Assessment of autophagy in plant cells

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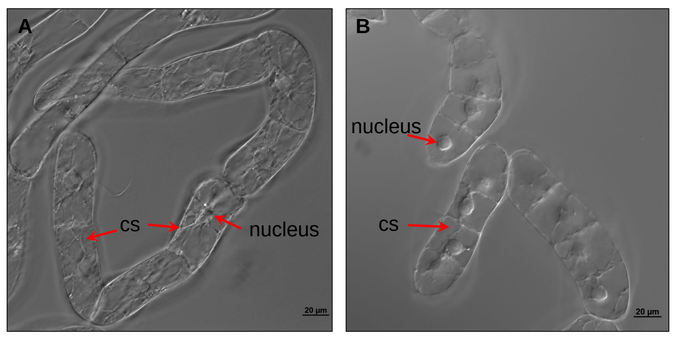
Autophagy is an evolutionary conserved catabolic process deemed to maintain or restore cellular and organismal homeostasis. In plants, basal autophagy is essential for growth and development; it is required for nutrient remobilization during senescence and nutrient deficiency, for removal of organelles and macromolecules formed during plant development or damaged by environmental stresses. The material to be degraded is delivered to the vacuole within the double–membrane vesicles - autophagosomes that are generated in the cytoplasm when macroautophagy is induced.
Polyamines (PAs) are ubiquitous cell components essential for normal growth of cells being implicated in cell division, differentiation and death during plant morphogenesis and in response to stresses. The direct relationship between PAs and autophagy has been acknowledged in the yeast, animal and human cells. We have studied the role of PAs in the process of autophagy in two plant model systems – tobacco BY-2 cell culture and Norway spruce embryogenic cultures.

