Stable transformation of *Opuntia ficus indica* callus cultures as evidenced by fluorescence of the tandem dimer Tomato gene

Peter Felker[1], Ron Bunch[1], John A. Tine[2], Guy R. Russo[2], Jean Gould[3], Marianne Arnold[3], Fang Wang[3], Ying Rong[4] and Martha Wright[5]

[1] D'Arrigo Bros, 21777 Harris Rd, Salinas, CA 93908.
[2] Center for Functional Genomics, University at Albany, SUNY, Health Sciences Campus, 1 Discovery Drive, Rensselaer, NY 12144.
[3] Department of Ecosystem Science and Management, Texas A&M University, College Station, TX 77843.
[4] Donald Danforth Plant Science Center, 975 N. Warson Rd. St. Louis, MO 63132.
[5] 7612 West 149th Street, Overland Park, KS 66223.

Corresponding author: peter_felker@hotmail.com

ABSTRACT

This communication provides a protocol for stable transformation of *Opuntia* callus cultures. It is a summary of ten years research from 2006 to 2016 of more than 340 experiments to obtain stable transformation and regeneration of five clones of *Opuntia ficus-indica*. Although regeneration was not achieved, stable transformation was achieved as evidenced by expression of a fluorescent marker gene six months after inoculation by soaking explants derived from unopened floral buds in *Agrobacterium tumefaciens* EHA 101. As cactus had too much auto fluorescence to permit use of even enhanced green fluorescent proteins, the fluorescent marker TdTomato with red/orange emission was used. To avoid consumer objections from antibiotic selective marker systems, the Phosphomannose Isomerase (PMI) gene that screens for growth on mannose was used. Explants that fluoresced grew well up to 10 g L^{-1} mannose, while explants that did not fluoresce senesced and died when cultured on 2 g L^{-1} mannose. The optimal basal media were found to be either Murashige-Skoog with 30 g L^{-1} sucrose, with either double the standard Ca concentration or Woody Plant media with an additional 2,000 mg L^{-1} KNO$_3$. Standard liquid shake, temporary immersion system or solid agar with 3 g L^{-1} gel rite was examined and the solid gel rite media was used. Previous reports that reported stable transformation of meristems by needle injection could not be repeated, possibly because those experiments were conducted with non-intron GUS that would have permitted the *Agrobacterium* to properly transcribe and splice transcripts for the *uidA* gene. Two independent reports of somatic embryogenesis Bouamama *et al.* (2011) and Gomez *et al.* (2006) in *Opuntia* were intensively examined, but could not be repeated. However smooth, green structures similar to "nodules" described by McGown *et al.* (1988) that can be induced to produce shoots were obtained but could not be induced to produce shoots. The hormone combinations that resulted in the greatest "structure" from immature floral explants were Zeatin (ZA) 0.2 to 0.75 mg L^{-1} with naphthalene acetic acid (NAA) 0.2 mg

L^{-1}, or Thidiazuron (TDZ) 0.75 with ZA 0.5 and NAA 0.4 mg L^{-1}, or metatopolin 1.5 with NAA 0.25 mg L^{-1}. Long-term culture on Picloram (PIC) resulted in cultures with a red tinge, thought to be stress-induced betalain production. However, the most promising hormone combination with (PIC) on floral explants was 0.01 TDZ/0.06 ZA/0.02 mg L^{-1} PIC. It is suggested that the most promising approaches to obtain shoots from these types of structures will come from transient or stable expression of the WUSCHEL and/or BABYBOOM regulatory genes in order to stimulate shoot development.

Keywords: Cactus, *de novo* shoot regeneration, somatic embryogenesis, transient *vs.* stable expression, fluorescent markers.

Abbreviations: BA-benzyladenine, GUS-β-glucuronidase, MS-Murashige Skoog, NAA - Naphthalene acetic acid, PIC-picloram, PMI-Phosphomannose Isomerase, TDZ-Thidiazuron, WP-Woody Plant Media, ZA- Zeatin.

INTRODUCTION

Opuntia ficus indica has the crassulacean acid metabolism (CAM) photosynthetic pathway that has several-fold greater conversion of water to dry matter than either the C_3 or C_4 photosynthetic pathways (Nobel, 1991, Han and Felker, 1997). *O. ficus indica* is a useful plant for arid lands whose fruits have a great diversity in color, size, brix, firmness, and spininess indicating great potential for breeding new combinations of these genes. The highly productive cladodes of some clones and species, both spiny and spineless, are used as livestock feed and as the young tender resprouts are used as a vegetable (i.e., "nopalitos") (FAO, 1995).

While improved progeny of controlled crosses have been reported by traditional crossing methods for cold hardy, spineless forage clones (Guevara *et al.*, 2011), and for high brix, high firmness fruits (Felker and Bunch, 2011), traditional sexual genetic improvement methods in *O. ficus indica* are complicated by the octoploid genome and the presence of apomixis (Mondragon, 2001). Multiple seedlings arise from the same seed due to apomixis, one of which is the zygotic embryo, while the apomictic seedlings are clonal copies of the female parent. After eliminating several of the smaller shoots from single germinated seeds, presumed to be the apomicts, five years later when it was possible to assess the phenotype from mature field plantings, more than 50% of the progeny in some crosses were deemed to be apomicts because of an identical phenotype to the female parent.

