Title page

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Molecular and physiological dissection of enhanced seed germination using short-term low-concentration salt seed priming in tomato

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Abstract

Seed germination is the initial step of plant development. Seed priming with salt promotes seed germination in tomato (*Solanum lycopersicum* L.); however, the molecular and physiological mechanisms underlying the enhancement of seed germination by priming remain to be elucidated. In this study, we examined the following in seeds both during and after priming treatment: the endogenous abscisic acid (ABA) and gibberellin (GA) concentrations; the expression of genes encoding ABA catabolic and GA biosynthesis enzymes, including 8'-hydroxylase (CYP707A), copalyl diphosphate synthase (CPS), GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox); and endosperm cap-weakening enzymes, including expansin (EXP), class Ⅰ β-1,3-glucanase (GulB), endo-β-mannanase (MAN) and xyloglucan endotransglucosylase (XTH). Tomato seeds were soaked for 24 hours at 25 ºC in the dark in 300 mM NaCl (NaCl-priming) or distilled water (hydro-priming). For both priming treatments, the ABA content in the seeds increased during treatment but rapidly decreased after sowing. Both during and after the priming treatments, the ABA levels in the hydro-primed seeds and NaCl-primed seeds were not significantly different. The expression levels of *SlGA20ox1*, *SlGA3ox1* and *SlGA3ox2* were significantly enhanced in the NaCl-primed seeds compared to the hydro-primed seeds. The GA_4 content was quantifiable after both types of priming, indicating that GA_4 is the major bioactive GA molecule involved in tomato seed germination. The GA_4 content was significantly higher in the NaCl-primed seeds than in the hydro-primed seeds 12 h

after sowing and thereafter. Additionally, the peak expression levels of *SlEXP4*, *SlGulB*, *SlMAN2* and *SlXTH4* occurred earlier and were significantly higher in the NaCl-primed seeds than in the hydro-primed seeds. These results suggest that the observed effect of NaCl-priming on tomato seed germination is caused by an increase of the GA_4 content via GA biosynthetic gene activation and a subsequent increase in the expression of genes related to endosperm cap weakening.

Keywords: seed priming, salt, abscisic acid, gibberellin, GA 20-oxidase, tomato

Abbreviations:

ABA, abscisic Acid

CPS, copalyl diphosphate synthase

CYP707A, ABA 8'-hydroxylase

EXP, expansin

GA, gibberellin

GA 2ox, GA 2-oxidase

GA3ox, GA 3-oxidase

GA20ox, GA 20-oxidase

GulB, class Ⅰ β-1,3-glucanase

LC-MS/MS, liquid chromatography-tandem mass spectrometry

MAN, endo-β-mannanase

qRT-PCR, quantitative real-time PCR

XTH, xyloglucan endotransglucosylase

Introduction

To improve seed-germination rates and field emergence under adverse environmental conditions, the pretreatment of seeds, termed seed priming, has been used in crop production. Appropriate priming treatments synchronise germination and improve seed performance in many crop species [1, 2]. By initiating germination processes before sowing, seed priming generally enhances germination and field emergence under adverse germination conditions [3]. Furthermore, treatment with an abiotic stress, such as sodium chloride (NaCl), before sowing enhances the tolerance of plants to saline [4]. In tomato, priming treatments using KNO_3, polyethylene glycol or NaCl have improved the germination, seedling emergence and the initial growth of plants [5]. According to a previous report, NaCl priming generally requires long-term treatment periods using solutions with relatively high concentrations of NaCl [6]; however, short-term seed priming with a low NaCl concentration also increases germination rates, field emergence and acquired stress tolerance [7].

The effect of priming on transcription at early stages of seed germination has been investigated in several plant species, including *Arabidopsis*, *Brassica oleracea* and tomato [8, 9, 10]. Furthermore, the antagonistic roles of the plant hormones abscisic acid (ABA) and gibberellin (GA) in regulating seed germination are known [11, 12, 13]. The molecular and physiological mechanisms underlying the effect of seed priming on germination in relation to plant hormones, however, are not

fully understood.

Regarding ABA function and metabolism in plants, 9-*cis*-epoxycarotenoid dioxygenase (NCED), which is the key enzyme in ABA biosynthesis, has been isolated [14, 15]. Genes encoding ABA catabolic enzymes have been isolated in *Arabidopsis* (*AtCYP707A1-4*) and barley (*HvABA8' OH1, 2*) [16, 17]. ABA is important for seed germination, as *AtCYP707A2*-knockout mutant seeds exhibit hyperdormancy and accumulate higher ABA levels than do wild-type seeds. In tomato, four predicted *CYP707A* genes have been identified. Among them, *SlCYP707A1* is up-regulated according with increased catabolism of ABA, and transgenic plants overexpressing *SlCYP707A1* have reduced ABA levels and exhibit ABA-deficient phenotypes, suggesting that this gene encodes a functional ABA 8'-hydroxylase [18]. However, the other three putative genes have not been examined.

GA is important for seed germination, as evidenced by the inability of GA-deficient tomato (*gib-1*) and *Arabidopsis* (*ga1-3*) mutants to germinate without exogenous GA [19, 20]. In *Arabidopsis* and other higher plants, genes encoding GA metabolic enzymes have been isolated, including copalyl diphosphate synthase (CPS), *ent*-kaurene synthase (KS), *ent*-kaurene oxidase (KO), *ent*-kaurenoic acid oxidase (KOA), GA 20-oxidase (GA20ox), GA 3-oxidase (GA3ox) and GA 2-oxidase (GA2ox) [21, 22]. The expression levels of these genes have been analysed during the germination process, as have endogenous GA concentrations [23], and several reports have shown

that their expression is regulated by both endogenous and environmental conditions (i.e., light and temperature) [24, 25, 26, 27]. In tomato, the GA biosynthetic genes *CPS*, *GA20ox*, *GA3ox* and *GA2ox* have been isolated [28, 29]. The process of fruit set in tomato involves GA_1 as the bioactive GA molecule and the early 13-hydroxylation pathway as the primary metabolic pathway [30, 29]. Pollination mediates fruit set through GA biosynthesis, which is mainly achieved by the up-regulation of *GA20ox* [29]. For seed germination in tomato, however, the relevant GA biosynthetic processes and changes in GA levels remain unclear.

The germination process of tomato seeds has been studied. The tomato embryo is surrounded by a rigid endosperm, and the endosperm region that encloses the radicle tip, the endosperm cap, weakens to allow the radicle to emerge [19]. Enzymes such as expansin (EXP), β-1,3-glucanase (GulB), endo-β-mannanase (MAN) and xyloglucan endotransglucosylase/hydrolase (XTH) are thought to be involved in the weakening of the endosperm cap, and the mRNA transcript levels of the genes encoding these enzymes are induced by GA. Even though radicle emergence is inhibited by ABA, the mRNA transcript levels of the genes encoding these enzymes (with the exception of *GulB*) are not inhibited by ABA, nor is the concomitant weakening of the endosperm cap [31, 32, 33, 34]. Moreover, it has been proposed that the weakening of the tomato endosperm cap is a biphasic process [35]. The first step is characterised by ABA-independent weakening of the endosperm cap that is associated with enzymes such as MAN. During the second step, ABA only inhibits radical

emergence. Therefore, the enzymes responsible for the weakening of the endosperm cap, a process related to germination, are regulated by the plant hormones ABA and GA.

To obtain a better understanding of the enhancement of tomato seed germination by NaCl priming, in this paper, we investigated the levels of plant hormones and of mRNA transcripts encoding enzymes required for plant hormone metabolism and endosperm cap weakening in germinating seeds. The results indicate GA_4 is the major bioactive GA in germinating tomato seed. NaCl-priming enhances GA accumulation and transcription of the gene for GA biosynthesis while it has little effect on ABA accumulation and its degradation during germination. The genes for endosperm cap weakening are also up-regulated by the priming in a manner closely correlating with the GA_4 accumulation. The molecular and physiological mechanisms in tomato that mediate enhanced seed germination when seeds are primed with salt are discussed.

2. Results

2.1. Germination of primed seeds

Short-term seed priming with a low salt concentration has effects similar to those of conventional priming methods [7]. In this study, we used a germination test to confirm the effects of seed priming. The time required for the initiation of germination and the final germination ratios were not different among the NaCl-, hydro- and GA-primed seeds (Fig. 1); however, the germination

rate of the NaCl- and GA-primed seeds were significantly higher between 36 and 60 h after sowing. In particular, the germination rate of the NaCl- and GA-primed primed seeds was 4.9 and 4.6 times higher than that of the hydro-primed seeds at 36 h after sowing (Fig. 1).

