Genes involved in ethylene and gibberellins metabolism are required for endosperm-limited germination of *Sisymbrium officinale* L. seeds

Germination in *Sisymbrium officinale* L. seeds

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Abstract The rupture of the seed coat and that of the endosperm were found to be two sequential events in the germination of *Sisymbrium officinale* L. seeds, and radicle protrusion did not occur exactly in the micropylar area but in the neighboring zone. The germination patterns were similar both in the presence of gibberellins (GA_{4+7}) and in presence of ethrel. The analysis of genes involved in GAs synthesis and breakdown demonstrated that (1) *SoGA2ox6* expression peaked just prior to radicle protrusion (20–22 h), while *SoGA3ox2* and *SoGA20ox2* expression was high at early imbibition (6 h) diminishing sharply thereafter; (2) the accumulation of *SoGA20ox2* transcript was strongly inhibited by paclobutrazol (PB) as well as by inhibitors of ET synthesis and signaling (IESS) early after imbibition (6 h), while *SoGA3ox2* and *SoGA2ox6* expression was slowly depressed as germination progressed; (3) ethrel and GA_{4+7} positively or negatively affected expression of *SoGA3ox2*, *SoGA20ox2*, and *SoGA2ox6*, depending on the germination period studied. Regarding genes involved in ET synthesis, our results showed that *SoACS7* was expressed, just prior to radicle emergence while *SoACO2* expression slowly increased as germination progressed. Both genes were strongly inhibited by PB but were almost unaffected by externally added ethrel or GA_{4+7}. These results suggest that GAs are more important than ET during the early stages of imbibition, while ET is more important at the late phases of germination of *S. officinale* L. seeds.

Keywords ACC- and GAs-oxidases · Endospermic seed · Ethylene · Germination · Gibberellins (GA_{4+7}) · Hedge mustard · Mucilage · Real-time PCR · *Sisymbrium*

Abbreviations

ACC	1-Aminocyclopropane-1-carboxylic acid
ABA	Abscisic acid
ET	Ethylene
GAs	Gibberellins
GAox	GA-oxidase
IESS	Inhibitors of ET synthesis and signaling
PB	Paclobutrazol

Introduction

Seed germination begins the postembryonic development of plants, this in turn determining successful seedling establishment and plant propagation. Therefore, germination is tightly controlled by diverse environmental conditions as well as by the developmental program, in which abscisic acid (ABA) and gibberellins (GAs) are some of the main regulators known (Kucera et al. 2005; Yamaguchi et al. 2007; Rodríguez-Gacio and Matilla 2009). A mature and viable seed that has overcome dormancy is prepared to germinate (Bewley 1997; Carrera et al. 2008). Triggered by gradual water uptake, germination occurs once high transcriptomic and proteomic activity are coordinated by the integration of environmental and internal signals, all this resulting in optimal growth (Koornneef et al. 2002; Finch-Savage and Leubner-Metzger 2006; Holdsworth et al. 2008). Germination ends with the onset of cell elongation in

the embryonic axis, for which mitosis is not essential. The process becomes visible when the radicle has protruded through two covering layers (i.e. testa or seed coat and a single endosperm layer in endospermic seeds) at the level of the micropylar region (Bewley 1997; Kucera et al. 2005; Finch-Savage et al. 2007). Two major forces play antagonistic roles in the germination: the growth potential of the radicle and mechanical resistance of the covering layers. In order to complete germination, the growth potential of the radicle must overcome the tissue resistance of the micropylar covering layers. This situation is regulated by hormonal signaling (Kucera et al. 2005; Rodríguez-Gacio and Matilla 2009). Weakening of the micropylar endosperm surrounding the radicle tip appears to be required for radicle protrusion, in which cell-wall hydrolytic enzymes are presumably involved (Finch-Savage and Leubner-Metzger 2006; Nonogaki et al. 2007). By contrast, in some hard-coated seeds the micropylar endosperm presents lower physical constraint against germination than does the lateral endosperm, and hence its structure is predisposed to rupture (Gong et al. 2005). For many endospermic species, seed-coat rupture and endosperm breakage are two sequential steps during germination, e.g., in tobacco (Solanaceae; Leubner-Metzger 2002; Petruzzelli et al. 2003) and in *Arabidopsis thaliana*, and *Lepidium sativum* (Brassicaceae; Liu et al. 2005; Müller et al. 2006). ABA inhibits endosperm breakage, but not seed-coat rupture, of after-ripened seeds in these three species. GAs are known to act as ABA antagonists during seed germination by increasing the potential growth of the embryo to overcome the tissue constraints (Kucera et al. 2005) and/or by weakening the endosperm (Bewley 1997; Müller et al. 2006). GAs promote endosperm rupture in the Brassicaceae plants *Arabidopsis*, *Lepidium*, and *Sisymbrium officinale* (Müller et al. 2006). The GAs–ABA antagonism has been investigated intensively and has become basic to seed biology (Bewley 1997; Finch-Savage and Leubner-Metzger 2006; Nonogaki et al. 2007).

By contrast, precise and confirmed information is lacking in relation to the role of ethylene (ET) in the transition from dormancy to germination and during germination itself (Kucera et al. 2005; Matilla and Matilla-Vázquez 2008). Although maximum ET production is usually detected after radicle emergence has ended, small amounts of gas are also produced in periods preceding protrusion. Moreover, there are seeds that cannot break dormancy by the supply of ET alone, while in other cases this gas is enough (Kucera et al. 2005; Finch-Savage and Leubner-Metzger 2006; Matilla and Matilla-Vázquez 2008). ET synthesis and cell sensitivity are two major factors for germination in *Arabidopsis* (Siriwitayawan et al. 2003; Holdsworth et al. 2008). Proteomic analysis in *Arabidopsis* has suggested an essential role of endogenous ET only after radicle protrusion (Gallardo et al. 2002). ET mutants have been generated, and genetic, physiological, and molecular characterization of these mutants is starting to shed light on the involvement of ET in the complex process by which germination is regulated (Matilla and Matilla-Vázquez 2008). Thus, seeds of *etr1* and *ein2* display enhanced primary dormancy, while *ctr1* seeds have slightly reduced dormancy compared to the wild type. The *etr1-2* mutation in *Arabidopsis* confers dominant ET-insensitivity and results in a great proportion of mature seeds that exhibit deep primary dormancy (Chiwocha et al. 2005). The complexity of hormonal responses and their functional overlap support the idea of intensive cross-talk between hormone-signaling pathways (Razem et al. 2006; Weis and Ori 2007). Cross-talk between ET and GAs appears to occur in *Arabidopsis* (de Grauwe et al. 2007, 2008; Feurtado and Kermode 2007), but this situation during seed germination needs to be investigated.

