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suicidal tomato cells:

programmed cell death in suspension-cultured tomato cells and ripening fruit

frank a. hoeberichts

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general introduction

Since both fresh and processed fruit form an important part of our diet, there is an ever-increasing demand, at least in western society, for improved quality of the fruit available. Commercial trade is dominated by a relatively small number of fruit, most importantly grape, banana, citrus, apple, and tomato (Table 1.1).

The botanical definition of a fruit is "a seed receptacle developed from an ovary". During fruit development (or maturation), seeds mature and the fruit reaches its final size. Subsequent fruit ripening often involves drastic changes in colour, flavour, and texture, aimed at making the mature fruit more attractive to consumers and thereby increasing chances for seed dispersal.

Climacteric and non-climacteric fruit ripening

The multiple changes that occur during fruit ripening require the synthesis of novel mRNAs and proteins as well as new pigments and flavour compounds. These processes require both energy and carbon skeleton building blocks that are supplied, just as in any other non-photosynthetic tissue, by respiration. Based on their respiration pattern during ripening, fruit can be classified as either climacteric or non-climacteric (Biale 1964). Climacteric fruit (such as avocado, pear, apple, and tomato) display a characteristic peak of respiratory activity during ripening, whereas non-climacteric fruit (such as orange, lemon, and strawberry) do not.

In climacteric fruit, a sharp increase in production of the gaseous hydrocarbon ethylene (C_2H_4), well known as a plant hormone (Bleecker and Kende 2000), is observed concomitantly with the respiratory upsurge. These fruit can also be induced to ripen by treatment with exogenous ethylene, whereas inhibition of

ethylene synthesis or perception delays the onset of ripening. Over the last few decades, it has become apparent that ripening, like any other plant developmental process, is under strict genetic control. Ethylene is believed to regulate fruit ripening by initiating and co-ordinating the expression of genes responsible for various aspects of the ripening process, such as the mentioned respiratory rise, chlorophyll degradation, carotenoid synthesis, conversion of starch to sugars, and cell wall degradation (Lelièvre et al. 1997; Giovannoni 2001).

	1998	1999	2000	2001
apples	56,893,409	58,173,751	58,960,716	60,237,466
avocado	2,267,634	2,352,951	2,485,135	2,552,556
banana	58,211,172	64,421,664	67,545,390	68,651,267
citrus	99,641,196	102,975,608	100,252,024	98,731,751
grape	57,010,146	60,890,266	64,029,460	61,949,961
kiwi	876,084	893,761	978,948	1,004,537
pear	15,279,336	15,751,121	16,960,660	17,321,837
tomato	94,338,747	104,366,671	101,975,637	100,259,346
total fruit	433,764,157	457,797,303	466,414,227	466,340,271

TABLE 1.1. *Estimated world-wide production of several kinds of fruit in (metric) tonnes (www.foa.org; June 2002).*

Ethylene biosynthesis and perception

It is believed that ethylene production in all plant tissues proceeds via a common biosynthetic pathway from methionine, via S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene (Figure 1.1). The two key enzymes for the biosynthesis of ethylene are ACC synthase (ACS) and ACC oxidase (ACO). Several members of their respective gene families have been cloned (reviewed in Fluhr and Mattoo 1996). Ethylene is perceived by a family of two-component histidine kinase-like receptors that negatively regulate ethylene responses (Figure 1.2). Various other proteins function downstream of these re-

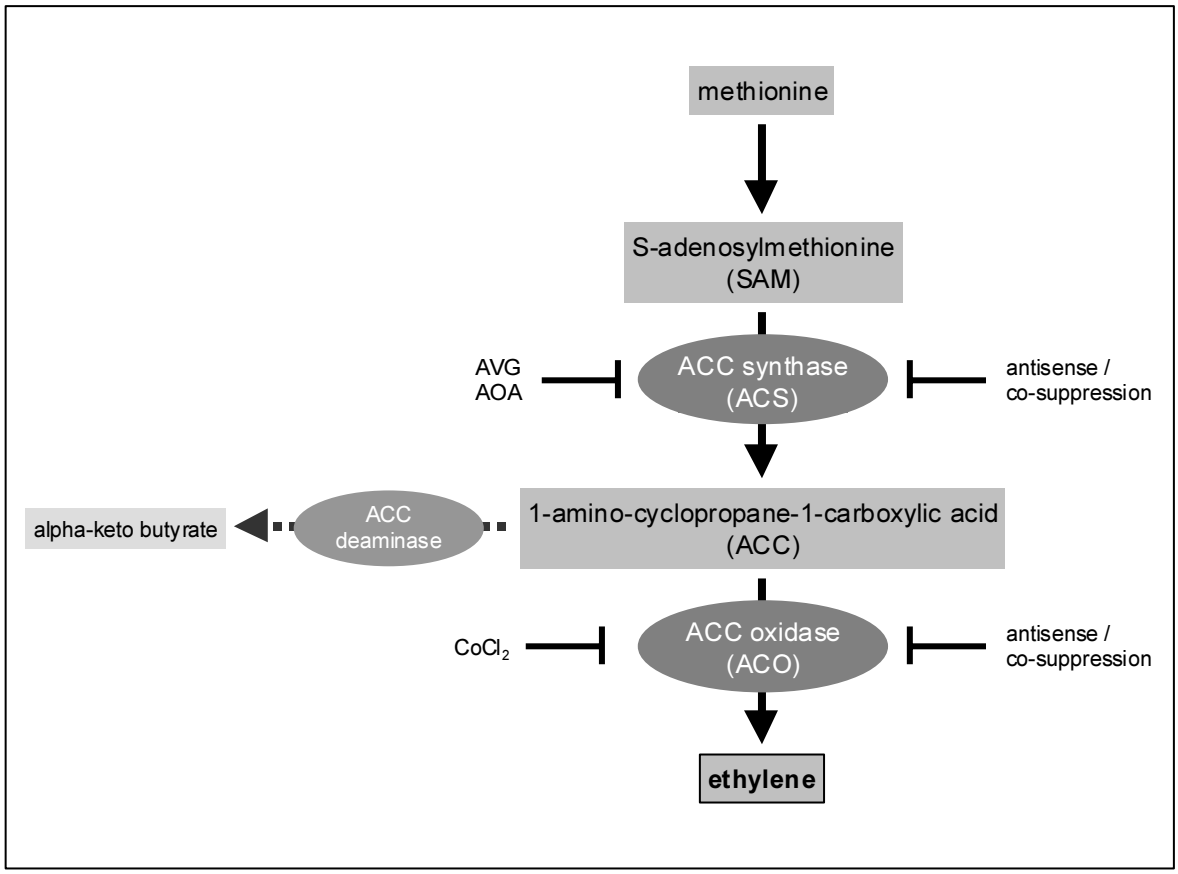


FIGURE 1.1. *Ethylene biosynthetic pathway in higher plants. Reacting compounds are depicted in square shapes, catalytic enzymes in rounded shapes. Chemical inhibitors of ethylene bio-synthetic enzymes are indicated on the left, genetic approaches to inhibit ethylene biosynthesis are depicted on the right. ACC deaminase is a bacterial enzyme that, when introduced into plant cells, is capable of metabolising ACC.*

ceptors, ultimately leading to regulation of gene transcription (Chang and Shockey 1999; Stepanova and Ecker 2000).

Molecular mechanisms of tomato fruit ripening

Of all climacteric fruits, arguably most is known about the molecular mechanisms controlling the ripening of the tomato. The relatively small genome (≈9.5 x 10^8 bp), the many single-gene mutants that are available (Grierson et al. 1987),

the fact that tomato plants can be transformed genetically and regenerated from tissue culture, combined with the commercial importance of the crop have facilitated research into the control mechanisms (Giovannoni 2001).

Major changes in the physiology and biochemistry of a mature green tomato start occurring at the onset of ripening. As stated above, ethylene plays an essential role in the initiation of ripening in all climacteric fruit, including tomato. In fact, the first detectable sign of ripening is an increase in the production of ethylene. Ripening-related increases in the levels of many mRNAs have been shown to be ethylene-dependent, emphasising that ethylene-dependent signal transduction is involved in the regulation of ripening (Lincoln et al. 1987; Maunders et al. 1987). Transgenic (antisense) tomato fruits revealed that besides this ethylene-dependent pathway, an ethylene-independent pathway is operational during tomato fruit ripening (Theologis 1992). Recently, a MADS box gene, *LeMADS-RIN*, necessary for fruit ripening was cloned. *LeMADS-RIN* antisense plants develop fruits that do not ripen, although plants exhibit normal ethylene sensitivity. This gene clearly demonstrates the importance of ethylene independent (developmental) regulation of tomato fruit ripening (Vrebalov et al. 2002).

Control of tomato fruit ripening

Because of the effects of ethylene on plant senescence in general, and fruit ripening in particular, large losses of fruit and vegetables occur annually worldwide. Consequently, it has always been a goal in commercial horticulture to prevent or delay fruit ripening in a reversible manner. Throughout the years, various methods for prolonging fruit shelf life have been investigated and employed, such as controlled atmosphere or the use of inhibitors of ethylene action (Beyer 1976; Sisler and Yang 1984; Sisler and Serek 1997). The cloning of genes induced during fruit ripening and of genes involved in ethylene biosynthesis and perception made it possible to control ripening in tomato using reverse genetics. Transgenic plants with altered ethylene production levels have been used to grow tomatoes with drastically changed ripening phenotypes (Hamilton et al. 1990; Klee et al. 1991; Oeller et al. 1991; Gray et al. 1992).

In some cases, however, these phenotypes suffer from side effects or too strong effects due to greatly reduced ethylene biosynthesis or perception. For

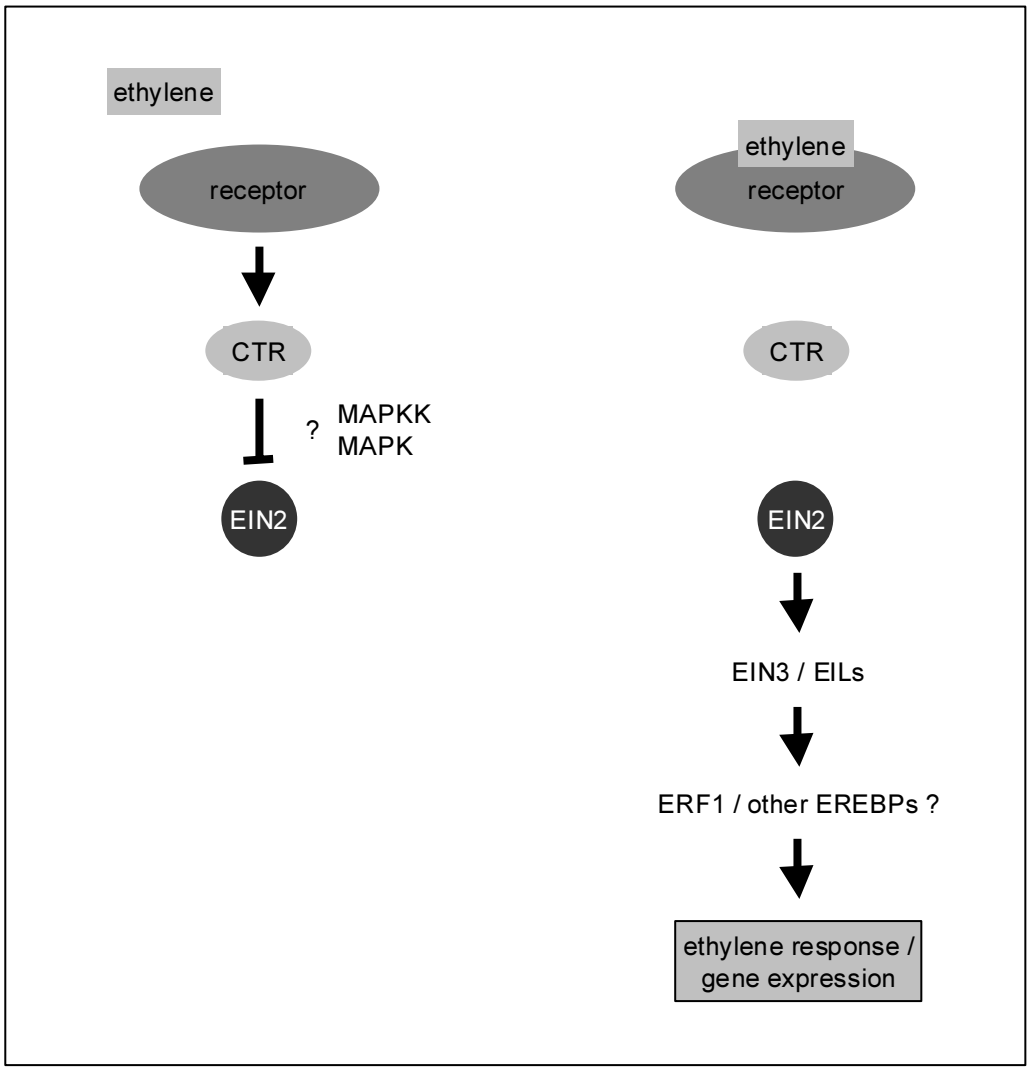


FIGURE 1.2. *Current view of the ethylene signal transduction pathway formulated on the basis of cloned A. thaliana genes. Left: in the absence of ethylene, receptors repress responses through activation of the down-stream negative regulator CTR, which, in turn, represses EIN2 through a MAPK cascade. Right: ethylene binding switches off receptor activation of CTR, resulting in derepression of the ensuing response pathway.*

example, introduction of a dominant mutant allele of the *Arabidopsis thaliana ETR1* gene encoding an ethylene receptor results in tomato plants that are inhibited not only in fruit ripening, but in virtually every ethylene response measurable, leading to a severely altered plant phenotype (Wilkinson et al. 1997). Expression the same gene construct in petunia greatly reduces adventitious root formation, limiting horticultural performance (Clark et al. 1999; Gubrium et al. 2000).

Improving the control of tomato fruit ripening by using new promoters

In general, the cauliflower mosaic virus (CaMV) 35S promoter is used to direct expression of the transgene in transgenic plants. This promoter is believed to express the corresponding transgene constitutively and at high levels in all plant cells (Odell et al. 1985). However, non-specific and constitutively high expression of (anti-sense) genes may be the cause of the too strong (side) effects mentioned above. Therefore, a transgenic approach to control tomato fruit ripening should better involve fruit-specific gene promoters. The presence of fruit- and ripening-specific gene promoters in the tomato genome has opened up the possibility for interfering with tomato fruit ripening in a way that is confined in both space and time using endogenous promoters. Several fruit-specific promoters have already been isolated from tomato, such as the *2A11* (Van Haaren and Houck 1991), *ACO1* (Blume and Grierson 1997), *E8* (Deikman et al. 1992), *E4* (Montgomery et al. 1993), and polygalacturonase *(PG)* promoter (Nicholass et al. 1995). Various studies illustrate the possibilities of using these fruit-specific promoters to obtain deviant tomato fruit (Good et al. 1994; Speirs et al. 1998; Chengappa et al. 1999; Griffiths et al. 1999; Sandhu et al. 2000; Lewinsohn et al. 2001).

The use of the appropriate fruit- and ripening-specific promoters could make it possible to affect fruit ripening specifically and only at the advanced stages. This strategy may have several advantages over the phenotypes that have been obtained so far. Fruit would first reach a certain degree of maturity, including the associated stages of coloration and taste development. Inhibition of further ripening or over-ripening at this point could result in tomatoes with both increased taste and increased shelf life. However, no promoters of genes expressed ex-

clusively during the advanced stages of tomato fruit ripening have been isolated yet. In fact, only a limited number of genes with the desired expression patterns have been described. Therefore, in order to obtain a suitable promoter, the isolation and characterisation of one or more tomato promoter regions is necessary.

Fruit specific gene expression of tomato CEL2 and EXP1

Tomato *CEL2* is a gene encoding an endo-ß-1,4-glucanase. *CEL2* is mainly expressed during the late stages of tomato fruit ripening, with transcript levels that become first detectable at the breaker stage and increase throughout further ripening. In experiments with 2,5-norbornadiene (NBD) it was shown that mRNA accumulation of *CEL2* in fruit is ethylene-dependent (Lashbrook et al. 1994; Gonzalez-Bosch et al. 1996). In *rin* and *Nr* mutant fruit, *CEL2* mRNA is absent or reduced.

During tomato fruit ripening both mRNA and protein levels of expansin 1 (EXP1) are strongly increased (Rose et al. 1997b; Brummell et al. 1999b; Rose et al. 2000). Expansins are extracellular proteins thought to function in cell wall loosening during growth or remodelling (Cosgrove 2000). *EXP1* mRNA can first be detected at the breaker stage of fruit ripening and its abundance increases dramatically at the turning stage, remaining high throughout ripening. *EXP1* is not expressed in roots, hypocotyls, stems and young leaves, suggesting its expression is fruit-specific (Rose et al. 1997b; Brummell et al. 1999b). Expression of *EXP1* is regulated by ethylene, and the fruit-ripening related increase in mRNA abundance is absent in *rin* and *nor* mutants (Rose et al. 1997b).

In conclusion, these two genes appear to have expression profiles that meet the described requirements and their promoters might be suitable for specific and timely expression of transgenes.

Improving the control of tomato fruit ripening: new target genes?

In addition to applying novel promoters, it may also be interesting to (re)consider the target genes used in strategies aimed at improving tomato fruit quality. So

far, the prolongation of shelf life has been accomplished by interference with ethylene signal transduction (Picton et al. 1995) or cell wall metabolism (Brummell et al. 2002).

In the approach proposed in the previous sections, it also seems obvious to use constructs that inhibit ethylene biosynthesis or perception, as they have previously turned out to be very effective. However, there is little information about the processes that play a role during late ripening and over-ripening, and about a possible role for ethylene in these processes. There are indications that the ripening process can be affected even by blocking ethylene perception at very late stages of ripening (Tucker and Brady 1987; Sisler and Lallu 1994). If so, limiting the expression of (anti-sense) genes that block ethylene biosynthesis or perception to the later stages of ripening (using the above described gene promoters) may indeed have the desired effects.

Besides using the suppression of ethylene genes to improve fruit quality, other genes might be suppressed (or over-expressed). As stated before, knowledge on the processes that play a role during late ripening and over-ripening is scarce. The involvement of additional processes and factors, other than ethylene, in the regulation of the later stages of ripening cannot be excluded. Here, we wish to examine if a process designated programmed cell death (PCD) might play a role in tomato fruit ripening. If so, identification of the mechanisms and regulating factors that underlie this process of cellular suicide may be a first step towards improving tomato fruit quality by targeting PCD.

PCD and fruit ripening

PCD is a process aimed at eliminating unnecessary or harmful cells during growth and development of multicellular organisms. It is indispensable for normal development and survival of plants, and plays an essential role in, for example, xylogenesis, the hypersensitive response (HR), aerenchyma formation, senescence, and embryogenesis (Greenberg 1996; Pennel and Lamb 1997; Richberg et al. 1998; Lam et al. 1999b). In animal cells, PCD is often associated with the occurrence of specific biochemical and morphological features such as condensation of the nucleus and cytoplasm, fragmentation of genomic DNA into large (50 to 300 kb) and subsequently small (200 bp) nucleosomal fragments (DNA

laddering), and fragmentation of the cell into membrane-confined vesicles (apoptotic bodies). The cell death process displaying such features is referred to as apoptosis (Steller 1995). Chapter 2 of this thesis contains a more elaborate introduction on animal apoptosis and plant PCD.

Currently, there are no experimental data supporting a role for PCD in (tomato) fruit ripening or during post-harvest senescence, although there are several examples of speculation on this subject (Hadfield and Bennett 1997; Dong et al. 1998; Xu and Chye 1999; Moriguchi et al. 2000). Molecular and biochemical similarities between developmental leaf senescence and postharvest changes of broccoli florets have been observed (Page et al. 2001). Both carpel and leaf (developmental) senescence is generally considered to involve PCD, a concept that is supported by the occurrence of genomic DNA fragmentation, a generally accepted marker of PCD (Orzáez and Granell 1997; Yen and Yang 1998). Moreover, DNA laddering has been demonstrated in harvested asparagus spears (Eason et al. 2002) and wilting petals of cut Freesia flowers (Yamada et al. 2001). PCD inevitably must be under strict genetic control, and if postharvest senescence of tomato fruit indeed involves PCD, the identification of PCD genes may allow future manipulation of postharvest quality attributes.

Considering the rather limited knowledge on plant PCD when the work on this thesis was initiated, it would have been a difficult task to verify if PCD occurs during the ripening process at all. Therefore, it was decided that PCD would first be studied in a model system of suspension-cultured tomato cells. The aim was to increase the general understanding of plant PCD and its regulatory mechanisms. The identification of PCD marker genes would not only be an initial step towards identifying components of the PCD machinery in plants, but also allow testing if PCD occurs during tomato fruit ripening.

Outline of the thesis

This thesis deals with PCD in a model system of suspension-cultured tomato cells, plant PCD in a more general perspective, and, to a lesser extent, with PCD during ripening and postharvest senescence of tomato fruit. Following **chapter 1** (general introduction), **chapter 2** gives a state-of-the-art overview of PCD in plants. Based on data available in the literature and data presented in this thesis,

it is argued that many cases of plant PCD proceed through cell death mechanisms that are functionally conserved between animals and plants. These mechanisms are, together with possible plant-specific PCD regulators, integrated in a model that describes plant PCD. In **chapter 3**, the establishment of a model system to study plant PCD is reported. This model system, that was used during much of the research carried out, consists of suspension-cultured tomato cells that can be induced to undergo apoptosis-like PCD by adding various chemicals. **Chapter 4** refers to the molecular cloning and characterisation of the tomato *DAD1* gene. It has been postulated that the DAD1 protein is an inhibitor of apoptosis in animals, and its sequence is conserved throughout the animal and plant kingdoms. Gene expression patterns of tomato *DAD1* during fruit ripening are presented. **Chapter 5** describes the isolation of genes that are differentially expressed during PCD in the tomato cell suspension model system. This work represents an initial step towards the identification of novel regulatory components of the PCD machinery in plants. **Chapter 6** focuses on the molecular cloning of the first metacaspase gene from tomato. Gene expression patterns of this metacaspase during PCD in the cell suspension model system and during infection of tomato leaves with the necrotrophic fungal pathogen *Botrytis cinerea* are shown.

Chapter 7 includes results from experiments to determine the role of ethylene during the advanced stages of tomato fruit ripening. It is shown what effects the application of an ethylene perception inhibitor has on physiological and molecular aspects of fruit ripening. In **chapter 8**, the isolation of the promoter regions of two fruit-specific genes from tomato is described. In addition, an attempt was made to analyse both sequences using particle bombardment.

Finally, **chapter 9** summarises the conclusions from all previous chapters. These conclusions are supplemented with some additional experimental data and subsequently used in a discussion that firstly deals with the regulatory mechanisms of PCD in the tomato cell suspension model system and of plant PCD in general. Secondly, it addresses the question whether PCD occurs during postharvest senescence of tomato fruit and discusses the implications with regard to the improvement of postharvest quality traits in tomato fruit.

2

multiple mediators of plant programmed cell death: interplay of conserved cell death mechanisms and plant-specific regulators

Frank A. Hoeberichts and Ernst J. Woltering. BioEssays: in press

Summary

Programmed cell death (PCD) or the ability to induce autodestruction of redundant, misplaced or damaged cells is essential to the construction, maintenance and repair of multicellular organisms. In contrast to the relatively well-described cell death pathways in animals, the mechanisms and regulation of plant PCD are still ill-defined. Several morphological and biochemical similarities between apoptosis and plant PCD have been described, including the degradation of genomic DNA, caspase-like proteolytic activity and cytochrome c release from mitochondria. Reactive oxygen species (ROS) have emerged as an important signal in plant PCD. ROS can activate plant PCD and the interaction between ROS accumulation and mitochondrial permeability provides a powerful feedback amplification loop. In addition, many plant hormones seem to have their respective effects on plant PCD through the regulation of ROS accumulation. The possible plant PCD regulators discussed in this review are integrated into a speculative model that combines mechanisms functionally conserved between animals and plants and plant-specific regulators.

2.1. Introduction

Programmed cell death (PCD) is a process aimed at eliminating redundant or harmful cells during the life cycle of multicellular organisms. For example, PCD is responsible for the removal of excess cells in the developing nervous system, or is activated in defence against infected or mutated cells, preventing further proliferation of a pathogen or disease. Deregulation of PCD is implicated in various human disorders ranging from cancer and autoimmune disorders to neurodegenerative diseases and AIDS (Thompson 1995).

In animal cells, PCD is often associated with the occurrence of specific biochemical and morphological features (Steller 1995) such as condensation of the nucleus and the cytoplasm, fragmentation of genomic DNA into large (50 to 300 kb) and subsequently small (200 bp) nucleosomal fragments (DNA laddering), and fragmentation of the cell into membrane-confined vesicles (apoptotic bodies). The cell death process displaying such features is called apoptosis. The core component of the apoptotic machinery is a proteolytic cascade involving a family of cysteine proteases named caspases. A schematic overview of the apoptotic process in animals is depicted in Figure 2.1. Specific "death receptors" can, upon activation, directly recruit caspase-activating multimeric protein complexes through what is called the extrinsic pathway. A diverse range of cellular stresses, including cytotoxic drugs and DNA damage, can trigger caspase activation via the intrinsic pathway, mediated by cytochrome c release from the mitochondria. Once activated, caspases may process and activate downstream caspases that cleave numerous cellular proteins, eventually leading to dismantling of the cell and the apoptotic phenotype (Wolf and Green 1999; Chang and Yang 2000; Grütter 2000; Hengartner 2000; Kaufmann and Hengartner 2001).

An important regulatory role in caspase activation is played by the BCL2-like protein (BLP) family of cytoplasmic proteins. Its members can either trigger or suppress PCD, and act via interference with caspase activation or via association with other BLPs. BLPs are also believed to govern caspase activation through effects on mitochondrial permeability (Adams and Cory 2001). Mitochondrial permeability is stimulated by various other signals, such as (stress-induced) calcium fluxes and increasing levels of reactive oxygen species (ROS). Reversely, loss of the mitochondrial transmembrane potential leads to excessive generation of ROS, thus providing a feedback amplification loop (Jabs 1999); not

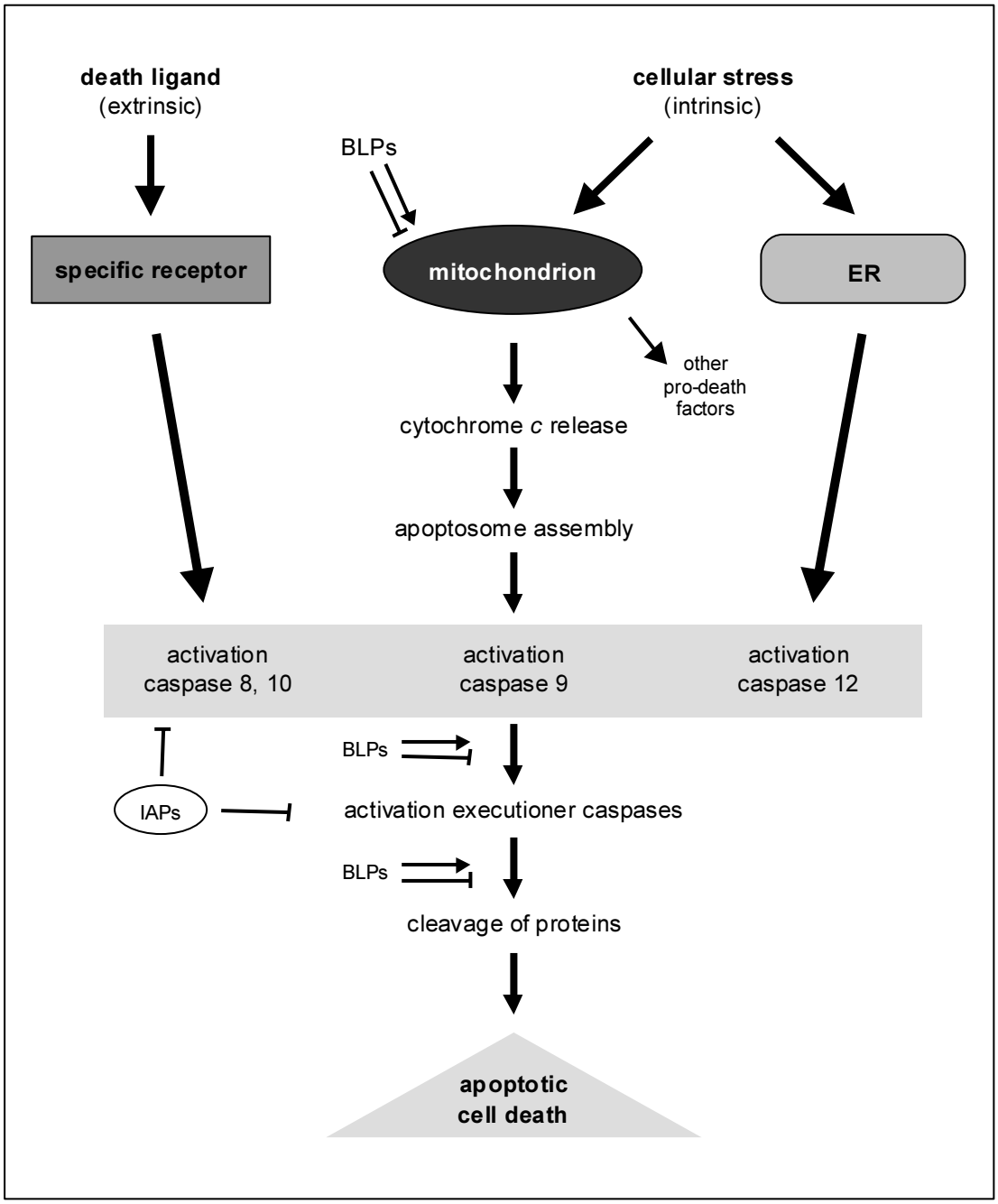


FIGURE 2.1. *Schematic overview of apoptosis in animals. For explanation, see text.*

indicated in Figure 2.1). A second family of apoptotic regulator are the inhibitor of apoptosis (IAP) proteins, believed to suppress apoptosis by deactivating caspases (Deveraux and Reed 1999).

In plants, as in animals, PCD is an essential process during growth and development (Lam et al. 1999b; Danon et al. 2000; Jones 2001). It is involved in for example xylogenesis (Fukuda 2000), aerenchyma formation (Drew et al. 2000b), petal senescence (Rubinstein 2000), and endosperm development (Fath et al. 2000; Young and Gallie 2000a). Furthermore, PCD is responsible for cell death in response to pathogens (Heath 2000; Shirasu and Schulze-Lefert 2000; Lam et al. 2001) and various abiotic stresses. In this review we will discuss the morphological, biochemical and molecular features of dying plant cells and we will highlight the putative functional conservation between elements of animal apoptosis and plant cell death. In addition, putative plant-specific modulators of cell death that have emerged so far (such as various plant hormones) are discussed. This information is then integrated in a speculative model describing the regulation of plant PCD.

2.2. Plant programmed cell death: functional conservation?

Apoptotic-like morphology and DNA laddering in dying plant cells

PCD research in plants was initially focused on identifying similarities with animal apoptosis. Indeed, a number of similarities was found between animal cells undergoing apoptosis and dying plant cells, such as condensation and shrinkage of the cytoplasm and nucleus (McCabe et al. 1997; De Jong et al. 2000), the formation of DNA-containing (apoptotic-like) bodies (Wang et al. 1996b; Danon and Gallois 1998; De Jong et al. 2000) and genomic DNA degradation. This process can be detected either by in situ labelling of 3'-hydroxyl termini (TUNEL) or by visualising nucleosomal fragments on an agarose gel (DNA laddering), and has been observed in plants during petal senescence (Orzáez and Granell 1997; Xu and Hanson 2000), leaf senescence (Yen and Yang 1998), endosperm development (Young and Gallie 2000b), HR (Mittler and Lam 1995; Levine et al. 1996;

Ryerson and Heath 1996), and various forms of abiotic stress (Ryerson and Heath 1996; Wang et al. 1996a; Katsuhara 1997; Danon and Gallois 1998; Gao and Showalter 1999; Stein and Hansen 1999; De Jong et al. 2000; LoSchiavo et al. 2000).

Degradation of nuclear DNA (nDNA) is catalysed by endonucleases, and indeed a number of nucleases have been implicated in plant PCD (reviewed in Sugiyama et al. 2000). No obvious similarities can be seen between the endonucleases involved in mammalian apoptosis and the ones nominated for plant PCD. However, both mammalian apoptosis and plant PCD can be regarded to employ a similar degradation procedure consisting of two distinct phases. In the first, autonomously proceeding phase, nDNA of dying cells suffers relatively limited fragmentation. In the second phase of apoptotic DNA degradation, dead cells are engulfed into macrophages and nDNA is hydrolysed by lysosomal activity which may correspond to the hydrolysis of nDNA by apoplastic or vacuolar endonucleases in plant PCD (Sugiyama et al. 2000).

Caspase-like proteolytic activity regulates plant cell death

In plants, proteolytic enzymes are known to be associated with both developmental PCD and pathogen- and stress-induced PCD (Beers et al. 2000). They are generally assumed to function in the autolysis of intracellular proteins, rather than as regulators. However, the evident participation of proteases, specifically caspases, in the regulation of animal PCD implies that plant proteases could be involved in the regulation of PCD likewise.

Indeed, there are several reports that link protease activity to the regulation of plant PCD. Proteasome inhibitors can prevent tracheary element (TE) differentiation in zinnia cell cultures when added at the time of culture initiation, whereas proteasome inhibition following commitment to differentiation only results in a delay. This suggests that proteasome function is required for induction of TE differentiation, but not for bulk autolysis during the final phases of TE differentiation (Woffenden et al. 1998). Furthermore, the appearance of a secreted protease is co-ordinated with secondary cell wall synthesis and cell death during TE differentiation. The protease and cell death both are inhibited by soybean trypsin inhibitor, whereas exogenous application of another serine protease prematurely trig-

gers cell death. These observations lead to the hypothesis that extracellular proteolysis triggers cell death (Groover and Jones 1999). Inhibitor studies also implicate serine proteases in signal transduction during elicitin-induced HR cell death (Yano et al. 1999; Sasabe et al. 2000). In soybean cells, PCD-activating oxidative stress induces a set of cysteine proteases. Inhibition of the induced cysteine protease activity by ectopic expression of cystatin, a cysteine protease inhibitor gene, can block PCD triggered either by an avirulent pathogen or by ROS (Solomon et al. 1999). These data suggest that the interplay between proteases and endogenous protease inhibitors is a way for plants to regulate cell death. It remains to be seen if this can be compared to the pivotal role that caspases and IAP proteins play in animal PCD.

To date, evidence for the existence of caspase-like proteins (CLPs) in plants is still indirect and mainly based on the inhibitory effects of caspase-specific inhibitors in plant cells. Such caspase-specific inhibitors can abolish bacteria-induced PCD in tobacco (Del Pozo and Lam 1998). In addition, chemical-induced PCD in tomato suspension cells can be inhibited by caspase-specific inhibitors (De Jong et al. 2000). Caspase-like activity has also been demonstrated in barley cell extracts and could only be inhibited by a specific caspase 3 inhibitor, not by cysteine protease inhibitors (Korthout et al. 2000). Micro-injection of caspase 3 substrate into living plant cells revealed that caspase-like activity is mainly present in the cytosol rather than in the vacuole (Korthout et al. 2000).

Proteolytic activity in plant cells undergoing PCD has also been studied using poly(ADP-ribose) polymerase (PARP), a well-characterised substrate for human caspase 3. Cleavage of endogenous PARP occurs during menadione-induced PCD in tobacco protoplasts (Sun et al. 1999a) and in heat shock-treated tobacco suspension cells (Tian et al. 2000). Exogenous (bovine) PARP is endoproteolytically cleaved in extracts of fungus-infected cowpea plants, and cleavage can be inhibited by caspase 3-inhibitor. Interestingly, a polypeptide (GDEVDGIDEV) mimicking the PARP human caspase-3 cleavage site (DEVD-G) partially inhibited PARP cleavage, whereas a modified peptide in which the essential aspartate was replaced by alanine (GDEVAGIDEV) did not affect PARP cleavage (D'Silva et al. 1998). However, cleavage of exogenous PARP in cowpea extracts results in fragments that are different from the fragments that remain after cleavage by a animal caspase (D'Silva et al. 1998). As it appears that the proteolytic activity detected in plants may have some different specificities from animal caspases,

interpretation of these data requires some caution.

In animals, the IAP protein family has been postulated to play its regulating role by inhibiting caspases (Deveraux and Reed 1999). IAP proteins, conserved between numerous organisms, are distinguished both by their ability to suppress apoptosis and by the presence of at least one baculoviral IAP repeat (BIR), required for their anti-death activity. It has been reported that *Agrobacterium*-induced PCD in maize cells can be suppressed by ectopic expression of an IAP from baculovirus (Hansen 2000). Likewise, transgenic expression of the baculovirus IAP in tobacco conferred resistance to several necrotrophic fungal pathogens that normally result in necrotic lesions (Dickman et al. 2001). The macromolecule p35 is another highly specific caspase inhibitor from baculovirus that is effective in inhibiting *Agrobacterium* -induced PCD in maize (Hansen 2000). Tobacco plants expressing *p35* are partially inhibited in HR cell death, whereas mutated versions of the p35 protein, impaired in caspase inhibition, are ineffective (Lam and Del Pozo 2000; Lam et al. 2001). These data point towards the existence of plant proteases that are able to recognise caspase specific inhibitors, and their involvement in cell death.

Recently, sequence comparison has revealed a group of CLPs, designated metacaspases, in fungi and plants. The universally conserved catalytic cysteine and histidine diad required for catalysis by cysteine proteases is present in these metacaspases (Uren et al. 1998). It has been shown that the only metacaspase present in yeast *(Saccharomyces cerevisiae)* displays a caspase-like proteolytic activity that is activated when yeast is stimulated by H_2O_2 to undergo apoptosis (Madeo et al. 2002). A second subgroup of caspase-related proteases are legumains, cysteine endopeptidases first identified in plants. Although legumains have a strict specificity for a asparagine (and not aspartate) residue immediately N-terminal to the substrate's cleavage site, they posses a protein fold similar to animal caspases and are believed to be evolutionarily related (Chen et al. 1998).

Taken together, these data suggest that caspase-like proteolytic activity plays a role during plant PCD. Considering the effectiveness of animal caspase inhibitors in blocking plant PCD, the observed cleavage of animal as well as endogenous PARP by activated plant proteases, the functioning of animal IAP proteins in plants, and identification of plant CLPs by sequence and secondary structure comparison, one could readily envision plant CLPs as proteins that possess the caspase fold and can recognise (some) animal caspase cleavage sites when ac-

tivated. Additional (as yet undetermined) plant-specific cleavage sites may be recognised in endogenous proteins. It will be interesting to see what proteins are cleaved during plant PCD. Besides PARP, lamin-like proteins have been reported to be cleaved during menadione-induced PCD in tobacco protoplasts (Sun et al. 1999b). Degradation of lamins is an important event in apoptosis, playing an essential role in chromatin condensation and breakdown of the nuclear envelope (Rao et al. 1996).

Role of mitochondria, cytochrome c, and BLPs

In animal systems, changes in mitochondrial membrane permeability, subsequent release of cytochrome c and the formation of the apoptosome play an important role in apoptosis. BLPs can act as regulators of apoptosis both by interference with caspase activation or through their effect on mitochondrial membrane integrity (Adams and Cory 2001; Adrain and Martin 2001; Ferri and Kroemer 2001).

