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

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A thesis submitted to the University of East Anglia for the degree of Doctor of Philosophy

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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 anthocyanin 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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# Table of Contents

Abstract ...................................................................................................................... i
Acknowledgements ..................................................................................................... ii
Table of Contents ....................................................................................................... iv
List of Figures ........................................................................................................... x
List of Tables ............................................................................................................ xii
Abbreviations ........................................................................................................... xiv

Chapter 1: General Introduction ............................................................................. 1
  1.1 Flavonoid biosynthesis ..................................................................................... 2
      1.1.1 phenylpropanoid pathway ...................................................................... 2
      1.1.2 Flavonoid biosynthesis .......................................................................... 4
      1.1.3 Transcriptional regulation of flavonoid biosynthesis .............................. 6
      1.1.4 Natural mutants affecting flavonoid production in tomato ..................... 9
      1.1.5 Enrichment of flavonoids in tomato by bio-engineering ......................... 10
  1.2 Tomato ripening and over-ripening ................................................................. 11
      1.2.1 Tomato fruit ripening process ............................................................... 12
          1.2.1.1 Ethylene signalling pathway .......................................................... 12
          1.2.1.2 Developmental and chemical changes during tomato fruit ripening process..... 15
      1.2.2 Tomato fruit over-ripening ................................................................... 16
      1.2.3 Strategies to extend tomato shelf life ..................................................... 17
  1.3 Plant—Pathogen Interactions ........................................................................... 19
      1.3.1 Pathogen lifestyles ................................................................................ 19
      1.3.2 Plant immune system—“zigzag” model ............................................... 21
      1.3.3 Role of ROS in plant pathogen response .............................................. 23
  1.4 Aims and goals ................................................................................................. 26

Chapter 2: General Materials and Methods ........................................................... 28
  2.1 Materials .......................................................................................................... 29
      2.1.1 Chemicals ............................................................................................. 29
      2.1.2 Antibodies ........................................................................................... 29
      2.1.3 Plant Materials ..................................................................................... 29
      2.1.4 Bacterial Strains .................................................................................. 30
      2.1.5 Plasmids .............................................................................................. 30
2.1.6 Medium recipes ........................................................................................................ 31

2.2 Methods .......................................................................................................................... 31
  2.2.1 Primer design ............................................................................................................... 31
  2.2.2 Polymerase chain reactions (PCR) ............................................................................. 31
  2.2.3 Purification of DNA from PCR reactions or agarose gels ........................................ 32
  2.2.4 E. coli Competent Cell for Heat Shock Transformation ........................................... 32
  2.2.5 Heat shock method of E. coli transformation ............................................................. 32
  2.2.6 Plasmid DNA isolation from E. coli .......................................................................... 33
  2.2.7 Quantification of DNA/RNA .................................................................................... 33
  2.2.8 Preparation of electrocompetent Agrobacterium (GV3101) cells ......................... 33
  2.2.9 Electroporation of Agrobacterium ............................................................................ 33
  2.2.10 DNA isolation from plants ...................................................................................... 34
  2.2.11 RNA isolation from tomato fruit ............................................................................ 34
  2.2.12 First strand cDNA synthesis .................................................................................. 35
  2.2.13 Real time quantitative PCR (RT-qPCR) ................................................................ 35
  2.2.14 Gateway Cloning ................................................................................................... 36
  2.2.15 Tomato transformation ............................................................................................ 36
  2.2.16 Trolox equivalent antioxidant capacity (TEAC) assay ............................................ 38
  2.2.17 Statistics .................................................................................................................. 39

Chapter 3: Enrichment of anthocyanins in tomato fruit delays processes late in ripening .... 40
  3.1 Introduction .................................................................................................................... 41

  3.2 Materials and Methods ............................................................................................... 43
    3.2.1 Storage tests ............................................................................................................ 43
    3.2.2 Texture analysis ...................................................................................................... 43
    3.2.3 Ethylene measurements and treatment .................................................................. 44
    3.2.4 Scanning electron microscopy of tomato cuticle .................................................... 44
    3.2.5 Measurements of cuticle thickness ....................................................................... 44
    3.2.6 Fourier Transform Infra-Red (FT-IR) spectroscopy of tomato peel ..................... 45
    3.2.7 Virus Induced Gene Silencing of tomato ............................................................... 45
    3.2.8 Microarray ............................................................................................................. 46
    3.2.9 Total Polygalacturonase (PG) and β-Galactosidase (β-Gal) Activity Measurements 47
    3.2.10 Malondialdehyde (MDA) measurements .............................................................. 48

