Research Article

Methods for suspension culture, protoplast extraction, and transformation of high-biomass yielding perennial grass *Arundo donax*

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Abbreviations: **PEG**, polyethylene glycol; **CIM**, callus induction media; **2,4-D**, 2, 4-dichlorophenoxyacetic acid; **BAP**, benzylaminopurine ; **PCV**, packed cell volume

Abstract

Arundo donax L. is a promising biofuel feedstock in the Mediterranean region. Despite considerable interest in its genetic improvement, *Arundo* tissue culture and transformation remains arduous. We developed methodologies for cell- and tissue culture and genetic engineering in *Arundo.* A media screen was conducted, and a suspension culture was established using callus induced from stem axillary bud explants. DBAP medium, containing 9 µM 2,4-D and 4.4 µM BAP, was found to be the most effective medium among those tested for inducing cell suspension cultures, which resulted in a 5-fold increase in tissue mass over 14 days. In contrast, CIM medium containing 13 µM 2,4-D, resulted in just a 1.4-fold increase in mass over the same period. Optimized suspension cultures were superior to previously-described solidified medium-based callus culture methods for tissue mass increase. Suspension cultures proved to be very effective for subsequent protoplast isolation. Protoplast electroporation resulted in a 3.3±1.5 % transformation efficiency. A dual fluorescent reporter gene vector enabled the direct comparison of the CAMV 35S promoter with the switchgrass ubi2 promoter in single cells of *Arundo.* The switchgrass ubi2 promoter resulted in noticeably higher reporter gene expression compared with that conferred by the 35S promoter in *Arundo*.

1 Introduction

In recent years there has been significant interest in harvesting renewable fuels and value-added chemicals from plant feedstocks, especially from perennial grasses such as *Arundo donax* L. [1,2,3]. Some reasons why perennial grasses are desirable as next-generation bioenergy feedstock are: 1) their low soil management requirements and growth on marginal land; 2) their ability to prevent soil erosion and increase soil organic carbon; 3) few natural pests; and 4) the capacity to reliably produce biomass [4]. In the U.S., switchgrass *(Panicum virgatum*) is a widely adapted biomass feedstock whereas in Europe, *Arundo* may fulfill an important role as a perennial grass feedstock [5,6]. Compared with switchgrass, *Arundo* has a much higher photosynthetic capacity, despite being a C3 plant [7,8], leading to the production of significant biomass [9]. In addition, *Arundo* is salt tolerant, and has been used for phytoremediation of soils contaminated with heavy metals [10,11]. Further, the cell wall composition of *Arundo* [12,13], along with thermochemical conversion into biofuel [14], has been characterized. The lignin present in the stems and leaves has a HGS-type structure, but is primarily composed of G subunits. Delignified stems have a highly heterogenous population of hemicelluloses in terms of sugar composition, molecular weight, and structure [13].

Despite the potential of *Arundo* as a bioenergy feedstock in Southern Europe [1], there are very few publications on tissue culture and transformation of this species. At present, *Agrobacterium*-mediated transformation of *Arundo* has not been demonstrated and limited success has been achieved using biolistics to transiently transform *Arundo* callus cultures [15]. A transient transformation protocol could provide crucial insight into gene expression [16], promoter screening [17], protein-protein interactions [18], transcriptional regulatory networks [19], and protein subcellular localization [20], as well as providing a necessary first step for the development of a stable transformation protocol for crop improvement [21]. As such, the goal of this work was to develop an *in vitro* culture system for *Arundo* to rapidly generate axenic protoplasts for subsequent transformation and analysis.

2 Materials and methods

2.1 Induction and maintenance of *Arundo* callus

Callus was induced using the protocol reported by Takahashi et al., [22] with some modification. Briefly, stem explants of *A. donax* were cut into approximately 30 cm long segments and surface sterilized with 70% (v/v) ethanol for 30 s and then transferred to a 4% (v/v) sodium hypochlorite solution, with one drop of Tween 20 (Sigma Aldrich, St. Louis, MO, USA), for 10 min prior to washing 3 times in sterilized distilled water. The cane internodes were then excised and placed on a basal Murashige and Skoog (MS) medium [23] supplemented with 0.05% Plant Preservative Mixture (PPM) (Plant Cell Technology, Washington, DC, USA) and 0.3% (w/v) Gelzan (Sigma Aldrich, St. Louis, MO, USA). The excised internodes were placed in a growth chamber at 25^{o}C under standard cool white fluorescent tubes with a flux rate of 35 µmol $s^{-1}m^{-2}$ and a 16-h photoperiod. Excised internodes were transferred onto CIM medium, MS medium supplemented with 3% sucrose (Fisher Scientific, Fair Lawn, NJ, USA), 9 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.7 g/L casein hydrolysate, 0.5 g/L L-glutamine, 1 g/L L-proline, and 0.4% Gelzan. These were placed in a growth chamber in the dark at 25°C for a month to induce calli. Once induced, the calli were maintained on the same medium, with 13 µM 2,4-D [22].

