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Brief Communication

Coordinated gene upregulation in maize through CRISPR/Cas-mediated enhancer insertion

Hannes Claeys[1,*], Eveline Neyrinck[1], Lies Dumoulin[1], Anne Pharazyn[1], Arne Verstichele[1], Laurens Pauwels[2,3], Michael L. Nuccio[4] and Frédéric Van Ex[1]

[1]*Inari Agriculture NV, Ghent, Belgium*
[2]*Ghent University, Department of Plant Biotechnology and Bioinformatics, Ghent, Belgium*
[3]*VIB Center for Plant Systems Biology, Ghent, Belgium*
[4]*Inari Agriculture Inc, Cambridge, Massachusetts, USA*

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**Correspondence* (Tel: +3292770383; email hclaeys@inari.com)

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Genome editing technologies have significantly improved our ability to precisely modify genomes and genes, opening new possibilities to engineer endogenous pathways and traits. In crops like maize, achieving small indels, base changes and structural variation has been demonstrated (Nuccio *et al*., 2021). However, while these edits typically lead to gene knock-out (KO) or knock-down, improvement of many agronomic traits requires higher gene expression, as evidenced by beneficial natural alleles and transgenes. Thus, crop improvement requires tools that allow predictable and tunable upregulation of multiple genes without the technical limitations and regulatory downsides of using transgenes. To develop a broadly applicable method to increase gene expression using editing, we searched for a small element native to maize that could be inserted into endogenous promoters to achieve upregulation. We identified a palindromic 12-bp sequence, GTAAGCGCTTAC ('plant enhancer', PE), in the maize genome that bears similarity to a known transcriptional enhancer element from the *Agrobacterium tumefaciens* octopine synthase promoter (Bouchez *et al*., 1989), and that also occurs in the genomes of other crops like soy, rice and barley. To insert PE into maize promoters during non-homologous end joining (NHEJ)-mediated repair of CRISPR/Cas-induced double-stranded breaks (Figure 1a), we bombarded immature maize embryos from a Cas9-expressing line (Lorenzo *et al*., 2022) with gold particles coated with (i) synthetic single guide RNA (sgRNA) targeting the core promoter of *GLUTAMINE SYNTHETASE1-3* (*Gln1-3*), (ii) PE trimer (3xPE) as a double-stranded oligodeoxynucleotide (dsODN) with two protective phosphorothioate bonds on both ends and without any target homology sequences, and (iii) plasmid carrying expression cassettes for a herbicide resistance marker and a fluorescent protein, allowing selection and visual screening during regeneration. 39% of regenerated lines carried dsODN-derived insertions in the target promoter. In addition to perfect 3xPE insertions, due to the imprecise nature of NHEJ, we also recovered alleles with small indels at the junctions, truncations leaving precisely one or two PE monomers or insertions of more than one 3xPE element (Figure 1b). Insertion alleles were usually present at 50% or 100% of amplicon sequencing reads, suggesting plants were not chimeric as previously reported in rice (Lu *et al*., 2020); sgRNAs and dsODNs are transiently delivered and likely rapidly disappear from cells through a combination of degradation and dilution after cell division, restricting editing to a brief window soon after delivery and leading to homogeneous plants. Testing progeny of five randomly chosen lines confirmed that insertions were heritable (Figure S1).

To confirm that 3xPE insertion reliably upregulates gene expression, we measured *Gln1-3* expression in segregating backcrossed T1 populations of three independent lines. In all three populations, there was a significant two-fold increase in *Gln1-3* expression in primary roots of individuals with a heterozygous insertion compared to siblings without insertion or to wild-type B104, with little variation between lines (Figure 1c). To confirm that 3xPE also works in other plant species, we transfected soy protoplasts with either a construct driving GFP from the wild-type GmFTL1b promoter or from a version with 3xPE inserted, showing that 3xPE increased fluorescence six-fold ($P = 0.001$, Student's *t*-test; Figure 1d).

We then extended our approach to 10 additional target genes (Table S1), generating maize lines with enhancer insertions in the promoter for each. Across all 11 targets, insertion efficiencies averaged 29% (range 6%–61%); different gRNAs targeting the same promoter had differing insertion efficiencies, suggesting that gRNA prescreening could lead to even higher success rates (Table S2). In all examined cases, enhancer insertions identified in T0 plants were inherited by T1 progeny. Expression analysis in resulting T1 populations demonstrated upregulation in leaves for all targets except one (Figure 1e), and in primary roots for all targets (Figure S2); even genes with high basal expression could be further upregulated. All experiments were performed with heterozygous enhancer insertions; in a hybrid crop like maize, achieving overexpression by a heterozygous edit is a major advantage. Nonetheless, homozygous insertion led to a doubling of *GS2* expression compared to heterozygous insertion (Figure 1f), indicating that expression level can be further tuned using zygosity. Finally, we also demonstrated that we could insert enhancer elements into multiple promoters simultaneously using gold particles loaded with four distinct sgRNAs. Most lines we regenerated contained insertions in more than one promoter, including lines with enhancer insertions in three promoters. Simultaneous upregulation of all three target genes was seen in both leaves and roots of resulting T1 plants (Figure 1g).

