DARWIN REVIEW

Xylem cell death: emerging understanding of regulation and function

Benjamin Bolhöner, Jakob Prestele and Hannele Tuominen*
Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, SE-90187 Umeå, Sweden
* To whom correspondence should be addressed. E-mail: hannele.tuominen@plantphys.umu.se

Received 5 October 2011; Revised 6 December 2011; Accepted 9 December 2011

Abstract

Evolutionary, as well as genetic, evidence suggests that vascular development evolved originally as a cell death programme that allowed enhanced movement of water in the extinct prodracheyophytes, and that secondary wall formation in the water-conducting cells evolved afterwards, providing mechanical support for effective long-distance transport of water. The extant vascular plants possess a common regulatory network to coordinate the different phases of xylem maturation, including secondary wall formation, cell death, and finally autolysis of the cell contents, by the action of recently identified NAC domain transcription factors. Consequently, xylem cell death is an inseparable part of the xylem maturation programme, making it difficult to uncouple cell death mechanistically from secondary wall formation, and thus identify the key factors specifically involved in regulation of cell death. Current knowledge suggests that the necessary components for xylem cell death are produced early during xylem differentiation, and cell death is prevented through the action of inhibitors and storage of hydrolytic enzymes in inactive forms in compartments such as the vacuole. Bursting of the central vacuole triggers autolytic hydrolysis of the cell contents, which ultimately leads to cell death. This cascade of events varies between the different xylem cell types. The water-transporting tracheary elements rely on a rapid cell death programme, with hydrolysis of cell contents taking place for the most part, if not entirely, after vacuolar bursting, while the xylem fibres disintegrate cellular contents at a slower pace, well before cell death. This review includes a detailed description of cell morphology, function of plant growth regulators, such as ethylene and thermospermine, and the action of hydrolytic nucleases and proteases during cell death of the different xylem cell types.

Key words: Autolysis, ethylene, fibre, metacaspase, protease, thermospermine, tracheary element, VND.

Introduction

A large proportion of the biomass on earth consists of dead but nevertheless functioning cells, the xylem elements. The death and complete clearing of xylem vessel elements and tracheids, commonly known as tracheary elements (TEs), is a prerequisite for the transport of water. Hollow conduits, supported by secondary wall thickenings, have been detected in fossils from the Mid-Silurian period, ~430 million years ago, and the water transport in these is believed to be one of the most important factors for the evolutionary success of vascular plants (Raven, 1993). A further improvement in water transport capacity occurred when the vascular cambium emerged, which allowed extensive lateral growth, in the form of the secondary xylem, in woody plant species. The gymnosperm secondary xylem is composed of tracheids, which contribute to both physical support and water transport. In angiosperms, functional diversification has occurred between different cell types of the secondary xylem. Water transport takes place in the vessel elements, whereas mechanical support is mainly provided by the predominant cell type of the xylem, the libriform or sclereid fibres (Esau, 1965). Although fibres do not transport water, they undergo cell death and continue to fulfil their structural purpose decades, or even centuries, after their cellular death.

The main pattern of cellular differentiation is identical between the different xylem cell types and involves initiation in the vascular cambium, rapid cell expansion, deposition of
the secondary cell wall, and finally cell death (Fukuda, 1996; Déjardin et al., 2010). However, there are significant differences in, for instance, cell morphology and the timing of these processes between the different xylem cell types (Fig. 1). Whereas vessel elements differentiate rapidly and die within a couple of days after their specification in the vascular cambium, fibres and tracheids stay alive substantially longer. The lifetime of both libriform fibres and tracheids was estimated to be ~1 month in trembling aspen (Populus tremula) and Norway spruce (Picea abies), respectively (JP, BB, and HT, unpublished). Further, whereas the final autolysis of the cell contents is rapid in the xylem vessel elements, it is slow in both the conifer tracheids and angiosperm fibres (Wodzicki, 1971; Skene, 1972; Courtois-Moreau et al., 2009). It therefore seems that conifer tracheids and angiosperm fibres share at least some parts of the cell death programme. In contrast, the vessel elements, despite being classed as TEs, seem to have evolved a distinct programme for cell death that is quite different from the ancient programme present in tracheids. Lastly, ray parenchyma cells contribute only marginally to the total number of xylem cells, but they are often the only living cells in the fully mature secondary xylem and can, depending on species, stay alive for decades before undergoing cell death (Nakaba et al., 2006, 2011). This review focuses on cell death of the water-transporting TEs and xylary fibres.

Fig. 1. Cell morphology in different stages of tracheary element (TE) and fibre (F) differentiation. TE differentiation includes: early differentiation in the cambial zone (TE1), cell expansion (TE2), secondary wall formation (TE3), changes in tonoplast permeability and vacuolar rupture (TE4), DNA degradation (TE5), final autolysis (TE6), and partial hydrolysis of the non-lignified primary cell walls (TE7). Fibre differentiation includes: early differentiation in the cambium (F1), cell expansion (F2), secondary wall formation (F3), loss of turgor (F4), disappearance of the organelles, starting autolysis and DNA degradation (F5), swelling of the remaining organelles and continued autolysis (F6), autolysis after vacuolar rupture (F7), and final clearing of the cell (F8). (n) nucleus, (v) vacuole, (o) organelles, (w) cell wall. Vacuolar disintegration seems to play a less important role in the death of fibres than in that of TEs because most of the cellular hydrolysis in xylem fibres occurs before the tonoplast breaks.
Morphological changes during xylem cell death

