Xylella fastidiosa Endoglucanases Mediate the Rate of Pierce's Disease Development in *Vitis vinifera* in a Cultivar-Dependent Manner

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Accepted 14 June 2019.

Xylella fastidiosa is a gram-negative bacterium that causes Pierce's disease (PD) in grapevine. *X. fastidiosa* is xylem-limited and interfaces primarily with pit membranes (PMs) that separate xylem vessels from one another and from adjacent xylem parenchyma cells. PMs are composed of both pectic and cellulosic substrates, and dissolution of PMs is facilitated by *X. fastidiosa* cell wall-degrading enzymes. A polygalacturonase, which hydrolyzes the pectin component of PMs, is required for both movement and pathogenicity in grapevines. Here, we demonstrate that two *X. fastidiosa* β -1,4-endoglucanases (EGases), EngXCA1 and EngXCA2, also play a role in how *X. fastidiosa* interfaces with grapevine PMs. The loss of EngXCA1 and EngXCA2 in tandem reduces both *X. fastidiosa* virulence and population size and slows the rate of PD symptom development and progression. Moreover, we demonstrate that single and double EGases mutants alter the rate of PD progression differently in two grapevine cultivars, Cabernet Sauvignon and Chardonnay, and that Chardonnay is significantly more susceptible to PD than Cabernet Sauvignon. Interestingly, we determined that there are quantitative differences in the amount of fucosylated xyloglucans that make up the surface of PMs in these cultivars. Fucosylated xyloglucans are targets of the *X. fastidiosa* EGases, and xyloglucan abundance could impact PM dissolution and affect PD symptom development. Taken together, these results indicate that *X. fastidiosa* EGases and the PM carbohydrate composition of different grape cultivars are important factors that influence PD symptom development and progression.

Keywords: cell wall-degrading enzymes, grapevine cultivar, pit membrane, xylem

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Funding: This work was supported by a grant awarded to C. Roper from the California Department of Food and Agriculture Pierce's disease and Glassy-Winged Sharpshooter Board (grant number 16-0510-SA) and the United States Department of Agriculture National Institute of Food and Agriculture Hatch Project 1002710.

*The e-Xtra logo stands for "electronic extra" and indicates that three supplementary figures and six supplementary tables are published online.

The author(s) declare no conflict of interest.

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Xylella fastidiosa is a gram-negative, xylem-limited bacterium that causes disease in many economically important crops across several continents. *X. fastidiosa* strains have been assigned to different subspecies that generally align with host specificity (Almeida and Nunney 2015; Schuenzel et al. 2005). In the United States, *X. fastidiosa* subsp. *fastidiosa* is the implicated causal agent of Pierce's disease (PD) of grapevine. In Central and South America, the citrus and coffee industries have been severely affected by *X. fastidiosa* subsp. *pauca*, which causes citrus variegated chlorosis and coffee leaf scorch, respectively (Chang et al. 1993; Li et al. 2001). Recently, *X. fastidiosa* subsp. *pauca* has been implicated as the causal agent of olive quick decline syndrome in southern Italy (Saponari et al. 2013) and is one of the first reports of *X. fastidiosa* in Europe. Symptoms of PD include leaf scorching, irregular lignification (green islands) and matchstick petioles, followed by fruit desiccation, cordon dieback, and vine death (Goheen and Hopkins 1988; Purcell 1986; Stevenson et al. 2005; Varela 1996). *X. fastidiosa* resides exclusively within the xylem of plant hosts, with symptoms similar to, but not exactly like, water stress imparted by xylem disruption (McElrone et al. 2001 and 2003; Thorne et al. 2006a). *X. fastidiosa* is transmitted by xylem-feeding hemipteran leafhopper insects, including *Graphocephala atropunctata*, the blue-green sharpshooter, and *Homalodisca vitripennis*, the glassy-winged sharpshooter (Hill and Purcell 1995).

PD symptom severity is highly correlated with the ability of the bacterium to proliferate within the xylem and systemically colonize the grapevine host (Chatterjee et al. 2008a; Sun et al. 2011). *X. fastidiosa* is strictly limited to the xylem tissue, a nutrient-poor niche with few available carbon sources dissolved as solutes in the xylem sap or the primary cell wall material that comprises pit membranes (PMs). The xylem in grapevines is built of many interconnected vessels that are finite, but variable in length, and vessels rarely span the entire length of the vine (Thorne et al. 2006b; Tyree and Zimmermann 2002). Xylem vessels are connected to each other by scalariform-bordered pits that contain PMs (Brett and Waldron 1996). PMs are porous primary cell wall-middle lamella structures that allow water and small solutes to pass between vessels but also serve to prevent the movement of disruptive elements such as air embolisms or pathogens (Stevenson et al. 2004; Tyree and Zimmermann 2002). *X. fastidiosa* must traverse PMs to move systemically within the grapevine xylem. However, grapevine PM pores have a diameter of 5 to 20 nm, and the rod-shaped *X. fastidiosa* cells, having a width of 250 to 500 nm and length of 1,000 to 4,000 nm, are too large to passively move through

these small pores (Buchanan et al. 2000; Choat et al. 2004; Mollenhauer and Hopkins 1974). Grapevine PMs are composed of cellulose microfibrils, xyloglucan (hemicellulose), and pectin (Brett and Waldron 1996; Dickson 2007). These polysaccharides can serve as the substrates of cell wall-degrading enzymes (CWDEs) produced by pathogenic bacteria and fungi as these invading pathogens navigate and proliferate within the xylem (Bateman 1976; Hématy et al. 2009; Popeijus et al. 2000; Ward et al. 1989). The *X. fastidiosa* genome encodes several genes annotated as CWDEs, including one polygalacturonase (PG), three β -1,4-endoglucanases (EC 3.2.1.4) (EGases), one cellobiohydrolase, and one xylanase (Simpson et al. 2000; Van Sluys et al. 2003). PGs facilitate the hydrolytic cleavage of pectin, and an *X. fastidiosa* mutant lacking one PG (PglA) failed to move beyond the point of inoculation and did not induce PD symptoms, indicating that the PG is required for systemic colonization (Roper et al. 2007).

EGases are hydrolytic enzymes that cleave β -1,4 glycosidic linkages, such as those found in cellulose, and the cross-linking glycans (hemicellulose), which are both constituents of the vascular plant cell wall (Buchanan et al. 2000; Larner 1960). Of the three annotated *X. fastidiosa* EGases, EngXCA2 (PD1851) is the only prototypical EGase composed of an N-terminal glycosyl hydrolase domain and a C-terminal carbohydrate binding module. *X. fastidiosa* EngXCA2 hydrolyzes cellulosic substrates, such as carboxymethylcellulose (CMC) and xyloglucan (Pérez-Donoso et al. 2010). EngXCA1 (PD1856) has a predicted N-terminal glycosyl hydrolase domain homologous to that of EngXCA2 and a predicted C-terminal expansin domain. The expansin domain is notable because expansins are considered to be plant proteins. Expansins are nonenzymatic and bind to cellulose and hemicellulose to facilitate cell wall expansion during plant cell growth and likely were acquired from plants via cross-kingdom horizontal gene transfer (Cosgrove 2000; Nikolaidis et al. 2014). Interestingly, with a few exceptions, expansins are found in genera of bacteria that dwell in plant xylem tissue, such as *Bacillus*, *Clavibacter*, *Ralstonia*, *Xanthomonas*, *Pectobacterium*, and *Dickeya*, suggesting that expansins are advantageous for bacteria inhabiting the xylem (Georgelis et al. 2014; Kerff et al. 2008; Tancos et al. 2018; Tovar-Herrera et al. 2018). The third EGase, Egl (PD2061), has a predicted N-terminal glycosyl hydrolase domain but does not have domains related to carbohydrate binding.

