CUCUMBER (*CUCUMIS SATIVUS* L.) FRUIT DEVELOPMENT: FACTORS INFLUENCING FRUIT SIZE, SHAPE, AND RESISTANCE TO *PHYTOPHTHORA CAPSICI*

By

Marivi Colle

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ABSTRACT

CUCUMBER (*CUCUMIS SATIVUS* L.) FRUIT DEVELOPMENT: FACTORS INFLUENCING FRUIT SIZE, SHAPE, AND RESISTANCE TO *PHYTOPHTHORA CAPSICI*

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Fruit size and shape are important quality traits in cucumber (*Cucumis sativus* L.) influencing market class and value, however the underlying mechanism driving variation is not known. Two sequenced cucumber cultivars representing extremes in fruit size and shape, 'Gy14' (pickling type) and '9930,' (Chinese long, CL) (long, narrow fruit) and their F_2 and RIL progeny were evaluated for ovule number, ovary length and diameter, fruit length and diameter, cell number and cell size from 7 days pre-anthesis (dpa) to 20 days post-pollination (dpp). Size and shape differences were influenced by numerous independent factors acting both pre-anthesis and post-pollination including the timing and orientation of cell division and cell expansion. Factors controlling fruit length were largely determined pre-anthesis, while factors regulating diameter were largely determined post anthesis. Expression of select marker genes and homologs of known fruit size genes were compared between CL and two pickling cucumber cultivars across fruit development, and were located with respect to fruit size QTL in cucumber. Several fruit growth related genes clustered in fruit size QTL in chromosomes 3 and 6. A cucumber homolog of Arabidopsis *ATHB-2*, a gene that controls direction of cell expansion, showed elevated expression earlier in CL relative to Gy14, correlating with longer cells in the longitudinal section. *ATHB-2,* which maps to major fruit size QTL *FS3.1*, had a deletion within the GAGA regulatory element in the 5' noncoding region of the CL allele.

Fruit development also influences susceptibility to infection by *Phytophthora capsici,* a major constraint in cucumber production. Our prior work showed that cucumber fruit (cv.

Vlaspik) exhibit age-related resistance (ARR) to *P. capsici.* Young fruits are highly susceptible, but as they reach the end of exponential growth (~10-12dpp), they become resistant. Screening of 8dpp and 16dpp fruit from 21 cucumber cultivars showed genetic variation in ARR expression to *P. capsici*. Crosses between ARR+ cultivars and Gy14 (ARR-) and their F_1 and F_2 progeny were used to examine inheritance of ARR in cucumber. F_1 fruits showed intermediate values between the parents. F_2 progeny showed a bimodal distribution suggesting one or more dominant factors regulating ARR. Our previous studies indicated that cucumber fruit surface was associated with ARR, suggesting possible physical or chemical components of resistance. Cucumber peels from 8dpp and 16dpp Vlaspik (ARR+) fruit were sequentially extracted with water and methanol, and a microtiter plate assay was developed to evaluate the antimicrobial activity of peel extracts against *P. capsici* by both visual growth and fluorescence assay. Greater inhibition of *P. capsici* growth was observed in wells treated with methanolic extracts from 16dpp fruit than 8dpp fruit. The aqueous extracts did not inhibit *P. capsici* growth.

Finally, in an effort to identify a source of resistance that would be expressed in very young fruit, a streamlined detached fruit method for high throughput screening was developed to test the U.S. cucumber Plant Introduction (PI) collection. A total of 1076 PI accessions, from 54 geographic locations, and the susceptible commercial cultivar, Vlaspik, were grown in the field over two seasons. Very young fruit (~4dpp) were tested for resistance to *P. capsici*. A set of 29 potentially resistant PIs was retested in the field. Three accessions, PI109483, PI178884 and PI214049, and their selfed progeny showed consistent, low disease ratings and may be considered useful for resistance breeding.

To my wonderful family and friends,
who never made me feel alone on this roller coaster ride of graduate school life,
thank you!
To my husband,
for the sacrifices you made, I give my deepest expression of love and appreciation…
this is for you!

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CHAPTER 1: Literature Review

INTRODUCTION

Cucumber (*Cucumis sativus* L., 2n=14) belongs to the Cucurbitaceae family, and is nested to the Asian/Australian clade of *Cucumis* (Renner et al., 2007). It has been cultivated since ancient times as a source of food and medicinal compounds (Janick et al, 2007). *Cucumis* species are suggested to be geographically centered in Africa except for *C. sativus* and *C. hystrix,* which are considered to be of Asian origin (Zhuang et al., 2006, Yang et al., 2012). Sequencing of 115 cucumber accessions from a core collection showed that cucumbers belong to four major geographic regions: East Asia, Eurasia, Xishuangbanna, and India (Qi et al., 2013). India and China are considered the first and second center of genetic diversification of cucumber, respectively (Sebastian et al 2010). Domestication of cucumber in India dates back to 3000B.C., and to 2000B.C. in China. Cucumber was introduced to Europe in the 13th century B.C. and in North America in the mid-16th B.C. (Staub et al., 1999, 2005, Paris et al., 2012).

Variation in cucumber fruit size and shape

The closest wild form of cucumber is found in India, the feral type *C. sativus* var. *hardwickii* with small and bitter fruits. Continued selection during domestication resulted in different cucumber cultivars showing an increase in variation in fruit size and shape. There are currently 1,486 cucumber plant introduction (PI) accessions listed in the USDA-ARS Germplasm Resources Information Network (GRIN), available in the North Central Regional Plant Introduction Station in Ames, Iowa (http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?12580#image) showing remarkable variation in fruit size and shape. Moreover, difference in fruit size and shape is also common among commercial varieties of cucumber being grown worldwide. In India, the most popular high-yielding commercial cultivar is Japanese Long Green, which is long and with even, dark-green color (Rai et al., 2008). In

China, the major cucumber market classes are the North China, which is about 30cm long and light colored, and South China type, which is ~40cm long and green (Jiang et al., 2015). Other major types of cucumber being cultivated worldwide include, Dutch Gherkin (~4-8cm long when harvested), the German Schalgurken type, English or European glasshouse type (~40cm long and thin), Mideast Beit Alpha type (similar with European glasshouse but shorter), Oriental trellis (Burpless) type, "lemon" cucumber (almost round shape), and the American processing and fresh market types.

In U.S., the two predominant types of cucumber commercially grown are pickling (~15 cm long and blocky), and fresh market/slicing types (~30cm long and thin). Michigan is the top producer of pickling cucumber in the country while the leading producers of slicing cucumber are Florida and Georgia (USDA 2013). Marketability of cucumber fruit depends on its quality as dictated by standards set by the USDA-Agricultural Marketing Service including specifications for length and diameter. Fruit size and shape are important quality traits in cucumber, and since the 1880s, fruit shape is one of the criteria used by breeders for selecting cultivars for quality and yield improvement (Robinson and Decker-Walters 1997).

Factors influencing fruit size and shape

Ovary Development

Fruit size and shape is the result of variation in general physiological processes involved in fruit development including ovary growth and fruit growth (Tanksley 2004, Johnson and Malladi 2011). Ovary development is regulated by a number of factors including hormones (i.e. ethylene, auxin, and cytokinin) and modulators of cell division and expansion (Gillaspy et al., 1993, Krizek 1999, Causier et al., 2002, Ozga and Reinecke 2003).

Cucumber has an inferior ovary usually containing three fused carpels, except for the "Lemon" cultivar with five carpels (Goffinet 1990; Robinson and Decker-Walters 1997). Increase in ovary size during development coincides with rapid cell division, however around anthesis, cell division slows down, and growth and development of the ovary typically ceases, causing senescence unless there is fertilization of the ovules (Ozga and Reinecke 2003, Machemer et al., 2011). For parthenocarpic cucumbers, the ovary continues to grow even in the absence of fertilization.

Reports on cucumber ovary development indicated that there is a correlation between size of ovary and mature fruits indicating that factors controlling fruit size and shape may exist early during initiation of ovary development (Goffinet 1990). Furthermore, QTL studies in melon showed that ovary shape is strongly correlated to fruit size and shape (Perin et al. 2002)

Cell division pre-anthesis

In early studies on ovary growth in cucurbits, it was observed that growth during early ovary development is due to an increase in cell number (Sinnott 1939). In tomato, cell division pre-anthesis was found to influence fruit size such that the number of cells in the ovary pericarp at anthesis served as the basis of the succeeding cell division upon pollination (Bohner and Bangerth, 1988). In blueberry, and olive, variation in cell number of different genotypes at anthesis indicated that cell division pre-anthesis has a major role in the differences in fruit size (Johnson and Malladi 2011, Rosati et al., 2011).

Ovule and seed development

Fruit size is also often a function of the number of successful fertilizations that have occurred in the ovary (Bohner and Bangerth, 1988). This is supported by observations in cucumber and tomato where the number of fertilized ovules determined the initial growth rate of

the ovary and amount of cell division (Varga and Bruinsma, 1986, Gillaspy et al., 1993). Early studies in cucumber indicated that fertilization occurs in ovules near the apical end of the ovary within 72 hours after pollination (Young 1943), and start of fruit growth is apparent as early as 24 hours after pollination (Fuller and Leopold 1975). Research by Varga and Bruinsma (1990) has shown that for cucumbers, fertilization is not necessary but the physical contact with the pollen tubes triggers ovary growth. However, ovary growth in a parthenocarpic cultivar occurs at a slower pace compared to pollinated cultivar, indicating that ovule and seed development is correlated with rapid fruit growth.

Carpel number

Carpel number has also been found to be associated with fruit size and shape. A study of *locule number* and *fasciated* in tomato showed that carpel number is a determining factor of fruit size (Cong et al., 2008, Munos et al., 2011). In cucurbits, a QTL experiment in melon showed that there is a strong correlation between carpel number and fruit shape involving the *pentamerous* gene, which has pleiotropic effects on fruit shape where five-carpel fruit are rounder than three-carpel fruit (Fernandez-Silva et al. 2010). However, effect of carpel number on fruit size of cucumber has not been reported yet.

Fruit growth post-pollination

Cell division and cell expansion post-anthesis

Fruit growth is driven both by increase in cell number and cell size (Gillaspy et al., 1993, Zhang et al., 2006). Fruit growth in cucumber is characterized by rapid cell division that occurs at 0-4 days post pollination (dpp) then slows down until 8dpp, followed by increase in cell size (Marcelis 1994, Boonkorkaew et al., 2008, Fu et al., 2008, Ando and Grumet 2010, Ando et al., 2012). Increase in fruit length in pickling cucumber cultivar ‘Vlaspik’, later in development,

coincides with increase in cell size (Ando and Grumet 2010). During fruit growth, mesophyll cells became vacuolated and cell walls of epidermal cells thickened (Ando and Grumet 2010). This was also observed during fruit development in watermelon where rapid cell division occurred during early fruit growth followed by cell expansion that resulted in the formation of large vacuolated cells (Wechter et al., 2008).

Prior studies on species exhibiting variation in fruit size have shown that cell number, not cell size, largely influence fruit size. In drupes such as sweet cherry, apricot, peach, and olive, variation in cultivar fruit size was due to the difference in mesocarp cell number (Hammami et al., 2011). This was also observed in different blueberry (Johnson and Malladi 2011) and strawberry genotypes (Cheng and Breen 1992). In melon, difference in pericarp cell number resulting from variation in period of cell division was associated with difference in fruit size (Higashi et al., 1999). On the other hand, a study in apple suggested that in addition to cell number, larger cell size and increased ploidy through endoreduplication were also considered contributing factors to the variation in fruit size (Malladi and Hirst 2010). Recent studies on Chinese cucumber cultivars also indicated that variation in fruit size was due to both cell number and cell size (Yang et al., 2013, Jiang et al., 2015).

Cell cycle-related genes involved in fruit growth

Following fertilization of the ovary, fruit development has two distinct phases – cell division and cell expansion (Gillaspy et al. 1993). Cell proliferation and growth is regulated by a number of factors controlling the different phases (G1, S, G2, and M) of the cell cycle (Inze and De Veylder, 2006, De Veylder et al., 2007). Different sets of genes such as cyclin-dependent kinases (CDKs), cyclins, CDK inhibitors, and CDK subunits regulate cell cycle. In Arabidopsis, tomato, maize, and rice, core cell cycle genes have been identified (Vandepoele et al., 2002,

Joubes et al., 2000, Menges et al., 2005, Rymen et al., 2007, Guo et al., 2007). Expression of different cyclin genes in tomato, and B-type CDKs and A2-, B1-, and B2-type cyclins in apple was associated with active cell division during early fruit growth (Joubes et al., 2000, Srivastava and Handa 2005, Malladi and Johnson 2011).

At the end of cell division, the cell expansion phase begins (Gillaspy et a., 1993). Core set of cyclins, CDKs, CDK inhibitors and other cell cycle genes were also associated with the regulation of cell expansion (Inze and De Veylder, 2006). In tomato, CDK inhibitors (e.g. *LeKRP1* and *LeKRP2)* and anaphase promoting complex activator (e.g. *SICCS52A)* are involved in regulating cell expansion through endoreduplication (Bisbis et al., 2006, Mathieu-Rivet et al., 2010, Nafati et al., 2011).

In cucumber, cyclins, cyclin-dependent kinases and kinesins that exhibit increased expression post-pollination or during early fruit growth were also identified (Fu et al., 2008, Ando et al., 2012). Recent studies had shown that expression of microtubule-associated genes, such as kinesins, correlated with fruit size variation in cucumber (Yang et al., 2013, Jiang et al., 2015).

Fruit size and shape genes in other plant systems

Several studies have examined the variation in fruit size and shape in tomato and have investigated the genetic basis of tomato fruit morphology (e.g. Frary et al., 2000, Tanksley 2004, Cong et al., 2008, Xiao et al., 2008, Rodriguez et al., 2011). Major genes that control fruit size and shape were identified, including *Fw2.2*, *OVATE*, *SUN*, and *FAS. Fw2.2* affects fruit size by negatively regulating cell number (Guo and Simmons 2011), and by controlling cell size through direct interaction with casein kinase II (Libault and Stacey 2010). Orthologs of *Fw2.2*, cell number regulator (*CNR*) in maize (Guo et al., 2010), avocado (Dahan et al., 2010) and cherry

(De Franceschi et al., 2013) have also been identified. The *OVATE* gene encodes a 60–70 amino acid C-terminal domain and a single mutation within the coding region resulted in a premature stop codon causing the transition of tomato fruit from round to pear-shaped (Liu et al. 2002). On the other hand, the *SUN* gene, which encodes a protein containing the IQ67 domain, controls elongated fruit shape by affecting the direction of cell division (Tanksley 2004, Xiao et al., 2008, Wu et al., 2011, Huang et al., 2013). The *fasciated* (*FAS*), and *locule number* (*LOC*) were found to control both fruit shape and size (Tanksley 2004, Rodriguez et al., 2011, Munoz et al., 2011, Huang et al., 2013). Mutation in *FAS* due to inversion of an ortholog of *YABBY2* resulted in increase in locule number, hence a flat-shaped tomato. In addition, recent report in tomato implicated a locus, *Solanum lycopersicum elongated fruit1* (*Slelf1*), on the increase in cell layer in the proximal region of the ovary resulting in elongated fruit shape (Chusreeaeom et al., 2014). In cucurbits, specific genes regulating fruit size and shape have not been described.

Genetic factors associated with fruit traits in cucurbits

Mapping studies have identified fruit size and shape QTLs in various cucurbit species. Tanaka et al. (1995) showed that fruit shape index of watermelon is influenced by a single incompletely dominant gene. In melon, 8 QTLs for fruit shape have been identified (Monforte et al., 2004) and loci for bigger fruit were shown to be dominant while loci for rounder fruit were additive or recessive (Fernandez-Silva et al., 2009). Moreover, Perin et al. (2002) showed that QTL for fruit shape and ovary shape co-segregate, indicating early control in fruit shape during ovary development. In addition, mapping study in melon also identified 42 QTLs associated with fruit shape (Diaz et al., 2011). Recent sequencing of the melon genome facilitated a comparative analysis of tomato gene families associated with fruit size and shape, and identified homologs for fruit size and shape genes in melon (Monforte et al., 2014).

Several studies have also examined the genetic factors controlling fruit related traits in cucumber. Yuan et al. (2008a,b) detected 37 QTLs for fruit length, fruit diameter, length/diameter ratio, fruit flesh thickness, and seed cavity diameter. Their research showed a high correlation between fruit length and fruit weight, and length/diameter ratio and fruit weight however, they did not find a significant correlation between length/diameter ratio and fruit diameter. Other QTL studies for fruit related traits in cucumber (i.e. Serquen et al., 1997, Fazio et al., 2003, Wang et al., 2005) have been conducted, however, relatively unsaturated maps were used for all of these studies due to the narrow genetic background of cucumber and low polymorphism (Bradeen et al., 2001, Heang et al., 2008, Yuan et al., 2008a,b). Further study is needed to identify factors regulating fruit size and shape in cucumber.

Available genomic resources for investigating fruit traits in cucumber

Cucumber has a small genome size of 376MB, it can be easily grown and has short life cycle. In the past few years, three different cucumber cultivars have been sequenced: Chinese long (North China fresh market type), Gy14 (North American pickling type), and North-European Borszczagowski cucumber cultivar (line B10) (Huang et al., 2009, Yang et al., 2012, Woycicki et al., 2011).

The availability of the cucumber genome sequence led to a dramatic increase in the genomic resources over the past years including large-scale identification of molecular markers and construction of high-resolution linkage maps using hundreds of SSR markers to identify fruit trait related QTLs (Ren et al. 2009, Cavagnaro et al., 2010, Yang et l., 2013). High-density genetic maps were also constructed using QTL-sequencing (QTL-seq), and Specific Length Amplified Fragment sequencing (SLAF-seq) to map major QTLs controlling flowering and fruit traits in cucumber (Xu et al., 2014, Wei et al., 2014, Lu et al., 2014). A recently developed single

nucleotide polymorphism (SNP) array has 45,000 SNPs with 4,000 to 6,000 SNPs per chromosome and additional 8,000 SNP from non-assembled scaffolds (Rubenstein et al., 2015). This tool, coupled with substantial information on fruit morphology and fruit development of a segregating cucumber population will be valuable in identifying fruit size QTLs and/or in cloning of specific genes controlling fruit size and shape.

Relationship between cucumber fruit development and *Phytophthora capsici* infection

Many of the cucumber growing areas in Michigan are contaminated with the oomycete pathogen *Phytophthora capsici* (*P. capsici)* resulting in considerable yield losses (Granke et al., 2012). Unlike in other hosts, *P. capsici* specifically infects fruits in cucumbers (Hausbeck and Lamour 2004).

Continuous spread of *P. capsici* infection among vegetable crops has been reported in the state. Major factors contributing to the spread of the disease include the use of irrigation water infested with the pathogen, specifically the zoospores which is the primary inoculum for spread throughout the growing season, and the ability of *P. capsici* oospores to survive in the soil for many years (Brasier 1992, Hausbeck and Lamour 2004, Padley et al., 2008, Granke et al., 2009, Lamour et al., 2011).

A number of methods have been used to manage disease occurrence. Some farmers plant their crops in a new location however, spread of *P. capsici* was still observed due to contaminated irrigation water or from dumping of fruits rejected from processing plants near the farm. Another method commonly used is crop rotation, however, the long-term survivability of oospores even in the absence of hosts limits its effectiveness. For example, Lamour and Hausbeck (2001) suggest that loss in squash production in 1999 was due to dormant oospores that were in the field 5 years prior to planting of squash. Other strategies of controlling disease

include planting into well-drained fields and well-raised beds whenever possible as well as application of fungicides, however certain strains of *P. capsici* have been reported to develop resistance to some fungicides (Babadoost 2004, Hausbeck and Lamour 2004, Meng et al., 2011). Yield loss due to *P. capisci* infection will be a continuing problem in cucumber production unless genetic resistance for the pathogen is developed.

Phytophthora capsici

Phytophthora capsici was first described by Leonian (1922) as the pathogen causing the disease in pods and branches of chili peppers (*Capsicum annuum* L.) in New Mexico, and disease occurrence usually happens during warm and rainy season. Modern classification of *P. capsici* indicates that it belongs to the group of oomycetes under Phylum Oomycota, kingdom *Straminipila/Straminopila* (Hausbeck and Lamour 2004, Lamour et al., 2011, Levesque 2011).

During the sexual stage, when A1 and A2 mating types are paired, oospores, which have a thick, multilayered wall containing β-glucan and cellulose, are produced (Hausbeck and Lamour 2004). *P. capsici* also produces thalli that give rise to lemon-shaped sporangia. *P. capsici* sporangia and spores are unlikely to be dispersed across fields through wind current. However, in the presence of water and favorable temperature, motile spores called zoospores, are released from the sporangia, which are capable of infecting plant parts.

Species of *Phytophthora* can infect host plants through hyphae, sporangia or zoospores (Robold and Hardham 2005, Hardham 2007). Zoospores increase the chance for the spread of the disease since they are motile, and are chemotactically and electrotactically attracted to potential infection sites on the surface of host plants. These tactic responses can either be non-specific or specific. For non-specific tactic response, zoospores are attracted to both host and non-host plants due to compounds such as sugars and amino acids diffusing from the plants, while for

specific tactic response, zoospores recognize specific chemoattractant from the host plant. After reaching the surface of a potential host plant, the swimming pattern of zoospores changes such that its ventral surface faces the plant, and this is followed by encystment. During encystment, their flagella detach, and materials are secreted from their vesicles that rapidly change their plasma membrane. Adhesion proteins are produced that prevent the spores from being dislodged from the plant surface, as well as facilitating reception of signals for the development and penetration of specialized infection structures such as hyphae or appressoria. *Phytophthora* hyphae may penetrate the plant surface either along anticlinal walls or directly through the outer periclinal wall (Robold and Hardham 2005, Hardham 2007, Hardham and Shan 2009).

Along with the penetration of hyphae, *Phytophthora* species secretes cell wall degrading enzymes. These enzymes include pectinases, and glucanases (Li et al., 2011). For necrotrophic species of *Phytophthora*, hyphae may grow intercellularly or intracellularly to absorb the required nutrients from dead and dying cells. On the other hand, for hemibiotrophic species such as *P. capsici*, hyphal growth is restricted to the apoplast and disruption of host cells is minimized, and acquisition of nutrients is through haustoria that form predominantly in mesophyll or cortical cells (Hardham 2007, Hardham and Shan 2009).

Early studies suggested that *P. capsici* is host specific to pepper however, it was found that this pathogen also infects other members of Solanaceae, Fabaceae, Pinaceae, Malvaceae, Euphorbiaceae, Proteaceae, Caricaceae family as well as Cucurbitaceae family including winter and summer squashes, pumpkin, zucchini, melon, and cucumber (Babadoost 2004, Li et al., 2011, Enzenbacher and Hausbeck 2012, Granke et al., 2012).

Genetic resistance to *P. capsici*

Developing high levels of resistance against *P. capsici* as a way to control disease incidence is the goal of breeding programs for many vegetable crops. In cucurbits, *Cucurbita pepo* accessions were screened for crown rot resistance to isolates of *P. capsici*. Eight accessions were observed to have low mean disease rating thus, have the potential to be used for breeding of lines and cultivars with resistance to *P. capsici* (Padley et al., 2008, Padley 2008). In addition, a Cucurbita breeding line, #394-1-27-12, developed in the University of Florida, showed resistance to the crown rot syndrome of *P. capsici*, and was used to determine the inheritance of resistance to this disease. Results indicated that resistance is conferred by three dominant genes (Padley and Kabelka 2009). In 2011, Chavez et al. identified five accessions in the *Cucurbita moschata* germplasm with resistance to Floridian isolates of *P. capsici*. Recently, two *C. pepo* accessions (PI 169417 and PI 181761) were identified to exhibit resistance to *Phytophthora* fruit rot (Krasnow et al., 2014). For cucumber, fruits at harvest stage of over 300 cucumber varieties and plant introduction accessions were screened for resistance to *P. capsici,* however, complete resistance was not observed (Gevens et al., 2006). Further screening needs to be done since not all of the cucumber accessions were tested against *P. capsici*. Currently, there are 1,486 cucumber plant introduction (PI) accessions listed in the USDA-ARS Germplasm Resources Information Network (GRIN), available in the North Central Regional Plant Introduction Station in Ames, Iowa (http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?12580#image) and possible source(s) of resistance may potentially come from plant introduction accessions that were not tested yet.

Age-related resistance (ARR)

When screening for cucumber fruit for resistance to *P. capsici*, it was observed that as fruits completed the period of rapid fruit elongation, they became less susceptible to *P. capsici* (Gevens et al., 2006, Ando and Grumet 2008, Ando et al., 2009). This transition from susceptibility to resistance usually occurred at around 10-12 days post-pollination. Developmentally regulated resistance, wherein resistance increases with plant or tissue age, is also referred to as age-related resistance (ARR) and has been observed in other host plant-pathogen interactions (Ficke et al., 2002, Panter and Jones 2002, Develey-Reviere and Galiana 2007).

ARR may be expressed at the whole plant level or in specific organs or tissues as a function of tissue maturity. An example of ARR in the whole plant level has been observed in pepper, wherein under controlled environmental conditions, as pepper plants became mature, they also became increasingly resistant to *P. capsici* (Kim et al., 1989). Similarly, pepper cultivars at 8-leaf were susceptible while 12-leaf stage plants were resistant when inoculated with *P. capsici* (Hwang et al., 1996). ARR in pepper was also observed at the whole plant level when inoculated with *Ralstonia solanacearum* (Lemessa and Zeller 2007), or with cauliflower mosaic virus (CMV) (Garcia-Ruiz and Murphy 2001). In cotton, mutant lines/varieties became increasingly resistant to cotton leaf curl virus (CLCuV) as plants aged (Akhtar et al., 2004). ARR was also observed in mature *Arabidopsis thaliana* when inoculated with *Pseudomonas syringae* pv. *tomato* (Al-Daoud and Cameron 2011).

In the other cases, specific organs develop resistance. Grapevine (*Vitis* spp.) berries exhibited ARR and became become nearly immune to infection by powdery mildew (*Uncinula necator*) within 4 weeks after fruit set (Gadoury et al., 2003, Ficke et al., 2003). The same

developmental resistance was also observed when mature grape berries were inoculated with *Guignardia bidwellii*, the pathogen causing grape black rot (Hoffman et al., 2002). Leaves of some plants also manifest ARR. For example, the fully expanded and mature leaves of two soybean cultivars Harosoy 63 and Harosoy exhibit resistance to *Phytophthora megasperma* (Bhattacharyya and Ward 1986, Ward 1989). ARR in leaves was also demonstrated in cowpea wherein increasing leaf age correlated with increase in resistance against race 1 of *Uromyces vignae* (Heath 1994). In pepper, infection of leaves by *C. coccodes* at the two-leaf stage resulted in massive colonization of all the leaf tissues including the vascular tissue, however penetration of *C. coccodes* was very limited in the older leaf tissues at the eight-leaf stage (Hong et al., 1998). In addition, mature leaves of wild-type rice plants (*Oryza sativa* L. cv. *Nipponbare*) are more resistant to blast fungus (*Magnaporthe grisea*) than new leaves (Xie et al., 2011).

ARR may also be a function of developmental transition in the plant life cycle such as transition to flowering or senescence (Panter and Jones 2002, Develey-Reviere and Galiana 2007). Effect of developmental transition was observed in *Corngrass1* mutant (*Cg1*) of corn (*Zea mays)* (Abedon and Tracy 1990). The juvenile-vegetative phase is extended in *Cg1* mutant. Inoculation of *Cg1* mutant with *Puccinia sorghi*, pathogen causing common rust in corn, showed that adult resistance to the pathogen was delayed wherein its mid-whorl leaves of the *Cg1* plants continue to display juvenile traits and susceptibility to *P. sorghi* while mid-whorl leaves of the wild type were resistant to the pathogen. In lentil cultivars, tissues below the top four or five nodes on the main stem and secondary branches were almost completely resistant to *Ascochyta fabae* and resistance was most apparent at the podding stage (Pedersen and Morrall 1994). In the case of potato, six varieties of potato exhibited maximum level of resistance to *P. infestans* when they were at the bud stage and just transitioning to flowering. This suggests that blight resistance

in potato is a developmentally regulated response as the plant transitions from vegetative to reproductive stage (Mutty and Hossenkhan 2008). The same was observed in Arabidopsis when resistance to *Pseudomonas syringae* coincided with the transition to flowering (Rusterrucci et al., 2005).

ARR can also influence race-specific resistance or can provide a broad spectrum of protection. In rice, expression of *Xa3* and *Xa21* against *Xanthomonas campestris* pv. *oryzae* and *Xanthomonas oryzae* pv. *oryzae*, respectively, was influenced by plant age and leaf maturity (Koch and Mew 1991, Century et al., 1999). Also, in wheat, resistance genes, *Lr 21* and *Lr* 22, were effective against *Puccinia recondita* at adult plant but not in seedling stage (Kumar et al. 1988). On the other hand, broad-spectrum resistance due to ARR was reported in tobacco wherein mature plants exhibited resistance to several pathogen including *Peronospora tabacina*, *Phytophthora parasitica,* and tobacco *mosaic virus* (Carviel et al., 2009).

Mechanisms of age-related resistance (ARR)

Development of resistance depends on the life cycle of the particular plant, however once it is acquired, resistance generally persists until senescence (Develey-Reviere and Galiana 2007). The mechanism involved in ARR is generally different from the response of plants to infection as a result of systemic acquired resistance (SAR) or induced systemic resistance (ISR), although there are some exceptions wherein ARR conforms to the gene-for-gene (*Avr*-*R*) model and showed similarity to SAR. In general, ARR in different pathosystems suggests that a variety of complex mechanisms are involved (Panter and Jones 2002, Develey-Reviere and Galiana 2007).

Anatomical and morphological changes during development

Fruit surface plays a role in plant development and provides protection against abiotic and biotic factors such as pathogens. Part of the structure of the fruit surface is the cuticle, which

serves as the first structural barrier that the pathogen must breakdown to gain access to the cells. In grape berries, cuticle thickness increased as the fruit matured however, the increase does not correlate with the degree of resistance in older berries (Ficke et al., 2002, 2004).

