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SHORT COMMUNICATION

The Tr-cp 14 cysteine protease in white clover (*Trifolium repens*) is localized to the endoplasmic reticulum and is associated with programmed cell death during development of tracheary elements

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Abstract Cysteine proteases are known to be associated with programmed cell death, developmental senescence and some types of pathogen and stress-induced responses. In the present study, we have characterized the cysteine protease Tr-cp 14 in white clover (*Trifolium repens*). Tr-cp 14 belongs to the C1A family of cysteine proteases with homology to XCP1 and XCP2 from *Arabidopsis thaliana* and p48h-17 from *Zinnia elegans*, which previously have been reported to be associated with tracheary element differentiation. The proform as well as the processed form of the protein was detected in petioles, flowers and leaves, but the processed form was more abundant in leaves and petioles than in flowers. The Tr-cp 14 protein was localized to differentiating tracheary elements within the xylem, indicating that the cysteine protease is involved in protein remobilization during tracheary element differentiation. Immunogold studies suggest that the protease prior to the burst of the vacuole was associated to the ER cisternae. After disruption of the tonoplast, it was found in the cytoplasm, and, in later stages, associated with disintegrating material dispersed throughout the cell.

Keywords Cysteine protease · Endoplasmic reticulum · Immunolocalization · Tracheary element differentiation · *Trifolium repens*

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Introduction

In plants, programmed cell death permits developmental plasticity and is involved in almost all phases of the plant life cycle including responses to pathogens and abiotic stress (Pennell and Lamb 1997). Hence, programmed cell death is an intrinsic part of cell differentiation programmes that during the life cycle of a plant lead to the production of different cell types. One example is the differentiation of parenchyma cells into highly specialized tracheary elements. The tracheary elements function as components of xylem vessels or tracheids to conduct long-distance, inter-organ water transport and confer mechanical strength onto a plant body (Kuriyama and Fukuda 2001).

Tracheary elements undergo a well-defined process of differentiation, which requires a tight coordination of secondary cell wall formation and programmed cell death (Groover and Jones 1999). At maturity, the tracheary elements lose their nuclei and other cellular contents, leaving a hollow tube through which water and nutrients pass. This process is coordinated such that neighbouring tracheary elements are joined together to form an interconnected tubular network (Fukuda 1996; Jung et al. 2008).

Tracheary element differentiation has been studied extensively in vitro using *Zinnia elegans* as a model system in which mesophyll cells from young leaves can be induced by hormones to differentiate into tracheary elements at high frequency without cell division (Fukuda and Komamine 1980a, b). By using molecular and physiological markers, the in vitro differentiation process has been divided into three stages: I (dedifferentiation), II (restriction of developmental potential) and III (tracheary element specific development) (for reviews see Fukuda 2000; Turner et al. 2007).

A characteristic feature of tracheary element cell differentiation is the collapse of the vacuole, i.e. the vacuolization of the cytoplasm (Groover and Jones 1999), that coincides with the degradation of the nucleus (Fukuda 2004; Turner et al. 2007). It has been suggested that the degradation of nuclear and chloroplast DNA is triggered by vacuole collapse and is completed within only 15 min after the vacuole collapse (Obara et al. 2001). The rupture of the vacuole is regarded to be required for the release of hydrolytic enzymes, including proteases, DNases and RNases, into the cytoplasm (Funk et al. 2002; Ito and Fukuda 2002; Lehmann et al. 2001).

