

RESEARCH PAPER

# Methionine synthase 1 provides methionine for activation of the GLR3.5 Ca<sup>2+</sup> channel and regulation of germination in Arabidopsis

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## Abstract

Seed germination is a developmental process regulated by numerous internal and external cues. Our previous studies have shown that calcium influx mediated by the Arabidopsis glutamate receptor homolog 3.5 (AtGLR3.5) modulates the expression of the *ABSCISIC ACID INSENSITIVE 4* (*ABI4*) transcription factor during germination and that L-methionine (L-Met) activates AtGLR3.1/3.5 Ca<sup>2+</sup> channels in guard cells. However, it is not known whether L-Met participates in regulation of germination and what cellular mechanism is responsible for Met production during germination. Here, we describe Arabidopsis methionine synthase 1 (AtMS1), which acts in the final step of Met biosynthesis, synthesizes the Met required for the activation of AtGLR3.5 Ca<sup>2+</sup> channels whose expression is up-regulated during germination, leading to the regulation of seed germination. We show that exogenous L-Met promotes germination in an AtGLR3.5-dependent manner. We also demonstrate that L-Met directly regulates the AtGLR3.5-mediated increase in cytosolic Ca<sup>2+</sup> level in seedlings. We provide pharmacological and genetic evidence that Met synthesized via AtMS1 acts upstream of the AtGLR3.5-mediated Ca<sup>2+</sup> signal and regulates the expression of *ABI4*, a major regulator in the abscisic acid response in seeds. Overall, our results link AtMS1, L-Met, the AtGLR3.5 Ca<sup>2+</sup> channel, Ca<sup>2+</sup> signals, and *ABI4*, and shed light on the physiological role and molecular mechanism of L-Met in germination.

**Keywords:** Calcium channels, AtGLR3.5, methionine, methionine synthase 1, seed germination.

## Introduction

Germination is a critical developmental process that transforms non-dormant seeds into a highly active state and plays a major role in the life cycle of the plant (Rajjou *et al.*, 2012). The germination process comprises three phases, namely imbibition by the dry seed, re-initiation of metabolic processes,

and the protrusion of the radicle through seed envelopes (Bewley, 1997; Penfield *et al.*, 2006; Holdsworth *et al.*, 2008; Weitbrecht *et al.*, 2011). The progression of germination is largely controlled by internal signaling cascades, such as those related to phytohormones including abscisic acid (ABA) and

gibberellins (GAs), as well as by environmental cues (Bewley, 1997; Holdsworth *et al.*, 2008; Weitbrecht *et al.*, 2011; Rajjou *et al.*, 2012; Han and Yang, 2015).

Numerous studies have examined the regulatory mechanisms that underlie seed germination (Gallardo *et al.*, 2001; Rajjou *et al.*, 2004; Nakabayashi *et al.*, 2005; Fait *et al.*, 2006; Catusse *et al.*, 2008; Sreenivasulu *et al.*, 2008; North *et al.*, 2010; Bassel *et al.*, 2011; Dekkers *et al.*, 2013; Bellieny-Rabelo *et al.*, 2016; Feenstra *et al.*, 2017) and have shown that it is collectively coordinated by temporally distinct transcriptomic, proteomic, and metabolic switches. Several studies have highlighted the role of the latter during germination (Fait *et al.*, 2006; Sreenivasulu *et al.*, 2008; Bellieny-Rabelo *et al.*, 2016; Feenstra *et al.*, 2017), with numerous pathways being associated with the turnover of proteins, polysaccharides, and lipids. It has also been reported that the contents of free amino acids are altered during the course of germination (Fait *et al.*, 2006; Dekkers *et al.*, 2013; Galland *et al.*, 2014; Angelovici *et al.*, 2017). The levels of amino acids such as Asp, Thr, and Ser significantly increase during the transition from stratification to germination *sensu stricto* in Arabidopsis seeds, although the amounts of Gly, Gln, Ile, and the non-proteogenic *r*-aminobutyrate are reduced (Fait *et al.*, 2006). Genes related to the biosynthesis of several amino acids are up-regulated during imbibition (Fait *et al.*, 2006). In addition to being the building blocks of proteins and the precursors of plant hormones and secondary metabolites (Amir, 2010), amino acids act as signaling molecules in diverse biological processes, including pollen-tube growth, plant-microbe interactions, stomatal movements, and defense responses (Michard *et al.*, 2011; Li *et al.*, 2013; Kong *et al.*, 2016; Toyota *et al.*, 2018). However, the physiological functions of amino acids and the molecular regulatory mechanisms that underlie the dynamic changes in their contents during seed germination remain largely unknown.

Our previous study demonstrated that the Arabidopsis glutamate receptor homolog 3.5 (AtGLR3.5, At2g32390) plays a critical role in seed germination through modulation of the cytosolic free Ca<sup>2+</sup> concentration, [Ca<sup>2+</sup>]<sub>cyt</sub>, and antagonization of the inhibitory effects of ABA via suppression of the transcription of *ABSCISIC ACID INSENSITIVE4* (*ABI4*), a key ABA-response regulator (Kong *et al.*, 2015). Consistent with the activation mechanism by ligands in animal glutamate receptors, several studies have demonstrated that amino acids such as L-Met, D-Ser, and Glu can activate plant GLR Ca<sup>2+</sup> channels (Qi *et al.*, 2006; Michard *et al.*, 2011; Vincill *et al.*, 2012; Tapken *et al.*, 2013; Kong *et al.*, 2016; Toyota *et al.*, 2018). For example, L-methionine (L-Met) activates guard cell heteromeric AtGLR3.1/3.5 Ca<sup>2+</sup> channels in the plasma membrane in order to regulate stomatal aperture (Kong *et al.*, 2016). In addition, a proteomic study reported that two house-keeping enzymes involved in methionine (Met) biosynthesis are temporally accumulated during radicle protrusion (Gallardo *et al.*, 2002), suggesting that Met has a role in seed germination. However, it remains unknown whether Met functions in AtGLR3.5-mediated germination control and which cellular mechanism is the source of the amino acid in the process.

In the present study, we demonstrate that L-Met promotes seed germination through up-regulation of the AtGLR3.5-mediated cytosolic Ca<sup>2+</sup> signal in Arabidopsis. We provide

evidence that L-Met positively regulates germination, and that this requires Arabidopsis methionine synthase 1 (AtMS1), activation of AtGLR3.5 Ca<sup>2+</sup> channels by L-Met, and modulation of the expression of *ABI4*.

