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**Note**

# The longer version of *Arabidopsis thaliana heat shock protein 18.2* gene terminator contributes to higher expression of stably integrated transgenes in cultured tobacco cells

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**Abstract** In order to achieve higher expression of a transgene, it is important to optimize not only the promoter, 5′-untranslated region (UTR), and localization signal, but also the transcriptional terminator. We previously reported that the terminator derived from the *Arabidopsis thaliana heat shock protein 18.2* gene (HSPT) increases gene expression in various plant species. In this study, with the goal of further increasing the expression level of transgenes, we evaluated a longer version of HSPT, corresponding to the 878 bp downstream of the stop codon, in cultured tobacco cells. The longer version of HSPT increased the expression levels of various genes including *Renilla reniformis* luciferase, *Photinus pyralis* luciferase, horseradish (*Armoracia rusticana*) peroxidase, and the non-toxic B subunit of Stx2e, a candidate vaccine protein for pig edema disease. This effect was not observed in a transient expression system. This element represents a useful tool for expression of transgenes integrated into the nuclear genome in plant cells.

**Key words:** *Arabidopsis thaliana heat shock protein 18.2* gene, horseradish peroxidase, luciferase, Stx2eB, transcriptional terminator.

Transgenic plants with improved agronomic traits are being put into commercial use, and plant-made vaccine antigens and biopharmaceuticals have also been developed. In May 2012, the United States Food and Drug Administration (FDA) approved the use of plant cell-based recombinant taliglucerase alfa for treatment of type 1 Gaucher disease; this was the first example of practical application of plant-made pharmaceuticals (PMPs) for human use (Maxmen 2012). In order to increase the accumulated levels of a recombinant protein, it is often necessary to optimize the expression cassette. At the translational level, the 5′-untranslated region (5′-UTR) plays an important role, because the nucleotides immediately upstream of the initiating AUG (−3 to −1) exert a considerable influence on translation initiation (Sugio et al. 2010), and some 5′-UTRs function as translational enhancers in plant cells (Matsui et al. 2012; Satoh et al. 2004; Sugio et al. 2008). At the post-translational level, targeting the protein into a vesicular transport pathway often leads to increased accumulation (Yoshida et al. 2004).

At the transcriptional level, both the promoter and the downstream region of the target gene, including the transcriptional terminator (polyadenylation signal), should be optimized. In a previous study, when a transgene without a terminator or with a poor terminator was introduced into a plant genome, aberrant transcripts without polyA tails accumulated, leading to induction of RNA-DEPENDENT RNA POLYMERASE6 (RDR6)-dependent RNA silencing (Luo and Chen 2007). Those authors also reported that silencing was relieved, and the expression level of the gene concomitantly increased, by a double terminator that promoted accurate polyadenylation of mRNA. We previously reported that the terminator derived from the *Arabidopsis thaliana heat shock protein 18.2* gene (*HSP*, At5g59720) (HSPT) increases gene expression; the HSPT increases mRNA levels of both transiently and stably expressed transgenes approximately 2-fold more than widely-used NOS (*nopaline synthase*) terminator (Nagaya et al. 2010). The HSPT is functional in a variety of plant cells including rice, *A. thaliana*, tomato, and lettuce (Hirai et al. 2011; Matsui et al. 2011; Nagaya et al. 2010). In this study, with the goal of further increasing the expression level of transgenes, we evaluated a longer version of HSPT.

We compared the expression levels obtained using the

Abbreviations: HRP, horseradish peroxidase; HSPT, transcriptional terminator derived from *A. thaliana heat shock protein 18.2* gene; LUC, luciferase; MAR, matrix attachment region; UTR, untranslated region.

