Reproduction and genetic accuracy during somatic embryogenesis in *Larix sibirica*

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DOI 10.18699/ICG-PlantGen2019-44

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Abstract: The quality of proliferative embryogenic cultures (ECs) and the genetic changes associated with somaclonal variations in the cell lines (Cls) and cloned plants of *Larix sibirica* were studied. Cls were obtained from zygotic embryos on medium AI, supplemented by plant growth regulators: (2,4-D:6-BAP, 2:0,5 and 2:1). All Cls actively formed embryonal-suspensor mass (ESM), in which globular embryos propagated through cleavage, budding formation and proliferation of embryonic tubes of the suspensor. Cytogenetic studies of proliferating Cls of Siberian larch showed that the cells of young cell lines (aged 1–2 years) contained mainly cells with the normal number of chromosomes for this species ($2n = 24$). Analysis of long-cultivated (7–9 years) Cls showed that their majority were genetically unstable and only one (Cl6) was characterized as being stability. The genetic stability of this line was confirmed by a microsatellite analysis of nine microsatellite loci. Molecular genetic studies of proliferating Cls, conducted using RAPD analysis, allowed us to obtain diversified line-specific PCR spectra that can be used as markers of ECs. Somatic embryos matured on the nutrient medium AI with ABA (32 mg L^{-1}). The number of mature somatic embryos in different cell lines varied from 9 (Cl 16.19) up to 1220 (Cl4) per gram of fresh weight of ESM. Somatic embryos germinated on the medium AI without hormones and rooted (5–15 %). Stable maturation and germination of SEs was observed in Cl6. For 7 years, cloned trees have been growing at the station "Pogorelsky Bor" IF SB RAS successfully. Microsatellite analysis of clones showed their full compliance to Cl6.

Key words: molecular-genetic markers; somatic embryogenesis; *Larix sibirica.*

1. Introduction

To solve the problem of larch species propagation, a number of programmers for microcloning via somatic embryogenesis have been developed (Park, 2004, 2014). The genetic instability of larch species *in vitro* cultures as well as of other plant taxa is well known (von Aderkas, Anderson, 1993; Burg et al., 2007). Nevertheless, some studies indicate that during somatic embryogenesis in some Pinaceae species genetic variation was not detected and regenerant genetics was the same as in the original explant (Mo et al., 1989; Helmersson et al., 2004; Arrillaga et al., 2014; Cabezas et al., 2016; Ahn, Choi, 2017).

The aim of this paper is to study of the genetic variability of long-proliferating cell lines producing somatic embryos for decades and cloned trees.

2. Materials and methods

Larix sibirica cones were collected to mid-July 2008–2018 from open- and cross-pollinated mother tree A4 in the arboretum of the V.N. Sukachev Institute of Forests (Krasnoyarsk). Eight embryogenic cell lines were obtained from immature zygotic embryos at the pre-cotyledonary stages: Cl4 (2009), Cl6 (2011), Cl107 (2013), Cl16.28 (2015), Cl 18.3 (2018) by open pollination; Cl5 (2009), by cross-pollination between *L. sibirica* Ledeb. and *L. sukaczewii*; Cl 17.7 (2017), by cross-pollination between *L. sibirica* and *L. sibirica*.

Explants were placed on the AI medium (Tretyakova, 2012) with sucrose (30 g L^{-1}), 2 mg L^{-1} 2.4-D and 0.5 / 1 mg L^{-1} 6-BAP. For proliferation of the obtained ESM, the basic AI nutrient medium, containing 2,4-D (2 mg L^{-1}), 6-BAP (0.5 mg L^{-1}) and sucrose (20 g L^{-1}) was used. The culture was incubated in the darkness at 24 ± 1 °C.

Experiments with maturating somatic embryos were carried out using the AI basic medium, containing 40 g L^{-1} sucrose, 32 mg L^{-1} abscisic acid (ABA), 0.2 mg L^{-1} indolebutyric acid (IBA) and 10 % polyethyleneglycol (PEG 8000). Gelrite (4 g L^{-1}) was used as a gelling agent. For germination of somatic embryos, hormone-free AI basic medium was used. Plantlets were transferred into glass flasks containing sterile soil substrate (sand/vermiculate/peat; 1:1:1; volume to volume), moistened with one-fourth strength AI media. Cultivation was carried out in the growth chamber.

