Chapter 19 - Ripening of tomato fruit and susceptibility to *Botrytis cinerea*

Barbara Blanco-Ulate, Estefania Vincenti, Dario Cantu, and Ann L.T. Powell

Abstract *Botrytis cinerea* causes gray mold decay in the ripe fruit of many plant species leading to significant economic losses for the producers, distributers and consumers of fresh and stored produce. This chapter summarizes current knowledge about the biology of a model fleshy fruit, tomato, infected with *B. cinerea*. The information presented emphasizes how ripening regulation and events in the host influence the outcomes of resistance when fruit are unripe and susceptibility when fruit are ripe. Fruit ripening regulators (e.g., transcription factors, epigenetic modifiers and hormones) and events unique to ripening that can impact the susceptibility of tomato fruit to *B. cinerea* are discussed. Understanding the processes in the fruit that underlie the shift from resistance to susceptibility during ripening and resolving how *B. cinerea* modifies its strategies of infection in response to the developmental changes of the host may guide efforts to improve the resistance of fruit not only to *B. cinerea*, but also to other fungal pathogens.

keywords: developmentally-regulated susceptibility, fruit ripening, plant hormones, fruit metabolism, plant defences

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19.1 Introduction

The interplay between tomato fruit and *Botrytis cinerea* is a prototype for climacteric fruit-necrotroph interactions (Powell et al. 2000; Flors et al. 2007; Cantu et al. 2008, 2009; Weiberg et al. 2013; Blanco-Ulate et al. 2013). Tomato fruit have been used extensively as a model system to understand the development and ripening of fleshy fruit organs. The acquisition of susceptibility to *B. cinerea* is a hallmark of tomato fruit ripening: unripe fruit are resistant to gray mold and become increasingly susceptible as they ripen. The abundance of molecular and genetic resources that have described the biology of fruit development and

ripening provide powerful experimental tools to understand the intersection between fruit ripening and susceptibility to *B. cinerea*. Complete genome sequences are available as well as large collections of spontaneous and induced mutations and high-quality "omics" datasets are frequently published (Carrari et al. 2006; Arie et al. 2007; Blanco-Ulate 2014; Cantu et al., 2009; Klee and Giovannoni 2011; Tomato Genome Consortium 2012; Osorio et al. 2012; Seymour et al. 2013). Fresh and prepared tomato fruit products are economically important and fungal pathogens, such as *B. cinerea*, cause losses of billions of dollars worldwide (Narayanasamy, 2006). Understanding the mechanisms that make ripe fruit susceptible will help breeders and postharvest technologists develop more effective approaches to reduce losses caused by *B. cinerea.*

19.1.1 Fruit ripening

Ripening is a complex and coordinated sequence of inter-related biochemical and physiological processes that transform the appearance, composition and characteristics of intact unripe fruit into a ripe fruit suitable for consumption by animals and humans (Seymour et al. 2013). Ripening is regulated by multiple interacting mechanisms and occurs in an organized and developmentally determined manner (Seymour et al. 2013). In tomato fruit, ripening begins at the distal end of the fruit (Cuong et al. 2014). Complex positive and negative regulatory processes occur at the onset of ripening and impact how ripening progresses. Networks of transcription factors constitute the core regulators of ripening, while hormones and other molecules act downstream to activate particular ripening pathways (Klee and Giovannoni 2011; Seymour et al. 2013). In addition, epigenetic and post-transcriptional modifications influence the expression of master regulators and specific ripening-related genes (Mohorianu et al. 2011; Karlova et al. 2013; Seymour et al. 2013; Zhong et al. 2013).

19.1.2 Unripe fruit become susceptible during ripening

Since tomato fruit become more susceptible as they ripen (Fig. 19.1), it had been thought that susceptibility is an inherent outcome of ripening (Prusky 1996; Klee and Giovannoni 2011). However, studies of the regulation of ripening and susceptibility have led to the conclusions that only selected ripening events and pathways are required to facilitate *B. cinerea* infections, and that *B. cinerea* modifies its infection strategy as fruit ripen (Cantu et al. 2008; Cantu et al. 2009; Blanco-Ulate et al. 2013 and 2014).

In the context of fruit-*B. cinerea* interactions, it is critical to distinguish between the processes of normal ripening and those that are needed to render ripe fruit susceptible. It is also necessary to determine how *B. cinerea* impacts ripening

by exploiting and diverting regulators and processes during fruit development. On unripe tomato fruit, *B. cinerea* accelerates selected aspects of ripening, thereby enhancing host susceptibility (Christescu et al. 2002; Cantu et al. 2009; Blanco-Ulate et al. 2013, 2014). How *B. cinerea* promotes susceptibility in fruit has not yet been determined, but toxins, plant hormone analogues, small RNAs and/or pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively) generated by *B. cinerea* during infections may be involved.

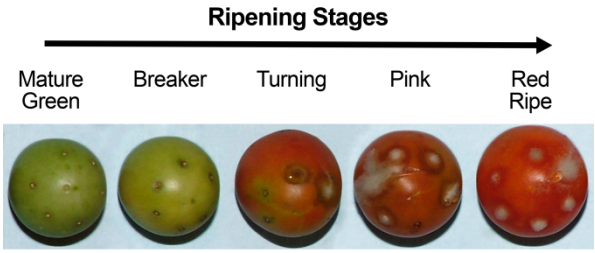


Fig. 19.1. Susceptibility of tomato fruit to *B. cinerea* as a function of ripening stage. Representative tomato fruit (cv. Ailsa Craig) at Mature Green, Breaker, Turning, Pink and Red Ripe stages at three days post-inoculation.Control of fruit ripening and susceptibility to *B. cinerea*

19.2 Control of fruit ripening and susceptibility to *B. cinerea*

19.2.1 Transcriptional regulators

Transcription factors are the central regulators of fruit ripening (Seymour et al. 2013). The intricate and dynamic relationships between the ripening-regulating transcription factors indicate that multiple coordinated inputs are addressed as fruit develop. These modulators could be perturbed by *B. cinerea* infections. Three tomato transcription factors, RIPENING-INHIBITOR (RIN), COLORLESS NON-RIPENING (CNR) and NON-RIPENING (NOR), control ripening up-steam of hormones and other regulatory molecules. Mutations in *CNR, RIN* and *NOR* result in fruit that do not ripen (Vrebalov et al. 2002; Manning et al. 2006; Seymour et al. 2013). Because susceptibility to *B. cinerea* differs among the fruit of the three non-ripening mutants, it has been concluded that *B. cinerea* infections require only specific aspects of ripening (Cantu et al. 2009, Blanco-Ulate et al. 2014).

