Significance of hydrolytic enzymes expressed during xylem cell death

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"If we are uncritical we shall always find what we want: we shall look for, and find, confirmations, and we shall look away from, and not see, whatever might be dangerous to our pet theories..."

(Sir Karl R. Popper CH, Austro-British Philosopher, 1902-1994)

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Abstract

Xylem is an inherent feature of all vascular plants and functions in water transport and mechanical support. In order to efficiently transport water, xylem cells are reinforced by secondary walls before they undergo programmed cell death and their cell contents are removed by autolysis to create a hollow tube. During their differentiation, xylem cells express various hydrolytic enzymes, such as proteases, nucleases and lipases, but only in a few examples has their role in xylem cell death been characterized. This thesis focuses on the regulatory aspects of xylem cell death and the autolytic cell clearance in vessel elements and fibers of hybrid aspen (*Populus tremula* L. x *tremuloides* Michx.) and in vessel elements of *Arabidopsis thaliana*. Using comparative transcriptomic analysis, candidate genes for fiber-specific cell death processes were identified. Further, a hypothesis is presented on the regulation of thermospermine levels in the vasculature by a negative feedback-loop involving auxin and the class III Homeodomain-Leucine Zipper (HD-ZIP III) transcription factor HOMEOBOX8 (PtHB8). The role of the Arabidopsis METACASPASE9 (AtMC9) in xylem cell death was characterized using molecular tools, such as reporter lines and fluorescent fusion proteins, and electron microscopy (TEM). This showed that cell death initiation is not controlled by AtMC9. Instead, evidence is presented for the involvement of AtMC9 in the *post mortem* autolysis of vessel elements that follows tonoplast rupture and leads to the formation of the hollow conduit. Cell death-associated genes were further observed to be expressed during the emergence of lateral roots in *Arabidopsis thaliana*. This led to the discovery that cells overlying a lateral root primordium undergo cell death, which was demonstrated by detection of DNA degradation and TEM analysis. It is concluded that cell death facilitates emergence of lateral roots through the overlying tissues in a concerted manner with cell wall remodelling. Together, these findings show that although individual hydrolytic enzymes may be dispensable for plant growth and development, their common regulators are the tool for understanding their function and importance.

Sammanfattning

Xylem är en karakteristisk vävnad i alla kärlväxter som leder vatten och mineraler samt har mekanisk stödfunktion. För att effektivt kunna transportera vatten förstärks xylemceller med sekundära cellväggar innan de dör genom programmerad celldöd. Deras cellinnehåll bryts ner genom autolys för att skapa ett ihåligt rör. Xylemceller uttrycker under sin differentiering olika hydrolytiska enzymer, såsom proteaser, lipaser och nukleaser, men bara för ett fåtal av dessa har funktionen under xylemcelldöd kartlagts. Denna avhandling fokuserar på reglering av xylemcelldöden och den autolytiska nedbrytningen av cellen, i såväl kärlelement och fibrer av hybridasp (*Populus tremula* L. x *tremuloides* Michx.) som i kärlelement av backtrav (*Arabidopsis thaliana*). Med hjälp av jämförande transkriptomanalys identifierades kandidatgener för fiber-specifika celldödsprocesser i hybridasp. Vidare utvecklades en hypotes om reglering av termosperminnivåer i vaskulaturen genom en negativ feedback-loop, som omfattar auxin reglering och klass III homeodomän-leucinzipper (HD-ZIP III) transkriptionsfaktorn HOMEOBOX8 (PtHB8). Funktionen av Arabidopsis METACASPASE9 (AtMC9) under xylemcelldöd karakteriserades med molekylära verktyg, såsom reporterlinjer och fluorescerande fusionsproteiner och elektronmikroskopi (TEM). Dessa analyser visade att celldödens initiering inte styrs av AtMC9. Istället presenteras bevis för en roll av AtMC9 i autolysen av kärlelement som sker *post mortem* efter att vakuolen har gått sönder och som slutför bildandet av det tomma kärlet. Genuttryck som associeras med celldöd observerades också under utvecklingen av laterala rötter i *Arabidopsis thaliana*. Detta ledde till upptäckten att celler som ligger ovanför ett lateralrotprimordium dör en programmerad celldöd och visar tecken på DNA-nedbrytning och autolys i TEM-analyser. Slutsatsen av denna studie är att celldöd i samspel med cellväggsmodifiering underlättar utväxten av laterala rötter genom de överliggande cellagren. Sammantaget tyder dessa upptäckter på att även om enstaka hydrolyserande enzymer inte är nödvändiga för växternas tillväxt och utveckling, så kan deras gemensamma reglering nyttjas för att förstå deras funktion och betydelse.

List of Papers

I
Charleen L. Courtois-Moreau, Edouard Pesquet, Andreas Sjödin, Luis Muñiz, **Benjamin Bollhöner**, Minako Kaneda, Lacey Samuels, Stefan Jansson and Hannele Tuominen (2009). A unique program for cell death in xylem fibers of *Populus* stem. *The Plant Journal* 58(2): 260-274.

II
Ana Milhinhos, Jakob Prestele, **Benjamin Bollhöner**, Andreia Matos, Francisco Vera-Sirera, Karin Ljung, Juan Carbonell, Miguel A. Blázquez, Hannele Tuominen and Célia M. Miguel (2013). Thermospermine levels are controlled by an auxin-dependent feedback-loop mechanism in *Populus* xylem. *The Plant Journal* 75(4): 685–698.

III
Benjamin Bollhöner, Bo Zhang, Simon Stael, Nicolas Denancé, Kirk Overmyer, Deborah Goffner, Frank Van Breusegem and Hannele Tuominen (2013). *Post mortem* function of AtMC9 in xylem vessel elements. *New Phytologist* 200(2): 498-510.

IV
Benjamin Bollhöner*, Ute Voß*, Jakob Prestele, Michael Wilson, Kim Kenobi, Corrado Viotti, Domenique André, Amnon Lers, Malcolm Bennett and Hannele Tuominen. Programmed Cell Death in Overlying Tissues Facilitates Lateral Root Emergence. *Manuscript*

*These authors contributed equally to the manuscript.

The papers will be referred to by their Roman numbers in the text.

Contributions as an Author

For **Paper I**, I performed and analysed the viability staining of stems (Figure 3i-k) and participated in design and analysis of the *in silico* comparative transcriptomics (Figure 8 and Table S2). I participated in discussion and writing.

For **Paper II**, I participated with AM and JP in the phenotyping of the hybrid aspens, grown in the greenhouse (Figure 2c-g, Figure 3) and performed viability stainings in stem sections. I participated in discussion of the results and interpretations and writing.

For **Paper III**, I performed the cloning for reporter lines (Figure 1), analysed the expression in reporter lines (Figure 1 and S3), characterised the *atmc9* mutants (Figure 2, S1a-c), analysed protoxylem cell death, and analysed the microarray experiment (all in Figure 2), performed and analysed the TEM analysis (Figure 3 and 4) and the subcellular localisation experiments (Figure 6). I was involved in design of all experiments and discussion of the results. I wrote the manuscript.

For **Paper IV**, I cloned the promoters, created and analysed the *promoter*::*GUS* lines (Figure 1b-e), co-performed and analysed the time lapse series (Figure 1f), designed, performed and analysed TUNEL stainings and TEM (Figure 2a-f), performed the EMS mutagenesis and performed and analysed LR staging assays (Figure 3 and S2) and draw the model (Figure 4). I wrote the manuscript.

Preface

Wood products are important parts of our everyday life. The interests in wood as a raw material dates back to when humans discovered the use of fire for heating and cooking. Nowadays, wood is used for construction and furniture but it is also an important source of biopolymers that have a broad range of industrial applications, ranging from paper products to a variety of chemicals. In more recent times, wood has become attractive as a carbon-neutral source of bioenergy and fuels and the demand for woody biomass is steadily increasing. At the same time, one should not forget the ecological importance of forests and their value for recreation, tourism and other economical uses of the forest and its products.

Understanding wood development may help to increase and optimise yields. The quantitative and qualitative aspects of wood are determined by the properties of the lignocellulosic secondary cell walls of the xylem cells that die a programmed cell death after secondary wall formation. The life time of the xylem cells is one of the limiting factors for secondary wall deposition, and regulation of xylem cell death may therefore offer ways to increase secondary wall thickness, and hence, wood density and biomass production. Knowledge on regulatory aspects of xylem development and cell death may in the future become useful in for instance genetic selection in forest tree breeding or in short rotation time plantations of woody energy crops.

Abbreviations

35S CaMV	35S Cauliflower Mosaic Virus (Promoter)
ACL5	ACAULIS5
Arabidopsis	*Arabidopsis thaliana*
ARF	AUXIN RESPONSE FACTOR
AtHB8	Arabidopsis thaliana HOMEOBOX8
AtMC9	Arabidopsis thaliana METACASPASE9
BFN1	BIFUNCTIONAL NUCLEASE1
BR	brassinosteroid
CesA	cellulose synthase
COMT1	CAFFEATE *O*-METHYLTRANSFERASE1
DNA	deoxyribonucleic acid
GFP	GREEN FLUORESCENT PROTEIN
GUS	ß-glucuronidase
HD-ZIP III	CLASS III HOMEO-DOMAIN LEUCINE ZIPPER
HR	hypersensitivity response
IDA	INFLORESCENCE DEFICIENT IN ABSCISSION
LR, LRP, LRE, LRI	lateral root, -primordium, -emergence, -initiation
LRR-RLK	leucin rich repeat-receptor like kinase
miR/miRNA	microRNA
MP	MONOPTEROS
mRNA	messenger RNA
NAC	NAM, ATAF1/2, CUC2
NBT	Nitro Blue Tetrazolium
ORE1	ORESARA1 (Korean for “long living)
ORF	open reading frame
PCD	Programmed Cell Death
PLCP	papain-like cysteine protease
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
SAC51	SUPPRESSOR OF ACAULIS5 1
SND1	SECONDARY WALL-ASSOCIATED NAC DOMAIN1
TE	tracheary element (vessel/tracheid)
TEM	Transmission Electron Microscopy
VND	VASCULAR RELATED NAC DOMAIN
VNI2	VND INTERACTING 2
XCP1/2	XYLEM CYSTEINE PEPTIDASE1/2
XND1	XYLEM NAC DOMAIN1
ZEN1	ZINNIA ENDONUCLEASE1

Introduction

Vascular plants (tracheophytes) are the dominating plants on earth and colonize most terrestrial ecosystems. In an aerial environment, the uptake of CO_2 through the stomata, which is required for photosynthetic carbon assimilation, is inevitably accompanied by transpiratory water loss. As a consequence, terrestrial plants are highly dependent on efficient water transport from the root-soil interface to the photosynthetic organs in the air. In vascular plants, water transport takes place in a specialized tissue, the xylem (after the Greek *xylon* = wood) that together with the assimilate-transporting phloem (after the Greek *phlios* = bark) forms the vasculature. The xylem can contain a few different cell types, such as tracheids, vessel elements, fibers, xylem parenchyma and ray cells (Esau 1965). The water conducting xylem cells, tracheids and vessel elements, commonly called as tracheary elements (TE), are dead at maturity and form hollow conduits that allow efficient water conductance. These conduits are reinforced against the mechanical forces of the transpiration stream by lignified secondary wall thickenings.

In addition, the reinforcements by secondary cell walls confer support to the entire plant body. In that way, the xylem serves height growth of plants in a dual way, mechanically and hydraulically. It gives the stability that allows plants to grow tall and enables long-distance transport of water, the prerequisite for hydration of the photosynthetic organs of a taller plant. Therefore, the development of the xylem is considered as one of the main factors for the evolutionary success of vascular plants on earth (Raven 1993).

Evolutionary Perspective of Vascular Development

When plants colonized the land, they had to adapt to a life in air. The new habitat they were facing required a large range of adaptations, as it did not provide physical support, was much drier and exposed to high solar radiation as well as rapid temperature fluctuations. One of the main limitations for plant growth was the availability of water in the gaseous atmosphere, and water transport in the plant body became a crucial feature. The evolution of the conduits that gave rise to the xylem of extant tracheophytes has been deduced from fossil data and integration of extant plant data (Friedman and Cook 2000). Understanding the evolution of early conduits evolution can help analysing the mechanisms that underlie xylem differentiation, as will be discussed later.