EGases are important enzymatic virulence factors for several bacterial phytopathogens. For necrotrophic bacteria, such as *Pectobacterium* (*Erwinia*) *carotovorum*, EGases contribute to virulence and nutrient acquisition by inducing soft rot (Barras et al. 1994; Walker et al. 1994). For bacteria with a significant xylem-dwelling phase, such as *Pantoea stewartii* subsp. *stewartii*, *Ralstonia solanacearum*, and *Xanthomonas campestris* pv. *campestris*, EGases play an important role in virulence and host colonization (Gough 1988; Mohammadi et al. 2012; Roberts et al. 1988; Saile et al. 1997). Moreover, it has been suggested that xylem-dwelling bacteria use EGases to degrade PMs as a means of acquiring nutrients (Fatima and Senthil-Kumar 2015; Pérez-Donoso et al. 2010; Pieretti et al. 2012).

The mechanisms by which *X. fastidiosa* acquires carbon in the xylem have not been fully elucidated, and it is unclear whether carbon released as a consequence of PM degradation is utilized by the bacterium as a nutrient source. In addition, differences in PD severity and symptom progression rate have been noted in different cultivars of *Vitis vinifera*, but little is known about the host physiological properties or bacterial molecular mechanisms underlying these observations. This study was structured to examine host PM composition as a factor in cultivar susceptibility, as well as to test the role of

X. fastidiosa EGases in plant colonization and disease symptom development. This work demonstrates that there are quantitative differences in the hemicellulosic component of PMs, namely the fucosylated xyloglucans, of two cultivars, Chardonnay and Cabernet Sauvignon, and that Chardonnay develops PD at a significantly faster rate than Cabernet Sauvignon. Furthermore, the virulence of an *X. fastidiosa* mutant lacking two of the EGases is severely impacted in both cultivars, but significantly more so in Cabernet Sauvignon. These results support our hypothesis that the composition of the hemicellulosic content of xylem PMs affects the rate of PD development and is one of the determinants of PD severity among grapevine cultivars. Collectively, two *X. fastidiosa* EGases (EngXCA1 and EngXCA2) also support bacterial growth in the xylem, suggesting EGases play a major role in nutrient acquisition in the xylem environment.

RESULTS

Grapevine cultivar is a factor in rate and severity of PD symptom development.

PD symptoms were monitored weekly for 20 weeks post-inoculation (WPI), and observed PD symptom scores were used to generate a generalized estimating equations (GEE) model to compare PD symptom development in Chardonnay and Cabernet Sauvignon. From the GEE model, PD symptom development was divided into stages: early-stage PD (5 WPI), mid-stage PD (10 WPI), late-stage PD (15 WPI), and the endpoint of the experiment (20 WPI). Wild type-inoculated Chardonnay vines exhibited significantly higher PD symptom scores than wild type-inoculated Cabernet Sauvignon vines at early-, mid-, and late-stage PD and at the endpoint of the experiment (Fig. 1A). To further understand the rate of disease manifestation, the GEE model was used to assess differences in the rate of PD symptom development in Cabernet Sauvignon and Chardonnay by calculating the change in PD symptom score over time between early-stage PD and late-stage PD (5 to 15 WPI) where symptom progression was linear (PD symptom progression rate). The PD symptom progression rate was significantly faster in Chardonnay than in Cabernet Sauvignon between early- and mid-stage PD (5 to 10 WPI), and between mid- and late-stage PD (10 to 15 WPI) (Table 1). However, wild-type titers at the point of inoculation (POI) and at 20 nodes distal to the POI were statistically similar in Chardonnay and Cabernet Sauvignon at the late stage of infection (Fig. 1B), indicating that symptom severity is decoupled from bacterial titer at this stage of disease. These trends were also seen in vines inoculated with Δ engXCA1, Δ engXCA2, or Δ engXCA1/2 (Table 1; Supplementary Fig. S1). All *P* values, estimated mean differences, and statistical significance values are summarized in Supplementary Tables S1 and S2.

The fucosylated xyloglucan component of xylem PMs differs between cultivars.

Immunogold-scanning electron microscopy (SEM) was used to detect fucosylated xyloglucans in the surface layer of inter-vessel PMs in Cabernet Sauvignon and Chardonnay grapevines to determine whether there were quantitative differences between the two cultivars with regard to the fucosylated xyloglucan component of PMs. After immunogold labeling, relatively few gold particles were bound to Cabernet Sauvignon PMs (Fig. 1C), indicating that the surface layer is sparsely populated with fucosylated xyloglucans. Conversely, many gold particles were detected for Chardonnay PMs (Fig. 1D), indicating that the surface layer is densely populated with fucosylated xyloglucans.

EGases are required for full virulence and affect rate of PD symptom development in a cultivar-dependent manner.

PD symptoms were monitored weekly over a 20-week period, and observed PD symptom scores were used to generate a GEE model to compare PD symptom development at early-, mid-, and late-stage PD in wild type and EGases mutant-inoculated vines (Supplementary Fig. S2). Subsequently, to better understand when EGases play the largest role, the GEE model was used to analyze the PD symptom progression rate between early- and late-stage PD (Tables 2 and 3). Cabernet Sauvignon vines inoculated with the $\Delta engXCA1/2$ double mutant had significantly lower PD symptom scores than wild type-inoculated vines during all stages of PD and at the 20-week

endpoint (Fig. 2A). Not only were symptoms less severe overall, but the PD symptom progression rate was significantly slower in $\Delta engXCA1/2$ -inoculated vines than in wild type-inoculated vines between early- and late-stage PD (Table 2), indicating that disease manifests more slowly in Cabernet Sauvignon vines inoculated with $\Delta engXCA1/2$.

To better understand how individual EGases contribute to disease, we also tested single $\Delta engXCA1$ and $\Delta engXCA2$ mutants in planta. In Cabernet Sauvignon, $\Delta engXCA1$ -inoculated vines had significantly lower disease ratings than wild type-inoculated vines during early-stage PD, but there were no differences in PD symptom scores during mid- and late-stage PD or at the endpoint (Fig. 2B). The PD symptom progression rate was not statistically different between $\Delta engXCA1$ -inoculated

Fig. 1. Grapevine cultivar affects Pierce's disease (PD) symptom development and is mediated by differences in pit membrane (PM) composition. **A**, Overall PD symptom severity is greater in cv. Chardonnay (green) than in cv. Cabernet Sauvignon (red). A generalized estimating equations model was used to determine the estimated mean PD symptom scores per week (dashed lines) and to calculate 95% confidence intervals (solid lines). **B**, *Xylella fastidiosa* titers at the point of inoculation (POI) and at 20 nodes distal to the POI are not significantly different between Cabernet Sauvignon and Chardonnay. Colony forming units were normalized per gram of petiole tissue and transformed using the natural log (ln). Statistical comparisons were made using a generalized negative binomial regression model. Error bars represent the standard error of the mean. **C**, Intervessel PMs of phosphate buffered saline (PBS)-inoculated Chardonnay have a dense distribution of silver-enhanced particles, indicating an abundance of fucosylated xyloglucans. **D**, Intervessel PMs of PBS-inoculated Cabernet Sauvignon have a moderate amount of silver-enhanced particles, indicating the presence of some fucosylated xyloglucans. Fucosylated xyloglucans were detected using the CCRC-M1 antibody, and intervessel PMs were imaged using immunogold-scanning electron microscopy. Scale bars are equal to 5 μ m.

vines and wild type-inoculated vines between early- and late-stage PD, indicating that although $\Delta engXCA1$ initially elicits fewer symptoms in planta during the early stage, PD develops at the same rate as in wild type-inoculated vines, with symptom severity eventually equaling wild-type levels (Fig. 2B; Table 2). $\Delta engXCA2$ -inoculated vines had significantly higher PD symptom scores than wild type-inoculated vines during all stages of PD and the endpoint, and the PD symptom progression rate was faster in $\Delta engXCA2$ -inoculated vines than in wild type-inoculated vines between early- and late-stage PD (Fig. 2C; Table 2).

