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Antiserum Preparation and Specific Detection of Banana RING-E3 Ubiquitin-Protein Ligase

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Abstract

According to the data of banana transcriptome sequencing, an E3 ubiquitin-protein ligase gene was cloned by RT-PCR method using the cDNA sample of banana leaves. The complete ORF of *E3 ubiquitin-protein ligase* is 681 bp long and its encoded protein showed 100% sequence identity to homologue RING-H2 finger protein (XP_009407047.1) of Musa_acuminata. Bioinformatic analysis indicated that E3 ubiquitin-protein ligase contains the Ring finger domain in C terminus and eight cross-brace motifs are found in the domain. The target gene was digested by EcoR V and EcoR I, and was inserted into prokaryotic expression vector pET-32a of the same digestions to obtain the plasmid pET32a-E3 ubiquitin-protein ligase. The recombinant plasmid was introduced into *Escherichia coli* strain BL21 (DE3), and induced at 25˚C with 0.4 mmol/L IPTG for 6 hours. The soluble fusion protein was expressed and high purity fusion protein was obtained by Ni^{2+}-NTA agarose affinity chromatography purification. The fusion protein was injected into mice 3 times to prepare the antiserum. Western blot analysis showed a specific protein band was detected in total protein sample of banana leaves, but not for the samples of wild-type *Nicotiana benthamiana* (N.B.) and wild-type *Arabidopsis thaliana* (A.T.), implying the antiserum was specific to banana E3 ubiquitin-protein ligase.

Keywords

Banana, E3 Ubiquitin-Protein Ligase, Antiserum Preparation, Polyclonal Antibody

1. Introduction

Ubiquitination is an important biological process in plants, such as physiological

activities of regulating plant cell cycle, plant flowering and plant disease resistance etc. [1]. Among them, the mechanism of ubiquitination responses to plant disease resistance is a hotspot in the current research. In recent years, many studies have shown that E3 ubiquitin-protein ligases (or E3 ubiquitin ligases) are involved in the regulation of plant disease resistance, and the ubiquitinated target proteins can mediate the transmission of disease resistance signaling. Meanwhile, some effector-proteins from pathogens are also present similar function of E3 ubiquitin-protein ligase, which can interfere with plant disease resistance [2].

Ubiquitin (Ub) is a highly conserved small molecule protein (containing 76 amino acids) that widely exists in living organisms, and is always involved in various biological processes in cells. Under the catalysis of a series of enzymes, ubiquitin covalently binds to the target protein for ubiquitination modification, which can alter the subcellular localization of the target protein, affect the protein activity, or promote the degradation of the target protein by the ubiquitin or 26S proteasome pathway [3]. The ubiquitination process is taken by the cascade reaction of E1 ubiquitin-activating enzymes (E1s), E2 ubiquitin-conjugating enzymes (E2s), and E3 ubiquitin ligases (E3s), respectively. Provided the energy from ATP, free ubiquitin molecules form a high-energy thioester bond through E1s, and then activate ubiquitin and transfer it to E2s. Subsequently, the ubiquitin molecule further transfers to target proteins for labeling through E3s. E3 ubiquitin ligase can specifically recognize one or a certain type of target proteins, which plays a decisive role in the ubiquitin pathway [4].

E3 ubiquitin-protein ligases are divided into four types, RING, HECT, U-box and CRLs, according to their characteristic of domains and the mechanism of ubiquitin delivery to substrate proteins. Among them, HECT-type, RING-type and U-box-type E3 ubiquitin ligases are single-subunit E3 ubiquitin ligases, while CRLs-type E3 ubiquitin ligases are multi-subunit E3 ubiquitin ligases [5]. The RING domain is a Cys-rich region composed of 40 - 60 amino acids, and contains eight spatially conserved Cys and His residues as metal ligands to chelate two zinc ions, and then promote ubiquitin transferring to the target protein [6].