The *rin* mutation results in full-sized firm tomato fruit that remain green (i.e. carotenoid compounds do not accumulate and chlorophylls are only slowly degraded). *rin* fruit do not produce or ripen in response to ethylene, a plant hormone involved in ripening (Vrebalov et al. 2002). *rin* fruit show the same susceptibility to *B. cinerea* as wild-type fruit (Fig. 19.2); they are resistant when unripe but become susceptible as they complete maturation. Thus, RIN is not

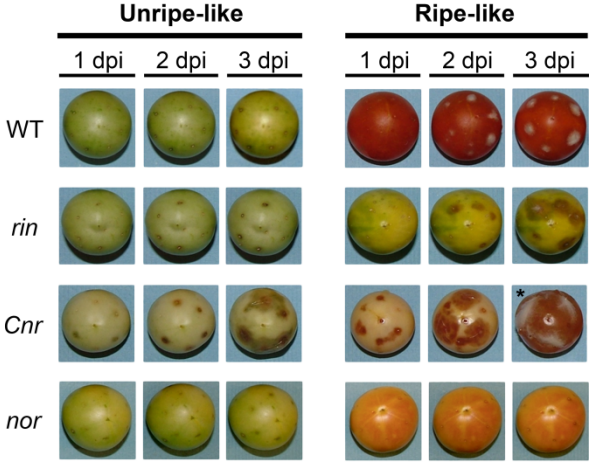
needed for the resistance of unripe fruit or the ripening-associated development of susceptibility (Cantu et al. 2009). RIN activates many aspects of ripening including: 1) ethylene synthesis and perception 2) cell wall disassembly; 3) carotenoid formation; 4) volatile aroma biosynthesis; 5) generation of ATP; and 6) suppression of auxin responses. RIN binds to the promoters of other ripening regulators, including *CNR* and *NOR* (Martel et al. 2011; Qin et al. 2012; Fujisawa et al. 2013; Zhong et al. 2013), and RIN regulation of ripening genes depends on CNR (Martel et al. 2011; Zhuo et al. 2013).

In contrast to *rin* and wild-type fruit, *Cnr* mutant fruit are hyper-susceptible to *B. cinerea,* even at the unripe stage (Fig. 19.2). The *Cnr* mutation leads to smaller colorless non-ripening fruit that lose chlorophyll normally but do not accumulate lycopene or soften or accumulate soluble sugars or synthesize ethylene (Fraser et al. 2001; Eriksson et al. 2004; Manning et al. 2006). The pericarp of the *Cnr* mutant fruit is mealy due to biochemical changes in the cell walls that lead to substantial loss of cell-to-cell adhesion (Orfila et al. 2002; Eriksson et al. 2004; Ordaz-Ortiz et al. 2009). Susceptibility-related genes targeted directly by CNR are unclear (Eriksson et al. 2004; Seymour et al. 2008), although it is known that the *Cnr* mutation results in the up-regulation of pathogenesis-related (PR) gene expression (Eriksson et al. 2004). Intriguingly, the disruption of normal ripening and the constitutive activation of PR genes in the *Cnr* mutant fruit is not sufficient to inhibit *B. cinerea* infections and in fact, infections are precociously robust, perhaps due to disturbances in adhesion between cells.

In contrast, fruit with the *nor* mutation, which also do not undergo most of the changes associated with ripening, are entirely resistant to *B. cinerea* infection at both the unripe-like and ripe-like stages (Fig. 19.2, Cantu et al. 2009). Thus, NOR promotes susceptibility and/or inhibits resistance during ripening. Osorio et al. (2011) identified some of the ripening processes that are reduced by the mutation of *NOR*, such as: 1) ethylene synthesis and perception, 2) expression of the ripening-related polygalacturonase, 3) levels of hexoses, carotenoid compounds and organic acids.

Transcription factors that influence fruit ripening downstream of RIN, CNR, and NOR, or interact with them to repress or activate ripening, may influence *B. cinerea* infections of fruit. The susceptibility of fruit with altered expression of the negative ripening regulators: SlMADS1, a tomato homolog of APETALLA SlAP2a and the ethylene response factor SlERF6 (Chung et al. 2010; Karlova et al. 2011 and 2013; Lee et al. 2012; Dong et al. 2013), or the positive regulators, SlNAC4, TOMATO AGAMOUS-LIKE 1 (SlTAGL1), the tomato FRUITFULL homologs (SlFUL1 and SlFUL2) and the HD-Zip homeobox transcription factor SlHB1 (Lin et al. 2008; Zhu et al. 2014; Fujisawa et al. 2014) has not been evaluated. Other transcription regulators related to fruit development, but not directly involved in ripening, could also impact the outcome of fruit-*B. cinerea* interactions. A Golden2-like (SlGLK2) and KNOX (SlTKN4 and SlTKN2) transcription factors and a response-regulator factor (SlAPRR2-like) regulate chloroplast development and chlorophyll accumulation prior to the onset of ripening (Powell et al. 2012; Pan et al. 2013; Nadakudti et al. 2014). *SlGLK2* over-

151 expression apparently favors *B. cinerea* infections of unripe fruit, hypothetically
152 because of the increased sugar contents of the fruit (A.L.T. Powell pers. comm.).



153

154 **Fig. 19.2.** Susceptibility of non-ripening tomato fruit mutants to B. cinerea. Tomato fruit of wild-
155 type (WT, cv. Ailsa Craig) and three isogenic non-ripening mutants (rin, Cnr and nor) at
156 equivalent ripening stages (Unripe-like: 31 days post-anthesis [dpa] and Ripe-like: 42 dpa) after
157 inoculation with B. cinerea (1-3 days post-inoculation, dpi). * Cnr red ripe-like fruit are dark at 3
158 dpi due to excessive B. cinerea growth.

