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RESEARCH PAPER

Members of the gibberellin receptor gene family *GID1* (*GIBBERELLIN INSENSITIVE DWARF1)* play distinct roles during *Lepidium sativum* and *Arabidopsis thaliana* seed germination

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Abstract

Germination of endospermic seeds is partly regulated by the micropylar endosperm, which acts as constraint to radicle protrusion. Gibberellin (GA) signalling pathways control coat-dormancy release, endosperm weakening, and organ expansion during seed germination. Three GIBBERELLIN INSENSITIVE DWARF1 (GID1) GA receptors are known in *Arabidopsis thaliana*: GID1a, GID1b, and GID1c. Molecular phylogenetic analysis of angiosperm GID1s reveals that they cluster into two eudicot (GID1ac, GID1b) groups and one monocot group. Eudicots have at least one gene from each of the two groups, indicating that the different GID1 receptors fulfil distinct roles during plant development. A comparative Brassicaceae approach was used, in which *gid1* mutant and whole-seed transcript analyses in *Arabidopsis* were combined with seed-tissue-specific analyses of its close relative *Lepidium sativum* (garden cress), for which three *GID1* orthologues were cloned. GA signalling via the GID1ac receptors is required for *Arabidopsis* seed germination, GID1b cannot compensate for the impaired germination of the *gid1agid1c* mutant. Transcript expression patterns differed temporarily, spatially, and hormonally, with *GID1b* being distinct from *GID1ac* in both species. Endosperm weakening is mediated, at least in part, through GA-induced genes encoding cell-wall-modifying proteins. A suppression subtraction hybridization (SSH) cDNA library enriched for sequences that are highly expressed during early germination in the micropylar endosperm contained expansins and xyloglucan endo-transglycosylases/hydrolases (XTHs). Their transcript expression patterns in both species strongly suggest that they are regulated by distinct GID1-mediated GA signalling pathways. The GID1ac and GID1b pathways seem to fulfil distinct regulatory roles during Brassicaceae seed germination and seem to control their downstream targets distinctly.

Key words: *Arabidopsis thaliana*, endosperm weakening, expansin, GIBBERELLIN INSENSITIVE DWARF1, *Lepidium sativum*, seed germination, xyloglucan endo-transglycosylase/hydrolase.

Introduction

Germination of endospermic seeds is a complex developmental process. It starts with water uptake by imbibition of the dry seed and ends when the radicle has protruded through all seed covering layers (Bewley, 1997*a*). In mature seeds of most angiosperm species, the embryo is encased by the endosperm and testa (seed coat) as covering layers (Linkies *et al.*, 2010*a*). The endosperm is therefore localized between the embryo and the testa and, in most mature seeds, the endosperm is a living tissue. Traditionally the endosperm was recognized primarily as a nutrient source

during seed development and seedling growth (Lopes and Larkins, 1993). However, evidence has accumulated over time (Linkies *et al.*, 2010*a*) that shows that the endosperm in mature seeds, in particular the micropylar endosperm covering the radicle also plays a major role in regulating germination timing (Ni and Bradford, 1993; Bewley, 1997*b*; Leubner-Metzger, 2003; Linkies *et al.*, 2009). For the completion of germination, the tissue resistance of the micropylar endosperm must be overcome by the growth potential of the radicle. For several species it has been shown that, during germination, weakening of the micropylar endosperm takes place, which decreases the threshold of the force needed for radicle protrusion through the endosperm layer to complete germination. This is the case in species with a thick endosperm, such as tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*), and coffee (*Coffea arabica*) and also for seeds with a thin endosperm, such as lettuce (*Lactuca sativa*) and garden cress (*Lepidium sativum*) (Ni and Bradford, 1993; Bewley, 1997*b*; Toorop *et al.*, 2000; Leubner-Metzger, 2003; da Silva *et al.*, 2004; Müller *et al.*, 2006). This supports the view that the endosperm, in particular the micropylar endosperm, participates in regulating germination timing.

Seed germination is inhibited by abscisic acid (ABA) and promoted by gibberellins (GA) (Kucera *et al.*, 2005). It has been shown that the induction of endosperm weakening requires an embryo signal which consists, at least in part, of bioactive GA or GA-biosynthesis precursors (Bewley, 1997*b*; Ogawa *et al.*, 2003). Treatment of 'isolated micropylar endosperm caps' with GA can replace the embryo signal to induce the onset and accelerate *L. sativum* endosperm weakening (Müller *et al.*, 2006). ABA-insensitive mutants like *Arabidopsis thaliana* (*Arabidopsis*) *abi3* and ABA-deficient mutants like *Arabidopsis aba1-1* and tomato *sitiens* show reduced seed dormancy (Groot and Karssen, 1992; Nambara *et al.*, 2000; Bassel *et al.*, 2006). Inhibition of endosperm weakening by ABA has been shown for *L. sativum* and other seeds (Bewley, 1997*a*; Müller *et al.*, 2006; Linkies *et al.*, 2009). Contrary to that, high GA contents and sensitivities lead to dormancy release and promotion of the germination process. GA-deficient mutants, such as *ga1-3* (*Arabidopsis*) and *gib1* (tomato), need an external supply of GA to induce and complete germination (Koornneef and Veen, 1980; Groot *et al.*, 1987). Endogenous GA is perceived by the GA receptors of the GID1 (GIBBERELLIN INSENSITIVE DWARF1) family, first identified in rice (OsGID1, *Oryza sativa*) (Ueguchi-Tanaka *et al.*, 2005). In *Arabidopsis*, three GID1-type GA receptors are known: AtGID1a, AtGID1b, and AtGID1c. After binding to its receptor, the GA–GID1 complex interacts with DELLA proteins which are negative regulators of the GA signalling pathway (Richards *et al.*, 2001). The F-Box protein SLY1/GID2, part of the $SCF^{SLY1/GID2}$ E3 ubiquitin ligase complex, recognizes the GA–GID1–DELLA complex and targets the DELLA repressors for degradation through the 26S proteasome. This leads to a de-repression and allows transcription of GA-responsive genes (Ueguchi-Tanaka *et al.*, 2007; Yamaguchi, 2008). GA treatment of the GA-deficient *Arabidopsis* mutant *ga1-3* results in the disappearance of the DELLA protein RGL2 (Tyler *et al.*, 2004) for which SLY1 is required (Ariizumi *et al.*, 2007). GA-insensitive *Arabidopsis* mutants, for example, *gid1*-receptor triple and *sly1-10* knockouts, and gain-of-function DELLA-mutants such as *gai*, show impaired germination (Steber *et al.*, 1998; McGinnis *et al.*, 2003; Dill *et al.*, 2004; Griffiths *et al.*, 2006; Iuchi *et al.*, 2007; Willige *et al.*, 2007). By contrast, loss-of-function DELLA-mutants such as *gai-t6* show enhanced germination (Kucera *et al.*, 2005). *L. sativum* endosperm weakening and its promotion by GA as a replacement for the embryo signal (Müller *et al.*, 2006) is thought to be achieved by cell-wall-modifications. The plant cell wall is a highly complex composite composed mainly of cellulose microfibrils embedded in a matrix of hemicellulosic and pectic polysaccharides (Cosgrove, 2005; Knox, 2008). Cell expansion growth is driven by water uptake and is restricted by the cell wall, whose structural properties and mechanical strength determine the shape, rate, and direction of growth of individual cells as well as of whole tissues (Cosgrove, 2005; Schopfer, 2006). Cell wall loosening is therefore an important process in all stages of plant development requiring elongation growth or tissue weakening, as it is the case during seed germination, where radicle growth and endosperm weakening take place. Some cell-wall modifying proteins are known to accumulate in a germination-specific and GA-induced manner. For endospermic Solanaceae seeds, these include β-1,3-glucanase (Leubner-Metzger *et al.*, 1995; Petruzzelli *et al.*, 2003) and β-1,4-mannanase (Bewley, 1997*b*; Nonogaki *et al.*, 2000). For endospermic Brassicaceae seeds, differential regulation of β-1,4-mannanase in the *L. sativum* micropylar endosperm and radical tissue has been shown by Morris *et al.* (2011) and *Arabidopsis* β-1,4-mannanase knockout mutants have a germination phenotype (Iglesias-Fernandez *et al.*, 2011). Isoforms of both enzymes accumulate specifically in the micropylar endosperm, covering the radicle tissue, but not in the non-micropylar endosperm. One of the most important stabilizing and tension-bearing interactions in the primary cell wall of eudicots is between cellulose microfibrils and the hemicellulose xyloglucan, on which xyloglucan endo-transglycosylases/hydrolases (XTHs) can act by transglycosylation (cleaving and reconnecting) or hydrolysis (Rose *et al.*, 2002). Another group of cell-wall-modifying proteins are expansins, thought to act by loosening of non-covalent bonds (Sampedro and Cosgrove, 2005). Both XTHs and expansins are induced by GA in the micropylar endosperm during tomato seed germination and proposed to facilitate endosperm weakening (Chen and Bradford, 2000; Chen *et al.*, 2002).

The bigger seeds of *L. sativum*, a close relative of *Arabidopsis*, were used to construct an endosperm-specific subtractive suppression hybridization (SSH) cDNA library to clone candidate genes involved in the early germination processes. Genes encoding cell-wall-modifying proteins including XTHs and expansins were over-represented in this library. Since GA is known to promote endosperm

weakening, this prompted us to investigate the transcript co-expression pattern of the GA receptors *GID1a*, *b*, and *c* and putative cell-wall-modifying genes. A combined approach was carried out with *Arabidopsis* mutants and seed and seed-tissue-specific analysis of *Arabidopsis* and *L. sativum* transcript expression. Our work shows that the eudicot GID1 receptors group into a GID1ac and a GID1b group, which, during seed germination, are regulated differentially and suggest two separate, but partially redundant pathways for GA responsiveness that have distinct downstream cell-wall-loosening genes as targets.

