Modification of plant regeneration medium decreases the time for recovery of *Solanum lycopersicum* cultivar M82 stable transgenic lines

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

28 Key Message: We decreased the time for recovery of tomato transgenic lines by 6 weeks through the addition of
29 indole-3-acetic acid to our standard plant regeneration medium.
30

31 **Abstract**

32 Tomato (*Solanum lycopersicum*) has rapidly become a valuable model species for a variety of studies including
33 functional genomics. A high-throughput method to obtain transgenic lines sooner than standard methods would
34 greatly advance gene function studies. The goal of this study was to optimize our current transformation method
35 by investigating medium components that would result in a decreased time for recovery of transgenics. For this
36 study, 6-day-old cotyledon explants from *Solanum lycopersicum* cultivar M82 *in vitro*-grown seedlings were
37 infected with the *Agrobacterium tumefaciens* strain LBA4404 containing the binary vector pBI121. This vector
38 contains the β-glucuronidase reporter gene and the neomycin phosphotransferase II selectable marker gene that
39 confers resistance to kanamycin. Modification of our standard plant regeneration medium with indole-3-acetic
40 acid (IAA) at concentrations of either 0.05 mg/l or 0.1 mg/l decreased the recovery time for transgenic lines by 6
41 weeks as compared to our standard medium that contains zeatin as the only plant growth regulator. We observed
42 50% and 54% transformation efficiency on plant regeneration medium containing 0.05 mg/l and 0.1 mg/l IAA,
43 respectively. Moreover, addition of 1 mg/l IAA to the root induction medium resulted in earlier root development
44 than medium that did not contain IAA. Addition of IAA to the plant regeneration and rooting media did not have
45 any negative effects on plant development. Recovery of transgenic lines in a shorter time results in higher
46 throughput for the introduction of gene constructs and has the potential to decrease the time and resources
47 needed to complete investigations of gene function.
48

49 Keywords: *Agrobacterium tumefaciens*; indole-3-acetic acid; *Solanaceae*; *Solanum pimpinellifolium*; tomato
50

51 **Introduction**

52 Tomato, *Solanum lycopersicum*, is a member of the *Solanaceae* family, which contains approximately 3,000 plant
53 species and includes some of the most economically important food crops. It is native to South America and was
54 brought to Europe in the 1500s and then to North America in the 1800s (Jones 1998). Tomato is a perennial plant
55 that has two different growth habits, determinate and indeterminate. There are two different market types of
56 tomatoes, fresh market and processing. According to the Agricultural Marketing Resource Center, in 2014 the US
57 dollar value for fresh market tomatoes was 1.14 billion and 1.325 billion for processing types, which are used to
58 make products such as juice, sauces, and ketchup [2]. In addition to being an economically important food crop,
59 tomato is an excellent source of health beneficial nutrients including beta-carotene and lycopene.

60 Over the years, utilization of tomato as a model plant species has increased because of readily available
61 resources such as mutant populations (Emmanuel and Levy 2002), bioinformatics tools (Bombarely et al. 2011),
62 and a high quality reference genome (Consortium 2012). In addition, since the very first report of *Agrobacterium*-
63 mediated transformation of tomato by McCormick et al. (Mccormick et al. 1986), there have been other reports of
64 successful transformations of different genotypes (Chyi and Phillips 1987; Fillatti et al. 1987; Frary and Earle 1996;
65 Park et al. 2003; Sun et al. 2006; Van Eck et al. 2006) and methods to improve transformation efficiency (Dan et al.
66 2016). A key aspect for the adoption of a model plant species is the availability of efficient transformation
67 methodology. This was certainly the case for *Arabidopsis*, which is by far the most widely used model for plant
68 research programs (Somerville and Koornneef 2002).

69 While there are several methods available for plant transformation, A*grobacterium tumefaciens*-mediated
70 transformation has become the most extensively used method (Gelvin 2003; Pitzschke and Hirt 2010). Despite its
71 effectiveness for gene transfer in tomato, there is still need for improvement. Improving methodology to decrease
72 the time from introduction of a gene construct of interest to recovery of stable transgenics would improve the
73 throughput and shorten the timeframe for studies that utilize tomato transgenic lines.

