1   Modification of plant regeneration medium decreases the time for recovery of *Solanum*

2   *lycopersicum* cultivar M82 stable transgenic lines

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10

11   **Abstract**

12   **Background:** Tomato (*Solanum lycopersicum*) has rapidly become a valuable model as a result

13   of the availability of a high quality reference genome, extensive genetic resources, and efficient

14   gene transfer methodology. A high-throughput method to obtain transgenic lines sooner than

15   standard methods has the potential to greatly advance gene function studies.

16

17   **Results:** The goal of this study was to optimize our current gene transfer (transformation)

18   method for tomato by investigating medium components that would result in a decreased time

19   for recovery of transgenic lines. The methodology reported here is based on infection of 6-day-

20   old cotyledon explants from *Solanum lycopersicum* cultivar M82 *in vitro*-grown seedlings with

21   *Agrobacterium tumefaciens* strain LBA4404 containing the binary vector pBI121. This vector

22   contains the β-glucuronidase reporter gene and the neomycin phosphotransferase II selectable

23   marker gene that confers resistance to kanamycin. Modification of our standard plant

24   regeneration medium by the addition of indole-3-acetic acid (IAA) at concentrations of either

25   0.05 mg/l or 0.1 mg/l greatly decreased the time for recovery of transgenic lines by 6 weeks as

26   compared to our standard medium that contains zeatin alone. Moreover, addition of 1 mg/l

27   IAA to the root induction medium resulted in faster root development. We observed 50% and

28   54% transformation efficiency when plant regeneration medium containing 0.05 mg/l and 0.1

29   mg/l IAA, respectively, was used for transformation experiments. Transgenic lines recovered

30   with the optimized method were similar in development to our standard method. No negative

31   effects were observed as a result of the addition of IAA to the medium.

32

33   **Conclusions:** The results showed that addition of IAA at concentrations of either 0.05 or 0.1

34   mg/l to the plant regeneration medium reduced the total time for recovery of stable transgenic

35   lines by 6 weeks. The ability to recover transgenic lines in a shorter time results in higher

36   throughput for the introduction of gene constructs and has the potential to decrease the time

37   needed to complete investigations of gene function. The ability to recover transgenic lines for

38   each gene construct in a shorter time saves labor and resource expenses because culture

39   maintenance is also reduced.

40

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

42   *pimpinellifolium*, tomato

43

44   **Background**

45   Tomato, *Solanum lycopersicum*, is a member of the *Solanaceae* family, which contains

46   approximately 3,000 plant species and includes some of the most economically important food

47   crops. It is native to South America and was brought to Europe in the 1500s and then to North

48   America in the 1800s [1]. Tomato is a perennial plant that has two different growth habits,

49   determinate and indeterminate. There are two different market types of tomatoes, fresh

50   market and processing. According to the Agricultural Marketing Resource Center, in 2014 the

51   US dollar value for fresh market tomatoes was 1.14 billion and 1.325 billion for processing

52   types, which are used to make products such as juice, sauces, and ketchup [2]. In addition to

53   being an economically important food crop, tomato is an excellent source of health beneficial

54   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 uE 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 uE 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$_2$HPO$_4$,

138   0.1 M NaH$_2$PO$_4$ 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.

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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 (%):
194   Total number of rooted shoots   x 100
195   Total number of cotyledon explants infected with *Agrobacterium*
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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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

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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.