2.2 Establishment and maintenance of *Arundo* suspension cultures

Cell suspension cultures were initiated from 10 month old embryogenic callus by adding 500 mg of calli to a 125 ml Erlenmeyer flask containing 25 ml of MS or B5 medium supplemented with 3% sucrose and various hormones. In order to test the effect of different media on initiation of suspension cultures, the hormone type and concentration were varied, along with the basal media as follows. DBAP medium was composed of MS salts, B5 vitamins, 9 µM 2,4-D and 4.4 µM 6-benzylaminopurine (BAP) (Sigma Aldrich, St. Louis, MO, USA) [24]. MSDP media was composed of MS salts and vitamins supplemented with 9 µM 2,4-D and 100 nM phytosulfokine-α (PSK-α) (PolyPeptide Group, Torrance CA, USA) [25]. B5G medium was composed of B5 salts and vitamins [26] supplemented with 0.7 g/L glutamine (Sigma Aldrich, St. Louis, MO, USA) [27]. After transfer of the calli to flasks, cultures were incubated in the dark at 28°C for 14 days on a gyratory

shaker at 80 rpm. Cultures were then filtered through a 100 µm Fisherbrand™ Cell Strainer (Fisher Scientific, Fair Lawn, NJ, USA), brought up to a volume of 25 ml with fresh medium, and transferred to a 125 ml flask. Three flasks, initiated separately from 3 different calli, were used for each medium and represented 3 independent biological replicates. Five milliliters of fresh medium was added to the cultures every 7 days for 6-8 weeks to compensate liquid evaporation. At the end of 8 weeks, cultures were considered to be established, as indicated by the presence of large cell clusters [29, 29] and the growth was assayed for another 21 days by measuring the packed cell volume (PCV) of the cultures [30]. Briefly, 5 ml of culture was transferred to a 15 ml conical centrifuge tube (Corning Incorporated) and allowed to settle for 10 minutes, at which point the PCV was measured. At the end of the experiments, the cultures were used to generate protoplasts for subsequent experiments.

2.3 Tissue culture compared with cell suspension culture

In order to determine the best strategy for rapidly attaining large amounts of *Arundo* tissue, we tested four culture conditions, with three independent replicates for each condition, all of which were initiated with 500 mg (fresh weight) of callus or cells. Treatment 1 (liquid DBAP to liquid DBAP) was initiated with 500 mg of an established suspension culture added to 25 ml of DBAP medium in an Erlenmeyer flask. Each replicate was initiated from a different flask of suspension cell culture. Treatment 2 (liquid DBAP to solid CIM) was initiated the same as Treatment 1, with the exception of the established suspension culture being added to solid CIM media. Treatment 3 (solid CIM to liquid DBAP) was initiated from callus grown on solid CIM media transferred directly into 25 ml of DBAP medium in an Erlenmeyer flask. Finally, Treatment 4 (solid CIM to solid CIM) was initiated from callus grown on solid CIM media transferred to solid CIM media. To determine the tissue mass at 1, 3, 5, 7, 10, 14, 18, and 21 days, the suspension cultures were filtered through a 70 µm Fisherbrand™ Cell Strainer (Fisher Scientific, Fair Lawn, NJ, USA) and the contents weighed, accounting for the mass of the cell strainer, and then returned to the culture. The mass of the calli grown on solid media, at the same time points, was measured by transferring calli to a Petri dish and taking fresh weight.

2.4 Protoplast isolation from suspension cell culture

Protoplasts of *Arundo* were isolated using a previously established method for isolation of protoplasts from switchgrass suspension cultures [31] with slight modifications. In addition to using switchgrass as a control, tobacco (*Nicotiana tabacum* L.) cv. Bright Yellow 2 (BY-2) suspension culture, cultured as previously described, was used as a positive control to ensure functionality of the enzymes [31,32,33]. Briefly, a 10 ml PCV of *Arundo* suspension culture collected during the exponential growth phase was used. Cell walls were digested with 20 ml of a solution (0.6 M mannitol, 10 mM MES pH 5, 1 mM $CaCl_2$, 0.1% BSA, 5 mM 2-mercaptoethanol) containing food grade enzymes (Rohament CL 15820 ECU, Rohapect 10L 10080 ADJU, and Rohapect UF 0.078 ADJU) (AB Enzymes, Darmstadt, Germany) was added to the cells and incubated at 37°C, for 2-3 hours with gentle shaking. After incubation, the solution was filtered through a 40 µm Fisherbrand™ cell strainer to remove cellular debris and centrifuged at 150 g for 10 min. Five volumes of W5 wash solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7) [34,35] was then added to the protoplasts. After resuspension, protoplasts were imaged using an Olympus IX 81 inverted microscope and counted using a hemocytometer. Protoplast diameter was measured using the ImageJ software package (US National Institutes of Health, Bethesda, Maryland, USA) calibrated with an ocular micrometer. The size distribution of the protoplasts was generated by grouping protoplasts in different size classes [36].