74 We were interested in finding an approach to decrease the time to obtain transgenic lines of the processing
75 type tomato M82 because this genotype is used for gene function studies in our lab as well as others (Brooks et al.
76 2014; Xu et al. 2015). We chose to start by investigating supplementation of our standard plant regeneration and
77 rooting media with a growth regulator that had the potential to speed up plant development (Van Eck et al. 2006).

78 Cytokinins and auxins are important hormones that influence growth and developmental processes in plants.
79 Interactions between cytokinins and auxins have been shown to be necessary for the shoot apex growth (Gupta
80 and Rashotte 2012; Shimizu-Sato et al. 2009). Auxin has also been shown to play a role in the specification of the
81 root apical meristem (Friml et al. 2003; Gupta and Rashotte 2012; Sabatini et al. 1999). The hormonal interactions
82 can be utilized in the area of tissue culture to leverage the presence of the hormones in the medium. In this study,
83 we report the effects of the addition of the auxin, indole-3-acetic acid (IAA) on the recovery time of M82
84 transgenic lines.
85
86 **Materials and methods**
87 *Plant material*
88 Seeds of *Solanum lycopersicum* cv M82 were surface sterilized in 20% (v/v) bleach solution containing Tween-20
89 for 20 min followed by 3 rinses in sterile water. Seeds were germinated in Magenta GA7 boxes (Caisson Labs,
90 Logan, UT) that contained 50 ml of Murashige and Skoog (MS) (Murashige and Skoog 1962) (Caisson Labs) based
91 medium containing 2.15 g/l MS salts, 100 mg/l myo-inositol, 2 mg/l thiamine, 0.5 mg/l pyridoxine, 0.5 mg/l
92 nicotinic acid, 10 g/l sucrose and 8 g/l Sigma agar (Sigma-Aldrich, St. Louis, MO). Cultures were maintained at 24°C
93 under a 16h light/8h dark photoperiod at 57 – 65 *u*E m^{-2} s^{-1}.
94 One day prior to infection with *Agrobacterium*, cotyledon explants and feeder layer plates were prepared.
95 Feeder layers were prepared before cutting the explants by dispensing 2 ml of a 1-week-old NT1 suspension
96 culture onto KCMS medium (4.3 g/l MS salts, 100 mg/l myo-inositol, 1.3 mg/l thiamine, 0.2 mg/l 2,4-
97 dichlorophenoxy acetic acid, 200 mg/l KH_2PO_4, 0.1 mg/l kinetin, 30 g/l sucrose, 5.2 g/l Agargel (Sigma Aldrich), pH
98 6.0. The suspension was covered with a sterile 7 cm Whatman filter paper. Explants were excised from 6-day-old
99 seedlings before the first true leaves emerged. To prepare the explants, seedlings were placed on a sterile paper
100 towel moistened with sterile water. Cotyledons were excised at the petioles, cut into approximately 1 cm sections,
101 placed adaxial side down on the KCMS feeder layer plates, and maintained at 24°C under a 16 h light/8 h dark
102 photoperiod at 57 – 65 *u*E m^{-2} s^{-1}.
103
104

105 *Bacterial strain and binary vector*

106 Electroporation was used to introduce the pBI121 vector (Chen et al., 2003) into the *Agrobacterium tumefaciens*
107 strain LBA4404. A single, well-formed colony from the selection plate was transferred to 50 ml of YEP selective
108 medium that contained 50 mg/l kanamycin and maintained in a shaking incubator at 28°C for 18 – 24 hrs or the
109 length of time needed to reach an OD_{600} of 0.6 - 0.7. The *Agrobacterium* suspension was centrifuged at 8000 rpm
110 for 10 min at 20°C. The pellet was resuspended in 50 ml of 2% MSO medium (4.3 g/l MS salts, 100 mg/l myo-
111 inositol, 0.4 mg/l thiamine, and 20 g/l sucrose) by vortexing.