  3.3 Results ........................................................................................................................... 49
    3.3.1 Anthocyanin enrichment doubles the shelf life of tomato ..................................... 49
3.3.2 Ethylene production and signaling were not impaired in the Del/Ros1 tomato …… 51
3.3.3 Cuticle composition and thickness were not changed in Del/Ros1 tomato …… 51
3.3.4 Delayed over-ripening was associated with anthocyanin accumulation …… 51
3.3.5 Microarray analysis indicates that the expression of ripening-related genes is suppressed in purple tomatoes………………………………………………… 55
3.3.6 Ripening-related cell wall degradation genes were suppressed in purple tomato ….. 55
3.3.7 Oxidative damage was reduced in purple tomato during over-ripening. ……… 58
3.4 Discussion…………………………………………………………………………………………….. 58
3.4.1 Accumulation of anthocyanins in tomato fruit doubles shelf life ………………… 58
3.4.2 Anthocyanin enrichment is the main reason for delayed over-ripening ……….. 60
3.4.3 Role of ROS in tomato ripening and over-ripening process …………………….. 61
Chapter 4: Enrichment of anthocyanins in tomato fruit can reduce susceptibility to the fungal pathogen, Botrytis cinerea …………………………………………………………… 63
4.1 Introduction …………………………………………………………………………………………… 63
4.2 Materials and Methods ……………………………………………………………………………… 66
4.2.1 B. cinerea growth and spore collection ………………………………………………………… 66
4.2.2 B. cinerea infection ……………………………………………………………………………… 67
4.2.3 Quantification of Botrytis growth on tomato using qPCR …………………….. 67
4.2.4 In vitro Botrytis growth test ………………………………………………………………… 67
4.2.5 3,3’-diaminobenzidine (DAB) staining for tomato ……………………………………… 68
4.3 Results ……………………………………………………………………………………………… 68
4.3.1 Purple MicroTom tomato has lower susceptibility to B. cinerea than WT red tomato ………………………………………………………………………………… 68
4.3.2 Purple MoneyMaker fruit have lower susceptibility to Botrytis cinerea than WT MoneyMaker fruit. ……………………………………………………………… 70
4.3.3 No ripening-related susceptibility to Botrytis cinerea was observed in Del/Ros1 tomatoes ………………………………………………………………………… 70
4.3.4 The reduced susceptibility to Botrytis cinerea is associated directly with accumulation of anthocyanins in purple tomatoes ……………………………… 74
4.3.5 Anthocyanins do not directly inhibit Botrytis cinerea growth in vitro………………… 77
4.3.6 ROS dynamics during pathogen infection were altered in purple tomatoes. ……… 77
4.4 Discussion…………………………………………………………………………………………… 80
4.4.1 Accumulation of anthocyanins in tomato fruit reduces the susceptibility to gray mould……………………………………………………………………………… 80
4.4.2 The ROS burst contributes positively to the susceptibility of ripe tomato fruit to infection by Botrytis………………………………………………………………… 81
Chapter 5: Accumulation of anthocyanins in the skin of tomato fruit is sufficient to extend shelf life .................................................................................................................. 84

5.1 Introduction .................................................................................................................. 85
5.2 Materials and Methods ............................................................................................... 86
  5.2.1 Storage tests ............................................................................................................ 86
  5.2.2 Measurements of cuticle thickness ...................................................................... 88
  5.2.3 Botrytis cinerea infection ..................................................................................... 88
  5.2.4 Staining of seed for proanthocyanidins ............................................................... 88
  5.2.5 Plasmid construction and tomato transformation ............................................... 89
5.3 Results .......................................................................................................................... 89
  5.3.1 Aft/Aft atv/atv tomato can be stored longer ......................................................... 89
  5.3.2 Aft/Aft atv/atv fruit have lower susceptibility to B. cinerea ................................. 92
  5.3.3 Accumulation of anthocyanins in the skin of tomato fruit extends shelf life ....... 96
5.4 Discussion ..................................................................................................................... 99
  5.4.1 Aft/Aft atv/atv fruit show extended shelf life ....................................................... 99
  5.4.2 Accumulation of anthocyanins in skin is sufficient to reduce pathogen susceptibility .................................................................................................................. 99
  5.4.3 Scientific findings from GM research can serve traditional breeding ............... 100
Chapter 6: Investigation of the function of different flavonoids in extension of shelf life in tomatoes ........................................................................................................... 101

