**PrCYP707A1**, an ABA catabolic gene, is a key component of *Phelipanche ramosa* seed germination in response to the strigolactone analogue GR24

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**Abstract**

After a conditioning period, seed dormancy in obligate root parasitic plants is released by a chemical stimulus secreted by the roots of host plants. Using *Phelipanche ramosa* as the model, experiments conducted in this study showed that seeds require a conditioning period of at least 4 d to be receptive to the synthetic germination stimulant GR24. A cDNA-AFLP procedure on seeds revealed 58 transcript-derived fragments (TDFs) whose expression pattern changed upon GR24 treatment. Among the isolated TDFs, two up-regulated sequences corresponded to an abscisic acid (ABA) catabolic gene, *PrCYP707A1*, encoding an ABA 8’-hydroxylase. Using the rapid amplification of cDNA ends method, two full-length cDNAs, *PrCYP707A1* and *PrCYP707A2*, were isolated from seeds. Both genes were always expressed at low levels during conditioning during which an initial decline in ABA levels was recorded. GR24 application after conditioning triggered a strong up-regulation of *PrCYP707A1* during the first 18 h, followed by an 8-fold decrease in ABA levels detectable 3 d after treatment. *In situ* hybridization experiments on GR24-treated seeds revealed a specific *PrCYP707A1* mRNA accumulation in the cells located between the embryo and the micropyle. Abz-E2B, a specific inhibitor of CYP707A enzymes, significantly impeded seed germination, proving to be a non-competitive antagonist of GR24 with reversible inhibitory activity. These results demonstrate that *P. ramosa* seed dormancy release relies on ABA catabolism mediated by the GR24-dependent activation of *PrCYP707A1*. In addition, *in situ* hybridization corroborates the putative location of cells receptive to the germination stimulants in seeds.

**Key words:** ABA, CYP707A inhibitor, parasitic plant, *Phelipanche ramosa*, seed germination, strigolactone

**Introduction**

Broomrape species (*Orobanche* spp. and *Phelipanche* spp.) are obligate root parasitic plants devoid of chlorophyll that exclusively depend on their hosts for their nutritional needs. Although most broomrape species develop in natural ecosystems

Abbreviations: ABA, abscisic acid; Abz, abscinazole; AEC; adenylate energy charge; AFLP, amplified fragment length polymorphism; RACE, rapid amplification of cDNA ends; SL, strigolactone; TDF, transcript-derived fragment.

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with no significant damage on their host plants, some are harmful parasitic weeds in important crops. These pests include Orobanche cumana on sunflower, Orobanche crenata and Orobanche foetida on legumes, and Phelipanche ramosa and Phelipanche aegyptiaca on tomato (Parker, 2009). They all have an extraordinary capacity for dissemination because each individual plant may produce up to 500 000 extremely small seeds (between 200 µm and 400 µm) containing an acotyledonous reduced embryo (Joel et al., 2011). The weedy life cycle of broomrapes is well-described with regard to its major host plants (Joel et al., 2007). Seed germination is induced by chemical signals exuded in the rhizosphere by host roots and leads to the emergence of a radicle that attaches to the host root surface. Most germination stimulants identified thus far belong to the strigolactone (SL) family (Yoneyama et al., 2010), although dehydrocostus lactone, polyphenols, and iso thiocyanates may be involved in the germination of O. cumana (Joel et al., 2011), O. foetida (Evidente et al., 2010), and P. ramosa (Auger et al., 2012), respectively.

Whatever the nature of the germination stimulant, several preparatory processes generally take place during a conditioning phase before the response to germination stimulants is possible (Joel et al., 1995). Seed hydration (Joel et al., 2007) and major metabolic pathways are initiated during seed conditioning, which thus displays a characteristic pattern of respiration, protein synthesis, and the utilization of reducing sugars (Bar-Nun and Mayer, 1993, 2002). However, some broomrape species may not require this conditioning phase (Plakhine et al., 2004). Seed hydration (Joel et al., 2007) and possible (Joel et al., 2007) and hydration (Plakhine et al., 2004) are initiated during seed conditioning, which thus displays a characteristic pattern of respiration, protein synthesis, and the utilization of reducing sugars (Bar-Nun and Mayer, 1993, 2002). However, some broomrape species may not require this conditioning phase (Plakhine et al., 2009).

SLs are a novel class of plant hormones involved in controlling shoot branching inhibition (Gomez-Roldan et al., 2008; Umehara et al., 2008). Several studies have investigated the SL signalling pathway in plants as well as the relationships between SLs and other phytohormones during the control of plant architecture. SLs interact with auxin and cytokinins (CK) in bud outgrowth control (Crawford et al., 2010; Dun et al., 2012) or during adventitious root initiation (Rasmussen et al., 2012). In addition, cross-talk can occur between SLs, auxin, and ethylene in the control of root hair elongation (Kapulnik et al., 2011). Finally, an ABC transporter has been shown to be a component of SL transport functioning as a cellular exporter (Kretzschmar et al., 2012). By contrast, although the key role of SLs as germination stimulants has been known for several decades (Cook et al., 2008), it has not been well studied due to the lack of genomic resources in parasitic plants. However, the Parasitic Plant Genome Project (PPGP) has made progress recently and ESTs from key developmental stages of S. hermonthica and P. aegyptiaca have been identified (Westwood et al., 2011). Here, the study starting from a genome-wide expression profiling (cDNA-AFLP) on P. ramosa—a closely related species to P. aegyptiaca—demonstrates the relationships between ABA catabolism and the expression of an ABA catabolism gene, PrCYP707A1, during the initiation of seed germination. The results indicate that PrCYP707A1 may be a major molecular component of the seed response to SLs in a root parasitic plant.