112

113 *Agrobacterium-mediated transformation*

114 Cotyledon explants were incubated in the *Agrobacterium*/2% MSO suspension for 5 min, transferred to a sterile
115 paper towel to allow excess suspension to briefly drain, placed back onto the feeder plates with the adaxial sides
116 down, and co-cultivated in the dark at 19°C for 48 hrs. Explants, adaxial side up, were transferred to our standard
117 plant regeneration selective medium designated 2ZK that contained 4.3 g/l MS salts, 100 mg/l myo-inositol, 1 ml/l
118 Nitsch vitamins (1000x), 20 g/l sucrose, 2 mg/l trans-zeatin, 75 mg/l kanamycin, 300 mg/l timentin, and 5.2 g/l
119 Agargel. One week later, the explants were transferred onto 2ZK medium containing IAA at either 0 mg/l, 0.01
120 mg/l, 0.05 mg/l, 0.1 mg/l, or 0.5 mg/l IAA.

121 After two weeks, explants were transferred onto 1ZK medium that contained 4.3 g/l MS salts, 100 mg/l myo-
122 inositol, 1 ml/l Nitsch vitamins (1000x), 20 g/l sucrose, 1 mg/l trans-zeatin, 75 mg/l kanamycin, 300 mg/l timentin,
123 5.2 g/l Agargel, and IAA at either 0 mg/l, 0.01 mg/l, 0.05 mg/l, or 0.1 mg/l IAA, or 0.5 mg/l in plates or Magenta
124 GA7 boxes depending upon the size of the shoots regenerating from the cotyledon explants.

125 When shoots were approximately 3 mm tall, they were excised from the cotyledon explants and transferred to
126 selective rooting medium designated RMK (4.3 g/l MS salts, 1 ml/l Nitsch vitamins (1000x), 30 g/l sucrose, pH 6.0, 8
127 g/l Difco Bacto agar (Becton, Dickinson and Company, Franklin Lakes, NJ), 75 mg/l kanamycin, 300 mg/l timentin,
128 and IAA at either 0 mg/l or 1 mg/l in Magenta GA7 boxes.

129 Unless otherwise noted, the pH of all media was adjusted to 5.8 before autoclaving. For all media, the trans-
130 zeatin, IAA, kanamycin, and timentin were dispensed from filter sterilized stock solutions into autoclaved medium

131 that was allowed to cool to 55°C. Cotyledon explant cultures were transferred to freshly prepared medium every
132 two weeks.
133
134 *GUS histochemical assay*
135 Histochemical assay of β-glucuronidase (GUS) activity was performed on leaves from putative transgenic and
136 control (non-transformed) plants. Leaves were vacuum infiltrated for 20 – 30 min in buffer (0.8 g/l 5-bromo-4-
137 chloro-3-indolyl-β–D-glucuronide (X-Gluc), 0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4 phosphate, 10 mM ethylenediamine
138 tetraacetic acid (EDTA), 1.6 mM potassium-ferricyanide and 1.6 mM potassium –ferrocyanide, 5% v/v Triton X-100,
139 and 20% v/v methanol) before incubation at 37°C overnight. The chlorophyll was removed from the leaves by 3 - 4
140 washes with 70% ethanol at room temperature. The leaves were examined with a Leica S8APO stereomicroscope
141 outfitted with a digital camera.
142
143 *Polymerase chain reaction analysis*
144 To confirm the presence of the neomycin phosphotransferase II selectable marker gene (*nptII*), DNA was extracted
145 from leaves of putative transgenic lines and controls (non-transformed) with the Qiagen DNeasy plant mini kit
146 (Hilden, Germany) as per the manufacturer's instructions. Primers used to detect *nptII* were forward 5'-GGC TGG
147 AGA GGC TAT TC-3' and reverse 5'-GGA GGC GAT AGA AGG CG-3'. The diagnostic amplicon size expected with
148 these primers is approximately 700 bp. The PCR program started with a one-step cycle of 2 min at 95°C, followed
149 by 29 cycles of 30 s at 94°C, 45 s at 57°C, 50 s at 72°C, and a 10 min final extension at 72°C. DNA was separated
150 and visualized by electrophoresis through a 1% agaraose, ethidium bromide-stained gel.
151
152 *Experimental Design*
153 A total of 5 different experiments were performed. Three biological replicates were used for each IAA
154 concentration in each experiment. A total of 750 cotyledon explants were used per IAA concentration
155 investigated. The standard error was calculated.
156