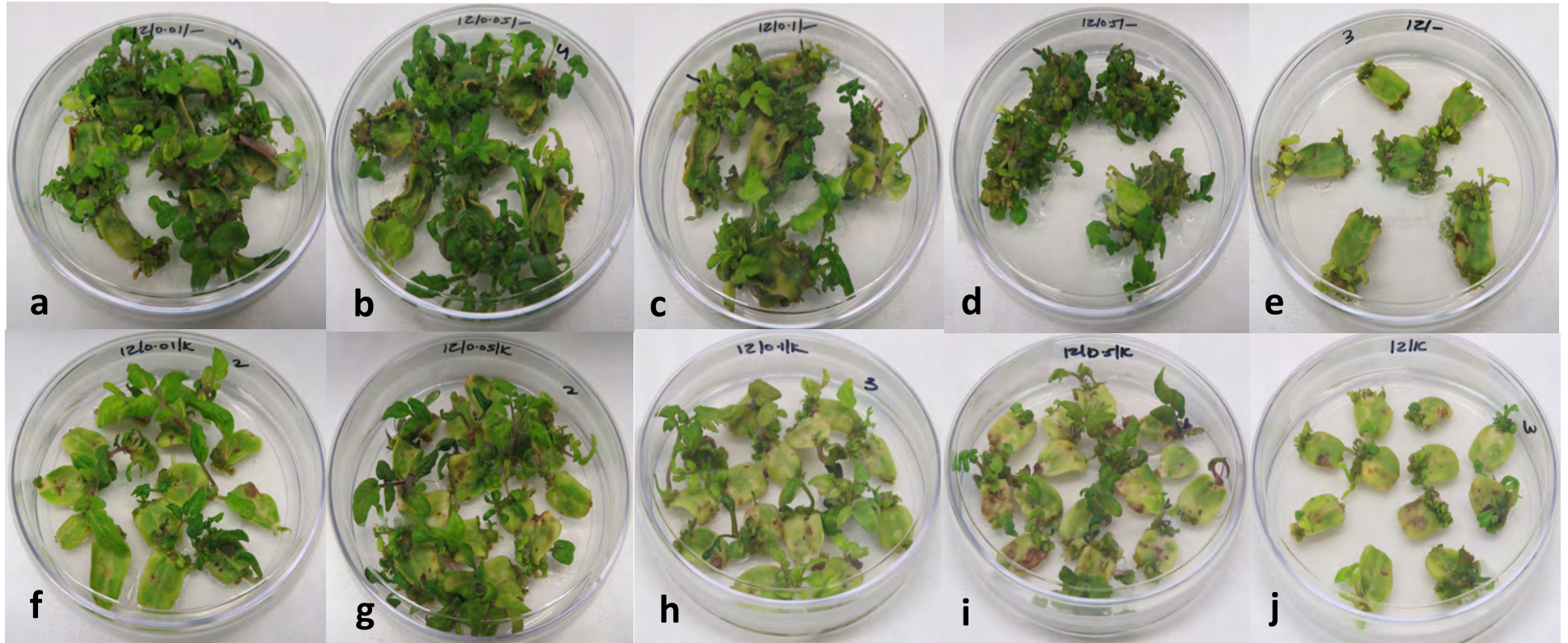427

428   **<u>Tables</u>**

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.**

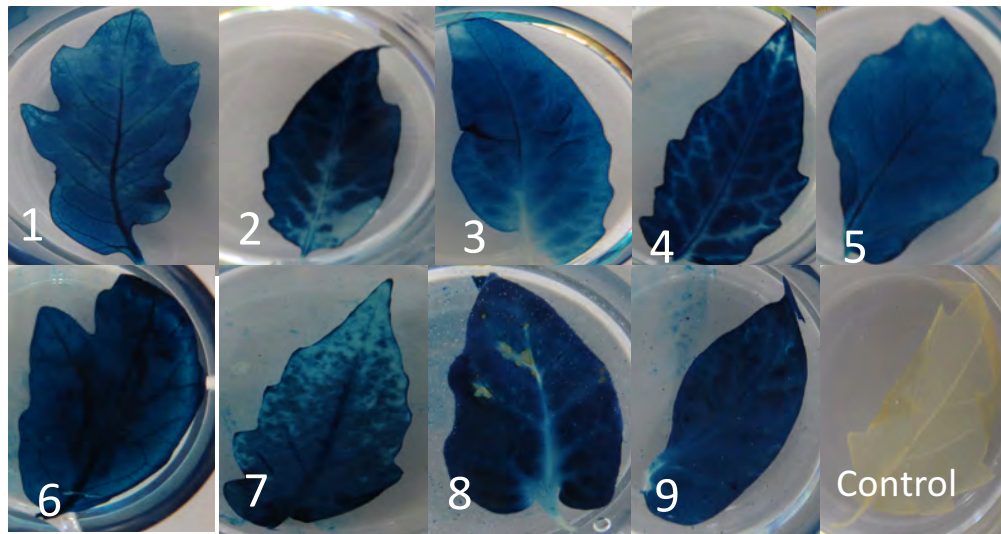| IAA (mg/l)	Total number explants	Total number rooted plants	Average transformation efficiency (+) SE*	Total time for recovery of transgenic lines (wks)
0	750	660	88 + 2.2	17
0.01	750	390	52 + 1.0	15
0.05	750	375	50 + 1.5	11
0.1	750	405	54 + 1.2	11
0.5	750	360	48 + 2.0	16

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

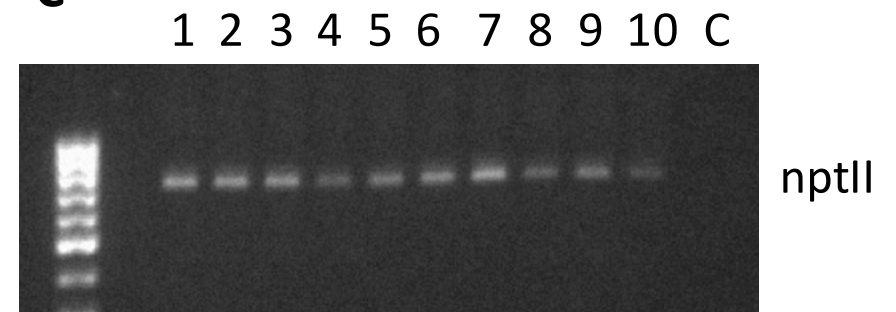436   standard error (SE) calculated from 3 biological replicates.

Gupta and Van Eck_Figure 1

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