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Identification of Resistance to Bacterial Leaf Blight in the U.S. Department of Agriculture Collard Collection

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***Abstract*. Bacterial leaf blight incited by *Pseudomonas cannabina* pv. *alisalensis* (*Pca*) is a devastating disease with incidence reports worldwide and a wide host range capable of infecting all commercially valuable *Brassica* crops. With no chemical control options available, the most effective form of disease control is host plant resistance, but thus far resistant germplasm has only been identified in *Brassica juncea* L. (mustard greens). We report the first screening of *Brassica oleracea* L. var. *viridis* germplasm, including leafy green collard and collard-like accessions, for resistance to bacterial leaf blight by artificial inoculation of *Pca* in greenhouse trials. All commercial cultivars tested displayed an intermediate disease response resulting in leaf lesion development that renders the product unmarketable. Two sources of significant resistance were identified in the U.S. Department of Agriculture (USDA) *viridis* collection, which provides a valuable source of resistance alleles for collard cultivar development and introgression into other *B. oleracea* crops.**

Bacterial blight emerged in the 1990s as a major disease of *Brassicaceae* crops and has since spread worldwide with disease incidence reports from Europe (Rubio et al., 2012; Sarris et al., 2010), Asia (Takahashi et al., 2013), Australia (Bull and Rubio, 2011), and both the East and West coasts of the United States (Cintas et al., 2002; Keinath et al., 2006; Wechter et al., 2010). Initial reports attributed the disease to *Pseudomonas syringae* pv. *maculicola* but were later determined to be incited by a new pathovar (pv. *alisalensis*; *Pseudomonas syringae* pv. *alisalensis*) responsible for bacterial blight of *Brassicas* (Bull et al., 2004; Cintas et al., 2002; Takahashi et al., 2013; Wechter et al., 2013). *Pseudomonas syringae* pv. *alisalensis* has since been reclassified as *Pca* (Bull et al., 2010a).

Pseudomonas cannabina pv. *alisalensis* is capable of infecting a wide range of *Brassicas*, including crop varieties of multiple species: *Brassica rapa* L. (Cintas et al., 2002; Keinath et al., 2006; Wechter et al., 2007), *B. juncea* (Keinath et al., 2006; Wechter et al., 2007, 2014), *Brassica napus* L. (Bull and Rubio, 2011; Koike et al., 2007), *B. oleracea* (Bull et al., 2010b; Cintas et al., 2002; Koike et al., 2000, 2006; Mauzey et al., 2011; Wechter et al., 2010), *Raphanus sativus* L. (Rubio et al., 2012), and *Eruca sativa* L. (Bull and DuToit, 2009; Bull et al., 2004; Sarris et al., 2010). A study comparing host plant resistance and the commonly used chemical bactericide acibenzolar-S-methyl found that host plant resistance was more effective at controlling bacterial blight in *B. juncea* (Keinath et al., 2016). However, levels of resistance to *Pca*, suitable for market production, have only been identified in *B. juncea* (Wechter et al., 2007, 2013), which led to the successful development of a resistant mustard green cultivar Carolina Broadleaf (Wechter et al., 2016). Herein, we report the first screening of *B. oleracea* germplasm for resistance to bacterial leaf blight incited by *Pca*.

Brassica oleracea consists of several economically important crop varieties, with a cash value of more than $2 billion from broccoli (var. *italica*), cabbage (var. *capitata*), cauliflower (var. *botrytis*), kale (var. *acephala*), Brussels sprout (var. *gemmifera*), and collard in the United States alone (U.S. Department of Agriculture, National Agricultural Statistics Service, 2014). Collard is a leafy green vegetable crop primarily produced in the southeastern United States with a yearly fresh market value of more than $125 million (U.S. Department of Agriculture, National Agricultural Statistics Service, 2014). Bacterial leaf blight was first found in collard in 2002 in commercial fields in South Carolina and has been detected every year since the initial report (Keinath et al., 2006). The disease is currently causing the highest yield losses of leafy green *Brassicas* in South Carolina (Keinath et al., 2006), and all tested commercial cultivars have been found to be susceptible to the pathogen (Keinath et al., 2011; Wechter et al., 2013). Thus, the objective of this study was to screen the USDA *viridis* PI collection for resistance to *Pca*, to identify resistant collard germplasm that can be used for collard cultivar development, and to provide resistance alleles for introgression into other economically important *B. oleracea* crop varieties.

