Genotypic and Phenotypic Variation among Pepper Accessions Resistant to Phytophthora capsici

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Abstract. Developing high levels of resistance to Phytophthora blight (Phytophthora capsici Leonian) of pepper (Capsicum annuum L.) is a goal of many pepper breeding programs. Genetic diversity and plant characteristics were evaluated on 21 pepper accessions previously identified as having high levels of Phytophthora root rot resistance. Accessions were evaluated for plant height, fruit size and shape, pericarp thickness, and pungency. Accessions varied widely from very unadapted material to those with more obvious breeding potential. Genetic diversity among the accessions was estimated using 22 simple sequence repeat (SSR) loci. Phytophthora capsici resistant accessions were identified that were not closely related to previously described sources of resistance. This information will allow breeders and researchers to further identify and incorporate novel sources of resistance to P. capsici into breeding programs. Accessions PI 201237 and PI 640532 appear to have the most potential for introgressing P. capsici resistance into bell pepper.

The oomycete Phytophthora capsici Leonian causes root and fruit rot, stem blight, and foliar blight in pepper (Capsicum annuum L.) (Leonian, 1922) causing global yield losses (Babadoost, 2004). Currently the disease is managed through the use of resistant cultivars, fungicides, and cultural practices such as crop rotation (Louw et al., 2008; Ristaino and Johnston, 1999). However, its widespread host range (Erwin and Ribeiro, 1996) spanning at least 14 families including economically important Cucurbitaceae, Solanaceae, and Leguminosae crops and many weeds makes control through crop rotation challenging. The additional development of resistance to fungicides (Cáffé-Filho and Ristaino, 2008; Hausbeck and Lamour, 2004) have led to more concerted efforts to develop P. capsici resistant pepper cultivars.

Resistance to P. capsici has been reported (Alcantara and Bosland, 1994; Bartaual et al., 1994; Bosland and Lindsey, 1991; Gil-Ortega et al., 1991; Guerrero-Moreno and Laborde, 1980) and is best described for the landrace CM-334 in which different genes control resistance to root rot, foliar blight, and stem blight (Sy et al., 2005; Walker and Bosland, 1999). Recently, Candole et al. (2010) described several new sources of resistance to P. capsici. Despite these sources of resistance, currently no bell or chile pepper cultivar is consistently resistant to all disease symptoms under field conditions (Oelke et al., 2003; Sy et al., 2008). Breeding cultivars resistant to P. capsici is not only complicated by the different disease symptoms, but also by the diversity of the pathogen itself. At least 20 P. capsici races have been identified using host differentials (Oelke et al., 2003; Sy et al., 2008) and host resistance to different races is often controlled by different genes (Monroy-Barbosa and Bosland, 2008). Breeders are therefore always on the lookout for new sources of resistance in the hope that it will provide resistance to additional P. capsici races and/or disease symptoms.

For breeders to make informed decisions about which resistant accession(s) to use in their breeding programs, it would be useful to have information related to important horticultural traits and the genetic diversity among these accessions. To shorten the time required to develop resistant cultivars, breeders would prefer to use resistant accessions that have similar horticultural characteristics to the cultivar being developed. Breeders are also interested in incorporating different resistance genes into cultivars (gene pyramiding) to obtain broader, more durable resistance (Stuthman et al., 2007). Without knowledge about the particular gene/alleles involved in resistance, breeders can incorporate resistance from different genetic backgrounds to increase the probability of broader resistance.

The traits important to pepper breeding will depend on the type of pepper the breeder is interested in developing. The specific goals in breeding bell pepper and chile pepper could vary substantially (Bosland and Votava, 2000). Fruit shape and size are important considerations to most breeders, although the specific goals differ, e.g., large blocky fruit for bell peppers and long narrow fruit for “Cayenne”. Pericarp thickness is another important trait with thick pericarp desirable for bell pepper but thin pericarp for peppers that are commonly dried (e.g., paprika types). Plant height may also have a significant influence with compact morphologies required for bell pepper, whereas larger plants are preferred for chile pepper (Crosby, 2008). Consumers expect a specific pungency level from certain pepper types, for example Jalapeño is expected to be less pungent than Cayenne. Moreover, bell peppers are non-pungent, whereas the degree of pungency in chile peppers varies greatly (Bosland and Votava, 2000; Crosby, 2008). The chemicals responsible for pungency are collectively called capsaicinoids, which can be measured using high-performance liquid chromatography (HPLC; Collins et al., 1995). Capsaicin and dihydrocapsaicin are responsible for 80% to 95% of total capsaicinoids and are therefore good indicators of fruit pungency (Bennett and Kirby, 1968; Cavett et al., 2004).

