Plant Growth Regul (2017) 81:175–181
DOI 10.1007/s10725-016-0189-4

BRIEF COMMUNICATION

Sucrose synthase *FaSS1* plays an important role in the regulation of strawberry fruit ripening

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Received: 14 January 2016 / Accepted: 11 June 2016 / Published online: 30 June 2016

Abstract Sugar content and composition are critical to fruit development. Sucrose, a photosynthate unloaded to the fruit, is metabolized by sucrose synthase, which might play a dominant role in sucrose accumulation during strawberry fruit ripening. However, substantial evidence regarding the molecular mechanism underlying sucrose accumulation in strawberry fruit development is lacking. Here, a strawberry sucrose synthase gene, *FaSS1*, was cloned and identified. Its 2421-bp cDNA includes an intact open reading frame and encodes an 806 amino acid protein, in which sucrose synthase-related conserved domains were predicted by a homology analysis. Using tobacco rattle virus-induced gene silencing, the downregulation of *FaSS1* transcripts significantly delayed fruit ripening, as evidenced by the changes of firmness, and soluble sugar and anthocyanin contents, as well as the transcripts of several ripening-related genes, including *PE1, PL1, XYL2, CHS, CHI*, and *DFR*. Furthermore, the mRNA expression level of *FaSS1* was inhibited by abscisic acid or sucrose, but not by glucose after fruit disc incubation in vitro. In conclusion, *FaSS1* plays an important role in the regulation of strawberry fruit ripening, and its expression could be inhibited by abscisic acid and sucrose.

Keywords Sucrose synthase gene *FaSS1* · Sucrose · Abscisic acid · Strawberry fruit ripening · Virus-induced gene silencing

Abbreviations

SS/Sus	Sucrose synthase
ABA	Abscisic acid
SqRT-PCR	Semiquantitative RT-PCR
TRV	Tobacco rattle virus
PG1	Polygalacturonase 1
PL1	Pectate lyase 1
XYL2	Xylitol dehydrogenase 2
CHS	Chalcone synthase
CHI	Chalcone isomerase
DFR	Dihydroflavonol 4-reductase

Introduction

Strawberry (*Fragaria ananassa*) is an economically important fruit crop. Sugar content and composition (sucrose, glucose, and fructose) are critical to fruit development, and are controlled by sugar transport, metabolism, and accumulation. Sucrose is unloaded into strawberry fruit through an apoplast pathway (Li et al. 2012). It is also suggested that

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Electronic supplementary material The online version of this article (doi:10.1007/s10725-016-0189-4) contains supplementary material, which is available to authorized users.

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sucrose synthase (SS/Sus, EC 2.4.1.13) might play a dominant role in sucrose accumulation during the fruit ripening (Tian et al. 2012). However, substantial evidence regarding the molecular mechanism underlying sucrose accumulation in strawberry fruit development is lacking.

Sus, which catalyzes the reversible conversion of sucrose to monosaccharose, is a key enzyme in sucrose metabolism. In plants, its main physiological function is to cleave sucrose to generate UDP-glucose, which plays an important role in the biosynthesis of storage products (Huber and Akazawa 1986; Sung et al. 1988). In Arabidopsis seed, sucrolysis (cleaving sucrose) by Sus is required for carbon partitioning and accumulation, which may provide energy for phloem transport in the silique wall, to a large extent, AtSus2 serves as a marker during the seed maturation (Baud et al. 2004; Fallahi et al. 2008; Núñez et al. 2008; Angeles-Núñez et al. 2010). The enzyme's key role, serving as a regulator in sink strength, has also been confirmed in cotton fiber formation (Jiang et al. 2012). In most grain crops, such as wheat (Hou et al. 2014), maize (Li et al. 2013), barley (Sreenivasulu et al. 2004), broad bean (Borisjuk et al. 2002), and rice (Karrer et al. 1992), the main function of Sus is played in starch biosynthesis.