2. Materials and Methods

2.1. Materials

The cDNA was reverse transcribed from total RNA of banana leaves in Haikou, Hainan and stored at −80˚C. Clone strains *E. coli* DH5α were purchased from Beijing TransGen Biotech Co., Ltd.; expression fungus *E. coli* BL21 (DE3), expression vectors pET-32a (+), wild-type *Nicotiana benthamiana* (N.B.) and wild-type *Arabidopsis thaliana* (A.T.) were kept in the Laboratory of Molecular Virology, Institute of Tropical Bioscience and Biotechnology (ITBB), Chinese Academy of Tropical Agricultural Sciences (CATAS). Ampicillin, rTaq DNA polymerase, Isopropyl *β*-D-Thiogalactoside (IPTG), dATP, *EcoR* V and *EcoR*I

were purchased from Dalian TaKaRa Co., Ltd.; DNA markers and protein marker were purchased from TransGen Biotech (Beijing) Co., Ltd.; chromogenic substrate kits, acrylamide, N,N'-methylene bisacrylamide and Tris (hydroxymethyl) aminomethane were purchased from Solarbio; sodium dodecyl sulfate (SDS), glycine and coomassie brilliant blue R-250 were purchased from Amresco; PCR products collection kits were purchased from BioDev-Tech; Goat Anti-mouse IgG-AP was purchased from Wuhan Boster Biological Technology Co., Ltd.; other chemical reagents (Analytical purity) was made in China.

2.2. Primers Design

According to the banana transcriptomic sequencing data (GenBank accession number is SRP129855) [7], specific pair-primers of E3 upl-F and E3 upl-R targeting E3 ubiquitin-protein ligase gene were designed for RT-PCR verification (Table 1). Based on the nucleotide sequences of E3 ubiquitin-protein ligase gene and pET32a vector, another specific pair-primers of E3 upl-F2 (containing EcoRV restriction enzyme site) and E3 upl-R2 (containing EcoRI restriction enzyme site) were designed for plasmid construction (Table 1). All primers were synthesized by Sangon Biotech (Shanghai) Technical Services Co. Ltd.

2.3. Gene Cloning and Prokaryotic Expression Plasmid Construction

The specific pair-primers of E3 upl-F and E3 upl-R were used to perform PCR amplification of the banana cDNA sample, and the PCR program was as follows: pre-denaturing at 98˚C for 1 min; 35 cycles of denaturing at 98˚C for 30 s, annealing at 57˚C for 15 s and extending at 72˚C for 1 min; and a final extension at 72˚C for 10 min. After gel purification (Bio-Rad, Beijing, China) and construction into pMD18-T vector (TaKaRa, Dalian, China), the inserted sequence was determined by bidirectional sequencing with three independently positive *Escherichia coli* Trans5a strains (TransGen, Beijing, China).

Meanwhile, the specific DNA fragment containing two restriction enzyme sites was amplified by PCR using the primers E3 upl-F2 and E3 upl-R2. The DNA fragment digested with *EcoR* V and *EcoR* I was cloned into a prokaryotic vector pET-32a (+) digested with the same enzymes to obtain the plasmid pET32a-E3 ubiquitin-protein ligase (designated as pET32a-E3 upl). The constructed plasmid pET32a-E3 upl was confirmed by restriction enzyme digestion and DNA sequencing.

Table 1. The list primers in this study.

Primer name	Primer sequence (5'-3')	Usage
E3 upl-F	CGACAAGAAGGCGATGGCGAC	PCR
E3 upl-R	TCTTCATCATTAGCAATATGCATTCC	
E3 upl-F2	**GATATC**GACAAGAAGGCGATGGCGAC (*EcoR* V)	pET32a
E3 upl-R2	**GAATTC**TCAGGGATCAGCAGAAGCAGG (*EcoR* I)	

2.4. Expression and Purification of Fusion Protein

The constructed plasmid pET32a-E3 upl was transformed into *Escherichia coli* BL21 competent cells. A positive single clone was inoculated into 250 ml LB liquid medium (containing 50 mg/L Ampicillin). The culture was incubated at 37˚C for 200 rpm until the OD_{600} reached 0.6 - 0.8. Subsequently, IPTG was added into a final concentration of 0.4 mmol/L, 25˚C, 200 rpm incubation for 6 hrs. 6000 rpm, centrifuge for 10 min at 4˚C, then discard the supernatant. The precipitate was dissolved in 25 ml 10 mM Tris-HCl (pH 8.0) solution, and was treated by ultrasonic wave (500W, 30 times, 10 seconds each time, 15 seconds interval). 10,000 rpm, centrifuge for 5 min at 4˚C, the supernatant was passed through a 0.45 μm filter to remove particulate matter and the soluble fused protein was obtained. Purification of the E3 ubiquitin-protein ligase fusion protein was conducted by the method of Ni^{2+}-NTA (His-Tag) agarose affinity chromatography [8].

