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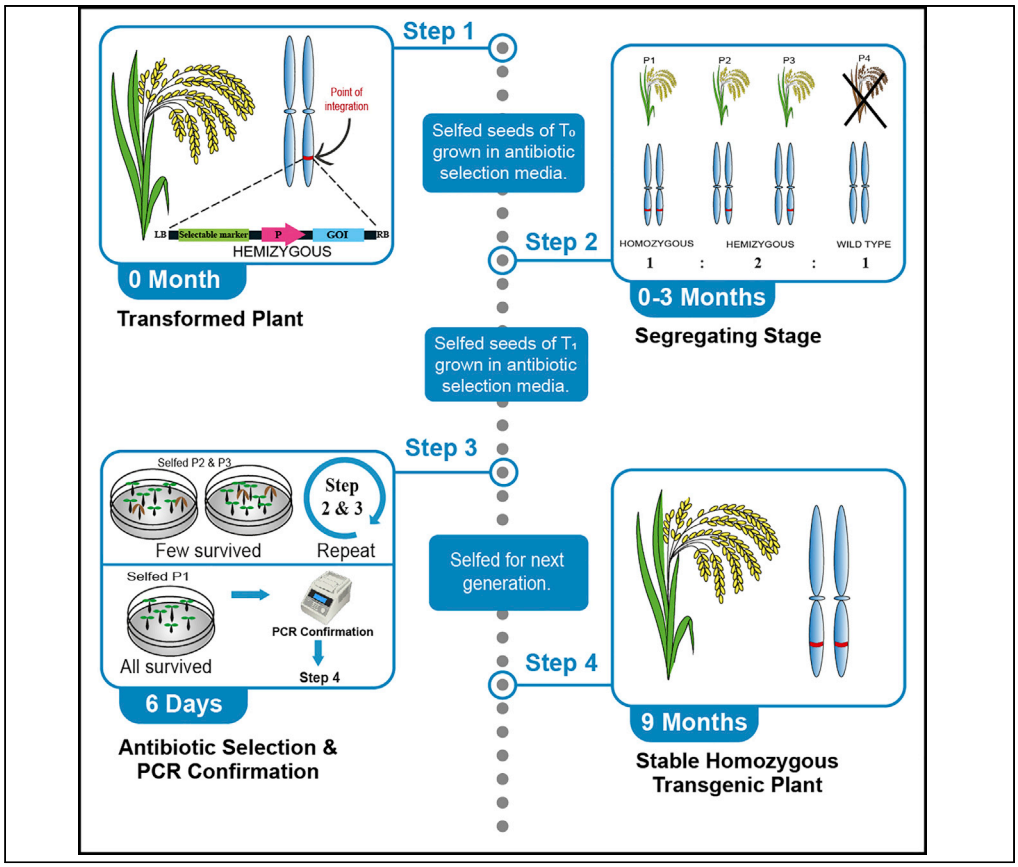
Assessment of homozygosity in transgenic plants using selectable markers



Production of homozygous transgenic plants is a prerequisite for the phenotypic analysis and/or for the commercial release of transgenic plants for cultivation. Here we present a simple protocol for the selection of homozygous transgenics using antibiotics as a selectable marker. The protocol has been used to select homozygous rice transgenic plants using hygromycin antibiotic. However, the described protocol can be used for selction of homozygous in any transgenic plants using a appropriate selectable marker.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Generation of transgenic plants using agrobacterium-mediated transformation

Selection of transformed plants on antibiotic medium

Confirmation of transgenics by using polymerase chain reaction

Identification of stable homozygous transgenic plants

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Assessment of homozygosity in transgenic plants using selectable markers

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SUMMARY

Production of homozygous transgenic plants is a prerequisite for the phenotypic analysis and/or for the commercial release of transgenic plants for cultivation. Here we present a simple protocol for the selection of homozygous transgenics using antibiotics as a selectable marker. The protocol has been used to select homozygous rice transgenic plants using hygromycin antibiotic. However, the described protocol can be used for selction of homozygous in any transgenic plants using a appropriate selectable marker.

