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Title:

The N-end rule pathway promotes seed germination and establishment through removal of ABA sensitivity in Arabidopsis.

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Summary:

The N-end rule pathway targets protein degradation through the identity of the amino-terminal residue of specific protein substrates. Two components of this pathway in *Arabidopsis thaliana*, PROTEOLYSIS6 (PRT6) and arginyl-tRNA:protein arginyltransferase (ATE) were shown to regulate seed after-ripening, seedling sugar sensitivity, seedling lipid breakdown and ABA sensitivity of germination. Sensitivity of *prt6* mutant seeds to ABA inhibition of endosperm rupture reduced with after-ripening time, suggesting that seeds display a previously un-described window of sensitivity to ABA. Reduced root growth of *prt6* alleles and the *ate1 ate2* double mutant was rescued by exogenous sucrose, and the breakdown of lipid bodies and seed-derived triacylglycerol was impaired in mutant seedlings, implicating the N-end rule pathway in control of seed oil mobilisation. Epistasis analysis indicated that PRT6 control of germination and establishment, as exemplified by ABA and sugar sensitivity, and storage oil mobilization, occurs at least in part via transcription factors ABI3 and ABI5. The N-end rule pathway of protein turnover is therefore postulated to inactivate as yet unidentified key component(s) of ABA signalling to influence the seed to seedling transition.

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Introduction:

The N-end rule pathway of protein degradation dictates the half life of proteins containing destabilising amino-terminal residues (1, 2). In Arabidopsis, several of the components of this pathway have been identified (3-5) (Fig. 1). Amino-terminal destabilising residues of proteins (N-degrons) are recognised by N-recognin E3 ligases (N-recognins), and are targeted for proteolysis via the 26S proteasome (2). In yeast and mammals, two classes of N-recognins (Type I and II) recognise either basic or hydrophobic amino-terminal amino acids, respectively. Yeast UBR1 was the first characterised E3 ligase with N-degron activity, and in mammals there are at least seven proteins that contain the characteristic UBR-box (2). In contrast, the Arabidopsis UBR-box-containing E3 ligase PROTEOLYSIS6 (PRT6) recognises only basic amino-acids, and PRT1, which does not contain a UBR-box, recognises aromatic amino acids (3, 4, 6). Other N-recognins must also exist in Arabidopsis since other N-degrons are unstable in *prt6 prt1* double mutants (3). N-degrons containing 1^o destabilising amino-terminal amino acids can also be created through the conversion of pre-N-degron 3^o or 2^o destabilising residues (Fig. 1). 3^o destabilising residues can be converted to 2^o through the action of N-terminal aminohydrolase (NTAN), or through chemical modification of cysteine, and 2^o to 1^o through the action of arginyl-tRNA:protein arginyltransferase (ATE1; Fig. 1). Arabidopsis contains two *ATE* genes, one of which (*ATE1*) has been implicated in leaf senescence (5). Model substrates starting with Asp or Glu were specifically stabilised in protoplasts of the *ate1* mutant, confirming a biochemical role in the N-end rule pathway (5). Key to the formation of N-degrons is the activity of specific endopeptidases that expose 1^o, 2^o or 3^o destabilising residues (Fig. 1). Degradation usually requires prior modification of substrates. For instance, Drosophila IAP 1 is cleaved by caspase to expose a destabilising residue, a process required for the correct regulation of apoptosis (7). Mammalian G-protein signalling components RGS4, 5 and 16 become substrates of the N-end rule pathway through oxidation and subsequent arginylation of Cys-2 exposed following MetAP activity (8), and Arabidopsis protein RIN4 is cleaved by a bacterial pathogenicity factor prior to rapid degradation (9) .