Materials and methods

Plant materials and germination assays

Mature seeds of *Arabidopsis thaliana* (L.) Heyhn. were harvested and stored for after-ripening at 25 °C, 51–54% relative humidity. The seeds were incubated on 1/10 Murashige-Skoog salts (medium) pH 7.0, solidified with 1% (w/v) agar-agar in continuous white light (*c*. 100 µmol s^{-1} m^{-2}) at 24 °C. Homozygous *gid1* mutant seeds were kindly provided by Professor Dr Claus Schwechheimer, ZMBP, Tübingen, Germany (Willige *et al.*, 2007) and by Shiori Ota, Bio Resource Centre RIKEN, Iberaki, Japan (Iuchi *et al.*, 2007). Testa rupture and endosperm rupture were scored using a binocular microscope as described by (Müller *et al.* (2006). After-ripened seeds of *Lepidium sativum* FR14 (Juliwa, Germany) were incubated in Petri dishes on two layers of filter paper with 6 ml 1/10 Murashige-Skoog salts medium in the light. Where indicated, gibberellin A_{4+7} (GA_{4+7}; Duchefa, The Netherlands) or *cis*-S(+)-abscisic acid (ABA; Duchefa) were added in the concentrations indicated.

RNA extraction and creation of the subtractive cDNA library

Total RNA from endosperm caps dissected from seeds after 8 h and 18 h imbibition was prepared as described by Cadman *et al.* (2006). A Suppression Subtraction Hybridization (SSH) library was constructed using the PCR Select™ cDNA Subtraction kit (Clontech, USA) as described in Linkies *et al.* (2010*b*). cDNA from micropylar endosperm dissected after 8 h imbibition of the whole seed was used as tester, subtracted with cDNA from endosperm caps dissected after 18 h imbibition as a driver. The resulting cDNA fragments were cloned in pUC19 and pCR®4-TOPO, multiplied in *E. coli* Top10 and sequenced (GATC, Germany). 184 cDNAs were sequenced and have been deposited as ESTs to GenBank, their accession numbers are listed in Supplementary Table S2 at *JXB* online.

Cloning of cDNA sequences from L. sativum

First strand cDNA was synthesized in 50 µl reactions from 5 µg total RNA from *L. sativum* FR14 seedlings, 2.5 µM oligo(dT)16, 2.5 µM random hexamers, using the Superscript III reverse transcriptase kit (Invitrogen, Germany) according to its instructions. PCR was performed using primers designed based on known *A. thaliana* sequences. At least three independent cDNA clones were sequenced to verify *L. sativum* sequences.

Analyses of transcript levels by quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from 200 *L. sativum* radicles and 1000 micropylar endosperm caps which were dissected from seeds at the times indicated, frozen in liquid nitrogen, and stored at –20 °C. RNA extraction of *Arabidopsis* was done using 200 whole seeds of WT or the *gid1* mutants. RNA extraction was carried out as described by Chang *et al.* (1993). Four biological replicate RNA samples of each time point and treatment were used for downstream applications. qRT-PCR was performed with first-strand cDNAs as templates that were obtained using the Superscript III reverse transcriptase kit (Invitrogen, Germany) with 0.3 nmol random 15-mers for reverse transcription of 5 µg RNA. Aliquots of 1 µl were then used for each quantitative RT-PCR reaction. Absolute QPCR SYBR Green ROX Mix (ABgene; UK) was used according to its instructions for quantification with the ABI PRISM 7300 Sequence Detection System (Applied Biosystems, UK). A melting curve confirmed single product amplification. Analysis of the raw data and calculation of the efficiency (E) for every single well was done using the software PCR miner (Zhao and Fernald, 2005). Relative expression for each well was calculated as $(1+E)^{-CT}$. Expression data for *Arabidopsis* and *L. sativum* was normalized by using the geometric mean (geomean) of validated housekeeping genes. Mean values ±SE shown were calculated from four biological replicates. Primers were designed using the bioinformatics software Geneious 4.7.5 (Biomatters, New Zealand) and are listed in Supplementary Table S3 at *JXB* online.

Histochemical GUS-staining and visualization

pAtGID1a::AtGID1a-GUS, pAtGID1b::AtGID1b-GUS, and *pAtGID1c::AtGID1c-GUS* reporter lines were kindly provided by Masatoshi Nakajima, University of Tokyo, Japan (Suzuki *et al.*, 2009). Seeds were placed in Petri dishes on two layers of filter paper with 1/10 Murashige-Skoog (MS) salts and imbibed for 20 h at 24 °C in continuous white light (*c*. 100 µmol s^{-1} m^{-2}). Where indicated, 5 µM ABA or 10 µM GA_{4+7} were added to the medium. 10 seeds with ruptured testa but intact endosperm were dissected into the embryo and the seed 'coats' (testa plus endosperm). Tissue fixation was done with 90% acetone for 20 min at room temperature. Afterwards, the seed tissues were incubated in 100 µl staining solution (50 mM phosphate-buffer (pH 7.2), 0.2% (v:v) Triton X-100, 10 mM EDTA (pH 8.0), and 2 mM X-gluc). Images were taken after 20 h of staining with the software IM1000 (Leica Microsystems, Wetzlar, Germany) and a Leica DCF480 digital camera attached to a Leica 12.5 binocular microscope.

Tetrazolium assay testing embryo viability

Testa and endosperm of imbibed seeds were removed and embryos were stained according to the procedure described by Graeber *et al.* (2010) in 1% (w/v) 2,3,5-triphenyltetrazolium chloride (Sigma, Germany) in phosphate buffer (pH 7) at room temperature for the time indicated. As negative controls, heat-killed seeds (dry seeds were incubated at 100 °C for 1 h) were used; wild-type seed served as positive controls. Two biological replicates of several seeds each were analysed per line.

Sequence alignments and molecular phylogenetic analysis

For sequence analysis, the bioinformatics software Geneious 4.7.5 (Biomatters, New Zealand) was used. Geneious Align was used for sequence alignments, with the BLOSUM62 matrix for alignments of protein sequences. Geneious Tree Builder was used for construction of the phylogenetic tree using the sequences of Supplemenatry Table S1 at *JXB* online.

Results

Three putative orthologues of the Arabidopsis GID1a, GID1b, *and* GID1c *genes are expressed in* L. sativum

Three *L. sativum GID1* cDNAs were cloned and analysed, including the complete coding sequences (cds) and the 5′ and 3′ untranslated regions (UTRs). Based on detailed

analysis (Fig. 1; see Supplementary Figs S1 and S2 at *JXB* online), these sequences of *L. sativum* were named *LesaGID1a*, *LesaGID1b*, and *LesaGID1c* according to the putative orthologous *AtGID1a*, *AtGID1b*, and *AtGID1c* genes of *Arabidopsis*, respectively, and have been submitted to GenBank (accession numbers HQ003455, HQ003456, HQ003457). Comparative sequence analysis between the cds of *GID1a*, *GID1b*, and *GID1c* of *Arabidopsis* and *L. sativum* showed 91.4, 89.4, and 91% pairwise identity (see Supplementary Fig. S1 at *JXB* online), which are values similar to known orthologues of *L. sativum* and *Arabidopsis* (Linkies *et al.*, 2009, 2010*b*; Graeber *et al.*, 2010). Pairwise comparative analysis on amino acid level showed that the putative GID1a, GID1b, and GID1c orthologous proteins of *L. sativum* and *Arabidopsis* displayed 96.2, 92.8, and 94.8% pairwise identity, respectively. In most cases, the observed amino acid changes did not affect protein hydrophobicity or polarity (see Supplementary Fig. S2 at *JXB* online). GID1 belongs to the family of hormone-sensitive lipases which are characterized by two conserved amino acid motifs: HGG and GXSXG (Østerlund, 2001; Hirano *et al.*, 2008). It was demonstrated that all three *Arabidopsis* GID1s bind GA and interact with DELLA proteins (Nakajima *et al.*, 2006; Suzuki *et al.*, 2009). From the crystal structures of the GA–GID1 and GA–GID1–DELLA complexes (Murase *et al.*, 2008; Shimada *et al.*, 2008) conserved amino acids for GA binding and DELLA repressor binding are known (for details see Supplementary Fig. S2 at *JXB* online). These conserved amino acids, as well as the HGG and GXSXG motifs are all present at the expected positions in the predicted GID1 proteins of *L. sativum* (see Supplementary Fig. S2 at *JXB* online). It is therefore proposed that LesaGID1a, LesaGID1b, and LesaGID1c are fully functional GA receptors.

