

PHYSIOLOGICAL AND MORPHOLOGICAL
CHANGES OF THE ABSCISSION ZONE
RELATED TO THE ETHYLENE
SIGNALLING AND PROGRAMMED CELL
DEATH DURING THE ABSCISSION OF
TOMATO LEAVES AND FLOWERS

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Physiological and morphological changes of the abscission zone related to the ethylene signalling and programmed cell death during the abscission of tomato leaves and flowers

Doctoral Dissertation

Fiziološke in morfološke spremembe abscizinskega območja, povezane z etilensko signalizacijo ter procesom programirane celične smrti med abscizijo listov in cvetov paradižnika

Doktorska disertacija

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To . . .

*Deborah, my dear, who stood beside me throughout this challenge.
Who made the difficult moments bearable and the good ones joyful.
Whatever comes next on this pages would have never been written if I
did not had your relentless and fearless support.*

As a smart person once said... Guts Over Fear...

I dedicate the next pages to you.

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Abstract

Ethylene has impact on several physiological plant processes, including abscission, during which plants shed both their vegetative and reproductive organs. Cell separation and programmed cell death are involved in abscission, and these have also been correlated with ethylene action. However, the detailed spatiotemporal pattern of the molecular events during abscission remains unknown. We examined the expression of two tomato *ACO* genes, *LeACO1* and *LeACO4* that encode the last enzyme in ethylene biosynthesis, 1-aminocyclopropane-1-carboxylate oxidase (ACO), together with the expression of other abscission-associated genes involved in cell separation and programmed cell death, during a period of 0 to 12 h after abscission induction in the tomato flower pedicel abscission zone and nearby tissues. In addition, we determined their localization in specific cell layers of the flower pedicel abscission zone and nearby tissues obtained by laser micro dissection before and 8 h after abscission induction. The expression of both *ACO* genes was localized to the vascular tissues in the pedicel. While *LeACO4* was more uniformly expressed in all examined cell layers, the main expression site of *LeACO1* was in cell layers just outside the abscission zone in its proximal and distal part. We showed that after abscission induction, ACO1 protein was synthesized in phloem companion cells, in which it was localized mainly in the cytoplasm. Samples were additionally treated with 1-methylcyclopropene (1-MCP), a competitive inhibitor of ethylene actions, and analyzed 8 h after abscission induction. Cell-layer-specific changes in gene expression were observed together with the specific localization and ethylene sensitivity of the hallmarks of cell separation and programmed cell death. While treatment with 1-MCP prevented separation of cells through inhibition of the expression of polygalacturonases, which are the key enzymes involved in degradation of the middle lamella, this had less impact on the occurrence of different kind of membrane vesicles and abscission-related programmed cell death. The study has successfully upgraded on the current knowledge on the role of ethylene during abscission and cell separation. Beyond that, our results will contribute to expand the knowledge about mechanisms that control abscission, which could be potentially used through biotechnology approaches to adapt the abscission process to the needs of the food industry.

Povzetek

Pri rastlinah, etilen vpliva na veliko različnih celičnih procesov, med katerimi je tudi abscizija. Abscizija je process, med katerim se rastline ločijo tako vegetativnih, kakor reproduktivnih organov. Ločitev celic in programirana celična smrt sta vključena v abscizijo in oba procesa sta pod vplivom etilena. Kljub temu pa časovni in prostorski vzorec poteka molekularnih dogodkov med abscizijo ostaja neznan. V naši raziskavi smo opazovali izražanje dveh paradižnikovih genov *ACO*, *LeACO1* in *LeACO4*, katera kodirata zadnji encim v sintezni poti etilena, 1-aminociklopropan-1-karboksilat oksidazo, skupaj z izražanjem drugih genov vključenih v ločevanje celic in programirano celično smrt, ki nastopajo v prvih dvanajstih urah po indukciji abscizije. Poleg izražanja, smo z pomočjo laserske mikrodisekcije, določili še njihovo lokacijo v specifičnih plasteh celic, katere sestavljajo abscizijsko cono paradižnikovega cveta. Izražanje obeh genov *ACO* je bilo omejeno na prevodno tkivo cvetnega peclja. Kljub enakomarni porazdelitvi izražanja *LeACO4* v vseh analiziranih tkivih, se je *LeACO1* izražal predvsem v zunanjih delih abscizijske cone, tako proksimalno kakor distalno. Pokazali smo, da se po indukciji abscizije, protein *ACO1* sintetizira v floemskih celicah spremljevalkah, in da se tam nalaga v citoplazmi. Vzorce smo dodatno še tretirali z 1-metilciklopropanom (1-MCP), kompetitivnim inhibitorjem etilenske vezave, ter analizirali tretirane vzorce pred in osem ur po indukciji. Opazili smo tkivno specifične razlike v izražanju genov ter občutljivosti ločevanja celic in programirane celične smrti na etilen. Medtem ko je tretiranje z 1-MCP onemogočilo ločitev celic preko inhibicije izražanja poligalakturonaz, ki so vključene v razgradnjo osrednje lamele, to ni preprečilo pojavljanja različnih membranskih veziklov in drugih celičnih znakov programirane celične smrti. Raziskava je na ta način nadgradila dosedanje znanje na področju abscizije ter vključenosti hormona etilena v sam proces ločevanja celic. Poleg tega bodo rezultati raziskave pomembno prispevali k širitvi znanja o mehanizmih nadzora in poteka abscizije in bodo omogočili uporabo v biotehnoloških pristopih za prilagajanje procesa abscizije zahtevam v pridelavi rastlin.

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Abbreviations

1-MCP	...	1-methylcyclopropene
ACC	...	1-aminocyclopropane-1-carboxylic acid
ACO	...	1-aminocyclopropane-1-carboxylate oxidase
AZ	...	Abscission zone
BFN1	...	Bifunctional nuclease 1
COX	...	Cyclooxygenase
DAPI	...	4'-6-diamidino-2-phenylindole
DMSO	...	Dimethyl sulfoxide
DNA	...	Deoxyribonucleic acid
EIN3	...	protein ethylene insensitive 3
ER	...	endoplasmic reticulum
ETR	...	Ethylene receptor proteins
FAA	...	Formalin acetic acid
FAOSTAT	...	Food and agriculture organization of the United States
FC	...	Fold change
FDR	...	False discovery rate
FFPE	...	Formalin-fixed, paraffin-embedded
LHCII	...	Light-harvesting complex of photosystem II
NADPH	...	Nicotinamide adenine dinucleotide phosphate
NEV	...	NEVERSHED
PBS	...	Phosphate-buffered saline
PCD	...	Programmed cell death
qPCR	...	Quantitative polymerase chain reaction
RNA	...	Ribonucleic acid
RT-qPCR	...	Reverse transcription real-time polymerase chain reaction
SEM	...	Standard error mean
TBA	...	Tert-butanol
TBN	...	Tomato bifunctional nuclease
TdT	...	Terminal deoxynucleotidyl transferase
TEM	...	Transmission electron microscopy
TUNEL	...	Terminal deoxynucleotidyl transferase dUTP nick end labeling
WT	...	Wild type

Chapter 1

Introduction

1.1 Tomato as food

Tomato is one of the most consumed vegetables around the world, and it represents a dietary source of vitamins, minerals and fibre, which are important for human nutrition and health. The fresh fruit are used in salads, for various culinary preparations, and as juices, while it is also processed in the form of purees, concentrates, condiments and sauces. Tomato plants are grown worldwide, both in the field and in greenhouses.

Tomatoes rank fourth among the leading world vegetables. In 2001, over 100 million metric tons were produced, with the 15 leading countries being (in descending order): China, the US, India, Turkey, Egypt, Italy, Spain, Brazil, Islamic Republic of Iran, Mexico, Greece, Russian Federation, Ukraine, Chile and Uzbekistan (FAOSTAT 2011). Interestingly, the countries that produce higher yields do not have the ideal climate for tomato crops, and also have less land area devoted to tomato production. Northern European countries, Canada and New Zealand produce most of their tomatoes under controlled greenhouse conditions.

Tomato consumption has also shown a general increasing trend of consumption for some time now. Tomatoes supply a mean of 12.1 kg/cap/yr, and tomato consumption is higher in Mediterranean and Arab countries (usually between 40 and 60 kg/cap/yr). Tomatoes are highly popular in Egypt, Italy, Israel, Lebanon, Turkey and United Arab Emirates (60-70 kg/cap/yr), whereas Greece and Libya have the highest consumption, of more than 100 kg/cap/yr. Tomatoes are also a popular food in South and North America (Peralta and Spooner, 2007).

Tomato is a rich source of nutrients. Fresh tomatoes and tomato juice are high in water and low in calories. Both are good sources of vitamins A and C, but unfortified tomato juice has about 2-3-fold the vitamin C content of raw, ripe (red) tomatoes. Similarly, canned tomatoes contain about 3-4-fold the vitamin C content of fresh ripe tomatoes. Ripe tomatoes contain 3-4-fold the vitamin A of mature green tomatoes, although otherwise red and green tomatoes are about equal in nutritional value (Ensminger et al., 1995). Tomato purée and plain types of tomato sauce have about twice the solids content and about double the nutritional value of fresh tomatoes and tomato juice. Tomato popularity and its high level of consumption make this vegetable one of the major sources of vitamins and minerals in the human diet (Peralta and Spooner, 2007).

1.2 Domestication

There have long existed controversies regarding the place of domestication, early history and taxonomy of tomato. The wild tomato species are native to western South America,

from southern Ecuador to northern Chile, and the Galapagos Islands. The putative progenitor of the cultivated tomato species is *Solanum lycopersicum* var. *cerasiforme*. It is currently widespread throughout warm regions of the world, but many of these are recent introductions (Peralta and Spooner, 2007).

There are two competing hypotheses for the place of domestication of tomato, one that indicates Peru, the other, Mexico. While the Mexican origin is reasonable, we cannot discount the Peruvian origin, or even parallel domestication in both areas. Tomatoes were first recorded outside the Americas in Italy in 1544. They were cultivated first as ornamental or curiosity plants, and were thought by many to be poisonous. It was first accepted as a vegetable crop in southern Europe during the late sixteenth century. The first European cultivars had yellow to red flattened fruits, with deep furrows, and flowers with stigmas that emerged from the anther tube. Derived cultivars had a wider range of fruit colours and shapes, smoother fruit, and stigmas included in the anther tube, which led to increased fruit set, but reduced the genetic variation of the crop (Peralta and Spooner 2007).

1.3 Taxonomy

The taxonomy of tomato has always been controversial. This controversy involves not only generic placement in *Lycopersicum* or *Solanum*, but also hypotheses on the interspecific relationships. Recent molecular data support the treatment of tomato as *Solanum*, and support allogamy, self-incompatibility, and green fruit as primitive tomatoes (Peralta and Spooner 2007).

Tomato also serves as a model organism to understand the basic genetics of diploid plants. Features that enhance the usefulness of tomatoes for genetic studies are: the naturally occurring variability in the species; self-pollination that leads to the expression of recessive mutations; the possibility of controlled hybridisation within and among species; the lack of gene duplication; and the possibility to easily identify the 12 chromosomes (Peralta and Spooner, 2007). In addition, tomato belongs to the extremely large family of Solanaceae, and it is closely related to many commercially important plants, such as potato, eggplant, peppers, tobacco and petunias. Knowledge obtained from studies conducted on tomato can be easily applied to these other plants, which makes tomato an important research material. On this basis, tomato serves as a model organism for the family Solanaceae, and specifically for fleshy fruited plants (Kimura et. al., 2008).

1.4 Abscission

Abscission in plants is a highly temporally and spatially regulated process in which various organs, including leaves, flowers and fruit are detached from the main plant body in a highly temporally and spatially regulated way (Roberts et al., 2002; Leslie et al., 2007). The occurrence of abscission has a large agricultural implication, both at the level of commodities growth and during storage and shelf life of fresh produce. For different agricultural commodities there is the need to either inhibit or induce abscission. The premature induction of abscission in trees, flowers and very young fruit that can result from extreme temperatures has a significant negative implication for yield and prices. Similarly, acceleration of abscission in different fresh produces during postharvest life and storage can result in significant loss of quality, as for example abscission of a cherry tomato from a bunch (BenMoualem et al., 2004) or from an inflorescence on an ornamental (van Doorn and Stead, 1997; van Doorn, 2002). To overcome these problems, many crops are treated after harvest with chemicals to delay or prevent abscission (van Doorn and

Woltering, 1991). On the other hand, in other agricultural commodities, acceleration of the abscission process is required at a given time, to be beneficial and allow improved mechanisation and labour saving. This can be seen for defoliation of cotton leaves before the boll harvest (Faircloth et al., 2004), or the weakening of fruit tree flowers or young fruitlets for thinning the fruit load (Link, 2000).

The basis for organ abscission is considered to be a cell separation process that occurs specifically in the preformed abscission-zone (AZ) tissue, which is located at the base of the organ that is to be shed (Osborne and Sargent, 1976; Roberts et al., 1984; Leslie et al., 2007; van Nocker, 2009). Additionally, it has been demonstrated that the activation phase of abscission of tomato leaves includes two spatially separated processes: the cell layers proximal to the AZ are characterised by several features of membrane vesicular trafficking and by induction of cell-wall-hydrolysing polygalacturonases; and the hallmarks of programmed cell death (PCD) occur primarily distal to the AZ (Bar-Dror et al., 2011; Dermastia et al., 2012).

The AZ is morphologically and physiologically distinct from neighbouring cells, and it includes smaller sized, cytoplasmically dense cells that form about 150 cell layers in different plants (Osborne and Sargent, 1976; Roberts et al., 1984; Leslie et al., 2007; van Nocker, 2009). In some plants, there is more than one AZ tissue, such as for tomato fruit, for which there is one AZ localised between the fruit itself and the pedicel, and a second AZ localised between the pedicel and the stem, to generate a joint (Divr et al., 2009). In tomato, the flower pedicel AZ is well defined, as a swollen node that is located in the middle of the pedicel, and which starts to form as soon as the sepals differentiate from the primordium. At anthesis, it is composed of six to eight layers of small cells, which traverse the pedicel at the site of an indentation of the epidermis (Tabuchi, 1999; Tabuchi & Arai, 2000; Tabuchi et al., 2001).

Although not much is known about the molecular regulation of abscission, based on knowledge accumulated from anatomical, physiological, genetic and molecular studies, the abscission process is suggested to require four successive phases for its successful execution (Roberts et al., 2000; Patterson, 2001; Taylor and Whitelaw, 2001; Jervis et al., 2003; Leslie et al., 2007). The first phase involves early differentiation of the cells localised at the site of the future organ separation into the definitive AZ. In the second phase, the differentiated AZ acquires competence to respond to the abscission signal(s), presumably via developmental and hormonal cues. The third phase is ethylene-mediated execution of abscission via a cell separation process, which involves major induction of cell wall modifying and hydrolytic enzymes. This causes degradation of the middle lamella between the AZ cells, to allow the physical separation of the abscised organ from the mother plant. The last phase follows detachment, and includes the development of a protective layer at the surface of the exposed tissue, which creates a scar on the plant body at the site of the organ detachment.

In tomato, the abscission process is induced by ethylene, while the rate and degree of abscission depends upon the endogenous balance between auxin and ethylene levels in the tissue (Patterson, 2001; Taylor and Whitelaw, 2001; Roberts et al., 2002; Meir et al., 2010, 2015; Nakano and Ito, 2013). Auxin concentrations must be reduced in the AZ to render it sensitive to ethylene, which promotes the advancement of abscission (Abeles and Rubinstein, 1964; Sexton and Roberts, 1982). Although a lot of studies have been carried out on the role of ethylene on the physiology of abscission, not much is known about the molecular and morphological dynamics that regulate this process. Large-scale analyses have been performed on tomato pedicel abscission, to define the roles of auxins, the developmental and defence-related proteins (Meir et al., 2010, 2011; Wang et al., 2013).

The last step in biosynthesis of ethylene is the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene, which is catalysed by the enzyme 1-aminocyclopropane-

1-carboxylate oxidase (ACO) (Van de Poel et al., 2012; Ruduś et al., 2013). Expression of the tomato *Solanum lycopersicum* (formerly *Lycopersicon esculentum*) *LeACO* genes is tightly linked to the ethylene levels produced by the plant (Van de Poel et al., 2012; Ruduś et al., 2013). In tomato, there are seven *LeACO* genes (Seymour et al., 2013), with *LeACO1* and *LeACO4* showing the highest expression in tomato flower and fruit tissues (The Tomato Genome Consortium, 2012; Tomato eFP Browser1). A transcript of *LeACO1* is induced early after flower removal in the pedicel AZ (Meir et al., 2010; Kim et al., 2015), while *LeACO4* expression is high in the pedicel AZ before abscission induction, after which it decreases (Kim et al., 2015). *LeACO5* has been shown to be expressed in the tomato flower pedicel distal to the AZ area (Nakano et al., 2013), as well as in the AZ (Meir et al., 2010). However, expression of *LeACO1* and *LeACO4* in specific cell layers of the flower pedicel AZ and the localisation of the ACO protein within the AZ are not known. Moreover, the subcellular localisation of the ACO protein remains unclear (Reinhardt et al., 1994; Ramassamy et al., 1998; Chung et al., 2002).

In addition, spatially and temporally regulated ethylene signalling can induce PCD in sensitised cells or tissues of many plants (Trobacher, 2009). In addition, spatially and temporally regulated ethylene signalling can induce PCD in sensitised cells or tissues of many plants (Trobacher, 2009). Based on their expression patterns and their modulation in transgenic plants, genes encoding polygalacturonases and β -1, 4- glucanases (cellulases) have been suggested to have central roles in this cell separation (Greenberg et al., 1975; Lashbrook et al., 1998; Brummell et al., 1999; Roberts et al., 2002; Bar-Dror et al., 2011; Dermastia et al., 2012).

EIN3 and EIN3-like (EIL) are a family of transcription factors that is central to ethylene signalling (Wang et al., 2002). They are functionally redundant and regulate multiple ethylene responses throughout plant development (Tieman et al., 2001). It has been shown recently in *Arabidopsis* that binding of the transcription factor EIN3 controls a multitude of downstream transcriptional cascades (Chang et al., 2013). This study also revealed that *ACO* genes are possible EIN3 targets. Furthermore, it has been shown that in melon, two orthologues of EIN3 function as transcriptional activators for the *ACO1* gene (Huang et al., 2010). The orthologues of the *Arabidopsis* *EIN3* genes in tomato are the *EIL* genes. *EIL2* is up-regulated in olive during abscission of the mature fruit (Parra-Lobato et al., 2011).

1.5 Programmed cell death

Programmed cell death is a genetically encoded, active process that leads to cell death. It is crucial for the development and survival of plants and it is regulated developmentally and environmentally (Gunawardena, 2008; Rogers, 2005; van Doorn and Woltering, 2005). Cell death mechanisms in plants have been suggested to be subdivided into two classes of PCD: autolytic and non-autolytic, which are analogous to autophagic PCD and necrosis in animals, respectively (van Doorn, 2011). PCD is involved in plant embryogenesis, self-incompatibility, xylogenesis and senescence, as well as in plant responses to biotic and abiotic stresses (Fukuda, 2004; Lam, 2004; Bozhkov et al., 2005; Van Breusegem and Dat, 2006).

It has been observed that a delay in tomato leaf abscission occurs when *LeLX* ribonuclease is inhibited (Lers et al., 2006). Tomato *LeLX* is a member of the T2/S-like RNases (Löffler et al., 1992) and it is an orthologue of *Arabidopsis* *RNS3* from a developmental PCD cluster (Olvera-Carillo et al., 2015). LX accumulates in the endoplasmic reticulum (ER) and its C-terminal amino-acid sequence, HDEF, has been demonstrated to function as an ER-retention signal (Lehmann et al., 2001; Kaletta et al.,

1998). Expression of the *LeLX* gene is highly induced during leaf and petal senescence, and it can also be induced in young leaves by ethylene (Lers et al., 1998). The involvement of *LeLX* in other PCD processes has been suggested by its induced expression during seed germination and xylogenesis (Lehmann et al., 2001).

The hallmarks of PCD have been observed for the distal side of the AZ in tomato leaves, and this includes loss of cell viability, altered nuclear morphology, DNA fragmentation, elevated levels of reactive oxygen species, and elevated enzymatic activities. Overexpression of the anti-apoptotic proteins p35 and SfiAP results in delay of flower abscission, which indicates the PCD requirement (BarDror et al., 2011). This also clearly demonstrates an asymmetry between the distal and proximal sides of the leaf AZ for the expression of *LeLX* and localisation of the LX protein. Specific induction of the LX protein has been detected for the distal side of the mature tomato AZ tissue and this was inactivated by pre-treatment with 1-methylcyclopropene (1-MCP), which is an inhibitor of ethylene perception (BarDror et al., 2011). 1-MCP effectively prevents ethylene effects across a broad range of fruit and vegetables by irreversibly binding to the active domain of the ethylene receptor protein (ETR) (Watkins, 2006). The distal side of the tomato flower AZ is also characterised by specific expression of the *Tomato bifunctional nuclease* (*LeTBN1*) gene, and by the activity of a nuclease that appears to be encoded by this gene (Bar-Dror et al., 2011). *LeTBN1* is an orthologue of *Arabidopsis Bifunctional nuclease 1* (*BFN1*), which is another gene that is included in a developmental PCD cluster (Olvera-Carrillo et al., 2015).

The requirement for PCD or abscission has also been supported by delayed abscission in tomato plants that overexpress genes for the anti-apoptotic proteins SfiAP and p35 (Bar-Dror et al., 2011). The induction of PCD usually includes increased levels of reactive oxygen species, which are mediators of stress signals (Petrov et al., 2015). As additional support for the association of PCD with abscission, following the induction of pedicel abscission, high levels of reactive oxygen species have been reported for the cells on the surface of the exposed AZ fractures (Bar-Dror et al., 2011). An important regulatory role of reactive oxygen species in abscission has been recently shown for cassava leaf abscission under water-deficit stress (Liao et al., 2016), and specifically NADPH oxidase inhibition was shown to mitigate leaf senescence, H₂O₂ elevation and the expression of senescence associated genes (Chen et al., 2013).

1.6 Research objectives

We hypothesised that the enzymatic machinery required for execution of cell separation is produced on the proximal side of the AZ, while ethylene biosynthesis, LX expression and PCD induction occur mainly on the distal side (Dermastia et al., 2012).

In the present study, the aim was to elucidate the spatiotemporal distribution of the key players involved in tomato flower pedicel abscission in the specific cell layers of the pedicel AZ and its close vicinity. We address the question of where the last enzyme in the ethylene biosynthetic pathway, ACO, is localised. We also examined the responsiveness to ethylene of several genes involved in cell separation and PCD during flower pedicel abscission.

For the specific induction of LX in the distal side, two hypotheses were evaluated:

- LX has a regulatory role in:
- modulation of the ethylene biosynthesis and perception pathways;
- induction of hormone levels during abscission.

For the role of ethylene and its synthesis during PCD and abscission, we defined the hypothesis:

- Ethylene biosynthesis occurs on the distal side of the AZ, where LX is also expressed and where PCD hallmarks are detected.

For the localisation of the enzymes needed for successful separation of cells during abscission, we defined the hypothesis:

- Poligalacturonases are expressed or localised preferentially on the proximal side of the AZ.

Experiments were performed to further characterise the differences between the proximal and distal sides of the asymmetry in the AZ during the different stages of the abscission process. Aspects to be characterised include:

- PCD occurrence, ethylene biosynthesis, cell-wall metabolism/ modification related gene expression, and global transcriptome changes;
- The consequences of efficient LX suppression on abscission, PCD and the observed asymmetry in the AZ.

Chapter 2

Materials and methods

2.1 Plant materials and growth conditions

2.1.1 Wild type tomato plants

Tomato plant seeds (*Solanum lycopersicum*) of cultivar ‘VF-36’ were obtained from Volcani Centre (Israel). The tomato cultivar ‘VF-36’ was first introduced in 1959, and it is a vine that is self-pollinating and has a sporophytic chromosome number of 24. These plants are commonly used as genetic stock in various types of research on tomato.

2.1.2 Transgenic tomato lines

Independent transgenic tomato lines 7-5, 20-2 and 1-7 were obtained from Volcani Centre, Israel. Transgenic tomato plants were generated for functional analysis of the *LeLX* ribonuclease in the background of the wild type (WT) line ‘VF36’. The plants were transformed with a transformation vector in which an RNAi construct (for construct sequence, see Appendix A.1) for inhibition of the endogenous *LeLX* gene expression was included. This *LeLXi* RNAi gene was under the control of the constitutive promoter, 35S, so the inhibition effects were maintained at all stages of the plant development and in all of the plant organs. The transformation was based on *Agrobacterium-tumefaciens*-mediated gene transfer, and for the selection of the transgenic plants, the kanamycin antibiotic resistance gene, *nptII*, was used. Transformation and regeneration of the plants was performed at the Volcani Centre of the Agricultural Research Organisation Facility in Israel during 2008. The only phenotypic consequence that has been identified so far in these transgenic plants is delay of abscission and senescence. Otherwise the plants are not phenotypically different from the WT, and they cannot be distinguished from it by the general developmental characteristics. The lines have varying degrees of inhibition of LX expression, and none of these had complete suppression, as there was still some protein detected during abscission induction.

2.1.3 Growth conditions

Seeds of the WT and transgenic tomato plants were planted in batches of 15-20 seeds per pot in commercial soil, and left to grow until the developing seedlings were about 5-6 cm in height. These seedlings were transplanted in larger pots, with 2 seedlings per pot, using the same commercial soil. The tomato plants were then grown in growth chambers at 25 °C and 75% relative humidity, and under a 16/8-h day/night cycle. They were watered once a day everyday with standard tap water. After 2-3 weeks, a slow release fertiliser

(Substral Osmocote) was added to the soil in the form of granules that were spread on top of the soil. Upon watering of the pots, the granules slowly dissolved and fertilised the soil. The tomato plants were kept under these conditions until the development of 4-5 leaves, when the plants were used for leaf abscission experiments, or until they started flowering, when they were used for flower abscission experiments.