Similar to what was observed in Cabernet Sauvignon, Chardonnay vines inoculated with the $\Delta engXCA1/2$ double mutant had significantly lower PD symptom scores than wild type-inoculated vines during all stages of PD (Fig. 2D), and the PD symptom progression rate in $\Delta engXCA1/2$ -inoculated vines was significantly slower than in wild type-inoculated vines between early- and mid-stage PD (Table 3). However, there was a notable difference in the rate at which PD symptoms developed in $\Delta engXCA1/2$ -inoculated Chardonnay compared with Cabernet Sauvignon. A continually lower rate of PD symptom progression was observed between early- and

Table 1. Comparison of the rate of Pierce's disease (PD) symptom progression in cv. Cabernet Sauvignon and cv. Chardonnay

Strain	PD stage interval ^a							
	Early stage to mid stage				Mid stage to late stage			
	Rate		Comparison		Rate		Comparison	
	Cabernet Sauvignon ^b	Chardonnay ^c	Rate difference ^d	P Value ^e	Cabernet Sauvignon ^b	Chardonnay ^c	Rate difference ^d	P Value ^e
Wild type	0.25	0.39	-0.14 ± 0.02	<0.0001 ***	0.27	0.32	-0.05 ± 0.02	0.0139 *
$\Delta engXCA1$	0.23	0.35	-0.12 ± 0.04	0.0013 **	0.28	0.40	-0.12 ± 0.04	0.0016 **
$\Delta engXCA2$	0.32	0.39	-0.07 ± 0.03	0.0087 **	0.34	0.39	-0.05 ± 0.03	0.0525 ns
$\Delta engXCA1/2$	0.02	0.17	-0.15 ± 0.02	<0.0001 ***	0.07	0.30	-0.23 ± 0.03	<0.0001 ***

^a Rates of PD symptom progression between early and mid stages of PD (5 to 10 weeks postinoculation) and between mid and late stages of PD (10 to 15 weeks postinoculation).

^b Estimated rate of PD symptom progression generated from the generalized estimated equations (GEE) model for each strain in Cabernet Sauvignon.

^c Estimated rate of PD symptom progression generated from the GEE model for each strain in Chardonnay.

^d Differences in the estimated rate of PD symptom progression (with standard error) in Cabernet Sauvignon and Chardonnay; negative values indicate that PD progresses slower in Cabernet Sauvignon than in Chardonnay, and positive values indicate that PD progresses faster in Cabernet Sauvignon than in Chardonnay.

^e P values of the estimated rate differences were generated using 95% confidence intervals in the GEE model: ns = no significance, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

Table 2. Comparison of the rate of Pierce's disease (PD) symptom progression for wild type and mutant strains in cv. Cabernet Sauvignon

Strain	PD stage interval ^a					
	Early stage to mid stage			Mid stage to late stage		
	Rate ^b	Rate difference ^c	P Value ^d	Rate ^b	Rate difference ^c	P Value ^d
Wild type	0.25	N/A	N/A	0.27	N/A	N/A
$\Delta engXCA1$	0.23	-0.02 ± 0.03	0.54 ns	0.28	0.01 ± 0.03	0.64 ns
$\Delta engXCA2$	0.32	0.06 ± 0.21	0.023 *	0.34	0.07 ± 0.03	0.0053 **
$\Delta engXCA1/2$	0.02	-0.23 ± 0.02	<0.0001 ***	0.07	-0.20 ± 0.02	<0.0001 ***

^a Rates of PD symptom progression between early and mid stages of PD (5 to 10 weeks postinoculation) and between mid and late stages of PD (10 to 15 weeks postinoculation).

^b Estimated rate of PD symptom progression generated from the generalized estimated equations (GEE) model for each strain.

^c Differences in the estimated rate of PD symptom progression (with standard error) for each mutant strain compared with wild type; negative values indicate rates for mutant strains are slower than wild type, and positive values indicate rates for mutant strains are faster than wild type.

^d P values of the estimated rate differences were generated using 95% confidence intervals in the GEE model: ns = no significance, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

Table 3. Comparison of the rate of Pierce's disease (PD) symptom progression for wild type and mutant strains in cv. Chardonnay

Strain	PD stage interval ^a					
	Early stage to mid stage			Mid stage to late stage		
	Rate ^b	Rate difference ^c	P Value ^d	Rate ^b	Rate difference ^c	P Value ^d
Wild type	0.39	N/A	N/A	0.32	N/A	N/A
$\Delta engXCA1$	0.35	-0.04 ± 0.03	0.0735 ns	0.40	0.08 ± 0.03	0.0023 **
$\Delta engXCA2$	0.39	0.00 ± 0.02	0.85 ns	0.39	0.07 ± 0.02	0.008 **
$\Delta engXCA1/2$	0.17	-0.22 ± 0.03	<0.0001 ***	0.30	-0.02 ± 0.03	0.46 ns

^a Rates of PD symptom progression between early and mid stages of PD (5 to 10 weeks postinoculation) and between mid and late stages of PD (10 to 15 weeks postinoculation).

^b Estimated rate of PD symptom progression generated from the generalized estimated equations (GEE) model for each strain.

^c Differences in the estimated rate of PD symptom progression (with standard error) for each mutant strain compared with wild type; negative values indicate rates for mutant strains are slower than wild type, and positive values indicate rates for mutant strains are faster than wild type.

^d P values of the estimated rate differences were generated using 95% confidence intervals in the GEE model: ns = no significance, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

Fig. 2. *Xylella fastidiosa* endoglucanases affect Pierce's disease (PD) severity and the rate of PD symptom development in a cultivar-dependent manner. Estimated mean PD symptom scores generated by the generalized estimating equations model for $\Delta engXCA1$ (yellow), $\Delta engXCA2$ (red), and $\Delta engXCA1/2$ (green) were compared with wild type (blue) in cv. Cabernet Sauvignon and in cv. Chardonnay at early, mid, and late stages of PD. **A**, $\Delta engXCA1/2$ induces milder PD symptoms than wild type in Cabernet Sauvignon. **B**, $\Delta engXCA1$ and wild type induce similar PD symptoms in Cabernet Sauvignon. **C**, $\Delta engXCA2$ induces more severe PD symptoms than wild type in Cabernet Sauvignon. **D**, $\Delta engXCA1/2$ induces milder PD symptoms than wild type in Chardonnay but to a lesser degree than in Cabernet Sauvignon. **E**, $\Delta engXCA1$ induces milder symptoms than wild type at the early and mid stages of PD but have symptom severity similar to wild type at the late stage of PD. **F**, $\Delta engXCA2$ and wild type induce similar symptoms at the early and mid stages of PD but induce more severe symptoms than wild type at the late stage of PD. Estimated mean PD symptom scores per week are represented by dashed lines, and 95% confidence intervals are represented by solid lines.

late-stage PD in Cabernet Sauvignon vines inoculated with $\Delta\text{engXCA1/2}$ compared with wild type-inoculated vines, whereas, in Chardonnay there was no difference in the rate of PD symptom development between mid- and late-stage PD (Tables 2 and 3). These data taken together indicate that *X. fastidiosa* virulence is dependent on EngXCA1 and EngXCA2 in both Chardonnay and Cabernet Sauvignon, but the rate at which PD symptoms develop in the absence of EngXCA1 and EngXCA2 is cultivar dependent.

