ahg12 is a dominant proteasome mutant that affects multiple regulatory systems for germination of Arabidopsis

Shimpei Hayashi1,† & Takashi Hirayama2

The ubiquitin-proteasome system is fundamentally involved in myriad biological phenomena of eukaryotes. In plants, this regulated protein degradation system has a pivotal role in the cellular response mechanisms for both internal and external stimuli, such as plant hormones and environmental stresses. Information about substrate selection by the ubiquitination machinery has accumulated, but there is very little information about selectivity for substrates at the proteasome. Here, we report characterization of a novel abscisic acid (ABA)-hypersensitive mutant named ABA hypersensitive germination12 (ahg12) in Arabidopsis. The ahg12 mutant showed a unique pleiotropic phenotype, including hypersensitivity to ABA and ethylene, and hyposensitivity to light. Map-based cloning identified the ahg12 mutation to cause an amino acid conversion in the L23 loop of RPT5a, which is predicted to form the pore structure of the 19S RP complex of the proteasome. Transient expression assays demonstrated that some plant-specific signaling components accumulated at higher levels in the ahg12 mutant. These results suggest that the ahg12 mutation led to changes in the substrate preference of the 26S proteasome. The discovery of the ahg12 mutation thus will contribute to elucidate the characteristics of the regulated protein degradation system.

As sessile organisms, plants cannot move and therefore need internal mechanisms to respond to environmental conditions. Accordingly, plants have developed unique response and adaptation systems to cope with the environment, including environmental stresses. Physiological and genetic studies have shown that plants are able to sense tiny environmental changes, such as fluctuations in light intensity, CO2 concentration, temperature, various chemicals, minerals, and water pressure, as well as touch and attack by pathogens or animals. The stimuli received from environmental changes are transduced to nuclei, where these multiple stimuli seem to be processed and integrated to evoke adequate and timely regulation of sets of genes for environmental adaptation.1–6

Recent studies on gene regulatory systems in plants have highlighted the importance of the ubiquitin-proteasome protein degradation system in the modulation of gene expression responding to environmental and developmental stimuli.7,8 Light is an important signal for photosynthetic organisms and represents one of the major developmental cues for plants, regulating germination, skotomorphogenesis, flowering, and senescence. The activity of key transcriptional factors, such as HY5 and PIFs, in the gene regulatory system responsive to light stimuli, is modulated by the ubiquitin proteasome system.9

Plant hormones also play pivotal roles in developmental processes and responses to environmental stresses. These compounds function as signaling molecules in the long-distance cell-cell communication systems in plants.10,11 In the last decades, the molecular mechanisms underlying the signaling pathways of plant hormones from signal perception to the resulting gene regulation have been elucidated significantly. Interestingly, proteasomal protein degradation is deeply involved in the all of the plant hormone signaling pathways.7,12 For example, indole acetic acid (IAA), or auxin, which is major developmental regulator, is recognized by a protein complex composed of a ubiquitin E3-ligase named TIR1 and its target protein AUX/IAA.13–15 IAA stabilizes the interaction

1Graduate School of Nanobioscience, Yokohama City University, 1-7-29 Suehiro, Tsurumi, Yokohama 230-0045, Japan. †Institute of Plant Science and Resources, Okayama University, 2-20-1 Chuo, Kurashiki, Okayama 710-0046, Japan. Correspondence and requests for materials should be addressed to T.H. (email: hira-t@okayama-u.ac.jp)
between TIR1 and AUX/IAA and facilitates the degradation of this inhibitory protein to activate auxin-responsive gene expression. In addition, EIN3, the pivotal transcription factor in the ethylene signaling pathway, is unstable and degraded by the ubiquitin-proteasome system in the absence of ethylene, whereas it becomes stable and activates ethylene-responsive genes in the presence of ethylene16,17. The proteasome is also involved in responses to abscisic acid. ABI5 and ABI3, important transcription factors involved in the abscisic acid response in the early germination stage, are targets of the ubiquitin-proteasome system18,19.

The proteasome is a very large protein complex composed of the catalytic 20S core particle (CP) and 19S regulatory particle (RP)20. The CP comprises four heptameric rings (two α1-α7 rings and two β1-β7 rings) forming a barrel-like structure, in which the substrate protein is degraded in an ATP-dependent manner. The RP consists of 19 subunits and can be subdivided into the lid subcomplex and the base subcomplex. The lid subdomain contains a barrel-like structure, in which the substrate protein is degraded in an ATP-dependent manner. The RP consists of non-ATPase subunit proteins RPN1-RPN13. Among them, RPN10 and RPN13 have the ability to bind ubiquitin while RPN11 removes ubiquitins from the ubiquitinated proteins21-23, suggesting that the lid subcomplex is involved in the recognition of ubiquitinated proteins. The base subcomplex consists of six homologous AAA-ATPases, RPT1-RPT6. These ATPases are thought to unfold the target proteins and deliver them to the CP24,25. Structural analyses of the RP have revealed the relative position of each component and are consistent with the predicted functions of these proteins26-28.