The probably simplest conduits are found in some bryophytes that can transport water over short distances in simple thin-walled tubular structures, called hydroids. It has, however, been suggested, that hydroids

and tracheids evolved independently and are not homologous (Ligrone *et al.* 2000, Ligrone *et al.* 2002). The extinct protracheophytes such as *Aglaophyton* were found to have a central vascular cylinder, but the conducting cells lacked secondary thickenings (Edwards 1986). As the protoplast of a living cell greatly impedes water conductance (Raven 1993), it can be assumed that those structures were dead empty cell corpses. Conduits that are supported by secondary cell wall thickenings, the characteristic of the tracheophytes, have been found in 430 million years old fossils from the Mid-Silurian period (Edwards *et al.* 1992). While the water transport in these structures was a prerequisite for the evolutionary success of vascular plants on the land surface, plants at that time were still small and herbaceous (Friedman and Cook 2000). Decreasing atmospheric carbon dioxide concentrations during the Early Devonian period (~400 million years ago) shifted the balance between water-loss and carbon dioxide uptake. This has been postulated to have driven improvements in water conductance and favoured the evolution of a vascular cambium and secondary xylem (Gerrienne *et al.* 2011). Secondary xylem evolved since then independently in several plant lineages (Spicer and Groover 2010). The secondary growth by a vascular cambium not only greatly improved water transport capacities, but also mechanical properties and hence, height growth of the plants. Together, this allowed trees to grow to heights of over 100 meters, up to the hydraulic limitations of their conduits (Koch *et al.* 2004).

In gymnosperms, tracheids are the main cell type of the xylem and function in both water transport and mechanical support. In the angiosperm lineage, xylem cell types underwent diversification, leading to a functional separation. Libriform fibers, which are the predominant cell type in secondary xylem of angiosperms, provide mainly mechanical support (Esau 1965). The end-to-end joining of vessel elements forms hollow tubes, the vessels, which transport water efficiently. Primitive vessels did, however, not improve water conductance per stem area (Sperry *et al.* 2007). Therefore, it has been suggested that the diversification of xylem cell types and the evolution of vessels may have been driven by advantages in the specialization of fibers for mechanical support (Sperry *et al.* 2007). As fibers do not function in water transport, the need for clearance of their cell contents is not obvious. But as tracheids, fiber cells undergo cell death after formation of their secondary walls and fulfill their structural purpose decades and centuries after their death.

Programmed Cell Death

Programmed cell death (PCD) is the genetically encoded process of cellular self-destruction. It has a central role in development, tissue homeostasis and integrity, but also during immune or defence responses of all multicellular organisms. But PCD has even been observed in protozoa and is generally considered to be an evolutionary conserved mechanism (Ameisen 2002). In contrast to passive, traumatic forms of cell death, such as necrosis, that result in early cell rupture and spilling of the cell contents, PCD is contained and damage to surrounding tissues does not occur. While "programmed" in PCD originally refers to the genetically encoded capability of each cell to commit this suicide (Ameisen 2002), it is often used to refer to the coordinated process observed upon initiation of the cell death "program" (Twumasi *et al.* 2010). In contrast, it does not refer to a "programmed" fate of a cell, although this might be assumed when referring to developmental PCD, where the death is a necessary and early determined part of the differentiation. In general, the initiation of a self-destruction pathway occurs in response to both external and internal stimuli, and depending on the stimuli can occur at almost any stage of any cells life. Each cell death process is characterized by a "point of no return" after which vital functions of the cellular machinery are irreparably damaged.

Apoptosis

The dominating form of PCD in animals is apoptosis, named after the "falling off" of autumn leaves, and is – in line with the origin of its name – a phenotypical description of a certain cell death process. Apoptosis is characterized morphologically by shrinkage of the cell, chromatin condensation and DNA degradation, blebbing of the plasma membrane and formation of apoptotic bodies. The apoptotic bodies are phagocytosed by macrophages and finally, the entire dying cell is eliminated (Ameisen 2002). In animals, this complete removal of cell remnants is required to prevent tissue inflammation and auto-immune responses (Pereira and Amarante-Mendes 2011).

Vacuolar Cell Death

Plants have cell walls, which prevent engulfment, and hence have no phagocytosis. Also, apoptotic bodies have never been observed in plant cell death, and neither are many other criteria of apoptosis fulfilled. Although the term is still occasionally used in plants, according to the morphological definition, apoptosis does not occur during plant PCD (van Doorn *et al.* 2011a). But plant cells have a large central vacuole, which plays an important

role in many cell death processes. These vacuole dependent forms of plant cell death have therefore been classified as vacuolar cell death (van Doorn *et al.* 2011a). Initially during vacuolar cell death, increasing parts of the cytoplasm are engulfed by lytic vacuoles, where the cytoplasmic contents are degraded. This leads to an increase in the vacuolar volume and a decrease of the cytoplasmic volume. Finally, and irreversibly, the tonoplast ruptures and releases the vacuolar hydrolases into the remaining cytoplasm. This causes a rapid degradation of organelles and finally the entire protoplast (van Doorn *et al.* 2011a). Without phagocytosis, the enzymes for the extensive autolysis have to be produced entirely by the cell itself prior to vacuolar rupture. After autolysis, the cell walls usually remain as an empty corpse, but also partial or total cell wall hydrolysis occurs in certain cases.

Xylem Development and Differentiation

Xylem is formed in different places of the plant body, but the development of a xylem cell follows in general the same pattern. The xylem cell originates from dividing procambial or preprovascular cells or from a vascular cambium. The cell fate is determined by integration of spatial information and continuously balanced against meristem maintenance (Miyashima *et al.* 2013). The differentiation starts by cell expansion, followed by secondary wall formation. After completion of secondary walls, the cells undergo programmed cell death and autolytic clearance of their cell contents. The lignification of the secondary walls, which started while the cell was still alive, continues after cell death (Pesquet *et al.* 2013).

Protoxylem cells differentiate during very early stages of plant primary growth and deposit annular or helical secondary wall thickenings. These allow passive cell elongation with continuing growth of the plant tissue. With ceasing primary growth, metaxylem cells differentiate and form secondary walls in a pitted or reticulate pattern, which does not allow further cell elongation. The procambium develops later into the vascular cambium, which gives rise to the secondary growth occurring massively in woody plants, but to a lesser extent also in most herbaceous species.

Vascular Patterning and Specification

The vasculature in the Arabidopsis root stele consists of an axis of xylem cells, flanked by two poles of phloem cells. The xylem axis consists typically of two peripheral protoxylem and three central metaxylem cells. Xylem and phloem are separated by cells of the procambium and together surrounded by the pericycle. The stele is bordered by the endodermis, which is followed by one cell layer each of cortex and epidermis (Dolan *et al.* 1993) (Figure 1).

Vascular patterns are established early during embryogenesis. Local auxin maxima induce a positive feedback loop, defining the position of the preprocambial tissue in the embryo. This feedback loop involves auxin induced expression of MONOPTEROS (MP/ARF5), which promotes expression of the auxin efflux carrier PIN-FORMED1 (PIN1). PIN1 transmits the auxin maximum to the next cell, which then will as well become a preprocambial cell and continue the self-inducing feedback loop mechanism. MP also activates expression of the *CLASS III HOMEO-DOMAIN LEUCINE ZIPPER* (*HD-ZIP III*) transcription factor gene *HOMEOBOX8* (*ATHB8*), one of the earliest preprocambial markers (Scarpella *et al.* 2006).

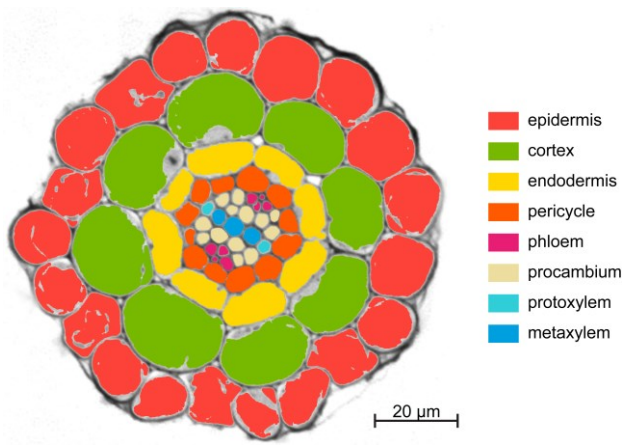

Figure 1. Arabidopsis primary root anatomy. Colourised picture of a cross section.

The preprocambial tissue gives later rise to the procambium, phloem and xylem initials (Caño-Delgado *et al.* 2010). The patterning of the vasculature is regulated by HD-ZIP III- and KANADI-family transcription factors that act antagonistically and are expressed in xylem or phloem precursor cells, respectively (Emery *et al.* 2003). The HD-ZIP III transcription factors consist in Arabidopsis of ATHB8, REVOLUTA (REV), PHABULOSA (PHB), PHAVOLUTA (PHV) and CORONA (CNA) and are required for xylem cell fate determination (Carlsbecker *et al.* 2010) and their levels are post-transcriptionally controlled by *miR165/166* (Emery *et al.* 2003). In the root, *mi165/166* expression is activated in the endodermis by the transcription factors SHORT ROOT (SHR) and SCARECROW (SCR). SHR originates from the vasculature but moves into the endodermis where it induces SCR expression. *miR165/166* move into the vascular cylinder where they control in a dose-dependent manner proto- and metaxylem differentiation via regulation of HD-ZIP III transcription factors (Carlsbecker *et al.* 2010). As already the provascular cells express SHR, which induces SCR in the neighbouring cell layer (Helariutta *et al.* 2000, Nakajima *et al.* 2001), it is

likely that this non-cell autonomous mechanism functions already in the vascular patterning in the early embryo (Lau *et al.* 2012).

Xylem Differentiation

While the concerted action of HD-ZIP III transcription factors in a dose dependent manner determines xylem cell fate (Carlsbecker *et al.* 2010), the link to the differentiation of the xylem cells is still missing. HD-ZIP III suppression by overexpression of *miR165* resulted in downregulation of *ACAULIS5* (*ACL5*), secondary wall-associated *CELLULOSE SYNTHASEs* (*CesA*s) and *XYLEM CYSTEINE PEPTIDASE1* (*XCP1*) and *XCP2*, but also auxin biosynthetic genes (Zhou *et al.* 2007). However, it remained unclear which of these transcriptional changes were causally related to the observed vascular defects in these plants and which were their consequence. The HD-ZIP III transcription factors have also recently been suggested to regulate ROS balance and meristem size (Roberts 2012).

A series of NAC domain transcription factors have been identified to be expressed specifically during xylem differentiation and hence named VASCULAR RELATED NAC-DOMAIN (VND). Special attention has been paid to VND6 and VND7 that are expressed specifically in developing meta- and protoxylem cells, respectively (Kubo *et al.* 2005). Overexpression of VND6 or VND7 alone is sufficient to induce ectopic formation of TE-like cells with meta- or protoxylem secondary wall patterning, respectively (Kubo *et al.* 2005, Yamaguchi *et al.* 2010a). Vice versa, dominant repression of VND7 or VND6 inhibited differentiation of proto- or metaxylem vessel elements, respectively, while single mutants did not show xylem defects (Kubo *et al.* 2005). However, VND7 appears to be the principal regulator of vessel differentiation, acting in different combinations of VND-family-heterodimers. VND7 stability has been suggested to be regulated by proteasome-mediated degradation (Yamaguchi *et al.* 2008).

VND7 mediated transcriptional activation has been shown to be suppressed by another NAC transcription factor, VND7 INTERACTING PROTEIN 2 (VNI2), which is targeted for degradation before VND7 activates TE differentiation (Yamaguchi *et al.* 2010b). VNI2 inhibits protoxylem vessel differentiation in Arabidopsis roots (Yamaguchi *et al.* 2010b), similar to what has been demonstrated for yet another xylem expressed NAC transcription factor, XYLEM NAC DOMAIN1 (XND1) (Zhao *et al.* 2005). XND1 overexpression strongly reduced xylem marker gene expression and suppressed secondary wall formation and cell death of vessel elements (Zhao *et al.* 2008). Vice versa, *xnd1* mutants had shorter TEs and showed increased sensitivity to the 26S proteasome inhibitor MG132 (Zhao *et al.* 2008). XND1 has further been demonstrated to be a direct target of VND7 in Arabidopsis protoplasts (Zhong *et al.* 2010b), suggesting it may act downstream of VND7

to fine-tune the rate of xylem differentiation. Interestingly, VND6 and VND7 directly activate transcription of not only secondary wall related genes but also cell death related genes, such as *XCP1*, *XCP2*, *BIFUNCTIONAL NUCLASE1* (*BFN1*) and *METACASPASE9* (*AtMC9*) (Ohashi-Ito *et al.* 2010, Yamaguchi *et al.* 2010b, Zhong *et al.* 2010b, Yamaguchi *et al.* 2011). These results suggest that a common signalling cascade via these NAC transcription factors can activate simultaneously secondary wall biosynthesis and cell death during TE differentiation (Figure 2).