157 **Results**

158 *Optimization of IAA concentration for recovery of stable transgenic lines*

159 After the co-cultivation period that followed infection with *Agrobacterium*, cotyledon explants were transferred to
160 our standard selective plant regeneration medium designated 2ZK that contains 2 mg/l trans-zeatin as the only
161 plant growth regulator. One week later, the explants were transferred to 2ZK supplemented with different IAA
162 concentrations (0 mg/l, 0.01 mg/l, 0.05 mg/l, 0.1 mg/l, 0.5 mg/l) to determine if the addition of IAA would
163 decrease the time from infection with *Agrobacterium* to recovery of stable transgenic lines. We continued to use
164 this same series of IAA concentrations in the subsequent selective plant regeneration medium designated 1ZK.

165 Medium supplemented with IAA resulted in shoots that were more fully developed earlier in the culture
166 process as compared to medium without IAA (Fig. 1A). In Figure 1A, cotyledon cultures shown in a – e represent
167 controls that were not infected with *Agrobacterium*. We observed that as the IAA concentration increased, the
168 level of plant regeneration from the controls decreased (Fig. 1A, a – d). Cotyledon explants infected with
169 *Agrobacterium* and cultured on medium containing IAA exhibited the same pattern of shoot development as the
170 cotyledons not infected, in that we observed more well-developed shoots at an early stage of culture post
171 infection (Fig. 11 f – i). For our standard method without IAA (Fig. 1A, e), the level of plant regeneration is
172 significantly less in comparison with medium that contained IAA.

173 In general, earlier emergence of well-developed shoots from *Agrobacterium*-infected cotyledon explants on
174 medium containing IAA translated to the recovery of whole rooted plants in less time as compared with medium
175 that did not contain IAA (Table 1). Medium containing either 0.05 mg/l or 0.1 mg/l IAA resulted in the shortest
176 time, 11 wks, for recovery of stable transgenic lines. There appeared to be a threshold of IAA concentration and
177 effect on recovery time because at 0.5 mg/l IAA the time was similar to our standard method. We observed a
178 similar decrease in time when transformations of other tomato genotypes were performed by different lab
179 members who tested plant regeneration medium that contained 0.1 mg/l IAA (data unpublished).

180

181 *Effect of IAA on transformation efficiency and rooting*

182 The formula below was used to calculate transformation efficiency (TE):

183

184 $$\frac{\text{Total number of rooted shoots}}{\text{Total number of cotyledon explants infected with } \textit{Agrobacterium}} \times 100$$
185
186

187 Overall, the TE was lower when medium containing IAA was used as compared to the TE of 88% when medium
188 containing trans-zeatin as the only growth regulator was used (Table 1).

189 When putative transgenic lines were approximately 3 - 4 cm tall, they were removed from the cotyledon
190 explants and transferred to either our standard selective rooting medium (RMK) without IAA or RMK
191 supplemented with 1 mg/l IAA designated RMIK. We chose this concentration based on previous work with
192 tomato transgenic lines recovered from a few genotypes that did not root as well as M82 on our standard rooting
193 medium (data not published). We observed that shoots cultured on RMIK resulted in the emergence of roots after
194 6 - 7 days as compared to 11 - 14 days on RMK. The addition of IAA to the medium did not result in any
195 phenotypic differences of the plants as compared to medium that did not contain IAA.

196

197 *Characterization of putative transgenic lines*

198 The first level of analysis to confirm the recovered plants from *Agrobacterium*-infected cotyledons were transgenic
199 was a histochemical assay for the GUS reporter protein. Whole leaves from plants rooted on RMK were used for
200 the analysis. All leaves exhibited GUS activity, although we observed variation in the level of intensity with some
201 leaves exhibiting a darker coloration than others (Fig 1B). GUS activity was not observed in leaves from non-
202 transformed control plants.

