Involvement of $O_2^{\cdot-}$ in the regulation of *Polycomb*, *Trithorax* and *LEC1*, *L1L*, *WUS*, *WOX5*, *STM* gene expression during somatic embryogenesis induction in *M. truncatula*

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Abstract

2,4-dichlorophenoxyacetic acid (2,4-D), an auxinic herbicide, in many in vitro somatic embryogenesis systems is used to induce embryogenesis. This compound present at a low concentration (0.5 μ M) during the induction phase, is necessary for *M. truncatula* embryogenic callus formation and, consequently, for embryo development. 2,4-D in higher concentration (5 μ M) causes an increase in $O_2^{\cdot-}$ accumulation which disturbs the callus and embryos formation. However, above processes are disturbed by DPI, an inhibitor of the NADPH oxidase responsible for the $O_2^{\cdot-}$ production. Thus, the aim of this study was to elucidate whether during first week of induction phase the change in the level of $O_2^{\cdot-}$ affects the expression of genes encoding Polycomb and Trithorax group proteins and the transcription level of some marker genes for SE. The expression of *Polycomb* and *Trithorax* group genes, and coding the transcription factors such as: *LEC1*, *L1L*, *WUS*, *WOX5* and *STM* depends on the $O_2^{\cdot-}$ accumulation in explant cells. Disorder of its level by manipulation of the concentration of 2,4-D or DPI disrupts expression of the above-mentioned genes and causes disturbances in the callus growth and development of somatic embryos.

Key message

This study provides new data regarding the inclusion of $O_2^{\cdot-}$ in the induction of SE; a suitable accumulation in tissues ensured by appropriate dose of 2,4-D is necessary for SE regulation at the epigenetic and transcriptional level.

Keywords Polycomb · Trithorax · Transcription factors · Legume · Superoxide radical

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
DPI	Diphenylene iodonium
ETC	Electron transport chain
IP	Induction phase
$O_2^{\cdot-}$	Superoxide radical
PcG	Polycomb group proteins
PGR	Plant growth regulator
PRC1	Polycomb Repressive Complex 1
PRC2	Polycomb Repressive Complex 2
ROS	Reactive oxygen species

SE	Somatic embryogenesis
SOD	Superoxide dismutase
TrxG	Trithorax group proteins

Introduction

Plants have the capacity of reproduction without egg fertilization from different kinds of somatic cells in the process known as somatic embryogenesis (SE). This multi-step process is initiated in vitro in differentiated cells directly or indirectly through a callus/pro-embryogenic cell mass and forms the basis of cellular totipotency in higher plants (Pasternak et al. 2002). It is accepted that induction of SE requires the action of various physical and chemical factors among which plant hormones and plant growth regulators (PGRs) are considered to be the most critical (Zavattieri et al. 2010; Rose et al. 2013; Rose 2019). The key regulators

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stimulate reprogramming of somatic cells and their dedifferentiation are auxins and cytokinins. The most commonly used PGR in in vitro culture is 2,4-dichlorophenoxyacetic acid (2,4-D), an herbicide with auxin-like properties (Grossmann 2010). 2,4-D induces process of SE and embryo multiplication, but it also inhibits embryos cell fate establishment; thus it is necessary to remove this compound from medium at the differentiation phase (Pasternak et al. 2002; Zavattieri et al. 2010). However, the induction of SE is more influenced by genotype than by any other factors; though, it is still not clear why this process is limited to certain genotypes, explants or cells (Neves et al. 1999; Rose et al. 1999). Almost three decades ago, it was commonly believed that in addition to genotype cellular stress caused by exposing plant explants to different stresses including supra-optimal growth regulators concentrations (e.g. 2,4-D) plays an important role in the cell fate switch, which lead to embryo development and suggest that SE is a developmental stress response (Dudits et al. 1991). This process starts with mechanical excision of the explant from the mother plant (wounding stress), subjecting it to chemical surface sterilization (oxidative stress) and putting it on artificial medium (dehydration/osmotic stress) (Nolan et al. 2006). The consequence of the above-mentioned stresses involves an increase in the production of reactive oxygen species (ROS) including many reactive oxidants such as a superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). A main source of ROS formed in plant cells as a result of stress factors is the NADPH oxidase; it produces $O_2^{\cdot-}$, that spontaneously or as a result of superoxide dismutase (SOD) transforms into H_2O_2 . It was suggested that ROS might function as a signal molecules to transduce external stimuli into cellular responses in *Medicago sativa* leaf protoplast-derived cells, and that ROS are involved in various hormone-dependent developmental processes (Pasternak et al. 2007; Fehér et al. 2008). In our most recent paper using two *Medicago truncatula* cv. Jemalong lines non-embryogenic (M9) and embryogenic (M9-10a) we found that the response (accumulation of $O_2^{\cdot-}$ and H_2O_2) to mechanical and chemical stress in leaf explants of both lines and the defense reaction such as antioxidant enzyme activity and expression of genes encoding them were similar; therefore, these stresses are not SE inducers (Orłowska and Kępczyńska 2020). However, a certain level of stress such as the presence of $O_2^{\cdot-}$, generated by 2,4-D presented at low concentration during the induction phase is necessary to induce SE in the *M. truncatula* M9-10a line. Recently it was suggested that 2,4-D, may induce a general tissue response manifested in the form of chromatin reorganization; thus, initiating a change in the expression of genes, which may trigger an embryogenic program in somatic cells (Garcia et al. 2019). This may result in the release of an embryogenic program which is normally inhibited by mechanisms silencing the expression of embryonic genes at the epigenetic