159 *19.2.2 Epigenetic changes and small RNAs*

160 When plants and pathogens interact, epigenetic modifications occur. These
161 epigenetic changes may include DNA methylation and modifications of histone
162 tails and lead to reprogramming of the transcription of genes involved in defence
163 responses (Dowen et al. 2012; Yang et al. 2013). Epigenetic changes could be a
164 means of inducing susceptibility in ripening fruit.
165 Throughout fruit development, epigenetic reprogramming occurs, but overall
166 DNA methylation declines once fruit begin to ripen (Zhong et al. 2013). Notably,
167 the promoters of tomato genes under the control of RIN are demethylated (Zhong
168 et al. 2013). Expression of *CNR* is epigenetically controlled (Manning et al. 2002;
169 Zhong et al. 2013). When seeds enclosed in the fruit flesh have developed full
170 viability and the fruit have reached their maximum size, the *CNR* promoter is
171 demethylated and subsequently a cascade of ripening events occurs. Although,
172 CNR is not the only catalyst of tomato fruit ripening, its activity coincides with
173 the demethylation of the promoters of genes regulated by RIN (Zhong et al. 2013;
174 Chen et al. 2015). Whether *B. cinerea* perturbs this form of ripening regulation has
175 not been explored, although *B. cinerea* does influence host gene expression for
176 susceptibility (Cantu et al. 2009).
177 Plant small RNAs (sRNAs), such as microRNAs (miRNAs) and small
178 interfering RNAs (siRNAs), are important means of regulating development,

defences and epigenetic modifications (Chen 2009; Ruiz-Ferrer and Voinnet 2009). In fruit, sRNAs influence the expression of transcription factors or proteins that contribute to key aspects of ripening (Moxon et al. 2008; Mohorianu, et al. 2011; Zuo et al. 2012, 2013; Karlova 2013).

miRNAs coordinate the onset and progression of ripening by integrating both transcriptional and hormonal controls (Moxon et al. 2008; Zuo et al. 2012; Karlova et al. 2013). For example, in tomato, miR156 and miR172 suppress the expression of *CNR* and *SlAP2a*, respectively (Karlova et al. 2013). Other miRNAs influence the expression of ripening genes downstream of these regulators, such as genes involved in ethylene synthesis and perception (Moxon et al. 2008; Mohorianu, et al. 2011; Zuo et al. 2012, 2013; Karlova et al. 2013). siRNAs are highly abundant in tomato fruit and particular clusters of siRNAs might regulate early development and ripening. Most siRNA targets have not been validated yet, but may include genes involved in lipid and polysaccharide metabolism, flavor and aroma compound biosynthesis and hormone signaling (Itaya 2008; Mohorianu et al. 2011; Zuo et al. 2013).

sRNAs may be involved in controlling fruit resistance and susceptibility. Studies on the roles of plant sRNA in the regulation of defence responses to *B. cinerea* have been done in vegetative tissues but not in fruit (Jin et al. 2012). Nevertheless, some miRNAs with putative roles in disease resistance (e.g., miR6022, miR6027 and miRZ8) have been detected in fruit (Karlova et al. 2013; Zuo et al. 2013). *B. cinerea* expresses sRNAs to regulate its own development and to suppress host immune responses (Weiberg et al. 2013). During infections of Arabidopsis and tomato leaves, *B. cinerea* introduces its sRNAs into the host where they act as effectors, which hijack plant sRNA-mediated mechanisms to silence genes involved in biotic stress signaling (Weiberg et al. 2013). *B. cinerea* sRNAs have been detected during fruit infections (Weiberg et al. 2013), but no connections have been drawn yet between them and the susceptibility of ripe fruit.

19.2.3 Hormones

Regulation involving the synthesis and perception of hormones impacts basal resistance and mediates plant defence responses. Interactions among hormone pathways provide the plant with adaptable defence strategies against different types of attackers, but these might also be vulnerable to exploitation by pathogens to favor infections (Van der Ent and Pieterse 2012). Plant hormones coordinate a variety of ripening events downstream of the main transcriptional controllers of ripening. In tomato fruit, *B. cinerea* infections cause transcriptional reprogramming of multiple plant hormone networks and depending on the developmental stage of the fruit, either resistance or susceptibility results (Blanco-Ulate et al. 2013).

Ethylene

Since the gaseous hormone, ethylene, is essential for the normal ripening of climacteric fruit (e.g., tomato, banana, apple, and peach), failure to synthesize or

perceive ethylene prevents ripening (Klee and Giovannoni 2011; Pech et al. 2012). Ripening of non-climacteric fruit (e.g., pepper, strawberry, grape and citrus) does not depend on ethylene, but this hormone can interact with other hormones and regulators to trigger particular ripening processes (Trainotti et al. 2005; Shemer et al. 2008; Lee et al. 2010; Böttcher et al. 2013). Ethylene influences the resistance and the susceptibility of plant tissues to pathogens. In cooperation with other hormones, ethylene activates defences against diverse pathogens, but ethylene also induces senescence or ripening, changes which facilitate susceptibility and infections by necrotrophs (Van Loon et al. 2006; Cantu et al. 2009; Van der Ent and Pieterse 2012).

Two systems, 1 and 2, are used to synthesize ethylene via the conversion of S-adenosyl methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) (Baur et al. 1971). Which system is used, depends on whether fruit ripen with climacteric or non-climacteric mechanisms and the developmental stage of the fruit. System 1 produces low levels of ethylene due to auto-inhibition of its biosynthetic genes and is active throughout early fruit development. Only System 1 is active when non-climacteric fruit ripen but System 2 is required and predominates when climacteric fruit ripen. System 2 generates high levels of ethylene by positive feedback regulation of its ethylene biosynthetic genes (Yokotani et al. 2009; Klee and Giovannoni 2011; Pech et al. 2012). In tomato fruit, the switch from System 1 to System 2 requires the transcription factor RIN and ethylene (Martel et al. 2011; Pech et al. 2012).

