Development of an efficient marker-free soybean transformation method using the novel bacterium *Ochrobactrum haywardense* H1

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Summary
We have discovered a novel bacterium, *Ochrobactrum haywardense* H1 (Oh H1), which is capable of efficient plant transformation. *Ochrobactrum* is a new host for *Agrobacterium*-derived vir and T-DNA-mediated transformation. Oh H1 is a unique, non-phytopathogenic species, categorized as a BSL-1 organism. We engineered Oh H1 with repurposed *Agrobacterium* virulence machinery and demonstrated Oh H1 can transform numerous dicot species and at least one monocot, sorghum. We generated a cysteine auxotrophic Oh H1-8 strain containing a binary vector system. Oh H1-8 produced transgenic soybean plants with an efficiency 1.6 times that of *Agrobacterium* strain AGL1 and 2.9 times that of LBA4404Thy-. Oh H1-8 successfully transformed several elite Corteva soybean varieties with T0 transformation frequency up to 35%. In addition to higher transformation efficiencies, Oh H1-8 generated high-quality, transgenic events with single-copy, plasmid backbone-free insertion at frequencies higher than AGL1. The SpcN selectable marker gene is excised using a heat shock-inducible excision system resulting in marker-free transgenic events. Approximately, 24.5% of the regenerated plants contained only a single copy of the transgene and contained no vector backbone. There were no statistically significant differences in yield comparing T3 null-segregant lines to wild-type controls. We have demonstrated that Oh H1-8, combined with spectinomycin selection, is an efficient, rapid, marker-free and yield-neutral transformation system for elite soybean.

Introduction
Plant transformation plays an important role in plant biology research and is the foundational technology enabling transgenic crop development. *Agrobacterium*-mediated transformation and particle bombardment are the most common DNA delivery methods. Whereas *Agrobacterium* remains the primary vector for DNA transfer, other bacteria have been shown to be capable of delivering DNA to plant cells. Hooykaas and co-workers (Hooykaas et al., 1977) introduced a Ti plasmid from *Agrobacterium tumefaciens* into *Rhizobium trifoli* and found that leaves of *Kalanchoe daigremontiana* infected with *R. trifoli* developed tumours containing octopine, suggesting DNA transfer. Similarly, *Phyllobacterium myrsinacearum* harbouring a Ti plasmid from *A. tumefaciens* could also induce tumour formation on *Kalanchoe* (van Veen et al., 1988). However, unequivocal molecular data confirming DNA transfer or integration were not presented in either of these studies. Broothaerts et al. (2005) showed that three non-*Agrobacterium* species, *Rhizobium* sp. NGR234, *Sinorhizobium meliloti* and *Mesorhizobium loti* (collectively called Transbacter™), were capable of genetically transforming *Ara- bidopsis thaliana*, *Nicotiana tabacum* and *Oryzia sativa*. This was accomplished by providing these bacterial species with a Ti plasmid carrying a set of virulence genes from *Agrobacterium*.

However, low rates of transformation were observed, and therefore, this technology has not been widely adopted. More recently, several plant species, including potato, tobacco, *Arabidopsis*, rice and cassava have been transformed with a rhizobial bacteria, *Ensifer adhaerens* OV14 (Rathore and Mullins, 2018). However, *Ensifer* also proved to be less virulent and less efficient at DNA transfer than *Agrobacterium*. Further, none of the alternative bacteria have been shown to be capable of efficiently transforming soybean. Our objective was to identify microbial alternatives capable of soybean transformation at an efficiency comparable to or better than *Agrobacterium*.

Soybean (*Glycine max* (L.)) is one of the most important crops and a significant source of protein for food and feed globally. Soybean seeds are not only rich in essential amino acids, but also rich in dietary minerals, vitamins, unsaturated fatty acids and isoflavones (Han et al., 2003). Genetically modified (GM) soybean is one of the earliest introduced GM crops for commercial cultivation and is the largest GM crop in terms of acreage planted worldwide (ISAAA, 2018). The product development process routinely involves testing many DNA vector designs to produce hundreds and sometimes thousands of transgenic events for characterization (Newell-McGloughlin and Burke, 2014). Therefore, efficient transformation methods are critical.
Over the last three decades, many different soybean transformation methods have been developed using Agrobacterium (Li et al., 2017). The key design elements of transformation systems are: (i) explant type, (ii) DNA delivery method, (iii) selectable marker system and (iv) tissue culture. Transformation systems can be evaluated on several key attributes: efficiency, speed, molecular quality of the insertion (e.g. copy number and intactness), occurrence of somaclonal variation, germplasm independence and the presence and function of selectable markers in the final transgenic insertion. Most methods used today have significant limitations in one or more of these areas.

Selectable marker genes are used in nearly all transformation procedures to simplify the identification of transgenic plants. Selectable markers, such as antibiotic or herbicide resistance genes, allow transformed cells to be easily identified (Sundar and Sakthivel, 2008) and enable easy tracking of transgene inheritance in subsequent generations (Altpeter et al., 2016). The ideal selectable marker gene allows for efficient and tight selection of transformed cells, quickly killing or inhibiting non-transformed cells but allowing rapid growth of transformed cells. The ideal marker is robust, works in a wide variety of germplasm and is effective across a range of concentrations. We selected the antibiotic resistance gene SpcN, which confers resistance to spectinomycin.

Although several bacterial alternatives to Agrobacterium have been reported since 1977 (Broothaerts et al., 2005; Hooykaas et al., 1977; Rathore and Mullins, 2018; van Veen et al., 1988), none of them were as efficacious as Agrobacterium and none were shown to be capable of transformation of the important crop species soybean (Glycine max). Here, we report a novel non-phytopathogenic bacterial, O. haywardense H1, as a new alternative host for Agrobacterium-derived vir and T-DNA vectors, capable of plant transformation and superior to Agrobacterium-mediated transformation for the production of high-quality soybean transgenic events. We also created a cysteine auxotrophic strain of O. haywardense H1-8 that effectively eliminated bacterial overgrowth in culture and was equivalent to the H1 strain. When combined with an embryonic axis tissue culture system, spectinomycin selection and inducible selectable marker excision, O. haywardense H1-8 resulted in a novel soybean transformation method that is rapid, efficient and marker-free in multiple elite soybean germplasm. In the future, this technology could be applied to other plant species as well.

Results

Bacteria screening using transient Ds expression in tobacco BY-2 suspension culture

We evaluated antibiotic resistance in 960 representative entries that are part of a large environmental collection of bacteria at Corteva. The proportion of isolates sensitive to gentamicin, hygromycin, kanamycin and spectinomycin at 100 mg/L in liquid LB medium was 255 (27%), 176 (18%), 226 (24%) and 213 (22%), respectively. Based on this, we selected gentamicin as a selectable marker for the T-DNA vector pH70365 harbouring a set of vir genes in a plasmid backbone (Table 6 and plasmid construction in Methods S1) to be introduced into the bacterial cells.