The $\Delta\text{engXCA1}$ single mutant also behaved differently in Chardonnay compared with Cabernet Sauvignon. $\Delta\text{engXCA1}$ -inoculated Chardonnay vines only displayed PD symptom scores similar to wild type-inoculated vines at late-stage PD, whereas PD symptom scores in $\Delta\text{engXCA1}$ -inoculated Cabernet Sauvignon vines were similar to wild type-inoculated vines at mid- and late-stage PD (Fig. 2B and E). Furthermore, the PD symptom progression rate for $\Delta\text{engXCA1}$ -inoculated Chardonnay vines was significantly faster between mid- and late-stage PD compared with wild type-inoculated vines (Table 3), whereas the PD symptom progression rate for $\Delta\text{engXCA1}$ -inoculated Cabernet Sauvignon vines was consistently similar to wild type-inoculated vines (Table 2).

$\Delta\text{engXCA2}$ -inoculated Chardonnay vines had PD symptom scores that were similar to those for wild type-inoculated vines during early- and mid-stage PD, but the $\Delta\text{engXCA2}$ mutant elicited significantly higher PD symptom scores than wild-type *X. fastidiosa* during late-stage PD and at the endpoint (Fig. 2F), which differed from its behavior in Cabernet Sauvignon, in which it was hypervirulent at all stages of PD (Fig. 2C). Moreover, PD symptoms in Chardonnay progressed at a similar rate between early- and mid-stage PD in $\Delta\text{engXCA2}$ - and wild type-inoculated vines, but progressed faster between mid- and late-stage PD in $\Delta\text{engXCA2}$ -inoculated vines, whereas in Cabernet Sauvignon PD symptoms progressed faster in $\Delta\text{engXCA2}$ -inoculated vines at all stages of PD (Tables 2 and 3).

In all trials, the disease ratings for phosphate buffered saline (PBS)-inoculated vines (negative controls) for each week were zero. All *P* values, estimated mean differences, and statistical significance values are summarized in Supplementary Tables S3 and S4.

EngXCA1 and EngXCA2 are required for complete PM degradation.

SEM was used to analyze the structural integrity of PMs in both Cabernet Sauvignon and Chardonnay vines inoculated with either wild type or $\Delta\text{engXCA1/2}$ during late-stage PD. At this stage of PD, the PMs of vines inoculated with wild type were completely dismantled in both cultivars (Fig. 3A and C). In contrast, the PMs of vines inoculated with $\Delta\text{engXCA1/2}$ were still present and were only partially degraded in both cultivars (Fig. 3B and D). The PMs of the PBS-inoculated control vines for both cultivars remained fully intact (Fig. 1C and D).

Bacterial colonization of the xylem is EngXCA1/EngXCA2-dependent.

In both Cabernet Sauvignon and Chardonnay, $\Delta\text{engXCA1/2}$ titers were significantly lower than wild-type titers at the POI and at 20 nodes distal to the POI, indicating that both EngXCA1 and EngXCA2 are concomitantly required to achieve maximal xylem colonization at both points. $\Delta\text{engXCA1}$ and $\Delta\text{engXCA2}$ single mutant titers at the POI and at 20 nodes distal to the POI were statistically similar to wild-type titers in both cultivars, indicating that deletion of EngXCA1 or EngXCA2 in singlet did not have a quantifiable impact on the ability of *X. fastidiosa* to proliferate at the POI or to move away from the POI during the late phase of infection (Fig. 4). In all trials, bacterial extractions from PBS-inoculated vines

(negative controls) did not yield any *X. fastidiosa* colonies. All *P* values, estimated mean differences, and statistical significance values are summarized in Supplementary Tables S5 and S6.

EngXCA1 has a marginal amount of EGase activity compared with EngXCA2.

Initial attempts to measure EGases activity from *X. fastidiosa* itself were unsuccessful; thus, recombinant expression was used to characterize the activities of EngXCA1 and EngXCA2. Previous studies indicated that recombinant EngXCA2 expressed in *Escherichia coli* possesses robust EGase activity in vitro when using both CMC and xyloglucan as substrates (Pérez-Donoso et al. 2010). A radial diffusion assay was used to determine the zones of CMC hydrolysis for recombinant EngXCA1 using differential staining with Congo red (Fig. 5A). EngXCA2 was included as a positive EGase control, and *E. coli* harboring the empty pET20b(+) plasmid vector served as a negative control. The area of the zone of hydrolysis for EngXCA1 was determined to be significantly different from that of the pET20b(+) empty vector control (*P* = 0.02217) and from that of EngXCA2 (*P* = 0.01881) (Fig. 5B). Additionally, the area of the zone of hydrolysis for EngXCA2 was determined to be significantly different from the pET20b(+) (negative controls) did not yield any *X. fastidiosa* colonies. All *P* values, estimated mean differences, and statistical significance values are summarized in Supplementary Tables S5 and S6.

Fig. 3. The $\Delta\text{engXCA1/2}$ double mutant is impaired in pit membrane (PM) dissolution. The integrity of intervessel PMs in cv. Chardonnay (A and B) and cv. Cabernet Sauvignon (C and D) grapevines inoculated with wild type (A and C) and $\Delta\text{engXCA1/2}$ (B and D) during late-stage infection was analyzed using immunogold-scanning electron microscopy. A and C, All of the intervessel PMs are completely degraded. B and D, Intervessel PMs are still in place but display partial degradation. Scale bar in each panel equals to 5 μm .

empty vector control ($P < 0.0001$). Western blotting confirmed the expression of recombinant EngXCA1 and EngXCA2, as well as the absence of expressed protein from the pET20b(+) empty vector control (Supplementary Fig. S3).

DISCUSSION

Xyloglucans are the most abundant portion of hemicellulose in dicotyledonous plants and can make up as much as 20% of the dry weight of the primary plant cell wall (Buchanan et al. 2000; Scheller and Ulvskov 2010). Xyloglucans are composed of 1,4-linked β -D-pyranosyl glucan backbone substituted with 1,6-linked xylose side chains. EGases can digest xyloglucans by cleaving linkages in the glucan backbone, and the *X. fastidiosa* EngXCA2 protein has shown EGase activity when using xyloglucan as a target substrate (Pérez-Donoso et al. 2010). The *X. fastidiosa* EGase mutants tested in this study, particularly the $\Delta\text{engXCA1/2}$ mutant, affected the rate of PD symptom progression in a cultivar-dependent manner, indicating that enzymatic targets of the EGases are a determining factor in the observed differences in cultivar susceptibility. Because the PM surface is a major interface between pathogen and host, we explored the content of potential

hemicellulosic target substrates of *X. fastidiosa* EGases in the xylem PMs of these two cultivars. Indeed, quantitative analyses of the PM surface layer of the highly susceptible Chardonnay cultivar and the more tolerant Cabernet Sauvignon cultivar indicate that Chardonnay PM surfaces contain a higher abundance of fucosylated xyloglucans than the PM surfaces in Cabernet Sauvignon, which contained a relatively low abundance of fucosylated xyloglucans. The rate of PD symptom progression and the overall PD symptom scores were significantly different between Chardonnay and Cabernet Sauvignon and quantitatively confirmed field observations that Chardonnay is significantly more susceptible to PD than is Cabernet Sauvignon.