2.2 Expression levels of CYP707A genes and endogenous ABA content during seed priming

ABA levels decline during seed imbibition in several plant species [36, 37]. To examine whether priming treatment influences this phenomenon, we measured the relative expression levels of *SlCYP707A* genes and endogenous ABA levels during seed priming (Fig. 2). The transcriptional analyses revealed that expression levels of *SlCYP707A1-4* genes during the priming period were similar to or lower than the expression observed in dry seeds immediately before priming (Fig. 2A-D). Endogenous ABA levels were not significantly different between the hydro- and NaCl-priming treatments. At 0 h, the ABA levels in the hydro- and NaCl-primed seeds were 2.2 and 2.5 times higher than the ABA content of dry seeds, respectively (Fig. 2E).

2.3 Expression levels of CYP707A genes and endogenous ABA content after sowing

To investigate endogenous ABA content further, we measured relative *CYP707A1-4* expression levels and endogenous ABA after sowing. The *CYP707A1-4* genes displayed different expression patterns (Fig. 3A-D). Relative to its level at the time of sowing, *SlCYP707A1* expression

dramatically decreased and almost disappeared by 24 h after the sowing (Fig. 3A). *SlCYP707A2* expression also decreased after sowing, but returned to the same level after 36 h and then decreased again (Fig. 3B) in both the hydro- and NaCl-primed seeds. With both treatments, the expression level of *SlCYP707A3* increased starting at 12 h after sowing; *SlCYP707A3* expression in hydro- and NaCl-primed seeds was 9.0- and 19.8-fold higher (respectively) at 48 h after sowing than at 0 h after sowing (Fig. 3C). The expression level of *SlCYP707A4* increased immediately, peaked 6 h after sowing and then decreased in both the hydro- and NaCl-primed seeds. Of the *SlCYP707A* genes, only *SlCYP707A4* tended to be affected by the NaCl treatment during the early period after sowing (Fig. 3D).

Endogenous ABA levels were not significantly different between the hydro- and NaCl-primed seeds after sowing. Relative to the time of sowing (0 h), ABA levels in the hydro- and NaCl-primed seeds declined to 85.7% and 47.2% (respectively) at 6 h after sowing, to 45.4% and 43.5% at 12 h after sowing and to 22.4% and 11.4% at 24 h after sowing (Fig. 3E).

2.4. Expression levels of GA biosynthetic genes and endogenous GA content after sowing

Bioactive GAs are important during the late phase of germination. We measured the relative expression levels of the GA biosynthesis genes *SlCPS; SlGA20ox1*, *-2 and -3;* and *SlGA3ox1* and *-2*, which were cloned by Rebers et al. [28]. We also measured endogenous GA levels after sowing. The

expression levels of *SlCPS,* whose protein product catalyzes the conversion to *ent*-copalyl diphosphate from geranylgeranyl diphosphate, decreased in the hydro- and NaCl-primed seeds after sowing (Fig. 4A). The genes encoding GA 20-oxidase displayed different expression patterns; of these genes, only *SlGA20ox1* was apparently affected by the NaCl-priming treatment (Fig. 4B, C and D). With both types of priming, the expression levels of *SlGA20ox1* increased during the first 24 h after sowing with a particularly rapid increase at 6 h after sowing. The levels temporarily decreased at 36 h after sowing and then increased again. The transcript levels of *SlGA20ox1* were significantly higher in the NaCl-primed seeds than in the hydro-primed seeds between 0 and 48 h after sowing (Fig. 4B). At 6 h after sowing, the levels of *SlGA20ox2* in the hydro- and NaCl-primed seeds decreased to less than a quarter and less than half, respectively, compared to those at the time of sowing (Fig. 4C). The expression levels of *SlGA20ox3* after sowing increased at 6 h, decreased at 12 h, increased again at 24 h and decreased again thereafter (Fig. 4D). *SlGA3ox1* showed stronger expression than *SlGA3ox2* in both the hydro- and NaCl-primed seeds (Fig. 4E and F). The expression levels of *SlGA3ox1* increased markedly in both the hydro- and NaCl-primed seeds at 24 h. *SlGA3ox1* expression peaked earlier in the NaCl-primed seeds (at 36 h) than in the hydro-primed seeds. The expression levels of *SlGA3ox2* increased in the hydro- and NaCl-primed seeds at 12 h and thereafter. The expression levels of *SlGA3ox2* were significantly higher in the NaCl-primed seeds than in the hydro-primed seeds starting at 12 h, except at the 36-h time point (Fig. 4F).

Regarding endogenous GAs (Fig. 5), the GA_{12} content was the same after both types of priming at the same time points and decreased up to 12 h after sowing. The levels of GA_{15} and GA_{53} increased up to 12 h. At 6 h after sowing, GA_{24}, GA_{44}, GA_{19} and GA_{20} levels increased gradually, and GA_9 levels increased rapidly. Endogenously bioactive GA_4 and GA_1 were quantifiable at 12 h and 36 h, respectively, and GA_4 was higher than GA_1. GA_4 rapidly increased at 24 h in both the hydro- and NaCl-primed seeds. GA_4 levels were significantly higher in the NaCl-primed seeds than in the hydro-primed seeds. At 12, 24 and 36 h, GA_4 levels were 1.36, 1.55 and 1.58 times higher, respectively, in the NaCl-primed seeds than in the hydro-primed seeds. The GA_1 content of the NaCl-primed seeds was 6.21 times that of the hydro-primed seeds at 36 h. GA_{34} and GA_8, catabolites of bioactive GAs, and GA_{51} and GA_{29}, catabolites of precursors of bioactive GAs, remained at low levels.

2.5. Expression levels of genes related to micropylar endosperm cap weakening

In tomato seed germination, the cell wall-loosening genes *SlEXP4*, *SlGulB*, *SlMAN2* and *SlXTH4* are related to micropylar endosperm cap weakening [32, 33, 31, 34], and cell wall loosening is a complex process. We measured the relative expression levels of all of these genes. The peak expression levels of *SlXTH4* in the NaCl- and hydro-primed seeds were at 12 h and 24 h, respectively, and those of *SlGulB* were at 36 h and 48 h. In contrast, *SlEXP4 and SlMAN2* peaked

simultaneously in both the NaCl- and hydro-primed seeds, at 24 h and 36 h, respectively (Fig. 6). For all of these genes, the maximum expression levels occurred earlier and were significantly higher in the NaCl-primed seeds than in the hydro-primed seeds (Fig. 6). The expression levels of *SlEXP4*, *SlMAN2* and *SlXTH4* were significantly higher in the NaCl-primed seeds than in the hydro-primed seeds at 0 h and rapidly increased at 12 h (Fig. 6A, C and D). The expression levels of *SlGulB* began to increase rapidly at 24 h (Fig. 6B).

3. Discussion

Seed-priming techniques have been used to improve both seed germination and seedling emergence in many crops [1]. Seed priming also makes germination more efficient under unfavourable conditions [38, 39]. The NaCl priming method improves germination, providing germination earlier than that of hydro-primed seeds in water-stressed conditions. In a previous study, we found that short-term seed priming with a lower salt concentration than in conventional priming methods enhanced seed germination [7]. Although the time required for the initiation of germination and the final germination ratios were not different among the priming treatments, the periods required for the field emergence and uniformity are obviously improved in NaCl-primed seeds compare to the hydro- or non-primed seeds (Fig. 1) [7]. Such an effective germination will contribute to improve the initial growth of seedling and eventually result in high quality and high

yield production especially under unfavourable conditions.

However, different seed lots responded differently to priming, even within the same cultivar [8]. For instance, primed tomato seeds from two seed lots exhibited vastly different germination responses [40]. The NaCl priming treatment used in this study was tested on different seed lots under both non-stress and stress conditions [7]. Because the influence of the NaCl priming treatment on germination was reproducible (Fig. 1), we conclude that it is effective.

ABA levels were two-fold higher in the hydro- and NaCl-primed seeds than in the dry seeds (Fig. 2E). The presence of salt did not influence the ABA levels, and they remained elevated during the priming period. ABA is known to regulate adaptive responses to a variety of environmental stresses [41]. Additionally, ABA induces the expression of several genes, including a gene encoding an ABA-responsive element binding protein that controls ABA-regulated gene expression to enhance stress tolerance [42]. Because there was no significant difference in ABA levels between the two priming treatments (Fig. 2E and Fig. 3E), we concluded that salt treatment has no affect on the ABA content of germinating tomato seeds. In both *Arabidopsis* and maize, dehydration-responsive element binding protein 2A (DREB2A) is induced by dehydration and salt stress independently of ABA to regulate stress-response genes [43, 45]. A signalling pathway similar to those in *Arabidopsis* and maize may exist in tomato, as NaCl and drought may induce DREB3 expression in tomato independently of ABA [44].