Tracheary elements

Early light microscopy analyses revealed that vessel elements or ‘wood pores’ were dead cells, but it was only with the invention of electron microscopy that detailed analyses of xylem differentiation and maturation became possible. Esau et al. (1963) were the first to describe the degradation of organelles and the protoplast in Cucurbita vessel elements. In maturing pine xylem, Wodzicki and Brown (1973) observed a gradual uptake of cell components into the vacuole, which they defined as autolysis that finally leads to breakdown of the vacuole. Autolysis of maturing vessels has since been documented in several different species (Srivastava and Singh, 1972; Esau and Charvat, 1978; Burgess and Linstand, 1984a). A detailed understanding of the autolytic processes of TEs has been provided by studies of cultures of Zinnia (Zinnia elegans) mesophyll cells, which can be induced to transdifferentiate into TEs in vitro in a semi-synchronized manner (Fukuda and Komamine, 1980). In this system, auxin and cytokinin are added to isolated mesophyll cells that initially dedifferentiate during the so-called stage I into procambial-like cells, followed by differentiation into TE precursor cells during stage II, and finally differentiation into TEs, including secondary wall formation and cell death, during stage III (Fukuda, 1996).

Morphologically, the first obvious indication of incipient cell death in Zinnia TEs is swelling of the vacuole, followed by changes in tonoplast permeability (Kuriyama, 1999) and rapid collapse of the vacuole (Groover et al., 1997). Although the cytoplasm has been reported to become less dense already during secondary wall biosynthesis (Groover et al., 1997), autolytic events are not believed to occur to any significant degree before vacuolar collapse. Cytoplasmic streaming ceases when the vacuole collapses, which is therefore considered as being the moment of cell death. Release of hydrolytic enzymes from the vacuole and activation of cytoplasmic enzymes by acidification of the cytoplasm is believed to induce swelling of organelles such as the endoplasmic reticulum (ER) and Golgi, and finally degradation of cellular contents (Fukuda, 1997). The pattern of DNA degradation is used for animal systems as well as plants as a basis to classify different types of cell death. The genetically programmed type of cell death, especially animal apoptotic cell death and plant vacuolar or autolytic cell death (van Doorn, 2011; van Doorn et al., 2011), often involves nuclear fragmentation, DNA degradation into a multitude of 180 bp fragments that generates the so-called ‘DNA ladder’, as well as positive staining with assays such as the TdT-mediated dUTP nick end labelling (TUNEL) assay. In the Zinnia TE system, nuclear DNA fragmentation has been visualized by TUNEL staining (Mittler and Lam, 1995; Groover et al., 1997; Twumasi et al., 2010). However, it has been demonstrated in Zinnia TEs that nuclear DNA is degraded very rapidly within 10–20 min after the rupture of the vacuole (Obara et al., 2001), suggesting that positive TUNEL staining of TEs might be related to post-mortem DNA degradation rather than any controlled degradation of DNA prior to cell death. It was also shown that the nucleus maintained a spherical shape while DNA degradation occurred, suggesting that no nuclear fragmentation occurs in Zinnia TEs in vitro (Obara et al., 2001). DNA laddering does not seem to occur either (Fukuda, 2000). In planta, electron microscopic analyses have revealed lobing of the nuclei in differentiating TEs (Esau et al., 1963; Lai and Srivastava, 1976; Burgess and Linstand, 1984b) (Fig. 2A). Fragmentation of the nucleus has also been observed prior to final autolysis in protoxylem elements of Arabidopsis roots expressing a nuclear-localized proAtMC9::GFP reporter construct (Fig. 2B). A positive TUNEL signal has been observed in the xylem vessel elements of pea roots (Mittler and Lam, 1995) and tomato leaves (Wang et al., 1996), but not in the secondary xylem of Populus stems (Courtois-Moreau et al., 2009). Based on the results of the Zinnia in vitro experiments and the in planta evidence, it appears that even though nuclear changes can be readily detected, DNA degradation is not causally related to TE cell death but occurs post-mortem during final autolysis of the cell contents.

After cellular hydrolysis, cell walls of TEs are modified by enzymes that are resistant to the highly lytic environment of dying TEs. Lignin deposition, that has been initiated prior to cell death, continues after TE cell death (Stewart, 1966, and references therein; Hergert, 1977; Burgess and Linstand, 1984b; Pesquet et al., 2010). In addition, primary walls are at least partially hydrolysed after loss of the plasma membrane in locations that are not protected by lignified secondary walls (O’Brien and Thimann, 1967; O’Brien, 1970; Burgess and Linstand, 1984a, b). This is also reflected as increased activity of cell wall hydrolytic enzymes of maturing Zinnia TEs in vitro (Ohdaira et al., 2002). Primary wall hydrolysis of lateral cell walls is believed to allow passive stretching of protoxylem vessel elements (O’Brien, 1970), while it is required in the end walls of all types of vessel elements for formation of the perforation plates.

