

The glutamate carboxypeptidase AMP1 mediates abscisic acid and abiotic stress responses in *Arabidopsis*

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Summary

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- *ALTERED MERISTEM PROGRAM1 (AMP1)* encodes a glutamate carboxypeptidase that plays an important role in shoot apical meristem development and phytohormone homeostasis.
- We isolated a new mutant allele of *AMP1*, *amp1-20*, from a screen for abscisic acid (ABA) hypersensitive mutants and characterized the function of *AMP1* in plant stress responses.
- *amp1* mutants displayed ABA hypersensitivity, while overexpression of *AMP1* caused ABA insensitivity. Moreover, endogenous ABA concentration was increased in *amp1-20*- and decreased in *AMP1*-overexpressing plants under stress conditions. Application of ABA reduced the *AMP1* protein level in plants. Interestingly, *amp1* mutants accumulated excess superoxide and displayed hypersensitivity to oxidative stress. The hypersensitivity of *amp1* to ABA and oxidative stress was partially rescued by reactive oxygen species (ROS) scavenging agent. Furthermore, *amp1* was tolerant to freezing and drought stress. The ABA hypersensitivity and freezing tolerance of *amp1* was dependent on ABA signaling. Moreover, *amp1* had elevated soluble sugar content and showed hypersensitivity to high concentrations of sugar. By contrast, the contents of amino acids were changed in *amp1* mutant compared to the wild-type.
- This study suggests that AMP1 modulates ABA, oxidative and abiotic stress responses, and is involved in carbon and amino acid metabolism in *Arabidopsis*.

Introduction

The *Arabidopsis AMP1* gene encodes an endoplasmic reticulum (ER) membrane-localized glutamate carboxypeptidase that has been implicated in the small peptide signaling process (Helliwell *et al.*, 2001; Vidaurre *et al.*, 2007). The orthologs of this presumptive glutamate carboxypeptidase – VP8 (*Zea mays*), PLA3 (*Oryza sativa*) and TRICOT (*Lotus japonicus*) – are involved in the regulation of phytohormone homeostasis (Suzuki *et al.*, 2008; Kawakatsu *et al.*, 2009; Suzuki *et al.*, 2012). VP8 modulates meristem development and seed maturation by controlling the accumulation of abscisic acid (ABA) and embryonic regulators such as *LEAFY COTYLEDON1 (LEC1)*/B3 domain transcription factors (Suzuki *et al.*, 2008). PLA3 regulates various developmental processes and plant hormone homeostasis. A *pla3* loss-of-function mutant maintains a slightly higher concentration of cytokinin but a lower amount of ABA than the wild-type, and it displays an ABA-insensitive phenotype (Kawakatsu *et al.*, 2009). In *Arabidopsis*, AMP1 regulates embryonic and postembryonic growth and development by affecting plant hormone biosynthesis and signaling (Chaudhury *et al.*, 1993; Vidaurre *et al.*, 2007). The *amp1-1* mutant was reported to have

increased zeatin content and leaf number, and an enlarged apical meristem in the shoot (Chaudhury *et al.*, 1993; Riou-Khamlichi *et al.*, 1999). Several alleles of *AMP1* that have pleiotropic phenotypes in response to different plant hormones have been isolated. A weak missense allele, *amp1-7*, exhibits decreased hypocotyl elongation when exposed to ethylene and GA₃ in light (Saibo *et al.*, 2007). Another *amp1* mutant was isolated as a suppressor of an *monopteros/auxin response factor 5 (mp/arf5)* mutant, suggesting a role for AMP1 in meristem-niche-associated auxin signaling (Vidaurre *et al.*, 2007). One recent study reported that the *amp1* mutation has different effects on dormancy and on ABA concentrations in different accessions (Griffiths *et al.*, 2011).

ABA regulates many important aspects of physiological processes, including seed dormancy and germination, vegetative growth and plant responses to environmental stresses (Leung & Giraudat, 1998; Finkelstein *et al.*, 2002). The biosynthesis of ABA involves five essential enzymes, encoded as *ABA1*, *ABA2*, *ABA3*, *NCED3* and *AAO3*. These genes can be rapidly induced by abiotic stress and exogenous ABA is capable to rescue their hypersensitivity phenotypes to freezing and salt stress of these ABA deficient mutants (Llorente *et al.*, 2000; Barrero *et al.*,

2006). By screening of ABA insensitive mutants, several key components in the ABA signaling pathway, including *ABI1* to *ABI5* (*ABA insensitive1-5*), have been characterized (Koornneef *et al.*, 1989; Finkelstein, 1994). *ABI1* and *ABI2* are PP2C (phosphatase type-2C) proteins with negatively regulatory roles in ABA signaling (Allen *et al.*, 1999; Merlot *et al.*, 2001). *ABI3* (a B3 domain transcription factor) and *ABI5* (a bZIP transcriptional factor) mainly function in ABA-dependent seedling growth arrest during seed germination and postgermination growth stages (Giraudat *et al.*, 1992; Finkelstein & Lynch, 2000b). *ABI4* is a member of the ERF/AP2 transcription factor family (Finkelstein *et al.*, 1998).

Unfavorable environments such as osmotic stress and salinity induce oxidative stress and promote reactive oxygen species (ROS) overproduction in chloroplasts, mitochondria and other cellular components (Miller *et al.*, 2008; Jaspers & Kangasjarvi, 2010; Suzuki *et al.*, 2012). Accumulated ROS are involved in various cellular responses and ultimately lead to cell death (Noctor *et al.*, 2007; Taylor *et al.*, 2009; Suzuki *et al.*, 2012). Besides its toxic effect, ROS also act as key molecules which can trigger the transcription of downstream stress response genes (Foyer & Noctor, 2005; Fujita *et al.*, 2006; Miller *et al.*, 2008; Jaspers & Kangasjarvi, 2010). To keep the balance of ROS and protect cellular homeostasis from membrane system injury and oxidative stress, the excessive ROS are scavenged by enzymatic and nonenzymatic antioxidants, such as superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, ascorbic acid and glutathione (Mittler *et al.*, 2004; Noctor *et al.*, 2007; Foyer & Noctor, 2009; Miller *et al.*, 2011). However, the precise mechanism of cellular ROS production in response to abiotic stresses is still unclear. Recent evidence shows that ROS generated from plasma membrane and mitochondria are involved in ABA signaling pathway to regulate the process of root growth, stomata movement and seed germination (Kwak *et al.*, 2003; Liu *et al.*, 2010; He *et al.*, 2012). Overexpression of a ROS-induced transcription factor *AtWRKY15* showed tolerance to salt and osmotic stress (Vanderauwera *et al.*, 2012). *Arabidopsis lsd1* and *chs2* mutants with ROS overaccumulation showed sensitivity to low temperatures (Huang *et al.*, 2010a,b). Oxidative stress occurrence in *Arabidopsis* cells are reported to affect carbon metabolism by inhibiting the TCA cycle in mitochondria, leading to decreased amino acid content and disturbed balance of metabolic coordination (Baxter *et al.*, 2007; Takahashi & Murata, 2008; Suzuki *et al.*, 2012).

In this study we report that *amp1* mutants displayed hypersensitivity to ABA and oxidative stress during germination and postgermination growth. Conversely, overexpression of *AMP1* resulted in early germination and insensitivity to ABA and oxidative stress. The concentration of ABA accumulation increased in *amp1*- and decreased in *AMP1*-overexpressing plants under osmotic stress. Consistently, loss-of-function of *AMP1* conferred enhanced freezing and drought tolerance. We also demonstrated that the accumulation of sugar and amino acids was affected in *amp1* mutants. Thus, our results suggest that *AMP1* is a novel component that is involved in ABA, oxidative and abiotic stress responses, and mediates carbon and amino acid metabolism in *Arabidopsis*.

Materials and Methods

Plant materials and growth conditions

The *Arabidopsis thaliana* (L.) Heynh ecotypes Columbia (Col-0) and Landsberg *erecta* (Ler) were used in this study. The mutants *aba2-1* (Leon-Kloosterziel *et al.*, 1996), *abi4-1* (Finkelstein *et al.*, 1998), *abi5* (Finkelstein & Lynch, 2000a), *pyr1 pyl1 pyl4*, *pyr1 pyl1 pyl2 pyl4* (Park *et al.*, 2009), *amp1-1* (CS8324), *amp1-13* (SALK_022988), *amp1-14* (SALK_087303), *amp1-22* (SALK_138749) are in the Col background, while *abi1-1* (Meyer *et al.*, 1994), *abi2-1* (Leung *et al.*, 1997) and *abi3-1* (Giraudat *et al.*, 1992) are in the Ler background. The *Arabidopsis* plants were grown at 22°C with a photoperiod of 16-h light : 8-h dark on MS medium (Sigma-Aldrich) containing 2% sucrose and 0.8% agar, unless otherwise indicated.

For the germination assay, sterilized seeds were plated on MS medium containing 0.8% agar, supplemented with different concentrations of ABA, Methyl violagen (MV) or glucose. Plates were stratified at 4°C in darkness for 3 d and then transferred to 22°C. Plants showing open green cotyledons were scored to calculate for germination rate at day 6 after the end of stratification. Three independent experiments were carried out and each experiment contained three replications with 30 seedlings for each replicate.

Screening of *amp1* and map-based cloning of *AMP1*

In order to isolate ABA hypersensitive mutants, T2 plant seeds from an *Arabidopsis* T-DNA insertion collection that was generated using the activation-tagging vector pSKI015 (Qin *et al.*, 2003) were planted on MS medium supplemented with 0.5 μM ABA, and grown at 22°C for 6 d. Plants that were hypersensitive to ABA were rescued on MS medium and transferred to soil to set seeds, and T3 seeds were used to recheck ABA sensitivity.

The *amp1-20* mutant with Col background was crossed to Ler, and the resulting F₂ seeds were collected. A total of 500 *amp1-20* mutant seedlings were chosen from the segregating F₂ population. Genomic DNA was extracted and used for PCR-based mapping with simple sequence length polymorphism (SSLP) and cleaved amplified polymorphic sequence (CAPS) markers. Candidate genes in this region were sequenced in *amp1-20* to identify the mutation.

Plasmid construction and plant transformation

For complementation of *amp1-20*, a 3.1-kb *AMP1* genomic fragment was amplified by PCR using the primers AMP1-3F and AMP1-3R (Supporting Information Table S1) and cloned into pCAMBIA1300 (CAMBIA, Canberra, Australia) containing the 2.3-kb promoter region of *AMP1*. To construct *pSuper:AMP1-GFP* and *pSuper:AMP1ΔN-GFP*, the *AMP1* cDNA fragment was amplified by PCR using the primers AMP1-4F and AMP1-4R and AMP1-5F and AMP1-5R, respectively, and subsequently cloned into a *pSuper*1300 vector (Yang *et al.*, 2010) containing a GFP tag.

All constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into plants via the floral dip transformation (Clough & Bent, 1998).

Freezing and drought tolerance assays

The freezing tolerance assay was carried out as described (Shi *et al.*, 2012). Briefly, 2-wk-old plants were placed in a freezing chamber set to -1°C and programmed to cool by decreasing the temperature at a rate of $-1^{\circ}\text{C h}^{-1}$. The plates were removed at -6°C , -7°C or -8°C , and incubated at 4°C for 12 h before transferred to 22°C . Survival seedlings were scored after a 2-d recovery period. For drought-tolerant assay, plants were grown in soil for 4 wk without watering. Then the plants were irrigated, and the survival rates were scored 4 d later.

Physiological analyses

Ion leakage and sugar content were measured as described (Lee *et al.*, 2002). Total soluble sugar (sucrose, glucose and fructose) content was measured as described (Stitt *et al.*, 1989; Strand *et al.*, 1999). Proline content was measured as described (Bates *et al.*, 1972). For the ABA content measurement, 2-wk-old seedlings grown at 22°C were treated with or without 40% PEG8000 for 6 h. ABA contents were determined as described previously (Kojima *et al.*, 2009). Total chlorophyll content was determined as described (Huang *et al.*, 2009).

RNA extraction and real-time PCR

Total RNA extraction from 2-wk-old plants and real-time PCR were performed as described (Huang *et al.*, 2010a). Quantitative real-time PCR (qRT-PCR) was performed using the SYBR Green PCR Master Mix kit (Takara, Tokyo, Japan). Three RT-PCR reactions were repeated independently using *Actin2/8* gene as an internal control. The relative expression levels were calculated as described (Huang *et al.*, 2010a). The primers used are listed in Table S1.