To look for PE insertions at non-target sites, high-coverage whole-genome sequencing data was generated for backcrossed T1s from five independent lines. Using a relaxed requirement of matching 1xPE, we recovered all 11 expected on-target insertions

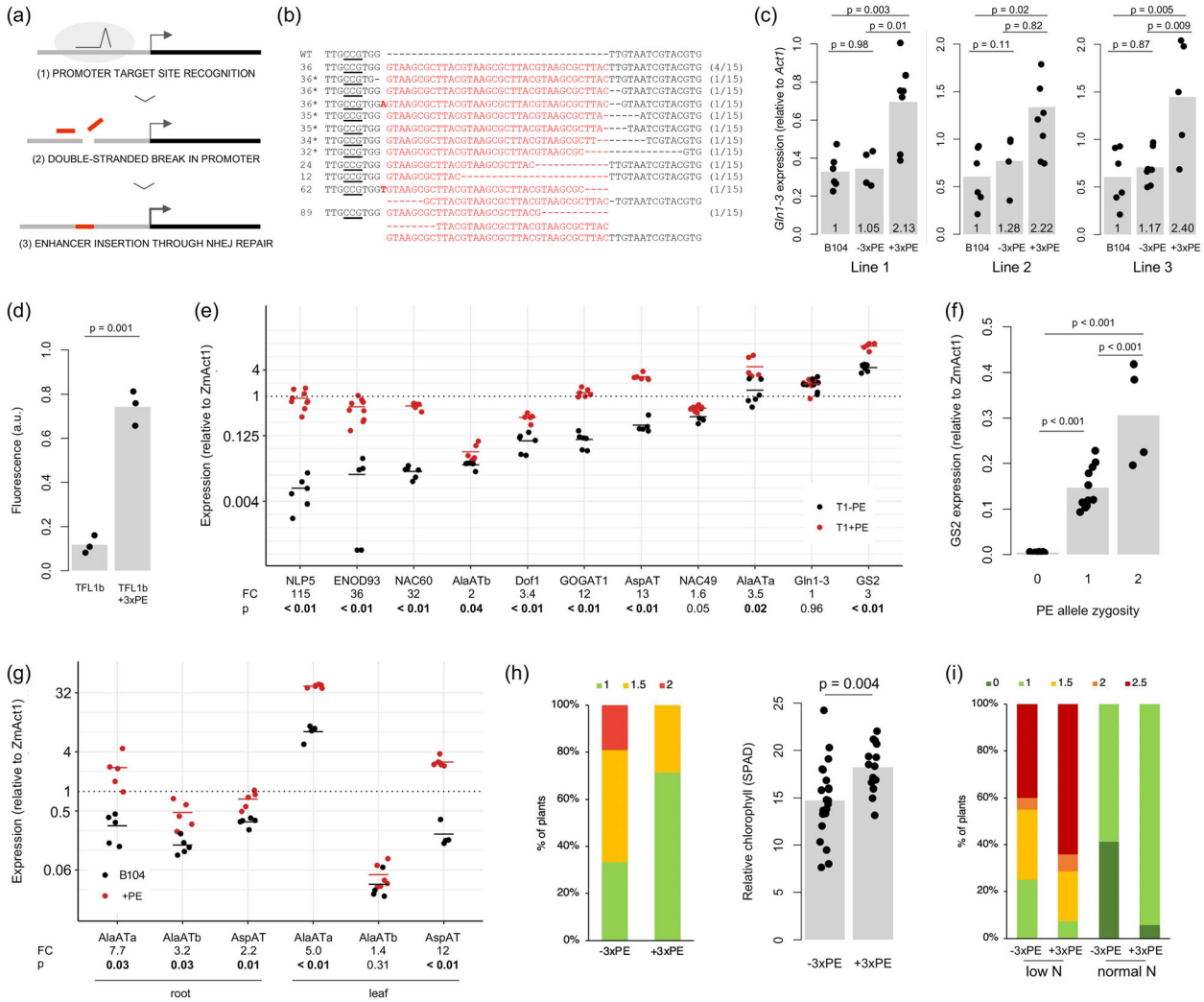

Figure 1 (a) Overview of the enhancer insertion process: CRISPR/Cas introduces a break in the promoter of a target gene, and dsODNs (red) are incorporated during the repair. (b) Sequences of recovered insertions (red) in the Gln1-3 target locus. PAM is underlined. Fractions on the right show the number of lines recovered for each allele. (c) Expression of *Gln1-3* in primary roots of three 3xPE insertion lines; *P*-values calculated using Tukey's HSD test, $P < 0.05$. (d) Fluorescence in soy protoplasts with GFP driven by TFL1b promoter versus TFL1b promoter with 3xPE inserted; *P*-value calculated using Student's *t*-test. (e) Gene expression in leaves of lines with 3xPE insertion in the corresponding promoter. (f) Expression of 3 target genes in plants with 3xPE insertions in all corresponding promoters. (e, f) FC, fold change. *P*-values calculated by Student's *t*-test. (g) GS2 expression in roots of individuals from a segregating population (0: wild-type, 1: heterozygous insertion, 2: homozygous insertion). *P*-values calculated by Tukey's HSD test. (h) Number of visibly senesced leaves in a population segregating for the presence of 3xPE in the AlaATa promoter, grown under low nitrogen (left). Relative chlorophyll of leaf 3 in the same segregating population (right; $N = 21;14$); *P*-value calculated by Student's *t*-test. (i) Number of visibly senesced leaves in a population segregating for the presence of 3xPE in the GS2 promoter, grown under either low nitrogen or normal nitrogen ($N = 20;14;17;18$). (c–h) -3xPE and + 3xPE indicate individuals with or without 3xPE from a segregating T1 population, B104 indicates wild-type plants grown alongside. Dots represent data points from individual plants. Averages are indicated by either grey bars or horizontal lines.

across these 5 lines, but only a single additional insertion, which was in an intergenic region (Table S3). The insertion locus showed no detectable homology to the gRNAs used, suggesting dsODN insertion during repair of a random double-stranded break. We did find plasmid fragment insertions that were often complex (Figure S3), as previously reported (Liu *et al.*, 2019). Nonetheless, two of the five lines had no detectable plasmid insertions, showing that it is possible to obtain transgene-free progeny after a single backcross.

We also tested an alternative delivery method to bypass the need for Cas-expressing lines. Therefore, we delivered dsODNs along with a plasmid encoding Cas9, sgRNA and a herbicide selection cassette. We found insertions in the target site in 3/28 resulting in calli (11%). These lower frequencies are likely the result of endogenous nuclease activity degrading dsODNs during the transcription/translation lag in CRISPR/Cas component production. To address this shortcoming, we co-delivered Cas RNPs with dsODNs and a selection plasmid. Analysis of calli harvested 4 weeks after bombardment indicated that 26/192 calli (14%) had PE insertions at the target locus, demonstrating the viability of this method.