## Materials and methods

### *Plant material and growth conditions*

The Arabidopsis genetic background Columbia (Col-0) was used as the wild-type. Seeds expressing *35S::Yellow Cameleon3.60* (*YC3.60*) were kindly provided by Dr Magaly Rincón-Zachary and Dr Elison B. Blancaflor (Samuel Roberts Noble Foundation, Oklahoma). The *AtGLR3.5*-RNAi lines and their lines expressing *YC3.60* have been previously described by Kong *et al.* (2015). The T-DNA insertion mutants *Atms1* (CS480822), *Atglr3.5-1* (Salk\_035264), and *Atglr3.5-2* (CS859735) were obtained from the Arabidopsis Biological Resource Center (ABRC; <https://abrc.osu.edu/>). Seeds were stratified at 4 °C for 3 d before sowing in soil and the plants were grown in controlled growth chambers at 22 °C under white fluorescent light (110 μmol m<sup>-2</sup> s<sup>-1</sup>) in long-day conditions (16/8 h light/darkness). Seed germination assays were performed as described previously (Kong *et al.*, 2015), with seeds sown on Murashige and Skoog (MS)-based media containing MS major salts with the indicated amounts of CaCl<sub>2</sub>, MS minor salts, vitamins, 1% (w/v) sucrose, and 0.8% (w/v) agar. Amino acids (L-Met, L-Asp, L-Thr, D-Ser, or L-Glu), LaCl<sub>3</sub> (a Ca<sup>2+</sup>-channel blocker), EGTA (a Ca<sup>2+</sup>-chelating agent), or DL-propargylglycine (PAG, a Met synthesis inhibitor; all Sigma Aldrich) were added to the MS media prior to sowing when required. Seeds harvested the same day were used for each germination experiment. For each sample, 80–100 seeds per treatment were carefully examined for germination, and three biological replicates were conducted.

### *Measurement of Ca<sup>2+</sup> levels in the cytosol*

*YC3.60*-based fluorescence resonance energy transfer (FRET)-sensitized emission imaging analysis was used to measure the level of cytosolic free calcium, [Ca<sup>2+</sup>]<sub>cyt</sub>, in the wild-type and *AtGLR3.5*-RNAi lines before and after L-Met or L-Glu treatment. The experiments were performed using 4-d-old seedlings expressing *YC3.60* placed under a TCS SP5 confocal laser-scanning microscope (Leica Microsystems) as described previously (Rincón-Zachary *et al.*, 2010; Kong *et al.*, 2015). Ten seedlings of each genotype were examined for each treatment.

### *Gene expression analysis*

Total RNA extraction, reverse-transcription, and quantitative PCR were carried out as described previously (Kong *et al.*, 2015). The gene-specific primers used for expression were as follows: *AtMS1*, 5'-CGTCGTGAGTACAAGGCCAAAAAG-3' and 5'-CAGTGAAGG CAAAACACAGACAACACTG-3'; *AtMS2*, 5'-GCCAGAGAAGGTGTT GAAGTTGACG-3' and 5'-GCCTTGCGCTAACTTCAGTTGT ACG-3'; and *AtMS3*, 5'-CCAACGTCCTATGAAGGGGATGC-3', and 5'-GGACGGCCAGTCAAGGTAAAAC-3'. The primer set used for *AtGLR3.5* is described by Kong *et al.* (2015) and the primers used for *ABI4* are as described by Footitt *et al.* (2011). Relative gene expression values were obtained by normalization to the housekeeping gene *ACTIN2*. Three biological repeats were used for each analysis.

### *Generation of Arabidopsis transgenes expressing AtMS1p::GUS*

The *AtMS1p::GUS* construct used for plant transformation was created using the Gateway method (Invitrogen). The 2-kb promoter region of *AtMS1* was PCR-amplified using the primers 5'-CACCGC AAAAGAGCTTATAACGTAGTTTATGC-3' and 5'-TTTGATTT TCTTTTACTGCATTAACAC-3'. The fragment was cloned into the pENTR/D-TOPO cloning vector and then fused to the GUS reporter gene in the binary vector pMDC163 (Karimi *et al.*, 2002). The resulting plasmid that harbored the GUS reporter driven by the *AtMS1*

promoter (*AtMS1p::GUS*) was introduced into the *Agrobacterium* strain GV3101, and then *Arabidopsis* transformation was performed using the floral dip method (Clough and Bent, 1998). Transgenic plants were identified through selection for hygromycin resistance. Homozygous T<sub>3</sub> lines carrying a single insertion were used for the analysis.

#### Histochemical staining with X-Gluc

GUS activity was examined as described by Jefferson et al. (1987). Seeds were incubated at 37 °C overnight in the dark in GUS staining solution containing 5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid (X-Gluc), as used previously by Kong et al. (2015), and were then washed three times in 100% ethanol and once in 70% ethanol (v/v). The seed coats were carefully removed under a SV6 dissection microscope and pictures taken with an Axio Cam ICC digital camera (both Zeiss).

#### Identification of *AtMS1* and *AtGLR3.5* T-DNA insertion lines

Three T-DNA insertion lines (SALK\_205174, CS828436, and CS480822) of the *AtMS1* gene (At5g17920) were obtained from ABRC, and homozygous seedlings were only identified in the CS480822 line. The two sets of primers used for genotyping this line were as follows: Sul2, 5'-GTGCAACCTTCAAAGCTGAAAGT-3'; Sul4, 5'-ATTTCA CACAGGAAACAGCTATGA-3'; 8474, 5'-ATAATAACGCTGCGGA CATCTACATTTT-3'; and TH39, 5'-GAGGCTGCACTTAGAGAA GGAATA-3'. For the primers used for the genotyping of *Atglr3.5-1* (Salk\_035264), the sequences of the left genomic primer (LP) and right genomic primer (RP) were 5'-TGAAGTTGC TGCAAATGTGAG-3' and 5'-TGTCGACATGTCCACAGCTAG-3'. The sequences of the LP and RP primers used for the genotyping of *Atglr3.5-2* (CS859735) were 5'-TTCAAAGCCAACCAATTTG-3' and 5'-CTGAAGATTGTGGACCAATGG-3'. The primer Lbb1.3 used in genotyping of both *Atglr3.5-1* and *Atglr3.5-2* lines was 5'-ATTTTGCCGATTTCCGGAAC-3'.

