Integrated phytohormone production by the plant growth-promoting rhizobacterium Bacillus tequilensis SSB07 induced thermotolerance in soybean

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ABSTRACT

As a result of climate change, crops often experience high-temperature stress that can drastically hinder plant growth and development. In soybean, an economically important crop that is highly sensitive to heat stress, the use of plant growth-promoting rhizobacteria (PGPRs) represents a promising strategy for countering the negative effects of heat stress. Accordingly, a novel strain of Bacillus tequilensis (i.e. SSB07) that grows actively at high temperatures was isolated, identified, and characterized in the present study. SSB07 improved the growth of Chinese cabbage seedlings and was shown to produce the gibberellins GA1, GA3, GA4, GA5, GA19, GA24, and GA53, as well as indole-3-acetic acid and abscisic acid. The application of B. tequilensis SSB07 was also found to increase the shoot length and biomass, leaf development, and photosynthetic pigment contents of soybean plants, and under heat stress, SSB07 inoculation significantly increased the endogenous jasmonic acid and salicylic acid contents of the phyllosphere and significantly down-regulated the production of stress-responsive ABA. Thus, B. tequilensis SSB07 shows promise for countering the negative effects of climate change on crop growth and development.

ARTICLE HISTORY
Received 17 November 2018
Accepted 25 June 2019

KEYWORDS
Plant growth-promoting rhizobacterium; thermotolerance; plant growth; Glycine max; phytohormone; Bacillus tequilensis; phyllosphere

Introduction

Plant growth-promoting rhizobacteria (PGPRs) are widely recognized for their ability to ameliorate abiotic stress in crops, thereby improving yield and resistance (Vessey 2003; Ahemad and Kibret 2014), through the solubilization, mobilization, and improved uptake of plant nutrients, as well as the modulation of phytohormones (i.e. phytohormone production; Vessey 2003; Bhardwaj et al. 2014). PGPRs are also known to regulate plant growth and development during pathogenesis by enhancing plant defense responses (Ahemad and Kibret 2014; De Coninck et al. 2015). Various genera of bacteria (e.g. Acetobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Klebsiella, Pseudomonas, and Serratia), have been reported to promote plant growth by enhancing seed emergence, plant biomass, and crop yield (Glick 2005; Jones et al. 2007). These effects have generally been attributed to the potential of PGPRs to produce extracellular enzymes and metabolites, including phytohormones.

The ability to produce phytohormones, like gibberellins (GAs), cytokinins, abscisic acid (ABA), and indole-3-acetic acid (IAA), has been reported for numerous bacterial species. Rhizobium phaseoli (Atzorn et al. 1988), B. pumilus (Gutierrez-Manero et al. 2001), B. macroides Cj-29 (Joo et al. 2005), and Acinetobacter calcoaceticus (Kang et al. 2012), for example, have been reported to produce GAs in culture media, and have the production of phytohormones by Bacillus species, like B. cereus, B. macroides, B. pumilus, B. macrolides, B. licheniformis, and B. subtilis, has been widely studied.

The application of phytohormone-producing PGPRs may be useful for improving crop physiology, biomass, and yield (Vessey 2003; Nilohimibere et al. 2011) by increasing the size, branch number, and surface area of host roots and leaves. Increases in leaf surface area facilitate sunlight absorption, whereas increased root branching and surface area facilitate nutrient uptake. Similar conclusions have been published regarding the roles of exogenous GA and IAA (Chen et al. 2014; Li et al. 2015; Wang et al. 2015). Phytohormones are key plant growth regulators that generally facilitate physiological processes under both normal and stressful conditions. The naturally occurring regulation of key phytohormones, such as GAs, ABA, salicylic acid (SA), and jasmonic acid (JA), can yield either positive or negative effects on plant growth during exposure to abiotic stresses (Miura and Furumoto 2013). GAs function as plant growth promoters, whereas ABA, JA, and SA are categorized as stress-related hormones (Kosová et al. 2012). Phytohormones interact, either synergistically or antagonistically, in order to modulate physiological processes under specific conditions (Pelegr and Blumwald 2011). During stress exposure, each phytohormone has a distinct regulating mechanism. For example, ABA and SA treatment have been reported to increase the heat stress tolerance in wheat and Arabidopsis (Khan et al. 2013; Suzuki et al. 2016). During temperature changes,
GAs function antagonistically, and the endogenous levels of both GAs and auxin are downregulated, eventually resulting in dwarfism (Achard et al. 2008; Miura and Furumoto 2013). Meanwhile, JA lessens the deleterious effects of temperature stress and improves the post-harvest storage of fruits (Rohwer and Erwin 2008; Wasternack, 2014).

