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Proanthocyanidins in seed coat's tegmen and endospermic cap inhibit seed germination in the bioenergy plant *Sapium sebiferum*

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Sapium sebiferum, a highly ornamental and bioenergy plant, is propagated by seed. Its seed coat contains germination inhibitors and needs long time stratification for germination. In this experiment, we discovered that *S. Sebiferum* seed coat (especially tegmen) and endospermic cap contained high levels of proanthocyanidins (PAs). Seed coat and endospermic cap removal induced seed germination whereas exogenous application with seed coat extract (SCE) or PAs significantly inhibited this process, suggesting that PAs in the seed coat played a major role in regulating seed germination in *S. sebiferum*. We further investigated how seed coat extract affected the expression of the seed germination-related genes. The results showed that SCE treatment upregulated the transcription level of the dormancy-related gene, abscisic acid (ABA) biosynthesis and signalling genes and gibberellins (GA) suppressing genes. SCE decreased the transcript levels of ABA catabolic, GA biosynthesis, reactive oxygen species (ROS) and nitrates signalling genes. Exogenous application of nordihydroguaiaretic acid (NDGA), gibberellic acid (GA_3), hydrogen peroxide (H_2O_2) and potassium nitrate (KNO_3) recovered seed germination in SCE supplemented medium. In this experiment, we highlighted the role of PAs, and its interactions with the other germination regulators, in the regulation of seed dormancy in *S. Sebiferum*.

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Abstract

Sapium sebiferum, a highly ornamental and bioenergy plant, is propagated by seed. Its seed coat contains germination inhibitors and needs long time stratification for germination. In this experiment, we discovered that *S. Sebiferum* seed coat (especially tegmen) and endospermic cap contained high levels of proanthocyanidins (PAs). Seed coat and endospermic cap removal induced seed germination whereas exogenous application with seed coat extract (SCE) or PAs significantly inhibited this process, suggesting that PAs in the seed coat played a major role in regulating seed germination in *S. sebiferum*. We further investigated how seed coat extract affected the expression of the seed germination-related genes. The results showed that SCE treatment upregulated the transcription level of the dormancy-related gene, abscisic acid (ABA) biosynthesis and signalling genes and gibberellins (GA) suppressing genes. SCE decreased the transcript levels of ABA catabolic, GA biosynthesis, reactive oxygen species (ROS) and nitrates signalling genes. Exogenous application of nordihydroguaiaretic acid (NDGA), gibberellic acid (GA_3), hydrogen peroxide (H_2O_2) and potassium nitrate (KNO_3) recovered seed germination in SCE supplemented medium. In this experiment, we highlighted the role of PAs, and its interactions with the other germination regulators, in the regulation of seed dormancy in *S. Sebiferum*.

Keywords: *Sapium sebiferum*, seed dormancy, tegmen, endospermic cap, proanthocyanidins, ABA, GA

Introduction

Seed germination is an important step of plant life-cycle because it decides the subsequent plant survival and its reproductive success. Seed coat plays an important role in retaining the dormancy. In many plant species, hard seed coat blocks the water uptake of seed (Baskin et al. 2000), restricts the gasses exchange and inhibits seed germination (Mcgill et al. 2017). Some plants like

Arabidopsis and *Rubus* contain phenolic compounds called proanthocyanidins (PAs) which inhibit the seed germination (Debeaujon & Koornneef 2000; Debeaujon et al. 2000; Jia et al. 2013; Jia et al. 2012; Liguo et al. 2012).

Chinese tallow (*Sapium sebiferum* L.) belongs to the Euphorbiaceae family and is native to eastern Asia (Esser 2002). It is popular because of its colourful autumn foliage (Zhao & Tao 2015). Tallow layer of its fruits contain highly saturated fatty acids, and highly unsaturated oil is found in the seed (Boldor et al. 2010). Tallow has been used for manufacturing soap, candles, cloth, and fuel, while the seed's oil can be used for making native paints and varnishes (Brooks et al. 1987; Jeffrey & Padley 1991). A single mature tree of *S. Sebiferum* produces many seeds. Estimated yield of *S. Sebiferum* tree is 4,700 litres of oil per hectare every year which far exceeds the average commercial yields those of traditional oilseed crops (Boldor et al. 2010; Webster et al. 2006).That is why, recently, *S. Sebiferum* has become a species of interest as a source of biodiesel (Gao et al. 2016)

Sexual propagation is an easy method of commercial propagation, and it's being used widely for the commercial propagation of a large number of plant species, including many bio-energy plants like *S. Sebiferum*. However, poor rate of seed germination due to deep dormancy has seriously limited its use (Li et al., 2011).

We observed that *S. Sebiferum* seeds have hard, dark brown to blackish seed testa and reddish brown tegmen. From the study of Debeaujon et al. (2000); Wada et al. (2011), we hypothesized that inhibitors found in *S. Sebiferum* could be the proanthocyanidins (PAs). PAs inhibit seed germination by influencing ABA, GA and ROS regulatory genes (Debeaujon & Koornneef 2000; Debeaujon et al. 2000; Jia et al. 2013; Jia et al. 2012; Liguo et al. 2012). Whether PAs response to nitrates signalling or not? It is not clear yet. Here we conducted several experiments to test our

hypothesis and to find answers to our above questions. In our experiment, seed coat especially its tegmen layer has high concentrations of PAs. Removal of seed coat can promote the seed germination. We found that intact and dormant seed accumulated PAs contents in the endospermic cap (ESC). We found dynamic changes in PAs contents in the endospermic cap during imbibition. Removal of endospermic cap gave maximum seed germination. Concentrated sulfuric acid broke the *S. Sebiferum* seed coat-imposed dormancy by degrading PAs of the seed coat. We tested the effect of *S. Sebiferum* seed coat extract (SCE) on seed germination and compared it with pure PAs (>95%), we found that germination inhibitory effect of the SCE was same as the effect of pure PAs. The SCE effected the transcriptional changes of dormancy-related genes, GA-, ABA-, ROS and nitrates-related genes. The seed primed with NDGA, GA_3, H_2O_2 and KNO_3 recovered the germination on SCE supplemented medium.

Materials and methods

Seed material collection and storage

S. Sebiferum seeds were harvested from the plants grown in the experimental field of Hefei Institute of Physical Science, Chinese Academy of Sciences (31° 52′ 0″ N, 117° 17′ 0″ E), Anhui, China. Seeds were filled in nylon bags and stored at room temperature.

Stock Solutions preparation

The GA_3 and NDGA were purchased from SIGMA-ALDRICH® while H_2O_2 and KNO_3 were purchased from Sangon Biotech Co. Ltd. The GA_3 and NDGA stock solutions were prepared by dissolving in 80% methanol. H_2O_2 and KNO_3 stock solutions were prepared by dissolving in distilled water. All stock solutions were diluted in distilled water for making a working solution.

Seed coat extraction, application and PAs analysis

Seed coat extract was prepared as described by Li et al. (2012) with little modification. In more detail: *S. Sebiferum* tree seed coats were ground into powder. Ten-gram seed coat powder was dissolved in 200 mL of 80% (v/v) aqueous methanol and placed in a refrigerator at 4 °C for 24 hours. After centrifugation at 4500 rpm at 4 °C for 10 minutes, the supernatant was evaporated under vacuum at 40 °C.

SCE and PAs were adjusted to 0.1%, 0.2%, and 0.3% in 0.5×MS containing 15mg/L sucrose and 8 g/L agar before sterilization. Media was autoclaved at 121 °C for 22 minutes and poured into 9 cm diameter Petri dishes (20 ml each) under laminar flow hood. Pure proanthocyanidins (UV≥95%, CAS 4852-22-6) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Endospermic cap and endospermic proanthocyanidins were analyzed by the previously used protocol as described by Xuan et al. (2014). Seed coat proanthocyanidins contents were analyzed conventional HCl–vanillin assay (Herald et al. 2014) and pure proanthocyanidins (UV≥95%) was used as a reference.

Pre-germination treatments and Germination conditions

Seeds were washed with 1% Sodium hydroxide (NaOH) for removing white tallow. Sulfuric acid scarification was done by dipping seed in 98.08% concentrated sulfuric acid (CAS 7664-93-9 purchased from Shanghai Chemical Reagent Co., Ltd.) at 4 °C. After sulfuric acid treatment, seeds were washed in running tap water for five times. Intact seed and scarified seed were sown in 10×10 cm pots containing peat moss. For seed coat extract's effect verification seed were carefully uncoated with a scissor. Uncoated seed kernels were sterilized by washing two times with 70% ethyl alcohol for 30 seconds and then incubated in 20% Sodium hypochlorite (NaClO) for 10

minutes. After rinsing NaClO off, the seeds were washed three times with autoclaved water and dried by blotting over sterilized filter papers. For endospermic cap (ESC) removing experiment, ESC was removed with the sterilized blade in the laminar flow hood.

