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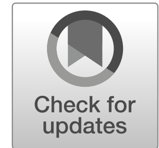
GENETIC TRANSFORMATION



Use of non-integrating *Zm-Wus2* vectors to enhance maize transformation

Non-integrating WUS2 enhances transformation

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Abstract
The use of *Baby boom* (*Bbm*) and *Wuschel2* (*Wus2*) has made maize transformation more efficient across an increasingly wide range of inbreds. However, the benefits have come with the requirement of excising these transformation helper components to enable plant regeneration, which adds size to the T-DNA, and complexity to the transformation system. A new system with the advantages of smaller size and simplicity for the selectable marker gene-containing T-DNA is described. First, expression of *Zm-Wus2* alone driven by the maize *Pltp* promoter (*Zm-Pltp$_{pro}$*), was determined to be sufficient to induce rapid somatic embryo formation from the scutella of maize immature embryos. It was also demonstrated that co-infecting with two strains of *Agrobacterium*, one with a *Wus2* expression cassette, and the other with a combination of both selectable and visual marker cassettes, produced transformed T0 plants that contained only a single copy of the selectable marker T-DNA, without the integration of *Wus2*. Furthermore, the process was optimized by varying the ratio of the two *Agrobacterium* strains, and by modulating *Wus2* expression to enable high-frequency recovery of selectable marker-containing T0 plants that did not contain *Wus2*. Several factors may have contributed to this outcome. *Wus2* expression in localized cell(s) appeared to stimulate somatic embryogenesis in neighboring cells, including those that had integrated the selectable marker. In addition, in cells in which the *Wus2* T-DNA did not integrate but the selectable marker T-DNA did, transient *Wus2* expression stimulated somatic embryo formation and regeneration of stable T0 plants that contained the selectable marker. In addition, augmenting the *Pltp* promoter with three viral enhancer elements to increase *Wus2* expression stimulated embryogenesis while precluding their regeneration. The phenomenon has now been designated as "altruistic transformation."

Keywords *Zm-WUS2* · somatic embryo formation · maize transformation · Agrobacterium

Introduction

The transcription factors *Baby boom* (*Bbm*) and *Wuschel2* (*Wus2*), which are also referred to as morphogenic genes, greatly enhance maize transformation (Lowe *et al.* 2016). Furthermore, spatio-temporal regulation of morphogenic gene expression by the maize *Pltp* promoter stimulate rapid (3–7 d post infection), and direct formation of somatic embryos from immature scutella (Lowe *et al.* 2018). The somatic embryos can be directly germinated into transgenic plants and bypass the need for callus initiation and maintenance. The use of *Nos$_{pro}$*::*Wus2* plus *Ubi$_{pro}$*::*Bbm* has been used to stimulate embryogenic callus formation in the public inbred B73 (Mookkan *et al.* 2017). More recently, *Axig1$_{pro}$*::*Wus2* plus *Pltp$_{pro}$*::*Bbm* has been shown by Lowe *et al.* (2018) to stimulate somatic embryo formation in B73, Mo17, and Fast Flowering Mini Maize (FFMM germplasm developed by McCaw *et al.* 2016). While resultant T0 plants that contained a single copy (SC) of the T-DNA with *Bbm* and *Wus2* were fertile and had a normal phenotype, ectopic expression of transcription factors can have subtle pleiotropic effects (Lowe *et al.* 2016, 2018). For example, expression of *CRC* (a fusion between two transcription factors, known as *R* and *C1*, which activates the anthocyanin pathway) in maize, induces the expression of hundreds of genes (Bruce *et al.*

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2000). Therefore, removal of the morphogenic genes is desirable for both transgene testing and commercial product development. Alternatively, transient expression of morphogenic genes from a T-DNA that is unlinked to a second T-DNA, which contains a selectable marker cassette and a visual marker cassette (referred to as the "Selectable" T-DNA), could offer a viable alternative to gene excision. This simplifies both vector construction and the downstream processes to produce transgenic or genome-edited plants.

Agrobacterium-mediated T-DNA transformation involves transient T-DNA gene expression within 36–48 h post infection, followed by stable T-DNA integration into the plant genome (Yoshioka *et al.* 1996; Gelvin 2003). This has been elegantly demonstrated using *CRE*-mediated excision of a genomic locus flanked by homologous loxP sites, without stable integration of the T-DNA harboring the *CRE recombinase* gene (De Buck *et al.* 2000). This was achieved by co-infection with two *Agrobacterium* strains, one containing a binary plasmid carrying the *CRE recombinase*, and the other strain carrying an excisable GUS construct linked to a *NPTII* cassette (De Buck *et al.* 2000).

In this study, a transformation method that exploits transient T-DNA expression to recover stable T0 plants using morphogenic genes is described. This development was predicated by the observation that a strong pulse of *Wus2* expression by the maize *Pltp* promoter was sufficient to rapidly stimulate somatic embryo formation and T0 plants. By using two *Agrobacterium* strains for transformation, one strain containing a T-DNA binary plasmid with a *Wus2* expression cassette, and a second strain containing a binary T-DNA plasmid with both a selectable and visual marker (referred to as the "Selectable T-DNA"), *Wus2*-mediated growth stimulation was provided *in trans* to cells containing the Selectable gene. Optimally, the strain containing *Wus2* T-DNA was present at a lower concentration relative to the strain containing the Selectable T-DNA. Using this mixture of *Agrobacterium* strains for transformation, transient expression of *Wus2* improved the frequency of regenerated stable T0 plants that contained only the Selectable T-DNA without stable integration of the *Wus2* transgene. The level of transient *Wus2* expression and the concomitant stimulation of somatic embryo formation in surrounding cells could be increased further, by positioning three viral enhancer elements in front of the *Pltp* promoter. This positioning provided the added benefit of increasing *Wus2* expression, which provided additional insurance that *Wus2*-expressing cells would inhibit their own regeneration. This method is hereby referred to as "altruistic transformation."

Materials and methods

Transformation and PCR analyses All transformation experiments and qPCR analyses were done as described previously (Lowe *et al.* 2018) with minor modifications.

Plant material The Pioneer maize (*Zea mays* L.) inbreds used in this study included both temperate inbreds (the non-stiff-stalk HC69, and stiff-stalks PH1V69 and PHW52), and tropical inbreds (non-stiff-stalks PH4BAH and PH2KD1, and stiff-stalks PH28SV, PH2Y8G, and PH4B9Z). These inbreds or suitable related germplasm will be provided under an applicable Material Release Agreement. All plants used for source immature embryos were grown in the greenhouse.

Culture media used for transformations and plant regeneration All media recipes are described by Lowe *et al.* (2016) and Jones *et al.* (2019), with minor modifications to media components as listed in Supplemental Table S-1, with all ingredients and sources listed in Table S-9. For selection, 0.1 mg L^{-1} imazapyr was present in the somatic embryo formation medium (13329) or 150 mg L^{-1} G418 was substituted for imazapyr.

***Agrobacterium*-mediated transformation** Expression components such as promoters, structural genes, terminators, and enhancer elements are listed in Supplemental Table S-2. All transformations were completed using the thymidine auxotrophic *Agrobacterium* strain LBA4404 THY, which contained pVIR9 (PHP71539), (Anand *et al.* 2018) at OD_{550} of 0.5. For mixtures of two *Agrobacterium* strains containing different T-DNAs, each strain was brought to the appropriate optical density and mixed at different ratios (1:1, 9:1, and 99:1), before infecting the immature embryos. *Agrobacterium* with different T-DNAs in the binary plasmid are referred to as "strains." Two selectable markers were used in experiments: a sulfonylurea herbicide resistance marker '*Hra*' (Green *et al.* 2009), driven by the sorghum ALS promoter (see Supplemental Table S-2), was used for selection with 0.1 mg L^{-1} imazapyr in both the maturation and rooting medium, or the *Ubi*$_{pro}$::*NPTII* gene was used with 150 mg L^{-1} G418 in both the maturation and rooting medium (13329 and 13158 media, respectively). Constructs used in this study are shown in Figs. 1, 2 , 4, 5, 6 and 7, and the individual components are described in Table S-2. The maize *Axig1* and *Pltp* promoters (auxin-induced and scutellum/callus-preferred, respectively) used in this study to drive expression of *Wus2* and *Bbm* have been previously described (Lowe *et al.* 2018). For two plasmids, PHP87598 and PHP88158, an expression cassette was added using a constitutive *Nos* promoter driving expression of *CRC*, which is a fusion of the maize *C1* and *R* transcription factors, which when expressed together activate the pathway for anthocyanin production (Bruce *et al.* 2000). T-DNA sequences for plasmids used in these experiments have been deposited in Genbank under the following accession numbers: PHP80770 (MN380778), PHP83623 (MN380779), PHP83027 (MN380780), PHP83621 (MN380781), PHP86491 (MN380782), PHP81561 (MN380783), PHP80912 (MN380784), PHP87078 (MN380785),