2.2 Induction of abscission

2.2.1 Induction of abscission in leaf AZ samples

Wild-type tomato plants and transgenic lines 1-7, 20-2 and 7-5 that showed inhibition of *LeLX* were used in the experiments on leaf abscission. For induction of abscission, the leaf blades of up to four lower leaves were removed (debladed; see Figure 1C) using a sharp razor, while leaving most of the petioles intact. Then, 36 h after the deblading, the plants were put into sealed Plexiglas containers. The airtight Plexiglas chambers were then filled with 50 ppm ethylene atmosphere and the plants were left inside the chambers for 24 h. Ethylene was applied to accelerate the abscission process. The plants that were used as controls were left intact and placed in the airtight chambers with a normal atmosphere for 24 h, before the sampling of the AZ tissue.

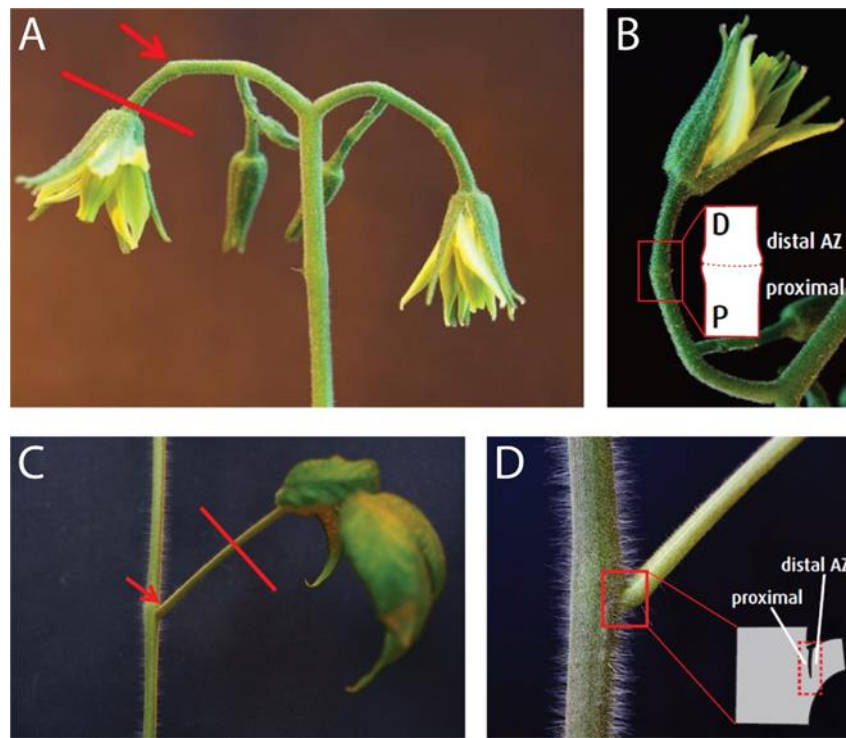


Figure 1: Abscission induction and sampling. (A) Tomato flower pedicel AZ: red line indicates where flowers were cut to induce abscission; arrow indicates flower pedicel AZ. (B) Schematic of AZ structure with the proximal and distal sides of the AZ marked. (C) Tomato leaf AZ: red line marks the region where the leaves were cut before induction of abscission; arrow indicates leaf AZ. (D) Schematic of leaf AZ structure, with proximal and distal AZ regions marked.

2.2.2 Induction of abscission in flower AZ samples

Wild-type tomato plants and transgenic lines 1-7, 20-2 and 7-5 were used in the experiments on flower abscission. A day before performing the experiments, all of the young flowers that had not reached full opening and the opened and senescence flowers were removed from the plants, to leave only the flowers that had opened the night before the initiation of the experiment. Using these flowers only, abscission was induced by removal of the flowers from their pedicels with a sharp razor blade (Figure 1A), as previously described by Meir et al. (2010). All of the experiments were performed using detached flower clusters with up to four flowers, which were kept in 150 mL water in Erlenmeyer flasks. The samples were collected at predefined times: 0 h, as before flower removal, and 2, 4, 6, 8, 10 and 12 h after flower removal.

2.2.3 1-methylcyclopropene treatment of flower AZ samples

The treatment of the detached flower clusters with 1-methylcyclopropene (1-MCP) was performed by exposing the plants to an atmosphere of 1,000 ppb 1-MCP in air-tight chambers for 2 h, prior to the induction of abscission. The 1-MCP was prepared from 0.14%

SmartFresh powder (AgroFresh Inc., Rohm and Haas Company) mixed with water, according to the manufacturer instructions. After the flower removal, the plants were again exposed to 1-MCP for the rest of the experiment. The sampling was performed as above during the period in which 1-MCP completely inhibited pedicel abscission, as described in Meir et al. (2010).

2.2.4 Sampling of leaf AZ tissue

Thin layers of tissue were cut from each side of the petiole AZ of the first three fully developed lower leaves of each tomato plant (i.e., L1, L2, L3, with L1 being the lowest leaf on the stem) using a scalpel. Figure 1D shows the regions of proximal and distal AZ that were sampled for each of the three leaves. For gene expression studies, samples were taken of the L1, L2 and L3 AZ in triplicates. After being cut from the plant, the distal and proximal sides for each of the three leaves were stored in separate plastic tubes and snap-frozen in liquid nitrogen. Samples were stored at -80 °C until total RNA was extracted.

2.2.5 Sampling of flower AZ tissue

For gene expression analysis, thin layers of AZ tissue were excised from each side of the pedicel AZ fracture (proximal and distal) using a sharp razor blade, as shown in Figure 1B. For each time point, three biological replicates were used. The tissue samples were then stored in separate plastic tubes (firma), snap-frozen in liquid nitrogen, and stored in a freezer at -80 °C until total RNA was extracted.

2.2.6 Sampling of flower and leaf AZ for microscopy studies

Median longitudinal sections of the stems and petioles containing the leaf AZ were excised using a razor blade (sample dimensions, approximately 10 x 10 x 2 mm) and fixed in formalin acetic acid (FAA). Flower pedicels containing the flower AZ were excised and fixed in FAA.

2.3 Plant material fixing and paraffin embedding

2.3.1 Tissue fixing

Formalin acetic acid is the most common fixative used in plant histology. It has a composition such that the effects on the tissue of each component in the fixative are balanced. Formalin forms crosslinks between the proteins in the tissue and prevents their precipitation in ethanol. However, together they do not preserve nucleoproteins very well, and therefore for the fixing of the nucleus, acetic acid is added. The fixative was prepared just before use and was cooled to 4 °C, or it was stored for a short period of time in a fridge at 4 °C.

Formalin acetic acid solution was prepared as follows:

- 100 ml 95% ethanol;
- 70 ml deionised (d)H₂O;
- 20 mL 37% formaldehyde solution (e.g., Sigma-Aldrich: #533998-500ML

Formaldehyde histological grade, 37 wt. % in H₂O, or Fluka: #47629

Formaldehyde solution);

- 10 mL glacial acetic acid (glacial = concentrated; Scharlau: #AC0352) - store at 4 °C.

For use, 10 mL of FAA fixative was aliquoted in cca. 20 mL glass vials, while keeping the vials on ice. Tissue samples of flower and leaf AZ (proximal and distal side separately), were cut with a razor blade so that at least one dimension of the sample was smaller than 2-3 mm, and immediately immersed in cold fixative. The vials were then exposed to a moderate vacuum for 15 min to pull the air out of the tissue. After the vacuum step, the fixative in the vials was replaced with fresh fixative. The samples were fixed overnight at 4 °C.

2.3.2 Dehydration in a series of tert-butanol

S Pure tert-butanol (TBA; Sigma-Aldrich: B85927 tert-butanol, 1L) was kept in a warm place before use, as it freezes below 25 °C.

The dehydrating solutions (series) were prepared as follows:

Step 1: 10 mL TBA, 40 mL 95% ethanol, 50 mL dH₂O

Step 2: 20 mL TBA, 50 mL 95% ethanol, 30 mL dH₂O

Step 3: 35 mL TBA, 50 mL 95% ethanol, 15 mL dH₂O

Step 4: 55 mL TBA, 45 mL 95% ethanol

Step 5: 75 mL TBA, 25 mL 95% ethanol

Step 6: 100 mL TBA

Step 7: 100 mL TBA

Step 8: 100 mL TBA

Plant tissue samples were dehydrated for 1-2 h for each step. Steps 1-5 were performed at room temperature, while steps 6-8 were performed in an incubation oven at 56-60 °C. At each step the previous solution was replaced with the next one, using a plastic pipette to transfer the liquids, and the progression was marked on the vial.

2.3.3 Embedding in paraffin

Molten Paraplast Plus (Sherwood Medical #8889-502005) and plastic pipettes were kept in an incubation oven prior to use. An equal volume of molten wax was added to the TBA in the vials. Paraplast Plus has a fine structure and contains additives (e.g., dimethyl sulphoxide) that gives it better penetration into the tissue, which is very important for trouble-free sectioning. The samples were kept in a 56-60 °C oven overnight. The next day, the TBA/wax mixture was poured away, fresh molten wax was added, and the samples were left in an oven overnight, so that all of the TBA evaporated. The wax was then changed for fresh wax several times (using a warm transfer pipette) or at least twice a day for 2 days.

2.3.4 Casting wax blocks

Equipment:

- hot plate (set on low – around 68 °C), with aluminium foil ‘tent’ to keep the heat inside;
- forceps;
- alcohol burner;

- metal or plastic moulds;
- embedding cassettes;
- a small beaker with molten Paraplast Plus;
- a tray with a thin layer of cold water (cooled by adding ice);
- an ice bucket filled with ice;
- a brass plate.

A small amount of the molten wax was kept in a beaker in the aluminium foil tent on the hot plate. The plate temperature was checked regularly, as prolonged temperatures over 62 °C damage the Paraplast Plus. If using metal moulds, it is important to keep them on a hot plate before using them. After taking the vials with samples out of the oven, they must be placed on the hot plate. Then molten wax was poured into the mould and a tissue sample was taken from the vial using forceps that were previously warmed using the alcohol burner. Samples of flower and leaf AZ tissue were orientated in the mould so that sections perpendicular to the AZ could be obtained. The mould was then carefully transferred to the brass plate so that the wax around the sample would solidify, to keep things in place. Afterwards, the mould was filled with hot wax to the edge and covered with a labelled embedding ring. The moulds were then transferred into the tray with ice-cooled water and left there until the wax was completely solid (cca. 10 min). Then the moulds were placed on ice, and after a few minutes it was possible to separate the wax blocks from the metal moulds. The samples embedded in wax blocks were kept in a refrigerator at 4 °C.

2.3.5 Sectioning

Equipment:

- rotary microtome;
- disposable microtome blades;
- a small brush;
- forceps with sharp points;
- a single-edged razorblade;
- dark cardboard plate;
- a stereomicroscope
- hot plate set on 40 °C;
- a small jar with distilled water;
- a plastic pipette;
- cleaned objective microscope slides, frosted on one edge;
- a pencil.

First, the wax blocks were trimmed using the razor in a way that left the upper and lower edges parallel, but left the right and left edges at an angle, so that the lower edge of the block was longer than the upper. The blocks must always be cut with the razor blade away from the sample, in clean cuts from the top to the bottom of the block. After being trimmed, the blocks were fixed onto the sample holder of the microtome. The thickness for the tissue sections was set to 12 µm for samples of tomato leaf AZ, and 10 µm for samples of flower pedicel AZ. The cutting angle was set to 7 degrees. Several sections were cut on a rotary microtome (Autocut 2040; Reichert-Jung) and placed on the dark cardboard, until it was certain that the region of interest had been cut. Then, with the help of a stereomicroscope, interesting sections were located. The selected sections were put in a hot bath to float, and were collected on Superfrost Ultra Plus slides (Menzel-Gläser) in pairs, consisting of three sections each so that one group could be used for the experiment and the other would be treated as the control. Sections were left on a hot plate overnight to dry and attach to the slides. They were stored in slide boxes in a refrigerator at 4 °C.

2.4 Immunolocalisation analysis

The sections were de-waxed in xylene (twice, 10 min each) and then rehydrated through a decreasing ethanol series (100%, 96%, 70%, 50%, distilled water) for 3 min at each concentration. Antigen retrieval, which is important to expose the protein epitopes after fixing, was performed by incubating the slides in sodium citrate buffer (10 mM sodium citrate, pH 6.0) for 15 min in a boiling water bath. Finally, the slides were taken to a solution of TBST (1× TBS pH 7.6, 0.25% TritonX-100) for 5 min to cool at room temperature. The sections were then incubated in blocking solution (TBST plus 5% normal donkey serum) for 30 min at room temperature.

TBST solution (1× TBS pH 7.6, 0.025% Triton-X 100) was prepared as follows:

- 40 mL 1 M Tris-HCl;
- 10 mL 1 M Tris-Base;
- 30 mL 5 M NaCl;
- 920 mL dH₂O;
- 250 µL Triton-X 100.

2.4.1 ACO1 immunolocalisation

Section rehydration was followed by incubation with the ACO1 primary antibody (Santa Cruz Biotechnology, aN-19, 1:100 dilution) in blocking solution, for 1 h at room temperature, in a wet chamber. The ACO antibody used was raised in a goat, against a sequence between amino acids 1-50 of ACO of *Arabidopsis thaliana* (accession #Q06588), which shares 80% identity with ACO1 of *S. lycopersicum*. Non-immune goat serum was used for the negative controls. After washing 3× for 10 min in TBST, the sections were incubated with alkaline-phosphatase-conjugated anti-goat antibodies (1:1000, in blocking solution; Jackson ImmunoResearch) for 1 h at room temperature, followed by washing the sections again as described above. The staining was developed in NBT/BCIP substrate solution (Roche Diagnostics) until the staining became visible (from 15 min, to 2 h), and the sections were washed with water, dehydrated rapidly through an increasing ethanol series (30 s at each step), followed by 2x 10 min in xylene solution (the second could be prolonged until the samples cleared up). When the samples were dehydrated and clear, they were mounted in Permount (Electron Microscopy Sciences).

2.4.2 TAPG4 immunolocalisation

Immunolocalisation of TAPG4 was performed essentially as described in 2.4.1, with affinity purified anti-TAPG4 polyclonal antibodies (BioGenes Antibodies) raised in rabbit against peptide sequence 306-320 (CPNHESCPNQSGVK) of TAPG4 (accession# Q96488). The blocking solution was TBST plus 5% normal goat serum, and the primary antibody was diluted 1:100 in blocking solution, and the secondary antibody was alkaline-phosphatase-conjugated anti-rabbit (1:1000, in blocking solution; Jackson ImmunoResearch). Washing and detection steps were performed as above.

2.4.3 LX immunolocalisation

For LX immunolocalisation, the blocking solution was replaced with anti-LX serum (Lers et al., 2006) diluted 1:10⁵ in blocking solution, and incubated overnight at 4°C. Pre-immune serum was used for the negative controls. Sections were washed the next day in 1× TRIS buffered saline for 5 min, followed by three washes in distilled water with 0.2% Tween 20. Sections were then incubated with alkaline phosphatase-labelled anti-rabbit antibodies

(Jackson ImmunoResearch), diluted 1:1000 in blocking solution, for 1 h at room temperature, followed by washing as described above. Staining was developed in nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl-phosphate substrate solution (Roche Diagnostics), following the manufacturer instructions, washed with water, dehydrated quickly through an ethanol series and xylene, and mounted in Permount (Electron Microscopy Sciences).

2.4.4 Microscopy analysis of stained sections and image collection

The stained sections were observed under a microscope (Zeiss AxioImager Z1) and photographed using a colour digital camera (AxioCam HRc). The purple precipitate at the sites of primary antibody binding to the LX protein was photographed under the microscope (Zeiss AxioImager Z1) and photographed using the colour digital camera (AxioCam HRc).

2.4.5 Detection of nuclear DNA fragmentation by TUNEL assays

Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assays (Gavrieli et al., 1992) were used to detect chromosomal DNA fragmentation, which is a process that occurs during PCD. In the reaction, the terminal deoxynucleotidyl transferase (TdT) binds labelled nucleotides on free 3'-OH ends of the DNA in a template-independent manner.

The tissue sections were de-waxed in xylene (twice, 10 min each) and rehydrated in a decreasing ethanol series (100%, 96%, 70%, 50%, and distilled water, for 3 min each). On each slide, two tissue sections were used for the experiment, and two for the negative control. Both pairs of sections were surrounded using a PEP pen, and Proteinase K (100 µg/mL; Qiagen) was added to the sections for 15 min at room temperature. This step makes the cell content more readily available to the reagents used in the TUNEL assays. Next the samples were washed (twice, 3 min each) in phosphate-buffered saline (PBS). The TUNEL assays were performed on the histological tissue sections, using ApopTag Fluorescein *In-Situ* Apoptosis Detection kits (Millipore). The rehydrated sections were incubated in a mixture of digoxigenin-labelled deoxynucleotides and terminal deoxynucleotidyl transferase for 60 min at 37 °C. This was followed by incubation with fluorescein-labelled anti-digoxigenin antibodies. The slides were washed with PBS and mounted using FluoroMount (Sigma-Aldrich) containing DAPI. The fluorescein-labelled nuclei were observed under blue-light excitation (excitation, 450-490-nm band-pass; emission, 515-nm long-pass). For the negative control section of the same slide, only the solution with labelled nucleotides was applied, while for the positive control, the samples were incubated with DNase I, washed in PBS, and then treated as normal samples. The slides were then incubated in a wet chamber for 60 min at 37 °C in the dark. After the incubation, the slides were washed in PBS buffer (3x for 3 min each).

2.4.6 DAPI staining

To differentiate between damaged and undamaged nuclei, when performing the TUNEL assay, the nuclei were also stained, using 600 nM 4'-6-diamidino-2-phenylindole (DAPI), which is a nucleus-specific stain. The fluorochrome DAPI binds to the DNA double chain, mainly to AT-rich sequences (Kubista, 1987). Upon binding to the DNA molecule, DAPI fluorescence increases about 20x (Barcellona et al., 1990). DAPI was added directly to the mounting medium used for the samples from the TUNEL assay. DAPI fluorescence of all of the nuclei was observed under UV excitation (excitation, 365/12-nm band-pass; emission, 397-nm long-pass).

Phosphate-buffered saline was prepared as follows (10x solution):

- 80 g NaCl;
- 2 g KCl;
- 11.45 g Na₂HPO₄;
- 2.4 g KH₂PO₄;
- 1000 mL dH₂O.

2.5 Laser microdissection

Laser microdissection of the tomato flower and leaf AZ was performed using a laser dissection microscope (PALM MicroBeam; Zeiss). The PALM MicroBeam uses a laser to cut different types of samples, and then collects the cut sections using laser pressure catapulting, which is achieved by shooting a defocused laser beam through the sample. The defocused beam pushes the sample upwards into a collection tube cap that is covered with silicon, which makes the sample stick on its surface.

The laser settings used to cut samples of both flower and leaf AZ were as follows:

- Laser speed: 30%;
- Laser focus: 80%;
- Cut energy: 55%;
- Laser pressure catapulting energy: 40%
- Delta: 2
- Cycles: 1

Tissue sections that were chosen for dissection were placed on specific slides that were covered with a special membrane that facilitates cutting and sampling of the tissue. The slides used in these experiment were MembraneSlide 1.0 PEN slides (Zeiss). Prior to sampling, the section were deparaffinised in xylene, and the water was removed from the tissue using 100% ethanol. This step is especially important, as any residual water in the tissue will negatively affect the laser catapulting step on smaller samples.

For the flower AZ samples, six regions of tissue were selected for sampling, as shown in Figure 2. These were named as the specific tissue regions of distal pedicel, distal AZ, proximal, proximal AZ, distal veins, proximal veins, distal pith and proximal pith.

All of the tissue regions of the flower AZ samples were sampled from five consecutive tissue slices from the AZ samples. These five dissected samples from each slice were pooled in the same AdhesiveCap 500 plastic tube (Zeiss) and snap frozen in liquid nitrogen. The tubes were then stored in a freezer at -80 °C.

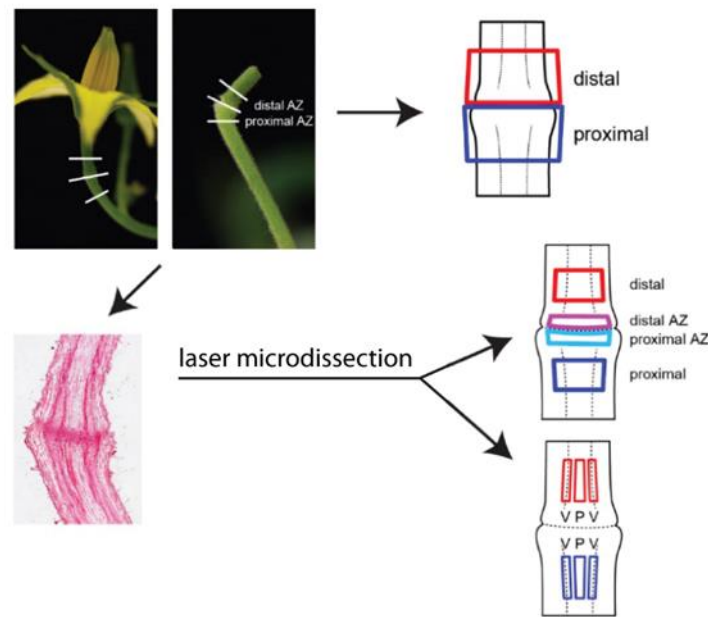


Figure 2: Scheme of the tissue sampling workflow for the hand-cut and laser microdissected AZ samples (V = vein tissue, P = pith tissue).

2.6 Transmission electron microscopy

Electron micrograph of tomato flower pedicel AZ and subcellular localisation of ACO1 proteins, where prepared by Magda Tušek Žnidarič at the National Institute of Biology (Ljubljana), according the following protocol:

The ultrastructural changes in the cells of the AZ were observed under transmission electron microscopy at 8 h after the induction of abscission, and without and with treatment with 1-MCP. One to three pedicels with AZs were cross-cut at about 1 mm on the proximal side and about 2 mm on the distal side, with each sampling time as described above. Some samples were cut longitudinally. The samples were fixed with 3% (w/v) glutaraldehyde in 0.08 M phosphate buffer, pH 7.2, post-fixed with 1% (w/v) osmium tetroxide in the same buffer, and embedded in Agar 100 resin (Agar Scientific). Ultrathin sections were stained with 1% (w/v) aqueous uranyl acetate and Reynolds lead citrate. The grids were examined under transmission electron microscopy (Philips CM 100), at an accelerating voltage of 80 kV. The images were recorded using a CCD camera (ORIUS SC 200), and the Digital Micrograph software was used (Gatan Inc.). Immunolocalisation of *LeACO1* was compared on ultrathin sections of control pedicels and pedicels at 8 h after abscission induction. The samples were fixed in 4% (w/v) paraformaldehyde and 0.25% (w/v) glutaraldehyde in 0.08 M phosphate buffer for 1 h, and embedded in LR White resin (London Resin Co.). The sections were treated with the ACO antibodies (Santa Cruz Biotechnology, aN-19; 1:100 dilution) for 1 h, and with gold-labelled (10 nm) protein A (Aurion) for 30 min. The control samples were treated only with gold-labelled protein A.

2.7 RNA isolation

2.7.1 Total RNA isolation from hand-cut samples of flower and leaf AZ

The samples used were those stored in FastPrep tubes and snap frozen in liquid nitrogen. Until Total RNA isolation they were stored at -80 °C. Before RNA isolation, a ceramic bead was added for homogenisation during the isolation protocol.

Total RNA was isolated with RNeasy Micro kits (Qiagen, No. 74004) and a protocol that was slightly modified compared to the original manufacturer instructions.

The isolation protocol used was as follows:

1. Prepare 2-mL FastPrep tubes with tissue samples and add 350 µL Buffer RLT;
2. Disrupt the tissue using the FastPrep-24 (MP Biomedicals);
3. Centrifuge the lysate for 3 min at maximum speed. Carefully remove the supernatant by pipetting it into a new 2 mL tube;
4. Add 1 volume 70% ethanol to the lysate (350-300 µL), and mix well by pipetting. Do not centrifuge. Proceed immediately to the next step;
5. Transfer the sample, with any precipitate, to an RNeasy MinElute spin column placed in a 2 mL tube. Centrifuge for 30 s at 8000x *g* and discard the flow through. Place the column in a new 2-mL tube;
6. Add 350 µL Buffer RW1 to the RNeasy MinElute spin column. Centrifuge for 30 s at 8000x *g* and discard the flow through. Place the column in a new 2-mL tube
7. Add 80 µL DNase I mix (which was obtained by mixing 10 µL DNase I stock solution with 70 µL RDD buffer) directly to the RNeasy MinElute spin column membrane. Incubate at room temperature for 15 min;
8. Add 350 µL RW1 buffer to the RNeasy MinElute spin column, and centrifuge for 30 s at 8000x *g* and discard the flow through. Place the column in a new 2-mL tube;
9. Add 500 µL RPE buffer to the spin column, and centrifuge for 30 s at 8000x *g* and discard the flow through. Place the column in a new 2-mL tube;
10. Add 500 µL 80% ethanol to the spin column, and centrifuge for 2 min at 8000x *g* and discard the flow through. Place the column in a new 2-mL tube;
11. Centrifuge the tubes with their lids open at full speed for 3 min. Ensure that the samples are completely dry and discard the collection tubes;
12. Place the RNeasy MinElute spin column in a new 1.5 mL collection tube. Add 16 µL pre-heated RNase free water from the isolation kit (80 °C) directly at the centre of the spin column membrane, and incubate the samples for 10 min at room temperature;
13. Centrifuge for 1 min at full speed, and discard the spin column;
14. If the samples are not used immediately after the isolation, store them at -80 °C

2.7.2 Total RNA isolation from microdissected samples of flower and leaf AZ

As the tissue was fixed in formalin and then embedded in wax for the microscopy analyses, the RNA extraction protocol used for the microdissected samples was different to that used for the hand-cut tissue samples.

First, as the microdissected tissue samples were not visible with the naked eye, no homogenisation of the tissue needed to be performed, however it is crucial that the tissue samples were incubated with Proteinase K, which lyses the tissues and makes the RNA molecules more readily available for purification.