In various plant systems, the release of cytochrome c from mitochondria into the cytosol precedes cell death (Balk et al. 1999; Stein and Hansen 1999; Sun et al. 1999a; Hansen 2000; Balk and Leaver 2001; Sagar Tiwari et al. 2002). In addition, apoptosis in nuclei from mouse cells can be induced in the cytosol of carrot cells by adding cytochrome c (Zhao et al. 1999), and HR-induced PCD is associated with the disruption of mitochondrial functions (Xie and Chen 2000). These findings might indicate a role for mitochondria and cytochrome c in plant cell death. However, cytochrome c is not released during petal cell death in (pollinated) petunia flowers (Xu and Hanson 2000), establishing at least one form of plant PCD in which cytochrome c release is not required. Nevertheless, the release of cytochrome c from plant mitochondria as caused by ROS, elevated calcium levels, or inhibition of electron transport, has been postulated to be a common means for integrating cellular stress and activating plant PCD (Jones 2000).

Evidence for a function of BLPs in plant PCD is accumulating. Initial indications, such as the detection of a BCL2 homologue in plant cells by immunoblotting (Dion et al. 1997), and the capability of animal BLPs to modify cell death processes in plants (Lacomme and Santa Cruz 1999; Lam et al. 1999a;

Mitsuhara et al. 1999; Dickman et al. 2001), are now supported by the isolation of homologues of human Bax inhibitor-1 from *A. thaliana* and rice (Bax is a pro-apoptotic member of the BCL2 family). Both clones, *AtBI1* and *OsBI1*, are capable of suppressing Bax-induced cell death in yeast (Kawai et al. 1999; Sanchez et al. 2000), whereas *AtBI1* is rapidly upregulated during wounding or pathogen challenge (Sanchez et al. 2000). In addition, overexpression of *AtBI1* can rescue plants expressing mammalian *Bax* from cell death (Kawai-Yamada et al. 2001). Furthermore, the *A. thaliana* genome contains two AtBI1 homologues, AtBI2 and AtBI3, and a newly identified family of 13 AtBI2-related (ABRs) genes encoding putative transmembrane proteins that could form macromolecular channels. Although their function remains to be elucidated, it has been suggested that these genes might represent functional equivalents of the mammalian BCL2 family (Lam et al. 2001).

R-genes, heat shock proteins and the apoptosome

Sequence alignments have uncovered significant similarity between regions from *C. elegans* CED4, its human counterpart APAF1 and several plant resistance *(R)* gene products (Van der Biezen and Jones 1998; Aravind et al. 1999). APAF1 represents one factor in a high molecular weight protein complex called the apoptosome. Once the apoptosome has been assembled in response to death-inducing stimuli, it recruits and activates caspases to initiate the cell death program. The functional significance of this homology is yet unclear. In contrast to the animal proteins, plant *R* gene products do not contain a caspase recruitment domain (CARD). However, this does not exclude that, in analogy to their animal counterparts, *R* gene products may function as controlling adaptors in plant protein complexes that become activated by pathogen-derived signals during HR-related PCD (Van der Biezen and Jones 1998). Indeed, the tomato resistance gene product Mi is involved in regulation of localised cell death (Hwang et al. 2000), whereas activation of a tobacco mitogen-activated protein kinase (MAPK) by tobacco mosaic virus depends on resistance gene *N* (Zhang and Klessig 2001).

It has been established that the survival-promoting effects of animal heat shock proteins (HSPs) can be partly attributed to the suppression of apoptosis

(Samali and Orrenius 1998). HSPs have been demonstrated to intervene at multiple points in the apoptotic pathway. These points include prevention of cytochrome c release and disruption of the apoptosome by binding to cytochrome c, inhibition of APAF1 oligomerisation, and suppression of caspase recruitment. HSP-mediated inhibition of cell death downstream of caspase activation and substrate cleavage has also been observed (reviewed in Beere and Green 2001). Recent experiments in our laboratory indicate that heat-treatments effectively protect tomato suspension cells against camptothecin-induced PCD (unpublished results). In view of the fact that this camptothecin-induced PCD involves caspase-like proteases (De Jong et al. 2000), it is tempting to speculate that HSPs have similar survival-promoting properties in plants as in animals.

Defender against apoptotic cell death (DAD1) is conserved among various organisms

The *DAD1* gene is highly conserved throughout both animal and plant kingdoms. This gene was originally isolated from a temperature-sensitive mutant hamster cell line that undergoes apoptotic cell death when incubated at non-permissive temperature (Nakashima et al. 1993), and encodes a protein that has been described to inhibit developmental PCD in *C. elegans* (Sugimoto et al. 1995). When various studies demonstrated substantial evolutionary and functional conservation (Sugimoto et al. 1995; Gallois et al. 1997; Tanaka et al. 1997), it was postulated that DAD1 is a universal negative regulator of PCD. Lead by sequence similarity between human DAD1 and *S. cerevisiae* OST2 (Silberstein et al. 1995), it was demonstrated that DAD1 represents an essential subunit of the mammalian oligosaccharyltransferase (OST), an enzyme complex functioning in N-linked glycosylation (Fu et al. 1997; Kelleher and Gilmore 1997; Sanjay et al. 1998). Recently, Makishima et al. (2000) showed that DAD1 interacts with MCL1 (a member of the BCL2 protein family), providing a new perspective on the putative role of DAD1 in apoptosis. However, experiments using truncated versions of the DAD1 protein show that DAD1-MCL1 interactions are not sufficient for complementation of the *dad1* mutant phenotype. Therefore, the exact function of DAD1 in apoptosis is still subject to speculation.

DAD1 homologues have been cloned from various plant species, and al-

though in some cases *DAD1* mRNA levels exhibit a (modest) downregulation during PCD in plants (Gallois et al. 1997; Orzaez and Granell 1997; Lindholm et al. 2000; Moriguchi et al. 2000; Hoeberichts et al. 2001), contradicting data make it hard to draw any general conclusions (Dong et al. 1998; Lindholm et al. 2000; Hoeberichts and Woltering 2001). Collectively, these data illustrate the obscurity that still surrounds the role of DAD1 in PCD, both in animals and plants.

Role of ROS and NO

Although ROS used to be regarded merely as toxic by-products of cellular metabolism, it is now recognised that molecules such as hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH), and superoxide ($\cdot O_2^-$) have a signalling role in many biological systems (Hancock et al. 2001; Vranová et al. 2002). Experimental data indicating that ROS can activate cell death programs, both in animal and plants, is accumulating (Jabs 1999).

In plant tissue, various conditions lead to accelerated generation and/or accumulation of ROS and subsequent PCD, for example ozone (O_3) fumigation (Rao et al. 2000a; Rao and Davis 2001), cold stress (Prasad et al. 1994), UV-radiation (Green and Fluhr 1995), senescence (Del Rio et al. 1998; Rubinstein 2000), and barley aleurone layer differentiation (Bethke and Jones 2001; Fath et al. 2001). The role of ROS in plant PCD has been most extensively studied during the HR to pathogen attack, when ROS are generated rapidly and transiently at the site of infection (Wojtaszek 1997; Dat et al. 2000). This process is generally referred to as the oxidative burst (Lamb and Dixon 1997). During the HR, ROS may possess direct antimicrobial activity and function in cell-wall reinforcing processes. As signal molecules, they are believed to induce PCD, and activate defence gene expression and systemic acquired resistance (SAR) (Alvarez et al. 1998; Orozco-Cardenas et al. 2001). Plant responses to ROS are dose dependent. High doses of ROS trigger HR-related PCD, whereas low doses induce antioxidant enzymes (Levine et al. 1994; Desikan et al. 1998), and block cell cycle progression (Reichheld et al. 1999). It has been postulated that through this dose-dependent action, ROS act as a trigger for PCD locally and as a diffusable signal for the induction of cell defences in neighbouring cells (Lamb and Dixon 1997).

Despite the recognition of ROS as signalling molecules in PCD, little is known about how these signals are perceived and transduced in plant cells. It has been reported that H_2O_2 is a potent activator of a MAPK cascade that induces specific stress-responsive genes in *A. thaliana* leaf cells, but represses auxin-inducible promoters (Kovtun et al. 2000). The activation of a redox signalling pathway possessing a MAPK module has also been reported in response to avirulent pathogen infection in *A. thaliana*. This signalling network functioned independent of the plant hormones ethylene, salicylic acid and jasmonic acid (Grant et al. 2000). However, ethylene plays a critical role in the release of H_2O_2 during PCD in tomato suspension cells, as inhibitors of ethylene biosynthesis or perception block H_2O_2 production and cell death (De Jong et al. 2002).

The free radical gas nitric oxide (NO), well characterised as mammalian signalling molecule, has also been recognised as a signal in plants (Durner and Klessig 1999; Neill et al. 2002). *A. thaliana* suspension cultures generate elevated levels of NO in response to avirulent bacteria. In this system, these elevated levels of NO were sufficient to induce cell death that involves caspase-like activity (Clarke et al. 2000). However, most data indicate that NO co-operates with ROS in the activation of HR cell death (Delledonne et al. 1998; Durner et al. 1998). Recently, it was demonstrated that the HR is triggered only by balanced production of NO and ROS. More specifically, dismutation of $\cdot O_2^-$ to H_2O_2 is required to activate cell death, which depends on synergistic interactions between NO and H_2O_2. Scavenging of $\cdot O_2^-$ by superfluous NO (or vice versa) disturbs the NO/H_2O_2 ratio, resulting in reduced cell death (Delledonne et al. 2001). Little is known about signalling pathways downstream of NO/H_2O_2. It has been shown that NO signalling during both PCD and defence responses requires cyclic GMP and cyclic ADP-ribose, two molecules that can serve as secondary messengers for NO signalling in mammals (Durner et al. 1998). Furthermore, NO activates MAP kinases in both *A. thaliana* and tobacco (Durner et al. 1998; Kumar and Klessig 2000). Collectively, these data indicate that NO is a ubiquitous signal in plants. Yet, it is apparent that the understanding of NO signalling in plant PCD is still at an early stage.

ROS, particularly H_2O_2, have been implicated in activation of the NF-κB signalling pathway that plays an essential role in regulating both immune and inflammatory responses, and tumour necrosis factor (TNF)-induced apoptosis in animal cells. Once activated and translocated to the nucleus, NF-κB can induce various

anti-apoptotic factors, including IAPs and BLPs. NF-κB activity is regulated by the family of ankyrin domain-containing IκB proteins that sequester NF-κB to the cytoplasm as an inactive complex (Foo and Nolan 1999; Perkins 2000). The human protein PIRIN, that has originally been isolated as a Nuclear Factor I associated protein (Wendler et al. 1997), is capable of binding to the ankyrin repeat domain of BCL3, a member of the IκB family. Together, NF-κB, BCL3 and PIRIN can form a protein complex that is capable of modulating NF-κB driven gene expression through interaction with an NF-κB DNA binding site (Dechend et al. 1999). A tomato homologue of human *PIRIN* is upregulated during camptothecin-induced PCD in tomato suspension cells. *LePIRIN* mRNA accumulation is also observed when cells are treated with the mycotoxin fumonisin B1 (Orzaez et al. 2001). Interestingly, the *A. thaliana NPR1/NIM1* gene that is required for systemic acquired resistance, a plant immune response, shows similarity to mammalian IκB (Cao et al. 1997; Ryals et al. 1997). Characterisation of the *A. thaliana* mutant *agd2* has uncovered that NPR1/NIM1 can suppress HR-induced cell death on the one hand, yet it promotes spontaneous cell death on the other hand (Rate and Greenberg 2001). Together, these data allow to speculate on the existence of an NF-κB/IκB-like signalling pathway in plants, and the possible regulatory role of LePIRIN as a mediator of protein-protein interactions during plant PCD. These PIRIN-dependent interactions could affect, either positively or negatively, the expression of anti-apoptotic genes.

Calcium signalling

Calcium (Ca^{2+}) is an almost universal intracellular messenger, controlling a broad range of cellular processes, including apoptosis (Krebs 1998). In plants, being no exception, calcium has also been recognised as a ubiquitous signal (Sanders et al. 1999). Thus it seems likely that the regulation of PCD in plants involves Ca^{2+} signalling.

Elevated calcium levels have been observed during tracheary element differentiation (Groover and Jones 1999), aerenchyma formation (He et al. 1996; Drew et al. 2000b), wheat aleurone differentiation (Kuo et al. 1996), the HR (Xu and Heath 1998), and leaf senescence (Huang et al. 1997). Furthermore, the Ca^{2+} channel blocker lanthanum chloride ($LaCl_3$) can inhibit H_2O_2-induced cell

death in soybean cells, bacteria-induced PCD in *A. thaliana*, and camptothecin-induced PCD in tomato cells. However, this inhibitor does not suppress the induction of more general stress or defence pathways (Levine et al. 1996; Mittler and Lam 1997; Hoeberichts et al. 2001), suggesting that Ca^{2+} fluxes are involved in signalling the activation of PCD, but not the activation of general stress or defence responses. Other experiments confirm a role for calcium signalling in pathogen defence. The *A. thaliana dnd1* mutant has been isolated as a line that failed to produce the HR in response to avirulent pathogen infection. Cloning of the corresponding *DND1/CNGC2* gene revealed that it encodes a cyclic nucleotide-gated ion channel that can allows passage of Ca^{2+}, K^{+} and other cations. (Clough et al. 2000). Expression studies have lead to speculation on a more general role for DND1/CNGC2 during PCD (Köhler et al. 2001). Another elicitor-activated Ca^{2+} permeable ion channel has been identified in parsley by patch-clamp analysis (Zimmermann et al. 1997).

Calcium-binding proteins interpret information contained in the temporal and spatial patterns of Ca^{2+} fluxes and accordingly bring about changes in metabolism and gene expression. Interestingly, plants contain a unique superfamily of calmodulin-like domain protein kinase (CDPKs) capable of activating protein phosphorylation cascades, a widely used mechanism by which extracellular stimuli are transduced into intracellular responses (Harmon et al. 2000). Various (putative) calcium binding proteins, among them several CDPKs, are induced during plant defence responses (Heo et al. 1999; Jakobek et al. 1999; Romeis et al. 2000; Murillo et al. 2001). It has been proposed that elevated Ca^{2+} levels can induce NADPH oxidase activity, either via CDPKs (Blumwald et al. 1998; Bolwell 1999), or directly (Sagi and Fluhr 2001).

Present data indicate that calcium signalling is an important mediator of plant PCD. The existence of the CDPK protein family indicates that plants have implemented certain plant-specific factors into this universally present signal transduction system.

2.3. Do plant-specific mediators of PCD act through modulation of ROS levels?

It is likely that, in addition to the putative regulators of PCD conserved throughout the animal and plant kingdoms, there are plant-specific mediators of PCD. Various plant hormones are strong candidates, and supporting evidence is starting to accumulate.

Salicylic acid

Salicylic acid (SA) is a key-signalling molecule in pathogen-induced disease resistance, but its function in relation to cell death is still poorly understood. The epistatic relationship between cell death and SA accumulation has been analysed in crosses between various *A. thaliana* mutants and the transgenic *nahG* line (depleted in SA). Whereas several mutants retain their spontaneous lesion phenotype in the *nahG* background, others display a reduction, delay or even abolition of their mutant phenotype. These data can only be explained if SA accumulation is placed both upstream and downstream of cell death, presumably as part of a feedback amplification loop (Alvarez 2000; Shirasu and Schulze-Lefert 2000).

Biochemical evidence suggests that the function of SA early in the HR might affect the phosphorylation status of a signalling pathway component that regulates the activation of a sustained oxidative burst (Draper 1997; Shirasu et al. 1997; Alvarez 2000). Fumonisin B1-induced cell death in *A. thaliana* protoplasts requires SA signalling (Asai et al. 2000), and transgenic *nahG* tobacco displays decreased lesion formation after O_3-treatment (Örvar et al. 1997), confirming a role for SA upstream of cell death. Conversely, ROS are capable of inducing SA accumulation (Chamnongpol et al. 1998) or even of directly stimulating SA synthesis (León et al. 1995), supporting the idea of a feedback amplification loop. One of the two MAPKs implicated in the activation of the HR in tobacco can be induced by SA (Zhang and Klessig 2001), providing a means by which SA could induce downstream phosphorylation.

In TMV-infected tobacco tissue that accumulate SA, a gradient of SA is estab-

lished along with lesion formation, with the highest levels of SA present in and around the necrotic lesions (Enyedi 1992). It has been postulated that low concentrations of SA might be inadequate for the induction of cell death but sufficient to activate survival signals and defence responses. This implicates low levels of SA in the regulation of lesion limitation, whereas high levels of SA actually induce cell death (Alvarez 2000). Interestingly, high levels of SA can rapidly inhibit mitochondrial functions by a mechanism that requires ROS (Xie and Chen 1999). Given the function of mitochondria in animal apoptosis (Green and Kroemer 1998), this effect of SA might contribute to the induction of cell death in response to pathogens.

Jasmonic acid

Jasmonic acid (JA) is a well-known signalling molecule in plant defence and stress responses. It has been implicated in O_3-induced hypersensitive cell death since O_3 induces JA biosynthesis within several hours of treatment (Koch et al. 2000; Rao et al. 2000b). Furthermore, treatment with exogenous methyl jasmonate inhibits propagation of O_3-induced cell death in O_3-sensitive *A. thaliana* plants. Accordingly, the jasmonate-insensitive mutant *jar1* displays increased spreading of cell death following exposure to O_3 (Overmyer et al. 2000). The highly O_3-sensitive *A. thaliana* ecotype Cvi-0 has greatly reduced JA sensitivity, whereas various other JA mutants show a similar high sensitivity to O_3 (Rao et al. 2000b). Together, these studies indicate that JA is an important component of a pathway that negatively regulates cell death and lesion formation. Interestingly, JA is believed to cause this effect by attenuating the O_3-induced ROS production, as wounding or treatment of plants with JA has been shown to reduce both O_3-induced cell death and O_3-induced ROS levels (Örvar et al. 1997; Schraudner et al. 1998; Overmyer et al. 2000). However, the precise mechanisms by which JA signalling regulates cell death are far from understood. Contradictory results show that viability of *jar1* mutant *A. thaliana* protoplasts is only marginally affected by fumonisin B1, whereas this fungal toxin induces apoptosis-like PCD in wild-type protoplasts. This indicates that JA-mediated signalling increases fumonisin B1-induced cell death (Asai et al. 2000).

Ethylene

Ethylene is a plant hormone well known for its role during plant senescence and cellular responses to numerous forms of stress and an additional regulatory role during plant PCD is emerging. Aerenchyma formation in hypoxic roots, one of the earliest examples of PCD recognised in plants, requires ethylene (Drew et al. 2000a). Epidermal cell death at the site of adventitious root formation, a response to submergence of the deepwater rice *O. sativa*, can be induced by application of the natural ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and it can be suppressed by inhibiting ethylene perception (Mergemann and Sauter 2000). During the development of cereal endosperm, ethylene is produced in two discrete peaks. Application of exogenous ethylene throughout seed development results in earlier and more extensive cell death and DNA fragmentation. Conversely, treatment with inhibitors of ethylene biosynthesis or ethylene perception reduces cell death and DNA fragmentation (Young and Gallie 2000b). There are also reports describing ethylene as a stimulant of senescence-associated PCD (Orzáez and Granell 1997; Navarre and Wolpert 1999). Furthermore, cell death induced by the mycotoxin fumonisin B1 seems to involve ethylene-mediated signalling pathways in both *A. thaliana* and tomato (Moore et al. 1999; Asai et al. 2000). Although ethylene alone is not sufficient to trigger camptothecin-induced PCD in tomato cell suspensions, cell death and the associated oxidative burst can be blocked by inhibition of ethylene signalling. Exogenous ethylene greatly stimulates camptothecin-induced H_2O_2 production and cell death (De Jong et al. 2002).

Studies of *A. thaliana* double mutants have provided additional evidence supporting a role for ethylene signalling in cell death. Crosses of the lesion-mimic mutant *acd5* and *ein2*, in which ethylene signalling is blocked, show decreased cell death (Greenberg et al. 2000). Ethylene insensitivity in double mutants of *ein2* and the O_3-sensitive *rcd1* blocks ROS accumulation that is required for lesion propagation, whereas exogenous ethylene increases ROS dependent cell death (Overmyer et al. 2000). In line with the suggested positive role for ethylene during propagation of ROS-dependent lesions (Overmyer et al. 2000), it has been proposed that limiting the spread of pathogen-induced cell death in tomato involves a downregulation of ethylene sensitivity (Ciardi et al. 2000).

Together, these data show that various forms of plant PCD require ethylene

signalling and, moreover, are accelerated by exogenous applied ethylene. It seems plausible that ethylene is required, though not sufficient, for regular PCD and that its function is linked to controlling the extent of cell death. The stimulation of PCD by ethylene seems associated with an increased production of ROS.

ABA and GA

Abscisic acid (ABA) has been implicated as a key regulator in cereal endosperm development. Cell death is accelerated in developing endosperm of ABA-insensitive or deficient maize mutants. It is believed that a balance between ABA and ethylene establishes the appropriate onset and progression of PCD during maize endosperm development (Young and Gallie 2000b). During germination, cell death in barley aleurone layers is induced by gibberellin (GA), whereas ABA antagonises this effect (Wang et al. 1996c; Bethke et al. 1999). It has been found that ROS are mediators of this hormonally regulated cell death pathway. Incubation of aleurone layers or protoplasts in H_2O_2-containing media results in death of GA-treated but not ABA-treated aleurone cells. Cells that are programmed to die are therefore less able to withstand ROS than cells that are programmed to remain alive (Bethke and Jones 2001), supposedly because ROS scavenging enzymes are strongly down-regulated in aleurone layers treated with GA, whereas ABA-treated cells maintain their ability to scavenge ROS (Fath et al. 2001; Fath et al. 2002). Wang et al. (1999) describe a similar protective role of ABA against cell death during androgenesis in developing barley anthers.

Lesion mimic mutants

Mutants that show spontaneous cell death in the absence of pathogens are called disease lesion mimics and are known to occur in a number of different plants, including *A. thaliana*, rice, maize, and barley (Dangl et al. 1996). Many of these mutants also exhibit increased expression of defence-related genes, elevated SA levels, and activation of SAR. It has therefore been proposed that lesion mimics represent defects in genes regulating HR-related cell death (Morel and Dangl 1997). A number of the lesion mimic genes have been cloned. The *A.*

thaliana LSD1 (lesion simulating disease1) gene encodes a zinc-finger protein needed to restrict lesion size during HR (Dietrich et al. 1997). LSD1 is believed to be a transcription factor that down-regulates or dampens cell death in plant cells surrounding the immediate site of infection. It has been proposed that LSD1 suppresses the engagement of a prodeath signalling pathway by antagonising NPR1 function, possibly at the level of transcription. Besides NPR1, this prodeath pathway requires ROS derived from directly challenged (neighbouring) cells and an SA-dependent amplification loop (Loake 2001; Aviv et al. 2002).

The maize *LLS1* (lethal leaf spot) gene encodes a putative dioxygenase required to limit the spread of cell death in mature leaves. It has been suggested that it functions to degrade a phenolic mediator of cell death, possibly SA (Gray et al. 1997). The *MLO* resistance gene from barley encodes a transmembrane protein of unknown function. In *mlo* mutant plants, the absence of MLO protein causes a leaf lesion phenotype and increased disease resistance, indicating that MLO negatively regulates both cell death and pathogen defence (Büschges et al. 1997).

The cloning of these (and other) genes and the study of their function is expected to help identify new regulatory components of cell death in plants. However, whereas some of these genes may indeed function as true regulators of hypersensitive cell death, others may merely perturb cellular metabolism in a way that promotes cell death. An example of this may be the *A. thaliana ACD2* gene that encodes a red chlorophyll catabolite reductase. Cell death in *acd2* mutant plants is believed to be caused by the accumulation of chlorophyll breakdown products (Mach et al. 2001).

2.4. Plant programmed cell death: hormonal control of a functionally conserved apoptotic cell death machinery

PCD is an intrinsic part of plant life. However, general, recurring (apoptotic?) processes and mechanisms in plant PCD have not yet clearly emerged. Chromatin degradation and the occurrence of DNA laddering, generally recognised as hallmarks of apoptotic cell death in animals, are found in many cases of plant PCD, although examples of non-apoptotic cell death in plant development have

also been described (Fath et al. 1999; Fukuda 2000). Rupture of the vacuolar membrane, preceded by loss of selective permeability, triggers rapid nuclear degradation during TE differentiation (Obara et al. 2001). This vacuolar collapse was suggested to be a singular event shared by all forms of plant PCD (Jones 2001). Yet, these features describe cell death from the execution phase and onwards. The molecular mechanisms leading to these events are poorly understood. Nevertheless, it seems likely that in plant cells, as in animal cells, once the cell death signal has been perceived, the various signalling pathways feed into a common (or limited number of) PCD pathway(s). The existence of and interactions between many of the putative mediators of plant PCD mentioned in this review suggest that part of this machinery is functionally conserved between animal and plant kingdoms.

Several recent studies suggest that recruitment of cysteine proteinases and mitochondria to the cell death machinery may be of a very ancient evolutionary origin, as a number of apoptotic enzymes have bacterial homologues (Koonin and Aravind 2002). Release of death-inducing factors from mitochondria has been observed in several unicellular eukaryotes (Lorenzo 1999; Arnoult et al. 2001; Arnoult et al. 2002), and, in addition, both ROS and a metacaspase are key regulators of apoptosis in yeast (Madeo et al. 1999; Madeo et al. 2002). Acquisition of these central apoptotic effectors appears to have been the result of mitochondrial endosymbiosis and subsequent additional symbiotic events during the evolution of primitive eukaryotes. At the same time, these core components have undergone specific proliferation and specialisation, with the outer layers of the cell death machinery gradually building up around them (Koonin and Aravind 2002).

A hypothetical model that incorporates the cell death mediators outlined in this review is depicted in Figure 2.2. Note that this model combines experimental data obtained from a broad variety of experimental systems, to acquire a general overview of the signalling events that could be involved in plant PCD. In this model, ROS, associated with many of the above-described examples of plant cell death, play a central role. Most likely, an NADPH oxidase complex (analogous to the oxidase in mammalian phagocytes) catalyses the reduction of oxygen to $\cdot O_2^-$, followed by dismutation of $\cdot O_2^-$ to H_2O_2. Indeed plant homologues of NADPH oxidase subunits have been cloned (Groom et al. 1996; Keller et al. 1998; Amicucci et al. 1999). A plant homologue of Rac, one of the cytosolic factors

required for animal NADPH activity, has been found to regulate ROS accumulation and cell death in rice (Kawasaki et al. 1999). In addition, NADPH oxidase activity is enhanced in TMV-infected tobacco cells (Sagi and Fluhr 2001).

NADPH-mediated generation of ROS may be stimulated by caspase(-like) activity, as caspase-inhibitors can prevent cell death and the preceding accumulation of ROS, both in animal and plant cells (Simizu et al. 1998; De Jong et al. 2002). Mitochondria provide an additional source of elevated ROS levels and, at the same time, are sensitive to oxidative stress. By analogy with animal cells, permeability of the mitochondrial membrane is regulated by ROS, calcium levels, and BLPs. These regulators can cause the release of death-inducing factors, most notably cytochrome c, into the cytosol, communicating the death program downstream. Strikingly, SA-dependent formation of ROS both triggers an increase in cytosolic Ca^{2+} (Kawano et al. 1998; Kawano and Muto 2000), and inhibits mitochondrial functions (Xie and Chen 1999). These could be telling examples of the role of ROS and mitochondria during plant PCD and, evenly important, of how they are connected. If endogenous plant BLPs exist, they can be readily envisioned as regulators of mitochondrial permeability, similar to animal BLPs. The finding that DAD1 can interact with a BLP (Makishima et al. 2000) for the first time makes it possible (yet highly speculative) to include this protein in a cell death model. All these regulators can cause the release of death-inducing factors, most notably cytochrome c, into the cytosol, communicating the death program downstream. Plant HSPs could function, in analogy to their animal counterparts, by repressing the activation of downstream proteases.

Plant hormones are strong mediators of plant PCD that often act in conjunction, like ABA and GA in barley aleurone layers, or SA and ethylene in HR-related cell death. As a general mode of action they may (indirectly) enhance or attenuate ROS. There are examples of hormones affecting the activity of the NADPH oxidase complex (reviewed in Draper 1997) or expression of genes coding for scavenging enzymes (Fath et al. 2001), but it has often not been determined how the relevant hormone exactly exerts its effect. Still, it is tempting to regard the control of ROS levels as an important seizing point for cell death regulation by hormones specific to plants. Conversely, ROS can stimulate SA biosynthesis (León et al. 1995), providing an example of a positive feedback effect.

Both the ankyrin repeats containing amino acid sequence of NPR1 and the emerging role of NPR1 in HR-related cell death have lead to speculation on an

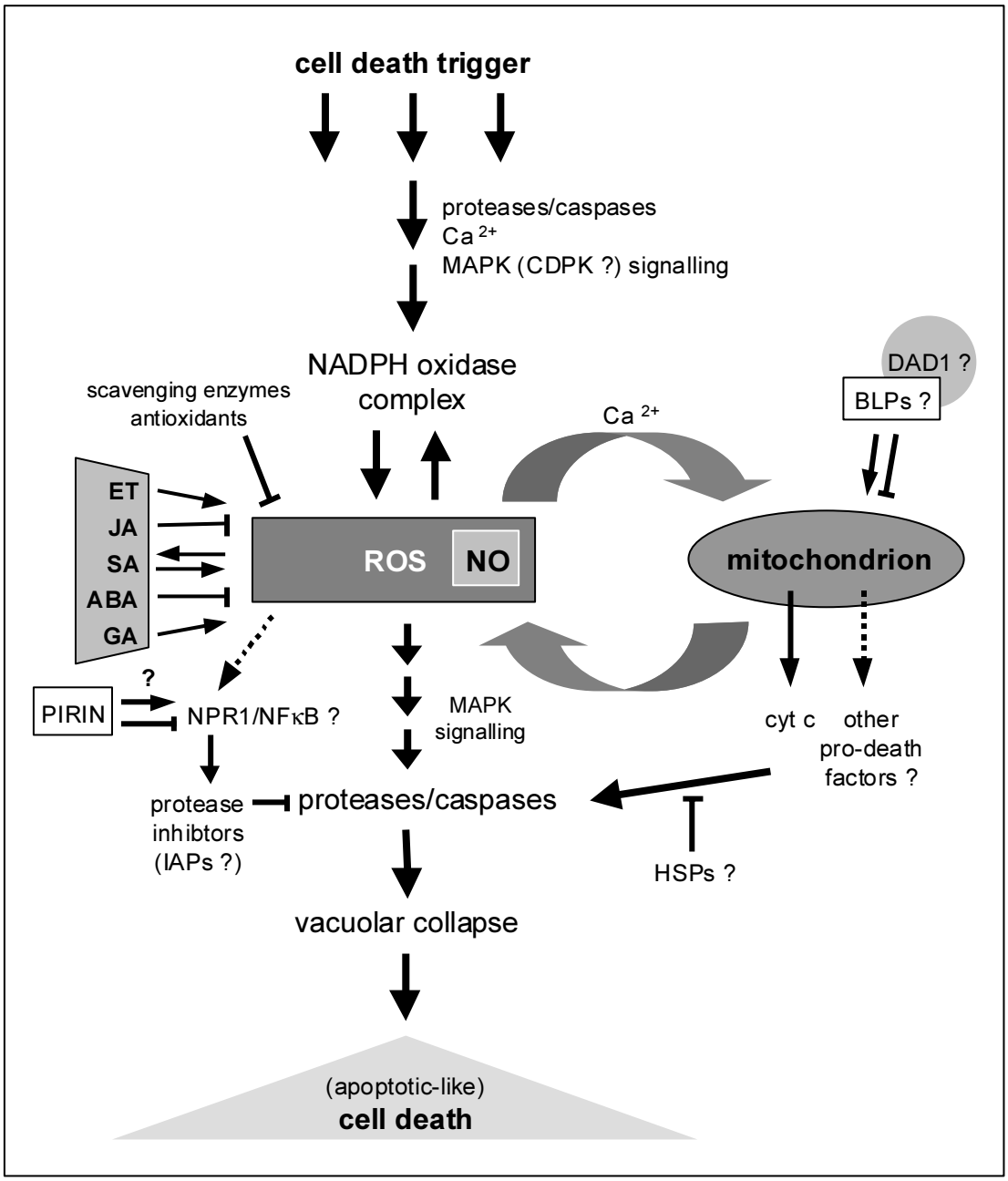


FIGURE 2.2. *Model of plant programmed cell death. For explanation, see text.*

NF-κB-like function of NPR1 in plants, suppressing cell death during HR (Cao et al. 1997; Ryals et al. 1997; Rate et al. 1999; Greenberg et al. 2000; Rate and Greenberg 2001). In animals, activation of NF-κB has been reported to occur in response to a wide range of stimuli, including oxidative stress (Perkins 2000). Analogously, plants might activate a NF-κB-like pathway in response to the oxidative burst, aimed at suppressing cell death, possibly through the activation of protease inhibitors.

2.5. Concluding remarks

Increasing evidence indicates that many cases of plant PCD proceed through a cell death mechanism that is functionally conserved between animals and plants. The plant hormones known to affect cell death in plants may act as mediators of this core pathway mainly by modulating ROS levels. Paradoxically, there is a "lack" of direct genetic evidence. This might be explained by low sequence similarity, obscuring possible functional analogy. Furthermore, plant PCD has undoubtedly adapted to the specific features of plant development and defence, integrating plant-specific mediators (such as various plant hormones) and plant-specific processes (such as remobilization or secondary cell wall synthesis). Thus, divergent mechanisms consistent with plant-specific aims of cellular suicide may also have evolved. This implies that plant PCD regulators cannot be readily identified by sequence comparison. “Novel” regulators may emerge from existing plant protein families. For example, 14-3-3 proteins, key regulators of numerous cellular processes (including apoptosis) in animals, also exist in *A. thaliana* (Rosenquist et al. 2001), and members of the plant-specific WRKY transcription factor family have already been implicated in senescence and defence responses (Hinderhofer and Zentgraf 2001; Robatzek and Somssich 2001).

3

chemical-induced apoptotic cell death in tomato cells: involvement of caspase-like proteases

Anke J. De Jong, Frank A. Hoeberichts, Elena T. Yakimova, Eugenia Maximova, and Ernst J. Woltering. Planta 211: 656-662 (2000)

Summary

A new system to study programmed cell death (PCD) in plants is described. Tomato (*L. esculentum* Mill.) suspension cells were induced to undergo PCD by treatment with known inducers of apoptosis in mammalian cells. This chemical-induced cell death is accompanied by characteristic features of apoptosis in animal cells, such as typical changes in nuclear morphology, fragmentation of the nucleus and DNA fragmentation. In search of processes involved in plant apoptotic cell death, specific enzyme inhibitors were tested for cell death inhibiting activity. Our results show that proteolysis plays a crucial role in apoptosis in plants. Furthermore, caspase-specific peptide inhibitors were found to be potent inhibitors of the chemical-induced cell death in tomato cells, indicating that, like in animal systems, caspase-like proteases are involved in the apoptotic cell death pathway in plants.

3.1. Introduction

Apoptosis or programmed cell death (PCD) is the active process of cell death that occurs during development and in response to environmental triggers in a wide variety of different biological systems. In plants, PCD is believed to be essential for development and survival. Apoptotic cell death occurs in two phases, first the commitment to cell death, followed by an execution phase characterised by morphological changes in cell and nuclear structure. The main morphological features of apoptosis, in mammalian cells, are chromatin condensation, cell shrinkage, systematic DNA cleavage with disintegration of the nucleus and fragmentation into discrete apoptotic bodies, and ultimately cell death (Martin et al. 1994). Such morphological features have also been observed in phytotoxin-treated tomato protoplasts, in sloughing root cap cells (Wang et al. 1996b) and in either senescing or chemical-treated tobacco protoplasts (O'Brien et al. 1998), indicating that a similar apoptotic pathway may be operative in plants.

Because molecular markers for apoptosis in plants are not available yet, the evidence for apoptosis in plants centres largely on chromatin condensation and DNA fragmentation. Fragmentation of DNA can be detected either by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) of DNA 3'-OH groups inside the nucleus (Gorczyca et al. 1993) or by detection of DNA laddering patterns on an agarose gel (Cohen et al. 1994). Besides mentioned examples where plant cell death is associated with typical apoptotic features, there are numerous other cases of PCD described in plants. PCD is part of the differentiation of *Z. elegans* parenchyma cells into tracheary elements (Mittler and Lam 1995; Fukuda 1997; Jones and Groover 1997), and it has been demonstrated in flower senescence (Orzáez and Granell 1997) and leaf senescence (Yen and Yang 1998). During interactions with the environment, cell death occurs in the so-called hypersensitive response (HR) to pathogen attack (for review, see Morel and Dangl (1997). PCD causes the deletion of aleurone cells (Wang et al. 1996c) and can also eliminate stamen primordia cells in female flowers of unisexual species (Dellaporta and Calderon-Urrea 1994). Cell death may also cause the formation of certain leaf lobes and perforations (Greenberg 1996). However, it is not clear whether in all these cases PCD follows an apoptotic pathway.

In mammalian cells caspases play a central role in the execution of pro-

grammed cell death. Caspases are cysteine proteases that have the ability to cleave adjacent to an aspartic acid residue. Caspases exist as dormant proenzymes in healthy cells and are activated through proteolysis. Once activated, caspases cleave a range of cellular substrates, leading to morphological hallmarks of apoptosis including DNA fragmentation (for review, see Nicholson and Thornberry 1997). One of the indications that caspases play a critical role in apoptosis in mammalian cells, is the observation that caspase specific peptide inhibitors block apoptosis (Nicholson et al. 1995; Schlegel et al. 1996). Caspase-specific inhibitors contain a peptide-recognition element corresponding to that found in endogenous substrates. Aldehyde and chloromethylketon derivatives of these peptides appear to be potent inhibitors of caspases. A first indication for the presence of caspase-like proteases in plants came from the work of Del Pozo and Lam (1998). They could detect caspase activity and showed that caspase-specific peptide-inhibitors could abolish bacteria-induced plant cell death. However, so far no caspase genes or caspase enzymes have been isolated from plants.

Our interest is to develop a model system to study the mechanisms of PCD in plants. Cell death was induced in tomato suspension cells by treatment with inducers of apoptosis in mammalian cells such as camptothecin, staurosporine and fumonisin B1. Camptothecin and staurosporine are inhibitors of topoisomerase I and protein kinase C, respectively and are known to induce apoptosis in a variety of mammalian systems (Jacobsen et al. 1996; Kaufmann 1998; Krohn et al. 1998). The mycotoxin fumonisin B1, a sphinganine analog, is an inducer of cell death in both mammalian and plant cells (Wang et al. 1996b). Treatment of tomato suspension cells with camptothecin, staurosporine or fumonisin B1 resulted in the occurrence of typical apoptotic-like ultrastructural changes and DNA fragmentation. The ability to inhibit chemical induced cell death with specific inhibitors makes this system an attractive tool to identify and to study processes involved in the apoptotic cell death pathway in plants.

3.2. Materials and methods

Chemicals

Fumonisin B1 was obtained from ICN Biochemicals and the caspase-inhibiting peptides

from Bachem AG (Bubendorf, Switzerland). Apoptag reagents (Oncor Appligene) were used for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL). Anti-digoxigenin-alkaline phosphatase Fab fragments were obtained from Boehringer Mannheim. All other chemicals were obtained from Sigma.

Cell culture

Tomato (*Lycopersicon esculentum* Mill.) cell suspension cultures, line Msk8 (Koornneef et al. 1987); kindly provided by T. Boller, Basel, Switzerland), were grown on a Murashige-Skoog type liquid medium supplemented with 5 µM α-naphtalene acetic acid, 1 µM 6-benzyladenine and vitamins as described by Adams and Townsend (1983). Cells were subcultured every 7 days by making a 1:4 dilution in 25 ml of fresh medium in 100 ml flasks with aluminium caps.