In this paper, we investigate the role of the N-end rule pathway in seed germination and establishment. Arabidopsis seed germination is regulated by intrinsic hormonal pathways and external environmental signals, which influence whether an imbibed seed completes germination or remains dormant (10-12). Dry arabidopsis seeds exhibit physiologically non-deep dormancy and following shedding, go through a period of after-ripening, through which the capacity for dormancy is lost (10, 13). The Arabidopsis embryo is surrounded by two structures: a single endosperm cell layer, and an external, dead testa layer, that is maternally derived (11). Germination proceeds through two stages: initially the testa layer ruptures and subsequently the endosperm ruptures opposite the emerging embryo radicle. Germination is said to be complete when the radicle has penetrated the endosperm (10, 11). The phytohormone Abscisic Acid (ABA) represses germination, and is presumed to function to stabilise the dormant state. Catabolism of ABA through ABA 8'-hydroxylase is required to reduce dormancy of imbibed seeds (14, 15), and many loci have been identified that enhance the desensitisation of seeds and seedlings to ABA or enhance the effect of ABA (11). In particular, the transcription factors ABI3, ABI4 and ABI5 enhance sensitivity to ABA (12). ABI3 has been shown to act upstream of ABI5 (16), and ABI4 has been implicated in the repression of seedling lipid degradation (17). The mechanisms through which ABA de-sensitisation occurs are not well understood. However, specific proteolysis is important during the seed to seedling transition, since E3 ligases (not associated with the N-end rule pathway) have been shown to have a small influence on germination potential in the presence of ABA, or to regulate seedling establishment following germination (18, 19). In addition, there is a relationship between ABA and sugar signalling and sensitivity during seedling establishment, such that ABA synthesis/signal transduction mutants are insensitive to exogenous sugars for establishment, although the physiological significance of these observations is still not clear (20, 21). In this paper, we show that ATE and PRT6 are major regulators of seed ABA sensitivity and seedling establishment, indicating a key role for the N-end rule pathway in the seed to seedling transition.

Results:

Influence of components of the N-end rule pathway on key aspects of germination and establishment

A genetic screen was carried out to identify Arabidopsis loci involved in the activation of processes associated with germination. Mutants were identified that showed reduced germination potential when after-ripening of wild-type (WT) seeds was complete (22). Positional cloning and complementation analysis with previously-published alleles identified the *PROTEOLYSIS6* (*PRT6*) gene as one of these loci (allele *prt6-4*, At5g02310; Supp. Fig. 1) (3). After-ripening (AR) of *prt6* seeds was substantially delayed, although moist chilling or exogenous gibberellic acid (GA) removed this block to germination (Fig 2A-C). The *prt6* alleles exhibited extreme ABA hypersensitive inhibition of germination, in comparison to previously published ABA hypersensitive mutants, or to transgenics ectopically expressing ABI3 and ABI5, positive components of the ABA signal transduction pathway (Fig. 2D,E, Fig. 4A, Supp. Fig. 2D). Endogenous ABA levels were not altered in *prt6-4*, or changed with after-ripening status (Supp. Fig 2A). ABA sensitivity of WT seeds did not change during after ripening (Fig.2F,G, Supp. Fig. 2C). In contrast, sensitivity to exogenous ABA decreased with dry storage time of mutant seeds (Fig. 2F). Previous studies have shown that Arabidopsis germination is composed of two phases, testa rupture, followed by endosperm rupture (reviewed in 11). Decreased germination potential of *prt6* seeds resulted from a defect subsequent to testa rupture as this was unaffected by storage time or in ABA sensitivity (Fig. 2G). Germination was also analysed using mutants of two other components of the N-end rule pathway: PRT1 (4) and ATE (Fig. 1). Arabidopsis contains two *ATE* genes: *ATE1* (At5g05700) and *ATE2* (At3g11240) (5) (Supp. Fig. 1). Neither *prt1-1*, nor the single *ate1-2* or *ate2-1* mutants demonstrated the extreme ABA hypersensitivity shown by *prt6* alleles, although *ate1-2* was as hypersensitive as some previously characterised mutants (Fig. 2D,E). However, *ate1-2 ate2-1* double mutant seed showed hypersensitivity similar to *prt6* alleles. In contrast to ABA hypersensitivity of germination, root growth of *prt6* was not hypersensitive to ABA inhibition of root elongation, as was also the case for another ABA hypersensitive mutant, *abh1* (Supp. Fig. 3).

Numerous genetic studies have indicated a role for ABA in sugar signalling during seedling establishment (20, 21). Therefore we analysed sugar sensitivity of establishment for N-end rule mutants in comparison to ABA signalling mutants (Fig. 2H, Supp. Fig. 4). *prt6-4* was hypersensitive to inhibition of establishment by sugars,

but not to sugar alcohols (Fig. 2H, Supp. Fig. 2B), and sucrose sensitivity was greater on water agarose compared to media supplemented with half strength MS salts, with IC50 of 0.27% and 1.4%, respectively (Fig. 3A).