Thus, it would be very useful if novel genes could be inserted into cactus by either *Agrobacterium* or Biolistics™ techniques. A useful tool to gauge DNA insertion and transcription is the use of genes whose translation products can be detected using visible screening approaches. Some enzymatic markers can be observed after the tissue has been killed and stained, for example in the case of the colorimetric substrate of β-glucuronidase (GUS) activity (Vancanneyt *et al.*, 1990). Fortunately, nondestructive visual marker gene products such as fluorescent proteins are available that provide continuous evaluation of transcription (Chiu *et al.*, 1996; Shaner *et al.*, 2004).

There are two potential problems using visible reporter genes as indicators of genetic transformation. If *Agrobacterium tumefaciens* is used to insert the *uidA* gene into the plant, the blue stain could be the result of either transcription by the bacteria or of the plant. This possibility has been eliminated by insertion of an intron into the sequence for the *uidA* gene (Vancanneyt *et al.*, 1990). Thus, the plant can splice out the intron and transcribe the gene, but the bacteria cannot. If plant tissues have intensive blue stain after *Agrobacterium* transformation, and an intron was not used in the *uidA* gene, it is possible that stable plant genetic transformation did not occur.

The second major problem may arise due to the differences between transient expression and long term stable expression. Intense expression of inserted color genes may occur 1 to 4 days after *Agrobacterium* or Biolistics™ due to transcription of the inserted DNA sequences by ribosomes in the cytoplasm, without insertion of the target DNA into the nuclear DNA (Saika *et al.*, 2012). If the DNA sequences are transported from the cytoplasm across the nuclear membrane and inserted into the genomic DNA then the genes will be permanently incorporated into the genome and will be permanently expressed. If intense color genes are only observed 1 to 4 days after DNA inoculation but not after 28 days, most likely the expression only occurred in the cytoplasm and this is termed transient expression. If the color genes are observed more than 28 days or for several years, then transformation is termed long term stable incorporation.

Frequently the selection system for transgenic tissues involves growth on antibiotic media. This implies the final transgenic tissues will have resistance to antibiotics. Due to adverse consumer perspectives on tissues containing antibiotic resistance, the mannose selective system (Negrotto *et al.*, 2000) was used. The transgenic marker gene (i.e. Phosphomannose Isomerase (PMI)), converts mannose to glucose. Thus, the media contains mannose but not glucose or sucrose. If the plants are not transformed with the PMI gene, they cannot convert mannose to glucose and the plants will die. The license holder for PMI gene and associated pNOV 2819 plasmid driven by the Cestum yellow leaf curling virus promoter, is available without charge to university research groups from SYNGENTA Licensing Manager, Traits Licensing, Syngenta Crop Protection, LLC, 9 Davis Drive, Research Triangle Park, NC 27709.

Llamoca-Zarate *et al.* (1998) were the first to report genetic transformation of *Opuntia* in cell suspension cultures using Biolistics™ and the GUS reporter gene on callus culture derived from cotyledons and hypocotyls. The friable callus cultures turned brown after 30-40 days and therefore long term stable transformation was not achieved. The use of hypocotyls and cotyledons as starting material for the callus would not be possible for existing elite clones, which is a disadvantage. Somewhat later the same research group reported transient expression in *Opuntia* using the GUS reporter gene followed by the use of Biolistics™ on tissue cultured shoots (Cruz *et al.*, 2009).

Due to the lack of regeneration techniques from undifferentiated callus in *Opuntia* (that is most commonly used for transformation) direct transformation of apical meristems by

injection of *Agrobacterium* as reported by the patent of Smith *et al*. (1991) seemed a logical approach. In spite of their report of transformation in various species of monocots and dicots, their work has not been confirmed. I was injected tissue cultured explants following the Smith *et al.* (1992) procedure with the pNOV 2819 plasmid, which were grown on mannose and analyzed more than 700 explants with GUS activity staining and by PCR. One PCR marker included sequences of the *Agrobacterium* that did not exist in the plant to ensure expression was not due to *Agrobacterium* contamination. None of these 700 explants were transformed.

With the publication of Silos *et al*. (2006) that reported stable transformation of *Opuntia* using apical meristem injection, it was reinitiated research on direct transformation of meristems using *Agrobacterium* injection. After two years of intensive research it was not possible to repeat this work. Possibly the GUS staining activity in the Silos *et al*. (2006) publication was the result of the fact their plasmid did not contain intron-containing *uidA* reporter gene resulting in *Agrobacterium* transcription/translation of the GUS.

Possible spiny tops of shoots or root structures with root hairs.

Possible torpedo Somatic embryo

Figure 1. Shoot like structures that occurred once after pre-incubating shoots on 2 mg L^{-1} ZA, then needle injection of *Agrobacterium* into the shoot apical meristem, co-cultivating for 4 days on 2 mg ZA plus 2 mg PIC, then the 1.5 mm shoot apex dissected and placed on 5 g L^{-1} mannose for transgenic selection and 2 mg L^{-1} PIC.