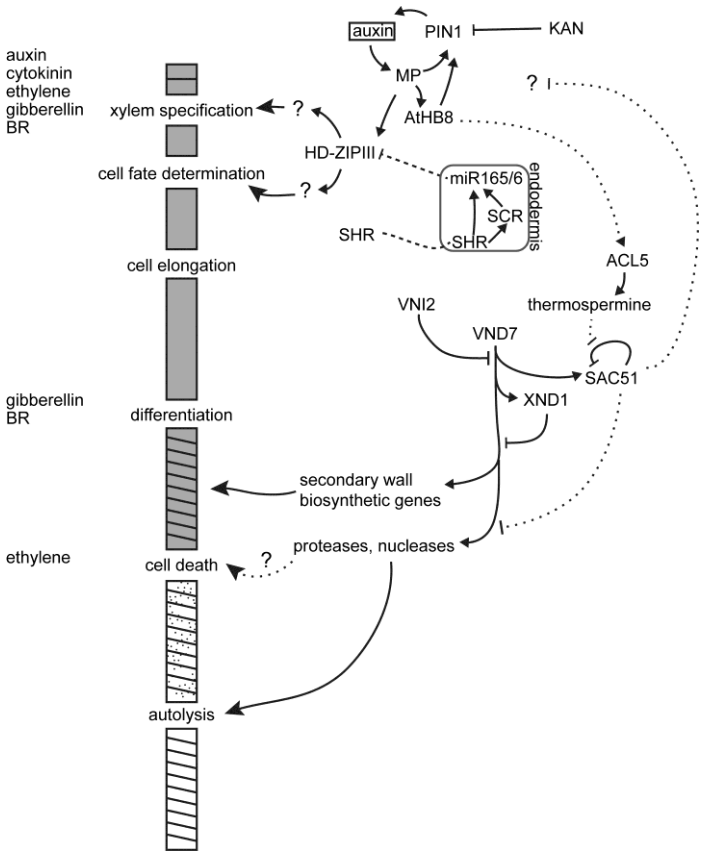

Figure 2. Signalling and regulatory pathways in xylem development. A simplified overview of the main steps of vascular specification, cell fate determination and differentiation. Hormones are listed to the left at the position of the differentiation process that they have been shown to be involved in. Dotted lines indicate hypothetical mechanisms.

In fibers of Arabidopsis, secondary wall formation is controlled by the NAC transcription factor SECONDARY WALL-ASSOCIATED NAC DOMAIN1/NAC SECONDARY WALL THICKENING PROMOTING FACTOR3 (SND1/NST3). Ectopic expression of SND1 generated cells with fiber morphology, while secondary wall formation was inhibited specifically

in fibers, both by dominant suppression of SND1 as well as in *snd1 nst1* double mutants (Zhong *et al.* 2006, Mitsuda *et al.* 2007, Zhong *et al.* 2007). In contrast to VNDs, SND1 seems to activate mainly transcription of genes for secondary wall biosynthesis and not cell death (Zhong *et al.* 2006, Ohashi-Ito *et al.* 2010). While this may explain why Arabidopsis fibers usually do not die after secondary wall formation, Zhong *et al.* (2010b) demonstrated that SND1 can induce expression of a few hydrolytic enzymes related to cell death, although to much lower extent than VND7. This suggests that Arabidopsis fibers instead may actively suppress the expression of cell death related genes by still unknown mechanisms.

Given the evolutionary importance of a TE differentiation program, it is not surprising that components of its transcriptional regulation are conserved within the tracheophytes (Zhong *et al.* 2010a, Zhong *et al.* 2010c, Ohtani *et al.* 2011). A recent analysis suggested that specification and radiation of the NAC transcription factors occurred during tracheophyte evolution (Yao *et al.* 2012). Furthermore, expansion of the VND family may have been important for vessel evolution in angiosperms (Nystedt *et al.* 2013)

Hormones in xylem differentiation and cell death

In contrast to their role in vascular patterning, little is known about direct effects of plant hormones on TE differentiation. Many hormones act during early xylem specification processes where they initiate the entire differentiation program, but less is known about their roles later on during the differentiation (Miyashima *et al.* 2013, Schuetz *et al.* 2013). The positioning of xylem cells as relatively few individual cells deep within other tissues hampers studying their differentiation *in planta*. Xylogenic cell culture systems of *Zinnia elegans*, but also Arabidopsis, have therefore been very instrumental in providing clues on xylem differentiation process on a cellular level, although these artificial systems cannot provide the cellular context that is important for many aspects of xylem differentiation. Furthermore, it has proven to be difficult to distinguish signals specific for cell death and autolysis from those specific for secondary wall biosynthesis. Most of the pharmacological agents that were able to block TE cell death *in vitro* blocked at the same time secondary wall formation (Woffenden *et al.* 1998, Groover and Jones 1999, Endo *et al.* 2001, Twumasi *et al.* 2010). This suggested early that these two processes are co-regulated during TE differentiation.

Auxins and cytokinins have been shown to affect TE differentiation *in vitro* (Fukuda and Komamine 1980), but seem to be required for basic transdifferentiation processes of mesophyll cells rather than specifically for TE differentiation (Milioni *et al.* 2001). Also the *in planta* evidence points

towards roles in early signalling of xylem differentiation and cambial maintenance for these hormones, rather than xylem maturation processes (Mähönen *et al.* 2000, Birnbaum *et al.* 2003, Bishopp *et al.* 2011).

Also brassinosteroid (BR) signalling functions in cell fate determination and has been shown to induce expression of HD-ZIP III genes (Ohashi-Ito *et al.* 2002, Ohashi-Ito and Fukuda 2003). BR levels increase in xylogenic cell cultures prior to TE differentiation (Yamamoto *et al.* 2001) and inhibition of BR synthesis in these cell cultures prevented secondary wall biosynthesis and cell death. Therefore, BR signalling has been suggested to induce the final stage of TE differentiation (Yamamoto *et al.* 1997).

Similarly, gibberellins have been shown to have such dual roles in vascular development. In xylogenic *Zinnia* cultures, inhibitors of gibberellin biosynthesis suppressed TE differentiation while addition of gibberellin was shown to promote lignification (Tokunaga *et al.* 2006). *In planta* studies suggest roles for gibberellins in regulation of cambial activity but also in fiber elongation (Mauriat and Moritz 2009, Ragni *et al.* 2011).

The gaseous plant hormone ethylene has a broad range of functions in plant development (Lin *et al.* 2009) but has also been shown to be involved in PCD processes (He *et al.* 1996, Tuominen *et al.* 2004, Volz *et al.* 2013). Ethylene treatment enhanced cambial activity in *Populus* stems (Love *et al.* 2009) and also maturing *Zinnia* TEs accumulate ethylene. Blocking of ethylene signalling by silverthiosulfate (STS) blocked lignification and cell death in the cell cultures, but apparently not the formation of secondary cell walls (Pesquet and Tuominen 2011). This indicates that ethylene signals may be related to initiation of the cell death program, and may act on e.g. vacuolar integrity.

Thermospermine

Polyamines play important roles in many cellular processes during development and in response to biotic and abiotic stresses (Takahashi and Kakehi 2010). The tetraamine thermospermine has been allocated roles during xylem differentiation, including auxin-cytokinin signalling, cell wall formation, and lignin biosynthesis (Ge *et al.* 2006, Cui *et al.* 2010, Vera-Sirera *et al.* 2010). The thermospermine synthase *ACAULIS5* (*ACL5*) is specifically expressed in Arabidopsis vessel elements prior to secondary wall deposition (Muñiz *et al.* 2008). *acl5* loss-of-function mutants are dwarfed and show vascular abnormalities, such as absence of metaxylem pattern (Clay and Nelson 2005, Muñiz *et al.* 2008). Premature expression of xylem cell death markers and, consequently, early vessel cell death in the *acl5* mutant suggested that thermospermine functions to prevent premature cell death and allow proper xylem differentiation (Muñiz *et al.* 2008).

A bHLH transcription factor, SUPPRESSOR OF ACAULIS51 (SAC51) has been suggested as a downstream target of thermospermine function. *SAC51* has several upstream open reading frames (uORF), which can act inhibitory on translation of the main ORF. A dominant mutation in one of these uORFs was shown to suppress the *acl5* phenotype completely in an *acl5 sac51* double mutant. It was therefore hypothesized that thermospermine may act on the uORF and thereby activate translation of SAC51 (Imai *et al.* 2006, Imai *et al.* 2008).

In the *acl5* mutant, the expression of *ACL5* (Hanzawa *et al.* 2000) and the *HD-ZIP III* genes is upregulated (Kakehi *et al.* 2010), suggesting a thermospermine-dependent negative feedback-loop controlling expression of *HD-ZIP III* genes and *ACL5* (Hanzawa *et al.* 2000). The expression levels were restored to wild type in the *acl5 sac51* mutant (Imai *et al.* 2006). Therefore, the negative feedback-loop was suggested to involve activity of SAC51 (Imai *et al.* 2006). The regulatory loop likely incorporates also *VND6* and *VND7* as both are upregulated in the *acl5* mutant (Muñiz *et al.* 2008) and *SAC51* was shown to be a direct target of VND7 (Zhong *et al.* 2010b).

ROS and Ca^{2+} and mitochondria

Ca^{2+} signals are centrally involved in many plant signalling pathways in plant growth and development as well as environmental perception and interaction (Dodd *et al.* 2010). Transient increases in cytoplasmic Ca^{2+} concentrations are found in response to a wide range of stimuli. In differentiating *Zinnia* TEs, Ca^{2+} influx has been causally linked to vacuolar collapse and DNA degradation (Groover and Jones 1999). A secreted serine protease has been proposed to accumulate during TE differentiation and to initiate the Ca^{2+} influx, leading to vacuolar collapse. Treatment of xylogenic cultures with trypsin induced a cell death via influx of Ca^{2+} that mimicked the naturally occurring PCD of TEs (Groover and Jones 1999).

Reactive oxygen species (ROS) are another cellular signal that can induce cell death, typically via an oxidative burst, a rapid increase in ROS levels (Van Breusegem and Dat 2006). ROS are closely linked to Ca^{2+} signalling and can stimulate Ca^{2+} influx, but also ROS production itself can be induced by Ca^{2+} signals (Monshausen and Haswell 2013). High levels of ROS have been found during TE differentiation in *Zinnia* cultures, however, no oxidative burst seems to occur (Groover *et al.* 1997, Gómez Ros *et al.* 2006). This seems contradictory to a role as a cell death inducing signal. Further, ROS are required for xylem lignification, both in *Zinnia* TEs and *in planta* (Karlsson *et al.* 2005, Srivastava *et al.* 2007). The non-differentiating parenchyma cells in TE cultures, as well as xylem parenchyma cells in intact plants, have been implicated as the main source of ROS for lignification of neighbouring vessel elements (Ros Barceló 2005, Gómez Ros *et al.* 2006).

Alterations in Ca^{2+}, pH and ROS levels can trigger formation of the mitochondrial permeability transition pore (PTP) prior to apoptotic cell death. This results in the release of proteins, such as cytochrome c, from the intermembrane space that can trigger caspases and induce apoptosis (Danial and Korsmeyer 2004). Also in plants, mitochondrial depolarization and morphological changes occur as fast responses in various PCD-inducing conditions (Vianello *et al.* 2007, Logan 2008). During *in vitro* differentiation of *Zinnia* TEs, depolarisation of mitochondrial membranes and cytochrome c release into the cytosol have been observed to precede vacuolar rupture (Yu *et al.* 2002). However, while TE differentiation and DNA degradation were inhibited by disruption of the PTP by cyclosporin A, this did not block cytochrome c release (Yu *et al.* 2002). Furthermore, it has been reported that cytochrome c alone is not sufficient to induce DNA degradation in plants (Yu *et al.* 2002, Balk *et al.* 2003). Therefore, it seems plausible that mitochondrial changes and cytochrome c release during xylem cell death may be a consequence of cell death but not represent a trigger for it.

Proteasome function

The proteasome is an enzyme complex that is in involved in many cellular proteolytic pathways, that function in protein degradation, signalling and regulatory mechanisms. It has been linked to TE differentiation on the basis of the effects that proteasome inhibitors had on TE differentiation. The proteasome inhibitors clasto-lactacystin β-lactone (LAC) and MG132 almost completely inhibited TE differentiation *in vitro* when given prior to cell differentiation (Woffenden *et al.* 1998), whereas LAC merely delayed TE differentiation induced by estrogen-controlled VND6 (Han *et al.* 2012). The effect of proteasome inhibitors on the early differentiation phase suggests an involvement of the proteasomal pathway in specification and early differentiation processes of TEs. This is further supported by the finding that a subunit of the 26S proteasome functions in auxin and brassinosteroid signalling (Jin *et al.* 2006). MG132 inhibits in addition to the proteasome also cysteine proteases that are expressed during later stages of TE differentiation. This effect has been concluded to delay the autolytic clearing of TE cell contents, which has been observed after a late treatment with MG132 after beginning of TE differentiation (Woffenden *et al.* 1998).