Sterilizes uncoated seed were sown in 0.5×MS containing 15 g/L sucrose for control treatment and same medium supplemented with 0.1%, 0.2%, and 0.3% SCE and PAs separately. For hormonal and signalling compounds treatments, priming was done in desirable concentration in distilled water for 12 hours at 25 °C. Germination conditions for all experiments were maintained as day/night temperatures of 25/20 °C, with 16- hours light/8 hours dark photoperiod, 150 µmol m^{-2} s^{-1} photosynthetic photon flux density and 70 % relative humidity. Protrusion of radical from micropyle was considered as the standard of seed germination. Germination data were recorded every day after germination start (5 days after imbibition). Shoot and root length were measured manually by the ruler. All seed germination pictures were taken by NIKON D90 containing NIKON DX AF-S NIKKOR 18-105mm lens. All vanillin essay's pictures were taken by Olympus SZX10 stereo microscope having TUCSEN 6.0 megapixel USB 2.0 colour camera.

Primer designing, RNA extraction, cDNA synthesis, and RT–qPCR conditions

The full sequences of all genes were obtained by local blasting Arabidopsis amino-acids sequence in blast-2.2.31. Local blast library was built by flower bud transcriptome (Accession: SRX656554, https://www.ncbi.nlm.nih.gov/sra/SRX656554) of *S. Sebiferum* (Yang et al. 2015). List of all genes full mRNA sequences is available in **S. Data 1**. Primers used for qPCR were designed by using primer premier 6. The Tm of the primers was between 59 and 61°C and list of all primers are given in **S. Table 1**. For gene expression analysis, seed samples were taken the 3rd and 6th day after imbibition. Samples were frozen in liquid nitrogen and stored at -80°C. RNA was extracted

by using E.Z.N.A® plant RNA extraction kit (OMEGA Pro -TEK) according to given protocol. 500 ng RNA of each sample was reverse transcribed using the cDNA synthesis SuperMix (TransGen Biotech.) according to given protocol. The cDNA samples were diluted 25X with sterile water. For each qPCR, 9µl of the sample, 10µl of the 2X QuantiNova SYBR Green PCR Master Mix (QIAGEN) and 0.5 µl of each primer was added to make a final volume 20µl. The RT–qPCRs were run on a Light Cycler®96 (Roche). The qPCR program run consisted of the first step at 95°C for 3 min and afterwards 45 cycles alternating between 15 s at 95°C, 15s at 60°C and 15s at 72°C.

Results

Sulfuric acid scarification is an efficient method to promote the seed germination by cracking seed coat and degrading the proanthocyanidins in seed coat in *S. sebiferum*.

S. Sebiferum has hard seed coat which is considered as a main factor of dormancy. We conducted an initial experiment to break *S. Sebiferum* seed dormancy. We treated *S. Sebiferum* seed with concentrated sulfuric acid from 10 to 60 minutes incubation time to investigate effects of sulfuric acid on the seed coat surface, PAs contents and water uptake of seed as well as seed germination and seedling growth. We found that sulfuric acid digested the seed coat external surface and caused cracks. The incubation time of the 10 and 20 minutes digested epidermal layer of the seed coat while the 30-, 40-, and 50 minutes incubation caused mild cracks in the seed coat. But the 60 minutes incubation in sulfuric acid caused deep cracks in seed coat **(Fig. 1A)**. When we measured the proanthocyanidins contents of 0-, 10-, 20-, 30-, 40-, 50-, and 60 minutes scarified seed, we found that sulfuric acid scarification degraded the PAs contents with incubation time **(Fig. 2)**.

We investigated the water uptake of intact seed and sulfuric acid scarified seed by (Li et al. 2012) method. Our results showed that water uptake gradually increased from untreated (control) 0 to 10 minutes, 20-, 30-, 40-, 50-, and 60 minutes. Water uptake percentage of 20-, 30-, 40-, 50-, and 60 minutes scarified seed was significantly different from control. But the water uptake percentage of 10 minutes scarified was not significantly different from control (**Fig. 1B).** From germination analysis, we found that 0-, 10-, 20-, 30-, 40-, 50-, and 60 minutes incubation showed germination of 2±0.4, 40±0.8, 45±4, 52±5.3, 68±2.4, 65±4 and 55±4% respectively (**Fig. 1C, S. Fig. 1**). Control and scarified seed's germination was significantly different (P=0.05).When we measured the root and shoot length of seedling of 45-day-old seedling, we found that the seedlings whose seeds were scarified 30-, 40- and 50 minutes incubation in sulfuric acid, have more root and shoot length than the seedlings of 0-, 10-, 20- and 60 minutes incubated seeds in sulfuric acid. (**Fig. 1 C and D**). These results suggested that sulfuric acid scarification promoted the seed germination by cracking the seed coat.

Tegmen and endospermic cap contained proanthocyanidins which inhibited seed germination.

We removed the seed coat and cultivated the uncoated seed on 0.5×MS medium. We found that the uncoated seeds showed 85±5% seed germination within seven days (**Fig. 3B and S. Fig. 2)**. Previous studies showed that *S. Sebiferum* seeds contain some inhibitors that can inhibit the cabbage seed germination (Li et al., 2012; Qian et al., 2016). We extracted the seed coat (Testa and tegmen combined in 80% (v/v) methanol, named the dry extract as SCE). To determine the impact of SCE on seed germination, we uncoated the seed, and sowed the uncoated seed on 0.5×MS (Murashige and Skoog medium) supplemented with 0.1%, 0.2% and 0.3% concentration of SCE. We found that SCE can inhibit seed germination (**Fig. 3B)**. Previous studies showed that

some plants seed coat contained proanthocyanidins that can inhibit seed germination (Jia et al. 2013; Wada et al. 2011). So we determined proanthocyanidins concentration in *S. Sebiferum* seed coat's testa and tegmen separately and also SCE by Vanillin assay. We found that testa and tegmen contain 3±2 and 65±5% (mean±sd) of proanthocyanidins respectively, while SCE contained 30±3% (mean±sd) proanthocyanidins (**Fig. 3A)**. We also tested seed germination in 0.1, 0.2 and 0.3% proanthocyanidins supplemented in 0.5×MS, the results showed that proanthocyanidins significantly inhibited the seed germination of *S. sebiferum* (**Fig. 3B**). These results suggested that the proanthocyanidins in the seeds of *S. sebiferum* played a major role in maintaining the seed dormancy of *S. sebiferum*.

Further, we found that the seed of *S. Sebiferum* contains dark brownish colour endospermic cap (ESC). When cultivated on 0.5×MS, we found that dark brownish ESC became darker in some dormant seeds (**Fig. 4A**). We removed that ESC very carefully with sterile surgery blade and cultivated those ESC removed seed on 0.5×MS. We found the seed without ESC showed 100% seed germination within five days which was significantly different than the seed with ESC (**Fig. 4B and 4C**). We hypothesized that the dark brownish ESC might accumulate proanthocyanidins, which inhibited seed germination. Then we determined proanthocyanidins by vanillin assay, interestingly, we found that ESC gives red colour which is an indication of proanthocyanidins. We also tested the dynamic changes in ESC of intact seeds cultivated in peat moss media. We found that the concentration proanthocyanidins of gradually decreased with imbibition time and after completely diminishing of proanthocyanidins in ESC, the seed showed the sign of germination (**Fig. 4D)**.

Effect of seed coat extract on the expression level of dormancy-related genes

We found the crosstalk effect of seed coat with dormancy and GA, ABA, ROS and nitrates related genes expression. To investigate that whether this effect in because of PAs, we sowed the uncoated seed on 0.5×MS (control), 0.5×MS supplemented with 0.3% seed coat extract (SCE) and 0.1% PAs separately. We compared the relative expression of GA, ABA, ROS, nitrates and dormancy related genes between the control, SCE and PAs treatments. It is very important to check the expression of dormancy specific genes while studying seed dormancy. *Delay of Germination 1 (DOG1)* is a dormancy specific gene which positively regulates the seed dormancy (Dekkers et al. 2016; Footitt et al. 2017). We found that the expression level of *SsDOG1* was significantly higher in SCE and PAs as compared to control on the 3rd and 6th day of imbibition. But the expression level of *SsDOG1* on both time points was not significantly different between SCE and PAs (**Fig. 5**).