Juicy fleshy fruit development is a distinctive process in plants. In *citrus*, Sus is a key enzyme for sugar accumulation, and of the six genes (*Sus1–6*), *CitSus1* and *CitSus2* were predominantly expressed in fruit juice sacs (Islam et al. 2014). In tomato fruit, the higher accumulation of hexose is correlated with an increased expression levels of both vacuolar *invertase* and *Sus* genes (Bastías et al. 2011), and interestingly, downregulation of the Sus activity inhibits the unloading capacity of sucrose (D'Aoust et al. 1999). Tian et al. (2012) suggested that sucrose accumulation during grape berry and strawberry fruit ripening might be controlled predominantly by Sus activity. In most fleshy fruits, such as pineapple (Zhang et al. 2012), grape (Martínez-Esteso et al. 2011), litchi (Yang et al. 2013), muskmelon (Wen et al. 2010), and tomato (Kortstee et al. 2007), the sucrolysis by Sus contributes to hexose accumulation and fruit quality formation.

As an important enzyme in plant growth and development, the regulation mechanisms of *Sus* expression were also studied in several plants. In grape berries, the *Sus* transcripts were repressed by glucose (Wang et al. 2014). In Arabidopsis seeds, *AtSUS2* expression could be induced by exogenous glucose but not by exogenous sucrose (Angeles-Núñez et al. 2012). The Sus activity may be regulated by tomato sucrose non-fermenting-1-related protein kinase 1 (Wang et al. 2012) and a rice calcium-dependent protein kinase (Asano et al. 2002).

Although strawberry is a model plant for study of non-climatic fruit ripening, the function and regulation of the *Sus* remains unclear. In the present study, a strawberry *Sus* gene, *FaSS1*, was cloned and silenced through virus-induced gene silencing. Additionally, its expression and regulation were also analyzed, finding that *FaSS1*, an abscisic acid/sucrose-inhibited gene, plays an important role in the regulation of strawberry fruit ripening.

Results

Transcriptome and SqRT-PCR analysis of *Sus* gene family in strawberry fruit development

Based on the description by Jia et al. (2011), four-stage fruits, including large green, white, initial red, and partial red fruits around the onset of ripening, were used for RNA-sequencing (RNA-seq). A total of eight *Sus* gene-like contigs were found and classified into three groups based on their predicted $\log_2$ expression levels (Fig. 1a). The contig comp64823_c0_seq1 and its complementary strand comp65427_c0_seq1 were expressed highest in large green fruits, then declined sharply and maintained relative high levels from white to partial red stages compared with other contigs, and thus the contig was named *FaSS1*

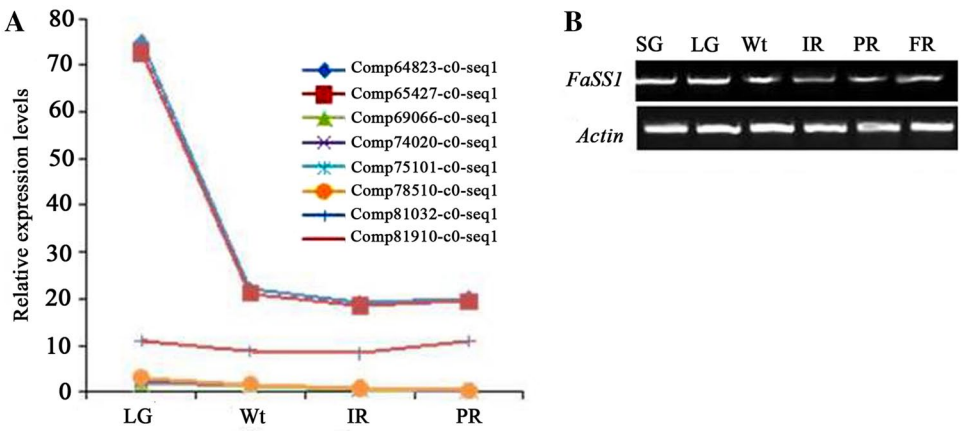

Fig. 1 Transcriptome data and SqRT-PCR analysis of sucrose synthase gene expression. **a** Eight contigs related to sucrose synthase were found and classified into three groups based on their $\log_2$ gene expression levels gained from RNA-seq data. **b** SqRT-PCR analysis of *FaSS*. *Actin* was used as internal control. *SG* small green fruits, *LG* large greening fruits, *Wt* white fruits, *IR* initial red fruits, *PR* partial red fruits, *FR* full red fruits

(loc101306372 in https://strawberry.plantandfood.co.nz/index.html). To further confirm the mRNA expression levels of *FaSS1* in developmental fruits, semiquantitative RT-PCR (SqRT-PCR) was also performed using the four-stage fruits. A similar trend of *FaSS1* expression was observed around the onset of the ripening (Fig. 1b), suggesting that the processes of the fruit ripening might be required for a high and stable expression of *FaSS1*.