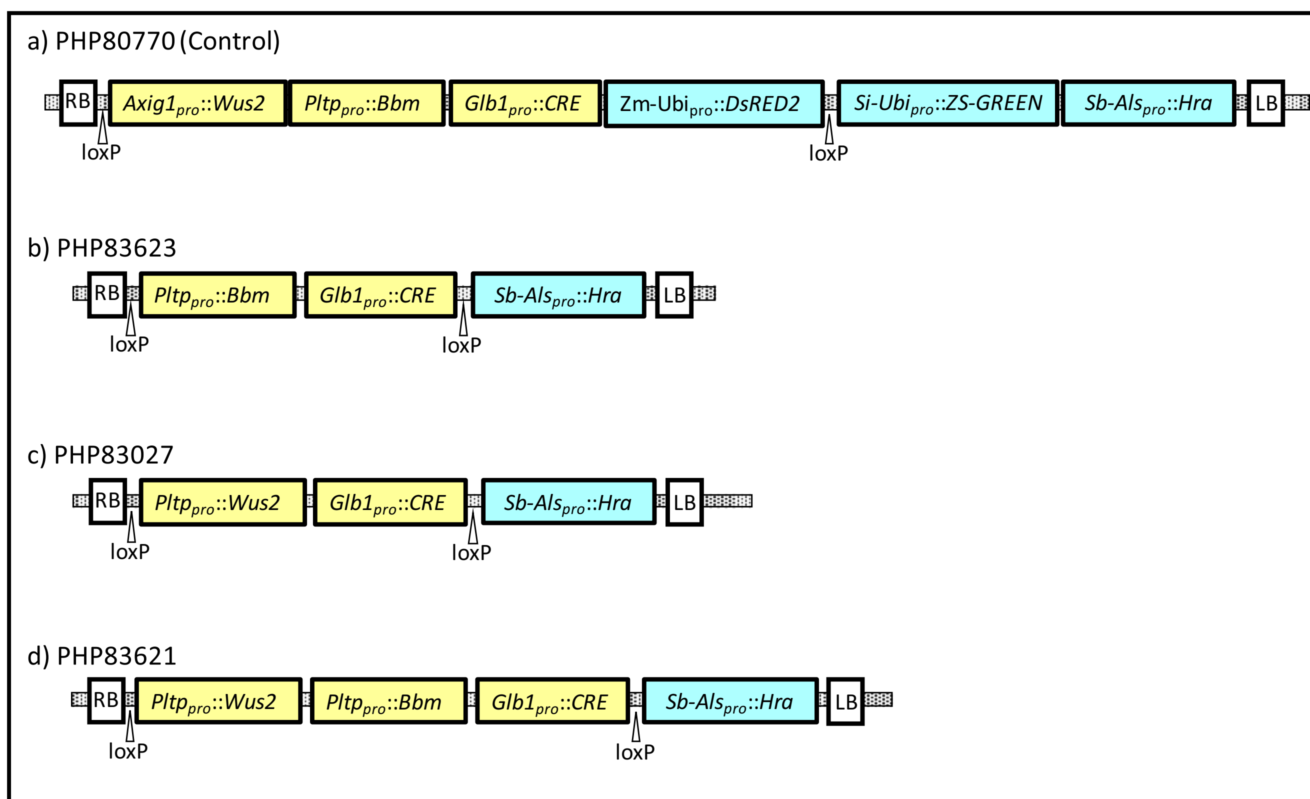

Figure 1. Constructs containing *Wus2* alone, *Bbm* alone, or in combination used in the experiments summarized in Table 1 below. *Yellow boxes* indicate excised using *CRE*/loxP, and *blue boxes* indicate no excision. *Axig1*$_{pro}$ is auxin-inducible. *Pltp*$_{pro}$ is expressed predominantly in the *Zea mays* (L) immature embryo scutellum and in callus. *Glb1*$_{pro}$ is a late-embryogenic promoter. *Ubi*$_{pro}$ and *Als*$_{pro}$ promoters are strong and weak constitutive promoters, respectively.

PHP85848 (MN380786), PHP87598 (MN380787), and PHP88158 (MN380788).

Agrobacterium cultures on solid medium were prepared to ensure that freshly cultured bacterial colonies were growing rapidly immediately before being used for transformation. The *Agrobacterium tumefaciens* strain LBA4404 THY, which contained both PHP71539 and a separate T-DNA-containing plasmid, was maintained as glycerol stocks and was periodically (every 2–3 wk) streaked out on fresh maintenance medium (Master Medium in Table S-1). The day before using the *Agrobacterium* for maize transformation, colonies were picked from the Master plate and streaked onto fresh plates containing YP medium (Ishida *et al.* 1996), on which the bacterium was grown overnight in the dark at 28°C. The following morning, colonies were collected and suspended in 700A liquid medium.

Maize immature embryos were harvested, typically at 10–12 d after pollination with an average length of approximately 1.5–2.0 mm. Immature embryos were isolated and placed into 700A medium. Once all of the embryos were harvested from the immature ear, the 700A medium was decanted, and the immature embryos were transferred into 700A medium

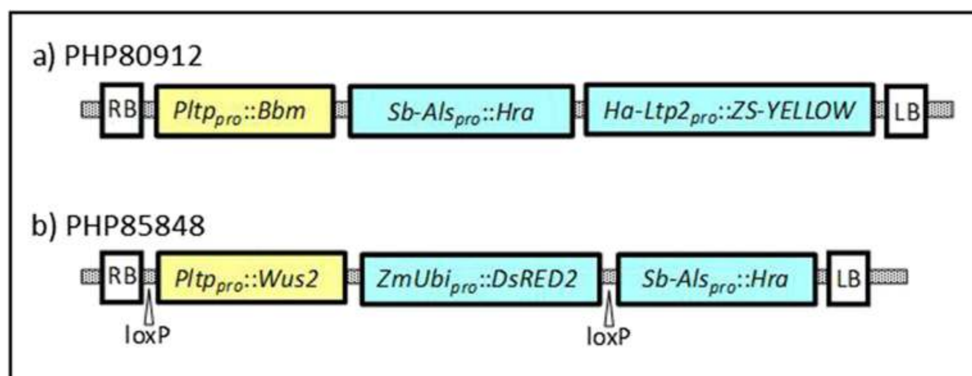

Figure 2. Contructs containing expression cassettes with the *Pltp*$_{pro}$ driving expression of *Bbm* (*a*) or *Wus2* (*b*) used in the experiment summarized in Figure 3. The *Bbm* and *Wus2* expression cassettes are highlighted in *yellow* because excision was not expected. *Ha-Ltp2*$_{pro}$ from barley is aleurone-specific.

containing freshly suspended *A. tumefaciens* strain LBA4404 THY (OD = 0.5 at 550 nm) or mixtures of strains. After 5 min in the liquid *Agrobacterium* suspension, the immature embryos were removed from the liquid and placed scutellum side up on 710I solid medium (co-cultivation medium), overnight at 21°C in the dark (for 700A, 700, and 710I media, see Zhao *et al.* 2002). The following morning embryos were moved onto 605G somatic embryo induction medium and cultured in dark at 28°C. After 6–7 d on 605G medium, the embryos were moved onto 13329 maturation medium, which contained either 0.1 mg L^{-1} imazapyr or 150 mg L^{-1} G418 (depending on the experiment). After 2–3 wk on 13329 medium, the embryos were moved to 13158 medium that also contained imazapyr (or G418) for rooting and placed under GE Ecolux (General Electric, Boston, MA) fluorescent lights (60 μmol m^{-2} s^{-1}), with a 16-h light/8-h dark photoperiod at 26°C.

Transformation frequency was defined as the number of imazapyr-resistant (or G418-resistant) T0 plants, relative to the total number of infected immature embryos. Imazapyr (or G418) selection was maintained during somatic embryo germination and rooting, to reduce the recovery of escapes (wild-type non-transgenic plants). Once shoots and roots had been established, plantlets were transferred to pots in the greenhouse (procedures for growing maize in greenhouse conditions are well established, see https://docs.lib.purdue.edu/pmcg/).

The JMP Pro 12.2.0 Statistical Discovery software package (SAS Institute Inc., Cary, NC) was used to conduct statistical tests. Data were transformed by arcsine square root transformation, and ANOVA was carried out with the Student's *t* test for each pair to identify significant differences between means.

Molecular analysis All molecular analyses were completed as described by Lowe *et al.* (2016). Molecular analysis for transgene copy number was accomplished using qPCR (Wu *et al.* 2014). In addition to copy number, qPCR data was also used to confirm recombinase-mediated excision based on the absence of loxP-flanked transgenes, and to screen for the presence of *Agrobacterium* binary vector backbone integration. To prepare genomic DNA samples, the extraction was performed using a single piece (200 ng) of fresh leaf tissue from each plant (Truett *et al.* 2000). Non-transgenic maize inbred lines were used as the negative controls. Quantification was based on detection of amplified gene sequences using gene-specific forward and reverse primers, along with the corresponding gene-specific FAM™ or Vic®-based MGB fluorogenic probes (Applied Biosystems, Waltham, MA). The 2−ΔΔCT method (Livak and Schmittgen 2001; ABI's user bulletin #2, www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf), was used to estimate copy number. For all maize transgenic plants, detection of *Agrobacterium* vector backbone was based on qPCR screening for sequences from five regions outside of the T-DNA (RB, virG, SPC, Tet, and LB). Plants with negative qPCR signals for all five regions were considered to be backbone-negative. Otherwise, the plants were classified as backbone-positive. Plants with intact single-copy T-DNA integrations without vector backbone were defined as Single-Copy (SC) events.

