THAÍS CRISTINA RIBEIRO DA SILVA

CELLULAR AND MOLECULAR MECHANISMS UNDERLYING ROOT SUCKER FORMATION IN *Arabidopsis lyrata*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 30 de maio de 2017.

Diego Silva Batista

Diego Ismael Rocha

Wagner Campos Otoni

Claudia Köhler

Leonardo Lopes Bhering
(Orientador)

“My soul honors your soul.
I honor the place in you where the entire universe resides.
I honor the light, love, truth, beauty and peace within you, because it is also within me.
In sharing these things, we are united,
we are the same,
we are one.”

Ao professor, orientador Wagner Otoni.
Dedico.

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Namastê.

BIOGRAFIA

Thaís Cristina Ribeiro da Silva, filha de Fernando Antonio Ribeiro da Silva e Gisele Cristina Gabrioli da Silva, nasceu em São Paulo – SP, no dia 09 de abril de 1988. Desde 1996 sua família reside em Pouso Alegre -MG, onde mora atualmente.

Em 2006 ingressou no curso de Ciências Biológicas na Universidade Federal de Viçosa (UFV), em Viçosa, MG. Escolheu como ênfase Genética e Biologia Molecular e, em julho de 2010, se graduou como bacharel, recebendo votos de louvor pelo desempenho acadêmico.

Durante o período de graduação foi bolsista de iniciação científica FAPEMIG no laboratório de Citogenética e Citometria do Departamento de Biologia Geral da UFV, onde desenvolveu projetos principalmente voltados para a área de cultura vegetal associando ferramentas citogenéticas e citométricas para determinação do conteúdo de DNA. Durante seis meses foi bolsista CNPq no Departamento de Estatística também da UFV.

Em agosto de 2010 inciou o mestrado no Programa de Genética e Melhoramento da UFV, atuando na mesma linha de pesquisa desevolvida durante a graduação.

Iniciou o doutorado em agosto de 2012, no mesmo programa do seu mestrado. Durante o doutorado trabalhou no Laboratório de Cultura de Tecidos (LCTII – BIOAGRO – UFV) sob orientação do professor Wagner Otoni. E em janeiro de 2015 foi aceita pelo programa Ciências Sem Fronteiras (bolsista CAPES) para desenvolver projeto do seu doutorado na Swedish Agricultural University (SLU, Uppsala, Suécia) sob a orientação da professora Claudia Köhler em Uppsala, Suécia onde desenvolveu essa pesquisa.

ÍNDICE

ABSTRACT

SILVA, Thaís Cristina Ribeiro da Silva, D.Sc., Universidade Federal de Viçosa, May, 2017. **Cellular and molecular mechanisms underlying root sucker formation in *Arabidopsis lyrata*.** Adviser: Leonardo Lopes Bhering.

Shoot organogenesis from roots (root suckers) allows vegetative propagation of *Arabidopsis lyrata*, the closest relative of *Arabidopsis thaliana,* in addition to sexual propagation and is an important trait associated with the root system. Using an *in vitro* system, we aimed to better understand the vegetative propagation in the model species *A. lyrata*, in what regards the morphological development of root suckers, the ability of vegetative propagation in different *in vitro* growth conditions, and identifying genes potentially involved in the formation of the new shoot apical meristem.Root sucker appearanceoccurred after30 days,most frequently in the axils of lateral roots. Root cross-sections showed a typical diarch primary structure and after 25 days, secondary root growth could be observed, as indicated by formation of the cambium. According to our data,root sucker emergence resembles the initiation of lateral roots from the pericycle, the tissue that gives rise to the vascular cambium during secondary growth. Regarding the *in vitro* growth conditions, full strength of MS induced the highest number of root suckers per plant, followed 3% of sucrose. However, light exposure and sucrose deprivation are not strictly required for sucker formation. Our data also revealed that auxin promotes root suckering. Vascular auxin response maxima are required to trigger lateral root initiation, suggesting that auxin-promoted sucker formation likely occurs by similar mechanisms. The evaluation of different shoot apical meristem related genes, suggests that the *STM* gene can be a potential marker to identify cells responsible in driving sucker formation. *Arabidopsis lyrata* proved to be an excellent model for further studies using root suckers, for example to study epigenetic marks throughout generations of clonal propagation.

RESUMO

SILVA, Thaís Cristina Ribeiro da Silva, D.Sc., Universidade Federal de Viçosa, maio de 2017. **Mecanismos celulares e moleculares subjacentes a formação de *root suckers* em *Arabidopsis lyrata***. Orientador: Leonardo Lopes Bhering.

A organogênese de brotos a partir de raízes (*root suckers*) permite a propagação vegetativa da *Arabidopsis lyrata*, o parente mais próximo da *Arabidopsis thaliana*. Utilizando um sistema *in vitro*, o presente estudo objetivou compreender melhor a propagação vegetativa nessa espécie modelo *A. lyrata*, no que se refere ao desenvolvimento morfológico de *suckers*, à capacidade de propagação vegetativa em diferentes condições de crescimento *in vitro* e à identificação de genes potencialmente envolvidos na formação do meristema apical dos brotos. O surgimento dos *suckers* ocorreu após 30 dias, mais freqüentemente na região axilar das raízes laterais. Os cortes transversais das raízes mostraram uma estrutura primária típica diarca e após cerca de 25 dias, pode-se observar o crescimento secundário da raiz, como indicado pela formação do câmbio. Conclui-se que a emergência do *sucker* assemelha-se à iniciação das raízes laterais a partir do periciclo, tecido que dá origem ao câmbio vascular durante o crescimento secundário. Em relação às condições de crescimento *in vitro*, a força total no meio MS induziu o maior número de *suckers* por planta, seguido por alta concentração de sacarose (3%). Exposição à luz e privação de sacarose não são estritamente necessários para a formação de *suckers*. Nossos dados também revelaram que a auxina promove a formação dos brotos. Máximas de auxina vascular são necessários para desencadear a iniciação da raiz lateral, sugerindo que a formação de *suckers* promovida por auxina ocorre provavelmente por mecanismos semelhantes. A avaliação de diferentes genes relacionados a meristema apical, demonstram que o gene *STM* pode ser um marcador para distinguir as células responsáveis pela formação de *suckers*. *Arabidopsis lyrata* provou ser um excelente modelo para estudos de organogênese em raíz e posteriores estudos usando esse sistema de reprodução para detectar marcadores epigenéticos através das várias gerações de propagação clonal.

1. INTRODUCTION

*Arabidopsis lyrata*L. belongs to the family Brassicaceae, together with many important crop plants including cabbage, mustard, as well as the model organism *Arabidopsis thaliana* (Rollins 1941). *A. lyrata* is the closest well-characterized relative of *A. thaliana* and is likely to have its status as a model plant. Unlike *A. thaliana*, *A. lyrata* is an outcrossing species and comprises a number of subspecies including ssp. lyrata distributed in North America and ssp. petraea, which has a patchy distribution in Europe. *A. lyrata* is believed to have diverged from *A. thaliana*between 3.8 and 5.8 million years ago (Kuittinen&Aguade 2000,Fobis-Loisy et al. 2007). The close evolutionary relationship between the two species, but also their differences in several aspects of the plant life-cycle and reproductive characteristics, makes this a unique and attractive system for evolutionary studies (Koch et al. 2000, Nasrallah2000,Clauss et al. 2002).

A.lyrata (formerly known as *Arabislyrata*, *Arabispetraea*, or *Cardaminopsispetraea*), has a genome of approximately twice the size of (1C approximately 245 Mb, 2n = 2X = 16) *A. thaliana*(1C approximately 157 Mb, 2n = 2X = 10) (Berr et al. 2006). The genes of the two *Arabidopsis*spp share a high degree of sequence similarity, allowing facile transfer of molecular markers and other data generated from the *A. thaliana* genome project to *A. lyrata* (van Treuren et al. 1997).

Despite being close relatives, from an evolutionary stand-point, *A. thaliana* and *A. lyrata* significantly diverge in their reproductive strategies. While *A. thaliana* is a selfing species, *A. lyrata* is an obligatory outcrosser. Moreover, some ecotypes of *A. lyrata*are known to reproduce asexually (Kusaba et al. 2001), a feature that was not been observed in *A. thaliana*.Some higher plants can propagate and multiply by vegetative reproduction in certain ecosystems, especially where sexual reproduction is hindered for reasons such as lack of pollinators or unfavorable climatic conditions.Therefore, plant reproductive adaptive responses have typically been classified dichotomously, separating species that survive and persist vegetatively (the resprouters) from those that perish and regenerate via formation of seeds (the seeders or non-sprouters) (Wells 1968, Midgley

1996).Among the species that propagate vegetatively, *A. lyrata* is characterized as a root-sprouter. Root-sprouters are plants with the ability to form adventitious buds (root suckers) on their roots in response to specific stimuli (Klimešová&Martínková 2004). Many root-sproutersdevelop shallow horizontal roots that sprout spontaneously, which can lead to varying degrees of clonal growth (Palacio et al. 2007,Klimešová&Martínková 2004).Taxonomical classifications taking into account the shoot-rooting and the root-suckering abilities of plants are particularly scarce. In fact, the formation of shoot-borne roots is often neglected in disturbance-response analyses, and most studies on resprouting do not discriminate between sprouts produced from roots, stems or stem-derived structures (e.g. stolons or rhizomes) (Koop 1987, Del Tredici 2001, Klimešová&Klimeš 2003).

