A novel petal up-regulated PhXTH7 promoter analysis in Petunia hybrida by using bioluminescence reporter gene

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Abstract  Flower opening is an important phenomenon in plant that indicates the readiness of the flower for pollination leading to petal expansion and pigmentation. This phenomenon has great impact on crop yield, which makes researches of its mechanism attractive for both plant physiology study and agriculture. Gene promoters directing the expression in petal during the petal cell wall modification and expansion when flower opens could be a convenient tool to analyze or monitor gene expression targeting this event. However, there are no reports of isolated gene promoters that can direct gene expression in petal or petal limb during the rapid cell wall dynamics when the flower opens. Xyloglucan endotransglucosylase/hydrolase 7 (XTH7), a cell wall modifying enzyme, was reported having up-regulated gene expression in the petal of Arabidopsis thaliana and Petunia hybrida. In this study, we fused a 1,904 bp length P. hybrida XTH7 promoter with a gene encoding a bright bioluminescent protein (Green enhanced Nano-lantern) to report gene expression and observed petal up-regulated bioluminescence activity by means of a consumer-grade camera. More importantly, this novel promoter demonstrated up-regulated activity in the petal limb of P. hybrida matured flower during flower opening. P. hybrida XTH7 promoter would be a useful tool for flowering study, especially for petal expansion research during flower opening.

Key words: bioluminescent plant, flower opening, petal up-regulated promoter, GeNL, luciferase, Petunia hybrida, xyloglucan endotransglucosylase/hydrolase (XTH).

Introduction

After the discovery by A. L. Jussieu in 1803 in South America, Petunia has become a model plant for flower development research. Having a relatively large flower size, short life cycle (3–4 months from seed to seed), easy laboratory cultivation, and well-established transformation protocol, Petunia has been used for pigmentation and flower architecture studies (Vandenbussche et al. 2016; Weiss 2000). Moreover, it belongs to the Solanaceae family, which includes important crop plants such as tobacco, tomato, and potato. This genetic relatedness makes Petunia applicable as a model for those crop studies and improvement in both research and agriculture.

Flower opening marks the readiness of plant for pollinations, which holds an important role in plant reproduction. Genetic tools such as petal specific or petal up-regulated promoters are useful for analyzing and manipulating flower development and flower opening. Promoters of chalcone synthases from Lilium and Gentiana triflora, involved in pigmentation of plant, were reported to have petal specific expression (Du et al. 2014; Kobayashi et al. 1998; Liu et al. 2011). These promoters were active in the petal, especially in pigmented tissue but not responsive to the flower opening. To our knowledge, no promoter has been reported to direct the gene expression in petal during cell wall modification that occurs in flower opening, which is convenient for petal expansion mechanism study.

Flower opening involves the dynamic regulation of cell division and expansion (van Doorn and van Meeteren 2003). During this phenomenon, cell division and expansion of the petal limb are strongly and rapidly induced leading to the cell wall relaxation and modification (Harada et al. 2011). The xyloglucan endotransglucosylase/hydrolase (XTH) is involved in cell wall modification, including cell wall expansion during...
plant growth and also in flower opening (Campbell and Braam 1999; Harada et al. 2011). XTHs have two catalytic activities: endo-transglucosylase and endo-hydrolase of the xyloglucan chains in cell wall (Eklöf and Brumer 2010). In Arabidopsis, XTH7 gene is associated with cell elongation and up-regulated under light (Liu et al. 2018) and in the petal of mature flower (Klepikova et al. 2016), revealed by transcriptome (RNA-seq) data. The function and expression pattern of the XTH7 suggest this gene may be involved in the flower opening process by regulating the division and expansion of the petal cell wall (Klepikova et al. 2016). Similarly, from analysis of RNA-sequencing data of P. hybrida, XTH7 gene expression was also up-regulated in post-flowering petal (Broderick et al. 2014). These evidences suggest XTH7 promoter activity might be up-regulated in petal during flower development, especially during flower opening.