Plant compounds with antimicrobial activity

The physiological/biochemical basis of resistance of plants to fungal, oomycete, and bacterial pathogens has been associated with both preformed and infection-induced antimicrobial compounds (Hammerschmidt 1999, Mert-Türk 2002). During development, plants may synthesize compounds with antimicrobial activity. For example, developmentally regulated preformed antifungal activity was found in flower tissue and in the achenes of green stage strawberry fruit (Terry et al., 2004). Extracts of strawberry flowers at post-anthesis showed greater antifungal activity than at white bud and full bloom stages. In maize, accumulation of phenolics and amides were also shown to be developmentally and spatially regulated (Le Clere et al., 2007). In grape, Ficke et al. (2004) suggested that a pre-formed biochemical compound near the cuticle surface was associated with resistance to *Uncinula necator* of grape berry.

While pre-formed/constitutively produced ARR-related compounds that inhibit *Phytophthora* have not yet been identified, anti-*Phytophthora* phytoalexins, which are defined as low-molecular-weight antimicrobial compounds that are induced after infection, have been observed. In pepper, there was a report on the role of post-infectionally formed capsidiol in the age-related resistance of pepper plants to *P. capsici* (Hwang and Kim 1990). Accumulation or elicitation competence of pterocarpan phytoalexin glyceollin in soybeans as a response to infection by *Phytophthora sojae* was found to be affected by age or developmental state of tissues wherein elicitation is maximal in 7-9 days old cotyledons (Abbasi and Graham 2001).

In cucumber, biochemical compounds associated with inhibition of *P. capsici* infection have not been identified yet. However, a number of studies have looked at the possible antimicrobial activity of some compounds in cucumber. Amine extracts from 10-day old seedlings of cucumber were shown to have inhibitory effect against *Staphylococcus aureus* and on *Pseudomonas aeruginosa* (Flayeh and Sulayman 1987). Analysis of the components of amine fraction indicated the presence of spermidine, putrescine, and 1,3-diaminopropane.

It was also reported that phytoalexin-like compounds are, in part, mediating the induced resistance to pathogens in cucumber (Daayf et al. 1997). In their study, p-coumaric acid methyl ester was found to increase markedly in plants infected with powdery mildew along with another phenolic compound. Assayed cucumber leaf tissue showed induced resistance to powdery mildew fungus *Podosphaera xanthii* due to phytoalexin compounds (McNally et al. 2003a). Fluorescence microscopy showed increase in the production of autofluorescent C-glycosyl flavonoid phytoalexins within the epidermal tissues of disease-resistant leaves. Moreover, laser scanning confocal microscopy revealed that the autofluorescent C-glycosyl flavonoid phytoalexins accumulated inside the haustorial complexes of the pathogen in the epidermal cells of resistant plants creating an incompatible reaction with the pathogen. Further analysis of the C-glycosyl flavonoid phytoalexins showed two major *C*-glycosyl flavonoid products namely cucumerin A and cucumerin B. Other compounds such as *C*-glycosyl flavonoids apigenin-8-*C*-*â*-D-glucopyranoside (vitexin), apigenin-6-*C*-*â*-D-glucopyranoside (isovitexin), luteolin-8-*C*-*â*-D-glucopyranoside (orientin), and luteolin-6-*C*-*â*-D-glucopyranoside (isoorientin), and 4-hydroxycinnamic acid (*p*-coumaric acid) were also found in high concentrations in resistant leaf tissues (McNally et al., 2003b).

Volatiles such as (*E,Z*)-2,6-nonadienal(NDE) and (*E*)-2-nonenal (NE) were also rapidly produced during wounding of cucumber (Cho et al. 2004). Aside from giving cucumber certain aroma, these volatiles also showed bactericidal activity against *Bacillus cereus, Escherichia coli* O157:H7, *Listeria monocytogenes,* and *Salmonella typhimurium*. Similar results were observed when antimicrobial properties of peel and pulp extract of Greek cucumber fruit were analyzed (Sotiroudis et al., 2010). Volatiles from peel extract showed the highest and widest spectrum of antimicrobial activities due to high (E,Z)-2,6-nonadienal (NDE) and (E)-2-nonenal (NE) content. Sphingolipids ((*2S,3S,4R,10E*)-2-[(2'*R*)-2-hydroxytetra-cosanoylamino]-1,3,4 octadecanetriol-10-ene, 1-*O*-*β*-D-glucopyranosyl (*2S,3S,4R,10E*)-2-[(2'*R*)-2-hydroxytetracosanoylamino]-1,3,4-octadecanetriol-10-ene, and soya-cerebroside I) were also isolated from crude methanol extract of cucumber stems and exhibited antifungal activity against *Pythium aphanidermatum*, *Botryosphaeria dothidea*, *Fusarium oxysporum* f.sp. *cucumerinum* and *Botrytis cinerea*, and antibacterial activity against *Xanthomonas vesicatoria*, *Pseudomonas lachrymans*, and *Bacillus subtilis* (Tang et al., 2010).

ARR in cucumber

The relationship between fruit age and susceptibility to *P. capsici* was manifested in both field- and greenhouse-grown cucurbits such as butternut squash, acorn squash, pumpkin, and in selected cucumber PI accessions and several cucumber cultivars (Gevens et al., 2006, Ando et al., 2009, Ando 2009). In all of the fruits tested, younger fruits were more susceptible to *P. capsici* than older fruits. However, among the cucurbits examined, cucumber exhibited the most striking effect of fruit age to disease susceptibility such that almost complete resistance to *P. capsici* was observed in older fruits (Ando et al., 2009). After 12dpp, cucumber fruits manifested lack of symptoms or a limited hypersensitive response (HR) (Ando et al., 2009). At 4 dpi, fruits

younger than 10dpp exhibited sporulation and tissue collapse, and fruits between 10-12dpp showed water soaking without sporulation. The transition from susceptible to resistant occurred when period of rapid elongation was completed (Gevens et al., 2006, Ando et al., 2009).

Preliminary examination of the factors that might be influencing ARR in cucumber showed that the fruit surface was associated with ARR (Ando and Grumet 2006). Exocarp sections (1-2mm) were exchanged between 8dpp and 15dpp fruits such that exocarp section of 8dpp was placed on top of 15dpp intact fruit and vice versa. Exocarp sections were then inoculated with *P. capsici*. The exocarp sections showed the same disease response as a whole fruit. In addition, no symptom development was observed in 8dpp fruit underneath 15dpp peel. These results indicate that the fruit surface of 15dpp fruit has inhibitory activity against *P. capsici* (Ando 2009).

Certain fruit surface properties of cucumber fruit also influenced formation of germ tube and appressoria of *P. capsici* zoospores (Ando 2009). When intact 8dpp and 16dpp fruits were inoculated with *P. capsici* zoospores, preliminary result showed that germination was not affected for both fruits. However, zoospores formed short germ tubes on the surface of 8dpp compared to the medium and long germ tubes in 16dpp. The pathogen was also able to form appressoria on 8dpp fruit whereas aberrant germ tubes were formed on 16dpp fruit. Formation of short germ tubes and development of appressoria is associated with successful penetration (Grenville-Briggs et al. 2008). These observations suggest that changes in exocarp properties of developing cucumber fruits influence resistance to *P. capsici*.

Components of fruit exocarp are also involved in signal transduction in host-pathogen interaction (Kolattukudy et al. 1995, Yakoby et al. 2002). Cuticular lipids play a role as messenger molecules during pathogen infection such as in the formation of appressoria and

initiation of penetration (Hwang et al., 1995, Patto and Niks 2001, Skamnioti and Gurr 2007, Feng et al., 2009, Uppalapati et al., 2012). Cutin monomers and lipid transfer proteins were shown to be involved in plant defense reactions (Fauth et al., 1998, Kauss et al., 1999, Kim et al., 2008, Carvalho and Gomes 2007, Kirubakaran et al., 2008, Lee et al., 2009, Kiba et al., 2012). Phenolics such as cinnamic acids and flavonoids present in the cutin matrix were shown to have antimicrobial activity (Muller and Riderer 2005, Dominguez et al., 2011). In cucumber, previous studies showed that methanol soluble compounds present in cucumber fruit peel can inhibit growth of a number of pathogens (Sotiroudis et al., 2010, Tang et al., 2010). Transcriptome study on cucumber fruits at different developmental stages showed that genes associated with cuticle biosynthesis are developmentally regulated (Ando et al., 2012). Peak of expression of homolog of the *SHINE 1* transcription factor (Aharoni et al.,2 004), and homologs of lipid transfer and *GDSL* motif lipase genes in cucumber fruit coincided with the exponential and post-exponential growth stages (8dpp, 12-16dpp).

Moreover, it was also observed that abiotic and biotic stress related genes including a variety of heat shock, redox, biotic defense and ethylene-related transcripts were highly represented in 12+16dpp age group compared to 0, 4 or 8dpp (Ando and Grumet 2008, 2010, Ando et al., 2012, Ando et al., 2015). Also, peak of expression of these genes coincided with increased resistance to *P. capsici* infection. In other plant systems such as grapes, pepper and tobacco, defense-related genes that are expressed late in plant development were identified and were shown to be associated with ARR (Hwang et al., 1991, Shibata et al., 2010, Ficke et al., 2002, Kus et al., 2002, Mutty and Hosenkhan 2008).

In general, genetic factors controlling ARR are not yet well understood. To our knowledge, studies of ARR have only been reported in two systems. A report in pepper

suggested that ARR is controlled by two major genes with epistatic interaction (Reifschneider et al., 1992). On the other hand, a study in sorghum showed that a dominant gene influences ARR expression (Tenkouano et al., 1998). In both of these cases, there appears to be simple genetic control of ARR.

Previous reports shown differences in the manifestation of ARR among cucurbit crops (Ando 2009, Meyer and Hausbeck 2013), however, variation in ARR expression among pure breeding cucumber cultivars has not yet been examined. The potential differences in ARR expression of different cucumber cultivars will facilitate the development of materials to study genetic factors regulating ARR.

Objectives of dissertation

This research was focused on two aspects related to cucumber fruit development, fruit size and shape, and resistance to *Phytophthora capsici*. This study aimed to examine whether the high variation in fruit size and shape observed among different cucumber genotypes is influenced by growth factors including ovary size, ovule number, cell number, cell size, period and rate of cell division and expansion pre- and post-anthesis. The role of these factors was examined using two sequenced cucumber genotypes with extreme differences in fruit size and shape, *C. sativus var. sativus* cv "Gy 14" (pickling) and *C. sativus var. sativus* cv "9930" (Chinese long) along with their F_1 and F_2 progenies. Moreover, a parallel study was also performed to identify fruit growth QTLs in a recombinant inbred line (RIL) population derived from a Gy14xCL cross, and high density SNP array developed in the laboratories of Y. Weng (Univ. Wisconsin), and R. Ophir and A.Sherman (ARO, Israel). My study also examined the expression of fruit growth marker genes and select fruit size and shape homologs in Chinese long and Gy14.

Establishment of effective control against *P. capsici* in cucumber has been difficult. The most effective approach is to identify a durable source of resistance against this pathogen. Thus, the goal of this study was to screen the full U.S. cucumber PI (Plant Introduction) collection for resistance to *P. capsici*. However, since fruit testing is labor and space intensive, using our prior knowledge regarding greater susceptibility of young cucumber fruits (Ando et al., 2009, Gevens et al., 2006), we developed a modified testing method to allow for a more efficient inoculation for high throughput screening using young fruit. This will also prevent miss-assessment of potential resistance that can occur as fruits become older.

Based on prior work in the lab showing that fruit surface plays a role in the manifestation of ARR (Ando 2009), I also sought to investigate the potential role of biochemical compounds in fruit peel in inhibiting *P. capsici* growth, and to examine the structural changes in fruit peel during development using cucumber fruit from susceptible and resistant ages of both ARR+ and ARR- cultivars. Since the genetic basis of ARR to *P. capsici* is not yet understood, this study also aimed to examine different inbred cucumber cultivars for ARR expression, and using selected cultivars that do and do not express ARR, examine the inheritance of ARR in cucumber.

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CHAPTER 2: Factors influencing fruit size and shape in cucumber

INTRODUCTION

Fruit size and shape are important quality traits in cucumber influencing market class and value (Robinson and Decker-Walters 1997). In the U.S., the two predominant types of cucumber commercially grown are pickling (short and blocky), and fresh market/slicing types (moderately long and thin). Marketability of cucumber fruit depends on quality as dictated by standards set by the United States Department of Agriculture/ Agricultural Marketing Service (USDA/AMS) including specifications for length and diameter. Therefore, fruit size and shape are important criteria for breeding improved cucumber cultivars.

In general, fruit size and shape results from the interplay among numerous physiological processes involved in both ovary and fruit development (Gillaspy et al., 1993, Tanksley 2004). Early studies on cucurbit fruit development indicate that difference in fruit size among cultivars is a function of ovary size, and factors controlling fruit size and shape may exist early during ovary development as suggested by the relationship between ovary size and mature fruits (Goffinet 1990). Ovary development is driven by factors involved in the initiation of ovary primordia, ovary growth and ovule development. Hormones (i.e. ethylene, auxin, cytokinin and brassinosteroids) and other factors in cell division are key regulators of ovary development (Gillaspy et al., 1993, Ozga and Reinecke 2003, Fanwoua et al., 2013). Increase in ovary size typically coincides with the period of rapid cell division (Gillaspy et al., 1993, Ozga and Reinecke 2003) and cell division pre-anthesis was found to influence size in fruits such as tomato (Bohner and Bangerth, 1998), blueberry (Johnson and Malladi 2011) and olive (Rosati et al., 2011).

During cucumber ovary development, cell division slows at anthesis and in the absence of pollination in non-parthenocarpic varieties, growth and development of the ovary typically

ceases, resulting in senescence (Varga and Bruinsma 1990). However, upon fertilization of the ovules, enlargement of the ovary continues and growth is apparent as early as 24 hours after pollination (Fuller and Leopold 1975). Rapid cell division occurs during the first few days post-pollination (0-4dpp) followed by increase in cell size (Marcelis 1994, Boonkorkaew et al., 2008, Fu et al., 2008, Ando and Grumet 2010, Ando et al., 2012). In tomato (Bohner and Bangerth, 1988; Tanksley 2004) and in some members of Rosaceae (e.g. Cheng and Breen 1992, Yamaguchi et al., 2002, Olsmtead et al., 2007, Johnson and Malladi 2011), variation in cultivar fruit size is due to difference in cell number. In melon, difference in pericarp cell number resulting from variation in period of cell division was associated with difference in fruit size (Higashi et al., 1999).

Our recent transcriptome study examining early fruit growth of cucumber showed genes that exhibit developmental specific patterns of expression (Ando et al., 2012). At 0+4dpp (cell division stage), genes that were highly represented included cell cycle-related genes such as cyclins and cyclin dependent kinases (Ando et al. 2012). High expression of cell cycle related genes also was observed for early fruit development in parthenocarpic cucumber (Fu et al., 2010). This is consistent with previous observations from other systems such as tomato and apple where rapid cell proliferation during fruit development was associated with high expression of core cell cycle genes (Joubes et al., 2000, Vandepoele et al., 2002, Menges et al., 2005, Rymen et al., 2007, Guo et al., 2007, Malladi and Johnson 2011).

Previous reports have described genes that are involved in regulating cell division and whose expression is correlated with fruit size and shape including *Fw2.2* and *SUN* (Frary et al., 2000, Tanksley 2004, Cong et al., 2008, Xiao et al., 2008, Xiao et al., 2009). *Fw2.2* negatively regulates cell number, thus influencing final fruit size in tomato (Libault and Stacey 2010, Guo

and Simmons 2011). Orthologs of *Fw2.2* have been identified in other species such as maize (Guo et al., 2010), avocado (Dahan et al., 2010), and cherry (De Franceschi et al., 2013). *SUN* gene controls fruit shape by regulating the direction of cell division (Tanksley 2004, Xiao et al., 2008, Wu et al., 2011). In melon, homologs of *SUN* were also identified (Monforte et al., 2014). Moreover, a fruit shape locus, *Slelf1*, was recently reported in tomato and this locus was associated with the increase in cell layer in the proximal region of the ovary resulting to elongated fruit shape (Chusreeaeom et al., 2014). In cucumber, variation in expression levels of kinesins in developing ovaries was correlated with differences in cell division among cultivars with different fruit size (Yang et al., 2013, Jiang et al., 2015).

Organ growth, especially towards the later part of development, is also driven by cell expansion (Gillaspy et al., 1993). Exponential growth of pickling cucumber fruit (*C. sativus* cv "Vlaspik") showed a rapid increase in fruit length which coincided with increase in cell size accompanied by vacuolization of mesophyll cells and thickening of epidermal cell walls (Ando and Grumet 2010). Developing watermelon fruit also exhibit rapid cell division during early fruit growth followed by cell expansion accompanied by the formation of large vacuolated cells (Wechter et al., 2008). Recent reports in cucumber also have implicated the involvement of kinesin genes in regulating cell expansion in developing fruit (Yang et al., 2013, Jiang et al., 2015). Moreover, studies in tomato have identified cell cycle related genes (e.g. *LeKRP1*, *WEE1* and *SICCS52A*) that control endoreduplication, hence affecting cell size in tomato lines with varying fruit size/weight (Bisbis et al., 2006, Gonzalez et al., 2007, Mathieu-Rivet et al., 2010, Nafati et al., 2011).

Other regulatory genes including *OVATE* and *FAS* were also shown to influence fruit shape in tomato and pepper (Cong et al., 2008, Moriguchi et al., 2011, Rodriguez et al., 2011,

Huang et al., 2013). Mutation in the *OVATE* gene led to elongated fruit shape in tomato (Liu et al. 2002, Huang et al., 2013) while mutation in *FAS*, and a domain that interacts with *FAS*, *locule number* (*LC*), resulted in a flat tomato with high locule number (Lippman and Tanksley 2001, Barrero et al. 2006, Cong et al. 2008, Huang and van der Knaap 2011). Recently, a homolog of *SUPERMAN* in cucumber was shown to partially complement flower and fruit development in Arabidopsis mutant, probably by regulating cell division (Zhao et al., 2014). However, to date, no specific genes that influence fruit size and shape in cucumber have been reported.

Previous studies mapping various fruit traits in cucumber were performed with low-density maps (Serquen et al., 1997, Fazio et al., 2003, Wang et al., 2005, Yuan et al., 2008a,b, Bradeen et al., 2001, Heang et al., 2008, Cavagnaro et al., 2010). The recent availability of genome assemblies of three cucumber cultivars, Chinese long '9930' (CL) (Huang et al., 2009), B10 (Woycicki et al., 2011), and Gy14 (Yang et al., 2012) facilitated the development of saturated linkage maps and increased the efficiency of identifying fruit-trait related QTLs (e.g. Wei et al., 2011, Miao et al., 2011, Zhang et al., 2011, Bo et al., 2014, Wei et al., 2014). Recently, a 45k-single nucleotide polymorphism (SNP) array in cucumber was developed drawing upon sequence data from CL and Gy14 (Rubinstein et al., 2015). This tool, together with extensive information on cucumber fruit development and morphology of a segregating population, will facilitate the construction of a high-density linkage map and the identification of fruit size and shape QTLs in cucumber.

In this study, we examined whether cucumber fruit size and shape is a function of the following growth-related factors: ovary size, ovule number, cell number, cell size and period and rate of cell division and expansion pre- and post-anthesis. The role of these factors was examined using two sequenced cucumber genotypes with extreme differences in fruit size and shape, *C.*

sativus var. sativus cv "Gy 14" (pickling) and *C. sativus var. sativus* cv "9930" (Chinese long) along with their F_1, F_2 and RIL progenies. The level and timing of expression of select marker genes and homologs of known fruit size genes were also compared between CL and two pickling cucumber cultivars across fruit developmental time points, and located with respect to the recently described fruit size QTL in cucumber (Weng et al., 2015). Our results suggest that factors controlling fruit length were largely determined pre-anthesis while factors regulating diameter were largely determined post anthesis, and that variation in fruit size and shape in CL and Gy14 is driven by a complex regulatory network controlling the timing and orientation of cell number and cell shape. Moreover, a number of fruit growth related genes mapped to the cucumber fruit size QTL and were specifically clustered in chromosomes 3 and 6 suggesting potential regulatory role in cucumber fruit size and shape.

MATERIALS AND METHODS

Analysis of fruit growth parameters in developing cucumber fruits

Two fruit growth experiments were performed in the greenhouse with 60 plants each of Gy14, Chinese long (CL), and their F_1 progeny. Seeds of Gy14 and CL were provided by Dr. Y. Weng, UW Madison. Progeny of Gy14 x CL and CL x Gy14 were made in the greenhouse. The plants were grown in ~4-L plastic pots with Suremix Perlite soil medium (Michigan Grower Product, Inc., Galesburg, MI) and fertilized once per week. Supplemental lights were used to provide an 18-h light period. Pest control was performed according to standard management practices in the greenhouse.

Pre-anthesis to anthesis

Developing floral buds from nodes 3, 4, and 5 from 10 plants each of Gy14, CL, and F_1 were measured daily for length and diameter from 7 days pre-anthesis to anthesis. At anthesis, 10 flowers from each genotype were dissected to count the ovule number.

To determine the rate and period of cell division and enlargement, cell number, and cell size during ovary growth, 3-5 developing floral buds were collected daily from each genotype from 7 days pre-anthesis to anthesis. The floral buds were immediately fixed in FAA solution (10% v/v formaldehyde; 5%v/v acetic acid; 50% v/v ethanol; 35% v/v water), dehydrated in ethanol grade series, stained with eosin dye, and embedded in wax as described by Jackson (1991). Longitudinal and transverse sections (10um) of each ovary were prepared using Leica rotary microtome (2125RT; Leica Microsystems, Buffalo Grove, IL). Tissue sections were viewed using light microscope with a Spot RT3 digital camera system (SPOT Imaging Solutions, Diagnostic Instruments, Inc., MI). To measure cell size, three boxes (30um x 30um) were drawn on each of the images. Cells inside the box were counted and the area of the box was divided by average cell number to determine average cell area. Cell number was determined using the formula of a circle for cross sections, and rectangle for longitudinal sections.

Post-pollination

To minimize environmental effects, 1-2 flowers from the third to fifth node were hand-pollinated on the same day on each of 60 plants for each genotype. To avoid the effects of interfruit competition, only one fruit was allowed to develop. Ten out of sixty fruits/genotype were measured daily for length and diameter until 20 days post-pollination (20dpp). To examine the cell number and cell size post-pollination, sets of 3-5 fruits were harvested at 2-day intervals (0, 2, 4, 6, 8, 10, 12, 14, 16 dpp) from each genotype based on a randomized complete block

design (RCBD). A wedge (~ 1cm^3) was cut from the mesocarp of the middle section of each fruit and was immediately fixed in FAA. Free-hand transverse and longitudinal sections (~10mm) of each wedge were prepared for microscopy per Ando and Grumet (2010). Images of the tissue sections were collected and measurement of cell number and cell size was performed as described above.

Analysis of fruit related traits in F_2 and RIL progeny

F_2 plants from reciprocal crosses of Gy14 x CL were grown in the greenhouse for two seasons (60-70 plants/experiment) as described above. One ovary from each F_2 plant was measured at anthesis for length and diameter. One-two flowers were hand pollinated for each plant, however only one fruit was allowed to develop. Fruits were measured daily for length and diameter until 20dpp. Fruits at 20dpp were dissected to count ovule/seed number.

Recombinant inbred lines (RILs) of Gy14xCL were planted for two seasons at the Horticulture Teaching and Research Center, Michigan State University. In the summer of 2011 and 2012, 123 lines (Gy14 X CL F_6:F_7) and 139 lines (Gy14 X CL F_7:F_8) were grown in the field, respectively, along with parental genotypes and F_1 progeny, in a randomized complete block design with three replications (5 plants/line per replication). Seeds were planted into 0.8 m wide plastic mulch with 1 m between rows and 0.3 m spacing within rows. Local standard commercial production guidelines were followed for fertilization and insect and weed control (Bird et al., 2005). Water was supplied by rain or by trickle irrigation to provide 25 mm per week. Pollination was facilitated by bees. Five flowers at anthesis were collected from each plot and ovary length, diameter and L/D were measured. To estimate the age of developing fruits, 1-2 flowers at anthesis on 2-3 plants in each plot were tagged. At maturity five fruits/rep were harvested from each plot (15 fruits/RIL) and evaluated for fruit length, diameter, L/D, ovule/seed

number, and carpel number. In 2012, mature fruits were also measured for flesh thickness and seed cavity diameter. Correlation analysis of fruit related traits and analysis of variance were performed using PROC CORR and PROC MIXED, respectively, in SAS software (SAS Institute Inc., Cary, NC).

Expression analyses of select developmental marker genes, and homologs of fruit size and shape genes

Transcriptome study on early fruit growth of pickling cucumber (cv. Vlaspik) indicated genes that exhibit developmental-specific patterns of expression (Ando et al., 2012). Genes that were highly expressed in 0+4dpp (i.e. cyclin dependent kinases, histone genes: *CycD3;1, histone 4*), 8dpp (i.e. lipid metabolism related genes: *LipidTr*, *GDSL-motif lipase/hydrolase*, *SHINE1*), and 12+16dpp (i.e. transcription factors: *ERF3*, *ATHB-2, WRKY70*) were selected for analysis. The expression of cucumber homologs of three fruit size and shape genes in tomato, *OVATE, FAS*, and *SUN* (Lippman and Tanskley 2001, Liu et al., 2002, Xiao et al., 2008) were also examined. Three fruits from CL, Gy14 and Vlaspik were collected at anthesis and 4, 8, 12, 16, and 20 dpp. Pericarp samples isolated from the middle part of the fruit were immediately frozen in liquid nitrogen. mRNA samples from the pericarp tissue were prepared using the Trizol method (Invitrogen, CA), followed by DNase I treatment and clean up (Qiagen, CA). Total RNA concentration was assessed using the nanodrop ND-1000 (Thermo Scientific, DE). First strand cDNA synthesis was performed using the High Capacity RNA-to-cDNA Kit (Invitrogen, CA). RT-PCR was done according to Ando and Grumet (2010). Gene-specific primers (Appendix Table 2.3) were designed using Primer Express software (Applied Biosystems). The ABI Prism 7900HT Sequence Detection System was used for qRT-PCR analysis. Revalution PCR Master Mix (Syzygy, MI) with ROX as reference dye was used for PCR quantification. PCR products

were quantified with reference to corresponding standard curves. *C. sativus polyubiquitin* (CuSa200910_13711) was used as an endogenous control for normalization. qRT-PCR was performed using cDNA of 3 fruits (3 biological replicates)/genotype with 3 technical replicates/biological replicate. Data were analyzed by analysis of variance (ANOVA) and Tukey HSD protocol in SAS (SAS Institute, Cary, NC).

Sequence analysis of fruit growth related genes

The coding sequences of the fruit growth marker genes were obtained from the cucumber fruit transcriptome data (Ando et al., 2012), and sequences of tomato fruit size and shape genes, *OVATE, FAS, SUN* and *Fw2.2* were derived from the Sol Genomics Network (solgenomics.net). Homology search was performed using Basic Local Alignment Search Tool (BLAST) and the TBLASTX platform in Chinese long genome database (www.icugi.org) and in Gy14 genome database (www.phytozome.net). The homologs with the highest bit score and E-value (1e-37 – 0.0) were selected. Pairwise comparisons of the coding and 5′ non-coding regions of fruit growth maker genes and homologs of fruit size and shape genes between pickling cultivars, Gy14 and Vlaspik, and between Gy14 and Chinese long were performed using BLAST software in NCBI (www.ncbi.nlm.nih.gov) and CLUSTAL OMEGA Global alignment tool (www.ebi.ac.uk).

RESULTS

Morphological differences in ovary and fruit size and shape are apparent throughout floral development and fruit growth. Chinese long (CL) flowers have more elongated ovaries compared to Gy14 (Figure 2.1a). This difference became more obvious post-pollination; CL had longer, thinner fruits, while Gy14 had shorter, thicker fruits. Difference in ovule number was also observed between the two parents, with CL having twice as many ovules as Gy14 (Figure

2.1b-c). The F_1 progeny showed intermediate ovary size, fruit size, and ovule number (Figure 2.1a-c).

Ovary growth pre-anthesis to anthesis

Differences in ovary size between CL and Gy14 were apparent as early as seven days pre-anthesis (Figure 2.2). The difference in length increased as the ovaries reached anthesis, ovaries of CL at anthesis were approximately 3x longer than Gy14 (Figure 2.2a). CL also had a larger diameter than Gy14 at 7 dpa, however, at anthesis their diameter was equivalent (Figure 2.2b). Also, CL showed a steady increase in the ratio of ovary length to diameter (L/D) over time, while the L/D of Gy14 remained constant (Figure 2.2c).

Cell number and rate of cell division pre-anthesis was higher for CL in the longitudinal direction, with an accelerated increase shortly before anthesis (Figure 2.2d). CL also had a greater number of cells than Gy14 in cross section at 7 dpa, however, the increase in rate of cell division in the radial orientation occurred earlier in Gy14, such that at anthesis the cell number in both genotypes was equivalent (Figure 2.2f). Cell length in longitudinal section and diameter in cross section pre-anthesis was comparable for CL and Gy14 throughout ovary development (Figure 2.2e,g).

