

Approaches we apply to study Norway spruce somatic embryogenesis

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INTRODUCTION:

One of the main objectives of COPYTREE is to share our expertise to tackle the challenges of *in vitro* cloning of woody plants. Here we present the methodological approaches we have implemented in studying *in vitro* mass propagation systems, mainly in Norway spruce somatic embryogenesis. According to the goal of knowledge transfer, we offer to share them in the COPYTREE network.

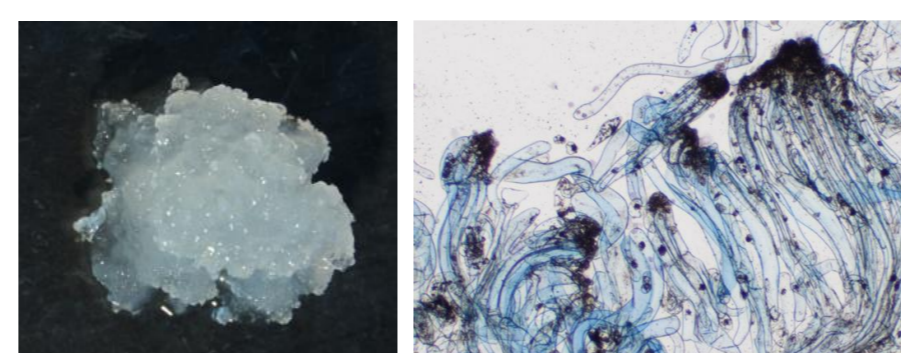
Our expertise cover methods of anatomy, histochemical detections, and indirect immunofluorescence, determination of the content of plant hormones, and analysis of gene expression. Newly we are also implementing methods of *in-vitro* virus detection. To control the developmental processes of *in-vitro* cultures, we use a broad spectrum of newly synthesized bioactive molecules as well as modulators of plant hormone metabolism and perception (e.g. anti-auxins, anti-gibberellins, cytokinin derivatives). Here we present the involvement of our methodological approaches in different projects realized in our laboratories.

Norway spruce somatic embryogenesis:

INDUCTION
from immature zygotic embryos; in the presence of auxins and cytokinins