2.5 Vector construction

Vector pTD-PvUbi2-35S was derived from pANIC10A [37] after several modifications. First, a unique restriction site, *Mlu*I, was inserted between the switchgrass polyubiquitin 1+3 (*PvUbi1+3*) promoter and the orange fluorescent protein, *pporRFP*. Since *PvUbi2* is a stronger promoter compared to *PvUbi1+3* [38], the former was PCR amplified from pANIC10A and used to replace *PvUbi1+3* using *Psp*XI and *Mlu*I-HF restriction enzymes. To compare the strength of the constitutive cauliflower mosaic virus *35S* promoter relative to *PvUbi2* in *A. donax*, a *35S-mGFP5ER-nos* cassette was PCR amplified from pBIN m-gfp5-ER [39] using the forward primer 5'-

taatctgcttggtaaccagattagccttttcaat-3' and reverse primer 5'-ttacttgttggcgcgcctcccgatctagtaacataga-3' (restriction sites are underlined) and inserted in pANIC10A using the restriction enzymes *Bst*EII and *Asc*I. The primers used for PCR amplification were designed in SnapGene and synthesized by Integrated DNA Technologies (Coralville, IA, USA). PCR was performed using Platinum *Taq* DNA polymerase (ThermoFisher Scientific, Waltham, MA, USA). To improve the efficiency of protoplast transformation, the size of the original vector was reduced by deleting unneeded sequences including 1) a 3,837 bp fragment encompassing 85% of the total length of ZmUbi1-Gateway-compatible ccdB cassette-OCS, using *Mfe*I, 2) a 3,405 bp sequence containing DNA required for *Agrobacterium*-mediated transformation, which included the right T-DNA border, the stability region, and 652 bp of the origin of replication, using *Fsp*I, and 3) a 2,977 bp sequence spanning *PvUbi2* promoter and hygromycin selectable marker, using *Pac*I and *Psp*XI. All PCR amplification and restriction digestion products were gel purified using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and quantified on a NanoDrop-1000 spectrophotometer (ThermoFisher) prior to cloning. Following *Pac*I and *Psp*XI digestion, the vector was blunt-ended and 5' phosphorylated using the Quick Blunting Kit (New England Biolabs, Ipswich, MA, USA). All ligation reactions were carried out using T4 DNA Ligase (New England Biolabs). Ligated constructs were transformed into either “One Shot ccdB survival 2 T1R” chemically competent cells or “One Shot Mach1 T1 phage-resistant” chemically competent cells (ThermoFisher) as per the manufacturer’s instructions and sequence confirmed. The final vector, pTD-PvUbi2-35S contains 8,690 bp (**Figure 1**) and features two independent fluorescent protein cassettes: the orange fluorescent protein *pporRFP* under the control of *PvUbi2* promoter and the green fluorescent protein *mGFP5-ER* under the control of CAMV *35S* promoter.

2.6 Polyethylene glycol (PEG)-mediated transformation

PEG-mediated DNA transformation was performed as previously described [35,21], with switchgrass protoplasts serving as a positive contol, with slight modifications. Protoplasts of *Arundo* were resuspended in MMg (0.4 M mannitol, 25–150 mM $MgCl_2$, 4 mM MES (pH 5.7)) at a concentration of 1×10^6 protoplasts/ml. Ten micrograms of pANIC10A-GFPstuffer [38] plasmid DNA

was then mixed with 200 µl of protoplasts (approximately 2×10^5 protoplasts). A 0–40 % PEG solution (0.6 M mannitol, 100 mM $CaCl_2$, 0–50 % PEG 4000 (Sigma–Aldrich, St. Louis, Missouri, USA) was added to the protoplasts to a final PEG concentration of 0, 10 or 20 %. After 20 min of incubation at room temperature (RT), protoplasts were washed twice with 1 and 4 ml of W5 and collected by centrifugation at 100 × g for 6 min. Protoplasts were then resuspended in 1 mL of WI (0.6 M mannitol, 4 mM KCl, 4 mM MES, pH 5.7), transferred to a 12-well plate (Corning Incorporated, Corning, NY, USA) and incubated at 28 °C in the dark for 20 h. As a positive control, switchgrass mesophyll protoplasts were transformed using PEG as previously described [31]. The transformation efficiency (%) was calculated by counting the number of protoplasts expressing pporRFP divided by the total number of protoplasts using a hemocytometer and multiplied by 100 as shown in the formula below:

$$\left(\frac{\#\ protoplasts\ expressing\ pporRFP}{total\ \#\ protoplasts}\right)\times 100 = \%\ transformation\ efficiency$$

To ensure the detection of all positive protoplasts, samples were collected from individual wells, centrifuged at 100 × g prior to resuspension in a minimal volume (~100 µl) and counted on a hemocytometer. Three independent biological replicates were analyzed for each tested condition.

2.7 Protoplast electroporation-mediated transformation

As an alternative to PEG-mediated transformation, electroporation was performed as previously described for tobacco BY-2 protoplasts with slight modifications [40]. One hour after isolation, protoplasts were centrifuged at 100 x g for 2 min and the W5 wash solution was removed. Protoplasts were then washed twice with electroporation buffer (0.4 M sucrose, 2.4 g/l HEPES, 6 g/l KCl, 600 mg/l $CaCl_2$ H_2O, pH 7.2), at 80 x g for 10 min without deceleration. A 500 µl aliquot containing 5-6×10^5 protoplasts in electroporation buffer was then placed into 0.4 cm gap electroporation cuvette (Bio-Rad Laboratories, Hercules, CA, USA). Forty micrograms of pTD-PvUbi2-35S DNA was then added to *Arundo* and tobacco BY-2 protoplasts and incubated at RT for 10-15 min, placing the cuvette horizontally. After incubation, electroporation of *Arundo* was conducted using a Bio-Rad Gene Pulser™ (Bio-Rad Laboratories, Hercules, CA, USA) at 130-300 V with a capacitance of 500 or 1000 µF. Tobacco BY-2 protoplasts were used as a positive control,

