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Optimisation of *Agrobacterium*-Mediated Seedling Leaf Transformation Across Multiple Sorghum Genotypes

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To Editor,

Genetic transformation in sorghum faces major challenges, including limited explant availability, phenolic compound accumulation, and strong genotype dependence. Morphogenic transcription factors (MTFs), such as *Baby boom* (*Bbm*) and *Wushel2* (*Wus2*), markedly improve transformation efficiency using seedling leaf whorls. The *Cre*/loxP system subsequently removes MTF genes after somatic embryogenesis, enabling fertile plant regeneration (Wang et al. 2023). This scalable strategy performs well in Tx430 (Butler et al. 2025), though phenolic interference continues to restrict broader genotype adoption.

In this study, we present an *Agrobacterium*-mediated MTF-assisted protocol using seedling leaf whorl in 13 economically important sorghum genotypes. This work includes the first successful report for several of these genotypes. Additionally, we demonstrate CRISPR-Cas9-mediated editing of the *glossy2* (*gl2*) gene using the same explant system. Finally, we evaluate alternate visual markers, *RUBY* (He et al. 2020) and free-use GFP (*fuGFP*; Coleman and Somerville 2019), for transformation tracking.

Thirteen sorghum genotypes were selected to evaluate the *Agrobacterium*-mediated seedling leaf whorl transformation protocol (Supporting Information). Tx430 served as a positive control due to its established transformability and low phenolic compound production; Tx623 represents the reference genome; IA100RPS and IA101RPS are sister inbred lines developed for biomass breeding in the northern latitudes (Salas-Fernandez and Kemp 2022). The remaining nine genotypes are part of the Sorghum Association Panel (SAP), representing broad genetic diversity and agronomic relevance.

Figure 1a summarises the transformation frequency (TF) and efficiency (TE) across the 13 genotypes. In this study, TF is the percentage of rooted regenerants per total number of infected leaf whorl explants. Using the standard eight-step procedure (Supporting Information; Figure S1), Tx430 consistently produced regenerants at a high frequency, and the transgene was stably inherited into the next generation (Figure S2). Across 10 independent experiments involving 115 explants, a total of 483 regenerants were obtained, yielding a TF range of 33% to 3620% with an average of 420% ± 230% (mean ± SE; Figure 1a). Similar trends were observed in IA101RPS, ICSV400 and SC51, with average TFs of 1269% ± 594%, 940% and 181% ± 89%, respectively (Figure 1a, easy).

However, the remaining nine genotypes exhibited variable tissue culture responses, primarily due to genotype-dependent production of phenolic compounds (Figure 1b; Figure S3). While the colour and intensity of phenolic compound accumulation varied among genotypes, the effects were consistently detrimental, leading to tissue browning, necrosis, and reduced regeneration potential. As a result, protocol optimisation was necessary to improve regeneration outcomes in these lines.

In CK60, SC329, SC673 and Tx623, explants produced moderate levels of phenolic compounds, causing partial tissue necrosis

and reduced regeneration, while some explants, calli, or regenerating shoots progressed to rooting. To boost survival, two extra subcultures were added to reduce the buildup of phenolic compounds. The second maturation step was shortened from 14 days to 10 days, and a third 10-day subculture was then implemented, continuing until shoots formed. Likewise, in the

(a)

Ease of Transformation	Genotype	# Infected seedlings†	# Subcultures‡	# Regenrants§	% Transf Frequency¶ (SE)	% Transf Efficiency††
Easy (Standard protocol)	IA101RPS	35	8	444	1269 (594)	159
	ICSV400	15	8	141	940	118
	SC51	16	8	29	161 (89)	23
	Tx430	98	8	483	420 (230)	53
Moderate (A few extra subcultures)	CK60	19	10	10	53 (10)	5
	SC329	6	10	1	17	2
	SC673	40	10	116	290 (90)	29
	Tx623	13	10	12	92	9
Difficult (Multiple Subcultures)	IA100RPS	51	8	0	0	0
	IA100RPS	13	14	6	46	1
	P898012	50	8	0	0	0
	P898012	49	14	197	532 (160)	38
	San Chi San	15	14	44	293	21
	SC1345	14	14	8	57	4
	SC265	5	13	13	260	20

†Total number of infected seedlings. A typical infection experiment uses 6 to 15 seedling leaf whorl explants.
‡Total number of subcultures from infection to rooting. The standard protocol has 8 subcultures.
§Total number of rooted independent regenerants.
¶The percentage of the total number of rooted regenerants per the total number of infected seedling explants. SE, standard error of the mean.
††Transformation frequency divided by the number of subcultures.

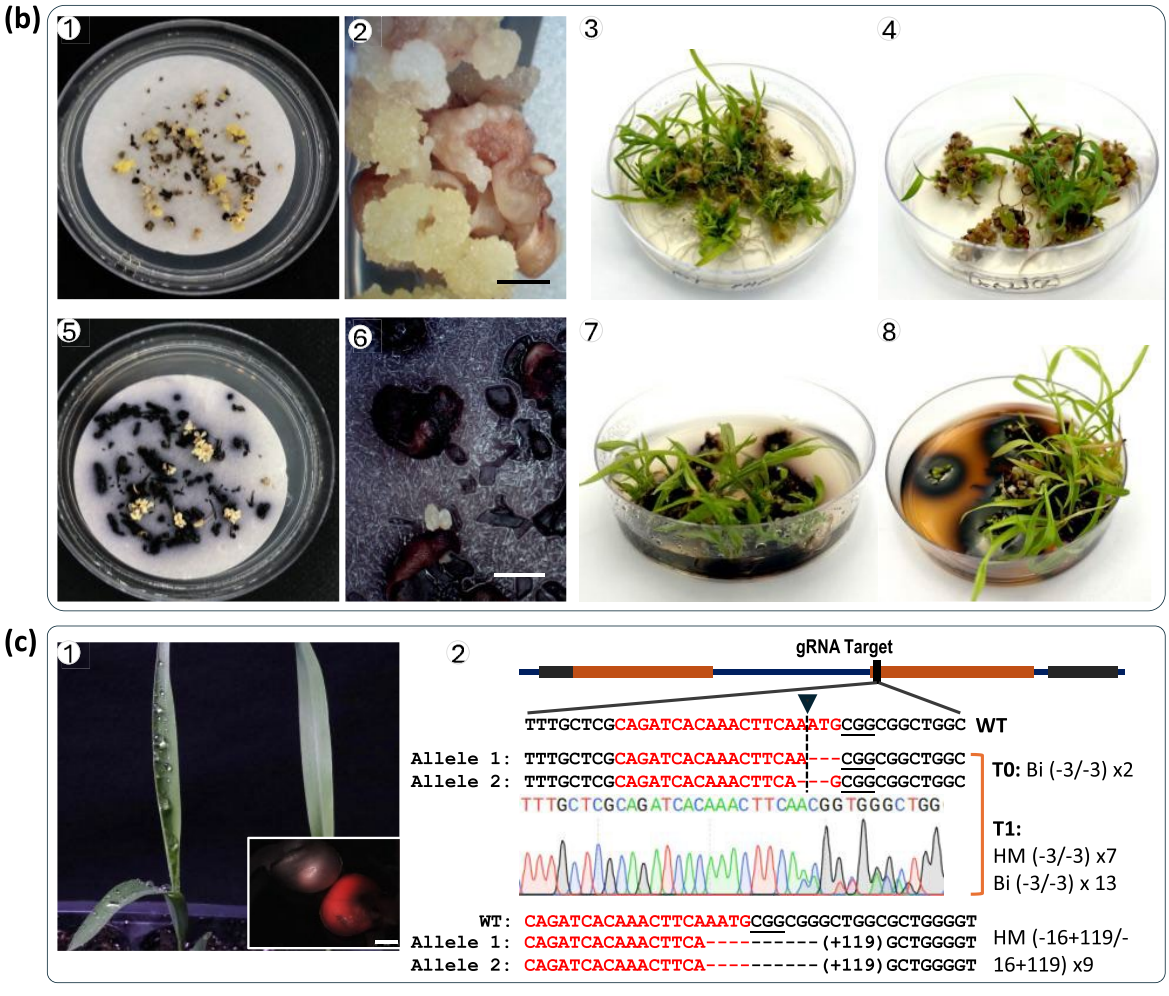

FIGURE 1 | *Agrobacterium*-mediated seedling leaf whorl transformation across thirteen diverse sorghum genotypes. (a) Summary of transformation frequency and varying efficiency based on the number of subcultures per genotype. (b1-b4) Tissue culture of sorghum in low to non-phenolic-producing genotypes: Tx430 (b1, b2), IA101RPS (b3), Tx623 (b4). (b5-b8) Tissue culture of sorghum in high phenolic-producing genotypes: P898012 (b5, b6, b8) and SC1345 (b7). (c) CRISPR/Cas9-mediated knockout of the *glossy2* gene showing a loss of function mutant phenotype (c1) in T1 seedlings.

rooting phase, a second subculture step was added after 10 days to mitigate phenolic-induced tissue necrosis. These modifications enhanced explant viability and regeneration, resulting in TFs ranging from 17% to 290% ± 90% across these four genotypes (Figure 1a, moderate).