Infections of unripe fruit by *B. cinerea* could induce ethylene production under System 1 and consequently trigger plant defence responses, but could also prematurely activate System 2 and the consequent climacteric ripening or, in non-climacteric fruit, initiate senescence or ripening pathways and result in enhanced susceptibility that overthrows ethylene-induced defences (Marcos et al. 2005; Swartzberg et al. 2008; Cantu et al. 2009; Blanco-Ulate et al. 2013). When *B. cinerea* infects unripe tomato fruit, expression of two *ACS* genes from System 2, *SlACS1a* and *SlACS2*, is precociously activated, suggesting that the pathogen activates ethylene synthesis by System 2 (Blanco-Ulate et al. 2013). *B. cinerea* itself is capable of synthesizing ethylene from methionine via the α-keto-γ-methyltiobutyric acid (KMBA) pathway (Chagué et al. 2002). Ethylene production by *B. cinerea* during infections (Christescu et al. 2002) may stimulate the production of host-derived ethylene and, thereby, promote premature ripening or senescence.

Salicylic acid

When examined in leaves, salicylic acid (SA) is primarily associated with basal host defences, effector-triggered immunity and local and systemic acquired resistance against biotrophic and hemi-biotrophic pathogens (Durrant and Dong 2004; Vlot et al. 2009). Because of mutual regulation, SA might enhance susceptibility to necrotrophs by antagonizing jasmonic acid (JA) and auxin signaling (Glazebrook 2005; Beckers and Spoel 2006; Spoel and Dong 2008). Synthesis and signaling by SA occur in fruit, but no roles for this hormone in regulating fruit development have been defined.

In tomato fruit, SA responses involving an NPR1-independent pathway appear

to be associated with resistance to *B. cinerea* infections in unripe fruit. SA induction of Mitogen-Activated Protein Kinase (MAPK) signaling via MAPK3 and MAPK6 may establish efficient defences against *B. cinerea* in unripe fruit, while NPR-1 related mechanisms or the absence of MAPK signaling in ripe fruit may be counterproductive for defence strategies later in ripening (Blanco-Ulate et al. 2013). Evidence that SA is important for unripe fruit defences comes from the increased susceptibility of unripe fruit from the *NahG* tomato line, which does not accumulate SA (Fig. 19.3, Cantu et al. 2009; Blanco-Ulate et al. 2013; Brading et al. 2000). Ripe *NahG* and wild-type fruit are equally susceptible, indicating that SA-related defences are unimportant in ripe fruit (Blanco-Ulate et al. 2013).

Jasmonates

JA and its derivatives are well-known participants in the induction of plant responses to insects and necrotrophic pathogens (Glazebrook, 2005; Browse 2009). As in infections of leaves, *B. cinerea* infections of tomato fruit increase JA biosynthetic gene expression (Wasternack 2007; Browse 2009; Blanco-Ulate et al. 2013). JAs are associated with the control of normal fruit ripening, possibly because JAs act synergistically with ethylene, (Peña-Cortés et al. 2004).

The efficiency of JA-mediated defence responses depends on interactions with other hormone pathways and signals derived from the pathogen itself (Rowe et al. 2010). For example, JA responses are inhibited by the NON-EXPRESSOR OF *PR* GENES 1 (NPR1), a key component of the SA signaling network, which prioritizes responses against biotrophs and necrotrophs (Pieterse et al. 2009; Van der Ent and Pieterse 2012). Infections by *B. cinerea* activate the NPR1-mediated pathway, which antagonizes JA-derived defences and promote susceptibility (El Oirdi et al. 2011). Ethylene and JA-mediated defences can act synergistically to impede necrotrophic infections (Glazebrook 2005). The simultaneous activation of ethylene and JA pathways impairs the NPR1-mediated suppression of JA-dependent defences, which results in the co-existence of resistance mechanisms against biotrophs and necrotrophs (Spoel et al. 2008; Leon-Reyes et al. 2010).

In unripe tomato fruit, the opportune activation of ethylene, SA and JA-mediated pathways could establish efficient defences to *B. cinerea*. However, as unripe fruit begin to ripen, ethylene-mediated induction of ripening and/or senescence probably results in a shift towards increased susceptibility by over-riding the defence pathways regulated by these hormones (Blanco-Ulate et al. 2013).

Abscisic acid

Abscisic acid (ABA)-mediated responses are essential for tolerance to abiotic stress (Fujita et al. 2006). ABA induces susceptibility to pathogens by antagonizing the SA- and JA/ethylene-dependent defences (Spoel and Dong 2008; Yasuda et al. 2008; Sánchez-Vallet et al. 2012). The effect of ABA on the outcome of particular plant-pathogen interactions depends on the organisms, the developmental stage of the host and/or the environmental conditions in which the interaction occurs (Ton et al. 2009; Robert-Seilaniantz et al. 2011). ABA regulates aspects of ripening in climacteric as well as non-climacteric fruit (Zhang et al. 2009; Koyama et al. 2010; Jia et al. 2011; Soto et al. 2013). In tomato fruit, ABA activates ripening because it enhances the expression of ethylene biosynthetic

genes from System 2 (Zhang et al. 2009). ABA also triggers senescence in vegetative tissues and possibly in fruit (Lee et al. 2011).

ABA contributes to the ripening-associated susceptibility of tomato fruit to *B. cinerea*. Ripe tomato fruit from the *sitiens* mutant, which does not synthesize ABA (Harrison et al. 2011), are less susceptible to *B. cinerea* than wild-type fruit (Fig. 19.3). A localized necrotic response that is typical of resistant unripe fruit develops in infected ripe fruit (Blanco-Ulate et al. 2013). Leaves from the *sitiens* tomato mutant also are more resistant to *B. cinerea* infections (Asselbergh et al. 2007; Curvers et al. 2010; Seifi et al. 2013a), although it is not clear whether the resistance mechanisms in *sitiens* leaves and fruit are similar. Characterization of infected leaves suggest that increased cell viability, induction of defence-related genes and increased oxidative burst limit *B. cinerea* growth (Curvers et al. 2010; Seifi et al. 2014).

