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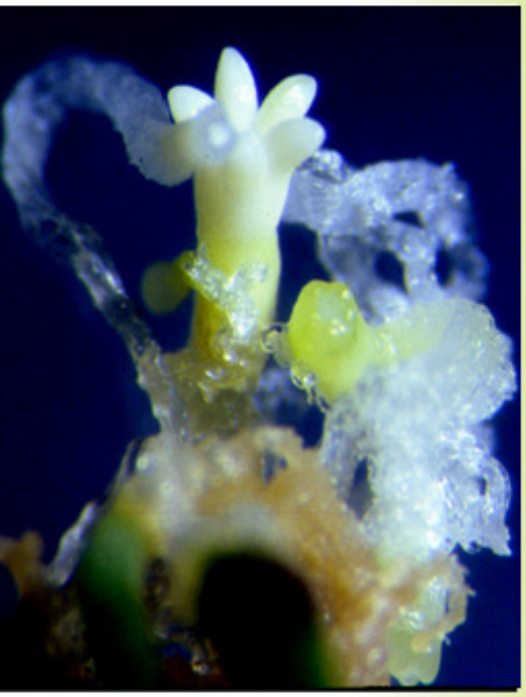

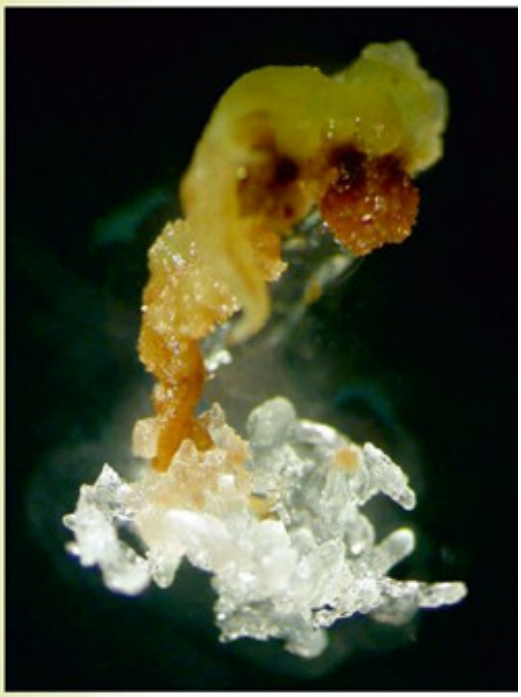

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Somatic embryogenesis as an effective regeneration support for reverse genetics in maritime pine: the Sustainpine collaborative project as a case study

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Background

Reverse genetics, defined as ectopic candidate gene expression or silencing, has become an indispensable tool for functional dissection of traits of interest in forest trees. In maritime pine as in other conifers, long generation time and long life span, high genetic loads as well as high genetic redundancy are major obstacles to perform standard genetic approaches including association genetics. Validating marker associations with specific properties before transferring into breeding selection models is still challenging. Stable *Agrobacterium*-mediated genetic transformation of maritime pine was first reported by Trontin *et al.* (2002) and developed in both France (FCBA, INRA) and Portugal (IBET) with sufficient refinement (reviewed in Trontin *et al.* 2007) to envisage practical application in reverse genetics as an attractive alternative to association studies. As a result, the technology has been recently implemented in French (GenoQB, 2006-2009) and transnational (Sustainpine, 2010-2013) or European initiatives (ProCoGen 2012-2016). Somatic embryogenesis (SE) was revealed as a key tissue culture system for achieving genetic transformation, easy cryopreservation of transgenic tissue and efficient transgenic plant regeneration in maritime pine. Much consideration is given in France, Portugal and Spain to apply SE as a clonal propagation system for maritime pine improvement and deployment strategies in multivarietal forestry (MVF, Park YS, reviewed in Klimaszewska *et al.* 2007). By using this technology it is expected that we will obtain greater genetic gain, high flexibility in deployment of tested embryogenic varieties and easy balancing of genetic gain and diversity in plantations. Both SE and *Agrobacterium*-mediated transformation methods developed at FCBA, INRA or IBET are being successfully transferred to different partners through running the Sustainpine project (http://www.scbi.uma.es/sustainpine/).