2.5. Antiserum Preparation and Specific Detection

Antibodies were generated against E3 ubiquitin-protein ligase by mixing purified fusion protein with an equal volume of Freund's adjuvant (Sigma) and the mixture used to immunize mice by subcutaneous injections at 7-day intervals. Mice were sacrificed after being anesthetized on the fifth day after the fifth immunization and the Anti-E3 ubiquitin-protein ligase serum was collected.

Total proteins were extracted from banana leaves, wild-type N.B. leaves and wild-type (A.T.) leaves to determine the specific of the antiserum by Western blot analysis. Equivalent amounts (10 μL) of protein extracts were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). Protein was detected using anti-E3 ubiquitin-protein ligase serum as the primary antibody (1:500 dilution), followed by alkaline phosphatase goat anti-mouse IgG (H + L) (Wuhan Boster Biological Technology Co., 1:2500 dilution) as the secondary antibody.

3. Results and Analysis

3.1. *E3 Ubiquitin-Protein Ligase* Gene Cloning and Its Protein Sequencing Analysis

The complete ORF of *E3 ubiquitin-protein ligase* is 681 bp long and encodes a protein with a predicted molecular mass of about 25 kDa (**Figure 1(a)**). NCBI blastp (https://www.ncbi.nlm.nih.gov) shows that E3 ubiquitin-protein ligase (Musa_acuminata-HK isolate) is 100% sequence identity to homologue RING-H2 finger protein (XP_009407047.1) in Musa_acuminata. Bioinformatic analysis indicated that E3 ubiquitin-protein ligase contains the Ring finger domain at 174 - 217 aa. In addition, eight cross-brace motifs (amino acid C or H) are found in the domain (**Figure 2**).

