Systems applied for plant regeneration are based either on organogenesis or on somatic embryogenesis (SE). The main goal of the project was to study and compare the involvement of polyamine metabolism and their cross-talk with different hormonal/cellular compounds during regeneration processes. For the examination of organogenesis a root culture based direct regeneration system was used based on the method of Rosspopoff et al. (2017) with a minor modification. As explants, 6 days old *Arabidopsis thaliana* seedlings were used. In this system auxin induced lateral root primordia (LRP) of arabidopsis roots could be converted to shoot meristem (SM) in the presence of cytokinin. Samples were collected after 24, 48, 72, and 96 h cytokinin induction. These time points mark the main stages of the cytokinin-induced conversion of LRP into SM. Conversion of LRP to SM starts with a mitotic pause (≈24 h cytokinin induction) during which cell divisions are transiently stoped in the competent roots. Following mitotic pause, LRPs go through a conversion phase (48 h cytokinin induction) and from converting organs early (72 h) and late (96 h) shoot promeristems develop. As control, six days old untreated seedlings were used. To study SE, as starting material, immature zygotic embryos were used and then samples were collected 10, 14 and 21 days after the induction of SE.

The level of the three main polyamines (putrescine (Put), spermidine (Spd) and spermine (Spm) changed differently during direct organogenesis. The level of Put increased transiently after 48 h cytokinin induction and decreased at later timepoints (at 72 and 96 h). Spermine level decreased during the conversion of LRP to SM. However, in contrast, Spd level showed a 3 fold increase in all three timepoints compared to the control plants. Exogenous application of Put, but mainly Spd enhanced regeneration efficiency which further verified the positive effect of these polyamines on direct shoot meristem formation. Interestingly, Put did not affect auxin transport and distribution. Cytokinin level in PTCS::GFP lines was also not affected by Put. Exogen spermin treatment did not affect the regeneration efficiency and did not cause any changes in auxin transport in PIN1::GFP lines.

To have a better insight into the regulation of endogenous polyamine levels during the direct shoot meristem formation, the expression of several genes coding for enzymes implicated in polyamine metabolism was investigated. Expression of *ARABIDOPSIS THALIANA ARGININE DECARBOXYLASE 1* (*AtADC1*) increased after 48h cytokinin induction. During the apparance of shoot promeristem (at 72-96 h), mRNA level of *AtADC1* was remained high but the expression level of *AtADC2* was also enhanced.

Since Put content was only increased after 48 h it can be hypothesized that Put produced by ADC1 was quickly metabolized by DAO or converted to Spd by Spd synthase. After 48 h cytokinin induction, but mainly during the formation of shoot promeristem, the mRNA level of *SPERMIDINE SYNTHASE (AtSPDS1)*, but mainly of *AtSPDS2* increased which results are in accordance with the 3-fold increase of Spd levels. In contrast, expression of *SPERMINE SYNTHASE (AtSPMS*) did not change significantly during the conversion of LRP to SM which is in agreement with the results in Spm content. mRNA level of *THERMOSPERMINE SYNTHASE (AtACL5*) enhanced after 72 h cytokinin induction and remained high until the formation of shoot promeristem. Furthermore, among the five paralogs *S-ADENOSYLMETHIONINE DECARBOXYLASE* (*AtSAMDC*) genes, mRNA level of *AtSAMDC2* and *AtSAMDC4* enhanced, while expression of *AtSAMDC1* and *AtSAMDC3* reduced during the direct SM formation compared to the control (Gémes et al., 2020., FESPB Congress, Gémes et al., 2021, XIII. Congress of Hungarian Society for Plant Biology¸ Kaszler et al., 2021). During SE, in contrast with organogenesis non of the three exogenously applied polyamines affected somatic embryo formation. Similar to organogenesis, among polyamine biosynthesis genes, expression of *AtADC1, AtSPDS2, AtSAMDC2* and *AtSAMDC4* increased. However, in contrast with direct SM formation, mRNA level of *AtADC2, AtSPDS1* and *AtACL5* decreased during SE.

Next, involvement of polyamine catabolism by polyamine oxidases was investigated. Application of the inhibitor of total PAO activity (Guazatine, Guaz) caused a delay in SM formation. After 48 h cytokinin induction expression of PIN1 reduced in Guaz treated seedling roots compared to the control. Auxin distribution was also influenced by Guaz during the conversion of LRP to SM in DR5::GFP lines. Furthermore, Guaz treatment influenced cytokinin level which was detected in PTCS::GFP lines. Among the 5 PAO genes only the mRNA level of *AtPAO5* increased after 48h cytokinin induction and remained high until the formation of late shoot promeristem. To verify the involvement of PAO5 in direct SM formation *AtPAO5* overexpressing transgenic plants (*35S::PAO5*) and *pao5-2* mutants were used, respectively. We have found that *35S::PAO5* transgenics had more while *pao5-2* mutants had less regenerants compared to the wild type (WT). Furthermore, expression level of the organogenesis marker genes, *ENHANCER OF SHOOT REGENERATION 1* and *2 (AtESR1,2)* were higher in transgenic (*35S::PAO5*) than in WT plants. These data indicate that PAO5 promotes the direct conversion of LRP to SM. Measuring the polyamine contents in