As a result of climate change, global air temperature is estimated to increase by 0.2°C/year for the next two decades (IPCC 2007). These increases may drastically affect the growth and yield of crop plants. Extreme temperatures can substantially alter the fluidity of membranes, structures of amino acids and proteins, and the concentrations of metabolites and osmolytes (Wahid et al. 2007; Zinn et al. 2010). Soybean [Glycine max (L.) Merr.], which is sensitive to high temperatures, shows a change in the metabolomics (Chebrolu et al. 2016) and antioxidant production (Sgobba et al. 2015).

Heat waves can also affect plant growth, photosynthesis, and pollination quite drastically (Sakata et al. 2014; Buchner et al. 2015; Bishop et al. 2016; Zhang et al. 2016).

The use of novel PGPRs to alleviate the negative effects of high temperatures on economically important plant species represents a potentially important and environmentally friendly strategy to counter climate change. PGPRs that are capable of producing GA, IAA, and ABA are rarely reported. Therefore, the present study isolated and identified a novel strain of B. tequilensis (i.e. SSB07), in order to assess its role as a PGPR. This study hypothesizes that phytohormone-producing PGPRs may maintain or even improve the growth of crop plants under high-temperature stress by modifying endogenous stress-related phytohormones.

Materials and methods

Isolation of bacteria from soybean rhizosphere

Rhizospheric soil samples were randomly collected from soybean fields in Danyang (Chungcheongbuk-do, Republic of Korea), as described by Barillot et al. (2013). Soil that was tightly adhered to the roots (i.e. rhizosphere) was separated using the method of Porcel et al. (2014). The pooled soil samples (5 g) were transferred to 50 mL sterile saline solution (0.85% NaCl), and the resulting suspensions were serially diluted (10⁻⁴). Aliquots of the diluted solutions (0.1 mL) were grown on Luria Broth (LB) agar plates (Merck Co., Germany), and the isolated bacterial strains were periodically inoculated onto new LB Petri plates until purification was confirmed (three subculture cycles), after which the strains were incubated for 48 h at 30°C. The cultures of individual bacteria strains were inoculated in LB media and incubated on a shaker at 150 rpm and 30°C.

Selection of plant growth-promoting strains

The isolated strains were screened for their ability to improve the germination of Chinese cabbage seed, and the promising strains were subsequently screened for their ability to promote the growth of Chinese cabbage seedlings. For the germination test, Chinese cabbage seeds were treated with 5% NaOCl for 10 min, rinsed with germ-free double-distilled water, and grown in autoclaved Petri dishes that were lined with two layers of sterilized Whatman No. 1 filter paper. Ten seeds were grown on each Petri dish, and three replicates were used for each treatment. Then, 10 mL of bacterial suspension (10⁷ CFU/mL) from each isolate was applied to their designated Petri plate. Sterilized LB media was used as a negative control, and double-distilled water was used as a positive control. The Petri plates were incubated in a growth chamber in the dark (14 h at 28°C, 10 h at 24°C; relative humidity 60–70%), and after 1 week, the total seedling length was recorded and the effects of the strains were examined.

To further distinguish and identify the ability of PGPRs to produce phytohormones, another bioassay was performed on gibberellin-deficient (Waiito-C; Mitsunaga and Yamaguchi 1993; Hossain et al. 2017) and normal rice (Whayoungbeyo) cultivars. Seeds of both rice cultivars were sterilized and treated as described above for soybean seeds. However, the inoculated seed (three replicates per treatment) was grown in autoclaved substrate for two weeks in a growth chamber with a day/night cycle of 14 h at 28 ± 0.3°C and 10 h at 25 ± 0.3°C, as well as a relative humidity 70%.