6.1 Introduction .................................................................................................................. 102
6.2 Materials and Methods ............................................................................................... 104
  6.2.1 Plant materials ..................................................................................................... 104
  6.2.2 Storage tests ........................................................................................................ 104
  6.2.3 Pre-treatment of tomato fruit before Botrytis infection ..................................... 104
  6.2.4 Supplementation with tomato juice and different flavonoids before inoculation with B. cinerea ................................................................................................. 105
  6.2.5 Plasmid construction .......................................................................................... 106
  6.2.6 Agroinfiltration ................................................................................................... 106
  6.2.7 Isolation of phenolic compounds and analysis by LC-MS ................................. 106
6.3 Results .......................................................................................................................... 107
  6.3.1 Flavonol-enriched tomatoes show delayed over-ripening ................................. 107
  6.3.2 The antioxidant compounds in AtMYB12 fruit are not as stable as in Del/Ros1 tomatoes. ............................................................................................................... 110
  6.3.3 AtMYB12 tomatoes are susceptible to the fungal pathogen, Botrytis cinerea .... 112
6.3.4 *AtMYB12* tomatoes have a reduced ability to alter the dynamics of the ROS burst during *Botrytis* infection...

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

6.3.6 Accumulation of different flavonoids in tomato using VIGS...

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

6.3.8 Susceptibility of VIGS fruit to *Botrytis* infection...

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

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

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

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...

6.4 Discussion...

6.4.1 *AtMYB12* tomato fruit have delayed over-ripening...

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

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

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

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

Chapter 7: Summary and Outlook...

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

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

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

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...

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

7.2.1 Application of flavonoids in other crops to extend shelf life...

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

7.2.3 Dissecting the mechanisms by which flavonoids affect pathogen susceptibility in the model plant, *Arabidopsis thaliana*...
Reference .......................................................................................................................................................... 149
Appendix 1. Recipes of mediums used in thesis ................................................................................................. 165
Appendix 2. Primers used in this thesis .............................................................................................................. 167
Appendix 3. 232 genes showed >3-fold change for at least two stages ......................................................... 169
List of Publications ........................................................................................................................................ 175
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic representation of the upstream and major steps of phenylpropanoid pathway</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>Schematic representation of the flavonoid biosynthetic pathway</td>
<td>5</td>
</tr>
<tr>
<td>1.3</td>
<td>Schematic representation of the regulation of flavonoid pathway</td>
<td>7</td>
</tr>
<tr>
<td>1.4</td>
<td>Schematic representation of ethylene signalling pathway during tomato fruit ripening</td>
<td>14</td>
</tr>
<tr>
<td>1.5</td>
<td>Zigzag Model for plant-pathogen interaction</td>
<td>22</td>
</tr>
<tr>
<td>1.6</td>
<td>Production and functions of reactive oxygen species (ROS) in plant pathogen responses</td>
<td>24</td>
</tr>
<tr>
<td>3.1</td>
<td>Accumulation of anthocyanins in tomato fruit delays late ripening and decreases pathogen susceptibility</td>
<td>42</td>
</tr>
<tr>
<td>3.2</td>
<td>Purple tomato show delayed over-ripening</td>
<td>50</td>
</tr>
<tr>
<td>3.3</td>
<td>Extended shelf life in purple tomato is not due to impaired ethylene production nor changes of cuticle structure</td>
<td>52</td>
</tr>
<tr>
<td>3.4</td>
<td>Delayed over-ripening in purple tomato was associated with anthocyanin accumulation</td>
<td>54</td>
</tr>
<tr>
<td>3.5</td>
<td>Late ripening-related processes are suppressed in anthocyanin-enriched tomato fruit</td>
<td>56</td>
</tr>
<tr>
<td>3.6</td>
<td>Cell wall degradation genes are suppressed in Del/Ros1 tomato</td>
<td>57</td>
</tr>
<tr>
<td>3.7</td>
<td>Oxidative damage is reduced in purple tomato compared to WT control tomato</td>
<td>59</td>
</tr>
<tr>
<td>4.1</td>
<td>Del/Ros1 MicroTom fruit have lower susceptibility than controls to Botrytis cinerea</td>
<td>69</td>
</tr>
<tr>
<td>4.2</td>
<td>Purple MicroTom fruit have lower susceptibility to Botrytis infection in spraying tests</td>
<td>71</td>
</tr>
<tr>
<td>4.3</td>
<td>Accumulation of anthocyanins in the MoneyMaker genetic background also reduces pathogen susceptibility</td>
<td>72</td>
</tr>
<tr>
<td>4.4</td>
<td>Ripening-related susceptibility to Botrytis cinerea in WT and Del/Ros1 MicroTom fruit</td>
<td>73</td>
</tr>
</tbody>
</table>
4.5 The strength of resistance to *B. cinerea* is associated with the levels of anthocyanins in fruits