### Materials and methods

#### Plant material and chemical treatments

P. ramosa L. Pomel seeds were collected in 2011 from mature flowering spikes growing on winter oilseed rape (Brassica napus L.) in Saint-Martin-de-Fraigneau, France, and stored at 25 °C in darkness. P. ramosa seeds were surface-sterilized for 5 min with 12% sodium hypochlorite and thoroughly rinsed three times with sterile distilled water. Seeds were then suspended in 1 mM Na/K phosphate buffer (pH 7.5) with a ratio of 10 mg seeds ml⁻¹. Seeds were then placed in the dark at 23 °C during the conditioning period. Unless otherwise mentioned, the conditioning period was 7 d. The conditioned seeds were stimulated by adding the synthetic SL GR24 at a final concentration of 10⁻⁷ M in 0.1% acetone. GR24 treatments were carried out at 21 °C in the dark. Corresponding control seeds were treated with 0.1% acetone. After these treatments, seeds were collected by filtration onto a 100 µm nylon mesh, blotted on absorbent paper and weighed. Seeds were then frozen in liquid nitrogen and stored at −80 °C before RNA, ABA or adenylate extraction. ABA 8'-hydroxylase (CYP707A) inhibitors abscinazole-E1 (Abz-E1) and abscinazole-E2B (Abz-E2B) (Okazaki et al., 2011, 2012) were solubilized in acetone and used for germination assays at various concentrations in 0.1% acetone.

#### Imbibition and adenylate energy charge determination

Seed imbibition was determined as described by Joel et al. (2011). Adenylate Energy Charge (AEC = ATP+0.5 ADP/AMP+ADP+ATP) was determined by quantifying adenine nucleotides extracted from 100 mg of seeds, essentially as described by Borisjuk et al. (2007). ATP, AMP, ADP, and ATP were separated by high-performance liquid chromatography.
(HPLC) on an IonPac AS11 column ( Dionex Corp., Sunnyvale, CA, USA) and quantification was done using a standard curve of known concentrations.

Germination assays

Seeds were conditioned by suspending around 100 sterilized seeds in 1 mM Na/K phosphate buffer (pH 7.5) and distributing them in a 96-well plate (Cell Culture Multiwell Plate Cellstar; Greiner Bio-One, Frickenhausen, Germany), and then stored for 7 d at 21 °C in the dark. Abz-E1, Abz-E2B and/or GR24 solutions were added to each well and volumes were adjusted to 100 µl with sterile distilled water to 0.2% acetonitrile (final concentration). A 0.2% acetonitrile solution was used as a negative control. Subsequently, plates were incubated for 3 d at 21 °C in the dark and germinated seeds were counted under a stereo microscope (Olympus SZX10; Olympus Europa GmbH, Hamburg, Germany). Seeds were considered as germinated when the radicle protruded out of the seed coat. Each germination assay was repeated at least three times.

For the ABA catabolism inhibitors (Abz-E1 and Abz-E2B) and GR24, IC$_{50}$ and EC$_{90}$ ± standard errors (SE), respectively, were determined from the dose–response curve $[g = f(c)]$, where g is the germination percentage as a function of (c) concentration of the compound tested and modelled with a four parameter logistic curve on at least three independent dilution ranges. Data were computed with SigmaPlot® V.10.0 (Systat Software Inc., San Jose, CA, USA). An analysis of variance was performed on the results using SigmaPlot version 10.0 ($P < 0.05$; Student–Newman–Keuls test, SNK).

For seed viability tests following the addition of Abz-E1 and Abz-E2B, treated seeds were washed three times with 100 µl of 1 mM Na/K phosphate buffer (pH 7.5) after an initial count. Then, 100 µl of 10$^{-9}$ GR24 in 0.1% acetonitrile were applied to the washed seeds. Plates were incubated as mentioned above prior to the determination of germination percentage.

cDNA-AFLP analysis

Total RNA was extracted from 100 mg of 7 d conditioned seeds (control) and conditioned seeds treated with GR24 for 2 h and 6 h, using the RNeasy Plant Mini Kit (Qiagen, Courtaboeuf, France). Extracts were treated with DNase I (0.02 U µl$^{-1}$, New England Biolabs, Ipswich, MA, USA). The integrity of total RNA was checked by electrophoresis on a 2% (w/v) agarose gel and RNA was quantified spectrophotometrically (A260/280; NanoDrop Spectrophotometer ND-1000, Labtech International Ltd, Rigmer, UK). Starting from 2 µg of total RNA, the AFLP-based transcript profiling (cDNA-AFLP) was performed as described by Vuylstee et al. (2007). All 32 possible primer combinations were performed. Selective [γ-$^{32}$P] ATP-labelled amplification products were separated on 8% polyacrylamide gels with the Model S2001 apparatus (Life Technologies, Paisley, UK). Dried gels were exposed to Biomax film (Sigma Aldrich, St Louis, MO, USA).