Heat-tolerance of selected PGPR

Isolate SSB07 was selected for further investigation, based on its promotion of host seedling length and enhancement of rice growth attributes. The selected bacterial isolate (SSB07) was grown in LB medium, and cultures were incubated on a shaker at 25, 30, or 35°C and 150 rpm for 48 h. The growth of the cultures was measured at 12 h intervals, using optical density at 600 nm, and the experiment was independently repeated three times, with five replicates each.

Molecular identification of selected PGPR

Isolate SSB07 was identified on the basis of its partial 16S ribosomal RNA (rRNA) gene sequence, using the primers reported by Lane (1991). For this purpose, total genomic DNA was isolated as described by Sambrook and Russel (2001), and the isolate’s nucleotide sequence homology was determined using BLAST. Sequences with the greatest homology and query coverage and lowest E-values were selected and aligned using ClustalW in MEGA (version 6.1), and a neighbour-joining phylogenetic tree was generated, using MEGA, with Planococcus maritimus as an outgroup.

Indole-3-acetic acid quantification

To characterize the ability of SSB07 to produce IAA, the strain was grown in LB medium and analysed as described by Kang et al. (2015). Briefly, the sample was extracted, dried, and methylated using diazomethane, and the IAA level of the broth was calculated by comparison to corresponding standards using gas chromatography-mass spectrometry (GC/MS) with selected ion monitoring (SIM; 6890N Network GC System and 5973 Network Mass Selective Detector, respectively; Agilent Technologies, Palo Alto, CA, USA).

Abscisic acid quantification

To characterize the ability of SSB07 to produce ABA, the strain was cultured in LB medium for 3 d, after which the resulting culture filtrate was partitioned using a 95:5 (v:v) solution of isopropanol and glacial acetic acid, and ABA in the culture medium was further extracted and quantified as described by Qi et al. (1998), using GC/MS-SIM.
Gibberellin quantification

To characterize the GA-producing ability of SSB07, GAs were extracted from pure culture filtrates, as described by Lee et al. (1998) and Khan et al. (2011). Briefly, LB was inoculated with SSB07, incubated in a shaker at 30°C and 120 rpm for 3 d, then partitioned by centrifugation (5000 × g at 4°C for 15 min). After adding deuterated GAs as internal standards and partitioning the GAs with ethyl acetate, the GA content of the supernatant (100 mL, pH 2.5) was analysed using column chromatography. The organic layer was vacuum dried, and the extracts were passed through a Davysil C18 column (90–130 μm; Alltech, Deerfield, IL, USA) and then subjected to HPLC using a 3.9 × 300 mm Bondapak C18 column (Waters Co., Milford, MA, USA). Forty-eight 1.0-mL fractions were collected and prepared for GC/MS—SIM, and 1 μL of each fraction was analysed by GC/MS. Peak area ratios (ng/100 mL) were calculated, and the analysis was repeated three times.

Soybean-PGPR interactions under heat stress

To investigate the ability of SSB07 to promote the growth and thermotolerance of soybean plants, we designed a growth chamber study in which plants were treated with SSB07 or one of two controls (Control 1 = water, Control 2 = sterilized LB media) and were grown in normal or heat-stress conditions. Each treatment group included 24 plants, and each experiment was performed in triplicate. All glassware, plastic germination trays, pots and soil substrate were sterilized prior to the experimentations as well as aseptic condition was maintained for germination and plant growth to eliminate the chance of contamination. Daewon soybean seeds were obtained from the soybean breeding laboratory of Kyungpook National University (South Korea), surface-disinfected in NaOCl (5%) for 10 min, and washed five times with autoclaved double-distilled water. The sterilized seeds were initially sown in plastic germination trays that contained soil substrate (detail forthcoming) and incubated. After 5 days, seedlings with similar growth rates were transferred to soil substrate (detail forthcoming) and incubated. After 5 days, seedlings with similar growth rates were transferred to soil substrate (detail forthcoming) and incubated. After 5 days, seedlings with similar growth rates were transferred to soil substrate (detail forthcoming) and incubated. After 5 days, seedlings with similar growth rates were transferred to soil substrate (detail forthcoming) and incubated. After 5 days, seedlings with similar growth rates were transferred to soil substrate (detail forthcoming) and incubated. After 5 days, seedlings with similar growth rates were transferred to soil substrate (detail forthcoming) and incubated. After 5 days, seedlings with similar growth rates were transferred to soil substrate (detail forthcoming) and incubated. After 5 days, seedlings with similar growth rates were transferred to soil substrate (detail forthcoming) and incubated. 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Bacillus tequilensis SSB07 cells were inoculated into LB media, and growth was measured at 12-h cycles, and then the effects of the bacteria, water, and LB media on the germination of Chinese cabbage seeds were compared. Values and error bars represent means ± SD. Different letter(s) indicate significant differences (p ≤ 0.05). Control 1 = water; Control 2 = sterilized LB media.