The endogenous ABA content of seeds rapidly declines upon imbibition during the early phase of germination in *Arabidopsis* and sugar beet [46, 47]. The expression of *AtCYP707A2* is responsible for the rapid decrease in ABA during seed imbibition [16]. In this study, *SlCYP707A4* expression increased rapidly after sowing, similarly to the transcriptional pattern observed for *AtCYP707A2*, and endogenous ABA levels actually declined with the initial increase (Fig. 3D and E). These results indicate that *SlCYP707A4* has the same role as *AtCYP707A2*; however, neither ABA levels nor *SlCYP707A4* transcript levels differed significantly between the hydro- and NaCl-primed seeds (Fig. 3D and E). These results are consistent with the expression pattern of *SlGulB*, which was inhibited by ABA [33] and showed no significant difference between the two treatment types up to 12 h (Fig. 6B). We conclude that the salt priming treatment does not influence the change in endogenous ABA levels and does not affect the promotion of seed germination (Fig. 1, 2E and 3E). Although the previous report [18] suggested *SlCYP707A1* encodes a functional ABA 8'-hydroxylase and *SlCYP707A3* showed the strongest transcriptional levels among *SlCYP707A* genes after 36 h after sowing (Fig. 3C), those genes would not participate in the ABA catabolism observed in the germinating seeds, because the transcriptional profiles were inconsistent with the declining pattern of ABA (Fig. 3A, C, and E).

In tomato fruit setting, the GAs of the early 13-hydroxylation pathway (GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_1, GA_8 and GA_{29}) are involved in the primary metabolic pathway [30], and GA_1 is the

bioactive GA that is most important for fruit setting [29]. Before germination, however, the concentrations of bioactive GA_4 and GA_9 (the precursor of bioactive GA_4) increased earlier than those of GA_1 and GA_{20} (the precursor of bioactive GA_1). Additionally, GA_4 levels were significantly higher than GA_1 levels in both the hydro- and NaCl-primed seeds, and exogenous GA_4 significantly improved seed germination rates compared to hydro-primed seeds (Fig. 1 and 5). These results indicate that GA_4 is important for tomato seed germination and that the GAs of the non-13-hydyoxylation pathway (GA_{12}, GA_{15}, GA_{24}, GA_9, GA_4, GA_{34} and GA_{51}) have dominant roles in tomato seed germination relative to the GAs of the early 13-hydyoxylation pathway. This finding is similar to observations for the hypocotyl in tomato and for Arabidopsis seed germination [48, 23].

In *Arabidopsis*, GA_{12} levels are relatively low, and some active GAs are present in dry seeds [23]. In tomato, however, GA mainly exists as GA_{12} soon after sowing, and thereafter, it seemed to be converted to GAs such as GA_9 and GA_4 (Fig. 5). These results indicate that downstream of the GA biosynthetic pathway, GA_{12} is suppressed during seed priming and becomes active after sowing (Fig. 5). There is evidence that the expression pattern of *SlGA20ox1*, the most strongly expressed *SlGA20ox* gene, is consistent with the metabolic order of GA intermediates (GA_{12}, GA_{15}, GA_{24}, GA_9, GA_{44}, GA_{19} and GA_{20}). The expression levels of *SlGA20ox1* were enhanced by NaCl-priming (Fig. 4B and 5). In *Arabidopsis*, bioactive GAs are regulated by the *GA20ox*, *GA3ox* and *GA2ox* pathways [49]. Our results indicate that, as in *Arabidopsis*, the metabolism downstream of GA_{12} is also

important for the biosynthesis of bioactive GAs in tomato seeds. In addition, *SlGA3ox1* and *SlGA3ox2* transcript levels were enhanced by NaCl-priming and were consistent with the changes in bioactive GA levels (Fig. 4E, 4F and 5). These results indicate that NaCl-priming increased *SlGA20ox1*, *SlGA3ox1* and *SlGA3ox2* transcript levels, which thereby caused bioactive GA levels to rise. In particular, the bioactive GAs associated with seed germination were found to be regulated by *SlGA3ox1* and *SlGA3ox2*.

SlEXP4, *SlGulB*, *SlMAN2* and *SlXTH4* encode genes related to the weakening of the micropylar endosperm cap in tomato seeds [32, 33, 31, 34]. The expression of these genes is induced by GA_{4+7}, and only *SlGulB* expression is inhibited by ABA [32, 33, 31,34]. Consistent with these previous reports, our data showed that in response to GA_4, *SlEXP4*, *SlMAN2*, and *SlXTH4* expression levels increased rapidly at 12 h, and *SlGulB* levels increased rapidly after a decrease in ABA (Fig. 3, 5 and 6). These results also indicate that GA_4 is important for seed germination in tomato. Independently of GA_4 and ABA, *SlEXP4*, *SlMAN2* and *SlXTH4* expression levels were significantly higher at 0 h in the NaCl-primed seeds than in the hydro-primed seeds (Fig 6A, C and D). In tomato seeds, the overexpression of *SlERF2*, encoding an ERF (ethylene response factor) protein and belonging to the ERF gene family, upregulates the expression of *SlMAN2*, suggesting that *SlERF2* stimulates seed germination by inducing the *SlMAN2* gene [50]. In addition to being induced by ethylene, ERF genes can also respond to jasmonate, ABA, salicylic acid and NaCl [51,

52, 53]. In this regard, *SlEXP4*, *SlMAN2* and *SlXTH4* expression immediately after sowing may have been influenced by transcription factors such as *SlERF2*. Our results suggest that the expression of these genes immediately after sowing, which occurs in a manner closely correlating with the GA_4, might be important for tomato seed germination.

In conclusion, our results demonstrate that short-term seed priming with a low salt concentration enhances the transcription of *SlGA20ox1*, *SlGA3ox1* and *SlGA3ox2* and increases GA_4 levels. The priming treatment also enhances the expression of genes encoding cell wall modification enzymes, suggesting the promoted endosperm cap weakening brings up the high-ratio and the uniform seed germination in the primed seeds and the increased GA_4 plays a key role in those events. The mechanism by which salt activates the expression of *SlGA20ox1*, *SlGA3ox1* and *SlGA3ox2* remains unclear and will be addressed in future studies.

4. Materials and Methods

4.1. Plant materials and priming treatments

Tomato seeds (*Solanum lycopersicum* L. cv. 'Momotaro Haruka') (Takii & Co., Ltd.) were used for all experiments. The seeds were soaked for 24 hours at 25 ºC in the dark in 300 mM NaCl (NaCl-priming), distilled water (hydro-priming) or 100 μM GA_{4+7} (GA-priming). After the priming treatment, the seeds were washed with distilled water and sown. During the priming and after

sowing, the seeds were immediately frozen in liquid N_2 and stored at -80 ºC.

4.2. Germination test

For the germination test, there were four replicates with 50 seeds per treatment. After priming, the seeds were placed evenly on two layers of filter paper (ADVANTEC No. 2; Toyo Roshi Kaisha, Ltd.) in a covered plastic case (19×13×6.5 cm). The seeds were moistened with 15.5 mL of distilled water, and the cases were maintained in an incubator in the dark in at 25 ºC. The number of germination events was counted at 12-hour intervals from the onset of germination. Seed germination was defined as the protrusion of the radicle through the seed coat.

4.3. Purification procedure for the quantification of endogenous GA and ABA

The purification procedure for quantifying endogenous GAs was modified from a previous report [54]. Lyophilised plant material (500 mg dry weight) was ground in 10 mL of acetone, and ^{2}H -labelled GAs (500 pg each; purchased from Lewis Mander, Australia National University) were added as internal standards. This mixture was incubated for 3 h at room temperature and centrifuged at 2000 x *g* for 15 min at 10 ºC. The supernatant was collected, and the pellets were rinsed with 10 mL of acetone and centrifuged at 2000 x *g* for 15 min at 10 ºC. The supernatant was combined with the first supernatant, concentrated to dryness and dissolved in 10 mL of aqueous acetonitrile (1:1, v/v).

The solution was partitioned against an equal volume of *n*-hexane, and the *n*-hexane phase was discarded. After removing the acetonitrile by drying, the pH was adjusted to 8.0 with 500 mM potassium phosphate buffer. The sample was loaded onto a polyvinylpyrrolidone (PVP, 500 mg; Tokyo Kasei, Japan) cartridge and eluted with 3 mL of 500 mM potassium phosphate buffer. The pH of this eluate was adjusted to 3.0 with HCl, and it was loaded onto a reverse-phase cartridge (Oasis HLB, 30 mg; Waters). After a washing step with 2 mL of water containing 2% formic acid, GAs were eluted with 2 mL of 80% acetonitrile containing 1% formic acid. The eluate was dried, dissolved in methanol and loaded onto an ion-exchange column (Bond Elut DEA, 100 mg; Varian). The GAs were eluted using 3 mL of methanol containing 0.5% acetic acid after washing with 3 mL of methanol. The elute was dried, dissolved in $CHCl_3$:ethylacetate = 1:1 (v/v) containing 1% acetic acid and loaded onto a SepPak silica cartridge (100 mg; Waters). The GAs were eluted with 3 mL of chloroform:ethylacetate = 1:1 (v/v), and the eluted GA-containing fraction was concentrated to dryness, dissolved in 20 μL of water containing 2% acetic acid and subjected to LC-MS/MS (liquid chromatography-tandem mass spectrometry) analysis. For ABA, [$^2H_6$]-ABA (Icon Services) was added as an internal standard. The purification procedure for ABA excluded the PVP cartridge and SepPac silica cartridge steps described for the GA purification.