#### Measurement of amino acid contents in seeds

Seeds that had been stratified for 3-d were incubated at 22 °C for 0 h or 24 h and collected for analysis. The amino acid contents were measured by OE BioTech (Shanghai, China) according to standard procedures for GC-MS. All chemicals and solvents used were of analytical grade. The analyses were carried out on a GC-MS system (Agilent, model 7890B) coupled with a mass-selective detector (Agilent, model 5977A). A DB-5MS fused-silica capillary column (30 m×0.25 mm×0.25 µm; Agilent J & W Scientific) was used to separate the derivatives. The analysis was performed under the following settings: Helium (>99.999%) was used as the carrier gas at a constant flow rate of 1 ml min<sup>-1</sup> through the column; the injector temperature was maintained at 260 °C; the injection volume was 1 µl by splitless mode and the solvent delay time was set to 5 min; the initial oven temperature was 60 °C, ramped to 125 °C at a rate of 15 °C min<sup>-1</sup>, to 210 °C at a rate of 5 °C min<sup>-1</sup>, to 270 °C at a rate of 10 °C min<sup>-1</sup>, to 305 °C at a rate of 20 °C min<sup>-1</sup>, and finally held at 305 °C for 5 min; the temperatures of the MS quadrupole and electron impact ion source were set to 150 °C and 230 °C, respectively; and the collision energy was 70 eV. Mass spectrometric data were acquired in full-scan mode (*m/z* 50–500).

#### Statistical analyses

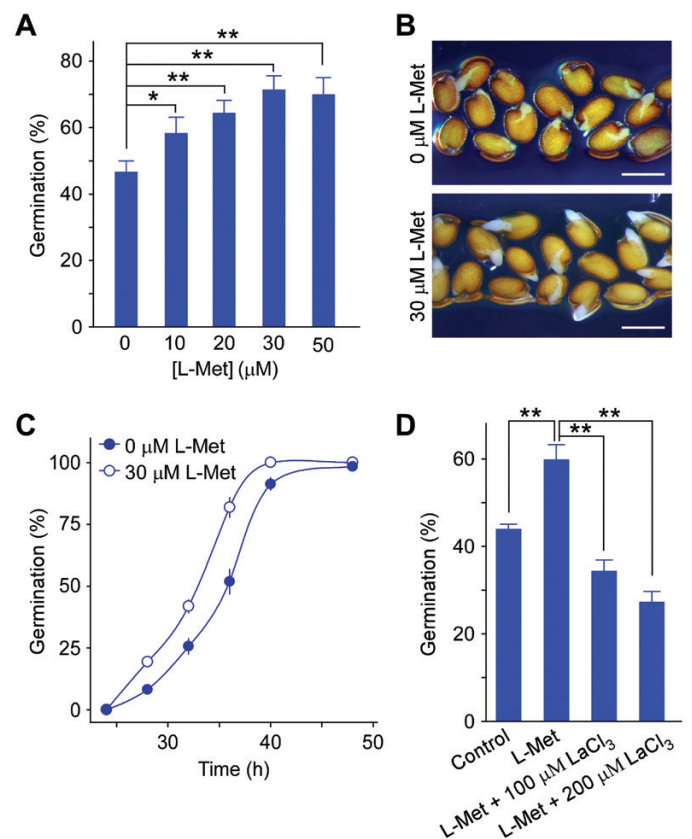
Student's *t*-test (one-tailed distribution) was used to determine the significance of the data.

## Results

### *L*-methionine promotes seed germination

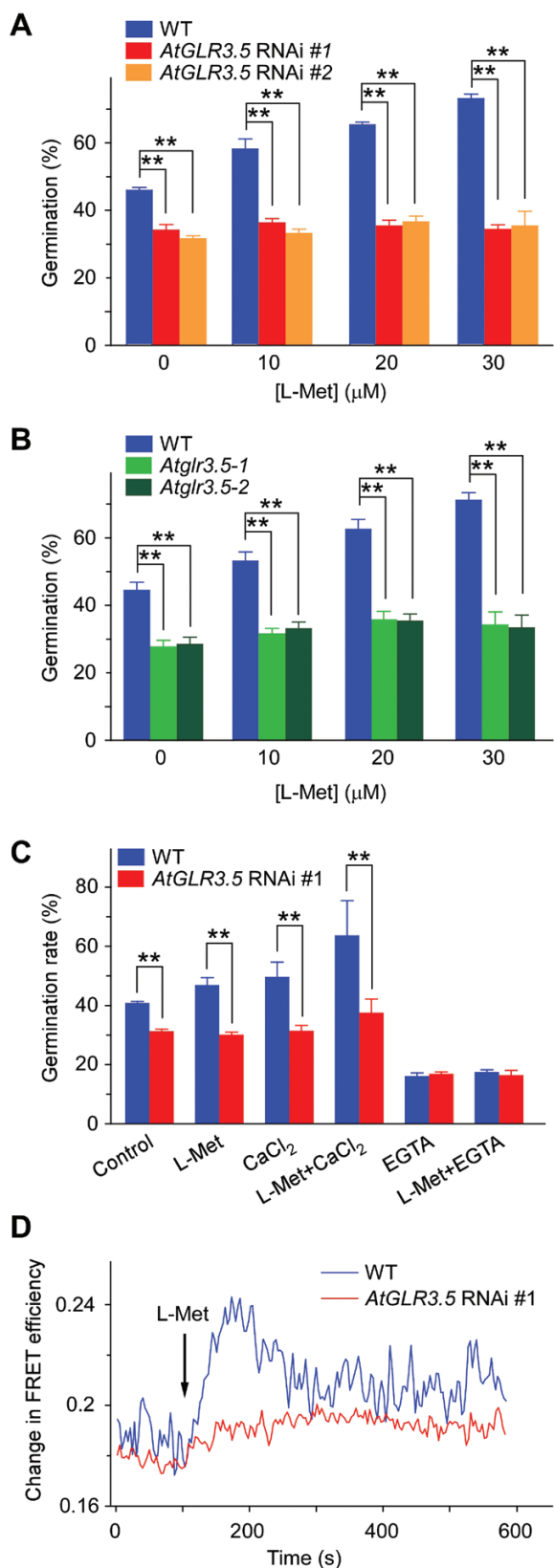
We have previously demonstrated that *AtGLR3.5* modulates [Ca<sup>2+</sup>]<sub>cyt</sub> and seed germination (Kong et al., 2015). To determine

how the ligand regulates *AtGLR3.5* activity during germination, we focused on amino acids whose levels are known to increase during germination (Fait et al., 2006) and that have been reported to activate plant GLR Ca<sup>2+</sup> channels (Michard et al., 2011; Li et al., 2013; Kong et al., 2016). We tested the effects of *L*-Met, *D*-Ser, *L*-Asp, and *L*-Thr at physiological concentrations (10 µM and 30 µM) on germination in *Arabidopsis* wild-type (WT) seeds at 36 h after sowing, at which time germination rates reach approximately half of their maximum value under our experimental conditions (Kong et al., 2015). In addition, the expression of *AtGLR3.5* is highly up-regulated between 24–36 h after imbibition, and knock-down of *AtGLR3.5* expression results in reduced germination at 36 h after imbibition (Kong et al., 2015). We observed that *L*-Met enhanced germination in a dose-dependent manner, with an optimal effect at 30 µM (Fig. 1A, B; Supplementary Fig. S1 at JXB online).