4.6 The reduced pathogen susceptibility to *Botrytis cinerea* is directly associated with anthocyanin accumulation in the fruit

4.7 Anthocyanins do not inhibit *B. cinerea* growth in vitro

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

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

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

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

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

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

5.6 The anthocyanin enriched regions of *Aft/Aft atv/atv* fruit have lower susceptibility to *B. cinerea* spraying infection

5.7 Phenotype of *PRD* fruit

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

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

6.2 Phenotypes of tomato fruit on the vine at different ripening stages

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

6.4 *AtMYB12* MicroTom tomatoes are susceptible to *Botrytis cinerea*

6.5 *AtMYB12* MoneyMaker tomato is susceptible to *B. cinerea*

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

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

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

6.9 Silencing of anthocyanin biosynthetic genes in *Del/Ros1* tomato alters
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.10</td>
<td>Silencing of anthocyanin biosynthetic genes in Del/Ros1 tomato</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>accumulates different flavonoid compounds</td>
<td></td>
</tr>
<tr>
<td>6.11</td>
<td>The length of viable storage period for VIGS fruit is positively</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>associated with their total antioxidant capacity</td>
<td></td>
</tr>
<tr>
<td>6.12</td>
<td>Susceptibility of different VIGS fruit to B. cinerea infection</td>
<td>127</td>
</tr>
<tr>
<td>6.13</td>
<td>Phenotypes of Del/Ros1 tomato crossed with anthocyanin biosynthetic</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>mutants</td>
<td></td>
</tr>
<tr>
<td>6.14</td>
<td>Crossing anthocyanin mutants with Del/Ros1 tomato generates hybrids</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>containing different flavonoid compounds</td>
<td></td>
</tr>
<tr>
<td>6.15</td>
<td>Shelf life of different natural mutants</td>
<td>131</td>
</tr>
<tr>
<td>6.16</td>
<td>Effects of supplements of flavonoids to B. cinerea inoculation on</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>lesion size in WT tomato fruit</td>
<td></td>
</tr>
<tr>
<td>6.17</td>
<td>Effects of flavonoid derivatives added to growth culture medium on</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>the growth of B. cinerea</td>
<td></td>
</tr>
<tr>
<td>6.18</td>
<td>Effects of flavonoid supplements to B. cinerea inoculation on growth</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>in WT Arabidopsis leaves</td>
<td></td>
</tr>
<tr>
<td>6.19</td>
<td>Effects of flavonoid supplements to B. cinerea inoculation on lesion</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>size in WT tobacco leaves</td>
<td></td>
</tr>
<tr>
<td>6.20</td>
<td>The scavenging ability of flavonoid compounds is associated with the</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>number of –OH groups on the B-ring</td>
<td></td>
</tr>
</tbody>
</table>
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Stock and Working Concentrations of Antibiotics</td>
<td>29</td>
</tr>
<tr>
<td>2.2</td>
<td>Vectors Used in This Thesis</td>
<td>30</td>
</tr>
</tbody>
</table>
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4CL</td>
<td>4-coumaroyl CoA ligase</td>
</tr>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>ae</td>
<td><em>entirely anthocyaninless</em></td>
</tr>
<tr>
<td>Aft</td>
<td><em>Anthocyanin fruit</em></td>
</tr>
<tr>
<td>ANS</td>
<td>anthocyanidin synthase</td>
</tr>
<tr>
<td>atv</td>
<td><em>atroviolacium</em></td>
</tr>
<tr>
<td>aw</td>
<td><em>anthocyanin without</em></td>
</tr>
<tr>
<td>BCP</td>
<td>1-bromo 3-chloropropane</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
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<td>C4H</td>
<td>cinamnate 4-hydroxylase</td>
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<td>CaMV</td>
<td>cauliflower mosaic virus</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<td>CHI</td>
<td>chalcone isomerase</td>
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<td>chalcone synthase</td>
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<td>cm</td>
<td>centimetre</td>
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<td>CoA</td>
<td>co-enzyme A</td>
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<td>Delila</td>
</tr>
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<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