PROLIFERATION
maintenance of cultures in the presence of auxins and cytokinins



MATURATION
5-6 weeks on medium supplemented with abscisic acid



DESICCATION
3 weeks of desiccation in high relative humidity



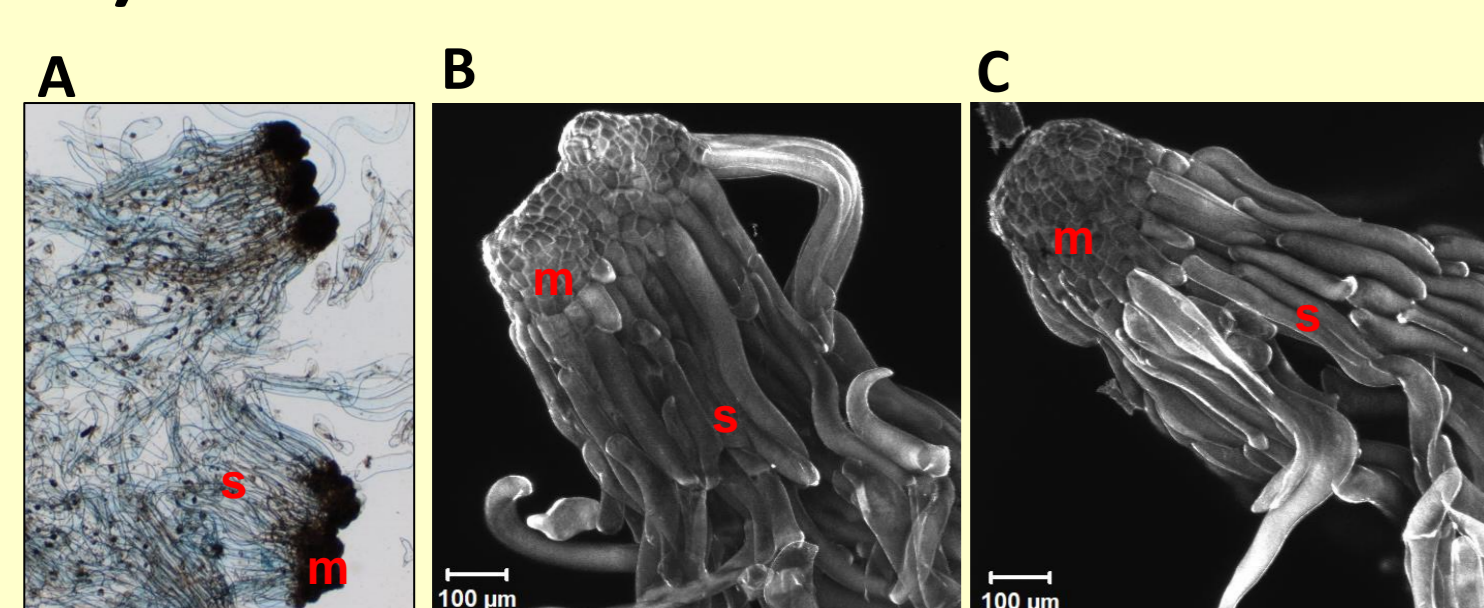
GERMINATION
without growth regulators



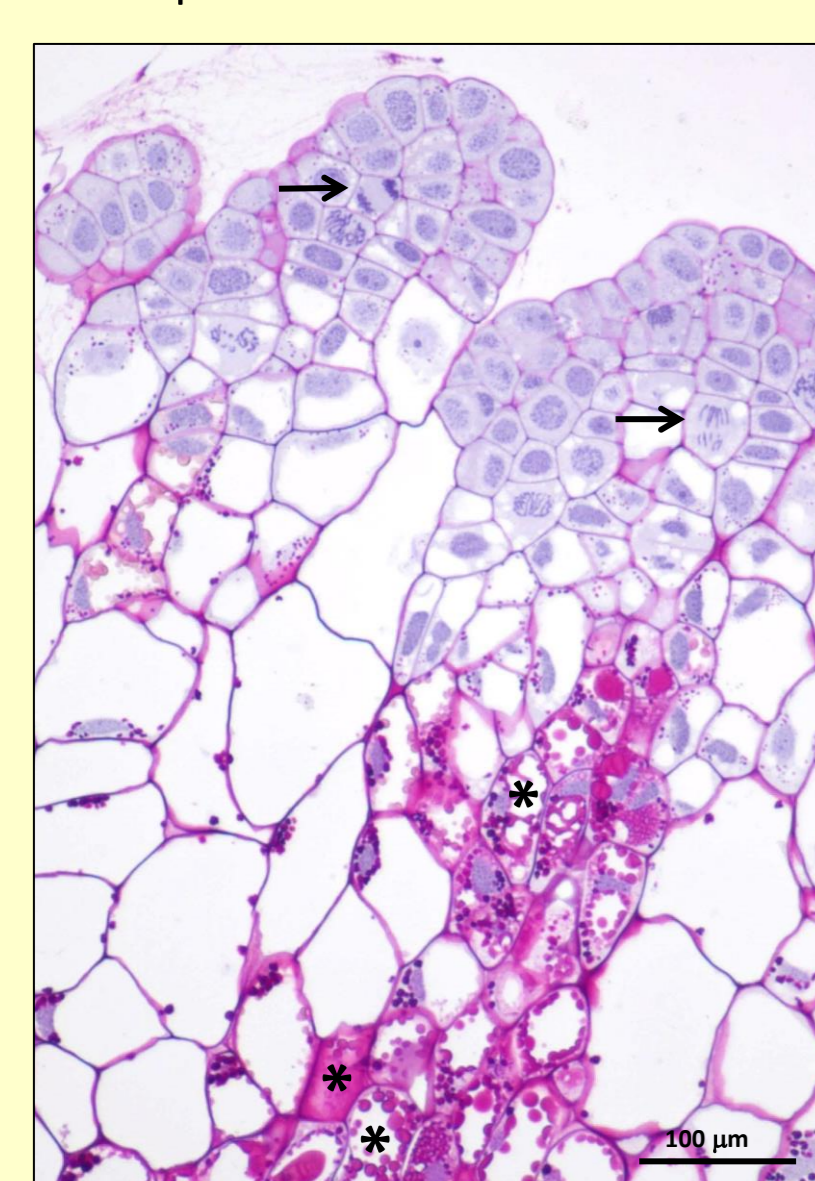
ANATOMY AND HISTOCHEMISTRY

We study the morphological and anatomical structure of plant material using stereomicroscopes and light, fluorescence, and confocal laser scanning microscopes. To assess cell viability, we use trypan blue or dual staining with fluorescein diacetate and propidium iodide to distinguish viable and dead cells. Proteins of interest are visualized using whole-mount indirect immunofluorescence labelling. To study cytology, localization of storage reserves, such as starch or proteins, or deposition of polyphenolic compounds, and to follow cell division we embed plant material into paraffin or resin (glycol methacrylate Technovit 7100) and prepare sections. Sections are stained with diverse dyes according to the structure we are interested in.