Accumulated evidence suggests that each subunit of RP has specific functions in plant physiological phenomena, presumably through the regulation of protein degradation. RPN10- and RPN12-defective mutants are altered in the responses to plant hormones such as auxin and cytokinin in Arabidopsis29,30. Mutations in RPN1 of Arabidopsis cause embryogenesis and growth defects31,32. Studies of RPT2 mutants indicated RPT2 is required for meristem maintenance and gametophyte or sporophyte development33,34 and that RPT2 is involved in gene silencing via DNA methylation35. A loss-of-function mutation of RPT5a, one of the two RPT5 genes in Arabidopsis, results also in defects in sporophyte development36. Furthermore, RPT2a and RPT5a are required for zinc deficiency-tolerance in Arabidopsis37. This non-redundancy in the function of RPT1-6 has also been reported in the yeast system25,38.

Here we report the novel RPT5a allele of Arabidopsis, ABA hypersensitive germination 12 (ahg12). The ahg12 mutant was isolated based on its weak ABA hypersensitivity at the germination stage39. In this study, we found that this mutant has a pleiotropic phenotype, including ethylene hypersensitivity and diminished dormancy. We found that the ahg12 mutation alters an amino acid residue in RPT5a. This amino acid residue is outside of the ATPase domain but is highly conserved among RPT5 orthologs. Recent structural analyses of the PAN complex of the archaea Methanocoldilinuss jannaschii and the RP complex of fission yeast revealed this residue to be in the L23 loop, which is predicted to form a pore structure of the base subcomplex40-42. Detailed analysis of the ahg12 mutation will provide information related to the function of RPTs and the RP subcomplex.

Results

ahg12 exhibits a pleiotropic phenotype in germination. Compared to the wild type, ahg12 mutant shows greater growth inhibition in response to exogenously applied ABA during seed germination using radicle emergence and post-germination growth as phenotypic markers (Fig. 1). It is unlikely that the enhanced ABA response in the mutant is conferred by an increased amount of endogenous ABA, because the ahg12 mutant was isolated originally from a mutagenized population of aba2-1 seeds that are deficient in ABA biosynthesis and is not the reversion mutant of aba2-1 regarding its mapped position39. Therefore, the ahg12 mutant is more likely to have changes in sensitivity to ABA. The ABA-hypersensitive phenotype was also observed in the F1 progeny of a cross between ahg12 and wild-type plants, indicating that the ahg12 mutation confers ABA hypersensitivity in a dominant manner. To investigate the ABA sensitivity of ahg12 at the seedling stage, we measured the root elongation rate. We found no significant differences between wild type and ahg12 in root growth rate at the seedling stage in the presence of various concentrations of ABA (Fig. S1). These findings suggest that the ABA-hypersensitive phenotype of ahg12 is restricted to the germination or early germination stage.

ABA is involved in the seed dormancy. In some cases, a positive correlation between ABA sensitivity and seed dormancy has been observed43,44. To investigate seed dormancy of the ahg12 mutant, we compared the effects of various stratification periods on seed germination between ahg12 and wild type using seeds harvested on the same date. Interestingly, without stratification, the ahg12 mutant showed higher germination rates than wild type (Fig. 2a). This result indicated that the ahg12 mutant displayed lower seed dormancy despite its enhanced ABA sensitivity. After a 2-day stratification, the seed germination rate of ahg12 was almost equal that of wild type.

The above results suggested that the physiological status of the ahg12 seed during germination is markedly different from that of wild type. Thus, the responses of ahg12 at the germination stages to other stimuli were also investigated. Light is an important stimulus that affects germination of Arabidopsis. Accordingly, we investigated the germination rates of stratified seeds exposed to light for different lengths of time. The ahg12 seeds required longer light exposure to reach a similar rate of germination as the wild-type seeds (Fig. 2b), indicating a reduced responsiveness of ahg12 to light during seed germination.

We next investigated responses of the ahg12 mutants to other phytohormones. The growth-inhibition or -promotion effects of auxin, cytokinin, and gibberellin were not markedly different between ahg12 and wild type in terms of hypocotyl length and root elongation rates (Fig. S2). Interestingly, there was a significant difference in sensitivity to ethylene. When grown on medium containing the ethylene precursor 1-aminoacyclopropene-1-carboxylic acid (ACC), the ahg12 mutant showed clearly shorter roots and dark-grown hypocotyls than wild type, indicating a hypersensitivity to ethylene in the mutant (Fig. 3).