Sequence analysis of TDFs

The GR24-regulated TDFs were recovered by PCR under the same conditions used for the pre-amplification. Purified PCR products were sequenced (Eurofins MWG Operon, Ebersberg, Germany) and a similarity search was done with BLASTN and BLASTX sequence alignments from the Parasitic Plant Genome Project (PPGP, http://ppgp.huck.psu.edu/) and The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/). Functional categorization of TDFs was done using the Blast2Go program (www.Blast2GO.de).

Cloning of PrCYP707A cDNAs

Total RNA isolated from 6 h GR24-treated conditioned seeds underwent a reverse transcription procedure. cDNAs were synthesized from 0.5 µg of total RNA using the Superscript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). Degenerate and specific primers corresponding to highly conserved regions between P. aegyptiaca and S. hermonthica CYP707A sequences were designed. After denaturation at 94 °C for 5 min, amplification consisted of 35 cycles of 45 s at 94 °C, 45 s at 55 °C, and 30 s, 45 s or 90 s at 72 °C. A final step of elongation was done at 72 °C for 5 min. The amplified DNA fragments were purified and cloned into a PGEM-T Easy vector (Promega, Madison, WI, USA). Recombinant plasmid DNAs were sequenced. Based on these partial CYP707A sequences, new primers were generated for rapid amplification of cDNA ends (RACE) of each fragment using the Genecaser kit (Invitrogen). RACE products corresponding to different CYP707A-encoding genes were cloned and sequenced. To amplify the PrCYP707A full-length cDNAs, specific primer pairs were designed. Sequence data from this article can be found in the GenBank/EMBL databases under accession numbers JQ838174 (PrCYP707A1) and JQ838175 (PrCYP707A2).

Real-time RT-PCR analysis

Total RNA was extracted from 200 mg of seeds and DNase I-treated using the same procedure as for the cDNA-AFLP analysis. cDNA was synthesised from 0.5 µg of total RNA using the Superscript II Reverse Transcriptase (Invitrogen) following the manufacturer’s instructions. Then, 5 ng of cDNA was used in 25 µl reactions containing 0.3 µM gene-specific primers and 6.25 µl platinum SYBR Green qPCR SuperMix with ROX (Invitrogen). PCR reactions for three biological replicates were performed each in triplicate with a 7300 real-time PCR system according to the manufacturer’s protocol (Applied Biosystems, Carlsbad, CA, USA). Fold change in mRNA expression was estimated using threshold cycles. The amplification of the constitutive elongation factor PrEF1α1 (forward, 5’-AGTGCTAGTGGTGTCTC3’ and reverse, 5’-CTGGGAGCAACACCTTAATTTCTT3’), which showed low cycle threshold (Ct) variation (standard deviation <1 Ct), was used as an internal control to normalize all the data (Péron et al., 2012). A control experiment without cDNA was included for each PCR mix. The following gene-specific primers were used: PrCYP707A1 (forward, 5’-GCCGCTCTCACAAGCTAAA3’ and reverse, 5’-TGTGACAGATTTGGGCTTTTGG3’) and PrCYP707A2 (forward, 5’-TCTCTTCCATGGAATTGTTT3’ and reverse, 5’-TGTGCTTGGACAGATCTTACTT-3’). An analysis of variance was performed on the results from qPCR analyses using SigmaPlot version 10.0. Means of three independent RNA isolations were tested at $P < 0.05$ (SNK test).

PrCYP707A1 in situ hybridization

Digoxigenin (DIG)-labelled RNA probes were prepared using an in vitro transcription kit (Riboprobe Combination Systems, Promega, Madison, USA) according to the manufacturer’s instructions. The riboprobes were synthesized from the full-length PrCYP707A1 clone. Antisense and sense probes were transcribed from SP6 or T7 RNA polymerase promoters after linearisation of the vector with Apal or Ndel, respectively. Samples of conditioned untreated or GR24-treated (6 h) seeds were prepared and in situ hybridization experiments were performed as previously described (Péron et al., 2012).

ABA quantification

ABA concentrations in seeds were determined according to Müller and Munné-Bosch (2011) with minor modifications. First, 200 mg of seeds were ground in liquid nitrogen and extracted in acetonitrile:water:acetic acid (49.5:49.5:1, by vol.). D$_2$-ABA [([S]-2-[H]6-[1-hydroxy-2,6,6-trimethyl-1-oxocyclohex-2-en-1-yl]-3-methyl-[22,4E]-pentadienoic acid)] was used as the internal standard and added in all samples (5 × 10$^{-9}$ mol) and in non-labelled ABA standard calibration solutions (5 × 10$^{-10}$ to 10$^{-6}$ mol$^{-1}$). Analysis was performed on a liquid chromatograph Agilent 1200 Series system (Agilent Technologies Inc, Santa Clara, CA, USA) coupled to a LTQ OrbitrapMS (Thermo Fisher Scientific, Waltham, MA, USA). A Hypersil GOLD column (100 × 2.1 mm, 1.9 µm) equipped with a guard column (Phenomenex, Le Pecq, France) was used. Gradient
elution was done with water:0.1% acetic acid (solvent A) and acetonitrile (solvent B). The gradient profile was linear and applied as follows: (t (min), % A): (0, 100%), (1, 100%), (10, 0%), (15, 0%), (18, 100%), (20, 100%). The flow rate was maintained constant at 0.5 ml min \(^{-1}\). ABA and \(\text{D}_6\)-ABA were ionized in an Atmospheric Pressure Ionization (API) source operated in the negative electrospray mode. Ion characterization was realized at a resolution better than 30 000 (FWHM). A mass accuracy better than 10 ppm was assured for parent ion (ABA: \(m/z\) 263.1277856; \(rt = 6.94 \pm 0.01\) min; \(\text{D}_6\)-ABA: \(m/z\) 269.166543; \(rt = 6.92 \pm 0.02\) min). An analysis of variance was performed on the results from ABA quantification using SigmaPlot version 10.0. Means of six independent metabolite extractions were tested at \(P < 0.05\) (SNK test).