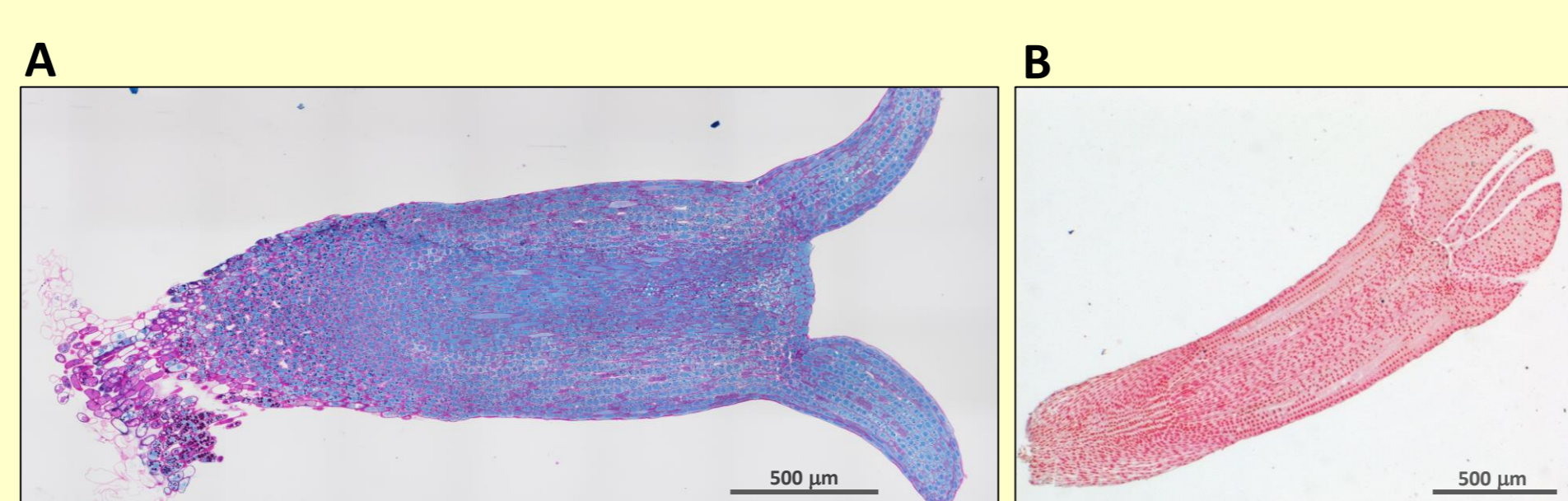
1) LIGHT MICROSCOPY



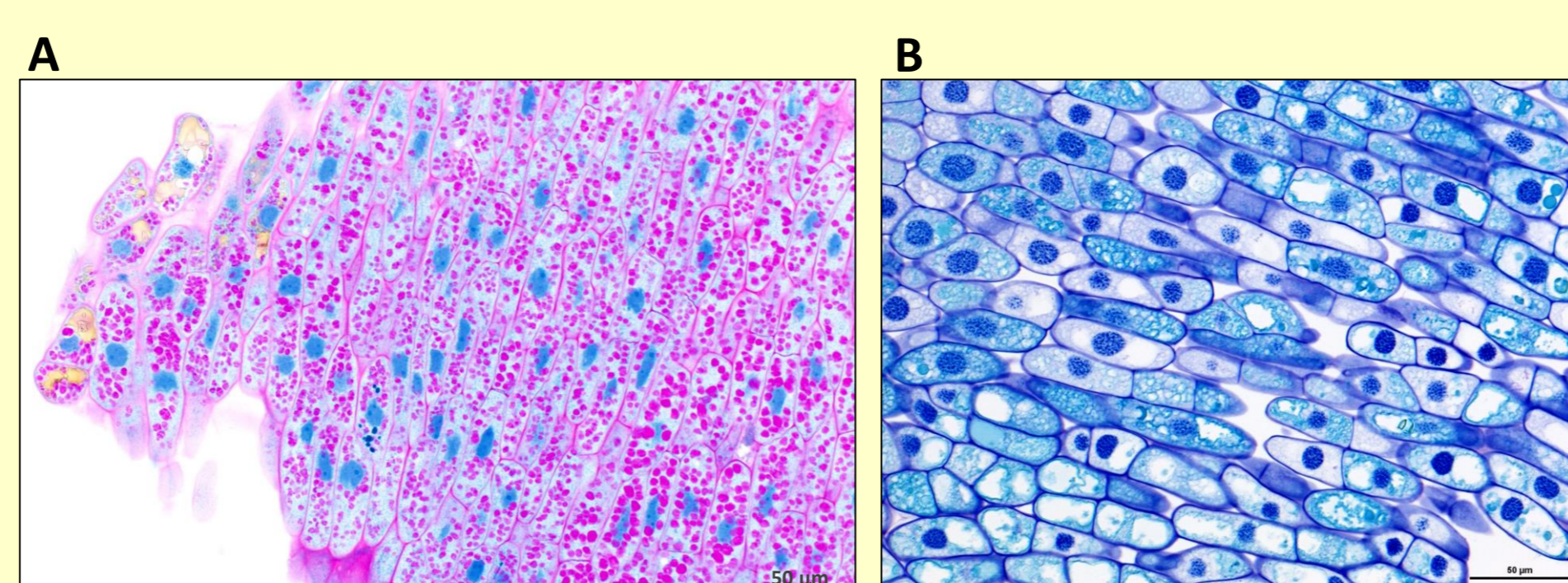
Early somatic embryos during proliferation (A,B) and embryo after one week of maturation (C). A - stained with trypan blue. B and C - cell walls were visualized with Calcofluor White; maximum intensity projection of confocal optical slices provided 3D reconstruction of the embryo morphology. m – meristematic cells; s – suspensor cells



Early somatic embryos in the proliferation phase; 4 μm resin sections were stained with periodic acid/Schiff reagent (PAS) and hematoxylin. PAS detected polysaccharides, such as starch; and hematoxylin stained nuclei (note cell divisions - arrows), cell walls, and slightly also cytoplasm (light grey-blue) visible mainly in the meristematic cells; PAS also stains in magenta polyphenolics (asterisk *)