at 300 V with a capacitance of 1000 µF. The time constant was recorded after each pulse to ensure the samples were uniform, and the pulse was delivered. After electroporation, samples were incubated for 40 min at RT in the cuvette, and then moved into a single well of a 12-well plate, followed by the addition of 2 ml of protoplast culture medium (4.3 g/l MS salts, 0.4 M sucrose, 2.5 mM MES hydrate, 5.4 mM $CaCl_2 \cdot H_2O$, 3.1 mM NH_4NO_3, pH 5.7). The remaining solution in the cuvette was mixed with 1 ml of protoplast culture medium and transferred into a second well. An additional 1 ml of protoplast culture medium was finally used to wash the cuvette and poured into the second well. The protoplast viability was monitored after electroporation using propidium iodide, working solution (1 mg/50 ml, Sigma–Aldrich, St. Louis, Missouri, USA) [31]. Similarly to PEG-mediated transformation, the transformation efficiency was measured at 14-16 hours after transformation by counting the number of protoplasts expressing mGFP5-ER and pporRFP and dividing by the total protoplast number. For this method efficiency of transformation was estimated directly in the well by counting 3 random fields of view. The efficiency of each field was calculated separately as described previously. Using this strategy, an average of about 300 protoplasts were counted per well. Three independent biological replicates, each the result of independent protoplast isolations and transformation, were analyzed for all conditions tested.

2.8 Statistical analysis

A completely random experimental design was used for media screening, growth analysis, and transformation. All experiments included at least three independent biological replicates, and were analyzed using mixed model ANOVA (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY, USA). Least significant differences (LSD) were used to determine significant differences among means when the ANOVA results were statistically significant ($P<0.05$).

3 Results

3.1 Establishment of suspension cultures of *Arundo*

To test various media, the PCV of cultures were analyzed over the course of 21 days (**Figure 2A**). Despite using solidified CIM medium for culturing the donor callus, liquid CIM resulted in the

poorest growth among the media tested (**Table 1**). Over the first 7 days, the PCV of the liquid CIM was below the limit of detection (50 µl), and reached a measurable level only at day 10. Moreover, there was no significant growth observed for the CIM cultures from day 10 to day 21 ($p=0.71$). Similar to CIM, suspension cultures initiated in MSDP medium showed no detectable growth over the first 10 days of culture, but reached a measurable level at day 14. From day 14 to the completion of the experiments at day 21, there was no significant growth ($p=0.13$). Significant growth was observed in the B5G and DBAP media, with the cultures reaching their peak growth at 7-10 days and 10-14 days respectively (**Table 1**). In B5G and DBAP, the PCV was measurable after only 1 day in culture, as opposed to 3 and 7 days for MSDP and CIM respectively. Between DBAP and B5G, there was no significant difference in growth over the first 3 days of culture ($p=0.65$); however, the PCV of the B5G culture was significantly greater at day 7 compared to DBAP ($p=0.024$). After 10 days in culture, the PCV of the suspension cultures in DBAP had increased 3.3-fold, while the B5G culture increased 2.8-fold compared with the initial amount. At this point, the DBAP culture surpassed the maximum PCV observed for the B5G culture. Furthermore, comparison of the PCV at day 10 (the maximum for both DBAP and B5G) across all of the media showed a significant difference between DBAP and B5G compared to the other two media tested (**Table 1**). After 10 days in culture, the PCV of the B5G culture decreased markedly. A similar trend was observed in the DBAP culture, but the decrease occurred after 14 days in culture. In both cases, the rapid cell growth in these cultures led to an observable decrease in the amount of liquid media in flasks. While we conclude that both DBAP and B5G were suitable for initiation and maintenance of *Arundo* cell suspension cultures, DBAP was chosen as the media for future experiments because of its higher PCV at 10 days of culture, indicating more biomass would be available sooner for follow-on experiments.

Prior to establishment of the cultures, the majority of the cells in DBAP were kidney-shaped, contained starch-rich plastids, and clearly-discernable nuclei (**Figure 2B**). After establishment, the suspension cultures mainly consisted of elongated cells devoid of vacuoles (**Figure 2C**). In addition, the established suspension cultures were a mix of individual embryogenic-like cells of

various size classes (**Figure 2E**). Once large (1-5 mm diameter) clusters were readily observable in the culture flasks, the cultures were considered suitable for subsequent experiments.

3.2 *Arundo* performs better in liquid culture than on solidified medium

A comparison of the mass of suspension cells and callus was also evaluated over the course of 21 days (**Table 2**, **Figure 2F**). At 14 days of culture, there was no significant difference in the amount of tissue generated from *Arundo* grown on solid media (maintained on solid CIM), a freshly incubated liquid culture (callus moved from solid CIM to liquid DBAP medium), or an established liquid culture transferred to solid media (liquid DBAP to solid DBAP) (**Figure 2D**). However, there was significantly more tissue generated from the established suspension cultures that were transferred to fresh liquid DBAP. In fact, 2.5 times more tissue was generated by cultures maintained in liquid DBAP compared to all other treatments at 14 days (**Table 2**). In addition, the established suspension cultures reached a maximum of 4.7 times the starting tissue mass over 21 days, whereas no other treatment exceeded 2 times the cell mass over the same time period. Based on these results, the established suspension cultures were used for the generation of protoplasts and transformation.