The main purpose of this work was to study the possible role of ET and its interactions with GAs during germination of *S. officinale* L. seeds, as a continuation of our previous work focused in the physiology of the after-ripening (AR) in these seeds (Iglesias-Fernández and Matilla 2009). Taking this into account, we selected several important genes implicated in the metabolism of both hormones. ET is produced in higher plants from methionine (Met) by a well-defined pathway, the last two steps involving (1) the conversion of *S*-adenosyl-Met (SAM) to 1-aminocyclopropane-1-carboxylic-acid (ACC), catalyzed by ACC synthase (ACS), a pyridoxal phosphate-dependent enzyme; and (2) the subsequent oxidation of ACC to ET, catalyzed by ACC-oxidase (ACO), an unusual plant dioxygenase that uses ascorbate instead of α-ketoglutarate as a reductant (Vandendussche et al. 2006). On the other hand, the last reactions of the GAs biosynthesis pathway are catalyzed by three soluble 2-oxoglutarate dependent dioxygenases, GA20ox (gibberellic acid 20-oxidase), GA3ox (gibberellic acid 3-oxidase), and GA2ox (gibberellic acid 2-oxidase) (Fagoaga et al. 2007). GA20ox and GA3ox catalyze the synthesis of bioactive gibberellins and GA2ox catalyzes the conversion of bioactive gibberellins to inactive ones. For all these reasons, we decided to analyze the expression pattern of genes involved in ET synthesis (*SoACS7* and *SoACO2*) as well as in GAs synthesis (*SoGA20ox2* and *SoGA3ox2*) and breakdown (*SoGA2ox2*) during the time course of germination in the presence of ET and GA_{4+7} and their inhibitors (IESS and PB, respectively).

Materials and methods

Plant material

Siliques of wild hedge mustard (*S. officinale* L.) were harvested in Galicia (north-western Spain in a place located at

latitude +45° 52′31″ and longitude −0 h 34 m 14 s at 240 m above sea level) on 20 July 2007, where weather conditions were as follows: 21.3°C as average temperature and 61% of relative humidity. These fruits provided dark-brown and light-brown dry seeds; the dark-brown seeds were selected for our experiments and were cleaned, pooled (mature fresh seeds) and stored for 6 months in a desiccator (30% humidity) at 21 ± 0.2°C to obtain fully after-ripened seeds.

Light microscopy

Seeds were fixed in 50 mM sodium phosphate buffer, pH 6.8, containing 2% (w/v) *p*-formaldehyde (Panreac Química, Barcelona, Spain) and 2% (v/v) glutaraldehyde (Merck, Darmstadt, Germany) for 2 days at 4°C. Fixation was followed by an ethanol dilution series as described Gong et al. (2005). Following dehydration, seeds were embedded in LR White Resin (Sigma-Aldrich Química, Madrid, Spain) and cut in 2-μm sections with a microtome with wet glass knives. Staining was carried out in 0.5% (w/v) periodic acid (Merck), Schiff's Reagent (Merck) and 1% (w/v) Naphthol Blue Black (Sigma-Aldrich Química). A Phase Contrast Attachment "Ph" for Optiphot-2 microscope (Nikon Inc., Melville, NY, USA) was used for bright field microscopy. Images were taken with Coolpix 8400 camera (Nikon).

Germination assays

Three replicates of 50 seeds were imbibed in 90-mm Petri dishes on two layers of filter paper (Whatman No. 1). In order to accelerate germination, Petri dishes always contained NO_3^- (3 ml of 20 mM KNO_3, according to Hilhorst and Karssen 1988; Hilhorst 1990; Iglesias-Fernández et al. 2007). Different hormonal treatments were applied: 100 μM GA_{4+7}, 10 μM ethrel (compound that releases ET in solution), 10 μM 1-aminocyclopropane-1-carboxylic acid (ACC; ET immediate precursor), 25 μM paclobutrazol (PB; well-known GAs biosynthesis inhibitor) or a mixture of synthesis inhibitors [100 μM aminoethoxyvinylglycine (AVG) and 1 mM cobalt chloride (Co_2Cl)], and signaling [(10 μM silver thiosulphate (STS)] of ET (hereafter IESS). All chemicals were from Sigma-Aldrich Química. Germination experiments were carried out in a growth chamber at 24°C with a 16-h photoperiod. Seeds were not surface-sterilized in order to avoid influencing their dormancy status; fungal infections were not detected by light microscope. Seeds were considered germinated when radicle protrusion was visible. The specificity of the ethrel effects in this study was checked as described in Calvo et al. (2004a). Germination tests were performed at least twice using three replicates.

Tetrazolium test and ruthenium red staining

Intact seeds were incubated in a 1% (w/v) aqueous solution of 2,3,5-triphenyltetrazolium chloride (Merck) at 30°C in darkness for 2 days. Tetrazolium salts were metabolically reduced to highly colored end products called formazans by NADH-dependent reductases of the endoplasmic reticulum (Berridge et al. 1996). Mucilage was detected in the seed basically as described by Western et al. (2000). In short, the whole seeds were allowed to imbibe on moist filter for between 5 min and 1 h, before the application of 0.2% (w/v) aqueous ruthenium red solution. Seeds were photographed with an Olympus B061 stereomicroscope.

Quantification of ACC

Tissue frozen at −80°C (50 mg) was homogenized at 4°C using a mortar and pestle with sterile distilled water at a 1:5 ratio (FW/v). The homogenate was centrifuged at 8,300*g* (Beckman Avanti J-25 minifuge, rotor JA-18.1 for Eppendorf tubes) for 15 min at room temperature, and the supernatant was assayed for ACC analysis using Lizada's method (Lizada and Yang 1979).

Total RNA isolation from seeds and cDNA synthesis

For RNA extraction, dark-brown seeds (Iglesias-Fernández et al. 2007) at 0, 6, 12, 18, 20, 22, and 26 h of germination were collected, immediately frozen in liquid N_2, and stored at −80°C until used. Three replicates were taken for each point. Seeds were finely ground in liquid N_2 using a micro-dismembrator S (Sartorius, Göttingen, Germany) at a shaking frequency of 1,500 min^{-1} for 2 min. Total RNA was isolated using the phenol extraction/lithium chloride precipitation method (Verwoerd et al. 1989). The integrity and purity of the RNA was checked both electrophoretically and by the 260/280 nm absorbance ratio. Total RNA samples were digested with DNase (DNase I recombinant, RNase-Free, Roche Diagnostics, Mannheim, Germany) following the manufacturer's directions. The RNA concentration was estimated by A_{260} measurement, and the samples were stored at −80°C. The reagents used in this protocol were supplied by Sigma-Aldrich Química, SA The cDNA was synthesized from 1 μg of total RNA using the First-Strand Synthesis kit for RT-PCR (Roche Diagnostics) using oligo-p (dT) as a primer, following manufacturer's directions. Samples were stored at −20°C.