Results

GR24 response, imbibition, and energy metabolism of seeds during conditioning

Prior to germination, broomrape seeds require a conditioning period with moist conditions and suitable temperatures to be receptive to germination stimulants (Joel et al., 1995). To evaluate the effect of conditioning period length on the GR24-triggered germination, conditioning periods ranging from 1–10 d were tested before seeds were treated with GR24 (Fig. 1A). Conditioning periods of 4 d or longer led to a statistically equivalent optimal germination response to GR24, ranging from 66 ± 3 to 78 ± 8% (ANOVA, \(P = 0.287\)), whereas shorter conditioning periods from 0–3 d hampered seed germination. When conditioning was sufficiently long, seed germination, corresponding to the radicle protrusion out of the seed coat, was synchronous and occurred 3 d after adding GR24.

Imbibition and AEC of seeds were determined during conditioning (Fig. 1B). \(P.\ ramosa\) seeds were fully imbibed after 1 h of soaking (189 ± 5% weight increase) and seed fresh weight remained constant over the next 10 d, in both untreated seeds and seeds treated with GR24 after the 7 d conditioning period. Along with rapid imbibition, a rapid and transient decrease in AEC was observed during the first hour of conditioning. AEC then increased and reached a maximum of 0.9 after 1 d of conditioning and remained constant for the next 9 d, for both GR24-treated seeds and the controls. These results indicate that, as of the first days of conditioning, the energy metabolism of seeds is adequate for germination.

![Fig. 1. Characterization of \(P.\ ramosa\) seed conditioning and germination. (A) Effect of the length of the conditioning period on seed germination. Germination percentages of seeds conditioned for different periods were determined 3 d after the addition of 1 nM GR24. Means are values ± SE. (B) Imbibition (squares) and adenylate energy charge (triangles) time-course during conditioning (open symbols and dotted line) and germination (filled symbols and solid line). Seeds were stimulated with 1 nM GR24 after a 7 d conditioning period. Means are values ± SE.](image-url)
day of the conditioning period, seeds were fully hydrated and not limited in terms of energy metabolism, but unable to respond to GR24 before 4 d of conditioning. A standard 7 d conditioning period was chosen arbitrarily for the subsequent experiments.

**Transcriptomic response of *P. ramosa* seeds to GR24**

To investigate the early molecular response of *P. ramosa* seeds to GR24, the transcriptomic profiles of GR24-treated seeds (2 h and 6 h treatments) and non-GR24-treated seeds (control) were compared using cDNA-amplified fragment length polymorphism (Vuylstee *et al.*, 2007). Both GR24-treatment triggered seed germination 3 d later. The 32 primer combinations produced some 250 TDFs, of which 58 showed an apparent differential expression between samples, including 43 up-regulated genes and 15 down-regulated genes when compared with control levels. Among the 58 sequenced TDFs, 44 showed a significant BLAST hit to sequences found in public databases (Table 1), with 12 annotated sequences according to biological function revealed that, nearly one-third of the sequences (12) encode proteins involved in ‘stress responses’, followed by sequences encoding proteins involved in metabolic processes (9), nucleotide binding (4), oxidation-reduction processes (2), translation (1), and, finally, unknown (3). Among the annotated TDFs, two (TDF30 and 37) corresponded to sequences encoding an ABA 8'-hydroxylase (cytochrome P450 CYP707A) that catalyses the C8'-hydroxylation of ABA to 8'-hydroxy-ABA and phaseic acid (Nambara and Marion-Poll, 2005). Compared with the control, both TDFs showed a strong up-regulation in seeds after 2 h and 6 h of GR24 treatment. Because CYP707A proteins belong to an enzyme family involved in the control of ABA levels during seed dormancy maintenance and breaking (Kushiro *et al.*, 2004; Saito *et al.*, 2004; Okamoto *et al.*, 2006), TDF30 and 37 were selected for further analysis.

**Molecular cloning of CYP707A homologues in *P. ramosa***

Sixty-three and 25 ESTs correspond to putative sequences encoding a cytochrome P450 CYP707A in *P. aegyptiaca* and

