the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2010) **61**, 792–803 doi: 10.1111/j.1365-313X.2009.04106.x

The *Mt*SNF4b subunit of the sucrose non-fermenting-related kinase complex connects after-ripening and constitutive defense responses in seeds of *Medicago truncatula*

William Bolingue[1], Claire Rosnoblet[2,†], Olivier Leprince[3], Benoit Ly Vu[3], Catherine Aubry[2] and Julia Buitink[1,*]

[1]*Institut National de la Recherche Agronomique, UMR 1191 Physiologie Moléculaire des Semences, IFR 149 QUASAV, 49045 Angers, France,*

[2]*Université d'Angers, UMR 1191 Physiologie Moléculaire des Semences, IFR 149 QUASAV, 49045 Angers, France, and*

[3]*Agrocampus Ouest, UMR 1191 Physiologie Moléculaire des Semences, IFR 149 QUASAV, 49045 Angers, France*

Received 12 October 2009; revised 27 November 2009; accepted 3 December 2009; published online 22 January 2010.

[*]For correspondence (fax +33 241 22 55 49; e-mail julia.buitink@angers.inra.fr).

[†]Present address: Interdisciplinary Research Institute (IRI) – CNRS USR 3078 – 59658 Villeneuve d'Ascq, France.

SUMMARY

Dormant seeds are capable of remaining alive in the hydrated state for extended periods of time without losing vigor, until environmental cues or after-ripening result in the release of dormancy. Here, we investigated the possible role of the regulatory subunit of the sucrose non-fermenting-related kinase complex, *Mt*SNF4b, in dormancy of *Medicago truncatula* seeds. Expression of *Mt*SNF4b and its involvement in a high-molecular-weight complex are found in dormant seeds, whereas imbibition of fully after-ripened, non-dormant seeds leads to dissociation of the complex. *Mt*SNF4b is capable of complementing the yeast Δ*snf4* mutant and of interacting with the *Mt*SnRK1 α-subunit in a double hybrid system. Transcriptome analyses on freshly harvested and after-ripened RNAi *Mtsnf4b* and wild-type embryos implicate *Mt*SNF4b in the defense response in hydrated dormant embryonic tissues, affecting the expression of genes encoding enzymes of flavonoid and phenylpropanoid metabolism, WRKY transcription factors and pathogenesis-related proteins. Silencing *MtSNF4b* also increased the speed of after-ripening during dry storage, an effect that appears to be related to a change in base water potential. No significant difference in ABA content or sensitivity was detected between mutant and wild-type seeds. Pharmacological studies using hexoses and sugar analogs revealed that mannose restored germination behavior and expression of the genes *PAL*, *CHR* and *IFR* in RNAi *Mtsnf4b* seeds towards that of the wild-type, suggesting that *Mt*SNF4b might act upstream of sugar-sensing pathways. Overall, the results suggest that *Mt*SNF4b participates in regulation of a constitutively activated defense response in hydrated, dormant seeds.

Keywords: SNF4b, seeds, *Medicago truncatula*, dormancy, biotic stress.

INTRODUCTION

The end of seed development is characterized by termination of seed filling, a decrease in seed water content and progressive arrest of metabolism. During this final phase, seeds such as those of *Medicago truncatula* acquire the ability to survive the dry state for long periods of time, also referred to as longevity, and enter into a dormant state. Dormancy is a genetically and environmentally determined developmental state that is imposed during imbibition of freshly harvested seeds, during which metabolism is active, but growth processes are repressed (Finch-Savage and Leubner-Metzger, 2006; Bradford and Nonogaki, 2007). Dormancy is gradually lost during the time-dependent process of after-ripening (AR) that occurs in the dry seed. In the dormant state, seeds are capable of remaining hydrated for extended periods of time without losing vigor (Finch-Savage and Leubner-Metzger, 2006; Bradford and Nonogaki, 2007). In addition, fluctuations of environmental conditions will cause the seeds to undergo hydration and dehydration cycles in the soil, which they can do without losing their ability to germinate. Dormant seeds have developed an array of protective mechanisms in order to remain viable until environmental cues are provided to

release dormancy, leading to germination and seedling establishment.

The sucrose non-fermenting related kinase (SnRK1) is known for its central role in energy metabolism, linking stress, sugar and developmental signals to regulate plant metabolism, energy balance, growth and stress responses (Schwachtje *et al.*, 2006; Ananieva *et al.*, 2008; Baena-Gonzalez and Sheen, 2008). The complex has also been shown to play a role during seed maturation, affecting accumulation of storage proteins and occasionally leading to precocious germination (Radchuk *et al.*, 2006; Rosnoblet *et al.*, 2007). The SnRK1 complex comprises an auto-inhibitory catalytic α subunit that is activated upon interaction with two other non-catalytic subunits, a β subunit (SnRKβ), which has been suggested to play an essential role in subcellular localization and the specificity of recognition between the kinase complex and its targets, and a γ subunit (SNF4/SnRKγ), which is thought to regulate the activity of the complex (Halford *et al.*, 2000; Kleinow *et al.*, 2000). Several regulatory β and γ subunits exist in plants, and are differentially regulated at the transcriptional level according to stress and developmental stage (Bouly *et al.*, 1999; Buitink *et al.*, 2004). In seeds, several SnRKβ and SnRKγ subunits are co-expressed, suggesting co-existence of several SnRK1 complexes formed by different regulatory subunits (Buitink *et al.*, 2004). This might explain the strong pleiotropic effects observed when the catalytic subunit is down-regulated (Radchuk *et al.*, 2006; Baena-Gonzalez and Sheen, 2008).

The expression of one subgroup of SnRKγ subunits, Pv42/LeSNF4/*Mt*SNF4b, is correlated with the final stage of maturation, when longevity and dormancy are acquired (Bradford *et al.*, 2003; Buitink *et al.*, 2004). *MtSNF4b* is expressed in parallel with the induction of desiccation tolerance, and its silencing affects oligosaccharide metabolism and longevity during maturation (Bradford *et al.*, 2003; Rosnoblet *et al.*, 2007). *LeSNF4* transcripts are ABA-inducible in tomato seeds, and their accumulation is linked to the inability of seeds to germinate, with levels remaining high when radicle protrusion is blocked by ABA, water stress, far-red light or dormancy (Bradford *et al.*, 2003).

In this study, we investigated the possible role of *Mt*SNF4b in seed germination and dormancy of *M. truncatula* seeds. We show that *Mt*SNF4b expression and complex formation are related to the AR status of the imbibed seeds. A transcriptomic analysis on imbibed mutant and wild-type embryos demonstrated that *MtSNF4b* silencing reduces gene expression related to biotic stress and secondary metabolism in dormant embryonic tissues. Silencing *MtSNF4b* also increased the speed of AR independently of ABA. A role of sugar sensing pathways downstream of SnRK1/SNF4b is suggested by the observation that mannose both delays germination and increases *PAL*, *CHR* and *IFR* expression in RNAi *Mtsnf4b* seeds. The results suggest that SNF4b plays a role in constitutive defense responses during hydration of dormant *M. truncatula* seeds.

RESULTS

*Mt*SNF4b expression and complex formation are related to dormancy

To investigate the role of *Mt*SNF4b in dormancy of *M. truncatula* seeds, we first characterized its dormancy behavior. Seeds of *M. truncatula* do not germinate readily under optimal conditions when imbibed 4 days after harvest, and exhibit non-deep dormancy comparable to that of *Nicotiana plumbaginifolia* (Grappin *et al.*, 2000; Faria *et al.*, 2005) (Figure 1a). Subsequent storage of freshly harvested seeds under controlled, dry conditions led to the gradual release of dormancy, referred to as AR, and a concomitant increase in germination speed (Figure 1a). Seeds that are imbibed after two weeks of AR take 10–14 days to germinate at 20°C in the dark, whereas breaking of dormancy either by one year of AR or an incipient cold imbibition (48 h at 4°C) results in a seed population that germinates within 24 h under similar imbibition conditions. Incubation in fluridone to block ABA and carotenoid synthesis released dormancy and increased the germination speed such that it was comparable with that of fully after-ripened seeds (Figure 1a). To investigate whether *MtSNF4b* gene expression is regulated

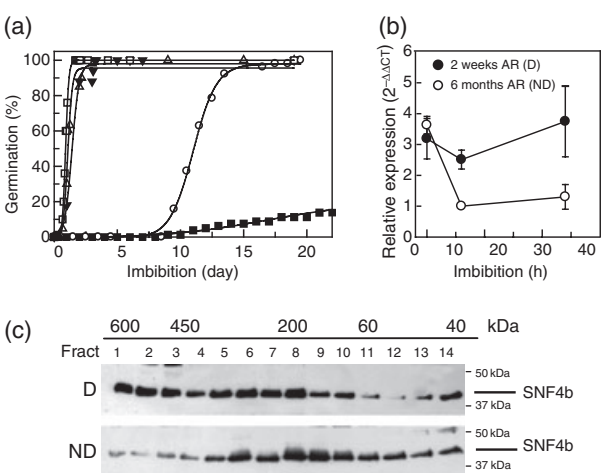

Figure 1. *MtSNF4b* expression and complex formation in *M. truncatula* seeds.