For promoter function or gene expression pattern studies in plant, a non-destructive, rapid response and high spatiotemporal resolution system is ideal to detect protein dynamics during growth and development. Conventionally, fluorescent or chemical-based reporters such as green fluorescent protein (GFP) or the β-glucuronidase (GUS), respectively are used for these purposes. However, fluorescent reporter application can be limited due to the high background signal from the autofluorescence in plant such as chlorophylls, polyphenols and lignin (DeBlasio et al. 2010; Donaldson 2020; Roshchina 2012). For GUS assay, formaldehyde and ferricyanide are essential in incubation and fixing solutions to visualize the GUS activity, which is destructive and toxic (Vitha et al. 1995). Therefore, gene expression reporter with a high spatiotemporal resolution, which allows rapid in planta response that will be convenient for promoter assay.

Bioluminescent reporter gene such as firefly luciferase (Fluc) is an alternative for fluorescent protein and GUS genes. Oxidation of the substrate, luciferin, catalyzed by the enzyme, luciferase, spontaneously emits light without any endogenous background autofluorescence (Kaskova et al. 2016). This fast response luminescent system does not require any excitation light like fluorescent proteins, and signal detection is much faster and less destructive in comparison to the GUS assay. One disadvantage of the bioluminescence emitted from the conventional Fluc, however, is the dim luminescence that limits the spatial resolution of imaging (Xie et al. 2019). In 2016, Green enhanced Nano-Lantern (GeNL), a novel bioluminescent protein was developed allowing bioimaging with high spatiotemporal resolution in mammalian cells (Suzuki et al. 2016). This bioluminescent protein consists of NanoLuc (NLuc), a luciferase derived from Oplophorus gracilirostris, and mNeonGreen (mNG), a green fluorescent protein emits a brilliant green bioluminescence by Förster resonance energy transfer from NLuc to mNG. In A. thaliana protoplast, GeNL exhibited 1,000-fold higher bioluminescence intensity compared to the conventional Fluc that allows signal detection by a consumer-grade camera (Furuhata et al. 2020). This data indicates high potential of GeNL as a high temporal and spatial resolution reporter gene for P. hybrida.

In this study, a 1,904 bp length P. hybrida XTH7 promoter was fused to GeNL and the transgene expression and bioluminescence pattern of different tissues (petal, leaf, and roots) were analyzed. Up-regulated expression and bioluminescence of GeNL driven by the XTH7 promoter was observed in the petal, especially high in the petal limb of mature P. hybrida flower. We believe this novel promoter would be a useful tool to study flower opening and flower opening mechanism.

Materials and methods

Plant materials

In this study, we used P. hybrida S5S17 strain that was kindly gifted from Dr. Ken-ichi Kubo from The University of Tokyo. Its leaves were sterilized by shaking in 10% antiformin and 0.05% tween 20 for 10 min and rinsed three times with double distilled water. Transformation and regeneration of Petunia is as follows the protocol of Khan et al. 2012 (Khan et al. 2012). Transgenic plants were moved to soil two weeks after root induction and screened for transgene expression based on its bioluminescence. All plants were grown in 24–26°C, long day condition (16 h light, 8 h dark).

Vector construction

Fluc, NLuc and GeNL genes were amplified by PCR to introduce EcoT22I and SacI restriction sites with primers: EcoT22I-Fluc-Fw and SacI-Fluc-Rv for Fluc, EcoT22I-NLuc-Fw and SacI-NLuc-Rv for NLuc, and EcoT22I-GeNL-Fw and SacI-GeNL-Rv for GeNL, respectively (Supplementary Table S1). PCR product was cloned into vector 35S::AtADH1::HSP::pUC19 by digestion and ligation with EcoT22I and SacI (the backbone vector was kindly gifted from Dr. Ko Kato from Nara institute of Science, Japan). The resulting 35S::AtADH1::UTR::Fluc::HSP, 35S::AtADH1::UTR::NLuc::HSP, and 35S::AtADH1::UTR::GeNL::HSP cassettes was cloned into binary vector pRI909 (TaKaRa) by treating with HindIII and EcoRI restriction enzymes and ligation using T4 DNA ligase (Promega).

