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Early events during the induction of somatic embryogenesis in genera *Medicago*

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Abstract

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In this review based on our data obtained in last 25 years we outline the early events of the induction of somatic embryogenesis in genera *Medicago* and in the model species - diploid *M. truncatula* and tetraploid *M. falcata.* Following the collected data considerable attention is paid on the factors affecting the process of the induction, activation of somatic cells for division by using an auxin 2,4-D (2,4-Dichlorophenoxyacetic acid) as a trigger, type of first cell division and further development. Based on recently established results the role of auxin in somatic embryo development is traced and confirmed by the localization of expression of the genes encoding an auxin influx carrier (*MtLAX3*) and the transcriptional factor an auxin response factor B3 (*MtARF-B3*) from the genome of *M. truncatula.* A brief consideration is paid to the role of genotype, explant pretreatment and genome size.

Keywords: somatic embryogenesis; *Medicago truncatula*; *Medicago falcata*; asymmetric division

Brief overview on genera Medicago

The genera *Medicago* is composed by annual and perennial species. They are diploid, tetraploid and polyploidy, wild and cultivated. The perennial species *M. sativa*, M. *falcata*, *M. varia, M. coerulea, M. arborea, M. glutinosa* are generally grouped as *M. sativa* complex. *M. sativa* for a long time has been the object of genetic cellular and molecular studies because of its good regeneration capacity *in vitro*. The reports on regeneration of *M. sativa* have been published, mostly by indirect somatic embryogenesis (Sanders and Bingham, 1975; Bingham et al., 1988; Arcioni et al., 1990; McKersie and Brown, 1996; Barbulova et al., 2002). Regeneration via direct somatic embryogenesis is also reported for *M. sativa* (Maheswaran and Williams, 1984) and *M. falcata* (Denchev et al., 1991). Annual diploid *Medicago* are closely related to alfalfa, they are self-pollinated and possess short life cycle. The first regeneration protocol for annual *M. truncatula* via indirect somatic embryogenesis is achieved by (Nolan et al., 1989) and a lot of protocols for this species have been reported (Chabaud et al., 1996; Hofman et al., 1997; Trinh et al., 1998). Protocols for regeneration of other annuals also have been created – *M. polymorpha* (Scarpa et al., 1993), *M. littoralis* (Zafar et al., 1995), *M. suffruticosa* (Li and Demarly, 1996), M. *lupulina* (Li and Demarly, 1995). Regeneration via direct somatic embryogenesis in liquid and solid media for *M. truncatula* (Iantcheva et al., 2001a; Iantcheva et al., 2005a) and for *M. littoralis, M. murex* and *M. polymorpha* also have been established (Iantcheva et al., 1999).

Type of somatic embryogenesis

Somatic embryogenesis (SE) is a process whereby a cell or group of cells from somatic tissue forms an embryo. Development of somatic embryos repeated the stages of zygotic embryo. There are two different types of somatic embryogenesis: direct somatic embryogenesis (DSE) and indirect somatic embryogenesis (ISE). DSE is characterized with the absence of formation of callus tissue and somatic embryos

appeared directly from the epidermal and sub-epidermal layers of starting explants tissue of *M. truncatula* (Iantcheva et al., 2001a) and *M. falcata* (Denchev et al., 1991), while in the ISE, process starts with formation of callus tissue and further embryo development. The indirect somatic embryogenesis system in genera *Medicago* (Barbulova et al., 2002; Iantcheva et al., 2005b; Svetoslavova et al., 2005; Iantcheva et al., 2009) are characterized by a sequence of events that includes the stimulation of cell proliferation, dedifferentiation, acquisition of embryogenic competence and the induction of embryogenesis. Treatment with an auxin (usually 2,4-D) is a main feature of the early stages of these procedures, but subsequent embryo development requires removal of exogenous auxin. The main feature of the indirect systems is that the initial activation of cell proliferation is temporally and physically separated from the induction of embryo specific cell division.