Fruit growth post-pollination

Following pollination, Gy14 and CL both exhibited a typical sigmoidal pattern of growth in length and diameter (Figure 2.3a,b). CL continued to increase in length until about 20 dpp with the greatest rate of increase occurring from approximately 6dpp-10dpp. Gy14 showed peak increase in length during the same period but at approximately half the rate (Figure 2.3a). On the other hand, the rate of increase in diameter of Gy14 was more than 2-fold greater than for CL (Figure 2.3b). The highest increase in diameter for Gy14 occurred from 4-9 dpp, while peak

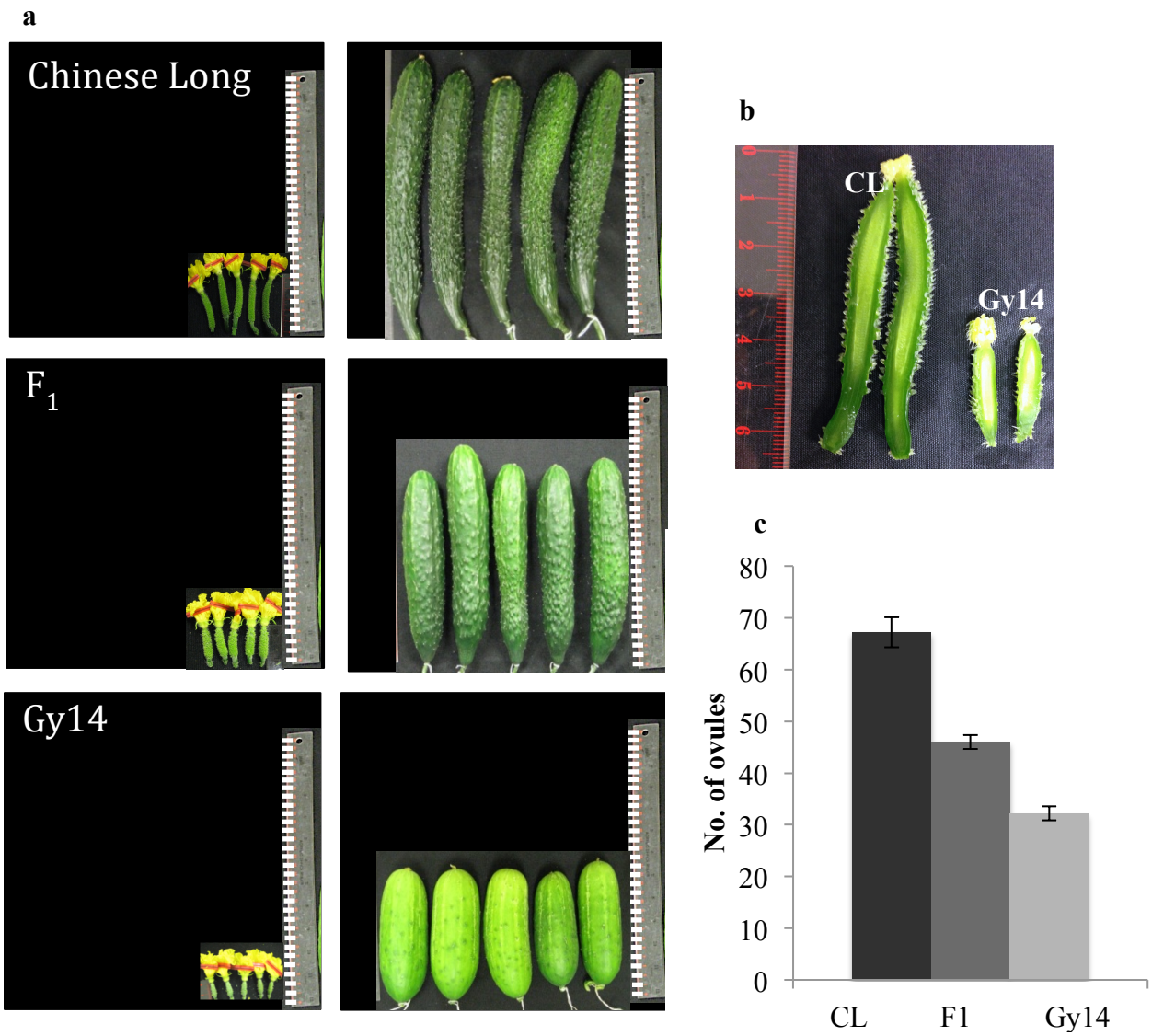

Figure 2.1. Phenotypic differences among the parents and F_1 progeny. (a) CL, Gy14 and F_1 progeny at anthesis (0dpp) and at full size (16dpp), (b) ovary of CL and Gy14 dissected longitudinally, and (c) ovule number (each value is the mean of 5-10 ovaries ± S.E).

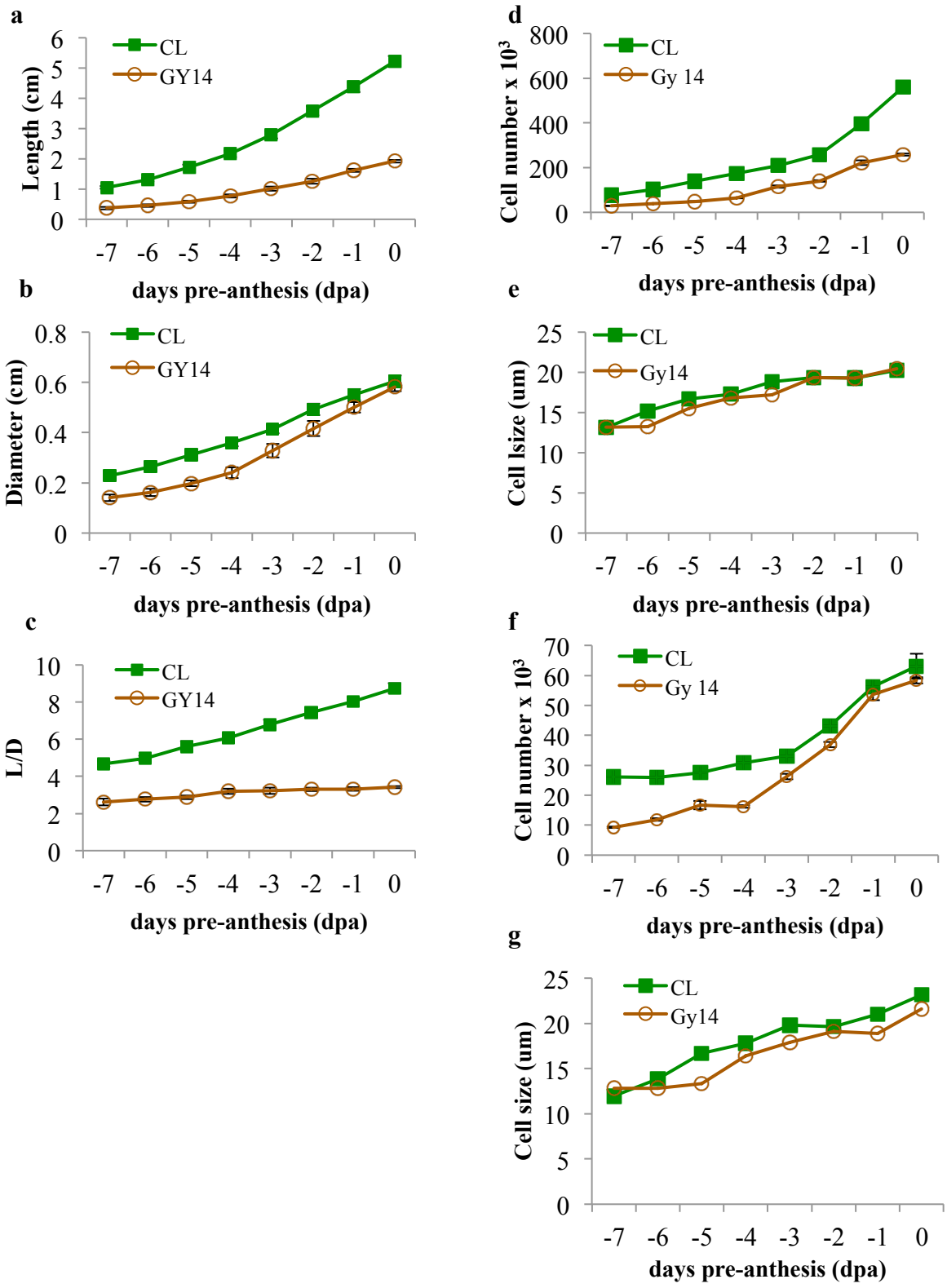

Figure 2.2. Ovary development of CL and Gy14. (a) Ovary length, (b) diameter, (c) L/D, cell number and cell length of CL and Gy14 in the longitudinal section (d-e) and cell number and cell diameter in transverse section (f-g). Each value is the mean of 5-10 ovaries ± S.E.

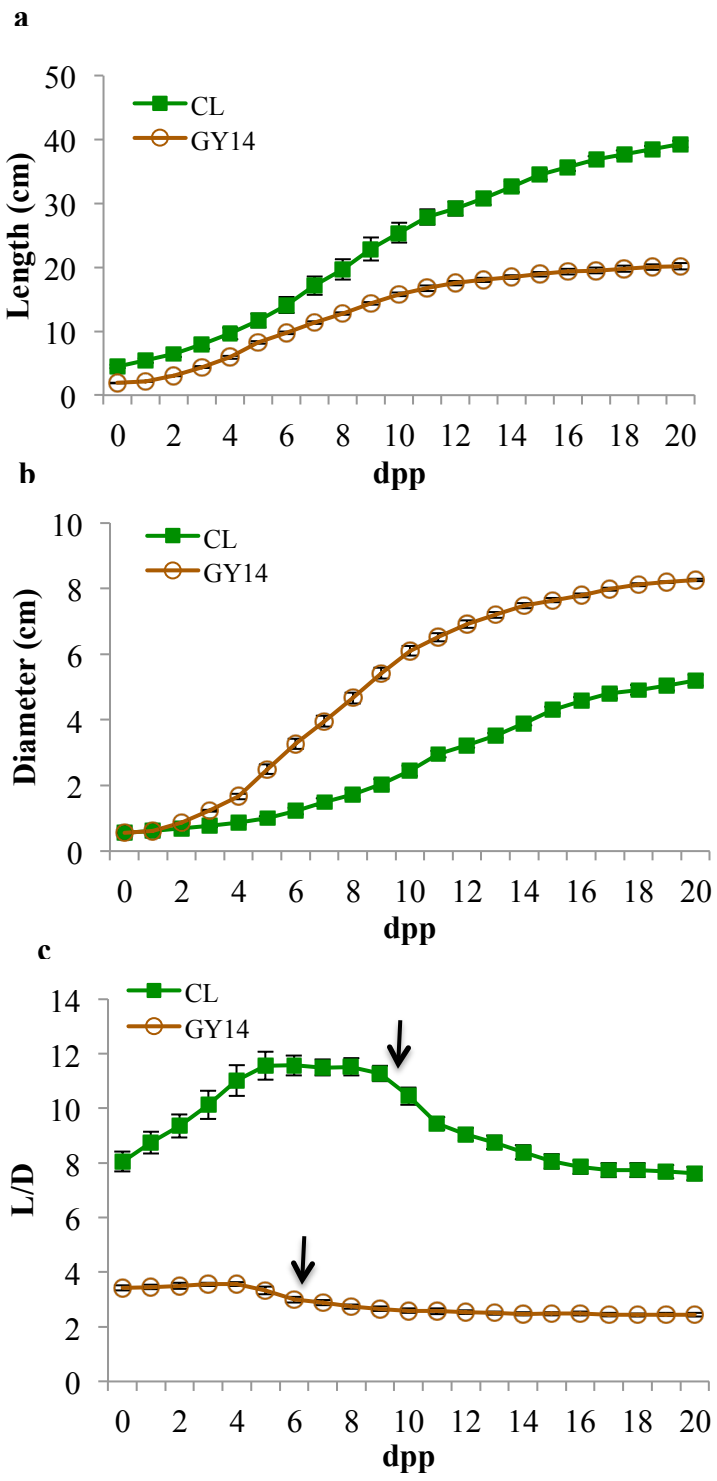

Figure 2.3. Fruit growth of Chinese long (CL) and Gy14 following pollination. (a) Fruit length (b) fruit diameter and (c) length/diameter ratio (L/D). Each value is the mean ±S.E. of 5-10 fruits/genotype.

increase in diameter growth of CL occurred later, approximately 9-11 dpp and was never as great as for Gy14 (Figure 2.3b). For CL and Gy14, the L/D ratio indicates that the rate of increase in length is initially greater than diameter but reverses as the fruit develops, however the transition occurred sooner and was less pronounced in Gy14 (Figure 2.3c). Collectively, these observations indicate differences in the magnitude, orientation, and developmental pattern of fruit growth.

Cell number in the longitudinal section increased at a similar rate but for several more days for CL than Gy14 (Figure 2.4a), however Gy14 showed markedly greater rate and a longer period of rapid cell division in the cross section (Figure 2.4b). Cells were longer in the longitudinal direction in CL than Gy14 ($p<0.05$, ANOVA, Tukey HSD) (Figure 2.4c). Examples of cross and longitudinal sections of developing ovary and fruit, from -4dpa to 16dpp, of CL and Gy14 are shown in Figure 2.5. An approximately 100-fold increase in cell area was observed for both cultivars during that time period.

Relationship of fruit growth factors in F_2 and RIL progeny

Greenhouse grown F_2 progeny of Gy14xCL and CLxGy14 showed a normal distribution for both fruit length and diameter, with parental types at the extremes (Figure 2.6). Ovule number (ON), ovary length (OL) and ovary L/D at anthesis were all highly correlated with each other, and with fruit length (FL) and fruit L/D at harvest stage (12dpp) and full size (20dpp), indicating that ovule number and ovary length are good predictors of fruit length (Table 2.1). In contrast, ovule number, ovary diameter, and ovary L/D were not correlated with fruit diameter at full size, suggesting that factors post-anthesis are more important in determining fruit diameter.

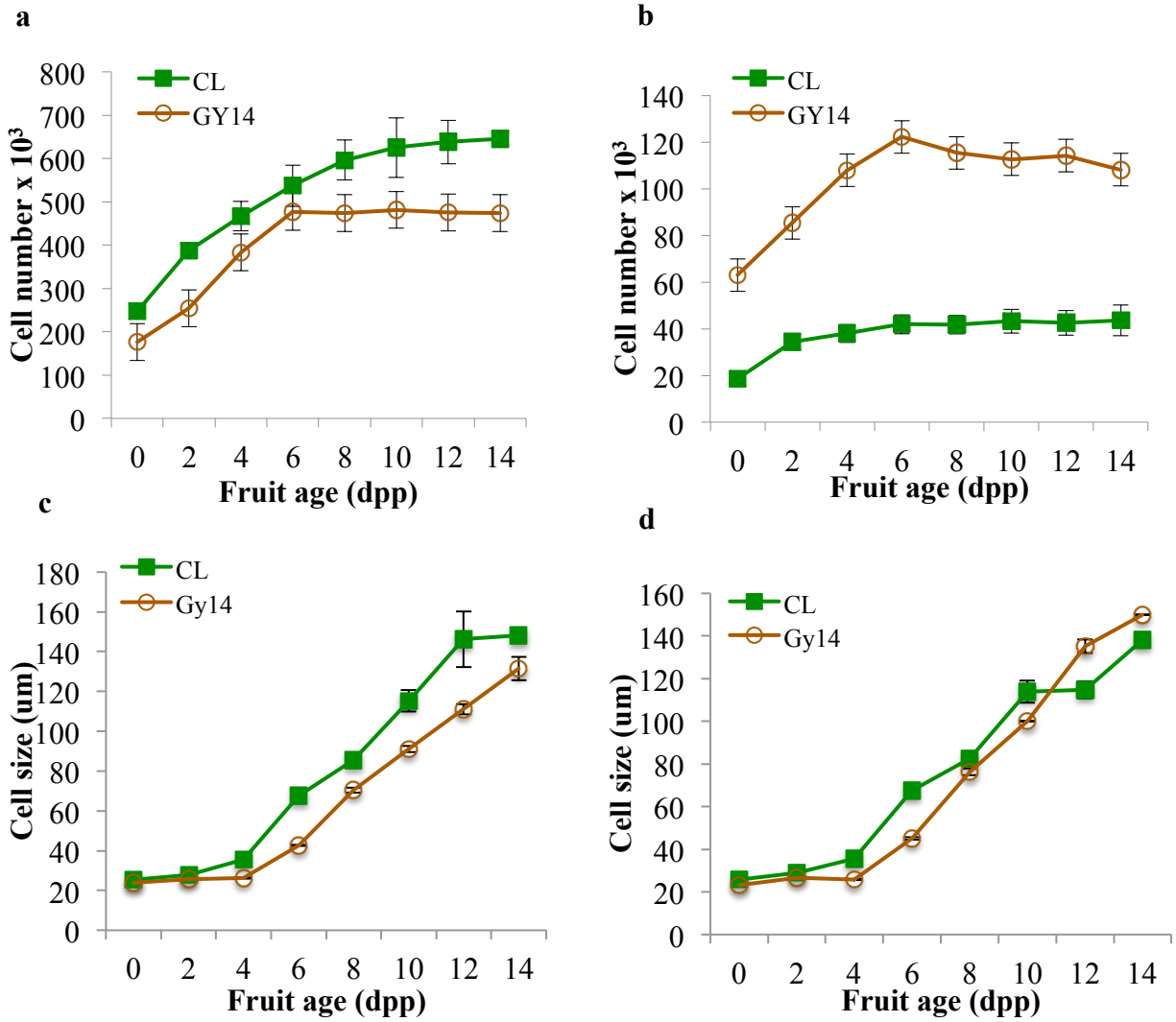

Figure 2.4. Fruit growth of Chinese long (CL) and Gy14 following pollination as a function of cell division and cell expansion. Cell number and cell size of CL and Gy14 in the longitudinal section (a & c) and cell number and cell size of CL and Gy14 in cross section (b & d). Each value is the mean ±S.E. of 3-5 fruits/genotype.

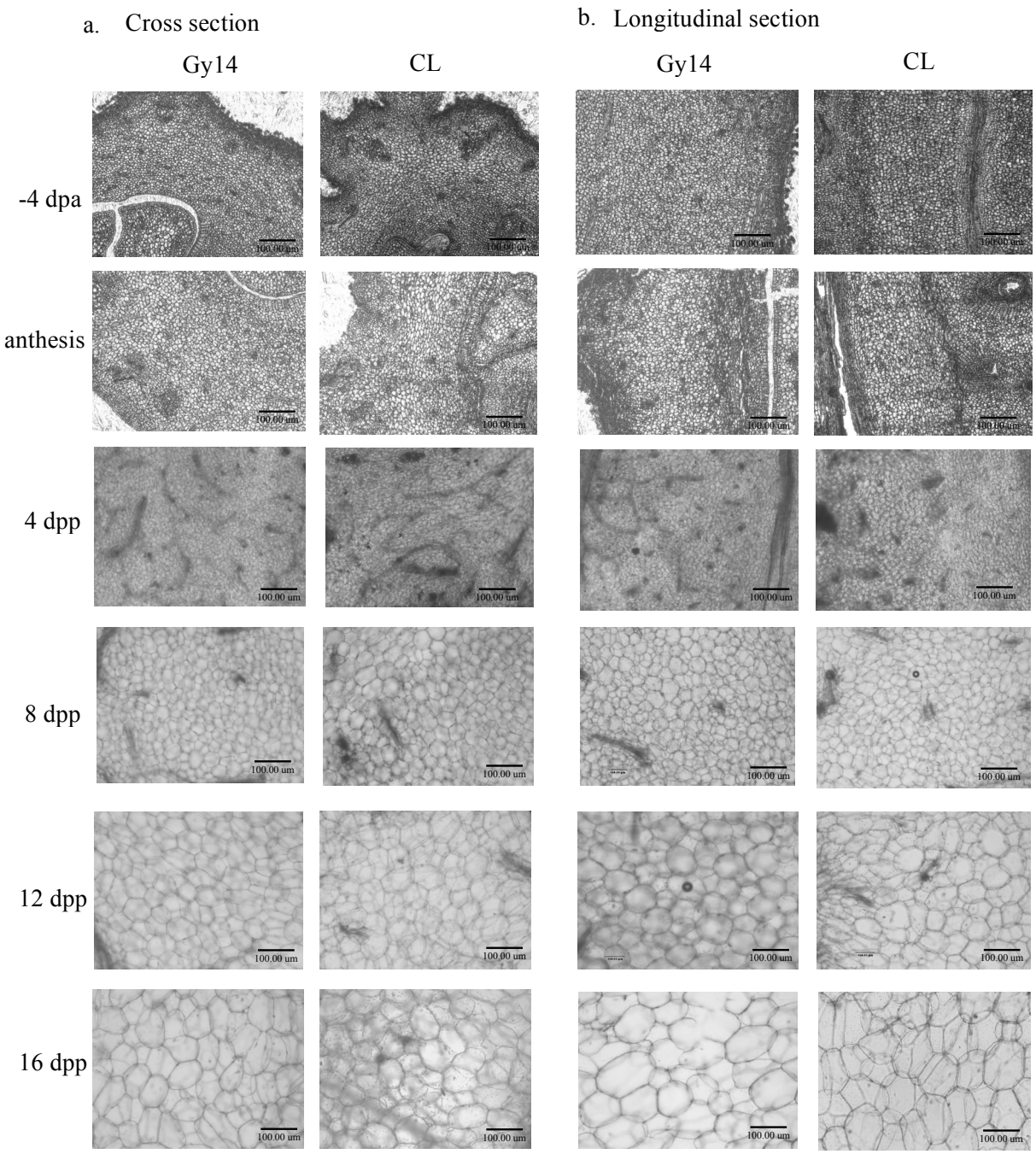

Figure 2.5. Examples of cross and longitudinal sections of ovaries and fruits of CL and Gy14 at different developmental stages.

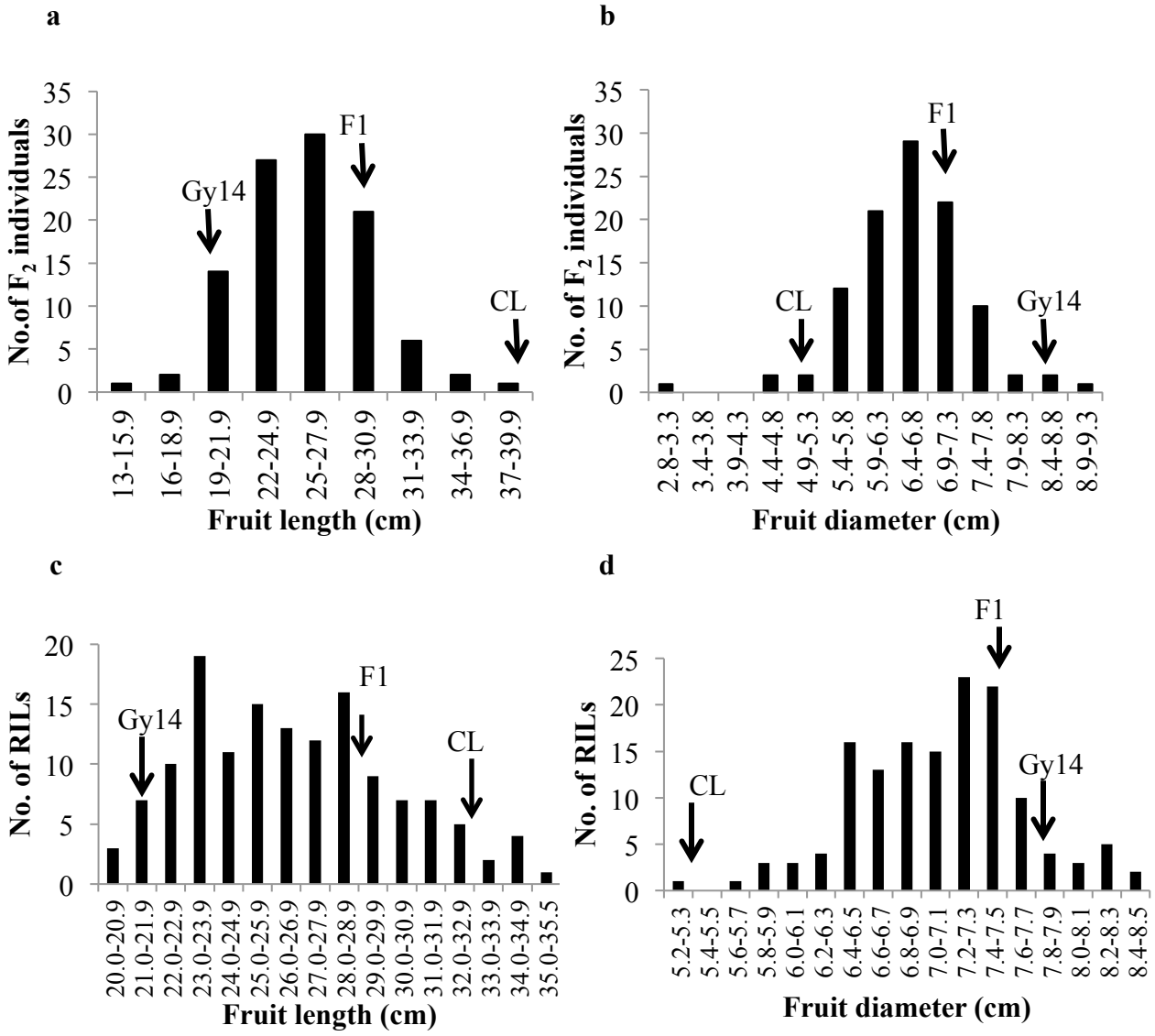

Figure 2.6. Distribution based on length and diameter of mature fruit of F_2 progeny, N=104 (a & b) and RILs, N=139, mean of five fruits/RIL (c & d). RIL data are from 2012, equivalent results were observed in 2011.

Table 2.1a. Correlation coefficients between ovary traits at anthesis and fruit traits at 20 days post pollination of (Gy14 X CL) F_2 progeny.

Trait	ON	OL	OD	OL/D	FLH	FDH	L/DH	FL	FD
Ovule number (ON)	-								
Ovary length (OL)	0.5***	-							
Ovary diameter (OD)	-0.23ns	0.24ns	-						
Ovary L/D (OL/D)	0.65****	0.76****	-0.43*	-					
Fruit length (harvest) (FLH)	0.61****	0.74****	0.10ns	0.77****	-				
Fruit diameter (harvest) (FDH)	0.09ns	0.33*	0.31*	0.1ns	0.52***	-			
Fruit L/D (harvest) (L/DH)	0.52***	0.50***	-0.36*	0.71****	0.54***	0.43**	-		
Fruit length at 20dpp (FL)	0.60***	0.80****	0.14ns	0.83****	0.97****	0.38*	0.71****	-	
Fruit diameter at 20dpp (FD)	-0.03ns	0.44*	0.35ns	0.18ns	0.36ns	0.58**	-0.05ns	0.39*	-
Fruit L/D (FL/D)	0.63***	0.59***	0.36ns	0.79****	0.83****	0.07ns	0.79****	0.84****	0.16ns

ns, *, **, ***, **** R value not significant, or significant at $p<0.05$, $p<0.01$, $p<0.001$, $p<0.0001$, respectively

Table 2.1b. Correlation coefficients between ovary traits at anthesis and fruit traits at maturity of (Gy14xCL) $F_{7:8}$ RILs (Michigan field trial 2012).

Trait	ON	OL	OD	OL/D	FL	FD	FL/D	Carp	SC
Ovule number (ON)	-								
Ovary length (OL)	0.58****	-							
Ovary diameter (OD)	0.14ns	0.54****	-						
Ovary L/D (OL/D)	0.57****	0.62****	-0.31***	-					
Fruit length (FL)	0.54****	0.60****	-0.04ns	0.70****	-				
Fruit diameter (FD)	-0.13ns	-0.15ns	0.09ns	-0.24**	0.13ns	-			
Fruit L/D (FL/D)	0.52****	0.62****	-0.07ns	0.76****	0.81****	-0.46****	-		
Carpel number (Carp)	0.15ns	0.38****	0.44****	0.03ns	0.19*	-0.15ns	0.26**	-	
Seed cavity diameter (SC)	-0.25*	-0.24**	0.34****	-0.56****	-0.27**	0.47****	-0.51****	0.24**	-
Flesh thickness (FT)	0.03ns	0.01ns	-0.15ns	0.14ns	0.34****	0.78****	-0.14ns	-0.33****	-0.19*

*, **, ***, **** R value significant at $p<0.05$, <0.01, <0.001, or <0.0001, respectively

The relationship among growth factors was further examined for two seasons in the field using segregating F_6/F_7 and F_7/F_8 RIL progeny of Gy14xCL (developed by Y. Weng, Univ. Wisconsin). The distribution frequency of RILs for the growth parameters measured was equivalent for both seasons and values for all traits were highly correlated between seasons (Appendix Table 2.4). All exhibited a normal distribution typical for quantitative traits. Examples of phenotypic distributions in 2012 are shown in Figure 2.6. Although some lines exhibited either shorter or longer/thinner or wider fruit than CL or Gy14, there was no significant transgressive segregation for fruit length and diameter (Figure 2.6, Appendix Table 2.5). Another factor that can influence fruit shape is carpel number as has been observed in melon (Fernandez-Silva et al., 2010). Interestingly, although both parents, CL and Gy14, had three carpels, segregating RIL progeny occasionally exhibited four to five carpels. However, carpel number could vary within a RIL family and even among fruits from the same plant, suggesting that environmental, rather than genetic factors played a key role in influencing carpel number.

As for F_2, strong correlations were observed among ovule number, ovary length, ovary L/D, fruit length and fruit L/D (Table 4, $R=0.50$-0.68, $P<0.0001$). On the other hand, ovary diameter was not correlated with ovule number, fruit diameter, and fruit L/D. This is consistent with lack of differences among genotypes for diameter at anthesis despite large differences at maturity (Appendix Table 2.5). There was also weak

correlation or no significant correlation between fruit length and diameter in both seasons (Table 2.1b; in 2011, $R=0.21$, $P<0.05$). The normal distributions and independent segregation of several components of fruit size and shape in the F_2 and RIL populations are consistent with complex quantitative traits under the control of multiple genetic factors.

Expression of growth marker genes and homologs of fruit size and shape genes

To gain insight into possible genes associated with fruit growth in cucumber, we examined data from our prior transcriptome analysis of fruit development (Ando et al., 2012), studies of cucumber fruit growth from the literature (Yang et al., 2013, Jiang et al., 2015), and a parallel study with RIL progeny of CL and Gy14 mapping fruit size and shape QTL (Weng et al., 2015).

The first few days post-pollination typically are marked by peak expression of cell division associated genes (Marcelis 1994, Boonkorkaew et al., 2008, Fu et al., 2010, Ando et al., 2012). Six cell division associated genes with peak expression immediately post-pollination, *histone 4* (*H4*), *histone 3.2* (*H3.2*), *Cyclin B1;4* (*CYCB1;4*), *Cyclin D1;1* (*CYCD1;1*), *Cyclin D3;1* (*CYCD3;1*), and *Cyclin dependent kinase 1* (*CDKE1*) (Ando et al., 2012), mapped to QTL regions that were associated with fruit size in RIL progeny of Gy14xCL (Weng et al., 2015) (Table 2.2). Each gene mapped to a QTL on separate chromosomes. All of the genes, with the exception of *CYCB1;4,* had 100% identity between CL and Gy14 (Table 2.2). A 158 bp sequence at the 5' end of the coding region of *CYCB1;4* was missing in CL while a 57 bp sequence in the middle of the CDS was not present in Gy14. In another pickling cucumber, Vlaspik, a 4 bp deletion at the 3' end of the coding region of *CYCB1;4* was also observed. Sequence comparisons of the 5' noncoding regions (~1500 bp upstream of transcription start site, TSS) showed that, except for *CDKE1*, there were variations in the sequence of each gene. *CYCD1;1* had 94% identity, with Gy14 showing a 88bp deletion in the TATA-rich region. The rest of the genes had 99% identity between CL and Gy14.