Cell death induction and inhibition

Cells were used for experiments 5 days after subculture. Cell death inducers and inhibitors were added simultaneously to 5 ml of suspension culture in 30 ml flasks with screw-caps. The viability was determined by staining with 0.002% fluorescein diacetate (FDA). Camptothecin, staurosporine and the peptides were applied in dimethylsulfoxide (DMSO) (final solvent concentration 0.1% v/v). DMSO had no effect on the viability of the cells. Fumonisin B1 was dissolved in water. Each putative cell death inhibitor was tested in at least three independent experiments.

Detection of nuclear changes and DNA fragmentation

To study nuclear morphology, cells were fixed in 5% buffered formalin (Sigma) and dried at microscope slides. The nuclei were stained with Hoechst 33258 and examined with a Zeiss axioplan microscope. For whole mount transferase-mediated dUTP nick-end labelling (TUNEL), cells were fixed in 4% formalin solution in phosphate buffered saline (PBS; 200 mM NaCl, 50 mM Na_2HPO_4, 50 mM NaH_2PO_4), pH 7.4 at room temperature for 30 min, washed in PBS, spotted on an amino-propyl-triethoxy silane-coated slide and dried at 30ºC. The cells were treated for 20 min at 37ºC (20 µg ml^{-1} proteinase K in 10 mM Tris pH 8.0) and washed with PBS. Subsequently the cells were subjected to TUNEL using digoxigenin-dUTP, according the protocol provided by Oncor Appligene. Anti-digoxigenin Fab fragments pre-conjugated to alkaline phosphatase (a 1:5000 dilution in PBS containing 0.1% Tween 20 and 200 µg ml^{-1} BSA) were used to visualise 3'-OH-labelled ends in the nuclei. The nuclei were counterstained with Hoechst 33258. The slides were viewed with a Zeiss axioplan microscope.

Isolation of DNA from tomato suspension cells for DNA laddering experiments was performed according to Wang et al. (1996c) with slight modifications. Several grams of

frozen cells were ground to powder in liquid nitrogen. The powder was mixed with 15 ml of hot (65°C) extraction buffer [0.1 M Tris pH 7.5; 50 mM EDTA; 500 mM NaCl; 10 mM ß-mercaptoethanol] and 1 ml of 20% SDS and mixed thoroughly. The mixture was incubated at 65°C for 20 min. Then, 5 ml of 5 M KAc was added, the samples were mixed, kept on ice for 30 min, and spinned down for 30 min. The supernatant was filtered through a tissue and collected in a clean tube, mixed with one volume of isopropanol, and immediately spinned down for 5 min (4°C). The pellet was briefly dried and dissolved in 300 µl CTAB buffer [0.2 M Tris pH 7.5; 50 mM EDTA; 2 M NaCl, 2% Cetyl-N,N,N triethyl ammonium bromide (CTAB)]. Samples were incubated for 15 minutes at 65°C and subsequently extracted with one volume of chloroform. The waterphase was precipitated with one volume of isopropanol, followed by centrifugation. Finally, the pellet was dissolved in 10 mM Tris pH 8.0, 1 mM EDTA and 0.1 µg μl^{-1} RNase. Agarose gel (1.8% agarose) electrophoresis was performed with 15 µg of DNA per lane.

3.3. Results

Induction of PCD in tomato suspension cells

Tomato suspension cells of line Msk8 were treated with 5 µM camptothecin, 2 µM staurosporine or 20 µM fumonisin B1. Camptothecin and staurosporine as well as fumonisin B1 induced cell death in tomato suspension cells, as determined by fluorescein diacetate (FDA) staining (Figure 3.1). FDA is a hydrophobic molecule which enters the cell passively and then is cleaved by cytoplasmic enzymes. It becomes fluorescent and charged after cleavage, which prevents it from leaving the cell. Under ultraviolet light a bright green fluorescence is observed in viable cells with an intact plasma membrane. An increase in number of dead cells was first detected after 8 hours of treatment. To find out whether the chemical-induced cell death in tomato cells shows similarities to mammalian apoptosis, the treated cells were analysed for the presence of some typical morphological characteristics of apoptosis. One of the characteristics of apoptosis is the occurrence of morphological changes of the nucleus (Lazebnik et al. 1993). In order to determine whether nuclear changes were induced by chemical treatment, the morphology of nuclei of treated and non-treated cells was com-

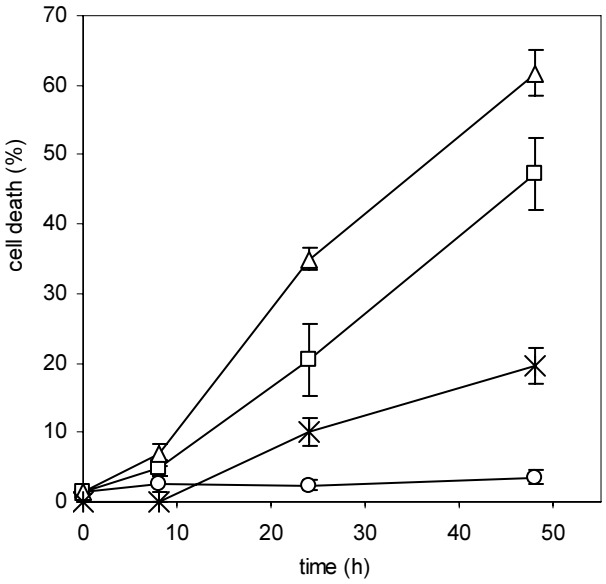
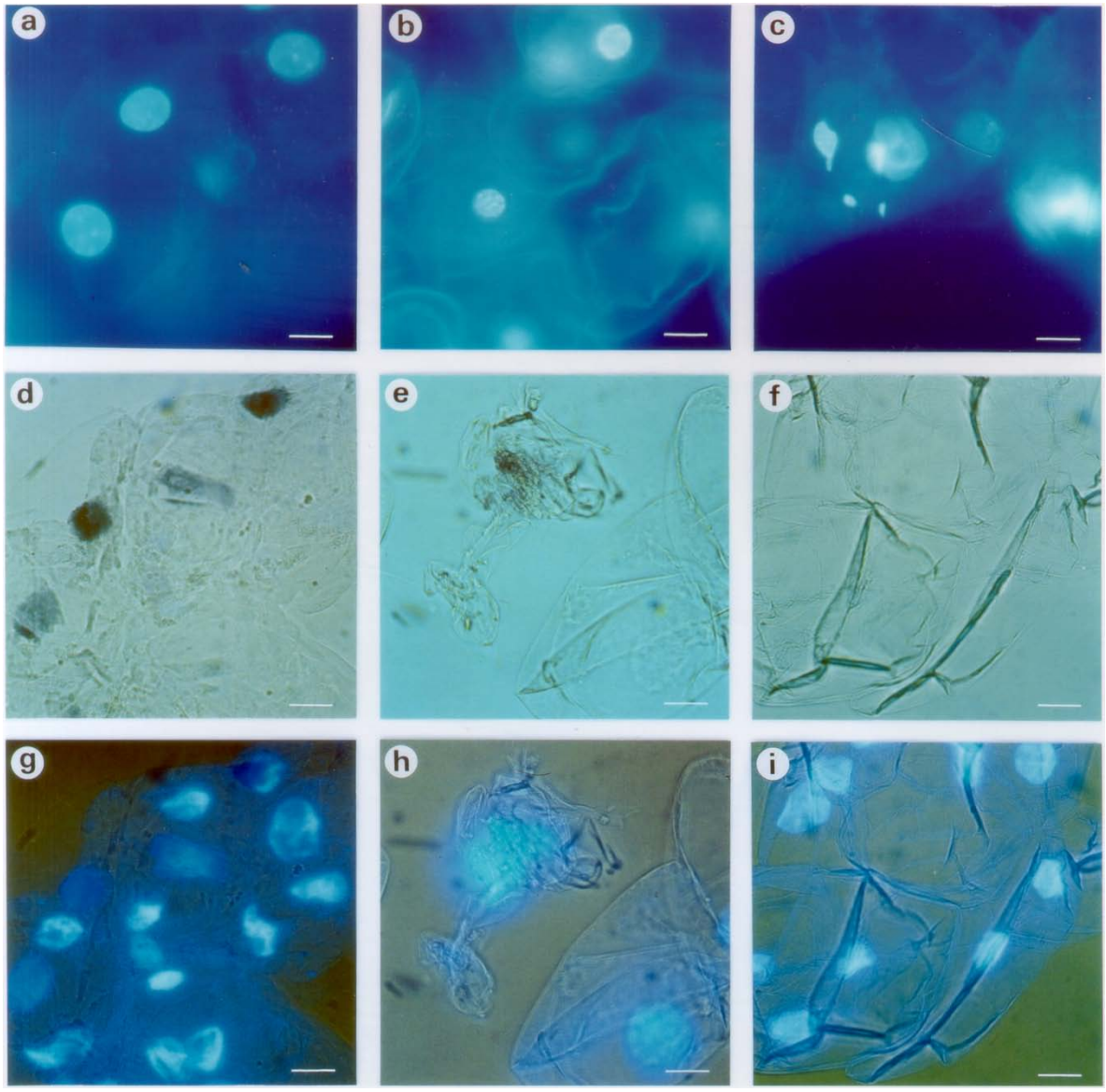


FIGURE 3.1. *Induction of cell death in tomato suspension cells. Cells were treated with 2 µM staurosporine (□), 5 µM camptothecin (∆) or 20 µM fumonisin B1 (x). Untreated control cells are denoted as (o). Cell death was determined by FDA staining. The standard error of at least three independent experiments using different batches of cells are indicated in the graphs.*

pared (Figure 3.2a and 3.2c). Camptothecin treatment consistently induced shrinkage of the nucleus and chromatin condensation, as visualised by the occurrence of intranuclear, punctuate structures in Hoechst-stained nuclei (Figure 3.2b). By contrast, in staurosporine-treated cells the above mentioned intranuclear punctuate structures were seldom observed. Instead, more often (in 2-5% of the cells), cells showed fragmented nuclei in which the formed bodies were in close association, or connected by DNA-containing threads, with the remaining nucleus (Figure 3.2c). Such fragmented nuclei were also observed in fumonisin

FIGURE 3.2. a-i*. Nuclear changes and DNA fragmentation in chemically-treated tomato suspension cells. The cells were treated with 5 µM camptothecin or with 2µM staurosporine for 48 h. Cells were analysed by light microscopy.* ***a-c****: Representative pictures from nuclear morphological studies. Fluorescence was used to visualise Hoechst 33258-stained DNA.* ***a****: Non-treated control cells;* ***b****: cells treated for 48 h with 5 µM camptothecin;* ***c****: cells treated for 48 h with 2 µM staurosporine.* ***d-i****: Assay for detection of DNA fragmentation in cells treated with 5 µM camptothecin for 48 h. Double labelled (TUNEL/Hoechst) cells are shown, using bright field illumination to detect DNA fragmentation (****d-f****) and fluorescence to detect nuclei labelled with Hoechst (****g-i****).* ***d, e****: Fragmentation of DNA in the nucleus, as detected by TUNEL assay, is visible as a purple precipitate.* ***g****: Due to the purple precipitate in TUNEL-stained cells, the Hoechst stain is less visible.* ***f, i****: TdT enzyme was omitted from the TUNEL reaction mixture. Bars = 25 µm (a-d, f, g, i) or 10 µm (e, h).*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B1-treated cells, but to a lesser extent (data not shown). Hence, these results show that chemical-induced cell death in tomato cells is accompanied by morphological changes of the nuclei that are characteristic for animal apoptotic cell death (Lazebnik et al. 1993).

Another specific feature of PCD is the cleavage of DNA at internucleosomal sites by DNA endonucleases. In order to detect DNA fragmentation in situ, fragmented DNA was end-labelled applying the TUNEL method. Treated cells were fixed after 48 hours of treatment and subjected to TUNEL. TUNEL-positive cells were found in cell cultures treated with camptothecin, staurosporine or fumonisin B1 (Figure 3.2d to 3.2i). The size, shape and location of the Hoechst-stained DNA in each of the TUNEL-positive cells corresponded with the location of the TUNEL staining. Some of the cells contained multiple TUNEL-positive, distinctly separated DNA-containing bodies (Figure 3.2e and 3.2h). When terminal deoxynucleotidyl transferase (TdT), the antibodies or the alkaline phosphatase substrate were omitted from the reaction mixture, no staining was observed (Figure 3.2f and 3.2i).

Because TUNEL may not unequivocally discriminate between internucleosomal DNA fragmentation associated with apoptosis and random DNA cleavage associated with necrosis, apoptotic DNA fragmentation have to be confirmed by other methods (McCabe et al. 1997; Loo and Rillema 1998). Therefore, DNA laddering patterns were visualised on agarose gels. DNA isolated from either camptothecin, staurosporine or fumonisin B1-treated cells revealed DNA ladders consisting of multiples of 180 bp when stained with ethidium bromide (Figure 3.3). This indicates that internucleosomal DNA cleavage occurs. Control cells did not show DNA fragmentation under these conditions (Figure 3.3, first lane). To show that cell death is not always accompanied with internucleosomal DNA cleavage, cells were treated with $FeSO_4$. $FeSO_4$ killed 90-100% of the cells, but a DNA smear was observed, rather than a DNA laddering pattern. Hence, these results show that DNA fragmentation is induced during chemical-induced cell death in tomato suspension cells. Together with the results of the studies on nuclear morphology, it is concluded that the chemical-induced cell death in tomato cells is apoptotic cell death.

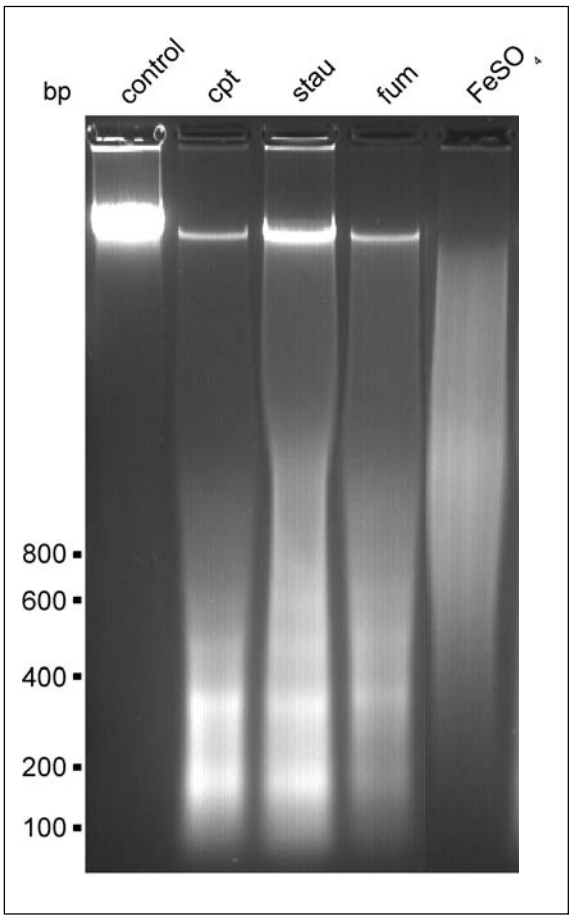

FIGURE 3.3. *Chemical-induced DNA fragmentation in suspension-cultured tomato cells. Suspension cells were treated with cell death inducing compounds for 48 hours. DNA was isolated, separated and stained with ethidium bromide. Cells were treated with 5 µM camptothecin (**cpt**), 2 µM staurosporine (**stau**), 20 µM fumonisin B1 (**fum**) or 100 mM $FeSO_4$.*

Protease inhibitors inhibit apoptosis in tomato suspension cells

Proteolysis is one of the main events during apoptosis in mammalian cells (Cohen 1997). To investigate the role of proteases in chemical-induced cell death in tomato, cysteine or serine protease inhibitors were applied simultaneously with camptothecin to tomato suspension cultures. After 24 hours of treatment the number of dead cells was determined. As shown in Figure 3.4a, the cysteine protease inhibitors N-ethylmaleimide and iodoacetamide inhibited cell death with 97% and 82%, respectively. The serine protease inhibitors Nα-p-tosyl-L-lysine chloromethylketone (TLCK) and 4- (2-aminoethyl) benzenesulfonylfluoride (AEBSF) inhibited camptothecin-induced cell death with 68% and 85%, respectively. TLCK was assayed for cell death inhibiting activity on either fumonisin B1 or staurosporine-induced cell death as well. TLCK inhibited both fumonisin B1

and staurosporine-induced cell death with about 67% and 73%, respectively. These results indicate that proteolysis is part of the chemical-induced cell death pathway in tomato suspension cells.

Caspase inhibitors inhibit apoptosis in tomato suspension cells

In mammalian cells caspases play a critical role in apoptosis. To determine whether caspase-like proteases are involved in camptothecin-induced apoptosis in tomato suspension cells, caspase-specific peptide inhibitors were applied together with camptothecin. The irreversible caspase-1 (ICE)-inhibitors Ac-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-CMK) and benzyloxycarbonyl-Asp-2,6-dichlorobenzoyloxymethylketone (Z-Asp-CH2-DCB), and the reversible inhibitors of caspase-1, Ac-Tyr-Val-Ala-L-aspartic acid aldehyde (Ac-YVAD-CHO), and of caspase-3, Ac-Asp-Glu-Val-L-aspartic acid aldehyde (Ac-DEVD-CHO), were tested for cell death-inhibiting activity. All caspase-specific peptide inhibitors caused about 85% inhibition after 24 hours (Figure 3.4b). The peptides acted optimal at a concentration of 100 nM, but Ac-YVAD-CHO acted at a concentration of 10 nM as well (data not shown). As a control for the specificity of the peptide sequences, two peptide-inhibitors with caspase-unrelated target preferences were tested. Both the peptides methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone (MeOSuc-AAPV-CMK) or H-Phe-CMK, which have the CMK-group in common with e.g. the caspase-specific peptide inhibitor Ac-YVAD-CMK, did not inhibit camptothecin-induced cell death (Figure 3.4b). This indicates that the inhibitory action of the caspase-specific peptide inhibitors on camptothecin-induced cell death in plant cells is determined by the peptide sequence and not by the CMK moiety. To determine whether the inhibitory action of caspase-inhibitors on programmed cell death is restricted to camptothecin-induced cell death, the inhibitors were assayed for their effect on either staurosporine or fumonisin B1-induced cell death. Addition of Ac-YVAD-CMK simultaneously with staurosporine or fumonisin B1 resulted in about 65% and 80% inhibition of cell death, respectively (Figure 3.4b).

So far, the effect of caspase-specific peptide inhibitors was assayed by FDA staining. In order to show that caspase-specific peptide inhibitors indeed inhibit programmed cell death in tomato cells, and not a necrotic type of cell death, the

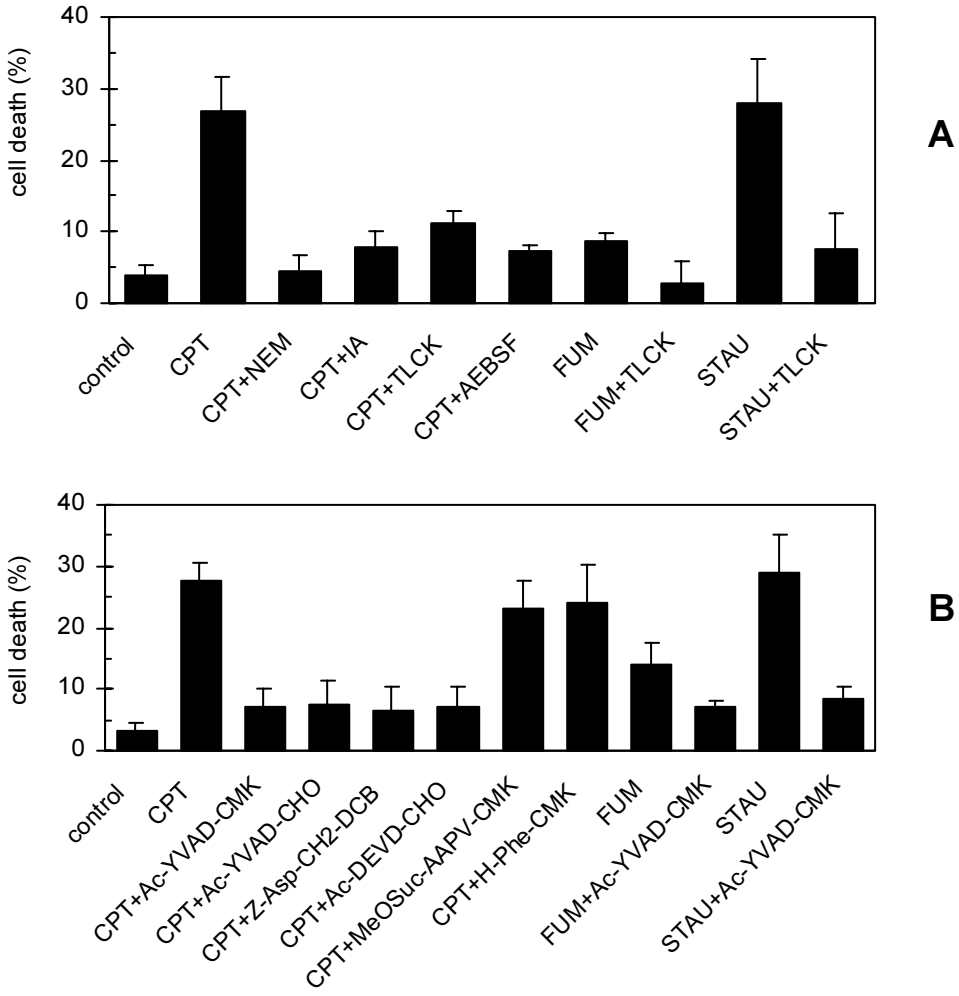

FIGURE 3.4. *Inhibition of chemical-induced cell death by protease inhibitors. Tomato suspension cells were treated with 5 µM camptothecin, 20 µM fumonisin B1 or 2 µM staurosporine, together with either protease inhibitors (**A**), or caspase-specific peptide inhibitors (**B**). After 24 hours the percentage of cell death was determined by FDA staining. The inhibitor concentration used was the lowest concentration that resulted in 50% inhibition or more. Data are the means; standard errors are of at least three independent experiments using different batches of cells. **A**: Treatments were with camptothecin (CPT), fumonisin B1 (FUM) or staurosporine (STAU), together with either 50 µM N-ethylmaleimide (NEM), 5 µM iodoacetamide (IA), 200 nM Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) or 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF). **B**: Treatments were with camptothecin (CPT), fumonisin B1 (FUM) or staurosporine (STAU), together with a caspase-specific peptide inhibitor, as indicated in the figure. The caspase-specific peptide inhibitors (Ac-YVAD-CMK, Ac-YVAD-CHO, Z-Asp-CH2-DCB, Ac-DEVD-CHO) and the control peptides (MeOSuc-AAPV-CMK, H-Phe-CMK) were applied at concentrations of 100 nM.*

effect of a caspase-specific peptide inhibitor on camptothecin-induced DNA fragmentation was studied. The fragmentation of DNA in camptothecin-treated cells was compared with DNA fragmentation in cells treated with camptothecin together with a caspase-specific peptide inhibitor (Ac-YVAD-CMK) or with a control peptide (MeOSuc-AAPV-CMK). As shown in Figure 3.5, cells treated with camptothecin together with Ac-YVAD-CMK reveal much less DNA fragmentation compared to cells treated with either camptothecin alone or together with the control peptide MeOSuc-AAPV-CMK. Taken together, these findings show that caspase-specific peptide inhibitors do interfere with the chemical-induced programmed cell death process in tomato cells. This suggests that the apoptotic cell death pathway induced by camptothecin, staurosporine as well as by fumonisin B1 involves caspase-like proteases.

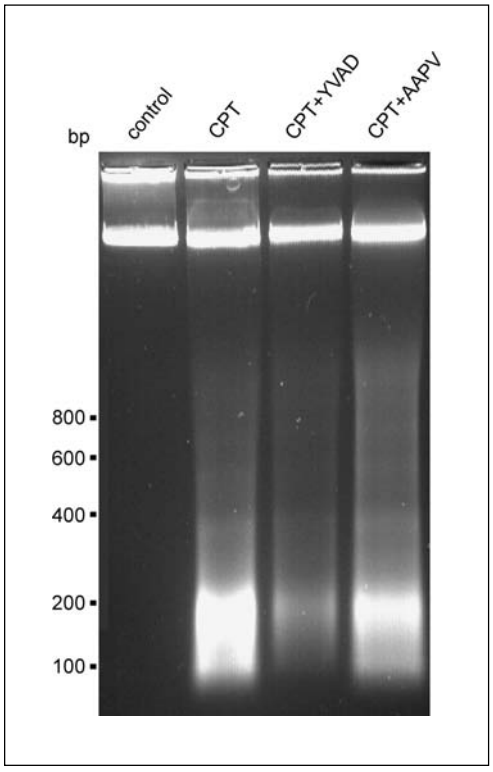

FIGURE 3.5. *Inhibition of DNA fragmentation by caspase-specific peptide inhibitors. Tomato suspension cells were treated with 5 µM camptothecin (CPT) alone, or with 5 µM camptothecin together with either 100 nM caspase-specific peptide inhibitor Ac-YVAD-CMK (CPT + YVAD) or 100 nM control peptide MeOSuc-AAPV-CMK (CPT + AAPV).*

Discussion

A model system was established to reproducibly induce apoptosis in tomato suspension cells. Treatment of tomato suspension cells with camptothecin, staurosporine or fumonisin B1 induces characteristic changes in nuclear morphology and internucleosomal DNA cleavage. These features are comparable with the morphological characteristics of apoptosis in mammalian cells (Martin et al. 1994). This indicates that camptothecin, staurosporine as well as fumonisin B1 induce apoptotic cell death in tomato suspension cells. Using specific enzyme inhibitors, the role of proteolysis in the apoptotic cell death pathway was investigated. Our results show the importance of proteolysis in plant apoptotic cell death and indicate that, apart from their regulatory role in animal cells, caspase-like proteases have a function in plant apoptosis.

In animal systems, many regulators and executors of apoptosis are known (Raff 1998). Classes of important physiological executors of apoptosis are the cysteinyl aspartate-specific proteases (caspases) and proteins of the BCL2-family. However, in contrast with animal systems, still not much is known about the regulators and executors of apoptosis in plants. Only a few plant genes homologous to PCD-related genes in animals have been found. Recently, a putative homologue of the human Bax-inhibitor-1 (BI-1) has been reported in *A. thaliana* (Xu and Reed 1998). The plant homologue of *DAD1* (Gallois et al. 1997; Orzaez and Granell 1997; Tanaka et al. 1997), which in animals defends against apoptotic cell death (Nakashima et al. 1993; Sugimoto et al. 1995), encodes for a subunit of the oligosaccharyltransferase (Kelleher and Gilmore 1997). However, the link between glycosylation and apoptosis, as well as the role of DAD1 in PCD, still remain to be established both in animals and plants. So far, no homologues of either caspase genes or genes of the *BCL2* family have been found in plants.

In mammalian systems, cysteinyl proteases are major executors of PCD (Cohen 1997), but also other classes of proteases have shown to be involved in PCD. A serine protease, Granzyme B, is able to trigger apoptosis by activating caspases (Yang et al. 1998), and the cathepsin D aspartic protease was shown to function as a positive mediator of apoptosis in HeLa cells (Deiss et al. 1996). Is proteolysis in plants as important as it is in mammalian apoptosis? In this work is shown that camptothecin-induced apoptosis can be inhibited by serine as well as cysteinyl protease inhibitors. These observations suggest that both serine

and cysteinyl proteases are involved in apoptosis in plants. Several observations support our finding that different classes of proteases play a role in plant PCD. Firstly, xylogenesis, the differentiation process of *Z. elegans* mesophyl cells into tracheary elements is associated with a 40-kD serine protease (Groover and Jones 1999). Also several cysteine and serine protease genes are induced during xylogenesis (Ye and Varner 1996). Secondly, phytepsin, a barley vacuolar aspartic protease, is highly expressed during autolysis of developing tracheary elements and sieve cells (Runeberg-Roos and Saarma 1998). Another example of an aspartic protease involved in PCD is the specific expression of an aspartic protease in barley nucellar cells during degeneration (Chen and Foolad 1997). Thirdly, in brinjal (*Solanum melongena*), the expression of a cysteine protease coincided with developmental events associated with PCD (Xu and Chye 1999) and cysteine proteases were shown to be involved in the regulation of oxidative stress-induced PCD in soybean cells (Solomon et al. 1999). Taken together, these examples illustrate that, in line with findings in mammalian systems, different classes of proteases are associated with PCD in plants.

In mammalian cells, both camptothecin and staurosporine-induced apoptosis can be suppressed by caspase-specific peptide inhibitors (Jacobsen et al. 1996; Kaufmann 1998). Our results show that camptothecin, staurosporine as well as fumonisin B1-induced apoptotic cell death can be suppressed with caspase-specific peptide inhibitors. These results suggest that camptothecin, staurosporine as well as fumonisin B1 activate similar pathways in plant cells as they do in animal cells. In camptothecin-treated tomato suspension cells, the caspase-specific peptide inhibitors act at concentrations of 10 to 100 nM. None of the tested protease inhibitors prevented camptothecin-induced apoptotic cell death in tomato suspension cells at similar low concentrations. Furthermore, in line with our findings is the observation that caspase-specific peptide inhibitors could abolish bacteria-induced plant PCD (Del Pozo and Lam 1998). This may indicate that, in analogy with mammalian systems, caspase-like proteases play a prominent role in PCD in plants. The active concentrations of 10 to 100 nM of the caspase-specific inhibitors Ac-YVAD-CHO and Ac-DEVD-CHO in tomato suspension cells correspond with active concentrations of the caspase-specific peptide inhibitor Ac-DEVD-CHO in mammalian cells. On the other hand, mammalian caspases are poorly inhibited by Ac-YVAD-CHO (Nicholson et al. 1995; Schlegel et al. 1996). This observation may suggest that the responsible plant enzymes

bear little resemblance to the known mammalian caspases. A biochemical approach will be required to establish which proteases are affected by caspase-specific peptide inhibitors in camptothecin-induced apoptotic cell death in tomato suspension cells.

Acknowledgements

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4

cloning and analysis of a defender against apoptotic cell death (dad1) homologue from tomato

Frank A. Hoeberichts and Ernst J. Woltering. Journal of Plant Physiology 158: 125-128 (2001)

Summary

A cDNA clone homologous to the human defender against apoptotic cell death (*DAD1*) gene, which is believed to be a conserved inhibitor of programmed cell death, was isolated from tomato (*L. esculentum* cv. Prisca). The 351 basepair open reading frame predicted a 116 amino acid protein sequence (LeDAD1) that showed high homology to other DAD1 proteins. Northern analysis revealed that *LeDAD1* was constitutively expressed during ripening of wildtype, *rin*, and *Nr* tomato fruit.

4.1. Introduction

Programmed cell death (PCD) plays an important role during the life of multicellular organisms, since it allows for the elimination of harmful and redundant cells (Raff 1998). In plants, PCD is important for normal development and survival, as it plays an essential role in, for example, xylogenesis, the hypersensitive response (HR), senescence, and embryogenesis (Lam et al. 1999b). However, relatively little is known about plant PCD pathways. There are several indications that plants employ a form of PCD similar to apoptosis, the most common form of PCD in animals that is defined by a distinct set of morphological and biochemical features (Steller 1995). The presence of genomic DNA fragmentation, a hallmark of apoptosis, has been reported in various plant systems (Wang et al. 1996b; Orzáez and Granell 1997; De Jong et al. 2000). Furthermore, key regulatory genes in animal apoptosis, such as caspases and members of the *BCL2* gene family, have been implicated in plant PCD as well (Del Pozo and Lam 1998; Lam et al. 1999a; De Jong et al. 2000).

The *DAD1* (defender against apoptotic cell death) gene was originally isolated during complementation studies on a mutant hamster cell line that undergoes apoptotic cell death when incubated at non-permissive temperature (Nakashima et al. 1993). A role for DAD1 as an apoptotic suppresser was suggested when a study of transgenic *C. elegans* showed that overexpression of *DAD1* is sufficient to prevent apoptosis during development of *C. elegans* embryos (Sugimoto et al. 1995). The cloning of *DAD1* homologues from other organisms, among them various plant species, and several complementation studies have demonstrated a substantial evolutionary and functional conservation (Sugimoto et al. 1995; Tanaka et al. 1997). In *A. thaliana*, *DAD1* was shown to be expressed in all organs tested, whereas transcript abundance is reduced during development of siliques (Gallois et al. 1997). *DAD1* transcript levels in pea decline during petal senescence, a process that involves PCD (Orzaez and Granell 1997). These data are in accordance with the suggested role of DAD1 as a PCD suppresser. However, *DAD1* expression in apple shows an induction of transcript levels during senescence of leaves, petals and fruit (Dong et al. 1998). Here we report the isolation of a *DAD1* homologue from tomato (*L. esculentum*), and determination of its expression during ripening of wildtype, *rin*, and *Nr* fruit.

4.2. Material and methods

Total RNA was extracted from mature tomato fruit as described (Chang et al. 1993). Total RNA samples were subjected to RT-PCR, using the Marathon cDNA Amplification kit (Clontech), according to the manufacturer's instructions. From the resulting cDNA a *DAD1* partial clone was amplified by PCR using two nested degenerated oligo nucleotides (5'-TYC THG CKG TKT GYY TIM GDA TWC AAG-3' and 5'-TYT WIC ICC DGA RMG RGC NTT TGC-3') in combination with two adapter primers (Clontech). For amplification KlenTaq LA Polymerase Mix (Clontech) was used under the following conditions: 1 min 94 °C followed by 35 cycles of 20 sec 94 °C, 30 sec 40 °C, and 50 sec 68 °C. A specific primer (5'-ATT GAT ATC TAG CCA AGA AAG TTC ATG ATC AC-3') designed using the partial clone was applied in subsequent 5' RACE reactions to obtain the full-length *DAD1* messenger. Fragments were cloned into pGEM-T Easy vector (Promega) and QIAfilter Plasmid Midiprep (Qiagen) purified plasmid DNA was used for sequencing. Sequence analysis was performed using the dideoxy chain termination method (AutoRead sequencing kit and ALF Express automated sequencer, AP Biotech). Sequence alignments were done using ClustalW software (European Bioinformatics Institute).

Genomic DNA was isolated from mature green tomato fruit. DNA was purified, digested and blotted according to Ausubel et al. (1995).

Northern blots were used to examine *LeDAD1* expression patterns in tomato pericarp tissue during ripening. Fruit from either the Pearson or the Ailsa Craig genotype were used for RNA extractions. The used *rin* mutant was in the Ailsa Craig background, whereas the *Nr* mutant was in the Pearson background. Electrophoresis, blotting and hybridisation of RNA were performed as described (Ausubel et al. 1995). For hybridisation, an RNA probe consisting of the complete *LeDAD1* coding region was used. Blots were exposed to phosphorus screens (Fuji) that were scanned using the STORM phosphor imager (Molecular Dynamics), and bands were quantified using Image-Quant software (Molecular Dynamics).

4.3. Results and discussion

A cDNA clone homologous to known *DAD1* genes was isolated from tomato (*L. esculentum* cv. Prisca) and named *LeDAD1* (*L. esculentum DAD1*). *LeDAD1* consists of 493 bp, excluding the poly(A) tail, and predicts a 351 bp open reading frame, encoding a 12.7 kDa protein of 116 aa long. The aa sequence of LeDAD1 aligned to various other DAD1 proteins is depicted in Figure 4.1. LeDAD1

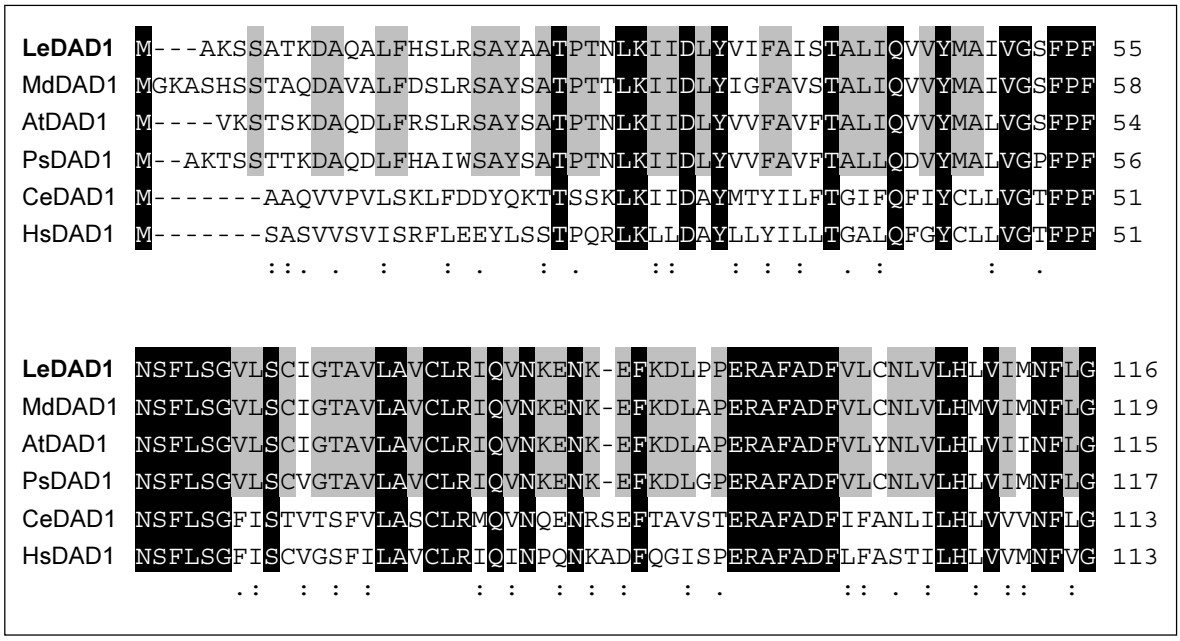

FIGURE 4.1. *Alignment of the predicted amino acid sequences from LeDAD1 and various other DAD1 proteins (Md: Malus domestica, accession number U68560; At: Arabidopsis thaliana, X95585; Ps: Pisum sativum, U79562; Ce: Caenorhabditis elegans, X89080; Hs: Homo sapiens, D15057). Residues that are highlighted in black are conserved in all sequences depicted. Residues highlighted in grey are conserved only in plant sequences. At the bottom of the sequence (**:**) indicates conserved amino acid substitutions, while (**.**) indicates semi-conserved substitutions. LeDAD1 sequence data were deposited in the GenBank database under accession number AJ250003. Sequences were aligned using ClustalX software (version 1.8).*

shares its highest homologies with *A. thaliana* (87% identical residues), apple (85%) and pea DAD1 (85%).

A *LeDAD1* cDNA derived RNA probe was hybridised to tomato genomic DNA digested with various restriction enzymes in order to estimate the number of *DAD1*-related sequences in the tomato genome. After using XbaI, BglII, EcoRI, and SphI, only one hybridising band was detected under low stringency washing conditions. Using SacI or BamHI resulted in the detection of two hybridising bands (data not shown). The *LeDAD1* cDNA clone does contain a BamHI restriction site, but does not contain a SacI site. However, PCR analysis of genomic tomato DNA indicates the *LeDAD1* gene contains at least one intron, possibly explaining the presence of a SacI restriction site (data not shown). So, tomato, in

contrast to *A. thaliana*, but similar to rice, presumably has only one *DAD1* gene present in its genome.

