Roles of Flavonoid Compounds in Determining the Shelf Life of Tomato Fruit

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Abstract

This thesis examines the role of flavonoid compounds in determining the shelf life of tomato fruit. Shelf life is one of the most important traits for tomato industry. Although there are many approaches to enhance the post-harvest performance of tomato fruit, most of them would have side effects such as reducing fruit quality.

My work showed that accumulation of anthocyanins in tomato fruit by over expressing Delila and Rosea 1, two transcriptional factors from *Antirrhinum majus*, can extend tomato shelf life significantly. Compared to WT tomato fruit, the anthocyanin-enriched purple fruit have double the shelf life including delayed over-ripening and reduced susceptibility to the fungal pathogen, *Botrytis cinerea*. My data indicate that the delayed over-ripening is associated with the high antioxidant capacity of purple tomato and the reduced susceptibility to *B. cinerea* is due to the high scavenging ability of anthocyanins.

Using *Aft/Aft atv/atv*, a conventionally bred tomato variety which accumulates anthoycanin predominantly in the skin, I showed that accumulation of anthocyanins in tomato skin can be sufficient to extend fruit shelf life. The scientific findings from transgenic crop studies can provide new strategies for conventional breeding improved fruit quality.

My research also revealed that, as a general principle, flavonoids extend tomato shelf life. For ripening and over-ripening, increasing total antioxidant capacity by accumulating flavonoids of different types can delay these processes. To reduce susceptibility to *B. cinerea*, however, the effects of different flavonoids depend on their scavenging abilities, which are determined by their molecular structures. Taken together these findings demonstrate that increasing total antioxidant capacity by accumulating different flavonoid compounds offers a general strategy to delay over-ripening of tomato fruit. However, to reduce susceptibility to *B. cinerea*, flavonoid compounds with high scavenging activity should be used.

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Abbreviations

4CL	4-coumaroyl CoA ligase
ABA	abscisic acid
ACN	acetonitrile
ae	*entirely anthocyaninless*
Aft	*Anthocyanin fruit*
ANS	anthocyanidin synthase
atv	*atroviolacium*
aw	*anthocyanin without*
BCP	1-bromo 3-chloropropane
bHLH	basic helix-loop-helix
C4H	cinanmate 4-hydroxylase
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
CHI	chalcone isomerase
CHS	chalcone synthase
cm	centimetre
CoA	co-enzyme A
Del	Delila
DEPC	Diethylpyrocarbonate
DFR	dihydroflavonol 4-reductase
dH2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
dpb	day(s) post breaker
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ETI	Effector-triggered immunity

F3’5’H	flavanone 3’5’-hydroxylase
F3’H	flavanone 3’-hydroxylase
F3H	flavanone 3-hydroxylase
FW	Fresh weight
h	hour
HPLC	high performance liquid chromatography
HR	Hypersensitive Response
JA	jasmonic acid
LC-MS	Liquid chromatography–mass spectrometry
M	Molar
MBW	MYB/bLHL/WDR40
MeJA	methyl jasmonate
mg	milligram
MG	mature green
min	minute
mL	millilitre
mM	milli molar
mm	millimetre
mol	Mole
mRNA	messenger RNA
ºC	degrees centigrade
OD	Optical Density
PAL	phenylalanine ammonia lyase
PAMP	Pathogen-associated molecular pattern
PCR	polymerase chain reaction
PDA	Potato dextrose agar
PG	Polygalacturonase
PLI	Promoter of Light Induced protein
PTI	PAMP-triggered immunity
RNA	ribonucleic acid
ROS	Reactive oxygen species

Ros1	Rosea 1
RT	Room Temperature
RT-qPCR	Real time quantitative PCR
s	second
SA	salicylic acid
SDS	sodium dodecylsulphate
SEM	Standard Error of the Mean
Taq	Thermophilus aquaticus
TBG	β-galactosidase
TE	tris / EDTA
TEAC	Trolox Equivalent Antioxidant Capacity
TRV	Tobacco Rattle Virus
µg	microgram
µL	microliter
µM	micro molar
µm	micrometer
VIGS	Virus Induced Gene Silencing

Chapter 1: General Introduction

1.1 Flavonoid biosynthesis

Flavonoids are a large group of organic compounds synthesized from phenylalanine in most plants, from bryophytes to angiosperms. These compounds contribute to nearly all aspects of plant responses to biotic and abiotic stresses: they are key players in regulating plant responses to pathogens (La Camera et al., 2004) and some are used as phytotoxins by invading plants to displace native plants (Bais et al., 2003). They have roles in plant reproduction by attracting pollinators (Shang et al., 2010) and act as signalling molecules during microbe—legume symbiosis (Hassan and Mathesius, 2012).

There are two groups of genes involved in flavonoid biosynthesis: the structural genes which are directly involved in the biosynthesis of flavonoids, and the transcriptional factors that control the transcriptional levels of those biosynthetic genes.

1.1.1 phenylpropanoid pathway

Flavonoids are all synthesized from the phenylpropanoid pathway and all general phenylpropanoids are synthesized from phenylalanine. The shikimate pathway supplies phenylalanine for phenylpropanoid metabolism. Erythrose-4-phosphate, produced from the oxidative pentose phosphate pathway and phosphoenolpyruvate (PEP) from glycolysis, are the precursors for this seven-step pathway (Herrmann and Weaver, 1999). All intermediate compounds in the shikimate pathway can be used in branch pathways for the synthesis of other secondary metabolites. Phenylalanine is one of the final products of this pathway. Other products include tyrosine and tryptophan (Herrmann and Weaver, 1999).

The general phenylpropanoid pathway starts from phenylalanine, and involves the activity of three enzymes, phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumaroyl CoA ligase (4CL). PAL catalyzes the deamination of phenylalanine to cinnamate. It directs the flux of carbon from the shikimate pathway to phenypropanoid metabolism. C4H generates 4-coumarate and

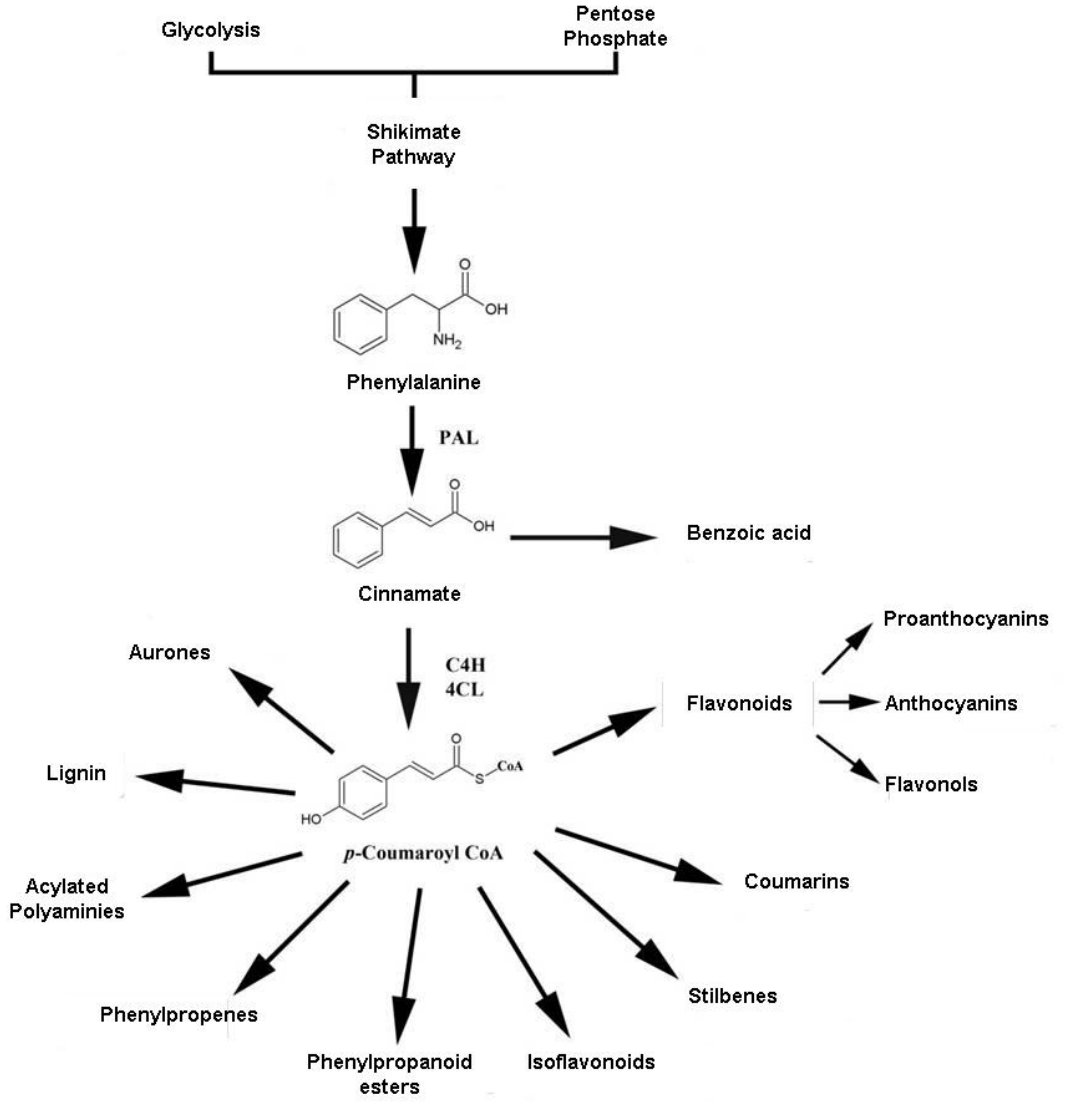

Figure 1.1 Schematic representation of the upstream and major steps of phenylpropanoid pathway. Phenypropanoids are synthesized from phenylalanine. In plants, phenylalanine is synthesized from shikimate pathway, which is downstream of glycolysis and pentose Phosphate pathway. The general phenylpropanoid pathway starts from phenylalanine and is catalyzed by phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumaryol CoA ligase (4CL) to generate the central metabolite, *p*-coumaroyl CoA. Adjusted from Vogt (2010).

4CL generates p-coumaroyl CoA, the activated intermediate for various branches of phenylpropanoid metabolism (Hamberger et al., 2007) (Fig 1.1).

1.1.2 Flavonoid biosynthesis

Flavonoids are a group of plant-specific phenylpropanoid compounds with multiple functions including stress responses and pigmentation. Two important compounds in this group are anthocyanins and flavonols (Winkel-Shirley, 2002).

Anthocyanins are water-soluble pigments responsible for the red, purple, and blue colours of many flowers and fruit (Grotewold, 2006). They are produced by plants to attract pollinators and seed dispersers (Shang et al., 2010). Anthocyanin production is also induced under stress conditions (Feild et al., 2001; Gould et al., 2002; Li et al., 1993; Olsen et al., 2009; Rubin et al., 2009) and infection by pathogens (Lorenc-Kukula et al., 2005). Besides their physiological roles in plants, dietary anthocyanins are associated with protection against certain cancers (Wang and Stoner, 2008), cardiovascular diseases (Tsuda et al., 2003) and other chronic human disorders (Tsuda et al., 2003). Although many studies attribute the protective effects of dietary anthocyanins to their antioxidant capacity, their bioavailability is low and they likely promote health by suppressing specialised signalling pathways involved in inflammation and disease development (Meiers et al., 2001).

The biosynthesis of anthocyanins is one of the best characterised secondary metabolism pathways of plants. The general phenylpropanoid pathway reaches a branch point with the production of 4-coumaroyl CoA. Chalcone synthase (CHS) is the first enzyme of flavonoid pathway. Condensation of one molecule of 4-coumaroyl CoA with three molecules of malonyl-CoA is catalysed by CHS to produce naringenin chalcone. Naringenin chalcone is the precursor for all flavonoids and its C-ring is closed by the activity of chalcone isomerise (CHI) to form naringenin. Flavanone 3-hydroxylase (F3H) catalyses the hydroxylation of the C-ring to make dihydrokaempferol. Additionally, hydroxyl groups can be added at the 3’ and 5’ positions of B-ring to generate dihydromyricetin (by flavanone 3’5’-hydroxylase, F3’5’H) and dihydroquercetin (by flavanone 3’-hydroxylase, F3’H).

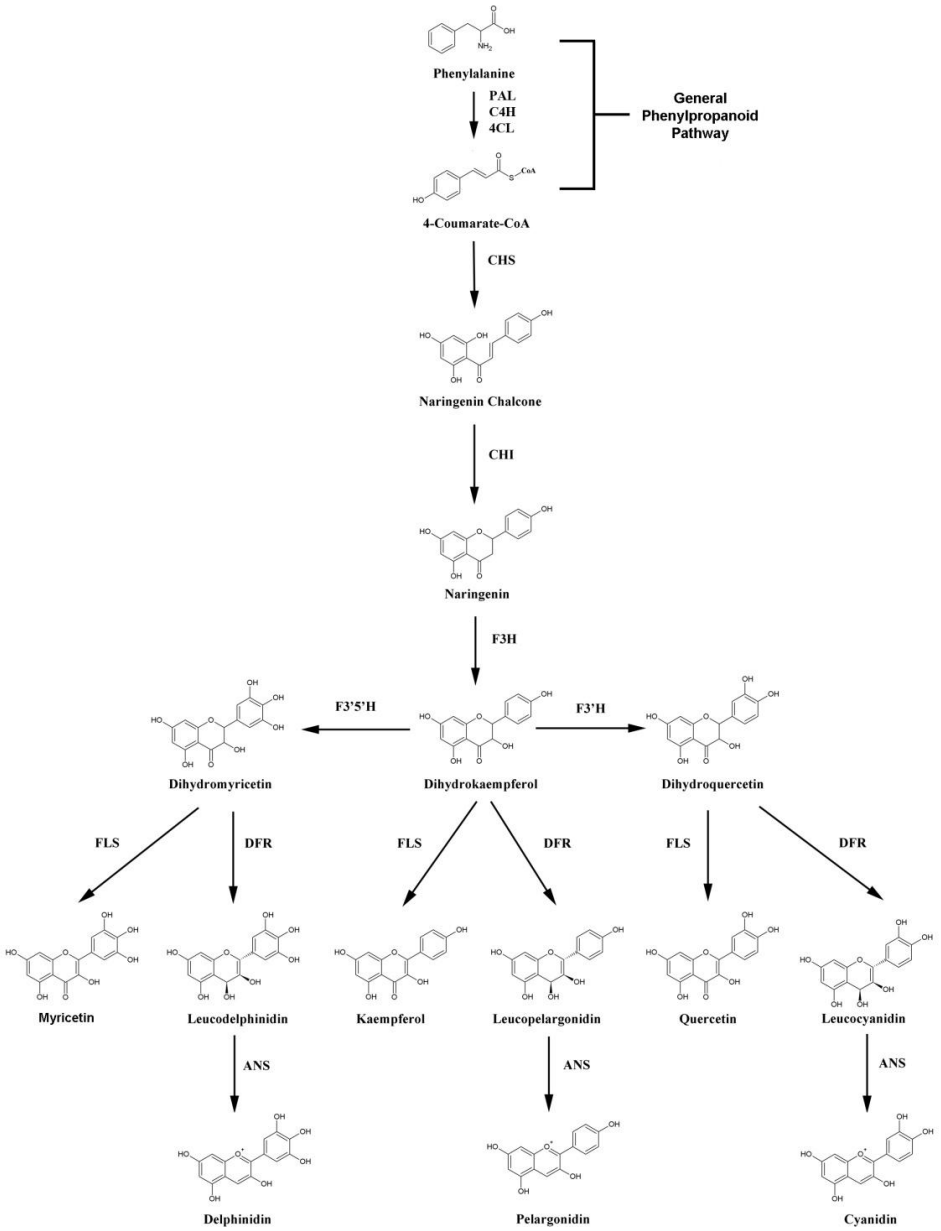

Figure 1.2 Schematic Representation of the Flavonoids Biosynthetic Pathway. The general phenylpropanoid pathway is catalyzed by phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumaryol CoA ligase (4CL). General flavonoid biosyntheis genes are chalcone synthase (CHS), chalcone isomerise (CHI), flavanone 3-hydroxylase (F3H), flavanone 3'5'-hydroxylase, (F3'5'H) and flavanone 3'-hydroxylase (F3'H). Anthocyanins are synthesized by dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS). Flavonols are synthesized by flavonol synthase (FLS).

The subsequent step is catalysed by dihydroflavonol 4-reductase (DFR) to convert dihydroflavonols into the corresponding leucoanthocyanidins. DFR is the first step committed to anthocyanin biosynthesis. Then anthocyanidin synthase (ANS, also known as leucoanthocyanidin dioxygenase (LDOX)) converts leucoanthocyanidins to their corresponding anthocyanidins. Anthocyanidins are stabilized by glycosylation of the 3-position of the C ring. Further decoration of anthocyanins by the addition of methyl, acyl or glycosyl groups affects the stability and color of anthocyanins, but these decoration steps vary widely between different plant species (Grotewold, 2006) (**Fig. 1.2**).

Flavonols are the most abundant subgroup of flavonoids in nature. Flavonols play important roles in plant development and stress responses. The early steps of flavonol biosynthesis are the same pathway as for anthocyanins (CHS, CHI, F3H and F3'H/F3'5'H). Flavonol synthase (FLS), a soluble 2-oxoglutarate-dependent dioxygenase, is the key branch-point enzyme. It catalyses the biosynthesis of flavonols from dihydroflavonols (Falcone Ferreyra et al., 2012). Different flavonols (myricetin, quercetin and kaempferol) are distinguished by the number of hydroxyl (-OH) groups on the B-ring. Flavonols are frequently glycosylated on the 3-position and may have additional glycosylation.

1.1.3 Transcriptional regulation of flavonoid biosynthesis

Genes involved in anthocyanin biosynthesis can be divided into following groups: the genes of general phenylpropanoid metabolism (*PAL, C4H, 4CL*), early biosynthesis genes (EBGs, including *CSH, CHI* and *F3H*) and late biosynthesis genes (LBGs, *DFR, ANS, 3GT, GST*). Previous studies indicate that the structural genes encoding the enzymes of the flavonoid biosynthesis pathway can be co-regulated. The co-regulation patterns of "early" biosynthetic genes (EBGs) and "late" biosynthetic genes (LBGs) are different (Jackson et al., 1992; Martin et al., 1991). The EBGs are upstream genes common to the different flavonoid branch pathways while LBGs are active in the branches specific to the synthesis of particular flavonoids.

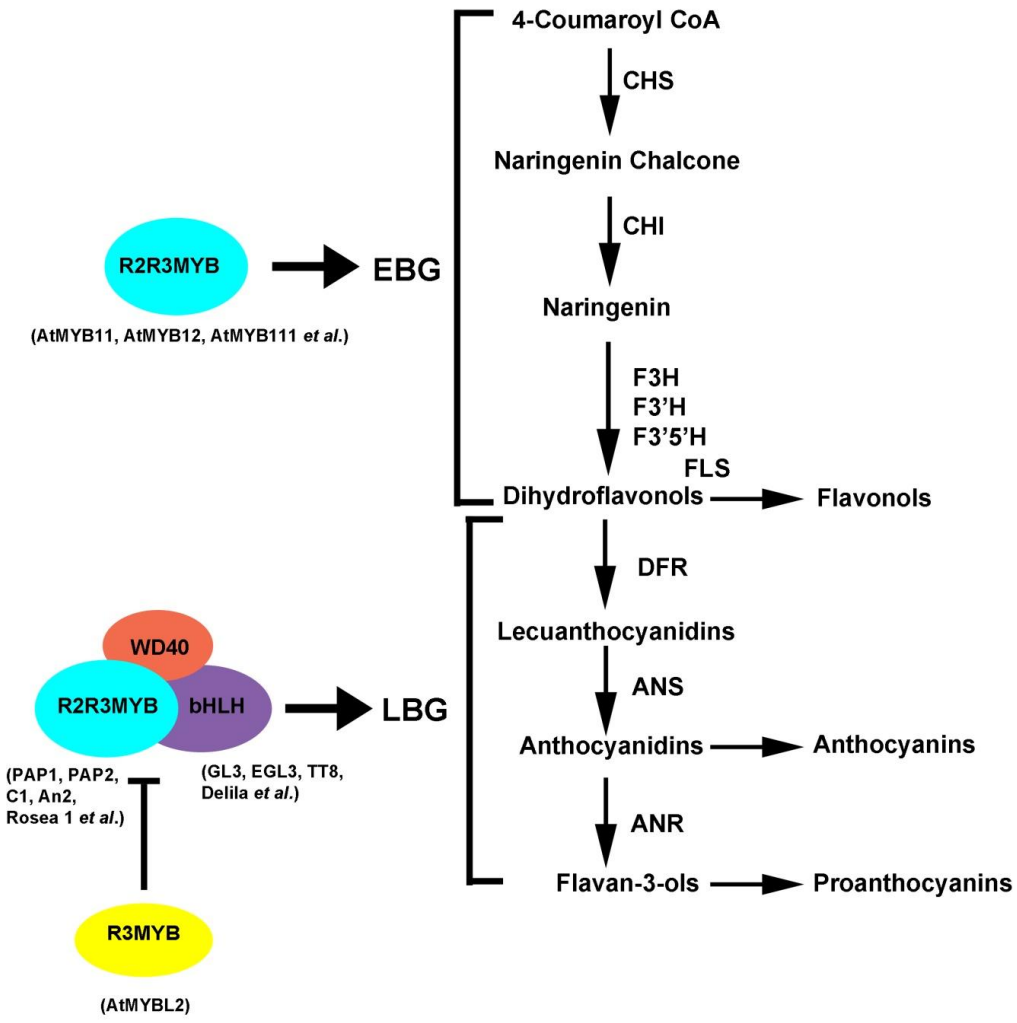

Figure 1.3 Schematic representation of the regulation of the flavonoid biosynthesis pathway. The flavonoid biosynthetic genes are divided into two groups: "early" biosynthetic genes (EBGs) and "late" biosynthetic genes (LBGs). For EBGs, single R2R3MYBs are key regulators while for LBGs, the MYB-bHLH-WD40 (MBW) complex is the major regulator (MBW is also found to regulate EBGs according to Butelli et al. (2008)). R3MYBs were found to compete with R2R3MYB to form MBW complex. Adjusted from Dubos et.al. (2008)

The EBGs are normally induced prior to LBGs, and can be found in many tissues. In most cases, single R2R3MYB transcription factors have been found to activate these genes. For example the MYB11, MYB12 and MYB111 TFs in *Arabidopsis* (Mehrtens et al., 2005; Stracke et al., 2007) and the P1 TF in maize (Grotewold et al., 1991). Recent studies show AtMYB12, a R2R3 MYB regulator, regulates flavonol biosynthesis, especially the EBGs. Although *FLS* is classified as a LBG, it is highly induced by AtMYB12. (Mehrtens et al., 2005) (**Fig. 1.3**).

A protein complex composed of MYB and bHLH transcriptional factors, together with WD40 repeat protein regulates anthocyanin production at the transcriptional level (Ramsay and Glover, 2005). The MYB-bHLH-WD40 (MBW) complex is conserved between species and it also regulates various cell fate pathways such as epidermal cellular diversity (Serna and Martin, 2006) (**Fig. 1.3**).

The MYB proteins involved in the MBW complex are R2R3MYB proteins which have two adjacent repeats (Ramsay and Glover, 2005). Many R2R3MYB genes that affect anthocyanin biosynthesis have been identified in *Arabidopsis* (*PAP1*), maize (*C1*), *Petunia hybrida* (*An2*) and *Antirrhium majus* (*Rosea1*, *Rosea2* and *Venosa*) (Borevitz et al., 2000; Paz-Ares et al., 1987; Quattrocchio et al., 1999; Schwinn et al., 2006). Recent studies suggest that the R2R3MYB proteins active in the MBW complex are the components most closely aligned with the expression of the flavonoid biosynthetic genes, limiting the production of flavonols and anthocyanins (Schwinn et al., 2006). Also associated with the MBW complex are R3MYB. Because of their interaction of bHLH proteins which act competitively with R2R3MYB, the R3MYB proteins may counteract the activity of the MBW complexes. Thus, R3MYBs act as negative regulators in flavonoid biosynthesis (Dubos et al., 2008; Matsui et al., 2008) (**Fig. 1.3**).

The bHLH proteins have a basic region that binds to DNA and the HLH domain interacts with other proteins (Toledo-Ortiz et al., 2003). The bHLH proteins regulate anthocyanin biosynthesis comprise a specific sub-family which can interact with MYB proteins through a conserved N-terminal domain (Goff et al., 1992). Examples of bHLH genes involved in regulating anthocyanin are *TT8*, *GL3* and *EGL3* in

Arabidopsis, *Lc* and *R* in Maize, *An1* in *Petunia hybrida* and *Delila* in *Antirrhinum majus* (Goodrich et al., 1992; Ludwig et al., 1989; Spelt et al., 2000; Zhang et al., 2003).

Previously, Delila (Del), a bHLH transcription factor, and Rosea1 (Ros1), a MYB transcriptional factor, were cloned from *Antirrhinum majus* (Goodrich et al., 1992; Schwinn et al., 2006). Delila was found to activate the LBGs in the corolla tube (Goodrich et al., 1992; Martin and Gerats, 1993; Martin et al., 1991). Rosea1, together with other MYB TFs Rosea2 and Venosa, influence the LBGs of anthocyanin biosynthesis in different ways, and can be used to explain the anthocyanin pigmentation variations between different types of anthocyanins (Schwinn et al., 2006)

WD40 repeat proteins are believed to facilitate interaction between bHLH and MYB proteins and have no direct DNA binding function (Ramsay and Glover, 2005). The WD40 protein in the MBW complex is *TTG1* in *Arabidopsis* as, in maize it is *PAC1* and in *Petunia hydrida* it is *AN11* (Carey et al., 2004; de Vetten et al., 1997; Walker et al., 1999).

1.1.4 Natural mutants affecting flavonoid production in tomato

Because of their potential health benefits, there is a growing interest in modulating flavonoid compounds in plants. Tomatoes provide a very good candidate to produce flavonoids, since it is a very popular and important crop for the daily diet. Although there are a variety of flavonoids in tomato seedlings, they accumulate only small amounts of naringenin chalcone and some flavonols in fruit (Gonzali et al., 2009).

Although not common, there are several tomato "mutants" that accumulate anthocyanins in their fruit (Al-sane et al., 2011). For example, the dominant gene *Anthocyanin fruit* (*Aft*) from *Solanum chilense* was introgressed into *Solanum lycopersicum* (domestic tomato). The *Aft/Aft* fruit showed light-dependant accumulation of anthocyanins in fruit (Jones et al., 2003; Mes et al., 2008). Another dominant gene, *Aubergine* (*Abg*), from *Solanum lycopersicoides* Dunal, can also induce anthocyanin accumulation in fruit peel when introgressed into domestic

tomato (Mes et al., 2008). A recessive gene, *atroviolacea (atv)*, derived from *Solanum cheesmaniae* (L. Riley) Fosberg causes accumulation of high amounts of anthocyanins in many tissues. Crossing *Aft/Aft* or *Abg/Abg* with *atv/atv* can generate tomato plants with high anthocyanin content in the fruit (Mes et al., 2008; Povero et al., 2010).

Compared with anthocyanin-enriched mutants, there are more anthocyanin-deficient mutants in tomato(Al-sane et al., 2011). For instance, the *anthocyanin without* (*aw*) mutant does not synthesis anthocyanins in vegetative tissues. Over-expression of *DFR* gene from normal tomato can complement the *aw* mutant, indicating the *aw* mutant lacks the DFR activities (Goldsbrough et al., 1994). Another well studied mutant is *entirely anthocyaninless* (*ae*), which lacks anthocyanidin synthase (ANS) activity (De Jong et al., 2004; Tanksley et al., 1992).

1.1.5 Enrichment of flavonoids in tomato by bio-engineering

Recently, various transgenic strategies have been used to generate high flavonoid tomatoes. In most cases, single structural or regulatory genes were transformed into tomato. However, in most cases, transgenic plants failed to accumulate significant amount of flavonoids in the fruit. For example, overexpression of the *Delila* gene in tomato results in the accumulation of anthocyanins only in vegetative tissue. Over expression of the *Arabidopsis PAP1* and the tomato *ANT1* gene can produces only spotted anthocyanin pigmentation in fruit (Mathews et al., 2003; Zuluaga et al., 2008).

In another study, two maize TF genes, *LC* (MYB-type) and *C1* (MYC-type) were over-expressed under the control of the fruit specific E8 promoter. Major flavonoid biosynthetic genes were up-regulated in *LC/C1* fruit, except *F3'5'H*. The *LC/C1* fruit were enriched with flavonols but lacked anthocyanins (Bovy et al., 2002). Further analysis indicate that the dihydroflavonol reductase (DFR) enzyme from tomato prefers dihydromyricetin as its substrate, while tomato FLS prefers dihydrokaempferol and dihydroquercetin as substrates. The activation of the *F3'5'H*

gene is a key step to produce dihydromyricetin and thus a key factor determining the production of anthocyanins in tomato fruit (Bovy et al., 2002).

Recently, the *Delila (Del)* and *Rosea1 (Ros1)* genes from the snapdragon *Antirrhinum majus* were expressed in tomato. By using the fruit-specific E8 promoter those two genes were expressed specifically in tomato fruits. Over-expression of *Delila* and *Rosea 1* genes together in tomato can activate all the general phenylpropanoid metabolism genes (*PAL,C4H, 4CL*) , as well as the EBGs (*CHS, CHI* and *F3H*) and the LBGs (*F3'5'H, ANS, 3-GT*) of anthocyanin biosynthetic pathway (Butelli et al., 2008) . As a result, especially due to the activation of *F3'5'H*, the anthocyanin levels of fruit were increased up to 2.83 ± 0.46 mg per g fresh weight in the transgenic lines (Butelli et al., 2008).

The *Arabidopsis MYB12* gene was also expressed fruit-specifically to induce the flavonol biosynthesis in tomato. The *AtMYB12* was found to activate flavonol biosynthesis in *Arabidopsis thaliana* (Mehrtens et al., 2005). Ectopic expression of *AtMYB12* in tomato fruits activates the biosynthesis of caffeic acid dervatives (CADs), in addition to flavonol biosynthesis (Luo et al., 2008).

1.2 Tomato ripening and over-ripening

Many plant species produce fleshy, delicious and colourful fruit to attract animals to eat and disperse their seeds. Fleshy fruit can be divided into two categories: climacteric and nonclimacteric. The major difference between these two groups is that at the initial stage of ripening, climacteric fruit require an increase in the levels of the plant hormone, ethylene, to progress normal ripening. Exogenous ethylene supplements can trigger the ripening process in climacteric fruit (Klee and Giovannoni, 2011). Nonclimacteric fruit, however, do not need increase in ethylene levels to trigger ripening, and application of exogenous ethylene to this type of fruit can not initiate or accelerate ripening (Klee and Giovannoni, 2011; Seymour et al., 2013).

Among all the climacteric fruit, tomato fruit ripening is very well studied because tomato is one of the most important vegetable crops in the world. The ripening of tomato fruit has a strict requirement for a burst of ethylene synthesis. Compared to other climacteric fruit, there are abundant genetic and molecular resources available for tomato. Many ripening mutants of tomato have been identified and characterized extensively (Giovannoni, 2007).

1.2.1 Tomato fruit ripening process

Tomato fruit development can be divided into five stages: fruit set (organogenesis), fruit expansion, fruit maturation, fruit ripening, and fruit over-ripening (senescence) (Alba et al., 2005; Gillaspy et al., 1993). Tomato ripening is a highly complicated and regulated process including softening, pigment accumulation, seed maturation as well as other metabolic changes (Klee and Giovannoni, 2011).

1.2.1.1 Ethylene signalling pathway

As a climacteric fruit, the ripening of tomato is strictly dependant on the production and signalling of ethylene. This requirement, as well as the existence of genetic recourses, makes tomato the perfect model for studying ethylene production and signalling (Giovannoni, 2004; Giovannoni, 2007). Ethylene synthesis is highly regulated. The pathway contains two key enzymes: ACC synthase (ACS) converts S-adenosylmethionine into 1-aminocyclopropane-1-carboxylate (ACC). ACC is then converted into ethylene by ACC oxidase (ACO). During the ripening, expression of two tomato *ACS* genes, *SlACS2* and *SlACS4* were significantly induced. Indicating ACC synthesis is the rate limiting step (Barry et al., 1996). Silencing of ethylene biosynthetic genes in tomato can significantly reduce ethylene production and delay the ripening process (Oeller et al., 1991) (**Fig. 1.4**).

The initiation of ethylene production during ripening is controlled by transcription factors. The recessive *rin* and dominant *Cnr* mutants in tomato can block the production of ethylene and cause the failure of fruit ripening (Manning et al., 2006; Vrebalov et al., 2002). RIN encodes a MADS-domain protein transcription factor

MADS-RIN (Vrebalov et al., 2002). MADS-RIN has been shown to interact with the CArG-box in the promoter of *ACS* genes. This indicates a direct activation function of MADS-RIN in ethylene biosynthesis (Fujisawa et al., 2011) MADS-RIN is also directly controls the expression of other ripening-related genes, such as other ripening-related TFs (NR, NOR and CNR), cell wall modification genes, carotenoid biosynthetic genes and even MADS-RIN itself (Fujisawa et al., 2011; Martel et al., 2011; Qin et al., 2012). *CNR* encodes a SQUAMOSA PROMOTER BINDING PROTEIN (SRBP) family transcription factor. CNR-SRBP is thought to act downstream of MADS-RIN in the ethylene production regulatory hierarchy (Manning et al., 2006) (**Fig. 1.4**).

Once ethylene production has been triggered, the ethylene signalling pathway is activated. So far, seven ethylene receptors (ETRs) have been identified in tomato (SlETR1, SlETR2, NR. SlETR4, SlETR5, SlETR6 and SlETR7) (Lashbrook et al., 1998; Tieman and Klee, 1999; Tieman et al., 2000). The ethylene receptors in tomato act as negative regulators of the ethylene signalling pathway: without ethylene, the receptors suppress the downstream pathway. With ethylene perceived, the inhibition of ethylene receptors is removed and downstream signalling is induced (Hua and Meyerowitz, 1998; Tieman et al., 2000) (**Fig. 1.4**).

Ethylene receptors physically interact with the CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), a negative regulator which suppresses downstream genes (Leclercq et al., 2002). At least three CTR1 family genes have been identified in tomato (Adams-Phillips et al., 2004) (**Fig. 1.4**).

Downstream of CTR1 are a set of transcription factors. Four Ein3-like transcription factors (EILs) have been identified in tomato (Tieman et al., 2001; Yokotani et al., 2003). On the absence of ethylene, ETRs interact with CTR1 to suppress EILs. Upon ethylene binding, ETR-CTR1 suppression is removed and EILs are activated (Kevany et al., 2007). EILs then activate downstream ethylene response factors (ERFs) as well as other ripening-related genes (Solano et al., 1998) (**Fig. 1.4**).

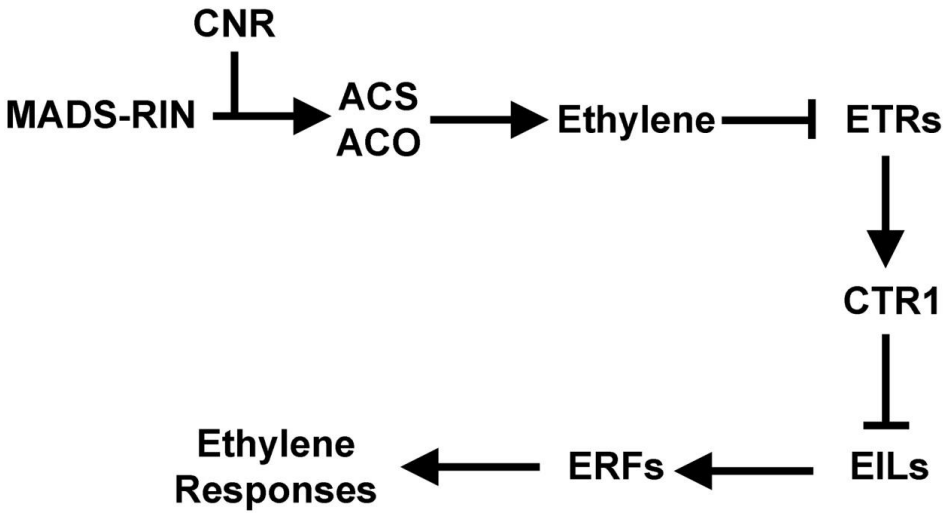

Figure 1.4 Schematic representation of ethylene signalling pathway during tomato fruit ripening.

At the beginning of ripening, transcription factors RIN and CNR activate the expression of ethylene biosynthetic genes. Without ethylene binding, the ethylene receptors (ETRs) interact with CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) to negatively regulate downstream Ein3-like transcription factors (EILs). Ethylene binding removes the inhibition of ETR-CTR1 on EILs. EILs activate downstream ethylene response factors (ERFs) to induce ethylene responses.

1.2.1.2 Developmental and chemical changes during tomato fruit ripening process

Tomato ripening process starts at breaker stage, when the yellow color becomes noticeable. During the ripening process, as the seed maturation finishes, tomato fruit undergo a set of changes to attract the seed dispersers. This process includes series of morphological and chemical changes.

The most important change in fruit appearance during ripening is the colour change. This is mainly caused by the conversion of chloroplasts to chromoplasts (Egea et al., 2010). This is accompanied by the accumulation of carotenoids (principally β-carotene and lycopene). Carotenoid accumulation changes the fruit colour from green to red (Klee and Giovannoni, 2011). Notably, the key carotenoid biosynthetic gene *Phytoene Synthase (PSY)* is a downstream target of ethylene signalling (Lee et al., 2012). This also indicates a direct role of ethylene in inducing tomato colour change during the ripening.

Another important change in tomato fruit during ripening is fruit softening. Softening and textural changes are caused largely by fruit cell wall disassembly. The activation of cell wall degrading enzymes during the ripening process is responsible for these changes (Brummell and Harpster, 2001).There are a large number of cell wall modification genes showing expression changes in tomato fruit during ripening process (Tomato Genome Consortium, 2012). The most important cell wall degradation enzymes are tomato polygalacturonases (PGs). PGs catalyse the hydrolytic cleavage of galacturonide linkages in homogalacturonans, an important component of cell walls (Carpita and Gibeaut, 1993). *SlPG* expression is very low in green fruit but is highly expressed at the onset of ripening (Dellapenna et al., 1989). PG activities increase rapidly during the ripening process (Grierson and Tucker, 1983) and keep on increasing as fruit become over-ripe (Tucker et al., 1980). Suppression of PG activity in tomato can reduce the softening of fruit and extend shelf life (Kramer et al., 1992; Langley et al., 1994). Other important cell wall enzymes include pectin methylesterase (PME) and β-galactosidase (TBG). PMEs catalyse the removal of pectin methylester groups from cell walls to help PGs access their substrates. PME is mainly activated at the green stage and suppressed by

ethylene signalling at breaker stage (Tieman et al., 1992). Silencing studies indicate that, *SlTBG4*, a ripening-related β -galactosidase gene, also has impact on fruit softening (Smith et al., 2002).

There are also other chemical changes besides pigmentation during tomato fruit ripening which impact fruit quality. For instance, α-tomatine is a defense compound which is highly accumulated in green fruit. It helps to prevent immature fruit from being eaten by seed dispersers at inappropriate stages of maturity. During the ripening, the amount of α-tomatine in fruit decreases significantly. This will increase the flavor of the mature fruit, and attracts seed dispersers (Rick et al., 1994). During ripening, the sugar contents (mainly glucose and fructose) of fruit also increase. This is mainly due to the increased degradation of starch. The pH of ripe fruit is usually around 4 (Klee and Giovannoni, 2011). There are also a set of derivatives from secondary metabolism which act as volatile chemicals to positively enhance fruit flavor (Tieman et al., 2012).

1.2.2 Tomato fruit over-ripening

Unlike ripening, the over-ripening (senescence) process of tomato fruit hasn't been studied extensively. There is no clear definition for fruit over-ripening so far. To some extent, the over-ripening of tomato fruit can be defined as programmed senescence of tomato fruit: over-ripening is the process whereby fully ripe tomato fruit begin to show symptoms which can shorten their shelf life. These include loss of flavour, over-softening and infection by post-harvest pathogens. The outcome of over-ripening is that the maximum shelf life is reached and fruit become inconsumable. Over-ripening process is of importance for tomato plants. If there are no proper seed dispersers, the plants have to use a backup strategy to release their seeds. Because the tomato seeds are inside the fruit, the fruit need to break down to release the seeds from inside. Thus the decaying of fruit after ripening is a necessary part of their development.

Several changes are notable during over-ripening. The first is the increased rate of softening. Since the beginning of the ripening process, the activities of different cell

wall enzymes increase through the ripening and over-ripening processes. This causes continues degradation of the cell wall matrix and leads to over-softening (Brummell and Harpster, 2001).

The antioxidant scavenging system of fruit also decreases activity during the ripening and over-ripening (Jimenez et al., 2002). At the breaker stage, the activity of antioxidants declines while oxidative processes, such as hydrogen peroxide production and lipid peroxidation, increase (Jimenez et al., 2002). The reduction in the antioxidant system and induction of the oxidative stress seem to play important roles in tomato ripening and over-ripening processes.

The susceptibility to postharvest pathogen also increases during the over-ripening of tomato fruit (Cantu et al., 2009). Plant cell walls are the first layer of defence. During the ripening and over-ripening processes, because of the activities of cell wall degradation enzymes, the dynamics of the cell wall matrix change. The degradation of defensive barriers gives the pathogen better opportunities to infect the fruit (Cantu et al., 2008a; Cantu et al., 2008b). The products from cell wall degradation can also serve as nutrients for the pathogen. Pathogens can even secret cell wall degradation enzymes as virulence factors to facilitate their infection (Jakob et al., 2007; Oeser et al., 2002; Shah et al., 2012). On the other hand, during the ripening and over-ripening processes, there are significant changes in fruit metabolism, and accumulated ripening-related changes may attenuate the defence systems in unripe fruit (Cantu et al., 2009).

1.2.3 Strategies to extend tomato shelf life

Tomato is one of the most important agricultural products in the world. It is also a very useful plant model for ripening and other applied agricultural research. One of the biggest challenges for the tomato industry is post harvest losses, which causes huge economic losses every year. Different means have been employed to extend tomato shelf life.

The easiest and most widely used strategy is to harvest the fruit at the mature green stage and store them at low temperature. Then, during transportation, the fruit are exposed to endogenous ethylene to induce the ripening. This can effectively reduce the post harvest damage of the fruit during the transportation. However, because the fruit are ripened artificially, they lose their flavour and become tasteless (Maul et al., 1998; Maul et al., 2000).

The accumulation of oxidative stress has been shown to be associated with the ripening and over-ripening (Jimenez et al., 2002). Postharvest application of antioxidant compounds can effectively extend tomato shelf life (Bhagwan et al., 2000). Extending shelf life by reducing reactive oxygen species has also been proved to be effective in other crops (Zidenga et al., 2012).

As ethylene signaling plays a very important role in tomato ripening, application of ethylene signaling inhibitors is an effective way to improve shelf life. For example, fruit harvested at breaker stage, stored at higher temperature but treated with ripening inhibitor 1-methylcyclopropene (1-MCP) can extend shelf life without loss of much flavor (Baldwin et al., 2011). However, compounds such as 1-MCP are still costly and cannot be applied in a large scale.

Another resource for extending tomato shelf life is the existence of large numbers of ripening-related mutants. By crossing ripening mutants with commercial varieties, fruit of hybrids heterozygous for ripening mutations can significantly extend shelf life (Kopeliovitch et al., 1979). So far, many ripening mutants have been introduced successfully into commercial varieties, such as *rin* (ripening inhibitor), *nor* (nonripening), *Nr* (never-ripe), and *alc* (Alcobaca) (Mutschler et al., 1992).

During the past 20 years, transgenic approaches have been widely used to improve tomato shelf life. There are several major targets for researchers to extend shelf life. The first group of targets are cell well degradation (CWD) enzymes. The strong activation of CWD genes at the climacteric is the major reason for fruit softening. Suppressing CWD genes can be used as a strategy to delay softening. Cell wall modification enzymes such as polygalacturonase (PG) and β-Galactosidase (TBG)

are involved in ripening-related softening (Brummell and Harpster, 2001). Suppression of such enzymes was found to effectively delay over-ripening process (Kramer et al., 1992; Meli et al., 2010). Another important group of targets are ripening-related. By silencing ripening inducers or over-expression ripening inhibitors, the shelf life of transgenic tomato fruit can be extended (Centeno et al., 2011). One of the best studied approaches has been to over express polyamines (PAs) in tomatoes. PAs have been found to decrease during the fruit ripening and accumulation of PAs in fruit can enhance shelf life (Mehta et al., 2002; Nambeesan et al., 2010).

1.3 Plant—Pathogen Interactions

Unlike animals which have complex immune systems, plants rely on innate immune systems to resist pathogen attack. There are multiple plant—pathogen interactions and the defence strategies are different when the plants are facing different pathogens.

1.3.1 Pathogen lifestyles

There are three major lifestyles for plant pathogen: biotrophic, hemi-biotrophic and necrotrophic. Biotrophic pathogens acquire nutrients from living host tissue. Nectrophic pathogens kill the host tissue at early stage of infection and derive nutrients from dead tissues. Some pathogens, however, switch their lifestyles during the infection and are so called hemi-biotrophic pathogens. In most cases, hemi-biotrophic pathogens exhibit as biotrophs during the early stages of infection. Once the infection progresses, these pathogens will switch to a nectrophic lifestyle to kill the host and spread (Glazebrook, 2005).

Important biotrophic pathogens include *Puccinia graminis* f. sp. *tritici* (*Pgt*) which causes wheat stem rust disease and *Blumeria graminis* which causes wheat powdery mildew disease (Dean et al., 2012). Normally, biotrophic pathogens grow between host cells. They use variety of approaches such as haustoria production to interact with the host cell and gain nutrients (Dodds and Rathjen, 2010).

The best studied hemi-biotrophic pathogens include *Pseudomonas syringae* and *Phytophthora infestans*. *P. syringae* previously has been recognized as biotrophic but later was re-defined as hemi-biotrophic (Thaler et al., 2004). At the early stage of *P. syringae* infection, host cell death was inhibited. However, during the late stage, the infected site showed necrosis. *P. infestans* is an oomycete which causes late blight, a serious potato disease. During the first 3-5 days after infection, the pathogen penetrates the host cell to gain nutrients. Then the pathogen switch to the nectrophic lifestyle to cause strong cell death and the necrotic lesions develop rapidly (Smart et al., 2003). Compared to *P. syringae*, the later necrosis symptoms in *P. infestans* infection are more obvious.

Typical necrotrophic pathogens are *Botrytis cinerea* and *Alternaria brassicicola*. Those pathogens normally induce host cell death at the early stages of infection. In order to induce stronger cell death, nectrophic pathogens usually produce different phytotoxins (Colmenares et al., 2002; Kim et al., 2008).

Because of their different lifetypes and infection mechanisms, there are contrasting defence mechanisms for biotrophic and necrotrophic pathogens. For biotrophic pathogen, R gene mediated resistance is the major defence mechanism. The production of reactive oxygen species (ROS) induces hypersensitive response (HR). HR triggers cell death at the infection site to stop the pathogen from extracting nutrients. In the mean time, the salicylic acid (SA)-dependent signalling pathway is activated, leading to the expression of pathogenesis-related (PR) proteins that contribute to resistance. For nectrophic pathogens, however, HR induced cell death facilitates the pathogen infection. In this situation, the SA-dependent signaling contributes negatively to pathogen resistance. Current observations suggest that different signalling pathways contribute to resistance to different pathogen. The SA signalling pathway mainly acts against biotrophic pathogens while ethylene (ET) and jasmonic acid (JA) signalling pathways contribute to resistance to nectrophic pathogen resistance (Dodds and Rathjen, 2010; Glazebrook, 2005).

1.3.2 Plant immune system—"zigzag" model

A "zigzag" model has been proposed to represent current view of plant immune system: at the first stage, pathogen-associated molecular patterns (PAMPs), such as flagellin (for bacteria) and chitin (for fungi), are recognized by pattern recognition receptors (PRRs). This recognition can result in PAMP-triggered immunity (PTI) to inhibit pathogen colonization (Jones and Dangl, 2006). Plants can recognize a wide range of PAMPs from bacteria and fungi via different PRRs (van de Veerdonk et al., 2008; Zipfel et al., 2006; Zipfel et al., 2004). PTI can trigger early responses to pathogens such as reactive oxygen species (ROS) burst, expression of defence genes, as well as callose formation (Chisholm et al., 2006; Nurnberger et al., 2004) (**Fig. 1.5**).