Table 2.2. Sequence analysis of fruit growth marker genes and homologs of known fruit size and shape genes in Chinese long and two pickling cultivars, Gy14 and Vlaspik.

Gene Annotation	Gene ID		Coding sequence (bp)	% Identity Coding sequence between pickling types (Gy14vsVlaspik)	% Identity Coding sequence (CLvsGy14)	Peptide sequence (aa)	% Identity Peptide sequence	%Identity of Noncoding sequence[d] upstream of TSS (CLvsGy14)	Scaffold		Nearest SNP position	Chromosome	QTL
	CL database	Gy14 database							ID	Position			
Cell division related genes													
Histone H4[a]	Csa1M084320	Cucsa.226890	312	100	100	103	100	99	scaffold01910	38277..38549	12780	1	***FS1.1***
Histone H3.2[a]	Csa4M290220	Cucsa.065360	411	100	100	136	100	99	scaffold00696	100045..100492	168010	4	***FS4.1***
CYCB1;4[a,c]	Csa6M428000	Cucsa.086730	1344	98	80	447	77	99	scaffold00873	136897..138925	167381	6	***FS6.1***
CYCD1;1[a,c]	Csa5M488810	Cucsa.275450	1008	99	100	335	100	94	scaffold02581	360802..361877	500985	5	***FS5.1***
CYCD3;1[a,c]	Csa3M199660	Cucsa.244590	1131	100	100	376	100	99	scaffold02135	453575..454475	285858	3	***FS3.2***
CDKE1[a]	Csa4M561190	Cucsa.357390	1455	99	100	484	100	100	scaffold03578	396070..407419	423465	4	***FS4.1***
Kinesins													
CsKF2[b,c]	Csa3M062600	Cucsa.042400	3420	poor Vlaspik seq	100	1139	100	94	scaffold00540	808095..814567	1032712	3	*mfl3.1*[f]
CsKF3[b]	Csa1M568560	Cucsa.051280	2832	poor Vlaspik seq	83	1094/943	83	poor Gy14 seq	scaffold00557	441146..447459	562239	1	***FS1.2***
CsKF4[b]	Csa6M499030	Cucsa.104020	3831	100	100	1276	100	100	scaffold00927	2647364..2665474	2839496	6	***FS6.2***
CsKF7[b,c]	Csa1M495290	Cucsa.213570	3156	100	99	1051	99	99	scaffold01543	1355893..1361913	266269	1	*mfl1.1*[f]
Late post-exponential growth related transcription factors													
ATEBP (ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEIN)[a]	Csa3M164580	Cucsa.252810	861	100	79	286	79	99	scaffold02229	1675348..1677319	1819375	3	***FS3.1***
ATHB-2 (ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 2)[a]	Csa3M019370	Cucsa.321080	828	100	100	275	100	97	scaffold03080	942,858..943,966	1061509	3	***FS3.1***
Dof-type zinc finger domain-containing protein[a]	Csa3M238150	Cucsa.032330	635	100	94	212	94	99	scaffold00429	1668528..1670302	1484523	3	*fl3.1*[g]
ERF3 (ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 3[a]	Csa3M073900	Cucsa.042770	645	100	100	214	100	poor Gy14 seq	scaffold00540	1115839..1116812	1032712	3	*mfl3.1*[f]
WRKY70[a]	Csa3M727990	Cucsa.177060	843	100	100	280	100	99	scaffold01225	1045077..1046536	1057405	3	***FS3.2***
Ovary growth related transcription factors													
Indehiscent (IND)[c]	Csa6M483450	Cucsa.101020	672	poor Vlaspik seq	95	223	95	99	scaffold00927	285353..286206	452585	6	**FS6.1/*mfd 6.2***[e]
Transcriptional regulator SUPERMAN, putative[c]	Csa3M141870	Cucsa.255130	564/503	poor Vlaspik seq	89	168	89	100	scaffold02229	3219209..3219828	3518579	3	***FS3.1***
Fruit size and shape homologs													
FAS	Csa6M426940	Cucsa.086660	522	100	100	173	100	99	scaffold00873	49793..56042	167381	6	***FS6.1***
Fw2.2-like	Csa3M002640	Cucsa.322780	1257	99	100	418	100	98	scaffold03080	2422514..2424398	2372961	3	*mfl3.1*[f]
OVATE	Csa6M454390	Cucsa.083680	405	poor Vlaspik seq	99	134	99	poor CL seq	scaffold00858	455066..455352	215910	6	***FS6.1*** */ mfd 6.2*[e]
SUN	Csa6M507030	Cucsa.338260	1107	poor Vlaspik seq	100	368	100	poor CL seq	scaffold00542	417641..422056	374797	6	***FS6.2***

Gene sequences were aligned against Gy14 database (http://phytozome.jgi.doe.gov/pz/portal.html#!search?show=BLAST), CL database (http://icugi.org/cgi-bin/ICuGI/tool/blast.cgi) and in Vlaspik fruit transcriptome data (Ando et al., 2012; Mansfeld et al., unpublished)

Pairwise alignment was performed using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and CLUSTALW Omega Global alignment (http://www.ebi.ac.uk/Tools/psa/)

[a] Fruit growth genes described in Ando et al (2012)

[b] Differentially expressed genes in cucumbers with varying fruit size (Yang et al. (2013)

[c] Differentially expressed genes in cucumbers with varying fruit length measured only at 4 days pre-anthesis (Jiang et al. (2015)

[d] Non coding sequence is 1500bp upstream of transcription start site

[e] Fruit trait related QTLs identified by Miao et al. (2011), [f]Bo e al. (2014), and [g]Wang et al. (2014) as described in Weng et al. (2015)

qRT-PCR analysis of two of the cell division genes, *his4* and *CYCD3;1,* did not indicate difference in level or timing of expression in CL, Gy14 and Vlaspik (Figure 2.7). Peak expression in all cultivars occurred from 0-4 dpp, coinciding with the period of rapid increase in cell number (Figure 4a,b) as had been observed previously (Ando et al., 2010, 2012). *His3.2*, *CYCB1;4, CYCD1;1,* and *CDKE1* remain to be tested.

The Vlaspik fruit transcriptome data also showed specific sets of genes that were highly expressed during peak exponential fruit growth including the cuticle-associated genes *GDSL-motif lipase/hydrolase* (*GDSL*), *lipid transfer protein* (*lipidTr*) and *SHINE1* (*SHN1*) (Ando et al., 2012). Expression level of these genes was relatively low pre-anthesis but peaked during exponential to late exponential growth (8-12dpp) for all three cultivars (Fig. 2.8a-f). However, consistent with thinner cuticle observed in CL in relation to Gy14 and Vlaspik (Chapter 3 Figure 3.6b), gene expression in CL was 3-10 fold lower compared to the two pickling cultivars.

Late/post-exponential fruit growth in Vlaspik was specifically marked by increased expression of a set of 18 genes annotated as transcription factor homologs (Ando et al., 2012), including five that mapped to fruit size QTL identified by Weng et al. (2015), Bo et al. (2014) and Wang et al. (2014): *ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEIN* (*ATEBP*), *HOMOEBOX PROTEIN 2* (*ATHB-2*), *Dof-type zinc finger domain-containing protein* (*DOF*), *ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 3* (*ERF3*), and *WRKY70* (Table 2.2, Figure 2.9). All clustered to a similar region of chromosome 3. *ATEBP* and *DOF* had 79% and 94% identity between CL and Gy14, respectively, while the rest of the genes had 100% identity. A 183 bp sequence at the 5′ end of the *ATEBP* CDS was not present in CL. For *DOF*, there was a

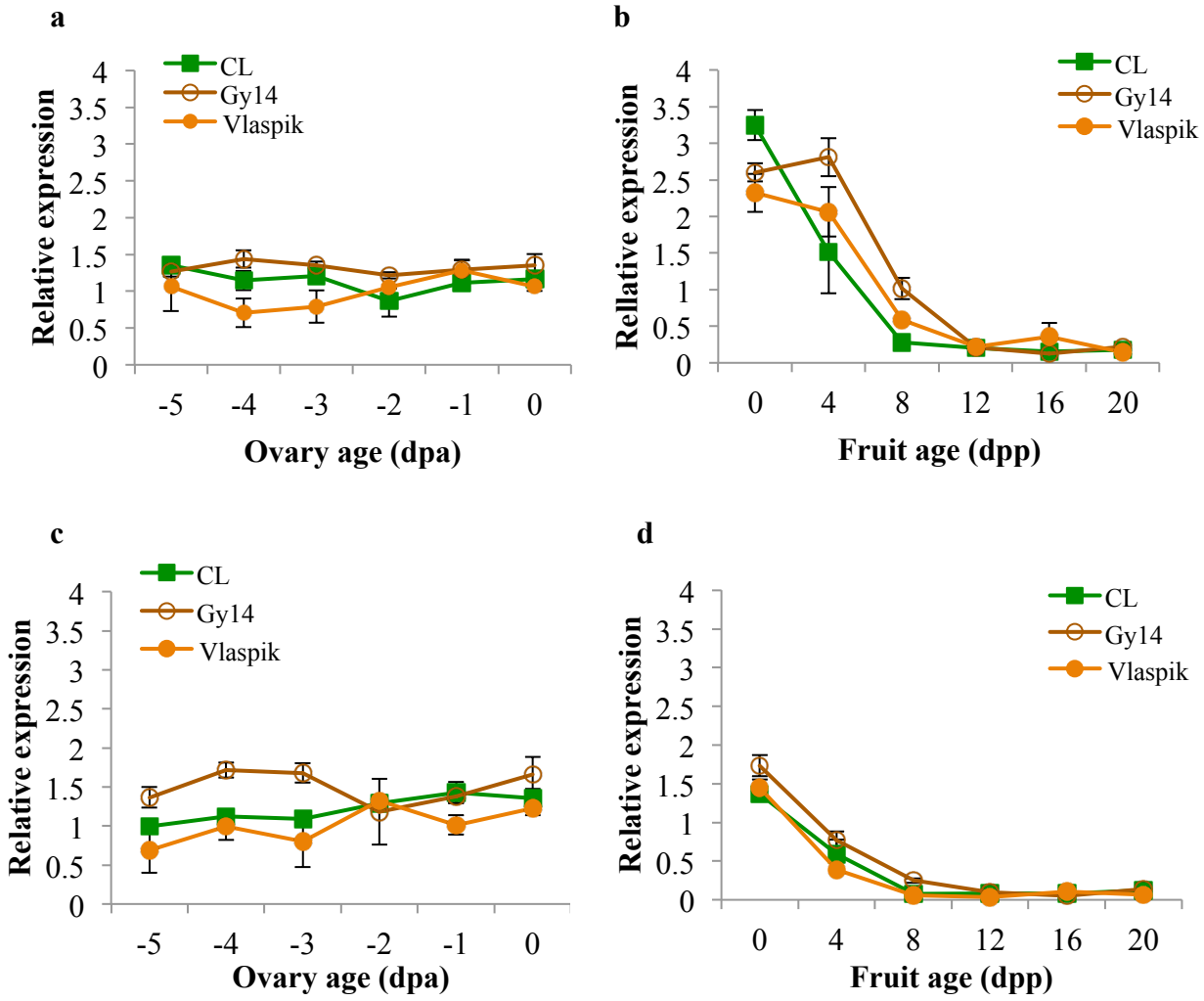

Figure 2.7. Expression of cell cycle-related genes [(a & b) *Histone 4*, (c & d) *CycD3;1*] in CL, Gy14 and Vlaspik at pre-anthesis to post-pollination, Each value is the mean of 3 biological replicates with 3 technical replicates/biological replicate ± S.E.

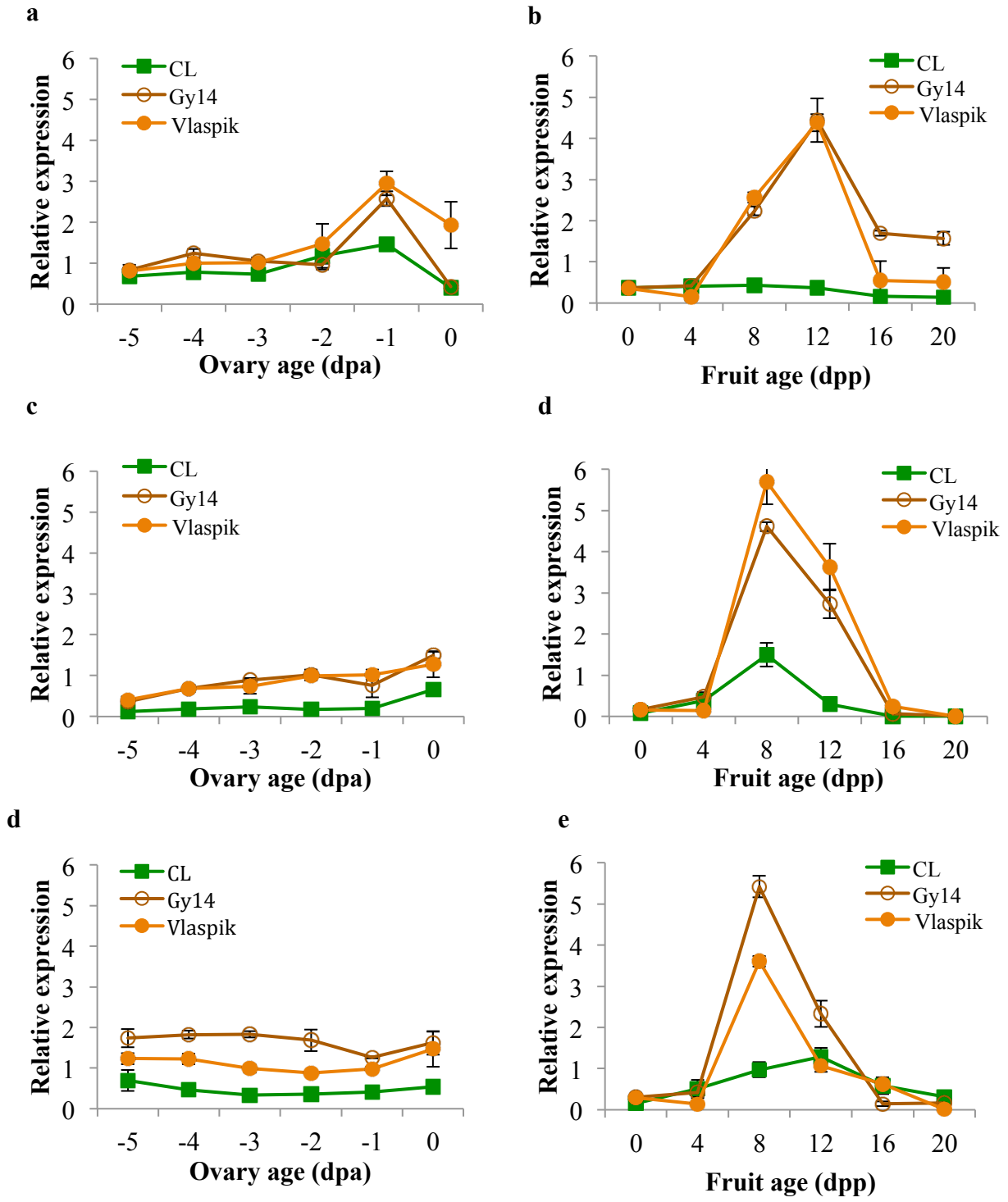

Figure 2.8. Expression of cuticle associated genes in CL, Gy14 and Vlaspik: (a & b) *LipidTr,* (c & d) *GDSL*, and (e & f) *SHINE* (Each value is the mean of 3 biological replicates with 3 technical replicates/biological replicate ± S.E.

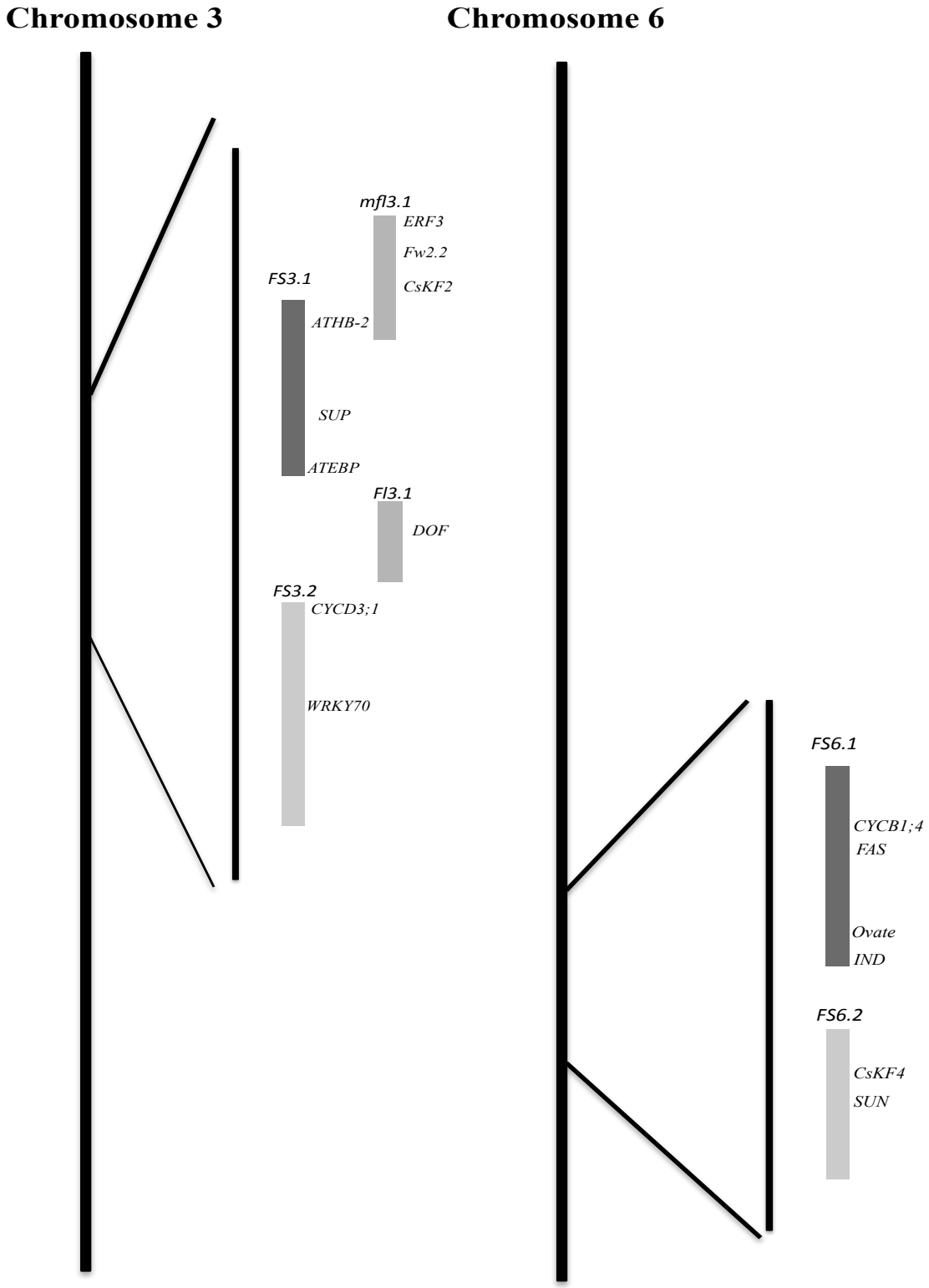

Figure 2.9. Approximate location on chromosomes 3 and 6 of fruit growth marker genes and homologs of known fruit size and shape genes in cucumber.

38-bp deletion at the 5′ end of the CDS in Gy14. Difference in the 5′ noncoding region was also observed for each of these genes. There were insertions/deletions and transitions/transversions within the sequence of either CL or Gy14. In the *ATHB-2* noncoding region, CL had an 8 bp and 12 bp deletion in the GAGA element and C-rich region, respectively. Sequence comparison of the 5′ noncoding region was not performed for *ERF3* due to poor sequence quality of Gy14. Notably, expression analysis of *ATHB-2* and *ERF3* showed that CL exhibited earlier and greater expression than either of the two pickling cultivars (Figure 2.10 e,f). Expression of WRKY70 did not differ between CL and the two pickling cultivars. Expression of *ATEBP* and *DOF* remains to be tested.

The cucumber cultivars also were examined for homologs of genes that have been identified to regulate fruit growth in tomato and other species: *Fw2.2*, *OVATE*, *FAS*, and *SUN* (Tanskley et al., 2004, Cong et al., 2008, Tsaballa et al., 2011, De Franceschi et al., 2013). In each case, the putative homolog with the highest homology was selected (E-value 1e-37 - 1e-83). All four putative homologs mapped to cucumber fruit size QTL. Homologs of *OVATE* and *FAS* genes mapped to *FS6.1* QTL on chromosome 6 (Table 2.2, Figure 2.9), and the homolog of *Sun* also was located on chromosome 6 but within the *FS6.2* QTL region. The *Fw2.2-like* gene did not map to any of the Gy14 and CL QTLs identified by Weng et al., (2015), however, it mapped to chromosome 3 in *mfl3.1* (Bo et al., 2014) along with *ERF3*. Except for *OVATE*, with 99% identity in the coding region, the rest of the homologs had 100% identity between CL and Gy14. A difference in amino acid was observed in position 47 in the peptide sequence of *OVATE* leading to a potentially conservative change in amino acid between asparagine in CL and histidine in Gy14. In the 5' noncoding region, homologs of *FAS* and *Fw2.2* had 99% and 98% identity, respectively, due to transitions/transversions. Identity of the noncoding sequence

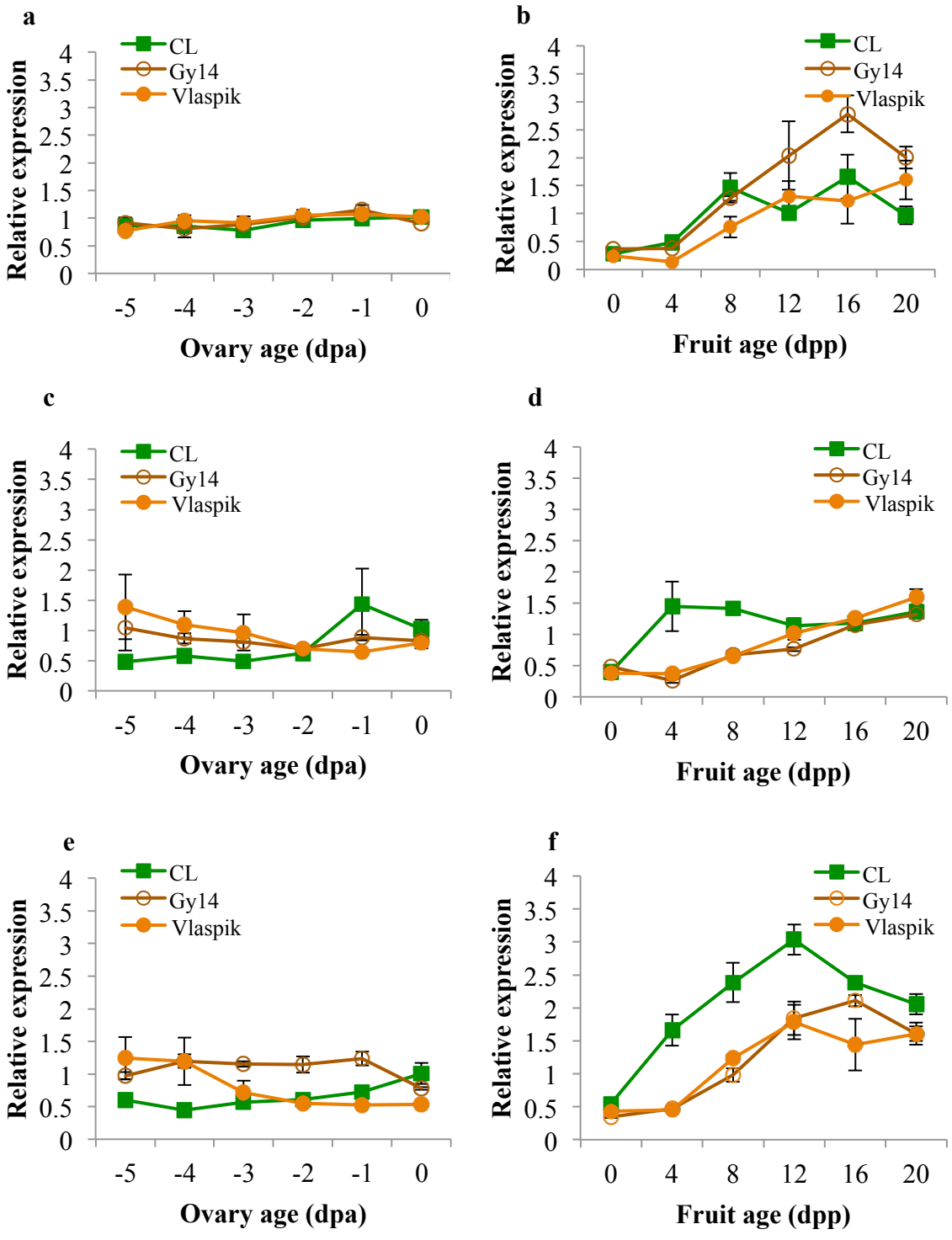

Figure 2.10. Expression of late/post-exponential fruit growth transcription factors in CL, Gy14 and Vlaspik: (a & b) *WRKY70*, (c & d) *ERF3* and (e & f) *ATHB-2* Each value is the mean of 3 biological replicates with 3 technical replicates/biological replicate ± S.E.

for the homologs of *Ovate* and *SUN* was not determined due to poor sequence quality of CL. qRT-PCR analysis of *OVATE*, *FAS*, and *SUN* did not show difference in expression between CL and the pickling types over the observation period of -5dpa to 20dpa (Appendix Figure 2.12). Expression of the homolog of *Fw2.2* remains to be tested. Recent report identified another fruit growth locus in tomato, *Slelf1,* however specific gene sequence is not yet available (Chusreeaerom et al., 2014), hence will be examined in the future.

DISCUSSION

Fruit size and shape are important determinants of market class and value in cucumber, however, key regulatory factors of fruit growth have not yet been identified in this species. Our data showed that CL and Gy14 exhibit dynamic differences in ovary and fruit size and shape throughout development. Evaluation of fruit growth-related factors in these two cultivars showed that there are a number of independent components, acting both pre- and post-anthesis, to influence fruit size and shape in cucumber.

Influence of ovary development on fruit growth

Prior analysis of fruit development in cucumber has been primarily focused on growth post-pollination (e.g. Marcelis and Hofmann-Eijer 1993, Fu et al., 2008, Ando et al., 2012, Yang et al., 2013), however, reports in other species, including other cucurbit crops, have shown that fruit growth can be influenced by ovary development (Perin et al., 2002, Johnson and Malladi 2011, Rosati et al., 2011). Difference in ovary length and diameter between CL and Gy14 was apparent as early as 7 days pre-anthesis with CL exhibiting longer ovaries and bigger ovary diameter than Gy14. During the period prior to anthesis, CL exhibited a more pronounced increase in ovary length while Gy14

exhibited a more rapid increase in diameter beginning at 4dpa. These differences in rates of growth in ovary length and diameter suggest that there are distinct factors controlling length and diameter acting early during development.

We further observed a relationship between ovary growth in the longitudinal direction pre-anthesis, and fruit length at maturity. Segregating F_2 and RIL progeny from reciprocal crosses of CL and Gy14, showed a strong positive relationship between ovary length and fruit length, and between ovary L/D and fruit L/D. These findings suggest that ovary length at anthesis is a good predictor of fruit length and genetic control of fruit shape acts prior to anthesis. Ovary length was also highly correlated with ovule number likely due to the linear arrangement of ovules within the cucumber ovary (Jing et al., 2000).

In contrast, ovary diameter was not correlated with fruit diameter, indicating that fruit width is primarily regulated post-anthesis. Consistent with this observation, multi-location and multi-season field trials of recombinant inbred line (RIL) populations of cucumber showed greater effect of environment on fruit diameter than length (Weng et al., 2015). Correlations among locations and seasons for ovary length, fruit length, and fruit L/D were all consistently high (0.64 to 0.86), but were lower for ovary diameter and fruit diameter (0.38 to 0.63).

Fruit growth post-pollination

Post-pollination, both CL and Gy14 exhibited a typical sigmoidal pattern of growth consistent with previous reports on parthenocarpic and non-parthenocarpic cucumber fruit growth (Marcelis and Hofmann-Eijer 1993, Ando and Grumet 2010). However, the rate of increase and timing of increase, both in length and diameter, differed between the two cultivars. For Gy14, peak increase in length and diameter

occurred at the same time. The growth pattern observed for length and diameter in Gy14 paralleled that previously observed for a second pickling type, cv "Vlaspik" (Ando and Grumet 2010). In contrast, the greatest increase in length for CL occurred earlier during fruit development followed by an increase in diameter later in development. Fruit growth in apple and in tomato cultivars with elongated fruit also showed initial increase in length followed by increase in width and the developmental timing of growth in length and diameter was attributed to differences in regulation of cell division (Van der Knaap and Tanksley 2001, Chang et al., 2014).

The difference in developmental rhythms for fruit length and diameter between CL and Gy14 was also manifested in L/D ratio, or fruit shape index. The L/D ratio peaked from 0-4dpp in Gy14 but for CL, L/D peaked at 5-9dpp, coinciding with the later shift in growth from length to diameter. Similar regulation of L/D ratio was observed in melon, wherein a decrease in fruit shape index coincided with the increase in diameter later in development (Monforte et al., 2014). Moreover, unlike CL where change in L/D ratio continued until around 16dpp, change in L/D ratio in Gy14 was apparent only until approximately 8dpp suggesting that the final fruit shape in Gy14 was achieved earlier during fruit development.