It was attempted to duplicate the work Gomes *et al*. (2006) who reported regeneration of *Opuntia* from dissected apical meristems with the goal of achieving both genetic transformation and regeneration. The Gomes *et al*. (2006) method consisted of dissecting 1.5 mm meristems from the tissue culture shoot apices that were placed on 4 mg L^{-1} picloram on MS media in the dark at 25°C for 5-6 weeks prior to evaluation. Gomes *et al*. (2006) stated

that after somatic embryos formed they were placed on embryo maturation media consisting of 0.01 mg L^{-1} picloram + 0.5 mg L^{-1} BA at 25°C in with 16-hour photoperiods. Interesting structures such as seen in Figure 1 were observed when shoots were pre-incubated for 10 days on MS with 2 mg L^{-1} Zeatin and then needle inoculated, co-cultivated for 4 days on 1 or 2 mg L^{-1} Picloram + 2 mg L^{-1} Zeatin and then 1.5 mm explants dissected and placed on 1 or 2 mg L^{-1} Picloram plus clavamox + 5 g L^{-1} mannose in the dark. After approximately one year's research this method was abandoned.

Bouamama *et al.* (2011) reported somatic embryogenesis and regeneration from *Opuntia* using immature anthers as explants. This procedure employed immature anther explants placed in the dark for four weeks on Chee and Poul basal nutrient media with 2.0 mg L^{-1} 24D and 2.5 mg L^{-1} thidiazuron (TDZ) and then sub-cultured in the light. They transferred what they reported as 3 mm somatic embryos to the light on hormone free media with charcoal to get shoots to develop somatic embryos. It was possible to obtain structures similar to Bouamana's (see Figure 3a, b, c and d) but it was not possible to generate shoots from our cultures after two years research. Figure 3e appears to be shoots derived from preexisting axillary meristems.

This communication reports constructs and non-destructive fluorescent methods to continuously monitor transient and stable transformation, inoculation methods to obtain stable transformation of callus, and results of non-successful hormonal trials to obtain regeneration from nodules or callus.

MATERIALS AND METHODS

Five *Opuntia ficus indica* spineless clones with orange fruit denoted 1-27-24, purple fruit denoted 1-21-27, pink fruit denoted 1-12-19, green fruit denoted 1-29-21 and red fruit denoted Comm were used in this study. The basal salt media used was either woody plant with an additional 2,000 mg L^{-1} KNO$_3$ or full strength Murashige Skoog (MS) with double the Ca concentration in full strength MS.

Immature pistil/filament explants were used as shown in Figure 2 essentially as described by Gonzalez-Melindi *et al.* (2005). In grape somatic embryogenesis, the union between the filaments and the pistil produced the most somatic embryogenesis (Dennis Gray, personal communication) and therefore this sector was also used for cactus.

The method for the stable transformation was as follows. Approximately 2 mm long pistil/filament segments were placed on Woody Plant Media with 15 g L^{-1} sucrose, 3 g L^{-1} gel rite with 2.0 mg L^{-1} 24 D and 2.5 mg L^{-1} TDZ. From one to 3 weeks after placing the explants on the media, callus approximately 5 mm in diameter was placed into 2 mL tubes and inoculated by soaking the explants in 800 µl of 0.25 OD *Agrobacterium* for 30 minutes. After 30 minutes the solutions were removed by blotting with filter paper. The calli were co-cultured on the filter paper for 1 hour, then washed twice with ½ MS and transferred to the rest media (no mannose) for 1 week, then 2 weeks on low selection media (1 g L^{-1} mannose), and then

on Woody Plant media with 21 g sucrose, 0.3 mg L^{-1} picloram PIC, 0.2 mg L^{-1} TDZ, 0.35 mg L^{-1} Zeatin and 10 g L^{-1} mannose. The time after placement on culture media before *Agrobacterium* inoculation was examined from 3 weeks to 3 months and 0–3 weeks produced the most transformation.
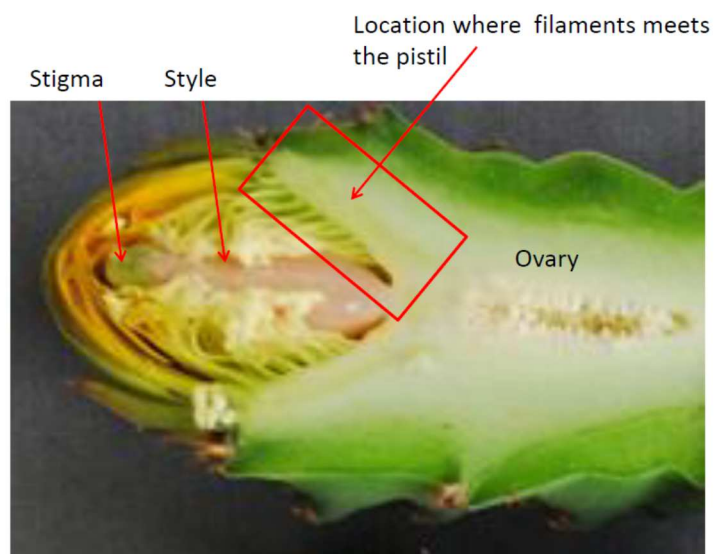

Figure 2. Longitudinal section of immature *Opuntia* floral buds that were "Stage 6" according to Bouamama *et al.* (2011) and Gonzalez-Melindi *et al.* (2005). The explants used to repeat Bouamana's results and the remaining experiments in this communication were taken from the region where the filaments attach to the pistil.

