**Enhanced RNAi stability through imperfect inverted repeats: nucleotide mismatches prevent intrinsic self-silencing of hpRNA transgenes in plants**

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**Supplementary Figures**

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**Figure S1**. Sequence alignment between modified *GUS* sequences with wild-type *GUS* sequence.

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**Figure S2.** Southern blot hybridization shows variable transgene insertions in sixteen independent hpGUS[G:U] transgenic lines. a, southern blot hybridization of hpGUS[G:U] transgenic lines. DNA was digested with *Hin*dIII prior to gel electrophoresis and probed with an OCS-T probe. b, GUS expression levels of the T1 transgenic lines analysed.

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**Figure S3**. Sequence alignments between G:U modified and WT sequences of *EIN2* and *PDS.*

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**Figure S4.** Average DNA methylation levels of 35S promoter in the four hpGUS[G:U] and hpGUS[1:4] lines based on the bisulfite sequencing data in Figure 4C (lower panel). Note the correlation between relatively low GUS activity or stronger RNAi (upper panel) and the relatively low methylation levels (lower panel) in the hpGUS[1:4] lines.

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**Figure S5**. a. *EIN2* RNAi phenotypes (hypocotyl length) of the 12 independent hpEIN2[WT] and hpEIN2[G:U] lines analysed by McrBC-digestion PCR in Figure 5A. The red asterisks indicate lines that were further analysed using bisulfite sequencing in Figure 5C. b. RNAi phenotypes of the 19 hpEIN2[WT] and hpEIN2[G:U] lines used for sRNA northern blot hybridization in Figure 7A. c. *GUS* RNAi phenotypes of the 19 independent hpGUS[WT] and hpGUS[G:U] lines used for sRNA northern blot hybridization in Figure S9A.

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**Figure S6**. RNAi phenotypes in primary (T1) hpPDS[WT] and hpPDS[G:U] lines (a) and in T2 hpEIN2[WT] and hpEIN2[G:U] plants (b) of the *nrpd1a-3* and *ocp11* mutant backgrounds. For hpPDS, each plant is an independent transgenic line; for hpEIN2, plants inside each rectangle represent siblings of an independent line. *EIN2* RNAi was assayed under light on ACC medium because many of the T2 *ocp11*/hpEIN2[G:U] seed showed slow and low rate of germination in the dark. The hpEIN2[G:U] plants showed stronger growth than the hpEIN2[WT] plants in both mutant backgrounds, indicating stronger *EIN2* RNAi. The *ocp11*/hpEIN2[G:U] plants showed the most vigorous root growth indicating the strongest *EIN2* RNAi.