Results

The *Pltp* promoter driving *Wus2* alone is sufficient to stimulate the direct initiation of somatic embryos Immature zygotic embryos from two maize inbreds HC69 and PH1V69, were transformed with either the control plasmid PHP80770 (*Zm-Axig1*$_{pro}$::*Wus2* + *Zm-Pltp*$_{pro}$::*Bbm*), or with the test plasmids PHP83623 (*Zm-Pltp*$_{pro}$::*Bbm*), PHP83027 (*Zm-PltpP*$_{pro}$::*Wus2*), and PHP83621 (*Zm-PltpP*$_{pro}$::*Wus2* + *Zm-Pltp*$_{pro}$::*Bbm*), as described in Fig. 1. The T-DNA in all four constructs also carried a *CRE* expression cassette under the control of the *Zm-Globulin1* (*Glb1*) promoter (Liu *et al.* 1998), to enable auto-excision of the morphogenic genes and the *CRE* gene flanked by directly oriented loxP sites (Fig. 1), which has been used previously for excision of the morphogenic genes *Zm-Wus2* and *Zm-Bbm* (Chu *et al.* 2019). Transformation results for the four vectors are shown in Table 1. Without the *Bbm* and *Wus2* expression cassettes, recovery of transgenic events (without a prolonged callus selection stage), for HC69 typically ranges between 0 and 2%, and for PH1V69 the frequency is 0, but for the purpose of this experiment, this treatment was not included.

In maize transformation experiments, differences between genotypes are a common observation, and the overall transformation frequencies for HC69 were higher than for PH1V69 (Table 1). For both maize inbreds, transformation frequencies for *Wus2* alone, or for both combinations of *Wus2* + *Bbm* produced transgenic events, and the *Pltp*$_{pro}$::*Wus2* treatment produced a similar transformation frequency as the *Axig1*::*Wus2* + *Pltp*$_{pro}$::*BBM* treatment for both inbreds. In contrast, using *Pltp*$_{pro}$::*Bbm* alone resulted in a significantly lower transformation frequency for HC69, or no recovery of transgenic events for PH1V69. The treatment in which both *Wus2* and *Bbm* expression were driven by *Pltp*$_{pro}$, produced significantly higher transformation frequencies for both inbreds (relative to the other three treatments). For all treatments in both inbreds, the SC frequencies were reduced relative to the total transformation frequencies, but still followed the same trend. When the test vectors were compared with the control plasmid in the two inbreds, the SC frequency ranged between 2 and 4% for the control, while no SC events were recovered from *Zm-Pltp*$_{pro}$::*Bbm*. In contrast, using *Zm-Pltp*$_{pro}$::*Wus2* produced SC event frequencies ranging from 5 to 10%. Similarly, the *Zm-Pltp*$_{pro}$::*Wus2* + *Zm-Pltp*$_{pro}$::*Bbm* also produced SC events,

Table 1. Transformation results for two maize inbreds using *Wus2* alone, *Bbm* alone, and combinations of *Wus2* plus *Bbm*. Immature zygotic embryos from two inbreds were transformed with the constructs shown in Fig. 1 (in order for each inbred). T0 plant number and transformation frequencies are shown, including both multi- and single-copy (SC) numbers. The SC event frequency (T0 plants containing a single copy of T-DNA without a vector backbone relative to the total number of immature embryos), were tabulated based on qPCR data. Transformation and single-copy data for individual replicates within each treatment are shown in Table S-3

Inbred	Promoters for Morphogenic Genes		Num. embryo	Num. T0 plants	Txn% ($\bar{x}\pm$ SD)	Sign. diff.	Num. SC	SC%
	Wus2	*Bbm*						
HC69	*Axig1*$_{pro}$	*Pltp*$_{pro}$	388	219	57.5 (6.1)	a	12	4
HC69		*Pltp*$_{pro}$	148	8	5.0 (2.0)	b	0	0
HC69	*Pltp*$_{pro}$		414	242	57.8 (3.4)	a	42	10
HC69	*Pltp*$_{pro}$	*Pltp*$_{pro}$	429	334	77.8 (6.0)	c	58	14
PH1V69	*Axig1*$_{pro}$	*Pltp*$_{pro}$	360	31	8.8 (2.7)	a	6	2
PH1V69		*Pltp*$_{pro}$	360	0	0	b	0	0
PH1V69	*Pltp*$_{pro}$		360	40	11.0 (6.1)	a	18	5
PH1V69	*Pltp*$_{pro}$	*Pltp*$_{pro}$	360	93	23.3 (9.5)	c	40	11

Num. embryo = the number of immature embryos infected by the *Agrobacterium*.

Num. T0 plants = the number of qPCR-confirmed independent T0 plants (transgenic events) recovered per treatment.

Txn% = the transformation frequency calculated as (# T0 plants/*Agrobacterium*-infected immature embryos) * 100.

Num. SC = the number of T0 plants (events) that contained a single copy of the *Wus2*/*Bbm* T-DNA.; SC% = the frequency of single-copy T0 plants relative to the starting number of *Agrobacterium*-infected immature embryos.

Sign. diff. = signifies whether the means for the treatments within an inbred are significantly different (different letters) or not (same letter) with $p = 0.05$

but at higher frequencies of 11–14% in the two different inbreds. The data suggested the following trends in the two inbreds: (1) transformation frequencies using the control vector (*Zm-Axig1*$_{pro}$::*Wus2* + *Zm-Pltp*$_{pro}$::*Bbm*), were comparable to those using *Zm-Pltp*$_{pro}$::*Wus2*, but the production of SC events was higher for *Zm-Pltp*$_{pro}$::*Wus2*; (2) both *Wus2* and *Bbm* under the control of the *Pltp* promoter resulted in higher transformation frequencies and SC event frequencies, and in contrast; and, (3) using *Zm-Pltp*$_{pro}$::*Bbm* resulted in the lowest transformation and SC event frequency compared with the other constructs.

Mixing an *Agrobacterium* strain containing *Zm-Pltp*$_{pro}$::*Wus2* with an *Agrobacterium* strain containing *Zm-Pltp*$_{pro}$::*Bbm* produced an unexpected response It was observed that a strong pulse of *Wus2* expression alone was enough to improve transformation and SC frequency (Table 1). The co-expression of the morphogenic genes from two different *Agrobacterium* strains was also evaluated, to characterize their effect on corn transformation. Immature embryos derived from PHIV69 were transformed with two *Agrobacterium* strains containing either PHP80912 (*Zm-Pltp*$_{pro}$::*Bbm* plus marker genes), or PHP85848 (*Zm-Pltp*$_{pro}$::*Wus2* plus marker genes) separately, or as mixtures. For the mixture, the two strains of *Agrobacterium* were individually adjusted to $OD_{550} = 0.5$ and mixed at a 1:1 ratio for immature embryo transformation. Embryos derived from four independent ears were split among the three treatments, using a minimum of 30 embryos/ear per treatment. Plants were regenerated and sampled for qPCR analysis for presence and copy number of *Wus2*, *Bbm*, or both (depending on the treatment). The frequency of recovering single-copy T0 plants (SC%) was measured for each treatment and is summarized in Fig. 3 (with data for replicates within each treatment shown in Table S-4).

As predicted, *Zm-Pltp*$_{pro}$::*Bbm* alone transformed poorly and no SC events were recovered. As expected, *Zm-Pltp*$_{pro}$::*Wus2* produced several SC events in each replicate ($\bar{x}$ = 14.6%), with 38 total SC plants recovered. When the two *Agrobacterium* suspensions containing these two plasmids were mixed together in a 1:1 ratio, 25 SC events were recovered that had only integrated the *Zm-Pltp*$_{pro}$::*Bbm* T-DNA ($\bar{x}$ = 11%), and 13 SC events were recovered that only contained the *Zm-Pltp*$_{pro}$::*Wus2* T-DNA ($\bar{x}$ = 5.3%). In the *Wus2* + *Bbm* treatment, no transgenic events were recovered that contained both T-DNAs. In summary, *Zm-Pltp*$_{pro}$::*Bbm* alone did not produce SC events, while *Zm-Pltp*$_{pro}$::*Wus2* effectively produced SC events, which was consistent with the data presented in Table 1. Unexpectedly, the mixed *Agrobacterium* treatment resulted in the recovery of stable SC plants that either contained only *Zm-Pltp*$_{pro}$::*Bbm* (with no *Zm-Pltp*$_{pro}$::*Wus2* integration), or contained *Zm-Pltp*$_{pro}$::*Wus2*. The frequency of SC *Zm-Pltp*$_{pro}$::*Bbm* plants was nearly twice the frequency of SC plants that contained *Zm-Pltp*$_{pro}$::*Wus2* alone. Based on these results, it was hypothesized that the pulsed expression of *Wus2* stimulated somatic embryogenesis of adjacent cells that had stably integrated *Zm-Pltp*$_{pro}$::*Bbm*, which resulted in stable SC plant recovery.