Direct somatic embryogenesis is characterized by the formation of embryos directly from differentiated tissue without the requirement for dedifferentiation stage involving disorganized cell proliferation. The model systems of somatic embryogenesis – tetraploid *M. falcata* (Denchev et al., 1991) and diploid *M. truncatula* (Iantcheva et al., 2001a; Iantcheva et al., 2005a) involved direct formation of embryos from young leaves, petioles and root explants in response to an induction treatment. There are different models to explain this phenomenon. At one of them it has been proposed that there are cells within the tissue, which are already embryogenically competent and only require the inductive signal to trigger direct embryo formation (Williams and Maheswaran, 1986; Carman, 1990). It has been argued that "direct" system for somatic embryogenesis does not differ significantly from "indirect" procedures at the molecular level and both proceed through similar stages of genetic re-programming at different rates (De Jong et al., 1993). In the direct model the inductive signal acts as a mitotic trigger and reactivates cell division in cells that are already competent to switch from somatic to embryogenic type and proceed into asymmetric cell division to form embryos. In the indirect model the induction of cell proliferation is required for dedifferentiation, which then permits the acquisition of embryogenic competence in certain cells.

Asymmetric division starts the process of embryo formation

To distinguish these models the investigation of induction of first cell division is studied in two single cell suspension culture systems for direct somatic embryogenesis in liquid media – *M. falcata* (Iantcheva et al., 2004a) and *M. truncatula* (Iantcheva et al., 2001b, 2006a, 2006b). Initial embryogenic cell division and embryogenic competence might be visualized and linked with the expression of reporter *GUS* gene under the control of promoters from cell cycle regulatory genes (*cyc A*, *cdc* 2a) and *gfp* reporter gene under 35S promoter. The expression pattern of the above reporter genes and behavior of single embryogenic cells in the condition of liquid culture confirms the asymmetry of first cell division that starts the process of direct somatic embryogenesis.

The induction of direct somatic embryogenesis in single cell suspension cultures of *M. falcata* and *M. truncatula* features the reactivation of the cell cycle in differentiated plant cells under the influence of external stimuli. The artificially induced series of cell divisions open the way to switch from somatic to embryogenic cell types. These systems for direct somatic embryogenesis from single cell in liquid media has been used for investigation of initiation of embryos and their further formation. One of the primary events in somatic embryogenesis is asymmetry of the first cell division (Dudits et al., 1995; Iantcheva et al., 2004a; Iantcheva et al., 2006a). In these cases treatment with an auxin 2,4-D, is a key element of embryo induction whereas no auxin is required for further embryo development and conversion to plants (Williams and Maheswaran, 1986; Carman, 1990). Acquired embryogenic competence and the initial embryogenic cell division in *M. falcata* system might be visualized and linked to the expression of certain cell cycle genes (such as cyclin dependent kinases and cyclins). In this investigation, the behaviour of single embryogenic cells in culture condition suitable for embryo induction, control and transgenic *M. falcata* plants are used. Localization of expression of reporter gene *gus A* under the control of two promoters from the cell cycle regulating genes Atpcdc2a and Atpcyc3a, as markers of cell division competence and activity is used to confirm asymmetry of the first cell division in embryogenic cells in *M. falcata*. Following the procedure starting with single cells (isolated on day 0) until day 15 the proportion of asymmetric divisions gradually increased. Up to this time symmetric divisions are only half as frequent.