*Arabidopsis lyrata*plants can undergo vegetative propagation and they have been previously described as a stoloniferous species (also called asubterranean runner) (Al-Shehbaz& O'Kane 2002,Gaudeul et al. 2007).Nonetheless, the anatomical and morphologicalcharacterization of *A.lyrata* roots is yet to be described, and thus there is no information on the origin of the new buds.

Root development is driven by the activity of meristems, which can also be responsible for giving rise to new plants. Within the root, the pericycle is a heterogeneous, meristematic, non-vascular tissue that is divided into two cell populations — one at the xylem pole and one at the phloem pole.In angiosperm roots, the pericycle is essential for lateral root initiation and is also involved in secondary root growth (Dubrovsky&Rost 2001, Beeckman&De Smet 2014). The pericycle plays an important role for the pluripotent capacity of plants and in the process of regeneration of shoots from root explants in *Arabidopsis thaliana* under *in vitro* conditions (Atta et al. 2009,Perianez-Rodriguez et al. 2014), given that adventitious shoots were shown to emerge from the xylem-pole pericycle cells on shoot-inducingmedium (Atta et al. 2009, Che et al. 2007). Anatomical studies of *Convolvulus arvensis*roots corroborated that the initial cell divisions of the pericycle cells, followed by their redifferentiation into founder cells, and the subsequent morphogenic events leading to primordium formation that precede shoot development, strongly resembled those occurring during

lateral root formation (Beijerinck 1887,Bonnett& Torrey 1966, Motte et al 2014.). Therefore, the origin of buds (suckers) in sprouter species has been proposed to be dependent on the pericycle cells.InAspen (*Populustremuloides*), for example, root suckers originate from primordia that are formed from meristematic cells during secondary growth of the root cork cambium (Schier 1973,Schier et al. 1985).On the other hand, in peach palm (*Bactrisgasipaes*Kunth) shoot apex explants, adventitious shoots initiate from the pre-procambium cells (Almeida et al. 2012, Frey 2003)

The origin of roots suckers being xylem-pole pericycle cells orthe pre-procambium might be an important decisive factor for the newly formed shoots to efficiently being able to capture water and nutrients from the explants via the already established vascular system. In fact, xylem-pole pericycle, pre-procambium, procambium, and cambium cells all have stem cell features. Procambium and cambium are pluripotent vascular meristemsinvolved in primary and secondary development of vascular tissues, respectively (Eloet al. 2009). It is possible that only stem cells are competent to regenerate roots or shoots via *de novo* organogenesis. Since *de novo* shoot organogenesis is initiated from the pre-procambium or xylem-pole pericycle cells, it seems that this regeneration process shares some similarities with that of lateral root formation, rather than lateral shoot formation. Lateral shoots originate from external cells on the adaxial side of leaf axils, while adventitious shoots formed via *de novo* shoot organogenesis initiate from internal xylem-pole pericycle or pre-procambium cells (Xu & Huang 2014).

Physiological conditions such as carbohydrate levels, nutrient status, or other growth-contributing resources within the root such as lipid concentrations have been shown to affect the number of suckers formed in diverse species (Bellingham & Sparrow 2000, Grime 2006).Klimeš&Klimešová (1999) observed that nutrient shortage facilitates the formation of root buds in *Rumexacetosella*, but their growth and transition to shoots are stimulated at higher nutrient levels. Nutrient availability may exert an important effect on the carbohydrate stores available for resprouting (Knox & Clarke 2005). However, the resprouting ability of the Mediterranean shrub *Erica australis* does not correlate withnitrogen content in the storage

organs (Cruz et al. 2003). Indeed, resprouters do not seem to accumulate more macronutrients than non-sprouters in their roots (Pate et al. 1990).

Previous studies have shown that lateral root formation is significantly affected by auxins, andindole-3-acetic acid (IAA), the major endogenous auxin in plants, is necessary for both the initiation and the later development of lateral roots (Celenza et al. 1995,Reed et al. 1998,Bhalerao et al.2002,Vilches-Barro et al. 2015).Auxins and cytokinins are also believed to play prominent roles in the root suckering process in trees and shrubs(Cline 1991). Interestingly, high root temperatures have been proposed to facilitate auxin degradation (Schier et al. 1985, Hungerford 1988), and to promote root growth and cytokinin synthesis (Hungerford 1988) and thereby stimulate sucker initiation.

Several molecular players have been shown to be involved in the genetic control of *in vitro* plant regeneration(Motte et al. 2014, Xu & Huang 2014). One such example is*WUSCHE*L (*WUS*), which codes for a transcription factor that plays a key role in the initiation of shoot organogenesis (Gallois et al. 2004, Gordon et al. 2007, Chatfield et al. 2013, Suet al. 2015). Similarly, *SHOOTMERISTEMLESS* (*STM*) codes for another transcription factor required for shoot regeneration *in vitro* (Barton &Poethig1993,Endrizzi et al. 1996,Hibara et al.2003) and has also been linked to the establishment of meristems in response to cytokinin (Brand et al. 2002, Scofield et al. 2013). Both *WUS* and *STM* transcripts over-accumulate in explants incubated on shoot inducingmedium (Chatfield et al. 2013, Gordon et al. 2007).

Given how little information is available on the mechanisms that lead to sucker formation, the present study aimed to better understand the vegetative propagation in the model species*Arabidopsis lyrata*, in what regards the morphological development of root suckers, the ability of vegetative propagation in different*in vitro* growth conditions, as well as to identifygenes potentially involvedin the formation ofthe new shoot apical meristem. Some questions were addressed: Where does the initiation of sucker development occur? Which growth conditions influence sucker initiation *in vitro*? Does auxin presence and transport affect formation of lateral roots and root suckers? Which genes are expressed during sucker

development?

2. MATERIALS AND METHODS

2.1. Plant Material and Growth Conditions

2.1.1. Plant Material

Seeds of *A. lyrata* from an Icelandic populationwere sterilized and rinsed in sterile water. Five seeds were transferred to Petri dishes (150x20mm, Sarstedt®) containing 100 mL of control culture medium consisting of half strength MS (Murashige and Skoog) medium supplemented with sucrose (1%) and agar (0.8%). The plates were sealed with Micropore® and kept in the dark in a cold room (4 ºC) for three days. The plates were transferred to a temperature-controlled growth room (22 ± 2 ºC) under 16-h photoperiod, irradiance of 110 µmol m^{-2} s^{-1} (day-light fluorescent lamp) for 45days. The subsequent experiments were performed with suckers (clones) from the same mother plant, in order to minimize genetic effects. The suckers were excised from the mother plant and transferred to new culture mediumuntil the appearance of new roots (approximately 10 days), after which they were utilized for further experiments.

2.1.2. Growth Conditions

Different growth conditions were tested alongside the control described above:twosucrose concentrations (0% and 3%), MS strength (full and 1/8), and light conditions (continuous light and continuous darkness). The latter was provided by covering the root cultures with aluminum foil.Other conditions were as described for the control. One plant (clone) was used per plate (92x16mm, Sarstedt®), and a total of 10 plants were assayed per treatment.The number of root suckers was scored after 30 days of *in vitro* growth.T-test was performed in order to compare the average of treatment and control.

2.1.3. IAA treatments

For auxin treatments, the control culture medium was supplemented with 0.1, 1 and 10 µM of Indole-3-acetic acid (IAA) (Sigma®) and square Petri dishes (120x120x17mm, Greiner Bio-one®) containing 100mL of medium were vertically kept under the conditions described above. Seven biological replicates were assayed per plate. The number of root suckers was scored after 15 days of *in vitro* culture. Petri dishes were analyzed under a Leica Z16 Apo Macroscope and the images were recorded using a Leica DFC490 camera with a 0.63x optical adapter.

2.1.4. Auxin transport and biosynthesis inhibitor treatments

For auxin inhibitor treatments,*A. lyrata* plants were grown in culture medium that was supplemented with either10 or 100 µM L-Kynurenine (KYN-Sigma®),or with 1 µM of Naphthylphthalamic acid (NPA-Sigma®)and 1 µM of NPA with 2.5 µM of IAA in square Petri dishes containing 100mL of MS-based medium, which werekept vertically under the same conditions described above.Seven biological replicates were used in each plate. The number of root suckers was scored after 30 days of *in vitro* culture. Petri dishes were analyzed under a Leica Z16 Apo Macroscope and the images were recorded using a Leica DFC490 camera with a 0.63x optical adapter.

