Characterization of tobacco cell lines transformed with the AtPIN5 gene from the auxin efflux carrier family of Arabidopsis

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PIN-FORMED (PIN) proteins represent family of transmembrane proteins with predicted secondary transporter function (Zažímalová et al., 2007). Several members of the family play critical role in polar auxin transport, i.e. spatio-temporally defined distribution of plant hormone auxin responsible for many key events in plant development. The 'traditional' well-characterised members of PIN family have been shown to be rate- and direction-limiting elements of auxin efflux complex.

*At*PIN5 (AT5G16530) is so far uncharacterised member of the family and together with *At*PIN8, the most dissimilar to the rest of the protein family. Particularly striking difference resides in almost complete lack of variable hydrophilic loop which connects two hydrophobic domains (Fig. 1) and could probably have rather regulatory than transport function (Matsuoka et al., 1993). The characterisation of *At*PIN5 function may help to understand the regulatory function of hydrophilic loop of the 'longer' ('traditional') PINs.

To test the function of *At*PIN5, well-characterised tobacco BY-2 cells (Nagata et al., 1992) have been used. After inducible overexpression of myc-tagged AtPIN5 (Fig. 2) indirect immunofluorescence visualisation using anti-myc antibody showed ER-like pattern in interphase cells (Fig. 3c). In contrast, other well-characterised PIN proteins localise preferentially on the transversal plasma membranes, determining the directionality to the flow of auxin through the file of cells (Figs 3a, b for PIN1 and PIN7, respectively). Moreover, in telophase, where PIN1 (Fig. 3d) and PIN7 (Fig. 3e) were localised into the newly formed cell plate, PIN5 was again reflecting the distribution of endomembranes (Fig. 3f).

While the activity of overexpressed *At*PIN7 protein led to symptoms of auxin starvation (Petrášek and Zažímalová, 2006; Fig. 4 a-c, e), after *At*PIN5 overproduction auxin starvation phenotype was not observed (fig 4d). Instead, part of the PIN5 overexpressing population started to die out (fig 4f, h). Interestingly, this effect of the action of *At*PIN5 can be partly rescued by the addition of synthetic auxin 2,4-D to the media (fig 4f, i). Cell death induced by the AtPIN5 overexpression was shown to have some characteristics typical for programmed cell death such as protoplast shrinkage, vacuome disintegration (Fig. 5a-g) and DNA fragmentation (Fig. 5h-m).

The model of function of various PINs is presented in Fig. 6.

PIN5 - the most distinct member of the PIN-FORMED (PIN) protein family - has a role in auxin redistribution, but it is quite different from the role of all so far characterised members of this family of auxin efflux carriers.

Fig. 1: *At*PIN5 protein represents the most distinct member of the PIN protein family