In animal systems, a range of proteolytic enzymes has been found to be key players in programmed cell death. Comparatively less is known about proteolysis and regulation of programmed cell death in plants. However, a range of plant studies are now available that document the involvement of cysteine proteases in programmed cell death, in particular with respect to tracheary element differentiation (Minami and Fukuda 1995; Ye and Varner 1996; Beers et al. 2004; Turner et al. 2007; Woffenden et al. 1998; Zhao et al. 2000). In the in vitro *Z. elegans* system, cysteine protease activity was shown to rapidly increase just before the lysis of the vacuole which in turn initiates the autolysis of the tracheary element cellular content in stage III (Minami and Fukuda 1995; Ye and Varner 1996; Yamamoto et al. 1997). In *Arabidopsis thaliana*, two papain-like cysteine proteases, XCP1 and XCP2, and one putative subtilisin-type serine protease, XSP1, have been identified from an enriched xylem cDNA library (Zhao et al. 2000), suggesting that these proteases might be associated with tracheary element differentiation. This was further confirmed by studies on *A. thaliana* transformed with either XCP1- or a XCP2 promoter-β-glucuronidase fusion constructs, where the GUS activity in the transgenic plants was confined to differentiating tracheary elements (Funk et al. 2002). In a similar approach, Pyo et al. (2004) found that the *Z. elegans* ZCP4 promoter drives GUS expression only in immature tracheary elements in *A. thaliana*. Subcellular analyses with immunogold labelling revealed that XCP1 and XCP2 in differentiating tracheary elements often were associated with the endoplasmic reticulum (ER), the Golgi vesicles and the intact central vacuole. This indicates a classical secretory pathway localization for the two cysteine proteases (Avci et al. 2008).

In this paper, we have characterized the cysteine protease Tr-cp14 that is associated with early tracheary element formation in *Trifolium repens*. It is highly identical to the paralogous XCP1 and XCP2 of *A. thaliana* and also appears to be secreted via the secretory pathway. However, in contrast to the two *Arabidopsis* cysteine proteases, Tr-cp14 could not be detected in the central vacuole prior to the implosion of the tonoplast. Rather, the cysteine protease Tr-cp14 was observed to accumulate in the endoplasmic reticulum, from where it appeared to spread throughout the cell during the collapse of the central vacuole.

Material and methods

Plant material

Throughout this study, *T. repens* cv. Rivendel were used. The procedures for single stolon growth, inoculation with *Rhizobium*, construction and screening of the genomic library have been described previously (Asp et al. 2004b).

Preparation and affinity purification of antibodies

A 980-bp fragment of the Tr-cp 14 cDNA clone encoding the pro-region and the catalytically active region of the cysteine protease (GenBank accession no. AY192361) was cloned into the pET21b vector (Novagen, Darmstadt, Germany) to overexpress the Tr-cp 14 polypeptide in *Escherichia coli*. The protein was expressed, purified and renatured as described by (Asp et al. 2004a). Polyclonal antibodies were raised in rabbits against the antigen by DakoCytomation A/S (Glostrup, Denmark). Affinity columns for purification of antibodies were obtained by coupling of the antigen to SulfoLink columns (Pierce, Rockford, IL, USA). The coupling of antigen, binding of antibodies, subsequent washing and elution of specific antibodies were performed as described in the SulfoLink® Kit manual provided by the manufacturer.

Immunoblot analysis

Equal amounts of proteins extracted from different *T. repens* tissues were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The proteins were subsequently transferred onto nitrocellulose membranes (Roth, Karlsruhe, Germany). The purified antibody directed towards Tr-cp 14 was used in a dilution of 1:1,000. The secondary antibody directed against rabbit immunoglobulins (GE Healthcare, Munich, Germany) was used in a dilution of 1:100,000. For detection of immunoreactive

proteins, the ECL Advance Western Blotting Detection Kit of GE Healthcare (Munich, Germany) was used.

Fixation and embedding

For ultrastructural and immunogold labelling analysis, tissue from petioles of *T. repens* plants was fixed in 2.5 % *v*/*v* glutaraldehyde and 1 % *v*/*v* formaldehyde in 0.1 M sodium cacodylate (pH 7.4) at 4 °C overnight, and postfixed for 2 h in buffered 1 % osmium tetroxide on ice. Samples for immunofluorescence were fixed in 2 % *v*/*v* formaldehyde only. Washing was with 0.1 M sodium cacodylate (pH 7.4). The specimens were dehydrated in a graded series of ethanol and embedded in LR White resin (London Resin Company, Reading, UK). Polymerization was in gelatine capsules at 50 °C overnight.