4.4 Quantification of endogenous GA and ABA

The GA and ABA measurements were performed using an LC-MS/MS system consisting of a quadruple/time-of-flight tandem mass spectrometer (Q-Tof Premier; Waters) and an Acquity Ultra Performance liquid chromatography system (Waters) equipped with a reverse-phase column (Acquity UPLC BEH-C18 Waters) as described by Varbanova et al. [54] and Saika et al. [55], respectively. To calculate the GA and ABA concentrations from the LC-MS/MS data, MassLynx ver. 4.1 software (Waters) was used.

4.5. RNA isolation, cDNA synthesis and quantitative RT-PCR

Total RNA was extracted from approximately 100 mg of the frozen samples using the Fruit-mate™ for RNA Purification reagent (Takara Bio Inc., Otsu, Japan) and the RNeasy plant Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturers' instructions. The extracted RNA was dissolved in RNase free-water and stored at -80 º. For cDNA synthesis, 1 μg of total RNA was reverse-transcribed with the RT reagent Kit with gDNA Eraser (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions.

Gene-specific primers were designed using Amplify ver. 3.1.4 (B Engels, University of Wisconsin, USA; http://engels.genetics.wisc.edu/amplify/) and were based on published sequences (Supplementary Table S1). Quantitative RT-PCR (qRT-PCR) reactions were performed using the Mx 3000P qRT-PCR system (Stratagene, San Diego, CA, USA). To normalise the qRT-PCR

reactions, the ubiquitin 7 gene was used as an internal standard (accession number AK246454; [18]). For all of the genes, the reaction cycles were as follows: 95 ºC for 10 min for the initial denaturation; 45 cycles of 95 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 30 s; and 1 cycle of 95 ºC for 30 s, 55 ºC for 30 s, and 95 ºC for 30 s. The specific amplification of a single transcript was confirmed with single dissociation peaks and calibration curves and by agarose gel electrophoresis. Gene expression levels were calculated relative to the transcript levels of the ubiquitin 7 gene according to the instructions provided by Stratagene and the method reported by Pfaffl [56].

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 21580075).

Supplementary material

Table S1. Primer sequences used for quantitative real-time PCR.

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Supplementary Table

Table S1 Primer sequences used for quantitative real-time PCR.

Gene	TC/GB number*	Primer (5'-3')	Reference
SlCPS	AB015675	5'-GGAAAATTGGCTACTGACGGTAGG-3'	Serrani et al. 2007
		5'-GGCATCCAATTCGGAAGCA-3'	
SlGA20ox1	AF049898	5'-CGATTTCACATGGCCTACTCTTC-3'	the present study
		5'-GTAGAAGCTAAGAGAACGTGTACG-3'	Serrani et al. 2007
SlGA20ox2	AF049899	5'-ACC AGA TCT TGC GTT AGG AAC TG -3'	Asahina et al. 2007
		5'-CCT GAG ACG TTG TCT TGA TGG A-3'	
SlGA20ox3	AF049900	5'-GGACAGGGCCTCATTGTGAT-3'	Asahina et al. 2007
		5'-AAACTTGAAGCCCACCAACACT-3'	
SlGA3ox1	AB010991	5'-CAGACCACATGAGCTTCGAGAA-3'	Asahina et al. 2007
		5'-CCTGATGGTGTCACTGGCTATG-3'	
SlGA3ox2	AB010992	5'-GTAACGGTTCCTCTCCTTCGC-3'	Serrani et al. 2007
		5'-ACCTACTTGGACGCCACTTTG-3'	
SlCYP707A1	EU183406	5'-AGAGAGGCTGTAGCTGAGTGG-3'	Nitsch et al. 2009
		5'-TTGGCAAGTTCATTCCCTGGAC-3'	
SlCYP707A2	TC177455	5'-GCAATGAAAGCGAGGAAAGAGC-3'	Nitsch et al. 2009
		5'-TCGAGCTGCAAAGATGACTCC-3'	
SlCYP707A3	AI484420	5'-CTAAGGTGGCAAGGAGGAAGC-3'	Nitsch et al. 2009
		5'-GTGTCCTGGGCAGCAAAGAG-3'	
SlCYP707A4	TC186477	5'-GAGCATTCAAACCCGAAGCC-3'	Nitsch et al. 2009
		5'-AATTGTACCCTGTTTCGAGCAC-3'	
SlMAN2	AF184238	5'-CCAGGATATAATCAAAATATACGC-3'	the present study
		5'-CATGGCATGAGACTGACTTGTA-3'	
SlXTH4	AF186777	5'-TCAGCCAATGAAGATCTACTCAAG-3'	the present study
		5'-AAACTTGTACCTCTTGTGGTGTGA-3'	
SlEXP4	AF059488	5'-TGTTCTTCTCTGTTTTCTCACTGC-3'	the present study
		5'-CTCCGTAACCTTGGCTGTATAAAT-3'	
SlGulB	M80608	5'-GGGTTCATTTAGGAACGATGTTAG-3'	the present study
		5'-TAGGTGCTGTAAAAAGAGCATACG-3'	
UBQ7	AK246454	5'-CCCTGGCTGATTACAACATTC-3'	Nitsch et al. 2009
		5'-TGGTGTCAGTGGGTTCAATG-3'	

*TC and GB indicate TIGR and GenBank, respectively.

Figure Captions

Figure 1. Germination profiles of primed tomato seeds. Open circles, black circles and black squares indicate the hydro-, NaCl- and GA-primed seeds, respectively. For priming (shaded area), the seeds were soaked for 24 h at 25 ºC in the dark in either distilled water (hydro-priming) or 300 mM NaCl (NaCl priming) or 100 µM GA_{4+7} (GA_{4+7} -priming). The horizontal axis based on 0 h of sowing and -24 h indicates dry seed immediately before seed priming. This experiment was repeated four times with 50 seeds per replicate for each treatment. Values indicate the means ±SE. The different letters indicate the statistical significance of the means at a given time point according to Tukey's test ($P < 0.01$).

Figure 2. Relative expression levels of *SlCYP707A* genes and the endogenous ABA content during seed priming. (A) - (D) indicate the relative mRNA transcript levels of *SlCYP707A1 - 4*, respectively. Transcriptional levels of the genes are presented relative to the level at -24 h (dry seed). The striped, open and shaded columns indicate dry seeds, hydro-primed seeds and NaCl-primed seeds, respectively. The horizontal axis in each graph based on 0 h of sowing and indicates the following time points: -24 h (dry seed and immediately before seed priming), -12 h (12 h after seed priming) and 0 h (completed seed priming for 24 h and immediately before sowing). Values indicate the means ±SD. The asterisks indicate significant differences according to Student's *t*-test (*$P < 0.05$).

(E) indicates the endogenous ABA levels. Samples from the same seed batch were measured three times. The open circles and black circles indicate the hydro-primed and NaCl-primed seeds, respectively. The horizontal axis and values are as described above. gdw: grams dry weight.

Figure 3. Relative expression levels of *SlCYP707A* genes and endogenous ABA contents during seed germination. (A) - (D) indicate the relative mRNA transcript levels of *SlCYP707A1 - 4*, respectively. Transcriptional levels of the genes are presented relative to the level at -24 h (dry seed). The open and shaded columns indicate hydro-primed seeds and NaCl-primed seeds, respectively. The horizontal axis in each graph indicates the following: 0 h (completed seed priming for 24 h and immediately before sowing) and the number of hours after sowing. Values indicate the means ±SD. The asterisks indicate significant differences according to Student's *t*-test (*P < 0.05, **P < 0.01). (E) indicates the endogenous ABA levels. Samples from the same seed batch were measured four times. The open circles and black circles indicate the hydro-primed and NaCl-primed seeds, respectively. The horizontal axis and values are as described above. gdw: grams dry weight.

Figure 4. Relative expression levels of GA biosynthetic genes during the germination of primed seeds. (A), (B), (C), (D), (E) and (F) indicate the relative mRNA transcript levels of *SlCPS*, *SlGA20ox1*, *SlGA20ox2*, *SlGA20ox3*, *SlGA3ox1* and *SlGA3ox2*, respectively. Transcriptional levels

of the same homologue genes are presented relative to *GA20ox1* and *GA3ox1* in the hydro-primed seed at sowing time (0 h), respectively. The open and shaded columns indicate the hydro-primed and NaCl-primed seeds, respectively. The horizontal axis in each graph indicates the following: 0 h (completed seed priming for 24 h and immediately before sowing) and the number of hours after sowing. Values indicate the means ±SD. The asterisks indicate the statistical significance of the means at each time point according to Student's *t*-test (*P < 0.05, **P < 0.01).