For total RNA purification from the FFPE samples, QIAGEN RNeasy FFPE kits (#73504) were used, with a modified version of the kit protocol provided by Zeiss Microscopy Labs. The incubation with Proteinase K in this modified protocol was significantly prolonged, and should produce better RNA yields compared to the original version. The time necessary for optimal Proteinase K digestion depends on factors like tissue type, fixing procedure, and element size of lifted material. An overnight digestion (cca.16 h) worked well for these samples.

The protocol used for total RNA purification from the FFPE samples was as described below:

1. Add 150 μ L Buffer PKD and 10 μ L Proteinase K to the tube containing the LCM elements in the AdhesiveCap, and invert the tube to obtain contact between the liquid and the adhesive surface;
2. Use an incubator to digest the samples in an “upside-down” position at 56 °C overnight, then vortex and heat at 80 °C for precisely 15 min in a heating block. Note: do not use any water bath for the upside-down incubation;
3. Incubate on ice for 3 min;
4. Add 16 μ L DNase Booster Buffer and 10 μ L DNase I stock solution. Mix gently by inverting the tube. Centrifuge briefly to collect the residual liquid from the sides of the tube;
5. Incubate at room temperature for 15 min;
6. Transfer the lysate to a new 1.5 mL microcentrifuge tube;
7. Add 320 μ L Buffer RBC to adjust the binding conditions, and mix the lysate thoroughly;
8. Add 720 μ L ethanol (100%) to the sample, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 9K
9. Transfer 700 μ L of the sample to an RNeasy MinElute spin column placed in a 2 mL collection tube. Close the lid gently and centrifuge for 15 s at $\geq 8000 \times g$ (≥ 10000 rpm). Discard the flow through. Reuse the collection tube in step 10;
10. Repeat step 9 until the entire sample has passed through the RNeasy MinElute spin column. Reuse the collection tube in step 11;
11. Add 500 μ L Buffer RPE to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 15 s at $\geq 8000 \times g$ (≥ 10000 rpm), to wash the spin column membrane. Discard the flow through. Reuse the collection tube in step 12. Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use.
12. Add 500 μ L Buffer RPE to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 2 min at $\geq 8000 \times g$ (≥ 10000 rpm), to wash the spin column. After centrifugation, carefully remove the spin column from the collection tube so that the column does not come into contact with the flow-through;
13. Place the RNeasy MinElute spin column in a new 2 mL collection tube, and discard the old collection tube with the flow through. Open the lid of the spin column and centrifuge at full speed for 5 min. Discard the collection tube with the flow through. It is important to dry the spin column membrane, as residual ethanol can interfere with the downstream reactions;
14. Place the RNeasy MinElute spin column in a new 1.5 mL collection tube. Add 14-30 μ L RNase-free water directly to the spin column membrane. Close the

lid gently and centrifuge for 1 min at full speed, to elute the RNA. The dead volume of the RNeasy MinElute spin column is 2 μ L. Elution with 14 μ L RNase-free water will result in 12 μ L eluate;

15. The RNA solution can be stored at -20°C or used directly for the reverse transcription.

After total RNA isolation, the samples were stored in the freezer at -80 °C

2.8 Gene expression analysis

2.8.1 Gene expression assays

The gene expression assays were designed using the Custom TaqMan Gene Expression Assays service (Applied Biosystems). The genes for which the assays were designed were: *LeACO1*, *LeACO4*, *LeEIL2*, *LeRBOH1*, *LeTAPG1*, *LeTAPG4*, *LeLX* and *LeTBN1*. All assays were supplied as a 20 $\times$ concentrated primer-probe mix, with primer pairs at 18 μ M, and probe at 5 μ M. The primer and probe sequences are listed in Table 1.

Table 1: Primer and probe sequences used for the gene expression analysis

Gene	Forward primer	Reverse primer	Probe	ITAG Identifier
LeACO1	AAGGGACTCCGC GCTCATA	CAAGTTGGTCACC AAGGTTAACC	TCGATGTTCTCCCA TGCGCC	Solyc07g049530
LeACO4	GGGAGAGCACTT TCTTCTGAAAC A	CTTCAGTGCAAAAT CTTTCATAACTTTC CT	CAGGTCAGGAAGTTC A	Solyc02g081190
LeEIL2	GTGTCTTCATTG CCCTCATAGTGA	TGATTGTCTCTGCT GGATCTGTCT	TTCGCAATGGTTTTTC C	Solyc01g009170
LeLX	CACTTGACTTCA AGACCAAATCTA ACC	AATTGCCTTTTTAA TGCTTTCTACACCA T	ATGCAGGGATTAAAC C	Solyc05g007940
LeTBN1	GAGGCAGCTCAT GCAGTCA	GGCCGATAAGTCA CCGTTAACATA	TCCGGCAACAACATC T	Solyc02g078910
LeRBOH1	AGTTTCGTCAGT TTTCACAGGAGT T	CTTTAAGCTCTTGC GAGAAATGCT	AACAGCCTCAGCTTT C	Solyc08g081690
LeTAPG1	ACGGATGTCATA ACGTAAAGCTTC A	CATGAATTCCATCT GTGTTGGGACTA	CTGGAGCGGATACCT T	Solyc02g067630

2.8.2 One-step reverse transcription real-time PCR

Gene expression in all of the AZ samples was followed using one-step reverse transcription real-time polymerase chain reaction (RT-qPCR). AgPath-ID™ One-Step RT-PCR kits were used. Here, the use of gene-specific primers was very important, as reverse transcription of formalin-fixed RNA with only standard oligo dT-primers would be very inefficient and strongly 3' biased due to the numerous strand breaks and modifications inflicted by the formalin fixing and paraffin embedding procedures. The reaction volume was 5 μ L, which was composed of 3 μ L AgPath-ID™ RT-PCR master mix without the »Detection Enhancer« solution, and 2 μ L of the specific dilution of the RNA sample. All reactions were performed in duplicate, and using two different dilutions of the RNA samples

(10x and 50x dilutions for hand cut samples, 5x and 15x dilutions for laser microdissected samples).

Quantitative reverse transcription real-time PCR (qPCR) was performed in a PCR machine (ABI ViiA 7™ Real-Time PCR System; Applied Biosystems). For all reactions, 384-well optical plates were used, covered with the appropriate adhesive foil (Applied Biosystems).

Cycling conditions were as follows:

- a) 10 min at 45 °C (reverse transcription step);
- b) 10 min at 95 °C (polymerase activation step);
- c) 40 cycles of:
 - i) 10 s at 95 °C (denaturation step);
 - ii) 1 m at 60 °C (extension step).

Baseline detection and the threshold value for each specific plate run were detected using the automatic option in the ViiA™ 7 RUO software (version 1.2.2, Applied Biosystems). Additional care was taken to make sure that the fluorescence threshold was always positioned in the exponential region of the amplification curve.

2.8.3 Relative quantification

To perform relative quantification after a qPCR experiment, serial dilutions (x1, x10, 100x, 1000x, 10000x, 100000x diluted) of a standard sample must be placed on the same plate as the samples that are to be analysed with a specific assay. It was of paramount importance that the standard sample contains enough of every target gene to be analysed. Therefore, the total RNA used as a standard sample was pooled from different abscission induced and control samples of either leaf or flower AZ. As for normal samples, each dilution of the standard sample was placed on the plate in duplicates.

Having a dilution series of a standard sample allowed a standard curve to be defined for each assay used. An arbitrary value was assigned to each dilution, taking care that the values followed the dilution steps used (e.g., 10-fold). This way a relative copy number could be calculated for each sample, which was further normalised to the amount of house-keeping gene, COX. All of the calculations used for relative quantification of the gene expression for both leaf and flower AZ samples were performed using the quantGenious software (<http://quantgenious.nib.si>), and the details for the specific steps and methodology used can be found in the software user manual (http://quantgenious.nib.si/documents/manual_external.pdf).

The parameters and thresholds that were set in the quantGenious software were as follows:

- Difference in Cq values between replicates should be <0.5;
- Sample efficiency should be between -2.8 and -4.2;
- Difference between the slopes of the sample dilutions and the standard curve for each assay should be <0.6;
- Quality control Cq for target gene was set to 28;
- Depending on the assay, LOQ was set based on the dilution curve, to the Cq value of the last dilution where the efficiency was still between -2.8 and -4.2;

For samples in which the lower dilution had Cq values below LOQ, but the higher dilution had values above, only the higher dilution was used in the calculation.

2.8.4 Statistical analysis

The relative expression values were exported from the quantGenious software for each sample and gene combination. The files were exported in TEXT format, and the data was imported and manipulated using the R statistical software. All statistical analyses and charts were produced using custom R scripts. Before analysis, the expression values were log-transformed (log base 2).

To analyse the differences in expression of each gene in the time series data of flower AZ, two-way ANOVA was used to examine the effects of time after induction (0, 2, 4, 6, 8, 10, 12 h) and AZ side (proximal vs. distal) on the expression of the selected genes. Significance levels of the main effects of the time point and zone factors on gene expression were tested, and also the interaction effects between time point and zone. For samples of flower abscission that were also treated with 1-MCP, two-way ANOVA was used to examine the effects of time after induction (0 and 8 h after induction), AZ side (proximal vs. distal) and presence or absence of 1-MCP treatment on the expression of the selected genes. Statistical significance was set also at $p < 0.05$.

Expression differences in leaf AZs samples were also tested using two-way ANOVA to evaluate the differences in expression of the selected genes between the proximal and distal sides of the leaf AZ, as sampled from the different leaves.

For all of the datasets, *post-hoc* analysis of the ANOVA results for gene expression data was performed using the Tukey's Honest Significant Difference method.

2.9 Microarray data analysis

Microarray data were provided by the Volcani Centre (Israel), where they used AZ-specific microarrays as described previously by Sundaresan et al. (2011). The samples used for the microarray analysis were of proximal AZ at 0 and 5 h post induction, and distal AZ, also at 0 and 5 h post induction of flower pedicel abscission. They used three biological repetitions per sample. The data they sent contained both the processed data and the raw signal values, which were put through a custom analysis pipeline. This analysis pipeline was performed with R script, which uses the R package »limma« to process the raw signal values and to calculate the differential expression of genes (contrasts) every non-redundant combination of samples. The methods used by the »limma« package are described in detail in Phipson et al. (2016). The resulting values after the data for a specific contrast was processed were logFC for each gene present in the microarray, plus other statistics that were used to assess the quality and significance of the corresponding data. The microarray features were filtered according to the Agilent quality control flags. Only features where none of the samples were compromised were retained for further analysis. The microarray data contained a total of 96,152 features and about 40% passed the quality control step in the analysis, which left 38,638 features, from which the contrast could be calculated. The false discovery rate (FDR) was controlled using the Benjamin-Hochberg correction and setting the threshold for the adjusted p-value at 0.05. The thresholds for up-regulation and down-regulation were set at 2 and -2 log fold change, respectively.

Chapter 3

Results

3.1 Leaf AZ

3.1.1 Gene expression dynamics of *LeACO1*, *LeACO4* and *LeEIL2* during tomato leaf abscission

In this study, we examined whether the distribution of the transcript abundances of *LeACO1*, *LeACO4* and *LeEIL2* varied during leaf abscission for WT tomato and for its transgenic lines that show inhibited *LeLX* expression. It has been demonstrated that for transgenic plants with inhibited LX expression, both leaf and flower abscission are significantly inhibited (Lers et al., 2006; Bar-Dror et al., 2011).

The expression of *LeACO1* was very low for the leaf AZ for both the WT and the transgenic tomato lines. However, *LeACO1* expression was increased significantly after induction of abscission for both WT and transgenic lines. There were no significant differences in *LeACO1* expression between the sides of the AZ fracture, and among the leaves at different developmental stages. However, there was a trend for a gradual increase in *LeACO1* expression from the oldest leaf (L1) towards the youngest leaf examined (L3) for the WT plants (Figure 3). Additionally, there was a trend of slightly higher *LeACO1* expression at the proximal side of the AZ fracture for the WT plants, which was seen only for the oldest leaves in the transgenic lines (Figures 4, 5, 6).

The initial levels of *LeACO4* expression were high before abscission induction. There was significantly more *LeACO4* transcript at the distal side of the AZ fracture for the L1 and L2 leaves for the WT (Figure 3), with a similar trend for the transgenic lines (Figures 4, 5, 6). For all of the examined WT leaves, *LeACO4* expression dropped 24 h after abscission induction (Figure 3), with a similar trend also observed for the transgenic plants (Figure 4, 5, 6). For the control WT plants, L1 showed a statistical difference in transcript distribution between the proximal and distal sides of the AZ, while L2 and L3 did not. In this case, the asymmetry indicates that *LeACO4* is expressed more on the distal side of the AZ in developmentally older leaves. When abscission was induced, *LeACO4* expression levels dropped on the distal side compared to the control samples, while on the proximal side for L1 and L2 there was little change in expression.

However, for the L3 leaves, *LeACO4* was indeed induced compared to the control samples, and considering the inhibition for L3 at the distal side, this produced an even greater asymmetry in this transcript distribution. Even if there was no significant change for the proximal side for L1 and L2, the inhibition of *LeACO4* at the distal side was strong enough to induce significant differences between the proximal and distal sides for both L1 and L2. Although not significant due to the higher variability in the samples for the

proximal side, a similar pattern of *LeACO4* expression was observed for the transgenic tomato line.

There was some correlation between the *LeEIL2* and *LeACO4* expression profiles for the control samples, as there was again significant difference in the transcript distribution for the older leaves, which was then evened out by the younger ones (Figure 3). Upon induction, *LeEIL2* expression was significantly lower only for the L1 and L2 leaves on the distal side of the leaf AZ, which also correlated with the *LeACO4* behaviour, although the magnitude of this inhibition was smaller. In the transgenic lines, we observed the same differences between the control and ethylene treated samples, with the exception that the differences for L3 were also significant.

Interestingly, *LeLX* levels in the transgenic plants were different, with lines 20-2 and 7-5 showing higher steady-state levels of the transcript (Figures 4, 5, 6). Nevertheless, upon induction of abscission, none of the transgenic lines showed the same distal increase in *LeLX* expression that was seen for the WT plants (Figure 3), which indicated that *LeLX* suppression was indeed successful in all three of these transgenic lines.

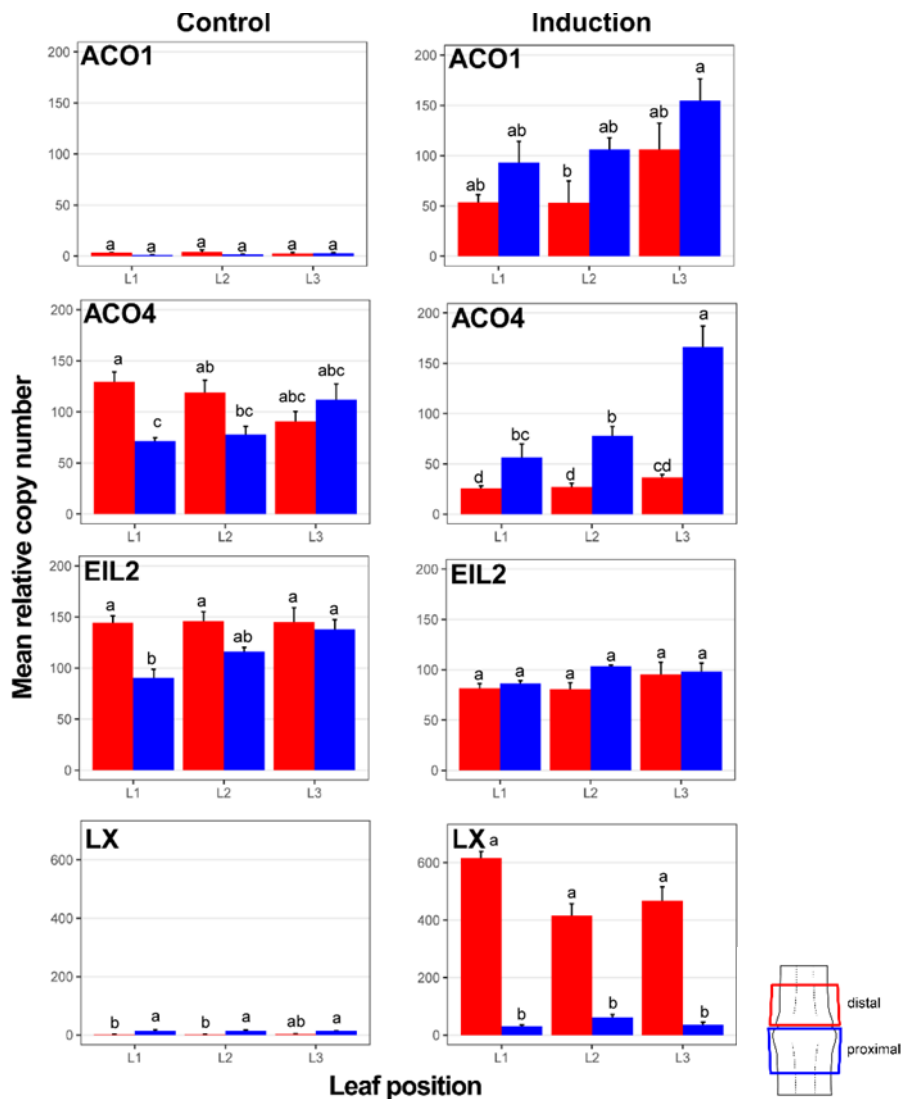


Figure 3: Gene expression profiles of *LeACO4*, *LeACO*, *LeEIL2* and *LeLX* in the leaf AZ of WT tomato plants ('VF36'). Control and ethylene-treated samples of the distal (red) and proximal (blue) AZ were analysed, with the data expressed as means $\pm$ SE (n=3).

Ethylene-treated plants were exposed to the ethylene atmosphere for 24 h, and the bottom three leaves were sampled (L1, L2, L3, where L1 was the oldest leaf).

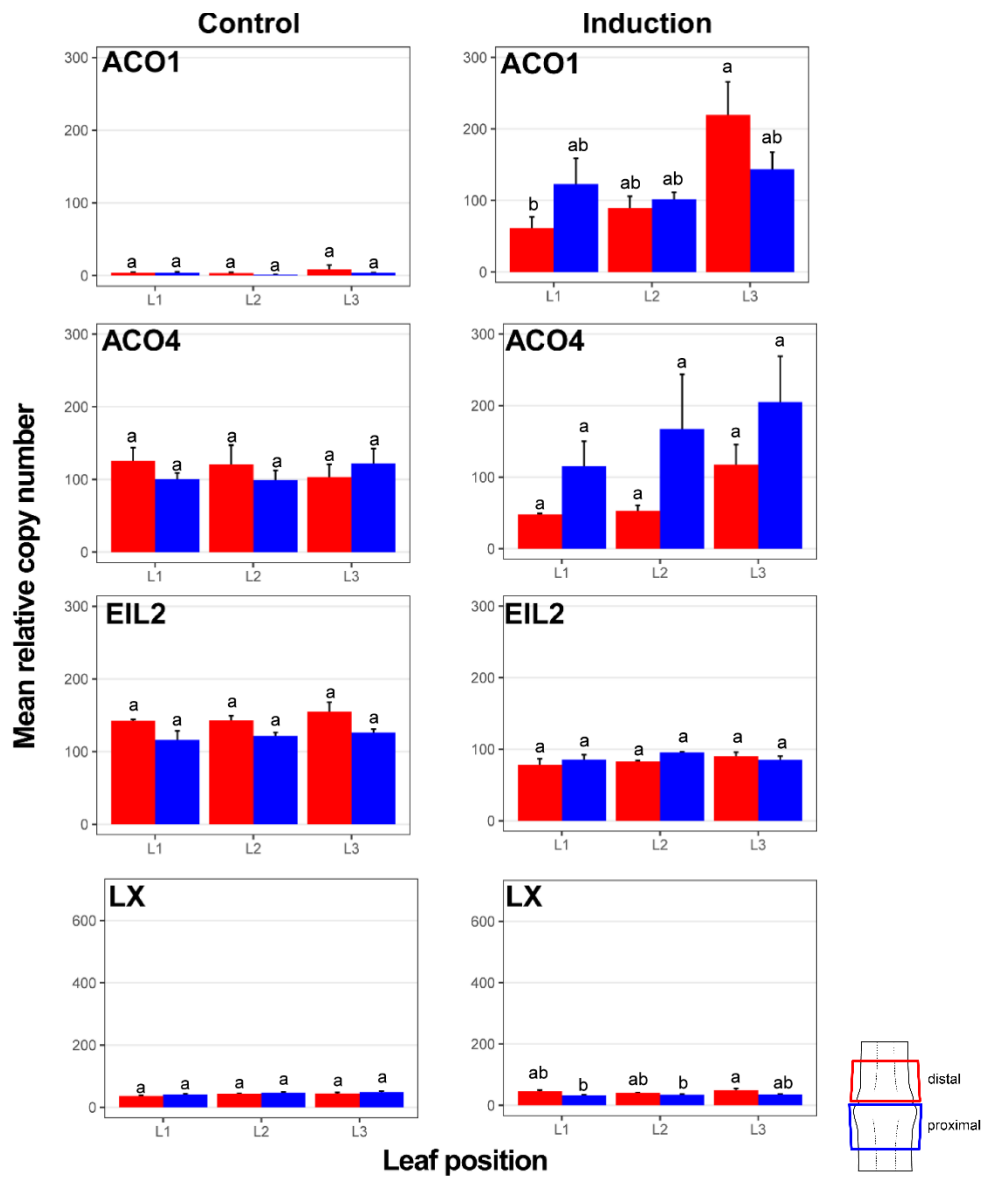


Figure 4: Gene expression profiles of LeACO4, LeACO, LeEIL2 and LeLX in the leaf AZ of tomato line 1-7 with inhibition of LeLX expression. Control and ethylene-treated samples of the distal (red) and proximal (blue) AZ were analysed, with the data expressed as means $\pm$ SE (n=3). Ethylene treated plants were exposed to the ethylene atmosphere for 24 h, and the bottom three leaves were sampled (L1, L2, L3, where L1 is the oldest leaf).

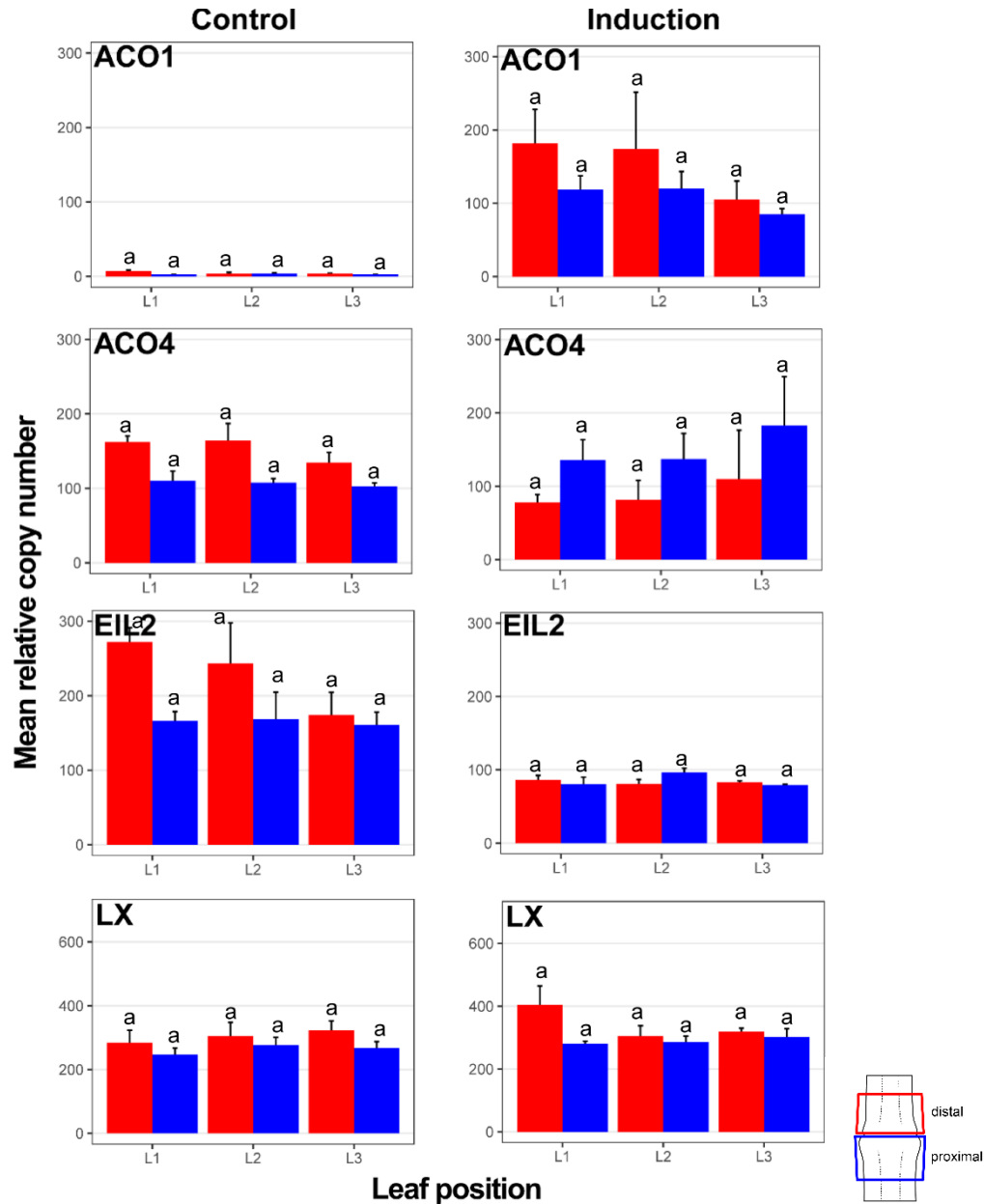


Figure 5: Gene expression profiles of LeACO4, LeACO, LeEIL2 and LeLX in the leaf AZ of tomato line 20-2 with inhibition of LeLX expression. Control and ethylene-treated samples of the distal (red) and proximal (blue) AZ were analysed, with the data expressed as means $\pm$ SE (n=3). Ethylene treated plants were exposed to the ethylene atmosphere for 24 h, and the bottom three leaves were sampled (L1, L2, L3, where L1 is the oldest leaf).