TE Cell Death Morphology

The overall purpose of TE cell death and autolysis is to create a hollow conduit that functions in water transport. Detailed descriptions of the degradative processes during TE cell death in various plant species were made possible by electron microscopy. First observations describe the

degradation of organelles and the protoplast during differentiation of *Cucurbita* vessels (Esau *et al.* 1963), and vessel maturation has since then been intensively studied (Srivastava and Singh 1972, Esau and Charvat 1978, Burgess and Linstead 1984a).

During autolysis of pine tracheids, cellular components are gradually taken up into the vacuole before the vacuole finally breaks down (Wodzicki and Brown 1973). In xylogenic cell cultures of *Zinnia elegans* (Fukuda and Komamine 1980), the first indication of cell death is the swelling of the vacuole, which is followed by changes in tonoplast permeability (Kuriyama 1999). The collapse of the vacuole occurs rapidly and at the same time, cytoplasmic streaming ceases (Groover *et al.* 1997). Therefore, tonoplast rupture is considered as the moment of death (Groover *et al.* 1997). The final autolysis is triggered by the release of hydrolytic enzymes from the vacuole and by acidification of the cytoplasm that activates further hydrolytic enzymes. During final autolysis, organelles such as the ER and Golgi have been observed to swell prior to degradation (Fukuda 1997). Also the degradation of DNA is triggered by the rupture of the vacuole and rapidly accomplished within 10 to 20 minutes (Obara *et al.* 2001). DNA degradation is a classical hallmark of cell death and as it is incompatible with cell survival it is generally regarded as a "point of no return" during PCD (Greenberg 1996). However, it seems that DNA degradation in TEs occurs solely after rupture of the tonoplast, or *post mortem*, during the final autolysis, instead of being part of the initiation of the cell death program (Obara *et al.* 2001). DNA laddering, characteristic for the action of certain nucleases in apoptosis, does not seem to occur in TE cell death (Fukuda 2000), during which instead S1-type nucleases degrade the chromosomal DNA. Also, lobing of the nucleus, indicating fragmentation, has been observed in differentiating TEs (Esau *et al.* 1963, Lai and Srivastava 1976, Burgess and Linstead 1984b).

Finally, to connect several cell corpses to form a functioning vessel unit, the end walls of the individual vessel elements are modified. End wall modifications vary dependent on the species, but range from scalariform perforations to complete removal of the wall (Jansen *et al.* 2004). Also in the *Zinnia* TEs *in vitro*, one of the end walls is removed, forming a perforation in the mature TE (Burgess and Linstead 1984b, Nakashima *et al.* 2000). The events that occur in TEs *post mortem* are difficult to study *in planta*, but it has been suggested, on the basis of microscopic evidence, that the cell wall matrix is enzymatically hydrolysed after loss of the plasma membrane (O'Brien 1970), and that remaining cellulose microfibrils are destroyed mechanically by the transpiration stream (O'Brien and Thimann 1967, Burgess and Linstead 1984a).

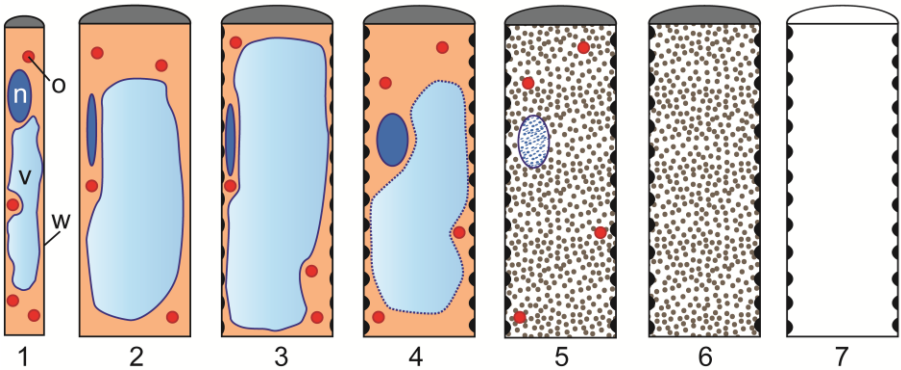

Figure 3. Morphological changes during differentiation of vessel elements. Early differentiation in the cambial zone (1) is followed by cell expansion (2) and secondary wall formation (3). Changes in tonoplast permeability lead to tonoplast rupture and the cytoplasmic streaming stops (4), and the cell is considered dead. The degradation of DNA occurs rapidly *post mortem* (5), and the final autolysis of the cell contents (6) and partial hydrolysis of primary end walls create the hollow conduit (7). vacuole; n, nucleus; o, organelle; w, cell wall. Image adapted from Bollhöner *et al.* (2012).

Autolytic Processes – Execution of Cell Death

The presence of cell walls has important consequences for plant cell death. In most plant cell death processes, the cells do not disappear entirely as animal cells do, but their cell walls remain. In some cases - such as the cell death of xylem cells - the main function of the tissue is actually carried out by the dead cell corpses. Furthermore, as plants to not have phagocytosis, the dying plant cell has to organize the removal of its own cell contents during a cell-autonomous self-digestion, called autolysis.

A large number of hydrolytic enzymes, such as proteases, lipases and nucleases are expressed during xylem differentiation. These enzymes are believed to be stored either as inactive zymogens or in compartments such as the vacuole (Funk *et al.* 2002) or the ER (Schmid *et al.* 1999, Farage-Barhom *et al.* 2011, Mulisch *et al.* 2013). The activation and the release of these enzymes result in hydrolysis of the cell contents.

It has been shown that protease activities increase during TE differentiation (Beers and Freeman 1997) and especially cysteine and serine proteases have frequently been identified in TEs (Minami and Fukuda 1995, Ye and Varner 1996, Beers and Freeman 1997, Yamamoto *et al.* 1997, Groover and Jones 1999). Interestingly, the pharmacological inhibition of cysteine proteases blocked not only cell death, but also secondary wall biosynthesis in *Zinnia* cell cultures if added at the start of the culture (Fukuda 2000, Twumasi *et al.* 2010). However, when the inhibitors were given after start of the differentiation, it delayed the degradation of the cell contents but could not block it (Woffenden *et al.* 1998). These results suggest

the function of a signalling cascade in xylem differentiation, which in similarity to the signalling cascade in apoptosis involves cysteine proteases.

Cysteine Proteases

Cysteine dependent aspartate proteases (caspases) are central players in the metazoan apoptotic pathway (Sanmartín *et al.* 2005, Fuchs and Steller 2011), but their role varies dependent on the species. In the nematode, caspases are required for initiation of the cell death process itself (Ellis and Horvitz 1986). In higher animals, their function is important for triggering the final degradative machinery, but inhibition of caspases cannot prevent the cell to die (Pereira and Amarante-Mendes 2011, Martin *et al.* 2012). Also plant extracts are able to cleave synthetic caspase-substrates and various apoptotic inhibitors work efficiently in modulating plant PCD (del Pozo and Lam 2003, Danon *et al.* 2004, Rotari *et al.* 2005). Hence, it was expected that plant caspases exist. That this was not the case became clear when plant genomes were sequenced and no caspase-homologous sequences could be identified. Therefore, the plant activities against caspase substrates are called caspase-like activities. The activities are often referred to using the tetrapeptide sequence that is cleaved, for instance as DEVD-, VEID- or YVAD-ase activity (Bonneau *et al.* 2008).

Metacaspases

Despite the lack of caspase homologues, there are ancestral relatives of caspases present in plants. On the basis of structural similarity, Uren *et al.* (2000) identified two groups of proteases that share with caspases the conserved catalytically active His-Cys diad and the structure of the caspase subunits p20 and p10. Based on these similarities, they were named para- and metacaspases and grouped into the clan CD of cysteine proteases.

Paracaspases are present in metazoans and slime molds, while metacaspases are specific to plants, fungi and protozoa. According to that study, metacaspases may most closely represent the ancestral eukaryotic protease that gave rise to these diverged protease families (Uren *et al.* 2000). Metacaspases were further classified as type I and type II, on the basis of their structure. Type I metacaspases have a proline rich N-terminal prodomain and type II metacaspases a long linker region between the protease domains. Furthermore, plant type I metacaspases have a Zn-finger motif, similar to the hypersensitivity response (HR)-related LESION SIMULATING DISEASE1 (LSD1) (Uren *et al.* 2000).

After this discovery, metacaspases turned into high-ranked candidates for plant caspase-like proteases. However, biochemical characterisation revealed that metacaspases have specificity towards the positively charged

amino acids arginine or lysine in the P1 position of the cleavage site (Vercammen *et al.* 2004, Bozhkov *et al.* 2005, Watanabe and Lam 2005). This is in striking contrast to caspases, which strictly require a negatively charged aspartate residue in the cleavage site. Therefore, metacaspases cannot cause the plant caspase-like activities. Despite their predicted common ancestor, metacaspases and caspases are biochemically distinct protease families (Vercammen *et al.* 2007, Bonneau *et al.* 2008).

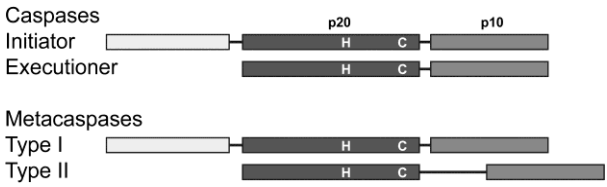

Figure 4. Comparison of the domain structures of caspases and metacaspases, with indicated catalytic H and C residues. Figure adapted from Vercammen *et al.* (2007).

Nevertheless, a number of plant metacaspases have - in functional analogy to caspases - been assigned roles in cell death processes. A spruce type II metacaspase (*mcIIPa*) is required for differentiation and cell death in the embryo suspensor, and has actually been suggested to function upstream of a caspase-like VEIDase activity (Suarez *et al.* 2004, Bozhkov *et al.* 2005). Arabidopsis has nine metacaspases, three type I (AtMC1-3) and six type II (AtMC4-AtMC9) metacaspases. AtMC9 is biochemically and structurally distinct from the other type II members that arose by a number of duplication events (Vercammen *et al.* 2004). Several of the Arabidopsis metacaspases have been shown to be involved in the control of cell death processes. These include cell death during disease resistance against a fungal pathogen (van Baarlen *et al.* 2007), cell death induced by UV-radiation and hydrogen peroxide (He *et al.* 2008) and biotic and abiotic stress (Watanabe and Lam 2011). Further, the type I metacaspases AtMC1 and AtMC2 function as positive and negative regulators, respectively, of cell death during the HR (Coll *et al.* 2010). *AtMC9* stands out as the so far only metacaspase with an acidic pH optimum (Vercammen *et al.* 2004). Its role has been uncharacterised, but it has been found to be specifically expressed during differentiation of xylem elements (Turner *et al.* 2007, Ohashi-Ito *et al.* 2010).

Although the preferred synthetic substrates have been identified for several metacaspases, very little is known so far about their natural biological substrates (Tsiatsiani *et al.* 2011). Recently, Tsiatsiani *et al.* (2013) reported on the identification of a large number of physiological substrates of AtMC9, identified *in vitro* and *in vivo*, that may eventually shed light on the function of this metacaspase. This study also supported the hypothesis that metacaspases may be more liberal in their substrate specificity than

caspases (Tsiatsiani *et al.* 2011). This could be indicative for a more degradative role of metacaspases, in contrast to the proteolytic signalling by specific caspase cleavage (Enoksson and Salvesen 2010).

The spruce mcIIPa was shown to cleave TUDOR STAPHYLOCOCCAL NUCLEASE (TSN) during cell death in the spruce embryo (Sundström *et al.* 2009). In Arabidopsis, TSN is required for stabilization of stress-regulated mRNAs that encode secreted proteins (dit Frey *et al.* 2010). Several of these proteins are protease inhibitors (dit Frey *et al.* 2010) that are involved in suppression of cell death (Solomon *et al.* 1999, Coffeen and Wolpert 2004). This led to the proposal of a model according to which metacaspases can promote the activity of cell death proteases by cleavage of TSN, resulting in decreased translation of protease inhibitors (Tsiatsiani *et al.* 2011). Interestingly, TSN was also identified as a target of the human caspase-3 during apoptosis, but cleavage patterns of plant and human TSN were totally different (Sundström *et al.* 2009). This indicates some evolutionary conservation of fundamental cell death mechanisms between plants and animals, by which the same downstream targets are processed by proteases with entirely different biochemical properties.