Interestingly, Sun et al. (2011) determined that the pectin component of Chardonnay PMs is almost exclusively weakly methyl-esterified. PGs are more effective at hydrolyzing pectin with low degrees of methyl esterification, and the effectiveness of PGs in hydrolyzing pectin is inversely proportional to the degree of methyl-esterification (Lionetti et al. 2012). Our data combined with the Sun et al. (2011) study indicate that Chardonnay PMs offer more available target substrates for *X. fastidiosa* CWDEs in the form of weakly methyl-esterified pectin and high quantities of fucosylated xyloglucans that are readily digested by *X. fastidiosa*. It is tempting to speculate that Cabernet Sauvignon PMs contain pectin with high levels of methyl-esterification and that this feature, coupled with low levels of fucosylated xyloglucans, leads to less efficient PM dissolution in Cabernet Sauvignon and could explain why this cultivar is significantly less susceptible to PD compared with Chardonnay. Scanning electron micrographs of PMs in vines inoculated with the $\Delta\text{engXCA1/2}$ mutant indicate that this mutant does not rupture PMs as readily as its wild-type parent, supporting our hypothesis that EGases target the hemicellulosic

Fig. 4. *Xylella fastidiosa* colonization is dependent on both EngXCA1 and EngXCA2. Colonization at the point of inoculation (POI) and at 20 nodes distal to the POI in **A**, cv. Cabernet Sauvignon (black), and **B**, cv. Chardonnay (white), by $\Delta\text{engXCA1}$ (triangle) or $\Delta\text{engXCA2}$ (square) is similar to wild type (circle), whereas colonization by $\Delta\text{engXCA1/2}$ (diamond) at both locations is significantly reduced. Colony forming units were normalized per gram of petiole tissue and transformed using the natural log (ln). Statistical comparisons were made using a generalized negative binomial regression model. Error bars represent the standard error of the mean.

Fig. 5. EngXCA1 has marginal endoglucanase activity. **A**, A radial diffusion assay shows that recombinant EngXCA1 is able to hydrolyze carboxymethylcellulose (CMC) but much less than the previously characterized *Xylella fastidiosa* endoglucanase, EngXCA2. **B**, Recombinant EngXCA1 hydrolyzes significantly more CMC than the empty vector control but significantly less than EngXCA2. Trials consisted of three biological replicates, each consisting of three technical replicates per treatment. Boxplot whiskers represent the minimum and maximum values per treatment, and black dots represent outliers. Letters indicate statistical significance.

content of PMs. Taken together, these data support previous findings that PG is the major enzymatic determinant for movement in grapevine xylem and that EGases are accessory enzymes that facilitate PM dissolution (Roper et al. 2007).

The bacterial titer at the POI for each mutant strain was similar to the respective bacterial titer 20 nodes distal to the POI, indicating that none of the EGase mutants were hindered in movement. However, these assays were performed during the late stage of PD, when the vines had a PD symptom score of ≥ 3 (all leaves have some scorching with a few matchstick petioles), which was at the end of the linear portion of the PD symptom curve. It is possible that movement could be delayed for certain EGase mutants during the earlier phases of infection and that the timing of the colonization assay did not capture this delay. Several attempts to isolate *X. fastidiosa* from grapevines at earlier time points were not successful. Our own previous work (Clifford et al. 2013; Wang et al. 2017) and work performed by other research groups indicates that *X. fastidiosa* cannot be consistently quantified in grapevines with PD symptom scores < 3 (Deyett et al. 2019).

Soluble carbohydrates in xylem sap are in relatively low abundance, and the carbohydrate composition of xylem sap fluctuates over the course of the growing season (Roper 2006). Thus, soluble carbohydrates are likely to be an inconsistent source of carbon for a microorganism living in the xylem (Andersen and Brodbeck 1989; Hardy and Possingham 1969; Loeschner et al. 1990). The carbohydrate content of PMs may serve as a reliable and sustainable source of carbon that *X. fastidiosa* utilizes to augment any carbon obtained from xylem sap. Neither $\Delta\text{engXCA1}$ nor $\Delta\text{engXCA2}$ were impaired in population growth compared with wild type in both cultivars, but proliferation of the $\Delta\text{engXCA1/2}$ mutant was significantly impaired within the xylem, supporting the hypothesis that these two EGases play a collective role in harvesting carbon in the xylem environment. However, despite this impairment, the $\Delta\text{engXCA1/2}$ mutant population did increase, indicating that the PG may also have a role in carbon acquisition. It is possible that the different roles of these EGases allow them to act synergistically to degrade PMs and harvest carbon. However, our data suggest that these EGases act in a redundant manner, in which one can compensate for the loss of the other.

In accordance with this carbon acquisition hypothesis, we expected that there would be a significant difference in bacterial titer in Chardonnay and Cabernet Sauvignon, with the less susceptible variety harboring less *X. fastidiosa*. However, both cultivars supported similar populations of bacteria at the POI and at 20 nodes distal to the POI. As mentioned above, this result is likely due to the inability to quantify *X. fastidiosa* titer in grapevine tissue during the early phases of *X. fastidiosa* infection when bacterial titer is below the threshold of reliable detection. Thus, we speculate that the *X. fastidiosa* titer may be lower in the less susceptible Cabernet Sauvignon cultivar at earlier time points.

EngXCA1 is predicted to be a chimeric protein comprised of an EGase domain and an expansin domain (Cosgrove 2017). There are only a few examples of bacterial expansins, and they are almost exclusively found in bacteria that have a xylem-dwelling component of their lifestyle and have varying roles in virulence (Cosgrove 2017; Nikolaidis et al. 2014). Deletion of one expansin in *Clavibacter michiganensis* subsp. *michiganensis* resulted in a hypervirulent phenotype in tomato, whereas deletion of one expansin in *Ralstonia solanacearum* resulted in a hypovirulent phenotype for root inoculations (Tancos et al. 2018). EngXCA1 digested a β -1,4 linked glucan backbone (CMC), indicating that it possesses EGases activity. However, the zone of hydrolysis of the CMC substrate was

significantly smaller relative to a lower concentration of EngXCA2, indicating that EngXCA1 is a less potent EGase than EngXCA2 in vitro. Additionally, the loss of only EngXCA1 had a small, but significant, impact on *X. fastidiosa* virulence in the early stages of infection in Chardonnay. This leads us to speculate that the primary role of EngXCA1 is to function as an expansin rather than an EGase in vivo. Expansins disrupt the bonds between hemicellulosic xyloglucans and the hydrophobic regions of cellulose microfibrils, allowing relaxation of the cell wall and subsequent extension (Cosgrove 2018). EngXCA1 could aid the PM degradation process by acting as an expansin and disrupting the bonds between cellulose and xyloglucans, thereby providing other CWDEs, such as EngXCA2 and PG, with easier access to target substrates. This effect would be most evident in highly susceptible varieties (e.g., Chardonnay) that contain high amounts of fucosylated xyloglucans in PMs. Deletion of EngXCA1 had little effect on PD severity in Cabernet Sauvignon, in which the PMs have a lower abundance of fucosylated xyloglucans relative to Chardonnay, supporting the hypothesis that the amount of cross-linking glycans mediates PM integrity.