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previously reported HSPT (HSPT250), a 250-bp of DNA element that includes the entire 3′-UTR and additional downstream sequence, to the levels obtained with a longer version of HSPT (HSPT878) corresponding to the 878 bp downstream of the stop codon. As a reporter, we initially used *Renilla reniformis* luciferase (*Rluc*). The expression cassettes were constructed using the binary vector pRI909 (Takara Bio Inc., Shiga, Japan) (Figure 1); detailed methods for the construction are described in Supplemental Figure S1. Cultured tobacco cells (*Nicotiana tabacum* L. cv. BY2) were transformed using *Agrobacterium tumefaciens* EHA105 harboring each construct. Five and four independent infections were performed for HSPT250 and HSPT878, respectively. From each infection, at least eleven kanamycin-resistant clones were randomly picked for measurement of Rluc activity (Figure 2A). For each plasmid, average Rluc activities were calculated for the clones from each infection, and then the average Rluc activity was calculated for all infection events (Figure 2B). Clones obtained from the HSPT878 infections tended to exhibit higher expression levels: the average activity for HSPT878 was almost double of HSPT250, and the difference was significant ($p=0.00046$). Next, we confirmed the effect of HSPT878 using firefly (*Photinus pyralis*) luciferase (Fluc) as a reporter. In this case, a translational enhancer derived from *A. thaliana alcohol dehydrogenase* (*AtADH*) was fused to Fluc. AtADH-Fluc-HSPT250 and AtADH-Fluc-HSPT878 were constructed, and stable transgenic BY2 cells were generated using these constructs. The average Fluc activity for AtADH-Fluc-HSPT878 was calculated in the same way as in the Rluc experiments. Once again, clones obtained from HSPT878 infections tended to exhibit higher levels of expression (Figure 2C), on average 1.3-fold higher than the Fluc activity obtained with AtADH-Fluc-HSPT250 (Figure 2D); however, the difference was of borderline statistical significance ($p=0.0504$). Possible explanations for the smaller effect than that obtained using Rluc reporter include that Fluc is very unstable in plant cells and elevated transcription did not simply lead to increased accumulation of the protein.

We next sought to determine whether the increased expression obtained with HSPT878 results from events that occur at the chromatin level. To this end we performed transient expression analysis, in which naked DNA without chromosomal proteins serves as a template for transcription. Protoplasts were prepared form BY2 cells as described previously (Matsui et al. 2011), and the Rluc-HSPT250 or the Rluc-HSPT878 was co-transfected with the expression plasmid for Fluc (AtADH-Fluc-HSPT250) for normalization of transfection

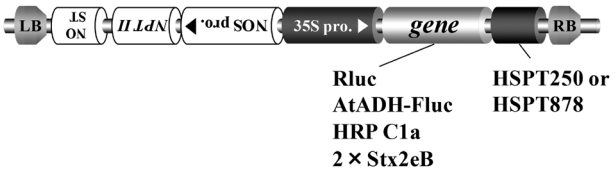


Figure 1. Schematic representation of the expression plasmid used in this study. 35S pro., Cauliflower mosaic virus 35S RNA promoter; NOST, transcription terminator from *A. tumefaciens nopaline synthase* gene; NOS pro., *A. tumefaciens nopaline synthase* gene promoter; NPTII, *neomycin phosphotransferase* gene for kanamycin resistance, derived from *E. coli*; RB, right border; LB, left border.

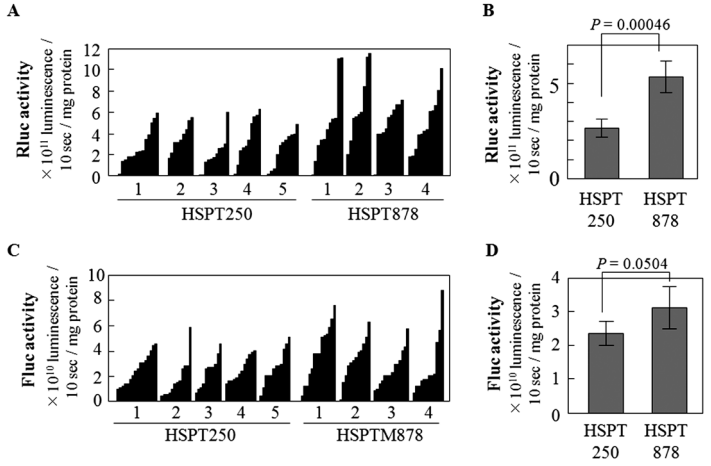


Figure 2. Expression of the Rluc and Fluc genes in transgenic BY2 cells. A. *Agrobacterium* infections were performed five and four times for Rluc-HSPT250 and Rluc-HSPT878, respectively. At least eleven kanamycin-resistant clones were picked for each infection and analyzed. Each bar represents data from one clone. B. For each plasmid, average Rluc activities were calculated for the clones from each infection, and then the average Rluc activity was calculated for all infection events. Standard deviations between infection events are shown. C. *Agrobacterium* infections were performed five and four times for AtADH-Fluc-HSPT250 and AtADH-Fluc-HSPT878, respectively. At least eleven kanamycin-resistant clones were picked for each infection and analyzed. Each bar represents data from one clone. D. For each plasmid, average Fluc activities were calculated for the clones from each infection, and then the average Fluc activity was calculated for all infection events. Standard deviations between infection events are shown.