In P898012, IA100RPS, San Chi San, SC265 and SC1345, explants produced markedly high levels of phenolic compounds, leading to severe browning and necrosis (Figure 1b, images 5–8). Initial attempts using the standard eight-step subculture protocol failed to yield regenerants in IA100RPS and P898012 (Figure 1b, images 5 and 6). To address this, the protocol was extended to a minimum of 14 subcultures or more to improve explant survivability. Specifically, explants were transferred to fresh selection media every 10 days, instead of 21-day intervals. In the maturation stage, subculture was performed every 7–10 days to reduce phenolic compound buildup. This strategy was carried out to the rooting stage. This labour-intensive but effective modification greatly improved callus survival and regeneration, yielding TFs of 46% (IA100RPS), 532% ± 160% (P898012), 293% (San Chi San), 57% (SC1345) and 260% (SC265), respectively (Figure 1a, Difficult).

Although the TF observed in genotypes like P898012 appears comparable to that of Tx430, achieving similar outcomes requires significantly more effort and resources. P898012 demands nearly twice the number of subcultures to reach equivalent TF levels, making it less efficient for transformation. To better assess genotype suitability, we introduce the metric of TE, defined as

$$\mathrm{TE} = \left(\frac{\text{Total Regenerants}}{\text{Total Explants} \times \text{Number of Subculture}} \right) \times 100\,\%$$

This metric helps researchers evaluate the practical feasibility of working with specific genotypes. For instance, P898012 achieved a TE of 38%, indicating that ~40% more effort is needed compared to Tx430, which had a TE of 53% (53/38 = 1.4; Figure 1a). Thus, while TF may appear similar, TE provides a more realistic measure of transformation practicality.

We next tested CRISPR-Cas9-mediated genome editing using the sorghum seedling leaf whorl as explants. A single gRNA construct, pKL2536 (Figures S4 and S5), targeting the second exon of the *gl2* gene in Tx430, was introduced into three LBA4404 derivative strains (Figure S6): (1) LBA4404Thy- (= LTA), (2) LBA4404T1 (= LT1A) and (3) an engineered *Agrobacterium* LBA4404Thy- strain carrying an insertional mutation in the *recA* gene to improve plasmid stability (= LTR1A; Method S1). These *Agrobacterium* strains were each carrying the virulence gene helper plasmid, pKL2299A (Aliu et al. 2024). During the preliminary experiment, targeted indel mutations were detected from 26% to 58% of the randomly selected transgenic calli samples (Figure S6). Two independent T0 plants were later regenerated from the calli transformed with the LTR1A strain. Sanger sequencing and ICE analysis (See Method S1) showed that both plants had short indel mutations (−3 bp/−3 bp) at the target site (Figure 1c, image 2). Due to the continuous Cas9 activity, additional indel mutations were observed in the T1 seedlings such as a 16 bp deletion plus a 119 bp insertion (55 bp + 59 bp + 5 bp) at the *gl2* target site (Figure 1c, image 2). *gl2* encodes a key enzyme involved in waxy cuticle biosynthesis on the juvenile leaf surface. As expected, biallelic and homozygous loss-of-function mutant T1 plants exhibited the *gl2* knockout phenotype, with water droplets adhering to leaf surfaces upon misting (Figure 1c, image 1).

Lastly, we demonstrated the successful expression of two alternative visual markers, *RUBY* and free-use GFP (*fuGFP*) in Tx430, confirming their utility for monitoring transformation events in sorghum seedling leaf whorls (Figure S7).

Author Contributions

K.W. conceived the project idea. M.K.A., K.L. and K.W. designed the project. K.L. designed and constructed the vectors and *Agrobacterium* strains. M.K.A. performed the sorghum transformations. M.K.A. and K.L. conducted molecular analysis. M.K.A., K.W. and K.L. wrote the manuscript.

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Data Availability Statement

The data that support the findings of this study is available in the Supporting Information of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** pbi70438-sup-0001-Supinfo.pdf.

Supporting Information for

Brief Communication

Optimization Of *Agrobacterium*-Mediated Seedling Leaf Transformation Across Multiple Sorghum Genotypes

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Methods S1

Thirteen sorghum genotypes were selected to evaluate the *Agrobacterium*-mediated seedling leaf whorl transformation protocol: CK60, IA100RPS, IA101RPS, ICSV400, P898012, San Chi San, SC1345, SC265, SC329, SC51, SC673, Tx430, and Tx623.

For genotype screening, we employed *Agrobacterium tumefaciens* strain LBA4404Thy- carrying T-DNA binary vector PHP101515 and ternary helper plasmid PHP71539 (Kumar and Azanu et al., 2025). The T-DNA construct PHP101515 includes two transgene cassettes: antibiotic resistance gene *NptII* (driven by the *Setaria italica* ubiquitin promoter and sorghum ubiquitin terminator) and fluorescent marker gene *ZsGreen1* (regulated by the sorghum ubiquitin promoter and rice ubiquitin terminator). Additionally, the construct contains three gene cassettes (*Bbm/Wus2/Cre*) flanked by two loxP sites: (1) Nos::Wus2, (2) 3xEnh::ZmUbi::Bbm, and (3) Rab17::Cre. The Cre recombinase, driven by the drought-inducible maize *Rab17* gene promoter, facilitates excision of the *Bbm/Wus2* expression cassettes following transformation. This excision can be triggered when transformed tissues are incubated in media containing abscisic acid (ABA) (Kumar and Azanu et al., 2025).

We adopted the leaf transformation protocol and media composition presented in Wang et al. (2023) with several modifications to enhance applicability across diverse sorghum genotypes (Methods). Our optimized seed surface sterilization protocol for field-grown sorghum seeds successfully eliminated fungal contamination during germination (Figure S1). The modified tissue culture procedure consists of eight sequential stages from *Agrobacterium* infection to regeneration: co-cultivation (1 day), resting (10 days), selection (21 days), first excision (4 days), first maturation (14 days), second excision (4 days), second maturation (7 days dark + 14 days light), and rooting (14 days). This streamlined workflow enables recovery of rooted transgenic events within 90 to 100 days from seed disinfection.

1. Molecular analysis of the Glossy2 target gene

1.1 Glossy2 gRNA sequence design

The *glossy2* (*gl2*) gene is reported to be conserved across various cereal crops (Zobrist et al, 2024). To ensure that a pre-existing gRNA in pKL2013 and pKL2359 (Lee et al., 2019, 2023) for maize could work for sorghum, the sequence for the *gl2* gene in sorghum (*Sbgl2*) was obtained from sorghum base (https://sorghumbase.org/). This was then aligned with the maize *gl2* gene sequence using SnapGene software (www.snapgene.com). Both sequences showed a conserved sequence for the gRNA target site at the second exon. Total genomic DNA was extracted from the leaf tissues of young sorghum seedlings of

Tx430, Tx623, and P898012. Primers spanning the gRNA target region, Sbgl2-F2 and Sbgl2-R2 (Table S1), were used to amplify the entire *Sbgl2* gene by PCR using high-fidelity Q5 DNA polymerase (NEB, Ipswich, MA, USA). Sanger sequencing analysis showed that the gRNA target was conserved across all the analyzed genotypes. Therefore, we used the same gRNA that showed highly efficient targeted mutagenesis in maize (Lee et al., 2019, 2023) and teosinte (Zobrist et al., 2024) to target the *Sbgl2* gene in Tx430.

1.2 Construct design for Gl2 editing and alternate visual markers

1.2.1 pKL2536 construct

The CRISPR/Cas9 construct pKL2536 (Figures S4A and S5) carries a red fluorescent protein marker *mCherry* driven by a 2x Cauliflower Mosaic Virus 35S promoter (2xP35S) and soybean vegetative storage protein gene terminator (Tvsp). It also carries *neomycin phosphotransferase II* (*NptII*) as a selectable marker that is driven by maize *Ubiquitin* promoter (ZmUbi) and *PinII* terminator. In addition to these, it harbors the maize codon optimized *SpCas9* (zCas9) that is driven by *Seteria Ubiquitin* promoter (SiUbi) and *rbc5-E9* terminator, followed by a *gl2* sgRNA (Sbgl2gRNA) driven by rice *U3* promoter (OsU3) and a rice *U6* terminator (TOsU6.2). Flanked by two loxP sites are expression cassettes of two morphogenic genes, *Baby boom* (*Bbm*) and *Wuschel2* (*Wus2*), and a Cre recombinase cassette driven by an ABA-inducible *Rap17* promoter (ZmRap17). The *Wus2* gene is driven by an *Agrobacterium nopaline synthase* promoter (NOS) and maize *In2* terminator. The *Bbm* gene is driven by a sorghum *Ubiquitin* promoter (SbUbi) whose expression is enhanced by 3 viral enhancers (3xEnh) and terminated by sorghum *Ubiquitin* terminator. During ABA-induced excision of the morphogenic genes, *ZmRap17* promoter drives the expression of *Cre*, which in turn induces the recombination of the two loxP sites, leading to their excision and leaving behind a T-DNA comprising of the *mCherry* visual marker, z*Cas9*, Sbgl2gRNA, and *NptII* selectable marker (Figures S4A & S5).