Fluorescence microscopy

Five-day-old *AMP1-OX* and *pSuper:GFP* seedlings were treated with 50 μM ABA for 6 h. GFP fluorescence in roots was imaged using a confocal laser scanning microscope (LSM510; Carl Zeiss, Oberkochen, Germany).

Protein preparation and immunoblot assay

Soluble and membrane proteins were isolated as described (Hua *et al.*, 2001). HSP90 monoclonal antibody was used as a soluble marker after fractionation.

For immunoblots, 10-d-old seedlings were treated with 500 μM CHX (cycloheximide; Berberich & Kusano, 1997) for 2 h, or with 50 μM ABA or 50 μM ABA plus 50 μM MG132 for 0–6 h. AMP1-GFP fusion proteins were detected by immunoblot using an anti-GFP antibody (1 : 1000 dilution;

Sigma-Aldrich). Rubisco stained by Ponceau S (GenView, CA, USA) was used as a loading control.

Amino acid measurement

Amino acids were prepared from fresh seedlings as described previously (Wu *et al.*, 1996). Briefly, c. 0.05 g seedlings were collected and extracted by adding 500 μl of HClO_4 extraction buffer (Perchloric acid), followed by centrifugation at 10 000 g for 10 min. Subsequently, 250 μl 2 M K_2CO_3 was added to the supernatant and centrifuged at 10 000 g for 10 min. The supernatant was used for subsequent fluorometric HPLC methods described (Wu *et al.*, 1997).

Measurement of ROS in plants

Nitroblue tetrazolium (NBT) and 3,3'-diaminobenzidine (DAB) staining with 2-wk-old seedlings was performed to detect superoxide as described previously (Shi *et al.*, 2007). H_2O_2 was measured using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich; Munemasa *et al.*, 2007). All experiments were repeated more than three times.

Microarray experiment

Affymetrix GeneChip ATH1 arrays representing 24 000 *Arabidopsis* genes were used for the analysis of whole genome gene expression profile in wild-type Col, *amp1-20* and *AMP1-OX2* overexpressing plants. Total RNA was extracted from 2-wk-old seedlings grown on MS medium containing 2% sucrose. The raw microarray data were analyzed using Gene Ontology Annotations (<http://www.arabidopsis.org/tools/bulk/go/index.jsp>) and visualized with MapMan software (<http://mapman.gabipd.org>; Ariel *et al.*, 2012).

Results

Disruption of *AMP1* causes hypersensitivity to ABA in *Arabidopsis*

In order to identify novel components involved in the plant response to ABA, mutants that were hypersensitive to high concentrations of ABA were isolated by screening individual lines derived from an *Arabidopsis* T-DNA insertion collection with Col-0 background (Qin *et al.*, 2003). One mutant, *amp1-20*, was selected for further study (Fig. 1a). Genetic analysis showed that no T-DNA insertions in *amp1-20* were linked to the mutant phenotype. Consequently, positional cloning was used to map the mutation locus to a 200-kb region on the bottom arm of chromosome 3 between the markers F24B22 and F28P10 (Fig. S1a). The sequencing of candidate genes in this region revealed a 37-bp deletion at the C-terminus of the *AMP1* gene (Chaudhury *et al.*, 1993; Hellwell *et al.*, 2001). This deletion causes a frameshift and generates a premature stop codon (Fig. S1b). RT-PCR showed that this deletion has no obvious effects on the transcription of the truncated *AMP1* fragment (Fig. S1c).

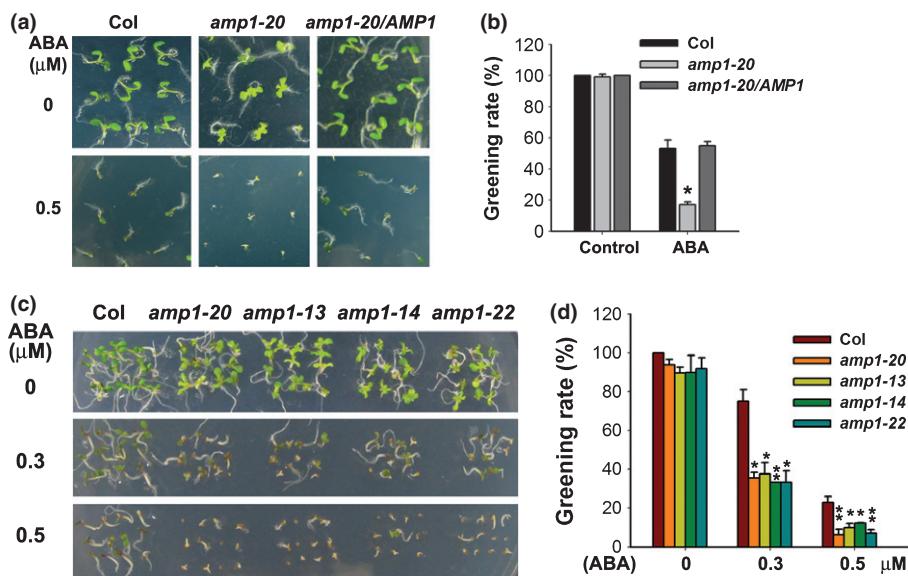


Fig. 1 Arabidopsis seed germination of *amp1* mutants in response to abscisic acid (ABA). (a, b) The wild-type, *amp1-20* and complement transgenic lines grown on MS medium supplemented with 0 or 0.5 μM ABA. Photographs were taken 6 d after the end of stratification (a). The greening rates (b) given are the means of three replicates ± SD ($n = 30$ for each). *, $P < 0.05$ (Student's *t*-test). Three independent experiments were carried out with similar results. (c, d) Seed germination of wild-type and *amp1* mutants on MS medium supplemented with 0, 0.3 or 0.5 μM ABA. Photographs were taken 6 d after the end of stratification (c). The greening rates (d) given are the means of three replicates ± SD ($n = 30$ for each replicate). **, $P < 0.01$; ***, $P < 0.001$ (Student's *t*-test). Three independent experiments were carried out with similar results.

In order to determine whether the hypersensitivity of *amp1-20* could be attributed to the loss of *AMP1* function, three T-DNA insertion lines, *amp1-13*, *amp1-14* (Griffiths *et al.*, 2011) and *amp1-22* (SALK_138749), were isolated (TAIR; <http://www.arabidopsis.org>). There was no detectable full-length *AMP1* transcript in any of these three mutants (Fig. S1c). All three mutants exhibited significant hypersensitivity to exogenous ABA in seed germination compared to the wild-type (Fig. 1c,d). Similar to *amp1-1* (Chaudhury *et al.*, 1993), *amp1-20* showed severe morphological defects, including more leaves, and shorter petioles and primary roots than the wild-type (Fig. S1d).

We next transformed *amp1-20* with a wild-type genomic fragment harboring the 5.4-kb *AMP1* genomic region. All of the transgenic lines that were obtained complemented the *amp1-20* phenotypes in terms of ABA sensitivity and morphology, as listed above (Fig. 1a), further confirming *amp1-20* as a new mutant allele of *AMP1*.

ABA response during germination in *amp1-20* and *AMP1* overexpressing plants

In order to further explore the role of *AMP1* in ABA response, we generated transgenic plants overexpressing *AMP1* fused with GFP (*AMP1-OX*) under the control of a Super promoter derived from octopine and mannopine synthase genes (Ni *et al.*, 1995) in the wild-type Col and *amp1-20* mutant. The *AMP1-OX* lines in *amp1-20* fully rescued the *amp1-20* morphological phenotypes (Fig. S1d), indicating the *AMP1-GFP* is functional. We chose two independent lines (*AMP1-OX2* and *AMP1-OX7*) in the Col background – in which *AMP1* protein was overexpressed (Fig. 2a) – for further analysis. These transgenic plants were indistinguishable morphologically from wild-type plants.

In contrast with *amp1*, the *AMP1-OX* lines displayed significantly early greening on ABA-free medium (Fig. 2b,c). Furthermore, the *AMP1-OX* lines showed increased ABA insensitivity in

assays of ABA-conferred inhibition of cotyledon greening (Fig. 2d,e). Taken together, these results indicate that *amp1* mutants are hypersensitive to ABA, while *AMP1* overexpression reduces ABA sensitivity; thus implicating *AMP1* plays a role in ABA response during the germination stage.

ABA response in seedling growth in *amp1-20* and *AMP1* overexpressing plants

Next, we investigated the function of *AMP1* in the response of postgermination growth to ABA. Seedlings were grown on MS medium for 4 d and subsequently transferred to MS medium containing 30 μM or 50 μM ABA for 10 d, wild-type seedlings showed an increasing inhibition of leaf emergence and primary root growth with increased amounts of ABA. Compared with wild-type plants, the primary root growth of *amp1-20* was significantly arrested by ABA. By contrast, the growth retardation by ABA was obviously suppressed in the *AMP1-OX2* line (Fig. 2f,g). These results indicate that *AMP1* is required for the ABA response at the seedling growth.

Global gene expression analysis in *amp1-20* and *AMP1*-overexpressing plants

Previous study indicated that the homologous genes of *AMP1* in several other species affect ABA metabolism (Suzuki *et al.*, 2008; Kawakatsu *et al.*, 2009). To further dissect the function of *AMP1* in *Arabidopsis*, we performed Affymetrix ATH1 Genome Array with *amp1-20*, *AMP1-OX2* and wild-type Col-0 plants. Then we analyzed microarray data to search for genes that are possibly regulated by *AMP1*. A total of 684 genes were upregulated in the *amp1-20* and downregulated in the *AMP1-OX2* line (Table S2), and 253 genes were downregulated in the *amp1-20* and upregulated in the *AMP1-OX2* line compared with the wild-type (more than two-fold changes, $P < 0.05$; Table S3). The *AMP1* gene was 26-fold upregulated in the *AMP1-OX2* line