We screened 2688 isolates. 718 out of the 2688 (26.7%) were sensitive to gentamicin. 179 of the 718 were successfully transformed with the transformation DNA vector pH70365. These 179 bacterial isolates carrying pH70365 were used to transiently transform tobacco BY-2 cells. 28 of the 179 bacterial isolates were transformation capable, as assessed by the observation of transient DsRED expression in BY-2 cells.

Bacteria identification by 16S rDNA sequencing and fatty acid profiling

We evaluated the 16S rDNA sequences of the 28 transformation-capable bacterial isolates. 16S rDNA sequences were PCR amplified, sequenced and used to search the NCBI nucleotide database. The best match for 16S rRNA sequence from one isolate, EP1A09, was with Ochrobactrum species (>97% identity). The best matches for the remaining 27 strains indicated they were Agrobacterium species (98% identity). The 16S rRNA sequence from EP1A09 is presented in Figure S1. Fatty acid methyl ester (FAME) profiling of EP1A09 revealed a species level match to O. anthropi, the only Ochrobactrum species in the FAME library. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis found a species level match to O. grignonense, but not to strains of O. anthropi, O. gallinifaecis, O. intermedium, Ochrobactrum sp. and O. tritici.

Genome assemblies and characterization of Ochrobactrum EP1A09 by 16S rRNA and Multilocus sequence analysis

To clarify the taxonomic position of the novel strain, draft genomic assemblies were constructed for EP1A09 and 15 culture collection strains of Ochrobactrum with known identities (Table S1). The 16S rRNA from these strains was aligned with other published 16S rRNA sequences from related Brucella and Agrobacterium species. EP1A09 had the highest (98.7%) 16S rRNA identity with five Ochrobactrum species that belong to clade 2 of Ochrobactrum, which contains mostly environmental species (Leclercq et al., 2020). In contrast, there was lower identity (95.1% to 98.2%) of 16S rRNA sequence with clade 1 of Ochrobactrum and Brucella species from animal isolates and opportunistic pathogens (Leclercq et al., 2020).

To improve the resolution of the phylogenetic comparison of EP1A09 to known Ochrobactrum and Brucella strains, we used multilocus sequence analysis (MLSA) as described by Romano et al. (2009). In this analysis, EP1A09 clustered with a group of environmental Ochrobactrum isolates (clade 2) that is unique from clade 1 as shown in Figure 1. Clade 1 includes Brucella and the opportunistic pathogens O. anthropi and O. intermedium (Leclercq et al., 2020). All members of clade 2 can be considered BSL-1, well-characterized agents not known to cause disease in healthy adult humans and being a minimal potential hazard to laboratory personnel and the environment (TRBA466, 2010). The MLSA sequences from EP1A09 had only 86.2% identity with the nearest known BSL-2 organisms O. anthropi and O. intermedium (Table S2). Based on this analysis, we classified EP1A09 as a BSL-1 organism. The closest MLSA match was to O. pituitosum at 99.6% identity. Based on this sequence similarity data, we propose that EP1A09 is a novel species of Ochrobactrum.

EP1A09 was deposited under Accession Number NRRL B-67078 with the Agricultural Research Service Culture Collection (NRRL) and was named Ochrobactrum haywardense H1, because it originated in a plant soil sample from Hayward, California. Electron microscopy showed that the morphology of O. haywardense H1 is short rods, straight or slightly curved with flagella.
Bush bean, *Nicotiana benthamiana* and sorghum infiltration for transient expression and *Arabidopsis thaliana* stable transformation

We have well-developed transient expression systems in our laboratory and used them to assess *O. haywardense* H1. *N. benthamiana* plants and leaf of bush bean (*Phaseolus vulgaris* L. variety Royal Burgundy) were infiltrated with *O. haywardense* H1 harbouring pPHD4673 (Table S3) and exhibited transient DsRED expression (Figure S2a and b). No DsRED expression was observed from explants infiltrated with buffer only and a strain *O. haywardense* H1 lacking a T-DNA binary vector. Similarly, sorghum leaf explants infiltrated with *O. haywardense* H1 (pPHP4674) showed transient DsRED expression (Figure S2c); no expression was observed when the leaf samples were infiltrated with an *O. haywardense* H1 lacking a T-DNA binary vector.

*Ochrobactrum haywardense* H1 (pPHP4673) was used to transform *A. thaliana* ecotype Col-0 with floral spray transformation (Chung et al., 2000) in four different experiments, generating kanamycin-resistant and DsRED-positive seedlings with frequencies ranging from 0.5, 1.0, 0.6 and 0.9, and an average transformation efficiency of 0.8% (Table S4). Kanamycin-resistant and DsRED-positive seedlings were transplanted to soil, and qPCR was conducted to determine the copy number of transgenes in transgenic events. We assayed 41 kanamycin-resistant T0 events selected randomly from 46 T0 events, all of which were qPCR positive. Ten events contained single copies of the DsRED and *nptII* genes, while 19 had two copies of one or both genes. The remaining 12 events had more than three copies of at least one gene. Our preliminary studies with bush bean, *N. benthamiana*, sorghum and *Arabidopsis* transformation have established a proof of principle for *Ochrobactrum* transformation, and further optimization of the methods could be developed.

**Soybean transformation**

Corteva Agriscience North America commercial soybean varieties P29T50, P33T50 and 93Y21 and Brazilian commercial soybean varieties DM118 and 98C21 were used for stable transformation. First, our elite, maturity group 3 variety 93Y21, was transformed with *O. haywardense* H1 harbouring vector pPHP82314 (Table S3). Following co-culture of the soy embryonic axes (EA) (Figure 2a) with *O. haywardense* H1 (pPHP82314) for 4–5 days, transient RFP expression was observed in the proximity of the apical meristematic region (Figure 2b), demonstrating efficient...
gene delivery in soybean EA explants. Spectinomycin-resistant adventitious shoots were produced from the shoot apical meristem of EAs in the selection medium containing 25 mg/L spectinomycin 10–14 days after transformation (Figure 2c). These shoots were green, in contrast to non-transformed tissues that were bleached by the spectinomycin selection. Most of the spectinomycin-resistant shoots showed RFP expression, and 100% of the bleached shoots had no RFP expression. By 24–28 days after transformation, spectinomycin-resistant and RFP-positive shoots elongated to 0.2–0.5 cm (Figure 2d). Wild-type untransformed shoots in selection-free medium exhibited no RFP expression (Figure 2e). Transformed, green, healthy shoots of 0.5–2 cm in height were produced within 5–6 weeks (Figure 2f, right), and almost 100% of the spectinomycin-resistant shoots showed RFP expression. Untransformed tissues derived from the embryonic axes, on the contrary, were bleached in selection medium containing spectinomycin at concentrations of 25 mg/L or higher (Figure 2f, left). Elongated green shoots were excised and transferred to rooting medium containing spectinomycin, 10 mg/L, for further elongation and rooting. T0 plants with well-developed root systems were subsequently transplanted to potting soil (Figure 2g) where over 90% of the plants developed normally to produce seeds.