203 To further confirm the recovered plants were indeed stable transgenic lines, we did PCR analysis for the
204 presence of the *nptII* selectable marker gene in plants found to be positive for GUS activity. Total genomic DNA
205 was isolated from the leaves of the GUS-positive lines and non-transformed control plants. PCR amplification of
206 the *nptII* gene was detected in plants that were also GUS positive. No amplified product was detected in DNA from
207 the control, (non-transgenic) plants (Fig. 1C).

208

209 *Modified protocol*

210 Based on our findings, we now follow a modified protocol as outlined in Figure 2 for *Agrobacterium*-mediated
211 tomato transformations. The modified protocol takes into account recovery time and TE. IAA concentrations of

212 0.05 and 0.1 mg/l IAA both resulted in a 6-week decrease for recovery of stable transformants, however, we chose
213 to use 0.1 mg/l IAA in our modified protocol because of the 54% TE (Table 1). In addition to M82, we have applied
214 this protocol to other tomato genotypes including the most closely related wild species, *Solanum pimpinellifolium*,
215 and also observed a decrease in time for recovery of transgenic lines as compared to our previous tomato
216 transformation methodology (data not published).
217
218 **Discussion**
219 For development of stable transformation methodology, the foremost factors to be considered are
220 transformation efficiency and the time from infection with *Agrobacterium tumefaciens* until the recovery of
221 transgenic lines. Various parameters have been investigated to reach a high transformation efficiency for tomato
222 including application of lipoic acid to reduce tissue necrosis caused by *Agrobacterium* infection of the MicroTom
223 genotype (Dan et al. 2016). Methods that provide both high efficiency and the shortest time to recovery of
224 transgenic lines lead to a high-throughput pipeline that allows earlier evaluation of gene function. In turn, a high-
225 throughput pipeline decreases the amount of labor and resources needed, which can translate into significant
226 financial savings.
227 The focus of our study was to investigate medium components that had the potential to decrease the time for
228 recovery of stable tomato transgenic lines. Our standard method, which has a high transformation efficiency at
229 approximately 90%, takes 17 weeks for recovery of transformants. The interest in optimization of our methods
230 stemmed from an increased need for transgenic lines because tomato has become the model species of choice for
231 many studies that include ripening, abiotic and biotic tolerance, and nutritional content (Gonzali et al. 2009;
232 Martel et al. 2011; Nguyen et al. 2010; Sun et al. 2010). In addition, with the recent demonstration of successful
233 genome editing by CRISPR/Cas9 in tomato, the interest in applying this technology for the study of gene function
234 will increase (Brooks et al. 2014; Ito et al. 2015). Therefore, a transformation methodology that can deliver
235 modified lines in a shorter time frame will help to advance these studies.
236 Our standard protocol is a modified version of methods reported by Fillatti et al. (1987) in which zeatin is the
237 only growth regulator incorporated into the plant regeneration medium (Van Eck et al. 2006). We chose to start
238 our investigation by examining additional growth regulators that, in combination with zeatin, would greatly reduce

239 the time for recovery of stable transgenic lines but not have a significant negative effect on transformation
240 efficiency. In a literature search, we found several reports that demonstrated a positive effect on tomato plant
241 regeneration and transformation efficiency when indole-3-acetic acid (IAA) was incorporated into zeatin-
242 containing plant regeneration medium (Gubis et al. 2004; Park et al. 2003; Yasmeen 2009). However, they did not
243 report any effects observed on the time required to recover transgenic plants.

244 We found that addition of either 0.05 or 0.1 mg/l IAA to our standard plant regeneration medium that
245 contains trans-zeatin as the only growth regulator decreased the time for recovery of stable transgenic lines from
246 17 to 11 weeks. Previous reports have demonstrated that shoot apical meristem development involves
247 interactions among cytokinin signaling pathway components, auxin, and several families of transcription factors
248 (Gupta and Rashotte 2012). It is possible that the addition of IAA to our standard plant regeneration medium
249 facilitates interactions among the cytokinin signaling and auxin regulated genes, which results in faster shoot
250 development from the cotyledon explants.

251 Although there was a reduction in transformation efficiency with the addition of IAA from approximately 90%
252 to about 50%, this level is acceptable considering transgenic lines can be evaluated significantly earlier than when
253 our standard method was used. This decrease in time allows researchers to test their material earlier and make
254 changes to their approaches sooner if results are unsatisfactory for their genes of interest.