Fruit ripening is a highly ordered developmental event that might involve PCD, although evidence for this has not been presented to date. Our research is aimed at investigating the possibilities of modifying tomato post-harvest characteristics by controlling PCD. Therefore, *LeDAD1* expression in ripening tomato fruit was studied. Fruit from two different cultivars, ranging from mature green to fully ripened stages, were used to analyse gene expression. In addition, *LeDAD1* transcript levels were studied during fruit ripening of two ripening impaired tomato mutants, *rin* and *Nr*. Fruit of the *rin* (ripening inhibitor) mutant fail to soften, do not accumulate carotenoid pigments, and eventually "ripen" to a yellow colour in a non-climacteric fashion. The ethylene-insensitive *Nr* (never ripe) mutant is characterised by delayed and incomplete ripening: *Nr* fruit mature to an orange

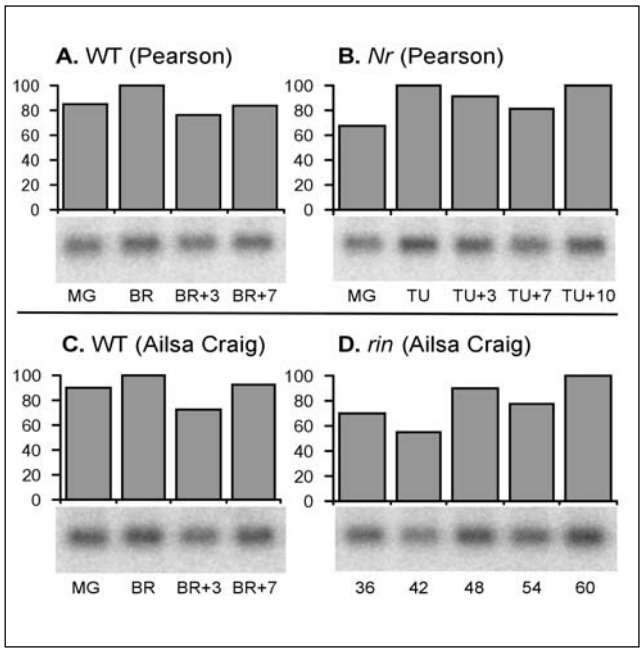

FIGURE 4.2. *Accumulation of LeDAD1 mRNA in wildtype (WT) Pearson (**A**), wildtype Ailsa Craig (**C**), Nr (**B**), and rin (**D**) tomato fruit during ripening. Wildtype tomato fruit were harvested at four stages: mature green (MG), breaker (BR), breaker +3 days (BR+3), and breaker +7 days (BR+7), the latter corresponding to fully red ripe fruit. Nr fruit were picked at five stages: MG, turning (TU), turning +3 days (TU+3), turning +7 days (TU+7), and turning +10 days (TU+10). Rin fruit were picked at various days post anthesis (dpa). A slight colour change was observed in these fruit at 54 dpa. Total RNA (10 µg) was extracted from pericarp tissue. Ribosomal bands were visualised on agarose to verify that equal amounts of RNA were loaded (data not shown). Bands were quantified and results (as % of maximum value) are depicted in the added graphs.*

colour and soften only moderately (Hobson and Grierson 1993). Rather than exhibiting a clear trend, the *LeDAD1* gene was constitutively expressed during ripening of wildtype tomato fruit (Figures 4.2A and 4.2C). In pericarp tissue from *rin* and *Nr* mutant fruit similar patterns of mRNA levels were detected (Figures 4.2B and 4.2D). The observed mRNA levels in both wildtype and mutant fruit suggest that LeDAD1 does not play a regulating role during ripening. The exact function of DAD1 has not yet been clearly established and is still subject to speculation. It has been found that DAD1 represents an essential subunit of the mammalian oligosaccharyltransferase (OST), an enzyme complex functioning in N-linked glycosylation (Kelleher and Gilmore, 1997). Recently, Makishima *et al.* (2000) showed that DAD1 interacts with MCL1 (a member of the BCL2 protein family), providing a new perspective on the putative role of DAD1 in apoptosis. Therefore, although it can not be excluded that *LeDAD1* transcript levels are differentially regulated during later stages of (over) ripening, one could conclude that tomato fruit ripening does not involve DAD1-regulated PCD.

4.4. Acknowledgements

The authors thank Bingye Xue (Shandong Institute of Pomology, P.R. China) for technical assistance and Cornelius Barry (University of Nottingham, U.K.) for kindly providing the Northern blots. This work was supported by grants from c-DLO and the European Union (FAIR CT95-0225).

5

changes in gene expression during programmed cell death in tomato cell suspensions

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Plant Molecular Biology 45: 641-654 (2001)

Summary

To identify genes involved in plant programmed cell death (PCD), changes in gene expression during PCD in a model system of suspension-cultured tomato cells were studied. Tomato homologues of *DAD1* and *HSR203*, two genes that have been implicated in PCD elsewhere, were isolated. During camptothecin-induced PCD tomato *DAD1* mRNA levels approximately halve, whereas tomato *HSR203* mRNA levels increase 5-fold. A differential display approach was used to identify novel genes that show changes in expression levels during camptothecin-induced PCD. This resulted in isolation of two up-regulated *(CTU1 and CTU2)* and four down-regulated *(CTD1, CTD2, CTD4, and CTD5)* cDNA clones. *CTU1* shows high homology to various glutathione S-transferases, whereas *CTU2* shows homology to human *PIRIN*. *CTD1* is highly similar to early-auxin-responsive *Aux/IAA* genes. *CTD2* corresponds to the tomato *RSI-1* gene, *CTD4* is an unknown clone, and *CTD5* shows limited homology with a proline-rich protein from maize. Addition of the calcium channel blocker $LaCl_3$ prevented camptothecin-induced cell death. The possible role of the various predicted gene products in plant PCD is discussed.

5.1. Introduction

Programmed cell death (PCD) is a process aimed at eliminating unnecessary or harmful cells during growth and development of multicellular organisms. It is now generally believed that a complex machinery of interconnected signal transduction pathways underlies this capability of cells to sacrifice themselves in response to changing cellular and environmental stimuli (Raff 1998). In animals, a large number of gene products involved in regulation and execution of the cell death process have already been identified. For example, a specific group of cysteine proteases named caspases are capable of activating and executing PCD, whereas members of the BCL2 protein family can either stimulate or suppress PCD. Many of these proteins are highly conserved among vertebrates and invertebrates (Hale et al. 1996; Wolf and Green 1999). In animals, most PCD is apoptosis, a specific form of PCD defined by a distinct set of morphological and biochemical features (Steller 1995).

As in animals, PCD is indispensable for normal development and survival of plants. It plays an essential role in, for example, xylogenesis, the hypersensitive response (HR), aerenchyma formation, senescence, and embryogenesis (Pennel and Lamb 1997; Richberg et al. 1998). In spite of this importance, detailed knowledge on how plant cells commit suicide is still, to a large extent, lacking. Various reports show that some morphological and biochemical characteristics of apoptosis (*e.g.* the occurrence of apoptotic nuclei and DNA fragmentation) are shared between animals and plants (Ryerson and Heath 1996; Wang et al. 1996b; Wang et al. 1996c; Orzáez and Granell 1997; Yen and Yang 1998; De Jong et al. 2000). Furthermore, caspase-specific peptide inhibitors have been shown to abolish bacteria-induced PCD in plants, and caspase-like proteolytic activity has been found in tobacco mosaic virus (TMV) infected plant cells undergoing HR-related PCD (Del Pozo and Lam 1998). The accumulating evidence for the capability of BCL2-like proteins from animals to function in plants (Lam et al. 1999a) is supported by the isolation of two homologues of human Bax Inhibitor-1 from *A. thaliana* (Sanchez 2000; Bax is a pro-apoptotic member of the BCL2 family). These data indicate that signalling pathways of cell death are conserved between animal and plant kingdoms.

To increase our understanding of plant PCD and its mechanisms of regulation, we are currently using suspension-cultured tomato (*L. esculentum* Mill.) cells

as a model system. Treatment of these suspension cultures with camptothecin, an inhibitor of topoisomerase I that induces apoptosis in a variety of animal systems (Kaufmann 1998), reproducibly causes cell death. This cell death is accompanied by typical apoptosis-related changes in nuclear morphology (*e.g.* chromatin condensation and segregation of DNA containing bodies) and internucleosomal DNA cleavage. Furthermore, application of specific caspase inhibitors effectively suppresses cell death in camptothecin-treated tomato suspension cells (De Jong et al. 2000).

An initial step towards identifying components of the PCD machinery in plants consists of assessing changes in gene expression in these suspension-cultured tomato cells undergoing camptothecin-induced PCD. For this purpose, a dual strategy was pursued. Firstly, expression patterns of two genes that have been implicated in PCD elsewhere were examined. Secondly, a differential screening was performed and PCD-related changes in gene expression of isolated novel clones were studied.

The first putative PCD gene studied is *DAD1* (defender against apoptotic cell death). This gene was originally isolated during complementation studies on a temperature-sensitive mutant hamster cell line that undergoes PCD when incubated at non-permissive temperature. The mutant DAD1 protein, which contains a single aa substitution, rapidly degrades upon shift to this non-permissive temperature. This degradation precedes the onset of apoptosis, suggesting that it may be the event that triggers PCD (Nakashima et al. 1993). Subsequent cloning of *DAD1* homologues from human (Nakashima et al. 1993), mouse (Apte et al. 1995), *A. thaliana* (Gallois et al. 1997), pea (Orzaez and Granell 1997), and many other species, has demonstrated a substantial evolutionary conservation. Moreover, the human, *A. thaliana*, rice, and *C. elegans* DAD1 protein can all efficiently rescue the mutant hamster cells from PCD (Sugimoto et al. 1995; Gallois et al. 1997; Tanaka et al. 1997). A role for *C. elegans* DAD1 as a PCD suppressor was suggested by studies of transgenic nematodes overexpressing *DAD1*. Expression of either human or *C. elegans DAD1* under control of a *C. elegans* heat-shock-inducible promoter is sufficient to suppress apoptosis during development of *C. elegans* embryos (Sugimoto et al. 1995). In plants, expression of *DAD1* has been studied during (natural) senescence of pea petals, a process that involves PCD. Upon pea flower anthesis and senescence, expression decreases dramatically, whereas petals of flowers in which senescence is de-

layed maintain high levels of *DAD1* mRNA transcripts (Orzaez and Granell 1997). These data support the suggested role of DAD1 as an inhibitor of PCD.

The second gene studied is *HSR203J* from tobacco. This gene is rapidly induced in cells activated to undergo HR during incompatible interactions between tobacco and various bacterial pathogens, but it does not respond to, for example, wounding (Pontier et al. 1994). A homologous gene from tomato named *LeHSR203* exhibits similar characteristics. It has been suggested that *HSR203(J)* might be an active participant in PCD during HR (Pontier et al. 1998).

For the second strategy, aimed at isolating novel clones and studying their PCD-related changes in gene expression, we made use of the differential display technique. This method, first described by Liang and Pardee (1992), has been employed successfully for isolating differentially expressed genes from a wide variety of species, involved in a wide variety of processes (for example tomato fruit ripening: Oh et al. 1995; Zegzouti et al. 1999).

In this paper, we describe gene expression patterns of tomato *DAD1 (LeDAD1)*, *HSR203 (LeHSR203)* and six other tomato cDNA clones during camptothecin-induced PCD in suspension-cultured tomato cells. mRNA levels of *LeDAD1*, a new member of the growing family of highly homologous *DAD1* genes, decrease in response to the camptothecin treatment, whereas *LeHSR203* mRNA levels rise. Transcript levels of four out of the six tomato cDNA clones, all isolated by means of differential display, are downregulated during camptothecin-induced cell death. Transcript levels of the remaining two cDNA clones are upregulated. Therefore, these clones were designated *CTD* (for CampTothecin Downregulated) and *CTU* (for CampTothecin Upregulated), respectively. Where possible, potential roles of the (putative) gene products in camptothecin-induced PCD of suspension-cultured tomato cells are discussed.

5.2. Material and Methods

Cell culture

Tomato (*L. esculentum* Mill.) cell suspension cultures were grown at 28 °C on Murashige-Skoog type liquid medium plus vitamins supplemented with 5 µM α-naphthalene acetic acid and 1 µM 6-benzyladenine. Cells were subcultured every 7 days by making a 1 to 4 dilution in 25 ml of fresh medium in 100 ml flasks, and used for experiments 5

days after subculturing. The various experiments were carried out either in 30 ml screw-capped bottles containing 5 ml of suspension culture or in 100 ml capped flasks containing 30 ml of suspension culture. The cell death inducer camptothecin was applied in a final concentration of 5 µM. As a stock solution 5 mM camptothecin dissolved in DMSO was used. DMSO alone had no effect on cell viability. Where applicable, $LaCl_3$ from a 0.1 M stock solution was added to a final concentration of 0.1 mM. Cell viability was determined by staining with 0.002% FDA.

DNA laddering

For extraction of genomic DNA from tomato suspension cells several grams of frozen cells were ground to powder in liquid nitrogen. The powder was mixed with 15 ml of hot (65 °C) extraction buffer (0.1M Tris pH 7.5; 50mM EDTA; 500mM NaCl; 10mM ß-mercaptoethanol) and 1 ml of 20% SDS, and mixed thoroughly. The mixture was incubated at 65 °C for 20 min. Then, 5 ml of 5M KAc were added, samples were mixed, kept on ice for 30 min, and spinned down for 30 min. The supernatant was filtered through a paper tissue and collected in a clean tube, mixed with one volume of isopropanol, and immediately spinned down for 15 min at 4 °C. The pellet was briefly dried and dissolved in 300 µl CTAB buffer [0.2M Tris pH 7.5; 50mM EDTA, 2M NaCl, 2% cetyl-N,N,N triethyl ammonium bromide (CTAB)]. Samples were incubated for 15 min at 65 °C and subsequently extracted with one volume of chloroform:isoamyl alcohol (24:1, v/v). The waterphase was precipitated with one volume of isopropanol, followed by centrifugation. Finally, the pellet was dissolved in 10 mM Tris pH 8.0, 1mM EDTA and 0.1 µg/µl RNase. Samples of 5 µg each were run on a 1.5% agarose gel and blotted to a nylon membrane. The DNA on the blot was hybridised with an [α^{32}P] dCTP random oligonucleotide priming-labelled probe composed of Sau3A digested genomic tomato DNA. Southern blotting and hybridisation (65 °C, 14 h) were performed as previously described (Ausubel et al. 1995). The blot was exposed to X-ray film (Kodak) for 24 h.

RNA isolation

Suspension cells were first harvested using a sieve and subsequently frozen in liquid nitrogen. Tomato fruits were kept on the bench at least 3 h after harvest, then fruits were cut and pericarp tissue was frozen in liquid nitrogen immediately. After that, RNA was extracted according to Chang et al. (1993).

Cloning of LeDAD1 and LeHSR203

RNA samples of mature green tomato fruit were subjected to RT-PCR, using the Marathon cDNA Amplification kit (Clontech), according to the manufacturer's instructions. From the resulting Marathon cDNA a *DAD1* partial clone was amplified by PCR using

two nested degenerated oligos (TYC THG CKG TKT GYY TIM GDA TWC AAG and TYT WIC ICC DGA RMG RGC NTT TGC) in combination with two adaptor primers (Clontech). For amplification KlenTaq LA Polymerase Mix (Clontech) was used under the following conditions: 1 min 94 °C followed by 35 cycles of 20 s 94 °C, 30 s 40 °C, and 50 s 68 °C. A specific primer (ATT GAT ATC TAG CCA AGA AAG TTC ATG ATC AC) designed using the partial clone was applied in subsequent 5' RACE reactions to obtain the full-length *DAD1* messenger. Fragments were cloned into pGEM-T Easy vector (Promega) and sequenced. *LeHSR203* was amplified from Marathon cDNA (Clontech) by PCR using one degenerate (ATG GTT CAT SAH AAG CAA GT) and one specific primer (TTA ATG CTT GTT GAT GAA CTC TGC). Primers were designed comparing a *LeHSR203* nucleotide sequence available from GenBank (accession number AB022689) and the protein sequence described by Pontier et al. (1998). PCR reactions were performed using *Taq* DNA polymerase (AP Biotech) under the following conditions: 1 min at 94 °C, followed by 36 cycles of 4 s 94 °C, 6 s 51 °C, and 35 s 72 °C. The PCR fragment was cloned into pGEM-T Easy vector (Promega) and subsequent sequence analysis revealed that indeed *LeHSR203* had been cloned.

Differential display

For first strand cDNA synthesis poly(A)$^+$ RNA was purified from total RNA samples using Dynabeads (Dynal). Two micrograms of poly(A)$^+$ RNA were heated at 72 °C for 10 min, cooled on ice for 5 min and then mixed with 2 µl 25mM dNTPs, 4 µl 0.1M DTT, 2 µl primer XSCVN (GAC TCG AGT CGA CAT CGA GCT_{17} VN), 8 µl of 5X first strand synthesis buffer (Gibco BRL), 1 µl RNAguard (AP Biotech), 12 µl of water and 1 µl of reverse transcriptase (Gibco BRL). The resulting solution was incubated for 2 h at 42 °C. After adding 2 µl of RNase H (AP Biotech) the mix was incubated for 20 min at 37 °C. The reaction was terminated by heating the sample at 70 °C for 15 min. DD-PCR amplification of cDNAs was performed following a modified protocol from Linskens et al. (1995). Two µl of cDNA were amplified using XSC (GAC TCG AGT CGA CAT CGA GC) as a reverse primer, and arbitrary 23 bp oligonucleotides as forward primers. Reaction mixtures were prepared using 1 µl of 0.1 M dNTPs, 2 µl of 10X KT buffer (150 mM KOAc, 35 mM $Mg(OAc)_2$, 750 µg/ml BSA, 400 mM tricine-KOH pH 9.2), 0.5 µl *Taq* DNA polymerase (AP Biotech), 0.1 µl of [α^{32}P] dCTP, 0.5 µl of 10 µM XSC primer, 2 µl of 10 µM arbitrary primers and 12 µl of H_2O. PCR conditions were: 2 min at 94 °C; 2 min at 45 °C; 5 min at 72 °C; 5 cycles of (15 s at 94 °C; 30 s at 52 °C; 50 s at 72 °C); 35 cycles of (15 s at 94 °C; 30 s at 56 °C; 50 s at 72 °C); 5 min at 72 °C. Reaction products were resolved in 6% polyacrylamide, 7 M urea sequencing gels. Gels were dried without fixation and exposed to X-ray films (Kodak). Bands of interest were cut from gel and gel pieces adjacent to the positive bands but corresponding to non-induced lanes were also

cut and used as controls for contamination. DNA from both positive and control gel pieces was isolated as described (Linskens et al. 1995) and pre-amplified by PCR. Conditions were similar to those used for DD PCR with the exception that only 10 cycles were performed and XSC primer was Cy5 Indocarbocyanin labelled. In order to eliminate false positives due to re-amplification of co-migrating bands, products from both positive and control pre-amplification reactions were electrophoresed under single strand conformational polymorphism (SSCP) conditions using a GenGelExcel 12.5 kit (AP Biotech). Cy5 labelled products were detected by phosphor imaging (Storm, Molecular Dynamics) and bands were excised and eluted. A final re-amplification of the recovered cDNAs was performed using similar conditions as for DD PCR. Products were cloned using the pGEM-T Easy vector system (Promega).

Northern blots
Electrophoresis, blotting, and hybridisation of RNA were performed as described (Ausubel et al. 1995). For hybridisation *LeDAD1* and *LeHSR203*, RNA probes consisting of the complete coding regions were used. For hybridisation of the isolated DD clones, whole DNA fragments were labelled. For each sample, 16 µg of total RNA was used. Exposure time of the blots varied from 6 to 24 h, with the exception of the *CTU2* blot, which was exposed for 5 days. All blots were exposed to phosphorus screens (Fuji) that were scanned using the STORM phosphor imager (Molecular Dynamics), and bands were quantified using ImageQuant software (Molecular Dynamics).

DNA sequencing
Template DNA was isolated using the QIAfilter Plasmid Midiprep kit or QIAprep Spin Miniprep kit (Qiagen). Nucleotide sequence analysis was performed using the dideoxy chain termination method (AutoRead sequencing kit and ALF Express automated sequencer, AP Biotech).

5.3. Results

Camptothecin-induced PCD

The effect of 5 µM camptothecin on cell viability of suspension cultured tomato cells is depicted in Figure 5.1. The first sample was taken just before adding

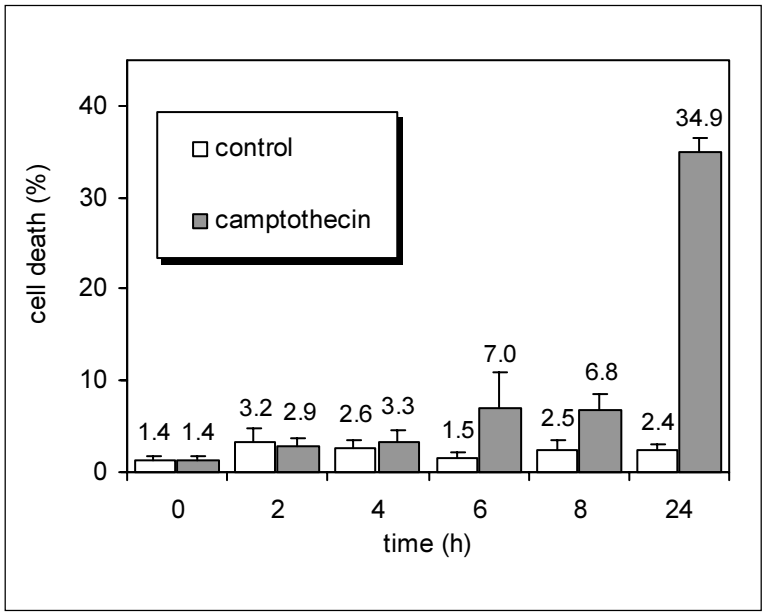


FIGURE 5.1. *Camptothecin-induced cell death in suspension-cultured tomato cells. Cells were either not treated (control) or treated with 5 µM camptothecin. Numeric values and error bars displayed in the graph result from a typical series of 3 experiments. The numbers of dead cells were determined by FDA staining and are expressed as percentages of the total amount of cells counted.*

camptothecin (0 h). Subsequent samples of control (non-treated) cells and camptothecin-treated cells were taken after 2, 4, 6, 8, and 24 h of further incubation. An increase in cell death was first detected after 6 to 8 h of camptothecin treatment. After 24 h of treatment, cell death had increased to 35%.

A characteristic feature of apoptosis, a specific form of PCD, is cleavage of chromosomal DNA at internucleosomal sites by specific DNA endonucleases. When run on an agarose gel and blotted, genomic DNA isolated from camptothecin-treated cells showed a ladder of DNA fragments that differed by around 180 bp. This DNA laddering was visible after 24 and 48 h of camptothecin treatment, but could not be detected in DNA samples taken from control cells (Figure 5.2).

Cloning and sequence analysis of tomato DAD1

A cDNA clone homologous to known *DAD1* genes was isolated from tomato (*L. esculentum* cv. Prisca) and named *LeDAD1* (for *L. esculentum DAD1*). *LeDAD1* consists of 493 bp, excluding the poly(A) tail, and predicts a 351 bp open reading frame (ORF), encoding a 12.7 kDa protein of 116 aa's long, with a calculated pI of 8.5. The aa sequence of LeDAD1 was compared to various other plant and

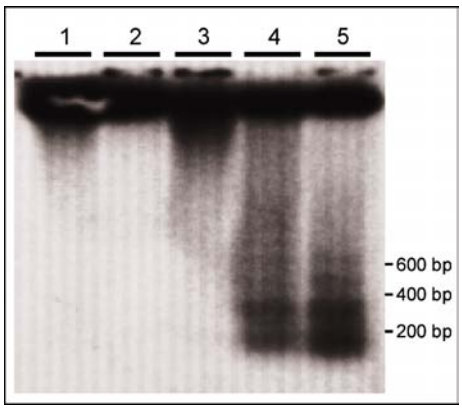


FIGURE 5.2. *Degradation of genomic DNA into nucleosomal fragments of distinct sizes (DNA laddering) visualised by southern blotting. Lanes 1, 2, and 3 correspond to DNA extracted from untreated cells after 0, 24, and 48 h respectively. DNA fragmentation becomes visible in cells treated with 5 µM camptothecin for 24 and 48 h (lanes 4 and 5). Fragment sizes are indicated on the right.*

animal DAD1 proteins. The results, shown in Table 5.1, demonstrate that Le-DAD1 shares highest homology (87% identical residues) with *A. thaliana* DAD1. There is around 45% identity with various animal DAD1 sequences, whereas Le-DAD1 and yeast OST2 are only 26% identical.

	Le	**At**	**Os**	**Md**	**Ps**	**Ce**	**Hs**	**Mm**	**Sc**	
Le		87	86	85	85	46	44	44	26	
At	93		82	84	87	47	46	46	26	
Os	92	89		78	78	45	45	45	27	
Md	90	89	84		80	45	44	44	27	
Ps	93	92	87	87		47	46	46	27	
Ce	64	68	66	64	63		61	61	31	
Hs	68	72	71	67	67	84		100	32	
Mm	68	72	71	67	67	84	100		32	
Sc	51	51	51	51	49	49	54	54		
% similarity										

% identity

TABLE 5.1. *Degree of identity and similarity of various DAD1 proteins, calculated using ClustalX software (version 1.8). The sequences used in this comparison are from: Lycopersicon esculentum (accession number AJ250003; Le), Arabidopsis thaliana (X95585; At), Oryza sativa (D89727; Os), Malus domestica (U68560; Md), Pisum sativum (U79562; Ps), Caenorhabditis elegans (X89080; Ce), Homo sapiens (D15057; Hs), Mus musculus (U83628; Mm), and Saccharomyces cerevisiae (U32307; Sc).*

Isolation of cDNA clones that show changes in expression during PCD

The RNA used in the differential display approach was isolated from camptothecin-treated and non-treated cells at two timepoints: 0 and 24 h after adding camptothecin. As yet, the differential display approach resulted in 6 cDNA clones, varying in length from 186 to 496 bp, that show either an increase or decrease in gene expression during camptothecin treatment. After the changes in mRNA levels had been confirmed using Northern blotting, two up-regulated clones were named *CTU1* and *CTU2* (for CampTothecin Up-regulated), and four down-regulated clones were named *CTD1*, *CTD2*, *CTD4*, and *CTD5* (for CampTothecin Down-regulated).

Sequence analysis of CTU and CTD clones

The first isolated clone described here is *CTU1*, a 496 bp long fragment that contains the 3' prime end of a previously undescribed tomato cDNA. The ORF within the fragment encodes an 111 aa long C-terminus. BLAST searches exposed high sequence similarity with tobacco 103-like genes and the potato *PRP1* gene that encode glutathione S-transferases (GSTs; Van der Zaal et al. 1987; Taylor et al. 1990). CTU1 shares 63% identity and 78% similarity on the aa level with tobacco 103-like sequence pCNT110 (accession number X56264). Homology with the potato PRP1 protein sequence (accession number J03679) amounts to 55% identity and 70% similarity. No significant homology could be established between *CTU1* and *Lepar*, the only described putative GST encoding partial cDNA clone from tomato. *Lepar* is believed to be a tomato homologue of a member of the tobacco *Par* gene family (Mito and Bennett 1995).

The *CTU2* cDNA fragment is 300 bp long. A BLAST search using the translated sequence revealed that the deduced aa sequence displays some homology to the human protein PIRIN. Human PIRIN is a nuclear protein capable of interaction with nuclear factor 1 (NF1; Wendler et al. 1997; accession number CAA69194)

The down-regulated clone *CTD1*, comprises 441 bp and is most similar to a nucleotide sequence from soybean *(Glycine max)* encoding the GH1 protein (Guilfoyle et al. 1993; accession number AF016633). *GH1* is a member of the

Aux/IAA gene family, that encode primary auxin-responsive gene products (Abel and Theologis 1996). The ORF corresponding to *CTD1* encodes 37 aa that are highly homologous (89% identity) to the C-terminal end of GH1. Other striking similarities (up to 81% identity) include several other protein members of the Aux/IAA family: *A. thaliana* IAA16 and IAA17/AXR3 (Kim et al. 1997; accession numbers U49072 and U49073), and tobacco IAA4.1 and IAA28 (Dargeviciute et al. 1998; accession numbers AF123509 and AF123508).

The cDNA fragment named *CTD2* consists of 231 bp. A BLAST search revealed that this fragment is 100% homologous to tomato *RSI-1*, a gene that is activated during auxin induced lateral root initiation in tomato seedlings (Taylor and Scheuring 1994).

The *CTD4* cDNA fragment is 186 bp long, and BLAST searches for homologous nucleotide or protein sequences did not yield any significant results.

The short 17 aa stretch encoded for by the 5' end of clone *CTD5* (300 bp) shares 65% (11 out of 17 aa) identity with the C-terminal end of an embryo-specific proline-rich protein from maize (Jose-Estanyol et al. 1992; accession number S44189). Similar homology was found with a 17 aa long stretch that appears twice in the ORF of a low temperature-regulated proline-rich protein from wheat (accession number U73214). The significance of these homologies remains unclear.

An overview of the isolated tomato CTU and CTD clones, including their GenBank accession numbers, is shown in Table 5.2.

mRNA levels of LeDAD1 and LeHSR203 during camptothecin-induced PCD

The levels of *LeDAD1* and *LeHSR203* mRNA transcripts were studied during camptothecin-induced PCD in tomato cell suspensions using Northern blotting. Samples used for extracting RNA were taken after 0, 1, 6, and 24 h of camptothecin treatment. Six hours after adding camptothecin to the cell cultures, *LeDAD1* mRNA quantities had decreased to 50% of the level in non-treated control cells. After 24 h, the decline was still around 50% (Figure 5.3A). *LeHSR203* mRNA levels on the other hand, rose during camptothecin-induced PCD. After adding camptothecin, mRNA levels remained relatively constant after 1 and 6 h, but after 24 h a 5-fold increase was detected (Figure 5.3B).

clone	bp	accession number clone	homologous gene	organism	accession number homologue	reference	gene product	$LaCl_3$ effect
CTU1	496	AJ250001	*pCNT110* *PRP1/GST1*	tobacco potato	X56264 J03679	Van der Zaal 1987 Taylor 1990	glutathione *S*-transferase	-
CTU2	304	AJ251752	*PIRIN*	human	CAA69194	Wendler 1997	nuclear factor	+
CTD1	441	AJ249996	*GH1*	soybean	AF016633	Guilfoyle 1993	GH1 protein	+
CTD2	231	AJ249997	*RSI1*	tomato	L22188	Taylor 1994	RSI1 protein	+
CTD4	186	AJ249999	-					+ / -
CTD5	300	AJ250000	*PRP* *WCOR518*	maize wheat	S44189 U73214	Jose-Estanyol 1992 -	proline-rich protein	-

TABLE 5.2. *Overview of CTU (for CampTothecin Up-regulated) and CTD (for Camp-Tothecin Down-regulated) clones isolated by means of differential display. An effect of $LaCl_3$ on camptothecin-induced gene expression is either present (+), unclear (+/-), or absent (-)*

mRNA levels of CTU and CTD clones during camptothecin-induced PCD

For each isolated cDNA clone, levels of mRNA extracted from non-treated suspension cultured cells were compared to transcripts levels in camptothecin-treated cells undergoing PCD. mRNA levels were determined 0, 1, 6, and 24 h after adding camptothecin.

CTU1 (the putative GST) showed low mRNA levels in control cells, but upon camptothecin treatment, levels rose rapidly. This was first observed after 1 hour and amounted to a 15-fold increase after 24 h, as shown in Figure 5.3C. Expression of *CTU2* (PIRIN) was detected only after 24 h of camptothecin treatment. Even then mRNA levels still appeared to be very low, since blots were exposed for 5 days in order to detect the signal (Figure 5.3D).

CTD1 (the putative *Aux/IAA* gene family member) showed a constant high level of expression in control cells. Camptothecin treatment induced a decrease first visible after 6 h and resulted in almost complete disappearance of the signal

after 24 h (Figure 5.3E). A very similar pattern of expression was observed with clone *CTD2* (tomato *RSI-1*): a 3-fold down-regulation first visible after 6 h, whereas after 24 h *CTD2* mRNA could barely be detected (Figure 5.3F). While CTD4 mRNA levels rose slightly in the control experiment, transcript levels in cells undergoing PCD decreased analogous to *CTD1* and *CTD*2. Although at 6 h the decrease was only around 30%, after 24 h *CTD4* mRNA could no longer be detected (Figure 5.3G). The level of *CTD5* (a putative proline-rich protein) mRNA was constantly high in control cells. The effect of camptothecin was first visible after 6 h, as transcript levels declined to around 50%. *CTD5* mRNA could no longer be detected after 24 h (Figure 5.3H).

Transcript levels of a gene encoding the tomato 60S ribosomal protein L17 did not show any strong changes, as can be seen in Figure 5.3I.

Effect of $LaCl_3$ on camptothecin-induced PCD

Lanthanum chloride ($LaCl_3$) is a plasma membrane Ca^{2+} channel blocker that inhibits Ca^{2+} fluxes, believed to be required for proper signal transduction during cell death (Richberg et al. 1998). The essential role of Ca^{2+} fluxes during early signalling in plant defence has been well documented (Grant and Mansfield 1999). $LaCl_3$ reduces camptothecin-induced cell death in our tomato cell suspensions to 5 to 10% after 24 hours (data not shown). Therefore, mRNA levels of all clones in cells that were simultaneously treated with camptothecin (5 µM) and $LaCl_3$ (0.1 mM) were compared to mRNA levels in untreated controls and camptothecin-treated samples.

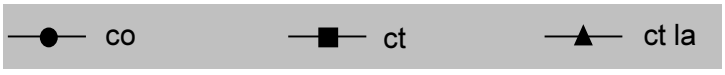
A small effect of $LaCl_3$ on the camptothecin-induced decrease in *LeDAD1* mRNA levels was visible after 6 and 24 hours, but this result might not be significant (Figure 5.3A). The effect on camptothecin-induced expression of *LeHSR203* on the other hand, was rather drastic. The strong induction of *LeHSR203* transcript levels visible after 24 h of camptothecin treatment had changed into a slight decrease when camptothecin and $LaCl_3$ were added concurrently, as can be seen in Figure 5.3B.

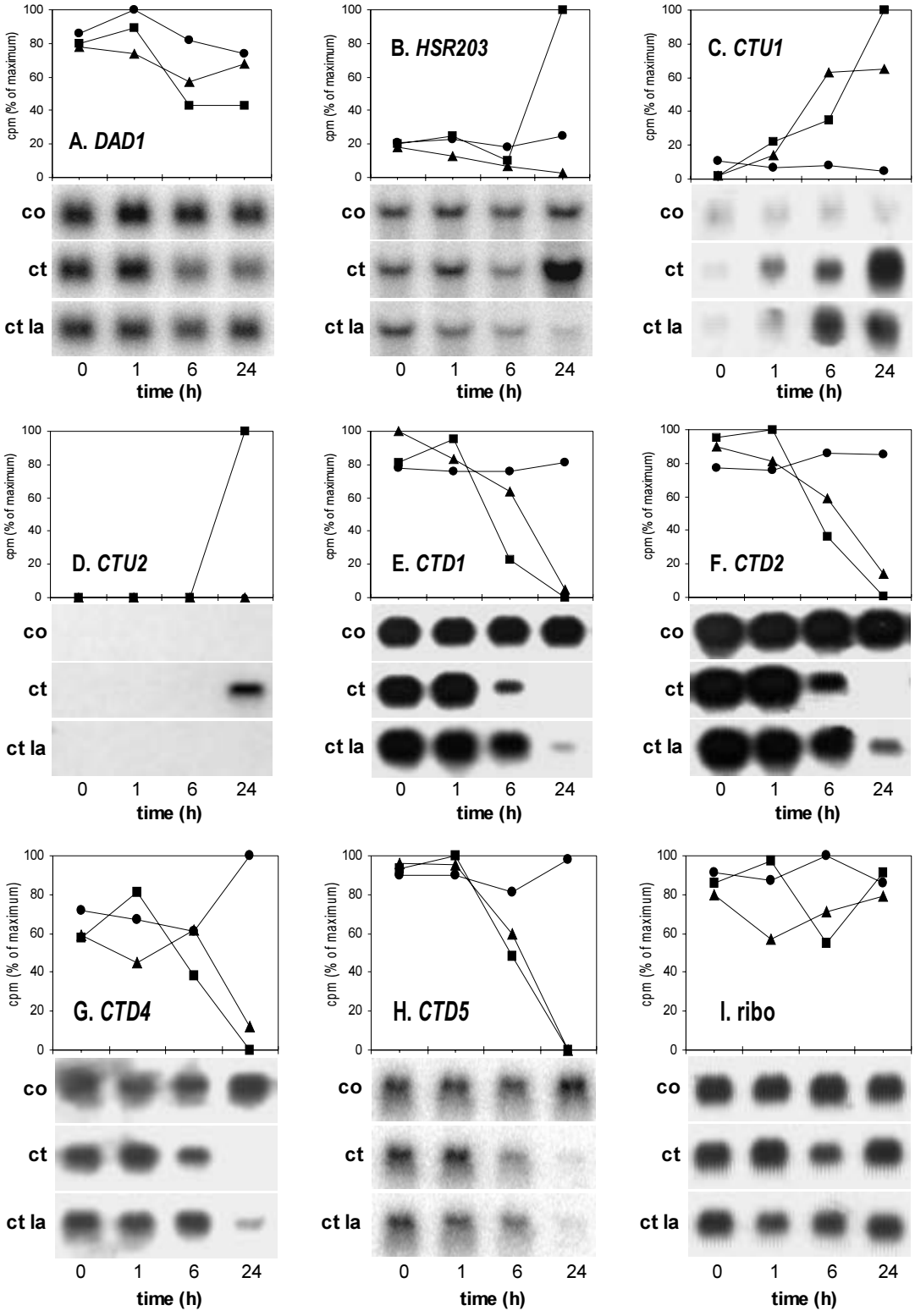
There appears to be little effect of $LaCl_3$ on the camptothecin-induced induction of *CTU1* mRNA levels (Figure 5.3C). The induction seems faster, yet less profound when camptothecin and $LaCl_3$ are added together. However, when

variations in intensity of the ribosomal bands (Figure 5.3I) are taken into account, differences are minimal. Conversely, the strong induction of *CTU2* mRNA levels due to camptothecin treatment was completely suppressed by adding $LaCl_3$ (Figure 5.3D). An effect of $LaCl_3$ was observed on *CTD1* transcript levels, since its camptothecin-induced down-regulation was substantially delayed. After 6 hours, mRNA levels in samples treated with camptothecin and $LaCl_3$ showed only a small decrease as compared to samples taken earlier or control samples. The decrease in *CTD1* mRNA levels that was clearly visible in camptothecin-treated samples after 6 h now became prominent only after 24 h (Figure 5.3E). $LaCl_3$ slowed down the camptothecin-induced decrease of *CTD2* to a similar extent (Figure 5.3F). The effect of $LaCl_3$ on the camptothecin-induced down-regulation of *CTD4* seemed to be limited to a small delay, but this is speculative (Figure 5.3G). There was no significant effect of $LaCl_3$ on either the camptothecin-directed transcript levels of *CTD5* (Figure 5.3H), nor on the tomato 60S ribosomal clone (Figure 5.3I).