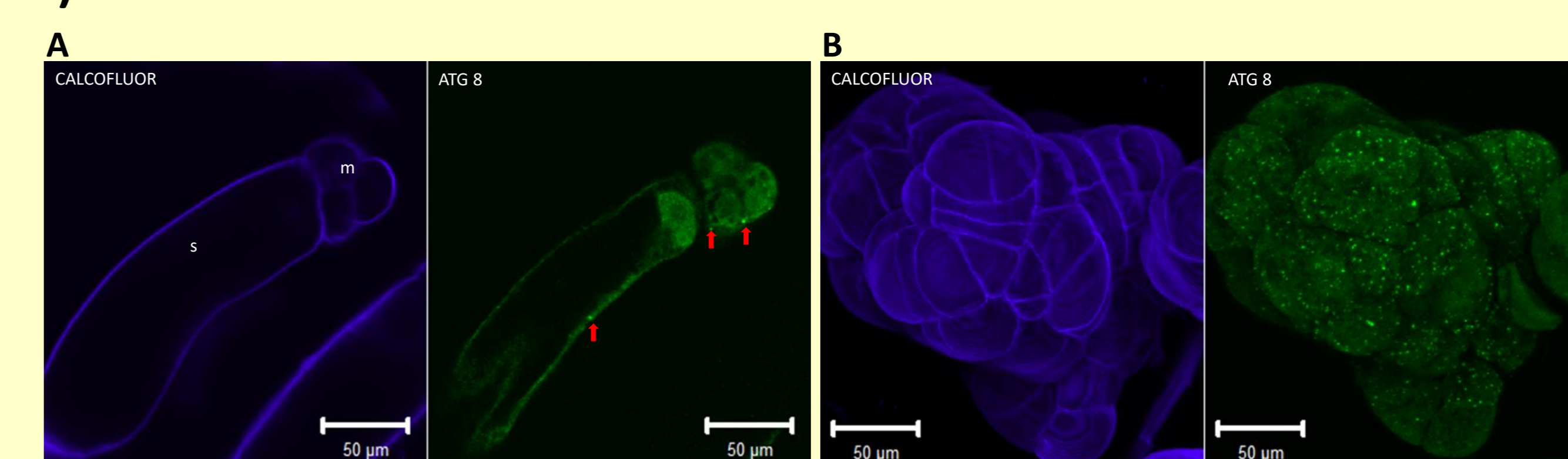


Cotyledonary somatic (A) and zygotic (B) embryo; resin-embedded sections were stained with PAS/Amido black (A) to detect polysaccharides and proteins, and with Nuclear Fast Red (B) to detect nuclei.

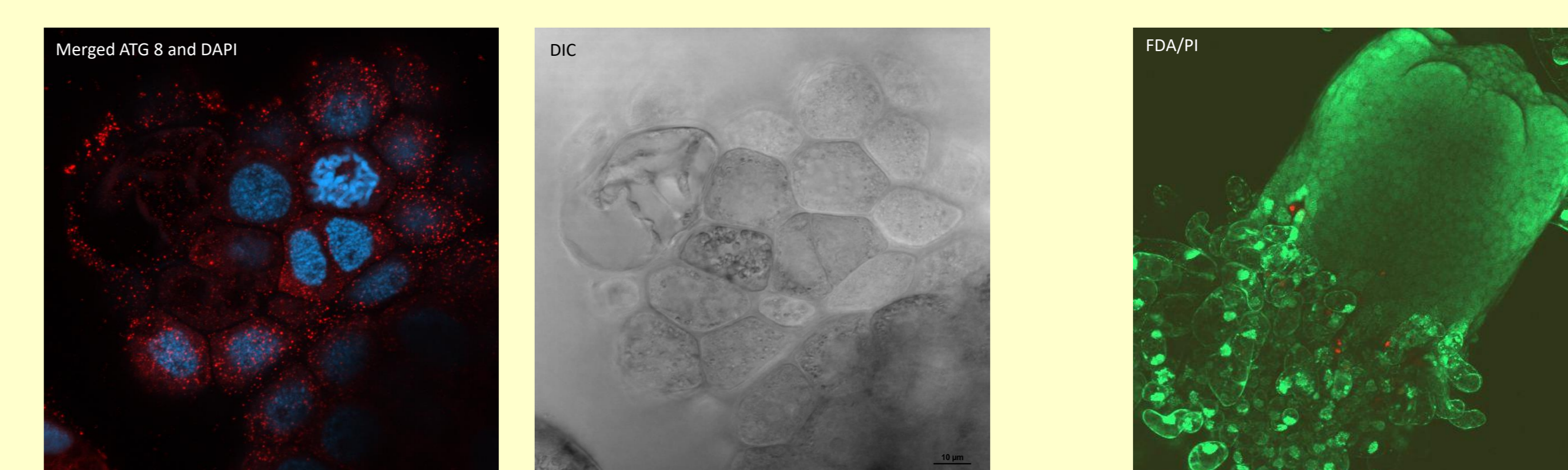


Details of the internal structure of different regions of cotyledonary embryos – root cap (A,B) and hypocotyl cortex (C); resin sections were stained: A with PAS/Amido black to detect polysaccharides (magenta - starch grains and cell walls) and proteins (blue) B with Toluidine blue to detect polyphenolic compounds (green blue) C with PAS/Amido black to detect polysaccharides and storage proteins