Immunofluorescence analyses

Semithin sections (1 μm) of the samples were cut with a diamond knife at a Leica Ultracut UCT ultramicrotome. The sections were collected on coated glass slides (Superfrost Plus, Menzel-Gläser, Braunschweig, Germany) and dried at 40 °C on a hot plate. Blocking was performed in Tris-buffered saline (pH 7.8) supplemented with 0.1 % (*v*/*v*) Tween-20 (TBST), to which 0.1 % (*w*/*v*) BSA and normal goat serum (1:30) were added (blocking buffer). The sections were then incubated overnight at 4 °C with the cysteine protease antibody in blocking buffer (1:10) and washed with TBST and blocking buffer. Incubation with anti-rabbit Alexa 594 was for 60 min at room temperature (Molecular Probes, Carlsbad, CA, USA), diluted (1:1,000) in blocking buffer. The slides were washed with TBST and water and were mounted with cover slips following addition of 3 % *w*/*v* *N*-propyl gallate [in 90 % (*v*/*v*) glycerol and 0.1 M Tris, pH 9.0]. Fluorescence emission was recorded with a Leica TCS SP confocal microscope at 600–670 nm for Alexa 594 fluorescence. Excitation was set to 543 nm.

Electron microscopy

For ultrastructural analyses, ultrathin sections of the specimens were cut with a diamond knife at a Leica Ultracut UCT ultramicrotome and placed on copper grids. Sections were stained with uranyl acetate (Reynolds 1963) and with lead citrate (Watson 1958) and observed in a FEI CM10 TEM.

For immunogold labelling, the sections were placed on nickel grids. As one control, we used the affinity-purified antibody against the large subunit of ribulose-1,5-bisphosphate-carboxylase/oxygenase (RbcL) from rice (Chiba et al. 2003), diluted 1:500. The secondary antibody was 15 nm gold-coupled goat anti-rabbit IgG (British Biocell International, Cardiff, UK), diluted 1:50 in blocking buffer. The sections were finally stained with uranyl acetate and observed in a Philips CM10 TEM.

Results

Nucleotide sequence and genomic organisation

A genomic library of *T. repens* cv. Rivendel was screened with a fragment of the coding region of the SAG12 cysteine protease from *A. thaliana*. A total of 16 clones were obtained, and comparative sequence analysis revealed that all were encoding putative cysteine proteases of the C1A family of papain-like proteases (van der Hoorn 2008). This paper presents the characterisation of one of the clones, designated Tr-cp 14 (GenBank accession no. AY192360).

The genomic clone of Tr-cp 14 was 4,410 bp and had a genomic organisation of three introns and four exons. The first intron was 215 bp, while the other two introns were 99 and 105 bp in length, respectively. The first exon was the largest and was predicted to be 462 bp, while the other three exons were 234, 141 and 216 bp in length, respectively (Online Resource 1a). The Tr-cp 14 gene was predicted to have a length of 1,472 bp with a 1,053-bp coding region (NetPlantGene World Wide Web Prediction Server, Hebsgaard et al. 1996), and by alignment with the corresponding cDNA sequence (GenBank accession no. AY192361).

Comparative sequence analysis

The amino acid sequence of the cysteine protease encoded by Tr-cp 14 was aligned to a selection of papain-like cysteine proteases of the C1A family from *A. thaliana*, *Lotus japonicus*, *Medicago truncatula*, *Zinnia violacea*, *Helianthus annuus* and *Z. elegans*, and a phylogenetic tree (Online Resource 1b) was constructed (Esteban-Garcia et al. 2010; Beers et al. 2004). The cysteine protease belongs to the highly conserved C1A-3 subfamily of cysteine proteases (Online Resource 2), and some members of the clade, such as e.g. XCP1 and XCP2 from *A. thaliana* (Zhao et al. 2000), have previously been shown to be involved in tracheary element differentiation (Online Resource 1b).

The C1A family of cysteine proteases are synthesized as preproenzymes with a signal peptide and a prodomain at the N-terminus. The autoinhibitory prodomain folds back onto the catalytic cleft and is removed during activation of the enzyme (Taylor et al. 1995; van der Hoorn 2008). Based on sequence information, a molecular mass of 40 kDa was predicted for the preproenzyme, while the activated enzyme was predicted to have a molecular weight of 32 kDa.

Immunolocalization of Tr-cp 14 during tracheary element differentiation

Proteins extracted from petioles, leaves and flower of *T. repens* were used for immunoblot analysis. By using the purified antibody, various amounts of proteins of the same size as the active and the proforms of Tr-cp 14 could be detected in different tissues. While the proform of the enzyme having a molecular weight of approximately 40 kDa was detectable in all tissues, the processed form having a molecular weight of approximately 32 kDa was clearly detectable in leaves and petioles, but almost undetectable in flowers (Fig. 1a, b).