level (Fehér 2005). A change in chromatin reorganization is carried out by among other means, Polycomb (PcG) and Trithorax (TrxG) group proteins. In plants, the Polycomb group proteins act as two conserved complexes: Polycomb Repressive Complex1 (PRC1) and the better known PRC2. PRC2 catalyzes the trimethylation of histone H3 lysine 27 (H3K27me3) through the SET-domain protein. PRC1 binds to the H3K27me3 marks on the target gene and ubiquitinates the lysine 119 of histone H2A (H2AK119ub) (Margueron and Reinberg 2011; He et al. 2012; Gleason and Kramer 2013). Ubiquitination results in the packing of the chromatin structure, making the gene in this area inaccessible to transcription factors and stably repressing its expression. In contrast to the Polycomb group, Trithorax proteins act as gene expression activators by catalyzing the trimethylation of histone H3 lysine 4 (H3K4me3) on the target gene (Schuettengruber et al. 2011). Both groups act as lysine histone methyltransferases (HKMT) and are critical modulators of cell identity, fate determination, differentiation and plant development from seed to seed (Hennig and Derkacheva 2009; Bratzel et al. 2010; de la Paz Sanchez et al. 2015). It has been shown that the acquisition of competence for embryogenesis by somatic cells of *M. truncatula* leaves is probably associated with a lower level of the *Polycomb* and *Trithorax* groups gene expression in primary explants of the embryogenic line compared with the expression in the non-embryogenic line (Orłowska et al. 2017; Orłowska and Kępczyńska 2018). Mořgová et al. (2017) showed that lack of PRC2 activity is required for the formation of somatic embryos from *Arabidopsis* zygotic embryos but is not sufficient. Thus, additional exposition to the herbicide 2,4-D or other abiotic stress are needed. These additional induction factors may affect the chromatin reorganization, which in consequence may change the gene expression pattern, including those encoding transcription factors (TFs). The TFs in turn switch the vegetative development program into an embryonic program (Zavattieri et al. 2010). There are several groups of genes whose expression during the induction phase are crucial for the process of SE in *M. truncatula* i.e. genes: encoding receptor kinases—*SOMATIC EMBRYO RECEPTOR-LIKE KINASE1* (*SERK1*) (Nolan et al. 2009); involved in stimulating cells to divide and differentiate into embryos—*BABY BOOM* (*BBM*) (Igielski and Kępczyńska 2017), *SOMATIC EMBRYO RELATED FACTOR1* (*SERF1*) (Mantiri et al. 2008); involved in the organization of meristematic centers: *WUSCHEL* (*WUS*) (Chen et al. 2009; Orłowska and Kępczyńska 2018; review Jha et al. 2020); *SHOOT MERISTEMLESS* (*STM*), *WUSCHEL-RELATED HOMEODOMAIN5* (*WOX5*) (Orłowska and Kępczyńska 2018), *WOX9* and *WOX9-1* (Kurdyukov et al. 2014; Tvorogova et al. 2019). Other genes are important for encoding transcription factors of the LAFL pathway: *LEAFY COTYLEDON1* (*LEC1*), *LEAFY COTYLEDON1-LIKE* (*LIL*) (Nolan et al.

