Plant Science 238 (2015) 251–261

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Competency for shoot regeneration from Arabidopsis root explants is regulated by DNA methylation

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A R T I C L E I N F O

Article history:
Received 6 May 2015
Received in revised form 8 June 2015
Accepted 14 June 2015
Available online 23 June 2015

Keywords:
WUSCHEL
Totipotency
Shoot organogenesis
Pericycle
Callus
Competence acquisition
Regeneration
cmt3

A B S T R A C T

Plants exhibit high capacity to regenerate in three alternative pathways: tissue repair, somatic embryogenesis and *de novo* organogenesis. For most plants, *de novo* organ initiation can be easily achieved in tissue culture by exposing explants to auxin and/or cytokinin, yet the competence to regenerate varies among species and within tissues from the same plant. In *Arabidopsis*, root explants incubated directly on cytokinin-rich shoot inducing medium (SIM-direct), are incapable of regenerating shoots, and a pre-incubation step on auxin-rich callus inducing medium (CIM) is required to acquire competency to regenerate on the SIM. However the mechanism underlying competency acquisition still remains elusive. Here we show that the *chromomethylase 3* (*cmt3*) mutant which exhibits significant reduction in CHG methylation, shows high capacity to regenerate on SIM-direct and that regeneration occurs *via* direct organogenesis. In WT, *WUSCHEL* (*WUS*) promoter, an essential gene for shoot formation, is highly methylated, and its expression on SIM requires pre-incubation on CIM. However, in *cmt3, WUS* expression induced by SIM-direct. We propose that pre-incubation on CIM is required for the re-activation of cell division. Following the transfer of roots to SIM, the intensive cell division activity continues, and in the presence of cytokinin leads to a dilution in DNA methylation that allows certain genes required for shoot regeneration to respond to SIM, thereby advancing shoot formation.

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1. Introduction

Plants are frequently subjected to herbivores and environmental effects leading to tissue damage, breakage and detached organs. To cope with injuries as well as to allow asexual reproduction, plants have developed a profound capacity to regenerate in three alternative pathways: tissue repair, somatic embryogenesis and *de novo* organogenesis, all of which are widely exploited in agriculture and plant biotechnology [1–4]. *De novo* organogenesis is the process through which wounded or detached organs or tissues form *de novo* meristems that further generate adventitious roots or shoots [5]. The regenerative potential varies within cells from the same plant and depends on their capability to respond to hormonal signals, their ability to enter the cell cycle and on their developmental potency. Plant cells that undergo endoreduplication which are considered to be irreversible differentiated are an example for the loss of the ability to renew cell cycle activity and therefore the capacity to regenerate [6,7]. For most plants, *de novo* organogenesis can easily be induced *in vitro* by culturing a detached organs or tissues explant on a medium supplemented with different hormones, most commonly auxin and cytokinine [3,8]. The nature of the newly formed organ is determined by the ratio between these two hormones [9]. A high auxin to cytokinin ratio promotes the formation of a proliferating mass of pluripotent cells termed callus, high auxin with no cytokinin induces root formation and high cytokinin to auxin ratio promotes shoot regeneration [10]. However, which organ will regenerate depends not only on the type and ratio of the applied exogenous hormones, but also on the potential of the explant cells to perceive or respond to these hormones [10,11].

Driven by a multitude of inputs, cells in multicellular organisms commit to their fate in a step-by-step differentiation process that is accompanied by epigenetic events to reinforce the commitment decisions [12,13]. These epigenetic changes include histone modifications, chromatin remodeling and DNA methylation, shutting off other developmental pathways to ensure that cell type specification will advance in one direction [13]. The epigenetic signature is stable and heritable through mitosis to allow lineage specific

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http://dx.doi.org/10.1016/j.plantsci.2015.06.015
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transcription profiles over many cell divisions, yet it can be reversible [14].

A prominent epigenetic modification is DNA methylation, which involves the addition of a methyl group to cytosine bases. This modification has a profound role in silencing transposable elements (TEs), regulating gene expression, guiding differentiation and controlling developmental potency [12,15,16]. In higher plants DNA methylation can occur in three sequence contexts: CG, CHG and CHH (where H is A, C or T), guided by distinct DNA methyltransferases (MTases) [15,17].

In many organisms, as embryonic cells differentiate, they gradually lose their totipotency, *i.e.*, the ability to give rise to all cell types that make up the body of the organism, and therefore they lose the capacity to regenerate the entire organism [18–20].

Plants however, maintain a source of pluripotent cells in structures called meristems as well as in tissue which maintains meristematic activity such as the pericycle, to allow continuous growth throughout their life cycle [21–24]. Under appropriate stimuli such cells can reactivate or increase their cell division rate leading to direct *de novo* organogenesis or to cell proliferation and the formation of pluripotent callus that can further regenerate.

In Arabidopsis, shoots can regenerate from root explants through a two-step process of pre-incubation on an auxin-rich callus induction medium (CIM) followed by culturing on a cytokinin-rich shoot induction medium (SIM) [25,26]. During the CIM pre-incubation stage, pericycle cells undergo cell divisions and acquire competence to respond to cytokinin signals and to form shoots [25]. Che et al. [25] showed that cell division activity is required for competency acquisition, and identified set of genes that are essential for *de novo* shoot formation, that are upregulated on SIM only if the root explants were incubated first on CIM [27–29]. Though, these authors have suggested that those genes are repressed by a process that can be unbound by pre-incubation on CIM [25], it is still unclear what is the nature of this repression, how it is released and what is the mechanism by which plant cells acquire competence to regenerate.