The localization of Tr-cp 14 was performed by immunofluorescence analyses with antibodies raised against the Tr-cp 14 protein (Fig. 1c) and by immunogold studies at the ultrastructural level of cells undergoing tracheary element differentiation (Fig. 1d–h). Differently fixed material and more than 50 sections were studied. There was no difference in labelling between samples either fixed with formaldehyde alone or postfixed with osmium tetroxide (results not shown). Because of better ultrastructural preservation of membranes, we focussed our analyses to the samples postfixed with osmium tetroxide. Tr-cp 14 was exclusively localized in a specific array of cells differentiating into tracheary elements within the xylem of the vascular tissue (Fig. 1c). In cells at an early stage of tracheary element differentiation, the Tr-cp 14 protein appeared to be unevenly distributed in the cytoplasm surrounding the intact vacuole (cells marked by stars in Fig. 1c, d), while at later stages, after collapse of the central vacuole, Tr-cp-14 was found in high amounts throughout the cell (cells marked by arrows in Fig. 1c, d). At later stages of tracheary element differentiation, when most of the cellular content had been degraded, Tr-cp 14 localized to the periphery of the cell adjacent to the secondary cell wall, where remnants of cytoplasm were detectable (Online Resource 3). Tr-cp 14 was not detected in cells where lysis was completed, suggesting that Tr-cp 14 is exclusively associated with early tracheary element differentiation.

In cells with an intact vacuole, most gold particles were associated with cisternae of the rough endoplasmic reticulum and with smooth vesicles and cisternae that locally were connected to the rER (Fig. 1e, f). Hence, most of the protease appeared to be bound to the membrane of the endoplasmic reticulum. After rupture of the tonoplast, Tr-cp 14 appeared to have been released from the cisternae, and many gold particles could be detected throughout the vacuolarized cytoplasm, inside the organelles and the nucleus (Fig. 1g). This pattern of labelling was specifically obtained with the antibody directed towards Tr-cp 14. When we used an antibody directed towards the large subunit of RbcL, no signals were observed to be bound to the nucleus in petiole cells of *T. repens* (Online Resource 4). At a later stage of cellular differentiation, when the cells had lost their structural integrity, Tr-cp 14 was found to be distributed throughout the cell, where it was mainly associated with electron-dense cellular material, and absent from regions of low electron density (Fig. 1h). Finally, when most of the cellular content had been degraded, the antibody was found to bind to residual material attached to the cell wall (Online Resource 3).

Discussion

A 4.4-kb genomic clone, designated Tr-cp 14, encoding a putative cysteine protease belonging to the C1A family was obtained by screening a *T. repens* genomic library with a probe from the SAG12 cysteine protease encoding gene from *A. thaliana* (Gan and Amasino 1995). Tr-cp 14 belongs to the C1A family of cysteine proteases and is an orthologue of p48h-17 from *Z. elegans* (Ye and Varner 1996) and XCP1 and XCP2 from *A. thaliana* (Zhao et al. 2000). The genomic architecture of Tr-cp 14 with four exons and three introns corresponds to that of XCP1 and XCP2 from *A. thaliana* as well as to the putative orthologues from *Lotus corniculatus* and *M. truncatula* and, thus appears to be conserved between species.

Immunoblot analyses with an antibody against the protein of Tr-cp 14 detected two proteins in leaves, petioles and flowers. The molecular weights could correspond to the proform and the active form of Tr-cp 14, whereby the putative active form was found to be most abundant in leaves and petioles. Immunohistochemical analysis showed that Tr-cp 14 was like 48h-17 in *Z. elegans* (Ye and Varner 1996) and XCP1 in *A. thaliana* (Funk et al. 2002) localized to cells developing into tracheary elements. During tracheary element differentiation, a number of hydrolytic enzymes, including DNAses, RNAses and proteases, have previously been shown to accumulate in the vacuole of differentiating cells (Kuriyama and Fukuda 2001; Fukuda 2000; Turner et al. 2007; Jung et al. 2008). It has been suggested that the hydrolytic enzymes degrading the cellular content are released from the vacuole into the cytoplasm (Kuriyama and Fukuda 2001; Fukuda 2000; Turner et al. 2007). In accordance with this concept, immunofluorescence studies identified XCP1 in vacuoles (Funk et al. 2002), and recent immunogold analyses indicated that XCP1 and XPC2 relocate from the endoplasmic reticulum to the vacuoles before rupture of the vacuole (Avci et al. 2008).