It has been reported that dormant seed has high levels of ABA (Millar et al. 2010). To find the transcriptional changes of ABA-related genes during different imbibition period of different treatments, we selected 9-cis-epoxycarotenoid dioxygenases 6 (*NCED6*), *INSENSITIVE3* (*ABI3*) *and CYP707A2* as ABA biosynthesis, signalling and catalyzing gene respectively (Dekkers et al. 2016; Footitt et al. 2011). We also found that PAs and SCE both promoted the expression level of *SsNCED6* on both 3rd and 6th day of imbibition. Expression levels of *SsABI3* were not significantly different between control, PAs and SCE on the 3rd day of imbibition. Interestingly, on the 6th day of imbibition, the *SsABI3* expression level was remained same in SCE and PAs but dropped in control. In SCE and PAs, the *SsCYP 707A2* expression decrease gradually with time, while in control the *SsCYP 707A2* expression is higher during both 3rd and 6th day of imbibition (**Fig. 5**).

We selected GA biosynthesis gene (*GA3OX1*), GA inactivating genes (*GA2OX*), signalling genes and negative regulator of GA like *GAI* (*GIBBERELLIC ACID INSENSITIVE*) and *RGL2*

(*REPRESSOR-OF-GA1 2*) (Lee et al. 2002; Matsushita et al. 2007; Ravindran et al. 2017; Rieu et al. 2008; Shen et al. 2016). PAs and SCE significantly repressed the *SsGA3OX1* expression level as compared to control on the 3rd while *SsGA3OX1* transcript remained unchanged on the 6th day in both SCE and PAs. In PAs and SCE, the expression level of *SsGA2OX* was increased on the 3rd day and then decreased non-significantly on the 6th day of imbibition. In control treatment, *SsGA2OX* transcription was decreased gradually with time. *SsRGL2* and *SsGAI* expression level were higher in SCE and PAs as compared to control during both 3rd and the 6th day of imbibition **(Fig. 6).**

Reactive Oxygen species are highly active during seed germination. *MITOGEN-ACTIVATED PROTEIN KINASE* (*MPKs*) protein regulated the ROS signalling. Among the *MPKs* genes, *MPK6* is highly active during seed germination (Oracz et al. 2009; Oracz & Karpinski 2016). In our experiment, effects of PAs and SCE were negative on *SsMPK6* transcription. Relative expression of the *SsMPK6* was decreased in the seed growing on SCE and PAs with time. On the other hands, in the control treatment, the transcription levels of the *SsMPK6* increased from the 3rd to the 6th day of imbibition (**Fig. 7).**

Among the soil nutrients, nitrates play an important role in seed germination with a specific molecular mechanism(Lara et al. 2014). To investigate the impact of seed coat on nitrates signalling, we selected nitrates signalling genes like *NIN-LIKE-PROTEIN 8* (*NLP8*) and *CBL-INTERACTING PROTEIN KINASE 23* (*CIPK23*) (Footitt et al. 2017; Yan et al. 2016). The transcript level of *SsNLP8* was higher in control as compared to PAs and SCE treatments in both 3rd and the 6th day of imbibition. PAs and SCE treatments decreased the expression level of *SsCIPK23* gradually as compared to control on both the 3rd and 6th imbibition day (**Fig. 7).**

To find out the crosstalk effect of seed coat's proanthocyanidins with GA, ABA, ROS, nitrates, we primed the uncoated seed in sterile water (for control), GA_3 (50 µM), nordihydroguaiaretic acid (NDGA, 'ABA biosynthesis inhibitor', 50 µM), H_2O_2 (20 mM) and 0.4% KNO_3 separately overnight at room temperature and sowed the primed seed in 0.5×MS medium supplemented with 0.3% SCE. After seven days imbibition, the control, GA_3, NDGA, H_2O_2 and KNO_3 priming showed 36±4.8, 97.22±4.8, 91.9±1, 93.44±3 and 97.22±4.8 (mean±sd) percent germination respectively on 0.5×MS medium supplemented with 0.3% SCE. We found that all of our treatments significantly (P=0.05) promoted germination as compared to the control (**Fig. 8 and S. Fig. 3).**

Discussion

Seed coat imposed seed dormancy

Seed dormancy is a condition in which a viable seed is unable to germinate even in favourable conditions (Bewley 1997; Leubnermetzger 2006). Seed coat plays an important role in retaining the dormancy. We found that *S. Sebiferum* seed has seed coat-imposed dormancy. We detected proanthocyanidins (PAs) in *S. Sebiferum* seed coat and endospermic cap. We found that PAs presented in seed coat's tegmen and endospermic cap reduced the seed germination. PAs play an important role in seed coat hardness and cause physical dormancy. PAs bind to the proteins and create the hard seed coats which may act as a mechanical barrier (Debeaujon et al. 2000; Wada et al. 2011). Sulfuric acid has been used for breaking seed dormancy (Statwick 2016). Application of concentrated sulfuric acid degrades the PAs of the seed coat (**Fig. 2**). For example, the plant species like *Rubus coreanus* and *Rubus hoffmeisterianus* have seeds with low concentrations of PAs, require less time of sulfuric-acid scarification, and have high germination rate among *Rubus*

261 species (Wada et al. 2011). Sulfuric acid scarification successfully broke the *S. Sebiferum* seed
262 dormancy. We also found that tegmen layer of the seed coat, which is tightly bound to testa,
263 contains an abundance of proanthocyanidins (**Fig. 3A**). In intact seed, tegmen layer tightly covers
264 the endosperm and which could be one of the main reasons of seed dormancy. Contrastingly, in
265 scarified seed, seed coat started to rupture on the second day of imbibition. Cracked seed coat split
266 from the endosperm, loosen the tegmen-endosperm contact that could induce the water uptake,
267 oxygen diffusion and also reducing mechanical resistance to radical emergence (Chaves et al.
268 2017; Schelin et al. 2003).

269 The seed coat reduces the water uptake of seed thus causes seed dormancy (Baskin et al., 2000).
270 In this study, we found that water uptake (%) was significantly lower in intact seed as compared
271 to 20-, 30-, 40-, 50-, and 60 minutes acid scarification (**Fig. 1A**). Water uptake (%) in 10 minutes
272 scarified seed and the intact seed was not significantly different but the PAs contents and seed
273 germination percentage were significantly different (**Fig. 1B, C and 2)**. It suggested that *S.*
274 *Sebiferum* seed coat permeability is enough for water uptake which is necessary for seed
275 germination. It is agreement with the results of Mcgill et al. (2017) which showed that the rapid
276 uptake of water within the first hour of imbibition indicates that the *M. hortensia* seed coat is not
277 acting as a water impermeable barrier preventing germination. *S. Sebiferum* seed coat extract
278 (SCE) significantly inhibit the seed germination of lettuce seed (Qian et al. 2016). Previously, it
279 has been found that PAs present in the seed coat of *Arabidopsis* and Rubus seed can prohibit the
280 seed germination (Debeaujon et al. 2000; Liguo et al. 2012; Wada et al. 2011). So, we also
281 compared seed germination between SCE and pure PAs (>95%). We found that SCE and PAs
282 significantly inhibited the seed germination of uncoated *S. Sebiferum* seed as compared to control
283 (**Fig. 3B and S. Fig. 2**). When we investigated the PAs contents in SCE, we found that SCE

contained 30% PAs (**Fig. 3A**). So the PAs present in SCE could be the germination inhibitory compound which could play a major role in *S. Sebiferum* seed dormancy. Sulfuric acid scarification significantly reduced the PAs contents in the seed coat with the incubation time. Thus the breaking dormancy effect of sulfuric acid scarification could be because of degradation of the PAs contents in seed coat **(Fig. 2 and S. Fig. 1)**.

S. Sebiferum seed accumulated PAs in endospermic coat which can have a role in dormancy

Endospermic cap (ESC) covers the Radical tip (RAD) in *S. Sebiferum* seed. The majority of key genes for seed germination are expressed in ESC and RAD (Morris et al. 2000). PAs can disturb the ABA/GA homoeostasis and modulate ROS level in radicle by disturbing the transcription of key genes involved in ABA, GA and ROS regulation (Debeaujon & Koornneef 2000; Jia et al. 2013; Jia et al. 2012; Liguo et al. 2012). ABA/GA homoeostasis and ROS activity cause rupturing of ESC by losing the ESC cells wall and also allow radical protrusion (Graeber et al. 2010; Muller et al. 2006; Voegele et al. 2012).In this experiment, we detected PAs contents in ESC of dormant seed **(Fig. 4A**).We found that ESC removal can give 100% germination on 4rth day of imbibition. In non-dormant seed, the endospermic ESC's PAs was dissolved gradually with imbibition time (**Fig. 4B, C and D**). And as the ESC's PAs were dissolved, the ESC started to rupture and let the radicle protrude. **(Fig. 4D**).