Caspase-like activities

Some of the enzymes causing the plant caspase-like activities have been identified and indicate a quite variable group of proteases (Bonneau *et al.* 2008). The group includes cysteine proteases such as VACUOLAR PROCESSING ENZYME (VPE) (Hatsugai *et al.* 2004, Rojo *et al.* 2004) and the proteasome (Hatsugai *et al.* 2009, Han *et al.* 2012), as well as the subtilisin-like serine proteases saspase (Coffeen and Wolpert 2004) and phytaspase (Chichkova *et al.* 2010). Although TE differentiation in *Zinnia* was impaired by synthetic caspase inhibitors and proteasome inhibitors (Woffenden *et al.* 1998, Twumasi *et al.* 2010), there is so far only little direct evidence for caspase-like activities during xylem cell death. Han *et al.* (2012) detected caspase-like DEVDase activity in the differentiating xylem of poplar, caused by the 20S proteasome subunits. Caspase-like activities in the xylem could also derive from VPEs that are expressed in developing xylem elements (Kinoshita *et al.* 1999). VPEs are vacuolar localised enzymes, which are responsible for maturation and activation of vacuolar proteins (Hara-Nishimura and Hatsugai 2011). VPE activity is required for tonoplast rupture during tobacco mosaic virus-induced cell death (Hatsugai *et al.* 2004). Despite their reported roles in pathogen resistance and cell death during seed coat formation (Hara-Nishimura and Hatsugai 2011), a full knock-out of all four *VPEs* did not show any developmental phenotype (Gruis *et al.* 2004). Hence, the role of VPEs during xylem differentiation remains unclear.

Papain-like cysteine proteases

The *XYLEM CYSTEINE PEPTIDASE1* (*XCP1*) and *XCP2* are papain-like cysteine proteases (PLCPs) that are expressed in xylem vessel elements (Zhao *et al.* 2000, Funk *et al.* 2002). Both XCP1 and XCP2 are located in the vacuole in vessel elements where they function redundantly in micro-autolysis in the intact vacuole. After rupture of the tonoplast, they function in mega-autolysis of cellular contents (Avci *et al.* 2008). XCP1 is autocatalytically activated at an optimal pH of 5.5, by cleavage of a prodomain to gain full enzymatic activity (Zhao *et al.* 2000). In the absence of XCP1 and 2, the autolysis of vessel elements is delayed. However, vessels are ultimately cleared and are expected to function normally, as the growth of the *xcp1 xcp2* double mutant is not affected (Avci *et al.* 2008).

Nucleases

Cleavage of the nuclear DNA deprives the cell of its basic information of life. DNA degradation by nucleases is one of the main characteristics of metazoan apoptosis. The DNA is first cut internucleosomally into fragments with multiples of 180 bp, causing characteristic DNA laddering, before it is finally degraded (Samejima and Earnshaw 2005). DNA laddering can occur in plant cell death as well (Reape and McCabe 2008), but has not been observed during xylem cell death.

In xylogenic *Zinnia* cultures, three main nuclease activities were identified in the differentiating TEs (Ito and Fukuda, 2002). One of them is the S1-type nuclease ZINNIA ENDONUCLEASE1 (ZEN1) that was assumed to localise to the vacuole (Thelen and Northcote 1989, Aoyagi *et al.* 1998). Knock-down of *ZEN1* did not affect the onset of TE cell death, but reduced nuclear DNA degradation (Ito and Fukuda 2002). This suggests that ZEN1 has a role in *post mortem* DNA degradation and that ZEN1 dependent DNA degradation is not linked to cell death initiation in TEs, which is in agreement with the observation that rapid nuclear degradation occurs not until the tonoplast ruptures (Obara *et al.* 2001).

The closest Arabidopsis homologue of *ZEN1* is the *BIFUNCTIONAL NUCLEASE1* (*BFN1*), which belongs to a small gene family (Ito and Fukuda 2002). *BFN1* expression was detected in senescing tissues and during developmental PCD (Pérez-Amador *et al.* 2000, Farage-Barhom *et al.* 2008). During senescence, *BFN1* expression is regulated by the NAC transcription factor ORESARA1 (ORE1) (Matallana-Ramirez *et al.* 2013) and during TE differentiation by the NAC transcription factor VND7 (Zhong *et al.* 2010b). The function of BFN1 for plant growth and development is not clear, due to absence of obvious phenotypes. However, during senescence, BFN1 moves within special ER compartments towards the nucleus and is

found to colocalise with fragmented nuclei of dead cells (Farage-Barhom *et al.* 2011). This implies a role in DNA degradation during the autolytic processes. ER-localisation has also been described for the tomato LX ribonuclease that is expressed during senescence and developmental PCD, including that of xylem cells (Lehmann *et al.* 2001). In addition, S1 nucleases have been suggested to require proteolytic cleavage for activity (Lesniewicz *et al.* 2013).

Aim

The overall aim of this thesis project was to understand regulatory aspects of the cell death processes forming the functional xylem tissue. The main focus was to study the function of cell death associated hydrolases, with special emphasis on the xylem specific metacaspase AtMC9. This thesis focusses on aspects of transcriptional control, regulation and execution of cell death and the final autolysis, but also on common aspects that may be shared with other developmentally regulated cell death programs.

Results and Discussion

In the following section, results and conclusion from the three articles and the manuscript this thesis is based on are presented and discussed with regard to their implications for the understanding of xylem cell death.

In **paper I** we describe how the cell death of fibers differs from that of vessel elements in hybrid aspen (*Populus tremula* x *tremuloides*) and identify fiber-specific components of the cell death process. This study led also to the identification of an AtMC9-homologous metacaspase, upregulated during xylem cell death.

Paper II describes an aspect of the complex regulatory mechanisms controlling xylem differentiation that was studied in hybrid aspen by overexpressing the *ACL5* orthologue *POPACAULIS5*. On the basis of these experiments a model is proposed of negative feedback-loop regulation of thermospermine levels, involving auxin and PtHB8.

Paper III focuses on the role of the metacaspase AtMC9 in xylem development of Arabidopsis. We show that AtMC9 is required during the autolytic processes, following cell death of vessel elements, but is dispensable for initiation of the cell death itself. The overall plant development is not affected by the delayed autolysis and the importance of autolytic clearance of vessel elements is discussed. The absence of xylem cell death-related phenotypes in various hydrolase mutants and the coupling of cell death to secondary wall formation are discussed from an evolutionary point of view.

In **paper IV**, we describe another developmentally regulated cell death process during the emergence of lateral roots, which involves the same hydrolytic enzymes that are expressed also during xylem development. The developmental cell death marker genes led to the discovery that cells overlying a lateral root primordium in Arabidopsis undergo cell death in addition to cell wall remodelling. Further, the regulation of these enzymes in the cell death during lateral root development was studied.

Xylem cell death was studied mainly in two model systems; the Arabidopsis root protoxylem and the secondary xylem of aspen stems. The Arabidopsis protoxylem cells allow detailed analyses of the spatial and temporal aspects of differentiation *in planta*. A major disadvantage is that the xylem cannot be specifically isolated for analyses. For this purpose, the secondary xylem of a tree, such as aspen, is more useful. The secondary xylem of aspen consists only of three cell types, vessel elements, fibers and rays parenchyma, all originating from the vascular cambium. This allows sampling of high amounts of xylem tissue, as well as tangential cryo-sectioning throughout the developmental stages of xylem formation.

Fiber and Vessel Element Cell Death Programs (I)

Fibers are the predominant cell type in the xylem of most angiosperms and especially woody angiosperms. Fibers are specialised to function in mechanical support and vessels have taken over the water conductance. Nevertheless, the general pattern of differentiation is similar between vessels and fibers. Both cell types originate from the vascular cambium, elongate and expand before they deposit secondary cell walls. Their development is terminated by cell death and clearance of the cell contents (Fukuda 1996, Déjardin *et al.* 2010). A comparison of the morphological features of vessel and fiber cell death (**Paper I**) revealed that these two processes are quite distinct despite the similar outcome. Most striking was the difference in the rate of degradation. In vessel elements, the rupture of the tonoplast is believed to initiate autolysis, which occurs then very rapidly (Groover *et al.* 1997, Obara *et al.* 2001). Fibers in contrast appeared to start degradation of cell contents, including DNA and organelles, prior to tonoplast rupture and were already largely autolysed when tonoplast rupture finalized their fate (Figure 4 and **Paper I**).

Cell death of fibers still occurs in a coordinated way synchronously around the stem circumference at a certain distance from the cambium (**Paper I, Fig 3**), suggesting that it is controlled by a genetically encoded program. Transcriptomic analyses revealed potential candidate genes involved in the xylem cell death program of *Populus* (**Paper I, Fig 7**). This led to identification of a metacaspase, homologous of *AtMC9*, that was upregulated during xylem maturation. Also nucleases, autophagy-related genes and other PCD related genes were upregulated. The indication of autophagy is interesting in the light of the slow degradation processes observed in fibers. Autophagy is a process that involves enclosure of cytoplasmic contents in autophagosomes and delivery to the vacuole for degradation. It is involved in nutrient recycling and cellular homeostasis, but also pathogen defence and development (Kwon *et al.* 2010, Hofius *et al.* 2011). In fibers, autophagy may therefore function in the gradual degradation of cell contents prior to the final degradation upon vacuolar rupture.

In order to identify components that are specific to fiber cell death, we performed a comparative transcriptomic approach (**Paper I, Fig 8**). A set of genes that were upregulated during *Populus* xylem maturation were compared against publicly available gene expression datasets from Arabidopsis (Zimmermann *et al.* 2004). To select for cell death and degradative processes, genes were required to be upregulated in a senescing cell culture experiment (Swidzinski *et al.* 2002), which was identified as the most interesting dataset, with upregulation of most of the known xylem cell death marker genes. To select for genes specifically expressed in fibers but not in vessel elements, genes were excluded if upregulated in datasets

representing gene expression during TE differentiation *in vitro* (Kubo *et al.* 2005), in maturing root xylem (Brady *et al.* 2007) or in the stele (Birnbaum *et al.* 2003). Further, only genes were selected that had been identified previously in Arabidopsis xylem development (Ko and Han 2004, Ehlting *et al.* 2005, Zhao *et al.* 2005) to filter against signal from the ray cells that are not found in Arabidopsis. Using these criteria, this method may allow identification of genes with fiber-specific expression, but it cannot reveal any common "core" cell death regulators that are shared with TE or other cell death processes.

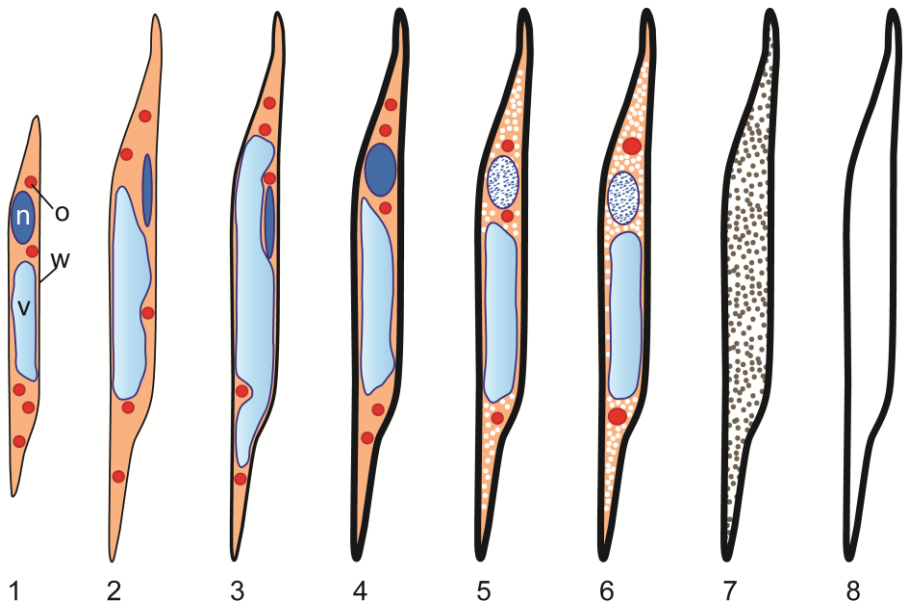

Figure 5. Morphological changes during differentiation of fibers. Fiber cells originate from the cambium (1) and differentiation leads to cell expansion (2) and secondary wall deposition (3). A loss of turgor pressure (4) precedes observation of beginning autolysis (5) that includes swelling of organelles (6). After vacuolar rupture, the cell is finally autolysed (7) and a cleared cell corpse remains (8). v, vacuole; n, nucleus; o, organelle; w, cell wall. Image adapted from Bollhöner *et al.* (2012).

In conclusion, this study showed that fibers and vessel elements die in a different pattern and that fiber cell death is distinct not only morphologically but also in its genetic control. Interestingly, the observed patterns of cell degradation in fibers are similar to what was described for the autolysis of tracheids in gymnosperms (Wodzicki 1971, Skene 1972). This suggests the presence of an ancient cell death program in gymnosperm tracheids that is shared by angiosperm fibers, despite their diverse functions. In contrast, the vessel elements, although classified as tracheary elements together with tracheids, seem to have evolved a distinct and very rapid cell death program.