Real-time quantitative PCR assays

Isolation of *SoGA3ox2*, *SoGA20ox2*, *SoGA2ox6*, *SoACO2*, and *SoACS7* partial-length cDNAs was carried out as described Iglesias-Fernández and Matilla (2009). PCR analysis was performed with the cDNA extracted at different

times over the germination process, as stated above, as a template. Specific primer design was performed using the sequences obtained for *SoGA3ox*, *SoGA20ox*, *SoGA2ox*, *SoACO*, and *SoACS*. The *18S-RNA* was used as a control since it was found to be expressed at constant levels throughout the study period (Supplementary Fig. S1). The PCR was performed in an iCycler iQ™ Real-time Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Specific primers used were *SoGA3ox2*, 5′ CTGTGGTTGG CATTAGGTTC 3′, 5′ GAGAGTTGAGTCGGTATGGG 3′; *SoGA20ox2*, 5′ GGTCTTGGTGAAGGATGG 3′, 5′ AAG ATCATGGAGCTTCTGG 3′; *SoGA2ox6*, 5′ GTAGAT GGACTTGAGATTTGC 3′, 5′ CAGTCACCGACCAATA CG 3′; *SoACO2*, 5′ GGTGATAACCAACGGCAAGT 3′, 5′ TGTAGAACGAGGCAATGGAC 3′; *SoACS7*, 5′ GGC TTCTATGTTGTCGGA 3′, 5′ CGATCCCTGCCTTCTTA 3′; *18sRNA*, 5′ GGCTCGAAGACGATCAGATA 3′, 5′ TC ATAAGGTGCCGGCGGAGT 3′. For each 25-µl reaction, 1 µl of sample cDNA was mixed with 12.5 µl of IQ™ SYBR® Green Supermix (Bio-Rad Laboratories), 0.5 µl of forward primer (12 µM, final concentration 240 nM), 0.5 µl of reverse primer (12 µM, final concentration 240 nM), and 10.5 µl of sterile water. Samples were subjected to thermal-cycling conditions of DNA polymerase activation at 95°C for 4 min, 40 cycles of 45 s at 95°C, 45 s at 52°C (for *SoGA20ox*, *SoGA2ox*) or 55°C (for *SoGA3ox*, *SoACO* and *SoACS*), 45 s at 72°C, and 45 s at 80°C; a final elongation step of 7 min at 72°C was performed. The melting curve was designed to increase 0.5°C every 10 s from 62°C (for *SoGA20ox*, *SoGA2ox*) or 65°C (for *SoGA3ox*, *SoACO* and *SoACS*). Real-time PCR analysis was performed with two different cDNAs from the same time-point (from two different RNAs), and each was carried out in triplicate. The amplicon was analyzed by electrophoresis and sequenced once for identity confirmation. Quantification was based on analysis of the threshold cycle (Ct) value as described by Pfaffl (2001).

Statistical analysis

The statistical treatment was based on a variance analysis and averages/means were compared using the least significant difference (LSD) test at $P < 0.05$ (Steel and Torrie 1982).

Results

Germination of *S. officinale* seeds

Histological alterations of seed-coat and endosperm during the imbibition and protrusion

The mature seeds of the Brassicaceae *Arabidopsis*, *Lepidium* (Liu et al. 2005; Müller et al. 2006) and *S. officinale* (Fig. 1) are endospermic. The tetrazolium test applied to the seeds used in this work showed that 100% were alive. The seed is surrounded by the seed coat, which contains the outer and inner integuments (Fig. 1a; general view of *S. officinale* dry seed). The outer integument includes (1) a mucilaginous cell layer enriched in columella that produce abundant mucilage enveloping the seeds within a few minutes after the beginning of imbibition; this myxospermic character was demonstrated with ruthenium red dye (Iglesias-Fernández et al. 2007); and (2) a palisade epidermal cell layer with thickened cell walls rich in pectin. The inner integument is composed of a thin endodermic layer without differences in the cell-wall thickness (Fig. 1b). The endospermic tissue, containing only one cell layer, appears alongside the inner integument. In dry seeds, these endospermic cells have abundant protein bodies and low cell turgor (Fig. 1b). After 4 h of imbibition (Fig. 1c), (1) the number and height of the columella decrease; (2) the endospermic protein bodies begin to degrade; (3) the cell turgor increases in the endospermic micropylar region; and (4) the onset of water uptake triggers cell elongation and greater pressure of the radicle against the surrounding envelopes. At the end of imbibition (18–19 h) the view is (Fig. 1d; general view) as follows: (1) the palisade cell layer corresponding to micropylar zone is destroyed; (2) the endospermic protein bodies in the micropylar and adjacent to zone are emptied; (3) the micropylar endosperm layer is thinner than at 4 h of imbibition; (4) the protein bodies begin to degrade in the lateral region of radicle; and (5) the seed-coat rupture occurs near the micropylar zone (Figs. 1d, e). Events described above indicate that the micropylar endosperm breaks and the radicle protrudes (Fig. 1e). Therefore, *S. officinale* constitutes a good system to study the hormonal control of Brassicaceae endosperm rupture during germination.

The effect of ethrel and GA_{4+7} pulses in the germination profile

In control (20 mM KNO_3), protrusion began at 19–20 h (2–4 ± 1% of germination) and increased rapidly until 25–26 h (100% germination). In the presence of 100 µM GA_{4+7} or 10 µM ethrel, the protrusion became detectable at 15–16 h (4–5 ± 1% of germination) and 100% was reached at 23 h. Quantitatively significant differences were not detected between ethrel and GA_{4+7} treatments (Fig. 2). In the presence of ACC, the germination percentages were similar to those found with ethrel (Table 1). When GA_{4+7} and ethrel were added together to the imbibition medium, no boosting of the germination percentage with respect to the individual hormone treatments was observed (Table 1). The maximum germination percentage was barely diminished by the ACC-synthase inhibitor AVG (10 or 100 µM), while the