Cytogenetic analyses were carried out using globular SEs at the proliferation stage. The material was treated with 0.2 % colchicine, was fixed in ethanol–acetic acid mixture (3:1) and was stained in 1 % aceto-hematoxylin solution. The cover glass was placed on the sample, avoiding the formation of bubbles and examined on a MIKMED-6 microscope (LOMO, Russia).

To genotype the microsatellite loci, DNA was isolated from needles of maternal trees A4, ESM and needles of 16 cloning trees. DNA was extracted using the CTAB method (Devey et al., 1996). The quality and quantity of the material obtained were checked using a Qubit 2 fluorimeter (Invitrogen, USA). The isolated DNA was used for PCR with 9 pairs of oligonucleotides designed previously for the microsatel-

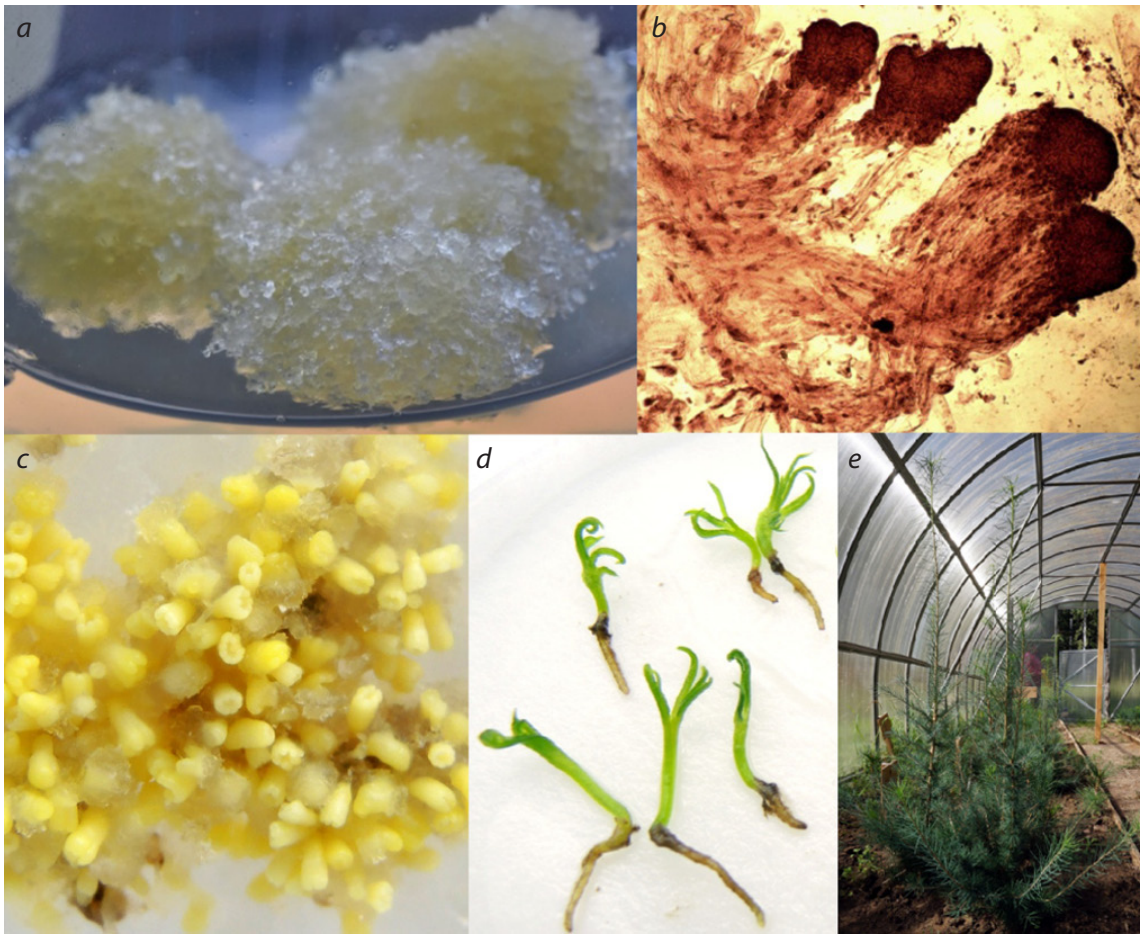

Figure 1. *Larix sibirica* Ledeb. plant regeneration via somatic embryogenesis: proliferating embryonal-suspensor mass (*a*), the multiplication of globular somatic embryos by cleavage (*b*), maturation of somatic embryos (*c*), plantlets derived via SE (*d*), cloned plants of *L. sibirica* in the greenhouse (*e*).

lite genotyping of different larch species (Krutovsky et al., 2014).

Statistical data analyses were carried out using standard techniques (Shmidt, 1984) by Microsoft Excel (Microsoft Corporation, USA) and STATISTICA 6.0 (Tulsa Scientific, USA). The reliability of the data obtained was assessed using one-way ANOVA test.

3. Results and discussion

Zygotic embryos of *Larix sibirica* from A4 genotypes obtained after open and cross-pollinations were introduced into the culture on the nutrient medium AI supplemented by growth regulators in 2009–2018. A cytological study demonstrated that ECs of larch included embryonal heads of globular somatic embryos and embryonal tubes (suspensors) (Figure 1, *a*, *b*). The number of globular SEs in the Cls tested in this study varied from 2040 (Cl6) to 4000 (Cl107) per gram fresh weight of ESM. Multiplication of somatic embryos has gone through the cleavage, budding of suspensor cells and suspensor protrusion.