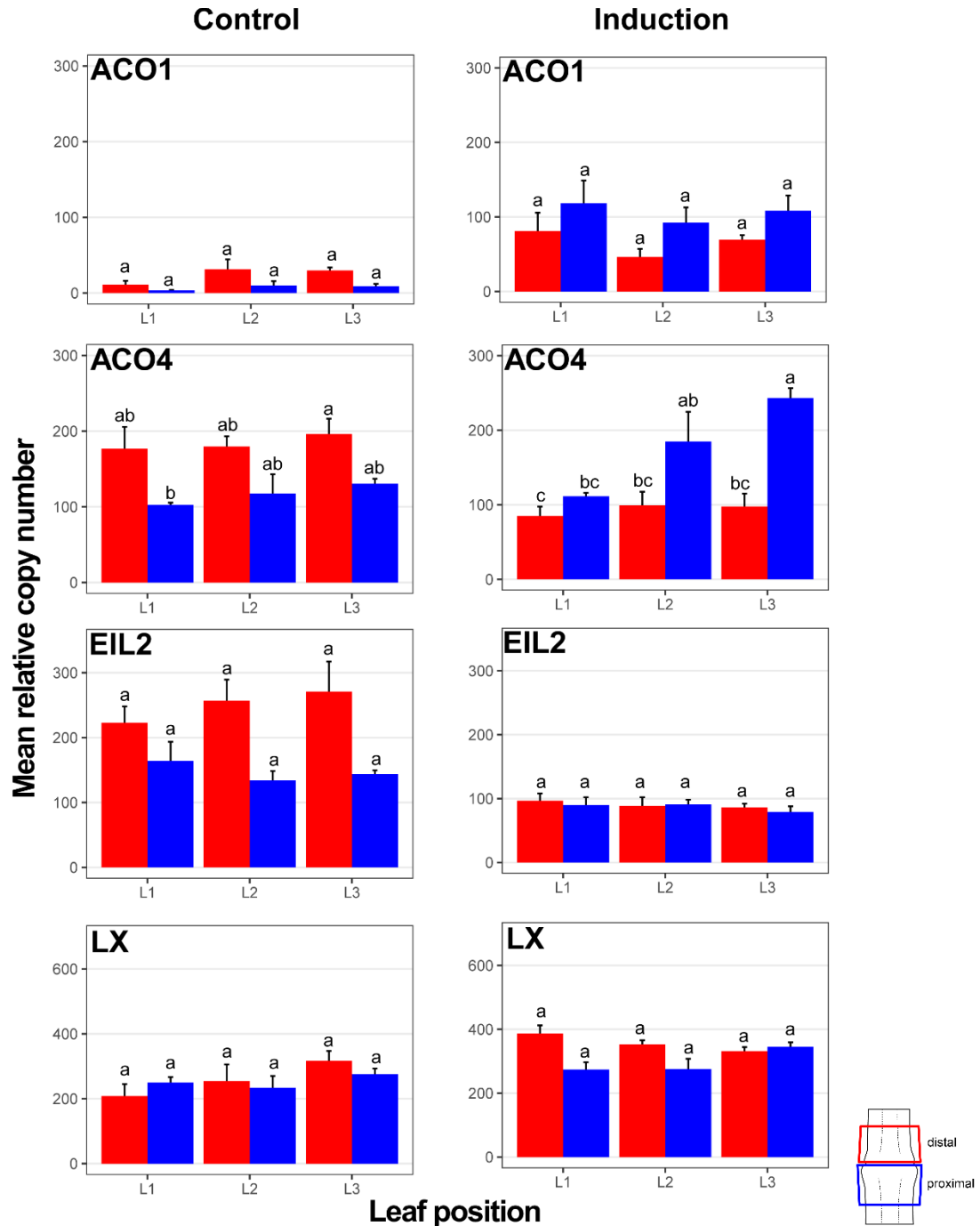


Figure 6: Gene expression profiles of LeACO4, LeACO, LeEIL2 and LeLX in the leaf AZ of tomato line 7-5 with inhibition of LeLX expression. Control and ethylene-treated samples of the distal (red) and proximal (blue) AZ were analysed, with the data expressed as means $\pm$ SE (n=3). Ethylene treated plants were exposed to the ethylene atmosphere for 24 h, and the bottom three leaves were sampled (L1, L2, L3, where L1 is the oldest leaf).

3.1.2 Immunolocalisation of LX and ACO in the leaf AZ

In the leaf AZ samples, the distribution of the LX and ACO proteins were analysed for both WT and LX-inhibited transgenic lines. The ACO protein was observed both for the

WT and the transgenic tomato plants, and was distributed preferentially towards the proximal side of the AZ fracture (Figure 7).

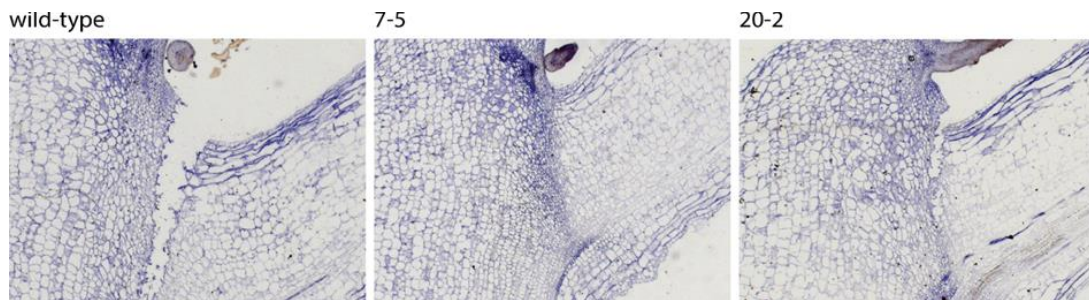


Figure 7: Immunolocalisation of the ACO protein in tomato leaf AZ. Samples of leaf AZ from WT and transgenic (lines 20-2, 7-5) tomato 24 h after induction with ethylene. NBT/BCIP staining (blue-purple signal) denotes the presence of the ACO protein.

LX was detected for the WT plants preferentially on the distal side of the AZ fracture, as was previously reported by Bar-Dror et al. (2011). In the present study, we performed LX RNase localisation also in LX-inhibited transgenic plants, based on the assumption that in these lines the protein content during abscission was lower than for the WT plants, but was nonetheless still present after abscission induction (personal communication). Although a clear abscission fracture was observed in the transgenic plants, there was no detectable signal of LX on the distal side, where usually the WT plants showed the strongest LX signal (Figure 8). There was, however, a faint signal in the epidermal tissue cells for both the WT plants and the transgenic lines.

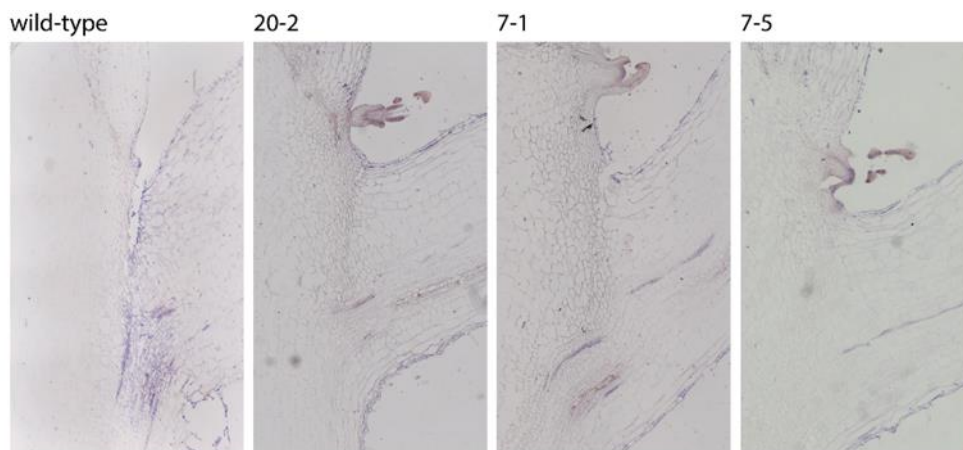


Figure 8: Immunolocalisation of LX in tomato leaf AZ. Samples of leaf AZ from WT and transgenic (lines 20-2, 1-7, 7-5) tomato 24 h after induction with ethylene. NBT/BCIP staining (Blue-purple signal) denotes presence of the LX protein.

3.2 Flower AZ

3.2.1 Changes in cellular morphology during abscission

Using transmission electron microscopy, the ultrastructural changes in the AZ were examined before induction of abscission and 8 h afterwards. Before induction, differentiated

cells in the pedicel AZ were morphologically and physiologically distinct from the neighbouring cells (Figure 9A). These cells were small and packed tightly together, without intercellular spaces. They had dense cytoplasm, with small vacuoles. Their nuclei were round to oval, and their chloroplasts were smaller (i.e., around $1.9 \times 0.9 \mu\text{m}$), in comparison to the chloroplasts in the pedicel cortex outside the AZ (i.e., $4.0 \times 2.2 \mu\text{m}$). The plasma membrane was tightly fitted to the cell wall. The nuclei of the sieve element companion cells in the phloem were similar to those in the other AZ cells, with heterochromatin at the border. Sieve tube elements contained endoplasmic reticulum and mitochondria near the plasma membrane, and in some regions, filaments of P-proteins were seen.

The abscission always started in the companion cells in the central AZ. One of the first signs of abscission was detachment of the plasma membrane from the cell wall (Figure 9B–D). Also, early in the process, many vesicle-like small vacuoles developed in the peripheral cytoplasm of the cells (Figure 9E). These were more abundant in the AZ and the tissues proximal to it, and they were commonly packed with deposits. In addition to these small vacuoles, the cells in the area of abscission were characterised by different kinds of vesicles, which were often packed in paramural bodies between the plasma membrane and the cell wall (Figure 9B). The nuclei of many cells in the area became electron dense (Figure 9E). In some cells, dilatation of the endoplasmic reticulum was observed. At this stage of abscission, the intercellular space was greatly increased between the cells in the AZ, and it commonly became filled with dense material (Figure 9C, D).

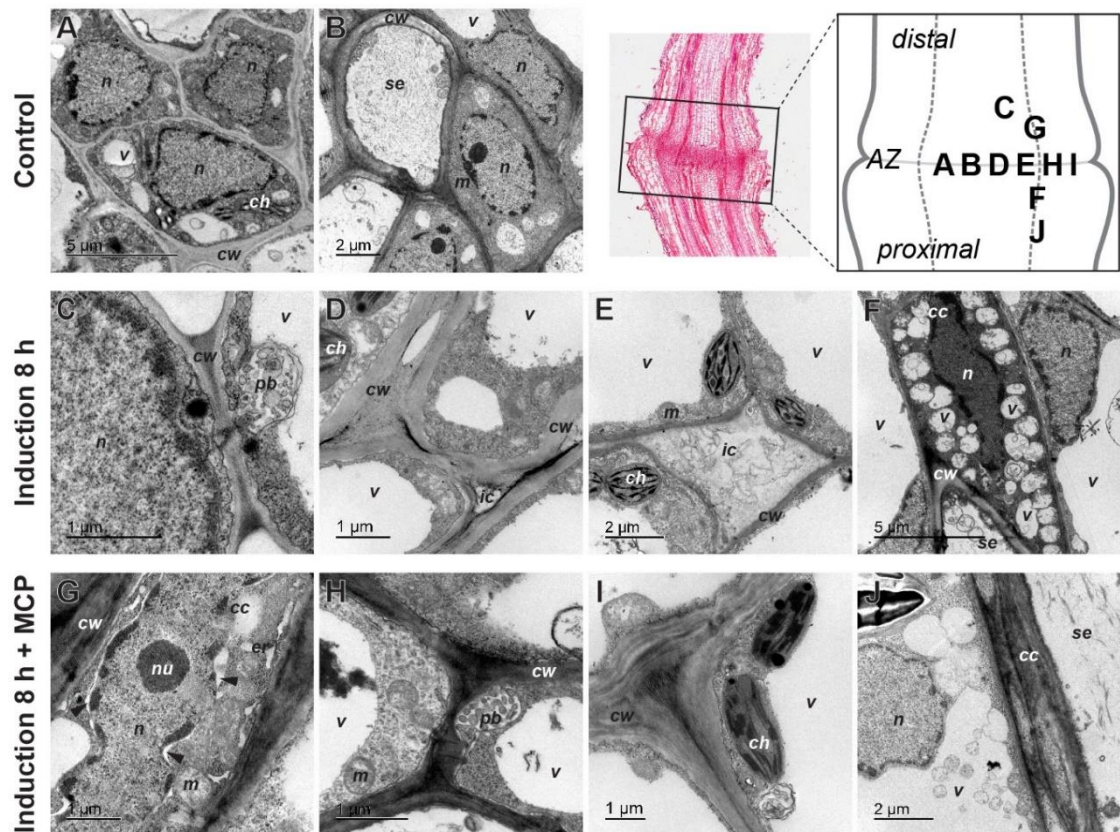


Figure 9: Ultrastructure of tomato flower pedicel cells before induction of abscission (A), before induction of abscission and pre-treated with 1-MCP (B), 8 h after induction (C–F) and 8 h after induction and 1-MCP treatment (F–J). (A, B) The cells in the AZ before abscission induction were small and tightly packed. (C) Formation of the paramural body. (D) Appearance of changes in the cell wall and the beginning of cell separation. (E)

Intercellular spaces filled with non-homogeneous material. (F) Nucleus in companion cell with partly condensed chromatin, in contrast to nucleus in adjacent cell and small vacuoles/ vesicles filled with material. (G) Nucleus with partly condensed chromatin, dilated nuclear membrane (arrowheads), and dilated endoplasmic reticulum, in a companion cell. (H) Paramural body. (I) Non-separated cells with some changes in cell-wall structure. (J) Fusion of small vacuoles into a large one. The part of the pedicel that was used to prepare sections is indicated by a rectangle in the histological section and the letters in the schematic AZ drawing indicate the places from where the individual micrographs were taken (bottom left). cc, companion cell; ch, chloroplast; cw, cell wall; er, endoplasmic reticulum; ic, intercellular space; m, mitochondrion; n, nucleus; nu, nucleolus; pb, paramural body; se, sieve tube element; v, vacuole/ vesicle.

To examine the association of the observed changes during pedicel abscission induced by ethylene, the plants were treated with 1-MCP. Eight hours after abscission induction, this treatment with 1-MCP had profound effects specifically on the separation of the cells. The cells stayed packed tightly together, and in general there was no enlargement of the intercellular space, although there were some changes in the cell wall structure (Figure 9G, H). On the other hand, the 1-MCP treatment did not affect the formation of membrane evaginations, and the formation of multivesicular and paramural bodies, as well as small vacuoles, which appeared in similar quantity in the 1-MCP untreated samples (Figure 9G, I). In addition, features associated with PCD in the distal part of AZ were not affected by the 1-MCP treatment (Figure 9F). Several cells had plasma membranes detached from the cell wall, dilated ER, and nuclei with condensed chromatin and dilated nuclear membrane (Figure 9F). Cells with similar characteristics also appeared in the proximal part of AZ, but their frequency was low.

3.2.2 Differential gene expression analysis of microarray data between proximal and distal flower AZ

3.2.2.1 Overview of differential expression results during abscission in the flower pedicel

As can be observed in Figure 10, time post induction was a more important factor in the guiding of differential expression than the AZ side (Figure 11). The differences in the percentages of differentially expressed genes were 2-fold higher when the time post induction was compared to the AZ side. Also, a larger fraction of genes was down-regulated over time (cca. 9%-10% of the genes analysed) than up-regulated (cca. 2%-3% of the genes analysed) on both the proximal and distal sides of the flower pedicel AZ (Figure 10). There was little difference in expression between the AZ sides at 0 h post induction (0.01%-0.02%), as the system was stable before abscission was induced. Even with the perturbation of the system, the number of differentially regulated genes between the AZ sides increased by a small margin (0.05%-0.06%) (Figure 11). This microarray experiment was designed to compare the specific changes between the proximal and distal AZ regions during abscission. Table 3 contains a list of all of the genes that were differentially regulated between the AZ regions.

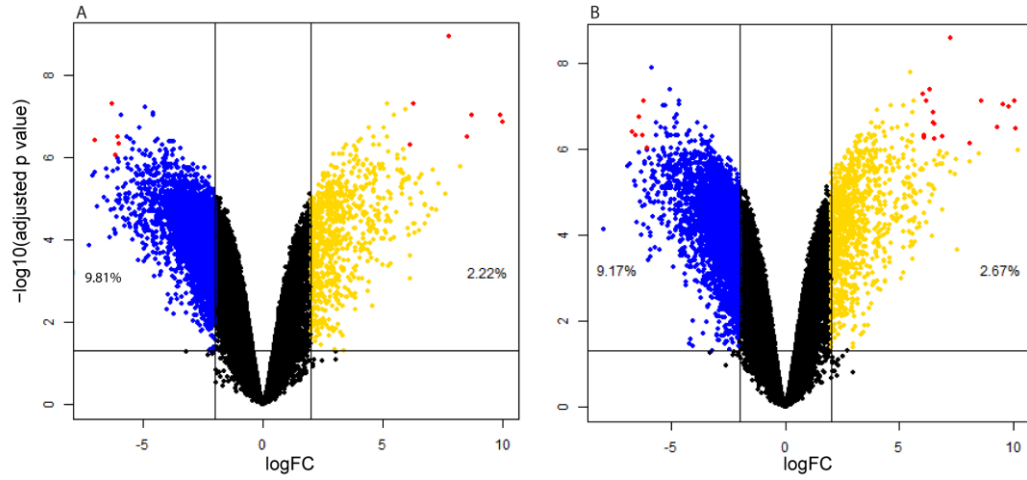


Figure 10: Differential gene expression during flower abscission. (A) Volcano plot for the contrast between samples of proximal AZ at 5 h after induction versus samples of proximal AZ at 0 h after induction (control samples). (B) Volcano plot for the contrast between the samples of distal AZ at 5 h after induction versus samples of distal AZ at 0 h after induction (control samples). Genes above the threshold values of $\logFC > 2$ and $\text{adjPval} < 0.05$ are marked as yellow dots. Genes below the threshold values of $\logFC > -2$ and $\text{adjPval} < 0.05$ are marked as blue dots. Genes above the threshold values of $\text{abs}(\logFC) > 6$ and $\text{adjPval} < 0.000001$ are marked as red dots. The list of genes that represented the red dots can be seen in Table 2. The percentages given below the dots represent the fraction of the microarray features that were above the above-mentioned thresholds (black lines), compared to all of the features that were retained after quality control (i.e., 38,638).

Table 2: The significantly differentially expressed genes during abscission. The ITAG identifiers and corresponding descriptions for the genes marked with red dots in Figure 10 are shown. The proximal and distal AZ at 5 h after abscission induction are compared to their respective controls at 0 h. These genes have $\logFC$ values that were above the thresholds of $\text{abs}(\logFC) > 6$ and the adjusted p values above $\text{adjPval} < 1 \times 10^{-6}$.

Region of AZ	ITAG ID	ITAG Description	Status
Proximal	Solyc12g042610	Serine/threonine-protein phosphatase	Up-regulated
	Solyc08g067700	Unknown protein	Up-regulated
	Solyc06g007180	Asparagine synthase	Up-regulated
	Solyc03g006700	Peroxidase	Up-regulated
	Solyc03g020050	Proteinase inhibitor II	Up-regulated
	Solyc01g057000	Universal stress protein family protein	Up-regulated
	Solyc11g065770	Cytochrome P450	Up-regulated
	Solyc02g085180	Catalytic/ hydrolase	Down-regulated
	Solyc10g080980	Nodulin MtN21 family protein	Down-regulated
	Solyc01g010970	ARGONAUTE 1	Down-regulated
	Solyc04g078200	Gibberellin-regulated family protein	Down-regulated

	Solyc03g096550	Wound/stress protein	Up-regulated
	Solyc06g007180	Asparagine synthase	Up-regulated
	Solyc05g054890	ABC transporter G family member 1	Up-regulated
	Solyc03g006700	Peroxidase	Up-regulated
	Solyc08g067630	Unknown protein	Up-regulated
	Solyc01g110000	Beta-galactosidase	Up-regulated
	Solyc03g020050	Proteinase inhibitor II	Up-regulated
	Solyc01g057000	Universal stress protein family protein	Up-regulated
	Solyc03g044300	AP2-like ethylene-responsive transcription factor	Up-regulated
Distal	Solyc02g067750	Carbonic anhydrase	Up-regulated
	Solyc10g083400	Cytochrome P450	Up-regulated
	Solyc11g065770	Cytochrome P450	Up-regulated
	Solyc05g053330	MYB transcription factor	Up-regulated
	Solyc09g084480	Proteinase inhibitor I	Up-regulated
	Solyc08g082250	Endoglucanase 1	Down-regulated
	Solyc08g066490	Receptor-like protein kinase	Down-regulated
	Solyc02g072540	CBL-interacting protein kinase 16	Down-regulated
	Solyc03g005360	Unknown protein	Down-regulated
	Solyc04g078200	Gibberellin-regulated family protein	Down-regulated

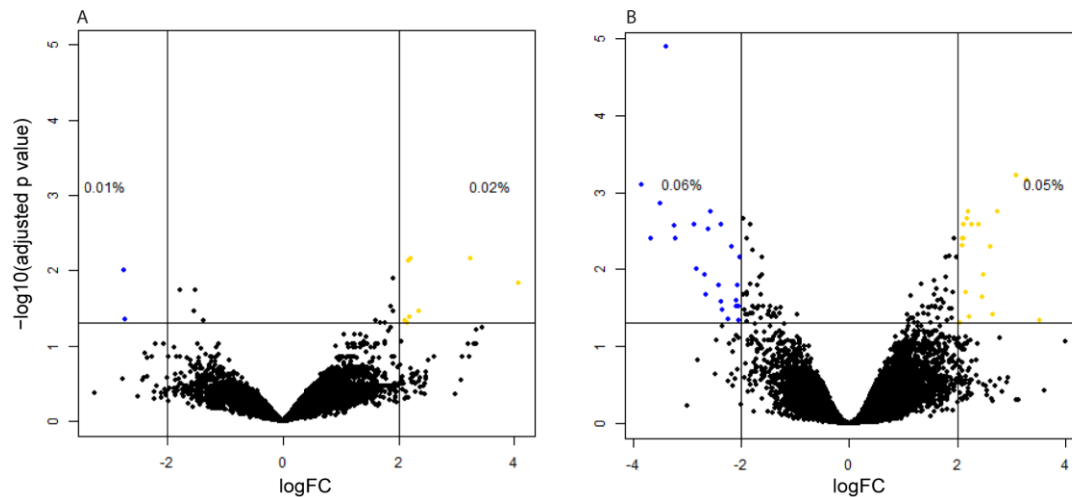


Figure 11: Differential gene expression between the proximal and distal AZ. (A) Volcano plot for the contrast between the proximal AZ at 0 h after induction versus samples of the distal AZ at 0 h after induction (control samples). (B) Volcano plot for the contrast between the proximal AZ at 5 h after induction versus distal AZ at 5 h after induction (control samples). Genes above the threshold values of $\log_{FC} > 2$ and $\text{adjPval} < 0.05$ are marked as yellow dots. Genes below the threshold values of $\log_{FC} > -2$ and $\text{adjPval} < 0.05$ are marked as blue dots. The list of genes representing the yellow and blue dots are given in Table 3. The percentages given above the dots represent the fraction of microarray

features that were above the before-mentioned thresholds (black lines), compared to all of the features that were retained after quality control (38,638).

3.2.2.2 Differential expression of genes related to specific processes during abscission

Groups of genes related to different biological processes were selected, as described by Kim et al. (2015). The focused here was mainly on genes related to ethylene regulation and synthesis, PCD, or senescence. In the next sections these groups of genes were visualised in the context of the microarray data that were analysed, taking into account the spatial and temporal differences in expression.

3.2.2.2.1 Differential expression of genes related to ethylene during abscission

In tomato pedicel abscission, ethylene production and signalling have major roles. The dataset was filtered using a list of ethylene-related genes, and the results were plotted using the volcano plots show in Figures 12 and 13. When comparing different times on the same side of the AZ, differential regulation of several genes can be observed (Figure 12, red dots). While the majority of the genes in the group did not pass the threshold for differential regulation, of those that did, more were down-regulated during abscission than were up-regulated. Table 4 shows the ITAG identifiers for the probes that were detected above the thresholds (red dots) and their respective descriptions.

Table 3: Differentially expressed genes during abscission. The ITAG identifiers and their corresponding descriptions for genes are marked with yellow and blue dots in Figure 11. Samples of proximal AZ are compared against samples of distal AZ (control samples) at 0 and 5 h post induction (HPI) of abscission, respectively. These genes have logFC values that were above the thresholds of $\text{abs}(\log\text{FC}) > 2$ and the adjusted p values above $\text{adjPval} < 0.05$.