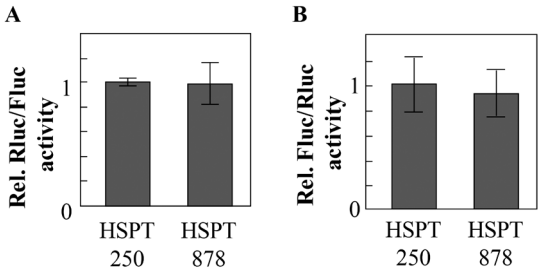


Figure 3. Transient expression of the Rluc and the Fluc genes in protoplasts derived from BY2 cells. A. Rluc-HSPT250 or Rluc-HSPT878 was co-transfected with the expression plasmid for Fluc (AtADH-Fluc-HSPT250) for normalization of transfection efficiency. The Rluc/Fluc activity was calculated for each transfection, and average Rluc/Fluc activities of three fully independent transfections were determined relative to the activity obtained with HSPT250. B. AtADH-Fluc-HSPT250 or AtADH-Fluc-HSPT878 was co-transfected with the expression plasmid for Rluc-HSPT250 for normalization of transfection efficiency. The Fluc/Rluc activity was calculated for each transfection, and average Fluc/Rluc activities of three fully independent transfections were determined relative to the activity obtained with HSPT250.

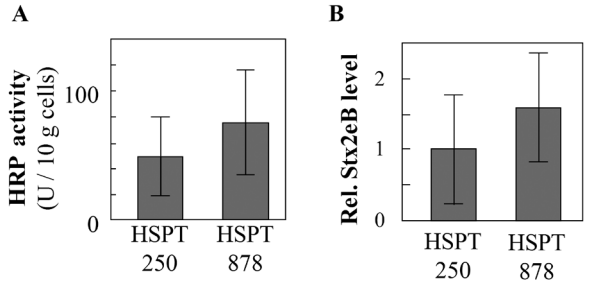


Figure 4. Production of useful proteins using HSPT878 in BY2 cells. Horseradish (*Armoracia rusticana*) peroxidase (HRP) (A) or the non-toxic B subunit of Stx2e, a vaccine antigen (B), was produced.

efficiency. The Rluc/Fluc activity was calculated for each transfection, and average Rluc/Fluc activities of three fully independent transfections were determined relative to the activity obtained with HSPT250 (Figure 3A). In transient expression, the Rluc/Fluc activities of HSPT878 and HSPT250 were almost the same. Transient expression analysis was also performed using the Fluc reporters; in this experiment, each Fluc construct was co-transfected with Rluc-HSPT250 for normalization. We confirmed that relative Fluc/Rluc activities of HSPT250 and HSPT878 were almost the same (Figure 3B). These data suggest that HSPT878 stimulates higher expression via a mechanism related to the chromosomal context.

Finally, we evaluated the utility of HSPT878 for the production of useful proteins. Horseradish (*Armoracia rusticana*) peroxidase (HRP) is used for a variety of detection procedures such as western blotting and enzyme-linked immunosorbent assay (ELISA). We previously reported high-level production of secreted HRP using BY2 cells (Matsui et al. 2012). Here we used an HRP-expression construct in which HRP was fused to a variant of the 5′-UTR of *Arabidopsis thaliana arabinogalactan-protein 21* (*AtAGP21*) containing a modification to the sequence context of the initiation codon (−3 to −1 bp before AUG). For these experiments, we generated plasmids in which this HRP-expression construct was followed by HSPT250 or HSPT878. Following infection by *A. tumefaciens* containing these plasmids, kanamycin-resistant BY2 cells were picked for analysis of HRP accumulation. Replacement of HSPT250 with HSPT878 increased the average accumulation level by about 1.5-fold (Figure 4A). Next, we used a similar strategy to express a candidate vaccine protein for pig edema disease, the non-toxic B subunit of Stx2e produced by the *Escherichia coli* strain that causes this illness (Sato et al. 2013). Again, replacing HSPT250 with HSPT878 increased the accumulation level by about 1.5-fold (Figure 4B). From these results, we can conclude that HSPT878 is more useful than HSPT250 for obtaining high-expression clones of stable transgenic BY2 cells. The HSPT878 will be a powerful tool for recombinant protein production as well as for basic sciences such as function analysis of plant genes.