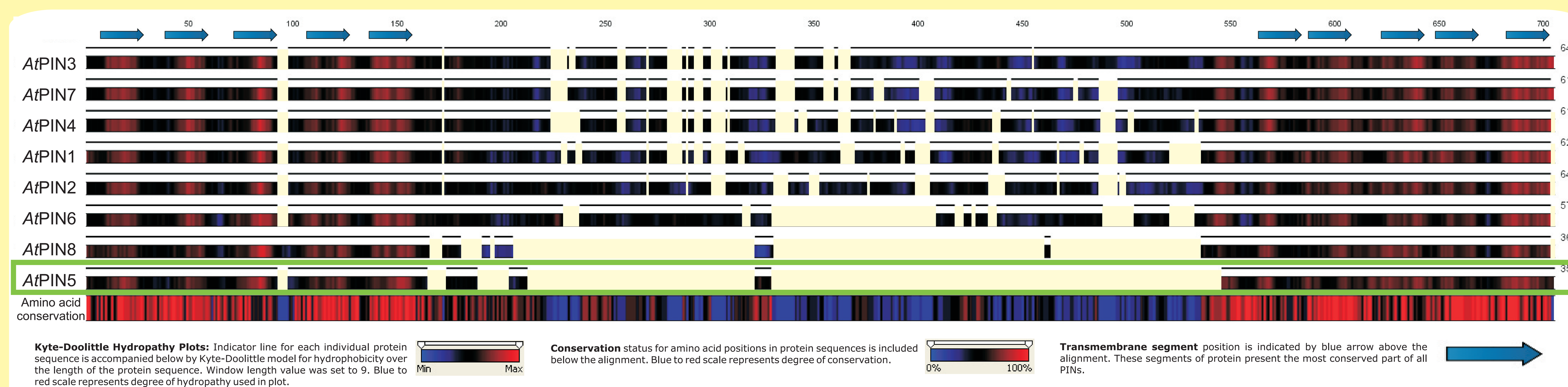


Fig. 2: RT-PCR of inducible *At*PIN5 in BY-2 cells

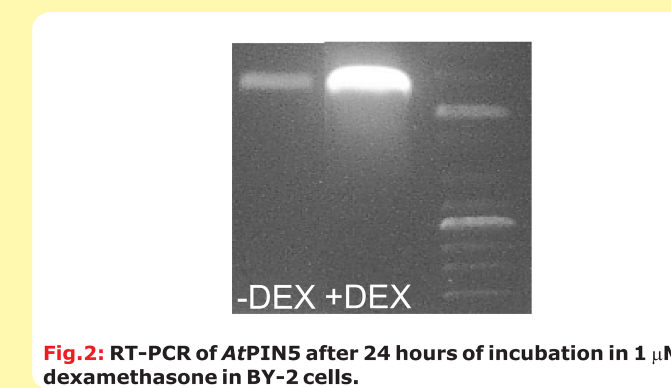


Fig. 2: RT-PCR of *AtPIN5* after 24 hours of incubation in 1 μM dexamethasone in BY-2 cells.

Fig. 3: *At*PIN5 protein localisation differs strikingly from Localisation of other PINs

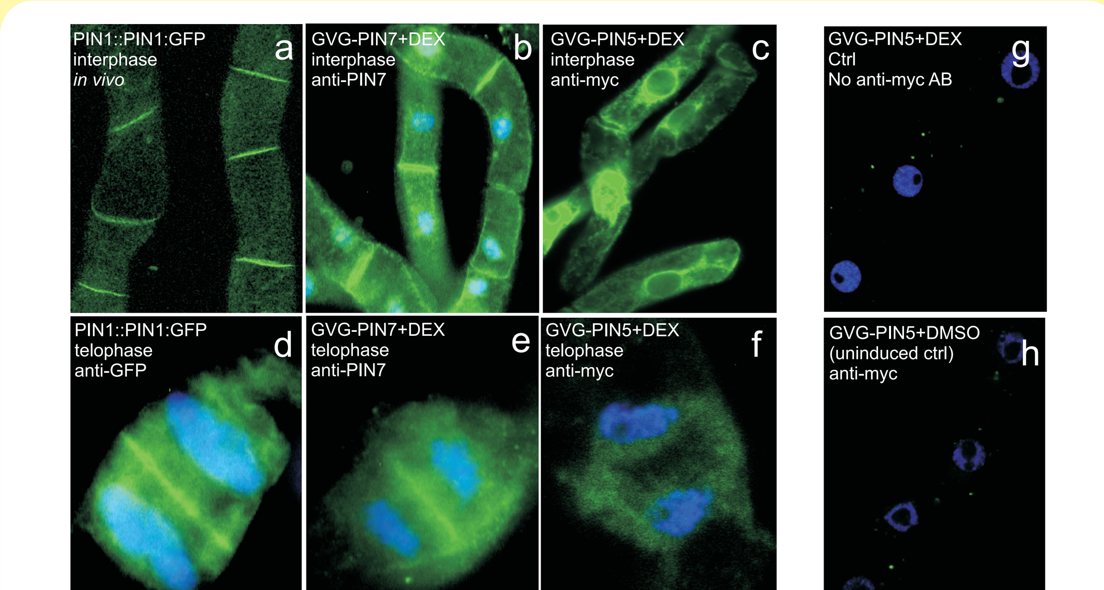


Fig. 3: Localisation of PINs in BY-2 cells. (a-c) Interphase cells, PIN1::PIN1:GFP (a), anti-PIN7 immunofluorescence in GVG-PIN7 cells (b), anti-myc immunofluorescence in GVG-PIN5 cells (c). (d-f) Telophase cells, PIN1::PIN1:GFP (d), anti-PIN7 immunofluorescence in GVG-PIN7 cells (e), anti-myc immunofluorescence in GVG-PIN5 cells (f). Control dexamethasone-induced GVG-PIN5 cells without anti-myc antibody (g). Control non-induced GVG-PIN5 cells with anti-myc antibody (h).