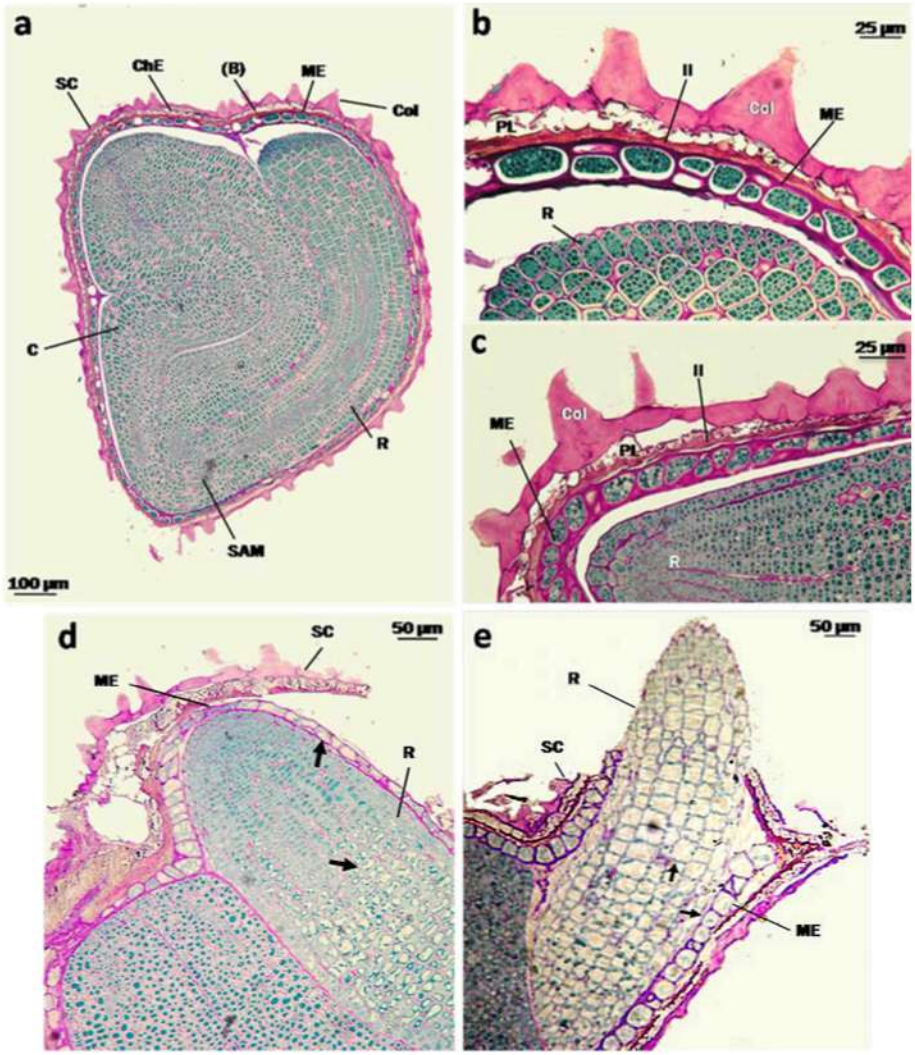

Fig. 1 Structure of a mature *S. officinale* L. seed. Bright field microscopy of longitudinal sections of seeds stained with PAS-Naphthol Blue Black. **a** Whole dry seed, showing the mature and fully differentiated embryo, the endosperm (aleurone layer), and the testa (seed coat). **b**, **c** Structure of micropylar zone enclosing the radicle tip in the dry seed at 4 h of imbibition,. **d** General view of a seed at 18–19 h of germination showing the testa disrupted and empty proteic bodies (*arrows*). **e** Radicle protrusion in germinating seed (22 h) showing the endosperm broken; *C* cotyledon, *ChE* chalazal endosperm, *Col* columella, *II* inner integument, *ME* micropylar endosperm, *PL* palisade layer, *R* radicle, *SAM* stem apical meristem, *SC* seed coat. The *letter* refers to the position of the close-up section. Experiments were carried out using at least 15 seeds. *Size bars* are given for each panel

ACC-oxidase inhibitor Co^{2+} (100 µM) slightly decreased it. All these inhibitions were amply overcome by GA_{4+7} and ethrel, whereas the inhibitor of bioactive GAs synthesis PB strongly prevented it (Table 2). We conducted pulse experiments to determine whether the time course of *S. officinale* seed germination was altered by ethrel or GA_{4+7} added at different times during the imbibition phase; the results referring to the time course may reflect the timing of the synthesis of ET or GAs during imbibition. For this purpose, ethrel or IESS (Fig. 2a–d) and GA_{4+7} or PB (Fig. 2e–h) were applied at 0, 4, 8, 12, and 16 h of imbibition, and the kinetics of induced germination were quantified until 27 h. The germination time course was altered, depending on the time at which the compounds were added. Thus, ethrel and GA_{4+7} stimulated radicle emergence with respect to control, only when applied early (i.e. 0, 4, and 8 h; Fig. 2a–c, e–g). On the contrary, the hormonal effect was null when ethrel or GA_{4+7} were added later (i.e. 16 h; Fig. 2d, h). However, the PB proved to be more efficient germination inhibitor when added early than IESS (Fig. 2a–g), but when they were applied after 16 h they showed a comparable level of imbibition (Fig. 2a–g).

The effect of ethrel and GA_{4+7} on ACC content and expression of *SoACS7* and *SoACO2* genes during seed germination

A slight peak of ACC coinciding with early radicle emergence was detected in control seeds (Fig. 3a). The acceleration of endosperm rupture caused by ethrel and GA_{4+7} also advanced the appearance of this peak that was quantitatively higher in the presence of ethrel than in that of GAs (Fig. 3b–d). The major levels of ACC observed in the presence of ethrel were perhaps a consequence of autocatalytic production. Moreover, the presence of GA_{4+7} provoked a sharp decline in ACC after reaching the maximum percentage of endosperm rupture; this was not observed in the presence of ethrel. The ACC content significantly declined with IESS after the imbibition phase, while PB caused the opposite effect (Fig. 3c, e). In the control and all studied