For complete details on the use and execution of this protocol, please refer to Passricha et al. (2016).[1]

BEFORE YOU BEGIN

In the recent decade, the production of transgenic plants is common for research and commercial purpose. In the study of transgenic plants, the selection and confirmation of homozygous, non-segregating, stable transgenic lines are a prerequisite for any downstream studies. A diploid plant is homozygous when the two identical alleles of the gene are present at the same locus of the homologous chromosome. The transgenic plant is called homozygous for a gene of interest (GOI) when both alleles of the GOI at a given locus are the same.

During transgenic plant production (Sahoo et al.),[2] after transforming the GOI into the plants either by Agrobacterium-mediated transformation or biolistic gun method, the regenerated plants obtained are called putative independent transgenic events (named as T_0 plants) and these will be hemizygous for the GOI (Figure 1). To maintain consistency of the transgene phenotype over subsequent generations it is essential to obtain stable homozygous transgenic lines for these transgenic events.

There is a range of conventional and advanced techniques described by Passricha et al.[1] to confirm the zygosity of the transgene. In our protocol, we will explain in detail a simple and cost-effective method of screening transgenic homozygous lines using antibiotics as a selectable marker. The basis of antibiotic selection is that the selectable marker (an antibiotic resistance gene) co-segregates along with the GOI and follows the Mendel law of segregation during progeny production. As shown in Figure 1, along with the GOI, the selectable marker will also integrate into the genome of the plant and the T_0 plants will be hemizygous for both the GOI and the antibiotic resistance gene. The self-pollinated T_0 plants produce the segregating population of the progeny (T_1 generation) with a phenotypic ratio of 3:1 and a genotypic ratio of 1:2:1 (Figure 2). Based on this principle, the collected T_1 seeds from the T_0 plants will be germinated on the MS medium containing the appropriate

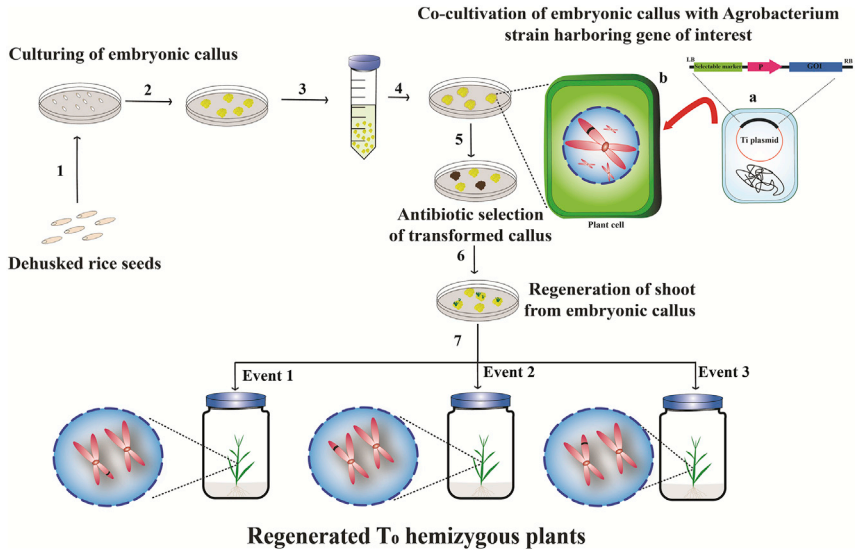

Figure 1. Illustration of Agrobacterium-mediated gene transfer in Nipponbare rice callus