Analysis of relationships among fruit traits in F_2 progeny showed that fruit length was strongly correlated with L/D, such that, longer fruit were generally thinner. Despite this general trend, varying combinations of length and diameter were observed. While most were either 'long and narrow' or 'short and wide' some were 'long and wide' and 'short and narrow,' giving a 20-fold range in volume of ~90 cm^3 to ~1,800 cm^3. The lower correlation observed between diameter and L/D, than length and L/D, is consistent with the QTL study in cucumber by Yuan et al. (2008a,b) wherein they found a

significant correlation between fruit L/D and length, but not L/D and diameter. The various combinations between fruit length and diameter indicate that length and diameter segregate independently and that there are different factors controlling length and diameter post-pollination. These observations also indicate that there is not a set fruit volume, and that fruit size is separable from fruit shape. Growth factors that control photosynthetic capacity and assimilate distribution or biomass allocation have been previously shown to influence the overall fruit size in cucumber (Marcelis 1991, 1993) and these factors may also affect the final fruit size of CL and Gy14.

Ovule number, which was highly correlated with fruit length, may also contribute to fruit growth post-pollination. Studies in tomato and in cucumber indicated that fertilized ovules could influence the rate of cell division and initial growth rate of the ovary and fruit, possibly by eliciting combined action of various hormones such as auxin, cytokinin, gibberellin and brassinosteroid (Bohner and Bangerth, 1988, Varga and Bruinsma 1990, Gillaspy et al., 1993, Boonkorkaew et al., 2008, Li et al., 2014). The specific regulator of ovule number is not described yet in cucumber. However, a recent report showed that homologs of *INDEHISCENT* (*IND*) and *SUPERMAN* (*SUP*), genes in Arabidopsis that regulate ovule development and direction of auxin transport, respectively, were differentially regulated in cucumber exhibiting variation in fruit length (Jiang et al., 2015).

Ovary and fruit size and shape as a function of cell number and cell shape

Cell number was associated with differences in ovary and fruit length and diameter during development. There was a markedly greater increase in cell number in the cross section of Gy14, while CL had greater cell number in the longitudinal direction throughout ovary and fruit development. These observations are consistent with previous

reports on the role of cell number in regulating ovary (e.g. Cheng and Breen 1992, Harada et al., 2005, Zhang et al 2006) and fruit size (e.g. Higashi et al., 1999, Tanksley 2004, Johnson and Malladi 2011) in other species. Here we examined the expression of developmental gene markers including cell cycle-related genes, *histone 4* (*H4*) and *cyclin D3;1* (*CycD3;1*), during fruit growth. Consistent with earlier studies of cell cycle genes (Fu et al., 2010, Ando et al., 2012, Cui et al., 2014), both *H4* and *CycD3;1* were most highly expressed at 0-4dpp in all cultivars, coinciding with the period of peak cell division during early fruit growth. However, although the expression level of the cell cycle related genes was equivalent for both CL and Gy14, indicating that cells were actively dividing for both cultivars, the orientation of cell division differed such that CL cells were actively dividing in the longitudinal direction while Gy14 cells were more actively dividing radially.

Several cell cycle related genes, cyclins, cyclin dependent kinases (CDKs), histones and kinesins that were upregulated during early fruit development (Ando et al., 2012) mapped to cucumber QTL regions associated with fruit size and shape. D type cyclins, such as *CycD;1* and *CycD3;1,* regulate the commitment of cells to the mitotic cell cycle (Mironov et al., 1999, Meijer and Murray 2000, Oakenfull et al., 2002). CycD3 activity was also associated with *H4* expression in the proliferating cells in the S phase of the cell cycle (Riou-Khamlichi et al., 1999). *H4* interacts with *H3.2* to modulate chromatin condensation during mitotic division (Henikoff and Smith 2015, Wilkins et al., 2015).

Type B cyclins are also involved in the CDK/CYC cell cycle complexes (Boruc et al., 2010). Differences in the coding region of *CycB1;4,* a gene shown to co-localize with the spindle in the cytoplasmic region during chromosome segregation (Bulankova et al.,

2013), were observed between CL and Gy14, suggesting possible variation in its activity between the two cultivars. In addition, three cell division-related kinesins, *CsKF2, CsKF3,* and *CsKF4,* that showed differential expression in developing ovaries at 4dpa in cucumber cultivars exhibiting variation in fruit length (Jiang et al 2015) also mapped to cucumber fruit size QTL. Kinesins are microtubule-based molecular motors required for centrosome separation during the mitotic phase (Nigg 2001, Smith 2001, Lee and Liu 2004).

Studies in tomato, avocado and cherry have identified specific genes involved in regulating cell division (*Fw2.2* and its homologs) that were associated with fruit size variation (Nesbitt and Tanksley 2001, Tanksley 2004, Dahan et al., 2010, De Franceschi et al., 2013). Also in tomato, the *SUN* gene has been shown to control fruit elongation by affecting the direction of cell division (Xiao et al., 2008, Xiao et al., 2009, Wu et al., 2011). High expression of *SUN* is associated with reduction in cell number in the transverse direction but increased cell number in the longitudinal direction. Although the cucumber homolog of *SUN* mapped to a fruit size QTL, there were no coding sequence differences, nor was there difference in expression between CL and Gy14. However, it is also possible that *SUN* could be under the control of a regulatory element that dictates the orientation of cell division; such differential spatial distribution would not be readily evident from total analysis of gene expression.

Cell number alone does not appear to be sufficient to account for the differences in length and diameter between CL and Gy14. The cross section of Gy14 fruit had 2.5x more cells than CL, but fruit diameter of Gy14 was only 1.7x larger than CL. On the other hand, CL fruit had only 1.4x more cells in the longitudinal section than Gy14, but CL fruit were 2.2x longer than Gy14. In other species, cell size has been implicated to

contribute to fruit size variation (Harada et al., 2005, Yang et al., 2013). Although, we did not observe significant difference in cell area between CL and Gy14, CL cells were more elongated in the longitudinal direction than in Gy14. This difference became apparent after 4dpp, i.e., the end of period of rapid cell division. The homolog of *Arabidopsis homeobox protein 2* (*ATHB-2*), a gene involved in the shift in orientation of cell expansion (Steindler et al., 1999), showed earlier and greater expression in CL than either of the pickling cultivars, Gy14 or Vlaspik, coinciding with the end of cell division in the lateral direction. Collectively, the timing and expression level of *ATHB-2* corresponded to the difference in cell shape and the onset of cell expansion between the two fruit types.

ATHB-2 mapped to *FS3.1*, one of the major QTLs for fruit size in cucumber (Weng et al., 2015). Sequence comparison of the coding region of *ATHB-2* between CL and Gy14 showed 100% sequence similarity. Examination of the 5′ non-coding region, revealed a deletion in the GAGA element in CL. GAGA elements have been shown to regulate gene expression in diverse systems such as soybean (Sangwan and O'Brian 2002) and Drosophila (Tsukiyama et al., 1994), possibly by nucleosome stabilization. Although, further fine mapping needs to be done, our results suggest a potential role of *ATHB-2* in fruit growth in cucumber, specifically on fruit elongation.

A homolog of *ERF3* also showed an expression pattern similar to *ATHB-2,* wherein it was upregulated sooner in CL than the pickling cultivars. *ERF3* did not map to any QTLs identified in the study by Weng et. al. (2015), however it mapped to a scaffold within *mfl3.*1(Bo et al., 2014), a previously identified cucumber fruit QTL. *ERF3* is a member of Class II ethylene response factors and acts as a repressor in the ethylene-dependent transcription process (Ohta et al., 2001, Koyama et al., 2003). While homologs of *ERF3* have been shown to be involved in defense response (Kitajima et al., 1998,

Ohme-Takagi et al., 2000) and have not been specifically implicated in growth regulation, a study in Arabidopsis showed that an *ERF*-like gene affects cell division and expansion, possibly by regulating specific cell cycle/expansion related genes and by altering hormone signaling (Marsch-Martinez et al., 2006, Strader et al., 2010, Pei et al., 2013). In cotton, ethylene was implicated in modulating cell elongation by influencing the orientation of microtubules and cellulose microfibrils (Shi et al., 2006, Qin and Zhu 2011).

Moreover, recent reports in cucumber suggested the involvement of two kinesin genes, *CsKF1* and *CsKF7*, in differential cell expansion among cucumber cultivars exhibiting different fruit size (Yang et al., 2013, Jiang et al., 2015). However, only *CsKF7* mapped to a cucumber fruit length QTL, *mfl1.1*. In Arabidopsis, a kinesin-like calmodulin binding protein gene was described to interact with *ANGUSTIFOLIA* (*AN*) (Smith and Oppenheimer 2005). The *an* mutant showed altered microtubule resulting in cell expansion defects manifested by narrow leaves and cotyledons. In addition, a cyclin dependent kinase, *CDKE1*, that showed high expression during early cucumber fruit growth (Ando et al., 2012) also mapped to a fruit size QTL*, FS4.*1. CDKE acts in cell expansion in leaves and floral cell-fate specification (Wang and Chen 2004, Inze and De Veylder 2006, Engler et al., 2009, Van Leene et al., 2011).

Expression of other fruit size and shape homologs in cucumber

In addition to *SUN* and *Fw2.2*, two regulatory genes, *OVATE* and *FAS,* which control fruit shape in tomato (Liu et al., 2002, Xiao et al., 2008, Lippman and Tansksley et al., 2001), also mapped to cucumber fruit size QTL *FS6.1*. The *OVATE* gene is primarily expressed in the ovary and is considered a negative regulator of growth (Liu et al. 2002). A premature stop codon in the tomato gene resulted in transition from round to

pear shape fruit (Liu et al. 2002), while a mutation in *FAS* resulted in increased locule number and a flat fruit shape in tomato (Lippman and Tanksley 2001, Barrero et al., 2006, Rodriguez et al., 2011, Huang et al., 2013). Analysis of *OVATE* and *FAS* homologs in cucumber showed that CL and the pickling type Gy14 and Vlaspik had equivalent expression throughout ovary and fruit development. The homolog of *OVATE* showed an initial high expression at anthesis followed by a decline in expression at 4dpp in both cultivars consistent with peak expression in tomato (Liu et al., 2001). The expression of the cucumber homolog of *FAS* was also similar to the expression pattern in tomato, wherein it is most strongly expressed during the first few days post-pollination (Cong et al., 2008, Xiao et al., 2009).

Interestingly, the majority of the genes examined clustered to a specific region of either chromosome 3 or 6. For example the cucumber homologs of *FAS, OVATE* and *SUN* are all located in on QTLs *FS6.1* and *FS6.2* while *Fw2.2* was located on *mfl3.1*. Clusters of *OVATE, SUN*, and *FAS* gene family members also have been observed in tomato (Huang et al., 2013). A recent study identified 34, 31 and 9 putative members of the *OVATE, SUN*, and *FAS (YABBY)* gene families, respectively (E-value: <1e-5). Tightly linked groupings including family members of all three genes were found in three chromosomal locations. None of these locations, however, include the original of *OVATE, SUN*, and *FAS* genes specifically identified to influence tomato fruit size and shape. The cucumber genome also contains 20,18 and 5 putative members of *OVATE, SUN*, and *FAS* gene families, respectively (Weng et al., 2015). The homologs shown in Figure 2.10 on chromosome 6 are those with the highest homologies to the known tomato fruit size genes; the *OVATE* and *SUN* homologs were both located on Gy14 scaffold 00542. However, pairings of other family members of *SUN* and *OVATE* (*OFP*) were

found in close association in three additional locations, including a region within *FS3.1* where they were again located on the same scaffold, emphasizing the importance of QTL fine mapping to further dissect the genomic regions influencing fruit size and shape.

Cucumber fruit growth

The key drivers of fruit growth, cell division and cell expansion, are the cumulative result of diverse regulatory networks influenced by genetic factors and the environment. In other systems such as tomato, fruit growth is driven by intensive cell division early in development followed by a period where both cell proliferation and expansion occur simultaneously until the proliferative activity stops, and cell expansion continues (Gillaspy et al., 1993, Bertin et al., 2003). Our data suggest that in cucumber, the relative periods of cell proliferation and expansion can drive variation between cultivars showing differences in size and shape during fruit growth as illustrated in Figure 2.11.

In CL cucumber, radial cell proliferation ended early in development, while cell division in the longitudinal direction continued for a longer period and overlapped with the onset of cell enlargement. The simultaneous cell proliferation and cell enlargement early in development appear to drive the rapid increase in fruit length in CL during this period. In contrast, the greatest increase in diameter occurred after the period of cell division, indicating that radial increase was primarily driven by cell expansion. This difference in pattern of cell division and expansion longitudinally and radially during fruit development correlated with the higher increase in absolute growth in fruit length than diameter in CL. On the other hand, in Gy14, the shift in cell division to cell

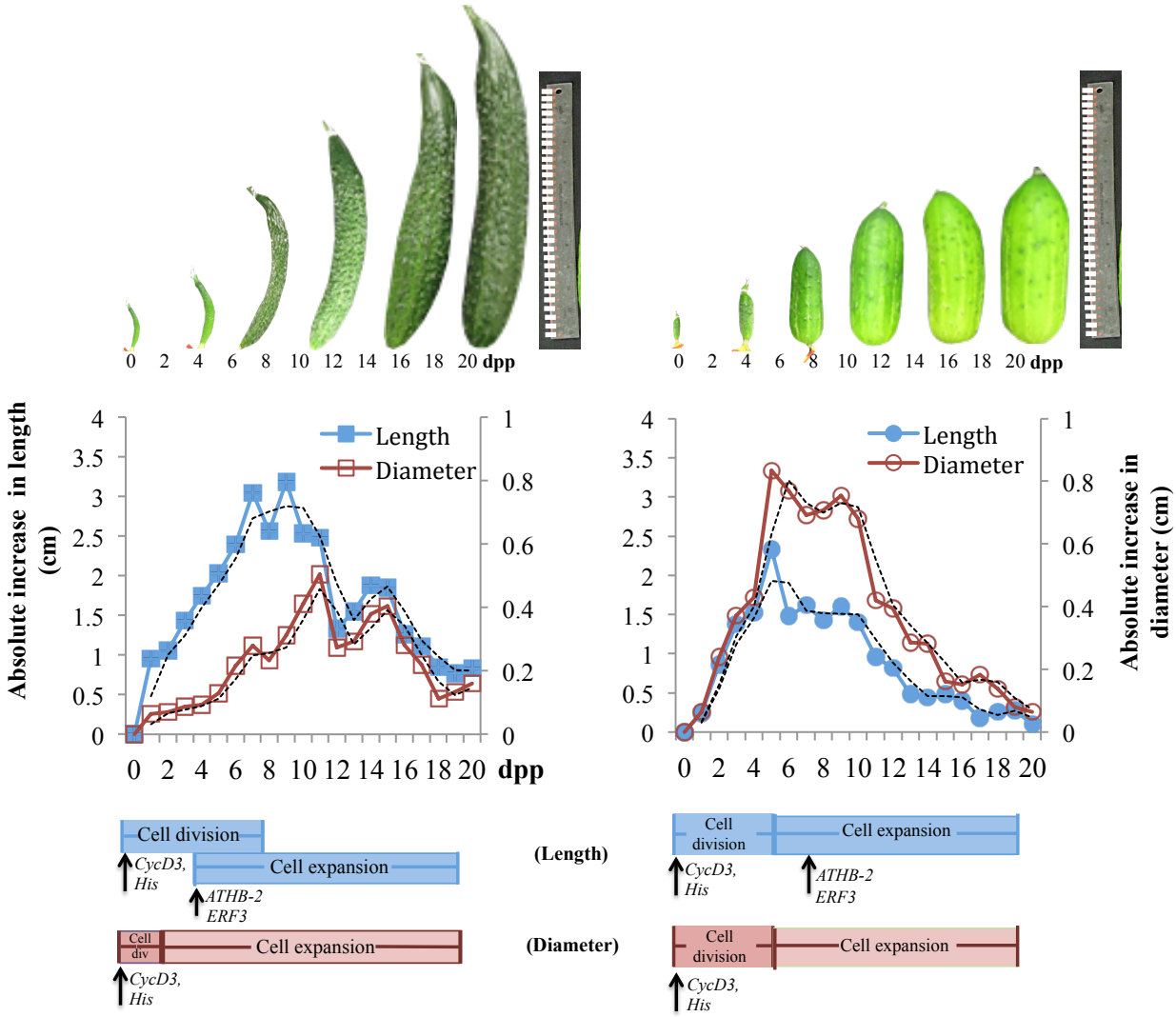

Figure 2.11. Fruit growth of Chinese long and Gy14.

expansion occurred during the same period both laterally and longitudinally. Although the period of cell proliferation was the same in both directions, there were more cells radially indicating preferential orientation of cell division in Gy14. For Gy14, the increase in fruit length and diameter followed the same pattern, however the absolute increase in diameter was more pronounced.

Despite differences in the pattern of cell division and expansion, both cultivars showed intensive cell division prior to cell expansion. The period of cell proliferation correlated with the level of expression of cell cycle related genes such as *CYCD3;1* and *His4*. Differential expression of the transcription factors, *ATHB-2* and *ERF3* between CL and Gy14, correlated with the difference in cell length in the longitudinal direction, suggesting that these genes may play a role in the regulation of cell expansion. Overall, the proposed model implicates that variation in fruit size and shape appears to result from a complex interplay between timing and orientation of cell division and expansion, and suggests potential involvement of numerous factors, all of which can influence the final growth pattern.

CONCLUSION

In this study, we examined ovary development and fruit growth between two cucumber genotypes showing distinct difference in fruit size and shape. Our findings indicate differences in numerous independent factors, both pre-anthesis and post-pollination, that can influence fruit size and shape, including: ovule number; differential rate and period of cell division in the longitudinal and transverse directions in both ovaries and fruit; differential timing and rate of fruit elongation and expansion; and cell shape. Among these factors, ovule number and ovary length were good predictors of length. The differential rate and period of cell division, and

orientation of cell shape appeared to be the primary drivers of fruit elongation. Moreover, the strong correlation between ovary L/D and fruit L/D indicates that key genetic factors regulating fruit shape act early during ovary development.

These results indicate that fruit size and shape of cucumber are controlled by multiple interdependent factors consistent with quantitatively inherited traits. Several studies in cucumber found a number of QTLs associated with fruit length, diameter and L/D (Serquen et al., 1997, Fazio et al., 2003, Yuan et al, 2008a,b, Miao et al., 2011, Wei et al., 2014, Bo et al., 2014), however, underlying factors that would explain the variation in fruit growth among cucumber cultivars with varying fruit size and shape have not been described yet. A recent study has identified cucumber fruit size and shape QTLs using a high-density linkage map based on a 45K SNP array in cucumber (Weng et al., 2015, Rubenstein et al., 2015). Examination of cell division-related genes, transcription factors, and homologs of known fruit size genes against the recently described cucumber QTL showed that many of these genes mapped to major QTL regions associated with fruit size and shape. Further examination showed that some of these genes were located on the same scaffolds within the same QTL region or are in QTL regions that are of close proximity within a specific chromosome segment. Although further fine mapping needs to be done to identify specific candidate genes, the co-localization of these genes with fruit size QTLs and their close association imply involvement in a regulatory mechanism controlling fruit size and shape in cucumber. Therefore, results from our fruit growth study both pre-anthesis and post-pollination, together with the fruit growth analysis of the RILs developed from CL and Gy14, and recent QTL data (Weng et al., 2015), should provide essential information that would facilitate the identification of underlying factors associated with fruit size and shape in cucumber.

APPENDIX

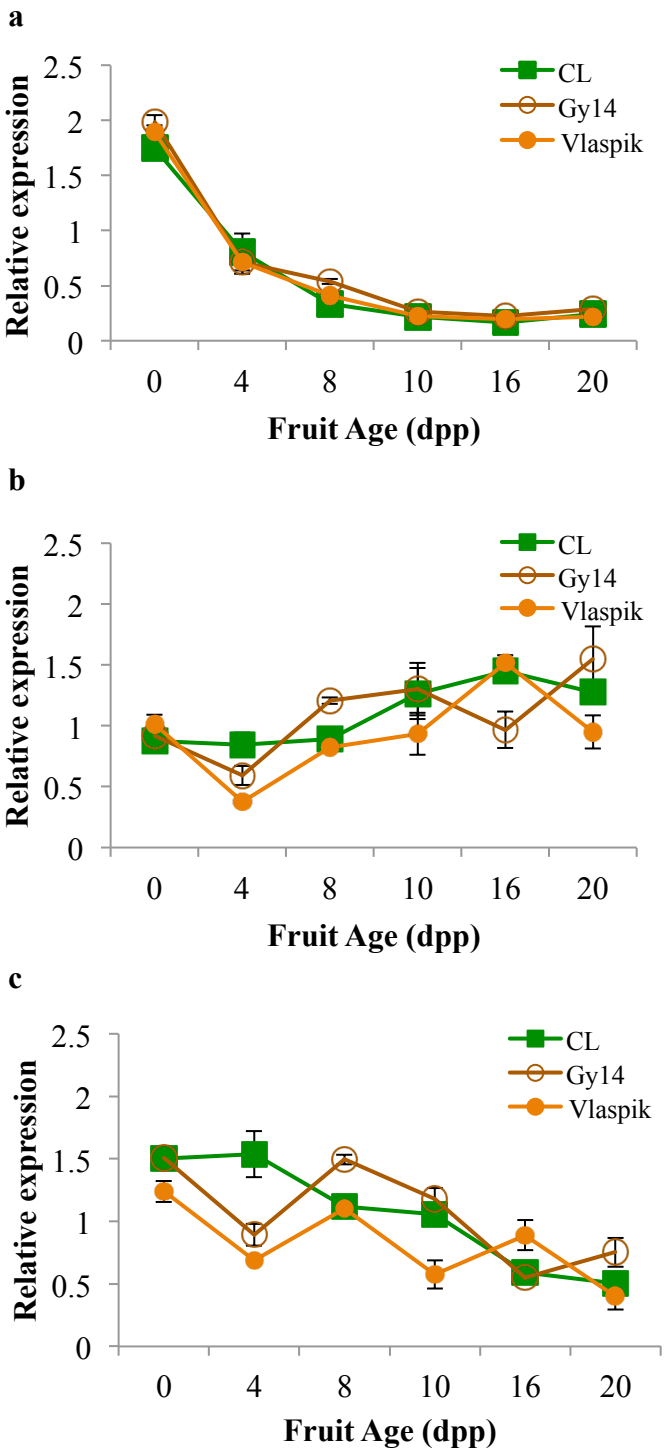

Figure 2.12. Expression of homologs of fruit size ad shape genes in CL, Gy14 and Vlaspik: (a & b) *Ovate*, (c & d) *FAS* and (e & f) *SUN* Each value is the mean of 3 biological replicates/technical replicate ± S.E.

Table 2.3. Primer sequences used in qRT-PCR analysis.

Genes	Primers	
	Forward (5' - 3')	Reverse (5' - 3')
CycD3;1	CCTCACAAACGCAAGCATGA	TGCGTCAATCACGCCATTT
Histone H4	AATGTGATTCGTGATGCTGTTACC	CCATAGCAGTCACCGTCTTCCT
GDSL-motif lipase/hydrolase	GGCCGCTTGGGTGTGTT	ACTGCCCGCCGCTACTT
SHINE1	GATATGGCTTGGGACGTTTGA	AGCTTGGTCGTACGCTTTGG
Lipid transfer protein	CGCACAACGCCGGATAG	GCCATTCACCAATGATTTCAAG
ATHB-2	CCACCCGGCCTTTCAAC	GCCCTGGGTAATGGGTTATTTAC
ERF3	CCACCTTCCGATCTTTTGATT	AGGCACAACGCGGTACATC
WRKY70	GATTGCTCCTGGCCTGACA	GCAATTCATCGGCTGCTTTT
OVATE	GTGGAGGGGAAAATCAGGGA	GCTCCAAATCCTTCTCCTCGAA
FAS	TCCTATTCGCCCACCAGAGA	GCTGCTGTGCTAAAGGCTTC
SUN	TCTGAGCATTCCTTGCCAAAC	CAATCATTGGAAGGCACTTGTCTA

Table 2.4. Correlations of RIL ovary and fruit growth traits between 2011 and 2012

Fruit trait	Correlation between Summer 2011 and 2012
Ovule number (ON)	0.64****
Ovary length (OL)	0.64****
Ovary diameter (OD)	0.38****
Ovary L/D (O L/D)	0.76****
Fruit length (FL)	0.80****
Fruit diameter (FD)	0.55****
Fruit L/D (FL/D)	0.78****

Table 2.5 Comparison of mean values for fruit growth parameters evaluated among parents and progeny in 2011 and 2012.

Genotype		MSU (2011+2012)					
		Anthesis			Mature		
		Length (cm)	Diameter (cm)	L/D	Length (cm)	Diameter (cm)	L/D
CL:	mean	3.35 a	0.50 a	6.82 a	30.29 a	5.39 d	5.70 a
	std.err.	0.09	0.02	0.38	0.73	0.16	0.2
	range	3.06 - 3.62	0.46 -0.54	5.72 - 8.28	26.78-32.00	4.95-5.91	5.00-6.32
Gy14:	mean	1.83 c	0.52 a	3.51 c	20.58 c	8.08 a	2.55 c
	std.err.	0.08	0.01	0.1	1.17	0.23	0.13
	range	1.62 - 2.10	0.49 - 0.55	3.23 - 3.59	17.9-26.4	6.94-8.58	2.19-3.20
F1:	mean	2.72 b	0.53 a	5.31 b	27.32 b	7.52 b	3.64 b
	std.err.	0.13	0.02	0.18	1.08	0.13	0.16
	range	2.32 - 3.24	0.47 - 0.63	4.95 -5.30	24.12-32.40	6.98-7.97	3.21-4.25
RILs:	mean	2.71 b	0.53 a	5.29 b	25.49 b	6.83 c	3.76 b
	std.err.	0.02	0.01	0.04	0.15	0.03	0.02
	range	1.40-4.85	0.30-0.91	2.55-10.71	17.62-38.00	4.74-9.12	2.44-6.59

Means with the same letter are not significantly different at p=0.05, LSD

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CHAPTER 3: Factors influencing age related resistance to *Phytophthora capsici* of cucumber fruit

INTRODUCTION

The oomycete pathogen, *Phytophthora capsici*, infects a broad range of crop species including several members of the Cucurbitaceae family, such as winter and summer squashes, pumpkin, zucchini, melon, and cucumber (Babadoost 2004, Li et al., 2011, Enzebacher and Hausbeck 2012, Granke et al., 2012). In cucumber, *P. capsici* primarily causes fruit rot (Gevens et al., 2006). Infected young fruits exhibit water soaking followed by sporulation and tissue collapse (Gevens et al., 2006, Ando et al., 2009). Many cucumber-growing areas are contaminated with *P. capsici* resulting in loss of productive land and rejection of loads of harvested cucumbers (Hausbeck and Lamour 2004).

P. capsici is capable of producing oospores which can survive in the soil for a long period of time (Hausbeck and Lamour 2004). *P. capsici* also produces thalli that give rise to lemon-shaped sporangia. In the presence of water and favorable temperature, motile zoospores are released from the sporangia. The primary inoculum throughout the growing season is zoospores; irrigation water contaminated with zoospores result in dispersal of the pathogen in different fields (Brasier 1992, Hausbeck and Lamour 2004, Granke et al., 2009, Lamour et al., 2011).

Zoospores are chemotactically and electrotactically attracted to potential infection sites on the surface of host plants. After reaching the surface of a potential host, zoospores encyst and adhesion proteins are produced that prevent the spores from being dislodged from the plant surface, as well as facilitate the reception of signals for the development and penetration of specialized infection structures such as hyphae or appressoria (Robold and Hardham 2005, Hardham 2007, Hardham and Shan 2009). *Phytophthora* hyphae may penetrate the plant surface either anticlinal walls or directly through the outer periclinal wall (Li et al., 2011). The hyphal

growth of hemibiotrophic species such as *P. capsici* is restricted to the apoplast, disruption of host cells is minimized, and acquisition of nutrients is through haustoria that form predominantly in mesophyll or cortical cells (Hardham 2007, Hardham and Shan 2009).

Findings from studies on both field- and greenhouse-grown cucurbits indicated an age-related resistance (ARR) of cucurbit fruit to *P. capsici* (Gevens et al., 2006, Ando et al., 2009, Ando 2009, Meyer and Hausbeck 2013). In all of the cucurbit fruit tested, younger fruits were more susceptible to *P. capsici* than older fruits. However, among the cucurbits examined, cucumber exhibited the most striking effect of fruit age to disease susceptibility such that almost complete resistance to *P. capsici* was observed in older fruits (Ando et al., 2009). Moreover, in cucumber, the transition from susceptible to resistant occurred when period of rapid fruit elongation was completed [approximately 10-12 days post pollination (dpp)] (Gevens et al., 2006, Ando et al., 2009).

Developmentally regulated resistance or ARR, wherein resistance increases with plant or tissue age, was also observed in other species and could be manifested either in the whole plant level, only in specific organs or during developmental transition (Ficke et al., 2002, Panter and Jones 2002, Develey-Reviere and Galiana 2007). In the pathosystem such as pepper – *P. capsici*, *Ralstonia solanacearum*, or cauliflower mosaic virus (CMV) (Kim et al., 1989, Hwang et al., 1996, Garcia-Ruiz and Murphy 2001, Lemessa and Zeller 2007); Arabidopsis - *Pseudomonas syringae* pv. tomato (Al-Daoud and Cameron 2011); and mutant cotton - cotton leaf curl virus (CLCuV) (Akhtar et al., 2004), the whole plant exhibited increasing resistance as the plant aged. Fruit of some species could also develop resistance as a function of tissue maturity as shown in grape - *Uncinula necator* (powdery mildew) and *Guignardia bidwellii*, (grape black rot) interactions (Hoffman et al., 2002, Gadoury et al., 2003). ARR was also manifested in older

leaves in several plant-pathogen interactions including soybean - *Phytophthora megasperma* (Bhattacharyya and Ward 1986, Ward 1989); cowpea - *Uromyces vignae* (Heath 1994); pepper - *C. coccodes* and rice -*Magnaporthe grisea* (blast fungus) (Hong et al., 1998, Xie et al., 2011). The transitions from juvenile to vegetative stage, and from vegetative stage to reproductive stage were also associated with development of resistance and this type of resistance was manifested in the interactions between corn - *Puccinia sorghi* (Abedon and Tracy 1990); lentil - *Ascochyta fabae* (Pedersen and Morrall 1994); potato - *P. infestans* (Mutty and Hossenkhan 2008); and Arabidopsis - *Pseudomonas syringae* (Rusterrucci et al., 2005).