Thermospermine in Xylem Differentiation Control (II)

Paper II focuses on a signalling aspect of xylem cell differentiation. The tetraamine thermospermine is a critical polyamine for vascular development and xylem cell specification and is synthesized by the thermospermine synthase ACAULIS5 (ACL5). According to the model proposed by Vera-Sirera *et al.* (2010), thermospermine is involved in a safeguard mechanism, that is activated simultaneously with the TE differentiation program and prevents premature cell death. Hence, overexpression of *ACL5* was hypothesized to prolong the differentiation and potentially enhance formation of secondary walls. As Arabidopsis does not allow isolation of large amounts of xylem tissue for analyses, this study utilised the secondary xylem of hybrid aspen. The *Populus* orthologue of the Arabidopsis *ACL5* was identified and named *POPACAULIS5* (**Paper II, Fig. 1**). POPACAULIS5 is able to synthesize thermospermine in yeast cells and is able to largely restore the growth of the Arabidopsis *acl5* mutant (Figure 6).

Figure 6. POPACAULIS5 restores stem elongation in the *acl5* mutant. A plant with restored stem elongation surrounded by *acl5* dwarfs during phenotype selection in the T1 generation after transformation of *acl5* (Col-0 background) with *35S::POPACAULIS5*.

35S-promoter driven overexpression of *POPACAULIS5* in hybrid aspen resulted in dwarfed plants and growth retardation to varying degrees. The severity of the phenotype correlated with the level of transgene overexpression (**Paper II, Fig. 2 and 4**) and only mild to medium level affected lines were able to root and grow in soil. When grown in the greenhouse, these trees showed a reduction in overall growth and minor alterations in xylem development. In the transgenic trees, final lignification and maturation of the xylem fibers appeared to be delayed, and some lines did not form at all the mature xylem normally seen in wild type trees. After final maturation, the fibers appear whiter or "drier" when observed in a fresh stem cross section. This appearance coincides with a strong increase in autofluorescence and phloroglucinol staining, and is therefore probably linked to lignin polymerisation. In wild type, this characteristic change in the xylem appearance coincides spatially with the death of the fibers (Figure 7, Bollhöner *et al.* 2012). However, the delay in maturation in the *35S::POPACAULIS5* transgenic lines was not matched by an equal delay in fiber cell death and the appearance of the mature, whitish fibers was spatially separated from fiber cell death. This suggests that *post mortem*

processes that play a role in xylem maturation were altered. The thickness of the zone of living fibers was not altered in the transgenics (**Paper II, Fig. 3**) but the trees showed a reduced stem diameter. With the assumption of a constantly reduced diameter growth rate, this may indicate that the fibers in the transgenic trees stay alive for a longer time.

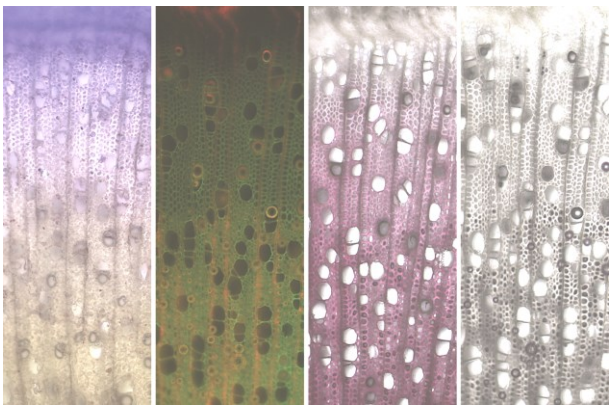

Figure 7. Observation of fiber cell death and lignification in fresh cross sections of hybrid aspen stems. (a) Nitro Blue Tetrazolium (NBT) viability staining indicates where fibers undergo cell death. (b) Autofluorescence, mainly caused by polymerised lignin, increases already during differentiation but most strongly after fiber cell death. (c) Also phloroglucinol staining increases further after cell death, indicating ongoing *post mortem* lignification. (d) Air bubbles (dark cells) in the dead, empty fibers of a fresh unstained section indicate the border between living and dead fibers

The initially puzzling observation, that *POPACAULIS5* transcript as well as thermospermine levels were significantly increased in leaves but not in the xylem of transgenic trees indicated suppression of the transgene expression specifically in xylem tissues. Further, decreased auxin levels in both leaves and xylem tissue of the transgenic trees suggested that a negative feedback loop would involve auxin to suppress *POPACAULIS5* transgene expression (**Paper II, Fig. 4 and 5**). The HD ZIP III transcription factor PttHB8 was studied in this context since it was predicted to regulate the expression *POPACAULIS5*. Overexpression of a gain-of-function version of PttHB8 resulted in upregulation of *POPACAULIS5* expression. Based on these results, a model was proposed where auxin regulates transcription of *POPACAULIS5* via PttHB8, but also stabilizes *POPACAULIS5* transcript (**Paper II, Fig. 9**). Increased thermospermine levels reduce auxin levels via a negative feedback loop, which in turn results in destabilization of *POPACAULIS5* transcript. This provides a mechanism that allows rapid changes in thermospermine levels in response to fluctuating auxin signals, but it also helps to maintain a stable thermospermine homeostasis, which is crucial for proper xylem development (Muñiz *et al.* 2008).

The Role of AtMC9, a Cell Death-Associated Hydrolytic Enzyme (III)

Paper III addresses the function of the Arabidopsis metacaspase AtMC9 during cell death of the xylem vessel elements. *AtMC9* had previously been identified as upregulated during TE differentiation *in vitro* (Turner *et al.* 2007) and was shown to be expressed in root xylem (Ohashi-Ito *et al.* 2010). We also identified its poplar homologue as upregulated during xylem cell death (**Paper I, Fig. 7**).

We obtained T-DNA insertion lines for AtMC9 and identified two knock-out mutants, GABI_540H06 and SALK_075814, referred to as *atmc9-1* and *atmc9-2*, respectively. These mutant plants grew completely normal and did not show any phenotypes in the xylem, either (**Paper III, Fig. S1**). Based on the function of other type II metacaspases in diverse forms of cell death (Suarez *et al.* 2004, He *et al.* 2008, Watanabe and Lam 2011), we hypothesized that AtMC9 may have a function in initiation of xylem cell death, i.e. upstream of the rupture of the vacuole. We used xylem specific cell death reporter lines (**Paper III, Fig. S3**) to monitor the viability of the protoxylem and, indirectly, the position of vacuolar rupture. These analyses showed that vacuolar rupture is not altered in *atmc9* mutants (**Paper III, Fig. 2**). Hence, we concluded that AtMC9 does not function in initiation of xylem cell death. We next tested whether AtMC9 functions after rupture of the vacuole, which would be *post mortem*, as the vacuolar burst is defined as the moment of death. After breakdown of the tonoplast, the mega-autolysis of the cell contents is rapidly clearing the vessel element. Not much is known about the autolytic machinery, but the PLCPs XCP1 and 2 have been shown to participate in mega-autolysis (Avci *et al.* 2008). As fluorescent marker proteins are unlikely to function properly in this highly autolytic environment, the progression of autolysis was followed in chemically fixed samples by transmission electron microscopy (TEM).

With the TEM analyses we could identify several stages of vessel autolysis. In some protoxylem cells, the tonoplast had just started to disintegrate and was still partly visible, this is likely to represent the beginning of the final autolysis. Cells that had progressed further showed the cytoplasm intermixed with the vacuolar space but with organelles still detectable. At later stages, cells were filled with a grainy mass without identifiable substructures, and finally an empty vessel remained (**Paper III, Fig. 3**). However, we never obtained several stages of autolysis from one and the same protoxylem cell file due to the fast rate of autolysis. TE cell death has been demonstrated to occur rapidly *in vitro*. After 6-8 hours of secondary wall deposition, vacuolar rupture occurred in less than 10 min and subsequent protoplast shrinkage took up to 4 hours (Pesquet *et al.* 2010). We could with the TEM observe ongoing autolysis *in vivo* on average in one

cell per protoxylem cell file in wild type. Assuming a root growth rate of 2 mm/day and a given cell length of 185 µm, about 11 protoxylem cells/day differentiate in a single protoxylem cell file. This allows estimating very roughly the time lapse between the differentiation of two consecutive protoxylem cells to approximately 2 hours. Finding on average one cell in autolysis per cell file implies that the autolysis is finished within 2 hours.

In the *atmc9-2* mutant, we confirmed by the TEM analyses that the timing of vacuolar rupture occurs as in wild type. But following vacuolar rupture, autolytic stages were detected up to three times more often than in wildtype (**Paper III, Fig. 4**). Therefore, we concluded that the absence of AtMC9 delayed the autolytic clearance and, hence, that AtMC9 functions in the *post mortem* degradation of the cell contents.

AtMC9 has a requirement for acidic pH for activation (Vercammen *et al.* 2004) in contrast to all other so far characterised metacaspases, which are active at physiological pH (Tsiatsiani *et al.* 2011). This is in agreement with the function of AtMC9 in the autolysis after tonoplast rupture, which leads to acidification of the cell lumen. Furthermore, we found that AtMC9 is localised in the cytoplasm in intact xylem vessels (**Paper III, Fig. 6**). This suggests that the protease is inactive in the cytoplasm until the pH drops after tonoplast rupture. Its proteolytic substrates may not be restricted to the cytoplasm or vacuole, but also extracellular targets are possible after degradation of the protoplast.

The Importance of Autolysis for Water Transport (III)

The efficient and rapid degradation of cell contents is generally assumed to be a requirement for proper vessel function. Remnants in the water conducting system are expected to cause clogging of vessels when accumulating at end plates or to nucleate cavitation in vessels under water stress (Groover and Jones 1999). However, a variety of mutants in genes encoding hydrolytic enzymes that are expressed during TE/xylem differentiation do not show any severe effect. The *xcp1 xcp2* double mutant showed a delay in autolysis similar to *atmc9-2* but grows completely normal as well (Avci *et al.* 2008). A quadruple *vpe* mutant did not cause obvious alterations in vegetative growth (Gruis *et al.* 2004) and nucleases do not seem to be critical for growth, either. Knock-down of the tomato ribonuclease LX delayed senescence, but did not affect plant growth (Lers *et al.* 2006). We could not detect growth abnormalities in mutants in the Arabidopsis S1-nuclease BFN1, which is expressed in xylem vessels and coregulated with AtMC9 (**Paper IV**).

These results suggest that the different hydrolytic enzymes either function somewhat redundantly or that they may have additional functions in the xylem vessels other than degradation of the cellular contents and that may

not be easily detected in controlled, optimal growth conditions. XCP1/XCP2 activities are for instance inhibited by the *Cladosporium fulvum* virulence factor Avr2, which interacts with plant proteins to prevent the plant defence response to the biotrophic pathogen (van Esse *et al.* 2008). XCP1/2 have also been identified in apoplastic preparations (Boudart *et al.* 2005) and in the xylem sap of *Brassica napus* (Kehr *et al.* 2005), suggesting that they may function even after completion of cellular autolysis.

We tested *atmc9* mutants for their response to a number of pathogens. While the mutants did not show an altered HR response, they were more sensitive to root infections with the xylem pathogen *Ralstonia solanacearum*. The wild type (Arabidopsis ecotype Col-0) is susceptible and dies completely within a few days after infection. The enhanced wilt response in the mutant correlated with an increased growth of the pathogen in the xylem (**Paper III, Fig. S2**). The *Ralstonia* infection process has recently been characterised and the invasion through the outer root tissues depends on plant defence. The spreading after the pathogen reached the dead xylem depends in contrast on cell wall properties, namely the resistance of the pectic middle lamella which is degraded by the pathogen (Digonnet *et al.* 2012).

As our experimental infection bypasses the natural invasion process and instead allows direct access to the xylem tissues, active plant defence responses are less likely to be involved in AtMC9 function during the *R. solanacearum* infection. Instead, it might be the xylem properties that allow faster spreading and enhanced growth of the invading pathogen in the *atmc9* mutants. This could have at least two reasons. First, the bacteria may somehow benefit from the remnants of the delayed autolysis, if they serve as nutrient or energy source. Secondly, the mutant may have alterations in xylem vessel cell wall properties that make for instance pectin more easily degradable and thereby allow the pathogen to spread faster between vessels and colonize the plant more rapidly.

Vessel elements have evolved a rapid differentiation process, so that they quickly can function in water transport. Maybe the rapid clearing of vessel elements is less important and a moderate autolysis is sufficient before cell contents can be flushed away with the onset of the transpiration stream.

TE Differentiation - a Program for Cell Death or Secondary Walls?

Living cells would regardless of secondary wall thickenings be very inefficient in water transport (Raven 1977). Dead cells, however, without the reinforcements of secondary walls would not be able to withstand the mechanical forces of a long-distance water transport. Therefore, it appears logical that secondary cell wall formation and cell death are tightly coupled

in the differentiation of xylem elements. The coupling has been observed experimentally, as a failure to functionally separate xylem cell death from secondary wall biosynthesis.