255 In addition to supplementation of the standard plant regeneration medium with IAA, we also investigated
256 effects of adding IAA to the rooting medium, which was not a component in our standard rooting medium.
257 Inclusion of IAA in *in vitro* rooting medium has been reported for tomato, however, it is not routinely added
258 because tomato readily develops roots in culture medium without growth regulators (Frary and Earle 1996). Our
259 interest was to determine if supplementation decreased the time to rooting, which we did observe. Auxin is
260 produced in both shoots and roots and the auxin produced in the roots helps in root development (Overvoorde et
261 al. 2010; Petersson et al. 2009; Stepanova et al. 2008). It is possible that IAA, when exogenously added, increases
262 the levels of auxin in the plants, hence resulting in the cells differentiating earlier to form roots. However,
263 research needs to be conducted to confirm this hypothesis.

264

265 **Conclusions**

266 Interest in tomato as a model has increased over the years and we have seen a rise in the number of research
267 groups that require stable transgenic lines for various studies. Modification of our standard plant regeneration
268 medium through the addition of either 0.05 or 0.1 mg/l AA shortened the recovery of transgenic lines by 6 weeks
269 for the M82 tomato cultivar. Application of this modification for transformation of other tomato genotypes in our
270 lab also resulted in a decreased time for recovery of stable transgenic lines.

271 A shorter recovery time for stable transgenic lines is highly desirable for functional studies to allow earlier
272 determination of the genes and networks involved in phenotypes of interest. A decrease in recovery time would
273 also provide a higher throughput process, which has the potential for cost savings related to labor and resources.
274 Optimization studies of standard transformation methodologies for different plant species should always be
275 considered in order to alleviate bottlenecks for generation of stable transgenic lines (Altpeter et al. 2016).
276 Availability of efficient transformation methods is especially critical with the rapid development of genome editing
277 technologies, which will result in an increased demand for generation of transgenic lines for basic research studies
278 that can lead to crop improvement.
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362

363 **<u>Figure Legends</u>**

364 **Figure 1**

365 **Results for the recovery of *Solanum lycopersicum* cv M82 stable transgenics from *Agrobacterium tumefaciens*-**

366 **infected cotyledon explants cultured on plant regeneration medium supplemented with different**

367 **concentrations of indole-3-acetic acid (IAA).** (A) *Agrobacterium tumefaciens*-infected cotyledon explants

368 (approximately 5 weeks post infection) cultured on selective plant regeneration medium containing the following

369 amounts of IAA in mg/l (f) 0.01, (g) 0.05, (h) 0.1, (i) 0.5, and (j) 0. Images a – e represent the corresponding non-

370 infected controls for each IAA concentration, respectively.

371 (B) Histochemical analysis for GUS expression in leaves taken from independent transgenic lines designated 1 - 9

372 recovered from selective plant regeneration medium that contained 0.1 mg/l IAA. GUS expression was not

373 observed in the non-transformed controls.

374 (C) Agarose gel of PCR products showing the expected ~700 bp product amplified from the *nptII* selectable marker
375 gene in 10 independent transgenic lines (lanes 1 – 10). These lines were recovered from selective plant
376 regeneration medium that contained 0.1 mg/l IAA. C = the control

377 **Figure 2**

378 **Schematic representation of the optimized *Agrobacterium tumefaciens*-mediated transformation methodology**
379 **for *Solanum lycopersicum* cv M82.** See the Materials and Methods for details on seed sterilization and all media
380 compositions.