35S::PAO5 plants before and during the formation of SM, we have found that the initial level of Spd (but not Put and Spm) was significantly higher in transgenic plants compared to the control. After cytokinin induction, the level of all three polyamines decreased at a higher rate in transgenic compared to the WT plants. These data suggest that the overexpression of *AtPAO5* influences the polyamine homeostasis. In addition, level of reactive oxygen species (ROS) was found to be different in *35S::PAO5* and WT plants during the conversion of LRP to SM. Interestingly, ectopic expression of *AtPAO5* resulted in elevated ROS level during direct SM formation. As PAO5 has dehydrogenase rather then oxidase activity and PAO5 has high affinity for T-Spm, we hypothesized that PAO5 regulates direct SM formation controlling T-Spm homeostasis and not increasing H_2O_2 level. As pao5 mutants has 2-fold higher T-Spm compared to the WT, to verify our hypothesis, *pao5-2* mutants were used. In agreement, *pao5-2* and WT plants did not show any differences in ROS level. Furthermore, not exogenously applied H_2O_2 but T-Spm decreased the direct SM formation compared to the untreated controls (Kaszler et al., 2021; 2023; Benkő et al., 2022). Since haemoglobins were reported to affect cytokinin sensitivity and PAO5 was demonstrated to influence cytokinin sensitivity in a T-Spm dependent manner, next the expression of *HAEMOGLOBIN 1 (AtGLB1), AtGLB2* and *TYPE A ARABIDOPSIS RESPONSE REGULATORS (A-ARRs*, which are the feed back repressors of cytokinin pathway) was investigated in WT and *pao5-2* mutants during the conversion of LRP to SM. We have found that mRNA levele of both GLB genes enhanced but the increase of the relative expression of *AtGLB1* started earlier and was more pronounced than that of *AtGLB2*. In WT plants which were treated with T-Spm and in *pao5-2* mutants which has higher T-Spm content the relative expression of both GLB genes decreased at all timepoints compared to the controls. Moreover, relative mRNA level of *A-ARRs (AtARR5, AtARR7, AtARR16)* increased in *pao5-2* mutants after 24 h cytokinin induction and remained high until the formation of late promeristem (96 h cytokinin induction) compared to the WT roots. Relative expression of *AtARR4* and *AtARR15* also enhanced in *pao5-2* mutants but only after 72 h cytokinin induction (at the formation of shoot promeristems). Altogether, these results indicate that PAO5 might control cytokinin sensitivity via T-Spm dependent *AtGLB1/2* expression (Kaszler et al., 2023). As haemoglobins are potent NO scavengers, next the involvement of NO in the cytokinin induced direct SM formation in PAO5 dependent manner was investigated. The mRNA level of *NITRATE REDUCTASE 1 (AtNIA1)* increased during the

conversion of LRP to SM both in WT and in *pao5-2* mutant roots. However, the increase was lower in *pao5-2* mutants at the formation of late promeristem than in WT plants. Interestingly, expression level of *AtNIA2* did not change during the direct formation of SM nor of WT and *pao5-2* mutants. Moreover, the expression of *S-NITROSOGLUTATHIONE REDUCTASE (AtGSNOR)* did not change during the conversion of LRP to SM neither in WT nor in *pao5-2* mutants. In agreement, only a limited and transient difference was detected at 48-72 h cytokinin induction in *in situ* NO level of WT and *pao5-2* mutants. Based on these results, the potential role of NO on cytokinin signaling during LRP-SM conversion can be excluded. Interestingly, exogenously applied NO donor GSNO decreased both the mRNA level of *AtPAO5* and the regeneration efficiency which results indicate that uncontrolled NO accumulation might act negativelly on the regeneration process upstream of *AtPAO5* (Kaszler et al., 2020, 2023; Gémes et al., 2021, XIII. Congress of Hungarian Society for Plant Biology; Gémes and Kaszler, 2021, 8th Plant Nitric Oxide Int. Meeting).

Among ethylene synthesis genes, mRNA level of 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE 8 (*AtACS8), ACC OXYDASE 2 (AtACO2)* and *AtACO3* increased after 48h cytokinin induction and remained high until the formation of late shoot promeristems in WT plants. Interestingly, in *pao5-2* mutants relative expression of both *AtACS8* and *AtACO2* decreased compared to the WT plants. Exogenous application of ACC significantly decreased both the expression of *AtPAO5* and the efficiency of direct SM formation compared to the untreated controls. These results indicate the involvement of the ethylene precursor ACC in the regulation of direct SM formation in PAO5 dependent manner.

