



***In vivo* bud Transformation (ISSA)**

Preparation and inoculation of dormant lateral buds.

Introduction to ISSA protocols

Dissecting the molecular basis of wood formation is confounded by many factors including the variability of wood properties within and between stems, long generation times, out-crossing mating systems, late age expression of commercially important traits, high heterozygosity, high genetic load, seasonal dormancy, long mature trait establishment periods and the sheer physical size of mature trees.

Induced Somatic Sector Analysis (ISSA) has been developed to overcome some of these problems and to aid the medium to high throughput functional analysis of genes with a suspected role in wood formation and wood quality determination. ISSA allows for the simultaneous creation of hundreds of independently transformed wood sectors (e.g. 30 sectors per plant = 300 in a 10-plant experiment); within a short period of time (e.g. 2-6 months, depending on the trait under investigation); within the tree species of interest (poplar, eucalypts, pines or others); within the tissue of interest (vascular cambium, developing wood or bark); without genetic variation; without environmental variation and at a relatively low cost.

This method sheet is intended to provide some practical advice and insight into our experiences in developing and using this method that goes beyond the peer-reviewed published information.

Background

In attempts to increase the size of somatic tissue sectors (over the same time period, relative to the *in vivo* stem method) we undertook experiments where meristematic tissue in dormant lateral buds was accessed via cutting and inoculated with *A. tumefaciens*. To date this method has been successfully applied in *Populus* only as other genera such as *Eucalyptus* and *Acacia* have been trialled, but with limited to no success. The structural properties of dormant lateral buds dictate their applicability to these methods. For undertaking these protocols we suggest that large, robust dormant lateral buds be used to increase the likelihood of success.



General Information

Constructs

- It is vital for these protocols that a reporter gene is present to identify transgenic sectors. A selectable marker is not necessarily required to undertake ISSA protocols. To date we have focused our efforts on using the GUS reporter and histological assays have been adapted for this reporter system in stem tissue (see “Sample harvesting and GUS histological assay” information sheet). We have been using pCAMBIA vectors, in particular the pCAMBIA 1305.1 harboring the GUS plus reporter with which we have had much success. GUS might not necessarily be the best reporter as we have found that it can influence cell wall biochemistry (unpublished). We are currently investigating the utility of fluorescence reporters system.

A. tumefaciens

- To date we have only attempted transformations using *Agrobacterium*-mediated transformation protocols. For this work we have used the *A. tumefaciens* strain AGL1. Other strains are likely to work.

Protocol

1. *Agrobacterium* preparation

(you probably have your own methods which could also be used – see notes below)

- Grow single colony of *A. tumefaciens* in appropriate media containing appropriate antibiotics for between 48-72 hours.
- Dilute approx 1:25 bacteria culture in fresh warm media containing appropriate antibiotics.
- Grow bacteria to an optical density (OD 600 nm) of between 0.4 and 0.6.
- Centrifuge bacteria (15 min, 1000 g, 4° C).
- Re-suspend in room temperature basal MS (Murashige and Skoog) media (including glucose).
- Bacteria is ready for inoculations (inoculation media).

Notes

- We have been using MS but other complex plant media could be used. The type of media used for inoculation should provide a favorable environment for tissue in the bud.

- We typically dilute 2 ml of bacteria culture in 40 ml and after appropriate growth period (OD 0.4-0.6) centrifuge and re-suspend the bacterial pellet in 1 ml of MS. This gives us a highly concentrated inoculation medium that we have found does not affect shoot viability and is likely to increase transformation efficiency (it increased transformation efficiency in the *in vivo* stem method).

2. Preparation and inoculation of dormant lateral buds

- Select plants with large, robust dormant lateral buds (see note below).
- With a sharp scalpel blade (No11 have worked the best) cut longitudinally through the centre of a dormant lateral bud exposing the internal meristematic tissue (Figure 1). This should penetrate both the dormant lateral bud as well as all the leaf scar at the base all the way to the stem proper.
- Add enough inoculation media to wet the internal tissue. We have found that between 2-5 μ l is sufficient per bud.
- Wrap dormant lateral buds firmly in parafilm or equivalent by pushing the two halves together to prevent desiccation (this is why large robust buds are better).
- When shoots have broken through the protective covering and have begun to grow remove it and allow shoot to grow.
- After sufficient growth harvest shoots and perform GUS assay.