Cloning of the *FaSS1* gene

To isolate the *FaSS1* gene, the sequence gained from RNA-seq was used as a query to search the strawberry gene library (https://strawberry.plantandfood.Co.nz/index.html) using the BLAST algorithm. A highly homologous sequence was found and the specific primers were designed. Finally, a 2421-bp sequence encoding 806 amino acids was cloned by RT-PCR. A protein domain search in the NCBI website (http://structure.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi) was performed using the amino acid sequence, in which a sucrose metabolism domain [GT1-sucrose-synthesis (Supplimentary Fig. 1A); red underlined (Supplimentary Fig. 1B)] and a sucrose-synthesis domain [sucrose-synth (Supplimentary Fig. 1A); green underlined (Supplimentary Fig. 1B)] were predicted. Thus, the putative strawberry *Sus* gene, *FaSS1*, was isolated successfully.

Functional analysis of *FaSS1* in strawberry fruit by virus-induced gene silencing

To investigate the role of *FaSS1* in developmental fruit, we generated tobacco rattle virus (TRV)-based pTRV1- and pTRV2-FaSS_{500} RNAi fruits using de-greened fruits according to the description by Jia et al. (2011). 2 weeks after inoculation, the surface of the control fruits inoculated with empty TRV vector only, turned fully red; in contrast, the inoculated sector on the surface of the RNAi fruits remained white (Fig. 2a).

To confirm the suppression of *FaSS1* expression at the molecular level, a SqRT-PCR analysis was performed using the RNAi and control fruits. The *FaSS1* transcripts were downregulated in the RNAi fruits in comparison with the control fruits (Fig. 2b, c). These results indicated that *FaSS1* plays a role in the regulation of strawberry fruit ripening.

Understanding the role of *FaSS1* in the regulation of fruit ripening

To further understand the role of *FaSS1* in the regulation of fruit ripening, we analyzed the soluble sugar contents, including sucrose, glucose, and fructose, together with the ripening-related events, including fruit firmness and anthocyanin contents in the RNAi and control fruits. We also assessed the transcripts of the ripening-related genes *polygalacturonase 1* (*PG1*), *pectate lyase 1* (*PL1*), and *xylitol dehydrogenase 2* (*XYL2*) for fruit firmness, and *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), and *dihydroflavonol 4-reductase* (*DFR*) for anthocyanin (Jia et al. 2011, 2013; Tian et al. 2012). The results showed that the sucrose contents were lower in RNAi fruits than in control fruits; however, the glucose and fructose contents were higher in RNAi fruits than in control fruits (Fig. 3a). Fruit firmness increased but the anthocyanin contents decreased in RNAi fruits compared with the control fruits (Fig. 3b).