For these experiments, the *Agrobacterium tumefaciens* strain EHA101 with the pNOV plasmid 2819 was used. As can be seen in Figure 3, we modified the pNOV 2819 plasmid using the CMP promoter to drive both dTomato gene and the PMI gene. Also, note that backbone contains an additional *VirG* gene that provides increased virulence leading to greater incorporation of this gene.

The protocol for the preparation of the *Agrobacterium* was as follows. Two days before inoculation, an *Agrobacterium* culture was grown overnight in Luria Broth (LB) media plus antibiotics at 200 rpm at 25-27°C. The antibiotics used for the *Agrobacterium* cultivation with EHA101: CMP-dTomato were Kanamycin 50 mg L^{-1} + Spectomycin 100 mg L^{-1}. One day before inoculation the culture from the 2-day culture was spun down and the culture resuspended in pre-induction media (i.e. ¼ Murashige-Skoog (MS) media with 20 g L^{-1} glucose, 20 mg L^{-1} acetosyringone in 75 mM MES buffer pH 5.4) with antibiotics. This was incubated overnight at 200 rpm at 25-27°C. On the day of inoculation, the *Agrobacterium* was spun down and resuspended in Vir Induction Media (VIM) (glucose 20 g L^{-1}, Acetosyringone 20 mg L^{-1} in a 75 mM MES buffer pH 5.4). Before inoculation the detergent Silwet L-77 was added to achieve a 0.01% solution and mixed well. The first small fluorescent spots were obtained about 30 days after inoculation.
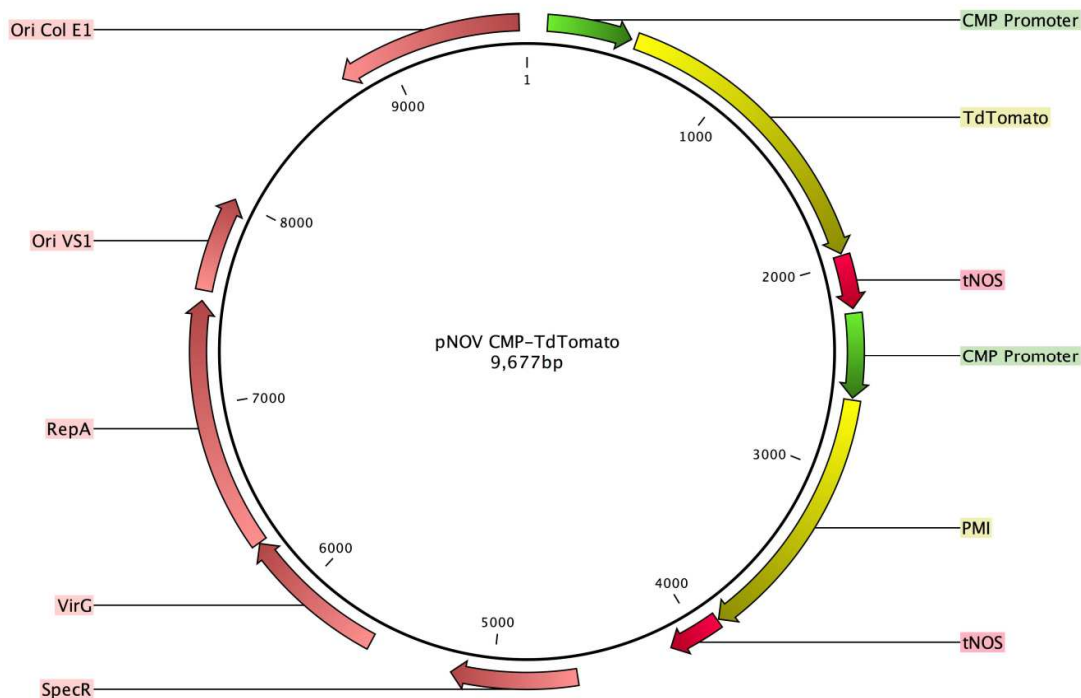
Figure 3. pNOV2819 plasmid modified to include the fluorescent tdTomato gene as well as the PMI gene. Both genes were driven by the same CMP promoter. Note the extra *VirG* gene at position 6,000, which provides additional virulence for infection and plasmid transfer.

Fluorescence assay methods

Initially it was used the GUS staining method to detect transformation from *Agrobacterium,* but as this was a destructive test, the inoculated tissues could not be followed continuously. It was initially tried a Green Fluorescent Protein and the improved Green Fluorescent Protein reported by Chiu *et al*. (1996). However, cactus produced too much background fluorescence to permit use of these proteins for critical evaluations. Therefore, it was evaluated the new generation of fluorescent proteins reviewed by Shaner *et al*. (2004) and selected the tdTomato fluorescent protein because it had longer wavelengths (554 nm excitation and 581 emission) outside of the problematic cactus auto fluorescence region and because tdTomato was the brightest of these new fluorescence proteins. The Texas A&M fluorescent microscope Zeiss, Model: Stemi SV11 Stereomicroscope used a Chroma cube filter set # 49010 (https://www.chroma.com/products/sets/49010-et-r-b-phycoerythrin-morange-mko), that excites in a very narrow range (546/10) with a dichroic at 560LPXR and an emission at 585/40.