HPI	ITAG ID	Description	Status
0	Solyc12g056460	MADS box transcription factor	Up-regulated
	Solyc10g085740	GDSL esterase/lipase At5g03820	Up-regulated
	Solyc09g089520	Proteinase inhibitor I	Up-regulated
	Solyc01g057910	MYB transcription factor	Up-regulated
	Solyc06g062670	Zinc finger family protein	Up-regulated
	Solyc11g066590	Lysosomal Pro-X carboxypeptidase	Down-regulated
	Solyc07g006860	Xyloglucan endotransglucosylase/hydrolase 3	Down-regulated
5	Solyc00g166690	Unknown protein	Up-regulated
	Solyc10g082020	Cytokinin riboside phosphoribohydrolase LOG	Up-regulated
	Solyc04g051540	WRKY family transcription factor	Up-regulated
	Solyc12g056460	MADS box transcription factor	Up-regulated
	Solyc06g050870	Hypoxia induced protein conserved region containing protein expressed	Up-regulated
	Solyc03g116070	Zinc finger-homeodomain protein 3	Up-regulated
	Solyc10g085740	GDSL esterase/lipase At5g03820	Up-regulated
	Solyc00g117450	Short internode related sequence 5	Up-regulated
	Solyc11g069890	BEL1-like homeodomain protein 8	Up-regulated

Solyc03g114860	Alpha-1 4-glucan-protein synthase	Up-regulated
Solyc01g060000	Mutator-like transposase	Up-regulated
Solyc06g062670	Zinc finger family protein	Up-regulated
Solyc12g005940	1-aminocyclopropane-1-carboxylate oxidase	Up-regulated
Solyc09g008830	Os01g0318400 protein	Up-regulated
Solyc03g033680	Short internode related sequence 5	Up-regulated
Solyc00g013290	LINE-type retrotransposon	Down-regulated
Solyc01g093960	MADS box transcription factor	Down-regulated
Solyc07g038110	Beta-galactosidase	Down-regulated
Solyc11g066590	Lysosomal Pro-X carboxypeptidase	Down-regulated
Solyc10g080980	Nodulin MtN21 family protein	Down-regulated
Solyc07g065940	Unknown protein	Down-regulated
Solyc05g006920	Peptide transporter	Down-regulated
Solyc02g089200	MADS-box transcription factor	Down-regulated
Solyc03g083770	Pectinesterase	Down-regulated
Solyc06g073920	YABBY-like transcription factor CRABS CLAW-like protein	Down-regulated
Solyc02g068280	Subtilisin-like serine protease	Down-regulated
Solyc02g087280	Peptide transporter	Down-regulated
Solyc11g017010	Sucrose transporter	Down-regulated
Solyc03g044300	AP2-like ethylene-responsive transcription factor	Down-regulated
Solyc07g062530	Phosphoenolpyruvate carboxylase 2	Down-regulated
Solyc05g015750	Transcription factor MADS-box 2	Down-regulated
Solyc07g056310	Glucan endo-1 3-beta-glucosidase 4	Down-regulated
Solyc06g066510	Avr9/Cf-9 rapidly elicited protein 146	Down-regulated
Solyc10g074920	Mannan endo-1 4-beta-mannosidase	Down-regulated
Solyc01g080260	At4g14280-like protein	Down-regulated
Solyc11g012930	Nodulin family protein	Down-regulated

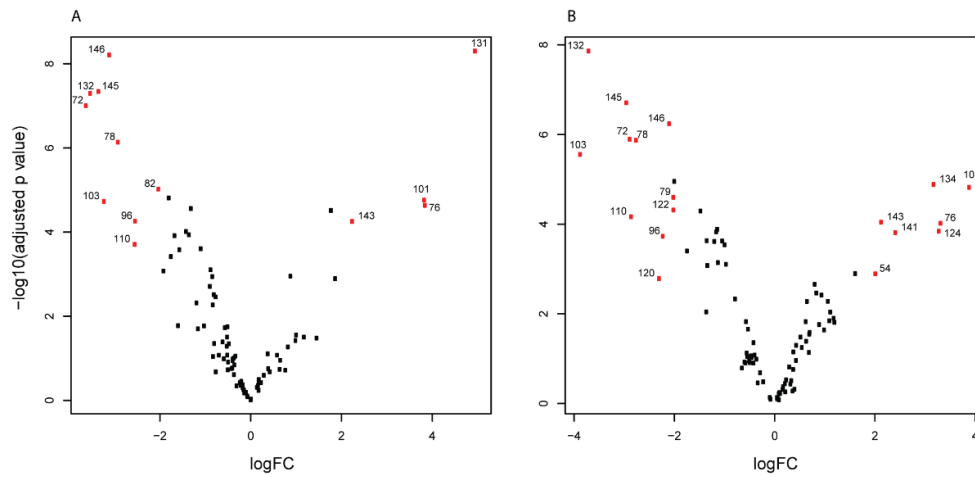


Figure 12: Differential expression of the subset of ethylene-related genes during abscission. (A) Volcano plot for the contrast between proximal AZ at 5 h after induction versus proximal AZ at 0 h after induction (control samples). (B) Volcano plot for the contrast between distal AZ at 5 h after induction versus distal AZ at 0 h after induction (control samples). Genes above the threshold values of $\text{abs}(\log_{2}(\text{FC})) > 2$ and $\text{adjPval} < 0.05$ are marked as red dots. Numbers above the red dots are the IDs for the specific probes in the ethylene dataset (see Table 4).

Only one gene in the whole list, *LeACO2*, was detected as differentially regulated between the AZ sides. At 5 h post induction the expression of *LeACO2* was higher for the proximal side of AZ compared to the distal side, by more than 2-fold. Other *LeACO* genes were differentially regulated only in terms of time after induction. Two genes, *LeACO4* and *LeACO6*, were down-regulated with time, while *LeACO1*, *LeACO2*, *LeACO3* and *LeACO5* were all up-regulated (Figure 12).

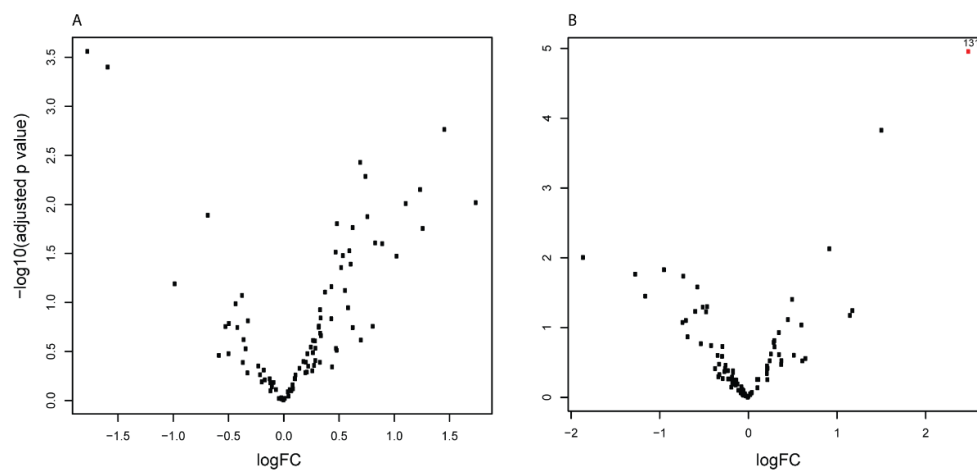


Figure 13: Differential expression of the subset of ethylene-related genes between the proximal and distal AZ. (A) Volcano plot for the contrast between proximal AZ at 0 h after induction versus distal AZ at 0 h after induction (control samples). (B) Volcano plot

for the contrast between proximal AZ at 5 h after induction versus distal AZ at 5 h after induction (control samples). Genes above the threshold values of $\text{abs}(\log\text{FC}) > 2$ and $\text{adjPval} < 0.05$ are marked as red dots. Numbers above red dots are IDs for the specific probes in the ethylene dataset (see Table 4).

Table 4: Gene IDs and descriptions for the significantly differentially expressed ethylene-related genes.

Table ID	ITAG ID	ITAG Description	UniProtKB Gene Name
54	Solyc07g049550	1-aminocyclopropane-1-carboxylate oxidase	<i>LeACO3</i>
72	Solyc02g081190	1-aminocyclopropane-1-carboxylate oxidase	<i>LeACO4</i>
76	Solyc01g095080	1-aminocyclopropane-1-carboxylate synthase	<i>LeACS2</i>
78	Solyc02g070040	Ethylene-responsive nuclear protein	
79	Solyc11g011740	Ethylene-responsive transcription factor 2	
82	Solyc08g079750	1-aminocyclopropane-1-carboxylate synthase	
96	Solyc07g056580	Ethylene receptor	<i>LeETR2</i>
101	Solyc07g026650	1-aminocyclopropane-1-carboxylate oxidase	<i>LeACO5</i>
103	Solyc02g091990	1-aminocyclopropane-1-carboxylate synthase	<i>LeACS3</i>
110	Solyc03g006320	Ethylene-responsive transcription factor 4	
120	Solyc01g090300	Ethylene responsive transcription factor 1b	
122	Solyc02g036350	1-aminocyclopropane-1-carboxylate oxidase	<i>LeACO6</i>
124	Solyc07g049550	1-aminocyclopropane-1-carboxylate oxidase	
131	Solyc12g005940	1-aminocyclopropane-1-carboxylate oxidase	<i>LeACO2</i>
132	Solyc01g090310	Ethylene responsive transcription factor 1a	
134	Solyc06g019200	Peptidase M50 family protein	
141	Solyc07g049530	1-aminocyclopropane-1-carboxylate oxidase	<i>LeACO1</i>
143	Solyc05g051200	Ethylene-responsive transcription factor 1A	
145	Solyc08g081540	1-aminocyclopropane-1-carboxylate synthase	<i>LeACS1B</i>
146	Solyc08g081550	1-aminocyclopropane-1-carboxylate synthase	

3.2.2.2.2 Differential expression of genes related to PCD during abscission

At the set threshold, six PCD-related genes were induced on both sides of the AZ during abscission (Figure 14), although there were no significant differences seen between the proximal and distal sides (Figure 15). Three of the six genes, were induced similarly on both sides: *LeLX*, *LeLE* and *LeTBN1*. The other three showed some side specificity, as two showed a greater increase in expression for the proximal side, and one for the distal side. The only one of these last three genes that was characterised was *LeRBOH1*, which showed

greater expression for the proximal side. The other gene with greater expression for the proximal side, Solyc01g099620, had the description of »Respiratory burst oxidase-like protein«. The uncharacterised gene that showed greater expression for the distal side was Solyc07g006560, with the description »Hypersensitive response assisting protein« (Table 5).

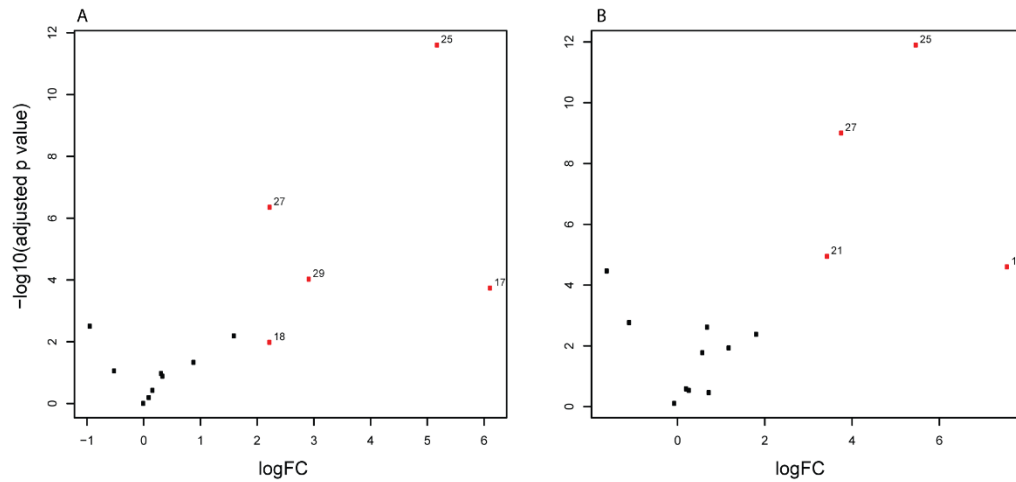


Figure 14: Differential expression of a subset of PCD-related genes during abscission. (A) Volcano plot for the contrast between proximal AZ at 5 h after induction versus proximal AZ at 0 h after induction (control samples). (B) Volcano plot for the contrast between distal AZ at 5 h after induction versus distal AZ at 0 h after induction (control samples). Genes above the threshold values of $\text{abs}(\log_{2}(\text{FC})) > 2$ and $\text{adjPval} < 0.05$ are marked as red dots. Numbers above red dots are IDs for the specific probes in the ethylene dataset (see Table 5).

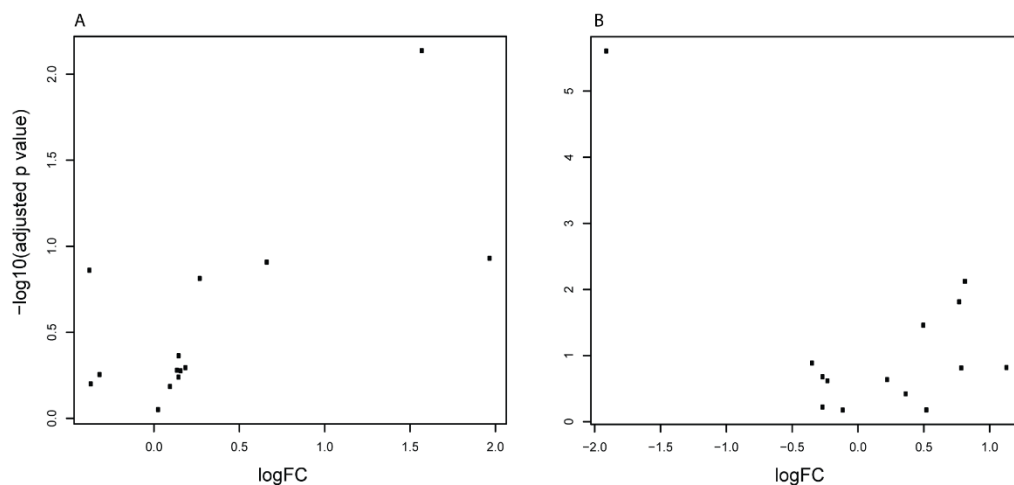


Figure 15: Differential expression of a subset of PCD-related genes between the proximal and distal AZ. (A) Volcano plot for the contrast between proximal AZ at 0 h after induction

versus distal AZ at 0 h after induction (control samples). (B) Volcano plot for the contrast between proximal AZ at 5 h after induction versus distal AZ at 5 h after induction (control samples). Genes above the threshold values of $\text{abs}(\log\text{FC}) > 2$ and $\text{adjPval} < 0.05$ are marked as red dots. Numbers above red dots are IDs for the specific probes in the ethylene dataset (see Table 5).

Table 5: Gene IDs and descriptions for significantly differentially expressed PCD-related genes.

Table ID	ITAG ID	ITAG Description	UniProtKB Gene Name
17	Solyc05g007950	Ribonuclease T2	<i>LeLE</i>
18	Solyc01g099620	Respiratory burst oxidase-like protein	
21	Solyc07g006560	Hypersensitive response assisting protein	
25	Solyc05g007940	Ribonuclease T2	<i>LeLX</i>
27	Solyc02g078910	Endonuclease	<i>LeTBN1</i>
29	Solyc08g081690	Respiratory burst oxidase	<i>LeRBOH1</i>

3.2.2.2.3 Differential expression of genes related to senescence during abscission

Induction of abscission in the flower pedicel was performed by removing the flowers from the pedicel, thus wounding the plant material. After wounding, senescence of the pedicel usually started on the distal side of the AZ. The microarray data were analysed and grouped into subsets using a list of genes related to the process of senescence. Here, there were both down-regulated and up-regulated genes after induction of abscission on either sides of the AZ (Figure 16), although again, none of the genes showed significant differential expression between proximal and distal sides of the AZ (Figure 17). Table 6 shows these senescence-related genes that were differentially regulated during abscission.

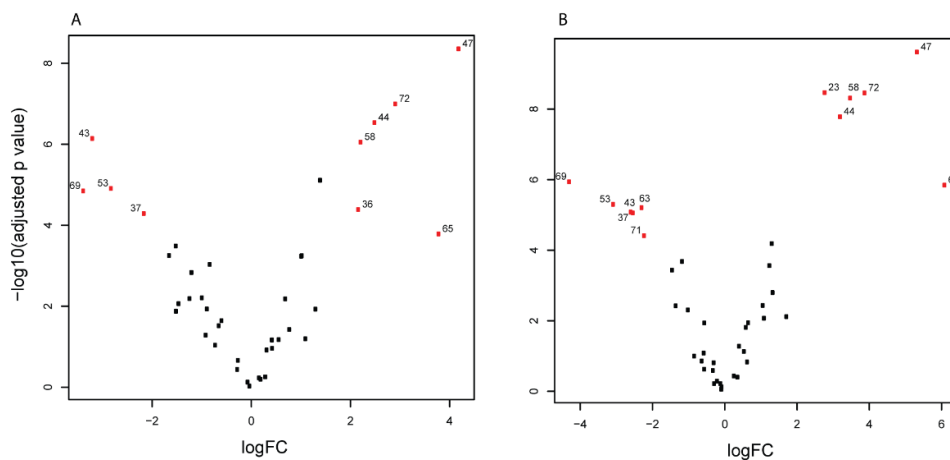


Figure 16: Differential expression of a subset of senescence-related genes during abscission. (A) Volcano plot for the contrast between proximal AZ at 5 h after induction *versus*

proximal AZ at 0 h after induction (control samples). (B) Volcano plot for the contrast between distal AZ at 5 h after induction *versus* distal AZ at 0 h after induction (control samples). Genes above the threshold values of $\text{abs}(\log\text{FC}) > 2$ and $\text{adjPval} < 0.05$ are marked as red dots. Numbers above red dots are IDs for the specific probes in the ethylene dataset (see Table 6).

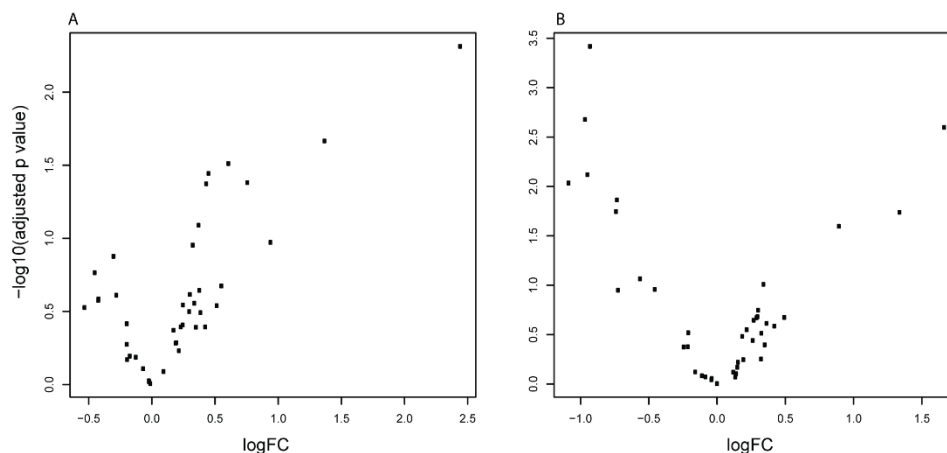


Figure 17: Differential expression of a subset of senescence-related genes between the proximal and distal AZ. (A) Volcano plot for the contrast between proximal AZ at 0 h after induction *versus* distal AZ at 0 h after induction (control samples). (B) Volcano plot for the contrast between proximal AZ at 5 h after induction *versus* distal AZ at 5 h after induction (control samples). Genes above the threshold values of $\text{abs}(\log\text{FC}) > 2$ and $\text{adjPval} < 0.05$ are marked as red dots. Numbers above red dots are IDs for the specific probes in the ethylene dataset (see Table 6).

Table 6: Gene IDs and descriptions for significantly differentially expressed senescence-related genes

Table ID	ITAG ID	ITAG Description	UniProtKB Gene Name
23	Solyc08g080090	Senescence-inducible chloroplast stay-green protein 2	<i>LeSGR1</i>
36	Solyc02g094400	Glycerophosphodiester phosphodiesterase gde1	
37	Solyc02g067660	Phytoalexin deficient 4	
43	Solyc02g071380	1-aminocyclopropane-1-carboxylate oxidase 3	
44	Solyc07g041900	Cathepsin L-like cysteine proteinase	<i>CYP-3</i>
47	Solyc02g071430	1-aminocyclopropane-1-carboxylate oxidase	
53	Solyc06g050760	Wound-induced protein 1	
58	Solyc08g080090	Senescence-inducible chloroplast stay-green protein 2	
63	Solyc11g011170	ATA15 protein	
65	Solyc12g056480	Senescence-inducible chloroplast stay-green protein	
69	Solyc02g077050	Cathepsin B-like cysteine proteinase	

71	Solyc02g071400	1-aminocyclopropane-1-carboxylate oxidase
72	Solyc07g041910	Cathepsin H

3.2.3 Gene expression dynamics of selected genes during tomato flower pedicel abscission

Gene expression for the selected genes was analysed at several time points after induction. Under these experimental conditions, the tomato pedicels were completely abscised at 12 h post induction, and for this reason, samples were collected every 2 h from the start to the 12th hour after induction. This approach provided reasonable gene expression profile resolution for good insights into the expression dynamics during abscission.

Changes in expression of *LeTBN1* were observed 2 h after induction of abscission (Figure 18). On the proximal side, the expression increased at a slower rate compared to the distal side. This steep increase in expression on the distal side of the AZ thus led to an asymmetric distribution of the amount of *LeTBN1* transcript between these two sides. On the other hand, *LeLX* was induced immediately after this induction of abscission, although the differences between the proximal and distal sides were significant only at 6 h after induction (Figure 18). On the distal side, *LeLX* expression steeply increased from 6 h after induction. Similarly, *LeRBOH1* expression was also increased at 6 h post induction but there are no differences in transcript amount between the proximal and distal sides of the pedicel AZ (Figure 18).

LeACO1 expression was increased rapidly after induction of abscission (Figure 18), and in the first 2 h post induction this reached a stable plateau that was maintained for the rest of the period. On the other hand, *LeACO4* expression was inhibited during the progression of abscission, although not as rapidly as the increase in *LeACO1* expression. *LeACO4* down-regulation was more prominent 2 h after induction of abscission, which was when *LeACO1* was at its peak expression (Figure 18). While the expression profiles of these two genes were completely opposite, there were no differences in gene expression between the two sides of the AZ. *LeEIL2* expression remained stable on both sides of the AZ throughout this period (Figure 18). The expression profiles of two polygalacturonases, *LeTAPG1* and *LeTAPG4*, were also re-examined. *LeTAPG1* expression was induced a few hours after the flowers were removed from the petiole, while *LeTAPG4* expression increased immediately (Figure 18). Although this pattern was confirmed here, there were no significant differences in gene expression between the proximal and distal sides of the AZ.

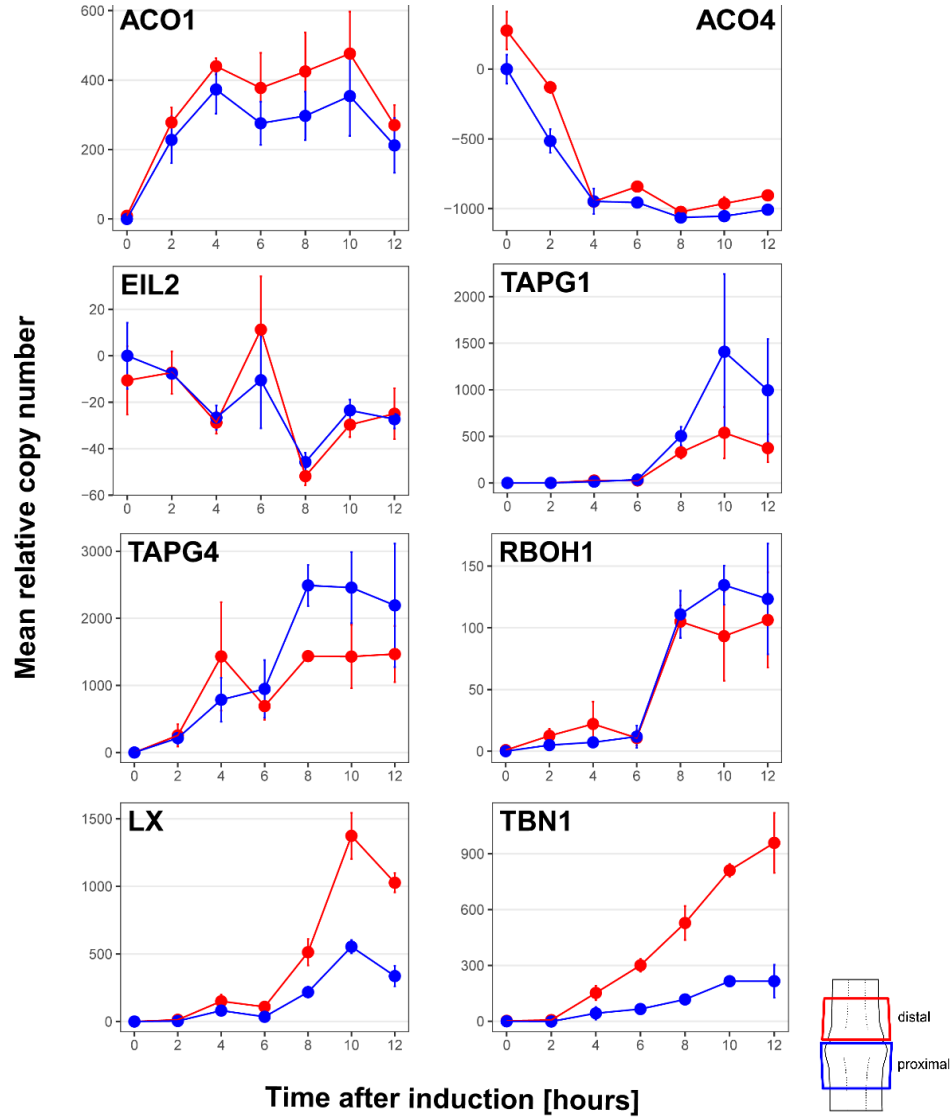


Figure 18: Gene expression dynamics and tissue-specific expression of the selected genes during flower pedicel abscission in WT plants. Tomato flower pedicels were sampled 0, 2, 4, 6, 8, 10 and 12 h after induction of abscission, and separated into the proximal (blue lines) and distal (red lines) sides at the abscission fracture plane, as shown schematically. The data are expressed as relative copy numbers $\pm$ SEM.