The concentration of 2,4-D (4 mg/l) in induction medium acts as an inductive signal for the cells which possess embryogenic potential and re-activates these cells for division. Cellular processes such as embryo-specific DNA methylation (Vergara et al., 1990), disruption of tissue continuity by interruption of cell-cell connections (Smith and Kirkorian, 1989) and establishment of cell polarity can be induced by auxin (Parrott, 1993). Of the three types of suspension cultures (control and transcriptional reporters pcyc3a::*gus A* and pcdc2a::*gus A*) pcyc3a::*gus A* was most suitable for observation of the process of embryo formation from first asymmetric division until plantlet formation as its expression is more

strictly associated with cell proliferation, whereas the *gus A* reporter gene under the control of pcdc2a promoter appears active in a broad range of cells. Indeed, the *gus* gene under the control of the promoter of a cyclin type A gene is typically expressed early in the cell cycle from G1 through S phase until entry into mitosis (Shaul et al., 1996; Fowler et al., 1998; Iantcheva et al., 2015). On the other hand the expression of *gus* under the control of the cdc2a promoter is observed not only in dividing cells but also in cells competent for division (Hemerly et al., 1993). In the control culture the observation of type of divisions is difficult and it is not possible to visualise cells, which are active and competent for division.

In model system diploid *M. truncatula* confocal microscopy observation of 35S *gfp* single cell fraction confirmed that the fraction is composed from the three types of cells – spheroid, ovoid and elongated (Iantcheva et al., 2006b). Transfer of these cells into a fresh induction medium supplemented with 2,4-D reactivates cell for division. Green fluorescent protein (*gfp*) was detected strongly in the nucleus where is tends to slowly accumulate. In cells competent for division nucleus is situated at the cell periphery and first asymmetric division is probably consequence of nuclear migration from central region to the periphery that also is observed in *M. sativa* mesophyll protoplast (Dijak and Simmonds, 1988). Further development of such asymmetrically divided cell continued with the formation of three cells proembryo. These data are based on confocal software, which offers possibility to depict *gfp* fluorescent profile in cells and structures. In the observed profiles peaks indicated that the highest level of *gfp* expression is concentrated into the nucleus. Two peaks confirm the presence of two nuclei with separation of the cell of two unequal parts. Three peaks corresponding to the three nuclei of three cell proembryo.

Transfer of cell suspension culture to developmental medium without 2,4-D led to a further development of already formed embryo structures - globular and torpedo and decrease in the number of both asymmetric and symmetric divisions. If the fraction of single cells is collected and transferred back to induction medium containing auxin the number of divisions increased again as cells possessed high embryogenic potential. The process of embryo formation could be repeated and the embryogenic potential could be kept for a long period. The phenomenon of cyclic production of embryos in suspension culture composed from single cells and small cell clusters makes embryogenesis a suitable system for mass propagation, gene transfer and functional genomics studies (Iantcheva et al., 2014). However, repetitive embryogenesis necessitates identification of the appropriate developmental stage that will allow maintenance of embryogenic potential for a long period.

Model cell suspension cultures *M. falcata* and *M. truncatula* are particularly suitable for studies of very early events of process of somatic embryogenesis like induction of embryogenic potential, primary and consequent divisions, development of somatic embryos from single cell to plantlets.

Auxin 2,4-D as a trigger of the process and further role in embryo development

There are numerous studies concerning the hormonal induction of SE in a wide range of species. In genera *Medicago* the main external stimuli able to induce an embryogenic pathway of plant development are plant growth regulators (PGRs) especially auxins and cytokinins, which are used to reactivate cell cycle and trigger cell divisions. In most of the cases high level of exogenously applied auxin in combination of low level of cytokinins are considered as one of the crucial factors for induction of embryogenic potential in cells (Sanders and Bingham, 1975; Brown and Atanassov, 1985; Nolan et al., 1989; Chabaud et al., 1996; McKersie and Brown, 1996; Pintos et al., 2002). The embryogenic effect of 2,4-D is well known in legumes and in genera *Medicago* (Denchev et al., 1991; Zafar et al., 1995; Trinh et al., 1998). 2,4-D can reach the highest intracellular concentration and usually results in high frequency embryo formation. The concentration of 2,4-D in the process of dedifferentiation and differentiation *in vitro* also plays an important role (Denchev and Atanassov, 1988). In the study of Barbulova et al. (2002) 2,4-D concentration at 5 mg/l 2,4-D or 2 mg/l 2,4-D together with macro and micro salts composition of callus induction media for five alfalfa (*Medicago sativa*) commercial cultivars results to production of more dense, necrotic and less embryogenic callus compared to the white soft and highly embryogenic callus obtained in medium with 1 mg/l 2,4-D. For these cultivars the lowest concentration of 2,4-D is the optimal one. According Vergara et al. (1990), the high concentration of 2,4-D at some point block the cell division and inactivate the cells that already possess the embryogenic potential. High frequency of direct somatic embryo formation in liquid medium for perennial *M. falcata* (Denchev et al., 1991) and annual species *M. truncatula* and *M. polymorpha* (Iantcheva et al., 2001a) is observed in the presence of 4 mg/l 2,4-D. The concentrations to 11 mg/l 2,4-D are able to induce somatic embryogenesis, while 40 mg/l of the auxin block the induction.

