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Technical note

Improved dual luciferase reporter (DLR) assay to determine the protein stability

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ABSTRACT

We developed a binary vector co-expressing *firefly luciferase* (*FF*) and *Renilla luciferase* (*REN*) to detect protein stability in response to different stimuli, and verified the functionality of the vector. The StrigoQuant-like reporter expressing *FF* and *REN* in one transcript is a sensitive tool for detecting protein abundance in different genotypes. However, we found that significant differences in the relative FF/REN ratio of empty StrigoQuant vector in different genotypes. Therefore, to determine the actual protein abundance, the relative FF/REN ratio of the protein of interest should be normalized to that of the empty vector.

1. Introduction

The stability of many proteins is fine-tuned in cells, where some proteins function in a spaciotemporal manner and are regulated by various signals. After achieving the desired function, proteins are degraded by various proteases.

Strigolacone (SL) is an important phytohormone that regulates diverse developmental processes, such as shoot branching and host-parasite interactions [1,2]. In the SL signaling pathway, the key repressor D53 (its Arabidopsis homolog SMXL6) is ubiquitinated and degraded in the presence of GR24 (a synthetic SL analog). In the dominant rice *d53* mutant, the d53 protein is more stable than the wild-type (WT) D53 following GR24 treatment. Compared with the WT plant, the D53 protein is more abundant in SL-deficient and insensitive mutants [1]. Developing tools and techniques to analyze protein stability is essential in many functional studies.

Isobaric tag for relative and absolute quantitation (iTRAQ) has been used widely to study differentially expressed proteins (DEPs) in the WT and mutants, and in plants subjected to various stimuli [3,4]. To verify iTRAQ results in terms of protein levels, Western blotting is the most direct and reliable method. However, it takes time to develop a specific antibody. Some studies have used quantitative PCR (qPCR) to confirm the results of iTRAQ [3]. However, there is no consistent correlation between gene transcription and protein levels. The dual luciferase assay is also commonly used to monitor gene expression regulated by other proteins or stimuli [5]. However, the vectors expressing *firefly luciferase* (*FF)* and the internal control *Renilla luciferase* (*REN)* are usually transformed into plant materials separately [6], which may cause systematic bias due to difference in transformation efficiency. Here, we developed a binary vector that co-expressed the genes coding *FF* and *REN*, and were driven by the *CaMV 35S* promoters, respectively. We validated the efficiency of this vector system by studying D53 protein stability in response to GR24 in tobacco leaves.

The StrigoQuant biosensor is an efficient tool for detecting the protein level in response to SL [7]. In a single construct, the sensor module (Arabidopsis SMXL6 fused to FF) is connected to the internal control (REN) via the self-processing 2A peptide, and is transcribed as a single transcript driven by the *CaMV 35S* promoter after transformation into plant protoplasts. GR24 treatment of the WT protoplasts transformed with the StrigoQuant sensor showed a significant concentration-dependent decrease in the FF/REN ratio. However, we found that this method has clear drawback in that the FF/REN ratio differs significantly among different genotypes (such as the WT and *d3* mutant [8]) when transformed with the empty vector, suggesting that the protein translational efficiency differs among the various genotypes. Therefore, to determine the actual abundance of the protein of interest in the different experimental materials, it is crucial that the FF/REN ratio of the protein of interest should be normalized to that of the empty

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vector.

2. Materials and methods

2.1. Plasmid construction

To construct a binary vector expressing both *FF* and *REN* driven by two independent *CaMV 35S* promoters, a DNA fragment containing the *CaMV 35S promoter*, *REN* coding sequence and *NOS* terminator was amplified from *pGREENII 0800* empty vector [9] using primers RlucSalF and RlucHindR, and inserted into the *Sal*I and *Hin*dIII sites of *pCAMBIA1200* to produce *pCAMBIA1*200-*REN*. Likewise, a DNA fragment harboring the *CaMV 35S* promoter, *FF* coding sequence and *NOS* terminator was amplified from the *pGEAD-LUC* empty vector using primers LucEcoF and LucBamR, and inserted between the *Eco*RI and *Bam*HI sites of *pCAMBIA1*200-*REN* vector to make *pCAMBIA1*200-*FF--REN* using an In-Fusion® HD Cloning Kit (Clontech). Table S1 shows the primers used. The StrigoQuant-like reporter [10] was a generous gift from Dr. Guosheng Xiong.

2.2. Transient transformation in tobacco leaves

Agrobacterium tumefaciens harboring the *pCAMIA1200-D53/d53-FF-REN* vector was injected into tobacco leaves. After a 72-h incubation, the leaves were injected with 1 μM GR24 (Coolaber) and incubated for different times. Dual luciferase assays (Promega) were used to detect the relative FF/REN of D53 and d53 [7].

