A protein-independent fluorescent RNA aptamer reporter system for plant genetic engineering

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Reporter systems are routinely used in plant genetic engineering and functional genomics research. Most such plant reporter systems cause accumulation of foreign proteins. Here, we demonstrate a protein-independent reporter system, 3WJ-4 × Bro, based on a fluorescent RNA aptamer. Via transient expression assays in both Escherichia coli and Nicotiana benthamiana, we show that 3WJ-4 × Bro is suitable for transgene identification and as an mRNA reporter for expression pattern analysis. Following stable transformation in Arabidopsis thaliana, 3WJ-4 × Bro co-segregates and co-expresses with target transcripts and is stably inherited through multiple generations. Further, 3WJ-4 × Bro can be used to visualize virus-mediated RNA delivery in plants. This study demonstrates a protein-independent reporter system that can be used for transgene identification and in vivo dynamic analysis of mRNA.
Plant genetic manipulation techniques are powerful tools in functional genomics research and crop genetic improvement. Using these techniques, target genes can be transformed into recipient plants to achieve various goals, such as gain or loss-of-function. Marker genes, which are critical to efficient transgene plant development, fall into two categories: selectable marker genes and reporter genes. Selectable marker genes, such as antibiotic resistance and herbicide resistance genes, are used to enrich positive transformed cells based on their ability to confer resistance to toxic substances.

Reporter genes do not provide a selective advantage to cells but rather are used to confirm transgenic events because they allow visual detection of transformed cells and tissues. Although numerous reporter genes, including those encoding fluorescent proteins (FPs), β-glucuronidase (GUS), and luciferase (Luc), have been employed to identify transgenic lines or visualize target gene expression patterns in plants, they all have limitations. For example, the green fluorescent protein gene (GFP) is commonly employed to track subcellular localization of proteins; however, the high autofluorescence of GFP in plant organs often makes it inappropriate for large-scale expression analysis at the whole-plant level. The GUS gene is widely used for tissue-specific expression analysis of target proteins because GUS is readily detectable by histochemical staining, but it cannot be used to measure the dynamic expression of target genes because of the destructive histochemical staining and destaining procedure involved in its detection. The Luc gene has also been frequently used to monitor real-time gene expression in plant tissues, whereas neither the reporter systems based on protein products, which cannot be used to report the expression of non-coding RNA. Consequently, there is a need for a reporter system to directly monitor the expression of different categories of target genes without the accumulation of foreign proteins.

To accomplish this, we focused on reporter systems acting at the transcriptional level. An RNA-based reporter system approach could circumvent some of the limitations of protein-dependent reporter systems. In creating such systems, the greatest challenge is that of exploiting unique markers to allow dynamic imaging of RNAs in living plant cells and tissues. Reif et al. fused the malachite green aptamer, the ribozyme and a siRNA to the bacteriophage phi29 packaging RNA three-way junction (3WJ) motif to generate RNA nanoparticles, which was demonstrated an excellent tool for monitoring RNA folding and degradation in real time in living cells. Besides, several RNA aptamers, with names such as Spinach and Broccoli, have been also created for live-cell imaging of RNA based on a small fluorophore whose fluorescence is activated upon binding and sequestration within the aptamer. Spinach and Broccoli both produce green fluorescence, but Broccoli is superior to Spinach due to the shorter sequence, brighter fluorescence, and higher affinity to fluorophore, DFHBI-1T. The spectra of all the 3WJ-nBro series had the same fluorescence signal, revealing 3WJ-4 × Broccoli to be a satisfactory aptamer for RNA imaging in live plant cells. Consequently, we set out to create fluorescent aptamers to apply the RNA aptamer approach to plants, aiming to develop a reporter system for plant genetic engineering at the transcriptional level.

In this study, we design a series of fluorescence aptamers based on the modified 3WJ scaffold and the optimized Broccoli sequence. To screen for an optimum aptamer with high specificity to the target gene, we employed the RNAi (RNA interference) tool for monitoring RNA folding and degradation in real time in living Arabidopsis thaliana cells using our reporter system combined with a modified N. benthamiana rattle virus (TRV) system. In summary, we report here the development of a protein-independent reporter system for plant genetic engineering, which should facilitate both functional genomics research and the genetic improvement of crops.

Results

Construction and screening of RNA fluorescence aptamer. Starting from the previously reported F30-Broccoli (F30-Bro), we modified the F30 structure to form a 3WJ and optimized the Broccoli sequence. To screen for an optimum aptamer with strong fluorescence and high stability, we created a series of 3WJ-nBro aptamers, 3WJ-Broccoli (3WJ-Bro), 3WJ-2 × Broccoli (3WJ-2 × Bro), 3WJ-4 × Bro, and 3WJ-6 × Broccoli (3WJ-6 × Bro), based on the modified 3WJ scaffold and two linkers (Supplementary Fig. 1). We then in vitro transcribed each member of the 3WJ-nBro series, as well as the original F30-Bro, individually with T7 RNA polymerase and incubated them with 10 μM DFHBI-1T. The spectra of all the 3WJ-nBro series had the same single emission peak at 527 nm and the same single excitation
peak at 488 nm, similar to the spectrum of the F30-Bro (Fig. 1a and Table 1). The extinction coefficient of all the 3WJ-nBro series was increased compared with F30-Bro, among which the extinction coefficient of 3WJ-4 × Bro was the highest, reaching 48390 M⁻¹ cm⁻¹ (2.32-fold higher than that of F30-Bro) (Table 1). All of the aptamer RNAs produced green fluorescence signals after excitation with 488-nm light, and the 3WJ-nBro series exhibited higher brightness than F30-Bro (Fig. 1b). Quantitative fluorescence data showed significantly higher fluorescence intensity for 3WJ-Bro compared with F30-Bro, indicating that our 3WJ scaffold improved the fluorescence intensity of Broccoli (Fig. 1c). Furthermore, the fluorescence intensities of 3WJ-2 × Bro and 3WJ-4 × Bro were 0.5-fold and 1.2-fold higher, respectively, than that of 3WJ- Bro, whereas that of 3WJ-6 × Bro was lower than that of 3WJ-4 × Bro but 0.7-fold higher than that of 3WJ-Bro. Thus, embedding multiple tandem-repeated Broccoli units in the 3WJ scaffold and linker did promote fluorescence intensity, and embedding four Broccoli tandem repeats in the scaffold was optimal (Fig. 1b, c). To compare the stability of the four 3WJ-nBro series and F30-Bro, we measured the fluorescence of each aptamer–DFHBI-1T complex at 488-nm excitation every hour for 5 h. The remaining fluorescence was higher for 3WJ-nBro series than for F30-Bro throughout the 5 h, and the fluorescence of 3WJ-4 × Bro was still 2981.68 a.u. after 5 h, which was sixfold and twofold higher than those of F30-Bro and 3WJ-2 × Bro, respectively (Fig. 1d). We also calculated the fluorescence decay rate after normalization to the corresponding initial fluorescence intensity. F30-Bro showed quicker fluorescence decay than the 3WJ-nBro series: after 5 h, 65% of the initial fluorescence remained for 3WJ-4 × Bro, but only 27% for F30-Bro (Fig. 1e). However, no significant difference of fluorescence decay rate was detected among the 3WJ-nBro series (Fig. 1e). Thus, all