**Fig. 1.** *L*-methionine (*L*-Met) promotes seed germination in *Arabidopsis*. (A) Seed germination at various concentrations of *L*-Met in the growth medium. Wild-type seeds were sown on half-strength MS medium supplemented with the indicated amounts of *L*-Met and incubated in the dark at 4 °C for 3 d before being transferred to a growth chamber at 22 °C for 36 h. (B) Germination phenotypes in the presence or absence of 30 µM *L*-Met at 36 h in the growth chamber. Representative images from three biological replicates are shown. Scale bars are 0.5 mm. (C) Time-course of seed germination in the presence or absence of *L*-Met (30 µM). Seeds were sown as described in (A) and incubated at 22 °C. (D) Effect of the Ca<sup>2+</sup>-channel blocker LaCl<sub>3</sub> on *L*-Met regulation of seed germination. Seeds were incubated on half-strength MS medium (control) or on medium supplemented with *L*-Met (10 µM), or *L*-Met (10 µM) plus LaCl<sub>3</sub> (100 µM or 200 µM) for 36 h and scored for germination. Data in (A, C, D) are means (±SE) from three independent replicates. Significant differences were determined using Student's *t*-test: \**P*<0.05, \*\**P*<0.01. (This figure is available in colour at JXB online.)





**Fig. 2.** L-methionine (L-Met) induces AtGLR3.5-dependent increases in  $[Ca^{2+}]_{cyt}$  in Arabidopsis. (A) Germination of wild-type (WT) and *AtGLR3.5*-RNAi seeds at different concentrations of L-Met. Seeds were sown on half-strength MS medium supplemented with L-Met and were scored for germination after incubation at 22 °C for 36 h. (B) Germination of WT and *AtGLR3.5* T-DNA insertion lines (*Atglr3.5-1* and *Atglr3.5-2*) at different concentrations of L-Met. Seeds were examined for germination after incubation at 22 °C

Time-course analysis further confirmed that there was more rapid seed germination in the presence of L-Met than in its absence (Fig. 1C). Since the positive effect of L-Met on germination was similar to what we had previously observed for Ca<sup>2+</sup> treatment (Kong *et al.*, 2015), we next tested whether the effect of L-Met was related to Ca<sup>2+</sup> or Ca<sup>2+</sup> channel(s). Seed germination rates were therefore examined following treatment with L-Met in combination with a Ca<sup>2+</sup>-channel blocker, LaCl<sub>3</sub>. We found that LaCl<sub>3</sub> eliminated the promotional effects of L-Met, resulting in a germination rate that was lower than that of the control (Fig. 1D). This suggested that the promotional effects of L-Met on germination were achieved via Ca<sup>2+</sup> channel(s), or that L-Met activated Ca<sup>2+</sup> channel(s) to promote germination.

#### *AtGLR3.5* mediates L-Met-dependent seed germination

Since *AtGLR3.5* is essential for Arabidopsis seed germination (Kong *et al.*, 2015) and functions as a Ca<sup>2+</sup> channel (Kong *et al.*, 2015, 2016), we investigated whether it was involved in the L-Met-modulated control of germination. We examined germination rates in the presence of L-Met in *AtGLR3.5*-RNAi lines and in *AtGLR3.5* T-DNA insertion lines (*Atglr3.5-1* and *Atglr3.5-2*), all of which have largely reduced *AtGLR3.5* transcript levels (Kong *et al.*, 2015; Supplementary Fig. S2). In contrast to the enhanced germination rates in WT seeds in response to L-Met, the two independent *AtGLR3.5*-RNAi and the two T-DNA insertion lines showed no apparent changes in germination under the concentrations of L-Met applied (Fig. 2A, B), which suggested a crucial role of *AtGLR3.5* in L-Met-mediated germination. The simultaneous application of L-Met and CaCl<sub>2</sub> led to greater enhancement of germination in WT seeds than when each was applied separately (Fig. 2C). In contrast, this enhanced germination was largely attenuated in the *AtGLR3.5*-RNAi lines, which have defects in the  $[Ca^{2+}]_{cyt}$  increase in response to CaCl<sub>2</sub> (Kong *et al.*, 2015). When the Ca<sup>2+</sup> chelating agent EGTA was added to medium containing L-Met, germination was severely inhibited in both the WT and the *AtGLR3.5*-RNAi lines (Fig. 2C). These results suggested that the regulation of seed germination by L-Met involved *AtGLR3.5* and a Ca<sup>2+</sup> signal.

To obtain direct evidence that L-Met regulates *AtGLR3.5*-dependent fluctuations in  $[Ca^{2+}]_{cyt}$ , we performed FRET-sensitized emission imaging analysis and monitored the dynamics of changes in  $[Ca^{2+}]_{cyt}$  using plants harboring the Ca indicator protein YC3.60 (Kong *et al.*, 2015). L-Met triggered a transient increase in  $[Ca^{2+}]_{cyt}$  in the root cells of WT seedlings,

for 36 h. (C) Germination of WT and *AtGLR3.5*-RNAi seeds in response to various treatments. Seeds were sown on modified MS media containing 0 mM CaCl<sub>2</sub> (control), 0 mM CaCl<sub>2</sub> and 10 μM L-Met, 1 mM CaCl<sub>2</sub>, 1 mM CaCl<sub>2</sub> and 10 μM L-Met, 0 mM CaCl<sub>2</sub> and 10 mM EGTA, or 0 mM CaCl<sub>2</sub> supplemented with 10 μM L-Met and 10 mM EGTA, and were scored for germination after incubation at 22 °C for 36 h. Data in (A–C) are means (±SE) from three independent experiments. Significant differences were determined using Student's *t*-test: \*\**P*<0.01. (D)  $[Ca^{2+}]_{cyt}$ -dependent FRET efficiency changes in WT and *AtGLR3.5*-RNAi plants in response to 1 mM L-Met. The primary roots of seedlings expressing yellow cameleon 3.60 (YC3.60) were used for the analysis and the time point when plants were treated with L-Met is indicated. Ten seedlings of each genotype were tested, and representative results are shown. (This figure is available in colour at JXB online.)