The production of T0 events, from initiation of transformation to transplanting to soil, typically required 9–11 weeks. T1 seeds from most transgenic events showed RFP expression under a fluorescence microscope (Figure 2h). Plant transformation frequencies in elite variety 93Y21 ranged from 10 to 18% (Table S5). In a separate set of experiments, transformation frequencies of four other Corteva elite soybean varieties (P29T50, P33T50, DM118 and 98C21) infected with O. haywardense H1 (pPHP82314) showed 21% (64 T0/169 EA), 3% (22/711), 2% (9/498) and 16% (94/600), respectively.

Transgene analyses in T0 and progeny
qPCR was conducted to determine the copy number of transgenes in T0 events. We assayed 34 spectinomycin-resistant T0 events, all of which were qPCR positive (Table S6). Fourteen events contained a single copy of the RFP and SpcN expression cassettes, while 13 had two copies of one or both genes, and the remaining 7 had more than three copies of at least one gene cassette. We did further molecular analysis to confirm T-DNA insertions were intact and to determine the site of integration in soybean chromosomal DNA. To further characterize the insertion site, Southern-by-Sequencing (SbS<sup>TM</sup>) data were analysed for junction regions flanking the left and right borders of 12 events (ESOY 7103.7.10, 7103.7.11, 7103.8.5, 7103.8.7, 7140.4.2, 7140.4.3, 7140.5.1, 7140.5.2, 7140.6.1, 7140.6.5, 7140.6.9 and 7140.6.10) that were randomly selected from 14 events that had previously been characterized as single-copy events by qPCR (Table S6). Nine (ESOY 7103.7.10, 7103.7.11, 7103.8.5, 7103.8.7, 7140.4.2, 7140.4.3, 7140.5.1, 7140.5.2 and 7140.6.10) out of 12 events had T-DNA flanking regions that

Figure 2 Developmental stages of transgenic shoots from apical meristem of soybean embryonic axis and the production of transgenic plants. (a) Soybean 93Y21 embryonic axis isolated from imbibed dry seeds. Bar is 0.4 mm. (b) Soybean embryonic axis explants 5 days after transformed with Ochrobactrum haywardense H1 (pPHP82314). Bar is 1 mm. (c) 10 days. Bar is 2 mm. (d) 28 days. Bar is 5 mm. (e) Wild-type untransformed shoots in selection-free medium. (f) Untransformed embryonic axis produced bleached primary shoots and no further shoot growth (left) and embryonic axis transformed with O. haywardense H1 (pPHP82314) produced vigorous growth of shoots in 1.5–3.0 cm height in 35 days after transformation in 25 × 100-mm Petri dishes. Bar is 2 cm. (g) T0 events in soil. Bar is 5 cm. (h) TagRFP expression in T1 progeny under RED filter combination with green LED illumination. Bar is 2 cm.
mapped to the 93Y21 genome, and each of the two flanking genome sequences mapped to the same chromosomal location (Table 1). Three (ESOY 7140.6.1, 7140.6.5 and 7140.6.9) out of 12 events had a complicated insertion patterns. Event ESOY 7140.6.1 contains two full copies of the T-DNA at a single locus with additional fragments of the right border at three separate loci in chromosome 5. Event ESOY 7140.6.5 contains a single locus with a fragment of the right border attached to the right border of a full copy of the T-DNA. ESOY 7140.6.9 contains a single locus with two partial fragments attached to the right border of a full copy of the T-DNA.

All 34 plants were self-pollinated, and T1 seeds were collected. T1 seeds collected from 34 events exhibited various levels of RFP expression under the fluorescence microscopy, and 100 T1 seeds were randomly selected from each transgenic line to determine the ratios of RFP expressing to non-expressing T1 seeds as shown in Figure 2c. The results, summarized in Table S7, are consistent with a 3 : 1 Mendelian segregation ratio for single-locus insert and a 15 : 1 Mendelian segregation ratio for a two-locus insert. Three (ESOY 7140.6.1, 7140.6.5 and 7140.6.9) events that contained complicated insertions at a single locus showed 3 : 1 Mendelian segregation ratio.

Marker-free T0 events by heat shock induction of Cre recombinase

To obtain transgenic soybean plants devoid of the marker gene used for selection, we developed a recombinase-mediated marker excision system consisting of the Cre recombinase gene under the control of the soybean heat shock-inducible promoter GmHSP17.3B. LoxP sequences, in direct repeat orientation, were used for selection, we developed a recombinase-mediated marker excision system. To obtain transgenic soybean plants devoid of the marker gene and the RFP expression cassettes between the repeated loxP sequences leaving transgenic plants with only the trait gene cassette and one loxP sequence. Excision was evaluated using a panel of qPCR assays that span the T-DNA insertion in 93Y21 and P29T50 T0 events (Table S8). All spectinomycin-resistant T0 events were qPCR positive indicating tight selection with the spectinomycin selection system. T0 events transformed with O. haywardense harboured an excision vector pPHP86194 showed successful removal of the Cre, SpcN and RFP expression cassettes and contained only the IPD083Aa trait gene cassette after heat shock treatment. The use of soybean GmHSP17.3B heat shock-inducible promoter (pPHP86194) resulted in frequencies of excision ranging from 36% to 49%. Single-copy events of the trait gene cassette only ranged from 12% to 26% after heat shock treatment (Table 2b). In total, of the 369 T0 events transformed with O. haywardense H1 (pPHP86194), 164 T0 events (44%) showed various excision patterns and 68 T0 events (18%) showed single-copy complete excision (SCCE) resulting in events containing only the