Table 1: The spectral characteristics of fluorescent RNA aptamers.

| Aptamers   | Optimal excitation (nm) | Optimal emission (nm) | Extinction coefficient (M⁻¹ cm⁻¹) |
|------------|-------------------------|-----------------------|-----------------------------------|
| F30-Bro    | 485                     | 525                   | 14,580                            |
| 3WJ-Bro    | 488                     | 527                   | 19,490                            |
| 3WJ-2 × Bro| 488                     | 527                   | 31,870                            |
| 3WJ-4 × Bro| 488                     | 527                   | 48,390                            |
| 3WJ-6 × Bro| 489                     | 527                   | 33,420                            |

Extinction coefficients were all measured at pH 7.4.
indicated that F30-Bro, 3WJ-Bro, and 3WJ-6 × Bro all had mRNA (DFHBI-1T. Scale bars, 25 μm. All experiments above were repeated three independent times. Source Data underlying b–e are provided as a Source Data file.

four 3WJ-nBro series produce more stable fluorescence than F30-Bro, and 3WJ-4 × Bro produces the most.

To assess whether the 3WJ-nBro series could be used to detect mRNA in vitro, we appended each 3WJ-nBro and F30-Bro downstream of the terminal codon of the target gene AtCLE as a means to transcribe the fusion mRNA in vitro. The target mRNA could be detected from the lighted-up fluorescence of aptamers after DFHBI-1T binding (Fig. 2a). We used the RNAfold web server to predict the minimum free energy (MFE) secondary structures of the five aptamer-tagged mRNAs, which suggested that these aptamers could keep their original MFE structures after being used to tag mRNAs (Supplementary Fig. 2). However, fluorescence imaging and quantification of the fusion mRNAs indicated that F30-Bro, 3WJ-Bro, and 3WJ-6 × Bro all had significantly decreased fluorescence intensity when attached to AtCLE mRNA, whereas 3WJ-2 × Bro and 3WJ-4 × Bro showed no significant effects on fluorescence intensity upon mRNA attachment (Fig. 2b and Supplementary Fig. 3). Comparison of the fluorescence intensities among the fusion mRNAs indicated that AtCLE mRNA tagged with 3WJ-4 × Bro (AtCLE-3WJ-4 × Bro) produced the strongest fluorescence signal, of up to 4300 a.u., followed by AtCLE-3WJ-2 × Bro, AtCLE-3WJ-6 × Bro, AtCLE-3WJ-Bro, and AtCLE-F30-Bro (Fig. 2b). We also assessed the stability of the fluorescence of free and mRNA-fused aptamers, as evidenced by fluorescence decay over 5 h. F30-Bro, 3WJ-Bro, and 3WJ-6 × Bro had distinctly lower fluorescence stability when appended to the 3’ terminus of AtCLE mRNA, whereas 3WJ-2 × Bro and 3WJ-4 × Bro showed similar fluorescence.
stability before and after fusion to the mRNA (Fig. 2c). Consequently, considering both fluorescence intensity and stability, 3WJ-4 × Bro showed the greatest potential for use in RNA imaging in vitro among the five aptamers tested.

To explore the performance of 3WJ-1Bro series in RNA imaging in vivo, we constructed a series of constructs expressing 3WJ-1Bro-tagged AtCLE mRNAs and tested them in both E. coli and N. benthamiana cells. Confocal microscopy imaging showed that more than 50% of E. coli cells expressing AtCLE-3WJ-2 × Bro, AtCLE-3WJ-4 × Bro, or AtCLE-3WJ-6 × Bro fluoresced, whereas <30% of cells expressing AtCLE-3WJ-Bro or AtCLE-F30-Bro showed detectable fluorescence (Fig. 2d and Supplementary Fig. 4). In addition, the cells expressing AtCLE-F30-Bro showed considerably weaker average fluorescence than those expressing any of AtCLE-3WJ-1Bro mRNAs (Fig. 2d and Supplementary Fig. 5), and cells expressing AtCLE-3WJ-4 × Bro showed the highest fluorescence. Moreover, in N. benthamiana cells, only cells expressing AtCLE-3WJ-4 × Bro mRNA exhibited bright fluorescence under the confocal microscope, whereas cells expressing any of the other aptamer-tagged mRNAs showed no detectable fluorescence (Fig. 2e). These results indicated that 3WJ-4 × Bro was the only fully competent aptamer that could be successfully used for mRNA imaging in both live E. coli cells and plant cells. Consequently, we used 3WJ-4 × Bro in constructing our transcriptional-level reporter system.