For cloning the XTH7 promoter, 1,904 bp upstream from the start codon of the XTH7 gene was cloned from the genomic DNA of P. hybrida using sequence information from Sol Genomic Network database (https://solgenomics.net/). Plant sample was crushed in Tris-EDTA buffer and XTH7 promoter was amplified by PCR by KOD FX Neo (TOYOBO) to introduce HindIII and KpnI restriction sites with HindIII-XTH7-Fw and KpnI-XTH7-Rv primers (Supplementary Table S1). KpnI was also introduced in the 5’ end of GeNL for

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XTH7::GeNL cassette construction by PCR with KpnI-GeNL-Fw and SacI-GeNL-Rv primers (Supplementary Table S1). XTH7::GeNL/prI909 was constructed following the same cloning procedure of 35S::GeNL construction, but using HindIII and KpnI.

**In vitro spectroscopy of GeNL and NLuc**

For GeNL and NLuc protein purification from E. coli, first GeNL/prSETB and NLuc/prSETB vectors were transformed into E. coli JM109 (DE3) competent cells (TaKaRa) by heat-shock transformation method following supplier protocol. Protein purification was as follows the protocol of Farhana et al. 2019 (Farhana et al. 2019).

Emission spectra of E. coli purified GeNL, NLuc proteins and transgenic P. hybrida GeNL and NLuc leaf extract in PBS buffer were measured with the imagenic multichannel analyzer PMA-12 (Hamamatsu Imagenics). For bioluminescence emission spectrum measurement of purified proteins, 1 µl of stock furimazine solution (Promega) was added to 200 µl of 100 nM protein in PBS buffer (pH 7.5). For plant samples, leaf was ground by mortar and pestle in liquid N2 and resuspended in 0.1% β-mercaptoethanol PBS buffer (200 mg·ml⁻¹ buffer) then centrifuged at 15,000 g in four min to collect the supernatant as the crude extract. The emission spectrum was measured by adding 1 µl of stock furimazine solution to 200 µl of crude extract.

**Bioluminescence intensity measurement**

Bioluminescence intensity from plant sample was measured using GRATING MICROPLATE READER SH-9000 Series (Corona Electric Co., Ltd.). 100 µl of crude extract of sample prepared by the same method as spectroscopy measurement was added to the microplate, then 100 µl of 1% furimazine in PBS buffer was added to measure the bioluminescence intensity at one second gate time.

**Bioluminescence imaging**

For macroscopic imaging, bioluminescence images of transgenic P. hybrida were taken by the Sony a7s camera. All images were taken with ISO 2500, and f-numbers of 3.2. Exposure time varied for different samples ranging from 5 to 30 s. For leaf sample, adaxial surface was abraded using sandpaper for improving the absorption of substrate solution. Substrate solution contained 1% furimazine (for NLuc and GeNL P. hybrida) or 1 µM D-luciferin (for Fluc P. hybrida), and 0.1% β-mercaptoethanol in PBS buffer. Substrate solution was brushed or sprayed onto samples before imaging in the dark chamber. For flower sample, 1% DMSO was added into the substrate solution then the flower was brushed with the substrate solution. Bioluminescence images of different stages of XTH7::GeNL flower were taken to evaluate XTH7 promoter activity: young flower bud (3 cm length from receptacle to bud tip, stage 1), mature bud (before flower opening, stage 2), opening flower (stage 3–4), fully opened flower (stage 5) and two days after blooming (stage 6). The duration between each stages were: stage 1 to stage 2, 2–3 days; stage 2 to stage 3, 12–24 h; stage 3 to stage 4, 30–60 min; stage 4 to stage 5, 30–60 min; stage 5 to stage 6, two days. Bioluminescence images in Figure 4C were converted into pseudo-color images by using “Fire” format of lookup table function in imageJ software to display the difference of bioluminescence intensity. For microscopic imaging, OLYMPUS microscope system with electron multiplying charge-coupled device (EMCCD) camera and OLYMPUS UPlanSapo 20X/0.75 dry objective lens was used. Samples were placed in MS medium containing 1% stock furimazine on glass slide. For 35S::Fluc P. hybrida, D-luciferin was added into MS medium to make 1 µM D-luciferin final concentration. Images were analyzed with ImageJ to evaluate the intensity from the pseudo-color. Exposure time is two seconds, maximum GAIN, BIN: 2.
RNA isolation, cDNA synthesis and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was extracted using ISOGEN RNA extraction solution from Nippon Gene Co., Ltd. 800 ng of total RNA was used for cDNA synthesis using Superscript III M-MLV reverse transcriptase kit (Thermo Fisher).