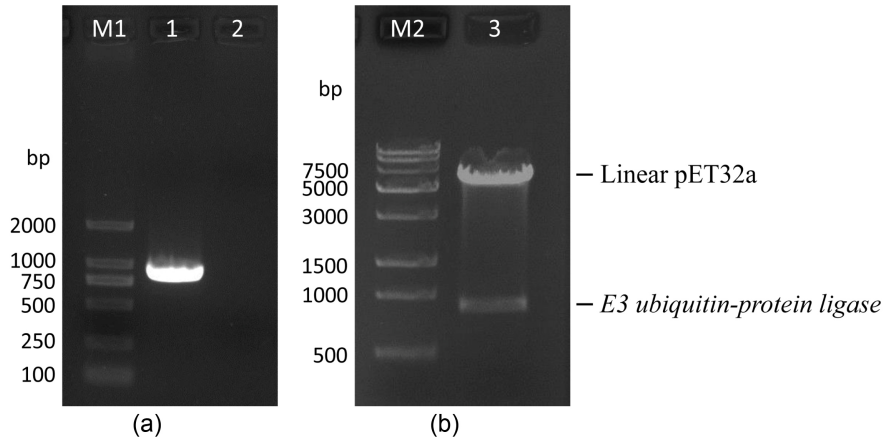

Figure 1. RT-PCR and Enzyme digestion of recombinant plasmid. M1: Trans2K DNA Marker; 1: RT-PCR fragment of *E3 ubiquitin-protein ligase*; 2: Negative control; M2: Trans15K DNA Marker; 3: pET32a-E3 upl plasmid digested by *EcoR* V and *EcoR* I.

```
Musa_acuminata-HK:MATMETTPLHGQADWVLRAYQTHGRPAWAPVVIKLGYTVMHILEGQVVEEPPRSYHIFLF: 60
XP_009407047.1    :MATMETTPLHGQADWVLRAYQTHGRPAWAPVVIKLGYTVMHILEGQVVEEPPRSYHIFLF: 60

Musa_acuminata-HK:TLGHFLHQASRRQAISTVLLIAGVYGYDICFAGRLERQLVAFCNYPVETALNSGNGFDMI:120
XP_009407047.1    :TLGHFLHQASRRQAISTVLLIAGVYGYDICFAGRLERQLVAFCNYPVETALNSGNGFDMI:120

Musa_acuminata-HK:VDVLLAHFPIDEEPSSDVEGVGENGNFGGIPASTDAVKELAVVKYERGGDVREESCIICF:180
XP_009407047.1    :VDVLLAHFPIDEEPSSDVEGVGENGNFGGIPASTDAVKELAVVKYERGGDVREESCIICF:180
                                Ring finger domain
Musa_acuminata-HK:EEFDEGVEVTRMPCKHAFHGGCLTRWLERSHACPLCRHAIPASADP:226
XP_009407047.1    :EEFDEGVEVTRMPCKHAFHGGCLTRWLERSHACPLCRHAIPASADP:226
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Figure 2. Multiple sequences alignment of E3 ubiquitin-protein ligase (Musa_acuminata-HK isolate) and its homologue RING-H2 finger protein (XP_009407047.1). Ring finger domain, 174 - 217 aa, is shown under the black line. Eight cross-brace motifs are shown with black shades.

3.2. Tertiary Structure Analysis of E3 Ubiquitin-Protein Ligase

The protein structure predicted by alphafold2 showed that tertiary structure of E3 ubiquitin-protein ligase consists of two relatively independent domains, the N-terminal domain and the C-terminal domain (Ring finger domain), which are connected by a linker. The N-terminal domain consists of two α-helixs and four β-sheets, while the Ring finger domain consists of two α-helixs. Further analysis showed that the eight cross-brace conserved amino acid are mainly distributed on both sides of one α-helix in the Ring finger domain, with 4 motifs (three Cs and one H residues) on each side (Figure 3).

3.3. Recombinant Plasmid Construction, Protein Expression and Purification

The recombinant plasmid pET32a-E3 upl was digested by *EcoR* V and *EcoR* I

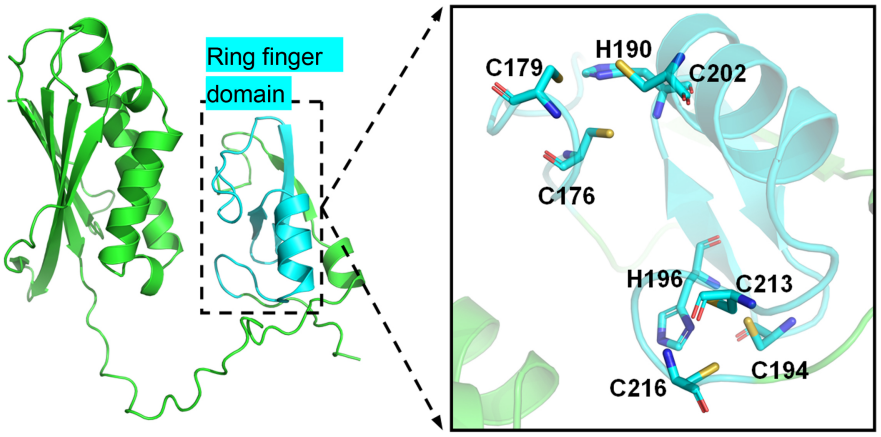

Figure 3. Predicted tertiary structure of E3 ubiquitin-protein ligase. Eight cross-brace motifs (C176, C179, H190, C194, H196, C202, C213, C216) are labeled.

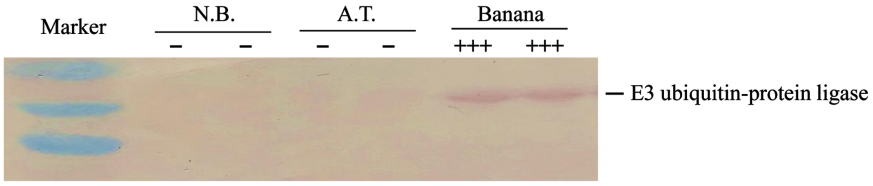

Figure 4. Specific detection of antiserum by Western blot analysis. Marker: ProteinRuler II; N.B.: total protein of wild-type N.B. leaves; A.T.: total protein of wild-type (A.T.) leaves; Banana: total protein of banana leaves.

and the result showed that the predicted size of *E3 ubiquitin-protein ligase* DNA fragment was obtained (**Figure 2(b)**). The inserted DNA sequences were further verified by DNA sequencing.

The fused protein E3 ubiquitin-protein ligase was induced by adding the IPTG and soluble fused protein was obtained by ultrasonic wave treatment. The high purity fusion protein was obtained by Ni^{2+}-NTA agarose affinity chromatography purification.

3.4. Specific Detection of the Antiserum

To detect the specific of the E3 ubiquitin-protein ligase polyclonal antibody, total proteins from different plant species leaves were analyzed by Western blot. The result showed that a specific immunoreactive band of approximately 25 kDa was detected in total protein of banana leaves, but not for the protein samples of N.B. or A.T. plants (**Figure 4**), implying the polyclonal antibody was specific to banana E3 ubiquitin-protein ligase.

4. Discussion