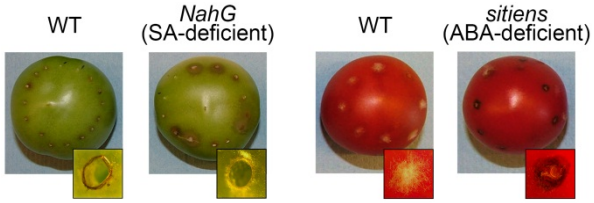


Fig. 19.3. Susceptibility of salicylic acid (SA) and abscisic acid (ABA) deficient tomato fruit to *B. cinerea.* Inoculated tomato fruit (3 days post-inoculation) of wild-type (WT, cv. Moneymaker), *NahG* (SA-deficient) and *sitiens* (ABA-deficient). Insets show a magnification of one of the inoculation sites. Unripe wild-type and ripe *sitiens* fruit have dark necrotic rings that limit infection symptoms; whereas unripe *NahG* fruit or ripe WT fruit have no necrotic zone (Reproduced, with permission, from Blanco-Ulate et al. 2013).

Auxins

Normal fruit development requires auxins and once ripening begins they are inactivated (Seymour et al. 2013). Indole-3-acetic acid (IAA), the most abundant auxin in plants, decreases just before ripening starts, and IAA-aspartate, the inactive conjugate, increases (Böttcher et al. 2010, 2013; Kumar et al. 2012). Auxin has been shown to be both a susceptibility or resistance factor: depending on the host tissue it can induce resistance or susceptibility (Sharon et al. 2007; Fu et al. 2011). IAA induces susceptibility to *B. cinerea* in Arabidopsis leaves by antagonizing oligosaccharide-induced resistance (Savatin et al. 2011), but in tomato leaves and eggplant fruit, pre-treatments with IAA or naphthaleneacetic acid (i.e. NAA, a synthetic auxin) reduce *B. cinerea* infections (Sharon et al. 2007). *B. cinerea* can produce auxins (Sharon et al. 2007), but whether *B. cinerea* produces these hormones *in planta* and how this production impacts the susceptibility of fruit is currently unknown.

19.3 Ripening fruit properties and susceptibility to *B. cinerea*

19.3.1 Tissue integrity and physical barriers

Tissue softening is an integral aspect of ripening in many fruit. Fruit softening occurs primarily as a result of the activities of plant cell wall modifying enzymes that disassemble the networks of polysaccharide in the wall. The composition and thickness of the fruit cuticle and the decline in cellular turgor pressure during ripening also influence the fruit's texture (Brummell and Harpster 2001; Shakel et al. 1991). Softening compromises the integrity of the physical barriers that fruit have against pathogens, and thereby, increases susceptibility to pathogen infections. Furthermore, softened fruit are more vulnerable to physical damage, such as bruising or cracking, which may provide entry points for pathogens (Brummell and Harpster 2001).

Plant cuticles are composite structures of cutin and cuticular waxes, which cover the epidermis of aerial organs. When reaching the plant surface, *B. cinerea* can secrete an assortment of cutinases and lipases to breach the cuticle and penetrate the host (Van Kan et al. 1997; Reis et al. 2005; Van Kan 2006; Leroch et al. 2013). The permeability of Arabidopsis and tomato leaf cuticles has been correlated with enhanced resistance to *B. cinerea*. Enhanced permeability of plant cuticles may favor the generation, movement and perception of pathogen-induced signals (e.g., reactive oxygen species, oligosaccharides, cutin monomers), which activate defence responses (Chassot et al. 2007; Bessire et al. 2007; Curvers et al. 2010; L'Haridon et al. 2011). However, defects in cuticle development, apart from permeability, could compromise defences against bacteria and *B. cinerea*, as reported in leaves of the Arabidopsis mutant *glabra1* (Xia et al. 2010).

When infecting unripe tomato fruit, *B. cinerea* expresses a putative cutinase (*cut-like1*) (Blanco-Ulate et al. 2014). Since mutations in the previously characterized *B. cinerea cutA* gene do not reduce the pathogen's virulence in tomato fruit (Van Kan et al. 1997), the requirement of other *B. cinerea* cutinases, such as *cut-like1*, for successful infections should be studied.

The properties of the cuticles of ripe tomato fruit influence *B. cinerea* infections. The fruit cuticles of the *delayed fruit deterioration* (*dfd*) tomato mutant are stiffer and have higher wax contents than wild-type cuticles. *dfd* mutant fruit have minimal loss of water due to transpiration, substantially elevated cellular turgor and reduced susceptibility to *B. cinerea* (Saladie et al. 2007). Fruit from *cutin deficient* mutants (*cd1-3*) are more susceptible to *B. cinerea* (Isaacson et al. 2009).

The implications of the disassembly or degradation of plant cell walls during *B. cinerea* infections of vegetative and fruit tissues are examined in detail in Chapter 18. Here we highlight a few relevant points to relate fruit ripening and cell wall integrity with the susceptibility of ripened fruit to *B. cinerea*. As fruit ripen, the structure and composition of their cell walls change. Hemicellulose and

cellulose networks loosen and are broken down, structural proteins associated with the cell wall are released or are no longer synthesized and pectins are degraded. As fruit ripen, these changes allow the walls around cells in the pericarp flesh and epidermis to expand and become hydrated, which increase the porosity of the extracellular matrix (Redgwell et al. 1997; Brummell and Harpster 2001; Vicente et al. 2007). The changes in fruit cell wall architecture during ripening may disrupt the deposition and localization of defence-related proteins and antimicrobial compounds, and is likely to favor the pathogen-induced degradation of wall polysaccharides. In unripe fruit, cells are enclosed within compacted walls. When infecting unripe tomato fruit, *B. cinerea* expresses a wide-range of cell wall modifying enzymes but *B. cinerea* also accelerates disassembly of the host cell wall by inducing expression of endogenous fruit proteins to disassemble the wall (Cantu et al. 2009; Blanco-Ulate et al. 2014). *B. cinerea* expresses hydrolases and induces the fruit softening enzymes in order to expose the carbon-rich nutrients of the wall polysaccharides and facilitate colonization.

19.3.2 Metabolism and metabolites

Transcription factors and other regulators of ripening influence basic metabolites, such as sugars, amino acids and organic acids, but these metabolites in turn also exert control over fruit development itself. For example, the accumulation of sucrose is needed for ABA synthesis and normal ripening in strawberries (Jia et al. 2013).