To permit routine lab screening for transgenic shoots at the whole plate level, an inexpensive (< USD $4,000) tdTomato monitoring system was developed using a black and white CCD camera, three 40-watt LED green lights, a narrow bandwidth interference filter for the CCD

camera and cutoff filters for the green excitation filters. The details of this system can be obtained from the senior author.

RESULTS

An example of tdTomato transient expression from needle injected *Agrobacterium* into a cactus shoot at the right and a non-injected shoot at the left shows the potential to examine at the whole plate level can be seen in Figure 4.
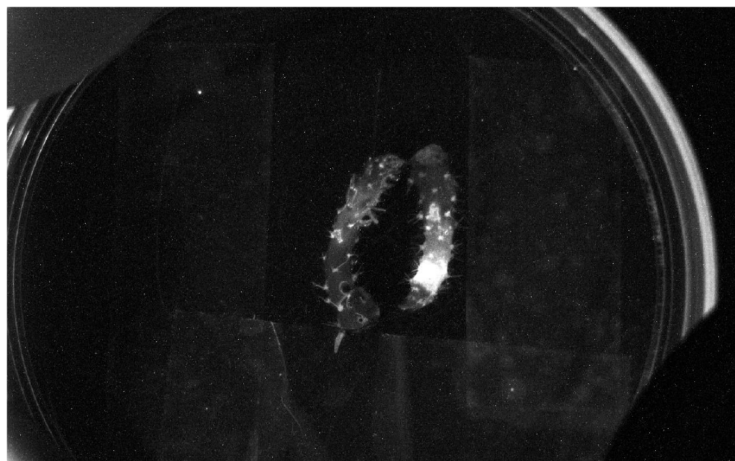

Figure 4. Example of "homemade" fluorescent system to visualize tdTomato fluorescence at the whole plate level. The shoot at the right was inoculated and the shoot on the left was a non-inoculated control. This photograph had a 3.97 second exposure with a gain of 40.

As stable meristem incorporation was not achieved, the hypothesis was considered that perhaps the CMP promoter was not functional in the meristem. For this reason, it was compared meristem-specific promoter of Shirasawa-Seo *et al*. (2005) to the strong potato ubiquitin promoter bul427 of Rockwell *et al*. (2008), the 35 S promoter and the CMPS promoter by ligating them to GUS and using Biolistics™ to shoot them into the apical meristem region of shoots. The result was that the CMP promoter of the pNOV plasmid had the strongest meristem activity of all the promoters tested and therefore lack of promoter activity was not responsible for the failure of our plasmid DNA from being stably incorporated into cactus shoot meristems.

Figure 5. Needle injected *Agrobacterium* strain EHA 101 with pNOV 2819 plasmid with the CMPS promoter driving both the PMI gene and the tdTomato fluorescence gene 4 days after inoculation. The fluorescence completely disappeared after 2 weeks and was thus transient and not stable transformation. The figure on the right was a 15 second exposure.

Several years of intensive research to obtain stable transformation by needle injection of *Agrobacterium* following the patent of Smith *et al.* (1992) and the paper of Silos *et al.* (2006), first using GUS and then tdTomato as marker eventually led to method that yielded intensive fluorescence in the tips of young shoots as seen in Figure 5. This required a 10 day pre-culture of tissue cultured shoots on MS media containing 2 mg L^{-1} ZA. However none of these shoots continued to fluoresce and it was deemed that tdTomato gene was never transported across the nuclear membrane and stably incorporated into the plants double stranded DNA.

An example of long-term stable transformation is shown in Figure 6 for variety 1-27-24. Such fluorescence was obtained for all five varieties for several years. The plate on the right was photographed with a conventional camera and the plate on the left photographed with homemade fluorescence system. These calli were inoculated on 10/5/2012, grown on 2.0 mg L^{-1} 2,4-D + 2.5 mg L^{-1} TDZ + sucrose 10 g L^{-1} + Vancomycin 250 mg L^{-1} + mannose 5 g L^{-1} + 3 g L^{-1} gel rite and photographed approximately 6 months later on 5/8/13. As non-inoculated controls do not fluoresce and as non-fluorescing calli eventually died on 2 g L^{-1} mannose, but strongly fluorescent calli were grown for more than 6 months on 10 g L^{-1} mannose, it is reasonable to believe that the calli were stably transformed.

Frequently sectors of explants that have been on 10 g L^{-1} mannose for many months are not fluorescent (such as the top right explant in Figure 6). Snapp (2009) reported that the half-life of many fluorescent proteins is on the order of 24 hours. Although the PMI gene and the fluorescence gene are driven in tandem by the same CMPS promoter, perhaps in 2-year-old