Fibres

Xylary fibres die in a coordinated fashion, which suggests an underlying genetic programme (Courtois-Moreau et al., 2009). Prior to cell death, the fibres deposit extensive secondary cell walls and, similar to TEs in vitro, the majority of lignification seems to occur after cell death (Fig. 2C–F). The main difference seems to be the rate of differentiation. An analysis of cellular ultrastructure, as well as nuclear integrity, has revealed that xylary fibres exhibit DNA breaks, most probably due to degradative processes in the nucleus, long before the cells die (Courtois-Moreau et al., 2009). This is in contrast to TEs, where DNA degradation is initiated only after vacuolar collapse (Obara et al., 2001). Moreover, the cytoplasmic contents of the fibres start to be hydrolysed gradually well ahead of
Fig. 2. Specific characteristics of xylem cell death. (A) Electron transmission micrograph of an Arabidopsis vessel element showing lobing of the nucleus. (B) Nuclear fragmentation in transmission micrograph of an specific characteristic of xylem cell death. (A) Electron micrograph of a protoxylem cell, visualized by confocal microscopy analysis of a nuclear-localized green fluorescent protein (GFP) under transcriptional control of the AtMC9 promoter. Arrows indicate fragments of the nucleus in a late maturing protoxylem cell. The arrowhead indicates an intact nucleus in the neighbouring protoxylem cell. Roots were counterstained with propidium iodide that reveals all cell walls as well as the spiral/annular secondary wall thickenings of protoxylem elements. (C–F) Bulk lignification and cell death in Populus fibres. Microscopy images of cross-sections of the stem show living cells of the stem staining blue after a viability staining (C) (Courtois-Moreau et al., 2009), lignin autofluorescence (D), lignin deposition by phloroglucinol staining (E), and a bright field image (F). Death of the fibres, indicated by an arrow in C, coincides with a massive increase in lignin autofluorescence (D), and phloroglucinol-detectable lignin accumulation (E) as well as occurrence of air in the dead fibres (F). (G) Appearance of nuclei in a radial section of Populus xylem stained with 4′,6-diamidino-2-phenylindole (DAPI) and visualized by epifluorescence microscopy. Progressing fibre maturation (from the left to the right) leads to longitudinally elongated nuclei that show relaxation, indicated by the asterisk, before they disappear. Ray cell nuclei are relaxed and elongated in the radial direction. (H) Absence of cell death in xylem fibres of Arabidopsis hypocotyls. An epifluorescence micrograph shows a DAPI-stained cross-section of a 2-month-old Arabidopsis Ler hypocotyl, where fibres develop thick secondary walls but retain their nuclei. Bars indicate 2 μm (A), 50 μm (B, G, H), or 200 μm (C–F).

Xylem cell death-related signalling

The signals related to initiation and execution of xylem cell death are poorly understood. This is partly due to difficulties in identifying signalling that is specifically related to cell death and not secondary cell wall formation. Most pharmacological agents that block xylem cell death also block secondary cell wall formation (Yamamoto et al., 1997; Groover and Jones, 1999; Yu et al., 2002; Twumasi et al., 2010), suggesting that the two processes are co-regulated. Co-regulation of xylem maturation has indeed recently been demonstrated to occur...
via the action of NAC transcription factors, as described later in this review. However, it is clear that even though the different phases of xylem maturation are jointly regulated by a few master switches, it is likely that the individual processes have separate controls as well. For instance, bursting of the vacuole must involve unique regulatory aspects to allow the correct timing of cellular autolysis in response to endogenous and exogenous stimuli.

**Plant growth regulators**

Auxins and cytokinins are prerequisites for TE differentiation *in vitro* (Fukuda and Komamine 1980), but it seems that their only function is the early reprogramming of mesophyll cells into the TE differentiation programme (Miliotti et al., 2001). brassinosteroids, on the other hand, are believed to play a role during late xylem maturation based on experiments with *Zinnia* TEs *in vitro*. brassinosteroid precursors have been shown to accumulate during TE differentiation, whereas inhibition of brassinosteroid synthesis in TE cultures undergoing differentiation prevented cells from maturing and undergoing cell death (Yamamoto et al., 1997). Ethylene is another hormone that deserves special attention based on its crucial function in other cell death processes (He et al., 1996; Tuominen et al., 2004). It has been shown that maturing *Zinnia* TEs accumulate ethylene (Pesquet and Tuominen, 2011), whereas blocking ethylene signalling using silver thiosulphate (STS) appears to block TE maturation (E. Pesquet and H. Tuominen, unpublished results). STS-induced changes in TE maturation are unique in the sense that TEs develop cellulosic secondary walls but do not lignify or die. It has been shown recently that cell death precedes bulk lignification in TEs *in vitro* (Pesquet et al., 2010), which means that the STS-mediated arrest of TE maturation is most probably due to blocking of cell death, which in turn blocks lignification. Therefore, it can be concluded on the basis of these experiments in the *Zinnia in vitro* system that ethylene seems to interfere with the cell death programme also in TEs. This conclusion is, however, not supported by *Arabidopsis* mutant analyses because no developmental defects have been reported for any of the dominant ethylene receptor or downstream signalling mutants, even though complete removal of ethylene biosynthesis is reportedly lethal (Tsuschisaka et al., 2009). It has to be emphasized that inhibitors like STS are never specific to one pathway (Strader et al., 2009). Nevertheless, it is possible that the *in vitro* system actually reveals some basic regulatory aspects of xylem differentiation that are masked or compensated for by the cellular context in intact vascular tissues.