In an ex vivo study in grapevine stem explants, purified recombinant *X. fastidiosa* EngXCA2 was required for PM dissolution in collaboration with a purified PG. Thus, it was surprising that the *X. fastidiosa* $\Delta\text{engXCA2}$ mutant was hypervirulent and had a faster PD symptom progression rate in both Cabernet Sauvignon and Chardonnay. Hypervirulent phenotypes have been documented for mutant strains of *X. fastidiosa* that do not aggregate or form biofilms and are locked in the planktonic, exploratory state in the xylem (Burbank and Stenger 2017; Chatterjee et al. 2008b; Gouran et al. 2016; Guilhabert and Kirkpatrick 2005; Newman et al. 2004). Interestingly, the $\Delta\text{engXCA2}$ mutant had a mucoid colony morphotype on solid medium compared with wild-type *X. fastidiosa* cells that have a comparatively drier colony phenotype. Furthermore, the $\Delta\text{engXCA2}$ mutant did not aggregate in liquid cultures (*data not shown*). Overproduction or deregulation of exopolysaccharides (EPS) can have an adverse effect on the ability of bacteria to attach to surfaces and self-aggregate, both of which are hallmarks of the regimented developmental processes related to biofilm formation (Dertli et al. 2015; Koutsoudis et al. 2006; Rendueles et al. 2013). Therefore, it is likely that excessive EPS accumulation in the $\Delta\text{engXCA2}$ mutant disrupts cell-to-cell adhesion and, subsequently, early biofilm developmental processes that are known to be an important factor in attenuating *X. fastidiosa* virulence early in the xylem colonization process (Guilhabert and Kirkpatrick 2005; Rapicavoli et al. 2018), which leads to the observed hypervirulent phenotype for the $\Delta\text{engXCA2}$ mutant. The *X. fastidiosa* EPS molecule is predicted to be composed of a β -1,4 linked glucan backbone, which could be a substrate of EngXCA2. Our data indicate that EngXCA2 could play a dual role in the biology of *X. fastidiosa*, one of which is that of a traditional plant CWDE that targets primary plant cell walls and the other in facilitating the turnover of EPS. Further studies on the role of EngXCA2 in EPS processing and biofilm development are warranted.

Our findings indicate PM composition is an important component of the plant-microbe interaction between cultivated grapevines and *X. fastidiosa*. In highly susceptible cultivars, the abundance of readily accessible carbohydrates in PMs that are compatible with *X. fastidiosa* CWDEs facilitates a greater PD symptom progression rate. In contrast, PD symptom progression is slower in more tolerant varieties that have lower abundances of substrates that are compatible with *X. fastidiosa* CWDEs. Little is known about PM composition in the many plant hosts in which *X. fastidiosa* resides as a commensal

bacterium without causing disease (Freitag 1951). Our data suggest that the carbohydrate composition of PMs in tolerant grapevines may be less compatible with *X. fastidiosa* CWDEs than the PM carbohydrate composition found in susceptible hosts. Furthermore, PM carbohydrate composition may be a factor in plant-microbe interactions that determines where bacteria fall on the symbiosis spectrum of commensalism or parasitism.

MATERIALS AND METHODS

Bacterial strains.

All bacterial strains, plasmids, and primers used in this study are listed in Table 4.

Media and bacterial growth conditions.

X. fastidiosa strains were grown at 28°C in PD3 broth and solid medium (Davis et al. 1981). *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium. Selection of *X. fastidiosa* transformants was performed on PD3 with kanamycin or gentamicin (5 µg/ml), and subculturing of transformants was performed on PD3 with kanamycin (30 µg/ml) or gentamicin (10 µg/ml), when appropriate. For selection of *E. coli* transformants, kanamycin (30 µg/ml) or gentamicin (10 µg/ml) was added.

Construction of the $\Delta engXCA1$, $\Delta engXCA2$, and $\Delta engXCA1/2$ mutants.

Overlap extension PCR was used to combine 5' and 3' flanking regions adjacent to the target gene (*engXCA1* or *engXCA2*) with an antibiotic resistance gene to create a deletion construct. Homologous recombination between flanking regions of the deletion construct and the same regions within the *X. fastidiosa* chromosome facilitated complete removal of the target gene and subsequent insertion of the antibiotic resistance gene. The 5' and 3' flanking regions adjacent to *engXCA1* were amplified from the *X. fastidiosa* chromosome via PCR using primer pairs *engXCA1_LF_fwd* and *engXCA1_LF_rev* and *engXCA1_RF_fwd* and *engXCA1_RF_rev*, respectively. The kanamycin resistance gene was amplified from pCR2.1 TOPO (Invitrogen) using primers *engXCA1_kan_fwd* and *engXCA1_kan_rev*. The 5' and 3' flanking regions adjacent to *engXCA2* were amplified from the *X. fastidiosa* chromosome via PCR using primer pairs *engXCA2_LF_fwd* and *engXCA2_LF_rev* and *engXCA2_RF_fwd* and *engXCA2_RF_rev*, respectively. The gentamicin resistance gene was amplified from the pBBR1-MCS5 plasmid (Kovach et al. 1995) using primers *engXCA2_gent_fwd* and *engXCA2_gent_rev*. Individual fragments for each deletion construct were amplified using *Ex Taq* DNA polymerase (Takara) and 30 PCR cycles: denaturation at 95°C for 1 min; primer annealing at 60°C for

Table 4. Strains, plasmids, and primer sequences used in this study

Strains, plasmids, and primers	Relevant characteristics or primer sequence ^a	Source
Strain		
<i>Escherichia coli</i> TOP10	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ (<i>araleu</i>)7697 <i>galIU galK rpsL</i> (<i>StrR</i>) <i>endA1 nupG</i>	Invitrogen
<i>Xylella fastidiosa</i> Temecula 1		Temecula, CA, U.S.A.
<i>Xylella fastidiosa</i> $\Delta engXCA1$	<i>engXCA1</i> deletion in Temecula 1 background	This study
<i>Xylella fastidiosa</i> $\Delta engXCA2$	<i>engXCA2</i> deletion in Temecula 1 background	This study
<i>Xylella fastidiosa</i> $\Delta engXCA1/2$	<i>engXCA1</i> and <i>engXCA2</i> deletions in Temecula 1 background	This study
Plasmid		
pCR8/GW/TOPO	pUC19 derivative, spectinomycin-resistant	Invitrogen
pCR2.1 TOPO	Kanamycin-resistant	Invitrogen
pBBR1-MCS5	Gentamicin-resistant	Kovach et al. 1995
BI1	pCR8/GW/TOPO with $\Delta engXCA1$ deletion construct	This study
BI2	pCR8/GW/TOPO with $\Delta engXCA2$ deletion construct	This study
BI3	pET20b(+) protein expression vector with <i>engXCA1</i> gene	This study
MCR7	pET20b(+) protein expression vector with <i>engXCA2</i> gene	Pérez-Donoso et al. 2010
Primer		
<i>engXCA1_LF_fwd</i>	CGATCCGCCACGCTTCAGTATTGCC	This study
<i>engXCA1_LF_rev</i>	GGGCTTCCCAACCTTGACCGTGACT	This study
<i>engXCA1_kan_fwd</i>	AGTCACGGTCAAGGTTGGGAAGCCC	This study
<i>engXCA1_kan_rev</i>	GCAACATCCGGTTGCTACTTTCCACAG	This study
<i>engXCA1_RF_fwd</i>	CTGGTGAAAGTAGCAACCGGATGTTGC	This study
<i>engXCA1_RF_rev</i>	CCACACTCGCAGTGGCGCTTATTGG	This study
<i>engXCA2_LF_fwd</i>	TGCGTCCATAGACGCTTGCCATC	This study
<i>engXCA2_LF_rev</i>	CCACGGTGTGCGTCTAAGTATCCTTAATAGG	This study
<i>engXCA2_gent_fwd</i>	CCTATTAAGGATACTTAGACGCACACCGTGG	This study
<i>engXCA2_gent_rev</i>	GGAATAGAAAATGCACTTTAGGTGGCGGTACTTG	This study
<i>engXCA2_RF_fwd</i>	CAAGTACCGCCACCTAAAGTGCATTTTCTATTCC	This study
<i>engXCA2_RF_rev</i>	GTTCGAGACCGAGAATACCGATGATGAG	This study
GW1	GTTGCAACAAATTGATGAGCAATGC	Invitrogen
GW2	TAATTGCTCATCAATTTGTTGCAAC	Invitrogen
<i>engXCA1_XfgenDNA_For_seq</i>	CCTCAGGCGATACAGGCTAATCAGC	This study
<i>engXCA1_XfgenDNA_Rev_seq</i>	CGCTCAGGTCAATTCGTGATACACC	This study
<i>engXCA1mut_Kan_For_seq</i>	ATCGCCTTCTTGACGAGTCTTCTTG	This study
<i>engXCA1mut_Kan_Rev_seq</i>	CTGCGTGCAATCCATCTTGTTCATC	This study
<i>Xf_engXCA2_PCR_fwd</i>	CCGGTTTCAAAGTCGGGATC	This study
<i>Xf_engXCA2_PCR_rev</i>	ATGTGCGAACCAGTTTTCC	This study
<i>Xf_engXCA2seq_92outLF</i>	CCTGCCCCTGAGTCAAAGAG	This study
<i>Xf_engXCA2seq_71intLF</i>	CCCTAACGGCGTTTGAATCAC	This study
<i>Gent_seq_fwd</i>	GACGCACACCGTGGAAACGGATGAAG	This study
<i>Xf_engXCA2seq_33intRF</i>	CGGAGTGTGTAACGATGGCAT	This study

