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

Background: Tomato (*Solanum lycopersicum*) has rapidly become a valuable model as a result of the availability of a high quality reference genome, extensive genetic resources, and efficient gene transfer methodology. A high-throughput method to obtain transgenic lines sooner than standard methods has the potential to greatly advance gene function studies.

Results: The goal of this study was to optimize our current gene transfer (transformation) method for tomato by investigating medium components that would result in a decreased time for recovery of transgenic lines. The methodology reported here is based on infection of 6-day-old cotyledon explants from *Solanum lycopersicum* cultivar M82 *in vitro*-grown seedlings with *Agrobacterium tumefaciens* strain LBA4404 containing the binary vector pBI121. This vector contains the β-glucuronidase reporter gene and the neomycin phosphotransferase II selectable marker gene that confers resistance to kanamycin. Modification of our standard plant regeneration medium by the addition of indole-3-acetic acid (IAA) at concentrations of either 0.05 mg/l or 0.1 mg/l greatly decreased the time for recovery of transgenic lines by 6 weeks as compared to our standard medium that contains zeatin alone. Moreover, addition of 1 mg/l IAA to the root induction medium resulted in faster root development. We observed 50% and 54% transformation efficiency when plant regeneration medium containing 0.05 mg/l and 0.1 mg/l IAA, respectively, was used for transformation experiments. Transgenic lines recovered with the optimized method were similar in development to our standard method. No negative effects were observed as a result of the addition of IAA to the medium.

Conclusions: The results showed that addition of IAA at concentrations of either 0.05 or 0.1 mg/l to the plant regeneration medium reduced the total time for recovery of stable transgenic lines by 6 weeks. The ability to recover transgenic lines in a shorter time results in higher throughput for the introduction of gene constructs and has the potential to decrease the time needed to complete investigations of gene function. The ability to recover transgenic lines for each gene construct in a shorter time saves labor and resource expenses because culture maintenance is also reduced.

Keywords: *Agrobacterium tumefaciens*, indole-3-acetic acid, *Solanaceae*, *Solanum pimpinellifolium*, tomato

Background

Tomato, *Solanum lycopersicum*, is a member of the *Solanaceae* family, which contains approximately 3,000 plant species and includes some of the most economically important food crops. It is native to South America and was brought to Europe in the 1500s and then to North America in the 1800s [1]. Tomato is a perennial plant that has two different growth habits, determinate and indeterminate. There are two different market types of tomatoes, fresh market and processing. According to the Agricultural Marketing Resource Center, in 2014 the US dollar value for fresh market tomatoes was 1.14 billion and 1.325 billion for processing types, which are used to make products such as juice, sauces, and ketchup [2]. In addition to being an economically important food crop, tomato is an excellent source of health beneficial nutrients including beta-carotene and lycopene.

55 Over the years, utilization of tomato as a model plant species has increased because of
56 readily available resources such as mutant populations [3], bioinformatics tools [4], and a high
57 quality reference genome [5]. In addition, since the very first report of *Agrobacterium*-
58 mediated transformation of tomato by McCormick et al. [6], there have been other reports of
59 successful transformations of different genotypes [7-12]. A key aspect for the adoption of a
60 model plant species is the availability of efficient transformation methodology. This was
61 certainly the case for *Arabidopsis*, which is by far the most widely used model for plant research
62 programs [13].

63 While there are several methods available for plant transformation, A*grobacterium*
64 *tumefaciens*-mediated transformation has become the most extensively used method [14, 15].
65 Despite its effectiveness for gene transfer in tomato, there is still need for improvement.
66 Improving methodology to decrease the time from introduction of a gene construct of interest
67 to recovery of stable transgenics would improve the throughput and shorten the timeframe for
68 studies that utilize tomato transgenic lines.

69 We were interested in finding an approach to decrease the time to obtain transgenic lines
70 of the processing type tomato M82 because this genotype is used for gene function studies in
71 our lab as well as others [16, 17]. We chose to start by investigating supplementation of our
72 standard plant regeneration and rooting media with a growth regulator that had the potential
73 to speed up plant development [12].