In stage 2, successful pathogens can secret effectors to suppress PTI. This is also called effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). For example, the fungal pathogen *Cladosporium fulvum* can produce effector Ecp6 (extracellular protein 6). Ecp6 contains LysM domains which are similar to the plant chitin receptor, CEBiP. Ecp6 can bind to CEBiP, preventing it from binding to chitin to trigger chitin-triggered immunity (de Jonge et al., 2010) (**Fig. 1.5**).

In stage 3, the pathogen effector is specifically recognized by a NB-LRR protein. This will induce effector –triggered immunity (ETI) (Jones and Dangl, 2006). Compared to PTI, ETI is a more accelerated and amplified process, which normally triggers strong responses such as hypersensitive response (HR). HR causes cell death at the infected site, which can effectively halt the growth and spreading of the pathogen (Spoel and Dong, 2012) (**Fig. 1.5**).

In stage 4, some pathogens have evolved to produce additional effectors or have modified their effectors to avoid ETI. As a result, natural selection drives plants to evolve new R proteins so ETI can be induced again (Jones and Dangl, 2006). This process repeats and drives the co-evolution of plants and their pathogens (**Fig. 1.5**).

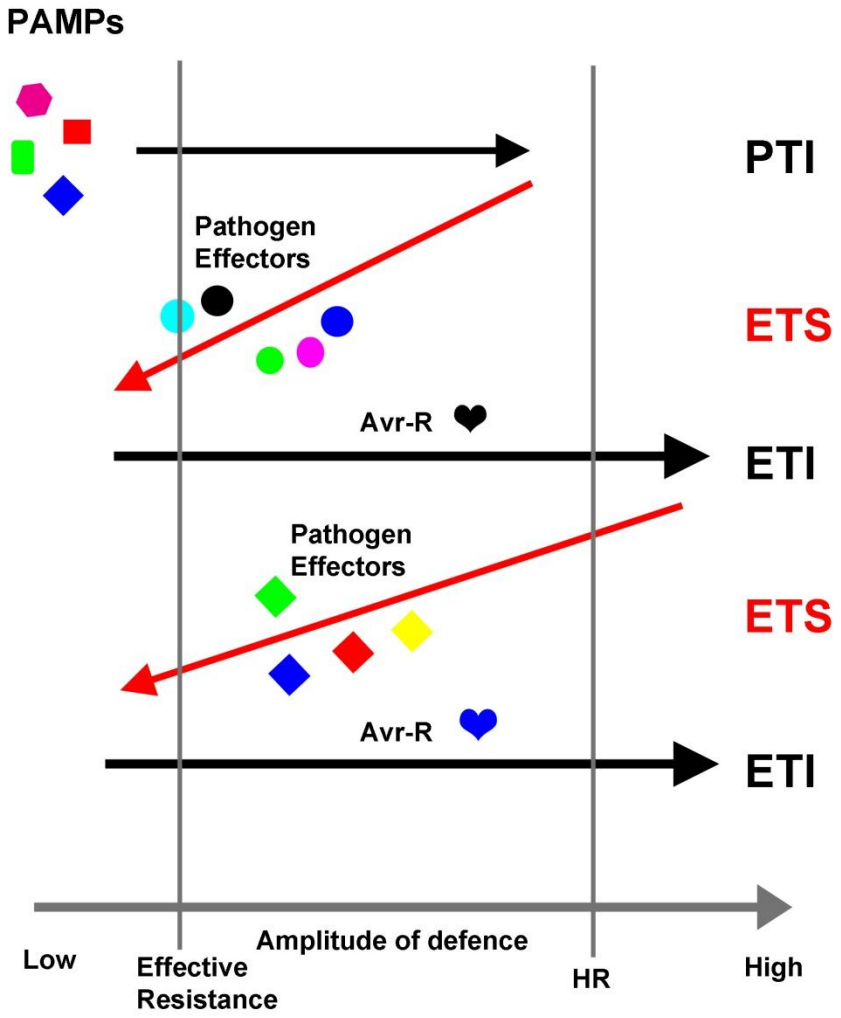

Figure 1.5 Zigzag Model for plant-pathogen interaction.

During plant-pathogen interactions, plants detect pathogen associated molecular patterns (PAMPs) to trigger PAMP-triggered immunity (PTI). Pathogens use effectors to inhibit PTI, resulting in effector-triggered susceptibility (ETS). Plants use R proteins to recognize pathogen effectors to induce effectors-triggered immunity (ETI). To overcome ETI, pathogens use another set of effectors to recreate ETS. Plants need to use new R proteins to recognize new effectors to achieve new ETI. Adjusted from Jones and Dangl (2006).

1.3.3 Role of ROS in plant pathogen response

The production of reactive oxygen species (ROS) is a very important response in plants during pathogen infection. After the recognition of a pathogen, plants generate superoxide (O_2^-) or hydrogen peroxide (H_2O_2) (Torres et al., 2006). The recognition of pathogen PAMPs or effectors by PRRs and R proteins, rapidly, generates low-amplitude and transient ROS production in plants. For avirulent pathogens, the first ROS accumulation is followed by the second phase of the ROS burst which is of much higher amplitude, and triggers other defence responses, HR included. For virulent pathogens, after the initial ROS accumulation, no further ROS burst is produced and the plants fail to trigger further defence response (Lamb and Dixon, 1997).

One key enzyme for ROS generation following pathogen recognition is the plasma membrane-located NADPH oxidase (also known as respiratory burst oxidase, RBO) which can be inhibited by diphenylene iodonium (DPI) (Grant et al., 2000). Another group of enzymes are cell wall peroxidases, which can be inhibited by cyanide or azide (Bolwell et al., 1998). Although both NADPH oxidases and peroxidases are involved in the production of ROS during responses to pathogen, recent research indicates that alternative mechanisms might be involved in ROS production during PTI, whereas the R protein-mediated ROS burst is dependant mainly on NADPH oxidases (Soylu et al., 2005) (**Fig. 1.6**).

ROS are involved not only in pathogen response, but also in other aspects of plant development and stress response. Because oxidative stress may be destructive to cells, it is vital for plants to maintain their oxidative status. In addition, due to their capacity to harvest light energy through photosynthesis, chloroplast represent a major source of ROS when light and dark reactions are not marked. Plants have established many mechanisms to scavenge ROS to avoid their damaging effects.

In plant, various ROS-scavenging systems maintain ROS homeostasis (**Fig. 1.6**) (Mittler et al., 2004). Major ROS-scavenging systems include different enzymes (superoxide dismutases, ascorbate peroxidase, catalase et.al.), as well as antioxidants such as ascorbic acid and glutathione (Mittler et al., 2004).

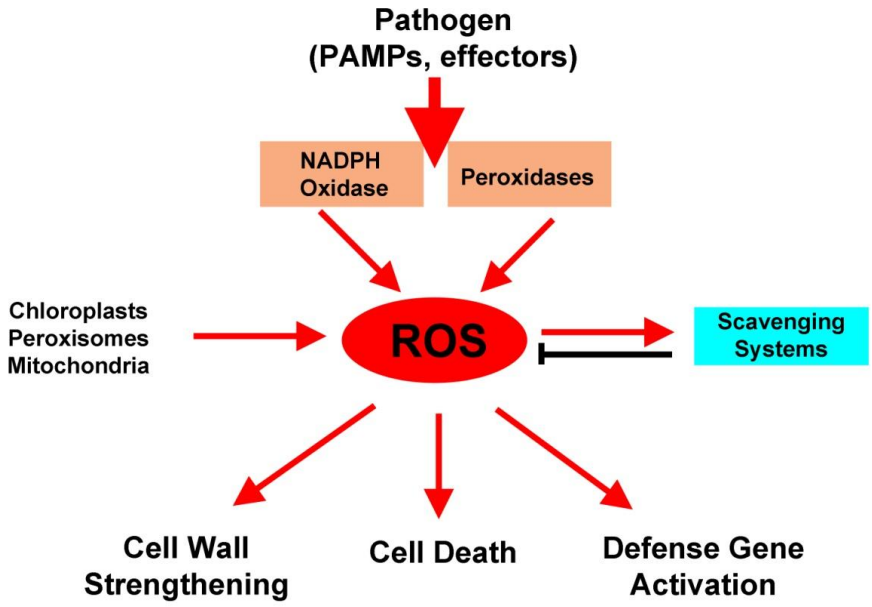

Figure 1.6 Production and functions of reactive oxygen species (ROS) in plant pathogen responses. In normal condidtion, ROS was generated in chloroplasts, peroxisomes and mitochondria. During the pathogen intection, pathogen PAMPs or effectors were recognized. Through NADPH oxidases or peroxidises, the plant generates ROS. ROS production can trigger pathogen response such as cell wall strengthening, cell death and defense gene activation. Plants have established many mechanisms to scavenge ROS. Adjust from Torres et.al. (2006)

Over expression of superoxide dismutases (SODs) in chloroplasts or mitochondria has been shown to reduce the oxidative damage effectively in transgenic tobacco (Bowler et al., 1991). Silencing of ascorbate peroxidase and catalase in transgenic tobacco can increase the HR response to pathogen infection (Mittler et al., 1999) (**Fig. 1.6**).

There are multiple functions of ROS in plant defense responses. First, ROS production is directly associated with the formation of defensive barriers. For example, in barley, ROS production is associated with the formation of defensive barriers during powdery mildew infection (Huckelhoven and Kogel, 2003). The second function of ROS is to trigger the programmed cell death (PCD) through the hypersensitive response (HR). H_2O_2 generated from the ROS burst can trigger PCD locally and act as a signal to activate the defence response in adjacent cells (Levine et al., 1994). Functional analysis using *Arabidopsis atrboh* mutants indicate that reducing ROS production will attenuate the HR response during the infection of avirulent pathogens (Torres et al., 2002). Third, ROS can also function as secondary messengers. Because of the potential of H_2O_2 to travel trough membrane, ROS can play important roles in rapid, long distance signalling during stress conditions (Miller et al., 2009). In addition, ROS can interact with salicylic acid (SA) to mediate the onset of systemic acquired resistance (SAR) (Durrant and Dong, 2004). Both the initial and secondary ROS bursts are required for the establishment of SAR (Alvarez et al., 1998) (**Fig. 1.6**).

Despite the fact that ROS are usually associated with successful pathogen responses, in certain circumstances ROS contribute negatively to pathogen resistance in plants. A strong ROS burst after pathogen recognition, can trigger HR to induce programmed cell death. For biotrophic and early stage hemi-biotrophic pathogens, cell death will halt their growth and spread effectively. However, for nectrophic pathogens which require host cell death to acquire nutrients and spread, strong host cell death can increase their pathogenesis. Some pathogens can even induce strong ROS burst in the host plant to facilitate their infection process (Govrin and Levine, 2000; Torres et al., 2006; van Kan, 2006). *Sclerotinia sclerotiorum*, a necrotrophic ascomycete fungus, secrets oxalic acid into the host to suppress the ROS burst at the

initial infection stage. After the initial establishment finished, the pathogen can induce strong ROS burst to induce cell death (Kim et al., 2008; Williams et al., 2011). *Botrytis cinerea* can manipulate the host antioxidant system to adjust ROS production for its own advantage (Kuzniak and Sklodowska, 2005). Indeed, *B. cinerea* carries two genes encoding NADPH oxidases that generate ROS during the infection process. Deletion of these two genes causes the nearly complete loss of pathogenesis (Segmuller et al., 2008). ROS play very important roles in both pathogen resistance and susceptibility which vary significantly between different types of pathogens.

1.4 Aims and goals

A significantly extended shelf life of purple high anthocyanin tomatoes was been observed post harvest and in fruit left on the vine. *Del/Ros1*fruits showed longer shelf life than the WT fruits. High anthocyanin fruit were associated with delayed ripening, resistance to opportunistic infection and, consequently, a considerably longer shelf life.

In this thesis, I start to investigate the mechanism underlying the extended shelf life of the anthocyanin-enriched, purple tomatoes. I addressed the following questions: how long and by what mechanism can anthocyanins delay over-ripening process of purple tomatoes? Does anthocyanin-enrichment enhance the pathogen resistance of tomato? If so, what is the mechanism of enhanced pathogen resistance? Can accumulation of anthocyanins provide a general target for extending tomato shelf life by conventional breeding? Do other flavonoid compounds have similar functions in extending tomato shelf life?

In chapter 3, I describe detailed storage tests to quantify the storage time of purple tomatoes compared to controls. I describe the correlation of anthocyanin production and delayed over-ripening in purple tomato. I also explain the role of antioxidants in tomato over-ripening and check the effects of increased antioxidant capacity on shelf life.

In chapter 4 I describe the pathogen resistance of high anthocyanin, purple tomatoes as another key factor in determining tomato shelf life. I investigate different ways of testing infection to measure the reduced susceptibility of purple tomato to *Botrytis cinerea*. I highlight the role of the ROS burst in *Botrytis* pathogenesis and how anthocyanins alter the dynamics of the ROS burst to reduce the susceptibility to this pathogen.

In chapter 5 I transfer the knowledge from the transgenic high anthocyanin, purple tomato to a purple tomato produced by conventional breeding. I show that accumulation of anthocyanins in tomato can be developed as a general strategy to extend tomato shelf life. Scientific findings from GM crop research can serve conventional breeding strategies.

In chapter 6 I extend my research from anthocyanins to other flavonoids and measure their effects on shelf life extension. By both transient silencing and stable mutants, I establish the roles of different flavonoids in extending tomato shelf life. I discuss the correlation between the structure of flavonoids and their ROS-scavenging ability, as well as their potential to reduce susceptibility to *Botrytis* tomato.

Chapter 2: General Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals used in this research were of molecular biology grade obtained from Invitrogen, Promega, Roche, Qiagen, Sigma, BioRad, Fluka and New England Biolabs. For HPLC, materials were obtained from Waters. Chemicals at HPLC grade were purchased from Fischer Scientific Ltd.

2.1.2 Antibodies

To select bacterial and transgenic plants, ampicillin, carbenicillin, chloramphenicol, gentamicin, kanamycin, rifampicin and streptomycin were used. The stock and working concentrations of each of the antibiotics used are shown in **Table 2.1**.

Table 2.1 Stock and Working Concentrations of Antibiotics

Antibiotic	**Solvent**	**Purpose**	**Stock Conc.**	**Working Conc.**
Ampicillin	H_2O	*E.coli* et.al. Selection	100 mg/mL	100 µg/mL
Chloramphenicol	Ethanol	Gateway cloning	34 mg/mL	34 µg/mL
Gentamicin	H_2O	Gateway cloning	100 mg/mL	100 µg/mL
Kanamycin	H_2O	Select transgenic plants and *E.coli*, *Agrobacterium*	100 mg/mL	100 µg/mL
Rifampicin	Ethanol	*Agrobacterium* Selection	50 mg/mL	50 µg/mL
Streptomycin	Ethanol	*B. cinerea* plate growth	100 mg/mL	100 µg/mL

2.1.3 Plant Materials

Tomato varieties MicroTom, MoneyMaker and Ailsa Craig are used as WT plants in different experiments. MicroTom was used for the *PRD* tomato transformation (see **section 5.2.4**). Sunblack (*Aft/Aft atv/atv*) tomato was provided by Prof. Pierdomenico Perata (Povero et al., 2010). Natural mutant of tomato, *aw* (LA3736) and *ae* (LA3612), in the Ailsa Craig genetic background were obtained from the Tomato Genetic Resource Centre (http://tgrc.ucdavis.edu/).

Transgenic tomato *E8:Del/Ros1* C, Y and N in MicroTom, *Del/Ros1* MoneyMaker, *AtMYB12* MicroTom, *AtMYB12* MoneyMaker and Indigo (*Del/Ros1* × *AtMYB12*) MicroTom were obtained from lab stocks (Butelli et al., 2008; Luo et al., 2008).

E8:Del/Ros1 N MicroTom was crossed with *aw* and *ae* mutants by Dr. Eugenio Butelli.

2.1.4 Bacterial Strains

For normal plasmid transformation, *E.coli* strain DH5α was used. For Gateway destination vector construction, *ccd* resistant *E.coli* strain DB3.1 was used. *Agrobacterium* strain GV3101 was used for transient VIGS in plants. For stable plant transformation, *Agrobacterium* strain AGL1 was used.

2.1.5 Plasmids

Vectors used and constructed in this thesis are listed in **Table 2.2**.

Table 2.2 Vectors Used in This Thesis

Name	Size (bp)	Purpose	Selective Antibiotics	Supplier/Reference
pGEM-T	3003	T/A cloning site	Ampicillin	Promega
pDONR207	5585	Gateway entry vector	Gentamicin, Chloramphenicol	Invitrogen
pTRV1	19370	VIGS	Kanamycin	Orzaez et al. 2009
pTRV2	9617	VIGS	Kanamycin	Orzaez et al. 2009
pTRV2-Del/Ros1	9899	VIGS	Kanamycin	Orzaez et al. 2009
pTRV2-GW	11326	VIGS, TRV2 followed by Gateway cassette	Kanamycin, Chloramphenicol	Orzaez et al. 2009
pTRV2-SlF3H	9938	VIGS, containing *SlF3H* fragment	Kanamycin	This thesis
pTRV2-SlDFR	9945	VIGS, containing *SlDFR* fragment	Kanamycin	This thesis
pTRV2-SlANS	9950	VIGS, containing *SlANS* fragment	Kanamycin	This thesis
PJAM1890	18441	Stable transformation (GW:Ros1/35S:Del)	Kanamycin, Chloramphenicol	Martin et al. 2012
pEF 1-PLI-4	3853	pDONR207 containing PLI promoter	Gentamicin	Estornell et al. 2009
PRD	18137	PLI:Ros1/35S:Del	Kanamycin	This thesis

2.1.6 Medium recipes

To grow bacterial, pathogen and transgenic plants, Luria Broth (LB), Murashige & Skoog (MS), Potato dextrose agar (PDA) and MEYYA mediums were used. Recipes for media used are shown in **Appendix 1**.

2.2 Methods

2.2.1 Primer design

Primer design was by using online software Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) or Primer 3 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) following several principles: the optimised length of primers should around 20-28 bp (except for primers for restriction and Gateway cloning). The Tm for primers should be around 60 ºC with GC content around 40-60%. Complementary sequences should be avoided in single primer or between forward and reverse primers. To stabilize the primers, the 3' of the primer were designed to terminate with G or C. For qPCR primers, the products were around 100-120 bp. Major genes and primers used in this thesis can be found in **Appendix 2**.

2.2.2 Polymerase chain reactions (PCR)

PCR reactions were undertaken by using G-Storm Thermal Cyclers (Kapa Biosystems). The reaction mixture was normally consisted of 10-20 ng of DNA template, 0.1 µM each of the forward and reverse primer, 100 µM of dNTPs, 1x concentration of *Taq* DNA polymerase and 1x concentration *Taq* buffer in a total volume of 15 µL. The standard PCR protocol was as follows: initial denaturation (4 min at 94°C), followed by 25-35 cycles of denaturation (45 seconds at 94°C), annealing (30 seconds at 60°C) and extension (60 seconds at 72°C), and final extension(5 minutes at 72°C).

For cloning PCR procedure, the protocol was similar to that described above except a tiny amount of bacterial colony was used to replace the template DNA

2.2.3 Purification of DNA from PCR reactions or agarose gels

To purify PCR products or restriction digestion products from 1% agarose gels, QIAquick Gel Purification Kit (Qiagen) was used following the supplier's instructions. To purify DNA from PCR reactions, a QIAquick PCR Purification Kit (Qiagen) was used following the supplier's instructions.

2.2.4 *E.coli* Competent Cell for Heat Shock Transformation

E.coli from a single colony was grown in 5 mL LB at 37 ℃, 200-220 rpm, over night. 1 mL of over night culture was added into 100 mL LB, 37 ℃, 200-220 rpm, and grown until OD_{600} ~ 0.35-0.4. The flask was then placed on ice to cool down the culture to 0℃. Chilled culture was transferred into pre-chilled 50 mL tubes. The culture was centrifuged at 4000 rpm, 4℃, for 10 mins. The supernatant was removed and the pellet was resuspend with 30 mL pre-cold 0.1M $CaCl_2$. The culture was incubated on ice for 30 mins and then centrifuged at 4000 rpm, 4℃, for another 10 mins. The supernatant was removed and the pellet was resuspend with 2 mL pre-cold 0.1M $CaCl_2$. Finally, 2mL 30% Glycerol was added into the culture (make final concentration of Glycerol to 15%).The culture was divided into 1.5 ml tubes (100-200 µL per tube). Competent *E.coli* cells could be kept at -80℃ for several months.

2.2.5 Heat shock method of *E.coli* transformation

Heat shock transformation was used to transfer competent *E.coli* cells with desired plasmids. *E.coli* competent cells from -80℃ were thawed on ice and 10-50 ng of the desired plasmids were added and mixed. Mixtures were kept on ice for 30 mins. Heat shock was given at 42℃ for 1 min. After heat shock, the tube was placed on ice to cool down. 900 µL LB medium was added and the mixture was incubated at 37℃ for one and a half hour. The cells were then pelleted by centrifugation at 8,000 rpm for 2 mins and resuspended in 100 µL of LB. These cells were divided into 20 µL and 40 µL portions and plated on LB agar with the desired antibiotics. The remaining 40 µL was kept at 4℃. The plates were incubated at 37˚C overnight. Colonies were checked and picked the next day.

2.2.6 Plasmid DNA isolation from *E. coli*

Plasmid DNA was isolated from 3-5 mL of *E.coli* cultures grown overnight under the appropriate antibiotic selection. Plasmid DNA isolation was done using QIAprep® Miniprep Kit (Qiagen) following the manufacturer's instructions. The principle strategies for this method are to lyse bacterial cells using alkaline lysis and to then absorb the DNA on silica in the presence of high salt (Vogelstein and Gillespie, 1979).

2.2.7 Quantification of DNA/RNA

The concentration of DNA and RNA were quantified by using a NanoDrop 2000C UV-Vis Spectrophotometer (Thermo) following the following the supplier's instructions.

2.2.8 Preparation of electrocompetent *Argobacterium* (GV3101) cells

Argobacterium (GV3101) cells from a single colony were inoculated in 10 mL LB supplement with 50 mg/L rifampicin at 28˚C overnight. 2 mL of overnight culture were inoculated into 100 mL LB at 28℃ until OD600~0.5-0.8 was reached. The culture was placed on ice to cool down and transferred into pre-cold 50 mL tubes. The tubes were centrifuged at 4000 rpm at 4℃ for 15min. The supernatant was removed and the pellet was resuspended with 50 mL of cold 10% glycerol. Centrifugation was repeated and the pellet was resuspended with 25 mL of cold 10% glycerol. Finally the culture was centrifuged at 4000 rpm, 4℃ for 15min and the pellet was resuspended in 2mL of cold 10% glycerol. The final culture was divided into 1.5 mL tubes (100 µL per tube) and kept at -80℃.

2.2.9 Electroporation of *Agrobacterium*

Competent *Agrobacterium* cells from -80 ℃ were thawed on ice and 200 ng of plasmid DNA were added. The mixture was kept on ice for one hour and transferred into a pre-cold electroporation cuvette. The cells were electrocuted using a BioRad Pulser (BioRad Laboratories) with the following settings: 400 Ω, 25 µFD and 2.5 kV. 1 mL liquid LB medium was added and the mixture was incubated at 28°C with

constant shaking (250 rpm) for 4 hours. The cells were then pelleted by centrifugation at 8,000 rpm for 2 min and resuspended in 100 µL of LB. These cells were divided into 20 µL and 40 µL portions and plated on LB agar with 50 mg/L rifampicin plus desired antibiotics. The remaining 40 µL were kept at 4℃. The plates were incubated at 28˚C. Colonies were checked after two days.

2.2.10 DNA isolation from plants

Young tomato leaves were harvested and placed into 1.5 mL tubes. Leaves were macerated with a sterilized plastic pestle. After grinding, 400 µL DNA extraction buffer (0.2M Tris-HCl pH8, 0.4M LiCl, 25mM EDTA and 1% SDS) was added and mixed by brief vortex. Samples were then centrifuged at 13000 rpm at RT for 5 mins. 300 µL of supernatant was transferred to a new tube and mixed with 300 µL isopropanol. After mixing, the tubes were centrifuged at 13000 rpm for 10 mins. Supernatant was removed and the pellet was washed with 500 µL 70% ethanol. The tubes were centrifuge at 13000 rpm for another 5 minutes. The supernatant was removed and the tube was air dried on the bench for another 5-10 mins. The pellet was finally eluted with 50 µL dH_2O and the DNA concentration by was measured using a NanoDrop spectrophotometer.

2.2.11 RNA isolation from tomato fruit

Tomato fruit RNA was isolated based on a modified Trizol extraction method. This method is based on the use of acid guanidinium thiocyanate-phenol-chloroform mixture to separate RNA/DNA (in the aqueous phase) from protein partitions (in the organic phase) (Chomczynski and Sacchi, 1987). Tomato seeds were removed and remaining fruit were homogenized under liquid nitrogen in a mortar. About 200mg fruit sample was added into one 2mL tube. 1.5 mL Trizol Reagent (TRI Reagent®, Sigma) was added and mixed thoroughly by vortexing vigorously. The sample was then incubated for 5 min at RT in dark while shaking. 150 µL of 1-bromo 3-chloropropane (BCP, Sigma) was added (BCP is used to help the separation of phenol from water). After mixing, samples were shaken vigorously for 15 seconds and incubated for another 10 min at RT while shaking. After incubation, samples were centrifuged for 10 min at 12,000 g at 4 ºC. The aqueous upper phase (~0.75

mL) was transferred to a fresh 1.5 mL eppendorf tube. 750 µL (or equal volume transferred in previous step) of Isopropanol was added. Samples were mixed well by turning or vortex at moderate speed for 10 seconds. The mixture was then incubated for 5 min at RT followed by centrifuging for 8 min at 12000 x g at 4 ºC. The supernatant was removed and the pellets were washed with 500 µL isopropanol. The samples were centrifuged for another 5 min at 7,500 x g at 4 ºC and supernatant was discarded. The pellets were washed with 1.0 mL 75 % ethanol. After centrifucation for 5 min at 7,500 x g at 4 ºC or at 12000 g, the pellets were washed again with 1.0 mL 75 % ethanol. The pellets were air dried at RT for 5 min and resuspended in 40 µL DEPC treated H_2O. After brief spin, the supernant (32 µL) was transferred to a new tube. 4 µL 10× DNAse1 buffer and 4 µL DNAse1 were added. The tube was incubated at RT for 45 min. The reaction was stopped by adding 4 µL 25mM EDTA and incubated at 65 ºC for 10min. RNA concrntration and quality were measured using a NanoDrop spectrophotometer.

2.2.12 First strand cDNA synthesis

First strand cDNA was synthesised using SuperScript™ III (Invitrogen). 1-2 µg of total RNA (add with DEPC-treated water to 18 µL), together with 1 µL primer mix (0.25 µg/uL oligodT (Progema) plus 0.25 µg/µL random primer (invitrogen)), 1 µL 10 mM dNTP were mixed and the reaction was incubated at 65°C for 5 mins and on ice for another 5 mins. 6 µL 5X first-strand reaction buffer, 2 µL DTT, 1 µL RNaseOUT (invitrogen) and 1 µL SuperScript™ III were added to the mixture and incubated for 50-60 min at 50°C. The reaction was terminated at 70°C for 15 min. The cDNA product was diluted to 10 ng/µg based on the initial amount of RNA.

2.2.13 Real time quantitative PCR (RT-qPCR)

RT-qPCR was done using SYBR® Green JumpStart™ *Taq* ReadyMix™ (Sigma). All RT-qPCRs were performed using an Opticon 2 Real Time PCR machine (MJ Research) using following protocol: 10 min at 95°C and then 40 cycles consisting of 20 sec at 95°C, 20 sec at 60°C and 20 sec at 72°C, followed by 10 min at 72°C. For tomato RT-qPCR, tomato *Ubiquitin 3* gene (Solyc01g056940) was used as reference. Details of primer sequences can be found in **Appendix 1**.

2.2.14 Gateway Cloning

PCR products amplified for Gateway cassettes were purified either by using a PCR purification kit or a gel purification kit. The PCR fragment with attB1 and attB2 cassettes were cloned into pDONOR-207 vector using BP clonase™ (Invitrogen) following the manufacturer's instructions. Recombination reactions were incubated at RT for 2 h or at 16°C overnight. The reaction was stopped by adding 1 μL of proteinase K and incubated at 37°C for 10 min. 5 μL of the BP reaction mix was used for DH5α transformation to make the entry vector.

The entry vector then could react with destination vector using LR clonase™ (Invitrogen) following the manufacturer's instructions. The reaction was stopped by adding 1 μL of proteinase K and incubating at 37°C for 10 min. 5 μL of the BP reaction mix was used for DH5α transformation to make the expression vector.

2.2.15 Tomato transformation

Tomato seeds were given a 70% EtOH treatment for 2 minutes to loosen gelatinous seed coats. EtOH was removed and the seeds were rinsed once with sterile water. Domestos/Vortex (10%) was added and the culture was shaken for 3 hours. The seeds were washed 4 times with water. About 20-30 seeds were put into tubs containing MS medium and left at 4°C until required. Seeds maybe left for up to 3 months at 4°C. After 3 weeks in the fridge, the seeds were germinated at the same time in the culture room (16 hour photoperiod, supplemented with Gro-Lux or incandescent light- especially important for regeneration). Seedlings were grown for 7-10 days. For transformation ideally cotyledons were young and still expanding, no true leaf formation was visible.

One day before transformation, inoculate 10mL of L medium containing the appropriate antibiotics was incoluated with the *Agl1* strain (50 μg/mL Rifampicin, and 100 μg/mL Spectinomycin). Cultures were grown under shaking at 28°C, over night. 1ml of fine tobacco suspension culture was aliquated on to plates containing the cell suspension medium solidified with 0.6% agarose or MS medium with

0.5mg/L 2,4-D, 0.6% agarose. Cell suspension culture was spread around to give an even layer to make the feeder plates. Place plates unsealed and stacked in the culture room in 1ow light.

On the day of transformation, a sterilized Whatman No.1 filter paper was placed on top of the feeder plates. Cotyledons were used because hypocotyls give rise to a high number of tetraploids. Cotyledons were cut under water and with a rolling action of a rounded scalpel blade to minimise damage to the tissue. In a petri dish, the tip of the cotyledon was cut off and two more transverse cuts were made to give two explants of about 0.5 cm long. The explants were transferred to a new petri dish of water to prevent any damage during further cutting..

The *Agrobacterium* culture was centrifuged and the pellet was resuspended in MS medium with 3% sucrose to an OD_{600} of 0.4-0.5. Bacterial suspension was placed in a petri dish and the explants were inmmersed. Explants were then removed from the culture and blotted on sterile filter paper. About 30-40 explants were placed on a feeder plate, abaxil surface uppermost (upside down). No particular period of time was required for immersion in the bacteria once the explants have been completely immersed. The plates were kept in the culture room with 1ow light.

After 48 hours' co-cultivation, the explants was removed from the feeder layers and put onto tomato regeneration plates containing Timentin at 320mg/L and Zeatin Riboside at 2mg/L and the appropriate antibiotic to select for the T-DNA transformation marker. Cotyledons were placed right side upwards so that they curled into the medium ensuring good contact between the cut edges of the leaf with the nutrients and antibiotics. Agargel was used as the setting agent produced a soft medium into which the pieces can be pushed gently. 12-16 pieces explants were placed per petri dish. Plates are left unsealed and returned to the culture room.

Explants were transferred to fresh medium every 2-3 weeks. Once regenerating material was too large for petri dish it was transferred into a larger screw capped glass jars, a petri dish lid was used to replace the plastic cap to allow better light penetration and better gas exchange.

Shoots were cut from the explants and put into rooting medium with reduced antibiotic concentrations, Timentin at 320mg/L and kanamycin at 50mg/L. If the explants did not root at first, they were recut and placed in fresh medium. If they still failed to produce roots they were probably escapes and were discarded. If using the kanamycin resistance gene as the selectable marker a simple ***npt II*** assay can be carried out to confirm the identity of true transformants.

To transfer to soil, the roots was gently washed under running water to remove as much of the medium as possible. Plants were carefully moved into hydrated, autoclaved Jiffy pots (peat pots) and the posts were enclosed to keep humidity high while in the growth room. Gradually the humidity was decreased. Once roots were seen to be growing through the Jiffy-pots, the plants can be moved to the glasshouse.

2.2.16 Trolox equivalent antioxidant capacity (TEAC) assay

The assay method was based on the ability of antioxidant molecules to quench the long-lived ABTS, Fluka)$^{\cdot+}$, a blue-green chromophore with characteristic absorption at 734 nm, compared with that of Trolox, a water-soluble vitamin E analog. The addition of antioxidants to the preformed radical cation reduces it to ABTS, determining a decolorization. A stable stock solution of ABTS$^{\cdot+}$ was produced by reacting a 7 mmol/L aqueous solution of ABTS with 2.45 mmol/L potassium persulfate (final concentration) in water and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. At the beginning of the analysis day, an ABTS$^{\cdot+}$ working solution was obtained by the dilution of the stock solution in ethanol to an absorbance of 0.70 ± 0.02 AU at 734 nm, verified by a Hewlett-Packard 8453 Diode Array spectrophotometer (HP, Waldbronn, Germany), and used as mobile phase in a flow-injection system, according to Pellegrini et al (Pellegrini et al., 2003).

5 mmol L^{-1} stock solution of Trolox was prepared in ethanol and stored at -20 °C for a maximum of 6 months. The stock solution of Trolox was diluted daily in ethanol at different concentrations (0-2 mmol/L) to obtain working solutions for building the

dose-response curve. After addition of 1.0 mL of diluted ABTS solution (A734nm 0.700±0.020) to 10 μL of antioxidant compounds or Trolox standards (final concentration 0–15 uM) in ethanol or PBS the absorbance reading was taken at 30°C exactly 1 min after initial mixing.

Wild type and transgenic tomatoes were washed, deprived of seeds and homogenized under liquid nitrogen in a mortar. One gram of the homogenized sample was extracted with 4 mL of water under agitation for 20 min at room temperature, centrifuged at 1000 x g for 10 min and the supernatant collected. The extraction was repeated with 2 mL of water and the two supernatants were combined. The pulp residue was reextracted by the addition of 4 mL of acetone under agitation for 20 min at room temperature, centrifuged at 1000 x g for 10 min and the supernatant collected. The extraction was repeated with 2 mL of acetone and the two supernatants were combined. Tomato extracts were immediately analyzed for their antioxidant capacity. Results were expressed as TEAC (Trolox equivalent antioxidant capacity) in mmol of Trolox per kg of fresh weight.

2.2.17 Statistics

Unless specifically described, paired or unpaired, two-tailed Student's t-tests were used to compare group differences throughout this thesis. p values less than 0.05 were considered significant.

Chapter 3: Enrichment of anthocyanins in tomato fruit delays processes late in ripening

3.1 Introduction

Previously, Eugenio Butelli produced tomato fruit with a high content of anthocyanins by transforming tomato (*Solanum Lycopersicum* var MicroTom) with genes encoding two transcription factors (Delila and Rosea 1) from snapdragon (*Antirrhinum majus*) that induce biosynthesis of anthocyanins (Butelli et al., 2008; Goodrich et al., 1992; Schwinn et al., 2006). Expression of *Delila* (*Del*) and *Rosea1* (*Ros1*), under the control of the fruit-specific E8 promoter, induced the expression of the genes required for anthocyanin biosynthesis to create intensely purple tomato fruit (Butelli et al., 2008).

While growing the purple tomatoes, we observed that they had improved shelf life compared to wild-type, red fruit. The shelf life of food is defined as the period during which a stored product remains suitable for consumption and is normally determined by the degree of softening, shrivelling and rotting of fruit. Two factors determine the shelf life: fruit softening late during ripening and pathogen susceptibility. Purple fruit from *Del/Ros1* tomato plants have normal size, shape, and number of seeds. However, purple fruit exhibit delayed ripening after breaker compared to red fruit. This was evident from the appearance of the purple fruit both on the vine and during postharvest storage and from a reduced level of fungal infection under either condition (**Fig 3.1A** and **B**).

Much work has been done to characterize the ripening process (from fertilisation to fully ripe) of tomato fruit (Klee and Giovannoni, 2011). However, there is limited research on over-ripening of tomato. A previous study indicated that during over-ripening, antioxidant levels decrease, free radical scavenging is reduced in activity and oxidative damage increases in tomatoes (Jimenez et al., 2002). One of the most significant features of the *Del/Ros1* tomato is its high antioxidant capacity due to the production of large amount of anthocyanins, which can act as antioxidants. I proposed the hypothesis that the accumulation of anthocyanins increased the antioxidant capacity of tomato fruit. Elevated antioxidant capacity in the fruit slows down the rise in oxidative damage, thus delaying the onset of over-ripening.

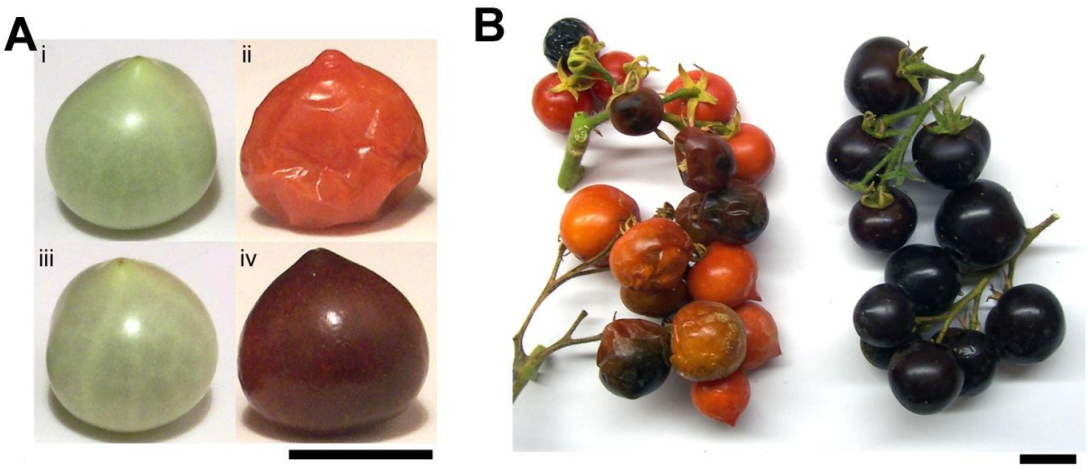

Figure 3.1 Accumulation of Anthocyanins in Tomato Fruit Delays Late Ripening and Decreases Pathogen Susceptibility.

(**A**) Wild-type, red (i and ii) and transgenic, purple (iii and iv) tomato fruits were tagged during the initial stages of development and harvested and photographed at the end of the green stage (i and iii). The same fruit, stored at room temperature, was re-photographed after 2 months (ii and iv). The scale bar represents 2 cm.

(**B**) Severe symptoms of opportunistic infection normally associated with over-ripe red, wild-type tomato fruit on the vine (left) were not observed in purple, *Del/Ros1* tomato fruit of the same age grown under identical greenhouse conditions (right). The scale bar represents 2 cm.

To test this hypothesis, I needed to investigate following questions: 1. How long can the anthocyanins of purple tomatoes extend shelf life? 2. Is the delay in over-ripening associated directly with the production of anthocyanins? 3. Is there any molecular evidence to support the apparent delay in over-ripening of purple tomatoes?

3.2 Materials and Methods

3.2.1 Storage tests

WT (red) and *Del/Ros1* (purple) MicroTom fruits were tagged at breaker stage and harvested at 14 days post breaker (d0=14dpb). All fruits were sterilized in 10% bleach for 10 minutes, followed by rinsing three times in sterilized water and air-drying. Ten fruits were placed in one sterilized glass jar and kept at 18°C in the dark. Every week, the total weight of 10 fruits was measured and proportion of fruits showing softening and tissue collapse was assessed. After each measurement, fruits were transferred to a new sterilized jar.

3.2.2 Texture analysis

Mechanical tests were carried out with a Stable Microsystems TaXT2 texture analyzer. A 0.5 mm diameter probe with a 45° conical tip was attached to the crosshead of the test machine. Skin penetration tests were carried out with a test speed of 0.1 mm/sec and a maximum penetration depth of 3 mm. Fruits were held in a small cup between two metal plates on the sample table.

The force-distance plots show typically two distinct regions. At the beginning there was an approximately linear increase in the force up to the bioyield point. At this point the skin was penetrated and the force reading suddenly dropped from its local maximum (fmax). After that, a second gradient was observed, which represents the penetration of the probe into the flesh.

The bioyield force (MPa) = $f_{max}/ (\pi R^2)$
R equals the radius of probe (0.25 mm)

To obtain the firmness of the fruit skin, the slope= $(f_{max}-f_0)/d_{max}$, (with: initial force (f_0), the bioyield force at the local maximum (fmax) and at distance (d_{max})) was divided by the area of the flat end of the probe tip.

The firmness of fruits (MPa/mm) was calculated as $(f_{max}-f_0)/(d_{max}\times \pi R^2)$

3.2.3 Ethylene measurements and treatment

Ethylene was measured from fruit harvested at or just before the breaker stage. Fruit were sealed in airtight tubes for 20 h after which a 1 mL sample of the headspace was taken and injected into a gas chromatograph (Shimadzu model GC-14B, Kyoto, Japan) equipped with a flame ionization detector. Samples were compared to a standard of known concentration and normalized for fruit mass. For ethylene treatment, ripe WT and *Del/Ros1* MicroTom tomato fruit were placed together in a sealed box. To consistently supply ethylene, ripe banana was put in the box. Fruits were checked after four weeks storage. The ethylene measurements were performed by Dr. Eugenio Butelli.

3.2.4 Scanning electron microscopy of tomato cuticle

Blocks of fruit pericarp tissue including peel were frozen in nitrogen slush at –190°C. Frozen samples were warmed to -100°C prior to fracture, and the specimens were then sputter-coated with platinum and examined using a Philips XL 30 FEG scanning electron microscope fitted with a cold stage. The scanning electron microscopy was done with the assistance of Prof. Cathie Martin.

3.2.5 Measurements of cuticle thickness

Cuticle thickness measurements (Yeats et al., 2012) involved slicing WT and purple fruit into 10-30 µm thick sections. Sections were stained with Sudan red and cuticle thickness was determined using a Leica DM6000 microscope, taking the average of 8-10 measurements. The average and standard error of the mean of three to five biological replicates were recorded.

3.2.6 Fourier Transform Infra-Red (FT-IR) spectroscopy of tomato peel

Tomato peel was obtained from ripe wild type and *Del/Ros1* tomato fruit, carefully removing any attached flesh material. The material was washed sequentially with 1% (w/v) SDS in 50 mMTris-HCL pH 7.2, water, 50% ethanol, acetone and then air dried at room temperature. FT-IR spectra were recorded on a BioRad FTS175C (BioRad, now Varian) spectrometer equipped with a MCT detector and a Golden Gate single-reflection diamond ATR sampling accessory (Specac). Both the outer and inner sides of the peel were measured. The dry samples were gently pressed onto the ATR crystal, with either the inside or outside in contact with the crystal. For each spectrum, 128 scans at 2 cm^{-1} resolution were averaged and referenced against the empty crystal. Data from FT-IR spectra were provided by Dr. Eugenio Butelli.

3.2.7 Virus Induced Gene Silencing of tomato

Virus-induced gene silencing (VIGS) is an excellent method for studying gene function by reverse genetics in tomato fruit (Orzaez et al., 2009). The principle of VIGS is to trigger the plant's defence response upon infection by tobacco rattle virus (TRV). Once the defence response has been triggered, the RNA-mediated silencing system can target the viral genome to specifically interfere with and degrade viral mRNA. By inserting target gene fragments from the host into the viral vector, the target host gene can be specifically silenced (Lu et al., 2003). A method for doing this effectively in fruit has been published by Orzaez et al., (2009).

The plasmids pTRV1 and pTRV2-*Del/Ros1* were provided by Dr. Diego Orzaez (Orzaez et al., 2009). Plasmids were transferred into *Agrobacterium* strain GV3101:pMP90.

Agroinfiltration was modified from the methods described previously (Orzaez et al., 2009; Orzaez et al., 2006). Two days before agroinfiltration, 10mL *Agrobacterium* culture was grown from single colonies at 28°C in LB medium supplemented with selective antibiotics. Overnight culture (1mL) was transferred into 100mL TY medium supplemented with selective antibiotics. The culture was grown overnight at 28°C.

On the day of agroinfiltration, the culture was centrifuged at 2000 rpm at 4°C for 10 min. Pellets were resuspended in 20mL infiltration medium (10 mM MES, 10 mM $MgCl_2$, 200 μM acetosyringone, pH 5.6) and incubated in the dark at RT with gentle shaking (20-30 rpm) for 2-3 hours. The OD600 was measured for each culture, and every culture was diluted to OD600=0.5.

To infiltrate the fruit, the pTRV1 culture and one pTRV2-derived culture were mixed in a 1:1 ratio. *Del/Ros1* MoneyMaker fruit at the mature green stage were selected. Fruit were labeled and injected with 0.5-1mL of bacterial mixture through the peduncule. Agroinfiltrated fruit were marked at the breaker stage and samples were collected at two weeks after breaker.

3.2.8 Microarray

Preparation of RNA for microarray analysis was performed by Dr Eugenio Butelli, and the arrays were hybridized in the laboratory of Prof. Antonio Granell.

VIGS-*Del/Ros1* fruit were tagged at the breaker stage. Total RNA was extracted from the red and purple sectors of *Del/Ros1* VIGS fruits, 8, 30 and 45 days after breaker following methods described previously (Bugos et al., 1995)

The TOM2 array was used to monitor changes in transcript levels. RNA amplification and aminoallyl labeling were performed by using the Message Amp™ aRNA kit (Ambion # AM1750). Microarray hybridisation, scanning and data analyses were performed as described previously (Lytovchenko et al., 2011): Telechem Hybridization Chambers (Corning) were employed for the manual hybridisation of labeled samples to the TOM2 long-oligo, 11,862 - gene, microarray. A GenePix 4000B scanner was used for scanning the microarray slides at 532 nm and 635 nm; with a resolution of 10 μm and 100% power. Images were quantified using GenePix Pro 4.1 image analysis software (Axon Instruments/Molecular Devices). Valid spots were defined as having intensity values ≥2-fold the mean background intensity in ≥1 channel.

Normalisation and calculation of differential expression were performed in R (http://cran.r-project.org) using Bioconductor libraries (http://www.bioconductor.org). Within-array normalisation was performed with the aim of making the background-subtracted log-ratios average to zero within each microarray. This was achieved by fitting a LOESS curve to each print tip (with the parameters smoothing filter = 0.4, iterations = 3; and $\delta = 0.01$). The resulting data were then subject to a between-array normalisation step where the average intensity values for each array were transformed such that they followed the same empirical distribution; while leaving the log-ratios unaffected. Differential expression was calculated by fitting a linear model for each gene across the microarrays (Smyth, 2004) (where the contrasts were parameterised as differential gene expression between purple and red sectors, at 8, 30 and 45 days post-breaker respectively); the estimated coefficients were, in turn, computed from the fit; followed by computation of moderated *t*-scores and log-odds by empirical Bayes shrinkage of the standard errors towards a common constant. Genes exhibiting a fold-change of ≥ 3 and Benjamini Hochberg-adjusted *p*-values ≤ 0.05 were selected for further scrutiny. Microarray data analysis was done by Dr. Andreas Magusin.

The GEO accession number for the TOM2 microarray data from this analysis is GSE46341 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46341).

3.2.9 Total Polygalacturonase (PG) and β-Galactosidase (β-Gal) Activity Measurements

The methods used to assay polygalacturonase and β-galactosidase were modified from those reported previously (Rodoni et al., 2010). Wild type and transgenic tomatoes were washed, deprived of seeds and homogenized in liquid nitrogen in a mortar. Three grams of the homogenized sample were extracted with 9mL of sodium acetate buffer (50 mM, pH 5.5, 1 M NaCl, 10 g/L polyvinyl-polypyrrolidone; PVPP) under agitation for 2h at 4°C, centrifuged at 1000 x g for 10 min and the supernatant collected. The supernatant was dialyzed (Spectrapor 8 kD cutoff tubing, Spectrum Laboratories Inc., Rancho Dominguez, CA) against sodium acetate buffer (50 mM, pH 5.0) overnight at 4°C, and the dialyzed samples were used to determine both

enzyme activities. All the steps during the extract preparation were carried out at 0-4°C.