To verify whether the observed inhibitory effect of $LaCl_3$ on changes in gene expression during camptothecin-induced PCD can not simply be explained by the effect that $LaCl_3$ itself has on mRNA, an additional experiment was performed. Figure 5.4 shows the effect of 0.1 mM $LaCl_3$ alone on transcript levels in suspension-cultured tomato cells. This concentration of $LaCl_3$ has no effect on cell viability (data not shown). Firstly, the clones that displayed what was believed to be a significant inhibitory effect of $LaCl_3$ on camptothecin-induced changes in mRNA levels (*HSR203*, *CTU2*, *CTD1*, *CTD2*) were examined. When fluctuations of

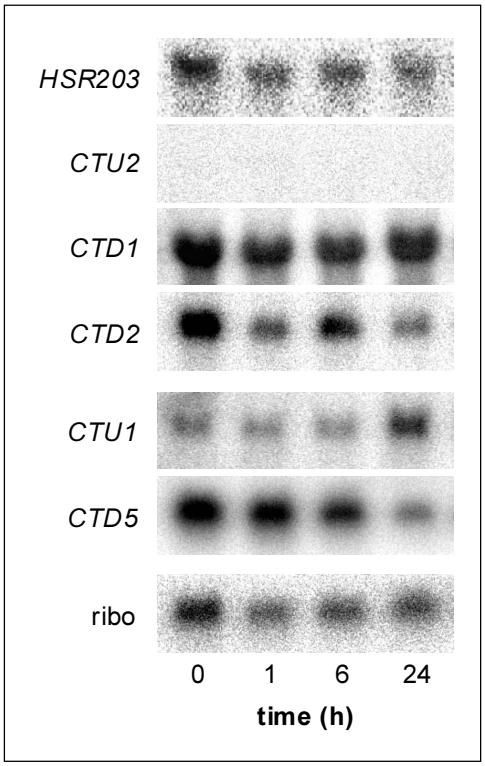
FIGURE 5.3. *Accumulation of mRNAs during camptothecin-induced PCD in tomato suspension cells. Total RNA was extracted from non-treated control samples (**co**), camptothecin-treated samples (5 µM, **ct**), and samples treated with 5 µM camptothecin and 0.1 mM $LaCl_3$ (**ct la**). Samples were taken after 0, 1, 6, and 24 h of the respective treatment. For each sample 16 µg of RNA was loaded. Bands were quantified using ImageQuant software and results for each clone are outlined in the added graphs. **A**, DAD1; **B**, HSR203; **C**, CTU1 (putative glutathione S-transferase); **D**, CTU2 (putative pirin); **E**, CTD1 (putative Aux/IAA gene); **F**, CTD2 (RSI-1); **G**, CTD4 (unknown); **H**, CTD5 (putative proline-rich protein); **I**, ribo (ribosomal protein L17).*




A. DAD1
B. HSR203
C. CTU1
D. CTU2
E. CTD1
F. CTD2
G. CTD4
H. CTD5
I. ribo
cpm (% of maximum)
co
ct
ct la
0 1 6 24
time (h)

the ribosomal clone were taken into account, changes in *HSR203* and *CTD1* mRNA levels never exceeded 30%. *CTU2* transcripts could not be detected, whereas 24 h of $LaCl_3$ treatment decreases *CTD2* levels to around 25%. *CTU1* and *CTD5*, the two clones that did not exhibit any $LaCl_3$ effect on camptothecin-induced changes in mRNA levels (Figure 5.3), did display changing mRNA levels due to $LaCl_3$ alone: CTU1 showed a 3-fold increase, whereas CTD5 showed a 4-fold decrease (Figure 5.4).

FIGURE 5.4. *Accumulation of mRNAs during lanthanum chloride treatment of tomato suspension cells. Samples were taken after 0, 1, 6, and 24 h of the 0.1 mM $LaCl_3$ treatment, a concentration that did not affect cell viability (data not shown). For each sample 16 µg of RNA was loaded. Names of the hybridising probes are depicted on the left: DAD1, HSR203, CTU1 (putative glutathione S-transferase), CTU2 (unknown), CTD1 (putative Aux/IAA gene), CTD2 (RSI-1), CTD4 (unknown), CTD5 (putative proline-rich protein), ribo (ribosomal protein L17).*



5.4. Discussion

PCD is an active, genetically controlled process that ultimately leads to the removal of cells no longer wanted by organisms. It occurs during normal growth and development, or in response to a wide variety of environmental triggers. This report describes the study of mRNA transcript levels of several tomato

genes during camptothecin-induced PCD in tomato cell suspensions. The clones studied have been isolated randomly by means of differential display, or were selected on the basis of their putative function in cell death suggested elsewhere.

The suspension-cultured tomato cells constitute a powerful model system for studying PCD in plants. Treatment of these cells with camptothecin induces cell death that is accompanied by the degradation of genomic DNA into small oligonucleosomal fragments (DNA laddering), a hallmark of apoptosis in animal systems (Steller 1995). Moreover, caspase-specific peptide inhibitors prevent camptothecin-induced cell death in this system (De Jong et al. 2000). These data indicate that camptothecin induces apoptotic cell death in suspension-cultured tomato cells.

A decrease in viability of the tomato cells can first be detected after 6 to 8 hours of treatment with camptothecin. Samples were taken after 0, 1, 6, and 24 hours of treatment, in order to detect: very early changes in mRNA levels (1 hour); changes in mRNA levels that precede the first detectable alteration in viability (6 hours); and changes that take place when PCD is in full progress (24 hours). Northern analysis showed that most changes in mRNA levels accompanying PCD take place within 6 hours of camptothecin treatment, and therefore precede the first detectable decrease in cell viability. The amount of isolated total RNA per gram of camptothecin-treated cells harvested at 0, 1, and 6 hours was similar to the RNA yield from control cells (data not shown). However, 24 hours after adding camptothecin, RNA yields had dropped 4-fold (data not shown). This decrease in RNA yield can be partly accounted for by the fact that one third of the harvested camptothecin-treated cells are dead (Figure 5.1), and most likely do not contain any RNA. An additional explanation could be a decreasing amount of total RNA in cells that undergo PCD, analogous to the decreasing total RNA content in cells from leaves undergoing senescence (Lers, 1998). Still, although expression data presented here were obtained using total amounts of RNA, an increase in one messenger's transcript level is unlikely to be the result of the decrease in transcript levels of all other messengers, since mRNA levels of a gene encoding a ribosomal protein showed only minor variations. Moreover, most changes in mRNA levels discussed here involve alterations larger than 4-fold.

The identified tomato homologue of DAD1 (LeDAD1) is highly similar to other plant DAD1 proteins. The *DAD1* gene was originally cloned from a temperature-

sensitive mutant hamster cell line that undergoes apoptosis when incubated at non-permissive temperature (Nakashima et al. 1993), and encodes a protein that has been described to inhibit developmental PCD in *C. elegans* (Sugimoto et al. 1995). Hence, DAD1 is believed to be a PCD suppressor. Protein sequence comparison revealed that human DAD1 is 40% identical to the *S. cerevisiae* OST2 protein. OST2 has been cloned as a subunit of the yeast enzyme oligosaccharyltransferase (OST), that catalyses the transfer of preassembled high mannose oligosaccharides to glycosylation sites of newly synthesised proteins (Silberstein et al. 1995). The assumption that DAD1 represents an essential subunit of mammalian OST has been confirmed (Fu et al. 1997; Kelleher and Gilmore 1997; Sanjay et al. 1998). This role in OST suggests that DAD1 may influence PCD only indirectly.

In animal systems, several attempts have been made to elucidate the role of DAD1 during PCD, but results have been inconclusive (Saeed et al. 1997; Nishii et al. 1999). In plants, data are no less contradictory. In *A. thaliana*, *DAD1* was shown to be expressed in all organs tested, whereas transcript abundance is reduced during development of siliques (Gallois et al. 1997). Furthermore, *DAD1* transcript levels decline during senescence of pea petals, a process that involves PCD (Orzaez and Granell 1997). These data would be consistent with a putative role as a PCD suppressor. However, *DAD1* expression in apple *(Malus domestica)* shows an induction during senescence of leaves, petals and fruit (Dong et al. 1998). Here, we present *DAD1* expression levels in tomato cells undergoing camptothecin-induced PCD. Transcript levels decrease half-fold within 6 h after adding camptothecin, to still clearly detectable levels. These data could be interpreted as down-regulation of a cell death suppresser, although the observed decrease is very limited.

The expression of *LeHSR203* clearly is induced by camptothecin treatment of tomato suspension cells. After 24 hours of treatment, *LeHSR203* mRNA levels have increased 5-fold as compared to the relatively constant mRNA levels in control cells. A strong correlation between tobacco *HSR203J* -induction and HR-related PCD has been described (Pontier et al. 1994), whereas specific caspase inhibitors can block the activation of HR-related cell death along with *HSR203J* transcript accumulation (Del Pozo and Lam 1998). Data presented here, showing that *LeHSR203* mRNA levels rise during camptothecin-induced PCD, are in line with the putative role of *HSR203* as an active participant in the cell death proc-

ess, as suggested by Pontier et al. (1994). The HSR203J protein has been identified as a serine hydrolase (Baudouin et al. 1997), and is believed to play a role in the limitation of cell death during HR (Pontier et al. 1999). Likewise, it could control the extent of cell death in tomato cell suspensions. Recently, it was reported that *HSR203J* mRNA expression is not induced during leaf senescence (Pontier et al. 1999). Thus, it seems that cell death during the HR and the cell death pathway activated by camptothecin share common signals, different from senescence-associated cell death.

The isolated clone named *CTU1* is rapidly up-regulated during camptothecin-induced PCD. The protein fragment encoded by this partial cDNA clone shows high homology to the C-terminus of tobacco 103-like proteins and the potato PRP1 protein. The mRNAs encoding 103-like proteins were first identified as members of a family of auxin-induced genes (Van der Zaal et al. 1991), and share high similarity with the potato *PRP1* gene. The mRNA of this latter gene accumulates rapidly after treatment of leaves with fungal elicitor, and thus behaves as mRNAs encoding pathogenesis-related (PR) proteins (Taylor et al. 1990). Both the NT103 and PRP1 proteins exhibit GST activity (Droog et al. 1993; Hahn and Strittmatter 1994). GSTs are believed to protect cells against the damaging effect of ROS that arise from various physiological processes or pathogen attack. They are also involved in detoxification of xenobiotic compounds such as herbicides. As some GSTs are capable of binding auxin or auxin analogues, they also have been implicated in auxin signalling, but this role is still speculative (Marrs 1996). The camptothecin-induced mRNA accumulation of *CTU1* can be explained by the need of the cell to deal with increased oxidative stress due to a PCD-associated oxidative burst. This generation of ROS is believed to play a regulatory role in PCD (Richberg et al. 1998). Our results indicate that camptothecin-triggered cell death in tomato suspension cells is also accompanied by a rise in ROS levels (De Jong et al. 2002).

To our knowledge, only one partial cDNA clone corresponding to a GST from tomato has been previously described. *Lepar* was cloned as a tomato homologue of the auxin-regulated tobacco Par genes (Mito and Bennett 1995). Protein translations of both clones overlap 24 aa, of which only 6 are identical. This shows that *CTU1* is different from *Lepar*.

Expression of the *CTU2* cDNA fragment shows a very strong induction after 24 hours of camptothecin treatment. The deduced partial sequence of *CTU2* is

homologous to human PIRIN, a NF1-interacting protein (Wendler et al. 1997). Recently, PIRIN has been shown to interact with the human transcription factor NF-κB (Dechend et al. 1999). NF-κB is involved in the tumour necrosis factor (TNF) signalling pathway and is partly responsible for the cellular changes observed during TNF-triggered apoptosis. Upon activation, NF-κB promotes the transcription of anti-apoptotic genes (Perkins 2000). Other components of the TNF signalling pathway have been identified and shown to interact with NF-κB, thereby regulating its ability to activate gene expression. Among them are the IκB proteins, a family of proteins containing several ankyrin domains that regulate NF-κB by sequestering it in the cytoplasm (Perkins 2000). Strikingly, the *A. thaliana* transcription factor NPR1/NIM1 shares homology with mammalian IκB proteins (Cao et al. 1997; Ryals et al. 1997). Taken together, these data hint towards the existence of a NF-κB/IκB-like signalling pathway in plants, and a possible role of a tomato PIRIN-like protein in the regulation of this pathway during camptothecin-induced PCD. Cloning of the full-length cDNA corresponding to CTU2 (as well as CTD4 and CTD5) may help to illuminate their potential roles in plant PCD.

The *CTD1* cDNA clone has been identified as the 3' end of a tomato gene belonging to the early-auxin-responsive *Aux/IAA* gene family. In a specific response to auxin, a plant hormone essential for cell elongation and division, steady-state mRNA levels of most *Aux/IAA* genes rapidly increase within 5 to 60 min. In *A. thaliana* this family comprises at least 25 members, and homologues have been cloned from many other plant species (Abel and Theologis 1996; Kim et al. 1997). These genes encode short-lived nuclear proteins (Abel et al. 1994), and it has been proposed they are transcription factors responsible for the regulation of expression of late auxin-regulated genes (Kim et al. 1997). One of the highly conserved putative nuclear localisation signals is conserved in CTD1. One partial *Aux/IAA* cDNA has been cloned from tomato, along with the earlier mentioned *Lepar*, and was designated *LeAux* (Mito and Bennett 1995). The two putative proteins only share 7 identical aa's in an 11 aa long region of overlap. Therefore, we believe *CTD1* is different from *LeAux*. It has been shown that a mitogen activated protein kinase (MAPK) cascade capable of repressing auxin-inducible promoters is activated by oxidative stress (Kovtun et al. 2000). Hence, the observed strong and swift down-regulation of CTD1 may be a result of the camptothecin-provoked oxidative burst (De Jong et al. 2002) and could be aimed

at suppressing auxin signal transduction, at times when growth-promoting properties are no longer needed.

CTD2 corresponds to tomato *RSI-1*, a gene that is activated within 4 h during auxin induced lateral root initiation in tomato seedlings (Taylor and Scheuring 1994). The down-regulation of *RSI-1* mRNA levels during PCD might be caused by oxidative signalling, similar to the down-regulation of *CTD1*. In fact, it could be a result of inactivation of factors such as CTD1.

$LaCl_3$ is a well-known plasma membrane Ca^{2+} channel blocker. The induction of PCD is often accompanied by an increase of cytosolic calcium, and it has been suggested that the elevation of cytosolic Ca^{2+} levels directly activates cell death effectors (Richberg et al. 1998). Inhibition of this Ca^{2+} flux by $LaCl_3$ prevents cell death in H_2O_2-induced PCD in soybean suspension cultures and blocks the evocation of apoptotic cell morphology induced by bacterial inoculation of soybean cells. In contrast, H_2O_2 induction of a GST is Ca^{2+} independent (Levine et al. 1996). This demonstrates that the signalling pathway leading to PCD can be independently blocked from the pathway leading to induction of GST expression.

Camptothecin-induced cell death in the tomato cell suspension system is also drastically reduced by adding $LaCl_3$ concurrently (data not shown), an observation that underlines the importance of Ca^{2+} fluxes during PCD. Northern blotting revealed that, in line with findings of Levine (1996), $LaCl_3$ has very little effect on the camptothecin-induced mRNA levels of *CTU1*, a putative *GST* and general stress-related gene (Figure 5.3C). However, blocking Ca^{2+} flux has a very strong effect on transcript levels of both *LeHSR203* and *CTU2* (Figures 5.5B and 5.5D). The induction visible 24 hours after adding camptothecin completely disappears, indicating that *LeHSR203* and *CTU2* activation can be blocked independently from *CTU1* activation. It is tempting to speculate that induction of *LeHSR203* and *CTU2* are part of a specific PCD pathway and can be uncoupled from the activation of general stress genes, such as *CTU1*.

An effect of $LaCl_3$ on the camptothecin-induced down-regulation of *CTD1* and *CTD2* is present, since the decrease in mRNA levels caused by adding $LaCl_3$ and camptothecin simultaneously is less profound and delayed (as compared to adding camptothecin alone; Figures 5.5E and 5.5F). Nevertheless, expression levels are still reduced (as compared to control samples), suggesting mRNA levels can be down-regulated via both Ca^{2+}-dependent and Ca^{2+}-independent path-

ways. Data for *CTD4* and *LeDAD1* are hard to interpret, as the visible effect of adding $LaCl_3$ might not be significant (Figures 5.5G and 5.5A). Transcription patterns of *CTD5* are not affected by adding both camptothecin and $LaCl_3$ (Figure 5.3H), and therefore *CTD5* might represent a gene affected through the same pathway that regulates general stress genes such as *CTU1*.

Since the counteracting effect of $LaCl_3$ on camptothecin-induced changes in gene expression might merely reflect the way $LaCl_3$ itself affects transcription, suspension cells were treated with $LaCl_3$ (Figure 5.4). $LaCl_3$ does not affect mRNA levels of *HSR203*, *CTU2*, and *CTD1*, thereby ruling out this explanation. Neither can the (changing) *CTD2* mRNA pattern displayed in Figure 5.4 account for the tempering effect of $LaCl_3$ on camptothecin-induced CTD2 down-regulation. On the other hand, the effect of $LaCl_3$ on transcript levels of *CTU1* and *CTD5* is very similar to the effect of camptothecin, supporting the concept that *CTU1* and *CTD5* are general stress genes induced by both camptothecin and $LaCl_3$.

PCD is an essential part of plant development and defence. A model system of suspension-cultured tomato cells, in which PCD can be induced by adding camptothecin, was used in an attempt to increase the understanding of PCD in plants. Future in planta validation of the results presented here, along with research on clones yet to be isolated, will help identifying PCD genes and elucidating the underlying regulatory mechanisms of plant PCD.

5.5. Acknowledgements

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6

a metacaspase is upregulated following infection of tomato leaves with the fungal pathogen botrytis cinerea

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Summary

Plant cells can undergo programmed cell death (PCD) accompanied by biochemical and morphological hallmarks similar to those of animal apoptosis. However, orthologs of animal caspases, cysteinyl aspartate-specific proteases that constitute the core component of animal apoptosis, have not yet been identified in plants. Here, we describe the isolation of *LeMCA1*, a (type II) metacaspase gene from tomato (*L. esculentum*) that encodes a protein with homology to mammalian caspases. Southern analysis indicates that there is at least one more metacaspase present in the tomato genome. *LeMCA1* gene expression is rapidly induced upon infection of tomato leaves with the fungal pathogen *B. cinerea*, and is constitutive in suspension-cultured tomato cells.

6.1. Introduction

Programmed cell death (PCD) is a process aimed at eliminating unnecessary or harmful cells during growth and development of multicellular organisms. In animals, PCD often takes the appearance of apoptosis, a form of PCD defined by a distinct set of morphological and biochemical features (Steller 1995). The core component of the apoptotic machinery is a proteolytic cascade that involves a family of cysteinyl aspartate-specific proteases named caspases. During apoptosis, initiator caspases activate downstream executioner caspases that process a variety of target proteins eventually leading to the apoptotic phenotype (Hengartner 2000).

In plants, PCD is equally important as in animals. It is involved in for example xylogenesis, plant reproduction, aerenchyma formation, and senescence. Furthermore, PCD occurs in response to pathogens and various abiotic stresses (Greenberg 1996). Several morphological and biochemical similarities between animal apoptosis and plant PCD have been described, including DNA laddering, caspase-like proteolytic activity, and cytochrome c release from mitochondria. Increasing evidence indicates that many cases of plant PCD proceed through a mechanism that is functionally conserved between animals and plants (Lam et al. 2001; Hoeberichts and Woltering 2002). However, to date no functional homologues of animal caspases have been identified in plants, even though a vast amount of biochemical evidence suggests the existence of caspase-like activity in plants, and its involvement in plant PCD (Woltering et al. 2002).

Recently, iterative homology searches have identified a family of putative cystein proteases distantly related to caspases, designated metacaspases. They are present in the genomes of both fungi and plants. For example, the genome of *A. thaliana* contains 9 metacaspase-like sequences. Modelling of the three-dimensional protein structure of metacaspases indicates significant tertiary structure homology to animal caspases (the caspase-hemoglobinase fold; Uren et al. 2000). Mutational studies in *Trypanosoma brucei* first suggested that metacaspases indeed function as cysteine proteinases (Szallies et al. 2002). Recently, it was shown that the only metacaspase present in *S. cerevisiae* displays a caspase-like proteolytic activity that is activated when yeast is stimulated by H_2O_2 to undergo apoptosis (Madeo et al. 2002).

In this paper, we report the cloning of a metacaspase from tomato named

LeMCA1. In addition, we describe gene expression patterns of *LeMCA1* during HR-related cell death after infection of tomato leaves with the fungal pathogen *Botrytis cinerea*, and during chemical-induced PCD in suspension-cultured tomato cells.

6.2. Material and methods

Cloning and analysis of LeMCA1
A cDNA library from camptothecin-treated tomato cells was made as described by Orzaez et al. (2001). Using two sets of degenerate primers designed using sequences present in the databases from *A. thaliana* and *Hevea brasiliensis*, a 194 bp long metacaspase fragment was cloned (Table 6.1 and Figure 6.1). Primers first PCR reaction: 1AaF and 1DaR; primers double nested PCR reaction: 1BaF and 1CaR. A second series of primers, specifically designed to clone a type I metacaspase were used unsuccessfully (primer series 2A, 2B, 2C, and 2D; see Table 6.1). Subsequently, specific primers in combination with AP1 and AP2 (Clontech) were used in rapid amplifications of cDNA ends (RACE) reactions. All PCR products were purified from gel and cloned in pGEM-T Easy (Promega). Nucleotide sequencing was performed by BaseClear (Leiden, The Netherlands). Protein alignments were made using Clustal X software (version 1.8).

Genomic DNA extraction and Southern blotting
Genomic DNA was extracted from tomato suspension cells as described previously (De Jong et al. 2000). DNA was then digested overnight with different restriction enzymes, separated by electrophoresis in an 1% agarose gel, and transferred to a nylon membrane. A 416 bp long fragment of the *LeMCA1* coding region (bp 22 to 438 relative to ATG) was labelled using PCR Digoxigenin (DIG) Labelling Mix Plus (Roche). The blot was hybridised overnight at 42 °C in Ultrahyb solution (Ambion) and then washed twice at 42 °C in 2x SSC, 0.1% SDS for 15 minutes. Fragments were detected using alkaline phosphatase labelled anti-DIG antibodies (Roche) and CDP-Star chemiluminescent substrate (Roche).

Cell cultures and treatments
Tomato (*L. esculentum* Mill.) cell suspension cultures were grown as described by De Jong et al. (2000). Cell viability was determined by staining with 0.002% FDA. Cells were treated with 5 µM camptothecin, 20 µM fumonisin B1, 10 µM methyl jasmonate, 10 µM salicylic acid, or 100 µl l^{-1} ethylene.

type II	forward	1A a F	5' - GGNTGYRTNAAYGACGT - 3'
	forward nested	1B a F	5' - CAYTAYAGYGGNCAYGGNAC - 3'
		1B b F	5' - CAYTAYTCNGGNCAYGGNAC - 3'
	reverse nested	1C a R	5' - TSDATDAGVCCRCCRCTRTG - 3'
		1C b R	5' - TSDATDAGVCCRCCNGARTG - 3'
	reverse	1D a R	5' - CCRCTNADNARWATNCC - 3'
		1D b R	5' - CCNGANADNARWATNCC - 3'
type I	forward	2A a F	5' - GGNTGYATCVWNGACGC - 3'
		2A b F	5' - GGNTGYATCVWNGATGC - 3'
	forward nested	2B a F	5' - ATHYTNATGYTNACNGAGGA - 3'
		2B b F	5' - ATHYTNATGYTNACNGAAGA - 3'
	reverse nested	2C a R	5' - ARTGRAASACNARNGARTC - 3'
		2C b R	5' - ARTGRAASACNARRCTRTC- 3'
	reverse	2D a R	5' - TGRTCYTCCCANWCGTA - 3'
		2D b R	5' - TGRTCYTCCCANWCATA - 3'

TABLE 6.1. *Sequences of the degenerate primers that were used to clone metacaspases from tomato. Primers were designed to either amplify a type I metacaspase fragment or a type II metacaspase fragment.*

Inoculation assays

Botrytis cinerea strain SAS56 was cultured as described by Van der Vlugt-Bergmans et al. (1997). Conidia were harvested from sporulating plates by washing with sterile water, containing 0.05% Tween 80. The conidia were filtered through glass-wool, washed three times by centrifugation (8 min, 800 rpm, 114 x g) and resuspending in sterile water, and finally resuspended in inoculum buffer. Tomato plants (cultivar Moneymaker genotype Cf4) were grown in soil in the greenhouse for approximately 7 weeks. Leaves were cut from the plant and inserted with their stems in a block of water-saturated florist's foam. The block was placed in a petridish in a plastic box with a grid on the bottom, such that the leaf was spread out over the grid. Wet filter paper was placed beneath the grid to obtain high humidity. Leaflets were inoculated with conidial suspensions on the upper side either by pipetting individual droplets or by spraying. Leaves were dried at room temperature for 30 minutes. The box was closed with a plastic transparent cover and incubated at 20 °C with a 16 h photoperiod.

RNA isolation and Northern blotting

RNA isolation from (treated) suspension cells, electrophoresis, and hybridisation were performed as described by Hoeberichts et al. (2001). RNA isolation from *B. cinerea* and (infected) tomato leaves, electrophoresis, and hybridisation were performed as described by Van der Vlugt-Bergmans et al. (1997). For hybridisation, the following DNA fragments were used: the *LeMCA1* probe described above (both DIG and radioactively labelled); a DIG labelled 346 bp fragment of the tomato 60S ribosomal L17 gene (accession number AJ250002); a radioactively labelled 730 bp EcoRI-HindIII fragment of the *B. cinerea* actA gene; a radioactively labelled 1.7 kb EcoRI fragment of the radish 18S rDNA gene (Benito et al. 1998).

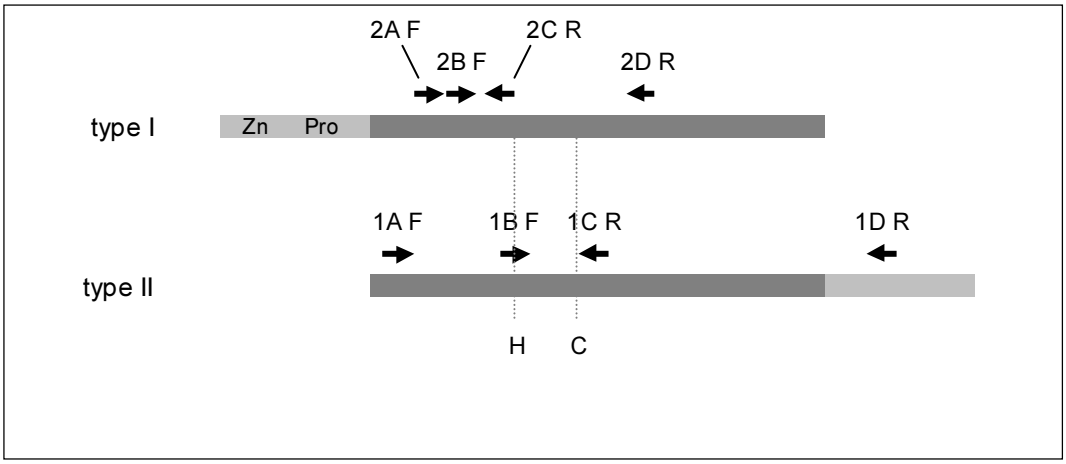

FIGURE 6.1. *Schematic aligment of type I and type II metacaspases. Plant type I metacaspases contain a prodomain that consists of a proline-rich region (Pro) and a zinc finger motif (Zn) similar to that of the plant HR protein LSD1. Type II metacaspases possess no obvious prodomain but have a conserved insertion of approximately 180 amino acids at their C terminus. The position of the conserved histidine (H) and cysteine (C) residues is indicated at the bottom. Primers that were designed to clone both type I and type II metacaspase fragments from tomato are depicted by arrows.*

6.3. Results and discussion

The cloned LeMCA1 gene is flanked by genes that have been linked to PCD

A 1540 bp long metacaspase cDNA fragment was cloned from tomato (accession number AY114141). While cloning of the full-length cDNA clone was in progress, the sequence of the tomato SW-5 locus was reported. This 35250 bp long locus (accession number AY007366), located on tomato chromosome 9, confers resistance to tospoviruses and contains the tospovirus resistance genes A (Sw5-a) and B (Sw5-b) (Spassova et al. 2001). The locus also contains, on the telomeric side of Sw5-a and Sw5-b, a putative open reading frame (ORF) that, excluding a 1300 bp long intron, displayed 99.3% identity with the cloned metacaspase fragment (Figure 6.2).

From the cloned cDNA fragment and the genomic sequence it was concluded that this tomato gene, named *LeMCA1* (for *L. esculentum* metacaspase 1), contains a 1251 bp long coding region, encoding a 416 amino acid long protein of approximately 45.6 kDa.

Plant metacaspases fall into two types. Type I metacaspases contain a prodomain that consists of a proline-rich motif and a zinc finger motif similar to that of the plant HR protein LSD1. Type II metacaspases contain no obvious prodo-

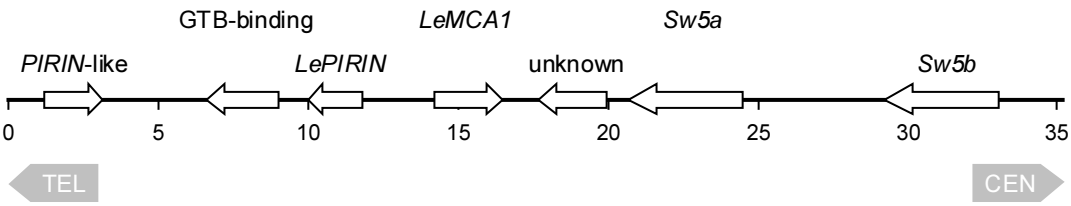

FIGURE 6.2. *Genetic organisation of the 35 kb fragment that contains the LeMCA1 gene. A total number of seven ORFs are present in this stretch of genomic DNA: a PIRIN-like gene, an ORF encoding a protein that resembles a tobacco GTP-binding protein, LePIRIN, LeMCA1, a gene encoding a protein of unknown function, Sw5a, and Sw5b (Spassova 2001).*

main but have a conserved insertion of approximately 180 amino acids between the regions corresponding to caspase p20 and p10 subunits (Uren et al. 2000; see Figure 6.1). The predicted LeMCA1 protein is a type II metacaspase.

Alignment with various full length metacaspase protein sequences present in the databases revealed that LeMCA1 is 75% identical and 87% similar to a metacaspase from rubber tree *(Hevea brasiliensis)* that was originally cloned as a latex-abundant protein (Shin et al. 1999), 67% identical and 80% similar to one of the *A. thaliana* metacaspases (accession number NP_178052), and 14% identical and 24% similar to the only metacaspase in *S. cerevisiae* (Table 6.2).

Strikingly, the tomato SW-5 locus, in addition to *LeMCA1*, also contains the *LePIRIN* gene and a second pirin-like gene. Moreover, *LeMCA1* and *LePIRIN* are adjacent genes that, due to their opposite orientation, share their 5' upstream regions. Their respective ATG start codons are separated by 1818 bp. *LePIRIN* was originally cloned as a gene that is upregulated during chemical-induced PCD in tomato suspension cells (Hoeberichts et al. 2001; Orzaez et al. 2001). It has been postulated that PIRIN plays a regulatory role as a mediator of protein-protein interactions during plant PCD (Orzaez et al. 2001).

Another remarkable observation is that SW5-a and SW5-b are members of a family of resistance (R) gene products that contain the nucleotide binding-ARC (NB-ARC) domain. This domain is also present in *C. elegans* CED4 and its human counterpart APAF1. CED4/APAF1 represents one factor in a high molecular weight protein complex called the apoptosome that activates caspases during animal apoptosis. The functional significance of this homology is yet unclear. By analogy, *R* gene products may function as controlling adaptors in plant protein complexes that become activated by pathogen-derived signals during HR-related PCD (Van der Biezen and Jones 1998).

Taken together, it can be concluded that five out of seven ORFs on a 35250 bp long genomic DNA fragment are somehow linked to programmed cell death (Figure 6.2).

Southern blotting reveals at least two metacaspases

A southern blot of digested tomato genomic DNA was hybridised with part of the *LeMCA1* coding region and washed at low stringency. This blot, in combination

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TABLE 6.2

TABLE 6.2. *Degree of identity and similarity of different metacaspases, calculated using Clustal X software (version 1.8). The sequences used in this comparison are from: Le Lycopersicon esculentum; Hb Hevea brasiliensis; At Arabidopsis thaliana; Sp Schizosaccharomyces pombe; Sc Sacharomyces cerevisiae; Tb Trypanosoma brucei.*

with the *LeMCA1* genomic sequence present in the database, establishes the presence of at least two (type II) metacaspases in the tomato genome (Figure 6.3). Considering the low degree of homology between type I and type II metacaspases (see Table 6.2), it is most likely that the *LeMCA1* probe used only hybridised with genes encoding type II metacaspases. Therefore, type I metacaspases that may be present in the tomato genome will not have been visualised.

Expression of LeMCA1 during infection of tomato leaves by Botrytis cinerea

Botrytis cinerea is a fungal pathogen that infects at least 235 hosts (Jarvis 1977). It is a typical necrotroph that kills plant cells and subsequently feeds on these dead cells. Infection of *A. thaliana* induces an oxidative burst and HR-releted cell death (Govrin and Levine 2000). The infection process is severely inhibited in HR-deficient *dnd1* mutant plants, suggesting that the induction of PCD as part of the HR, contributes to the sustained infection of *B. cinerea* in *A. thaliana* (Govrin and Levine 2000). Likewise, transgenic tobacco plants expressing, human *BCL2* or *BCL-xl*, nematode *CED9*, or baculovirus *Op-IAP* (genes that negatively regulate animal apoptosis) display increased resistance to *B. cinerea*, supporting a crucial role for host-activated HR cell death in susceptibility of plants to *B. cinerea* (Dickman et al. 2001).

Transcriptional regulation of caspases, in addition to the known regulatory mechanisms at the protein level, is believed to be essential for several examples of animal apoptosis (Chin et al. 1997; Von Mering et al. 2001; Cakouros et al. 2002). To reveal a possible correlation between *LeMCA1* gene expression and HR cell death in tomato we compared *LeMCA1* and *LeHSR203* mRNA levels during infection of tomato leaves with *B. cinerea*. Detached tomato leaves were

enzyme(s)	fragment(s) in bp
-	undigested (**1**)
KpnI	>29000 (**2**)
KpnI HindIII	3011 (**3**); 638 (**4**)
HindIII	3011 (**3**); 638 (**4**)
EcoRI	4799 (**5**)
BamHI	12539 (**6**); 6249 (**7**); 60
BamHI NcoI	4593 (**8**); 378 (**9**); 60
NcoI	5031 (**10**)

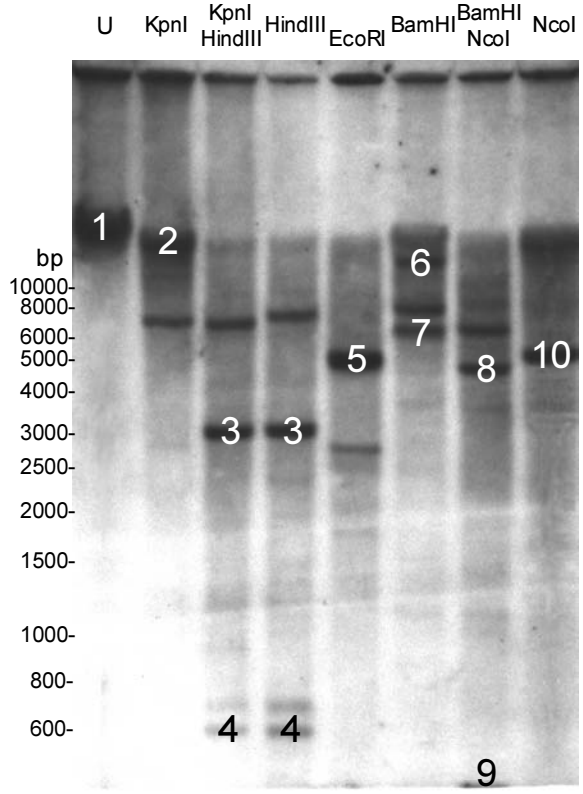

FIGURE 6.3. *Southern blot of digested genomic tomato DNA hybridised with part of the LeMCA1 coding region and washed at low stringency. Genomic DNA was digested with (combinations of) various restriction enzymes. Calculated fragment sizes (using the genomic sequence) are depicted in the added table. Numbers on the blot correspond to the numbers assigned to each fragment.*

inoculated as described (Benito et al. 1998). At 16 hours post inoculation (hpi) necrotic lesions appear. Following lesion appearance, infections seem to be stopped untill 48-72 hpi. Then, approximately 5-10% of the lesions start to expand, eventually covering the whole leaf. This process is accompanied by an increase in mRNA levels of the *B. cinerea* actin gene *BcactA*, which serves as a marker for actively growing *B. cinerea* (Benito et al. 1998). In this experiment, the *Bcact* messenger was detected at 16 hpi, with an increase at 96 and 120 hpi (Figure 6.4).

Transcript levels of *LeHSR203* could already be detected at low levels in uninfected material. These levels were higher, yet stable, throughout infection (Figure

6.4), providing an indication that *B. cinerea* induces a HR in tomato, like in tobacco and *A. thaliana*. *LeMCA1* mRNA was undetectable in uninfected leaves and levels increased upon infection in a way very similar to *LeHSR203* (Figure 6.4). This indicates that expression of *LeMCA1* is correlated with the formation of primary necrotic lesions (16 hpi) and remains at similar levels throughout infection.

Previously, the effects of ethylene and SA on resistance in tomato against *B. cinerea* have been described. Transcript levels of certain Pathogenesis Related (PR) protein genes are induced upon pretreatments with 1 µl l^{-1} ethylene or 4 mM SA (Díaz et al. 2002). However, *LeMCA1* did not display any induction in leaves after these pretreatments (data not shown).

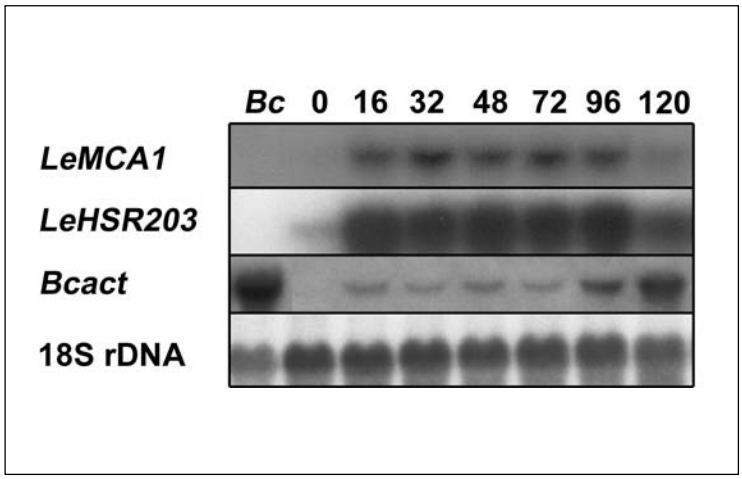

FIGURE 6.4. *Expression of LeMCA1, LeHSR203, and Bcact (actin probe of B. cinerea reflecting fungal biomass) following B. cinerea infection of tomato leaves at 20 °C. RNA was isolated from B. cinerea cells (Bc) and from samples taken after 0 to 120 hours post inoculation. Per sample, 20 µg total RNA was loaded on gel.*

Expression of LeMCA1 during chemical-induced PCD

Northern analysis was used to determine mRNA abundance in tomato suspension cells undergoing chemical-induced PCD. This cell death is accompanied by typical apoptosis-related morphological changes and internucleosomal DNA degradation (De Jong et al. 2000).