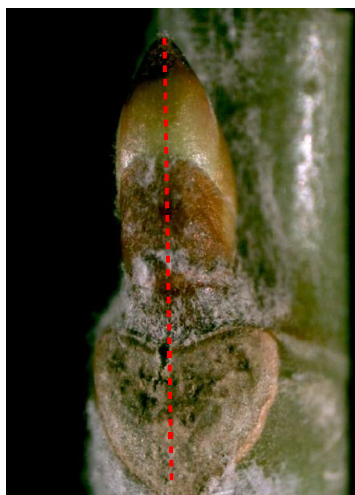


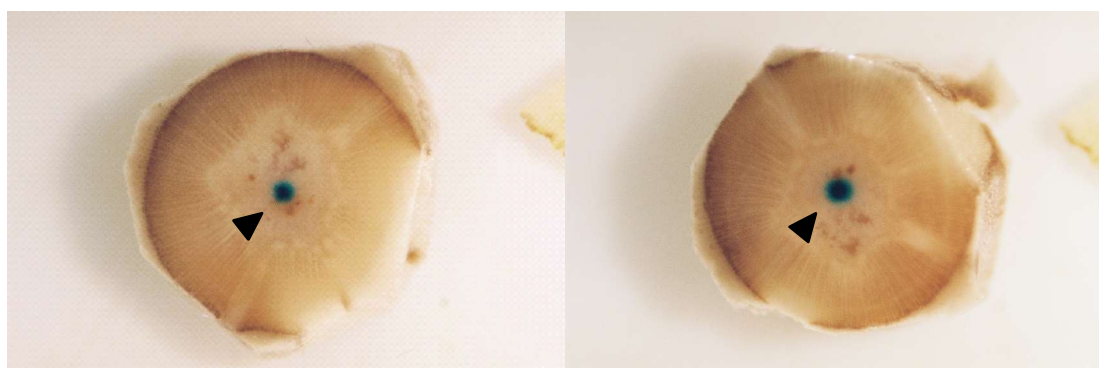
Figure 1: Line indicating the orientation of the cut that severs the dormant lateral bud.

Notes

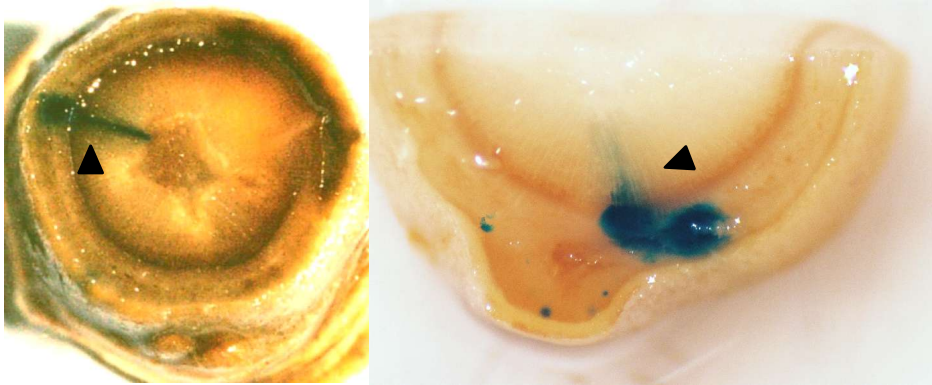
- We have found that transformation efficiency can be higher in dormant lateral buds that have just broken dormancy and are in the early stages of shoot development (i.e. bud beginning to expand but has not burst). To achieve this we subject plants to glasshouse conditions conducive to bud burst several days prior to inoculation.
- We also take growth measurements at the beginning and at harvest. Initially we measure the diameter of the stem @ 10 cm stem height, while at harvest we measure the stem diameter again, as well as individual shoot diameter at 1 cm from stem and the shoot length. We also note the number of dormant lateral buds inoculated and the number that develop into shoots.
- During experimentation we make a note of the number of deformed leaves on each individual shoot and look for evidence of stem scars on the shoot. These are the result of the cutting procedure.