Total PG activity was measured in a mixture containing 50 mM sodium acetate buffer pH 5.0, 0.15% (w/v) polygalacturonic acid, and 1 mL of enzymatic extract, in a total volume of 3mL. The mixture was incubated at 37°C, aliquots of 300 µL were taken at different times, mixed with 1mL Borate buffer pH9 and 200uL 1% 2-cyanoacetamide (Sigma-Aldrich, St. Louis,MO). The mixture was immersed in a boiling bath for 10 min, then cooled down to room temperature (RT) and the OD 276 nm was measured. Results were expressed as delta OD in 1s under the assay conditions per kilogram of fresh fruit.

For total β-Gal activity, the reaction mixture consisted of 0.5 mL of 0.1 M citrate (pH 4.0), 0.4 mL of 0.1% BSA, 0.1 mL of enzyme extract, and 0.5 mL of 10 mM *p*-nitrophenyl-β-galactoside (substrate). After 15 min at 37°C, the reactions were terminated by the addition of 2mL of 0.4 M sodium carbonate, and the liberated *p*-nitrophenol was measured at 420nm. Results were expressed as delta OD in 1s under the assay conditions per kilogram of fresh fruit.

3.2.10 Malondialdehyde (MDA) measurements

Tomato fruit seeds were removed, and 2.5 gram of fruits at the same stage of ripeness were ground in 10mL of 10 mM sodium phosphate buffer, pH 7.2 (adding acid-purified sand to help grinding). The extract was centrifuged at 2000 x *g* for 10 min and the supernatant was taken. An aliquot (100µL) was added to a 2mL tube containing 0.4mL of distilled water, 0.25 mL of 20% (w/w) trichloroacetic acid, and 0.5 mL of 10 mM thiobarbituric acid. A control was run for each sample in which thiobarbituric acid was replaced by an equal volume of distilled water. The mixture was heated in a boiling water bath for 30 min and then centrifuged for 10 min at 2000 x *g* to remove haziness. The cleared samples were allowed to equilibrate to room temperature before the absorption at 532 nm was measured. When the OD was being measured exactly 1000uL was put into the cuvette. The concentration of malondialdehyde (MDA) was calculated using its molar extinction coefficient of 156

mM^{-1} cm^{-1}. Results were presented as mmol MDA production per mg protein (mmol MDA per mg protein).

3.3 Results

3.3.1 Anthocyanin enrichment doubles the shelf life of tomato.

For each line, 40 fruits (four jars) were harvested and entered the storage tests. For the fresh weight loss, there was no significant difference between WT and *Del/Ros1* MicroTom fruit during the first five weeks of storage. After five weeks, however, the fresh weight of WT fruits decreased very fast and the decrease of *Del/Ros1 N* fresh weight was relatively slower. After 10 weeks of storage, there were significant differences in fresh weight between WT and *Del/Ros1* fruits (**Fig 3.2A**). After four weeks storage, all of the WT fruits had become completely collapsed and inconsumable while the purple fruits remained in good condition (**Fig 3.2B**).

Over-ripening was significantly delayed in *Del/Ros1* fruit compared to WT. For WT fruit, after five weeks' storage, nearly all the fruits showed over-ripening symptoms (visual softening and collapse on the fruit tissues). For the *Del/Ros1* MicroTom fruit, however, the over-ripening processes were significantly delayed: after five weeks' storage, only about 20% *Del/Ros1*fruits showed softening symptoms. By the end of ten weeks' storage, most of the *Del/Ros1* fruits showed over-ripening symptoms (**Fig 3.2C**). The time for *Del/Ros1* tomatoes to show a particular degree of over-ripening was twice as long as for WT MicroTom tomatoes to reach the same stage.

The skin strength of both WT and purple tomatoes during the storage tests was also measured. At harvest (14-16 dpb), the skin strength of WT and *Del/Ros1* MicroTom fruits were not very different. However, during storage, the skin strength of WT fruit, measured by texture analysis, went down quickly while the process in *Del/Ros1* tomatoes was much slower (**Fig 3.2D**).

The WT MicroTom tomato could be kept for about 35 days at 18°C in the dark before it became totally inconsumable. However, for the *Del/Ros1* tomatoes, the time needed to see a similar level of over-ripening was extended to 70 days. All measurements supported this conclusion. All data indicated that it requires twice as

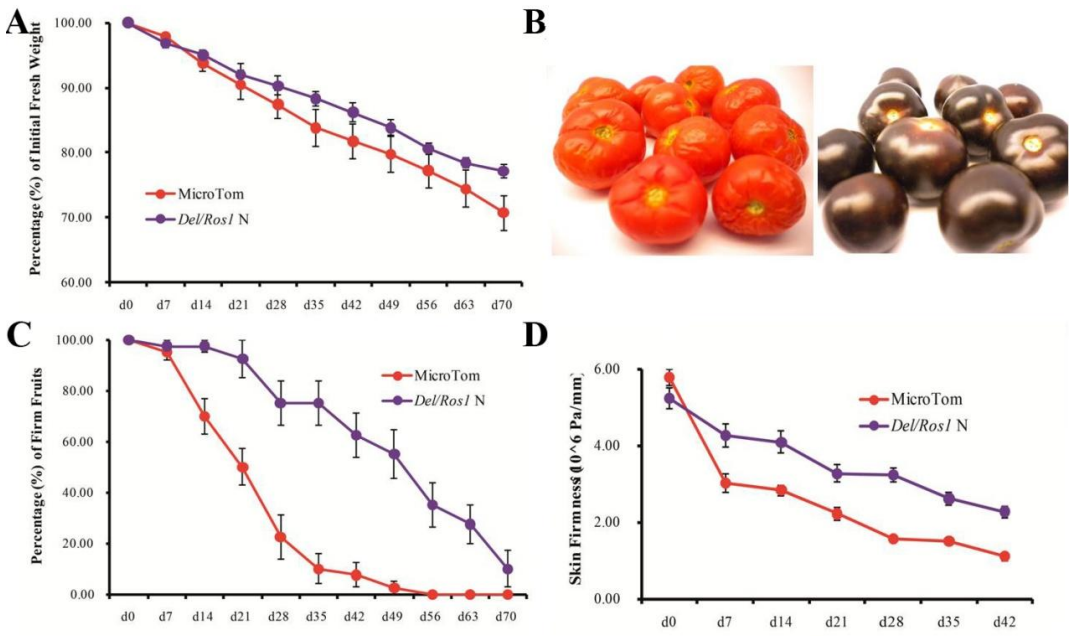

Figure 3.2 Purple tomato show delayed over-ripening.

(**A**) Fresh weight loss during the storage test for WT MicroTom and *Del/Ros1* fruit. The fresh weight of ten fruits in the same jar was calculated, Average values were obtained from four different groups of ten fruits, error bars indicate standard errors of the mean (n=4). Fruit were harvest at 14 days post breaker (d0=14 dpb).

(**B**) WT and *Del/Ros1* MicroTom fruits after four-week's storage.

(**C**) Percentage of WT and *Del/Ros1* MicroTom fruit showing over-ripening symptoms by visual observation during storage tests. Mean values were obtained from four different groups and error bars indicate standard errors of the mean (n=4).

(**D**) Texture changes in MicroTom and *Del/Ros1* fruit during the storage tests. Average values were calculated for at least eight individual fruits and error bars indicate the standard errors of the mean.

long for purple tomato to achieve a similar degree of over-ripening as WT tomato: the purple tomato has double the shelf life of WT tomatoes.

3.3.2 Ethylene production and signaling were not impaired in the *Del/Ros1* tomato

Production of ethylene, required for full ripening in climacteric fruit such as tomato, was transiently increased 2-fold in purple transgenic fruit compared to controls (**Fig 3.3A**), suggesting that the effects of elevated flavonoids on fruit ripening were not due to reduced ethylene production. In addition, application of ethylene to both WT and purple tomato induced over-ripening, indicating that ethylene signaling is not impaired in purple tomatoes. These results showed that the extended shelf life of purple tomatoes is due neither to impaired ethylene production nor impaired ethylene signaling.

3.3.3 Cuticle composition and thickness were not changed in *Del/Ros1* tomato

Measurement of cuticle thickness indicated no significant differences between WT and purple tomatoes (**Fig 3.3 B-D**). In addition, Fourier transform infrared (FT-IR) spectroscopy revealed there were no significant cell wall compositional changes between purple tomato peel and red tomato peel, one week after breaker (**Fig 3.3E**). These data implied that the extended shelf life of purple tomato was not due to changes in the cuticle or peel thickness and composition as a result of high anthocyanin production.

3.3.4 Delayed over-ripening was associated with anthocyanin accumulation

To determine the effects of anthocyanins on delaying over-ripening at the molecular level, virus induced gene silencing (VIGS) was used to silence the expression of *Del* and *Ros1* genes in purple MoneyMaker fruit. VIGS-*Del/Ros1* silenced fruit had red and purple sectors on the same fruit. In the red sectors, *Del* and *Ros1* expression was silenced and anthocyanin production was low. In old fruit, the red sectors were clearly softer than the purple sectors, indicating that not producing anthocyanins reduced the extended shelf life of purple fruit (**Fig. 3.4**).

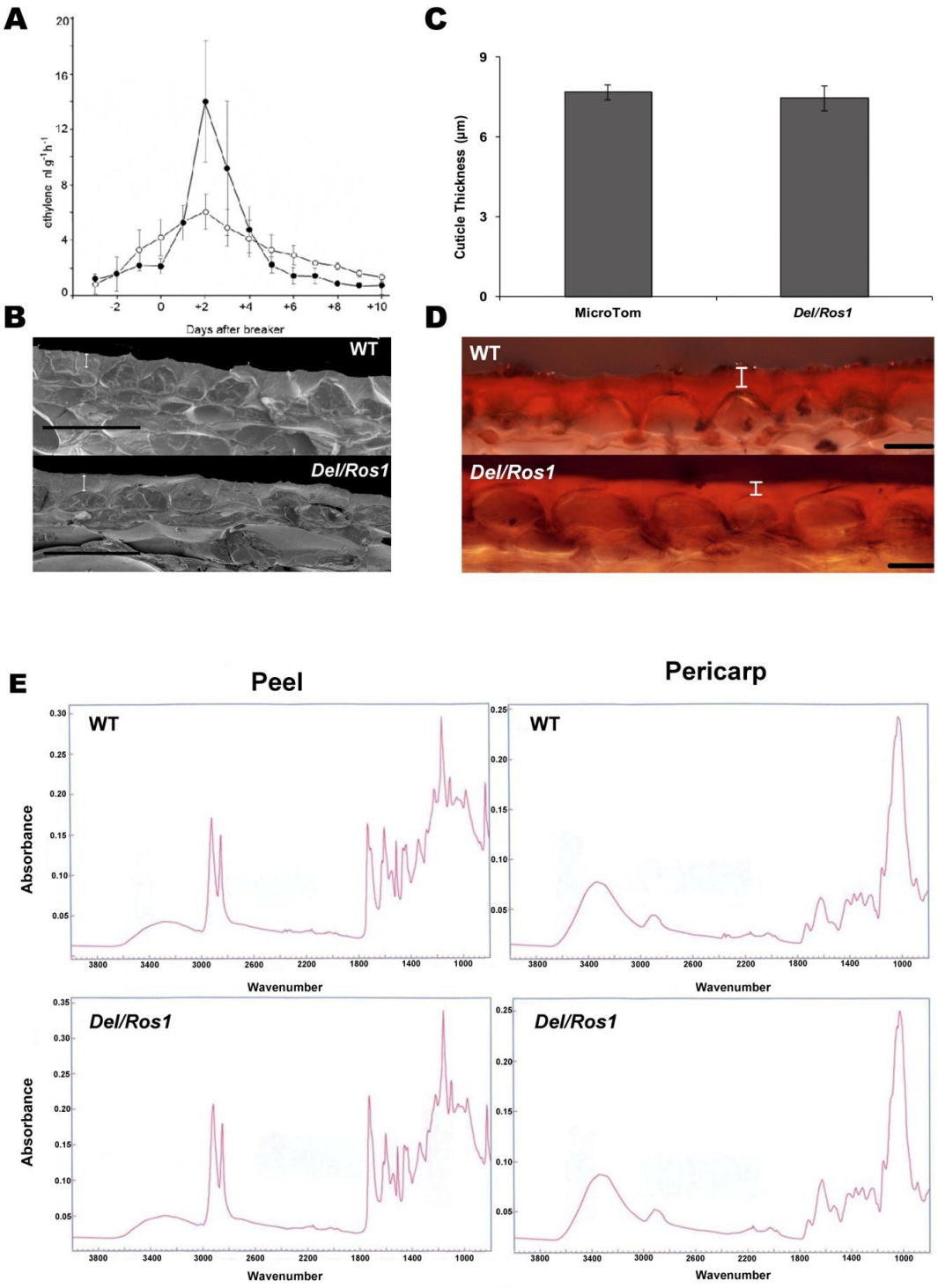

Figure 3.3 Extended shelf life in purple tomato is neither due to impaired ethylene production nor changes of cuticle structure.

(A) Ethylene production in red (○) and purple (●) tomato fruit during ripening. Data represent mean values ± se of at least ten individual fruits for each genotype.

(**B**) Freeze-fracture scanning electron microscopy (SEM) indicated no significant morphological changes to the thickness of the cuticle in the peel of purple fruit. Arrows indicate cuticle. Scale bars represent 50µm.

(**C**) Cuticle thickness of purple and red tomatoes. Measurements were made above the centre of each epidermal cell as indicated by bars in (**D**). Error bars show the standard error of the mean ($n \geq 3$ biological replicates, each replicates at least 8 single cells were counted).

(**D**) Light micrographs of sections of the fruit surface of tomato stained with Sudan Red. Bars indicate cuticle thickness. Scale bars show 20 µm.

(**E**) FT-IR (Fourier Transformed InfraRed) spectra of wild type (**upper panels**) and *Del/Ros1* (**lower panels**) tomato. The analysis of the peel (**left**) and pericarp (**right**) of the fruit is shown.

Figure 3.4 Delayed over-ripening in purple tomato was associated with anthocyanin accumulation.

VIGS-*Del/Ros1* tomato fruit were kept until 6 weeks after breaker. The red (low anthocyanin) sectors showed severe over-ripening symptoms while the purple (high anthocyanin content) sectors remained firm.

3.3.5 Microarray analysis indicates that the expression of ripening-related genes is suppressed in purple tomatoes

The expression profiles of purple and red sectors on the same VIGS-*Del/Ros1* fruit were compared at different time points late in ripening. RNA samples were isolated from purple and red sectors at 8, 30 and 45 days after breaker. 3-fold differences in expression levels (Purple vs Red) were set as standard for significant differences. 232 genes showed >3-fold difference in transcript levels between purple and red sectors over at least two stages (**Fig 3.5A** and **Appendix 3**). Functional annotation revealed many of these genes to be involved in primary and secondary metabolism, cell wall modification, oxidative stress and pathogen resistance (**Fig 3.5B** and **Appendix 3**). At each stage, excluding genes involved in phenylpropanoid pathway, there were always more genes that were suppressed in purple sectors compared to red sectors (**Fig 3.5C** and **Appendix 3**), indicating that the suppression of ripening-related genes in purple tomato might be a reason for the extended shelf life.

3.3.6 Ripening-related cell wall degradation genes were suppressed in purple tomato

To confirm the suppression of ripening-related genes in purple tomato, WT and *Del/Ros1* MicroTom fruit were sampled at different ripening stages to check the ripening related expression of enzymes known to be involved in over-ripening and the activities of these enzymes. Obvious suppression of transcript levels of genes encoding enzymes involved in cell wall degradation was found during all the ripening stages of purple tomatoes: In WT tomatoes, polygalacturonase (PG) gene expression (*SlPG2a*) reached its highest transcript level three days after breaker and then declined quickly. In purple tomatoes, however, there was less induction of *SlPG2a* during ripening (**Fig 3.6A**). A similar result was observed for the β-Galactosidase (TBG) gene (*SlTBG4*), another ripening related gene encoding a cell wall modification enzyme (**Fig 3.6B**).

Changes in the levels of total polygalacturonase and β-Galactosidase activities were measured during ripening at MG, breaker , B+3, B+7 and B+14 in WT and *Del/Ros1* fruit. Significant differences in enzyme activities were observed in *Del/Ros1* fruit pericarp compared to wild type. PG activity increased significantly in WT fruit

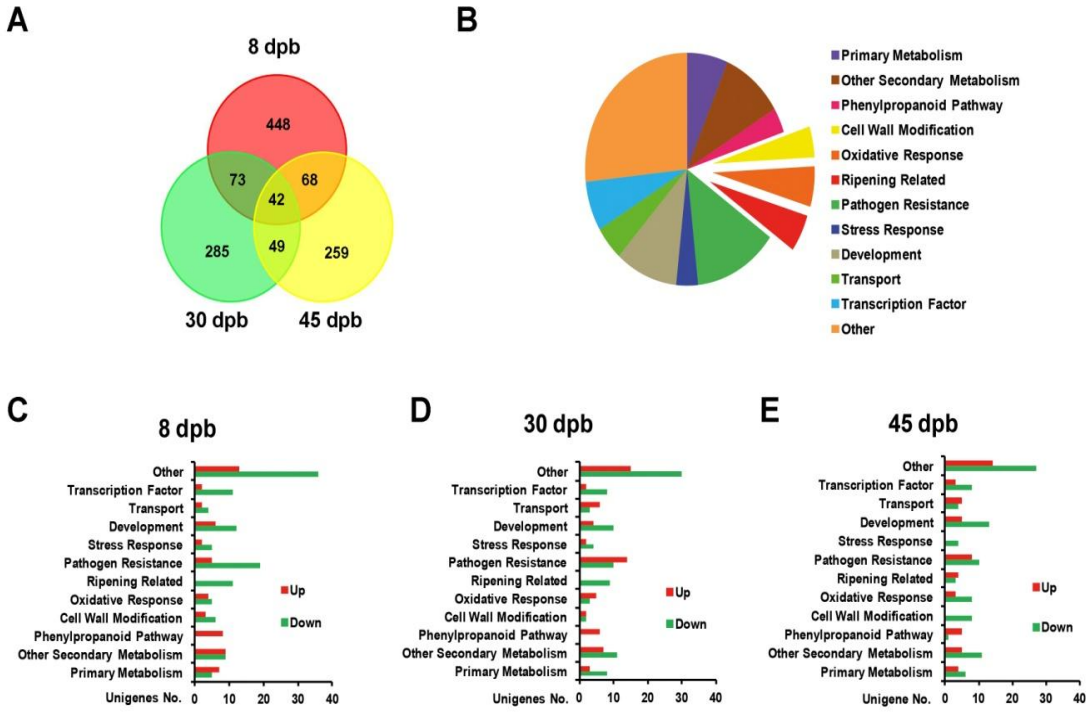

Figure 3.5 Late ripening-related processes are suppressed in anthocyanin-enriched tomato fruit

(**A**) Genes were selected that showed >3-fold differences in expression between the purple and red sectors of VIGS-*Del/Ros1* silenced fruit. 232 genes showed significant differences over at least two time points.

(**B**) Functional annotation of the 232 genes showing >3-fold differences in expression between purple and red sectors over at least two time points.

(**C**) Functional classification of selected differentially-expressed genes at 8 dpb, 30 dpb and 45 dpb.

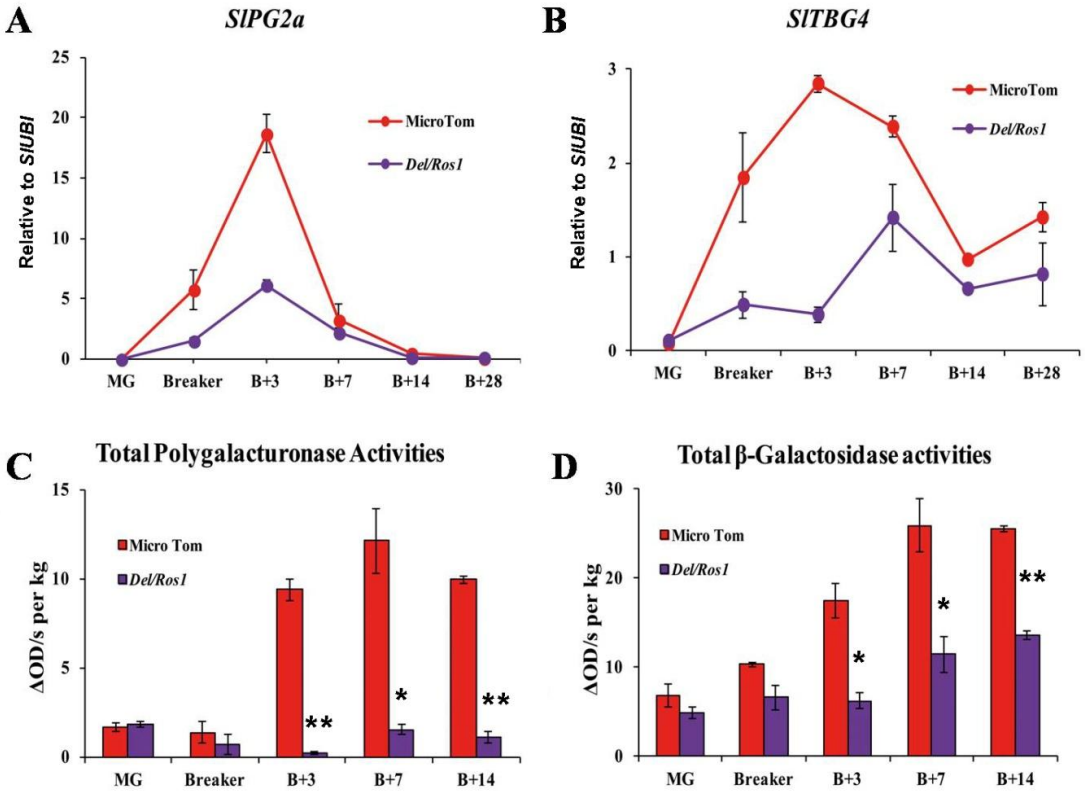

Figure 3.6 Cell wall degradation genes are suppressed in Del/Ros1 tomato.

(**A** and **B**) RT-qPCR analysis of genes encoding cell wall modifying enzymes in WT and *Del/Ros1* fruits during ripening. (**A**) polygalacturonase 2a (*SlPG2a*) and (**B**) β-galactosidase 4 (SlTBG4). Error bars show standard error of the mean (n = 3 biological replicates).

(**C** and **D**) Total polygalacturonase (**C**) and β-galactosidase (**D**) activities in red and purple fruit at different stages during ripening. Error bars show the standard error of the mean (n=3 biological replicates). * ($p<0.05$) and **($p<0.01$) compared to WT at the same stage.

shortly after breaker, whereas in *Del/Ros1* fruit, PG activity remained low up to two weeks after breaker (**Fig 3.6C**). For total TBG activity, induction after breaker was observed in WT fruit while in purple tomatoes the activity was lower (**Fig 3.6D**). These results indicate that ripening related processes are suppressed in purple tomatoes during over-ripening.

3.3.7 Oxidative damage was reduced in purple tomato during over-ripening.

During the early stages of ripening, antioxidant systems protect the fruit from oxidative damage. However, during late ripening, oxidative damage increases due to reduced activities of ROS scavenging enzymes (Mondal et al., 2004). In order to check the oxidative damage in the fruit, the content of malondialdehyde (MDA) in both purple and WT tomatoes at different stages was measured. MDA is a byproduct of lipid peroxidation and can be used to measure damage resulting from oxidative stress during tissue senescence (Dhindsa et al., 1981).

In WT tomato, MDA levels increased late in ripening. In purple tomatoes, however, MDA levels did not increase significantly up to 4 weeks after breaker (**Fig 3.7A**). Lower oxidative damage in purple tomato was associated closely with increased total antioxidant capacity during over-ripening, which resulted principally from the accumulation of anthocyanins (**Fig 3.7B**).

3.4 Discussion

3.4.1 Accumulation of anthocyanins in tomato fruit doubles shelf life

During the storage tests, because the fruit were sterilized and every operation was done in a flow cabinet, I effectively eliminated pathogen infection. Without pathogen infection, the only determining factor for shelf life is the rate of over-ripening. For the WT MicroTom tomato, it took about 21 days for 50% of the fruit to show over-ripening symptoms. For purple, *Del/Ros1* tomatoes, however, it took about 49 days to see a similar degree of over-ripening (**Fig. 3.2C**). Also, for the texture analysis experiment, I observed a reduction of about 50% in tissue strength for WT fruit after 14 days storage. For purple tomatoes, however, it took about 28-35 days to see a similar reduction in strength (**Fig. 3.2D**). All these data indicate that under the same, sterile conditions, when both purple and red fruit are harvested fully ripe (two weeks

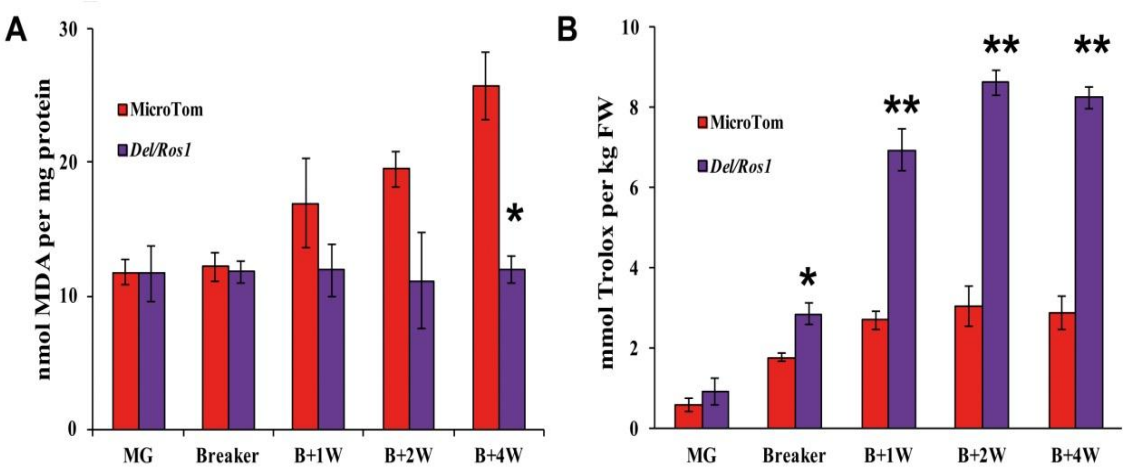

Figure 3.7 Oxidative damage is reduced in purple tomato compared to WT control tomato.

(**A**) Malondialdehyde levels in pericarp of red and purple Microtom fruit during ripening. Error bars show the SEM (n = 3). *p < 0.05 compared with WT, red fruit at same stage**.**

(**B**) Trolox equivalent total antioxidant capacity (TEAC) of water extracts from red and purple tomatoes during ripening. Error bars show the SEM (n = 3). *p < 0.05 and **p < 0.01 in comparison to WT, red fruit at the same stages.

after breaker), the purple tomatoes can be kept twice as long as the WT, red tomatoes.

Previously, several studies have been done to examine the extended shelf life of different tomato varieties (Mehta et al., 2002; Nambeesan et al., 2010). Compared with other studies, I undertook a more comprehensive analysis to the shelf life of *Del/Ros1* tomato: by doing all the operation under sterilized condition, I can better analysis the over-ripening process without the interference of pathogen infection; by labeling fruit at breaker stage, I can choose fruit at the same stage to do the experiment; by doing multiple measurements (fresh weight measurement, visual symptom inspection and texture analysis), I obtained consistent results showing the purple tomato doubles the shelf life.

In addition, compared to previous studies which can only improve shelf life for several days (Mehta et al., 2002; Nambeesan et al., 2010), our *Del/Ros1* tomato can significantly delay the over-ripening process by doubling the length of storage. Storage time is important for the tomato industry and extensions of just one day can have significant commercial impact.

3.4.2 Anthocyanin enrichment is the main reason for delayed over-ripening

The application of VIGS technology on *Del/Ros1* tomato provided direct evidence for the roles of anthocyanins in delaying over-ripening. By making chimeric fruit, I was able to compare anthocyanin-enriched and -deficient sectors under exactly the same conditions. On old VIGS-*Del/Ros1* fruit, significant differences in over-ripening were observed between red and purple sectors. The red sectors showed obvious symptoms of over-ripening whereas the purple sectors on the same fruit remained firm (**Fig. 3.4**). On the same fruit, the purple and red sectors were exactly the same age and developed under exactly the same conditions. The only difference between them was the anthocyanin content. Thus anthoycanin enrichment is the cause of the delayed over-ripening in the purple sectors.

The correlation of anthocyanin production and shelf life extension has been reported in other species. For instance, a previous study indicates gamma irradiation can induce anthocyanin production of grape and extend shelf life (Ayed et al., 1999).

Although anthocyanins have been reported to have multiple functions, their potential to extend shelf life has not been reported widely. This might be due to the scarcity of such high levels of anthocyanins in nature. There are crops which accumulate small amounts of anthocyanins. However, it is unusual for natural varieties to accumulate levels of anthocyanins as high as in *Del/Ros1* tomatoes. Anthocyanin accumulation in vegetative tissues has been found to slow normal growth rates. Over-expression of *Del* and *Ros1* in tobacco plants under 35S promoter causes accumulation of high levels of anthocyanins throughout the plant. Compared to WT tobacco plants, the growth of *Del/Ros1* tobacco is extremely slow (personal communication from Dr. Kalyani Kallam). In our *Del/Ros1* tomato, *Del* and *Ros1* are expressed only in fruit from breaker onwards, so there is no growth inhibition during the early growth phase. At the ripe stage, high levels of anthocyanins accumulate and impact shelf life significantly. Using these transgenic tomatoes, we discovered the role of anthocyanins in extending shelf life, which has not been reported for natural varieties of tomato. I want to check whether this finding can also be verified in natural varieties. This part of work is described in Chapter 5.

3.4.3 Role of ROS in tomato ripening and over-ripening process

The ripening and over-ripening of tomato fruit is complicated. It involves multiple chemical and morphological changes. Among all these changes, the roles of antioxidants and free radical scavenging systems have been widely studied. During ripening and over-ripening, the scavenging ability of the fruit antioxidant system declines (Jimenez et al., 2002). The production of reactive oxygen species (ROS) is important for plant development and stress responses. Also, because high levels of ROS are destructive for plant cells, it is important to maintain the balance between the generation and scavenging of ROS.

ROS play very important roles in fruit ripening and over-ripening. During the early expansion stages, the fruit undergo photosynthesis and respiration, as well as other physiological processes to meet their requirements for size expansion. During these processes, ROS are generated and accumulate. Once ROS accumulate to a certain level, together with other signals, they can trigger the initiation of ripening. The fruit stop expanding and proceed to ripening stages. During ripening and over-ripening,

because of the decreasing activity of antioxidant scavenging systems, ROS accumulation increases (Jimenez et al., 2002; Xu et al., 2012). The high levels of ROS then trigger further senescence activities.

In normal tomato fruit, in the early green stage, ROS generated from developmental activities can be effectively scavenged by their own antioxidant system. As fruit growth continues, more ROS are generated, breaking the balance between ROS generation and removal by antioxidants. Then the fruit stops expanding and triggers ripening. During ripening, the activity of antioxidant system begins to decline (Jimenez et al., 2002) and the rate of fruit respiration increases (Xu et al., 2012). More ROS accumulate and trigger further ripening-related changes including cell wall degradation and cell senescence. The balance between antioxidant activity and ROS generation is the key factor regulating tomato fruit ripening and over-ripening.

In *Del/Ros1* tomato, because of the E8 promoter, the expression of Del and Ros1is induced only at breaker. Before breaker, *Del/Ros1* fruit undergo similar development to WT fruit. Their ROS dynamics are very similar to WT fruit. After breaker, due to the accumulation of anthocyanins, the antioxidant capacity of *Del/Ros1* fruit increases significantly (**Fig. 3.7B**). As a result of the higher hydrophilic antioxidant capacity, the accumulation of ROS and oxidative damage is slowed (**Fig. 3.7A**), which slows the rate of late ripening.

To conclude, ROS play important roles in ripening and over-ripening. Alterations in ROS dynamics can delay ripening and over-ripening to extend shelf life (Zidenga et al., 2012). The accumulation of antioxidants during ripening provides a novel strategy to extend shelf life. To test this hypothesis, more experiments will be described in Chapter 6.

Chapter 4: Enrichment of anthocyanins in tomato fruit can reduce susceptibility to the fungal pathogen, *Botrytis cinerea*

4.1 Introduction

One important cause of tomato post-harvest losses is pathogen infection. During the processes of ripening and over-ripening, tomato fruit become more susceptible to

pathogen infection than early stage, unripe green fruit (Cantu et al., 2009; Prusky, 1996). Infection by pathogens of fruit during over-ripening can help to break down fruit and release mature seeds (Gillaspy et al., 1993).

One of the most important post harvest pathogens of tomato fruit is *Botrytis cinerea*, a necrotrophic fungal pathogen which can infect a wide range of host species. *B. cinerea* is found to be more destructive on mature or senescent tissues. Infection of *B. cinerea* on fruit causes gray mould symptoms. The pathogen enters plants during the early stages of fruit development and remains quiescent. Once environmental and physiological conditions change after ripening, the pathogen begins to germinate and cause infection. This usually happens during the post-harvest period of crop products. Consequently, *B. cinerea* is considered to be a post-harvest pathogen. (Williamson et al., 2007).

Infection by *B. cinerea* of vegetative tissues has been studied extensively, especially its interaction with model plant, *Arabidopsis thaliana*. Current studies indicate complicated cross-talk between different signalling pathways, to regulate *B. cinerea* resistance in *Arabidopsis*. In general, resistance to *B. cinerea* requires the induction of the jasmonic acid (JA) signalling pathway, whereas the salicylic acid (SA) signalling pathway negatively contributes to *B. cinerea* resistance (El Oirdi et al., 2011). SA signalling is accompanied by a ROS burst which induces the hypersensitive response (HR). HR activates programmed cell death. For biotrophic pathogens, host cell death will cut off the supply of nutrients and so halt the growth and spread of the pathogen. However, for nectrophic pathogens, host cell death can facilitate infection (Glazebrook, 2005). Previous studies indicate that application of ROS inhibitors can significantly reduce the susceptibility of *Arabidopsis* leaves to *B. cinerea*, whereas pre-treatment with ROS inducers increases susceptibility to this pathogen (Govrin and Levine, 2000).

Unlike *Arabidopsis* leaf infection, little is known about *B. cinerea* infection of tomato fruit. Previous studies indicate the susceptibility of tomato fruit to *B. cinerea* increases during ripening. At the green stage, tomato fruit show good resistance to *B. cinerea* infection. However, after breaker, the susceptibly to infection by this

pathogen increases significantly (Cantu et al., 2009; Cantu et al., 2008a; Cantu et al., 2008b).

One reason for the increased susceptibility during ripening is the changes to cell walls. As the primary defence barrier, the degradation of cell walls during ripening reduces their physical strength for defence (Cantu et al., 2008b). In addition, supporting evidence shows that the products from cell degradation are important resources for pathogen growth (Cantu et al., 2008a). *B. cinerea* can produce its own cell wall degrading enzymes during infection as virulence factors (Have et al., 1998). Inhibition of cell wall degrading enzyme activities is found to improve the resistance to this pathogen (Powell et al., 2000).

Another important factor in the susceptibility to infection is the changing of physiological conditions of fruit during ripening. Recent proteomics studies indicate that there is not much difference in the protein complement between green and ripe tomatoes infected with *B. cinerea* (Shah et al., 2012). The difference in susceptibility between green and ripe tomatoes is not due to the differential expression patterns of pathogen response genes, nor to the different virulence factors the pathogen uses. Indeed, the difference in responses to pathogens during ripening might be caused by the changes in the physiological condition of the host. During ripening, there are significant changes in physiological status of tomato fruit. Consequently, even though both the pathogen and host use similar strategies in infecting green and ripe fruit, susceptibility increases significantly in ripe fruit (Shah et al., 2012).

There are several ways to improve resistance of tomato to *B. cinerea*, post-harvest. For instance, storing of mature green or pink tomato fruit at 38°C for 3 days can effectively reduce *B. cinerea* infection. Heating can directly kill or reduce the activity of *B. cinerea* that has entered the fruit during early growth stages, and thus can significantly reduce infection during storage (Fallik et al., 1993). Different chemical treatments can also effectively reduce *B. cinerea* infection (Liu et al., 2007).

While growing *Del/Ros1* tomato in the greenhouse, it was noticed that, compared to red tomatoes, purple tomatoes showed much less opportunistic infection (**Fig. 3.1**). This indicated that *Del/Ros1* tomatoes might have lower susceptibility to post-harvest pathogens. In this section, I measured the susceptibility of purple tomatoes to pathogens using different approaches. I used *B. cinerea*, the major tomato postharvest pathogen, for these studies. Several questions were addressed: 1. Do purple tomato fruit have decreased susceptibility to *B. cinerea*? 2. Is reduced pathogen susceptibility associated directly with anthocyanin accumulation? 3. How do anthocyanins reduce susceptibility to *B. cinerea*?

4.2 Materials and Methods

4.2.1 *B. cinerea* growth and spore collection

B. cinerea (strain B05.10) was grown from a stock stored at -80 °C, on PDA plates (see **Appendix 1**). Plates were incubated at 20°C, with 12h light/12h dark. After one week. *B. cinerea* mycelium was cut from an initial PDA plate and plated into MEYAA Medium (see **Appendix 1**). This plate was kept for another 10-14 days at 20°C, with 12h light/12h dark until large amounts of spores could be seen under the microscope.

To collect the spores, 14 mL of 0.05% Tween 80 in sterile water was poured onto the culture on the MEYAA medium. The agar was scraped carefully with a bent and sealed glass Pasteur pipette, until all the spores were detached. Spores were transferred with a sterile 5 mL pipette to glass wool filters (made in plugged 5 mL pipette tips) placed in a 15 mL centrifuge tube, to collect the spores and retain the mycelium. The tubes with the tips were centrifuged for 5 min at 800 rpm/114 x g. The supernatant was discarded carefully, and the spores were washed and then resuspended in sterile water (10mL). An aliquot of the spore suspension was diluted 10 fold in an Eppendorf tube with water, to provide a sample for counting. Spores were counted using a haemocytometer (Thoma cell, depth 0.1 mm) under microscope. Spores were then centrifuged again for 5 min and resuspended at a final concentration of $2.5x10^7$/mL. These spores could be kept at 4 °C for up to two weeks. For long term storage, the culture was mixed with an equal volume of 30% glycerol (in ddH_2O) and stored at -80 °C.

4.2.2 *B. cinerea* infection

For spray inoculation, red and purple tomatoes were harvested 14 days after breaker and surface sterilized. Intact red and purple fruits were sprayed thoroughly with spores (2.5×10^5 spores/mL) three times in the fume cabinet and kept at 20°C, in high humidity. Infection symptoms were monitored at 3dpi, 4dpi and 5dpi.

For wound inoculation, the fungal culture was diluted with medium to 5×10^4 spores/mL (for MicroTom fruit) or 1×10^5 spores/mL (for MoneyMaker fruit) and inoculated for 1.5 h to stimulate germination. The spore innoculum (5µL) was added to each wound. Lesion diameter was measured 24, 48 and 72 hours after inoculation.

4.2.3 Quantification of *Botrytis* growth on tomato using qPCR

For infections in MicroTom, at 3 days post inoculation, a half fruit (containing the inoculation site) was harvested and seeds were removed. For innoculations of globe tomatoes in the MoneyMaker background, 1.5 cm of tissue around the inoculation site were excised). Harvested samples were kept in 2mL tubes and frozen in liquid N_2. Then, samples were freeze-dried over night. The next day small iron balls were put into each tube, samples were blended into a fine powder by a QIAGEN Tissuelyser LT (http://www.qiagen.com) (pre-chilled on dry ice). Total DNA was extracted from the finely ground samples using a QIAGEN DNAse Plant Mini Kit (http://www.qiagen.com) following the manufacturer's instructions. DNA concentrations were measured using a Nanodrop spectrophotometer and adjusted to a final DNA concentration of 10ng/uL. qPCR was undertaken using total DNA and the ratio of *B. cinerea Cutinase A* gDNA to tomato *ACTIN* gDNA was measured. The primers for the tomato actin gene and for the *B. cinerea Cutinase A* gene were:

SlACT-q-F (ACAACTTTCCAACAAGGGAAGAT),

SlACT-q-F (TGTATGTTGCTATTCAG GCTGTG),

BcCutA-q-F (ATTCCACAATATGGCATGAAATC)

BcCutA-q-R (ATGTTATCTCATGTTATCTC).

4.2.4 *In vitro Botrytis* growth test

Growth tests of *B. cinerea* were performed on PDA plates. PDA medium was made up with 50% red or purple tomato juice, prepared by homogenising whole fruit and then centrifugation. As a negative control, 15mg/mL Triademinol was added to PDA medium. Blocks of B05.10 mycelium (5mm diameter) were cut from a *B. cinerea* plate and placed in the centre of the test plates. Mycelial growth was measured daily. Streptomycin and ampicillin (100mg/L each) were added to all the plates to prevent infection by other fungi or bacteria, respectively.

4.2.5 3,3'-diaminobenzidine (DAB) staining for tomato

3,3'-diaminobenzidine (DAB) forms an insoluble polymer in the presence of H_20_2 and peroxidase activity. So H_20_2 can be localised in tissues by DAB staining (Thordal-Christensen et al., 1997). Staining solution was made by dissolving 5 mg/mL DAB-HCl (Sigma D-5637) in water (adjusted to pH 3.8 with 1 M KOH). DAB was infiltrated into excised fruit pericarp via the transpiration stream or by vacuum infiltration following immersion of cut petioles in DAB solution, three times, 1 min for each immersion. Infiltrated tissues were kept in DAB solution and incubated at RT in the dark overnight.

4.3 Results

4.3.1 Purple MicroTom tomato has lower susceptibility to *B. cinerea* than WT red tomato

When MicroTom fruit were wound-inoculated with *Botrytis* spores, the diameter of the lesions did not increase significantly one day post inoculation (1dpi) on either WT or purple fruit, suggesting that the fungus needs about 24 hours to germinate following inoculation. From 2 dpi, however, there was greater spread of infection in red fruit than in purple fruit. At 3 dpi, the average size of the lesions in purple tomatoes was significantly smaller than in red fruit, indicating reduced susceptibility to *B. cinerea* infection (**Fig. 4.1A**). qPCR was done by comparing total *Botrytis* DNA with total tomato DNA at 3dpi. For *Del/Ros1* MicroTom fruit, there was significantly less *Botrytis* growing on purple fruit compared to WT red fruit (**Fig. 4.1B**).

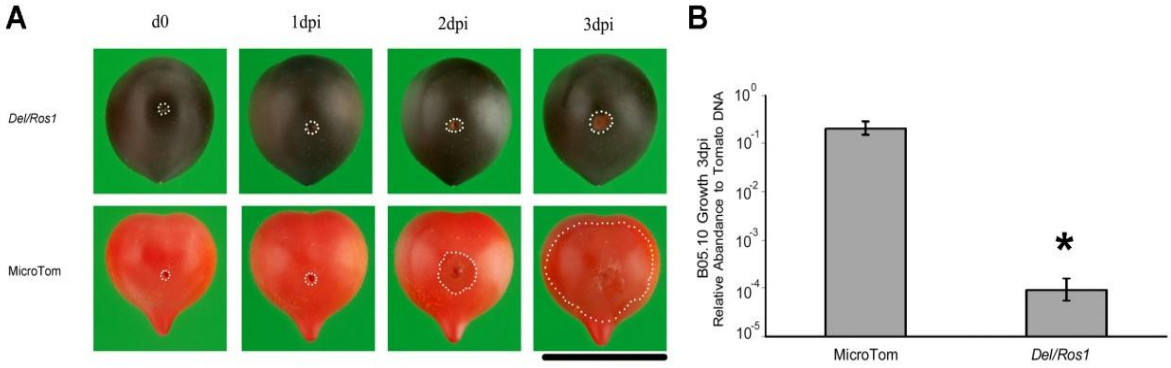

Figure 4.1 *Del/Ros1* MicroTom fruit have lower susceptibility than controls to *Botrytis cinerea*.

(A) *Botrytis*-infected purple tomatoes showed slower spreading of infection lesions compared to red, WT tomatoes. White dots lines indicate lesion margins. Scale bar indicates 2 cm.

(B) Quantitative PCR revealed more *Botrytis* growing on the WT tomatoes than on purple fruit, 3 dpi. *Botrytis* growth was calculated by comparison of the ratio of *Botrytis* DNA to tomato DNA. Error bars show the SEM (n = 3). * indicates $p < 0.05$ compared to control red tomato.

For the spraying tests, intact fruits were treated with *Botrytis cinerea* culture. Fruit were divided into three different categories, dependent on their symptoms: the **resistant** (R) group had no infection on the fruit; the **partially resistant** (PR) group

had lesions on the fruit surface but the lesions didn't spread later and the **susceptible** (S) group had spreading lesions (**Fig. 4.2A**). After five days, there were more resistant *Del/Ros1* fruit than WT MicroTom fruit and the percentage of partially resistant and susceptible fruits was lower for *Del/Ros1* fruit (**Fig 4.2B**) than for control fruit. Intact *Del/Ros1* fruit had lower susceptibility when sprayed with *Botrytis cinerea* culture. The purple tomatoes had lower opportunistic infection and the expansion of lesions was suppressed in purple fruit compared to controls.

All these data indicated that anthocyanin-enriched purple MicroTom tomatoes had lower susceptibility to *Botrytis cinerea* than WT red tomatoes.

4.3.2 Purple MoneyMaker fruit have lower susceptibility to *Botrytis cinerea* than WT MoneyMaker fruit.

Experiments on larger sized tomatoes using the Del/Ros1 trait introgressed into the Money Maker background also showed lower susceptibility of *Del/Ros1* fruit compared to red MoneyMaker tomatoes to *Botrytis cinerea* (**Fig. 4.3**). All these results indicated that the lower susceptibility of anthocyanin-enriched purple tomatoes to *Botrytis cinerea* is not specific to any one genetic background.

4.3.3 No ripening-related susceptibility to *Botrytis cinerea* was observed in *Del/Ros1* tomatoes

In order to see whether there were ripening-related changes in susceptibility to pathogens in purple tomatoes, WT and *Del/Ros1* MicroTom fruit were harvested at different time points (MG, breaker, B+1W, B+2W, B+4W and B+8W). Each fruit was sterilized, wounded and inoculated with *Botrytis cinerea* (B05.10) as described before. Lesion diameters were measured at 3dpi.

WT MicroTom fruit at the early stages (MG and Breaker) showed good resistance to *B. cinerea* as did purple, *Del/Ros1* fruit. However, one week after breaker, WT fruit increased in their susceptibility as they became fully ripe (**Fig. 4.4**). *Del/Ros1* fruits,

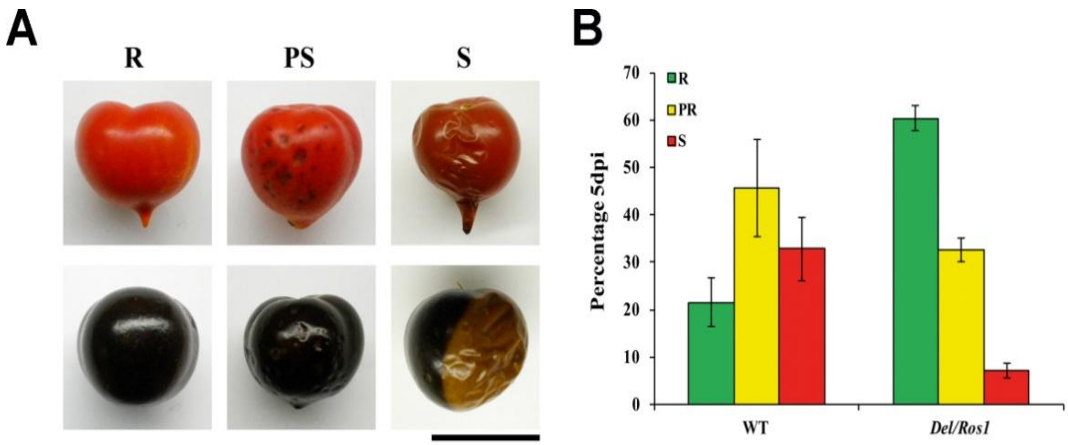

Figure 4.2 Purple MicroTom fruit have lower susceptibility to *Botrytis* infection in spraying tests.

(A) Phenotypes of fruits showing resistance (**R**), partial susceptibility (**PS**) and susceptibility (**S**) when sprayed with spores of *B. cinerea*. Pictures were taken at 5dpi. Scale bar represents 2 cm.

(**B**) Different degrees of susceptibility to *B. cinerea* shown by red and purple fruit in spraying tests. Fruit were checked 5 days after inoculation. Error bars represent the standard error of the mean for three independent assays.