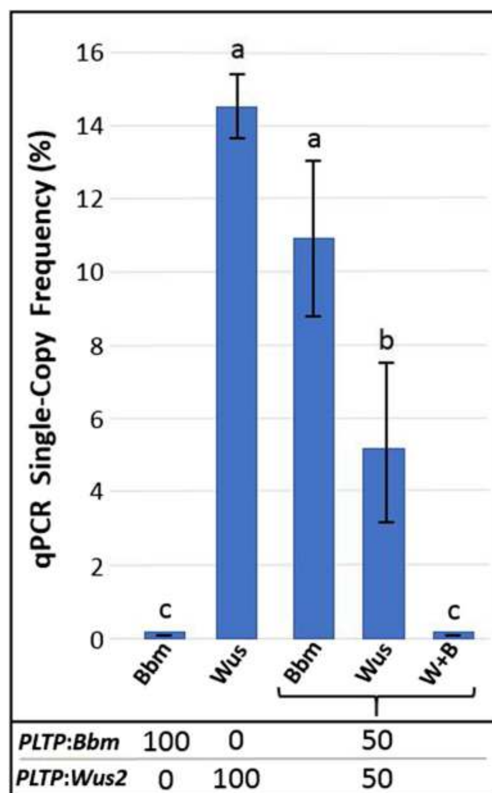

Figure 3. Single-copy frequencies for integrated T-DNAs containing either the *Bbm* gene (Bbm), the *Wus2* gene (Wus), or the *Bbm* and *Wus2* T-DNAs (W+B) in T0 plants for maize inbred PH1V69. *Zea mays* (L) immature embryos from four ears were aliquoted into three batches, and transformed with *1)* a single *Agrobacterium* strain containing PHP80912 (*Zm-Pltp*$_{pro}$::*Bbm*); *2)* a single *Agrobacterium* strain containing PHP85848 (*Zm-Pltp*$_{pro}$::*Wus2*); or *3)* with a 1:1 mixture of the two *Agrobacterium* strains. The three treatments are designated by the numbers at the bottom; Treatment 1 (100% *Bbm*:0% *Wus2*), Treatment 2 (0% *Bbm*:100% *Wus2*), and Treatment 3 (50% *Bbm*:50% *Wus2*). qPCR was used to determine the numbers of recovered T0 plants that were single copy (SC) for *Bbm* and *Wus2*, and the mean frequencies relative to the starting number of immature embryos are shown. Underneath the bars, acronyms indicate qPCR for *Bbm* and *Wus2* for the various treatments. W+B stands for a single copy for both *Bbm* and *Wus2*. Letter designations above the bars signify whether the means for the treatments are significantly different (*different letters*), or not (*same letter*) with $p = 0.05$. Single-Copy Transformation data for individual replicates within each treatment are shown in Table S-4.

Transformation with a mixture of one *Agrobacterium* strain containing an herbicide resistance marker and another *Agrobacterium* strain containing Zm-*Pltp*$_{pro}$::Wus2 resulted in the recovery of events that contained only the selectable marker in a ratio-dependent manner To test the hypothesis that *Wus2* stimulated somatic embryo formation in neighboring cells that had received only *Bbm*, the experiment described previously was repeated, only replacing *Bbm* with the Selectable expression cassettes. To this end, a second *Agrobacterium* strain was engineered, which contained a T-DNA binary plasmid harboring the *Hra* and *Zs-GREEN* expression cassettes (PHP86491, Fig. 4), without any morphogenic genes (no *Bbm* or *Wus2*). To improve the morphogenic stimulation, three caulimoviral enhancers from the Fig Wart Mosaic Virus, the Peanut Chlorotic Streak Virus, and the Mirabalis Mosaic Virus (the tandem enhancer being abbreviated as 3xENH), were placed upstream to increase expression from the *Zm-Pltp*$_{pro}$::*Wus2* expression cassette in the altruistic T-DNA (in PHP87078, see Fig. 4).

Agrobacterium strains containing either *Hra* + *Zs-GREEN* (Selectable), or 3xENH- *Zm-Pltp*$_{pro}$::*Wus2* (*Wus2*) were suspended in liquid, adjusted to the same density, and used with the Selectable alone (1:0 treatment), or in mixed ratios of 1:1, 9:1, and 99:1 (Selectable:*Wus2*). Next, the mixtures were used to transform HC69 immature embryos. As shown in Table 2, for the four treatments (1:0 Selectable alone, 1:1 Selectable to *Wus2* ratio, 9:1 Selectable to *Wus2* ratio, and 99:1 Selectable to *Wus2* ratio), the overall transformation frequencies were 2.2, 9.2, 24.2, and 20.8%, respectively [(# Multi-copy + # Single-copy/# Immature embryos)*100]. SC frequencies followed the same trend (2.7, 5.9, 12.6 and 6.8%). All of the SC T0 plants contained only a single copy of the Selectable T-DNA without *Wus2*. Based on a confidence interval of 0.05, all three *Agrobacterium* mixture ratios (1:1, 9:1, and 99:1), resulted in SC% values that were similar to each other but significantly greater than the control (100% Selectable vector). Co-integration frequencies were low to non-existent in the three treatments with mixed strains. In the first three treatments (Selectable alone, 1:1 and 9:1), wild-type non-transgenic plants were produced ("Num. Escape" in Table 2). In contrast, no escapes were observed in the 99:1 treatment.

In summary, the following trends were observed: (1) the addition of altruistic *Wus2* increased the SC transformation frequencies of the stably integrated Selectable-containing (*Hra*) transgene for all the *Agrobacterium* mixtures when compared with Selectable T-DNA alone; (2) while all three dilutions were not statistically different, the mean SC% for the 9:1 mixture appeared to provide the most practical treatment for further experimentation; and (3) very few of the plants were co-transformed (contained both T-DNAs). These data clearly demonstrated that strong transient expression of *Wus2* from the altruistic T-DNA stimulated somatic embryo formation in cells in which *Wus2* did not integrate, which resulted in the recovery of only Selectable T-DNA-containing T0 plants.

Trans acting recombinase activity indicated co-residence of both T-DNAs in a high frequency of "Selectable only" events When the two *Agrobacterium* strains were mixed at a ratio of 9:1 (*Hra*:*Wus2*), there were at least two possible mechanisms that could result in high-frequency somatic embryo formation containing only the selectable marker. Transient *Wus2* expression could occur in a cell receiving both T-DNAs with only the selectable marker integrating, and/or WUS2 protein could move from one cell into another cell containing only the Selectable T-DNA. To test this theory, an *Agrobacterium*

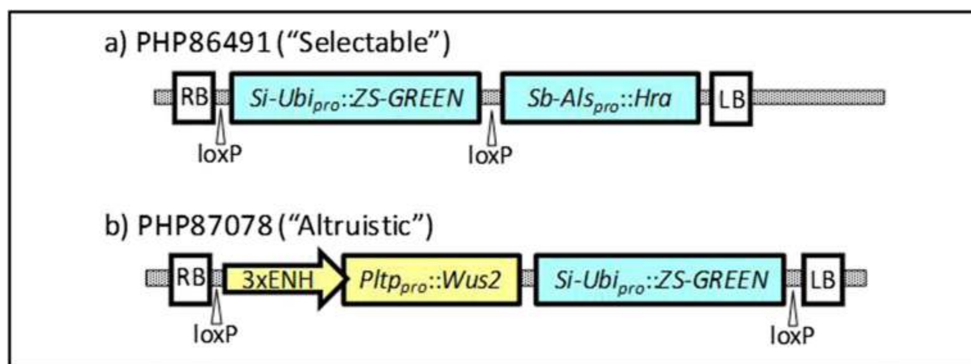

Figure 4. Constructs used in the experiment summarized in Table 2 below. The T-DNA within PHP86491 (*a*) contained constitutive *ZS-GREEN* and an *Hra* expression cassette in which the *Highly resistant Acetolactate synthase gene* (*Hra*) was expressed behind the sorghum Als_{pro}. The T-DNA within PHP87078 (*b*) contained a *Wus2* expression cassette using the *Zm-Pltp*$_{pro}$ with three viral enhancer element upstream (abbreviated 3xENH), and the contituitive *ZS-GREEN*. The *WUS2* expression cassette in the Altruistic T-DNA is highlighted in yellow, and due to the absence of *CRE*, no excision was expected.

culture harboring the altruistic T-DNA (loxP + 3xENH:: *Zm-Pltp*$_{pro}$::*Wus2* + *Zm-Hsp26*$_{pro}$::CRE + *Nos*$_{pro}$::*CRC* + loxP, PHP87598), and an *Agrobacterium* strain harboring a T-DNA with *Hra* plus a loxP-flanked *Zs-GREEN* (PHP86491, Fig. 5), were normalized to equal cell densities and mixed at ratios of 1:0, 1:1, and 9:1 before infecting HC69 immature embryos. As the *Wus2*-containing *Agrobacterium* was diluted with increasing amounts of Selectable T-DNA-containing *Agrobacterium*, a corresponding increase in the transformation frequency (Table 3) was observed. The highest dilution (9:1), resulted in a significantly higher frequency in T0 plants that contained a single copy (SC) of only the Selectable T-DNA, relative to the *Hra*-alone (1:0) treatment (Table 3). Plants that contained only one copy of the *Hra* with or without *Zs-GREEN* (excised), and without the *Agrobacterium* backbone were scored as SC (Table 3). The frequency of SC events ranged from 1.6–6%, with the highest frequency resulting from the highest dilution of the altruistic *Agrobacterium*. Co-transformed events were recovered in both the 1:1 and 9:1 treatments. Escapes were observed in all three treatments, but were readily eliminated before the plants were transferred to the greenhouse based on qPCR screening.