1. Place dehusked mature rice seeds onto the callus induction media, **2**. Incubate seeds for callus induction, **3.** Co-cultivate the callus with agrobacterium containing the gene of interest (GOI) in MS bacterial suspension media, **4.** Place the co-cultivated callus on the medium and allow it for agrobacterium infection (a. Agrobacterium harbor gene of interest, b. Infection and transferring of gene of interest in plant cell) **5.** Selection of transformed callus on antibiotic selection media (media with hygromycin), **6.** Shoot regeneration from different transformed calli on antibiotic media **7.** Transferring each shoot arising from a different callus into rooting media these regenerated T_0 plants with different events were further taken for PCR screening for GOI. **Events 1,2,3:** These are the individual transgenic plants generated from independent callus where the point of integration GOI would be in a different location of the chromosomes. P- Promoter, GOI- Gene of interest. For more details on rice transformation please refer to Sahoo et al.[2]

antibiotic (Based on the type of antibiotic resistance gene present on the vector used in GOI transformation) for further selection.

Antibiotic selection media preparation

Timing: 2 h

Weigh 2.2 g MS (Sigma-Aldrich, Cat# M0404) and dissolve in 1 L of distilled water to get a half-strength MS medium.

1. Add 5 g of Agar (0.5%) (Himedia, GRM666-500G).
2. Autoclave at 121°C for 20 min at 15 lbs pressure (Equitron Autoclave).
3. Add hygromycin (50 mg/L) (Himedia PCT1503-20ML) to the media when it is warm (45°C).
4. Mix well and pour it into Tarson's plantons (glass container) and allow it for solidification.

Note: 1. Since antibiotics (hygromycin/kanamycin) are heat sensitive, make sure to do filter sterilization using a 0.2 μm sterile filter) and store them under −20°C. 2. Add antibiotic after autoclaving.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Murashige and Skoog basal medium with Gamborg's vitamins	Sigma-Aldrich,	Cat# M0404

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bleach (6%)	Oxford Chemicals	Cat# 08148
Tween-20	Sigma-Aldrich	Cat# P1379-100ml
Ethanol 99.9%	Changshu Hongsheng Fine Chemical Co., Ltd	Lot No. 20220303
Agar-Agar	Himedia	Cat# GRM666500G
Hygromycin B Solution	Himedia	Cat# PCT150320ML
Biological samples		
Matured rice seed (*Oryza sativa* cv. Nipponbare)	https://www.ars.usda.gov	N/A
Agrobacterium strain with construct	(Pospíšilová et al.)[3]	N/A
Other		
3M micropore tape	3M Healthcare	Cat# 1530
Microtips	Tarsons	Cat# T521000
Micropipette	Tarsons	Cat# 030090
Nylon syringe filters (0.2 μm)	Himedia	Cat# SF127-50NO
Paper bag	N/A	N/A
Centrifuge tubes-50 mL	Tarsons,	Cat# T546041
Stainless forceps LA821-2no	Tarsons	Cat# LA821-2NO
Reagent bottle-1000 mL	Borosil	Cat# 1501029
Blotting paper		
Plant growth chamber	Conviron	GEN-1000
Shaker incubator	Scigenics Biotech	Orbitek incubator
Laminar hood	Microfilt	N/A
Weighing balance	Shimadzu	AUX220
Autoclave	Equitron medica private limited	N/A
Polyhouse	Dept of Biotechnology & Bioinformatics, JSSAHER, Mysuru	N/A

MATERIALS AND EQUIPMENT

Antibiotic selection media		
Reagent	Final concentration (per liter)	Amount
MS basal salts (4.4 g/L)	Half strength	2.2g
Agar agar	0.5%	5g
Hygromycin B stock (50 mg/mL)	50 mg	1 mL
ddH_2O	N/A	999 mL
Total	**N/A**	**1,000 mL**

⚠ CRITICAL: Add hygromycin to the media after autoclaving.

STEP-BY-STEP METHOD DETAILS

T_0 generation

Timing: 3 months

This section describes the production and confirmation of T_0 transgenic plants.

Select the transformed calli in a growth medium containing antibiotic (Figure 1, step 5).