Table 1

| Event ID | Insertion sites in chromosomal DNA | Genomic Seq_Construct Seq (RB) | Construct Seq (LB)_Genomic Seq |
|----------|----------------------------------|-------------------------------|--------------------------------|
| ESOY 7103.7.10 Chr2 | 20935390 | TATGCTGATAGGATGGGAGAGAGAGGATCGTGGTGTGAGATGATCATACCAT | TATGCTGATAGGATGGGAGAGAGAGGATCGTGGTGTGAGATGATCATACCAT |
| ESOY 7103.7.11 Chr14 | 11230307 | TGGATCTGAGGATGCTGGTGTGAGATGATCATACCAT | TGGATCTGAGGATGCTGGTGTGAGATGATCATACCAT |
| ESOY 7103.8.5 Chr20 | 34652379 | AATCAGAATAAGGACAGAGGAAAGAGGATCGTGGTGTGAGATGATCATACCAT | AATCAGAATAAGGACAGAGGAAAGAGGATCGTGGTGTGAGATGATCATACCAT |
| ESOY 7103.8.7 Chr10 | 4354542 | TTGAATGATATATTTCTCAACATTCTATATAAATAAACATTCAATTCACAAG | TTGAATGATATATTTCTCAACATTCTATATAAATAAACATTCAATTCACAAG |
| ESOY 7140.4.2 Chr9 | 1275457 | AAAATTGATGTAATATTTCTCAACATTCTATATAAATAAACATTCAATTCACAAG | AAAATTGATGTAATATTTCTCAACATTCTATATAAATAAACATTCAATTCACAAG |
| ESOY 7140.4.3 Chr10 | 39649379 | AGTTAGGATAGTATATTTCTCAACATTCTATATAAATAAACATTCAATTCACAAG | AGTTAGGATAGTATATTTCTCAACATTCTATATAAATAAACATTCAATTCACAAG |
| ESOY 7140.5.1 Chr7 | 5475354 | TTTATGATGATGTAATATTTCTCAACATTCTATATAAATAAACATTCAATTCACAAG | TTTATGATGATGTAATATTTCTCAACATTCTATATAAATAAACATTCAATTCACAAG |
| ESOY 7140.5.2 Chr2 | 15245273 | TTCATATAATATTTCTCAACATTCTATATAAATAAACATTCAATTCACAAG | TTCATATAATATTTCTCAACATTCTATATAAATAAACATTCAATTCACAAG |
| ESOY 7140.6.10 Chr12 | 3629935 | AAGTCTGAGGATGCTGGTGTGAGATGATCATACCAT | AAGTCTGAGGATGCTGGTGTGAGATGATCATACCAT |

Chromosome mapping is shown for each event border sequence. Genomic mapping of the left and right border sequences in red font for each event to the Williams B2 Assembly 2 genomic reference.
analysed by SbS to confirm T-DNA insertions were intact and to determine the site of integration in soybean chromosomal DNA and the confirmation that the transgenic event that has undergone loxP excision. Seven (44%) out of 16 SCCE T0 events had T-DNA flanking region that mapped to the 93Y21 and P29T50 genome build, and each of the two flanking genome sequences mapped to the same chromosomal location (Table S9) and showed clean excision at loxP sites (Figure S4). We evaluated Mendelian transgene inheritance by analysing T1 progeny using qPCR. T1 seedlings from five self-pollinated T0 plants (ESOY 8111.1.1, 8237.1.9, 8243.1.2, 8402.4.1 and 8402.4.6) were randomly selected from 7 events that had previously been characterized as SCCE events by SbS analysis (Table 2b and Figure S4). Thus, for all five events, segregation was consistent with the expected 1 : 2 : 1 segregation pattern (P < 0.05) as shown in Table S10. In total, these results demonstrated that this Cre-based excision system is an effective tool for generating marker-free events with expected single-locus Mendelian inheritance.

### Field agronomic characterization

Field performance of T3 null-segregant lines of elite varieties 93Y21, P29T50 and P33T50 was evaluated in a multi-location study. T3 null-segregant lines from *O. haywardense* H1 (pPHP82314) transformation showed statistical equivalence for relative maturity, total-plot-gap (an estimate of emergence and plant stand) and seed yield across backgrounds. When observing results by genetic background, maturity was significantly different in 9 of the 27 null-segregant lines. Maturity differences of approximately 2 days are considered agronomically acceptable. Only 2 lines exhibited maturity delays of two or more days compared with the wild type. Total-plot-gaps, a measure of stand emergence, increased in only one of the null-segregant lines indicating most lines had no difference in plant emergence. Seed yield was equivalent across all null-segregant lines. These data support the conclusion that transgenic soybean events transformed with *O. haywardense* H1 have little to no impact on the agronomics of elite soybean varieties.

### Frequencies of *O. haywardense* H1-8, *A. tumefaciens* AGL1 and LBA4404Thy- strains in soybean transformation

Field performance of T3 null-segregant lines of elite varieties 93Y21, P29T50 and P33T50 was evaluated in a multi-location study. T3 null-segregant lines from *O. haywardense* H1 (pPHP82314) transformation showed statistical equivalence for relative maturity, total-plot-gap (an estimate of emergence and plant stand) and seed yield across backgrounds. When observing results by genetic background, maturity was significantly different in 9 of the 27 null-segregant lines. Maturity differences of approximately 2 days are considered agronomically acceptable. Only 2 lines exhibited maturity delays of two or more days compared with the wild type. Total-plot-gaps, a measure of stand emergence, increased in only one of the null-segregant lines indicating most lines had no difference in plant emergence. Seed yield was equivalent across all null-segregant lines. These data support the conclusion that transgenic soybean events transformed with *O. haywardense* H1 have little to no impact on the agronomics of elite soybean varieties.

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### Frequencies of *O. haywardense* H1-8, *A. tumefaciens* AGL1 and LBA4404Thy- strains in soybean transformation

Side-by-side comparisons in two transformation experiments of soybean embryonic axis (EA) transformations were carried out using wild-type *O. haywardense* H1 (pPHP82314) and the cysteine auxotrophic strain *O. haywardense* H1-8 (pPHP82314). Eight out of nine plates containing 30 EAs/plate transformed with wild-type *O. haywardense* H1 (pPHP82314) showed bacterial overgrowth in transformation #1, and all plates (7/7) were contaminated with *O. haywardense* H1 (pPHP82314) overgrowth in transformation #2 after 5–6 weeks of cultures in shoot induction medium. None of the plates (0/16) transformed with the cystine auxotrophic strain *O. haywardense* H1-8 (pPHP82314) showed bacterial overgrowth in transformation #1 or #2 after 5–6 weeks, and up to 10 weeks, of culture. Transformation frequencies of wild-type *O. haywardense* H1 (pPHP82314) and the cystine auxotrophic strain *O. haywardense* H1-8 (pPHP82314) were 18% (82 T0/450 EA) and 19% (89/482), respectively.