2014; Orłowska et al. 2017), *LEC2*, *FUSCA3* (*FUS3*) and *ABSCISIC ACID-INTENSIVE3* (*ABI3*) (Barreto et al. 2019).

To the best of our knowledge, there are not studies on the effect of ROS on the expression of genes encoding the proteins from PRC2, PRC1 and TrxG or coding the transcription factors characteristic for SE induction. Previously, it was shown that in the presence of 2,4-D during somatic embryogenesis in *Daucus carota* L. (LoSchiavo et al. 1989), *Cucurbita pepo* L. (Leljak-Levanić et al. 2004), *Acca sellowiana* Berg (Fraga et al. 2012) the level of DNA methylation increased.

Therefore, the aim of this study was to determine whether manipulation of $O_2^{\cdot-}$ levels by 2,4-D and diphenylene iodonium (DPI), an inhibitor of among others, NADPH oxidase, during the first week of the induction phase (IP) in the embryogenic line of *M. truncatula* affects: (a) the expression of genes encoding Polycomb (PRC1 and PRC2) and Trithorax group proteins; (b) and the transcription level of key genes such as *LEC1*, *LIL*, *WUS*, *WOX5* and *STM* for the induction of somatic embryogenesis.

Material and methods

Tissue culture protocol

Tissue culture was conducted using the embryogenic (M9-10a) line of *M. truncatula* Gaertn. cv. Jemalong. For donor plant production, we used seeds of this line, kindly provided by Professor Pedro Manuel Fevereiro, ITQB, Portugal (Neves et al. 1999). Primary leaf explants were placed on the SH medium (Schenk and Hildebrandt 1972) supplemented with 1 μ M zeatin and one of the following compounds: 0.5 μ M 2,4-D (standard medium); 5 μ M 2,4-D; 1 μ M DPI + 0.5 μ M 2,4-D and 10 μ M DPI + 0.5 μ M 2,4-D. After 21 days of induction callus tissue was transferred to MS (Murashige and Skoog 1962) differentiation medium free of growth regulators and DPI for 14 days. Accumulation of $O_2^{\cdot-}$ in explants cells incubated in presence of the above-mentioned compounds were analyzed according to protocol described previously (Orłowska and Kępczyńska 2020). In situ localization of $O_2^{\cdot-}$ was carried out after staining explants in a solution of 6 mM nitro blue tetrazolium (NBT) in 10 mM Tris-HCl (pH 7.4) for 10 min at 25 °C in the dark. Pale yellow NBT reacts with $O_2^{\cdot-}$ to form dark blue insoluble formazan (Fryer et al. 2002).

Gene expression analysis

Total RNA was extracted from the leaf explants at two time points (induction day 2 and 7) using the Direct-zol™ RNA-MiniPrep Kit (ZymoResearch). Molecular analysis was described in detail previously (Orłowska and Kępczyńska

2018). qPCR was performed with the 5 × HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne) using the STEP ONE Real-time PCR System (Life Technologies), following the manufacturer's instructions. All experiments were carried out in triplicate. Gene expression was normalized to *ACTIN2* and expressed as a fold change to the obtained value in 0.5 μ M 2,4-D taken as 1. The results are expressed as mean $\pm$ SD. Statistical analyses were performed using ANOVA followed by Tukey's HSD post-hoc test. Differences between the mean values were considered to be significant at $p < 0.01$ or $p < 0.05$.

Results

$O_2^{\cdot-}$ is present in explants during the first week of the SE induction phase and 2,4-D and DPI are modulators of its accumulation (Fig. 1), which finally significantly affects the callus growth of *M. truncatula* M9-10a line and somatic embryo formation (Fig. 2). It was therefore interesting to check whether these compounds affect the transcription of the genes encoding histone methyltransferases represented by the Polycomb and Trithorax group proteins in tissues of this line. For this purpose we compared *PRC1*, *PRC2* and *TrxG* genes expression in tissues incubated on standard medium (0.5 μ M 2,4-D) supplemented with a tenfold

Fig. 1 Effect of 2,4-D (0.5 and 5 μ M) and DPI (1 and 10 μ M) on superoxide radical accumulation in the tissue of embryogenic line (M9-10a) of *Medicago truncatula* during the induction phase on SH medium