Taken together, our results demonstrate that ahg12 is a mutant with a unique combination of phenotypes: increased sensitivity to ABA, decreased dormancy, decreased responsiveness to light during seed germination, and increased sensitivity to ethylene.
Figure 1. Seed germination of *ahg12* is hypersensitive to ABA. (a) Germinating seeds of *ahg12* in the presence of ABA. Imbibed and stratified seeds of wild type (WT), *ahg12*, and F1 progeny between *ahg12* and WT were grown on plates containing ABA for 7 days. (b) Germination rate of *ahg12* in the presence of ABA. Imbibed seeds (>50) of WT and *ahg12* were stratified and then sown on plates containing ABA. Seeds that showed radicle emergence or post-germination growth (expansion of green cotyledons) were counted. The data are mean of three independent experiments. Error bars indicate standard deviation.
The ahg12 mutation localizes to the pore structure of the RP complex. To identify the ahg12 mutation, genetic linkage analysis of the F2 generation (approximately 800 lines) of a test cross between ahg12 (Col) and wild type (Ler) was undertaken. The ABA-hypersensitive phenotype was linked to a genomic region containing 35 genes on chromosome 3. Using DNA sequencing analysis, a point mutation consistent with effect of the EMS mutagen used to generate these mutants was identified in the At3g05530 gene, which encodes the proteasome RP AAA-ATPase 5a (RPT5a) (Fig. 4a,b). The mutation was located in the third exon of the RPT5a gene and caused an amino acid substitution, Ser112 to Phe. Amino acid sequences of RPT5 orthologs are conserved in eukaryotes such as yeasts and animals, and this Ser112 residue is exceptionally highly conserved (Fig. 4c). However, mutants altered at the corresponding amino acid residue have not been reported previously.

To confirm that this mutation is responsible for the ABA hypersensitivity, we generated the transgenic plants expressing the RPT5a gene with the identified mutation and investigated their ABA sensitivity in seed germination. In spite of possessing a wild-type copy of RPT5a as an endogenous gene, the transgenic plants expressing the ahg12 allele showed higher sensitivity to ABA than wild type (Fig. 4d). This dominant effect of the transgene was
Figure 3. ahg12 is hypersensitive to ethylene. (a) Etiolated seedlings of ahg12 grown on plates containing ethylene precursor. Imbibed seeds of wild type (WT) and ahg12 were stratified for 2 days and then grown on plates with or without 1-aminocyclopropane-1-carboxylic acid (ACC, 10 μM) for 4 days. (b) Hypocotyl length of the etiolated ahg12 seedlings grown on plates containing ethylene precursor. Hypocotyl lengths of the etiolated seedlings (>20) grown on plates containing various concentrations (0, 0.1, and 10 μM) of ACC for 4 days were measured. (c) Root growth rate of ahg12 seedlings grown on plates containing ethylene precursor. Root length of 4-day-old seedlings grown on normal plates were measured, and seedlings were transferred to ACC-containing plates. After 4 days, root length was measured again to calculate the growth rate. The data are means of three independent experiments. Error bars indicate standard deviation. Asterisks indicate significant differences between the corresponding values. (*P < 0.05; **P < 0.01; t-test after arcsine-transformation).
Figure 4. *ahg12* is a novel mutant allele of *RPT5a*. (a) Schematic representation of the *AHG12* gene and the *ahg12* mutation site. A transition mutation (C to T) was detected at codon 112 of the *At3g05530* gene in *ahg12*. White and black boxes indicate untranslated regions and exons, respectively. (b) Schematic representation of *AHG12/RPT5a* protein. The approximate position of the *ahg12* mutation site is shown. (c) Alignment of the polypeptide sequences around the amino acid residues corresponding to the *ahg12* mutation site (red square) of RPT5 from various organisms. Identical amino acid residues are shown with a black background. (d) ABA sensitivity of transgenic plants expressing a modified *RPT5a* gene and T-DNA disruptants of *RPT5a* (*rpt5a*-4). Genomic *RPT5a* with the *ahg12* mutation containing putative promoter and terminator regions was introduced into WT plants (WT + *RPT5a*<sup>S112F</sup>). Imbibed and stratified seeds were sown on plates with or without ABA (0.3 μM) and grown for 7 days.
consistent with the dominance of ahg12, demonstrating that the identified mutation is responsible for the ABA hypersensitivity of ahg12. We also investigated the ABA sensitivity of a T-DNA insertional disruptant mutant of RPT5a (rpt5a-4, SALK_046321, Fig. S3) as a second allele of ahg12. However, the rpt5a-4 mutant did not show ABA hypersensitivity (Fig. 4d). This difference between phenotypes suggests that ahg12 does not cause inactivation of RPT5.