1.2.2 pKL2555 construct

The *fuGFP* construct pKL2555 has the same *Bbm*, *Wus2*, *Cre*/loxP, and *NptII* cassettes found in pKL2536. Instead of the CRISPR/Cas9 and sgRNA cassettes, pKL2555 carries the *fuGFP* (Coleman and Somerville, 2019) as a visible marker driven by a *Seteria Ubiquitin* promoter (SiUbi) and Tvsp terminator (Figure S4B).

1.2.3 pKL2557 construct

The *RUBY* construct pKL2557 also carries identical *Bbm*, *Wus2*, *Cre*/loxP, and *NptII* cassettes found in pKL2536 and pKL2555. Like pKL2555, pKL2557 carries another visible marker *RUBY*, which produces vivid purple color for easy tracking of the transformation process (He et al., 2020; Lee et al., 2023; Pramanik *et al.,* 2024). A CaMV 35S promoter (P35S) and Arabidopsis *AtHSP18.2* terminator drive the expression of three key enzymes *CYP76AD1*, *DODA,* and *Glucosyl transferase*, to convert tyrosine into purple pigment betalain (Figure S4C; He et al., 2020).

1.2.4 Generation of recombination-suppressed Agrobacterium *LBA4404Thy- strain*

To enhance plasmid vector stability within *Agrobacterium*, the *recA* gene, which plays a critical role during DNA repair and homologous recombination (Farrand et al., 1989), was mutagenized using the CRISPR RNA-guided INTEGRATE-mediated transposon insertion (Aliu et al., 2022). The resulting *recA*-deficient strain was designated as LBA4404Thy-RecA-1 (= LTR1). pKL2555 and pKL2557 were transformed into this strain carrying a ternary virulence gene helper plasmid pKL2299A (Aliu et al., 2024). The CRISPR/Cas9 construct pKL2536 was introduced into three strains, all with the pKL2299A helper plasmid: 1) LBA4404Thy- (= LTA; Ranch et al., 2012), 2) LBA4404T1 (= LT1A; Aliu et al., 2024), and LBA4404Thy-RecA-1 (= LTR1A).

2. Sorghum Seedling leaf whorl-based *Agrobacterium*-mediated transformation

The method described here is based on the leaf transformation method as reported in Wang et al. (2023) with some modifications.

2.1 *Equipment and supplies*

2.1.1 Growth medium preparation

- 2L and 4L Erlenmeyer glass flasks
- Aluminum foil
- Autoclave
- Autoclave indicator tape
- Benchtop Magnetic stirrer
- Graduated measuring beakers
- Magnetic stirring rod
- Measuring cylinder
- Millipore water
- pH meter
- Weighing boats/sheets

2.1.2 Explant Material Preparation

- 50 mL centrifuge tubes
- 60 mm x 15 mm petri dish
- 70% Ethanol
- 80% Ethanol
- Autoclaved 7 cm diameter Whatman filter paper (Catalog number 1001-070, Millipore Sigma, Burlington, MA, USA)
- Autoclaved deionized water (dH_2O)
- Beaker
- Bleach (6% Sodium hypochlorite)
- Forceps
- Light incubator (28°C, 16 h/8 h light/dark, with light intensity at 80-100 µmol/m^2/s)
- Clear plastic boxes with lids (12-5/16" × 9-1/16" × 4"; Catalog # 295C, Pioneer Plastics, Dixon, KY, USA)
- Sorghum seeds

2.1.3 Seedling leaf whorl explant transformation

- 1.5 mL centrifuge tubes
- 100 µm cell strainers (Catalog number 22363549, Thermo Fisher Scientific, Washington DC, USA)
- Bead sterilizer
- Cuisinart Mini-food processor (DLC-122, Cuisinart®, USA).
- Cuvettes
- Forceps
- Paper towels
- Parafilm
- Percival chamber (28°C, 16 h/8 h light/dark, with light intensity at 80-100 µmol/m^2/s)
- Percival chamber (28°C, Dark)
- Petri dishes (100 mm x 15 mm)
- Petri dishes (100 mm x 25 mm)
- Scalpel with #11 surgical blades
- Spectrophotometer

2.1.4 Plant Growth Supplies

- 2.5-gallon pots (Nursery Supplies Inc. Chambersburg, PA, USA)

- Clear Plastic Humi-dome (Hummert International, Earth City, MO, USA)
- Full mix (15-5-15) fertilizer
- Growth Chamber or greenhouse for regenerant acclimatization and seed production (12 h/12 h light/dark)
- Plastic insert (1801 deep inserts, T.O. Plastic, Clearwater, MN, USA)
- Plastic label
- Soil mix (Sungro Professional Growing Mix Sunshine Mix #1, Sun Gro Horticulture, Agawam, MA, USA)
- Standard Flat Trays with drain holes (STF-1020-OPEN, T.O. Plastic, Clearwater, MN, USA)
- Tassell bags (Lawson Pollinating Bags, Northfield, IL, USA)

2.1.5 Fungicides

PINK Fungicide

To make about 0.5 L of PINK fungicide, mix the following ingredients in order, in a beaker with stirring

- 75 mL Cruiser 5FS (insecticide)
- 37 mL Vibrance Cinco (5 fungicides)
- 162 mL water
- 10 mL Color Coat Red (colorant)
- 10 mL CF Clear (surfactant)
- 162 mL water

This fungicide is mainly used for maize seed treatment with about 17 µL per seed, which was administered using a Dubois AccuCoat 250. However, it helped with sorghum seed pre-treatment as well.

BLUE Fungicide

For the BLUE treatment fungicide, about 0.554 L is made, which is enough for pre-treatment of about 45 kg of seeds.

To make 0.554 L of the BLUE fungicide, mix the following ingredients in order, in a beaker with stirring.

- 14.75 mL Keystone Blue (colorant)
- 2.4 mL Maxim 4FS (fungicide)
- 19 mL Concep III (herbicide safener)
- 19 mL Apron XL (fungicide)
- 488 mL water
- 11 mL CF Clear (surfactant)

2.2 *Tissue Culture Media*

2.2.1 Agrobacterium Mother Plate (AB +Thy) Medium

Prepare 1 L of *Agrobacterium* Mother Plate by dissolving 5 g of glucose in 800 mL of deionized water (dH_2O). Adjust the pH to 6.8 with NaOH and top up the volume to 900 mL with dH_2O. Pour the solution in a 2 L Erlenmeyer glass flask and add 15 g Bacto agar. Autoclave for 30 min, cool to 55°C then add the following:

50 mL 20x AB Salts[1]
50 mL 20x AB Buffer[2]
2 mL 5x $FeSO_4 \cdot 7H_2O$ stock
2 mL thymidine (25 mg/mL)
*0.5 mL spectinomycin (100 mg/mL)
1 mL gentamicin (50 mg/mL)

Mix uniformly on the magnetic stirrer or by swirling the flask gently on the flow bench. Using an autoclaved beaker, pour 20 mL of the media into 100 mm x 15 mm petri dishes.

*In place of spectinomycin (for PHP101515), 1 mL of kanamycin (50 mg/mL) was added for *Agrobacterium* strains carrying pKL2536, pKL2555, or pKL2557.

[1]20x AB Salts: 20 g/L ammonium chloride (NH_4Cl), 6 g/L magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 3 g/L potassium chloride (KCl), and 0.228 g/L calcium chloride ($CaCl_2$). Dissolve in 1 L autoclaved dH_2O. Filter sterilize or autoclave, and store at 4°C.
[2]20x AB Buffer: 60 g/L potassium phosphate dibasic (K_2HPO_4), and 20 g/L sodium phosphate monobasic (NaH_2PO_4). Dissolve in 1 L autoclaved dH_2O. Filter sterilize or autoclave, and store at 4°C.

2.2.2 Working Plate (YEP +Thy) Media

Prepare 1 L of Working Plate media by dissolving 10 g Peptone, 5 g Yeast Extract, and 5 g NaCl in 800 mL dH_2O. Adjust the pH to 6.8 using NaOH and top up the volume to 1 L using dH_2O. Pour the solution into a 2 L Erlenmeyer glass flask and add 15 g Bacto agar. Autoclave for 30 min, cool to 55°C, then add the following:
2 mL thymidine (25 mg/mL)
*0.5 mL spectinomycin (50 mg/mL)
1 mL gentamycin (50 mg/mL)

Mix the media uniformly on a magnetic stirrer or by swirling the flask. Using an autoclaved beaker, pour 20 mL of the media into 100 mm x 15 mm petri dishes.

*In place of spectinomycin (for LBA4404Thy-/PHP101515+PHP71539), 0.5 mL of kanamycin (50 mg/mL) was added for *Agrobacterium* strains carrying pKL2536, pKL2555, or pKL2557.