Surprisingly, when the results for the two "Mixed-Agro" treatments were combined, the *Zs-GREEN* marker was excised in approximately 42% of the recovered events that were SC for the Selectable T-DNA (based on the total number of SC-*Hra* (No ZSG), relative to the total number of SC-*Hra* (both with and without ZSG). Because there were no *Wus2* or *CRE* sequences present in these cells, excision must have

Table 2. Transformation data and the frequency of recovering SC integration events in Pioneer maize inbred HC69, after transformation with an *Agrobacterium* strain harboring a plasmid with a Selectable T-DNA (PHP86491), or with a mixture of Selectable T-DNA *Agrobacterium* (*Hra*) and a second *Agrobacterium* strain harboring a T-DNA containing plasmid with *Wus2* (PHP87078). Transformation and single-copy data for individual replicates within each treatment are shown in Table S-5

Agro. strain(s)	Agro. ratio	Number imm. embryo	Num. plants	Determined using qPCR				SC% $\overline{x}$ ± (SD)	Sign. diff.
				Num. escape	Num.MC	Num. Co-Tx	Num. SC		
Hra	1:0	923	29	8	8	NA	13	2.7 (3.1)	a
Hra + *Wus2*	1:1	260	44	16	11	4	13	5.9 (6.1)	b
Hra + *Wus2*	9:1	289	84	12	42	2	28	12.6 (4.2)	b
Hra + *Wus2*	99:1	230	48	0	30	0	18	6.8 (5.3)	b

Agro ratio = ratio of *Hra*-Agro:*Wus2*-Agro used in the *Agrobacterium* mixture for transformation

Number imm. embryo = the number of immature embryos infected by the *Agrobacterium*

Num. plant = the total number of plants recovered per treatment; Num. escapes = the number of non-transgenic plants recovered per treatment

Num. MC = the number of plants containing multiple copies of transgenes and/or Agrobacterium plasmid backbone sequence

Num. Co-Tx = the number of plants containing both the Selectable (*Hra*) and Altruistic (*Wus2*) T-DNA sequences

Num. SC = the number of T0 plants (events) that contained a single copy of the *Wus2*/*Bbm* T-DNA

SC% = the frequency (mean ± standard deviation) of single-copy T0 plants relative to the starting number of *Agrobacterium*-infected immature embryos (based on replicates in Table S-5)

Sign. diff. = signifies whether the means for the treatments within an inbred are significantly different (different letters), or not (*same letter*) with $p = 0.05$

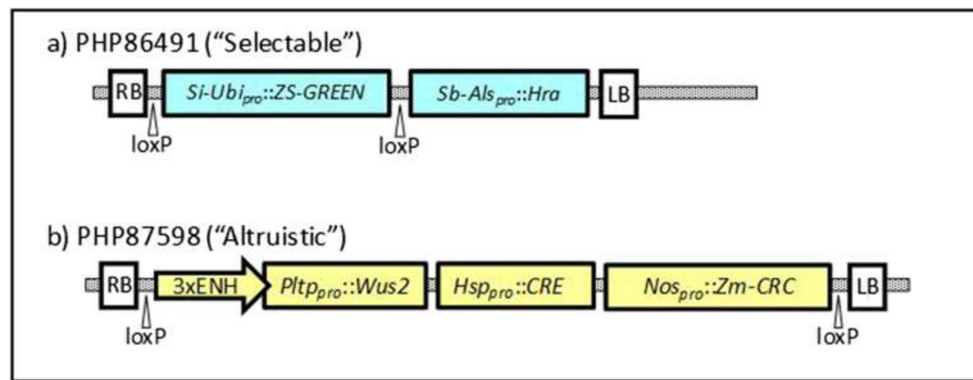

Figure 5. Constructs used in the experiment summarized in Table 3 below. The Selectable T-DNA within PHP86491 (*a*) contained *ZS-GREEN* (flanked by loxP recombination sites) and *Hra* (outside the loxP sites). The T-DNA within PHP87598 (*b*) contained a *Wus2* expression cassette using the 3xENH:*PLTP$_{pro}$*, a Heat Shock Protein promoter (*Hsp$_{pro}$*) driving expression of the *CRE* recombinase and a constitutive promoter (*Nos*) driving expression of *CRC*. Expression cassettes within the Altruistic T-DNA that could potentially be excised with exposure to CRE recombinase are highlighted in *yellow*. The feature labeled 3xENH is a fusion of three enhancer elements from plant viral promoters described in Table S1.

occurred even though the *Wus2*/*CRE*-containing T-DNA did not integrate. However, the presence of the three strong viral enhancer elements in the T-DNA, while not immediately adjacent to the *Hsp26$_{pro}$*, could have enhanced activity of the promoter *in trans*, as has been concluded in other studies (Weigel *et al.* 2000; Yoo *et al.* 2005; Gudynaite-Savitch *et al.* 2009). It should be emphasized that no heat treatment was applied during this experiment (which normally stimulates the *Zm-Hsp26$_{pro}$*). This suggested that *CRE* recombinase activity had to be present due to transient expression from a non-integrated altruistic T-DNA. The remaining 58% of cells received the loxP-flanked *Zs-GREEN* T-DNA and produced transgenic plants. There are two explanations to describe this observation: 1) CRE

Table 3. Transformation data for maize inbred HC69 after infection with an *Agrobacterium* strain containing PHP86491, or after co-infection with mixtures of two *Agrobacterium* strains, one containing PHP86491, and the second containing PHP87598 (mixed at two different ratios). Transformation and single-copy data for individual replicates within each treatment are shown in Table S-6

Agro. strain(s)	Agro. ratio	Num. embryos	Reg. plants	Determined by qPCR								SC%	Sign. diff.
				Number escapes	Number T0 PCR+	SC-HRA + SC-ZSG	SC-HRA (no ZSG)	Number MC	WUS only	Co-Txn	SC-HRA BB-		
HRA	1:0	321	61	39	22	6	0	16	NA	NA	6	1.6 (1.4)	a
HRA:WUS	1:1	191	34	11	23	5	6	6	1	5	11	3.8 (5.4)	a,b
HRA:WUS	9:1	551	123	39	84	20	12	26	0	26	32	6.0 (3.7)	b

Agro ratio = ratio of HRA-Agro:WUS-Agro used in the *Agrobacterium* mixture for transformation

Num. embryo = the number of immature embryos infected by the *Agrobacterium*

Reg. plant = the number of plants recovered per treatment

Number. escapes = the number of non-transgenic plants recovered per treatment (containing no T-DNA sequences)

Number T0 PCR+ = the number of qPCR-positive plants for any of the transgenes within the two T-DNA sequences

SC-HRA + SC-ZSG = the number of plants containing single copies of both *Hra* and *Zs-GREEN*

SC-HRA (no ZSG) = the number of plants containing a single copy of *Hra* and no sequence from the *Zs-GREEN* T-DNA

Number. MC = the number of plants containing multiple copies of transgenes and/or *Agrobacterium* plasmid backbone sequence

WUS only = number of plants with only the *Wus2* expression cassette (possible due to T-DNA truncation)

Co-Tx = the number of plants containing both the Selectable (*Hra*) and Altruistic (*Wus2*) T-DNA sequences, including both single- and multicopy integrations

SC-HRA BB- = the number of T0 plants (events) that contained a single copy of the Selectable T-DNA (*Hra*) with no Altruistic (*Wus2*) sequence and no *Agrobacterium* backbone sequence

SC% = the frequency of single-copy T0 plants relative to the starting number of *Agrobacterium*-infected immature embryos (mean ± standard deviation, based treatment replicates in Table S-6)

Sign. diff. = signifies whether the means for the treatments are significantly different (different letters) or not (same letter) with $p = 0.05$

was insufficiently expressed in the co-infected cell, or the Selectable T-DNA-containing cell was not co-infected by the altruistic T-DNA; or 2) the Selectable-containing cell was stimulated to produce somatic embryos due to movement of WUS2 protein from adjoining cells, or its downstream mobile signaling pathway.

Adding *CRC* to the *Wus2* helper vector eliminated co-transformed events without excision Encouraged by the results, an altruistic T-DNA vector was constructed (PHP88158), in which the *CRE* expression cassette had been removed (Fig. 6*b*), but the 3xENH: *Zm-Pltp*$_{pro}$::*Wus2* and a *Nos*$_{pro}$::*CRC* expression cassettes remained, with *CRC* acting both as a color marker and a counter-selective agent. In addition to providing expression of *Wus2* at levels above *Pltp* alone, it was speculated that the 3xENH would also increase *CRC* expression.

This new construct was tested in six new recalcitrant Pioneer inbreds (all normally non-transformable). Two liquid suspensions, one with the Selectable T-DNA-containing *Agrobacterium* strain (harboring PHP86491, Fig. 6*a*), and the other with the *Wus2*-containing *Agrobacterium* strain (harboring PHP88158), were normalized to an OD_{550}= 0.5. The two *Agrobacterium* suspensions were then mixed together at a ratio of 9:1 (Selectable:*Wus2*) and used to infect immature embryos from six different Pioneer maize inbreds, including one Pioneer temperate inbred (PHW52), two inbreds that are intermediate between temperate and tropical (PH2KD1 and PH28SV), and three tropical inbreds (all six inbreds not transformed previously). For all six inbreds, when a Selectable T-DNA-containing (such as *Hra* or *NPTII*) *Agrobacterium* was used alone (without a *Wus2 Agrobacterium* strain), no transgenic events could be produced in these genotypes (data not shown). However, when a 9:1 mixture of *Agrobacterium* strains (Selectable/*Wus2*) was used, transformation frequencies for these inbreds ranged from 1.2 to 46%, and T0 plants that contained a single copy for the selectable T-DNA ranged from 0 to 19.6% (Table 4, and Table S-7). Co-transformation frequencies were low for all six inbreds. The few co-transformed plants obtained were easily identified by their deep red color. While unable to develop into healthy, fertile plants due to the presence of *Wus2* and *CRC*, abnormal plantlets with small, red leaves were sampled in culture for qPCR, which provided the co-transformation data.