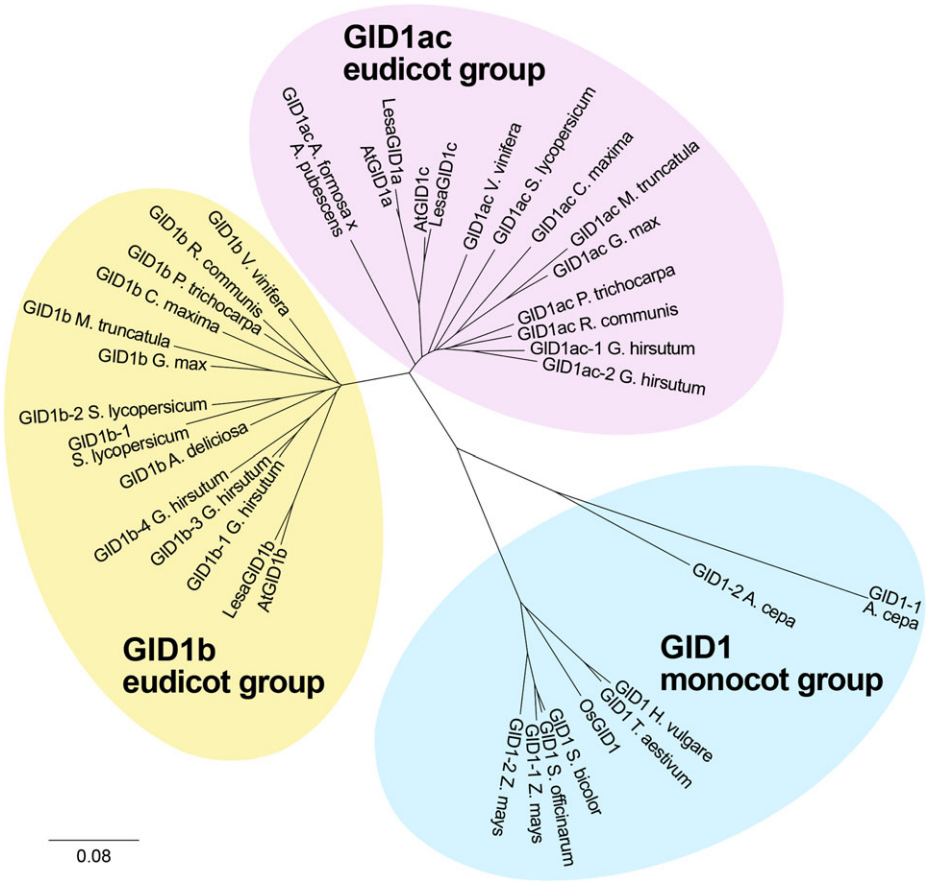

Fig. 1. Molecular phylogenetic analysis of the angiosperm GID1 receptor family reveals individual clustering into three groups: eudicot GID1ac, eudicot GID1b, monocot GID1. Translated protein sequences of 37 available GID1 full-length or near full-length cDNA sequences from different species were aligned into a phylogenetic unrooted tree as described in the methods. A. cepa=*Allium cepa*; A. deliciosa=*Actinidia deliciosa*; A. formosa=*Aquilegia formosa*; A. pubescens=*Aquilegia pubescens;* At=*Arabidopsis thaliana;* Lesa=*Lepidium sativum;* C. maxima=*Cucurbita maxima;* G. hirsutum=*Gossypium hirsutum*; G. max=*Glycine max*; H. vulgare=*Hordeum vulgare*; M. truncatula=*Medicago truncatula*; Os=*Oryza sativa*; P. trichocarpa=*Populus trichocarpa*; R. communis=*Ricinus communis*; S. bicolor=*Sorghum bicolor*; S. lycopersicum=*Solanum lycopersicum*; S. officinarum=*Saccharum officinarum*; T. aestivum=*Triticum aestivum*; V. vinifera=*Vitis vinifera*; Z. mays=*Zea mays*. Sequence accession numbers are listed in Supplementary Table S1 at *JXB* online. Note that, in addition to tomato, various other asterids (*Lactuca* and *Helianthus* species) have at least one GID1ac and one GID1b gene. They are listed in Supplementary Table S1 at *JXB* online, but were not included in the phylogeny as no full-length sequences are available.

Angiosperm GID1 gene family members fall into three distinct phylogenetic groups: eudicot GID1ac, eudicot GID1b, and monocot GID1

As *Arabidopsis* and *L. sativum* have several GID1 genes, it was investigated whether this is a general feature of the eudicots and compared it with the monocots, in which, for example, the rice genome contains only one GID1 gene (*OsGID1*). Thirty-seven angiosperm GID1 cds were used to carry out a molecular phylogenetic analysis (Fig. 1). Alignment of all predicted proteins showed that the eudicot GID1 genes cluster into two distinct groups: the 'GID1ac' and the 'GID1b' group, which were named according to the corresponding *Arabidopsis GID1* genes that they contain. Almost all of the included core eudicot species (rosids and asterids) have at least one GID1 member of each group. An exception is *Actinidia deliciosa* for which only one GID1 was found, which may be due to insufficient sequence availability. In addition to the asterid species included in the phylogenetic analysis (Fig. 1), EST sequences were also found that demonstrate that several *Lactuca* and *Helianthus* species have at least one GID1 from each group (see Supplementary Table S1 at *JXB* online). It is an interesting finding that the monocot GID1 proteins (in contrast to the core eudicots) cluster into one separate group with no further split, even when they contain more than one *GID1* gene (Fig. 1).

Comparison of GID1 transcript secondary structures and stability motifs suggests high mRNA turnover, and GID1 transcript expression during Arabidopsis *seed germination is differentially regulated by GA and ABA*

The predicted secondary structures differed between the six *GID1* mRNA molecules of *L. sativum* and *Arabidopsis*, but in all cases one intron splice site just downstream the start of the coding sequence and relatively long 5' and 3' UTRs are evident (see Supplementary Figs S1 and S3 at *JXB* online). The average 5' and 3' UTR lengths from all annotated genes of the *Arabidopsis* genome are 0.13 kb and 0.24 kb, respectively (Haas *et al.*, 2003). With 0.35–0.49 kb, all the *GID1* 3' UTRs except *LesaGID1c* are longer than average (see Supplementary Fig. S1 at *JXB* online) and this is often associated with high mRNA turnover (Garneau *et al.*, 2007). mRNA half-lives of *AtGID1a* and *AtGID1b* determined by Narsai *et al.* (2007) in *Arabidopsis* cell cultures are short with $t_{1/2}$ <1 h, whereas *AtGID1c* transcripts are of moderate stability. Together with mRNA secondary structure (see Supplementary Fig. S3 at *JXB* online; Hofacker and Stadler 2006), various global motifs associated with mRNA destabilization, identified by Narsai *et al.* (2007) and Jiao *et al.* (2008) are prominent in the *GID1* 3' and 5' UTRs (see Supplementary Figs S3 and S4 at *JXB* online). Therefore, *GID1* transcripts of both species may not only be highly regulated, but also possess conserved regulatory patterns as well as differences between the two species. As considerable amounts of bioactive GA_4 are already present in dry *Arabidopsis* seeds (Ogawa *et al.*, 2003), it is expected that GA responsiveness of the seed tissues is induced immediately upon imbibition and that its regulation during germination depends, at least in part, on the expression of the three *AtGID1* genes. The *AtGID1a*, *AtGID1b*, and *AtGID1c* transcript expression patterns were analysed during the germination of unstratified *Arabidopsis* seeds by quantitative real-time RT-PCR (qRT-PCR, Fig. 2): The transcript abundance at 30 h imbibition of *AtGID1a* and *AtGID1c* was high compared with *AtGID1b*, with *AtGID1a* transcripts being twice as abundant as *AtGID1c* transcripts (CON, Fig. 2C). This difference in transcript abundance, *AtGID1a*>*AtGID1c*>>*AtGID1b*, is already evident in dry seeds and during the early germination phase, as shown in Fig. 2B, based on *in silico* analysis of microarray expression data (Preston *et al.*, 2009) with the *Arabidopsis* eFP browser (Winter *et al.*, 2007). Furthermore, the hormonal regulation of the *AtGID1* transcript expression was analysed by qRT-PCR (Fig. 2C): GA exerts a negative feedback mechanism causing about a 2-fold down-regulation of the transcript levels of *AtGID1a* and probably also *AtGID1c*, but did not affect *AtGID1b* expression. In contrast to GA, ABA did not exert any significant effect on the transcript levels of *AtGID1a, AtGID1b* or *AtGID1c* (Fig. 2C). The differential transcript regulation in seeds, especially the higher abundance and the GA-triggered negative feedback loop associated only with the two *GID1ac* group members, but not with *GID1b*, suggests that the two groups of *GID1* receptors may play distinct roles during the germination process.

The *pAtGID1::AtGID1-GUS* reporter gene lines from Suzuki *et al.* (2009) were used to investigate the spatially and hormonally regulated expression patterns of the three individual *GID1* genes in different *Arabidopsis* seed tissues, i.e. the embryo and the endosperm, during germination. Transcription of the three *pAtGID1::AtGID1-GUS* reporter constructs is controlled by *c.* 3 kb of *AtGID1a*, *AtGID1b*, and *AtGID1c* 5' regulatory region containing the corresponding *GID1* gene promoters, and in the following text the three transgenic lines are referred to with the abbreviations 'PGID1a', 'PGID1b', and 'PGID1c', respectively. In agreement with the qRT-PCR results on whole *Arabidopsis* seeds imbibed in medium without the addition of hormones (control, CON), the histological GUS staining of PGID1a and PGID1c embryos was much stronger compared with PGID1b (CON in Fig. 3A). As the GID1 gene promoter activity is an approximate estimate for GID1 gene transcription and, based on the roughly equal distribution of blue staining over the entire embryos, it is concluded that GID1 gene transcription is not confined to a highly localized zone. There are, however, clear differences in the strength of the three GID1 promoter activities that suggest that the high GID1ac and low GID1b transcript abundances in CON-seeds (Fig. 2) are the result of higher-level GID1ac and low-level GID1b gene transcription in CON-embryos (Fig. 3A). To investigate the effects of ABA and GA, seeds of the different transgenic lines were also imbibed with the hormones added. In agreement with