2) FLUORESCENCE MICROSCOPY



Detection of autophagosomes in proembryo (A) and early somatic embryo (B). Autophagosomes (green dots; arrows in A) are detected using whole-mount indirect immunofluorescence (primary antibody anti-ATG8, secondary antibody DyLight488). Cell walls are stained with Calcofluor White. s – suspensor, m - meristem

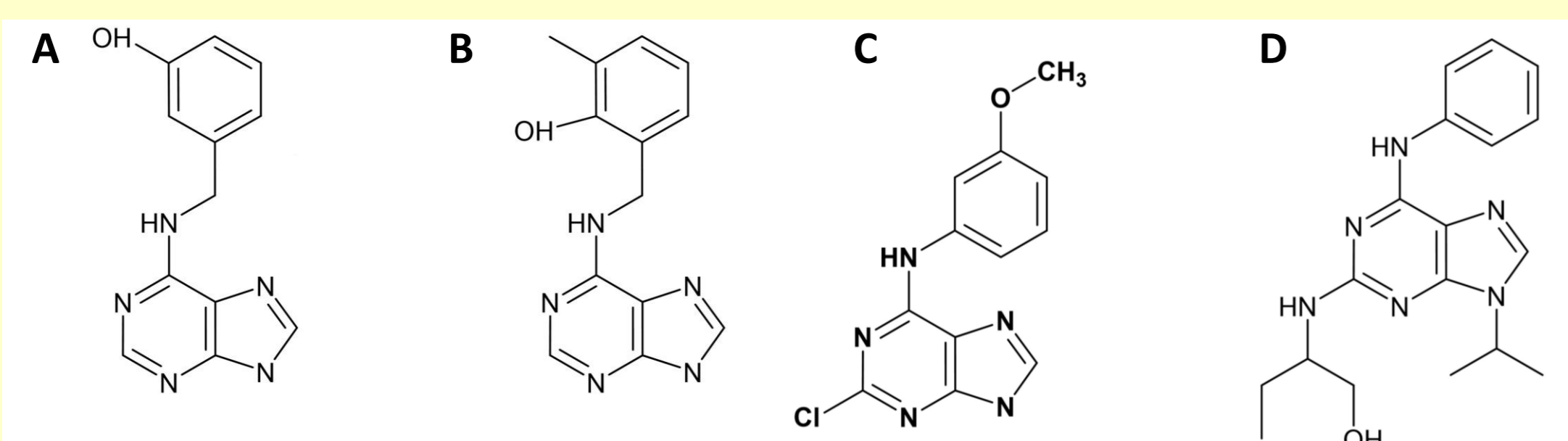


Detection of autophagosomes in early somatic embryo. Autophagosomes (red dots) are detected using whole-mount indirect immunofluorescence (primary antibody anti-ATG8, secondary antibody AlexaFluor564). In the right image, cells are visible in Nomarski contrast (DIC). Viability assessment in the early cotyledonary embryo of Norway spruce (fluorescein diacetate (FDA) and propidium iodide (PI) staining; green – viable cells, red – dead cells)

BIOACTIVE MOLECULES

In IEB we have derived a wide range of biologically active molecules with a broad spectrum of activities: anti-gibberellins, anti-auxins, and others; applied in various *in-vitro* systems (e.g. Smýkalová et al. 2019). Specifically, we show the chemical structures of selected cytokinin derivatives, that are or going to be applied in Norway spruce somatic embryogenesis during the currently running project.

Cytokinin derivatives



A 6-(3-hydroxybenzylamino)purine, *meta-topolin*, a novel type aromatic cytokinin

B 6-(2-hydroxy-3-methylbenzylamino)purine, *PI-55*, anti-cytokinin (Spíchal et al., 2009)