Characteristics of 3WJ-4 × Bro as an in vitro mRNA reporter. The detection of most endogenous RNAs requires highly sensitive imaging probes due to their low expression level. To confirm the detectable level of the 3WJ-4 × Bro marker, we designed a series of concentrations for in vitro transcrpts of the 3WJ-4 × Bro-tagged AtCLE (AtCLE-3WJ-4 × Bro), incubated the dilutions with 10 μM DFHBI-1T in 50 μL HEPS, and imaged them. Fluorescence signals could be observed when the transcript content was as low as 9.375 nmol, indicating that 3WJ-4 × Bro has high detection sensitivity in mRNA imaging (Supplementary Fig. 6). Furthermore, linear regression showed that the fluorescence intensity increased linearly with increasing transcript contents from 9.375 to 300 nmol (Fig. 3a).

To determine whether 3WJ-4 × Bro was a useful marker for reporting the expression of different target genes, we tested three genes of different sequence lengths: AtCLE, mCherry, and NtTub. We fused 3WJ-4 × Bro to the 3′-untranslated region (3′-UTR) of each test gene to create a chimeric gene series (AtCLE-3WJ-4 × Bro, mCherry-3WJ-4 × Bro, and NtTub-3WJ-4 × Bro). In vitro imaging of equimolar amounts of transcripts of the three genes showed similar fluorescence levels for each (Fig. 3b), and quantification data showed average fluorescence intensities for each 3WJ-4 × Bro-tagged mRNA of more than 4000 a.u., with no significant difference among the three (Fig. 3c), suggesting that 3WJ-4 × Bro is sufficiently stable to avoid interference from the different target mRNAs. Results from a fluorescence decay assay showed that 3WJ-4 × Bro had greater fluorescence stability after attachment to the 3′-UTR of each of the three mRNAs for 5 h after adding DFHBI-1T. In addition, we observed an increased fluorescence stability for 3WJ-4 × Bro with increasing length of the mRNA sequence over 5 h, indicating that mRNAs with higher molecular weights may enhance the stability of the 3WJ-4 × Bro tag (Fig. 3d). These favorable detection sensitivity and stability characteristics make 3WJ-4 × Bro a noteworthy potential marker for tracking different mRNAs.

Performance of 3WJ-4 × Bro in a prokaryotic expression system. To explore the capability of 3WJ-4 × Bro as a marker for reporting functional gene expression in prokaryotic cells, we transformed the constructs harboring the three chimeric genes (AtCLE-3WJ-4 × Bro, mCherry-3WJ-4 × Bro, and NtTub-3WJ-4 × Bro) into E. coli BL21 cells (Supplementary Fig. 7). Confocal imaging showed robust green fluorescence in E. coli cells expressing each of the three 3WJ-4 × Bro-tagged mRNAs. As a comparison, cells containing either untagged mRNA or DFHBI-1T alone showed no detectable fluorescence signal (Fig. 4a and Supplementary Fig. 8). We also assessed the detection efficiency based on the ratio of fluorescent cells to total cells. Approximately 80% of cells expressing each of the three mRNAs tagged with 3WJ-4 × Bro fluoresced, indicative of high detection efficiency for 3WJ-4 × Bro in reporting the transcription of different target genes in E. coli cells (Fig. 4b). In addition, quantitation of the fluorescence of cells expressing the three 3WJ-4 × Bro-tagged mRNAs showed similar fluorescence intensities in all three, suggesting that the 3WJ-4 × Bro marker did not perturb mRNA transcription of any of the three target genes in living E. coli cells (Fig. 4c).

Most heterologous RNA aptamers are cleaved by RNase in live cells, causing them to dissociate from the target RNAs. To ensure that the fluorescence observed in E. coli precisely reflected the distribution of the mRNA, rather than free 3WJ-4 × Bro, we conducted an integrity analysis of the 3WJ-4 × Bro-tagged mRNAs by in-gel imaging of fluorescent RNAs, in which total cellular RNAs from induced E. coli cells were separated in a urea denaturing gel and stained with DFHBI-1T. The gel image showed a single band of the expected size for each sample (Fig. 4d). Furthermore, when we excised the RNA bands, eluted them from the gel, and subjected them to reverse transcription sequencing, the cDNA sequence corresponded to the full-length sequence of each 3WJ-4 × Bro-tagged mRNA (Supplementary Fig. 9). These results confirmed that the fluorescence signal seen in E. coli cells came only from the intact 3WJ-4 × Bro-tagged mRNAs, indicating that 3WJ-4 × Bro detection accurately reports the expression level of functional genes in prokaryotic cells.

In addition, to explore the effect of 3WJ-4 × Bro on the translation activity of target mRNAs, we transformed the different test genes, with and without the 3WJ-4 × Bro marker, into E. coli strain Rosetta (DE3) cells for protein expression analysis. The results of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining showed that cells harboring the test genes both with and without the 3WJ-4 × Bro marker expressed target proteins of the expected size and showed the same distribution of total protein on the gel (Supplementary Fig. 10). For further verification, we performed immunoblotting. The target proteins expressed from 3WJ-4 × Bro-tagged mRNAs had the same molecular weights and expression levels as those from untagged mRNAs (Fig. 4e). These data confirmed 3WJ-4 × Bro as an excellent gene marker for reporting the expression of different target genes in prokaryotic systems that does not perturb either transcription or translation.