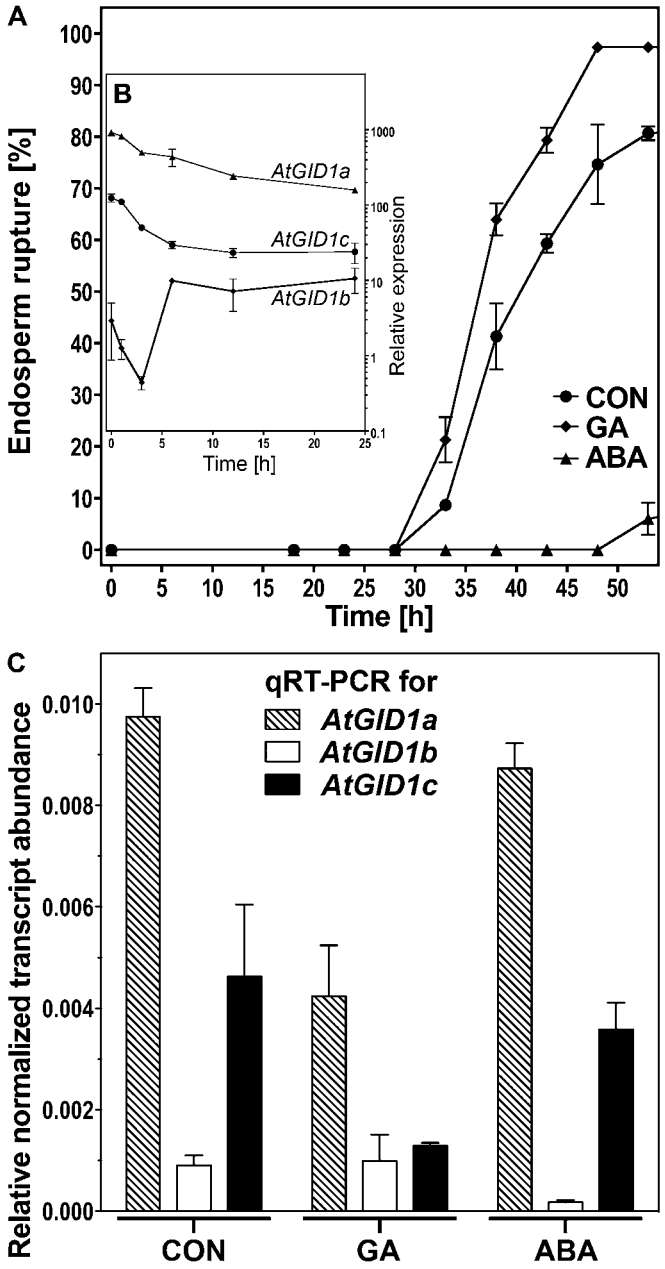

Fig. 2. Transcript abundances in whole seeds of the three *Arabidopsis thaliana GID1* genes during germination. (A) The time-course of endosperm rupture of *A. thaliana* (Col) seeds on medium without (CON) or with 10 μM GA_{4+7} (GA) or 1 μM ABA added. Afterripened seeds were incubated at 24 °C in continuous light without preceding stratification. Mean values ±SE of 3×50 seeds. (B) *In silico* analysis on *GID1* transcript abundance during early germination of unstratified *Arabidopsis* (Col) seeds based on eFP-Browser microarray data; Relative expression values ±SD for *AtGID1a* (At3g05120), *AtGID1b* (At3g63010), and *AtGID1c* (At5g27320). (C) *GID1* transcript abundance pattern determined by qRT-PCR in whole seeds of *Arabidopsis* (Col) at 30 h, i.e. at 0% endosperm rupture for CON (see A). ddCt expression values relative to validated constitutive transcripts are presented. Medium additions as in (A). Mean values ±SE of 4× >1000 seeds.

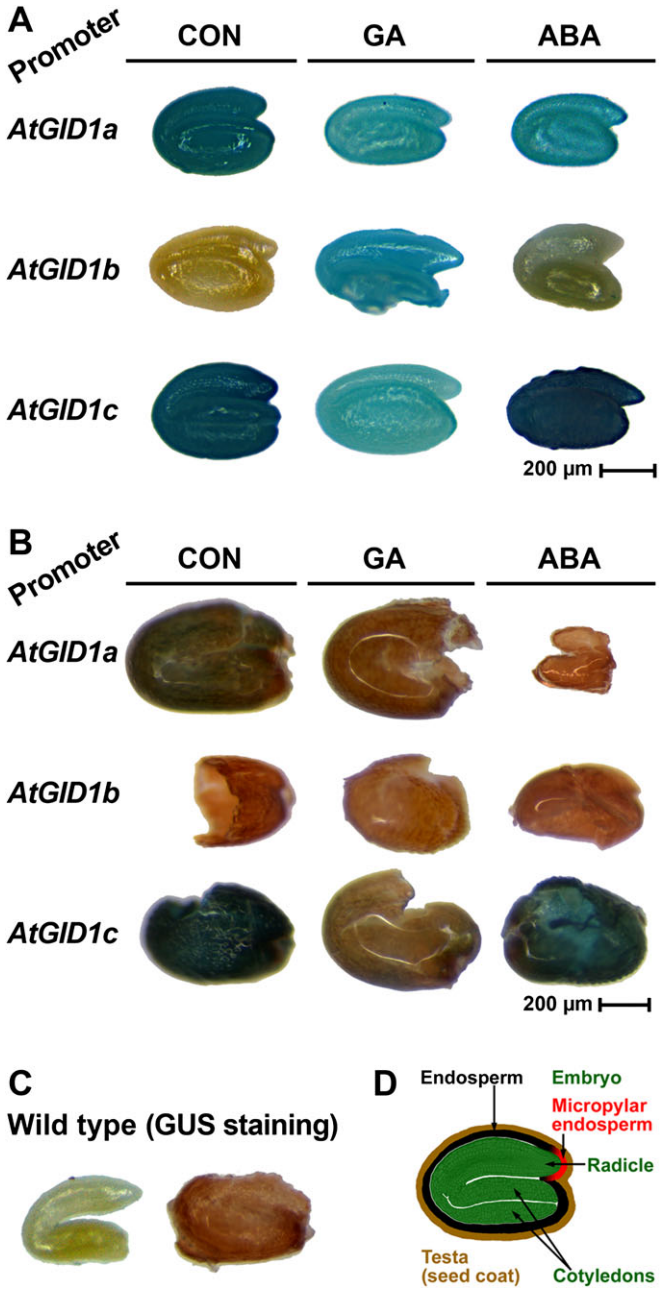

Fig. 3. Histochemical analysis of gene promoter activities in of the three *Arabidopsis thaliana GID1* genes during seed germination. Seeds of GUS reporter lines in which the three *GID1* gene promoters, *AtGID1a, AtGID1b,* and *AtGID1c* control the transgene transcription were imbibed in medium without (CON) or with 10 μM GA_{4+7} (GA) or 5 μM ABA added. After seed dissection into embryo and 'coat' (testa plus endosperm) at 20 h, histochemical GUS analysis was performed. (A) Histochemical GUS analysis of isolated embryos from *GID1* reporter gene seeds. (B) Histochemical GUS analysis of isolated 'coats' from *GID1* reporter gene seeds. (C) Negative control for the histochemical GUS staining with isolated wild-type embryo and 'coat'. (D) Cross-section of *A. thaliana* seed indicating the specific seed tissues analysed used in the experiment.

a GA-mediated down-regulation of the GID1ac transcript abundances in the qRT-PCR analysis (Fig. 2), GA addition decreased the staining intensity of the PGID1a and PGID1c embryos (Fig. 3A). In contrast to the roughly equal

transcript abundances for *GID1b* in CON and GA (Fig. 2), the blue staining is stronger in embryos from GA-treated seeds compared with CON (Fig. 3A). This suggests as a possible explanation that, for *GID1b*, the transcript degradation is of utmost importance for the GA-mediated down-regulation of the transcript abundances. Neither GA nor ABA changed the spatial expression patterns in embryos for the three *GID1* genes. While GA affected the staining intensities, ABA did not appreciably change the staining intensities of the embryos compared with CON (Fig. 3A). The staining was slightly stronger for PGID1c-compared to PGID1a-embryos for all three treatments (Fig. 3A), whereas, for the transcript abundances, it was the other way around. This implies that transcript degradation may be more important for regulating the GID1c transcript abundances. As for the embryo, the staining intensities in the endosperm were PGID1c>>PGID1a> PGID1b for the CON-series (Fig. 3B). Interestingly, the effects of the hormones on the staining intensities differed between embryo and endosperm: In contrast to the embryo, GA-treatment did not cause increased staining in the PGID1b endosperm. But, as in the embryo, GA-treatment caused decreased staining for PGID1a and PGID1c. For the ABA-treatment the decreasing effect on the PGID1a staining in the embryo was even more pronounced in the endosperm, while for PGID1b and PGID1c the ABA effects did not differ between embryo and endosperm (Fig. 3). Taken together, the embryo and the endosperm of *Arabidopsis* seeds (Fig. 3D) exhibited similar and distinct aspects regarding the spatial and hormonal regulation of the three *GID1* gene promoter activities. This regulation was more similar for *GID1a* and *GID1c*, but considerably different between *GID1b* and *GID1ac*., and further support the view that the two groups of *GID1* receptors may play distinct roles during the germination process.

Germination kinetics of Arabidopsis gid1 *mutants reveal different relevance of the three GID1 receptors during seed germination*

To investigate the individual roles and importance of the three GID1 receptors in the germination process, phenotypic analyses of *Arabidopsis gid1a, gid1b*, and *gid1c* single- and double-knockout mutants were carried out. Endosperm rupture of the seed populations was scored over time on medium without (CON) and with GA or ABA added (Fig. 4). Without hormonal addition none of the single-knockout mutants exhibited an appreciable phenotype, although *gid1c* completed germination slightly later compared with the wild type (WT) and the other single mutants (Fig. 4A). A very striking phenotype was observed for *gid1agid1c*: only 4% of the seeds completed germination, and this happened only very late (Fig. 4A). The viability of the *gid1agid1c* seeds was confirmed using the tetrazolium assay (Fig. 4D). The tetrazolium assay is a standard test for seed viability and is widely used for this purpose (Graeber *et al.*, 2010). Also *gid1bgid1c* completed germination later than the WT and the single mutants.

Treatment with GA did not appreciably alter the germination kinetics of the knockout mutants (Fig. 4B). Only 11% of the *gid1agid1c* seeds completed germination, demonstrating that most seeds are GA-insensitive and that AtGID1b cannot replace the AtGID1ac group receptors that appear to be required for *Arabidopsis* seed germination. Treatment with ABA strongly delayed endosperm rupture and completely inhibited *gid1agid1c* seed germination (Fig. 4C). The strong inhibition of *gid1agid1c* germination and delayed germination of seeds containing the *gid1c* mutation was also evident with an independent second set of T-DNA insertion single and double knockout mutants (data not shown). Taken together, the three *GID1* genes of *Arabidopsis* seem to have distinct importance during seed germination: At least one GID1ac group member is required with AtGID1c being more important than AtGID1a, while AtGID1b seems to play a minor role.