2.2. Histological analyses

For anatomical and ultrastructural characterization of *Arabidopsis lyrata* roots,roots with and without suckers were fixed in FAA (10:2:1,Formalin – acetic acid – alcohol) solution (Karnovsky 1965),and embedded in Historesin Leica®.For the anatomical studies, fixed samples were dehydrated in a graded ethanol series and embedded in methacrylate (Historesin, Leica Instruments, Germany). Transverse and longitudinal sections (4µm thick) were obtained with an automated advance rotating microtome (Microm HM 355 S) and stained with toluidine blue at pH 3.2 (O'Brien & McCully 1981).

Slides were analyzed under a Zeiss Axioscope A1 microscope with differential interference contact (DIC) optics and theimages were recorded using a Leica DFC295 camera with a 0.63x optical adapter.

2.3. Quantification of SAM related genes expression

2.3.1. Primer design

To identify *A. lyrata* genes and their homologs, we performed a TBLASTX search using the *Arabidopsis thaliana* sequences as queries against the Phytozome databases (http://www.phytozome.net/). Primers were designed using the Primer-BLAST software for the amplification of gene fragments around 100 bp in length (Table 1) and an annealing temperature of 60°C. To test primer specificity, PCR products were visualized after electrophoresis on 1% (w/v) agarose gels containing 0.02% (v/v) EtBr in 0.5× TBE buffer. Primer sequences and amplicon length are shown in Table 1.

Table 1. Primer sequences and amplicon sizes for the reference genes *ELF1* and the target genes *STM*, *WOX4* and *WOX1*

Gene	Forward primer	Reverse primer	Amplicon size (pb)
ELF1	F 5´TGGTGACGCTGGTATGGTA	R 5´GGTCTGCCTCATGTCCCTAA	100
STM	F 5´AGCTCCCTAAAGAAGCTCGC	R 5´TGGTCCAGCCCTGTTGATTC	115
WOX4	F 5´TCTCTAACCCGAGTGTAGCAACG	R 5´TTCTCCACCATGGATGCTCTCCTG	116
WOX1	F 5´CGTACCAGAGAAGCAGCGAA	R 5´ ATCCGGTTCACCGTTAGAGC	93

2.3.2. RNA extraction and cDNA synthesis

Roots of three *Arabidopsis lyrata*plants germinated from seeds, which had been grown under normal *in vitro* conditions, were used. The samples were harvested and classified as with or without root suckers, in three diferrent time points, at 25, 45, and 70 days (totalizing 9 plants, 3 for each time point). Total RNA was extracted using the Qiagen RNeasy kit, followed by DNase I treatment (Qiagen). cDNA was synthesized using an oligo-dT

primer,from total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Fisher Scientific, Sweden).

2.3.3. RT-qPCR and Data analysis

Maxima SYBR Green qPCR Master Mix (Thermo Scientific) was used to perform the qPCR in an iQ5 qPCR system (Bio-Rad Laboratories AB, Sweden) with three technical replicates. The primers used for the RT-qPCR are described in Table 1. *ELF1* was used as the reference gene. Relative quantification of gene expression was performed as described in Pfaffl(2001).

3. RESULTS

3.1. Root suckering characterization

As is the case for many species that produce shoots from roots (Schier& Campbell 1976, Guerrero-Campo et al. 2006,Souza et al. 2009, Decombeix et al. 2011,Fawa et al. 2014), the root-to-shoot capacity of *Arabidopsis lyrata*may represents a strategic adaptive mechanism for vegetative reproduction.

Plants of a natural population of *A. lyrata*spp*. lyrata* from Iceland cultivated under greenhouse conditions developed several shoots along the main root (Figure S1). Shoot primordia and shoots were, most of the time, positioned close to a lateral root primordia. The new shoots arise not only close to the soil surface, but also 15 cm beneath the soil surface, indicating the light is not required for the sucker formation (Figure S1b).

In order to identify the developmental origin of root suckers in *A. lyrata*, sucker development was followed under *in vitro* growth conditions. Roots growing on hormone-free medium and standard light conditions were white and thin (Figure 1a). But after 25 days of *in vitro* growth, the roots started becoming greener and thickened, and shoot buds started emerging after 30 days, approximately (Figure 1b). Sucker formation occurred most frequently in the close vicinityto the lateral roots (Figure 1b), but could also be observed between lateral roots (Figure 1c). The first suckers emerged on the upper part of the root in close vicinity to the mother plant, while later on also on distal parts of the root (data not shown).

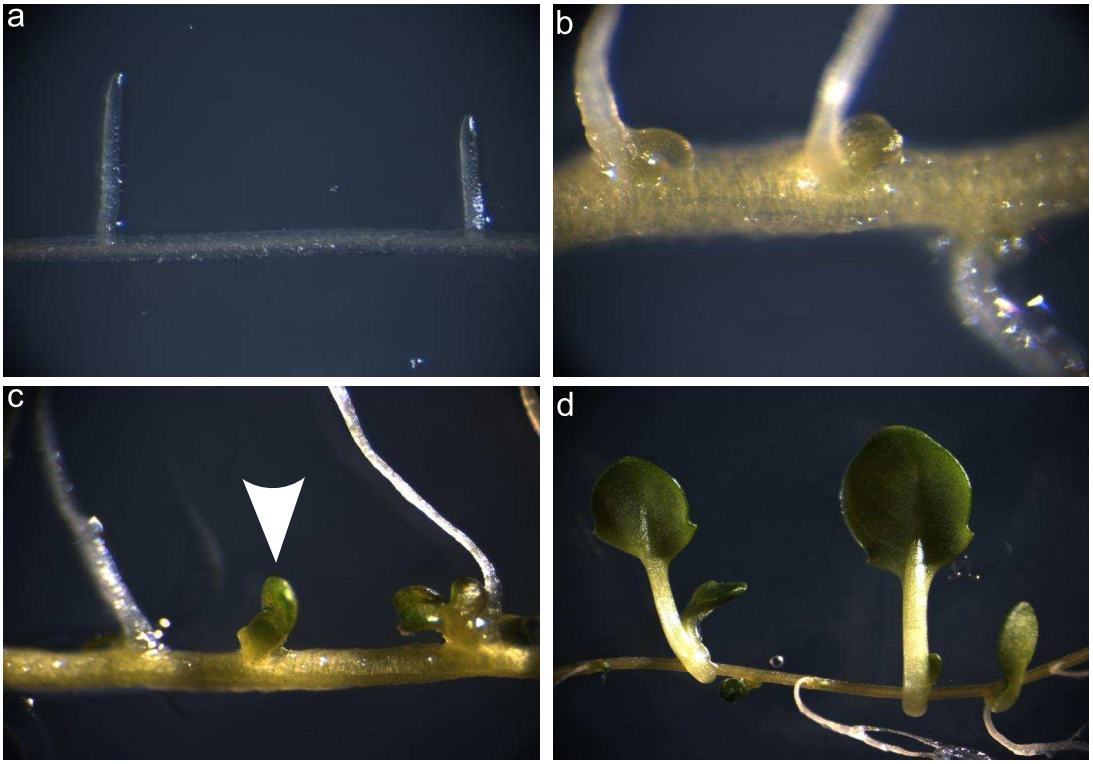

Figure 1. Developmental stages of *A. lyrata*roots growing on hormone-free medium after a) 15 days, with no observable suckers, b) 30 days, with buds emerging at lateral root axils, c) 40 days, with new buds emerging also in the internodes (arrowhead) and d) after 40 days, where in some cases well developed leaves can already be observed.