Fig. 2 Effect of 2,4-D and DPI on the ability to form callus tissue and somatic embryos in the embryogenic line (M9-10a) of *Medicago truncatula*

higher 2,4-D concentration (5 μM) and DPI (1 and 10 μM) during the first week of the IP. These times were chosen, because in a previous study key changes in the expression of the above-mentioned genes were detected in this period (Orłowska et al. 2017; Orłowska and Kępczyńska 2018, 2020). *PRC2* complex genes expression was performed for the *CLF*, *MSII*, *FIE* and *VRN2* genes (Fig. 3a). Previously, we showed that their expressions differs in the M9 and M9-10a lines (Orłowska et al. 2017). A tenfold increase in 2,4-D concentration (5 μM) compared with standard medium (0.5 μM) resulted in an increase in the transcription level of all analyzed *PRC2* genes in tissues both after the second (except *VRN2*) and seventh day of the IP. The highest increase was recorded for the *CLF* and *FIE* genes, both of which were about twofold higher than the control. An even greater increase in expression was noted after the addition of 1 μM DPI to standard medium. On the second and seventh day of the IP, *CLF* showed 5- and 6.5-fold higher transcription levels, respectively, and *FIE* showed 2- and six-fold higher transcription levels, respectively. A significant, although slight increase in *MSII* and *VRN2* expression was noted. However, the addition of DPI at a concentration of 10 μM to the induction medium inhibited the transcription of all analyzed genes encoding the *PRC2* complex proteins. Increasing the 2,4-D concentration in SH medium from 0.5 μM (standard medium) to 5 μM had no significant effect on the expression of two (*LHP1* and *VRN1*) of the three analyzed genes encoding proteins of the *PRC1* complex (Fig. 3b). However, the transcription level of *RING1* was elevated on the second and seventh days of the IP (about 2.5-fold). DPI at a concentration of 1 μM in medium caused about a 3.5- and 5-fold increase the transcription of *LHP1* after the second and seventh days of the IP, respectively, and an even higher expression of the *RING1* gene (five and six-fold, respectively). This inhibitor did not significantly affect

VRN1 gene expression. In presence of DPI at a 10 μM concentration no expression of the three tested genes encoding the *PRC1* complex proteins was detected. In contrast to the *PRC2* and *PRC1*, an increase of the 2,4-D concentration in the induction medium from 0.5 to 5 μM had no effect on the expression of genes encoding proteins belonging to the Trithorax group, i.e. *ATX2*, *ATX3.1* and *ATX3.2* (Fig. 3c). In turn, DPI, at both a 1 and 10 μM concentration had no effect or inhibited transcription of the above genes, with the exception of *ATX2*. Expression of this gene in tissues incubated in the presence of 1 μM DPI increased on the second day of the IP (about a fivefold increase compared with standard medium).

Increasing of the 2,4-D concentration during the first week of IP and lowering the production of $\text{O}_2^{\cdot-}$ by DPI, had an effect on changes in the expression of *PRC2*, *PRC1* and *TrxG* genes. Thus, we determined that it was useful to investigate whether these changes at the epigenetic level, are accompanied by changes in the expression of genes encoding transcription factors, such as *LEC1*, *LIL*, *WUS*, *WOX5* and *STM*, which are directly involved in somatic embryogenesis (Orłowska and Kępczyńska 2018). Analysis of the *LEC1* transcription level showed that a tenfold higher 2,4-D concentration (5 μM) in induction medium increased the transcription level of *LEC1* more than 18-fold in the tissues of the M9-10a line on the seventh day of the IP (Fig. 4a). On the second day, DPI present during the IP in

Fig. 3 Effect of 2,4-D (0.5 and 5 μM) and DPI (1 and 10 μM + 0.5 μM 2,4-D) on the expression of the genes encoding *PRC2* (a), *PRC1* (b) and Trithorax (c) group proteins at 2 and 7 day of induction phase in the embryogenic line (M9-10a) of *Medicago truncatula*. Gene expression was normalized to *ACTIN2* and expressed as fold change to the obtained value in 0.5 μM 2,4-D taken as 1. Statistical analyses were performed using ANOVA with confidence interval 0.05 and Tukey's HSD post-hoc test. Bars show standard deviation