(a) Germination curves at 4 days of after-ripening (AR) (solid squares), at 2–3 weeks of AR (open circles) imbibed first for 48 h at 4°C (open squares) or imbibed in 100 μM fluridone (open triangles), and imbibed at 6 months AR at 20°C in the dark (closed triangles). Data are significantly different when they differ by 18% or more (χ^2 test, $P < 0.05$).

(b) Relative transcript levels (±SE) of *MtSNF4b* in seeds during imbibition at 20°C after 2–3 weeks AR (dormant, D, closed symbols) or 6 months AR (non-dormant, ND, open symbols).

(c) Western blot analysis of *Mt*SNF4b after 1–2 weeks of AR and 48 h of imbibition at 20°C (D) or 4°C (ND) after separation of the protein extracts according to their molecular weight using gel filtration chromatography as described by Rosnoblet *et al.* (2007).

in relation to dormancy, as found for the *LeSNF4* gene of tomato (Bradford *et al.*, 2003), its expression was analyzed by quantitative RT-PCR. *MtSNF4b* transcript levels in embryos remain high in freshly harvested, dormant seeds (Figure 1b). In contrast, transcript levels decreased in imbibed non-dormant seeds prior to germination.

Previous studies using yeast and plant native protein extracts demonstrated, using chromatography techniques, that the catalytic subunits, together with their regulatory counterparts, were present in high-molecular-weight fractions, and that this assembly corresponded to the activity of the SNF1 complex (Estruch *et al.*, 1992; Sugden *et al.*, 1999). We previously demonstrated that *Mt*SNF4b is incorporated into a high-molecular-weight complex during the final stages of maturation (Rosnoblet *et al.*, 2007). To investigate the link between *Mt*SNF4b complex formation and dormancy, soluble proteins were extracted from hydrated dormant seeds after imbibition for 48 h at 20°C, or from hydrated seeds in which dormancy was released by imbibition at 4°C. No seeds had germinated after 48 h under either imbibition condition, but cold-treated seeds germinated within 12 h compared to 12–14 days for seeds that were imbibed directly at 20°C. Freshly extracted native proteins were separated by gel filtration, after which the presence of the SNF4b protein in the various fractions was analyzed by Western blot (Figure 1c). SNF4b was not only visible in those fractions corresponding to its own molecular weight (approximately 42 kDa), but a high-molecular-weight complex of 400–600 kDa containing SNF4b (fractions 1–4) was also detected in dormant seeds. In contrast, imbibition of seeds in which dormancy was released resulted in partial dissociation of the complex, as evidenced by the almost complete absence of SNF4b in these same fractions. Further imbibition of non-dormant seeds was previously found to lead to complete disappearance of SNF4b in fractions >200 kDa (Rosnoblet *et al.*, 2007). This suggests that the SNF4b-associated complex remains intact during hydration of dormant seeds.

*Mt*SNF4b is a functional SNF4 subunit and interacts with the α subunit of *Medicago truncatula*

To verify that the complex in which *Mt*SNF4b participates is a SnRK1 complex, we first tested whether *MtSNF4b* indeed encodes a functional homolog of the corresponding yeast gene by complementation of a mutant deficient in SNF4 (*snf4Δ2*). In yeast, the SNF4 protein is required for activation of SNF1 kinase, which is essential for de-repression of sugar-metabolizing enzymes in the absence of glucose. As a consequence, *snf4Δ2* mutants are able to grow on glucose but not on galactose or other carbohydrate sources (Figure 2a,b). Expression of *MtSNF4b* in a yeast strain with a deletion in the *SNF4* gene restored growth on galactose to a level essentially equivalent to that in the wild-type (Figure 2a).

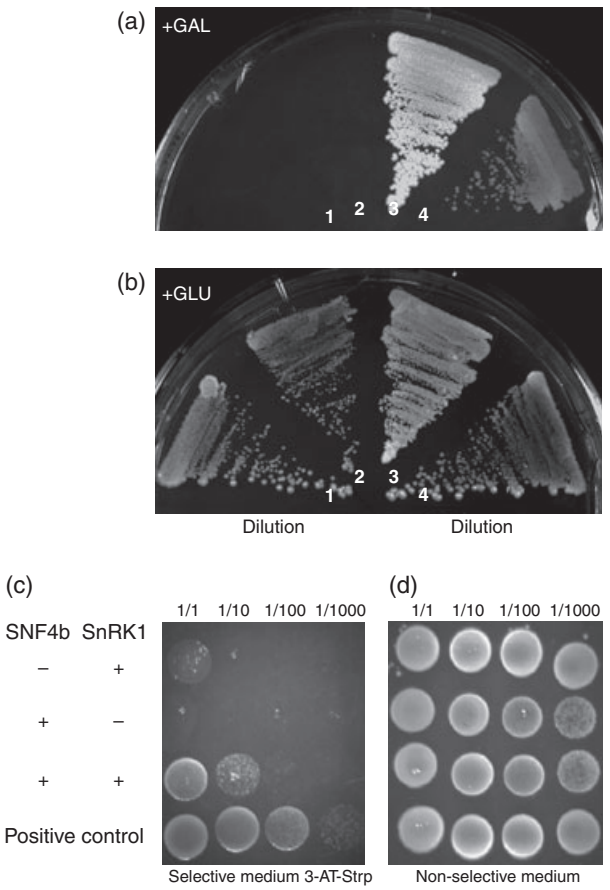

Figure 2. *MtSNF4b* is a functional SNF4 subunit that interacts with *Mt*SnRK1.
(a,b) Complementation of yeast *snf4*. Sector 1, yeast *snf4Δ2*; sector 2, yeast *snf4Δ2* transformed with the empty vector; sector 3, yeast *snf4Δ2* transformed with *MtSNF4b*; sector 4, wild-type strain. Colonies were grown at 28°C on selective YNB medium containing 2% galactose (a) or 2% glucose (b) for 7 and 3 days, respectively.
(c,d) Interaction between *Mt*SNF4b and *Mt*SNF1 assayed by a bacterial two-hybrid system. Reporter strains were co-transformed with pBTLGF2/pTRGMtSnRK1 and pBTMtSNF4b/pTRG as negative controls, with pBTMtSNF4b/pTRGMtSnRK1, and with pBTLGF2/pTRGGal11 as positive control. Transformed strains were plated on selective medium (3-amino-1,2,4-triazole and streptomycin, 3-AT-Strp) (c) or non-selective medium (d).

In addition, a possible interaction with the catalytic α subunit (SnRK1) was tested using a bacterial double hybrid system. Screening of the EST databank Medicago truncatula Gene Index (MTGI) as well as the *Medicago truncatula* genome revealed the presence of one SnRK1 gene with 96 and 92.8% similarity to AKIN10 (Q38997) and AKIN11 (P92958) of *Arabidopsis thaliana*, respectively, and 96.6% similarity with the SnRK1 of tomato (Q9M726, LeSNF1). The sequence of this *SnRK1*, hereafter named *MtSnRK1*, was cloned into the pTRG vector, and the *MtSNF4b* sequence into the pBT vector. No auto-activation of either subunit could be detected, but *Mt*SNF4b and *Mt*SnRK1 were capable of interacting with each other (Figure 2c). These data suggest that, in *M. truncatula* seeds, *Mt*SNF4b interacts

with the SnRK1 subunit and is likely to play a role in activation of the SnRK1 catalytic function. These data confirm the results obtained for another member of the PV42/SNF4b family, LeSNF4, which is capable of complementing yeast *snf1* and *snf4* mutants and physically interacting with LeSNF1 and LeSIP1 in a glucose-dependent manner in yeast two-hybrid assays (Bradford *et al.*, 2003).

Transcriptome profiling indicates a role for *Mt*SNF4b in gene regulation as a function of after-ripening time

To detect genes that are potentially regulated by *Mt*SNF4b, we made use of previously produced lines in which *MtSNF4b* was silenced by RNAi (Rosnoblet *et al.*, 2007), together with a set of controls to verify the effects of transformation and RNAi-mediated effects. Silencing *MtSNF4b* reduced protein levels of SNF4b to almost undetectable levels compared to wild-type seeds (Figure S1) (Rosnoblet *et al.*, 2007). A transcriptome analysis was performed on 6 h-imbibed embryos of wild-type and RNAi *Mtsnf4b* lines at 2–3 weeks of AR, referred to as the fresh (F) dataset. As the SNF4b remained complex only in freshly harvested imbibed seeds, we also compared wild-type versus RNAi *Mtsnf4b* seeds at 6 months of AR, in which the complex dissociated upon imbibition (referred to as the stored, S, dataset). A third analysis was added to identify the genes that are differentially expressed in dormant (freshly harvested) versus non-dormant seeds (after AR), as this dataset is not yet available for *M. truncatula* seeds. For this purpose, a comparison was made between wild-type seeds after 2–3 weeks of AR versus 6 months of AR, using RNA from the F and S datasets (referred to as the dormancy, D, dataset). Sets of genes that showed enhanced or decreased expression in each of the three datasets were identified, and their overlap is presented in a Venn diagram (Figure 2 and Appendix S1).

A total of 217 genes are present in the F dataset, compared to only 37 genes in the S dataset, confirming a dormancy-related link as found for *Mt*SNF4b expression and complex formation (Figure 3). Comparison between the F and S datasets showed that, irrespective of the duration of AR, only seven genes were differentially up-regulated in both datasets, such as 12-oxophytodionoate (OPDA) reductase, a *Myb* transcription factor and two genes encoding plant lipid transfer proteins (*MtN5*). Interestingly, when a threshold *P* value of 0.05 was used instead of 0.01, an additional two genes, encoding 12-OPDA reductase aand glutathione-*S*-transferase, were detected.