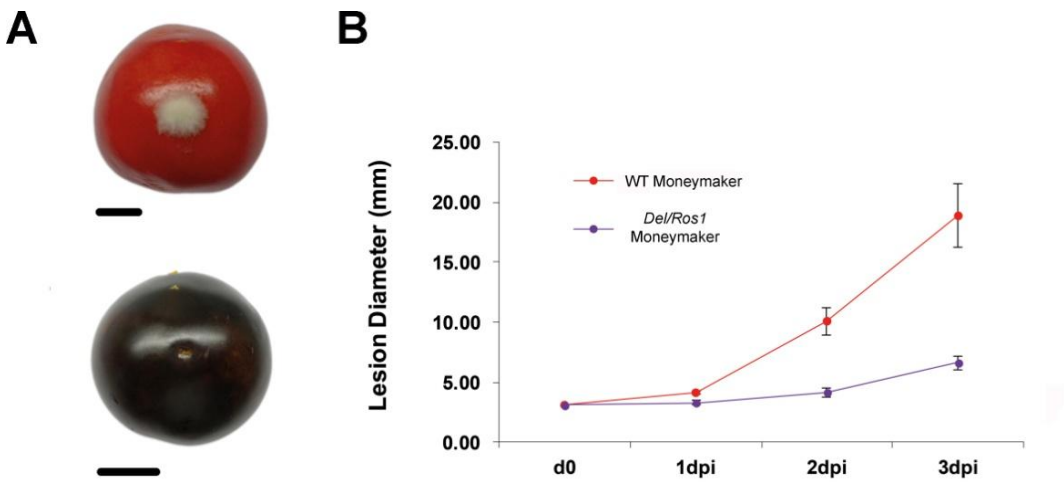

Figure 4.3 Accumulation of anthocyanins in the MoneyMaker genetic background also reduces pathogen susceptibility.

(**A**) Phenotype of WT and *Del/Ros1* MoneyMaker fruit after *Botrytis* wound inoculation. Picture was taken at 3dpi. Scale bars represent 2cm.

(**B**) Lesion development following *Botrytis* wound inoculation of red and purple fruits. Error bars indicate the standard error of the mean (n≥3).

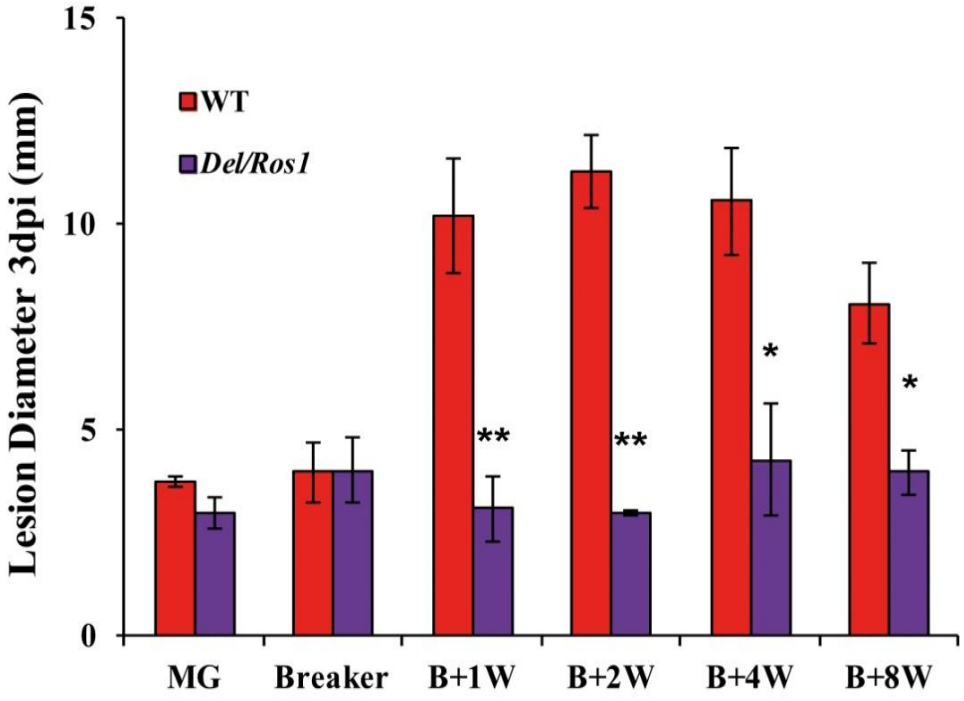

Figure 4.4. Ripening-related susceptibility to *Botrytis cinerea* in WT and *Del/Ros1* MicroTom fruit.

Lesion diameters were measured at 3dpi for tomatoes at different stages of fruit ripening. Error bars indicate the standard errors of mean ($n \geq 3$). * $p < 0.05$ and ** $p < 0.01$ for values for purple tomatoes compared to red tomatoes at the same stage

however, showed low susceptibility at all ripening stages. After 4 weeks, as the WT fruits decayed and most likely due to the loss of nutrients, the growth of *Botrytis cinerea* decreased a little (**Fig. 4.4**). These changes in ripening-related susceptibility to a nectrophic pathogen were not observed in *Del/Ros1* tomatoes. The differences in pathogen susceptibility between WT and *Del/Ros1* tomatoes started after breaker,

when anthocyanins began to accumulate in purple tomatoes. This suggests a direct role for anthocyanins in reducing susceptibility to pathogens in tomato fruit.

4.3.4 The reduced susceptibility to *Botrytis cinerea* is associated directly with accumulation of anthocyanins in purple tomatoes

In order to check whether the reduced susceptibility of purple tomatoes to *B. cinerea* is caused specifically by accumulation of anthocyanins in purple tomato, I repeated the inoculation tests on different *Del/Ros1* tomato lines. The *Del/Ros1* C and Y lines contained less anthocyanin than the N line (**Fig. 4.5A**), and also had lower antioxidant capacities (**Fig. 4.5B**) (Butelli et al., 2008). While lines C and Y showed lower susceptibility to *Botrytis cinerea* than WT tomatoes, their infection lesions were greater than those in *Del/Ros1* N line fruit (**Fig. 4.5C**). These results show that the strength of resistance to *Botrytis cinerea* is associated with the content of anthocyanins in tomato fruits.

I then used the VIGS-*Del/Ros1* fruit to test susceptibility to the pathogen by inoculating *Botrytis cinerea* spores on both purple, non-silenced (NS) and red, silenced (S) sectors. The red sectors showed increased susceptibility as the lesion diameter increased very fast (**Fig 4.6B**). The purple sectors, however, remained resistant to lesion development (**Fig. 4.6A**). As the accumulation of anthocyanins was inhibited in the red sectors of VIGS-*Del/Ros1* fruits, susceptibility to the pathogen was increased significantly.

All these data indicated that accumulation of anthocyanins in purple tomato is likely the direct cause of the reduced susceptibility to the pathogen.

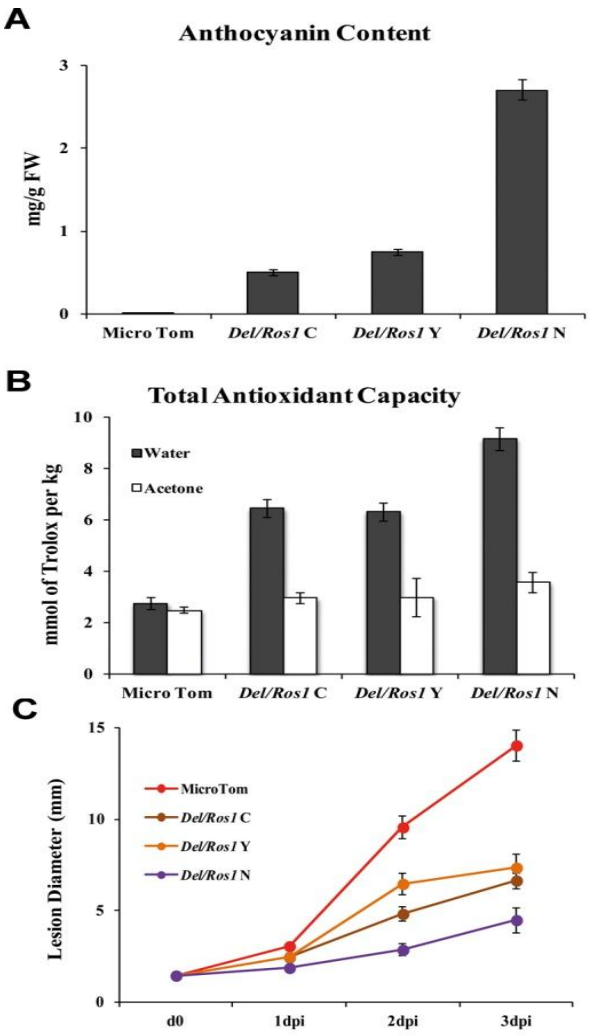

Figure 4.5 The strength of resistance to *B. cinerea* is associated with the levels of anthocyanins in fruits.

(**A**) Anthocyanin contents in different transgenic lines. Error bars represent the standard error of the mean (n=3).

(**B**) Trolox equivalent total antioxidant capacity of different transgenic lines. Error bars represent the standard error of the mean (n=3).

(**C**) Lesion development following *B. cinerea* inoculation. Error bars show the standard error of the mean (n=3)

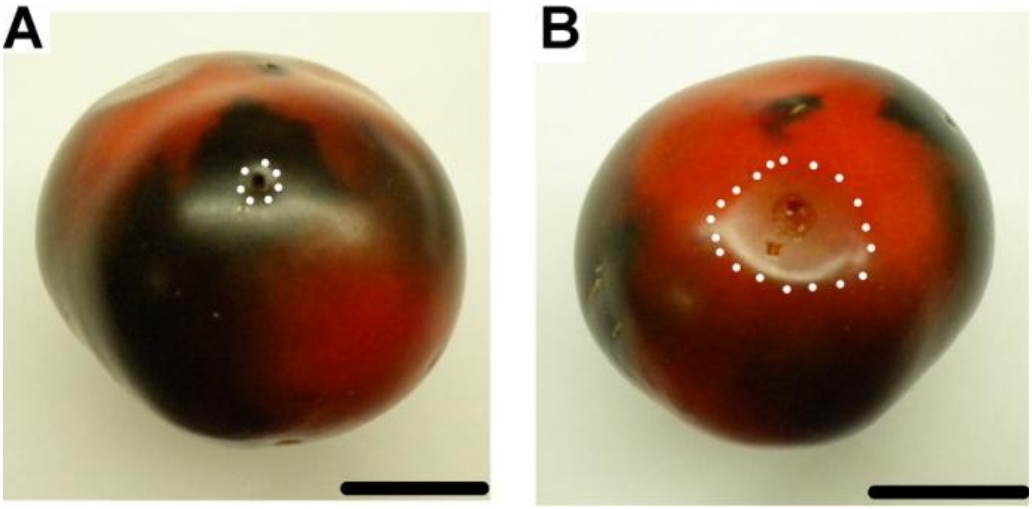

Figure 4.6 The reduced pathogen susceptibility to *Botrytis cinerea* is directly associated with anthocyanin accumulation in the fruit. Both high anthocyanin content, purple (**A**) and low anthocyanin content, red (**B**) sectors of the same VIGS-*Del/Ros1* fruit were inoculated with *Botrytis cinerea*. Pictures were taken at 3dpi. White dots lines showed lesion margin. Scale bars show 2cm.

4.3.5 Anthocyanins do not directly inhibit *Botrytis cinerea* growth *in vitro*

In order to investigate the mechanism of reduced pathogen susceptibility brought about by anthocyanin accumulation, I checked whether anthocyanins have direct inhibitory effects on growth of *B. cinerea*.

When *B. cinerea* was grown on standard PDA plates, the diameter of mycelium increased steadily and reached the margin of the plate after three days. When Triademinol, a *B. cinerea* inhibitor, was added to the PDA plate, there was no growth of *B. cinerea* mycelium after three days, at all. When *B. cinerea* was grown on PDA plates, supplemented with equal amounts of WT or purple tomato juice, the mycelium growth rate was similar to that on standard PDA plates (Fig. 4.7B). After three days of incubation, the mycelium size was similar in both WT and *Del/Ros1* plates (Fig. 4.7A). These data indicated that neither WT juice nor *Del/Ros1* juice can inhibit *B. cinerea* growth, directly, and consequently, anthocyanins do not inhibit the growth of *Botrytis* directly. The reduced susceptibility of high anthocyanin tomatoes to *B. cinerea* may require living cells.

4.3.6 ROS dynamics during pathogen infection were altered in purple tomatoes.

One of the most important traits for purple tomato is the high antioxidant capacity of the fruit because of the accumulation of antioxidant compounds—anthocyanins (Butelli et al., 2008). The reduced susceptibility to *B. cinerea* in purple tomato might be the result of the high scavenging ability of anthocyanins.

To test this hypothesis, 3,3'-Diaminobenzidine (DAB) staining was used to visualize ROS dynamics during infection. Previous data indicated that significant differences in infection patterns were seen in red and purple tomato between 24 to 48 hours post inoculation (Fig 4.1). DAB staining during this period might provide direct evidence of differences in ROS levels related to the final infection status of fruit.

For both WT and *Del/R*os1 MicroTom fruit, wounding without pathogen inoculation produced only small ROS bursts around the wound sites. For WT tomato, infection of *B. cinerea* generated a large ROS burst. The ROS burst was seen not only around the lesion, but also spread across the fruit tissue (**Fig. 4.8A**). This spreading ROS burst was associated with the rapid spread of the infected lesion (**Fig. 4.1A**).

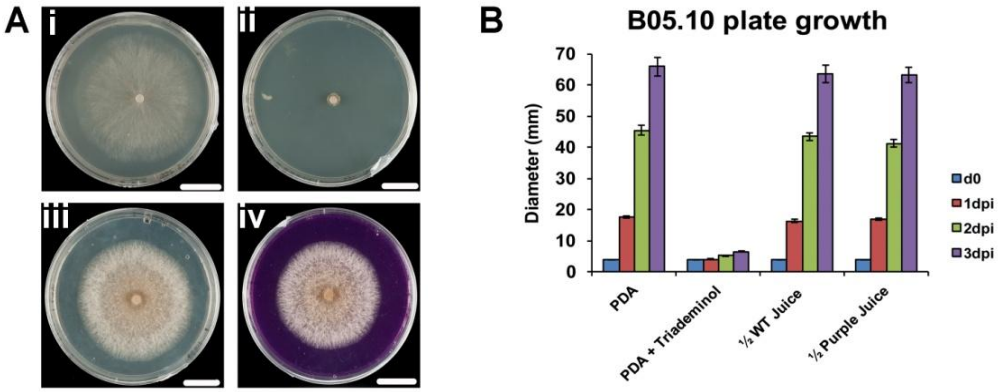

Figure 4.7 Anthocyanins do not inhibit *B. cinerea* growth *in vitro*.
(**A**) Addition of juice from either red or purple tomatoes to the growth medium had no effect on growth of *B. cinerea*. PDA medium (i) and PDA with 15mg/L Triademinol (an inhibitor of fungal growth) (ii) PDA supplemented with 50% red juice (iii) and with 50% purple juice (iv). Pictures were taken three days after plate inoculation. Scale bars, 2cm.

(**B**) Measurement of mycelium diameter during the growth test. Error bars show SEM (n=3).

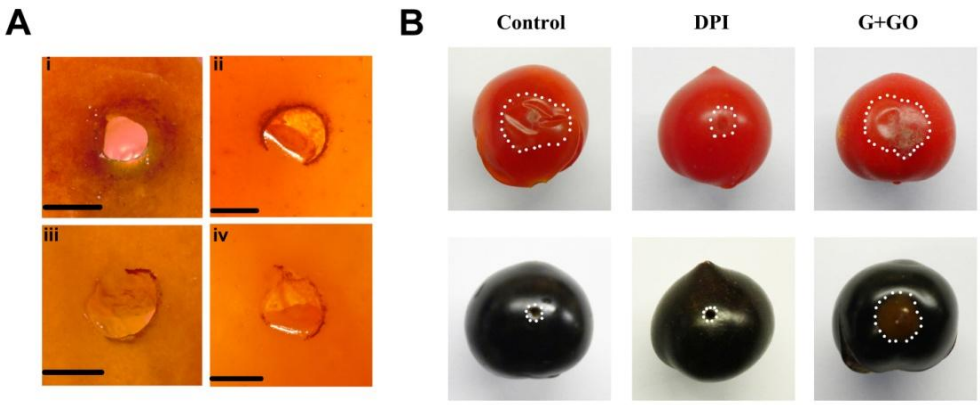

Figure 4.8 Anthocyanins inhibit spreading of *B. cinerea* on fruit by altering the dynamics of the ROS burst during infection.

(**A**) 3,3'-Diaminobenzidine (DAB) staining of hydrogen peroxide produced 24 h after inoculation of *B. cinerea*: red (i) and purple (ii) fruits stained with DAB, 24 h after inoculation, wound only red (iii) and purple (iv) fruit stained 24 h after wounding. Scale bars, 1mm.

(**B**) The levels of ROS in red and purple tomatoes were altered by infiltration of a water control, 10 mM diphenyleneiodonium chloride (DPI, ROS inhibitor) or 50 units/mL glucose oxidase plus 1% glucose (G+GO, ROS inducer). Fruits were wounded and infiltrated one hour prior to *B. cinerea* inoculation. Pictures were taken 3dpi. White dotted lines represent lesion margins.

For *Del/Ros1* tomatoes, although inoculation with *Botrytis* also generated a strong ROS burst around the infection site, the ROS burst failed to spread across the fruit (**Fig. 4.8A**). This limited ROS burst was matched by the limited infection area in purple fruit (**Fig. 4.1A**).

To characterize better the importance of the ROS burst to *B. cinerea* infection, both ROS inhibitors and inducers were introduced into the fruit prior to pathogen inoculation. Pre-infiltration of WT fruit with the ROS inhibitor, diphenyleneiodonium chloride (DPI), an NADPH oxidase inhibitor, can reduce the ROS burst. Compared to the water-treated control, DPI-treated WT fruit were less susceptible to *B. cinerea* than mock-infiltrated fruit (**Fig. 4.8B**). When *Del/Ros1* tomato fruit were pre-infiltrated with a ROS inducer, glucose oxidase plus glucose (G+GO), the fruit became susceptible to *B. cinerea* infection, compared to *Del/Ros1* fruit treated with water control. These data indicated that the ROS burst contributes positively to susceptibility to *B. cinerea* in ripe tomato fruit. Anthocyanins may inhibit the spreading of *B. cinerea* infections by altering the dynamics of the ROS burst during *B. cinerea* infection.

4.4 Discussion

4.4.1 Accumulation of anthocyanins in tomato fruit reduces the susceptibility to gray mould.

My data indicate a direct role of anthocyanins in reducing susceptibility to *Botrytis* in ripe tomato fruit. Previous data indicated that anthocyanin accumulation is associated with reduced susceptibility to *B. cinerea* in grape (Iriti et al., 2004). In my experiments, I observed that accumulation of anthocyanins in tomato fruit can significantly reduce susceptibility to *Botrytis cinerea*, in both wounding and spraying tests **(Fig. 4.1** and **4.2**). I also showed that the reduced pathogen susceptibility in anthocyanin-enriched fruit is not specific to particular genetic backgrounds. In both MicroTom and MoneyMaker, the *Del/Ros1* fruit were always less susceptible to *Botrytis* infection than WT fruit (**Fig. 4.3**). In addition, I demonstrated that the strength of resistance to *B. cinerea* is associated with the levels of anthocyanins in fruit (**Fig. 4.5**). Silencing of anthocyanin production directly enhances the pathogen susceptibility in *Del/Ros1* tomatoes (**Fig. 4.6**). These data, together with previous

results from research on grape, suggest that anthocyanins may offer protection to fruit from *Botryti*s infection.

There two main types of compounds which can contribute to pathogen resistance in plants. The first type includes compounds which can inhibit the growth of the pathogen directly. The other type includes compounds which can indirectly disrupt normal infection processes or enhance the resistance response. When high anthocyanin tomato juice was added to the medium to test growth of *Botrytis* in plate assays, there were no significant effects on the growth of the fungus (**Fig. 4.7**), indicating that anthocyanins do not inhibit *Botrytis* infection, directly. Anthoycanins might contribute to lower pathogen growth by indirectly affecting susceptibility infection.

4.4.2 The ROS burst contributes positively to the susceptibility of ripe tomato fruit to infection by *Botrytis*.

The dual functions of the ROS burst in plant defence are well established. For biotrophic pathogen infection, the ROS burst induces HR which can trigger programmed cell death (PCD). PCD can effectively cut off the supply of nutrients to the pathogen, and thus halt pathogen growth and spread. For necrotrophic pathogens, however, PCD can facilitate infection, as they require dead tissue to acquire nutrients (Glazebrook, 2005). *B. cinerea* is a typical necrotrophic fungal pathogen. Infection by *B. cinerea* of *Arabidopsis* leaves requires the HR to achieve pathogenesis (Govrin and Levine, 2000). *Botrytis* can even produce its own ROS inducer, to induce strong HR during the infection (Segmuller et al., 2008).

My data indicate that during the *B. cinerea* infection of WT, ripe tomato, a strong ROS burst is induced around the infection site. The ROS burst then spreads to trigger HR in adjacent cells (**Fig. 4.8A**). This matches the rapid spread of the pathogen on WT fruit (**Fig. 4.1A**). For *Del/Ros1* tomatoes, although a strong ROS burst is induced around the infection site, it fails to induce HR in the adjacent cells (**Fig 4.8A**) and the infection lesion does not spread quickly (**Fig 4.1A**). The ROS burst contributes negatively to *B. cinerea* resistance and, in *Del/Ros1* tomatoes, the ROS burst is effectively scavenged. The alteration of ROS dynamics during *B. cinerea*

infection is the main reason for the reduced pathogen susceptibility of *Del/Ros1* tomatoes.

During infection of tomato leaves, activities of enzymes in the peroxisomal antioxidant system are altered by *B. cinerea* infection (Kuzniak and Sklodowska, 2005). This indicates the potential role of antioxidant systems in response to *B. cinerea*. During fruit ripening, the activities of antioxidants decline. This leads to an accompanying decline in scavenging ability. Previous studies show that there is no significant difference in protein profiles between green and ripe fruit infected with *B. cinerea* (Kuzniak and Sklodowska, 2005). This indicates that the different susceptibilities of green and ripe tomatoes to *B. cinerea* are not due primarily to the differential expression of pathogen response genes.

Cell wall dynamics and antioxidant capacity of the fruit change significantly during ripening. In purple tomatoes, the activities of cell wall degrading enzymes are suppressed (**Fig. 3.6**). Also, the total antioxidant capacity is increased as a result of the accumulation of anthocyanins (**Fig. 3.7B**). Thus, ripening-related susceptibility is abolished in purple tomato (**Fig. 4.4**). When I altered the ROS dynamics in the WT and *Del/Ros1* tomato prior to pathogen infection, I observed significant changes to pathogen susceptibility (**Fig. 4.8B**). As the ROS inducer/inhibitor treatments are short, they are unlikely to cause significant changes in cell wall composition. However, these treatments altered the susceptibility of WT and *Del/Ros1* tomato to *B. cinerea* infection. These data indicate that it is the altered ROS dynamics in *Del/Ros1* tomatoes that is the main reason for the reduced susceptibility to *B. cinerea*.

Anthocyanins are antioxidants which can effectively scavenge ROS (Rice-Evans et al., 1996). Enrichment of anthocyanins in tomato fruit increases antioxidant capacity. During *B. cinerea* infection, although the pathogen can generate a strong ROS burst, there are sufficient anthocyanins to quench the ROS burst. By doing this, the purple tomato can increase its tolerance to the ROS burst and does not become more susceptible to *B. cinerea*.

Application of antioxidants has been proved to be effective in inhibiting *B. cinerea* infection in various crops (Elad, 1992). Here I show that enrichment of anthocyanins in tomato fruit can complement the losses of antioxidant capacity that occur during over-ripening. The high antioxidant capacities of anthocyanins can effectively scavenge the ROS burst during *B. cinerea* infection. Increasing tomato fruit scavenging ability by metabolic engineering can reduce susceptibility to pathogens.

Chapter 5: Accumulation of anthocyanins in the skin of tomato fruit is sufficient to extend shelf life

5.1 Introduction

Because of their health benefits, there is a growing interest in enriching vegetable and fruit crops in flavonoids. Among all crops, tomato is a good candidate for such enrichment as it is a very important commercial fruit all over the world (Al-sane et al., 2011; Gonzali et al., 2009).

In plants, there are two classes of genes that regulate flavonoid biosynthesis by inducing the expression of the structural genes, which encode the enzymes that are directly involved in flavonoid biosynthesis. The genes encoding these transcription factors (TFs) control the transcriptional activity of structural genes (Grotewold, 2006). In normal tomato, there is an intact set of structural genes for flavonoid biosynthesis, although their expression is very low, especially in tomato fruit (Gonzali et al., 2009).

During the past decade, there have been two main approaches to enrich the content of flavonoids in tomato fruit:

1) Transgenic approaches involving over-expression of either transcriptional or structural genes from other species (Bovy et al., 2002; Butelli et al., 2008; Muir et al., 2001), or
2) Conventional breeding to introduce different natural mutants that enhance flavonoid levels.

There are several 'mutations' in tomato that result in enhanced synthesis of anthocyanins in fruit or vegetative tissues (Al-sane et al., 2011). The tomato accession LA1996 which carries the dominant *Anthocyanin fruit* (*Aft*) gene, introgressed from *S. chilense*, expresses anthocyanins in fruit skin upon activation by high light (Jones et al., 2003). The tomato accession LA0797 carrying the recessive *atroviolacea (atv)* gene, introgressed from *S. cheesmaniae* (L. Riley) Fosberg, accumulates anthocyanins particularly in vegetative tissues (Mes et al., 2008). An *Aft/Aft atv/atv* line of tomato was obtained by crossing these two lines (LA1996 x LA0797) and shows intense pigmentation on tomato fruit skin (Mes et al., 2008; Povero et al., 2010). Like *Aft/Aft* fruit, *Aft/Aft atv/atv* fruit accumulate anthocyanins in a light-dependant manner (Mes et al., 2008).

Using genetically modified purple tomatoes, I found that anthocyanin enrichment in fruit can double the shelf life by delaying over-ripening and reducing susceptibility to *Botrytis cinerea*. In order to check whether this finding can serve as a strategy to create tomato fruit with longer shelf life, *Aft/Aft atv/atv* fruit were investigated to determine their shelf life.

5.2. Materials and Methods

5.2.1 Storage tests

Since near-isogenic lines for both *Aft/Aft* and *atv/atv* mutations were not available among the *S. lycopersicum* varieties, *S. lycopersicum* cv. Ailsa Craig as chosen as a control tomato line for all the analyses. This choice was made because, unlike the mutant lines selected for this study, Ailsa Craig does not produce anthocyanins in fruit skin, but it shows the same vegetative and fruit characteristics, such as the morphology of the plant and fruit, the size of mature tomatoes, and their ripening time (time from anthesis to the different ripening stages). All of these parameters are quite similar between Ailsa Craig and the mutant lines, creating conditions to perform valid comparisons (Povero et al., 2010).

WT (cv. Ailsa Craig) and *Aft/Aft atv/atv* fruits were labeled (when the color of WT fruit and the low-anthocyanin regions of *Aft/Aft atv/atv* fruit began to turn yellow) at breaker stage and harvested at seven days post breaker. In order to induce anthocyanin production in *Aft/Aft atv/atv*, extra light was supplied in the greenhouse at different angles. The resulting *Aft/Aft atv/atv* fruit accumulated anthocyanins all over their skin (**Fig. 5.1**). Fruit were sterilized in 10% bleach for 10 minutes, followed by rinsing at least three times in ddH_2O and air-drying in a flow cabinet. Each fruit was placed in a clean jar and kept at 17°C or at RT in semi-sterile conditions. Every week, the fresh weight of each fruit was measured and the proportion of fruit showing the symptoms of softening and collapse were assessed. All measurements were carried out in a flow cabinet to avoid contamination. After measurement, fruit were transferred to a clean, new jar.

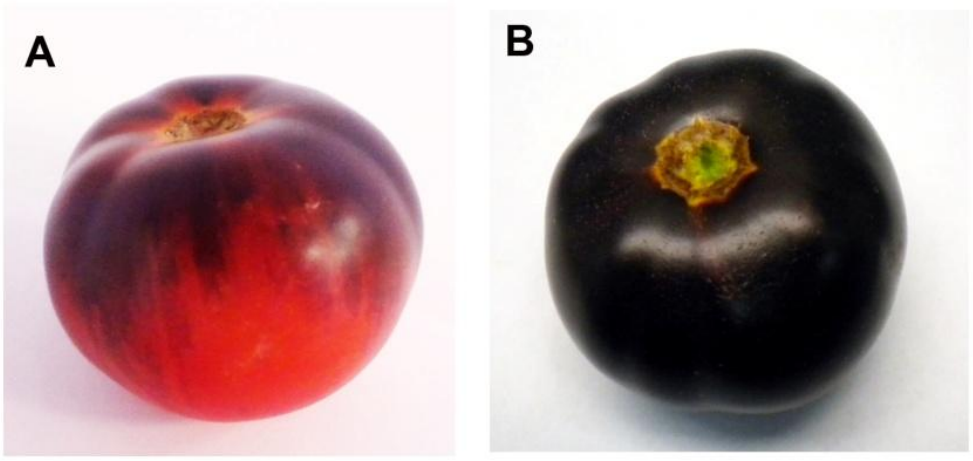

Figure 5.1 Supplementation with high light can induce stronger and more uniform anthocyanin production in the skin of *Aft/Aft atv/atv* fruit.

(A). *Aft/Aft atv/atv* fruit grown under natural light. (B) *Aft/Aft atv/atv* fruit grown with supplemental light. Pictures were taken at 7 dpb.

5.2.2 Measurements of cuticle thickness

Cuticle thickness was measured as described by Yeats et al. (2012) (see section **3.2.5**). Fruit were sampled at 7 days after breaker. WT Ailsa Craig (*aft/aft Atv/Atv*), red regions and purple regions of *Aft/Aft atv/atv* were sliced into 10-30 μm thick sections by VIBRATOM® 1000. Each section was then stained with Sudan red (Fluka) and cuticle thickness was determined by using a Leica DM6000 microscope, taking the average of 8-10 measurements. The average and SEM of at least three biological replicates were calculated.

5.2.3 *Botrytis cinerea* infection

B. cinerea (B05.10) was grown and collected as previously described (Stefanato *et al*., 2009) (see section **4.2.1**). Both WT Ailsa Craig and *Aft/Aft atv/atv* tomatoes were harvested at 14 days after breaker and surface sterilized. Intact wild type and *Aft/Aft atv/atv* fruits were sprayed thoroughly with spores (2.5×10^5 spores/mL) three times and kept at 20°C, in high humidity. Infection symptoms were observed at 4dpi.

For wound inoculations, fruit were wounded using sterilized 200 μL tips. B05.10 fungal culture was diluted with ¼ PDB to 5×10^4 spores/mL (for MicroTom background) or 2.5×10^5 spores/mL (for WT Ailsa Craig and *Aft/Aft atv/atv* fruits) and incubated at RT for 1.5 h prior to inoculation. For each wound site, 5 μL of spores were added. Lesion diameter was measured at 3dpi.

To quantify *Botrytis* growth using qPCR, tissues within 1 cm of the initial infection site were harvested three days after inoculation. Pericarp tissue was freeze dried and total DNA was isolated using a Qiagen DNeasy Plant Mini Kit following the protocol from the manufacturer. qPCR was performed as described in Chapter 4 (see section **4.2.3**).

5.2.4 Staining of seed for proanthocyanidins

Tomato seed were stained for proanthocyanidins using 4-dimethylaminocinnamaldehyde (DMACA) as described by Abeynayake et al. (2011). The presence of proanthocyanidins and flavan-3-ols in seeds were stained using 0.01% (w/v) 4-dimethylaminocinnamaldehyde (DMACA) in absolute ethanol (0.8%

w/v HCl). Seeds were stained for 2 hrs and then transferred to 100% ethanol and incubated at 4°C over night. Pictures of stained seeds were taken the next day.

5.2.5 Plasmid construction and tomato transformation

The promoter of the *PLI* gene (*pPLI*) contained in pEF 1-PLI-4 and was kindly provided by Dr. Diego Orzaez (Estornell et al., 2009). Using Gateway cloning, the PLI promoter was first recombined into the pDONR 207 vector to create pENTR-pPLI. The pPLI fragment was then inserted into a binary vector pJAM1890 (GATEWAY:Ros1/35S:Del) (Martin et al., 2012) using the LR recombination reaction to make pPLI:Ros1/35S:Del (PRD).

The PRD plasmid was transferred into *Agrobacterium tumefaciens* strain AGL1 by triparental mating. Tomato variety MicroTom was transformed by cotyledon-dipping (Fillatti et al., 1987) (see **Section 2.2.15**).

5.3. Results

5.3.1 *Aft/Aft atv/atv* tomato can be stored longer

Storage tests at 17°C indicated that, compared to WT Ailsa Craig fruit, *Aft/Aft atv/atv* fruit showed delayed over-ripening. Compared to WT fruit, the fresh weight reduction of *Aft/Aft atv/atv* fruit during storage was slower (**Fig. 5.2A**). In addition, for WT Ailsa Craig fruit, 42 days of storage was required to see 50% of the fruit showing over-ripening symptoms (visual rotting and collapse on the fruit surface). For *Aft/Aft atv/atv* fruit, however, 65 days were required to see a similar degree of over-ripening (**Fig. 5.2B**).

Storage tests were repeated at room temperature (RT) and showed similar results: *Aft/Aft atv/atv* fruit showed slower over-ripening and fresh weight reduction than WT Ailsa Craig fruit (**Fig. 5.2 C** and **D**).

During the RT storage test, at 42 days, the seed in Ailsa Craig fruits showed viviparous germination while *Aft/Aft atv/atv* tomatoes did not (**Fig. 5.3A**). The absence of precocious germination in the *Aft/Aft atv/atv* tomatoes was probably due to the elevated anthocyanin levels in the seed of *Aft/Aft atv/atv* plants (**Fig. 5.3B**).

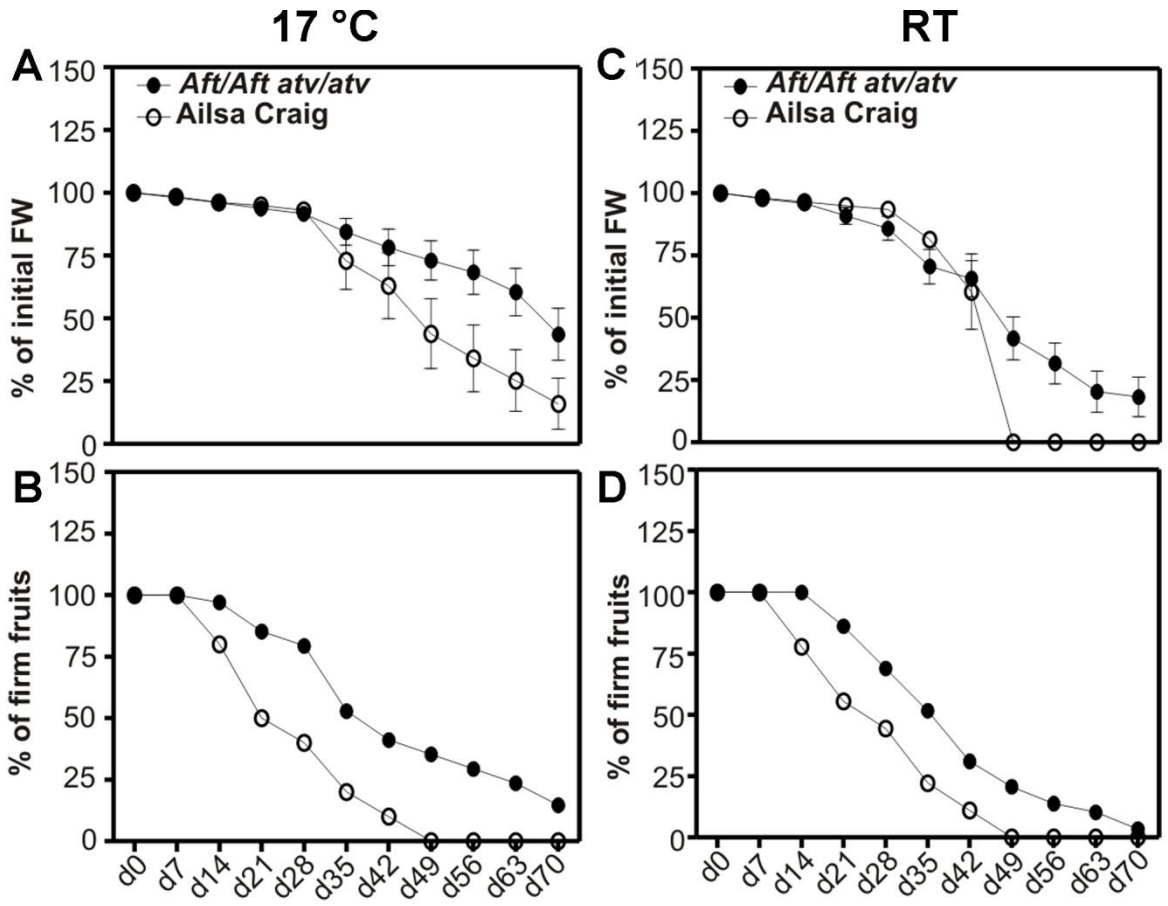

Figure 5.2 Accumulation of anthocyanins in *Aft/Aft atv/atv* tomatoes delays late ripening.

WT Ailsa Craig and *Aft/Aft atv/atv* tomato fruits were stored at 17°C (**A** and **B**) or at room temperature (**C** and **D**). Fruits were harvested at 7 days post breaker (d0=7dpb). (**A** and **C**) Fresh weight was represented as the ratio to the initial weight. Error bars show the standard error of the mean (n≥8). (**B** and **D**) The percentages of fruit showing over ripening symptoms (softening and shriveling) were assessed every week during storage tests.

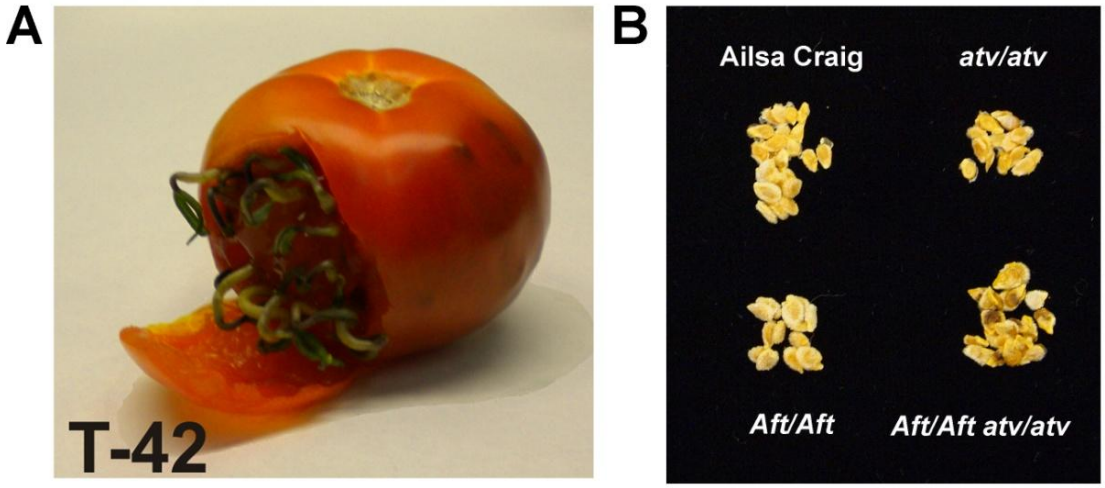

Figure 5.3 *Aft/Aft atv/atv* seeds accumulate anthocyanins to prevent viviparous germination

(**A**) Seeds of WT Ailsa Craig fruit showed viviparous germination during the storage test. Picture was taken at 42 days after storage started.

(**B**) *Aft/Aft atv/atv* seeds accumulate anthocyanins.

On the same *Aft/Aft atv/atv* fruit grown under natural light condition, I also noticed more rapid over-ripening of red regions than purple regions (**Fig. 5.4A**). Previous data had indicated that enrichment of tomatoes with anthcoyanins increases the total antioxidant capacity of fruit and delays over-ripening. Measurement of the total antioxidant capacity of different sectors on the same fruit of *Aft/Aft atv/atv* tomatoes indicated that anthocyanin enrichment increased the antioxidant capacity of the fruit tissues containing anthocyanins (**Fig. 5.4B**). Cuticle thickness is thought to affect the shelf life of tomato (Yeats et al., 2012). Measurement of cuticle thickness of both WT and *Aft/Aft atv/atv* fruit showed there was no significant difference in the thickness of the cuticle between red and purple regions (**Fig. 5.3C**). Taking all these results together, accumulation of anthocyanins in skin of *Aft/Aft atv/atv* tomatoes appears to delay their over-ripening. The delayed over-ripening was not due to the altered cuticle thickness of *Aft/Aft atv/atv* fruit.

5.3.2 *Aft/Aft atv/atv* fruit have lower susceptibility to *B. cinerea*

The susceptibility of *Aft/Aft atv/atv* and WT Ailsa Craig fruit to postharvest pathogen infection was investigated by infecting wounded or intact fruit with *B. cinerea* spores. Both the red and purple regions of *Aft/Aft atv/atv* fruit were wounded and infected with *B. cinerea* spores. Compared to WT Ailsa Craig fruit, the purple sectors of *Aft/Aft atv/atv* tomatoes showed significantly smaller lesion sizes. The red sectors on the same fruit, however, had similar lesion sizes to the WT fruit (**Fig. 5.5A** and **C**). qPCR indicated that *B. cinerea* growth was significantly slower on the purple regions compared to that on red regions of the same fruit and compared to growth on WT Ailsa Craig (**Fig 5.5B**). All these data indicated that the anthocyanin-enriched, purple regions of *Aft/Aft atv/atv* fruit have reduced susceptibility to *B. cinerea* wound infection.

When intact fruit were sprayed with *B. cinerea* spores, there were severe infection symptoms on the WT Ailsa Craig fruit. This indicated that the WT Ailsa Craig fruit are highly susceptible to *B. cinerea* infection. For *Aft/Aft atv/atv* fruit, however, distinct differences were seen between different regions: the purple regions showed less infection while the red regions had severe infection (**Fig. 5.6**).

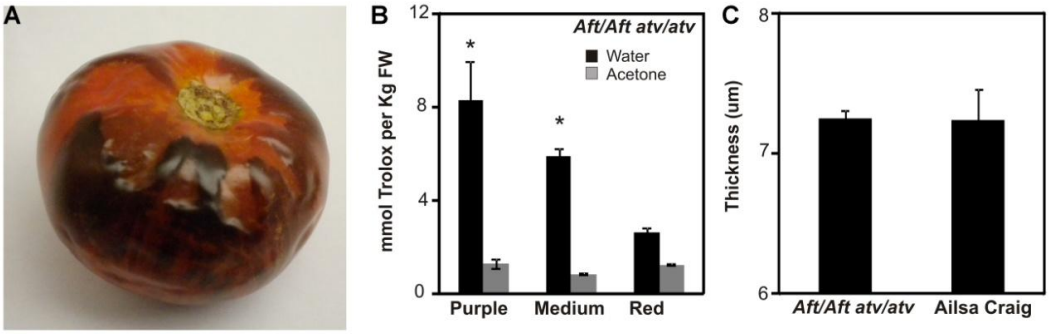

Figure 5.4 High antioxidant capacity of anthocyanins is the main reason for the delayed over-ripening in *Aft/Aft atv/atv* tomatoes

(**A**) The purple regions showed slower over-ripening than the red regions on the same fruit.

(**B**) Trolox equivalent total antioxidant capacity (TEAC) of water and acetone extracts from purple, medium and red regions of *Aft/Aft atv/atv* tomato peel during ripening. Error bars show the standard error of the mean (n=3). * ($p<0.05$) values for purple regions compared to red regions at the same stage.

(**C**) Cuticle thickness of WT Ailsa Craig and the purple region of *Aft/Aft atv/atv* fruit. Measurements were made above the centre of each epidermal cell. Error bars show the standard error of the mean ($n \geq 3$).

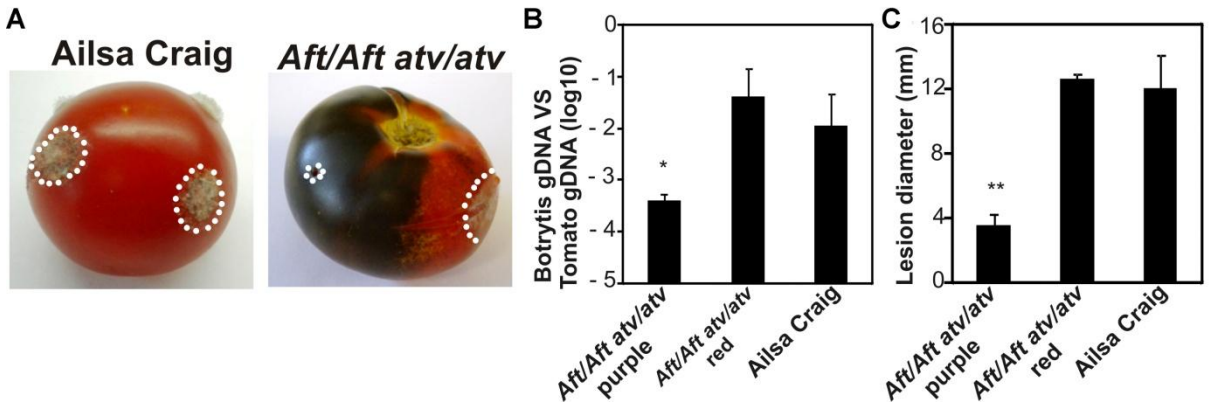

Figure 5.5 Anthocyanin enriched regions of *Aft/Aft atv/atv* fruit have lower susceptibility to *B. cinerea* wound infection.

(**A**) Symptoms of wounded WT Ailsa Craig, purple and red regions of *Aft/Aft atv/atv* tomatoes after inoculation with *B. cinerea* B05.10. Pictures were taken at 3 dpi.

(**B**) Quantitative PCR indicates there was significantly less *B. cinerea* growing on the purple regions of *Aft/Aft atv/atv* fruit at 3 dpi compared to red regions. *Botrytis* growth was calculated by comparing the ratio of *Botrytis* DNA to tomato DNA. Error bars show the standard error of the mean (n=3). * ($p<0.05$) compared to WT fruit.

(**C**) Lesion size was significantly smaller in *Aft/Aft atv/atv* purple regions. Lesion diameter was measured 3dpi. Error bars show the standard error of the mean ($n \geq 3$). ** ($p<0.01$) compared to WT Ailsa Craig.

Figure 5.6 The anthocyanin enriched regions of *Aft/Aft atv/atv* fruit have lower susceptibility to *B. cinerea* spraying infection. Pictures were taken at 5 dpi.

All these data indicated that the anthocyanin-enriched parts of *Aft/Aft atv/atv* fruit are less susceptible to *B. cinerea*.

5.3.3 Accumulation of anthocyanins in the skin of tomato fruit extends shelf life.

In order to determine whether the extended shelf life of *Aft/Aft atv/atv* is directly associated with anthocyanin accumulation (which in *Aft/Aft atv/atv* tomatoes is restricted to the skin), MicroTom was transformed with a *Del/Ros1* gene construct designed to induce skin-specific accumulation of anthocyanins in fruit.

The promoter of the early light-inducible protein (PLI) has been reported to be light-dependant and is highly expressed in tomato fruit skin (Estornell et al., 2009). I expressed *AmRosea1*, a *MYB*-like gene, under the control of the PLI promoter in tomato together with *AmDelila* under the control of the CaMV35S promoter (PRD). Forty-nine T0 plants were obtained and 13 lines were carried through to the T1. The 13 PRD lines showed varying amounts of anthocyanins in skin and small amounts in flesh in the most pigmented lines (Figure 5.6A). Compared to the previously reported E8:*Del/Ros1* line, the *PRD8-2* line produced more anthocyanins in the skin than the *Del/Ros1* line C but lower than the *Del/Ros1* line N. The *PRD17-2* line produces similar amount of anthocyanins in the skin to the *Del/Ros1* line C. Both *PRD8-2* and *PRD17-2* contain substantially less anthocyanins in the flesh than E8:*Del/Ros1* lines (**Fig. 5.7A** and **B**). The *PRD8-2* and *PRD17-2* lines were carried on to T2 generation to test their susceptibility to *Botrytis cinerea*.