prt6 and *ate1-2 ate2-1* mutant seeds retain oil bodies following germination

Roots of single *prt6* and double *ate1-2 ate2-1* mutants were shorter than WT on 1/2MS media but exhibited enhanced growth in the presence of 0.5% sucrose (a concentration non-inhibitory to establishment, Fig. 3A), indicating that seedlings may be energetically challenged (Fig. 3B). Hypocotyls and endosperms of these mutants retained oil bodies well beyond the time that these had disappeared in the respective WT seedlings (Fig. 3C,D, Supp. Fig. 5). Accordingly, analysis of fatty acid composition demonstrated that mutant seedlings retained eicosenoic acid (20:1), a fatty acid considered to be diagnostic of Arabidopsis seed triacylglycerol (23) (Fig. 3E). However, the capacity for beta-oxidation of the model substrate, 2,4-dichlorophenoxybuytric acid (2,4-DB) was not markedly reduced in these mutants, as judged by root elongation inhibition assays (Supp. Fig. 6), suggesting that the affected step occurs prior to peroxisome import and beta oxidation of fatty acids.

Genetic interactions of *PRT6* with seed ABA and GA signal transduction components

Both ABA and GA have been shown to play antagonistic roles in the regulation of germination. Therefore we analysed the genetic interactions between *PRT6* and genes associated with signalling and synthesis of these hormones. The RGL2 DELLA protein is a negative regulator of germination (24). We observed that *rgl2-1* seeds did not exhibit marked sensitivity to ABA, whereas *rgl2-1 prt6-4* seeds were hypersensitive (Fig. 4A). Freshly-harvested *rgl2-1 prt6-4* seeds showed a reduction in germination potential in comparison to *rgl2-1* seeds (Fig. 4C), and seeds were also hypersensitive to sucrose for establishment (Fig. 4D, Supp. Fig. 4).

Mutations in ABA biosynthesis (*aba1-1*) and signal transduction (*abi1-1*) reduce seed dormancy and sensitivity of establishment to exogenous sugar, and in addition, *abi1-1* reduces sensitivity of germination to exogenous ABA (25, 26). Whereas *aba1-1* seeds were similar in ABA sensitivity to WT, *aba1-1 prt6-4* double mutant seeds exhibited hypersensitivity similar to *prt6-4*. Both *abi1-1* and *abi1-1 prt6-4* seeds showed highly ABA insensitive germination potential (Fig. 4A). Although

freshly-harvested *abi1-1 prt6-4* seeds were non-dormant (Fig. 4C), *aba1-1 prt6-4* seeds did retain some dormancy in comparison to *aba1-1*. Both *abi1-1* and *aba1-1* showed sucrose insensitivity of establishment (Fig. 4D), however, only *abi1-1* seeds retained this phenotype in the *prt6* background.

The transcription factors ABI5 and ABI3 participate in germination-related ABA signalling (27, 28). We used two alleles of ABI3 (*abi3-8*, missense mutation in the B1 domain; *abi3-10*, missense mutation in the B2 domain) and one allele of ABI5, all of which show highly reduced sensitivity of germination to ABA (27, 28). In all cases, double mutants containing *prt6-4* also showed ABA insensitive germination (Fig. 4B). Freshly harvested seeds of both *abi5-1* and *abi3-8* exhibited high levels of primary dormancy, whereas *abi3-10* seeds were non-dormant (Fig. 4C), and the *abi3-10 prt6-4* double mutant seeds showed reduced dormancy. Whilst *prt6-4* partially increased sucrose hypersensitivity for *abi3-8* and *abi3-10* seeds, it did not affect sensitivity in the *abi5-1* background (Fig. 4D). Light microscopic analysis demonstrated that that oil bodies were not retained in establishing seedlings of *abi3-10 prt6-4* and *abi5-1 prt6-4*, in contrast to *prt6-4* (Fig. 4E).

Discussion

In this paper we describe the identification of a major function for the N-end rule pathway of protein degradation in the regulation of the seed to seedling transition. We show that this pathway is required for key aspects of seed after-ripening, ABA sensitivity, lipid degradation and seedling establishment.

The *PRT6* gene was identified as a regulator of germination potential. Mutant seeds exhibited a greatly increased after-ripening time (Fig. 2), suggesting that PRT6 function is required to potentiate after-ripening. Prolonged after-ripening may be due to enhanced ABA signalling, since germination was shown to be highly sensitive to exogenous ABA, and ABA sensitivity of *prt6-4* reduced with seed storage time, as germination potential of untreated seeds increased. ABA sensitivity occurred following testa rupture, indicating action late in phase II of germination, although ABA levels in imbibed *prt6* seeds were similar to those of WT. The *prt6* alleles exhibited an extreme sensitivity of germination to ABA, suggesting that this protein is key to the removal of ABA sensitivity immediately prior to germination. In agreement with a role in germination, analysis of publicly available expression data indicated that all three

components are expressed at low levels in embryo and endosperm of germinating seeds (Supp. Fig. 7).