The typical feature of RING E3 ubiquitin ligases contains the ring finger domain, which is an important factor for the function of ubiquitin pathway. The common amino acid sequences of RING domain are $Cys\text{-}X_2\text{-}Cys\text{-}X_{(9\text{-}39)}\text{-}Cys\text{-}X_{(1\text{-}3)}\text{-}His\text{-}X_{(2\text{-}3)}\text{-}Cys\text{-}X_2\text{-}Cys\text{-}X_{(4\text{-}48)}\text{-}Cys\text{-}X_2\text{-}Cys$, where X

can be any amino acid [9]. Depending on the fifth conserved amino acid, RING fingers are mainly classified as C3H2C3 (RING-H2)-type or C3HC4-type (RING-CH) [10]. In this study, banana E3 ubiquitin-protein ligase contained the C3H2C3 (RING-H2)-type in the Ring finger domain. Studies have shown that RING E3 ubiquitin-protein ligases are involved in plant immune response. For example, *ATL* (*Arabidopsis toxicos para levadura*) genes encoding RING-type E3 ubiquitin-protein ligases play a role in plant immune responses [11]. *ATL9* gene in *Arabidopsis* was found to regulate plant resistance to *Golovinomyces cichoracearum*, a biotrophic fungal pathogen [12]. The ATL-related gene *RFP1* (RING-finger protein 1) in tomato was also resistant to *Phytophthora infestans*, a hemibiotrophic pathogen [13].

Studies have shown that many E3 ubiquitin-protein ligases are involved in plant resistance to viral diseases. For example, OsRFPH2-10, RING-H2 finger E3 ubiquitin-protein ligase, which plays a key role of ubiquitination system in rice, is involved in rice antiviral defense in the early stages of *Rice dwarf virus* infection [14]. Meanwhile, viral pathogens can interfere with plant resistance by acting on the ubiquitination system. The C2 protein encoded by *Beet severe curly top virus* (BSCTV) can interact with the beet SAMDC1 protein, thereby inhibiting the degradation of SAMDC1 by the ubiquitin/26S proteasome, resulting in reduced resistance to the virus that suppressing host gene silencing of methylation-regulated genes [15].

In this study, banana E3 ubiquitin-protein ligase contains the Ring finger domain at C terminus, and eight cross-brace motifs are mainly distributed on both sides of the α-helix, with 4 motifs (three Cs and one H residues) on each side. The purified fusion protein was used to prepare the specific polyclonal antibody, which specifically recognized banana E3 ubiquitin-protein ligase, but not for the E3 ubiquitin-protein ligase of N.B. or A.T. plants, implying the polyclonal antibody was specific to banana E3 ubiquitin-protein ligase. The study is a basis for the protein-protein interaction between BBTV encoded proteins and banana E3 ubiquitin-protein ligase, and the mechanism of banana E3 ubiquitin-protein ligase involves in anti-*Banana bunchy top virus*.

5. Conclusion

In conclusion, our results showed that a specific polyclonal antibody of banana E3 ubiquitin-protein ligase, which contains a Ring finger domain, was prepared. Furthermore, the polyclonal antibody was specifically recognizing the banana E3 ubiquitin-protein ligase, which is a basis for the study of E3 ubiquitin-protein ligase antiviral disease caused by *Banana bunchy top virus*.

Acknowledgements

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Ethics Approval and Consent to Participate

Preparation of antibody in mice has approval from the Institutional Animal Care and Use Committee (IACUC) of the Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences (ITBB[2020]63).

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Zhou, B. and Zeng, L. (2017) Conventional and Unconventional Ubiquitination in Plant Immunity. *Molecular Plant Pathology*, **18**, 1313-1330. https://doi.org/10.1111/mpp.12521