RPT5 is a subunit of the 26S proteasome complex. The dominant effect of ahg12 implied that the mutation affected some function of this complex. To investigate whether the ahg12 mutant is deficient in the protein degradation mediated by the proteasome, the mutant’s sensitivity to canavanine was evaluated. Canavanine, an analog of arginine, is incorporated into newly synthesized proteins and changes physicochemical properties of the proteins. Mutants that are deficient in the ubiquitin-proteasome system show increased sensitivity to the toxicity of proteins containing canavanine\(^2\). Consistent with previous studies, rpt5a-4 showed higher sensitivity to canavanine than wild type, probably due to a lower capacity for removal of toxic proteins (Fig. 5a). This growth-inhibitory effect was prominently visible in the root tissues, which were directly in contact with the medium containing canavanine. By contrast, the canavanine sensitivity of ahg12 was almost the same as that of wild type. This result suggests that the proteasome-mediated protein degradation system is essentially functional in the ahg12 mutant. Consistent with this, the levels of total polyubiquitinated proteins determined by immunoblot analysis using with an antibody recognizing ubiquitinated proteins were similar between ahg12 and wild type (Fig. 5b).

Arabidopsis has two genes that encode RPT5: RPT5a and RPT5b (Fig. S4). To investigate the possibility that the presence of RPT5b contributed to the ABA hypersensitivity in ahg12, we generated a double mutant between ahg12 and a T-DNA disruption mutation in RPT5b (rpt5b-3). The double mutant showed ABA hypersensitivity during seed germination similar to that of the ahg12 single mutant (Fig. 5c, Fig. S5), demonstrating that the ABA-hypersensitive phenotype in ahg12 is independent of RPT5b. It was reported that dysfunction of both RPT5a and RPT5b leads to sterility\(^2\). However, we successfully obtained the double mutant between ahg12 and rpt5b. This result supports the idea that the RPT5a\(^{46}\) is functional as RPT5.

To examine potential functional differences between RPT5a and RPT5b, a recombinant RPT5b gene with an ahg12-like mutation (Ser111 to Phe, RPT5b\(^{S111F}\), Fig. S4) was introduced into wild-type plants. However, unlike the case of ahg12, the obtained transgenic plants, in which RPT5b\(^{S111F}\) is the major RPT5b transcript, did not show any ABA hypersensitivity during seed germination (Fig. S6). This result suggests that there are functional differences other than the gene expression pattern between RPT5a and RPT5b.

Since serine is the major target for protein phosphorylation in eukaryotes, we speculated that the Ser112 of RPT5a might be a phosphorylation site. To investigate this possibility, we generated a construct for recombinant RPT5a in which Ser112 was converted to aspartic acid to mimic phosphorylation and introduced it to wild-type plants. We obtained several independent transgenic lines but did not detect any abnormal ABA sensitivity in germination (Fig. S6).

Based on structural data for the 26S proteasome derived from Saccharomyces cerevisiae\(^2\), the ahg12 mutation site, Ser112, was expected to face toward the pore through which substrate proteins are introduced into the CP (Fig. 7). This information implied that ahg12 might affect the molecular mechanism for substrate uptake in the proteasome. ABA, ethylene, and light responses, which were changed in ahg12, are regulated by key regulators ABI5, EIN3, and PIL5, respectively\(^4\). The activities of these transcriptional regulators are modulated by protein degradation through the ubiquitin-proteasome system, responding to environmental stimuli\(^18,25\). Therefore, as next step, we investigated whether the ahg12 mutation affects accumulation of these signaling components. For this purpose, the ORFs of these transcriptional factors were fused to luciferase (LUC) and the resulting recombinant proteins were transiently expressed in protoplasts derived from ahg12 and wild-type plants. This assay allowed us to quantify the protein levels objectively\(^5\). The results of this assay demonstrated that the ahg12 cells had higher levels of ABI5, EIN3, and PIL5 proteins than did the wild-type cells (Fig. 6). This result suggests that the ahg12 mutation, which converts serine to the more bulky phenylalanine at the pore, decreases the degradation efficiency of the proteasome for some proteins, including ABI5, EIN3, and PIL5.

**Discussion**

We isolated ahg12 as an ABA-related mutant that showed a unique combination of phenotypes, with increased ABA and ethylene sensitivity and decreased light sensitivity at germination but also decreased seed dormancy. The ahg12 mutation was found to create a novel amino acid substitution mutation in RPT5, which is a component of the RP of the 26S proteasome.