Other components of the N-end rule pathway have been identified in Arabidopsis, including PRT1 (4, 6) and ATE (5). It is unlikely that this pathway acts through bulk degradation of proteins in imbibed seeds, since we did not observe any influence of the *prt1* mutant on ABA or sucrose sensitivity. However, germination of double mutant *ate1-2 ate2-1* seeds was highly sensitive to exogenous ABA. Moreover, *ate1-2 ate2-1* double mutant seeds also showed prolonged after-ripening (data not shown). This observation confirms the importance of the N-end rule pathway in seed ABA signalling, since ATE provides arginylation of secondary destabilising residues for PRT6. The similarity of the phenotypes of *prt6* and *ate1-2 ate2-1* mutants demonstrates that arginylation of a 2^o destabilising residue is required for the substrate(s), and also indicates that the relevant substrate(s) continue to be functional throughout processing to their arginylated form (cf. Fig. 1). In common with some other ABA hypersensitive mutants (but not *aip2* or *abh1*), *prt6* and *ate1-2 ate2-1* were also hypersensitive to sucrose for seedling establishment (as were transgenic seedlings ectopically expressing ABI3 and ABI5). Sugar sensitivity of establishment was specific to endogenous mono- and disaccharides but not sugar alcohols, discounting an osmotic effect.

Remarkably, the N-end rule mutants retained oil bodies after they had been degraded in WT seedlings, indicating that this pathway influences many aspects of development from imbibition to establishment. The inhibition of lipid degradation in these mutants was an unexpected finding, since *prt6* and *ate1-2 ate2-1* seedlings are able to complete establishment in the absence of exogenous sucrose, a process which is considered to require seed oil mobilisation (29). Beta-oxidation of the model substrate 2,4-DB was unaffected, consistent with an early block in lipid mobilisation, or in an associated pathway (30). Further studies are required to characterise the precise mechanism by which this pathway regulates lipid breakdown and to explore its biotechnological potential.

Analysis of the genetic relationship between *PRT6* and components of ABA/GA pathways indicated that PRT6 function is closely associated with ABA, but not GA signalling. Assay for the effect of exogenously-applied ABA on the repression of early endosperm rupture in moist pre-chilled seeds indicated an interaction between *PRT6* and the ABA signalling components *ABI1*, *ABI3* and *ABI5*. The *abi1-1*

mutation dominantly represses ABA signalling, thereby bypassing the requirement for PRT6, although the dominant negative nature of this mutation means that it is difficult to provide a straightforward explanation of the associated signalling hierarchy. In the absence of activity of the ABI3 or ABI5 transcription factors, PRT6 function is not required, since ABA sensitivity is removed. RGL2 is one member of the DELLA family of proteins, that has been shown to negatively regulate germination (24, 38). Removal of RGL2 activity is required for activation of GA-regulated responses (24, 39). Mutation of *RGL2* did not alter ABA hypersensitivity of *prt6-4*, suggesting that PRT6 function to remove ABA sensitivity is required before that of GA signalling. Assay for the effect of genetic interactions on primary dormancy showed that the dominant *abi1-1* mutation bypasses a requirement for *PRT6*. Interestingly, both *abi5-1* and *abi3-8* exhibited high levels of primary dormancy, whereas *abi3-10* did not. Dormancy in *abi3-10 prt6-4* seeds was increased, suggesting that *PRT6* function may be required before that of *ABI3* in the removal of ABA signal transduction. Alternatively, *abi3-10* may represent a weaker allele that may still provide some protein function in the absence of PRT6 activity in the double mutant. Although *aba1-1* seeds are in a low dormancy state, dormancy is increased in combination with *prt6-4*. This may be due to the failure to degrade PRT6 substrate(s) in a *prt6* background, an effect which could be enhanced through low levels of remaining ABA. Similarly, *rgl2* seeds exhibited a low dormancy state, in this case due to lack of repression of GA signalling. Stabilisation of PRT6 substrate in the double mutant may partially overcome this reduced dormancy, as suggested above for *aba1-1 prt6-4*, by increasing the strength of ABA signalling, in opposition to GA activation of germination. Assay for the sensitivity of seedling establishment to exogenous sucrose also demonstrated a genetic interaction between *PRT6* and components of ABA signalling.