Molecular genetic studies of proliferating Cls, conducted using RAPD analysis, allowed us to obtain diversified line-specific PCR spectra that can be used as markers of ECs. Microsatellite analysis performed on nine loci showed a weak genetic variability of Cls. Cytogenetic studies showed that younger cell lines (Cl17.7 and Cl16.28) have the somatic chromosome number typical of *Larix sibirica* ($2n = 24$). The analysis of long-cultivated Cls (Cl5, Cl4, Cl12 and Cl107) showed them cytogenetically instable and only Cl6 remained cytogenetically stable throughout 7 years of cultivation. Somatic embryos were allowed to mature on the AI media with ABA for 20–60 days.

After one week of maturation, somatic embryos of Cls separated from polyembryogenic complexes. Changes in anatomical structure were clearly visible after two to three weeks of maturation. Suspensors degenerated during maturation of somatic embryos at the cotyledonary stage of embryogenesis via the programmed cell death (PCD) mechanism. Somatic embryos having reached 1.1–1.5 mm in size and having a bipolar structure were considered mature (Figure 1, *c*). Different types of morphogenetic changes were observed during the maturation of somatic embryos. Well-developed somatic embryos were observed in Cl4; most of them (83.3 %) formed roots which stopped growing after 14 days. All embryos obtained from the maturation experiments have been used for germination tests. For 5 weeks, the regenerants elongated,

formed cotyledons, hypocotyls and roots (Figure 1, *d*). Plantlets without developmental deviations (5 to 15% of total Cls) were cultivated using a soil substrate in growth chambers. CL6 demonstrated stable maturation and germination of embryos (13 regenerants 1 g^{-1}, ESM).

CL6 cloned trees are growing successfully in the soil of the nursery of the Pogorelsky Bor of the Institute of Forest (Figure 1, *e*) during 7-years. These somatic plants are genetically stable and can be recommended as planting material for the plantation growing of larch in Siberia.

4. Conclusions

Our observations generally show that the obtained Cls of Siberian larch has been successfully proliferating on the AI medium for nine years. Microsatellite analysis of proliferating Cls showed their low variability. According to cytogenetic studies, only young (one- to two-year-old) cell lines were genetically stable. The genotyping of seven-year-old cloned larch trees showed full compliance with Cl6 from which they were obtained.

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Acknowledgements. The work is supported by the RFBR according to the research project No.18-54-00010 Bel_a.

Conflict of interest. The authors declare no conflict of interest.