As both PAO and ethylene may affect NO metabolism, next the possible influence of ACC on NO metabolism genes was investigated. ACC enhanced the expression of *AtNIA1* and decreased the mRNA level of *AtGLB1* and *AtGLB2* during the conversion of LRP to SM compared to the untreated control. In accordance, ACC enhanced NO level after 48h CK induction and remained high until the formation of late shoot promeristems (Kaszler et al., 2020, FIBOK; Kaszler et al., 2022, VISCEA).

In contrast with organogenesis, expression level of AtPAO1 and AtPAO4 but not AtPAO5 increased during SE. However, *pao1* and *pao4* mutants, respectively, did not show any differences in the efficiency of somatyc embrio formation compared to the WT. Application of Guaz inhibited SE. These results indicate that AtPAO1 and AtPAO4 regulates collectively but not respectively the formation of somatic embryos in

arabidopsis. mRNA level of *AtNIA1* and *2* and both GLB genes increased during the formation of somatic embryos. NO level was also slightly enhanced after 10 days induction but decreased at later timepoints. Interestingly, both the induction and inhibition of NO by exogenously applied NO donor (SNAP) or scavenger (cPTIO), respectively, decreased the efficiency of SE.

Monitoring the effect of NADPH oxidases and its possible cross-talk with polyamine oxidases (PAOs) we have found that mRNA level of *AtRBOHs* did now show any changes during the formation of SM in WT plants. However, interestingly, regeneration efficiency was higher in *atrbohd* mutant plants than in WT. Furthermore, expression level of *AtESR1* and *AtESR2* also increased in *atrbohd* mutants at all timepoints compared to the WT. Moreover, in *pao5-2* mutants and in T-Spm treated WT plants, mRNA level of *AtRBOHD* was significantly higher during the cytokinin induced SM formation compared to the WT palnts. These results indicate a cross talk between PAO5 and RBOHD during the conversion of LRP to SM.

In contrast with organogenesis, during somatic embryo formation, expression of both *AtRBOHD* and *AtRBOHF* enhanced. However, interestingly, the efficieny of SE did not decreased in *atrbohd*, *atrbohf* and *atrbohdf* mutants compared to the WT. ROS level decreased in all mutants but expression level of embryogenesis marker (AtLEC1, AtFUS3) genes was not affected. Both the inhibition of NADPH oxidases by diphenilene iodinoim (DPI) and the inhibition of PAOs (Guaz) inhibited SE both in WT and in mutant plants. In addition, WT and mutant plants did not show any differences in NO production during the formation of SEs.

As a conclusion of the project we can say that polyamines, mainly Spd involved in the cytokinin induced direct conversion of LRP to SM. In agreement, mRNA level of the genes coding Spd biosynthesis (*AtADC1,2; AtSAMDC2,4; AtSPDS1,2)* and the back-conversion of T-Spm to Spd (*AtPAO5*) enhanced during the formation of SM. The AtPAO5 maintained T-Spm homeostasis is required of cytokinin induced direct SM formation. Expression level of GLB genes (*AtGLB1,2*) were negativelly controlled by high level of T-Spm. In details, in the absence of AtPAO5, level of T-Spm enhanced, mRNA level of GLB genes decreased, while expression level of the feed back repressor of cytokinin signalling (*A-ARRs*) which are coding indirectly by GLBs, were higher. Although, GLBs are the scavengers of NO, we cold not find evidence that GLBs affect cytokinin sensitivity through its NO scavenging activity, but we found that

uncontrolled and higher NO accumulation inhibited *AtPAO5* expression and direct SM formation.

Furthermore, AtPAO5 and AtRBOHD cross talked during direct SM formation. The absence of AtRBOHD, increased the mRNA level of *AtPAO5* and regeneration efficieny. Furthermore, high level of T-Spm enhanced the relative expression of *AtRBOHD* and decreased the conversion efficiency of LRP to SM.

In contrast with organogenesis, SE is not regulated by polyamines directly. AtPAO1 and AtPAO4 involved in the induction of SE but they regulated not respectively but collectively the formation of somatic embryos. In agreement with organogenesis, NO level slightly increased during SE which was related with the increased expression level of *AtNIA1* and *AtNIA2*. mRNA level of GLBs also enhanced during the process. Somatic embryo formation was inhibited by the application of NO donor, similarly to organogenesis, Among RBOHs, AtRBOHD and AtRBOHF regulated SE collectively, but in contrast with organogenesis, cross talk between RBOHs and PAOs could be not proved during the formation of somatic embryos.