When wounded fruit were infected with a *B. cinerea* culture, all transgenic lines showed smaller lesion sizes at 3dpi compared to WT fruits (**Fig. 5.8A**). In spraying test, the proportion of fruit showing severe infections was always lower for the transgenic PRD lines than for WT fruits (**Fig. 5.8B**). Susceptibility was inversely correlated with anthocyanin content; *E8:Del/Ros1* N and *PRD8-2* tomatoes, which had the highest levels of anthocyanins, were less susceptible to *B. cinerea* than *PRD17-2* and *Del/Ros1* C. These results showed that the reduced pathogen susceptibility in *Aft/Aft atv/atv* fruit is directly associated with anthocyanin production. Also, skin-specific accumulation of anthocyanins in tomato is sufficient to reduce the susceptibility of fruit to *B. cinerea*.

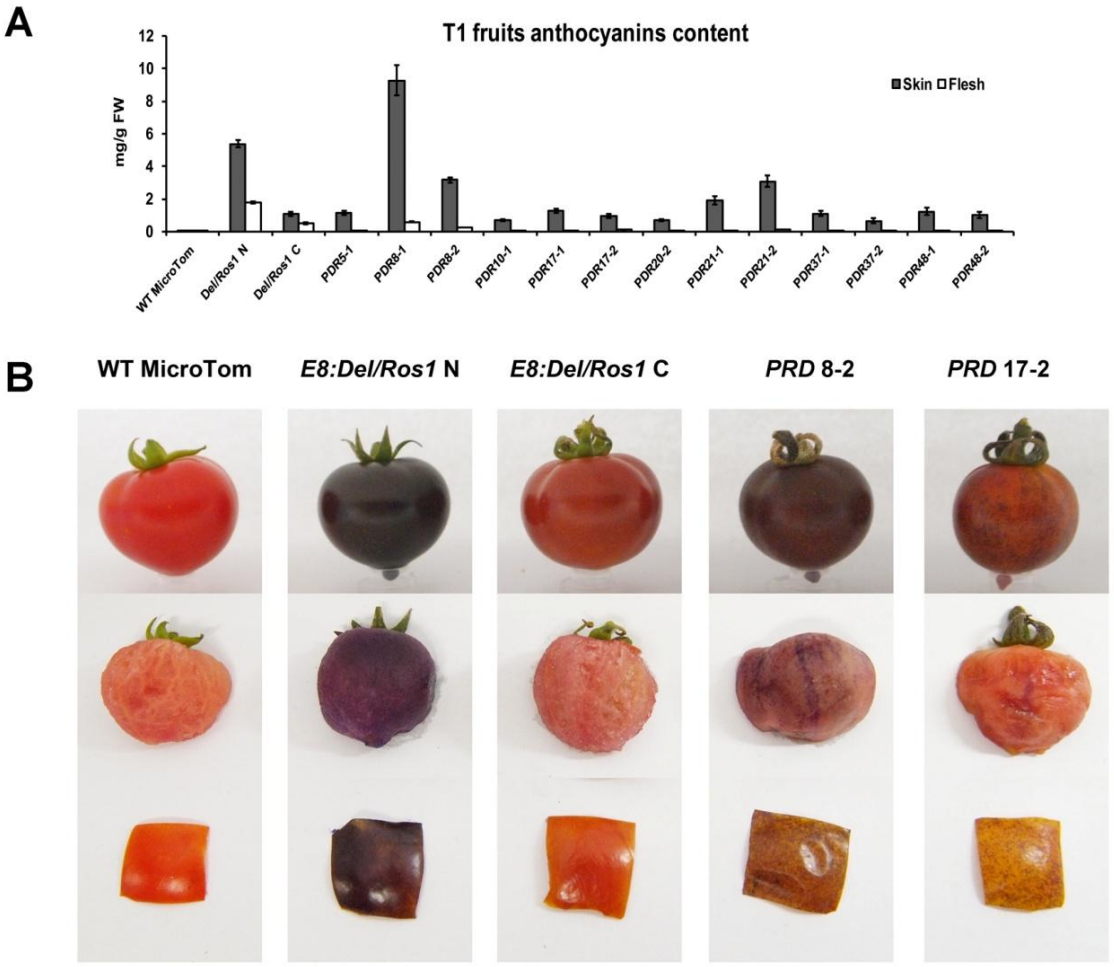

Figure 5.7 Phenotype of *PRD* fruit.

(**A**) Anthocyanin contents of the skin and flesh of different fruit lines. WT MicroTom and E8:*Del/Ros1* N and C lines were used as controls. Error bars show SEM (n=3).

(**B**) Phenotype of *PRD8-2* and *PRD17-2* fruit. Fruit were harvest at two weeks after breaker. Each fruit was photographed before and after the skin peeled. The bottom line showed the peeled skin of each line.

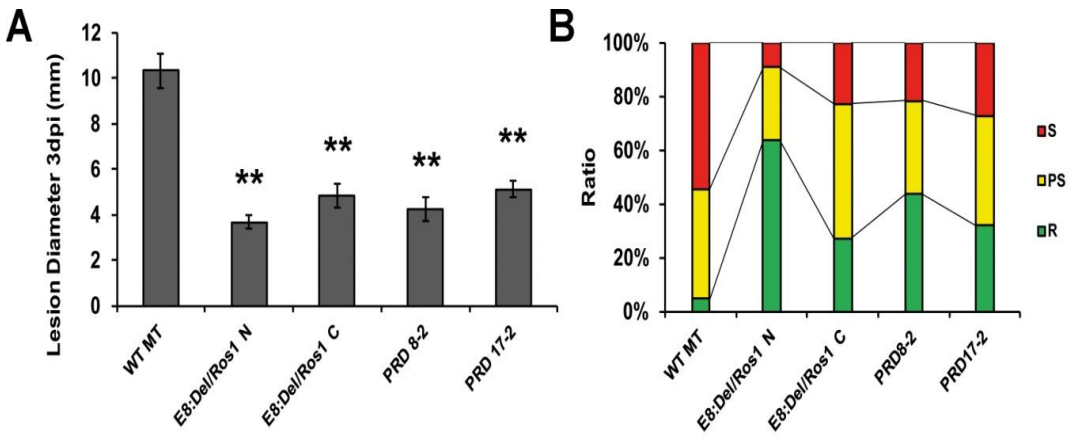

Figure 5.8 Accumulation of anthocyanins in skin is sufficient to reduce susceptibility to *Botrytis cinerea* infection.

(**A**) Lesion diameter of *B. cinerea* infection at 3 dpi. Error bars show SEM (n=18), ** indicates $p<0.01$ compared to WT fruit.

(**B**) Different degrees of susceptibility to *B. cinerea* shown by WT and transgenic fruit in spraying tests. Fruit were checked 5 days after inoculation. Resistance (R), partial susceptibility (PS) and susceptibility (S).

5.4 Discussion

5.4.1 *Aft/Aft atv/atv* fruit show extended shelf life

Postharvest losses caused by over-ripening and pathogen infection are the most important problem for the tomato industry. Current attempts to extend tomato shelf life often have negative effects to the flavor, aroma, and texture (Baldwin et al., 2011). Anthocyanins have been reported to contribute to longer shelf life in other species (Ayed et al., 1999). We also showed that enrichment of anthocyanins in tomatoes by genetic modification can double the shelf life of fruit.

Aft/Aft atv/atv, a tomato variety obtained by introgression, also has delayed over-ripening and reduced pathogen susceptibly. The extended shelf life of *Aft/Aft atv/atv* is associated directly with anthocyanin production in the skin: compared to red, low anthocyanin regions on the same fruit, purple, high anthocyanin regions showed delayed over-ripening and reduced susceptibility to *B. cinerea* (**Fig 5.3, 5.4** and **5.5**). Cuticle measurements showed there to be no differences in cuticle thickness between *Aft/Aft atv/atv* fruit and WT fruit. The only difference between different regions on the same *Aft/Aft atv/atv* fruit is anthocyanin accumulation, which is limited to the fruit skin. Compared to the purple sectors on the same fruit, the red sectors of *Aft/Aft atv/atv* show the same, quick over-ripening as WT fruit (Fig. 5.3A). This indicates that the normal ripening process is retained in the red parts of *Aft/Aft atv/atv* fruit. All these data indicate that anthocyanin accumulation in skin is the main reason for the extended shelf life in *Aft/Aft atv/atv* fruit.

5.4.2 Accumulation of anthocyanins in skin is sufficient to reduce pathogen susceptibility

When sprayed with *B. cinerea* spores, on the same *Aft/Aft atv/atv* fruit, the red sectors showed severe infection symptoms. The purple, anthocyanin-enriched sectors, however, showed much less susceptibility (**Fig. 5.5**). When purple sectors were wounded and infected with *B. cinerea*, they also showed less susceptibility to infection than the red sectors (**Fig 5.4**). The different sectors on the same fruit were under the same conditions, the only difference between them was the accumulation of anthocyanins in skin.

The *PRD* tomatoes accumulate high levels of anthocyanins in their skin. However, compared to previously reported E8:*Del/Ros1* fruit, they have very low anthocyanin levels in their flesh. Like the *Aft/Aft atv/atv* fruit, *PR*D fruit showed lower susceptibility to *Botrytis cinerea* compared to WT controls. The strength of the reduction in susceptibility is also associated with the amount of anthocyanins produced (**Fig. 5.6** and **5.7**). All these data support the conclusion that accumulation of anthocyanins in skin is sufficient to reduce the susceptibility of fruit to *Botrytis cinerea*.

5.4.3 Scientific findings from GM research can serve traditional breeding.

In most cases, scientists study the function of a gene/compound based on initial observations from breeders. For anthocyanin-enriched tomatoes produced by conventional breeding, their shelf life extension had not previously been reported, perhaps because of the low amount of anthocyanin in *Aft/Aft atv/atv* tomatoes. Because of the very high anthocyanin content of GM purple tomatoes, we identified a significant extension to the shelf life of their fruit. A variety producing anthocyanin in the skin of its fruit, produced by conventional breeding also has an extended shelf-life, although not as great as that of the GM purple tomatoes. Using GM crops we have therefore identified and dissected biological processes which have escaped the notice of breeders using natural variation. The findings from such research can, in turn, serve conventional breeding for improved varieties.

Chapter 6: Investigation of the function of different flavonoids in extension of shelf life in tomatoes

6.1 Introduction

Among the flavonoids, there are two major groups of compounds: anthocyanins and flavonols. Due to their health benefits, in recent years, there is growing interest to engineer enrichment of anthocyanins or flavonols in crops (Bovy et al., 2002; Luo et al., 2008; Muir et al., 2001; Winkel-Shirley, 2001).

Previously, tomato fruit with a high content of flavonols were produced by fruit-specific expression of the *Arabidopsis* transcription factor MYB12, (Luo et al., 2008). Due to the high antioxidant capacities of flavonols, *AtMYB12* tomato fruit have high total antioxidant capacity compared to WT fruit (Luo et al., 2008).

In addition to transgenic tomato lines accumulating high levels of flavonoids, there are many flavonoid biosynthesis mutants indentified in tomato (Al-sane et al., 2011). For instance, the *anthocyanin without* (*aw*) mutant can not synthesise anthocyanins. Over-expression of a wild type tomato *DFR* gene in the *aw* mutant can rescure anthocyanin production. This indicates the *aw* mutant lacks DFR activity (Goldsbrough et al., 1994). Another important mutant is *entirely anthocyaninless* (*ae*), which also does not produce anthocyanins.

Virus Induced Gene Silencing (VIGS) has proved to be an efficient tool to analyse gene function in tomato fruit (Orzaez et al., 2006). Previous studies indicate that *Del/Ros1* tomatoes provide a visual marker for transient gene silencing in fruit (Orzaez et al., 2009). Using VIGS, a specific association between anthocyanin accumulation and extended shelf life in purple tomato was established. The anthocyanin biosynthetic pathway in *Del/Ros1* tomato is highly induced (Butelli et al., 2008), making the purple tomato perfect to study the effects of intermediate flavonoid compounds by VIGS.

Longer shelf life, together with lower pathogen infection, is one of the most important traits for the tomato industry. Previously, we showed that accumulation of anthocyanins in tomato fruit can extend shelf life. Both the GM *Del/Ros1* tomato and the *Aft/Aft atv/atv* tomato (from conventional breeding) have delayed over-ripening and reduced susceptibility to *B. cinerea*. The extended shelf life of anthocyanin-

enriched tomatoes is associated with the high antioxidant capacity of anthocyanins. Oxidative stress accumulation is a key factor during ripening and over-ripening of fruit. Accumulation of anthocyanins can increase the total antioxidant capacity of tomato fruit, thus slowing the increase in oxidative stress and delaying ripening and over-ripening. The ROS burst induced by pathogen infection is thought to contribute negatively to resistance to *B. cinerea*. The high scavenging ability of anthocyanins can effectively alter ROS dynamics during *B. cinerea* infection and reduce susceptibility to the pathogen.

A common feature shared by different flavonoid compounds is their high scavenging ability (Heim et al., 2002). The high free radical scavenging capacity of flavonoids is predominantly attributed to the high activities of their hydroxyl substituents to ROS. *In vitro* analysis indicates that the structure of the B-ring is an important factor determining the antioxidant capacity of flavonoids (Sekher Pannala et al., 2001). The number of free hydroxyl groups on the B-ring determines the antioxidant capacity (Burda and Oleszek, 2001; Rice-Evans et al., 1996). Among the different positions in natural flavonoids, a free –OH on the C-3' position is the most powerful group contributing to scavenging ability (Burda and Oleszek, 2001).

Our previous data indicated that their high antioxidant capacity determines the extended shelf life of anthocyanin-enriched tomatoes. Tomato lines accumulating different flavonoid compounds have been engineered (Butelli et al., 2008; Luo et al., 2008). As different flavonoid compounds have different structures, their antioxidant capacities may be different. Tomato fruit accumulating different flavonoid compounds may have different degrees of shelf life extension.

To analyse the roles of different flavonoids in extending shelf life, *AtMYB12* tomatoes fruit were compared to WT and *Del/Ros1* tomatoes using both storage tests and pathogen infection tests. To screen the actives of different polyphenol compounds in determining tomato shelf life, VIGS fruit with different flavonoid biosynthetic genes silenced were also analyzed. The results from VIGS test were confirmed using natural mutants. The correlations between the structure of the compounds and the shelf life extensions they confer, are discussed.

6.2 Materials and Methods

6.2.1 Plant materials

The *AtMYB12* tomatoes have been described by (Luo et al., 2008). A new line of tomato in the MicroTom genetic background was made by crossing *Del/Ros1* N MicroTom (Butelli et al., 2008) with *AtMYB12* MicroTom. The resulting line, named Indigo, contains high amounts of both anthocyanins and flavonols.

Two natural mutants of tomato, *aw* (LA3736) and *ae* (LA3612), in the Ailsa Craig genetic background were obtained from the Tomato Genetic Resource Centre (http://tgrc.ucdavis.edu/). The *aw* mutant lacks DFR activity (Goldsbrough et al., 1994) while the *ae* mutant is deficient in ANS activity (De Jong et al., 2004; Tanksley et al., 1992). Both *aw* and *ae* mutants were crossed with *Del/Ros1* N MicroTom tomato (Butelli et al., 2008). The $aw^{-/-}$ *Del/Ros1* and $ae^{-/-}$ *Del/Ros1* lines were selected from a segregating F2 population. A control (with functional *Aw* and *Ae* genes) carrying *Del/Ros1* was also selected from these F2 populations.

6.2.2 Storage tests

Storage tests for tomatoes in the MicroTom genetic background were undertaken as described previously (see **Section 3.2.1**). WT, *Del/Ros1*, *AtMYB12* and Indigo tomatoes were tagged at breaker and harvested two weeks after breaker. Fruit were washed with water and sterilized with 10% bleach for 20 min. After surface sterilization, fruit were rinsed with sterilized water, three times and air-dried in a clean flow cabinet. Ten fruit from the same line were placed in one clean Phytatray II™ (Sigma-Aldrich) and stored in the dark at 17°C. Each week, the proportion of fruit showing over-ripening symptoms (visual softening and collapse of the surface) was calculated for each box, and the fresh weight was measured. After inspection, the fruit were transferred to a new Phytatray.

6.2.3 Pre-treatment of tomato fruit before *Botrytis* infection

MicroTom fruit were harvested two weeks after breaker and surface sterilized. Fruit were inoculated with the following solutions for 16 hour:

1). water,
2). 50 µM DPI,
3). 100 unit/ml glucose oxidase + 1% glucose (GO+G),
4). 5 mM methyl jasmonate (MeJA),
5). 5 mM Salicylic acid (SA),
6). 50µM abscisic acid (ABA),
7). 2 mM ethephon (ET).

After innoculation, fruit were air-dried and kept in sealed boxes with high humidity for a further 4 hours. *B. cinerea* spores were collected according to the methods described in previous chapters (see **Section 4.2.1**). Aliquots of 5 µL containing 5×10^4 spores/mL culture were inoculated into wounded fruit and lesion size was measured at 3dpi.

6.2.4 Supplementation with tomato juice and different flavonoids before inoculation with *B. cinerea*

B. cinerea spores were collected by methods described in previous chapters (see **Section 4.2.1**). For juice supplementation, *B. cinerea* spores were first diluted to 1.25×10^6 spores/mL in ¼ strength PDB and incubated at RT for 2 hours. One volume of this initial culture was mixed with 9 volumes of juice prepared from WT, *AtMYB12*, *Del/Ros1* N, *DelRos1* C and Indigo tomatoes (to make the final concentration to 1.25×10^5 spores/mL). As a control, one volume of the initial culture was mixed with 9 volumes of ¼ strength PDB. Six wound sites were made on each WT MoneyMaker fruit and each site was inoculated with 10 µL of juice plus *Botrytis* culture. Inoculated fruit were stored in boxes with high humidity. Every 24 hour, 10 µL of juice (or water for controls) was added to the inoculation site. Lesion sizes were assessed at 3 dpi.

For supplementation with specific compounds, the *B. cinerea* culture was diluted to 1.25×10^5 spores/mL in ¼ strength PDB, supplemented with purified compounds to different final concentrations (for control, 5% EtOH was added to ¼ strength PDB). Culture (10 µL) was inoculated onto each site. Solutions of compounds in water (10

µL) were supplied to the inoculation sites every 24 hours. Lesion sizes were assessed at 3 dpi.

6.2.5 Plasmid construction

A Gateway destination vector pTRV2-GW was kindly provided by Dr. Diego Orzaez (Orzaez et al., 2009). Fragments of target genes (200-300bp of the cDNA) (see **Appendix 1** for information on genes and primers) were amplified with Gateway compatible primers and recombined into pDONR207 by the BP reaction to generate an Entry clone. The Entry vector was then recombined with the pTRV2-GW Destination Vector using an LR reaction to make Expression clones pTRV2-*SlCHS1*, pTRV2-*SlCHI*, pTRV2-*SlF3H*, pTRV2-Sl*DFR*, pTRV2-*SlANS*. The sequenced Expression vectors were then transferred into *Agrobacterium tumefaciens* strain GV3101:pMP90 by electro-poration.

6.2.6 Agroinfiltration

Agroinfiltration was performed as described in **section 3.2.7**.

6.2.7 Isolation of phenolic compounds and analysis by LC-MS

Sectors of VIGS fruit or natural mutants were ground into fine powder in liquid nitrogen. To isolate phenolic compounds, one gram of ground tissue was extracted in 10 mL 50% MeOH (containing 2% Formic Acid) in the dark, at 4°C, overnight with gentle agitation (30 rpm). Samples were then centrifuged for 10 min at 4000 rpm, at 4°C. Supernatants were collected and then cleared by filtration through a 0.22 mm membrane filter (Millipore).

All samples were analysed on a Surveyor HPLC system attached to a DecaXPplus ion trap MS (both Thermo), using 10µL injections. Phenolics were separated on a 100×2mm 3µ Luna C18(2) column (Phenomenex) using the following gradient of acetonitrile versus 0.1% formic acid in water, run at 300µL min^{-1} and 30°C: 0 min, 1% acetonitrile (ACN); 4 min, 1% ACN; 23 min, 30% ACN; 30 min, 70% ACN; 30.5 min, 1% ACN; 37 min, 1% ACN.

Phenolics were detected by light absorbance, collecting full spectra from 200-600 nm, and chromatograms at 280 nm and 500-550 nm both with 19 nm band width. For positive electrospray detection, spectra from *m/z* 100-2000 and data-dependent MS2 of the most abundant precursor ions at collision energy of 35% and an isolation width of *m/z* 4.0 were collected. Dynamic exclusion was used to ensure that after two spectra had been collected for a precursor ion, it would be ignored for 0.5min in favour of the next most abundant ion. Spray chamber conditions were 50 units sheath gas, 5 units aux gas, 350°C capillary temperature, and 3.8kV spray voltage using a steel needle kit.

6.3 Results

6.3.1 Flavonol-enriched tomatoes show delayed over-ripening.

When stored in sterile conditions, *AtMYB12* tomatoes showed delayed over-ripening compared to WT tomatoes. Fifty percent of WT fruit showed over-ripening symptoms after storage for 2 weeks. For *AtMYB12* tomatoes, however, the date for a similar level of over-ripening was delayed to 3-4 weeks after the start of storage. For *Del/Ros1* and Indigo (contains *Del/Ros1* and *AtMYB12*) tomatoes, longer storage times (5-6 weeks) were needed to see the same degree of over-ripening (**Fig. 6.1A**). The fresh weight of the four lines showed the same rate of reduction over the first four weeks of storage. However, beyond four weeks, the fresh weight reduction in *AtMYB12* increased significantly while other lines maintained the same rate of reduction (**Fig. 6.1B**).

The delayed over-ripening for *AtMYB12* tomatoes compared to WT was also observed in fruit kept on the vine. Over-ripening can be seen on WT fruit from four weeks after breaker. For *AtMYB12* tomatoes, however, 6-8 weeks after breaker were needed to observe the same degree of over-ripening. For most purple *Del/Ros1* and indigo tomatoes, a similar degree of over-ripening was seen at 8-10 weeks after breaker (**Fig. 6.2**).

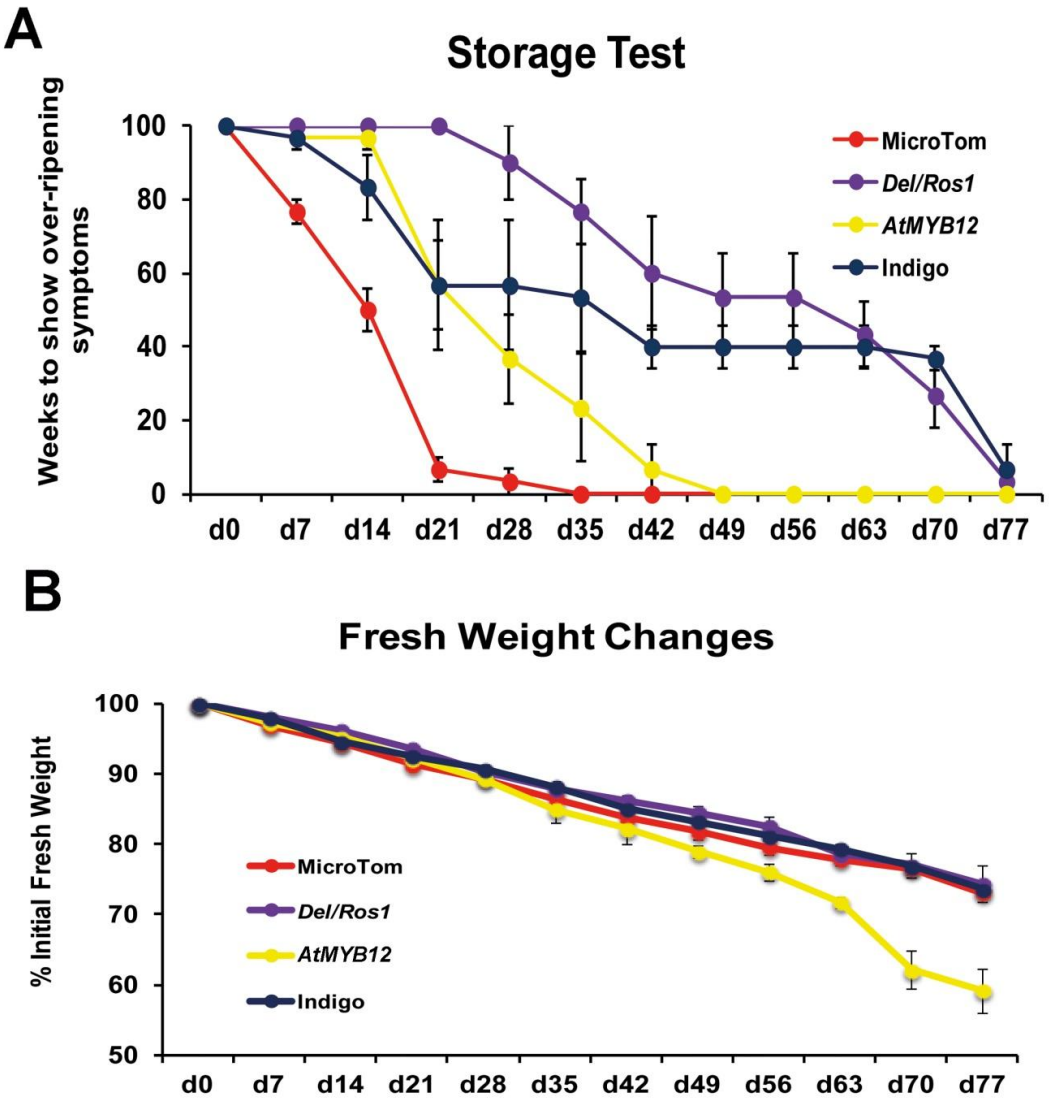

Figure 6.1 *AtMYB12* tomatoes store for longer than WT MicroTom but for less time than *Del/Ros1* tomatoes.

Fruits were harvested at 14 days post breaker (d0 = 14 dpb).

(**A**). Percentages of fruit showing over-ripening symptoms were assessed every week during storage tests.

(**B**) Fresh weight of different fruit during the storage. The total weight of the ten fruit in the same jar was calculated. Error bars in (**A**) and (**B**) show the SEM (n = 3).

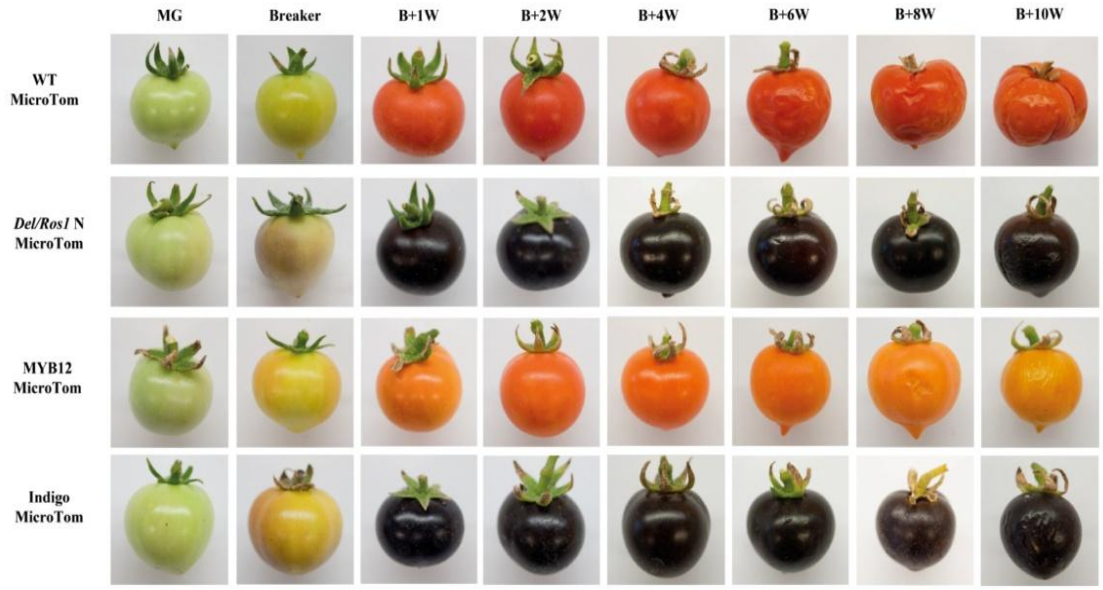

Figure 6.2. Phenotypes of tomato fruit on the vine at different ripening stages. For each stage, 8-12 fruit were checked and the most representative fruit are shown.

Collectively these data indicate that compared to WT tomatoes, *AtMYB12* tomatoes have delayed over-ripening. However, the length of the delay is not as great as for purple *Del/Ros1* or Indigo *Del/Ros1 AtMYB12* tomatoes.

6.3.2 The antioxidant compounds in *AtMYB12* fruit are not as stable as in *Del/Ros1* tomatoes.

My previous data indicated that the high antioxidant capacity of anthocyanin-enriched tomatoes reduces the oxidative damage to fruit tissues during the ripening, and consequently delays over-ripening. I checked the antioxidant capacities of the four lines used in the storage tests, at different stages of over-ripening. For all the transgenic lines (*Del/Ros1*, *AtMYB12* and Indigo), the antioxidant capacity of hydrophilic compounds was found to be increased significantly at breaker (**Fig. 6.3A**). This was due to the induction of the E8 promoter at breaker. The expression of *Del/Ros1* and *AtMYB12* transcription factors enhanced the production of flavonoids, which increased the antioxidant capacities of the fruit.

During over-ripening, the total antioxidant capacity of *AtMYB12* tomatoes showed more rapid declines than *Del/Ros1* and Indigo tomatoes: significant reductions in the antioxidant capacity of *AtMYB12* fruit came 8 weeks after breaker. However, for *Del/Ros1* and Indigo tomatoes, significant reductions were not seen until 10 weeks after breaker (**Fig. 6.3A**). This indicates that the compounds in *AtMYB12* tomatoes are likely less stable than in *Del/Ros1* and Indigo tomatoes.

It seemed that the shorter shelf life of *AtMYB12* tomatoes compared to *Del/Ros1* tomatoes was likely due to the lower stability of the antioxidant compounds in *AtMYB12* tomatoes. To test this hypothesis, production of malondialdehyde (MDA), a by-product of lipid peroxidation which can be used as a marker for the damage resulting from oxidative stress during senescence, was measured for all the tomato lines. For WT MicroTom fruit, MDA production kept on increasing through the entire late ripening period. However, for all the transgenic lines, the MDA levels remained low up to four weeks after breaker. For the *AtMYB12* line, the MDA levels increased at six weeks after breaker, when the total antioxidant capacity of *AtMYB12* began to decrease significantly. For *Del/Ros1* tomatoes, however, the increase in

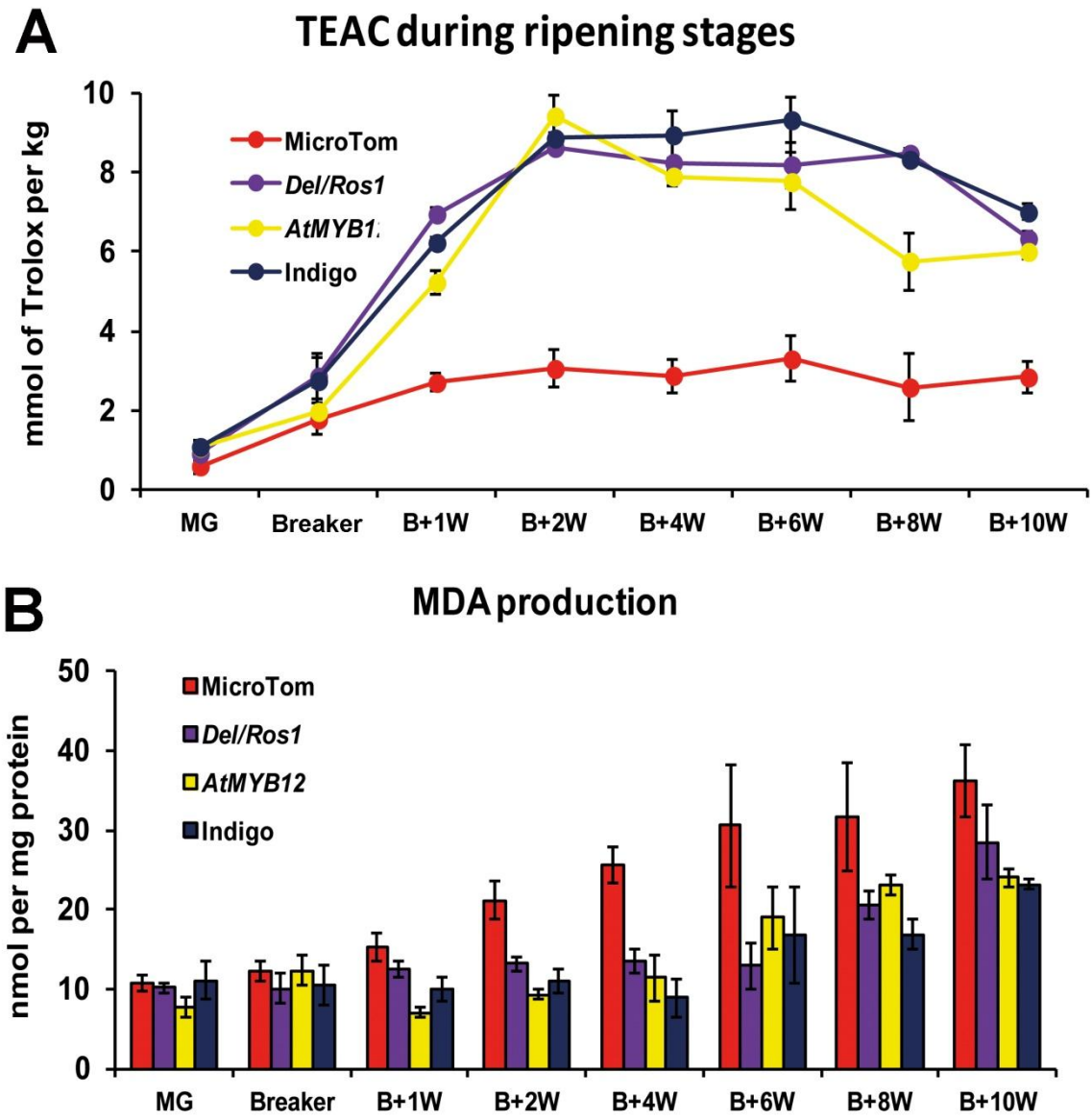

Figure 6.3. The high antioxidant capacity of *AtMYB12* tomatoes cannot be maintained as long as for *Del/Ros1* fruit.

(**A**) Total antioxidant capacity of hydrophilic compounds in different tomato lines during ripening.

(**B**) MDA content of different tomato lines during ripening. Error bars show SEM (n=3).

MDA levels came 8 weeks after breaker (**Fig. 6.3B**). This matched the late reduction of antioxidant capacity in *Del/Ros1* fruit.

6.3.3 *AtMYB12* tomatoes are susceptible to the fungal pathogen, *Botrytis cinerea*.

Previous data indicated that anthocyanin-enriched *Del/Ros1* tomatoes have lower susceptibility to *Botrytis cinerea*, one of the most important postharvest pathogens of tomato fruit. In order to check the pathogen susceptibility of *AtMYB12* tomatoes, both wounded and intact fruit were inoculated with *B. cinerea*.

When intact fruit were sprayed with *B. cinerea* spores, at 5 days post-inoculation (5dpi), the percentage of fruit showing severe symptoms was high for both WT and *AtMYB12* tomatoes. However, very a few of *Del/Ros1* and Indigo fruit showed severe infection (**Fig. 6.4A**). This indicated that *AtMYB12* fruit are as susceptible as WT fruit *to B. cinerea* infection by spraying.

When wounded fruit were inoculated with *B. cinerea* spores, at 1 dpi, the size of the lesions did not increase in any fruit type. From 2 dpi, however, there was greater spread of infection in WT and *AtMYB12* fruit than in *Del/Ros1* and Indigo fruit. At 3 dpi, the average size of the lesions in WT and *AtMYB12* tomatoes was significantly larger than in *Del/Ros1* and Indigo fruit, indicating they are more susceptible to *B. cinerea* infection (**Fig. 6.4B**). Quantitative PCR with total DNA extracted from infected tomatoes confirmed that there was significantly more *Botrytis* growing on WT and *AtMYB12* fruit than on *Del/Ros1* and Indigo fruit at 3 dpi (**Fig. 6.4C**).

Previous data had shown that the susceptibility of tomato fruit to necrotrophic pathogens increases as the ripening process progresses (Cantu et al., 2009; Cantu et al., 2008a). When fruit at different stages were infected by *B. cinerea*, a correlation between ripening and increased susceptibility was seen in both WT and *AtMYB12* lines. However, in *Del/Ros1* and Indigo lines, there was no significant increase in susceptibility to *B. cinerea* after the breaker stage (**Fig. 6.4D**).

AtMYB12 was also introduced into the MoneyMaker background (normal size fruit) as described by (Luo et al., 2008). *AtMYB12* MoneyMaker fruit were also very

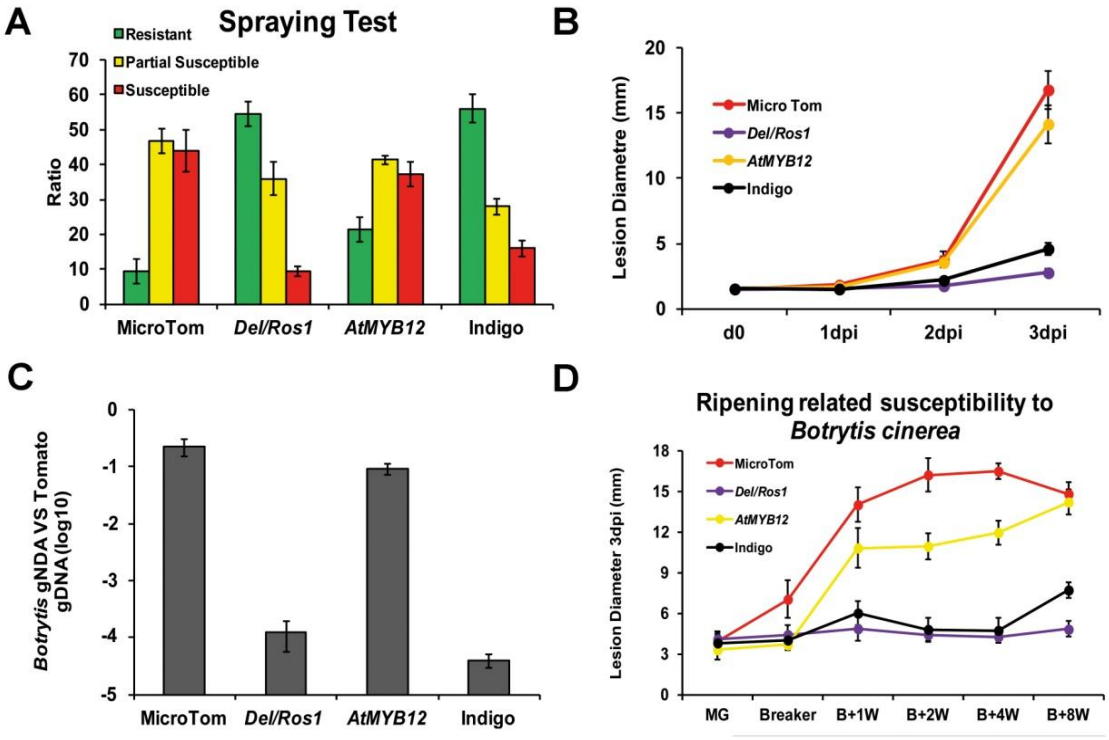

Figure 6.4 *AtMYB12* tomatoes are susceptible to *Botrytis cinerea*.

(**A**) Different degrees of susceptibility to *B. cinerea* were shown by red and purple fruit in spraying tests. Fruit were checked 5 days after inoculation. Error bars represent the SEM for three independent assays. Resistance (R), partial susceptibility (PS) and susceptibility (S).

(**B**) Lesion development of wounded fruit infected with *B. cinerea*. Error bars represent the SEM (n=6).

(**C**) Quantitative PCR revealed *Botrytis* growing on wounded, infected fruit 3 dpi. *Botrytis* growth was calculated by comparison of the ratio of *Botrytis* DNA to tomato DNA. Error bars show the SEM (n = 3).

(**D**) Ripening related susceptibility to *Botrytis cinerea*. Error bars represent the SEM for three independent assays.

Figure 6.5. *AtMYB12* MoneyMaker tomato is susceptible to *B. cinerea*. From left to right, WT, *Del/Ros1* and *AtMYB12* MoneyMaker tomato infected with *Botrytis cinerea*. White dots represent lesion margins. Fruit were harvest at 7 dpb. Pictures were taken at 3 dpi.

susceptible to infection by *B. cinerea* (**Fig. 6.5**). Collectively these data indicated that, unlike *Del/Ros1* tomatoes, *AtMYB12* tomatoes are as susceptible as WT tomatoes to post-harvest infection by *B. cinerea*.

6.3.4 *AtMYB12* tomatoes have a reduced ability to alter the dynamics of the ROS burst during *Botrytis* infection

In order to investigate the high susceptibility of *AtMYB12* tomatoes to *B. cinerea* infection, RT-qPCR was undertaken to check the expression of important pathogen response genes before and after inoculation. Expression of major pathogen response genes (*SlChi*, *SlGLU*, *SlMCA7* and *SlPR1*) was induced in WT and *AtMYB12* tomato following inoculation, as well as in *Del/Ros1* tomatoes (**Fig. 6.6A-D**). This indicates that normal defense responses were operational in all the lines. However, a hypersensitive response gene, *SlHSR203*, was highly expressed in WT and *AtMYB12* tomatoes after inoculation while in *Del/Ros1* tomatoes, the induction was lower (**Fig. 6.6E**). *HSR203* expression is associated with hypersensitive response (HR) triggered cell death (Pontier et al., 1998; Tronchet et al., 2001). These data suggest that the HR in *AtMYB12* tomatoes is very strong following infection with *B. cinerea*.

To test this hypothesis, the total antioxidant capacities of both infected and wound-only fruit were measured. Data showed that after *B. cinerea* infection, the total antioxidant capacity of WT and *AtMYB12* fruit declined compared to fruit without infection. For *Del/Ros1* tomatoes, however, the total antioxidant capacity was stable following inoculation with *B. cinerea* (**Fig. 6.6F**). Although *AtMYB12* fruit have high antioxidant capacity compared to WT fruit before pathogen infection, they have reduced ability to alter the dynamics of the ROS burst during *Botrytis* infection compared to *Del/Ros1* tomatoes.

My previous data indicated that anthocyanins alter the dynamics of the ROS burst during the *B. cinerea* infection of tomato fruit, thus altering the HR to reduce the susceptibility to infection. In order to check the influence of ROS dynamics on pathogen susceptibility of tomato fruit, fruit from different lines were pre-treated with DPI (ROS inhibitor) and GO + G solution (ROS inducer), as well as resistance-

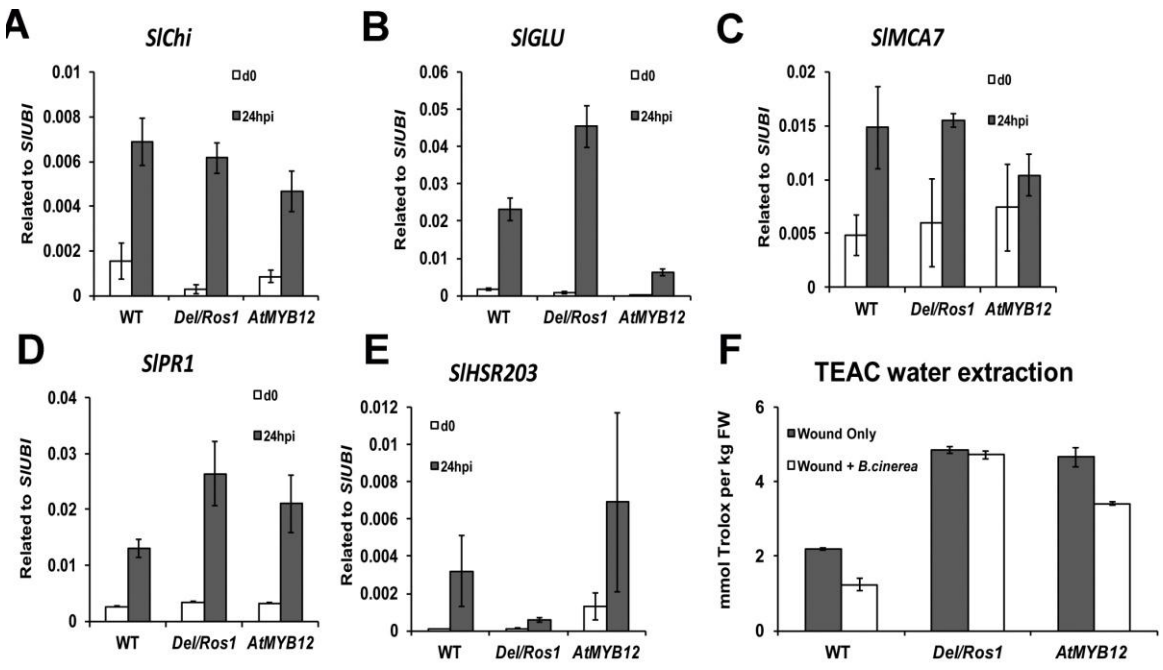

Figure 6.6 The hypersensitive response was high in WT and *AtMYB12* tomatoes.

(**A**)-(**E**) RT-qPCR of pathogen response genes before and after *B. cinerea* infection. (**A**) *Chitinase* (*SlChi*), (**B**) *β -1,3-glucanase* (*SlGLU*), (**C**) *Metacaspase 7(SlMCA7)*, (**D**) *Pathogenesis-related protein 1*(*SlPR1*) and (**E**) *Hypersensitivity-related gene 203* (*SlHSR203*).

(**F**) The total antioxidant capacity of uninfected and infected fruit. Both wound only and wound + infection fruit were analyzed at 3 dpi. Error bars show the SEM, n=3.

related plant hormones/precursors: methyl jasmonate (MeJA), salicylic acid (SA), abscisic acid (ABA) and ethephon (ET).

Compared to the water-treated fruit, DPI treated WT and *AtMYB12* fruit had smaller lesion sizes at 3dpi. For *Del/Ros1* and Indigo tomatoes, however, GO+G treatment increased their susceptibility to *Botrytis* (**Fig. 6.7**). These data indicate that the ROS burst contributes positively to susceptibility to *Botrytis* infection. DPI treatment reduced the susceptibility of *AtMYB12* tomatoes to *Botrytis* infection, indicating that *AtMYB12* tomatoes have a lower ability to alter ROS dynamics than *Del/Ros1* purple tomatoes, during pathogen infection.

I also found that ABA and ET pretreatment could increase the susceptibility of *Del/Ros1* and indigo fruit to *Botrytis* infection, while SA increased the susceptibility of Indigo fruit, only. This showed that both lines are sensitive to ABA and ethylene and that ABA, ethylene and SA positively affect the susceptibility of ripe fruit to pathogens like *Botrytis*. MeJA treatment, however, reduced the susceptibility of *AtMYB12* tomatoes to *Botrytis*, indicating that JA signaling contributes to determining the resistance of ripe fruit to *Botrytis*.

6.3.5 Specific compounds contribute to the lower susceptibility of *Del/Ros1* tomatoes to *B. cinerea*

When fruit juice from different lines was added to media to assay growth of *Botrytis* on plates, no inhibition of *Botrytis* growth was seen for any of the juice-supplements. Compared to normal PDA plates, the growth of *Botrytis* mycelium was greater on juice-supplemented plates (**Fig. 6.8A**). These results indicated that for all the juices, there was no direct inhibition of growth of *B. cinerea*. Indeed, it seems likely that the reduced susceptibility of *Del/Ros1* purple and Indigo tomatoes to *B. cinerea* infection requires living tomato cells for its manifestation.

The total antioxidant capacity of *AtMYB12* tomato juice was as high as that of the *Del/Ros1* N line (initially used in this research) and the Indigo line. The *Del/Ros1* C line, another independent transgenic line contains less anthocyanins than line N, and has intermediate antioxidant capacity (**Fig. 6.8B**).