Fig. 3 (continued)

both concentration, 1 and 10 μ M, also increased the *LEC1* transcription level by about eleven and sixfold, respectively, compared with its expression observed in explants incubated on standard medium free from this inhibitor. After 1 week, the transcription level of *LEC1* increased tenfold in presence of 1 μ M DPI, but at a tenfold higher concentration of this NADPH oxidase inhibitor its expression was below the detection level. The expression of *LIL*, the *LEC1* homologue, was also influenced by 2,4-D; 5 μ M of this compound in the induction medium resulted in an increase in expression of this gene on the seventh day compared with its transcription level in the control tissues (0.5 μ M) by as much as 11-fold (Fig. 4b). Therefore, it is clearly seen that the expression of this gene is associated with the presence of auxinic 2,4-D and with increased $O_2^{\cdot-}$ accumulation after first week (Fig. 1). Reducing the production of this radical by DPI, caused drastic changes in the *LIL* expression; the transcription level of this gene increased 20- and 145-fold on the second and seventh days of the IP, respectively. After using DPI at a higher concentration (10 μ M), the decrease in *LIL* expression that occurred on the seventh day was below the detection level.

We also recorded the expression of genes usually involved in the organization of the meristematic centers in embryos e.g., *WUS*, *WOX5* and *STM* in leaf explants incubated in the presence of 5 μ M 2,4-D and DPI (1; 10 μ M) during the first week of IP (Fig. 4c–e). Transcription of the *WUS* gene was detected in leaf explants at the beginning of the IP and, after the second day; 5 μ M 2,4-D caused a tenfold decrease in expression of this gene compared with the control (Fig. 4c), but 1 μ M DPI increased its expression 1.5-fold. DPI at a concentration of 10 μ M on the second and seventh days of the IP reduced expression of this gene below detection level. On the seventh day, the transcription level of *WUS* was not observed. At a concentration of 5 μ M 2,4-D, in contrast to *WUS*, increased the *WOX5* and *STM* expression (Fig. 4d, e); the transcription level of both genes was approximately 4.5-fold higher compared with their expression in control tissues after the second day and almost two and threefold after the seventh day, respectively. DPI at both concentrations inhibited the expression of these genes.

Fig. 4 Effect of 2,4-D (0.5 and 5 μ M) and DPI (1 and 10 μ M+0.5 μ M 2,4-D) on the expression of the genes encoding *LEC1* (a), *LIL* (b), *WUS* (c), *WOX5* (d) and *STM* (e) group proteins at 2 and 7 day of induction phase in the embryogenic line (M9-10a) of *Medicago truncatula*. Gene expression was normalized to *ACTIN2*

and expressed as fold change to the obtained value in 0.5 μ M 2,4-D taken as 1. Statistical analyses were performed using ANOVA with confidence interval 0.05 and Tukey's HSD post-hoc test. Bars show standard deviation

Discussion

Callus formation from explants requires changes in cell identities and growth patterns and these changes of their developmental fates under the influence of environmental and hormonal cues are accompanied by changes in the expressions of numerous genes. The altered gene expression is at both the transcriptional and epigenetic levels (review: Fehér 2014; Rose 2019). Increased production of ROS by abiotic stress e.g. wounding, sterilization and auxinic herbicide 2,4-D is considered one of the most important factors initiating SE (Dudits et al. 1991; Fehér et al. 2003; Zavattieri et al. 2010; Fehér 2014; Elhiti and Stasolla 2015). However, in our last paper we showed that accumulation of $O_2^{\cdot-}$ and H_2O_2 in primary explants of non-embryogenic and embryogenic lines of *M. truncatula* under the influence of chemical (surface sterilization) and mechanical (wounding) stresses was similar (Orłowska and Kępczyńska 2020). Moreover, the response to these abiotic stresses during the first week of the induction phase i.e. the activity of antioxidant enzymes such as SOD, catalase and ascorbate peroxidase, and the expression of genes encoding these enzymatic proteins were similar in the tissues of both lines. Furthermore, 2,4-D present at a low concentration during the induction phase is necessary for embryogenic callus formation in the *M. truncatula* M9-10a line and consequently for embryo development; its absence during the IP resulted in the blocking of embryogenic callus formation. A certain level of $O_2^{\cdot-}$ generated by 2,4-D is necessary to induce this process but using it at a higher concentration causes an increase in the accumulation of this radical, which disturbs these processes that have mentioned above. Inhibiting or blocking the induction of SE by DPI, an inhibitor of NADPH oxidase responsible for the production of $O_2^{\cdot-}$, the first from ROS to appear in tissues under stress, clearly show that adequate its level is necessary for the induction of this process in *M. truncatula* (Orłowska and Kępczyńska 2020). Thus we continued the research that is presented in this paper to determine whether the observed disorders of the SE process that result from manipulating of the $O_2^{\cdot-}$ level using different 2,4-D and DPI concentrations are associated with changes at the epigenetic and transcriptional level.