slow growing cultures there is enough low-level transcription of the PMI gene present to prevent mannose toxicity, but not enough transcription to visualize the fluorescent protein.
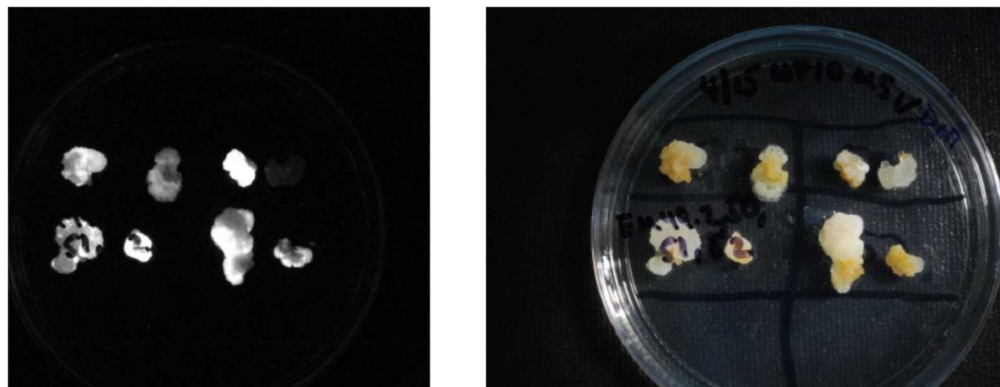

Figure 6. Callus from clone 1-27-24. These calli were inoculated on 10/5/2012, grown 2.0 mg L^{-1} 2,4-D + 2.5 mg L^{-1} TDZ + sucrose 10 g L^{-1} + Vancomycin 250 mg L^{-1} + mannose 5 g L^{-1} + 3 g L^{-1} gel rite and photographed approximately 6 months later on 5/8/2013. The photo on the right was taken with a conventional camera and the photograph on the left with the "home made" fluorescent set up.

A mannose concentration of 5 g L^{-1} was sufficient to kill non-fluorescent (i.e. non transformed) cactus and cactus grew very well on 10 g L^{-1} mannose. In the pursuit of somatic embryos following Bouamana's procedure with 2.0 mg L^{-1} 2,4-D combined with 2.5 mg L^{-1} TDZ it was observed green, round, smooth, globular structures that would not germinate with standard somatic embryo hormone media (Figure 7). Merkle, who viewed the structures, suggested that were not somatic embryos, but rather nodules first described by McGown *et al.* (1988).

Nodules differ from somatic embryos. While they cannot form a root and shoot, with correct hormone treatments, they can be induced to produce shoots. Cross sections of these nodules (Figure 8) revealed dividing centers of tracheid like cells, but not a typical shoot meristem. Kim *et al.* (1999) of Merkle's group were able to obtain regeneration and transformation of sweet gum from nodules using liquid culture, but not solid culture. As Scherer *et al.* (2013) obtained rapid propagation from nodules of another CAM plant, pineapple using the Temporary Immersion System; this seemed a logical approach for use with *Opuntia*. It was tried the previously reported somatic embryogenesis on agar, traditional liquid shake culture and in the Temporary Immersion Systems, but none of the round green structures germinated to form shoots.

A literature search of hormones to obtain shoots from nodules found that either a cytokinin such as BA or Zeatin was used in the range of 0.2 to 4 mg L^{-1}, in combination with an auxin, almost always NAA, in the range of 0.05 to 0.4 g L^{-1}. These cytokinin/NAA combinations were sometimes supplemented with TDZ. Various trials on cactus found that Zeatin was a stronger

stimulus for morphogenesis in cacti than benzyladenine or 2 isopentenyl adenine (2iP) and, therefore, we routinely used ZA instead of BA. Sixteen factorial combinations of either NAA/Zeatin or TDZ/NAA/Zeatin were examined numerous times with the goal to obtain shoot emergence from nodules, albeit without success. At the very end of this project due to a recent report (Chauhan and Taylor, 2016) that the cytokinin metatopolin stimulated *de novo* shoot production from a wide range of recalcitrant species, this cytokinin was compared to ZA.
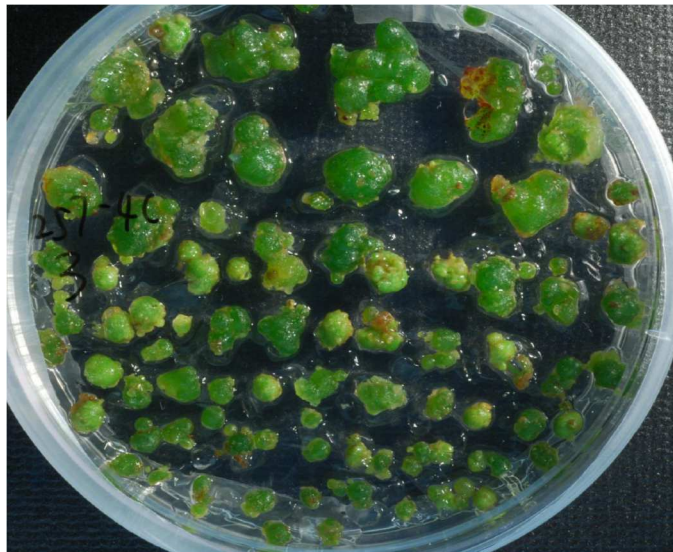
Figure 7. Example of nodules cultured from floral explants the first 4 weeks on 0.5 mg L^{-1} 24D, with 0.5 mg L^{-1} Benzyladenine and then 1 mg L^{-1} NAA with 5 mg L^{-1} TDZ for the next 64 weeks.
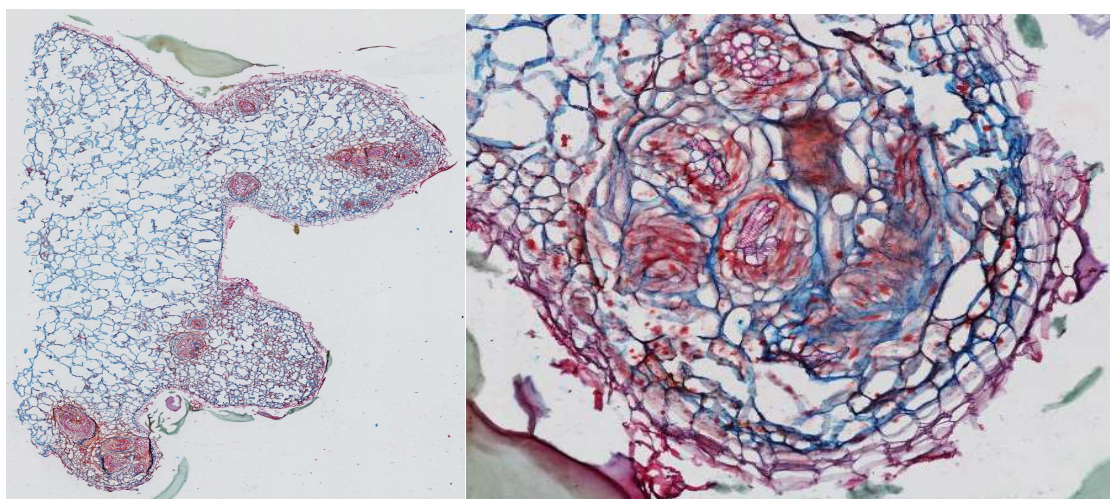