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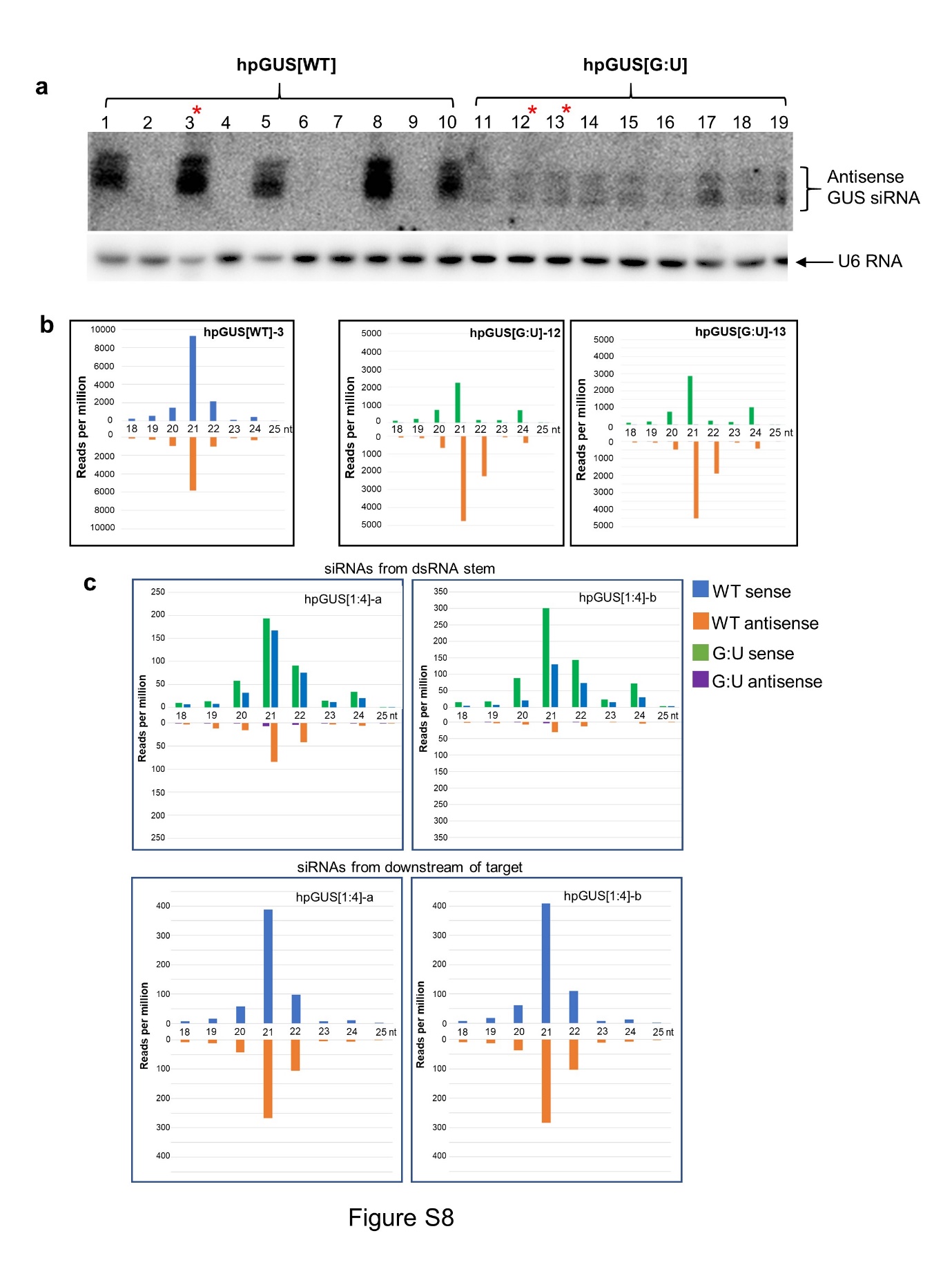
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**Figure S7**. hpPDS[WT] transgene shows strong DNA methylation in the IR region (left panel) but induces low levels of methylation at the target PDS genomic sequence compared to the hpPDS[G:U] transgene (right panel) in the Arabidopsis RdDM mutants *nrpd1a-3* and *ocp11*. A. hpPDS[WT] induces uniform but weaker photobleaching phenotypes than hpPDS[G:U] in *nrpd1a-3* and *ocp11*. The plants are primary T1 transgenics. Note the extreme photo bleaching of the *ocp11*/hpPDS[G:U] plants indicating extreme *PDS* RNAi. B. Bisulfite sequencing results. The yellow-highlighted areas represent the hpPDS IR (left) and the hpRNA-targeted PDS genomic sequence (right).

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**Figure S8**. Average levels of IR and target gene methylation in the hpEIN2[WT] and hpEIN2[G:U] lines shown in Figure 6. Note the reduction in target sequence methylation in *nrpd1a* compared to Col-0 backgrounds for the hpEIN2[WT] but not the hpEIN2[G:U] lines..



**Figure S9**. a. Northern blot hybridisation to detect antisense siRNAs in T0 hpGUS[WT] and hpGUS[G:U] lines using the 200 bp sense *GUS* target sequence as probe. b. Summary of sRNA deep sequencing data of T0 hpGUS[WT] and hpGUS[G:U] lines. c. Size distribution of sense and antisense siRNAs from the dsRNA stem region of two independent strongly silenced hpGUS[1:4] lines (a and b; with <10% GUS activity of the untransformed control plants) (upper panel) and profiles of siRNAs from the *GUS* mRNA region downstream of the 200 bp region targeted by hpGUS[1:4].

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**Figure S10**. a. Northern blot analysis of small RNAs in hpEIN2[WT] and hpEIN2[G:U] plants in Col-0 (T2 generation) and *nrpd1* and *ocp11* backgrounds (T1 primary transformants). Note that RNA samples of the two lines in Col-0 background were depleted of large RNA therefore enriched for sRNA, whereas the rest of the samples were total RNA extracted with Trizol reagent. Consequently the northern blot cannot be used to compare siRNA abundance between Col-0 and the mutants. Note that G:U hpRNA-derived siRNAs showed faster gel mobility than traditional hpRNA-derived siRNAs in all backgrounds. Also note that the tasiR255 band showed faster mobility than that of miR168, and overlaps with the 21 nt band of hpEIN2[G:U]-derived siRNAs (bottom panel). b. hpEIN2[G:U]-derived siRNAs showed equal or stronger signals on northern blot (lower half) but lower numbers of sRNA reads in sRNA sequencing data (upper half), suggesting that G:U hpRNA-derived siRNAs are under-represented in sRNA sequencing.