381

382 **Tables**

383 **Table 1: Results for recovery of stable transgenic lines of *Solanum lycopersicum* cv M82 from *Agrobacterium***
384 ***tumefaciens*-infected cotyledon explants cultured on selective plant regeneration medium supplemented with**
385 **different indole-3-acetic acid (IAA) concentrations.**

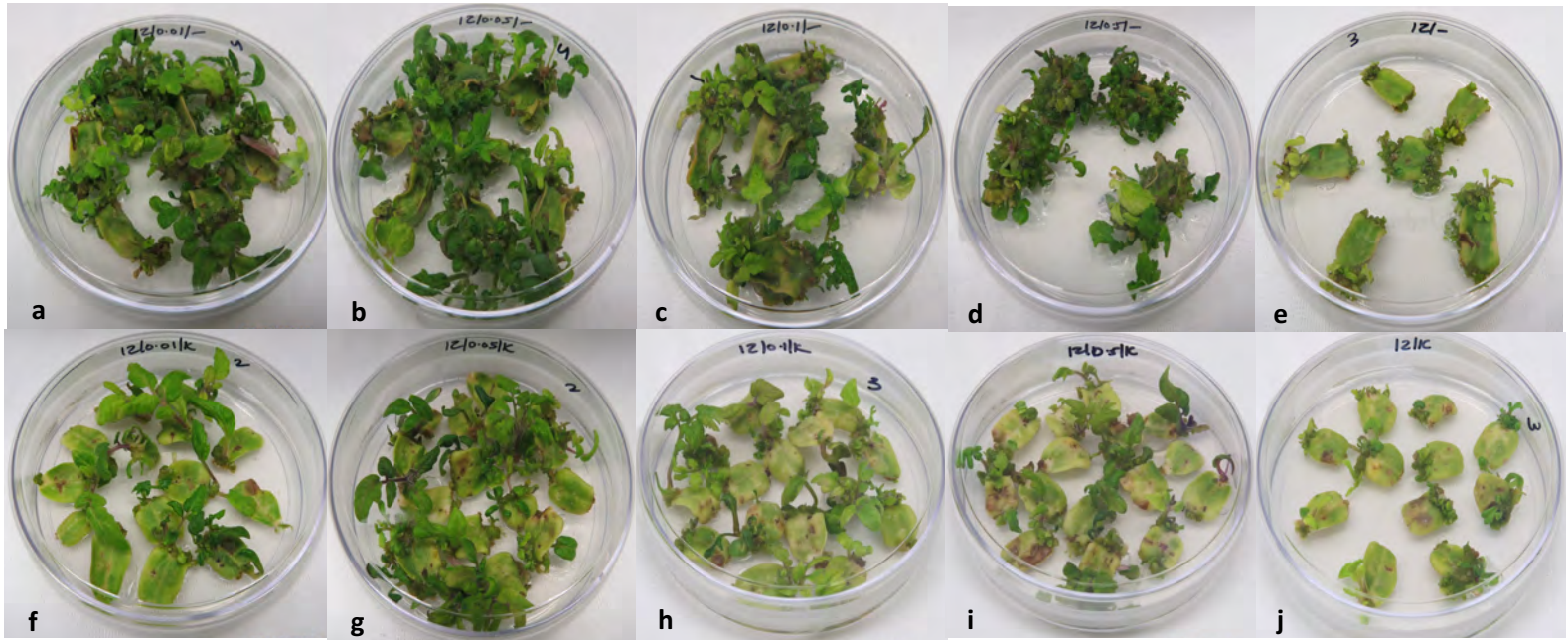
IAA (mg/l)	Total number explants	Total number rooted plants	Average transformation efficiency (±) SE*	Total time for recovery of transgenic lines (wks)
0	750	660	88 ± 2.2	17
0.01	750	390	52 ± 1.0	15
0.05	750	375	50 ± 1.5	11
0.1	750	405	54 ± 1.2	11
0.5	750	360	48 ± 2.0	16

386

387 *Average transformation efficiency was calculated as percent of stable transgenic lines recovered from the total
388 number of cotyledon explants infected with *Agrobacterium tumefaciens*. Transformation efficiency values shown
389 are the average from 5 experiments ± the standard error (SE) calculated from 3 biological replicates.