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<td>DFR</td>
<td>dihydroflavonol 4-reductase</td>
</tr>
<tr>
<td>dH2O</td>
<td>distilled water</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphates</td>
</tr>
<tr>
<td>dpb</td>
<td>day(s) post breaker</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ETI</td>
<td>Effector-triggered immunity</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>LC-MS</td>
<td>Liquid chromatography–mass spectrometry</td>
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<td>PAL</td>
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<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PDA</td>
<td>Potato dextrose agar</td>
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<td>PG</td>
<td>Polygalacturonase</td>
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<td>PLI</td>
<td>Promoter of Light Induced protein</td>
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<td>PTI</td>
<td>PAMP-triggered immunity</td>
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<td>RNA</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>Abbreviation</td>
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<td>salicylic acid</td>
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<td>sodium dodecylsulphate</td>
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<td>Thermophilus aquaticus</td>
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<td>β-galactosidase</td>
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<td>TEAC</td>
<td>Trolox Equivalent Antioxidant Capacity</td>
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<td>VIGS</td>
<td>Virus Induced Gene Silencing</td>
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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
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-coumaryl CoA ligase (4CL) to generate the central metabolite, p-coumaroyl CoA. Adjusted from Vogt (2010).

Figure 1.1 Schematic representation of the upstream and major steps of phenylpropanoid pathway. Phenylpropanoids 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-coumaryl 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-coumaryl CoA ligase (4CL). General flavonoid biosynthesis genes are chalcone synthase (CHS), chalcone isomerase (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 hybrida 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, *atroviacea* (*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 Rosea1 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 derivatives (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 \( \beta \)-carotene and lycopene). Carotenoid accumulation changes the fruit colour from green to red (Klee and Giovannoni, 2011). Notably, the key carotenoid biosynthetic gene \textit{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). \textit{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 \( \beta \)-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, \textit{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 wall 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 necrotrophic 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, necrotrophic 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 necrotrophic 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 necrotrophic 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 condition, ROS was generated in chloroplasts, peroxisomes and mitochondria. During the pathogen infection, pathogen PAMPs or effectors were recognized. Through NADPH oxidases or peroxidases, 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$_2$O$_2$ 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$_2$O$_2$ 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 necrotrophic 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**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Solvent</th>
<th>Purpose</th>
<th>Stock Conc.</th>
<th>Working Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>H2O</td>
<td><em>E.coli</em> et.al. Selection</td>
<td>100 mg/mL</td>
<td>100 μg/mL</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Ethanol</td>
<td>Gateway cloning</td>
<td>34 mg/mL</td>
<td>34 μg/mL</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>H2O</td>
<td>Gateway cloning</td>
<td>100 mg/mL</td>
<td>100 μg/mL</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>H2O</td>
<td>Select transgenic plants and <em>E.coli</em>, <em>Agrobacterium</em></td>
<td>100 mg/mL</td>
<td>100 μg/mL</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Ethanol</td>
<td><em>Agrobacterium</em> Selection</td>
<td>50 mg/mL</td>
<td>50 μg/mL</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Ethanol</td>
<td><em>B. cinerea</em> plate growth</td>
<td>100 mg/mL</td>
<td>100 μg/mL</td>
</tr>
</tbody>
</table>