Figure 5. Endogenous GA levels during primed seed germination. Samples from the same seed batch were measured four times. The open circles and black circles indicate the hydro-primed and NaCl-primed seeds, respectively. The horizontal axis in each graph indicates the following: 0 h (completed seed priming for 24 h and immediately before sowing) and the number of hours after sowing. Values indicate the means ±SD. The asterisks indicate the statistical significance of the means at each time point according to Student's *t*-test (*P < 0.05, **P < 0.01). a and b indicate the two and three times, respectively, when the levels were not quantifiable because they were undetectable or unreliable due to a low abundance or due to co-migration of impurities. The letters to the upper and lower right of the number of hours indicate the hydro- and NaCl-primed seed samples, respectively. gdw: grams dry weight.

Figure 6. Relative expression levels of genes associated with endosperm cap weakening. (A), (B), (C) and (D) indicate the relative mRNA transcript levels of *SlEXP4*, *SlGulB*, *SlMAN2* and *SlXTH4*, respectively. Transcriptional levels of the genes are presented relative to that in the hydro-primed seed at sowing time (0 h). The open and shaded columns indicate the hydro-primed and NaCl-primed seeds, respectively. The horizontal axis in each graph indicates the following: 0 h (completed seed priming for 24 h and immediately before sowing) and the number of hours after sowing. Values indicate the means ±SD. The asterisks indicate the statistical significance of the means at a given time point according to Student's *t*-test (*$P < 0.05$, **$P < 0.01$).

Fig 1

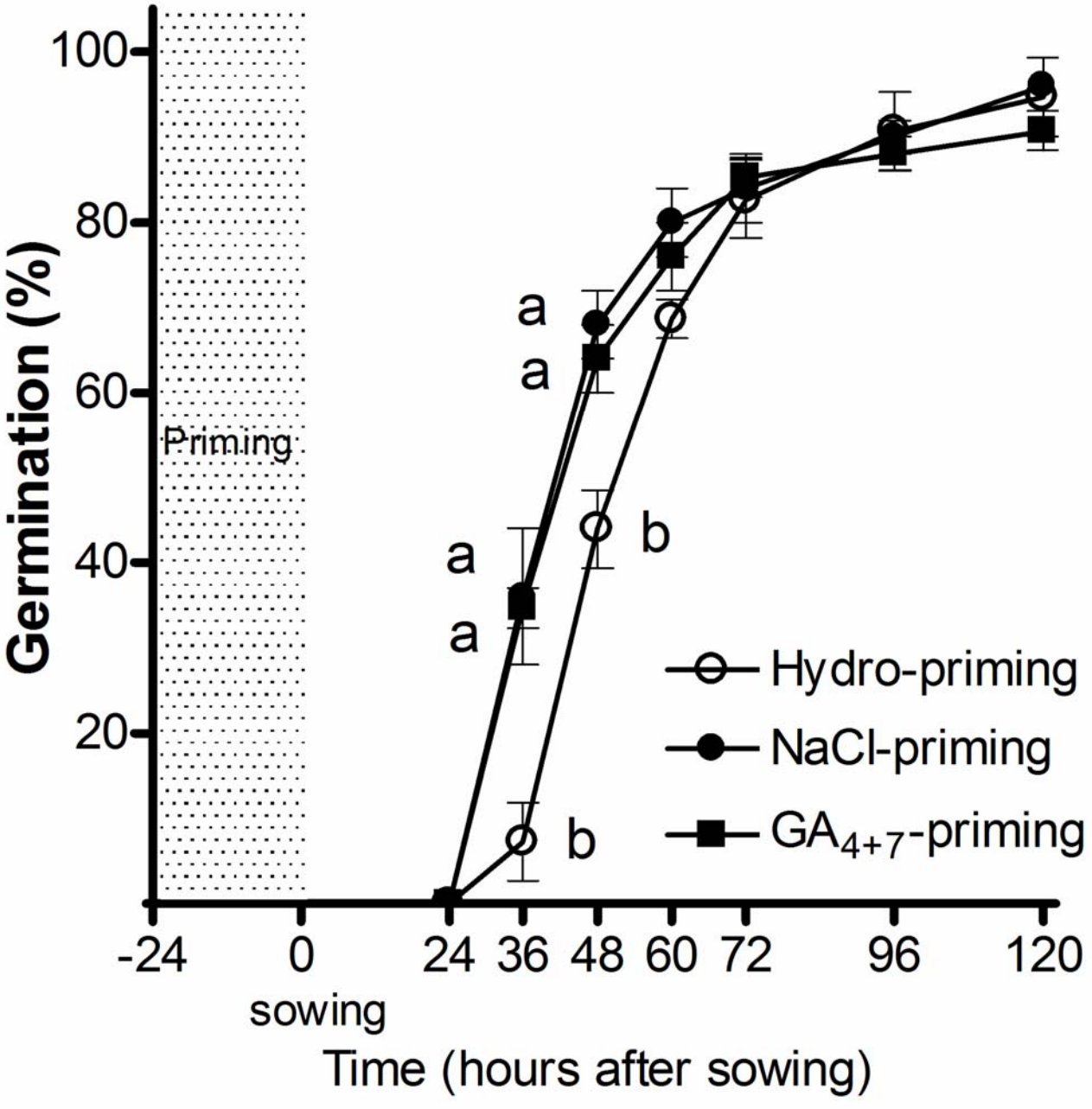
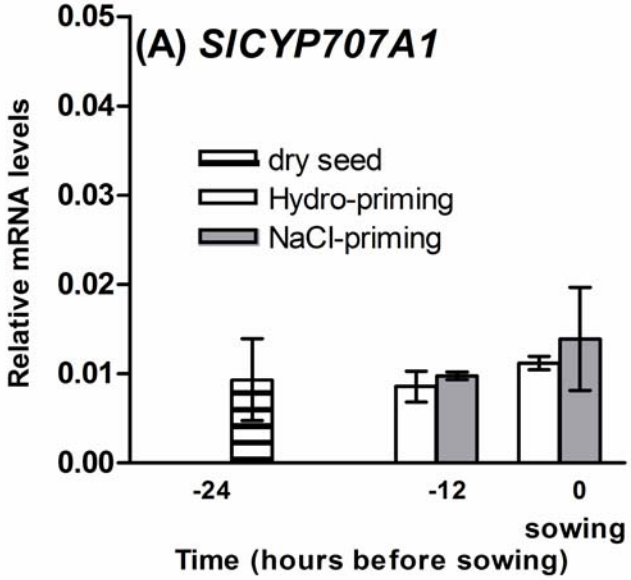
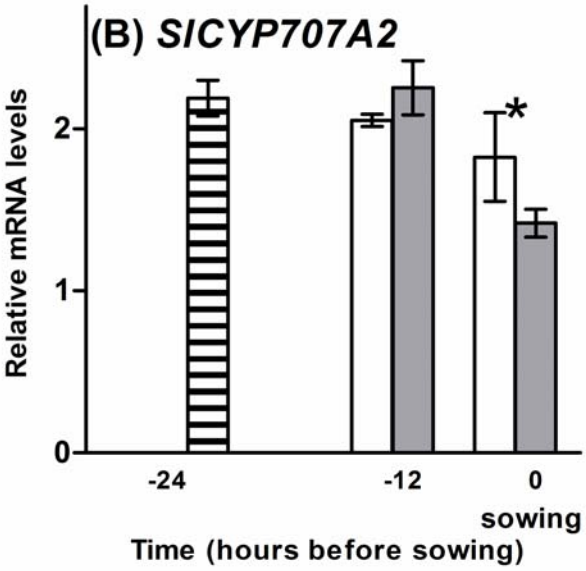
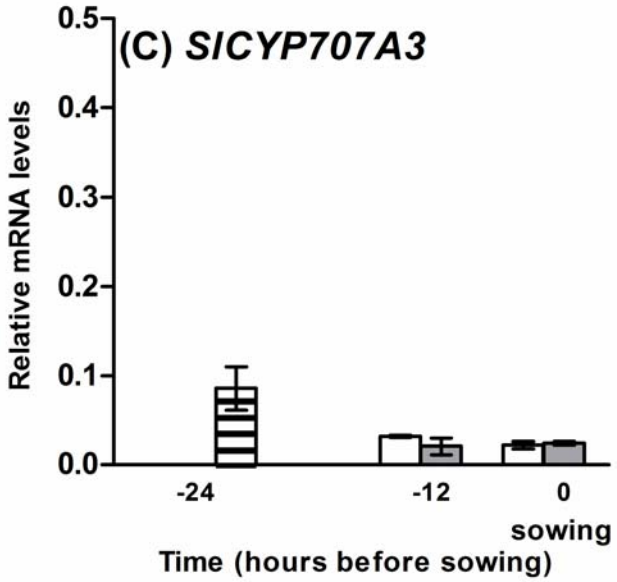
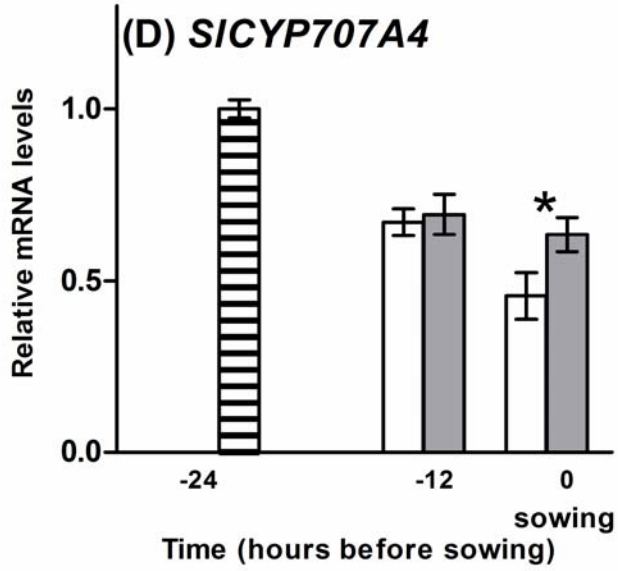