SE in model line PN519: effective transfer at different labs

Using published protocols from FCBA, INRA and IBET (Klimaszewska *et al.* 2007), most SE steps could be

completed by Sustainpine partners for the selected embryogenic line provided by FCBA (PN519). A 100% recovery and multiplication of embryogenic tissue was achieved from a shared FCBA cryopreserved stock. PN519 line maturation required only minor adaptations at different labs with yields established in the range 60-221 embryos per g fresh mass (f.m.) tissue (FCBA/INRA reference yield: 85 ± 16 embryos g^{-1} f.m.). Progressive loss of embryogenic ability as a function of line ageing (usually within 6 months propagation) could explain the lower yields obtained in some cases (< 20 embryos g^{-1} f.m.). Efficient handling of embryogenic line post-reactivation using a combination of adapted culture practices is a key point for successful regeneration of transgenic plants. Cotyledonary embryos could be converted into acclimatized plantlets at a rate of 31-60% (INRA/FCBA reference data: 35-45%).

Genetic transformation of PN519 with reference, control binary vector

pCbar, a binary vector provided by FCBA and derived from pCambia1301 (Roberts *et al.* 1998) and pAHC20 (Christensen and Quail 1996), was used as a reference to test for genetic transformation of PN519 at different labs. Considering published papers (reviewed in Trontin *et al.* 2007) and unpublished data, a common Sustainpine protocol based on phosphinothricin (PPT) selection was established by FCBA and INRA. Considering FCBA/INRA reference data for this line (89.6 ± 18.4 PCR-positive, PPT-resistant lines g^{-1} f.m), protocol transfer was considered effective for most partners with a transformation rate established in the range of 19.0-78.4 PCR-positive, PPT-resistant lines g^{-1} f.m. However it is still a tricky step that required technical adaptations at some labs. A key consideration for successful transgenic plant regeneration was to avoid PN519 line ageing and associated decrease in both transformation rate and embryogenic ability.