The ahg12 mutation affected Ser112 of RPT5a. Although this amino acid residue is highly conserved among RPT5 proteins of eukaryotes, there has been no mutation around this residue reported previously. Structural studies of the Methanococcales\(^2\) proteasome complex, which is the archaean counterpart of the eukaryotic proteasome complex, revealed that the residue corresponding to Ser112 of RPT5a is localized in loop L23 near the pore structure of the OB fold of the base subcomplex of RP\(^{\text{P}}\) (Fig. 7). It is likely that the conversion of an amino acid residue in this vicinity to the pore would affect the activity of the proteasome. Indeed, exchanging other residues in this loop of the PAN complex compromised PAN activity\(^4\). Since most of the proteasome structure of fission yeast overlaps with the PAN structure\(^4\), it is plausible that ahg12, wherein the Ser residue is converted to bulky Phe, affects RP activity in plants.

A question arises as to why the ahg12 mutation affects restricted plant biological phenomena such as responses to ABA, ethylene, and light. It is possible that this mutation alters the substrate preference of the proteasome, and thereby decreases the degradation efficiencies of specific substrates including ABI5, EIN3, and PIL5. Functional asymmetry among RPT AAA-ATPases has been demonstrated in budding yeast\(^25,34\), in which a defect in each RPT somehow causes different effects on proteasome function. By analogy, ahg12 in RPT5a might affect the degradation of a set of targets. This idea is consistent with the distinct effects of the rpt2 and rpt5 mutants of
Figure 5. The 26S proteasome in ahg12 remains essentially functional. (a) Canavanine sensitivity of ahg12. Imbibed and stratified seeds of WT, ahg12, and disruptant of RPT5a (rpt5a-4) were grown on plates containing 8 μM canavanine for 4 weeks. (b) Ubiquinated proteins in ahg12 seedlings. Total proteins extracted from 2-week-old seedlings of WT, ahg12, and rpt5a-4 were resolved by SDS-PAGE and ubiquitinated proteins were detected by immunoblot. (c) ABA sensitivity of the ahg12 and ahg12rpt5b-3 mutants at germination. Imbibed and stratified seeds of WT, ahg12, and ahg12rpt5b-3 were sown on plates with or without ABA (0.3 μM) and grown for 7 days.
Arabidopsis33–37. The ahg12 mutation might affect substrate preference due to a change in the pore structure of the RP as discussed above. It is also possible that ahg12 slightly decreases the degradation efficiencies of all substrates. In this case, the phenotype related to the responses to ABA, ethylene, and light might be prominent in ahg12 because these responses presumably require particularly drastic degradation of signaling components compared with other phenomena at germination. However, the fact that the ABA-hypersensitive phenotype was not observed in the null mutant of RPT5a, in which protein degradation capacity was probably lower than in ahg12, does not seem to support the latter possibility. In any case, isolation and characterization of ahg12 demonstrated again that the responses to ABA, ethylene, light signals at germination are dependent on the protein degradation ability of the proteasome. Taken all together, our study on ahg12 emphasizes the importance of protein degradation in the regulation of plant biological phenomena including germination, and confirms the necessity of the OB fold loop structure of the RP complex for proteasome activity.

The transient expression assays with LUC-fused proteins quantitatively demonstrated that the ahg12 cells showed greater accumulation of signaling components that are involved in the physiological phenotypes of ahg12 (Fig. 6). The difference in the accumulated ABI5 between ahg12 and wild type was not particularly large in the leaf cell protoplasts, considering the clear ABA hypersensitive phenotype of ahg12. It is possible that the difference in ABI5 level is much larger in germinating seeds, where ABI5 naturally functions. It is likely that the stability of

**Figure 6.** ABI5, EIN3, and PIL5 accumulate to a higher level in ahg12. Accumulation of LUC-fused proteins transiently produced in Arabidopsis protoplasts. The vectors for expressing LUC-fused proteins shown in the figure were co-transfected with the vector for expressing GUS. LUC activity in each sample was normalized to GUS activity. The data are means of three independent experiments. Error bars indicate standard deviation. (**P < 0.01; t-test).**

**Figure 7.** Predicted spatial localization of ahg12. Three-dimensional structure of the RP complex extracted from a published structural data of the 26S proteasome complex derived from budding yeast (PDB ID: 4CR2) (left panel: top view, right panel: side view). The amino acids corresponding to the ahg12 mutation site of yeast RPT5 (Ser122) are represented with space-filling model (blue). The graphics were drawn with Molmil software.
other substrates of the proteasome is also affected by ahg12 and that these in turn presumably contribute to the phenotypes of this mutant. To examine what happens in the mutant in more detail, a comprehensive proteomic analysis is required.