*P. hybrida* XTH7 and GeNL gene expression was quantified by qRT-PCR. For XTH7 gene, qRT-PCR-XTH7-Fw and qRT-PCR-XTH7-Rv primers were used (Supplementary Table S1). For GeNL gene, qRT-PCR-GeNL-Fw and qRT-PCR-GeNL-Rv primers were used (Supplementary Table S1) Tubulin 1 (TUB) gene expression served as a housekeeping gene with qRT-PCR-TUB1-Fw and qRT-PCR-TUB1-Rv primers used in previous report (Supplementary Table S1) (Kretzschmar et al. 2012). SYBR Green PCR Master Mix (Applied Biosystems) was used for qRT-PCR. For each sample, three technical replicates were analyzed. qRT-PCR was performed on a StepOne Fast Real-Time PCR System (Applied Biosystems). For quantification, comparative CT (ΔΔCT) method was applied (Livak and Schmittgen 2001).

Results

Expression of GeNL in Petunia system

GeNL bioluminescence was evaluated in comparison with Fluc and NLuc in *P. hybrida*. Gene expression cassettes were constructed with plant constitutive CaMV 35S promoter, *AtADH* 5′ untranslated region (UTR) for gene expression enhancement and *AtHSP18.2* terminator (Figure 1A). Six transgenic lines of Fluc, four transgenic lines of NLuc and ten transgenic lines of GeNL were obtained.

Bioluminescence spectrum and intensity of each construct was assessed to compare their activities in *P. hybrida*. Bioluminescence emission spectrum of NLuc and GeNL in *P. hybrida* was analogous with *E. coli* expressed and purified proteins, which indicates NLuc and GeNL is functional in *P. hybrida* with distinct emission peaks of NLuc at 460 nm and mNG at 520 nm (Figure 1B). The bioluminescence intensity of the Fluc lines was too low to measure the emission spectrum.
All of GeNL lines and NLuc lines demonstrated higher bioluminescence intensity compared to all Fluc lines (lowest line and highest line of GeNL is 1.8-fold and 189.5-fold higher than the highest line of Fluc construct in term of bioluminescence intensity) (Figure 1C). Similar bioluminescence intensity was observed between NLuc and GeNL (Figure 1C).

Performance of GeNL bioluminescence as a marker was examined in comparison with Fluc and NLuc by imaging at microscopic and macroscopic level. For microscopic inspection, root and leaf epidermal cell bioluminescence were observed using electron multiplying charge-coupled device (EMCCD) camera. Cell outline could be observed in the root and leaf of NLuc and GeNL lines whereas cell shape was indistinguishable due to weak bioluminescence intensity of Fluc lines (Figure 2A, B). Macroscopic imaging was performed with the leaf of the brightest line of each construct by digital single-lens reflex (DSLR) camera in the dark chamber to examine whether GeNL bioluminescence is apparent for a quick and easy detection. As expected, NLuc and GeNL exhibit strong bioluminescence that was visible by the camera, even with naked eye observation, when the substrate was infiltrated or applied on abraded leaf, whereas very dim bioluminescence was detected in the substrate infiltrated Fluc leaf, and no bioluminescence could be detected in abraded Fluc leaf (Figure 2C). The bioluminescence of other tissues including root, stem, and flower of GeNL P. hybrida was also visible and detectable by DSLR camera even in small tissues (Figure 2D). Strong fluorescence could also be detected from GeNL line flower under blue light excitation (Supplementary Figure S1).