Knowledge of the genetic diversity among P. capsici resistant accessions and commercial cultivars would benefit plant breeders. The P. capsici resistant cultivar CM-334 has been extensively used in breeding programs (Monroy-Barbosa and Bosland, 2008; Ortega et al., 1991). Breeders wanting to introduce new P. capsici resistance genes into their programs probably want to use a source that is not genetically closely related to CM-334. The use of SSR markers to determine genetic variation and uniformity within and between accessions are well established in many crops including pepper (Hanáček et al., 2009; Kwon et al., 2005; Portis et al., 2007; Tam et al., 2005; Tommasini et al., 2003). Knowledge of genetic diversity among resistant accessions would help breeders to choose accessions based on this information.

The aim of this study is to determine phenotypic variation of horticulturally important traits and genetic variation among the P. capsici resistant accessions identified by Candole et al. (2010).

Materials and Methods

Plant material. Seeds from 21 accession lines previously reported to be resistant to P. capsici (Candole et al., 2010) were planted in seedling trays at the University of Georgia South Milledge greenhouses in Athens, GA on 5 May 2009. The word “line” is used as described in Candole et al. (2010) to indicate selfed seed from an individual plant of
Table 1. Simple sequence repeat primers used in this study.

<table>
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<tr>
<th>Primer name</th>
<th>Sequence 5’-3’</th>
<th>Allele size range (bp)</th>
<th>Annealing temp (°C)</th>
<th>No. of alleles</th>
<th>Polymorphism information content</th>
<th>Reference</th>
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autosampler and column compartments, and a photodiode-array detector (PDA), all controlled by Chemstation (revision B.03.01) software package. Solvents used were A: water and B: acetonitrile run isocratically at 60% B at a flow rate of 1.00 mL·min⁻¹. The autosampler compartment was maintained at 4°C. The volume of injection for both samples and standards was 10 μL. The capsaicinoid compounds were retained using an Agilent Eclipse XDB-C18 column (150 mm × 4.6 mm × 5 μm; Foster City, CA) protected by a guard column (12.5 mm × 4.6 mm × 5 μm) of the same phase, all held at 30°C within the column compartment. Eluted compounds were detected using the PDA equipped with a semimicro flow cell with a full spectral scan set from 190 to 350 nm with an agarose gel. Three primer pairs (Table 1) were used in this study. Scoville heat units (SHUs; Scoville, 1912) were calculated by multiplying the sum of ppm capsaicin and dihydrocapsaicin by 15 (Bosland and Votava, 2000).

Genetic diversity and linked markers. Three leaf disks were collected from three plants of each accession/cultivar using the lid of a microfuge tube as a punch. Samples were immediately frozen in liquid nitrogen and stored at −80°C until processing. Samples were ground with 5-mm stainless steel beads (Qiagen, Inc., Waltham, MA) and diluted if necessary.

Twenty-two previously published SSR primer pairs (Table 1) were used in this study. The reaction contained between 10 and 100 ng template DNA, 0.4 μM of a fluorescently labeled (6FAM, HEX, or 6TAM) forward and unlabeled reverse primer, 400 μM of each dNTP, 0.6 U Taq DNA polymerase (NEB; Standard Taq buffer (NEB) in 20 μL total reaction volume. Polymerase chain reaction (PCR) products were amplified in 96-well plates on a MyCycler (Bio-Rad, Hercules, CA) using 1-min initial denaturation followed by one cycle of 25 s at 95°C, 25 s at 56°C, and 30 s at 72°C. In every following cycle, the denaturing temperature was reduced by 1°C until it reached 53°C. The subsequent 36 cycles consisted of 25 s at 95°C, 20 s at 53°C, and 30 s at 72°C. For some primers, a base annealing temperature of 50°C was used instead of 53°C (Table 1), and in such cases, the initial stepdown was carried out for six cycles followed by 33 cycles at the base annealing temperature. The amplification was followed by a final extension step of 15 min at 72°C.