2.2.3 Seed Germination Media

Prepare 1 L of Seed Germination media by dissolving 2.165 g MS Basal Salt Mixture (M524, Phytotechlab) and 20 g sucrose in 800 mL deionized water. Adjust the pH to 5.6 using NaOH and top up the volume to 1 L with dH_2O. Pour the solution into a 2 L Erlenmeyer glass flask and add 5 g Sigma Agar (A7921, Sigma-Aldrich). Autoclave for 30 min, cool to 55°C, then add the following:
1.7 mL ancymidol (2 mg/mL)[1]
1 mL benomyl (50 mg/mL)[2]
1 mL meropenem (10 mg/mL)[3]
Mix the media uniformly on the magnetic stirrer or by swirling the flask. Using an autoclaved beaker, pour 70 mL of the media into 100 mm x 25 mm petri dishes.
[1]ancymidol (A9431, Sigma).
[2]benomyl (Sigma product methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate; #381586).
[3]meropenem trihydrate (AM16380, Carbosynth).

2.2.4 Infection 700J Solution

Prepare 1 L of Infection 700J solution by dissolving 1.204 g magnesium sulfate (anhydrous) and 5 g maltose in 800 mL deionized water. Adjust the pH to 5.6 using NaOH and top up the volume to 1 L with dH_2O. Pour 0.5 L of the solution into 1 L bottles with caps. Autoclave for 30 min, cool to room temperature and store at 4°C.

2.2.5 700F Liquid Solution

Make fresh on the day of infection and adjust volume based on the number of experiments and explants.

Make 100 mL 700F Liquid solution by adding together:
100 mL 700J
0.2 mL thymidine (25 mg/mL)
0.1 mL acetosyringone (200 mM)

*0.2 mL Break-Thru (10% v/v)

*In place of Break-Thru, Silwet (L-77, Cat No. VIS-01) can be used at the same concentration.

2.2.6 710N Co-cultivation Media

Prepare 1 L of 710N co-cultivation media by dissolving the following chemicals in 800 mL deionized water:

4.33 g MS Basal Salt (M524, Phytotechlab)
0.1 g myo-inositol
0.7 g L-proline
20 g maltose
10 g glucose
0.5 g 2-(N-morpholino)ethanesulfonic acid (MES)
0.5 mL nicotinic acid (1 mg/mL)
0.5 mL pyridoxine (1 mg/mL)
2.5 mL thiamine (0.4 mg/mL)
0.049 mL cupric sulfate (100 mM)
4 mL 2,4-dichlorophenoxyacetic acid (2,4-D) (0.5 mg/mL)

Adjust the pH to 5.6 using NaOH, top up the volume to 1 L with dH_2O. Pour the solution into a 2 L Erlenmeyer glass flask and add 8 g Sigma Agar (A7921, Sigma-Aldrich). Autoclave for 30 min, cool to 55°C, then add the following:
1 mL acetosyringone (100 mM)
1 mL ascorbic acid (10 mg/mL)
1.7 mL silver nitrate (2 mg/mL)
0.01 mL 6-Benzylaminopurine (BAP) (1 mg/mL)
2 mL thymidine (25 mg/mL)

Mix the media uniformly on a magnetic stirrer or by swirling the flask. Using an autoclaved beaker, pour 20 mL of the media into 100 mm x 15 mm petri dishes.

2.2.7 605B Resting Media

Prepare 1 L of 605B resting media by dissolving the following chemicals in 800 mL dH_2O:

4.33 g MS Basal Salt (M524, Phytotechlab) *
[1]60 mL N6 Macronutrient Stock*
[2]0.6 mL B5H Minor salts (1000x)*
[3]6 mL NaFe EDTA for B5H (100x)*
[4]0.4 mL Eriksson's vitamins*
0.6 g S&H vitamins powder*
1.68 g potassium nitrate*
0.5 mL thiamine (0.4 mg/mL)*
1.98 g L-proline*
0.3 g casein hydrolysate
1.6 mL 2,4-D (0.5 mg/mL)
0.049 mL cupric sulfate (100 mM)
20 g sucrose
0.6 g glucose

Adjust the pH to 5.6 using NaOH and top up the volume to 1 L with dH_2O. Pour the solution into a 2 L Erlenmeyer glass flask and add 6 g TC Agar (A296, Phytotechlab). Autoclave for 30 min, cool to 55°C, then add the following:
1.2 mL dicamba (1 mg/mL)
1.7 mL silver nitrate (2 mg/mL)

2 mL meropenem (5 mg/mL)
Mix the media uniformly on a magnetic stirrer or by swirling the flask. Using an autoclaved beaker, pour 30 mL of the media into 100 mm x 25 mm petri dishes.

*NB: In place of MS Basal salt, N6 Macronutrient, B5H minor salts, NaFe EDTA for B5H, Eriksson's vitamins, S&H vitamins, potassium nitrate, proline, and thiamine, 11 g of a pre-mixed 605B stock powder (M5605, Phytotechlab) can be used.

[1]N6 Macro Nutrient Stock: Make 1 L of N6 Macro Nutrient stock by dissolving 1.66 g of $CaCl_2 \cdot 2H_2O$; 4.62 g of $(NH_4)_2SO_4$; 4 g of KH_2PO_4; 1.85 g of $MgSO_4 \cdot 7H_2O$ and 28.3 g of KNO_3 in autoclaved dH_2O. Filter sterilize and store at 4 °C for 2-3 months.

[2]B5H Minor Salts (1000x): Make 1 L of B5H minor salts by dissolving 3 g boric acid; 10 g of $MnSO_4 \cdot H_2O$; 0.25 g of $Na_2MoO_4 \cdot 2H_2O$ and 0.75 g of potassium iodide (KI) in autoclaved dH_2O. Filter sterilize and store at 4 °C for 2-3 months.

[3]NaFe EDTA for B5H (100x): Make 1 L of NaFe EDTA for B5H by dissolving 3.7 g of EDTA-$Na_2 \cdot 2H_2O$ and 2.79 g of $FeSO_4 \cdot 7H_2O$ in autoclaved dH_2O. Filter sterilize and store at 4 °C for 2-3 months.

[4]Eriksson's vitamins stock (2500x): Make 1 L of Eriksson's vitamins stock by dissolving 2 g glycine, 0.5 g niacin, 0.5 g pyridoxine and 0.5 g thiamine-HCl in autoclaved dH_2O. Filter sterilize and store at 4 °C for 2-3 months.

2.2.8 G418 Selection media

Prepare 1 L of 605B media as explained above, autoclave for 30 min, cool to 55°C then add the following:
1.2 mL dicamba (1 mg/mL)
1.7 mL silver nitrate (2 mg/mL)
2 mL meropenem (5 mg/mL)
1 mL G418 (150 mg/mL; G810, Phytotechlab)

Mix the media uniformly on a magnetic stirrer or by swirling the flask. Using an autoclaved measuring beaker, pour 30 mL of the media into 100 mm x 25 mm petri dishes.

2.2.9 Abscisic acid (ABA) Excision Media

Prepare 1 L of 605B media as explained above, autoclave for 30 min, cool to 55°C, then add the following:
1.2 mL dicamba (1 mg/mL)
1.7 mL silver nitrate (2 mg/mL)
2 mL meropenem (5 mg/mL)
0.5 mL ABA (0.1 M)

Mix the media uniformly on a magnetic stirrer or by swirling the flask. Using an autoclaved beaker, pour 30 mL of the media into 100 mm x 25 mm petri dishes.

2.2.10 Maturation Media 404

Prepare 1 L of maturation media by dissolving the following in 800 mL deionized water:
0.61 g Modified MS Basal Salts (M407, Phytotechlab)
0.1 g myo-inositol
0.7 g L-proline
85 g sucrose
5 mL *MS Vitamin Corteva Stock
1.25 mL cupric sulfate (1 mg/mL)
1.2 g ammonium nitrate
2.52 g potassium nitrate
0.193 g sodium phosphate monobasic
0.06 mL zeatin(trans) (0.5 mg/mL)

Adjust the pH to 5.6 using NaOH and top up the volume to 1 L with dH_2O. Pour the solution into a 2 L Erlenmeyer glass flask and add 6 g TC Agar (A296, Phytotechlab). Autoclave for 30 min, cool to 55 °C, then add the following:

2 mL indole-3-butyric acid (IBA) (1 mg/mL)
1 mL 6-(3-hydroxybenzylamino)purine (meta-topolin) (0.5 mg/mL)
0.1 mL thidiazuron (1 mg/mL)
1 mL ABA (0.1 mM)
1 mL BAP (1 mg/mL)
2 mL indole-3-acetic acid (IAA) (0.5 mg/mL)
2 mL meropenem (5 mg/mL)

Mix the media uniformly on a magnetic stirrer or by swirling the flask. Using an autoclaved beaker, pour 30 mL of the media into 100 mm x 25 mm petri dishes.

*MS Vitamin Corteva Stock (200x): Prepare 1 L of MS Vitamin Corteva stock by dissolving 0.4 g/L glycine, 0.1 g/L nicotinic acid, 0.1 g/L pyridoxine HCl, and 0.02 g/L thiamine·HCl in autoclaved dH_2O. Filter sterilize and store at 4 °C for 2-3 months.