Fig. 4: Cell fate after overexpression of *At*PIN5 in BY-2 is different from the effect of other 'traditional' PINs.

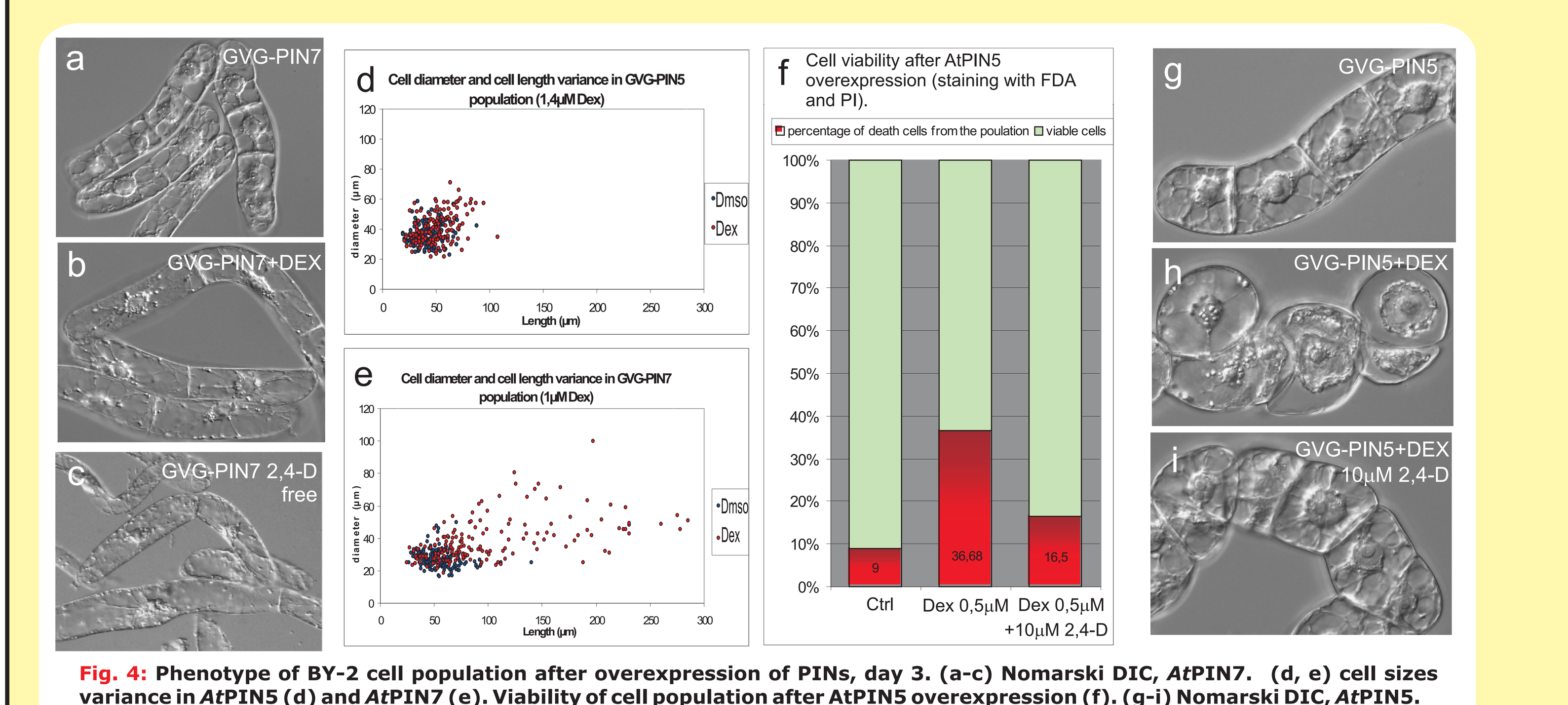
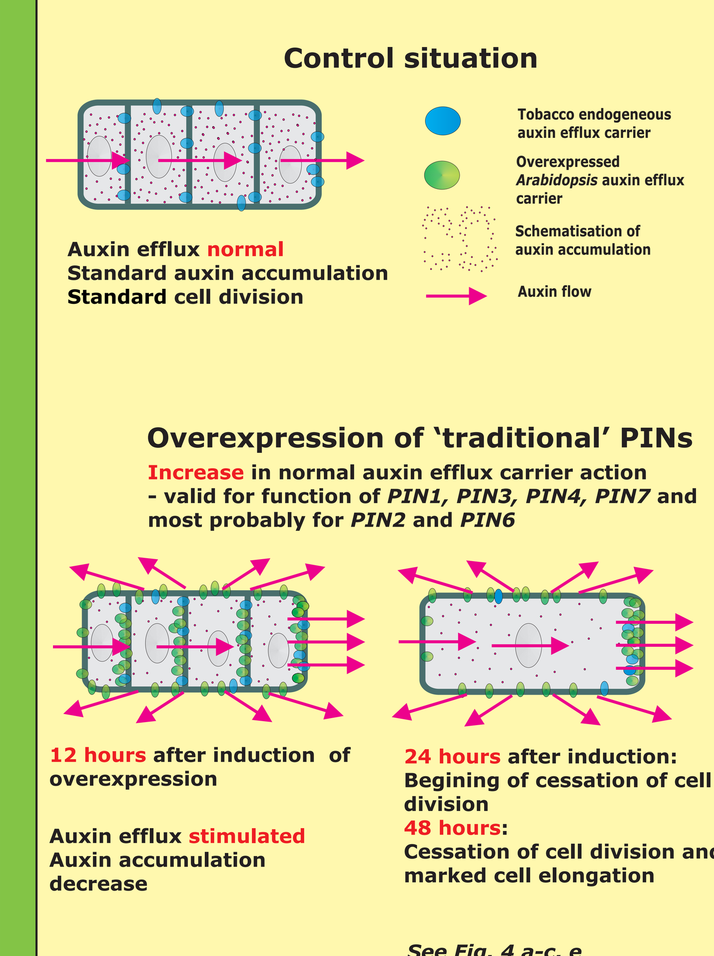
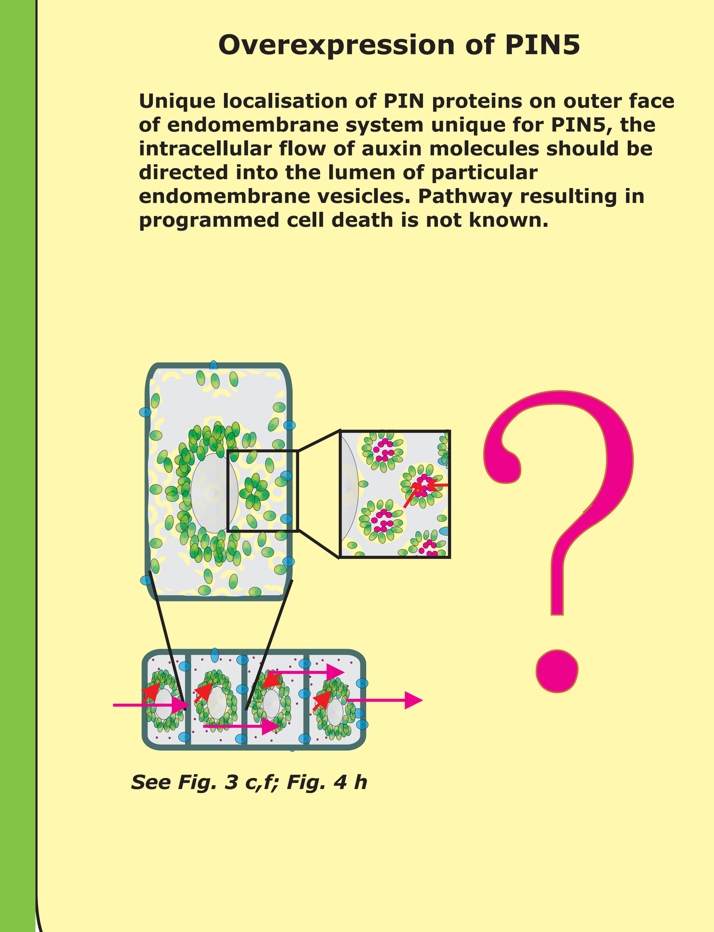


Fig. 4: Phenotype of BY-2 cell population after overexpression of PINs, day 3. (a-c) Nomarski DIC, AtPIN7. (d, e) cell sizes variance in *At*PIN5 (d) and *At*PIN7 (e). Viability of cell population after AtPIN5 overexpression (f). (g-i) Nomarski DIC, *At*PIN5.