Sugars

The main apoplastic sugars in tomato fruit are glucose and fructose; sucrose is found in lower concentrations (Ruan et al. 1996). Soluble galacturonic acid, monosaccharides derived from pectin breakdown, is detected in the ripe tomato fruit apoplast (Chun and Huber 1998). One might expect that soluble sugars in fruit cells can be carbon nutrients for pathogens. However, in tomato fruit, sugar accumulation is not a major factor in susceptibility, since *Cnr* mutant fruit, which are very susceptible to *B. cinerea*, have low levels of sugars. The susceptibility of *Cnr* mutant fruit may be mostly promoted by changes in the cell wall architecture.

In response to microbial infections, plant cells increase extracellular invertases to enhance cleavage of sucrose to hexoses in the apoplast, ensuring that sufficient sugars are available as energy sources for the synthesis of defence responses, known to demand high levels of energy. Hexose sugars released by disassembly of the cell wall can also act as signals to induce the expression of defence-related genes and to repress photosynthesis (Roitsch et al. 2003). SlLIN5, an extracellular tomato fruit invertase, has been shown to alter hormone synthesis and signaling networks (e.g., ABA, JA, ethylene) and responses to pathogens (Zanor et al. 2009). Infections of unripe tomato fruit by *B. cinerea* induce the expression of *SlLIN5* and *SlLIN6*, which presumably alter hexose accumulation and impact JA-mediated responses (Cantu et al. 2009; Hyun et al. 2011).

Botrytis cinerea has hexose transporters involved in the uptake of fructose (i.e. Ftr1/Hxt13) and galacturonic acid (Hxt15 and Hxt19). Mutants in these genes, including the double mutant *Δhxt15/Δhxt19*, show normal development and virulence on leaves when compared to the wild-type strain, suggesting that additional hexose transporters or other mechanisms may transport sugars (Doehlemann et al. 2005; Zhang et al. 2013b). The role of *B. cinerea*'s hexose transporters during infections of fruit has yet to be described. *B. cinerea* has two functional hexokinases, Hxk1, and the glucokinase, Glk1. These enzymes phosphorylate hexoses and may participate in carbon source sensing. Hxk1 is needed during *B. cinerea* infections of tomato and apple fruit, host tissues which have higher levels of fructose compared to vegetative tissues (Rui and Hahn 2007).

Botrytis cinerea can metabolize the galacturonic acid released by the disassembly of pectin polysaccharides (e.g., as fruit soften or are macerated by the fungus). Mutants in each step of the galacturonic acid catabolism pathway, *Δgar1/Δgar2*, *Δlgd1* and *Δlga1*, have reduced virulence on tobacco and Arabidopsis leaves (Zhang and Van Kan, 2013), but no differences in virulence has been observed between the mutants and the wild-type strain on tomato leaves or apple and pepper fruit (Zhang et al. 2011). Preliminary results indicate that only the *Δlgd* mutant, affected in galactonate dehydratase activity, is less virulent in tomato fruit (A. L. T. Powell and B. Blanco-Ulate, pers. obs.).

pH

Most ripe fruit (e.g., tomato, strawberry, grape, apple, cherry and apricot) are acidic (Manteau et al. 2006). As tomato fruit ripen, the pH of the apoplastic fluid decreases from 6.7 in unripe to 4.4 in ripe fruit (Almeida and Huber 1999). The acidic environment of the fruit favors the production of proteases, polygalacturonases, laccases, extracellular polysaccharides and oxalic acid by *B. cinerea* (Manteau et al. 2006). However, acidification can also activate plant defences as reactive oxygen species are generated (Schaller and Oecking 1999; Nurnberger and Scheel 2001), so changes in intra- and extra-cellular pH alter host responses as well as infection functions of *B. cinerea* (Prusky et al. 2013).

Organic acids

As tomato fruit develop before ripening, carboxylic acids from the TCA cycle raise the osmotic potential that drives cell growth and they are major sources of energy during ripening (Liu et al. 2007; Morgan et al. 2013). Increased citric acid in ripe fruit contributes to the synthesis of important biochemical intermediates and energy-rich molecules via the TCA cycle (Morgan et al. 2013). A role of citric acid during plant-*B. cinerea* interactions has not been described.

Malic acid/malate regulates starch biosynthesis and the accumulation of total soluble solids during tomato fruit ripening (Centeno et al. 2011). Ripe tomato fruit with high levels of malic acid (i.e. by suppression of malate dehydrogenase) have elevated water loss by transpiration, significantly reduced soluble sugars (glucose, fructose and sucrose) and enhanced susceptibility to *B. cinerea*. The altered osmotic potential in these lines seems to be the cause of both the fruit's water loss phenotype and the consequent pathogen susceptibility (Centeno et al. 2011),