Modulation of the $O_2^{\cdot-}$ level during first week of IP changes the expression of *PRC2*, *PRC1* and *TrxG* genes

In our previous research we showed the involvement of the *PRC2*, *PRC1* and *TrxG* genes during prime events of SE in *M. truncatula* (Orłowska et al. 2017; Orłowska and Kępczyńska 2018). Therefore, it was appropriate to check the expression of the above-mentioned genes during the

transition of leaf somatic to embryogenic cells that occur during early events of the IP in conditions of varied $O_2^{\cdot-}$ accumulation.

Increased accumulation of this radical during the first week of the IP under the effect of 2,4-D at a concentration elevated tenfold was accompanied by an increase in the expression of the four tested genes i.e. *CLF*, *MSI1*, *FIE* and *VRN2*, of the PRC2 complex, which may be the cause of disorders in the callus and embryo development in *M. truncatula*. The presence of 2,4-D in the induction medium at higher concentration than in standard medium disturbs the production of highly embryogenic callus and formation of fully developed embryos; only globular embryos are formed (Orłowska and Kępczyńska 2020; Fig. 2). Mozgowa et al. (2017) using *Arabidopsis clf swm* mutants established that reduced activity of the PRC2 complex during the 2,4-D inductive treatment (5 μ M) is necessary and sufficient for SE. At the same time we reported that the lower expression of the genes encoding the PRC2 proteins in *M. truncatula* leaf primary explants of the embryogenic line, compared with their expression in the non-embryogenic line, probably allows the initiation of dedifferentiation processes and embryo development (Orłowska et al. 2017). The observation in this study (Fig. 2) was that the development of somatic embryos was stopped after reaching the globular stage under the influence of higher 2,4-D concentration during the IP. This resulted in increased expression of *PRC2* genes, which may indicate that an adequate level of PRC2 activity may be important for the proper development of embryos. Reduction of $O_2^{\cdot-}$ production in the presence of 1 μ M DPI during the first week of the IP and a drastic reduction in callus production and consequent embryo formation is accompanied by increased expression of *PRC2* genes, mainly *CLF* and *FIE*. A tenfold increase in DPI concentration, completely blocking somatic cell differentiation and callus formation, stopped the expression of these genes. These results indicate that adequate $O_2^{\cdot-}$ accumulation in tissues during the first week of the IP is necessary to ensure the proper course of SE in *M. truncatula* and that this ROS regulates the gene expression of the PRC2 complex. Previously, Nic-Can et al. (2013) showed that the somatic cells of *Coffea canephora* can be reprogrammed epigenetically through dynamic changes in global histone H3 methylation (H3K4me3, H3K9me2, H3K27me3 marks) and global DNA methylation. They reported that during the beginning (between 0 and 7 day) of the 56-day course of the SE process in *C. canephora* low levels of H3K9me2 and H3K27me3 marks were accompanied by a gradual increase in total DNA methylation. However, later during transition from somatic cells into somatic embryos global histone methylation changed together with DNA methylation (either decreased or increased).