Treatment with camptothecin resulted in 13% and 51% of dead cells, and treatment with fumonisin B1 resulted in 7% and 30% of dead cells after 12 and 48 hours respectively (data not shown). Cell death in all other samples, including controls, never exceeded 6.5%. *LeMCA1* mRNA levels did not significantly

change during PCD induced by either camptothecin or fumonisin B1, and were comparable to mRNA levels in control cells (Figure 6.5A). This differs from the expression patterns of *LeHSR203* and *LePIRIN*, two genes that are both upregulated during chemical-induced PCD (Hoeberichts et al. 2001; Orzaez et al. 2001). *HSR203* has been correlated to HR-related cell death in both tobacco and tomato, and does not respond to various stress conditions such as wounding (Pontier et al. 1994; Pontier et al. 1998). Furthermore, tomato suspension cells were exposed to 10 µM methyl jasmonate (MJ), 10 µM salicylic acid (SA) or 100 µl l^{-1} ethylene for 24 hours. Treatment with both MJ and SA caused a slight decrease in mRNA levels, whereas ethylene caused a slight increase (Figure 6.5B).

Blots were also probed with a specific *LeMCA1* 3' untranslated region DNA fragment. This probe resulted in a somewhat lower signal overall, but similar relative intensities (data not shown), indicating that Figure 6.5 indeed reflects *LeMCA1* mRNA levels.

LeMCA1 transcript levels in control cell suspensions are relatively high in comparison to the levels in uninfected leaves, that were undetectable (compare Figure 6.4 to Figure 6.5). It can not be excluded that these levels reflect a constant induction as the suspension cells are constantly liable to a certain amount of stress.

6.4. Concluding remarks

Plant metacaspases are strong candidates for a caspase-like role during plant PCD, particularly now the only metacaspase in yeast has been shown to function as a bona fide caspase (Madeo et al. 2002). Here, we show that a tomato metacaspase, *LeMCA1*, is induced during infection of tomato leaves with *B. cinerea*. This necrotrophic pathogen induces HR-related cell death in *A. thaliana* (Govrin and Levine 2000), tobacco (Dickman et al. 2001), and tomato (supported by the observed increase in *LeHSR203* transcript abundance). However, *LeMCA1* mRNA levels are not induced during chemical-induced PCD in suspension-cultured cells. It can be concluded that only one of the two forms of PCD investigated is accompanied by an increase in *LeMCA1* mRNA levels. As post-translational regulation of caspases plays a crucial role in various examples of animal apoptosis, it will be interesting to determine LeMCA1 protein levels during

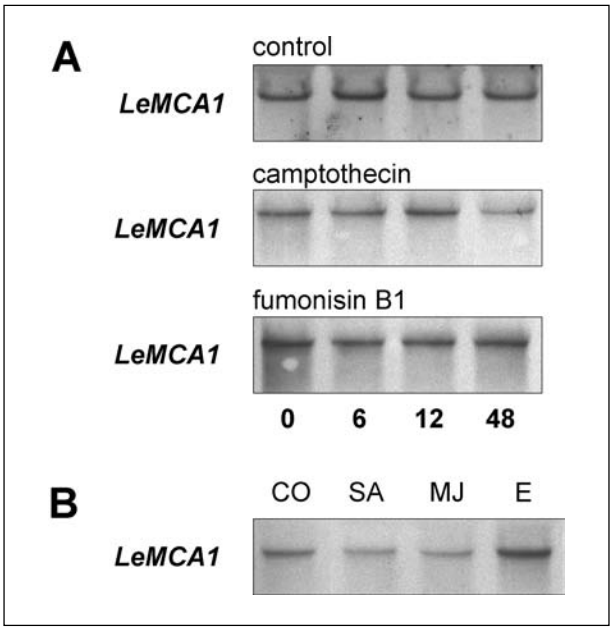

FIGURE 6.5. *LeMCA1 mRNA levels in tomato suspension cells. Total RNA was extracted from control cells and cells undergoing PCD after 0, 6, 12, and 48 hours of camptothecin or fumonisin B1 treatment (**A**). In addition, samples treated for 24 hours with 10 µM salicylic acid (SA), 10 µM methyl jasmonate (MJ), and 100 µl/l ethylene (E) were compared to samples taken from non-treated control (CO) cells (**B**). Per sample, 16 µg total RNA was loaded on gel.*

PCD and investigate the possible role of protein processing. The tomato genome contains at least two type II metacaspases (Figure 6.3) and the *A. thaliana* genome contains three metacaspase-like sequences of type I and six of type II (Table 6.2). One could speculate that different metacaspases display different expression patterns and have different functions during plant PCD. In this regard it is important to mention that the yeast metacaspase is of type I. Our efforts to clone type I metacaspases from tomato have so far been unsuccessful.

The *LeMCA1* gene in located in the close vicinity of two PIRIN genes and two disease resistance genes that both contain an NB-ARC domain. Although intriguing, the biological significance of this observation remains unknown.

6.5. Acknowledgements

The authors thank Rina Michaeli for technical assistance. We are indebted to Prof. Linus van der Plas and Dr. Sander van der Krol for critically reading the manuscript and stimulating discussions. This work was supported by the Dutch Ministry of Agriculture, Nature Management and Fisheries.

7

ethylene perception is required for the expression of tomato ripening-related genes and associated physiological changes even at advanced stages of ripening

Frank A. Hoeberichts, Linus H. W. van der Plas and Ernst. J. Woltering
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Summary

Treatment of tomato fruit (*L. esculentum* L. cv Prisca) with 1-methylcyclopropene (1-MCP), a potent inhibitor of ethylene action, delayed colour development, softening, and ethylene production in tomato fruit harvested at the mature green, breaker, and orange stages. 1-MCP treatment also decreased the mRNA abundance of *PSY1*, *EXP1*, and *ACO1*, three ripening-related tomato genes, in mature green, breaker, orange, and red ripe fruit. These results demonstrate that the ripening process can be inhibited both on a physiological and molecular level, even at very advanced stages of ripening. The effects of 1-MCP on ripening lasted 5 to 7 days and could be prolonged by renewed exposure. Furthermore, it is shown that in fruit matured on the vine, ethylene production rates, *ACO1* and *EXP1* transcript levels reached significantly higher levels than during postharvest ripening of fruit detached at the MG stage.

7.1. Introduction

The ripening of tomato fruit corresponds to a series of biochemical, physiological and structural changes, aimed at making the fruit attractive to consumers and thereby increasing chances for seed dispersal. The ripening process is controlled by an increase in ethylene production at the onset of ripening (Lelièvre et al. 1997). In commercial horticulture, it has always been a goal to control tomato fruit ripening. Large annual losses due to spoilage make a means to control ripening of great economical importance. In this regard, elucidation of the role of ethylene in tomato fruit ripening has brought powerful tools, such as chemical inhibitors of ethylene perception (Beyer 1976; Sisler and Yang 1984; Sisler and Blankenship 1993; Sisler and Serek 1997) and transgenic plants with altered ethylene production levels (Hamilton et al. 1990; Klee et al. 1991; Oeller et al. 1991).

It has been suggested that ethylene acts as a rheostat rather than as a trigger for fruit ripening, which implies that ethylene must be present continuously in order to maintain transcription of the necessary genes (Theologis 1992). If this were true, interfering with ethylene biosynthesis or perception would affect the progression of any stage of ripening. In most studies, mature green (MG) tomatoes were used in which ripening can be completely inhibited by blocking ethylene perception (Hobson et al. 1984; Davies et al. 1990; Sisler and Blankenship 1993). Only few studies describe the effect of ethylene inhibitors on tomatoes that have already initiated ripening. Davies et al. (1988) showed that treating breaker (BR) tomato fruit with silver ions can prevent the appearance of ripening-related mRNAs and proteins. Silver thiosulphate (STS), NBD, and diazocyclopentadiene (DACP) are capable of inhibiting various ripening-related biochemical changes such as polygalacturonase (PG) activity or lycopene accumulation in tomato fruit tissue after ripening has commenced (Tucker and Brady 1987; Liu et al. 1989; Sisler and Lallu 1994).

In an attempt to bring together and increase the rather fragmented information presently available, particularly concerning the role of ethylene during the advanced stages of ripening, we present here an integrated study of the effects of 1-MCP on the physiological and molecular aspects of tomato fruit ripening. This gaseous compound presumably binds to the ethylene receptor (Sisler and Serek 1997) and its effectiveness in blocking various ethylene-regulated re-

sponses has been described (Serek et al. 1994; Hall et al. 2000). In the present study, the objective was to investigate the effects of 1-MCP on colour development, fruit softening and ethylene production at various ripening stages. Furthermore, the effect of 1-MCP on mRNA levels of three ripening-related genes, phytoene synthase 1 *(PSY1)*, expansin 1 *(EXP1)*, and ACC oxidase 1 *(ACO1)*, was examined. In addition, differences in ripening behaviour between fruit detached from the plant at the MG stage and subsequently allowed to ripen in air, and fruit matured on the vine were studied.

7.2. Materials and methods

Tomato plants
Tomatoes (*L. esculentum* L. cv. Prisca) grown in a greenhouse were collected and sorted into four groups according to the Dutch C.B.T. coloration index: mature green (MG), colour stage 1, 28 tomatoes; breaker (BR) colour stages 2 to 4, 16 tomatoes; orange (OR), colour stages 6 to 8, 21 tomatoes; and red ripe (RR), colour stages 10 to 12, 31 tomatoes. Before sorting, fruit were kept overnight to ensure wound-responses would not interfere with the experiment. During the further course of the experiment tomatoes were kept in air at 20 °C and 70% relative humidity.

1-MCP treatment
Tomato fruit were treated with 1-MCP in 70 liter stainless steel containers for 20 hours in the dark. Control tomatoes were kept under identical conditions in air. 1-MCP was obtained by dissolving 0.1 gram of EthylBloc (FloraLife Inc.) in 10 ml of water in a closed 100 ml bottle. Three 10 ml samples of the bottle's headspace were injected into the containers. Samples taken from each container were analysed for 1-MCP, using an United Technologies Packard model 437A GC equipped with a stainless steel column filled with Alumina GC (Chrompack) and a flame ionisation detector. 1-MCP concentrations, quantified using isobutane as a calibration gas, were determined at the start and at the end of the 1-MCP treatment, and declined from 150 to 50 nl l^{-1} during the 20 hour treatment.

Determination of colour, firmness and ethylene production
Colour was assessed using a Minolta 200CR colorimeter in terms of the lightness (*L*), a green (negative values) to red (positive values) scale (a), and a blue (negative values) to

yellow (positive values) scale (b). Two readings were recorded per fruit. The formula $2000a/L(a^2+b^2)^{1/2}$ produces a tomato colour index (Richardson and Hobson 1987). Fruit firmness was determined by recording the compression distance of whole fruit as a result of an applied force of 3 Newton, using an Instron 4300 series Universal Testing Machine (Polderdijk et al. 1993). Four measurements were performed per fruit. Ethylene production of ripening fruit was measured by enclosing them in airtight 2 liter glass vials, allowing ethylene to accumulate for 1 hour. Samples of the headspace were analysed for ethylene using a Chrompack type 437A GC, equipped with a stainless steel column filled with Alumina GC (Chrompack) and a flame ionisation detector.

Northern blotting

Tomato pericarp tissue was sampled at designated timepoints and frozen immediately in liquid nitrogen. Tissue was taken from two randomly chosen tomatoes. RNA was extracted as described by Chang et al. (1993). Contaminating carbohydrates were removed by an additional precipitation with 35% (v/v) ethanol. Sixteen µg of total RNA were fractionated on 1.2% agarose gels containing 2.2 M formaldehyde. The fractionated RNA was transferred to positively charged nylon membranes (Roche) according to standard procedures (Ausubel et al. 1995). Membranes were hybridised at 42 °C using Ultrahyb hybridisation buffer (Ambion) according to the manufacturer's instructions. Random prime ^{32}P-labelled DNA probes were generated using Hexanucleotide Mix (Roche).

All probes were derived from cloned cDNA sequences of the respective genes. cDNA was made from fruit RNA using the Marathon cDNA Amplification Kit (Clontech). Fragments were amplified by PCR using specific primers for each sequence (see below) and cloned into pGEM-T Easy (Promega). Plasmid DNA was isolated by miniprep (Qiagen), and used in subsequent PCR reactions to obtain template DNA fragments for random prime labelling reactions.

The EXP1 probe used was derived from the *EXP1* coding region. Two specific synthetic oligonucleotides (forward: 5'-ATG GGT ATC ATA ATT TTC ATC C-3'; reverse: 5'-TAA GTT ACA GGA ACA ATG CCA GCG-3') were used to generate a 470 bp fragment corresponding to the 5'-end of the *EXP1* coding region (Rose et al. 1997b; GenBank accession number U82123). The same primers were used for subsequent amplification of template DNA fragments. The *ACO1* gene-specific probe was derived from the cloned 3'-untranslated region. Two synthetic oligonucleotides (ACO1/F3UTR: 5'-TGC AAG TGC TTA GAT CCC AAT TC-3'; oligo dT) were used to generate a 226 bp fragment that was identical to the fragment described by Barry et al. (1996) (accession number A35021). Fragments used in labelling reactions were amplified with ACO1/F3UTR and M13Forward. The *PSY1* gene-specific probe was derived from the cloned 3'-

untranslated region. Two synthetic oligonucleotides (PSY/F3UTR: 5'-TCA AAG ATA AAG CAT GAA ATG AAG-3'; oligo dT) were used to generate a 200 bp fragment corresponding to the 3'-end of *PSY1* mRNA (Ray et al. 1987; *pTOM5*; accession number A21360). Fragments used in labelling reactions were amplified with PHY/F3UTR and M13Forward. As a control for RNA loading, all blots were hybridised with a DNA probe derived from a gene encoding the tomato 60S ribosomal protein L17. Membranes were washed at a final stringency of 0.2 x SSC, 0.1% SDS, 42 °C. Hybridisation signals were measured with a STORM 860 phosphor-imager (Molecular Dynamics) and quantified using ImageQuant software (Molecular Dynamics). The quantified hybridisation signals depicted in Figures 7.2 and 7.3 were corrected using the ribosomal signal as a reference.

7.3. Results

Colour development

At harvest, mature green (MG) tomatoes had a tomato colour index (TCI) value of around –16 (Figure 7.1.A1), breaker (BR) fruit had a TCI value of about –10 (Figure 7.1.A2), orange (OR) fruit a value of 18 (Figure 7.1.A3), and red ripe (RR) fruit a value of 37 (Figure 7.1.A4).

1-MCP almost completely inhibited colour development of MG fruit, with the first signs of coloration occurring 15 days after the start of the experiment, i.e. one week after the second 1-MCP treatment (Figure 7.1.A1). After 18 days, treated tomatoes had not yet reached the OR stage, while control fruit had almost obtained the red colour associated with the RR stage. Colour development of BR fruit was also inhibited by 1-MCP, although these fruit reddened slightly during the first 15 days. Control BR fruit reached a red colour after 8 to 11 days (Figure 7.1.A2). 1-MCP treatment significantly delayed further colour development of OR fruit (Figure 7.1.A3). RR tomatoes did not show any further colour development, irrespective of treatment (Figure 7.1.A4).

Fruit softening

1-MCP delayed softening of MG (Figure 7.1.B1) and BR fruit (Figure 7.1.B2). Firmness, of MG fruit was similar to that of BR fruit (compare Figure 7.1.B1 to 7.1.B2). Softening of OR tomatoes, with a slightly higher initial compression value, was also delayed when treated with 1-MCP (Figure 7.1.B3). It is difficult to assess whether 1-MCP inhibited softening of RR fruit, as measurements were scattered and showed high standard errors of the mean values. However, from day 8 on, the average compression value of 1-MCP treated tomatoes was always lower than that of control tomatoes (Figure 7.1.B4).

Ethylene production

In 1-MCP treated MG fruit, the occurrence of a rise in ethylene production was delayed and production rates remained less than 0.2 nl g^{-1} h^{-1}. The inhibition of ethylene production disappeared after 15 days (8 days after the second treatment). Ethylene production of control MG fruit peaked at day 8, with an ethylene production rate of 4.5 nl g^{-1} h^{-1} (Figure 7.1.C1). BR tomatoes displayed high initial ethylene production rates that decreased after 1-MCP treatment, but which increased again at day 15. Ethylene production rates of control BR tomatoes initially increased to a peak value of 4.9 nl g^{-1} h^{-1} at day 5, but then gradually decreased (Figure 7.1.C2). OR fruit produced 7.4 nl g^{-1} h^{-1} ethylene when harvested, but ethylene production rates decreased during the experiment, both in treated and non-treated tomatoes. However, in 1-MCP treated fruit, a much sharper initial decrease, followed by an increase at days 15 and 18, was observed (Figure 7.1.C3). No clear differences were observed between ethylene production of 1-MCP treated and control RR tomatoes (Figure 7.1.C4).

FIGURE 7.1. *Colour development (**A**), firmness (**B**), and ethylene production (**C**) during ripening of tomato fruit harvested at the mature green (MG; **1**), breaker (BR; **2**), orange (OR; **3**), or red ripe stage (RR; **4**) in non-treated fruit (●) and 1-MCP treated fruit (▲). Arrows indicate the timepoints of 1-MCP treatments. The standard error of the means is represented by vertical bars. Increasing values of the tomato colour index (TCI) indicate decreasing green and increasing red; increasing values of compression (in mm) indicate increased softening of the fruit.*

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FIGURE 7.1

PSY1 mRNA levels

Induction of *PSY1* was clearly visible during post-harvest ripening of MG tomatoes, with peak mRNA levels detected after 11 days. In MG fruit treated with 1-MCP, however, *PSY1* induction was blocked until day 14. At day 18, *PSY1* mRNA levels in treated fruit were comparable to maximum levels observed in control fruit (Figure 7.2.A1). Control BR fruit already contained relatively large amounts of *PSY1* mRNA. Transcript levels peaked at day 4 after harvest and then gradually decreased. Treatment with 1-MCP downregulated *PSY1* drastically, but mRNA levels increased again after 11 days (Figure 7.2.A2). Transcript levels in OR tomatoes were relatively high and stayed at comparable levels for 6 days. Although less profound and abrupt, 1-MCP treatment downregulated *PSY1* mRNA levels in OR fruit (Figure 7.2.A3). RR control fruit showed a 3 to 4-fold increase in *PSY1* mRNA within 4 days after harvest, but at day 6 transcript levels had dropped again to basal levels. This increase was absent in RR fruit treated with 1-MCP (Figure 7.2.A4).

EXP1 mRNA levels

EXP1 mRNA levels in MG control tomatoes increased about 1.5 fold shortly after harvest. There was no increase visible in 1-MCP treated MG fruit until 11 days after harvest (Figure 7.2.B1). Control BR fruit showed gradually increasing transcript levels up to 6 days after harvest, then levels rapidly dropped. In BR fruit treated with 1-MCP, *EXP1* transcript levels initially decreased, but had raised 11 and 18 days post-harvest (Figure 7.2.B2). OR tomatoes displayed gradually in

FIGURE 7.2. *mRNA levels of phytoene synthase (PSY1; **A**), expansin (EXP1; **B**), and ACC oxidase (ACO1; **C**) during fruit ripening in tomatoes harvested at the mature green (MG; **1**), breaker (BR; **2**), orange (OR; **3**), and red ripe stage (RR; **4**) in non-treated samples (black bars) and 1-MCP treated samples (grey bars). Northern blot hybridisation signals were quantified, corrected using signals obtained with a ribosomal probe, and expressed as percentage of the maximum value for each fruit stage. Timepoints of 1-MCP treatments were as indicated in Figure 7.1.*

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FIGURE 7.2

creasing levels of *EXP1* mRNA, both when treated and not treated with 1-MCP. However, the increase observed in treated tomatoes was delayed (Figure 7.2.B3). Control RR tomatoes showed an almost 6-fold increase in *EXP1* mRNA levels after 6 days. This increase had disappeared after 18 days. RR fruit treated with 1-MCP first showed reduced mRNA levels, and then (after day 6) increased mRNA levels (Figure 7.2.B4).

ACO1 mRNA levels

ACO1 transcript levels in MG control tomatoes were high until 11 days after harvest, whereas 1-MCP treated fruit exhibited an immediate decrease in mRNA levels, followed by a modest increase at days 11 and 14 (Figure 7.2.C1). *ACO1* mRNA levels in untreated BR tomatoes were high until 6 days after harvest. In contrast, BR fruit treated with 1-MCP showed an immediate and drastic decrease in *ACO1* mRNA levels. At day 6, immediately before the second 1-MCP treatment, transcript levels showed a modest rise (Figure 7.2.C2). In control OR fruit, *ACO1* transcript levels gradually declined. The decline observed in 1-MCP treated fruit, however, was immediate and total (Figure 7.2.C3). Expression levels of *ACO1* in RR control fruit did not exhibit any clear trends. However, mRNA levels in 1-MCP treated samples were always lower than in their control counterparts, with the exception of day 18 (Figure 7.2.C4).

Ripening on the vine versus ripening off the vine

During the experiments described, it was noted that ethylene production of fruit picked at the OR stage was 1.6 times higher than the maximum value observed in tomatoes that had been harvested at the MG stage and ripened further in air (7.4 nl g^{-1} h^{-1} versus 4.5 nl g^{-1} h^{-1} respectively; compare Figure 7.1.C3 to Figure 7.1.C1). Several additional, independent experiments were performed and confirmed this difference in ethylene production rates: the maximum ethylene production of tomatoes detached from the plant at the MG stage varied between 4 and 6 nl g^{-1} hr^{-1}, whereas in fruit that had matured on the vine ethylene production reached values between 7 and 11 nl g^{-1} hr^{-1} (data not shown).

These differences in ethylene production rates were supported by RNA accumulation patterns. To visualise this, the first RNA samples (day 0) of each series (harvested at MG, BR, OR, and RR) were loaded side by side on a separate Northern blot. The detected hybridisation signals, plotted against the corresponding colour values, were compared to the signals of untreated MG fruit (harvested at the MG stage and ripened in air) depicted by the black bars in Figures 7.2 A1, B1, and C1. *ACO1* mRNA levels in attached BR fruit increased 7-fold when compared to initial values in MG tomatoes. Transcript levels in detached fruit hardly increased at all during further ripening (Figure 7.3A). Essentially the same was observed for *EXP1*. *EXP1* transcript levels in detached fruit hardly rose, whereas a 3-fold increase was detected in attached fruit (Figure 7.3B). In contrast, there was little difference between maximum *PSY1* mRNA levels in detached fruit and in fruit that ripened on the vine (Figure 7.3C).

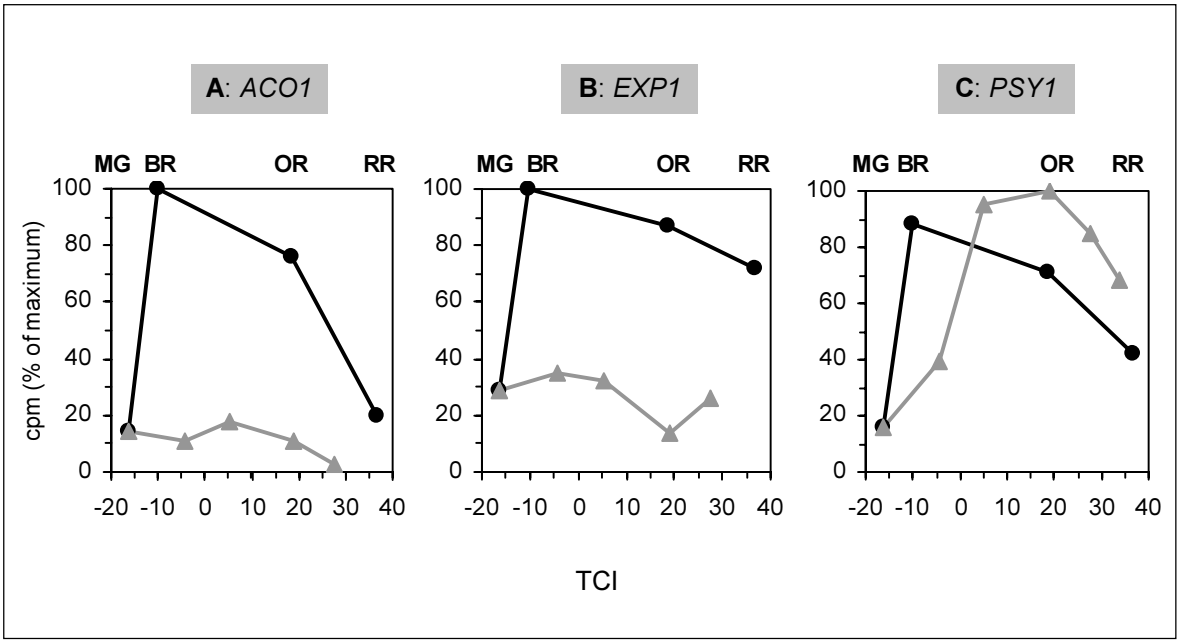

FIGURE 7.3. *Accumulation of ACO1 (**A**), EXP1 (**B**), and PSY1 (**C**) mRNA during fruit ripening in tomatoes attached to vine (●), or detached from the plant at the MG stage and ripened in air (▲). Fruit were classified according to their colour (TCI), and corresponding ripening stages are indicated on top of the graph. Data are presented as percentage of the maximum hybridisation signal observed.*

7.4. Discussion

The effects of 1-MCP, an inhibitor of ethylene action, on several fruit ripening processes of tomato have been examined. Results show that 1-MCP treatment of MG fruit almost completely inhibited colour development, fruit softening and ethylene production (Figures 7.1 A1, B1, and C1), as shown in previous studies (Lelièvre et al. 1997). 1-MCP also displays inhibitory effects on (further) ripening when applied to BR or even OR tomatoes (Figures 7.1 A2, A3, B2, B3, C2 and C3). These results indicate that ethylene perception is required for continuation of the ripening process when it has just been initiated (BR), but also when it has progressed to advanced stages (OR). However, whereas ripening of MG tomatoes could almost be completely blocked for 18 days, further ripening of OR tomatoes was only delayed. These results are consistent with reports describing the effect of various inhibitors of ethylene perception on several other ripening parameters (Tucker and Brady 1987; Sisler and Lallu 1994), and support the concept of ethylene being a rheostat rather than a trigger for fruit ripening (Theologis 1992).

1-MCP altered mRNA patterns of three ripening-related genes (Figure 7.2). The three genes were selected because of their (putative) roles in each of the described physiological changes associated with tomato fruit ripening: colour development, softening and the increase in ethylene production. *PSY1* was chosen since its mRNA levels have been reported to increase during tomato fruit ripening (Giuliano et al. 1993) and are regulated by ethylene (Picton et al. 1993). PSY catalyses an early step in lycopene biosynthesis, the carotenoid responsible for the red colour of tomato fruit (Bartley and Scolnik 1995). *EXP1* was selected as a molecular marker for firmness. Expansins are believed to be involved in cell wall loosening during growth or remodelling (Cosgrove 2000). During tomato fruit ripening *EXP1* mRNA and protein levels are strongly increased (Rose et al. 1997b), and suppression of *EXP1* in transgenic tomato plants results in firmer fruit, whereas overexpression gives rise to softer fruit (Brummell et al. 1999a). *ACO1* was chosen because it is the major ACC oxidase (ACO) transcript accumulating during fruit ripening (Barry et al. 1996). ACO catalyses the oxidation of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene, the last step in the ethylene biosynthesis pathway (Lelièvre et al. 1997).

The observed mRNA levels of *PSY1*, *EXP1*, and *ACO1* in non-treated ripening

tomatoes are largely in line with expression patterns described previously. The most apparent differences concern the relatively high initial expression levels of *EXP1* and *ACO1* in MG fruit (Figs. 2B1 and 2C1). Although *EXP1* and *ACO1* mRNA levels are generally believed to be very low in pre-climacteric MG fruit (Maunders et al. 1987; Barry et al. 1996; Rose et al. 1997b), relatively high transcript levels in MG tomatoes have been reported before (Nakatsuka et al. 1997; Brummell et al. 1999b). This variation may be caused by differences in the actual ripening stage of the used MG fruit, as reflected by differences in initial ethylene production rates. Alternatively, observed discrepancies could be the result of using different tomato varieties. Finally, it cannot be excluded that, in the case of *EXP1*, the relatively high hybridisation signal detected in the MG sample was caused by cross-hybridisation. The probe used contains stretches of sequence that share more than 80% homology with *EXP5* and *EXP6*, two members of the expansin gene family that exhibit expression during fruit development and the earliest stages of fruit ripening. During the remaining stages of ripening, however, *EXP1* is the sole expansin transcript detectable (Brummell et al. 1999b).

The expression of *PSY1*, *EXP1*, and *ACO1* during tomato fruit ripening is believed to be stimulated by ethylene. Various inhibitors of ethylene action have been used to repress ripening-related expression of *PSY1* (*TOM5*), *EXP1* and *ACO1* (*TOM13*) in MG fruit (Davies et al. 1988; Rose et al. 1997b; Nakatsuka et al. 1998). Here, it is shown that *PSY1*, *EXP1*, and *ACO1* transcript levels were suppressed following 1-MCP treatment not only of MG and BR fruit, but also of OR and RR fruit.

The effect of 1-MCP on *ACO1* mRNA levels was greater in OR than in MG fruit. Treatment of MG fruit roughly results in a 3-fold reduction of *ACO1* transcripts, whereas treatment of OR fruit decreases (initially higher) mRNA levels over 40-fold. Eventually, both reductions result in comparable absolute *ACO1* mRNA levels, suggesting that during early (pre-climacteric) stages of ripening *ACO1* expression is relatively independent of ethylene, whereas during later stages of ripening ethylene is a strong inducer of *ACO1* expression.

A significant difference in ethylene production during fruit ripening of tomatoes attached to the vine or detached from the plant at the MG stage was observed (data not shown). These differences in ethylene production are supported by molecular data. Detachment of fruit from the plant not only lowers ethylene

production rates, but also decreases the maximum amounts of *ACO1* and *EXP1* mRNAs detected. Transcript levels of EXP1 in attached and detached fruit have been described before. EXP1 mRNA seemed to reach slightly higher levels in fruit ripened on the vine, but these differences were not commented upon (Rose et al. 1997b). *PSY1* mRNA levels during ripening in attached fruit, however, were similar to the levels observed in harvested fruit (Figure 7.3).

Comparable observations have been made in low-ethylene transgenic fruit. Physical detachment of tomatoes from plants expressing ACC deaminase or from *ACO1*-antisense plants at the onset of ripening markedly exaggerates the slow-ripening phenotype (Klee 1993; Picton et al. 1993). Lower internal ethylene concentrations in detached fruit are likely to play a role in this. Ethylene synthesised during ripening can more easily diffuse out of the fruit when picked, since the stem scar is estimated to be approximately 1000-fold more permeable to gas exchange than the tomato skin (Cameron and Yang 1982). As a result, detached fruit contain less internal ethylene which (considering its autocatalytic nature) has a negative effect on the ethylene synthesis rate, as well as *ACO1* and *EXP1* mRNA levels. In this regard, the lower ethylene levels must be sufficient to normally induce PSY1 mRNA levels, indicating that different genes require different levels of ethylene for ripening-related expression.

Differences in ripening behaviour between attached and detached fruit from *ACO1*-antisense plants have also led to speculations about a "ripening-factor-X" (Picton et al. 1993). This factor, associated with attachment to the plant, can modulate ripening in conjunction with low levels of ethylene, and provides an alternative explanation for the observed differences in *ACO1* and *EXP1* mRNA levels. So-called developmental factors (not ethylene) are already held responsible for the upregulation of *ACO* and *ACS* at the onset of ripening, thereby initiating autostimulatory ethylene production and, as a consequence, climacteric fruit ripening (Oeller et al. 1991; Barry et al. 2000).

In conclusion, physiological changes associated with tomato fruit ripening can be halted or delayed by inhibiting ethylene perception, even when the fruit has reached advanced stages of ripening. Recent additional data showing that application of 1-MCP to ripe tomatoes results in an increase in postharvest life based on fruit appearance (Wills and Ku 2002) confirm these findings. Suppressed transcript levels of various ripening-related genes may underlie these 1-MCP induced effects on postharvest development. Although interrupting the rip-

ening process early inhibits development of the fruit more strongly, this can have negative effects on sensory characteristics such as volatile formation, as was shown in ACO antisense melon (Ayub et al. 1996). Therefore, it might be of interest to direct attempts at interfering with the ripening process (by using either ethylene action inhibitors or transgenic approaches) towards later stages of ripening.

7.5. Acknowledgements

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8

cloning of the promoter regions of two fruit-specific genes from tomato and their functional analysis by particle bombardment

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Summary

Tomato fruit ripening involves a highly regulated series of biochemical, physiological, and structural changes controlled by the plant hormone ethylene. During the advanced stages of ripening, genes encoding for an endo-ß-1,4-glucanase (CEL2) and an expansin (EXP1) are highly expressed. The 5' flanking regions of these two fruit-specific genes were cloned. The 1341 bp long *EXP1* promoter fragment contained a 19 bp stretch also present in the promoter of fruit-specific polygalacturonase (PG) gene and two putative ethylene responsive elements (EREs). The 660 bp long *CEL2* promoter fragment did not contain any putative EREs. Several promoter deletion constructs were transiently expressed in fruit tissue by particle bombardment, using firefly luciferase as a reporter gene. Some positively and negatively regulating regions could be identified, but it was concluded that this method is not suitable to readily analyse promoter activity in tomato fruit tissue.

8.1. Introduction

As both fresh and processed fruit form an important part of our diet, there is an ever-increasing demand, at least in western society, for improved fruit quality. Tomato has long been the subject of extensive research efforts aimed at improving fruit quality. Most effort has been put into attempts to prevent or delay fruit ripening. Tomato fruit ripening corresponds to a highly regulated series of biochemical, physiological and structural changes. This process is believed to be initiated and controlled by ethylene (C_2H_4), a gaseous plant hormone that is involved in numerous aspects of plant growth and development (Lelièvre et al. 1997). The application of inhibitors of ethylene action (Beyer 1976; Sisler and Yang 1984; Sisler and Serek 1997) has been used over the years to prevent tomato fruit from ripening.

The cloning of genes involved in ethylene biosynthesis (reviewed by Lelièvre et al. 1997) and perception (reviewed by Chang and Shockey 1999) allowed a molecular biological approach. Transgenic plants with altered ethylene production levels have been used to grow tomatoes with drastically changed ripening phenotypes (Hamilton et al. 1990; Klee et al. 1991; Oeller et al. 1991). Several other fruit quality characteristics, such as firmness (Schuch et al. 1991; Kramer and Redenbaugh 1994), colour (Bird et al. 1991), flavour (Speirs et al. 1998; Lewinsohn et al. 2001), and provitamin A content (Römer et al. 2000), have also been modified using transgenic plants.

In most cases, expression of the transgene is driven by the CaMV 35S promoter. This promoter is believed to constitutively express the corresponding transgene at high levels in all plant cells (Odell et al. 1985). However, non-specific, constitutively high expression of transgenes may cause too strong effects and/or side effects. For example, tomato plants expressing the antisense *ACS2* gene show a 99.5% inhibition of ethylene production, resulting in fruits that never turn red and never grow soft or develop an aroma (Oeller et al. 1991). In melon, expression of antisense *ACO* using the CaMV 35S promoter has negative effects on sensory characteristics such as volatile formation (Ayub et al. 1996). Expression of a dominant mutant allele of the *A. thaliana ETR1* gene encoding an ethylene receptor in both tomato and petunia greatly reduces adventitious root formation, limiting horticultural performance (Clark et al. 1999; Gubrium et al. 2000).

Therefore, the transgenic approach to control tomato fruit ripening should better involve fruit- and ripening-specific gene promoters. The presence of such promoters in the tomato genome, opens up the possibility for interfering with tomato fruit gene expression in a way that is confined in both space and time. Several fruit-specific promoters have already been isolated from tomato, such as the *2A11* promoter (Van Haaren and Houck 1991), the *ACO1* promoter (Blume et al. 1997; Blume and Grierson 1997), the *E8* promoter (Deikman et al. 1992), the *E4* promoter (Montgomery et al. 1993), and the *PG* promoter (Nicholass et al. 1995). Various studies illustrate the possibilities of using these fruit-specific promoters to obtain deviant tomato fruit (Good et al. 1994; Speirs et al. 1998; Chengappa et al. 1999; Griffiths et al. 1999; Sandhu et al. 2000; Lewinsohn et al. 2001). In addition, the isolation and characterisation of additional fruit- and/or ripening-specific promoters will add to the understanding of the regulation of ripening-related gene expression.

Expansins are extracellular proteins thought to function in cell wall loosening during growth or remodelling (Cosgrove 2000). During tomato fruit ripening both expansin 1 *(EXP1)* mRNA and protein levels are strongly increased (Rose et al. 1997b; Brummell et al. 1999b; Rose et al. 2000). *EXP1* transcripts can first be detected at the breaker stage of fruit ripening and its abundance increases dramatically at the turning stage, remaining high throughout ripening. *EXP1* is not expressed in roots, hypocotyls, stems and young leaves, suggesting its expression is fruit-specific (Rose et al. 1997b; Brummell et al. 1999b). Expression of *EXP1* is regulated by ethylene, and the fruit-ripening related increase in mRNA abundance is absent in *rin* and *nor*, two ripening-impaired tomato mutants (Rose et al. 1997b).

Cellulases or endo-ß-1,4-glucanases (EGases) are a second family of enzymes implicated in ripening-associated fruit softening (Rose et al. 1997a). Two family members, cellulase 1 *(CEL1)* and *CEL2*, have been cloned from tomato. Although their patterns of mRNA accumulation are somewhat overlapping, *CEL1* mRNA predominates in abscission zones and anthers, whereas *CEL2* predominates in ripening fruit. *CEL2* is mainly expressed during the late stages of tomato fruit ripening, with transcript levels that become first detectable at the breaker stage and increase throughout further ripening. In experiments with NBD it was shown that mRNA accumulation of *CEL2* in fruit is ethylene dependent. In *rin* fruit, *CEL2* mRNA is virtually absent and transcript levels are not restored by

ethylene treatment (Lashbrook et al. 1994; Gonzalez-Bosch et al. 1996).

Here, we report the isolation and sequencing of 5' flanking regions of two tomato genes, *CEL2* and *EXP1*. In addition, we have functionally analysed both promoter regions using transient gene expression of promoter-luciferase fusions. Various deletion constructs were introduced into red ripe (RR) fruit pericarp, mature green (MG) fruit pericarp, and tomato leaves by particle bombardment.

8.2. Material and methods

Plant material

Tomato (*L. esculentum* c.v. Tradiro) plants were grown under standard greenhouse conditions. Tomato fruit were harvested at two stages: mature green (MG; fully developed green fruit) and red ripe (RR; fully red and ripe fruit). Tomato leaves were all harvested when fully expanded, yet showing no signs of senescence.

Cloning of EXP1 and CEL2 5' upstream regions

The 5' upstream regions of *EXP1* and *CEL2* were cloned using the Universal GenomeWalker kit (Clontech). First, adaptor-ligated GenomeWalker Library DNA was constructed according to the manufacturer's instructions. Per gene, two nested gene-specific primers were designed (EXP1/R1: 5'-AACCACCAGAGTAAACACCAGG-3'; EXP1/R2: 5'-AAACAGGCTCCACAACTTAATCC-3'; CEL2/R3: 5'-ATATCACTCCATCTCTCG CCG-3'; CEL2/R4: 5'-AAGGAGTTGAAGAGAAGGAAGAG-3') and used in subsequent PCR reactions in combination with adapter primers AP1 or AP2 (Clontech) and the constructed adaptor-ligated GenomeWalker Library DNA.