3. Post harvest sector identification

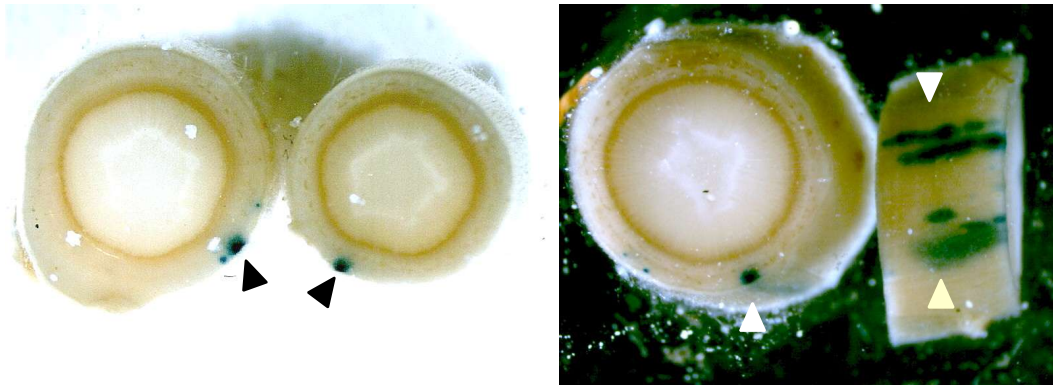
- After harvest and GUS assaying wood pieces are now ready for assessment under a microscope. The sector types are outlined below including brief descriptions. For further information regarding sector types and their definitions please see the material in the “references” section below.
 - **Pith sector** – A pith sector (PS) can be found specifically in pith parenchyma cells in the centre of the stem, appearing as globular or longitudinally elongated sector.



- **Cambial sector** – A cambium sector (CS) can be comprised of many cells types, all of which originate from cambial or procambial initials (including all secondary xylem and phloem derivatives). Transversely, such a sector appears as a wedge starting at the pith/xylem boundary and extending in the radial direction increasing in width to the cambium and then into the phloem.



- **Outer cortex sector** – Outer cortex sectors (OCS) occur in outer cortical tissue appearing as a group of cells extending to 10 mm longitudinally and to 1 mm tangentially.



Other experimental notes

- For any work involving functional gene testing ensure that you also inoculate separate dormant lateral buds with a blank vector (ie use the same vector but without the candidate gene) as a control. Differences in the frequencies of particular sector types could give insights into the effects of candidate genes on different stem tissue types, e.g. tissue specific lethality.



References

Spokevicius A.V. (2006) The use of induced somatic sectors for the elucidation of gene function and developmental patterns in xylogenic tissue. PhD Thesis, The University of Melbourne

You can find this online here; <http://eprints.infodiv.unimelb.edu.au/archive/00002300/>

Spokevicius A.V., Van Beveren K.S. and G. Bossinger (2006) *Agrobacterium*-mediated transformation of dormant lateral buds in poplar trees reveals developmental patterns in secondary stem tissue. *Functional Plant Biology*, 33, 133-139.

Further Reading

Spokevicius, A.V., Southerton, S., MacMillan, C.P., Qui, D., Gan, S., Tibbits, J.F.G, Moran, G.F and G. Bossinger (2007) β -tubulin affects cellulose microfibril orientation in plant secondary fibre cell walls. *The Plant Journal*, 51: 717-726.

Spokevicius, A.V., Tibbits, J.F.G. and G. Bossinger (2007) Whole plant and plant part transgenic approaches in the study of wood formation – benefits and limitations. *Transgenic Plant Journal*, 1: 49-59.

Van Beveren, K. S., Spokevicius, A. V., Tibbits, J., Wang, Q. and G. Bossinger (2006) Transformation of cambial tissue *in vivo* provides efficient means for Induced Somatic Sector Analysis (ISSA) and gene testing in stems of woody plants species. *Functional Plant Biology*, 33: 629-638.

Spokevicius, A.V., Van Beveren, K., Leitch, M. M. and G. Bossinger (2005) *Agrobacterium*-mediated *in vitro* transformation of wood-producing stem segments in eucalypts, *Plant Cell Reports*. 23: 617-624.

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