Advanced trait engineering for complex traits will require combining multiple types of edits. To enable simultaneous targeting of certain genes for KO and others for upregulation,

we co-delivered synthetic sgRNAs and dsODNs for upregulation targets with a plasmid encoding a gRNA for a KO target to Cas-expressing embryos, hypothesizing that dsODNs would be partly degraded by the time this KO gRNA has been transcribed, leading to indels without dsODN insertion. We regenerated several lines with a dsODN-derived insertion in the promoter of the upregulation target and small indels in the KO target site (Figure S4); none of the 12 regenerated lines had an enhancer insertion in the KO target. Some lines showed continued editing (Table S4), raising the possibility of additional KOs in the next generation.

Finally, we performed small-scale phenotyping experiments with selected lines as proof of concept that enhancer insertions lead to phenotypes. Increased expression of alanine aminotransferase (AlaAT) has been reported to increase nitrogen use efficiency in cereals (Tiong *et al.*, 2021), while increased expression of GS2 increases nitrogen uptake in wheat (Hu *et al.*, 2018). In our experiments, plants with increased *AlaAT* expression showed a tendency towards less leaf senescence ($P = 0.06$; chi-square test) and higher leaf relative chlorophyll content ($P = 0.004$; Student's *t*-test) when grown in limited nitrogen compared to siblings without the enhancer allele (Figure 1h), suggesting higher tolerance to low nitrogen. Conversely, plants with higher *GS2* expression had increased leaf senescence, especially under normal nitrogen levels ($P = 0.02$; chi-square test; Figure 1i).

In summary, we provide a method to achieve efficient and consistent upregulation of target genes using editing, thereby engineering agronomically relevant traits and moving towards breeding by editing. This method could easily be extended to more sophisticated applications such as insertion of elements conferring tissue-specific or stress-responsive expression, or the ability to reprogram developmental transcription patterns through, for example, insertion of native transcription factor binding sites, enabling next-generation trait engineering.

Author contributions

H.C., M.L.N. and F.V.E. designed experiments and wrote the paper. E.N., L.D. and A.P. executed experiments. A.V. analysed data. L.P. provided materials.

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Conflicts of interest

H.C., E.N., L.D., A.P., A.V., M.L.N. and F.V.E. are employees of Inari Agriculture, a for-profit company that has filed patent applications on technology and findings described in this manuscript.

Data availability statement

Data available on request from the authors.

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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–S4 Supplementary Figures.
Table S1–S6 Supplementary Tables.
Data S1 Supporting Information.