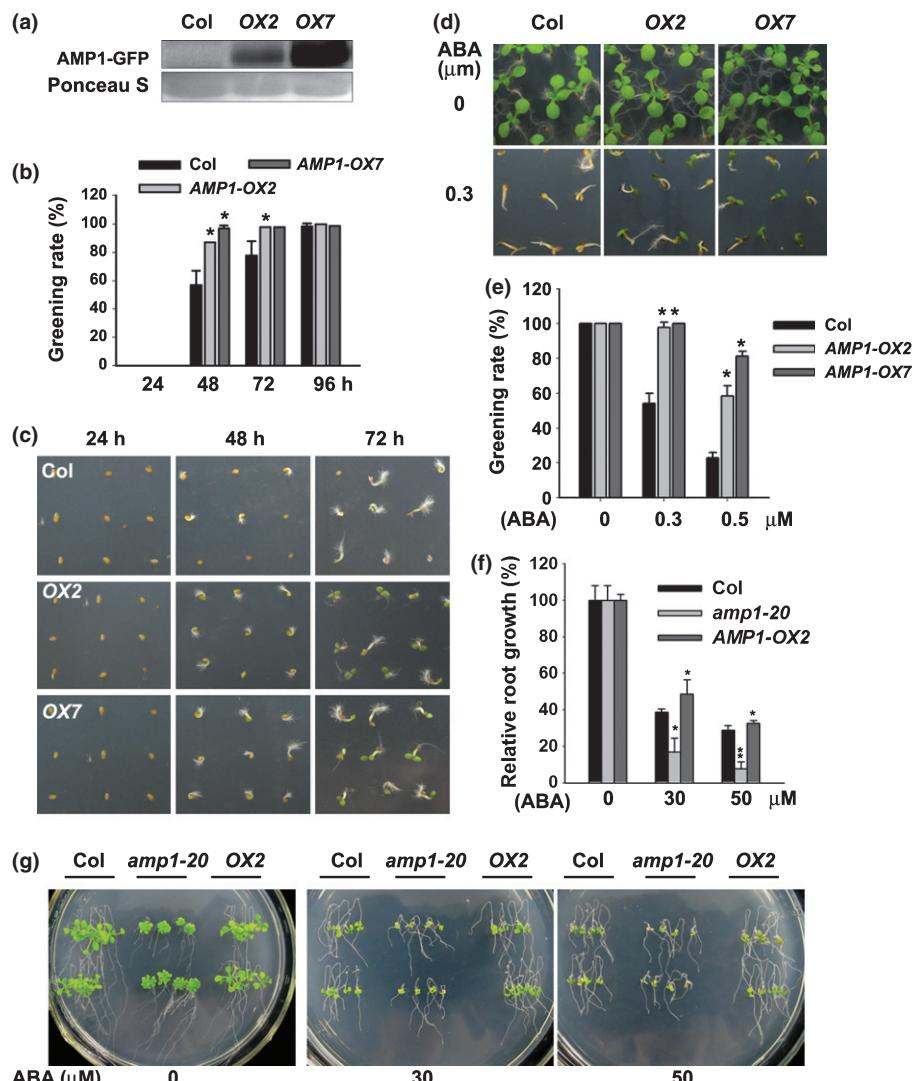


Fig. 2 Arabidopsis seed germination and postgermination growth of *AMP1*-overexpressing lines in response to abscisic acid (ABA). (a) Immunoblot analysis of *AMP1* in 2-wk-old *AMP1*-OX lines with an anti-GFP antibody. (b, c) Seed germination of wild-type and *AMP1*-OX lines on MS medium. The greening rates given are the means of three replicates \pm SD ($n = 30$ for each replicate) (b). *, $P < 0.05$ (Student's *t*-test). (d, e) Seed germination of *AMP1*-OX lines on MS media supplemented with 0 or 0.3 μ M ABA. Photographs were taken 6 d after the end of stratification (d). The greening rates given are the means of three replicates \pm SD ($n = 30$ for each replicate) (e). *, $P < 0.05$ (Student's *t*-test). Three independent experiments were carried out with similar results. (f, g) The effect of ABA on the seedling growth of *amp1* mutants and *AMP1*-OX lines. Seedlings were grown on MS media for 4 d and transferred to MS medium supplemented with 0, 30 or 50 μ M ABA. Relative root growth (f) was measured and photographs (g) were taken 10 d after ABA treatment. The data presented are the means of three replicates \pm SD ($n = 30$ for each replicate) (f). *, $P < 0.05$; **, $P < 0.01$ (Student's *t*-test). Three independent experiments were carried out with similar results.

compared with wild-type, indicating that our microarray experiment is efficient. In total, there were 937 genes whose expression was changed oppositely in the *amp1-20* and *AMP1*-OX2 lines. Amongst these genes, we identified 30 cytochrome P450 genes and six amylase related genes, which are consistent with the previous study of Helliwell *et al.* (2011). We classified these genes with microarray data analysis tool Mapman (Ariel *et al.*, 2012; Fig. S2). The most significantly changed expressing genes in phytohormone categories belong to the IAA, ABA and ethylene pathways (Fig. S2a). A group of endoplasmic reticulum (ER) stress-associated genes such as heat shock protein (*Hsp*) genes dramatically decreased; while cold and drought stress-related gene as well as dismutase/catalase genes increased in *amp1-20* mutant (Fig. S2b,c). Three enzymatic families were also changed obviously, which include UDP glucosyl and glucuronyl transferases, cytochrome P450 families and glutathione-S-transferases (Fig. S2d). Based on the transcriptomic analysis, we suggest *AMP1* affects many metabolic processes including phytohormones, oxidative stress, UDP glucosyl and glucuronyl transferases, and amylase metabolisms.

ROS accumulation in the *amp1-20* mutant

It has been reported that osmotic stress stimulates ROS production to regulate seed germination and stress response via activating the ABA signalling pathway (Mustilli *et al.*, 2002; Kwak *et al.*, 2003). We therefore monitored the accumulation of ROS in the *amp1-20* mutant. Two-week-old mutant and wild-type seedlings were treated with NBT or DAB, which (respectively) stain two major ROS, superoxide (O_2^-) and hydrogen peroxide (H_2O_2). NBT staining revealed a higher concentration of O_2^- accumulated in *amp1-20* seedlings (Fig. 3a). By contrast, DAB staining showed that H_2O_2 accumulation in mutant and wild-type was at similar, very low concentrations (Fig. S3a). We then detected H_2O_2 by fluorescence using DCFH-DA staining. To our surprise, there was less extensive fluorescent spotting observed in the *amp1-20* mutant than in the wild-type leaves (Fig. S3b). In plants, O_2^- molecules are catalyzed by the main antioxidant enzymes superoxide dismutases (SODs) to form H_2O_2 . Next we examined the SOD activities of *amp1*, *AMP1*-OX and wild-type seedlings. SOD activities were decreased in *amp1* mutants but

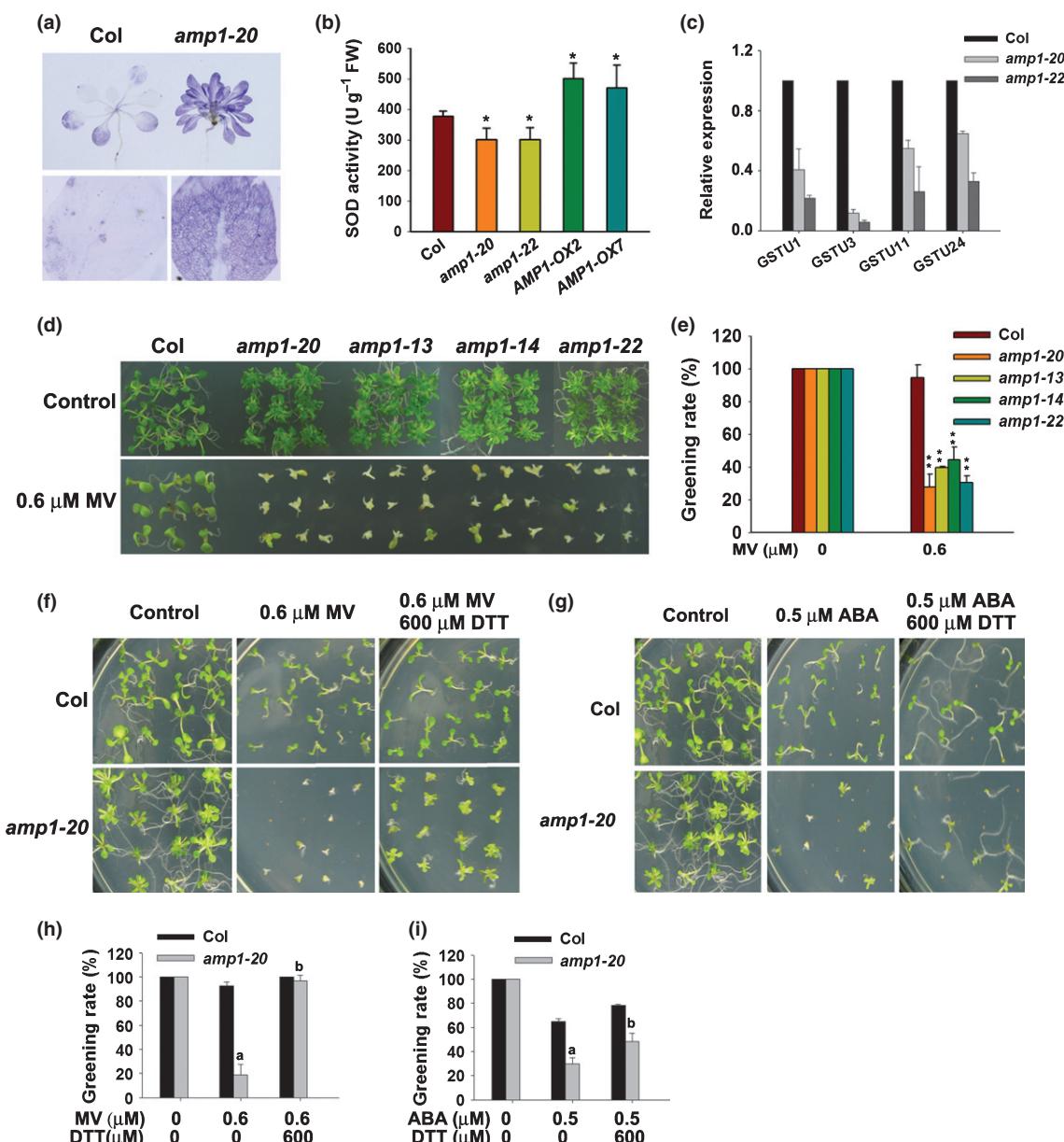


Fig. 3 Phenotype of *amp1*- and *AMP1*-overexpressing *Arabidopsis* plants in response to oxidative stress. (a) NBT staining of 2-wk-old *amp1-20* and wild-type seedlings. (b) SOD enzyme activity of *amp1* and *AMP1-OX* seedlings. The data presented are the means \pm SD. *, $P < 0.05$ (Student's *t*-test). Three independent experiments were carried out with similar results. (c) Expression of *GST* genes by qRT-PCR. Data represent the means of three replicates \pm SD. *, $P < 0.05$ (Student's *t*-test). Three independent experiments were carried out with similar results. (d, e) Seed germination on MS medium supplemented with 0 or 0.6 µM MV. Photographs were taken 12 d after the end of stratification (d). The greening rates given are the means of three replicates \pm SD ($n = 30$ for each) (e). ***, $P < 0.01$ (Student's *t*-test). Three independent experiments were carried out with similar results. (f, g) Seed germination of *amp1-20* and wild-type on 0.6 µM MV (f), or 0.5 µM ABA (g) medium supplemented with or without 600 mM DTT. (h, i) Quantification of seed germination of *amp1-20* and wild-type described in (f, g). The data presented are the means of three replicates \pm SD ($n = 30$ for each replicate). Samples of *amp1-20* with different letters are significantly different. $P < 0.05$ (Student's *t*-test). Three independent experiments were carried out with similar results.

increased in *AMP1-OX* lines compared with the wild-type (Fig. 3b). This result suggests that AMP1 might affect the activity of SOD enzymes, which results in the over accumulation of O_2^- in *amp1* mutants.

Glutathione S-transferases (GSTs) are thought to play major roles in oxidative stress. One of the *Arabidopsis* GSTs named GUST17 has been recently reported to affect the accumulation

of GSH and ABA, mutations of *GUST17* exhibit ABA hypersensitivity during seed germination (Chen *et al.*, 2012). Using qRT-PCR, we found that expression of a set of *GST* genes significantly decreased in the *amp1-20* mutant compared with the wild-type (Fig. 3c), which is consistent with our microarray data analysis (Fig. S2d). Moreover, we examined total glutathione ((GSH) + (GSSG)) content and reduced glutathione ratio

((GSH) : ((GSH) + (GSSG))) in *amp1-20* and *AMP1-OX2* seedlings. Less total glutathione accumulated in *amp1-20*, while more total glutathione accumulated in the *AMP1-OX2* seedlings than in the wild-type (Fig. S3c). However, compared with the wild-type, no significant difference of the (GSH) : ((GSH) + (GSSG)) ratio was observed in *amp1-20* and *AMP1-OX2* seedlings (Fig. S3c).

Methyl viologen (MV) is an electron donor that induces oxidative stress to damage plant photosynthesis. In a seed germination assay, we found that the cotyledon greening rates of *amp1-20* were significantly decreased in the presence of 0.6 µM MV (Fig. 3d,e). These results indicate that *amp1* mutants are hypersensitive to oxidative stress.

Next we asked whether the increased ROS concentrations in *amp1* mutants were correlated with their MV and ABA hypersensitive phenotypes. In the presence of the ROS scavenging agent dithiothreitol (DTT), the MV-conferred cotyledon greening inhibition of *amp1-20* was almost completely rescued (Fig. 3f,h). The ABA-conferred cotyledon greening inhibition of both *amp1-20* and the wild-type was partially compromised by DTT (Fig. 3g,i). Moreover, the relative rescue degree of *amp1-20* by DTT was higher than that of wild-type (Fig. 3g,i). These results suggest that AMP1 mediates ABA and MV responses at least partially dependent on ROS.

The freezing tolerance of the *amp1-20* mutant

Based on the results that the endogenous homeostasis of ABA and ROS has been disrupted in *amp1-20*, we wondered whether its responses to abiotic stress are affected. Therefore, we examined the freezing tolerance of *amp1*. Intriguingly, *amp1-20* exhibited constitutively freezing-tolerant phenotypes. Nonacclimated *amp1-20* mutants were more tolerant to freezing at -6°C than wild-type seedlings (Fig. 4a,b). To determine whether the *amp1* mutation also affects cold acclimation, we tested the freezing tolerance of seedlings pre-grown at 4°C for 4 d and then treated them at -8°C for 1 h. The *amp1* mutants showed higher survival rates at -8°C than the wild-type seedlings (Fig. 4a,b). These results demonstrate that *amp1* mutations affect both the basal and acquired freezing tolerances of plants. Besides *amp1-20* mutant, several other *amp1* mutants exhibited the enhanced freezing tolerance (Fig. 4c).