Performance of 3WJ-4 × Bro in plant transient expression system. The spatiotemporal expression and dynamics of mRNAs play critical roles in directly and indirectly regulating complex biological processes in plants. To further explore the utility of 3WJ-4 × Bro as a genetically encoded marker in excised plant cells, we conducted a protoplast transient expression assay. We expressed each of the three 3WJ-4 × Bro-tagged mRNAs (AtCLE-3WJ-4 × Bro, mCherry-3WJ-4 × Bro, and NtTub-3WJ-4 × Bro) in protoplasts under the control of the CaMV 35S promoter (Supplementary Fig. 11a). Confocal imaging of the protoplasts showed bright fluorescence in those expressing 3WJ-4 × Bro-tagged mRNAs after incubation with 10 μM DFHBI-1T but no signal in the absence of either 3WJ-4 × Bro or DFHBI-1T (Fig. 5a and
To further assess the performance of 3WJ-4 × Bro in tracking target gene expression in plant tissues, we introduced the mCherry-3WJ-4 × Bro as a favorable marker for reporting mRNA expression in excised plant cells. To monitor the dynamic process of mRNA synthesis and translation in plant cells, we introduced the chimeric genes (AtCLE-3WJ-4 × Bro, mCherry-3WJ-4 × Bro, and NtTuba-3WJ-4 × Bro) into N. benthamiana leaf tissue by Agrobacterium-mediated transformation for transient expression. Confocal imaging showed bright fluorescence in the leaves expressing each of the three target genes with the 3WJ-4 × Bro marker after incubation with DFHBI-1T, whereas leaves expressing marker-free genes or not incubated with DFHBI-1T showed no fluorescence (Fig. 5c and Supplementary Fig. 13), confirming that the 3WJ-4 × Bro/DFHBI-1T system could be used to report mRNA transcription in plant tissue in vivo. Furthermore, obvious cytoplasmic and nuclear localization signals were observed in leaf cells, demonstrating the distribution of three target mRNAs (Fig. 5c). Scanning of intracellular and extracellular fluorescence intensity (marked by blue lines in Fig. 5c) showed high S/B in the green fluorescence channel (Fig. 5d), which aided the accuracy of the 3WJ-4 × Bro/DFHBI-1T system.

To ensure accurate assessment of the expression levels of target mRNAs, we also detected the integrity of the 3WJ-4 × Bro-tagged mRNAs through an in-gel imaging assay. We observed a single expected band on the gel for each 3WJ-4 × Bro-tagged mRNA (Supplementary Fig. 14). Sequencing results of the excised and eluted RNA bands revealed the full-length sequences of each 3WJ-4 × Bro-tagged RNA, indicating that the 3WJ-4 × Bro/DFHBI-1T system accurately reported the expression and localization of the target mRNA in plant tissue.

Next, to further investigate the effect of the 3WJ-4 × Bro tag on the translation of the fusion mRNAs in plant, we visualized mCherry-3WJ-4 × Bro mRNA and its protein product in the leaves simultaneously through confocal microscopy. The bright green and red fluorescence signal were simultaneously observed in the mCherry-3WJ-4 × Bro channel and the mCherry channel, respectively, indicating that 3WJ-4 × Bro did not affect the function of mRNA as a genetically encoded template for protein translation in the plants (Fig. 5e). Moreover, we found no cells showing only green or red fluorescence alone, which indicated that 3WJ-4 × Bro was reliably linked to the mRNAs and vice versa. Thus, the spectroscopically and biochemically favorable characteristics of 3WJ-4 × Bro/DFHBI-1T, which include bright, stable fluorescence, high S/B contrast, and robust reporting of mRNA expression without perturbation of the target RNA’s transcription, localization, or translation, qualify this system as an excellent protein-independent reporter system at the transcriptional level for plant genetic transformation.

To monitor the dynamic process of mRNA synthesis and transfer in plant cells, we transformed the construct expressing AtCLE-3WJ-4 × Bro into N. benthamiana leaf cells by Agrobacterium infiltration and then incubated leaf samples with 10-μM DFHBI-1T at 0, 24, 48, and 72-h post transformation, respectively. The imaging results showed no detectable fluorescence at 0-h post infiltration. AtCLE-3WJ-4 × Bro fluorescence...
Fig. 4 Performance of 3WJ-4 × Bro in E. coli cells for reporting the expression of various genes. a Representative images of E. coli cells expressing three mRNAs tagged with 3WJ-4 × Bro after incubation with 10 μM DFHBI-1T. Scale bars, 25 μm. b The percentage of detectable fluorescing cells to the 500 total cells expressing each 3WJ-4 × Bro-tagged mRNAs. Error bars, mean ± s.d. (n = 6). c Fluorescence quantification of mRNAs tagged with 3WJ-4 × Bro in cells. Error bars, mean ± s.d. (n = 9). d Assessment of the integrity of three mRNAs with the 3WJ-4 × Bro tag in E. coli cells. Total cellular RNA was extracted and separated by urea denaturing gel electrophoresis. After washing, the gel image was stained with DFHBI-1T and photographed. Red lines indicate the locations of marker bands on the gel. e Immunoblot analysis of target proteins translated from 3WJ-4 × Bro-tagged mRNAs. Source data are provided as a Source Data Underlying e. All experiments were repeated three independent times with the same conclusion. Source Data are provided as a Source Data file.

Detection of virus-mediated RNA delivery in plant using 3WJ-4 × Bro. The virus-mediated RNA delivery system, which bypasses the requirement for producing transgenic plants, is widely used for gene overexpression and genome editing. TRV has been the most useful vector for this approach. However, the detection of RNA delivery still depends on RT-PCR, which is time consuming and inefficient. Consequently, we proposed a more efficient method for detecting RNA delivery using the 3WJ-4 × Bro/DFHBI-1T system. Starting from a recombinant vector derived from the RNA2 genome of TRV, we created a vector to express mCherry-3WJ-4 × Bro mRNA under the control of the pea early browning virus (PEBV) promoter (Fig. 6a). Subsequently, the Agrobacterium cells respectively harboring the RNA1 genome and RNA2 vector were mixed and infiltrated into N. benthamiana leaves to reconstitute the TRV virus. We then detected fluorescence signals in both infiltrated and systemic leaves (non-infiltrated leaves of the same plant) under ultraviolet light by confocal microscopy at 5 days after infiltration.

After permeation of DFHBI-1T, infiltrated leaves showed visible green fluorescence upon exposure to ultraviolet light, but interestingly, weak fluorescence was also observed in systemic leaves (Fig. 6b), indicating that the mCherry-3WJ-4 × Bro-tagged mRNAs were delivered from the infiltrated leaves to systemic leaves by TRV. The fluorescence microscopy images further showed that mCherry-3WJ-4 × Bro-tagged mRNAs accumulated in the cytoplasm and nucleus of leaves of both types (Fig. 6b). The
red signal in the mCherry channel showed that mCherry protein was translated from mCherry-3WJ-4 × Bro and was mainly distributed in cytoplasm of both types of leaves (Fig. 6b). To further validate mRNA delivery, we performed RT-PCR amplification of mCherry-3WJ-4 × Bro from both inoculated and systemic leaves. A single expected band was detected in each leaf sample (Fig. 6c), and the sequencing data identified the same sequence of mCherry-3WJ-4 × Bro in two types of leaves.