Tissue-specific analysis of L. sativum *seeds reveals spatial, temporal, and hormonal differences of GID1 transcript expression*

The different GID1 GA receptors seem to play distinct roles during plant development. For germination their function during the process of endosperm weakening is of special interest and was examined in individual tissues for this purpose. The bigger seed size and the temporal and ABA-related separation of testa and endosperm rupture of *L. sativum* facilitate analysis of defined seed tissues (Fig. 5), such as the micropylar endosperm and the radicle. Preparation of samples was done by removing the testa covering the hypocotyl- and radicle part of the seed, cutting off the radicle (lower 1/3 of the hypocotyl-radicle axis) and detaching the micropylar endosperm and the radicle tissue. Transcript expression patterns of *LesaGID1a*, *LesaGID1b*, and *LesaGID1c* in the micropylar endosperm and in the radicle after 8 h and 18 h of imbibition of *L. sativum* were analysed by qRT-PCR (Fig. 5C, D). Those times were selected in order to capture the early germination phase of *Lepidium sativum*, just after testa rupture at the onset of endosperm weakening, and the late germination phase, just prior to endosperm rupture. Without hormone treatment (CON), the transcript expression levels at 8 h of *LesaGID1a* and *LesaGID1c* were similar and about twice that compared with *LesaGID1b*. The levels of all three transcripts decline to very low and roughly equal levels from the early to the late germination phase in both tissues (Fig. 5C, D).

GA treatment slightly promoted *L. sativum* seed germination and triggered a negative feedback loop that resulted in reduced transcript levels of *LesaGID1a* and *LesaGID1c* at 8 h compared with CON (Fig. 5C, D). While transcript abundance of *LesaGID1a* was reduced to about 30% upon GA treatment, *LesaGID1c* mRNA became undetectable in both tissues at 8 h. In contrast to the transcripts of the *L. sativum* GID1ac group members, GA treatment caused a 14.3-fold (micropylar endosperm) and a 7.2-fold (radicle) increase in *LesaGID1b* transcript abundance at 8 h compared with CON. *LesaGID1b* transcript levels declined from

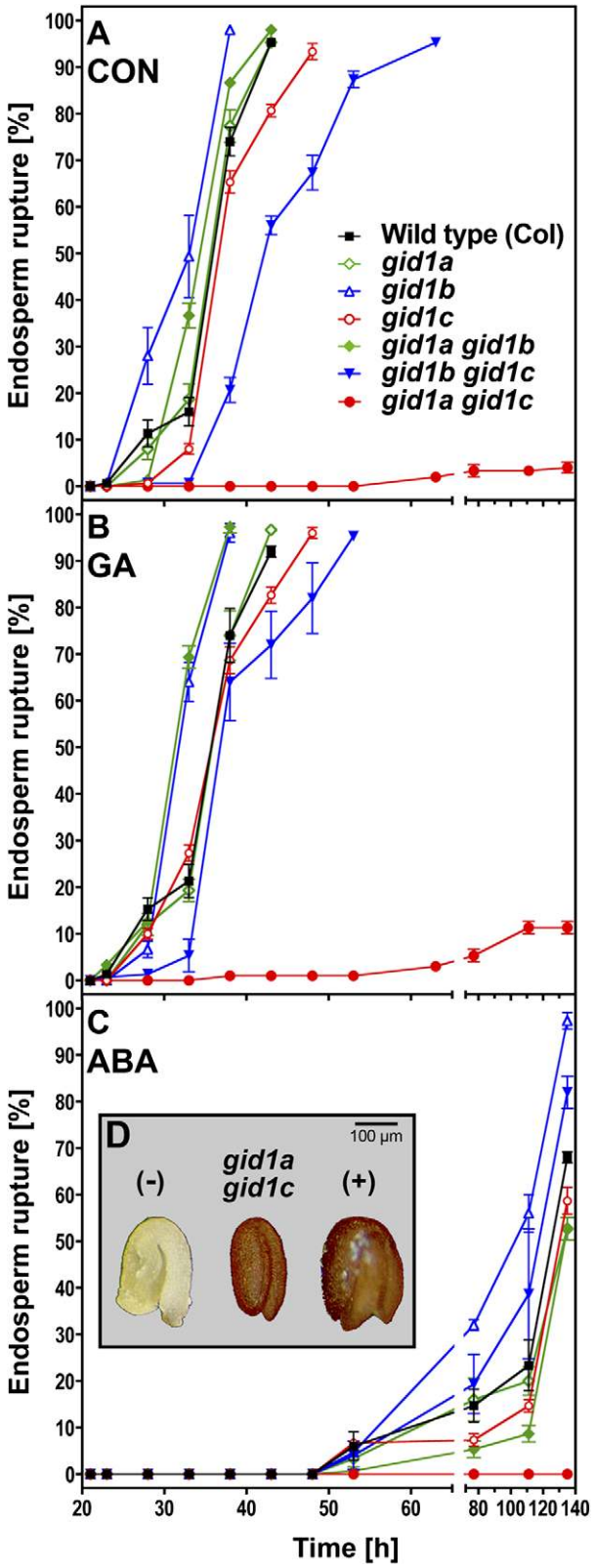

Fig. 4. The effect of the three GID1 receptors on the seed germination of *Arabidopsis thaliana* studied with mutants for AtGID1a, AtGID1b, and AtGID1c. Time-course analysis of endosperm rupture of after-ripened seeds of *Arabidopsis* single (*gid1a, gid1b, gid1c*) and double (*gid1agid1b, gid1bgid1c, gid1agid1c*) mutants imbibed at 24 °C in continuous light without preceding stratification. The results presented were obtained with the mutant set by Iuchi *et al.* (2007), the same results were obtained with an independent mutant set by Willige *et al.* (2007). (A) Medium without hormones added (control, CON). (B) 10 µM GA_{4+7} (GA). (C) 1 µM ABA. (D) Tetrazolium-assay to confirm viability of *gid1agid1c*; (–) and (+): negative (seed killed by preceding heat treatment, 1 h at 100 °C) and positive wild-type seed controls. Mean values ±SE of 3×50 seeds are presented.

the early (8 h) to the late (18 h) germination phase to low levels upon GA treatment as in CON.

ABA treatment delays endosperm rupture (Fig. 5). Comparison of CON and ABA at 8 h shows in the radicle about a 2-fold increase of the *LesaGID1a* and *LesaGID1c* transcript levels, but no difference for *LesaGID1b.* As in CON and GA, upon ABA treatment the expression of all three transcripts was also down-regulated to very low levels at 18 h. However, at 96 h ABA, where the percentage of germinated seeds is comparable with 18 h CON (Fig. 5A), in the micropylar endosperm the *LesaGID1c* transcript levels were up-regulated roughly 3-fold compared with 8 h ABA (Fig. 5C). In the radicle, *LesaGID1b* and *LesaGID1c* transcripts levels (96 h versus 8 h ABA) were up-regulated 5-fold to roughly equal values (Fig. 5D). By contrast, the *LesaGID1a* transcript levels remained low at 96 h ABA in both tissues (Fig. 5C, D). Taken together, a GA-triggered negative feedback loop on transcript expression of the GID1ac group members is evident in the *L. sativum* micropylar endosperm and the radicle. In addition, a GA-triggered positive feedback loop on *LesaGID1b* was found during *L. sativum* seed germination, and the ABA regulation of the *GID1* transcripts showed complex temporal and tissue-specific patterns.

A subtractive suppression hybridization (SSH) cDNA library from micropylar endosperm tissue of germinating seeds of L. sativum *contains candidate genes for endosperm weakening*

An SSH library from micropylar endosperm tissue was constructed enriched for transcripts at 8 h (early germination) compared with 18 h (late germination). This library contains transcripts that are more highly abundant or unique during the early germination phase and, therefore, are likely to be important for the germination process. 184 clones were sequenced and the partial sequences were given a putative function based on their highest BLAST hit (see Supplementary Table S2 at *JXB* online). About 10% of the transcripts were coding for ribosomal proteins, indicating the importance of protein biosynthesis for germination. This is further supported by the presence of the universal translation initiation and elongation factors. The subtractive cDNA library also contained transcripts of proteins involved in mRNA splicing, binding, and turnover. Furthermore, a clone was found for an RCAR/PYR/PYL-type ABA receptor and many of the library clones are GA regulated (see Supplementary Table S2 at *JXB* online). An analysis of Gene Ontology (GO) classes in our subtractive