3.3 Protoplast preparation and transformation.

Protoplasts of *Arundo* were isolated from 4 to 7 day old suspension cell cultures (**Figure 2G**), with an average of $3.8\times10^5 \pm 2.5\times10^5$ protoplasts/ml of PCV. Morphologically, the protoplasts ranged in size from 4.9 to 53.4 µm in diameter with an average size of 20.7±12.3 µm. The majority of the protoplasts ranged from 4 to 20 µm in diameter (**Figure 2H**). PEG- and electroporation transformation experiments were performed on protoplasts. Initial transformation experiments were conducted using the pANIC10A-GFPstuffer [31], which expresses *pporRFP* driven by the switchgrass ubiquitin promoter, *PvUbi1+3* [38]. All attempts to transform *Arundo* protoplasts with PEG (varying DNA quantity, $MgCl_2$ concentration, PEG% solution) failed, despite the success with the switchgrass positive controls. However, electroporation at 130 V and 1000 µF attained a transformation efficiency of 3.3±1.5%. When the voltage was increased to 300 V, with the

capacitance remaining the same, there was a decrease in protoplast viability from 67.7±3.0% to 11.9±4.5% and no transformation was detected. When the capacitance was decreased to 500 µF, at 130 and 300 V, the efficiency of transformation was reduced to 1.0±0.2 and 0.3±0.4%, respectively. In these treatments, the viability remained high, 82.±1.5% at 130 V and 80±2.4% at 300 V, despite the lower transformation efficiency. Not surprisingly, the switchgrass *PvUbi2* [38] promoter yielded higher marker gene expression than the *35S* promoter in *Arundo* protoplasts using the pTD-*PvUbi2-35S* binary vector (**Figure 3A-D)**. As expected, tobacco BY-2 protoplasts, used as a control, showed high expression of the mGFP5-ER fluorescent marker driven by the *35S* promoter, but only weak expression of the pporRFP fluorescent marker driven by the *PvUbi2* promoter (**Figure 3E-H**).

4 Discussion

Previous work on tissue culture of *Arundo donax* used solid CIM medium for callus induction and maintenance [22]. Unfortunately, *Arundo* callus grown on this medium is relatively slow-growing, making it difficult and time-consuming to generate enough tissue for subsequent transformation. Of particular importance, *Arundo* callus growth on CIM appears to be suboptimal compared with other relevant plant species. For example, *Sorghum* callus grow 1.7 times faster than *Arundo* on solidified CIM [41]. For these reasons, a suspension culture system for *Arundo* was pursued as a mean to increase the amount of tissue produced. The results from the *Arundo* suspension cultures established in DBAP demonstrate that cultivation in liquid medium produces far more tissue than solid culture with CIM. Similar results have been found in other species, such as cotton (*Gossypium hirsutum* L.) and broccoli (*Brassica oleracea*) [42-43]. The difference in *Arundo* growth in liquid culture may be the result of greater nutrient availability, a more gradual pH change, or lower production of toxic compounds [42].

Despite the significant increase in tissue mass for the suspension cultures of *Arundo*, the amount obtained was still relatively low when compared to suspension cultures of other biofuel crops. Suspension cultures of sugarcane (*Saccharum officinarum*) have been shown to increase by 6

times the starting inoculum in only 10 days [44]. Similarly, switchgrass suspension cultures have been shown to increase 8.6 times over 14 days of culture [24]. In both of these systems, the increase in fresh weight is ~2 times greater than *Arundo*. These data suggest that further optimization of the culture media may be necessary for *Arundo*, as other bioenergy crops have placed extensive effort in this direction [45,46,47]. Also, the ability of plant species to be manipulated *in vitro* is strongly genotype-dependent [48,49], therefore more effort is needed to screen different accessions and identify genotypes more amenable to *in vitro* cultivation.

One unique characteristic of *Arundo* compared with other bioenergy crops is that it does not sexually reproduce; therefore there is no seed production [50,51]. As such, *Arundo* is not amenable to improvement through standard breeding practices. The lack of seed production, however, is a highly advantageous trait when considering bioconfinement and restriction of gene flow, especially transgene flow [52]. In either case, genetic improvement of *Arundo* requires a reliable method for transformation to introduce novel genes, or inhibit/overexpress native ones [53,54]. An effective transformation method is also essential for screening of gene expression, validation of gene silencing or genome-editing vectors, and general cell and molecular assays. Considering that only there have been few RNA sequencing studies [55,56] and no whole genome information is presently available for *Arundo*, it is essential that screening be conducted at early stages, prior to developing transgenic plants. Currently, transformation of *Arundo* callus has only been achieved in one study, in which particle bombardment was used to introduce exogenous DNA into callus [15]. While effective, particle bombardment requires specialized equipment and is costly with regards to consumables. Further complicating transient-, and to a greater extent, stable transformation, callus growth is very slow, leading to a long lag time prior to when screening can be performed. As an alternative, PEG-mediated transformation and electroporation of *Arundo* protoplasts was evaluated in our project. High molecular weight PEG solutions have proven to be toxic to protoplasts isolated from some plant species [57]. In addition, the presence of Ca^{2+} or Mg^{2+} and the ratio of cations : PEG are critical factors for optimizing protoplast transformation [58,59]. While we did vary these parameters, we never achieved PEG-mediated transformation of *Arundo*