**Performance of 3WJ-4 × Bro in plant stable transformation system.** Based on the outstanding performance of the 3WJ-4 × Bro/DFHBI-1T reporter system in plant transient transformation, we planned to use this reporter system for transgene identification and expression analysis of target genes in stable transformation. We selected NtTub as a target gene and marked it with 3WJ-4 × Bro at its 3′-UTR. We then stably transformed the fusion gene NtTub-3WJ-4 × Bro into A. thaliana. We screened 6000 transgenic T1 seeds, which were germinated on MS culture medium containing 50 μg/mL kanamycin, resulting in 50 kanamycin-resistant T1 transgenic seedlings. Leaves from kanamycin-resistant seedlings were excised for positive identification by genome PCR (gPCR) and fluorescence imaging after infiltration with 30-μM DFHBI-1T. gPCR analysis indicated that 45 of the 50 kanamycin-resistant seedlings were positive transgenic plants, and fluorescence imaging showed that 44 of these 50 seedlings had a bright green fluorescence (Fig. 7a, b). Moreover, all 44 fluorescent seedlings were gPCR-confirmed positive seedlings, but one of 45 gPCR-confirmed seedlings (designated N-F1) showed no fluorescence under the confocal microscope (Fig. 7b). To clarify this result, we assessed NtTub-3WJ-4 × Bro expression in N-F1 seedlings by RT-PCR. No NtTub-3WJ-4 × Bro expression was detected (Supplementary Fig. 15), indicating that NtTub-3WJ-4 × Bro was successfully integrated into the genome but with its expression silenced. Thus, the 3WJ-4 × Bro/DFHBI-1T system can simultaneously and accurately report both the presence and expression of the target gene in transgenic plants.

To assess the genetic stability of 3WJ-4 × Bro in transgenic offspring, we grew six T2 transgenic families derived from six T1 plants in soil and subjected 766 T2 seedlings to fluorescent identification. The results showed that 572 T2 seedlings were fluorescent and 194 T2 seedlings showed no fluorescence (Table 2). A χ² test showed that the ratio of fluorescent plants to nonfluorescent plants fit the model of 3:1 Mendelian segregation in every T2 family, indicating that all six T1 transgenic plants harbor a single integrated copy of NtTub-3WJ-4 × Bro.
Fig. 6 Detection of long-distance RNA delivery by 3WJ-4 × Bro in N. benthamiana. a Schematic diagram of the Agrobacterium binary vector system containing the TRV RNA1 and RNA2 genomes with some modification, as well as the experimental scheme of the TRV-mediated target RNA delivery. RNA1 contains LB (left border), 2 × 35S (2 × CaMV 35S promoter), RdRNAP (encoding RNA-dependent RNA polymerase), MP (encoding movement protein), 16k (encoding a cysteine-rich protein), Rz (self-cleaving ribozyme), Tnos (nopaline synthase terminator), and RB (right border). RNA2 contains LB, p35S, CP (encoding coat protein), PEBV (pea early browning virus promoter), Rz, Tnos, and RB. mCherry-3WJ-4 × Bro was cloned under the control of PEBV. The flask, tube, and syringe are drawn by software Photoshop CS6. b Fluorescence signals of N. benthamiana leaves under ultraviolet and confocal microscopes. Agrobacterium cultures carrying the engineered TRV RNA2 and RNA2 vectors were co-infiltrated into N. benthamiana leaves to express and transmit 3WJ-4 × Bro-tagged RNA (mCherry-3WJ-4 × Bro). At 5 days after Agrobacterium infection, both infiltrated and systemic leaves were sampled and then incubated with 10-μM DFHBI-1T at room temperature for 20 min. Scale bars, 75 μm. Three independent trials showed the same results. c Confirmation by RT-PCR of the presence of the fusion RNA (mCherry-3WJ-4 × Bro) in both infiltrated and systemic leaves. Source Data underlying b are provided as a Source Data file.
Broccoli units (four copies), while 3WJ-6 × Bro showed lower fluorescence compared to 3WJ-4 × Bro, which is possibly explained by that the 3WJ structure and linker 2 are not stable enough to effectively fold six copies of Broccoli. In addition, 3WJ-4 × Bro showing highest fluorescence stability is possibly attributed to its spatially stable structure enhanced by utilizing linker 1.

To investigate the aptamers’ ability to image mRNA in vivo, we fused each aptamer to the 3′-UTR rather than 5′-UTR of target genes to avoid the possibility that the presence of the aptamer tertiary structure fused to the 5′-UTR could abolish target gene translation by hindering ribosome complex assembly. Upon fusion with the mRNA AtCLE, we observed a significant decrease in fluorescence for F30-Bro, 3WJ-Bro, and 3WJ-6 × Bro, but not 3WJ-4 × Bro and 3WJ-2 × Bro, indicating that only 3WJ-4 × Bro and 3WJ-2 × Bro were stable enough to tag mRNA in vitro. In addition, in vivo assays revealed that although 3WJ-2 × Bro, 3WJ-4 × Bro, and 3WJ-6 × Bro all showed good potential for mRNA imaging in E. coli, only 3WJ-4 × Bro showed detectable fluorescence in N. benthamiana, indicating that stability of a given aptamer differed in different types of cells. This is probably the

Table 2 The identification of transgenic Arabidopsis thaliana T2 plants by fluorescence and RT-PCR.

| T2 transgenic family | Total plant number | F-P plant number | F-N plant number | N-P plant number | N-N plant number | \( \chi^2 \) value for 3:1 |
|----------------------|--------------------|------------------|------------------|------------------|------------------|--------------------------|
| #1                   | 122                | 93               | 0                | 0                | 29               | 0.098                    |
| #2                   | 114                | 86               | 0                | 0                | 28               | 0.012                    |
| #3                   | 133                | 100              | 0                | 0                | 33               | 0.003                    |
| #4                   | 139                | 105              | 0                | 0                | 34               | 0.022                    |
| #5                   | 116                | 86               | 0                | 0                | 30               | 0.046                    |
| #6                   | 142                | 102              | 0                | 0                | 40               | 0.761                    |
| Total                | 766                | 572              | 0                | 0                | 194              | 0.044                    |

F-P indicates both fluorescent and RT-PCR positive plants; N-N indicates neither fluorescent nor RT-PCR positive plants; F-N indicates fluorescent but RT-PCR negative plants; N-P indicates nonfluorescent but RT-PCR positive plants.