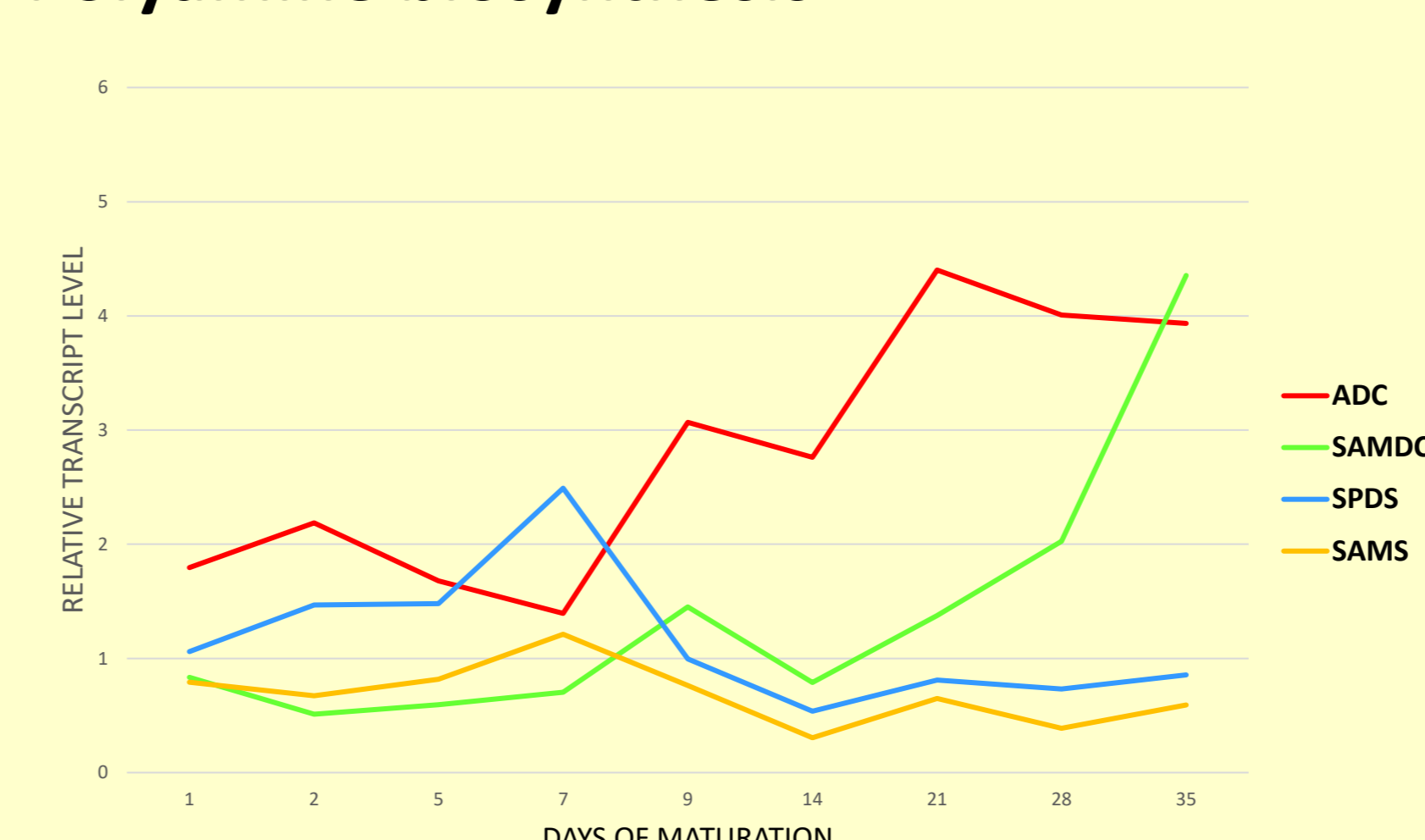
C 2-chloro-6-(3-methoxyphenyl)aminopurine, *INCYDE*, an inhibitor of cytokinin degradation, an inhibitor of cytokinin oxidase/dehydrogenase (Zatloukal et al., 2008)

D (2R)-2-[[6-(Benzylamino)-9-(propane-2-yl)-9H-purin-2-yl]amino]butan-1-ol, *roscovitine*, an inhibitor of cytokinin glucosylation (Blagojeva et al., 2004)