2.2.11 Rooting Media

Prepare 1 L of rooting media by dissolving the following in 800 mL dH_2O:
4.33 g MS Basal Salt Mixture (M524, Phytotechlab)
0.1 g myo-inositol
40 g sucrose
5 mL MS Vitamin Corteva Stock

Adjust the pH to 5.6 using NaOH and top up the volume to 1 L with dH_2O. Pour the solution in a 2 L Erlenmeyer glass flask and add 6 g *Bacto Agar (BD Difco 214010, Fisher Scientific). Autoclave for 30 min, cool to 55°C, then add the following:
0.5 mL IBA (1 mg/mL)
2 mL meropenem (5 mg/mL)

Mix the media uniformly on a magnetic stirrer or by swirling the flask. Using an autoclaved measuring beaker, pour 30 mL of the media into 100 mm x 25 mm petri dishes.

*Bacto Agar (BP1423, Fisher Scientific) can be substituted for Difco Agar.

2.3 *Methods*

2.3.1 Sterilization of greenhouse or field-grown sorghum seeds for leaf whorl explants

Although the process of generating leaf whorl explants for sorghum transformation is readily accessible to all researchers, the seeds used for generating these explants, as reported so far, were mostly sourced from greenhouse-grown plants. To ensure that this protocol is easily replicable by research labs that may not readily access greenhouse or growth chamber spaces, there is a need to create a robust seed surface sterilization protocol for field-grown seeds.

Sorghum seeds do not have secondary protection, such as husks or pods, to shield the seeds from adverse biotic or abiotic factors (Silva *et al*., 2022). When placed side by side, Tx430 seeds harvested from the field looked significantly different from those collected from the greenhouse (Figure S1). Some of these differences include severe coloring and cracking of the seeds obtained from the field. These could be due to their direct contact with uncontrolled environmental factors such as pest infestations, severe rain and wind, and direct intense sunlight. Due to this, the level of sterilization required for generating aseptic

seedling leaf whorl explants from field seeds varies greatly from the method of sterilization for seeds derived from greenhouse conditions. Attempts to use the sterilization protocol for greenhouse-derived seeds on the field-grown seeds resulted in severe contamination and loss of explant materials (Figure S1). To overcome this challenge, we optimized the seed sterilization protocol for field-grown sorghum seeds. The two different seed sterilization protocols are outlined below.

2.3.2 Seed sterilization for greenhouse-grown seeds

1. Tx430 seeds that were sourced from the greenhouse were first observed for any cracks and then cleaned of any debris. About 15 to 30 seeds were placed into a 50 mL centrifuge tube with a screw cap.
2. About 10 mL of 80% ethanol was then added to the seeds in a laminar flow hood such that the seeds were completely submerged in the ethanol. The tubes were gently swirled and allowed to stand for 2 min. The ethanol was discarded, and the seeds were rinsed once with 30 mL of autoclaved deionized water with gentle shaking.
3. The water was poured out, and 20 mL of 50% bleach solution (3% Sodium hypochlorite final concentration) with 2-3 drops of Tween-20 (0.1%) was added to the seeds. The tubes were capped and sterilized on a bench-top shaker at 70 rpm for 20 min.
4. The bleach solution was poured out and safely discarded, and the seeds were rinsed 3 times, one minute each, with autoclaved distilled water. The seeds were then sown on the seed germination medium.
5. About 10 seeds were placed on each seed germination medium, with the embryo side up and the seeds slightly embedded in the medium for good contact of the root after sprouting.
6. The germination plates with seeds were covered individually with their lids to control cross-contamination between plates and placed into an enclosed clear plastic box with lid and dimensions 12-5/16" × 9-1/16" × 4" (Catalog # 295C, Pioneer Plastics, Dixon, KY, USA).
7. The plastic box was kept in a Percival growth chamber set at 28°C, 16h/8h light/dark and light intensity of 80-100 µmol/m^2/s.
8. The individual petri dish lids were removed on the third day after sterilization. The seedlings were allowed to grow for 10 to 12 days after sterilization and then used as explants for *Agrobacterium*-mediated transformation.

2.3.3 Sterilization of field-grown seeds.

1. The field-grown seeds go through multiple cleaning and selection steps to eliminate microbial contamination. Firstly, well dried field-harvested seeds were carefully examined for cracks as well as any leftover debris.
2. The seeds were next treated with either PINK or BLUE fungicides (see Section 2.1.5 Fungicides), to kill any microbes that may grow on the germination media. About 200 seeds were placed in a beaker, and about 3.4 mL of the fungicide was slowly added onto the seeds, while slightly shaking the beaker with the seeds at the same time, to ensure that all the seed surfaces were fully coated with the fungicide. The seeds were then allowed to dry briefly, after which they were ready for sterilization or stored at 4°C.
3. For a single leaf transformation experiment, about 20 fungicide pre-treated seeds were selected, and observed once more for any cracks (Figure S1). The seeds were then soaked in autoclaved sterile water in a 60 mm x 15 mm petri dish for about 2 to 3 h, to soften the seed coat. Following this, a pair of forceps was used to gently peel off the softened seedcoat starting from the back of the seed and peeled over the surface of the embryo, so that the forceps do not directly pierce the surface of the embryo, to cause seed damage.
4. Seeds with removed seedcoat were examined for severe coloring of endosperm or contamination of embryo area. Seeds with severe brown blotches on endosperm or embryo area were discarded and those with clear embryo surface appearance and minimal seed spotting were moved to the next sterilization phase.

5. These seeds were then placed into a 50 mL tube containing autoclaved deionized water, until all the seedcoats were removed. The water was then drained off and 50% bleach solution (3% Sodium hypochlorite) with 0.1% Tween-20 was added to the seeds and incubated for 20 min while shaking at 70 rpm. No ethanol pre-sterilization step is performed on the field seeds with removed seedcoats.
6. Following sterilization, the bleach solution was appropriately discarded, and the seeds were rinsed three times, 1 minute each with autoclaved deionized water. The seeds were then sown on seed germination media as described above.

Note: Following sterilization and prior to sowing, the field-grown seeds were further examined once again to ensure that only clean-looking seeds were placed on the seed germination media. Additionally, tests were conducted to determine if field-grown seeds could be grown aseptically without fungicide application or seedcoat removal. However, our field-grown seeds showed microbial contamination without either fungicide pre-treatment or seedcoat removal (Figure S1). Therefore, we recommend that all the field-grown seeds go through fungicide pre-treatment and seed coat removal for optimum results.

2.3.4 Preparation of Agrobacterium *Mother and Working Plates*

1. About 7 days after seed sterilization, *Agrobacterium* mother plates were prepared. The glycerol stocks were taken out from the -80°C freezer and streaked on *Agrobacterium* mother plate media (AB+Thy), containing the appropriate antibiotics.
2. The mother plates were then sealed with parafilm and incubated upside down in a dark incubator set at 28°C for 48 to 72 h. The mother plates were then stored at 4°C refrigerator and were used to streak working plates a day before infection.
3. The day before the seedling leaf whorls were due for infection, multiple *Agrobacterium* colonies were collected from the mother plate with an inoculation loop and spread evenly onto an appropriate working plate (YEP+Thy+antibiotics). The plates were sealed with parafilm and incubated upside down in a dark incubator at 28°C for about 18 h prior to infection.

2.3.5 Preparation of the sorghum leaf whorl explants for transformation

1. About 10 to 12 days after seed sterilization, the sorghum seedlings were harvested for leaf infection. Prior to infection, the enclosed plastic box together with the seedlings were pre-heat-treated at 45°C and at a relative humidity of 70% for 2 h. The box with seedlings was then externally disinfected with 70% ethanol spray and placed into a sterile laminar flow hood for the leaf infection process.
2. To prepare each leaf whorl segment, the 2 to 3 outer leaves on each seedling were peeled off using 2 pairs of forceps, to expose the inner greener and much younger leaf whorls.
3. Holding the crown of the seedling with a pair of forceps in one hand, a sharp scalpel of razor blade was used to cut the seedling segment at the base of the stem, closest to the root and above the media.
4. The cut leaf whorl segment was then placed on a petri dish lid, and the crown portion, about 3 cm above the base of the stem is cut off and discarded, leaving behind the 3cm mid-stem portion as the explant. The leaf whorl explant was then placed in a closed 100 mm x 25 mm petri dish containing 700J liquid media at room temperature until all the leaf segments were collected. About 10 to 15 seedling leaf whorls are sufficient for experimental treatment.
5. A Cuisinart mini food processor was prepared for cutting up the leaf segments into smaller pieces. The motor of the processor was surface sterilized with 70% ethanol followed by wiping and then placed in the laminar flow hood. The bowl part of the processer together with its lid and the grinding blade were thoroughly surface sterilized with 70% ethanol and then placed in the laminar flow hood. The ethanol on the surface of the food processer bowl and blade was allowed to air dry and these were rinsed three times, with vigorous shaking using autoclaved distilled water.
6. About 50 mL to 100 mL of 700J liquid media (or until one of the Cuisinart blades was submerged in the solution) was added into the Cuisinart.
7. Each seedling whorl explant was then cut vertically into 2 equal halves and placed into the food processor. Using the lower processing control on the motor, and with about 7 to 10 short pulses, the leaf segments were cut into smaller pieces of about 3 mm to 5 mm each. The leaf pieces were

then strained out using 100 µm cell strainer. The cell strainer with leaf explants were placed into a petri dish with lid, to prevent dehydration.