The expression of this selected group of genes was tested in three independent transgenic lines, as 1-7, 20-2 and 7-5, under inhibition of expression of the LX protein, to test the hypothesis that the delay of abscission caused by LX suppression (Lers et al., 2006) is in any way related to changes in their expression profiles. *LeLX* expression was lower in the transgenic lines than in WT plants, as was expected, although this was exceptionally low in the 1-7 line than in the other two lines (20-2, 7-5), which showed similar basal levels of *LeLX* expression. Other genes did not show any specific changes of their expression profiles when compared to those in WT plants (Figures 19, 20, 21). The only exception was *LeRBOH1*, which showed lower overall expression in all three of these transgenic lines. Again, the only gene that showed any clear difference in expression between the proximal and distal sides of the AZ was the PCD marker gene *LeTBN1* (Figures 19, 20, 21).

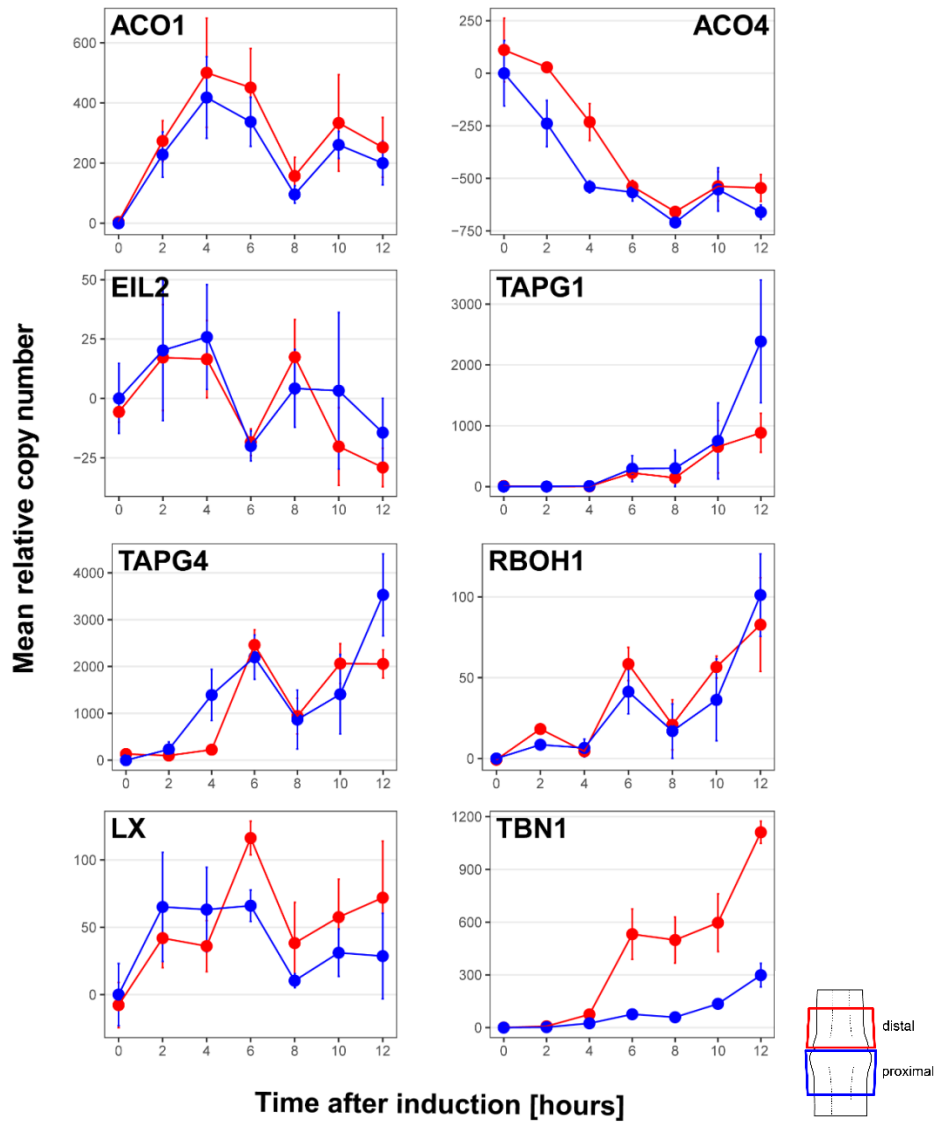


Figure 19: Gene expression dynamics and tissue-specific expression of the selected genes during flower pedicel abscission in transgenic tomato line 1-7. Tomato flower pedicels were sampled 0, 2, 4, 6, 8, 10 and 12 h after induction of abscission, and separated into proximal (blue lines) and distal (red lines) sides at the abscission fracture plane, as shown schematically. The data are expressed as relative copy numbers $\pm$ SEM.

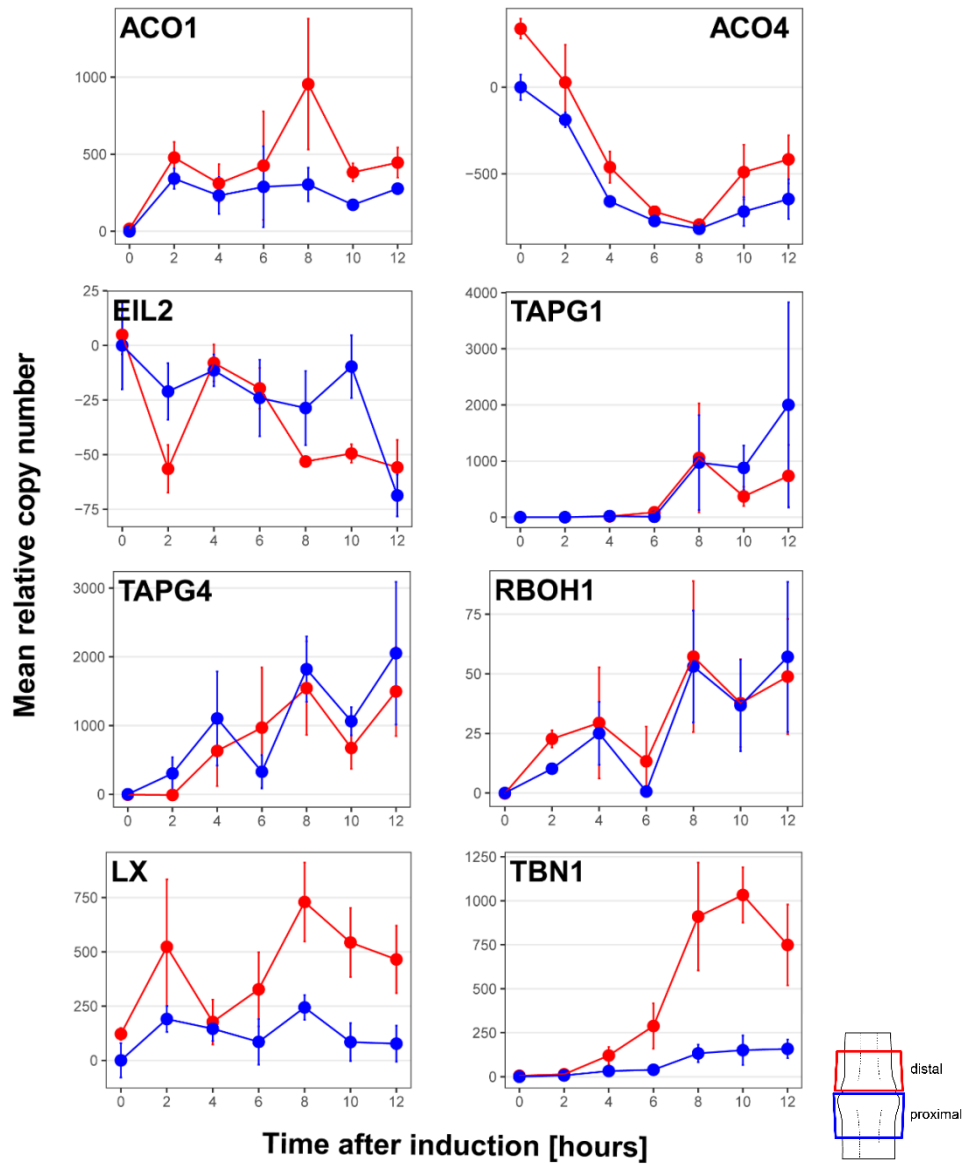


Figure 20: Gene expression dynamics and tissue-specific expression of the selected genes during flower pedicel abscission in transgenic tomato line 7-5. Tomato flower pedicels were sampled 0, 2, 4, 6, 8, 10 and 12 h after induction of abscission, and separated into proximal (blue lines) and distal (red lines) sides at the abscission fracture plane, as shown schematically. The data are expressed as relative copy numbers $\pm$ SEM.

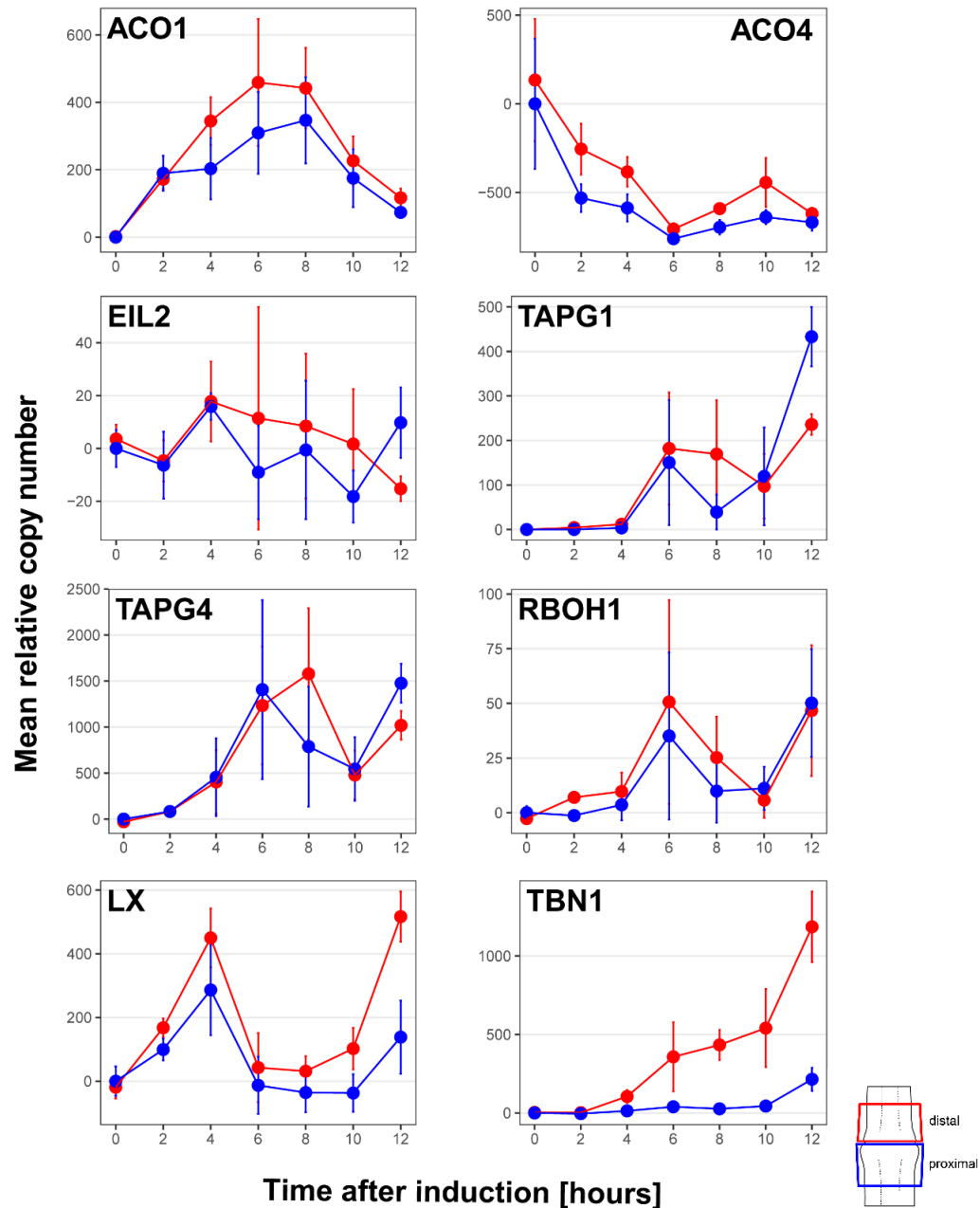


Figure 21: Gene expression dynamics and tissue-specific expression of the selected genes during flower pedicel abscission in transgenic tomato line 20-2. Tomato flower pedicels were sampled 0, 2, 4, 6, 8, 10 and 12 h after induction of abscission, and separated into proximal (blue lines) and distal (red lines) sides at the abscission fracture plane, as shown schematically. The data are expressed as relative copy numbers $\pm$ SEM.

3.2.4 Influence of 1-MCP treatment on gene expression of selected genes

To determine the role that ethylene signalling has on molecular changes that occur during abscission, experiments were performed where the plants were treated with 1-MCP. Figure 22 shows the gene expression profiles for selected genes at 0 h and 8 h for control and 1-MCP-treated flower pedicels. While 1-MCP treatment did not significantly influence the expression levels of any of these genes prior to induction, this did inhibit the expression of

several of these genes at 8 h post induction. The *LeACO1*, *LeLX* and *LeTAPG1* transcripts showed the greatest degree of inhibition on both sides of the AZ. On the other hand, *LeEIL2* showed greater expression at 8 h post induction with 1-MCP treatment. Other genes, like *LeTAPG4* and *LeRBOH1*, were significantly inhibited only on the proximal AZ side, or in the case of *LeACO4*, the inhibition was significantly less on the distal side of the AZ. The gene that showed the lowest response to 1-MCP treatment was one of the two PCD markers, *LeTBN1*. Differences between the proximal and distal side were more noticeable for the untreated flower pedicels. *LeACO1*, *LeACO4*, *LeLX* and *LeTBN1* showed an asymmetric distribution towards the distal side of the AZ, while *LeRBOH1*, *LeTAPG1* and *LeTAPG4* were expressed more on the proximal side of the AZ. *LeEIL2* showed an overall equal distribution for both sides of the AZ.

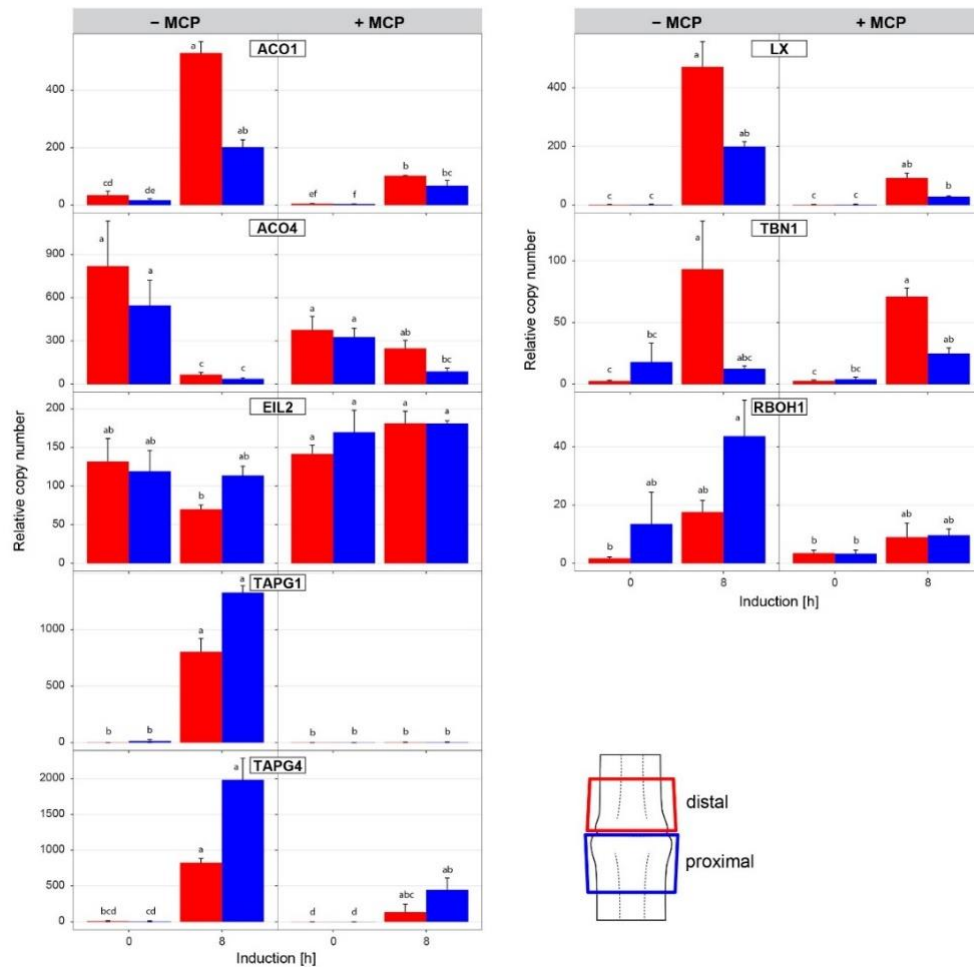


Figure 22: Gene expression in hand-cut tomato flower pedicels before and after abscission induction and without (-MCP) and with (+MCP) 1-MCP treatment. Different letters indicate significantly different expression levels ($p \leq 0.05$) between the leaf positions and the side of the AZ. Below: The location of the sampling sites of the distal (red) and proximal (blue) flower AZ.

3.2.5 Laser microdissection and gene expression analysis in four different tissue regions of the tomato flower pedicel

On the basis of the differences in gene expression between the two sides of the AZ, gene expression was analysed at the tissue level using laser micro-dissected samples. The proximal and distal sides of the AZ were thus divided into two parts each. The distal side was divided in the distal AZ, which was the part of the tissue closest to the actual abscission fracture, and distal part that was located further up the flower petiole (see legend in Figure 23). The same was done for the proximal side. To go into further detail, the distal and proximal sides were divided into the vein tissue and the pith tissue that was located between the veins. The expression analysis showed a more complex picture this time, as the genes were differentially expressed even considering the different tissues on the same side of the AZ.

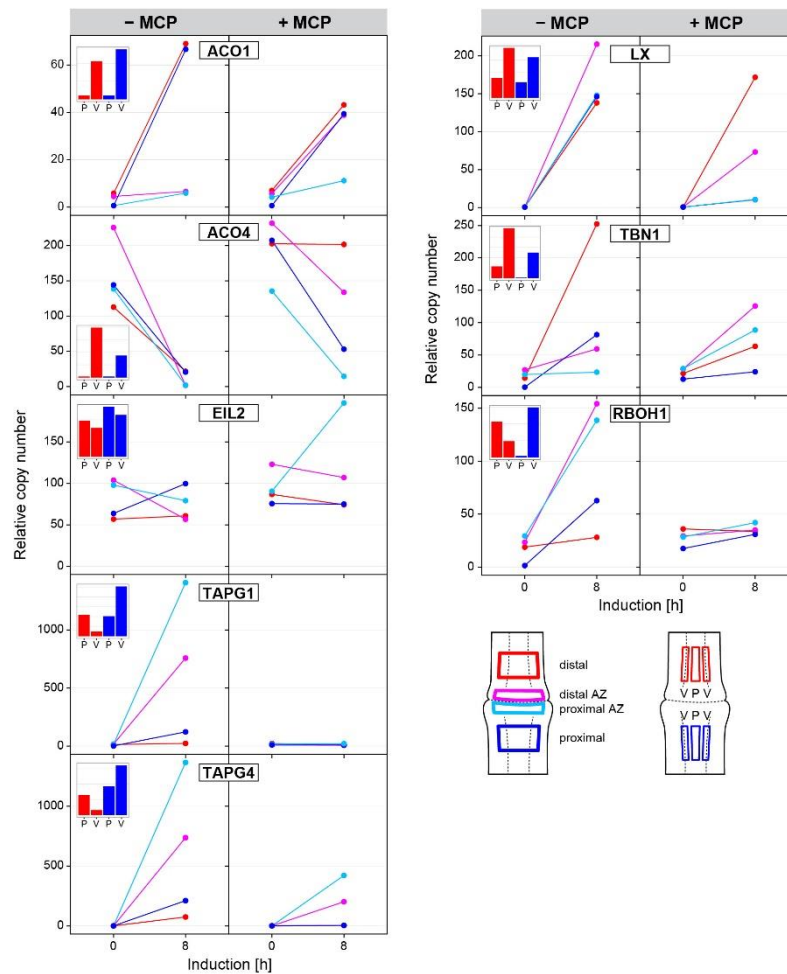


Figure 23: Tissue-specific gene expression of selected genes during tomato flower pedicel abscission. The gene expression analysis for tomato pedicel AZ was divided into four regions using laser micro-dissection, as shown schematically: distal, distal AZ, proximal AZ, and proximal. Untreated (-MCP) and 1-MCP treated (+MCP) samples were taken 0 h and 8 h after induction of abscission. Additionally, samples of vein and pith tissue were analysed in -MCP abscission induced pedicels. The insets show the relative expression between the pith (P) and vein (V) tissues for the proximal (blue) and distal (red) sides of the AZ.

Programmed cell death marker gene expression showed some additional spatial information at this tissue level. *LeTBN1* was expressed in the distal and proximal tissues at 8 h post induction more than in the proximal and distal AZ. In the 1-MCP treated samples, on the other hand, expression was higher in the proximal and distal AZ. 1-MCP thus appears to inhibit *LeTBN1* expression for the distal and proximal tissues, rather than around the AZ fracture, where the expression actually increased (Figure 23). At 8 h after induction of abscission, inhibition of the signal on the distal side was observed, specifically in the vein tissue where the transcript expression was also the highest, while the signal around the fracture remained. Tissue-directed gene expression analysis of *LeTAPG4* showed that this enzyme was expressed preferentially on the proximal side of the AZ, while the signal on the distal side was lower (Figure 23). Even without 1-MCP treatment, *LeTAPG1* and *LeTAPG4* showed low expression levels for proximal and distal tissues, while for the proximal and distal AZ their expression was high. In the controls and outside the AZ tissue, *LeTAPG4* was not detected (or it was detected in very low amounts). *LeLX* expression was highest in the tissue from the distal AZ at 8 h post induction, compared with the other tissue regions. Upon 1-MCP treatment, the expression at 8 h post induction dropped in all tissue regions, except for the distal tissue, where the expression remained stable (Figure 23).

3.2.6 ACO1 and TAPG4 localisation during tomato flower abscission

As ethylene regulation and signalling have a major role in tomato petiole abscission, the expression and localisation of ACO1 were followed at the tissue and cellular levels. This approach would provide relative precision for pinpointing the tissues in which ethylene was produced during abscission, and would indicate what kind of changes occurred during inhibition of the ethylene signalling pathway.

Immunolocalisation of ACO1 in untreated tissues showed that it was produced when abscission was induced (Figure 24A), and that it was located mainly in the vascular tissue present on both sides of the AZ. Although the signal was present on both sides of the AZ, it was strongest in the veins of the distal side. This immunolocalisation was confirmed also by analysing the transcript expression pattern of *LeACO1* in these different tissue regions of the tomato petiole (Figure 25). Both the distal and proximal sides outside of the AZ region had high expression of *LeACO1* at 8 h post induction. Further separation of these regions in the vascular and pith tissues showed that the expression was much higher in the vascular tissue. Immunolocalisation at the cellular level using gold particles showed that ACO1 proteins can be found mainly in the cells that surround the vascular tissue of the proximal and distal sides of the AZ (Figure 24B).

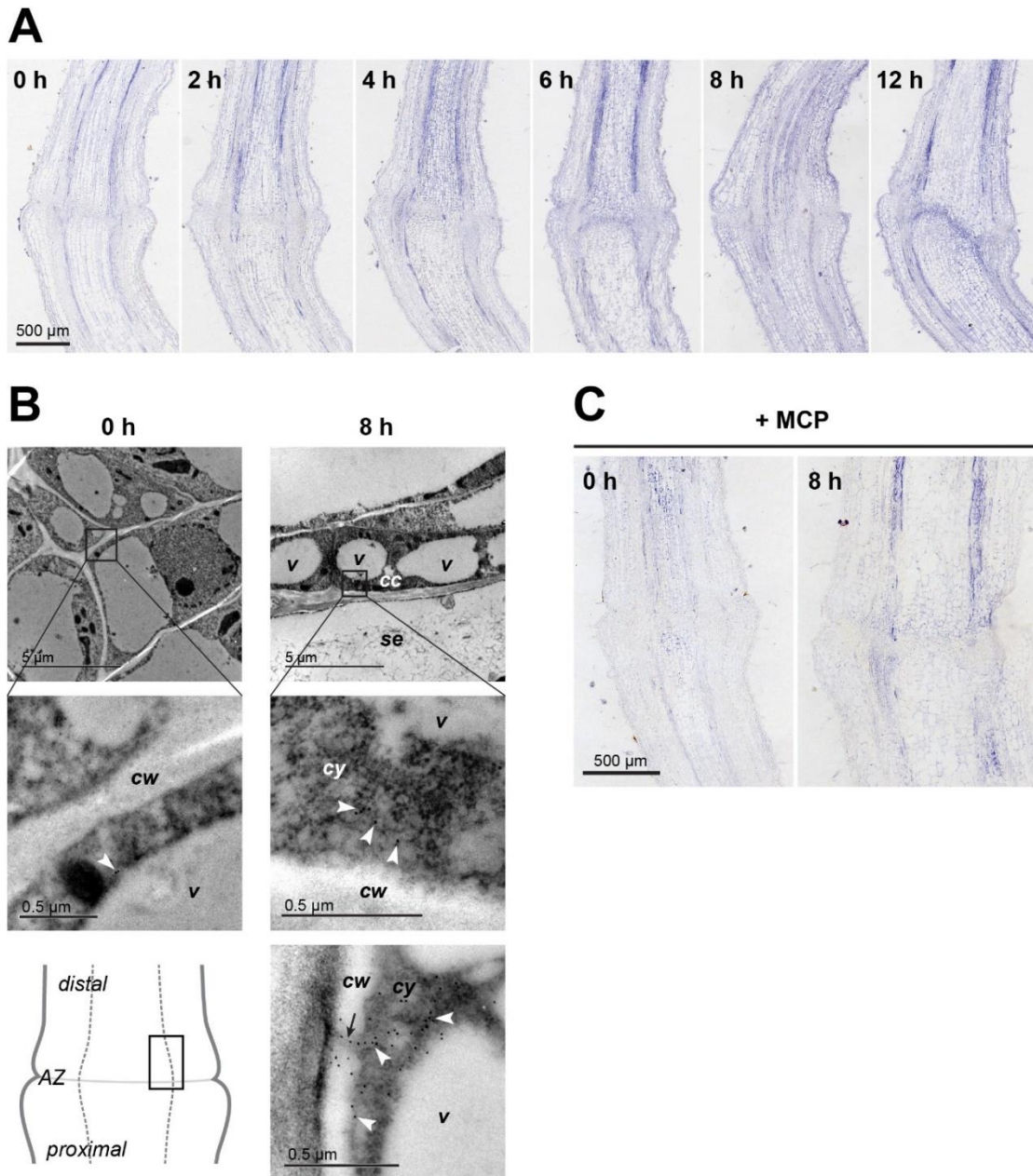


Figure 24: Localisation of the LeACO protein in the flower pedicel AZ. (A) Immunolocalisation of LeACO in tissue collected 0, 2, 4, 6, 8 and 12 h after induction of abscission, as seen under the light microscope. (B) Immunogold localisation of LeACO in the pedicel AZ at the transmission electron microscopy level. White arrowheads indicate gold particles in the cytoplasm; cc, companion cell; cw, cell wall; cy, cytoplasm; se, sieve element; v, vacuole. (C) Immunolocalisation of LeACO in 1-MCP treated pedicels, before and 8 h after abscission induction.