Figure 8. (a) Section of developing nodule. (b) Dividing tracheid cells in a possible pre-meristematic region.

There were very marked behaviors in the hormonal response of the five clones tested, including the response to Picloram. The green 1-29-21 clone seemed to possess considerable endogenous auxins resulting in stimulation of large amounts of amorphous growth, while in contrast the purple clone 1-21-27 produced very little amorphous growth in low auxin media and required higher concentrations of cytokinins or auxins for development. An example of those response differences is shown in Figure 9.

Figure 9. Comparison of structure *versus* amorphous growth for clone 1-29-21 which is in the top of both plates to 1-21-27 at bottom of each plate at 24 weeks in culture on MS media with double Ca. The hormones for the top plate were PIC/0.1, TDZ 0.1, ZA/0.125 mg L^{-1} while the hormones for the bottom plate were PIC/0.075, TDZ/0.025, ZA/0.06 mg L^{-1}. Note clone 1-29-21 has extensive amorphous growth in the top plate while the 1-21-27 has good structure with high hormones. In contrast at the lower plate clone 1-29-21 has good structure without amorphous growth while clone 1-29-21 does not have sufficient hormones for development.

At the highest TDZ and auxin concentrations smooth roundish "meganodules" in the terminology of McGown *et al.* (1988) were observed, but these never germinated and usually

senesced and died within a year of culture. The lower the hormone concentrations tested, the greater were the number of small round dividing structures. Examples of both TDZ/NAA/ZA and only NAA/ZA are shown in Figures 10-11. Only results for the green clone 1-29-21 are shown as it was the most responsive.
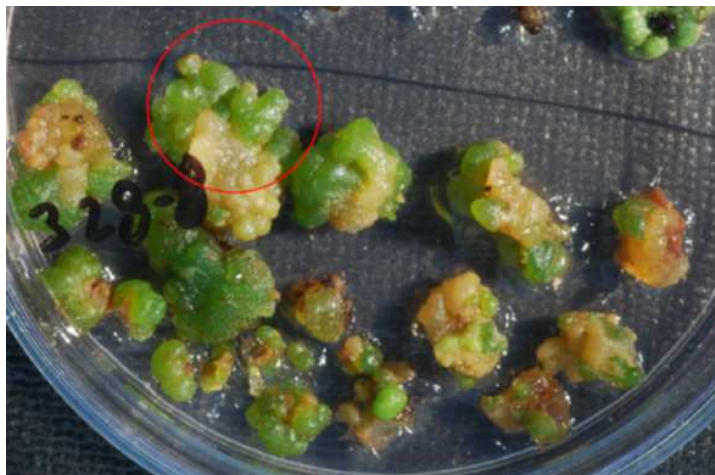

Figure 10. Example of optimum structured callus using TDZ with ZAA and NAA. The 24-week-old clone 1-29-21 floral explants were grown on MS with 30 g L^{-1} sucrose and TDZ 0.75, ZA 0.5 and NAA 0.4 mg L^{-1}. The zone circled in red appeared most promising.
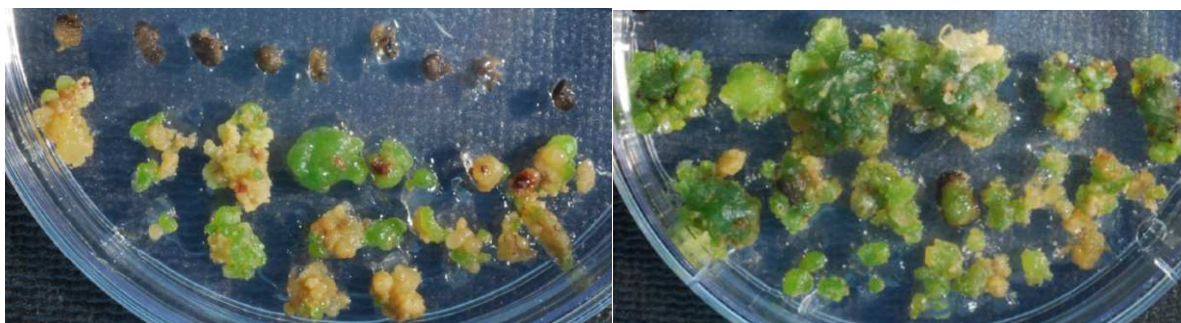