Seed coat extract may regulate the expression of *DOG1*, a key gene involved in seed dormancy

A high proportion of the genome information, approximately 78,154 transcripts of the mature dry seed of *S. Sebiferum* were identified (Divi 2016). Proteomic studies revealed that all the proteins

translation required for seed germination are translated from stored mRNAs (Sano et al. 2012). Seed coat can also regulate seed germination by altering the transcription levels of stored mRNAs (Jia et al. 2012). *DOG1* is a key gene required for the induction of dormancy; acts as a timer for seed dormancy release and it is abundance in freshly harvested seeds (Dekkers et al. 2016; Nakabayashi et al. 2012; Nguyen et al. 2012). We found that *SsDOG1* transcriptional level was higher in PAs and SCE than control. But in both PAs and SCE, the *SsDOG1* expression levels decreased from 3rd to the 6th day of imbibition, which is suggesting that effect of proanthocyanidins on *SsDOG1* is temporary. So, the seed sowed in SCE and PAs also started to show a low percentage of germination (**Fig. 4D, 5 and S. Fig. 2**). Which is suggesting that for breaking dormancy, the decrease of *SsDOG1* is necessary (Footitt et al. 2017). Hence, PAs in seed coat may regulate dormancy specific genes and are responsible for seed coat-imposed dormancy.

Seed coat extract induced the seed dormancy by influencing the expression of ABA, GA, ROS and nitrates related genes

Seed coat plays a key role in seed embryo protection and keeps it dormant under unfavourable conditions. It has been suggested that seed coat induced the seed dormancy by influencing on transcription levels of ABA and GA biosynthesis or degradation genes and unbalance between GA/ABA homeostasis (Debeaujon & Koornneef 2000; Debeaujon et al. 2000; Liguo et al. 2012). In our study, the *SsGA3OX1* expression level was decreased in SCE and PAs treatments as compared to control on the 3rd day of imbibition.While on the 6th day of imbibition, the transcription level of *SsGA3OX1* remained unchanged in PAs and SCE treatments. Which is suggesting that PAs of SCE inhibit the expression level of GA biosynthesis gene. PAs and SCE regulated the transcription levels of *SsGA2OX*, *SsRGL2* and *SsGAI* positively **(Fig. 6).** The *GA2OX*, *RGL2* and *GAI* are the repressors of seed germination. Gibberellins improve the seed

329 germination by downregulating the *RGL2* and *GAI* which are the main factors repressing seed
330 germination by negatively regulating GA response (Lee et al. 2002; Ravindran et al. 2017)**.**
331 Exogenous application of GA_3 recovered the seed germination of SCE supplemented medium (**Fig.**
332 **8 and S. Fig. 3**) which agreed with the results of Debeaujon & Koornneef (2000).

333 We found that PAs and SCE both promoted the expression level of *SsNCED6* on the 3rd day
334 of imbibition and on 6th day *SsNCED6* expression level decreased, which is showing that PAs
335 regulate the seed dormancy my maintaining the high levels of ABA biosynthesis in imbibed seed
336 (Millar et al. 2010)**.** The expression level of *SsABI3* in PAs and SCE was almost three folds higher
337 than control on the 6th day of imbibition, which is suggesting that PAs may regulate ABA
338 signalling in the imbibed seed. In PAs and SCE treated seed the *SsCYP 707A2* expression decrease
339 on the 3rd day of imbibition. Interestingly, on the 6th day of imbibition, the *SsCYP 707A2*
340 expression in control was significant than SCE, but not significantly different than the PAs (**Fig.**
341 **5**). Which is revealing that the SCE suppressed the *SsCYP707A2* transcription level more than
342 PAs. The *S. Sebiferum* SCE may contain seed germination inhibitory compounds including PAs.
343 These inhibitory compounds and PAs upregulate the ABA biosynthesis and signalling genes while
344 down-regulating the ABA catabolic gene thus suppress the seed germination (Debeaujon &
345 Koornneef 2000; Jia et al. 2012; Liguo et al. 2012; Wada et al. 2011). In our experiment, the seed
346 primed in NDGA recovered the seed germination when grew on SCE supplemented medium (**Fig.**
347 **8 and S. Fig. 3**). NDGA inhibits ABA accumulation in seed by suppressing the ABA biosynthesis
348 (*NCEDs*) genes activity (Han et al. 2004).

349 ROS and nitrates are also important players of seed germination due to their role in the
350 maintenance of ABA/GA homeostasis (Debeaujon & Koornneef 2000; Jia et al. 2012; Lara et al.
351 2014; Liu et al. 2010; Yan et al. 2016; Zhou et al. 2015). In our experiment, exogenous application

of PAs and SCE in growing medium significantly reduced the transcription level of *SsMPK6* **(Fig. 7)**. H_2O_2 is well-known for promoting seed germination which activates the *MPK6* expression level (Wang et al. 2010). In this experiment, H_2O_2 primed seed recovered the germination on SCE containing a medium (**Fig. 8 and S. Fig. 3**). Previously, it has been found that mutation of Arabidopsis transparent testa 8 (*TT8*) lacked PAs accumulation in its testa and produced a high level of H_2O_2 after imbibition (Jia et al. 2013; Liu et al. 2010). H_2O_2 is the main kind of ROS in plants which regulate seed germination through GA / ABA metabolism and signalling, (Jia et al. 2013; Jia et al. 2012; Liu et al. 2010). Our RT-PCR analysis revealed that relative expression of nitrates regulatory genes like *SsNLP8* and *SsCIPK23* increased along with *SsCYP707A2* in control as compare to SCE and PAs **(Fig. 7)**. KNO_3 priming promoted the seed germination percentage on SCE supplemented medium (**Fig. 8 and S. Fig. 3**). KNO3 is a source of nitrates which activate the expression of nitrates signalling genes *NLP8* and *CIPK23* which induce seed germination. *NLP8* binds directly to the promoter of *CYP707A2* and reduces the abscisic acid levels in the nitrate-dependent manner (Footitt et al. 2017; Liu et al. 2010; Yan et al. 2016). KNO3 is also involved in the production of nitric oxide by regulating the activity of nitrate reductase enzyme in nitric oxide biosynthesis which removes the seed dormancy (Lara et al. 2014).

Conclusion

In this experiment, we found that the *S. Sebiferum* seed contains a plenty of PAs in the tegmen layer of the seed coat. The tegmen layer tightly binds to endosperm and may regulate the ABA/GA homeostasis level in seed by influencing the transcription of ABA and GA regulatory genes. Endosperm absorbs PAs which may interact with ROS and nitrates signalling genes, which may regulate the key genes of GA/ABA homeostasis in the imbibed seed (Debeaujon & Koornneef 2000; Jia et al. 2012; Lara et al. 2014), and caused dormancy in *S. Sebiferum* seed. Further studies

of molecular levels are needed to find out the relationship between ABA, GA, ROS, nitrates regulatory enzymes and PAs of seed coat's tegmen. Endospermic cap accumulates PAs which also may counteract the ROS and GA related enzymes those are necessary for the radical tip to weaken and rapture endospermic cap, and help to protrude the radical tip. In our experiments, we found the dynamic changes in PAs levels of the endospermic cap but which specific environment or enzymes are required to catabolize PAs, it remained a debate for the future. To generalize the phenomena, especially, "endospermic cap accumulates PAs and its dynamic changes during germination", more investigations from other species seed are required.

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537

Figure 1

Sulfuric acid (SA) scarification significantly promoted the seed germination of *Sapium sebiferum*.

A, Effect of SA scarification time on seed coat, red arrows indicate the bruises, scars and cracks caused by SA. Bars 1 mm. **B,** SA impacts on water uptake in the seed. **C,** SA induced seed germination of *S. Sebiferum*. **D** and **E,** Impact of SA on shoot and root length of seedlings respectively. Shoot and root length was measured after 45 days of imbibition. Data shown are means±sd (n=3). Means with different letters are significantly different at $P < 0.05$ using Duncan's multiple range HSD Post hoc test.