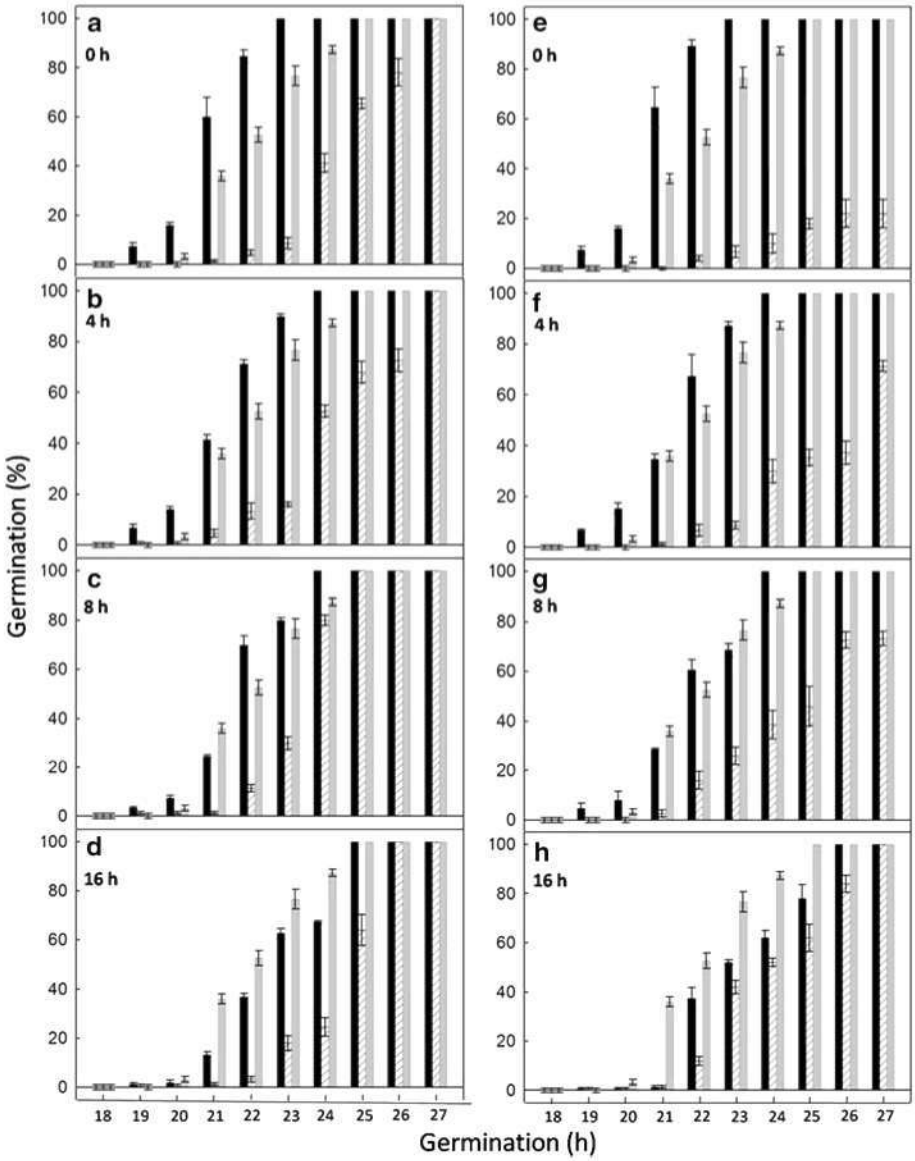

Fig. 2 Germination time course of *Sisymbrium officinale* seeds under indicated pulses of 10 μM ethrel (*black bars*), IESS (*scratched bars*) and control (*gray bars*) (**a–d**); and 100 μM GA_{4+7} (*black bars*); 25 μM PB (*scratched bars*) and control (*gray bars*) (**e–h**). The specificity of ethrel was from ethylene and not from an acid effect (Gallardo et al. 1991; Calvo et al. 2004a). Data are mean ± standard error (SE) of five independent experiments

Table 1 Effect of GA_{4+7} and ethrel on endosperm rupture of after-ripened seeds of *S. officinale*

Treatment	Endosperm rupture (%)				
	15 h	18 h	20 h	22 h	26 h
Control	–	–	9 ± 2b	51 ± 5f	100
GA_{4+7}	4 ± 2a	10 ± 3b	25 ± 4d	92 ± 7 h	100
Ethrel	–	8 ± 2b	24 ± 3d	89 ± 6 h	100
ACC	2 ± 1a	10 ± 1b	26 ± 2d	90 ± 1 h	100
GA_{4+7} + ethrel	–	5 ± 1a	21 ± 1d	85 ± 3 h	100

Data are mean values of three independent experiments ± SD. Significant differences between values as assessed by LSD test ($P > 0.05$) are shown as different letters (Steel and Torrie 1982)

treatments, a notable decrease in ACC content was observed during the onset of imbibition. A possible explanation for this sharp decline is that the ACC was referred to FW, which augmented markedly in the early stages of imbibition. In other seeds (e.g. chick-pea; Gallardo et al. 1991), a strong amount of ACC and 1-(malonylamino) cyclopropane-1-carboxylic acid (MACC) was lost into the germination medium during the initial stages of soaking. However, the percentage of ACC that can be reabsorbed by the seed is not known.

During imbibition, *SoACS7* expression was not detected in control under any of the treatments. However, notable levels of expression were found in the control when endosperm rupture reached 50–100% (Fig. 3a). This expression was hardly affected by ethrel (Fig. 3b), while it was detected earlier with GA_{4+7} (Fig. 3d), and IESS caused higher levels of expression at 26 h of germination (Fig. 3c). The presence of PB completely eliminated *SoACS7* expression (Fig. 3e). However, *SoACO2* expression, which was barely detected in control seeds during the first 6 h of

Table 2 Effect of AVG, Cl_2Co, PB, and IESS treatments on maximum germination percentage (26 h) of *S. officinale* after-ripened seeds

Control	100
AVG	87 ± 2
AVG + ethrel	100
AVG + GA_{4+7}	99 ± 1 c
Cl_2Co	62 ± 3 b
Cl_2Co + ethrel	98 ± 2c
Cl_2Co + GA_{4+7}	97 ± 2 c
PB	2 ± 1 a
PB + ethrel	95 ± 4 c
PB + GA_{4+7}	100
IESS	4 ± 2 a
IESS + ethrel	100
IESS + GA_{4+7}	99 ± 1 c

Data are mean values of three replicates ± SE. Significant differences between values as assessed by LSD test ($P > 0.05$) are shown as different letters (Steel and Torrie 1982)

imbibition, rose sharply during the germination process (Fig. 3f). It was stimulated by ethrel and GA_{4+7} (Fig. 3g, i), and was strongly inhibited by PB (Fig. 3j) but less by IESS (Fig. 3h).

Effect of ethrel and GA_{4+7} on expression of gibberellin-metabolism genes during seed germination

SoGA20ox2 and *SoGA3ox2* were strongly expressed during the first 6 h of imbibition (Fig. 4a, f). The level of *SoGA20ox2* transcripts between 12 and 26 h was 5–6 times lower than at 6 h, while the SoGA3ox-mRNA levels from 12 to 26 h were similar and about half the values registered at 6 h (Fig. 4a, f). The notable and early *SoGA20ox2* and *SoGA3ox* expression was markedly inhibited by ethrel (Fig. 4b, g) and GA_{4+7} (Fig. 4d, i), and was scarcely detected in the presence of ethrel and GA_{4+7} added together (Iglesias-Fernández and Matilla 2009). Likewise, the level of *SoGA20ox2* transcript was inhibited by IESS (Fig. 4c) and strongly by PB (Fig. 4e), and this inhibitory effect was stronger for *SoGA3ox2*, for which the transcript was not detected at 6–18 h (Fig. 4h, j). The profiles of *SoGA3ox2* expression were very similar in the presence of ethrel and GA_{4+7} and quantitatively increased with the germination process (Fig. 4g, i).

Contrary to *SoGA3ox* and *SoGA20ox* expression, the *SoGA2ox* transcript was hardly detectable at 6 h but peaked around the time of endosperm rupture (i.e. 18–20 h; Fig. 4k). This maximum was strongly inhibited by ethrel and GA_{4+7} (Fig. 4l, n) as well as by IESS and PB, strongly inhibiting *SoGA2ox* expression throughout germination (Fig. 4m, o). Curiously, GA_{4+7} and ethrel notably raised the levels of *SoGA3ox2*-mRNA at 6 h (Fig. 4l, n). A plausible explication for this may be that endogenous GA_{4+7} and ET induced the synthesis of bio-active GAs through *SoGA3ox2* (Fig. 5), thereby exceeding the threshold level of GAs needed at 6 h; these GAs induced *SoGA2ox6* expression to degrade them, thereby acquiring again the physiological levels of GAs. By contrast, ethrel and GA_{4+7} added together inhibited the *SoGA2ox6* expression to barely detectable levels (Iglesias-Fernández and Matilla 2009).