74 Cytokinins and auxins are important hormones that influence growth and developmental
75 processes in plants. Interactions between cytokinins and auxins have been shown to be
76 necessary for the shoot apex growth [18, 19]. Auxin has also been shown to play a role in the

77 specification of the root apical meristem [18, 20, 21]. The hormonal interactions can be utilized
78 in the area of tissue culture to leverage the presence of the hormones in the medium. In this
79 study, we report the effects of the addition of the auxin, indole-3-acetic acid (IAA) on the
80 recovery time of M82 transgenic lines.
81

82 **Methods**

83 ***Plant material***

84 Seeds of *Solanum lycopersicum* cv M82 were surface sterilized in 20% (v/v) bleach solution
85 containing Tween-20 for 20 min followed by 3 rinses in sterile water. Seeds were germinated in
86 Magenta GA7 boxes (Caisson Labs, Logan, UT) that contained 50 ml of Murashige and Skoog
87 (MS) [22] (Caisson Labs) based medium containing 2.15 g/l MS salts, 100 mg/l myo-inositol, 2
88 mg/l thiamine, 0.5 mg/l pyridoxine, 0.5 mg/l nicotinic acid, 10 g/l sucrose and 8 g/l Sigma agar
89 (Sigma-Aldrich, St. Louis, MO). Cultures were maintained at 24°C under a 16h light/8h dark
90 photoperiod at 57 – 65 *u*E m^{-2} s^{-1}.

91 One day prior to infection with *Agrobacterium*, cotyledon explants and feeder layer plates
92 were prepared. Feeder layers were prepared before cutting the explants by dispensing 2 ml of
93 a 1-week-old NT1 suspension culture onto KCMS medium (4.3 g/l MS salts, 100 mg/l myo-
94 inositol, 1.3 mg/l thiamine, 0.2 mg/l 2,4-dichlorophenoxy acetic acid, 200 mg/l KH_2PO_4, 0.1 mg/l
95 kinetin, 30 g/l sucrose, 5.2 g/l Agargel (Sigma Aldrich), pH 6.0. The suspension was covered
96 with a sterile 7 cm Whatman filter paper. Explants were excised from 6-day-old seedlings
97 before the first true leaves emerged. To prepare the explants, seedlings were placed on a
98 sterile paper towel moistened with sterile water. Cotyledons were excised at the petioles, cut

99 into approximately 1 cm sections, placed adaxial side down on the KCMS feeder layer plates,

100 and maintained at 24°C under a 16 h light/8 h dark photoperiod at 57 – 65 *u*E m^{-2} s^{-1}.

101

102 ***Bacterial strain and binary vector***

103 Electroporation was used to introduce the pBI121 vector into the *Agrobacterium tumefaciens*

104 strain LBA4404. A single, well-formed colony from the selection plate was transferred to 50 ml

105 of YEP selective medium that contained 50 mg/l kanamycin and maintained in a shaking

106 incubator at 28°C for 18 – 24 hrs or the length of time needed to reach an OD_{600} of 0.6 - 0.7.

107 The *Agrobacterium* suspension was centrifuged at 8000 rpm for 10 min at 20°C. The pellet was

108 resuspended in 50 ml of 2% MSO medium (4.3 g/l MS salts, 100 mg/l myo-inositol, 0.4 mg/l

109 thiamine, and 20 g/l sucrose) by vortexing.

110

111 ***Agrobacterium-mediated transformation***

112 Cotyledon explants were incubated in the *Agrobacterium*/2% MSO suspension for 5 min,

113 transferred to a sterile paper towel to allow excess suspension to briefly drain, placed back

114 onto the feeder plates with the adaxial sides down, and co-cultivated in the dark at 19°C for 48

115 hrs. Explants, adaxial side up, were transferred to our standard plant regeneration selective

116 medium designated 2ZK that contained 4.3 g/l MS salts, 100 mg/l myo-inositol, 1 ml/l Nitsch

117 vitamins (1000x), 20 g/l sucrose, 2 mg/l trans-zeatin, 75 mg/l kanamycin, 300 mg/l timentin,

118 and 5.2 g/l Agargel. One week later, the explants were transferred onto 2ZK medium

119 containing IAA at either 0 mg/l, 0.01 mg/l, 0.05 mg/l, 0.1 mg/l, or 0.5 mg/l IAA.

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

125 Shoots were excised and transferred to selective rooting medium designated RMK (4.3 g/l
126 MS salts, 1 ml/l Nitsch vitamins (1000x), 30 g/l sucrose, pH 6.0, 8 g/l Difco Bacto agar (Becton,
127 Dickinson and Company, Franklin Lakes, NJ), 75 mg/l kanamycin, 300 mg/l timentin, and IAA at
128 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
130 media, the trans-zeatin, IAA, kanamycin, and timentin were dispensed from filter sterilized
131 stock solutions into autoclaved medium that was allowed to cool to 55°C. Cotyledon explant
132 cultures were transferred to freshly prepared medium every two weeks.