The one-sided \( \chi^2 \) test for 3:1 of F-P plants to N-N plants were conducted with significance level at 0.01, \( \chi^2_{1,0.01} = 6.63 \).
main reason that, although aptamers such as Mango and Corn have been used for RNA imaging in E. coli and mammalian cells, there have been no reports of their application in plant cells.

To use 3WJ-4 × Bro as a marker for reporting gene expression, we investigated effects of different mRNAs on 3WJ-4 × Bro in vitro and in vivo. We established that 3WJ-4 × Bro could be used to image different mRNAs in both E. coli and plant cells, and the target proteins were detected in E. coli by immunoblotting and in plant cells by mCherry fluorescence (Figs. 4 and 5). Furthermore, no cleavage occurred between the mRNA and the tag.

In our study, the obvious and in plant cells by mCherry fluorescence accurately reflects the expression and distribution of mRNA in live cells. By contrast, F29- and F30-scaffold Broccoli are reported to undergo differential cleavage in E. coli and mammalian cells, which implies that the structure of the 3WJ scaffold plays a pivotal role in maintaining the stability of both the Broccoli aptamer and RNAs of interest. Thus, 3WJ-4 × Bro is a favorable marker for reporting gene expression in plant cells without perturbation of the transcription or translation of target genes. Furthermore, our transgenic expression assay showed that the nuclear localization fluorescence signal of 3WJ-4 × Bro-tagged mRNAs was easily detected in N. benthamiana leaves, but not in N. benthamiana protoplasts. This difference was probably due to insufficient DFHBI-1T infiltration into protoplast nuclei during the relatively short incubation time required to avoid protoplast destruction.

To assess the detection sensitivity of the 3WJ-4 × Bro/DFHBI-1T reporter system, we imaged the NTtubα mRNA tagged with 3WJ-4 × Bro and NTtubα protein with GFP under the same microscope and observed similar fluorescence intensities for both systems.

To assess the application of the 3WJ-4 × Bro/DFHBI-1T system, we used it to track an RNA virus infection (Fig. 6), a process that poses severe hazards to the growth and development of plants, including crop plants. Although numerous molecular mechanisms of virus infection have been described, direct imaging of RNA virus dynamics is still lacking. In our study, we successfully detected the transfer of RNAs expressed by TRV from an inoculated leaf to other leaves, validating the utility of 3WJ-4 × Bro as a direct imaging tool for investigating RNA virus invasion and simultaneously facilitating research into the regulatory mechanism of long-distance transport of RNA. Homoplastically, the 3WJ-4 × Bro/DFHBI-1T system could also be employed to identify and monitor protein–RNA interactions in real time in vivo. Moreover, the system is well suited for identifying transgenic plants (Fig. 7), for which it could be a competitive alternative to traditional diagnostic PCR. During the traditional transgenic generation screening process, qPCR identification is indispensable after antibiotic selection to ensure the insertion of the complete T-DNA into the plant chromosome as opposed to vector backbone integration, which may result from target gene escape. In addition, qPCR analysis is required to evaluate silencing efficiency and/or transcript level in the transgenic plants. However, these molecular genetic tests are costly and time consuming. Although GFP can be used as a marker for the rapid visual screening of transgenic plants, this approach requires constructing a fusion protein, which may negatively affect the biological function of target proteins. By contrast, 3WJ-4 × Bro provides a convenient means both to identify transgenic plants at the transcriptional level, without affecting target gene expression, and to estimate expression levels on the basis of fluorescence intensity as an alternative to qPCR. It could also be used to monitor the spatiotemporal-specific expression of a target gene during the course of a plant’s lifetime.

The application of this system mainly depends on fluorescence intensity and stability. In our study, the obvious fluorescence was observed in plant cells, which is partly explained by the high accumulation transcripts of 3WJ-4 × Bro-tagged gene constitutively expressed under CaMV 35S. However, endogenous gene showed a physiologically normal expression under the control of native promoter. Consequently, different types of endogenous promoter will be used to confirm potential utility of our system as a transcriptional reporter in plants in our further work. In addition, the fluorescence intensity is partly affected by the infiltration efficiency of DFHBI-1T into plant cell, which may be a limitation when comparing across different tissues or plants. This limitation could be got around if (i) plants cell membrane permeability could be changed by different solvents of DFHBI-1T solution; (ii) a derivative of DFHBI-1T, with high fluorescence and permeability, is developed.

In conclusion, we report protein-independent reporter system using a designed aptamer, 3WJ-4 × Bro, and its fluorophores, DFHBI-1T, for transgene identification and gene expression analysis in plants. The 3WJ-4 × Bro aptamer showed high folding efficiency and stability in plant cells, integrated into the plant genome with its target genes and co-expressed with them, and was stably inherited through generations of transgenic lines through co-segregation with its target gene during the meiosis. Together, these properties make 3WJ-4 × Bro a desirable marker for plant genetic engineering and a useful tool for various types of functional genomics studies, including explorations of protein–RNA interaction, RNA–RNA interaction, RNA virus invasion, and promoter function.