Materials and Methods

Plant materials. Germplasm for the disease screen included the USDA collection of *viridis* group PIs that are currently available from the National Plant Germplasm System in Geneva, NY, and 24 additional *B. oleracea* PIs of various varieties that produce collard-like leafy greens (germplasm information available from Dryad data repository, doi:10.5061/dryad.4851qq0). The Brassica leafy green commercial cultivars (obtained from Abbott and Cobb Seed Co., Feasterville, PA), 'Topper' (*B. rapa* turnip green) and 'Top Bunch' (*B. oleracea* var. *viridis* collard green), were used as susceptible controls (Wechter et al., 2013). 'Carolina Broadleaf' (*B. juncea* mustard green cultivar) and PI 195553 (*Brassica* spp. PI) were used as resistant controls based on previous reports of low disease occurrence in response to inoculation with *Pca* in both greenhouse and field studies (Wechter et al., 2007, 2013, 2016).

Initial disease screen and retests of selections. The initial disease screen of the collard PI collection was completed as a series of five greenhouse tests of 19–24 PIs each between Oct. 2015 and Feb. 2016 in Charleston, SC. Each test included the same four controls ('Carolina Broadleaf', PI 195553, 'Topper', and 'Top Bunch') and followed a randomized complete block design with two replications of four plants per replication. Seeds were planted into Metro-Mix 200 soil mix (The Scott's Co., Maryville, OH) in 5-cm square pots and thinned to one plant per pot after 10 d. At 7 d postinoculation, the two most affected leaves per plant were visually evaluated for percentage of leaf area with blight symptoms (necrosis, chlorosis, or both; Fig. 1). Each plant was given a score of 0% to 100% disease severity (DS), in 5% increments, based on the two-leaf average (Wechter et al., 2013).

Collard PIs with an average DS of less than 25% were chosen for retesting to confirm resistance to *Pca*. In addition, PIs with at least four individual plants displaying less than 25% DS were moved outside for vernalization. Vernalized selections were selfed through hand pollination, given a new ID (PI#-plant#), and the resulting progeny included in retesting along with the original PIs. Five additional lines were created by selfing and collecting seeds from individual selections from two PIs [PI 662820 (2) and PI 662833 (3)]. The most highly susceptible PI from the initial screening (PI 662819) was used as the susceptible control for the retests and 'Carolina Broadleaf' as the resistant control. Two commonly grown commercial

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Fig. 1. Collard leaves with bacterial leaf blight symptoms depicting disease severity scores of 85% (**A**) and 5% (**B**). Images were taken 7 d postinoculation with *Pseudomonas cannabina* pv. *alisalensis* from PIs screened in greenhouse trials.

collard cultivars, Top Bunch and Blue Max, were included in the retests to determine whether any of the PIs exhibited enhanced resistance as compared with commonly grown cultivars. After the initial screening of the collard PI collection, two retests of putatively resistant accessions were completed in Nov. 2016 and Apr. 2017 following the same protocols as described for the preliminary screening.

Bacterial isolate and inoculations. Isolate T3C of *Pca* (Wechter et al., 2007, 2013) was used for all assays. Isolate T3C was streaked from a –80 °C glycerol stock onto *Pseudomonas* agar F (PAF) medium (Difco; Becton Dickenson and Co., Franklin Lakes, NJ) and grown for 24 h at 25 °C. A single colony was restreaked on a PAF plate and grown for 48 h at 25 °C. A 10-µL loop of cells was taken from the PAF plate and inoculated into 200 mL of King's B broth (Difco; Becton Dickenson and Co.) and grown on a rotary shaker at 200 rpm at 25 °C for 24 h. Cell culture turbidity was determined using a biophotometer (Eppendorf, Hauppauge, NY) at a wavelength of OD_{600}. Cell suspension was diluted to a final concentration of $\approx 2 \times 10^6$ colony-forming units (cfu) using sterile distilled water; based on previous studies, an OD_{600} of 0.1 is $\approx 1 \times 10^6$ cfu (data not shown). Cell suspension was applied to the adaxial and abaxial surfaces of the leaves using a Paasche airbrush (Paasche Airbrush Co., Chicago, IL) at a pressure of ≈160 kPa. Inoculum was applied from a distance of 15–20 cm from the leaf surface until incipient runoff. The plants were then placed in a dew chamber at 100% humidity and 25 to 27 °C for 24 h and then transferred to the greenhouse. The plants were left in the greenhouse for an additional 6 d and rated 7 d postinoculation.

