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Brief Communication

Seeing is believing: a visualization toolbox to enhance selection efficiency in maize genome editing

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The emerging and promising biotechnology methodology of CRISPR/Cas9 gene editing is revolutionizing crop improvement. However, the low-efficiency, time-consuming and labour-intensive nature of positive or/and negative selections during or after transformation and subsequent identification of mutations are major challenges for its agricultural application, from upstream (high-throughput) mutant screening to downstream commercial production (He and Zhao, 2020). Although visual markers, and in particular fluorescent markers including green fluorescent protein (GFP) and red fluorescent protein (RFP), have been employed for the rapid visualization of transgenic material (Qi *et al.,* 2020), the integration of cost-effective and non-invasive CRISPR-separation gene-editing tools is still in its infancy but remains critical for crop genetics and breeding (Callaway, 2018). Furthermore, special light sources are required to visualize fluorescent signals, which increases the cost and inconvenience of the application of fluorescence markers, especially in field conditions. We have developed a visualization toolbox, ViMeBox (VIsual Maize Editing toolBox), for the selection of positive transformants in maize (*Zea mays*). In the ViMeBox system, the vector expressing Cas9 includes a gene cassette containing a visible marker expressed from a tissue-specific promoter; Cas9-free kernels that are easy to separate by the visible marker are also undergoing gene-editing. ViMeBox offers two advantages: (i) It enables enhanced expression of DsRED2, which makes seeds containing Cas9 visible to the naked eye in natural light, and does not affect genome-editing efficiency or plant development. (ii) It is effective for different seed tissues, for example, using embryo-specific promoters or promoters preferentially expressed in the embryo or aleurone. Furthermore, ViMeBox has potential applications in diverse additional scenarios.

To drive DsRED2 expression, we employed three tissue-specific promoters, the aleurone-expressed promoter of barley (*Hordum vulgare*) *HvLtp2* (Kalla et al., 1994), the embryo-expressed promoter of maize *ZmESP* (Liu *et al.,* 2014), and the embryo/aleurone-expressed promoter of maize *ZmGlb1* (Liu *et al.,* 1998). We also included the CaMV35S enhancer to increase fluorescent protein expression so that it is visible to the naked eye. These three engineered promoter-driven DsRED2 fluorescent labelling systems were then integrated into a CRISPR/Cas9 vector separately and were named ViMeRed1–3 (Figure 1a–c).

The expression of all constructs was analysed in the maize inbred line KN5585, which has been demonstrated to have a high-transformation efficiency (Liu *et al.,* 2020). Notably, although seed-specific promoters were used, red fluorescence was also clearly observed for ViMeRed1 in differentiating callus under natural light (Figure 1e). As expected, in harvested ears that expressed ViMeRed1 (aleurone-specific) transformation events, we could separate Cas9-transformed (red) and Cas9-free (yellow) kernels (T_1) under natural light and fluorescence excitation wavelengths, and the association between the presence of red colour and Cas9 transformation was confirmed by PCR assays using 20 randomly selected Cas9-free kernels for each transgenic event. Sanger sequencing further identified that the mutation rate at target loci was approximately 80%. We also tested other fluorescent markers, including GFP, YFP and AmCyan. Although tissue-specific expression was also driven by eCaMV35S-HvLtp2, signals were not visible in natural light and could only be distinguished with fluorescence excitation, using the AmCyan labelling system (ViMeCyan) as an example (Figure 1d,f).

Similar to ViMeRed1, ViMeRed2 and ViMeRed3 were also visible under natural light but was more easily observed by fluorescence excitation (Figure 1g–i). Embryo-specific red fluorescence was visible exclusively in ViMeRed2 seeds and red signals were observed clearly in both embryo and aleurone in ViMeRed3 seeds. However, the red signal was preferentially observed in the aleurone of ViMeRed1-seeds, with a small amount of leakage in the embryo.

We next test if the integration of the construct had any effect on Cas9 activity and editing efficiency. In total, 21 ViMeRed1, 14 ViMeRed2, and 15 ViMeRed3 independent T_0 events were generated, and Sanger sequencing revealed a mean mutation rate of 80%, which was not significantly different from that observed without labelled fusion proteins (Liu *et al.,* 2020). A high-mutation rate was also observed in ViMeCyan T_0 events (Figure 1e,f,j).