Discussion

The *S. officinale* seed is composed of the embryo, surrounded by two covering layers (i.e. seed coat and endosperm), and cotyledons (Fig. 1a, d). The micropylar endosperm surrounding the radicle tip is composed by one cell layer. In *A. thaliana* a monostratified micropylar endosperm also exists (Liu et al. 2005), but one or two layers appear in *L. sativum* (Müller et al. 2006) and a few in *L. virginicum* (Nguyen et al. 2000). As in *Arabidopsis* and *Lepidium* (Müller et al. 2006; Bethke et al. 2007; Piskurewicz et al. 2008), the germination of *S. officinale* seeds consists of two sequential phases (i.e. testa rupture followed by endosperm rupture) (Fig. 1e, f). Petruzzelli et al. (2003) showed that two-step-type germination (testa and endosperm rupture) was found in the Cestroidea sub-family of Solanaceae (e.g. *Nicotiana* and *Petunia*); but not in the Solanoideae sub-family (e.g. *Lycopersicon*) (Leubner-Metzger et al. 1998). The role of the testa as a germination constraint has been studied by using *Arabidopsis* mutants (Koornneef et al. 2002), but the intervention of micropylar endosperm in the seed-coat rupture is still unclear. (1) The secretion of hydrolytic enzymes, for which the substrates are structural components of the cell4 wall (i.e. loosening), and/or (2) the increase in the cellular expansion to cause the physical rupture of the testa, might be possibilities. Thus, the micropylar endosperm in *Arabidopsis* is considered to be a barrier for radicle protrusion and a determinant factor of coat dormancy (Müller et al. 2006; Bethke et al. 2007). In larger seeds, direct biomechanical measurements of the weakening are possible, showing that ABA inhibits and GAs promote this process during *Lepidium* seed germination (Müller et al. 2006). In tomato and coffee seeds, endosperm weakening appears to be biphasic, and only the second phase leading to endosperm rupture was found to be inhibited by ABA, and it was also demonstrated that ABA inhibits the growth potential of the embryo (Toorop et al. 2000; da Silva et al. 2004). GAs are known to act as ABA antagonists during seed germination by increasing the growth potential of the embryo to overcome the tissue constraints (Kucera et al. 2005) and/or by promoting endosperm weakening (Bewley 1997; Müller et al. 2006). Likewise, GAs can promote the endosperm rupture of the

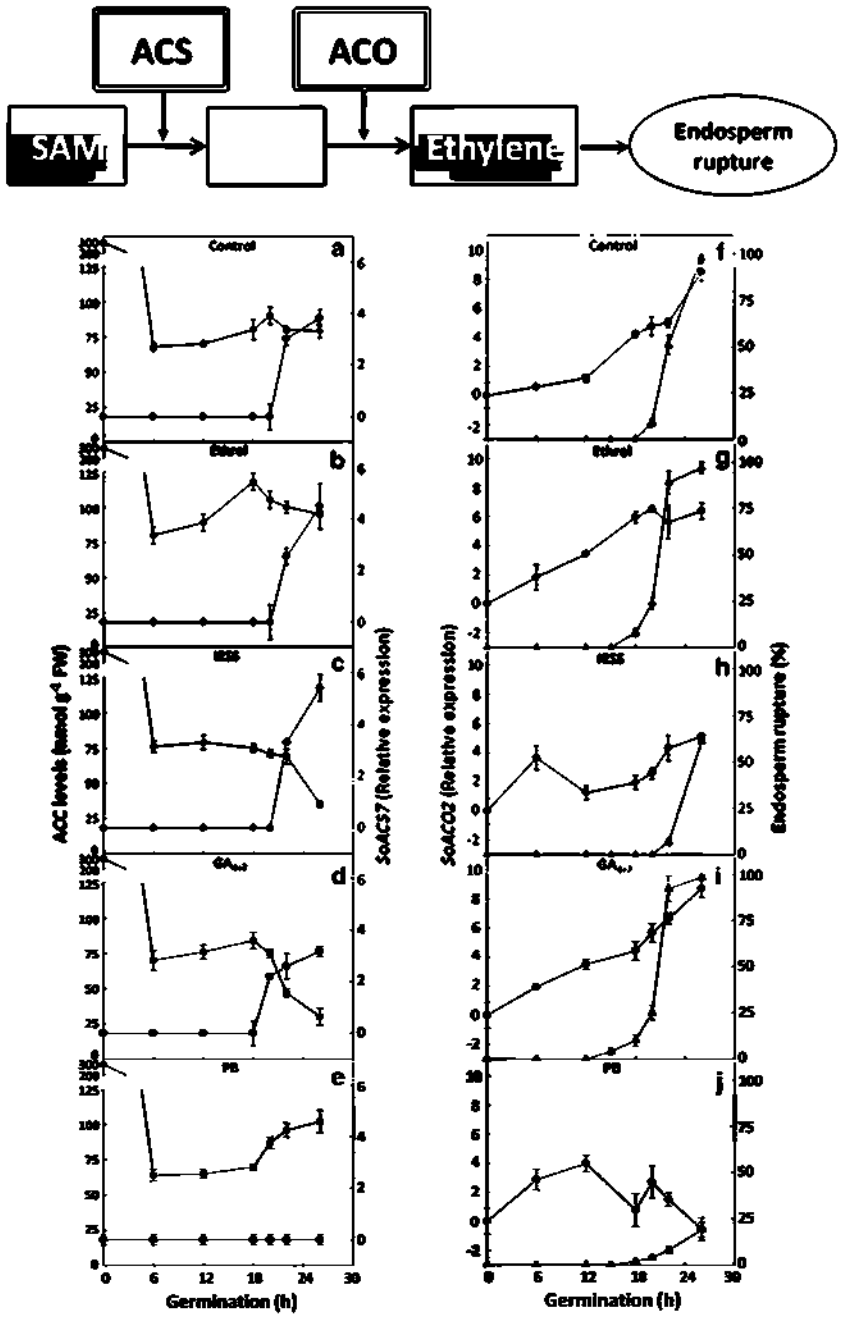

Fig. 3 Quantification of ACC levels and expression of *SoACS7* and *SoACO2*, analyzed by real-time qPCR, during germination of *S. officinale* seeds. **a–e** ACC levels (*squares*) and *SoACS7* (*circles*) expression during germination in presence of different treatments (control, ethrel, IESS, GA_{4+7}, and PB). **f–j** *SoACO2* (*circles*) expression during germination in presence of different treatments (control, ET, IESS, GA_{4+7}, and PB) and endosperm rupture rate (*triangles*). Note that the initial decrease in ACC/FW content (0–6 h) was related to the fact that FW was used and seed imbibition ended during the first few hours. *Error bars* represent the standard error of three (ACC quantification and gene expression) and five (germination percentage) independent experiments

Brassicaceae relatives *Arabidopsis*, *Lepidium*, and *S. officinale* (Müller et al. 2006). Here, we demonstrate that the covering tissues of S. *officinale* seeds surrounding the radicle apex are structurally altered during the early imbibition. Thus, the palisade layer is disorganized; the cell volume increases in the endosperm layer, and the abundant protein bodies begin to degrade; finally the protein bodies corresponding to sub-apical zone of radicle are also degraded. Similar structural changes were also previously documented (Bewley 1997; Toorop et al. 2000; da Silva et al. 2004; Bethke et al. 2007). Therefore, our results strongly suggest that endosperm weakening occurs in *S. officinale*.