Statistical analysis. Each test of the initial screen was analyzed separately because of heterogeneity of variances (P value $<2.2 \times 10^{-16}$) as determined by Levene's test (Levene, 1960) in the car package (Fox and Weisberg, 2011) of R (R Core Team, 2016). The two retests were analyzed both separately and in combination because they had homogeneity of variances (P value = 0.30). Analyses of variance (ANOVAs) of DS were conducted for all tests separately ($N = 7$) and the retests combined using a general linear model in the aov package (Chambers et al., 1992) in R. Models of the individual tests included genotype, replication, and plants nested within replication as fixed effects. The same model was used for analysis of the combined retests with the addition of test and the interaction of genotype and test as fixed effects. Fisher's protected least significant differences (LSDs) between genotypes were determined with the agricolae package (Mendiburu, 2016) in R with an alpha of 0.05. Accession means for each test (five total) of the initial screen and the two retests were calculated in R. Correlation between accession means of the retests was assessed by calculating Pearson's correlation coefficient (r) using the stats package of R.

Results

Disease screen. The USDA *viridis* PI collection ($N = 110$) was screened for resistance to bacterial leaf blight following inoculation with *Pca*. The results associated with the screening of the PI collection, including ANOVA tables, LSD, and DS of each accession tested, have been deposited in the Dryad data repository (doi:10.5061/dryad.4851qq0). Accession was the most significant factor (P value from 9.3×10^{-13} to 2×10^{-16}) in all five tests of the screening. Significant differences were found among accessions evaluated in each test, with LSD values ranging from 16.8% to 24.4% DS. Disease severity of the accession means within each test varied widely, with a minimum difference between the most and the least resistant of 76%. Both cultivars had an intermediate disease response, with the mean DS of 'Top Bunch' ranging from 18.1% to 55.6% and 'Topper' from 11.3% to 35.6%. The resistant controls, 'Carolina Broadleaf' and PI 195553, had the lowest mean DS in every test, and eight accessions (PI 662586, PI 662668, G 30859, PI 662796, G 32556, PI 662805, G 32768, and G 33036) displayed similar results with no significant differences from these two resistant controls.

Retests of the most resistant PIs. Fifteen of the most resistant PI accessions (mean DS <25%) and five selfed progeny were included in two additional tests (Table 1). Accession means across the two additional tests ranged from 2.6% to 39.7%. Accession means were significantly correlated between the tests with a P value of 0.02 and an r of 0.47. The commercial collard cultivars (Blue Max and Top Bunch) displayed an intermediate response to *Pca* as their DS means (17.5% and 16.9%, respectively) were significantly different from both the resistant and susceptible controls (Table 1). Three accessions (PI 662820-1, PI 662820-2, and G 33036) were identified as not significantly different from the resistant control with a mean DS of 9% to 11%. Both G 33036 and PI 662820 (the PI from which two selections were made) are classified as collards (var. *viridis*), but they have very different horticultural characteristics (Fig. 2). G 33036, named Purple Curly Collard, had a lower mean DS but produces a kale-like plant, undesirable for collard production. This accession also exhibited wide segregation for leaf size, shape, curliness, and color. Two resistant selections were derived from PI 662820, named Lyda Gibbs after the originating seed saver, which produces uniform, high-yielding horticulturally desirable collard plants with large, bright-green leaves.

Discussion

The rapid worldwide spread of *Pca*, combined with the devastating potential losses caused by bacterial leaf blight on *Brassicas*, highlights the importance of identifying sources of resistance in *Brassica* crops. In the studies described herein, resistance to *Pca* was found in two collard PI accessions, both collected in North Carolina, that vary drastically from one another in terms of horticultural characteristics. Selections from PI 662820 have resistance to *Pca* and produce horticulturally desirable leaves.