[2] Popovic, D., Vucic, D. and Dikic, I. (2014) Ubiquitination in Disease Pathogenesis and Treatment. *Nature Medicine*, **20**, 1242-1253. https://doi.org/10.1038/nm.3739

[3] Tian, A.M., Yu, H. and Cao, J.S. (2020) Classification and Function of E3 Ubiquitin Ligase in Plants. *Chinese Journal of Cell Biology*, **42**, 907-915.

[4] Nakamura, N. (2018) Ubiquitin System. *International Journal of Molecular Sciences*, **19**, Article No. 1080. https://doi.org/10.3390/ijms19041080

[5] Zheng, N. and Shabek, N. (2017) Ubiquitin Ligases: Structure, Function, and Regulation. *Annual Review of Biochemistry*, **86**, 129-157. https://doi.org/10.1146/annurev-biochem-060815-014922

[6] Deshaies, R.J. and Joazeiro, C.A. (2009) RING Domain E3 Ubiquitin Ligases. *Annual Review of Biochemistry*, **78**, 399-434. https://doi.org/10.1146/annurev.biochem.78.101807.093809

[7] Yu, N.T., Xie, H.M., Zhang, Y.L., Wang, J.H., Xiong, Z. and Liu, Z.X. (2019) Independent Modulation of Individual Genomic Component Transcription and a CIS-Acting Element Related to High Transcriptional Activity in a Multipartite DNA Virus. *BMC Genomics*, **20**, Article No. 573. https://doi.org/10.1186/s12864-019-5901-0

[8] Yu, N.T., Feng, T.C., Wang, J.H., Zhang, Y.L. and Liu, Z.X. (2011) Cloning and Prokaryotic Expression of Rep Gene of the Haikou Isolate of Banana Bunchy Top Virus and Preparation of Polyclonal Antiserum against Rep. *Plant Protection*, **37**, 38-43.

[9] Cockram, P.E., Kist, M., Prakash, S., Chen, S.H., Wertz, I.E. and Vucic, D. (2021) Ubiquitination in the Regulation of Inflammatory Cell Death and Cancer. *Cell Death & Differentiation*, **28**, 591-605. https://doi.org/10.1038/s41418-020-00708-5

[10] Lim, S.D., Hwang, J.G., Jung, C.G., Hwang, S.G., Moon, J.C. and Jang, C.S. (2013) Comprehensive Analysis of the Rice RING E3 Ligase Family Reveals Their Functional Diversity in Response to Abiotic Stress. *DNA Research*, **20**, 299-314. https://doi.org/10.1093/dnares/dst011

[11] Zeng, L.R., Vega-Sánchez, M.E., Zhu, T. and Wang, G.L. (2006) Ubiquitination-Mediated Protein Degradation and Modification: An Emerging Theme in Plant-Microbe Interactions. *Cell Research*, **16**, 413-426. https://doi.org/10.1038/sj.cr.7310053

[12] Berrocal-Lobo, M., Stone, S., Yang, X., Antico, J., Callis, J., Ramonell, K.M. and Somerville, S. (2010) ATL9, a RING Zinc Finger Protein with E3 Ubiquitin Ligase Activity Implicated in Chitin- and NADPH Oxidase-Mediated Defense Responses. *PLoS ONE*, **5**, Article ID: e14426. https://doi.org/10.1371/journal.pone.0014426

[13] Ni, X.M., Tian, Z.D., Liu, J., Song, B.T. and Xie, C.H. (2010) Cloning and Molecular Characterization of the Potato RING Finger Protein Gene *StRFP1* and Its Function in Potato Broad-Spectrum Resistance against *Phytophthora infestans*. *Journal of Plant Physiology*, **167**, 488-496. https://doi.org/10.1016/j.jplph.2009.10.019

[14] Liu, L.F., Jin, L., Huang, X.H., Geng, Y.T., Li, F., Qin, Q.Q., Wang, R., Ji, S.Y., Zhao, S.S., Xie, Q., Wei, C.H., Xie, C., Ding, B. and Li, Y. (2014) OsRFPH2-10, a Ring-H2 Finger E3 Ubiquitin Ligase, Is Involved in Rice Antiviral Defense in the Early Stages of *Rice dwarf virus* Infection. *Molecular Plant*, **7**, 1057-1060. https://doi.org/10.1093/mp/ssu007

[15] Zhang, Z., Chen, H., Huang, X., Xia, R., Zhao, Q., Lai, J., Teng, K., Li, Y., Liang, L., Du, Q., Zhou, X., Guo, H. and Xie, Q. (2011) BSCTV C2 Attenuates the Degradation of SAMDC1 to Suppress DNA Methylation-Mediated Gene Silencing in *Arabidopsis*. *The Plant Cell*, **23**, 273-288. https://doi.org/10.1105/tpc.110.081695