Despite early reports on developmentally regulated resistance in a number of pathosystems, in general, the genetic basis of ARR is not well understood. Only two studies, to our knowledge, have examined inheritance of ARR. A study in sorghum using F_2 generation and $F_{2:3}$ families indicated that developmental related resistance against *Colletotrichum graminicola* was controlled by dominance at a single multiallelic locus (Tenkouano et al., 1998). On the other hand, inheritance study for ARR against *P. capsici* in pepper suggested that ARR is controlled by two major genes with epistatic interaction (Reifschneider et al., 1992). These findings indicate that ARR seems to be under simple genetic control.

The mechanism involved in ARR was suggested to be, in most cases, different from the response of plant to infection as a result of systemic acquired resistance (SAR) or induced systemic resistance (ISR) and in general, ARR in different pathosystems suggests that a variety of complex mechanisms are involved (Panter and Jones 2002, Develey-Reviere and Galiana 2007). In cucumber, factors contributing to ARR expression are not yet described. However, investigation of cucumber fruit - *P. capsici* interaction revealed a potential role of fruit peel in ARR. Examination of *P. capsici* zoospore germination on the surface of cucumber fruit

harvested at 8 and 16 dpp showed short germ tubes and a higher number of appresoria on 8 dpp fruit and more frequent occurrence of long or aberrant germ tubes on 16 dpp fruit (Ando 2009). Long germ tubes and aberrant appressoria-like structure have been associated with inability of the pathogen to infect the host plant (Grenville-Briggs, 2008). When peels of 15 dpp fruit were placed on top of 8 dpp intact fruit and inoculated with *P. capsici*, the 15 dpp fruit surface section showed resistance similar to intact 15 dpp fruit and protected the underlying 8 dpp fruit from infection (Ando 2009). These results suggest that the fruit peel has properties that inhibit *P. capsici* growth.

Part of the structure of the fruit peel is the cuticle, which serves as the first structural barrier that the pathogen must breakdown to gain access to the cells. In grape berries, cuticle thickness increased as the fruit matured however, the increase does not correlate with the degree of resistance in older berries (Ficke et al., 2002). On the other hand, components of cuticle were associated with signal transduction upon pathogen infection (Kolattukudy et al. 1995, Yakoby et al. 2002). Studies indicated that cuticular lipids play a role as messenger molecules during pathogen infection such as in the formation of appressoria and initiation of penetration (Kolattukudy et al. 1995, Yakoby et al. 2002, Hwang et al., 1995, Patto and Niks 2001, Skamnioti and Gurr 2007, Feng et al., 2009, Uppalapati et al., 2012). Cutin monomers and lipid transfer proteins were also suggested to be involved in plant defense reactions (Fauth et al., 1998, Kauss et al., 1999, Kim et al., 2008, Carvalho and Gomes 2007, Kirubakaran et al., 2008, Lee et al., 2009, Kiba et al., 2012). Phenolics such as cinnamic acids and flavonoids present in the cutin matrix were also shown to have antimicrobial activity (Muller and Riderer 2005, Dominguez et al., 2011).

The physiological basis of resistance of plants to various pathogens has been associated with both pre-formed and infection-induced antimicrobial compounds (Hammerschmidt, 1999, Mert-Türk, 2002). For example, in maize, accumulation of phenolics and amides were shown to be developmentally and spatially regulated (Le Clere et al., 2007). Developmentally regulated preformed antifungal activity was also found in flower tissue and in the achenes of green stage strawberry fruit (Terry et al., 2004). In addition, there are also reports on the role of infection-induced compounds in resistance to pathogens. For example, infection-induced phytoalexins were identified in pepper-*P. capsici* pathosystem; capsidiol formed after infection was associated with age-related resistance (Hwang and Kim 1990). Accumulation of pterocarpan phytoalexin glyceollin in soybeans, after *Phytophthora sojae* infection, was also affected by age or developmental state of tissues wherein elicitation is maximal in 7-9 days old cotyledons (Abbasi and Graham 2001). In cucumber there are no reports yet on developmentally regulated biochemical compounds conferring resistance against *P. capsici*. However, several studies have demonstrated antimicrobial activity of biochemical compounds in cucumber, including methanol-soluble p C-glycosyl flavonoid phytoalexins which increase markedly in leaves infected with *Podosphaera xanthii* (powdery mildew) leading to an induced resistance against the fungus (McNally et al., 2003a,b; Fofana et al., 2005). Glycoside-linked phenolic compounds from cucumber leaves (Lin et al., 2009), sphingolipids isolated from crude methanol extract of cucumber stems (Tang et al., 2010), and volatiles from cucumber fruit (Sotiroudis et al., 2010) also have been shown to inhibit pathogen growth.

Indeed, expression of ARR may be influenced by a number of factors and different mechanisms may be involved in regulating ARR. In this study, we examined the potential role of biochemical compounds in fruit peel in inhibiting *P. capsici* growth using cucumber fruit from

susceptible and resistant ages of both ARR+ and ARR- cultivars. Results of bioassay indicated that biochemical compounds from cucumber fruit peel might confer ontogenic resistance against *P. capsici*. Moreover, we also examined different inbred cucumber cultivars for ARR expression, and using selected cultivars, we also examined inheritance of ARR in cucumber. Our results indicate that not all cucumber cultivars manifest ARR. In addition, our findings also suggest that ARR may be inherited as a dominant trait.

MATERIALS AND METHODS

Screening of cucumber cultivars for ARR

A set of 22 cucumber cultivars was grown in the greenhouse following the procedure described by Ando et al. (2012). Hand pollinations were performed on 1-2 flowers per plant. To avoid the effects of interfruit competition, only one fruit per plant was allowed to develop. Three to ten fruits at 16dpp were collected from each cultivar and examined for ARR. The detached fruit method by Gevens et al. (2006) was used to screen the cultivars but with some modifications. Zoospore suspensions were prepared from 7-day old cultures of *P. capsici* isolate OP97 (Gevens et al., 2006) or NY0664-1 expressing either GFP or RFP (Dunn et al., 2013) grown on diluted V8 media and flooded with 6-10 ml sterile distilled water to release zoospores as described by Gevens et al. (2006). After surface sterilization, each fruit was inoculated with two droplets (30ul/droplet) each of *P. capsici* OP97 and NY0664-1 zoospore suspension with a concentration of $1x10^5$ zoospores/ml. Inoculated fruits were placed on a humid chamber lined with wet paper towel and trays were incubated at 25-26^oC under constant light. Development of disease symptoms such as water soaking and mycelial growth on each fruit was monitored daily for ten days. Fruits were evaluated using a disease rating in scale of 1-9 (1=no symptom;

9=tissue collapse) as shown in Figure 3.1. ARR and non-ARR expressing genotypes were selected and used as parents to develop a segregating population for ARR inheritance study.

Examination of cucumber fruit peel

Cucumber cv. 'Vlaspik' fruit at 0, 4, 8, and 16dpp were collected and exocarp sections (2-3mm) were excised from the middle section of each fruit. Sample preparation and imaging was performed by the Center for Advanced Microscopy of Michigan State University. Exocarp tissues were fixed in glutaraldehyde solution and dried in Balzers Model 010 critical point dryer (Balzers Union Ltd., Balzers, Liechtenstein). After drying, the samples were mounted on aluminum stub using high vacuum carbon tabs (SPI supplies, West Chester, PA) and coated with osmium using a NEOC-AT osmium coater (Meiwafosis Co. Ltd., Osaka, Japan. Processed exocarp tissues were then examined in a JEOL JSM-7500F scanning electron microscope (JEOL Ltd., Tokyo, Japan).

To determine if there was a difference in cuticle accumulation among the cucumber cultivars 'CL' and the two pickling types, 'Gy14' and 'Vlaspik', peel sections were collected from the middle part of 16dpp fruit of each cultivar. Thin cross sections (1-2mm) of the peel were prepared and were stained with Sudan IV (Buda et al., 2009). Peel sections were viewed through a light microscope with Spot RT3 digital camera system (SPOT Imaging Solutions, Diagnostic Instruments, Inc., MI).

Preparation of peel extracts

Pickling cucumber plants cv. 'Vlaspik' (ARR expressing) and breeding line 'Gy14' (non-ARR expressing) were grown in the greenhouse as described above. Hand pollinations were performed sequentially to allow for simultaneous harvest of fruit at 8 and 16 dpp. Fruit exocarp

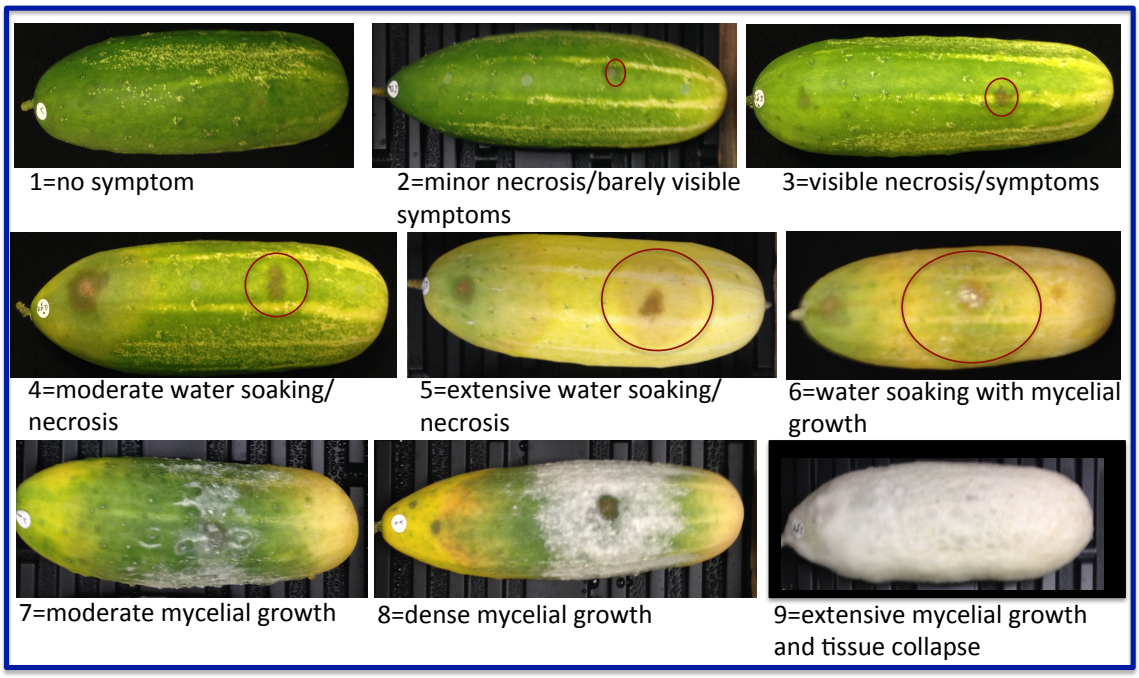

Figure 3.1. Disease rating scale used in the evaluation of cucumber fruit for ARR.

(1-2mm thick) was collected from the middle section of each fruit by razor blade. Frozen peel samples from fruits of the same developmental stage were pooled and used immediately for sequential extraction with water followed by methanol (Figure 3.2, Table 3.1) based on the procedure by Jayaprakasam et al. (2003). Each extract was concentrated by rotary evaporation (BUCHI Rotavapor, BUCHI, Corp., Newcastle, DE) and freeze-dried using Genesis Pilot Freeze Dryer (SP Scientific Industries, Stoneridge, NY) .

Bioassay of peel extracts

The aqueous and methanolic extracts (Table 3.1) were redissolved in water and 10% methanol, respectively, to a final concentration of 25 µg/ul. A 96-well clear (Thermo Fischer Scientific Inc., Waltham MA) or black microtiter plate (Griener Bio-One, Orlando, FL) was prepared with 200 µl clarified V8 media (centrifuged at 10,000 rpm for 10 min) per well. Samples were treated with 10 µl crude extract solution or solvent controls, and inoculated with 20 µl of $1x10^5$ zoospores/ml suspension of either *P. capsici* isolate OP97, NY0664-1G (GFP) or NY0664-1R (RFP) prepared as described above and incubated at 25^oC with a 16h light/ 8h dark cycle for 72 hours. Visual ranking was performed on a 1-5 scale as illustrated in Figure 3.6. Fluorescence values were measured at 485nm (excitation) and 530nm (emission) for NY0664-1G (GFP) and at 530nm (excitation) and 590nm (emission) for NY0664-1R (RFP) using SpectraMax M2e (Molecular Devices, Sunnyville, CA) at 0, 24, 48 and 72hrs post inoculation. Mean fluorescence measurements from the media and extract controls in the absence of pathogen were subtracted from the mean fluorescence values for the corresponding treatments. Each experiment was repeated two or three times with five replicate samples per treatment. Data were analyzed by ANOVA using the SAS program 9.1 (SAS Institute Inc., Cary, NC) with mixed procedures.

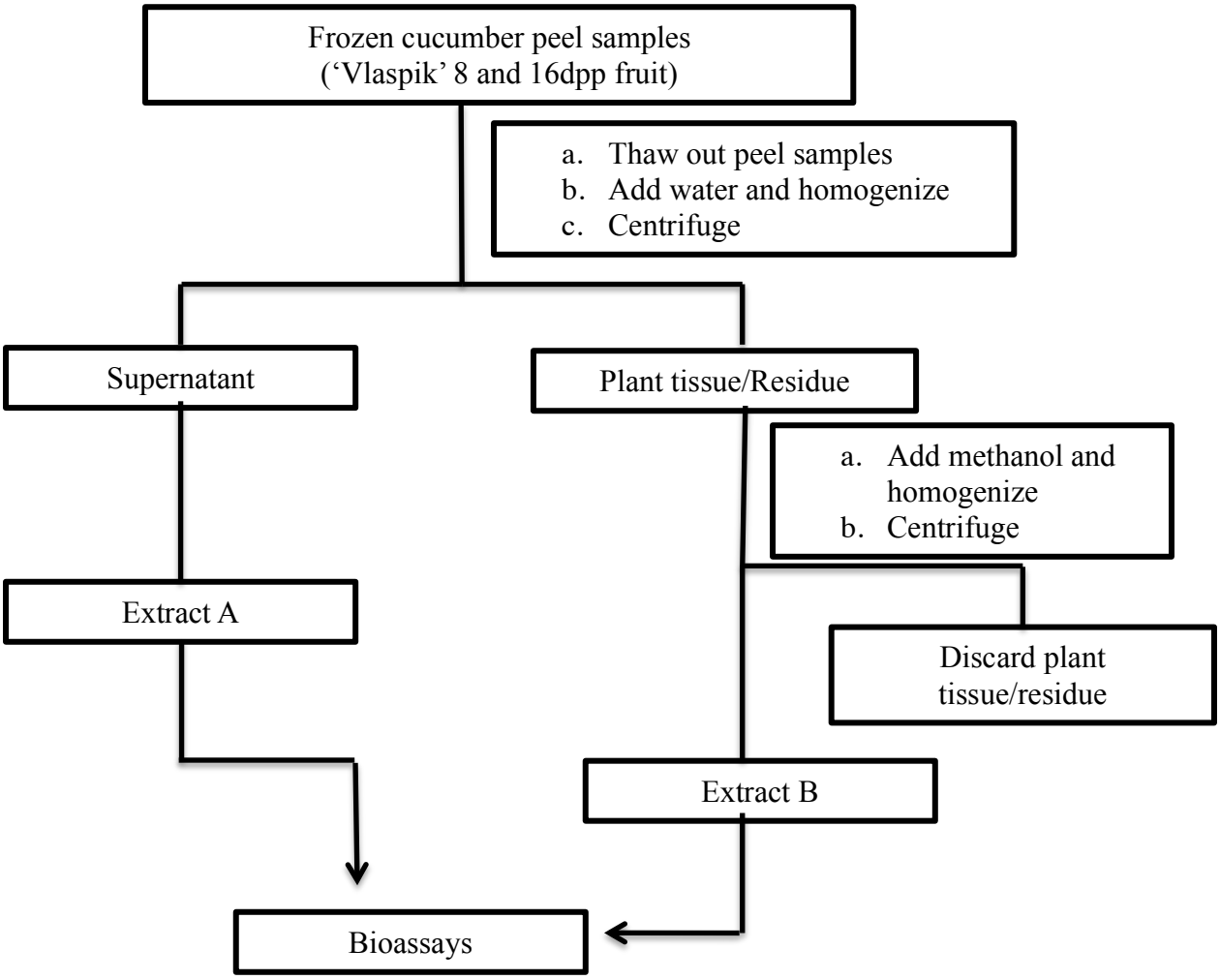

Figure 3.2. Schematic of extraction protocol for cucumber peel samples.

Table 3.1. Aqueous and methanolic extracts from 8dpp and 16dpp 'Vlaspik' fruit peel.

Fruit age	Fruit peel (g)	Water (ml)	Methanol (ml)	Aqueous extract (mg/ml)	Methanolic extract (mg/ml)
8dpp	14.5	150	150	3.47	0.80
16dpp	138.2	400	350	3.10	1.49

Examination of the genetic basis of ARR expression

To understand the genetic basis of ARR expression, 10 P1 (Vlaspik, ARR+), 10 P2(Gy14, ARR-), 26 F_1 and 161 F_2 progeny from reciprocal crosses between Vlaspik and Gy14 were grown in the greenhouse. One fruit from each plant was harvested at 16dpp and evaluated for ARR as described above.

RESULTS AND DISCUSSION

Expression of ARR to *P. capsici* in cucumber cultivars

A set of 22 cultivars was examined for ARR by comparing response to zoospore inoculation of 8dpp and 16dpp fruits. Symptoms were evaluated using a 9-point disease rating scale as illustrated in the Methods section. Ratings of 1-3 were considered resistant with no symptom or symptoms limited to the site of inoculation; 4-6=moderately susceptible; and 7-9=highly susceptible (Figure 3.1). As previously described, the cultivar 'Vlaspik' showed strong expression of ARR (Gevens et al., 2006, Ando 2009). The 8dpp fruit were highly susceptible with severe symptoms and extensive pathogen growth (disease rating of 8.0±0) but the 16dpp fruit exhibited resistance with slight necrotic spots limited to the site of inoculation (disease rating of 3.0±0.9) (Figure 3.3a,b). In addition to 'Vlaspik', two cultivars, 'Pointsett 76' and 'Long Green Improved', also exhibited ARR with mean disease score of 3.0±0.2 and 2.5±0.3 at 5dpi (16dpp fruit), respectively (Table 3.2, Figure 3.4). The rest of the cultivars tested did not show ARR (range of mean disease score: 4.4± 0.5 - 8.0± 0.0) such that both 8dpp and 16dpp fruit were susceptible to *P. capsici*. Previous studies showed differences in ARR expression in different cucurbit crops (Ando 2009, Meyer and Hausbeck 2013). For example, zucchini and summer squash did not manifest ARR as strongly as the other cucurbits, while melon and

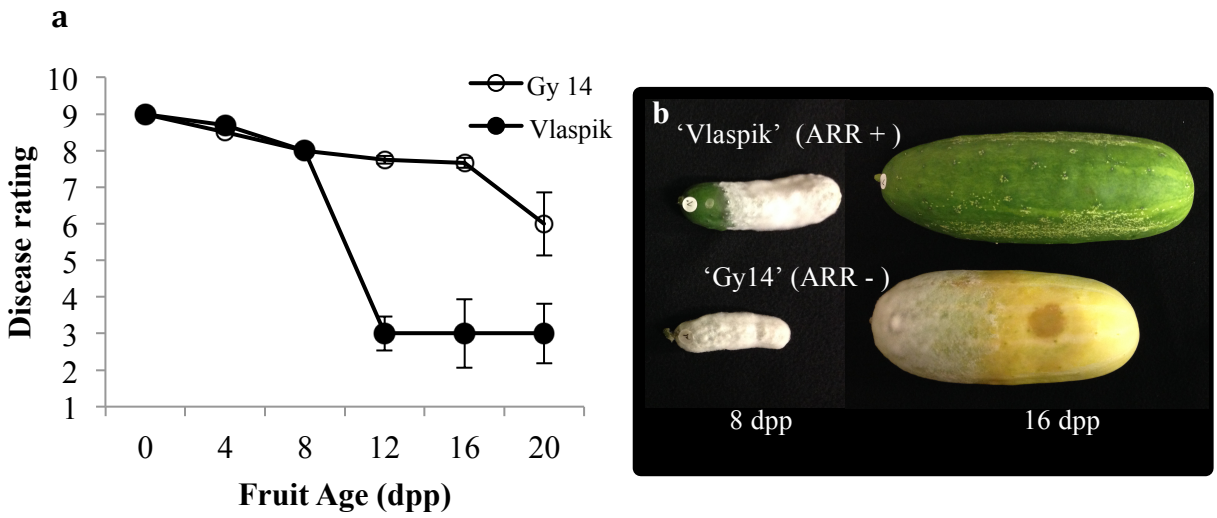

Figure 3.3. (a) Disease rating at 5 dpi of 'Vlaspik' (ARR+) and 'Gy14' (ARR-) fruits at different developmental stages. (b) Disease development of 8 and 16 dpp 'Vlaspik' and 'Gy14' fruits. Each value is a mean of 3-5 fruits ± S.E. Photo taken at 5dpi.

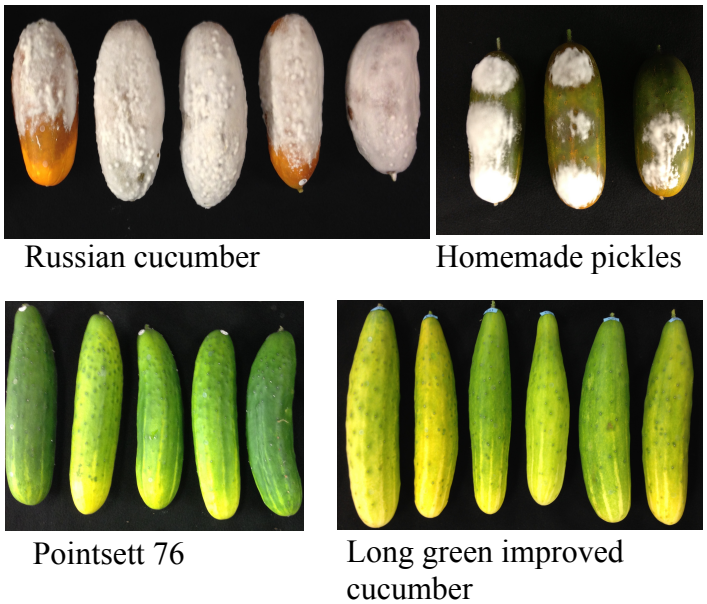

Figure 3.4. Examples of ARR and non-ARR-expressing cucumber cultivars. Fruits were harvested at 16 dpp . All fruits harvested at 8dpp were susceptible. Photos taken at 10 days post inoculation.

Table 3.2. Cucumber cultivars tested for ARR.

Cucumber Varieties	Disease rating	ARR (R/S)	
		8 dpp	16 dpp
Ashley	7.0±0.0	S	S
Boston Pickling	5.0±0.7	S	S
Boston Pickling Improved	7.0±0.0	S	S
Certified Organic Boothby's	8.0±0.0	S	S
Chinese Long	6.5±0.5	S	S
Delikatesse	7.0±0.0	S	S
Gy14	7.7±0.1	S	S
Homemade Pickles	5.1±0.1	S	S
Long Green Improved	2.5±0.3	S	**R**
Miniature White	7.6±0.4	S	S
Muncher	7.5±0.3	S	S
National Pickling	7.3±0.3	S	S
Parisian Pickling	7±0.0.0	S	S
Pointsett 76	3.0±0.2	S	**R**
Rhinish Pickle	4.5±1.0	S	S
Russian Cucumber	6.1±0.8	S	S
Spacemaster 80	6.5±0.4	S	S
Tanja	8.0±0.0	S	S
Tendergreen Burpless	8.0±0.0	S	S
Vlaspik	3.0±0.9	S	**R**
White Wonder	7.3±0.2	S	S
Zarnista	4.4±0.5	S	S

Each value is a mean of 3-10 fruits ± S.E.

and watermelon did not exhibit ARR at all (Ando 2009). Variation in the expression of ARR was also observed between a processing pumpkin cultivar and a winter squash cultivar (Meyer and Hausbeck 2013). The observed variability in ARR expression among different cucumber cultivars will enable the development of materials to study genetic factors regulating ARR.

Analysis of cucumber fruit peel in relation to fruit age and ARR

Our previous studies in cucumber indicated that fruit surface plays an important role in ARR to *P. capsici* (Ando 2009, Ando et al., 2015). Therefore, we examined changes during development of the physical and biochemical components of the fruit peel that may influence the manifestation of ARR.

Structural changes in cucumber fruit peel during development

During the susceptible ages when fruit were rapidly growing (0-8dpp), the fruit surface underwent dramatic changes with regard to glandular trichomes, warts and epidermal cell structure (Figure 3.5a). The epidermal cells rapidly expanded, with approximately 50-fold increase in cell size. The most marked difference in cell surface between 8ddp (susceptible age) and 16dpp (resistant age) fruit was in cuticle accumulation. At 16dpp, a thick layer of cuticle was present on the upper epidermal layer and was heavily intercalated between the epidermal cells.

Comparison of fruit peels between ARR- 'Gy14' and 'CL,' and ARR+ 'Vlaspik' cultivars at 16dpp showed that epidermal cell type and cuticle thickness varied among the cucumber cultivars (Figure 3.5b). However, the structural differences were related to market type and not ARR to *P. capsici*. The pickling type 'Gy14' (ARR-) and 'Vlaspik'(ARR+) had similar type of columnar epidermal cells and showed comparable accumulation of cuticle manifested by the darker stain around the cells. On the other hand, the epidermal cells of CL (also ARR-) were less elongated and showed minimal accumulation of cuticle. Thus, although structural changes

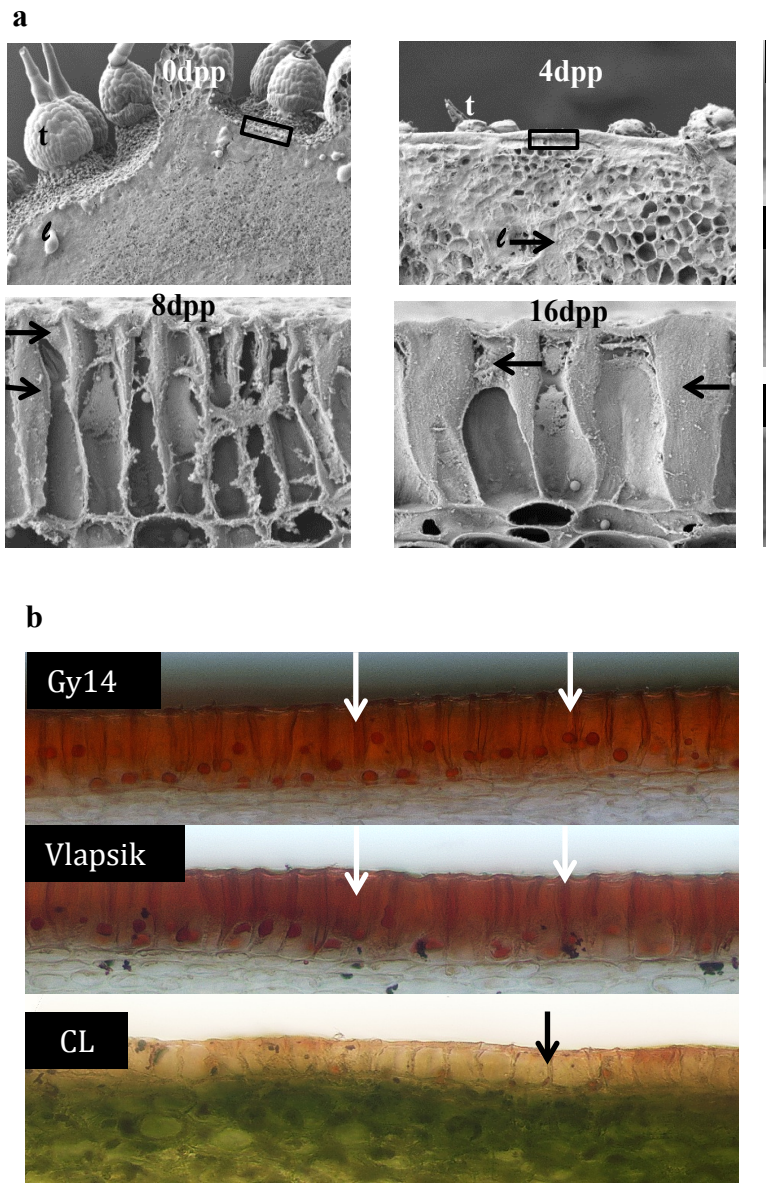

Figure 3.5. (a) Scanning electron microscopy of cross sections of Vlaspik (ARR+) fruit peel at different developmental stages. Cuticle accumulation between epidermal cells in 8 and 16 dpp peel are denoted by the arrows; box in 0dpp and 4dpp indicate epidermal cell layer; t=trichomes; l=latex. All sections were viewed at the same magnification (3,200x). (b) Cross sections of peel from 16 dpp fruit of Vlaspik (ARR+), Gy14 (ARR-) and Chinese long (ARR-). Cuticle accumulation between epidermal cells is denoted by the arrows. Peel sections were stained using Sudan IV and viewed at 200x magnification.

such as cuticle deposition may enhance the physical barrier against *P. capsici*, there must be other factors contributing to ARR expression in cucumber peels. A study in grape also showed that cuticle thickness in mature berry fruit did not confer ARR (Ficke et al., 2002).

Bioassay of compounds from cucumber fruit peel

Previous reports have indicated that compounds present in the cutin matrix such as cinnamic acids and flavonoids can exhibit antimicrobial activity (Muller and Riderer 2005, Dominguez et al., 2011). Moreover, studies in other species such as citrus (Ben-Yehoshua et al., 1992, Oliveira and Furlong, 2008), avocado (Adikaram et al., 1992), mango (Druby and Prusky 1986), and pomegranate (Dahham et al., 2010) have demonstrated that fruit peel extracts have antifungal activity against specific pathogens. A study in melon identified compounds in the fruit rind that exhibit antifungal activity against *Fusarium oxysporum* f. sp. *melonis* (Kumar and McConchie, 2010). Cucumbers also are capable of producing antimicrobial compounds. Previous reports have indicated that methanol-soluble volatiles from cucumber fruit (Sotiroudis et al., 2010), and sphingolipids isolated from crude methanol extract of cucumber stems (Tang et al., 2010) exhibit antimicrobial activity. Methanolic extracts from cucumber leaves were shown to also inhibit *Cladosporium cucumerinum* (Daayf et al., 1997). In addition, induced accumulation of methanol-soluble C-glycosyl flavonoid and other phenolics in cucumber leaves upon infection of *Podosphaera xanthii* (powdery mildew) was associated with resistance to the fungus (McNally et al., 2003a,b; Fofana et al., 2005). Moreover, a study in cucumber indicated that there is a correlation between leaf age and increase in the production of inhibitory glycoside-linked phenolic compounds in the cells beneath penetrating appressoria of *Colletotrichum orbiculare* (Lin et al. 2009). However, developmentally regulated biochemical compounds with antimicrobial activity have not been reported yet in cucumber fruit peel.