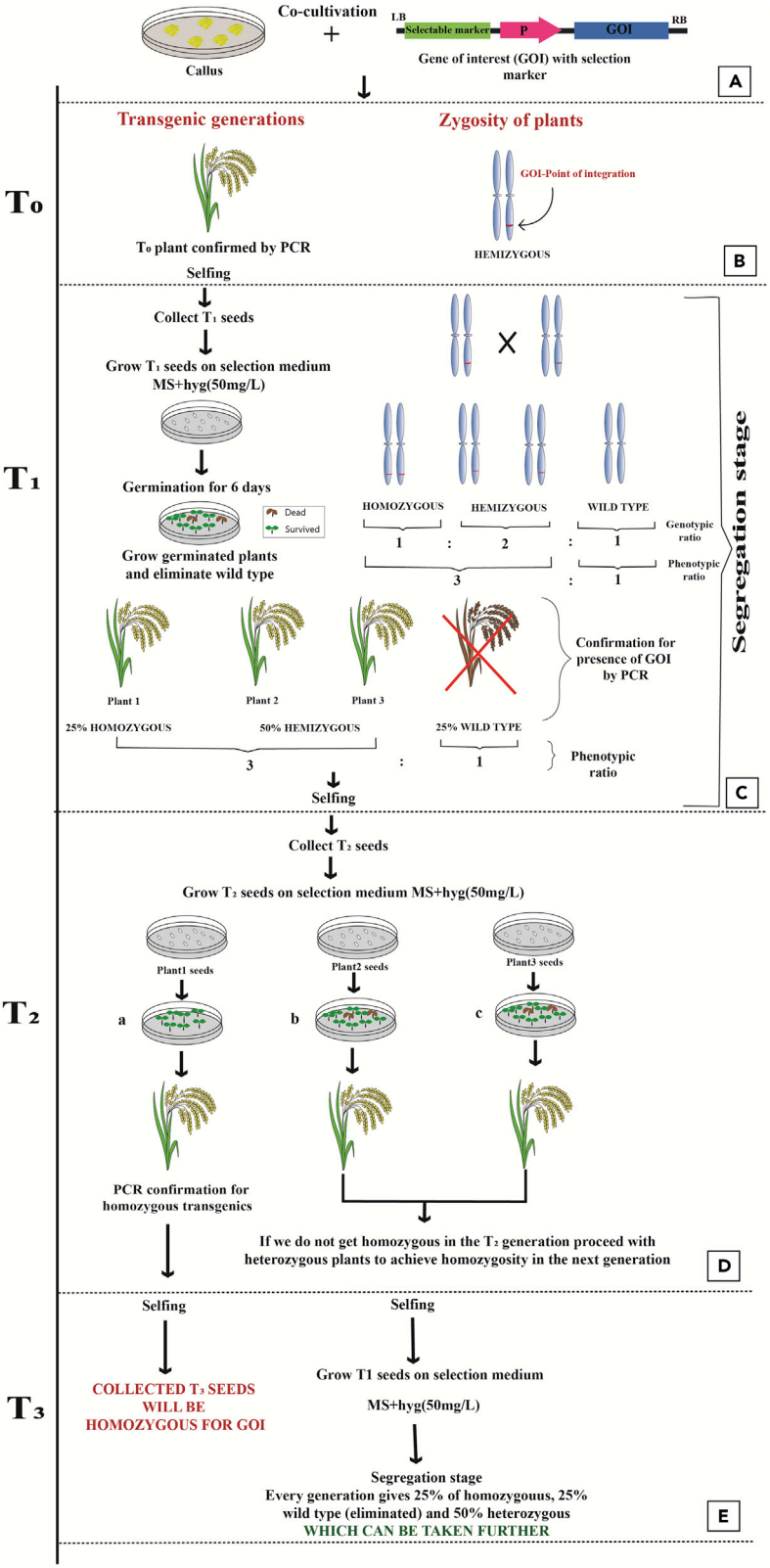
Co-cultivation
+
LB
Selectable marker
P
GOI
RB
Callus
Gene of interest (GOI) with selection marker
A
Transgenic generations
Zygosity of plants
T0
T0 plant confirmed by PCR
GOI-Point of integration
HEMIZYGOUS
Selfing
B
Collect T1 seeds
Grow T1 seeds on selection medium MS+hyg(50mg/L)
T1
Germination for 6 days
Dead
Survived
Grow germinated plants and eliminate wild type
HOMOZYGOUS
HEMIZYGOUS
WILD TYPE
1 : 2 : 1
Genotypic ratio
3 : 1
Phenotypic ratio
Segregation stage
Plant 1
Plant 2
Plant 3
Confirmation for presence of GOI by PCR
25% HOMOZYGOUS
50% HEMIZYGOUS
25% WILD TYPE
3 : 1
Phenotypic ratio
Selfing
C
Collect T2 seeds
Grow T2 seeds on selection medium MS+hyg(50mg/L)
T2
Plant1 seeds
Plant2 seeds
Plant3 seeds
a
b
c
PCR confirmation for homozygous transgenics
If we do not get homozygous in the T2 generation proceed with heterozygous plants to achieve homozygosity in the next generation
D
Selfing
Selfing
T3
COLLECTED T3 SEEDS WILL BE HOMOZYGOUS FOR GOI
Grow T1 seeds on selection medium
MS+hyg(50mg/L)
Segregation stage
Every generation gives 25% of homozygouus, 25% wild type (eliminated) and 50% heterozygous
WHICH CAN BE TAKEN FURTHER
E

