Improving the efficiency of embryogenesis in elite cotton cultivars

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Introduction

One step in the production of transgenic cotton is the regeneration of whole plants from undifferentiated cells (calli) in which the gene of interest has been inserted. The first stage is a crucial one and involves producing plant embryos from callus cells (somatic embryogenesis). The frequency of embryo formation is low and the process is poorly understood. Of the cultivars that can form embryos, most are no longer grown commercially (e.g., Coker). To introduce a gene into commercially important cultivars, the standard international practice is to transform a Coker cultivar, produce plants that are homozygous for the gene inserted and then enter these plants into a backcross breeding program (Wilkins *et al.* 2000). This process delays the commercial release of transgenic cotton by several years. A method that allows commercial cotton cultivars to be transformed and regenerated directly would be a significant advance in cotton biotechnology (Rajasekaran *et al.* 2001).

In the last decade, there has been compelling evidence that somatic embryogenesis in species such as carrot and spruce is promoted by a class of plant molecules known as arabinogalactan-proteins (AGPs) (Kreuger and van Holst 1993, Egertsdotter and von Arnold 1995, van Hengel *et al.* 2001). The mechanism is not understood. In preliminary experiments, we discovered several AGPs that are produced specifically by cotton cells undergoing somatic embryogenesis. The aim of our research is to identify these AGPs and to test whether they can improve the efficiency of embryogenesis in elite cotton cultivars.

Results and Discussion

AGPs present in embryogenic calli are more abundant than, and different to, those in non-embryogenic calli

We extracted AGPs from embryogenic and non-embryogenic Coker calli of the same age. The average yield of AGPs from embryogenic calli (8.8 mg/g freeze-dried calli)

was significantly greater than that from non-embryogenic calli (3.4 mg/g freeze-dried calli). This is consistent with what has been found in spruce and chicory (Egertsdotter and von Arnold 1995, Chapman *et al.* 2000). The AGPs were then analysed by reversed phase-high performance liquid chromatography (HPLC) (Figure 1). The profiles show that embryogenic calli AGPs consist of four major peaks, three of which are relatively hydrophobic whereas non-embryogenic calli AGPs comprise a single, relatively hydrophilic peak. In our experience, each peak is likely to consist of multiple AGP species.

This data shows that cotton cells undergoing somatic embryogenesis produce specific AGPs that are not produced by non-embryogenic cells and is consistent with the hypothesis that AGPs play a role in somatic embryogenesis.

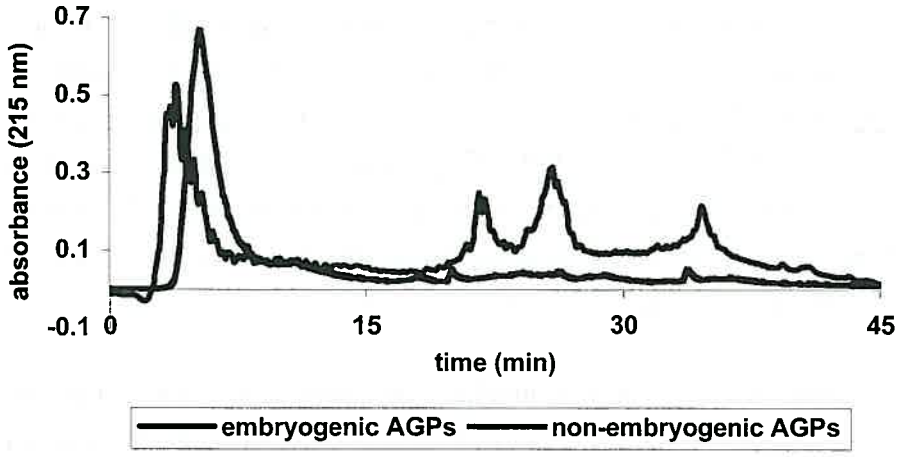

Figure 1. Reversed phase-HPLC profiles of AGPs extracted from embryogenic and non-embryogenic cotton calli (Coker 315). AGPs are detected by their absorbance at 215 nm.

Embryogenic AGPs promote embryogenesis in Coker 315

We have developed a bioassay to test whether AGPs extracted from embryogenic calli can promote embryogenesis when incorporated back into the growth medium. Briefly, this bioassay involves the growth of hypocotyls segments on standard media for five weeks. The explants are then transferred to fresh media with or without embryogenic AGPs that have been incorporated into it and grown for a further four weeks. At this stage, the number of explants that have produced embryogenic calli are tallied and all material is transferred again to fresh media of the same composition. Embryogenic calli is tallied again after a further two and four weeks.

A typical result from Coker 315 is shown in Figure 2. It shows that when embryogenic AGPs are present in the growth medium, an increase in the number of explant lines producing embryogenic calli results. These results have been repeated more than eight times and on each occasion, embryogenic AGPs have had a promotive effect on the amount of embryogenesis. Individual trials cannot be combined to give mean data due to variability in the amount of embryogenesis from trial-to-trial that occurs when explants are derived from different batches of seed or seed that is of different age. We have had experiments where the percentage of lines producing embryogenic calli has reached around 80 % in the presence of embryogenic AGPs.