### Table 1. List of TDFs modulated in *P. ramosa* conditioned seeds treated for 2 h or 6 h with GR24

| TDF no. | Regulation* | Best Arabidopsis hit (Accession no.) | Functional category | E value |
|---------|-------------|-------------------------------------|---------------------|--------|
| 1       | +           | Sulphite reductase (NP_196079)       | Oxidation-reduction processes | 2.00E-28 |
| 3       | +           | 60S ribosomal protein L8-3 (NP_195336) | Translation | 3.00E-12 |
| 5       | +           | Sucrose synthase 3 (NP_192137)       | Carbohydrate metabolic processes | 6.00E-38 |
| 6       | −           | High mobility group (HMG1/2) domain-containing protein (NP_586578) | Nucleotide binding | 8.00E-11 |
| 7       | O           | Ninja-family protein AFP3 (NP_189598) | Nucleotide binding | 7.00E-18 |
| 8       | O           | Peptidyropyl isomerase ROF2 (NP_199668) | Response to stress | 6.00E-28 |
| 10      | O           | Heat shock protein 81.4 (NP_200411)  | Response to stress | 3.00E-45 |
| 13      | O           | PPPDE putative thiold pepitidase family protein (NP_187365) | Unknown | 1.00E-28 |
| 14      | O           | Rossmann-fold NAD(P)-binding domain-containing protein (NP_175552) | Oxidation-reduction processes | 1.00E-16 |
| 15      | O           | Aldolase-type TIM barrel family protein (AED97862) | Response to stress | 8.00E-25 |
| 16      | O           | Phosphatidylethanolamine-binding protein (NP_195750) | Secondary metabolic processes | 0.006 |
| 20      | O           | Trans-cinnamate 4-monooxygenase (NP_180607) | Carbohydrate metabolic processes | 3.00E-26 |
| 26      | O           | HIPL2 protein (NP_201069)            | Carbohydrate metabolic processes | 3.00E-23 |
| 28      | O           | Methionine synthase 2 (NP_001118664) | Protein metabolic processes | 3.00E-21 |
| 30      | O           | Abscisic acid 8'-hydroxylase 1 (NP_974574) | Protein metabolic processes | 0.091 |
| 31      | O           | Acetylornithine decasylate (NP_001190758) | Protein metabolic processes | 1 |
| 32      | O           | 26S proteasome regulatory subunit 4-A (NP_194633) | Nuclear binding | 2.00E-25 |
| 33      | O           | RNA recognition motif-containing protein (NP_197436) | Response to stress | 0.073 |
| 36      | O           | Heat shock 70kDa protein 1 (NP_195870) | Response to stress | 0.001 |
| 37      | O           | Abscisic acid 8'-hydroxylase 1 (NP_974574) | Response to stress | 3.00E-23 |
| 38      | O           | B1-type protein (NP_567468)          | Unknown | 3.00E-18 |
| 40      | O           | Heat shock protein 81-1 (NP_200076)  | Response to stress | 3.00E-23 |
| 41      | O           | Beta-glucosidase 44 (NP_188436)      | Transport | 6.00E-20 |
| 42      | O           | 5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase (NP_197294) | Carbohydrate metabolic processes | 5.00E-41 |
| 44      | O           | Glutathione S-transferase PM24 (NP_192161) | Cellular amino acid metabolic processes | 1.00E-16 |
| 45      | O           | Heat shock protein 21 (NP_194497)    | Response to stress | 6.00E-26 |
| 48      | O           | Splicing factor U2af large subunit B (NP_176287) | Nuclear binding | 0.02 |
| 49      | O           | Catalase 2 (NP_195235)              | Response to stress | 1.00E-26 |
| 50      | O           | Heat shock protein 70B (NP_173055)   | Response to stress | 1.00E-20 |
| 51      | O           | Phenylalanine ammonia-lyase 3 (NP_001190223) | Response to stress | 6.00E-10 |
| 58      | O           | Putative xyloglucan glycosyltransferase 8 (NP_180039) | Carbohydrate metabolic processes | 1.00E-26 |

*Compared with the non-treated control sample, (+) corresponds to an up-regulation, (−) a down-regulation, and (O) no change.
GR24 induces PrCYP707A1 the first 18 h of GR24 treatment (Fig. 2). After conditioning, the expression patterns of PrCYP707A1 and PrCYP707A2 were examined in detail during a 3 d GR24 treatment (Fig. 2). While levels of PrCYP707A2 mRNA were low and did not change upon GR24 treatment (data not shown), the expression level of PrCYP707A1 showed a rapid and strong significant increase as of 1 h (Fig. 2A) and reached a peak 18 h (Fig. 2B) after the addition of GR24. After 24 h, PrCYP707A1 mRNA levels dropped, reaching a value similar to that observed the first 30 min, and remained stable for the next 48 h.

Change in PrCYP707A1 gene expression in P. ramosa seeds after GR24 treatment

After conditioning, the expression patterns of PrCYP707A1 and PrCYP707A2 were examined in detail during a 3 d GR24 treatment (Fig. 2). While levels of PrCYP707A2 mRNA were low and did not change upon GR24 treatment (data not shown), the expression level of PrCYP707A1 showed a rapid and strong significant increase as of 1 h (Fig. 2A) and reached a peak 18 h (Fig. 2B) after the addition of GR24. After 24 h, PrCYP707A1 mRNA levels dropped, reaching a value similar to that observed the first 30 min, and remained stable for the next 48 h.

PrCYP707A1 is up-regulated by GR24 after a minimum conditioning period

First, the expression patterns of PrCYP707A1 and PrCYP707A2 were examined during conditioning. No change in mRNA accumulation of either gene was recorded (data not shown). Because P. ramosa seeds were receptive to GR24 after a minimum 4 d conditioning period (Fig. 1A), the expression of PrCYP707A1 and PrCYP707A2 were examined in 6 h GR24-treated seeds after various conditioning periods (1, 3, 5, and 7 d) (Fig. 3). In seeds that underwent 1 d and 3 d of conditioning, GR24 triggered neither germination nor PrCYP707A1 mRNA accumulation. By contrast, PrCYP707A1 was significantly up-regulated in GR24-stimulated seeds that were conditioned for 5 d and 7 d, with germination percentages of 72±6% and 77±2%, respectively. Whatever the conditioning period, PrCYP707A2 did not exhibit any major change in its expression level.