2.3.6 Transformation of leaf pieces with Agrobacterium

1. Fresh 700I infection solution was made on the day of infection such that, about 20 to 30 mL 700I solution was used per transformation.
2. About 0.5 mL of the 700I solution was pipetted onto the *Agrobacterium* working plate. Using an inoculating loop in circular motions, the *Agrobacterium* cells were suspended in the 700I solution on the plate. Using a 1 mL pipette, the *Agrobacterium* cell suspension was collected and re-suspended into a 50 mL tube containing the whole infection solution. Using the 700I solution only as a blank, OD of the *Agrobacterium* suspension was measured and adjusted until it was within a range of 0.5 to 0.6 at 550 nm.
3. The chopped leaf pieces in the strainer were then gently transferred into the *Agrobacterium* infection solution and infected for 20 min either with a gentle rotation on the benchtop shaker or on the laminar flow hood with hand shaking every 5 min.
4. Following the infection, the leaf pieces were strained through the 100 µm cell strainer to remove the *Agrobacterium* infection solution. The strainer was then dabbed briefly on sterile paper towel to remove any excess solution.
5. The infected leaf pieces were then evenly distributed onto a 7 cm diameter sterile Whatman filter paper that had been placed on 710N co-cultivation media plates at room temperature. The leaf pieces were spread evenly across the filter paper on the media such that approximately 1.5 to 2 seedlings were spread per plate. For example, when 10 seedlings were used for preparation of leaf explants, about 5 to 7 co-cultivation plates were used to distribute the explants. This is to avoid over-crowding of the calli that would be produced later during the tissue culture process.

2.3.7 Tissue culture and Regeneration Process of Transgenic Events

1. Following spreading of the leaf pieces on the 710N culture media with filter paper, the plates with lids were placed into a dry sterile secondary box and incubated at 20°C in the dark overnight for co-cultivation.
2. The next day, 2 pairs of forceps, with a pair in each hand, were used to gently lift the filter paper on the 710N media, onto 605B resting media at room temperature. The plates were incubated in the enclosed plastic box for 7 to 10 days at 28°C in the dark, after which the filter paper with calli were transferred onto a G418 selection media plate for 3 weeks, also at 28°C in the dark. At this stage, visible calli pieces are formed and fluorescent visual marker genes can be observed using a hand-held fluorescent flashlight with goggles or under a fluorescent dissecting microscope.
3. At the end of the selection period, calli pieces were transferred individually, using sterile forceps onto ABA induction plates under the laminar flow hood. These plates were made freshly, a day before use and used within a week after preparation to ensure optimum efficacy of the ABA. The ABA plates with calli were then kept in the enclosed plastic box at 28°C in the dark for 4 days. To ensure good media contact, the forceps were first used to slightly break the surface of the ABA media before the calli were placed there.
4. The calli were transferred onto maturation media at 28°C in the dark for 2 weeks, followed by a second ABA treatment on new ABA media plates for 4 days. The calli were then transferred onto a second maturation media and kept in the dark at 28°C for one more week, and then moved into light at 28°C, 16h/8h day/night with cool fluorescent light at an intensity of 60-80 µmol/m^2/s.
5. Regenerating shoots and greening calli that started to form about a week or two after transfer to light were immediately transferred onto rooting media for about 7 to 10 days for better shoot establishment and root development.
6. The regenerants from calli pieces came in three different forms: 1) single independent event per individual callus piece, 2) multiple shoots clustered on the same callus piece and not easily separable, and 3) multiple independent events on the same calli but at different foci that are easily detectable by the presence of calli barriers separating each regenerant. The regenerants from each

unique foci can be easily separated from other unique events using a pair of forceps to gently tease them apart. Each unique event can then be transferred into a plate for further shoot growth and root development.

2.3.8 Acclimatization and transfer to soil

1. About 14 to 21 days following transfer of regenerating calli onto rooting media, the shoots with well-established roots were transferred into soil. The roots of the plantlets were first rinsed gently in deionized water to remove leftover media and calli. The plantlets were then placed into plastic inserts containing pre-wetted soil mix. The plastic inserts with plantlets were covered with humi-dome to prevent dehydration.
2. The plantlets were next acclimated in a lighted growth chamber set at 28°C with 12h/12h day/night and light intensity under fluorescent lights at 250 to 320 µmol/m^2/s for 7 to 10 days. About 4 days following the initial transfer, the humi-dome was partially opened and by 7 to 10 days, the humi-dome was taken off to enhance the acclimatization process. Throughout this period, the plantlets were monitored daily for optimum moisture supply, such that they were neither overwatered nor underwatered to avoid dehydration while promoting good root development

2.3.9 Growth to Maturity

1. After the initial acclimatization of 10 to 14 days, the transgenic sorghum plantlets were moved into bigger pots. 2.5-gallon pots were filled with Sungro soil media to the top. Each pot was then wetted maximumly with water. A hole was made in the center of each pot of soil such that the hole was about halfway into the soil. This ensures that the roots are well established in the pot and the lower portion of the shoots are also held well in an upright position as the plant develops, to reduce shoot bending.
2. Each acclimated plantlet in seed flat was gently carried with the soil around it and placed into the 2.5-gallon pot with holes. The plantlets were covered to the base of the shoot.
3. The plantlets and pots were monitored regularly, such that drying soil was lightly watered with fertilizer water (Macro 15-5-15) until the panicle was formed.
4. The watering continued all through pollination until seeds were fully formed. Once the seeds began to dry, watering ceased.
5. Once fully dried, the panicles with seeds were collected by gently twisting the panicle above the flag leaf from side to side to break or by use of scissors to gently detach the panicle from the dry stem. The harvested panicle with seeds were stored in a large tassel bag, taped to seal and stored at room temperature for short storage and at 4°C for longer storage.

2.3.10 Glossy2 knockout mist test on T1 seedlings.

1. Well-dried, harvested T1 seeds were individually and randomly handpicked from the harvested T0 knockout panicles. The seeds were cleaned of any debris.
2. Sterile 7 cm diameter Whatman filter paper was placed into an empty 100 mm x 25 mm petri dish with lid. The filter paper was wetted with autoclaved deionized water, and the seeds were placed on the filter paper. Each plate was then sealed with micropore tape, and the plate was placed in a dark drawer or covered with aluminum foil at room temperature. Three days later, the germinated T1 seedlings were transferred into pre-wetted soil in seedling flats as described above. The seedlings were watered as desired and allowed to develop further for about 7 to 10 days.
3. To perform the *gl2* knockout test, both wild-type and knockout seedlings were misted with water from a spray bottle. The seedlings were then observed for the knockout phenotype of water droplets adhering to the leaf surfaces (knockout mutant) and the water droplets falling off completely (wild type).

3. Supplementary Reference

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Table S1. Primers for *Sbgl2* PCR and sequencing analysis.

Primer name	Description	Sequence (5' - 3')
Sbgl2 Forward	Sequencing Primer to determine editing within gRNA cut site	ATTTCGGGGTGAACTAAACA
Sbgl2 Forward 2	PCR primer to amplify sorghum *glossy2* for genotyping analysis	GATCAGCAGCGGTCTTAACT
Sbgl2 Reverse 2	PCR primer to amplify sorghum *glossy2* for genotyping analysis	TGACGTGGAAGGAGTAGCAC

Supplementary Figures

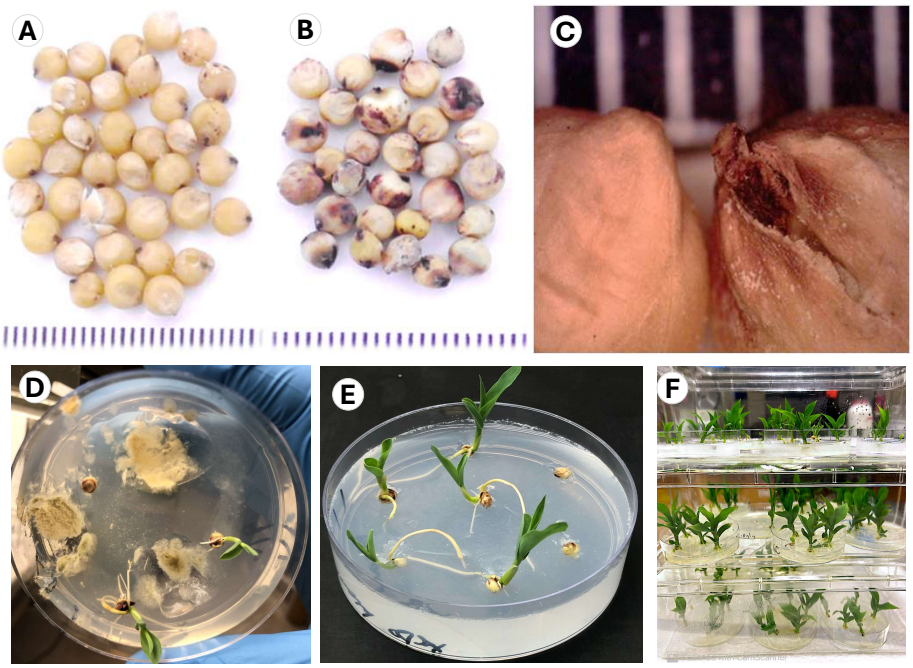


Figure S1. Optimization of the seed surface sterilization protocol for field-grown seeds. Tx430 seeds grown in the greenhouse (A) and open field (B). (C) Close up view of intact (left) and cracked (right) sorghum seeds. (D) Germinating field-grown seeds before the protocol optimization. (E, F) Germinating field-grown seeds disinfected with the optimized protocol.