However, a few mutants suggested that the opposite, TE cell death in absence of secondary wall formation, seems to be possible. The *gapped xylem* (*gpx*) mutant has empty spaces in the position of xylem vessels (Turner and Hall 2000). The biogenesis of these holes has been compared to the formation of lysigenous aerenchyma, which were suggested to be formed by a cell death program similar to that in TEs (Kozela and Regan 2003, Mühlenbock *et al.* 2007). Another mutant, *wee1*, suffers from premature death of provascular root cells that neither form secondary cell walls (Cools *et al.* 2011).

These mutants suggest, although not being further characterised, that TE cell death can occur without preceding secondary wall deposition. This is interesting in the light of the evolution of vascular plants. In fact, thin-walled dead cells can function as conduits in short-distance transport that generates only small negative pressure. Examples for this kind of transport are found in the hydroids of certain mosses and in the thin-walled conduits of extinct protracheophytes like *Aglaophyton* (Edwards 1986, Sperry 2003). These simple conduits may not present the direct precursors of tracheids as the phylogenetic relationships of mosses, protracheophytes and extant vascular plants are somewhat unclear (Kenrick 2000). But this indicates nevertheless, that water transport in thin-walled dead cells preceded that in secondary-wall-enforced tracheids (Friedman and Cook 2000). Hence, an ancient conduit differentiation program may have primarily controlled the autolysis of the cell, and later adopted the regulation of secondary wall biosynthesis which led to the evolution of tracheids. Therefore, TE differentiation may in fact represent a cell death program that controls additionally secondary wall biosynthesis (Twumasi *et al.* 2010, Bollhöner *et al.* 2012).

This scenario could explain why it has been almost impossible to uncouple cell death from secondary wall formation experimentally. In other forms of plant cell death, caspase inhibitors usually affect cell death signalling, possibly *via* a functionally conserved caspase-like cascade (Vartapetian *et al.* 2011). But in TEs, these inhibitors rather have general inhibitory effects on the entire process of TE differentiation (Twumasi *et al.* 2010), suggesting that - if it exists - a conserved caspase-like death cascade may act upstream of TE differentiation. Hence, successful inhibition of the cell death trigger may not have necessarily been recognized as such, as an inhibition of secondary wall formation is phenotypically masking the effects on cell death and autolysis.

Consequently, it could be suggested, that the autolytic processes after completion of the secondary walls could be the organised disposal of the cell contents, only completing a cell death program that had been switched on

before the formation of the secondary walls. Manipulating the involved degradative enzymes can slightly change the course of cell degradation (Woffenden *et al.* 1998, Avci *et al.* 2008) (**Paper III**), but these changes seem to be of minor importance for plant growth and survival (Avci *et al.* 2008) (**Paper III**). Further, the actual decision switch for death may not be the activation of certain proteases expressed during differentiation, but instead be found in the very early signalling for xylem differentiation.

In fact, also caspases have been proposed to rather regulate the way of cell disposal during apoptosis than the death itself (Pereira and Amarante-Mendes 2011). While caspases clearly are the key switches for cell death in the nematode *Caenorhabditis elegans* and a mutation in a single gene is sufficient to prevent the worm's cells from undergoing developmental cell death (Ellis and Horvitz 1986), the inhibition of executioner caspases in mammalian cells does not lead to increased survival of cells. Instead, cells undergo a morphologically different death, which led to the conclusion that in mammals the decision about death is made further upstream of the caspase pathway and that caspases instead have an important role in executing the cell death in a distinct way, which is safe for the organism. This includes for instance attracting macrophages to engulf the cellular remnants, avoiding inflammatory responses and potential risks of auto-immune reactions, many of which have been shown to be connected to incorrect cell disposal (Pereira and Amarante-Mendes 2011).

The *Populus* Metacaspases

Upon identification of a metacaspase being upregulated during xylem maturation in *Populus* (**Paper I**), we aimed to analyse its role during cell death of the secondary xylem. The *Populus* genome encodes 14 metacaspases, 10 of these are type I and four are type II metacaspases (Figure 8). This prevalence of type I over type II numbers is a common pattern in plant genomes (Tsiatsiani *et al.* 2011). Type II metacaspases are plant specific and interestingly, all so far sequenced embryophytes, or land plants, appear to have at least two type II metacaspases (Tsiatsiani *et al.* 2011). Possibly, this is linked to the two different pH optima of type II metacaspases that may be conserved across land plants.

The *Populus* genome underwent a whole genome duplication event (Tuskan *et al.* 2006), and hence, there are many times two homologous *Populus* genes found for each Arabidopsis gene. This is, however, not the case in the metacaspase family, due to repeated duplications of the Arabidopsis type II metacaspase genes (Vercammen *et al.* 2004) and obviously some additional duplication of *Populus* type I metacaspase genes (Figure 8).

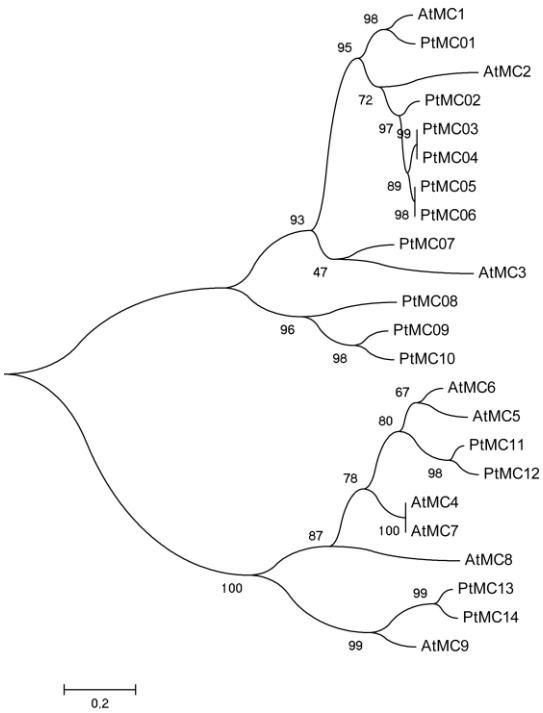

Figure 8. Phylogenetic analysis of metacaspases from Arabidopsis and *Populus*. *Populus* metacaspases were named according to their position in the phylogenetic tree. The evolutionary history was inferred by using the Maximum Likelihood method and the tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Analyses were conducted in MEGA5 (Tamura *et al.* 2011).

We studied the expression of the two AtMC9-homologous metacaspases *PttMC13* and *PttMC14* in the secondary xylem, using promoter::GUS reporter constructs in transgenic hybrid aspen. This revealed that *PttMC13* is expressed in both vessels and fibers during late maturation stages, while *PttMC14* expression is specific to late maturing vessels, only (Figure 9). The distinct expression patterns of *PttMC13* and *PttMC14* in both vessels and fibers or only vessels, respectively, support the hypothesis that fiber and vessel cell death programs are mechanistically different (**Paper I**). Furthermore, it suggests that the homologous gene pairs in *Populus* may have evolved slightly different functions to fulfil their roles in the two distinct forms of cell death occurring in the xylem cells.

With intend to understand their function during xylem cell death in aspen we targeted *PttMC13/14* for suppression by RNA interference (RNAi). Three different promoters were used to control tissue specificity and timing of the RNAi; the endogenous *PttMC13* promoter, the fiber-specific promoter of the Caffeate *O*-methyltransferase1 (*PtCOMT1*) (Tiimonen *et al.* 2007) and the *35S CaMV* promoter. This yielded transgenic lines that not only showed strongly reduced mRNA levels, but virtually lacked PttMC13/14 protein, based on Western Blot analyses (Figure 10a,b). Nevertheless, those lines grew normally and neither did show any xylem related phenotypes, when xylem anatomy, wood density or wood chemistry were analysed. The lifetime

of the fibers in the PttMC13/14-downregulated trees was not affected (Figure 10c), suggesting that PttMC13 does not act as initiator of fiber cell death. This is in agreement with the function of its homologue AtMC9 in Arabidopsis vessel element cell death (**Paper III**).

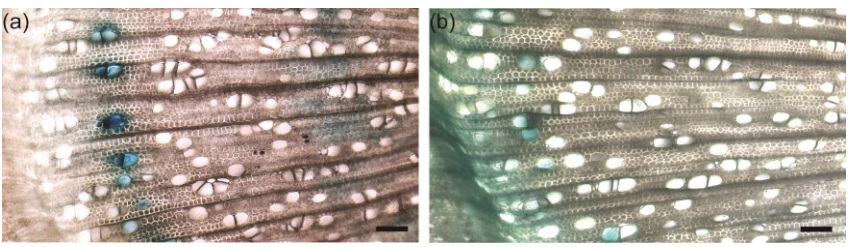

Figure 9. Expression pattern of (a) *PttMC13* and (b) *PttMC14* in the developing xylem. Histochemical GUS staining of *Populus* stems transgenic for *promoter*::*GUS* fusions of the two metacaspase genes. Scale bars indicate 100 µm. Figure adapted from Bollhöner *et al.* (2012).

Physiological Meaning of Fiber Cell Death

The death of tracheids and vessels with removal of the protoplast is necessary for efficient water transport. The physiological meaning of fiber cell death is less clear. Evolutionary, as fibers are believed to origin from tracheids, it is possible that fibers just did not evolve mechanisms to protect themselves from the fate of all xylem cells. This is supported by recent discoveries on transcriptional regulation coupling secondary wall biosynthesis and cell death support (Ohashi-Ito *et al.* 2010, Zhong *et al.* 2010b).

But there are also potential advantages in killing fiber cells: For their function in mechanical support, only the cell wall but not the protoplast is needed and keeping them alive would require a tremendous amount of energy considering the amount of fibers in a full grown tree. Instead, autolytically degrading fiber cell contents may allow the plant to recycle nutrients and use these for further growth and reproduction. This is supported by the finding that most cysteine and aspartic proteases upregulated during *Populus* fiber cell death also were upregulated in senescing leaves (Moreau *et al.* 2005), suggesting similar recycling and degradation processes taking place. Also, lignification appears to benefit from cell death, although the exact mechanism of this relationship is not fully understood (Pesquet and Tuominen 2011, Pesquet *et al.* 2013).

Another advantage could be that dead and autolysed cells are less attractive to pathogens. The lignified cell wall is chemically protected and may not profit from a living protoplast. Fiber cell death and autolysis depletes the cells of nutrients and in that way creates an environment, unfavourable for many pathogens (Yadeta and Thomma 2013).

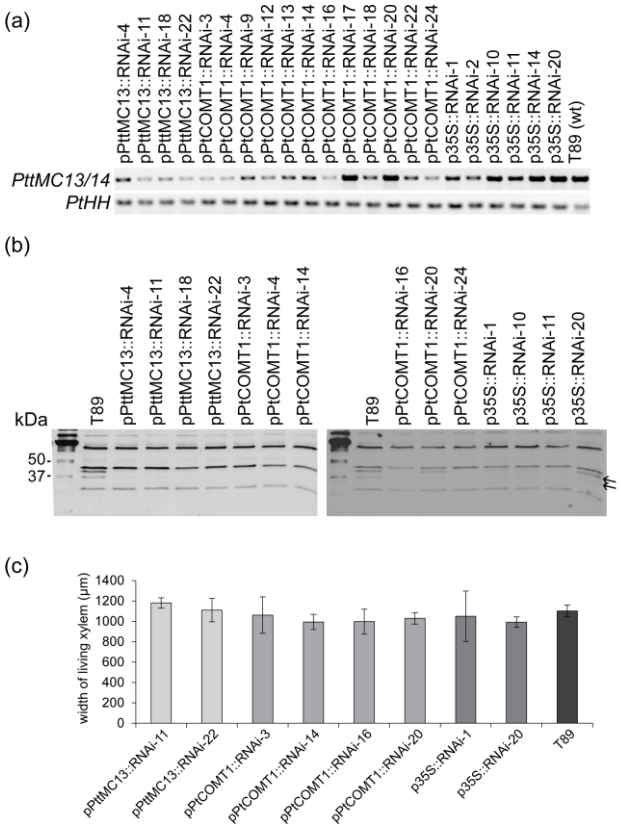

Figure 10. Downregulation of *PttMC13* and *PttMC14* expression by tissue specific RNAi. (a) RT-PCR showing suppressed mRNA levels of *PttMC13/14* in independent stable transgenic lines. Three different promoters were used to drive expression of the RNAi inverted repeat construct; the endogenous promoter *pPttMC13*, the lignin biosynthesis-related promoter *pPtCOMT* and the constitutive *35S CaMV* promoter. The primer pair used does not distinguish between PttMC13 and PttMC14. Reference gene *PtHH* Potri.005G072300 (Histone 3). (b) Suppression of PttMC13/PttMC14 protein expression in RNAi lines. Western-Blot with an anti-PttMC13/14 peptide antibody. Arrows indicate the PttMC13/14 specific bands at approximately 37 and 43 kDa, that are not detected in most RNAi lines. (c) Suppression of PttMC13/14 does not affect initiation of fiber cell death. NBT viability staining of stem cross sections, NBT stained living xylem area was measured from the cambium.