**XTH7 promoter function investigation with GeNL**

About 2 kb genomic DNA region upstream of the TSS of XTH7 gene was subcloned from P. hybrida as previous studies have indicated many promoter cis-elements are found within 2 kb from the transcription start site (TSS) (Lieberman-Lazarovich et al. 2019; Potenza et al. 2004). This DNA fragment was cloned into GeNL expression cassette to test the promoter activity based on the bioluminescence activity (Figure 1A, Supplementary Data S1). Five transgenic lines of XTH7::GeNL P. hybrida were obtained. Evaluation of the bioluminescence intensity in petal, leaf and root of three random XTH7::GeNL lines (Line 1, 2 and 3) compared with the 35S::GeNL lines demonstrated the petal up-regulated expression pattern of GeNL protein in P. hybrida (Figure 3A). Comparable with the bioluminescence data, expression level of GeNL gene driven by the XTH7 promoter was also up-regulated in the petal with the highest level in the petal limb in these lines, which had similar expression pattern to the endogenous XTH7 gene (Figure 3B).

**XTH7 promoter activity**

The XTH7::GeNL line petal was detectable with DSLR camera whereas very dim bioluminescence was observed in leaf (Figure 4A), which agrees with petal up-regulated expression pattern of GeNL protein in P. hybrida (Figure 3A). This promoter was active in all flower components, however, very low bioluminescence intensity was observed in the sepal (Figure 4B). Bioluminescence of different stages of flower was also examined with DSLR camera to analyze the XTH7 promoter activity during flower development. In the young flower bud, XTH7 promoter was active in the whole flower, but decreased as the flower bud reached maturity (Figure 4C). Notably, the bioluminescence intensity was highest in the petal limb of the mature flower after it fully bloomed (stage 5) and still active in the petal limb after two days (stage 6) (Figure 4C).
promoter (Supplementary Table S2). This promoter contains several motifs including enhancer, typical TATA box, abscisic acid, auxin, methyl jasmonate and light responsive motifs, but no tissue specific motif was found.

Discussion
Up-regulated activity of PhXTH7 promoter in petal limb of P. hybrida mature flower was demonstrated successfully with bioluminescence imaging by fusing this novel promoter with GeNL reporter gene. GeNL exhibits sufficient bioluminescence that allows high spatial resolution in planta imaging. Luminescent signal outlining the shape of each cell in the root and leaf epidermis was detected, which was unachievable with Fluc bioluminescence (Figure 2A, B). Recently, single cell bioluminescence imaging of plant cells using Fluc demonstrated low spatiotemporal-resolution image that distinctive single cell could not be observed (Muranaka and Oyama 2020), which agrees with our result in root and leaf cell bioluminescence imaging of Fluc P. hybrida. For macroscopic imaging, GeNL enables in planta detection with DSLR camera even in small tissues such as anthers or stigma whereas very low spatial resolution images were achieved with Fluc (Figure 2C, D). Additionally, NLuc could also be another choice as a reporter gene since it demonstrates comparable bioluminescence intensity to GeNL with a different emission wavelength (460 nm emission peak of NLuc). Different emission spectra of GeNL and NLuc will allow microscopic imaging of different target molecules simultaneously at high spatial

Figure 4. Bioluminescence images of XTH7::GeNL P. hybrida. BF: Bright field. BL: Bioluminescence. Scale bar: 1 cm. (A) Bioluminescence images of leaf and flower of 35S::GeNL and XTH7::GeNL P. hybrida, exposure time: 5 s for leaf and 30 s for flower image. Leaf was abraded with sandpaper. (B) Bioluminescence images of flower compartments. Exposure time: 5 s. Wild type flower was included as a negative control (C) bioluminescence images of different stages of flower buds and mature flower of XTH7::GeNL P. hybrida. Exposure time: 30 s. Pseudo-color image scale from 0 to 255 represents the bioluminescence intensity.
resolution in plants, or macroscopically using relatively inexpensive equipment.