The best-characterized cold signaling pathway in plants is the CBF/DREB1 transcriptional regulatory cascade (Thomashow, 1999). We next examined whether the cold-regulated genes in the CBF pathway are involved in the freezing tolerance of *amp1-20*. qRT-PCR analysis showed that the levels of *CBF1-CBF3* in *amp1-20* were similar to those in wild-type plants before or after cold treatment (Fig. S4a). However, the expression of *RD29A* in *amp1-20* was consistently higher than in the wild-type with or without cold treatment (Fig. S4a,b). *COR47* was also upregulated in *amp1-20* under cold stress (Fig. S4a). Our microarray analysis also showed that several *COR* genes, including *COR15a*, *COR15b*, *RD29B*, and *COR78* were upregulated in *amp1-20* (Table S2). Therefore, constitutive expression of *COR* genes could be attributable to the enhanced freezing tolerance of *amp1-20*.

The drought tolerance of *amp1-20* and *AMP1-OX* plants

We also explored whether *amp1* and *AMP1-OX* plants have an altered response to drought stress. Wild-type, *amp1* and *AMP1-OX* plants were grown for 2 wk in soil and then subjected to dehydration treatment for an additional 2 wk. After rewatering, the survival rate of the wild-type was 44% and nearly all *amp1-20* plants survived; however, the survival rates for *AMP1-OX2* and *AMP1-OX7* plants were only 3% and 8%, respectively (Fig. 4d,e). These results demonstrate that *amp1* mutants enhance drought tolerance, while overexpression of *AMP1* confers reduced drought tolerance.

The expression of ABA biosynthesis and signaling genes in *amp1-20* and *AMP1-OX* plants

Based on the above observations, we hypothesized that the altered ABA sensitivity of *amp1* and *AMP1-OX2* seedlings might be attributed to the regulation of the expression of ABA biosynthesis or signaling genes by *AMP1*. To test this hypothesis, we evaluated the transcript levels of ABA biosynthesis genes by qRT-PCR in *amp1* and *AMP1-OX2* plants. In the absence of ABA, the transcript levels of *ABA1*, *ABA3* and *NCED3* were clearly higher in *amp1* than in wild-type plants (Fig. 4f). Moreover, the ABA-induction of *ABA1*, *ABA3* and *NCED3* was more pronounced in *amp1* than in the wild-type.

The expression of ABA signaling (*ABI1* and *ABI5*) and ABA responsive (*ABF3*) genes was also examined in *amp1* and *AMP1-OX2* seedlings. The ABA-induced expression of *ABI1* and *ABI5* was increased in *amp1*, and decreased in *AMP1-OX2* seedlings compared to the wild-type (Fig. 5a). Consistently, the transcript levels of *ABF3* induced by ABA were consistently greater in *amp1* compared to the wild-type (Fig. 4f). Therefore, *AMP1* negatively regulates the expression of genes in ABA biosynthesis and signaling pathway.

ABA concentrations in *amp1-20* and *AMP1-OX* plants under stress

In order to further examine whether AMP1 plays a role in the regulation of ABA biosynthesis, we measured the endogenous ABA contents of *amp1-20* and *AMP1-OX2* seedlings. Under normal conditions, low concentrations of ABA were found in both *amp1-20* and *AMP1-OX2* seedlings, similar to the concentration observed in wild-type plants. The endogenous ABA content was dramatically elevated after 40% PEG treatment for 6 h. However, the ABA content in *amp1-20* was higher than that in the wild-type under the same conditions. By contrast, the ABA content in *AMP1-OX2* seedlings was lower than that in the wild-type (Fig. 4g). These results indicate that *AMP1* negatively regulates stress-induced ABA concentrations in *Arabidopsis*.

The effect of ABA on AMP1 at the mRNA and protein levels

Because the *amp1* mutants were hypersensitive to ABA, we asked whether the *AMP1* transcript level was regulated by ABA.

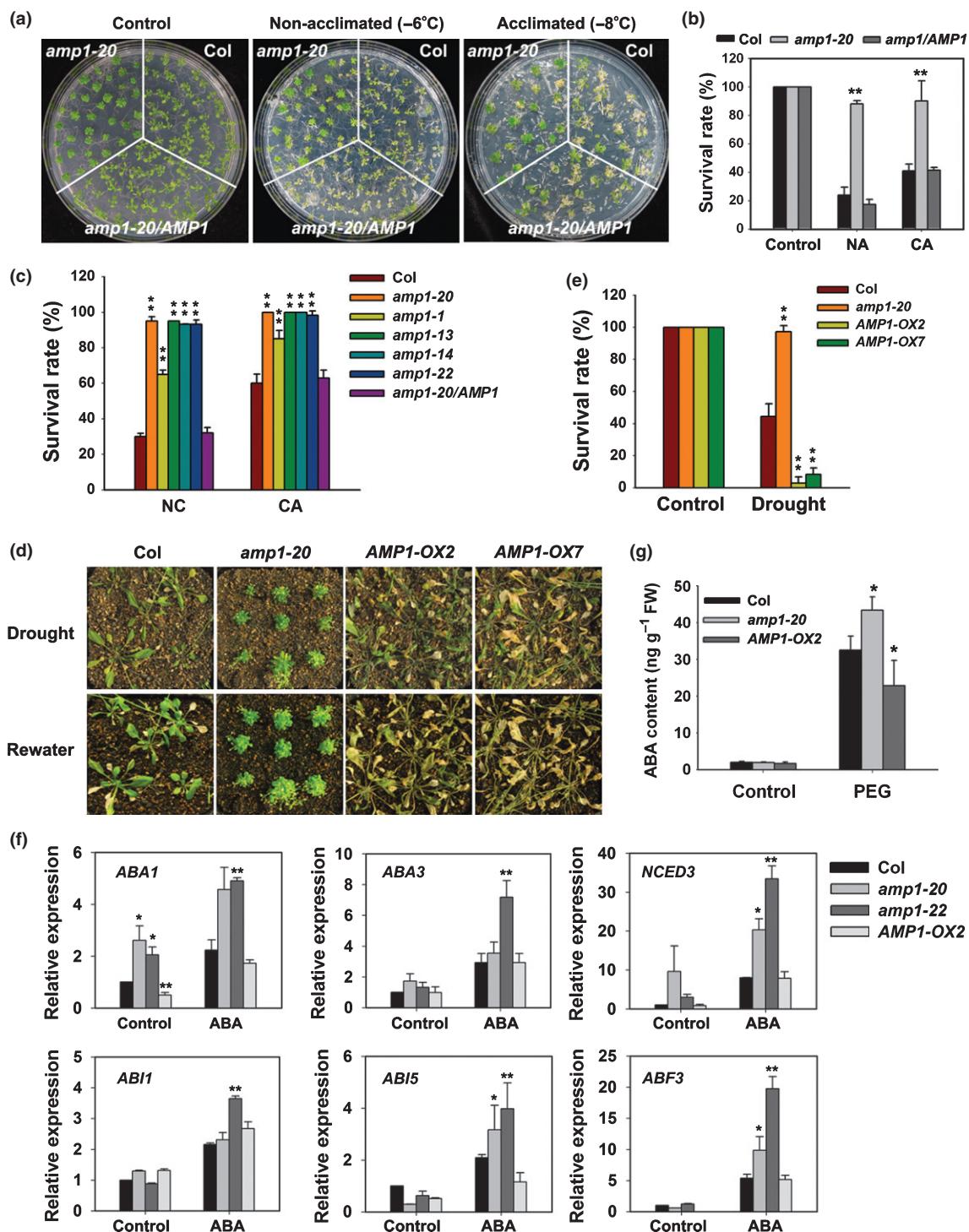


Fig. 4 Freezing and drought tolerance in *amp1* mutants. (a, b) Freezing assay of *amp1-20*. Two-week-old light-grown *Arabidopsis* seedlings were treated at -6°C for 1 h (nonacclimated (NA)) or -8°C for 1 h after 4°C treatment for 4 d (acclimated (CA)). Photos were taken after 2 d of incubation at 22°C (a), and survival seedlings that can re-grow were scored after 2-d recovery (b). The data shown represent the means of three replicates \pm SD ($n = 30$ for each replicate). **, $P < 0.01$ (Student's *t*-test). Similar results were observed in four independent experiments. (c) Survival rate of *amp1* T-DNA insertion lines after freezing treatment. The data shown represent the means of three replicates \pm SD ($n = 30$ for each replicate). **, $P < 0.01$ (Student's *t*-test). Similar results were observed in three independent experiments. (d, e) Survival rates of *amp1-20* and *AMP1-OX* plants under drought stress. Plants were grown in soil for 4 wk without application of water. The photos were taken at 4 d after rewetting (d). The data presented are the means of three replicates \pm SD ($n = 30$ for each replicate). **, $P < 0.01$ (Student's *t*-test). Similar results were observed in three independent experiments. (f) The effect of *AMP1* on expression of abscisic acid (ABA)-related genes. Two-week-old seedlings were treated with $50 \mu\text{M}$ ABA for 6 h. Transcription levels were determined by qRT-PCR. The data represent the means of three replicates \pm SD. Similar results were observed in at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$ (Student's *t*-test). (g) ABA content in *amp1-20* and *AMP1-OX2* plants. Two-week-old seedlings were treated with 40% PEG 8000 for 6 h. The data presented are the means of three replicates \pm SD. *, $P < 0.05$ (Student's *t*-test). Similar results were observed in three independent experiments.

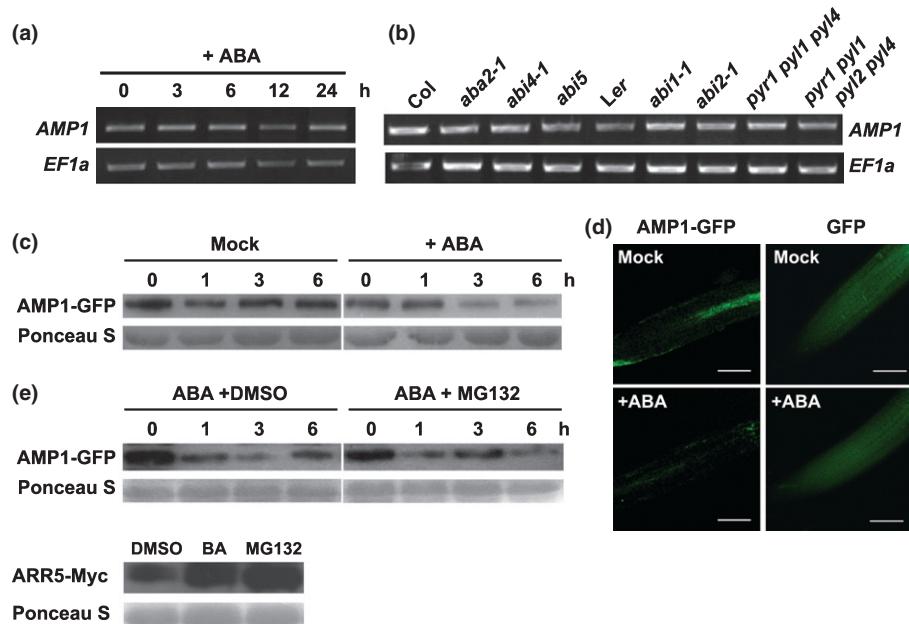


Fig. 5 *AMP1* gene and protein expression in response to abscisic acid (ABA). (a) *AMP1* gene expression in response to ABA. Two-week-old *Arabidopsis* seedlings were treated with 50 μM ABA for the indicated times. (b) *AMP1* gene expression in mutants deficient in ABA biosynthesis and signaling. (c) *AMP1* protein expression in response to ABA. Ten-day-old *AMP1-OX2* transgenic seedlings were treated with 500 μM CHX for 2 h, followed by 0 or 50 μM ABA for 0–6 h, and then subjected to immunoblot assays with an anti-GFP antibody. (d) Visualization of the *AMP1-GFP* in roots. Five-day-old *AMP1-OX2* and *Super:GFP* (as a control) seedlings were treated with 50 μM ABA for 6 h. Bars, 100 μm. (e) The analysis of *AMP1* protein concentrations in the presence of MG132. Ten-day-old *AMP1-OX2* seedlings (upper panel) were treated with 500 μM CHX for 2 h, followed by 50 μM ABA plus 50 μM MG132 or DMSO for the indicated times. 35S:ARR5-Myc plants (Ren *et al.*, 2009) (lower panel) treated with 50 μM MG132, 50 μM BA or DMSO for 6 h were used to test the efficacy of the proteasome inhibitor MG132. The total proteins were then subjected to immunoblot assays with an anti-GFP or anti-Myc antibody. Similar results were repeated in three independent experiments.

qRT-PCR showed that *AMP1* expression in wild-type plants was not affected by exogenous ABA (Fig. 5a). We next analyzed the expression of *AMP1* in mutants deficient in ABA biosynthesis and signaling, including *aba2-1*, *abi4-1*, *abi5*, *abi1-1*, *abi2-1*, *pyr1 pyl1 pyl4* and *pyr1 pyl1 pyl2 pyl4* (Park *et al.*, 2009). *AMP1* expression was consistently unchanged in the genotypes tested compared to the wild-type (Fig. 5b), indicating that the transcript level of *AMP1* is not regulated by endogenous ABA biosynthesis and signaling.