Methods

Design and clone of aptamer-related sequences. Based on the previously reported F30-Broccoli (F30-Bro), we modified the structure of the F30 scaffold to form a 3WJ scaffold and optimized the sequence of Broccoli (Supplementary Fig. 1). We designed a series of 3WJ-nBroyoaptamers by inserting Broccoli into two different specific sites of the 3WJ scaffold to construct 3WJ-2 × Bro and 3WJ-4 × Bro (Fig. 2). To further improve the structure and function of the 3WJ scaffold, we investigated effects of different mRNAs on 3WJ-4 × Bro/DFHBI-1T system, for which it could be a competitive alternative to traditional diagnostic PCR. During the traditional transgenic generation screening process, qPCR identification is indispensable after antibiotic selection to ensure the insertion of the complete T-DNA into the plant chromosome as opposed to vector backbone integration, which may result from target gene escape. In addition, qPCR analysis is required to evaluate silencing efficiency and/or transcript level in the transgenic plants. However, these molecular genetic tests are costly and time consuming. Although GFP can be used as a marker for the rapid visual screening of transgenic plants, this approach requires constructing a fusion protein, which may negatively affect the biological function of target proteins. By contrast, 3WJ-4 × Bro provides a convenient means both to identify transgenic plants at the transcriptional level, without affecting target gene expression, and to estimate expression levels on the basis of fluorescence intensity as an alternative to qPCR. It could also be used to monitor the spatiotemporal-specific expression of a target gene during the course of a plant’s lifetime.

In vitro transcription and fluorescence measurement. 3WJ-4 × Bro, 3WJ-6 × Bro, 3WJ-3 × Bro, and 3WJ-2 × Bro were respectively transcribed in vitro using T7 RNA polymerase at 37 °C for 16 h in 40 mM Tris-HCl, 30 mM MgCl2, 2 mM spermidine, 1 mM dithiothreitol (DTT), 5 mM NTPs, 1 U/μL inorganic pyrophosphatase and 4 U/μL T7 RNA polymerase (pH 7.9). The transcripts were purified on a denaturing 6% polyacrylamide gel (acrylamide: bisacrylamide = 29:1) containing 6 M urea in TBE (Tris, borate, ethylenediaminetetraacetic acid (EDTA) buffer). The RNA was excised and eluted in RNA elution buffer (40 mM Tris-HCl, pH 8.0, 0.5 M sodium acetate, 0.1 mM EDTA) and precipitated with ethanol. All transcripts were dissolved in DEPC-purified water, and the purity and concentration of RNAs were measured with a NanoDrop ND-2000 spectrophotometer. Subsequently, 1 μg transcribed RNA was incubated with 100 ng of fluorescent probe DFHBI-1T (5'-fluorescein dihydrobenzylidenyl-2'-methyl-1-(2,2,2-triluoroethyl)-1H-imidazol-5(4H)-one) in 50 μL 1 × HEPEs buffer (pH 7.4) in a centrifuge tube at 25 °C for 20 min. DFHBI-1T was purchased
from L. aerogenes. The extinction spectra of the complexes were scanned from 400- to 550-nm wavelengths at 527-nm emission and the emission spectra of that were excited at 400 nm in 1-mM MES (pH 5.7), 0.5 M ammonium, 20 mM KCl and incubated at room temperature for 12 h. The protoplasts were centrifuged at 100 x g for 2 min to remove the supernatant and 200 μl W1 solution was added to resuspend the protoplasts. DHFBI-1T at a final concentration of 10 μM was added into the protoplast solution for 10 min, followed by incubation of 1-mM MES (pH 5.7) and then the fluorescent intensity at 25 °C was measured at 488-nm excitation and 527-nm emission wavelengths.

Expression of 3WJ-4 × Bro-tagged mRNA in N. benthamiana leaves. Recombinant plasmid binary was constructed (Supplementary Fig. 22) and transformed into Agrobacterium tumefaciens strain GV3101. Positive clones were picked from YEP agar plates containing 50 μM kanamycin and 35 μM rifampicin and were determined by PCR, and were inoculated into LB medium containing the same antibiotics for incubation at 28 °C overnight. A total of 1 μl of the transformed protoplast solution was transferred to a 384-well plate, and the fluorescence intensity at 25 °C was measured at 488-nm excitation and 527-nm emission wavelengths.

Detection of virus-mediated RNA delivery by the receiver system. The sequence of mCherry-3WJ-4 × Bro was amplified from recombinant plasmid bPlasmid II SK-mCherry-3WJ-4 × Bro by specific primers TRV2-mC F and TRV2-mC R (Supplementary Table 2) and subcloned into pTRV2 plasmid between mCherry sequence of mCherry-c2x plasmids between Sac I and Xba I site (Supplementary Fig. 20). The recombinant mCherry-c2x were transformed individually into E. coli strain Rosetta (DE3). Single positive clones identified by PCR were inoculated into Luria Broth liquid medium containing 50 μg/ml ampicillin and grown overnight at 37 °C at 200 rpm when the OD600 reached 0.8. The cells were collected at room temperature using a conical microfuge objective x63, N.A. 1.46, excitation 488 nm, emission 527 nm) and analyzed with LAS AF Lite software.

Prokaryotic expression and immunoblotting. Agro-3WJ-4 × Bro, mCherry-3WJ-4 × Bro, and NiTuba-3WJ-4 × Bro were respectively amplified from recombinant plasmids pBluescript II SK-mCherry-3WJ-4 × Bro and pBI221 plasmids between the a dual expression vector for N. benthamiana plants using a syringe (objective HCX PL APO CS 20x 0.70 DRY UV, excitation 488 nm, emission 527 nm).