Why are the genes encoding RPT proteins duplicated (e.g., RPT5a and RPT5b) in many plants? Why do various mutants of proteasome subunits show different phenotypes? These are major questions in the study of plant proteasomes. The amino acid sequence of RPT5b is quite similar to that of RPT5a, suggesting that these two RPT5 proteins could share functions to a large degree. In fact, a previous study reported that RPT5b can compensate for the absence of RPT5a in some cases36. In our study, the transgenic plants overexpressing RPT5b with an ahg12-like mutation did not show ABA hypersensitivity (Fig. S6), suggesting that there are some functional differences between RPT5a and RPT5b. It is possible that the slight differences in amino acid sequence are responsible for these distinct functions. Domain-swapping experiments between RPT5a and RPT5b might provide crucial data. In the future, we also plan to generate modified plants in which other RPT members have ahg12-like mutations and to evaluate the resulting phenotypes and proteasome substrate specificities. The identification of this dominant RPT5a mutant allele thus represents an important step toward addressing long-standing questions in proteasome biology.

**Methods**

**Oligonucleotides.** Oligonucleotides used in this study are listed in Table S1.

**Plant materials and growth conditions.** *Arabidopsis thaliana* (L.) Heynh, ecotypes Columbia (Col) and Landsberg erecta (Ler) were used. Plants were grown on MS plates (1 × Murashige and Skoog salt mix, 2% sucrose, 2.5 mM MES (pH 5.8) and 0.8% agar) or on soil at 23 °C under 16 h light/8 h dark cycles. Seeds were first imbibed at 4 °C for 2 d before transfer to a growth chamber, unless otherwise noted. The ahg12 mutant was isolated as described previously39 and the *aba2-1* mutation was removed by crossing with wild-type Columbia twice. The *RPT5a* T-DNA insertion line (rt5a-437, SALK_004632) was obtained from TAIR52 and given from Dr. Sakamoto (Tokyo University of Science). The *RPT5b* T-DNA insertion line (rt5b-337) was given from Dr. Yamaguchi (Hokkaido University).

**Mapping the ahg12 locus.** Rough mapping for the ahg12 locus was described previously39. Genetic linkage analysis indicated that ahg12 is located on chromosome 3. For fine mapping, genomic DNAs were extracted from approximately 800 F2 progeny between ahg12 and Ler, and genotyping was performed using PCR markers on chromosome 3. F3 seeds were obtained independently, and their ABA sensitivities were evaluated. Genotypes at the ahg12 locus in the F2 individuals were determined based on the segregation ratio in F3 populations. Genes in the genomic region completely linked with the ABA-hypersensitive phenotype were analyzed by DNA sequencing.

**Generation of transgenic plants.** The genomic *RPT5a/AHG12* gene with putative promoter and terminator regions was amplified from genomic DNA of the ahg12 mutant by PCR with specific primers (Table S1). The DNA segment was cloned into a pGreenII 0129 binary vector54. The open reading frame of *RPT5b* was amplified from cDNA and then an ahg12-like mutation (Ser111 to Phe) was introduced into the *RPT5b* gene by PCR. Agrobacterium strain GV3101 was transformed with the plasmids and used for the transformation of Arabidopsis plants by the flower-dipping method54. Transgenic lines were screened by hygromycin tolerance.

**Immunoblot analysis.** Total proteins were extracted from 2-week-old seedlings. Equal amounts of proteins were resolved by SDS-PAGE and transferred to PVDF membranes. Ubiquitinated proteins were detected by a polyclonal anti-ubiquitin antibody (BML-U95910, Enzo Life Science) and HRP-conjugated anti-rabbit IgG antibody (W4011, Promega Corp.).

**Transient expression analysis of LUC-fused proteins.** *ABI5, EIN3,* and *PIL5* cDNAs from the initiation codon to the last codon were amplified using specific primers (Table S1) and cloned into a plant expression vector to generate a fusion to the *LUC* gene under the control of the 35SCaMV promoter. These plasmids and a control plasmid containing a 35SCaMV promoter–GUS fusion were transfected into mesophyll protoplasts derived from the leaves of three-week-old ahg12 and wild-type plants. The protoplasts were incubated at 22 °C under dark conditions for 24 h and harvested for LUC and GUS assays. The LUC and GUS activities were measured as described previously55.