**In vitro tolerance of SSB07 to temperature**

The number of SSB07 cells observed in the cultures grown at 35°C was greater than those grown at 25 or 30°C at all four time intervals (12, 24, 36, and 48 h; Figure 2), and the greatest number of SSB07 cells was observed on 2.55 ± 0.1 OD at 35°C after 48 h.

**Molecular identification and phylogenetic analysis of SSB07**

BLAST analysis identified the SSB07 16S rRNA sequence as *Bacillus tequilensis*, and phylogenetic analysis placed the isolate in a clade with *B. tequilensis* KCTC AYT 001000043 (Figure 3). Furthermore, the SSB07 16S rRNA sequence was submitted to NCBI GenBank (accession number KP860637).

**Phytohormone production ability of SSB07**

SSB07 cultures contained both bioactive and inactive GAs, including GA1, GA3, GA5, GA6, GA19, GA24, and GA53, as well as IAA and ABA (Table 1). The control (sterilized LB media) was also analysed as a reference for the presence of any kind of phytohormones, however, not a single type was traced (data not shown).

**SSB07-induced thermotolerance**

Under heat-stress conditions, SSB07 inoculation significantly enhanced the thermotolerance of soybean plants, as demonstrated by the retention of shoot length, leaf width, shoot weight, and chlorophyll content, when compared to Control 1 and Control 2 plants (Table 2). However, no significant difference was noted between the leaf lengths of the SSB07-inoculated and control treatment plants. Under normal conditions, the shoot length, leaf length, shoot weight, and chlorophyll content of the SSB07-inoculated plants was significantly greater than those of Control 1 and Control 2 plants.

In addition, scanning electron microscopy (Figure 4) confirmed the presence of SSB07 on the root surfaces of inoculated soybean plants grown under both normal and heat-stress conditions, whereas no SSB07 or other microbes were found on the root surfaces of Control 1 and Control 2 plants, regardless of growth conditions.

**Effect of SSB07 on endogenous phytohormone levels**

Under heat-stress conditions, the ABA content of SSB07-inoculated plants (4.44 ± 0.09 ng/g DW) was significantly lower than that of the Control 1 (4.95 ± 0.03 ng/g DW) and Control 2 (4.91 ± 0.03 ng/g DW) plants (Figure 5), and under normal conditions, the ABA content of the SSB07-inoculated plants (4.76 ± 0.02 ng/g DW) was significantly greater than that of the Control 1 (4.41 ± 0.12 ng/g DW) and Control 2 (3.95 ± 0.03 ng/g DW) plants.

Meanwhile, the JA content (Figure 5) of the SSB07-inoculated plants (28.77 ± 0.66 ng/g DW) was higher than that of the Control 1 (25.19 ± 1.25 ng/g DW) and Control 2 (22.05 ± 0.25 ng/g DW) under heat stress, and under normal conditions, the JA contents (Figure 5) of the Control 1 (33.71 ± 0.20 ng/g DW) and Control 2 (31.57 ± 0.24 ng/g DW) plants were significantly greater than those of the SSB07-inoculated plants (30.61 ± 0.08 ng/g DW). SSB07 inoculation also increased endogenous levels of SA (Figure 5), under both heat-stress (1.15 ± 0.02 µg/g DW) and normal conditions (1.11 ± 0.001 µg/g DW), when compared to the Control 1 (1.10 ± 0.002 µg/g DW) and Control 2 (1.08 ± 0.002 µg/g DW) treatments.