To evaluate an alternative to *Hra* as the selectable marker, an *Agrobacterium* strain containing *NPTII* (PHP81561) in the Selectable T-DNA (Fig. 7*a*) was tested, along with the *Agrobacterium* strain carrying *Wus2* (PHP88158), in the Altruistic T-DNA (Fig. 7*b*). After transformation, the transformed cells were selected on media supplemented with 150 mg L^{-1} G418. As shown in Table 5, all six inbreds could be transformed using G418 selection. Compared with *Hra* (Table 4), the overall transformation frequency in all six inbreds appeared to be higher when *NPTII* was used as the selectable marker (Table 5), and SC T0 plants could be regenerated from all six inbreds. Importantly, while the use of *NPTII* and G418 selection improved transformation for all of the inbreds, the co-transformation frequency remained low (Table 5).

The growth response responsible for the observed low co-transformation frequencies shown in Tables 4 and 5 (using PHP88158), is shown in Fig. 8. Seven days after *Agrobacterium* infection, numerous multicellular clusters of cells on the scutellar surface accumulated anthocyanin (Fig. 8*a*), while somatic embryo formation in surrounding tissues also occurred (yellow arrows) due to inferred expression of *Wus2* from the anthocyanin co-expressing sectors. When transferred to embryo maturation medium, the somatic embryos continued to develop and regenerate (Fig. 8*b*) and produced vigorously growing plantlets that were anthocyanin-free, while additional growth in tissues with accumulated anthocyanin was inhibited. Therefore, the cells that integrated the *Wus2*/*CRC* T-DNA could produce somatic embryos but were incapable of regenerating normal plants, which was likely due to the enhanced expression of the transcription factors.

Successfully growing tropical plants (both wild-type and transgenic progeny) in the greenhouse is a challenge, because their photoperiod requirements are so different from the light conditions in a temperately located facility. Nonetheless, fertile T0 plants were produced in all six maize inbreds. For example, in the inbred PH2KD1, which is adapted to photoperiods that are intermediate compared with temperate or tropical inbreds,

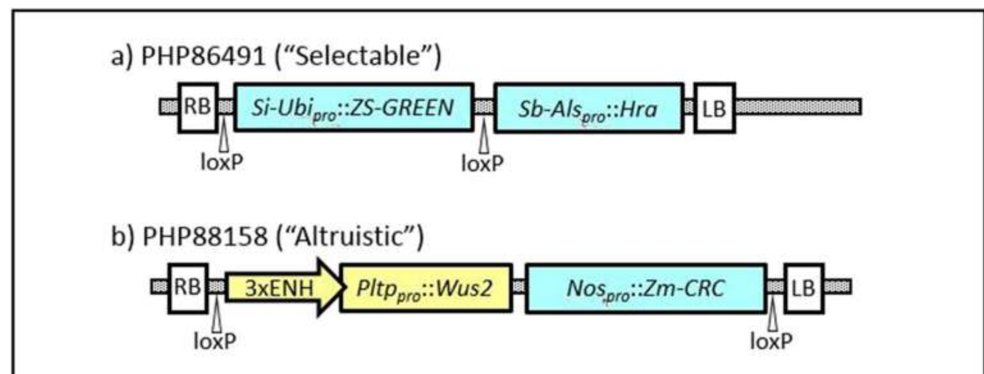

Figure 6. Contructs used in the experiment summarized in Table 4 below. The T-DNA within PHP86491 (*a*) contained ZS-GREEN and *Hra,* while PHP88158 (*b*) contained 3xENH:*Plt*$_{pro}$::*Wus2* and a constitutive promoter (*Nos*) driving expression of *CRC.*

Table 4. Transformation frequency, single-copy frequency, and the number of co-transformed T0 maize plants recovered. Txn% represents the frequency of T0 plants recovered (relative to the number of immature embryos infected), which contained only the *Hra*-containing T-DNA, after using a mixture of two *Agrobacterium* strains at a 9:1 ratio; the first with *Hra* for selection (in PHP86491), and the second that contained 3xENH:: *Zm-Pltp$_{pro}$*::*Wus2* in the T-DNA of PHP88158. Transformation data was based on both imazapyr-resistance and qPCR, while SC and Co-Txn determinations were based on qPCR data. Transformation and single-copy data for individual replicates within each treatment are shown in Table S-7

Inbred	Immature Embryos treated	Determined using qPCR				
		Number of HRA+ T0 plants	Txn% $\bar{x}$ ± (SD)	Number single-copy (SC)	SC% $\bar{x}$ ± (SD)	Number Co-Txn
PHW52	339	157	46 (16)	56	12.4 (8.8)	0
PH2KD1	178	75	39 (34)	38	19.6 (18.2)	2
PH28SV	61	1	1.2 (1.1)	0	0	0
PH4BAH	72	22	34 (30)	8	11.1 (5.8)	0
PH2Y8G	130	19	14 (9)	3	1.9 (2.6)	0
PH4B9Z	109	7	4.5 (6.2)	1	1 (1.9)	1

SC% = Single-Copy Frequency for the Selectable T-DNA (*Hra*), relative to the Number of Immature Embryos

Number Co-Txn = the number of T0 plants that had integrated both the *HRA*- and *Wus2*-containing T-DNAs

29 T0 plants from the NPTII/G418 experiment, and 29 plants from the *Hra/*imazapyr experiment were grown to maturity in the greenhouse. Of this total number for each set of plants, 21/29 and 25/29 plants set seed (respectively). Of the 21 (*NPTII*) and 25 (*Hra*) fertile ears, seed set was greater than 100 kernels/ear in 21 (16 selfs and 5 pollinated with WT pollen), and 22 ears (13 selfs and 9 pollinated with WT pollen), respectively. For a second maize inbred (PH4BAH), which is a true tropical line, 18 plants in the NPTII experiment were grown in the greenhouse. Of these plants, 14 set seed, and six plants produced over 100 kernels/ear. For the *Hra* experiment, 10 plants were sent to the greenhouse, nine produced seed, and eight T0 plants had seed sets >100 kernels/ear. Robust fertility is a good indicator of overall plant vigor after transformation. Inbreds from temperate regions are adapted to the light conditions in the greenhouses used in this experiment, and plant vigor and fertility was very good, as has been previously reported (Lowe *et al.* 2016, 2018).

Discussion

When virulent *Agrobacterium* strains were used for plant transformation, it was observed that normal untransformed shoots and shooty teratomas would occasionally arise from *Agrobacterium*-derived tumors cultured on hormone-free medium (Aerts *et al.* 1979). Presumably, untransformed cells were able to proliferate on hormone-free medium due to stimulation by enhanced hormone production (auxin and cytokinin) from neighboring cells containing the T-DNA genes. These results were further exploited in transformation by mixing either a wild-type "armed," tumor-producing *Agrobacterium* strain (Depicker *et al.* 1985) or a partially armed *Agrobacterium* strain (that produced shooty growths), in combination with a disarmed *Agrobacterium* strain with or without a selectable marker (Brasileiro *et al.* 1991; Zambryski *et al.* 1984; Kuchuk *et al.* 1990; Aronen *et al.* 2002; Mihálka

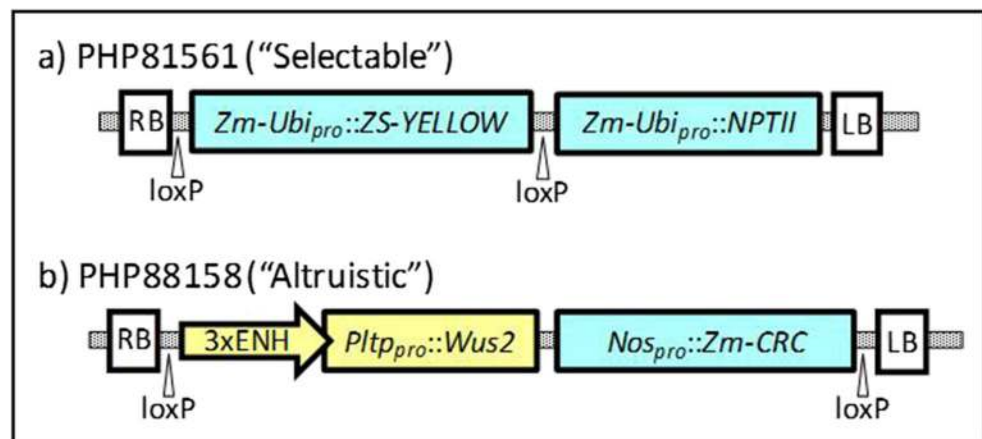

Figure 7. Constructs used in the experiment summarized in Table 5 below. The T-DNA within PHP86491 (*a*) contained *ZS-YELLOW* and *NPTII*, while PHP88158 (*b*) contained 3xENH:*Plt$_{pro}$*::*Wus2* and *Nos*::*CRC*.