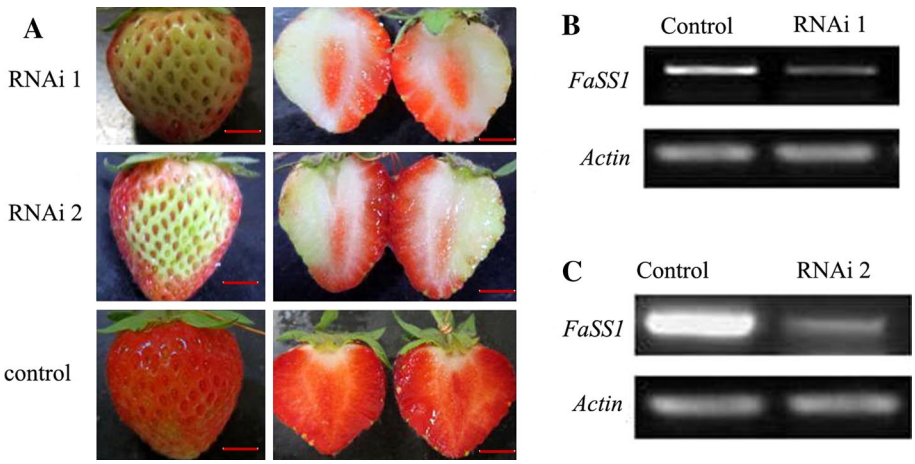

Fig. 2 Downregulation of *FaSS1* transcripts using virus-induced gene silencing in strawberry fruits. **a** 2-week-old large green fruits still attached to the plants were used for inoculation. The control fruits were inoculated with *Agrobacterium* containing the TRV only (control), while treated fruits were inoculated with *Agrobacterium* containing the TRV carrying a *FaSS1* fragment (RNAi1 and RNAi2). 2 weeks after inoculation, phenotypes were observed for the control fruits and RNAi fruits. **b, c** *FaSS* transcripts of the control and RNAi fruits were detected by SqRT-PCR analyses. *Actin* was used as internal control. *Red bars* 1.0 cm

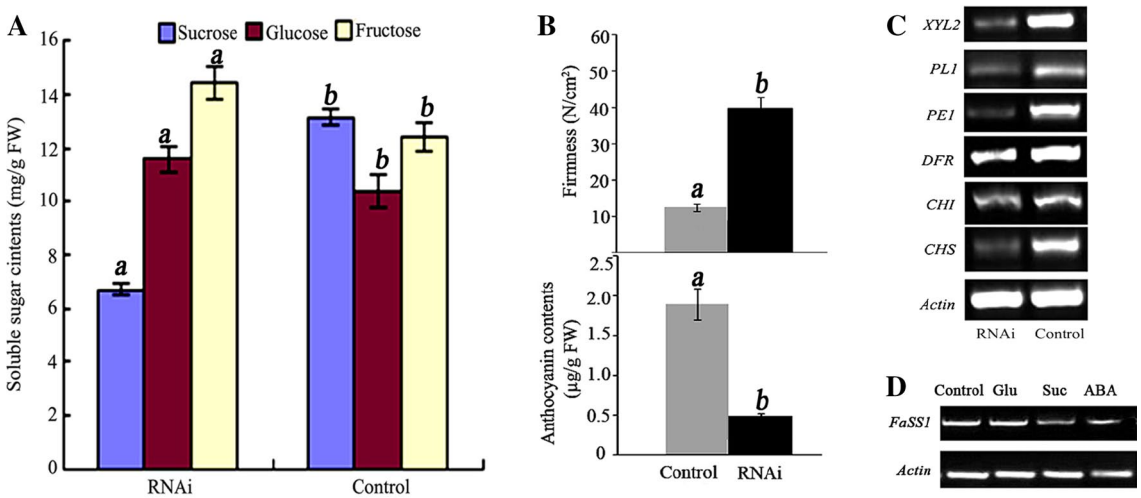

Fig. 3 Changes of the ripening-related parameters in RNAi fruit and effects of sugar and ABA on *FaSS1* transcripts in vitro. **a** Determination of soluble sugar contents by HPLC. The standard samples used were D-(+) glucose, D-(−) fructose, and sucrose (Sigma–Aldrich). **b** Determination of firmness and anthocyanin contents. **c** SqRT-PCR determination of transcripts of the ripening-related genes: *PG1, PL1*, and *XYL2* for firmness; *CHS, CHI*, and *DFR* for anthocyanin. **d** Fruit discs were treated by exogenous sucrose, glucose, and ABA according to the description by Jia et al. (2013). *FaSS1* transcripts were detected by SqRT-PCR after 2-h incubation. *Actin* was used as internal control. *Error bars* represent the SE (n = 3). *Different letters* indicate statistically significant differences at $P < 0.05$ as determined by Duncan's test

The SqRT-PCR analysis showed that the transcripts of the ripening-related genes, including *PG1, PL1, XYL2, CHS, CHI*, and *DFR*, were all significantly downregulated in RNAi fruits compared with the control fruits (Fig. 3c). The changes in the ripening-related events and gene transcript levels in RNAi fruits are consistent with a role of *FaSS1* in the regulation of the fruit ripening.

To explore the regulation of *FaSS1* expression, effects of ABA, sucrose (Suc), and glucose (Glu) on the mRNA expression levels of *FaSS1* were analyzed using a fruit-disc-incubation test in vitro. The SqRT-PCR analysis showed that, compared with the control incubated with 4% mannitol, 100 μM ABA or 4% sucrose significantly inhibited the mRNA expression levels of the *FaSS1* gene, respectively; in contrast, 4% glucose had no effect on the gene transcripts (Fig. 3d). The results demonstrated that *FaSS1* expression could be inhibited by ABA and sucrose.