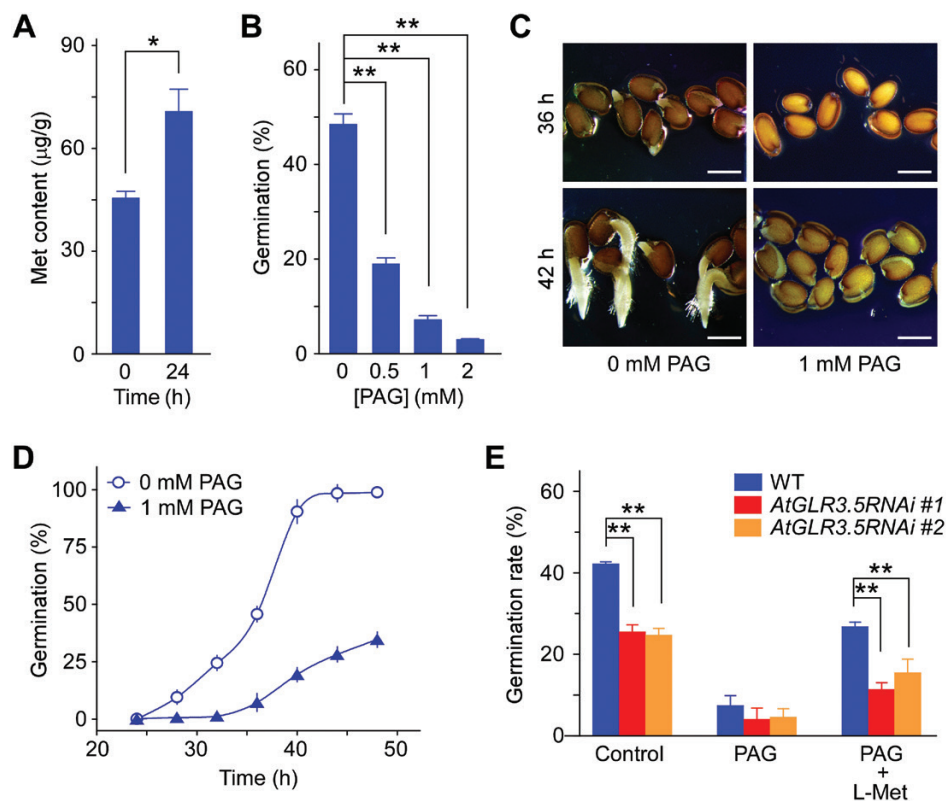
whilst this response was largely eliminated in the *AtGLR3.5*-RNAi seedlings (Fig. 2D). In contrast, L-Glu induced a rise in  $[Ca^{2+}]_{\text{cyt}}$  (Qi *et al.*, 2006; Rincón-Zachary *et al.*, 2010; Toyota *et al.*, 2018) in both the WT and *AtGLR3.5*-RNAi seedlings (Supplementary Fig. S3), indicating that changes in  $[Ca^{2+}]_{\text{cyt}}$  triggered by L-Glu did not require *AtGLR3.5*. Consistent with these results, L-Met stimulated germination in the WT but not in *AtGL3.5*-RNAi and *AtGL3.5* T-DNA insertion lines (Fig. 2A, B), whereas L-Glu had no obvious effects on germination in any of the genotypes (Supplementary Figs S4, S5). Taken together, these data indicated that L-Met activated the *AtGLR3.5*  $Ca^{2+}$  channel and caused an increase in  $[Ca^{2+}]_{\text{cyt}}$  during seed germination.

#### *AtGLR3.5* function is regulated by Met that is biosynthesized via Met synthase 1

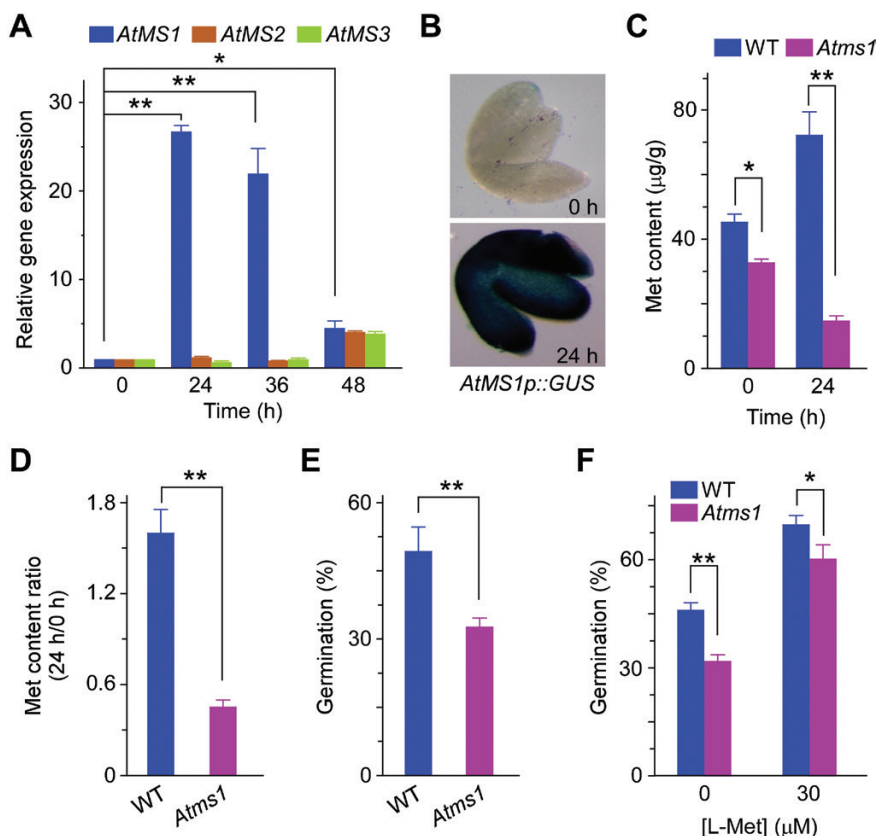
Since it promoted seed germination (Fig. 1A–C), we examined Met contents in germinating seeds at 0 h and 24 h after transfer to germination conditions. The Met content in the WT increased ~1.6-fold during this period (Fig. 3A). We then investigated the source of the Met that induced the *AtGLR3.5*-mediated increases in  $[Ca^{2+}]_{\text{cyt}}$  during germination. Met in plants can be produced either via *de novo* biosynthesis or via recycling

(Gallardo *et al.*, 2002; Amir, 2010; Willke, 2014). Given that germination is the earliest physiological step in the life cycle of a plant, we focused on biosynthesis. Pharmacological analyses showed that PAG, a Met-synthesis inhibitor (Thompson *et al.*, 1982; Ravanel *et al.*, 1998), retarded germination in a dose-dependent manner (Fig. 3B). As reported previously (Gallardo *et al.*, 2002), PAG dramatically impeded seed radicle protrusion and germination (Fig. 3C, D), and this impairment could to some extent be lessened by supplementation with L-Met (Fig. 3E); however, this effect was much lower in *AtGLR3.5*-RNAi seeds. These data indicated that the *de novo* biosynthesis of Met was critical for *AtGLR3.5*-mediated germination.