A 1341 bp long 5'-upstream region of the *EXP1* gene was amplified as follows: 1 µl adaptor-ligated GenomeWalker Library DNA was added to 49 µl PCR mix 1 [2.6 units Expand Long Template DNA polymerase mix (Boehringer Mannheim), 1x PCR Buffer 1 (containing 1.75 mM $MgCl_2$; Boehringer Mannheim), 0.35 mM dNTPs, 0.3 µM primer AP1, 0.3 µM primer EXP1/R2] and amplified using the following PCR conditions: 30 sec 94 °C; 7 cycles of [3 sec 94 °C, 3 min 72 °C]; 32 cycles of [3 sec 94 °C, 3 min (+ 10 sec added every cycle) 68 °C; 10 min 68 °C. For the nested PCR reaction 1 µl of 50x diluted primary PCR mix was used as a template and added to 49 µl PCR mix 2 [2.6 units Expand Long Template DNA polymerase mix (Boehringer Mannheim), 1x PCR Buffer 1 (containing 1.75 mM $MgCl_2$; Boehringer Mannheim), 0.35 mM dNTPs, 0.3 µM primer AP2, 0.3 µM primer EXP1/R1]. The nested PCR conditions used were: 30 sec

94 °C; 7 cycles of [2 sec 94 °C, 3 min 68 °C]; 33 cycles of [2 sec 94 °C, 15 sec 65 °C, 3 min (+ 10 sec added every cycle) 68 °C]; 10 min 68 °C.

A 660 bp long 5' upstream region of the *CEL2* gene was amplified as follows: 1 µl adaptor-ligated GenomeWalker Library DNA was added to 49 µl PCR mix 1 (CEL2/R3; as described above) and amplified using the following PCR conditions: 30 sec 94°C; 7 cycles of [3 sec 94°C, 3 min 72°C]; 32 cycles of [3 sec 94°C, 3 min (+ 10 sec added every cycle) 68°C]; 10 min 68°C. For the nested PCR reaction 1 µl of 50x diluted primary PCR mix was used as a template and added to 49 µl PCR mix 2 (CEL2/R4; as described above) and amplified using the following PCR conditions: 30 sec 94°C; 6 cycles of [3 sec 94°C, 3 min 68°C]; 32 cycles of [3 sec 94°C, 5 sec 62°C, 3 min (+ 10 sec added every cycle) 68°C]; 30 min 68°C. Fragments were purified from gel (QIAquick Gel Extraction kit, Qiagen) and cloned into pGEM-T Easy (Promega).

DNA sequencing

DNA was isolated using either the QIAfilter Plasmid Midiprep kit or the QIAprep Spin Mini-prep kit (Qiagen). Nucleotide sequence analysis was performed using the dideoxy chain termination method (AutoRead sequencing kit and ALF Express automated sequencer, AP Biotech).

Analysis of promoter sequences

The promoter fragment was analysed for the presence of previously characterised regulatory elements using the PLACE database accessible through the world wide web (Higo et al. 1999).

Cloning of constructs

Promoter deletion constructs (translational fusions) were made with fragments directly amplified from genomic DNA using specific PCR primers (see below) and Expand Long Template DNA polymerase (Boehringer Mannheim). Fragments were cloned into pGL3 Enhancer Vector (Promega) using the pGL3 Enhancer Vector multiple cloning site and restriction sites present in the specific primers. The integrity of each open reading frame was verified by sequencing. Primers and restriction sites used for each construct: p660CEL2-LUC; CPF1 (5'-GTTTTTAA GCGGCCGC AAGCTT TTCATTTA-TATTTGAGTACGTAG-3') and CPR3 (5'-TATTAG CCATGG TATTTGGTTGTGAACTGAGG-3'); HindIII and NcoI. p233CEL2-LUC; CPF2 (5'-GATATATA GCGGCCGC AAGCTT TCAAAAG-TAGGAAAAAAAACCCG-3') and CPR3; HindIII and NcoI. *CEL2* constructs were cloned in front of the ATG start codon of the luciferase coding region using the NcoI restriction site present in the pGL3 Enhancer Vector. p1341EXP1-LUC; EPF2 (5'-GGG AAGCTT GAGCTC AAAGAAAATAAAGAAAAAAGGGAG-3') and EPR3 (5'-TAGGTG AAGCTTCTTAA-

GATTTAATGGAGTTGAGG-3'); SacI and HindIII. p1180EXP1-LUC; EPF3 (5'-GGG AAGCTT GAGCTC GCAGCTAAGTTGGCAAAAAGTG-3') and EPR3; SacI and HindIII. p660EXP1-LUC; EPF4 (5'-GGC AAGCTT GAGCTC TAGTGGGAGCGGAAAATTCG-3') and EPR3; SacI and HindIII. p358EXP1-LUC; EPF5 (5'-GGC AAGCTT GAGCTC TTTAGTTGAACCTCAAATGCG-3') and EPR3; SacI and HindIII. *EXP1* constructs were cloned in front of the ATG start codon of the luciferase coding region using the HindIII restriction site present in the pGL3 Enhancer Vector. As a result, a 34 bp long part of pGL3 Enhancer Vector remained between the end of the *EXP1* 5'UTR and the ATG start codon of the luciferase coding region. The pE35S-GUS plasmid (pAPP34) consists of a duplicated CaMV35S promoter (749 bp plus 851 bp) cloned in front of the *uidA* gene (Romano et al. 2001). All plasmids were propagated in *E. coli* DH5α and purified for bombardment using the QIAfilter Plasmid Midiprep kit (Qiagen).

Particle bombardment

Leaves and fruit were harvested and surface sterilised as described (Montgomery et al. 1993). Leaf discs (about 2 cm in diameter) were directly placed on agar plates containing 0.8% (w/v) agar in H_2O. Fruit discs (about 1 cm thick and 2 cm in diameter) were cut from pericarp tissue, soaked for 5 minutes in CPW12 (Baum et al. 1997), and placed on agar plates containing 0.8% (w/v) agar in CPW12. An amount of 3.3 mg of gold particles (1.6 micron; Biorad) were coated with 6.25 µg DNA consisting of luciferase reporter plasmid DNA and GUS reference plasmid DNA in a 1.5:1 ratio essentially as described previously (Montgomery et al. 1993). Plant material was bombarded with 8 µl (550 µg) DNA-coated gold particles by a helium driven Biolystics system (Biorad) with 60 hPa vacuum. For fruits, a pressure of $9.0x10^3$ hPa was used, for leaves $7.5x10^3$ hPa. Fruit discs were bombarded a second time after flipping the discs on the plate. Bombarded tissue was incubated 24 hrs at 24 °C. Each construct was used in two independent experiments and each experiment included six replica bombardments.

In vivo luciferase activity measurements

Fruit discs were sprayed with a luciferin solution (1mM firefly D-luciferin, sodium salt, 0.01% Tween 80) by using an air-brush dispenser, 1 hour before starting the measurement of luciferase activity. Luciferase activity was imaged with a 2D-luminometer consisting of a liquid nitrogen cooled slow-scan CCD camera (512-TKB, Princeton Instruments). Photon emission was quantified by computer (Metamorph 4.1, Universal Imaging Corp.). Luciferase activity was quantified in relative light units per pixel. Measurements lasted 6 to 12 hours and integration intervals were 30 minutes.

GUS assay

Plant tissue was frozen in liquid nitrogen and pulverised using mortar and pestle. Lysis buffer (1x Cell Culture Lysis Reagent (Promega), 0.275 mM Tris-phosphoric acid (pH=7.8)) was added (0.3 ml per gram of tissue), and the mixture was homogenised in an eppendorf tube using a small pestle. The extract was cleared by centrifugation (20.000xg, 20 min, 4 °C) and assayed for GUS activity as described (Montgomery et al. 1993). Fluorescence was determined with a Biolumin 960 apparatus (Molecular Dynamics).

Determination of expression ratio's

The total integrated luciferase signal during the second half of the experiment was subtracted from the total integrated luciferase signal during the first half of the experiment, rendering an initial total luciferase signal. The average initial total luciferase signal of tissues that had not been shot (n=6) was subtracted from each individual initial total luciferase signal for each construct, rendering the corrected luciferase signal. Individual, corrected GUS activities were obtained by subtracting the average total GUS signal of tissues that had not been shot (n=6) from each individual GUS signal. Ratios of the corrected luciferase signal over the corrected GUS signal were calculated for each individual bombardment. Averaging all ratio's per construct (n=6) resulted in the expression ratio.

8.3. Results

LeCEL2 5' region

A 660 bp long 5' *CEL2* gene flanking region was isolated and sequenced. A putative TATA motif (TATATAAA) was identified at position -84 (relative to the ATG start codon), preceded by a CCAAT motif at position -112 bp. The longest *CEL2* cDNA present in the public databases (accession number U13055) contains 47 bp upstream of the ATG start codon. The promoter fragment was analysed for the presence of (other) previously characterised regulatory elements. A putative ACGT containing ABA-responsive element (ABRE) was identified at position –147 (ACGTGTC; Hattori et al. 2002). This motif partially overlaps with a hexameric

motif called the G-box (CACGTG) that can interact specifically with a family of bZIP transcription factors. G-box elements have been demonstrated to be essential functional components of many stimulus-responsive promoters (Menkens et al. 1995). At position –198 a putative W-box (TTGACC; reverse complement) was found. This elicitor-response element is implicated in the regulation of pathogenesis-related gene expression and specifically binds WRKY transcription factors (Eulgem et al. 1999). The cloned CEL2 5' region also contains a repeated element. The 38 bp long stretch from –660 to –623 is repeated from –492 to –455 (36 out of 38 identical nucleotides).

LeEXP1 5' region

A 1341 bp long 5' *EXP1* gene flanking region was isolated and sequenced. A putative TATA motif (TTATTT) can be identified at position –149 (relative to the ATG start codon), preceded by a CCAAT motif at position –193 (reverse complement). The promoter fragment was analysed for the presence of (other) previously characterised regulatory elements. Two putative ethylene-responsive enhancer elements are present at positions –417 (ATTTCAAA) and –678 (AATTCAAA; reverse complement) (Montgomery et al. 1993; Itzhaki et al. 1994). At position –767, a putative Z-DNA-forming sequence is present (ATACGTGT). This element is believed to be involved in light-dependent developmental expression of the *A. thaliana CAB1* gene (Ha and An 1988). A putative regulatory element identical to the TATCCAY-motif involved in feed-back sugar repression of cereal alpha-amylase gene expression (Hwang et al. 1998; Lu et al. 1998; Toyofuku et al. 1998) is found at position –1267 (reverse complement) relative to the ATG start codon.

BLAST homology searches revealed that a short stretch of 19 bp at position –1212 bp is identical to part of the tomato PG promoter. In addition, slightly modified versions of this stretch are present in the promoters of tomato *ACS2* and *Phaseolus vulgaris* polygalacturonase-inhibiting protein 1 *(PGIP1)*. Alignment, orientation and location of this sequence in each of the mentioned promoter regions are depicted in Figure 8.1.

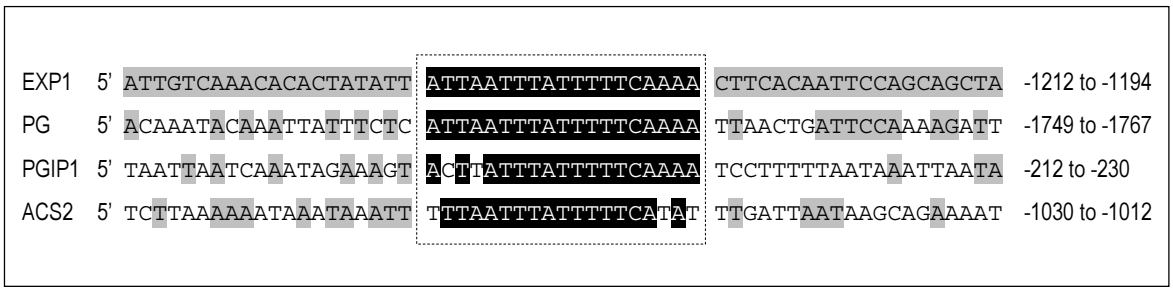

FIGURE 8.1. *Alignment of promoter regions from tomato EXP1, PG (accession number X80908), ACS2 (X59139), and Phaseolus vulgaris PGIP1 (X78417). A 19 bp stretch that is present in both the EXP1 and PG promoter, and identical nucleotides in the PGIP1 and ACS2 promoters are shaded black. Similarities between flanking sequences of EXP1 and the other three sequences are indicated by gray shading. The position of each 19 bp stretch (relative to its respective ATG start codon) is indicated on the right.*

Functional analysis of the promoter regions

In order to functionally analyse the promoter regions of tomato *CEL2* and *EXP1*, a series of 5' deletion constructs (translational fusions) were made. Two *CEL2* deletion constructs, named p660CEL2-LUC and p233CEL2-LUC (Figure 8.2A) and four *EXP1* deletion constructs, named p1341EXP1-LUC, p1180EXP1-LUC, p660EXP1-LUC, and p358EXP1-LUC, were made (Figure 8.2B). The pE35S-GUS construct was used as the reference gene in each bombardment. Luciferase and GUS activity were determined in RR tomato fruit, MG tomato fruit, and tomato leaves. As a control, a construct containing only the luciferase coding region without promoter region (pGL3 Enhancer Vector; pLUC hereafter) was used. Ratios of luciferase to GUS activities for each bombardment are depicted in Figure 8.3.

In RR tomato fruit bombardment with pLUC resulted in a ratio of 0.2. Activities directed by the 660 bp (p660CEL2-LUC) and the 233 bp (p233CEL2-LUC) long *CEL2* promoter regions were 0.4 and 1.1 respectively. In MG fruit, p660CEL2-LUC gave a ratio of 8.0 and p233CEL2-LUC of 9.3, compared to ratio of 0.5 for pLUC. In leaf, bombardment with p660CEL2-LUC resulted in an activity (2.6) lower than that of pLUC (3.2), whereas activity of p233CEL2-LUC was

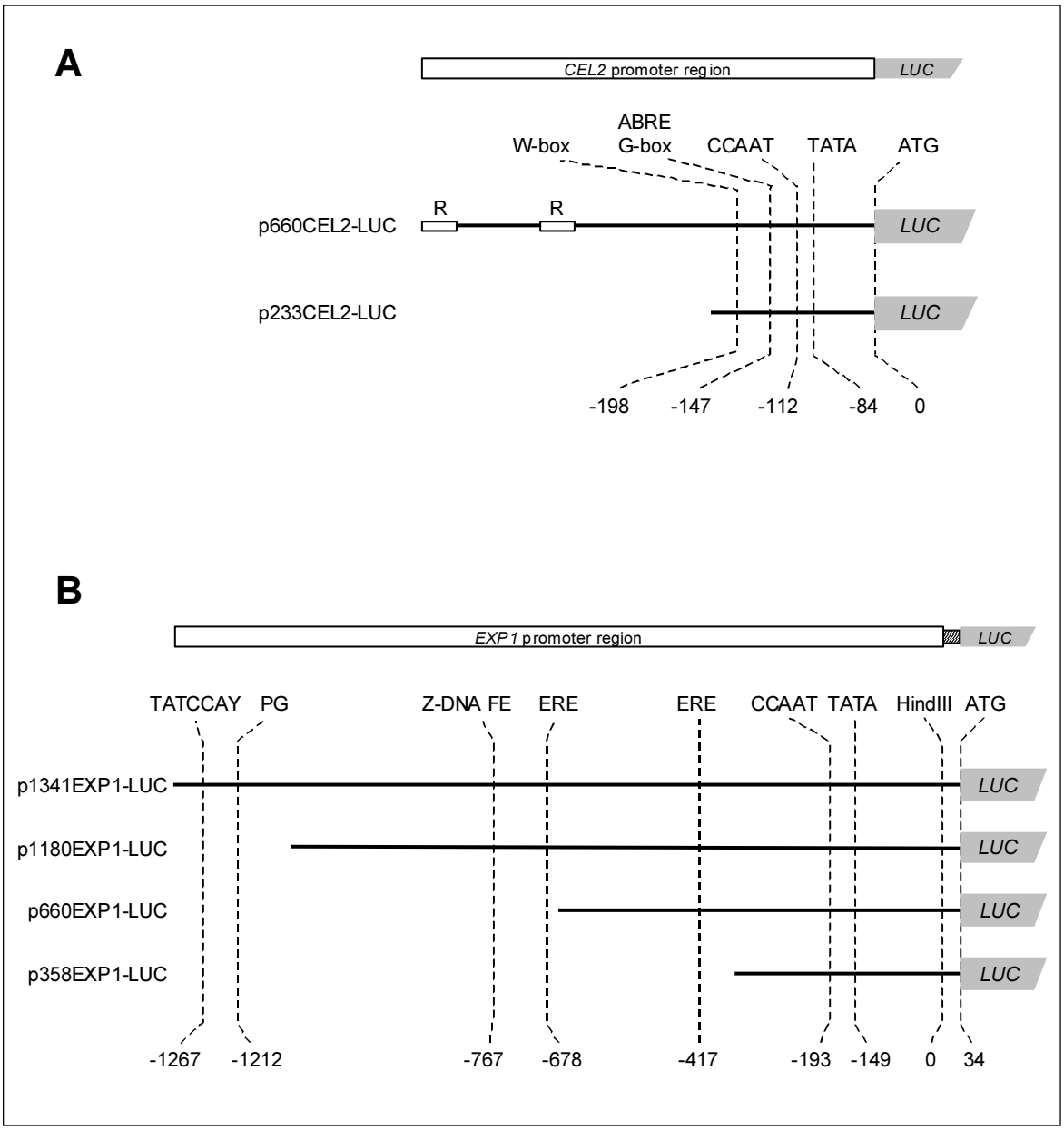

FIGURE 8.2. *CEL2 (**A**) and EXP1 (**B**) promoter deletion constructs (translational fusions). Putative elements are indicated on top, positions relative to the 3' end of the promoter region at the bottom. In between the luciferase ATG start codon and the 3' end of the EXP1 promoter region remained a 34 bp long piece of the pGL3 Enhancer cloning vector. R: repeated element; ABRE: abscisic acid responsive element; ERE: ethylene responsive element; PG: 19 bp stretch identical to corresponding stretch in polygalacturonase promoter; Z-DNA FE: Z-DNA forming element.*

8.7 (Figure 8.3A).

The longest *EXP1* promoter construct, p1341EXP1-LUC resulted in a ratio of 0.5 in RR fruit. Ratios of all other *EXP1* constructs, p1180EXP1-LUC, p660EXP1-LUC, and p358EXP1-LUC, did not exceed the ratio of pLUC in RR fruit (0.2). However, in MG fruit p1341EXP1-LUC resulted in a ratio of 1.6, p1180EXP1-LUC in a ratio of 0.7, p660EXP1-LUC in a ratio of 2.6 and p358EXP1-LUC in a ratio of 3.7. These values all exceed the pLUC ratio of 0.5. In leaf, bombardment with the two shortest *EXP1* constructs, p660EXP1-LUC and p358EXP1-LUC, results in ratio's similar to that of pLUC (3.6 and 3.2 compared to 3.2 respectively), whereas activities directed by p1341EXP1-LUC (0.7) and p1180EXP1-LUC (1.8) were lower than the pLUC ratio (Figure 3B).

8.4. Discussion

Promoter regions from two tomato fruit ripening genes, *CEL2* and *EXP1*, were isolated. Sequence analysis revealed that the *CEL2* 5' flanking region of 660 bp contains a number of putative regulatory elements (Figure 8.2). However, a putative ethylene-responsive element (ERE) as found in the promoter regions of carnation *GST1* (Itzhaki et al. 1994), tomato *E4* (Montgomery et al. 1993) and *ACO1* (Blume and Grierson 1997) is not present in this sequence, even though the *CEL2* gene is clearly ethylene-responsive (Lashbrook et al. 1994; Gonzalez-Bosch et al. 1996). Considering the rather short length of this cloned promoter region, it can not be excluded that one or more EREs are located further upstream. Alternatively, ethylene-responsiveness can be comprised by other, uncharacterised regulatory elements.

The cloned *EXP1* 5' flanking region does contain two putative EREs that could be involved in its observed ethylene-responsiveness (Rose et al. 1997b). Additionally, the *EXP1* promoter region contains a 19 bp sequence element found also in the tomato PG promoter (Figure 8.1). This short sequence is located in a 3.4 kb long region of the PG promoter that directs ripening-specific expression in either orientation. PG is a cell wall degrading enzyme, its mRNA levels increase from the onset of ripening and the gene is not expressed in other organs (Nicholass et al. 1995). This stretch is not part of the *Sol3* transposon that has been identified in promoters of various genes including tomato *PG* and *ACS7*

(Oosumi et al. 1995; Shiu et al. 1998). Slightly modified versions of it can be found in two additional promoter sequences, tomato *ACS2* and *Phaseolus vulgaris* (bean) *PGIP1*. Tomato *ACS2* mRNA, undetectable in MG fruit, is strongly induced in RR fruit. Transcripts were also detected at the onset of petal senescence (Rottmann et al. 1991). Transcripts of the PGIP1 gene accumulate upon

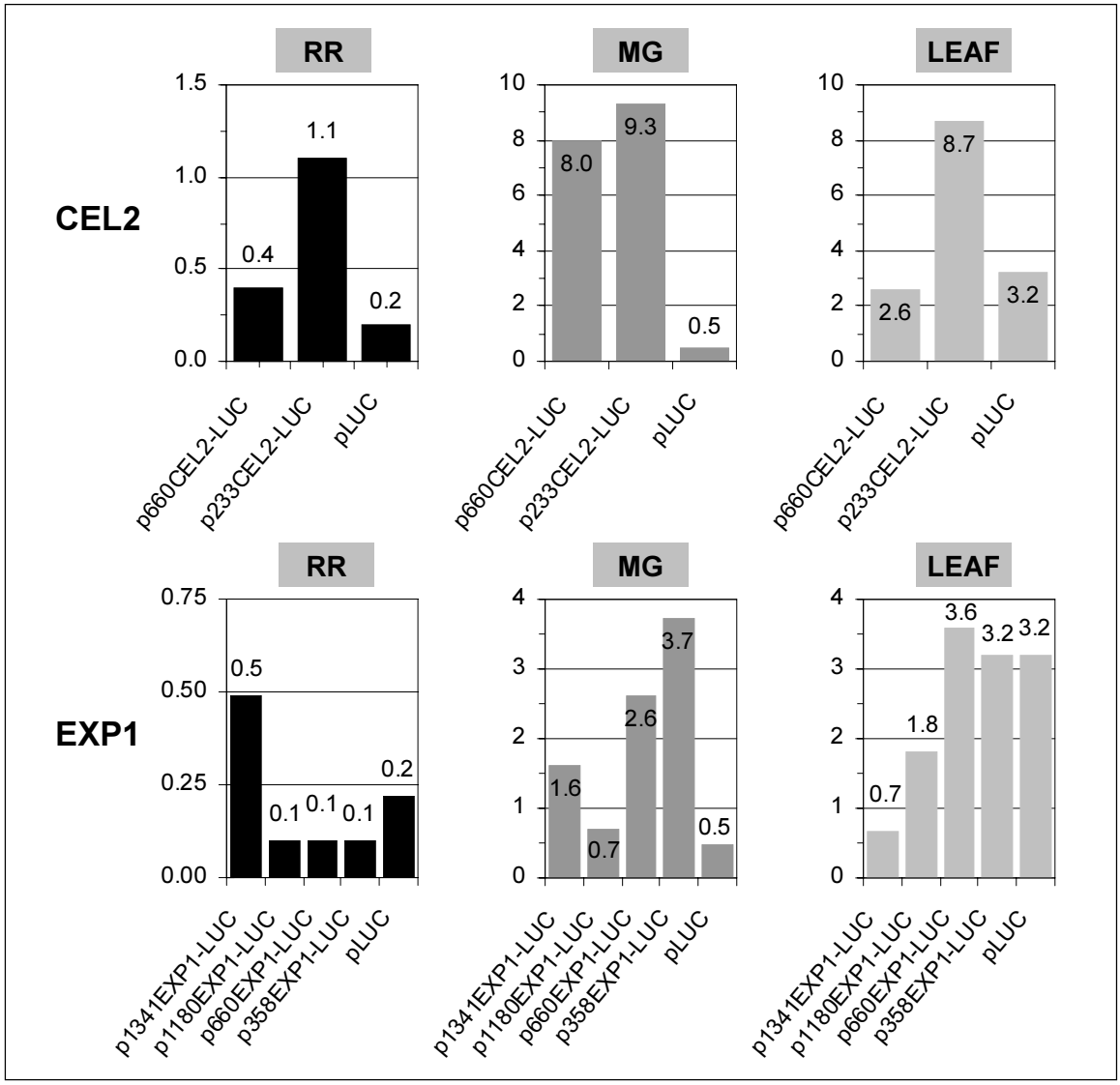

FIGURE 8.3. *Expression ratios of CEL2 and EXP1 promoter deletion constructs in leaf tissue (**LEAF**) and in mature green (**MG**) and red ripe (**RR**) tomato fruit. Ratios were calculated by dividing the measured activity of promoter-luciferase fusions by the measured activity of E35S-GUS.*

wounding and elicitor treatment (Devoto et al. 1998).

Using a transient gene expression system, we have analysed the cloned 5' flanking regions from tomato *CEL2* and *EXP1*. It was determined that in RR tomato fruit tissue, 660 bp of *CEL2* 5' flanking sequence could direct luciferase reporter gene expression that was two-fold higher than expression from the promoterless reporter gene vector pLUC (Figure 8.3A). Expression directed by 233 bp of *CEL2* 5' flanking sequence was almost six times higher (relative to pLUC), indicating that the region between –233 and –660 contains a negative regulatory element.

In MG fruit, expression directed by the 233 bp of *CEL2* 5' flanking sequence was only slightly higher than that of the 660 bp construct (Figure 8.3A). Noteworthy is that ratio's of both constructs in MG fruit are roughly ten times higher than ratios of the same constructs in RR fruit. This in contrast to expression data obtained by Northern analysis, describing that mRNA abundance in RR fruit is markedly higher than in MG fruit (Lashbrook et al. 1994; Gonzalez-Bosch et al. 1996). A possible explanation for this would be the presence of important regulatory element(s) upstream of position -660.

The expression ratio of p660CEL2-LUC in leaf tissue does not exceed the ratio of pLUC, indicating that expression is fruit-specific. However, 233 bp of *CEL2* 5' flanking sequence does direct a relatively low level of expression in leaf tissue (Figure 8.3A). A regulatory element located between position –233 and –660 could be responsible for the observed difference between expression of the 630 bp *CEL2* construct in fruit and in leaf tissue.

Only the longest of the *EXP1* constructs, p1341EXP1-LUC, was able to direct expression of the luciferase reporter gene in RR fruit tissue (Figure 8.3B). It is interesting to see that truncating the region between position –1180 and –1341 results in reporter gene expression ratio lower than that of pLUC. This region contains the 19 bp region that is shared with the tomato PG promoter (Figure 8.1).

Activity of the -1341 *EXP1* promoter construct in MG fruit is comparable to that in RR fruit (relative to activity of the promoterless pLUC construct). This suggests the presence of additional regulatory elements outside the cloned region, as *EXP1* mRNA levels increase drastically during ripening (Rose et al. 1997b; Brummell et al. 1999b). As in RR fruit, deletion of 161 bp, including the 19 bp sequence, results in decreased promoter activity in MG fruit (Figure 8.3b;

compare p1341EXP1-LUC to p1180EXP1-LUC). Therefore this region may be involved in fruit-specificity of the promoter, rather than in ripening-specificity. Further shortening of the promoter (by deletion of the regions harbouring each of the putative EREs) causes drastic increases in expression ratio's in MG fruit, indicating that both regions contain regulatory elements that negatively regulate *EXP1* expression in MG fruit.

The fact that none of the *EXP1* constructs could direct significant expression in leaf tissue corresponds with the fruit-specific pattern of expression of the *EXP1* gene as determined by Northern analysis (Rose et al. 1997b; Brummell et al. 1999b; Hoeberichts et al. 2002). However, it implies that even the shortest promoter construct (358 bp) is sufficient to prevent expression in leaf tissue.

Particle bombardment of ripe tomato fruit tissue with promoter-luciferase fusions as a transient gene expression system has been described before (Montgomery et al. 1993). Several improvements on the original protocol have been published and subsequently used (Manzara et al. 1994; Daraselia et al. 1996; Baum et al. 1997). In our hands, this system generally resulted in very low luciferase activities that could only be detected using very sensitive in vivo luciferase measurements. What is more, adding homogenised tomato fruit tissue to homogenised transgenic tomato leaf tissue has a negative effect on (in vitro determined) LUC activity (data not shown). This suggests that assay conditions in tomato fruit tissue are not optimal.

On average, GUS activity levels (coming from a CaMV35S-GUS construct) determined in leaf tissue were about tenfold higher than in MG or RR tissue, reflecting that transformation efficiency in fruit tissue is relatively low (data not shown). In addition, transformation efficiencies varied greatly per bombardment, as could be seen by differences in GUS activity.

Although low luciferase activity might be due to "lack of strength" of the promoters under study, the low transformation efficiency of tomato fruit by particle bombardment, its variability, and non-optimal assay conditions in fruit tissue, suggest that this method is not suitable for the characterisation of fruit-specific promoter regions.

8.5. Acknowledgements

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9

general discussion

The research described in this thesis was aimed at providing new tools for the improvement of quality and shelf-life of tomato fruit. We focused on strategies that, on the one hand would allow the fruit to ripen and develop the desired taste and flavour, yet on the other hand block the later stages of ripening and deterioration.

Cloning of the promoter regions from two fruit-specific genes

Transgene constructs that are used to improve tomato fruit quality seldom contain tissue and/or timing-specific promoters. The universally used CaMV35S promoter directs expression of target genes at high levels in all tissues (Odell et al. 1985). This often leads to undesirable side effects. A number of fruit- and ripening-specific promoters have been previously isolated from tomato, such as the *2A11*, *ACO1*, *E4*, *E8*, and *PG* promoter. Expression of the accompanying genes, however, is induced during or before the mature green stage (Dellapenna et al. 1986; Lincoln and Fischer 1988; Pear et al. 1989; Barry et al. 1996). We have therefore concentrated on finding new promoters that could serve our specific purposes.

Two ripening specific genes, *LeCEL2* and *LeEXP1*, were selected on the basis of their expression patterns described elsewhere (Lashbrook et al. 1994; Gonzalez-Bosch et al. 1996; Rose et al. 1997b). The *EXP1* promoter contained two putative EREs and a 19 bp long stretch that is also present in the promoter of the tomato PG gene (chapter 8). It will be interesting to see whether (multiple copies of) this sequence can confer fruit-specificity to a minimal CaMV35S promoter.

Functional analysis of these promoters was initiated. Several promoter deletion constructs were transiently expressed in fruit tissue using particle bombardment (chapter 8). Although some positively and negatively regulating regions could be identified, it was concluded that this method is not suitable to readily analyse promoter activity in tomato fruit tissue, contrary to previous reports describing the use of this method (Montgomery et al. 1993; Manzara et al. 1994; Baum et al. 1997). Signal intensities were very low in general and highly variable between repeated bombardments. This is most likely due to the low transformation efficiency of tomato fruit tissue caused by the high water content of fruit tissue. Although more time-consuming, transgenic plants containing a series of deletion constructs for each promoter will be a better tool to functionally analyse fruit-specific promoters.

Besides particle bombardments, an attempt was made to obtain transgenic tomato plants expressing a construct that contained translational fusions of the cloned 670 bp long *CEL2* promoter fragment in front of the firefly luciferase *(LUC)* reporter gene. Such plants would reveal in vivo reporter gene expression directed by the cloned *CEL2* promoter fragment. Unfortunately, only four transgenic plants could be obtained. Fruit from three of these plants did not display any LUC activity, whereas one plant displayed very high constitutive LUC activity throughout the whole plant. These plants could therefore not be used to study the isolated promoter region (data not shown).

Ethylene perception is required at advanced stages of tomato fruit ripening

In addition to new promoters that may be used in improving tomato fruit quality, we have also looked for appropriate genes to use as a target. Most approaches to inhibit tomato fruit ripening are directed towards blocking ethylene biosynthesis or perception. Ethylene plays a very important regulatory role in climacteric fruit ripening. Its function in co-ordinating the onset of fruit ripening by inducing the expression of numerous ripening-related genes is well described. However, much less is known on the role of ethylene during the later stages of ripening. It has been postulated that ethylene is needed throughout the ripening process (Theologis 1992). The evidence published so far is strongly supported and further extended with molecular data by the observations described in chapter 7.

Ethylene-inducible genes such as *PSY1*, *EXP1*, and *ACO1* constantly need ethylene in order to remain transcriptionally active during fruit ripening. Along with downregulation of gene expression caused by inhibiting ethylene perception, (further) development of fruit colour, softening, and climacteric ethylene production are inhibited or delayed (chapter 7). Inhibition of (further) ripening by blocking ethylene biosynthesis or perception at the advanced stages is therefore feasible and could potentially combine improved flavour and aroma with improved shelf-life.

Besides genes involved in ethylene signalling, other possibilities were considered. It was hypothesised that PCD might play a role during tomato fruit ripening, particularly at the late stages. The occurrence of DNA laddering, an apoptotic hallmark, during postharvest senescence of asparagus and petals of cut freesia flowers has been reported (Yamada et al. 2001; Eason et al. 2002). There is also evidence that postharvest-senescence in broccoli shows a number of similarities with developmental senescence, a process that involves PCD (Page et al. 2001). Targeting PCD regulatory genes by specific inhibitors or through transgenic approaches could subsequently become a new means to improve fruit quality.

Establishment of tomato cell suspensions as a plant PCD model system

It is clear that PCD is an intrinsic part of many (developmental) processes in plants. One could argue that all cell death preceded by gene transcription or de novo protein synthesis per definition is PCD. However, the existence of general, recurring (apoptotic?) processes and mechanisms in plant PCD is much less clear. It seems likely that in plant cells, as in animal cells, once the cell death signal has been perceived, the various signalling pathways feed into a common (or limited number of) PCD pathway(s). In chapter 2, it is outlined that there is a large amount of data suggesting that certain cell death mechanisms are conserved between animals and plants. Assuming that plant PCD does use a conserved mechanism, cell death activated in a cell culture will share fundamental similarities with whole-plant developmental or defence-activated cell death. Therefore, understanding of the cell death program in cell cultures (in vitro) might be directly relevant to understanding the regulation and mechanisms of

plant PCD during growth and development. The establishment of such a model system is described in chapter 3.

There are a number of reasons that make cell cultures an attractive system to study the mechanisms of PCD. Cell cultures can be easily used to generate large quantities of cells. It is relatively simple to examine cell death quantatively by using various vital stains. Compounds can be easily added (or removed) from cultures, and cells are relatively accessible for analysis. Cell cultures also give rise to a relatively homogeneous populations of cells. Whereas PCD in planta often occurs in a limited number of cells among large numbers of surviving cells, cell death in cell cultures can be induced in a relatively synchronised fashion. This results in a high proportion of cells that actually undergo PCD relative to cells that do not.

Biochemistry of chemical-induced PCD in tomato cell suspensions

Chemical-induced PCD in tomato cell suspensions is accompanied by morphological and biochemical features characteristic for animal apoptosis (chapter 3). Moreover, cell death can effectively be blocked by specific inhibitors of animal caspases, indicating that caspase-like proteolytic activity is involved in this apoptotic cell death pathway (chapter 3). This demonstrates that PCD in our model system shares important features with animal apoptosis.

Further studies have revealed that cell death is preceded by a transient increase in H_2O_2 production. Both cell death and H_2O_2 release can be blocked by the NADPH oxidase inhibitor diphenyl iodonium (De Jong et al. 2002). Inhibition of either ethylene synthesis or ethylene perception blocks H_2O_2 production and PCD. In itself, ethylene is insufficient to trigger H_2O_2 production and cell death, yet exogenous ethylene greatly enhances chemical-induced release of H_2O_2 and subsequent PCD (De Jong et al. 2002). Interestingly, a serine protease inhibitor and a mammalian caspase inhibitor both inhibit (camptothecin-induced) cell death, but not (camptothecin plus) ethylene-stimulated cell death (De Jong et al. 2002). Ethylene-stimulated cell death, on the other hand, could be blocked by a mammalian MAPK inhibitor (PD98059).

The plasma membrane calcium channel blocker $LaCl_3$ is also capable of blocking H_2O_2 release and cell death (chapter 5; De Jong et al. 2002). Moreover,

it prevents the accompanying changes in gene expression (chapter 5). Conversely, calcium influx in combination with ethylene treatment is sufficient to induce cell death (De Jong et al. 2002). Furthermore, preliminary results demonstrate that, although expressed at very low levels, a tomato gene encoding a CDPK is upregulated during chemical-induced PCD (J. Leclercq and F.A. Hoeberichts, unpublished results). CDPKs can activate protein phosphorylation cascades, thereby providing a means for downstream signal transduction (Harmon et al. 2000). The possibility that elevated Ca^{2+} levels induce NADPH oxidase activity during plant-pathogen interactions has been brought forward more than once (Blumwald et al. 1998; Bolwell 1999). These observations strongly suggest a regulatory role for calcium signalling during PCD in our model system.

This model system thus provides a relatively simple tool for straightforward biochemical studies aimed at identifying plant PCD signal transduction pathways. In the future, transgenic approaches will also be used to uncover or confirm the involvement of (additional) signalling pathways.

Gene expression during chemical-induced PCD

The model system also proved to be a rapid and reliable means to determine mRNA accumulation patterns of known genes that had been implicated in PCD previously.

As described in chapter 2, it has been suggested that DAD1 is a conserved suppresser of PCD, both in animals and plants (Sugimoto et al. 1995). However, the exact function and role of DAD1 in PCD is still subject to speculation. Tomato also possesses a *DAD1* gene that encodes for a protein highly homologous to other known DAD1 proteins (chapter 4). During chemical-induced PCD in tomato suspension cells, the expression of *DAD1* shows a very limited decrease to still clearly detectable levels (chapter 5). This does not suggest a significant role for DAD1 as a suppresser of PCD in our model system.

Expression of *LeHSR203* is upregulated during chemical-induced PCD (chapter 5). A strong correlation between tobacco *HSR203J* induction and caspase-dependent HR-related PCD has been described (Pontier et al. 1994; Del Pozo and Lam 1998). The results presented in chapter 5 are in line with the putative role of HSR203J as an active participant in the cell death process, as suggested

by Pontier et al. (1994). The HSR203J protein has been identified as a serine hydrolase (Baudouin et al. 1997), and was suggested to play a role in the limitation of cell death during the HR (Pontier et al. 1999). Likewise, it could control the extent of cell death in tomato cell suspensions.

Metacaspases, a family of proteins distantly-related to animal caspases, have recently emerged as candidate plant caspases. The identification of a metacaspase gene family in the fully sequenced genome of *A. thaliana* (Uren et al. 2000) immediately gave rise to speculation on a putative role as regulators of PCD. By that time, a vast amount of biochemical evidence had already demonstrated the existence of caspase-like activity in plants, and its involvement in plant PCD (reviewed in Woltering et al. 2002). A key paper describing that the *S. cerevisiae* metacaspase displays a caspase-like proteolytic activity and that this activity is induced when yeast is stimulated by H_2O_2 to undergo apoptosis (Madeo et al. 2002), demonstrated unequivocally that metacaspases indeed can function as caspases.

The tomato genome also contains at least two metacaspases (chapter 6). Transcripts of *LeMCA1*, a type II metacaspase from tomato, do not accumulate during chemical-induced PCD in tomato cells (chapter 6). Interestingly, *LeMCA1* mRNA does accumulate during infection of detached tomato leaves with *B. cinerea* (chapter 6). It has been established that this necrotrophic pathogen induces HR-related cell death in *A. thaliana* (Govrin and Levine 2000) and tobacco (Dickman et al. 2001). The upregulation of *LeHSR203*, that occurs simultaneously with the induction of *LeMCA1*, supports the assumption that *B. cinerea* causes an HR-like response in tomato as well (chapter 6). Infection of plant leaves with *B. cinerea* involves rapid production of ROS by the host (Tiedemann 1997). Since the yeast metacaspase is induced by H_2O_2, one could argue that *LeMCA1* expression is induced by the host's oxidative burst. Although release of H_2O_2 also occurs during chemical-induced PCD in tomato suspension cells, the levels of H_2O_2 might be insufficient to induce *LeMCA1* gene expression. It remains to be seen whether LeMCA1 plays a regulatory role during PCD similarly to the role of the yeast metacaspase and the role of mammalian caspases in PCD. It should be noted that the tomato genome contains at least two and possibly more metacaspase genes that could encode proteins with distinct functions. Moreover, LeMCA1 belongs to a different metacaspase subfamily (type II) than the yeast metacaspase (type I).