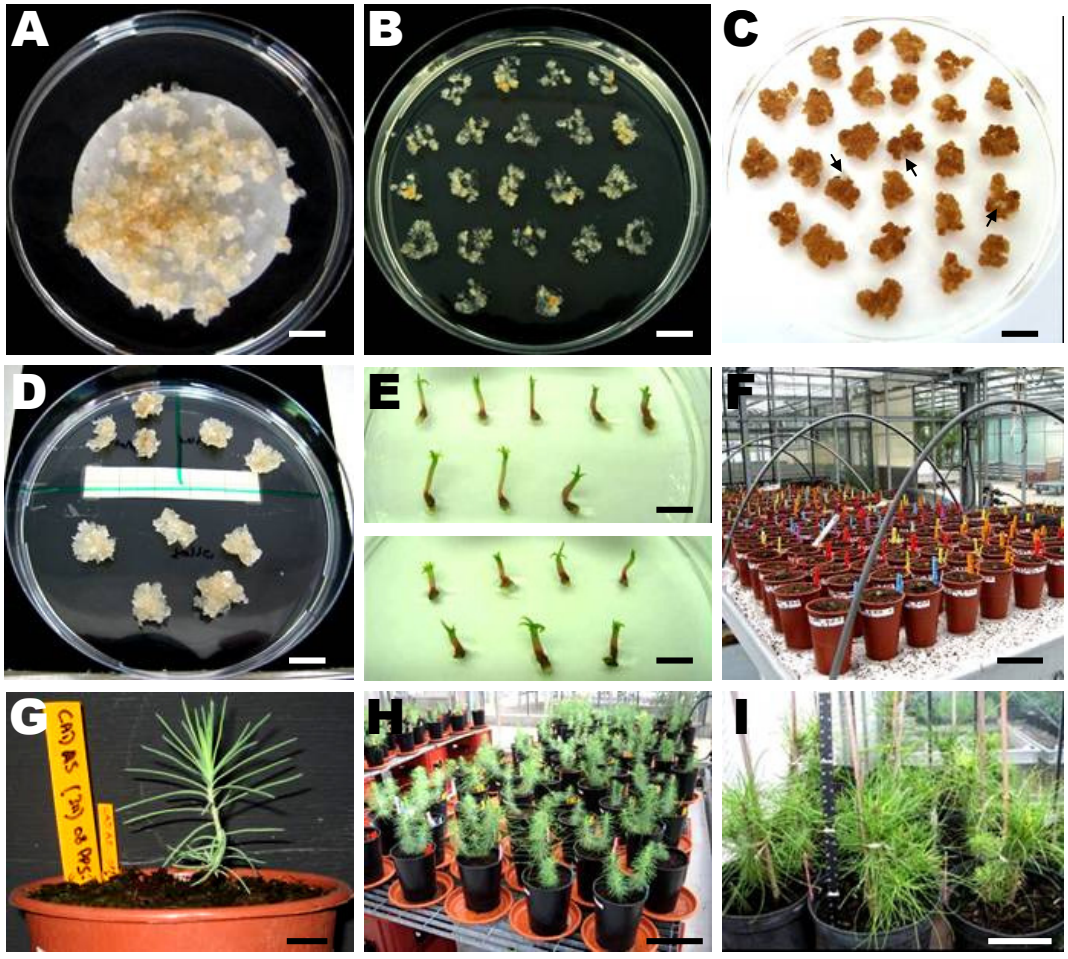

Fig. 1 *Agrobacterium-mediated genetic transformation and plant regeneration through somatic embryogenesis in maritime pine.* ***a*** *Cocultivated embryogenic tissue from line PN519 (1 week after cocultivation with Agrobacterium tumefaciens).****b*** *Small embryogenic cell clumps on selective medium (phosphinothricin) for 1 week.* ***c*** *Culture behaviour after selection for 7 weeks. Note the discreet phosphinothricin-resistant embryogenic whitish tissue (arrows) escaping from the surrounding, browning non-transformed cells.* ***d*** *Phosphinothricin-resistant embryogenic lines propagated on selection medium.* ***e*** *Germinating cotyledonary somatic embryos from transgenic (upper photo) and non-transformed control (lower photo).* ***f*** *Acclimatized plantlets in the greenhouse.* ***g*** *Young plantlet (2 months old post acclimatization) expressing an ihpRNA targeting the CAD gene.* ***h*** *One-year-old transgenic plants expressing ihpRNA constructs targeting the CAD, GRP, KOR or MYB14 gene.* ***i*** *Two-year-old transgenic plants overexpressing the GS1a gene. Bars = 1cm (a-e, g) or 10 cm (f, h, i). Picture origin: Sustainpine project (a-e), GenoQB project (f-h), FCBA/Univ. Málaga collaboration (i)*

Transformation rate of PN519 with selected overexpression and RNAi vectors

Ubiquitin promoter-based binary vectors developed by PSB/Gent University (Karimi *et al.* 2002) were selected for constitutive overexpression (OE, pMBb7Fm21GW-UBIL) or downregulation of gene expression through RNAi (ihpRNA: intron-spliced hairpin RNA strategy, pBb7GW-I-WG-UBIL). Standard protocols for maritime pine transgenesis using PPT as selective agent resulted in a quite low transformation rate (PCR-positive, PPT-resistant lines g^{-1} f.m.) for both the OE (4.0-8.0) and RNAi vector (3.0-8.7) as compared with reference pCbar. The number of transgenic lines produced in standard transformation experiment is sufficient to fulfil the requirement for biological repeats, i.e. 3-10 independent transgenic lines per construct. However validation of alternative, improved selection schemes is underway.

Conclusion

Previous expertise in maritime pine SE and genetic transformation (FCBA, INRA Orléans, IBET) has been transferred and is currently jointly developed with 3 other partners (Univ. of Alcalá, Univ. of València, Humboldt Univ. of Berlin). This transnational task force developed in the frame of Sustainpine (workpackage 3) is supporting one of the greatest efforts in conifers worldwide towards reverse genetics for functional candidate gene analysis (39 genes investigated). Biological outcomes in maritime pine will be facilitated by integrated analysis (collaboration between 9 Sustainpine partners) of new and previously created transgenic resources (cryopreserved embryogenic lines and somatic plants up to several years old).

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