Met is synthesized in plants from O-phosphohomoserine (OPH) via three successive enzyme reactions, with vitamin B12-independent Met synthase being responsible for the final step (Ravanel *et al.*, 1998; Gallardo *et al.*, 2002). The Arabidopsis genome harbors three functional isoforms of Met synthase (*AtMS*) genes (Ravanel *et al.*, 2004). To identify which of them was responsible for the production of the Met required for seed germination, we performed quantitative reverse-transcription PCR (qRT-PCR) analysis and found large changes in expression of *AtMS1* (At5g17920) during germination (Fig. 4A). Transcript levels of *AtMS1* peaked 24 h after the onset of germination and then decreased gradually until its completion. In



**Fig. 3.** The biosynthesis of methionine (Met) plays a role in *AtGLR3.5*-modulation of seed germination in Arabidopsis. (A) Met contents in wild-type (WT) seeds at 0 h and 24 h after incubation of the seeds under germination conditions. (B) Germination of WT seeds with different concentrations of the Met-biosynthesis inhibitor DL-propargylglycine (PAG). Seeds were sown on half-strength MS media supplemented with the indicated concentrations of PAG and scored for germination after incubation at 22 °C for 36 h. (C) Germination phenotypes of WT seeds in the presence or absence of PAG. Seeds were sown on half-strength MS media supplemented with 0 mM or 1 mM PAG and were incubated at 22 °C for 36 h (top) or 42 h (bottom). Representative images of three biological replicates are shown. (D) Time-course of seed germination in the presence or absence of PAG. Seeds were sown in the same medium as in (C) and incubated at 22 °C. (E) Germination of *AtGLR3.5*-RNAi seeds in the presence of PAG (1 mM) or PAG plus Met (30 µM). Seeds were scored for germination after incubation at 22 °C for 36 h. Data in (A, B, D, E) are means ( $\pm$ SE) from three independent experiments. Significant differences were determined using Student's *t*-test: \**P*<0.05, \*\**P*<0.01. (This figure is available in colour at JXB online.)



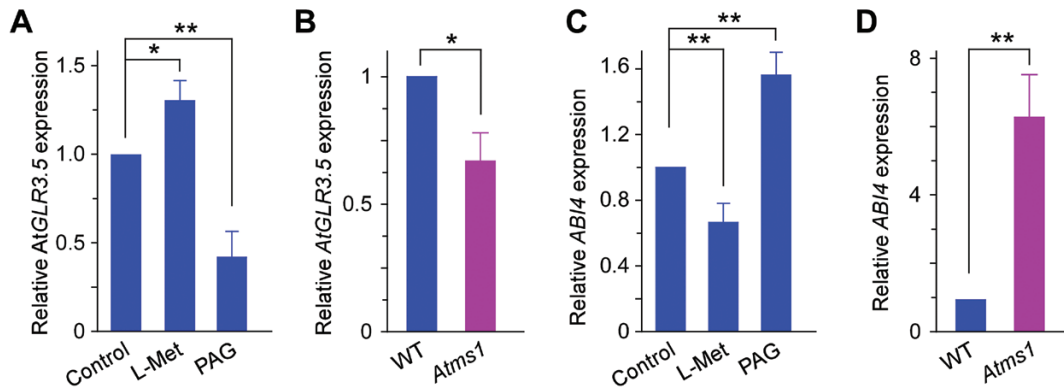
**Fig. 4.** Methionine (Met) derived from *AtMS1* positively regulates seed germination in *Arabidopsis*. (A) qRT-PCR analysis of *AtMS1*, *AtMS2*, and *AtMS3* expression at 0–48 h during seed germination. Wild-type (WT) seeds were incubated in the dark at 4 °C for 3 d and transferred to a growth chamber at 22 °C for 0–48 h prior to the analysis. Expression is relative to that of *ACTIN2*. (B) Expression patterns of *AtMS1* in seed embryos as indicated by *AtMS1pro::GUS*. The seeds were stratified and incubated in a growth chamber at 22 °C for 0 h or 24 h before GUS staining, and images were taken after the removal of seed coats. Representative images are shown. (C) Met contents in WT and *Atms1* seeds. Seeds were incubated at 22 °C for 0 h or 24 h prior to the analysis. (D) The ratio of Met content at 24 h versus at 0 h at 22 °C in WT and *Atms1* seeds based on the results in (C). (E) Germination of WT and *Atms1* seeds. Germination was scored after incubation at 22 °C for 36 h. (F) Germination of WT and *Atms1* seeds with or without treatment with L-Met. Seeds were scored for germination after incubation at 22 °C for 36 h. All data are means ( $\pm$ SE) from three replicates. Significant differences were determined using Student's *t*-test: \* $P < 0.05$ , \*\* $P < 0.01$ . (This figure is available in colour at JXB online.)

line with the qRT-PCR data, transgenic seeds expressing the GUS reporter gene driven by a 2.0-kb promoter sequence of *AtMS1* exhibited a significant increase in GUS activity after 24 h incubation under germination conditions (Fig. 4B). The *AtMS1* transcription data are consistent with previous results from a proteomic study that showed that levels of the *AtMS1* protein greatly increase 1 d after imbibition (Gallardo *et al.*, 2002). To further assess the role of *AtMS1* in germination, we isolated a T-DNA insertion mutant, *Atms1*, that has a reduced level of *AtMS1* transcripts (Supplementary Fig. S6). The *Atms1* mutant produced lower amounts of Met than WT seeds at both 0 h and 24 h under germination conditions (Fig. 4C), thus indicating a role of *AtMS1* in Met biosynthesis during germination. Interestingly, in contrast to the WT (Fig. 3A), the Met content in the mutant decreased between 0 h and 24 h (Fig. 4C), which may have been attributable to consumption of a significant amount of Met during germination and the low amounts of Met biosynthesized in the mutant. The ratio of Met content at 24 h versus 0 h was much lower in the mutant than that in the WT (Fig. 4D), suggesting that the mutant had lower amounts of Met available for facilitating germination. In line with this result, the germination rate of *Atms1* seeds was reduced relative to the WT (Fig. 4E). The germination rate

in both the WT and the mutant was increased in the presence of exogenously supplemented L-Met (30  $\mu$ M), but the effect was proportionally greater in *Atms1* seeds (Fig. 4F), which implied that the reduced level of Met was the cause of lower germination in the mutant. The results from these experiments, together with those relating to the reduced sensitivity of *AtGLR3.5*-RNAi seeds to L-Met (Fig. 2A, B) and the reduced effect of L-Met applied to PAG-treated *AtGLR3.5*-RNAi seeds (Fig. 3E) compared to the WT, demonstrated that *AtMS1*-derived Met played an essential role in the regulation of seed germination by *AtGLR3.5*.