Germination (%)
100
80
60
40
20
Priming
a
a
a
a
b
b
Hydro-priming
NaCl-priming
GA4+7-priming
-24
0
sowing
24
36
48
60
72
96
120
Time (hours after sowing)

Fig 2


(A) SICYP707A1
dry seed
Hydro-priming
NaCl-priming
Relative mRNA levels
0.05
0.04
0.03
0.02
0.01
0.00
-24
-12
0
sowing
Time (hours before sowing)


(B) SICYP707A2
Relative mRNA levels
2
1
0
*
-24
-12
0
sowing
Time (hours before sowing)


(C) SICYP707A3
Relative mRNA levels
0.5
0.4
0.3
0.2
0.1
0.0
-24
-12
0
sowing
Time (hours before sowing)


(D) SICYP707A4
Relative mRNA levels
1.0
0.5
0.0
*
-24
-12
0
sowing
Time (hours before sowing)

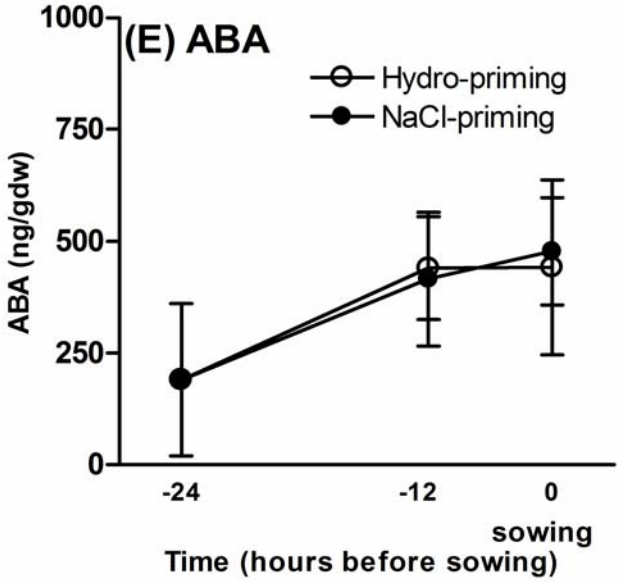
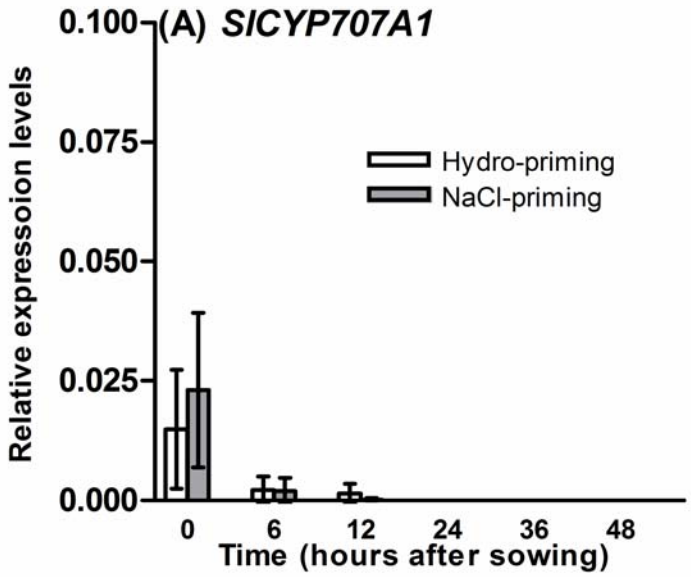
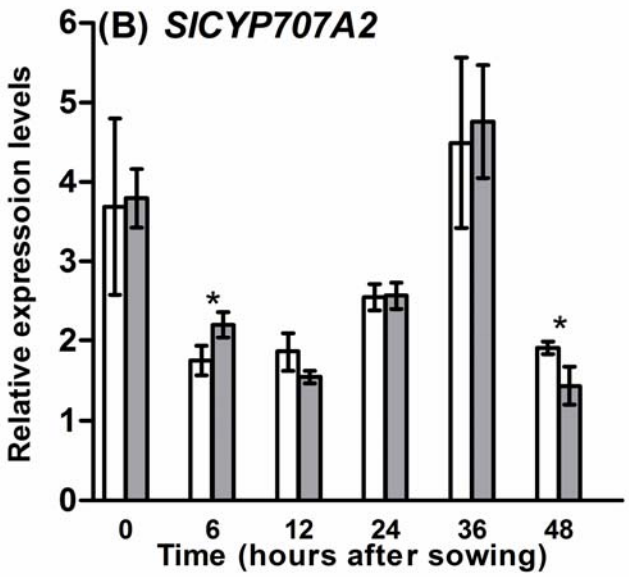
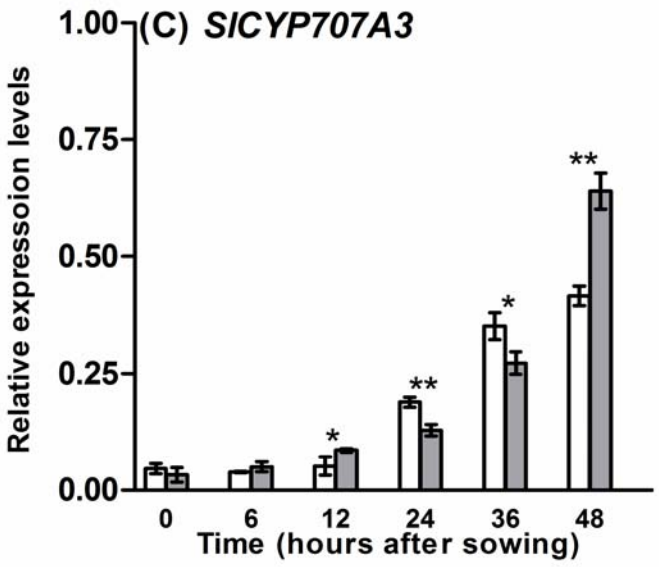
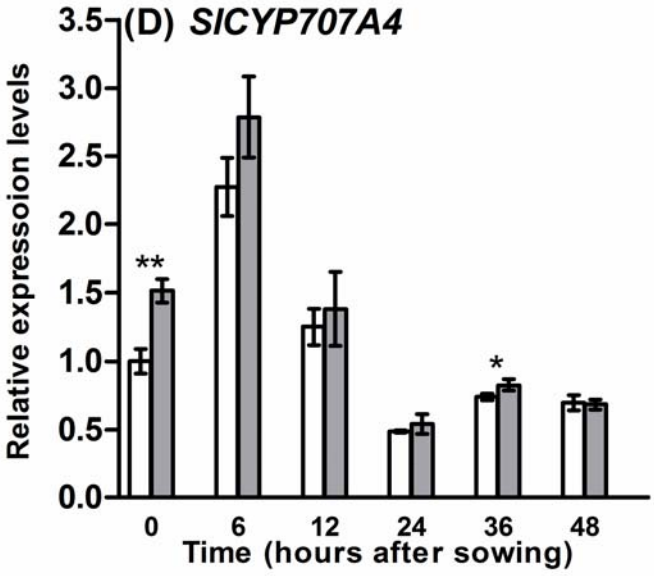

(E) ABA
Hydro-priming
NaCl-priming
ABA (ng/gdw)
1000
750
500
250
0
-24
-12
0
sowing
Time (hours before sowing)