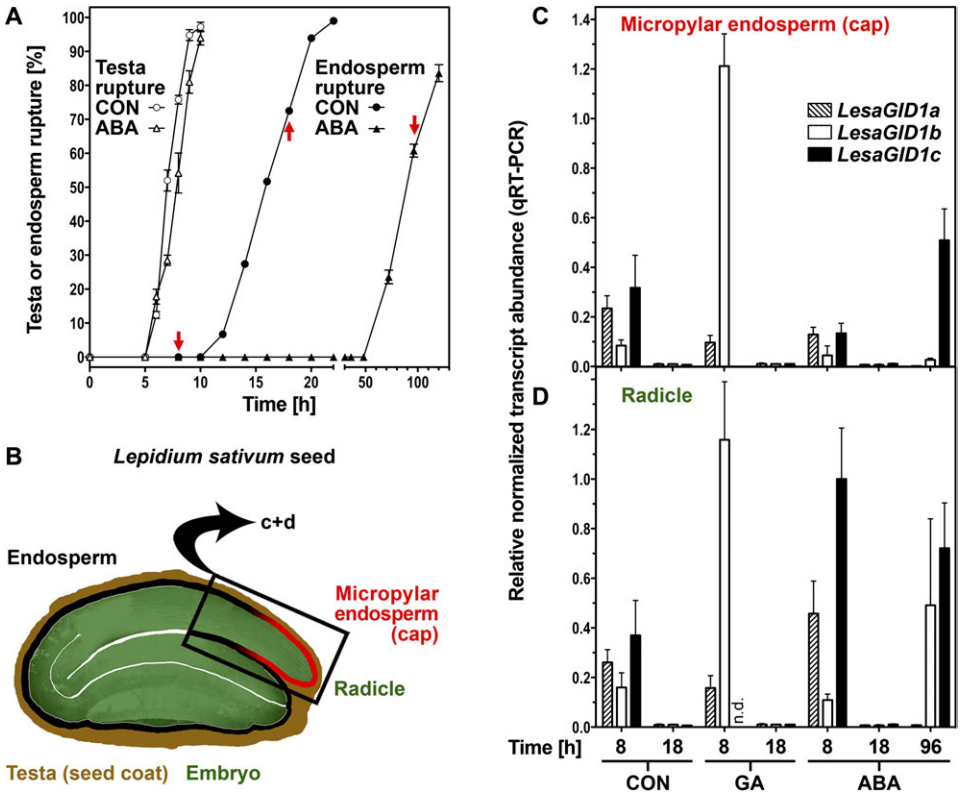

Fig. 5. Transcript abundance patterns of the *LesaGID1a, LesaGID1b*, and *LesaGID1c* receptor genes in specific seed tissues of *Lepidium sativum* during germination. A, Time course analysis of testa and endosperm rupture of *L. sativum* FR14 seeds in medium without (CON) and with ABA added. Continuous light, 18 °C. Mean values ±SE of 3×50 seeds are presented. Red arrows indicate sampling time points for the qPCR. (B) Cross-section of *L. sativum* seed indicating the specific seed tissues used in the experiment: micropylar endosperm and radicle (lower 1/3 of the hypocotyl-radicle axis). (C, D) qRT-PCR analysis of *LesaGID1a, LesaGID1b*, and *LesaGID1c* transcript abundances for micropylar endosperm (C) and radicles (D) at 8, 18, and 96 h imbibition. ddCt expression values relative to validated constitutive transcripts are presented. Medium additions, as indicated: 10 μM GA_{4+7}, 10 μM ABA. Mean values ±SE of 4×1000 micropylar endosperms and 4×200 radicles are presented; n.d.=not detectable.

cDNA library compared with the whole set of GOs annotated on TAIR by the software GOstat (Beißbarth and Speed, 2004) showed that cDNAs annotated as 'cell wall' (GO:0005681) or 'external encapsulating structure' (GO:0030312) were significantly over-represented (P <0.001). They contained genes such as the putative orthologues of *EXLA1* (an expansin-like A, At3g45970) and *EXPA9* (an α-expansin, At5g02260) from the expansin superfamily, and *XTH18* (At4g30280) and *XTH19* (At4g30290) from the xyloglucan endo-transglycosylases/hydrolases (XTHs) family. They are potential candidates to be involved in early GA-induced weakening of the endosperm tissue and their transcript expression was therefore analysed further.

Analysis of expansin and XTH transcript expression patterns in individual seed tissues of L. sativum *and in the* gid1 *single and double mutants of* Arabidopsis *during germination assigns them to distinct regulatory GID1 pathways*

The transcript expression of *EXLA1, EXPA9, XTH18*, and *XTH19* in the micropylar endosperm and the radicle of *L. sativum* during germination was determined by qRT-PCR (Fig. 6). All genes showed higher expression at 8 h compared with 18 h (the two time points used for library construction) in the micropylar endosperm (Fig. 6A), which confirms the reliability of the data obtained from the SSH library. At 8 h, the transcript abundances of all four genes was higher in the micropylar endosperm compared witho the radicle (Fig. 6). The expression levels of all four transcripts declined from the early (8 h) to the late germination (18 h) phase to very low and roughly equal levels in both tissues. Interestingly, upon GA treatment *EXLA1* and *EXPA9* displayed a distinct pattern compared to both XTH genes during early germination. The transcript levels of *XTH18* and *XTH19* declined below the detection limit in 8 h GA-imbibed seed tissues, while transcript levels of *EXLA1* and *EXPA9* remained high upon GA treatment during early (8 h) germination. At 18 h with GA treatment all four transcripts showed similar low transcript abundance. This distinct effect of GA on the 8 h transcript expression levels, with a GA-triggered down regulation affecting the XTHs, but not the expansins, shows that, during early germination, they are regulated by distinct pathways regarding GA, but during late germination

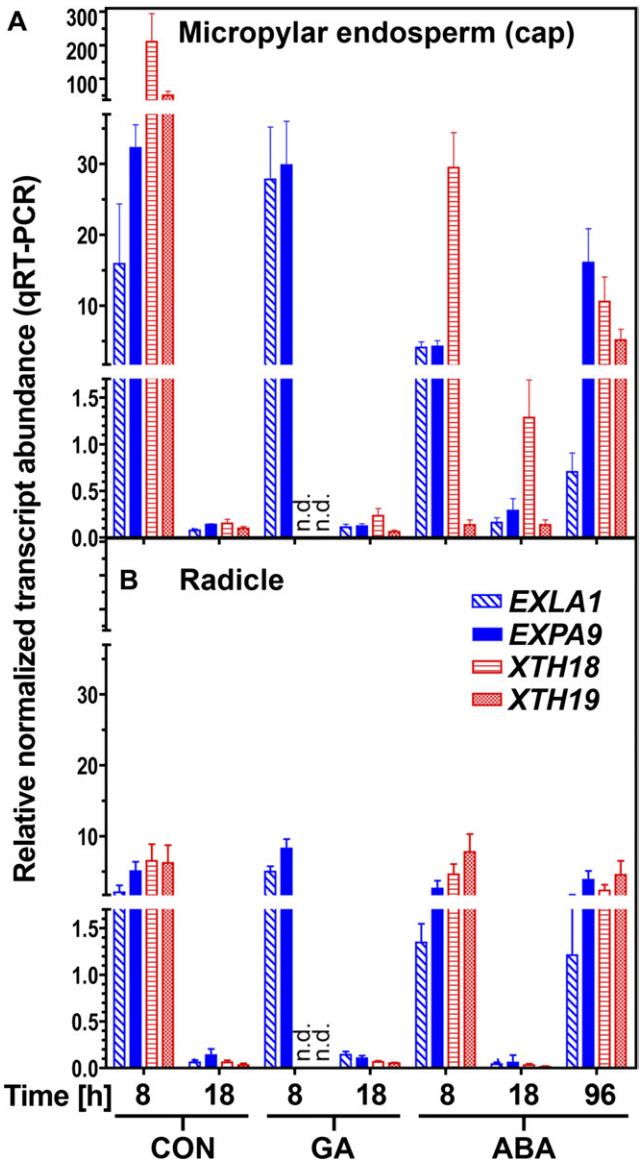

Fig. 6. Transcript abundance patterns of putative cell-wall-loosening genes (expansins and XTHs) in specific seed tissues of *Lepidium sativum* during germination. qRT-PCR analysis of *EXLA1, EXPA9*, *XTH18*, and *XTH19* transcript abundances for (A) micropylar endosperm and (B) radicles at 8, 18, and 96 h imbibition. ddCt expression values relative to validated constitutive transcripts are presented. For time-courses of testa and endosperm rupture in continuous light (18 °C) see Fig. 5A. Medium additions, as indicated: 10 μM GA_{4+7}, 1 μM ABA. Mean values ±SE of 4×1000 micropylar endosperms and 4×200 radicles are presented; n.d.=not detectable.

equally regarding the down-regulation. Since ABA strongly delays endosperm rupture, an additional time point very late during germination was investigated (96 h) which, in terms of endosperm rupture, corresponds to 18 h CON and for which transcripts of *LesaGID1c* and *LesaGID1b* accumulated (Fig. 6). Upon ABA treatment there is an up-regulation of all four candidate cell-wall-modifying protein transcripts in the micropylar endosperm and the radicle very late during germination (at 96 h; Fig. 6). Taken together, this shows that the XTHs and expansins in *L. sativum* are regulated differentially: Both XTHs appear to be in a GID1ac pathway, since *LesaGID1a, LesaGID1c,* as well as *XTH18* and *XTH19* are down-regulated by GA during early germination (Figs 5C, 6). Contrary to that, both expansins are not down-regulated by GA during early *Lepidium* germination, which assigns them to a different pathway.

The *XTH18* and *XTH19* transcript levels are up-regulated upon imbibition of *Arabidopsis* wild-type (WT) seeds, based on comparing dry seeds with 30 h-imbibed seeds (Fig. 7), i.e. a timepoint during germination that corresponds physiologically to the 8 h CON timepoint in *L. sativum*. Treatment of *Arabidopsis* WT with GA did not affect the relative transcript abundances of both XTHs in whole seeds as it was evident in specific seed tissues in *L. sativum.* If, in *Arabidopsis*, the *XTH18* and *XTH19* transcripts are regulated by the GID1ac pathway, their regulation should be impaired in the *gid1agid1c* mutant which also exhibits a germination phenotype (Fig. 4). To test if, in *Arabidopsis*, the *XTH18* and *XTH19* transcripts are also regulated by the GID1ac pathway, their expression was compared in seeds of the *gid1* single and double mutants (Fig. 7). In contrast to the WT and most *gid1* mutants, the *XTH18* and *XTH19* transcript levels remained low in imbibed *gid1agid1c* mutant seeds (Fig. 7). As the relative transcript abundances of both XTHs were up-regulated during germination in WT and in the *gid1* single and double knockout mutants, except for the *gid1agid1c* mutant, *XTH18* and *XTH19* regulation in *Arabidopsis* can be assigned to the GID1ac pathway.