Identification of stably transformed A. thaliana by the reporter system. Agrobacterium GV3101 containing recombinant binary plasmid pBI121-NiTuba-3WJ-4 × Bro were grown to ODmax of 1.6 at 28 °C at 180 rpm in a shaker. The cells were pelleted by centrifugation and resuspended in infiltration medium (5% sucrose, 0.02% Silwet L-77) to a final ODmax of 1.0 and then incubated at room temperature in darkness for 2 h. A. thaliana (Colombia ecotype) inflorescences were immersed in Agrobacterium solution for 10 s twice and covered with plastic bags to retain humidity. After 16 h of cultivation in darkness, the plants were transferred to a light chamber (with a 16-h light/8-h dark photoperiod) for further growth. The seeds of T0 generation were obtained screened on the solid MS medium containing 30 μg/ml kanamycin. Two-week-old kanamycin-resistant seedlings were respectively transferred to soil and MS medium for 5 days of growth. The tissues (leaf, root elongation zone, and root tip) excised for gPCR and RT-PCR identification. The primers were designed to detect the presence of mRNA were detected on the basis of their fluorescence signals using a confocal microscope (objective HCX PL APO CS 20x 0.70 DRY UV, excitation 488 nm, emission 527 nm).

Integrity analysis of mRNAs with 3WJ-4 × Bro tag. The integrity of the transcripts of 3WJ-4 × Bro-tagged genes expressed in live cells was detected by in-gel imaging of fluorescent RNAs. Total RNA was extracted from live cells (e. coli, A. thaliana and N. benthamiana) and processed as described by Song et al. [44]. The gel was washed three times with RNase-free water for 15 min to remove the area, and stained for 20 min at room temperature in 10 μM DHFBI-1T in 1 × HEPES (pH 7.4). The fluorescent intensity was measured with a fluorescence plate reader: 100 μl of the treated protoplast solution above was transferred to a 384-well plate, and the fluorescence intensity at 25 °C was measured at 488-nm excitation and 527-nm emission wavelengths.
7.4). The gel was imaged on a ChemiDoc MP (Bio-Rad) with a 488-nm excitation and 527-nm emission. The expected band was excised from the gel and the eluted mRNA was reverse transcribed to cDNA using a High Capacity CDNA Reverse Transcription Kit (ThermoFisher). The cDNA was then sequenced to confirm the integrity of the mRNA with 3WJ-4× Bro tag.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Data supporting the findings of this work are available within the paper and its Supplementary Information file. A reporting summary for this Article is available as a Supplementary Information file. The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request. The data that support the findings of this study are available from the corresponding author upon reasonable request. The data underlying Figs. 1, 2b, and 3 were generated using an RNA-Binding Sensor for in vitro quantification and live-cell localization of small RNAs. Nucleic Acids Res 45, e130 (2017).

Shu, D., Khisamutdinov, E. F., Zhang, L. & Peixuan, G. Programmable folding of fusion RNA in vivo and in vitro driven by PRNA 3WJ motif of phi29 DNA packaging motor. Nucleic Acids Res 42, e10 (2014).

Ponchon, L. & Dardeil, F. Recombinant RNA technology: the tRNA scaffold. Nat. Methods 4, 571–576 (2007).

Svensen, N. & Jaﬀrey, S. R. Fluorescent RNA aptamers as a tool to study RNA-modifying enzymes. Cell Chem. Biol. 21, 415–425 (2016).

Alam, K. K. et al. A fluorescent split aptamer for visualizing RNA assembly. Viro. Ac Synth. Biol. 6, 1710–1721 (2017).

Trachman, R. J. et al. Structure and functional resolution of the Mango-III fluorogenic RNA aptamer. Nat. Chem. Biol. 15, 472–479 (2019).

Ana, M. M. H. & Baulcombe, D. C. Tobacco rattle virus 16-kilodallon protein encodes a suppressor of RNA silencing that allows transient viral entry in meristems. J. Virol. 82, 4064–4071 (2008).

DineshKumar, S. P. et al. Virus-induced gene silencing. Methods Mol. Biol. 718, 287–294 (2011).

Carlsson, A., Letarte, I., Chen, J. & Kashk, K. Visual screening of microspore-derived transgenic barley (Hordeum vulgare L) with green-fluorescent protein. Plant Cell Rep. 20, 331–337 (2001).

Yuviene, N. O. et al. An assay for express screening of potato transformants by GFP fluorescence. Moc. Univ. Biol. Sci. Bull. 73, 69–75 (2018).

Autauro, A., Westhof, E. & Rycckelynck, M. Spinach: a fluorogenic RNA aptamer optimized for in vitro applications. Nucleic Acids Res 44, 2491–2500 (2016).

Warnier, K. D. et al. A homodimer interface without base pairs in an RNA mimic of red fluorescent protein. Nat. Chem. Biol. 13, 1195–1201 (2017).

Wang, Z. et al. In situ spatial complementation of aptamer-mediated recognition enables live-cell imaging of native RNA transcripts in real time. Angew. Chem. Int. Ed. 130, 984–988 (2018).

Song, W., Strack, R. L., Svensen, N. & Jaﬀrey, S. R. Plug-and-play fluorophores extend the spectral properties of spinach. J. Am. Chem. Soc. 136, 1198–1201 (2014).

Filonov, G. S. & Jaﬀrey, S. R. RNA imaging with dimeric broccoli in live bacterial and mammalian cells. Curr. Protoc. Chem. Biol. 8, 1–28 (2018).

Farinaz, R. & Majid, M. B. High efficient prokaryotic expression and purification of bioactive human growth hormone using a cleavable self-aggregating tag. Gene Rep. 2018, 128–131 (2018).

Yoo, S. D., Cho, Y. H. & Jen, S. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat. Protoc. 2, 1565–1572 (2007).

Atul Jani, R., Nag, S. & Setty, S. R. G. Visualization of intracellular tyrosinase activity in vitro. Bio. Protoc. 6, 1–6 (2016).

Ali, Z. et al. Efﬁcient virus-mediated genome editing in plants using the CRISPR/Cas9 system. Mol. Plant 8, 1288–1291 (2015).

Clough, S. J. & Bent, A. F. Floral dip: a simpliﬁed method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Planta 16, 735–743 (1998).

**Author contributions**

Conceptualization, Y.Z. and C.F.; funding acquisition, Y.Z. and R.W.; investigation, J.B., C.F., W.W. and M.W.

**Competing interests**

The authors declare no competing interests.