Figure 11. Example of structured callus with clone 1-29-21 with only Zeatin and NAA after 44 weeks after culture on MS with 30 g L^{-1} Sucrose. The top panel is ZA (0.75 mg L^{-1}) and NAA (0.2 mg L^{-1}) and the lower panel is ZA (0.20 mg L^{-1}) and NAA (0.25 mg L^{-1}).

In the very last phases of this research, the Cytokinin metatopolin was compared to Zeatin and as can be seen in Figure 12, metatopolin appears to stimulate at least as much morphogenesis as Zeatin.
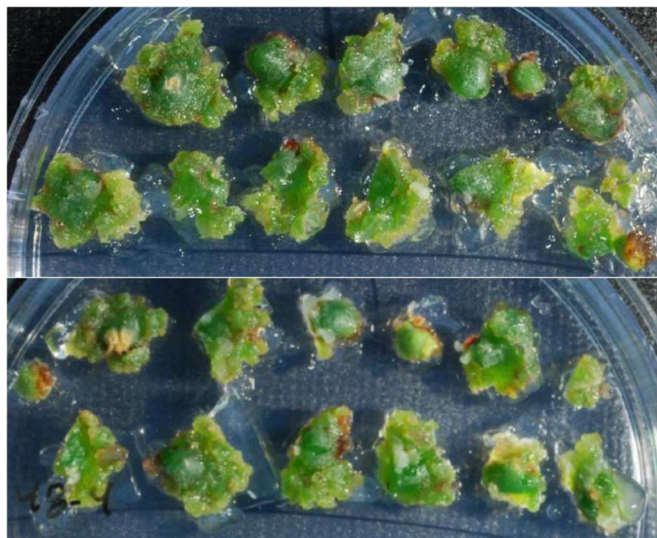
Figure 12. Example of structured callus at short times after explant initiation (8 weeks) comparing ZA vs. metatopolin with clone 1-29-21 on MS. The optimal Zeatin treatment (0.5 mg L^{-1}) is in the upper panel and the optimum metatopolin (1.5 mg L^{-1}) is in the lower panel. Both contained NAA at 0.25 mg L^{-1}.

DISCUSSION

Despite more than 350 experiments over a 10-year period, some lasting more than two years, stable incorporation of the tdTomato gene was accomplished, but regeneration from callus was not. The initial grids started at 0.2 to 4.0 mg L^{-1} auxin with 0.05 to 4.0 mg L^{-1} NAA and over the course of the years were gradually shifted to lower and lower concentrations as these gave the most structured callus. Perhaps some of the lowest hormone concentrations will ultimately be successful in achieving regeneration. Alternatively, perhaps mitochondrial biomarkers that have been shown to be strongly related to stress induced somatic embryogenesis may be helpful (Arnholdt-Schmitt *et al.*, 2016).

However, of all the possible techniques to stimulate shoot formation in recalcitrant species, perhaps the most promising is the use of shoot inducing regulatory genes such as BABYBOOM or WUSCHEL that when expressed in the plant can simulate undifferentiated callus to form shoots. Based on the pioneering patent by Bruce *et al.* (2005) that used WUSCHEL to stimulate shoots, DuPont-Pioneer has developed protocols to permit development of transformed and regenerated maize plants from seeds to plants with tassels in less than 70 days (Lowe *et al.*, 2016). Cao *et al.* (2015) have recently reviewed the involvement of WUSCHEL in shoot apical meristem formation. Similarly, Florez *et al.* (2015) have recently used BABYBOOM to enhance somatic embryogenesis in cacao. Gordon-Kamm (2016 per. comm.) has found that sometimes these regulatory genes can stimulate shoot development via transient expression produced by Biolistics™ or needle injection without the need to stably incorporate these genes in the tissue, thus greatly reducing

approving from regulatory agencies. Another alternative to obtain transient expression would be to insert the WUSCHEL or BABYBOOM genes in the triple block protein of a Potex virus as described by Baulcomb *et al.* (1995). Sanches *et al.* (2015) reported 4 sequences of Potex viruses from symptomless plants that's should be ideal vectors for WUSCHEL transient expression. When transient expression using Biolistics™ or needle injection is used (Gordon-Kamm, 2016 pers. comm.), recommended that a strong promoter be used. It was prepared inserted BABYBOOM and WUSCHEL in separate pNOV2819 plasmids that are driven by the strong CMPS promoter. If approval is obtained by the SYNGENTA licensing office, these constructs are available from John Cushman at University of Nevada Reno.

ACKNOWLEDGEMENTS

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