Table 5. Transformation frequency, single-copy frequency, and the number of co-transformed T0 maize plants recovered. Txn% represents the frequency of T0 plants recovered (relative to the number of immature embryos infected), which contained only the *NPTII*-containing T-DNA, after using a mixture of two *Agrobacterium* strains at a 9:1 ratio; the first with *NPTII* for selection (in PHP81561), and the second that contained 3xENH:: *Zm-Pltp*$_{pro}$::*Wus2* in the T-DNA of PHP88158. Transformation data was based on both G418-resistance and qPCR, while SC and Co-Txn determinations were based on qPCR data. Transformation and single-copy data for individual replicates within each treatment are shown in Table S-8

Inbred	Immature embryos treated	Determined using qPCR				
		Number of NPTII+ T0 plants	Txn% $\overline{x} \pm$ (SD)	Number single-copy (SC)	SC% $\overline{x} \pm$ (SD)	Number Co-Txn
PHW52	331	223	77 (32)	45	7.4 (7.4)	1
PH2KD1	167	89	53 (32)	32	16.2 (17.3)	0
PH28SV	62	7	16 (9)	2	4.3 (2.9)	0
PH4BAH	77	27	36 (18)	14	20.1 (16.5)	0
PH2Y8G	135	10	7 (6)	1	0.5 (0.9)	0
PH4B9Z	102	48	54 (23)	15	17.4 (9.2)	0

* SC% = single-copy frequency for the Selectable T-DNA (*NPTII*), relative to the number of immature embryos. Number Co-Txn = the number of T0 plants which had integrated both the *NPTII*- and *Wus2*-containing T-DNAs

et al. 2003). This allowed the recovery of T0 plants that contained only the T-DNA from the disarmed strain. As suggested by Mihálka *et al.* (2003), the recovery of transgenic events could have occurred from the transient expression of the T-DNA genes on the armed (or partially armed) strain without its stable integration. Whereas Mihálka *et al.* (2003) used partially disarmed strains containing T-DNA with either auxin or cytokinin genes for transformation, in this study a disarmed *Agrobacterium* strain containing a T-DNA plasmid harboring the maize *Wus2* expression cassette was used to stimulate somatic embryogenesis in neighboring cells and recover stable transgenic events.

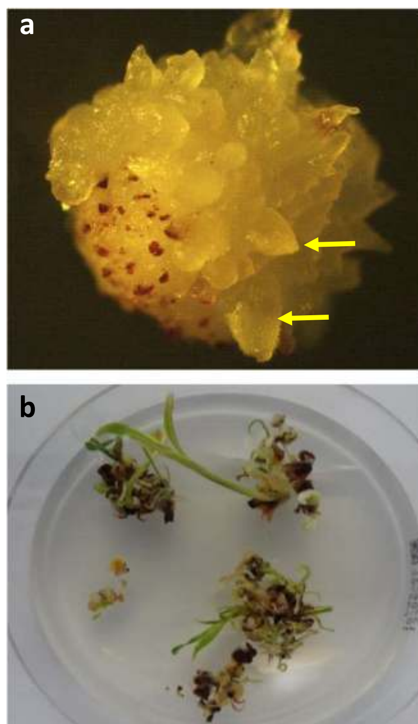

Figure 8. After co-transformation of tropical maize inbred PH2KD1 with two *Agrobacterium* strains at a ratio of 90% PHP 81561 and 10% PHP88158, multicellular clusters that contained anthocyanins due to *CRC* expression can be observed in the lower left-hand quadrant of the originally transformed zygotic immature embryo (*a*) with non-red somatic embryos forming nearby (*yellow arrows*). When transferred to embryo maturation medium, plantlets with no anthocyanin readily regenerated, while the growth of anthocyanin-containing tissues was inhibited (*b*).

As reviewed by Yau and Stewart (2013), mixed *Agrobacterium* strains have primarily been used to produce marker-free transgenic events. This has been accomplished either through co-transformation, in which the two independent transgenic loci would be segregated away from each other later in the process (Miller *et al.*; 2002; Komari *et al.* 1996), or through transient expression of the altruistic T-DNA (either hormone genes or selectable markers), for the direct production of marker-free transgenic events (Dutt *et al.* 2008; Gleave *et al.* 1999; Park *et al.* 2004). While the use of hormone-producing *Agrobacterium* strains to improve transformation has been useful in dicots, the utility in cereals has been minimal, due to the limited effect of cytokinins on somatic embryogenesis (the major route for cereal transformation).

Alternatively, transient expression of transcription factors to elicit an embryogenic response represents a viable alternative. Accordingly, Florez *et al.* (2015) used transient delivery of a transcription factor to stimulate embryo proliferation by introducing a T-DNA containing a *Tc-Bbm* expression cassette into *Theobroma cacao* cotyledons cultured on hormone-free medium. Using this method, the authors elicited an embryogenic response, which ultimately regenerated non-transgenic plants (which was not possible without *Tc-Bbm*). Because phenotypically normal non-transgenic plants were produced,

the authors speculated that a co-transformation approach could work to produce transgenic plants.

In maize, direct induction of somatic embryos capable of rapidly germinating from immature embryos (without a callus phase), using the auxin-inducible promoter *Axig1* driving *Wus2* in combination with *Bbm* driven by a maize *Pltp* promoter was reported previously (Lowe *et al.* 2018). This study demonstrated that the *Pltp* promoter driving *Wus2* alone (with no *Bbm*), is sufficient to rapidly induce somatic embryo formation on the scutellum of transformed zygotic immature maize embryos. As previously observed, ectopic expression of *Wus2* in maize scutella leads to cell divisions in neighboring cells and creates elongated meristem-like projections with the *Wus2* expressing cells embedded in the apex (Lowe *et al.* 2016). Based on these observations, it was hypothesized that transient *Wus2* expression could stimulate growth in a non-cell autonomous manner, and lead to embryogenesis and plant regeneration, making *Wus2* an attractive candidate for use in an "altruistic transformation" approach.

The *Pltp* promoter has unique properties that make it desirable to drive morphogenic genes. It has been shown to be strongly expressed in the scutellar epithelial layer of embryos at the stage used for transformation, but is not expressed in early-stage embryos, meristems, roots, or reproductive tissues (Lowe *et al.* 2018). In previous studies, we used the *Pltp* promoter to express *Bbm* along with an auxin-inducible *Wus2* gene. In addition, the current results demonstrate that *Zm-Pltp*$_{pro}$::*Wus2* alone, is sufficient to directly produce somatic embryos without *Bbm* (Table 1).

The benefit of using *Wus2* in an altruistic T-DNA was first recognized when maize immature embryos were co-transformed with a mixture of two *Agrobacterium* strains; the first containing *Zm-Pltp*$_{pro}$::*Wus2*, and the second containing *Zm-Pltp*$_{pro}$::*Bbm* in their respective T-DNAs. When strains containing either *Zm-Pltp*$_{pro}$::*Wus2* or *Zm-Pltp*$_{pro}$::*Bbm* were used, only the *Zm-Pltp*$_{pro}$::*Wus2* produced a high frequency of single-copy T0 plantlets, while few to none were produced by *Zm-Pltp*$_{pro}$::*Bbm* alone (Table 1). However, when the two *Agrobacterium* strains were mixed at a 1:1 ratio, only the T-DNA from the *Agrobacterium* strain containing *Zm-Pltp*$_{pro}$::*Bbm* was integrated into a majority of the recovered plants. This result confirmed the hypothesis that expressed WUS2 protein was able to stimulate growth either through transient expression or by the movement of WUS2 into neighboring cells. The synergistic stimulation of somatic embryogenesis was demonstrated in previous studies, when *Wus2* and *Bbm* integrated and expressed in the same cell (Lowe *et al.* 2016, 2018). This study demonstrated that the co-integration of the *Bbm* and *Wus2* expression cassettes in the same cell is not necessary. Through co-transformation experiments, it was shown that expression of *Zm-Pltp*$_{pro}$::*Wus2* alone was sufficient to produce somatic embryos and plants that only contained *Zm-Pltp*$_{pro}$::*Bbm*. Based on this result, transient expression of *Wus2* alone could be used to recover T0 plants that contained other genes (without containing *Wus2*).

To test this hypothesis, an altruistic T-DNA vector was designed with high *Wus2* expression that contained three strong viral enhancer sequences (from the Fig Wart Mosaic Virus, the Peanut Chlorotic Streak Virus, and the Mirabalis Mosaic Virus) upstream of the *Zm-Pltp*$_{pro}$::*Wus2* cassette, with no selectable marker in the T-DNA (PHP87078). It was previously determined that these viral enhancers in the proximity of *Zm-Pltp*$_{pro}$::*Wus2* resulted in 'morphogenic toxicity' in the transgenic sectors expressing this cassette, which exhibited abnormal development that precluded further regeneration. Despite the abnormal growth patterns, it was hypothesized that the strongly enhanced promoter driving *Wus2* expression would result in high concentrations of WUS2 protein, which would readily move into adjacent cells, and provide an even greater zone of regeneration competence. In addition to the *Zm-Pltp*$_{pro}$::*Wus2*-containing *Agrobacterium*, *Hra* or *NPTII* was substituted in place of *Bbm*, to illustrate that using *Wus2* in a mixed-strain experiment can successfully be used to recover *Wus2*-free events. As shown in Table 2, transgenic events were recovered from all of the dilutions tested (mixing two *Agrobacterium* strains containing either PHP86491 or PHP87078), and although the numbers were small, the ratio of 9:1 (*Hra*/*Wus2*) produced the greatest increase in overall transformation frequencies and the number of single-copy events (SC%), which contained only the Selectable T-DNA (from PHP86491). Interestingly, when other researchers used mixtures of a hormone-producing "shooty" strain with a disarmed strain that contained a T-DNA, one group found that the ratio of disarmed strain to "shooty" strain was optimally 10:1 in poplar and wild cherry (Brasileiro 1991), while a similar comparison by another group using the same two "shooty" and disarmed *Agrobacterium* strains found that the control treatment, in which only the disarmed strain was used, was optimal in silver birch (Aronen *et al.* 2002).