In *Arabidopsis* it was also found that, as in *L. sativum*, the expansins exhibited a distinct regulation of their transcript expression during seed germination for WT and the individual *gid1* single- and double-mutants (Fig. 7). It is known that transcripts of α-expansins like *EXPA9* accumulate strongly during the early phase of *Arabidopsis* seed germination and that this induction is promoted by GA, is not inhibited by ABA, and is mainly localized in the endosperm (Holdsworth *et al.*, 2008*a*; Preston *et al.*, 2009). In agreement with this, it was found that *EXPA9* transcripts accumulate in whole seeds of *Arabidopsis* during early germination, and that this induction is promoted by GA, but not inhibited by ABA (Fig. 7). GA treatment also promoted the transcript expression of the expansin-like A gene *EXLA1*. GA addition therefore leads to higher transcript levels of both expansins but does not affect the XTH transcript levels. Based on the decrease in *AtGID1A* and *AtGID1c*, but not *AtGID1b*, transcript abundance through the addition of GA (Fig. 2) this suggests that both expansins in *Arabidopsis* are regulated in a pathway involving GID1b. In contrast to the two XTHs, relative transcript abundances of *EXPA9* were not affected in the *gid1agid1c* mutant and was roughly constant in imbibed seeds of WT and all of the *gid1* mutants (Fig. 7). In summary, evidence is provided from two Brassicaceae species that, during seed germination, the GID1ac pathway and the GID1b pathway have different roles and regulate different downstream genes.

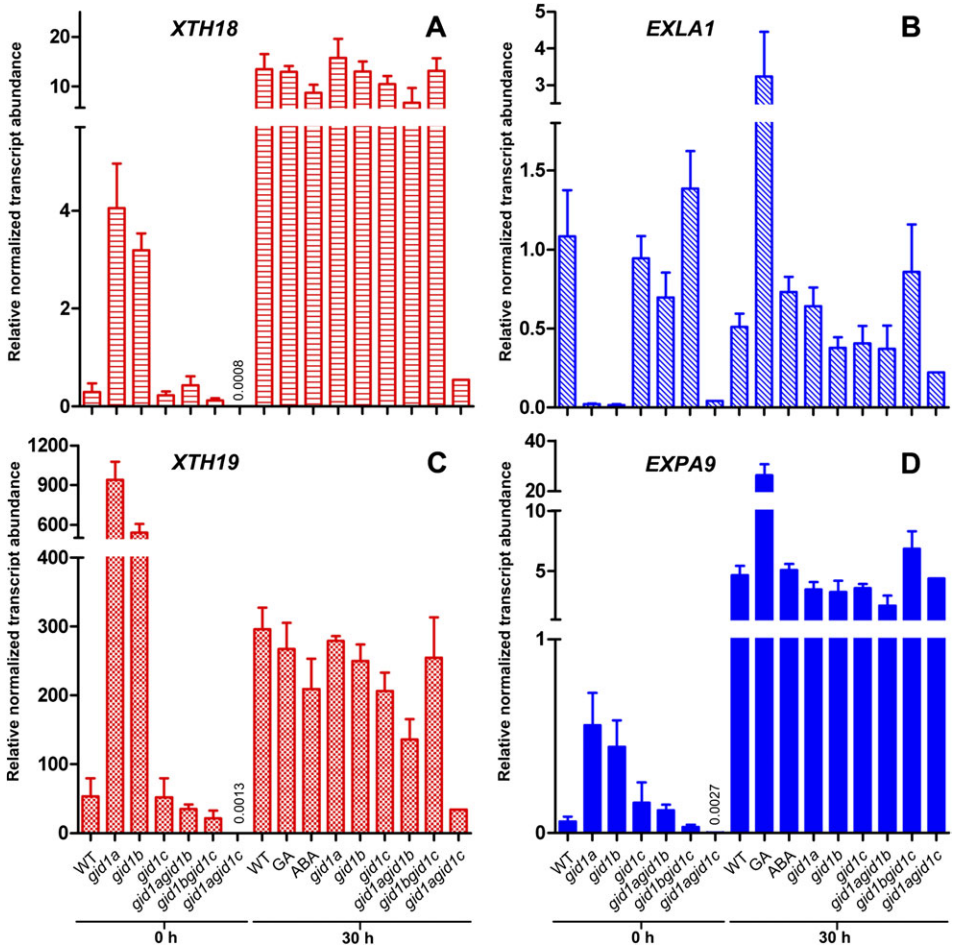

Fig. 7. Transcript abundances of *XTH18*, *XTH19*, *EXLA1*, and *EXPA9* in *Arabidopsis gid1* mutant seeds. qRT-PCR analysis of (A) *XTH18* (At4g30280), (B) *EXLA1* (At3g45970), (C) *XTH19* (At4g30290), and (D) *EXPA9* (At5g02260) in wild-type (WT) and *gid1* single and double mutants in dry seeds and after 30 h imbibition. ddCt expression values relative to validated constitutive transcripts are presented. Medium additions, as indicated: 10 μM GA_{4+7}, 1 μM ABA. Mean values ±SE of 4×200 seeds are presented.

Discussion

Eudicots contain at least one GID1 gibberellin receptor from each of the two distinct groups, GID1ac and GID1b, which are associated with two distinct regulatory pathways

Our molecular phylogenetic analysis showed that the known angiosperm GID1 receptors fall into three distinct groups: eudicot GID1ac, eudicot GID1b, and monocot GID1 (Fig. 1). The finding that the core eudicot species always have at least one GID1 gene from each of the two eudicot groups supports the view that the two distinct groups fulfil some distinct developmental functions required for all angiosperms and suggests distinct regulatory pathways. That the individual GID1 receptors of *Arabidopsis* (AtGID1a, AtGID1b, AtGID1c) display partial redundancy and have functional specificities for regulating the GA-responsiveness of different developmental processes has been postulated before (Griffiths *et al.*, 2006; Iuchi *et al.*, 2007). Hirano *et al.* (2007) investigated GA signalling components in a broad evolutionary context by phylogenetic comparison of the GID1 proteins of the two angiosperms, *Arabidopsis* and rice, to pine as a gymnosperm, *Selaginella moellendorffii* as a lycophyte and *Physcomitrella patens* as a moss representative. They showed that the GID1 proteins of pine, the lycophyte, and the moss, cluster further away from the GID1 group with the two angiosperms. In agreement with this, when putative GID1 coding sequences of *Selaginella moellendorffii*, *Physcomitrella patens*, and the three gymnosperms *Picea glauca*, *Picea sitchensis*, and *Pinus taeda* are included in our phylogenetic analysis, they also clustered as a distinct group (data not shown) apart from our comprehensive eudicot GID1ac, eudicot GID1b, and monocot-GID1 clusters that are based on comparing the sequences of 36 angiosperm species (Fig. 1; see Supplementary Table S1 at *JXB* online). The distinction between two eudicot groups (GID1ac and GID1b) and the monocot group is not only supported by the molecular phylogeny, but also by biochemical evidence in studies with the three *Arabidopsis* GID1s: Nakajima *et al.* (2006) showed that AtGID1a and AtGID1c bind GA_4 and GA_3 with a *c.* 10-fold lower affinity compared with AtGID1b. All three

Arabidopsis GID1 receptors can interact with all five *Arabidopsis* DELLA proteins (GAI, RGA, RGL1, RGL2, RGL3) targets (Nakajima *et al.*, 2006; Willige *et al.*, 2007; Suzuki *et al.*, 2009). However, Suzuki *et al.* (2009) showed that differential *in vivo* interaction affinities exist: the major preference of AtGID1b is GAI and RGA, while the major preferences of AtGID1a and AtGID1c are RGL1 and RGL3; AtGID1a has, in addition, RGL2 as a second major preference. These biochemical features, combined with distinct tissue-specific and hormonal expression patterns of the different GID1 receptors and DELLA repressors, can therefore define distinct GID1ac and GID1b pathways in eudicots. In contrast, in monocot species, only one group of GID1 receptors seems to exist, even when more than one GID1 homologue is present within a species (Fig. 1). This might be linked to the fact that, in monocot species, fewer DELLA proteins exist: one DELLA protein in rice (*Oryza sativa*; SLR1) and barley (*Hordeum vulgare*; SLN1) and a putative second homologue in maize (*Zea mays*; D8 and D9), and, therefore, less specialization of the GID1s is needed (Peng *et al.*, 1999; Ikeda *et al.*, 2001; Gubler *et al.*, 2002; Weston *et al.*, 2008). Differential expression and distinct patterns of degradation of different DELLA repressors has been shown in seeds (Bassel *et al.*, 2004; Ariizumi *et al.*, 2008; Piskurewicz *et al.*, 2008, 2009; Piskurewicz and Lopez-Molina, 2009). In an evolutionary context a separation between an GID1ac-type (interacting preferentially with the RGL1/RGL2/RGL3 group of DELLA repressors) and a GID1b-type (interacting preferentially with the GAI/RGA group of DELLA repressors) pathway hints at a greater specialization of eudicot GID1-mediated GA signalling, while such a partial functional separation has not occurred within the monocots. It has to be noted, though, that less work has been carried out on monocot species seed germination so far, and that the degree of partial redundancy between the two eudicot pathways may differ depending on the developmental process.