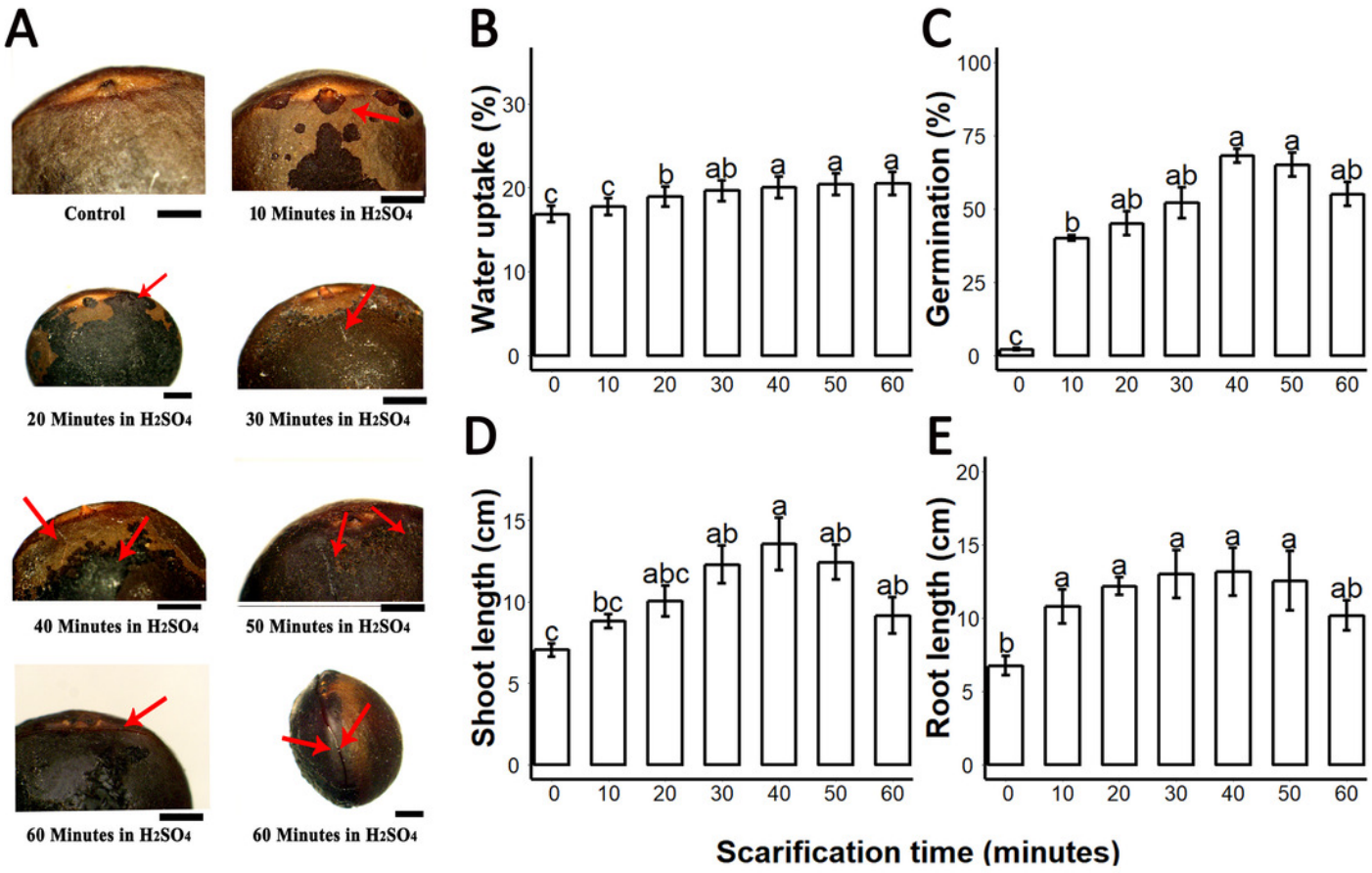

Figure 2(on next page)

Impact of sulfuric acid scarification on PAs contents of *S. Sebiferum* seed coat.

Seeds of *S. Sebiferum* were dipped in concentrated sulfuric acid for 10, 20, 30, 40, 50, and 60 minutes separately. PAs contents of acid scarified were determined by vanillin assay. Data shown are means±sd (n=3). Means with different letters are significantly different at $P < 0.05$ using Tuckey's HSD Post hoc test.

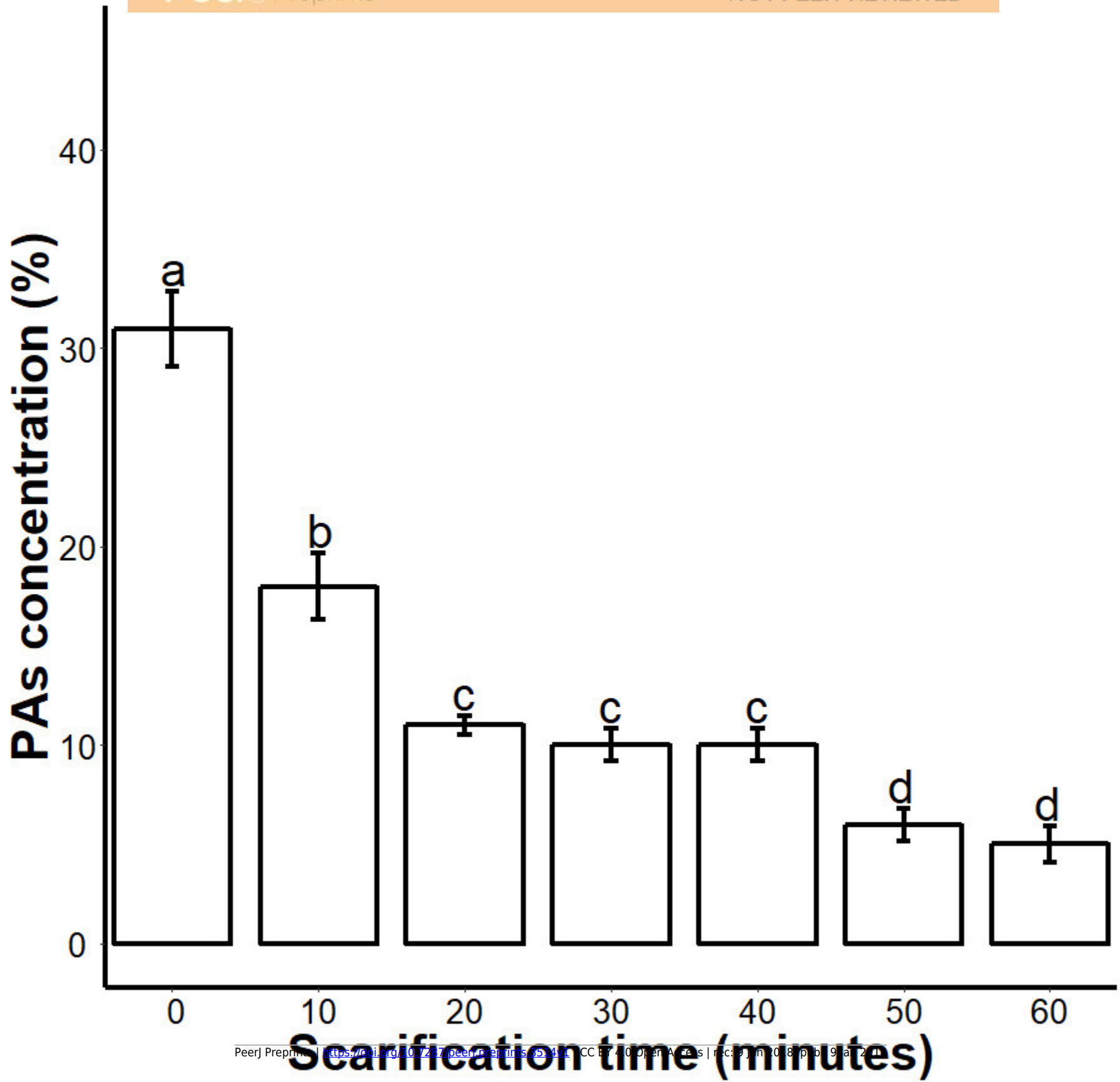

Figure 3(on next page)

Impacts of exogenous application of SCE and PAs on seed germination.

A, PAs contents in SCE, tegmen and testa of *S. Sebiferum* seed coat. **B,** Impact of different concentrations of SCE and PAs on seed germination. Data shown are means±sd (n=3). Means with different letters are significantly different at $P < 0.05$ using Tuckey's HSD Post hoc test.