Soybean EA transformation was carried out using (i) *O. haywardense* H1-8 harbouring a helper plasmid pPHP85634 plus binary vector pPHP7910, (ii) AGL1 (Lazo et al., 1991) harbouring binary vector pPHP7910 and (iii) LBA4404Thy- auxotrophic strain (WO2010078445A1) harbouring the helper plasmid

| Table 2 | Frequency and excision efficiencies in soybean 93Y21 transformation |
|---------|---------------------------------------------------------------|
|         | Number of embryonic axis (%) | Number of T0 plants (%) | Transformation frequency (%) |
| Replicates | 1 | 2 | 3 | 4 | 5 | Mean |
| (a) pPHP85797 | 1 | 76 | 16 | 21 | 11 | 12 | 21 |
| (Cre-) | 2 | 90 | 11 | 23 | 26 | 17 | 20 |
| (b) pPHP85797 | 1 | 228 | 50 | 22 | 18 | 19 | 20 |
| (Cre-) | 2 | 210 | 38 | 18 | 20 | 21 | 20 |

(c) Frequency of SCCE events in soybean 93Y21 T0 plants

| Replicates | 1 | 2 | 3 | 4 | 5 | Mean |
| (a) pPHP85797 | 1 | 48 | 0 | 0 | 48 | 100 |
| (Cre-) | 2 | 49 | 0 | 0 | 49 | 100 |
| (b) pPHP85797 | 1 | 54 | 0 | 0 | 54 | 100 |
| (Cre-) | 2 | 65 | 0 | 0 | 65 | 100 |
| (c) pPHP85797 | 1 | 73 | 0 | 0 | 73 | 100 |
| (Cre-) | 2 | 58 | 0 | 0 | 58 | 100 |

(d) Frequency of excision after heat shock treatments in soybean 93Y21 T0 plants

| Replicates | 1 | 2 | 3 | 4 | 5 | Mean |
| (a) pPHP85797 | 1 | 48 | 0 | 0 | 48 | 100 |
| (Cre-) | 2 | 49 | 0 | 0 | 49 | 100 |
| (b) pPHP85797 | 1 | 54 | 0 | 0 | 54 | 100 |
| (Cre-) | 2 | 65 | 0 | 0 | 65 | 100 |
| (c) pPHP85797 | 1 | 73 | 0 | 0 | 73 | 100 |
| (Cre-) | 2 | 58 | 0 | 0 | 58 | 100 |

(e) Frequency of excision after heat shock treatments in soybean 93Y21 T0 plants

IPD083Aa gene after heat shock treatment. On the contrary, of 289 T0 events transformed with *O. haywardense* H1 harbouring pPHP85797 and lacking a Cre cassette, none were excised after heat shock.

Seven 93Y21 T0 events and nine P29T50 T0 events that had been identified as SCCE events by qPCR (Tables S8 and S9) were further analysed by SbS to confirm T-DNA insertions were intact and to

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pPHP71539 plus the binary vector pPHP97910 (plasmid construction in Methods and Table S3) in a side-by-side comparison experiment with three replicates. Soybean transformation was carried out essentially as described in the Methods. EAs of 93Y21 transformed with O. haywardense H1-8 (pPHP85634 + pPHP97910) exhibited more vigorous, faster shoot formation compared with either AGL1 or LBA4404Thy-. Most shoots displayed DsRED-positive expression and EAs transformed with O. haywardense H1-8 lacking a T-DNA binary vector became completely bleached in same culture condition as shown in Figure 2f. Three replicates were carried out as side-by-side comparison transformations on the same day, respectively, and all three replicates showed consistent results. Bars are 2 cm.

**Discussion**

A novel bacterial strain O. haywardense H1 capable of plant transformation was discovered by screening a library of bacterial strains with a high-throughput screen based on transformation of tobacco BY-2 cells. O. haywardense H1 is a Gram-negative bacterium initially identified by biochemical tests as a member of the Ochrobactrum species. O. haywardense H1 is a non-phytopathogenic species in the same clade as other Ochrobactrum environmental isolates and is a BSL-1 or Risk Group 1 organism, similar to Agrobacterium. Ochrobactrum is in the order Rhizobiales, Brucellaceae family, and is not regarded as a plant pathogen. Based on phylogenetic and biochemical analysis, EP1A09 was determined to be a novel species of Ochrobactrum EP1A09 and was named O. haywardense H1. Both genera, Agrobacterium and Ochrobactrum, belong to the same Risk Group and BSL classifications (BSL Level 1) and can be handled similarly in the laboratory. The Risk Group classification is based on pathogenicity to humans and animals, and BSL classification refers to the related laboratory guidelines, important considerations from the organism’s handling perspective.

*Agrobacterium tumefaciens* genetically transforms host plants by transferring and integrating a segment of its own DNA into the genome of its host cells (Lacroix and Citovsky, 2013). The innate ability of members of the Rhizobiales family to mediate bacterium-to-eukaryote DNA transfer is nearly unique. Recently, Lacroix and Citovsky (2016) demonstrated close homologs of the vir genes are encoded by the p42a plasmid of Rhizobium etli. This microorganism is related to Agrobacterium and may have acquired the vir genes by horizontal gene transfer from Agrobacterium, but R. etli is known only to be a symbiotic bacterium that forms nitrogen-fixing nodules in several species of beans. However, the transformation efficiency mediated by R. etli was much lower than with A. tumefaciens in tobacco (N. tabacum) transformation. Weller et al. (2004) reported that several...
bacteria, including strains of *Rhizobium* sp. and *Ochrobactrum* sp. that harboured Ri plasmids, transformed hydroponically grown cucumber and tomato plants, leading to a hairy root phenotype. However, the presence of *Agrobacteria* was not ruled out as a possibility, complicating the analysis, and it was concluded that *Ochrobactrum* CSL 2573 was incapable of transforming plants and inducing root-mat symptoms (Weller et al., 2005).

Over the last three decades, the most frequently employed plant genetic transformation methods are *Agrobacterium*-mediated transformation and particle bombardment. Soybean transformation was first reported in 1988 using *A. tumefaciens*.

### Table 3
Comparison of *O. haywardense* H1-8, *A. tumefaciens* AGL1 and LBA4404Thy- strains in soybean 93Y21 transformation