For experimental procedure, GeNL establishes a more rapid and less laborious assay as a reporter gene in comparison with GUS. Substrate solution could be applied directly onto samples or into the imaging medium and bioluminescence can be observed in seconds manners, which is hours long for GUS detection because of sample fixing and staining procedures. Plant viability was maintained after bioluminescence detection after rinsing with water, which is also an advantage over GUS assay. In addition to A. thaliana (Furuhata et al. 2020), the high spatiotemporal-resolution and non-destructive imaging method using GeNL adds another advantage to bioluminescence reporter genes among the selection of available reporter genes that can be used for plant research.

However, one drawback of this method is the problem of substrate penetration in thick tissues, for example in leaf and stem tissues. Their cuticle layer obstructs the substrate penetration, which has to be removed by abrasion to observe luminescence. To further improve luminescence, β-mercaptoethanol was added to the substrate as a reducing agent to prevent the polyphenols oxidation effects on luciferase activity in P. hybrida leaf (Varma et al. 2007). This point should be considered when using GeNL in other species that have high concentration of polyphenols. On the other hand, root bioluminescence is less challenging compared to the shoot since the liquid substrate can be absorbed passively into the root system. For the flower of P. hybrida, additional DMSO can also improve permeation of the substrate without abrasion to reduce the damage to the tissue.

As another technical advantage, the mNG fluorescent protein moiety in GeNL emits detectable fluorescence (Supplementary Figure S1) upon irradiation with UV/blue light that could be convenient for screening transgenic shoots or seedlings in aseptic conditions in a translucent container, as demonstrated in this study. Furthermore, fluorescence imaging could also be conducted in parallel with bioluminescence imaging for inspection of expression in deep tissue, which cannot be seen with bioluminescence imaging. The fusion of the fluorescent protein in GeNL makes it a convenient tool for screening, and cellular imaging for tissue specific analysis.

A novel 1,904 bp length promoter region of the XTH7 gene in P. hybrida was successfully cloned exhibiting petal up-regulated expression supported by qRT-PCR and bioluminescence assay data. XTH7 promoter activity is found to be up-regulated in the cross sectional expanding or spherical tissues like stem/pedicel, ovary, anther and petal (Figure 4A, B) or in young flower bud or in petal limb in matured flower (Figure 4C), which seems to be related to the cell wall dynamics in flower development. This finding agrees with previous report of similar function of XTH18, XTH19 and XTH20 genes that are responsive to mechanical or turgor pressure that leads to cell wall modification (Miedes et al. 2013). Moreover, XTH7 promoter contains methyl jasmonate, light and auxin response cis elements (Supplementary Table S2), which are also related to flowering or cell wall elongation (Cheng and Zhao 2007; Majda and Robert 2018; Radhika et al. 2010) that could be convenient to study the cell wall dynamics during flower development. Further applications in petal shape modification and pigmentation could also be considered as this novel promoter is up-regulated in petal limb during flower opening.

In this study, a new XTH7 promoter in P. hybrida that is up-regulated in petal tissue during flower opening was isolated and characterized. The petal up-regulated and flower opening induced expression pattern of this novel promoter was demonstrated by the bright bioluminescence of GeNL in P. hybrida. GeNL demonstrated great potential as a rapid-response, non-destructive and high spatiotemporal resolution reporter gene for promoter assay in P. hybrida, which is detectable by DSLR consumer-grade camera. PhXTH7 promoter is the first promoter that is reported to direct transgene expression in petal limb during the flower opening in P. hybrida. We believe this promoter would contribute to flowering study as a useful tool that can target the petal expression, especially in petal expansion.

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