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>
<thead>
<tr>
<th>Name</th>
<th>Size (bp)</th>
<th>Purpose</th>
<th>Selective Antibiotics</th>
<th>Supplier/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM-T</td>
<td>3003</td>
<td>T/A cloning site</td>
<td>Ampicillin</td>
<td>Promega</td>
</tr>
<tr>
<td>pDONR207</td>
<td>5585</td>
<td>Gateway entry vector</td>
<td>Gentamicin, Chloramphenicol</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pTRV1</td>
<td>19370</td>
<td>VIGS</td>
<td>Kanamycin</td>
<td>Orzaez et al. 2009</td>
</tr>
<tr>
<td>pTRV2</td>
<td>9617</td>
<td>VIGS</td>
<td>Kanamycin</td>
<td>Orzaez et al. 2009</td>
</tr>
<tr>
<td>pTRV2-Del/Ros1</td>
<td>9899</td>
<td>VIGS</td>
<td>Kanamycin</td>
<td>Orzaez et al. 2009</td>
</tr>
<tr>
<td>pTRV2-GW</td>
<td>11326</td>
<td>VIGS, TRV2 followed by Gateway cassette</td>
<td>Kanamycin, Chloramphenicol</td>
<td>Orzaez et al. 2009</td>
</tr>
<tr>
<td>pTRV2-SIF3H</td>
<td>9938</td>
<td>VIGS, containing SIF3H fragment</td>
<td>Kanamycin</td>
<td>This thesis</td>
</tr>
<tr>
<td>pTRV2-SIDFR</td>
<td>9945</td>
<td>VIGS, containing SIDFR fragment</td>
<td>Kanamycin</td>
<td>This thesis</td>
</tr>
<tr>
<td>pTRV2-SIANS</td>
<td>9950</td>
<td>VIGS, containing SIANS fragment</td>
<td>Kanamycin</td>
<td>This thesis</td>
</tr>
<tr>
<td>PJAM1890</td>
<td>18441</td>
<td>Stable transformation (GW:Ros1/35S:Del)</td>
<td>Kanamycin, Chloramphenicol</td>
<td>Martin et al. 2012</td>
</tr>
<tr>
<td>pEF 1-PLI-4</td>
<td>3853</td>
<td>pDONR207 containing PLI promoter</td>
<td>Gentamicin</td>
<td>Estornell et al. 2009</td>
</tr>
<tr>
<td>PRD</td>
<td>18137</td>
<td>PLI:Ros1/35S:Del</td>
<td>Kanamycin</td>
<td>This thesis</td>
</tr>
</tbody>
</table>
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 °C 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 °C, 200-220 rpm, over night. 1 mL of over night culture was added into 100 mL LB, 37 °C, 200-220 rpm, and grown until \( \text{OD}_{600} \approx 0.35-0.4 \). The flask was then placed on ice to cool down the culture to 0°C. Chilled culture was transferred into pre-chilled 50 mL tubes. The culture was centrifuged at 4000 rpm, 4°C, 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°C, 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°C 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°C 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°C 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°C 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°C. 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 *Agrobacterium (GV3101)* cells

*Agrobacterium* (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°C 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°C 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°C 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°C.

2.2.9 Electroporation of *Agrobacterium*

Competent *Agrobacterium* cells from -80 °C 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°C. 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 dH2O 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 °C. 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 °C. 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 °C and supernatant was discarded. The pellets were washed with 1.0 mL 75 % ethanol. After centrifugation for 5 min at 7,500 x g at 4 °C 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 H2O. After brief spin, the supernatant (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 °C for 10 min. RNA concentration 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/µL oligoT (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 inoculated with the AglI 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 aliquoted 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 low 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 OD600 of 0.4-0.5. Bacterial suspension was placed in a petri dish and the explants were immersed. 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 low 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 pots 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)·⁺, 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·⁺ 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·⁺ 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 μM) 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 of the curve was calculated as \( \frac{f_{\text{max}} - f_0}{d_{\text{max}}} \) (with: initial force \( f_0 \), the bioyield force at the local maximum \( f_{\text{max}} \) and at distance \( d_{\text{max}} \)) was divided by the area of the flat end of the probe tip.