Figure 2. Schematic representation of a selection of stable homozygous transgenic rice plants using antibiotic as a selectable marker

(A) Coinfection/Cocultivation of callus with agrobacterium containing gene construct results in the generation of transformed callus (T_0) on antibiotic selction medium.

(B) T_0 plants are in hemizygous condition and are self-pollinated to the next generation.

(C) T_1 seeds are collected from selfed T_0 plants and selected to eliminate wild type on antibiotic selection media (1/2MS+ 50 mg/L hyg).

(D) Selected T_1 seeds are germinated on antibiotic selection medium and plants grown (T_1 plants) are self-pollinated to get T_2 seeds. T_2 seeds are collected from T_1 plants and selected on antibiotic media (1/2MS+ 50 mg/L hyg) for a zygosity test (hemizygous or homozygous seeds will germinate while wild-type seeds will be eliminated).

(E) Germinated T_2 seeds are grown and self-pollinated for the next generation to give T_3 seeds. If all T_2 seeds were germinated on antibiotic media then T_3 seeds are considered as stable homozygous for the GOI. If only a few T2 seeds germinate then it indicates heterozygous conditions (segregating line). These segregating T2 plants are self-pollinated and taken to the next generation for getting homozygous plants for the GOI. GOI- Gene of interest, 1/2MS-half-strength Murashige and Skoog, hyg- Hygromycin, LB: left border, RB-right border, P-promoter.

1. Grow the antibiotic-resistant calli on the regeneration medium to obtain T_0 plants (Figure 1, steps 6 and 7).
2. Transfer the two weeks old putative transgenic seedlings to polyhouse for further growth.
3. Extract the DNA from T_0 plants by using the CTAB method or by using a commercial plant DNA extraction kit.
   a. Use the extracted DNA to confirm the presence of the transgene (GOI) in the T_0 plants by PCR using specific primers as described in Abdalla.[4]
   b. Use any constitutively expressed gene (e.g., Actin/Ubiquitin) as an endogenous positive control.

Note: During gene transformation in plants the transgene can integrate randomly into the host plant genome. The inserted GOI can interfere with other parts of the host genome; coding regions, non-coding regions, epigenetic regions etc. Also, the expression level of the transgene varies with its location (Euchromatin or Heterochromatin) of the insertion. Thus, it is highly important to select at least 3–6 independent transgenic events (Figure 1, step 7) to obtain homozygous lines to make sure the phenotype is due to the GOI and not because of the above-mentioned possibilities of interference by GOI.