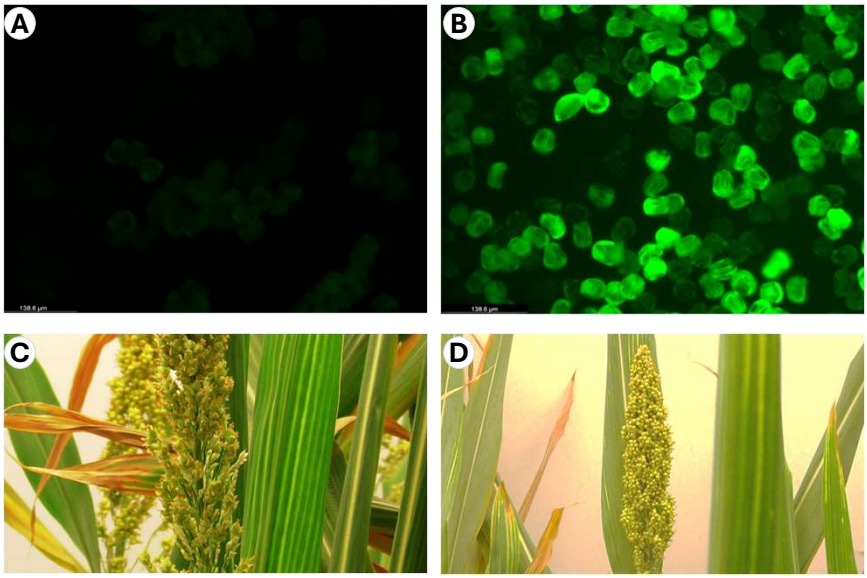


Figure S2. Production of T1 seeds from transgenic Tx430 T0 plants. Pollens from (A) wild-type Tx430 plant, and (B) Transgenic T0 plants produced by PHP101515 construct (Kumar and Azanu et al., 2025). (C, D) Seed sets from two transgenic T0 plants.

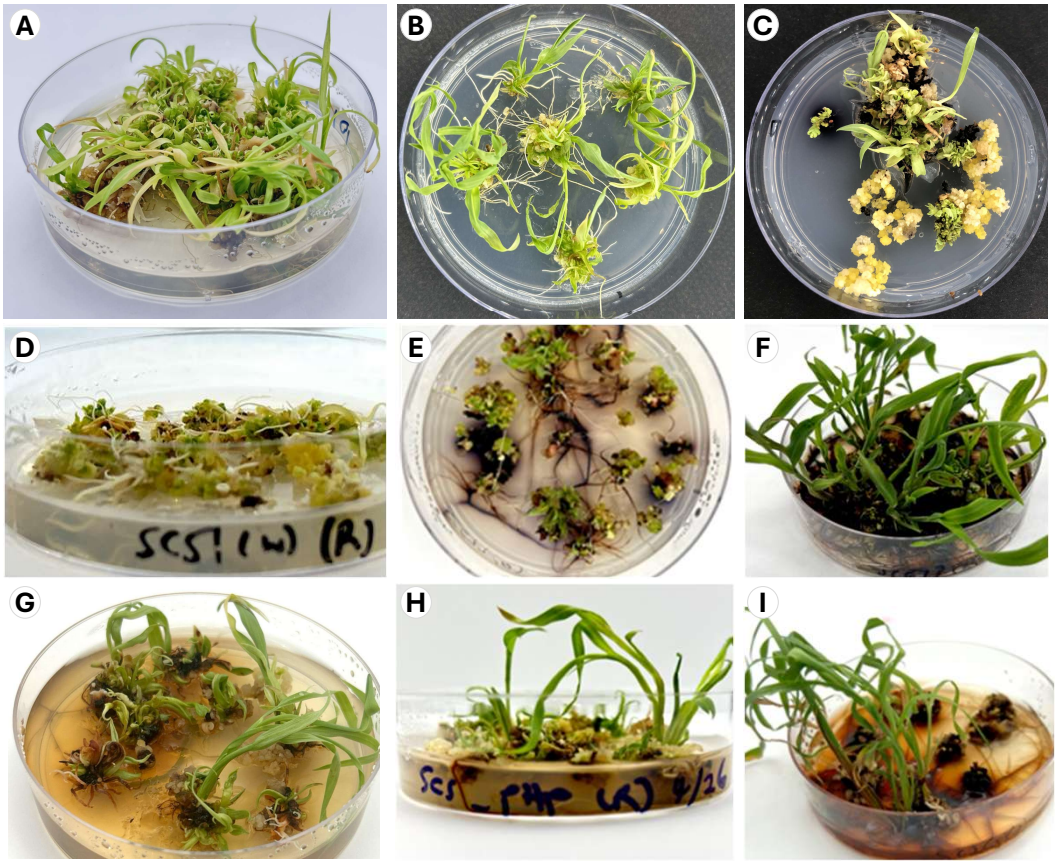


Figure S3. Presence or absence of phenolic compounds at varying levels among nine sorghum genotypes at the rooting stage. (A) IA101RPS; (B) ICSV400; (C) Tx430; (D) SC51; (E) CK60; (F) SC673; (G) IA100RPS; (H) San Chi San; and (I) SC265.

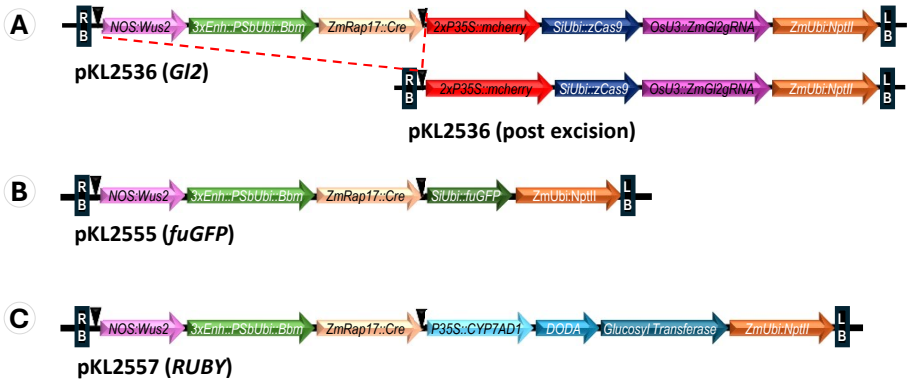


Figure S4. Schematic illustration of the T-DNA regions of the three constructs used in this study. (A) CRISPR/Cas9 construct pKL2536 targeting *SbGl2* before (top) and after ABA-induced excision (bottom). (B) *free use GFP* (*fuGFP*) construct pKL2555. (C) *RUBY* construct pKL2557.

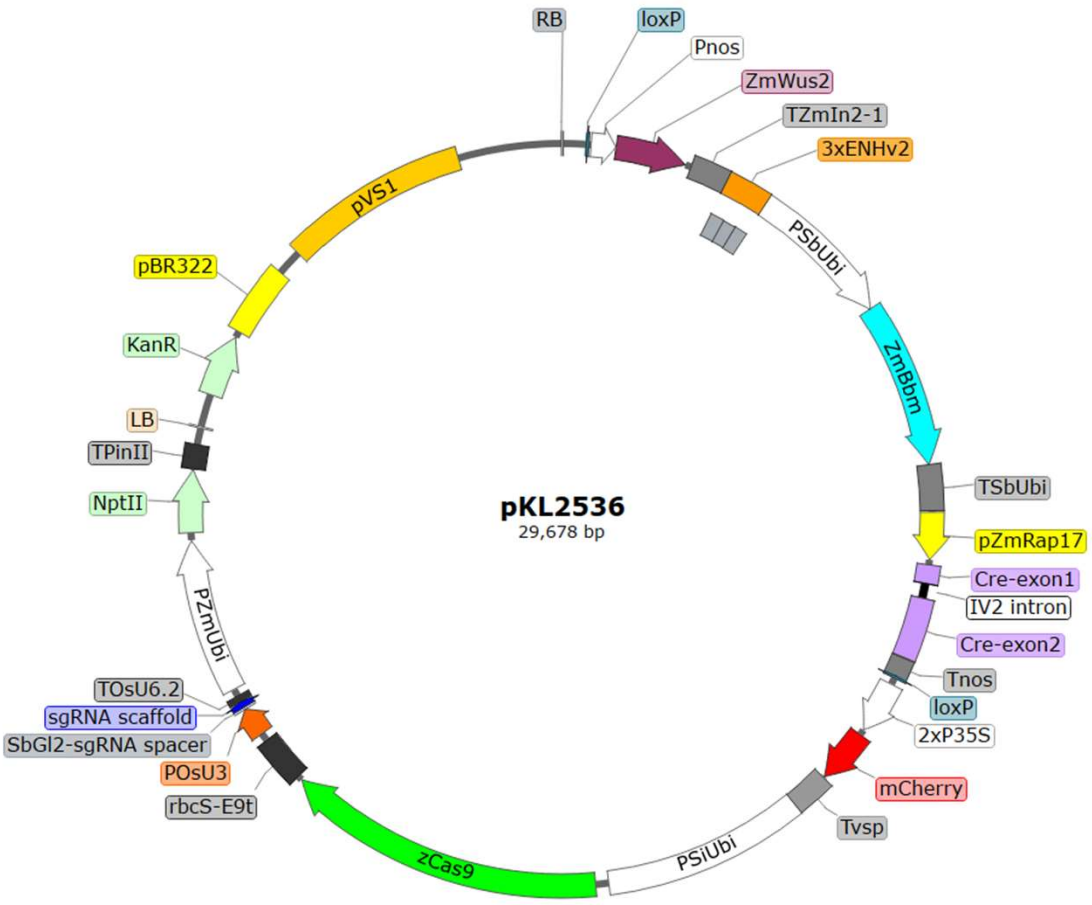
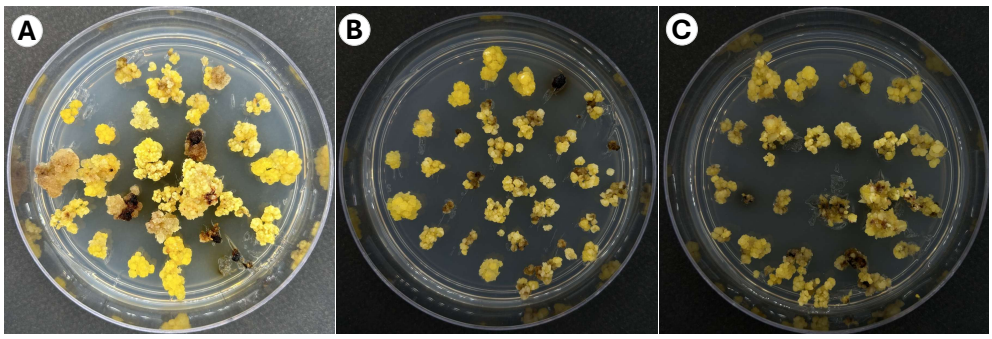