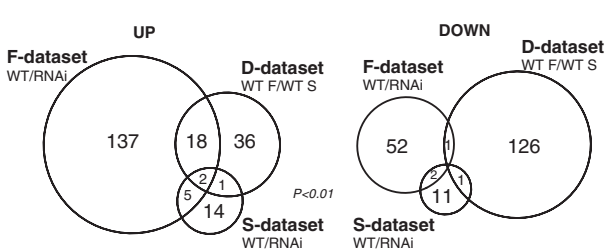

Figure 3. Venn diagram comparing overlapping genes that are up-regulated or down-regulated in wild-type/RNAi *Mtsnf4b* embryos at 2–3 weeks of AR (F dataset), at 6 months of AR (S dataset), or in wild-type embryos at 2–3 weeks of AR/6 months of AR (D dataset). AR, after-ripening; WT, wild-type; F, fresh; S, stored; D, dormant. $P < 0.01$.

Twenty-one genes overlapped between the D dataset and the F dataset. Twenty were up-regulated in wild-type seeds at 2–3 weeks of AR, encoding several pathogenesis-related proteins (ABR17/18) that are developmentally regulated during seed development (Iturriaga *et al.*, 1994), as well as genes encoding chalcone synthase and chalcone reductase, peroxidases or caffeoyl CoA 3-*O*-methyltransferase (Appendix S1). Only one gene overlapped between the down-regulated F and D datasets, encoding a small heat-shock protein. An additional 175 genes were found in the D dataset that were not detected in the F dataset, including legumins and caleosins amongst the up-regulated genes and 126 down-regulated genes, such as ribosomal RNAs or several phosphatase 2C proteins that function as negative regulators in ABA signaling (Figure 3 and Appendix S1) (Gosti *et al.*, 1999). Silencing *MtSNF4b* changes the expression of a subset of genes that are regulated prior to AR.

MtSNF4b silencing reduces gene expression related to secondary metabolism and biotic stress

From the comparison between the datasets, it is evident that the largest set of genes that are regulated differentially between mutant and wild-type are up-regulated genes of the F dataset (Figure 3). Silencing *MtSNF4b* results in a decrease in the expression of these genes when seeds are not yet fully after-ripened. The main classes characterizing the 162 up-regulated genes in the F dataset are related to secondary metabolism, biotic stress, the cell wall, transcription factors and transport (Table 1). A large number of genes are implicated in secondary metabolism, encoding enzymes related to flavonoid and phenylpropanoid biosynthesis. A closer look among them revealed that 27 genes encode various isoforms of 11 of the 13 enzymes involved in medicarpin biosynthesis (Appendix S2 and Figure S3). The class of transcription factors contains five WRKY transcription factors and two ethylene-responsive transcription factors. In addition, four genes related to protein fate encode RING-H2 finger proteins that belong to the ATL sub-family, which has been suggested to be involved in the early steps of the plant defense signaling pathway (Serrano and Guzman, 2004). These results were confirmed for several genes by quantitative RT-PCR (Figures 4 and S2). In wild-type seeds, transcript levels of two genes found in the F dataset, chalcone reductase (*CHR*) and a pathogenesis-related protein (*PR10*), increased sharply during imbibition after only 2 weeks of AR, whereas transcript levels remained low when seeds were imbibed after complete AR (Figure 4a,d). In addition, for both *CHR* (Figure 4a–c) and *PR10* (Figure 4d–f), the quantitative RT-PCR analysis confirmed reduced expression

Table 1 Classification of up-regulated or down-regulated genes in wild-type/RNAi *Mtsnf4b* embryos at 2–3 weeks of AR (F dataset)

Classification	Number of genes Fold change (WT/RNAi) >1.5 ($P < 0.01$)	Fold change (WT/RNAi) >−1.5 ($P < 0.01$)
Metabolism	48	4
Secondary metabolism	35	2
Amino acid metabolism	3	1
Lipid, fatty acid and isoprenoid	4	0
Hormone metabolism	4	0
Carbohydrate metabolism	1	1
Cell rescue	22	17
Disease, virulence and defense	19	0
Detoxification	2	4
Stress response	1	13
Cell wall	13	2
RNA regulation of transcription	9	1
Cellular transport	10	1
Energy	1	0
Protein synthesis	1	0
Protein fate	5	0
Development	2	0
Signal transduction	1	1
Unclassified proteins	51	29

in seeds of three independent RNAi lines compared to wild-type seeds and seeds transformed with a vector containing RNAi construction targeted against the proximal region of the MtENOD40-1 promoter, which serves as control for RNAi-induced silencing. Comparable observations were made on a number of other genes involved in secondary metabolism (*IFR*, *IFS* and *PAL*) at two harvests before and after AR (Figure S2). Using a PR10 family-specific antibody (Ramos and Bisseling, 2003), it was verified that differences in *PR10* gene expression resulted in differences at the protein level (Figure 3g). For three independent biological replicates, Western blots confirmed that the PR10 protein level was higher in control seeds than in mutant seeds.

Classification of the 55 down-regulated genes in the F dataset revealed that 26 genes are related to abiotic stress, encoding eight heat-shock proteins, five late embryogenesis abundant (LEA) proteins and four detoxification enzymes (Table 1 and Appendix S2). It is possible that the absence of SNF4b provokes a stress response, which is also evident from the up-regulation of a heat shock transcription factor in the mutant seeds. Overall, these data suggest a role for *Mt*SNF4b in activation of constitutive defense responses to biotic stress when seeds are in a dormant state.

Silencing *MtSNF4b* affects the speed of AR of freshly harvested seeds

Given the potential link of *Mt*SNF4b with dormancy, we tested the effect of *MtSNF4b* silencing on germination speed in relation to AR. Seeds of various lines grown under similar conditions were imbibed in the dark at 20°C for various times (Figure 5a–d). For wild-type seeds, freshly harvested seeds germinated only partially (>20%) over 3 weeks of imbibition, whereas seed lots that were after-ripened for 2–3 weeks germinated within 8–16 days. Compared to wild-type seeds, RNAi *Mtsnf4b* seeds germinated approximately twice as fast after 2–3 weeks of AR, but were equally dormant when imbibed straight after harvest. This difference in germination speed after 2–3 weeks of AR was observed for several

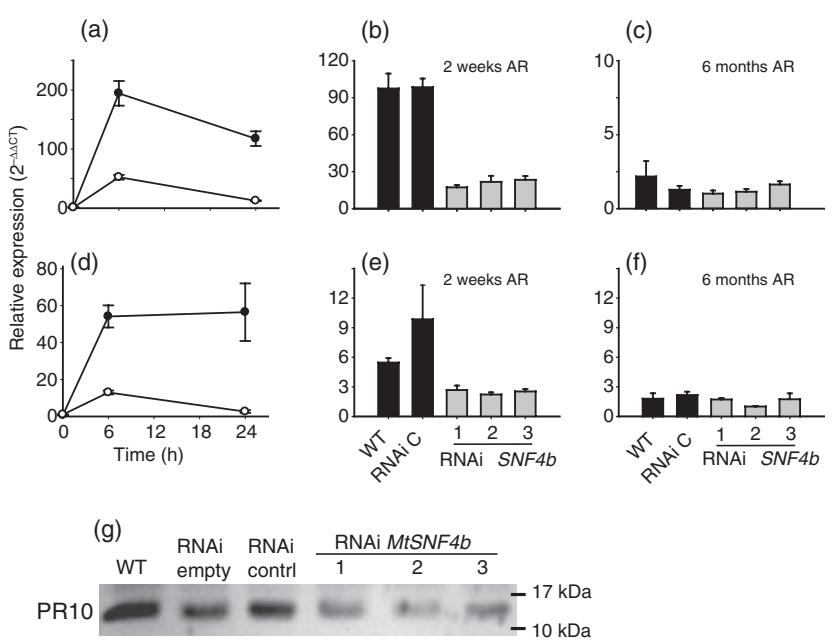

Figure 4. Effect of AR on the relative expression of *CHR* (TC100400) and *PR10* (TC109466) in relation to *MtSNF4b*.
(a,d) Quantitative RT-PCR of *CHR* (a) and *PR10* (d) during imbibition of WT embryos after 2 weeks (closed symbols) or 6 months (open symbols) of AR. Values are the means of three replicates ± SE.
(b,c,e,f) Quantitative RT-PCR of *CHR* (b,c) and *PR10* (e,f) in 6 h-imbibed embryos of WT and RNAi *Mtsnf4b* lines at 2 weeks (b,e) or 6 months (c,f) of AR. Values are the means of three replicates ± SE.
(g) Western blot analysis of 6 h-imbibed embryos of WT and RNAi *Mtsnf4b* lines using a PR10 antibody.
WT, wild-type; RNAi empty, seeds transformed with the empty plasmid; RNAi C/contrl, the same plasmid containing an RNAi construct targeted against the proximal region (1.2 kb) of the *MtENOD40-1* promoter. *CHR*, chalcone reductase.