Polyamines are implicated in several different processes during xylem differentiation, including cell wall formation, lignin biosynthesis, and auxin–cytokinin signalling (Ge et al., 2006; Cui et al., 2010; Vera-Sirera et al., 2010). Interestingly, *ACAUILL5 (ACL5)*, which encodes the biosynthetic enzyme for the synthesis of a recently identified tetra-amine, thermospermine, is specifically expressed in *Arabidopsis* vessel elements prior to secondary wall deposition (Muñiz et al., 2008). *acl5* loss-of-function mutants exhibit incorrect or incomplete secondary cell wall formation as well as early expression of xylem cell death markers, and consequently early vessel cell death compared with the wild type, suggesting that thermospermine has a protective role against premature xylem maturation and cell death (Muñiz et al., 2008). Exogenous thermospermine has been shown to inhibit *Zinnia* TE differentiation almost completely (Kakehi et al., 2010), which could be due to accentuated protection against premature TE maturation, resulting in complete arrest of TE differentiation. Genetic analyses have further indicated a basic helix–loop–helix (bHLH) transcription factor SUPPRESSOR OF *ACAUILL5* (SAC51) as a target of ACL5 function. ACL5 or thermospermine is believed to activate translation of SAC51 by inhibiting one of the negatively acting upstream open reading frames of SAC51 (Imai et al., 2006, 2008). SAC51 has been shown to be a direct target of one of the NAC transcription factors (VND7) (Zhong et al., 2010b), and it is possible that SAC51 coordinates signals coming from the NAC transcription factors and ACL5 to control the rate of differentiation specifically in differentiating TEs (Fig. 3).

**Other signalling components**

It has been suggested that calcium ions regulate vacuolar integrity during TE maturation. An increase in Ca$^{2+}$ influx appears to accompany cell death differentiation of TEs. Both chelation of extracellular Ca$^{2+}$ and blocking of Ca$^{2+}$ influx channels have been shown to suppress vacuolar

---

**Fig. 3.** Transcriptional regulation of tracheary element cell death. Cell death is regulated as an integral part of the xylem maturation programme by the activity of the NAC transcription factors VND6 and VND7 that induce expression of both cell death- and secondary wall-related genes. Thermospermine synthase ACL5 is proposed to impede the rate of xylem maturation by activating translation of SAC51 (Vera-Sirera et al., 2010) even though it is not clear how SAC51 mediates inhibition of xylem maturation. Expression of SAC51 as well as XND1, that is another rate-inhibitory factor, is induced by VND7 (Zhong et al., 2010b). Lignification requires activity of second level transcription factors (Zhong et al., 2010a) that are activated by the NAC master switches. TE cell death is required for bulk lignification (Pesquet et al., 2010).
rupture and DNA degradation in differentiating *Zinnia* TEs (Groover and Jones, 1999). It has also been proposed that Ca$^{2+}$ influx is controlled by the activity of a secreted 40 kDa serine protease, which continuously accumulates in the extracellular space, inducing a massive Ca$^{2+}$ influx and TE cell death above a certain critical level of the protease (Groover and Jones, 1999). Reactive oxygen species (ROS) are important signalling compounds in various cell death processes both in animals and in plants. In *Zinnia* in vitro cultures, it has been found that differentiating TEs are constantly exposed to a highly oxidative environment, but no bursts of rapidly increasing ROS levels seem to occur (Groover et al., 1997; Gómez Ros et al., 2006). Inhibition of ROS production by diphenyleneiodonium does not influence TE cell death either (Groover et al., 1997). However, ROS levels have been correlated with the extent of xylem lignification in planta (Srivastava et al., 2007), and ROS production has been shown to be required for lignification in *Zinnia* TEs (Karlsson et al., 2005). It seems, therefore, that ROS are involved in regulation of TE lignification but not cell death.

During apoptotic cell death in animals, changes in Ca$^{2+}$, pH, and ROS production can trigger formation of the mitochondrial permeability transition pore (PTP), leading to release of proteins, such as cytochrome *c*, from the intermembrane space (Danial and Korsmeyer, 2004). Mitochondrial depolarization and morphological changes have also been observed as fast responses to various cell death-inducing conditions in plants (Logan, 2008). Also in differentiating *Zinnia* TEs, it has been reported that mitochondrial membranes are depolarized and cytochrome *c* is released into the cytosol prior to vacuolar rupture (Yu et al., 2002). However, cyclosporin A, which blocks apoptosis in animals probably by disrupting the PTP, has been shown to inhibit *Zinnia* TE formation and to block DNA degradation and cell death induced by the anticancer drug BetA without blocking cytochrome *c* release (Yu et al., 2002). It can therefore be concluded that cytochrome *c* alone is not sufficient to induce DNA degradation and that the mitochondrial changes, observed prior to vacuolar bursting, appear to be a side effect rather than an evolutionarily conserved trigger of TE cell death.

### Autolytic processes during xylem cell death

During differentiation of xylem elements, a large array of proteases, lipases, and nucleases are believed to be produced and stored in various compartments, such as the vacuole, until they are released, activated, and mixed with the cytoplasmic contents, resulting in hydrolysis of cellular contents. Experimental evidence for this cascade of events is rather scarce, but it has been shown in *Zinnia* cell cultures that proteinase activity increases during TE differentiation (Beers and Freeman, 1997), and several serine and cysteine proteases with TE-specific expression patterns have also been identified (Minami and Fukuda, 1995; Ye and Varner, 1996; Beers and Freeman, 1997; Yamamoto et al., 1997; Groover and Jones, 1999). Furthermore, pharmacological inhibition of cysteine protease activity in *Zinnia* cell cultures reportedly blocks both secondary wall formation and cell death if added at the start of the culture (Fukuda, 2000), or delays degradation of the cell contents if added after commitment to TE differentiation (Woffenden et al., 1998). On the basis of these results, and others described in more detail below, it seems that the final execution of xylem cell death is similar to apoptotic cell death in the sense that degradative enzymes act downstream of a signalling cascade. An interesting aspect in plants is the participation of several different organelles in the pre-death storage of the degradative enzymes. Whereas the vacuole is believed to be the most important storage organelle (see, for instance, Funk et al., 2002), hydrolytic enzymes have also been shown to be localized in the ER in xylem elements and senescing cells (Farage-Barhom et al., 2011) as well as in dying endosperm cells (Schmid et al., 1999).