EXPRESSION ANALYSIS

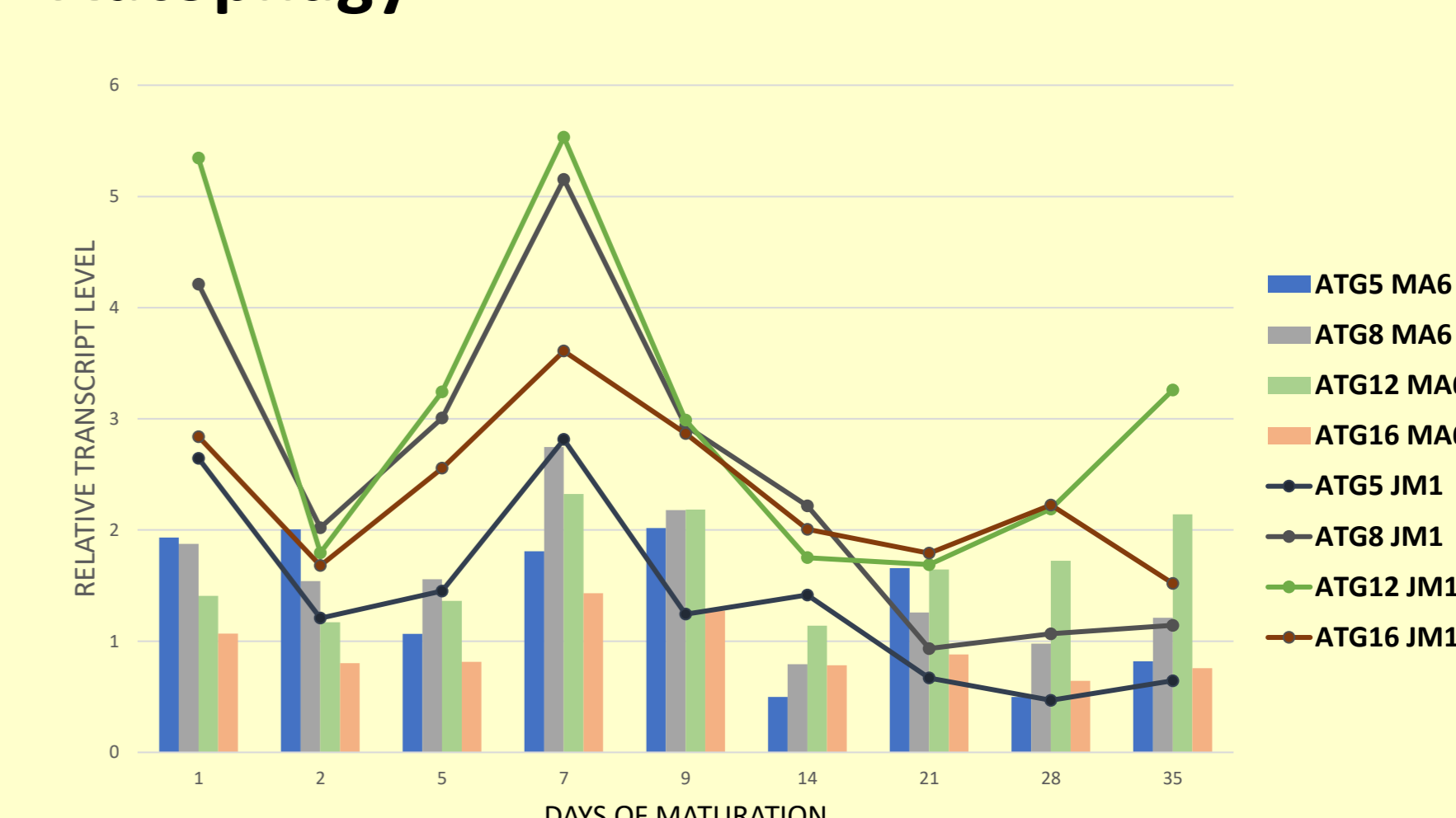
The expression of genes of interest is analyzed by quantitative real-time PCR.

Polyamine biosynthesis



The expression levels of polyamine biosynthetic enzyme genes during the maturation phase of somatic embryo development: ADC arginine decarboxylase; SAMDC S-adenosylmethionine decarboxylase; SPDS spermidine synthase; SAMS S-adenosylmethionine synthase.

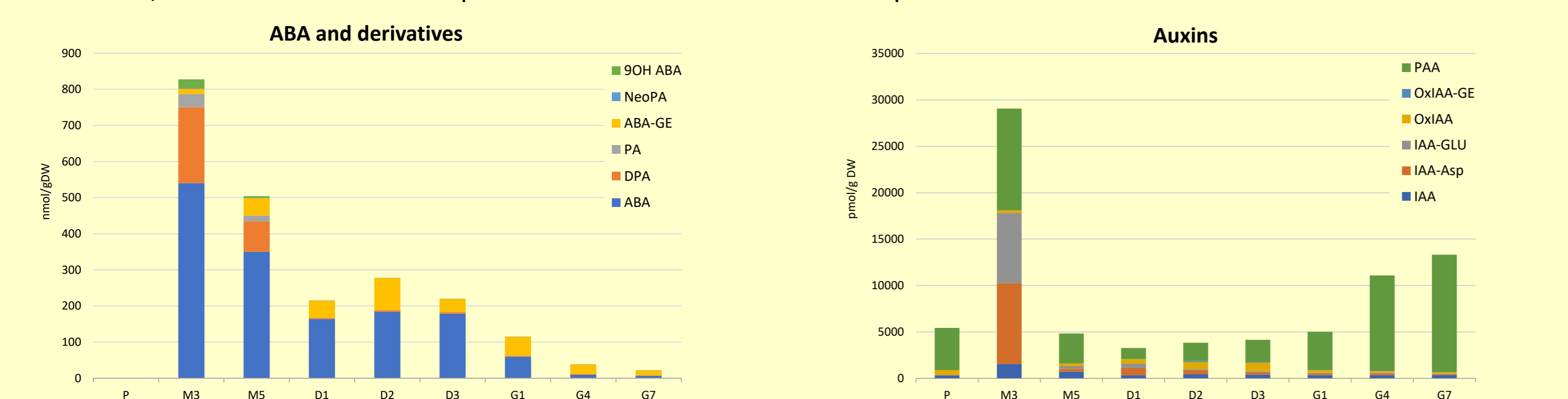
Autophagy



The expression levels of Autophagy related genes *ATG5*, *ATG8*, *ATG12*, and *ATG16* during the maturation phase of somatic embryo development in two embryogenic lines (embryogenic culture JM1 – lines, and embryogenic culture MA6 – bars).