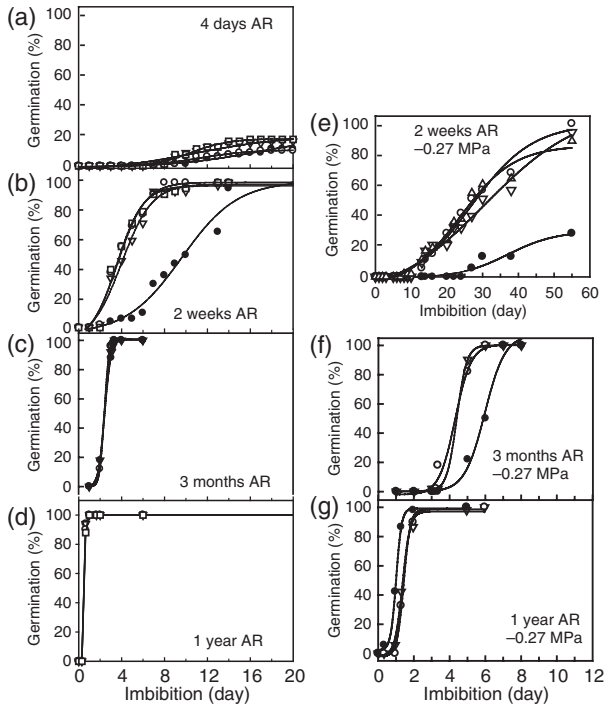

Figure 5. Effect of *MtSNF4b* silencing on dormancy.
Germination curves of wild-type (solid symbols) and RNAi *Mtsnf4b* seeds (open symbols) imbibed at 20°C in the dark in water (a–d) or in polyethylene glycol at −0.27 MPa (e–g) for indicated durations of after-ripening (AR). (a) 4 days of AR, (b,e) 2–3 weeks of AR, (c,f) 3 months of AR, (d,g) 1 year of AR. Data are significantly different when they differ by 18% or more (χ^2 test, $P < 0.05$).

independent harvests in a reproducible manner (data not shown). AR durations of 3 months and 1 year led to a comparable germination speed between mutant and control lines (Figure 5c,d).

Progress towards completion of germination is a function of the mean base water potential [ψ_b(g)] during imbibition (Finch-Savage and Leubner-Metzger, 2006; Bradford and Nonogaki, 2007). To assess how sub-optimal water conditions influence germination time during the course of AR, wild-type and mutant seeds after various durations of AR were imbibed at −0.27 MPa using a polyethylene glycol solution (Figure 5e–g). Imbibition of seeds after 2–3 weeks of AR under these conditions exacerbated the differences in germination behavior between wild-type and mutant seed batches (Figure 5e). This difference was still observed but to a lesser extent after 3 months of AR, and had completely disappeared after 1 year of AR (Figure 5f,g). These data suggest that silencing *MtSNF4b* might be related to a change in ψ_b(g). While weakening of enclosing tissues is a prerequisite for germination, embryo growth potential is a second factor that determines whether a seed germinates or not (Finch-Savage and Leubner-Metzger, 2006; Bradford and Nonogaki, 2007). No difference in the penetration of tetrazolium was detected between mutant and wild-type seeds (data not shown), discarding the hypothesis that cell-wall permeability is at the origin of the reduced dormancy phenotype, as found for Arabidopsis *testa* mutants (Debeaujon *et al.*, 2000). Collectively, these data indicate that the phenotype observed as a result of silencing *MtSNF4b* is under the influence of a time-dependent component controlled by dry storage and thus AR.

Action of SNF4b occurs downstream of ABA

Given the difference in dormancy and speed of AR induced by silencing *MtSNF4b*, we investigated the possible link between SNF4b and ABA signaling pathways. ABA concentrations above 10 μM induced rapid accumulation of the SNF4b protein in immature seeds (Figure 6a). ABA contents (Figure 6c,d) were assessed in seeds obtained from harvests at 1 week and 8 months of AR, respectively, with the corresponding germination behavior shown in Figure 6(b). No significant difference in ABA content after 1 week of AR was detected between wild-type or RNAi seeds, regardless of whether they were dry or imbibed for 24 h (Figure 6c), although a twofold difference in germination speed was observed. In addition, in the batch of seeds obtained after 8 months of AR, which almost completely relieved dormancy, no difference between wild-type and mutant ABA content was observed either. Imbibition of these seeds resulted in a slight but significant decrease in ABA for the wild-type and one of the RNAi lines (Figure 6d), but no large degradation of ABA was detected as found for non-dormant seeds of *N. plumbaginifolia* (Grappin *et al.*, 2000). ABA sensitivity was studied by evaluating the final percentage germination of seed lots after 8 months of AR as a function of ABA concentration. A comparable sensitivity to ABA is observed for two independent seed lots of RNAi *Mtsnf4b* lines (Figure 4e). Fluridone was found to efficiently break dormancy of both wild-type and mutant *M. truncatula* seeds, decreasing the time for 50% germination (T_{50}) from 152 to 20 h for wild-type seeds, and from 80–102 to 20–23 h for two RNAi *Mtsnf4b* lines (Table S1). Incubation in paclobutrazol (100 μM), a gibberellic acid (GA) inhibitor, doubled the germination speed in both wild-type and mutant lines. In contrast, GA_3 up to concentrations of 100 μM had little effect on germination speed and did not change the germination behavior between mutant or wild-type seeds (Figure S4). Thus, ABA is capable of inducing gene and protein expression of *Mt*SNF4b, but signaling pathways downstream of *Mt*SNF4b are not implicated in changing the content or sensitivity of ABA during imbibition of *M. truncatula* seeds.

Role of MtSNF4b in sugar signaling

We next investigated a possible role of *Mt*SNF4b in sugar sensing pathways. Mannose and 2-deoxyglucose, glucose analogs that are phosphorylated by hexokinase but not further metabolized, cause repression of photosynthetic gene

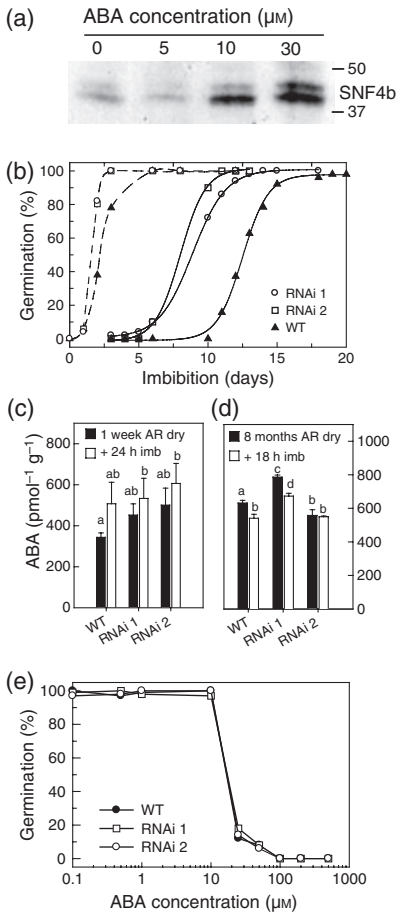

Figure 6. Relationship between ABA and SNF4b.
(a) Western blot analysis using an anti-SNF4b antibody of 16-day-old immature embryos imbibed for 6 h in the indicated concentrations of ABA.
(b) Germination curves for seeds of WT (solid symbols) and two RNAi *Mtsnf4b* lines (open symbols) after-ripened for 1 week (dashed lines) or 8 months (solid lines) that were used for ABA determination. Data are significantly different when they differ by 22% or more (χ^2 test, $P < 0.05$).
(c,d) ABA concentration in dry seeds and seeds imbibed for the indicated times after 1 week (c) or 8 months of AR (d). Values are means ± SE of three replicates. Different letters represent significant differences after multiple comparison of the means using the Student–Newman–Keuls test ($P < 0.05$).
(e) The effect of ABA on the final percentage germination of seeds of WT (solid symbols) and two RNAi *Mtsnf4b* lines (open symbols) after 8 months of AR.
AR, after-ripening; WT, wild-type.

expression at low physiological levels, and inhibit germination in Arabidopsis (Pego *et al.*, 1999). Glucose produced via a glycolysis-dependent pathway is capable of inducing the expression of *PR1* and *PR5*, which belong to the family of pathogenesis-related proteins (Xiao *et al.*, 2000; Jossier *et al.*, 2009). 3-*O*-methylglucose (3-OMG), another glucose analog, is taken up by the plant with the same efficiency as glucose, but, unlike glucose, mannose or 2-deoxyglucose, cannot be phosphorylated by hexokinase. This analog can be used to activate a hexokinase-independent signaling pathway via sugar uptake that activates expression of genes such as *PAL* (Rolland *et al.*, 2006). As several *PAL* genes were differentially expressed between mutant and wild-type embryos (Appendix S1), we first determined the effect of 3-OMG on germination and gene expression in wild-type seeds. Surprisingly, 3-OMG was capable of further delaying the germination of dormant, 2-week AR seeds, but had no further effect on germination of non-dormant seeds (Figure S5). Seeds with an intermediate dormancy level showed an intermediate delay of germination by 3-OMG. Similarly, 3-OMG repressed the expression of *PAL*, *CHR* and *IFR* in dormant seeds, but increased their expression in non-dormant seeds (Figures 7a and S6a,g). The delay of germination in 2-week AR seeds of RNAi *Mtsnf4b* lines by 3-OMG was comparable to that of wild-type seeds (Figure S7). 3-OMG has no significant effect on gene expression (Figures 7b and S6b,h), comparable to that of 3-OMG on gene expression in wild-type seeds at intermediate dormancy level.