### Cysteine proteases XCP1, XCP2, and VPE

**XYLEM CYSTEINE PEPTIDASE1** (XCP1) and **XCP2** were originally identified in an *Arabidopsis* xylem cDNA library (Zhao et al., 2000), and later shown to be expressed specifically in xylem vessel elements (Funk et al., 2002). XCP1 and XCP2 are papain-like cysteine proteases, which have 71% similarity to each other at the nucleotide level and 60–65% similarity to other members of the papain-like cysteine protease subfamily C1A. It has been shown that XCP1 is synthesized with a pro-domain that is autocatalytically cleaved at an optimal pH of 5.5 to gain full enzymatic activity (Zhao et al., 2000). Both XCP1 and XCP2 are located in the vacuole where they function redundantly to control micro-autolysis in the intact vacuole and mega-autolysis of cellular contents after rupture of the tonoplast (Avci et al., 2008).

Vascular-processing enzymes (VPEs) are cysteine proteases that are responsible for maturation of vascular proteins (Hara-Nishimura et al., 1991). VPEs have been shown to contribute to resistance against several different types of pathogens in both tobacco and *Arabidopsis* (Hatsugai et al., 2004; Rojo et al., 2004; van Baarlen et al., 2007), and VPE has been implicated in a developmental cell death programme during seed coat formation in *Arabidopsis* (Nakaune et al., 2005). One of the four *Arabidopsis* VPE genes, **xVPE**, was shown to be expressed specifically in developing xylem elements of the root (Kinoshita et al., 1999). In addition, it has been observed that several different VPE genes are expressed in late-maturing xylem fibres of *Populus* stem (Moreau et al., 2005; Courtois-Moreau et al., 2009), supporting the idea that VPEs are also involved in xylem cell death. Strikingly, both the *Arabidopsis* xVPE and tobacco VPEs were shown to have caspase-1-like activity and to be inhibited by caspase-1 inhibitors (Hatsugai et al., 2004; Rojo et al., 2004). Therefore, it is believed that a large proportion of, if not all, caspase-1-like activities in plants can be attributed to the activity of VPEs. Furthermore, Hatsugai et al. (2004) demonstrated that full VPE activity is required...
for tonoplast rupture during tobacco mosaic virus induced cell death, suggesting that VPEs control tonoplast integrity during vacuolar cell death. This is particularly interesting with regard to TE cell death, where tonoplast rupture is clearly critical for the activities of many hydrolytic enzymes. Identification of VPE targets is needed to shed further light on the signalling pathways involved in the control of tonoplast integrity.

Metacaspases and caspase-like activities

The cysteine protease family of caspases are central constituents of an apoptotic cell death pathway that is conserved in mammals, flies, and nematodes. Caspases are required either for the cell death process itself (Ellis and Horvitz, 1986) or for triggering the final degradative processes (Riedl and Shi, 2004). For a long time, caspase homologues were assumed to exist in plants as well, based on the ability of some plant extracts to cleave synthetic caspase substrates (for a summary, see Bonneau et al., 2008) and the effectiveness of various apoptotic effectors in modulating plant cell death processes (del Pozo and Lam, 2003; Danon et al., 2004; Rotari et al., 2005). However, sequencing of plant genomes has revealed that plants do not have homologues of caspases, and, more recently, several plant enzymes have been identified as being responsible for the caspase-like activities observed in plants. These include the cysteine proteases VPE (Hatsugai et al., 2004; Rojo et al., 2004) and a proteasome subunit (Hatsugai et al., 2009), as well as the subtilisin-like serine proteases saspase (Chichkova et al., 2010). There is, however, no evidence of caspase-like activities during xylem cell death, but synthetic caspase inhibitors as well as proteasome inhibitors have been shown to reduce Zinnia TE differentiation (Woffenden et al., 1998; Twumasi et al., 2010).