Discussion

Sugar accumulation plays an important role in fruit development and quality, and sucrose, an end product of photosynthesis, is metabolized by Sus, which is as an initial step in the biosynthesis of storage products in many plant sink organs, including starch biosynthesis (Karrer et al. 1992; Borisjuk et al. 2002; Sreenivasulu et al. 2004; Li et al. 2013; Hou et al. 2014) and hexose accumulation (Kortstee et al. 2007; Wen et al. 2010; Martínez-Esteso et al. 2011; Li et al. 2013; Zhang et al. 2012; Yang et al. 2013). Although the Sus is suggested to play a dominant role in sucrose accumulation during strawberry fruit ripening (Tian et al. 2012), substantial evidence is yet lacking.

In the present study, we have provided physiological and molecular evidence to confirm the following hypothesis: (1) *FaSS1* transcripts are stably and constantly expressed during the red-color development and enlargement of the fruits (Fig. 1); (2) downregulation of *FaSS1* transcripts inhibits the fruit ripening, as evidenced by changes in its mRNA expression levels (Fig. 2) and the ripening-related parameters, including fruit firmness, and the anthocyanin and soluble sugar contents, as well as the variations in transcripts of the ripening-related genes, including *PG1, PL1, XYL2, CHS, CHI*, and *DFR* (Fig. 3); and (3) *FaSS1* expression could be inhibited by ABA and sucrose (Fig. 3).

Plant hormones and sugar accumulation are critical to fruit ripening (Jia et al. 2011; Chai et al. 2013; Sun et al. 2015). It was previously reported that both ABA and soluble sugar contents increase, on the whole, during strawberry fruit development, and this is especially true for sucrose, which increases slowly during de-greening and then increases rapidly during red-color formation (Jia et al. 2011). In the present study, *FaSS1* transcripts were most highly expressed in large green fruits, and then expression declined sharply and was maintained at a relatively high level after the white stage (Fig. 1). The rapid increase in the sucrose contents might result from the sharp decrease in *FaSS1* expression, suggesting a sucrolysis role of *FaSS1* in the developmental fruits. The rapid decline in *FaSS1* transcripts might result from the rapid increase in both ABA and sucrose contents, in that *FaSS1* expression could be inhibited by ABA and sucrose (Fig. 3). In the *FaSS1*-RNAi fruits,

the sucrose contents were significantly downregulated (Fig. 3), which might result from that the downregulation of *FaSS1* expression could inhibit phloem transport capacity for sucrose, similar to a result reported by Fallahi et al. (2008). Given that sucrose functions as a signal involved in the regulation of strawberry fruit development and ripening (Jia et al. 2013), as a result, the decreased sucrose in RNAi fruits might lead to unripening fruits. Our results have demonstrated that a high, constant and stable expression of *FaSS1* is required for the strawberry fruit ripening after white stage.

Materials and methods

Plant materials

Octaploid strawberry (*F. ananassa* cv. 'Beinongxiang') fruits were used in this study. Strawberry plants were grown under a glasshouse environment (20–25 °C, 70–85 % relative humidity, and a 14/10 h light/dark regime) in springtime during 2014–2015. Three hundred flowers on 40 strawberry plants were tagged during anthesis. Different developmental stages of fruits, including small green, big green, white, initial red, partially red and fully red fruits, were collected at 6, 15, 22, 24, 26, and 29 days after anthesis, respectively. Twenty fruits with uniform size were sampled at every stage (one replication), and quickly frozen in liquid nitrogen and stored at −80 °C until use.

RNA extraction, library construction, and RNA-seq

RNA was extracted using the RNeasy plant mini kit (Qiagen, Dusseldorf, Germany) from mixed receptacles (n = 3) for every stage fruit above. DNase digestion with RNase-Free DNase (Qiagen) was performed to remove contaminating DNA, and the RNA samples were then processed using the RNA library prep kit (New England BioLabs, Ipswich, MA, USA) and sequenced on the Illumina HiSeq2000 platform (Illumina, USA).