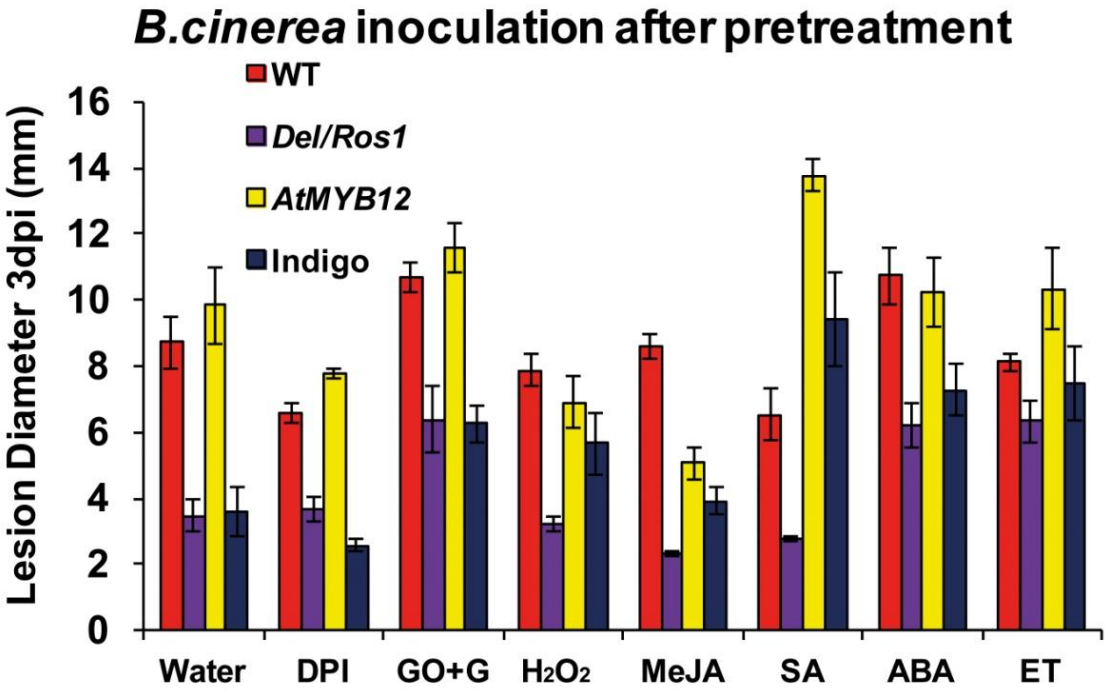

Figure 6.7 *B. cinerea* infection of pre-treated fruit.

Fruit were pre-treated with water, 50 µM DPI, 100 unit/ml glucose oxidase + 1% glucose (GO+G), 5 mM methyl jasmonate (MeJA), 5 mM Salicylic acid (SA), 50µM abscisic acid (ABA) and 2 mM ethephon (ET) before *B. cinerea* infection. Lesion diameter was measured 3 dpi. Error bars show the standard error of the mean (SEM) ($n \geq 6$).

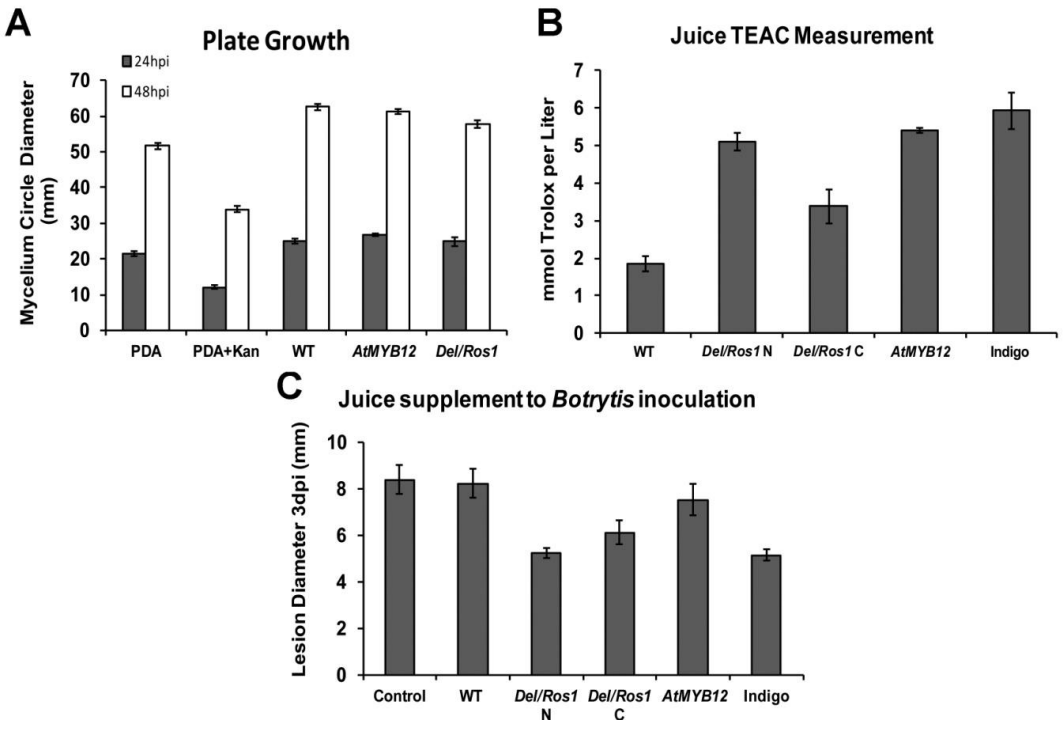

Figure 6.8 Different tomato juices have different effects to *B. cinerea* infection.

(**A**) Addition of juice from different tomatoes to the growth medium had no direct effect on the growth of *B. cinerea*. Juices were isolated from different lines at 14 dpb. Error bars show SEM (n=3).

(**B**) Total antioxidant capacity of different tomato juices. Error bars show SEM (n=3).

(**C**) Juice supplements for *Botrytis* inoccula on WT MoneyMaker fruit affect susceptibility. Different juices were supplied to different inoculation sites on fruit. Error bars show SEM (n=12).

When different fruit juices were added to the *B. cinerea* inoculations on WT MoneyMaker tomatoes, WT MicroTom and *AtMYB12* tomato juice did not reduce lesion development compared to water control supplements. However, juice from *Del/Ros1* N, *Del/Ros1* C and Indigo lines did reduce lesion size, significantly (**Fig. 6.8C**). Juice from fruit enriched in different flavonoids had no direct inhibitory effects on *B. cinerea* growth *in vitro*, and all juices had high antioxidant capacity. However, when supplied with *B. cinerea* in inoculations, juice from *Del/Ros1* and Indigo lines could effectively reduce the susceptibility of fruit to *B. cinerea*. These data indicate that specific compounds in *Del/Ros1* (and Indigo) tomatoes contribute to the reduced susceptibility to *Botrytis*.

6.3.6 Accumulation of different flavonoids in tomato using VIGS.

Previously, by using virus induced gene silencing (VIGS), we successfully silenced the expression of *Delila* and *Rosea1* genes in purple tomatoes to produce purple fruit with red sectors which contain low levels of anthocyanins. We found that on the same VIGS-*Del/Ros1* fruit, although red sectors were of exactly the same age as purple sectors, the red sectors showed more rapid over-ripening. In addition, the red sectors lost their resistance to *Botrytis cinerea* while the purple sectors remained resistant. These results indicated that the delayed over-ripening and reduced pathogen susceptibility are directly associated with the accumulation of anthocyanins.

To analyse in more detail which compounds contribute directly to the extended shelf life, three key anthocyanin biosynthetic genes (*SlF3H, SlDFR* and *SlANS*) were silenced separately in purple tomatoes.

Silencing of *SlF3H, SlDFR* and *SlANS* by VIGS can effectively block the production of anthocyanins in *Del/Ros1* MoneyMaker fruit. The silenced sectors accumulated no anthocyanins (**Fig. 6.9A**). RT-qPCR of cDNA from both non-silenced and silenced sectors on the same fruit indicated that silencing of anthocyanin biosynthetic genes did not affect the expression of *Del/Ros1*. When *SlF3H* was silenced, expression of early anthocyanin genes (*SlPAL, SlCHS1* and *SlCHI*) as well as *SlDFR* was reduced (**Fig. 6.9B**). When *SlDFR* was silenced, there was no

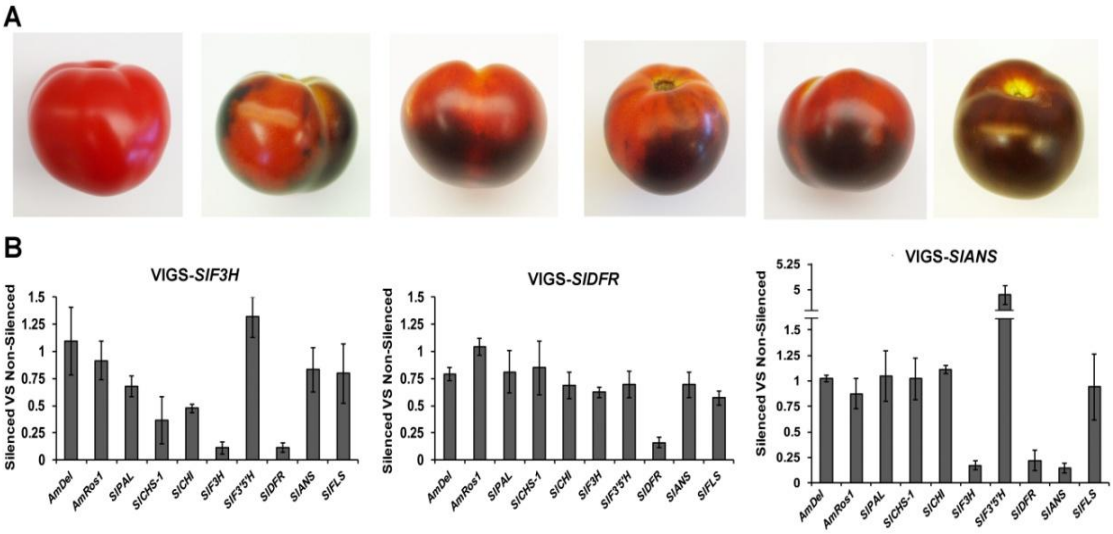

Figure 6.9 Silencing of anthocyanin biosynthetic genes in *Del/Ros1* tomato alters anthocyanin biosynthetic pathway.

(**A**). From left to right, phenotype of WT, VIGS-*Del/Ros1*, VIGS-*SlF3H*, VIGS-*SlDFR*, VIGS-*SlANS* and *Del/Ros1* MoneyMaker fruit. Pictures were taken at two weeks after breaker. (**B**) Expression of anthocyanin biosynthetic genes in VIGS fruit. Data are represented by comparing the expression of silenced sectors to the non-silenced sectors on the same fruit. Error bars show SEM (n≥2).

significant reduction in the expression levels of other genes (**Fig. 6.9B**). Interestingly, in VIGS-*SlANS* silenced sectors, although late anthocyanin biosynthetic genes (*SlF3H*, *SlDFR* and *SlANS*) were silenced, a significant induction of *SlF3'5'H* was found (**Fig. 6.9B**).

F3'5'H is a P450 which catalyses the addition of –OH groups to the C-3' and 5' positions on the B-ring. When *F3'5'H* expression was induced, flavonoids with three –OH groups on the B ring were produced. LC-MS data indicated that very little anthocyanin was present in any of the silenced sectors of VIGS-*SlF3H*, VIGS-*SlDFR* and VIGS-*SlANS* fruit (**Fig. 6.10A**). However, the VIGS-*SlANS* silenced sectors contained increased amounts of myricetin (a flavonol which has 3 –OH groups on its B-ring) derivatives (compound 8, **Fig. 6.10B**) which are likely to be the result of induction of *F3'5'H* expression.

6.3.7 The shelf life of VIGS fruit is positively correlated with the total antioxidant capacity of the silenced sectors.

The total antioxidant capacity of silenced sectors on different VIGS fruit was changed because these sectors accumulated different compounds. Compared to WT MoneyMaker fruit, the antioxidant capacity of hydrophilic compounds was about 3-fold higher in *Del/Ros1* MoneyMaker fruit. However, in silenced sectors of different VIGS fruit, the total antioxidant capacities were decreased. Silencing of *Del/Ros1* in purple tomato caused the loss of most of higher antioxidant capacity. The VIGS-*Del/Ros1* fruit had only marginally higher TEAC values than WT fruit. Silencing of *SlF3H* and *SlDFR* resulted in the absence of most of the anthocyanins (present in the purple, non-silenced sectors) and their TEAC values were reduced to only 2-fold higher than WT tomatoes. For VIGS-*SlANS* fruit, although silenced sectors did not have most of the anthocyanins present in purple sectors, enrichment in myricetin derivatives compensated, in part, and the TEAC values were not much lower than in purple sectors. The silenced VIGS-*SlANS* sectors had TEAC values 2.5-fold higher than those of WT fruit.

Storage tests of ripe fruit showed a good correlation between total antioxidant capacity and storage time. VIGS-*SlF3H* fruit showed ripening defects (the silenced

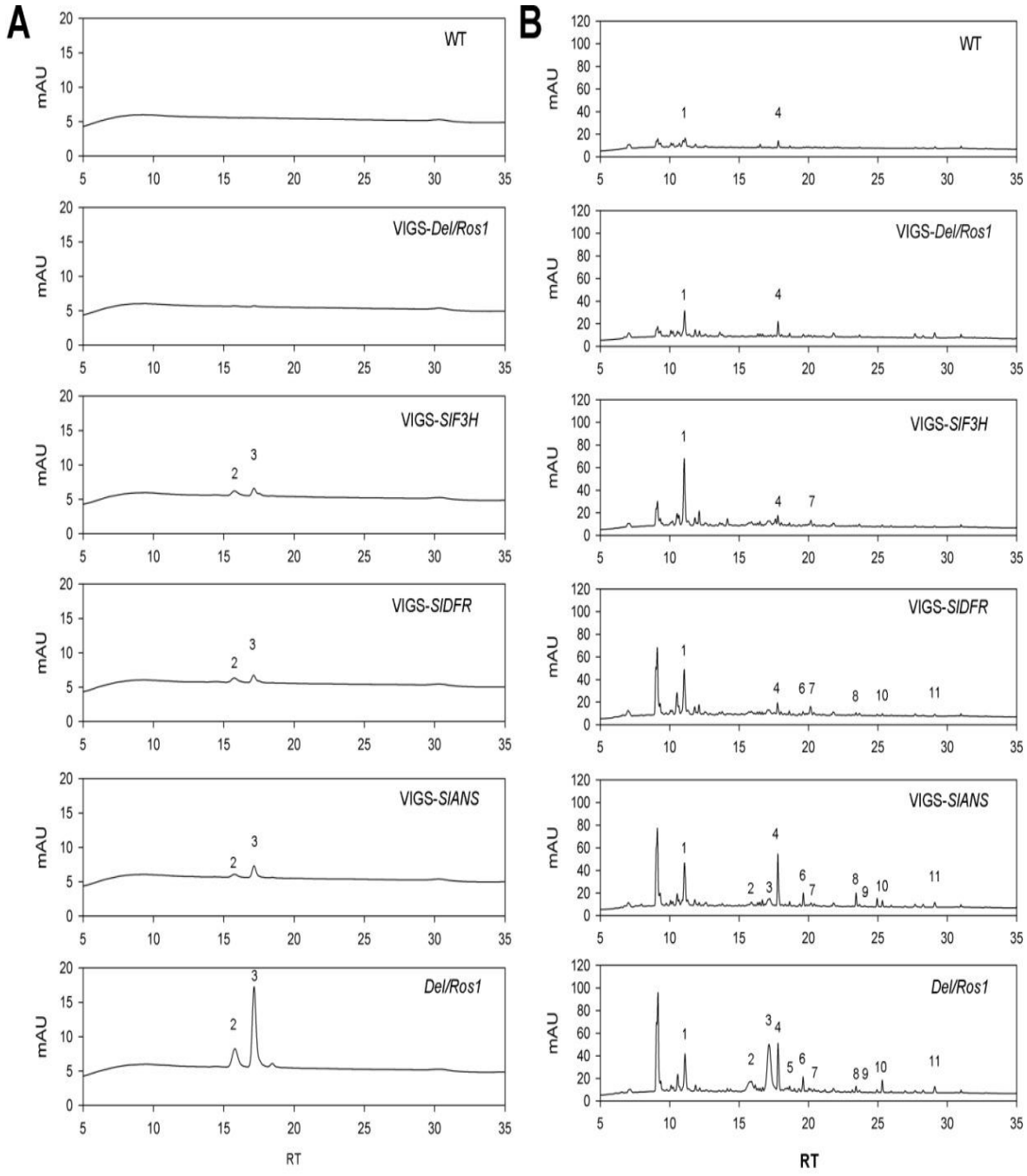

Figure 6.10 Silencing of anthocyanin biosynthetic genes in *Del/Ros1* tomato accumulates different flavonoid compounds

(**A**) Comparative HPLC analysis of methanol extracts of tomato fruit recorded at 525 nm showing the accumulation of anthocyanin compounds in different fruit.

(**B**) Comparative HPLC analysis of methanol extracts of tomato fruit recorded at 280 nm showing the accumulation of phenylpropanoid compounds in different fruit.

Compounds indentified are: 1. Cholorgenic acid, 2. Delphinidin 3-(trans-coumaroyl)-rutinoside-5-glucoside (Del-Cou-Rut-Glc), 3. Petunidin 3-(trans-coumaroyl)-rutinoside-5-glucoside (Pet-Cou-Rut-Glc), 4. Rutin, 5. Malvidin- coumaroyl)-rutinoside-glucoside (Mal-Cou-Rut-Glc), 6. Kaempfero- rutinoside, 7. Naringin, 8. Myricetin-coumaroyl-rutinoside (Myr-Cou-Rut), 9. Methyl myricetin- coumaroyl-rutinoside (MeMyr-Cou-Rut), 10. Quercetin-coumaroyl-rutinoside (Que-Cou-Rut). 11. Naringenin.

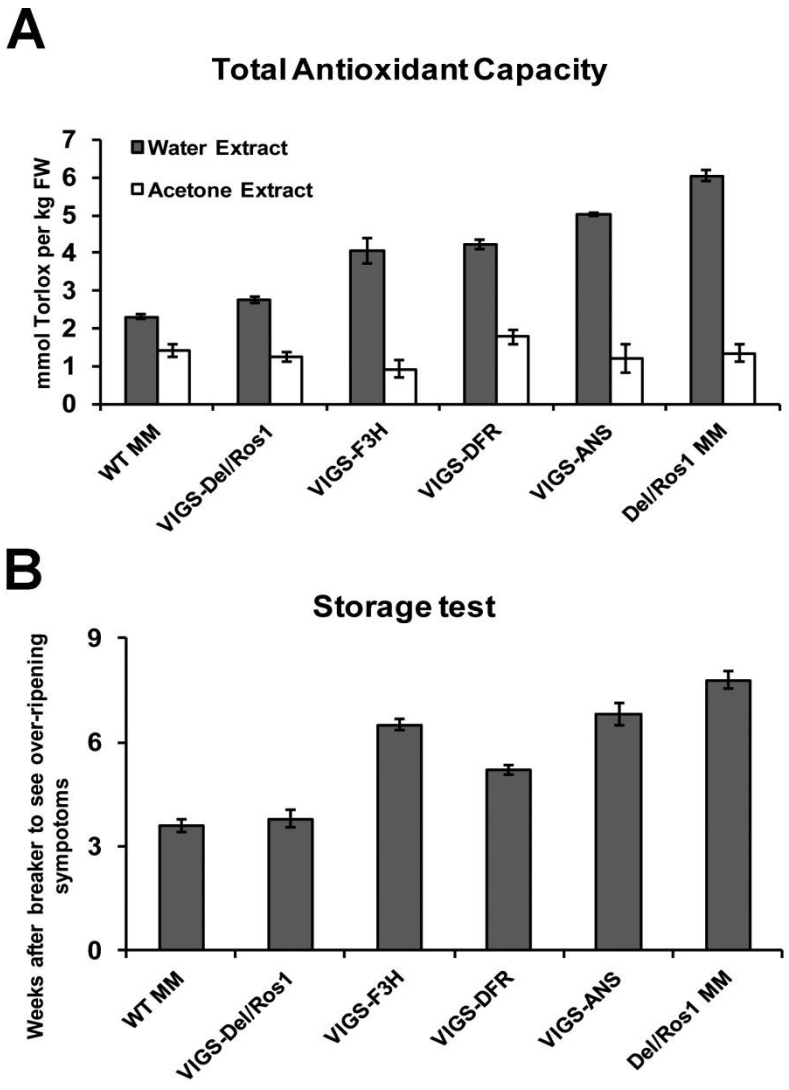

Figure 6.11 The length of viable storage period for VIGS fruit is positively associated with their total antioxidant capacity.

(**A**) Total antioxidant capacity of water and acetone extracts from WT and different VIGS fruits. Error bars show SEM (n=3).

(**B**) Times of storage length of different VIGS fruits to see over-ripening symptoms. Fruits were harvested 2 weeks after breaker, and the times to show overripening symptoms (visual rotting and collapse of fruit) were recorded. Error bars show the SEM, n =10.

parts showed un-even ripening and the fruit tissues became very hard). Apart from this, the viable storage time of different fruit increased as their total antioxidant capacity increased (**Fig. 6.11B**). These data suggest that increased antioxidant capacity is the main cause of the delay in over-ripening in purple tomatoes.

6.3.8 Susceptibility of VIGS fruit to *Botrytis* infection

When the silenced sectors of different VIGS fruit were innoculated with *Botrytis* and compared with normal *Del/Ros1* tomatoes at 3dpi, *SlANS*-silenced fruit showed nearly the same level of resistance to infection as purple *Del/Ros1* fruit. However, *SlDF*R-silenced tomatoes lost this resistance and became as susceptible as the VIGS-*Del/Ros1 fruit* (**Fig. 6.12**). Silencing of *SlF3H* also reduced the pathogen resistance although this remained better than that of VIGS-*SlDFR* fruit (**Fig. 6.12**). This might be due to the impaired ripening observed in VIGS-*SlF3H* fruit.

Compared to normal *Del/Ros1* MoneyMaker tomatoes, the silenced sectors of VIGS-*SlDFR* fruit lost most of their anthocyanins (delphinidin derivatives and petunidin derivatives) and their pathogen resistance was abolished. This indicates that delphinidin and petunidin derivatives in purple tomato contribute specifically to pathogen resistance. Although the silenced sectors of VIGS-*SlANS* fruit, lost most of their anthocyanins, they accumulated increased amounts of myricetin derivatives and still had good resistance to *B. cinerea*. These data suggest that myricetin derivatives in tomato also contribute to resistance.

6.3.9 Natural mutants confirm the importance of specific flavonoid compounds to susceptibility to *B. cinerea*.

In order to confirm my results from *Botrytis* infection of silenced sectors of VIGS-*SlDFR* and VIGS-*SlANS* fruit, two natural anthocyanin mutants in the Ailsa Craig genetic background, *aw* (LA3281) and *ae* (LA3612), were crossed with *Del/Ros1* tomato. The *aw* mutant is a natural DFR-deficient mutant and *ae* lacks ANS activity.

Compared to *Del/Ros1* Ailsa Craig fruit, $aw^{-/-}$ *Del/Ros1* fruit produced no anthocyanins but were enriched with kaempferol derivatives (**Fig. 6.13** and **Fig. 6.14**). The hydrophilic antioxidant capacity was two-fold higher than that of WT

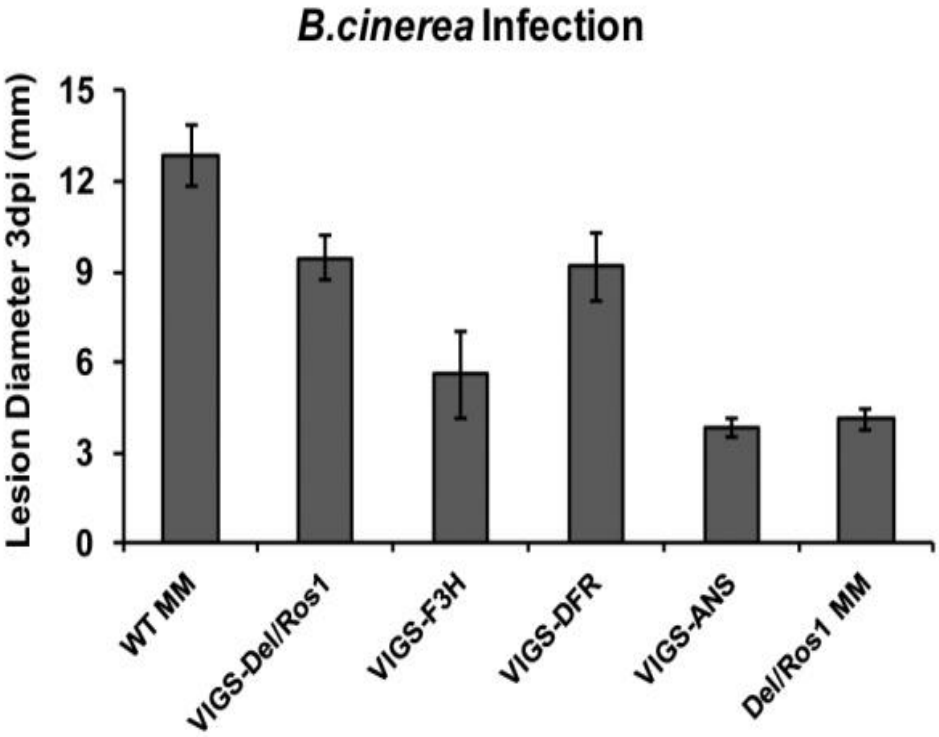

Figure 6.12. Susceptibility of different VIGS fruit to *B.cinerea* infection.

Lesion diameters were measured at 3 dpi. Error bars indicate SEM (n=10).

Figure 6.13 Phenotypes of *Del/Ros1* tomato crossed with anthocyanin biosynthetic mutants.

From left to right: WT Ailsa Craig, *Del/Ros1* Ailsa Craig, *aw*$^{-/-}$ *Del/Ros1* and *ae*$^{-/-}$ *Del/Ros1*. Fruit were harvest at 14 dpb.

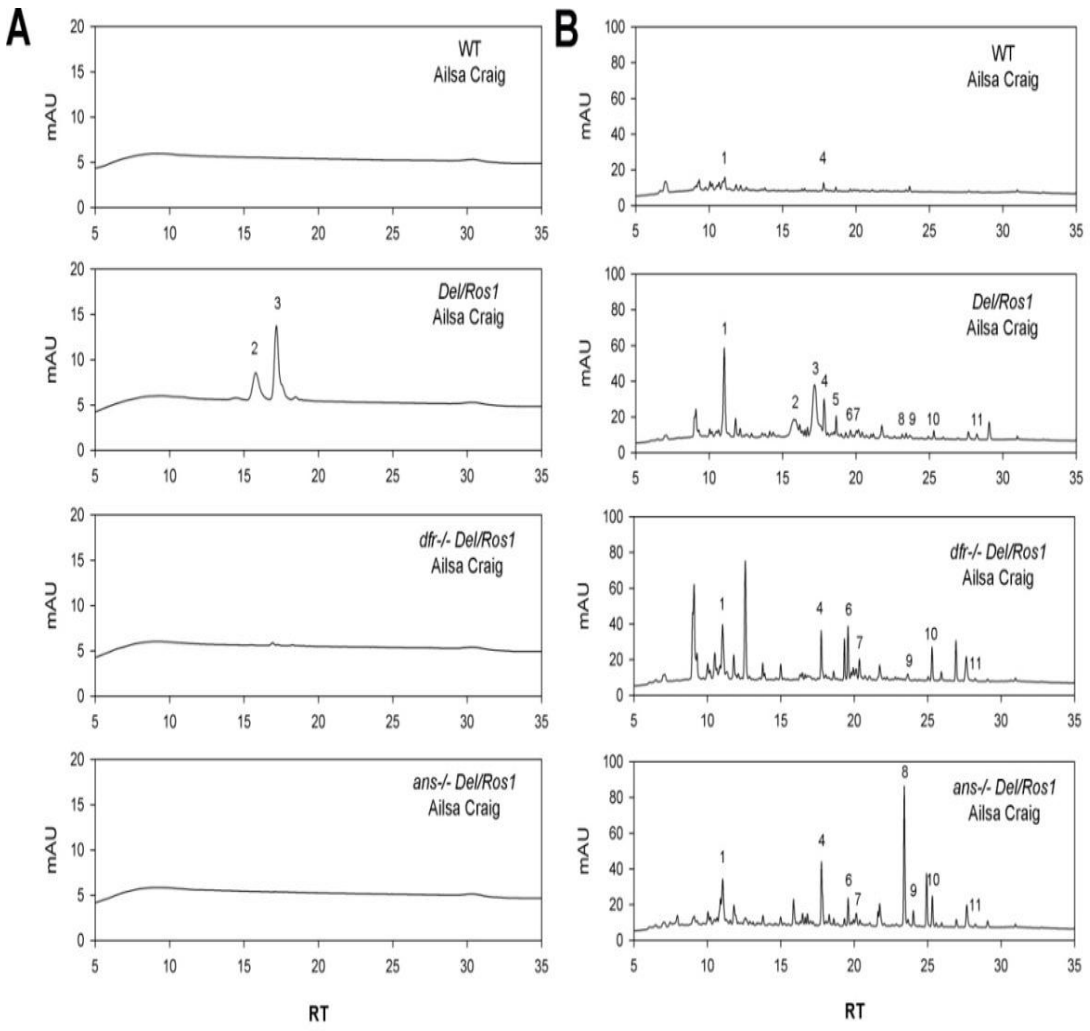

Figure 6.14 Crossing anthocyanin mutants with *Del/Ros1* tomato generates hybrids containing different flavonoid compounds.

(**A**) Comparative HPLC analysis of methanol extracts of tomato fruit recorded at 525 nm showing the accumulation of anthocyanin compounds in fruit of different lines.

(**B**) Comparative HPLC analysis of methanol extracts of tomato fruit recorded at 280 nm showing the accumulation of phenylpropanoid compounds in different fruit. Annotation of compound is the same as in **Figure 6.10**.

Ailsa Craig fruit (**Fig. 6.15A**). For *ae*$^{-/-}$ *Del/Ros1* fruit, however, the content of myricetin derivatives was increased (**Fig.6.14**) and the TEAC value was about three fold that of WT (**Fig.6.15A**).

Storage tests indicated that both *aw*$^{-/-}$ *Del/Ros 1* and *ae*$^{-/-}$ *De1/Ros1* fruit can be stored longer than WT Ailsa Craig fruit. However, compared to *ae*$^{-/-}$ *Del/Ros1* fruit which can be stored more than twice as long as WT fruit, *aw*$^{-/-}$ *Del/Ros1* could only be stored about 1.5 times longer than WT Ailsa Craig fruit (**Fig. 6.15B**). This matched the TEAC values of these lines (**Fig. 6.15A**). All these data support my previous conclusion that the strength of delay in over ripening is dependent on the total antioxidant capacity of the tomato fruit.

When different fruit were inoculated with *B. cinerea* spores, the infection lesions on *ae*$^{-/-}$ *Del/Ros1* and *Del/Ros1* fruit were significantly smaller than those on WT and *aw*$^{-/-}$ *Del/Ros1* fruit at 3dpi. This indicated that *ae*$^{-/-}$ *Del/Ros1* fruit still have resistance to *B. cinerea* while the *aw*$^{-/-}$ *Del/Ros1* fruit lose their resistance (**Fig. 6.15C**). These results from natural mutants, combined with the results from fruit VIGS, indicated that the silencing of DFR activity in purple tomatoes increases pathogen susceptibility whereas silencing of ANS activity maintains pathogen resistance. The *Del/Ros1* fruit lacking ANS activity make myricetin derivatives and still have resistance to *B. cinerea* infection. These data suggest that myricetin derivatives, as well as delphinidin and petunidin derivatives, can contribute to resistance to *B. cinerea* in tomato fruit.

6.3.10 Supplementation of compounds during *B. cinerea* inoculation of tomato affects the susceptibility to the pathogen

To confirm different effects of flavonoids on susceptibility to *Botrytis*, pure flavonoids were purchased. The two major anthocyanins (Delphinidin 3-o-(coumaroyl) rutinoside, 5-o-glucoside (Del-Cou-Rut-Glc) and Petunidin 3-o-(coumaroyl) rutinoside, 5-o-glucoside (Pet-Cou-Rut-Glc)) were purified from the *Del/Ros1* purple tomatoes by HPLC purification by Katharina Bulling.

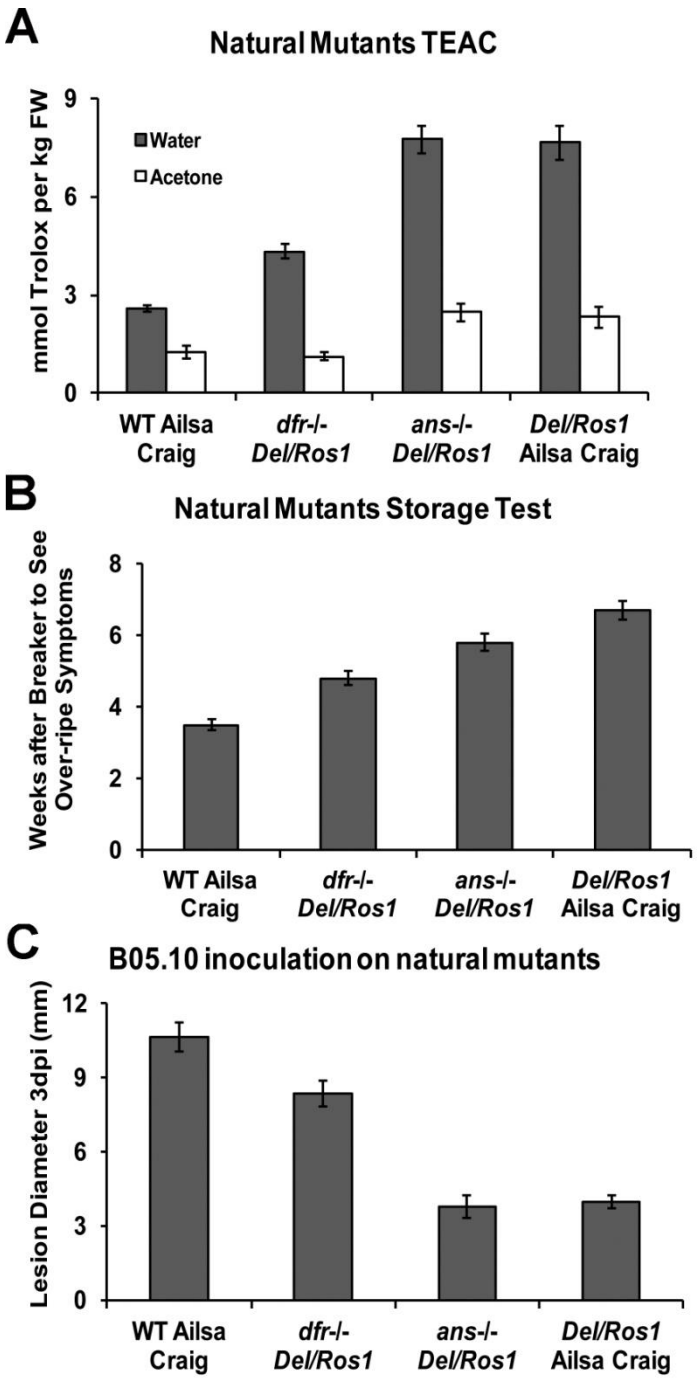

Figure 6.15 Shelf life of different natural mutants

(**A**) Total antioxidant capacity of different fruit.

(**B**) Storage test of different fruit. Fruits were harvested 2 weeks after breaker, and the times to show over ripening symptoms (visual rotting and collapse of fruit) were recorded. Error bars show the SEM (n =10).

(**C**) Susceptibility of different fruit to *B.cinerea infection*. Lesion diameters were measured at 3 dpi. Error bars indicate SEM (n=10).

My previous data indicated that supplementation of tomato juices during inoculation with *B. cinerea* can affect the susceptibility of WT tomatoes to the pathogen (**Fig. 6.8C**). To screen for the compounds that influence susceptibility to *Botrytis*, standard compounds at different concentrations were supplied to inoculation sites on WT tomatoes.

Supplementation with the two anthocyanins purified from tomato, as well as with myricetin and quercetin, showed dose-dependent inhibition of *B. cinerea* lesion development. For kaempferol, rutin and chlorogenic acid supplements, no significant inhibition was seen at final concentrations of 1 mM (**Fig. 6.16**). These data indicate that anthocyanins, as well as myricetin and quercetin, can reduce the susceptibility of tomato fruit to *B. cinerea* infection.

In vitro growth assays showed that supplementation of delphinidin, petunidin, quercetin and myricetin derivatives in medium did not inhibit the normal growth of *Botrytis* (**Fig. 6.17**). This indicated that the effects of these compounds on *Botrytis* infection were not due to their direct inhibition of *Botrytis* growth.

6.3.11 Supplementation of flavonoids during *B. cinerea* inoculation of leaves also affects the susceptibility to the pathogen.

In order to confirm the inhitory effects of different purified compounds, four-week old Arabidopsis plants grown in short days were inoculated with *B. cinerea* spores containing different amounts of flavonoid compounds. Similar dose-dependent inhibition was seen with supplementation with myricetin, quercetin, petunidin and delphinidin derivatives in infection of leaves by *Botrytis* (**Fig. 6.18**).

Flavonoid supplements to inoculations of *Botrytis* on 5 week old tobacco plants grown in short days gave similar results. Compared to control inoculations on the same leaf, Pet-Cou-Rut-Glc, Del-Cou-Rut-Glc, myricetin and quecertin supplements significantly inhibited lesion development on leaves (**Fig. 6.19**).

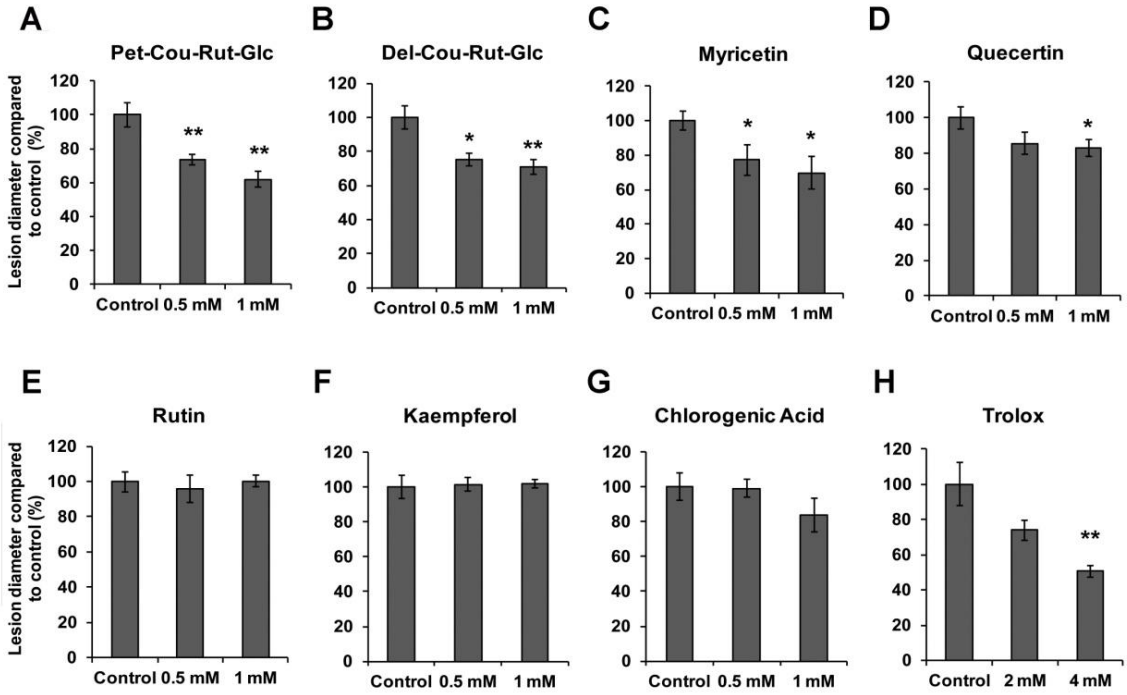

Figure 6.16 Effects of supplements of flavonoids to *B.cinerea* inocula on lesion size in WT tomato fruit.

Compounds at different concentrations, as well as control (5% EtOH) were supplied to different inoculation sites on the same fruit. Data are presented as the ratio of lesion diameter to the average lesion diameter of control treatments. Error bars show SEM (n=6). * and ** indicate $p<0.05$ and $p<0.01$.

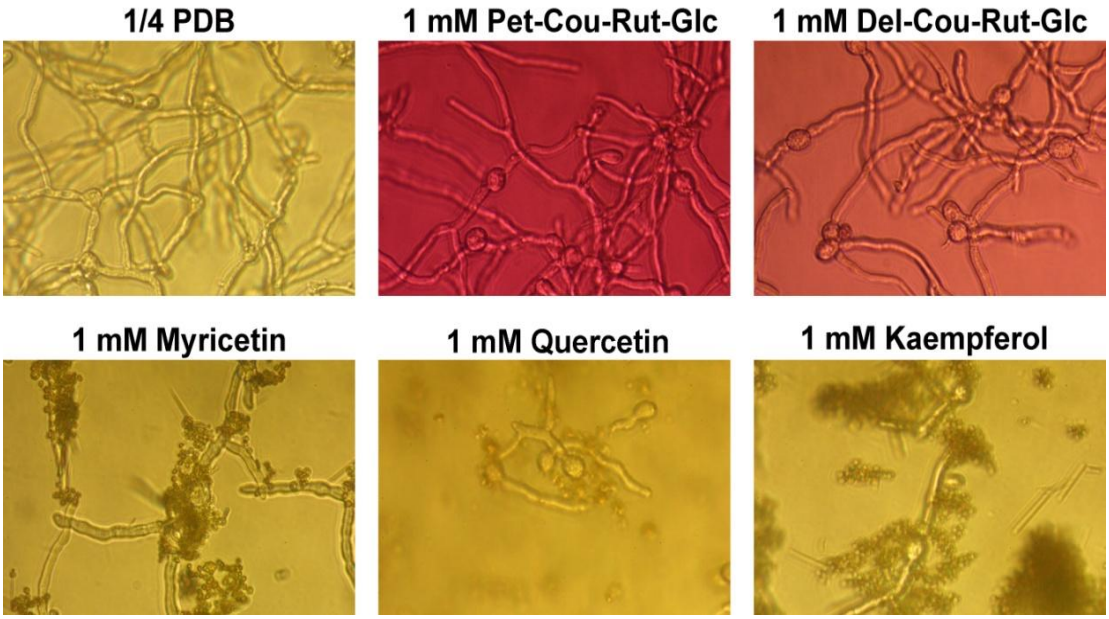

Figure 6.17 Effects of flavonoid derivatives added to to growth culture medium on the growth of *B.cinerea*.

1 mM of different compounds was added to the standard ¼ strength PDB medium containing 5×10^4 spores/ml *B.cinerea* spores. Pictures were taken at 16 hpi.

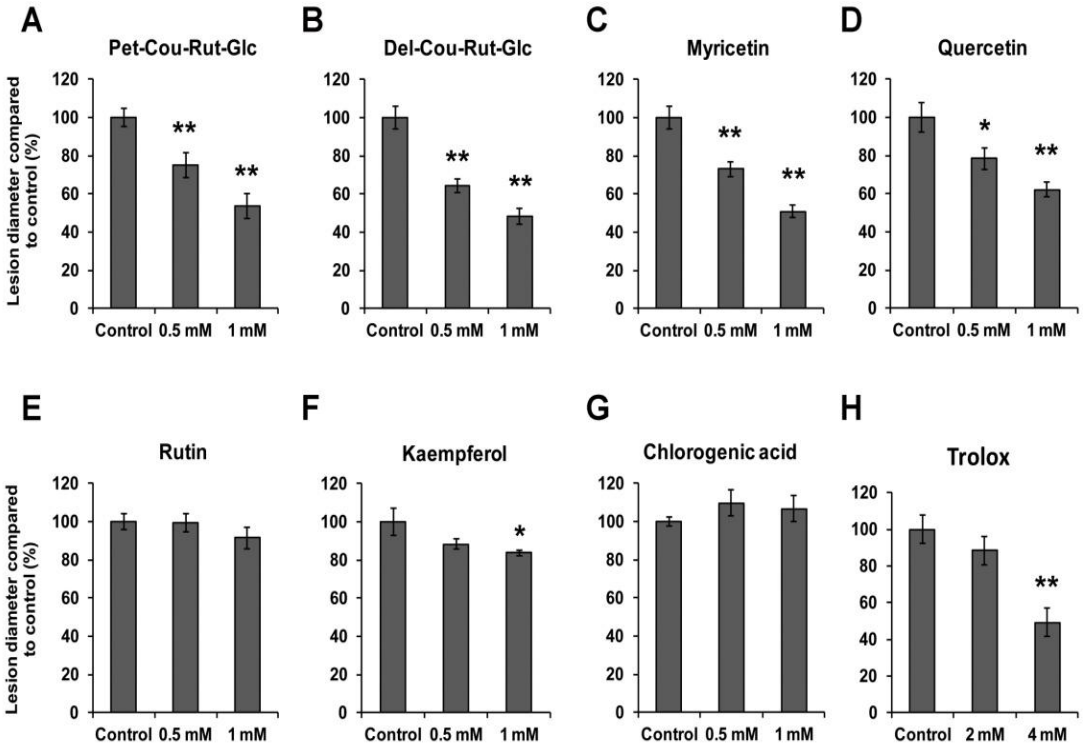

Figure 6.18 Effects of flavonoid supplements to *B.cinerea* inocula on growth in WT *Arabidopsis* leaves.

Compounds at different concentrations, as well as control (5% EtOH) were supplied to different inoculation sites on the same plant. Data are presented as the ratio of lesion diameter to the average lesion diameter of control treatments. Error bars show SEM (n=12). * and ** indicate $p<0.05$ and $p<0.01$.

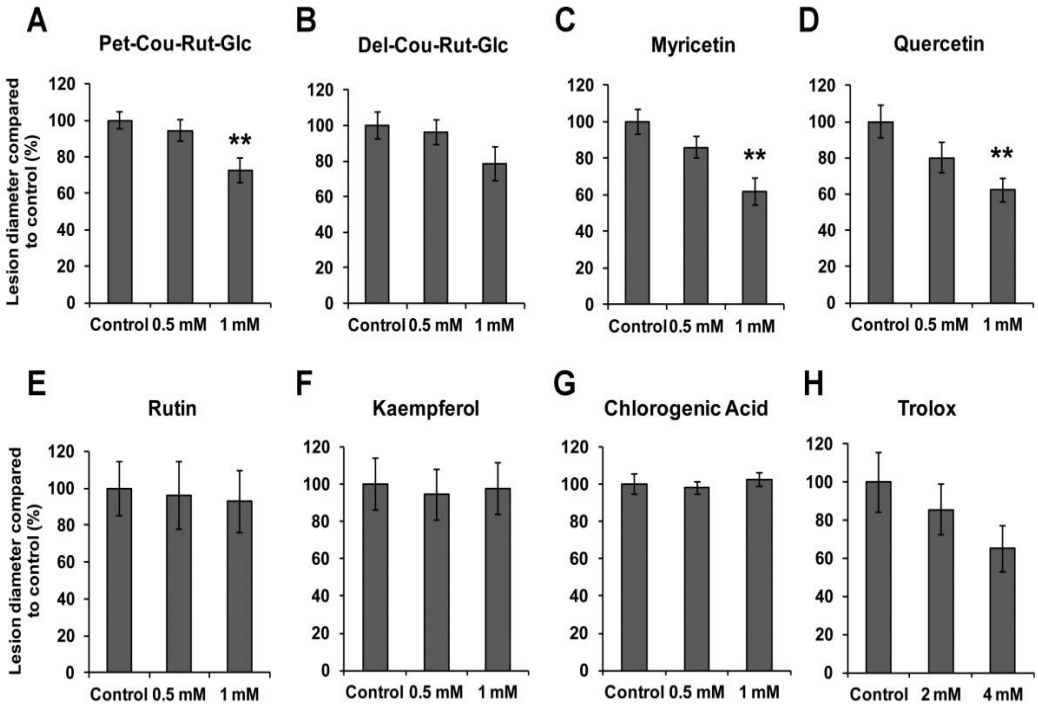

Figure 6.19 Effects of flavonoid supplements to *B.cinerea* inocula on lesion size in WT tobacco leaves.

Compounds at different concentration, as well as control (5% EtOH) were supplied to different inoculation sites on the same leaf. Data are presented as the ratio of lesion diameter to the average lesion diameter of control treatment. Error bars show SEM (n=6). ** indicates $p<0.01$.

6.3.12 –OH groups on the B-ring determine the scavenging ability of flavonoids, as well as the capacity to decrease susceptibility to *Botrytis* infection.