Double mutants *abi3 prt6* and *abi5 prt6* did not retain oil bodies, suggesting a role for ABA signalling in the repression of lipid degradation. Previous work has shown that ABI4, not ABI5 is required for lipid breakdown in seedlings (17), however, the experimental system employed was different to that reported here, involving application of very high levels (20μM) of ABA to mutant seeds.

Mechanisms regulating after-ripening are not well understood (11, 13). Previously, it has been shown that catabolism of ABA in imbibed seeds is an

important regulator of germination potential, and that seeds lacking ABA 8'-hydroxylase activity exhibit enhanced dormancy (14, 15). However, ABA catabolism alone cannot explain reduced functioning of ABA pathways in the repression of germination because there is only a slight increase in dormancy of these mutant seeds. Mechanisms for the reduction of sensitivity to ABA must also be important. Many proteins, representing different biochemical pathways, are involved in the desensitisation of seeds to ABA (11), but the function of these proteins in after-ripening has not been reported. The N-end rule components, PRT6 and ATE play key roles in the removal of ABA sensitivity. After-ripening of *prt6-4* was delayed and sensitivity to ABA reduced with after-ripening time, suggesting that the N-end rule pathway may be responsible for desensitising seeds during a "window" of ABA responsiveness (10, 12). This pathway may function through the ABA signalling components ABI3 and ABI5, although it is not possible from genetic evidence to state whether these are direct targets, or whether they function upstream or downstream of the N-end rule pathway. Germination potential following imbibition is signalled by after-ripening, and it is likely that both ABA catabolism (through ABA 8'-hydroxylase activity) and desensitisation (through the N-end rule pathway and also through other more minor components) play important roles in removing the capacity for ABA to repress germination and maintain a dormant state. As after-ripening is a key ecological trait, it will be interesting to understand the influence of the environment on these two components. Recent work has shown that light influences ABA 8'-hydroxylase function (31).

The discovery of new roles for the N-end rule pathway in plants poses many intriguing questions. For example, nitric oxide (NO) can remove dormancy in imbibed seeds (32), and we note that NO has been shown to function within the N-end rule pathway (2), raising the possibility that NO operates through this pathway. The pathway requires a specific endopeptidase-substrate interaction or action of MetAPs (Fig. 1), and it has been shown that many peptidases (including MetAPs) are induced following after-ripening (13). Although ABI3 and/or ABI5 are possible substrates, ectopic expression of these transcription factors did not result in the extreme ABA sensitivity observed for *prt6* and *ate1/2* mutants (Supp. Fig. 2D), nor did it result in marked retention of oil bodies (data not shown). The future identification of the substrate(s) for this pathway in imbibed seeds should shed further light on the

regulation of ABA desensitisation, the mechanism(s) through which after-ripening signals germination potential, and the regulation of lipid degradation.

Materials and Methods

Plant material and physiological analysis

Arabidopsis genetic resources were obtained from NASC (UK) or individual researchers. Plant growth and germination/seedling establishment assays were carried out as before (13, 33). Unless otherwise stated, seed germination/establishment was assayed at 7 days' imbibition at 22^{o}C: radicle protrusion through the endosperm was used as the criterion for germination; cotyledon greening for establishment (establishment assayed on water agarose containing 1% (w/v) sucrose). Where indicated in figure legends seeds were exposed to 2 days moist chilling at 4^{o}C prior to transfer to 22^{o}C to remove residual dormancy.

Lipid analysis

Observation of lipid bodies was carried out following staining with the lipophilic dye Nile Red by using a Nikon eC1/TiU Inverted Confocal Microscope, seedling images (Fig. 4) were obtained by differential interference contrast microscopy using a Nikon OptiPhot 2 Upright Microscope. Fatty acids were analyzed by gas chromatography following conversion to methyl esters (FAMEs) (23) adapted for small tissue samples using 17:0 as an internal standard.

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Figure legends:

Figure 1

Diagrammatic representation of the N-end rule pathway associated with PRT6 function.