#### *Met differentially regulates the expression of AtGLR3.5 and ABI4 in germinating seeds*

Our results indicated that Met induced *AtGLR3.5*-mediated increases in  $[Ca^{2+}]_{cyt}$  (Fig. 2D) and that it modulated *AtGLR3.5*-dependent seed germination (Fig. 2A). To further investigate the regulation by Met, we examined its effect on *AtGLR3.5* expression. As shown in Fig. 5A, addition of L-Met up-regulated *AtGLR3.5* expression, while the Met biosynthesis inhibitor PAG reduced its expression. In the *Atms1* mutant with lower Met content, *AtGLR3.5* transcript levels were



**Fig. 5.** Methionine (Met) modulates the expression of *AtGLR3.5* and the key seed germination regulator *ABI4* in Arabidopsis. (A) qRT-PCR analysis of *AtGLR3.5* expression in wild-type (WT) seeds in response to treatment with L-Met (30  $\mu$ M) or PAG (1 mM). (B) Expression of *AtGLR3.5* in WT and *Atms1* seeds. (C) Expression of *ABI4* in WT seeds in response to L-Met (30  $\mu$ M) or PAG (1 mM). (D) *ABI4* expression in WT and *Atms1* seeds. All seeds were incubated in the dark at 4 °C for 3 d and then transferred to 22 °C for 36 h prior to the analysis. Expression is relative to that of *ACTIN2*. Data are means ( $\pm$ SE) from three replicates. Significant differences were determined using Student's *t*-test: \* $P < 0.05$ , \*\* $P < 0.01$ . (This figure is available in colour at JXB online.)

significantly lower than in the WT (Fig. 5B). These data suggested that in addition to activation of the *AtGLR3.5* channel, L-Met up-regulated *AtGLR3.5* expression in germinating seeds. In our previous study, we observed that an *AtGLR3.5*-mediated increase in  $[Ca^{2+}]_{\text{cyt}}$  suppresses *ABI4* expression in germinating seeds (Kong et al., 2015). Therefore, we examined the effect of Met on *ABI4* expression. L-Met decreased the expression of *ABI4*, while PAG increased its expression (Fig. 5C). In addition, the transcript levels of *ABI4* in the *Atms1* mutant were ~6-fold higher than in the WT (Fig. 5D). Overall, these results suggested that L-Met acted upstream of the *AtGLR3.5*– $Ca^{2+}$ –*ABI4* cascade in seed germination and that it antagonistically regulated the functions of *AtGLR3.5* and *ABI4*.

## Discussion

Since the discovery of homologs of animal glutamate receptor  $Ca^{2+}$  channels in plant genomes (Lam et al., 1998), plant GLRs have been expected to function similarly to  $Ca^{2+}$  channels (Lacombe et al., 2001; Ward et al., 2009; Hedrich, 2012). Numerous studies have demonstrated the involvement of plant GLRs in  $Ca^{2+}$ -associated developmental and physiological processes (Kim et al., 2001; Kang et al., 2004; Meyerhoff et al., 2005; Li et al., 2006, 2013; Cho et al., 2009; Michard et al., 2011; Mousavi et al., 2013; Vincill et al., 2013; Kong et al., 2015, 2016; Toyota et al., 2018). In addition, several reports have demonstrated that plant GLRs can be gated by amino acids such as D-Ser and L-Met, in a manner similar to their counterparts in animals (Qi et al., 2006; Michard et al., 2011; Tapken et al., 2013; Forde and Roberts, 2014; Kong et al., 2016). We have previously observed that *AtGLR3.5* modulates  $[Ca^{2+}]_{\text{cyt}}$  and is essential for Arabidopsis seed germination (Kong et al., 2015), and therefore in the present study we investigated whether it is regulated by some amino acid(s). Using a pharmacological approach, we first demonstrated that regulation of seed germination by L-Met required *AtGLR3.5* and  $Ca^{2+}$  signaling (Fig. 2A, B). Furthermore, using plants harboring the calcium marker protein YC3.60, we found that L-Met triggered a transient rise in  $[Ca^{2+}]_{\text{cyt}}$  in the primary roots of WT seedlings but that this was largely abolished in *AtGLR3.5*-RNAi seedlings

(Fig. 2D). Together with the results from our previous study (Kong et al., 2016), this indicated that L-Met activates the *AtGLR3.5*-dependent increase in  $[Ca^{2+}]_{\text{cyt}}$ , thereby regulating germination. The positive effect of L-Met that we observed is consistent with previous studies of other plant GLRs (Tapken et al., 2013; Kong et al., 2016). Using the *Xenopus* oocyte heterologous expression system, Tapken et al. (2013) investigated the relative efficacy of all 20 common amino acids on *AtGLR1.4* channel activity and found that L-Met was the most effective. Our recent *in planta* study showed that L-Met acts as an agonist on *AtGLR3.1/3.5* heteromeric  $Ca^{2+}$  channels in the stomatal movement process (Kong et al., 2016). Our current findings, together with those of previous studies, highlight the regulation of plant GLR channel activity by ligand amino acids.

Our germination data (Fig. 2A, B) suggested that an *AtGLR3.5*-dependent increase in  $[Ca^{2+}]_{\text{cyt}}$  triggered by L-Met occurred in germinating seeds, and direct evidence for this could be obtained by monitoring the changes in  $[Ca^{2+}]_{\text{cyt}}$  in the embryo during seed germination. However, to our knowledge, no suitable technique is available to monitor  $[Ca^{2+}]_{\text{cyt}}$  in a tissue enveloped in the seed coat. Given that we and others have successfully recorded changes in  $[Ca^{2+}]_{\text{cyt}}$  in response to  $CaCl_2$  in the primary roots of young seedlings containing YC3.60 (Rincón-Zachary et al., 2010; Kong et al., 2015), we examined changes in response to L-Met treatment in seedling roots as a proxy for the germinating seeds. In this way, we demonstrated a role of exogenous L-Met in the *AtGLR3.5*-mediated increase in  $[Ca^{2+}]_{\text{cyt}}$ . These results together with the promotion by L-Met of *AtGLR3.5*-dependent seed germination (Fig. 2A, B) lead to the proposition that L-Met activation of *AtGLR3.5* activity occurs *in vivo* during germination. However, because Met can be converted to other metabolites during germination, we cannot exclude the possibility that they might play a role.