One possible explanation for the increased expression levels obtained with HSPT878 is that this element contains a matrix attachment region (MAR). In chromosomal contexts, MARs contribute to increased expression in plant cells (Abranches et al. 2005; Butaye et al. 2004; Fukuda and Nishikawa 2003; Halweg et al. 2005; Mankin et al. 2003; Nowak et al. 2001; Xue et al. 2005), but do not have this effect in transient expression systems (Fukuda and Nishikawa 2003). In the presence of a MAR, adjacent DNA regions adopt a more open conformation, potentially leading to elevated transcription (Fukuda and Nishikawa 2003; Zhang et al. 2009). This might also be the case with the HSPT878, and this possibility should be analyzed in future studies.

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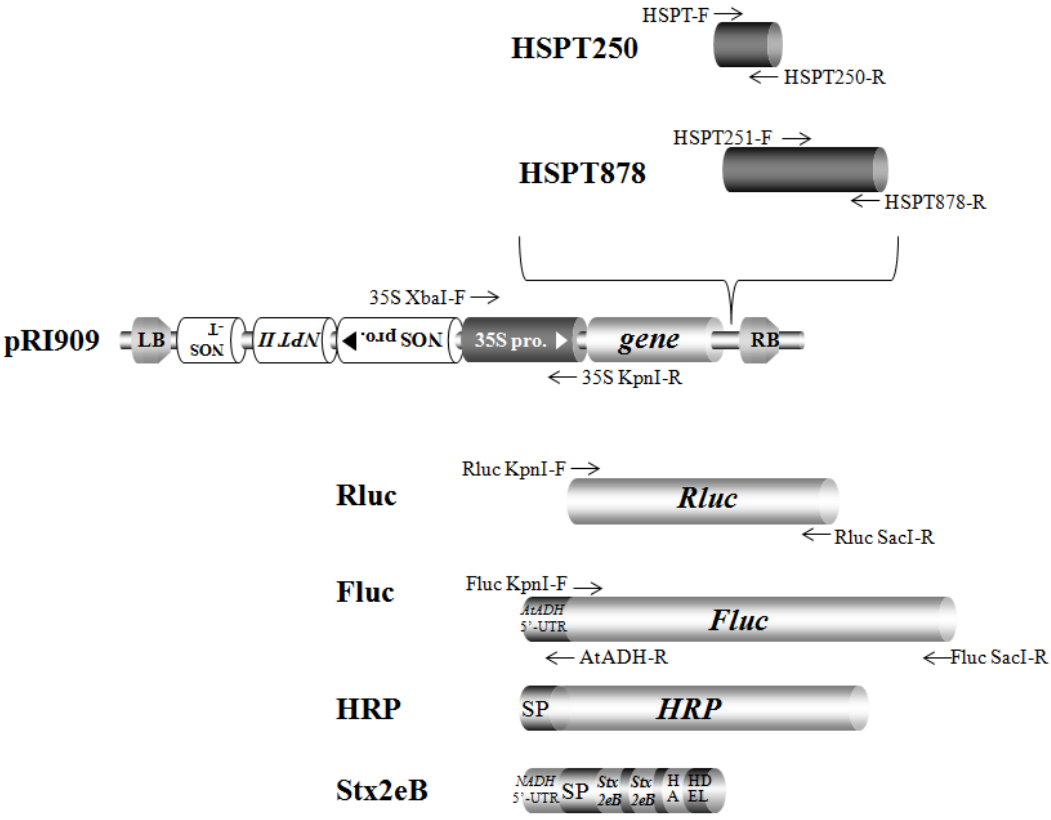


Supplemental Fig. 1 Construction of plasmids

A DNA fragment containing the cauliflower mosaic virus 35S promoter (35S pro.) was PCR-amplified using primers 35S *Xba*I-F (5′-aaatctagattagccttttcaatttcag-3′; *Xba*I site underlined) and 35S *Kpn*I-R (5′-aaaggtacccgggcgtgttctctccaaatgaaa-3′; *Kpn*I site underlined) and *CaMV35S-Rluc-HSP* (Nagaya et al. 2010) as the template. The resultant fragments were digested with *Xba*I and *Kpn*I, and inserted into the *Xba*I–*Kpn*I gap of pRI909 (TaKaRa Bio Inc., Shiga, Japan). A DNA fragment containing the *luciferase* gene from *Renilla reniformis* (*Rluc*) was PCR-amplified using primers Rluc *Kpn*I-F (5′-aaaggtaccatggcttccaaggtgtacgac-3′; *Kpn*I site underlined) and Rluc *Sac*I-R (5′-aaagagctcttactgctcgttcttcagc-3′; *Sac*I site underlined) and *CaMV35S-Rluc-HSP*