475 although the direct influence of malate on *B. cinerea* virulence and metabolism
476 has not been determined.
477 Oxalic acid (OA) accumulates as tomato fruit ripen. Changes in the
478 concentrations of OA can cause changes in the levels of citric, malic and ascorbic
479 acids. OA is a strong acid and a powerful Ca^{2+} chelator (Chakraborty et al. 2013).
480 *B. cinerea* secretes significant amounts of OA when infecting leaves (Van Kan
481 2006). OA modifies the host environment and contributes to pathogenesis by: 1)
482 acidification that favors *B. cinerea* virulence factors production; 2) strong Ca^{2+}
483 chelation that weakens pectin interactions within the cell wall and inhibits some
484 defence signaling pathways; and 3) inhibition of callose deposition. OA also may
485 alter the production of ROS in a pH-dependent manner, inactivating host
486 responses or, alternatively, inducing programmed cell death and susceptibility to
487 *B. cinerea* (Prusky et al. 2013).
488 **Amino acids**
489 Efficient regulation of host carbon/nitrogen metabolism is required for the
490 accurate activation of defence-related pathways and to avoid unnecessary
491 consumption of energy. In tomato leaves, increased cell viability and effective
492 resistance against *B. cinerea* infections is achieved by replenishment of the TCA
493 cycle by γ-aminobutyric acid (GABA) and glutamate metabolism (Seifi et al.
494 2013a and 2013b). *B. cinerea* can alter primary amino acid metabolism in leaf
495 hosts to induce susceptibility. It has been demonstrated that induction of
496 asparagine synthetase after *B. cinerea* infections of tomato leaves promotes
497 susceptibility by accumulation of asparagine, a rich source of nitrogen for the
498 pathogen and by facilitating pathogen-induced host senescence (Seifi et al. 2014).
499 The levels of free amino acids increase as fruit ripen and GABA and glutamate
500 are the most abundant amino acids in tomato fruit (Boggio et al. 2000; Rolin et al.
501 2000; Akihiro et al. 2008; Sorrequieta et al. 2010). The highest levels of GABA
502 are in unripe fruit and then they decrease rapidly (Carrari et al. 2006; Mounet et al.
503 2007). GABA is a non-protein amino acid that regulates the cytosolic pH, protects
504 against oxidative stress and contributes to resistance against pathogens and insects
505 (Akihiro et al. 2008).
506 Glutamate is the main free amino acid in ripe tomato fruit and provides the
507 characteristic 'umami taste' of tomato (Bellisle 1999). The metabolism of
508 glutamate is important for: 1) the transport of nitrogen via the glutamine
509 synthetase and glutamine-oxoglutarate aminotransferase cycle, 2) cellular redox
510 and 3) the reprograming of TCA-dependent energy (Seifi et al. 2013b). The
511 changes in amino acid metabolism in tomato fruit during *B. cinerea* infections
512 have not yet been investigated, but will be informative for determining whether
513 similar mechanisms exist at least in resistant unripe fruit.
514 **Reactive oxygen species, antioxidants and nitric oxide**
515 *B. cinerea* causes an oxidative burst as it penetrates the cuticle layer and
516 colonizes hosts (Van Kan 2006). Hydrogen peroxide (H_2O_2) accumulates in the
517 plasma membrane of invading hyphae and in the fungal cell wall (Schouten et al.
518 2002). *B. cinerea* not only produces reactive oxygen species (ROS) itself but also
519 induces free radical production by the host tissue (chapter 14). Plants commonly
520 respond to infections with a rapid oxidative burst that induces localized cell death

(e.g., the hypersensitive response), which can help to constrain infections of biotrophic pathogens, but also of *B. cinerea* in some settings such as unripe fruit (Cantu et al. 2008, 2009). On susceptible hosts, this oxidative burst potentiates infections especially by necrotrophic pathogens, such as *B. cinerea* (Lyon et al. 2007).

During tomato fruit ripening, the levels of ROS, lipid peroxidation and protein oxidation increase, while the expression and activity of the principal scavenging enzymes, such as superoxide dismutase, catalase and those associated with the ascorbate-glutathione cycle decrease (Lacan and Baccou 1998; Jimenez et al. 2002; Qin et al. 2009). In ripe tomato fruit, the accelerated accumulation of antioxidants, such as ascorbic acid and carotenoid pigments (e.g., lycopene, γ-carotene and β-carotene), may partially neutralize the increase in oxidative processes during ripening (Jimenez et al. 2002; Mondal et al. 2004).

Even though *B. cinerea* may take advantage of ROS production by the fruit to generate widespread oxidative stress and promote susceptibility (Heller and Tudzynski, 2011; chapter 14), precisely timed and localized generation of ROS can limit *B. cinerea* infections (Asselbergh et al. 2007). On unripe tomato fruit, localized accumulation of H_2O_2 is detected as a consequence of *B. cinerea* inoculation, but this response is not observed on ripe tomato fruit or as result of mechanical wounding. The accumulation of H_2O_2 however correlated with resistance in unripe fruit (Cantu et al. 2008, 2009).

The accumulation of free radicals during *B. cinerea* infections depletes the antioxidant pool in the host tissues (Lyon et al. 2007). Thus, it can be anticipated that increasing antioxidants in fruit may limit the oxidative stress generated by *B. cinerea* infections and promote resistance (Van Baarlen et al. 2007). Interestingly, tomato fruit that are purple due to the engineered accumulation of the antioxidant, anthocyanin, (Butelli et al. 2008) are resistant to *B. cinerea* at all ripening stages and the fruit senesce extremely slowly. This delay in senescence and softening-related events in the purple tomato fruit probably also contributes to their improved resistance (Zhang et al. 2013a).

Nitric oxide (NO) is a diffusible free radical that delays fruit ripening and senescence in both climacteric and non-climacteric fruit by inhibiting ethylene pathways (Singh et al. 2013). In general, endogenous levels of NO are significantly higher in unripe fruit than in ripe fruit (Leshem et al. 1998; Leshem 2000). During plant-pathogen interactions, NO cooperates with other signaling agents (e.g., Ca^{2+}, SA, JA and ethylene-mediated pathways) to induce programmed cell death and systemic acquired resistance (Mur et al. 2006; Bellin et al. 2013). In tomato fruit, MAPK kinases are involved in NO signaling during infections. In unripe tomato fruit, up-regulation of the transcripts encoding SlMAPK1, SlMAPK2 and SlMAPK3 is observed in response to *B. cinerea* infections and might be involved in establishing NO-induced responses (Zheng et al. 2014). However, it is not resolved whether NO is involved in the resistance of unripe tomato fruit to *B. cinerea*-infections.

19.3.3 Defences in ripening fruit

Most plant tissues utilize some form of preformed and inducible defences to protect themselves against pathogen attack. The resistance of unripe fruit is associated with preformed antimicrobial compounds, inducible phytoalexins and PR proteins, which mostly accumulate in the cuticle or cell walls. During fruit ripening, most of these compounds either decline or become ineffective, perhaps also by losing their association with the wall (Prusky et al. 2013).

Secondary metabolites

The leaves and peel of unripe tomato fruit are rich in the toxic steroidal alkaloid α−tomatine, a saponin that disrupts plasma membranes, causing leakage of electrolytes and depolarization of membrane potential (Itkin et al. 2011, Mintz-Oron et al. 2008). α-tomatine levels decline as tomato fruit ripen (Friedman 2002) and might inhibit growth of *B. cinerea* mycelia in unripe tomato fruit (Verhoeff and Liem 1975), however most *B. cinerea* strains can detoxify α−tomatine by hydrolysis of its sugar adduct (Quidde et al. 1998). Thus, α-tomatine may partially restrict *B. cinerea* infections of unripe tomato fruit, but it is not the only mechanism fruit use for resistance. Early studies reported that the sesquiterpene, rishitin, is produced in unripe tomato fruit as a consequence of *B. cinerea* infection and that it may boost the deleterious effects of α-tomatine on the fungus (Glazener and Wouters 1981).