RNA-seq data analysis

The raw reads were filtered with the FASTQ_Quality_Filter tool from the FASTX-toolkit, and more than 35 bp and score higher than 20 were kept (Ming et al. 2012). All valid reads were combined to perform de novo splicing by the paired-end method with Trinity software (Wang et al. 2010). The longest transcripts per locus were used as a unigene. The bowtie2-2.2.2 software was used to compare reads with unigenes using the single-end mapping method (Wang et al. 2010; Ben et al. 2012). To compare the levels of unigene expression among the four libraries, the transcript level of each expressed unigene was calculated and normalised to the reads per kilobase of the exon model per million mapped reads (RPKM, Mortazavi et al. 2008). The significance of differential unigene expression was determined by using the Chi-squared test with a threshold of P = 0.05. P values were adjusted to account for multiple testing by using the false discovery rate (FDR) and assigned error ratio Q values (<0.05) (Benjamini et al. 2001). The unigenes with an adjusted P value of <0.05 and an absolute value of $\log_2$ (expression fold change) >1 were deemed to be differentially expressed, while the unigenes with an FDR-adjusted P value of <0.05 were considered statistically significant.

Cloning of *FaSS1* gene and SqRT-PCR analysis

The cDNA obtained above was used as a template for amplifying the *FaSS1* gene with primers designed from a strawberry gene library (https://strawberry.plantandfood.co.nz/index.html) (forward, 5′-ACCCACATCCCATTTCTCTCT-3′; reverse, 5′-CTCAGCCAACAGATTGCTTCT-3′). PCR was performed under the following conditions: 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for an additional 10 min. The PCR products were ligated into a T1-Sample vector and subsequently transformed into *Escherichia coli* DH5a. Positive colonies were selected using kanamycin (100 μg/ml), and sequenced by Huada China (Beijing, China). The primers for SqRT-PCR were designed as showed in Supplimentary Table 1. The entire process was repeated three times.

Construction of the virus vector and *agrobacterium*-mediated infiltration

The pTRV1 and pTRV2 vectors (Liu et al. 2002) were kindly donated by Dr. Liu Yu-le, Qinghua University. A 424-bp cDNA fragment of *FaSS1* was amplified using primers (sense, 5′-CGAGTTCCACACCTACCGTG-3′; and antisense, 5′-GAACAGCCGCGTGTCCTCCTC-3′). The amplified fragment (from 114 to 537 bp) was cloned into the T1-Sample vector (Trans), digested by *Xba*I and *Sma*I, and subsequently cloned into the virus vector *Xba*I-*Sma*I-cut pTRV2. The *Agrobacterium* strain GV3101 containing pTRV1, pTRV2, or the pTRV2 derivative pTRV2-*FaSS1* was used for RNAi. *Agrobacterium*-mediated TRV infiltration by syringe injection with a needle into strawberry fruits was performed as described by Fu et al. (2005).

Determination of firmness and anthocyanin and ABA contents

Three uniform strawberry fruits were used for the detection of firmness and anthocyanin and ABA contents,

respectively. Flesh firmness was analyzed on two sides of each fruit using a GY-4 fruit penetrometer (Digital Force Gauge, Shanghai, China). The strength of flesh firmness was recorded as $N \cdot cm^{-2}$. The anthocyanin content was analyzed using a spectrophotometer according to the methods described by Villarreal et al. (2010). The ABA content was analyzed using HPLC (high performance liquid chromatography) according to the methods described by Jia et al. (2011). The entire process was repeated three times.

Fruit disc incubation in vitro

Incubation of fruit disc with sucrose, glucose, and ABA in vitro was done as described by Jia et al. (2013): the discs were divided into four sections, one section was incubated in equilibration buffer with 4 % mannitol as control, the other were incubated in equilibration buffer with 4 % sucrose, 4 % glucose, and 100 μM ABA, respectively. The four sections were placed in 250 ml flask and shaking at 25 °C for 2 h. After washing with double distilled water, the tissues at each time points were frozen in liquid nitrogen and kept at –80 °C until use. The entire process was repeated three times.

Acknowledgments This work was supported the China National Science Foundation (Project 31471837, 31272144, 41473004) and the National Key Technology Supported Program of China (Project 2011BAD32B03), the Project of Construction of Innovative Teams and Teacher Career Development for Universities and Colleges under Beijing Municipality (Grant No. IDHT20140509), One Hundred Talent Program of Beijing Science and Technology (Grant No. LIRC201612), and Beijing Municipal Education Commission (Grant No. CEFF-PXM2016-014207-000038).

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