2.3. Rice protoplast transformation

Rice protoplasts were isolated and transformed as described previously [8]. After incubation for 16 h, the protoplasts were treated with 0.1 μM GR24 (Coolaber) and incubated for another 2 h.

3. Results

3.1. Designing a binary vector expressing both FF and REN

Many studies need to detect changes in protein abundance in response to various stimuli. In the traditional dual luciferase assay, with one vector harboring the coding sequence of the protein of interest translationally fused to FF and another containing the *REN* coding sequence, the vectors are usually transiently co-transformed into tobacco leaves or protoplasts. The REN activity is used as an internal control to eliminate any difference in transformation efficiency. However, the transformation efficiency may differ since the two plasmids are delivered as two events. The concentration, purity, and size of plasmids significantly affect the transformation efficiency, especially for protoplasts [11]. To avoid differences in the transformation efficiency of two independent plasmids, we constructed a binary vector containing the *FF* coding sequence driven by a *CaMV 35S* promoter, multiple clone sites, and the *REN* coding sequence driven by another *CaMV 35S* promoter (Fig. 1).

3.2. Detecting the D53 or d53 protein stability in response to GR24 in tobacco leaves

To test the functionality of the constructed vector, the SL signaling repressor D53, or its stabilized mutant form d53, was fused to the C terminus of FF in this vector. The construct was transformed into tobacco leaves and incubated for 3 days. The tobacco leaves were then injected with GR24 and incubated for 6 or 24 h. The FF/REN ratio of D53 was decreased after incubation with GR24 for 6 and 24 h. The D53 protein level was significantly reduced, from 58% at 6 to 38% at 24 h (Fig. 2A). By contrast, the stabilized d53 mutant protein level was reduced only slightly after incubation with GR24 for 10 h (Fig. 2B). These results were consistent with our results (Supplementary Fig. S1) and previous Western blotting findings obtained using anti-D53 antibody [1], suggesting that this vector reflects the actual response of D53 to GR24 treatment in a transient expression system.

3.3. Comparing D53 abundance between the WT and SL-insensitive mutant d3 using the StrigoQuant-like reporter transient expression assay

The StrigoQuant biosensor is a useful quantitative tool for specifically and sensitively detecting the degradation of SMXL6 in response to SL in *Arabidopsis* [7]. SMXL6 was replaced by D53 to generate the StrigoQuant-like reporter [10]. However, we found that the StrigoQuant-like reporter transient expression assay in rice does not reflect the actual difference in the abundance of D53 between the WT and SL-insensitive *d3* mutant. The relative FF/REN ratio (representing D53 abundance) did not show more D53 accumulation in the *d3* mutant than in the WT (Fig. 3A), which is not consistent with the Western blotting results obtained using anti-D53 antibody [1]. We assumed that the transcription or translation efficiency of FF and REN from the same plasmids may be different. To test this, the empty vector with the *FF* and *REN* coding sequence driven by the same *35S* promoter was transformed into WT and *d3* mutant protoplasts. The qPCR showed no significant difference in *FF* and *REN* transcript levels between the WT and *d3* mutant (Supplementary Fig. S2), while the relative FF/REN ratio of the empty vector in the *d3* mutant was obviously lower than that in the WT (Fig. 3B), suggesting that the translation efficiency is lower in the *d3* mutant. To eliminate the difference in translation efficiency in different genotypes, i.e., the WT and *d3* mutant, the relative FF/REN of D53 was normalized to that of empty vector. The normalized FF/REN ratio of D53 in the *d3* mutant was significantly higher than that in the WT (Fig. 3C). These results suggest that the relative FF/REN ratio of the protein of interest must be normalized to the relative of FF/REN ratio of the empty vector to show the actual abundance of a protein in different genotypes.

4. Discussion

Many studies use iTRAQ to identify DEPs in the WT and mutants, or to compare DEPs in materials with or without stimuli. In the traditional method used to verify the results, specific antibodies are developed to determine the protein abundance in different samples. However, antibody development is time-consuming and it is impossible develop specific antibodies for some proteins owing to their primary sequence or secondary structure. Therefore, a reliable, sensitive way to detect protein abundance independent of antibodies is needed. The Improved StrigoQuant-like reporter and binary vector co-expressing *FF* and *REN*

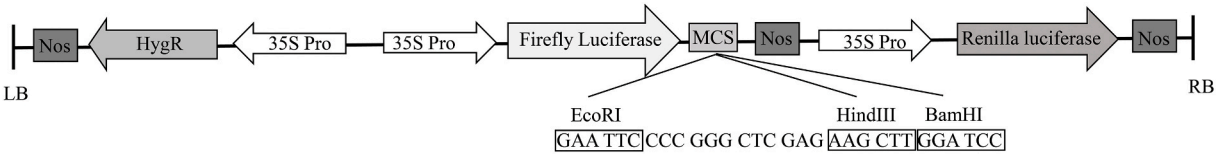

Fig. 1. The binary vector co-expressing *FF* and *REN*. 35S Pro, *CaMV 35S promoter*; MCS, multiple cloning sites. LB, T-DNA left border; RB, T-DNA right border.