133

134 ***GUS histochemical assay***

135 Histochemical assay of β-glucuronidase (GUS) activity was performed on leaves from putative
136 transgenic and control (non-transformed) plants. Leaves were vacuum infiltrated for 20 – 30
137 min in buffer (0.8g/l 5-bromo-4-chloro-3-indolyl- β —D-glucuronide (X-Gluc), 0.1 M Na_2HPO_4,
138 0.1 M NaH_2PO_4 phosphate, 10 mM ethylenediamine tetraacetic acid (EDTA), 1.6 mM
139 potassium-ferricyanide and 1.6 mM potassium –ferrocyanide, 5% v/v Triton X-100, and 20% v/v
140 methanol) before incubation at 37°C overnight. The chlorophyll was removed from the leaves

141 by 3 - 4 washes with 70% ethanol at room temperature. The leaves were examined with a Leica
142 S8APO stereomicroscope outfitted with a digital camera.
143
144 ***Polymerase chain reaction analysis***
145 To confirm the presence of the neomycin phosphotransferase II selectable marker gene (*nptII*),
146 DNA was extracted from leaves of putative transgenic lines and controls (non-transformed)
147 with the Qiagen DNeasy plant mini kit (Hilden, Germany) as per the manufacturer's
148 instructions. Primers used to detect *nptII* were forward 5'-GGC TGG AGA GGC TAT TC-3' and
149 reverse 5'-GGA GGC GAT AGA AGG CG-3'. The diagnostic amplicon size expected with these
150 primers is approximately 700 bp. The PCR program started with a one-step cycle of 2 min at
151 95°C, followed by 29 cycles of 30 s at 94°C, 45 s at 57°C, 50 s at 72°C, and a 10 min final
152 extension at 72°C. DNA was separated and visualized by electrophoresis through a 1%
153 agaraose, ethidium bromide-stained gel.
154
155 ***Experimental Design***
156 A total of 5 different experiments were performed. Three biological replicates were used for
157 each IAA concentration in each experiment. A total of 750 cotyledon explants were used per
158 IAA concentration investigated. The standard error was calculated.
159
160
161
162

163 **Results**

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

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

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

181 In general, earlier emergence of well-developed shoots from *Agrobacterium*-infected
182 cotyledon explants on medium containing IAA translated to the recovery of whole rooted plants
183 in less time as compared with medium that did not contain IAA (Table 1). Medium containing
184 either 0.05 mg/l or 0.1 mg/l IAA resulted in the shortest time, 11 wks, for recovery of stable

185 transgenic lines. There appeared to be a threshold of IAA concentration and effect on recovery
186 time because at 0.5 mg/l IAA the time was similar to our standard method. We observed a
187 similar decrease in time when transformations of other tomato genotypes were performed by
188 different lab members who tested plant regeneration medium that contained 0.1 mg/l IAA
189 (data unpublished).
190

191 **Effect of IAA on transformation efficiency and rooting**

192 The formula below was used to calculate transformation efficiency (TE).

193 Transformation efficiency (%):

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

194
195
196

197 Overall, the TE was lower when medium containing IAA was used as compared to the TE of 88%
198 when medium containing trans-zeatin as the only growth regulator was used (Table 1).
199 When putative transgenic lines were approximately 3 - 4 cm tall, they were removed from
200 the cotyledon explants and transferred to either our standard selective rooting medium (RMK)
201 without IAA or RMK supplemented with 1 mg/l IAA. We chose this concentration based on
202 previous work with tomato transgenic lines recovered from a few genotypes that did not root
203 as well as M82 on our standard rooting medium (data not published). We observed that shoots
204 cultured on RMK supplemented with IAA resulted in the emergence of roots after 6 - 7 days as
205 compared to 11 - 14 days on medium without IAA. The addition of IAA to the medium did not
206 result in any phenotypic differences of the plants as compared to medium that did not contain
207 IAA.
208

209 ***Characterization of putative transgenic lines***

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

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

222

223 ***Modified protocol***

224 Based on our findings, we now follow a modified protocol as outlined in Figure 2 for all
225 *Agrobacterium*-mediated tomato transformations. IAA concentrations of 0.5 and 0.1 mg/l IAA
226 both resulted in a 6-week decrease for recovery of stable transformants, however, we chose to
227 use 0.1 mg/l IAA in our modified protocol because of the 54% transformation efficiency (Table
228 1). In addition to M82, we have applied this protocol to other tomato genotypes and also
229 observed a decrease in time for recovery of transgenic lines as compared to our previous
230 tomato transformation methodology (data not published).