**Discussion**

PGPRs regulate the phyllosphere continuum of plants, with ameliorative effects reported for the anthosphere (flowers), phylloplane (leaves), caulosphere (stems), and carposphere (fruits). The phyllosphere is essential for the economic and food-related benefits of most plants, including soybean. For example, maintaining and improving stem and leaf growth can ensure higher yields. Indeed, the results of the present study demonstrate that the application of *B. tequilensis*
SSB07 to the rhizosphere of soybean plants significantly improves plant growth traits and induces physiochemical changes in the phyllosphere (Figure 4, Table 2). The improvement in plant growth was evident in the greater shoot length, shoot biomass, leaf area, and photosynthetic pigments of the inoculated plants, under both heat-stress and normal conditions. Similar effects have also been reported for a multiple numbers of bioactive PGPR applications in a wide variety of other crops, including cucumber, pepper, and Chinese cabbage (Ruzzi and Aroca 2015). Heat stress severely damages the biophysicochemical properties of crops, often affecting crop production drastically. Such stress has been perceived as an increasing problem, owing to changes in climatic conditions, especially global warming (Sgobba et al. 2015; Chebrolu et al. 2016; Tewari et al. 2016). Heat stress inhibits leaf development by inhibiting the maintenance of water balance, in turn affecting photosynthetic pigments and their functions, as well as plant height and biomass. Such effects were observed in the control plants of the present study but were reduced by *B. tequilensis* SSB07 inoculation. Similarly, Ngumbi and Kloepper (2016) proposed that PGPRs could impart multifaceted benefits, including thermotolerance, to economically important crop plants, and Grover et al. (2011) reported that PGPRs can improve the growth and development of crop plants and can help maintain sustainable supplies of

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**Table 1. Levels of gibberellins (GAs), indole-3-acetic acid (IAA) and abscisic acid (ABA) produced by SSB07.**

| GA1 (µg/mL) | GA3 (µg/mL) | GA5 (µg/mL) | GA8 (µg/mL) | GA19 (µg/mL) | GA24 (µg/mL) | GA53 (µg/mL) | IAA (µg/mL) | ABA (ng/mL) |
|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| 0.24 ± 0.02 | 0.81 ± 0.01 | 0.43 ± 0.03 | 0.62 ± 0.05 | 2.25 ± 0.01 | 1.28 ± 0.06 | 0.52 ± 0.02 | 0.42 ± 0.05 | 0.43 ± 0.09 |

Values in columns represent mean ± SD (n = 3).

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**Table 2. Effect of SSB07 application on the growth characteristics of soybean plants.**

| Treatment | Shoot length (cm) | Leaf length (cm) | Leaf width (cm) | Shoot weight (g) | Chlorophyll (SPAD) |
|-----------|------------------|-----------------|-----------------|-----------------|-------------------|
| Non-heat Stress (30°C) | | | | | |
| Control 1 | 23.2 ± 2.1b | 6.3 ± 0.6ab | 4.9 ± 0.4a | 7.19 ± 1.1b | 27.3 ± 2.0ab |
| Control 2* | 21.4 ± 1.5c | 7.7 ± 0.4ab | 5.0 ± 0.2a | 7.31 ± 0.3b | 26.3 ± 1.6ab |
| *B. tequilensis* SSB07 | 27.4 ± 1.4a | 8.4 ± 0.7a | 5.2 ± 0.4a | 8.81 ± 1.3a | 30.6 ± 2.0a |
| Heat Stress (38°C) | | | | | |
| Control 1 | 18.0 ± 1.5ab | 5.7 ± 0.4a | 4.0 ± 0.4b | 3.24 ± 0.3b | 22.6 ± 2.3b |
| Control 2* | 17.9 ± 1.4b | 5.6 ± 0.6ab | 3.6 ± 0.5b | 3.39 ± 0.3b | 22.5 ± 3.4b |
| *B. tequilensis* SSB07 | 20.4 ± 2.0d | 6.3 ± 0.2a | 4.8 ± 0.2a | 4.26 ± 0.2a | 28.3 ± 1.6a |

*Control 1 = Water; *Control 2 = Sterilized LB media.
§Fully expanded uppermost youngest leaves were used for its measurement of length, width and chlorophyll contents.
Different letter(s) indicate significant differences (p < 0.05; n = 3), as evaluated by the Duncan Multiple Range Test (SAS 9.0).
agriculture products in changing climatic conditions. Furthermore, Pasala et al. (2016) suggested that the negative effects of abiotic stresses, such as heat, drought, and salinity, can be minimized by inoculating crops with bio-regulators, such as PGPRs.