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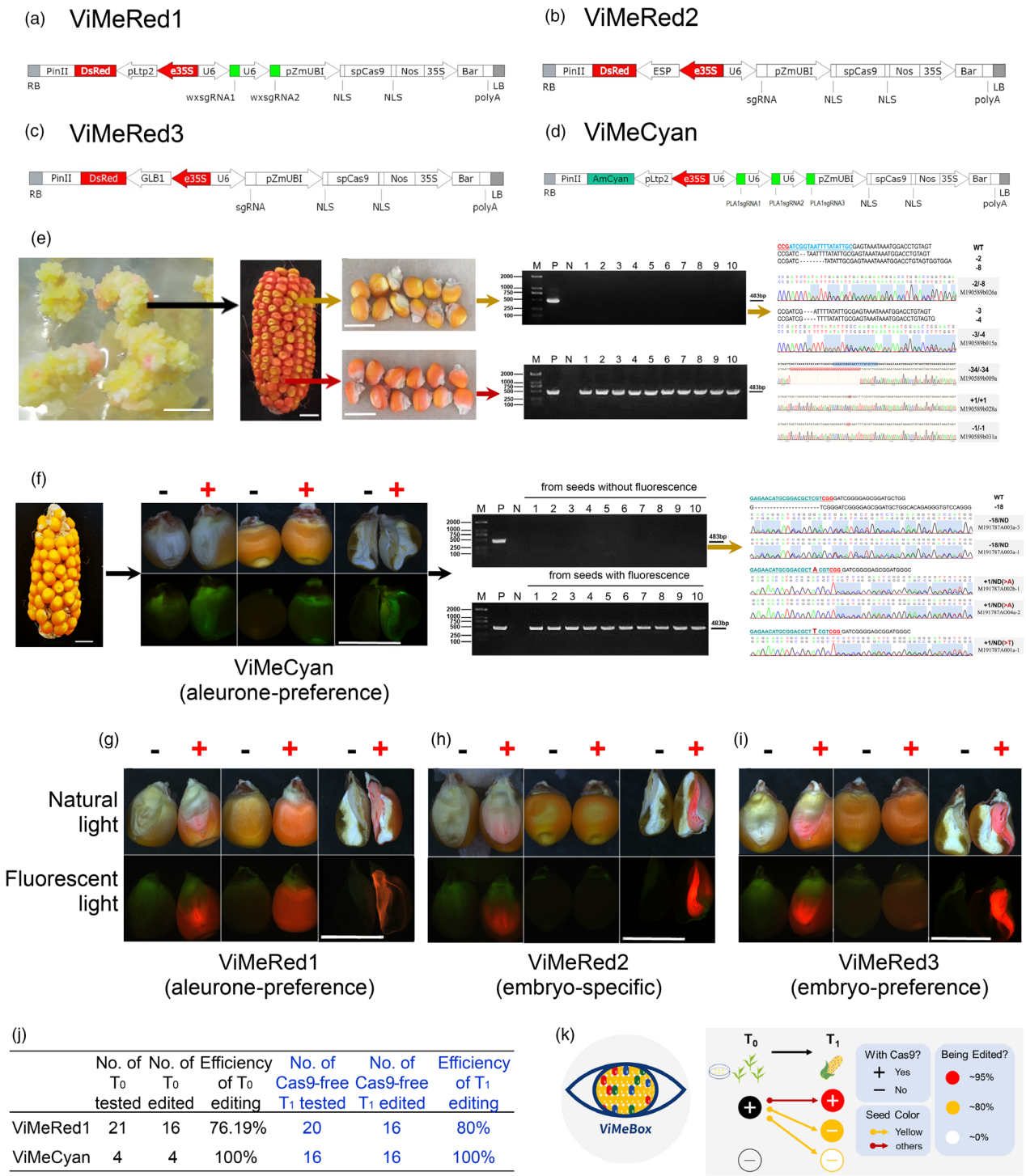

(j)

	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
ViMeRed1	21	16	76.19%	20	16	80%
ViMeCyan	4	4	100%	16	16	100%

Figure 1 Demonstration and application of ViMeBox. (a–c) The ViMeBox toolbox consisting of three ViMeRed vectors. (d) The ViMeCyan vector. (e) Schematic diagram of typical ViMeRed expression (exemplified by ViMeRed1), including resistant callus, ear and seeds, under natural light. PCR was used to validate the presence or absence of Cas9 in different coloured kernels in harvested ears of T_0 plants transformed with ViMeRed1 (M, marker; P, positive control = ViMeRed1 plasmid; N, negative control = wild-type DNA). Sequences for mutant alleles are also shown. (f) Schematic diagram of the ViMeCyan system, showing a harvested ear under natural light and seeds under natural/fluorescent light in the absence ("−") or presence ("+") of Cas9. The validation of Cas9 mutant alleles is shown (M, marker; P, positive control = ViMeCyan plasmid; N, negative control = wild-type DNA). (g–i) The absence ("−") or presence ("+") Cas9 in three ViMeRed systems under natural light and fluorescent light. (j) Gene-editing efficiency of T_0 seedlings and T_1 Cas9-free seeds. (k) The application of ViMeBox tools in the positive or/and negative selection of transgenic insertions.

In summary, we have established ViMeBox, a comprehensive visual toolbox for maize genome editing that uses different fluorescent markers to specifically label two seed tissues. This set of tools makes the positive selection of transgenic events cost-effective and rapid (Figure 1k). The ability to accurately visualize fluorescence with the naked eye is the major advantage of the

system compared with the use of other fluorescent markers, such as GFP, RFP, or mCherry, and anthocyanin or betalain pigment-based systems that colour the whole plant (He *et al.,* 2020; He and Zhao, 2020), the fluorescence-derived tool is suitable for both positive and negative selection, and because it does not affect plant development or seed germination, it is also suitable for other crops. ViMeBox could also be combined with other emerging technologies. For example, the use of Hi-Edit (Kelliher *et al.,* 2019) or IMGE (Wang *et al.,* 2019), which use Cas9-carried haploid inducer lines to produce gene-edited progeny, in combination with ViMeRed2, could increase the efficiency of screening haploid material by removing diploids that produce visibly red embryos. In the future, ViMeBox will be improved and could easily be extended to other seed crops to accelerate agricultural innovations.

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Conflict of interest

X.J. and H.B. are employees of WIMI Biotechnology Company.

Author contributions

X. L. and J. Y. designed the research; J. X., Y. Y., B. H., Z. C. performed experiments; L. J analysed the gene-editing data. J. X. and X. L. wrote the manuscript. All authors participated in discussion and revision of the manuscript.

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