In this review we are focused on the role of the genes encoding an Auxin influx carrier transmembrane transporter (*MT3G072870*, Plaza 2.5, *MtLAX3*) and a transcriptional factor an auxin response factor, containing a DNA-binding pseudobarrel and B3-binding domains (*Mt5g040880*, PLA-

ZA 3.0 Dicots, *MtARF-B3*), from genome of *M. truncatula*, in the process of indirect somatic embryogenesis (Revalska et al., 2015, 2017). These genes are initially identified by a reverse genetic approach in a population of *Tnt*1 retrotransposon-tagged mutants of *M. truncatula* (Revalska et al., 2011). Collaborative efforts of the Samuel Roberts Noble Foundation and partners in European groups of FP 6 integrated project GLIP have enabled the generation of *Tnt1*-insertion mutant collections for *M. truncatula* (d'Erfurth et al., 2003; Tadege et al., 2008; Iantcheva et al., 2009). To identify the disrupted genes in different *Tnt1* mutants, flanking sequence tag (FST) information has been generated for many of the lines and deposited for public use at the Samuel Roberts Noble Foundation (http://bioinfo4.noble.org/ mutant/). Two FSTs partially correspond to the genes encoding an Auxin influx carrier protein, *LAX3* and transcriptional factor *ARF-B3*. The genes and their promoters are cloned, and later on in the generation of stable overexpressed, knockdown and transcriptional reporters transgenic plants, their transcription profiles are evaluated and its expression pattern are assessed by the β-glucuronidase reporter gene (GUS).

Auxin is an important signalling molecule that elicits diverse plant processes from cell division, differentiation, cell elongation, root initiation, and apical dominance to tropic responses (Swarup et al., 2005; Overvoorde et al., 2010; Peret et al., 2013). Auxin also plays a major role in zygotic embryogenesis (Wolters et al., 2011). Significant amount of published data on auxin biosynthesis, metabolism, transport and development of somatic embryos shows that auxin plays important roles during the induction, embryo formation and in the subsequent embryo development (Feher et al., 2003; Yang and Zhang, 2010). The exogenously applied and endogenous auxin which is mainly synthesized in the young leaves and apical meristem of the shoot and roots (Ljung et al., 2005; Chen et al., 2014) is taken up by cells by a combination of carrier-mediated uptake or diffusion of the dissociated lipophilic acid and it is transported acropettaly from shoot to the root (Ljung et al., 2005; Friml et al., 2003; Teale et al., 2006). In long distance, most auxin is transported throughout the plant by young leaves and flowers by unregulated flow in the mature phloem (Swarup et al., 2001; Marchant et al., 2002). In short distance, auxin move from cell to cell by forming local maxima and create a gradient. This movement mechanism is controlled by the coordinated action of influx and efflux carriers and it is called polar auxin transport – PAT (Friml et al., 2002; Benkova et al., 2003; Vieten et al., 2007; Vanneste and Friml, 2009). It has been proved, that PAT inside the plant tissue is unique and it is not detected for other signaling molecules (Petrasek and Friml, 2009). In model plant *A. thaliana* three main classes of auxin transporters have been identified - like-aux 1 (AUX/LAX), pin formed proteins (PIN) and p-glycoproteins (PGP) ABC transporter family. LAX proteins are auxin influx carriers (Bennett et al., 1996; Palme and Gälweiler, 1999). The first putative efflux carrier to be characterized was *AtPIN1* (Gälweiler et al., 1998; Saini et al., 2013). *AtPIN* is a member of family auxin efflux transporters polar localized in plasma membrane with a central role in many plant processes (Firml et al., 2003; Paponov et al., 2005). Influx and efflux auxin transporters are with asymmetric cellular localization and their dynamic action are required for maintaining the PAT (Estell, 2001; Muday et al., 2003). It has been shown that in protophloem cells, *AUX*1 and *PIN1* show localization at opposite sides of the same cell. This suggests that *AUX1* and *PIN1* are targeted by divergent vesicle trafficking pathways and their establishment at opposite sides of the completed cell wall (Kleine-Vehn et al., 2006).