protoplasts. However, while PEG-mediated transformation failed, electroporation was successful in generating transient expression of the fluorescent reporter genes. While, similar to particle bombardment, electroporation requires specialized equipment, its throughput can be markedly increased using plate-based assays. In addition, screening after electroporation can be conducted in less than 24 hours, providing a rapid assessment of gene expression. To demonstrate the functionality of the *Arundo* protoplast transformation system, the expression of fluorescent reporters driven by monocot (*PvUbi2*) and dicot (*35S*) promoters were evaluated using the pTD-*PvUbi2-35S* vector developed in this work. The results demonstrated the utility of screening promoters/gene expression in a rapid protoplast system: *35S* promoter imbued low expression in *Arundo* protoplasts. This finding was in contrast to previous work using particle bombardment to transform *Arundo* callus [15]. However, protoplast systems are much more sensitive for measuring fluorescent protein expression compared with callus-based systems [29]. The identification of *PvUbi2* as a strong promoter for *Arundo* has implications in the development of future vectors aimed at the generation of stably-transformed *Arundo*. For instance, a strong promoter should be chosen to drive the plant resistance cassette in order to impose a strong selection and eliminate the majority of false positives/escapes [60,61]. Assays such as the one conducted in this work may help to overcome current limitations in the generation of transgenic *Arundo*, by providing data on promoter activity prior to regeneration [62].

In conclusion, in our project, a cell suspension culture system using DBAP medium was established for *Arundo*, which yielded up to 6-fold tissue-mass increase relative to callus culture systems. In addition, we developed a low-cost protoplast isolation system from suspension cultures that appear to have utility for assays for gene expression, and promoter screening. They could be useful for genome editing and stable transformation. Finally, the transient transformation of *Arundo* protoplasts using electroporation was achieved and used to screen a representative monocot and dicot promoter. These experiments indicated that *PvUbi2* was a strongly-active promoter in *Arundo*, whereas 35S was a weak promoter, thus informing the design of future vectors for *Arundo* biotechnology.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Table 1. Media screen for the establishment of suspension cultures over a 21-day culture period. Cell growth was monitored for 21 days after filtration through a 100 µm cell strainer by measuring the packed cell volume (PCV). Three independent replicates were measured for each medium.

Media	Packed cell volume (µl) measured by day					
	1	3	7	10	14	21
DBAP	900 ±10	100 ±20	130 ±30	280 ±30	280 ±30(a) [(a)]	90 ±10
CIM	N.D.[(b)]	N.D.	N.D.	60 ±20	60 ±10 (b)	60 ±40
MSDP	N.D.	N.D.	N.D.	N.D.	60 ±20 (b)	200 ±100
B5G	80	90 ±10	220 ±30	220 ±30	120 ±30 (b)	90 ±10

[(a)] Letter designations at 14 days indicates the results of Tukey's HSD analysis, with common letters among treatments representing no significant difference from 3 biological replicates.

[(b)] N.D. indicates that the packed cell volume was below the detection limit (50 µl).

Table 2. Comparison of various cell- and tissue culture regimes to generate tissue mass over a 21-day culture period. The starting material for each replicate was 500 mg with the treatment time course (day 0) beginning with the second medium in each treatment. Three independent replicates were measured for each treatment.

Treatments	Fresh weight (mg) per day							
	1	3	5	7	10	14	18	21
Liquid DBAP to liquid DBAP	500 ±10	700 ±70	730 ±50	940 ±70	1500 ±130	2400 ±380 (a) [(a)]	2300 ±570	2000 ±600
Solid CIM to solid CIM	510 ±10	540 ±30	580 ±40	630 ±10	730 ±70	720 ±20 (b)	720 ±210	730 ±200
Solid CIM to liquid DBAP	510 ±10	720 ±30	740 ±150	780 ±180	750 ±230	740 ±240 (b)	830 ±290	660 ±280
Liquid DBAP to solid CIM	500	660 ±50	720 ±40	820 ±20	860 ±90	940 ±140 (b)	980 ±70	970 ±80

[(a)] Letter designations at 14 days indicates the results of Tukey's HSD analysis, with common letters between treatments representing no significant difference from 3 biological replicates.

Figure and table legends

Table 1. Media screen for the establishment of suspension cultures over a 21-day culture period. Cell growth was monitored for 21 days after filtration through a 100 µm cell strainer by measuring the packed cell volume (PCV). Three independent replicates were measured for each medium.