| Replicates | Number of embryonic axis | Number of T0 plants | Transformation frequency %a |
|------------|--------------------------|---------------------|----------------------------|
| (a) O. haywardense H1-8 (pPHP85634+pPHP97910) | #1 143 | 50 | 35 |
| #2 150 | 39 | 26 |
| #3 157 | 54 | 34 |
| Mean 150 | 48 | 32 |
| #1 200 | 51 | 26 |
| #2 150 | 21 | 14 |
| #3 360 | 69 | 19 |
| Mean 237 | 47 | 20 |
| A. tumefaciens AGL1 (pPHP97910) | #1 200 | 22 | 11 |
| #2 150 | 15 | 10 |
| #3 360 | 43 | 12 |
| Mean 237 | 26.7 | 11 |
| A. tumefaciens LBA4404Thy- (pPHP71539+pPHP97910) | #1 200 | 12 (24) | 19 (38) | 7 (14) |
| #2 150 | 8 (21) | 15 (39) | 16 (41) | 5 (13) |
| #3 360 | 15 (28) | 17 (32) | 22 (41) | 16 (30) |
| Mean 48 | 12 (24) | 17 (36) | 19 (40) | 9 (20) |
| A. tumefaciens AGL1 (pPHP97910) | #1 51 | 10 (20) | 11 (22) | 30 (59) | 33 (65) |
| #2 21 | 3 (14) | 4 (19) | 14 (67) | 14 (67) |
| #3 50 | 9 (18) | 20 (40) | 21 (42) | 24 (48) |
| Mean 41 | 7 (17) | 12 (27) | 22 (56) | 24 (60) |
| A. tumefaciens LBA4404Thy- (pPHP71539+pPHP97910) | #1 22 | 5 (23) | 8 (36) | 9 (41) | 6 (27) |
| #2 15 | 5 (33) | 6 (40) | 4 (27) | 3 (20) |
| #3 43 | 9 (21) | 21 (48) | 13 (30) | 5 (12) |
| Mean 27 | 6 (26) | 12 (41) | 9 (33) | 5 (20) |
particle bombardment (McCabe et al., 1988). While the use of non-Agrobacterium species for transformation of soybean has been previously described (US9365859B2), the transformation frequencies reported were very low (0.04%–0.05%) and they are not a viable alternative to A. tumefaciens. In this report, we show that O. haywardense H1 and H1-8 strains were able to transform soybean with frequencies to 35% at the T0 plant level, 700–800 times better than other Rhizobium-mediated soybean transformation systems (US9365859B2).

We have developed a simple and highly efficient soybean transformation system using the novel bacterium O. haywardense H1 to infect soybean EA. The EA explants are advantageous in that they can be easily obtained as starting material from mature dry seeds by a simple protocol, the meristem maintains mitotic activity and responds well to tissue culture, and it takes less time to generate transgenic soybeans compared with other methods. We optimized many parameters of the transformation process, including method of co-cultivation, selection agent, medium composition and vector system, thereby significantly improving transformation frequencies. Our highest transformation efficiency was up to 35% based on regenerated T0 plants, over twice the efficiency of previously reported efficiencies of 15.8% (Liu et al., 2004) and 16.4% efficiencies previously reported for Agrobacterium-mediated soybean (O’Hoft et al., 2003).

The presence of marker genes in the construct has been shown to affect expression of nearby trait genes (Botts et al., 2019). In addition, many selectable marker genes being used confer resistance to antibiotics. Therefore, it is desirable to have a transformation system that does not retain marker genes in the final product. Several molecular tools for the removal of marker genes from transgenic clones have been described including co-transformation (Sripriya et al., 2011), transposon-mediated repositioning of the gene of interest (Charg et al., 2008), exploitation of multi-auto-transformation system (Khan et al., 2011) and site-specific recombinase systems (Sengupta et al., 2010). Marker-free plastidic lettuce plants expressing pectinases and coagulation factor CTB-FIX were also created by the removal of the antibiotic resistance gene by homology-based marker excision (Daniell et al., 2019; Srinivasan et al., 2021). The use of a co-transformation and site-specific Cre/lox recombination system (Joubès et al., 2004; Ov, 2002) with the Cre recombinase gene where sequences we intended to excise, including the antibiotic selectable marker gene SpcV and the Cre gene, were placed between two directly repeated loxP sites. The Cre gene was under the control of a heat shock-inducible GmHSP17.3B promoter, and heat shock treatment of T0 plantlets triggered excision.

Phenotypic variation in plants regenerated from cell or tissue culture has been observed (Phillips et al., 1994). Heritable variation induced as a consequence of cell or tissue culture has been referred to as somaclonal variation. This culture-associated variation induced as a consequence of cell or tissue culture has been observed (Phillips et al., 1994). T0 plantlets triggered excision. Ochrobactrum haywardense H1 strain showed partial resistance to various β-lactam antibiotics such as timentin, carbenicillin and cefotaxime, commonly used antibiotics to eliminate bacteria from cultures, resulting in bacteria overgrowth and significant loss of cultures in the process of plant transformation. We successfully generated β-lactam antibiotic-sensitive and cytochrome axotrophic mutant strains of O. haywardense H1-8 that facilitated the removal of bacteria after co-cultivation. The auxotrophic O. haywardense H1-8 strain was unable to grow without cytochrome supplementation, virtually eliminating bacterial overgrowth completely in tissue culture conditions. A. tumefaciens AGL1 (Lazo et al., 1991) is a super virulent strain and has been used widely for plant transformation. The LBA4404 Thy-106 vectors harbouring helper pPHP71539, showed high transformation frequency and high-quality event production in maize inbreds (Anand et al., 2018). Unitary vectors pPHD4673, pPHD4674, pPHP70365, pPHP82314, pPHP85797 and pPHPB6194 used during the development of the methodology in this study contain -23.5 kb of vir genes, two origins (ColEl1, pvS1) for stable replication in both E. coli and Ochrobactrum, a bacterial selectable marker in plasmid backbone and a T-DNA encoding a plant selectable marker, a visual fluorescent protein marker, Cre-lox excision cassette and trait gene. The large size of the plasmids makes the T-DNA challenging to clone during the vector construction and confirming structural confirmation of the plasmid. Thus, we developed a ‘binary vector system’ having two constituents (a T-DNA component and a vir helper component), each located on a separate plasmid in O. haywardense H1-8. The helper vector pPHP85634 has a RK2full PARDE origin (Roberts et al., 1994; US20180216123A1) for stable replication in a broad range of bacteria and contains -23.5 kb of vir genes from the hypervirulent pTiBoS42 Ti (Hood et al., 1986). The binary vector pPHP97910 has two origins (ColEl1, pvS1) and contains T-DNA harbouring plant selectable marker and gene of interest. The trans acting virulence (vir) functions are encoded by the helper and having the cis acting T-DNA border in the separate binary vector simplified vector construction and structural confirmation of the plasmid. Both the RK2full PARDE ori helper plasmid and the pvS1 ori binary vector were very stable in terms of plasmid compatibility in Ochrobactrum. To test plasmid stability, colonies of (i) O. haywardense H1-8 harbouring the helper plasmid pPHP71539 (pvS1 ori) plus the binary vector (pvS1 ori) as described above and (ii) helper pPHP85634 (RK2full PARDE ori) plus the binary vector (pvS1 ori) were streaked on LB medium containing 100 mg/L of gentamicin, kanamycin and cytochrome. Gentamicin- and kanamycin-resistant single colonies were then cultured on antibiotic-free LB cytochrome medium for 3 days (Ochrobactrum doubling time is ~2.5 h), and then individual single colonies were replicated to medium with and without antibiotic selection. As expected, 190/190 colonies of O. haywardense H1-8 (pPHP71539 + pPHP97910) and all 194/194 colonies of O. haywardense H1-8 (pPHP85634 + pPHP97910) grew on antibiotic-free medium. However, 102/190 (54%) of the O. haywardense H1-8 colonies containing pPHP71539 + pPHP97910 (pvS1 + pvS1 ori, respectively) grew on selection medium indicating a loss of plasmid. In contrast, 186/194 (96%) of replicated O. haywardense H1-8 colonies containing
evaluation. We have demonstrated that drag in 27 T3 null-segregant lines tested in multi-location field phenotypically stable transgenic events that exhibited no yield gene of interest only. This method produced genotypically and free production of genetically modified soybean containing the remove selectable and screenable markers that allows for marker-frequency. An efficient heat-inducible system was developed to capable of producing single transgene copy events at high simple and highly efficient soybean transformation system and molecular engineering of the bacteria, we developed a very that is capable of plant transformation. By a combination of multiple soybean genotypes and is superior to bacteria from a backyard plant soil sample, O. haywardense that often accompany inoculation with reactions and pathogen-associated molecular pattern (PAMPs) plants may not respond to it with the same necrotic defence ments and because derived plant material in the laboratory and controlled environ-...
Bacterial identification with 16S rDNA sequence, fatty acid methyl esters (FAME) and matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)

