Journal of Integrative Agriculture 2026, 25(2): 820–823

Available online at ScienceDirect

Journal of Integrative Agriculture



LETTER

# From seed to whole plant: An innovative visual marker system to enhance selection efficiency in soybean genome editing

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**Highlights**

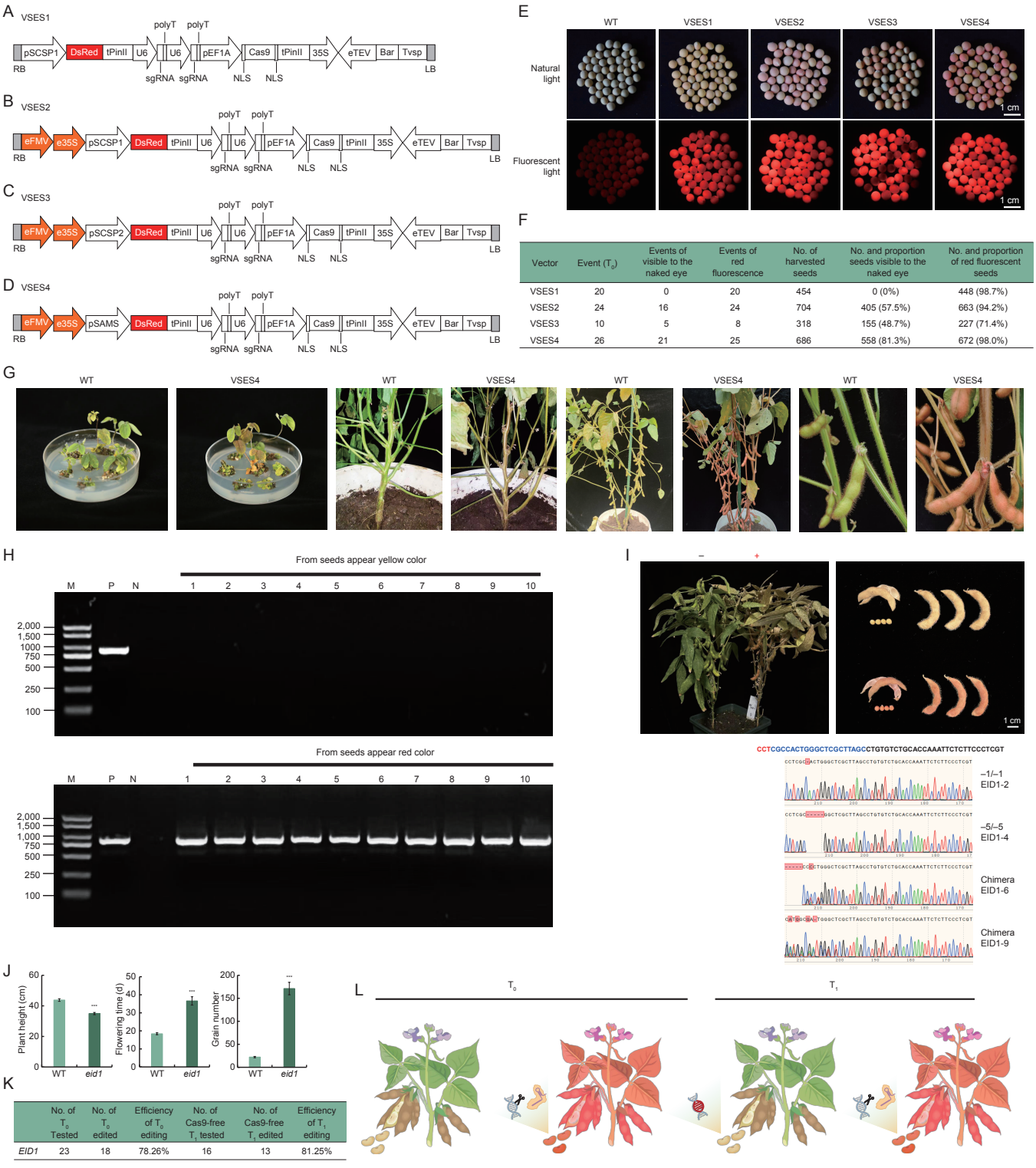
- The VSES2-4 systems enabled rapid identification of transgenic soybean plants under natural light.
- Using the VSES4 system, transgenic plants could be distinguished at various developmental stages without compromising genome editing efficiency.
- The VSES4 system significantly accelerates transgenic line screening and reduces labor-intensive procedures in soybean genome editing.