To identify the cell layer giving rise to root suckers, we analyzed cross and longitudinal sections of *A. lyrata* roots before and during sucker initiation (Figure 2). Root cross-sections showed a typical diarch primary structure at approximately 15 days of growth in hormone-free medium (Figure 2a). After about 25 days secondary root growth could be observed, as indicated by formation of the cambium andphellogen (Figures 2b, c), which follows a similar pattern to that described for secondary root growth in *A. thaliana* (Dolan et al. 1993, Dolan & Roberts 1995). Sucker initiation was observed on roots that had entered secondary growth (Figure 2d-f). Cross and longitudinal sections suggest that suckers originate from the vascular cambium, the meristem giving rise to secondary xylem and phloem and associated tissues (Dolan & Roberts, 1995) (Figure 2d, e). Interestingly, sucker emergence resembles the initiation of lateral roots from the pericycle, the tissue that gives rise to the vascular

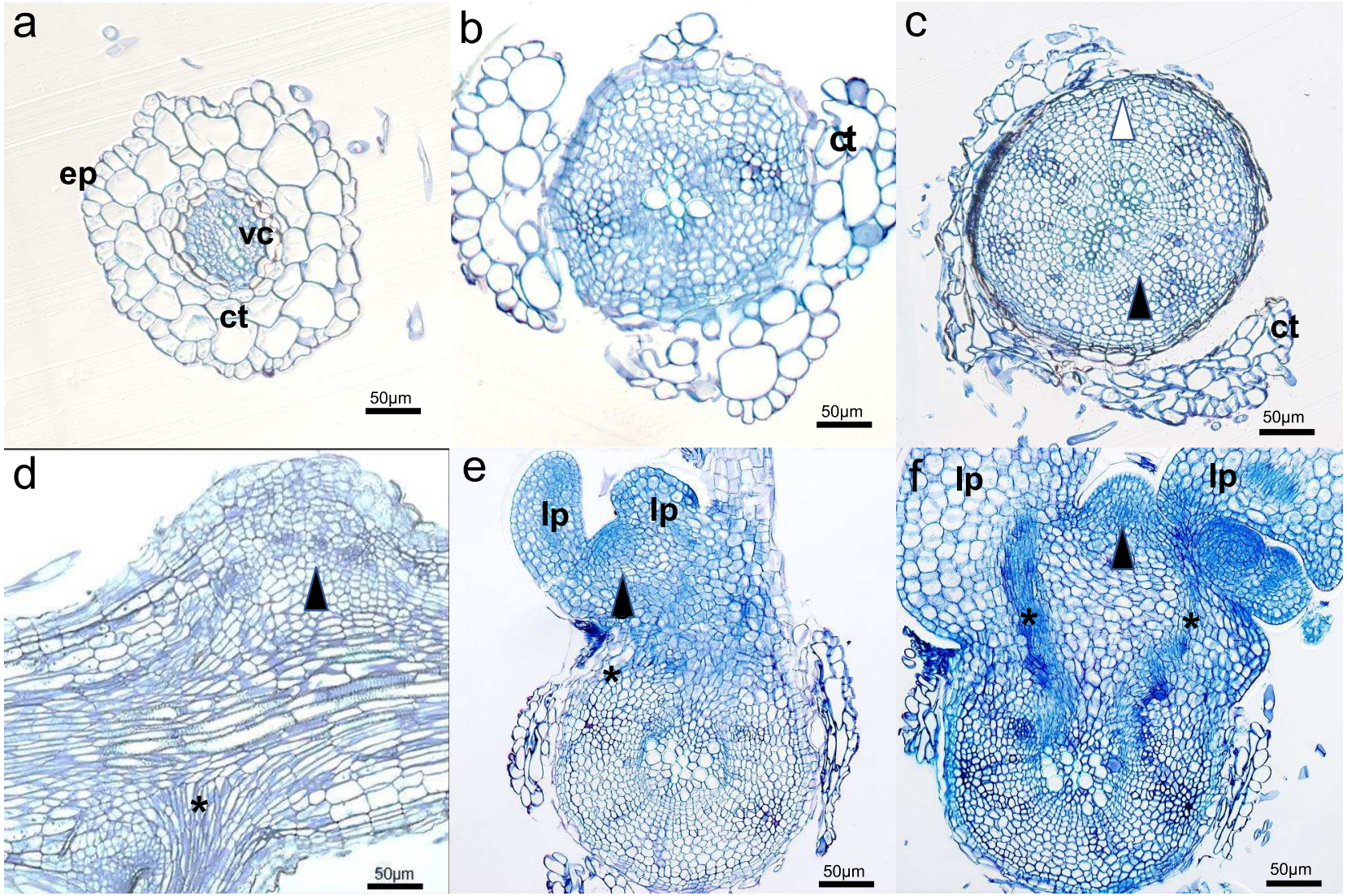
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Figure 2. Morphology of root sucker initiation in *Arabidopsis lyrata*. (a-c, e,f) Cross and (d) longitudinal sections. a) 15-day-old primary root. (b) 25-day-old root showing initial secondary growth. (c) Progressive secondary growth in 30-day-old roots. Note the presence of the cambium (dark arrowhead) and phellogen (white arrowhead) with initial suber formation. (d) Longitudinal section of a 20-day-old root with an emerging sucker bud (arrowhead) and a lateral root primordium (asterisk). (e, f) Sucker buds in 35- (e) and 40- (f) day-old roots. Leaf primordia (lp) and shoot apical meristem (SAM) (arrowhead) are clearly visible. Vascular connections (asterisks) between the original root explant and sucker are formed (f). Abbeviations: ep = epidermis; ct = cortex; lp = leaf promordia; vc = vascular cylinder. Scale bar = 50 µm.

cambium during secondary growth (Dolan & Roberts 1995). But instead of following a root fate, a shoot apical meristem (SAM) is established forming leaf primordia (Figure 2e) and progressively a vascular continuity is established (Figure 2f).

3.2. Sucker formation under different *in vitro* conditions

Previous work proposed that carbohydrate levels, nutrient status, or other growth-contributing resources within the root affected sucker initiation (Frey et al. 2003). Thus, we tested whether different *in vitro* growth conditions have an impact on sucker initiation. We analyzed sucker formation in 10 *A. lyrata* clones under different treatments for 30 days (Figure 3). Under control conditions (1/2 MS, 1% sucrose, 16:8 photoperiod), each plant gave rise to an average of 35 suckers. Full strength of MS induced the highest number of root suckers per plant (70), followed by high concentration of sucrose (3%) (50). Conversely, low concentration of MS nutrients and darkness significantly reduced sucker formation to 20 and 15 suckers per plant, respectively. Nonetheless, plants in culture media without sucrose or continuous light conditions did not show significant differences in comparison to the control. These observations suggest that the availability of nutrients, light and sucrose positively correlate with the presence of suckers, but that light exposure and sucrose deprivation are not strictly required for sucker formation.

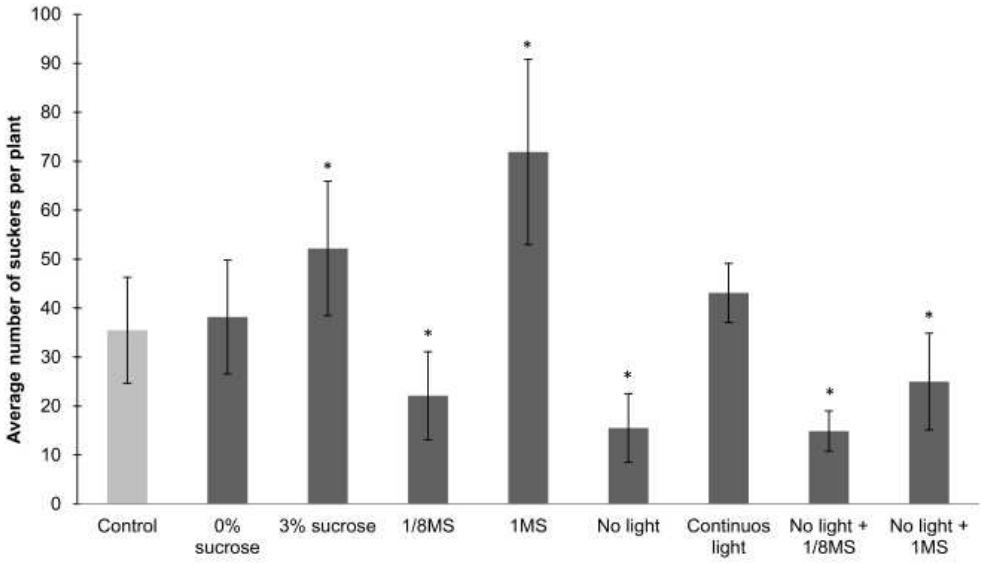

Figure 3. Average number of root suckers per plant under different growth conditions. 10 plants were analyzed per condition. Error bars represent standard deviation. Asterisks mark significant differences compared to the control (t-test, $p<0.05$).

We further tested whether deprivation of light and nutrients would have an additive effect on sucker formation, but no differences were observed when compared to deprivation of light alone. Furthermore, we observed that constant darkness suppressed the increase of sucker formation induced by high nutrient concentrations (No light+full MS), suggesting that light conditions have a dominant effect on sucker formation in *A. lyrata*. Nevertheless, plants grown on soil produced etiolated suckers, indicating that light promotes sucker formation, but is not essential for this process (Figure S1b).