The search for plant caspase genes resulted in the discovery of structurally related proteins, called metacaspases (Uren et al., 2000). Metacaspases have a cleavage site specificity towards arginine or lysine in the P1 position, in contrast to caspases, which have a strict requirement for an aspartate residue in the cleavage site (Vercammen et al., 2004; Bozhkov et al., 2005; Watanabe and Lam, 2005). However, similar to caspases, metacaspases have a conserved cysteine–histidine dyad. They are also separated into two groups depending on the presence (type I metacaspases) or absence of a pro-domain (type II metacaspases). Furthermore, the function of metacaspases appears to be analogous to those of caspases. A spruce metacaspase (mecIPa) has been shown to be required for differentiation and cell death in the embryo suspensor (Suarez et al., 2004). In Arabidopsis, several different type II metacaspases have been implicated in the control of various cell death processes, such as disease resistance towards a fungal pathogen (van Baarlen et al., 2007), UV radiation- and hydrogen peroxide-induced cell death (He et al., 2008), and biotic and abiotic stress (Watanabe and Lam, 2011). The type I Arabidopsis metacaspases AtMC1 and AtMC2 have been identified as positive and negative regulators, respectively, of the hypersensitive cell death response (Coll et al., 2010). The Arabidopsis AtMC9 is specifically expressed in differentiating xylem elements (Turner et al., 2007; Ohashi-Ito et al., 2010), and a homologue of AtMC9 was specifically up-regulated during xylem maturation in Populus (Courtois-Moreau et al., 2009). Populus has two homologues of AtMC9, which are both expressed in the xylem. One of them is specifically expressed in late maturing vessel elements, whereas the other is expressed in both vessel elements and maturing fibres prior to cell death (Fig. 4D, E). The function of AtMC9 or its homologues has not yet been demonstrated, but the expression at the very last stages of xylem maturation strongly suggests an involvement in xylem cell death.

Nucleases

In metazoans, apoptotic cell death involves various nucleases, including Ca2+/Mg2+-, Mg2+-dependent, and cation-independent forms that are responsible for a gradual degradation of genomic DNA. After initial cleavage into high molecular weight fragments, DNA is cut internucleosomaly into fragments with multiples of 180 bp, causing characteristic DNA laddering, before it is finally degraded (Samejima and Earnshaw, 2005). There is no evidence for this kind of DNA degradation pattern during xylem cell death (Fukuda, 2000), even though it seems to occur during some forms of plant cell death (Reape and McCabe, 2008). In Zinnia cell cultures, three main nucleases—a 24 kDa Ca2+/Mg2+-dependent nuclease and two Zn2+-dependent nucleases with approximate sizes of 40 kDa and 43 kDa—appear specifically in differentiating TEs (Ito and Fukuda, 2002). The 43 kDa Zn2+-dependent nuclease has been identified as the S1-type nuclease ZINNIA ENDONUCLEASE1 (ZEN1), which is most probably localized to the vacuole (Thelen and Northcote, 1989; Aoyagi et al., 1998). Interestingly, a knock-down of ZEN1 expression reduced, or at least delayed, degradation of the nucleus in differentiating TEs in vitro, without affecting the moment of cell death (Ito and Fukuda, 2002), demonstrating the importance of ZEN1 in post-mortem DNA degradation. ZEN1 is most similar to the Arabidopsis BIFUNCTIONAL NUCLEASE1 (BFN1), which belongs to a small gene family composed of five members (Ito and Fukuda, 2002). BFN1 is expressed in tissues undergoing senescence and developmental cell death (Pérez-Amador et al., 2000; Farage-Barhom et al., 2008). A recent study has shown that BFN1 is localized to special ER compartments and moves towards the nucleus in senescing leaf mesophyll cells, finally co-localizing with fragmented nuclei of dead cells (Farage-Barhom et al., 2011). Similarly, an ER-localized tomato LX ribonuclease is expressed in senescing cells and differentiating xylem elements (Lehmann et al., 2001). These results suggest that degradation of nuclear DNA and RNA takes place in TEs only after cell death, which is supported by morphological analyses of TE nuclei. The situation is somewhat different in the xylem fibres where at least partial DNA degradation.
occurs well before cell death, and therefore other nucleases are expected to be involved, such as those proposed recently (Courtois-Moreau et al., 2009).

What are the hydrolytic enzymes needed for in TEs?

Mutations in the genes encoding hydrolytic enzymes in xylem TEs were expected to have serious effects on the normal growth pattern of the plants because complete and rapid autolysis of TEs is believed to be required for normal water transport (Groover and Jones, 1999). However, several studies have indicated this is not the case. A knockdown of the tomato LX ribonuclease was found to have no effect under optimal growth conditions, but merely delayed senescence (Lers et al., 2006). Similarly, it has been shown that loss of function of the Arabidopsis S1-nuclease BFN1 does not affect the overall pattern of plant growth (BB and HT, unpublished results). The xcp1 xcp2 double mutant reportedly grows completely normally (Avci et al., 2008), and even complete abolishment of the whole VPE gene family in Arabidopsis appears to cause no obvious alterations in the vegetative growth pattern of the plants (Gruis et al., 2004). Except for the xcp1 xcp2 double mutant, none of these mutants has been characterized in detail with regard to xylem death, and it is possible that minor changes in xylem cell death occur in these mutants without affecting the water transport capacity or overall growth pattern. However, there are other possible reasons for the lack of phenotypic alterations in these mutants. It is quite likely that functional redundancy occurs, not just within each gene family but also more widely within the large protease families. Also, phenotypic changes may only be apparent when the plants are exposed to competition for nutrients and to biotic or abiotic stresses under natural conditions.