GR24 induces PrCYP707A1 mRNA accumulation in cells close to the micropyle

Seeds strongly accumulated PrCYP707A1 transcripts during the first 18 h of GR24 treatment (Fig. 2). In situ hybridization experiments were performed on longitudinal sections of 6 h GR24-treated seeds to localize this accumulation spatially (Fig. 4). To allow better visualization and identification of the different parts of 6 h GR24-treated seeds (cf. Joel et al., 2011), a seed section was stained with toluidine blue (Fig. 4A). Positive hybridization with the specific antisense probe indicated PrCYP707A1 transcript accumulation (Fig. 4C, 4D), whereas no signal was observed after hybridization with the sense probe (Fig. 4B). In 7 d conditioned seeds, transcripts accumulated mainly in the embryo cells facing the micropyle (Fig. 4C). By contrast, PrCYP707A1 mRNA accumulated markedly in the cells near the micropyle in 6 h GR24-treated seeds, whereas no staining was detected in the embryo cells (Fig. 4D). According to Joel et al. (2011), these stained cells may correspond to perisperm tissue. These results indicate that GR24 induced a change in the spatial localization of PrCYP707A1 expression in seeds.

Change in seed ABA levels during conditioning and GR24 treatment

ABA levels in seeds were determined during the 7 d conditioning period and the following 3 d GR24 treatment. A 6.3-fold decrease in ABA content occurred during the first day of conditioning (Fig. 5). Although no significant decrease was observed in seeds during the next 6 d of conditioning, a second, 8-fold drop in ABA content was observed specifically in 3 d GR24-treated seeds compared with 10 d conditioned seeds. Interestingly, this decline in ABA levels in GR24-treated seeds followed the up-regulation of PrCYP707A1 occurring during the first 18 h of GR24 treatment (Fig. 2).

Abz-E1 and Abz-E2B inhibit GR24-triggered seed germination of P. ramosa

The preponderant role of ABA catabolism in the GR24-dependent germination of P. ramosa seeds was confirmed by using two CYP707A inhibitors, Abz-E1 and Abz-E2B (Okazaki et al., 2011, 2012). When 7 d conditioned seeds were treated for 3 d with 10−4 M GR24 together with Abz-E1 or Abz-E2B at various concentrations, seed germination was inhibited in a concentration-dependent manner. Inhibition was maximal at 100 µM, 84±5% and 90±3% for Abz-E1 or Abz-E2B, respectively, with IC50 reaching 30±16 µM and 17±9 µM, respectively (data not shown). The most effective inhibitor, Abz-E2B was used for further experiments. Using GR24 concentrations ranging from 10−12 to 10−7 M with Abz-E2B concentrations ranging from 10−6 to 10−4 M, no GR24 concentration was able to overcome Abz-E2B inhibition (Fig. 6A). IC50 values ranged insignificantly from 8.3 µM and 17 µM according to GR24 concentration (ANOVA, P=0.877). Germination inhibition was total with 100 µM Abz-E2B and 10−12 M GR24. Increasing concentrations of Abz-E2B did not significantly modify the EC50 of GR24 (values ranging from 0.9 nM to 11.3 nM; ANOVA, P=0.181) (Fig. 6B). This indicates that Abz-E2B did not interfere with the perception of the germination stimulant. Significant reduction in the maximum germination percentage was observed with Abz-E2B concentrations of 5 µM or higher (ANOVA, P<0.001). To ensure that the Abz-E2B inhibition of germination was not due to seed mortality,
treated seeds were subsequently washed with distilled water and treated once again with GR24 in the absence of the inhibitor. Once washed, the seeds showed a germination rate similar to that of untreated seeds (data not shown). Taken together, these results demonstrate that, with regard to germination, Abz-E2B acts as a non-competitive antagonist of GR24 with reversible inhibitory activity.

**Discussion**

Germination of obligate root parasitic plants is stimulated by the perception of secondary metabolites released from the roots of a potential host plant (Estabrook and Yoder, 1998). Among the germination stimulants identified so far, SLs have been the most extensively studied (Yoneyama et al., 2010). In addition to their capacity to induce broomrape seed germination at nanomolar concentrations, SLs are host recognition signals for symbiotic arbuscular mycorrhizal fungi (Besserer et al., 2006) and constitute new plant hormones that inhibit shoot branching (Gomez-Roldan et al., 2008; Umehara et al., 2008).

Although much progress has been made on the signalling activities of SLs in non-parasitic plants, the signalling pathway triggered by SLs leading to broomrape seed germination remains unclear. Here, early transcriptome modifications of *P. ramosa* seeds triggered by a synthetic SL, the germination stimulant GR24, were studied using an AFLP-based transcript profiling procedure (Vuylsteke et al., 2007). The overall results from the cDNA-AFLP experiments indicate that GR24 does not induce massive modification of the transcriptome because, among the 2500 TDFs visualized on gels, only 58 showed significant differential expression between the control and the 2h and 6h GR24-treated samples. Two of the most distinct TDFs, in terms of expression pattern and biologically significant association with germination, corresponded to a *CYP707A* gene encoding
an ABA 8'-hydroxylase (Table 1). Interestingly, an AtCYP707A gene has been already shown to be up-regulated in Arabidopsis seedlings treated with GR24 (Mashiguchi et al., 2009). Based on both TDF sequences, a search in the Parasitic Plant Genome Project databank uncovered three distinct CYP707A sequences in P. aegyptiaca and S. hermonthica. RACE-PCR strategies revealed two CYP707A full-length cDNAs, named PrCYP707A1 and PrCYP707A2.