In dormant and non-dormant wild-type seeds, mannose did not affect germination or gene expression (Figures 7e, S5 and S6e,k). However, when *MtSNF4b* was silenced, mannose delayed germination and increased gene expression in 2-week AR seeds. Likewise, glucose increased gene expression in RNAi *Mtsnf4b* seeds but had no effect in wild-type seeds (Figures 7c and S6c,i). The effect of glucose on germination was dependent on the dormancy level, resulting in an increase in the speed and homogeneity of germination in both dormant wild-type and RNAi seeds at 2 weeks of AR (Figures S5 and S7). Glucose had no effect on germination in non-dormant wild-type seeds, as is the case for Arabidopsis (Pego *et al.*, 1999). The fact that these observations did not result from an osmotic effect brought about by the sugars was verified by using a polyethylene glycol solution with a comparable osmotic water potential (Figure S8).

DISCUSSION

This analysis shows that *Mt*SNF4b, a functional subunit of the SnRK1 complex in *M. truncatula*, is involved in seed dormancy-related events. Silencing *MtSNF4b* affects the expression of biotic stress-related genes that are expressed in seeds during imbibition only when they are dormant. In addition, silencing of *MtSNF4b* also resulted in faster AR of freshly harvested seeds. We previously demonstrated a role for *Mt*SNF4b during maturation (Rosnoblet *et al.*, 2007), and the present study indicates an additional role for *Mt*SNF4b during the germination phase of seeds. At the final stages of maturation, concomitant with the formation of a SnRK1/SNF4b complex, oligosaccharide accumulation was affected in RNAi *Mtsnf4b* seeds, in addition to the longevity of mature seeds (Rosnoblet *et al.*, 2007). The effect of *MtSNF4b* on AR may have an indirect origin via modification of cell walls through activation of biotic defenses. In *Mt*SNF4b-deficient embryos, transcriptomic analysis revealed an up-regulation of genes involved in cell-wall metabolism and rigidity, such as enzymes involved in the breakdown of galactomannans (α-galactosidase and β-mannosidase), as

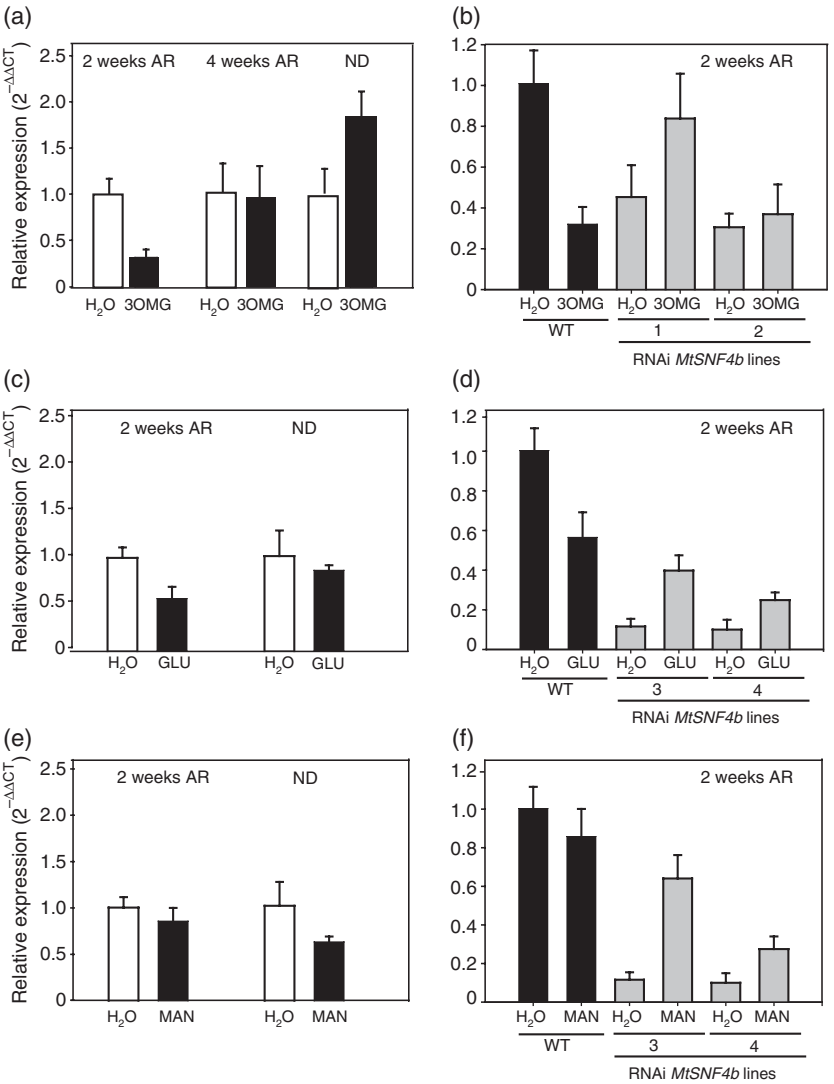

Figure 7. Effect of hexose and sugar analogs on *PAL* (TC106609) expression in relation to AR and *MtSNF4b*.
(a,c,e) Quantitative RT-PCR of *PAL* in WT embryos imbibed for 20 h at 20°C in the dark in 50 mM 3-*O*-methylglucose (3OMG, a), 60 mM glucose (GLU, c) or 20 mM mannose (MAN, e) after 2 weeks, 4 weeks and 1 year of AR (ND).
(b,d,f) Quantitative RT-PCR of *PAL* in embryos of WT and two RNAi *Mtsnf4b* lines imbibed for 20 h at 20°C in the dark in 50 mM 3-OMG (b), 60 mM GLU (d) and 20 mM MAN (f) after 2 weeks of AR.
Values are the means of three replicates ± SE. PAL, phenylalanine ammonia lyase; AR, after-ripening; ND, non-dormant.

well as down-regulation of a polygalacturonase inhibitor (Table 1 and Appendix S1). However, several studies have suggested a role for SNF4b in dormancy or AR (Bradford *et al.*, 2003; Argyris *et al.*, 2008; Carrera *et al.*, 2008). A homolog of *MtSNF4b* in tomato, *LeSNF4*, was previously identified as being highly expressed in non-germinating tissues, and was suggested to indicate a high effective ABA/GA ratio in seeds (Argyris *et al.*, 2008). Indeed, genes associated with the synthesis and deactivation of GA and ABA are reciprocally regulated in association with the imposition and release of dormancy (Cadman *et al.*, 2006). Although we confirmed that the regulatory subunit SNF4b is ABA-inducible, our study failed to demonstrate a direct downstream link with ABA, either by pharmacological approaches or transcriptomic analyses (Figure 6 and Appendix S1). Recently, a study was performed with the aim of separating the action of ABA in seed dormancy from AR-regulated gene expression using *Arabidopsis thaliana* mutants defective in ABA synthesis or perception (Carrera *et al.*, 2008). Interestingly, the homolog of *MtSNF4b* in Arabidopsis, At1g15330, was identified as one of the 103 genes that were down-regulated by AR independently of ABA signaling (Carrera *et al.*, 2008). These data support the suggestion that SNF4b plays a role in AR rather than dormancy, and confirm the ABA-independent pathway downstream of *Mt*SNF4b.

Given the suggested link between SnRK1 and sugar signaling pathways (Tiessen *et al.*, 2003; Jossier *et al.*, 2009), it is possible that the altered soluble sugar composition in RNAi seeds observed by Rosnoblet *et al.* (2007) is related to the phenotypes observed in this study. We first verified whether this difference in sugar content had an effect on osmotic potential, which might explain the germination phenotype. However, when expressed on a molar basis, no difference exists in sugar content between mutant and wild-type seeds, indicating that the final difference in osmotic pressure due to differences in sugar composition is negligible. Our pharmacological studies and quantitative RT-PCR experiments indicated a complex relationship between sugar

perception, signaling and dormancy. Whereas glucose and mannose delay seed germination of Arabidopsis (Pego *et al.*, 1999), this is not the case for *M. truncatula*, raising the question of whether differences in mechanisms of sugar sensing exist between species. After-ripening modified the perception of 3-OMG in *M. truncatula* seeds. In non-dormant seeds, 3-OMG induced expression of *PAL*, *CHR* and *IFR*, whereas expression of these genes was repressed by 3-OMG in dormant seeds. It remains to be investigated whether this effect implies sugar sensing via a hexokinase-independent pathway or perturbed metabolism due to a putative accumulation of 3-*O*-methylglucose-6-phosphate (Cortès *et al.*, 2003), which may have a different effect on dormant versus non-dormant seeds. The effect of 3-OMG on gene expression appears to be independent of *Mt*SNF4b. The difference between wild-type and RNAi seeds is most likely due to a lower dormancy level in the 2-week AR RNAi seeds, which is comparable to that of wild-type seeds after 4 weeks of AR.