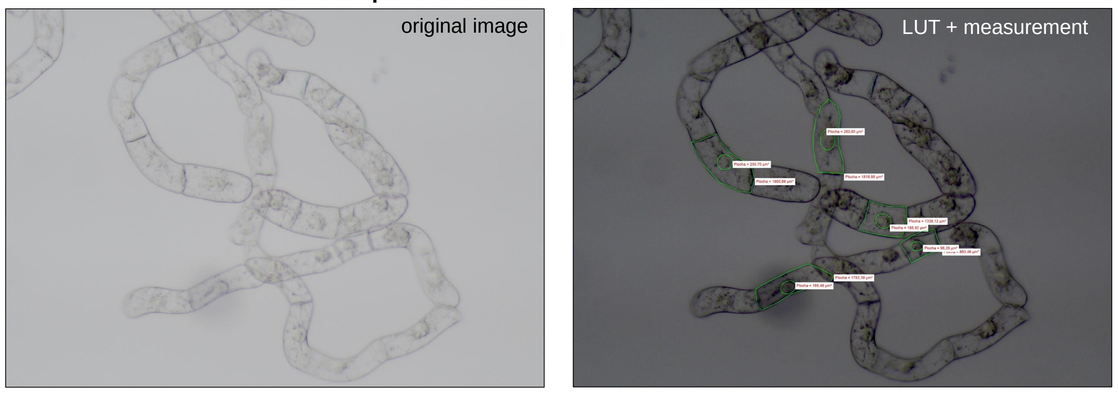
Tobacco BY-2 cell culture



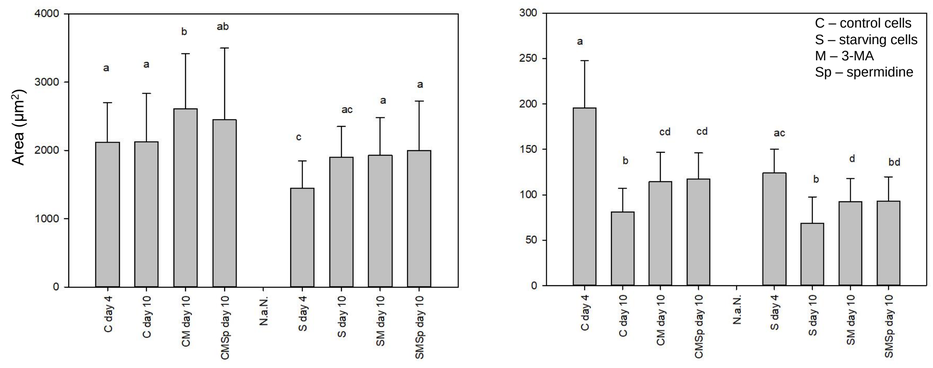
(A) Well-growing non-treated BY-2 cells are characterized by dense network of cytoplasmic strands.
(B) Tobacco cells exposed to sucrose starvation in order to induce autophagy. Cytoplasmic strands (cs) disappeared, starving cells cells were less metabolically and mitotically active.

To describe the effect of starvation and applied spermidine (one of higher PAs) and/or 3-methyladenine (3-MA, an inhibitor of autophagosome formation), we measured the area of cells and cell nuclei using the image analysis software NIS-elements (Laboratory imaging, Prague, Czech Republic). At first, we manually measured, after contrast enhancement, 5 randomly selected cells and their nuclei on 10 images at each variant.