For DNA analysis, genomic DNAs were prepared using MasterPure™ genomic DNA Purification Kit (Cat No. MCD85201, Epibio, Madison, WI). PCR was performed with a PTC-100TM Programmable Thermal Controller (MJ Research, Inc., San Francisco, CA) using genomic DNAs extracted from 28 bacteria. The universal primers used for 16S rDNA amplification were:

SEQ ID NO: 1 16S-F 5'-AGAGTTTGATCCTGGCTCAG 3'
SEQ ID NO: 2 16S-R 5'-ACGGCTACCTTGTTACGACTT 3'

The resulting PCR products were sequenced by Elim Biopharmaceuticals (Hayward, CA), and these sequences were used to search the NCBI GenBank nucleotide sequence database.

Fatty acid (FAME) analyses were performed using gas chromatographic analytical system according to MIDI Labs standard procedures (Newark, DE) and these sequences were used to search the NCBI GenBank nucleotide sequence database.

Generation of *O. haywardense* H1-8 mutant strain

For β-lactam antibiotic-sensitive and cystine auxotrophic *O. haywardense* H1-8 mutant strain, β-lactamase genes (SFO-1 (ΔblaA), Class B Zn-metalloenzyme (ΔblaB) and serine acetyltransferase (ΔCysE) were sequentially deleted from wild-type *O. haywardense* H1 through direct transformation based allele replacement (WO2005056757A2).

In short, both upstream and downstream sequences (between 1 and 2 kbps) of the gene/locus to be deleted were generated by PCR and seamlessly cloned into the linearized pGP704 sacB suicide plasmid. The resulting ‘knock-out’ plasmid is transformation into wild-type *O. haywardense* H1 by an electroporation method. The plasmid was then integrated into the chromosome by homologous recombination, and deletion mutants were identified by sacB-mediated sucrose counter-selection. A fraction of the sucrose-resistant candidate colonies from each allele replacement reaction were subjected to PCR with the primers (Table S11) flanking each gene to determine whether it had been deleted. Through four rounds of ‘knock-out’ processes, *O. haywardense* H1-8 with all three β-lactamase genes (SFO-1 (ΔblaA), Class B Zn-metalloenzyme (ΔblaB), OXA-1 (ΔblaD)) and serine acetyltransferase (ΔCysE) were deleted from *O. haywardense* H1.

The genome sequences of isolates were determined using illumina sequencing technology (Illumina, San Diego, CA). *O. haywardense* H1-8 was confirmed β-lactam antibiotic-sensitive and exhibited auxotrophy for cysteine.

Transient expression in bush bean, *Nicotiana benthamiana*, sorghum leaf discs and stable transformation in *Arabidopsis*

Bush bean (Phaseolus vulgarus L. variety Royal Burgundy), *N. benthamiana* (cv. TW17, US Nicotiana Germplasm Collection, North Carolina State University, Raleigh, NC, USA) and sorghum (Sorghum bicolor Pioneer genotype TX430) infiltrations were carried out using modified protocols according to Kapila et al. (1997) and Siehl et al. (2014). In short, overnight culture of *O. haywardense* H1 lacking a T-DNA unitary vector and *O. haywardense* H1 harbouring pPHP70365 (Table S3) was pelleted and resuspended to an OD 600 = 1.0 in 10 mM MgCl2 + 400 μM acetylsyringone (pH 5.6). Bacteria were delivered into the underside of top three leaves in 8-day-old bush bean plantlets and 4-week-old *N. benthamiana* plantlets using a 1-mL blunt-tipped plastic syringe (FX2379ACS, Daigger & Company, Buffalo Grove, IL). Post infiltration, plants were moved to the growth chamber.

*Sorghum bicolor* (DuPont Pioneer TX430) seedling plants were grown in growth chambers with 16-h light at 375–450 μEjm2s1, 26 °C day and 22 °C night. Leaves of 3-week-old sorghum seedlings were infiltrated with *O. haywardense* H1 lacking a T-DNA unitary vector and *O. haywardense* H1 harbouring pPHP4674 (Table S3). The levels of expression for DsRED fluorescent protein were observed under the Leica M165FC fluorescence stereomicroscope (Leica, Wetzlar, Germany) equipped with a filter set for excitation at 530–560 nm and emission at 590–650 nm.

Floral spray transformation of *A. thaliana* ecotype Col-0 was carried out using protocol according to Chung et al. (2000).

Soybean transformation

Various Corteva Agriscience elite soybean varieties were used. Mature dry seeds were surface sterilized for 16 h using chlorine gas, produced by mixing 3.5 ml of 12 N HCl with 100 ml of commercial bleach (5.25% sodium hypochlorite) as described by Di et al. (1996). Disinfected seeds were imbibed on solid agar medium (about 100 seeds in a 25 × 100 mm Petri dish) containing 5 g/L sucrose and 6 g/L agar at room temperature for 6–8 h in the dark, and then the seeds were soaked in distilled sterile water for overnight at room temperature in the dark. Intact embryonic axes were isolated using a scalpel blade.