Fig. 6: Model of auxin flow through the cells





Methods:

Plant material:
Cells of tobacco (*Nicotiana tabacum* L. cv Bright-Yellow 2) line BY-2 (Nagata et al., 1992) were cultivated in darkness at 25°C on an orbital incubator (IKA KS501, IKA Labortechnik, Staufen, Germany; 120 rpm, orbital diameter 30 mm) in liquid medium (3% [w/v] sucrose, 4.3 g.l^{-1} Murashige and Skoog salts, 100 mg.l^{-1} inositol, 1 mg.l^{-1} thiamin, 0.2 mg.l^{-1} 2,4-dichlorophenoxyacetic acid, and 200 mg.l^{-1} KH_2PO_4, pH 5.8) and subcultured weekly. Stock BY-2 calli were maintained on media solidified with 0.6% (w/v) agar and subcultured monthly. Transgenic cells and calli were maintained on the same media supplemented with 100 mg.ml^{-1} cefotaxim and 100 mg.ml^{-1} kanamycin or 20 mg.ml^{-1} hygromycine. Cultivation conditions and media for Arabidopsis cells were same as for tobacco cells.
All chemicals were obtained from Sigma (St. Louis) unless otherwise stated. Expression of inducible AtPIN genes was induced in cell suspensions of transformed cell lines by the addition of dexamethasone (DEX; 0,5 or 1 μM, from 100 mM stock solution in DMSO) at the beginning of the subcultivation period.

Alignment of protein sequences:
Alignment of protein sequences was constructed with program CLC Main Workbench 4,0 using cost value 10 for gap opening and 1 for gap extension.

DNA constructs and transformation of BY-2 cells:
Arabidopsis PIN5 coding sequences were cloned into the binary transformation plasmid pTA7002 containing the complete two-component glucocorticoid-inducible system (Aoyama and Chua, 1997) and construct was named pTA-AtPIN5. BY-2 cells were transformed according to An et al. (1985) as described in Petrášek *et al* (2003). PTA-AtPIN5 was introduced into Agrobacterium tumefaciens strain C58C1 or GV2260 and then into tobacco cells by co-cultivation. Lines with the most obvious phenotypical response to overexpression induced by DEX were selected and named GVG-PIN5.

Cell viability
Changes in cell viability were monitored after Fluorescein diacetate and Propidium iodide (both Biochemika) staining.
TUNEL method was used to detect free 3´OH termini in nuclear DNA - TMR red (red fluorescence) In Situ Cell Death Detection Kit (Roche Diagnostic). The procedure was exactly performed according to the method described by Jones *et al* (2001).

Immunofluorescence
PIN proteins were visualized according modified protocol for tubulin visuailisation as described in Petrášek *et al.* (2003). 100μM MBS (3-maleimidobenzoic acid N-hydroxysuccinimide ester) was used for prefixation. Monoclonal mouse anti-*c-Myc* antibody (Sigma) was used at 1:500, polyclonal rabit anti-PIN7 antibody at 1:2000 and monoclonal anti-GFP20 (Sigma) at 1:1000 dilution.

Microscopy
Nikon Eclipse E600 epifluorescence microscope equipped with appropriate filter sets and Nomarski DIC was used. DIC images were taken with color digital camera (DVC 1310C, USA) and digitally stored. Confocal microscopy was performed on Zeiss LSM 5 Duo.

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Acknowledgements

We are thankful to Jiří Friml who kindly provided PIN expression constructs, to Markéta Pařezová and Nikola Drážná for their excellent technical assistance and to Sibu Simon for help with RT-PCR and innumerable small helps.
This work was supported by the Grant of the Ministry of Education, Youth and Sports of the Czech Republic, project no. LC06034 and by the Grant Agency of the Academy of Sciences of the Czech Republic, project no. KJB600380604.