ABA is known to play a major role in seed dormancy and germination (Koornneef et al., 2002). Its hormonal action is controlled by a fine-tuned balance between biosynthesis and catabolism (Nambara and Marion-Poll, 2005). Seed dormancy maintenance involves ABA synthesis (Finkelstein et al., 2008) whereas a
decrease in ABA content triggered by after-ripening, stratification, and other dormancy-releasing mechanisms promote the germination process in dormant seeds (Gubler et al., 2005). Thus, dormancy release relies mainly on ABA catabolism by specific ABA 8'-hydroxylases encoded by the cytochrome P450 CYP707A family (Kushiro et al., 2004; Saito et al., 2004; Okamoto et al., 2006). ABA 8'-hydroxylases catalyse ABA hydroxylation and produce 8'-hydroxy ABA which is then spontaneously isomerized to phaseic acid (Nambara and Marion-Poll, 2005). CYP707A-related sequences have been characterized in many plant species. For instance, four genes encoding CYP707A activity have been identified in Arabidopsis (Kushiro et al., 2004; Saito et al., 2004) and two in barley (Millar et al., 2006). Among the four Arabidopsis sequences, AtCYP707A2 is up-regulated in association with a rapid decrease in ABA content during seed imbibition. Seeds of a cyp707a2 mutant were hyperdormant and accumulated 6-fold higher ABA levels than the wild type (Kushiro et al., 2004; Millar et al., 2006), whereas constitutive expression of AtCYP707A1 in Arabidopsis results in decreased ABA levels in seeds along with dramatically reduced dormancy (Millar et al., 2006). Similarly, ABA content in barley is higher in embryos of after-ripened dormant seeds than of non-dormant seeds in association with higher HvCYP707A1 expression levels in non-dormant compared to dormant seeds (Millar et al., 2006). Altogether, these results highlight the major role of CYP707A genes in regulating the ABA level during seed dormancy and release.

In root parasitic plants, the control of ABA levels is also thought to be involved in the seed germination process (Zehhar et al., 2002; Chae et al., 2004). In the present study, a strong decrease in ABA levels in P. ramosa seeds occurred during the first day of conditioning (Fig. 5). This decrease was maintained to a lesser extent for the next 6 d of conditioning. Interestingly, neither gene, PrCYP707A1 nor PrCYP707A2, exhibited any change in their expression levels during the 7 d conditioning period (data not shown). Based on these results, the decrease in ABA levels in conditioned P. ramosa seeds does not seem to be
associated with ABA catabolism, but rather with ABA release in the medium as previously demonstrated in the 1 d conditioned seeds of *O. minor* (Chae *et al.*, 2004).

The present study shows that GR24 treatment, following conditioning, induced a second decrease in the ABA levels in seeds (Fig. 5). These results suggest that GR24 promotes a sufficient and necessary reduction in ABA content for *P. ramosa* seed germination. Accordingly, when exogenous ABA is applied during conditioning and GR24 treatments, germination of *P. ramosa* seeds is inhibited (Zehhar *et al.*, 2002). Here, the second ABA reduction in response to GR24 was associated with the strong and rapid up-regulation of *PrCYP707A1* that started as early as 1 h after the addition of GR24 and persisted with increasing intensity for 18 h (Fig. 2). By contrast, *PrCYP707A2* did not show any change in its expression during the 72 h GR24 treatment. These findings indicate that conditioned *P. ramosa* seeds can germinate only after endogenous ABA content reaches a sufficiently low level through CYP707A-dependent catabolism triggered by GR24. The important role of *CYP707A* genes in the regulation of ABA levels and dormancy release in response to environmental cues has already been investigated in non-parasitic plant: exogenous nitrate and light, two seed dormancy releasing stimuli.
in *Arabidopsis*, induce an up-regulation of *AtCYP707A2* (Seo et al., 2006; Matakadiis et al., 2009). A PrCYP707A1-dependent release of dormancy in *P. ramosa* seeds appears to rely on the perception of another environmental cue: the exogenous germination stimulant produced by the host plant. This primordial role of CYP707A1 in *P. ramosa* seed germination was confirmed by the application of specific inhibitors of ABA 8'-hydroxylase, Abz-E1 and Abz-E2B (Okazaki et al., 2011, 2012) on conditioned seeds together with GR24 (Fig. 6). These inhibitors prevented seed germination. Abz-E1 and Abz-E2B correspond to structural analogues of uniconazole, which has been shown to inhibit the germination of *P. ramosa*, *O. aegyptiaca*, and *O. minor* seeds when applied during conditioning (Zehhar et al., 2002; Song et al., 2005; Uematsu et al., 2007). This study now provides evidence that uniconazole inhibited broomrape seed germination by inhibiting both gibberellin synthesis and ABA catabolism.