Amplifications were carried out separately for each primer pair; products were diluted either 2.5 × 5 or 5 × 10 depending on observed band intensity on an agarose gel. Three primer pairs (one for each fluorescent label) were pooled...
together. The diluted products were combined with a GeneScan-500 ROX internal-lane size standard (ABI; Applied Biosystems by Life Technologies Corporation, Carlsbad, CA) and 50% formamide before analysis on the Applied Biosystems 3730xl 96-capillary DNA Analyzer (ABI) at the Georgia Genomics Facility. GeneMapper software Version 4.0 (ABI) was used for allele scoring and size estimation.

PowerMarker Version 3.25 (Liu and Muse, 2005) was used to calculate frequency-based distances (Nei et al., 1983) and draw a tree based on the unweighted group method with arithmetic mean (Sneath and Sokal, 1973) that was visualized in Tree View (Page, 1996). Heterozygosity (Weir, 1996) and polymorphism information content (PIC; Botstein et al., 1980) were also calculated using PowerMarker, whereas genotype diversity was calculated as described by Tommasini et al. (2003).

The DNA used for SSR analysis was also used for amplification of the D04 sequence characterized amplified region (SCAR) marker reported to be linked to the Phyto 5.2 resistance locus of CM-334 (Quirin et al., 2005). The reaction contained between 10 and 100 ng template DNA, 0.4 μM of each primer (Quirin et al., 2005), 400 μM of each dNTP, 0.6 U Taq (NEB; New England Biolabs), and 1 × standard Taq buffer (NEB) in 20 μL total volume. PCR products were amplified using 90 s initial denaturation followed by 35 cycles of 15 s at 95 °C, 20 s at 55 °C, and 30 s at 72 °C followed by a final single elongation step of 15 min at 72 °C. In addition, all samples were amplified with the ITS-A and ITS-B primers described by Blattner (1999) using identical conditions as described previously to ensure that fragments could be amplified from all samples (data not shown).

All amplified fragments were separated using 1.5% agarose gel electrophoresis and Benchtop PCR markers (Promega Corporation, Madison, WI) were included to estimate fragment size.

Results and Discussion

The resistant accessions show a large amount of both phenotypic and genetic diversity among accessions (Table 2; Figs. 1 and 2).

Horticultural characteristics. The accessions generally have a tall growth habit compared with commercial bell pepper cultivars, similar to ‘Early Jalapeno’, but some accessions (e.g., PI 593564) were even taller (Table 2). Compact size is usually desired in commercial bell pepper cultivars. PI 201239 and PI 593564 were two of the most compact accessions investigated.

Fruits were generally small and elongated with a thin pericarp (Table 2). PI 640560 had the largest fruit and, along with PI 640532, and PI 640581 also had relatively thick pericarp. Both erect and pendant pedicel positions were observed. Except for PI 201231, PI 593572, and PI 640581, which had orange, olive/brown, and orange/red mature fruit color, respectively, all accessions had red mature fruit. Depending on the breeding objectives, breeders should choose resistant accessions that have horticultural characteristics suitable to their requirements.

The phenotypic data were collected in a single greenhouse experiment and it is expected that absolute values for traits like plant height, fruit size, and fruit weight will be different under field conditions. However, it is likely that general trends will hold true, for example that PI 201239, PI 593572, and PI 640480 will have heavier fruit than PI 511883, PI 593573, and PI 640833 and that PI 640480 and PI 640581 will have more elongated fruit than PI 201237 and PI 640532.

Pungency. The levels of capsaicin and dihydrocapsaicin varied widely among accessions (Table 2). The pungency (SHU) of the different accessions ranged from trace amounts in PI 640532 and PI 640641 to ≈23,000 SHUs in PI 224438 and PI 593564 (Fig. 1). Because capsaicinoids are produced only in the placenta, larger fruit with thick pericarp tend to have lower capsaicinoid levels as a result of the dilution effect of the inclusion of pericarp and seeds (Ben-Chaim et al., 2006). Indeed, PI 640532 and PI 640560 and other accessions with relatively large fruit and thick pericarp (Table 2) had low pungency (Fig. 1).
The presence or absence of pungency is controlled by a single gene, *Pun1*, coding for a putative acyltransferase (Deshpande, 1935; Stewart et al., 2005; Webber, 1911). The loss of function allele at this locus leads to absence of pungency in the homozygous recessive state (Stewart et al., 2005). When capsaicinoids are present, the amount of capsaicinoids is a quantitative trait controlled by several loci (Ben-Chaim et al., 2006; Blum et al., 2003; Zewdie and Bosland, 2000a). Breeders should select resistant accessions with pungency levels that would best fit their ultimate breeding goals. However, it should be noted that pungency is influenced by the environment (Zewdie and Bosland, 2000b) and therefore values may be different under field conditions.