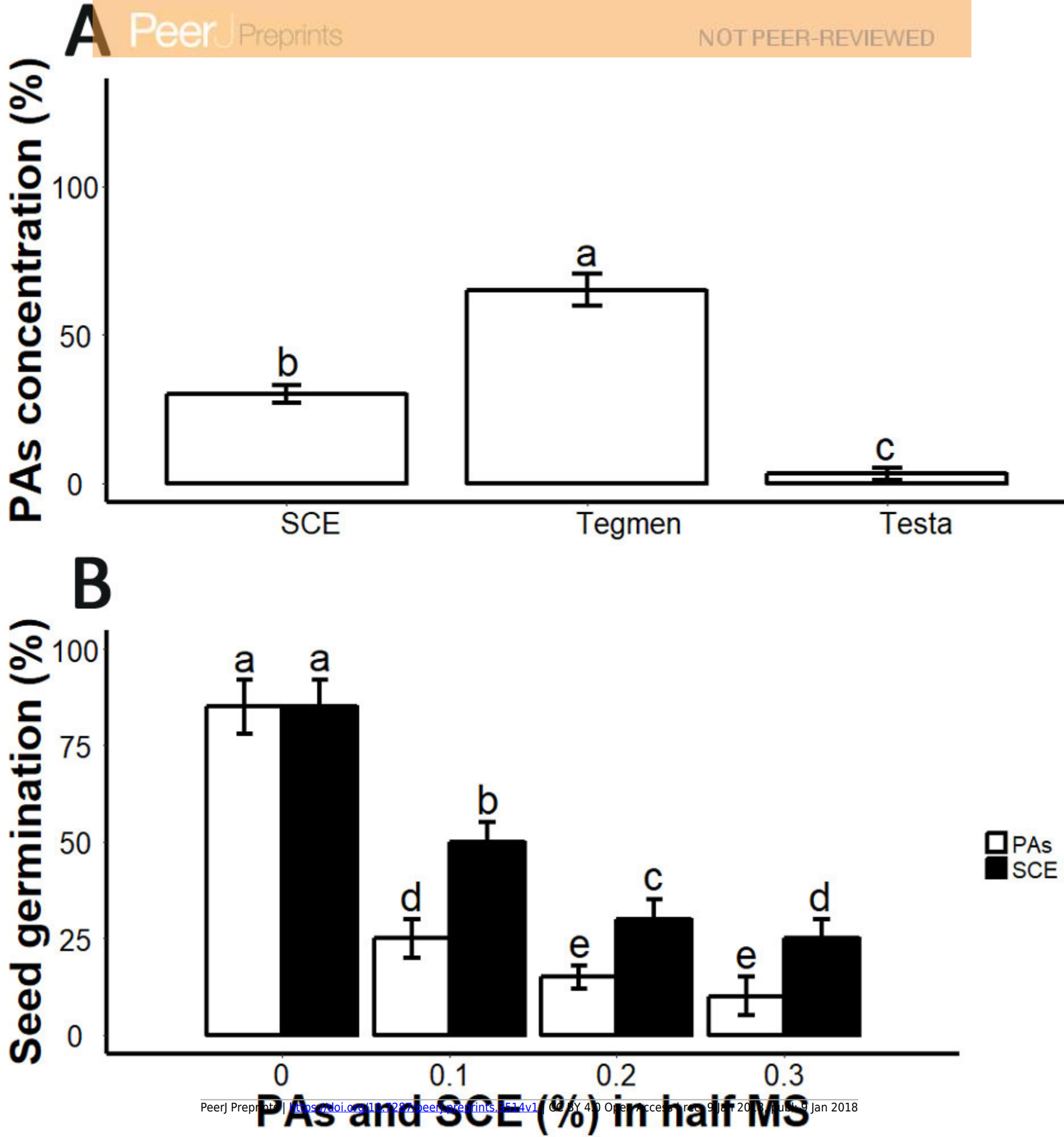

Figure 4

PAs in the endospermic cap affected the seed germination.

A, Accumulantion of PAs in endospermic cap of dormant seed. **B and C,** Decaping of endospermic cap significantly promoted seed germination as compared to control (with endospermic cap). **D,** Dynamic changes of PAs in the endospermic cap of non-dormant seed. Bars in **A** and **D** 2mm. **B** 1cm

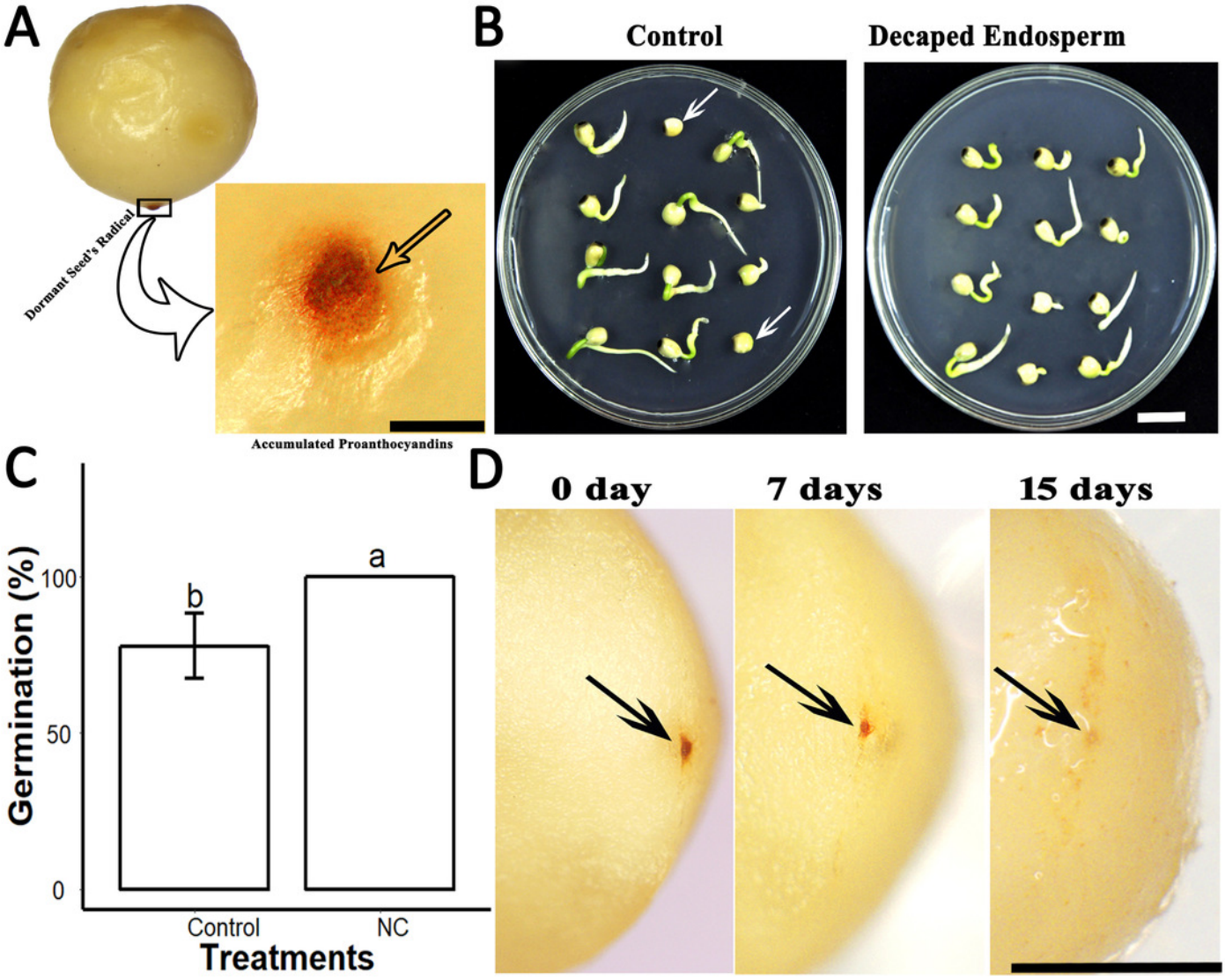

Figure 5(on next page)

Effect of SCE on the expression of seed dormancy-related gene (*SsDOG1*) and ABA-related genes.

The expression of *SsDOG1*, *SsNCED6*, *SsCYP707A2* and *SsABI3* were determined by qRT-PCR on 3rd and 6th day after treatment. *SsACTIN* was used as the reference gene. **Control**, seed grown in half MS medium. **PAs**, proanthocyanidins supplemented half MS medium. **SCE**, half MS medium supplemented with seed coat extract. Data shown are means±sd (n=3). Means with different letters are significantly different at $P < 0.05$ using Tuckey's HSD Post hoc test.

