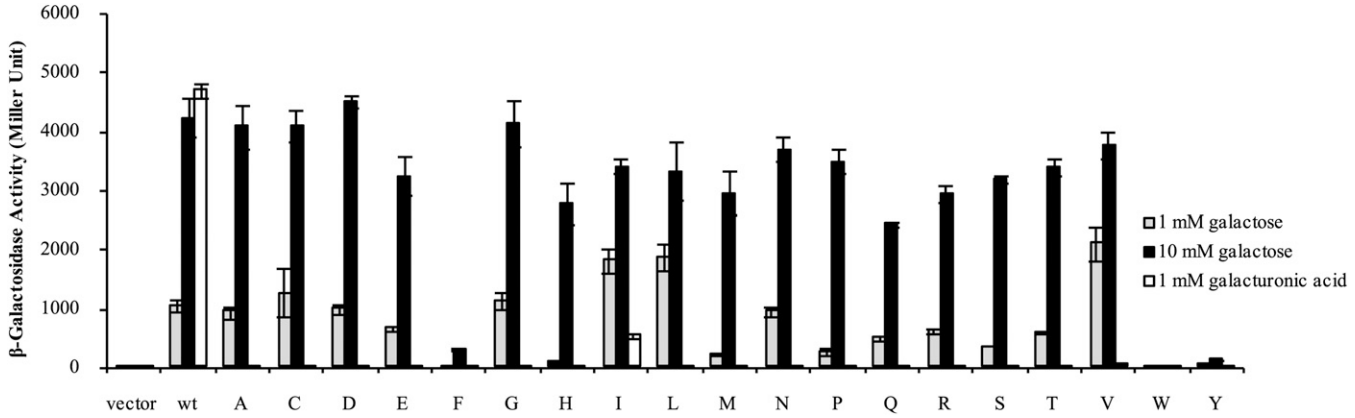

Fig. S6. β-Galactosidase assays of K262 random mutants in response to different sugars. Effects of mutation on induction of P*virB-lacZ* in the presence of different sugars. The sugar concentration was used as 1 mM galactose, 10 mM galactose, and 1 mM galacturonic acid. Glycerol (0.25%) was also added. Samples were assayed in triplicate, and results are plotted as means with SDs.

Table S1. Isothermal calorimetry (ITC) experiments of ChvE with galactose and glucuronic acid at different pHs

Protein	Sugar	pH	*N*	K_a, M^{-1}	ΔH, cal/mol	ΔS, cal·mol^{-1}·deg^{-1}	K_d, M
ChvE	Galactose	4.6	0.9	2.97E+06	−1.57E+04	−23.1	3.37E-07
ChvE	Galactose	5.5	0.8	3.49E+06	−1.66E+04	−25.7	2.87E-07
ChvE	Galactose	6.5	0.9	7.07E+06	−1.62E+04	−22.8	1.41E-07
ChvE	Galactose	7.5	0.8	7.94E+06	−1.95E+04	−33.9	1.26E-07
ChvE	Glucuronic	4.6	0.9	1.48E+05	−1.4E+04	−23.9	6.76E-06
ChvE	Glucuronic	5.5	0.9	3.77E+04	−1.1E+04	−16.2	2.65E-05
ChvE	Glucuronic	6.5	1.0	2.93E+03	−7.5E+03	−9.27	3.41E-04
ChvE	Glucuronic	7.5	1.0	7.54E+02	−7.0E+03	−10.3	1.33E-03

Themodynamics of binding of galactose and glucuronic acid to ChvE as a function of pH as determined by ITC. The buffer conditions for these experiments are as follows: (*i*) 25 mM NaAc, 50 mM NaCl, pH 4.6; (*ii*) 25 mM Mes, 50 mM NaCl, pH 5.5; (*iii*) 25 mM Mes, 50 mM NaCl, pH 6.5; and (*iv*) 25 mM phosphate buffer, 50 mM NaCl, pH 7.5.

Table S2. Thermodynamics of binding of various sugars to ChvE at pH 5.5

Protein	Sugar	pH	*N*	K_a, M^{-1}	ΔH, cal/mol	ΔS, cal·mol^{-1}·deg^{-1}	K_d, M
ChvE	Galactose	5.5	1.2	2.99E+06	−1.77E+04	−29.6	3.34E-07
ChvE	Arabinose	5.5	1.1	1.69E+06	−1.86E+04	−33.7	5.92E-07
ChvE	Glucose	5.5	0.9	1.39E+06	−2.10E+04	−42.7	7.19E-07
ChvE	Glucuronic	5.5	1.1	3.20E+04	−1.25E+04	−21.3	3.13E-05
ChvE	Galacturonic	5.5	1.0	7.53E+03	−1.60E+04	−35.9	1.33E-04

Thermodynamics of binding of various inducing sugars to ChvE determined by ITC. The ChvE concentration was 20 μM for neutral sugars (250 μM) and 50 μM for sugar acids (1 mM) in 25 mM Mes, 50 mM NaCl, pH 5.5, 25 °C.

Table S3. X-ray diffraction data collection and refinement

	Crystal	
	ChvE with galactose	ChvE with glucuronic acid
Data collection		
Resolution range, Å	42.577–1.801	45.477–1.75
Total reflections	1,196,383	493,103
Unique reflections	57,571	62,168
Completeness	94.6% (96.9*)	94.8% (62.2*)
R_{merge}	0.082 (0.494*)	0.059 (0.543*)
Space group	P65	P65
Unit cell dimensions	$a = b$ = 130.076 Å, c = 64.676 Å, α = β = 90, γ = 120	$a = b$ = 130.488 Å, c = 63.422 Å, α = β = 90, γ = 120
Refinement		
Resolution, Å	1.80	1.75
R	0.15	0.15
R_{free}	0.19	0.19
rmsd bond length (mc), Å	0.004	0.004
rmsd bond angle (mc)	0.891	0.859
Total no. of atoms	5,729	5,893
Average B factor for all atoms, Å^2	31.74	26.87

*Indicates the highest resolution shell.

Table S4. Tumor formation on tobacco leaf explants in response to *A. tumefaciens* strains carrying mutant *chvE* alleles

Strain AB300 (Δ*chvE*) plus	Tumors/leaf explant*
Experiment 1	
Vector (pBBR5)	0.4 ± 0.1
chvE	5.2 ± 0.5
$chvE^{R17A}$	4.2 ± 0.7
$chvE^{K262L}$	5.3 ± 0.8
Experiment 2	
Vector (pBBR5)	0.2 ± 0.1
chvE	7.2 ± 1.3
$chvE^{K262S}$	2.0 ± 0.4

*Shown are ±SE (n = 12).