T_1 generation

Timing: 8 days

This section describes the segregation pattern in T_1 seeds.

4. From the PCR confirmed independent events of T_0 plants, collect independently the matured T_1 seeds from each plant (Figure 2).
5. Incubate the collected T_1 seeds for drying at 37°C for 48 h and store them in a paper bag at room temperature with proper labeling till further use.

⚠ CRITICAL: While harvesting seeds from different plants, to avoid any cross-contamination of seeds, make sure to label each paper bag properly with details including the name of the gene construct, transgenic generation, and collection date.

6. Prepare about 100 mL $^1/_2$ strength MS media with 0.5% agar) in 2 Tarson's plantons (glass container each and autoclave. After the autoclave, add the appropriate antibiotic (hygromycin-50 mg/L) and mix properly and allow for solidification.
7. Take about 30 T_1 dehusked (manually remove the husk from the seed without damaging the embryo) seeds from independent events and 10 wild Type (WT) dehusked seeds in separate 15 mL tubes.

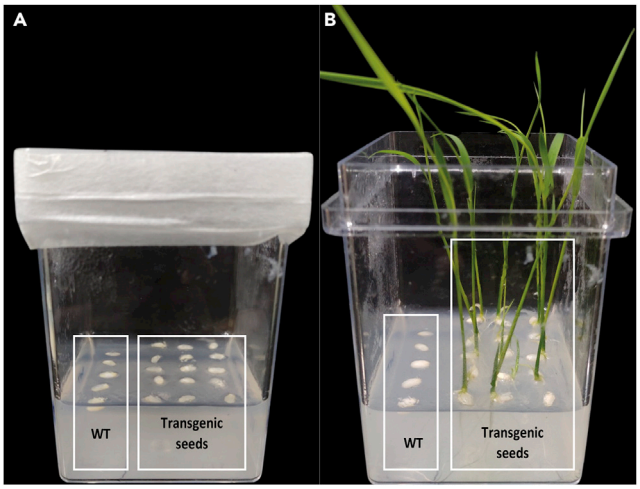

Figure 3. Phenotype of WT and transgenic plants on selection medium

(A) Sterilized WT and transgenic seeds placed on selection media containing antibiotic.

(B) 10 days old survived transgenic plants on selection medium.

8. Surface sterilize the seeds with 70% ethanol for 90 s followed by 2% of sodium hypochlorite with 0.05% of Tween 20. Keep the tubes on a rotary shaker for 1 h at 180 rpm. Rinse the seeds with sterile distilled water 3–4 times to remove sodium hypochlorite completely. Dry the seeds on sterile blotting paper to remove the moisture content.
9. Place the sterilized T_1 seeds and WT seeds (as a negative control) in the plantons containing selection media $^1/_2$ MS + hygromycin 50 mg/L (Figure 3A).

⚠ CRITICAL: Ensure forceps used for placing sterile seeds are sterilized properly to avoid contamination.

10. Keep the plantons for seed germination at 27°C for 6 days under controlled conditions in the plant growth chamber (light: dark period 10:14 h, relative humidity 60%, and light intensity 230 $\mu M/s^{-1}$).
11. After 6 days count the number of germinated seedlings of the T_1 population (Figure 3B).
    a. As described above, if the antibiotic-resistant gene follows Mendelian segregation, 70%–80% of the seedlings' will survive on the selection plate (In our case we observed that out of 30 seeds 22 survived and the rest along with WT did not germinate/survive i.e., a 73% survival rate was observed).
    b. If all the T_1 seeds survive on the selection medium, such an event should be considered as multiple transgene insertion lines.