We then investigated whether ABA influences the *AMP1* protein concentration. *AMP1-OX2* seedlings were treated with 50 μM ABA and concentrations of *AMP1-GFP* were detected by immunoblot analysis. The *AMP1* protein concentration decreased after 3 h of ABA treatment, but remained unchanged in the absence of ABA treatment (Fig. 5c). Consistently, the *AMP1-GFP* signal was strongly reduced in the root after 6 h of ABA treatment in *AMP1-OX2* plants (Fig. 5d). Thus, ABA may affect *AMP1* accumulation at the post-transcriptional concentration.

In order to determine whether the *AMP1* protein degradation occurs via the 26S ubiquitin-proteasome pathway, we treated *AMP1-OX2* seedlings with the proteasome inhibitor MG132 (Smalle & Vierstra, 2004). MG132 treatment did not inhibit the ABA-induced *AMP1* degradation (Fig. 5e). These results suggest that the ABA-induced *AMP1* protein degradation is not mediated by the 26S proteasome pathway.

ABA hypersensitivity and freezing tolerance of *amp1 abi* double mutants

In order to further dissect the genetic interactions between *amp1* and the ABA signaling pathway, we generated double mutants of *amp1-20* with five ABA signaling mutants: *abi1-1* (Meyer *et al.*, 1994), *abi2-1* (Leung *et al.*, 1997), *abi3-1* (Giraudat *et al.*, 1992), *abi4-1* (Finkelstein *et al.*, 1998) and *abi5* (Finkelstein & Lynch, 2000a). As *abi1-1*, *abi2-1* and *abi3-1* are in the *Ler* background; the double mutant combinations of these *abi* mutants with *amp1-20* are in the mixed background. The *abi* mutants partially to fully rescued the ABA hypersensitive phenotype of *amp1-20* during germination (Fig. 6a,b). This result indicates that the ABA hypersensitivity of *amp1-20* was at least partially suppressed by these *abi* mutants.

In order to decipher the correlation between the ABA signaling and freezing tolerance of *amp1-20*, the phenotypes of the *amp1 abi* double mutant were analyzed under freezing conditions. Among these double mutants, the *amp1 abi1* and *amp1 abi3* seedlings showed survival rates intermediate between the *amp1-20* mutant and the *abi* single mutants (Fig. 6c,d). Accordingly, the ion leakages of *amp1 abi1* and *amp1 abi3* double mutants were higher than that of the *amp1* seedlings, but lower than that of the *abi* mutants after freezing treatment (Fig. 6e), indicating that *abi1* and *abi3* partially compromised the freezing tolerance of *amp1-20* seedlings. These results suggest that the freezing

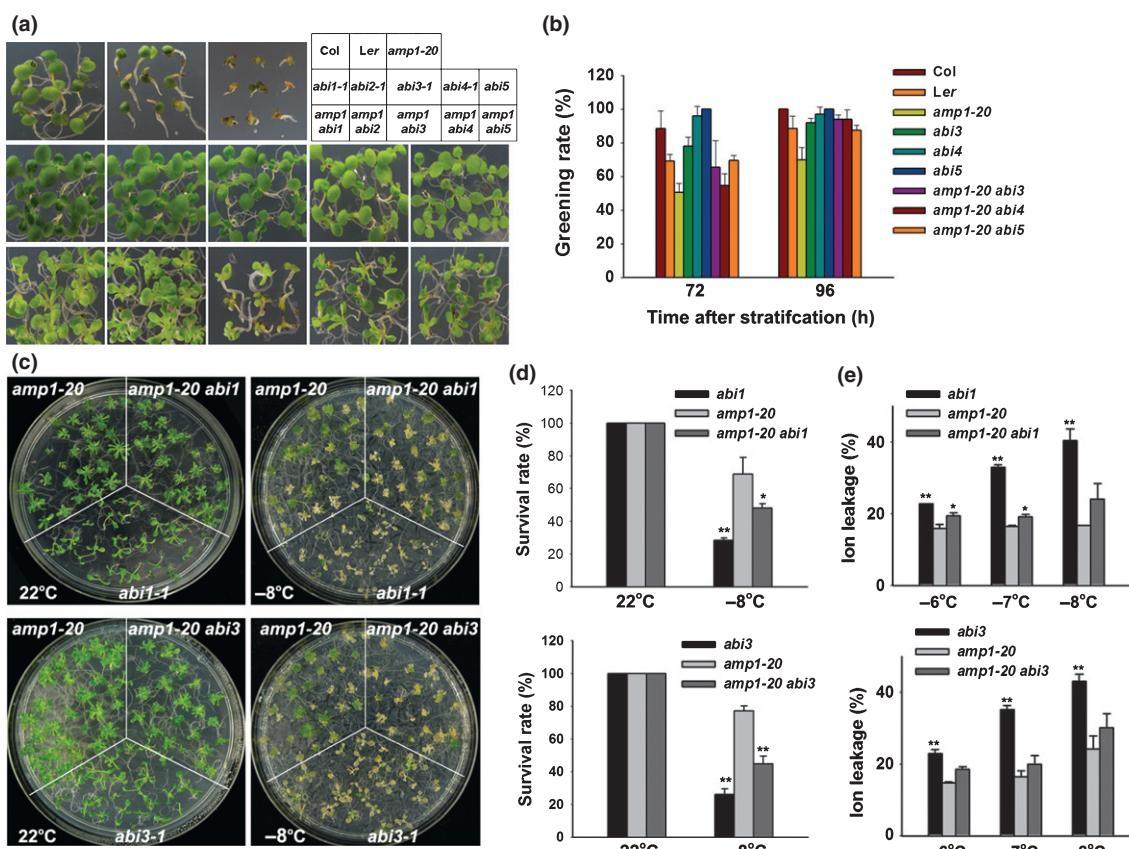


Fig. 6 Genetic interactions between *amp1-20* and *abi* mutants in response to abscisic acid (ABA) and freezing stress. (a) Arabidopsis seed germination in *amp1-20 abi* double mutants grown on MS medium supplemented with 0.5 µM ABA. The photographs were taken 10 d after ABA treatment. (b) The greening rates for the plants in (a) were measured. The data presented are the means of three replicates ± SD ($n = 50$ for each replicate). Similar results were observed in three independent experiments. (c) Phenotypes of seedlings after freezing treatment at -8°C. Two-week-old seedlings of *abi1*, *amp1-20*, and *amp1-20 abi*, were treated at -8°C for 1 h, followed by recovery at 22°C for 4 d. (d) Survival rates of *amp1 abi1* and *amp1 abi3* double mutants in (c). (e) Ion leakage of *amp1 abi1* and *amp1 abi3* double mutants after freezing treatment. The data represent the means of three replicates ± SD. Similar results were observed in four independent experiments. *, $P < 0.05$; **, $P < 0.01$ (Student's *t*-test), indicating a significant difference when compared to the *amp1-20* mutant.

tolerance of *amp1-20* seedlings is partially dependent on ABA signaling.

Deletion of membrane domain affects AMP1 function

AMP1 protein is reported to localize to the ER membrane (Vidaurre *et al.*, 2007). To determine whether the membrane localization is important for AMP1 function, we generated a truncated form of AMP1 fused to GFP under the control of a Super promoter (*AMP1ΔN-GFP*), in which the N-terminal membrane-localization signal peptide was deleted, and transformed this construct into *amp1-20* to generate *AMP1ΔN-GFP/amp1-20* plants (Fig. 7a). The *AMP1ΔN-GFP* protein was not localized at the membrane any more (Fig. 7b). All the *AMP1ΔN-GFP/amp1-20* transgenic plants showed growth defects and ABA hypersensitive phenotypes which are similar to *amp1-20* (Fig. 7c,d), indicating that the membrane localization of AMP1 is required for its functions.

In plants, increasing evidence indicates that abiotic stress-associated ROS production induces ER stress in plant cell (Chu *et al.*, 2010; Jaspers & Kangasjarvi, 2010). To determine whether AMP1 can affect plant ER-stress response, we tested the

phenotype of the *amp1-20* and *AMP1-OX2* lines in the presence of 0.01 nM ER stress-inducer tunicamycin (TM). Wild-type and *AMP1-OX2* seedlings exhibited the same growth inhibition after germination for 2 wk; however, the growth of the *amp1-20* mutant was totally arrested under the same conditions (Fig. S5). Together with the effect of *amp1* mutations on ROS accumulation, these results suggest that a lack of AMP1 contributes to ER stress via disruption of ROS homeostasis.

Changes in AMP1 expression affect sugar accumulation and amino acid metabolism

Sugar plays an important role during plant growth and development, and moderates abiotic stress by regulating carbohydrate metabolism. High sugar accumulation is reported to be increased under freezing stress to protect enzymes and cell membrane from dehydration (Price *et al.*, 2004). The concentration of total soluble sugar was two-fold higher in *amp1* mutants than in the wild-type at 22°C (Fig. 8a).

In our microarray data, a set of amylase related genes were upregulated in the *amp1-20* mutant (Table S2). The enzyme

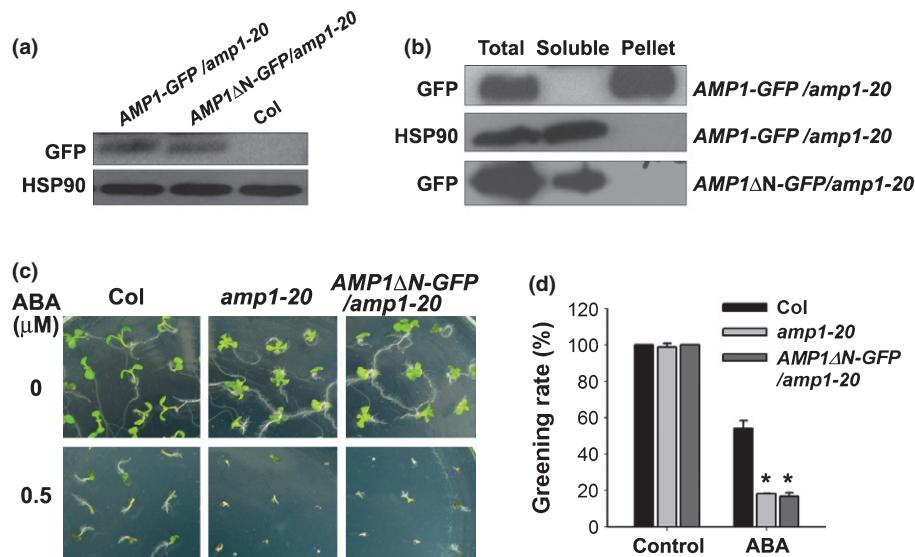


Fig. 7 The membrane localization of AMP1 is required for its function in response to abscisic acid (ABA). (a) AMP1 expression in *AMP1ΔN-GFP amp1-20* Arabidopsis plants by immunoblot analysis with an anti-GFP antibody. Ponceau S was used as a loading control. (b) Expression of *AMP1ΔN-GFP* in *AMP1ΔN-GFP amp1-20* plants. The soluble and pellet fractions were separated by ultracentrifugation, and then subjected to immunoblot assays with an anti-GFP or anti-HSP90 antibody. (c, d) Response of *AMP1ΔN-GFP amp1-20* plants to ABA. The wild-type, *amp1-20*, *AMP1-GFP amp1-20* and *AMP1ΔN-GFP amp1-20* mutants were grown on MS medium supplemented with 0 or 0.5 μM ABA. Photographs (c) were taken 6 d after the end of stratification. The greening rates (d) given are the means of three replicates ± SD ($n=30$ for each replicate). *, $P<0.05$ (Student's *t*-test).