It cannot be excluded that the hydrolytic enzymes are present in the TEs for purposes other than or additional to the autolysis of cellular contents. This is supported by a recent finding on the inhibitory effect of the Cladosporium fulvum virulence protein Avr2 on the activities of XCP1 and XCP2 (van Esse et al., 2008). Cladosporium fulvum is a biotrophic pathogen that spreads in the apoplast of leaves. Its virulence factors interact with plant proteins, such as proteases, to prevent the defence responses of the plant. The interaction of Avr2 with XCP1 and XCP2 suggests that they have a role in resistance against xylem resident pathogens. It is possible that XCP1 and XCP2 remain in the xylem sap even after the death of the xylem vessel elements as a safety mechanism against eventual pathogen invasion. Proteomics studies have identified XCP1/XCP2 in apoplastic preparations (Boudart et al., 2005) and in the xylem sap of Brassica napus (Kehr et al., 2005). To elucidate the function of the hydrolytic enzymes further, it is important to create mutants where whole protease gene families are knocked out. An alternative approach is to study xylem maturation in response to xylem-specific expression of protease inhibitors, such as the Avr2 virulence protein, which are expected to suppress the activities of several different cysteine proteases (Rooney et al., 2005; van Esse et al., 2008).
Transcriptional regulation

Positive regulators

TE cell death appears to be co-regulated with secondary wall formation based on a large number of genetic and pharmacological experiments where it has been impossible to separate these two processes. However, occurrence of TE cell death without any secondary wall formation has been reported in at least two Arabidopsis mutants. Dead cells, or rather empty holes, have occasionally been observed in locations where TEs are supposed to be formed in a gapped xylem (gpx) mutant (Turner and Hall, 2000). Premature death of provascular root cells which do not form secondary cell walls has been observed in the weel mutant during replication stress (Cools et al., 2011). The opposite, secondary wall formation that is not accompanied by cell death, has been observed in TEs in response to STS. This kind of cascade has come from the analyses of the control the two processes separately. Further evidence for a common signalling cascade, diverting at some point to secondary cell walls has been observed in the secondary cell walls of xylem (xylem) mutant (Turner and Hall, 2000). Premature death of provascular root cells which do not form secondary cell walls has been observed in the weel mutant during replication stress (Cools et al., 2011). The opposite, secondary wall formation that is not accompanied by cell death, has been observed in TEs in response to STS treatment, as described above, and in ectopic TEs of Arabidopsis plants overexpressing the NAC transcription factor genes NST1, NST2, and NST3/SND1 (Mitsuda et al., 2005; Zhong et al., 2006). Therefore, it seems probable that secondary wall formation and cell death are induced through a common signalling cascade, diverting at some point to control the two processes separately. Further evidence for this kind of cascade has come from the analyses of the NAM, ATAF1/2, CUC2 (NAC) domain transcription factors VASCULAR-RELATED NAC-DOMAIN6 (VND6), VND7, and the NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1), NST2, and NST3/SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1 (SND1). VND6, VND7, and SND1 are all expressed in TEs, and overexpression of each of them induces ectopic secondary wall formation in a striated pattern that is reminiscent of TEs (Kubo et al., 2005; Mitsuda et al., 2005, 2007; Zhong et al., 2006; Yamaguchi et al., 2008). Vice versa, dominant repression of each of these factors leads to inhibition of secondary wall formation in specific cell types (Kubo et al., 2005; Zhong et al., 2006, 2007; Mitsuda et al., 2007). These data, together with the ability of these factors to induce expression of secondary wall-related genes, have clearly established these transcription factors as master switches of secondary wall formation. Surprisingly, post-translational activation of both VND6 and VND7 resulted in transcriptional activation of genes involved in not only secondary wall formation but also cell death, such as XCP1, XCP2, and AtMC9 (Ohashi-Ito et al., 2010; Yamaguchi et al., 2010, 2011; Zhong et al., 2010b). The activation was mediated by the binding of VND6 and VND7 to promoter regions carrying the tracheary-element-regulating cis-element (TERE) (Ohashi-Ito et al., 2010; Yamaguchi et al., 2011). These results suggest that, firstly, in addition to secondary wall formation, the NAC master switches VND6 and VND7 also regulate TE cell death and, secondly, that cell death is an integral part of the TE maturation programme. This programme seems to be highly conserved between different tracheophytes (Zhong et al., 2010a, c; Ohtani et al., 2011).

The xylem fibres of Arabidopsis normally do not die (Fig. 2H), and genes encoding cell death-related hydrolases are not expressed in these cell types (Fig. 4A–C). Hence, it is not clear whether Arabidopsis possesses a transcriptional programme for cell death in xylary fibres. Nevertheless, it seems clear that the secondary wall formation of xylary fibres is controlled by SND1. SND1 is highly expressed in xylary fibres and both a dominant repression of SND1 function and a simultaneous knock-out in SND1 and NST1 inhibit secondary wall formation specifically in xylary fibres (Zhong et al., 2006; Mitsuda et al., 2007; Zhong et al., 2007). As expected, based on its function in non-dying cells, post-translational activation of SND1 does not induce expression of cell death-related hydrolytic enzymes in Arabidopsis xylogenic cultures (Ohashi-Ito et al., 2010). Zhong et al. (2010b), however, have demonstrated that SND1 induces expression of some hydrolytic enzymes, albeit to a much lower level than VND7, and mapped the secondary wall NAC-binding element (SNBE) as the consensus sequence necessary for SND1 binding in Arabidopsis protoplasts. Hence, it is possible that xylary fibres in Arabidopsis have the capacity to express genes required for cell death by the activity of SND1 but that their expression and therefore cell death is normally inhibited in planta by still unknown factors. Identification of Arabidopsis mutants or ecotypes with pronounced fibre cell death would help to clarify this issue.