The auxin influx carrier protein AUXIN-RESISTANT1 (*AUX1*) belongs to the amino acid permease family of proton-driven transporter and plays a role in the uptake of the Trp-like auxin molecule indole-3-acetic acid (Bennett, 1998). In *A. thaliana* genome there are four genes AUXIN RESISTANT1 (*AUX1*) and LIKE AUX1 (*LAX1*), *LAX2*, and *LAX3* (Parry et al., 2001). *M. truncatula* contains a family of five genes related to *AUX1* of *A. thaliana* (*MtLAX*) (Schnabel and Frugoli, 2004). *MtLAX* genes are involved in local auxin transport, development of lateral roots and root nodules. It has been shown that during lateral root and nodule development *MtLAX* genes are expressed in the primordia and in the regions of the developing organs where the vasculature arises in later stages (De Billy et al., 2001).

We obtained transcriptional reporter plants by introducing the construct of *MtLAX3* promoter fused to GUS-GFP reporters, into wild type *M. truncatula* (*pMtLAX3::GUS-GFP*), to follow the expression pattern of *MtLAX3* during somatic embryogenesis (Revalska et al., 2015). The T_1 progeny of *M. truncatula* positive transgenic plants, are used for histochemical analyses. Localization of gene expression is traced in the process of indirect somatic embryogenesis in the model species. GUS activity was observed as a spots in the initial callus tissue of the explants, but became stronger in the formed globular embryos and especially in torpedo stage (Fig. 1a, b). GUS activity is subsequently detected in early and late cotyledonary stages (Fig. 1c). *It is known t*he combined action of auxin eflux carriers PIN1, PIN4 and PIN7 play essential role in auxin transport, cell division and auxin distribution during all stages of zygotic embryogenesis (Bassuner et al., 2007; Firml et al., 2002; Jenic and Barton, 2005). Because it is well known that auxin cell to cell transport is mediated by auxin influx (LAX proteins) and ef-

flux (PIN proteins) carriers (Saini et al., 2013) and the plant hormone auxin plays important role in every aspect of plant growth and development, including embryogenesis (Davies, 2010; Quint et al., 2005), we confirmed the involvement of *MtLAX3* in somatic embryo development.

Transcription factors (TFs) that bind specifically to 5'-TGTCTC-3' auxin response elements in the promoter, upstream of auxin-activated genes, are called auxin response factors (ARFs) (Ulmasov et al., 1997; Hagen and Guilfoyle, 2002; Guilfoyle and Hagen, 2007; Tiwari et al., 2003). ARFs control the expression of several plant genes and contain a highly conserved plant-specific B3-type domain (Guilfoyle and Hagen, 2001; Yamasaki et al., 2004, Revalska et al., 2017). Analysis of GUS activity in transcriptional reporter plants (*pMtARF-B3::GUS-GFP*) of *M. truncatula*, *Lotus japonicas* and *Arabidopsis thaliana* confirmed localization of *MtARF-B3* expression during the process of indirect somatic embryogenesis of these three model species (Revalska et al., 2016a, 2016b, 2017). In *M. truncatula* a slight GUS signal was observed in the initial callus tissue on the explants which is more pronounced in formed globular embryos (Fig. 1d) and later on in torpedoes, and subsequent early and late cotyledonary stages (Fig. 1e). Localized GUS expression in plantlets is less pronounced trough whole habitus but clear confirming well shaped bipolar structure (Fig. 1f).