β-amylase is essential for the breakdown of starch to provide an immediate source of soluble sugars during cold stress (Kaplan & Guy, 2004; Kaplan *et al.*, 2007). We analyzed the expression of β-amylase genes, including *BAM3* and *BAM9*, in *amp1* seedlings grown at 22°C. Expression of *BAM3* and *BAM9* was higher in *amp1* mutants than that in the wild-type; however, *BAM3* expression in *AMP1-OX2* plants was lower than that in the wild-type (Fig. 8b). This finding is consistent with a previous study showing that β-amylase genes are upregulated in plants bearing *amp1/pt* alleles (Helliwell *et al.*, 2001). Moreover, the expression of the galactinol and raffinose synthase genes *AtGolS1*, *AtGolS2* and *AtGolS3* were also upregulated in *amp1* mutants, while *AtGolS2* and *AtGolS3* were downregulated in *AMP1-OX2* plants (Fig. 8b).

We further investigated whether *amp1* and *AMP1-OX* lines exhibited an altered sugar response. With 5% and 7% glucose, the relative primary root lengths were much shorter in the *amp1* mutants and higher in the *AMP1-OX* lines compared to the wild-type (Fig. 8c,d). These results indicate that AMP1 negatively regulates sugar accumulation and sugar sensitivity. Furthermore, the *amp1 abi3*, *amp1 abi4*, and *amp1 abi5* double mutants showed dramatically decreased sensitivity to glucose during greening (Fig. S6), demonstrating that *abi3*, *abi4* and *abi5* could at least partially rescue the sugar sensitivity of *amp1-20*.

Proline (Pro) is an osmolyte that accumulates under abiotic stress to protect plants. We tested whether *amp1-20* accumulates more Pro than the wild-type. Intriguingly, the accumulation of Pro dramatically decreased before and after 4°C treatment (Fig. S4c). This observation prompts us to propose that AMP1 as a small peptidase may also modulate amino acid metabolism. To test this hypothesis, total amino acids were extracted from

2-wk-old *Arabidopsis* seedlings, and were measured by HPLC. Twelve out of 23 amino acids measured, including Arg, Cit, Gly, His, Leu, Lys, Orn, Phe, Ser, Trp, Tyr and Val, significantly decreased in *amp1* mutants compared to the wild-type. By contrast, Asn, Glu and Try increased in the *AMP1-OX* lines compared to the wild-type (Fig. 8e). Taken together, our results suggest that AMP1 is associated with sugar and amino acid metabolism.

Discussion

Glutamate carboxypeptidases are ubiquitous in various species of eukaryotes (Rawlings & Barrett, 1997; Barinka *et al.*, 2008), but the functions of AMP1 are not yet well characterized in plants. Two homologs of AMP1, VP8 in maize and PLA3 in rice, have been shown to regulate the homeostasis of ABA, and loss-of-function in these two genes results in an ABA-deficient phenotype in seeds, such as viviparity and insensitivity to ABA stress (Suzuki *et al.*, 2008; Kawakatsu *et al.*, 2009). One recent study showed that the ABA levels of three *amp1* alleles in three different accessions are different, but do not correlate with the level of seed dormancy (Griffiths *et al.*, 2011). In this study, we isolated a new allele of *AMP1* and made a series of new observations of loss-of-function *amp1* mutants especially in response to abiotic stress. Also we identified some antagonistic phenotypes of *AMP1*-overexpressing lines. These findings shed more light on understanding the functional mechanism of *AMP1* gene in *Arabidopsis*.

At present, the diverse biological functions of AMP1 and homologs in different plant species are still elusive due to their unknown substrates. *Arabidopsis amp1* mutants were

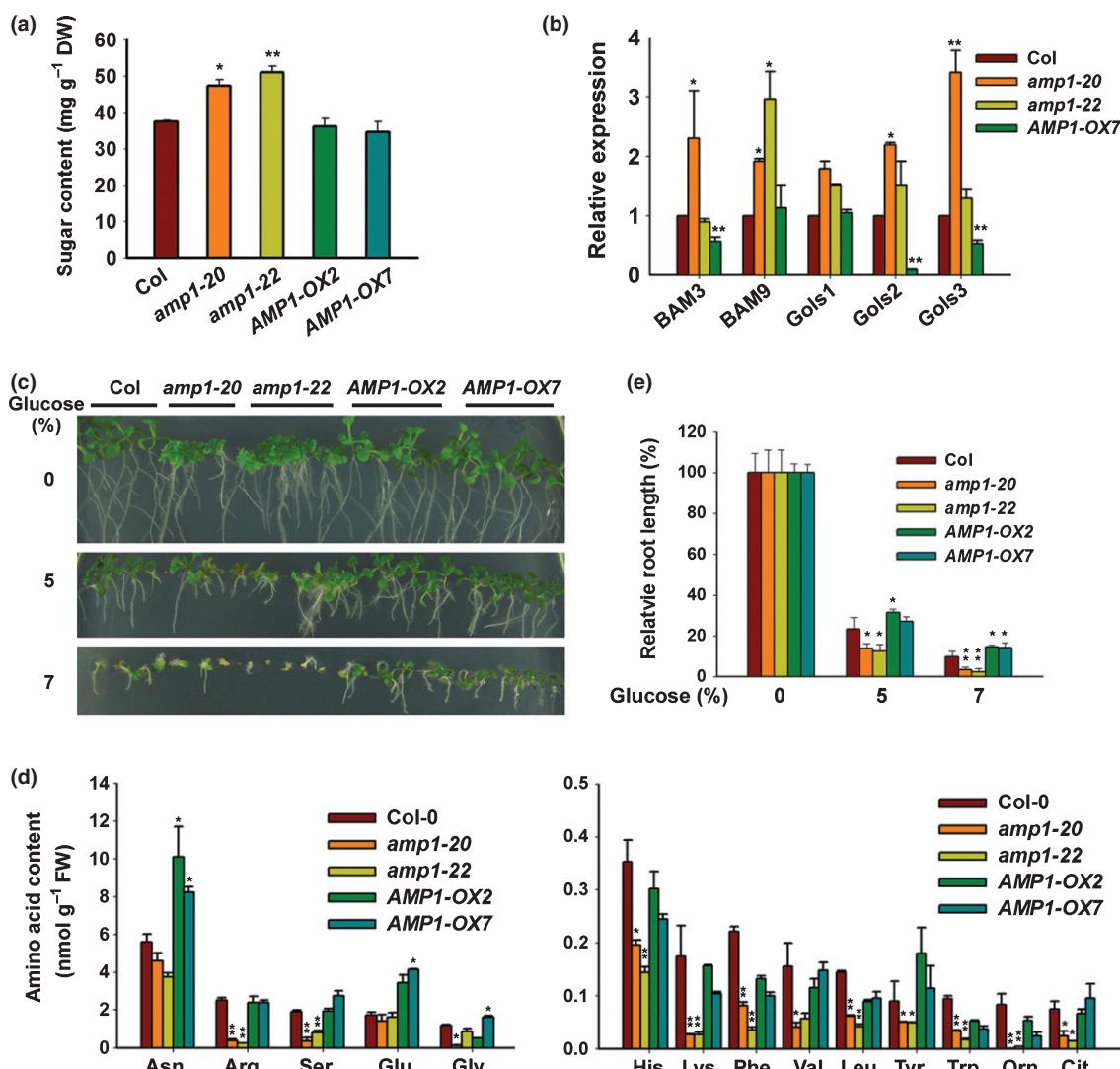


Fig. 8 Sugar and amino acid content of *amp1* and *AMP1*-overexpressing plants. (a) Sugar content in 2-wk-old plants grown at 22°C. The data presented are the means of three replicates \pm SD. *, $P < 0.05$; **, $P < 0.01$ (Student's *t*-test). Similar results were observed in three independent experiments. (b) The expression of sugar biosynthesis genes in 2-wk-old plants grown at 22°C. The data represent the means of three replicates \pm SD. Similar results were observed in at least three independent experiments. (c, d) Primary root growth of *amp1* and *AMP1*-OX plants on MS media supplemented with different concentrations of glucose (5–7%). Photographs (c) were taken and primary root growth (d) was measured on 10 d after glucose treatment. The data presented are the means of three replicates \pm SD ($n = 30$ for each experiment). *, $P < 0.05$; **, $P < 0.01$ (Student's *t*-test). (e) Amino acids were prepared from 2-wk-old seedlings and measured by HPLC. The data presented are the means of three replicates \pm SD. *, $P < 0.05$; **, $P < 0.01$ (Student's *t*-test).

hypersensitive to ABA during seed germination and postgermination growth. These phenotypes of *amp1* in *Arabidopsis* are opposite with the phenotypes in monocots (Suzuki *et al.*, 2008; Kawakatsu *et al.*, 2009). Conversely, the overexpression of *AMP1*-induced early germination and ABA insensitivity during germination and postgermination growth. Moreover, elevated endogenous ABA was detected in *amp1* under osmotic stress, which is consistent with the upregulation of genes involved in ABA biosynthesis, signaling and ABA-associated abiotic stress. Intriguingly, *AMP1* protein might be influenced by stress-induced ABA accumulation because the exogenous ABA treatment negatively regulated *AMP1* protein concentration. As expected, the *abi* mutants, including two dominant negative mutations in *ABI1* and *ABI2*, and the mutations in *ABI3*, *ABI4*

and *ABI5*, obviously rescued the ABA hypersensitivity and freezing tolerance of *amp1-20*, which further indicates that the phenotype of *amp1-20* is dependent on ABA signaling. Thus, we conclude *AMP1* is a negative regulator of the ABA biosynthesis pathway under stress conditions in *Arabidopsis*.

ABA stimulates the accumulation of ROS, especially H₂O₂ and superoxide. It has been shown that *atrbohD/atrbohF* double mutants display ABA insensitive phenotype during seed germination and root growth due to their impaired stress-induced ROS production and ABA signaling pathway (Kwak *et al.*, 2003). In our study, we interestingly found that superoxide was overproduced in *amp1* mutants, but less H₂O₂ was detected in *amp1* mutants. This is probably due to the reduced activity of SOD in *amp1* mutants. Therefore, *AMP1* probably mediates cellular

ROS detoxification in *Arabidopsis*. This hypothesis is also supported by the hypersensitive response of *amp1* mutants to MV-induced oxidative stress. Application of exogenous ROS scavenging agent DTT alleviates growth inhibition of *amp1* and wild-type by ABA, with more alleviation trends in *amp1*. Thus, the mutation of *AMP1* probably stimulates the production of ROS, thereby impairing the plant response to ABA. However, ROS imbalance in *amp1* is not the only determinant for its ABA-hypersensitive phenotype, because application of DTT could not totally restore *amp1-20* to the wild-type phenotype in response to ABA.

Plants exhibiting enhanced freezing and drought tolerance often accumulate more osmolytes, such as soluble sugar and proline. Consistent with its freezing and drought tolerance, an increased concentration of soluble sugar was detected in *amp1* mutants. The inhibition of growth by high sugar concentration was also observed in *amp1* mutants. Previous studies indicate that sugar has a tight connection with ABA during seed germination. Screening for sugar-insensitive mutants identified several ABA signaling or biosynthesis deficient mutants, including *aba2*, *abi4* and *abi5* (Arenas-Huertero *et al.*, 2000; Laby *et al.*, 2000). Indeed, the primary root length inhibition of *amp1* by high concentration of glucose could be compromised by five *abi* mutations. These results suggest that the high concentrations of sugar in *amp1* could account for the hypersensitivity of ABA.