Fig 3


(A) SlCYP707A1
Relative expressoion levels
0.100
0.075
0.050
0.025
0.000
Hydro-priming
NaCl-priming
0
6
12
24
36
48
Time (hours after sowing)


(B) SlCYP707A2
Relative expressoion levels
6
5
4
3
2
1
0
*
*
0
6
12
24
36
48
Time (hours after sowing)


(C) SlCYP707A3
Relative expressoion levels
1.00
0.75
0.50
0.25
0.00
*
**
*
**
0
6
12
24
36
48
Time (hours after sowing)


(D) SlCYP707A4
Relative expressoion levels
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
*
0
6
12
24
36
48
Time (hours after sowing)

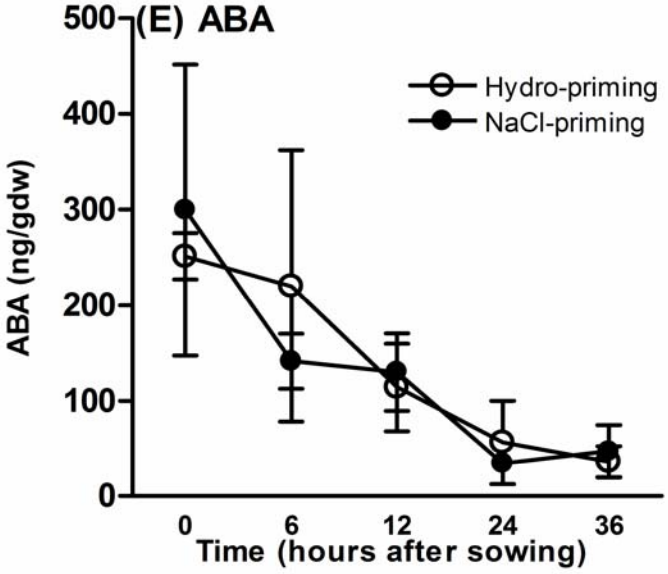
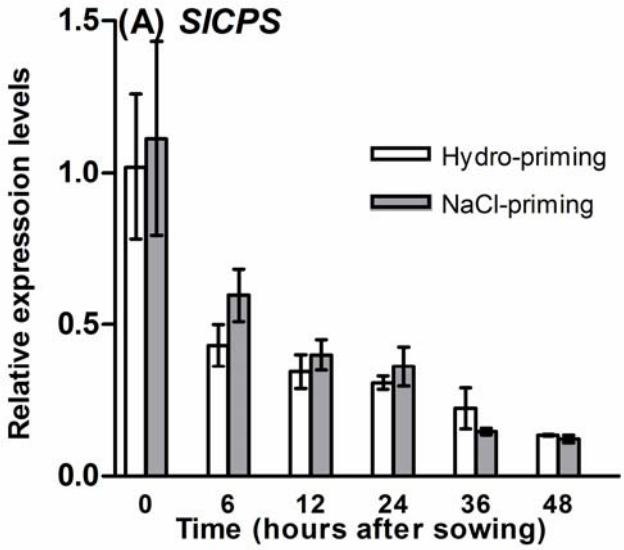
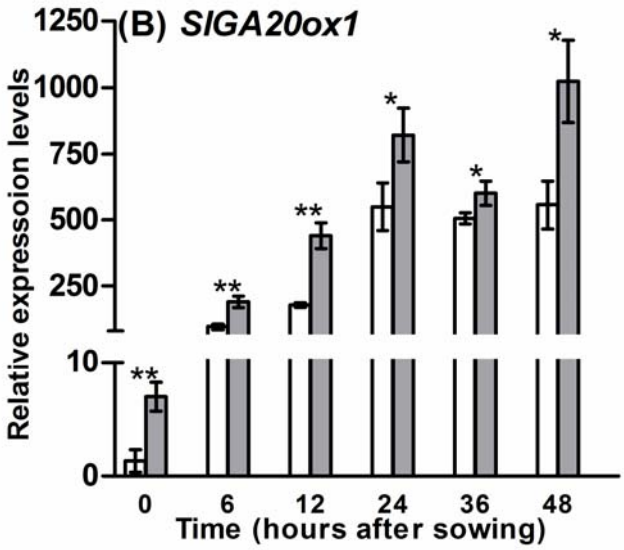
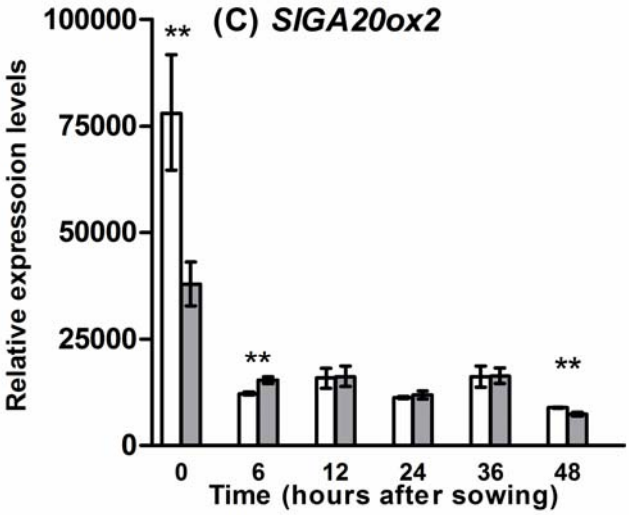
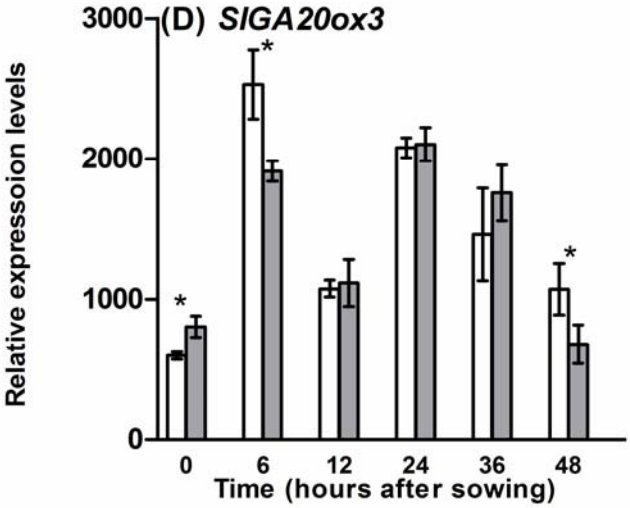

(E) ABA
ABA (ng/gdw)
500
400
300
200
100
0
Hydro-priming
NaCl-priming
0
6
12
24
36
Time (hours after sowing)

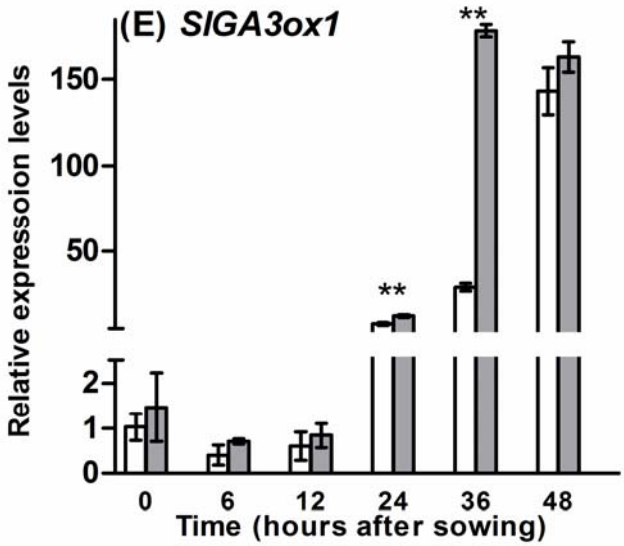
Fig 4


(A) SlCPS
Hydro-priming
NaCl-priming
Relative expressoion levels
1.5
1.0
0.5
0.0
0
6
12
24
36
48
Time (hours after sowing)


(B) SlGA20ox1
Relative expressoion levels
1250
1000
750
500
250
10
0
0
6
12
24
36
48
Time (hours after sowing)


(C) SlGA20ox2
Relative expressoion levels
100000
75000
50000
25000
0
0
6
12
24
36
48
Time (hours after sowing)


(D) SlGA20ox3
Relative expressoion levels
3000
2000
1000
0
0
6
12
24
36
48
Time (hours after sowing)


(E) SlGA3ox1
Relative expressoion levels
150
100
50
2
1
0
0
6
12
24
36
48
Time (hours after sowing)