Emerging and powerful genome editing tools, particularly CRISPR/Cas9, are facilitating functional genomics research and accelerating crop improvement (Jiang *et al.* 2021; Cao *et al.* 2023; Chen C *et al.* 2023; Liu *et al.* 2023a). However, the detection and screening of transgenic lines remain major bottlenecks, being time-consuming, labor-intensive, and inefficient during transformation and subsequent mutation identification. A simple and efficient visual marker system plays a critical role in addressing these challenges. Recent studies demonstrated that the *GmW1* and *RUBY* reporter systems were used to obtain visual transgenic soybean (*Glycine max*) plants (Chen L *et al.* 2023; Chen *et al*. 2024). However, these systems are still in their infancy in soybean. Although visual markers, such as green fluorescent protein (GFP) and red fluorescent protein (DsRed2), have been widely used in soybean, rice, maize, and other plants, achieving rapid visualization of transgenic plants (Quaedvlieg *et al.* 1998; Nishizawa *et al.* 2006; Xu *et al.* 2021), the application of these visual markers and integration of non-invasive, efficient and convenient CRISPR/Cas9-separation genome editing tools remain challenging in soybean. We have developed a novel visual system, visual soybean editing system (VSES), in which the Cas9-expressing vector incorporates a DsRed2 expression cassette driven by a constitutive promoter, enabling straightforward identification of transgenic soybean plants. The VSES offers three significant advantages: (i) Enhanced *DsRed2* expression enables Cas9-containing seeds to be visually identified under natural light, removing the requirement for specialized equipment; (ii) transgenic plants can be rapidly distinguished during the seedling stage based on stem and leaf color; (iii) the VSES does not compromise genome editing efficiency.

To drive *DsRed2* expression, we used three distinct promoters: two soybean seed coat-specific promoters (SCSP1 and SCSP2) derived from seed storage protein genes, and a constitutive promoter from the *S-adenosylmethionine synthetase* (SAMS) gene (Yi and Yu 2006; Fan *et al.* 2009; Wei *et al.* 2010). Furthermore, we incorporated two consecutive viral enhancers (2xEnh) from *Cauliflower mosaic virus 35S* (CaMV35S) and *Figwort mosaic virus* (FMV) to enhance fluorescent protein expression. Subsequently, the pSCSP1-DsRed2, 2xEnh-pSCSP1-DsRed2, 2xEnh-pSCSP2-DsRed2 and 2xEnh-pSAMS-DsRed2 expression cassettes were respectively integrated into the CRISPR/Cas9 vector and named VSES1-4 (Fig. 1-A–D). Expression of the above four vectors was evaluated in the soybean cultivar Dongnong 50.

Received 3 March 2025; Received in revised form 15 April 2025; Accepted 29 April 2025; Available online 3 June 2025
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doi: 10.1016/j.jia.2025.06.010




| Vector | Event ($T_0$) | Events of visible to the naked eye | Events of red fluorescence | No. of harvested seeds | No. and proportion seeds visible to the naked eye | No. and proportion of red fluorescent seeds |
|---|---|---|---|---|---|---|
| VSES1 | 20 | 0 | 20 | 454 | 0 (0%) | 448 (98.7%) |
| VSES2 | 24 | 16 | 24 | 704 | 405 (57.5%) | 663 (94.2%) |
| VSES3 | 10 | 5 | 8 | 318 | 155 (48.7%) | 227 (71.4%) |
| VSES4 | 26 | 21 | 25 | 686 | 558 (81.3%) | 672 (98.0%) |

| | No. of $T_0$ Tested | No. of $T_0$ edited | Efficiency of $T_0$ editing | No. of Cas9-free $T_1$ tested | No. of Cas9-free $T_1$ edited | Efficiency of $T_1$ editing |
|---|---|---|---|---|---|---|
| *EID1* | 23 | 18 | 78.26% | 16 | 13 | 81.25% |

**Fig. 1 Demonstration and application of the visual marker system.** A–D, visual soybean editing system (VSES)1–4 consisting of the pSCSP1-DsRed2, 2xEnh-pSCSP1-DsRed2, 2xEnh-pSCSP2-DsRed2, and 2xEnh-pSAMS-DsRed2 expression cassettes, respectively. LB, left border; RB, right border. E, schematic illustration of the VSES1–4 expression system, showing harvested seeds under natural and fluorescent light. WT, wild type. F, statistical analysis of the proportions of red fluorescent seeds and naked-eye visible seeds in VSES1–4 transgenic plants. G, representative images comparing WT and VSES4 transgenic plants at seedling, flowering and maturity stages. H, PCR validation of Cas9 presence or absence in differently colored seeds harvested from $T_0$ plants transformed with VSES4. M, marker; P, positive control (transgenic plant with Cas9); N, negative control (WT plant). I, transgenic plants and seeds from $T_1$ generations with Cas9 (+) or without Cas9 (–) targeting *GmEID1*. Sequence chromatograms of homozygous *eid1* mutants (without Cas9) and chimeras (with Cas9) are also shown. J, comparative analysis of plant height, flowering time, and grain number between WT and *eid1* mutants. Bars mean SD. Statistical significance was determined by Student's *t*-tests (***, $P<0.001$, $n=10$). K, genome editing efficiency in $T_0$ seeds and $T_1$ Cas9-free seedlings. L, application of VSES4 in soybean genome editing, demonstrating its utility in visually distinguishing Cas9-free from Cas9-transformed plants.