^a Primer sequences are presented 5' to 3'.

1 min; extension at 72°C for 2 min. The three fragments for each deletion construct were assembled by overlap extension PCR, and the resulting products were cloned into pCR8/GW/TOPO (Invitrogen) following the manufacturers' instructions to create plasmids pBI1 and pBI2. The *ΔengXCA1* construct was verified by sequencing using primers GW1 and GW2 (Invitrogen) and *engXCA1_kan_fwd* and *engXCA1_kan_rev*. The *ΔengXCA2* construct was verified by sequencing using primers GW1 and GW2 (Invitrogen) and *engXCA2_gent_fwd* and *engXCA2_gent_rev*. Either pBI1 or pBI2 was transformed into *X. fastidiosa* by electroporation. Electrocompetent *X. fastidiosa* cells were prepared according to Matsumoto et al. (2009), and 200 ng of pBI1 or pBI2 and 1 μl of TypeOne restriction inhibitor (Epicentre) were electroporated (3.0 kV, 300 Ω, 25 μF) into *X. fastidiosa* using a 0.2-cm cuvette (Bio-Rad) and the GenePulser X-cell (Bio-Rad). Positive transformants were validated via subculturing onto PD3 agar containing the appropriate antibiotics. Genomic DNA from *X. fastidiosa ΔengXCA1* and *X. fastidiosa ΔengXCA2* positive transformants was extracted using the DNeasy blood and tissue kit (Qiagen). *ΔengXCA1* was verified by sequencing using primers *engXCA1_XfgenDNA_For_seq*, *engXCA1_XfgenDNA_Rev_seq*, *engXCA1mut_Kan_For_seq*, and *engXCA1mut_Kan_Rev_seq*. *ΔengXCA2* was verified by sequencing using primers *Xf_engXCA2_PCR_fwd*, *Xf_engXCA2_PCR_rev*, *Xf_engXCA2-seq_92outLF*, *Xf_engXCA2seq_71intLF*, *Gent_seq_fwd*, and *Xf_engXCA2seq_33intRF*. The *X. fastidiosa ΔengXCA1/2* double mutant strain was created by transforming electrocompetent *ΔengXCA1* cells with 200 ng of the pBI2 plasmid and 1 μl of TypeOne restriction inhibitor (Epicentre). Positive transformants were obtained and screened as described above. All relevant primers can be found in Table 4. Complementation tools are limited for *X. fastidiosa*, and repeated attempts to obtain transformants for complementation of the EGase mutants were unsuccessful.

Recombinant EngXCA1 and EngXCA2 protein expression.

The *engXCA1* gene was inserted into the pET20b(+) protein expression vector to create pBI3 (Genscript USA Inc.). The gene was inserted between the *SacI* and *XhoI* restriction sites, and the codon sequence was aligned to include a histidine (His) tag and the periplasmic localization signal peptide. The pBI3 plasmid was used to transform *E. coli* BL21 DE3. Single colonies of *E. coli* harboring either pBI3, pMCR7 (Pérez-Donoso et al. 2010), or empty vector (pET20b(+)) were grown in 5 ml of liquid LB media containing ampicillin (100 μg/ml) at 37°C while shaking at 200 rpm overnight. Each overnight culture (4 ml) was added to 400 ml of liquid LB media containing ampicillin (100 μg/ml), and each culture was incubated at 37°C while shaking at 200 rpm until each had an optical density (OD₆₀₀) = 0.5. The cultures were centrifuged at 5,000 rpm for 10 min, the supernatant was discarded, and cell pellets were resuspended in 400 ml of fresh LB media containing ampicillin (100 μg/ml) and 0.6 mM isopropyl β-D-1-thiogalactopyranoside to induce protein production. The cultures were incubated at 4°C while shaking at 200 rpm overnight. After protein induction, the cultures were split in half to assess both protein expression via Western blotting and EGase activity.

Cell cultures (200 ml) expressing recombinant EngXCA1, recombinant EngXCA2, or the pET20b(+) empty vector were centrifuged at 5,000 rpm for 10 min. Cell pellets were freeze-thawed twice at -80°C, then lysed using the B-PER Complete lysis reagent (Thermo Scientific), following the manufacturers' instructions. A protease inhibitor was added during cell lysis to prevent protein degradation. Lysed samples were mixed 1:1 with 2× Laemmli buffer (Bio-Rad), boiled for 5 min, and

subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel. The protein was then transferred to a 0.45-μm nitrocellulose membrane overnight. The membrane was blocked with 5% nonfat milk and then probed with an α-His tag primary monoclonal antibody (Thermo Scientific) followed by a goat α-mouse secondary polyclonal antibody conjugated with alkaline phosphatase (Thermo Scientific). The blot was developed using the AP development kit (Bio-Rad), following the manufacturers' instructions.

EGase activity assay.

Cell cultures (200 ml) expressing recombinant EngXCA1, recombinant EngXCA2, or the pET20b(+) empty vector were centrifuged at 5,000 rpm for 10 min, and cell pellets were each resuspended in 1 ml of 1× PBS (pH 7.4). Each cell resuspension (50 μl) was placed onto the center of a respective plate containing 1% agarose (medium EEO, Fisher Scientific) and 0.2% CMC (Sigma-Aldrich) in phosphate buffer (pH 6.0) and incubated at 37°C for 48 h. After incubation, the perimeter of each cell droplet was marked, the diameter was measured using calipers, and then the cells were removed with copious amounts of water. Each plate was flooded with 0.1% Congo red dye (Sigma-Aldrich), followed by flooding with 1 M sodium chloride twice, and then flooding with 5% acetic acid (Carder 1986). The perimeter of each zone of hydrolysis was marked, and the diameter was measured using calipers. The area of each zone of hydrolysis was calculated and normalized by subtracting the area of the associated cell droplet. The assay was performed with three biological replicates containing three technical replicates of each treatment. Statistical significance between treatments was determined using the Kruskal-Wallis rank sum test for nonparametric data. Pairwise comparisons were made using Dunn's multiple comparison test, and *P* values were adjusted using the Benjamini-Hochberg false discovery rate method.

In planta virulence assays.