During our investigation on early events in somatic embryogenesis we confirm that exogenously applied auxin (in our case 2,4-D) activates cell cycle and trigger cell division. The auxin enter in the plant cell by transmembrane auxin transporter (LAX3). 2,4-D is a synthetic auxin analog, mainly used as a herbicide, which is absorbed through the leaves and is translocated into the plant's meristem (Cobb and Reade, 2010; Simon and Petrasek, 2011). 2,4-D is a substrate for LAX transporters and about 75% of it requires an influx carrier to enter the cell, especially if its concentration outside the cell is low (Dellbare et al., 1996). Auxin elicits gene expression responses by binding to the F-box ubiquitin protein TIR1, hence increasing the affinity of TIR1 for the Aux/IAA family of transcriptional inhibitors. The ubiquitination of the Aux/IAA transcriptional inhibitors and their further degradation by the 26S proteasome lead to activation of the auxin response factors (Woodward and Bartel, 2005; Teale et al., 2006) which specifically bind to the auxin responsive element in the promoter of auxin inducible genes and activate their expression. Auxin signalling alter the expression of genes families AUX/IAA and ARFs (Teale et al., 2006) and influx and efflux transmembrane carriers LAX and PIN proteins which are asymmetrically localized in such a way maintain the polar auxin transport.

We are able to detect the reactivation of cell cycle by marker genes *cyc3A* and *cds2a* after 2,4-D treatment during the induction of embryogenic competence in the single cells of *M. falcata,* which leads to high proportions of asymmetric divisions and starts the process of embryo formation. The influence of 2,4-D treatment only for 1 hour activates cells for division in root tips of transgenic *M. falcata* plants expressing *gus* gene under cell cycle promoters - cyc A and cyc B (Iantcheva et al., 2004b). Therefore genes involved in the cell cycle are also induced by auxin treatment in early events of induction of somatic embryogenesis.

Role of other PGRs for induction of somatic embryogenesis

For some annual *Medicago* species α-Naphthaleneacetic acid (NAA) is essential for indirect somatic embryogenesis initiation *M. polymopha* (Scarpa et al., 1993), *M. rigidula* and *M. orbicularis* (Ibragimova and Smolenskaya, 1997*), M. truncatula* (Nolan et al., 1989). The molecular mechanisms involved in the induction of this process are still not fully understood. Somatic Embryogenesis Receptor Kinase (SERK) gene from *M. truncatula* (MtSERK1) is cloned and its expression examined in culture (Nolan et al., 2003). The auxin (NAA) stimulates MtSERK1 expression but its expression is significantly higher when medium is supplemented with cytokinin 6-Benzylaminopurine (BAP). The effect of cytokinin in indirect somatic embryogenesis systems is more pronounced. Induction of callus tissue and further embryo formation is observed in *M. truncatula* and *M. sativa* when culture medium is supplemented with BAP (Trinh et al., 1998). Formation of callus tissue and induction of embryogenic potential among different species of genera *Medicago* require different cytokinins kinetin, BAP, zeatin (Denchev et al., 1991; Nolan et al., 1989; Ding et al, 2003; Chabaud et al., 2004, Kim et al., 2004).