To be receptive to germination stimulants, broomrape seeds require a preparatory phase of several days called the conditioning period (Joel et al., 1995). The present study demonstrated that *P. ramosa* seeds require a minimum of 4 d of conditioning to allow optimal germination in response to GR24 (Fig. 1A). At first glance, this result contradicts a previous study that concluded that *P. aegyptiaca*, a closely related species, does not need conditioning to respond to germination stimulants (Plakhine et al., 2009). However, non-conditioned *P. aegyptiaca* seeds stimulated by GR24 only germinate after 7 d, a period that may correspond to a 4 d conditioning period and a 3 d germination process, as reported for *P. ramosa* seeds here. This study showed that the conditioning period starts with seed imbibition that takes around 1 h and optimal AEC (0.9) is reached as of first day of conditioning (Fig. 1B). This rapid imbibition is obtained by water entering the seed through the micropyle which opens after 30 min (Joel et al., 2011). Thus, the inability of *P. ramosa* seeds to respond to GR24 during the first 4 d of conditioning cannot be attributed to a defect in seed hydration nor in energy status of the embryo. An analysis of the expression pattern of PrCYP707A1 in seeds treated for 6 h with GR24 demonstrated that PrCYP707A1 expression was not up-regulated until a conditioning period of 5 d or more (Fig. 3). Taken together, these results suggest that *P. ramosa* seeds do not have physical dormancy and that the minimal conditioning period may correspond to physiological processes resulting in the set-up of the machinery needed for GR24 perception and signalling leading to PrCYP707A1 over-expression.

A recent study on the inheritance of the germination control in *P. aegyptiaca* seeds suggests that receptors of germination stimulants are located in the living perisperm cells beneath the micropyle (Plakhine et al., 2012). The *in situ* hybridization experiments support this hypothesis since, upon GR24 treatment, PrCYP707A1 mRNA accumulated rapidly and specifically in similar cells in *P. ramosa* seeds (Fig. 4). Moreover, such rapid accumulation triggered 1 h after GR24 application can be attributed to the location of these cells, which are readily accessible to the germination stimulant entering through the micropyle.

One question that remains is how the germination stimulant activates the signalling pathway leading to rapid transcriptional activation of the PrCYP707A1 gene. Expression of *AtCYP707A2* has been shown to be regulated by exogenous nitrate, which releases seed dormancy in *Arabidopsis* (Matakadiis et al., 2009). The nitrate control of seed dormancy is known to proceed via the production of nitric oxide (NO) (Bethke et al., 2006). In this context, Liu et al. (2009, 2010) demonstrated in *Arabidopsis* that hydrogen peroxide (*H*$_2$O$_2$) and NO are involved in the up-regulation of the *AtCYP707A2* gene and the subsequent decrease in ABA levels during seed imbibition. Interestingly, the cDNA-AFLP procedure also identified two TDFs (44 and 49) putatively encoding a GST and a catalase in *P. ramosa* GR24-treated seeds, suggesting that oxidative stress may have occurred upon GR24 stimulation. The possible involvement of NO and *H*$_2$O$_2$ in SL signalling and PrCYP707A1 activation, as well as the occurrence of oxidative stress during *P. ramosa* seed germination, are currently under investigation.

The cDNA-AFLP procedure proved to be a powerful tool to identify candidate genes involved in the response of *P. ramosa*, a non-model plant, to the germination stimulant GR24. In addition to PrCYP707A1, other revealed TDFs may correspond to genes putatively involved in this process (Table 1). For instance, the *Arabidopsis* genes similar to TDF11-39 and TDF36-50 encode heat shock proteins, HSP90 and HSC70, respectively, known to form a molecular complex that modulates ABA-dependent physiological responses such as stomatal closure and seed germination (Clément et al., 2011). Interestingly, the *Arabidopsis* proteins peptidylprolyl isomerase RFO1 and ROF2, also called AtFKBP64 and AtFKBP65, corresponding to TDF8, have been shown to bind to HSP90 (Aviezer-Hagai et al., 2007). Similarly, FK506 binding proteins (FKBP) are thought to play a major role in seed germination of sorghum (Sharma and Singh, 2003). Moreover, TDF7 may correspond to a member of a small plant-specific protein family, ABI five binding proteins (AFPs), which interact with the transcription factor ABA-insensitive5 (ABI5), a key regulator of ABA signalling and stress response in *Arabidopsis* seeds (Garcia et al., 2008). Finally, TDF20 and TDF51 correspond to two sequences encoding a cinnamate 4-hydroxylase (*C4H*) and a phenyl ammonia lyase (*PAL*), respectively, involved in phenylpropanoid synthesis. In *Arabidopsis*, both *C4H* and *PAL2* genes have been shown to be transiently induced in seeds exposed to the germination stimulants karrikins identified in smoke from wildfires (Nelson et al., 2010). Interestingly, karrikins and GR24 signalling require the same F-box protein MAX2 in *Arabidopsis* germination and shoot branching processes, respectively (Nelson et al., 2011). Thus, all these putative candidate genes deserve further study to investigate their potential implication in *P. ramosa* seed germination.

In summary, GR24 triggered the dormancy release of *P. ramosa* seeds by activating a strong and rapid up-regulation of an ABA-catabolic gene PrCYP707A1 that occurs in association with a reduction in ABA levels. However, release from dormancy was shown to require a minimum conditioning period since germination and the activation of PrCYP707A1 expression only occurred 4 d post imbibition. The results on the spatial and temporal expression of PrCYP707A1 corroborate previous studies suggesting that putative receptors of parasitic plant germination stimulants are effective following a conditioning period and are located in the cells between the embryo and the micropyle. How GR24 triggers the ABA decline leading to the *P. ramosa*
seed germination requires further study, as does the possible implication of gibberellins since antagonism between ABA and GA plays a key role in controlling seed germination.

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