3.3. Auxin promotes root suckering

Auxin is a key regulator of lateral root development (Lavenus et al. 2013). Since most suckers were formed in the axils of lateral roots, we asked

whether auxin could also have a role in sucker formation. To test this, *A. lyrata*clones were grown under three different concentrations of IAA and the number of suckers per plant was determined after 15 days of treatment (Figure 4). Strikingly, in the IAA-treated samples the average number of suckers per plant was two-fold higher compared to the control. However, no significant differences were observed between the different concentrations of IAA used (Figure 4e)

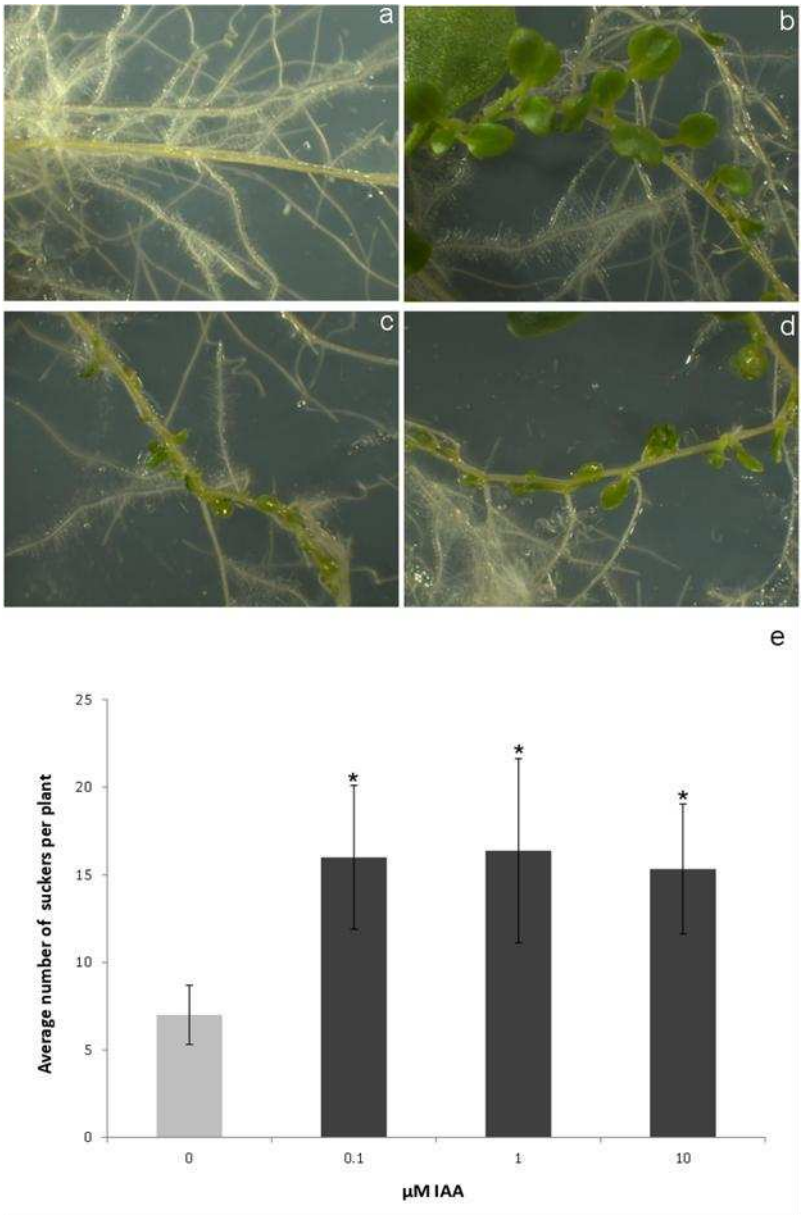

Figure 4.*A. lyrata* root phenotypes in 15-day-old plants grown under control conditions (a) or on media supplemented with IAA (b-d). b) 0.1 µM IAA; c) 1 µM IAA; d) 10 µM IAA. e) Average number of suckers per plant grown under control conditions or on media supplemented with IAA. Error bars represent standard deviation. Asterisks mark significant differences compared to the control (t-test, $p<0.05$).

We tested whether auxin is a necessary condition for sucker development, by investigating the effect of KYN and NPA that either block auxin biosynthesis or polar transport auxin transport, respectively (Figure 5) (Reed et al. 1998,Yang et al. 2014). Inhibition of auxin biosynthesis or transport affects lateral root formation in *A. thaliana* (Reed et al. 1998,Casimiro et al. 2001) and it also can be seen in *A. lyrata*(Figure S2) under KYN (Figure S1a,b) and NPA (Figure S1c) treatments the number of lateral roots was visibly decreased, and recovered when the medium was supplemented with IAA (Figure S2d).

While under the NPA treatment the total number of suckers per plant decreased, we could observe sucker formation throughout the entire root, and not only close to the mother plants as observed in control conditions (Figure 5a, b). Furthermore, while in thecontrol sucker initiation occurred predominantly in the axils of lateral roots, it frequently occurred in between lateral roots upon NPA treatment. Treatment with NPA also caused morphological changes of the new shoots that formed cup-shaped like structures (Figure 5b) lacking vascular connections with the roots (data not shown). Nevertheless, these suckers were able to germinate, and a subsequently underwent normal development. Treatments with KYN significantly reduced the number of suckers per plant, but the morphology of the suckers did not differ compared to the control (Figure 5c). Importantly, the number of suckers per plant decreased upon blocking auxin biosynthesis and transport (Figure 5e), andconsistently the number of lateral roots also decreased in all treatments (Figure 5f).

Altogether, our data suggests that auxin promotes root suckering. Vascular auxin response maxima are required to trigger lateral root initiation (Benková et al. 2003), suggesting that auxin-promoted sucker formation likely occurs by similar mechanisms.

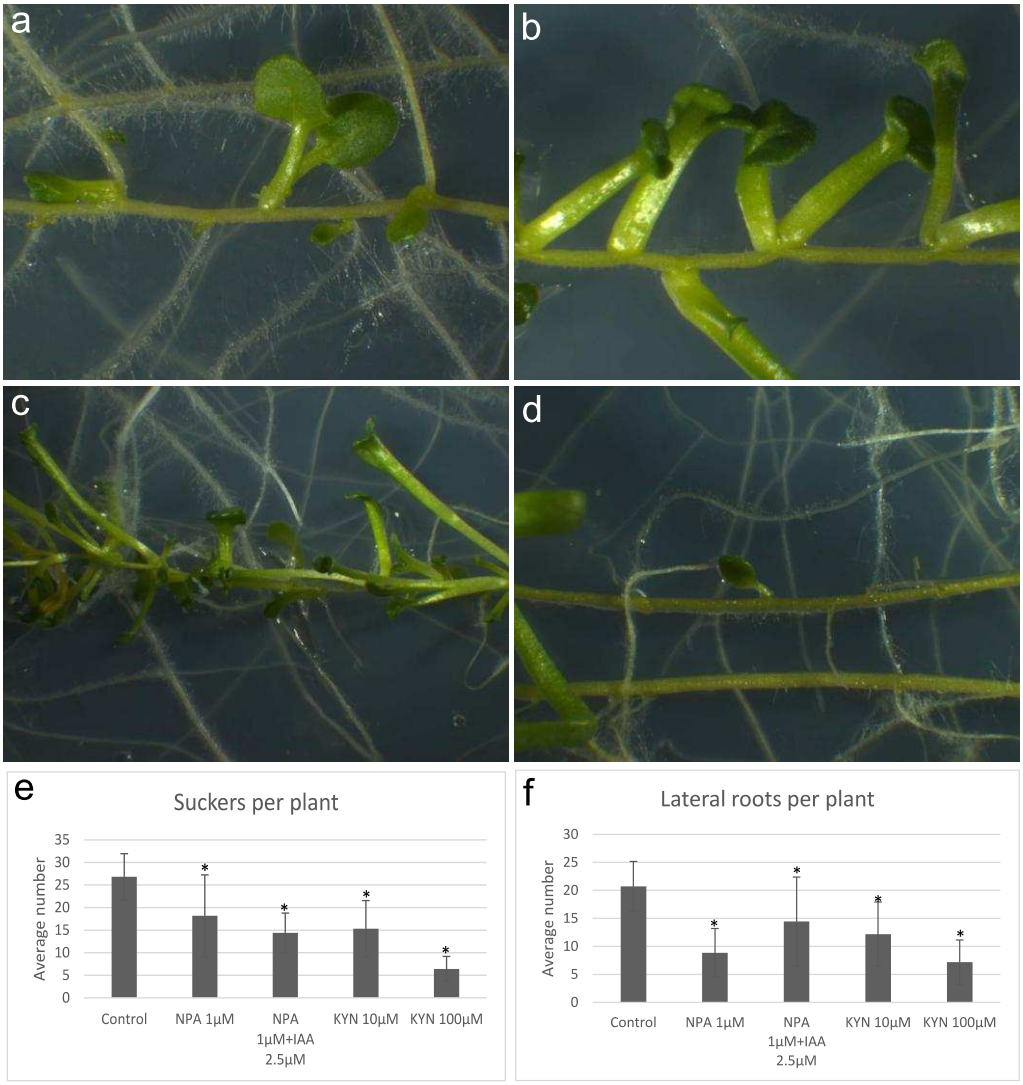

Figure 5. *Arabidopsis lyrata* root phenotypes in 30-day-old plants grown under control conditions (a) or on media supplemented with auxin inhibitors (b-d). (b) 1µM NPA. (c) 1 µM NPA, + 2.5 µM IAA (d) 100 µM KYN. Average number of suckers (e) and lateral roots (f) per plant grown under control conditions or on media supplemented with auxin inhibitors. Error bars represent standard deviation. Asterisks mark significant differences compared to the control (t-test, $p<0.05$).