Components for which orthologous Arabidopsis genes have been identified are highlighted. Protein substrate (represented as cylinder) is cleaved by a methionine aminopeptidase (MetAP) or a specific endo-peptidase (Endo-P) at a unique amino-acid sequence (dotted curve). Cleavage reveals destabilising amino acid residues, which may be further modified by enzymatic (NTAN, N-terminal amidohydrolase; ATE1/2 Arg-tRNA-protein transferase) or chemical (O_2, NO: nitric oxide) action. 1^o, 2^o, 3^o indicate primary, secondary and tertiary destabilising N-terminal residues,

respectively. Generation of a primary destabilising residue (1^{o}) leads to targeting by E3 ubiquitin ligases PROTEOLYSIS6 (PRT6) or PRT1, depending on the nature of the destabilising residue. C*, oxidised derivative of cysteine.

Figure 2

ABA sensitivity of germination, after-ripening and sucrose sensitivity of establishment.

A. After-ripening of WT (L*er*) and *prt6-4* seeds assayed on water agarose after 7 days.

B. Germination potential of seeds imbibed for 7 days on water-agarose media, after increasing periods of cold, moist chilling.

C. Germination potential of seeds imbibed for 7 days on agarose media containing 1/2MS in the absence (black bars) and presence (white bars) of 100 µM GA_3.

D, E. Germination potential after 7 days, of WT, mutant and transgenic seeds. Assayed on 1/2MS media in the presence of exogenous ABA following 2 days moist chilling. Seeds were stored for 2 months prior to assay. Respective WT and mutant seeds are: Col-0, *prt1*-1*, prt6-1*, *prt6-2*, *ate1-2*, *ate2-1*, *abh1*, *ahg1, 2, 3* (3-5, 34-37). Data points for *ate2-1* are slightly obscured by those of *prt1*. Results obtained for ABI3OX, ABI5OX and *aip2* are presented in Supp. Fig. 2D.

F, G. Change in sensitivity of endosperm rupture (F) and testa rupture (G) to exogenous ABA with time of dry storage of cold chilled WT (L*er*) and *prt6-4* seeds, assayed on 1/2MS media, measured at 7 days following 2 days moist chilling.

H. Sucrose sensitivity of establishment (greening of cotyledons) of WT, transgenic and mutant seedlings grown on water agarose supplemented with 1% (w/v) sucrose, measured at 7 days, following 2 days moist chilling. Dose response curves are presented in Supp. Fig. 4.

Data represent means +/- SE of the mean.

Figure 3

Influence of *PRT6* and *ATE1/2* on lipid degradation during establishment.

A. Establishment of *prt6-4* and WT (L*er*) seedlings after 7 days growth on increasing concentrations of sucrose either in the presence (1/2MS) or absence (water) of 1/2 MS following 2 days moist chilling.

B. Influence of exogenous sucrose on root length of *prt6* and *ate1* and *2* alleles in relation to WT, measured at 7 days imbibition on 1/2MS media following 2 days moist chilling.

C, D. Representative confocal microscopy images of hypocotyl epidermal (C) and endosperm (D) cells (5 days imbibition on 1/2MS, no added sucrose, following 2 days moist chilling) stained with Nile Red to reveal oil bodies, which can be seen as red-staining spherical inclusions in *prt6-1* and *ate1 ate2* but not in Col-0. Bar indicates 20 microns. Bright field images are presented in Supp. Fig. 5.

E. Eicosenoic acid (20:1) content of 5 day old seedlings grown on 1/2MS media following 2 days moist chilling.

Data represent means +/- SE of the mean.

Figure 4

Genetic interactions of *PRT6* with components of ABA and GA biosynthesis and signalling.

A, B. Germination potential after 7 days, of WT mutant and transgenic seeds on 1/2MS media in the presence of exogenous ABA, following 2 days moist chilling. *abi1-1* (L*er* background) (26), *aba1-1* (L*er*) (25), *rgl2-1* (L*er*) (24), *abi3-8* (Col-0), *abi3-10* (Col-0) (28), *abi5-1* (Ws) (27).

C. Germination potential after 7 days, of freshly-harvested seeds on water agarose.

D. Seedling establishment after 7 days on water agarose containing 1% (w/v) sucrose, following 2 days moist chilling (dose response curves are presented in Supp. Fig. 4.).

E. Light micrographs of hypocotyl epidermis of single and double mutant seeds showing retention of lipid bodies in *prt6-4* but not in single or double mutant combinations with *abi3-10* or *abi5-1*, on 1/2MS media after 5 days, following 2 days moist chilling. Bar indicates 20 microns.

Data represent means +/- SE of the mean.