Although we cannot rule out that a minor amount of the Tr-cp 14 protease is located inside the vacuole, immunogold labelling showed that the major fraction was located in the ER and was found to spread throughout the vacuolarized cytoplasm after rupture of the tonoplast. Rather, the protein

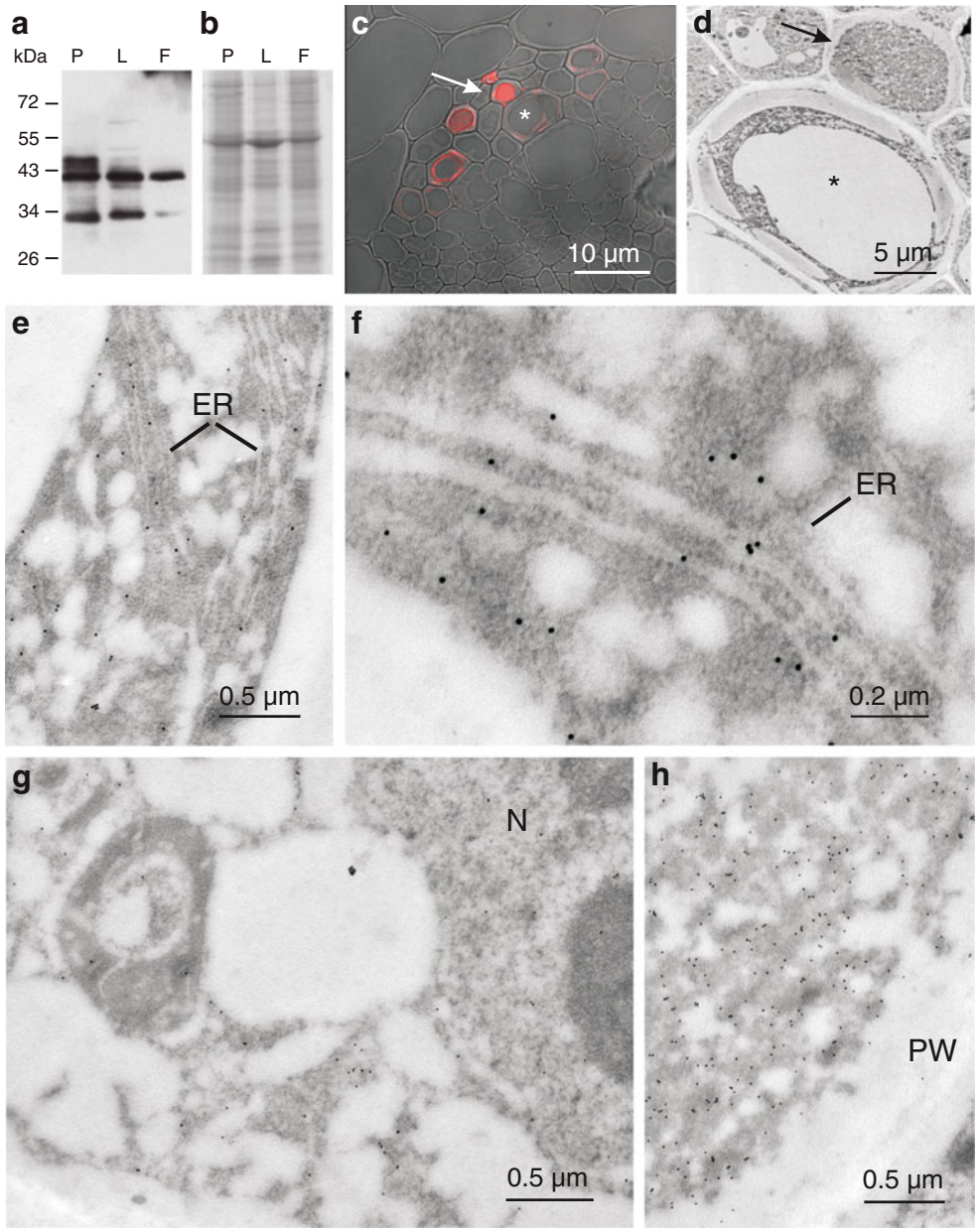

Fig. 1 **a** Immunological detection of the Tr-cp 14 protein in total protein extracts of petiole (*P*), leaf (*L*) and flower (*F*) of *T. repens*. **b** Coomassie-stained SDS–PAGE gel of total protein extracts from *T. repens* used for immunological detection of the Tr-cp 14 protein. **c** Localization of the Tr-cp 14 protein in tracheary element differentiating cells in the xylem of petiole of *T. repens* by immunofluorescence labelling. The Tr-cp 14 protein can be detected in the cytoplasm of cells differentiating into tracheary elements. During the early stages of tracheary element differentiation (cell marked by *star*), the Tr-cp 14 protein was detectable in the cytoplasm surrounding the central vacuole. After rupture of the tonoplast, strong labelling was observed throughout the cell (cell marked by *arrow*). After digestion of the cellular content, a weak label can be detected at the cellular periphery. This image is a single confocal laser scanning microscopy image, where the bright field image has been overlaid with the immunofluorescence signals. **d** TEM micrograph of cells undergoing tracheary element differentiation in the xylem of the petiole of *T. repens*. Cells before (*star*) and after (*arrow*) collapse of the vacuole. **e–h** Immunogold localization of Tr-cp 14 in tracheary element differentiating cells in petiole one of *T. repens*. **e**, **f** Before collapse of the central vacuole, the Tr-cp 14 protein was detectable in association with rough and smooth cisternae of the endoplasmic reticulum (*ER*). **g** During collapse of the central vacuole, many gold particles can be found in the cytoplasm and in the nucleus (*N*). **h** After the vacuole has collapsed, the Tr-cp 14 protein can be identified in cytoplasmic residues throughout the cell. The cell walls (*PW* primary wall) are not labelled