Induction of somatic embryogenesis by cytokinin alone is relatively very rare among legumes and especially in genera *Medicago*. In legumes somatic embryogenesis induce by cytokinin is established for *Trifolium repence* (Maheswaran and Williams, 1985), *Phaseolus* (Malik and Saxena, 1992). In annual *Medicago – M. trunacatula, M. littoralis*, *M. murex* and *M. polymorpha* direct induction of somatic embryos is achieved on solid media in the presence only of N-Phenyl-N1-1,2,3,-thiadiazol-5-ylurea, thidiazuron (TDZ) (Iantcheva et al., 1999). In this system the whole process of embryogenesis from induction to maturation is completed on medium containing cytokinin as well this system is species independent. TDZ is a plant growth regulator which possess cytokinin like activity and able to induce direct somatic embryogenesis in other legumes (Saxena et al., 1992, Murthy et al., 1995). This growth regulator re-activates cell cycle and is

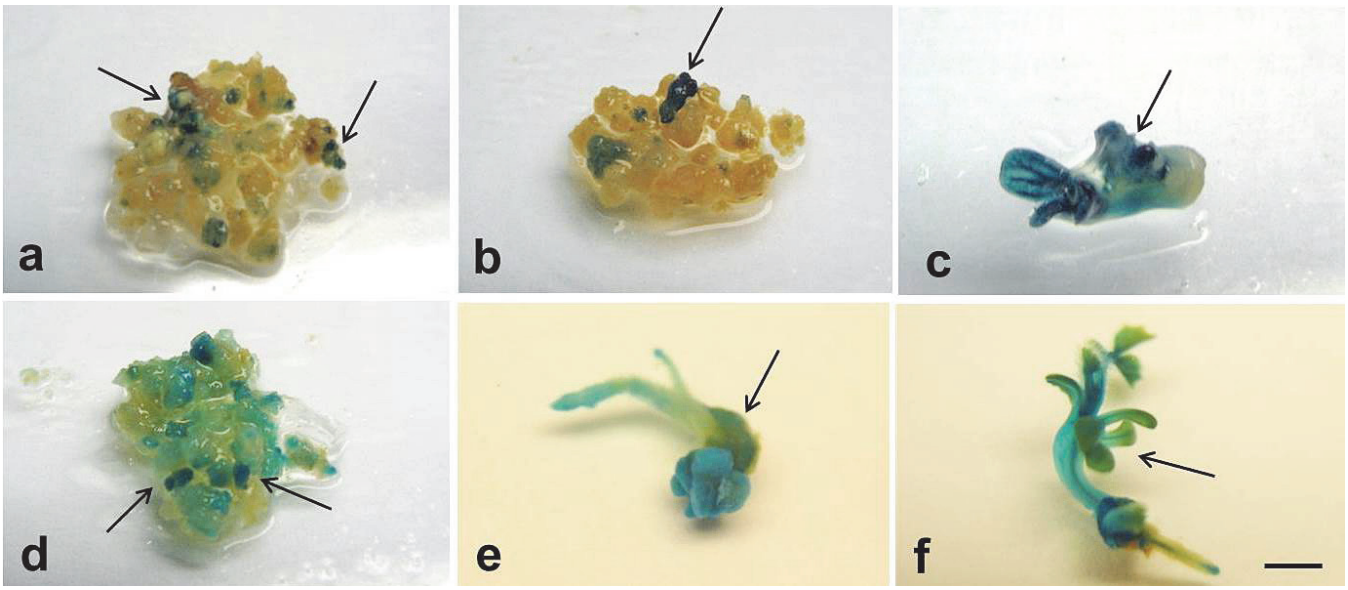

Fig. 1. Expression pattern of p*MtLAX3*::GUS (a, b, c) and p*MtARF-B3::*GUS (d, e, f) during different stages of indirect somatic embryogenesis in *M. truncatula*: *a*) GUS activity in callus and formed globular embryos; *b*) strong signal in embryos in torpedo stage; *c*) GUS activity detected in late cotyledonary stage; *d*) GUS activity in callus and formed globular embryos; *e*) slight signal in early cotyledonary stage; *f*) localized GUS expression in well-shaped plantlet

found to be stronger then 2,4-D. After 1 hour treatment with MS medium supplemented with 1 mg/l TDZ root tips cells of transgenic *M. falcata* plants are competent for division and strongly express GUS reporter gene under promoters from cell cycle regulating genes – *cyc* A and *cyc* B (Iantcheva et al., 2004b).