Proline is proven to be an important osmolyte (Nanjo *et al.*, 1999). Despite its enhanced freezing tolerance, *amp1* contained much less proline than the wild-type. Moreover, proline is not the only amino acid with a lower content in *amp1*. Of 23 amino acids measured, most decreased significantly in *amp1*, except for Cys, Met and a further nine amino acids. Thus, *amp1* seems to be amino acid starving and disturbs the balance of carbon and nitrogen (C/N) metabolism. Carbon assimilation is required for amino acids biosynthesis, and C/N metabolism is subject to ABA regulation and ROS generation (Foyer *et al.*, 2003; Taylor *et al.*, 2004). Previously, *Arabidopsis* glutamate receptors (AtGLR1.1) have been shown to coordinate C/N metabolism and ABA synthesis; the loss-of-function of *AtGLR1.1* results in sensitivity to elevated exogenous C:N ratio and ABA (Kang & Turano, 2003; Kang *et al.*, 2004). It has also been reported that altered carbon metabolism in both chloroplasts and mitochondria could generate excessive ROS (Suzuki *et al.*, 2012). Enzymes involved in TCA cycle are sensitive to oxidative stress, which leads to the decrease in amino acids (Baxter *et al.*, 2007). Thus, the imbalance of C/N metabolism might induce ROS accumulation in *amp1* and, in turn, causes its sensitivity to oxidative stress. Considering the potential peptidase activity of AMP1, it is tempting to speculate that AMP1 and/or its natural substrates might be responsible for the activity of N-metabolic enzymes.

In summary, we proposed a working model depicting the action of AMP1 in the regulation of abiotic stress responses (Fig. 9). AMP1 protein is negatively regulated by abiotic stress, possibly through ABA. The *amp1* mutations disturb the balance of C/N metabolism, which may promote ABA biosynthesis and impair ROS scavenging system. On the one hand, ABA

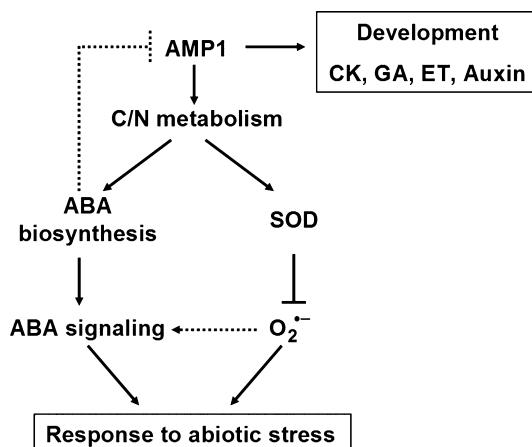


Fig. 9 The proposed working model of AMP1 in abiotic stress. AMP1 acting as an ER-located carboxypeptidase is important in regulating the balance of carbon (C)/nitrogen (N) metabolism. In *amp1* mutants, imbalanced energy metabolism disturbs the cellular hormone homeostasis via affecting abscisic acid (ABA) biosynthesis and signaling. The stability of AMP1 protein is negatively regulated by ABA. In addition, activation of reactive oxygen species (ROS) signaling also occurs due to the disturbance of ROS scavenging system in *amp1* mutants. Hence, AMP1 serves as a negative regulator to modulate freezing and drought stress responses. CK, cytokinin; GA, gibberellin; ET, ethylene.

biosynthesis and signaling activate transcription of stress responsive genes and enhance abiotic stress tolerance of *amp1*; on the other, imbalance of ROS scavenging system fails to protect cells from oxidative stress, which may induce cellular ROS burst and ER-stress. This finding raises the possibility that AMP1 catalyzes its natural substrates to regulate C/N metabolism, thereby modulating ABA and abiotic stress.

Acknowledgements

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Phenotypes of the *amp1* mutants.

Fig. S2 Hormone pathways, stress and metabolic process regulated in *amp1-20* and *AMP1-OX2* plants.

Fig. S3 ROS accumulation in *amp1* mutant.

Fig. S4 Relative expression of cold responsive genes and proline content in *amp1-20*.

Fig. S5 The response of *amp1-20* to TM.

Fig. S6 Postgermination growth of *amp1-20* and *amp1 abi* double mutants on MS media supplemented with different concentrations of glucose.

Table S1 Gene-specific primers used in this study

Table S2 Differentially expressed genes in *amp1-20* and *AMP1-OX2* plants

Table S3 Differentially expressed genes in *amp1-20* and *AMP1-OX2* plants

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Supporting information

Shi et al. (2013) The glutamate carboxypeptidase AMP1 mediates ABA and abiotic stress responses in *Arabidopsis*

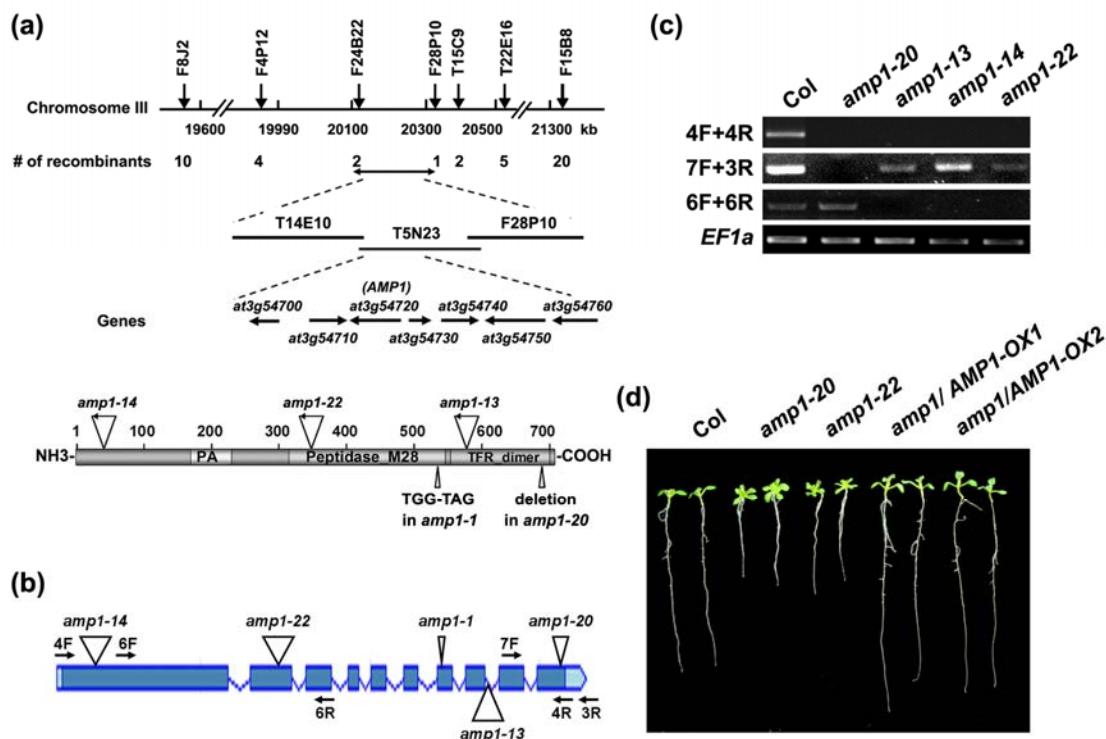


Fig. S1 Phenotypes of the *amp1* mutants.

(a) Positional cloning of the *AMPI* gene. The positions of the markers used for mapping are indicated above the line. The corresponding nucleotide positions are numbered in kilobases below the line. A schematic diagram of the AMP1 protein, indicating the PA (protease associated), Peptidase M28, and TFR (transferrin receptor) dimer domains, is indicated in the bottom panel. The mutation positions of the *amp1* mutants are shown.

(b) Schematic diagram of genomic fragment of the *AMPI* gene. Exons are represented by dark blue boxes, UTR regions are presented by light blue boxes, and intron regions

are presented by lines. The primer positions used in Fig. S1b are shown with arrows.

(c) *AMP1* expression in *amp1* mutants by qRT-PCR.

(d) Morphology of *amp1* mutants and *AMP1-OX* transgenic plants in *amp1-20* background.

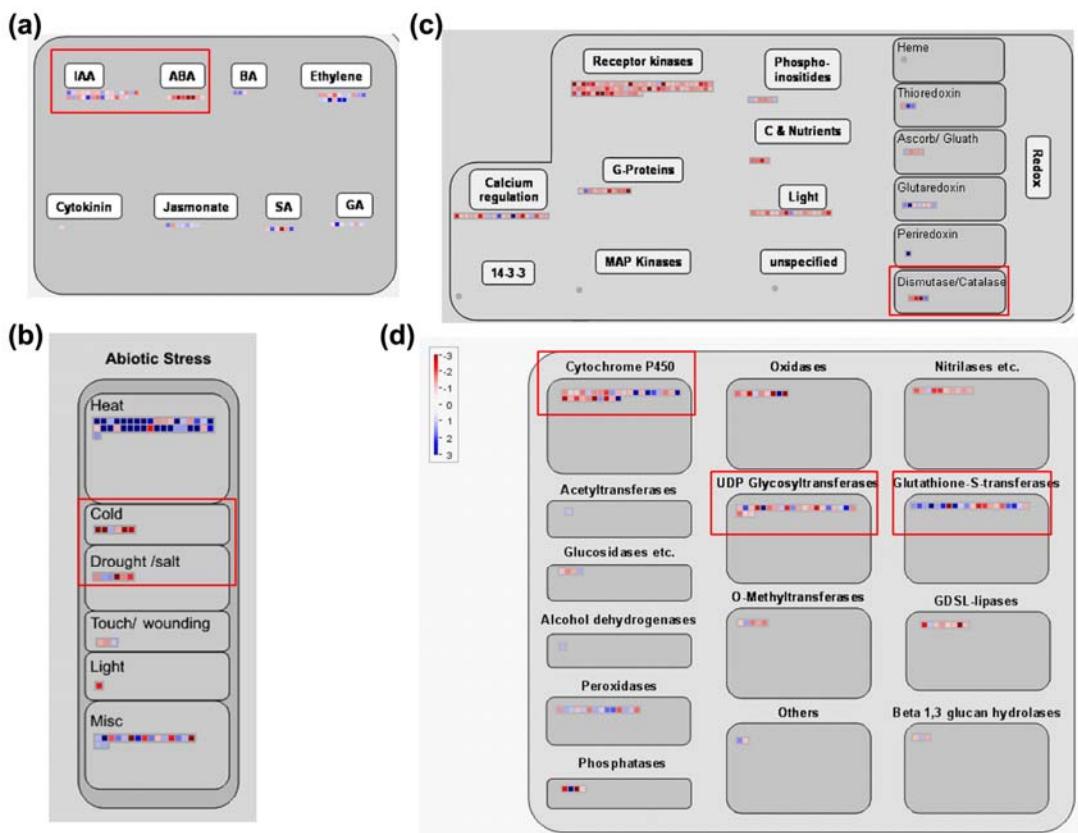


Fig. S2 Hormone pathways, stress and metabolic process regulated in *amp1-20* and *AMP1-OX2* plants.

Totally 360 genes regulated by *AMP1* (> 2 -fold change, $P < 0.05$) were loaded into Mapman software. Changed genes are shown with the color code scale bar (based on log₂ ratios) (blue: induction, and red: repression). (a) Mapman hormonal “Regulation Overview” pathway. (b) Mapman “Cellar response overview” pathway. (c) Mapman “Regulation Overview” pathway. (d) Mapman “Large Enzymatic Families” pathway.

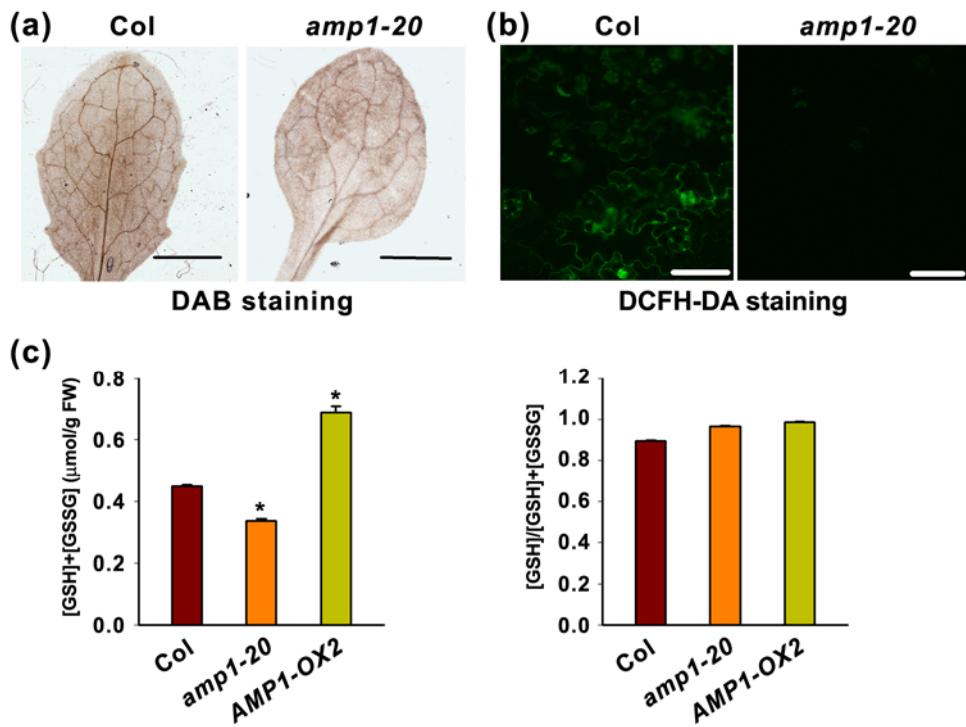


Fig. S3 ROS accumulation in *amp1* mutant.