En example of the cell /nucleus area measurement

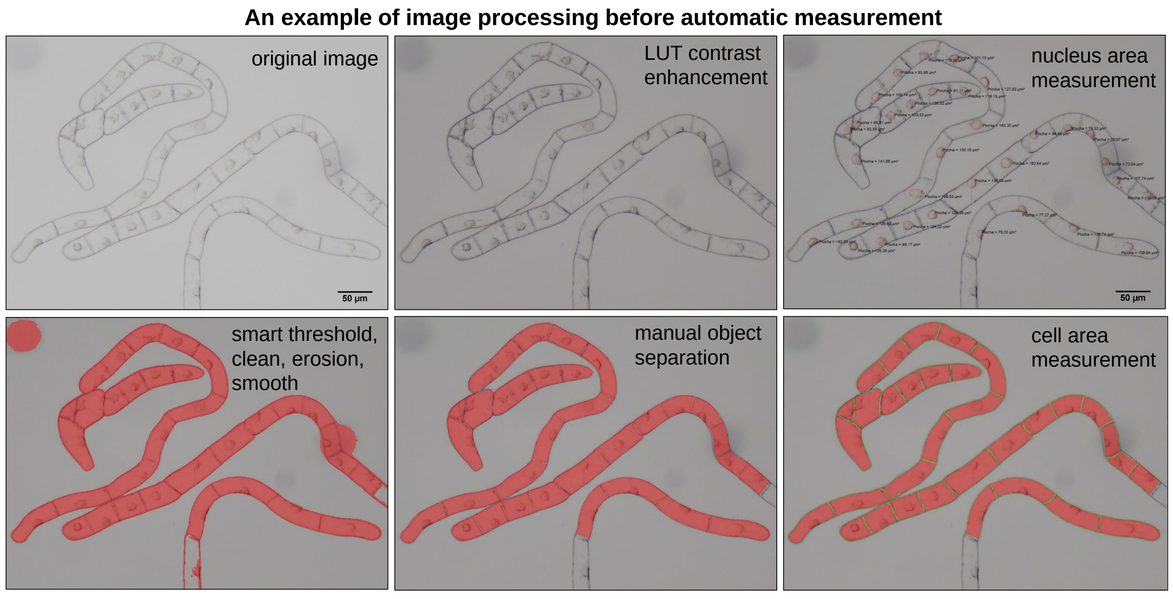


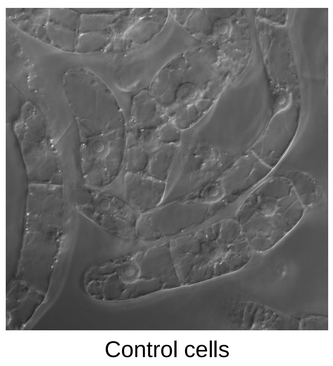
Results of the measurement



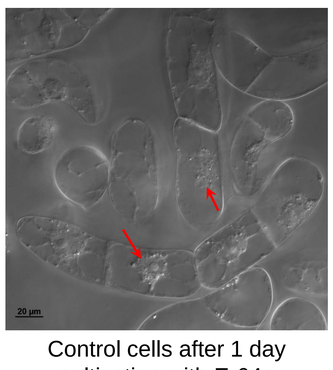
Even though this simple analysis has proved the statistically significant differences in both cell and nucleus areas after 4 days of starvation as compared to control we are afraid that this approach could provide biased results. To obtain more unbiased results we used smart thresholding and basic tools of mathematical morphology to adjust the binary image before automatic measurement of the cell areas. Threshold of nuclei is difficult, we measured area of nuclei manually. Evaluation of the results is in the process.