Gupta and Van Eck_Figure 1

A



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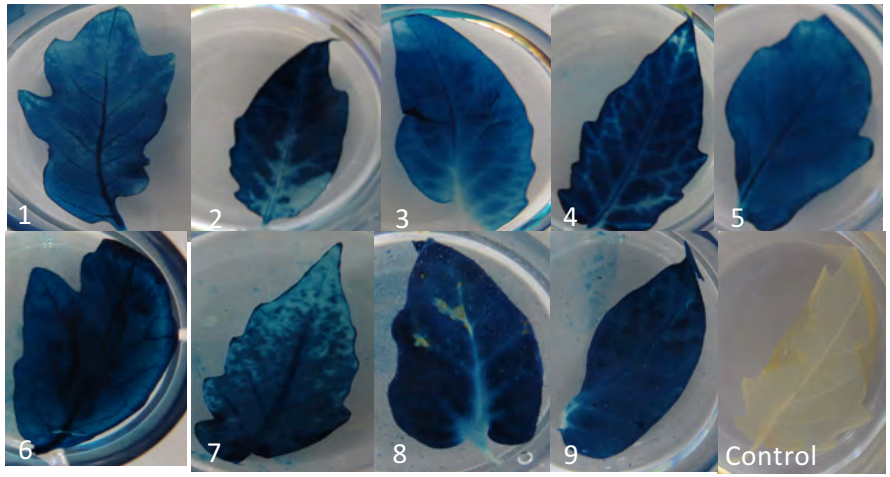

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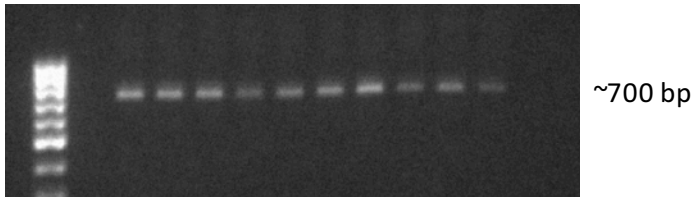
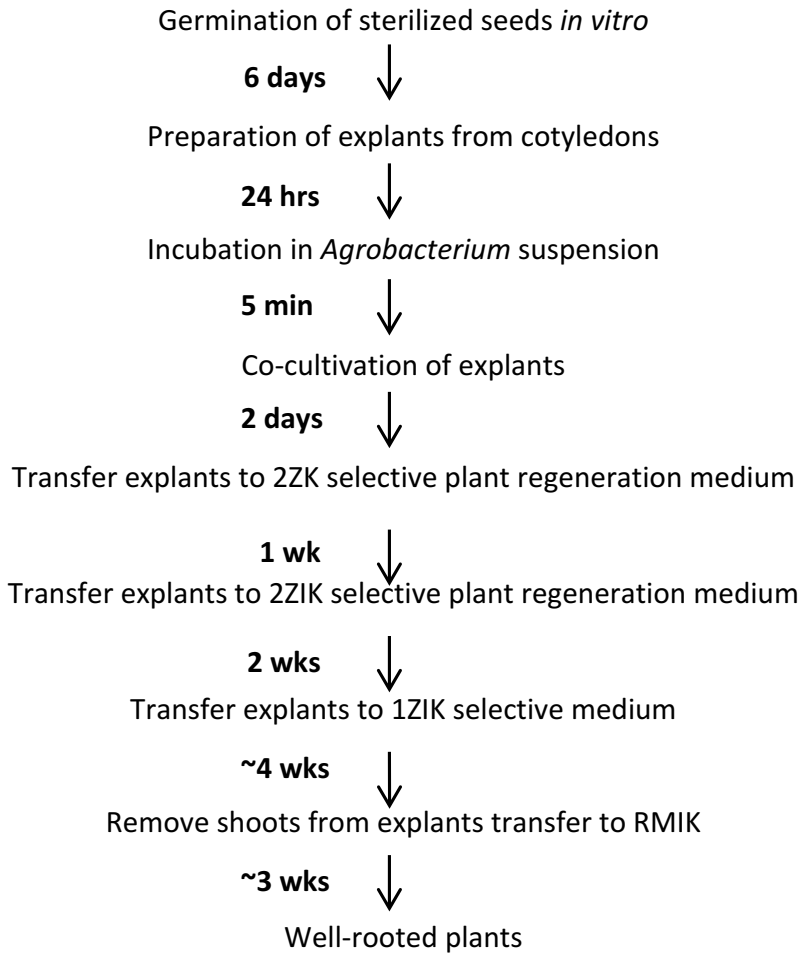
Gupta and Van Eck_Figure 2