Previous data have shown that the scavenging ability of flavonoids is mainly determined by the –OH groups on the B-ring (Burda and Oleszek, 2001; Rice-Evans et al., 1996). Compared to kaempferol which only has one –OH on the 4' position of the B-ring, compounds such as myricetin and quecertin have an extra –OH on the C-3' position of the B-ring. Thus they have higher scavenging ability. Myrecetin has two pairs of hydroxyl groups in *cis* on the B-ring, further increasing its ability to act as an antioxidant.

Measurements of total antioxidant capacity indicated that compounds that have 2 or 3 –OHs on the B-ring have higher antioxidant capacity than compounds that have only one –OH (**Fig. 6.20B**). Compared to a Trolox standard, myricetin, delphinidin and petunidin derivatives have 2.5 fold higher antioxidant capacity. For quercetin, the antioxidant capacity was even higher, reaching 3 fold more than the Trolox standard. For kaempferol, which contains just one –OH group on the B-ring, the antioxidant capacity is lower than for compounds with 2 or 3 –OH groups on the B-ring (kaempferol antioxidant capacity showed no sigificant difference to the Trolox standard) (**Fig. 6.20B**). The antioxidant capacity of rutin, which is the rutinoside of quercetin, is very low despite it having two –OH groups on its B-ring. This might be the result of the glycosylation on the 3 position on the C ring.

A good correlation between the scavenging ability of the compounds and their ability to inhibit *Botrytis* infection *in vivo* was observed. Flavonoid compounds with –OH groups on the C-3' position of the B-ring have higher scavenging ability and appear to contribute better to reducing susceptibility to *B. cinerea*.

6.4 Discussion

6.4.1 *AtMYB12* tomato fruit have delayed over-ripening

My previous data suggested that tomato fruit with higher antioxidant capacity show slower over-ripening. *AtMYB12* tomatoes have increased levels of flavonols and their antioxidant capacity is about 3 times higher than WT tomato (Luo et al., 2008). At the beginning of over-ripening, *AtMYB12* tomatoes show delayed over-ripening

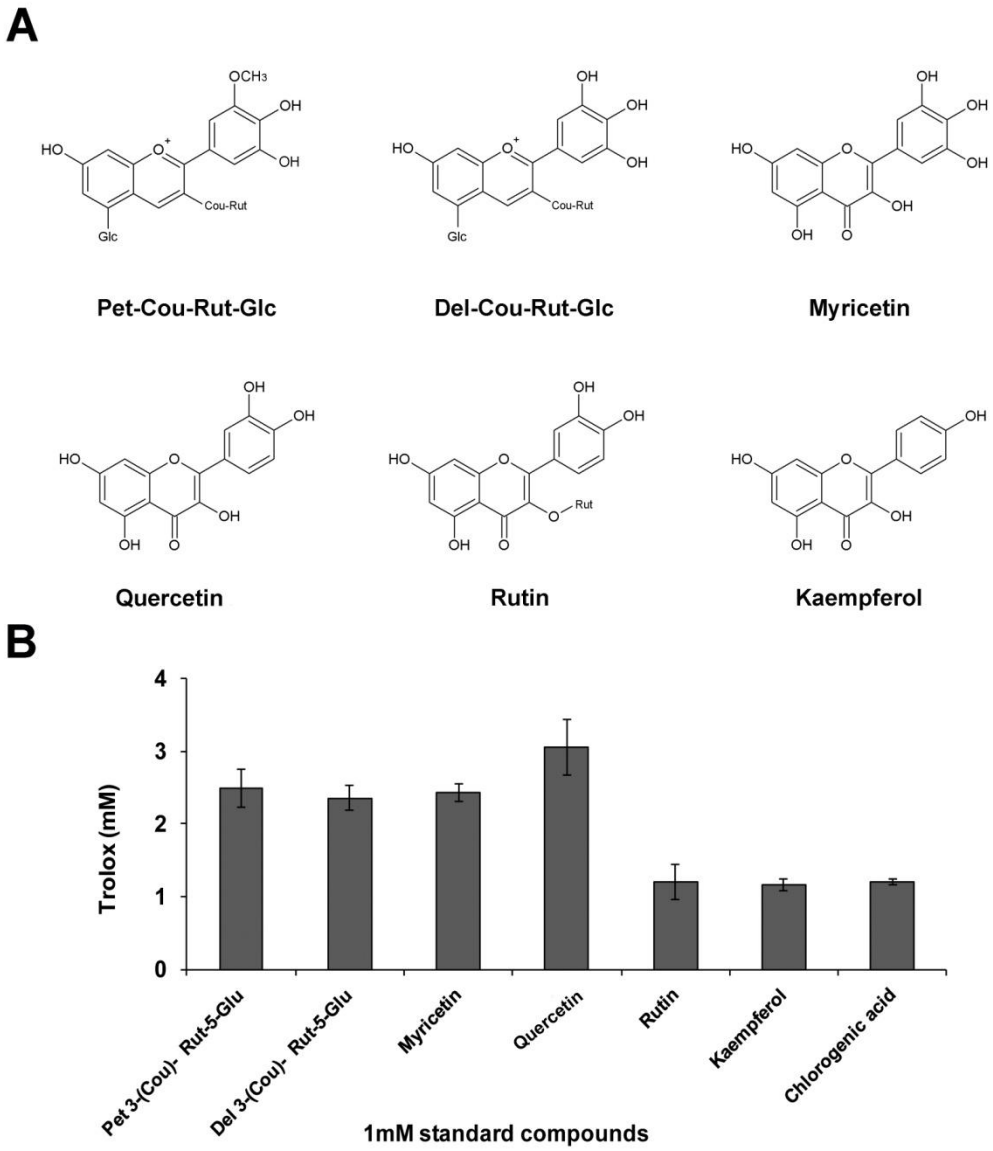

Figure 6.20 The scavenging ability of flavonoid compounds is associated with the number of –OH groups on the B-ring.

(**A**) Structure of different flavonoid compounds.

(**B**) Trolox equivelant total antioxidant capacity of different compounds. Data are shown as the antioxidant capacity equivalent to that of 1 mM Trolox . Error bars show SEM (n=3).

compared to WT fruit. This was due to their higher antioxidant capacities. However, my data indicate that the effect of flavonols in *AtMYB12* fruit was not as durable as the effect of anthocyanins in *Del/Ros1* tomatoes. After 3-4 weeks storage (6 weeks after breaker), there were significant reductions in the hydrophilic antioxidant capacity and increased production of MDA in *AtMYB12* tomato fruit (**Fig. 6.3**). The reasons for rapid loss of hydrophilic antioxidant capacity late in ripening in *AtMYB12* tomato fruit need to be investigated further.

6.4.2 High hydrophilic antioxidant capacity is the factor determining delayed over-ripening.

In both VIGS fruit and storage tests of natural mutants, a good correlation between the length of over-ripening and antioxidant capacity of fruit was found. When compared within the same background, fruit with higher antioxidant capacity could be stored longer than those with lower antioxidant capacities (**Fig. 6.11** and **6.15**).

We suggest that oxidative stress is a key factor in determining the rate of over-ripening of tomato fruit. During normal fruit ripening, oxidative stress increases as the fruit undergo different metabolic and physiological changes. Once oxidative stress reaches a certain level at which the scavenging system can no longer work effectively, oxidative damage will accumulate. Once oxidative damage reaches a certain point, it may directly or indirectly trigger downstream signaling pathways which accelerate over-ripening. For fruit with low antioxidant capacity, oxidative damage accumulates rapidly post-breaker (**Fig. 6.3B**). Thus over-ripening comes strongly and quickly in regular fruit. Fruit with higher hydrophilic antioxidant capacity, have a better ability to scavenge free radicles and to reduce oxidative stress. So, in these fruit, oxidative damage accumulates more slowly and over-ripening is delayed and weaker.

6.4.3 *AtMYB12* has a low ability to alter ROS dynamics during *B. cinerea* infection.

RT-qPCR results indicate that there is no significant difference in expression of these genes between WT and *AtMYB12* tomato fruit (**Fig 6.6A** to **D**). However, the expression of the HR response gene, *SlHSR203,* was highly induced in WT and

AtMYB12 tomatoes after *B. cinerea* inoculation (**Fig. 6.6E**). Activation of *HSR203*, was found to be correlated with the programmed cell death triggered by HR (Pontier et al., 1998). These data suggest that *AtMYB12* tomatoes have a stronger HR response to *B. cinerea* infection than anthocyanin-enriched *Del/Ros1* tomatoes.

Although there were differences between the responses of different lines to pretreatment with plant hormones, the responses of the different lines to ROS inducer/inhibitor pretreatment were consistent: pretreatment with a ROS inducer can increase the susceptibility of fruit to *B. cinerea*, while ROS inhibitor treatments reduce this susceptibility (**Fig 6.7**). These data indicate an important role for ROS dynamics in infection of ripe fruit by *B. cinerea*.

Previous data indicate that the ROS burst negatively contributes to resistance to *Botrytis* (Govrin and Levine, 2000). HR responses triggered by the ROS burst provide an efficient way for plants to resist biotrophic pathogens. However, nectrophic pathogens such as *B. cinerea*, benefit from the death of the host cell (Glazebrook, 2005). During infection, necrotrophic pathogens can even produce effectors to activate the ROS burst to promote pathogenesis (Alkan et al., 2009; Kim et al., 2008). Previous data showed that tomato fruit that have high anthocyanin contents can better alter the ROS dynamics during *B. cinerea* infection, thus suppressing lesion development. For *AtMYB12*, although the total hydrophilic antioxidant capacity of the fruit is high, its ability to quench any ROS burst is low. This indicates that, unlike the delayed over-ripening, the ability for fruit to scavenge ROS during pathogen infection is not determined by the general antioxidant capacity, and instead, the scavenging ability of specific compounds is more decisive.

6.4.4 VIGS provides excellent platform to investigate the roles of different polyphenol compounds in tomato.

In normal tomato fruit, there is no anthocyanin accumulation. Therefore it is impossible to check the function of different anthocyanin biosynthetic genes and related compounds in tomato fruit. The *Del/Ros1* purple tomato is an excellent tool for research on anthocyanin biosynthesis. My data indicate that particular anthocyanin biosynthesis genes can be silenced effectively by VIGS. The purple

tomato provides a visual system enabling one to distinguish the silenced parts of fruit by visual inspection. By blocking particular genes, the biosynthetic pathway can be stopped at certain steps. HPLC data indicate that the metabolic profiles match the gene expression profiles. This system can be used to accumulate intermediate compounds in flavonol biosynthesis, with a visual marker. I have used this system to dissect out the role of different compounds in the extended shelf life of purple tomatoes.

6.4.5 –OH group number on the B-ring of flavonoids is associated with reduced susceptibility to *Botrytis*.

Using VIGS and natural mutants, I found that silencing of *DFR* and *ANS* had different effects on susceptibility to *Botrytis*, in *Del/Ros1* tomato. Silencing of *SlANS* in *Del/Ros1* tomatoes, reduced the production of anthocyanins in silenced sectors, but maintained the reduced susceptibility to *B. cinerea*. LC-MS data indicate myricetin derivatives, a group of flavonols which have three –OH groups on the B-ring, accumulated in VIGS-*SlANS* tomatoes. This was due to the induction of *SlF3'5'H* expression in the VIGS-*SlANS* fruit.

In the Aisa Craig genetic background, HPLC data indicated that normal purple tomato fruit are enriched with delphinidin and petunidin (both have 3 –OH groups on B ring). For the *ans*$^{-/-}$ *Del/Ros1* mutant, although it lacks anthocyanin, the myricetin (3 –OHs on B ring) content is increased. For *aw*$^{-/-}$ *Del/Ros1* fruit, however, instead of myricetin, kaemperfol (with just one –OH group on its B ring) is enriched (Fig.13). Like the VIGS fruit, *aw*$^{-/-}$ *Del/Ros1* Ailsa Craig fruit are susceptible *to B. cinerea* while *ae*$^{-/-}$ *Del/Ros1* fruit show reduced susceptibility: the number of –OH groups on the B ring seems to be associated with susceptibility to *Botrytis*.

To sum up, increasing the general antioxidant capacity of tomato fruit is an efficient way to delay over-ripening. Reduced susceptibility to *Botrytis* seems to require specific flavonoid compounds. My previous data indicated that anthocyanins suppress ROS to inhibit HR during the period of *Botrytis* spread. The ability to scavenge ROS is associated with the number of –OH groups on the B ring. Previous data indicated that the –OH groups on the B ring are important for the scavenging

ability of hydrophilic polyphenol compounds. Compounds with more –OHs on their B ring have superior scavenging ability (Burda and Oleszek, 2001; Rice-Evans et al., 1996). My data suggested that accumulation of flavonoid compounds with three –OH groups on their B-ring can decrease the susceptibility of tomato to *Botrytis*. This highlights the importance of the structure of the flavonoids that accumulate in determining susceptibility to *Botrytis*.

Chapter 7: Summary and Outlook

7.1 Roles of flavonoid compounds in determining the shelf life of tomato fruit

Shelf life extension is one of the most important challenges for the modern tomato industry. Although there are already many different approaches being developed to extend shelf life, most of them inevitably cause losses in fruit quality (Baldwin et al., 2011; Vicente et al., 2007).

Previously, by expressing *Delila* and *Rosea 1*, two transcription factors from snapdragon (*Antirrhinum majus*), under the control of the fruit-specific E8 promoter, Butelli and co-workers produced tomato fruit with a high content of anthocyanins (Butelli et al., 2008; Goodrich et al., 1992; Schwinn et al., 2006). While growing the purple tomatoes, we noticed they had extended shelf life compared to WT fruit. The shelf life of tomato fruit is defined as the period during which the tomatoes remain consumable and is normally determined by the degree of softening, shrivelling and rotting of fruit. Two factors determine shelf life: fruit softening late during ripening and susceptibility to pathogens, normally necrotrophic rotting pathogens. The purpose of my PhD research was to explore the mechanisms determining the extended shelf life in the anthocyanin-enriched purple tomatoes. I also showed that enrichment of anthocyanins in tomato skin by conventional breeding can extend the shelf life of tomato fruit. In addition, I extended the knowledge of shelf life extension to other flavonoid compounds.

7.1.1 Accumulation of anthocyanins can extend the shelf life of tomato fruit

Results presented in Chapter 3 and 4 imply that anthocyanin accumulation in *Del/Ros1* tomato can extend shelf life by delaying over-ripening and reducing pathogen susceptibility to *Botrytis cinerea*.

My data showed that anthocyanin accumulation in tomato fruit can double the the time for over-ripening. In purple, *Del/Ros1* tomato, it takes twice as long to see the a similar degree of over-ripening to those in the red, WT fruit (**Fig 3.2**). During normal ripening and over-ripening, the activity of the fruit antioxidant systems declines while the oxidative stress accumulates (Jimenez et al., 2002) (**Fig. 3.7A**). Accumulation of anthocyanins in *Del/Ros1* tomatoes increases the total antioxidant

capacity of fruit significantly (**Fig. 3.7B**). The increased antioxidant capacity slows down the accumulation of oxidative damage and ROS, which are thought to be signals in ripening and over-ripening. Slower ROS accumulation can cause the suppression of ripening-related genes and thus slows down over-ripening (**Fig 3.5** and **3.6**).

Enrichment of anthocyanins in purple tomato can significantly reduce the susceptibility to *B. cinerea* (**Fig. 4.1-4.4**). Anthocyanins do not directly suppress the growth of *B. cinerea* (**Fig. 4.7**). However, they can alter the dynamics of the ROS burst during the *B. cinerea* infection of tomato fruit (**Fig. 4.8**). The ROS burst is thought to contribute negatively to *B. cinerea* resistance (Glazebrook, 2005; Govrin and Levine, 2000) and alteration of ROS dynamics can change the susceptibility of tomato to *B. cinerea* (**Fig. 4.8** and **6.7**).

7.1.2 Findings from GM crop research can provide new strategies for conventional breeding.

Besides GM anthocyanin-enriched tomatoes, there are several tomato varieties produced by conventional breeding that accumulate anthocyanins in fruit. Data from Chapter 5 indicate that accumulation of anthocyanins in the skin of *Aft/Aft atv/atv* fruit, a conventionally bred variety, also extends shelf life.

To confirm that accumulation of anthocyanins in tomato skin is sufficient to extend shelf life, I expressed the *Ros1* gene predominantly in tomato skin by using the PLI promoter. Accumulation of anthocyanins in *PRD* tomato skin reduced susceptibility to *B. cinerea.*

My data indicate that scientific findings from research on GM crops can inform conventional breeding strategies. From the GM *E8*:*Del/Ros1* tomato, I found that anthocyanins can extend tomato shelf life. I hypothesized that varieties which accumulate anthocyanins in fruit might also have extended shelf life. Using the conventionally bred variety *Aft/Aft atv/atv*, I tested my hypothesis. In addition, I showed that accumulation of anthocyanins in tomato skin is sufficient to extend shelf life. Using new GM *PRD* tomato line, I confirmed this new hypothesis.

7.1.3 Scavenging ability decides the role of flavonoid compounds in shelf life extension and the scavenging ability of flavonoid compounds is determined mainly by their chemical structure.

Anthocyanins belong to a larger group of plant secondary metabolic compounds called flavonoids. There are significant structural similarities between anthocyanins and other flavonoids, so enrichment of other flavonoids in tomato fruit may also contribute to shelf life extension. Data from Chapter 6 show that accumulation of flavonols, a group of flavonoid compounds, in *AtMYB12* tomato can also delay over-ripening of fruit. However, compared to anthocyanin-enriched *Del/Ros1* tomatoes (**Fig. 6.1-6.4**), *AtMYB12* tomatoes are susceptible to *B. cinerea* (**Fig. 6.4** and **6.5**).

Using VIGS and natural mutants, I then showed that delayed over-ripening is associated with the high total antioxidant capacity of flavonoids in enriched tomatoes. Accumulation of flavonoids in tomato fruit can be used as a strategy to delay over-ripening. However, in reducing susceptibility to *B. cinerea*, different flavonoids have different effects. Assays using purified flavonoids indicated that the number of hydroxyl groups on the B-ring is a key factor to determining the scavenging ability of flavonoids, and this scavenging ability directly determines the ability of flavonoids to reduce susceptibility to *B. cinerea* in tomato. My data establish the correlation between structure, chemical characters and the biological functions of flavonoids.

7.2 Prospects of understanding the roles of flavonoids in shelf life extension.

My data indicate the important roles of flavonoids in determining the shelf life of tomatoes. However, as tomato fruit is not a very good model for pathogen studies, there remain several questions to be addressed. It will be interesting to see whether the roles of flavonoids in shelf life extension are species specific.

7.2.1 Application of flavonoids in other crops to extend shelf life

My data indicate that in tomato, flavonoids can increase the antioxidant capacity of the fruit tissue. Increased antioxidant capacity can slow down the accumulation of

oxidative damage and thus slow down over-ripening. It will be interesting to test whether this finding can be applied to other climacteric fruit such as apple, melon and peach. There are several apple and peach anthocyanin mutants (Chagne et al., 2013; Espley et al., 2009; Espley et al., 2007; Werner et al., 1998), it will be interesting to test whether these varieties have extended shelf life.

Suppression of oxidative processes has been established as an efficient way to extend shelf life of other crops. For instance, suppression of ROS accumulation can significantly extend the shelf life of cassava (Zidenga et al., 2012). It will be interesting to see whether we can alter the ROS dynamics of other plants during ripening or post-harvest storage by other approaches, and whether these treatments will in turn extend the shelf life of a wide range of crops.

7.2.2 Purify more flavonoid compounds directly from tomato fruit and test their activities

Supplementation of two *Del/Ros1* tomato anthocyanin compounds Delphinidin 3-o-(coumaroyl) rutinoside, 5-o-glucoside (Del-Cou-Rut-Glc) and Petunidin 3-o-(coumaroyl) rutinoside, 5-o-glucoside (Pet-Cou-Rut-Glc) can significantly reduce the susceptibility of tomato fruit to *Botrytis cinerea*. However, due to limitations of time and technology, I did not have the opportunity to purify other flavonoids directly from *Del/Ros1* tomatoes or natural mutants crossed with *Del/Ros1* tomato. I already showed that the decoration of flavonoids has a significant impact on the compounds activities on susceptibility to *Botrytis* (see the difference between standard quercetin and rutin in **Fig. 6.17**-**6.20**). In my thesis, the major flavonols tested were standard compounds purchased commercially. However, in tomato fruit, they are present in other derivative forms. Thus their activities might be different from the standard compounds, tested. To better analyse the roles of different flavonoids in tomato, it will be necessary to purify the major flavonol derivatives from natural mutants or *AtMYB12* tomato. These purified compounds, can then be tested with the two anthocyanin derivatives to analyse their roles in determining the susceptibility to *Botrytis* in tomato fruit.

7.2.3 Dissecting the mechanisms by which flavonoids affect pathogen susceptibility in the model plant, *Arabidopsis thaliana*

Arabidopsis thaliana is the best model to study plant-pathogen interactions. My preliminary data showed that supplementation of different flavonoids in inoculations of *Botrytis* can significantly change susceptibility to the pathogen in WT *Arabidopsis* plants (**Fig. 6.18**). As there are many pathogenesis-related mutants available in *Arabidopsis*, the same screening test can be repeated to further dissect the mechanisms by which flavonoids influence susceptibility.

Using standard and plant-derived compounds, the activities of different flavonoids in determining susceptibility to *Botrytis* can be screened in WT *Col-0* plants. This will help to establish the association between the structure of flavonoids and resistance to pathogens. Once an interesting compound has been identified, it can be re-screened using different *Arabidopsis* pathogen resistance mutants (ROS-, ethylene-, JA-, SA- and ABA-related *etc.*). The candidate pathways potentially controlling this process will be examined for responses to different flavonoids.

There are more pathogens available for study in *Arabidopsis*. My data show that flavonoids can reduce the susceptibility only to *Botrytis cinerea* in tomato fruit. I tried other pathogens of tomato but most of them were not virulent even on ripe WT fruit (data not shown). Using *Arabidopsis*, we can check for the effects of flavonoids on other nectrophic pathogens, for example, *Alternaria brassicicola*, as well as on biotrophic pathogens such as *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis.* Using pathogens of different life-types, we can establish a more comprehensive understanding of the roles of flavonoids in pathogen resistance.

Reference

Abeynayake, S.W., Panter, S., Mouradov, A., and Spangenberg, G. (2011). A high-resolution method for the localization of proanthocyanidins in plant tissues. Plant methods *7*, 13.

Adams-Phillips, L., Barry, C., Kannan, P., Leclercq, J., Bouzayen, M., and Giovannoni, J. (2004). Evidence that CTR1-Mediated Ethylene Signal Transduction in Tomato is Encoded by a Multigene Family Whose Members Display Distinct Regulatory Features. Plant molecular biology *54*, 387-404.

Al-sane, K.O., Povero, G., and Perata, P. (2011). Anthocyanin tomato mutants: Overview and characterization of an anthocyanin-less somaclonal mutant. Plant Biosystems *145*, 436-444.

Alba, R., Payton, P., Fei, Z., McQuinn, R., Debbie, P., Martin, G.B., Tanksley, S.D., and Giovannoni, J.J. (2005). Transcriptome and selected metabolite analyses reveal multiple points of ethylene control during tomato fruit development. Plant Cell *17*, 2954-2965.

Alkan, N., Davydov, O., Sagi, M., Fluhr, R., and Prusky, D. (2009). Ammonium secretion by Colletotrichum coccodes activates host NADPH oxidase activity enhancing host cell death and fungal virulence in tomato fruits. Molecular plant-microbe interactions : MPMI *22*, 1484-1491.

Alvarez, M.E., Pennell, R.I., Meijer, P.J., Ishikawa, A., Dixon, R.A., and Lamb, C. (1998). Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell *92*, 773-784.

Ayed, N., Yu, H.L., and Lacroix, M. (1999). Improvement of anthocyanin yield and shelf-life extension of grape pomace by gamma irradiation. Food Research International *32*, 539-543.

Bais, H.P., Vepachedu, R., Gilroy, S., Callaway, R.M., and Vivanco, J.M. (2003). Allelopathy and exotic plant invasion: from molecules and genes to species interactions. Science *301*, 1377-1380.

Baldwin, E., Plotto, A., Narciso, J., and Bai, J. (2011). Effect of 1-methylcyclopropene on tomato flavour components, shelf life and decay as influenced by harvest maturity and storage temperature. J Sci Food Agric *91*, 969-980.

Barry, C.S., Blume, B., Bouzayen, M., Cooper, W., Hamilton, A.J., and Grierson, D. (1996). Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. Plant J *9*, 525-535.

Bhagwan, A., Reddy, Y.N., Rao, P.V., and Mohankumar, K.C. (2000). Shelf life extension of tomato fruits by postharvest antioxidant application. Journal of Applied Horticulture *2*, 88-91

Bolwell, G.P., Davies, D.R., Gerrish, C., Auh, C.K., and Murphy, T.M. (1998). Comparative biochemistry of the oxidative burst produced by rose and french bean cells reveals two distinct mechanisms. Plant Physiol *116*, 1379-1385.

Borevitz, J.O., Xia, Y., Blount, J., Dixon, R.A., and Lamb, C. (2000). Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell *12*, 2383-2394.

Bovy, A., de Vos, R., Kemper, M., Schijlen, E., Almenar Pertejo, M., Muir, S., Collins, G., Robinson, S., Verhoeyen, M., Hughes, S., *et al.* (2002). High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes LC and C1. Plant Cell *14*, 2509-2526.

Bowler, C., Slooten, L., Vandenbranden, S., De Rycke, R., Botterman, J., Sybesma, C., Van Montagu, M., and Inze, D. (1991). Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. The EMBO journal *10*, 1723-1732.

Brummell, D.A., and Harpster, M.H. (2001). Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. Plant molecular biology *47*, 311-340.

Bugos, R.C., Chiang, V.L., Zhang, X.H., Campbell, E.R., Podila, G.K., and Campbell, W.H. (1995). RNA isolation from plant tissues recalcitrant to extraction in guanidine. Biotechniques *19*, 734-737.

Burda, S., and Oleszek, W. (2001). Antioxidant and Antiradical Activities of Flavonoids. Journal of Agricultural and Food Chemistry *49*, 2774-2779.

Butelli, E., Titta, L., Giorgio, M., Mock, H.P., Matros, A., Peterek, S., Schijlen, E.G., Hall, R.D., Bovy, A.G., Luo, J., *et al.* (2008). Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. Nat Biotechnol *26*, 1301-1308.

Cantu, D., Blanco-Ulate, B., Yang, L., Labavitch, J.M., Bennett, A.B., and Powell, A.L. (2009). Ripening-regulated susceptibility of tomato fruit to Botrytis cinerea requires NOR but not RIN or ethylene. Plant Physiol *150*, 1434-1449.

Cantu, D., Vicente, A.R., Greve, L.C., Dewey, F.M., Bennett, A.B., Labavitch, J.M., and Powell, A.L. (2008a). The intersection between cell wall disassembly, ripening, and fruit susceptibility to Botrytis cinerea. Proc Natl Acad Sci U S A *105*, 859-864.

Cantu, D., Vicente, A.R., Labavitch, J.M., Bennett, A.B., and Powell, A.L. (2008b). Strangers in the matrix: plant cell walls and pathogen susceptibility. Trends in plant science *13*, 610-617.

Carey, C.C., Strahle, J.T., Selinger, D.A., and Chandler, V.L. (2004). Mutations in the pale aleurone color1 regulatory gene of the Zea mays anthocyanin pathway have distinct phenotypes relative to the functionally similar TRANSPARENT TESTA GLABRA1 gene in Arabidopsis thaliana. Plant Cell *16*, 450-464.

Carpita, N.C., and Gibeaut, D.M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J *3*, 1-30.

Centeno, D.C., Osorio, S., Nunes-Nesi, A., Bertolo, A.L., Carneiro, R.T., Araujo, W.L., Steinhauser, M.C., Michalska, J., Rohrmann, J., Geigenberger, P., *et al.* (2011). Malate Plays a Crucial Role in Starch Metabolism, Ripening, and Soluble Solid Content of Tomato Fruit and Affects Postharvest Softening. Plant Cell.

Chagne, D., Lin-Wang, K., Espley, R.V., Volz, R.K., How, N.M., Rouse, S., Brendolise, C., Carlisle, C.M., Kumar, S., De Silva, N., *et al.* (2013). An ancient duplication of apple MYB transcription factors is responsible for novel red fruit-flesh phenotypes. Plant Physiol *161*, 225-239.

Chisholm, S.T., Coaker, G., Day, B., and Staskawicz, B.J. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. Cell *124*, 803-814.
Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical biochemistry *162*, 156-159.

Colmenares, A.J., Aleu, J., Duran-Patron, R., Collado, I.G., and Hernandez-Galan, R. (2002). The putative role of botrydial and related metabolites in the infection mechanism of Botrytis cinerea. Journal of chemical ecology *28*, 997-1005.

De Jong, W.S., Eannetta, N.T., Jong, D.M., and Bodis, M. (2004). Candidate gene analysis of anthocyanin pigmentation loci in the Solanaceae. Theoretical and Applied Genetics *108*, 423-432.

de Jonge, R., van Esse, H.P., Kombrink, A., Shinya, T., Desaki, Y., Bours, R., van der Krol, S., Shibuya, N., Joosten, M.H., and Thomma, B.P. (2010). Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. Science *329*, 953-955.

de Vetten, N., Quattrocchio, F., Mol, J., and Koes, R. (1997). The an11 locus controlling flower pigmentation in petunia encodes a novel WD-repeat protein conserved in yeast, plants, and animals. Genes Dev *11*, 1422-1434.

Dean, R., Van Kan, J.A., Pretorius, Z.A., Hammond-Kosack, K.E., Di Pietro, A., Spanu, P.D., Rudd, J.J., Dickman, M., Kahmann, R., Ellis, J., *et al.* (2012). The Top 10 fungal pathogens in molecular plant pathology. Molecular plant pathology *13*, 414-430.

Dellapenna, D., Lincoln, J.E., Fischer, R.L., and Bennett, A.B. (1989). Transcriptional Analysis of Polygalacturonase and Other Ripening Associated Genes in Rutgers, rin, nor, and Nr Tomato Fruit. Plant Physiol *90*, 1372-1377.

Dhindsa, R.S., Plumb-Dhindsa, P., and Thorpe, T.A. (1981). Leaf Senescence: Correlated with Increased Levels of Membrane Permeability and Lipid Peroxidation, and Decreased Levels of Superoxide Dismutase and Catalase. Journal of Experimental Botany *32*, 93-101.

Dodds, P.N., and Rathjen, J.P. (2010). Plant immunity: towards an integrated view of plant-pathogen interactions. Nature reviews Genetics *11*, 539-548.

Dubos, C., Le Gourrierec, J., Baudry, A., Huep, G., Lanet, E., Debeaujon, I., Routaboul, J.M., Alboresi, A., Weisshaar, B., and Lepiniec, L. (2008). MYBL2 is a new regulator of flavonoid biosynthesis in Arabidopsis thaliana. Plant J *55*, 940-953.

Durrant, W.E., and Dong, X. (2004). Systemic acquired resistance. Annu Rev Phytopathol *42*, 185-209.

Egea, I., Barsan, C., Bian, W., Purgatto, E., Latché, A., Chervin, C., Bouzayen, M., and Pech, J.-C. (2010). Chromoplast Differentiation: Current Status and Perspectives. Plant and Cell Physiology *51*, 1601-1611.

El Oirdi, M., El Rahman, T.A., Rigano, L., El Hadrami, A., Rodriguez, M.C., Daayf, F., Vojnov, A., and Bouarab, K. (2011). Botrytis cinerea Manipulates the Antagonistic Effects between Immune Pathways to Promote Disease Development in Tomato. Plant Cell *23*, 2405-2421.

Elad, Y. (1992). The use of antioxidants (free radical scavengers) to control grey mould (Botrytis cinerea) and white mould (Sclerotinia sclerotiomm) in various crops. Plant Pathology *41*, 417-426.

Espley, R.V., Brendolise, C., Chagne, D., Kutty-Amma, S., Green, S., Volz, R., Putterill, J., Schouten, H.J., Gardiner, S.E., Hellens, R.P., *et al.* (2009). Multiple repeats of a promoter segment causes transcription factor autoregulation in red apples. Plant Cell *21*, 168-183.

Espley, R.V., Hellens, R.P., Putterill, J., Stevenson, D.E., Kutty-Amma, S., and Allan, A.C. (2007). Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. Plant J *49*, 414-427.

Estornell, L.H., Orzaez, D., Lopez-Pena, L., Pineda, B., Anton, M.T., Moreno, V., and Granell, A. (2009). A multisite gateway-based toolkit for targeted gene expression and hairpin RNA silencing in tomato fruits. Plant biotechnology journal *7*, 298-309.

Falcone Ferreyra, M.L., Casas, M.I., Questa, J.I., Herrera, A.L., Deblasio, S., Wang, J., Jackson, D., Grotewold, E., and Casati, P. (2012). Evolution and expression of tandem duplicated maize flavonol synthase genes. Frontiers in plant science *3*, 101.

Fallik, E., Grinberg, S., Lomaniec, E., Lurie, S., and Lalazar, A. (1993). Effect of postharvest heat treatment of tomatoes on fruit ripening and decay caused by Botrytis cinerea. Plant Disease *77*, 985-988.

Feild, T.S., Lee, D.W., and Holbrook, N.M. (2001). Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of red-osier dogwood. Plant Physiol *127*, 566-574.

Fillatti, J.J., Kiser, J., Rose, R., and Comai, L. (1987). Efficient Transfer of a Glyphosate Tolerance Gene into Tomato Using a Binary Agrobacterium Tumefaciens Vector. Nature Biotechnology *5*, 726 - 730.

Fujisawa, M., Nakano, T., and Ito, Y. (2011). Identification of potential target genes for the tomato fruit-ripening regulator RIN by chromatin immunoprecipitation. BMC plant biology *11*, 26.

Gillaspy, G., Ben-David, H., and Gruissem, W. (1993). Fruits: A Developmental Perspective. Plant Cell *5*, 1439-1451.

Giovannoni, J.J. (2004). Genetic regulation of fruit development and ripening. Plant Cell *16 Suppl*, S170-180.

Giovannoni, J.J. (2007). Fruit ripening mutants yield insights into ripening control. Current opinion in plant biology *10*, 283-289.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol *43*, 205-227.

Goff, S.A., Cone, K.C., and Chandler, V.L. (1992). Functional analysis of the transcriptional activator encoded by the maize B gene: evidence for a direct functional interaction between two classes of regulatory proteins. Genes Dev *6*, 864-875.

Goldsbrough, A., Belzile, F., and Yoder, J.I. (1994). Complementation of the Tomato anthocyanin without (aw) Mutant Using the Dihydroflavonol 4-Reductase Gene. Plant Physiol *105*, 491-496.

Gonzali, S., Mazzucato, A., and Perata, P. (2009). Purple as a tomato: towards high anthocyanin tomatoes. Trends in plant science *14*, 237-241.

Goodrich, J., Carpenter, R., and Coen, E.S. (1992). A common gene regulates pigmentation pattern in diverse plant species. Cell *68*, 955-964.

Gould, K.S., McKelvie, J., and Markham, K.R. (2002). Do anthocyanins function as antioxidants in leaves? Imaging of H2O2 in red and green leaves after mechanical injury. Plant, Cell & Environment *25*, 1261-1269.

Govrin, E.M., and Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen Botrytis cinerea. Curr Biol *10*, 751-757.

Grant, J.J., Yun, B.W., and Loake, G.J. (2000). Oxidative burst and cognate redox signalling reported by luciferase imaging: identification of a signal network that functions independently of ethylene, SA and Me-JA but is dependent on MAPKK activity. Plant J *24*, 569-582.

Grierson, D., and Tucker, G. (1983). Timing of ethylene and polygalacturonase synthesis in relation to the control of tomato fruit ripening. Planta *157*, 174-179.
Grotewold, E. (2006). The genetics and biochemistry of floral pigments. Annu Rev Plant Biol *57*, 761-780.

Grotewold, E., Athma, P., and Peterson, T. (1991). Alternatively spliced products of the maize P gene encode proteins with homology to the DNA-binding domain of myb-like transcription factors. Proceedings of the National Academy of Sciences *88*, 4587-4591.

Hamberger, B., Ellis, M., Friedmann, M., de Azevedo Souza, C., Barbazuk, B., and Douglas, C.J. (2007). Genome-wide analyses of phenylpropanoid-related genes in Populus trichocarpa, Arabidopsis thaliana, and Oryza sativa: the Populus lignin toolbox and conservation and diversification of angiosperm gene familiesThis article is one of a selection of papers published in the Special Issue on Poplar Research in Canada. Canadian Journal of Botany *85*, 1182-1201.

Hassan, S., and Mathesius, U. (2012). The role of flavonoids in root-rhizosphere signalling: opportunities and challenges for improving plant-microbe interactions. Journal of experimental botany *63*, 3429-3444.

Have, A.t., Mulder, W., Visser, J., and van Kan, J.A.L. (1998). The Endopolygalacturonase Gene Bcpg1 Is Required for Full Virulence of Botrytis cinerea. Molecular Plant-Microbe Interactions *11*, 1009-1016.

Heim, K.E., Tagliaferro, A.R., and Bobilya, D.J. (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. The Journal of Nutritional Biochemistry *13*, 572-584.

Herrmann, K.M., and Weaver, L.M. (1999). The Shikimate Pathway. Annual review of plant physiology and plant molecular biology *50*, 473-503.

Hua, J., and Meyerowitz, E.M. (1998). Ethylene responses are negatively regulated by a receptor gene family in Arabidopsis thaliana. Cell *94*, 261-271.

Huckelhoven, R., and Kogel, K.H. (2003). Reactive oxygen intermediates in plant-microbe interactions: who is who in powdery mildew resistance? Planta *216*, 891-902.

Iriti, M., Rossoni, M., Borgo, M., and Faoro, F. (2004). Benzothiadiazole enhances resveratrol and anthocyanin biosynthesis in grapevine, meanwhile improving resistance to Botrytis cinerea. J Agric Food Chem *52*, 4406-4413.

Jackson, D., Roberts, K., and Martin, C. (1992). Temporal and spatial control of expression of anthocyanin biosynthetic genes in developing flowers of Antirrhinum majus. The Plant journal : for cell and molecular biology *2*, 425-434.

Jakob, K., Kniskern, J.M., and Bergelson, J. (2007). The role of pectate lyase and the jasmonic acid defense response in Pseudomonas viridiflava virulence. Molecular plant-microbe interactions : MPMI *20*, 146-158.

Jimenez, A., Creissen, G., Kular, B., Firmin, J., Robinson, S., Verhoeyen, M., and Mullineaux, P. (2002). Changes in oxidative processes and components of the antioxidant system during tomato fruit ripening. Planta *214*, 751-758.

Jones, C.M., Mes, P., and Myers, J.R. (2003). Characterization and Inheritance of the Anthocyanin fruit (Aft) Tomato. Journal of Heredity *94*, 449-456.

Jones, J.D., and Dangl, J.L. (2006). The plant immune system. Nature *444*, 323-329.
Kevany, B.M., Tieman, D.M., Taylor, M.G., Cin, V.D., and Klee, H.J. (2007). Ethylene receptor degradation controls the timing of ripening in tomato fruit. Plant J *51*, 458-467.

Kim, K.S., Min, J.-Y., and Dickman, M.B. (2008). Oxalic Acid Is an Elicitor of Plant Programmed Cell Death during Sclerotinia sclerotiorum Disease Development. Molecular Plant-Microbe Interactions *21*, 605-612.

Klee, H.J., and Giovannoni, J.J. (2011). Genetics and control of tomato fruit ripening and quality attributes. Annual review of genetics *45*, 41-59.

Kopeliovitch, E., Rabinowitch, H.D., Mizrahi, Y., and Kedar, N. (1979). The potential of ripening mutants for extending the storage life of the tomato fruit. Euphytica *28*, 99-104.

Kramer, M., Sanders, R., Bolkan, H., Waters, C., Sheeny, R.E., and Hiatt, W.R. (1992). Postharvest evaluation of transgenic tomatoes with reduced levels of polygalacturonase: processing, firmness and disease resistance. Postharvest Biology and Technology *1*, 241-255.

Kuzniak, E., and Sklodowska, M. (2005). Fungal pathogen-induced changes in the antioxidant systems of leaf peroxisomes from infected tomato plants. Planta *222*, 192-200.

La Camera, S., Gouzerh, G., Dhondt, S., Hoffmann, L., Fritig, B., Legrand, M., and Heitz, T. (2004). Metabolic reprogramming in plant innate immunity: the contributions of phenylpropanoid and oxylipin pathways. Immunological reviews *198*, 267-284.

Lamb, C., and Dixon, R.A. (1997). The Oxidative Burst in Plant Disease Resistance. Annual review of plant physiology and plant molecular biology *48*, 251-275.

Langley, K.R., Martin, A., Stenning, R., Murray, A.J., Hobson, G.E., Schuch, W.W., and Bird, C.R. (1994). Mechanical and optical assessment of the ripening of tomato fruit with reduced polygalacturonase activity. Journal of the Science of Food and Agriculture *66*, 547-554.

Lashbrook, C.C., Tieman, D.M., and Klee, H.J. (1998). Differential regulation of the tomato ETR gene family throughout plant development. Plant J *15*, 243-252.

Leclercq, J., Adams-Phillips, L.C., Zegzouti, H., Jones, B., Latche, A., Giovannoni, J.J., Pech, J.C., and Bouzayen, M. (2002). LeCTR1, a tomato CTR1-like gene, demonstrates ethylene signaling ability in Arabidopsis and novel expression patterns in tomato. Plant Physiol *130*, 1132-1142.

Lee, J.M., Joung, J.G., McQuinn, R., Chung, M.Y., Fei, Z., Tieman, D., Klee, H., and Giovannoni, J. (2012). Combined transcriptome, genetic diversity and metabolite profiling in tomato fruit reveals that the ethylene response factor SlERF6 plays an important role in ripening and carotenoid accumulation. Plant J *70*, 191-204.

Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. (1994). H2O2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell *79*, 583-593.

Li, J., Ou-Lee, T.M., Raba, R., Amundson, R.G., and Last, R.L. (1993). Arabidopsis Flavonoid Mutants Are Hypersensitive to UV-B Irradiation. Plant Cell *5*, 171-179.
Liu, J., Tian, S., Meng, X., and Xu, Y. (2007). Effects of chitosan on control of postharvest diseases and physiological responses of tomato fruit. Postharvest Biology and Technology *44*, 300-306.

Lorenc-Kukula, K., Jafra, S., Oszmianski, J., and Szopa, J. (2005). Ectopic expression of anthocyanin 5-o-glucosyltransferase in potato tuber causes increased resistance to bacteria. J Agric Food Chem *53*, 272-281.

Lu, R., Martin-Hernandez, A.M., Peart, J.R., Malcuit, I., and Baulcombe, D.C. (2003). Virus-induced gene silencing in plants. Methods *30*, 296-303.

Ludwig, S.R., Habera, L.F., Dellaporta, S.L., and Wessler, S.R. (1989). Lc, a member of the maize R gene family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcriptional activators and contains the myc-homology region. Proc Natl Acad Sci U S A *86*, 7092-7096.

Luo, J., Butelli, E., Hill, L., Parr, A., Niggeweg, R., Bailey, P., Weisshaar, B., and Martin, C. (2008). AtMYB12 regulates caffeoyl quinic acid and flavonol synthesis in tomato: expression in fruit results in very high levels of both types of polyphenol. Plant J *56*, 316-326.

Lytovchenko, A., Eickmeier, I., Pons, C., Osorio, S., Szecowka, M., Lehmberg, K., Arrivault, S., Tohge, T., Pineda, B., Anton, M.T., *et al.* (2011). Tomato fruit photosynthesis is seemingly unimportant in primary metabolism and ripening but plays a considerable role in seed development. Plant Physiol *157*, 1650-1663.

Manning, K., Tor, M., Poole, M., Hong, Y., Thompson, A.J., King, G.J., Giovannoni, J.J., and Seymour, G.B. (2006). A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. Nature genetics *38*, 948-952.

Martel, C., Vrebalov, J., Tafelmeyer, P., and Giovannoni, J.J. (2011). The tomato MADS-box transcription factor RIPENING INHIBITOR interacts with promoters involved in numerous ripening processes in a COLORLESS NONRIPENING-dependent manner. Plant Physiol *157*, 1568-1579.

Martin, C., and Gerats, T. (1993). Control of Pigment Biosynthesis Genes during Petal Development. Plant Cell *5*, 1253-1264.

Martin, C., Prescott, A., Mackay, S., Bartlett, J., and Vrijlandt, E. (1991). Control of anthocyanin biosynthesis in flowers of Antirrhinum majus. Plant J *1*, 37-49.

Martin, C., Zhang, Y., Tomlinson, L., Kallam, K., Luo, J., Jones, J.D.G., Granell, A., Orzaez, D., and Butelli, E. (2012). Colouring up Plant Biotechnology. In Recent Advances in Polyphenol Research (Wiley-Blackwell), pp. 131-142.

Mathews, H., Clendennen, S.K., Caldwell, C.G., Liu, X.L., Connors, K., Matheis, N., Schuster, D.K., Menasco, D.J., Wagoner, W., Lightner, J., *et al.* (2003). Activation tagging in tomato identifies a transcriptional regulator of anthocyanin biosynthesis, modification, and transport. Plant Cell *15*, 1689-1703.

Matsui, K., Umemura, Y., and Ohme-Takagi, M. (2008). AtMYBL2, a protein with a single MYB domain, acts as a negative regulator of anthocyanin biosynthesis in Arabidopsis. Plant J *55*, 954-967.

Maul, F., Sargent, S., Balaban, M., Baldwin, E., Huber, D., and Sims, C. (1998). Aroma Volatile Profiles from Ripe Tomatoes are Influenced by Physiological Maturity at Harvest: An Application for Electronic Nose Technology. J AmSocHort Sci *123*, 1094–1101.

Maul, F., Sargent, S.A., Sims, C.A., Baldwin, E.A., Balaban, M.O., and Huber, D.J. (2000). Tomato Flavor and Aroma Quality as Affected by Storage Temperature. Journal of Food Science *65*, 1228-1237.

Mehrtens, F., Kranz, H., Bednarek, P., and Weisshaar, B. (2005). The Arabidopsis transcription factor MYB12 is a flavonol-specific regulator of phenylpropanoid biosynthesis. Plant Physiol *138*, 1083-1096.

Mehta, R.A., Cassol, T., Li, N., Ali, N., Handa, A.K., and Mattoo, A.K. (2002). Engineered polyamine accumulation in tomato enhances phytonutrient content, juice quality, and vine life. Nat Biotechnol *20*, 613-618.

Meiers, S., Kemeny, M., Weyand, U., Gastpar, R., von Angerer, E., and Marko, D. (2001). The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth-factor receptor. J Agric Food Chem *49*, 958-962.

Meli, V.S., Ghosh, S., Prabha, T.N., Chakraborty, N., Chakraborty, S., and Datta, A. (2010). Enhancement of fruit shelf life by suppressing N-glycan processing enzymes. Proc Natl Acad Sci U S A *107*, 2413-2418.

Mes, P.J., Boches, P., Myers, J.R., and Durst, R. (2008). Characterization of Tomatoes Expressing Anthocyanin in the Fruit. Journal of the American Society for Horticultural Science *133*, 262-269.

Miller, G., Schlauch, K., Tam, R., Cortes, D., Torres, M.A., Shulaev, V., Dangl, J.L., and Mittler, R. (2009). The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. Sci Signal *2*, ra45.

Mittler, R., Herr, E.H., Orvar, B.L., van Camp, W., Willekens, H., Inze, D., and Ellis, B.E. (1999). Transgenic tobacco plants with reduced capability to detoxify reactive oxygen intermediates are hyperresponsive to pathogen infection. Proc Natl Acad Sci U S A *96*, 14165-14170.

Mittler, R., Vanderauwera, S., Gollery, M., and Van Breusegem, F. (2004). Reactive oxygen gene network of plants. Trends in plant science *9*, 490-498.

Mondal, K., Sharma, N.S., Malhotra, S.P., Dhawan, K., and Singh, R. (2004). Antioxidant Systems in Ripening Tomato Fruits. Biologia Plantarum *48*, 49-53.

Muir, S.R., Collins, G.J., Robinson, S., Hughes, S., Bovy, A., Ric De Vos, C.H., van Tunen, A.J., and Verhoeyen, M.E. (2001). Overexpression of petunia chalcone isomerase in tomato results in fruit containing increased levels of flavonols. Nat Biotechnol *19*, 470-474.