An example of image processing before automatic measurement

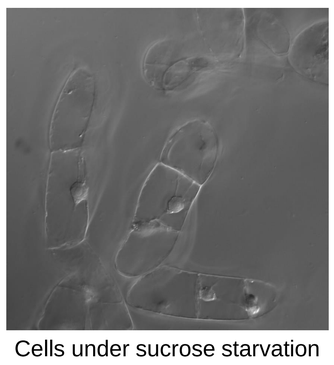




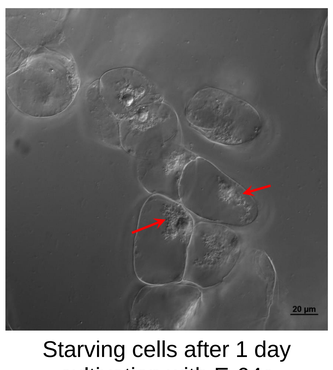
Control cells



Control cells after 1 day cultivation with E-64c



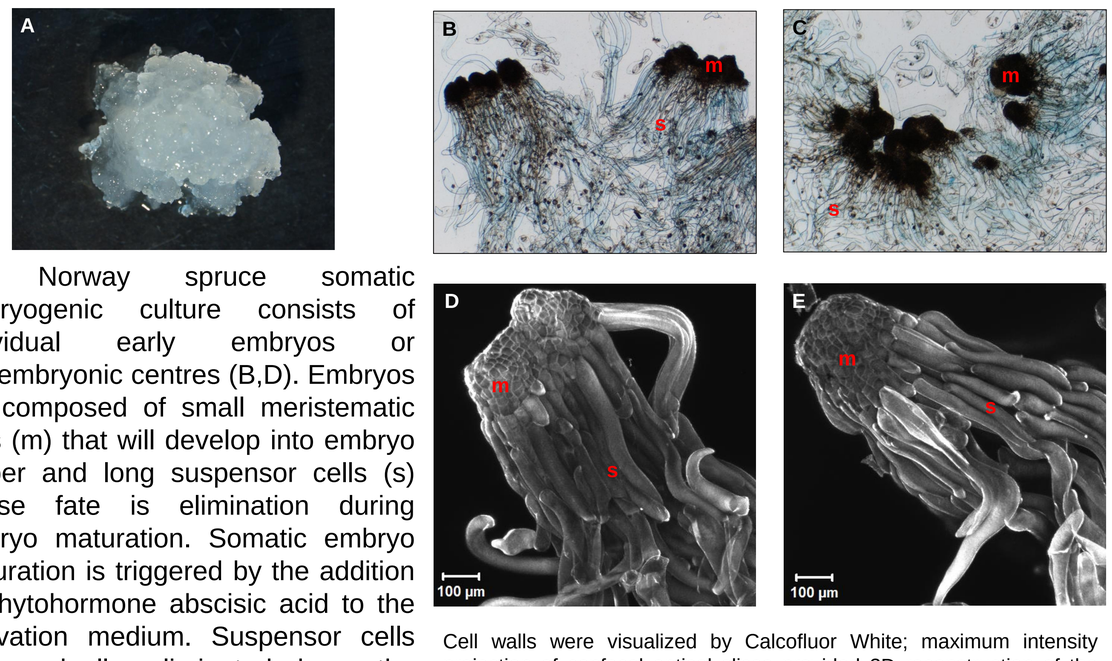
Cells under sucrose starvation



Starving cells after 1 day cultivation with E-64c

Application of the inhibitor E-64c
Under sucrose starvation condition, tobacco cells carry out degradation of cellular proteins which is performed by autophagy. Addition of cysteine protease inhibitor E-64c to the cultivation medium retarded the digestion process and led to the accumulation of vesicles (arrows) that could be endosomes, autolysosomes or autophagosomes around nuclei in both control and starving cells. It seems that the vesicle accumulation is higher in starving cells. We would like to assess the area that is occupied by vesicles around the nucleus.

Norway spruce somatic embryos



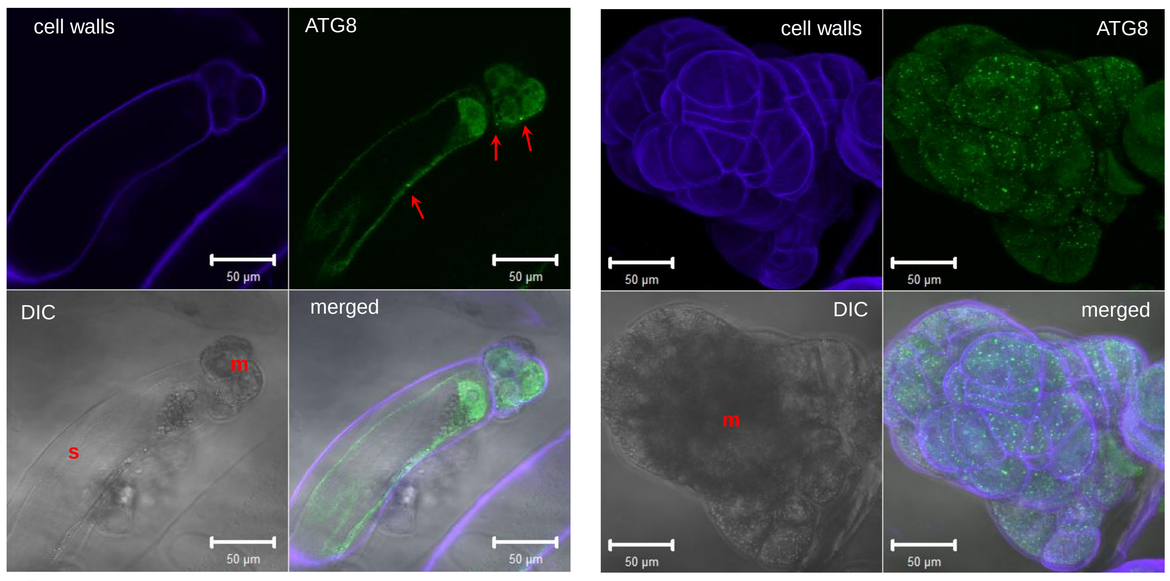
(A) Norway spruce somatic embryogenic culture consists of individual early embryos or polyembryonic centres (B,D). Embryos are composed of small meristematic cells (m) that will develop into embryo proper and long suspensor cells (s) whose fate is elimination during embryo maturation. Somatic embryo maturation is triggered by the addition of phytohormone abscisic acid to the cultivation medium. Suspensor cells are gradually eliminated by the programmed cell death, in which autophagy plays crucial role.

Cell walls were visualized by Calcofluor White; maximum intensity projection of confocal optical slices provided 3D reconstruction of the embryo morphology.