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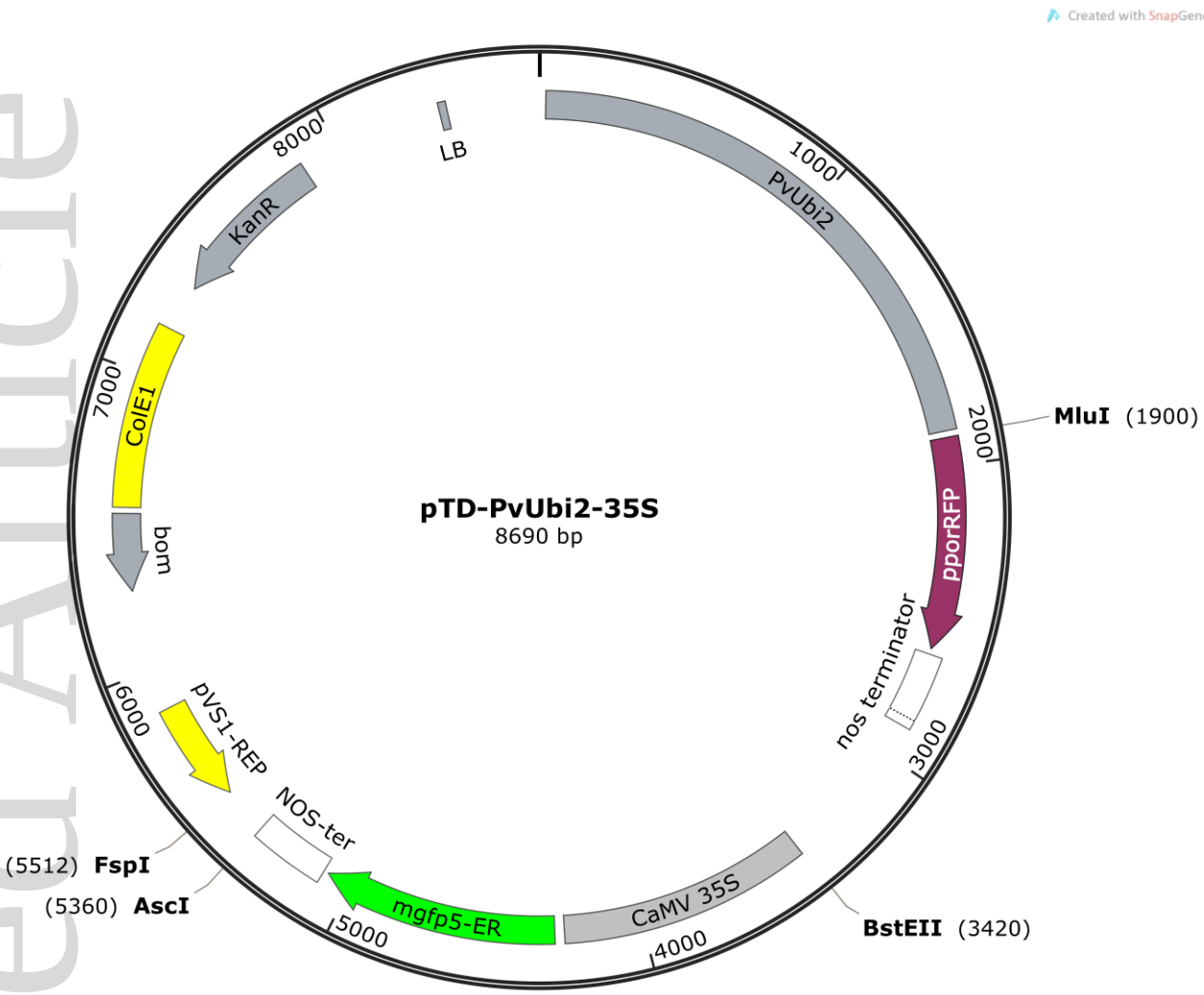

Figure 1. Map of pTD-*PvUbi2-35S vector*. Abbreviations: *PvUbi2* (*Panicum virgatum* polyubiquitin *2* promoter and intron), *pporRFP* (*Porites porites* red fluorescent protein coding region), *nosT* (*Agrobacterium tumefaciens* nopaline synthase terminator), CaMV 35S (cauliflower mosaic virus 35S promoter), mgfp5-ER (modified green fluorescent protein with an endoplasmic reticulum targeting sequence), ColE1 (origin of replication in *E. coli*), bom (origin of transfer in *E. coli*), KanR (kanamycin bacterial resistance marker), LB (T-DNA left border). Unique restriction sites used for vector construction are also indicated.