(Nagaya et al. 2010) as the template. The resultant fragments were digested with *Kpn*I and *Sac*I, and inserted into the *Kpn*I–*Sac*I gaps of pRI909 containing 35S pro. A DNA fragment for the transcriptional terminator derived from the *Arabidopsis thaliana heat shock protein 18.2* gene (HSPT250), corresponding to the 250 bp downstream of the stop codon, was PCR-amplified using primers HSPT-F (5′-aagagctcatatgaagatgaagatgaa-3′; *Sac*I site underlined) and HSPT250-R (5′-aagaattcactagtcttatctttaatcata-3′; *Eco*RI site underlined, and *Spe*I site double-underlined). The resultant fragments were digested with *Sac*I and *Eco*RI, and inserted into the *Sac*I–*Eco*RI gap of pUC118. This plasmid was treated with *Hin*dIII followed by treatment with T4 DNA polymerase, resulting in destruction of the *Hin*dIII site in HSPT250. A *Pst*I site was then destroyed in a similar fashion. The *Sac*I–*Eco*RI fragment containing HSPT250 was then inserted into the *Sac*I–*Eco*RI gap of pRI909 containing 35S pro. to generate Rluc-HSPT250. A DNA fragment corresponding to the region 251–878 bp downstream of the stop codon of the *AtHSP18.2* gene was PCR-amplified using primers HSPT251-F (5′-atctagatgggctcatagagtaaaacgaggcgagg-3′; *Xba*I site underlined) and HSPT878-R (5′-agaattcaaactagtgtacagatatatgttgaattatt*c*agctcttc-3′; *Eco*RI site underlined, and *Spe*I site double-underlined; "g" was changed to "c" [in italics] in order to destroy a native *Sac*I site). The resultant DNA fragments were digested with *Xba*I and *Eco*RI, and inserted into the *Spe*I–*Eco*RI gap of Rluc-HSPT250 to generate Rluc-HSPT878.

Plasmids for firefly (*Photinus pyralis*) *luciferase* (*Fluc*) were generated as follows: a DNA fragment containing *Fluc* was PCR amplified using primers Fluc-F (5′-aggtacc*atggaagacgtc*aaaaacataaa-3′; *Kpn*I site underlined, and *Aat*II site double underlined; nucleotides corresponding to the *Fluc* gene are in italics) and Fluc *Sac*I-R

(5′-agagctcactagtttacacggcgatctttccgc-3′; *Sac*I site underlined). In the corresponding Fluc protein, Ala4 (gcc) was changed to Val (gtc) in order to introduce an *Aat*II site (gacgtc, "c" was changed to the underlined "t"). A DNA fragment containing 35S pro., the 5′-UTR of the *A. thaliana alcohol dehydrogenase* gene (*AtADH* 5′-UTR), and the 5′-terminal part of *Fluc* containing the AatII site was PCR-amplified using primers 35S XbaI-F and AtADH-R (5′-*tttgacgtcttccat*tatcaacagtgaagaacttgcttttg-3′; nucleotides corresponding to *Fluc* are in italics; *Aat*II site underlined) and *AtADH-GUS-HSP* (Matsuura et al. 2013) as the template. These two fragments were ligated via their *Aat*II sites, and inserted into the *Xba*I–*Sac*I gaps of Rluc-HSPT250 and Rluc-HSPT878 to generate AtADH-Fluc-HSPT250 and AtADH-Fluc-HSPT878, respectively.

The expression plasmid for Stx2eB containing HSPT250 (Stx2eB-HSPT250) is the same as plasmid 2BH in our previous report (Matsui et al. 2011). A *Sac*I–*Eco*RI fragment containing HSPT878 was inserted into the *Sac*I–*Eco*RI gap of Stx2eB-HSPT250 to generate Stx2eB-HSPT878. The expression plasmid for HRP containing HSPT250 (HRP-HSPT250) is same as *AtAGP21*mod 5′-UTR HRP C1a in our previous paper (Matsui et al. 2012). A *Sac*I–*Eco*RI fragment for HSPT878 was inserted into the *Sac*I–*Eco*RI gap of HRP-HSPT250 to generate HRP-HSPT878.