A preliminary experiment testing different solvents, ethanol, isopropanol, acetone, methanol, and water, was performed to extract compounds from the exocarp of 4, 8, and 16dpp cucumber fruits. Methanol extracts from 16dpp fruit peel inhibited *P. capsici* mycelial growth relative to 4dpp and 8dpp fruit peels as evidenced by the number of sporulating mycelial rings (5 rings for 4dpp and 8dpp; 2 for 16dpp fruit peel extract) formed at 5dpp (Figure 3.6a). To verify these initial observations, a microtiter plate assay was developed to provide a replicable and quantitative method to test the effects of cucumber fruit peel extracts on growth of *P. capsici* (Figure 3.6b).

Bioassay of peel compounds from 'Vlaspik' fruit at 8dpp and 16dpp showed that methanolic extracts from cucumber fruit peels could inhibit *P. capsici* growth in vitro as evidenced by visual pathogen growth or fluorescence assay at 48-72 hours post-inoculation (hpi) (Figure 3.6c,e). Greater inhibition on *P. capsici* growth was observed in fruit peel of resistant age (16dpp) than susceptible age fruit (8dpp). Wells treated with aqueous extracts were comparable to the controls and were characterized by cottony appearance indicating extensive mycelial growth (Figure 3.6b,c). These results indicate that pre-formed methanol-soluble compounds from 16dpp fruit peel are associated with the developmentally regulated resistance to *P. capsici* in cucumber.

Previous work in cucumber identified sets of genes that were upregulated during fruit development including increased expression of a variety of heat shock, redox, biotic defense and ethylene-related genes in resistant age (12+16dpp) fruit (Ando et al., 2012). Moreover, transcriptome analysis comparing fruit peel and pericarp tissue in 'Vlaspik,' revealed that several

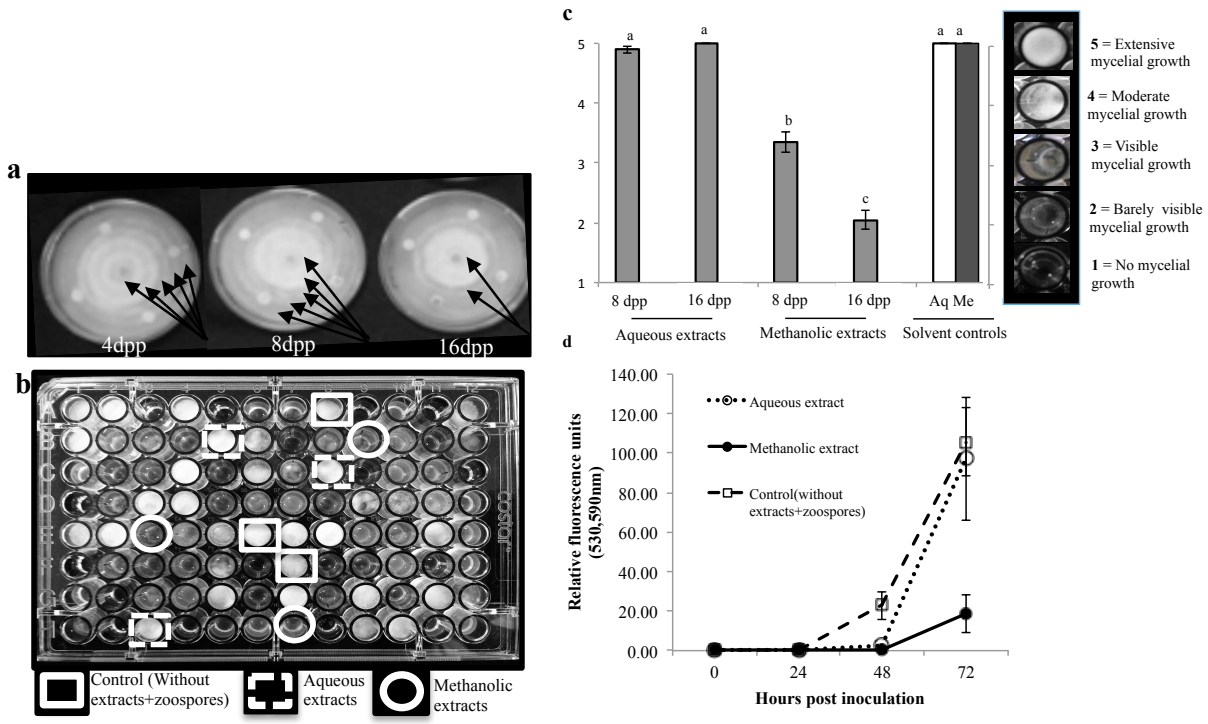

Figure 3.6. (a) Methanolic extracts of peel sections from 4dpp, 8dpp, and 16 dpp cucumber 'Vlaspik' fruit. Extracts from 16dpp fruit inhibited the growth of *P. capsici* relative to extracts from 4 and 8 dpp fruit. Arrows indicate rings of sporulating hyphae (5 rings for 4 and 8dpp, and 2 for 16dpp). Photos were taken at 5 days post inoculation (dpi). (b-e) Effect of aqueous and methanolic extracts from cucumber fruit peel on growth of *P. capsici* in vitro. (b) Photograph illustrating microtiter plate assay *P. capsici* growth response to fruit peel extracts. (c) Effect of 8 dpp and 16 dpp fruit peel extracts of 'Vlaspik' on growth of isolate OP97. (d) Visual rating scale for *P. capsici* growth. (e) Effect of 8 dpp and 16 dpp fruit peel extracts of 'Vlaspik' on growth of isolate NY0664-1RFP. Each value is the mean of 4-5 replicate samples ± S.E. Bars marked with different letters are significantly different (LSD, $P<0.05$). Each experiment was performed twice with equivalent results.

genes in the flavonoid synthesis pathway were specifically up-regulated in peel tissue of 16 dpp fruit (Ando et al., 2015). Expression of flavonoid biosynthetic genes has been associated with resistance in other pathosystems. Accumulation of epicathechin in avocado lines exhibiting resistance to fruit decay fungus (*Colletotrichum gloeosporioides*) was correlated with increased expression of flavonoid biosynthesis enzyme-encoding genes *Phenylalanine ammonia lyase* (*PAL*) and *F3H* (Ardi et al., 1998). High gene expression of an *F3H* homolog in chickpea recombinant inbred lines was associated with resistance against *Ascochyta rabiei* (Cho et al., 2005), and linkage mapping in soybean supported the role of *F3H* in resistance to soybean mosaic virus (Cheng et al., 2009). Induction of FLS expression was observed in apple infected with *Erwinia amylovora* (Venisse et al., 2002) and *Venturia inaequalis* (Slatnar et al., 2010) and higher expression was observed in a variety of grape resistant to multiple pathogens (Ali et al., 2011).

Development of segregating populations for genetic analyses of ARR in cucumber

Reciprocal crosses were made between ARR+ Vlaspik (V) and ARR- Gy14 (G) to study inheritance of ARR. For both parents and their F_1 progeny, fruit harvested at 8dpp showed strong symptoms as early as 3dpi and continued to progress, in each case, to a mean disease score of 9.0± 0 at 10dpi (Figure 3.7a). However, 16dpp fruit of 'Vlaspik'(ARR+) remained resistant showing only minimal necrosis at the site of inoculation by the end of the rating period (mean disease score of 3.4±0.8). The F_1 progeny also showed age-related resistance but to a reduced extent relative to 'Vlaspik.' The 16dpp F_1 fruit initially responded similarly to 'Vlaspik' however, from 5-10dpi, they showed intermediate values between the two parents (Figure 3.7a,b). There was an increase in symptom development in the F_1 progeny fruit leading to extensive water soaking with very minimal visible mycelial growth at 10dpi (mean disease rating

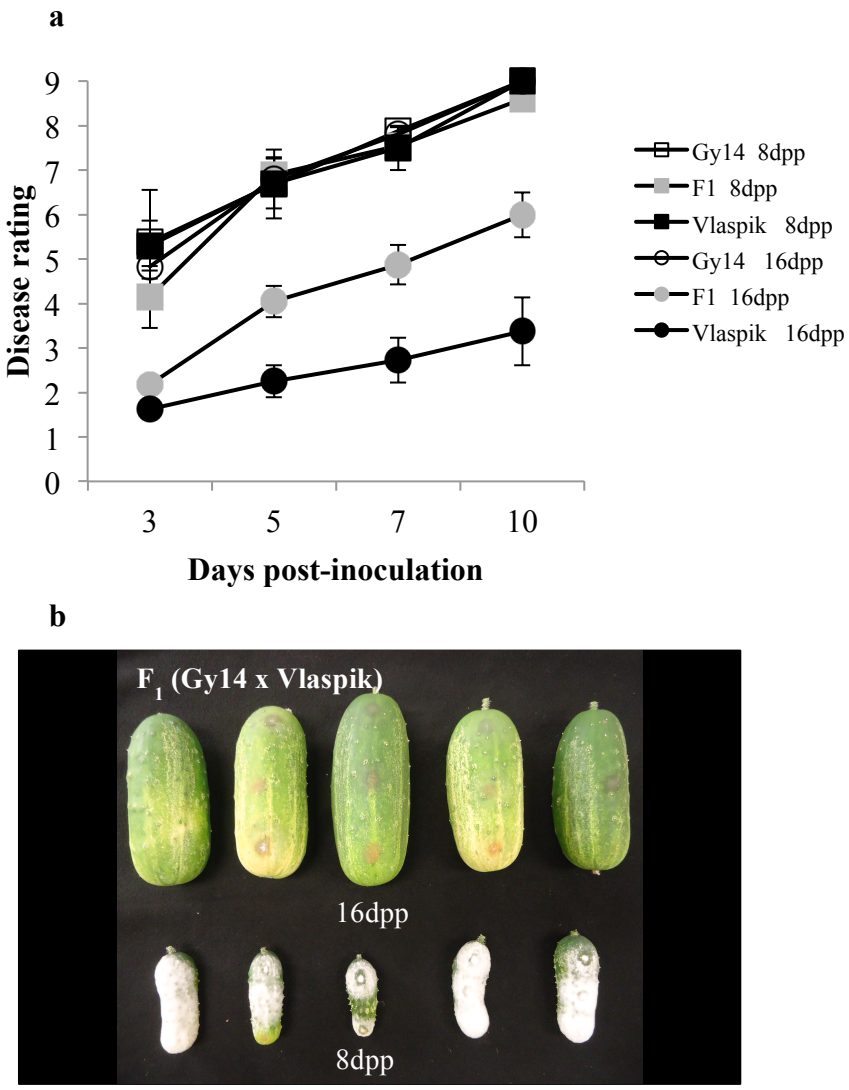

Figure 3.7. (a) Disease rating of 'Vlaspik' (ARR+), 'Gy14' (ARR-) and F_1 progeny at different time points. (b) Symptom development in 8dpp and 16dpp F1 fruit at 5dpi. Each value is a mean of 5-10 fruits ± S.E. (Photo taken at 5 days post inoculation).

of 5.9±0.5). The F_1 progeny from the reciprocal crosses of VxG and GxV showed equivalent disease response ($p<0.05$). On the other hand, 16dpp fruit of Gy14 (ARR-) remained susceptible (mean disease rating of 9.0± 0).

The frequency distribution of F_2 progeny was skewed toward low disease ratings such that majority of the F_2 fruits were resistant to *P. capsici* at 16dpp (Figure 3.8). Although the symptom severity increased over time, the relationships among 'Vlaspik', 'Gy14', and F_1 remained constant, with a high correlation of disease ratings over time (R=0.92-0.94). Fruits that showed signs of successful *P. capsici* infection at 3dpi continued to succumb to fruit rot, while fruits that did not show symptoms (e.g. score of <1.5) at 7dpi remained symptom free throughout the disease rating period. This suggests that we can select true resistant and susceptible plants as early as 7dpi. Accurate phenotyping is extremely important for QTL analysis. A previous report found that QTLs associated with fruit rot resistance in pepper varied depending on the period when disease rating was taken (Naegele et al., 2014).

Initial segregation analysis of the F_2 population and the response of F_1 fruit to *P. capsici* infection indicate that ARR may have a dominant component, possibly under the control of a major gene. There are examples from other pathosystems where ARR appears to be under simple genetic control. ARR to *P. capsci* in pepper is controlled by two major genes (Reifschneider et al., 1992) and ARR to *Colletotrichum graminicola* in sorghum is under the control of a single dominant gene (Tenkouano et al., 1998).

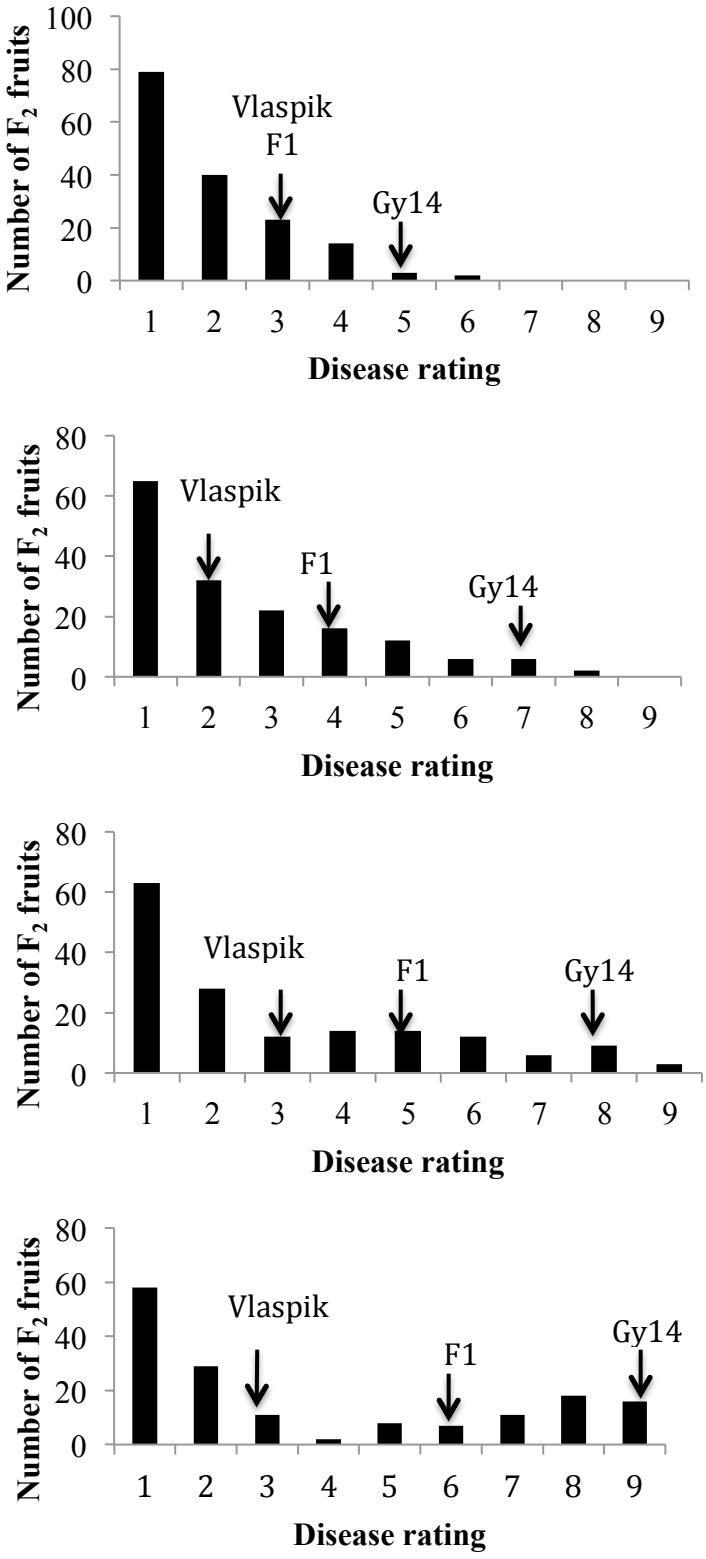

Figure 3.8. Distribution of F_2 fruits based on disease rating taken at different time points: (a) 3dpi, (b) 5dpi, (c) 7dpi, and (d) 10dpi, N=161

CONCLUSION

In this study, we examined the role of fruit surface in ARR in cucumber. Our findings showed that structural changes occurred in the fruit peel during development. Cuticle deposition and thickened epidermal cell walls may provide a physical barrier against *P. capsici*. Our results also showed that methanol-soluble biochemical compounds from cucumber fruit peel are capable of inhibiting *P. capsici* growth and are possibly associated with ARR in cucumber. Moreover, we developed segregating populations to examine inheritance of ARR. Although further investigation needs to be done, our initial results showed that ARR may be controlled by a major dominant gene. Further evaluation of additional crosses using other ARR+ cucumber cultivars will facilitate the analysis of inheritance as well as the identification of ARR-associated QTLs in cucumber. Results of QTL analyses will provide better understanding of the mechanism regulating ARR expression in cucumber.

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CHAPTER 4: Screening the cucumber plant introduction collection for young fruit resistance to *Phytophthora capsici*

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INTRODUCTION

Cucumber (*Cucumis sativus*) production in the eastern and midwestern U.S. is subject to severe losses due to fruit rot caused by the soil borne oomycete pathogen, *Phytophthora capsici* (Granke et al., 2012; Sonogo and Ji, 2012). The disease causes commercial loads of harvested cucumbers to be rejected for sale and farmland to be removed from cucumber production. *P. capsici* has tremendous reproductive potential, allowing for rapid spread both within and between fields (Granke et al., 2012). The sporangia, which are continuously produced throughout the growing season, release motile infective zoospores upon contact with water and provide a constant source of inoculum for new infections. The pathogen also produces sexual oospores which serve as long-lived overwintering structures. *P. capsici* is notable for its wide host range including numerous solanaceous, cucurbit and legume crops (Hausbeck and Lamour, 2004; Tian and Babadoost, 2004). The combined effects of broad host range, spread of the disease through infested irrigation water, and the ability of *P. capsici* oospores to survive in the soil for many years, makes control by cultural practices very difficult (Gevens et al., 2007; Granke et al., 2012; Sonogo and Ji, 2012). Furthermore, several strains of *P. capsici* isolated from states in Eastern, Southern and Midwestern U.S. have developed resistance to key fungicides, reducing usefulness of some chemical controls (e.g., Café and Ristaino, 2008; Dunn et al., 2010; Jackson et al, 2012; Lamour and Hausbeck, 2000). Collectively these factors dictate that yield losses due to *P. capisci* infection will be a continuing problem in cucumber production unless genetic resistance is developed.

Several recent studies have searched for sources of host plant resistance to crown rot and fruit rot caused by *P. capsici* in cucurbit crops. Screening of *Cucurbita pepo* accessions for crown rot resistance led to identification of eight accessions with low mean disease ratings

(Padley et al., 2008). Inheritance studies using a *Cucurbita* breeding line indicated that resistance is conferred by three dominant genes (Padley et al., 2009). Five accessions of *Cucurbita moschata* were identified with resistance to Floridian isolates of *P. capsici* (Chavez et al., 2011); high levels of seedling stage crown rot resistance were reported in S_1 progeny of three melon (*Cucumis melo*) introductions (Donahoo et al., 2013); and seedling resistance was observed in two accessions of watermelon (*Citrullus lanatus* var. *lanatus*) (Kim et al., 2013). Several bottle gourd (*Lagenaria siceraria*) rootstocks used for grafting with watermelon also were found to confer crown rot resistance (Kousik et al., 2012b). Screening for sources of resistance to *Phytophthora* fruit rot in watermelon identified four *Citrullus lanatus* var. *lanatus* accessions along with a *Citrullus lanatus* var. *citroides* and a *Citrullus colocynthis* accession (Kousik et al., 2012a).

The primary losses caused by *P. capsici* infection of cucumber result from fruit rot (Hausbeck and Lamour, 2004). *P. capsici* preferentially infects cucumber fruits, while leaves and vines remain healthy (Ando and Grumet 2006; Grumet et al., 2013). Thus it is essential that screening for resistance is performed directly on fruit. A prior study (Gevens et al., 2006) tested more than 300 cucumber varieties and plant introductions (PIs), including 100 genotypes selected to provide a representative sample of genetic variance in the cucumber germplasm as determined by Knerr et al. (1989). That study did not identify a suitable source of genetic resistance.

In the process of that screening, which was performed on harvest-stage fruit, we observed that larger fruit appeared to be less susceptible than smaller fruit. Analysis of hand pollinated fruit of known ages ranging from 0-16 days post pollination (dpp), showed that very young fruit (e.g., 0-4 dpp) were most highly susceptible (Gevens et al., 2006). As fruits completed the

period of rapid fruit elongation, at approximately 10-12 dpp, they became less susceptible, and were essentially resistant by 16 dpp. Developmentally-regulated, or age-related resistance, wherein resistance increases with plant or tissue age, has been observed in other host plant-pathogen interactions, including *P. capsici* infection of pepper, and subsequent studies with *P.capsici* infection of other cucurbit fruits (Develey-Reviere and Galiana 2007; Ando et al., 2009; Meyer and Hausbeck, 2013; Hwang et al., 1996). These results have implications for disease control strategies including appropriate location and timing of fungicide applications. They also indicate that it is critical to screen the highly susceptible, young cucumber fruit when testing for resistance to *P. capsici*.

Fruit testing is time, labor and space intensive. The objectives of this study were to develop a modified testing method to allow for a more efficient inoculation for high throughput screening utilizing young cucumber fruit, and to screen the full U.S. cucumber PI (Plant Introduction) collection for resistance to *P. capsici*. We used knowledge gained in our prior studies regarding greater susceptibility of floral ovaries and very young fruit, and lack of difference in susceptibility between pollinated and parthenocarpic fruit (Ando, 2009; Ando et al., 2009; Gevens et al., 2006), to develop a more streamlined fruit screening method and prevent mis-assessment of potential resistance that can occur as the fruits become older. Screening of the cucumber PI collection identified three accessions as potential sources for young fruit resistance to *P. capsici*.

MATERIALS AND METHODS

Seed of 1297 cucumber PI accessions was provided by the North Central Regional Plant Introduction Station, Ames, Iowa (http://www.ars-grin.gov/cgi-

bin/npgs/html/taxon.pl?12580#image). Of those, 1076 PIs were not previously screened for resistance to *P. capsici*. The 1076 accessions were planted in small plot, unreplicated trials of 3 plants/plot at the Michigan State University Horticulture Teaching and Research Center, East Lansing, MI, in the summers of 2011 and 2012. Seeds were planted into 0.8 m wide plastic mulch with 2 m between rows and 1 m spacing within rows. Local standard commercial production guidelines were followed for fertilization and insect and weed control (Bird et al., 2005). Water was supplied by rain or by trickle irrigation to provide 25 mm per week. Pollination was facilitated by bees. Once the period of fruit setting began, fruit were harvested two or three times a week until ten fruit had been collected from each PI. Fruit were collected on 15 dates in 2011 and 20 dates in 2012. Very young fruits, estimated to be approximately 3-4 days post pollination based on fruit size and blossom appearance, were harvested and brought to the laboratory for inoculation.

The harvested fruit were washed, surface sterilized by brief immersion in a 5% sodium hypochlorite solution, rinsed with water several times, and allowed to air dry. A modified inoculation procedure based on the methods of Gevens et al. (2006) was developed to streamline the screening process. *Phytophtora* zoospore suspensions were prepared from *P. capsisi* isolate OP97 mycelia cultured on diluted V8 agar media as per Gevens et al. (2006). After seven days of culture, the plates were flooded with 6 ml sterile distilled water to release zoospores. A 20 ul aliquot was removed for quantitation by hemocytometer. The remainder was diluted to a concentration of 1 x 10^5 zoospores/ml; 30 ul of the zoospore suspension was applied to the center of each fruit. Incubation was performed under constant light at 23 – 25^{o}C in covered trays lined with wet paper towels to maintain high humidity as described by Gevens et al. (2006). Fruit from the susceptible control Vlaspik were included at each harvest to ensure effectiveness of the

inoculation procedure. On rare occasions (<1% of fruit tested) the droplet did not remain on the surface of the fruit. In those cases, an additional 1 or 2 droplets were applied; in most cases the droplet stayed on the surface following the repeat application. In those cases where the droplet still fell off the surface, the combined droplets created a 'pool'. The fruits were then placed on top of the pool for 24 hours, a time that our prior methods development tests had shown was sufficient to establish infection. The fruits were then rotated so that the surface that was in contact with the inoculum was visible for scoring. In no case was potential resistance associated with failure of the droplet to remain on the fruit surface. In any case where it was not clear if the droplet ran off the fruit, the fruit was discarded from analysis.

The fruit were monitored daily for symptom development and obvious pathogen growth for a period of at least five days. All disease ratings used for analysis were taken at 5 days post inoculation. In 2011 the fruit responses were scored using a disease rating scale of 1-5 defined as: 1- no symptoms; 2 - mild water soaking; 3 - water soaking with necrosis; 4 - extensive water soaking (may also include necrosis and/or obvious mycelium growth); 5 - tissue collapse (with or without obvious mycelium growth). In 2012 the rating system was modified to a 1-9 scale (illustrated in Figure 4.1) to better capture the range of symptoms observed among the diverse genotypes. Responses with scores of 1-3 (i.e., no symptoms or minor symptoms limited to the point of inoculation) were considered resistant; 4-6 (i.e., moderate to extensive water soaking and/or limited necrosis or mycelial growth), moderately susceptible; and 7-9 (i.e., moderate to extensive mycelium growth, sporulation, necrosis and tissue collapse), highly susceptible.

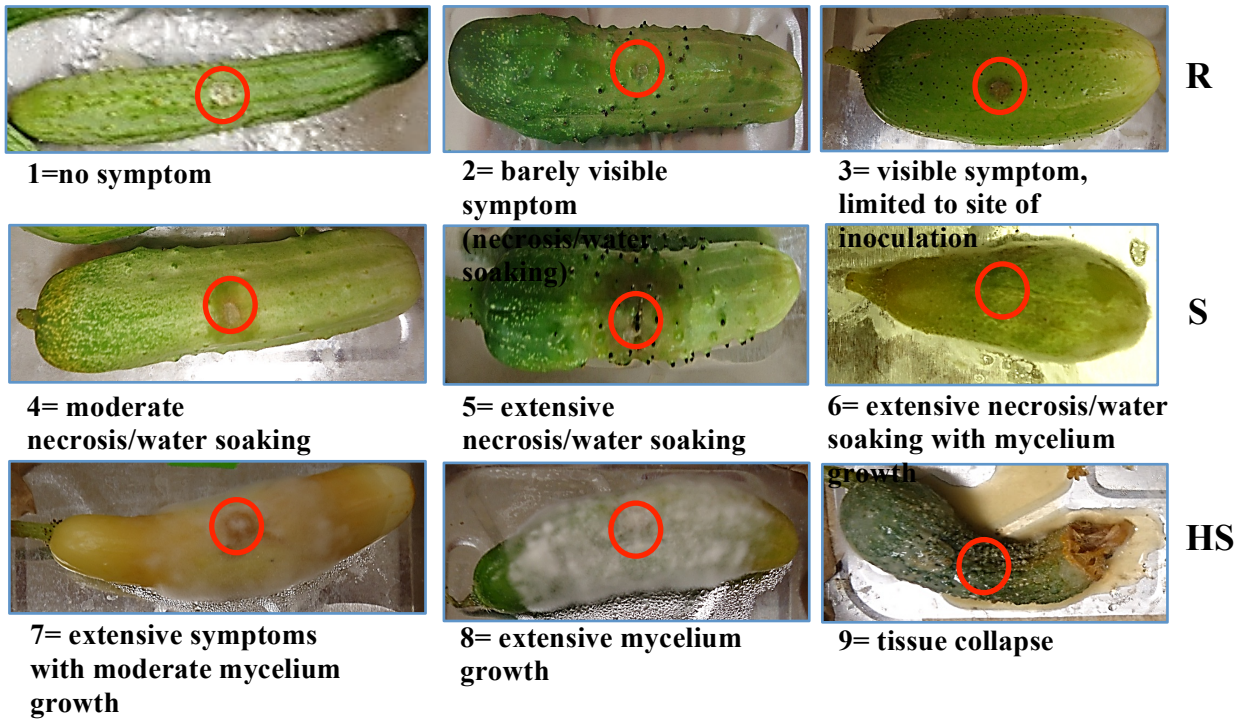

Figure 4.1. Symptom rating scale for young cucumber fruit response to inoculation with *P. capsici*. R-resistant, S-susceptible, HS-highly susceptible.

Based on the initial screen of the full PI collection, 28 accessions were selected for further testing for reproducibility of observed potential resistance. The selected PIs had mean disease scores <2 in 2011 or <4 in 2012. An additional 16 accessions from the moderate disease rating group that appeared to be segregating for resistance were also included for further testing.

To account for the possibility of segregation within the PI sample, subsequent testing of the putatively resistant PIs in 2013 was performed in the greenhouse and/or field using individual plants. To increase the number of samples that could be tested per plant in the greenhouse, a preliminary study was performed to verify correspondence between the response of un-pollinated ovaries from female flowers at anthesis with that of young fruit (data not shown). For the greenhouse trials, 5-10 un-pollinated female flowers were collected from each plant at anthesis and ovaries inoculated with zoospore suspensions as described above. Progeny were produced on three accessions in the greenhouse for which individual plants had been verified to produce resistant fruit: PI109483, PI175693, and Ames 26084. One or two female flowers from those individual plants were hand-pollinated using male flowers from the same plant. Mature fruits were collected at 30-35 days post pollination and seed extracted for field planting in 2013.