The 1-MCP treatment reduced the expression and immunolocalisation signal of ACO1 for both sides of the AZ (Figure 25). This provided additional confirmation of the 1-MCP effects on *LeACO1* synthesis, as its expression had already been inhibited by this treatment in hand-cut samples of the distal and proximal AZ (Figure 22).

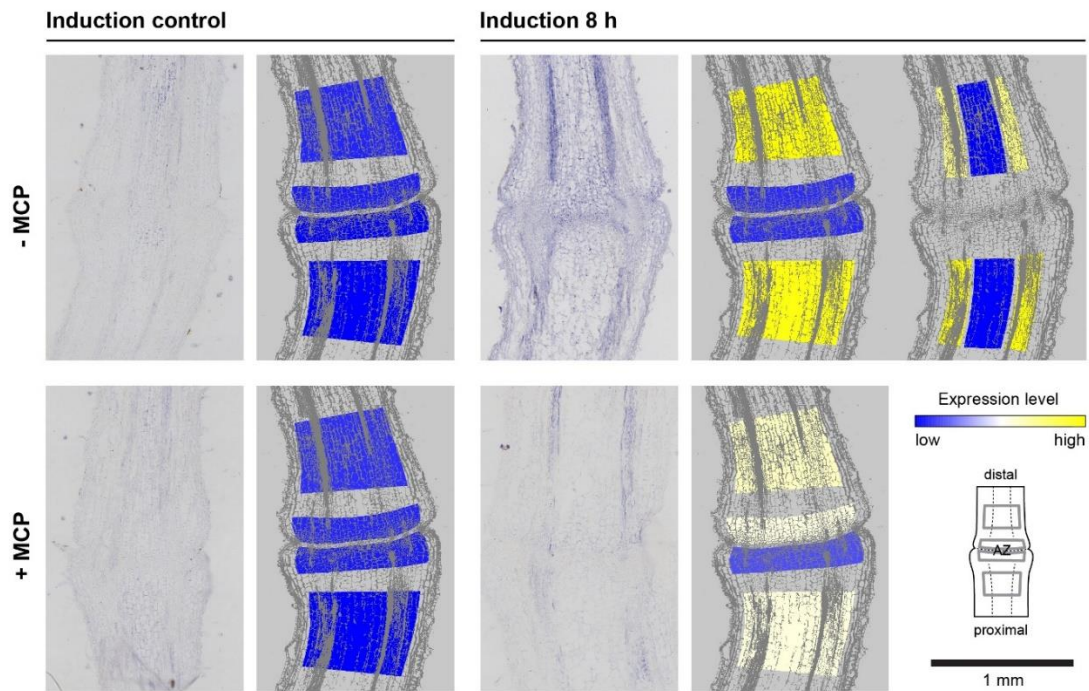


Figure 25: Immunolocalisation and tissue-specific expression of LeACO1 during flower pedicel abscission. Immunolocalisation of LeACO following abscission as seen at the light microscope level. As shown schematically, the gene expression analysis of LeACO1 in tomato pedicel AZ was divided into four regions using laser microdissection: distal, distal AZ, proximal AZ and proximal. Untreated (-MCP) and 1-MCP-treated (+MCP) samples were taken 0 h and 8 h after induction of abscission. Additionally, vein and pith tissue were analysed in untreated abscission-induced pedicels. Expression levels for each gene are illustrated according to a colour scale.

The expression of *LeTAPG4* transcripts in the specific tissue regions correlated with the immunolocalisation results for this protein, as the strongest signal was indeed detected in the induced tissues, and specifically for the proximal edge of the AZ fracture (Figure 26B). The treatment of the pedicels with 1-MCP inhibited this expression, and though a very low amount of transcript was present, the immunolocalisation level did not detect any specific signal. Even when present, the *LeTAPG4* signal did not appear outside the AZ, and was restricted to the vicinity of the AZ fracture (Figure 26A).

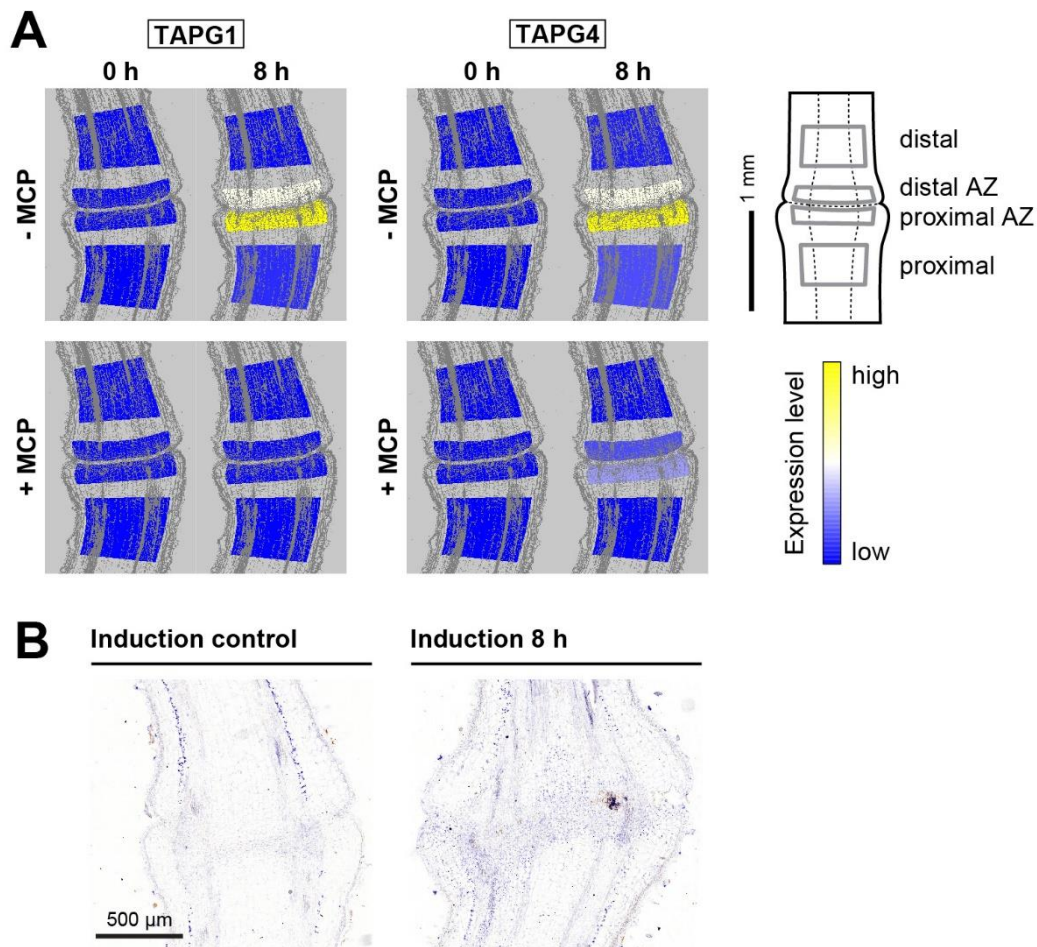


Figure 26: Tissue-specific expression of polygalacturonase genes and localisation of LeTAPG4. (A) Tomato pedicel AZ was divided into four regions using laser microdissection, as shown schematically. Untreated (-MCP) and 1-MCP treated (+MCP) tissues were sampled 0 h and 8 h after induction of abscission. Expression levels for each gene are illustrated according to a colour scale. (B) Immunolocalisation of LeTAPG4 before and 8 h after the abscission induction.

3.2.7 Inhibition of DNA degradation after 1-MCP treatment

To follow PCD at the cellular level, terminal dUTP nick-end labelling (TUNEL) assays were used, which selectively stain damaged DNA, a common feature of PCD. TUNEL assays on tissue sections of the flower AZ showed prevalent staining of nuclei (green) on the distal side at 8 h post induction of abscission (Figure 27), which was the same pattern that was observed with *LeLX* and *LeTBN1* expression (Figure 23). Also, the signal on the distal side was much stronger for the vascular tissue, which also correlated with the expression of both *LeTBN1* and *LeLX*. To test the effects of ethylene and its signalling during these processes, the AZ samples were treated with 1-MCP. These data showed that although some of the signal around the vascular tissue specifically in the AZ was retained when abscission was induced, the strong signal on the distal side that was seen in the untreated plants was not present (Figure 27). This pattern of tissue expression, however, might only be observed for the localisation of *LeTBN1* transcript expression under the 1-MCP treatment, where some of the signal was also retained for the distal AZ (Figure 23).

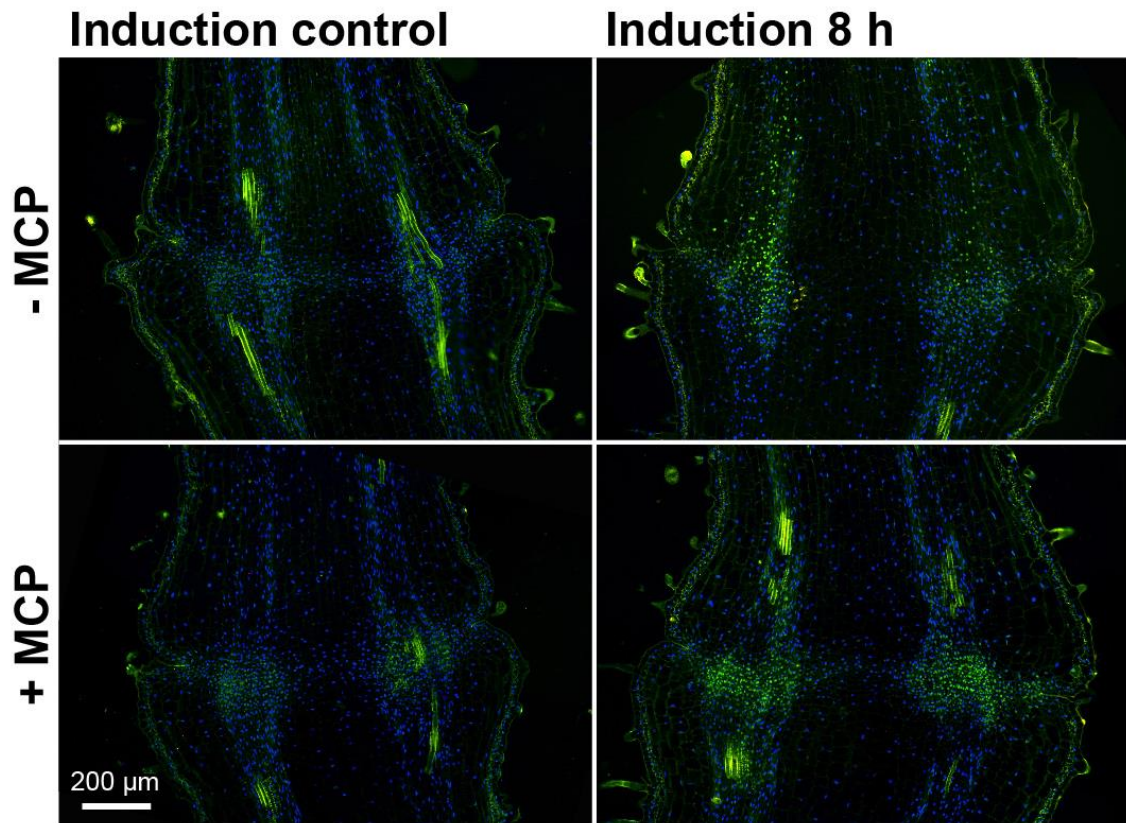


Figure 27: DNA fragmentation in tomato flower pedicel AZ. Tissue sections of 1-MCP untreated (-MCP) and treated (+MCP) samples were taken at 0 h (induction control) and 8 h after induction of abscission, and were stained for the TUNEL assay. TUNEL-positive nuclei with fragmented DNA are green, while nuclei with non-fragmented DNA are blue, with the DAPI staining labelling all of the nuclei. The green signal outside the nuclei is due to autofluorescence of the cell walls. Images are composites of two fluorescence channels.

Figure 29 shows images of the flower petiole AZ after abscission induction where a variation of the TUNEL assay was used, in which the signal was produced using the enzyme alkaline phosphatase. Without the autofluorescent noise from the cell walls, it can be clearly seen that the treatment of the plants with 1-MCP inhibited DNA degradation in the nuclei of the vascular tissue distal to the AZ.

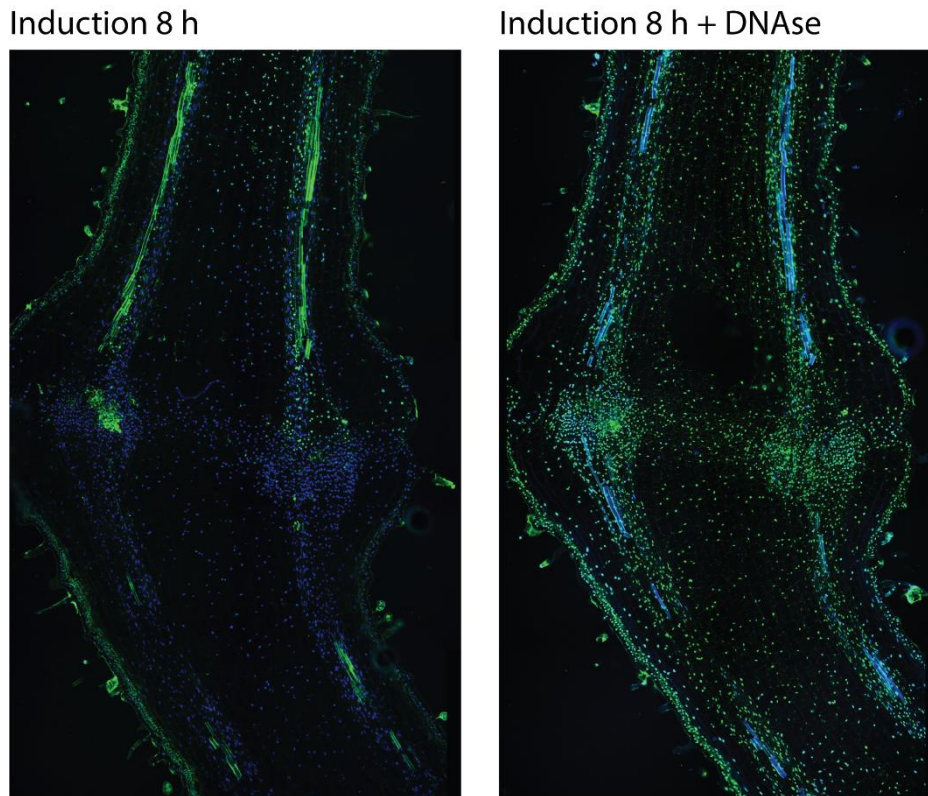


Figure 28: Positive control for DNA fragmentation in the tomato flower pedicel AZ. Tissue sections 8 h after induction of abscission were treated with DNase (+DNase) and stained with the TUNEL assay. TUNEL-positive nuclei with fragmented DNA are green, while the nuclei with non-fragmented DNA are blue, with the DAPI staining labelling all of the nuclei. The green signal outside the nuclei is due to the autofluorescence of the cell walls. Images are composites of two fluorescence channels.

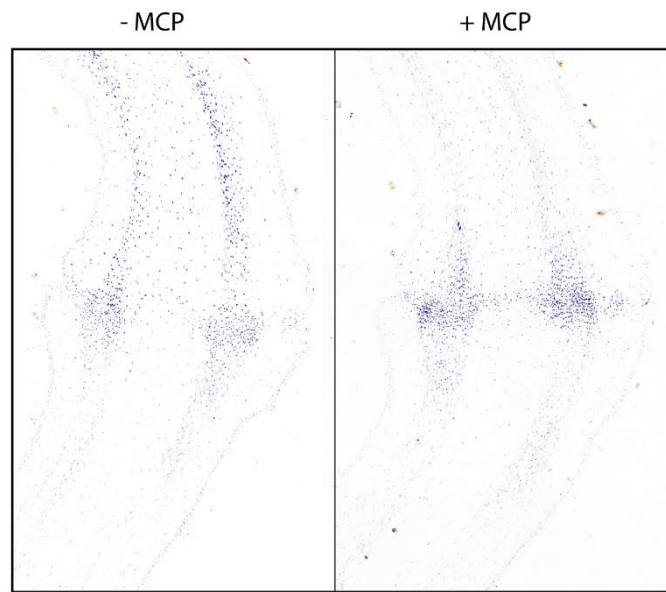


Figure 29: TUNEL assay on tissue sections of the WT flower AZ. 1-MCP untreated (-MCP) and treated (+MCP) samples of tomato flower pedicel 8 h post induction of abscission. The NBT/BCIP staining (blue-purple signal) indicates fragmented DNA in the stained nuclei.

Discussion

By analysing in detail the expression and localisation patterns of key enzymes related to ethylene production, cell separation, and programmed cell death, we wanted to gain some insight into if and how these processes are related to each other. As the AZ of the tomato pedicel and leaf are relatively complex both in structure and function, it is paramount to merge both molecular and spatial data, as these processes might vary greatly in the range of a few layers of cells. Laser microdissection coupled with qPCR provides a powerful tool to analyse tissue-specific gene expression. In the flower AZ, specific expression patterns were only visible when moving from whole tissue samples to microdissected layers of cells. The coupling of this methodology with immunolocalisation techniques at the tissue and sub-cellular level has allowed us to precisely pinpoint the origins of physiological processes related to the morphological changes observed during abscission. In this study, special attention was dedicated to genes related to ethylene production and signalling (i.e., *LeACO1*, *LeACO4*, *LeEIL2*), cell separation (i.e., *LeTAPG1*, *LeTAPG4*), PCD (i.e., *LeLX*, *LeTBN1*), and the formation of reactive oxygen species (i.e., *LeRBOH1*).

4.1 Ethylene-related changes in cell morphology during tomato flower pedicel abscission

The AZ cells are morphologically and physiologically distinct from their neighbouring cells. More specifically, they are small and cytoplasmically dense, interconnected with plasmodesmata, and resemble undifferentiated cells with meristematic potential (Roberts, 2000; Leslie et al., 2007; van Nocker, 2009; Bar-Dror et al., 2011). In addition, several genes associated with organ differentiation in meristems are expressed in the AZ before induction of abscission (Nakano et al., 2013; Wang et al., 2013).

After induction of tomato pedicel abscission, the morphology of the AZ cells changes rapidly. Vesicle formation in the cytoplasm and between the plasmalemma and cell wall can be observed throughout the tissue. At the same time, widening of extracellular spaces and softening of cell walls can be observed, further confirming that the vesicle formation is due to an extensive exocytosis process (Figure 9) (Dermastia et al., 2012). These changes are probably related to the loading of the extracellular space with degrading enzymes and other secondary metabolites that will help to form a protective layer after the separation process is completed (Winder et al., 2015).

It is worth mentioning too that the ability to follow the morphological changes during tomato pedicel abscission at a cellular level can be relatively difficult, as the process is particularly rapid, as the most significant changes happen during the first 8 h after induction. Also, AZ samples are subject to some biological variability, which makes it difficult to precisely define the exact succession of morphological changes and cellular processes that occur during the beginning phases of abscission.

In contrast to the leaf AZ (Bar Dror et al., 2011), where the cell-wall structure remained intact during abscission, in the pedicel AZ, cell-wall modification/expansion is often observed (Figure 9). The same expansion has already been described during abscission (Roberts et al., 2002), and it is thought to be involved in facilitating the cell separation process and the breakage of the xylem vessels that cross the fracture plane.

Chloroplast and nucleus morphology undergoes significant early changes during the abscission process, and while treatment with 1-MCP largely prevented chloroplast

degradation, chromatin condensation and nuclear membrane dilatation remained prominent features in these treated tissues. Therefore these data appear to strongly indicate that changes regarding chloroplast degradation during tomato pedicel abscission are ethylene regulated. PCD hallmarks like vesicle formation, chromatin condensation, and nuclear membrane dilatation are initially visible in parenchymal cells of the vascular tissue inside the AZ. As for tomato leaf abscission (Bar-Dror et al., 2011), in the pedicel the PCD signal spreads from the vascular tissue through the entire AZ tissue. The major difference is that while in the leaf AZ this PCD is mainly located on the distal side of the fracture, in the pedicel, cells that undergo PCD can be observed throughout the AZ region on both the proximal and distal sides. However, PCD is much more widespread on the distal side of the petiole outside the AZ, and the strongest signal of fragmented nuclei was observed towards the wound created upon induction of abscission through flower removal (Figure 29).

4.2 Ethylene synthesis enzymes are expressed in the vascular tissue

4.2.1 Expression profiles and localisation of ethylene-related genes during tomato pedicel abscission

We have previously shown that changes in cell morphology and asymmetric localisation of molecular processes are intrinsic to the abscission process in tomato leaf (Bar-Dror et al., 2011). Specifically, the synthesis and perception of endogenous ethylene is of great importance in tomato flower abscission, as pedicels treated with 1-MCP fail to abscise for at least 30 h after induction (Meir et al., 2010). However, no method for measuring ethylene concentrations are currently suited for the tissue-directed measurements required here (Cristescu et al., 2012).

The ACO proteins are enzymes that catalyse the last step in ethylene biosynthesis, and their expression is directly correlated with the rate of ethylene production (Van de Poel et al., 2012). Monitoring the expression and localisation of these proteins can be performed in tissue and even at the cellular level. Although not a direct measurement of ethylene concentrations in plant tissues and cells, the localisation of the ACO proteins provides a good approximation of the location of ethylene evolution during specific physiological processes. Previous data relating to the intracellular localisation of the ACO proteins have been contradictory. While studies of suspension-cultured tomato cells and apple fruit pericarp tissue have shown ACO in the cytoplasm (Reinhardt et al., 1994; Chung et al., 2002), immune-cytolabelling of apple fruit protoplasts demonstrated LeACO located at the external side of the plasma membrane (Ramassamy et al., 1998).

In the present study, we have demonstrated for the first time the location where the ACO enzyme is expressed during flower abscission. The expression of both of the *LeACO* genes analysed in this study, *LeACO1* and *LeACO4*, occurred almost exclusively in the vascular tissues. In agreement with the *LeACO1* expression pattern, the majority of the LeACO1 protein was in the phloem-cell cytoplasm, although some signal was seen in the area of the phloem-cell plasmodesmata and the cell walls (Figures 24, 25). As ACO catalyses the last step in ethylene biosynthesis, it is rational to predict from the data here that ethylene biosynthesis takes place in the phloem, from where it diffuses to the other tissues for its actions. It remains enigmatic if and how this is related to several suggestions of diffusible signals derived from vascular tissue that are required for abscission (McManus, 2008; Tucker and Yang, 2012). A seminal study from Thompson and Osborne (1994) on

abscission in bean, hypothesised the presence of a specific signal that spreads from the vascular tissue during abscission and induces cell separation throughout the AZ. On the other hand, other studies on tomato have shown that in this plant system, the cells that form the AZ are themselves already competent to separate, regardless of the presence of the vascular tissue (Tucker and Yang, 2012; Sexton, 1979). Endogenous ethylene is a good candidate for such a signal in tomato, as it originates from the vascular tissue during abscission, and also the inhibition of its signalling pathway leads to a severe inhibition of cell separation (Meir et al., 2010).

The present findings combined with some of the literature data (Meir et al., 2010) indicate that pedicel abscission follows the broadly accepted concept of two systems of ethylene biosynthesis described during climacteric fruit development and ripening (McMurchie et al., 1972; Van de Poel et al., 2012). As a part of system 1, *LeACO4* expression before abscission induction was high, and it decreased simultaneous with the induction of high expression of *LeACO1* (present study) and *LeACO5* (Meir et al., 2010) in system 2, at 2 h after abscission induction. Similar expression patterns of the *LeACO* genes were demonstrated here for the leaf petiole AZ (Figure 3).

The *LeACO1* and *LeACO4* profiles during abscission are similar to their profiles detected during tomato fruit ripening (Van de Poel et al., 2012), but on a much shorter timescale. It is currently not known if every ACO isoform actually synthesises ethylene or if they only have a regulatory role in ethylene synthesis.

Microarray data suggest that during abscission, two of the isoforms, *LeACO4* and *LeACO6*, are down-regulated, while four others, *LeACO1*, *LeACO2*, *LeACO3* and *LeACO5*, are up-regulated (Figure 12). The specific patterns observed might be related to their phylogenetic relationships, as *LeACO4* and *LeACO6* are closely related in tomato (Figure 30). Therefore, they might have specific functions, or better, they might be induced during specific processes in the plant. In contrast, during tomato fruit ripening (Van de Poel et al., 2012), *LeACO2* was shown to be down-regulated, whereas in the present microarray data, *LeACO2* was up-regulated.