BIOCHEMICAL ANALYSES

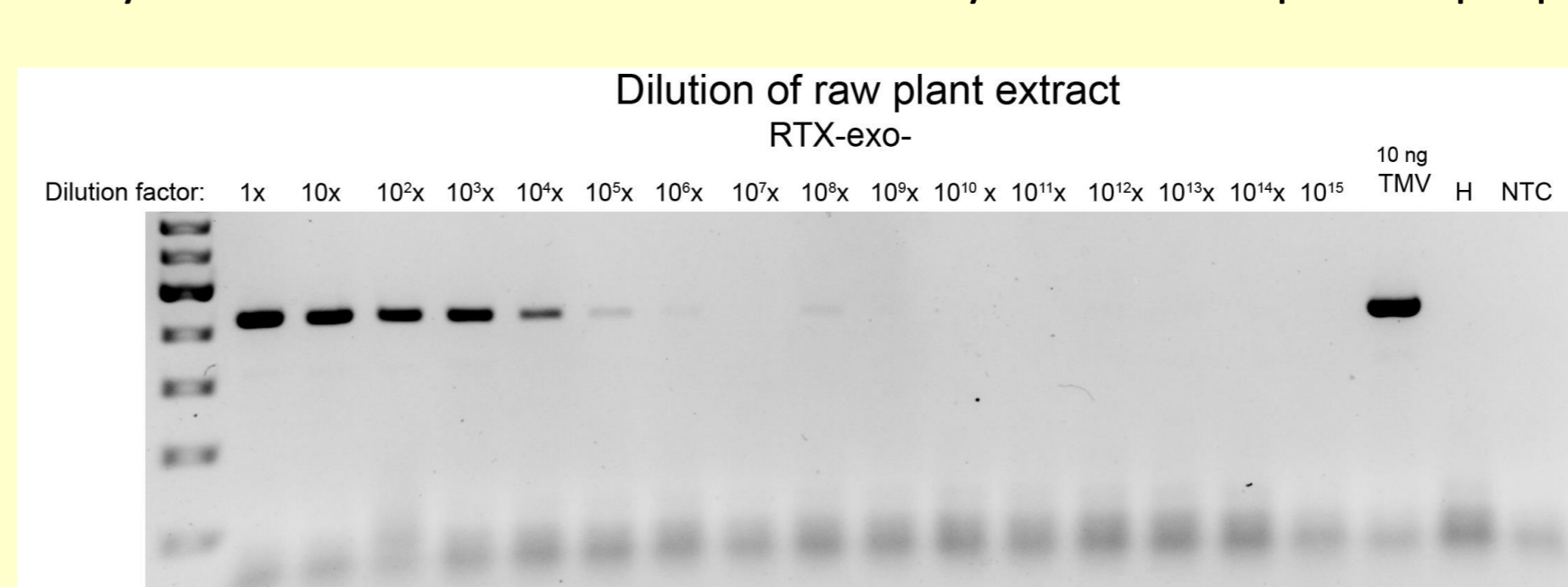
Analyses are performed using a UHPLC –MS/MS system, we are able to detect a broad spectrum of biologically active compounds, and our aim is specially targeted to **PHYTOHORMONES**. We are able to detect cytokinins (bioactive forms - free bases, transport forms - ribosides, storage forms - O-glucosides, deactivation forms - N-glucosides, and the immediate biosynthetic precursors - CK phosphates), as well as gibberellins, jasmonates, salicylates, and also auxins, and ABA with their derivatives, which content in our experimental material we show as representative below.



The content of ABA and their derivatives and auxins during the whole Norway spruce somatic embryo development process. P - proliferation, M - maturation, D - desiccation, G - germination (M3, M5, D1, D2, D3 - weeks of the process; G1, G4, G7 - days of the process). Vondráková et al. 2018

VIRUS DETECTION

Viruses and other pathogens can be sensitively detected using PCR and isothermal DNA/RNA amplification methods. For routine medium to high throughput detection, sample preparation and RNA isolation is time-consuming, increases the risk of cross-contamination, and can be expensive. We are developing innovative methods that allow sensitive direct detection of viral and other pathogens in unpurified plant extracts. For this purpose, we use commercial enzymatic mixtures as well as novel enzymes with improved properties.



RT-PCR virus detection in crude extracts of *Nicotiana benthamiana*. Assays were performed using RTX exo-polymerase. Based on the dilution series of purified TMV virus in raw plant extracts, the detection limit ranges from 0.1 to 0.01 pg of TMV virus per reaction.



Isothermal detection of TMV virus using RT-LAMP. Similar to RTX-PCR, crude plant extract can be used directly in the reaction RT-LAMP. No PCR cyclers and gel-electrophoresis device are required for the reaction. However, the primer design could be more complicated than the design of primers for PCR.

References:

Blagojeva et al. (2004). Cytokinin N-glucosylation inhibitors suppress deactivation of exogenous cytokinins in radish, but their effect on active endogenous cytokinins is counteracted by other regulatory mechanisms. *Physiologia Plantarum* 121(2), 215-222.
Smýkalová et al. (2019). The effects of novel synthetic cytokinin derivatives and endogenous cytokinins on the *in vitro* growth responses of hemp (*Cannabis sativa* L.) explants. *Plant Cell, Tissue and Organ Culture* 139(2), 381-394.
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Zatloukal et al. (2008). Novel potent inhibitors of A. thaliana cytokinin oxidase/dehydrogenase. *Bioorganic & Medicinal Chemistry* 16(20), 9268-9275.