The secretion of mucilage, an extremely hydrophylic pectin-rich polymer, is abundant in imbibed *S. officinale* seeds, completely encircling the seed coat (Fig. 1a–d). The mucilage-producing cells in mixospermous seeds such as *S. officinale* are located in the epidermal tissue of the testa (Western et al. 2004). Because the imbibition process must be tightly regulated in order to start the normal germination process, the mucilage may be involved in slow and controlled water uptake (Penfield et al. 2001). The fruit of *S. officinale* contains two kinds of seeds with respect to the color of their testa (i.e. dark-brown, used here, and light-brown). The dark-brown seeds have a greater capacity to

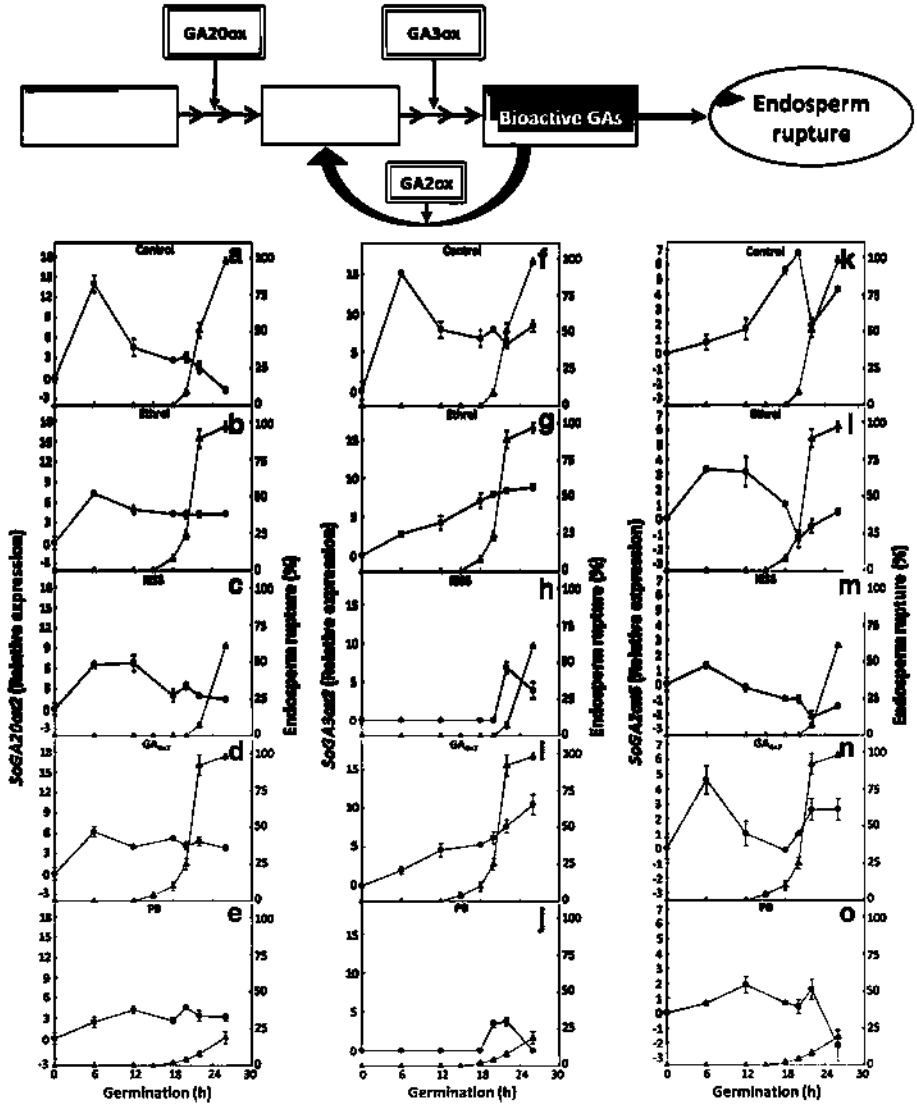

Fig. 4 Expression profile of *SoGA20ox2*, *SoGA3ox2*, and *SoGA2ox6*, analyzed by real-time qPCR, during germination of *S. officinale* seeds. **a–e** *SoGA20ox2* (*circles*) expression during germination in presence of different treatments (control, ethrel, IESS, GA_{4+7}, and PB) and endosperm rupture rate (*triangles*). **f–j** *SoGA3ox2* (*circles*) expression during germination in presence of different treatments (control, ethrel, IESS, GA_{4+7}, and PB) and endosperm rupture rate (*triangles*). **k–o** *SoGA2x6* (*circles*) expression during germination in presence of different treatments (control, ethrel, IESS, GA_{4+7}, and PB) and endosperm rupture rate (*triangles*). *Error bars* represent the standard error of three independent experiments

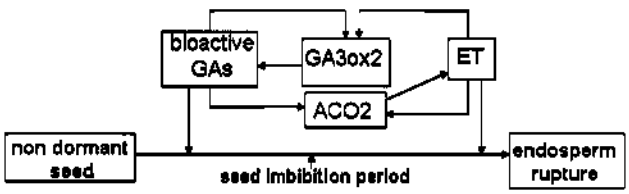

Fig. 5 Model for interaction between ET and GAs during the *S. officinale* seed germination. According to the interaction, ET and GAs positively act on *SoACO2* and *SoGA3ox2*. The model was based on Rodriguez-Gacio and Matilla (2009) and the results shown in this work

secrete mucilage, a slower water-uptake rate, and a faster germination rate (Iglesias-Fernández et al. 2007). Therefore, the mucilage might work in *S. officinale* to enhance and control the initial imbibition and therefore the germination process.

The functional overlap between different hormonal responses support the idea of an interaction between the signaling pathways (Brady and McCourt 2003; Chiwocha et al. 2005; De Grauwe et al. 2007, 2008; Holdsworth et al. 2008). The seeds of *S. officinale* are highly sensitive to ET and GA_{4+7}. The germination profiles in its presence were similar and these patterns were not altered by the addition of both hormones together. PB and IESS sharply inhibited the effect induced by GA_{4+7} and ET, and this inhibition was reversed by GA_{4+7} and ET, respectively (Iglesias-Fernández and Matilla 2009). Depending on developmental and environmental cues, ET and GAs have additive or synergistic (i.e. one hormone increases the responsiveness to the other) effects (Saibo et al. 2003; Vriezen et al. 2004; de Grauwe et al. 2007). Even though the intervention of GAs in seed germination appears to be beyond any doubt, data on the participation of ET are still scarce (for a review see Matilla and Matilla-Vázquez 2008). In the present work, although similar profiles of germination were induced by ET and GA_{4+7} added to the beginning of germination, a

detailed analysis of the germination time-courses shown in Fig. 2 (i.e. experiment of hormonal pulses) suggests a greater need for exogenous GA_{4+7} than ET. The germination kinetics determined in the presence of PB was consistent with this observation. Thus, the addition of PB at the beginning of imbibition led to an abrupt slowdown of radicle emergence, and the germination percentage was higher when the inhibitor was applied later. These data aim to a main GAs implication in the early imbibition period, although their synthesis is necessary during the whole germination period. In addition, the reduced germination profiles in the presence of IESS suggest that ET synthesis and signaling are also involved throughout the imbibition period.