Fiber cells have recently been shown to be able to form unnaturally thick secondary walls using an artificial positive feedback loop (APFL) (Yang *et al.* 2013). This may serve as a proof of concept that fiber cells have potential for drastically enhanced secondary wall deposition, without negatively affecting plant growth. However, this study was performed in Arabidopsis where fiber specific NAC transcription factors have been shown to mainly regulate secondary wall biosynthetic genes but not cell death related genes (Zhong *et al.* 2006). In aspen, it is likely that secondary wall biosynthesis and cell death are coregulated also in fibers (Ohtani *et al.* 2011). Hence, in a similar

APFL approach in a tree like aspen, the enhancement of the entire program may also lead to faster fiber death. Therefore, understanding the regulation of fiber cell death initiation is crucial in order to delay it. Interestingly, there are cases of fibers that naturally do not undergo cell death after secondary wall formation and at least stay alive for very long times. In many shrubs but also some trees, some or even all xylary fibers can retain their protoplast (Fahn and Leshem 1963). As none of those species has been analysed more in detail, nothing is known how their fiber survival is regulated, but studying cases of naturally inhibited xylem cell death may reveal yet unknown regulatory steps, that may prove useful in order to prolong fiber life time with the intent to enhance biomass production.

A Cell Death Program in Lateral Root Development (IV)

When studying the xylem expression of the cell death reporter line *pBFN1*::*nGFP* (**Paper III, Fig. S3)**, we observed occasionally in little older roots a highly specific signal that did not arise from the xylem. The signal was present only in a small number of cells and the shape and the pattern suggested that it occurred in connection to a lateral root primordium (LRP). This observation was supported by the detection of *BFN1* upregulation in a time course transcriptomics dataset from tissues involved in lateral root (LR) development, collected at the Centre for Plant Integrative Biology (CPIB) at the University of Nottingham (**Paper IV, Fig. 1a**). To identify genes with a similar expression profile as *BFN1* during LR development, a correlation analysis of all expression profiles in this dataset was performed. The list of highest ranked genes includes several other cell death candidates and was headed by *AtMC9* (**Paper IV, Table S1**). Several of these genes were shown to be expressed in the tissues overlying an LRP (**Paper IV, Fig. 1b-e**).

We could then show by a time-lapse analysis, that the *pBFN1*::*nGFP* expression is induced by the formation of a LRP and that different cells overlying the LRP express the reporter during different phases of the LR development process (**Paper IV, Fig. 1f**). In total, 5-6 cells directly in front of the LRP, but belonging to the different tissue layers, showed a GFP signal at some point during the LR emergence (LRE) process. The analysis further suggested that the expression is dependent on the progression of the LRP through the overlying tissues. The inner cells of the endodermis started expression early, when the LRP was at stage I or II, while cortex and epidermal cells followed at later stages.

The hydrolytic activity of nucleases and proteases has been implicated in cortical cells of maize during emergence of lateral roots (Bell and McCully 1970). In maize, cell separation and cell death seem to work simultaneously during LRE. Cortical cells separate upon enzymatic degradation of the middle lamella, but the cortical cell layers also gradually collapse (Bell and

McCully 1970, Karas and McCully 1973). Nuclease activity has also been detected in soya bean root cortex cells that stained TUNEL-positive in front of a LRP (Kosslak *et al.* 1997). Cortical cell death in reaction to a growing lateral root primordium has furthermore been observed in detailed electron microscopic studies in *Convolvulus arvensis* (Bonnett 1969).

In Arabidopsis, enzymatic degradation of the middle lamellae occurs in the LRP overlying tissues (Laskowski *et al.* 2006) and cell separation is assumed to be sufficient for LR emergence (LRE) (Jansen *et al.* 2013). Cell death has not been reported to occur in Arabidopsis during LRE. In Arabidopsis, the LRP only encounters one layer of cortex cells, together with one layer each of endodermis and epidermis. All the species where cortical cell death during LRE has been observed previously have a several layered root cortex. This may increase the resistance that the LRP has to grow against, and hence, the reactions in the overlaying tissues may be more pronounced but also different (Jansen *et al.* 2013). But it could also just simplify the observation of processes that may occur only in very few cells in Arabidopsis.

In fact, we could show that DNA degradation, a hallmark of programmed cell death, occurs also in Arabidopsis in a few cells overlying the LRP by TUNEL staining (**Paper IV, Fig. 2a** and **S1**). This indicates the actual activity of nucleases like BFN1, which we observed to be expressed in these cells. Furthermore, our TEM studies of developing LR showed that the cells in the overlying tissues indeed die and undergo autolysis (**Paper IV, Fig. 2b-f**).

LR development is in all its phases dependent on auxin signalling (Lavenus *et al.* 2013), but auxin also regulates the reaction of the overlaying tissues to facilitate emergence of the LRP (Swarup *et al.* 2008, Péret *et al.* 2012, Kumpf *et al.* 2013). Auxin released from the LRP has recently been suggested to induce expression of the floral organ abscission peptide INFLORESCENCE DEFICIENT IN ABSCISSION (IDA), which after secretion upregulates via binding to its receptors expression of cell wall remodelling genes in the overlying tissues (Kumpf *et al.* 2013). Auxin accumulation in cortex and epidermal cells is achieved by auxin-induction of the auxin influx carrier LIKE AUX1 3 (LAX3) (Swarup *et al.* 2008). By coordinating aquaporin expression in both LRP and overlaying tissues, auxin also regulates tissue hydraulics and facilitates emergence (Péret *et al.* 2012). Together, these mechanisms enable a concerted reaction of specific cells overlaying the LRP, which is responsible for mechanically shaping the LRP (Lucas *et al.* 2013).

While various auxin related mutants show drastic phenotypes including block or strong reduction of LR initiation and emergence (Péret *et al.* 2009), mutants that are suggested to be affected in the mechanisms of LR emergence show only rather mild phenotypes (Swarup *et al.* 2008, Péret *et*

al. 2012, Kumpf *et al.* 2013). The single mutants in cell death related genes, that are expressed in the LR overlying tissues, did not show any delay in LRE in the staging assays performed on gravitropically induced LR (**Paper IV, Fig. 3** and **S2**). Hence, the selected genes are not individually required for LRE. So far, none of these genes has been assigned a role in cell death initiation; rather they may represent the autolytic machinery degrading the cell contents of the dying cells. Therefore, it is unlikely that the initiation of cell death would be affected in any of the analysed single mutants.

Interestingly, BFN1 expression during senescence was recently shown to be controlled by the NAC transcription factor ORE1/ANAC092/AtNAC2 (Matallana-Ramirez *et al.* 2013). The set of genes upregulated by ORE1 showed large overlap with the list of genes correlated with the expression pattern of *BFN1* during LR development. Additionally, we found large overlap with a set of genes coregulated with AtMC9 in the dataset from a high-resolution spatiotemporal map of the Arabidopsis root (Brady *et al.* 2007, Hruz *et al.* 2008). ORE1 had previously been implicated in lateral root development and suggested to integrate auxin and ethylene signals (He *et al.* 2005). Lateral root number was reported to be increased in ORE1 overexpressors but unaffected in *ore1* mutants (He *et al.* 2005). This data suggested that ORE1 may act as a transcriptional regulator also of the cell death genes during lateral root development, similar to the role of VND7 in xylem vessel differentiation and cell death.

We found in an LR staging assay, that LR development is normally initiated in *ore1*, but that the emergence is slightly but significantly delayed (**Paper IV, Fig. 3a,c**). This confirms the role of ORE1 in lateral root development and suggests that it functions in the emergence process by regulating expression of cell death related genes. A more pronounced phenotype became obvious in EMS mutants of the *pAtMC9*::*nGFP* reporter line that we created originally to identify upstream regulators of *AtMC9* expression during xylem differentiation. Two of the lines with strongly reduced or absent xylem GFP-signal showed defects in LR development. LR staging assays revealed that both LR initiation (LRI) and emergence were delayed in these mutants (**Paper IV, Fig. 3b,e-f**). Most of the mutants affected in LRI are auxin-related (Péret *et al.* 2009) and our mutants may help in identifying the upstream signalling, that connects auxin with the LR-cell death pathway.

Epidermal cell death in rice, associated with adventitious root formation, is induced by mechanical forces and is dependent on ethylene signalling (Steffens *et al.* 2012). This suggests that also the cell death in overlying tissues during LRE may be mechanically induced, in response to the pressure created by the growing LRP.

Furthermore, it is unlikely that the death of overlying cells during LRE is required to physically remove the cells in order to create space for the

growing LRP. The programmed cell death of plant cells does not involve phagocytosis and complete cell removal including the cell walls is rare. With a few exceptions, plant cell death is not a way of organ shaping (Gunawardena 2008). But the cell death in overlying tissues will certainly abolish any turgor pressure in these cells, which has been postulated to mechanically facilitate LRE (Péret *et al.* 2012). It is possible that the physiological meaning of the cell death actually is tightly coupled to the cell wall remodelling enzymes. The cell death itself may not be mechanically required, but could function to quickly and massively release cell wall remodelling enzymes, such as the pectic hydrolases.

Conclusions and Future Perspectives

Cell death is necessary for development and survival of all multicellular organisms and the specific execution of cells needs to be highly regulated and controlled to prevent tissue damage. Plant cell death is fundamentally different from animal cell death in that plants do not have phagocytes that engulf and remove dying cells. Instead, plant cells have to organize their removal themselves, which requires autolytic cell degradation to an extent not found in animals.

When this project started in the beginning of 2008, basically nothing was known about the physiological function of the many proteases that had been identified in differentiating xylem. Intuitively, many people assumed especially cysteine proteases to be cell death regulators. Metacaspases were due to their relationship to apoptotic caspases regarded with special interest.

We identified as upregulated during xylem maturation in *Populus* stems a large set of proteases, including many cysteine proteases and among those a metacaspase. The role of this metacaspase was studied both in *Populus* and Arabidopsis and we have shown that *AtMC9* is the only metacaspase differentially expressed during xylem differentiation in Arabidopsis. We found that it functions *post mortem* in the final autolysis of xylem vessel elements. In the meantime, a similar function had been demonstrated for the papain-like cysteine proteases XCP1 and XCP2.

These findings together have important consequences for the xylem cell death field. They show that the putative cell death regulators in fact are degradative enzymes of the final autolysis, but that they are not required for cell death regulation. Also for several of their coregulated genes, mutants did not show any developmental phenotype. This indicates that instead of cell death regulators, autolytic cleaners are selected by the criteria of upregulation during late xylem differentiation.

The identification of the mechanistic trigger that regulates cell death initiation remains a future challenge for xylem cell death research. The results from this thesis and other studies imply that such a trigger may not be found within the differentially expressed hydrolases. Two alternative scenarios for the regulation of xylem cell death are possible. In the first, proteases that are not differentially, but rather constitutively expressed act in perception of yet unidentified cell death signals and may initiate a proteolytic cascade that results in cell death, i.e. vacuolar rupture. In the second scenario, the signalling for xylem cell death occurs early in xylem specification and execution of cell death is only transiently inhibited while secondary walls are formed: My hypothesis, based on the results of this thesis and other studies, is that xylem differentiation presents a cell death program that adopted the formation of secondary walls. This has major

implications for the understanding of cell death regulation, as in this scenario cell wall regulation would be expected to be subordinated to a cell death regulation. Consequently, NAC transcription factors would be positioned as transcriptional regulators of the cell death program, and their upstream regulation is certainly of critical importance. An interesting link to cell death regulation may be the HD ZIP III transcription factors that have recently been suggested to regulate ROS homeostasis, a known player in cell death signalling.

We demonstrated in *Populus* the role of a HD ZIP III transcription factor in an auxin dependent negative feedback-loop that regulates thermospermine levels. The effect of this feedback-loop on xylem differentiation and late maturation supports the second scenario. It will be interesting in future projects to characterise the molecular function of thermospermine and to identify how it regulates xylem differentiation.

Yet another aspect of this thesis supports that cell death regulation may lie further upstream and not in the processing and activation of the hydrolytic enzymes differentially expressed during late phases of xylem differentiation. We demonstrated that cells in front of a lateral root primordium undergo cell death after expressing hydrolytic enzymes – the same enzymes expressed in differentiating xylem cells. While these genes individually were shown to be dispensable for lateral root development, we showed that upstream regulators of BFN1 and AtMC9 indeed play a role for lateral root emergence, likely due to their upstream regulatory function on cell death.

This study further showed that cell death and cell wall remodelling apparently act closely together. Cell wall modifications occur together with cell death as well during TE differentiation, in the abscission zone and in lateral root cap cells, a striking similarity offering a target for future studies.

This and other fundamental similarities between different developmental plant cell death processes may provide a strong basis for future plant cell death research, instead of somewhat shaky comparisons with an animal cell death, that despite some similarities has now been shown to be executed in a totally different manner.

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