Gene expression during chemical-induced PCD: novel genes

The cell suspension system was also used to identify novel genes that are differentially expressed during chemical-induced PCD. Chapter 5 describes the cloning of several cDNA fragments derived from genes that are up- or downregulated during PCD.

Clone *CTU2* is strongly induced during chemical-induced PCD in tomato suspension cells. Expression can be inhibited by the calcium channel blocker $LaCl_3$ and a mammalian caspase inhibitor, whereas exogenous ethylene applied in combination with the cell death inducing-chemical stimulates expression. *CTU2*, or *LePIRIN* as the full length clone was designated, displays significant homology to the human *PIRIN* gene (chapter 5; Orzaez et al. 2001). Human PIRIN is a nuclear factor that has the ability to form quaternary complexes with the oncogene BCL3, NF-κB, and the NF-κB DNA binding domain (Dechend et al. 1999). NF-κB is responsible for the cellular changes observed during TNF-triggered apoptosis. Upon activation, NF-κB promotes the transcription of anti-apoptotic genes (Foo and Nolan 1999; Perkins 2000). It is tempting to speculate about the possibility that LePIRIN functions in mediating protein-protein interaction between transcription factors in an NF-κB/IκB-like signalling pathway during plant PCD. The existence of such a pathway in plants has also been suggested elsewhere (Cao et al. 1997; Ryals et al. 1997; Rate and Greenberg 2001; Yan et al. 2002; chapter 2).

A second upregulated cDNA clone is highly homologous to various GSTs. GSTs are believed to protect cells against the damaging effect of ROS that arise from various physiological processes or pathogen attack (Marrs 1996). The mRNA accumulation of CTU1 observed here most likely is a consequence of increased (oxidative) stress.

One of the downregulated clones identified, *CTD1*, is highly similar to early-auxin-responsive *Aux/IAA* genes (chapter 5). These genes encode transcription factors responsible for the regulation of expression of late auxin-regulated genes (Kim et al. 1997). It has been shown that a mitogen activated protein kinase (MAPK) cascade capable of repressing auxin-inducible promoters is activated by oxidative stress (Kovtun et al. 2000). Again, the observed down-regulation of this auxin-responsive factor may be induced by ROS and could be aimed at suppressing auxin signal transduction, at times when growth-promoting properties are no longer needed. *CTD2* corresponds to tomato RSI-1, an auxin-inducible

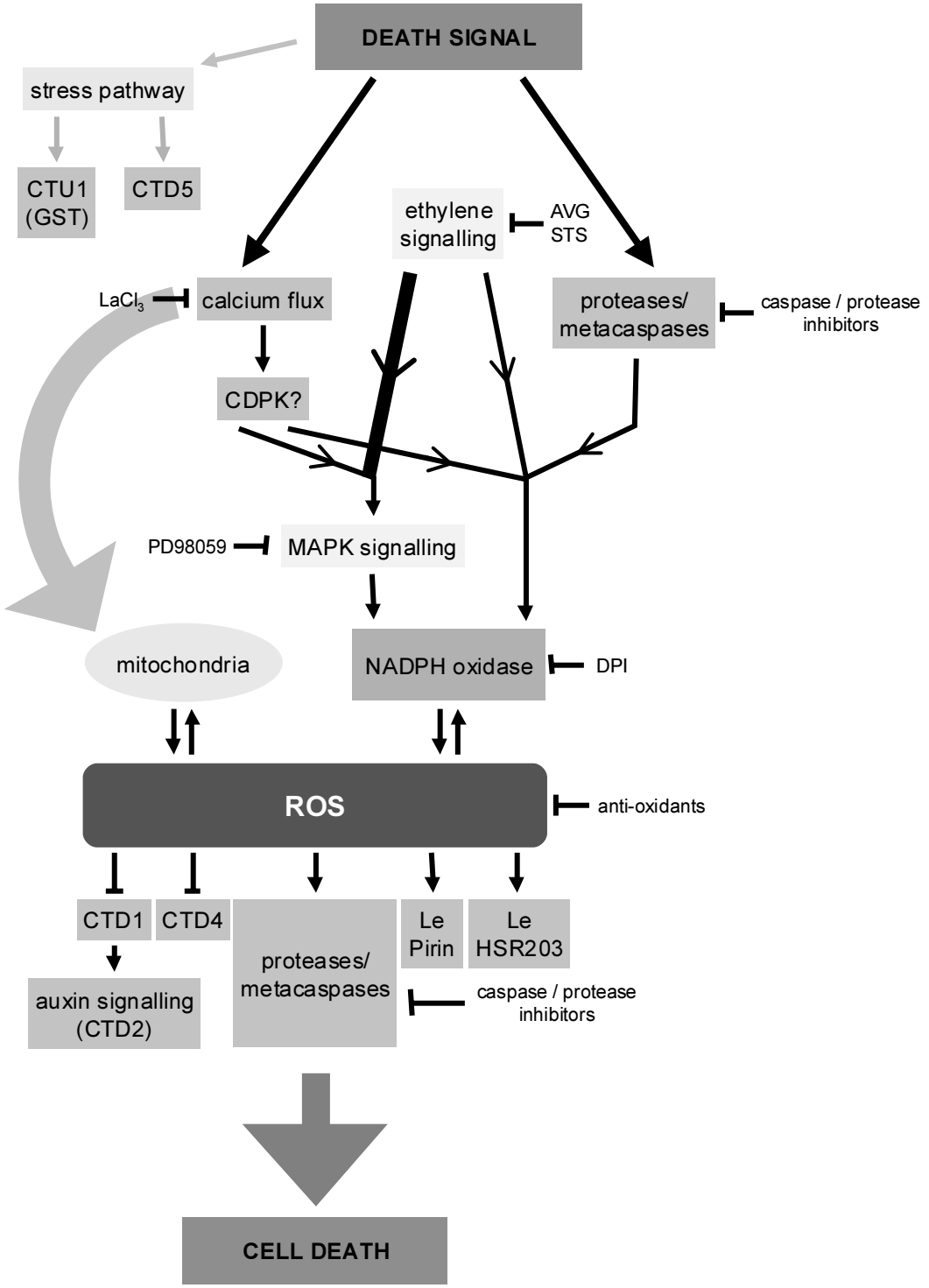
gene (Taylor and Scheuring 1994), and its downregulation could be a result of inactivation of factors such as *CTD1*.

Two more downregulated genes were cloned, *CTD4* and *CTD5*. *CTD4* does not show significant homology to any gene present in the databases, whereas *CTD5* shows limited homology with a proline-rich protein from maize.

The expression studies described in chapter 5 show that adding $LaCl_3$ does not alter the induction of *CTU1* (the GST) and *CTD5*, indicating that this induction is calcium-independent. These data confirm that the signalling pathway leading to cell death can be uncoupled from the activation of more general stress genes, as suggested by Levine et al. (1996). Thus, $LaCl_3$ can be used to distinguish between PCD and stress genes.

A (hypothetical) scheme of the signal transduction routes that are activated during chemical-induced PCD in tomato suspension cells is depicted in Figure 9.1. This scheme includes results from both biochemical and molecular studies. Taken together, the tomato cell suspensions provide a useful model system. Both biochemical pathways involved and differentially expressed genes can be readily identified. Subsequent characterisation of the factors involved may lead to the identification of general regulators of plant PCD. As explained, LePIRIN seems to be an interesting candidate.

FIGURE 9.1. *Signal transduction during chemical-induced PCD in suspension-cultured tomato cells. Cell death-inducing chemicals presumably induce both caspase-like proteolytic activity (possibly metacaspases) and calcium influx (possibly including subsequent CDPK signalling). Together with basal levels of ethylene, these factors induce the NADPH complex resulting in ROS production. In addition, calcium fluxes are known to stimulate ROS production by the mitochondria. Note that ROS may stimulate additional ROS production by both the NADPH complex and the mitochondria in a feedback amplification loop. In the case of high ethylene levels, the NADPH complex is also activated in a protease-independent way, possibly through a MAPK signalling cascade. ROS function as triggers for subsequent downstream events, including suppression of auxin signalling, protease activation, and increased expression of LePIRIN and LeHSR203, that eventually result in apoptotic cell death. The calcium-independent pathway by which CTU1 (a putative GST) and CTD5 are induced is indicated at the top left.*


DEATH SIGNAL
stress pathway
CTU1 (GST)
CTD5
ethylene signalling
AVG
STS
LaCl3
calcium flux
proteases/ metacaspases
caspase / protease inhibitors
CDPK?
PD98059
MAPK signalling
mitochondria
NADPH oxidase
DPI
ROS
anti-oxidants
CTD1
CTD4
proteases/ metacaspases
Le Pirin
Le HSR203
auxin signalling (CTD2)
caspase / protease inhibitors
CELL DEATH

Implications for plant PCD in general

Cell death in our suspension cell model system shares morphological and biochemical characteristics with animal apoptosis. Moreover, LePIRIN and LeMCA1, two putative regulators of plant PCD, are homologous to factors that are involved in animal PCD. Accumulating evidence supports the notion that part of the PCD machinery is functionally conserved between animal and plant kingdoms. Indeed, recruitment of cysteine proteinases to the cell death machinery may be of a very ancient evolutionary origin (Koonin and Aravind 2002). In addition, both ROS and a metacaspase are key regulators of apoptosis in yeast (Madeo et al. 1999; Madeo et al. 2002). ROS are emerging as important PCD signalling molecules, both in our model system and in systems studied by others (Jabs 1999; Vranová et al. 2002).

At the same time, it is obvious from our model system that these core components are under the control of plant-specific regulators, for example ethylene or CDPKs. In other words, they have undergone specific proliferation and specialisation, adapted to plant-specific needs. These needs may not only vary between species, but also from one example of PCD to another, depending on biological context. For example, remobilisation of nutrients is an important aspect of leaf senescence that must be taken into account by the plant. Cell death can only be initiated after remobilisation has taken place. During the HR, however, remobilisation is not an issue, and cell death probably occurs rapidly in an attempt to halt spreading of the pathogen. Thus, biological context may determine specific factors of PCD regulation and is likely to be a cause for the observed differences between various forms of PCD.

PCD during tomato fruit ripening

Having established that DNA laddering, a generally recognised hallmark of apoptosis, occurs during chemical-induced PCD in tomato cells, we tried to show that DNA laddering occurs during post-harvest senescence of tomato fruit. Various samples were analysed, including pericarp tissue from BR, OR, and RR tomatoes, locular gel tissue from BR, OR, and RR tomatoes, and columnella tissue from BR and OR tomatoes. All tomatoes used had been stored for several days

prior to the extraction of genomic DNA. In none of these samples DNA laddering could be detected (data not shown).

Increased or decreased expression of a number of genes has been found to correlate with cell death in the model system of suspension-cultured tomato cells (see above). The expression of some of these genes was studied during post-

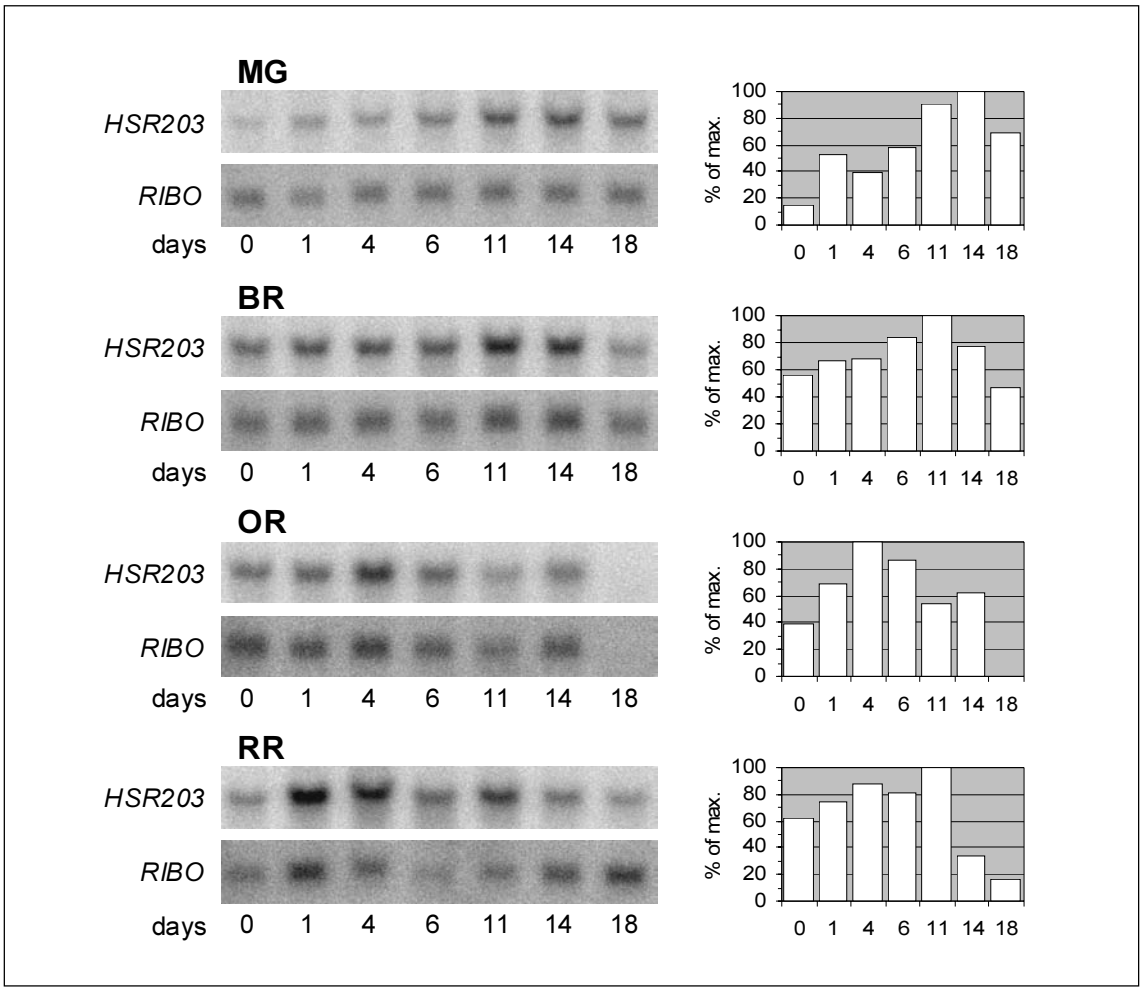


FIGURE 9.2. *Accumulation of LeHSR203 mRNA during postharvest senescence of tomatoes. Fruit were harvested at the MG, BR, OR, or the RR ripening stage and subsequently stored at 20 °C for the indicated numbers of days. Total RNA was isolated from pericarp tissue, blotted and hybridised as described in chapter 7. Sixteen µg of total RNA were used per sample, and the complete LeHSR203 coding region was used as a probe. Pictures are accompagnied by graphs representing quantified signals (normalised using the ribosomal signal).*

harvest senescence of tomato fruit using Northern analysis. *LePIRIN* mRNA could not be detected (data not shown). *LeHSR203* transcripts were clearly detectable in tomato fruit (Figure 9.2). Transcript levels significantly increased during postharvest senescence of MG fruit. The same is true for fruit harvested at the BR, OR, and RR stage. Initial *LeHSR203* mRNA levels were higher in BR, OR or RR fruit than in MG fruit (Figure 9.2). *CTD1* (IAA/Aux factor) mRNA was detected at very low levels in ripening tomato fruit. However, unlike during chemical-induced PCD, transcript levels did not decrease during the course of ripening (data not shown).

Taken together, we did not find sound evidence supporting the notion that PCD occurs during postharvest senescence of tomato fruit. Although *LeHSR203* gene expression patterns during postharvest senescence of tomato fruit were similar to those observed during various forms of plant PCD, those of *LePIRIN* and *CTD1* were not. Moreover, the degradation of genomic DNA (DNA laddering) could not be detected. This may be due to the small number of cells that is actually undergoing PCD at a given point in time. Both genomic DNA degradation and differences in mRNA levels may be obscured by gene expression in the bulk of cells that are still viable.

Future perspectives

The findings presented here imply few possibilities for the improvement of postharvest quality of tomato fruit by targeting plant PCD signalling pathways, as the occurrence of PCD during tomato fruit ripening or postharvest senescence could not be established. However, improvement of tomato fruit quality through inhibition of ethylene biosynthesis or perception at the later stages of ripening seems feasible. The fruit-specific promoters that were isolated for this purpose need to be characterised in more detail.

The model system of suspension-cultured tomato cells has been succesfully established and subsequently used to study PCD in plant cells. In the future, this system will be used further to obtain additional information regarding signalling pathways and regulatory factors of plant PCD. Perhaps that novel and more sensitive markers allow to determine a role for PCD in ripening and postharvest senescence of tomato fruit.

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abbreviations

A. thaliana	=	Arabidopsis thaliana
aa	=	amino acid
ABA	=	abscisic acid
Ac	=	acetate
Ac-DEVD-CHO	=	Ac-Asp-Glu-Val-L-aspartic acid aldehyde
Ac-YVAD-CMK	=	Ac-Tyr-Val-Ala-Asp-chloromethylketone
ACC	=	aminocyclopropane-1-carboxylic acid
ACD	=	accelerated cell death
ACO	=	ACC oxidase
ACS	=	ACC synthase
AEBSF	=	4-(2-aminoethyl)benzenesulfonyl fluoride
APAF	=	apoptotic protease activating factor
BIR	=	baculoviral IAP repeat
BLP	=	BCL2-like protein
B. cinerea	=	Botrytis cinerea
bp	=	basepair
C. elegans	=	Caenorhabditis elegans
CaMV	=	cauliflower mosaic virus
CARD	=	caspase recruitment domain
CDPK	=	calmodulin-like domain protein kinase
CLP	=	caspase-like protein
CTU	=	camptothecin up-regulated
CTD	=	camptothecin down-regulated
DAD	=	defender against apoptotic cell death
DD	=	differential display
DMSO	=	dimethyl sulfoxide
DNA	=	deoxyribonucleic acid
EIL	=	EIN3-like
EIN	=	ethylene-insensitive
ERE	=	ethylene-responsive element
EREBP	=	ERE binding protein
ERF	=	ethylene-responsive factor
FDA	=	fluorescein diacetate
GA	=	gibberellin
GST	=	glutathione S-transferase
HR	=	hypersensitive response

HSP	=	heat shock protein
IAP	=	inhibitor of apoptosis
JA	=	jasmonic acid
L. esculentum	=	Lycopersicon esculentum
LSD	=	lesion simulating disease
M	=	molar
MAPK	=	mitogen-activated protein kinase
MCP	=	methylcyclopropene
MeOSuc-AAPV-CMK	=	methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone
mRNA	=	messenger RNA
NB-ARC	=	nucleotide binding - APAF1, R genes, CED4
NBD	=	2,5-norbornadiene
nDNA	=	nuclear DNA
NF	=	nuclear factor
Nr	=	never ripe
ORF	=	open reading frame
OST	=	oligosaccharyltransferase
PARP	=	poly(ADP-ribose) polymerase
PCD	=	programmed cell death
PCR	=	polymerase chain reaction
PG	=	polygalacturonase
PR	=	pathogenesis-related
rin	=	ripening inhibitor
RNA	=	ribonucleic acid
ROS	=	reactive oxygen species
RT-PCR	=	reverse transcriptase-polymerase chain reaction
S. cerevisiae	=	Saccharomyces cerevisiae
SA	=	salicylic acid
SAM	=	S-adenosylmethionine
SAR	=	systemic acquired resistance
SSCP	=	single strand conformational polymorphism
TE	=	tracheary element.
TLCK	=	Nα-p-tosyl-L-lysine chloromethylketone
TMV	=	tobacco mosaic virus
TNF	=	tumour necrosis factor
TUNEL	=	terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
dUTP	=	2'-deoxyribo-uridine triphosphate
Z-Asp-CH2-DCB	=	benyloxycarbonyl-Asp-2,6-dichlorobenzoyloxymethylketone
Z. elegans	=	Zinnia elegans

summary

Tomato fruit ripening involves a series of highly organised biochemical, physiological and structural changes that are under strict genetic control. The plant hormone ethylene (C_2H_4), in synergy with certain developmental cues, regulates fruit ripening by initiating and co-ordinating the expression of genes responsible for different aspects of the ripening process, such as the respiratory rise, chlorophyll degradation, carotenoid biosynthesis, conversion of starch to sugars, and cell wall degradation. The economical importance of tomato as a crop has resulted in substantial research efforts aimed at the improvement of fruit quality traits, such as shelf life. Inhibition of ethylene action or perception, either by the use of inhibitors or by transgenic approaches, has been proved to be a powerful means to control ripening.

In this thesis, research aimed at providing new tools to further improve tomato fruit quality is described. It was hypothesised that fruit quality could be improved if (further) ripening was inhibited only after the fruit had reached a certain degree of maturity, including the associated stages of coloration and taste development. Genes that encode for proteins involved in ethylene biosynthesis or perception are potential targets for a transgenic approach within this strategy. It was believed that ethylene acts as a rheostat rather than a trigger during tomato fruit ripening, which means that ethylene signalling is required throughout the complete course of ripening. Indeed, treatment of tomato fruit with 1-MCP, a potent inhibitor of ethylene action, delayed colour development, softening, and ethylene production not only in tomato fruit harvested at the mature green stage, but also in the breaker, and orange stages. 1-MCP treatment decreased the mRNA abundance of *PSY1*, *EXP1*, and *ACO1*, three ripening-related tomato genes, in mature green as well as in breaker, orange, and red ripe fruit. These results demonstrate that the ripening process can be inhibited both on a physiological and molecular level, even at very advanced stages of ripening.

In plant transgenic approaches, the most widely used promoter is the CaMV 35S promoter. It expresses the corresponding transgene constitutively and at

high levels in all plant cells. A number of endogenous, fruit-specific tomato promoters, such as the *2A11*, *ACO1*, *E8*, *E4*, and polygalacturonase (PG) promoter, have also been successfully used to obtain transgenic fruit. These promoters are generally active throughout ripening. To obtain DNA sequences that potentially could direct expression of a transgene towards the later stages of ripening, two new promoters were cloned. *CEL2* (encoding an endo-ß-1,4-glucanase) and *EXP1* (an expansin) are specifically and highly expressed during the advanced stages of ripening. The 1341 bp long *EXP1* promoter fragment contains a 19 bp stretch also present in the promoter of fruit-specific polygalacturonase (PG) gene, and two putative ethylene-responsive elements (EREs). The 660 bp long *CEL2* promoter fragment does not contain any putative EREs. Several promoter deletion constructs were transiently expressed in fruit tissue by particle bombardment, using firefly luciferase as a reporter gene. Some positively and negatively regulating regions could be identified, but it was concluded that this method is not very suitable to determine promoter activity in tomato fruit tissue.

In addition to disrupting ethylene biosynthesis or perception, new possibilities to inhibit tomato fruit ripening were searched for. It was postulated that programmed cell death (PCD) might play a role during the advanced stages of ripening and postharvest senescence. PCD is a process aimed at eliminating unnecessary or harmful cells during growth and development of multicellular organisms. It is indispensable for normal development and survival of plants. To study a possible role for PCD in tomato fruit ripening and postharvest senescence, first, a model system of suspension-cultured tomato cells was established. These cells can be induced to undergo PCD by treatment with chemicals that are known to induce a specific form of PCD in animal cells, named apoptosis. This chemical-induced cell death in tomato is accompanied by characteristic features of animal apoptosis, such as typical changes in nuclear morphology, the fragmentation of the nucleus and genomic DNA degradation. Moreover, inhibitor studies suggest that, like in animal systems, caspase-like proteases are involved in this apoptotic-like cell death pathway.

To identify genes potentially involved in plant PCD, changes in gene expression during chemical-induced PCD were studied. Tomato homologues of *DAD1* and *HSR203*, two genes that had been implicated in PCD previously, were isolated. *LeDAD1* mRNA levels are reduced by approximately 50%, whereas

LeHSR203 mRNA levels increase 5-fold during chemical-induced PCD tomato. A differential display approach, used to identify novel genes, resulted in isolation of two up-regulated *(CTU1* and *CTU2)* and four down-regulated *(CTD1, CTD2, CTD4,* and *CTD5)* cDNA clones. *CTU1* shows high homology to various gluthatione S-transferases, whereas *CTU2* shows homology to human *PIRIN*. *CTD1* is highly similar to early-auxin-responsive *Aux/IAA* genes. *CTD2* corresponds to the tomato *RSI-1* gene, *CTD4* is an unknown clone, and *CTD5* shows limited homology with a proline-rich protein from maize. A tomato metacaspase gene, designated *LeMCA1*, that encodes a protein homologous to mammalian caspases was also cloned. Caspases are cysteinyl aspartate-specific proteases that constitute the core component of animal apoptosis. Southern analysis indicates that there is at least one more metacaspase present in the tomato genome. Unexpectedly, *LeMCA1* gene expression is constitutive in suspension-cultured tomato cells. However, *LeMCA1* is rapidly induced upon infection of tomato leaves with the fungal pathogen *Botrytis cinerea*, suggesting a possible role in disease-related cell death.

Taken together, the data derived from the model system of tomato suspension cells suggest that plants make use of cell death pathways that share homology with animal apoptotic pathways. These conserved mechanisms, however, can be activated and regulated by plant-specific factors such as plant hormones

The occurrence of PCD during fruit ripening and postharvest senescence was investigated using DNA laddering and the expression of several genes as markers. No DNA laddering could be detected in tomato fruit tissue. The tomato homologue of a putative negative regulator of apoptosis, *LeDAD1*, is constitutively expressed during ripening of wildtype, *rin*, and *Nr* tomato fruit. *LePIRIN* (a tomato homologue of a human gene encoding a putative nuclear factor that is believed to be involved in NF-κB/IκB signalling) mRNA could not be detected in fruit tissue. *LeHSR203* (correlated with HR-like cell death) transcripts were clearly detectable in tomato fruit and significantly increased during postharvest senescence of MG, BR, OR, and RR fruit. *CTD1* (IAA/Aux factor) mRNA was detected at very low levels in ripening tomato fruit, but, unlike during chemical-induced PCD, transcript levels did not decrease during the course of ripening.

In conclusion, these data do not support the hypothesis that PCD occurs during postharvest senescence of tomato fruit. Although *LeHSR203* gene ex-

pression patterns during postharvest senescence of tomato fruit were similar to those observed during various forms of plant PCD, those of the other genes under investigation were not. In addition, the degradation of genomic DNA (DNA laddering) could not be detected. The results up to now did not lead to novel opportunities for the improvement of tomato fruit quality by targeting plant PCD signalling pathways. However, improvement of tomato fruit quality through inhibition of ethylene biosynthesis or perception at the later stages of ripening seems feasible.

samenvatting

Tijdens de rijping van tomaten *(Lycopersicon esculentum)* vindt er een geordende reeks van biochemische processen plaats die de vrucht doen veranderen wat betreft kleur, hardheid en smaak. Het belang van de tomaat als landbouwproduct heeft ervoor gezorgd dat het begrijpen en het beïnvloeden van de rijping onderwerp van wetenschappelijk onderzoek zijn geworden. Met name het verbeteren van de houdbaarheid geniet als onderzoeksdoel al geruime tijd de aandacht.

De rijping van tomaten wordt gereguleerd door het gasvormige plantenhormoon ethyleen (C_2H_4). Nadat een tomaat zich heeft ontwikkeld tot een volgroeide (maar nog volledig groene) vrucht begint hij grote hoeveelheden ethyleen te produceren. Het ethyleen vormt een signaal voor de vrucht om te gaan rijpen, en het verstoren van het vermogen van een tomatenplant om ethyleen te synthetiseren of waar te nemen heeft tot gevolg dat de vruchten niet rijpen.

De biosynthese en perceptie van ethyleen in de plant is afhankelijk van de aanwezigheid en werking van specifieke eiwitten. Specifieke remmers zijn in staat om de aanmaak of de effecten van ethyleen, en daarmee de rijping, te verhinderen. Voorts is het ook mogelijk om de rijping te belemmeren door, met behulp van transgene technieken, de met deze specifieke eiwitten geassocieerde genen uit te schakelen. Als de rijping echter in een te vroeg stadium wordt geremd, komen smaak en kleur onvoldoende tot ontwikkeling.

Dit proefschrift beschrijft onderzoek naar het vinden van nieuwe mogelijkheden om de kwaliteit van tomaten te verbeteren. Uitgangspunt was de hypothese dat de kwaliteit van tomaten kan worden verbeterd door de vruchten eerst een zekere mate van rijpheid (inclusief de bijbehorende kleur en smaak) te laten bereiken, om ze vervolgens te blokkeren in hun verdere (over)rijping. Zo zou een goede smaak met een goede houdbaarheid gecombineerd kunnen worden. Genen die betrokken zijn bij de biosynthese en perceptie van ethyleen vormen een voor de hand liggend doel bij deze aanpak, aangezien vermoed werd dat ethyleen niet alleen een doorslaggevende rol speelt bij de initiatie van fruitrijping, maar ook onontbeerlijk is voor het continueren van het rijpingsproces. De in hoofdstuk 7

gepresenteerde gegevens laten zien dat het blokkeren van de ethyleenperceptie inderdaad de verdere fruitrijping remt. Tomaten behandeld met 1-MCP, een krachtige remmer van de ethyleenperceptie, worden minder snel rood en zacht en synthetiseren minder ethyleen dan niet-behandelde vruchten, zelfs als de tomaten voor hun behandeling al gedeeltelijk gerijpt zijn. De expressie van drie rijpings-geassocieerde genen *(LePSY1*, *LeEXP1* en *LeACO1)* blijkt onder invloed van 1-MCP sterk verlaagd.

In transgene planten wordt meestal gebruikt gemaakt van de CaMV 35S promotor. Deze promotor zorgt voor een doorlopende hoge expressie van het transgen in alle verschillende plantenweefsels. Het is ook mogelijk om met behulp van fruit-specifieke, uit de tomaat zèlf afkomstige promotoren een transgen aan te sturen. Echter, om DNA sequenties in handen te krijgen die een transgen uitsluitend tijdens de latere stadia van de fruitrijping tot expressie brengen, zijn er twee nieuwe fruit-specifieke promotoren geïsoleerd en gekarakteriseerd (hoofdstuk 8). De met deze promotoren verbonden genen, genaamd *LeCEL2* en *LeEXP1*, komen alleen tijdens de latere fasen van de fruitrijping tot expressie. Er kwam naar voren dat beide promotoren potentiële regulerende elementen bevatten. Bovendien bevat de *LeEXP1* promotor een gedeelte dat identiek is aan een gedeelte uit de promotor van het *PG* gen. Deze korte DNA sequentie is gelokaliseerd in een gedeelte van de *PG* promotor dat geassocieerd wordt met zijn fruit-specifieke karakter. Tevens werd een begin gemaakt met het karakteriseren van verschillende stukken van beide promotoren met behulp van de "particle bombardment" techniek. De resultaten duidden er echter op dat deze techniek niet geschikt is om promotor-activiteit in rijpend tomatenfruit te testen. Expressie studies in transgene tomatenplanten zal moeten uitwijzen of de twee promotoren geschikt zijn voor bovenstaande doeleinden.

Behalve naar het remmen van de biosynthese of perceptie van ethyleen werd naar andere mogelijkheden gezocht om de fruitrijping van tomaat te beheersen. Daarom werd onderzocht of geprogrammeerde celdood (programmed cell death; PCD) een rol speelt tijdens de vruchtveroudering. PCD is een proces gericht op het elimineren van overbodige of schadelijke cellen tijdens de groei en ontwikkeling van multi-cellulaire organismen. Aan de basis van deze cellulaire zelfmoord ligt een genetisch programma dat zorgt voor een stapsgewijze ontmanteling van de cel(len) in kwestie. Indien PCD ook een rol speelt tijdens de

(over)rijping van tomaten, dan zou het beïnvloeden van dit proces nieuwe mogelijkheden bieden om de fruitkwaliteit te verbeteren.

Omdat de kennis van PCD in planten bij aanvang van dit onderzoek beperkt was, is gekozen voor het opzetten van een modelsysteem. De karakterisering van een modelsysteem bestaande uit tomaten-celsuspensies staat beschreven in hoofdstuk 3. Deze suspensiecellen ondergaan PCD na het toevoegen van specifieke chemicaliën. Het PCD proces gaat gepaard met een aantal morfologische en biochemische veranderingen die ook optreden tijdens de meest voorkomende vorm van PCD in dieren, genaamd apoptose. Dit betreft morfologische veranderingen in de celkern, fragmentatie van de celkern en afbraak van genomisch DNA (DNA laddering). Bovendien zijn bepaalde remmers van caspases, specifieke cysteine-proteases die een cruciale rol spelen tijdens dierlijke apoptose, in staat PCD in de celsuspensies te verhinderen.

Om genen te vinden die een rol spelen bij de regulatie van PCD in planten werden veranderingen in genexpressie tijdens PCD in het modelsysteem bestudeerd (hoofdstuk 5). Allereerst werd gekeken naar de genexpressie van twee tomatengenen die homoloog zijn aan genen, *DAD1* en *HSR203*, die vermoedelijk een rol spelen tijdens PCD. Het expressieniveau van *LeDAD1* halveert tijdens PCD, terwijl de hoeveelheid *LeHSR203* mRNA vijfvoudig toeneemt. Vervolgens werd een aantal nieuwe genen geïsoleerd op basis van hun differentiële expressiepatroon. Twee genen *(CTU1* en *CTU2)* vertonen een afnemende expressie tijdens PCD, vier genen *(CTD1*, *CTD2*, *CTD4* en *CTD5)* een toenemende expressie. *CTU1* vertoont veel overeenkomst met genen die coderen voor gluthation S-transferases en is vermoedelijk betrokken bij het onschadelijk maken van oxidatieve stress. *CTU2* vertoont overeenkomst met een humaan gen genaamd PIRIN waarvan vermoed wordt dat het een rol speelt bij eiwit-eiwit interacties tijdens humane apoptose. *CTD1* is sterk homoloog aan *Aux/IAA* genen die coderen voor transcriptiefactoren die de respons van planten op veranderingen in auxine-concentraties reguleren. *CTD2* correspondeert met het tomaat *RSI1* gen, *CTD4* is afkomstig van een onbekend gen en *CTD5* vertoont beperkte gelijkenis met een gen dat codeert voor een proline-rijk eiwit uit maïs.

In hoofdstuk 6 staat de isolatie beschreven van een tomatengen dat codeert voor een metacaspase. Metacaspases zijn een familie van eiwitten die evolutionair verwant zijn aan dierlijke caspases. Het is bekend dat een metacapase in gist *(Saccharomyces cerevisiae)* een caspase-achtige rol speelt tijdens PCD. Het

geïdentificeerde tomaat-metacaspase komt verhoogd tot expressie tijdens infectie van tomatenblad met de schimmel *Botrytis cinerea*, maar niet tijdens PCD in het celsuspensie-systeem. Of metacaspases een rol spelen tijdens PCD in planten blijft vooralsnog onduidelijk.

De gepresenteerde gegevens suggereren dat PCD in planten fundamentele overeenkomsten vertoont met PCD in dieren. In hoofdstuk 2, tevens een uitgebreide introductie over PCD in planten, wordt dit inzicht verder uitgewerkt aan de hand van gegevens uit de literatuur en dit proefschrift. Er wordt beargumenteerd dat de regulering en uitvoering van PCD in planten tot op zekere hoogte verwantschap vertonen met die in dieren. Deze geconserveerde mechanismen kunnen tevens worden aangestuurd door plant-specifieke factoren, zoals bijvoorbeeld ethyleen.

Het optreden van PCD tijdens de vruchtveroudering werd bestudeerd door vruchtweefsel te onderzoeken op DNA laddering. Er kon niet worden aangetoond dat DNA laddering plaatsvindt in rijpende tomaten (hoofdstuk 9). Voorts werd de expressie van een aantal genen tijdens de rijping vergeleken met de expressie tijdens PCD in het modelsysteem. De expressie van *LeDAD1*, een potentiële negatieve regulator van celdood, blijft onveranderd tijdens de rijping van wildtype tomaten, en tomaten van de rijpingsmutanten *rin* en *Nr* (hoofdstuk 4). *LePIRIN* mRNA kon niet worden gedetecteerd in vruchtweefsel. Het expressiepatroon van *LeHSR203* tijdens de vruchtveroudering vertoont wel overeenkomst met het expressiepatroon tijdens PCD. Van *CTD1* mRNA werden wel lage hoeveelheden gedetecteerd in fruit, maar deze nemen niet af tijdens de vruchtveroudering.

Er werd dus vooralsnog geen bewijs geleverd voor het optreden van PCD tijdens de rijping van tomaten. Het lijkt echter wel mogelijk om de fruitkwaliteit te verbeteren door de ethyleenbiosynthese of -perceptie te remmen tijdens de latere stadia van de vruchtrijping.

nawoord

Met deze laatste alinea's van dit proefschrift wil ik graag allen bedanken die mij hebben geholpen bij het tot stand komen van mijn promotie. Slechts een klein aantal van al deze mensen kan ik hier met naam en toenaam noemen.

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Mariëtte, je hebt slechts een gedeelte van mijn promotie van heel dichtbij meegemaakt. Maar dat was wel het laatste en misschien dus wel het zwaarste gedeelte! Bedankt voor de tijd die we niet samen doorbrachten en nog veel meer voor de tijd die we wèl samen doorbrachten. Ik ben gek van je en ik zal er alles aan doen om ervoor te zorgen dat we samen heel ver komen.

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Frank

curriculum vitae

Ik werd geboren op 28 september 1969 in het limburgse plaatsje Horn. Na de plaatselijke lagere school te hebben doorlopen bezocht ik 3 jaar lang de Middenschool te Heythuysen. Deze vorm van middelbaar onderwijs, destijds experimenteel van aard, heeft inmiddels opgehouden te bestaan. Vervolgens stroomde ik door naar het atheneum op het Bisschoppelijk College te Roermond.

In 1988 begon ik met de studie Scheikunde aan de Universiteit van Amsterdam. Tijdens het derde jaar van deze studie koos ik voor de biochemie en de moleculaire biologie. Mijn afstudeerstage bestond uit onderzoek aan de moleculaire mechanismen van peroxisoom-biogenese in bakkersgist *(S. cerevisiae)*. De stage vond plaats op het voormalige E.C. Slater Instituut, thans onderdeel van het Swammerdam Institute for Life Sciences, onder leiding van Prof. Dr. H.F. Tabak. Mijn afstudeerscriptie vormde een actueel overzicht van DNA helicases en hun rol in het repareren van DNA.

Na mijn afstuderen in 1994 kwam ik terecht bij het toenmalige ATO-DLO, waar ik mijn vervangende dienstplicht kon vervullen. Onder de supervisie van Dr. A.D. de Boer kwam ik voor het eerst in aanraking met de moleculaire biologie van planten. Het onderzoek behelsde het karakteriseren van de moleculaire effecten van een koude-behandeling op tulpenbollen. In 1996 startte ik met mijn promotietraject, met Prof. Dr. L.H.W. van der Plas als promotor en Dr. Ing. E.J. Woltering als copromotor. Het onderzoek maakte deel uit van een omvangrijk E.U. project met de naam “Controlled ripening and increased storage life in fruits and vegetables through ethylene control”. In het jaar 2000, na het aflopen van mijn AIO contract, kreeg ik een deeltijdcontract bij het ATO aangeboden. Drie dagen per week heb ik sindsdien gewijd aan het vinden van genen die de bloemveroudering van anjer en iris markeren. De overige twee dagen besteedde ik aan het voltooien van dit proefschrift.