3.4. The expression of shoot apical meristem-related genes in roots changes during the plant development

The SAM possesses an organizing centre (OC) that acts to instruct and maintain pluripotency in the overlying stem cells of the central zone (CZ).

The homeodomain transcription factor WUSCHEL (WUS) is required for stem cell activity (Mayer et al. 1998) and is the founding member of the WUS-homeobox (WOX) family, which plays important roles in diverse plant stem cell systems (Nardmann&Werr 2006, Lu et al. 2015).

In parallel to this local regulatory system that maintains stem cell identity, cells are kept in an undifferentiated state throughout the SAM by the activity of SHOOTMERISTEMLESS (*STM*), encoded by a member of the KNOTTED-like homeobox (*KNOX*) gene family (Long et al. 1996, Byrne et al. 2002, Belles-Boix et al., 2006). STM represses the activity of the differentiation genes *ASYMETRIC LEAVES 1* (*AS1*) and *AS2*, which in turn form a dimer and repress *KNOX* gene expression to promote cell differentiation (Byrne et al. 2000, 2002, Guo et al. 2008).

To test whether these genes may be involved in sucker formation, we evaluated the expression of the SAM-related genes*STM*, *WOX1* and *WOX4* in *A. lyrata* roots before, during, and after sucker initiation. The number of suckers per plant during development was scored (table 2) and correlated with gene expression (Figure 6).

Table 2. Number of suckers per plant at different time points (T1:25 days; T2: 45 days, T3: 70 days) the roots were harvested for the RNA extraction from a representative replicate for each time.

	25 days	45 days	T3 days
STM	0	15	55
WOX1	0	10	50
WOX4	0	10	50

Relative expression of *STM* substantially increased over time, correlating with the number of suckers formed (Figure 6a). Expression of *STM* was detected before outgrowth of suckers was visible, rendering *STM* a suitable marker for sucker initiation. Like for *STM,* expression of *WOX1* correlated with sucker initiation (Figure 6b). The apparent decrease of *WOX1*expression levels in older roots is likely a consequence of increasing numbers of leaf primordia formed in 70 day-old roots, diluting *WOX1* mRNAs. In contrast, expression levels of *WOX4* were higher in the root samples

before sucker initiation, suggesting that *WOX4* expression decreased when root meristematic fate switched to SAM identity (Figure 6c).

Other genes involved in SAM development in *A. thaliana* were also tested, namely *CLV3* (Yadav et al. 2009), but *CLV3* expression did not correlate with sucker initiation (data not shown). Genes involved in root meristem development, such as *WOX5* and *SHR* (Sugimoto et al. 2010), which have also been implicated in lateral root formation, were also tested and they did not show any trend that could be related to root sucker formation (data not shown).

Altogether, expression of *STM* and *WOX1* positively correlate with sucker initiation and could serve as marker genes to trace the onset of sucker initiation.

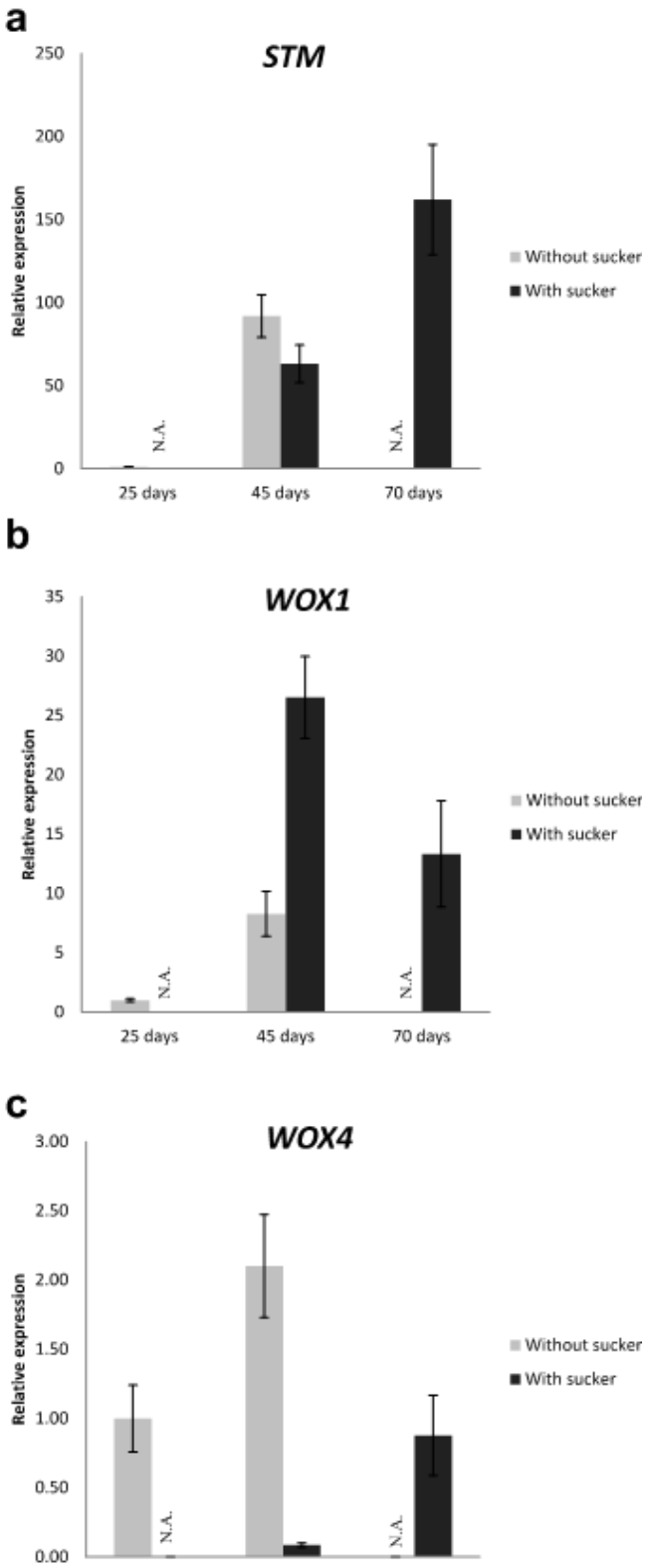

Figure 6. Relative expression of genes related to SAM activity in *A. lyrata* roots of 25-, 45- and 70-day-old plants. The genes assayed were (a) *STM*, (b) *WOX4,* and (c) *WOX1* Expression was normalized to *ELF1*. Roots with and without visible sucker formation were sampled. At 25 days after germination only roots without suckers were sampled and at 70 days only roots with

suckers were sampled. The assay was done in triplicate and the results from a representative replicate are shown. Error bars represent standard deviation.

4. DISCUSSION

4.1. A first description of *Arabidopsis lyrata* root anatomy

Here we report for the first time the anatomical characterization of vegetative propagation of *A. lyrata* via root suckering under *invitro* conditions. Plants of *Arabidopsis lyrata* cultivated *in vitro* have the ability to form suckers, even in the absence of added plant growth regulators, similarly to what happens in natural field conditions. Our findings suggest that *A. lyrata* roots undergo secondary growth as early as after 25 days of *in vitro* culture and that the meristematic activity of the vascular cambium is likely to be involved in the formation of the root suckers, in a similar fashion to what has been previously described for certain species of trees (Frey et al. 2003,Wan et al. 2006,Shah et al. 2015). We also observed that *A. lyrata*plants form root suckers when grown in soil, but that process, as well as the number of suckers formed, remains undetermined in field conditions.

Arabidopsis lyrata root sucker development occurs both naturally and under hormone free *in vitro* conditions. Conversely, other species like Seabuckthorn (*Hippophaerhamnoides* L.) produces new shoots from roots naturally, but under *in vitro* conditions it is strickly dependent on plant growth regulator supplementation (Shah et al. 2015). The formation of new organs is controlled in complex processes by the relative concentrations of hormones and the purpose of adding plant growth regulators to a medium used *in vitro* is to alter the natural gradients of hormones in planta (REF)

The anatomy of the *Arabidopsis lyrata* primary root has a similar tissue organization to that described for other members of the Brassicaceae (Peterson 1967,Kuras 1980, Dolan et al. 1993,Scheres et al. 2002). In other words, the mature vascular cylinder shows a diarchsimetry with an almost constant number of cells, and a characteristic orientation of pericycle cells facing the protoxylem and protophloem elements with regard to the surrounding endodermal cells. The cortex of an *Arabidopsis* root consists of a single layer of cells (Scheres et al. 2002), which is distinct from other members of this family that have thicker roots, like *Brassica napus* and

Sinapis alba, where there are two concentric rings of cortical cells (Kuras 1980).