Figure S5. Map of the CRISPR/Cas9 construct pKL2536, targeting sorghum *Glossy2* gene. RB, right T-DNA border; Pnos, *nopaline synthase* gene promoter from *Agrobacterium*; *ZmWus2*, maize *Wus2* gene; TZmIn2-1, transcription terminator from maize *In2-1* gene; 3xENHv2, triple viral enhancers; *ZmBbm*, maize *Bbm*; TSbUbi, sorghum *ubiquitin* gene terminator; PZmRap17, maize *Rap17* gene promoter; *Cre*, Cre recombinase; IV2 intron, potato IV2 intron; Tnos, *nopaline synthase* gene terminator from *Agrobacterium*; 2xP35S, 2x Cauliflower Mosaic Virus 35S promoter; *mCherry*, red fluorescent protein gene; Tvsp, soybean *vegetative storage protein* gene terminator; PSiUbi, *Seteria Ubiquitin* promoter (SiUbi); zCas9, maize codon-optimized *SpCas9*; rbcS-E9t, Pea RBCS E9 terminator; POsU3, rice *U3* promoter; SbGl2-sgRNA spacer, gRNA spacer targeting sorghum *Gl2* gene; TOsU6.2, rice *U6* terminator; PZmUbi, maize *ubiquitin* gene promoter; *NptII*, *neomycin phosphotransferase II* gene; TpinII, *potato proteinase inhibitor II* gene terminator; LB, left T-DNA border; KanR, kanamycin resistance gene fro bacterial selection; pBR322, high-copy number origin of replication for *E. coli*; pVS1, origin of replication from Pseudomonas for *Agrobacterium*.



LTA/pKL2536 LT1A/pKL2536 LTR1A/pKL2536

D **Analysis of editing efficiency in Tx430 calli with pKL2536 in three *Agrobacterium* strains**

Strain	Calli Genotype	Indel Mutations	# Calli analyzed
LTA	WT	(0/0)	17
	HM	(+1/+1)	1
	BI	(0/+1) x3; (+1/0) x1; (+1/-7) x1	5
LT1A	WT	(0/0)	10
	HM	(+1/+1) x1; (-27/-27) x1	2
	BI	(0/+1) x5; (+1/0) x3; (+2/0) x1; (+1/+18) x1; (-9/0) x1; (0/+15) x1	12
LTR1A	WT	(0/0)	16
	HM	-	0
	BI	(0/+1) x5; (+4/-14) x1; (+6/+1) x1; (+5/+1) x1	8

Figure S6. CRISPR/Cas9-mediated *SbGl2* editing using three *Agrobacterium* strains derived from LBA4404. (A-C) Maturation media plates for each *Agrobacterium* strain showing the ability to produce multiple calli. (D) Summary of *SbGl2* genotyping analysis using Sanger sequencing and ICE analysis (Conant et al., 2022). WT: Calli with wild-type sequence (no editing); HM: calli with homozygous mutation. BI: Calli with bi-allelic mutation.

1) Strain LTA: This is a thymidine auxotrophic strain (LBA4404Thy-) generated via *thyA* gene deletion by Corteva Agriscience; it contains vir gene helper plasmid pKL2299A (Aliu et al., 2024).
2) Strain LT1A; This is a thymidine auxotrophic strain (LBA4404T) generated via insertional mutagenesis of the *thyA* gene by our group; it also contains the vir gene helper plasmid pKL2299A
3) Strain LTR1A: This is the same as LTA, except the *recA* gene in this LBA4404 was mutated via insertional mutagenesis by our group. The *recA*-deficient LTR1A strain was used to enhance plasmid stability within Agrobacterium cell, especially can be useful for large plasmids as it lacks the *recA* gene that mediates homologous recombination thus suppress DNA rearrangements.

The transformation described in the table was from one experiment. Among the tested strains, LT1A showed highest editing efficiency (58%, 14/24), followed by LTR1A (33%, 8/24) and LTA (26%, 6/23). However, the difference was not statistically significant as the date was derived from one experiment.

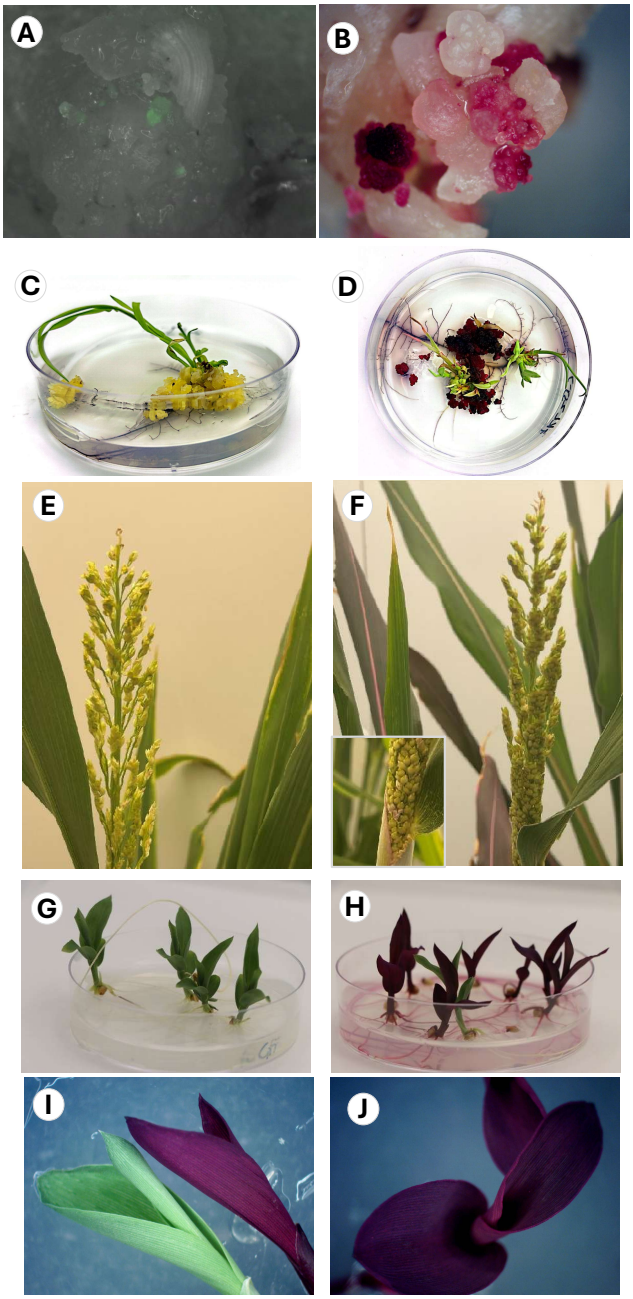


Figure S7. Test of alternate visual markers *fuGFP* and *RUBY* for sorghum transformation. Callus induction and selection: (A) *fuGFP* and (B) *RUBY*. T0 plantlets on rooting media: (C) *fuGFP* and (D) *RUBY*. Viable T0 plants at flowering: (E) *fuGFP* and (F) *RUBY*. T1 seedlings on germination media: (G) *fuGFP* and (H) *RUBY*. (H-J) Segregation of *RUBY* transgene in T1 seedlings.