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**Figure S11.** Summary of sRNA reads from the different regions of hpGUS and hpEIN2 transgenes and the respective target genes.The table on the top is the summary of the 1st depp sequencing data, and the table below is of the second deep sequencing data. Note thathpGUS[1:4] lines contain higher proportions of transitive siRNAs than other hpRNA lines. There was almost no sRNA reads from the upstream region of the target mRNA so the numbers were not listed

**Supplementary Tables**

**Supplementary Table 1.** Primers to make hpGUS[WT], hpGUS[G:U], hpGUS[1:4] and hpGUS[2:10] constructs.

|  |  |
| --- | --- |
| Primer | Sequence |
| GUS-WT-F | cctcgaggatccTCGCGTCGGCATCCGGTC |
| GUS-WT-R | gggtaccaagcttCGTAAGGGTAATGCGAGGTA |
| GUS-GU-F | ccctcgagTTGTGTTGGTATTTGGTTAGTGGTAGTGAAGGGTGAATAGTTTTTGATTAATTATAAATTGTTTTATTTTATTGGTTTTGGTTGTTATGAAGATGTGGATTTGTGTGGTA |
| GUS-GU-R | ggggtaccCATAAAAATAATACAAAATACAATAAAAATTAACCCCAATCCAATCCATTAATACATAATCATACACCATCAACACATTATCAAATCCTTTACCACACAAATCCACATCT |
| GUS-4M-F | ccctcgagTCGgGTCcGCAaCCGcTCAcTGGgAGTcAAGcGCGtACAcTTCgTGAaTAAgCACtAACgGTTgTACaTTAgTGGgTTTcGTCcTCAaGAAcATGgGGAgTTGgGTGcCA |
| GUS-4M-R | ggggtaccgGTAtGGGaAATcCGAcGTAgGGTtGGAcTTGcCCCgAATgCAGaCCAaTAAaGCGaGGTgGTGgACCtTCAcCACcTTAaCGAtTCCaTTGgCACcCAAcTCCcCATgT |
| GUS-10M-F | ccctcgagTCGCGTCGcgATCCGGTCtcTGGCAGTGttGGGCGAACtcTTCCTGATatACCACAAAggGTTCTACTaaACTGGCTTacGTCGTCATctAGATGCGGtgTTGCGTGGgt |
| GUS-10M-R | ggggtaccgcTAAGGGTAtaGCGAGGTAgcGTAGGAGTacGCCCCAATggAGTCCATTttTGCGTGGTgcTGCACCATgtGCACGTTAagGAATCCTTacCCACGCAAcaCCGCATCT |
| EIN2wt-F | CCTCGAGGATCCTCTAGACCTCAGCTAGGGTTTATC |
| EIN2wt-R | GGGTACCAAGCTTAACGCTTATGCGAGCTGCAA |
| EIN2-GU-F | CCTCGAGTCTAGATTTTAGTTAGGGTTTATTTAGAGAATGGTTTTTGTTTTATTTTTTGTTTTTTTGGTTTTTGTTGGATATATTATTTTGGGAAATGGGTTGTAAATATTGAAGGAG |
| EIN2-GU-R | GGGTACCAACACTTATACAAACTACAACATATTAACATAAAATAACAACAAAATTAAAAAACAAAATAATTACCACCAAATCATACCCAAAACAAACACCTCCTTCAATATTTACAACC |

**Supplementary Table 2:** DNA fragments of 450 bp wild-type and C-to-T converted sequence of *PDS* cDNA (red letter are introduced nucleotides for restriction sites)

|  |  |
| --- | --- |
| DNA fragment | Fragment sequence |
| PDS-WT | **CCTCGAGGGATCCGAATTCATCGAT**GAAAATAAAGTTTGCTATTGGACTTTTGCCAGCCATGGTCGGCGGTCAGGCTTATGTTGAGGCCCAAGATGGTTTATCAGTCAAAGAATGGATGGAAAAGCAGGGAGTACCTGAGCGCGTGACCGACGAGGTGTTTATTGCCATGTCAAAGGCGCTAAACTTTATAAACCCTGATGAACTGTCAATGCAATGCATTTTGATAGCTTTGAACCGGTTTCTTCAGGAAAAACATGGTTCCAAGATGGCATTCTTGGATGGTAATCCTCCGGAAAGGCTTTGTATGCCAGTAGTGGATCATATTCGATCACTAGGTGGGGAAGTGCAACTTAATTCTAGGATAAAGAAAATTGAGCTCAATGACGATGGCACGGTTAAGAGTTTCTTACTCACTAATGGAAGCACTGTCGAAGGAGACGCTTATGTGTTTGCCGCTCCAGTCGATATCCTGAA**TCTAGAGAATTCAAGCTTGGTACCc** |
| PDS-CT | **cCTCGAGAAGCTT**GAAAATAAAGTTTGTTATTGGATTTTTGTTAGTTATGGTTGGTGGTTAGGTTTATGTTGAGGTTTAAGATGGTTTATTAGTTAAAGAATGGATGGAAAAGTAGGGAGTATTTGAGTGTGTGATTGATGAGGTGTTTATTGTTATGTTAAAGGTGTTAAATTTTATAAATTTTGATGAATTGTTAATGTAATGTATTTTGATAGTTTTGAATTGGTTTTTTTAGGAAAAATATGGTTTTAAGATGGTATTTTTGGATGGTAATTTTTTGGAAAGGTTTTGTATGTTAGTAGTGGATTATATTTGATTATTAGGTGGGGAAGTGTAATTTAATTTTAGGATAAAGAAAATTGAGTTTAATGATGATGGTATGGTTAAGAGTTTTTTATTTATTAATGGAAGTATTGTTGAAGGAGATGTTTATGTGTTTGTTGTTTTAGTTGATATTTTGAA**GGATCCGGTACCc** |