The ameliorative benefits of PGPRs are the result of the intrinsic ability of PGPRs to produce plant growth regulators, such as GAs, auxin, and ABA. The similar may also assumed in current experiment, that production of the same phytohormones by *B. tequilensis* SSBO7 may have increased plant growth and that is how they increased survivability of soybean in a heat stress situation. Previous reports, such as those of Cohen et al. (2015), Porcel et al. (2014), Spence et al. (2015), and Gutierrez-Manero et al. (2001), have found the beneficial effects of such regulators production and have concluded that the secretion of IAA and GAs by certain PGPRs improve the ability of crop plants to combat both biotic and abiotic stresses. Such studies have especially recognized the ability of plant growth-promoting *Bacillus* strains to produce IAA, but less so for GAs. The present study indicated that *B. tequilensis* SSBO7 produces IAA, ABA, and a variety of GAs (Table 1), which suggests that these biosynthesis pathways could be available; however, further elucidation is needed at molecular levels. This is the first study of our new isolate that has shown that *B. tequilensis* SSBO7 can also produce GAs (Table 1). These findings suggest that *B. tequilensis* SSBO7 produces GA19 and GA3 in higher amounts in culture media. GA3 is a physiologically active gibberellin that is recognized for its ability to promote plant growth during biotic and abiotic stresses, including heat stress (Chen et al. 2014; Khan et al. 2015; Li et al. 2015). GAs are also produced by other *Bacillus* species, including *B. pumilus*, *B. licheniformis* (Gutierrez-Manero et al. 2001), and *B. macrolides* (Joo et al. 2005), and IAA biosynthesis is relatively well-known in the genus, as well (Ali et al. 2009; Khan et al. 2015). The present study reports, for the first time, that *B. tequilensis* also produces ABA, which is consistent with the results of the study by Spence et al. (2015), who reported that some microbes can produce ABA in their culture media. However, the potential of PGPR strains to synthesize ABA in culture media is poorly understood and needs further elucidation at molecular levels.

The stress resistance provided by PGPRs may result from biochemicals and the regulation of phytohormones, such as ABA, JA, and SA, responding to defence and growth of the plant (Vessey 2003; Pieterse et al. 2009; Khan et al. 2014; Carvalhais et al. 2015; Venturi and Keel 2016). In the present study, we found that the endogenous levels of JA and SA were significantly upregulated in heat-stressed, SSBO7-inoculated
soybean plants, whereas the endogenous level of ABA was downregulated (Figure 5). The opposite effect was observed in non-inoculated control plants. JA and SA, which are defence-related phytohormones, increments have rescued the soybean plant growth dynamics. Though the cross-talk between JA and SA has often been reported to occur in response to abiotic stresses, this can vary, depending on the type of plant-microbe interaction, as demonstrated by Khan et al. (2015). On the contrary, the stress-responsive hormone ABA content of soybean plants was significantly downregulated by PGPR-inoculation in the present study, which suggests that PGPR reduced the stress level of the plants. A similar conclusion was drawn by Khan et al. (2015), who applied endophytic microbes to tomato plants under heavy metal stress. In the present study, the PGPR-induced activation of JA and SA may have triggered soybean defence responses, in order to counteract heat stress.

In conclusion, the present study demonstrated that B. tequilensis SSB07 has a strong ability to produce biologically active metabolites, such as gibberellins, indole-3-acetic acid, and abscisic acid. This is the first study of our new isolate showing such abilities for this strain. SSB07 inoculation improved the shoot length, biomass, leaf area, and photosynthetic pigments of soybean plants under normal growth conditions, as well as under heat stress. This improvement in plant growth was coupled with changes in the endogenous levels of several phytohormones, i.e. ABA, JA, and SA.

Acknowledgements
SMK, ALK, MW, and SA developed the research question, designed the experiment, and wrote the manuscript. SMK conducted the experiment and collected the data. KEL, YGP, AYK, MAK, and YHY helped conduct the experiment. IIL supervised the project and provided both financial support and mentorship.

Disclosure statement
No potential conflict of interest was reported by the authors.

Funding
This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education [grant number 2016R1A6A1A0501910].

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