The vascular cambium, a secondary meristem, which originates from the pericycle, is the driving force behind secondary growth, which increases the girth of plant organs, by generating new vasculature (secondary xylem and phloem). In the model plant *Arabidopsis thaliana,* secondary growth occurs in the root, as well as in the stem and in the hypocotyl. In the latter and to a lesser extent in the root, a cylinder of wood is formed, resembling the process of secondary growth in *Arabidopsis lyrata* and also of most angiosperm trees (REF).

Regarding to the origin of root suckers, this is a topic well studied in*Populus*, a major model angiosperm tree (Mellerowicz et al. 2001) and resemble to *A. lyrata* root sucker development.Poplar (*Populus*spp) and Aspen (*Populustremuloides*Michx.) are clonal tree species that commonly regenerates via root suckering after disturbance (Frey et al. 2003). Most suckers that arise on the undisturbed roots of *P. angustifolia*, *P. deltoids*, and *P. balsamifera* after death or injury of aboveground parts appear to arise from suppressed buds embedded in the periderm. Only when roots are damaged, exposing the cambium, is it likely that suckers will arise from newly formed buds, similar to those developing at the ends of roots segments. (Schier&Campbel1976).

Unlike poplar root buds, aspen shoot primordia appear to arise at any time during root growth after the formation of a phellogen, probably in response to an outside stimulus. Also, compared to poplar root buds, they may be relatively short lived, if they do not develop into shoots, they may become a part of the phellem as successive phellogen are formed. (REF)

Vinocur et al. (2000) showed that all buds of *Populustremula* L. originated in close proximity to lateral roots that emerged from the pericycle in the main root, similarly to *A. lyrata* root suckers. Regenerated bud primordia, which formed a multi-layered meristem, appeared at the base a developing lateral root. In beech (*Fagus grandifolia*), root suckers arise from adventitious buds formed on callus tissues that develop following root injury (Jones &Raynal 1986, Beaudet&Messier 2008).

As previously mentioned, *Arabidopsis thaliana* plants are not described to undergo asexual reproduction in natural conditions. However, it is possible to regenerate shoots from *A. thaliana* roots and hypocotyl explants under *in vitro* conditions, in medium supplemented with growth regulators. Many lines of evidence support the understanding that pericycle and vascular parenchyma cells are intrinsically prone to undertake different morphogenetic pathways (De Smet et al. 2006, Atta et al. 2009, Sugimoto et al. 2010, Pulianmackal et al. 2014). In this case, the adventitious shoots emerge from the xylem-pole pericycle cells (Atta et al. 2009, Che et al. 2007). On the other hand, in peach palm shoot apex explants, adventitious shoots initiate from the pre-procambium cells (Almeida et al. 2012). The fact that root-derived-shoots originate in the xylem-pole pericycle and the pre-procambium might be important for the newly formed shoots to capture water and nutrients from the explants via the vascular system.

It is possible that only stem cells are competent to regenerate roots or shoots in *de novo* organogenesis. And, since *de novo* shoot organogenesis is initiated from the pre-procambium or xylem-pole pericycle cells, it seems that this regeneration process shares some similarities with that of lateral root formation, rather than lateral shoot formation. Lateral shoots originate from external cells on the adaxial side of leaf axils, while adventitious shoots formed via *de novo* shoot organogenesis initiate from internal xylem-pole pericycle or pre-procambium cells.

In *A. lyrata*,however, obtaining root sections of very early stages in sucker bud formation proved to be a major challenge since these buds originate from tissues which are already meristematic, meaning it is hard to identify these cells early during the development. Therefore, further studies should be carried that include molecular markers to be able to point outwhich root cells are able to develop shoot apical meristem characteristics and thus give rise to root suckers.

4.2. Nutrients and light trigger sucker formation

Our observations strongly indicate that nutritive factors, namely macro and micronutrients present in the MS medium (Murashige& Skoog 1962)

influence root suckers development. Plants grown under full-strength MS medium produced the highest number of suckers per plant, while the opposite was observed for plants grown under low MS concentration (Figure 3). This is in line with previous observations that major and minor concentration of inorganic salts are required for bud and shoot growth (Ramage &Willians 2002).

Mineral nutrients are a significant component of culture media but are often overlooked as possible morphogenic elicitors. The nutrient combinations used for a particular plant species and for certain developmental pathways are usually determined by the empirical manipulation of one or a combination of existing published formulations (Ramage &Willians 2002). Macronutrients such as nitrogen, phosphorus, and sulfur are important components of macromolecules such as proteins and nucleic acids, as well as constituents of many small molecules. Micronutrients are required in much smaller quantities than macronutrients and function in various roles such as enzyme cofactors or components of electron transport proteins (Marschner 1995). Given these important roles that essential minerals play in cellular processes, it is not surprising that the supply of these minerals is a determinant factor for morphogenesis. For instance, Hardy & Thorpe (1990) observed increased nitrogen metabolism during de novo shoot organogenesis in *calli* of *Nicotiana tabacum* and a follow-up study indicated that this increase occurred prior to meristemoid formation (Joy et al. 1994). Futhermore, Drew et al. (1973) showed that localized supply of nitrate, ammonium, and inorganic phosphorus could stimulate root branching in barley (*Hordeum vulgare*) and the work by Martínková et al. (2004) supported the hypothesis that the release of adventitious buds and growth of new adventitious shoots is facilitated at higher nutrient levels in *Rorippapalustris* (Brassicaceae).

So far, no studies have been done to test the effects of different concentrations of MS medium componentson root sucker formation. However, the effects of mineral medium strength (T medium)have been testedduring microtuber induction in *Dioscorea. alata* and *D. bulbifera* (Mantell& Hugo 1989). Furthermore,the effect of the supply of isolated nutrientshas beentested in a few species. For example,Shah et al. (2015) showed thatthe

formation of shoots from roots inseabuckthorn (*Hippophaerhamnoides* L.)was inhibited under low phosphorus concentrations and stimulated in both high phosphorus concentration and in the presence of IAA. Additionally, Wan et al (2006) propose that nutrients, such as inorganic nitrogenous compounds,help induce root suckering in aspen (Farmer 1962,Eliasson1971).

Our results suggest that when grown under a high sucrose concentration, *A. lyrata* plantsproduce more suckers than in control conditions (Figure 3).Readily available non-structural carbohydrate (NSC) reserves have previously been shown to influence suckering performance of aspen roots (Landhäusser&Lieffers2002,Schier&Zasada 1973), which is in line with our observations and indicates that external energy sources can directly impact on this form of asexual reproduction. In aspen, although NSC concentrations do not appear to influence the sucker initiation, they are important for early sucker shoot expansion and growth (Schier&Zasada 1973, Landhäusser&Lieffers 2002, Frey et al. 2003, Wachowski et al. 2014). As a result, root NSC concentrations might be pivotal for the viability of suckers when they originate from greater soil depths or are slowed by physical barriers (Landhäusser et al. 2007,Renkema et al. 2009).

Finally, we observed that the presence of light is not a strict requirement for sucker formation, since etiolated suckers could be observed emerging from underground roots, as far as 15 cm from soil surface. However, we did observe that roots deprived of light formed a significantly lower number of suckers (Figure 3), indicating that light stimulates sucker initiation. Light conditions are known to influence meristem activity and their absence can lead to arrested growth of tomato shoot apical meristems due to the lack of photosynthetic energy production (Yoshida et al. 2011). Furthermore, light influences the formation of indole-3-acetic acid (IAA) in germinating *Arabidopsis* seedlings (Bhalerao et al. 2002), which may have a role in lateral root development and, perhaps, in root suckers as well.

4.3. Auxin is a determinant factor driving sucker formation

Given that root suckers in *A. lyrata*originate from buds that colocalize with lateral roots, and the lateral roots development has been previously

shown to be highly dependent on auxin metabolism (Blakely et al. 1982,Laskowski et al. 1995), we hypothesized that auxin could be also involved in driving sucker development. Indeed, we observed that IAA induced the formation of suckers, increasing the number of suckers per plant independently of the concentration used (Figure 4). These results are in line with the work of Eliasson et al. (1971), who showed that auxin produced in the growing shoot apex of aspen is the main factor in the control of both axillary bud growth and sucker formation in the roots. However, Farmer (1962) and Schier (1981) demonstrated that sucker initiation is reduced in root cuttings of Aspentreated with auxin, or stimulated by applying auxin inhibitors such as α-(p-chlorophenoxy)isobutyric acid (Schier 1975), which suggests that auxin may have alternative roles in driving sucker development in different species.The hormone auxin is a major player in driving cell proliferation, meristem growth and organ formation (Sassi&Vernoux 2013) and it can act locally in roots to trigger lateral root formation in a process associated with local auxin concentration maxima (Dolan et al. 1993,De Smet et al. 2007,Garay-Arroyo et al. 2012, Lavenus et al. 2013, Möller et al. 2017). Therefore, the auxin concentration maxima also trigger the meristematic root cells of *A. lyrata* to form suckers.