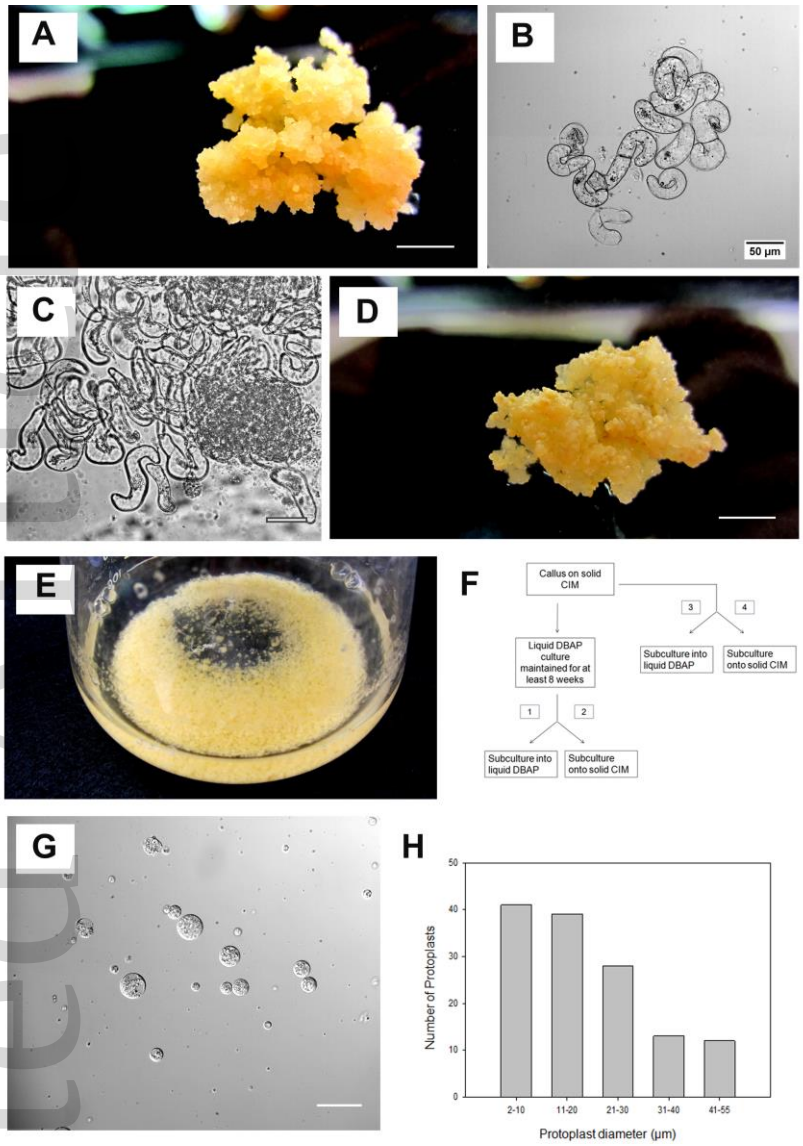

Figure 2. Suspension cell culture establishment and protoplast isolation. (A) One month-old callus grown on solid CIM medium. (B) Cells from seven day-old filtrated suspension cell culture in liquid DBAP medium. (C) Cells from fourteen day-old established suspension cell culture in DBAP medium. (D) Callus from one month-old suspension cell culture regrown on CIM solidified medium. (E) Large cell clusters (1-5 mm) after 1 week of subculture of 5 ml of PCV in 20 ml of fresh DBAP medium in a 125 ml Erlenmeyer flask. (F) Experimental scheme for comparing tissue culture and suspension cell culture. All treatments were initiated from callus on solid CIM: (1) liquid DBAP to liquid DBAP (2) liquid DBAP to solid CIM (3) solid CIM to liquid DBAP (4) solid CIM to solidified CIM medium. (G) Protoplasts isolated from a 5 day-old suspension cell culture. (H) Distribution of protoplast size in 5 samples of isolated protoplasts. Scale bar in A and D is 500 µm, scale bar in B, C and G is 50 µm.

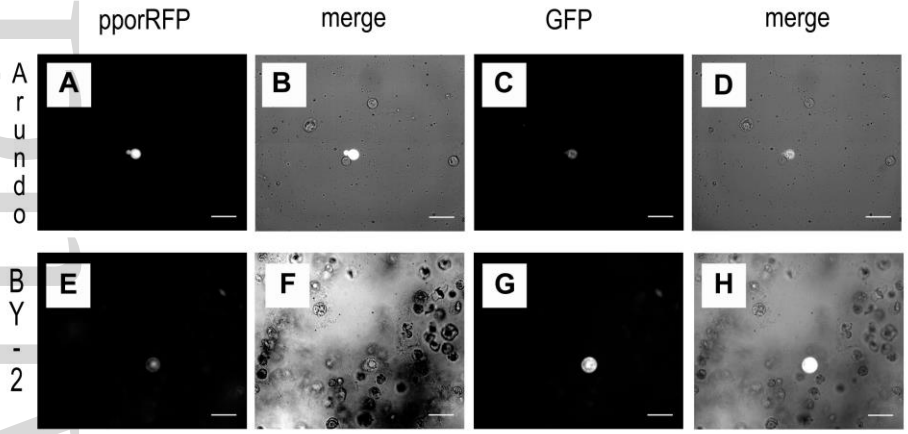

Figure 3. Transient expression of GFP and pporRFP fluorescent proteins in *Arundo* and tobacco BY-2 protoplasts transformed with pTD-*PvUbi2-35S* by electroporation. (A, B, C, D) *Arundo* protoplast. (E, F, G, H) tobacco BY-2 protoplast. Visualization of transformation using a TdTomato filter set: 545/30 nm excitation and 605/50 nm band pass emission (A, B, E, F) and GFP filter set: 470/30 nm excitation and 525/50 nm band pass emission (C, D, G, H). (B, D, F, H) represent the merge of fluorescent and bright field images. Exposure time was 300 ms and the scale bar is 50 µm in all pictures.