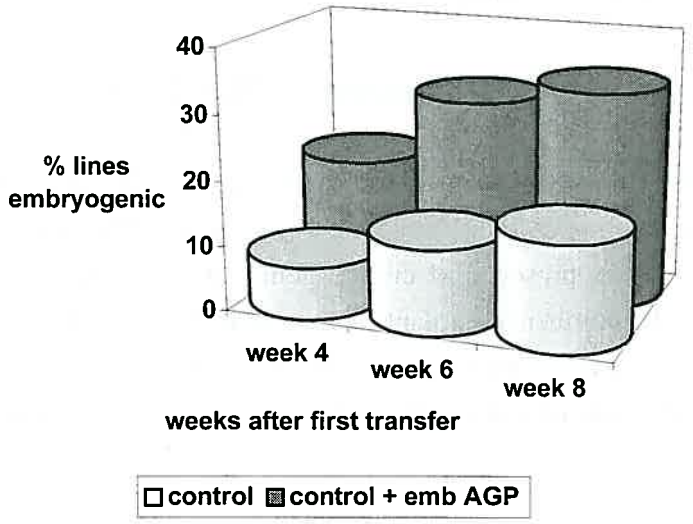

Figure 2. Typical bioassay results showing the effect of embryogenic AGPs on the rate of embryogenic calli formation in Coker 315. In this experiment, there were 102 independent explants in each group. The concentration of added embryogenic AGPs was 1 mg/L. After 8 weeks, the percentage of lines producing embryogenic calli does not increase.

We have also performed bioassays that show there is a dose dependent effect in adding embryogenic AGPs. A greater promotive effect occurs when the concentration of added embryogenic AGPs is increased from 1 to 2 mg/L.

We have also fractionated the embryogenic AGPs by reversed phase-HPLC and tested the resulting four fractions (Figure 1) individually. Preliminary results show that the most "active" AGP fraction is the first hydrophobic peak.

Embryogenic calli produced using added embryogenic AGPs in the growth media have been used to generate embryos that have been successfully germinated. These have resulted in numerous regenerated whole plants that are phenotypically normal and

fertile. This shows that embryogenic AGPs can be successfully used to promote the regeneration of whole cotton plants.

Cloning of genes encoding embryogenic AGPs

We have so far cloned two full-length genes encoding putative embryogenic AGPs by reverse-transcriptase PCR. These clones will be used to express individual AGP protein backbones recombinantly for testing in the bioassay, allowing us to determine whether they are the active, embryogenesis-promoting component. We also intend to express these genes constitutively in transgenic cotton to determine whether this improves the rate of somatic embryogenesis and therefore, the rate of regeneration.

Embryogenic AGPs promote embryogenesis in Siokra 1-4

We have tested whether embryogenic AGPs from Coker 315 calli can promote embryogenesis in the Australian cultivar, Siokra 1-4 (Figure 3). The results from three individual trials show that the presence of embryogenic AGPs in the growth medium results in an increase in the number of explant lines producing embryogenic calli. This shows that the promotive effects of embryogenic AGPs are not restricted to Coker 315. We are currently testing a range of other elite Australian cultivars in our bioassay.

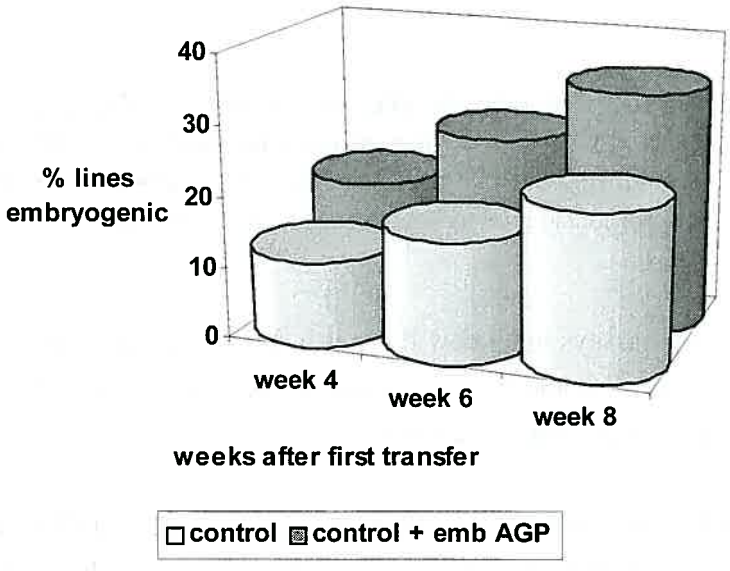

Figure 3. Typical bioassay results showing the effect of embryogenic AGPs on the rate of embryogenic calli formation in Siokra 1-4. In this experiment, there were 105 explants in each group. The concentration of added embryogenic AGPs was 2 mg/L. After 8 weeks, the percentage of lines producing embryogenic calli does not increase.

Conclusions

We have shown that arabinogalactan-proteins extracted from embryogenic cotton calli improve the efficiency of embryogenesis in Coker 315 and Siokra 1-4 when incorporated back into the growth medium. Therefore, the current regeneration protocols involving Coker can be improved by this technology and the direct transformation and regeneration of at least one Australian cultivar may be possible. Current work is focused on testing whether embryogenic AGPs can promote embryogenesis in other elite Australian cultivars and identifying the active AGP component(s).

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Acknowledgments

We would like to acknowledge Hexima Limited and the Cotton Research and Development Corporation for funding this project (HEX1C) and the University of Melbourne for providing the infrastructure for this project.