For seeds expressing VSES1, Cas9-transformed and Cas9-free seeds could be separated under fluorescence excitation wavelengths (Fig. 1-E). However, under natural light, all seeds appeared yellow, making visual distinction unfeasible. In contrast, VSES2-expressing transformation events significantly enhanced fluorescent protein expression, making Cas9-containing (red) seeds visible to the naked eye (Fig. 1-E). Red fluorescent seeds constituted 94.2% of harvested seeds, but only 57.5% were visible under natural light (Fig. 1-F). Similar results were observed with VSES3, where 48.7% of seeds were visible under natural light (Fig. 1-E and F). Notably, VSES4-expressing events displayed the highest proportion of visible red seeds under natural light, reaching 81.3% (Fig. 1-F). VSES4-expressing plants could be readily identified based on red stems and leaves during the seedling, flowering, and maturity stages (Fig. 1-G). However, transgenic seedlings with excessive accumulation of VSES4 transcripts exhibited various developmental abnormalities and caused complete lethality. The association between the red color phenotype and Cas9-transformation was validated through PCR analysis of 10 randomly selected seeds per transgenic event (Fig. 1-H). In addition, the correlation between the proportion of visible red seeds and *DsRed2* expression levels was confirmed by RT-qPCR analysis in VSES1-4 (Appendix A). These results demonstrate that the VSES4 is a simple and efficient visualization system for soybean genome editing.

We further constructed a CRISPR-Cas9 vector utilizing the VSES4 and designed two sgRNAs targeting *GmEID1* (Qin *et al.* 2023). In the $T_1$ generation, two Cas9-free homozygous *eid1* mutants (with 1- and 5-bp deletions, respectively) were identified from plants with green stems and leaves. In contrast, plants with red stems and leaves exhibited chimeric *GmEID1* mutations (Fig. 1-I). Compared to the wild type (WT), the mutant plants showed reduced height, delayed flowering, and significantly increased yield (Fig. 1-J). A total of 23 independent $T_0$ transformation events were generated, with a mutation rate of 78.26%, which was comparable to the 81.25% mutation rate observed in systems without DsRed2 expression cassettes (Fig. 1-K). Thus, the VSES4 does not affect Cas9 activity and can be widely used in soybean genome editing.

In conclusion, VSES4 enables rapid and efficient screening of $T_0$ Cas9-transformed plants based on seedling stem and leaf color (Fig. 1-L). In the $T_1$ generation, Cas9-free gene-edited plants were identified based on green seedling stems and leaves (Fig. 1-L). The principal advantage of this system lies in its capacity for visual screening of transgenic plants at the seedling stage without compromising genome editing efficiency. Although excessive accumulation of VSES4 transcripts induced developmental abnormalities in some transgenic plants, this phenotypic constraint can be effectively addressed through CRISPR/Cas9-mediated genome editing tools, which ultimately generate transgene-free edited plants. Meanwhile, tissue-specific promoters may be used to reduce the interference of constitutive expression on overall growth in the future. In addition, the VSES2/3-expressing plants showed no adverse effects on growth, and transgenic seeds were partly visible under natural light. In the future, the VSES2/3 can be applied to soybean gene function verification, seed production technology (SPT), and multi-control sterility (MCS) technology for rapid screening of transgenic and non-transgenic seeds. Potential integrations of VSES4 with emerging technologies are also promising. For instance, the 2xEnh-pSAMS-DsRed2 expression cassette could be combined with base editor (BE) systems to enable visualization-coupled precision editing (Niu *et al.* 2024). Similarly, co-transformation with developmental regulators such as *GRF5* or *GRF3-GIF1* may enhance transformation efficiency for producing visually identifiable gene-edited plants (Kong *et al.* 2020; Zhao *et al.* 2024). When combined with haploid inducer lines, the efficiency of haploid identification could be improved by visually eliminating diploids displaying red stems and leaves (Liu *et al.* 2023b; Wang *et al.* 2023). Future efforts will focus on optimizing the VSES4 system and extending its application to other crops, thereby advancing agricultural innovation and crop improvement.

## Acknowledgements

This work was supported by the Jilin Science and Technology Development Program, China (20240602032RC), the Jilin Agricultural Science and Technology Innovation Project, China (CXGC2024ZD001), the Jilin Agricultural Science and Technology Innovation Project, China (CXGC2024ZY012), and the Jilin Province Development and Reform Commission — Project for Improving the Independent Innovation Capacity of Major Grain Crops, China (2024C002).

## Declaration of competing interest

The authors declare that they have no conflict of interest.

## Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

**Appendix** associated with this paper is available at https://doi.org/10.1016/j.jia.2025.06.010

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