**Supplementary Table 3.** Primers for McrBC-PCR, bisulfite sequence and RT-PCR.

|  |  |  |
| --- | --- | --- |
| Primer | Sequence | Function |
| Link-35S- F2 | YYATYATTGYGATAAAGGAAAG | McrBC-PCR for junction region of hpEIN2[WT] and hpEIN2[G:U] lines |
| Link-EIN2- R2 | TAATTRCCACCAARTCATACCC |
| 35S-F3 | TGGCTCCTACAAATGCCATC | McrBC-PCR for junction of hpGUS[WT], hpGUS[1:4] and hpGUS[G:U] lines |
| UP-GUSwt-R1 | RRARTTRRCCCCAATCCARTCC |
| UP-35S-F2 | AGAAAATYTTYGTYAAYATGGTGG | McrBC-PCR for hpPDS[WT] lines |
| UP-35S-R2 | TCARTRRARATRTCACATCAATCC |
| Link-35S- F2 | YYATYATTGYGATAAAGGAAAGG | Bisulfite sequencing for hpEIN2[WT] and hpEIN2[G:U] |
| Link-EIN2- R1 | CCRAAACRARCACCTCCTTC |
| Link-EIN2- R2 | TAATTRCCACCAARTCATACCC |
| Link-35S-F1 | GAYAGTAGAAAAGGAAGGTGG | Bisulfite sequencing for hpGUS[WT] |
| GUSwt-R2 | CARRAACTRTTCRCCCTTCAC |
| Link-35S-F2 | YYATYATTGYGATAAAGGAAAGG |
| GUSwt-R1 | RCCCTTCACTRCCACTRACC |
| Link-35S-F1 | GAYAGTAGAAAAGGAAGGTGG | Bisulfite sequencing for hpGUS[1:4] |
| GUS-4m-R2 | CACRAARTRTACRCRCTTRAC |
| Link-35S-F2 | YYATYATTGYGATAAAGGAAAGG |
| GUS-4m-R1 | RCRCTTRACTCCCARTRARC |
| UP-35S-F1 | GGAGTYTAAGATTYAAATYGAGG | Bisulfite sequencing for hpGUS[G:U] |
| GUSgu-R2 | CAAAAACTATTCACCCTTCAC |
| UP-35S-F2 | AGAAAATYTTYGTYAAYATGGTGG |
| GUSgu-R1 | ACCCTTCACTACCACTAACC |
| PDSgen-bisF1 | TAAGYTGYAAYAGATGGTTAGG | Bisulfite sequencing for hpRNA-targeted *PDS* genomic region |
| PDSgen-bisF2 | ATGGTTAGGATATTGTGAATTTG |
| PDSgen-bisR1 | RACTACAARAAAAACATAAACAAC |
| PDSgen-bisR2 | AACATATTAATAARCARRATACC |
| hpPDS-bisF1 | TYTYYAYTGAYGTAAGGGATG | Bisulfite sequencing for hpPDS |
| hpPDS-bisF2 | AAGTTYATTTYATTTGGAGAGG |
| hpPDS-bisR1 | TTTRACATRRCAATAAACACCTC |
| hpPDS-bisR2 | TCACRCRCTCARRTACTCCC |
| EIN2gen-bisF1 | YTGAGTAATTATATTATYAGATAG | Bisulfite sequencing for hpRNA-targeted *EIN2* genomic region |
| EIN2gen-bisF2 | TATYAGATAGATTTAGGATGG |
| EIN2gen-bisR1 | ARAARRTRRTTARTARAATTCCAC |
| EIN2gen-bisR2 | TRARAAARTRACAARACAACCAC |