Negative regulators

VND-INTERACTING2 (VNI2) is a NAC transcription factor that seems to inhibit TE cell death on the basis of its inhibitory effect on the expression of XCP1 (Yamaguchi et al., 2010). An inhibitory role in TE cell death has also been assigned to yet another NAC transcription factor, XYLEM NAC DOMAIN1 (XND1). Overexpression of XND1 has been shown to suppress secondary wall formation and cell death of vessel elements, suggesting a role for XND1 in negative regulation of terminal TE differentiation (Zhao et al., 2008). A loss-of-function mutation in XND1 results in somewhat shorter vessel length, supposedly due to an enhanced rate of differentiation. Similar changes in cell size and rate of differentiation have been observed in the acl5 mutant (Muñiz et al., 2008), suggesting that XND1 and ACL5 function in the same pathway. XND1 has been demonstrated to be a direct target of SND1 and VND7 in Arabidopsis protoplasts (Zhong et al., 2010b). It contains a predicted bHLH consensus sequence in its promoter (http://www.Arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl), and it is therefore possible that ACL5 and its putative target SAC51, a bHLH transcription factor, act upstream of XND1 to control the rate of TE differentiation.

Is it a programme for secondary wall formation or cell death?

The coupling of secondary wall formation and cell death makes sense, as neither a thin-walled dead cell nor a thick-
walled living cell would be able to serve in long-distance water transport. However, whereas living cells are completely inefficient with regard to water transport, dead cells with thin walls are able to function as conduits in short-distance water transport where only a small negative pressure is generated, as for example in hydroids of mosses and thin-walled conduits of extinct protracted phyletic species (Sperry, 2003). Although phylogenetic relationships between mosses, protracted phyletic, and extant vascular plants are somewhat unclear (Kenrick, 2000), it is tempting to speculate that cell death may have preceded secondary wall formation during the evolution of vascular plants, leading to a scenario where the regulation of secondary wall formation was adopted into an existing cell death programme. One could imagine that when secondary wall formation evolved, the cell death process had to adapt and slow down to enable appropriate thickening of the cell wall, a function that has been suggested for thermospermine synthase ACL5 (Muñiz et al., 2008). Finally, what is observed after completion of the secondary wall could just be organized disposal of the cell contents, completing a cell death programme that had been switched on far earlier.

Concluding remarks

Regarding cell morphology, xylem vessel cell death in angiosperms can clearly be defined as a vacuolar (van Doorn et al., 2011) or autolytic type of cell death (van Doorn, 2011). Vessel elements typically contain large central vacuoles, which are involved in both storage of pro-death factors and the actual killing of the cell after rupture of the vacuolar membrane. The central vacuole is also important for angiosperm xylem fibres and the ancient type of TE is the gymnosperm tracheids, even though loss of its integrity is not causally related to cell death in these particular cell types since substantial amounts of degradation and/or remobilization of the cell contents occurs prior to the vacuolar bursting. Concerning cell death morphology, vessel elements seem to have adopted a cell death programme, which is probably not the most energetically efficient way of remobilizing cellular contents but ensures rapid cell death. In contrast, fibres seem to utilize autophagy to remobilize the cellular contents perhaps in a more controlled fashion.

Recent data on transcriptional regulation of xylem differentiation have indicated that cell death is transcriptionally regulated as a part of an overall xylem maturation programme, which includes secondary cell wall formation. This emphasizes the importance of cell death inhibition during xylem maturation. If the components required for cell death are synthesized simultaneously with the enzymes responsible for secondary cell wall synthesis, it is crucial for the cell to prevent premature activity of these components efficiently. Some of the mechanisms for this, such as inhibition of the hydrolytic enzymes by specific inhibitors and storage in inactive forms in safe compartments, are known. However, there must be additional mechanisms at play, for example to keep the vacuole intact until the secondary walls are correctly assembled in vessel elements and until the cell contents are appropriately remobilized in xylem fibres. Currently, little is known about the factors controlling timing of the vacuolar rupture. In xylem fibres, the time of vacuolar rupture varies significantly depending on the activity of the cambium and several external factors, and the lifetime of fibres is dramatically increased, for instance, during tension wood formation. In some species, xylem fibres retain their protoplast indefinitely (Fahn and Landeshem, 1963). Identification of factors that allow such dramatic increases in the longevity of the xylem fibres is likely to reveal completely new factors with the potential of inhibiting cell death even after completion of secondary walls.

Acknowledgements

The authors thank The Swedish Research Council VR and The Swedish Governmental Agency for Innovation Systems Vinnova (support of UPSC Berzelii Centre for Forest Biotechnology), The Swedish Research Council Formas (support of FuncFiber and BioImprove programs), and Umeå University for financial support to HT. Eric Beers is thanked for the gift of the Arabidopsis proXCP2::GUS seeds.

References


Funk V, Kositsup B, Zhao C, Beers EP. 2002. The Arabidopsis xylem peptidase XCP1 is a tracheary element vacuolar protein that may be a papain ortholog. Plant Physiology 128, 84–94.


of Arabidopsis with three Botrytis species: an important role for cell death control. Molecular Plant Pathology 8, 41–54.


Watanabe N, Lam E. 2011. Arabidopsis metacaspase 2d is a positive mediator of cell death induced during biotic and abiotic stresses. The Plant Journal 66, 969–982.


Zhao C, Avci U, Grant EH, Haigler CH, Beers EP. 2008. XND1, a member of the NAC domain family in Arabidopsis thaliana, negatively regulates lignocellulose synthesis and programmed cell death in xylem. The Plant Journal 53, 425–436.