**References**

1. Jiao, Y., Lau, O. S. & Deng, X. W. Light-regulated transcriptional networks in higher plants. *Nat. Rev. Genet.* 8, 217–230 (2007).
2. Ma, J. F. Plant Root Responses to Three Abundant Soil Minerals: Silicon, Aluminum and Iron. *Crit. Rev. Plant Sci.* 24, 267–281 (2005).
3. Jones, J. D. G. & Dangl, J. L. The plant immune system. *Nature* 444, 323–329 (2006).
4. Munns, R. & Tester, M. Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* 59, 651–681 (2008).
5. Hirayama, T. & Shinozaki, K. Research on plant abiotic stress responses in the post-genome era: past, present and future. *Plant J.* 61, 1041–1052 (2010).
6. Engineer, C. B. et al. CO2 Sensing and CO2 Regulation of Stomatal Conductance: Advances and Open Questions. *Trends Plant Sci.* 21, 16–30 (2016).
7. Vierstra, R. D. The ubiquitin-26S proteasome system at the nexus of plant biology. *Nat Rev Mol Cell Biol* 10, 385–397 (2009).
8. Stone, S. L. The role of ubiquitin and the 26S proteasome in plant abiotic stress signaling. *Plant Genet. Genomics* 5, 135 (2014).
9. Leivar, P. & Quail, P. H. PIs: pivotal components in a cellular signaling hub. *Trends Plant Sci.* 16, 19–28 (2011).
10. Santner, A., Calderon-Villalobos, L. I. A. & Estelle, M. Plant hormones are versatile chemical regulators of plant growth. *Nat Chem Biol* 5, 301–307 (2009).
11. Santner, A. & Estelle, M. Recent advances and emerging trends in plant hormone signalling, *Nature* 459, 1071–1078 (2009).
12. Feuillet, C., Leach, J. E., Rogers, J., Schnable, P. S. & Eversole, K. Crop genome sequencing: lessons and rationales. *Trends Plant Sci.* 16, 77–88 (2011).
13. Dharmasiri, N., Dharmasiri, S. & Estelle, M. The F-box protein TIR1 is an auxin receptor. *Nature* 435, 441–5 (2005).
14. Kepinski, S. & Leyser, O. The Arabidopsis F-box protein TIR1 is an auxin receptor. *Nature* 435, 446–51 (2005).
15. Tan, X. et al. Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature* 446, 640–5 (2007).
16. Guo, H. & Ecker, J. R. Plant Responses to Ethylene Gas Are Mediated by SCFEBF1/EBF2-Dependent Proteolysis of EIN3 Transition Factor. *Cell* 115, 667–677 (2003).
17. Potuschak, T. et al. EIN3-Dependent Regulation of Plant Ethylene Hormone Signaling by Two Arabidopsis F Box Proteins: EBF1 and EBF2. *Cell* 115, 679–689 (2003).
18. Lopez-Molina, L., Mongrand, S. & Cha, N.-H. A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. *Proc Natl Acad Sci USA* 98, 4782–4787 (2001).
19. Zhang, X., Garreton, V. & Cha, N.-H. The AIP2 E3 ligase acts as a novel negative regulator of ABA signaling by promoting ABI3 degradation. *Genes Dev.* 19, 1532–1543 (2005).
20. Kish-Trier, E. & Hill, C. P. Structural Biology of the Proteasome. *Annu. Rev. Biophys.* 42, 29–49 (2013).
21. Deveraux, Q., Ustrell, V., Pickart, C. & Rechsteiner, M. A 26 S protease subunit that binds ubiquitin conjugates. *J. Biol. Chem.* 269, 7059–7061 (1994).
22. Hsu, J. et al. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Plant J.* 5301–5310 (2012).
23. Solano, R., Stepanova, A., Chao, Q. & Ecker, J. R. Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. *Genes Dev.* 12, 2723–2737 (2008).
24. Shinn, H., Moon, J. & Huq, E. PIF1 is regulated by light-mediated degradation through the ubiquitin-26S proteasome pathway to require the ABI5 transcription factor in Arabidopsis. *Plant J.* 55, 403–407 (2002).
25. Sauer, R. T. & Baker, T. A. AAA+ Proteases: ATP-Fueled Machines of Protein Destruction. *Annu. Rev. Biochem.* 80, 587–612 (2011).
26. Erales, J., Hoyt, M. A., Troll, F. & Coffino, P. Functional Asymmetries of Proteasome Translocase Pore. *J. Biol. Chem.* 287, 18535–18543 (2012).
27. Ehlinger, A. & Wallers, K. J. Structural Insights into Proteasome Activation by the 19S Regulatory Particle. *Biochemistry (Mosc.)* 52, 3618–3628 (2013).
28. Beuming, S. et al. Deep classification of a large cryo-EM dataset defines the conformational landscape of the 26S proteasome. *Proc. Natl Acad. Sci. USA* 111, 5544–5549 (2014).