231 **Discussion**

232 For development of stable transformation methodology, the foremost factors to be
233 considered are transformation efficiency and the time from infection with *Agrobacterium*
234 *tumefaciens* until the recovery of transgenic lines. Methods that provide both high efficiency
235 and the shortest time to recovery of transgenic lines lead to a high-throughput pipeline that
236 allows earlier evaluation of gene function. In turn, a high-throughput pipeline decreases the
237 amount of labor and resources needed, which can translate into significant financial savings.

238 The focus of our study was to investigate medium components that had the potential to
239 decrease the time for recovery of stable tomato transgenic lines. Our standard method, which
240 has a high transformation efficiency at approximately 90%, takes 17 weeks for recovery of
241 transformants. The interest in optimization of our methods stemmed from an increased need
242 for transgenic lines because tomato has become the model species of choice for many studies
243 that include ripening, abiotic and biotic tolerance, and nutritional content [23-26]. In addition,
244 with the recent demonstration of successful genome editing by CRISPR/Cas9 in tomato, the
245 interest in applying this technology for the study of gene function will increase [16, 17, 27].
246 Therefore, a transformation methodology that can deliver modified lines in a shorter time
247 frame will help to advance these studies.

248 Our standard protocol is a modified version of methods reported by Fillatti et al. [8] in
249 which zeatin is the only growth regulator incorporated into the plant regeneration medium
250 [12]. We chose to start our investigation by examining additional growth regulators that, in
251 combination with zeatin, would greatly reduce the time for recovery of stable transgenic lines
252 but not have a significant negative effect on transformation efficiency. In a literature search,

253 we found several reports that demonstrated a positive effect on tomato plant regeneration and
254 transformation efficiency when indole-3-acetic acid (IAA) was incorporated into zeatin-
255 containing plant regeneration medium [10, 28, 29]. However, they did not report any effects
256 observed on the time required to recover transgenic plants.

257 We found that addition of either 0.05 or 0.1 mg/l IAA to our standard plant regeneration
258 medium that contains trans-zeatin as the only growth regulator decreased the time for
259 recovery of stable transgenic lines from 17 to 11 weeks. Previous reports have demonstrated
260 that shoot apical meristem development involves interactions among cytokinin signaling
261 pathway components, auxin, and several families of transcription factors [18]. It is possible that
262 the addition of IAA to our standard plant regeneration medium facilitates interactions among
263 the cytokinin signaling and auxin regulated genes, which results in faster shoot development
264 from the cotyledon explants.

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

270 In addition to supplementation of the standard plant regeneration medium with IAA, we
271 also investigated effects of adding IAA to the rooting medium, which was not a component in
272 our standard rooting medium. Inclusion of IAA in *in vitro* rooting medium has been reported
273 for tomato, however, it is not routinely added because tomato readily develops roots in culture
274 medium without growth regulators [9]. Our interest was to determine if supplementation

275 decreased the time to rooting, which we did observe. Auxin is produced in both shoots and
276 roots and the auxin produced in the roots helps in root development [30-32]. It is possible that
277 IAA, when exogenously added, increases the levels of auxin in the plants, hence resulting in the
278 cells differentiating earlier to form roots. However, research needs to be conducted to confirm
279 this hypothesis.
280

281 **Conclusions**

282 Interest in tomato as a model has increased over the years and we have seen a rise in the
283 number of research groups that require stable transgenic lines for various studies.
284 Modification of our standard plant regeneration medium through the addition of either 0.05 or
285 0.1 mg/l AA shortened the recovery of transgenic lines by 6 weeks for the M82 tomato cultivar.
286 Application of this modification for transformation of other tomato genotypes in our lab also
287 resulted in a decreased time for recovery of stable transgenic lines.
288 A shorter recovery time for stable transgenic lines is highly desirable for functional studies to
289 allow earlier determination of the genes and networks involved in phenotypes of interest. A
290 decrease in recovery time would also provide a higher throughput process, which has the
291 potential for cost savings related to labor and resources. Optimization studies of standard
292 transformation methodologies for different plant species should always be considered in order
293 to alleviate bottlenecks for generation of stable transgenic lines [33]. Availability of efficient
294 transformation methods is especially critical with the rapid development of genome editing
295 technologies, which will result in an increased demand for generation of transgenic lines for
296 basic research studies that can lead to crop improvement.