Mutschler, M.A., Wolfe, D.W., Cobb, E.D., and Yourstone, K.S. (1992). Tomato Fruit Quality and Shelf Life in Hybrids Heterozygous for the alc Ripening Mutant. HortScience *27*, 352-355.

Nambeesan, S., Datsenka, T., Ferruzzi, M.G., Malladi, A., Mattoo, A.K., and Handa, A.K. (2010). Overexpression of yeast spermidine synthase impacts ripening, senescence and decay symptoms in tomato. Plant J *63*, 836-847.

Nurnberger, T., Brunner, F., Kemmerling, B., and Piater, L. (2004). Innate immunity in plants and animals: striking similarities and obvious differences. Immunological reviews *198*, 249-266.

Oeller, P.W., Lu, M.W., Taylor, L.P., Pike, D.A., and Theologis, A. (1991). Reversible inhibition of tomato fruit senescence by antisense RNA. Science *254*, 437-439.

Oeser, B., Heidrich, P.M., Müller, U., Tudzynski, P., and Tenberge, K.B. (2002). Polygalacturonase is a pathogenicity factor in the Claviceps purpurea/rye interaction. Fungal Genetics and Biology *36*, 176-186.

Olsen, K.M., Slimestad, R., Lea, U.S., Brede, C., Lovdal, T., Ruoff, P., Verheul, M., and Lillo, C. (2009). Temperature and nitrogen effects on regulators and products of the flavonoid pathway: experimental and kinetic model studies. Plant Cell Environ *32*, 286-299.

Orzaez, D., Medina, A., Torre, S., Fernandez-Moreno, J.P., Rambla, J.L., Fernandez-Del-Carmen, A., Butelli, E., Martin, C., and Granell, A. (2009). A visual reporter system for virus-induced gene silencing in tomato fruit based on anthocyanin accumulation. Plant Physiol *150*, 1122-1134.

Orzaez, D., Mirabel, S., Wieland, W.H., and Granell, A. (2006). Agroinjection of tomato fruits. A tool for rapid functional analysis of transgenes directly in fruit. Plant Physiol *140*, 3-11.

Paz-Ares, J., Ghosal, D., Wienand, U., Peterson, P.A., and Saedler, H. (1987). The regulatory c1 locus of Zea mays encodes a protein with homology to myb proto-oncogene products and with structural similarities to transcriptional activators. EMBO J *6*, 3553-3558.

Pellegrini, N., Del Rio, D., Colombi, B., Bianchi, M., and Brighenti, F. (2003). Application of the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation assay to a flow injection system for the evaluation of antioxidant activity of some pure compounds and beverages. J Agric Food Chem *51*, 260-264.

Pontier, D., Tronchet, M., Rogowsky, P., Lam, E., and Roby, D. (1998). Activation of hsr203, a plant gene expressed during incompatible plant-pathogen interactions, is correlated with programmed cell death. Molecular plant-microbe interactions : MPMI *11*, 544-554.

Povero, G., Gonzali, S., Bassolino, L., Mazzucato, A., and Perata, P. (2010). Transcriptional analysis in high-anthocyanin tomatoes reveals synergistic effect of Aft and atv genes. J Plant Physiol.

Powell, A.L.T., van Kan, J., ten Have, A., Visser, J., Greve, L.C., Bennett, A.B., and Labavitch, J.M. (2000). Transgenic Expression of Pear PGIP in Tomato Limits Fungal Colonization. Molecular Plant-Microbe Interactions *13*, 942-950.
Prusky, D. (1996). Pathogen quiescence in postharvest diseases. Annu Rev Phytopathol *34*, 413-434.

Qin, G., Wang, Y., Cao, B., Wang, W., and Tian, S. (2012). Unraveling the regulatory network of the MADS box transcription factor RIN in fruit ripening. Plant J *70*, 243-255.

Quattrocchio, F., Wing, J., van der Woude, K., Souer, E., de Vetten, N., Mol, J., and Koes, R. (1999). Molecular analysis of the anthocyanin2 gene of petunia and its role in the evolution of flower color. Plant Cell *11*, 1433-1444.

Ramsay, N.A., and Glover, B.J. (2005). MYB-bHLH-WD40 protein complex and the evolution of cellular diversity. Trends in plant science *10*, 63-70.

Rice-Evans, C.A., Miller, N.J., and Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free radical biology & medicine *20*, 933-956.

Rick, C.M., Uhlig, J.W., and Jones, A.D. (1994). High alpha-tomatine content in ripe fruit of Andean Lycopersicon esculentum var. cerasiforme: developmental and genetic aspects. Proc Natl Acad Sci U S A *91*, 12877-12881.

Rodoni, L., Casadei, N., Concellon, A., Chaves Alicia, A.R., and Vicente, A.R. (2010). Effect of short-term ozone treatments on tomato (Solanum lycopersicum L.) fruit quality and cell wall degradation. J Agric Food Chem *58*, 594-599.

Rubin, G., Tohge, T., Matsuda, F., Saito, K., and Scheible, W.R. (2009). Members of the LBD family of transcription factors repress anthocyanin synthesis and affect additional nitrogen responses in Arabidopsis. Plant Cell *21*, 3567-3584.

Schwinn, K., Venail, J., Shang, Y., Mackay, S., Alm, V., Butelli, E., Oyama, R., Bailey, P., Davies, K., and Martin, C. (2006). A small family of MYB-regulatory genes controls floral pigmentation intensity and patterning in the genus Antirrhinum. Plant Cell *18*, 831-851.

Segmuller, N., Kokkelink, L., Giesbert, S., Odinius, D., van Kan, J., and Tudzynski, P. (2008). NADPH oxidases are involved in differentiation and pathogenicity in Botrytis cinerea. Molecular plant-microbe interactions : MPMI *21*, 808-819.

Sekher Pannala, A., Chan, T.S., O'Brien, P.J., and Rice-Evans, C.A. (2001). Flavonoid B-Ring Chemistry and Antioxidant Activity: Fast Reaction Kinetics. Biochemical and biophysical research communications *282*, 1161-1168.

Serna, L., and Martin, C. (2006). Trichomes: different regulatory networks lead to convergent structures. Trends in plant science *11*, 274-280.

Seymour, G.B., Ostergaard, L., Chapman, N.H., Knapp, S., and Martin, C. (2013). Fruit development and ripening. Annu Rev Plant Biol *64*, 219-241.

Shah, P., Powell, A.L., Orlando, R., Bergmann, C., and Gutierrez-Sanchez, G. (2012). Proteomic analysis of ripening tomato fruit infected by Botrytis cinerea. Journal of proteome research *11*, 2178-2192.

Shang, Y., Venail, J., Mackay, S., Bailey, P.C., Schwinn, K.E., Jameson, P.E., Martin, C.R., and Davies, K.M. (2010). The molecular basis for venation patterning of pigmentation and its effect on pollinator attraction in flowers of Antirrhinum. New Phytologist *189*, 602-615.

Smart, C.D., Myers, K.L., Restrepo, S., Martin, G.B., and Fry, W.E. (2003). Partial Resistance of Tomato to Phytophthora infestans Is Not Dependent upon Ethylene, Jasmonic Acid, or Salicylic Acid Signaling Pathways. Molecular Plant-Microbe Interactions *16*, 141-148.

Smith, D.L., Abbott, J.A., and Gross, K.C. (2002). Down-regulation of tomato beta-galactosidase 4 results in decreased fruit softening. Plant Physiol *129*, 1755-1762.
Smyth, G.K. (2004). Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Statistical applications in genetics and molecular biology *3*, Article3.

Solano, R., Stepanova, A., Chao, Q., and Ecker, J.R. (1998). Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev *12*, 3703-3714.

Soylu, S., Brown, I., and Mansfield, J.W. (2005). Cellular reactions in Arabidopsis following challenge by strains of Pseudomonas syringae: From basal resistance to compatibility. Physiological and Molecular Plant Pathology *66*, 232-243.

Spelt, C., Quattrocchio, F., Mol, J.N., and Koes, R. (2000). anthocyanin1 of petunia encodes a basic helix-loop-helix protein that directly activates transcription of structural anthocyanin genes. Plant Cell *12*, 1619-1632.

Spoel, S.H., and Dong, X. (2012). How do plants achieve immunity? Defence without specialized immune cells. Nature reviews Immunology *12*, 89-100.

Stracke, R., Ishihara, H., Huep, G., Barsch, A., Mehrtens, F., Niehaus, K., and Weisshaar, B. (2007). Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the Arabidopsis thaliana seedling. Plant J *50*, 660-677.

Tanksley, S.D., Ganal, M.W., Prince, J.P., de Vicente, M.C., Bonierbale, M.W., Broun, P., Fulton, T.M., Giovannoni, J.J., Grandillo, S., Martin, G.B., *et al.* (1992). High density molecular linkage maps of the tomato and potato genomes. Genetics *132*, 1141-1160.

Thaler, J.S., Owen, B., and Higgins, V.J. (2004). The role of the jasmonate response in plant susceptibility to diverse pathogens with a range of lifestyles. Plant Physiol *135*, 530-538.

Thordal-Christensen, H., Zhang, Z., Wei, Y., and Collinge, D.B. (1997). Subcellular localization of H2O2 in plants. H2O2 accumulation in papillae and hypersensitive

response during the barley—powdery mildew interaction. The Plant journal : for cell and molecular biology *11*, 1187-1194.

Tieman, D., Bliss, P., McIntyre, L.M., Blandon-Ubeda, A., Bies, D., Odabasi, A.Z., Rodriguez, G.R., van der Knaap, E., Taylor, M.G., Goulet, C., *et al.* (2012). The chemical interactions underlying tomato flavor preferences. Curr Biol *22*, 1035-1039.

Tieman, D.M., Ciardi, J.A., Taylor, M.G., and Klee, H.J. (2001). Members of the tomato LeEIL (EIN3-like) gene family are functionally redundant and regulate ethylene responses throughout plant development. Plant J *26*, 47-58.

Tieman, D.M., Harriman, R.W., Ramamohan, G., and Handa, A.K. (1992). An Antisense Pectin Methylesterase Gene Alters Pectin Chemistry and Soluble Solids in Tomato Fruit. Plant Cell *4*, 667-679.

Tieman, D.M., and Klee, H.J. (1999). Differential expression of two novel members of the tomato ethylene-receptor family. Plant Physiol *120*, 165-172.

Tieman, D.M., Taylor, M.G., Ciardi, J.A., and Klee, H.J. (2000). The tomato ethylene receptors NR and LeETR4 are negative regulators of ethylene response and exhibit functional compensation within a multigene family. Proc Natl Acad Sci U S A *97*, 5663-5668.

Toledo-Ortiz, G., Huq, E., and Quail, P.H. (2003). The Arabidopsis basic/helix-loop-helix transcription factor family. Plant Cell *15*, 1749-1770.

Tomato_Genome_Consortium (2012). The tomato genome sequence provides insights into fleshy fruit evolution. Nature *485*, 635–641.

Torres, M.A., Dangl, J.L., and Jones, J.D. (2002). Arabidopsis gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc Natl Acad Sci U S A *99*, 517-522.

Torres, M.A., Jones, J.D., and Dangl, J.L. (2006). Reactive oxygen species signaling in response to pathogens. Plant Physiol *141*, 373-378.

Tronchet, M., Ranty, B., Marco, Y., and Roby, D. (2001). HSR203 antisense suppression in tobacco accelerates development of hypersensitive cell death. Plant J *27*, 115-127.

Tsuda, T., Horio, F., Uchida, K., Aoki, H., and Osawa, T. (2003). Dietary cyanidin 3-O-beta-D-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia in mice. J Nutr *133*, 2125-2130.

Tucker, G.A., Robertson, N.G., and Grierson, D. (1980). Changes in Polygalacturonase Isoenzymes during the 'Ripening' of Normal and Mutant Tomato Fruit. European Journal of Biochemistry *112*, 119-124.

van de Veerdonk, F.L., Kullberg, B.J., van der Meer, J.W., Gow, N.A., and Netea, M.G. (2008). Host-microbe interactions: innate pattern recognition of fungal pathogens. Current opinion in microbiology *11*, 305-312.

van Kan, J.A. (2006). Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends in plant science *11*, 247-253.

Vicente, A.R., Saladié, M., Rose, J.K.C., and Labavitch, J.M. (2007). The linkage between cell wall metabolism and fruit softening: looking to the future. Journal of the Science of Food and Agriculture *87*, 1435-1448.

Vogelstein, B., and Gillespie, D. (1979). Preparative and analytical purification of DNA from agarose. Proc Natl Acad Sci U S A *76*, 615-619.

Vrebalov, J., Ruezinsky, D., Padmanabhan, V., White, R., Medrano, D., Drake, R., Schuch, W., and Giovannoni, J. (2002). A MADS-box gene necessary for fruit ripening at the tomato ripening-inhibitor (rin) locus. Science *296*, 343-346.

Walker, A.R., Davison, P.A., Bolognesi-Winfield, A.C., James, C.M., Srinivasan, N., Blundell, T.L., Esch, J.J., Marks, M.D., and Gray, J.C. (1999). The TRANSPARENT TESTA GLABRA1 locus, which regulates trichome differentiation and anthocyanin biosynthesis in Arabidopsis, encodes a WD40 repeat protein. Plant Cell *11*, 1337-1350.

Wang, L.S., and Stoner, G.D. (2008). Anthocyanins and their role in cancer prevention. Cancer Lett *269*, 281-290.

Werner, D., Creller, M., and Chaparro, J. (1998). Inheritance of the Blood-flesh Trait in Peach. HortScience *33*, 1243-1246.

Williams, B., Kabbage, M., Kim, H.J., Britt, R., and Dickman, M.B. (2011). Tipping the balance: Sclerotinia sclerotiorum secreted oxalic acid suppresses host defenses by manipulating the host redox environment. PLoS pathogens *7*, e1002107.

Williamson, B., Tudzynski, B., Tudzynski, P., and Van Kan, J.A.L. (2007). Botrytis cinerea: the cause of grey mould disease. Molecular plant pathology *8*, 561-580.
Winkel-Shirley, B. (2001). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol *126*, 485-493.

Winkel-Shirley, B. (2002). Biosynthesis of flavonoids and effects of stress. Current opinion in plant biology *5*, 218-223.

Xu, F., Yuan, S., Zhang, D.-W., Lv, X., and Lin, H.-H. (2012). The role of alternative oxidase in tomato fruit ripening and its regulatory interaction with ethylene. Journal of experimental botany *63*, 5705-5716.

Yeats, T.H., Buda, G.J., Wang, Z., Chehanovsky, N., Moyle, L.C., Jetter, R., Schaffer, A.A., and Rose, J.K. (2012). The fruit cuticles of wild tomato species exhibit architectural and chemical diversity, providing a new model for studying the

evolution of cuticle function. The Plant journal : for cell and molecular biology *69*, 655-666.

Yokotani, N., Tamura, S., Nakano, R., Inaba, A., and Kubo, Y. (2003). Characterization of a novel tomato EIN3-like gene (LeEIL4). Journal of experimental botany *54*, 2775-2776.

Zhang, F., Gonzalez, A., Zhao, M., Payne, C.T., and Lloyd, A. (2003). A network of redundant bHLH proteins functions in all TTG1-dependent pathways of Arabidopsis. Development *130*, 4859-4869.

Zidenga, T., Leyva-Guerrero, E., Moon, H., Siritunga, D., and Sayre, R. (2012). Extending Cassava Root Shelf Life via Reduction of Reactive Oxygen Species Production. Plant Physiol *159*, 1396-1407.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T., and Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell *125*, 749-760.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D., Felix, G., and Boller, T. (2004). Bacterial disease resistance in Arabidopsis through flagellin perception. Nature *428*, 764-767.

Zuluaga, D.L., Gonzali, S., Loreti, E., Pucciariello, C., Degl'Innocenti, E.D., Guidi, L., Alpi, A., and Perata, P. (2008). Arabidopsis thaliana MYB75/PAP1 transcription factor induces anthocyanin production in transgenic tomato plants. Functional Plant Biology *35*, 606-618.

Appendix 1. Recipes of mediums used in thesis

1. Lysogeny Broth (LB)

To prepare 1L LB liquid, add 10 g tryptone, 5 g yeast extract, and 10 g NaCl Adjust the pH of the medium to 7.0 using 1N NaOH and bring volume up to 1 liter. To prepare LB agar medium, Prepare LB liquid medium as above, but add 15 g/L agar before autoclaving.

2. Potato dextrose agar (PDA)

Potatoes, Infusion	200	g
Dextrose	20	g
Agar	15	g
Distilled water	1 L	(total volume)

Autoclaved at 121ºC, 15 minutes

3. Malt Extract Agar (MEA)

Malt extract	20	g
Peptone	1	g
Dextrose	20	g
Agar	15	g
Distilled water	1 L	(total volume)

Autoclaved at 121ºC, 15 minutes

4. MEYYA

Normal MEA plus 2 g/L yeast extract and 5 g/L extra agar

5. Murashige & Skoog (MS)

4.33 g Murashige and Skoog basal medium (Sigma M5519)

20 g sucrose

Add reagents to 900 mL of H_2O and stir until dissolved. Adjust the pH to 5.7 with 2 N KOH. Adjust the final volume to 1 L with H_2O. Sterilize by filtration at room temperature.

Appendix 2. Primers used in this thesis

Gene/Plasmid	Locus	Name	Purpose	Seq
pDONR207	pDONR207	pDONR207-seq-F pDONR207-seq-R	Sequencing	TCGCGTTAACGCTAGCATGGATCTC GTAACATCAGAGATTTTGAGACAC
pTRV2	pTRV2	pTRV2-seq-F pTRV2-seq-R	Sequencing	CTCAAGGAAGCACGATGAG CGATCAATCAAGATCAGTCGAG
B. cinerea cutinase A	Z69264	BcCutA-g-F BcCutA-g-R	qPCR	ATTCCACAATATGGCATGAAATC ATGTTATCTCCAGCGTGACAAAT
SlACTIN	Solyc11g005330	SlACTIN-g-F SlACTIN-g-R	qPCR	ACAACTTTCCAACAAGGGAAGAT TGTATGTTGCTATTCAGGCTGTG
SlANS	Solyc08g080040	SlANS-attB1 SlANS-attB2	GW Cloning	GGGGACAAGTTTGTACAAAAAAGCAG GCTTAGTCCCCAACCAGAACTAG GGGGACCACTTTGTACAAGAAAGCTG GGTATCTTCTCCTTTGGAGGCTC
SlDFR	Solyc02g085020	SlDFR-attB1 SlDFR-attB2	GW Cloning	GGGGACAAGTTTGTACAAAAAAGCAG GCTTAATTGATTTCATTAGCATC GGGGACCACTTTGTACAAGAAAGCTG GGTACTGGCCATTTCTGTCGCAC
SlF3H	Solyc02g083860	SlF3H-attB1 SlF3H-attB2	GW Cloning	GGGGACAAGTTTGTACAAAAAAGCAG GCTTATGGATAGGTGTAACTGAG GGGGACCACTTTGTACAAGAAAGCTG GGTAATTATCTTTAGTGGCTTG
Ubiquitin 3	Solyc01g056940	SlUBI-RT-F SlUBI-RT-R	RT-qPCR	GCCAAAGAAGATCAAGCACA TCAGCATTAGGGCACTCCTT
Polygalacturonase-2a	Solyc10g080210	SlPG2a-RT-F SlPG2a-RT-R	RT-qPCR	ATCTGGACAAGCTAGCAACATCAA TATACATGGTTCAACTCGATCACAA
beta-galactosidase 4	Solyc12g008840	SlTBG4-RT-F SlTBG4-RT-R	RT-qPCR	CTTGGCGAAACAGAAATGGT ACCTCGAACCCATTCAACAG
Delila	M84913	AmDel-RT-F AmDel-RT-R	RT-qPCR	AGATTACTTGAGAGGGCTTGAGAGG TGGCATCGTGTAGTTTAGTTTTTGT
Rosea1	DQ275529	AmRos1-RT-F AmRos1-RT-R	RT-qPCR	TGGTCGCTGATTGCTGGTAG ATCGTTCTCCATCCTCGCCTA
SlPAL	Solyc00g282510	SlPAL-qRT-F SlPAL-qRT-R	RT-qPCR	AATTGCTTCGAGTCGTGGATAG ACAAGGACTTGTCTCAGCTTCTG
SlCHS1	Solyc09g091510	SlCHS1-RT-F SlCHS1-RT-R	RT-qPCR	CCTTTATTTGAACTCGTCTCAGC CAGGAACATCCTTGAGTAAGTGG
SlCHI	Solyc05g010320	SlCHI-qRT-F SlCHI-qRT-R	RT-qPCR	TTGTCAACTCGGTCTAATGTGTC TAAAGTGGGACCTTATTGCACAC
SlF3H	Solyc02g083860	SlF3H-RT-F SlF3H-RT-R	RT-qPCR	ATGGATGAGCCGATTACATTTG TGGCCTCTTCAGTTTGTATCTTC
SlF3'5'H	Solyc11g066580	SlF3'5'H-RT-F SlF3'5'H-RT-R	RT-qPCR	CTCAACGCCACTAAATCTCCCTA TTGCCCATATGTTGACACTAAGC
SlFLS	Solyc11g013110	SlFLS-RT-F SlFLS-RT-R	RT-qPCR	TGTCCCATATCACCCTTCTTGTC TCACCAATGTGGACAATTATAGCA
SlDFR	Solyc02g085020	SlDFR-qRT-F SlDFR-qRT-R	RT-qPCR	GACTTGCCGACAGAAGCAAT GTGCATTCTCCTTGCCACTT

Gene/Plasmid	Locus	Name	Purpose	Seq
SlANS	Solyc08g080040	SlANS-qRT-F SlANS-qRT-R	RT-qPCR	ACGAACAGGATTTTGCTGCT TTTGAGCTCAGCAACTGCAT
Chitinase	Solyc02g082920	SlChitinase-qRT-F SlChitinase-qRT-R	RT-qPCR	GAGGACCTATCCAATTGACACACC CGCAACTAAATCAGGGTTGTTCAC
β -1,3-glucanase	Solyc01g060020	SlGLU-qRT-F SlGLU-qRT-R	RT-qPCR	GGATCGCGTCAATATAGGAACTT TTCCTACAGATCCTCCTCCTGTT
Metacaspase 7	Solyc09g098150	SlMCA7-qRT-F SlMCA7-qRT-R	RT-qPCR	AGGAGGTTTATGCCGGTTCAGG TTGGTCTGTTTGGCACCCACTG
Pathogenesis-related protein 1	Solyc09g007010	SlPR1-qRT-F SlPR1-qRT-R	RT-qPCR	ATACTCAAGTAGTCTGGCGCAAC CAGTTGCCTACAGGATCGTAGTT
Hypersensitivity-related gene 203	Solyc02g069800	SlHSR203-qRT-F SlHSR203-qRT-R	RT-qPCR	TTCAGTAGACCGGACATGGACTGG AAGTCGTCATGCGGTGGAACAG

Appendix 3. 232 genes showed >3-fold change for at least two stages.

Values were represented as the purple sectors VS. the red sectors on the same fruit. Only data >3 or <-3 were shown.

No.	Locus	Unigene ID	Type	D (8 days)	B (30 days)	C (45 days)
1	Solyc01g100030	SGN-U577318	Primary metabolism	17.9425505	5.418203367	-3.383655501
2	Solyc01g110360	SGN-U578807	Primary metabolism	-3.25568375	-8.046331047	
3	Solyc02g086970	SGN-U584014	Primary metabolism		-5.948060326	-5.654142501
3	Solyc02g086970	SGN-U584013	Primary metabolism		-8.526165044	-6.470265944
3	Solyc02g086970	SGN-U584019	Primary metabolism		-8.479029488	-7.036967819
4	Solyc02g093210	SGN-U565041	Primary metabolism	3.88304345	3.024594263	
5	Solyc03g114360	SGN-U565956	Primary metabolism	-9.38443594	-5.56635276	
6	Solyc03g114500	SGN-U577852	Primary metabolism	15.2468252		3.081647136
7	Solyc04g076880	SGN-U591468	Primary metabolism	-6.53002105	-6.467828431	
8	Solyc06g084460	SGN-U570071	Primary metabolism	5.01203326	15.21618238	
9	Solyc08g078090	SGN-U565342	Primary metabolism	-7.63259505	-5.774901119	3.648573976
10	Solyc09g011080	SGN-U580240	Primary metabolism		-13.61797796	-5.122952423
11	Solyc12g009000	SGN-U571966	Primary metabolism	-8.46863815	-4.786191868	3.408770511
12	Solyc12g056960	SGN-U571967	Primary metabolism	25.0514082		-13.70581164
13	Solyc12g099260	SGN-U566946	Primary metabolism	20.4343483		7.806454934
14	Solyc12g100270	SGN-U577635	Primary metabolism	15.465028		-16.13611035
15		SGN-U565448	Primary metabolism		-3.606059283	-8.185134658
16	Solyc01g103390	SGN-U579789	Other Secondary Metabolism		-4.287556538	-3.476723687
17	Solyc01g107050	SGN-U586560	Other Secondary Metabolism	-3.06101118	-5.995567494	-5.966697297
18	Solyc02g065220	SGN-U568891	Other Secondary Metabolism	8.17436161	-4.414555426	
19	Solyc03g078780	SGN-U572895	Other Secondary Metabolism	-20.210098	-10.22655024	-3.67319191
20	Solyc03g118470	SGN-U580078	Other Secondary Metabolism	-6.03143102	-6.705579095	5.592571039
21	Solyc03g122350	SGN-U573255	Other Secondary Metabolism	-3.61131598	3.241307553	
22	Solyc04g016230	SGN-U579259	Other Secondary Metabolism		-9.187724218	-12.33046698
23	Solyc04g070980	SGN-U583188	Other Secondary Metabolism	4.9616794	5.074151221	
24	Solyc04g082630	SGN-U578628	Other Secondary Metabolism		-4.309810277	-6.202489985
25	Solyc05g055700	SGN-U584869	Other Secondary Metabolism	25.2709594	3.294058594	7.377241361
26	Solyc06g009820	SGN-U566192	Other Secondary Metabolism	23.7808026		11.83012246
27	Solyc08g005610	SGN-U583027	Other Secondary Metabolism	-3.15138618	-3.156882642	
27	Solyc08g005610	SGN-U583024	Other Secondary Metabolism	-3.92911743	-3.373293846	
28	Solyc08g068610	SGN-U577168	Other Secondary Metabolism	-6.96980867		-3.053155428
29	Solyc08g068780	SGN-U578852	Other Secondary Metabolism	-3.32586003		-5.034019015
30	Solyc08g075320	SGN-U583028	Other Secondary Metabolism	3.08330374		3.374775364
31	Solyc09g010380	SGN-U568784	Other Secondary Metabolism		-5.120801292	-6.549774267

No.	Locus	Unigene ID	Type	D (8 days)	B (30 days)	C (45 days)
32	Solyc09g011550	SGN-U578895	Other Secondary Metabolism		-4.750813358	-13.26314305
33	Solyc09g098080	SGN-U564324	Other Secondary Metabolism	-3.40377184	8.380151165	-30.99181668
33	Solyc09g098080	SGN-U570171	Other Secondary Metabolism	-3.777499	7.154853513	
34	Solyc10g005060	SGN-U582403	Other Secondary Metabolism	6.66194182		-4.75833067
35	Solyc10g009430	SGN-U586391	Other Secondary Metabolism	5.07448373	-8.192040023	
36	Solyc10g078240	SGN-U576405	Other Secondary Metabolism	8.75383877	3.704611572	
37	Solyc12g015860	SGN-U580757	Other Secondary Metabolism	4.84586982	3.150177594	
38	Solyc12g044990	SGN-U565859	Other Secondary Metabolism		24.06297487	4.758982151
39	Solyc02g083860	SGN-U563669	Phenylpropanoid Pathway	8.86834587	7.870502637	3.586063715
40	Solyc05g056170	SGN-U577267	Phenylpropanoid Pathway	12.3018804	4.924856796	3.891272905
40	Solyc05g056170	SGN-U572140	Phenylpropanoid Pathway	15.7141943	4.109012205	3.274519411
41	Solyc06g068650	SGN-U577586	Phenylpropanoid Pathway	4.86803625		-16.34143933
41	Solyc06g068650	SGN-U577586	Phenylpropanoid Pathway	3.49199517		-20.78818321
42	Solyc09g007910	SGN-U580612	Phenylpropanoid Pathway	3.92518028	3.387677902	
43	Solyc09g007920	SGN-U577677	Phenylpropanoid Pathway	6.10261464		7.048110086
44	Solyc09g059170	SGN-U580217	Phenylpropanoid Pathway	14.9828613	7.846087703	
45	Solyc09g091510	SGN-U580262	Phenylpropanoid Pathway	15.0966642	13.72058825	16.24764126
45	Solyc09g091510	SGN-U579222	Phenylpropanoid Pathway	16.7750368	22.24203908	16.70309284
46	Solyc10g083440	SGN-U563149	Phenylpropanoid Pathway	4.8223612	9.168967001	3.861230052
47	Solyc01g104950	SGN-U581436	Cell Wall Modification	6.37805049		-4.886590309
48	Solyc03g031800	SGN-U577491	Cell Wall Modification	4.19734733		-3.125408084
49	Solyc03g114860	SGN-U577156	Cell Wall Modification		5.215171956	-9.687802978
50	Solyc04g082140	SGN-U568737	Cell Wall Modification	-9.99181955		-8.581412294
51	Solyc05g005560	SGN-U586540	Cell Wall Modification	4.15379219	3.86207272	
52	Solyc06g034370	SGN-U584317	Cell Wall Modification	-4.12476454		-5.816015547
53	Solyc07g055990	SGN-U592646	Cell Wall Modification	-4.29882525	-5.831065178	
54	Solyc08g082250	SGN-U567168	Cell Wall Modification	-3.48270067		-8.833368936
55	Solyc11g018800	SGN-U579084	Cell Wall Modification	-12.0575874		-5.667758703
56		SGN-U586326	Cell Wall Modification	-5.42195376	-15.36148494	-47.65437763
57	Solyc01g101050	SGN-U563367	Oxidative Response	14.0434255	12.07342496	5.319183548
58	Solyc02g084780	SGN-U564303	Oxidative Response		-161.0242052	-11.82499323
59	Solyc03g033710	SGN-U564304	Oxidative Response		-5.535363804	-8.230336569
60	Solyc04g054690	SGN-U581990	Oxidative Response	-3.1691638		3.082368809
61	Solyc06g008050	SGN-U584082	Oxidative Response	3.05663111	5.378418338	
62	Solyc06g071070	SGN-U571018	Oxidative Response	-3.09543603		-3.989842388
63	Solyc07g005390	SGN-U591185	Oxidative Response	3.19440104	3.01582299	
64	Solyc08g079090	SGN-U579163	Oxidative Response	-3.50674832	-3.268233937	-5.190719134
65	Solyc10g076240	SGN-U576375	Oxidative Response	-3.3279313		-6.288537853
66	Solyc12g008900	SGN-U585983	Oxidative Response	7.92415713		-23.8389344

No.	Locus	Unigene ID	Type	D (8 days)	B (30 days)	C (45 days)
67	Solyc12g100250	SGN-U574775	Oxidative Response		5.88532414	-3.199103443
68		SGN-U574780	Oxidative Response		4.384152652	-4.573619164
69		SGN-U583233	Oxidative Response	-11.8373669		3.161250042
70	Solyc01g095080	SGN-U567978	Other Ripening Related	-4.24222423		-12.30979832
71	Solyc01g095140	SGN-U577990	Other Ripening Related	-19.8440363		-3.742869918
72	Solyc01g111660	SGN-U581283	Other Ripening Related	-3.9336563		-3.908856225
73	Solyc03g026280	SGN-U570402	Other Ripening Related	-7.38254597	-5.403439595	5.04634212
74	Solyc03g124110	SGN-U570401	Other Ripening Related		-3.345780194	3.191735871
75	Solyc06g053140	SGN-U570344	Other Ripening Related	-3.07270376	-3.346654738	
76	Solyc07g006280	SGN-U578908	Other Ripening Related	-3.44743731	-8.499175915	3.822501865
77	Solyc09g075420	SGN-U584756	Other Ripening Related	-3.5734108	-3.25199107	
78	Solyc09g082690	SGN-U578107	Other Ripening Related	-3.84439611	-3.07021791	
79	Solyc10g009110	SGN-U564955	Other Ripening Related	-4.34940757	-3.112972123	
80	Solyc10g050970	SGN-U586437	Other Ripening Related	-5.93454546	-12.98411455	
81	Solyc12g009240	SGN-U562994	Other Ripening Related	-8.5866452	-4.09018759	5.289468254
82	Solyc01g005160	SGN-U565388	Pathogen resistance	-65.2943638	-5.943203435	
83	Solyc01g006050	SGN-U564684	Pathogen resistance		-22.35630145	-6.843278727
84	Solyc01g006320	SGN-U565020	Pathogen resistance	-6.25722729	3.514294115	
85	Solyc01g006550	SGN-U582497	Pathogen resistance	-6.85117167	3.159168249	
86	Solyc01g007020	SGN-U582344	Pathogen resistance	-32.7425772	-15.28870338	
87	Solyc01g009810	SGN-U569924	Pathogen resistance		4.475931397	-9.803499972
88	Solyc01g091590	SGN-U582992	Pathogen resistance	-7.18201961	-3.803942593	3.880869643
89	Solyc01g101180	SGN-U566179	Pathogen resistance	22.7848928		-4.993695893
90	Solyc02g069060	SGN-U572083	Pathogen resistance	-4.73293236	-23.09636768	
91	Solyc02g077060	SGN-U574985	Pathogen resistance	-11.8001493		3.197389761
92	Solyc02g087350	SGN-U571118	Pathogen resistance	-5.98006258	-4.324869107	4.187387101
93	Solyc03g006080	SGN-U604046	Pathogen resistance		3.006559678	-6.101978978
94	Solyc03g020060	SGN-U577965	Pathogen resistance	-6.85116613	-3.617239564	
95	Solyc03g044200	SGN-U565492	Pathogen resistance	16.7453484		4.761374053
96	Solyc03g114160	SGN-U576694	Pathogen resistance	-6.01551312	-4.491921139	3.465732988
97	Solyc03g118510	SGN-U579644	Pathogen resistance	7.04572381	4.860420957	
98	Solyc03g122350	SGN-U573255	Pathogen resistance	-3.71715101	3.236401066	
99	Solyc04g025300	SGN-U572465	Pathogen resistance	-4.6275159	73.72888442	
100	Solyc04g064870	SGN-U564929	Pathogen resistance	-3.13022868	5.128899954	
101	Solyc04g072000	SGN-U567041	Pathogen resistance	-3.01769088	-3.337145897	-213.0864248
102	Solyc04g077390	SGN-U566481	Pathogen resistance		13.83045067	3.744561406
103	Solyc06g082080	SGN-U575768	Pathogen resistance	-5.47092557		3.132015317
104	Solyc07g006110	SGN-U575382	Pathogen resistance	5.03003242	17.64674417	
105	Solyc07g007750	SGN-U577872	Pathogen resistance		3.594219394	4.300823318
106	Solyc07g008590	SGN-U571332	Pathogen resistance	-4.65355109		-9.66748307
107	Solyc07g008600	SGN-U562726	Pathogen resistance	-8.14034675		-17.197042
108	Solyc07g041900	SGN-U577702	Pathogen resistance		21.33473077	-6.379785104
109	Solyc08g016310	SGN-U572574	Pathogen resistance	-7.06143481	3.097208038	

No.	Locus	Unigene ID	Type	D (8 days)	B (30 days)	C (45 days)
110	Solyc10g084320	SGN-U581628	Pathogen resistance		3.009248719	-6.329855867
111	Solyc11g056680	SGN-U578838	Pathogen resistance	96.6404247	-12.56632119	
112		SGN-U580916	Pathogen resistance	-5.41156465		-25.33515081
113	Solyc01g108240	SGN-U586438	Stress Response	-39.987139	-5.673342009	
114	Solyc05g053310	SGN-U578281	Stress Response	4.38594526	3.305304487	-11.34386561
115	Solyc09g011560	SGN-U578097	Stress Response	-3.49442496		-12.95934055
116	Solyc09g084450	SGN-U578279	Stress Response		-4.568807842	-4.292554748
117	Solyc09g092260	SGN-U581004	Stress Response	4.91757698	5.540348049	
118	Solyc10g084000	SGN-U565610	Stress Response	-12.993853	-3.783318473	
119	Solyc11g007400	SGN-U575319	Stress Response	-10.0455165		-7.108165754
120	Solyc12g042830	SGN-U563517	Stress Response	-3.27864141	-7.777491765	
121	Solyc01g087970	SGN-U586216	Development	-10.6324954	-8.173669956	-6.259991937
122	Solyc01g108570	SGN-U568100	Development	4.3200343		3.328623957
123	Solyc02g064830	SGN-U585261	Development	-19.264482		-5.422769314
124	Solyc02g092110	SGN-U562944	Development	-10.6189368		-3.028306554
125	Solyc02g092120	SGN-U585798	Development	-8.25274151	-3.146597535	-4.552533226
126	Solyc02g092820	SGN-U573534	Development	-4.89478819		-21.18685736
126	Solyc02g092820	SGN-U573534	Development	-6.5457137		-28.6038243
127	Solyc03g034370	SGN-U577200	Development	-3.05607765	3.632638999	
128	Solyc04g007470	SGN-U568303	Development		-3.261679201	3.389617819
129	Solyc05g009430	SGN-U570863	Development		-6.635827429	-3.861139303
130	Solyc05g014370	SGN-U585808	Development	3.50346168		3.155108147
131	Solyc06g074030	SGN-U578982	Development	-4.38851321	-3.528814185	
132	Solyc07g005110	SGN-U584463	Development	6.54465146		-4.543903794
133	Solyc07g008020	SGN-U563561	Development	-37.5261379		-4.291296904
134	Solyc07g052610	SGN-U575911	Development	17.5759496	8.177003548	8.155827109
135	Solyc07g063850	SGN-U568361	Development		-7.818375475	-9.051208112
136	Solyc08g013690	SGN-U573406	Development	-8.33822823		5.095879063
137	Solyc08g076220	SGN-U580944	Development	-4.22983714	-4.986989291	
138	Solyc08g077910	SGN-U577404	Development		-9.024862707	-4.705818146
139	Solyc10g011660	SGN-U583984	Development	22.3924403	-3.98062605	
140	Solyc10g017520	SGN-U573093	Development		-3.501098264	-18.8332852
141	Solyc11g010600	SGN-U581437	Development	-3.45751156		-4.232305745
142		SGN-U578793	Development	3.66634948	7.450338567	-11.53859432
143		U11861	Development	-3.77861399	3.566880774	
144	Solyc02g063020	SGN-U582003	Transport	8.80615529	5.464757028	3.367538914
145	Solyc02g078450	SGN-U569469	Transport	-3.75326642	3.747692716	14.62417287
146	Solyc02g079330	SGN-U578370	Transport		-3.26771165	-3.797777767
147	Solyc02g086310	SGN-U579675	Transport		-7.439673452	-6.723556942
148	Solyc03g013440	SGN-U570219	Transport	3.74475869	4.487970092	
149	Solyc08g062360	SGN-U581117	Transport		5.828008591	3.153308235
150	Solyc08g082990	SGN-U584113	Transport		3.718543469	6.231963988
151	Solyc09g065560	SGN-U586390	Transport	-4.70813637	-6.175658833	

No.	Locus	Unigene ID	Type	D (8 days)	B (30 days)	C (45 days)
152	Solyc09g075820	SGN-U579712	Transport	-4.27987793		-4.563951722
153	Solyc09g090730	SGN-U571393	Transport	-3.1512367		-11.7782859
154	Solyc10g075150	SGN-U584460	Transport		6.812911671	3.422511444
155	Solyc01g095100	SGN-U565154	Transcriptional Factor	-3.63704664		-7.37820795
156	Solyc01g106460	SGN-U572160	Transcriptional Factor		4.671982026	-13.45396093
157	Solyc04g077980	SGN-U578910	Transcriptional Factor	-8.39614265	-3.129590822	4.410371353
158	Solyc05g006340	SGN-U583051	Transcriptional Factor	-3.2692592		-3.014300733
159	Solyc05g009660	SGN-U564702	Transcriptional Factor		-52.69880098	-20.74813469
160	Solyc05g052410	SGN-U565259	Transcriptional Factor	-3.9057349	-6.036513324	
161	Solyc05g052570	SGN-U578418	Transcriptional Factor	-3.57682945		3.330398753
162	Solyc05g053380	SGN-U577936	Transcriptional Factor	-5.44428729	-3.478742507	
163	Solyc05g054650	SGN-U585104	Transcriptional Factor	-45.9779858		-3.057086279
164	Solyc07g051840	SGN-U576772	Transcriptional Factor	3.38465302	-3.496861217	
165	Solyc07g053230	SGN-U589670	Transcriptional Factor	-18.2741226	-6.397994054	4.973977473
166	Solyc09g008250	SGN-U571257	Transcriptional Factor	3.31764223		-3.285065751
167	Solyc09g015770	SGN-U591364	Transcriptional Factor	-5.87200597	-3.001280264	
168	Solyc09g097870	SGN-U583138	Transcriptional Factor	-3.39603088		-4.101348368
168	Solyc09g097870	SGN-U583137	Transcriptional Factor	-3.2627182		-3.469280954
169	Solyc10g054010	SGN-U578320	Transcriptional Factor		7.882901915	-5.671989387
170		SGN-U577538	Transcriptional Factor	-11.6446215	-5.233021623	
171	Solyc01g009780	SGN-U573162	Other	-5.57763003	-7.016069604	4.166944614
172	Solyc01g073820	SGN-U568007	Other	-5.15267152		-29.18115098
173	Solyc01g073860	SGN-U563108	Other		-4.162716951	-69.26436678
174	Solyc01g073890	SGN-U584635	Other	-53.5933431		-18.23096546
175	Solyc01g102390	SGN-U582451	Other	-28.8267491		-11.45633314
176	Solyc01g102400	SGN-U582452	Other	-6.07016158		-6.035332118
177	Solyc01g102910	SGN-U564896	Other		3.800216127	-3.158777067
178	Solyc01g108170	SGN-U575520	Other	-92.4772109	-11.40950679	
179	Solyc01g111450	SGN-U580870	Other	-4.50602085		-3.125521028
180	Solyc02g022850	SGN-U576747	Other		3.768974386	-3.272843644
181	Solyc02g070990	SGN-U577305	Other	-5.20443364	5.522653872	-6.210383521
182	Solyc02g086980	SGN-U582911	Other		-4.735070201	-3.485377907
183	Solyc02g089620	SGN-U581540	Other	4.15698173		-4.725335294
184	Solyc02g089630	SGN-U578070	Other	3.78876224		-3.273739202
185	Solyc02g090890	SGN-U569421	Other	-4.04990027		-3.507188467
186	Solyc03g044900	SGN-U581994	Other		3.849237819	8.13719294
187	Solyc03g083910	SGN-U580076	Other	-3.80514063		3.462635116
188	Solyc03g098050	SGN-U577637	Other	-3.53532268		3.447337314
189	Solyc03g118060	SGN-U582268	Other	10.8598043	-3.595830277	
190	Solyc03g120620	SGN-U575946	Other	6.07264233	3.671161262	
191	Solyc04g007250	SGN-U583871	Other	-3.88483065	-3.298498701	
192	Solyc04g011750	SGN-U568388	Other	-5.08866525		-5.94884201
193	Solyc04g064670	SGN-U562775	Other	-3.69424921	-3.328947403	

No.	Locus	Unigene ID	Type	D (8 days)	B (30 days)	C (45 days)
194	Solyc04g074770	SGN-U585123	Other	19.2834868	5.773753586	
195	Solyc04g081960	SGN-U579515	Other	-12.7392794	-3.000526082	
196	Solyc05g008220	SGN-U570227	Other	-6.65036708		-15.7691775
197	Solyc05g009310	SGN-U573004	Other	-3.23047784	4.717293201	
198	Solyc05g013180	SGN-U564806	Other		-9.009342586	-4.905592985
199	Solyc05g055080	SGN-U562700	Other	-4.17438248	-4.092506482	5.369830405
200	Solyc06g007190	SGN-U586055	Other	-8.49257598	-4.786920295	4.79020824
201	Solyc06g060340	SGN-U578579	Other		-3.975749814	-5.09283484
202	Solyc07g007350	SGN-U568783	Other	15.8154254		12.7415231
203	Solyc07g008240	SGN-U567304	Other		-18.57622637	-4.362203387
204	Solyc07g055690	SGN-U570753	Other		-6.085579264	-45.36798337
205	Solyc09g005570	SGN-U581705	Other	-7.66254362		-6.258633521
206	Solyc09g042710	SGN-U567414	Other	8.18215985	4.229497122	
207	Solyc09g075890	SGN-U579991	Other	-3.81238143	-6.394361217	
208	Solyc09g083390	SGN-U570804	Other		-4.147468402	-3.981612317
209	Solyc10g006800	SGN-U565661	Other	-5.36995695	-4.590891396	4.514566886
210	Solyc10g008910	SGN-U581228	Other	13.191254		-4.509629411
211	Solyc10g009380	SGN-U563190	Other	6.83205975	5.61456846	
212	Solyc10g011740	SGN-U577667	Other	-6.05335151	-6.524856918	5.57932962
213	Solyc10g076610	SGN-U570742	Other		-11.68351533	-6.072795954
214	Solyc10g084960	SGN-U563898	Other	-4.77129495		-3.404153198
215	Solyc11g013330	SGN-U568890	Other		4.818769881	9.745513413
216	Solyc11g072860	SGN-U577239	Other	9.85150523	3.647654468	-6.11552632
217	Solyc12g011310	SGN-U579446	Other	-7.47035954	-9.877474692	
218	Solyc12g099260	SGN-U566946	Other	54.5742354	4.124565598	9.342628899
219		SGN-U573440	Other	-11.9684538	8.951847139	8.312701537
220		SGN-U564510	Other	-5.50737265	-4.000630545	
221		SGN-U570660	Other	-3.07248559	-3.721125704	
222		SGN-U571453	Other	-3.33190003	-3.089398779	
223		SGN-U571462	Other	-5.87735032	-3.26975972	
224		SGN-U578256	Other	-6.76074246	-6.97537963	
225		SGN-U585425	Other	-3.49398865	-4.468193295	
226		SGN-U592454	Other	6.0541032	3.940348284	
227		SGN-U591317	Other	-4.56910064	-3.322658379	
228		SGN-U581005	Other	8.87382349		-7.228758124
229		SGN-U565058	Other		6.380741637	5.196398113
230		SGN-U572585	Other	-8.60874818	-3.401030157	
231		empty	Other	-6.74536273	-6.208276077	15.31948109
232		unknown	Other	-39.5906726	-30.51731218	-239.6832808

List of Publications

* These authors contributed equally.

1. BUTELLI, E., LICCIARDELLO, C., **ZHANG, Y**., LIU, J., MACKAY, S., BAILEY, P., REFORGIATO-RECUPERO, G. & MARTIN, C. 2012. Retrotransposons control fruit-specific, cold-dependent accumulation of anthocyanins in blood oranges. *Plant Cell*, 24**,** 1242-55

2. MARTIN, C., **ZHANG, Y**., TOMLINSON, L., KALLAM, K., LUO, J., JONES, J. D. G., GRANELL, A., ORZAEZ, D. & BUTELLI, E. 2012. Colouring up Plant Biotechnology. *Recent Advances in Polyphenol Research.* Wiley-Blackwell.

3. MARTIN, C., **ZHANG, Y**., TONELLI, C. & PETRONI, K. 2013. Plants, diet, and health. Annu Rev Plant Biol, 64, 19-46.

4. **ZHANG, Y**., BUTELLI, E., DE STEFANO, R., SCHOONBEEK, H.-J., MAGUSIN, A., PAGLIARANI, C., WELLNER, N., HILL, L., ORZAEZ, D., GRANELL, A., JONES, JONATHAN D. G. & MARTIN, C. 2013. Anthocyanins Double the Shelf Life of Tomatoes by Delaying Overripening and Reducing Susceptibility to Gray Mold. *Current Biology* 23**,** 1094-1100.

5. BASSOLINO, L.*, **ZHANG, Y.***, SCHOONBEEK, H.-J., KIFERLE, C., PERATA, P. & MARTIN, C. 2013. Accumulation of anthocyanins in tomato skin extends shelf life. *New Phytologist* 200, 650-655.

Other publication from rotation period:

1. TURNER, A. S., FAURE, S., **ZHANG, Y**. & LAURIE, D. A. 2013. The effect of day-neutral mutations in barley and wheat on the interaction between photoperiod and vernalization. Theor Appl Genet, 126, 2267-77.