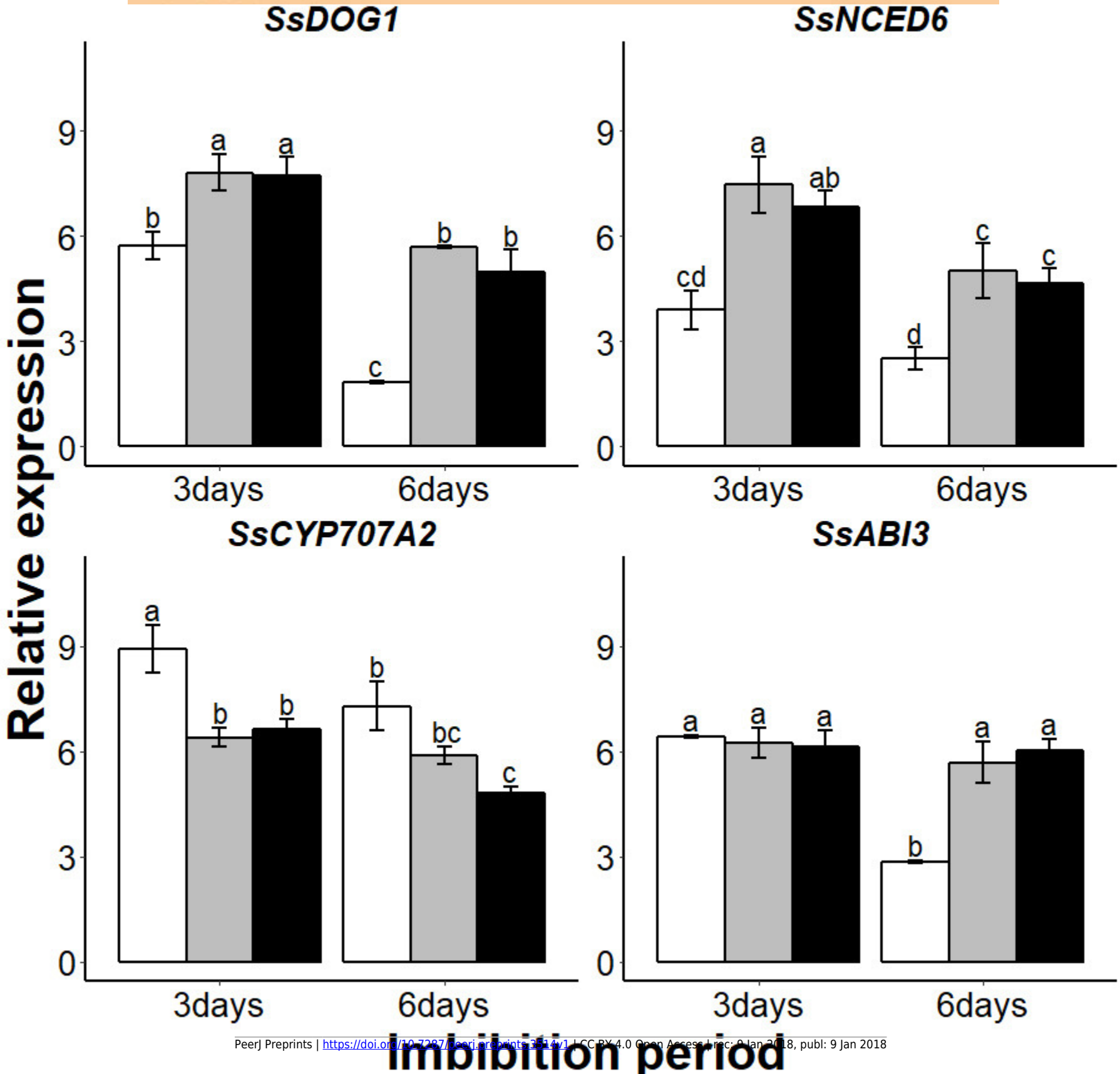

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Figure 6(on next page)

Effect of SCE on the expression of GA-related genes.

The expression of *SsGA3OX1, SsGAOX*, *SsRGL2*, and *SsGAI* was determined by qRT-PCR on 3rd and 6th day after treatment. *SsACTIN* was used as the reference gene. **Control**, seed grown in half MS medium. **PAs**, proanthocyanidins (0.1%) supplemented half MS medium. **SCE**, half MS medium supplemented with seed coat extract (0.3%). Data shown are means±sd (n=3). Means with different letters are significantly different at $P < 0.05$ using Tuckey's HSD Post hoc test.

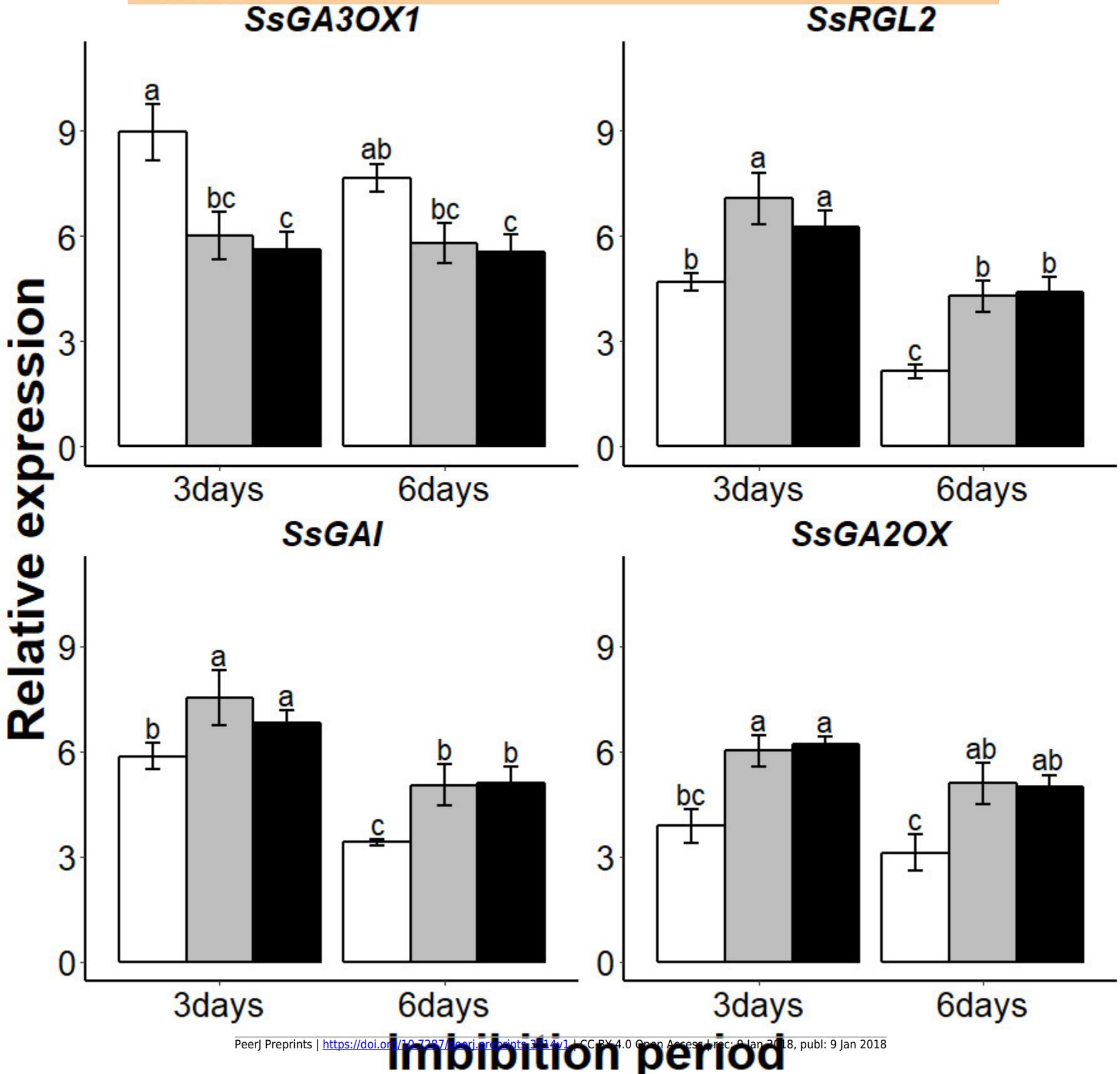

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Figure 7 (on next page)

Impact of SCE on expression levels of ROS and nitrates signalling genes.