- (a) DAB staining of 2-week-old *amp1-20* and wild type seedlings. Bars = 0.2 cm
- (b) DCFH-DA staining of 2-week-old *amp1-20* and wild type seedlings. Bars = 50 μm
- (c) Levels of glutathione in *amp1-20* and *AMP1-OX2* seedlings (2-week-old). Data represent the means of three replicates ± SD. * $P<0.05$ (Student *t*-test).

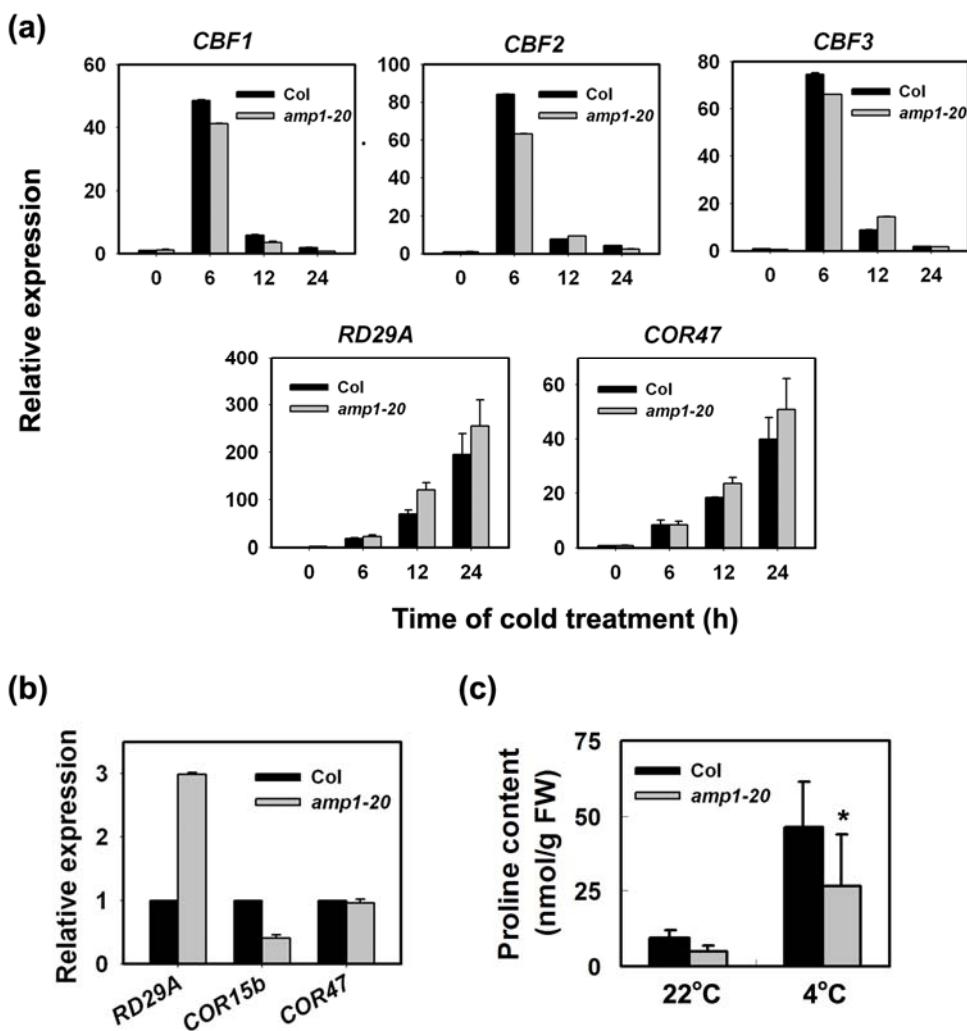


Fig. S4 Relative expression of cold responsive genes and proline content in *amp1-20*.

(a, b) Relative expression of cold responsive genes in *amp1-20* with (a) and without (b) cold treatment. Two-week-old seedlings grown at 22°C were treated at 4°C for the indicated times. Transcription levels were determined by quantitative RT-PCR. The data represent the means of three replicates \pm SD. (c) Proline content in the *amp1-20* mutant. The plants grown at 22°C for 2 weeks were treated at 4°C for 6 d. Data represent means of three replicates \pm SD. * $P < 0.05$ (Student's *t*-test). Similar results were observed in three independent experiments.

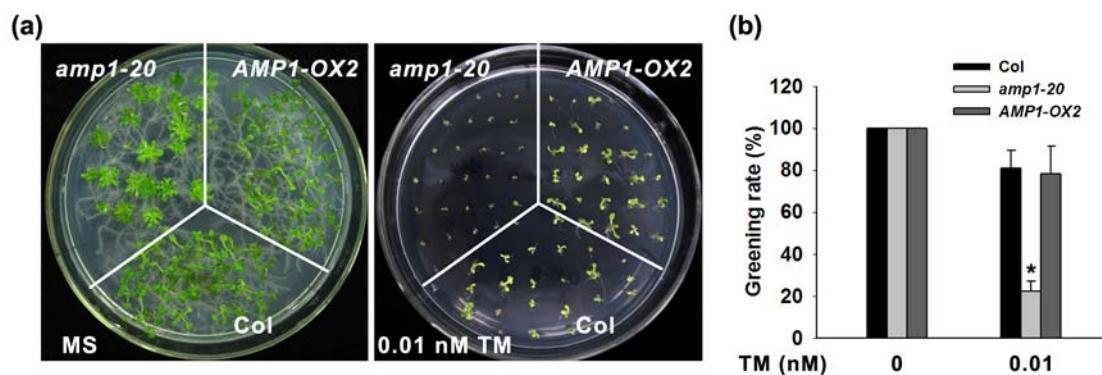
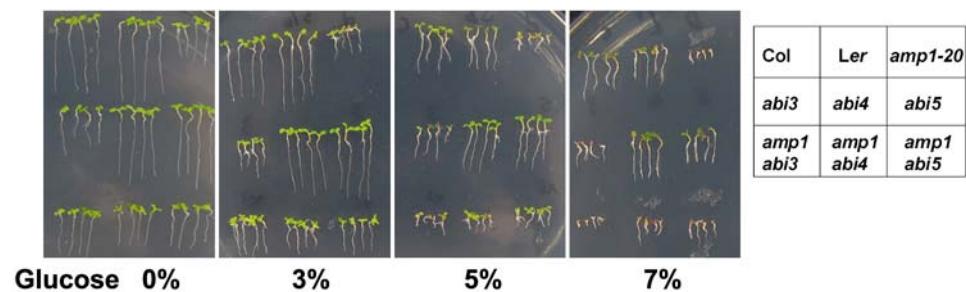


Fig. S5 The response of *amp1* to tunicamycin.

The wild-type, *amp1-20* and *AMP1-OX2* transgenic plants grown on MS medium supplemented with 0 or 0.01 nM tunicamycin (TM). Photographs were taken at 14 d after the end of stratification (a). The greening rates (b) given are the means of three replicates \pm SD (n=30 for each). *P < 0.01 (Student's *t*-test). Three independent experiments were done with similar results.

(a)



(b)

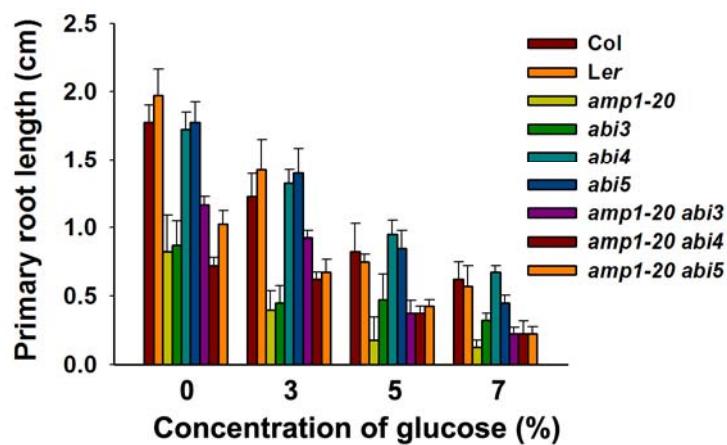


Fig. S6 Post-germination growth of *amp1-20* and *amp1 abi* double mutants on MS media supplemented with different concentrations of glucose.

- (a) Seedling growth of double mutants of *amp1-20* with *abi* on MS medium supplemented with 0%, 3%, 5% and 7% glucose. The pictures were taken after 6 d of treatment.
- (b) Primary root length for the plants mentioned in (a). Percentages are means of three replicates \pm SD.

Table S1. Gene-specific primers used in this study.

Primer name	Primer sequence (5'-3')
For genotyping and cloning	
<i>AMP1-1F</i> (For <i>amp1-20</i> genotyping)	GAAGGGATCAAAGGGAAGGA
<i>AMP1-1R</i>	CGCTAGGTCTCGTCGAATC
<i>AMP1-3F</i> (for complementation)	CATCCTCATGGTGAGGAAAC
<i>AMP1-3R</i>	GTTAGTCTTGAATATGATTAGA
<i>AMP1-4F</i> (for <i>pSuper:AMP1-GFP</i>)	GACGTC ATGTCACAACCTCTCACCA
<i>AMP1-4R</i>	GGTACC TGTGAAACCTCCTTAAGAGC
<i>AMP1-5F</i>	CTGCAG ATGCCAGACGCCGTTACTCCTCC
<i>AMP1-5R</i>	CTGCAG TGTGAAACCTCCTTAAGAGC
For RT-PCR	
<i>AMP1-6F</i>	CCATGTCGCCGAATACGAAGCT
<i>AMP1-6R</i>	CGACAAGAACCGTCTAAT
<i>AMP1-7F</i>	GAAGGGATCAAAGGGAAGGA
For Real-time-PCR	
<i>COR47</i> (At1g20440)- <i>F</i>	GAAGCTCCCAGGACACCACGAC
<i>COR47</i> - <i>R</i>	CAGCGAATGTCCCCTCTCCAC
<i>RD29A</i> (At5g52310)- <i>F</i>	ATCACTTGGCTCCACTGTTGTT
<i>RD29A</i> - <i>R</i>	ACAAAACACACATAAACATCCAAAGT
<i>COR15b</i> - <i>F</i>	CTCACTGACAAAGCCGATGA
<i>COR15b</i> - <i>R</i>	AGACGAAGGGATCACAATGC
<i>AtABA1</i> (At5g67030)- <i>F</i>	GAGGATTACAAACGGGCTCA
<i>AtABA1</i> - <i>R</i>	GGGCTCATTGGATTCTTCA
<i>AtABA3</i> (At1g16540) - <i>F</i>	TCAAAACCGGTGAATCAACT
<i>AtABA3</i> - <i>R</i>	TTCGTCTCCTCTCCTCCTTC
<i>AtNCED3</i> (At3g14440) - <i>F</i>	AGAGTGTGCCTTGTATGGTTTA
<i>AtNCED3</i> - <i>R</i>	CATCCTCTCTACAATAGTTCGCT
<i>ABII</i> (At4g26080)- <i>F</i>	CAATAAGAGAGGGATAGCGAACGAG
<i>ABII</i> - <i>R</i>	CGTCCATTGCTGTCTCCTCCA
<i>ABI5</i> (At2g36270)- <i>F</i>	CTTTGTTGATGGTGTGAGTGAG
<i>ABI5</i> - <i>R</i>	GTGTTCCACTATTACCATTGC
<i>ABF3</i> (At4g34000)- <i>F</i>	GGTAACATTGTGCTCAGTGGTGG
<i>ABF3</i> - <i>R</i>	AACGACCTTAATCTCATGCTGC
<i>ACTIN2/8</i> - <i>F</i>	
<i>ACTIN2/8</i> - <i>R</i>	