297 **Competing interests**

298 The authors declare that they have no competing interests.

299

300 **Authors' contributions**

301 SG and JVE designed the experiments, SG performed the experiments, SG and JVE wrote the

302 manuscript. Both authors read and approved the manuscript.

303

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309

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398

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400

401 **Figure Legends**

402 **Figure 1**

403 **Results for the recovery of *Solanum lycopersicum* cv M82 stable transgenics from**

404 ***Agrobacterium tumefaciens*-infected cotyledon explants cultured on plant regeneration**

405 **medium supplemented with different concentrations of indole-3-acetic acid (IAA).** (A)

406 *Agrobacterium tumefaciens*-infected cotyledon explants (approximately 5 weeks post infection)

407 cultured on selective plant regeneration medium containing the following amounts of IAA in

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

409 infected controls for each IAA concentration, respectively.

410 (B) Histochemical analysis for GUS expression in leaves taken from independent transgenic lines

411 designated 1 - 9 recovered from selective plant regeneration medium that contained 0.1 mg/l

412 IAA. GUS expression was not observed in the non-transformed controls.

413 (C) Agarose gel of PCR products showing the expected ~700 bp product amplified from the *nptII*

414 selectable marker gene in 10 independent transgenic lines (lanes 1 – 10). These lines were

415 recovered from selective plant regeneration medium that contained 0.1 mg/l IAA. C = the

416 control

417

418

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420

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422

423 **Figure 2**

424 **Schematic representation of the optimized *Agrobacterium tumefaciens*-mediated**

425 **transformation methodology for *Solanum lycopersicum* cv M82.** See the Materials and

426 Methods for details on seed sterilization and all media compositions.

427

428 **Tables**

429 **Table 1: Results for recovery of stable transgenic lines of *Solanum lycopersicum* cv M82 from**

430 ***Agrobacterium tumefaciens*-infected cotyledon explants cultured on selective plant**

431 **regeneration medium supplemented with 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

432

433 *Average transformation efficiency was calculated as percent of stable transgenic lines

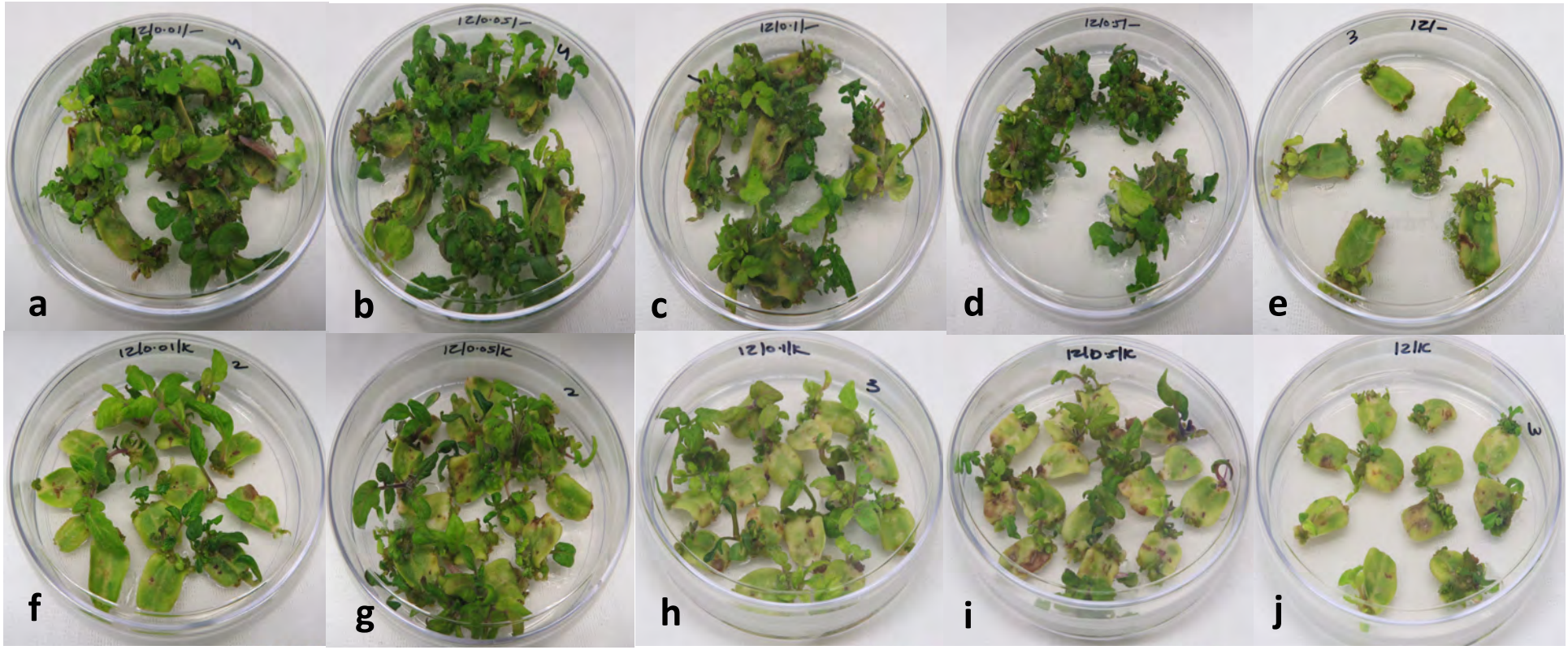
434 recovered from the total number of cotyledon explants infected with *Agrobacterium*