The expression of *SsMPK6, SsNLP8* and *SsCIPK23* were determined by qRT- PCR on the 3rd and 6th day after treatment. *SsACTIN* was used as the reference gene. **Control**, seeds grown in half MS medium. **PAs**, proanthocyanidins supplemented half MS medium. **SCE**, seeds cultivated on half MS medium supplemented with seed coat extract. Data shown are means±sd (n=3). Means with different letters are significantly different at $P < 0.05$ using Tuckey's HSD Post hoc test.

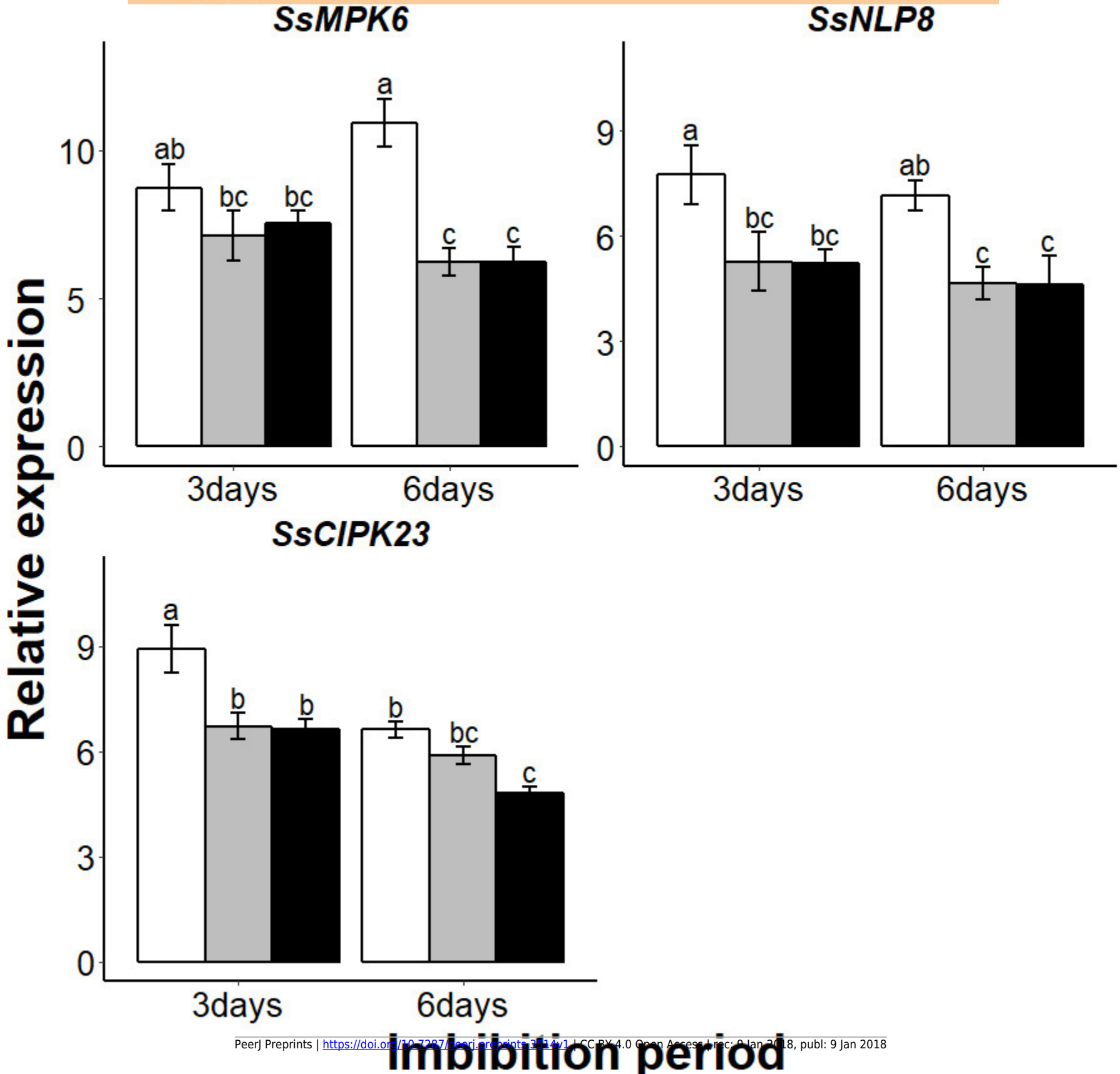

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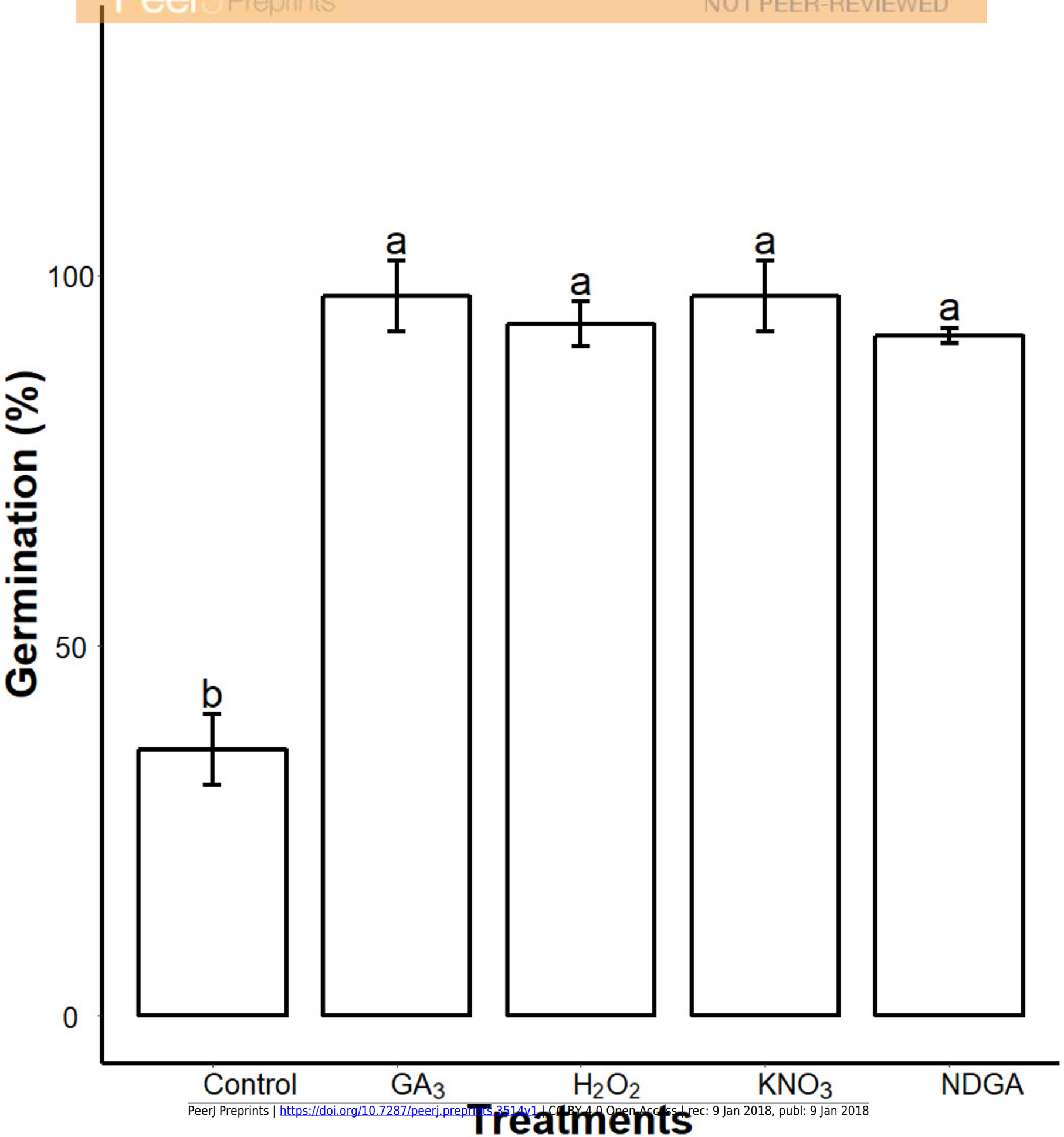
Figure 8(on next page)

Inhibitory effects of SCE on seed germination was alleviated by GA_3, NDGA, H_2O_2 and KNO_3.

Seeds were primed in double distilled water (Control), 50 μM GA_3, 50 μM NDGA, 20 mM H_2O_2 and 0.4% KNO_3 and were sowed in 0.3% SCE supplemented half MS medium. The seed germination rate was recorded 7 days after imbibition. Data shown are means±sd (n=3). Means with different letters are significantly different at $P < 0.05$ using Tuckey's HSD Post hoc test.



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