Our results rule out the possibility that a hexose signaling pathway acts upstream of *Mt*SNF4b, because this implies that these sugars would activate sugar signaling pathways in wild-type seeds but not in RNAi *Mtsnf4b* seeds. However, glucose and mannose appear to act downstream of *Mt*SNF4b in activating gene expression. Both sugars are capable of restoring *PAL*, *CHR* and *IFR* expression in *Mtsnf4b* mutants to levels comparable to those of wild-type seeds after 2 weeks of AR. Also, mannose delays germination of RNAi seeds but not of wild-type seeds. These results suggest that activation of a hexokinase-mediated signaling pathway is capable of restoring the phenotype of the mutants and coupling germination behavior with defense gene expression. However, glucose does not delay germination, leaving the possibility open that AR/germination and gene expression are controlled by two independent signaling pathways that are both influenced by *Mt*SNF4b. An alternative explanation is that mannose de-regulates carbon and energy metabolism more profoundly when *MtSNF4b* is silenced.

The effect of *Mt*SNF4b on biotic stress-related gene expression during hydration of dormant seeds appears to be uncoupled from the maturation phenotype. We showed that expression of these genes is activated during imbibition of freshly harvested dry wild-type seeds, and not the result of accumulation of transcripts during maturation. In SNF4b-deficient seeds, this activation is largely impeded. The genes that are differentially expressed as a result of silencing *MtSNF4b* are classified in the groups 'disease, virulence and defense', 'cell wall' and 'secondary metabolism'. *MtSNF4b* silencing-induced reduction in *PR10* transcripts was also found at the translational level. However, the role for PR10 proteins in biotic stress is circumstantial. *PR10* genes are known to be induced by pathogens as well as abiotic stresses (McGee *et al.*, 2001; Hashimoto *et al.*, 2004). Silencing of *PR10* resulted in an increased resistance to *Aphanomyces euteiches*, probably due to increased expression of PR-5b proteins (Colditz *et al.*, 2007). On the other hand, overexpression of pea *ABR17* in Arabidopsis rendered the seeds and seedlings more tolerant to abiotic stresses (Srivastava *et al.*, 2006). The functionality of the largest group of genes, which are related to phenylpropanoid metabolism (*PAL*, *C4H* and *CCoAOMT*) and flavonoid and isoflavonoid biosynthesis genes (*CHS*, *CHR*, *CHI*, *IFS*, *HID*, *IFR* and *VR*), remains to be tested in *M. truncatula* seeds. Most of these genes were found to be induced by pathogens or elicitors in *M. truncatula* and correlate with the accumulation of medicarpin (Deavours and Dixon, 2005; Dhaubhadel *et al.*, 2007; Naoumkina *et al.*, 2007). Medicarpin is an anti-fungal pterocarpan phytoalexin that is produced by many legumes (Mundodi *et al.*, 2001). Furthermore, medicarpin has been found to delay alfalfa seed germination (Miller *et al.*, 1988; Dornbos *et al.*, 1990). It may be that this phytoalexin is present in seeds primarily to avoid predation or microbial infections, but also has an effect on the regulation of germination in legume seeds.

What might be the downstream targets of the SnRK1/SNF4b complex that are responsible for the differential gene expression in relation to biotic stress? Direct targets are most likely (de)activated through phosphorylation by the catalytic α subunit and will not be identified by the transcriptome analysis. Nonetheless, expression of several *WRKY* genes in the F dataset points suggests possible downstream regulation by *Mt*SNF4b. A recent study on a kinase–protein interaction in rice identified SnRK1 as a potential phosphorylation candidate of WRKY (Ding *et al.*, 2009). WRKY proteins are involved in the regulation of plant defense responses (Eulgem and Somssich, 2007). A massive induction of *WRKY* transcription factor genes is correlated with the induction of genes involved in the central phenylpropanoid pathway and the downstream steps in the biosynthesis of medicarpin (Naoumkina *et al.*, 2007). Over-expression of the same *WRKY* genes as those found in our transcriptomic analysis (W100630 and W108715) in transgenic tobacco enhances typical defense responses such as PR protein production and accumulation of soluble and wall-bound phenolic compounds (Naoumkina *et al.*, 2008). In addition, they were shown to be induced by either yeast elicitor or methyl jasmonate (Naoumkina *et al.*, 2008). Our transcriptomic analysis also revealed a link to jasmonic acid (JA). Three 12-OPDA reductase genes are down-regulated by silencing *Mt*SNF4b, irrespective of the AR status of the seeds. These genes are responsible for the reduction of 12-OPDA to form OPC-8:0, which is subjected to β-oxidation to yield JA. JA and OPDA are both active as signaling molecules and induce the expression of overlapping but distinct sets of genes involved in biotic stresses (Taki *et al.*, 2005).

Although a number of genes regulated by *Mt*SNF4b are also found in the D dataset, additional genes related to defense are also differentially regulated when *MtSNF4b* is

silenced, suggesting that this subset of genes is directly related to the action of SNF4b and not simply to the reduced dormancy status of the mutant embryos. Imbibed dormant *M. truncatula* seeds have seed coats that remain devoid of any contaminating fungi and bacteria for months, whereas isolated seed coats are readily infected (data not shown). Expression studies in dormant Arabidopsis seeds demonstrated that genes related to defense and protection are also highly expressed (Cadman *et al.*, 2006). It remains to be tested whether the activation of defense gene expression by the SNF4b/SnRK1/β complex in seeds renders them more resistant to pathogen attack. An interesting parallel can be made with the developmentally induced dormancy in the drought-tolerant plant *Retama raetam*, a desert legume (Pnueli *et al.*, 2002). Dormancy in this plant is accompanied by the accumulation of transcripts encoding PR10-like proteins and WRKY transcription factors, whereas non-dormant plants subjected to stress conditions contained transcripts encoding small heat shock proteins.

EXPERIMENTAL PROCEDURES

Detailed experimental procedures used in this study are supplied in Appendix S3.

Plant material and treatments

Seeds of *Medicago truncatula* Gaertn. (R108) were harvested at the same time for all genotypes from plants that were grown side by side under identical environmental conditions as described by Rosnoblet *et al.* (2007). After-ripening occurred during storage at 60% RH, 20°C in the dark. After various durations of AR, batches of 50–100 seeds were scarified using sand paper and imbibed in water or in a polyethylene glycol solution (−0.27 MPa) at 20°C in the dark, and were considered to have germinated when the radicle protruded from the surrounding envelope. Statistical analysis of the germination data was performed using the χ^2 test, and differences were considered significant at $P < 0.05$. To release dormancy, seeds were either imbibed for 48 h at 4°C and subsequently transferred to 20°C, or imbibed in 100 μM fluridone [1-methyl-3-phenyl-5-(3-trifluoromethyl-(phenyl))-4-(1H)-pyridinone] (Duchefa, http://www.duchefa.com). ABA sensitivity was tested on seeds after 8 months of AR that were imbibed at various concentrations of ABA (Sigma, http://www.sigmaaldrich.com/) (control in 0.1% EtOH) in the dark at 20°C, and the final percentage germination was determined. For the microarray analysis, seeds at 2–3 weeks and 6 months of AR were imbibed in the dark for 6 h. For pharmacological studies, seeds were imbibed at 20°C in the dark in water or 50 mM 3-*O*-methylglucose (Sigma), 60 mM glucose (Sigma), 20 mM mannose (Sigma) or 40 mM glucosamine (Sigma) at various durations of AR. For quantitative RT-PCR studies, 20 h-imbibed embryos were removed from the seed coat and endosperm and frozen in liquid nitrogen.

Plasmid construction and plant transformation

SNF4b RNAi plasmid construction using the binary vector pFGC5941/Gateway (pFRB), transformation of the plasmid into *M. truncatula* and selection of positive lines were performed as described by Rosnoblet *et al.* (2007). Four homozygous lines (T_2) were retained for further analysis. Two additional transformants were used as negative controls: an R108 line transformed with the empty vector (RNAi empty) and an R108 line transformed with the pFGC5941 vector containing an RNAi construct targeted against the proximal region (1.2 kb) of the *MtENOD40-1* promoter (RNAi control). All studies were performed on seeds of the T_2 or T_3 generation.

Gel filtration chromatography and Western blot

Seeds at 1–2 weeks of AR were imbibed for 48 h at 4 or 20°C in the dark, after which native proteins were extracted and separated according to their molecular weight as described by Rosnoblet *et al.* (2007). Fractions were analyzed by Western blotting using an anti-SNF4b antibody as described by Rosnoblet *et al.* (2007). To study the effect of ABA, immature embryos at 16 days after pollination were incubated for 6 h in various concentrations of ABA. Soluble proteins were extracted and analyzed by Western blotting (10 μg/lane) using the anti-SNF4b antibody. For PR10 protein induction, soluble proteins were extracted from 36 h imbibed embryos after 2 weeks of AR and analyzed by Western blotting using an antibody against pea PsRH2 that recognizes MtPR10-1 (Ramos and Bisseling, 2003).

Complementation assay

The cDNA of *MtSNF4b* was cloned into pENTR/D-TOPO and recombined with pYES-DEST52 (Invitrogen, http://www.invitrogen.com/). The plasmid pYES-DEST52-MtSNF4b and the empty plasmid were introduced into the Δ*snf4* mutant of *Saccharomyces cerevisiae* MCY2634 (MATa his3-Δ200 leu2-3112 ura3-52 snf4Δ2). Strains were plated on yeast nitrogen base (YNB)/2% glucose or YNB/2% galactose.