In this study we also found that a change in $O_2^{\cdot-}$ accumulation in leaf explants of the *M. truncatula* embryogenic line during the first week of the IP causes changes in the expression of genes encoding proteins of the PRC1 complex. Of the three genes from this complex that were analyzed in the M9-10a line, only *RING1* expression was up-regulated by an elevated accumulation of $O_2^{\cdot-}$ caused by a higher concentration 2,4-D. Reducing the $O_2^{\cdot-}$ accumulation in leaf tissues by DPI differently modifies *PRC1* genes expression; DPI at a lower concentration increased the transcription level of *LHP1* and *RING1*, but at a higher concentration it blocked the expression of all tested genes of the *PRC1* complex. Interestingly, neither 2,4-D nor DPI changed the transcription of the *VRN1* gene. In a previous study, we showed that this gene among the five genes in the *PRC1* complex did not change its expression during the 21 days of the IP in the non-embryogenic line; however, in the embryogenic line, as a massive callus tissue gradually developed, only its transcription level increased until the end of the IP (Orłowska and Kępczyńska 2018). As in the case of the *PRC2* complex genes as well as in those of the *PRC1* complex, the expression of the *PRC1* genes in primary explants in the M9-10a line was lower than in the M9 line, which probably promoted the embryogenic pathway in *M. truncatula*. Of the five genes tested in the 21 day duration of the IP, only *RING1* expression increased until the seventh day, characterized by a lower expression in the tissues of M9-10a compared with its expression in the tissues of the M9 line (Orłowska and Kępczyńska 2018). In *Arabidopsis* mutants a lack of the *RING1a-b* genes expression results in the initiation of an embryogenic callus and embryo-like structures on various regions of the plant (Chen et al. 2010).

Because certain genes of the *Trithorax* group identified in the *M. truncatula* genome probably play a role in the acquisition of cell embryogenicity by the M9-10a line (Orłowska and Kępczyńska 2018), we checked whether their expression is modulated by the changing $O_2^{\cdot-}$ accumulation during the primary events of SE. In contrast to the *Polycomb* group, the *Trithorax* group genes (*ATX2*, *ATX3.1*, *ATX3.2*) did not change their expression under the influence of increased 2,4-D concentration and thus under increased $O_2^{\cdot-}$ accumulation. However, the decrease in the production of this ROS by DPI caused blocking of genes expression or had no effect; only the *ATX2* gene in the presence of a low DPI concentration was characterized by increased expression. Previous reports have shown that certain genes of the *Trithorax* group (*ATX2*, *ATX3.1*, *ATX3.2*) play a role in acquisition of the ability for embryogenicity in cells of embryogenic line of *M. truncatula* (Orłowska and Kępczyńska 2018); therefore, in this study we checked whether the expression of these genes is modulated by the changing $O_2^{\cdot-}$ accumulation in tissues during the first week of the IP. These genes do not appear to be influenced by an increased 2,4-D concentration. Interestingly, the decrease in $O_2^{\cdot-}$ production by DPI at both the concentrations that were

used in this study caused blockage of both *ATX3.1* and *ATX3.2* transcription after the second day of the IP; however, after first week, the $O_2^{\cdot-}$ production had no effect or inhibited the expression of *ATX2* and *ATX3.1* depending on DPI concentration. Furthermore, even at a low concentration, the transcription of the *ATX2* gene was stimulated. All these results shows that increasing $O_2^{\cdot-}$ accumulation with an elevated concentration of auxinic 2,4-D has no effect on the regulation of the expression of genes encoding TrxG proteins, while lowering the level of this ROS by DPI generally blocks or does not affect the expression of these genes.

Changes in gene expression of transcription factors in response to different $O_2^{\cdot-}$ production in explant cells

In addition to above dynamic changes in *PRC2*, *PRC1*, *TrxG* genes expression in presence of 2,4-D and DPI we found also that manipulation of the level of $O_2^{\cdot-}$ accumulation change the expression pattern of genes that characteristically induce SE, such as *LEC1*, *LIL*, *WUS*, *WOX5* and *STM*. *LEC1* and *LIL* are marker genes of the late developmental stages and are associated also with the acquisition of embryogenic competency by somatic cells during early events in *M. truncatula* SE induction (Orłowska et al. 2017). Manipulation of the $O_2^{\cdot-}$ level using an increased dose of 2,4-D or using DPI at both concentrations significantly changed the expression of these genes. 2,4-D significantly increased the expression *LEC1* and *LIL*, but only after seven days. It has been previously shown that *LEC1*, like *LEC2*, is involved in auxin metabolism/signaling (Junker et al. 2012; Wójcikowska et al. 2013). In *Arabidopsis* (Ledwoń and Gaj 2011) and alfalfa (Domoki et al. 2006) the expression of *LEC1* is stimulated by auxin, which is consistent with the present study's observed increase of this gene and its close *LIL* homolog under the influence of a high dose of 2,4-D. Lowering the accumulation of $O_2^{\cdot-}$ further modifies the expression of these genes; on day 2, a low concentration of DPI very clearly increases the expression of both of these genes, which in the case of the *LIL* gene is more than sevenfold higher after first week. Blocking $O_2^{\cdot-}$ production by DPI (10 μ M) which caused inhibition of the embryogenic callus formation was accompanied by inhibition of *LEC1* and *LIL* expression; after first week their expression was not detected. Thus, it is clear that the expression of these genes is modified by the presence of auxinic 2,4-D, which is responsible for the accumulation of the appropriate level of $O_2^{\cdot-}$, and lowering the $O_2^{\cdot-}$ level causes disturbances in the expression of *LEC1* and *LIL*, which are recognized as markers of embryogenicity in *M. truncatula* (Orłowska et al. 2017).