We followed up a core component of autophagy machinery - autophagy–related protein ATG8 using indirect immunofluorescence in early embryos (B,D) and in embryos after one week of maturation (C,E). Anti-ATG8 antibody labelled both free ATG8 and ATG8 localized in the autophagosome membranes as we observed diffused fluorescence signal in the cytoplasm and bright spots of different size.

Early embryos

In early embryos we found autophagosomes in both meristems and suspensors.

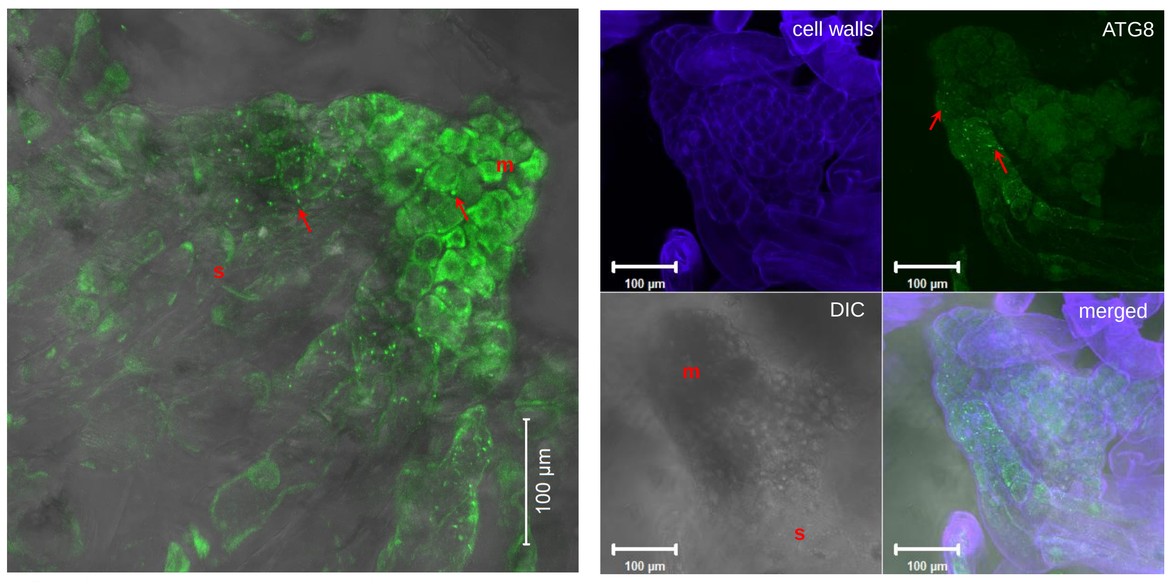


Small proembryo with several autophagosomes (arrows) in both suspensor cell (s) and meristem (m). Cell walls labelled with Calcofluor White; DIC – differential interference contrast; one optical slice.

Meristem of early embryo with plenty of autophagosomes (bright dots); cell walls labelled with Calcofluor White; DIC – differential interference contrast; Maximum intensity projection.

Start of maturation

In embryos cultivated for one week on the maturation medium we found less autophagosomes in meristems and more in suspensor cells localized below meristematic cells.



Developing embryo with individual autophagosomes (arrows) in the meristem (m) and big amount of them in suspensor cells (s) below meristem; Maximum intensity projection

Meristem and part of suspensor of the developing embryo with autophagosomes (arrows) in the region that could be eliminated during embryo development; cell walls labelled with Calcofluor White; DIC – differential interference contrast Maximum intensity projection.

We would like to assess the amount of autophagosomes in different parts of embryos on the either individual optical slices or Maximum intensity projections of confocal microscope. The problem of evaluation consists in the embryo size, bad penetration of chemicals inside the large embryo complexes that causes weak labelling of inner cells or complicated threshold because of diffuse fluorescence in the cytoplasm of meristematic cells, which is in some cases quite bright and cover the fluorescence signal of the small particles.

Acknowledgements:
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