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

### 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/RosI 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/RosI 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 MgCl₂, 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 peduncle. 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 δ = 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 ≥ 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/RosI 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/RosI 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/RosI 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/RosI 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 Ros1 is 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 susceptibility to infection by this
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 $2.5 \times 10^7$/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 ddH2O) 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 (2.5×10⁵ 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 5×10⁴ spores/mL (for MicroTom fruit) or 1×10⁵ spores/mL (for MoneyMaker fruit) and inoculated for 1.5 h to stimulate germination. The spore inoculum (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 inoculations 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 N₂. 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 (ACAACTTTCCAACAAGGAAGAT),
*SlACT*-q-F (TGTATGTGTGCTATTCAGGCTGTG),
*BcCutA*-q-F (ATTCCACAATATGGCATGAAATC)
*BcCutA*-q-R (ATGTTATCTCAGTTATTC).

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\textsubscript{2}O\textsubscript{2} and peroxidase activity. So H\textsubscript{2}O\textsubscript{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≥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 necrotrophic 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/Ros1 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 *Botrytis* 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. Anthocyanins 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 \textit{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 \textit{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 ddH₂O 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 Afl/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 anthocyanins 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 Afl/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 Afl/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 Afl/Aft atv/atv tomatoes appears to delay their over-ripening. The delayed over-ripening was not due to the altered cuticle thickness of Afl/Aft atv/atv fruit.

5.3.2 Afl/Aft atv/atv fruit have lower susceptibility to B. cinerea
The susceptibility of Afl/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 Afl/Aft atv/atv fruit were wounded and infected with B. cinerea spores. Compared to WT Ailsa Craig fruit, the purple sectors of Afl/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 Afl/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 Afl/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 ≥ 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 ≥ 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 harvested 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 susceptibility. 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, PRD 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 rescue 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 inoculation, 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 $5 \times 10^4$ 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 $1.25 \times 10^6$ spores/mL in $\frac{1}{4}$ 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 $1.25 \times 10^5$ spores/mL). As a control, one volume of the initial culture was mixed with 9 volumes of $\frac{1}{4}$ 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 $1.25 \times 10^5$ spores/mL in $\frac{1}{4}$ strength PDB, supplemented with purified compounds to different final concentrations (for control, 5% EtOH was added to $\frac{1}{4}$ 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-\textit{SICHSI}, pTRV2-\textit{SICHI}, pTRV2-\textit{SIF3H}, pTRV2-\textit{SIDFR}, pTRV2-\textit{SANS}. The sequenced Expression vectors were then transferred into \textit{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 0.5 min 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 3.8 kV 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 harvested 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 *SlPRI*) 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 ≥ 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* inocula 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 (*SipAL, 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-SIF3H, VIGS-SIDFR, VIGS-SIANS 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-SIANS silenced sectors, although late anthocyanin biosynthetic genes (SIF3H, SIDFR and SIANS) were silenced, a significant induction of SIF3’5’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-SIF3H, VIGS-SIDFR and VIGS-SIANS fruit (Fig. 6.10A). However, the VIGS-SIANS 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 SIF3H and SIDFR 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-SIANS 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-SIANS 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-SIF3H 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.

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 inoculated 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, SlDFR-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<sup>−</sup> Del/Ros1 and ae<sup>−</sup> Del/Ros1. Fruit were harvested 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/Ros1 and $ae^{+/−}$ Del/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 inhibitory 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 quercetin 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 growth culture medium on the growth of *B. cinerea*.

1 mM of different compounds was added to the standard ¼ strength PDB medium containing $5 \times 10^4$ 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 quercetin 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 significant 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 equivalent 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 radicals 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, necrotrophic 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


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


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


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


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)

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Autoclaved at 121ºC, 15 minutes

3. Malt Extract Agar (MEA)

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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 H₂O and stir until dissolved. Adjust the pH to 5.7 with 2 N KOH. Adjust the final volume to 1 L with H₂O. Sterilize by filtration at room temperature.
## Appendix 2. Primers used in this thesis

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Transport: 145
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Stress Response: 78
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* These authors contributed equally.


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