Note: Mendelian law of segregation: Plant transformation results in the development of different types of transgenic events including, the production of hemizygous condition for the gene of interest (GOI), multiple insertions of the transfer DNA (t-DNA), lethality, male gametophytic lethal, and female gametophytic lethal phenotypes. All these different types of events can give rise to different mendelian segregation. This protocol holds good for single-copy gene insertion which would be in hemizygous status. Please note, this protocol cannot be used if the transgene insertion leads to lethality in the target plant.According to the law of segregation, 'A diploid individual possesses a pair of an allele for any particular trait and each parent passes the alleles randomly to its next generation. Here GOI corresponds to the dominant trait and therefore its inheritance to the next generation leads to the formation of a 1:2:1 phenotype ratio and genotypic ratio of 3:1. This ratio gives 25% homozygous for GOI, 50% hemizygous for GOI (segregating population), and 25% for the homozygous wild type.

12. The 70%–80% of survived seedlings contain a mixture of homozygous (25%) and hemizygous seedlings (50%) in the ratio 1:2:1 (Figure 2).

T_2 generation

Timing: 3 months

This step explain the procedure to differentiate and select homozygous plants on selction medium.

13. Grow the surviving T_1 seedlings (about 20) individually (Label them as Plant 1(P1) to plant 20 (P20) in the polyhouse and collect the next generation seeds (T_2) separately from the individual T_1 plants.
14. Grow the collected T_2 seeds (About 30–40) from the individual T_1 plants (P1-P20) separately along with the WT seeds on the antibiotic selection medium (Repeat steps 7–9).
15. After 6 days of incubation in the plant growth chamber, count the number of surviving seeds from the individual plants. Look for the selection plate where all the seeds have survived (100% survival).
16. Out of 20 T_2 seed populations, at least 6–7 of them will show 100% survival on the selection medium.
17. The Petri plate in which all the T2 plants survived can be considered as the homozygous transgenic line for the GOI.

T_3 generation

Timing: 3 months

This step is used for mass multiplication of selected homozygous plants.

18. The T_2 homozygous plants (PCR confirmed) will be grown in the greenhouse for seed multiplication and to collect the homozygous stable T_3 transgenic seeds.

EXPECTED OUTCOMES

In the T_2 generation, stable homozygous transgenic plants are expected. If they are not in the T_2 generation, hemizygous plants are selected and taken for subsequent generations to get homozygous plants.

LIMITATIONS

This protocol holds good if there is only single gene insertion that follows the mendelian law of segregation. End user may require performing NGS (Next generation sequencing) to rule out the possibilities that plants does not contain any other pieces of the T-DNA elsewhere in the genome without the antibiotic resistant gene.

TROUBLESHOOTING

Problem 1

Wild-type seeds showing germination on antibiotic selection media.

Potential solution

Always use fresh antibiotic stock and recheck the proper concentration of the antibiotic used in the preparation of selection media.

Make sure that you use fresh stock of WT seeds and that they are truly WT i.e., they are not mixed up with any other transgenic events.

Problem 2
No transgenic seeds are showing germination.

Potential solution
Probably the insertion of GOI has caused the gametophytic lethality. Therefore, it is recommended to have enough (at least 3–6) 3 independent transgenic events.

Problem 3
Sterile seeds may sink to the bottom of the glass bottle as they are being placed on the selection media.

Potential solution
The concentration of agar in the medium is critical. It must be in a semi-solid state. Appropriate agar concentration in the medium should be 0.5%–0.6%.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr Mohan T. C mohantc@jssuni.edu.in.

Materials availability
The current study has not been deposited in a public repository because the study is in progress, but related information is available from the corresponding author on request.

Data and code availability
This study did not generate/analyze [datasets/code].

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AUTHOR CONTRIBUTIONS
M.T.C. and C.R. designed the experiments and wrote the manuscript. S.S.K. performed the experiments. M.T.C., C.R., and S.S.K. analyzed the data. S.S.K., A.R.B., and S.H. contributed to the illustrations. All the authors have read and approved the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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