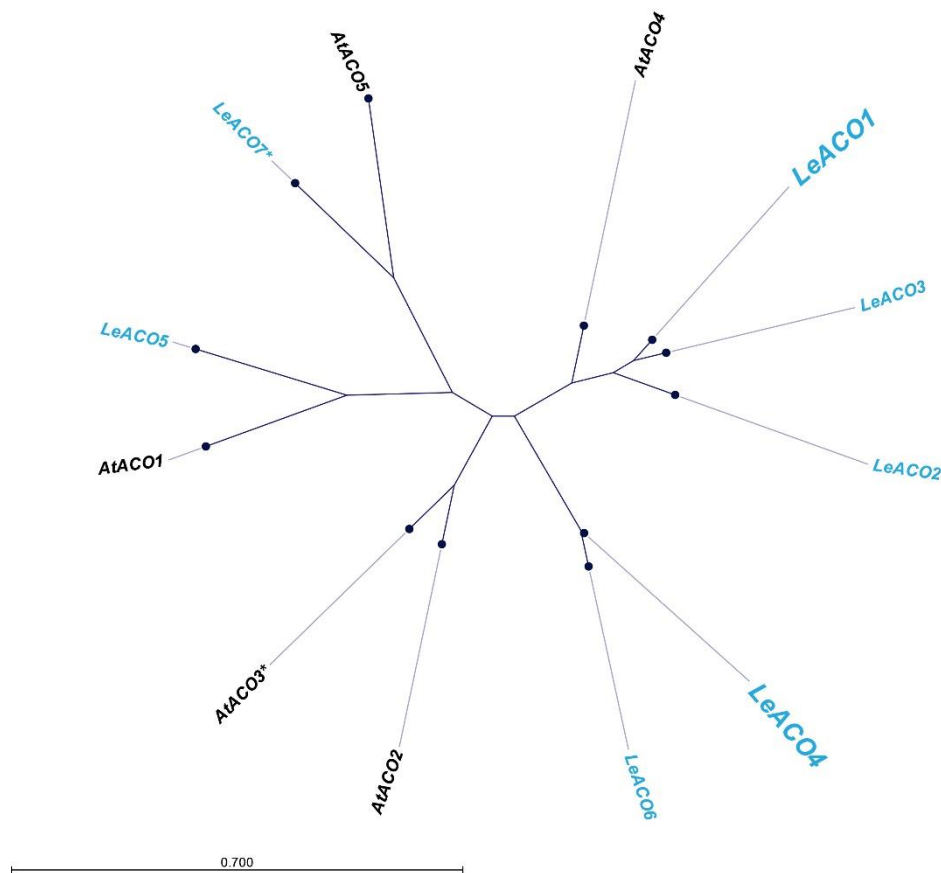


Figure 30: Maximum likelihood phylogenetic tree for *Arabisodopsis* and tomato ACO genes.

A tissue specific expression pattern for different ACO isoforms has also been suggested (Barry et al., 1996), but in the case of abscission, we found the same expression patterns both for the flower and the leaf (Figures 3, 18). The present study shows that *LeACO1* is highly expressed during abscission, and that its protein localises in the phloem companion cells, where ethylene is most likely to be synthesised, and then transported through the phloem to other tissues as a signal for abscission. Tissue-directed expression analysis showed that *LeACO4* also has a preferential localisation in vein tissue of tomato pedicel during abscission, but further work should be carried out at the cellular and protein levels to confirm or reject the concept that the protein is also expressed specifically in phloem companion cells.

While this might appear to contradict the recently published transcriptome data of Kim et al. (2015) that show *LeACO1* expression in the pedicel AZ, Kim et al. (2015) also pointed out that collection of abscission data is not uniform across all studies. Accordingly, after microdissection, *LeACO1* expression was seen outside the AZ specified in the present study, although inside the AZ area defined by Kim et al. (2015). On the other hand, *LeACO4* expression before abscission induction was more synchronised between these two studies, due to abundant *LeACO4* expression inside the AZ (Figure 23).

Of note, after the treatment with the competitive inhibitor of ethylene action, 1-MCP, the decrease in *LeACO4* expression that was seen in the untreated samples 8 h after abscission induction was prevented in the distal AZ, as well as in tissues distal to this (Figure 23). At the same time, the *LeACO1* expression pattern was only partially affected in most of the tissues examined, with slightly induced levels in the distal part of the AZ.

These data complement a previous microarray study (Meir et al., 2010), and suggest that during tomato pedicel abscission, *LeACO1* expression is not completely dependent on ethylene-related signalling.

LeEIL2 is a member of the EIN3-like family of transcription factor and it is one of the two orthologues of the *Arabidopsis* EIN3 gene, which had been shown to have multiple targets in the ethylene signalling pathway (Chang et al., 2013). In the present study, *LeEIL2* did not show any highly significant changes in expression during flower pedicel abscission induction, except for a small reduction in its expression. Its expression also showed only slight up-regulation during 1-MCP treatment of these tissues (Figure 23). The *EIL* genes are supposed to be a key transcription factor in the regulation of the response to ethylene perception, and it has been shown that their abundance is regulated post-transcriptionally, rather than at the gene expression level (Guo and Eckert, 2003; Cho and Yoo, 2014). Although the EIN3 and EIL proteins are part of a relatively large family of transcription factors in plants, it has also been shown that their function is mostly redundant (Tieman et al., 2001). Also, *LeEIL2* was equally distributed throughout the different tissues of the tomato pedicel, although as it is regulated post transcriptionally, further studies need to be performed to determine whether this holds true also at the protein level.

4.2.2 Expression profiles and localisation of ethylene-related genes during tomato leaf abscission

The expression and localisation patterns between the proximal and distal sides of the leaf were analysed here for the AZ, for *LeACO1*, *LeACO4* and *LeEIL2*. The AZs of the first three leaves were sampled, from the bottom of the plant and separately, so that the samples represented a variation in the leaf developmental stages. Older leaves at the bottom of the tomato plant are usually more prone to abscission (Burg, 1968), especially if they already show early signs of senescence.

It has been shown that during abscission of the tomato flower pedicel, *LeACO1* expression increases very rapidly after abscission induction, and the flower drops off soon afterwards (Figure 18; Meir et al. 2010). Would it be reasonable to think, therefore, that the situation is similar for the leaf petioles? Accordingly, *LeACO1* expression might increase gradually from L1 to L3 after abscission induction, and might subsequently decrease in the same manner. Therefore, our data for the L1 leaves at 24 h after abscission induction might reflect the already decreasing phase of the *LeACO1* expression (Figure 3). Additionally, there was a trend to slightly higher *LeACO1* expression for the proximal side of the AZ fracture in the WT and in some transgenic plants (Figures 3, 4, 6, 7), which was seen only for the oldest leaves in the transgenic lines. The preferential proximal localisation was supported also by immunolocalisation analysis of the ACO1 protein. This result is in contrast with the immunolocalisation results for the flower AZ, where the ACO1 protein was localised prevalently to the distal side of the AZ (Figure 24A).

The asymmetric nature of the physiological processes that occur during abscission (Bar-Dror et al., 2010) was again seen with regards to *LeACO4* expression. The transcript abundance was already high in the control samples, and in the older leaves this was distributed preferentially on the distal side of the AZ. Upon induction of abscission, *LeACO4* levels on the distal side dropped, while on the proximal side they remained stable, or even increased (Figure 3). A similar pattern was observed during fruit ripening in tomato, which was the main system in which genes from the *LeACO* family have been studied (Barry et al., 1996; Nakatsuka et al., 1998; Van de Poel et al., 2014), and where the transcript level of *LeACO4* also dropped rapidly at the onset of ripening (Van de Poel

et al., 2012). To some extent, *LeACO4* also appears to be developmentally regulated, as significant differences between the different leaves were observed here. Indeed, younger leaves showed greater abundance of the *LeACO4* transcript on the proximal side of the AZ, and even more so when abscission was induced. Younger leaves do abscise slower *in vitro*, but further studies are needed to determine whether proximal *LeACO4* expression is in any way connected with the rate of abscission. The speed at which an organ abscises was previously linked to a change in the auxin gradient through the AZ tissue, or in some cases to the absolute auxin concentration (Tucker and Kim, 2015; Jin et al., 2015). Also, in tomato, ethylene was shown to alter the expression of auxin-related genes, and since in tomato *LeACO4* expression during abscission follows the same profile as auxin-related genes (Meir et al., 2010), it is possible that as *LeACO4* expression remains high, so does the concentration of auxins, which in turn inhibits abscission.

On the other hand *LeACO1* transcripts accumulate during ripening (Van de Poel et al., 2012), and as we have shown in the present study, also during tomato leaf abscission. But unlike *LeACO4*, there is no significant asymmetry in its localisation (Figure 3). *LeACO1* expression was previously shown to highly correlate with ethylene production (Van de Poel et al., 2012), and in general, expression of *LeACO* genes was shown to be a good indicator of ethylene levels produced by different tissues (Rudus et al., 2012). To date, except for *LeACO1*, where its ability to produce ethylene has been demonstrated *in vivo* (Van de Poel et al., 2014), there has been no comprehensive analysis of the enzymatic activity of the *LeACO* family members. Interestingly, when testing *LeACO1* activity and a possible role for an inhibitor protein E8, Van de Poel et al. (2014) did not realize that the protein named E8 was a member of the *LeACO* family, namely *LeACO3*, which in the activity assay by itself did not produce ethylene (Van de Poel et al., 2014). It is thus possible that not all of the members of the *LeACO* family in tomato have an active role in ethylene production, or that their performances differ. Further analysis of the activities and/or the other possible regulatory roles of members of the *LeACO* gene family is needed to elucidate both their roles and the roles of ethylene in different physiological processes.

During leaf abscission, *LeEIL2* did show a decrease in abundance that might have been related to the post transcriptional regulation of this protein. Indeed, it was shown that signalling is inhibited by protein degradation rather than transcriptionally, which means that transcription does not change a lot during regulation (Guo and Ecker, 2003). In the present case, expression was decreased, and mainly on the distal side of the AZ (Figure 3), the same as for *LeACO4* expression. If similar factors modulate both expression of *LeACO4* and *LeEIL2*, or if *LeEIL2* negatively regulates *LeACO4*, remain to be determined.

4.2.3 The Influence of LeLX silencing on ethylene-related genes during abscission

In the present study, the hypothesis that LX inhibition can alter the expression of ethylene-related genes was also examined. As LX is both induced by ethylene and involved in PCD (Bar-Dror et al., 2010), it might represent a link between ethylene signalling and PCD. Although delay of abscission is caused by LX inhibition (Lers et al., 2006), no particular differences in *LeACO1*, *LeACO4* or *LeEIL2* expression were observed in any of the LX-inhibited transgenic lines, for both flower and leaf AZ (Figures). The general patterns of localisation and degree of expression remained the same. Which means that the delay in abscission seen in the *LeLX*-inhibited mutants was probably not caused by the influence of this protein any of the mentioned proteins or pathways that regulate them. LX is probably placed somewhere downstream in the ethylene regulation pathway, as it is itself induced by ethylene treatment (Bar-Dror et al., 2011).

LX localisation in leaf AZ tissue showed the absence of detection of LX-specific signal in all of the transgenic lines analysed, which as expected confirms the actual silencing of the protein in the mutants. However, fracture formation can still be observed in the transgenic lines, for samples that were collected at the same time post induction (Figure 8). It should be noted that the delay of abscission in the transgenic line tested was difficult to observe in the experiments that were performed, as it generally appeared that a common variability between biological replicates has a bigger impact on the progression of abscission in both flower and leaves. Of note, the mutants used in this study were different from those ones described by Lers et al. (2006), and it is thus difficult to directly compare the results of that study to the present observations.

4.3 Programmed cell death and cell separation in the pedicel AZ are differentially regulated by ethylene.

The two main processes involved in tomato flower pedicel abscission are cell separation and PCD (Bar-Dror et al., 2011). The results of this study show that these processes share a common origin in the vascular tissues inside the AZ. Cell separation in the flower pedicel AZ starts in the phloem companion cells in the vascular tissues that cross the swollen node, which defines the AZ. From there, the cell separation spreads along a very narrow line of cell layers towards both of the edges of the AZ. This cell separation progression follows the temporal expression patterns of the two polygalacturonase genes *LeTAPG1* and *LeTAPG4* (Kalaitzis et al., 1997; Meir et al., 2010; Bar-Dror et al., 2011), which encode the main cell-wall-hydrolysing polygalacturonase enzymes that are responsible for the dissolving of the middle lamellae that keep cells together. Both *LeTAPG1* and *LeTAPG4* expression, and the *LeTAPG4* protein, were limited to the flower pedicel AZ, with their prevailing expression at the proximal side of the AZ (Figure 26).

On the other hand, the morphological hallmarks of PCD are seen to spread from their start in the vascular tissues in the AZ towards the distal side of the flower pedicel. In agreement with this, the prevailing expression of two PCD marker genes, *LeLX* and *LeTBN1* (Olvera-Carrillo et al., 2015), was in the vascular tissues in the distal AZ and distal to it (Figure 23). Moreover, in both processes, the signal for PCD initiation arises from the vascular tissues, as shown by the gene expression and TUNEL analysis (the present study; Bar-Dror et al., 2011). It remains to be determined if and how the distal spread of the TUNEL signal (Figure 27) and the expression of the PCD markers are related to senescence caused by mechanical injury of the tissue during induction.

Analysis of the AZ cell ultrastructure during abscission showed that there was a clear discrepancy between the timing of the cell-wall dissolution and programmed cell death when ethylene signalling is inhibited in plants under 1-MCP treatment. Cell separation processes appear to be affected more by 1-MCP treatment than PCD-related processes (Figures 22, 23). Tissues in which ethylene signalling was inhibited showed a discrepancy between the onset of PCD hallmarks, such as condensed nuclear chromatin and dilation of nuclear membranes and the ER, and the loading of the extracellular spaces and the cell wall degradation (Figure 9).

4.3.1 Cell separation

The *LeTAPG4*, *LeTAPG1*, *LeCel1* and *LeCel5* genes that encode cell-wall hydrolysing enzymes were almost completely inhibited by 1-MCP (Figure 23; Meir et al., 2010). As has also been shown previously, mRNA of the polygalacturonase-encoding gene *LeTAPG4* was detected much earlier than *LeTAPG1* mRNA (Bar-Dror et al., 2011; Kalaitzis et al., 1997;

Meir et al., 2010) and *LeCell* and *LeCel5* expression, which encode cellulases (Meir et al., 2010; Bar-Dror et al., 2011). Additionally, the present study shows expression of the TAPG4 protein in the proximal AZ (26). The ultrastructural images of the flower pedicel AZ at 8 h from abscission induction support these data, as they showed the increased intercellular space filled with deposits. At the same time after abscission induction, formation of the intercellular space was completely prevented in the tissues treated with 1-MCP. Expression of *LeTAPG1* and *LeTAPG4* was also inhibited during 1-MCP treatment, which alone could be the reason for the delay in cell-wall separation and extracellular loading in the treated samples. It thus appears reasonable to assume that formation of the intercellular space at 8 h after abscission induction is a result of polygalacturonase activity, and that other cell-wall hydrolysing enzymes are needed for the completion of abscission.

4.3.2 Programmed cell death

On the other hand, the sensitivity to ethylene of the PCD marker genes *LeLX* and *LeTBN1* (Olvera-Carrillo et al., 2015) and *LeRBOH1* depends on the gene location in the specific cell layer inside the AZ, or in its vicinity (Figure 22, 23). Nevertheless, 1-MCP had stronger effects on PCD-related gene expression in cells in the distal. AZ *LeTBN1* expression was less affected by 1-MCP treatment than any other gene we have analysed in hand-cut tissues. Interestingly if we look at specific tissues, as with tissue-specific gene expression, we can see that *LeTBN1* expression was indeed inhibited, but only in the distal part of the tomato petiole. This result correlates well with the TUNEL assay results, which showed the same situation, as the signal from positive nuclei was much lower on the distal side of 1-MCP treated samples, while in the AZ, the signal was the same as in the non-treated samples (Figure 27). These results might indicate that PCD outside the AZ is actually ethylene regulated, while other signals drive AZ-specific PCD. What those signals might be still remains to be defined, and further research into the involvement and function of PCD during abscission is needed.

An interesting observation at the subcellular level was the formation of the paramural bodies between the plasma membrane and the cell wall, which was not affected by the 1-MCP treatment (Figure 9). Similar structures have been reported for the leaf AZ that were strictly associated with the proximal side of the AZ fracture (Bar-Dror et al., 2011; Dermastia et al., 2012). It has been suggested that these vesicles have a role during the development of a protective layer at the surface of the exposed tissue, following the release of the cells at the site of leaf detachment from the plant body (Bar-Dror et al., 2011; Dermastia et al., 2012). Similar structures have also been observed in *Arabidopsis* mutants, in which the shedding of the floral organ can be blocked by disruption of the function of the ADP-ribosylation factor and GTPase-activating protein NEVERSHED (NEV) (Liljegren et al., 2009). Thus, it has been suggested that NEV activity is required for correct trafficking of cargo molecules during cell separation, and that the formation of paramural bodies is associated with trafficking defects (Liljegren et al., 2009). Although the role of NEV homologs has not been explored in tomato, the studies on tomato flower pedicel and leaf petiole abscission (Bar-Dror et al., 2011) indicate that the formation of paramural bodies is an essential part of organ abscission in this plant. So far there is not a lot of information available regarding the role of PCD and its involvement in cell separation during abscission and it is still not clear whether PCD has an essential role in the overall process, or whether it is due to natural senescence of the organ that is going to be shed from the plant.

In contrast with previous results on leaf abscission in tomato (Bar-Dror, 2011), where PCD was preferentially localised on the distal part of the AZ, in tomato pedicel abscission

we did not observe such a marked asymmetry in PCD localisation between the two sides of the fracture zone. At a cellular level, PCD was observed on both the proximal and distal sides of the AZ fracture, where there was also a strong signal from TUNEL positive nuclei (Figures 27, 29). On the other hand, *LeTBN1* expression was highest on the distal side (Figures 18, 22, 23), which also showed senescence during abscission.

Beside PCD related genes, also genes involved in plant senescence are induced during flower pedicel abscission (Figure 16,17). Indeed, the distal part of the pedicel does senesce after the flower is removed (data not shown), thus it would be reasonable to think that the TUNEL signal observed distally in the flower pedicel is caused by the general degradation of the tissue. Senescence was shown to be delayed in ethylene-deficient tomato (John et al., 1995), and in our experiments 1-MCP treatment inhibited the distally located TUNEL signal (Figure 29). Taken together, our results suggest that the bulk of the DNA degradation signal and PCD on the distal side of the abscission zone are probably caused by the senescence process that occur after the tissue is wounded.

Interestingly, *LeRBOH1* expression was highest for the proximal side in hand-cut tissues (Figure 22). Tissue-directed gene expression later showed that during abscission, the highest expression of *LeRBOH1* was in the AZ tissue, although still, if only the proximal and distal sides are compared, it was again higher on the proximal side (Figure 23). *LeRBOH1* also appeared to be highly affected by 1-MCP treatment, specifically in the AZ tissue, where PCD processes were still active, even after 1-MCP treatment. The temporal profile of *LeRBOH1* expression and the vascular tissue localisation of this concurred with that of *LeLX* expression, and also roughly for *LeTBN1* expression. It remains under debate whether the NADPH-oxidase encoded by *LeRBOH1* and the generation of superoxide on the apoplastic side are crucial to the triggering of developmental PCD (De Pinto et al., 2012). However, the spatio-temporal pattern of *LeRBOH1* expression during flower abscission, and the similar time for induction of *LeTBN1* and *LeLX* expression appear to support the suggestion of its involvement in PCD during abscission. In transgenic lines with inhibited LX, a general reduction in *LeRBOH1* expression was also noted (Figures 19, 20, 22), which might be linked to the inhibition of abscission reported for these transgenic lines (personal communication).

It has been shown that, NADPH oxidases in plants are involved in the processes of stomatal regulation and cell elongation through the activation of Ca^{2+} channels (Foreman et al., 2003). As several studies have connected calcium concentrations and signalling to the process of abscission (Poovaiah et al., 1972, 1973; Trewavas et al., 1984), the specific *LeRBOH1* expression that is observed inside the AZ tissue (Figure 23) might also be related to the regulation of Ca^{2+} channels during abscission.

Conclusions

The outcomes of the present study show that the production of ethylene, cell separation, and the onset of PCD originate from the same tissue during tomato pedicel abscission, although both cell separation and PCD are differentially affected by a lack of ethylene perception. PCD localisation in the tomato pedicel during abscission is not as asymmetric as it is in the leaf AZ, at least in the AZ-specific tissue. The amount of observed PCD signal on the distal side, away from the AZ, was probably due to normal tissue senescence and was regulated by ethylene. At the cellular and molecular levels, cell separation appears to be more influenced by the disruption of the ethylene signalling pathway than the PCD related process, but this is only true specifically in the AZ tissue.

Production of endogenous ethylene might be a candidate for a diffusible signal that can regulate the cell separation processes that occur in the tomato pedicel AZ tissue, by potentially regulating the exocytosis of enzymes and secondary metabolites. The temporal expression pattern of *LeACO1* and *LeACO4*, which encode the last enzyme in the ethylene biosynthetic pathway, follows the concept of two systems of ethylene biosynthesis that have been described during climacteric fruit development and ripening. In agreement with the site of *LeACO1* gene expression, the encoded protein was localised mainly to the cytoplasm of the phloem companion cells just outside the AZ. These results suggest that the abscission-induced ethylene synthesis also occurs in vascular tissues out of the AZ. The inhibition of ethylene action with 1-MCP prevented the separation of cells in the AZ, apparently by inhibition of cell-wall hydrolysing enzymes. On the other hand, 1-MCP only had moderate effects on the expression of the two marker genes of PCD, *LeLX* and *LeTBN1*, and the appearance of the hallmarks of PCD. In addition, the formation of paramural bodies appears to be an important part of abscission in tomato. It appears unlikely, however, that ethylene also regulates the onset of and progression of PCD, or at least that present specifically in the AZ cells, as PCD in these cells remains unaffected by 1-MCP treatment. Further analyses of the signals regulating PCD during abscission are needed to define the role that this cellular process has during abscission.

Tissue-directed analysis, such as immunolocalisation and laser microdissection, might prove to be extremely useful in such studies, as the different tissues included in the AZ might have drastically different physiological profiles and roles during abscission. In some cases, resolution is needed even down to the cellular level, as in the present case with *LeACO1* immunolocalisation, which was shown to be specific to phloem companion cells. This result indicated that in the study of abscission, it would not be a bad idea to performed gene expression analysis and other types of analyses at the cellular level, as the averaging of the signals due to tissue pooling might hide important aspects in this process.

To date, the role that LX has on progression of abscission remains elusive. No significant changes were observed in LX-inhibited mutant plants regarding the expression of the genes analysed in this study. Further studies will be needed to determine its role in the abscission process, and why its inhibition leads to delay of abscission.

Taken together, the cell-layer-specific spatiotemporal patterns of abscission events and their responsiveness to ethylene might have applicable value for the fine-tuning of the timing of abscission. This might represent an alternative strategy for the prevention of preharvest fruit drop and for extending the lifetime of cut flowers, and also for promoting early abscission of buds and fruit to stimulate more vigorous plant growth and higher quality fruit and flowers.

Appendix A

RNAi construct

A.1 RNAi construct present in the transgenic tomato lines

The nucleotide sequence for the RNAi construct with which the lines 1-7, 7-5 and 20-2 were transformed is given below.

>LeLX_RNAi_construct

```
GCCAAACTACAAAGATGGTAAATGGCCACAAAATTGTGATAGGGAAAGCTCT
TTGGATGAATCTGAGTTCTCAGATCTTATAAGCACAATGGAAAAGAATTGGC
CATCACTTGCTTGCCCTAGTAGTGATGGTTTAAAATTTTGGAGTCATGAATGG
CTAAACATGGCACTTGTTTCAGCTCTTAACCAACATGCCTATTTCCAAACTGC
ACTTGACTTCAAGACCAAATCTAACCTTCTTCAAAATCTTAACAATGCAGGGA
TTAAACCAAGGAATGGAGATTATTATGGTGTAGAAAGCATTAAAAAGGCAAT
TGAAAAAGGAGTTGGACACACACCATTTATAGAGTGCAATGTTGATTCACAA
GGGAACCATCAATTATACCAAGTTTACCTATGTGTGGATTCTTCTGCAT
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Bibliography

Publications Related to the Thesis

Journal Articles

Chersicola, M., Kladnik, A., Tušek Žnidarič, M., Mrak, T., Gruden, K., & Dermastia, M. (2017). 1-Aminocyclopropane-1-Carboxylate Oxidase Induction in Tomato Flower Pedicel Phloem and Abscission Related Processes Are Differentially Sensitive to Ethylene. *Frontiers in Plant Science*, 8, 464

Biography

Marko Chersicola was born the 6th June of 1986 in Koper, Slovenija. He started his undergraduate studies at the Biotechnical Faculty of University of Ljubljana in 2006. His interest in plant physiology led him to the collaboration with the Department of Plant Physiology, where he worked as a volunteer on the effects of nanoparticles on plant growth and development. He conducted the experimental work for his bachelor thesis at the National Institute of Biology (NIB), where he was involved in studying the mechanisms of plant gene silencing, using both bioinformatics and functional genomics approaches. He successfully defended his thesis in 2012, and received the Bachelor Degree in Biology. After that he worked as a professional associate at the NIB, where he was involved in analyzing the complex interactions of defense signaling pathways in potato-PVY interactions. The same year he enrolled in the postgraduate programme “Information and communication technologies” at the International Postgraduate School of Jožef Štefan” and started to work as a young researcher at NIB. For several years, he was involved in a research project on tomato abscission, with the aim of understanding the complicate and intricate interactions between ethylene, programmed cell death and cellular processes that occur during abscission. His work was focused on the analysis of specific genes and their respective localization and expression patterns during abscission. Using microscopy, micro-dissection, immuno-localization and real-time PCR, he and his colleagues were able to demonstrate, for the first time, the specific location of synthesis of a key enzyme involved in ethylene evolution during abscission. During the years, the results his research were showcased both on national and international conferences and finally published as a research article in 2017.