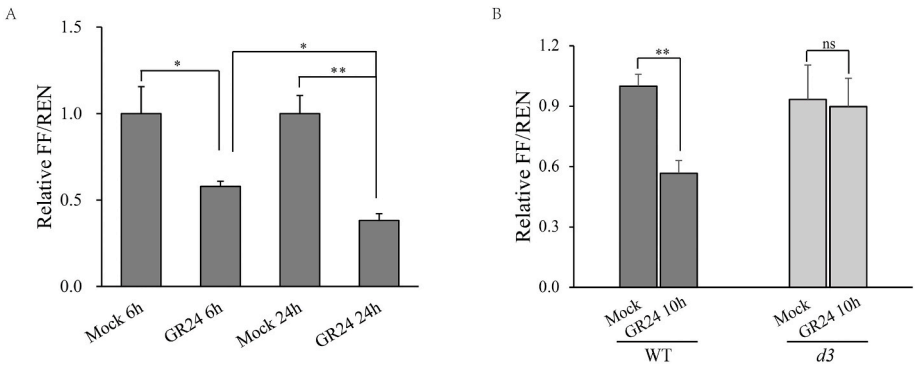

Fig. 2. The stability of D53 and d53 in response to GR24 in tobacco leaves using the binary vector. Stability of D53 (A), and the D53 its mutant form, d53 (B), in response to GR24. Data are the mean ± standard error (n = 3). *$p \leq 0.05$; **$p \leq 0.01$ with one-way ANOVA; ns, not significant.

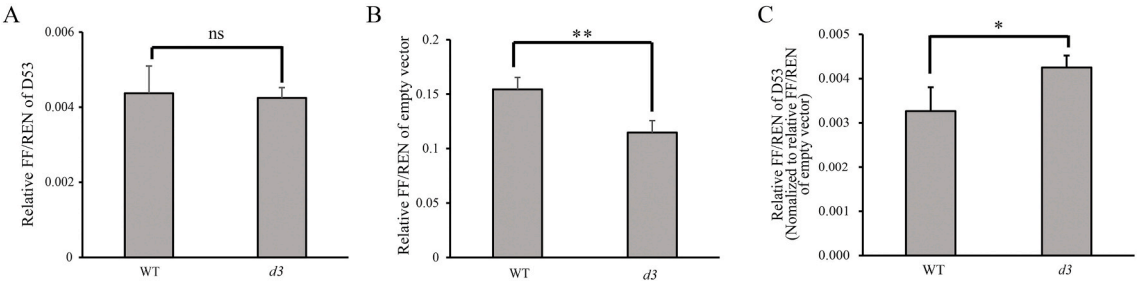

Fig. 3. The abundance of D53 in the WT and *d3* mutant in StrigoQuant-like reporter transient assay. The relative FF/REN ratio of D53 (A), empty vector (B), and D53 normalized to that of the empty vector (C) in the WT and *d3* mutant. Data are the mean ± standard error (n = 6). *$p \leq 0.05$; **$p \leq 0.01$ with one-way ANOVA; ns, not significant.

developed here may be powerful tools for detecting protein abundance in different genotypes, or the stability of proteins of interest responding to various stimuli.

The StrigoQuant biosensor can be used to compare protein stability in samples treated with or without stimuli. For example, the StrigoQuant biosensor has been used to compare the stability of Arabidopsis SMXL6 between WT and SL-insensitive mutants in response to SL treatment [7]. In that case, the relative FF/REN ratio of SMXL6 in protoplasts treated by SLs was normalized to that of mock treatment of the same genotype, which eliminated the difference in translational efficiency between the WT and mutants. However, when comparing the steady-state abundance of the protein of interest in different genotypes (such as the WT and mutant), the relative FF/REN ratio of that protein should be normalized to that of the empty vector to exclude differences in translational efficiency.

CRediT authorship contribution statement

Yinglu Sun: Formal analysis, Performing the experiment, data analysis. **Jinfeng Zhao:** Writing - original draft, Designing the experiment. **Suyash B. Patil:** Writing - review & editing. **Jingjing Fang:** Comments. **Jun Liu:** Writing - review & editing. **Xueyong Li:** Writing - review & editing.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2020.114021.

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