In this study we adopted the two-step regeneration system to study the involvement of DNA methylation in cell competency acquisition for regeneration. We show that incubation of root explants on CIM, drives pericycle cells to divide after 8 h and that cell division activity continued when explants were transferred to SIM. We demonstrate that root explants from mutants aberrant in specific DNA methylation context can regenerate shoots when cultured directly on SIM (SIM-direct), and that the shoot regenerates *via* direct organogenesis. We propose that re-activation of mitotic activity in WT root explants followed by culturing on SIM, advance cell division activity at the presence of cytokinin, which leads to a dilution in DNA methylation. This DNA re-programming allows certain genes that are required for shoot regeneration to respond to SIM and further promote shoot formation.

2. Materials and methods

2.1. Growth conditions and plant materials

The plant materials used in this study were: Arabidopsis accession Columbia-0 (Col-0), *met1-6*, *drm1xdrm2xcmt3-11* [30], *cmt3-11*, *cmt3-7* [31], (CS23895) and *CYCB1;1::GFP*, all of which were obtained from the Arabidopsis Biological Resource Center (ABRC). Seeds were surfacesterilized and sown on 20 cm × 20 cm plates containing Murashige and Skoog medium (Duchefa M0256). The plates were placed for 3 days at 4 °C and then transferred to a long day (16 h light/8 h dark) growth room at temperatures of 18–22 °C and placed vertically. The roots from 9-day-old seedlings were separated from the shoots and transferred directly to plates containing Gamborg's B5 medium (Sigma–Aldrich G5893) with 0.5 g/L MES (Sigma-Aldrich M2933), 20 g/L dextrose, 0.4% Phytagel (Sigma–Aldrich P8169) supplemented with either 0.1 mg/L kinetin (Sigma–Aldrich K3253) (0.5 mg/L 2,4-D for callus inducing medium (CIM) or with 0.894 mg/L 2ip (Sigma–Aldrich D7660) and 0.093 mg/L NAA (Sigma–Aldrich N1641) for shoot inducing medium (SIM). For the CIM–SIM treatment root explants were incubated for the designated time on CIM and then transferred to SIM.

2.2. Imaging and microscopy

Root explants images were captured using an Olympus SZX7 Stereomicroscope equipped with DP73 camera. Fluorescent images were captured using a Leica TCS SP8 confocal microscope. dsRED and GFP were viewed at excitation wavelengths of 552 nm and 488 nm, respectively. Fluorescence emission was collected at 580–620 nm for dsRED and between 500 and 530 nm bandpass for GFP. Live imaging video were captured using EVOS® FL Cell Imaging System equipped with CCD camera. Scanning electron microscopy was performed as described before [32] using a Hitachi 4700 scanning electron microscope.

2.3. RT-PCR and mRNA-seq analyses

Total RNA was isolated for mRNA-seq and RT-PCR analyses in the same way. For each sample 80 roots growing on 8 individual plates were pooled and total RNA was extracted using RNeasy Plant mini kit (Qiagen). For RT-PCR analysis cDNA synthesis was performed with the Invitrogen SuperScript II Reverse Transcriptase, using 1 μg of RNA. Primers used were as follows: *WUS*: F-TCGTGAGCGTCAGAAGAAGA, R-CCATCCTCCACCTACGTTGT with expected product of 352 bp for cDNA and 1045 bp for genomic DNA; *UBQ 21:* F-GCGCGACTGTTTAAAGAATACA; R-TGAACCCTCTCACATCACCA.

The specific primers used for the 14 tested genes for validation are listed in Supplemental material mRNA-seq analysis was done in a single replicate. mRNA was used to prepare libraries using TruSeq™-RNA and single-end sequencing was performed by multiplexing on Illumina HiSeq-2000 at the Technion Genome Center. Raw sequence reads (Fastq files) were quality assessed using FastQC software before and after trimming any remaining adapters using Cutadapt software. The filtered reads were then aligned to the indexed Arabidopsis TAIR10 reference genome using TopHat2, which was run with the −G option to specify known exon boundaries. The resulting alignment file (in BAM format) was converted to SAM format using Samtools software, and all the reads aligned to the chromosomal and mitochondrial genomes were filtered out. The resulting SAM file (containing only reads aligned to the nuclear genome) was converted again to BAM format and used as input for the cuffdiff program of the Cufflinks version 2.1.1 package, which was run with the −M option to discard read alignments to rRNA and tRNA genes. The resulting gene_exp.diff file was parsed to select genes that were differentially expressed between selected pairs of samples. The Fragments Per Kilobase of transcript per Million fragments mapped (FPKM) value for each gene in each genotype is presented in Supplemental Table S1.

2.4. Venn diagram and Gene Ontology analyses

Venn diagram was produced using Venny 2.0 at http://bioinfogp.cnb.csic.es/tools/venny/

Gene Ontology (GO) analysis on unique expressed genes was performed using the BiNGO 2.3 plugin tool version 3.2.0 [33]. Annotation data for *Arabidopsis thaliana* obtained from the Gene

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