Conditions for the 2013 field test were as described above with the exception that ten individuals of each potentially resistant PI were planted 2 m apart within a row, 3 m between rows, to allow testing of fruit from each plant separately. Harvesting of very young fruits and inoculation with *P. capsici* was performed as described above. Fruit were harvested on 13 dates. In most cases 10-15 fruit were tested per plant, with 100-200 fruit tested per PI or family sampled over multiple harvest dates.

Data were analyzed by Kruskal Wallis test in SAS (SAS Institute, Cary, NC) followed by multiple comparisons using the Dunn method.

RESULTS AND DISCUSSION

Sampling of very young fruits from the field, or ovaries from unpollinated flowers at anthesis in the greenhouse, combined with a revised zoospore inoculation procedure, allowed us to more quickly prepare and apply inoculum, reduce space needed to perform the inoculation experiments, and prevent mis-assessment of potential resistance that can occur as the fruits increase in age. The vast majority of the tested PIs were susceptible or highly susceptible to *P. capsici* (Table 4.1, Figure 4.2A,B). By 5 days post inoculation, nearly 99% (1064/1076) of the accessions had mean symptom ratings $\geq$2.0/5.0 scale in 2011 or $\geq$4.0/9.0 scale in 2012 indicating effectiveness of the screening method in causing infection. The mean disease rating for the population was 4.5/5.0 in 2011 and 7.3/9.0 in 2012. The control cultivar Vlaspik had ratings of 5.0 and 8.0, in 2011 and 2012, respectively. The very small number of potentially resistant PIs is consistent with the previous screening study where all of the tested accessions were susceptible to fruit infection by *P. capsici* (Gevens et al., 2006).

Prior studies by Gevens et al. (2006) and Enzenbacher and Hausbeck (2012) tested several *P. capsici* isolates for virulence on cucurbits. With the exception of one that was less severe than the OP97 isolate used in this study, all were comparable to OP97 for infectivity on cucumber fruit. The virulence of OP97 was further demonstrated by the highly susceptible responses of the great majority of tested accessions (Table 4.1). These results also indicate effectiveness of the zoospore inoculation procedure, which in addition to greater ease of application for very large numbers of samples, more closely resembles the primary mode of inoculation in the field.

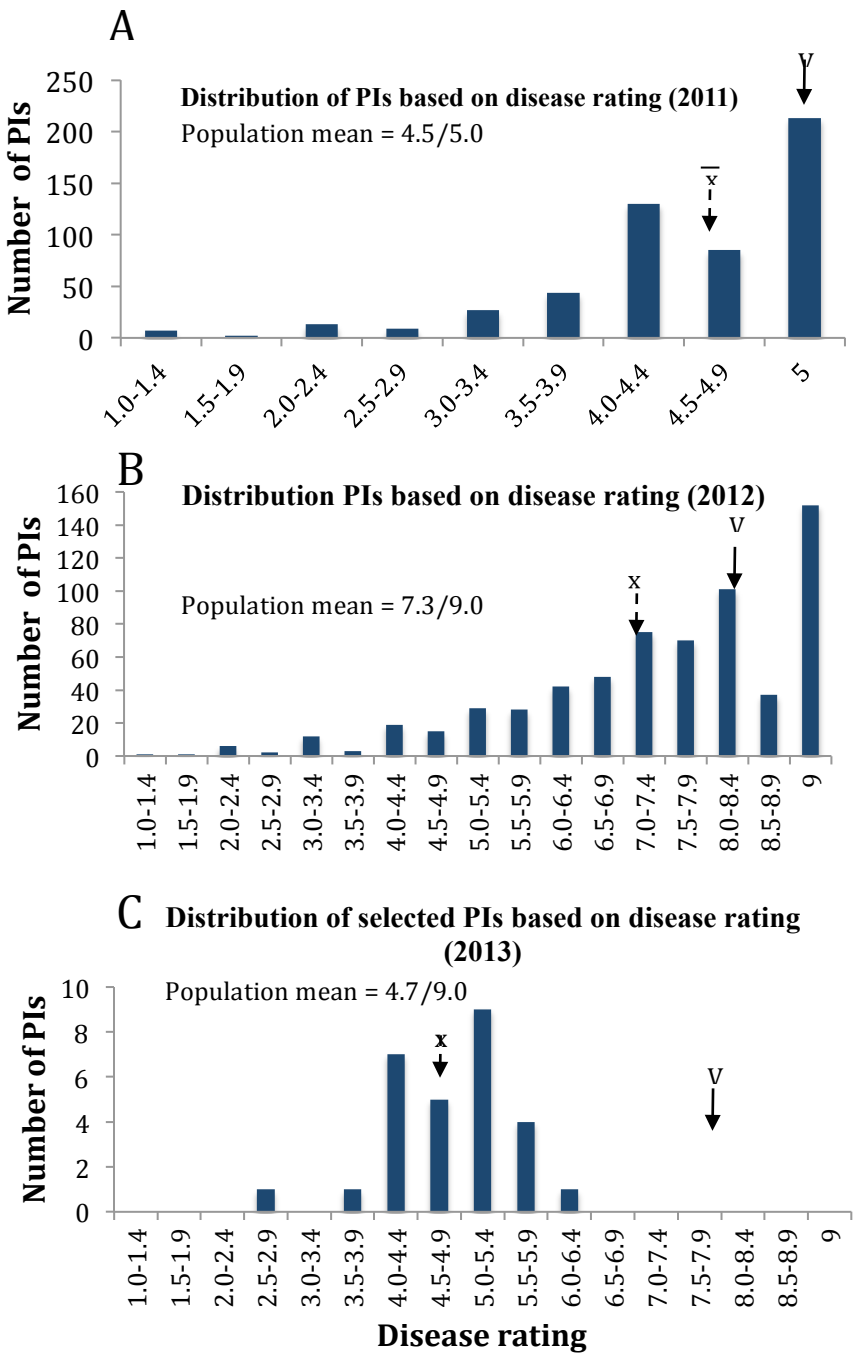

Figure 4.2. Distribution of disease scores for cucumber PIs screened for young fruit resistance to *P. capsici*. (A, B) Distribution of disease scores of PIs from *P. capsici* inoculation of field-grown fruit in 2011 and 2012. Value for each PI is the mean of 5-10 fruit at five days post inoculation (dpi). (C) Distribution of disease scores of PIs selected for potential fruit resistance based on screens in 2011 and 2012 and tested as individual plants in 2013. With the exception of 3 PIs, the value for each PI or S1 family is the mean of 80-200 fruit from 8-10 plants. The disease rating for the susceptible control Vlaspik is indicated by the solid arrows; the mean disease rating for the set of tested PIs is indicated by the dashed arrows.

Table 4.1. Preliminary disease scores of the cucumber PI collection for fruit response to inoculation by *P. capsici*. A total of 1076 were screened. Disease ratings are the mean of 5-10 young fruit/PI inoculated as described in methods. **Bold** indicates PIs selected for further testing.

Resistant Mean disease rating <2.0 (2011) or <4.0 (2012) **N=12 (1.1%)**	Susceptible Mean disease rating 2.0-2.99 (2011) or 4.0-6.99 (2012) **N=181 (16.8%)**						Highly susceptible Mean disease rating >3 (2011) or >7 (2012) **N=883 (82.1%)**
Ames: **26084**	Ames:7753	176520	357831	**432896**	605961	618874	
PI: 174166	**7785**	176521	357832	436609	605963	618875	
175693	12782	176523	357838	436648	605964	618881	The list of the 883 highly susceptible PIs is provided in Appendix Table 4.3.
206425	13257	176525	357847	436649	605967	618899	
214049	13353	176526	357851	436672	605968	618911	
285608	13357	**178884**	357852	458848	605972	618915	
357830	19222	178887	357858	458849	605973	618923	
432865	19227	179678	357861	458850	**605981**	618930	
605945	19229	181752	357862	478366	605982	618933	
605947	22384	188807	357866	481616	605984	618944	
605979	**22385**	197087	368549	481617	605987		
606013	**23612**	205996	**368552**	483339	605988		
	25936	206953	368553	483343	605989		
	PI: **92806**	206954	368554	508459	605992		
	109483	209066	368560	511818	605993		
	163214	224668	370022	511821	605997		
	163216	227664	370447	512595	**605998**		
	163223	255934	370448	512597	606000		
	164734	255935	372900	512598	606001		
	164951	263079	372905	512599	606007		
	165506	264231	378066	512609	606008		
	169351	267742	379279	512616	606009		
	169380	279464	379282	512618	606010		
	169389	281448	379283	532520	606011		
	169390	283901	379286	532521	606012		
	169395	321010	**385967**	**532522**	606014		
	169401	338235	390250	532523	606020		
	171604	339248	390256	605934	606023		
	173889	344347	414159	605936	606037		
	173893	344348	419078	605948	606042		
	175111	344432	422177	605949	606045		
	175679	344433	432854	605952	606048		
	175681	**344434**	432859	605953	606050		
	175691	355052	432860	605954	618866		
	175692	356832	432894	605959	618871		

Of the PIs with very low symptom ratings at 5 dpi, there appeared to be two types of responses. Some exhibited delayed and much reduced symptom development (i.e., only some water soaking without sporulation). Others did not produce symptoms or only showed small, localized necrosis limited to the site of inoculation (e.g., score of 2 or 3 on 9 point scale, Figure 4.1), possibly indicative of hypersensitive response. In some cases we observed a mixture of fruit within the same PI sample that exhibited resistant and susceptible responses. Since the close spacing of plants within the initial trials did not allow for differentiation among fruit produced by individual plants, it is possible that mixed disease response could result from variability within the PI sample. Variability within cucurbit PI accessions for disease resistance responses has been observed frequently (e.g., Donahoo et al., 2013; Wechter et al., 2011; Davis et al., 2007), possibly due to a mixed initial sample, or cross pollination prior to, or following initial collection.

Based on the screens in 2011 and 2012, 28 PIs were chosen for further testing in the greenhouse or field in 2013. In addition to PIs showing resistant phenotypes (mean disease scores <2 in 2011 or <4 in 2012), 16 accessions from the moderate disease rating group that appeared to be segregating for resistance were included for further testing (Table 4.1). The majority (61%) of the selected PIs were collected from Turkey or India (Table 4.2). Although there were a greater number of accessions from China in the PI collection, only one showed potential resistance in the 2011-2012 screens. This distinction among different geographical regions is consistent with population structure analysis indicating that cucumber germplasm comes from three distinct populations: China; India and Xishuangbanna; and Europe, American, and Central and West Asia (Lv et al., 2012). It appears likely that the resistance arose in Indian and/or West Asian germplasm.

Table 4.2. Retest of cucumber PIs selected for potential fruit resistance to *P. capsici*, 2013. Field-grown young fruits were harvested for each plant separately and inoculated in the laboratory as described in methods.

PI #	Origin	No. plants tested	No. fruit tested	Date of first fruit harvested	Range of plant mean disease scores	PI/family mean ± SE	P value (Dunn)	% fruit score <4
PI 214049	India	3	24[b]	8-13	2.0 - 3.0	2.59 ± 0.52	0.0014[c]	90%
PI 109483-2[a]	Turkey	8	83	7-26	2.5 - 4.7	3.45 ± 0.30	<0.0001	80%
PI 178884	Turkey	10	165	7-17	2.8 - 4.4	3.53 ± 0.19	<0.0001	80%
PI 109483-5[a]	Turkey	10	141	7-26	2.0 - 6.1	3.70 ± 0.47	<0.0001	72%
Ames 22385	Nepal	10	120	7-17	2.8 - 5.1	4.10 ± 0.22	<0.0001	59%
PI 605981	India	9	186	7-29	3.3 - 5.7	4.12 ± 0.22	0.0001	64%
PI 175679	Turkey	10	141	7-18	3.4 - 5.3	4.18 ± 0.19	<0.0001	62%
PI 109483-3[a]	Turkey	10	153	7-26	2.5 - 6.2	4.21 ± 0.38	0.0002	58%
PI 285608	Poland	5	86	7-17	2.8 - 5.3	4.30 ± 0.44	0.0096	63%
PI 368552	F. Serbia/Mont	10	133	7-17	3.3 - 7.3	4.40 ± 0.40	0.0002	55%
PI 606103	India	10	149	7-17	3.6 - 5.3	4.44 ± 0.23	0.0009	58%
PI 605979	India	9	101	8-6	2.1 - 6.5	4.46 ± 0.42	0.0022	55%
Ames 26084-2[a]	US	10	197	7-26	3.4 - 6.2	4.53 ± 0.31	0.0010	55%
PI 605998	India	10	145	7-17	3.5 - 4.6	4.71 ± 0.20	0.0088	54%
PI 169387	Turkey	10	125	7-17	3.5 - 6.1	4.82 ± 0.28	0.0160	44%
PI 357830	F. Serbia/Mont	9	110	7-17	3.7 - 6.0	4.87 ± 0.28	0.0289	44%
PI 432896	China	10	120	7-17	3.9 - 6.9	4.98 ± 0.28	0.0394	47%
PI 435946	F. Soviet Union	10	157	7-17	3.3 - 7.2	5.00 ± 0.43	0.0228	47%
PI 532522	Japan	9	138	7-29	3.0 - 6.5	5.06 ± 0.37	0.1090	49%
PI 169389	Turkey	10	126	7-29	4.2 - 6.9	5.13 ± 0.34	0.0567	42%
PI 605948	India	9	91	7-29	3.0 - 7.9	5.39 ± 0.47	0.3912	41%
PI 206425	Turkey	8	178	7-17	3.2 - 7.5	5.41 ± 0.44	0.6568	41%
PI 175693-5[a]	Turkey	9	122	7-17	2.8 - 6.8	5.41 ± 0.36	1	35%
PI 605945	India	5	33[b]	8-15	4.2 - 6.3	5.54 ± 0.46	1	32%
PI 605947	India	9	133	8-8	5.3 - 7.2	5.66 ± 0.25	1	29%
PI 175693-3[a]	Turkey	10	199	7-17	4.3 - 7.9	5.91 ± 0.37	1	31%
PI 344434	Iran	9	130	7-17	4.3 - 9.0	6.35 ± 0.51	1	22%
Vlaspik		5	64	7-17	7.4 - 8.0	7.70 ± 0.19	-	0%

To account for possible variability within the seed sample, subsequent testing of the putatively resistant PIs in 2013 was performed on individual plants. A small number were tested in the greenhouse in the spring of 2013; the majority were tested in the field. S_1 progeny were produced on three PIs in the greenhouse. In most cases 10-15 fruit were tested per plant, with a total of 100-200 fruit tested for each PI or S_1 family. The PIs re-tested in the field in 2013 had much lower disease ratings than the full collection of PIs (t-test, $P<0.00001$), as evidenced by a shift in the population distribution and a mean disease rating of 4.7, indicating general reproducibility of resistance for the selected PIs (Figure 4.2C, Table 4.2). The susceptible check, Vlaspik had a rating of 7.7, consistent with results in 2012. In some cases there was a range in mean disease scores for fruit from individual plants within a given accession or family, e.g., PI605979, for which single plant means ranged from 2.1 - 6.5, suggesting genetic variability or segregation for resistance within the accession (Table 4.2).

Three accessions (PI 109483 and PI 178884 collected from Turkey and PI 214049 from India) had low PI or family means; multiple plants with mean fruit scores <3.5; and 70-90% of total fruit with disease scores <4 (Table 4.2). In the case of PI 109483, scores from individual fruit in 2012 suggested segregation within the PI seed sample (Table 4.1). Self-pollinated progeny from greenhouse-grown individual plants with resistant fruit provided several S_1 families that showed resistance. Disease progression lines for PIs 109483, 178884 and 214049 showed a slow development of necrosis limited to the region of inoculation (Figure 4.3). Observation of the fruit for an additional 2-3 days did not show further disease development. Although promising for resistance, PI 214049 was slow to produce female flowers and fruit in Michigan growth conditions (Table 4.2).

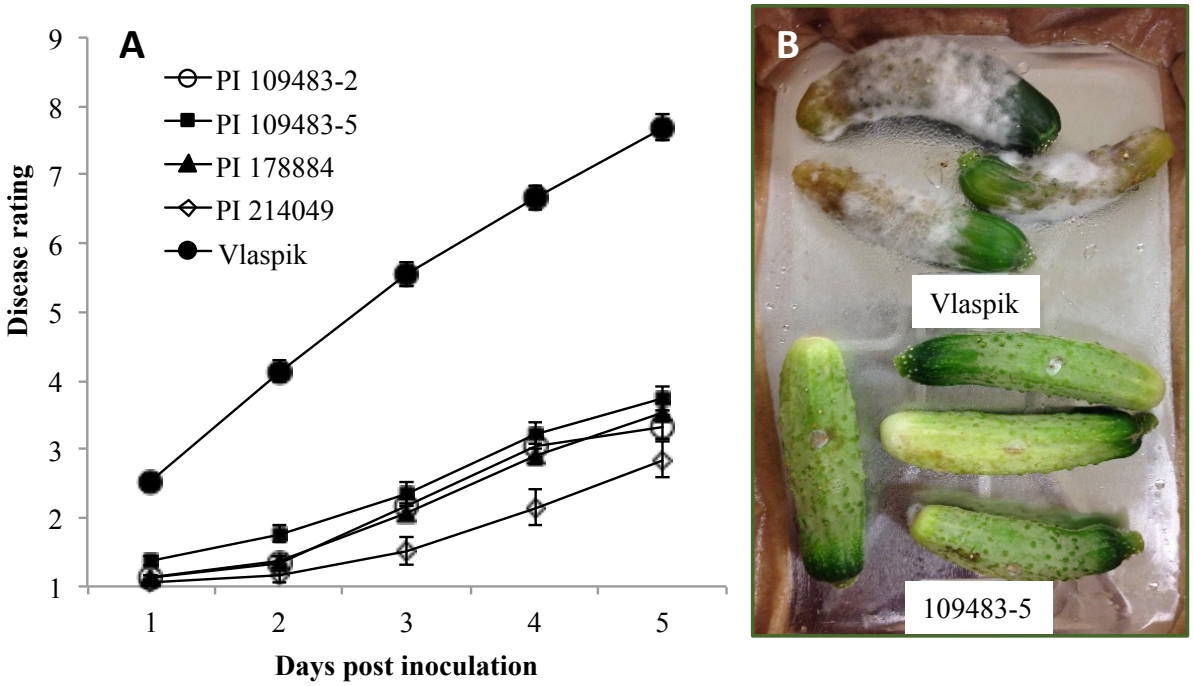

Figure 4.3. Response of young fruit of PIs or S1 progeny of 109483, 178884 and 214049 to inoculation by *Phytophthora capsici*. (A) Disease development curves following inoculation. Disease rating scale is as described in Figure 1; numbers of plants and fruits sampled are indicated in Table 4.2. (B) Example of disease response of Vlaspik (top) and PI109483-5 S1. (158-5, plant #9). The photograph was taken 5 days post inoculation. The disease responses of PI109483 fruit are limited to the site of inoculation (i.e., disease scores of 2 and 3).

Based on these studies, PI109483, PI178884 and PI214049 may be considered as possible sources of resistance to young cucumber fruit infection by *P. capsici*. Evaluation of the S_1 progeny of PI109483 indicates that the resistance is heritable and should allow for development of useful breeding materials that can be used for developing *P. capsici* resistant cucumber cultivars. Due to the possible variation or segregation within accessions it will be important to develop true-breeding resistant stock lines to facilitate future breeding efforts.

APPENDIX

Table 4.3. Cucumber PIs that were rated as highly susceptible (mean disease rating >3 in 2011 or >7 in 2012). Disease ratings are the mean of 5-10 young fruit/PI inoculated as described in methods.

Ames:	19218	118279	169399	176952	211983	264230	339245	379284	427089
1760	19219	135122	169402	176953	211984	265887	339246	379285	427090
2353	19220	135345	169403	176954	211985	267086	339247	379287	427230
2354	19221	137835	171600	176956	211986	267087	339250	390238	430585
3941	19223	137839	171601	176957	212599	267088	342951	390239	432848
3942	19224	137844	171602	177359	212985	267197	343451	390242	432849
3947	19225	137847	171603	177360	214155	267741	343452	390243	432850
3949	19226	137848	171605	177361	217946	267942	344349	390244	432852
3951	19228	137856	171606	178885	218036	269482	344353	390245	432853
4421	19230	163221	171607	178886	218199	271331	344437	390246	432855
4832	19231	163222	171608	178888	220169	271334	344438	390247	432856
5732	20149	164284	171609	179260	220171	271337	344439	390248	432857
5739	20151	164465	171610	179263	220338	271753	344440	390251	432858
5740	20206	164670	171611	179259	220789	275410	344441	390252	432861
5754	21694	164679	171612	179921	220790	275411	344442	390253	432862
7730	21695	164743	171613	181753	220791	277741	344443	390257	432864
7731	21696	164819	172839	181756	221440	279463	344444	390258	432866
7735	21698	164950	172840	181910	222243	279465	351139	390259	432868
7736	21761	164952	172841	181940	222244	279469	355053	390260	432870
7737	22250	165029	172842	182188	222782	279807	357835	390261	432871
7739	22386	165046	172843	182189	222783	283899	357837	390263	432872
7740	23007	165499	172844	182190	222985	283902	357840	390265	432873
7741	23008	167043	172845	182192	222986	285603	357841	390266	432874
7742	25154	167050	172846	183056	223437	285606	357842	390267	432875
7745	25155	167052	172847	183127	226509	285607	357843	390268	432876
7749	25156	167079	172848	183231	227013	285609	357844	390269	432878
7750	25699	167134	172849	183677	227208	285610	357845	390951	432879
7751	25929	167198	172851	183967	227210	288237	357846	390952	432880
7752	25930	167358	172852	188749	227235	288991	357848	390953	432881
7755	25931	167389	173674	193497	228344	288992	357850	391568	432882
7758	25932	169315	173892	197086	229309	288993	357853	391569	432883
7760	25933	169319	174160	200818	233932	288994	357854	391571	432884
12781	25934	169328	174167	202801	234517	288995	357855	391572	432885
13334	25935	169334	174172	204567	248778	288996	357856	391573	432886
13335	25937	169350	174173	204568	249550	289698	357860	392292	432887
13336	25938	169352	174174	204569	250147	292010	357863	400270	432888
13338	26049	169353	174177	204690	251028	292011	357868	401732	432889
13339	26085	169377	175120	204692	251520	292012	368548	401733	432890
13341	26086	169378	175121	205181	255933	296120	368550	406473	432891
13342	26507	169381	175680	205995	255938	296121	368551	418963	432892
13345	26916	169382	175683	206952	257286	296387	368555	418964	432893
13346	26917	169383	175686	207476	257494	302443	368557	418989	432895
13347	26918	169384	175688	209064	261608	304803	368558	419010	432897
13348	28156	169385	175690	209068	263046	306179	370019	419017	435946
13349	28184	169386	175694	209654	263049	321006	370449	419040	436608
13350	28956	169387	175696	211589	263078	321009	370450	419041	436610
13351	32744	169388	175697	211728	263080	321011	370643	419077	436673
13352	34596	169391	176517	211943	263081	324239	373917	419079	451975
13355	PI:	169392	176518	211962	263082	326595	373918	419108	451976
13356	105263	169393	176524	211975	263084	326596	374694	419135	458845
13358	105340	169394	176924	211977	263085	326598	376064	419136	458846
19038	109481	169396	176950	211978	264228	338236	379280	419182	458847
19039	113334	169397	176951	211980	264229	339241	379281	419183	458851

Table 4.3. (cont'd).

458852	500366	512625	535881	605976	606068	618926
458853	500370	512626	540414	605977	606539	618927
458854	502331	512627	540415	605978	618860	618928
458855	504561	512628	540416	605980	618863	618929
458856	504562	512631	561144	605983	618864	618934
462369	504563	512632	561145	605986	618865	618936
464873	504564	512633	561146	605990	618867	618937
466922	504565	512634	561147	605991	618868	618938
466923	504566	512635	601338	605995	618869	618939
478364	504567	512636	605911	605996	618870	618940
478365	504568	512637	605912	605999	618872	618941
478367	504569	512638	605913	606003	618873	618942
481612	504570	512639	605914	606004	618876	618943
481614	504571	512640	605915	606005	618877	618945
482412	504572	512641	605916	606006	618878	618946
482463	504573	512644	605917	606015	618879	618947
483341	504813	518848	605918	606016	618880	618948
483342	504814	518849	605919	606017	618882	618949
483344	504815	518850	605920	606018	618883	618950
487424	504816	518851	605921	606019	618884	618951
489752	506461	518852	605922	606021	618885	618952
489753	506462	518853	605923	606022	618886	618953
489754	506463	518854	605924	606024	618888	618954
490996	506464	525075	605925	606026	618889	618955
500359	506465	525150	605926	606027	618891	618956
500360	507874	525151	605927	606028	618892	618957
500361	507875	525152	605928	606029	618893	618958
500365	507876	525153	605929	606030	618894	618959
500366	508454	525154	605930	606031	618895	618961
500370	508455	525155	605932	606033	618896	
502331	508457	525156	605933	606034	618897	
504561	508458	525157	605935	606035	618898	
504562	511817	525158	605937	606036	618900	
504563	512336	525159	605938	606038	618901	
504564	512594	525161	605939	606039	618902	
504565	512596	525162	605940	606040	618903	
504566	512600	525163	605941	606041	618904	
504567	512601	525165	605942	606043	618905	
504568	512602	531308	605943	606044	618906	
504569	512603	531309	605944	606046	618907	
504570	512604	531310	605946	606047	618908	
504571	512605	531312	605950	606049	618909	
504572	512606	531313	605951	606051	618910	
504573	512607	531314	605955	606052	618912	
504813	512608	532160	605956	606053	618913	
504814	512610	532161	605957	606054	618914	
504815	512613	532162	605958	606055	618916	
504816	512614	532519	605962	606056	618917	
489754	512615	534539	605966	606057	618918	
490996	512617	534540	605969	606058	618919	
500359	512619	534541	605970	606060	618920	
500360	512620	534543	605971	606064	618921	
500361	512623	534545	605974	606065	618922	
500365	512624	535880	605975	606067	618924	

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CONCLUSIONS AND FUTURE WORK

Fruit size and shape are important determinants of market class and value in cucumber. However, the underlying factors regulating fruit size and shape have not been determined. Using two sequenced cucumber cultivars, Gy14 and Chinese long, which exhibit differences in fruit size and shape, this study has identified numerous factors acting both pre-anthesis and post-pollination that influence variation in fruit size and shape in cucumber, including the interplay of complex regulatory mechanisms controlling the timing and orientation of cell number and cell shape. Expression and gene sequence analyses of fruit growth marker genes revealed the potential role of *ATHB-2* in cell elongation in Chinese long. In addition, examination of several fruit growth related genes showed that they co-localized with cucumber fruit size QTLs (Weng et al., 2015) and were in close proximity with each other on either chromosome 3 or 6.

Analysis of different fruit growth traits in segregating F_2 and RIL populations indicated that ovule number and ovary length are good predictors of length, and factors regulating fruit shape act prior to anthesis while diameter is largely regulated post-pollination. Furthermore, variation in fruit volume suggests that fruit size is separable from fruit shape. Collectively, our findings showed that, consistent with quantitative trait, multiple factors regulate cucumber fruit size and shape. Although, we gathered substantial information from the analyses of fruit growth from pre-anthesis to post-pollination of CL and Gy14, fruit traits examined in segregating populations, and from the recently described cucumber fruit size QTL (Weng et al., 2015), we have not yet identified the specific genes contributing to fruit size and shape variation in cucumber. Therefore, by taking advantage of the highly dense SNP markers, fine mapping needs to be done to facilitate the identification of candidate genes that regulate fruit size and shape in cucumber. Moreover, once identified, expression and functional analyses of the candidate genes should be

performed. In parallel with the fine mapping study, expression analysis of homologs of fruit size genes that were found to co-localize with the fruit size QTL may also be carried out. Furthermore, to gather additional information on the potential role of *ATHB-2* in fruit elongation, further examination of gene expression of *ATHB-2* could be done using cucumber cultivars exhibiting variation in fruit size and shape.

Fruit development also influences response to infection by the oomycete pathogen, *Phytophthora capsici*. Previous work in our lab showed that cucumber fruit (cv. Vlaspik) exhibit an age-related resistance (ARR) to *P. capsici* (Ando et al., 2009). Young fruit are highly susceptible, but as the fruit transition away from exponential growth, at approximately 10-12dpp, they become resistant. Moreover, our previous studies also indicated that cucumber fruit surface was associated with ARR (Ando 2009), suggesting possible physical or chemical components of resistance. In this study, we examined the potential role of biochemical compounds in fruit peel in inhibiting *P. capsici* growth using cucumber fruit of Vlaspik (ARR+). Results of bioassay indicated that methanolic extracts from 16dpp fruit peel exhibit greater inhibition on *P. capsici* growth than 8dpp fruit peel. Moreover, we also examined different inbred cucumber cultivars for ARR expression, and using selected cultivars, we examined inheritance of ARR in cucumber. Our results indicate that there is variation in ARR expression among different cucumber cultivars. In addition, our findings also suggest that ARR may be inherited as a dominant trait and maybe under the control of one or more dominant factors. However, further evaluation of additional crosses using other ARR+ cucumber cultivars needs to be done to facilitate the analysis of inheritance of ARR in cucumber. Moreover, using segregating populations developed from an ARR+ and ARR- cucumber cultivars, a bulked segregant analysis (BSA) coupled with QTL-Seq approach could enable the identification of ARR-associated QTLs in cucumber.

Findings from such study, coupled with the RNASeq analysis currently undergoing in the laboratory, would provide better understanding of the underlying mechanism regulating ARR in cucumber.

Lastly, to find a more stable solution to the problem caused by *P. capsici* in cucumber production, source of genetic resistance must be identified. Thus, we screened a total of 1076 PI accessions for resistance to *P. capsici*. Using our prior knowledge regarding greater susceptibility of floral ovaries and very young fruit, and lack of difference in susceptibility between pollinated and parthenocarpic fruit (Ando 2009, Ando et al., 2009, Gevens et al., 2006), we developed a more streamlined fruit screening method to prevent mis-assessment of potential resistance that can occur as the fruits become older. From our tests, we selected 29 potentially resistant accessions and retested them in the field for two seasons. Three accessions were identified, PI109483, PI178884 and PI214049 that may be considered as possible sources of resistance to young cucumber fruit infection by *P. capsici*. However, we observed heterogeneity within the accessions, thus it will be important to develop true-breeding resistant stock lines to facilitate inheritance studies and future breeding efforts.

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