Even greater changes as a result of the manipulation of $O_2^{\cdot-}$ accumulation levels were found in the transcript level of

genes marking root identity (*WOX5*) and shoot apical meristem identity (*WUS* and *STM*). The response to auxinic 2,4-D treatment at a higher concentration was an increased expression of *WOX5*, which confirmed previous data obtained by Chen et al. (2009) that *WOX5* expression was auxin-dependent. Interestingly, the increase in the 2,4-D concentration in medium during the first week of IP increases *STM* gene transcription, but lowers *WUS* expression; both of these genes participate in the organization of the shoot meristematic centers (Sarkar et al. 2007). In a previous study, Zuo et al. (2002) showed that during the first week of the IP in presence of 2.5 μ M of 2,4-D in *Arabidopsis* root explants *WUS* expression, but not *LEC1* expression occurred; however, later (between days 14 and 28) no expression of *WUS* but *LEC1* was evident. Therefore, they concluded that *WUS* represses *LEC1* during SE. Later, we showed that during the early events in the *M. truncatula* SE induction, *WUS* was the first gene to be expressed. On the second day it reached its maximum level of expression and between days 7 and 21, it was not detected. Furthermore, the expression of *LEC1*, *LIL*, *STM* and *WOX5* just started to increase from the second day and reached a very high level where it remained until the end of the IP (Orłowska et al. 2017; Orłowska and Kępczyńska 2018). In this study it was confirmed that on the second day expression of *WUS*, in contrast to *LEC1* and *LIL*, takes place. Moreover, of the five tested genes that encode transcription factors after the second day of the IP, only the expression of three of the genes are regulated by 2,4-D, i.e., *WUS* (downregulated), *WOX5* and *STM* (upregulated). Chen et al. (2009) showed that, unlike *WUS*, the expression of *WOX5* is stimulated by auxins, which is consistent with the results obtained in this study. Decreased *WUS* expression on the second day by a high 2,4-D concentration may be associated with an increased expression of genes of the *PRC2* (*CLF*, *MSI1*, *FIE*) and *PRC1* (*RING1*) complex.

In summary, the results presented here show the essential role of $O_2^{\cdot-}$ in *M. truncatula* leaf explants during primary events of SE induction in the regulation of genes expression from the *Polycomb* and *Trithorax* groups, and from those encoding transcription factors such as: *LEC1*, *LIL*, *WUS*, *WOX5* and *STM*. Increasing $O_2^{\cdot-}$ accumulation by 2,4-D concentration that is too high, or lowering it by DPI, changes the expression of the above-mentioned genes and probably disturbs the balance between the PcG and TrxG proteins suitable for SE induction. These changes in the expression of genes encoding selected histone methyltransferases appear to directly affect the level of transcription of the genes encoding the tested TFs or they can be regulated by other epigenetic factors such as DNA methylation or the action of microRNA. These results indicate that an adequate level of $O_2^{\cdot-}$ is necessary to maintain an appropriate balance at the epigenetic and genetic level and induce the proper course of SE process.

Finally, it should be mentioned that DPI is a general flavoproteins inhibitor and inhibit not only NADPH oxidase but also proteins of the mitochondrial electron transport chain (ETC; Altenhöfer et al. 2015). It is possible that both, NADPH oxidase and mitochondrial ETC are more important at different times of culture as ROS level are modulated. NADPH oxidase begin on plasma membrane is nevertheless likely particularly important. However, this requires additional research indicating its direct involvement in the SE process.

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Compliance with ethical standards

Conflict of interest The authors declare that they have not conflict of interest.

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