Bacterial two-hybrid analysis

MtSNF4b was cloned into the pBT vector fused with a lamba-cl DNA binding site and *MtSNF1* (TC116507) was cloned into the pTRG vector fused with RNA polymerase-α. Reporter competent cells were co-transformed with both vectors using bacterioMatch II (Stratagene, http://www.stratagene.com/) according to the manufacturer's protocol, and spotted on selective (3-amino-1,2,4-triazole and streptomycin) and non-selective media by 20 μl of saturated culture (1/1, 1/10, 1/100, 1/1000) and incubated at 37°C for 1 and 2 days, respectively. Negative controls were bacteria co-transformed with pTRGGal11 and pBTMtSNF4b or pTRGMtSNF1 and pBT vectors.

ABA measurements

ABA content was measured in dry and imbibed seeds at 1 week and 8 months of AR, using two harvests. Imbibition times were 24 h (1 week AR) or 18 h (8 months AR). Extraction was performed on three biological replicates of 25 seeds as described by Jiang and Zhang (2001). ABA concentration was determined using the Phytodetek ABA immunoassay kit according to the manufacturer's instructions (Idetek).

Isolation of total RNA and mRNA purification

Total RNA was isolated as described by Verwoerd *et al.* (1989). Purification of mRNA was performed using a PolyATract mRNA isolation system III kit according to the manufacturer's protocol (Promega, http://www.promega.com/). The quality of mRNA was assessed using an Agilent 2100 bioanalyzer (Agilent, http://www.agilent.com).

Microarray analysis

Three microarray experiments were conducted to compare the transcriptome in embryos of (i) wild-type versus two RNAi lines at

2–3 weeks of AR, (ii) wild-type versus one RNAi line at 6 months of AR, and (iii) wild-type embryos at 2 weeks versus 6 months of AR (dormant versus nondormant). Microarray analysis replicates consisted of three independent biological replicates of 50 embryos, obtained from different plants. Probe labeling using the CyScribe cDNA post-labeling kit (GE Healthcare, http://www.gelifesciences.com), hybridization and image acquisition were performed as described by Buitink *et al.* (2006). Dye-specific variations were accounted for using a dye swap between the various wild-type RNA samples. Hybridization was performed using Mt16kOLI1Plus chips (Hohnjec *et al.*, 2005). Image data processing was performed using GENESPRING software (Agilent). Genes with a significant difference (P value <0.01) and >1.5 fold change in expression were considered.

Real-time quantitative PCR analysis

Reverse transcription reactions were performed on 1 μg of total RNA using a QuantiTect reverse transcription kit (Qiagen, http://www.qiagen.com/). Quantification of cDNA by quantitative RT-PCR was performed using an ABI PRISM 7100 sequence detection system (Applied Biosystems, http://www.appliedbiosystems.com/) and SYBR Green. Values are based on three repetitions. The constitutively expressed mRNA *Msc27* was used as a housekeeping gene for standardizing data and to assess the efficiency of quantitative RT-PCR (Gallardo *et al.*, 2007). Amplification primers are listed in Table S2. Changes in transcript abundance were estimated as the fold change relative to control conditions (Livak and Schmittgen, 2001).

ACKNOWLEDGEMENTS

We thank Dr Marten Denekamp (Syngenta Seeds, Enkhuizen, the Netherlands) for providing the pFRB RNAi binary vector and Dr P. Ratet (Institut des Sciences Vegetales, Gif-sur-Yvette, France) for the gift of seeds of *Medicago truncatula* cv. R108. We are indebted to Dr H. Franssen (Wageningen University, the Netherlands) for providing the PsRH2 antibody to perform the PR10 analysis. Lysianne Brocard and Martin Crespi (Institut des Sciences Végétales, CNRS, Gif sur Yvette, France) are gratefully acknowledged for the gift of mutants containing an RNAi construct targeted against the proximal region (1.2 kb) of the *MtENOD40-1* promoter. The yeast *Δsnf4* strain MCY2634 was kindly provided by Dr Maria Carlson (Columbia University, NY, USA).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Western blot analyses of four independent RNAi *Mtsnf4b* lines and three controls for the expression of SNF4b protein.

Figure S2. Confirmation of microarray data by expression analysis using quantitative RT-PCR of phenylalanine lyase, chalcone synthase, isoflavone synthetase and isoflavone reductase.

Figure S3. Changes in differential expression of genes encoding enzymes of the medicarpin pathway that were identified in the F dataset.

Figure S4. Effect of gibberellic acid and paclobutrazol on germination of wild-type and RNAi *Mtsnf4b* seeds after 2–3 weeks of AR.

Figure S5. Effect of hexoses and sugar analogs on the germination of wild-type seeds after indicated durations of after-ripening.

Figure S6. Effect of hexose and sugar analogs on *IFR* (a–f) and *CHR* (g–i) expression in relation to after-ripening in wild-type and *MtSNF4b* RNAi seeds.

Figure S7. The effect of hexoses and sugar analogs on the germination of seeds after 2 weeks of after-ripening in relation to *MtSNF4b* silencing.

Figure S8. The effect of a low water potential (−0.13 MPa) during imbibition on the expression of phenylalanine lyase, chalcone reductase and isoflavone reductase in dormant and non-dormant embryos.

Table S1. Effect of fluridone on germination of wild-type and RNAi *Mtsnf4b* seeds at 2–3 weeks of after-ripening.

Table S2. Primer sequences used for quantitative RT-PCR experiments.

Appendix S1. Sets of genes that showed enhanced or decreased expression in the three dataset analysed using 16K MtOLI1plus microarrays.

Appendix S2. Classification of genes that are differentially expressed in the F dataset.

Appendix S3. Additional details of experimental procedures.

Please note: As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

REFERENCES

Ananieva, E.A., Gillaspy, G.E., Ely, A., Burnette, R.N. and Erickson, F.L. (2008) Interaction of the WD40 domain of a myoinositol polyphosphate 5-phosphatase with SnRK1 links inositol, sugar, and stress signaling. *Plant Physiol.* **148**, 1868–1882.

Argyris, J., Dahal, P., Hayashi, E., Still, D.W. and Bradford, K.J. (2008) Genetic variation for lettuce seed thermoinhibition is associated with temperature-sensitive expression of abscisic acid, gibberellin, and ethylene biosynthesis, metabolism, and response genes. *Plant Physiol.* **148**, 926–947.

Baena-Gonzalez, E. and Sheen, J. (2008) Convergent energy and stress signaling. *Trends Plant Sci.* **13**, 474–482.

Bouly, J.P., Gissot, L., Lessard, P., Kreis, M. and Thomas, M. (1999) *Arabidopsis thaliana* proteins related to the yeast SIP and SNF4 interact with AKINα1, an SNF1-like protein kinase. *Plant J.* **18**, 541–550.

Bradford, K.J. and Nonogaki, H. (2007) *Seed Development, Dormancy and Germination*. Oxford: Blackwell Publishing.

Bradford, K.J., Downie, A.B., Gee, O.H., Alvarado, V., Yang, H. and Dahal, P. (2003) Abscisic acid and gibberellin differentially regulate expression of genes of the SNF1-related kinase complex in tomato seeds. *Plant Physiol.* **132**, 1560–1576.

Buitink, J., Thomas, M., Gissot, L. and Leprince, O. (2004) Starvation, osmotic stress and desiccation tolerance lead to expression of different genes of the regulatory β and γ subunits of the SnRK1 complex in germinating seeds of *Medicago truncatula*. *Plant Cell Environ.* **27**, 55–67.

Buitink, J., Leger, J.J., Guisle, I. *et al.* (2006) Transcriptome profiling uncovers metabolic and regulatory processes occurring during the transition from desiccation-sensitive to desiccation-tolerant stages in *Medicago truncatula* seeds. *Plant J.* **47**, 735–750.

Cadman, C.S., Toorop, P.E., Hilhorst, H.W. and Finch-Savage, W.E. (2006) Gene expression profiles of Arabidopsis Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism. *Plant J.* **46**, 805–822.

Carrera, E., Holman, T., Medhurst, A., Dietrich, D., Footitt, S., Theodoulou, F.L. and Holdsworth, M.J. (2008) Seed after-ripening is a discrete developmental pathway associated with specific gene networks in Arabidopsis. *Plant J.* **53**, 214–224.

Colditz, F., Niehaus, K. and Krajinski, F. (2007) Silencing of PR-10 proteins in Medicago truncatula results in an antagonistic induction of other PR proteins and in an increased tolerance upon infection with the oomycete aphanomyces euteiches. *Planta*, **226**, 57–71.

Cortès, S., Gromova, M., Evrard, A., Roby, C., Heyraud, A., Rolin, D.B., Raymond, P. and Brouquisse, R.M. (2003) In plants, 3-*O*-methylglucose is phosphorylated by hexokinase but not perceived as a sugar. *Plant Physiol.* **131**, 824–837.

Deavours, B.E. and Dixon, R.A. (2005) Metabolic engineering of isoflavonoid biosynthesis in alfalfa. *Plant Physiol.* **138**, 2245–2259.

Debeaujon, I., Leon-Kloosterziel, K.M. and Koornneef, M. (2000) Influence of the testa on seed dormancy, germination and longevity in Arabidopsis. *Plant Physiol.* **122**, 403–414.