Inoculations of tomato fruit with *B. cinerea*, up-regulate three hydroxycinnamoyl-CoA:tyramine N-(hydroxycinnamoyl) transferases (THTs) (Cantu et al. 2009), which are required for the production of *p*-coumaroyloctopamine and *p*-coumaroylnoradrenaline, two secondary metabolites with antimicrobial qualities (von Roepenack-Lahaye et al. 2003). The levels and effect of these metabolites during tomato fruit-*B. cinerea* interactions are not known.

In vitro and *in planta* studies have shown that volatile compounds produced by ripe fruit can inhibit *B. cinerea* growth (Vaughn and Gardner 1993; Vaughn et al. 1993). The major flavor volatiles, hexanal, 1-hexanol, (*E*)-2-hexen-1-ol, (*Z*)-6-nonenal, (*E*)-3-nonen-2-one, methyl salicylate and methyl benzoate are used for antifungal postharvest treatments of strawberry, blackberry and grape berries (Archbold et al. 1997)(chapter 11).

Pathogenesis-related proteins

Botrytis cinerea infections induce the expression and accumulation of host PR proteins in plant cell walls and vacuoles (Van Loon 1999). PR proteins hydrolyze fungal cell walls (e.g., chitinases, β-1,3-glucanases, mannanases), inhibit fungal enzymes (e.g., polygalacturonase-inhibitor proteins), have contact toxicity (e.g., thaumatin-like proteins and thionins) and signal for defences (e.g., PR-1 and the defensin PDF1.2) (Van Loon et al. 2006; Van Baarlen et al. 2007).

In contrast to other plant organs, uninfected fruit accumulate high concentrations of certain PR-like proteins (e.g., chitinases) as a consequence of ripening (Derckel et al. 1998; Cantu et al. 2009). During tomato fruit-*B. cinerea* interactions, PR proteins are induced both by ripening and infections. A β-1,3-

glucanase gene (e.g., *TomQ'a*) is induced one day post-inoculation when unripe fruit is infected but no induction occurs in infections of ripe fruit, or as consequence of ripening (Cantu et al. 2009). Later in infections (3 days post-inoculation), enhanced expression and accumulation of PR-proteins, such as the putative thaumatin-like protein *SlPRP-23*, the possible chitinase, *SlChi-like1,* and the β-1,3-glucanases, *TomQ'b* and *TomB13GLUB*, was detected in both unripe and ripe fruit (Cantu et al. 2009; Shah et al. 2012). PR-proteins in fruit may not be effective in controlling *B. cinerea* infections since the *Cnr* mutation induces PR gene expression in fruit but the fruit are hyper-susceptible to *B. cinerea*. It has not been resolved whether the retention of PR proteins is impacted by disassembly of the host cell walls, by for example, ripening or *B. cinerea* infections.

19.3.4 Botrytis cinerea toxins in fruit

Botrytis cinerea kills host plant cells not only through its extensive disassembly of the host cell wall matrix and its active release of ROS, but also through its production of toxins that lead to death of host cells (Chapter 15). *B. cinerea* can secrete toxic bicyclic sesquiterpenes (Collado et al. 1996; Rebordinos et al. 1996). Botrydial is a well-studied *B. cinerea* sesquiterpene, but other toxins with a botryane skeleton have also been described (Duran-Patron et al. 1999). From *in vitro* and *in planta* phytotoxicity assays, botrydial has the highest activity and effectively induces chlorosis and cell collapse in hosts (Colmenares et al. 2002). Botrytidial was detected when *B. cinerea* infected the ripe fruit of sweet pepper (Deighton et al. 2001). Whether fruit have the means to cope with the toxicity of botrydial is not known and more research is needed to understand whether these compounds have a role in susceptibility of fruit.

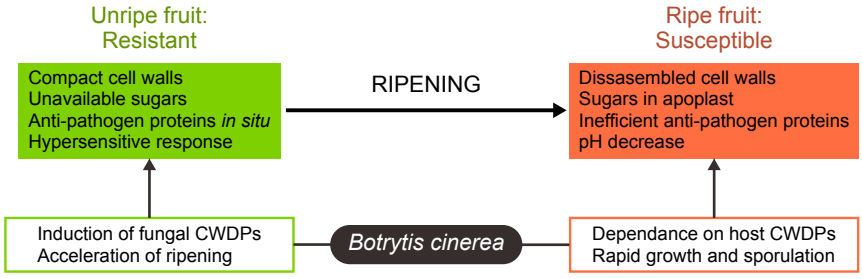


Fig. 19.4. Model depicting host and pathogen-derived regulators during ripening tomato fruit infections by *B. cinerea*. Events and conditions in unripe and ripe fruit that impact susceptibility are enumerated. Differences in *B. cinerea* infection strategies on unripe and ripe fruit are also shown.

19.4 Perspectives

The aim of understanding the increase in susceptibility that accompanies ripening is to improve the quality of ripened fruit. However, it is clear that the ripening fruit-*B. cinerea* interaction is dynamic, involving multiple inputs and responses by the host as well as the pathogen (Fig. 19.4). By understanding what distinguishes ripening from susceptibility, new varieties can be developed which ripen acceptably but are not predisposed to decompose due to *B. cinerea* infections. Since there are examples of non-ripening mutant fruit that are at least as susceptible to *B. cinerea* as fruit that ripen normally and because not all ripening processes are induced by infections of fully-developed, but non-ripe, fruit, only some aspects of ripening are necessary for susceptibility. Targeted reverse genetic approaches and evaluations of large mapping populations are needed to genetically dissect these complex traits in the fruit hosts. It will be especially important to identify how *B. cinerea* interferes with the regulation of these traits that occurs when healthy fruit ripen and how *B. cinerea* adapts to changing conditions in the host. Examining susceptibility in ripening fruit provides novel information about events that occur uniquely in fruit, but not in vegetative tissues, and has expanded knowledge about the versatility of *B. cinerea*-plant interactions.

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