To advance the understanding of the participation of ET and GAs during the germination of *S. officinale* seeds, we studied the expression of two genes involved in the ET synthesis (*SoACS7* and *SoACO2*) and three genes involved in GAs synthesis (*SoGA20ox2*, *SoGA3ox2*) and degradation (*SoGA2ox6*). GA biosynthesis takes place during early seed imbibition because germination can be inhibited at that time by GA-biosynthesis inhibitors (Pen and Harberd 2002; Ogawa et al. 2003; Fig. 2e, f of this work). Bioactive-GAs are required for seed germination, as demonstrated by the non-germinating phenotype of the *ga1-3 Arabidopsis* mutant (Koornneef et al. 2002; Ogawa et al. 2003). Likewise, the fact that (1) both *SoGA20ox2* and *SoGA3ox2*-mRNAs strongly increase in *S. officinale* during early imbibition (6 h); and (2) the *SoGA2ox6* expression involved in GAs deactivation is meager at 6 h in comparison to the rest of the germination period, suggests the involvement of GA synthesis in the preparation for germination of *S. officinale* seeds. Thus, *SoGA3ox2* is probably more involved than *SoGA20ox2* in the synthesis of bioactive-GAs during *S. officinale* seed germination, since the levels of *SoGA20ox2* transcripts are less abundant after 6 h. Given the wide expression of *SoGA2ox6* that takes place during the progression of radicle emergence, we can conclude that the level of bioactive-GAs during *S. officinale* seed germination is tightly controlled by the modulation of both their synthesis and catabolism.

The interactions between GAs and ET has been demonstrated to control several pathways of plant growth and development in *Arabidopsis* (Ogawa et al. 2003; Chiwocha et al. 2005; Weis and Ori 2007; De Grauwe et al. 2008; Dugardeyn et al. 2008). Recently, it was found that hypocotyl elongation induced by ET was dependent on, but not mediated by GAs (Vandenbussche et al. 2007). The Chiwocha's results suggest that ET signaling modulates the metabolism of several plant-hormone pathways in seeds (Chiwocha et al. 2005). In *S. officinale* seeds, *SoACS7* is expressed very late in the germination process, perhaps because the endogenous ACC levels are sufficient to supply *SoACO2*, the expression of which was noticeable during protrusion. It is possible that *SoACS7* is not the only member of the *SoACS* family related to germination; but we failed to isolate others. In this sense, the presence of ethrel or GA_{4+7}, which accelerated radicle emergence in *S. officinale*, positively altered the ACC levels and the *SoACO2*-expression pattern before protrusion. By contrast, in the presence of PB and IESS, the germination percentage, the ACC content, and the *SoACO2* expression were markedly inhibited. The addition of ET triggers a positive feedback that raises the expression of *PsACO1* in pea seeds (Petruzzelli et al. 2000). Likewise, the application of GA_3 also increased ACC content, ACC-oxidase, *FsACO1* expression, and ET synthesis in *Fagus sylvatica* seeds, suggesting some GAs-ET cross-talk (Calvo et al. 2004a). During GA_4 treatment of *ga1-3* seeds, which stimulated the germination, the expression of *AtACO* was also increased (Ogawa et al. 2003). Also, the treatment of *ga1-3* seeds with ET induced its germination (Kucera et al. 2005). When *AtACO2* expression was studied, ET was found to be induced in a feed-forward mechanism, the gene serving as a positive control for ET responses (De Paepe et al. 2004). Likewise, the expression of three *ACOs* genes was altered by ET (De Paepe et al. 2004). Taken together, the results shown in Fig. 3, and the notable levels of ACC existing under all treatments studied here, allow us to conclude that the immediate ET precursor ACC was not a limiting factor and hence the major regulation came via *SoACO2* expression, which ethrel and GA_{4+7} promoted (for hypothesis, see Fig. 5).

Notably, GAs and PB have an opposite effect on the expression of *SoACS7* and *SoACO2*, but a similar effect on the expression of GAs metabolism genes (*SoGA20ox2* and *SoGA3ox2*). Stimulation and inhibition in expression of *SoACS7* and *SoACO2*, caused by GAs and PB (respectively), could reflect the cross-talk GAs-ET. Meanwhile, the inhibition in expression of GAs metabolism genes caused by GAs could be generated by a negative feedback in the metabolism pathway. However, the inhibition provoked by PB could be a result of the total inhibition of GAs synthesis pathway from ent-kauren oxidase.

Our results indicate an interaction between GAs and ET signaling, since (1) the *SoGA3ox2* expression was strongly inhibited by PB and IESS, and the hormones studied; (2) ethrel and GA_{4+7} decreased two times the level of *SoGA20ox2*-mRNA during early imbibition and raised that level during germination; (3) PB markedly inhibited the expression of *SoGA20ox2* at 6 h of imbibition, whereas both PB and IESS slightly raised the levels of this transcript between 18 and 26 h; (4) *SoGA2ox6* expression was severely inhibited during germination by PB and IESS, and by GA_{4+7} and ethrel between 18 and 26 h, but GA_{4+7} and ethrel stimulated *SoGA2ox6* expression at the onset of

imbibition. Taking all the results together, we suggest that the regulation of the synthesis of bioactive-GAs involved in the germination of *S. officinale* seeds is subjected to a strong control by ET and GAs, and, if the threshold level of necessary GAs to prompt germination is surpassed (e.g. endogenous synthesis or exogenous GAs), a destruction mechanism exists (i.e. *SoGA2ox6*) to respond to this unnecessary synthesis. As an alternative proposal, and based on the study of the impact of the *etr1-2* mutation during the germination of *A. thaliana* (Chiwocha et al. 2005), it is possible in *S. officinale* that, (1) PB and IESS cause hormonal compensatory responses, or alternatively, (2) other hormonal pathways are usually regulated, either directly or indirectly, by GAs and ET in a positive or negative way. Germinating beechnut seeds evidenced a regulation cross-talk of *FsGA20ox1* expression by GAs and ET (Calvo et al. 2004b). GAs up-regulate the *ACO* and ET-inducible genes such as *HLS1* (Lehman et al. 1996) and an ET receptor gene *ERS1* (Hua and Meyerowitz 1998), suggesting that GAs activate ET biosynthesis and/or response. More recently, an up- and down-regulation by ET of different GAs-metabolism genes was also demonstrated; this finding is also related to ET-GA cross-talk (Dugardeyn et al. 2008).

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