Dhaubhadel, S., Gijzen, M., Moy, P. and Farhangkhoee, M. (2007) Transcriptome analysis reveals a critical role of CHS7 and CHS8 genes for isoflavonoid synthesis in soybean seeds. *Plant Physiol.* **143**, 326–338.

Ding, X., Richter, T., Chen, M. *et al.* (2009) A rice kinase–protein interaction map. *Plant Physiol.* **149**, 1478–1492.

Dornbos, D.L. Jr, Spencer, G.F. and Miller, R.W. (1990) Medicarpin delays alfalfa seed germination and seedling growth. *Crop Sci.* **30**, 162–166.

Estruch, F., Treitel, M.A., Yang, X.L. and Carlson, M. (1992) N-terminal mutations modulate yeast Snf1 protein-kinase function. *Genetics*, **132**, 639–650.

Eulgem, T. and Somssich, I.E. (2007) Networks of WRKY transcription factors in defense signaling. *Curr. Opin. Plant Biol.* **10**, 366–371.

Faria, J.M., Buitink, J., van Lammeren, A.A. and Hilhorst, H.W. (2005) Changes in DNA and microtubules during loss and re-establishment of desiccation tolerance in germinating *Medicago truncatula* seeds. *J. Exp. Bot.* **56**, 2119–2130.

Finch-Savage, W.E. and Leubner-Metzger, G. (2006) Seed dormancy and the control of germination. *New Phytol.* **171**, 501–523.

Gallardo, K., Firnhaber, C., Zuber, H., Héricher, D., Belghazi, M., Henry, C., Küster, H. and Thompson, R. (2007) A combined proteome and transcriptome analysis of developing *Medicago truncatula* seeds: evidence for metabolic specialization of maternal and filial tissues. *Mol. Cell Proteomics*, **6**, 2165–2179.

Gosti, F., Beaudoin, N., Serizet, C., Webb, A.A., Vartanian, N. and Giraudat, J. (1999) ABI1 protein phosphatase 2C is a negative regulator of abscisic acid signaling. *Plant Cell*, **11**, 1897–1910.

Grappin, P., Bouinot, D., Sotta, B., Miginiac, E. and Jullien, M. (2000) Control of seed dormancy in *Nicotiana plumbaginifolia*: post-imbibition abscisic acid synthesis imposes dormancy maintenance. *Planta*, **210**, 279–285.

Halford, N.G., Bouly, J.-P. and Thomas, M. (2000) SNF1-related protein kinases (SnRKs) – regulators at the heart of the control of carbon metabolism and partitioning. *Adv. Bot. Res.* **32**, 405–434.

Hashimoto, M., Kisseleva, L., Sawa, S., Furukawa, T., Komatsu, S. and Koshiba, T. (2004) Novel rice PR10 protein, RSOsPR10, specifically induced in roots by biotic and abiotic stresses, possibly via the jasmonic acid signaling pathway. *Plant Cell Physiol.* **45**, 550–559.

Hohnjec, N., Vieweg, M.F., Pühler, A., Becker, A. and Küster, H. (2005) Overlaps in the transcriptional profiles of *Medicago truncatula* roots inoculated with two different *Glomus* fungi provide insights into the genetic program activated during arbuscular mycorrhiza. *Plant Physiol.* **137**, 1283–1301.

Iturriaga, E.A., Leech, M.J., Barratt, D.H. and Wang, T.L. (1994) Two ABA-responsive proteins from pea (*Pisum sativum* L.) are closely related to intracellular pathogenesis-related proteins. *Plant Mol. Biol.* **24**, 235–240.

Jiang, M. and Zhang, J. (2001) Effect of abscisic acid on active oxygen species, antioxidative defence system and oxidative damage in leaves of maize seedlings. *Plant Cell Physiol.* **42**, 1265–1273.

Jossier, M., Bouly., J.P., Meimoun, P., Arjmand, A., Lessard, P., Hawley, S., Hardie, D.G. and Thomas, M. (2009) SnRK1 (SNF1-related kinase 1) has a central role in sugar and ABA signalling in *Arabidopsis thaliana*. *Plant J.* **59**, 316–328.

Kleinow, T., Bhalerao, R., Breuer, F., Umeda, M., Salchert, K. and Koncz, C. (2000) Functional identification of an Arabidopsis Snf4 ortholog by screening for heterologous multicopy suppressors of *snf4* deficiency in yeast. *Plant J.* **23**, 115–122.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, **25**, 402–408.

McGee, J.D., Hamer, J.E. and Hodges, T.K. (2001) Characterization of a PR-10 pathogenesis-related gene family induced in rice during infection with *Magnaporthe grisea*. *Mol. Plant–Microbe Interact.* **14**, 877–886.

Miller, R.W., Kleiman, R., Powell, R.G. and Putnam, A.R. (1988) Germination and growth inhibitors of alfalfa. *J. Nat. Prod.* **51**, 328–330.

Mundodi, S.R., Watson, B.S., Lopez-Meyer, M. and Paiva, N.L. (2001) Functional expression and subcellular localization of the *Nectria haematococca* Mak1 phytoalexin detoxification enzyme in transgenic tobacco. *Plant Mol. Biol.* **46**, 421–432.

Naoumkina, M., Farag, M.A., Sumner, L.W., Tang, Y., Liu, C.J. and Dixon, R.A. (2007) Different mechanisms for phytoalexin induction by pathogen and wound signals in *Medicago truncatula*. *Proc. Natl Acad. Sci. USA*, **104**, 17909–17915.

Naoumkina, M.A., He, X. and Dixon, R.A. (2008) Elicitor-induced transcription factors for metabolic reprogramming of secondary metabolism in *Medicago truncatula*. *BMC Plant Biol.* **8**, 132.

Pego, J.V., Weisbeek, P.J. and Smeekens, S.C.M. (1999) Mannose inhibits Arabidopsis germination via a hexokinase-mediated step. *Plant Physiol.* **119**, 1017–1023.

Pnueli, L., Hallak-Herr, E., Rozenberg, M., Cohen, M., Goloubinoff, P., Kaplan, A. and Mittler, R. (2002) Molecular and biochemical mechanisms associated with dormancy and drought tolerance in the desert legume *Retama raetam*. *Plant J.* **31**, 319–330.

Radchuk, R., Radchuk, V., Weschke, W., Borisjuk, L. and Weber, H. (2006) Repressing the expression of the SUCROSE NONFERMENTING-1-RELATED PROTEIN KINASE gene in pea embryo causes pleiotropic defects of maturation similar to an abscisic acid-insensitive phenotype. *Plant Physiol.* **140**, 263–278.

Ramos, J. and Bisseling, T. (2003) A method for the isolation of root hairs from the model legume *Medicago truncatula*. *J. Exp. Bot.* **54**, 2245–2250.

Rolland, F., Baena-Gonzalez, E. and Sheen, J. (2006) Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annu. Rev. Plant. Biol.* **57**, 675–709.

Rosnoblet, C., Aubry, C., Leprince, O., Ly Vu, B., Rogniaux, H. and Buitink, J. (2007) The regulatory γ subunit SNF4b of the sucrose non-fermenting-related kinase complex is involved in longevity and stachyose accumulation during maturation of *Medicago truncatula* seeds. *Plant J.* **51**, 47–59.

Schwachtje, J., Minchin, P.E.H., Jahnke, S., Van Dongen, J.T., Schittko, U. and Baldwin, I.T. (2006) SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. *Proc. Natl Acad. Sci. USA*, **103**, 12935–12940.

Serrano, M. and Guzman, P. (2004) Isolation and gene expression analysis of *Arabidopsis thaliana* mutants with constitutive expression of *ATL2*, an early elicitor-response RING-H2 zinc-finger gene. *Genetics*, **167**, 919–929.

Srivastava, S., Rahman, M.H., Shah, S. and Kav, N.N.V. (2006) Constitutive expression of the pea ABA-responsive 17 (ABR17) cDNA confers multiple stress tolerance in *Arabidopsis thaliana*. *Plant Biotechnol. J.* **4**, 529–549.

Sugden, C., Crawford, R.M., Halford, N.G. and Hardie, D.G. (1999) Regulation of spinach SNF1-related (SnRK1) kinases by protein kinases and phosphatases is associated with phosphorylation of the T loop and is regulated by 5′-AMP. *Plant J.* **19**, 433–439.

Taki, N., Sasaki-Sekimoto, Y., Obayashi, T. *et al.* (2005) 12-oxo-phytodienoic acid triggers expression of a distinct set of genes and plays a role in wound-induced gene expression in Arabidopsis. *Plant Physiol.* **139**, 1268–1283.

Tiessen, A., Prescha, K., Branscheid, A., Palacios, N., McKibbin, R., Halford, N.G. and Geigenberger, P. (2003) Evidence that SNF1-related kinase and hexokinase are involved in separate sugar-signalling pathways modulating post-translational redox activation of ADP-glucose pyrophosphorylase in potato tubers. *Plant J.* **35**, 490–500.

Verwoerd, T.C., Dekker, B.M. and Hoekema, A. (1989) A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Res.* **17**, 2362.

Xiao, W., Sheen, J. and Jang, J.-C. (2000) The role of hexokinase in plant sugar signal transduction and growth and development. *Plant Mol. Biol.* **44**, 451–461.