*Ochrobactrum haywardense* H1 lines containing the vectors listed in Table S3 were used for transformation. In a comparison, experiment between *O. haywardense* H1 and *Agrobacterium*, *Agrobacterium* strains AGL1 (Lazo et al., 1991) and LBA4404Thy (WO2010078445A1) was used. A volume of 15 mL of *O. haywardense* H1 suspension (OD 0.5 at 600 nm) in infection medium composed of 1/10X Gamborg B5 basal medium, 30 g/L sucrose, 20 μM MES, 0.25 mg/L GA3, 1.67 mg/L BAP, 200 μM acetylsyringone and 1 mM dithiothreitol in pH 5.4 was added to about 200–300 EAs, and they were plated on a 25 × 100 mm deep Petri dish. The plates were sealed with parafilm (Cat No. 528588, ‘Parafilm M’ VWR) and then sonicated (Sonicator-VWR model 50T) for 30 s. After sonication, EAs were incubated for 2 h at room temperature. After inoculation, excess bacterial suspension was removed and about 200–300 EAs were transferred to a single layer of autoclaved sterile filter paper (Cat No. 28320-020, VWR) in 25 × 100 mm Petri dish. The plates were sealed with...
Micropore tape (Cat No. 1530-0, 3M, St. Paul, MN) and incubated under dim light (1–2 µE/m²/s), cool white fluorescent lamps for 16 h at 21 °C for 3 days. After co-cultivation, the base of each embryonic axis was embedded in shoot induction medium (R7100, PhytoTech Labs) containing 30 g/L sucrose, 6 g/L agar and 25 mg/L spectinomycin (5742, PhytoTech Labs) as a selectable agent and 500 mg/L cefotaxime (GoldBio, ST Louis, MO) in pH 5.7. Shoot induction was carried out in a Percival Biological Incubator (Percival Scientific, Perry, IA) or growth room at 25 °C with a photoperiod of 16 h and a light intensity of 60–100 µE/m²/s. The levels of expression for DsRED or TagRFP fluorescent protein were observed under the Leica M165FC fluorescence stereomicroscope (Leica, Wetzlar, Germany) equipped with a filter set for excitation at 530–560 nm and emission at 590–650 nm.

After 4–6 weeks in selection medium, the spectinomycin-resistant shoots were cut and transferred to 2 strength MS rooting medium (M404, PhytoTech Labs) containing 15 g/L sucrose, 10 mg/L spectinomycin and 250 mg/L cefotaxime for further shoot and root elongations. Transformation efficiency was calculated based on the number of positive transgenic soybean T0 plants divided by the total number of EAs. Transgenic soybean plantlets were transferred to moistened Berger BM2 soil (Berger, Saint-Modeste, QC, Canada) and kept enclosed in clear plastic tray boxes until acclimatized in growth room at conditions of 16-h photoperiod at 250–350 µE/m²/s, 26/24 °C day/night temperatures. Hardened plantlets were potted in 2-gallon pots containing moistened SunGro 702 and grown to maturity for harvest in a greenhouse.

qPCR and Southern-by-Sequencing (SbS) analysis of transgenic soybean

Molecular analysis was done as described by Lowe et al. (2016) for qPCR analysis and Zastrow-Hayes et al. (2015) for Southern-by-Sequencing (SbS) TM. Genomic DNA extracted from fresh leaf punches derived from T0 events and non-transgenic wild-type plants was subjected to qPCR assays for detecting the presence/absence of the expression cassette components in the T-DNA. SbS analysis was carried out to confirm single-copy events, as determined by qPCR, on the nucleotide sequence level.

Generation of marker-free transgenic soybean plants by the Cre-lox site-specific recombination system under heat shock treatments

For heat shock treatment of soybean, 2–4 cm T0 plantlets with roots in 100 × 25 mm Petri dishes or magenta boxes on spectinomycin free-rooting medium were transferred into a Percival incubator (Percival Scientific, Perry, IA) at 45 °C, 70% humidity for 2 h in the dark. Non-heat shock-treated T0 plantlets were used as a control. After the heat shock treatment, T0 plantlets were transferred to moistened Berger BM2 soil (Berger, Saint-Modeste, QC, Canada) and kept enclosed in clear plastic tray boxes in a Percival incubator at 26 °C with a 16-h photoperiod at 250–350 µE/m²/s. 2–4 leaf punch samples were collected for qPCR and SbS analyses from newer growth 2 weeks after acclimatization of T0 events.

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Conflict of interest

The authors declared that they have no conflict of interest to this work. This work was funded by Corteva Agriscience, a for-profit agricultural technology company, as part of its research and development program. All authors were employees of Corteva Agriscience at the time of their contributions to this work. Patents have been filed related to this work.

Authors’ contributions

H-I.C. conceived the original project, designed, supervised and performed experiments, analysed data and wrote the manuscript. T.K., J.B. A.K. and T.J. supervised the project; Y.M., N.R. and J.Y. performed the experiments; L.C. conducted field trials and analysed data; C.H., M.B., L.C., T.J. and N.D.C. wrote the manuscript.

Data availability statement

Availability of materials described in this paper to academic investigators for non-commercial research purposes under an applicable material transfer agreement will be subject to proof of permission from any third-party owners of all or parts of the material and to government regulation considerations. Materials reported in this paper may contain components subject to third party ownership (e.g., TagRFP and DsRED). Transgenic and genome edited materials may be subject to governmental regulations. Obtaining the applicable permission from such third-party owners will be the responsibility of the requestor. Transgenic materials reported in this paper may only be made available if in full accordance with all applicable governmental regulations.

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Supporting information
Additional supporting information may be found online in the Supporting Information section at the end of the article.
Figure S1 1337 bp of 16S rDNA sequence of strain EP1A09.
Figure S2 Transient DsRED expression in N. benthamiana, P. vulgarus (bush bean) and sorghum leaves.
Figure S3 Schematic diagram of the T-DNA region of the vectors used in soybean transformation.
Figure S4 Schematic illustration of LoxP excision.

Table S1 Per cent identity between 16S rRNA sequences of EP1A09 and other members of the Ochrobactrum genus.
Table S2 Per cent identity between concatenated MLSA sequences of EP1A09 compared with other members of the Ochrobactrum genus.
Table S3 Vector list. Genetic components used to construct expression cassettes within the T-DNAs.
Table S4 Transformation efficiencies showing kanamycin-resistant and DsRED-positive Arabidopsis seeds transformed with O. haywardense H1 harbouring pPHP82314.
Table S5 Frequency of O. haywardense H1 (pPHP82314)-mediated soybean transformation in variety 93Y21.
Table S6 Presence and copy number of transgenes in transgenic soybean 93Y21 T0 events transformed with O. haywardense H1 (pPHP82314) by qPCR.
Table S7 Progeny analysis from self-pollinated soybean 93Y21 T0 events.
Table S8 qPCR analysis of T0 events transformed with O. haywardense H1 harbouring Cre minus pPHP85797 and Cre excision vector pPHP86194, respectively, after heat shock treatment.
Table S9 Junction sequence showing 50 bp soybean 93Y21 and P29T50 genome sequences, and right (upper) and left border (lower) 30 bp of construct (yellow) with right/left border sequences highlighted in blue for each event.
Table S10 Progeny analysis from self-pollinated soybean T0 events by qPCR.
Table S11 Sequence of primer pairs for amplifying target gene.
Methods S1 Introduction of plasmid by electroporation.