Fig. 5: The cell phenotype after the overexpression of *At*PIN5 in BY-2 has some characteristics of programmed cell death

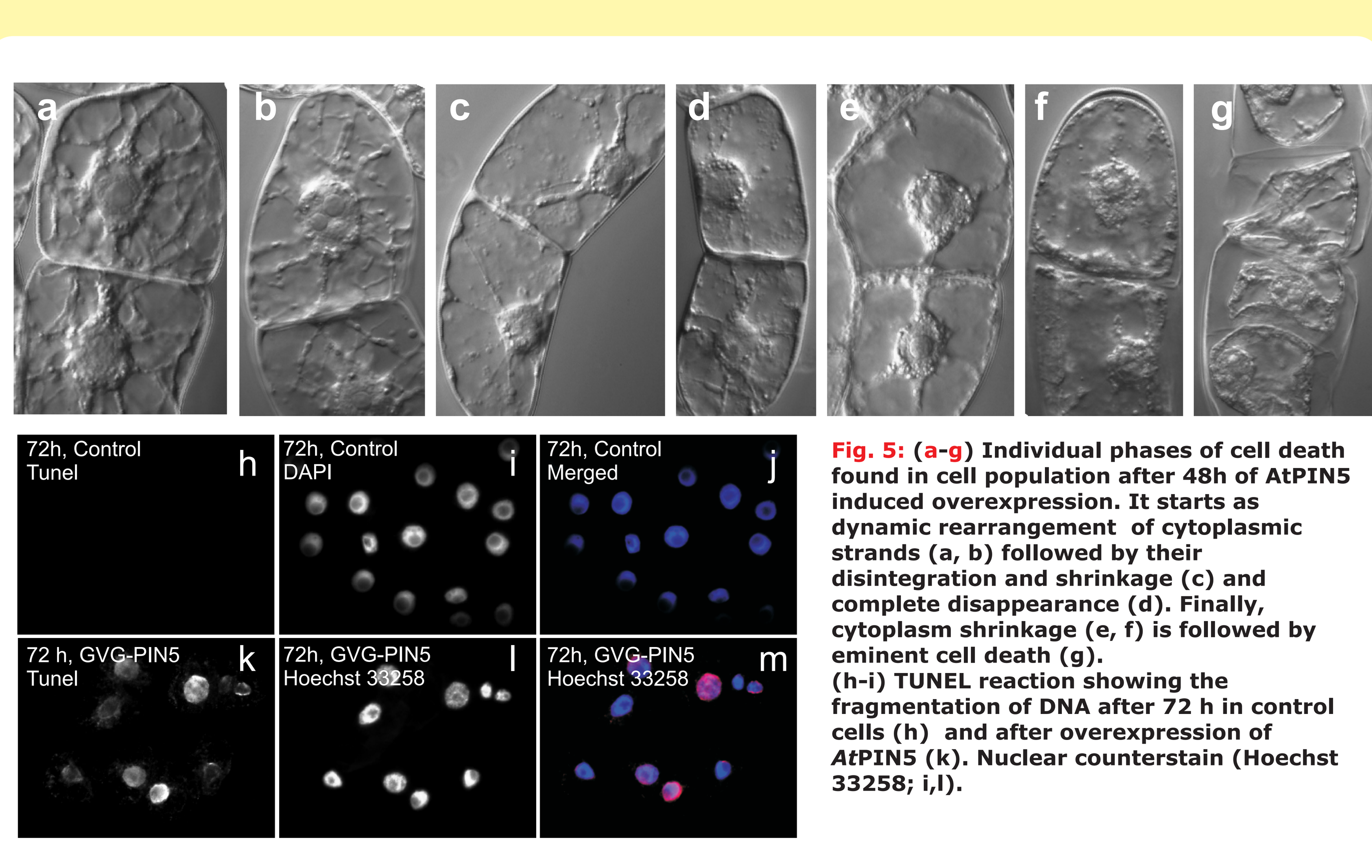

Fig. 5: (a-g) Individual phases of cell death found in cell population after 48h of AtPIN5 induced overexpression. It starts as dynamic rearrangement of cytoplasmic strands (a, b) followed by their disintegration and shrinkage (c) and complete disappearance (d). Finally, cytoplasm shrinkage (e, f) is followed by eminent cell death (g). (h-i) TUNEL reaction showing the fragmentation of DNA after 72 h in control cells (h) and after overexpression of *At*PIN5 (k). Nuclear counterstain (Hoechst 33258; i,l).