435 *tumefaciens*. Transformation efficiency values shown are the average from 5 experiments ± the

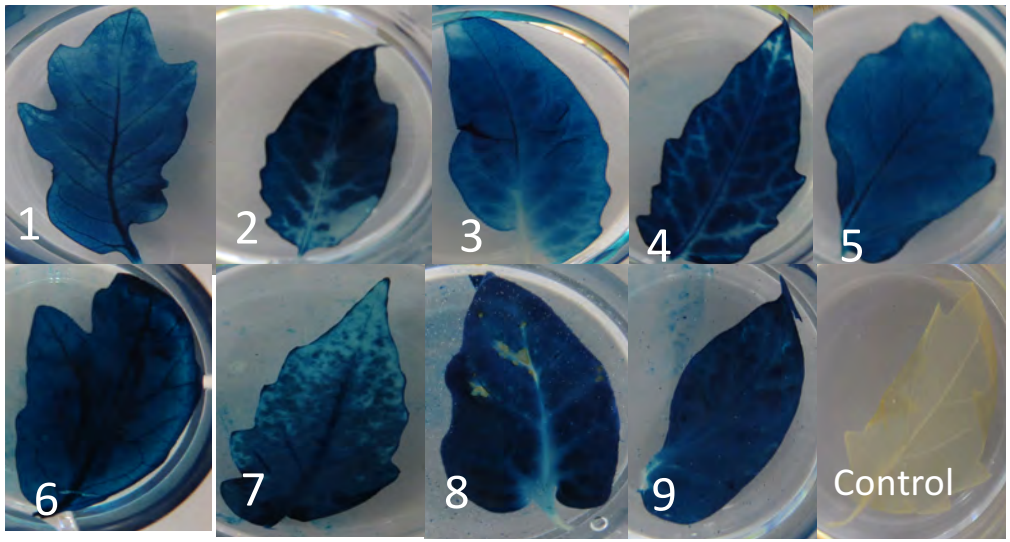
436 standard error (SE) calculated from 3 biological replicates.

Gupta and Van Eck_Figure 1

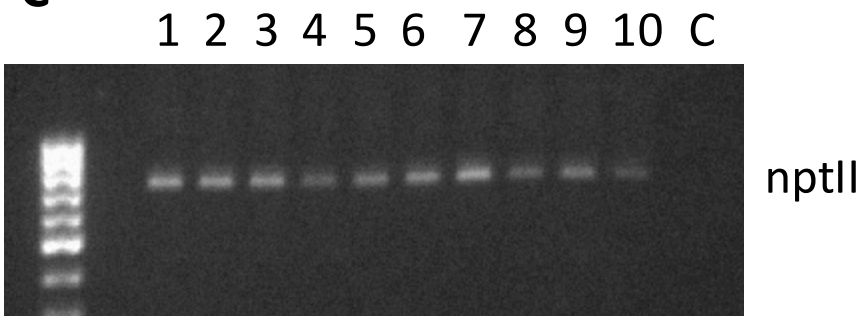
A



B



C



Gupta and Van Eck_Figure 2

Germination of sterilized seeds *in vitro*

6 days ↓

Preparation of explants from cotyledons

24 hrs ↓

Incubation in *Agrobacterium* suspension

5 min ↓

Co-cultivation of explants

2 days ↓

Transfer explants to 2ZK selective plant regeneration medium

1 wk ↓

Transfer explants to 2ZIK selective plant regeneration medium

2 wks ↓

Transfer explants to 1ZIK selective medium

~4 wks ↓

Remove shoots from explants transfer to RMIK

~3 wks ↓

Well-rooted plants