Vitis vinifera cv. Cabernet Sauvignon and cv. Chardonnay were propagated from cuttings (generously provided by Foundation Plant Services (UC Davis)). Vines were mechanically inoculated following the methodology outlined by Purcell and Saunders (1999). Briefly, a 20-μl drop with 10⁸ CFU/ml of *X. fastidiosa* inoculum was placed on the stem between the third and fourth nodes. The stem was pierced through the drop using a 20-gauge needle, and the drop was drawn into the xylem. Grapevines were inoculated with wild type, *ΔengXCA1*, *ΔengXCA2*, or *ΔengXCA1/2* *X. fastidiosa* strains. Grapevines inoculated with 1× PBS served as negative controls. Within trials, each strain was inoculated into 10 vines and the experiment was repeated three times for a total of 30 replicates per strain (or 1× PBS) inoculation. All trials were randomized in the greenhouse, and vines were given a weekly PD symptom score based on the established rating scale from 0 to 5 as described in Guilhabert and Kirkpatrick (2005), where 0 = a healthy vine, 1 = one or two leaves with scorching at the margins, 2 = two or three leaves with more developed scorching, 3 = all leaves with some scorching and a few matchstick petioles, 4 = all leaves with heavy scorching and many matchstick petioles, and 5 = a dead or dying vine. Each trial compared PD symptom scores over a 20-week period in Cabernet Sauvignon and Chardonnay grapevines inoculated with either wild type or one mutant strain. Each trial was completed twice over a 2-year period, for a total of six pathogenicity assays: two comparing wild type and *ΔengXCA1*, two comparing wild type and *ΔengXCA2*, and two comparing wild type and *ΔengXCA1/2*. Because wild type was present in every trial, data for wild type-inoculated vines was combined for each

vine type and used as a reference to make comparisons to data from vines inoculated with each of the mutants across multiple trials and years. The mean PD symptom score per week was calculated, and comparisons between wild type and each of the mutants were made at 5 WPI (early-stage PD), 10 WPI (mid-stage PD), 15 WPI (late-stage PD), and 20 WPI (endpoint). The rate of PD symptom progression was assessed within the linear portion of the disease curve (5 to 15 WPI) by calculating the slopes between early- and mid-stage PD (5 to 10 WPI) and mid- and late-stage PD (10 to 15 WPI).

Local and distal xylem colonization assays.

Grapevines were inoculated as described above. To determine the contribution of EGases in local xylem colonization, wild-type, $\Delta\text{engXCA1}$, $\Delta\text{engXCA2}$, and $\Delta\text{engXCA1/2}$ populations per gram of tissue were quantified by isolating *X. fastidiosa* from the first intact petiole closest to the POI. Additionally, isolations were performed on petioles from the twentieth node distal to the POI to determine whether the EGases (singly or in tandem) had an impact on movement. Petioles were collected from every vine in each of the six trials after the late-stage infection time point, surface-sterilized (30 s in 70% ethanol, 30 s in 10% bleach, and twice for 1 min in sterile deionized water), and ground in 2 ml of sterile 1× PBS. The resulting suspension was diluted, and the dilution series was plated on solid PD3 medium according to standard methods (Roper et al. 2007). *X. fastidiosa* titers were normalized by dividing colony counts (colony forming units) by the weight of the corresponding petiole (colony forming units per gram of tissue). The titers for wild type-inoculated vines from all trials were combined for each vine type and used as a reference, and wild-type titers were compared with the titers of each mutant.

Integrity and polysaccharide composition of intervessel PMs.

An immunogold SEM technique was used to reveal intervessel PM structure and immuno-localized fucosylated xyloglucans on the PMs of Chardonnay and Cabernet Sauvignon vines at the late-stage time point of PD symptom development. Details of the technique can be found in Sun et al. (2017). In brief, the third internode above the inoculated internode of each vine was fixed in 4% paraformaldehyde in PEM buffer (50 mM PIPES, 5 mM EGTA, 5 mM MgSO_4 , pH 6.9) for at least 24 h. Each internode was then trimmed into 3 mm wide × 5 mm long × 1 mm thick longitudinal segments, exposing either a radial or tangential surface of the secondary xylem. Trimmed specimens were washed twice with 50 mM PIPES and once with 0.2 M PBS with 3% nonfat milk powder (MP/PBS, pH 7.4) for 30 min for each wash. Subsequently, specimens were divided into three groups, with one as the immune-localization treatment and the other two as controls. A monoclonal antibody (mAb, CCRC-M1) and a corresponding secondary Ab (gold-conjugated goat antimouse IgG) were used to detect fucosylated xyloglucans on intervessel PMs. Specimens for each treatment were incubated at 4°C overnight in a threefold dilution of the original mAb mouse hybridoma supernatant in 3% MP/PBS. After three PBS washes of 10 min each, specimens were incubated at room temperature (RT) for 1 h with a 50-fold dilution in 3% MP/PBS of the original colloidal gold-conjugated goat antimouse IgG. Incubation was followed by three 10-min washes of deionized water and one 15-min silver enhancement treatment. Silver-enhanced specimens were washed twice in deionized water (10 min each), and dehydrated via an ethanol series. Dehydrated specimens were critical-point dried, sputter-coated with gold-palladium, and then examined at an accelerating voltage of 5 kV or 8 kV under a Hitachi 3400N SEM in the University of Wisconsin Stevens Point Biology Department. The other two

sets of specimens used for controls were processed in the same way described above, except that the mAb or secondary Ab incubation step was replaced with the 3% MP/PBS incubation within either of the two sets of specimens, respectively. Silver-enhanced gold particles appear as bright particles and indicate the presence, quantity, and distribution of fucosylated xyloglucans on intervessel PMs of the polysaccharide group. Specimens of either treatment or controls also were examined with SEM to reveal structural features of intervessel PMs.

Statistical analyses.

Longitudinal disease ratings are discrete, non-Gaussian, observations. Therefore, a GEE model was used for this analysis (Liang and Zeger 1986). The time trend was captured using a quadratic spline with knots at 5 weeks and 10 weeks. A GEE model with a quadratic spline time trend provided a robust approach to the data analysis. A full model with year, cultivar, strain, and time as main effects, cultivar × strain, cultivar × time, and strain × time as two-factor interactions, and cultivar × strain × time as a three-factor interaction was fit to the complete data set of disease ratings. The three-factor interaction was significant in the model, and therefore, least squares means (LSM), standard errors, and 95% confidence intervals for the mean disease rating for each combination of cultivar, strain, and time were obtained.

For each strain, contrasts between cultivars at each time point were carried out using two-sided asymptotic Z-tests based on the LSM. Within a cultivar, the three mutants were contrasted with wild type at each time point using a two-sided asymptotic Z-test. The LSM were used to estimate rates of PD symptom progression as the slopes between times 5 and 10 weeks, and between times 10 and 15 weeks. The two slopes were similarly contrasted between cultivar and within cultivar using two-sided asymptotic Z-tests.

Bacterial titer is an integer-valued count that was highly variable and contained several zeros. Therefore, a negative binomial generalized linear model (GLM) was used to analyze bacterial titer (McCullagh and Nelder 1989). Between-cultivar comparisons utilized a negative binomial GLM for each combination of strain and colonization location, and which coded cultivar using two dummy variables. Cultivars were compared using a two-sided asymptotic Wald test. Within-cultivar comparisons utilized a negative binomial GLM for each colonization location, and which coded strain with four dummy variables. Because mutant counts were expected to be lower than wild-type counts, contrasts of each mutant with wild type were carried out with lower one-sided asymptotic Wald tests.

All analyses were performed in SAS 9.4 (SAS Institute) using the GENMOD procedure.

ACKNOWLEDGMENTS

Grapevine cuttings used in this work were provided by Foundation Plant Services, University of California, Davis. The authors also thank P. Rolshausen for thoughtful discussions on cultivar susceptibility to vascular diseases.

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