The regulation of the GID1 pathways and its roles during seed germination differ from other developmental processes and may be conserved among the Brassicaceae

Our analysis of the *Arabidopsis* knockout mutants for the three GID1 receptors clearly showed that the AtGID1b receptor is not able to compensate for the phenotype of the *gid1agid1c* double mutant, i.e. GA signalling via the GID1ac receptors is required for seed germination (Fig.4). By contrast, the AtGID1c and AtGID1a receptors are partially redundant and can compensate for a lack of AtGID1b. Based on the seed germination phenotypes of the *Arabidopsis* knockout mutants and the ABA-related transcript expression pattern in the micropylar endosperm and the radicle of *L. sativum*, the GID1c receptors may be more important for Brassicaceae seed germination compared with the GID1a receptors. While transcript abundances in the order *AtGID1a>AtGID1c>AtGID1b* were evident in dry and imbibed seeds (Cadman *et al.*, 2006; Preston *et al.*, 2009), the order *AtGID1a>AtGID1b>AtGID1c* was evident throughout plant development in many other tissues and in seedlings (Griffiths *et al.*, 2006). This further indicates that there is a different importance of the individual GID1 receptors during seed germination compared with other developmental processes. The hypothesis of the different importance of the individual *GID1* genes during seed germination, with *GID1b* being distinct from *GID1ac* and having less importance, is also supported by the stronger GUS staining of the PGID1ac reporter gene lines compared with PGID1b. This finding is further supported by the fact that Griffiths *et al.* (2006) found negative feedback-regulation by GA for all three *GID1* transcripts in seedlings, while in our transcript analyses and GUS reporter line staining results, combined with *in silico* analysis with the eFP browser, down-regulation by GA during the germination of unstratified *Arabidopsis* seeds was evident only for the *AtGID1a* and *AtGID1c* transcripts, but not for the *AtGID1b* transcripts. It is therefore proposed that, in germinating seeds, *AtGID1b* transcript abundance is not negatively regulated by GA. An alternative interpretation would be a higher GA sensitivity of the negative feedback mechanism on *AtGID1b* transcript abundance compared with *AtGID1a* and *AtGID1c* as it was proposed by Iuchi *et al.* (2007). The endogenous GA content would then already be sufficient to decrease *AtGID1b* transcript abundance. However, in support of our proposal, the GA-triggered negative feedback loop on *AtGID1a* and *AtGID1c*, but not on *AtGID1b*, was also evident in 9 h-imbibed *ga1-3* L*er* seeds (Ogawa *et al.*, 2003). In agreement with this, during *L. sativum* seed germination, a GA-triggered negative feedback loop in the micropylar endosperm and the radicle was only evident for the *LesaGID1a* and *LesaGID1c* transcripts. This strongly suggests that a GA-triggered negative feedback loop during seed germination exists for *GID1a* and *GID1c* in Brassicaceae seeds, while *GID1b*-type transcripts are not down-regulation targets. Besides that, in both species expression patterns are similar regarding transcript abundance during early germination (8 h in *L. sativum* versus 30 h *Arabidopsis*), in which the *GID1ac* transcript levels are usually higher compared to *GID1b*, and no significant regulation by ABA during early germination takes place.

A subtractive cDNA library identified candidate genes for transcript splicing and turnover, translation and proteolysis, hormone signalling, and weakening in the micropylar endosperm

Weakening of the micropylar endosperm of *L. sativum* progresses between the early (8 h) and late (18 h) time points used in our germination experiments (CON), and is promoted by GA and inhibited by ABA (Müller *et al.*, 2006). Using a subtractive cDNA library of the micropylar endosperm, transcripts that are more highly expressed or unique during early germination were identified. The over-representation of the GO categories 'ribosomal protein'

(GO:0003735) and 'ribosome' (GO:0005840) (GOstat; *P*-value <0.001; Beißbarth and Speed, 2004) indicates intense protein biosynthesis during early germination. The importance of *de novo* protein biosynthesis for the germination process of *Arabidopsis* has been shown by Rajjou *et al.* (2004). It seems that *de novo* synthesis of ribosomal components is needed for germination and subsequent seedling growth (Holdsworth *et al.*, 2008*b*). The importance of early translation is further indicated by the occurrence of a translation initiation factor (*EIF3G*) and a translation elongation factor (*EF1-α*) in our library.

In addition to protein synthesis, *UBQ11* and three protease clones (see Supplementary Table S2 at *JXB* online), support our earlier finding that proteolysis in the micropylar endosperm is important (Müller *et al.*, 2010). The micropylar endosperm library also contains transcription and splicing factors indicating *de novo* mRNA biosynthesis and processing, as well as RNA binding proteins and putative ribonucleases that may be involved in diverse pathways of mRNA decay, in particular, the polyA-binding protein RBP31 (Ni *et al.*, 2010) and a core protein of the evolutionarily conserved CCR4-NOT complex (At2g32070), both involved in the deadenylation pathway of mRNA decay (Denis *et al.*, 2003; Garneau *et al.*, 2007).

The SSH library also contains a clone encoding PYL6, an RCAR/PYR/PYL-type ABA receptor (Nambara *et al.*, 2010; Raghavendra *et al.*, 2010). In germinating *Arabidopsis* seeds (eFP browser; Toufighi *et al.*, 2005); *PYL6* transcripts accumulate during imbibition, are down-regulated by ABA, and up-regulated by GA. In our tissue-specific transcriptome analysis of *L. sativum* seed germination (Linkies *et al.*, 2009) transcripts of the putative *PYL6* orthologue are far more abundant in the micropylar endosperm compared to the non-micropylar endosperm and the radicle. The RCAR/PYR/PYL-type and the GID1-type receptors may, therefore, be crucial components in regulating ABA and GA responsiveness important for endosperm weakening.

Two XTHs and two expansins are regulated differentially with expression patterns that assign them to distinct hormonal regulation

The two XTHs that were found in our subtractive library are putative orthologues of *AtXTH18* and *AtXTH19*, two phylogenetically closely related group-2-type XTHs (Rose *et al.*, 2002; Osato *et al.*, 2006). Several members of the XTH-family are known to be positively regulated on both the transcriptional and activity levels by growth-promoting plant hormones such as auxin and GA. For example, GA stimulates leaf elongation and XTH expression and activity in barley, while dwarf mutants with dysfunctional GA signal transduction show reduced XTH activities and shorter leaves (Smith *et al.*, 1996). XTHs are implicated in cell-wall hemicellulose remodelling leading to loosening (Van Sandt *et al.*, 2007) or stiffening (Maris *et al.*, 2009). The two expansins in our subtractive library, putative orthologues of *AtEXLA1* (an expansin-like A) and *AtEXPA9* (an α-expansin), may be involved in cell expansion and/or weakening of the micropylar endosperm during germination. Expansins loosen cell walls at acidic pH of around 4.5–6, which corresponds to the pH in the apoplast (Cosgrove, 2005; Sampedro and Cosgrove, 2005). No enzymatic activity has been found for expansins, and it has been hypothesized that they act on non-covalent bonds such as hydrogen bridges between cellulose microfibrils and hemicelluloses. Cell-wall loosening activity of α-expansins, but not of expansin-like proteins, has been demonstrated. In the seed, tissue-specific transcriptome analysis of *L. sativum* transcripts of the putative EXPA9 orthologue are far more abundant in the micropylar endosperm and the radicle compared with the non-micropylar endosperm (Linkies *et al.* 2009; Morris *et al.* 2011). This suggests that the EXPA9 proteins are involved in micropylar endosperm weakening and in radicle growth.

Expansins and XTHs have both been described in the context of GA and ABA regulation during seed germination (Finkelstein *et al.*, 2008; Holdsworth *et al.*, 2008*a*). In our tissue-specific analysis of the XTH and expansin transcript expression in *L. sativum*, their higher expression during early (8 h) germination indicates the necessity of early transcription of cell-wall-modifying genes before the onset of endosperm weakening. The temporal, spatial, and hormonal (GA and ABA) co-expression patterns (Figs 5, 6) of the *XTH18*, *XTH19*, and *LesaGID1* transcripts suggest that these XTHs are regulated in a *LesaGID1ac* involving pathway. Both during seed germination of *Arabidopsis* (whole seeds; Fig. 2) and *L. sativum* (specific seed tissues; Fig. 5) the GA-triggered negative feedback loop affected only the *GID1ac* orthologues, while transcripts of the GID1b orthologues are not down-regulated by GA in *Arabidopsis* and are strongly up-regulated in *L. sativum*. By contrast, the expression patterns of the *EXPA9* and *EXLA1* transcripts assign them to a different pathway, not involving *LesaGID1ac*. This is supported by the fact that, in 30 h-imbibed *Arabidopsis* seeds, *AtGID1b* expression is not affected by GA, while *AtGID1a* and *AtGID1c* transcripts are down-regulated by GA (Fig. 2). At the same time, GA induces expression of *EXLA1* and *EXPA9*, but not of the two XTH genes (Fig. 7), which indicates that the main regulation of both expansins during *Arabidopsis* germination is in a GID1b-involving pathway, while the two XTHs are in another pathway. In support of the association of the two XTHs with a GID1ac signalling pathway, the *Arabidopsis gid1agid1c* double mutant is impaired in the accumulation of transcripts upon imbibition for the two XTHs, but *EXPA9* transcripts accumulate as in WT. The *gid1agid1c* double mutant also has a seed germination phenotype (Fig. 4). Thus, although for *Arabidopsis* whole seeds and for *L. sativum* specific seed tissues were analysed and not every aspect of the regulation is equal in both species, as a general conclusion the cross-species approach provided support for the hypothesis that, during germination of both Brassicaceae species, the two XTHs appear to be regulated by the GID1ac pathway, while the expansins seem to be associated with the GID1b pathway.

In conclusion, eudicot species contain at least one *GID1ac* and one *GID1b* group member. GA responsiveness is similarly important and regulated during seed germination of different Brassicaceae species [e.g. *Arabidopsis thaliana*: Ogawa *et al.* (2003), *Lepidium sativum*: Müller *et al.* (2006), *Sisymbrium officinale*: Hilhorst *et al.* (1986); Iglesias-Fernández and Matilla (2010)] and in seeds from other eudicot families [e.g. Solanaceae: Ni and Bradford (1993); Toorop *et al.* (2000); Leubner-Metzger (2002, 2003); Petruzzelli *et al.* (2003); Asteraceae: Argyris *et al.* (2008); Rubiaceae: da Silva *et al.* (2004)]. Distinct, but partially overlapping roles are proposed for the GID1ac and GID1b pathways and their downstream target genes during seed germination that may be conserved among Brassicaceae (this work) or even among eudicots.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. GID1 transcript sequence alignments of *L. sativum* and *Arabidopsis*.

Supplementary Fig. S2. GID1 protein sequence alignments of *L. sativum* and *Arabidopsis*.

Supplementary Fig. S3. Comparison of *L. sativum* and *Arabidopsis* GID1 transcript secondary structures and stability motifs in 5' and 3' UTRs.

Supplementary Fig. S4. *L. sativum* and *Arabidopsis* GID1 5' and 3' UTRs motifs associated with mRNA stability.

Supplementary Table S1. Angiosperm phylogeny and GID1 receptor sequence accession.

Supplementary Table S2. cDNA clones obtained of the early micropylar endosperm SSH library.

Supplementary Table S3. Primer sequences used for quantitative PCR analyses.

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