Even minor blemishes on collard leaves caused by bacterial leaf blight can result in an unmarketable product. Although the mean DS of the PI 662820 selections (≈10%) may be too high for fresh market production, previous experience with other *Brassica* species indicates that additional rounds of

Table 1. Mean disease severity (DS) of accessions across two retests after inoculation with *Pseudomonas cannabina* pv. *alisalensis*.

Accession	Taxonomy[z]	DS (%)[y]
PI 662819	*Brassica* spp.*	39.7 a
PI 662833-3	*viridis*	30.0 b
PI 662668	*costata*	27.8 bc
PI 662796	*viridis*	24.0 bcd
PI 662799	*viridis*	25.0 bcd
PI 662803	*viridis*	24.3 bcd
PI 662805	*viridis*	25.0 bcd
G 32768	*viridis*	20.0 cde
G 30859	*medullosa*	19.6 cdef
G 32556	*viridis*	19.3 cdef
PI 662833-1	*viridis*	19.4 cdef
PI 662802	*viridis*	19.1 def
PI 662801	*viridis*	18.1 defg
Blue Max	*viridis*	17.5 defgh
Top Bunch	*viridis*	16.9 defgh
PI 662586	*viridis*	16.6 defgh
PI 662797	*viridis*	16.9 defgh
PI 662820	*viridis*	16.6 defgh
PI 662833	*viridis*	17.6 defgh
PI 662833-2	*viridis*	14.5 efgh
PI 662820-2	*viridis*	10.9 fghi
G 33036	*viridis*	10.1 ghi
PI 662820-1	*viridis*	9.4 hi
Carolina Broadleaf	*Brassica juncea**	2.6 i

[z]Taxonomy describes the variety of *Brassica oleracea* with the exception of those with an * symbol, which are listed as species.

[y]Disease severity (scored as percentage of diseased leaf tissue) averaged over two retests. Ratings with the same letter are not significantly different (P value ≤ 0.05) as determined by Fisher's protected least significant difference.

selection should provide gains in bacterial blight resistance. Indeed, a single round of selection has already decreased DS, with both PI 662820 selfed selections (-1 and -2) testing more resistant than the original PI. This significant gain from selection suggests polygenic inheritance for resistance in this *B. oleracea* germplasm that may be similar to what was found in *B. juncea* (Wechter et al., 2013). Additional generations of selection and selfing could be expected to increase the level of resistance in the PI 662820 lines. This strategy has already proven effective in the development of the cultivar Carolina Broadleaf (Wechter et al., 2016), which has marketable levels of resistance to *Pca* even though it originated from a PI that originally exhibited DS of more than 10% (Wechter et al., 2007). Inheritance of resistance in *B. juncea* is likely controlled by multiple recessive genes or by a single recessive gene with interaction of modifier genes (Wechter et al., 2013).

Genetic and cultural evidence suggest that collard may be most closely related to cabbage (Farnham et al., 2005, 2008; Pelc et al., 2015), which has a yearly cash value of almost half a billion dollars in the United States (U.S. Department of Agriculture, National Agricultural Statistics Service, 2014). In 2008, bacterial blight from *Pca* infection was found for the first time in commercial cabbage fields in Monterey County, CA, which caused reduced quality and marketability of the cabbage heads (Mauzey et al., 2011). The resistant germplasm identified here may provide alleles for the ready introgression of disease resistance into cabbage cultivars because of the close relationship between these crops (Farnham et al., 2005).

Here, we report the first screening of *B. oleracea* germplasm for resistance to bacterial leaf blight incited by *Pca*. We identified two significant sources of resistance within the USDA *viridis* PI collection, which is particularly important in light of the intermediate, but unmarketable, disease response of the commercial collard cultivars tested. Future breeding efforts will focus on continued rounds of selection from the initial selfed plants of PI 662820 to both attempt additional genetic gains and fix resistance to *Pca* for germplasm or cultivar release.

Fig. 2. Photographs of identified sources of *Pseudomonas cannabina* pv. *alisalensis* resistance, PI 662820 (**A**) and G 33036 (**B**), taken from a noninoculated field, lacking bacterial blight symptoms. Shown only to demonstrate the horticultural differences between the PIs. (Photo credit: Zachary Stansell.)

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