*Genetic diversity and linked markers.*

Broadly the accessions can be classified into two main clades (Fig. 2). Two accessions, PI 511883 and PI 640461, are genetically different from other accessions and each other. PI 511883 is a chile pepper that was purchased from a market in the Rio Balsas region of Mexico, and PI 640461 is a Cayenne cultivar from Indonesia.

PI 593573, one of the most resistant accessions under field conditions (Candole et al., 2010), is genetically (Fig. 2) and phenotypically (Table 2) very similar to the known resistant CM-334 and this increases the likelihood that these two accessions have the same resistance genes/alleles. If breeders want to increase the probability of introducing several resistance genes/alleles into their breeding material, it would be prudent to introduce resistance from accessions that are not closely related.

In an effort to further elucidate potential new sources of resistance, markers previously described to be linked to resistance loci in CM-334 were investigated. Two SSR primer pairs, CAMS163 and CAMS390, previously reported to be linked to two separate loci associated with *P. capsici* resistance in CM-334 (Minamiyama et al., 2007) were included in this study. CAMS163 was not very informative (PIC = 0.29; Table 1) and all accessions except for ‘Early Jalapeno’, PI 201237, PI 201239, and PI 593572 shared at least one allele at this locus with CM-334. CAMS390 was more polymorphic (PIC = 0.65; Table 1) with only PI 593573, PI 439273, and PI 566811 sharing alleles with CM-334 at this locus (data not shown). Quirin et al. (2005) developed a SCAR marker that is linked to the *Phyto* 5.2 resistance locus of CM-334, the same association product was observed in PI 593573, the resistant accession closely related to CM-334. The presence of a marker allele associated with a resistance locus in a particular accession/population does not necessarily imply the same association in different accessions. To determine whether D04 SCAR is associated with the *Phyto* 5.2 resistance locus in other accessions, linkage has to be shown in segregating populations. However, until such information becomes available, breeders who want to increase the probability of introducing several resistance genes/alleles into their breeding material should take genetic diversity among accessions (Fig. 2) into account.

Genotype diversity within accessions is a measure of uniformity - how similar individual plants within an accession are to each other (Tommasini et al., 2003). A value of 0 indicates no variation within accessions (all individuals have identical genotypes), whereas a value of 1 indicates that all individuals have different genotypes. PI 439273 had the highest genotype diversity, whereas several accessions show no genotype diversity (Table 2). For accessions that show high genotype diversity, breeders have to take into consideration that these lines are likely to show higher phenotypic variation within lines. There did not seem to be a direct association between genotype diversity scores and the variation of root rot severity reported for these lines by Candole et al. (2010). For example, PI 439273, PI 566811, and PI 640461 had the highest levels of genotype diversity and resistance ranges of 1 to 3, 0 to 2, and 1 to 4, respectively. In comparison, PI 631147, PI 640516, and PI 640560 had very low levels of genotypic diversity but resistance ranges of 1 to 9, 0 to 5, and 1 to 9, respectively. This is probably the result of a combination of the inherent variation typical of resistance screens and the small number of plants per accession used in the current study. The genotypic and phenotypic variation within each accession cannot be fully captured using only three plants, leading to an underestimation of variation within accessions.

Although *C. annuum* are generally considered to be self-pollinating, cross-pollination does occur (Pickersgill, 1997). In self-pollinating crops, low levels of heterozygosity would be expected. The majority of accessions show low levels of heterozygosity, with the exception of PI 439273, PI 566811, and PI 640461 (Table 2). Most of the accessions that show high genotype diversity also show high heterozygosity, indicating that outcrossing took place in these accessions. The cultivars Aristotle and Camelot show low genotype diversity and high heterozygosity as would be expected of F1 hybrids from inbred parents.

The information provided by this study will help breeders choose the most appropriate resistant accession(s) to incorporate into their program to achieve their ultimate breeding goals. Two particularly interesting accessions are PI 201237 and PI 640532. These accessions have relatively large fruit, thick pericarps, and high and uniform levels of root rot resistance (Candole et al., 2010). These accessions can potentially be useful for introgressing *P. capsici* resistance into bell pepper.

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