During *in vitro* shoot organogenesis of*Arabidopsis thaliana*, the endogenous auxin level of the explant has a significant influence on its regeneration capacity. Indeed, *Arabidopsis* lines or mutants with a high expression of the auxin biosynthetic *YUCCA* (*YUC*) genes have a high regeneration capacity even in protocols that bypass the auxin-supplemented callus-inducing medium (Zhao et al. 2001, 2013). In contrast, potato plants with a naturally high endogenous auxin concentration produce only calliin conventional regeneration protocols and require an anti-auxin for regeneration (Pal et al. 2012). Thus, screening novel auxins, auxin-like compounds, and/or auxin transport modulators for the efficient establishment of multiple auxin maxima might be a successful approach for improving regeneration efficiency; (Motte 2014) and finally the ability to produce root suckers.

Our results also indicate that polar auxin transport plays an important role during the root suckering process, given that auxin transport inhibitors

negatively affected root sucker formation. Wan et al (2006) also investigated the role of NPA in the control of suckering of 3-year old seedlings *Populustremuloides*. In this case, the authors applied NPA to the abraded periderm of the stem but thisdid not affect the number of root suckers or basal stem sprouts. However, application of NPA to the exposed xylem promoted the development of sucker buds and growth of root suckers, but did not influence basal stem sprouting. This suggests that polar auxin transport can have distinct role for different species.

4.4. STM is related to sucker formation

The gene expression studies in *A. lyrata*roots with and without suckers showed a correlation between *STM* expression androot sucker initiation. The *STM* gene encodes a homeodomain protein of the KNOTTED1 class and its mRNA accumulates in cells predicted to form the embryonic SAM (Long et al. 1996). *KNOTTED1-like homeobox* (KNOX) genes are thought to be involved in SAM formation and its maintenance; the *KNOTTED1* gene of maize and a related *Arabidopsis* gene, *KNAT1*, are expressed in undifferentiated cells in the meristem and downregulated from leaf primordia (Jackson et al. 1994, Lincoln et al. 1994,Long et al. 1996, Sato et al. 1996, Vollbrecht et al. 2000, Ohmori et al. 2013, Tsuda et al. 2011). Thus, *STM* is only expressed during shoot development and it is generally used as marker for shoot development during organogenesis (Cary et al. 2002). In *A. lyrata*roots, *STM* starts being expressed even before suckers can be visible, which means that this gene is a good candidate for the signalling pathways leading to sucker initiation and also that its expression can potentially be used as a marker for sucker formation.

Besides the STM-based KNOX pathway, which regulates meristem cell fate, preventing meristematic cells from adopting organ-specific cell fates, the WUS pathway is also requiredto modulate initial specification of stem cell identity in the central zone of the meristem (Kim et al. 2007,Zhang et al. 2014). Thus, the combined synergistic activities of these pathways are required for proper meristem organization and function.Distinctly from the KNOTTED1 class, *WUS* encodes a homeodomain protein of a distinct class

and is expressed in a group of cells underneath the stem cells of the SAM. WUS is postulated to affect stem cell fate in a non-cell-autonomous manner (Mayer et al. 1998). Studies on the biological function of plant HD proteins, designated *WUSCHEL HOMEOBOX* (*WOX*) proteins, have revealed that they are transcription factors and are involved in the regulation of various developmental processes (Lohmann et al. 2001,Haecker et al. 2004, Qu and Zhu 2006, van der Graaff et al. 2009, Zhang et al. 2010).Our search for a gene homologous to *WUS* in *A. lyrata*proved unfruitful, which may suggest that the *WUS* function is carried out by a different gene in this species. As an alternative, we tested the expression of its homologous genes *WOX1* and *WOX4*, becauseboth are involved in meristem maintenance (Zhang et al. 2011, Ohmori et al. 2013)

Interestingly, we observed a similar expression pattern of *WOX1* and *STM* (Figure 6).However,the expression of *WOX1* in roots with suckers was higher than that of roots without suckers in the 45d sample. This observation may be due to the fact that leaf primordia are already present in some root suckersat that stage andit has been previously reported that *WOX1* is expressed in initiating vascular primordia of the cotyledons during heart and torpedo embryo stages (Wu et al. 2005). Alternatively, it is possible that *WOX1* is expressed during SAM development in *A. lyrata*, but its expression starts at a later developmental stage, when compared to *STM*.

Finally, the *WOX4* gene of *A. lyrata* showed the opposite expression pattern of *WOX1* or *STM*: it was more strongly expressed in roots without visible suckers and its expression was reduced after sucker formation. This observation may have to do with the fact that WOX4 promotes proliferation of stem cells in the procambium/cambium in *A. thaliana* (Hirakawa et al. 2008, 2010, Ji et al. 2010), and could therefore be involved the early stages of secondary development of *A. lyrata* roots. Thus, once the meristematic cells differentiated, this may have led to a decrease in *WOX4* expression.

During lateral root development, when auxin accumulates in thepericycle cells, the root quiescent center marker WUSCHEL-RELATEDHOMEOBOX5 (WOX5) is expressed in the founder cells (Ditengouet al. 2008). However, we tested the WOX5 gene expression, but it has not shown any trend to correlate with sucker formation.

The shoot from root regeneration has been studied in *A. thaliana* and Shemer et al. (2015) has proved the role of CGH methylation regulating cell potency and competency to regenerate shoots from roots. Therefore, besides gene expression, methylation pattern of involved genes would be interesting.

5. CONCLUSIONS

Arabidopsis lyrata plants under *in vitro* conditions, with no plant growth regulators, seem to behavior similarly to plants on soil conditions, reproducing vegetativelythrough root suckers. The inner cells, probably related to pericycle, originate the new buds appeared to be sensitive tonutritive factors, light and to auxin, since they increase the number of root suckers.These observations reinforced the concept of the pericycle as an 'extended meristem' and the pluripotency of its cells.Findings from the current study can be implemented in basic studies of *de novo* shoot organogenesis and applied to *in vitro* propagation of different species. The evaluation of different SAM related genes, demonstrate that STM gene can be a marker to distinguish the proper cells responsible to sucker formation.*Arabidopsis lyrata* proved to be an excellent model for further studies using root suckers, for example to detect epigenetic markers through the several generations of *in vitro* culture.

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7. SUPPLEMENTARY FIGURES

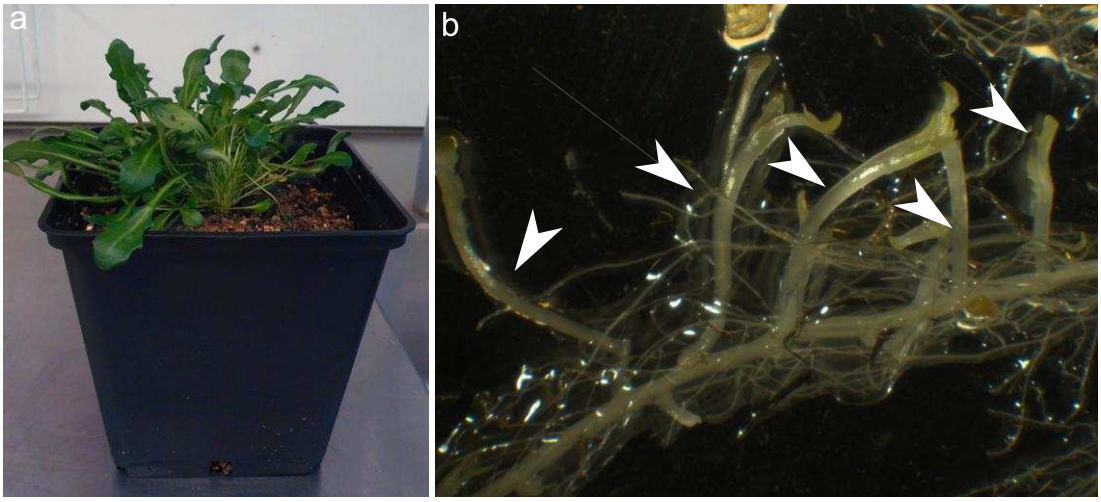

Figure S1*Arabidopsis lyrata* plant at 30 days after germination grown on soil. (a) whole plant overview, (b) etiolated root suckers (arrowheads).

Figure S2. Root suckers (clones) grown in (a) hormone free medium (control), (b) medium supplemented with100 µM L-KYN, (c) medium supplemented with 1 µM NPA, and (d) medium supplemented with 1 µM NPA + 2.5 µM IAA.