The experiment in which the altruistic plasmid contained *CRE recombinase* on the T-DNA helped discern how the *Wus2* altruistic method was working Table 3. In this experiment, the altruistic plasmid that contained 3xENH: *Zm-Pltp*$_{pro}$::*Wus2*, was followed by a heat-inducible promoter driving *CRE recombinase* plus the *Nos*$_{pro}$::*CRC* expression cassette (PHP87598), and all three expression cassettes were located within the flanking loxP sites. The Selectable T-DNA contained a loxP-flanked *Zs-GREEN* along with a non-excisable *Hra*. When *CRE* was expressed, cassettes flanked by the loxP sites were excised and resulted in events with only the Selectable T-DNA. Consistent with these data low co-transformation frequencies were observed in this experiment, and there was no indication of the presence of the altruistic T-DNA. It was observed that 3xENH not only stimulated the *Pltp* promoter but also presumably activated the downstream

heat shock promoter driving *CRE recombinase*, which resulted in the self-excision of the altruistic components. The contribution of the individual plasmids in the regenerating plantlets was determined using qPCR. Due to the auto-excision of loxP +3xENH: *Zm-Pltp*$_{pro}$::*Wus2* + *Hsp26*$_{pro}$::*CRE* + *Nos*$_{pro}$::*CRC* + loxP in the altruistic T-DNA, events were regenerated that were co-transformed with both T-DNAs, which predominantly contained only the portions of the altruistic T-DNA remaining outside of the loxP sites after excision. Selectable events were also recovered, in which roughly half contained *Hra*, but had lost *Zs-GREEN* due to excision (with no detectable integration of the altruistic T-DNA containing an excision footprint). Because the CRE protein cannot move from cell-to-cell (Ströh *et al.* 2013; Martin-Ortigosa *et al.* 2014), the excised events that did not contain the *CRE* expression cassette (originally from the altruistic T-DNA), could only have occurred if the T-strand did not integrate and *CRE* was expressed transiently. As speculated for other T-DNA configurations containing the 3xENH elements, these viral enhancers probably stimulated the *Hsp26* promoter immediately when introduced into the cell, which was terminated during excision. This is consistent with observations by De Buck *et al.* (2000), in which transient *CRE* expression after co-transformation with two T-DNAs resulted in excision without the integration of *CRE*. This data with the *Wus2*-containing altruistic T-DNA vector, suggests two contributing modes of action for *Wus2*-stimulated somatic embryogenesis (Fig. 9). First, when cells transformed with either the *Wus2* or the *Hra* T-DNAs are in close proximity,the movement of WUS2 protein into the *Hra* T-DNA-containing cells may stimulate somatic embryogenesis *in trans* (Fig. 9*a*). Movement of WUS protein has been demonstrated as an important aspect of its morphogenic influence in the meristem (Yadev *et al.* 2011), but other localized changes such as alterations in growth regulators may also occur. Alternatively, when both T-DNAs are present in the same cell, transient *Wus2* expression from the non-integrated *Wus2*-containing T-strand provides the necessary stimulation of somatic embryogenesis (Fig. 9*b*). This data is consistent with both explanations.

In the description of methods, chemical selection was applied during somatic embryo maturation and then continued into the germination and rooting stages of plantlet development, to reduce the recovery of wild-type non-transgenic (escape) plants. However, escapes were observed in Tables 2 and 3, but were not observed in Tables 4 and 5. This was a reflection of the baseline tissue culture response of the inbreds that were used in these experiments. HC69 (Tables 2 and 3) responds well in tissue culture, and this tendency is accentuated in non-transgenic cells by Altruistic *Wus2* expression from neighboring cells, even though a chemical selection is being applied. Higher concentrations of selective agents (or increasing the duration of selection) could eliminate this background, but were not tested in this study. In contrast, for the recalcitrant inbreds used in Tables 4 and 5, wild-type immature embryos did not produce a baseline culture response. While Altruistic *Wus2* expression was still stimulating growth

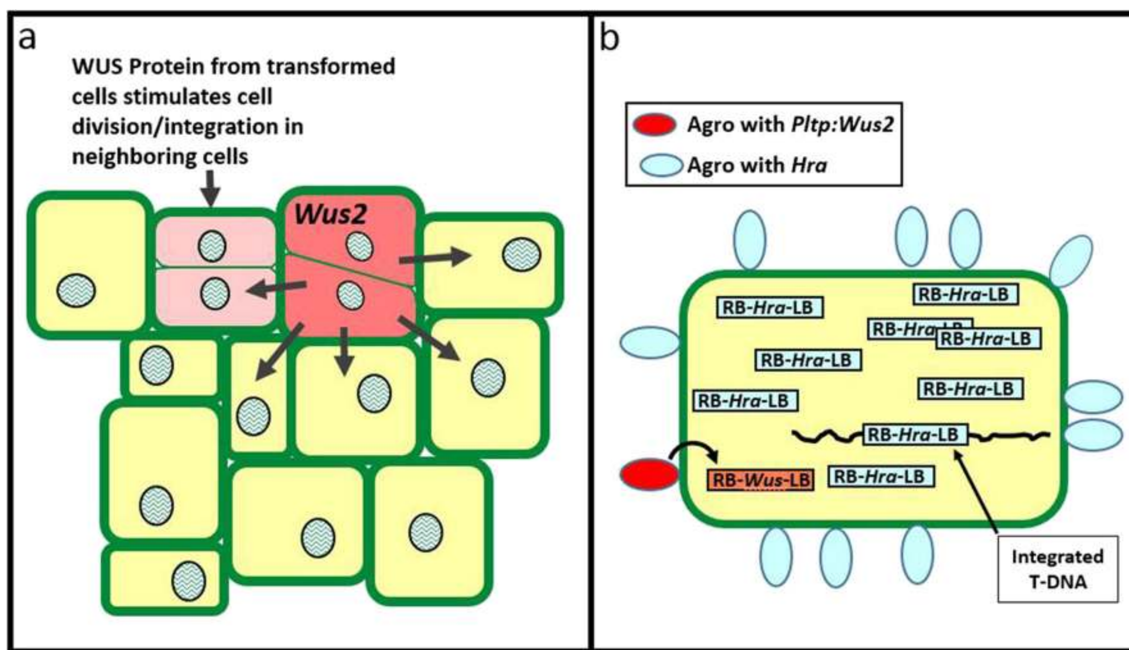

Figure 9. Two models for the mode-of-action of altruistic *Wus2 Zea mays* (L) transformation. (*a*) Movement of WUS protein stimulates cell division of neighboring cells that have independently integrated the Selectable T-DNA. (*b*) T-strands from both strains enter the cell, but only a fraction integrate. The ratios of the two Agrobacterium strains (9:1), favor integration of the T-DNA that contains the selectable marker, while the lower abundance of Wus2-containing T-strand permits transient Wus2 expression.

in non-*Wus2* cells in these six inbreds, the addition of chemical selection was adequate to control background growth, and no escapes were recovered.

To ensure that recovered transgenic plants did not contain the *Wus2/CRC* T-DNA, an altruistic T-DNA was designed with a Nos_{pro}::*CRC* expression cassette, in addition to the 3xENH: *Zm-Pltp*$_{pro}$::*Wus2* expression cassette without CRE-mediated excision. Normally, Nos_{pro}::*CRC* acts as a simple color marker as it directs the synthesis of red anthocyanin pigmentation. However, in this vector, it was expected that the expression of the *CRC* expression cassette would be enhanced due to the nearby presence of the three viral enhancers. The high levels of anthocyanin produced with this construct were inhibitory and development was arrested. In conjunction with the negative pleiotropic effects due to *Wus2* expression, the added stress imposed by anthocyanin accumulation resulted in an effective counter-selection against *Wus2*-containing cells (or somatic embryos), which helped to reduce the background of 'Selectable T-DNA' transformed lines that were also transformed with altruistic T-DNA. This altruistic transformation system is effective in sorghum (manuscript in progress) and, in principle, may be extended to any plant species that responds in a similar manner to over-expression of *Wus2*.

Conclusions

Despite improvements in transformation technologies for a small subset of plant species (such as maize), the availability of efficient transformation methods remains one of the remaining challenges for the plant transformation community, and represents the major impediment to genome editing in many crops (Altpeter *et al.* 2016). The use of morphogenic genes in both monocots and dicots has the potential to dramatically improve this situation (Gordon-Kamm *et al.* 2019). However, to date, strategies have focused on the use of single, complex T-DNAs that contain the morphogenic genes, and other genes such as selectable markers and/or traits, and components that will either excise or turn off the morphogenic genes later. These have been important first-generation methods that have demonstrated both increased transformation rates and extended genotype ranges. This study has demonstrated a viable second-generation alternative, which uses a mixture of an altruistic *Wus2*-containing *Agrobacterium*, and a separate selectable marker-containing *Agrobacterium*. This new method greatly simplifies vector construction, the tissue culture process, and downstream analysis of transgenic (or genome-modified) plants, and provides a more modular system to switch between different *Wus2*-containing and selectable marker-containing plasmids.

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