An intriguing finding of our study was that although both L-Met and L-Glu triggered increases in  $[Ca^{2+}]_{\text{cyt}}$  in WT seedlings (Fig. 2D; Supplementary Fig. S3), only L-Met stimulated germination (Fig. 1; Supplementary Figs S4, S5). According to the eFP Browser (<http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>; Winter et al., 2007), *AtGLR3.5* and *AtGLR3.7* are the



two *AtGLR*s that are highly up-regulated during the transition from dry to imbibed seeds. Histochemical analysis of transgenic seeds harboring *AtGLR3.5p::GUS* has shown that *AtGLR3.5* is indeed highly up-regulated in imbibed seeds, suggesting that it plays a crucial role in germination (Kong *et al.*, 2015). In contrast, *GLR3.3* and *GLR3.6*, which are reported to be required for systemic increases in [Ca<sup>2+</sup>]<sub>cyt</sub> triggered by Glu (Toyota *et al.*, 2018), show very low expression levels in imbibed seeds according to the eFP Browser. This is probably why the L-Glu treatment did not enhance seed germination despite the increases in Ca in the seedling roots (Supplementary Figs S3–S5). Furthermore, L-Met and L-Glu acted differently in WT and *AtGLR3.5*-RNAi plants: L-Met failed to trigger an increase in [Ca<sup>2+</sup>]<sub>cyt</sub> in *AtGLR3.5*-RNAi plants, whereas L-Glu triggered increases up to the WT level (Fig. 2D, Supplementary Fig. S3). These results indicate that the increase in [Ca<sup>2+</sup>]<sub>cyt</sub> evoked by L-Glu is independent of *AtGLR3.5* and thus has no effect on seed germination.

Met is one of the essential amino acids and is of great nutritional significance, so its level determines the quality of crops and cereal-based human diets (Hesse *et al.*, 2004; Galili and Amir, 2013). As a building-block for protein synthesis and a precursor of several key metabolites (Amir, 2010), Met is tightly intertwined with numerous indispensable cellular processes. The amount of synthase protein that catalyses the last step in Met biosynthesis increases greatly in 1-d imbibed seeds but rapidly decreases back to the dry seed level upon drying of the seeds, indicating the importance of Met in seed germination (Gallardo *et al.*, 2002). However, the molecular function of Met in seed germination and the underlying signaling networks have remained largely unclear. Our study shows that L-Met acts as a positive regulator of seed germination: L-Met enhanced *AtGLR3.5*- and Ca<sup>2+</sup>-dependent seed germination (Figs 1D; 2A, B), triggered *AtGLR3.5*-mediated increases in [Ca<sup>2+</sup>]<sub>cyt</sub> (Fig. 2D), and repressed the expression of *ABI4* (Fig. 5C), a component acting downstream of *AtGLR3.5* in seed germination (Kong *et al.*, 2015). Together with our previous work (Kong *et al.*, 2015), this current study links Met to *AtGLR3.5*-mediated Ca<sup>2+</sup> signaling and *ABI4* during seed germination. In addition to its nutritional value and its role as a fundamental metabolite, we have shown that Met plays a signal role in modulating cellular events that facilitate plants to achieve the initial step in their life cycle, seed germination.

*De novo* biosynthesis provides a critical source of the Met required for germination. A previous proteomic study identified two Arabidopsis enzymes that are differentially accumulated during germination, both of which are associated with Met biosynthesis (Gallardo *et al.*, 2002). Our findings further point to the significance of Met biosynthesis during germination. The fact that exogenously supplied Met promoted seed germination in a dose-dependent manner (Fig. 1A–C) suggested that there was an increase in Met content during germination, which was confirmed by GC-MS analysis (Figs 3A; 4C, D). In addition, treatment with the Met synthesis inhibitor PAG resulted in reduced germination (Fig. 3B–D), implying that *de novo* synthesis of Met is important for germination. We also demonstrated that the mRNA levels of *AtMS1* were greatly increased (~25-fold) as seed germination began, reaching a

maximum level at 24 h and then decreasing through to 48 h (Fig. 4A, B). Knocking out *AtMS1* impaired Met biosynthesis as well as seed germination (Fig. 4C, E). Furthermore, our expression analysis data were consistent with a previous proteomics study that showed that Met synthase is induced during the first day of imbibition, prior to radicle emergence, but decreases on the second day of imbibition when the protrusion of the radicle occurs (Gallardo *et al.*, 2002). Notably, the drastic change in *AtMS1* expression levels (Fig. 4A) were very similar to the expression pattern of *AtGLR3.5* during germination (Kong *et al.*, 2015), suggesting a functional correlation between the two proteins in the control of seed germination. Our finding that addition of L-Met led to a lower degree of recovery in germination in the presence of PAG in the *AtGLR3.5*-RNAi plants than in the WT (Fig. 3E) further supports the importance of Met biosynthesis in the *AtGLR3.5*-dependent regulation of germination. Overall, we have demonstrated that Met biosynthesis by *AtMS1* positively regulates the *AtGLR3.5* channel during germination.

Met is not the only amino acid that increases during germination in Arabidopsis. Asp and Thr (which belong to the Asp family together with Met) also exhibit significant increases (>40-fold) during the transition from stratification to germination (Fait *et al.*, 2006). Enhancing the metabolism of Lys, another member of the Asp family, alters the levels of other amino acids during early seed germination (Angelovici *et al.*, 2011). It has also been reported that Met content is positively correlated with the levels of Lys or Thr in plants (Amir, 2010). In our present study, knocking-out of *AtMS1* not only lowered the amounts of Met, but also reduced the levels of other Asp family amino acids (Supplementary Fig. S7). Since Thr also displayed a certain degree of promotional effects on germination (Supplementary Fig. S1), future examination of its role, as well as those of other amino acids not tested here, would provide more comprehensive insights into their functions during seed germination.

Given the wide range of roles of plant GLR channel-mediated Ca<sup>2+</sup> signals in responses to various biotic and abiotic stimuli (Kim *et al.*, 2001; Meyerhoff *et al.*, 2005; Li *et al.*, 2013; Mousavi *et al.*, 2013; Ortiz-Ramírez *et al.*, 2017; Cheng *et al.*, 2018; Toyota *et al.*, 2018) and the potential role of amino acids in stress resistance (Slama *et al.*, 2015), the L-Met–*AtGLR3.5*–*ABI4* cascade may function in response to external signals to control seed germination. Future studies are needed to elucidate the molecular mechanisms of amino acid and Ca<sup>2+</sup> signals in the adaptive responses of plants under stress conditions.

## Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Effects of different amino acids on seed germination.

Fig. S2. Genotyping of *AtGLR3.5* T-DNA insertion mutants and relative expression of *AtGLR3.5* in the two independent alleles.

Fig. S3. Changes in [Ca<sup>2+</sup>]<sub>cyt</sub> in WT and *AtGLR3.5*-RNAi plants in response to 1 mM L-Glu as determined by changes in FRET efficiency.



Fig. S4. Germination of seeds of the WT and two independent *AtGLR3.5*-RNAi lines at different concentrations of L-Glu.

Fig. S5. Germination of WT, *Atglr3.5-1*, and *Atglr3.5-2* seeds at different concentrations of L-Glu.

Fig. S6. Genotyping of the *Atms1* mutant and relative *AtMS1* transcript levels in the WT and mutant.

Fig. S7. Contents of Asp, Thr, and Lys in WT and *Atms1* seeds.

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