Other factors important for positive embryogenic response

During the years work on somatic embryogenesis in genera *Medicago* we indicated the role of genotype on positive embryogenic response. Successful induction and frequency of obtained embryos are highly variable among different species of genera *Medicago* and inside of the cultivars (Brown and Atanassov, 1985; Chen et al., 1987). We are able to observe significant variation in embryogenic capacity between individuals of one cultivar or species (Iantcheva et al., 2005). Genotype dependent embryogenic response is widely reported for *M. sativa* (Mitten et al., 1984; Nagarajan et al., 1986; Chen et al., 1987; Seitz Kris and Bingham, 1988; Ivanova et al., 1994; Barbulova et al., 2002) mainly for its heterogenous character (polyploidy, open-pollinated). Large screening of germplasm is successful prerequisite for isolation of regenerable genotype (Mitten et al., 1984; Brown and Atanassov, 1985; Wolton and Brown, 1988; Barbulova et al., 2002). Type of explant, age of plant tissue are other factors which would be considered for establishing of embryogenic competence. Preliminary check for ploidy level of starting tissue is preferred requirement for omitting polisomaty of initial explant which predisposes to ploidy variation in obtained regenerants. In general plant tissues are composed of the cells with different ploidy level (polysomaty), which is proved in a study of Iantcheva et al. (2001a) where more uniform monosomatic tissue dominated by 2C nuclei is selected as an initial explant for induction of embryogenic potential. The acquisition of embryogenic competence and direct formation of somatic embryos are in relationship with genome size. After examination of genome size of several annual species of *Medicago* it is proved that these with smallest genome size are characterized with faster formation of somatic embryos and high number of embryos per explant compared to those with the biggest genome size (Iantcheva et al., 2003; Fyad-Lameche et al., 2016).

Pretreatment of an initial explant as stress stimuli could also lead to acquisition of embryogenic competence. Application of an osmotic pretreatment with 1M sucrose of the initial root explant of *M. truncatula* is positively influenced on embryogenic response during different stages of somatic

embryogenesis - induction, maturation and conversion to plants (Iantcheva et al., 2005a). High osmotic stress activates predetermined embryogenic cells to switch from somatic to embryogenic type followed by cell division. We confirmed that pretreatment of explants with an osmotic is related to the accumulation of high level of endogenous ABA. The positive influence of osmotic pretreatment only for 1 hour with 1 M sucrose is found to activate cells for division in root tips of transgenic *M. falcata* plants expressing *gus* gene under cell cycle promoters - *cyc* A and *cyc* B (Iantcheva et al., 2004b). Short-term osmotic stress is also found to be related to the accumulation of free proline (Gangopadhyay et al., 1997) and this could be connected with the improvement of somatic embryogenesis. The positive role of proline for the induction and development of somatic embryos of alfalfa is reported by (Shetty and McKersie, 1993; Barbulova et al., 2002).

Conclusions

Somatic embryogenesis in genera *Medicago* is direct way to regenerate plant from single somatic cell. Early events of the induction could be traced and observed and turn light to process of cell cycle activation, induction and asymmetry of first cell division, reprogramming of cells from somatic to embryogenic type. This process offer cloning and characterization of genes involved in wounding, hormone activation, cell division, differentiation and developmental processes. During the last 40 years considerable advances in understanding mechanism of somatic embryogenesis system in genera *Medicago* are occurred. Developed protocols offers the exploitation of this technique for mass propagation of valuable genotypes, application of gene transfer methods for crop improvement, functional genomics and metabolomics studies and know how to explore cellular plasticity.

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