29. Bhattacharyya, S., Yu, H., Mim, C. & Matouschek, A. Regulated protein turnover: snapshots of the proteasome in action. *Nat. Rev. Mol. Cell Biol.* 15, 122–133 (2014).
30. Smalle, J. et al. Cytokinin Growth Responses in Arabidopsis Involve the 26S Proteasome Subunit RPN12. *Plant Cell* 14, 17–32 (2002).
31. Smalle, J. et al. The Pleiotropic Role of the 26S Proteasome Subunit RPN10 in Arabidopsis Growth and Development Supports a Substrate-Specific Function in Abscisic Acid Signaling. *Plant Cell* 15, 965–980 (2003).
32. Wang, S., Kurepa, J. & Smalle, J. A. The Arabidopsis 26S Proteasome Subunit RPN1a is Required for Optimal Plant Growth and Stress Responses. *Plant Cell Physiol.* 50, 1721–1725 (2009).
33. Ueda, M. et al. The HELTED ROOT gene encoding the 26S proteasome subunit RPT2a is essential for the maintenance of Arabidopsis meristems. *Development* 131, 2101–2111 (2004).
34. Lee, K.-H. et al. The RPT2 Subunit of the 26S Proteasome Directs Complex Assembly, Histone Dynamics, and Gametophyte and Sporophyte Development in Arabidopsis. *Plant Cell Online* 23, 4298–4317 (2011).
35. Sako, K. et al. Arabidopsis RPT2a, 19S Proteasome Subunit, Regulates Gene Silencing via DNA Methylation. *Plas ONE* 7, e37086 (2012).
36. Gallois, J.-L. et al. The Arabidopsis Proteasome RPT5 Subunits Are Essential for Gametophyte Development and Show Accession-Dependent Redundancy. *Plant Cell* 21, 442–459 (2009).
37. Sakamoto, T. et al. Arabidopsis thaliana 26S Proteasome Subunits RPT2a and RPT5a Are Crucial for Zinc Deficiency-Tolerance. *Biosci. Biotechnol. Biochem.* 75, 561–567 (2011).
38. Beckwith, R., Estrin, E., Worden, E. J. & Martin, A. Reconstitution of the 26S proteasome reveals functional asymmetries in its AAA+ unfoldase. *Nat. Struct. Mol. Biol.* 20, 1164–1172 (2013).
39. Murayama, M. et al. Isolation of Arabidopsis ahg11, a weak ABA hypersensitive mutant defective in nad4 RNA editing. *J. Exp. Bot.* 63, 5301–5310 (2012).
40. Zhang, F. et al. Structural Insights into the Regulatory Particle of the Proteasome from Methanocaldococcus jannaschii. *Mol. Cell* 34, 473–484 (2009).
41. Zhang, F. et al. Mechanism of Substrate Unfolding and Translocation by the Regulatory Particle of the Proteasome from Methanocaldococcus jannaschii. *Mol. Cell* 34, 485–496 (2009).
42. Bohn, S. et al. Structure of the 26S proteasome from Schizosaccharomyces pombe at subnanometer resolution. *Proc Natl Acad Sci USA* 107, 20992–20997 (2010).
43. Finkelstein, R., Reeves, W., Arizumi, T. & Steber, C. Molecular Aspects of Seed Dormancy*. Annu. Rev. Plant Biol.* 59, 387–415 (2008).
44. Nambara, E. et al. Abscisic acid and the control of seed dormancy and germination. *Seed Sci. Res.* 20, 55–67 (2010).
45. Yan, N., Doelling, J. H., Falbel, T. G., Durski, A. M. & Vierstra, R. D. The Ubiquitin-Specific Protease Family from Arabidopsis. *Annu. Rev. Plant Biol.* 55–67 (2010).
46. Palazzo, J. P., Edwards, E. A., Leyland, N. R., Bean, S. & Mullineaux, P. M. pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. *Plant Mol. Biol.* 42, 819–832 (2000).
47. Clough, S. J. & Bent, A. F. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J.* 16, 735–743 (1998).
48. Yoo, S.-D., Cho, Y.-H. & Sheen, J. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat Protoc.* 2, 1565–1572 (2007).
Acknowledgements
We thank Dr. Junji Yamaguchi for the rpt5b-3 mutant seeds, Dr. Takuya Sakamoto for the rpt5a-4 mutant seeds, and Ms. M. Fujii and M. Kato for their experimental assistance. We also thank laboratory members for their fruitful comments and discussions. This work was supported by Grants-in-Aid for Scientific Research (B) (24370023) (MEXT) to T.H.

Author Contributions
S.H. and T.H. conceived the study. S.H. performed most of the experiments and T.H. did several supportive experiments. S.H. and T.H. wrote and edited the manuscript.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Hayashi, S. and Hirayama, T. ahg12 is a dominant proteasome mutant that affects multiple regulatory systems for germination of Arabidopsis. Sci. Rep. 6, 25351; doi: 10.1038/srep25351 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/