appeared to accumulate and to be retained in the endoplasmic reticulum until tonoplast rupture. Although the genomic structure and the expression characteristics suggest that the Tr-cp 14 protease is closely related to XCP1, XCP2 and p48h-17 proteases, there is a distinct difference in the amino acid sequence which could be responsible for differences in subcellular distribution. Tr-cp 14 is the only protease of these three closely related enzymes which has a tri-Lys

motif at the C-terminus (Online Resource 2) (Asp et al. 2004a). C-terminal tri-Lys motifs have been identified as efficient ER retrieval and retention signals (Horak and Wenthold 2009).

It may thus be hypothesized that the Tr-cp 14 cysteine protease is stored as a preproenzyme in the ER where the pH is approximately 7.0 (Kim et al. 1998) and is activated by low pH after rupture of the central vacuole. The transient ER localization step followed by secretion via Golgi-dependent and Golgi-independent pathways is a regular feature of many other cysteine proteases such as members of the closely related KDEL group, for example the SH-EP of V*igna mungo* seedlings that is stored in KDEL-tailed cysteine protease-accumulating vesicles (KV) (Tsuru-Furuno et al. 2001) where after the SH-EP are transported to protein storage vacuoles by the Golgi-independent pathway (Toyooka et al. 2000; Okamoto and Minamikawa 1998). Other examples are the cysteine proteases of ER-derived ricinosomes of the endosperm of germinating castor bean (*Ricinus communis*) (Gietl and Schmid 2001). These proteases are released from ricinosomes of dying cells of the endosperm and degrade residual cellular material (Schmid et al. 1999). During activation of these enzymes, the KDEL sequence is removed (Gietl and Schmid 2001; Okamoto et al. 2001).

Tr-cp 14 is to our knowledge the first ER-residing protease of the C1A family involved in cell death during tracheary element formation. By storage of the inactive form in the ER, the cell might ensure that the enzyme is already spread throughout the cell when the vacuole ruptures. Tr-cp 14 hence might belong to the cell death-inducing proteins stored inside the endoplasmic reticulum which was shown to play an important role in several plant cell death processes (Cacas 2010).

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Conflict of interest The authors declare that they have no conflict of interest.

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