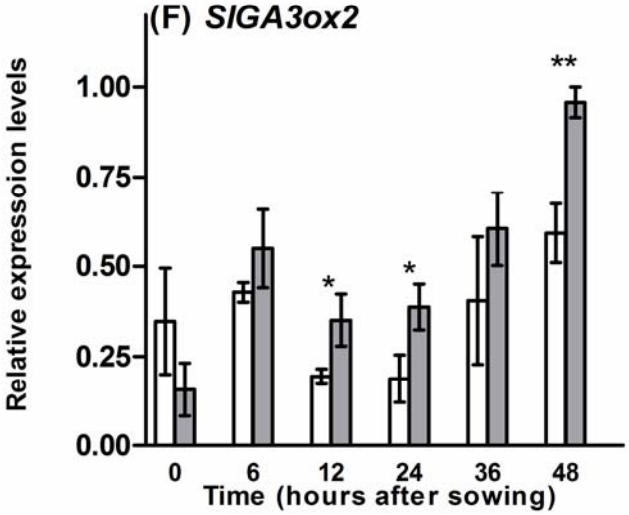

(F) SlGA3ox2
Relative expressoion levels
1.00
0.75
0.50
0.25
0.00
0
6
12
24
36
48
Time (hours after sowing)

Fig 5

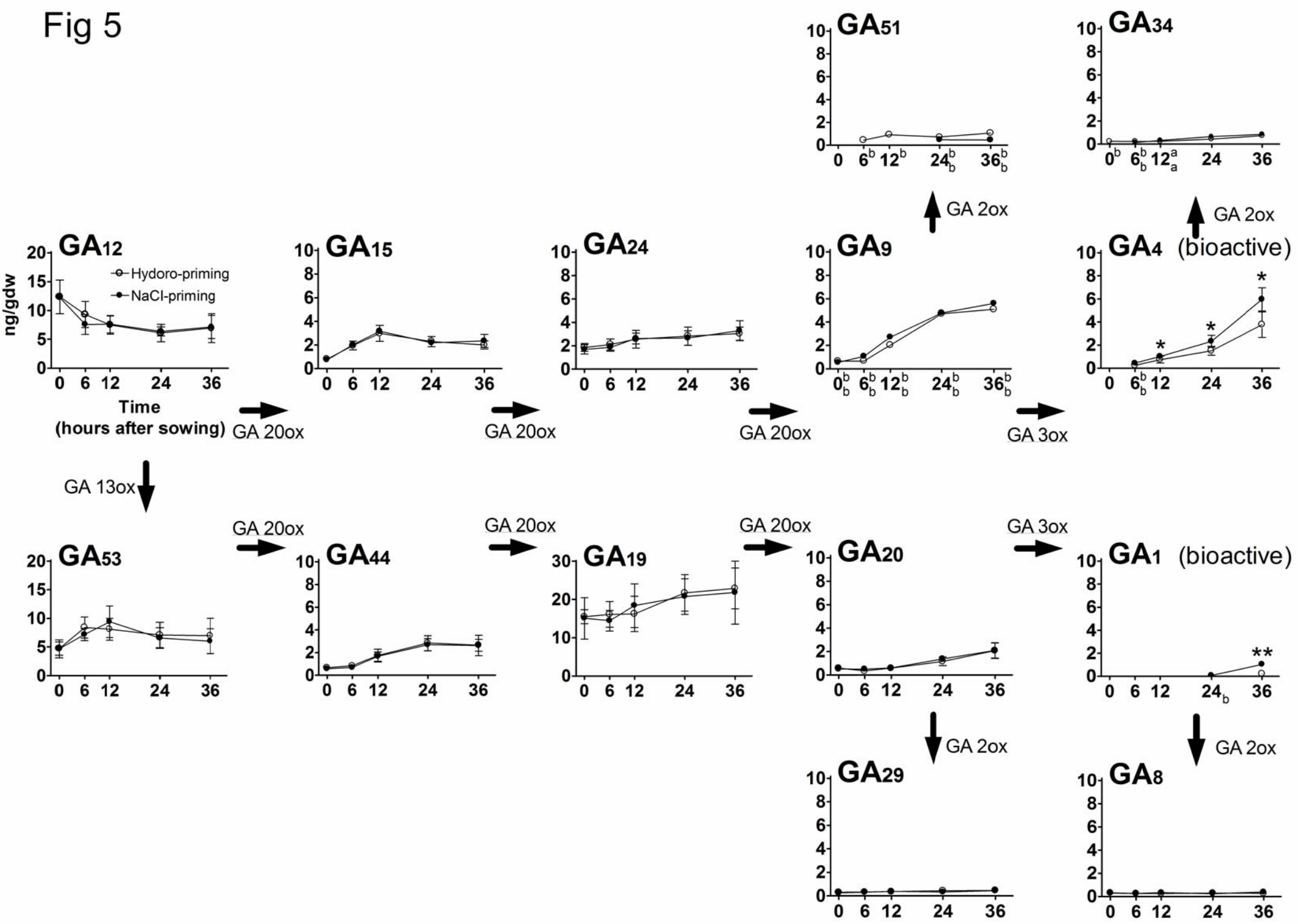
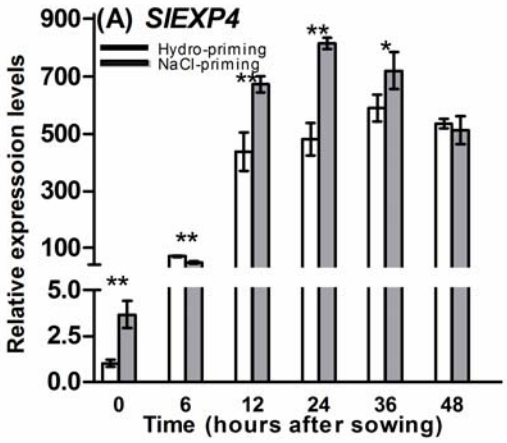
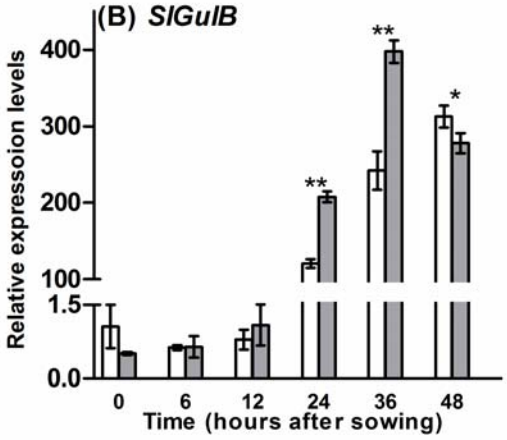
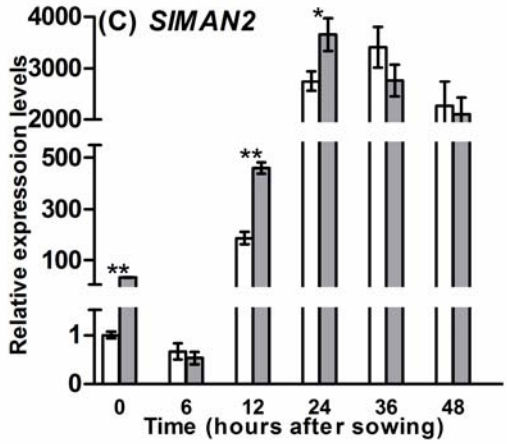
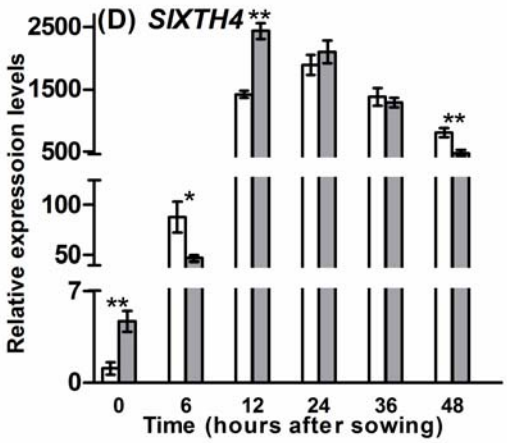

GA12
Hydoro-priming
NaCl-priming
ng/gdw
Time
(hours after sowing)
GA15
GA24
GA9
GA4 (bioactive)
GA51
GA34
GA53
GA44
GA19
GA20
GA1 (bioactive)
GA29
GA8
GA 20ox
GA 3ox
GA 13ox
GA 2ox

Fig 6


(A) SlEXP4
Hydro-priming
NaCl-priming
Relative expressoion levels
900
700
500
300
100
5.0
2.5
0.0
0
6
12
24
36
48
Time (hours after sowing)


(B) SlGulB
Relative expressoion levels
400
300
200
100
1.5
0.0
0
6
12
24
36
48
Time (hours after sowing)


(C) SlMAN2
Relative expressoion levels
4000
3000
2000
500
300
100
1
0
0
6
12
24
36
48
Time (hours after sowing)


(D) SlXTH4
Relative expressoion levels
2500
1500
500
100
50
7
0
0
6
12
24
36
48
Time (hours after sowing)